

Therapeutic Effect of *Broussonetia papyrifera* and *Lonicera japonica* in Ovalbumin-induced Murine Asthma Model

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Broussonetia papyrifera (L.) Vent. and *Lonicera japonica* Thunb. have been used in recent medicinal research for their antioxidative and anti-inflammatory properties. The present study investigated the therapeutic efficacy of *B. papyrifera* and *L. japonica* ethanolic extracts in a murine model of ovalbumin-induced asthma, in which intra-peritoneal (IP) injections and aerosol ovalbumin delivery were used to induce allergic asthma. Bronchioalveolar lavage fluid (BALF), serum samples, lungs and livers were collected from the experimental groups. In the groups treated with *B. papyrifera* and *L. japonica* extracts, CD3, CD4, serum IgE and IL-4 levels; activities of matrix metalloproteinase (MMP)-2 and MMP-9; and eotaxin levels in the BALF significantly decreased to near normal levels. Results of a histopathological analysis showed that the level of inflammation and mucous secretions reduced in the treated groups compared to the corresponding levels in the other groups. Moreover, results of a serum enzymatic analysis showed the non-toxic nature of the extracts in the *B. papyrifera* and *L. japonica* treated groups. Taken together, these results clearly indicate that the *B. papyrifera* and *L. japonica* extracts may be very effective against asthma and inflammation related diseases.

Keywords: Asthma, Mouse asthma model, *Broussonetia papyrifera*, *Lonicera japonica*, Inflammation.

The prevalence of asthma is increasing [1, 2]. Asthma is an allergic respiratory disease characterized by airway obstruction, bronchial hyper-responsiveness, mucus over-production and eosinophilic infiltration. Activation of T cells, especially T helper 2 (Th2)-type T cells, drives asthmatic allergic responses, including production of allergic cytokines and T lymphocyte recruitment to the lung [3].

Interleukin-4 (IL-4) is a cytokine critical to the differentiation of Th2-type T cells from naïve T lymphocytes (Th0) [4]. These Th2-type T cells produce IL-4, IL-5, IL-9 and IL-13 [5]. Enhanced release of Th2 cytokines triggers an increase in IgE levels, mucus secretion, eosinophilia and T cell activation [6]. Some of these effects are mediated by IL-4, which induces isotype switching of B lymphocytes to IgE-producing cells [7] and inhibits apoptosis of eosinophils and induces eosinophil activation by increasing eotaxin expression [8]. In patients showing atopy, Th2 as well as CD4+ T cells produce IL-4 and IL-5 [6]. In fact, persistent asthma is correlated with inflammation and deposition of extracellular matrix proteins and it may mediate release of matrix metalloproteinases (MMPs) [9]. MMPs are strongly expressed at sites of inflammation, and both MMP-2 and MMP-9 are predominantly secreted by T cells [10].

Many medications have been used in asthma treatment, including beta2-agonists and inhaled corticosteroids, but these have limitations and cause side effects in patients. Interestingly, leukotriene antagonists and corticosteroids are commonly used in the treatment of asthma. However, they have too many side effects, including severe adrenal suppression. Recently, the trend worldwide has been to use phytochemical therapeutics, because they are both economical and largely free from adverse effects.

B. papyrifera (L.) Vent. belongs to Moraceae family. Papyriflavonol A (PapA), isolated from the root bark of *B. papyrifera* (L.) Vent. shows activity against pathogenic bacteria and fungi [11]. It also prevents passive cutaneous anaphylaxis [12]. *Lonicera japonica* Thunb. belongs to Caprifoliace family and is also called Japanese honeysuckle or Jin Yin Hua. *L. japonica* also has anti-inflammatory and analgesic activities [13]. The combination of *B. papyrifera* and *L. japonica* total fractions exhibits strong anti-inflammatory effects [14]. Therefore, we selected these two bioactive extracts for this study, the major objective of which was to determine the efficacy of the combination of *B. papyrifera* and *L. japonica* as an anti-asthma agent.

To relieve the symptoms of asthma, beta2-agonists, such as salbutamol, albuterol and long-acting beta2-agonists (LABA), are

used as the first-line therapy [15]. Omalizumab, a humanized monoclonal anti-IgE antibody, is used to treat severe allergic asthma [16], and can reduce asthma exacerbations due to inhaled corticosteroid therapy [17]. However, these medications have limitations and cause side effects in patients. Local side effects of inhaled corticosteroids include oral candidiasis, hoarseness and dysphonia, along with systemic side effects including easy bruising, reduction in growth velocity and reduced bone mineral density [18], and adrenal suppression [19].

