

Inhibition of Mite-Induced Immunoglobulin E Synthesis, Airway Inflammation, and Hyperreactivity by Herbal Medicine STA-1

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The availability of STA-1 in suppressing allergen-induced immunoglobulin E (IgE) synthesis, airway inflammation and hyperreactivity in a murine model was investigated. The mice were intraperitoneally sensitized with *Dermatophagoides pteronyssinus* group 5 allergen (Der p 5) and orally treated with 300 mg/kg of STA-1 every other day for 14 days. The Der p 5-specific immunologic responses including changes of specific immunoglobulin G and E, cells in the broncholarvage fluid, and airway hyperreactivity were measured when mice received inhalation challenge with Der p 5 after sensitization for 21 days. By comparing with sham-treated groups, the synthesis of Der p 5-specific IgE was downregulated while the influx of eosinophils and neutrophils in the airway were remarkably reduced. In addition, Der p 5-induced airway hyperreactivity also was significantly eliminated by STA-1 treatment. These results showed that STA-1 could effectively suppress the Der p 5-induced allergic reactions, and the availability of STA-1 for the treatment of allergic asthma was demonstrated in this study.

Keywords Airway Hyperreactivity, Allergy, Immunoglobulin E, Traditional Chinese Medicine.

INTRODUCTION

The increasing incidence and prevalence of asthma in the world has made it a global health concern,⁽¹⁾ not to mention that the morbidity and mortality of asthma have increased in the past two decades.^(2,3) A consensus definition for asthma recognizes this disorder to be a chronic inflammatory disorder of the airways in which many cells and cellular elements including mast cells, eosinophils, T lymphocytes, neutrophils, epithelial cells, and macrophages play important roles in the inflammatory airway process. The inflammation also relatively heightens the existing bronchial hyperresponsiveness to a variety of stimuli.^(3,4) Unfortunately, current treatments for allergic diseases are not satisfactory. The most common treatment nowadays is the use of anti-inflammatory drugs, including steroids. However, long-term steroid therapy is often associated with multiple debilitating effects.^(2,5)

Traditional Chinese medicine (TCM), used in Asia for centuries, thus has become attractive in as a source of alternative or complementary therapies for asthma.^(6–8) For example, xiao-qing-long tang was shown to suppress airway inflammation by decreasing the number of total cells and eosinophil infiltration in the bronchoalveolar lavage fluid (BALF) of allergen-challenged mice.⁽⁹⁾ Another remedy, ding-chuan-tang, exhibits significantly inhibited asthmatic responses, and airway hyperreactivity and reduced the percent eosinophils in BALF on sensitized guinea pigs.⁽¹⁰⁾

A TCM formula, STA-1 (Table 1), was modified from mai-men-dong-tang (MMDT) and lui-wei-di-huang-wan (LWDHW) that were originally formulated by two outstanding physicians: Zhang Zhong-Jing (Han Dynasty, 150–219 AD) and Qian Yi (Song Dynasty, 1035–1117 AD). Traditionally, practitioners of Chinese medicine believed that these two formulaes could treat various lung disorders, especially bronchial asthma. In clinical practice, MMDT has been used as an expectorant, anticough agent, for bronchitis, and as a remedy

Table 1: Composition of STA-1 and STA-2.

Medicinal plants (weight ratio)	Family	Species
Radix Rehmanniae Preparata(8)	Scrophulariaceae	<i>Rehmannia glutinosa</i> Libosch.
Cortex Moutan Radicis(3)	Ranunculaceae	<i>Paeonia suffruticosa</i> Andr.
Radix Ophiopogonis(8)	Liliaceae	<i>Ophiopogon japonicus</i> (Thumb.) <i>Ker-Gawl</i>
Tuber Pinellia(2)	Araceae	<i>Pinellia ternate</i> (Thumb.) Breit.
Fructus Corni(4)	Cornaceae	<i>Cornus officinalis</i> Sieb. et Zucc.
Poria(3)	Polyporaceae	<i>Poria cocos</i> (Schw.) Wolf
Radix Panacis Quinquefolii(3)	Araliaceae	<i>Panax quinquefolium</i> L.
Rhizoma Alismatis(3)	Alismataceae	<i>Alisma orientalis</i> (Sam.) Juzep.
Radix Dioscoreae(4)	Dioscoreaceae	<i>Dioscorea opposita</i> Thumb.
Radix Glycyrrhizae(2)	Leguminosae	<i>Glycyrrhiza uralensis</i> Fisch.

for asthma.^(11,12) According to basic TCM theory, LWDHW was used to treat asthmatic children who were diagnosed as “deficiency of kidney energy” by Chinese doctors⁽¹³⁾ and can enhance the effect of lung meridian drug, such as MMDT, in asthma patients. Previous reports also showed that LWDHW and MMDT had wide pharmacological effects in modulating immune responses, providing improvement in lung function, and relieving asthma symptoms.^(11–15) Thus, the combination of these two prescriptions is likely to satisfy the needs in asthma treatment.

Dust mites have been the center of many researches because house dust mites have been regarded as the major source of indoor allergens.^(16,17) The *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* are two of the important clinical mite species as ~60% of the mite-allergic patients tested positive for *Dermatophagoides pteronyssinus* group 5 allergen (Der p 5).^(18,19) Allergic sensitization plays a significant role in the development of asthma in many patients.⁽²⁰⁾ Also, patients IgE levels correlated with asthma severity and bronchial hyperresponsiveness.⁽²¹⁾ Therefore, the development of a treatment that interrupts this pathway is particularly desirable to prevent downstream events.

In this study, our murine model⁽²²⁾ established for asthma studies was used to examine the immunomodulatory effect of STA-1 on allergen-induced immunologic responses. These effects were changes of specific immunoglobulin G and E, cells in the bronchoalveolar fluid, and airway hyperreactivity of sensitized mice.

MATERIALS AND METHODS

Animals

Specific pathogen-free, female BALB/c mice aged between 6 and 8 weeks were obtained from the animal breeding center of the College of Medicine, National Taiwan University (originated from the Jackson Laboratory, Bar Harbor, ME, USA). All experimental animal care and treatment followed the guidelines set up by the National Science Council of the Republic of China. Mice were age- and sex-matched for each experiment.

Sensitization

Mice were initially intraperitoneally (ip) sensitized with 10 µg of Der p 5 with 4 mg of aluminium hydroxide (Wyeth Pharmaceuticals, Punchbowl, Australia). Recombinant Der p 5 protein was prepared as described.⁽²²⁾ Then 14 days after sensitization, mice were injected with booster shots of Der p 5. Next, 7 days after the booster injection, mice were anesthetized 1P by 0.1 mg of PromAce® (Ayerst Laboratories, NY, USA). Additionally, sera were collected from the tail vein each week after sensitization.

Herbal Preparation and Dispensing

The pharmaceutical composition that consists of 10 herbs as listed in Table 1 was prepared by two different methods.

The first method required grinding 6 herbs (LWDHW): *Radix Rehmanniae Preparata*, *Cortex Moutan Radicis*, *Fructus Corni*, *Poria*, *Rhizoma Alismatis* and *Radix Dioscoreae*, and passing them through a sieve to produce herbal powder. Then 4 herbs (MMDT)—*Radix Ophiopogonis*, *Radix Glycyrrhizae*, *Radix Panacis Quinquefolii* and *Tuber Pinellia*—were stirred and soaked in distilled water for 30 min at room temperature, then boiled for 1 hr, and condensed to form a part B mixture. Last, the part B mixture was dried in a fluidized apparatus and mixed with the part A powder. The mixture produced by this method is STA-1

The second method required the herbs of LWDHW and MMDT to be stirred and soaked in distilled water for 30 minutes at room temperature, then boiled for 1 hr, and condensed to form part C and part D mixture, respectively. Then we mixed the two condensed mixtures, and dried at in a fluidized apparatus to form dry powder. The powder produced by this method is STA-2 The dried herbal powder contained 2–4% of water. All materials were condensed at a temperature of 50 to 60°C under vacuum condition with pressure between 400 and 650 mmHg to obtain a liquid condensate with a desirable concentration.

The standard herbal formulations (STA-1, STA-2) were prepared and donated by Sun-Ten Pharmaceutical. (Taipei, Taiwan). The plant was identified and authenticated by professor C.C. Chen, School of Chinese Medicine Resources, China Medical University. A voucher specimen was deposited at the Graduate Institute of Chinese Medical Science, China Medical University. The quality of each component herb in the regimen was checked by high pressure liquid chromatography to ensure compliance with government standard.

First, 50 mg of STA-1 or STA-2 with 20 µl of Tween 80 were homogenized and added to PBS to final volume 2 ml (25 mg/ml). Then, mice received STA-1 or STA-2 300 mg/kg every other day for 14 days by a gastric tube. The control group also was sensitized with allergen but fed with PBS alone.

