



Selaginella uncinata flavonoids ameliorated ovalbumin-induced airway inflammation in a rat model of asthma

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ABSTRACT

Ethnopharmacological relevance: *Selaginella uncinata* (Desv.) Spring, known as “Cuiyuncao”, is a perennial herb widely distributed in the Southeast Asian countries. In the folk medicine, the local minority commonly use it to treat cough and asthma for centuries.

Aim of the study: This study was carried out to investigate the protective mechanisms of total flavonoids from *S. uncinata* (SUF) on airway hyperresponsiveness, cytokine release and bitter taste receptors (T2Rs) signaling with emphasis on inflammatory responses in a rat model of ovalbumin (OVA)-induced asthma.

Materials and methods: Rats were sensitized and challenged with OVA to induce typical asthmatic reactions. Pathological changes of lung tissue were examined by HE staining. The serum levels of T cell-associated cytokines (IFN- γ , IL-4, IL-5 and IL-13), total IgE and OVA-specific IgE were determined by enzyme-linked immunosorbent assay (ELISA). Gene expressions of T2R10, IP3R1 and Orai1 in lung tissue were assayed by fluorescence quantitative real-time polymerase chain reaction (FQ-PCR) while protein expressions of NFAT1 and c-Myc were assayed by western blot analysis. The activation of SUF was investigated on transgenic T2R10-GFP HEK293 cells.

Results: SUF treatment attenuated airway hyperresponsiveness and goblet cell hyperplasia compared with OVA-challenged asthmatic rats. The serum levels of IL-4, IL-5 and IL-13 as well as total and OVA-specific IgE were decreased while serum IFN- γ was increased in SUF-treated rats. SUF treatment significantly up-regulated T2R10 gene expression, down-regulated IP3R1 and Orai1 gene expression. SUF further suppressed eotaxin, NFAT1 and c-Myc protein expression in lung tissues of OVA-challenged rats.

Conclusions: These results imply that SUF exerts anti-inflammatory function through the T2R10/IP3R1/NFAT1 dependent signaling pathway, and may warrant further evaluation as a possible agent for the treatment of asthma.

1. Introduction

Asthma is a chronic inflammatory disease characterized by airway inflammation and remodeling, bronchial hyper-responsiveness, variable airflow obstruction, and mucus hypersecretion (Gergen and Togias, 2015). The prevalence of asthma is rapidly increasing around the world, especially in young children, and it has become a significant cause of morbidity and mortality in developed countries (Ding et al., 2015). Inhaled and nebulized racemic adrenaline commonly used in the world appears (clinically) to be effective in patients with acute bronchiolitis (Carlsen and Carlsen, 2000), but β_2 -agonists have obvious questionable symptomatic effects (Waldeck, 2002; Mullane, 2011). The

frequent use of β_2 -agonists induces the issues of bronchodilator and bronchoprotective tolerance. After β_2 -agonist treatment, all patients achieved complete remission of cough, catarrh and asthma, but increased allergen responsiveness and masked inflammation have not been improved (Cockcroft and Sears, 2013). This underlines much impetus for urgent need of available anti-asthma agents.

Bitter taste receptors (T2Rs) have been proposed to be emerging targets for novel therapy of respiratory diseases (Deshpande et al., 2010; Shaik et al., 2016). After stimulated by T2R agonists chloroquine and denatonium in airway cells, T2Rs which couple with phospholipase C could generate the second messenger inositol 1,4,5-trisphosphate (IP3), and subsequently induce the release of Ca^{2+} from endoplasmic

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reticulum; meanwhile the extracellular Ca^{2+} entries through the pore-forming subunit of the CRAC channels Orai1. The increase of intracellular Ca^{2+} stores activate the Ca^{2+} -dependent transcription factor NFAT, which regulate expression of genes encoding chemokines and cytokines, such as TNF- α , IL-1 β , IL-6, G-CSF, IL-2 and MCP-1 (Jairaman et al., 2016; Samanta et al., 2014; Spinelli and Trebak, 2016). Clinical studies and animal models have demonstrated the role of genetic variation in T2Rs and its impact on disease susceptibility in airway remodeling, airway immunity, chronic rhinosinusitis cancer and breast (Mfuno Endam et al., 2014; Shaik et al., 2016; Workman et al., 2015; Yoon et al., 2016).

Selaginella uncinata (Desv.) Spring, known as “Cuiyuncao” in China and “Spring blue spikemoss” in Europe, is a perennial herb widely distributed in the Southeast Asian countries (Tsuji et al., 2007; Gu et al., 2013). It is described as one of the major anti-inflammation and pectoral medicines used in traditional Chinese therapy. Its dose is 10–20 g daily, which is recorded in the authoritative medical book of Ancient China “Compendium of Materia Medica” and the 2011 version of the “Chinese medicine processing regulations of Fujian Province”. In folk medical practices, it is believed by traditional Chinese medicine physicians that the herb, which can relieve a cough and ameliorate inflammation stasis, was extensively used as herbal medicine for the treatment of jaundice, edema, pulmonary tuberculosis and pertussis (Zou et al., 2015). Especially in Guangxi, Fujian, Taiwan and other coastal areas, infusions and decoctions of *S. uncinata* leaves are popularly used for treatment of cough and asthma for centuries (Zheng et al., 2011; Li, Lei and Chen, 2014). Recently, increasingly considerable attention has been given to the antiviral (Yin et al., 2014), antitumor (Li, Lei and Chen, 2014), antioxidant (Zheng et al., 2008), anti-inflammatory (Yoon and Baek, 2005) and anti-anoxic (Zheng et al., 2013) substances contained in the plant. The main polyphenolic compounds, such as flavonoids (Zheng et al., 2008; Zou et al., 2014) and steroidal saponins (Zheng et al., 2013), had been isolated and identified from *S. uncinata* ethanolic extracts. It has been recently reported that biflavonoids of *S. uncinata* have cytotoxic activities and inhibit the survival of human cancer cells (Li, Lei and Chen, 2014). Amentoflavone, the main biflavonoid in the *S. uncinata* extract, markedly decreased the mRNA expression of inflammatory mediators (MMP-2, MMP-9, prolyl hydroxylase, lysyl oxidase, VEGF, ERK-1, ERK-2, TNF- α , IL-1 β , IL-6, and GM-CSF) in lung tissues (Guruvayoorappan and Kuttan, 2008). This biflavonoid also exerted suppression of PGE2 biosynthesis via downregulation of COX-2/iNOS expression on A549 cells (Banerjee et al., 2002). Those properties may be indicative of the potential benefit in the pulmonary tract. However, its folk use on inflammatory diseases (such as asthma) has not been fully understood. In the present study, we evaluated the protective effects of *S. uncinata* flavonoids (SUF) in a murine model of ovalbumin (OVA)-induced asthma.

