



Anti-allergic and anti-inflammatory effects of bakkenolide B isolated from *Petasites japonicus* leaves



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ABSTRACT

Aims of the study: To elucidate the anti-allergic and anti-inflammatory effects of *Petasites* genus, we studied the effects of several compounds isolated from *Petasites japonicus* leaves.

Materials and methods: Bakkenolide B was isolated from *Petasites japonicus* leaves. Antigen-induced degranulation was measured in RBL-2H3 mast cells by measuring β -hexosamidase activity. Induction of inducible nitric oxide synthase and cyclooxygenase 2 was measured by Western blotting in peritoneal macrophages. Ovalbumin-induced asthma model was used for in vivo efficacy test of bakkenolide B.

Results: We found that bakkenolide B, a major component of the leaves, concentration-dependently inhibited RBL-2H3 mast cell degranulation. Bakkenolide B also inhibited the gene inductions of inducible nitric oxide synthase and cyclooxygenase 2 in mouse peritoneal macrophages. Furthermore, in an ovalbumin-induced asthma model, bakkenolide B strongly inhibited the accumulation of eosinophils, macrophages, and lymphocytes to bronchoalveolar lavage fluid.

Conclusion: Bakkenolide B has suppressive properties for allergic and inflammatory responses and may be utilized as a potent agent for the treatment of asthma.

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1. Introduction

Extracts from *Petasites* genus have been shown to have anti-allergic and anti-inflammatory effects (Fiebich et al., 2005; Lee et al., 2011; Zhang et al., 2011). Furthermore, lipophilic extracts of the rhizomes of *Petasites hybridus* L., Asteraceae (the butterbur) have been used to treat asthma (Danesch, 2004; Brattstrom et al., 2010). Extracts of the aerial parts of *Petasites japonicus* have been shown to have a type I anti-allergic property and to inhibit mast cell degranulation (Shimoda et al., 2006) and fukinolic acid, chlorogenic acid, caffeic acid and other components have been identified in the extracts (Shimoda et al., 2006). Pharmacological studies on the mechanism responsible for the effects of active ingredients have suggested that petasin and s-petasin inhibit Ca^{2+} channels, leading to a subsequent decrease in intracellular Ca^{2+}

concentration ($[\text{Ca}^{2+}]_i$), which inhibits the synthesis of leukotriene B_4 and cysteinyl leukotrienes in eosinophils and neutrophils (Bickel et al., 1994; Thomet et al., 2001; Wang et al., 2002, 2010; Fiebich et al., 2005).

Rhizomes of *Petasites hybridus* L. contains petasin, s-petasin, and related compounds and extracts derived from this plant are used as medicines in the West. On the other hand, in Korea, the leaves of *Petasites japonicus* are eaten. In this study, we sought to identify the active ingredients in *Petasites japonicus* leaves and to test them for anti-allergic and anti-inflammatory effects. Of the compounds isolated from *Petasites japonicus*, we found that bakkenolide B, a major component in its leaves showed significant effects in an ovalbumin-induced asthma model (Bakkenolide B structure in Fig. 1).

2. Materials and methods

2.1. Materials

Petasites japonicus leaves were collected in June 2012 in Chungdo province (South Korea). A voucher specimen (accession

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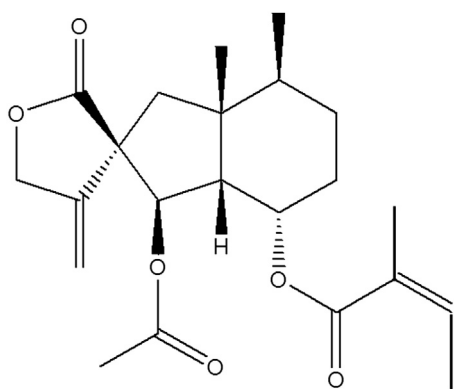


Fig. 1. Structure of bakkenolide B.

number MW-PRDR-11) was deposited at the Herbarium of Pusan National University. Fura 2-AM was obtained from Calbiochem (Darmstadt, Germany) and dexamethasone and other materials were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Isolation of bakkenolide B

