Anti-inflammatory effects of *Dendropanax morbifera* in lipopolysaccharide-stimulated RAW264.7 macrophages and in an animal model of atopic dermatitis

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Abstract. *Dendropanax morbifera* (*D. morbifera*), known as Dendro, means 'omnipotent drug' (Panax), and has been called the panacea tree. Various studies on *D. morbifera* are currently ongoing, aiming to determine its medicinal uses. The present study investigated the anti-inflammatory effects and under-lying mechanism of a natural extract of *D. morbifera* leaves (DPL) in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. In the present study, the following assays and models were used: MTT assay, nitric oxide (NO) assay, western blotting, ELISA and mouse models of atopic dermatitis. DPL extract markedly reduced the production of NO, inducible NO synthase and interleukin-6, as well as the nuclear translocation of nuclear factor-κB (NF-κB). Additionally, the LPS-induced activation of extracellular signal-regulated kinase 1/2 (ERK1/2), P38 and c-Jun N-terminal kinase (JNK) was suppressed by DPL extract. Taken together, these results indicate that NF-κB, ERK1/2, P38 and JNK may be potential molecular targets of DPL extract in the LPS-induced inflammatory response. Subsequently, the present study investigated the effects of DPL extract in a 2,4-dinitrochlorobenzene-induced atopic derma-titis mouse model. Ear thickness, serum immunoglobulin E levels and histological analysis revealed that the DPL extract was effective in attenuating the inflammatory response. These results indicate that DPL extract has anti-inflammatory poten-tial and may be developed as a botanical drug to treat atopic dermatitis.

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Introduction

Recently, more studies investigating natural materials have been conducted in response to the increased public interest in healthy skin. There is particular interest in the preven-tion and treatment of various skin diseases, including atopic dermatitis and inflammatory acne (1). Atopic dermatitis (AD), also known as inflammatory dermatitis, is characterized by severe itching, chronic edema and skin rashes (2). Although the etiology is not clearly understood, it is known that AD is associated with immunological abnormalities, abnormal skin barriers, and genetic and environmental factors (3). Exposure to allergens induces atopy, leading to an imbalance between T helper cell (Th) 1 and Th2 cytokines, and the overproduction of Th2 cytokines stimulates B-cells to increase the generation of immunoglobulin E (4). Increased immunoglobulin E (IgE) induces the exocytosis of compounds such as histamine in skin mast cells, causing edema and itching, thereby aggravating AD (4). Treatment methods for dermatitis include moisturizing dry skin, the use of steroids as anti-inflammatory agents, the application or administration of antihistamines and immuno-suppressive agents. Although these drugs may relieve certain symptoms, long-term use of topical steroids may additionally lead to thinning of the skin with subsequent bleeding (5,6). Therefore, a number of ongoing studies are attempting identify functional substances from safe and effective natural products to prevent AD without side effects (7,8).

The inflammatory response is a physiological protective activity in the human body that recognizes external physical and chemical stimuli, and employs a defensive mechanism to restore damaged tissues (9). Inflammatory reactions are categorized as acute or chronic depending on their activation and duration (10). When an inflammatory reaction occurs, inflammatory mediators such as nuclear factor-κB (NF-κB), nitric oxide (NO) and inflammatory cytokines are secreted. NF-κB is widely distributed and is known as a regulator of immune and inflammatory responses (11,12). NO, an indi-cator of the inflammatory response, is synthesized by NO synthase (NOS) from L-arginine. There are three types of NOS: Endothelial, neuronal and inducible NOS (iNOS). Of these, NO produced by iNOS serves an important pathological

role (13). Excessive NO production by iNOS induced by lipopolysaccharide (LPS) or inflammatory cytokines exacerbates the inflammatory response, resulting in tissue damage, gene mutations and nerve damage (14). Cyclooxygenase (COX) is an enzyme that catalyzes the conversion of arachidonic acid to prostaglandin. COX-2 is induced by various stimuli including inflammation, growth factors and cytokines released by cancer cells (15). The proinflammatory cytokines that are enriched during inflammation include tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β, and are secreted by macrophages to mediate various inflammatory responses that induce NO generation. Excessive production of NO and cytokines can lead to genetic alterations, and tissue and nerve damage, with fatal consequences for the host (16). Therefore, regulation of the inflammatory response is important for maintaining homeostasis, and an anti-inflammatory effect may be achieved by inhibiting the production of mediators such as NO and cytokines, which are produced in inflammatory reactions (17).

Other signaling molecules involved in inflammatory reactions include mitogen-activated protein kinases (MAPKs), which can be characterized as three different types of serine/threonine protein kinases: Extracellular signal-regulated protein kinase (ERK), stress-activated c-Jun N-terminal kinase (JNK) and P38 kinases. Each type of kinase has a different activity, and all 3 types are known to carry out signal transduction in various cellular activities, including proliferation, differentiation, cell death and inflammation (18). Efficient inhibition of these inflammatory mediators may be an important strategy in the development of anti-inflammatory drugs.

Dendropanax morbifera (*D. morbifera*) is one of the world's rarest warm climate trees. There is only one species in one genus in Korea, and it is found across a broad area of western and southern coastal Korea encompassing Jeju Island, Wando, Geomundo and Haenam; however, the native distribution has been impacted by overharvesting and urbanization, and *D. morbifera* now only grows in a limited area (19). *D. morbifera*, known as Dendro, means 'omnipotent drug' (Panax), and has been called the panacea tree. Various ongoing studies on *D. morbifera* are aiming to determine its medicinal uses (20-24). *D. morbifera* leaves are composed of 70.2% water, 1.2% protein, 2.7% fat, 1.7% ash, 56.9 mg% vitamin C and 746 mg% water soluble tannins; 32 types of substances have been identified with gas chromatography-mass spectrometry. Among these substances are β-selinene and capnellene-8-one, which are sesquiterpenes with two ring structures, as well as many unidentified volatile components (20). When *D. morbifera* bark is scarred, it produces a golden resinous solution that contains benzoic acid, a unique aromatic component (21,22).

Recent studies have revealed that polyacetylene compounds isolated from *D. morbifera* leaves have anti-complement activity, whereas ethanol extracts promote the actions of B- and T-cells (20,23). *D. morbifera* sap inhibited melanin biosynthesis by reducing the expression of tyrosinase in melanin synthesis, and the ethyl acetate fraction extracted from *D. morbifera* leaves also exhibited cell protective and skin whitening effects, such as antioxidant properties and tyrosinase inhibition (24). However, few studies or animal experiments have been conducted on the mechanism underlying its anti-inflammatory properties.