2. Materials and methods

2.1. Preparation of SUF and its quality control

The dried herb was obtained from Chinese Medicine Herbal Factory (Zhejiang, China), and identified by Professor Bing Yu, Zhejiang Chinese Medical University. The duplicate sample (No. 130526Y1) was at College of Pharmacy, Zhejiang Chinese Medical University.

The extraction and purification of total flavonoids from *S. uncinata* (SUF) were carried out according to the previous report (Chen et al., 2013). Briefly, powdered plant materials (2.0 kg) were extracted one time with ethanol (70%, v/v) under heating (90 °C) for 60 min. The extract solution was condensed and added into column loading-treated D101 macroporous resin for adsorption for 24 h. Then the resins were washed with water to get rid of polar impurities. SUF in the column was eluted with 70% ethanol, and the eluting solution was dried in vacuum condition until yellow powders were achieved. Prior to

use in assays *in vitro*, SUF were autoclaved and then handled with 0.22 μm filter membrane under sterile conditions using sterile vessels.

The standards (amentoflavone, hinokiflavone and isocryptomerin) were obtained from Herbest Bio-Tech Co. Ltd, China and their purities were more than 98%. The contents of those biflavonoids in SUF samples were determined by High Performance Liquid Chromatography (HPLC) using the previous method (Yin et al., 2014). HPLC was performed by using a Agilent 1200 HPLC term, including a vacuum degasser, binary pump, an autosampler and a photodiode array detector, controlled by Class-up station (Agilent Co. Ltd., USA). The HPLC column was a Diamonsil™ C18 (250 mm $\times$ 4.6 mm, 5 μm). The gradient mobile phase consisted of acetonitrile (A) and 0.5% acetic acid (B) was used to elute for 45 min with a gradient program as follows: 0–10 min, 36:64 (A: B, v/v); 10–28 min, linear gradient 36:64 $\rightarrow$ 50:50 (A: B, v/v) A; 28–29 min, linear gradient 50:50 $\rightarrow$ 58:42 (A: B, v/v) A; 29–35 min, 58:42 (A: B, v/v) A; and 35–45 min, linear gradient 58:42 $\rightarrow$ 85:15 (A: B, v/v), and the flow rate was kept at 1.0 ml/min. Column temperature was kept constantly at 25 °C. The UV absorbance was monitored at 370 nm. All injection volumes of sample and standard solutions were 20 μL . The HPLC analysis of standards (amentoflavone, hinokiflavone and isocryptomerin) and all the samples were carried out under the established experimental condition as mentioned above.

2.2. Animals and experimental design

Male special pathogen-free (SPF) SD rats weighing 180–220 g and male SPF Balb/c mice (body weights 20–24 g) were both purchased from Zhejiang Animal Experimental Center, China. Animals were housed in groups of five per standard cage, on 12 h light/dark cycle; and air temperature was maintained at 22 ± 2 °C. Each group was caged and artificial fed, with free access to water and normal food. Experiments were carried out in accordance with local guidelines and were approved by the ethics committee.

2.2.1. Rat model of ovalbumin-induced airway inflammation

The rats were divided into 6 groups (n =10): normal control group (NC), model control group (MC), positive control group (treated with dexamethasone (Dex) 1 mg/kg), SUF40 group (treated with SUF 40 mg/kg), SUF80 group (treated with SUF 80 mg/kg), and SUF120 group (treated with SUF 120 mg/kg). The rat airway inflammation was induced by chicken OVA (Grade III; Sigma-Aldrich, US) using the method described previously (Misaka et al., 2010; Deng et al., 2013). Briefly, fifty rats were immunized by intraperitoneal (i.p.) injection of 20 μg of OVA with 1 mg of aluminum hydroxide on day 1st and day 14th. The OVA-challenged rats were exposed to inhalation with 5% ovalbumin solution aerosolized using an ultrasonic nebulizer (Yuyue 4200, Kunshan, China) for 1 h per day from days 21th to 27th after the second sensitization. Three groups of asthma rats were orally administered once daily with 40, 80 and 120 mg/kg of SUF, respectively, and other ten asthma rats were i.p. injected with 1 mg/kg of Dex from the day 27th to 32th. Ten rats as the normal group were only sensitized and challenged with PBS.

2.2.2. Mice model of airway hyperresponsiveness

The mice were divided into 6 groups (n =10) and the animal models were prepared as above mentioned in 2.2.1 section. The drugs were also orally treated once daily from the day 27th to 32th after first OVA-challenge. Airway hyperresponsiveness (AHR) of each group was measured 2 h after the last drug treatment at the 32th day. Mice were placed in a barometric plethysmographic chamber (BUXCO, USA) and baseline readings were taken and averaged for 3 min. Aerosolized methacholine (Mch) in increasing concentrations (from 5 to 20 mg/ml) was then nebulized through an inlet of the main chamber for 3 min, readings were taken and were averaged for 3 min after each nebulization. The bronchopulmonary resistance was expressed as enhanced

pause (Penh) according to the manufacturer's protocol. Results were expressed as the percentage increase of Penh following challenge with each concentration of Mch, where the baseline Penh (after saline challenge) was expressed as 100%.

2.3. Cytokine analysis

Diets were removed from the cages 12 h and body weight was measured before the rats were sacrificed 24 h after the last treatment. Blood samples were collected and centrifuged at 3000g for 15 min to obtain serum, and stored at 4 °C until use. The levels of serum IL-4, IL-5, IL-13, IFN- γ , total IgE and OVA-specific IgE were determined by enzyme linked immunosorbent assay (ELISA). All commercially available kits used in the study were purchased from Boster Bioengineering Institute, Wuhan, China.

2.4. Histological analysis

Lung tissues were detached from the rats and fixed with Carnoy's solution overnight at 4 °C. The fixed tissues were embedded in paraffin and cut into 4- μ m sections with a microtome (Leica, Germany). The sections were placed on slide glasses, deparaffinized, and stained with hematoxylin and eosin (HE) in order to examine the cells that had infiltrated into the peribronchial and perivascular connective tissues. The degree of peribronchial and perivascular inflammation was scored on a subjective scale of 0 (no) to 4 (severe) in a blind manner (Jung et al., 2016; Lee et al., 2005; Yang et al., 2008). The grading scale was scored as follows: 0= no peribronchial or perivascular stain; 1= marginal peribronchial and/or perivascular stain; 2= slight, consistent increase in peribronchial and/or perivascular depth; 3= increased, uniform stain depth throughout all airways and vessels; and 4= dramatically increased depth of stain in all airways and vessels.

