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Suppressive Effects of *Benincasae hispida* on Allergic Inflammation

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Abstract

The effects of an extract of *Benincasae hispida* on allergic inflammation were examined in terms of histamine and β-hexosaminidase release, serum IgE level and inflammatory cytokine level. The *B. hispida* extract inhibited the release of histamine and β-hexosaminidase, a degranulation marker, from rat basophilic leukemia cells (RBL-2H3). When mice were first ovalbumin-challenged and then treated with *B. hispida* extract, there was a significant decrease in the IgE level in the mouse serum. The extract treatment reduced the serum IgE level prominently, compared with the ovalbumin-challenged mice. The extract also significantly reduced the TNF-α and IL-4 levels in the BAL fluid when challenged with antigen. Taken together, the *Benincasae hispida* extract may be efficacious against allergic inflammation.

Keywords: *Benincasae hispida*, Allergic inflammation, Histamine release, Inflammatory cytokine, Serum IgE level

Allergic inflammation is an important pathophysiological condition and includes asthma, atopic dermatitis, allergic rhinitis, and several ocular allergic diseases[1]. Generally, the pathophysiology of allergic responses may be divided into two components, the early- and late-phase reactions. The early phase can either subside or progress into a late-phase reaction, which can prolong the symptoms of response substantially and result in tissue damage.

The early-phase reaction is generally referred to as the immediate or type I allergic reaction, caused by the release of histamine and by activated mast cells following the cross-linking of allergen-specific IgE bound to mast cell $Fc_{\varepsilon}RI$ receptors[2]. It is well known that $Fc_{\varepsilon}RI$ aggregation triggers sequence of biochemical events leading to cell degranulation. Engagement of the receptor leads to the activation of tyrosine kinases, activation of phospholipase C (PLC), increased diacylglycerol, and mobilization of Ca^{2+} from internal stores. This is followed by the activation of protein kinase C (PKC), accompanied by an increase in mitogen-activated protein kinase (MAPK) activity and a Ca^{2+} influx. In $Fc_{\varepsilon}RI$ signaling, tyrosine phosphorylation of proteins is considered to play an essential role[3]. Recent studies have shown that in Syk kinase-deficient mast cells, there is complete abrogation of degranulation, elevation of Ca^{2+} influx, and activation of the ERK and JNK MAP kinase pathways. These observations indicate that Syk kinase is essential for the $Fc_{\varepsilon}RI$-mediated degranulation signal transduction[4,5].

Activated mast cells can produce a wide variety of inflammatory mediators, such as eicosanoids, proteoglycans, proteases, and several proinflammatory and chemotactic cytokines, like TNF-α, IL-4, IL-6, IL-13, and TGF-β[6]. Mediators of allergic inflammation affect nerve cells, causing itching[7], smooth muscle cells, causing contraction, leading to the airway narrowing in allergic asthma[8], and endothelial cells, causing vasodilation and edema[7]. Typically, the T-cells recruited in the early-phase reaction are of the Th2 variety, which belong to a subset of T-cells that produce IL-4 in allergic responses.

Benincasae hispida (Thunb). Cogn, commonly known as white pumpkin, wax gourd, or ash gourd, is cultivated primarily in China, India, and other semi-tropical countries for its edible fruit that has notable medicinal value[9]. According to an old Korean medical encyclopedia, the 'Donguibogam', which is listed in the "Memory of the World Register" by UNESCO, *Benincasae hispida* is efficacious against diabetes, edema, dropsy, diseases related to the liver, leucorrhea, and good for the detoxication of minerals, removal of fever, and to strengthen the function of the bladder and intestines[10]. Moreover, *in vitro* and *in vivo* studies have shown that the seeds of *Benincasa hispida* have an expectorant[11], anti-angiogenic[12], anti-oxidative[13],

gastroprotective[14], anti-ulcer[15], and anti-inflammatory effects in diabetic vascular complications[9]. The chemical components of the seeds[15-17] include saponin, urea, citrulline, linoleic acid, oleic acid, and fatty acids, as well as proteins, such as trigonelline, coffearin, and osmotin, as well as phytochemicals, such as triterpenes, sterols, flavonoid C-glycosides, acylated glucose, and benzyl glycoside.

To date, there has been no reported research on the suppressive effects of *Benincasae hispida* on allergic inflammation. Thus, we examined the inhibitory effects of *Benincasae hispida* on allergic inflammation by measuring serum IgE level, histamine release, and inflammatory cytokine production.

