

Original Article

Siraitia grosvenorii residual extract attenuates ovalbumin-induced lung inflammation by down-regulating IL-4, IL-5, IL-13, IL-17, and MUC5AC expression in mice

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ABSTRACT

Background: *Siraitia grosvenorii* fruits are used in traditional medicine to treat cough, sore throat, bronchitis, and asthma.

Purpose: This study aimed to investigate the anti-inflammatory and anti-asthmatic effects of *S. grosvenorii* residual extract (SGRE) on ovalbumin (OVA)-induced asthma in mice.

Methods: Asthma was induced in BALB/c mice by systemic sensitization to OVA, followed by intratracheal, intraperitoneal, and aerosol allergen challenges. SGRE was orally administered for four weeks. We investigated the effects of SGRE on airway hyper-responsiveness, OVA-specific IgE production, histological analysis of lung and trachea, immune cell phenotyping, Th1/Th2 cytokine production in bronchoalveolar lavage fluid (BAL) fluid and splenocytes, and gene expression in the lung.

Results: SGRE ameliorated OVA-driven airway hyper-responsiveness, serum IgE production, and histopathological changes in the lung and trachea. SGRE reduced the total number of cells in the lung and BAL, the total number of lymphocytes, neutrophils, monocytes, and eosinophils in the lung and BAL, the absolute number of CD4⁺/CD69⁺ T cells in the lung, and the absolute number of CD4⁺/CD8⁺ T cells and CD11b⁺/Gr-1⁺ granulocytes in the lung and BAL. SGRE also reduced Th2 cytokines (IL-4, IL-5, and IL-13) and increased the Th1 cytokine IFN- γ in the BAL fluid and supernatant of splenocyte cultures. SGRE decreased the OVA-induced increase of IL-13, TARC, MUC5AC, TNF- α , and IL-17 expression in the lung.

Conclusion: SGRE exerts anti-asthmatic effects via the inhibition of Th2 and Th17 cytokines and the increase of Th1 cytokines, suggesting that SGRE may be a potential therapeutic agent for allergic lung inflammation, such as asthma.

Introduction

Asthma is an inflammatory disease of the lung characterized by airway hyper-responsiveness, airflow obstruction, mucus hypersecretion due to goblet cell hyperplasia, bronchial eosinophilic inflammation, and airway remodeling (Lee et al., 2012). Multiple cell types of the innate immune system including mast cells, eosinophils, basophils, neutrophils, dendritic cells, and innate lymphoid cells, play roles in the initiation and maintenance of asthma, and Th2/Th17 lymphocytes of the adaptive immune system contribute to the pathogenesis of asthma by producing adaptive cytokines such as Th2 cytokines [interleukin

(IL)-4, IL-5, IL-13], and IL-17 (Schuijs et al., 2013). IL-4 and IL-13 elicit mucus hypersecretion, airway hyper-responsiveness, and immunoglobulin E (IgE) production via isotype switching in B cells and upregulation of adhesion molecules for eosinophil infiltration, and IL-5 promotes the differentiation, activation, and survival of eosinophils (Hall and Agrawal, 2014). The Th1 cytokine IFN- γ , which is secreted by Th1 cells, acts in conjunction with Th2 cytokines in maintaining the chronic inflammatory response in allergic disease, such as asthmatic airway inflammation (Ngoc et al., 2005).

Siraitia grosvenorii Swingle (Cucurbitaceae family) is a unique plant found mainly in the Guangxi province, China (Li et al., 2017). The fruit

Abbreviations: MLN, mesenteric lymph node; Mon, Montelukast; OVA, ovalbumin; PBMC, peripheral blood mononuclear cells; PLT, platelets; RBC, red blood cells; SGRE, *S. grosvenorii* residual extract; WBC, white blood cells