The fresh leaves of *P. japonicus* (425.36 g) were chopped to a fine particle with an electric mixer (HMF-3100 S, Hanil Electric, Seoul, Korea) and then extracted at room temperature with 75% EtOH. The EtOH was then removed using a rotary evaporator and the remaining aqueous extract was fractionated successively with BuOH, EtOAc, and *n*-hexane. The hexane extract (2.6728 g) so obtained was evaporated in vacuo, and the residue was chromatographed on a silica gel (40 μ m, Baker, NJ) column (100 $\times$ 4.0 cm) using a step gradient 2.5%, 15%, 25% acetone in dichloromethylene and 15% and 25% MeOH in chloroform to obtain 62 fractions. Fraction 9 (MWLSH9, 304.9 mg) was separated on a Sephadex column (100 $\times$ 3.0 cm) using MeOH as eluant to obtain 7 fractions. The fraction 3 (MWLSH9IC, 209.7 mg) was further separated on a Sephadex column (100 $\times$ 3.0 cm) using MeOH to obtain five fractions. Fractions 2 and 3 (MWLSH9ICIB, 202.3 mg) were passed through a silica gel column (100 $\times$ 4.0 cm) using 1.5% acetone in CH_2Cl_2 as eluant to yield bakkenolide B (173.8 mg). Pure bakkenolide B was identified by HPLC on a Phenomenex Luna C18 column (Phenomenex, 150 $\times$ 4.6 mm ID; 5 μ m particle size) using an acetonitrile-water reagent alcohol gradient at a flow rate of 1.0 ml per minute.

Bakkenolide B isolated from *P. japonicus* leaves was identified using ^1H , ^{13}C , and distortionless enhancement of polarization transfer nuclear magnetic resonance spectroscopy in CDCl_3 by comparison with previously reported spectral data (Naya et al., 1972; Dong et al., 2010).

2.3. Cell culture

Rat RBL-2H3 mast cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA), cultured at 37 $^\circ\text{C}$ in a 5% CO_2 humidified incubator, and maintained in high glucose DMEM, containing 10% (v/v) heat-inactivated fetal bovine serum, 100 units/mL penicillin, 50 $\mu\text{g}/\text{mL}$ streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate (Dearman et al., 2005).

2.4. Isolation and culture of mouse peritoneal macrophages

Mouse peritoneal macrophages were isolated from the peritoneal cavity of 3% thioglycol-treated C57BL/6 mouse and cultured at 37 $^\circ\text{C}$ in a 5% CO_2 humidified incubator, as previously described (Michaud et al., 2010). Isolated macrophages were maintained in

RPMI1640 containing 10% (v/v) heat-inactivated fetal bovine serum, 100 units/mL penicillin, 50 $\mu\text{g}/\text{mL}$ streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate for 24 h and then incubated in 0.5% FBS-containing media for 18 h. Macrophages were treated with bakkenolide B and 1 h later LPS (100 ng/ml) was added. Total proteins were sampled after 24 h of LPS treatment.

2.5. Measurement of intracellular Ca^{2+} concentrations

Cells were trypsin-digested, allowed to sediment, resuspended in HEPES-buffered medium (HBM), consisting of 20 mM HEPES (pH 7.4), 103 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 0.5 mM CaCl_2 , 25 mM NaHCO_3 , 15 mM glucose and 0.1% bovine serum albumin (fatty acid free), and then incubated for 40 min with 5 μM of fura 2-AM for subsequent $[\text{Ca}^{2+}]_i$ measurements. $[\text{Ca}^{2+}]_i$ levels were estimated by measuring changes in fura 2 fluorescence (Yun et al., 2004) using an emission wavelength of 510 nm and two and excitation wavelengths (340 nm and 380 nm) every 0.1 s using a F4500 fluorescence spectrophotometer (Hitachi, Japan). The ratios of fluorescence intensities ($\lambda_{340}/\lambda_{380}$) at these two wavelengths were used as a surrogate of $[\text{Ca}^{2+}]_i$, as previously described (Chang et al., 2006).