2.5. Immunohistochemistry analysis

Expression of Eotaxin in the lung tissues was detected by using corresponding immunohistochemistry detection kit (Boster Bioengineering Institute, Wuhan, China) following the manufacturer's instructions. The percentage areas of positive protein staining slides were analyzed using Image Pro Plus version 6.0 (Media Cybernetics, US) with 10 discontinuous, randomly chosen histological fields at 200 $\times$ magnification objective lens for each slide.

2.6. Fluorescence quantitative RT-PCR (FQ-PCR) analysis

Samples of lung tissues were homogenized, and total RNA was extracted from the lung of the rats in the experimental groups with the use of a Trizol reagent (Shanghai Biotechnology Co., Ltd., China). The synthesis of cDNA was performed according to the kit protocol (TaKaRa, Dalian, China). The specific designed primers pairs [Bitter taste-sensing type 2 receptor 10 (T2R10), inositol 1,4,5-triphosphate receptor 1 (IP3R1), Orai1 and 18 S] used for analysis were shown in Table 1 and were synthesized by Shanghai Biotechnology Co., Ltd., China. In this study, the eukaryotic 18 S rRNA was selected as the housekeeping gene, primarily because it shows limited change in expression under different experimental conditions.

The amplification reaction was conducted in a 25 μ L glass capillary containing 2 μ L of cDNA, 0.5 μ L each of the forward and reverse primers (final concentration of each, 0.5 μ M), 12.5 μ L 2 $\times$ SYBR Green and enough nuclease-free water for a final volume of 25 μ L for each reaction. The following thermal profile for RFQ-PCR, assays was used for all primer sets: 95 °C for 1 min followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 55 °C and elongation at 64 °C for 25 s with acquisition of fluorescent data. The relative expression levels of genes were calculated using the $2^{-\Delta\Delta C_t}$ method.

Table 1

Primer sequences for quantitative PCR.

Gene	Primer Sequences (5' to 3')	Size (bp)	Annealing (°C)
T2R10	AAGATGGAGGTCAATGCCAAGG TGCATACAGCAGCAGGAAGGAGA	96	63
IP3R1	GAGCATTGTACCACTTCTTCAG GGCTTTCTGGCTCGGCATCAAC	142	63
Orai1	CCGTCATCGGGACGCTGCTT GATGCCACCGTTGTGTCTGTT	155	62
18 S	GAATTCACAGTAAGTCCGGGCATCA CGAGGGCCTCACTAAACCATC	105	62

2.7. Western blot analysis

Proteins were extracted from samples of lung through using the commercial kits (Boster Bioengineering Institute, Wuhan). Protein levels in the supernatants were determined using the BCA assay kit (Boster Bioengineering Institute, Wuhan, China). Each sample (60 μ g) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane by electrophoretic transfer (Bio-Rad Laboratories, Inc., USA). The membrane was then probed with antibody reactive with nuclear factor of activated T cells 1 (NFAT1) and c-Myc (Santa Cruz, USA) at 4 °C overnight. After three washes with TBST, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Then blots were developed by a SuperSignal® West Dura Extended Duration Substrate (Pierce, USA). The protein β -actin (Santa Cruz, USA) was used as the loading control.

2.8. In vitro fluorometric assay

HEK293 cells stably expressing recombinant human T2R10 and the chimeric GFP protein were generated by the transgenic method described previously (Suzuki et al., 2006). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Invitrogen), 1% L-glutamine, 1% penicillin and 1% streptomycin in a humidified atmosphere containing 5% CO₂. GFP protein expression was induced by addition of chloroquine (3–90 μ g/ml) or SUF (3–90 μ g/ml) 24 h prior to performing follow-up experiments and the value of fluorescent intensity of each sample was determined by multifunctional microplate reader (MD, USA).

Then, cell viability assay was carried out to test cytotoxic effects of SUF by replacing the medium with 50 μ L of medium containing 5 mg/ml MTT (Sigma-Aldrich, USA). After 3 h, 150 μ L of dimethyl sulfoxide was added into each well and mixed thoroughly. In the end, the optical density of each well was quantified at 570-nm wavelength.

2.9. Statistical analysis

All statistical analyses were performed using the SPSS software (Version 13.0). All data were expressed as mean $\pm$ standard deviation. One-way analysis of variance was used to detect significant differences between the control and treatment groups. Tukey's test was used for multiple comparisons. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Quality evaluation of SUF

Three biflavonoids (amentoflavone, hinokiflavone and isocryptomerin) as showed in Fig. 1 in SUF were well identified by comparing both the retention times and UV spectra of the reference standards. By

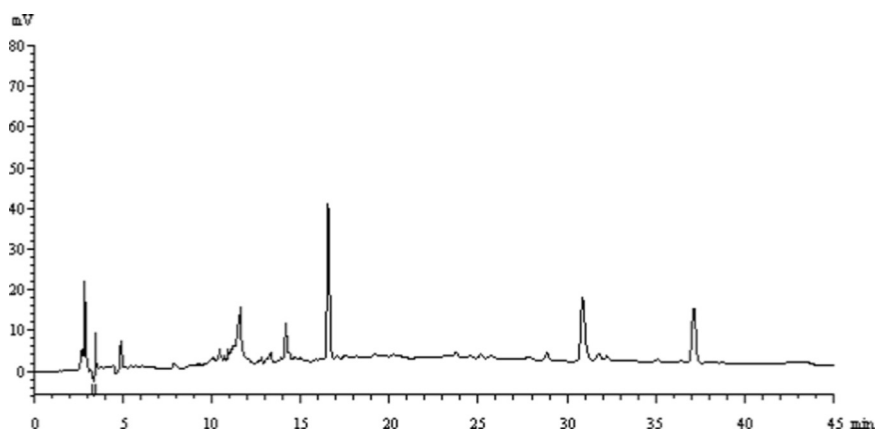


Fig. 1. HPLC analysis of SUF. The compounds (a. amentoflavone; b. hinokiflavone; c. isocryptomerin) were identified in the extract by comparing retention times of their standards. The chromatograms were obtained at a wavelength of 370 nm.

HPLC analysis, the contents of amentoflavone, hinokiflavone and isocryptomerin in the extract were detected to be 137.1 mg/g, 80.3 mg/g and 24.0 mg/g, respectively. Using lutein as the standard, the content of total flavonoids in SUF was detected to be 87.2% by colorimetric method (Yin et al., 2014).

