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Aqueous extract of the *Helianthus annuus* seed alleviates asthmatic symptoms *in vivo*

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Abstract. Molecular inflammation is a pivotal process in various degenerative immune diseases, including asthma and atopic dermatitis. In this study, we examined the effects of *Helianthus annuus* seed (HAS) aqueous extract on an *in vivo* anti-asthmatic model. Ovalbumin-induced mice were orally administered the aqueous extract of *Helianthus annuus* seeds, and their lungs were assessed by hematoxylin and eosin staining. Moreover, the expression levels of IL-4/IL-13 cytokines and IgE were determined. HAS extract induced a decrease in $CD4^+$ cell number, IL-4/IL-13 expression, and IgE secretion levels in the lungs. Our findings collectively suggest that the HAS extract has considerable potential in reducing the asthma-like symptoms induced by a mouse ovalbumin challenge model. However, further isolation and purification of the extract is required to determine the specific factor(s) responsible for its anti-asthmatic activity.

Introduction

While oxygen is a prerequisite for life, concentrations above the physiological limit are dangerous for cells (1). It is critical for the lungs, which are directly exposed to oxygen, to possess defense systems against oxidative attack. Antioxidants can alleviate hazardous symptoms induced by a wide variety of radicals (2). Free radical species, from metabolic reactions, are detoxified by antioxidants from food sources. In human degenerative disorders, such as asthma and chronic obstructive pulmonary disease, free radicals are the main cause of symptoms (3). Cellular antioxidants are required to attack the remaining number of radicals (4). Radicals trigger chronic obstructive pulmonary diseases, such as asthma, in the case of epithelial cell attack near the bronchoalveolar lavage (5).

Asthma is an inflammatory disorder of the airways, and the current focus in its management is the control of molecular inflammation (6). In bronchial asthma, various factors result in the infiltration of mast cells, eosinophils and Th2 lymphocytes into lesions with downstream mediators, leading to the formation of asthmatic phenotypes, such as mucous hyperproduction, airway hyper-responsiveness, and submucosal thickness (7).

Helianthus annuus (HA) has been an important resource of natural oil and lipid-rich nutrients for centuries. Additionally, HA is used as a preventive medicine against diuresis, diarrhea, and several inflammatory diseases (8). Recently, we showed that HAS inhibits cell damage induced by hydrogen peroxide or amyloid ß-peptide in SH-SY5Y neuroblastoma cells (9).

Sunflower-processing workers are exposed to pollen with high allergenic potential, particularly in the case of close contact (10). However, *Compositae* plants contain helianol as the predominant component (29-86%) in triterpene alcohol fractions, which exhibits anti-inflammatory activity against 12-O-tetradecanoylphorbol-13-acetate-induced inflammation in mice (11).

Here, further studies on the mechanisms of HAS reveal that it possesses potent anti-asthmatic activity *in vivo*. The major finding of this report is that HAS exhibits both antioxidant and anti-asthmatic activities *in vitro*, as confirmed by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric ion-reducing antioxidant power (FRAP) assays. The IL-4, IL-13, IgE, and $CD4^+$ levels *in vivo* were evaluated by immunohistochemistry.

Materials and methods

Cell culture. For the MTT assay, murine melanoma B16 cells were cultured at 37˚C in a humidified atmosphere of 5% CO_2

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Abbreviations: HAS, *Helianthus annuus* seeds; DPPH, 1,1-diphenyl-2-picrylhydrazyl; Fe(III)-TPTZ, ferric tripyridyltriazine; EDTA, ethylenediamine tetraacetic acid; FRAP, ferric ion-reducing antioxidant power; MMP, matrix metalloproteinase; IL, interleukin

Key words: Helianthus annuus seed, anti-asthmatic, ovalbumin-induced, interleukin-4, interleukin-13, IgE

and 95% air in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/ml of penicillin plus streptomycin.

Chemicals. 1,1-Diphenyl-2-picrylhydrazyl (DPPH; Sigma, St. Louis, MO) and ferric tripyridyltriazine (Fe(III)-TPTZ; Sigma) were employed for antioxidant assays. All other materials were of commercial grade.