Table 1: Groups of experiment.

Groups	Number of Mice
Naive control (Not treated)	7
Negative control (Alum)	7
Vehicle control	7
Extracts of BP and Lj (100 mg/kg)	7
Extracts of BP and Lj (200 mg/kg)	7
Extracts of BP and Lj (400 mg/kg)	7
Positive control (Montelukast) 10 mg/kg	7

BP: *Broussonetia papyrifera*, LJ: *Lonicera japonica*.

Albuterol is a short-acting beta2 agonist that causes hyperlactic acidosis [20]. Cardiovascular side effects or even sudden death can result from asthmatic hypoxia combined with use of beta2 agonists [21]. In recent research, some extracts of natural product showed either anti-inflammatory or antioxidative stress potency [22, 23]. For these reasons, in this study, we focused on natural products that might have fewer side effects.

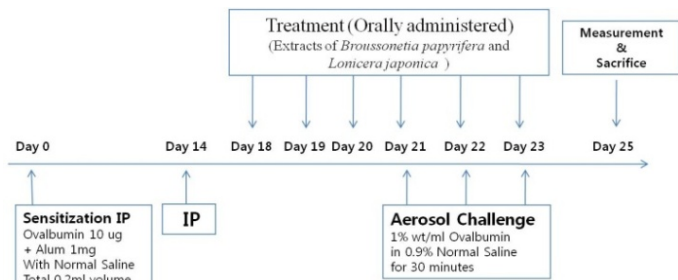


Figure 1: Induction of mouse asthma model and various treatment amounts with *Broussonetia papyrifera* and *Lonicera japonica*. IP: Intra-Peritoneal injection.

In a previous study, a total *B. papyrifera* flavonoid fraction, in combination with *L. japonica* fraction, showed anti-inflammatory activity [14]. In this study, we studied the therapeutic effects of these herbal extracts on allergic asthma. Among 7 experimental groups, the montelukast (a leukotriene antagonist) group was set as a positive control because of its bronchodilatory and anti-inflammatory effects [24]. In addition, montelukast treatment combined with that of an induced nitric oxide synthase (iNOS) inhibitor showed reduction in inflammation and oxidative stress [25]. First, we sensitized mice by intraperitoneal injection of ovalbumin (Days 0 and 14), and then administered ovalbumin inhalation for 3 days continuously (Days 21-23). On Days 18-23, extract of either *B. papyrifera* and *L. japonica* or montelukast were also administered to the mice (Figure 1).

Increase in the number of cells in the BALF is a significant marker for inflammation, and also suggests asthma induction [26]. Therefore, we counted the total number of cells in BALF samples. In induced (i.e., ovalbumin-challenged and ovalbumin-inhaled) but untreated groups (Alum and Vehicle groups), total cell numbers were significantly increased. In contrast, total cell numbers in treated groups (groups treated with 100, 200, and 400 mg/kg of extract, and montelukast groups) significantly decreased compared to those in the induced but untreated groups (Alum and Vehicle groups) (Figure 2).

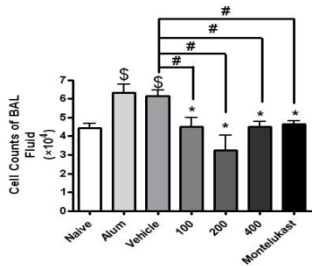


Figure 2: Total cell counts of BALF (Bronchoalveolar lavage fluid) of the mice lungs for the analysis of inflammatory status. Cells were counted in at least five squares of a hemocytometer using a light microscope after staining with trypan blue reagent. (n=4, ^s P<0.05, significantly different from Naive group; [#] P<0.05, significantly different from vehicle group; ^{*} P<0.05, significantly different from Alum group).

To determine the efficacy of *B. papyrifera* and *L. japonica* extracts in the asthma model, we used fluorescence-activated cell sorting (FACS), ELISA, and Western blot analyses to evaluate immune reactions as therapeutic markers, including cluster of differentiation (CD) proteins and cytokines. FACS revealed significantly decreased numbers of CD3 and CD4 lymphocytes in all of the treated groups (groups treated with 100, 200 and 400 mg/kg of extract, and montelukast groups) compared to those in the Alum and Vehicle groups (Figure 3A, B). CD3 and CD4 counts in the treated groups, especially in the 200 and 400 mg/kg groups, were either the same or lower than 50% of those in the untreated group numbers. When CD3 and CD4 lymphocytes were analyzed simultaneously, the proportion of activated lymphocytes in the treated groups also significantly decreased compared with those in the untreated groups (Figure 3C). A previous study showed significantly reduced activation of CD3 and CD4 T-lymphocytes in asthma patients treated with the test drug [27]. Therefore, our results suggest that the *B. papyrifera* and *L. japonica* extracts exhibit anti-inflammatory therapeutic effects related to T-lymphocyte immunity.