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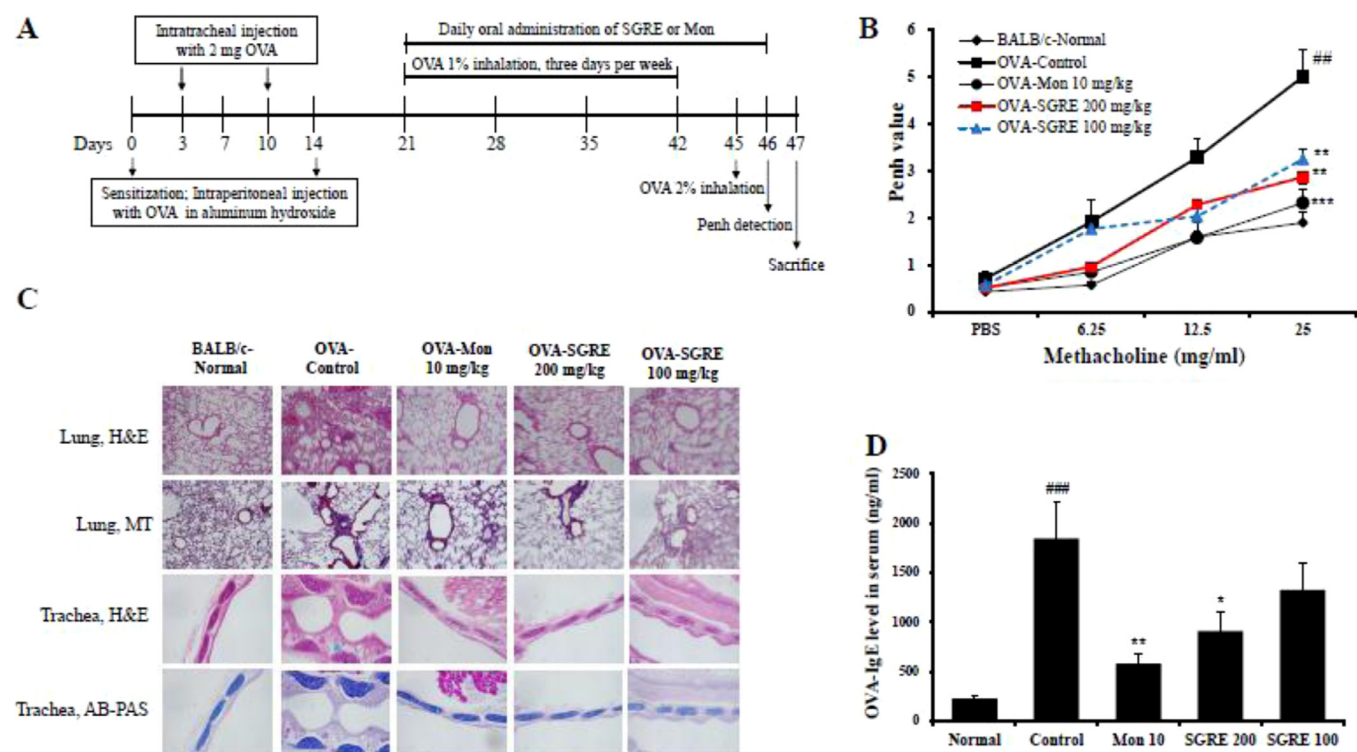


Fig. 1. The effect of *Siraitia grosvenorii* residual extract (SGRE) on airway hyper-responsiveness and serum IgE levels. (A) Schematic diagram. (B) Airway responsiveness to aerosolized methacholine (Penh) in each treatment group. (C) Histopathology of lung and trachea tissues of ovalbumin (OVA)-induced mice. H&E, MT, and AB-PAS staining of lung and trachea sections from normal and asthmatic mice treated with OVA (control), Mon (10 mg/kg), or SGRE (100 or 200 mg/kg). H&E: Hematoxylin-eosin; MT: Masson's trichrome; AB-PAS: Alcian blue-periodic acid Schiff. (D) OVA-specific IgE levels in the serum of OVA-induced mice. The results are expressed as the mean $\pm$ standard error of the mean (SEM; $n = 10$ per group). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ indicate statistically significant differences between the normal and asthmatic control mice. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ indicate statistically significant differences between the asthmatic control mice and the Montelukast (Mon)- or SGRE-treated mice.

of the *S. grosvenorii* has been used as traditional Chinese medicine to treat cough, sore throat, bronchitis, asthma, acute gastritis, and constipation; it is also used as a natural sweetener (Murata et al., 2010; Committee of National Pharmacopoeia, 2010). Previous studies have shown that both *S. grosvenorii* extract and its main component, mogroside, have anti-inflammatory, anti-diabetic, anti-carcinogenic, and anti-oxidative properties (Di et al., 2011; Qi et al., 2008; Yasuno et al., 2008). Recently, it was reported that mogroside has protective effects on bleomycin-induced pulmonary fibrosis and lipopolysaccharide (LPS)-induced acute lung injury in mice (Shi et al., 2014; