



DFSG, a novel herbal cocktail with anti-asthma activity, suppressed MUC5AC in A549 cells and alleviated allergic airway hypersensitivity and inflammatory cell infiltration in a chronic asthma mouse model

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ABSTRACT

Aim of the study: To develop a novel anti-asthma drug. DFSG is a novel herbal cocktail composed of 4 types of herbal medicines. This study explored whether DFSG has the potential to attenuate asthma symptom severity and aimed to determine the immunomodulatory mechanism of DFSG using a chronic asthmatic mouse model induced by repeated challenges with *Dermatogoides pteronyssinus* (Der p).

Materials and methods: BALB/c mice were intratracheally inoculated with Der p (50 µl, 1 mg/ml) once a week for 5 weeks. In addition, 30 min before Der p challenge, the mice were orally administered 1x DFSG (1 g/kg) or 1/2x DFSG (0.5 g/kg). Three days after the final challenge, the mice were sacrificed to evaluate inflammatory cell infiltration, lung histological features, blood total IgE, and cytokine levels in pulmonary alveolar lavage fluid. Furthermore, 30 min after the addition of DFSG, caffeic acid, p-coumaric acid or chlorogenic acid to A549 cells, 10 ng/ml IL-1β was added to evaluate the effect of the drug on mucin 5AC (MUC5AC) gene expression after stimulation of A549 cells by IL-1β.

Results: DFSG significantly reduced Der p-induced airway hyperresponsiveness, bronchial inflammatory cell infiltration, and total IgE and IgG1 serum levels. Furthermore, DFSG significantly inhibited T_H2 cytokines and increased the expression of T_H1 cytokines. In addition, immunohistochemical staining demonstrated that DFSG inhibited MUC5AC expression in the bronchial epithelial cells. DFSG and a mixture of caffeic acid, p-coumaric acid, and chlorogenic acid inhibited MUC5AC gene expression in A549 cells after stimulation with IL-1β.

Conclusion: These results suggest that by regulating T_H1 and T_H2 cytokines and MUC5AC expression, DFSG exhibits anti-airway inflammatory cell infiltration and anti-hyperresponsiveness activity and inhibits specific immunity in a chronic asthmatic mouse model. Therefore, DFSG has potential for development into a drug for chronic asthma treatment.

1. Introduction

Asthma is a complicated chronic disease affecting individuals ranging from children to older adults [1]. This airway inflammatory disease is characterized by eosinophilia, mucus hypersecretion by goblet cells, collagen deposition, and hyperresponsiveness to inhaled allergens and nonspecific stimuli [2]. Asthma can be controlled with an inhaler and by avoiding known allergens. Currently, β-activator and corticosteroid inhalers are primarily used to control symptoms [3]. However,

asthma still affects 300 million people worldwide, resulting in 180,000 deaths and the loss of 15 million disability-adjusted life years each year [4]. The European Union estimates an annual cost of 7.2 billion Euros caused by direct, indirect and productivity loss related to asthma [5]. In the United States, approximately 6.3 million children and 17.7 million adults have asthma. From 2006–2010, 50 % of adults and 38 % of children with asthma suffered from uncontrollable symptoms [6].

The earliest use of a treatment cocktail was performed for acquired immunodeficiency syndrome (AIDS) treatment [7]. Chinese herbal

Abbreviations: Der p, *Dermatogoides pteronyssinus*; IHC, immunohistochemical; PAS, Periodic acid-Schiff; FBS, fetal bovine serum; P.S., penicillin/streptomycin; AHR, airway hyperresponsiveness; MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

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Table 1

The components and dosage of herbal medications in DFSG.

TCM materia medica	Botanic family	Botanical nomenclature	Plant part	Amount (g)
1. Du Zhong (杜仲)	Eucommiaceae	<i>Eucommia ulmoides</i> Oliv.	Cortex	4
2. Fu Tzu (附子)	Ranunculaceae	<i>Aconitum carmichaeli</i> Debx.	Root	2
3. Shan Chu Yu (山茱萸)	Cornaceae	<i>Cornus officinalis</i> Sieb. et Zucc.	Fruit	4
4. Gou Qi Zi (枸杞子)	Solanaceae	<i>Lycium chinense</i> Mill.	Fruit	4
Total amounts				12

medicine has long been used to treat various malignancies, such as cancer [8], diabetes and inflammation [8], and for improving a patient's physical strength [9]. Although single herbs are seldom used alone, appropriately mixed herbal cocktails are often used. These cocktails exploit interactions between numerous phytochemicals present in different herbs to modulate various cellular pathways and achieve maximum therapeutic efficacy by targeting multiple pathological processes while minimizing side effects [10]. A major problem in current cancer chemotherapy is multidrug resistance and various adverse side effects; therefore, chemotherapy is gradually changing from single-agent treatment to a drug cocktail strategy of multidrug therapy. In addition, drug cocktail therapy has been demonstrated to have greater treatment effect than single drugs [11].

DFSG comprises 4 types of herbal medicines: *Eucommia ulmoides* Oliv., *Aconitum carmichaeli* Debx., *Cornus officinalis* Sieb. et Zucc. and *Lycium chinense* Mill. *Eucommia ulmoides* Oliv has pharmacological effects on hyperglycemia, hypertension, Alzheimer's disease, aging, sexual dysfunction, diabetes, obesity, and osteoporosis [12]. *Aconitum carmichaeli* Debx. is typically used to treat various diseases, for example cardiovascular disease, tumors, skin wounds, rheumatoid arthritis, gastroenteritis, edema, diarrhea, and depression [13]. Modern pharmacological studies have shown that *Cornus officinalis* Sieb. et Zucc and its active ingredients exhibit a wide range of pharmacological activities against diseases, such as anti-diabetes, neuroprotective, anti-inflammatory, antioxidative, anti-aging, cardioprotective and antibacterial effects [14]. *Lycium chinense* Mill has anti-aging, immune modulation, anti-fatigue, anti-tumor and male fertility-enhancing properties [15]. However, to date, no study has shown whether a combination of these 4 herbs has a therapeutic effect on asthma.

Airway mucus has key functions in host defense. Harmful substances induce goblet cell hyperplasia, which provides a frontline protective effect and clears toxic substances and dead cells in the airway [16]. However, excessive mucus production leads to coughing and wheezing and can damage mucociliary clearance, leading to retention of pathogens and toxic particles, and also cause airway obstruction, leading to ventilation-perfusion mismatch [16]. Inflammation not only stimulates activation of epidermal growth factor receptor but also converts IL-13-induced Clara and ciliated cells to goblet cells, thus leading to mucin 5AC (MUC5AC) expression and mucin overproduction and eventually to excessive mucus secretion [16]. Chronic airway diseases, such as asthma, chronic obstructive pulmonary disease and cystic fibrosis, are related to a mucus hyperproduction phenotype. Persistent goblet cell hyperplasia can result in airway obstruction and significantly increase the incidence and death rates of chronic airway diseases [16]. Therefore, inhibition of mucus secretion or production can be a therapeutic treatment for chronic airway diseases.