Reduction of Serum IgE Concentration

Elevated levels of IgE are associated with asthma, atopic dermatitis, and allergic rhinitis. In general, unusual IgE production is regulated primarily by Th2 cells. When Th2 cells are activated, IL-4, IL-5, IL-9, and IL-13 are secreted[18-20]; among these, IL-4 and IL-13 play key roles in IgE hyperproduction.

To investigate the effect of *B. hispida* extract on serum IgE levels, the OVA-specific IgE levels in mice treated with vehicle or *B. hispida* extract were examined (Figure 1). The IgE levels increased by approximately 4-fold in the OVA-challenged mice, compared with the saline-challenged mice. The oral administration of *B. hispida* extract during the OVA-challenge period significantly prevented the rise in serum total IgE levels. The *B. hispida* extract reduced the serum IgE level by 42%, compared with OVA-challenged mice. These results indicate that the *B. hispida* extract might contain inhibitors of serum IgE elevation.

Reduction of Inflammatory Cytokines in BAL Fluid

Cell components of the lower respiratory tract can be obtained from bronchoalveolar lavage (BAL) during respiratory diseases. BAL fluid has been used to assess biochemical and inflammatory changes in the interstitial lung tissue. Especially during asthma attacks, the expression of Th2 cytokines in BAL plays an important role in causing allergic inflammation, via enhanced IgE production[21]. Furthermore, IL-4 induces VCAM-1 (vascular cell adhesion molecule-1) gene expression in the endothelium and gathers eosinophils to the inflammatory lesions, causing increased infiltration, leading to chronic inflammation.

TNF-α is a pivotal proinflammatory cytokine that increases during allergic inflammation. Elevated TNF-α levels are frequently observed in the BAL fluid of asthmatic subjects undergoing allergen challenge[6]. TNF-α expression mediates neutrophil migration and

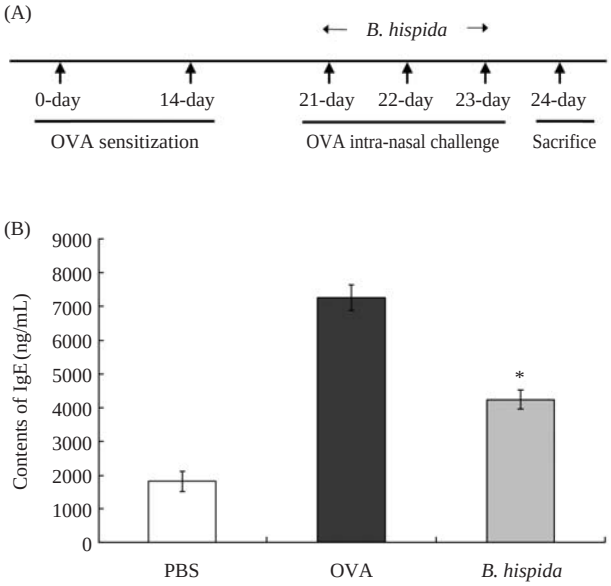

Figure 1. (A) Experimental protocol for the protective effect of *Benincasae hispida* extract. (B) Inhibition of the elevation in the serum IgE level by treatment with *Benincasae hispida* extract. $*P < 0.05$, significantly different from the OVA sensitized value.

infiltration; furthermore, it increases particle-induced cytotoxicity and also regulates neutrophil apoptosis in acute inflammation.

Changes in the IL-4 levels in the BAL of ovalbumin-challenged mice are presented in Table 1. The IL-4 level of the ovalbumin-challenged group was about 2-fold higher than that of the PBS group. Interestingly, the increased IL-4 level was lowered significantly, to the PBS-treated level, after *B. hispida* treatment. The TNF-α level of the ovalbumin-challenged group was about 3-fold higher than the PBS-treated group, and this value decreased by about 68%, compared with the ovalbumin-challenged group after 2% *B. hispida* treatment (Table 1). These results demonstrated that the *B. hispida* extract effectively lowered IL-4 and TNF-α in ovalbumin-challenged BAL fluid. *B. hispida* extract appeared to provide notable ameliorative benefit for allergic symptoms, by modulating IL-4 and TNF-α production in BAL fluid.

Histamine and β-hexosaminidase Release

Mast cells and basophils play essential roles in the pathogenesis of allergic reactions, such as atopic dermatitis and asthma. The rat basophilic leukemia cell line RBL-2H3, a tumor analog of mast cells, are mucosal mast cells that express the immunoglobulin Fc epsilon receptor I ($Fc_{\varepsilon}RI$). Stimulation of IgE-sensitized RBL2H3 cells with specific antigen triggers a cascade

Table 1. Effect of *B. hispida* on IL-4 and TNF-α levels in the BAL fluids of mice.