3.2. SUF ameliorated OVA-induced AHR

OVA-induced asthma is characterized by AHR and inflammation of the Airway responsiveness. In this study, AHR was assessed as a percent increase of Penh in response to increasing doses of methacholine. In the asthma rats, the dose-response curve of percent Penh kept rising compared with that of normal control (NC) group (Fig. 2). In addition, the percent Penh produced by methacholine administration (at doses from 5 to 20 mg/ml) increased significantly in the OVA-sensitized/challenged rats compared with the NC rats. The model rats treated with SUF showed a dose-response curve of percent Penh that was slowly but steadily rising compared with that of untreated rats. And the therapeutic effect of SUF at the doses of 40–120 mg/kg was dose-dependent. These results indicated that SUF had inhibitory effects on OVA-induced AHR.

3.3. SUF reduced the degree of pathological alteration in the lung tissues of asthma rats

In the present study, we investigated the effects of SUF on the

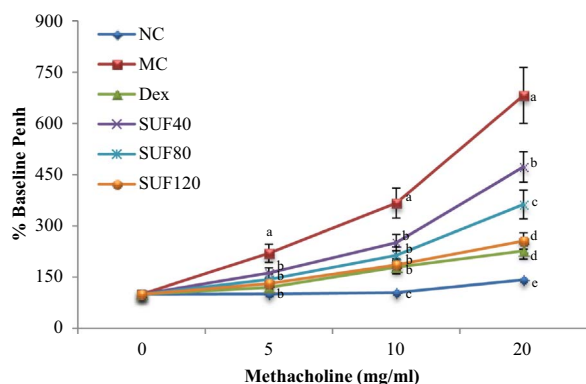


Fig. 2. Effects of SUF on airway responsiveness in OVA-challenged asthma mice. Airway responsiveness was measured at 2 h after the last drug treatment. Airway responsiveness to aerosolized methacholine was measured in unrestrained, conscious mice. Mice were placed into the main chamber and were nebulized first with saline, then with increasing doses (5–20 mg/ml) of methacholine for 3 min for each nebulization. Breathing parameters were taken for 3 min after each nebulization when Penh values were determined. Each value indicated the mean $\pm$ SD ($n = 10$). Different letters indicated statistically significant differences, $P < 0.05$ (Tukey's test).

morphological changes of lung tissues in asthma rats. The lung tissue of OVA-induced asthma rats was characterized by dense peribronchial and perivascular inflammation due to the typical allergic inflammation reaction, such as the accumulation and infiltration of lymphocytes and plasma cells, bronchial dilatation and cavity secretion (Fig. 3B). It was consistent with the results as reported previously (Li et al., 2016; Wang et al., 2016). However, these pathological changes were hardly observed in the NC group (Fig. 3A). Treatment with Dex (Fig. 3C) and SUF (Fig. 3D–3F) reversed OVA-induced allergic inflammation response around the bronchial trees and blood vessels. To further estimate the severity of leukocyte infiltration, inflammation score was used according to the five point scoring system. The higher inflammation score represented the more serious of the degree of pathological inflammation. By the histologic scoring, the inflammation scores in the NC group came almost to be zero, indicating peribronchial and perivascular inflammatory cells were hardly found in the lung tissues of normal rats. However, Compared with the MC group, the scores of the SUF-treated groups were significantly decreased in a dose-dependent manner ($P < 0.05$), indicating that SUF reduced the degree of inflammatory cell infiltration in the lung tissues (Fig. 3G). Treatment with Dex also reduced the degree of inflammatory cell infiltration, resulting in the decrease of its inflammatory score.

3.4. SUF inhibited inflammatory mediator production

To determine the pro-inflammatory cytokine levels in response to OVA sensitization, levels of IL-4, IL-5, and IL-13 in serum were measured by ELISA. As shown in Table 2, those inflammatory cytokines in the MC group were found to be dramatically increased compared with those in the NC group ($P < 0.05$). SUF (40–120 mg/kg) and dexamethasone intervention suppressed the production of IL-4, IL-5, and IL-13 compared with those in the MC group ($P < 0.05$). On the other hand, IFN- γ is well-known as an indicator of Th1 cells and its level in serum were markedly decreased after OVA challenge while SUF treatment could reverse the trends. But there was no significant difference on IFN- γ levels between the MC group and Dex-treated group ($P > 0.05$).

In view of the finding that antigen-specific Th2 responses trigger IgE antibody production (Lee et al., 2012), total and OVA-specific IgE production in the allergic asthma rat model was investigated. Total and OVA-specific IgE levels in serum were markedly increased in OVA-treated rats ($P < 0.05$) compared with PBS-treated rats (Fig. 4). Notably, SUF administration suppressed both total and OVA-specific IgE levels in serum ($P < 0.05$) compared with those in the OVA-induced group.