2. Materials and methods

2.1. Mice and reagents

Pathogen-free, male, 6- to 8-week-old BALB/c mice were purchased

from the National Laboratory Animal Center, Republic of China. Animal care and all experimental treatments followed the guidelines of the National Science Council of the Republic of China. The animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the China Medical University (No. 103-163-N). House dust mites *Dermatoglyphus pteronyssinus* (Der p) were purchased from Allergon, Sweden. Crude mite extract was prepared with ether and dialyzed with sterile double-distilled water [2]. Chlorogenic acid, caffeic acid and *p*-coumaric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. DFSG preparation

DFSG is a mixture of 4 common Chinese herbal medicines prepared according to a specific weight proportion as indicated in Table 1. Jason Tsai (a Quality Assurance Manager from Koda Pharmaceuticals Co., Ltd.) was entrusted to identify the origins of the plants. In total, 343.2 g of herbal medicine was used in this study. The medicines were extracted by boiling in 2.4 l of water for 1 h. After removal of the liquid boiled extract, the herbal medicines were extracted again with 1.7 l of boiled water for 1 h. The first and second batches of boiled extract were filtered using a 100-mesh filter. Then, the filtered extract was collected for drying. The DFSG powder was dissolved in sterile water to prepare a specific concentration to feed to the mice.

2.3. DFSG compound identification and content determination

2.3.1. Standards, reagents, and materials

Standard compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, formic acid and acetonitrile (HPLC grade) were bought from Merck (Darmstadt, Germany). Millipore Milli-Q purification system (Millipore, Bedford, MA, USA) was used to prepare deionized water.

2.3.2. Preparation of standard solutions and samples

Standard stock solutions (approx. 1 mg/ml) were prepared individually by dissolving an accurately weighed amount of standard in methanol. Then, reference solutions were prepared using a mixture of all standards, which were serially diluted with methanol to obtain different reference concentrations to plot standard curves. All of the prepared solutions were stored at 4 °C before analysis. Protein precipitation was performed with each sample solution in a 2x volume of methanol, followed by centrifugation at 15,000 g for 10 min, and the supernatant was diluted 10x with methanol and then vortexed and filtered through a 0.22-μm microporous membrane before injection. All samples were stored at 4 °C prior to analysis. To develop a sensitive and accurate quantitative method, tandem mass spectrometry (MS/MS) fragmentation was investigated for each analyte by direct infusion of a single standard solution into the mass spectrometer using a (-)-electrospray ion (ESI) and (+)-ESI source to optimize MS parameters, including the product ion, declustering potential, and collision energy.

2.3.3. Liquid chromatography

Chromatographic analysis was performed with an Agilent 1260 HPLC system equipped with an online degasser, a binary pump, and an autosampler. Chromatographic separation was carried out on an Agilent Zorbax Eclipse XDB-C8 column (150 × 4.6 mm i.d., 5.0 μm). The mobile phases consisted of acetonitrile (B) and 0.1 % formic acid in water (A). The flow rate was maintained at 0.5 ml/min, and the injected sample volume was 5 μl.

2.3.4. Mass spectrometry

Tandem mass spectrometry was performed on an AB Sciex QTRAP 5500 mass spectrometer equipped with an ESI source. The mass spectra were obtained in multiple reaction monitoring (MRM) mode. Positive and negative modes were applied as appropriate. Nitrogen was selected as the heating gas, collision gas, and nebulizer gas. The mass conditions were capillary voltages of 5500 V (+ mode) and −4500 V (− mode) and a source temperature of 550 °C.

2.4. Der p-induced model of chronic asthma

The 24 male, 6- to 8-wk-old BALB/c mice were randomly divided into 4 groups. The mice in the Der p-challenged group were intratracheally inoculated with Der p (50 μl, 1 mg/ml) once a week for a 5-week interval, with a total of 6 challenges, to induce a chronic asthmatic mouse model. The mice were orally administered sterile water 30 min before the Der p challenge. The mice in the 1x and 1/2x DFSG groups were fed 1 g/kg and 0.5 g/kg of DFSG, respectively, 30 min before the Der p challenge. The mice in the PBS group were challenged with PBS instead of dust mites, and sterile water was orally administered. At 72 h after the last challenge, the mice were sacrificed via intraperitoneal injection of xylazine (200 μg/mouse) and ketamine (2 mg/mice).² Serum samples were collected and stored at −80 °C until assayed. In addition, pulmonary alveolar lavage samples were collected to determine the total white blood cell count and measure cytokine levels, and lung tissue was collected for pathological biopsy.

2.5. Measurement of airway resistance

A single-chamber, whole-body plethysmograph (Buxco Electronics, Inc., Troy, NY) was used to systematically measure airway resistance in the male mice. Airway resistance is expressed as Penh (enhanced pause). The operation steps of the device were in accordance with the manual. First, each male mouse was placed in an individual chamber, and ultrasonic oscillation was used to convert methacholine liquid to gas, which was fed into the chamber through a connection tube, thus forcing the male mouse in the chamber to breathe the gas. Increasing doses of methacholine were administered every 3 min. The methacholine doses were 0, 3.125, 6.25, 12.5, 25 and 50 mg/ml.

2.6. Measurement of serum total IgE, total IgG1 and total IgG_{2a/2b}

A total of 100 μl of serum was added to a 96-well plate, and the total IgE level was measured using an IgE-specific ELISA kit (BD Pharmingen) according to the manufacturer's instructions. Before measuring total IgG1 and total IgG_{2a/2b}, the surface of each 96-well plate was coated with total IgG1 or total IgG_{2a/2b} antigens. After incubation overnight at 4 °C, the uncombined antigens were washed and incubated with biotinylated rat anti-mouse IgG1 or IgG_{2a/2b} mAb (2 μg/ml; BD Pharmingen) for 1 h. After the combined reaction, the unbound specific antibody was washed with buffer, followed by the addition of a TMB microwell peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD). After the streptavidin-HRP conjugate (1:1000 dilution, BD Pharmingen) reaction was completed and the color was generated, the plates were read with an ELISA plate reader at an optical density (OD) of 450 nm.

2.7. Measurement of cytokine levels in pulmonary alveolar lavage fluid

Cytokine concentrations in pulmonary alveolar lavage fluid were measured with an IL-5, IL-13, IL12 and IFN-γ ELISA Ready-SET-Go! Kit (eBioscience, San Diego, CA) according to the manufacturer's protocol.