Preparation and fractionation of samples. *Helianthus annuus* seeds were obtained from a farm in Chilgok, Korea. Seeds were placed in an oven at 60˚C for 12 h. Dried seeds were incubated in water (1:2; w/v) for 18 h, and centrifuged for 10 min at 10,000 rpm to collect supernatant fractions which were stored at -70˚C. The supernatant fractions were used for various *in vitro* and *in vivo* assays (data not shown). Plants were collected between October and November 2005, and identified by a senior member of the Department of Biological Sciences, Kyungpook National University, Daegu, Korea. The voucher specimens of plants have been deposited in the Enzyme Biotechnology Laboratory, KNU (# 2005-11).

Antioxidant assays. A DPPH assay was performed using a commercially available free radical ($DPPH^{\cdot+}$, 2,2 diphenyl-1-picrylhydrazyl), which is soluble in 50% methanol (12). Antioxidant activity was measured by a decrease in absorbance at 515 nm (13). An FRAP assay was developed to measure the ferric-reducing ability of plasma at a low pH (14). Reduction of the ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex into the ferrous (Fe^{2+}) form was accompanied by an intense blue color. Absorption was recorded at 593 nm.

T-bet promoter assay. A T-bet promoter assay was performed to evaluate whether HAS upregulates promoter activity (15). In brief, a T-bet promoter region (from positions -1836 to +192) was cloned and inserted into the pGL2-neo vector using two restriction enzyme sites (*Xho*I and *Hind*III). The neomycin region of pGL2-neo was cloned into the pGL2 luciferase reporter vector (Promega, Madison, WI, USA). The pGL2-neo/T-bet promoter vector was transfected into Raw 264.7 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and selected with G418 (700 μg/ml). Raw 264.7 cells containing the pGL2-neo/T-bet promoter were serially diluted in 96-well plates, and a single colony was selected by repeated experiments. We assessed the luciferase activity patterns of selected cells stimulated with PMA, LPS and Con A (as mitogens) at 20 h or left untreated. We selected LPS as the optimal T-bet promoter activator, and performed subsequent promoter experiments to determine HAS extract activity using LPS.

Protection against cell death by extracts. Protection against cell death was assayed using a CCK-2 kit (Dojindo, Gaithersburg, MD), as follows: cells ($5x10^5$/ml) were plated in 96-well plates, and incubated for 24 h in 100 μl of RPMI medium. Various concentrations of extract were added to the cells and incubated for an additional 48 h. Next, 10 μl of MTT solution (5 mg/ml MTT in PBS) was added to each well, followed by incubation at 37˚C for 4 h. To terminate the reaction, 100 μl of 0.04 M HCl was added in isopropanol with vigorous mixing. Absorbance was determined with a Victor multilabel counter (Wallac, Turku, Finland) at 564 nm.

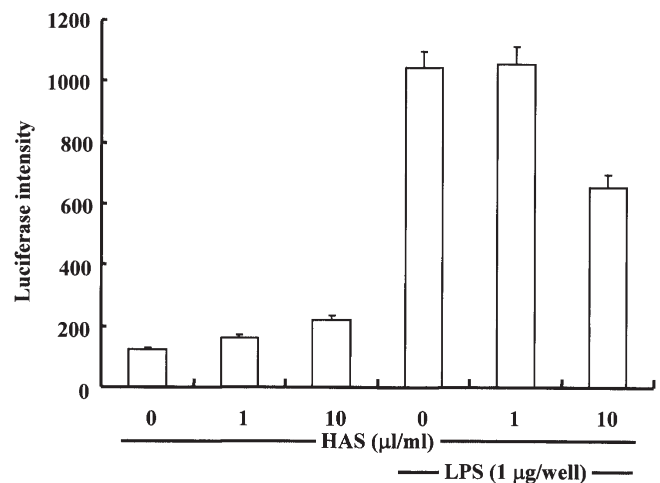

Figure 1. T-bet promoter assay used to determine activity of the water-extracted fraction of *Helianthus annuus* seed. A T-bet promoter region (from positions -1836 to +192) was cloned and inserted into the pGL2-neo vector using two restriction enzyme sites (*Xho*I and *Hind*III), as described in Materials and methods. The vector was cloned into the pGL2 luciferase reporter vector (Promega, Madison, WI, USA), and the pGL2-neo/T-bet promoter vector was transfected into Raw 264.7 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and selected with G418 (700 μg/ml). Raw 264.7 cells containing the pGL2-neo/T-bet promoter were serially diluted in 96-well plates, and a single colony was selected by repeated experiments as described in Materials and methods.