In the present study, the effects of *D. morbifera* leaf (DPL) extract on the production of NO, cytokine secretion and inflammation-associated protein expression was investigated in activated RAW264.7 macrophages by inducing inflammation with LPS. The anti-inflammatory effect of DPL was also determined in a 2,4-dinitrochlorobenzene (DNCB)-induced AD animal model.

Materials and methods

Chemicals, drugs and antibodies. Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, and 10% fetal bovine serum (FBS) were purchased from Welgene (Gyeongsan, Korea). LPS, MTT and dimethyl sulfoxide (DMSO) were purchased from Merck KGaA (Darmstadt, Germany). The nitrate/nitrite colorimetric assay kit was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). The mouse TNF ELISA (cat. no. 560478) and mouse IL-6 ELISA kits (cat. no. 555240) were purchased from BD Biosciences (San Jose, CA, USA). β-actin (cat. no. 4967), iNOS (cat. no. 2982), COX-2 (cat. no. 4842), phosphorylated (p)-NF-κB-p65 (cat. no. 3033), NF-κB-p65 (cat. no. 8242), p-NF-κB inhibitor-α (IκB-α; cat. no. 4812), IκB-α (cat. no. 5209) p-ERK1/2 (cat. no. 4376), ERK1/2 (cat. no. 9194), p-P38 MAPK (cat. no. 9211), P38 MAPK (cat. no. 8690), p-JNK (cat. no. 4668), JNK (cat. no. 9252) and anti-rabbit horseradish peroxidase (HRP; cat. no. 7074) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Plant materials and extraction. The *D. morbifera* leaves used in the experiments were collected from the native areas of Jeju Island. The collected *D. morbifera* leaves (100 g) were pulverized and then fermented with 70% ethanol and tertiary distilled water at a ratio of 4:6 for 10 days at room temperature, then subjected to hydrothermal extraction. The extracted solution was filtered using Whatman No. 1 disc paper. Then the extract was concentrated under reduced pressure using a rotary vacuum evaporator (R-220; BUCHI Corporation, New Castle, DE, USA), and the remaining solution was boiled again to obtain an extract (25). The extracts were mixed with distilled water (DW) and refrigerated.

Cell culture and stimulation. The RAW264.7 macrophage line was obtained from the Korean Cell Line Bank (Korean Cell Line Research Foundation, Seoul, Korea), and maintained in DMEM supplemented with 5% FBS/1% penicillin-streptomycin at 37°C in a 5% CO_2 humidified air environment. The cells were incubated for 24 h in medium supplemented with 10% FBS. Subsequently, the RAW 264.7 cells were pre-treated with doses of DPL (0, 100, 200, 300, 400 and 500 μg/ml) for 2 h at 37°C and treated with LPS (1 μg/ml) for 24 h at 37°C, in serum-free media.

Cell viability assay. RAW264.7 cells were seeded in a 96-well plate at a density of $1x10^5$ cells/ml and a volume of 200 μl/well. Following incubation for 24 h at 37°C, the cells were treated with DPL extract (mixed with DW) and negative control (DW) at various concentrations (DPL 0, 100, 200, 300, 400 and 500 μg/ml) for 24 h at 37°C, followed by the addition of 5 mg/ml MTT solution to each well, and the plates were further incubated for 2 h at 37°C. The supernatant

was removed and 200 μl DMSO was added to each well to solubilize the water-insoluble purple formazan crystals. The absorbance at a wavelength of 595 nm was measured using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The percentage of viable cells compared with that of untreated control cells was then estimated.

Measurement of NO. RAW264.7 cells were seeded ($1x10^5$/ml) and cultured in 96-well plates. Following incubation for 24 h at 37˚C, the cells were treated with DPL extract at the indicated concentrations (DPL 0, 100, 200, 300 and 400 μg/ml) for 2 h in serum-free medium prior to the addition of LPS (1 μg/ml). Following a 24-h incubation at 37˚C, the supernatants were measured for NO production using the nitrate/nitrite assay kit (Cayman Chemical Company). NO was measured as the accumulation of nitrite and nitrate reductase, which were determined spectrophotometrically using Griess reagent, included in the nitrate/nitrite assay kit, at an optical density of 540 nm.

Determination of TNF-α and IL-6 production. RAW 264.7 cells were pre-treated with doses of DPL (0, 100, 200, 300 and 400 μg/ml) for 2 h and treated with LPS (1 μg/ml) for 24 h at 37˚C. Subsequently, production of the proinflammatory cytokines TNF-α and IL-6 in the culture medium was determined using commercially available ELISA kits (BD Biosciences), according to the manufacturer's protocol.

Western blot analysis. Cells were preincubated with various concentrations of DPL extract (0, 200 and 400 μg/ml) for 2 h prior to a 24-h incubation at 37˚C with LPS (1 μg/ml), and subsequently harvested. Western blot assays were performed as previously described (26). The membranes were incubated with the primary antibodies specific for β-actin (1:10,000), p-NF-κB-p65 (1:1,000), NFκB-p65 (1:1,000), p-IκB-α (1:1,000), IκB-α (1:1,000) p-ERK1/2 (1:1,000), ERK1/2 (1:1,000), p-P38 MAPK (1:1,000), P38 MAPK (1:1,000), p-JNK (1:1,000) and JNK (1:1,000) overnight at 4˚C with gentle shaking. Following incubation with the primary antibodies, the membranes were incubated with HRP-conjugated anti-rabbit IgG secondary antibodies (1:1,000) for 2 h at room temperature with gentle shaking. The membranes were washed three times for 10 min in TBS containing 0.1% Tween-20. The bands were detected using enhanced chemiluminescence western blotting detection reagents (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. β-actin was used as a loading control. Band density was measured using ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA).