2.8. Staining method

2.8.1. Periodic acid-Schiff (PAS) staining

The lungs of mice were removed and stored in 4 % paraformaldehyde fixative. After being progressively dehydrated using alcohol and embedded in paraffin, the mouse lungs were sliced into 0.5-mm-thick sections and affixed to glass microscope slides. After the fixed tissue slices were dewaxed and washed, they were immersed in distilled water, placed in 0.5 % periodic acid for 15 min and washed with tap water for 5 min. After immersion in distilled water, the slides were stained with Schiff's reagent for 5 min, washed with tap water for 5 min, dehydrated, and sealed. Finally, the fixed tissue slices were assessed under a light microscope. The glycoproteins and neutral mucus appeared red, and the nuclei were stained blue.

2.8.2. Immunohistochemical (IHC) staining

Tissue specimens were fixed with 10 % neutral buffered formaldehyde solution and embedded in paraffin. Then, 4- to 5-μm sections were dewaxed and immersed in

0.3 % hydrogen peroxide for 30 min to interrupt endogenous peroxidase activity. Next, the sections were immersed in normal goat serum (1.5 %) for 20 min to interrupt nonspecific binding sites, and mouse monoclonal IgG1 (specific for MUC5AC (45M1); Thermo Fisher Scientific, San Jose, CA, USA) diluted 1:150 was applied for immunostaining at 4 °C overnight. Then, a biotinylated secondary antibody (Nichirei Co., Ltd., Tokyo, Japan) diluted 1:200 was used to process the sections, and avidin-biotin-peroxidase complex solution and 3,3'-diaminobenzidine (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were applied for color generation.

2.9. Cell viability test (MTT assay)

A549 cells were inoculated into a 96-well plate. Each well contained 5×10^4 cells/90 μl culture medium (F12 (Gibco) + 10 % FBS + 1 % penicillin/streptomycin (P.S.)). Then, the cells were incubated in a 37 °C, 5 % CO₂ incubator for 24 h. Afterward, the cells were challenged with difference drugs for 24 h. After a 24-h challenge, 5 μl Presto blue (Invitrogen) was added to each well to react for 1.5 h. Then, the OD at 570 and 600 nm was measured.

2.10. Cell cultivation and drug treatment

A549 cells were seeded into 6-well plates. Each well contained 1×10^6 cells/3 ml culture medium (F12 + 10 % FBS + 1 % P.S.). Then, the cells were cultured in a 37 °C, 5 % CO₂ incubator for 24 h. The cells were treated with different concentrations of DFSG and compounds and then 30 min later were challenged with 10 ng/ml IL-1β for 4 h. All cells were collected to evaluate MUC5A gene expression. Cells treated with 100 nM dexamethasone were used as the control group.

2.11. Real-time PCR

Total RNA was extracted from A549 cells using the TRIzol reagent (Invitrogen Life Technologies) and converted to cDNA using an ImProm-II Reverse Transcription System kit (Promega). Real-time PCR was performed with a Roche LightCycler FastStart DNA Master SYBR Green I Kit according to the operation manual. A Roche LightCycler 1.5 instrument was used to perform real-time PCR. The results were analyzed using the CP value. β-Actin was used as the control. The gene-specific primers (sense and antisense) for each gene were follows: β-

actin, 5'-GGAAATCGTGCGTGACA-3' and 5'-CACAGGATTCCATACCC AAG-3'; MUC5AC, 5'- TGC GTC CCA CGA CAT CTG -3' and 5'- CAG GTG AAT GGG CAC ATG TG-3'.

2.12. Statistical analysis

The data were analyzed using Student's *t*-test to compare differences among groups. The results are expressed as the means $\pm$ SD. A *P* value < 0.05 was considered to indicate statistical significance.

3. Results

3.1. DFSG reduced airway hyperresponsiveness and inflammatory reactions in Der p-induced chronic asthmatic mice

When asthma patients inhale allergens, they often experience dyspnea. Therefore, to determine whether DFSG attenuates dyspnea, we intratracheally inoculated mice with Der p repeatedly to establish a chronic asthmatic mouse model. The chronic asthmatic mice obtained through this method have clinical symptoms similar to those of asthma patients, such as airway hyperresponsiveness (AHR), airway inflammation, airway remodeling and high blood concentrations of total IgE [17]. The male mice were randomly divided into 4 groups. Compared to the PBS group, the Penh values were significantly increased in the Der p group (for various methacholine concentrations). However, in the Der p-challenged 1/2x and 1x DFSG groups, the Penh values were significantly reduced (for the 25 and 50 mg/ml methacholine concentrations) compared to the Der p group (Fig. 2). In particular, the 1x DFSG group showed significant attenuation of dyspnea. Furthermore, analysis of the total number of inflammatory cells in pulmonary alveolar lavage fluid from mice in different groups showed a significantly higher number of cells in the Der p group than in the PBS group. However, the total number of inflammatory cells was significantly reduced in the 1x DFSG group compared to the Der p group (Fig. 3)

3.2. The effect of DFSG on regulating blood total IgE, total IgG1 and total IgG_{2a/2b} levels in Der p-induced asthmatic mice

Clinical evidence shows that serum total IgE in most asthma patients is higher than that in non-asthma patients. To determine whether DFSG has the ability to regulate humoral immunity, we collected blood samples from mice and analyzed them via ELISA. The results showed that the serum total IgE in the Der p group was significantly higher than that in the PBS group, while the total IgE induced by the Der p challenge was effectively reduced in the mice fed 1x DFSG. In addition, chronic asthmatic mice can produce high total IgG1 levels associated with the T_H2 immune response due Der p challenge, and feeding of 1x DFSG significantly reduced the total IgG1 level (Fig. 4). In addition, the asthmatic mice fed 1x DFSG had a significantly increased total IgG_{2a/2b} level, which is associated with the T_H1 immune response, compared to mice in the Der p group (Fig. 4).

3.3. DFSG improves lung sputum and the degree of MUC5AC expression in Der p-induced chronic asthmatic mice

In the lungs of asthmatic patients, sputum is hypersecreted by goblet cells. Therefore, to determine whether DFSG can attenuate hypersecretion of sputum by goblet cells, we performed a PAS staining analysis of pathological sections. The results showed that mice in the PBS group did not have sputum secretion; however, hypersecretion of sputum by goblet cells could clearly be observed around the lung and trachea tissues in the Der p group mice. However, compared to the Der p group, the 1/2x and 1x DFSG groups exhibited significantly reduced sputum production in a dose-dependent manner (Fig. 5a). Furthermore, MUC5AC protein expression was demonstrated in the lung tissue via IHC staining, and a significant decrease in MUC5AC expression was

observed in the lung tissue of mice fed DFSG (Fig. 5b).