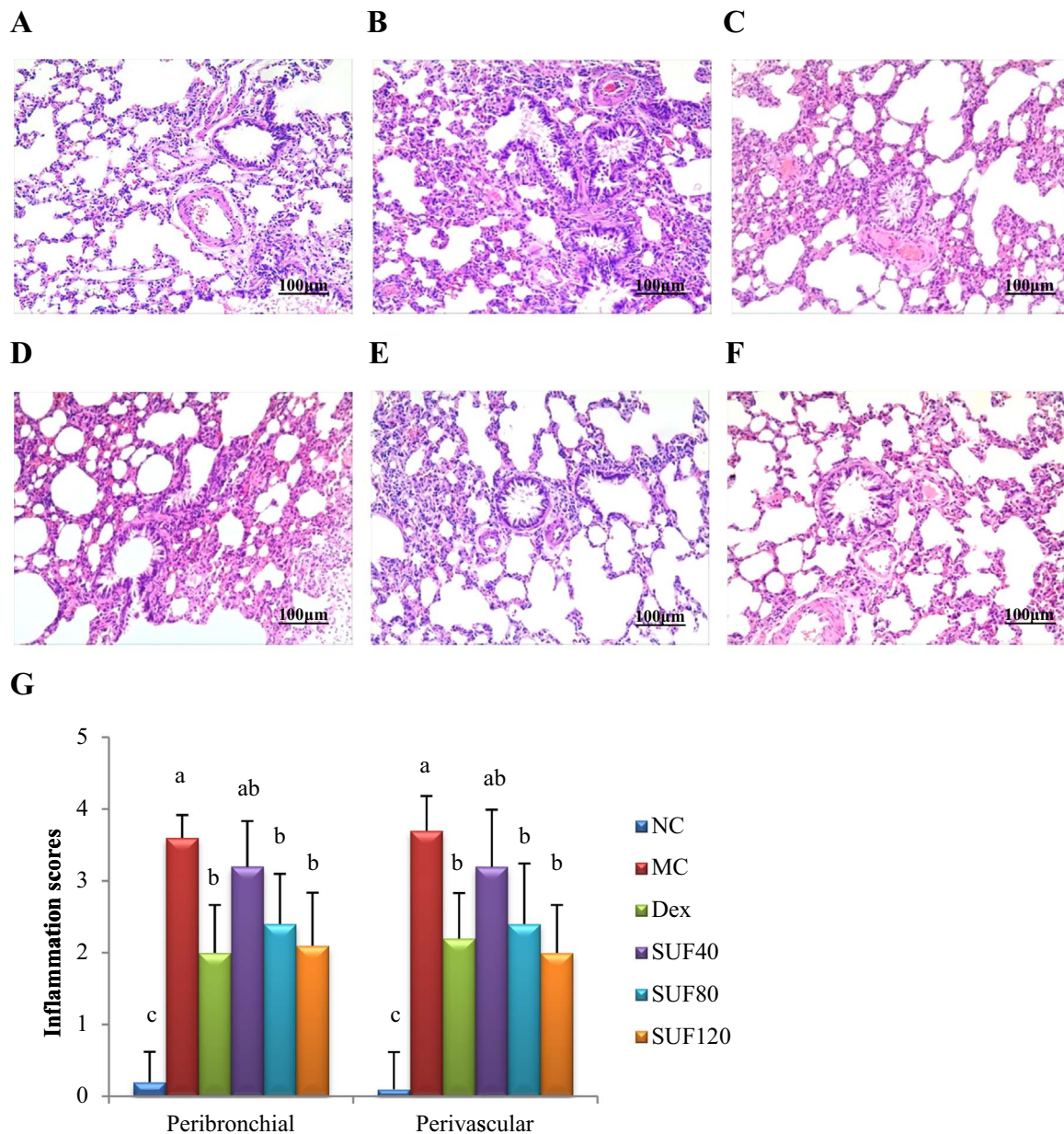


Fig. 3. Effects of SUF on OVA-challenged lung histopathologic changes. Lungs from each experimental group were processed for histological evaluation: (A) the lung section from the normal control (NC) group; (B) the lung section from model control (MC) group; (C) the lung section from model rats treated with 1 mg/kg of Dex. (D, E and F) the lung section from model rats treated with 40, 80 and 120 mg/kg of SUF, respectively (abbreviated as SUF40, SUF80 and SUF120, respectively). Representative histological section of the lungs was stained by HE. (G) Scoring of the inflammatory cell infiltration in the lung tissues. Each value indicated the mean $\pm$ SD ($n=10$). Different letters indicated statistically significant differences, $P < 0.05$ (Tukey's test).

Table 2
Effects of SUF on serum cytokines in OVA-challenged rats.

Group	Dose (mg/kg)	IFN- γ (pg/ml)	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-13 (pg/ml)
NC	–	146.9 $\pm$ 10.2 ^b	8.68 $\pm$ 0.95 ^c	16.15 $\pm$ 1.23 ^c	18.47 $\pm$ 2.48 ^c
MC	–	125.8 $\pm$ 13.8 ^c	10.82 $\pm$ 0.98 ^a	22.46 $\pm$ 1.34 ^a	53.75 $\pm$ 4.80 ^a
Dex	1	133.6 $\pm$ 11.2 ^{bc}	9.68 $\pm$ 0.99 ^b	17.61 $\pm$ 0.83 ^c	23.52 $\pm$ 3.59 ^d
SUF40	40	161.2 $\pm$ 7.73 ^a	8.70 $\pm$ 1.07 ^{bc}	18.25 $\pm$ 0.80 ^b	44.17 $\pm$ 4.23 ^b
SUF80	80	162.7 $\pm$ 10.3 ^a	8.00 $\pm$ 0.96 ^{cd}	16.90 $\pm$ 1.04 ^{bc}	31.19 $\pm$ 3.78 ^c
SUF120	120	169.5 $\pm$ 11.3 ^a	7.75 $\pm$ 0.81 ^d	16.18 $\pm$ 1.01 ^c	23.83 $\pm$ 2.27 ^d

NC, normal control group; MC, model control group; Dex, model rats treated with dexamethasone (1 mg/kg); SUF40, model rats treated with SUF (40 mg/kg); SUF80, model rats treated with SUF (80 mg/kg); SUF120, model rats treated with SUF (120 mg/kg). Data denoted were means $\pm$ SD ($n=10$). Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

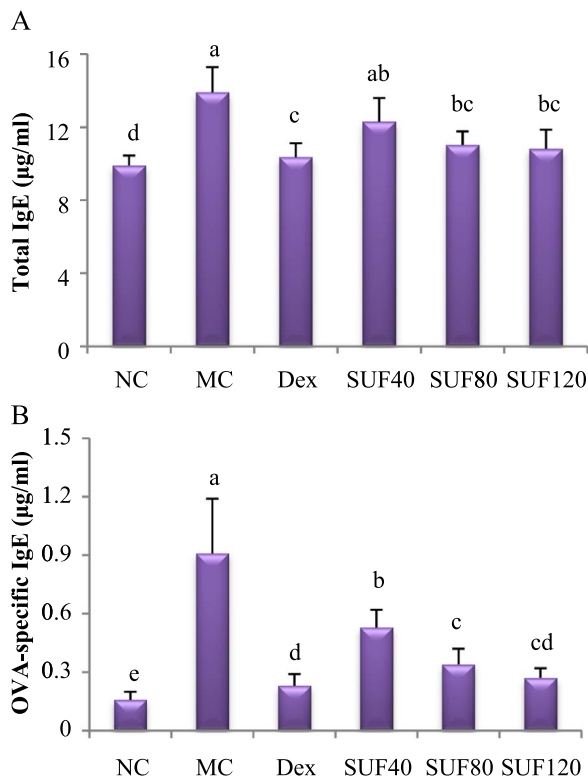


Fig. 4. Effects of SUL on total IgE (A) and OVA-specific IgE (B) production. Each value indicated the mean $\pm$ SD ($n = 10$). Different letters indicated statistically significant differences, $P < 0.05$ (Tukey's test).

3.5. SUL suppressed eotaxin expression in lung tissue

The immunostaining in Fig. 5 showed the expressions of eotaxin proteins in the lung tissues. Positive cells stained brown or yellow in the lung tissues. Eotaxin proteins had very little expression in lung tissue of the NC group while that was significantly increased ($P < 0.05$) in the MC group. After the treatment of SUL, the expression of eotaxin was much lower ($P < 0.05$) than that in the MC group.