Determination of Der p 5-Specific IgG2a and IgE

The amount of Der p 5-specific IgG2a and IgE were determined by ELISA. Protein high binding plates were coated with 100 µl of purified Der p 5 diluted in coating buffer (0.1M NaHCO₃, pH 8.2) to a concentration of 5 µg/ml. After overnight incubation at 4°C, plates were washed three times and blocked with 3% (wt/vol) BSA-PBS buffer for 2 hr at 25°C. Sera were used at 1:100 dilution for IgG measurement and 1:10 dilution for IgE measurement in duplicate. After overnight incubation at 4°C, either biotin-conjugated monoclonal rat antimouse IgE mAb or rat antimouse IgG mAb diluted in 0.05% gelatin buffer

was added for an additional hour. Avidin-alkaline phosphatase (1:1000) was then added and incubated for 1 hr at 25°C, followed by 6 washes. The color reaction was developed with the addition of phosphatase substrate p-nitrophenyl phosphate, disodium. Plates were read in a microplate autoreader (Metertech, Taiwan) at 405 nm. Readings were referenced to commercial isotype standards that were mouse anti-TNP mAb, IgG2a (G155–178), and IgE (IgE-3)

Aerosol Exposure and Analysis of Pulmonary Resistance

Mice were challenged with ultrasonic nebulization of 0.1% of Der p 5 diluted in PBS 21 days after sensitization. The inhalation challenge was performed in 1-L chamber connected to a DeVilbiss pulmosonic nebulizer (model 2512; DeVilbiss Corp., Somerset, PA, USA) that generated an aerosol mist. Then 8 to 18 hr after the aerosol exposure, mice were anesthetized with Promaz ip and then intubated with a 20-gauge tracheal cannula. Changes in esophageal pressure, an indication of pleural pressure, were measured using a saline-filled catheter (PE60) and a differential pressure transducer (DP45–14, Validyne Engineering Corp., Northridge, CA, USA). The esophageal catheter was advanced into the esophagus of the mouse until a clear cardiac artifact was discernible. Ventilatory flow was monitored at the trachea by a pneumotachograph (Fleisch 00000, Zabona, Basel, Switzerland) connected to a differential pressure transducer (MP45–14, Validyne). The signals from the transducer were connected to a digital electronic pulmonary monitoring system (Mumed, London, U.K.), that integrates flow to obtain volume change and calculates lung resistance (R_L) and dynamic compliance (D_{dyn}) in real time.

Experimental data were stored electronically, and experimental traces or processed data were plotted on a laser printer as required. Acetylcholine (Ac) was administered intravenously with a starting dose of 1.25 mg/kg. The average volume per Ac dose was 10 μ l. Then 2-fold-increase concentrations of Ac were administered ~5 min apart and only after transpulmonary pressure and volume had returned to within 10% of the baseline from the previous dose and before the next dose was administered. Intravenous saline in 10 μ l was administered before the first dose of Ac to establish baseline values. Ac dose-response curves for Der p 5-sensitized or sham-sensitized groups were obtained by calculating the mean \pm standard error for the percent change from baseline for individual animals at each Ac dose. PC_{100} were calculated by interpolation of the last 4 data points on the concentration-response curve.

Bronchoalveolar Lavage and Cell Counting

After lung-function parameters were measured, the mice were lavaged with 5 \times 0.5-ml aliquots of 0.9% sterile saline through a polyethylene tube introduced through tracheotomy. Lavage fluid was centrifuged (500 g for 10 min at 4°C), and the cell pellet was resuspended in 0.5 ml of Hank's balanced salt

solution (HBSS, Gibco). Total cell counts were made by adding 10 μ l of the cell suspension to 90 μ l of 0.4% trypan blue and counted under a light microscope in a Neubauer chamber. Differentiated cell counts were made from cytospin preparations stained by Leu's stain. Cells were identified and differentiated into eosinophils, lymphocytes, neutrophils, and macrophages by standard morphologic techniques. Fully 500 cells were counted under 400-fold magnification, and the percentage and absolute number of each cell type were calculated.

Safety Testing/LD₅₀ Evaluation

The toxicity of STA-1 was tested using male BALB/c mice obtained from the animal-breeding center when they were about 7 weeks old and housed *ad libitum* until they were 8 weeks old and ~20 g in weight.

Two experimental designs were used to study the toxicity of STA-1 in animal models: a single dosage group study and a multiple dosage group study. Each study group contained 10 mice. In the single dosage group, the mice were orally administered 5000 mg/kg of STA-1 or STA-2 and observed for 72 hr and then for 14 days. The total number of mice that died during the experimental period was counted to determine LD₅₀. In the multiple dosage group, the mice were orally administered 5000 mg/kg STA-1 or STA-2 for 28 consecutive days. At the end of the 28-day experiment, total number of mice that died during the experimental period was counted to determine LD₅₀. Mice fed with PBS served as placebo (sham).

Statistical Analysis

To assess changes of Der p 5-specific IgG and IgE, pulmonary resistance, and cell count after Der p 5 challenge, repeated measures for ANOVA were performed to compare the differences among groups. Following analysis of variance, Duncan multiple range test was used to differentiate differences between experimental and control groups. A $p < 0.05$ was used to indicate statistical significant difference.

RESULTS

Inhibition of Der p 5-Specific IgE Synthesis

The animals were immunized by peritoneal injection of 10 μ g of recombinant Der p 5 in conjunction with aluminum hydroxide. Serum collected from animals before Der p 5 exposure contained no Der p 5-specific IgE or IgG antibodies. Der p 5-specific IgE antibody could be detected 2 weeks after immunization. To investigate whether STA-1 formula can decrease the synthesis of Der p 5-specific IgE, mice were treated with 7 doses of STA-1 in 14 days (300 mg/kg each dose). The OD value for IgE in STA-1, STA-2, and placebo group were

0.0038 \pm 0.0542, 0.5802 \pm 0.2318, and 1.0198 \pm 0.1502, respectively. The OD value for IgG in STA-1, STA-2, and placebo group were 0.5731 \pm 0.1257, 0.5595 \pm 0.0131, and 0.6414 \pm 0.0363, respectively.

We found that Der p 5-specific IgE decreased significantly after the treatment of STA-1, compared with STA-2 or placebo group ($p < 0.05$). On the other hand, the serum levels of Der p 5-specific IgG did not show any difference among groups (Figure 1).

Decrease of Airway Hyperreactivity (AHR) in Vivo

The efficacy of STA-1 in the suppression of the synthesis of Der p 5-specific IgE synthesis prompted us to test whether oral administration of STA-1 can suppress the allergen-induced AHR. We determined the pulmonary resistance after aerosol challenge with 0.1% Der p 5-GST (Glutathion S-transferase) fused protein from ultrasonic nebulizer 21 days after sensitization. Pulmonary resistance and bronchoaveolar lavage were both performed after the inhalation challenge. Our data demonstrated that airway hyperreactivity decreased significantly 8 to 18 hr after inhalation challenge in the STA-1 and STA-2 treated animals, as compared with the sham-treated mice ($p < 0.05$) (Figure 2). Taken together, in addition to suppression of allergen-induced IgE synthesis, oral administration of STA-1 and STA-2 also can suppress allergen-induced AHR.

Suppression of Der p 5-Induced Airway Inflammation

To further characterize the effects of STA-1 on the inflammation of airway induced by Der p 5, mice were sacrificed after Der p 5 inhalation challenge

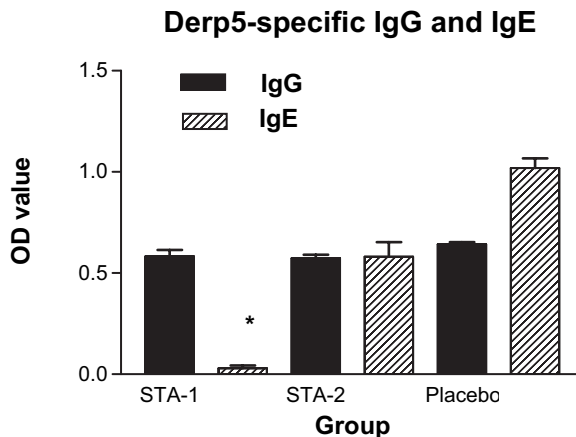


Figure 1: ELISA of Der p 5-specific IgG and IgE antibody after Der p 5 inhalation challenge. Der p 5 10 μ g adsorbed with alum were injected intraperitoneally into mice. Sera were collected for ELISA measurement 21 days after inhalation challenge (BALB/c, $n = 12$). Solid bar-IgG, Hatch bar-IgE, * $p < 0.05$.