Animals. A total of 30 BALB/c female mice (age, 4 weeks; body weight, 16-18 g) were purchased from the Nara Biotech, Co., Ltd. (Seoul, South Korea) and maintained at 23±5˚C at 40±10% relative humidity with a 12-h light/dark cycle (artificial lighting from 8:00 a.m. to 8:00 p.m.) in facilities approved by the Companion and Laboratory Animal Science Department of Kongju National University (Chungnam, Korea). The animals were housed in cages and allowed access to sterilized water and commercial rodent chow (Biopia, Seoul, Korea) *ad libitum*. All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of Kong-Ju National University (approval no. KNU_2017-10; Yesan, Korea) and the institutional guidelines were adhered to.

Induction of AD. AD was induced in BALB/c mice as previously described, with minor modifications (27). Briefly, BALB/c mice were divided into seven groups (n=5/group): Group I (control treatment; 3:1 acetone/olive oil solution; 20 μl/ear), group II (1% DNCB; 20 μl/ear), groups III (dexamethasone; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany; 50 μg/20 μl/ear; positive control), group IV (DPL high; 20 μl/ear, undiluted solution), group V (DPL medium; 20 μl/ear, 0.5x undiluted solution/DW) and group VI (DPL low; 20 μl/ear, 0.25x undiluted solution/DW) received DPL extract or combinations as indicated. To induce AD, the surfaces of both ears of the mice (not anesthetized) were stripped with surgical tape. Following this, 1% DNCB dissolved in a 3:1 acetone/olive oil solution was painted on each ear. The DPL extract was applied at three concentrations: High (undiluted solution mixed with an equal volume of 3:1 solution of acetone and olive oil, v/v), medium (0.5x undiluted solution/DW mixed with an equal volume of 3:1 solution of acetone and olive oil, v/v) or low (0.25x undiluted solution/DW mixed with an equal volume of 3:1 solution of acetone and olive oil, v/v). DNCB was applied every 2 days starting from 3 days prior to measurements. Dexamethasone and DPL extract were applied every 2 days from day 0. Following 24 h of DNCB application, the thickness of the ears was measured with a vernier caliper (Mitutoyo, Kawasaki, Japan). The mice were sacrificed on Day 10. Blood samples were then collected from the abdominal aorta, and the plasma was stored at -70˚C until further analysis. Following sacrifice, the ears were excised and subjected to histopathological analysis.

Measurement of Ig levels. Blood samples were obtained from each treatment group 10 days following AD induction. Total serum IgE levels were measured using an ELISA kit (cat. no. K3231082; Komabiotech, Seoul, Korea), according to the manufacturer's protocol.

Histological observations. The ears were immediately fixed in 10% formaldehyde (1 week at room temperature) following excision, and embedded in paraffin. Blocks were then cut into 5-μm-thick slices. To measure epidermal thickening, hematoxylin and eosin (H&E) staining was performed (6 h at room temperature). To evaluate mast cells, the skin sections were stained with toluidine blue (TB; 6 h at room temperature). The sections were examined under a light microscope (magnification, x200; Olympus CH30; Olympus Corporation, Tokyo, Japan).

Statistical analysis. The results are expressed as the mean ± standard deviation. The experiments were repeated three times. Statistical analyses were performed using Prism (version 5; GraphPad Software, Inc., La Jolla, CA, USA) Differences between the mean values for the individual groups were assessed by one-way analysis of variance with Dunnett's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of DPL extract on the viability of RAW264.7 macrophages. To investigate the effect of DPL extract on the survival of RAW264.7 macrophages, an MTT assay was performed. Cell viability was measured once RAW264.7 cells were treated with 0, 100, 200, 300, 400 and 500 μg/ml DPL extract and cultured for 24 h. There were no differences in cell viability at any concentration, confirming that the DPL extract did not affect the survival of RAW264.7 cells (Fig. 1). However, cell viability following treatment with DPL at a concentration of 500 μg/ml was decreased compared with other concentrations. Therefore, concentrations of 100, 200, 300 and 400 μg/ml were used.

Effects of DPL extract on LPS-induced NO production. NO is an inorganic compound produced by NO synthase. It is involved in many biological processes including the immune response, cytotoxicity and vascular relaxation, and it also maintains cell function and cytotoxicity depending on the concentration (28). The present study selected the concentration of DPL extract that had no effect on the survival of RAW264.7 macrophages and performed a NO assay. RAW264.7 cells were treated with DPL extract (100, 200, 300 and 400 μg/ml) for 2 h before treatment with LPS (1 μg/ml), and the concentration of NO was measured (Fig. 2A). The NO concentration in RAW264.7 cells stimulated with LPS was ~5 times higher compared with that observed in the untreated control group. In the groups treated with 100, 200, 300 and 400 μg/ml DPL extract with LPS, the NO concentrations were 68.9, 51.2, 28.1 and 24.4%, respectively, indicating that the NO concentration decreased in a DPL concentration-dependent manner when compared with the group treated with LPS alone (Fig. 2A).

Effects of DPL extract on LPS-induced iNOS and COX-2 protein expression. The protein expression levels of iNOS and COX-2, which are known to activate inflammation, were assessed by western blotting. The protein expression levels of iNOS and COX-2 were increased in the LPS-treated group when compared with the control group, and these levels were significantly decreased in a concentration-dependent manner in the groups treated with different concentrations of DPL (Fig. 2B and C).

Effects of DPL extract on LPS-induced proinflammatory cytokine production. The expression of TNF-α and IL-6 was quantified by ELISA to assess the anti-inflammatory effects of DPL extract. When compared with the expression levels in the group treated with LPS alone, the expression of TNF-α was significantly decreased following treatment with 300 and 400 μg/ml DPL, and the expression of IL-6 was significantly decreased in a concentration-dependent manner (Fig. 3).

Effects of DPL extract on the activation of NF-κB in LPS-stimulated RAW264.7 macrophages. NF-κB is activated by IκB-α and acts as an inflammatory mediator (11). To investigate the activation of the well-known inflammatory mediators, NF-κB and IκB-α, the present study performed western blot analysis. The expression of NF-κB and IκB-α was increased in the LPS-treated group when compared with the untreated
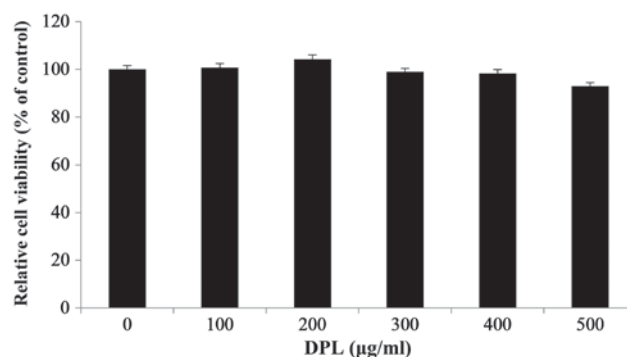

Figure 1. Effects of DPL extract on the viability of RAW264.7 cells. RAW264.7 cells (1x10^5 cells/ml) were treated with 100, 200, 300, 400 and 500 μg/ml DPL extract for 24 h, and cell viability was determined by an MTT assay. Each bar represents the mean ± standard deviation calculated from three independent experiments. DPL, *Dendropanax morbifera* leaf.

control group. Comparisons of the LPS and 200 or 400 μg/ml DPL extract groups and the LPS-only group revealed that the activation of NF-κB was significantly decreased with DPL extract treatment, whereas the activation of IκB-α was not significantly changed (Fig. 4).

Effects of DPL extract on LPS-induced NF-κB and MAPK phosphorylation. MAPK is a typical signaling molecule that affects the activation of NF-κB. When MAPK is phosphorylated and activated in the cell, it influences the production of various inflammatory mediators. When inflammation occurs, MAPKs such as P38 and JNK are activated in macrophages, further activating the inflammatory response (18). To confirm if the expression of MAPKs is involved in the inflammatory response, the present study performed western blot analysis. Comparing the LPS-only group with the untreated control group, the expression of p-ERK was not significantly changed, but the expression levels of p-JNK and p-P38 were increased (Fig. 5). When assessing DPL extract treatment, compared with the group treated with LPS-only, the expression levels of p-ERK, p-JNK and p-P38 were significantly downregulated in the 400 μg/ml DPL-treated group; the levels of p-JNK and p-P38 were also significantly downregulated in the 200 μg/ml DPL-treated group. Therefore, p-JNK and p-P38, in particular, exhibited more significant decreases in expression than p-ERK.

Effect of DPL extract on DNCB-induced AD-like skin lesions in BALB/c mice. DNCB is a typical compound with a benzene ring that triggers a local immune response to induce inflammatory dermatitis (25,29,30). Therefore, to determine the effect of DPL extract on induced AD, the present study measured ear thickness in mice following treatment with DNCB and DPL extract. The DPL extract was applied to the ears at three concentrations: High (undiluted solution), medium (1:2 dilution), or low (1:4 dilution). A schematic of the experimental procedure is presented in Fig. 6A. The ear thickness of the DPL-treated group was decreased when compared with that of the DNCB-induced AD group (Fig. 6B). In addition, the AD symptoms were more effectively reduced in the DPL low group than in the DPL high and medium groups. Next, the present study measured the morphological changes in the lymph nodes of induced AD mice. The results revealed that
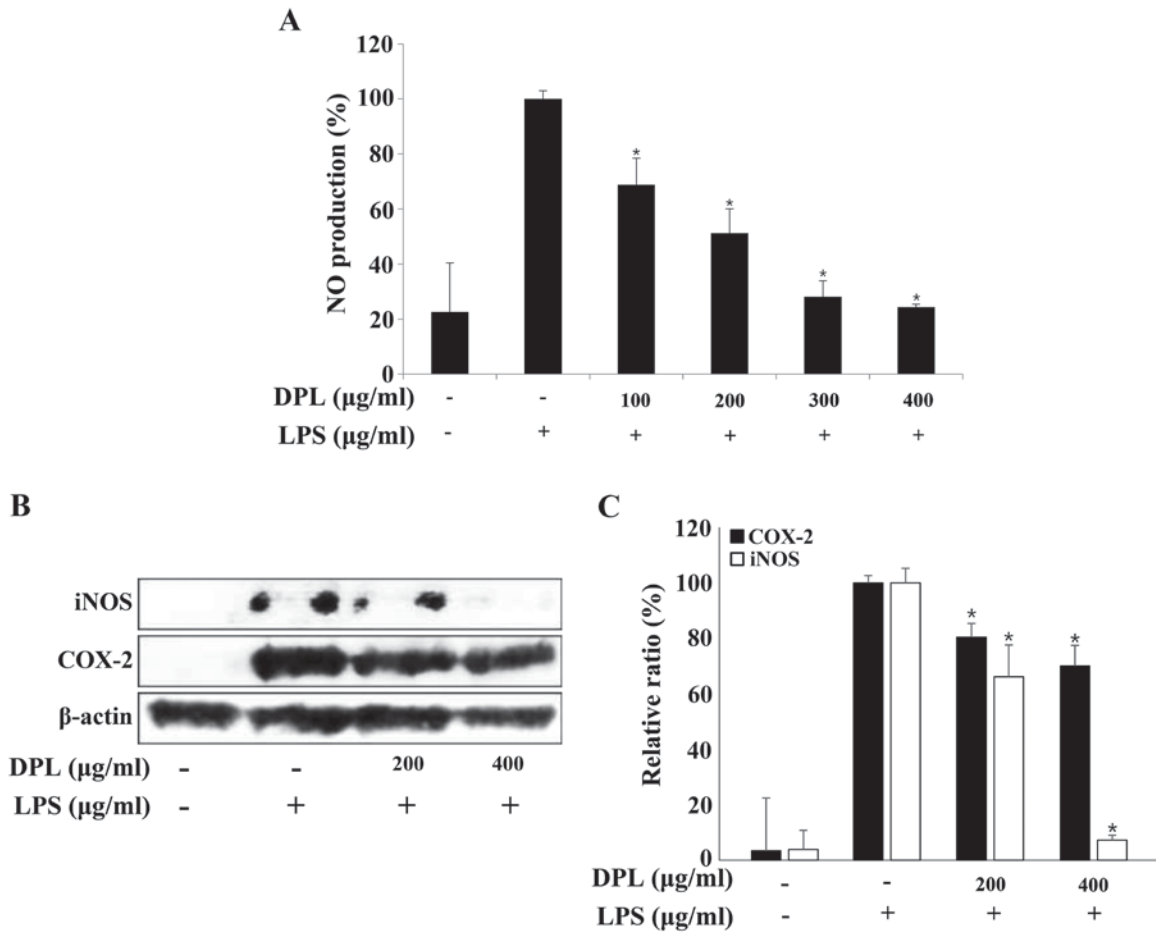
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Figure 2. Effects of DPL extract on LPS-induced iNOS and COX-2 protein levels and NO production in RAW264.7 cells. (A) RAW264.7 cells were pre-incubated with 100, 200, 300, and 400 μg/ml DPL extract for 2 h, and then treated with 1 μg/ml LPS for an additional 24 h. NO was measured using the Griess reaction. (B) The cells were sampled and lysed following 24 h treatment, and iNOS and COX-2 protein levels were determined by western blotting. β-actin was used as a loading control. (C) Data analysis was performed using ImageJ software by measuring the integrated band densities following background subtraction. Each bar represents the mean ± standard deviation calculated from three independent experiments. *P<0.05 vs. LPS only treatment. DPL, *Dendropanax morbifera* leaf; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; NO, nitric oxide; COX2, cyclooxygenase 2.
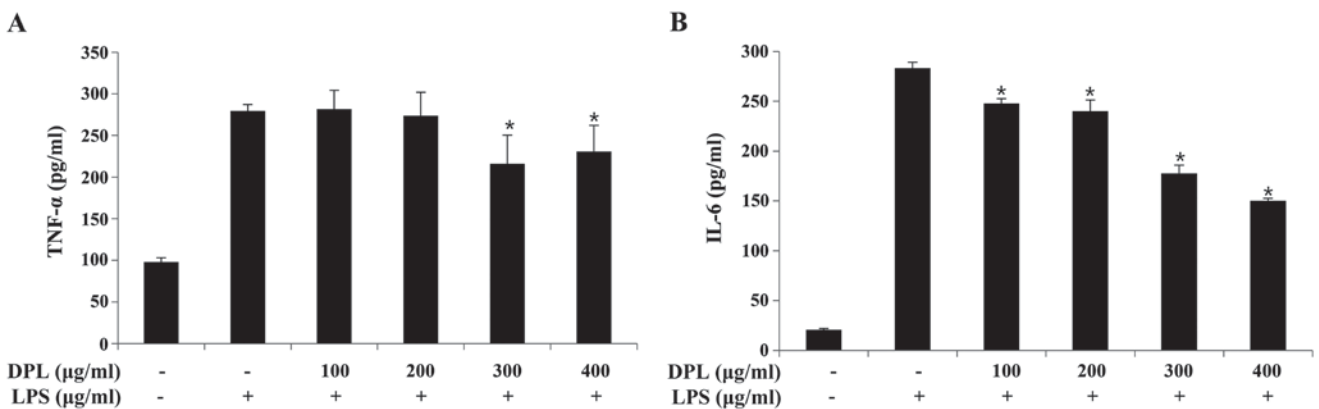
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Figure 3. Effects of DPL extract on LPS-induced pro-inflammatory cytokine production in RAW264.7 cells. RAW264.7 cells were treated with LPS (1 μg/ml) in the presence or absence of DPL extract at the indicated concentrations for 24 h. (A) TNF-α and (B) IL-6 in the culture supernatant were measured by ELISA. Each bar represents the mean ± standard deviation calculated from three independent experiments. *P<0.05 vs. LPS only treatment. DPL, *Dendropanax morbifera* leaf; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; IL, interleukin.