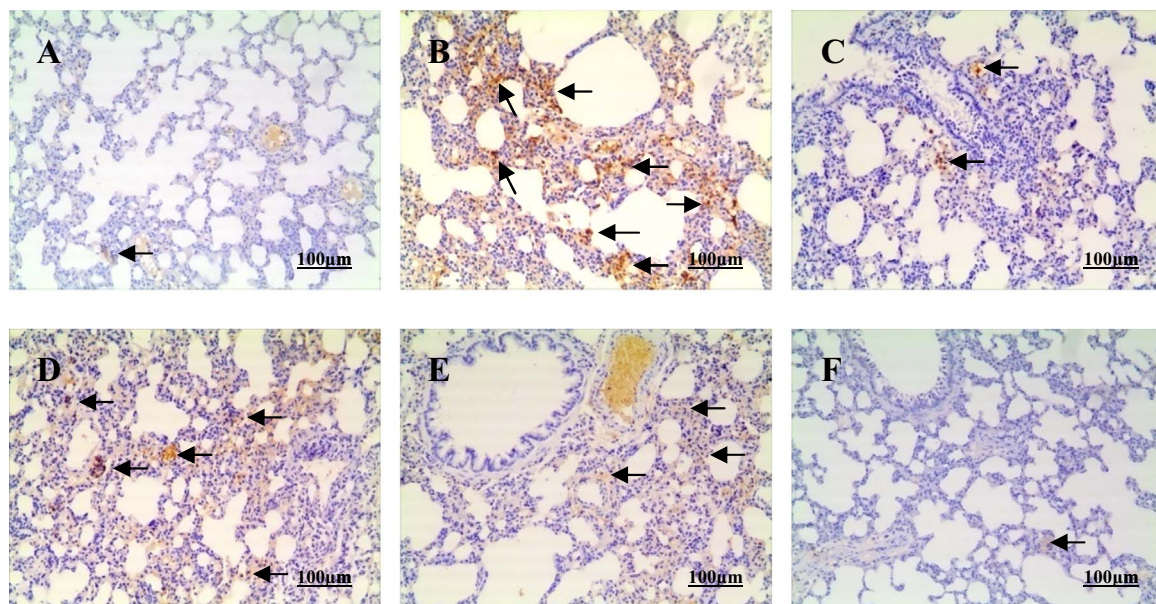


Fig. 5. Effects of SUL on eotaxin expressions in lung tissues of asthma rats. Lungs from each experimental group were processed for evaluation of eotaxin expressions: (A) the lung section from the normal control (NC) group; (B) the lung section from model control (MC) group; (C) the lung section from model rats treated with 1 mg/kg of Dex. (D, E and F) the lung section from model rats treated with 40, 80 and 120 mg/kg of SUL, respectively (abbreviated as SUL40, SUL80 and SUL120, respectively). Representative histological section of the lungs was stained by immunohistochemistry. Black arrow showed the positive expression of eotaxin proteins in the lung tissues.

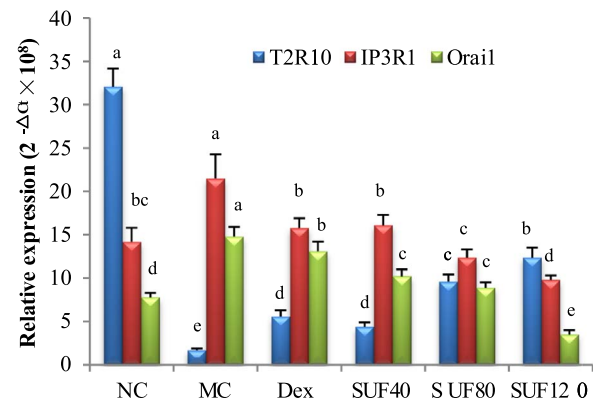


Fig. 6. Levels of key effectors in taste receptor signaling pathway. RFQ-PCR analysis of key genes (T2R10, IP3R1 and Orai1) in the lung tissues. Data denoted were means $\pm$ SD ($n = 10$). Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

3.6. SUL regulated expressions of T2R10 and its downstream effectors in taste receptor signaling pathway

The expression of T2R10 mRNA was obvious in lung tissue of the NC group while it were significantly decreased ($P < 0.05$) in the MC group. In contrast, the expressions of IP3R1 and Orai1 mRNA were significantly increased ($P < 0.05$) in the MC group compared with those in the NC group. However, the treatment with SUL at the doses of 40–120 mg/kg could reverse those changes (Fig. 6). It increased the expression of T2R10 mRNA but decreased IP3R1 and Orai1 in the lung tissues of asthma rats. There were significant differences among MC group and SUL-treated groups. And the regulating actions of SUL were found to proportionally increase in a dose-dependent manner.

As shown in Fig. 7, the expressions of NFAT1 and c-Myc proteins were increased approximately 1.5-fold more than those in the NC group ($P < 0.05$). After treatment of Dex or SUL, those protein expressions in the lung tissues of asthma rats were significantly reduced ($P < 0.05$). These results suggested that SUL had marked inhibition on inflammatory gene expressions.

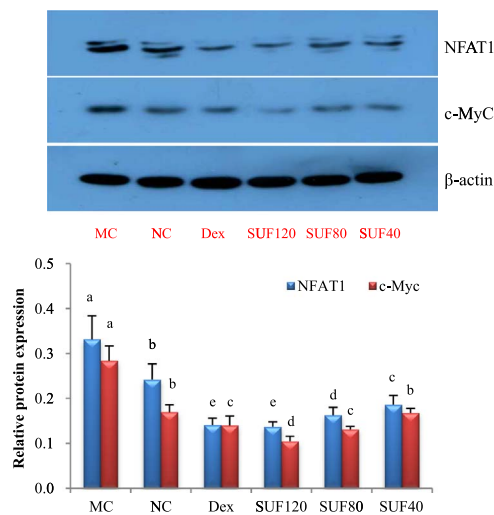


Fig. 7. Expression of downstream effectors NFAT1 and c-Myc in taste receptor signaling pathways. Western blotting analysis of NFAT1 and c-Myc protein in the lung tissues. Data denoted were means $\pm$ SD (n=10). Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

3.7. SUF induced the expression of T2R10

In this study, T2R10 gene, which has high expression in the alveolar epithelial cells (Deshpande et al., 2010), and GFP gene were co-transfected into HEK293 (in which T2R10 and GFP were not expression). Fluorescence intensity of GFP and T2R10 expression were positively associated. Rise in green fluorescence intensity intuitively reflected the activation of T2R10 in HEK293 transgenic cells. As shown in Fig. 8A, SUF induced the expression of GFP in both dose- and time-dependent manners, indicating that it upregulated the expression of T2R10 and might be a potential T2Rs agonist. Moreover, cytotoxic effects of SUF on HEK293 cells were tested by MTT assay as shown in