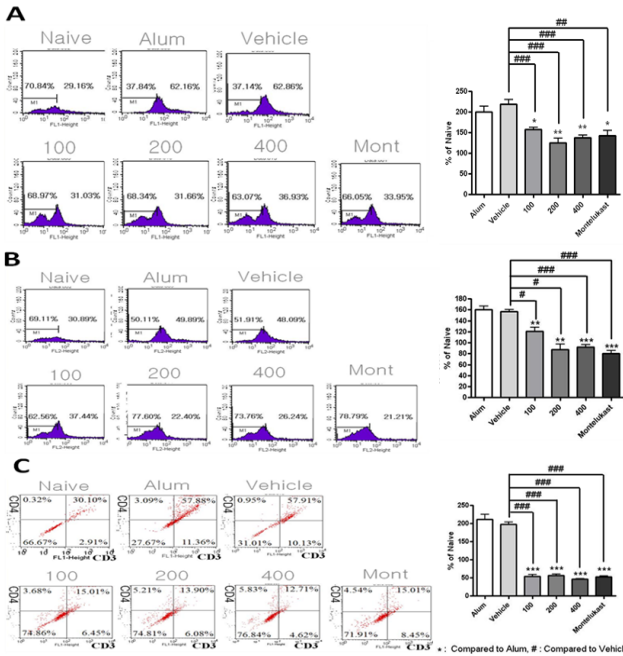


Figure 3: Flow cytometry analysis of T-lymphocyte related anti-inflammatory efficacy of *Broussonetia papyrifera* (Bp) and *Lonicera japonica* (Lj) in BALF. (A) CD3 lymphocytes analysis. (B) CD4 lymphocytes analysis. (C) CD3 and CD4 lymphocytes. Statistical analysis (n=4, [#] P<0.05, ^{##} P<0.01, significantly different from Vehicle group; ^{*} P<0.05, ^{**} P<0.01, significantly different from Alum group).

We next measured the concentrations of IL-4 and eotaxin in BALF and serum IgE levels in mice. In the development of allergic

inflammations such as asthma, IL-4 is a key cytokine because it induces B lymphocyte isotype switching to an immunoglobulin (Ig)-E secreting isotype [28], increases the expression of eotaxin [29], and activates mast cells and mucin hypersecretion [30]. Furthermore, IL-4 induces the differentiation of Th0 lymphocytes into Th2 lymphocytes [4]. Our results showed that IL-4 levels in BALF in most treatment groups significantly decreased compared to those in the Alum and Vehicle groups (Figure 4A). Compared to the untreated groups, the 400mg/kg and montelukast groups showed significantly decreased serum IgE levels (Figure 4B). These results indicate that asthma was alleviated, especially in the groups treated with 400 mg/kg extract. Furthermore, compared to the Vehicle group, the treated groups showed decreased eotaxin levels in BALF. The average eotaxin levels in the Alum group tended to be higher than all the treated groups; however, the differences were not statistically significant (Figure 4C). The groups treated with the combination of *B. papyrifera* and *L. japonica* extracts also showed lower levels of IL-4, eotaxin, and IgE in BALF or serum than the corresponding levels shown by the Alum and Vehicle groups. Taken together, these data indicate that allergic symptoms related to Th2-type T lymphocytes and cytokines were significantly ameliorated in the groups treated with 400 mg/kg extract and montelukast.

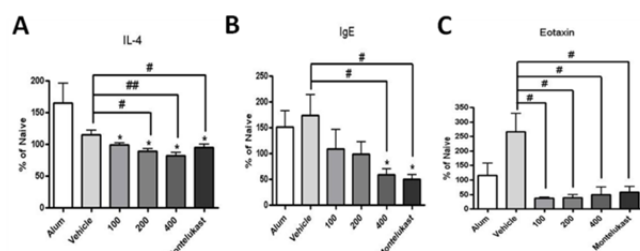


Figure 4: Enzyme-linked immunosorbent assay (ELISA) analysis of Interleukin-4 (IL-4) and Eotaxin in BALF, Immunoglobulin E (IgE) in serum for the anti-inflammatory efficacy analysis of *Broussonetia papyrifera* (Bp) and *Lonicera japonica* (Lj). (A) Statistical analysis of IL-4 in BALF. (n=4, * $P < 0.05$, # $P < 0.01$, significantly different from Vehicle group; * $P < 0.05$, significantly different from Alum group). (B) Statistical analysis of IgE in Serum. (n=4, * $P < 0.05$, significantly different from Vehicle group; * $P < 0.05$, significantly different from Alum group). (C) Statistical analysis of Eotaxin in BALF. (n=4, * $P < 0.05$, significantly different from Vehicle group).

Inflammatory cytokines such as IL-4 stimulate the secretion of MMPs in asthma status [31], thereby leading to high levels of expression at inflamed sites. In particular, MMP-2 levels increase in cases of inflammatory injuries [32]; further, in sputum samples of asthma patients, higher gelatinolytic activities related to MMP-2 and MMP-9 were noted [33]. In this study, Western blots were performed to determine the expression levels of MMP-9 in homogenized lung tissue samples. MMP-9 levels in the groups treated with 200 and 400 mg/kg extract significantly decreased compared to the levels in the Vehicle group and the group treated with 100 mg/kg extract. Moreover, compared to the group treated with 200 mg/kg extract, the group treated with 400 mg/kg extract showed a significant decrease. MMP-9 protein expression was measured by densitometry of the bands-of-interest in the Western blots (Figure 5). MMP-2 activity was also assessed by the zymography method. The groups treated with 200 and 400 mg/kg extract showed higher MMP-2 gelatinolytic activities (Figure 5A). Our data show lower secretion of MMP-2 and MMP-9 after treatment with the *B. papyrifera* and *L. japonica* extracts, most likely a result of the inhibition of IL-4 cytokine secretion and decrease in T lymphocyte activation described earlier.

To determine the therapeutic efficacy of the extracts in the lungs, we performed a histopathological examination using hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining. H&E

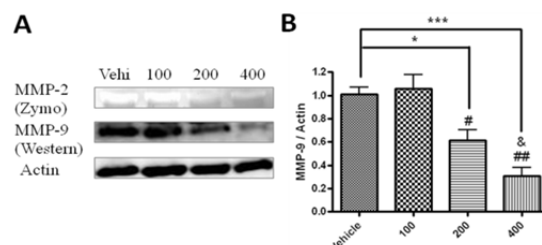


Figure 5: Analysis of *Broussonetia papyrifera* and *Lonicera japonica* effects on matrix metalloproteinase (MMP)-2, MMP-9 activities. (A) MMP-2 zymography (50 μ g) and MMP-9 Western blot (50 μ g) (B) Statistical analysis of MMP-9 (n=4, * $P < 0.05$, *** $P < 0.001$, significantly different from Vehicle group; * $P < 0.05$, # $P < 0.01$ significantly different from 100 mg/kg group, & $P < 0.05$ significantly different from 200 mg/kg group.)

staining revealed that inflammatory cells appearing to be neutrophils (among other types) infiltrated the lung tissues in the non-treated groups. In contrast, the extract-treated groups demonstrated less infiltration (Figure 6A and B). PAS staining revealed that mucus production significantly decreased in the extract treated groups. In the non-treated groups most of the epithelial cells were covered by mucus, an effect ameliorated in the extract-treated groups (Figure 6C).

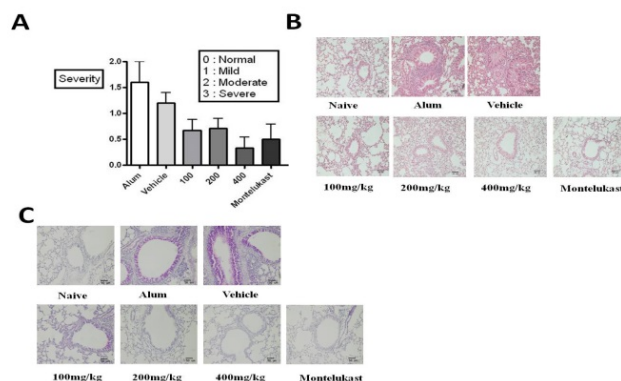


Figure 6: Histopathological analyses of lung. (A) Hematoxylin-eosin stain (H&E) scoring (n=4). (B) Image of H&E. (C) Periodic acid-Schiff (PAS) staining of lung tissues for the anti-inflammatory effect determination of *Broussonetia papyrifera* and *Lonicera japonica*.