the lymph nodes were increased in the DNCB-treated group when compared with the untreated control group, whereas, the DPL-treated groups exhibited a decreased size compared with DNCB, particularly in the DPL low group (Fig. 6C). In the

induced AD mice, IgE levels were significantly decreased in the DPL-treated groups when compared with the DNCB-treated group (Fig. 6D). These results confirmed that DPL extracts attenuated the symptoms of AD in mice.
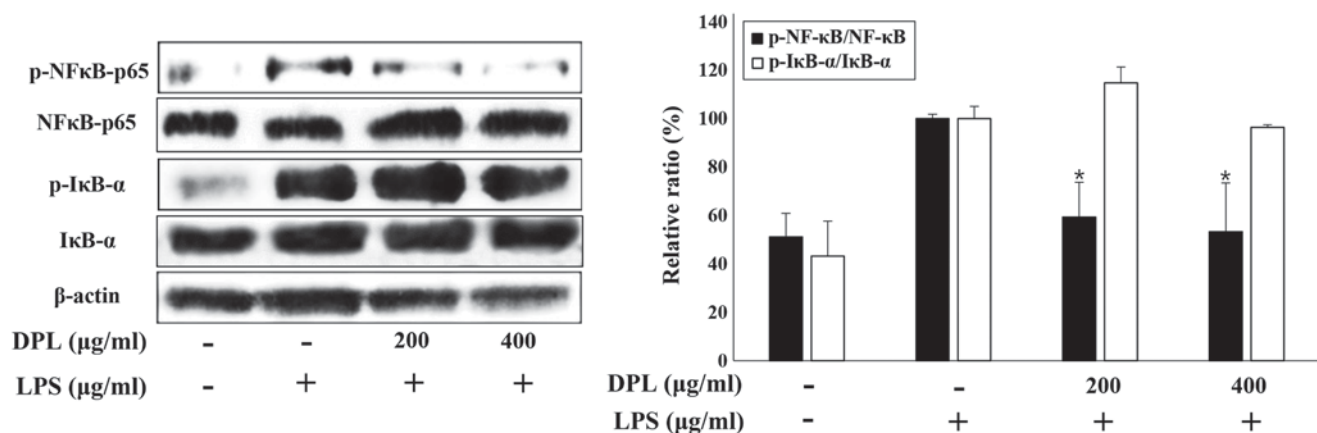
Figure 4. Effects of DPL extract on the degradation or phosphorylation of NF-κB/p65 and IκB-α protein in LPS-induced RAW264.7 cells. RAW264.7 cells were treated with LPS (1 µg/ml) in the presence or absence of DPL extract at the indicated concentrations for 24 h. NF-κB phosphorylation and IκB-α degradation were assessed by western blotting. Data analysis was performed using ImageJ software by measuring the integrated band densities following background subtraction. Each bar represents the mean ± standard deviation calculated from three independent experiments. *P<0.05 vs. LPS only treatment. DPL, *Dendropanax morbifera* leaf; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; IκB-α, NF-κB inhibitor-α; p-, phosphorylated.
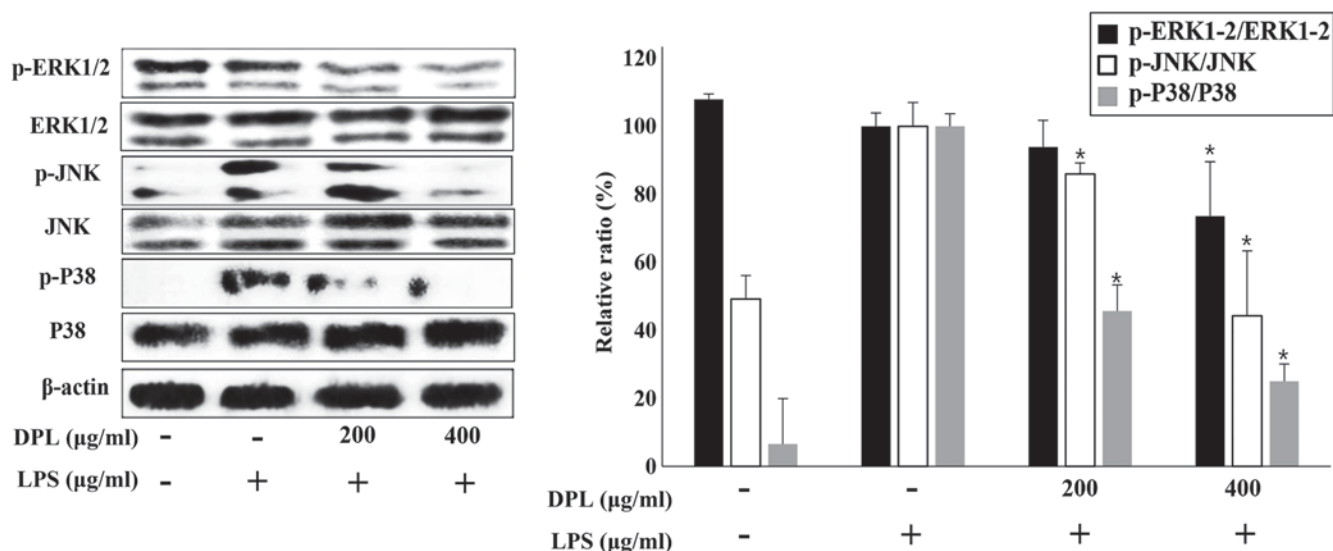