	IL-4 (pg/mL)	TNF-α (pg/mL)
PBS	51.9±7.0	75.01±4.1
OVA	110.7±8.1*	229.5±33.0*
B. hispida	52.2±2.6**	74.3±4.1**

The mice were treated with *B. hispida* and cytokine levels in the BAL fluid were measured as described in the Materials and methods. Results are expressed as mean±SEM (n=6 in each group).
*$P<0.05$ as compared to PBS treated group; **$P<0.05$ as compared to OVA treated group.

of events leading to degranulation, mediator release, activation of mitogen-activated protein kinase (MAPK), tyrosine kinase, and phospholipase C, increased reactive oxygen species production, calcium influx, and cytokine production. The secretion of histamine and β-hexosaminidase from RBL-2H3 is a hallmark of the allergic reaction resulting from allergen exposure. Histamine is released from intracellular secretory granules, induced by elevation of intracellular Ca^{2+} concentrations and the activation of protein kinase C[22]. The role of histamine in allergic inflammation is supported by several types of evidence, including the reproduction of features of allergic inflammation by injected or inhaled histamine, the reduction of allergic inflammation by histamine receptor antagonists, and more recently by the demonstration that mice genetically modified to make less histamine have a diminished capacity to develop allergic inflammation[23]. β-hexosaminidase is also stored in secretory granules of mast cells and is released concomitantly with histamine when mast cells are immunologically activated. Thus, β-hexosaminidase activity in the medium is also used as a marker of mast cell degranulation[24].

The degranulating effect was measured by assaying histamine release from allergen-sensitized RBL-2H3 cells (Figure 2). About 50% of the histamine was induced and released after DNP-BSA challenge. However, upon treatment with 0.0625% *B. hispida* extract, histamine release was reduced by 44%, compared with DNP-BSA sensitized cells. Treatment with 0.125% of *B. hispida* extract displayed a 54% inhibitory effect on the histamine release, compared with DNP-BSA sensitized cells.

After stimulation with antigen, cells also release β-hexosaminidase, another marker of mast cell degranulation. Thus, to determine whether *B. hispida* extract could modulate Ag-induced β-hexosaminidase release, IgE stimulated RBL-2H3 cells were treated with *B. hispida* extract and then challenged with DNP-BSA. The degranulating effect of *B. hispida* extract, measured by β-hexosaminidase release from allergen-

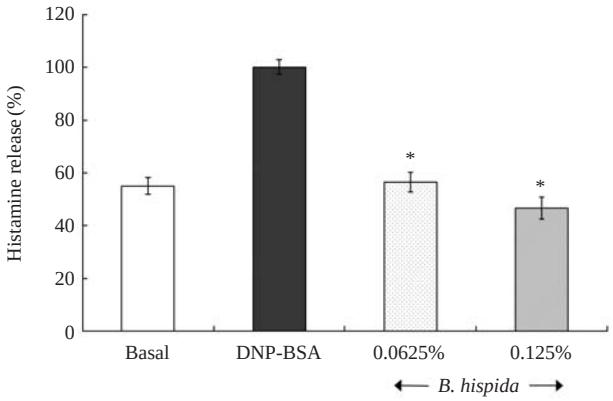

Figure 2. Inhibition of histamine release by treatment with *Benincasae hispida* extract in RBL-2H3 cells. *$P<0.05$, significantly different from the DNP-BSA challenged value.

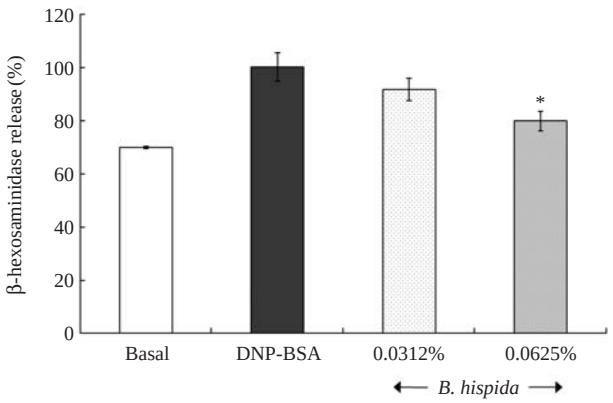

Figure 3. Inhibition of β-hexosaminidase release by treatment with *Benincasae hispida* extract in RBL-2H3 cells. *$P<0.05$, significantly different from the DNP-BSA challenged value.

sensitized RBL-2H3 cells, is depicted in Figure 3. About 30% of the β-hexosaminidase was induced and released after DNP-BSA challenge. Upon treatment with 0.0312% *B. hispida* extract, however, β-hexosaminidase release was reduced by 10%, compared with DNP-BSA sensitized cells. Treatment with 0.0625% of *B. hispida* extract showed a 20% inhibitory effect on the β-hexosaminidase release, compared with DNP-BSA sensitized cells.