Determining IL-4, IL-13, and IgE levels by ELISA. IL-4 and IL-13 levels were determined by sandwich ELISA, with slight modifications (16). Cytokine levels in serum were expressed as pg/mg of the total protein amount. The serum IgE level was measured with a commercial ELISA kit (17).

Ovalbumin-induced animal model. Mice underwent ovalbumin (OVA) sensitization and challenge according to previous protocols, with slight modifications (18-20). OVA (100 μg/ml in a saline, sterile-filtered) was mixed with an equal volume of 10% (w/v) aluminum potassium phosphate (alum; Sigma), and its pH was adjusted to 6.5 with 10 N NaOH.

Histopathology and immunohistochemistry. Tissues were embedded in paraffin, cut into 4- to 6-mm sections, and set overnight on a slide warmer at 37˚C. Paraffin was removed by dipping in xylene 3 times for 10 min. Xylene was removed by dipping serially in 70-100% ethanol for 60 min. After washing, peroxidase bleaching was performed via 1% BSA/PBST for 30 min at 4˚C. Hematoxylin and eosin (H&E) staining was performed according to previous reports (21,22). For each mouse, eight randomly selected airways of the left lung were analyzed. Lung tissues were fixed for 24 h in 10% neutral buffered formalin solution, and routinely processed for immunohistochemical analysis. Paraffin sections were placed on Probe-On slides, and incubated with methanol containing 3% hydrogen peroxide to inhibit endogenous peroxidase activity. Tissue sections were treated with 10% normal goat serum for 1 h at room temperature to block non-specific binding. Slides were subsequently incubated overnight at 4˚C

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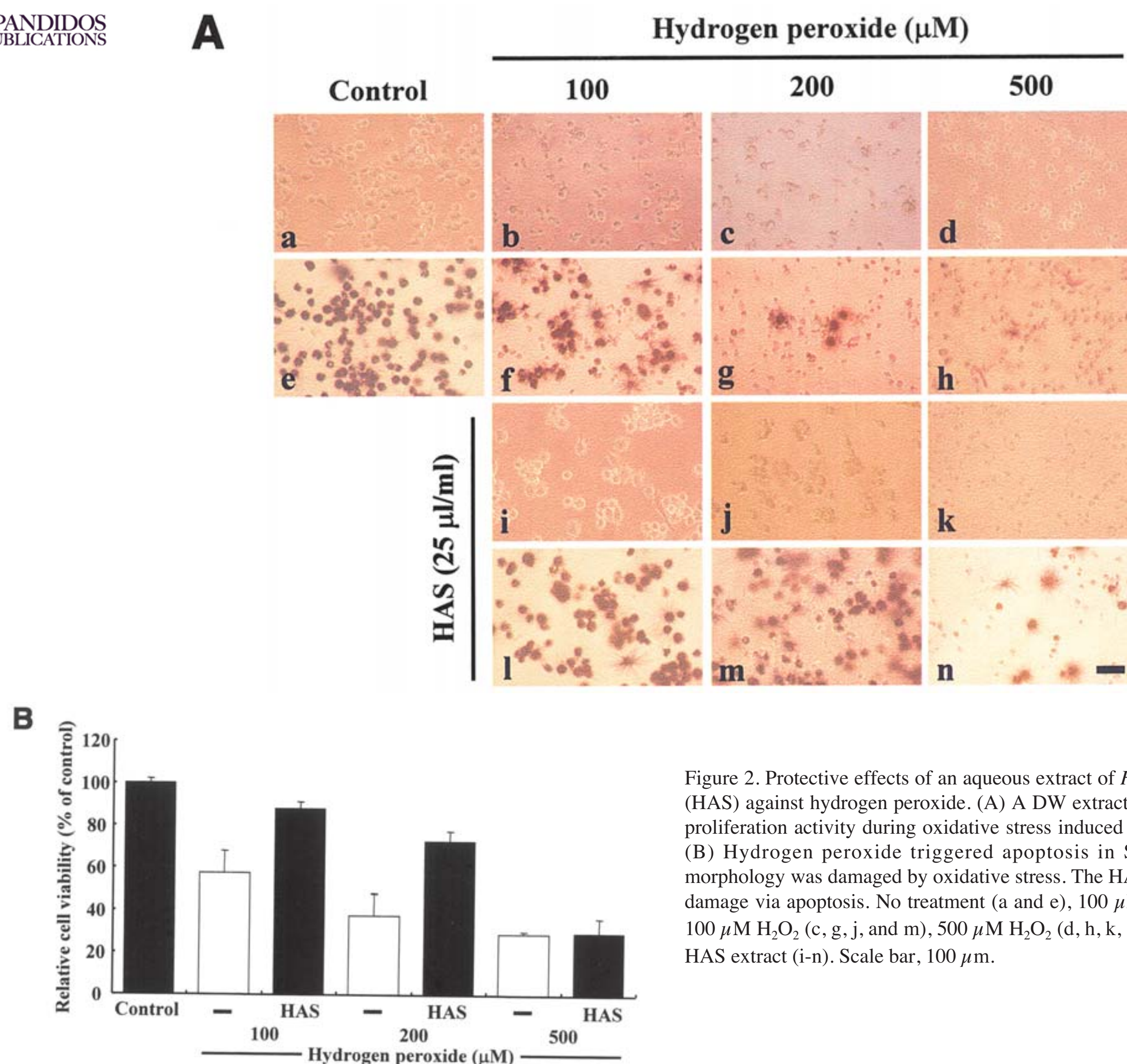