3.4. DFSG regulates T_H1 and T_H2 cytokine levels in pulmonary alveolar lavage fluid from Der p-induced chronic asthmatic mice

We previously demonstrated that exposure to house dust mites as a crude extract increased the T_H2 polarized allergic reaction in mice, and this reaction is a characteristic of bronchial asthma.² To further investigate the mechanism by which DFSG regulates cytokines in the lungs, the levels of T_H1 and T_H2 cytokines in pulmonary alveolar lavage fluid were analyzed. ELISA results showed that the levels of the T_H1 cytokines IL-12 β and IFN- γ and the T_H2 cytokines IL-5 and IL-13 in the Der p group were significantly higher than those in the PBS group, while the levels of T_H2 cytokines were significantly decreased in mice fed a 1x dose of DFSG, but those of T_H1 cytokines were significantly increased. All of the cytokine levels were modulated in a dose-dependent manner (Fig. 6).

3.5. The effect of DFSG on MUC5AC expression in lung tissue

The IHC experiment results showed that DFSG can indeed reduce MUC5AC expression in lung tissues. To determine whether DFSG can reduce sputum secretion in lung tissues by inhibiting expression of the MUC5A gene in epithelial cells, IL-1 was used to stimulate A549 lung epithelial cells to activate MUC5A gene expression. The results showed that DFSG significantly inhibited MUC5A gene expression in a dose-dependent manner (Fig. 7). Further study found that DFSG produced a 54 % inhibition rate against MUC5AC gene expression at a 10 μ g/ μ L concentration.

At 10 μ g/ μ L, DFSG contains 1.47 μ M caffeic acid, 3.58 μ M p-coumaric acid, and 3.12 μ M chlorogenic acid (Fig. 1). However, when administered separately, 1.47 μ M caffeic acid, 3.58 μ M p-coumaric acid, and 3.12 μ M chlorogenic acid had no significant inhibitory effect on MUC5AC gene expression. However, when 1.47 μ M caffeic acid, 3.58 μ M p-coumaric acid, and 3.12 μ M chlorogenic acid were combined and used to treat epithelial cells, the inhibitory effect on MUC5AC gene expression was similar to that of 10 μ g/ μ L DFSG (Fig. 7).

4. Discussion

In the present study, we provide evidence for the immunoregulatory effects of DFSG on allergen-induced airway hypersensitivity and airway inflammation in an established murine model of chronic allergic asthma. DFSG possesses anti-inflammatory effects as indicated by the reduction in the total number of cells in bronchoalveolar lavage fluid (BALF) relative to that observed in Der p group mice. Moreover, DFSG also decreased the quantity of hyperplastic goblet cells as evidenced by histology analysis of lung specimens. Our findings provide a new herbal cocktail for asthma treatment.

The correlation between elevated serum IgE levels and the incidence or severity of asthma is well established [2]. Therefore, inhibiting IgE activity or interfering with IgE synthesis has recently become a novel approach for asthma drug development, such as omalizumab (Xolair) [2]. In the present study, DFSG treatment significantly decreased the total IgE level in serum. Furthermore, we found that DFSG treatment resulted in a reduction in IgG1 levels and a slight elevation in IgG_{2a/2b} levels.

Evidence indicates enhanced development of the CD4 + T_H2 lymphocyte subset in allergic processes, while the responses of CD4 + T_H1 lymphocytes may be repressed, which may represent a key mechanism in the development of allergic asthma [18]. T_H2 cells produce T_H2 cytokines, such as IL-5 and IL-13. However, IL-5 is an important cytokine for eosinophil differentiation, proliferation, and migration to the airways. Eosinophilia in blood and tissue is a hallmarks of allergic asthma, and eosinophils released granule-stored cationic proteins and proinflammatory mediators, thus causing the symptomatology of

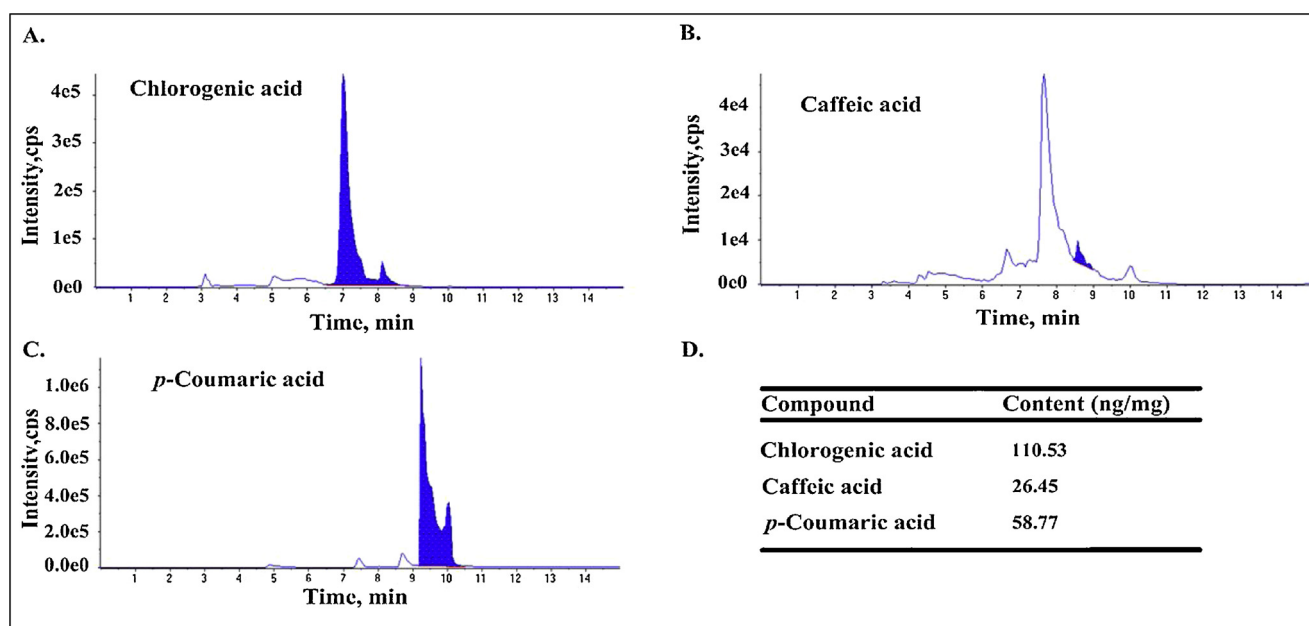


Fig. 1. (a–c) LC-mass spectrum of DFSG (d) The composition of DFSG - caffeic acid, p-coumaric acid, and chlorogenic acid (unit: ng/ DFSG ex. 1 mg).

asthma [2]. The US FDA approved two humanized monoclonal antibodies, Reslizumab and Mepolizumab that bind to IL-5, for patients with severe, eosinophilic asthma. Moreover, Benralizumab binds to the IL-5 receptor and is undergoing clinical development. IL-13 has many pathogenic effects in asthma, including IgE synthesis, bronchial fibroblast proliferation, AHR induction, increased mucus production, eosinophil and basophils recruitment [19] and airway remodeling [20]. Moreover, reducing IL-13 signaling abolishes allergen-driven responses [21]. Two drugs in clinical development, lebrikizumab and tralokinumab, are IL-13 antagonists [1]. Thus, suppression of these cytokines may be effective in alleviating allergic asthma. In our present study, we showed that DFSG treatment decreased IL-5 and IL-13 secretion in BALF. Therefore, we speculate that DFSG treatment reduces the pathological features of asthma by modulating T_H2 responses at the molecular level.