These results demonstrate that the allergic inflammation-mediating histamine and β-hexosaminidase release were markedly inhibited by treatment with *B. hispida* extract, suggesting that the extract may be a candidate anti-allergic agent. Tea polyphenols[25] and xanthones from *G. mangostana*[26], and guarana seed extract[27] and acanthopanax[28], have been previously reported to suppress mast cell degranulation.

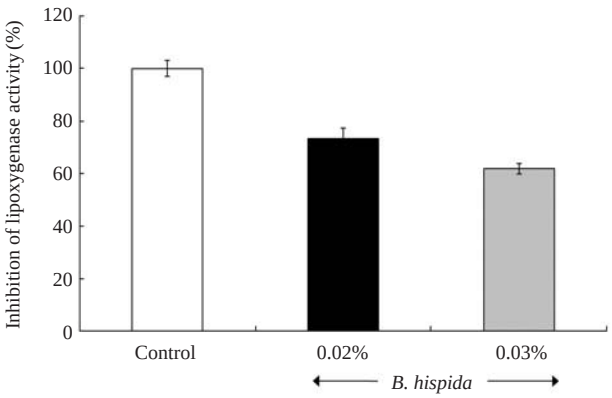

Figure 4. Inhibition of lipoxygenase activity by treatment with *Benincasae hispida* extract.

Lipoxygenase Inhibition *in vitro*

The effect of the aqueous extract of *B. hispida* on *in vitro* lipoxygenase activity was examined (Figure 4). Lipoxygenases comprise a family of non-heme iron-containing dioxygenases, representing the key enzymes in the biosynthesis of leukotrienes that have been postulated to play an important role in the pathophysiology of several inflammatory and allergic diseases[29]. Lipoxygenase activity was reduced by approximately 26 and 38% by treatment with 0.02 and 0.03% *B. hispida*, respectively. The results indicated that the *B. hispida* extract had an inhibitory effect on lipoxygenase activity.

Mast cells produce and release leukotrienes and prostaglandins, as well as histamine. The release of leukotrienes and prostaglandins is caused by the activation of phospholipase A_2, an intracellular Ca^{2+}-dependent enzyme, an effect that is followed by the oxidation of fatty acids with lipoxygenase. Several lipoxygenase inhibitors reduce the release of both leukotriene and histamine from mast cells. Tea polyphenols have been suggested to suppress leukotriene release through the inhibition of lipoxygenase activity[30].

Discussion

To treat allergic inflammation, several antagonistic drugs are used to block the action of allergic mediators, or to prevent the activation of cells and degranulation processes. These include antihistamines, steroids, such as cortisone, dexamethasone, and hydrocortisone, epinephrine, and antileukotrienes. In alternative medicine a number of allergy treatments, particularly from traditional herbal medicine, are known.

In conclusion, our study demonstrated that *B. hispida* extract was capable of alleviating IgE mediating secretion of histamine and β-hexosaminidase from mast cells. It is also capable of reducing IgE levels in mouse serum and decreasing IL-4 and TNF-α levels in bronchoalveolar lavage fluid. These results show that *B. hispida* extract has a notable suppressive effect on allergic inflammation. Thus, *B. hispida* extract may be useful in alleviating a number of allergic inflammations, including atopic dermatitis, asthma, and rhinitis. However, it is not clear whether the inhibitory effect of the extract on allergic inflammation was due to some unidentified component, which displayed an inhibitory effect on the degranulation of mast cells and in preventing increase in serum IgE levels. Thus, it is necessary to further characterize the active components responsible for the anti-allergic inflammatory action and elucidate the targets of those components, as well as the detailed mechanism by which the *B. hispida* extract suppresses allergic inflammation.

Materials & Methods

Preparation of *Benincasae hispida* Extract

Benincasae hispida was provided by Susin Ogapy Co. Ltd. (Cheonan, Chungnam, Korea). The whole fruit was freeze-dried, and the dried powder was then dissolved in sterile phosphate-buffered saline (2% w/v). After centrifugation (12,000 × *g*, 5 min), the supernatant was collected and used in this experiment.