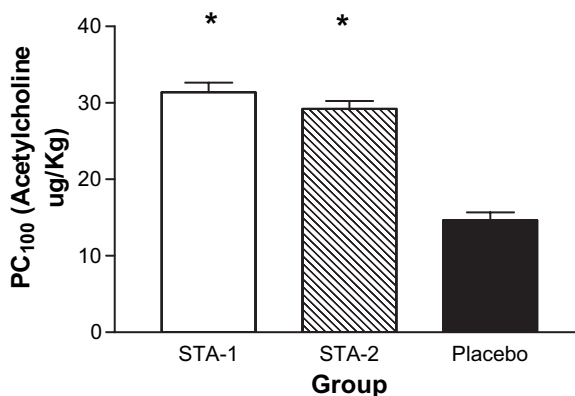


Figure 2: Mice ($n = 12$ per group) were sensitized with Der p 5 and received inhalational treatment as described. Changes of pulmonary resistance were determined and expressed as PC₁₀₀ that were calculated by interpolation of the last 4 data points on the acetylcholine concentration-response curve. * $p < 0.05$, blank bar-STA-1 group, hatched bar-STA-2 group, solid bar-placebo group.

and measurement of pulmonary resistance. Bronchoalveolar lavage fluids were collected to calculate the differential count of inflammatory cells. The results demonstrated that both neutrophils and eosinophils decreased significantly in those mice treated with STA-1, as compared with that of the STA-2 and sham-treated groups ($p < 0.05$). In contrast, there were no differences among the counts of lymphocytes and pulmonary macrophages among the three groups (Figure 3). In addition, both STA-2 formula and placebo could not suppress

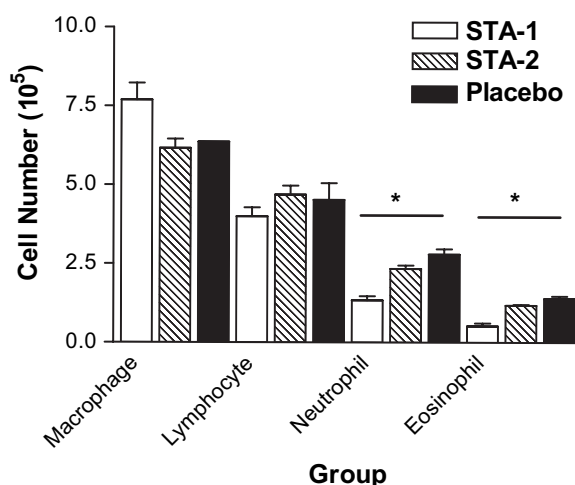


Figure 3: Inflammatory cell composition of bronchoalveolar lavage fluids. Der p 5-sensitized mice ($n = 12$ per group) were treated with STA-1, STA-2, or placebo as described. Cell differential percentages were determined by light microscopic evaluation of cytopsin preparations. Data are expressed as absolute numbers of cells. * $p < 0.05$, Blank bar-STA-1 group, hatched bar-STA-2 group, solid bar-placebo group.

the influx of neutrophils and eosinophils into the airway. We therefore concluded that oral administration of STA-1 could effectively downregulate the Der p 5-induced airway inflammation.

Safety of STA-1 and STA-2

Results in both single dosage and multiple dosage groups show that the LD₅₀ of STA-1 and STA-2 were estimated to be greater than 5,000 mg/kg. No mouse died within 72 hr after feeding 16 times the effective mouse daily dose of STA-1, STA-2, and placebo ($n=10$), or did any die during the following weeks. No mice died within 28 consecutive days after feeding on the same dose of STA-1, STA-2, and placebo ($n=10$), too. No observable abnormal clinical signs were attributable to the STA-1 and STA-2 dosing, and there was neither loss of body weight nor grossly abnormal findings. This shows that STA-1 and STA-2 had low toxicity so that STA-1 and STA-2 could be referred to as a safe medicine.

DISCUSSION

There is a need for new or alternative approaches to control allergic asthma on top of the long-term systemic steroid therapy disease.⁽⁵⁾ Complementary or alternative medicine approaches such as traditional Chinese medicine are increasingly viewed in western countries as possible additional treatments for a variety of diseases, including asthma.⁽⁶⁻⁸⁾ In this study, we investigated the *in vivo* effects of STA-1, a TCM formula, on allergen-induced bronchial inflammation and hyperreactivity in mite-sensitized mice. We demonstrated that STA-1 could reduce the synthesis of Der p 5-specific IgE tremendously, significantly downregulate the influx of neutrophils and eosinophils into airway, and decrease allergen-induced specific airway hyperreactivity.