The cytokines produced by T_H1 and T_H2 cells can antagonize and cross-regulate each other's development and activity [18]. The T_H1

cytokines IL-12 and IFN- γ can be considered "anti-inflammatory" because they inhibit certain responses caused by T_H2 cell activation [18]. Vaccination and therapeutic strategies against asthma are based on the induction of IFN- γ and IL-12 secretion, which shift the immune system toward T_H1 responses [22]. T_H1 cells secrete IFN- γ , which activates macrophages and dendritic cells and in turn induces their production of IL-12 [18]. IFN- γ participates in T_H1 -mediated inhibition of T_H2 cell activity, such as bronchial hyperresponsiveness induction, goblet cell proliferation, and eosinophil recruitment [18]. Moreover, IFN- γ can inhibit IL-4-induced IgE and IgG4 synthesis by B cells with strong specificity [18]. In addition, T cells reduce the production of IFN- γ in patients with asthma according to disease severity [23]. Nebulized IFN- γ decreases the number of eosinophils in BALF, indicating the therapeutic potential of IFN- γ in asthmatic patients [24]. However, the asthmatic airway exhibits increased IFN- γ expression after corticosteroid treatment [25]. In addition, allergen immunotherapy in patients with asthma results in greater IFN- γ production by circulating T cells

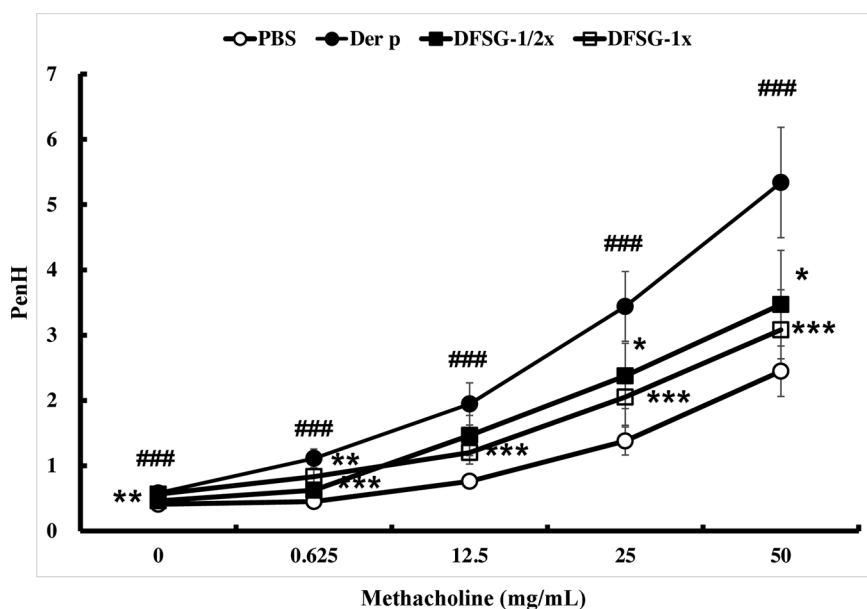


Fig. 2. The effect of DFSG on airway hyperresponsiveness in Der p-induced asthmatic mice. Evaluation of airway hyperresponsiveness 2 days after the final Der p challenge. The values represent the means $\pm$ SDs of 6 mice. # $P < 0.05$ (versus the PBS group); * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (between the nontreated and DFSG-treated groups).

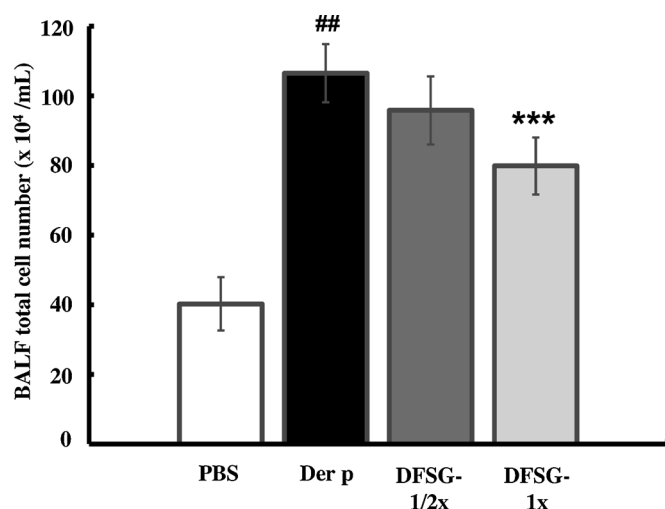


Fig. 3. The effect of DFSG on inflammatory cell infiltration into the lung in Der p-induced asthmatic mice. Calculation of the total number of inflammatory cells in pulmonary alveolar lavage fluid obtained from mice sacrificed 3 days after the final Der p challenge. The values represent the means $\pm$ SDs of 6 mice. ^{##} $P < 0.01$ (versus the PBS group); ^{***} $P < 0.001$ (between the nontreated and DFSG-treated groups).

[26] and increased IFN- γ -producing T cells in the nasal cavity [27]. IL-12 plays a major role in allergic inflammation and inhibition of inappropriate IgE synthesis as a result of allergen exposure [18]. The whole-blood cultures from asthmatic patients showed greater IL-12 production than those from nonatopic control subjects [28]. In our present study, we showed that DFSG treatment increased IFN- γ and IL-12 secretion in BALF. Therefore, we speculate that DFSG could modulate T_H1/T_H2 responses at the molecular level.

In healthy individuals, a viscous, viscoelastic, protective mucus gel is present in the airway, the biophysical properties of which largely depend on the interaction between MUC5AC and MUC5B [29]. The airway mucosa responds to inflammation and infections in various manners, including hyperplasia and hypertrophy of surface mucus (goblet) cells and submucosal cells and excessive mucus secretion [29]. Excessive secretion of airway mucus and mucus retention are major problems in patients with chronic airway disease [29]. Therefore, drugs that improve mucus clearance will provide relief for millions of people around the world [29]. In this study, DFSG was found to inhibit MUC5AC gene expression in epithelial cells (Fig. 5b), thereby reducing pulmonary tissue excretion of MUC5AC (Fig. 5a). Further study showed that DFSG inhibits MUC5AC expression through the synergistic effect of its contained compounds (caffeic acid, p-coumaric acid, and chlorogenic acid) (Fig. 7).

