



Betula platyphylla attenuated mast cell-mediated allergic inflammation in vivo and in vitro

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ABSTRACT

Aims

Betula platyphylla (*B. platyphylla*) has traditionally been used in Korea to treat inflammatory diseases. However, the exact mechanism that accounts for the anti-inflammatory effect of *B. platyphylla* is not completely understood. The aim of the present study is to elucidate whether and how *B. platyphylla* modulates the mast cell-mediated allergy inflammation in vitro and in vivo.

Main methods

We investigated to ascertain the pharmacological effects of *B. platyphylla* on both compound 48/80 or histamine-induced scratching behaviors and 2, 4-dinitrochlorobenzene (DNFB)-induced atopic dermatitis in mice. Additionally, to find a possible explanation for the anti-inflammatory effects of *B. platyphylla*, we evaluated the effects of *B. platyphylla* on the release of histamine in compound 48/80-induced rat peritoneal mast cells (RPMCs), production of inflammatory mediators and activation of nuclear factor- κ B (NF- κ B) and caspase-1 in phorbol 12-myristate 13-acetate plus calcium ionophore A23187 (PMACI)-stimulated human mast cells (HMC-1).

Key findings

The finding of this study demonstrated that *B. platyphylla* reduced compound 48/80 or histamine-induced scratching behaviors and DNFB-induced atopic dermatitis in mice. Additionally, *B. platyphylla* inhibited the release of histamine in RPMC and production of inflammatory cytokines as well as the activation of NF- κ B and caspase-1 in stimulated HMC-1.

Significance

Collectively, the findings of this study provide us with novel insights into the pharmacological actions of *B. platyphylla* as a potential molecule for use in the treatment of allergic inflammatory diseases.

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Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by eczematous inflammation of the skin (Buske-Kirschbaum et al., 2001). The incidence of this disease has increased steadily over recent years. AD is known to be the result of an immune system dysregulation, ultimately resulting in allergic inflammation (Gold and Kemp, 2005).

Mast cells are tissue-based inflammatory cells of bone marrow origin, which respond to the danger signals of innate and acquired immunity with both immediate and delayed releases of inflammatory

mediators. It has been previously reported that mast cells can be found in larger numbers in AD lesional skin. In response to various stimuli, mast cells generate a variety of cytokines, including interleukin (IL)-6, and tumor necrosis factor (TNF)- α (Bradding et al., 1993). TNF- α has an important amplifying effect in asthmatic inflammation and stimulates airway epithelial cells to produce cytokines, including IL-6, IL-8 and GM-CSF (Boero et al., 2010). IL-6 is an important co-factor in IL-4 dependent IgE synthesis. IL-6 is also produced from mast cells and its local accumulation is associated with a local allergic reaction. As the chemokine, IL-8 acts on target cells such as neutrophils, T-lymphocytes, and eosinophils and plays a role in activation of inflammatory effector cells (Sengupta et al., 2004). The release of these cytokines may be of major importance in the development of a variety of inflammatory skin disorders (Trefzer et al., 2003). Therefore, the inhibition of cytokine secretion can aid in the development

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of a useful therapeutic strategy for allergic inflammatory diseases such as AD.

Nuclear factor-kappa B (NF- κ B) performs a crucial function in the expression of many of the genes involved in immune and inflammatory responses (Tegeder et al., 2001). In the nucleus, NF- κ B activates gene transcription; thus, NF- κ B performs a pivotal function in the regulation of immune and inflammatory responses, occurring via the control of the transcription of inflammatory cytokine genes (Gadaleta et al., 2011). An increase in NF- κ B activity associated with the secretion of high levels of IL-6 and TNF- α has also been noted in the context of allergic inflammatory responses (Mukaida, 2000). The results of those studies demonstrated that NF- κ B activation and the subsequent activation of pro-inflammatory cytokine gene expression are critically important in the initiation and perpetuation of allergic inflammation.

Caspase-1 is a member of a family of caspases with large prodomains, and its activation is involved in both apoptosis and inflammation (Lee et al., 2001; Wang et al., 2005). Specific three adaptors have been reported to regulate the activation of caspase-1. They are receptor interacting protein (RIP)-2, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), a PYRIN-caspase recruitment domain (CARD) protein (Srinivasula et al., 2002; Stehlik et al., 2003; Mariathasan et al., 2004), and Ice-protease activating factor (Ipaf) (Poyet et al., 2001). Rip2, ASC, and Ipaf are all CARD-containing proteins; their CARD domains bind to the CARD domain of caspase-1 prodomain via CARD–CARD interactions, induce caspase-1 oligomerization, and promote caspase-1 activation. Activation of caspase-1 induces inflammatory response via the production of pro-inflammatory cytokines and the recruitment of neutrophils (Faubel et al., 2007). These results have implicated caspase-1 activation as an attractive target for the treatment of inflammatory diseases.

Traditional medicine has been the subject of increased interest for its potential in the treatment of inflammation. Although traditional herbal medicines have long been used effectively in treating diseases, the pharmacologic mechanisms of most herbal medicines have not been elucidated. *Betula platyphylla* (*B. platyphylla*) has been employed as a natural drug in Asia and specifically in Korea. Recently, several studies have shown that *B. platyphylla* have an anti-oxidant effect in lung fibroblast cells and an anti-cancer effect in human promyelocytic leukemia (Ju et al., 2004). *B. platyphylla* inhibited the development of AD-like skin lesions in NC/Nga mice induced by picryl chloride (Kim et al., 2008). However, the precise molecular mechanisms of *B. platyphylla* on mast cell-mediated allergic inflammation have yet to be clearly elucidated.

In this study, we attempted to ascertain the mechanisms underlying the pharmacological effects of *B. platyphylla* on both compound 48/80 or histamine-induced scratching behaviors and 2, 4-dinitrochlorobenzene (DNCB)-induced atopic dermatitis in mice. Additionally, to find a possible explanation for the anti-inflammatory mechanisms of *B. platyphylla*, we evaluated the effects of *B. platyphylla* on the release of histamine in compound 48/80-induced rat peritoneal mast cells (RPMCs) and production of inflammatory cytokines and activation of NF- κ B and caspase-1 in phorbol 12-myristate 13-acetate plus calcium ionophore A23187 (PMA/CI)-stimulated human mast cells (HMC-1).

Materials and methods

Reagents

Compound 48/80, PMA, calcium ionophore A23187, avidin peroxidase (AP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DNCB were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Iscove's Modified Dulbecco's Media (IMDM) was purchased from Gibco BRL (Grand Island, NY). Fetal bovine serum

(FBS) was purchased from JR Scientific, Inc. (Woodland, CA, USA). Anti-human TNF- α /IL-6/IL-8, recombinant TNF- α /IL-6/IL-8, biotinylated TNF- α /IL-6/IL-8, anti-mouse IgE, recombinant IgE and biotinylated IgE were purchased from Pharmingen (San Diego, CA, USA). NF- κ B, and histone antibodies (Abs) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Animals

The original stock of male ICR mice (5 weeks, 25–30 g), BALB/c mice (5 weeks, 19–20 g) and SD rats (7 weeks, 250–300 g) was purchased from Orient Co., Ltd, a branch of Charles River Laboratories (Seoul, Korea). Animals were housed 10 per cage, allowed access to water and food ad libitum, and maintained at a constant temperature ($24 \pm 1^\circ\text{C}$) and humidity ($60 \pm 10\%$) under a 12-h light/dark cycle (light on 08:00:20:00 h). The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in Daegu Haany University guidelines.

Preparation of *B. platyphylla*

The dried *B. platyphylla* were purchased from the Human herb (Gyeongbuk, Korea). The roots (100 g) were chopped using a blender with 1 L of 70% ethanol solution under room temperature for 24 h and then concentrated under a vacuum. Then the extract solution obtained was filtered, concentrated on a water bath under vacuo, frozen and lyophilized to yield ethanol extracts (yield: 10.7%). Dilutions were made in saline and filtered through 0.22- μm syringe filter.

Scratching behavioral experiment

Before the experiment, the ICR mice ($n=6$) were put into acrylic cages ($22 \times 22 \times 24$ cm) for about 30 min for acclimation. The behavioral experiments were performed according to the method of Sugimoto et al. (1998). The rostral part of the skin on the back of mice was clipped, and compound 48/80 (50 $\mu\text{g}/\text{kg}$) or histamine (100 $\mu\text{g}/\text{kg}$) for each mouse was intradermally injected. The scratching agents were dissolved in tween 80 and then used. Control mice received a tween 80 injection in place of the scratching agent. Immediately after the intradermal injection, the mice (one animal/cage) were put back into the same cage for the observation of scratching. Scratching of the injected site by the hind paws was counted and compared with that of the other sites, such as the ears. Each mouse was used for only one experiment. The mice generally showed several scratches for 1 s, and a series of these behaviors was counted as one incident of scratching for 30 min. *B. platyphylla* (400 mg/kg) was orally administered 1 h before the scratching agents.

DNCB-induced atopic dermatitis

Experiments were conducted in accordance with a previously described protocol (Gao et al., 2005). The dorsal skin of the BALB/c mice ($n=6$) was shaved and treated with a depilatory prior to the experiment. The mice were sensitized with 100 μl of 0.15% DNCB in acetone-olive oil (3:1) applied to the dorsal skin twice per week for 5 weeks. Control mice received vehicle (acetone/olive oil = 3:1). After 3 weeks, *B. platyphylla* (400 mg/kg) was orally administered 2 weeks until the end of the experiment.

Preparation of RPMC

RPMCs were isolated as previously described (Hong et al., 2001). In brief, rats were anesthetized by ether and injected with 30 ml of Tyrode buffer B (137 mM NaCl, 5.6 mM glucose, 12 mM NaHCO_3 , 2.7 mM KCl, and 0.3 mM Na_2HPO_4) containing 0.1% gelatin into the

peritoneal cavity, and the abdomen was gently massaged for about 2 min. The peritoneal cavity was carefully opened, and the fluid containing peritoneal cells were aspirated with Pasteur pipette. Thereafter, peritoneal cells were sedimented at $150\times g$ for 10 min at room temperature and resuspended in Tyrode buffer B. To separate mast cells from the major components of rat peritoneal cells, i.e., macrophages and small lymphocytes, suspended peritoneal cells were layered on 2 ml of percoll and centrifuged at $400\times g$ for 10 min at room temperature. The cells in the pellet were washed and resuspended in 1 ml of Tyrode buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl_2 , 1 mM MgCl_2 , 5.6 mM glucose, and 0.1% bovine serum albumin) containing calcium. Mast cells were about 95% pure as assessed by toluidine blue staining.

Histamine assay

RPMC suspensions (2×10^5 cells/ml) were incubated for 15 min at 37°C before the addition of compound 48/80 for stabilization. The cells were incubated with the *B. platyphylla* (1 mg/ml) for 1 h, and then incubated for 30 min with compound 48/80 (6 $\mu\text{g}/\text{ml}$). The reaction was stopped by cooling the tubes in ice. The cells were separated from the released histamine by centrifugation at $400\times g$ for 5 min at 4°C . The histamine levels were measured by ELISA using a histamine assay kit (Stressgen Biotechnologies, USA) according to the manufacturer's directions. Duplicate aliquots of supernatant were measured for each sample.

Cell culture

Human mast cell line, HMC-1, cells were grown in IMDM medium supplemented with 100 IU/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 10% heat-inactivated FBS at 37°C in 5% CO_2 .