Figure 5. Effects of DPL extract on the MAPK signaling pathway in LPS-induced RAW264.7 cells. RAW264.7 cells were treated with LPS (1 µg/ml) in the presence or absence of DPL extract at the indicated concentrations for 24 h. MAPKs were assessed by western blot analysis of whole cell lysates. Each bar represents the mean ± standard deviation calculated from three independent experiments. *P<0.05 vs. LPS only treatment. DPL, *Dendropanax morbifera* leaf; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1/2; JNK, c-Jun N-terminal kinase; p-, phosphorylated.

Effects of DPL extract on DNCB-induced immune cell infiltration in BALB/c mice. When AD is induced, the secretion of vasodilators such as proteases and histamine by mast cells increase (31). The ears of the induced AD mice were excised to examine the thickness of the ear tissue and mast cell secretion. Firstly, the morphological changes in the ear tissues were evaluated by H&E staining. The ear dermal thickness of induced AD mice were increased, whereas the ear dermal thickness was significantly decreased in the DPL-treated group (Fig. 7A and C). Additionally, the present study measured mast cell secretion by TB staining, which revealed that mast cell secretions were increased in AD mice, whereas mast cell secretions were significantly decreased in the DPL-treated group (Fig. 7B and D). These results indicate that DPL reduces inflammation and mast cell secretions, and suppresses AD symptoms.