There is growing evidence that the gut microbiota plays a vital role in treatments with herbal medicines because of the complex interactions between the gut microbiota and the ingredients in herbal medicines [30]. This interaction involves the following activities: (1) the gut microbiota bioconverts some of the ingredients in herbal medicines into metabolites that have different bioavailability and biological activity from their precursors; (2) some chemicals in herbal medicines can improve the composition of the gut microbiota, thereby improving the pathology caused by a dysfunction of the gut microbiota; (3) the gut microbiota can mediate the interaction between various ingredients contained in herbal medicines [30]. However, some ingredients in herbal medicines retain their original structure in the blood, such as caffeic acid, p-coumaric acid, and chlorogenic acid [31–33]. Meanwhile, the gut microbiota also plays an important role in maintaining human immunity, and animal studies support this view because germ-free mice (lacking any microorganisms) and young mice receiving antibiotics exhibit exaggerated allergic airway inflammatory responses

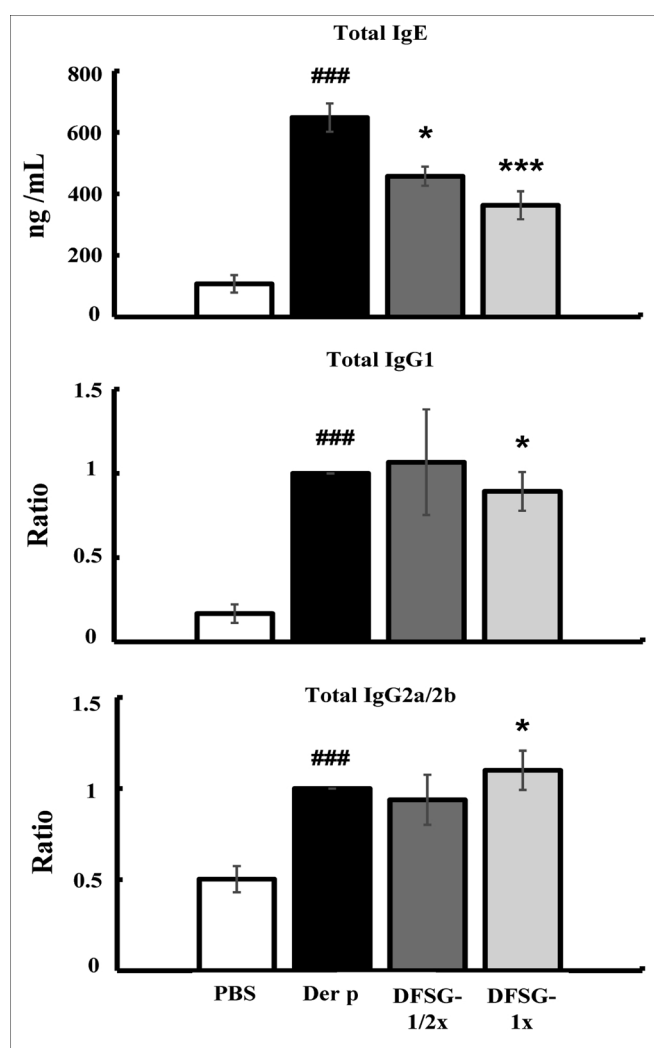


Fig. 4. The effect of DFSG on serum antibodies in Der p-induced asthmatic mice. DFSG significantly reduced serum total IgE and total IgG1 levels and significantly increased the serum total IgG_{2a/2b} level. The values represent the means $\pm$ SD of 6 mice. ^{###} $P < 0.001$ (versus the PBS group); ^{*} $P < 0.05$ and ^{***} $P < 0.001$ (between the nontreated and DFSG-treated groups).

[34]. Furthermore, Kao et al. used three models to study the effects of XQLT on allergen-induced airway inflammation [35], and after oral administration of Chinese herbal medicines for 1 h, high doses of caffeic acid, p-coumaric acid, and chlorogenic acid appeared in the blood [31–33]. We speculate that the effective bioactive ingredients in DFSG need to be taken up through gut microbiota reactions before reaching the lungs through the blood, the effective bioactive ingredients were need 30–60 min from the mouth to the lungs, after which time the effective bioactive ingredients work synergistically on different targets (Fig. 8). Therefore, from the perspective of preventive medicine, it is recommended that this medication should be taken prophylactically to prevent allergen stimulation.

5. Conclusion

DFSG is an herbal cocktail with multicomponent and multitarget effects, which balance the expression of T_H1 (IL-12/IFN- γ) and T_H2 (IL-5/IL-13) cytokines through coordinated activity of its ingredients. DFSG can inhibit MUC5AC expression in lung epithelial cells through the synergistic effects of its included compounds (caffeic acid, p-coumaric acid, and chlorogenic acid), thus ameliorating the increase in pulmonary sputum production. Therefore, DFSG employs the above