MTT assay

To test the cell viability by each concentration of *B. platyphylla*, the MTT colorimetric assay was performed. Briefly, HMC-1 cells (3×10^5 cells/well) were incubated with *B. platyphylla* (0.01–1 mg/ml) for 12 h. After the addition of MTT solution, the cells were incubated at 37°C for 4 h. The crystallized MTT (formazan) was dissolved in dimethyl sulfoxide and the absorbance was measured at 540 nm.

Assay of cytokines

TNF- α , IL-6, and IL-8 secretions were measured by modification of an enzyme-linked immunosorbent assay (ELISA). 96 well plates were coated with 100 μl aliquots of anti-human TNF- α , IL-6, and IL-8 monoclonal Abs at 1.0 $\mu\text{g}/\text{ml}$ in PBS at pH 7.4 and were incubated overnight at 4°C . After additional washes, 100 μl of cell medium or TNF- α , IL-6, and IL-8 standards were added and incubated at 37°C for 2 h. After the 2 h incubation at 37°C , the wells were washed and then 0.2 $\mu\text{g}/\text{ml}$ of biotinylated anti-human TNF- α , IL-6, and IL-8 was added and again incubated at 37°C for 2 h. After washing the wells, AP was added and plates were incubated for 30 min at 37°C . Wells were again washed and ABTS substrate was added. Color development was measured at 405 nm using an automated microplate ELISA reader. A standard curve was run on each assay plate using recombinant TNF- α , IL-6, and IL-8 in serial dilutions.

Preparation of cytoplasmic and nuclear extract

Nuclear and cytoplasmic extracts were prepared as described previously (Schoonbroodt et al., 1997). Briefly, after the cells were activated with PMACI and then washed with ice-cold phosphate-buffered saline (PBS). These cells were resuspended in 60 μl of buffer A (10 mM HEPES/KOH, 2 mM MgCl_2 , 0.1 mM EDTA, 10 mM KCl, 1 mM

DTT, and 0.5 mM PMSF, pH 7.9). The cells were allowed to swell on ice for 15 min, lysed gently with 2.5 μl of 10% Nonide P (NP)-40, and centrifuged at 2000 g for 10 min at 4°C . The supernatant was collected and used as the cytoplasmic extracts. The nuclei pellet was resuspended in 40 μl of buffer B (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, and 0.5 mM PMSF, pH 7.9), left on ice for 20 min, inverted and the nuclear debris was spun down at 15,000 g for 15 min to remove nuclear debris. The supernatant (nuclear extract) was collected, frozen in liquid nitrogen and stored at -70°C until ready for analysis.

Western blot analysis

The protein was mixed with SDS sample buffer, boiled for 5 min and then separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were blocked with 10% skim milk for 1 h, washed with PBST/0.1% Tween 20 and incubated 1 h with primary antibodies. After three washes in PBST/0.1% Tween 20 for 30 min, the membranes were incubated for 30 min with secondary Abs. After three washes in PBST/0.1% Tween 20 for 30 min, the protein bands were visualized by an enhanced chemiluminescence detection system according to the recommended procedure (Amersham Corp. Newark). The quantity of protein was evaluated by using a bicinchoninic acid (BCA) protein assay (Sigma, St. Louis, MO, USA).

NF- κB immunofluorescence

Cells were fixed with 4% paraformaldehyde and incubated with 5% BSA in PBS for 60 min. The preparation was incubated for 1 h at room temperature with NF- κB Abs diluted in 0.1% BSA (1:200). Next, the preparation was washed 3 times with PBS and exposed to the secondary Abs (fluorescein isothiocyanate-conjugated anti-rabbit IgG at 1:200 and 0.1% BSA/PBS) for 60 min. The fluorescent image was viewed using an Olympus confocal microscope (New Hyde Park, NY, USA).

Assay of caspase-1 activity

The enzymatic activity of caspase-1 was assayed using a caspase colorimetric assay kit (R&D System Inc., MN, USA) according to the manufacturer's protocol. The lysed cells were centrifuged at 14,000 rpm for 5 min. The protein supernatant was incubated with 50 μl reaction buffer and 5 μl caspase substrate at 37°C for 2 h. The absorbance was measured using a plate reader at a wavelength of 405 nm. Equal amounts of the total protein from each lysate were quantified using a BCA protein assay.

Statistical analysis

The experiments show a summary of the data from at least-three experiments and presented as the mean $\pm$ S.E.M. Statistical evaluation of the results was performed by independent *t*-test. For all experiments, $P<0.05$ was considered significant.

Results

Effect of *B. platyphylla* on scratching behaviors in mice

The anti-pruritic effects of *B. platyphylla* were investigated on the compound 48/80-induced scratching behavior animal model. When the *B. platyphylla* was orally administered 1 h before compound 48/80 injections, the scratching behaviors were reduced. The inhibition rate of *B. platyphylla* (400 mg/kg) was approximately 41.21% (Fig. 1A). Terfenadine was used as a positive control in this study. In addition, we investigated the contribution of *B. platyphylla* in

histamine-induced scratching behavior. As shown in Fig. 2, orally administered *B. platyphylla* inhibited the scratching behaviors by 47.31% (Fig. 1B).

Effect of *B. platyphylla* on DNCB-induced atopic dermatitis and IgE levels in serum

In order to evaluate the regulatory effects of *B. platyphylla* in an atopic dermatitis in vivo model, DNCB was administered to BALB/c mice. As shown in Fig. 2A, when the mice were treated for 2 weeks with *B. platyphylla*, the atopic dermatitis was recovered to a significant extent. To evaluate the effects of *B. platyphylla* on IgE levels in serum, blood samples were collected. The levels of IgE were measured via ELISA. The results showed that IgE levels were increased as the result of DNFB exposure, but this phenomenon was significantly reduced in the *B. platyphylla* group (Fig. 2B).