To examine the toxicity of the extract, the highest administered dose of 400 mg/kg extract was selected, and serum levels of alanine transaminase (ALT) and aspartate transaminase (AST) were quantified. In the extract treated groups, ALT and AST levels were not significantly changed compared to the corresponding levels in the treatment of Naïve group. Moreover, the test group showed ALT and AST levels similar to the corresponding levels in the montelukast group (Figure 7A and B). For these reasons, *B. papyrifera* and *L. japonica* extracts appear to be biocompatible treatments for asthma.

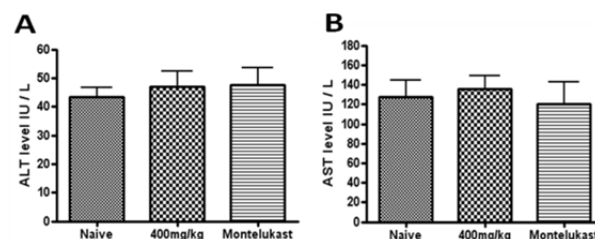


Figure 7: Toxicity test of *Broussonetia papyrifera* and *Lonicera japonica* for the evaluation of biocompatibility. (A) Alanine transaminase (ALT). (B) Aspartate transaminase (AST) (n=4).

In conclusion, in this study, we used the latest assays to elucidate the potential mechanisms through which *B. papyrifera* and

L. japonica extracts exert their therapeutic effects. Our results suggest that the application of these extracts can be recommended because of their potent antioxidant, anti-inflammatory, and anti-asthmatic activities; very low toxicity; and cost effectiveness. This work has added importance since there is a recent worldwide trend of the use of natural therapeutics.

Experimental

Materials: Ovalbumin was purchased from Pierce (Rockford, IL) and alum from Thermo Scientific (Bremen, Germany). Dimethyl sulfoxide (Sigma, St Louis, MO) was used to prepare the extract of *Bp* and *Lj*. Zoletil® (tiletamine hydrochloride and zolazepam hydrochloride) was purchased from Virbac (Carros, France). Miliplex mouse cytokine panel I -5 plex kit (Millipore, USA) was used to detect IL-4 and Eotaxin, IgE ELISA kit was purchased from USCN Life Science Incorporation (China). FITC-conjugated anti-murine CD3 and PE-conjugated anti-murine CD4 antibodies were purchased from BD biosciences (San Jose, CA), MMP-9 antibody from Abcam (Cambridge, UK) and actin antibody from SantaCruz (CA, USA).

Preparation of *Broussonetia papyrifera* and *Lonicera japonica* extract: *Broussonetia papyrifera* (*Bp*) dried root bark was extracted with 100% ethanol according to the ethanol thermal extraction method. The extract was dried under vacuum and then dissolved in water. After fractionation with ethyl acetate, the fraction was subsequently dried and used in the experiment. The yield of *Bp* dried fraction was approximately 4.2%, w/w. Papyriflavonol A and broussonetichalcone A were found in this fraction with contents of 0.33% and 1.03%, respectively, according to HPLC analysis. Dried *Lonicera japonica* (*Lj*) was extracted with 70% ethanol at room temperature. The extract was dried under vacuum and used in the experiment. The final yield of *Lj* was approximately 9.7%, w/w. Loganin and swerosid, analyzed by HPLC, formed 4.19% and 3.30%, respectively. The detailed content, characterization and information of extracts have been described in the works of Jin and colleagues [14].

Animals and induction of mouse asthma model: Six-week-old female BALB/c mice were purchased from Joongang Laboratory Animal Inc. (Seoul, Korea) and maintained in the laboratory animal facility with temperature and relative humidity maintained at 23±2°C and 50±20%, respectively, and a 12 h light/dark cycle. Mice were randomly divided into 7 groups (n=7); summarized in Table 1. A mouse asthma model was induced [34]. Intra-peritoneal (IP) injections of ovalbumin (20 µg per mouse) with alum (2 mg / mouse) in normal saline, total volume 0.2 ml, were made twice (days 0, 14) to sensitize the mice to allergen. Ovalbumin (1% wt/mL ovalbumin in 0.9% normal saline inhalation for 30 min) was delivered for 3 days continuously (day 21, 22, and 23). At the same time, extract of *Bp* and *Lj* was orally administered for 6 days continuously (day 18, 19, 20, 21, 22 and 23). Extracts of *Bp* and *Lj* 100, 200 and 400 mg/kg administered groups were orally administered in a dose-dependent manner. Montelukast was orally administered at 10 mg/kg to the appropriate group. The naïve group was challenged with normal saline. After repeated inhalations and treatments, 48 h later (day 25), mice were sacrificed and BALF, serum, lungs and livers were collected (Figure 1). All methods used in this study were approved by the Animal Care and Use Committee at Seoul National University (SNU-100222-1).