Figure 2. Protective effects of an aqueous extract of *Helianthus annuus* seed (HAS) against hydrogen peroxide. (A) A DW extract of HAS increased cell proliferation activity during oxidative stress induced by hydrogen peroxide. (B) Hydrogen peroxide triggered apoptosis in SH-SY5Y cells. Cell morphology was damaged by oxidative stress. The HAS extract repaired cell damage via apoptosis. No treatment (a and e), 100 μM H_2O_2 (b, f, i, and l), 100 μM H_2O_2 (c, g, j, and m), 500 μM H_2O_2 (d, h, k, and n), and 25 μl/ml of HAS extract (i-n). Scale bar, 100 μm.

with rabbit anti-IL-4 or anti-IL-13 antibody (Santa Cruz Biotechnology; 1:200). Periodic Acid Schiff (PAS) staining was performed using a previous protocol (20).

Statistical analysis. Data were expressed as the means ± standard deviation. Statistical significance was determined by the Student's t-test for independent means using the Microsoft Excel program (23). The critical level for significance was set at $P<0.05$.

Results and discussion

In this study, we established that the aqueous extract of *Helianthus annuus* seed (HAS) inhibits oxidation, and consequently asthmatic activity, both *in vitro* and *in vivo*. *Helianthus annuus*, a plant grown worldwide, is used for industrial purposes, such as oil extraction, nutritional supplements, and food resources. Reports demonstrate that compounds found in the leaves and/or flowers of *Helianthus annuus* inhibit fever and rheumatoid arthritis (24). Moreover, in Asian countries, the seed has long been regarded as a diuretic. *Helianthus annuus* is considered a nutraceutical supplement in the development of functional food due to its efficacy in treating specific ailments and its unsaturated fatty acid content. The extracts can be used in food sub-ingredients. Further studies on the *in vivo* sub-acute or chronic toxicological properties and efficacy of HAS extracts are required to evaluate their suitability as a functional food. We have already shown that HAS protects against cell damage induced by hydrogen peroxide or amyloid ß-peptide in SH-SY5Y neuroblastoma cells (9). At a dose range of 6 g to 60 kg/day, HAS did not induce morphological toxicity at the tissue level (data not shown). The enhanced cell protection capability of HAS in an *in vitro* cell damage assay system(s) supports its potential application as a protective agent. However, there have been no reports on other biological activities of HAS extracts. With the aid of various *in vitro* and *in vivo* anti-asthmatic assays, we determined the specific activities of HAS extracts in this study. Our data showed that HAS extracts exert potent anti-asthmatic effects, based on antioxidant activity.

Levels of oxidative stress increase in children and adults with asthma, both in the circulation and lungs. Oxidative stress is the final result of numerous molecular events and mechanisms in which the endogenous antioxidant defense systems are overcome by free radicals, resulting in the classical physiological asthma symptoms (2). Therefore, to develop novel effective anti-asthmatic therapies, clarification of the molecular mechanisms underlying the molecular inflammatory process and oxidative stress is essential (25).