Effects of *B. platyphylla* on compound 48/80-induced histamine release from RPMC

To investigate the regulatory effects of *B. platyphylla* on histamine release from RPMC, we measured histamine levels using the

histamine assay kit. Compound 48/80 induced the histamine release from RPMC. However, *B. platyphylla* (1 mg/ml) significantly inhibited the histamine release. The inhibition rate reached up to 38.41% (Fig. 3).

Effect of *B. platyphylla* on the inflammatory cytokine production in PMACI-stimulated HMC-1 cells

In an effort to determine the molecular mechanism of *B. platyphylla*, the human mast cell line, HMC-1, was employed in this study. We determined whether *B. platyphylla* modulates the PMACI-induced production of TNF- α , IL-6, and IL-8. The levels of TNF- α , IL-6 and IL-8 in culture supernatants were measured via ELISA. As is shown in Fig. 4, the production of TNF- α , IL-6, and IL-8 in response to PMACI was inhibited as the result of pre-treatment with *B. platyphylla* in a dose-dependent manner. The maximal rates of TNF- α , IL-8, and IL-6 inhibition by *B. platyphylla* (1 mg/ml) were approximately $50.52 \pm 4.3\%$, $35.33 \pm 4.1\%$, and $48.64 \pm 3.7\%$, respectively. Additionally, we observed that *B. platyphylla* did not affect cell viability (data not shown).

Effect of *B. platyphylla* on NF- κ B activation in the nuclei of PMACI-stimulated HMC-1 cells

As the suppression of NF- κ B activation has been linked with anti-inflammation, we theorized that the effects of *B. platyphylla* might be mediated, at least in part, via the suppression of NF- κ B activation. Additionally, because NF- κ B activation requires the nuclear translocation of the RelA/p65 subunit of NF- κ B, we evaluated the effects of *B. platyphylla* on the nuclear pool of RelA/p65 protein via Western blot analysis. In PMACI-stimulated cells, the levels of Rel/p65 were increased, but *B. platyphylla* reduced these enhanced nuclear levels of Rel/p65 (Fig. 5A). The relative levels of NF- κ B (in nucleus) were represented in Fig. 5B. Fig. 5C displays the results of immunofluorescence staining for the distribution of NF- κ B (red). PMACI induced translocation of NF- κ B into the nucleus, but *B. platyphylla* inhibited this phenomenon.

Effect of *B. platyphylla* on caspase-1 activation in PMACI-stimulated HMC-1 cells

Activation of caspase-1 induces inflammatory response via stimulation of inflammatory cytokine. In order to determine the regulatory mechanism of *B. platyphylla* on allergic inflammation, we evaluated the effects of *B. platyphylla* on PMACI-induced caspase-1 activation. We showed that the enhanced caspase-1 activity induced by PMACI was significantly reduced by *B. platyphylla* in a dose-dependent manner (Fig. 6). The maximal inhibition rates of *B. platyphylla* (1 mg/ml) were approximately $30.11 \pm 2.1\%$.

Discussion

Recently, herbal medicine has been the subject of increased interest for its potential in the treatment of inflammation. However, the precise pharmacologic mechanisms of most herbal medicines have not been elucidated. In this study, we demonstrated the molecular mechanisms of *B. platyphylla* on mast cell-mediated allergic inflammation in vivo and in vitro. The findings of this study show that *B. platyphylla* attenuated the compound 48/80 or histamine-induced scratching behaviors and inhibited DNCB-induced atopic dermatitis under in vivo conditions. Additionally, *B. platyphylla* inhibited the release of histamine from RPMC and production of TNF- α , IL-6, and IL-8, and also inhibited the activation of NF- κ B and caspase-1 in PMACI-stimulated HMC-1 cells.

AD is a chronic inflammatory skin disease and is characterized by erythema, edema, and scaling (Leung and Bieber, 2003). Generally,

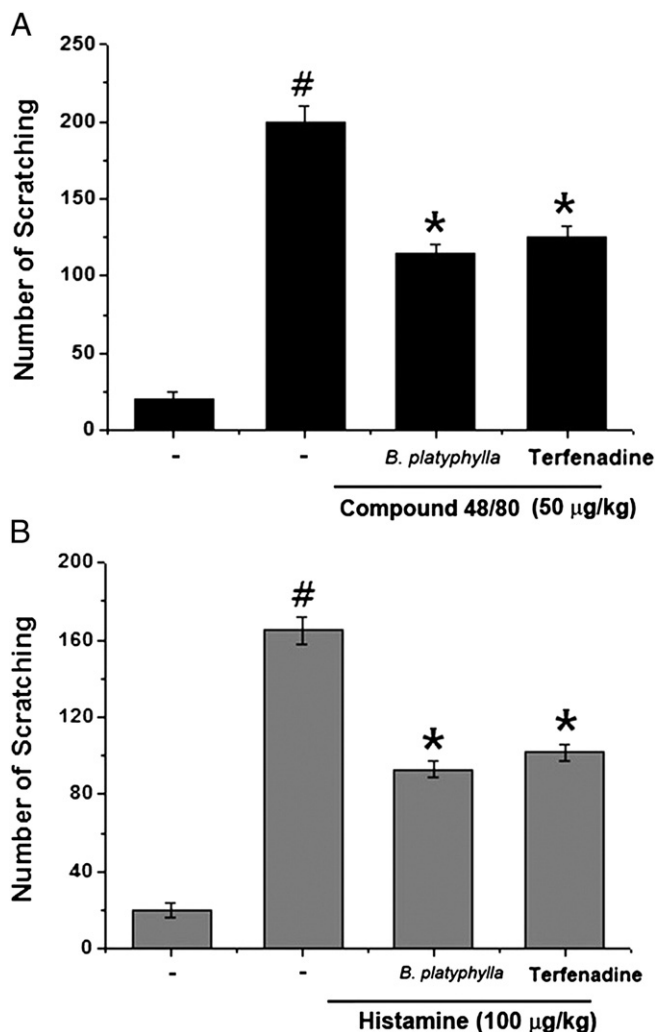


Fig. 1. The effect of *B. platyphylla* on scratching behavior in ICR mice. *B. platyphylla* (400 mg/kg) was orally administered 1 h before compound 48/80 (50 µg/kg) or histamine (100 µg/kg) intradermal injection. Scratching behavior was counted as one incident of scratching for 30 min. Each datum represents the means $\pm$ S.E.M. of three independent experiments. ([#] $P < 0.05$ vs. control group, ^{*} $P < 0.05$ vs. compound 48/80 or histamine-treated group).

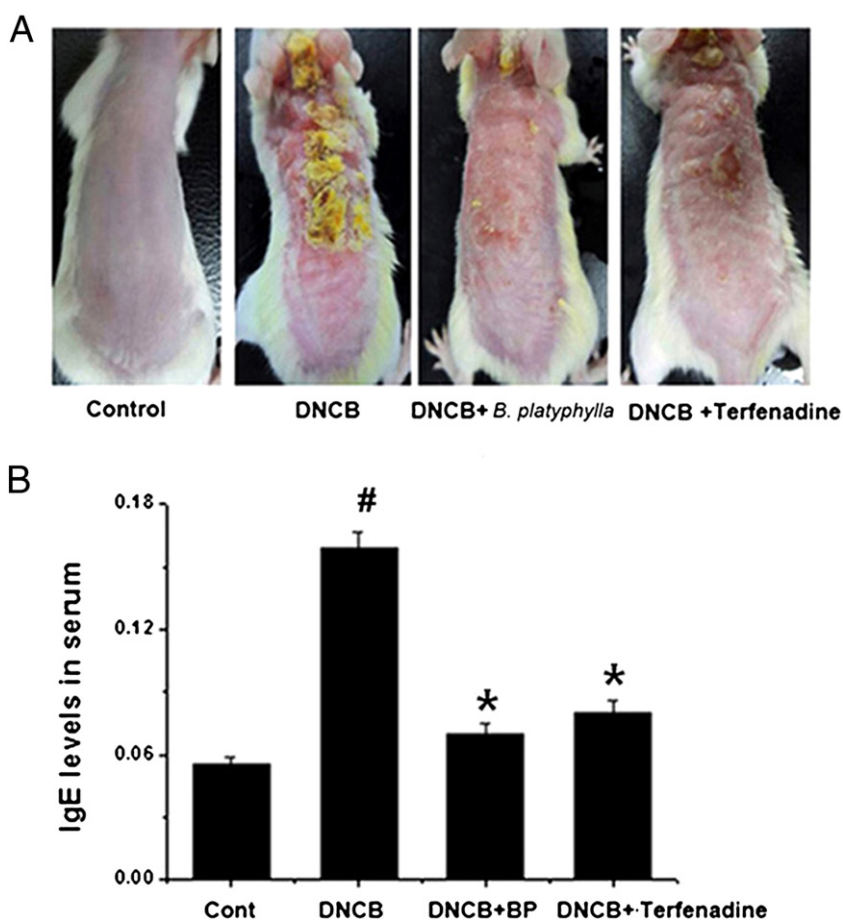


Fig. 2. The effect of *B. platyphylla* on the DNCB-induced dermatitis and serum IgE level. (A) The BALB/c mice ($n=6$) were sensitized with 100 μ l of 0.1% DNCB in acetone–olive oil (3:1) or vehicle (acetone/olive oil = 3:1) applied to the dorsal skin twice each week for a total period of 5 weeks. After 3 weeks, *B. platyphylla* (400 mg/kg) was orally administered 2 weeks prior to the end of the experiment. (B) Blood samples were collected and then levels of serum IgE in the indicated groups were measured using the ELISA method (* $P<0.05$ vs. control group, # $P<0.05$ vs. DNCB-treated group).

steroid therapy is a crucial factor in the treatment of AD, but it cannot be administered over the long-term, owing to its deleterious side-effects. Therefore, several researchers have attempted to find a new drug, which is effective in the treatment of AD (Shiohara et al., 2004). The AD was characterized by a potent skin inflammation associated with an elevated level of IgE against many types of allergens (Allam and Novak, 2006; Brenninkmeijer et al., 2008). On the base

of these studies, we have focused to evaluate the effects of *B. platyphylla* on DNCB-induced allergic reactions in vivo. The findings of this study revealed that *B. platyphylla* significantly reduced DNCB-induced atopic dermatitis. Additionally, *B. platyphylla* caused a reduction in IgE levels in serum induced by DNCB. These results demonstrate *B. platyphylla*'s potential effect on anti-allergic responses via the regulation of IgE levels.

In pathological skin conditions, histamine is involved in the induction of itching and edema (Minami and Kamei, 2004). This study focused on the manner in which *B. platyphylla* regulate the levels of histamine from mast cells and histamine-induced scratching behaviors in mice. We showed that *B. platyphylla* inhibited the histamine release from RPMCs and histamine or compound 48/80-induced scratching behaviors in mice.