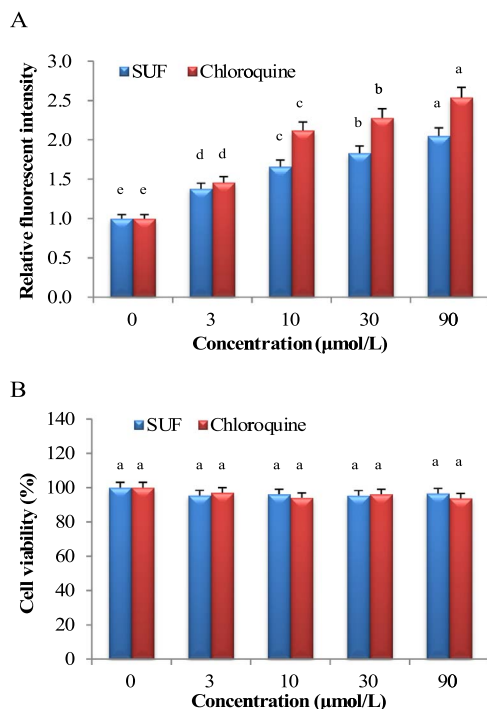


Fig. 8. SUF increased T2R10 protein expression. (A) Effects of SUF on T2R10 protein expression in T2R10-GFP transgenic HEK293T cells. (B) Cytotoxic effects of SUF in normal HEK293T cells. Each value indicated the mean $\pm$ SD (n = 5). Different letters indicated statistically significant differences, $P < 0.05$ (Tukey's test).

Fig. 8B. And SUF had caused barely a ripple on cell viability ($P > 0.05$).

4. Discussion

Glucocorticoids (such as Dex, prednisone and hydrocortisone) are first-line drugs for controlling airway inflammation by suppressing the mucus hypersecretion. Although mechanisms underlying the action of glucocorticoids are varied and complex, they are strictly controlled for persistent use due to its serious side effects, including immune suppression, secondary infections and muscle atrophy (Cockcroft and Sears, 2013). Thus, new therapeutic approaches are necessary. The herbal extracts exhibit various biological activities, including antibacterial, antifungal, anti-inflammatory, antioxidant, and antitumor effects, which have potential for asthma treatment. In this study, we focused on the potential of SUF as a therapeutic agent for treating asthma using a rat model. The above results demonstrated that: (1) SUF significantly ameliorated OVA-induced AHR and reduced the degree of lung pathological alteration; (2) SUF decreased serum levels of T-helper type2 (Th2) cytokines and IgE but increased IFN- γ ; and (3) SUF up-regulated T2R10 expression and down-regulated its downstream effectors.

Flavonoids, which are polyphenolic plant secondary metabolites ubiquitously present in vegetables, fruits and beverages, have a positive influence on various diseases. These effects were not only attributed to their direct antioxidant properties, but also their direct inhibition of inflammatory mediator production (Kang and Kim, 2016; Saqib and Janbaz, 2016; Terahara, 2015). They could inhibit IL-4-induced signal transduction and affect the differentiation of naïve CD4⁺ T cells through their inhibition on the activation of the aryl hydrocarbon receptors (Jang et al., 2012; Tanaka and Takahashi, 2013; Nyanhanda, Gould and Hurst, 2014). Various studies of flavonoids in IL-4-sensitized cell models and asthmatic rodent models have also demonstrated their beneficial effects (Bao et al., 2011; Li and Zhang, 2013; Yang et al., 2013). Clinical studies suggest that an increase in flavonoid intake can ameliorate the airway inflammation and bronchial hyper-responsiveness of asthmatic patients (Arts and Hollman, 2005; Li and Brown, 2009; Nurmatov, Devereux and Sheikh, 2011). After enrichment by D101 macroreticular resin, the content of total flavonoids in SUF was detected to be 87.2% by colorimetric method, indicating the simple and efficient method for preparative separation of total flavonoids from *S. uncinata*. Despite the mass bioactive components in the plant extracts, in this study, three commercially available standards (amentoflavone, hinokiflavone and isocryptomerin) were used for quality control of SUF by HPLC analysis. Since the total content of three biflavonoids in SUF was only about 24%, more investigations about developed HPLC detection method and indicative components in SUF should be further considered for quality evaluation of SUF.

Asthma is a chronic inflammatory disease characterized by airway inflammation and remodeling, bronchial hyper-responsiveness, and variable airflow obstruction (Lambrecht andammad, 2014; Yoon, 2014). AHR is a measure of the bronchial constriction commonly found in asthmatics. In our study, the effect of SUF on airway responsiveness was obtained by monitoring the enhanced pause (Penh) for 3 min as shown in Fig. 2. In addition, AHR had been associated with sub-epithelial fibrosis, which was observed as airway wall thickening during the remodeling process. Thus, the finding that the degrees of lung pathological changes were significantly improved by SUF could be explained, in part, by reduction in AHR.

It has been well-known that Th1/Th2 cells imbalance is considered to be the immunological cause of asthma, led by the dominant upregulation of Th2 cells in immune response (Polikepahad, Moore and Venugopal, 2006; Ismaila et al., 2013). In allergic diseases such as bronchial asthma, the activity of Th1 cells (which product IFN- γ) is reduced, while Th2 cell activity is activated, resulting in Th2-dominated immune responses. Aberrant production of the prototypical type 2 cytokines, IL-4, IL-5 and IL-13, has long been associated with the