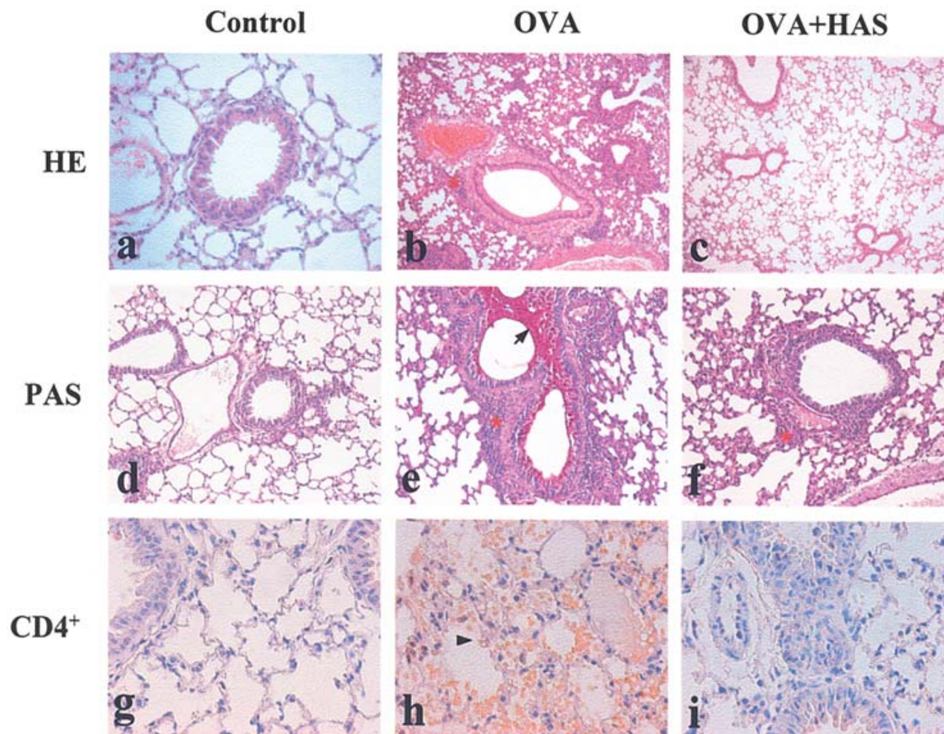

Figure 3. Aqueous extract of *Helianthus annuus* seed (HAS) inhibits the immune response in an ovalbumin-induced asthma model. Immunohistochemistry of an asthmatic animal model shows that HAS extracts inhibit $CD4^+$ cells by controlling immune cell proliferation in lung tissue. The lung tissues of asthma animal models revealed $CD4^+$ cells (g-i), PAS-positive cells (d-f), and hematoxylin and eosin (HE)-stained positive cells (a-c) in the presence or absence of HAS. Part of the immune response (arrowhead in h) is shown to convey the exact distribution of asthma-related immune cells.

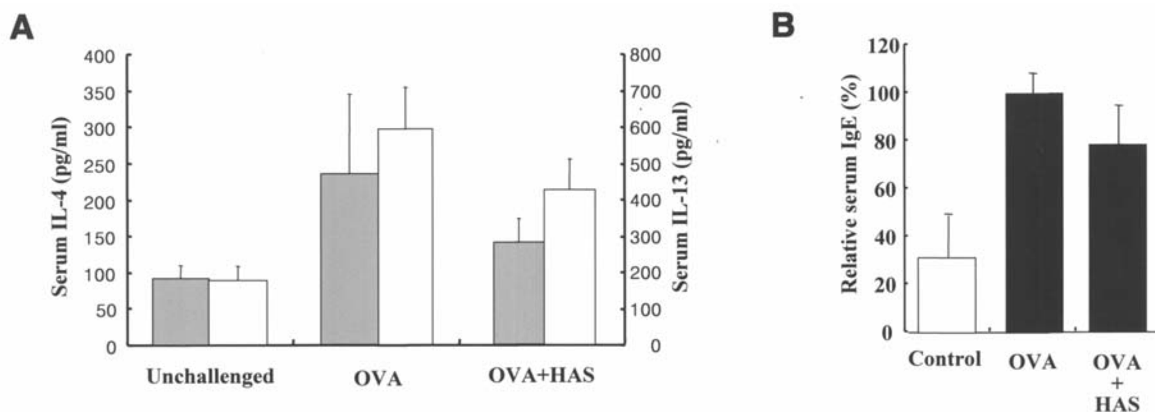

Figure 4. Inhibitory effects of aqueous extracts from *Helianthus annuus* seed (HAS) in an OVA-challenged asthmatic animal model. Serum IL-4 (A, shaded columns), IL-13 (A, white columns) and IgE (B) levels were determined using ELISA kits, as described in Materials and methods.