To gain further insights into the mechanisms of *B. platyphylla*-mediated inhibition of PMACI-induced inflammatory mediators (TNF- α , IL-6 and IL-8), we examined the regulatory effect of *B. platyphylla* on intracellular signaling molecules involved in the PMACI signaling pathways in HMC-1. Mast cells play differential roles in the inflammation by initiating and regulating immune responses by the release of various cytokines and chemokines via differential intracellular signal transduction pathways (Harvima and Nilsson, 2011). HMC-1 cell line was a useful cell for studying cytokine activation pathways (Sillaber et al., 1993). In response to different stimulations, mast cells release an array of cytokines especially TNF- α , IL-6 and IL-8 with the potential to cause inflammation (Galli et al., 2005). Therefore, the development of new biological therapies for inflammatory diseases has generally focused on the blockage of

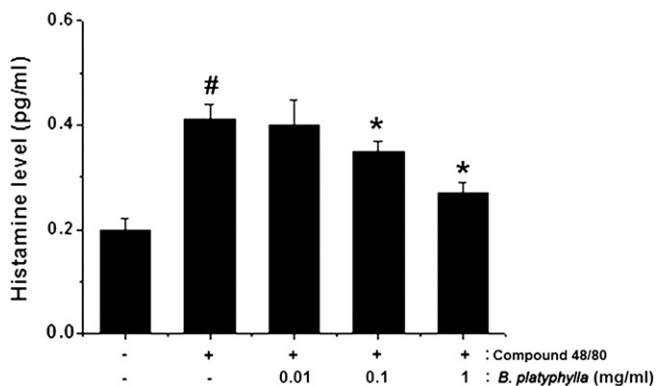


Fig. 3. The effect of *B. platyphylla* on histamine release from RPMCs. RPMCs (2×10^5 cells) were pretreated with various concentrations of *B. platyphylla* (0.01–1 mg/ml) for 1 h and then treated with compound 48/80. The histamine content was measured by the histamine assay kit as described under the Materials and methods section. All data were represented in the mean $\pm$ S.E.M. of triplicate determinations from triplicate separate experiments (* $p<0.05$ vs. control, # $p<0.05$ vs. compound 48/80 alone).

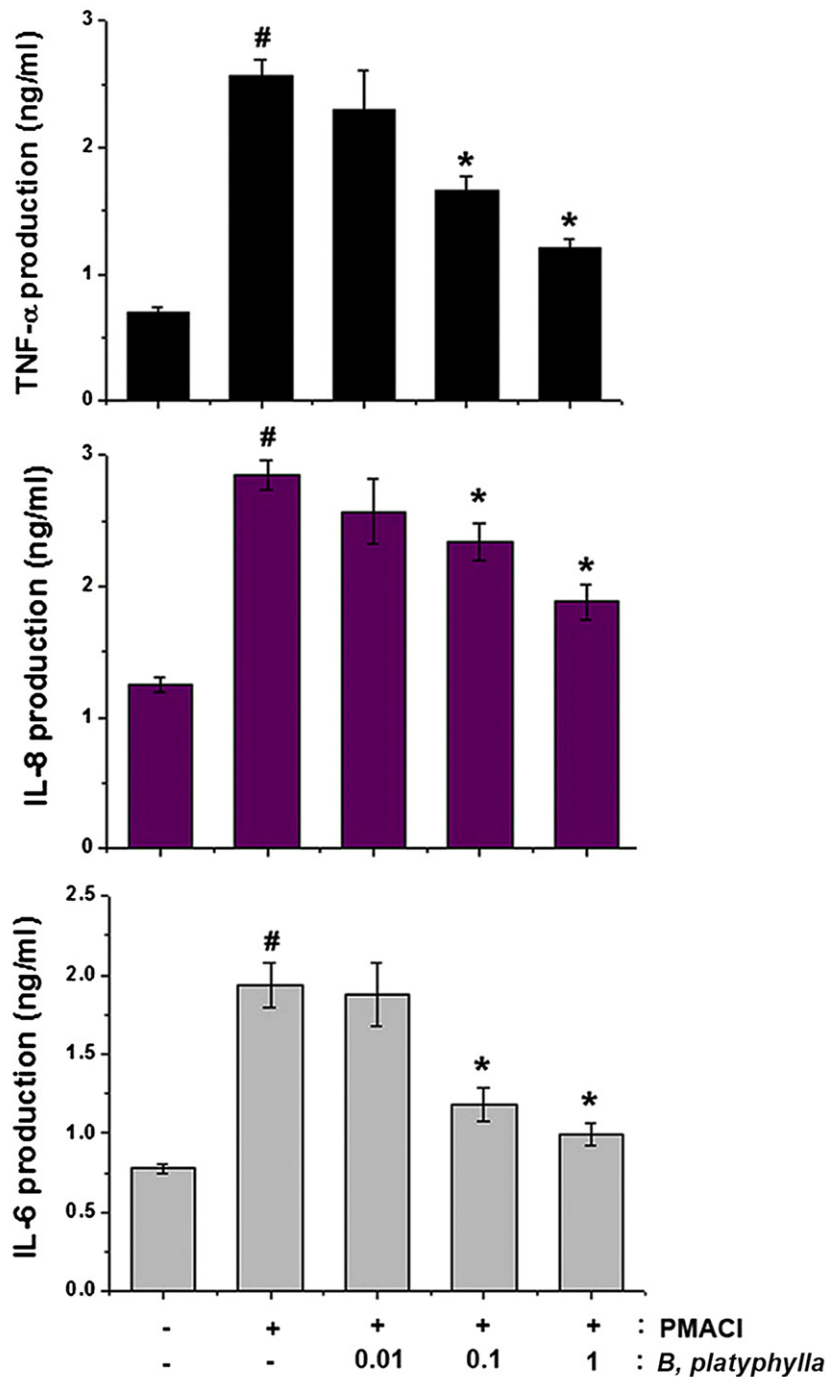


Fig. 4. The effects of *B. platyphylla* on the production of inflammatory cytokines in PMACI-stimulated HMC-1 cells. HMC-1 cells were pre-treated with *B. platyphylla* (0.01–1 mg/ml) for 1 h and then stimulated with PMACI for 12 h. The levels of inflammatory cytokines (TNF- α , IL-8 and IL-6) were measured from cell supernatant using ELISA. All data were represented in the mean $\pm$ S.E.M. of triplicate determinations from triplicate separate experiments ([#] $p < 0.05$ vs. control, ^{*} $p < 0.05$ vs. PMACI alone).

members of the inflammatory cascade, such as cytokines. Cyclosporin A has been employed previously in the treatment of atopic dermatitis, owing to the suppression of IL-6 and IL-8 production noted in cases of severe pediatric atopic dermatitis (Bunikowski et al., 2001). In this research, we demonstrated that *B. platyphylla* inhibited the secretion of TNF- α , IL-6, and IL-8 in PMACI-stimulated HMC-1 cells. The maximal rates of TNF- α , IL-8, and IL-6 inhibition by *B. platyphylla* (1.0 mg/ml) were approximately $50.52 \pm 4.3\%$, $35.33 \pm 4.1\%$, and $48.64 \pm 3.7\%$, respectively. These results demonstrate that *B. platyphylla* exerts an anti-inflammatory effect via the regulation of inflammatory cytokine production.