Sensitization and Challenge

BALB/c male mice were obtained from Orient Bio Co. Ltd (Seoul, Korea). Mice, 7 weeks old, weighing 18-19 g, were acclimatized for 2 days at 25 ± 2°C and a normal day/night cycle before starting the experiment. The mice were sensitized with 0.2 mL of normal saline containing 500 μg/mL ovalbumin (OVA; Sigma, St. Louis, MO, USA), adsorbed on 100 mg/mL aluminum hydroxide, intraperitonially (i.p.) on days 0 and 14. Seven days after the final i.p. injection, the mice were given the sample orally on days 21, 22, and 23. At 30 min after oral administration, 100 μL OVA (150 μg/100 μL) inhalation was performed.

All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Soonchunhyang University.

Measurement of OVA-specific IgE

On day 24, each mouse was sacrificed and a blood sample was collected. The sera were separated by centrifugation (13,000 × *g*, 10 min) and kept at −70°C until analysis for IgE. OVA-specific IgE levels were measured by enzyme-linked immunosorbent assay (ELISA). The results are expressed as ng/mL of serum.

The plates were coated with diluted anti-mouse IgE overnight at 4˚C and then 1 : 250 diluted sera were added to the wells and the plates were incubated for 2 h at room temperature. The bound IgE was detected with biotinylated anti-mouse IgE antibodies and the plates were developed by the addition of horseradish peroxidase and 3,3′,5,5′-tetramethylbenzidine, and measured using a plate reader at 450 nm.

Cytokine Determination by ELISA

Shortly after exsanguination, the trachea was cannulated and 1 mL of saline was used per lavage and repeated four times for each mouse. About 4 mL of bronchoalveolar lavage (BAL) fluid was centrifuged ($12,000 \times g$, 10 min) and the supernatant was kept at −70˚C until analysis for cytokines. IL-4 and TNF-α in the BAL fluid were measured using a modified ELISA method, as described in our previous report.

MTT Assay for Cell Viability

The MTT assay was used to determine the maximum concentration of the extract that did not affect cell viability, as described previously[28,31].

Histamine and β-hexosaminidase Release

The rat basophile leukemia cell line RBL-2H3 was maintained in DMEM with 10% fetal bovine serum and 100 unit/mL penicillin/streptomycin at 37˚C and 5% CO_2[32]. Cells (2×10^4 cells/well) were precultured at 37˚C for 24 h in 0.1 mL of medium per well in a 96-well plate. The supernatants were discarded and the cells were incubated at 37˚C for 2 h with DMEM containing 2% FBS and anti-DNP IgE. The cells were washed three times with HEPES buffer. After incubating in 0.1 mL of HEPES buffer containing *Benincasae hispida* extract at 37˚C for 10 min, the cells were challenged with DNP-BSA (4 μg/200 μL) at 37˚C for 35 min. The plate was placed at 4˚C to stop the reaction. Next, 0.2 mL of 1 N NaOH and 0.1 mL 1% *o*-phthalaldehyde (OPT) was added at room temperature, 5 min after the supernatant was removed to a 24-well plate. The reaction was stopped with 0.2 mL/well of 0.1 N HCl. Signals were quantitated using a fluorometer (405 nm excitation and 450 nm emission). Results are expressed as a percentage of the total release minus the spontaneous release. Analyses were performed in triplicate.

β-hexosaminidase assays were performed on the same cell culture conditions as those used for the histamine assay. After stimulation by DNP-BSA, the cells were centrifuged ($5,000 \times g$, 1 min) and the supernatants were collected and chilled on ice. Then, 50 μL of each sample was incubated with 50 μL of 1 mM ρ-nitrophenyl-β-acetyl-D-glucosamide (Sigma), dissolved in 0.1 M citrate buffer (pH 5), in a 96-well plate at 37˚C for 1 h. The reaction was stopped by the addition of 200 μL/well of 0.2 M glycine buffer (pH 10.7). The absorbance of the samples was measured using a plate reader at 407 nm.

Lipoxygenase Activity Determination

The assay mixture contained a sufficient amount of soybean lipoxygenase and 0.02% or 0.03% *B. hispida* extract in 0.1 M borate/NaOH buffer (pH 9.0). Next, 500 μM linoleic acid was added after pre-incubation at 30˚C for 10 min. The reaction was started by the addition of the enzyme, and increases in UV absorption at 234 nm were measured at 25˚C for 1 min. One unit (U) of activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of hydroperoxy linoleate/min under the assay conditions. The ε value used for the calculations was 25,000 mol^{-1}/L cm^{-1}[33].

Statistical Analysis

Statistical analyses were performed using SPSS statistical software (SPSS vesion 13.0). Treatment effects were analyzed using one-way ANOVA, followed by Duncan's multiple range tests. $P < 0.05$ was used to indicate significance.

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