pathogenesis of asthma (Ingram, and Kraft, 2012). These interleukins mediate the important pro-inflammatory function in asthma, including induction of the IgE isotype switch, increased expression of vascular cell adhesion molecules, stimulation of mucus production and Th2 lymphocyte differentiation, thereby releasing IL-4, IL-5 and IL-13 (Kirstein et al., 2010). Mice over-expressing IL-13 in the lung develop several characteristics of asthma such as pulmonary eosinophilia, airway epithelial hyperplasia, airways obstruction and hyper-responsiveness to cholinergic stimulation. Although both IL-4 and IL-13 are capable of inducing asthma like phenotype, the activity of IL-13 appears to be greater than that of IL-4 (Huang et al., 2016; Qaseem et al., 2013). In this study, SUF reduced the levels of IL-4, IL-5 and IL-13, consistent with levels of total and OVA-specific IgE in serum. On the other hand, IFN- γ is the prominent Th1 effector and is implicated in both acute severe and chronic stable asthma. It inhibits allergen-induced eosinophil recruitment and IgE release via down-regulating GATA-3, IL-4 and IL-5 expression in the lung tissues of asthma model (Finkelman et al., 2010; Kim, DeKruyff and Umetsu, 2010; Lu et al., 2016). Previous researches have shown conflicting results that IFN- γ over-expression in the airways also induces the non-eosinophilic inflammation, enhanced smooth muscle contractility and lung fibrosis (Kobayashi et al., 2012; Raundhal et al., 2015; Yu et al., 2011). In the study, the level of IFN- γ was also significantly elevated by 40–120 mg/kg SUF. Treatment with Dex also inhibited the production of inflammatory mediators IL-4, IL-5 and IL-13, but could not significantly improve the decreased levels of serum IFN- γ due to the well-known immunosuppression of Dex. These findings indicated that SUF acted through modulating both Th1 and Th2 cytokines responsible for eosinophil recruitment into lung tissue. Recently, many studies have demonstrated that amentoflavone exhibited the protective effects against acetic acid-induced ulcerative colitis and LPS-induced neuroinflammation via down-regulating iNOS and COX-2 expressions, modulating oxidant/anti-oxidant balance, inhibiting pro-inflammatory cytokine profiles in NF- κ B signal pathways (Ishola et al., 2012, 2013; Sakthivel and Guruvayoorappan, 2013; Saroni Arwa et al., 2015; Wansi et al., 2016). This biflavonoid induced apoptosis of hypertrophic scar fibroblasts and inhibited angiogenesis of endothelial cells (Zhang et al., 2014). It also significantly enhanced innate immune responses by improving immune organs as well as stimulating the production of IL-2 and INF- γ both in normal and tumor bearing animals (Guruvayoorappan and Kuttan, 2007). These results indicated that biflavonoids, especially amentoflavone, may play multiple roles in the anti-asthma effects of SUF. Further evaluation on the anti-inflammatory and immunomodulatory activities of SUF should be done in order to determine their application in medicinal field.

Eotaxin, produced by airway epithelium, endothelial cells, lymphocytes, macrophages, and eosinophils, is the strongest and most specific factor, affecting eosinophil function. It is strongly involved in asthmatic inflammation (Raby et al., 2006). This chemokine causes the release of progenitors of eosinophils from the bone marrow and could increase the count of mature eosinophil forms in peripheral blood. Moreover, eotaxin can elicit migration of mast cells and basophils (Pease and Williams, 2001). The histopathological study also confirmed the foregoing findings. Histopathological alterations, such increased production of Eotaxin and infiltration of inflammatory cells in lung tissue, are observed in asthmatic conditions, which are closely related to the Th2 cytokine and IgE levels (Adar et al., 2014). After 2 weeks of OVA-sensitization/challenge, the similar histopathological changes were observed in lung tissue. However, the symptoms of these alterations were significantly improved in Dex- or SUF-treated rats. Both Dex and SUF prevented the development of eosinophil infiltration, lung inflammation, and the decreased expression of eotaxin protein in lung tissue. These results were in agreement with the data of Th2 cytokine changes obtained in serum. And it indicated that SUF could attenuate the inflammatory response in OVA-induced asthma via regulation of inflammatory chemokines.

Bitter taste receptors (T2Rs), a subfamily of G-protein coupled receptors (GPCRs), are one of the basic taste receptors in the tongues and protect against consuming potentially harmful substances, which are often bitter. Recently, T2Rs are also found to be expressed in many cell types of the airways, including solitary chemosensory cells, ciliated epithelial cells and smooth muscle cells of airways (ASM) (Coghlan, 2010; Kinnamon, 2012). Bitter substances can activate T2Rs in airway smooth muscle to cause greater bronchodilation than β_2 adrenergic receptor agonists, the most commonly used bronchodilators. Leukocytes and mast cells from children with severe asthma have increased expression of T2Rs (Ekoff et al., 2014) while the T2Rs agonists (such as chloroquine and denatonium) could cause relaxation of isolated ASM and dilation of airways (Manson et al., 2014). When asthma happened or treated with bronchoconstrictors, global intracellular content of Ca^{2+} ($[\text{Ca}^{2+}]_i$) is obviously increased in ASM and T cells (Zhang et al., 2013). These Ca^{2+} changes in cells are mediated via activation of the canonical bitter taste signaling cascade (i.e., T2R-gustducin-phospholipase C β [PLC β]-inositol 1,4,5-triphosphate receptor [IP3R]). Activation of T2Rs fully reverses the increase in $[\text{Ca}^{2+}]_i$ induced by bronchoconstrictors, and this lowering of the $[\text{Ca}^{2+}]_i$ is necessary for bitter tastant-induced ASM cell relaxation (Robinet et al., 2014). Thus, T2Rs may be potential targets for the development of a new class of more efficacious agonists for bronchodilation and treatment of asthma (Deshpande et al., 2010). In this study, anti-asthma mechanism of SUF was investigated in transgenic HEK293 (T2R10-GFP) cell model and OVA-induced asthmatic rodent model. SUF activated the expression of T2R10 in both dose- and time-dependent manners *in vitro*. The results of FQ-PCR analysis also showed that SUF up-regulated the T2R10 mRNA levels but inhibited its key downstream targets (IP3R1) in the lung tissue of asthmatic rats. Moreover, SUF could inhibit the Orai1 mRNA expression, which regulates Ca^{2+} entry via governing Ca^{2+} release-activated Ca^{2+} (CRAC) channel (Shim, Tirado-Lee and Prakriya, 2015). Treatment with SUF down-regulated the protein levels of NFAT1 and c-Myc. As the most downstream effectors in the T2Rs pathway, the transcription factor NFAT1 and c-Myc both cooperates with Ras/MAPK pathway to induce cell death and inflammation in different cell types, such as T lymphocytes, Burkitt's lymphoma and fibroblasts (Robbs, Lucena and Viola, 2013; Fletcher and Prochownik, 2015). These results were consistent with the inhibition of SUF on inflammatory cytokine (IL-4, IL-5 and IL-13) production. It indicated that SUF could ameliorate OVA-induced inflammation via T2R10/IP3R1/NFAT1 signaling pathway.

5. Conclusion

In conclusion, this study demonstrates that SUF inhibits airway inflammation, ameliorates AHR, and suppresses Th2 cytokines and IgE levels through regulating T2R10/IP3R1/NFAT1 pathway. It may warrant further evaluation as a possible agent for the treatment of asthma.

Conflict of interest

The authors have declared that no competing interests exist and publishing such observations doesn't interfere with the rights of the indigenous people.

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