This cytokine production is associated with increased activation of the gene transcription regulators NF- κ B (Gilmore and Garbati, 2011). After a variety of stimuli, the I κ B proteins are phosphorylated, and degraded, allowing for NF- κ B to translocate into the nucleus where it can bind specific DNA sequences located in the promoter regions of target genes and activate gene transcription, thereby indicating its pivotal function in the regulation of inflammatory responses, via the control of the transcription of inflammatory cytokine genes. From this, inhibition of NF- κ B activation has been suggested as an anti-inflammatory strategy in AD. Therefore, we attempted to determine whether the anti-inflammatory effect of *B. platyphylla* is through

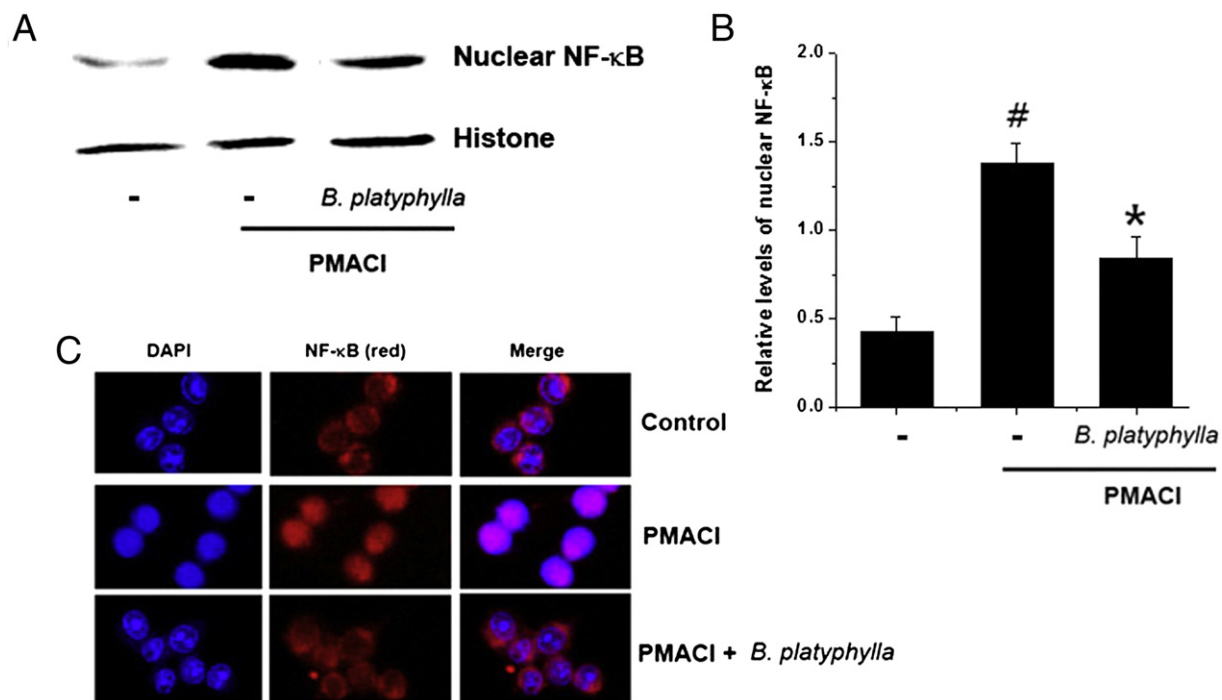


Fig. 5. The effect of *B. platyphylla* on the NF-κB activation in the nuclei of PMACI-stimulated HMC-1 cells. HMC-1 cells were pre-treated with *B. platyphylla* (1 mg/ml) for 1 h and then stimulated with PMACI for 2 h. (A) Nuclear extracts were prepared as described in the Materials and methods section and evaluated for RelA/p65 via Western blot analysis. (B) The relative levels of NF-κB were represented. (C) The cells were fixed, and stained with TRITC-conjugated phalloidin (NF-κB, red) and DAPI (nuclear, blue), and examined under an Olympus microscope (Magnification $\times 100$). All data were represented in the mean $\pm$ S.E.M. of triplicate determinations from triplicate separate experiments ($^{\#}p < 0.05$ vs. control, $^{*}p < 0.05$ vs. PMACI alone).

the regulation of NF-κB activation. The results demonstrated that *B. platyphylla* inhibited the NF-κB translocation into nucleus in stimulated HMC-1 cells. Additionally, we confirmed the effect of *B. platyphylla* on NF-κB translocation into nucleus via immunofluorescence staining for the distribution of NF-κB. Therefore, we hypothesized that *B. platyphylla* might exert anti-inflammatory effects via NF-κB activation. Although *B. platyphylla* attenuated the activation of NF-κB, the effect of *B. platyphylla* on the pathways involving NF-κB (phosphorylation of IκB-α and IKK activation) was not determined. Therefore, further studies will be necessary in order to clarify more precisely the role of *B. platyphylla* on the NF-κB pathway in mast-cell mediated inflammation.