Collection of bronchioalveolar lavage fluid (BALF): Mice were sacrificed using an overdose (500 µL) of Zoletil® (tiletamine

hydrochloride and zolazepam hydrochloride, Virbac, France) / xylazine. After tracheotomy, 0.5 mL of PBS was instilled into the lungs and the first supernatant of BALF (400 × g, 10 min) was collected for the measurement of cytokine and stored at -70°C. Further BALF was collected an additional 4 times by aspiration; a total of approximately 4 mL was collected and centrifuged. All cell pellets were collected and re-suspended in red blood cell (RBC) lysis buffer for 5 min. After RBC lysis, cells were washed 3 times and re-suspended in PBS. Total cell numbers were counted using a hemocytometer for the relationship between increased cell numbers in BALF and inflammation.

Measurement of IL-4 in BALF and IgE, eotaxin in serum: Levels of IL-4 in BALF, and IgE and eotaxin in serum were measured using an enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions.

Zymography: MMP-2 activity was assessed by a gelatin zymography method. Lung tissues were homogenized in Passive Lysis Buffer® (Promega) and centrifuged (13200 g, 4°C, 30 min). Fifty µg of protein were loaded for gelatin zymography. Gels were composed of 10% SDS-PAGE containing 1% gelatin. After electrophoresis (80V), gels were washed twice in 2.5% Triton X-100 for 30 min and then incubated in collagenase buffer (1M Tris-HCl pH 7.6, NaCl, CaCl₂ and Brij-35) for 24 h. After incubation, gels were stained with Coomassie brilliant blue reagent and destained with 10% methanol and 10% acetic acid solution. Gelatinase activities were assessed by observing white bands on gels.

Flow cytometry: BAL fluid cells were re-suspended in PBS and stained with FITC-conjugated anti-murine CD3 mAb and PE-conjugated anti-murine CD4 mAb in a separate reaction. Cells were washed with PBS and fixed in 4% paraformaldehyde overnight. Flow cytometry was performed using a FACS Calibur analytical flow cytometer (BD biosciences) and 1×10⁴ cells were used for analysis.

Western blot analysis: Fifty µg of lung homogenate protein was analyzed on SDS-PAGE. Proteins were separated and transferred to nitrocellulose membranes and blocked for 1 h in 5% skim milk. The nitrocellulose membranes were incubated overnight with corresponding primary antibodies in 5% skim milk at 4°C. After that, the membranes were incubated with horse radish peroxidase conjugating secondary antibodies (Invitrogen) for either 3 h at room temperature or overnight at 4°C. After washing with TTBS (Tris-Buffered Saline + Tween 20) solution for 30 min, bands were detected using a luminescent image analyzer LAS-3000 (Fujifilm, Tokyo, Japan). Quantification of Western blot bands was performed by Multi Gauge version 2.02 software (Fujifilm, Tokyo, Japan).

Tissue histopathology: Lung tissues not used for BALF collections were fixed in 10% formalin and embedded in paraffin, sectioned at 3 µm thickness, and stained with hematoxylin (Sigma GHS-116), eosin (Sigma HT-110116) for H&E staining and periodic acid-Schiff (PAS) (Sigma 395B) to detect mucus production. Tissues were mounted and cover slipped using Dako mounting medium (Dakocytomation, Denmark, CA).

Detection of ALT and AST levels in serum: Alanine transaminase (ALT) and aspartate transaminase (AST) levels in serum were analyzed by a ChemaDiagnostica automatic analyzer (Olympus, JP/AU400) according to the manufacturer's instructions for the examination of liver toxicity.

Statistical analysis: Data are expressed as mean values $\pm$ standard error. Graphs were drawn using GraphPad Prism V4.03 (GraphPad Software, San Diego, CA, USA) and statistical significances were analyzed using Microcal Origin student's t-test two populations (Microcal Software, Northampton, MA, USA). The significance was set at $P < 0.05$ and $P < 0.01$.