We hypothesized that comparison of the T-bet promoter activity between the control and HAS-treated samples should disclose the indirect biological activity of HAS *in vivo*, via examination of asthmatic symptoms. Accordingly, a vector containing the T-bet promoter insert (pGL2-neo/T-bet promoter) was introduced into the Raw 264.7 cell line. LPS-treated cells exhibited higher luciferase intensity indicative of T-bet promoter activity, whereas LPS-treated cells incubated with HAS displayed suppressed promoter activity in a dose-dependent manner (Fig. 1). In the absence of LPS, cells displayed a mild increase in luciferase intensity. These results suggest that HAS exerts dual effects by increasing or decreasing the promoter activity in the presence or absence of LPS. T-bet promoter activity was reconfirmed using antioxidant assays, such as DPPH and FRAP (data not shown). Next, we treated cells with hydrogen peroxide (0-500 μM) with or without the extracts. SH-SY5Y neuroblastoma cells were grown up to 24 h after cell splitting, and the viability was measured following hydrogen peroxide damage (Fig. 2). Cell morphology findings revealed that hydrogen peroxide induced cell death in a concentration-dependent manner (Fig. 2Aa-d), which was clearly confirmed with the MTT assay (Fig. 2Ae-h). Upon the addition of HAS (25 μl/ml), viable cell numbers were significantly restored (up to 70% at a concentration of 100 μM HAS) following hydrogen peroxide onset. The level of protection was

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...d again in Fig. 2B. The protection percentage was ...ith 200 μM hydrogen peroxide, whereas no protection was evident with 500 μM. This protection was confirmed by experiments showing that HAS alleviates classical apoptotic cell death in a concentration-dependent manner (data not shown).

Next, we investigated the *in vivo* protective effects with an ovalbumin-induced asthma model. All mice were sensitized intraperitoneally with 100 μg/head of OVA on days 0 and 12. Mice were exposed to aerolized OVA or saline for 1 h on days 21 and 24. Following treatment with HAS extract (50 μg/ml) every 2 days for 2 weeks, histopathological examination of tissue samples was performed. H&E staining revealed clear tissue in the control (Fig. 3a), whereas several immune-positive cells were evident around the small parts of the lungs in OVA-treated tissue (Fig. 3b). PAS staining disclosed similar patterns to those obtained with the H&E stain (Fig. 3d and e). However, the number of total leukocytes from the OVA-challenged group treated with HAS (25 μl/ml) decreased significantly, compared with that of OVA-challenged lung tissues (Fig. 3c and f). We assessed the $CD4^+$ cell content by immunohistopathological staining, as shown in Fig. 3g-i. No changes were evident in lung tissues.

To evaluate the effects of HAS on molecular markers related to asthma, we measured the IL-4 and IL-13 content with ELISA kits. The IL-4 content was ~106.3±8.9 pg/ml in the control group, but ~250.2±35.5 in the OVA-challenged group. This increase in the IL-4 level may explain why the OVA-induced immune response in leukocytes increased up to 5.5-fold. However, treatment with HAS suppressed the IL-4 level to ~42% (Fig. 4A). We assumed that the IL-13 level would similarly decrease in the HAS-treated group. Consistent with this theory, 30% IL-13 expression was observed in the OVA plus HAS-treated group (Fig. 4A). This phenomenon was also evident in HAS-treated lung tissues (Fig. 4C). ELISA data were confirmed by IgE expression (Fig. 4B), a final event of molecular inflammation by mast cells.

In conclusion, this is the first report to show that HAS extracts exhibit anti-asthmatic activity *in vitro* and *in vivo* via a IL-4/IL-13-dependent mechanism. Manipulation of anti-asthmatic activity by regulation of the asthma-related molecular signal(s) may allow elucidation of the precise atopic dermatitis mechanism. Future studies will focus on the purification and characterization of the potent anti-asthmatic factor(s) of HAS.

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