Discussion

AD is an inflammatory skin disease that causes symptoms such as severe itching, chronic edema and skin rash. The pathogenesis of this disease has not been elucidated to date, but immunological abnormalities, and environmental and genetic factors are known to be involved (3). Contact with atopic allergens alters the balance of inflammatory cytokines and stimulates B-cells to produce IgE, which mediates the mast cell secretion of histamine in the skin, causing edema, itchy skin, inflammatory reactions and the release of inflammatory mediators such as NF-κB, NO and inflammatory cytokines (4). Immunosuppressive agents such as steroids or antihistamines are used to treat inflammatory skin diseases, but owing to the induced side effects, these agents are inadequate for long-term treatment. In this regard, studies
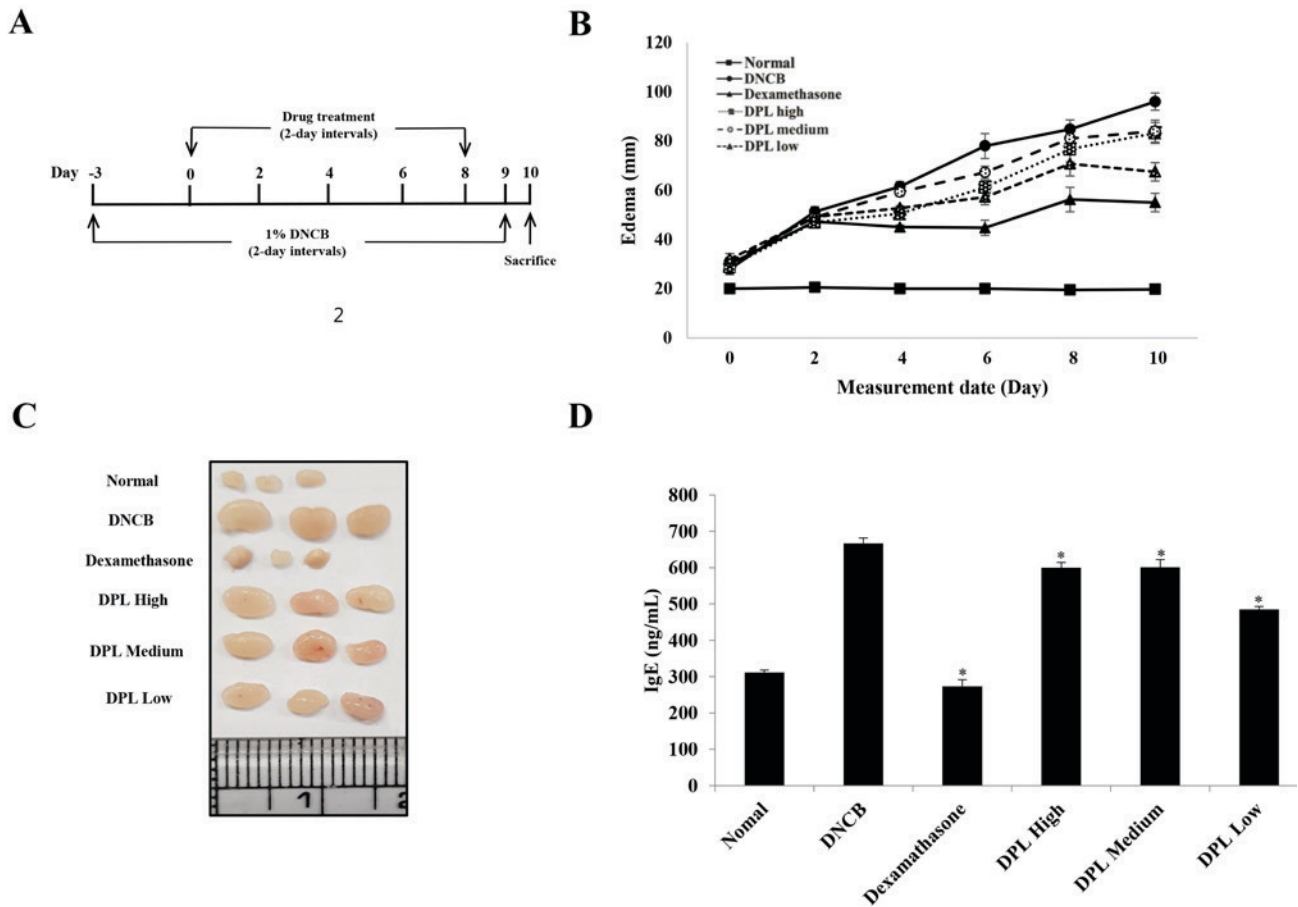
Figure 6. Effects of DPL extract on DNCB-induced AD in BALB/c mice. (A) Schematic diagram presenting the timeline employed for the induction of AD and treatment with DPL extract. (B) Ear thickness during the course of AD. (C) The lymph nodes were photographed to record morphological changes. (D) Levels of serum IgE were measured by ELISA. Blood samples were collected on day 10 post-induction. Data are expressed as the mean ± standard error of the mean. *P<0.05 vs. DNCB. DPL, *Dendropanax morbifera* leaf; LPS, lipopolysaccharide; DNCB, 2,4-dinitrochlorobenzene; AD, atopic dermatitis; IgE, immunoglobulin E.
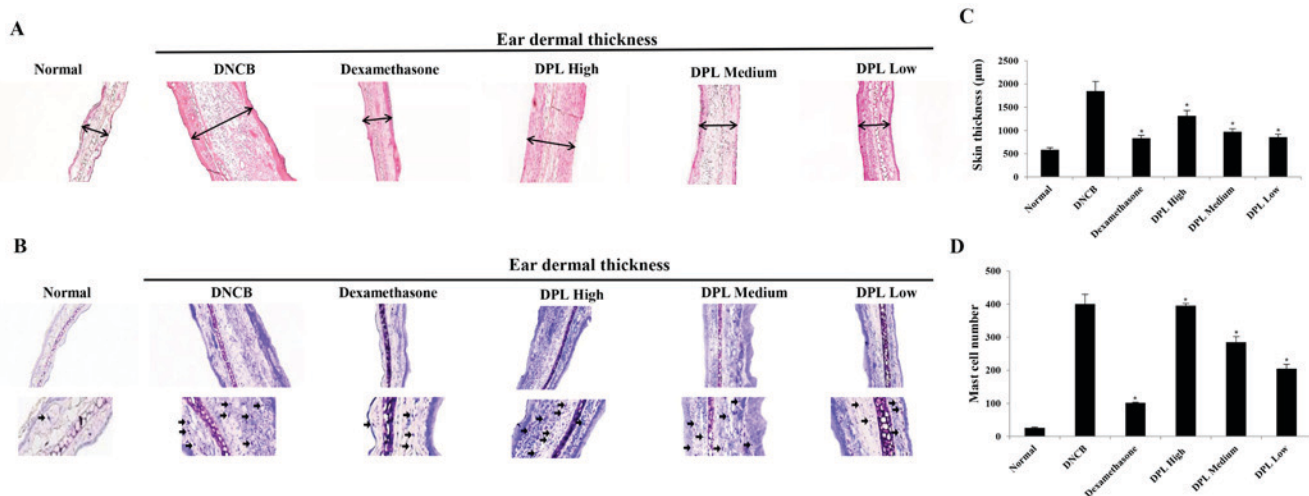

Figure 7. Effects of DPL extract on tissue inflammation and the infiltration of immune cells in atopic dermatitis mice. (A) Hematoxylin and eosin (red) and (B) toluidine blue (blue) staining was performed, and the cells were examined under a light microscope (magnification, x200). The arrows indicate mast cells. (C) Skin thickness and (D) the numbers of mast cells were measured. Data are expressed as the mean ± standard error of the mean. *P<0.05 vs. DNCB. DPL, *Dendropanax morbifera* leaf; LPS, lipopolysaccharide; DNCB, 2,4-dinitrochlorobenzene.

are being conducted to identify potentially safe and effective natural products with no side effects (5,6).

Among such natural sources, *D. morbifera*, which means 'omnipotent drug (Panax)', has been called a panacea, and has

been the subject of various studies investigating medicinal plants (20-24). As a result, 32 different substances have been identified in *D. morbifera*. Extracts from *D. morbifera* leaves are known to activate B- and T-cells (20,23), inhibit the synthesis of melanin, have antioxidant properties, and protect and whiten skin (24). Although *D. morbifera* has exhibited potential anti-inflammatory effects, few studies have been conducted to identify a clear mechanism relevant to AD. The present study investigated the effects of DPL extract on the production of NO, cytokine secretion and inflammation-associated protein expression in activated RAW264.7 macrophages by inducing inflammation with LPS, and investigated its anti-inflammatory effects in DNCB-induced AD.

Firstly, the toxicity of DPL was evaluated in RAW264.7 macrophages by MTT assay. No cytotoxicity was observed at a concentration of 500 μg/ml. This has already been reported (32), and further experiments were conducted at concentrations of 400 μg/ml or less.