2.6. Measurement of degranulation

Degranulation was estimated by measuring β -hexosaminidase release as previously described by Dearman et al. (Dearman et al., 2005). RBL-2H3 cells (2×10^5 cells/well in 24-well plates) were sensitized with 0.5 $\mu\text{g}/\text{mL}$ monoclonal anti-dinitrophenyl specific mouse IgE (DNP-IgE, D8406, Sigma, St. Louis, MO) overnight at 37 $^\circ\text{C}$ in a 5% CO_2 incubator. Cells were washed twice with PIPES buffer (pH 7.2), containing 25 mM PIPES, 110 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM MgCl_2 , 0.1% BSA, and 1 mM CaCl_2 to remove DNP-IgE before stimulation, and then incubated in PIPES buffer in 500 μL . Different concentrations of bakkenolide B were then added to each well, incubated at 37 $^\circ\text{C}$ for 30 min, and then 10 $\mu\text{g}/\text{mL}$ of human dinitrophenyl albumin (DNP-hAb, A6661 Sigma, St. Louis, MO) was added and cells were incubated for a further 30 min at 37 $^\circ\text{C}$ to induce degranulation. Aliquots (50 μL) of medium was then transferred to a 96-well microplate and incubated with 50 μL of 1 mM 4-nitrophenyl N-acetyl- β -D-glucosaminide (N9376, Sigma, St. Louis) in 0.1 M citrate buffer (pH 4.5). Cells were lysed with an equal volume of 0.5% Triton X-100 at 37 $^\circ\text{C}$ for 1 h. Total hexosaminidase activity from RBL-2H3 mast cells was measured at the same rate. The reaction was terminated by adding 250 μL of 0.05 M sodium carbonate buffer (pH 10.0, 0.05 M Na_2CO_3 /0.05 M NaHCO_3). The absorbance (OD) at 410 nm was measured using a microplate reader. Release (%) was calculated by the ratio of release in stimulated cells to total hexosaminidase activity.

2.7. Western blot

Macrophages were harvested and resuspended in lysis buffer. Concentrations of proteins were determined using a BCA protein assay (Thermo Scientific, Rockford, IL, USA) and equal amounts of proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 5% skim milk, and then incubated with specific rabbit antibody recognizing COX-2 and iNOS (Cell Signaling Technology, Danvers, MA, USA). Anti-rabbit horseradish-linked IgG was used as the secondary antibody. Signals were developed using an enhanced chemiluminescence system (Advant, USA) (Kimura et al., 2010).

2.8. Induction of asthma in BALB/c mice and drug administration

Six-week-old male BALB/c mice were obtained from Hyochang Science (Daegu, Korea) and adapted for a week beforehand in the Laboratory Animal facility at Pusan National University. The mice were divided into three groups ($n=7$ /group) that is PBS-injected control group, ovalbumin-injected asthma group, and bakkenolide B-treated asthma group (1 mg/kg), and asthma was induced by the intraperitoneal injection of ovalbumin and alum on the 1st and 14th days. From the 28th day, mice were exposed to nebulized ovalbumin for 10 min for three consecutive days (Aoki et al., 2010). Two days after treatment with nebulized ovalbumin, BALF were collected from lungs and the cells in BALF were stained and counted.

2.9. Statistics

Results are expressed as means $\pm$ SEs for the indicated numbers of determinations. The statistical significances of differences were determined by ANOVA, and Statistical significance was accepted for p values < 0.05 .

3. Results

3.1. Bakkenolide B inhibited antigen-induced degranulation in RBL-2H3 mast cells

The degranulation of histamine, leukotrienes, and prostaglandins in antigen-exposed mast cells is a key step of allergic responses. Therefore, we utilized rat RBL-2H3 mast cells to find active components in *Petasites japonicus*. Degranulation was measured by assessing β -hexosaminidase activity in media after antigen exposure (Dearman et al., 2005). The antigen-induced release of β -hexosaminidase was inhibited by bakkenolide B in a concentration-dependent manner (Fig. 2).

3.2. Bakkenolide B did not inhibit antigen-induced $[Ca^{2+}]_i$ increases in RBL-2H3 mast cells

Degranulation is evoked via an elevation of $[Ca^{2+}]_i$ after antigen-exposure in mast cells. Therefore, we measured $[Ca^{2+}]_i$ increases induced by antigen exposure and investigated whether bakkenolide B inhibited these increases (Lee et al., 2005). Antigen exposure was found to induce $[Ca^{2+}]_i$ increases (Fig. 3), but bakkenolide B had no effect, indicating that the degranulation inhibitory mechanisms of bakkenolide B and petasin differ, as

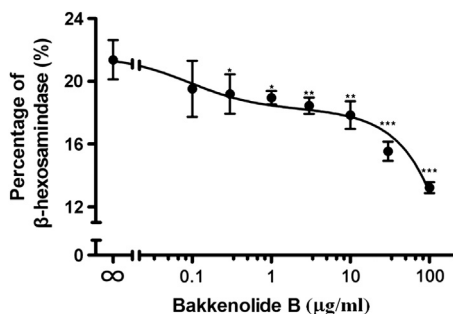


Fig. 2. Bakkenolide B inhibited antigen-induced β -hexosaminidase release from RBL-2H3 mast cells in a concentration-dependent manner. RBL-2H3 cells were sensitized overnight with anti DNP-IgE, treated with different concentrations of bakkenolide B, and 30 min later challenged with DNP human serum albumin. Antigen-induced degranulation was measured by released β -hexosaminidase activity. Bakkenolide B inhibited antigen-induced degranulation in RBL-2H3 mast cells in a dose-dependent manner. Results are the means $\pm$ SEs of three independent experiments.