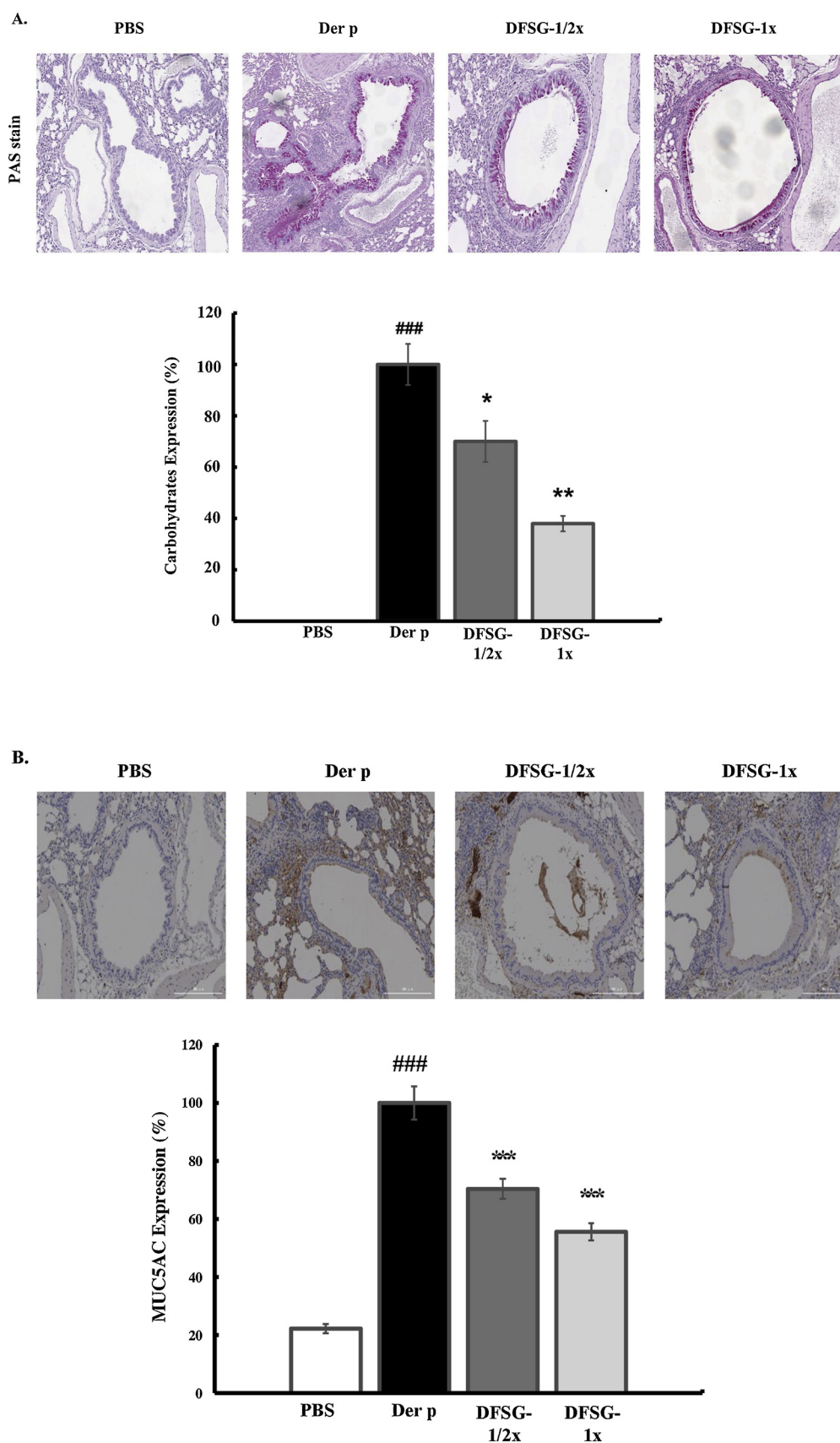


Fig. 5. The effects of DFSG on pulmonary histology in Der p-induced asthmatic mice. (a) Histopathology studies of goblet cell hyperplasia and mucus secretion around the trachea (PAS stain; original magnification, $\times 100$). (b) IHC staining showed that DFSG inhibited the expression of MUC5AC in tracheal epithelial cells (MUC5AC; $\times 100$). The values represent the means $\pm$ SD of 6 mice. ### $P < 0.001$ (versus the PBS group); * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (between the nontreated and DFSG-treated groups).

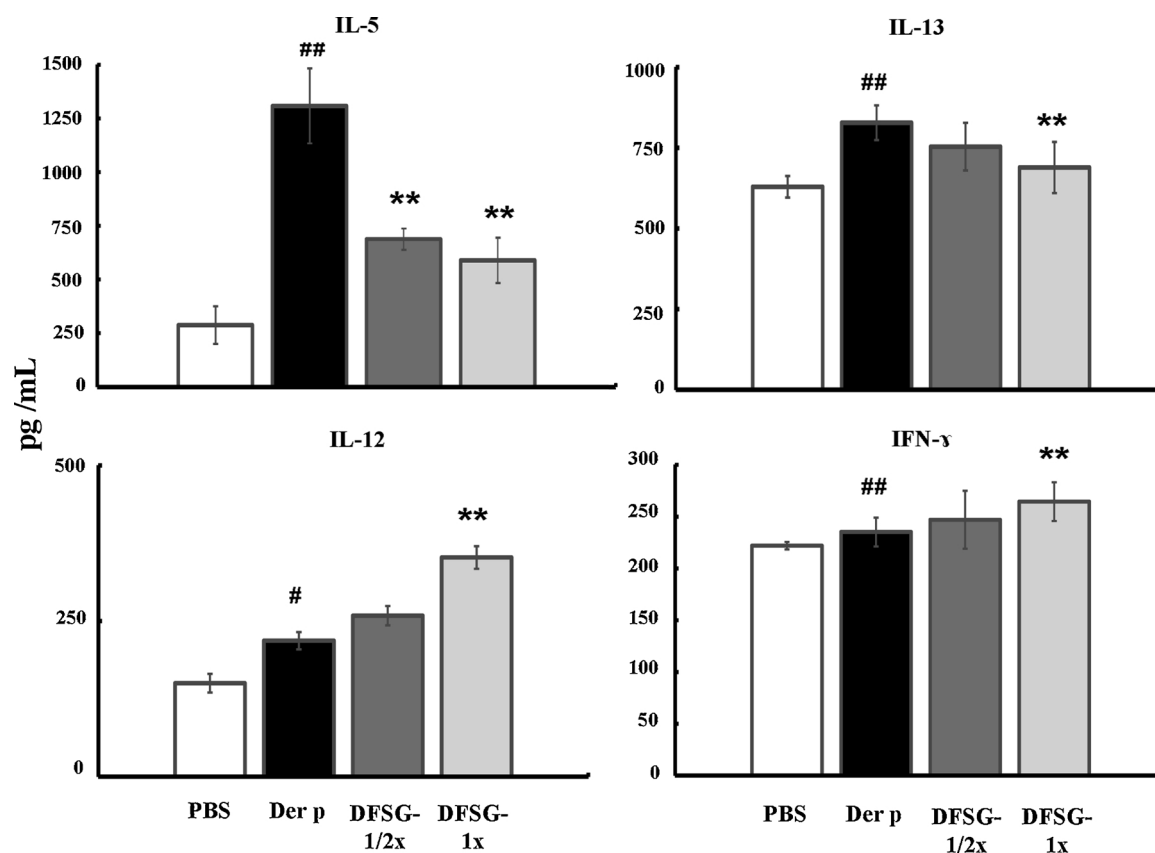


Fig. 6. The effects of DFSG on cytokine levels in pulmonary alveolar lavage fluid from Der p-induced asthmatic mice. DFSG significantly reduced levels of the T_H2 cytokines IL-5 and IL-13 and significantly increased levels of the T_H1 cytokines IL-12 and IFN- γ . The values represent the means $\pm$ SD of 6 mice. # $P < 0.05$ and ## $P < 0.01$ (versus the PBS group); ** $P < 0.01$ (between the nontreated and DFSG-treated groups).

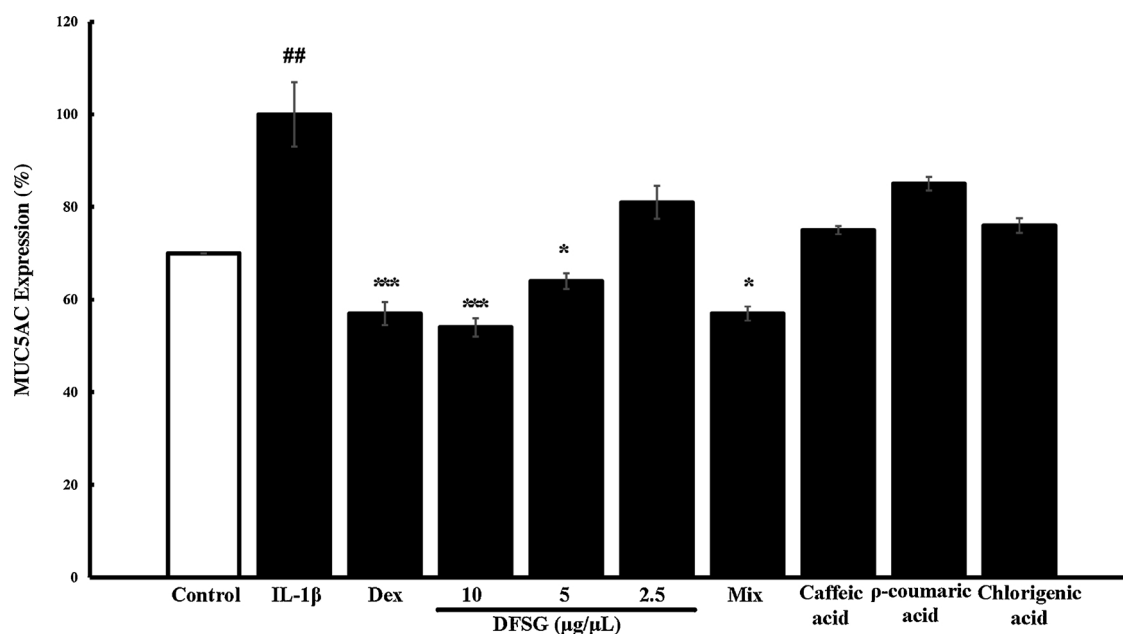


Fig. 7. The effect of DFSG, caffeic acid, p-coumaric acid, and chlorogenic acid on A549 pulmonary epithelial cells stimulated by IL-1 β . The inhibitory effect of DFSG on MUC5AC expression was dose dependent. Furthermore, a mixture containing 1.47 μ M caffeic acid, 3.58 μ M p-coumaric acid, and 3.12 μ M chlorogenic acid (according to the weight composition in DFSG) had an inhibitory effect similar to that of DFSG. The gene profile shown is representative of three independent experiments. ## $P < 0.01$ (versus the control group); * $P < 0.05$ and *** $P < 0.001$ (between the IL-1 β and drug-treated groups).

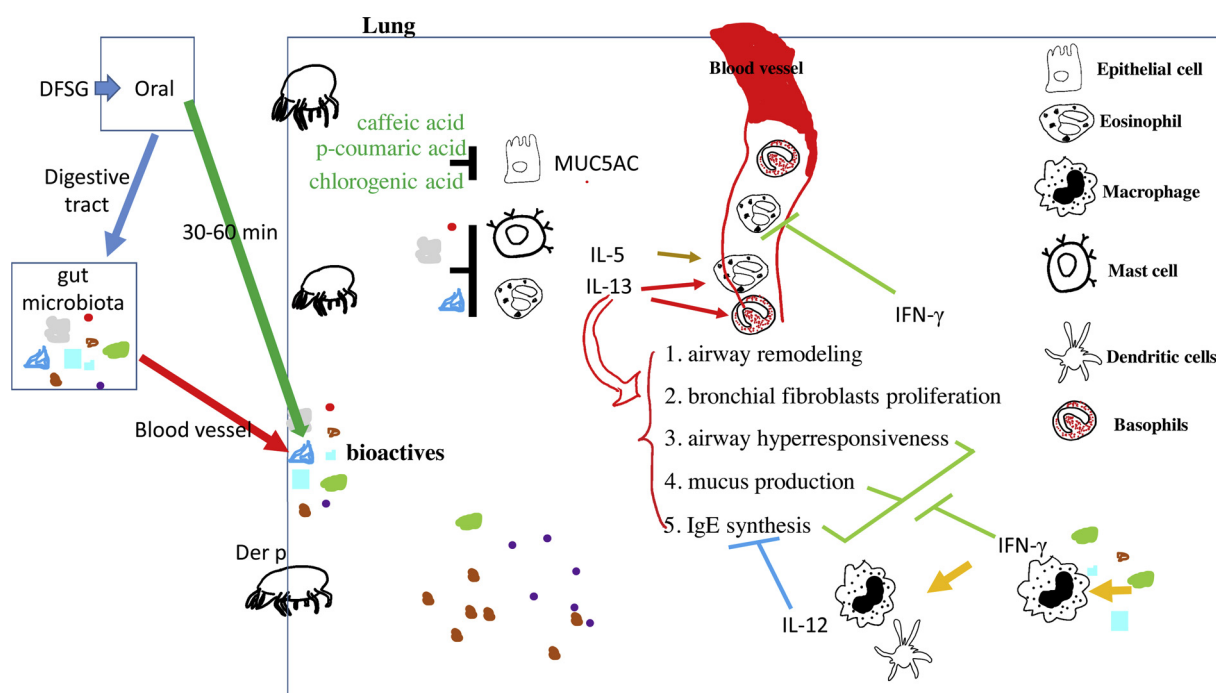


Fig. 8. The proposed mechanism of DFSG. Der p enters the lungs via the airway, and some of the effective bioactive ingredients inhibit the release of IL-5 and IL-13 from eosinophils and mast cells. Other bioactive ingredients promote the release of IFN- γ from macrophages. IFN- γ stimulates macrophages and dendritic cells to secrete IL-12. Caffeic acid, p-coumaric acid, and chlorogenic acid synergistically inhibit epithelial cell secretion of MUC5AC to reduce sputum production. Released IL-5 and IL-13 cause eosinophils to return to the lungs, while IL-13 also causes basophils to return to the lungs. IL-13 promotes IgE synthesis, induces the proliferation of bronchial fibroblasts, induces airway hyperresponsiveness and enhanced mucus production, and promotes airway remodeling. IFN- γ inhibits the migration of eosinophils back into the lungs, as well as IgE synthesis, airway hyperresponsiveness, and mucus production. IL-12 inhibits IgE synthesis.

molecular mechanisms to reduce airway resistance, total serum IgE/IgG1 and the total number of inflammatory cells in the lung. Thus, DFSG has potential for development into an asthma drug.

Author contributions

Li-Jen Lin contributed to study design and data interpretation. Li-Jen Lin and Hui-Ying Huang performed the experiments, analyzed the data, and interpreted the results of experiments. Li-Jen Lin supervised the work, evaluated the data, and wrote the manuscript for publication. All authors discussed the results and contributed to the final manuscript.

Declaration of Competing Interest

The authors declare no competing financial interest. Jason Tsai is currently employed at Koda Pharmaceuticals Co., Ltd. and no conflict of interest.

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