Der p 5-specific IgE accounted for one-quarter of the total house dust mite-specific IgE.⁽¹⁸⁾ This indicates that Der p 5 is a clinically significant allergen in mite allergy. Total and house dust mite allergen-specific IgE levels were correlated with the intensity of asthma severity.⁽⁴⁾ Inhibition of IgE as a therapeutic agent is welcome because the inhibition of IgE can lead to the downregulation of both high- and low-affinity IgE receptors, thereby resulting in decreased mediator release.^(21,23) In the airway these mediators rapidly elicit bronchial mucosa edema, produce mucus, and cause smooth muscle constriction and eventually recruit an inflammatory infiltrate.⁽¹³⁾

Both STA-2 and placebo groups did not show any significant effects in the reduction of Der p 5-specific IgG2a and IgE antibody after Der p 5 inhalation challenge whereas Der p 5-specific IgE decreased from 1.0198 ± 0.1502 to 0.0038 ± 0.0542 for the STA-1 group compared with the placebo group. Also, it inhibited significantly allergen-induced specific airway hyperreactivity. Coyle

et al.⁽²⁴⁾ observed that anti-IgE treatment could block both eosinophilic inflammation and Bronchohyperreactivity (BHR) in mice after active immunization with allergen. Whether the reduction of IgE played an important role in suppressing AHR or not in this model remained unknown. STA-1 treatment significantly downregulates the synthesis of Der p 5-specific IgE level and is believed to be the basis of its therapeutic action. These findings provide experimental evidence that STA-1 exhibits an immunoregulatory effect that may be favorable for the treatment of allergic diseases.

Findings at autopsy in patients with fatal asthma showed submucosa of airway were markedly infiltrated by neutrophils and eosinophils.⁽⁴⁾ The mature eosinophil has dense intracellular granules that are sources of inflammatory proteins, including eosinophil-derived neurotoxin and cationic protein, can directly damage airway epithelium, and intensify bronchial responsiveness. These effects increase the severity of asthma.⁽²⁵⁾ The development of the late asthmatic response is associated with recruitment of eosinophils into the airway which presumably causes cellular activation and bronchoconstriction.⁽²⁶⁾ Previous studies also reported that a reduction in BALF eosinophils was accompanied by markedly reduced AHR after a variety of interventions.⁽²⁷⁾

Neutrophilic inflammation in the airway also was demonstrated during exacerbations of asthma and with status asthmaticus.^(28,29) This study showed that STA-1 significantly decreased the airway inflammatory cells infiltration, especially in eosinophils and neutrophils, and at the same time modified AHR. We hypothesize that STA-1 must regulate some cytokine or inflammation mediator expression related to eosinophils and neutrophils and can benefit in asthma treatment. Further studies are needed to verify the precise role of this herbal medicine to regulate these molecular mechanism during allergen-induced inflammation.

The only difference between STA-1 and STA-2 is the preparation procedure in six main ingredients: *Radix Rehmanniae Preparata*, *Cortex Moutan Radicis*, *Fructus Corni*, *Poria*, *Rhizoma Alismatis*, *Radix Dioscoreae*, the six ingredients of LWDHW. Our results demonstrated that STA-1 is more effective than STA-2 on allergen-sensitized mice. There are some volatile compounds obtained from plants that possess some biological activity.⁽³⁰⁾ Nevertheless, these molecules are well known to be thermally sensitive and vulnerable to chemical changes.⁽³¹⁾ We believe that some active components are lost in the preparation process of LWDHW. It is worthwhile to pay more attention to essential oil of LWDHW on allergy. Therefore, it is reasonable to hypothesis that some volatile compounds have anti-inflammatory effect in LWDHW.

Also, the result suggest that the manufacturing process might largely affect the clinical availability of STA-1. The pharmacologically active components of STA-1 have not yet been characterized. However, some of the herbs in this formula are reported to exhibit anti-inflammatory and antiallergic properties. Further fractionation of STA-1 extract, purification, and identification

of active components will provide the scientific basis of this herbal medicine in the treatment of asthma.

CONCLUSION

In our study suggested that STA-1 could inhibit the synthesis of allergen-specific IgE, airway inflammation, and hyperreactivity and may be available for the treatment of allergic asthma. Some volatile compounds have an anti-inflammatory effect in LWDHW, but the bioactive components are still unclear. Although the complexity of traditional Chinese herbal formulas containing many constituents makes standardization of herbal products difficult, further investigations on their usage in developing new asthma and allergy medications still remain.

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