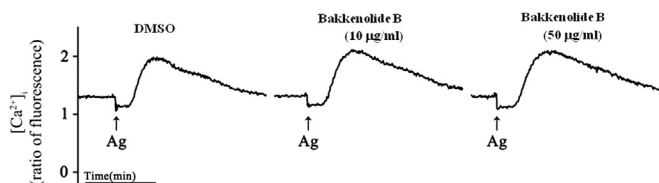


Fig. 3. Effects of bakkenolide B on antigen-induced $[Ca^{2+}]_i$ increases in RBL-2H3 mast cells. RBL-2H3 cells were sensitized overnight with anti DNP-IgE, loaded with Fura-2 AM for 45 min, and washed twice with PBS. The cells were then preincubated with bakkenolide B for 10 min and challenged with DNP human serum albumin. Arrows indicate when the antigen was added. Antigen induced increase of intracellular Ca^{2+} concentration and this increase was not inhibited by bakkenolide B upto 50 μ g/ml. The data shown are representative of three independent experiments.

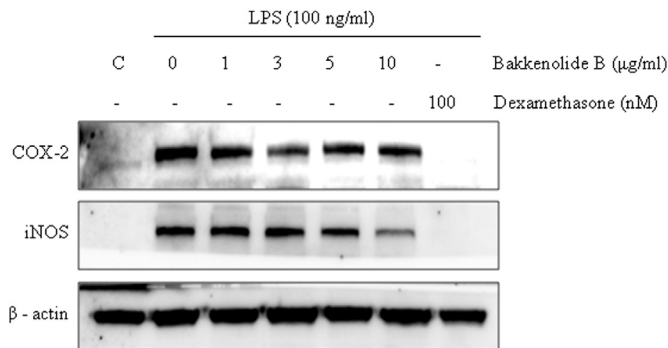


Fig. 4. Bakkenolide B inhibited inducible NOS expression in RBL-2H3 mast cells. Mouse peritoneal macrophages were treated with bakkenolide B or dexamethasone and 1 h later stimulated with LPS. LPS induced the expression of inducible NOS and COX-2. Bakkenolide B dose-dependently inhibited inducible NOS and COX-2. Dexamethasone was used as a positive control. Results are representative of three independent experiments that yielded similar results.

petasin is known to block voltage-dependent Ca^{2+} channels (Wang et al., 2010) (Fig. 3).

3.3. Bakkenolide B inhibited the induction of inducible NOS and COX-2 in peritoneal macrophages

Inflammatory responses are also involved in allergic response, and thus, we measured the effects of bakkenolide B on the expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) in mouse peritoneal macrophages. LPS induced the expression of both iNOS and COX-2 in macrophages (Fig. 4), and bakkenolide B strongly and concentration-dependently inhibited these inductions (Fig. 4).

3.4. Bakkenolide B inhibited the accumulation of eosinophils, macrophages, and lymphocytes in bronchoalveolar lavage fluids

The anti-allergic effect of bakkenolide B observed in RBL-2H3 cells in vitro was further examined in the ovalbumin-induced mouse asthma model. Bronchoalveolar lavage fluids (BALF) of ovalbumin-induced asthma mice showed accumulation of eosinophils, macrophages, and lymphocytes (Fig. 5), and bakkenolide B (1 mg/kg) inhibited these migrations into bronchoalveolar lavage fluid (Fig. 5). Bakkenolide B was treated before sensitization with nebulized ovalbumin, because it did not inhibit the maturation of immature dendritic cells (data not shown).