NO, a known indicator of the inflammatory response, is synthesized by NOS from L-arginine. There are three types of NOS: Endothelial, neuronal, and iNOS, and among these, NO produced by iNOS serves an important pathological role (13). In the present study, the NO production in LPS-induced RAW264.7 cells was significantly reduced by treatment with different concentrations of DPL. A previous study also demonstrated that NO expression was inhibited in extracts of *D. morbifera* stems (25). These results suggest that DPL extract inhibited the LPS-induced expression of NO in RAW264.7 cells.

iNOS and COX-2 serve important roles in the production of NO, and the regulation of iNOS and COX-2 expression is a known strategy for alleviating inflammatory diseases (14,15). In the present study, western blotting was performed to examine the expression of these proteins. The expression levels of iNOS and COX-2 were significantly increased in LPS-stimulated RAW264.7 cells when compared with untreated cells, whereas in the group treated with 200 and 400 μg/ml DPL the expression levels of iNOS and COX-2 were decreased. In particular, the expression of iNOS was decreased in a concentration-dependent manner. A previous study also reported significant inhibition of COX-2 and iNOS expression, consistent with the results obtained in the present study (33). These results suggest that DPL extracts may regulate the expression of the inflammatory mediators iNOS and COX-2, and regulate the expression of iNOS, thereby suppressing NO production and alleviating the inflammatory response.

The inflammatory cytokines TNF-α and IL-6 serve important roles in the inflammatory process, causing inflammation through trauma and stress, and regulating the expression of inflammatory mediators (34). In the present study, the expression of TNF-α and IL-6 was measured, revealing that TNF-α expression was significantly decreased with high concentrations of DPL, and the expression of IL-6 was decreased in a concentration-dependent manner when compared with RAW264.7 cells stimulated with LPS and DPL extract. Previous studies have also reported significantly reduced IL-6 expression following treatment with different concentrations of *D. morbifera* extract (20,25). These results indicate that DPL extract may inhibit the inflammatory response by regulating the expression of IL-6 in RAW264.7 cells stimulated with LPS.

TNF-α and IL-6 activate inflammation by activating NF-κB attached to IκB-α (11). Western blotting was performed to examine the activation of NF-κB and IκB-α. The induction of p-NF-κB appeared to be increased following treatment with LPS; by contrast, the induction of p-NF-κB was lower in the groups treated with 200 and 400 μg/ml DPL than in the group stimulated with LPS. Yu *et al* (34) demonstrated that olefolioside A, a component of the *D. morbifera* extract, inhibited the activation of NF-κB and IκB-α. These results suggest that DPL inhibited the activation of NF-κB, which induced an inflammatory response in RAW264.7 macrophages stimulated with LPS.

MAPK, a typical signaling molecule that affects the activation of NF-κB, is phosphorylated and activated in cells, and influences various inflammatory mediators. When inflammatory responses are induced, MAPK activators such as P38 and JNK are activated in macrophages (18). In the present study, RAW264.7 cells stimulated with LPS were treated with different concentrations of DPL extract and the activation of ERK, P38, and JNK was assessed. The expression of p-ERK, p-P38 and p-JNK was significantly decreased by 400 μg/ml DPL treatment. A previous study also revealed that *D. morbifera* extract modulates the expression of MAPK (34). These results suggest that DPL extract may modulate the signaling pathway of MAPK and inhibit the inflammatory response in LPS-stimulated macrophages.

BALB/c mice were treated with DNCB, which is known to induce inflammatory dermatitis, to induce AD. DPL extract was then applied to the ear and the thickness of the ear was assessed. Ear thickness was significantly lower in the DPL-treated group than in the group treated with DNCB. The ears in the DPL low-dose group exhibited a larger decrease in thickness than the medium- and high-dose groups. This suggested that the higher the concentration of DPL extract (the closer to undiluted solution), the higher the viscosity, which decreased its penetration; thus, groups treated with lower concentrations of DPL exhibited higher effects owing to the low viscosity. DPL was less effective than dexamethasone, which was used as a positive control. However, the dexamethasone groups observed cutaneous atrophy and erythema. This indicated that DPL may have potential for therapeutic use with fewer side effects (cutaneous atrophy and erythema) than dexamethasone with repeated use. There were significantly fewer morphological changes in the lymph nodes of the DPL-treated group than in the DNCB-treated group, and IgE was significantly decreased in the DPL-treated group. Additionally, morphological changes in the epidermis and corium of ear tissues, and the secretion of immune-activated mast cells were measured by H&E and TB staining. The ears of DNCB-treated mice were thicker than those of DPL-treated mice, and the mast cell distribution was significantly decreased in the DPL-treated group. Lee *et al* (35) reported that *Dendropanax* exhibited anti-inflammatory effects in pneumonia, and Jung *et al* (36) confirmed that *Dendropanax* relieves nephritis. These results suggest that DPL extract alleviated DNCB-induced atopic skin disease.

In conclusion, the present study investigated the anti-inflammatory effects of DPL extract. It was observed that *D. morbifera* regulated the expression of NO and IL-6, and the signaling pathway of NF-κB and MAPK in LPS-stimulated

RAW264.7 macrophages *in vitro*. The present study also confirmed that DNCB-induced inflammatory dermatitis was mitigated by DPL *in vivo*, without side effects. These results suggest that DPL extract merits future research and development as a natural anti-inflammatory or functional drug. In addition, the investigation of the components of *D. morbifera* will be required in the future as well as studying the mechanisms underlying its anti-inflammatory effects.

Acknowledgements

Availability of data and materials

Not applicable.

Authors' contributions

GSC, DPL, SMK, CHK and JYJ conceived and designed the experiments. GSC, ESY, SHK and JSW performed the experiments. GSC, DPL, ESY and JYJ analyzed the data. DPL, CHK, HJK and SMK contributed in analyzing the data and provided reagents and materials. GS wrote the manuscript.

Ethics approval and consent to participate

All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of Kong-Ju National University (approval no. KNU_2017-10; Yesan, Korea) and the institutional guidelines were adhered to.

Patient consent for publication

Not applicable.

Competing interests

The authors Dong-pyo Lim, Sae-man Kim and Chang-hyun Kim are all affiliated with MBG group (Daejeon, Republic of Korea); this company also provided financial support for the present study. The authors declare that they have no competing interests.

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