Caspase-1 is involved in apoptosis and inflammation. Caspase-1 activation regulates inflammation via the production of inflammatory cytokines and the recruitment of neutrophils. It has also been reported previously that caspase-1^{-/-} mice exhibit reduced IL-6 production (Kuida et al., 1995). RIP-2 is a CARD-containing protein and regulates caspase-1 activation. CARD domain of RIP-2 binds to the CARD domain of caspase-1 via CARD-CARD interactions, which induce caspase-1 activation. This interaction of RIP-2/caspase-1 induced IKK phosphorylation and IκB-α degradation. Thus, released NF-κB translocates to the nucleus, where it induces gene transcription (Lamkanfi et al., 2004). It was reported that complex of caspase-1/RIP2 activates NF-κB and MAPK-signaling pathways leading to

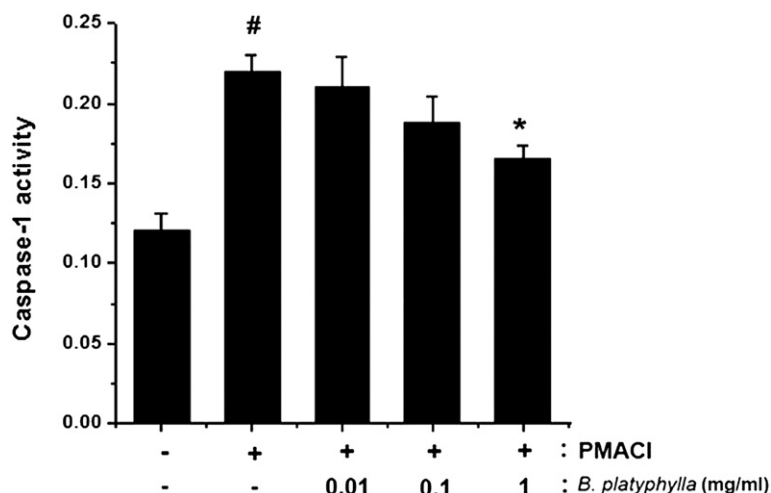


Fig. 6. The effect of *B. platyphylla* on caspase-1 activation in PMACI-stimulated HMC-1. The cells were pretreated with *B. platyphylla* (0.01–1 mg/ml) for 1 h prior to PMACI stimulation for 4 h. The enzymatic activity of caspase-1 was tested by a caspase-1 colorimetric assay. All data were represented in the mean $\pm$ S.E.M. of triplicate determinations from triplicate separate experiments ($^{\#}p < 0.05$ vs. control, $^{*}p < 0.05$ vs. PMACI alone).

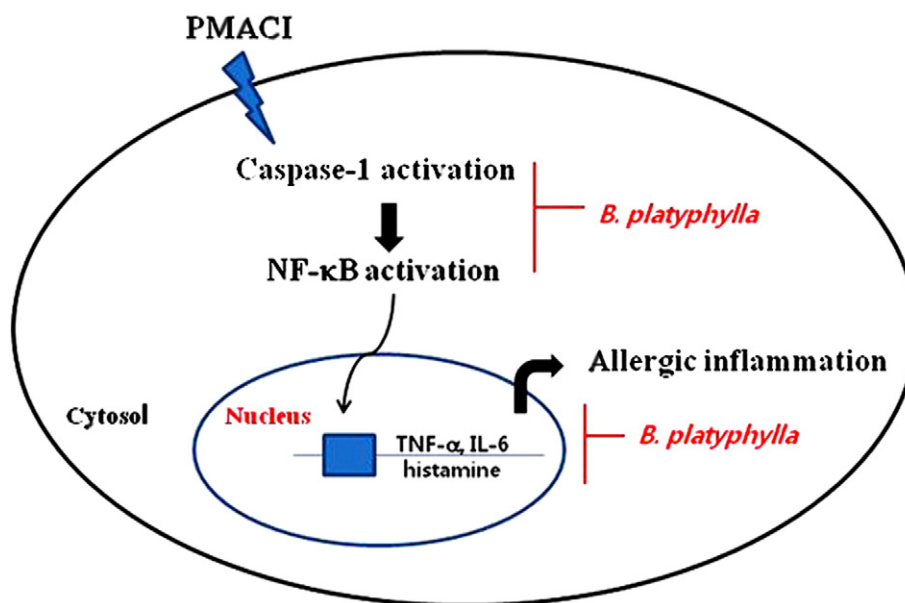


Fig. 7. Proposed anti-inflammatory mechanism of *B. platyphylla* in PMACI-stimulated HMC-1.

activation of JNK, ERK2 and p38 (McCarthy et al., 1998; Navas et al., 1999). Therefore, we postulated that *B. platyphylla* mediates its effects at least partly through suppression of caspase-1 activation. In this research, we noted that *B. platyphylla* suppressed the PMACI-induced activation of caspase-1. This finding demonstrated that the inhibitory effects of *B. platyphylla* on mast cell-mediated inflammation might derive from the regulation of caspase-1 activation. A model of the anti-inflammatory mechanism of *B. platyphylla* is provided in Fig. 7. These results suggested that down-regulation of caspase-1 by *B. platyphylla* might reduced NF-κB activation, and then ultimately suppressed the levels of inflammatory cytokines.

In conclusion, *B. platyphylla* can regulate the allergy response in vivo, including compound 48/80 or histamine-induced scratching behaviors and DNCB-induced atopic dermatitis. Additionally, we demonstrated in this study that the anti-inflammatory activities of *B. platyphylla* could be attributed, at least in part, to the inhibition of histamine and inflammatory cytokine production (TNF-α, IL-8, and IL-6). These effects of *B. platyphylla* are caused by the inhibition of PMACI-induced the activation of NF-κB and caspase-1 activation in mast cells. These results provide experimental evidence demonstrating that *B. platyphylla* may prove useful in the treatment of allergic inflammatory diseases.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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