4. Discussion

The genus *Petasites* (Astraceae) has been reported to be a rich source of sesquiterpene lactones (Liao et al., 1997; Wu et al., 1999;

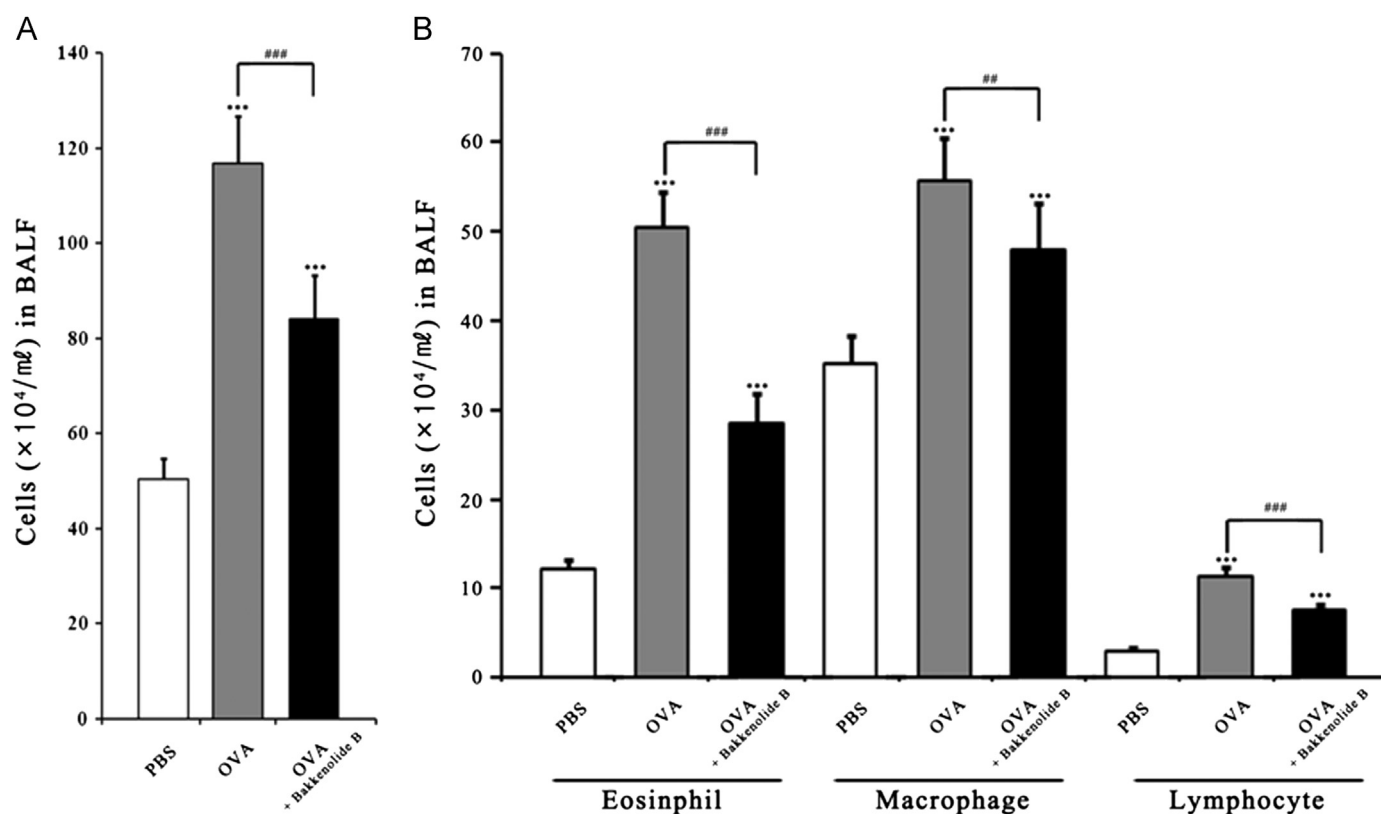


Fig. 5. Effect of bakkenolide B on lymphocyte accumulation in the BALF samples of ovalbumin-induced asthma model mice. Mice were immunized with ovalbumin twice by intraperitoneal injection (on day 1 and 14) and then sensitized to nebulized ovalbumin on days 28, 29, and 30. Bakkenolide B (1 mg/ml) was administered by intraperitoneal injection 1 h before ovalbumin nebulization on days 28, 29, and 30. Cells in BALF samples were stained using the May-Grunwald method and counted. (A) Total cell counts in the BALF samples of control mice (PBS), ovalbumin-induced asthma mice (OVA), and bakkenolide B (1 mg/ml)-treated asthma mice (OVA+bakkenolide B). (B) Cells counts of eosinophils, macrophages, and lymphocytes from the BALF samples of the PBS, OVA, and OVA+bakkenolide B (1 mg/ml) treated groups. Three-day exposure to ovalbumin through airways induced accumulation of eosinophils, macrophages, and lymphocytes in BALF. However, in bakkenolide B-treated mice group, accumulation of the cells was significantly reduced. Statistical significance: ** $P < 0.01$, *** $P < 0.001$ vs. PBS-treated mice group and ## $P < 0.01$, ### $P < 0.001$ vs. OVA-treated mice group.

Wang et al., 2009; Dong et al., 2010; Zhang et al., 2011), for example, (Wu et al., 1999) isolated 32 bakkenolides from *Petasites formosanus*. In addition, bakkenolide-type sesquiterpenes isolated from this genus have been reported to exhibit anti-platelet aggregation activity (Liao et al., 1997). Furthermore, bakkenolide B has to be non-cytotoxic to HepG2, KB, CCM2, and P338 cancer cell lines (Wu et al., 1999), but to be weakly cytotoxic to Hela, MCF-7, and LLC cell lines with IC_{50} values of 53.1, 45.9, and 147.2 μM , respectively (Dong et al., 2010). Moreover, Xu et al., (2011) found that bakkenolide B from *Valeriana jatamansi* has a weak neuroprotective effect against MPP^{+} -induced neuronal cell death in dopaminergic neuroblastoma SH-SY5Y cells (Xu et al., 2011).

Regarding anti-allergic effects, a total bakkenolide fraction of *Petasites tricholobus* has been reported to ameliorate allergic rhinitis in ovalbumin-sensitized Wistar rats (Zhang et al., 2011), and reduced serum levels of histamine and IL-4 were observed in bakkenolide-treated rats with reduced numbers of infiltrating eosinophils in nasal mucosa and diminished nose rubbing frequencies (Zhang et al., 2011). Shimoda et al. found that extracts of *Petasites japonicus* exhibited a type I anti-allergic property and inhibited mast cell degranulation (Shimoda et al., 2006), and Lee et al. found that *Petasites japonicus* extract had a suppressive effect on ovalbumin-induced airway inflammation in an asthmatic model (Lee et al., 2011). However, the components responsible for these effects have not been clearly reported.

In the present study, we found that bakkenolide B inhibited antigen-induced degranulation in RBL-2H3 mast cells and the induction of inducible NOS and COX-2 in mouse peritoneal

macrophages. In addition, bakkenolide B inhibited the accumulation of eosinophils, macrophages, and lymphocytes in the BALF samples of ovalbumin-induced asthma mice. Therefore, we conclude that bakkenolide B in *Petasites japonicus* leaves has anti-allergic and anti-inflammatory effects.

Significant effects of bakkenolide B have been observed in 10 $\mu\text{g}/\text{ml}$ and lower concentrations in RBL-2H3 mast cells and peritoneal macrophages. Considering that the molecular weight of bakkenolide B is 390.46, 10 $\mu\text{g}/\text{ml}$ of bakkenolide B equals to 25.6 μM . That is lower than the IC_{50} values in Hela, MCF-7, and LLC cell lines (Dong et al., 2010). Furthermore, 1 mg/kg of bakkenolide B was effective in ovalbumin-induced asthma model. Therefore, bakkenolide B may be used as a potent agent for the treatment of asthma.

Bronchial asthma is a common chronic inflammatory disease of the lung (Wills-Karp, 2004). Airway hyperresponsiveness, reversible airflow obstruction, and bronchial hyperresponsiveness are characteristic features of asthma (Busse and Rosenwasser, 2003). The pathogenesis of asthma is associated with increased infiltration of inflammatory cells and excessive mucus secretion into airway (Wills-Karp, 2004). A variety of cell types such as lymphocytes (Th2), mast cells, and eosinophils are involved in asthma. T lymphocytes play a pivotal role in the pathogenesis by producing Th2 pattern of cytokines (Wills-Karp, 2004). Those cytokines play in orchestrating the complex series of events leading to immunoglobulin E production, and development, recruitment, and activation of the primary effector cells of the allergic response, mast cells and eosinophils (Wills-Karp, 2004). As shown in Fig. 5,

the number of leukocytes, particularly eosinophils, in BALF was increased in the ovalbumin-treated group, and the infiltration was diminished by bakkenolide B treatment.

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