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References

- [1] Burr ML, Wat D, Evans C, Dunstan FD, Doull IJ. (2006) Asthma prevalence in 1973, 1988 and 2003. *Thorax*, **61**, 296-299.
- [2] Centers for Disease Control and prevention (CDC). (2011) Vital signs: asthma prevalence, disease characteristics, and self-management education: United States, 2001--2009. *MMWR. Morbidity and Mortality Weekly Report*, **60**, 547-552.
- [3] Romagnani S. (2000) The role of lymphocytes in allergic disease. *Journal of Allergy and Clinical Immunology*, **105**, 399-408.
- [4] Hsieh CS, Heimberger AB, Gold JS, O'Garra A, Murphy KM. (1992) Differential regulation of T helper phenotype development by interleukins 4 and 10 in an alpha beta T-cell-receptor transgenic system. *Proceedings of the National Academy of Sciences of the United States of America*, **89**, 6065-6069.
- [5] Jutel M, Pichler WJ, Skrbic D, Urwyler A, Dahinden C, Muller UR. (1995) Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN-gamma secretion in specific allergen-stimulated T cell cultures. *Journal of Immunology*, **154**, 4187-4194.
- [6] Elias JA, Lee CG, Zheng T, Ma B, Homer RJ, Zhu Z. (2003) New insights into the pathogenesis of asthma. *Journal of Clinical Investigation*, **111**, 291-297.
- [7] Larche M, Robinson DS, Kay AB. (2003) The role of T lymphocytes in the pathogenesis of asthma. *Journal of Allergy and Clinical Immunology*, **111**, 450-463.
- [8] Hoontrakoon R, Kailey JM, Bratton DL. (1999) IL-4 and TNF alpha synergize to enhance eosinophil survival. *Journal of Allergy and Clinical Immunology*, **103**, S239-S239.
- [9] Kelly EA, Busse WW, Jarjour NN. (2000) Increased matrix metalloproteinase-9 in the airway after allergen challenge. *American Journal of Respiratory and Critical Care Medicine*, **162**, 1157-1161.
- [10] Goetzl EJ, Banda MJ, Leppert D. (1996) Matrix metalloproteinases in immunity. *Journal of Immunology*, **156**, 1-4.
- [11] Sohn HY, Kwon CS, Son KH. (2010) Fungicidal effect of prenylated flavonol, papyriflavonol A, isolated from *Broussonetia papyrifera* (L.) Vent. against *Candida albicans*. *Journal of Microbiology and Biotechnology*, **20**, 1397-1402.
- [12] Kwak WJ, Moon TC, Lin CX, Rhyn HG, Jung H, Lee E, Kwon DY, Son KH, Kim HP, Kang SS, Murakami M, Kudo I, Chang HW. (2003) Papyriflavonol A from *Broussonetia papyrifera* inhibits the passive cutaneous anaphylaxis reaction and has a secretory phospholipase A2-inhibitory activity. *Biological and Pharmaceutical Bulletin*, **26**, 299-302.
- [13] Ryu KH, Rhee HI, Kim JH, Yoo H, Lee BY, Um KA, Kim K, Noh JY, Lim KM, Chung JH. (2010) Anti-inflammatory and analgesic activities of SKLJI, a highly purified and injectable herbal extract of *Lonicera japonica*. *Bioscience, Biotechnology, and Biochemistry*, **74**, 2022-2028.
- [14] Jin JH, Lim H, Kwon SY, Son KH, Kim HP. (2010) Anti-inflammatory activity of the total flavonoid fraction from *Broussonetia papyrifera* in combination with *Lonicera japonica*. *Biomolecules and Therapeutics*, **18**, 197-204.
- [15] Muller V, Galfy G, Eszes N, Losonczy G, Bizzi A, Nicolini G, Chrystyn H, Tamasi L. (2011) Asthma control in patients receiving inhaled corticosteroid and long-acting beta2-agonist fixed combinations. A real-life study comparing dry powder inhalers and a pressurized metered dose inhaler extrafine formulation. *BMC Pulmonary Medicine*, **11**, 40.
- [16] Pelaia G, Gallelli L, Renda T, Romeo P, Busceti MT, Grembiale RD, Maselli R, Marsico SA, Vatrella A. (2011) Update on optimal use of omalizumab in management of asthma. *Journal of Asthma and Allergy*, **4**, 49-59.
- [17] Chipps BE, Fligliomeni M, Spector S. (2012) Omalizumab: An update on efficacy and safety in moderate-to-severe allergic asthma. *Allergy and Asthma Proceedings*, **33**, 377-385.
- [18] Chung KF, Caramori G, Adcock IM. (2009) Inhaled corticosteroids as combination therapy with beta-adrenergic agonists in airways disease: present and future. *European Journal of Clinical Pharmacology*, **65**, 853-871.
- [19] Bruni FM, De Luca G, Venturoli V, Boner AL. (2009) Intranasal corticosteroids and adrenal suppression. *Neuroimmunomodulation*, **16**, 353-362.
- [20] Kovacevic A, Schwahn B, Schuster A. (2010) Hyperlactic acidosis as metabolic side-effect of albuterol and theophylline in acute severe asthma. *Klinische Pädiatrie*, **222**, 271-272.
- [21] Burggraaf J, Westendorp RG, in't Veen JC, Schoemaker RC, Sterk PJ, Cohen AF, Blauw GJ. (2001) Cardiovascular side effects of inhaled salbutamol in hypoxic asthmatic patients. *Thorax*, **56**, 567-569.
- [22] Shin IS, Lee MY, Lim HS, Ha H, Seo CS, Kim JC and Shin HK. (2012) An extract of *Crataegus pinnatifida* fruit attenuates airway inflammation by modulation of matrix metalloproteinase-9 in ovalbumin induced asthma. *PLoS One*, **7**, e45734.
- [23] Panahi Y, Tavana S, Sahebkar A, Masoudi H, Madanchi N. (2012) Impact of adjunctive therapy with *Chlorella vulgaris* extract on antioxidant status, pulmonary function, and clinical symptoms of patients with obstructive pulmonary diseases. *Scientia Pharmaceutica*, **80**, 719-730.
- [24] Diamant Z, Mantzouranis E, Bjermer L. (2009) Montelukast in the treatment of asthma and beyond. *Expert Review of Clinical Immunology*, **5**, 639-658.
- [25] de Souza FC, Gobbato NB, Maciel RG, Prado CM, Martins MD, Leick EA, Tiberio IF. (2013) Effects of corticosteroid, montelukast and iNOS inhibition on distal lung with chronic inflammation. *Respiratory Physiology and Neurobiology*, **185**, 435-445.
- [26] Roh SS, Kim SH, Lee YC, Seo YB. (2008) Effects of radix adenophorae and cyclosporine A on an OVA-induced murine model of asthma by suppressing to T cells activity, eosinophilia, and bronchial hyperresponsiveness. *Mediators of Inflammation*, **781425**.
- [27] Majori M, Piccoli ML, Bertacco S, Cuomo A, Cantini L, Pesci A. (1997) Inhaled beclomethasone dipropionate downregulates CD4 and CD8 T-lymphocyte activation in peripheral blood of patients with asthma. *Journal of Allergy and Clinical Immunology*, **100**, 379-382.
- [28] Coffman RL, Ohara J, Bond MW, Carty J, Zlotnik A, Paul WE. (1986) B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. *Journal of Immunology*, **136**, 4538-4541.
- [29] Doucet C, Brouty-Boye D, Pottin-Clemenceau C, Jamin C, Canonica GW, Azzarone B. (1998) IL-4 and IL-13 specifically increase adhesion molecule and inflammatory cytokine expression in human lung fibroblasts. *International Immunology*, **10**, 1421-1433.
- [30] Dabbagh K, Takeyama K, Lee HM, Ueki IF, Lausier JA, Nadel JA. (1999) IL-4 induces mucin gene expression and goblet cell metaplasia *in vitro* and *in vivo*. *Journal of Immunology*, **162**, 6233-6237.
- [31] Parks WC, Shapiro SD. (2001) Matrix metalloproteinases in lung biology. *Respiratory Research*, **2**, 10-19.
- [32] Tan RJ, Fattani CL, Niehouse LM, Tobolewski JM, Hanford LE, Li Q, Monzon FA, Parks WC, Oury TD. (2006) Matrix metalloproteinases promote inflammation and fibrosis in asbestos-induced lung injury in mice. *American Journal of Respiratory Cell and Molecular Biology*, **35**, 289-297.

- [33] Cataldo D, Munaut C, Noel A, Franken F, Bartsch P, Foidart JM, Louis R. (2000) MMP-2- and MMP-9-linked gelatinolytic activity in the sputum from patients with asthma and chronic obstructive pulmonary disease. *International Archives of Allergy and Immunology*, **123**, 259-267.
- [34] Lee MY, Lee NH, Jung D, Lee JA, Seo CS, Lee H, Kim JH, Shin HK. (2010) Protective effects of allantoin against ovalbumin (OVA)-induced lung inflammation in a murine model of asthma. *International Immunopharmacology*, **10**, 474-480.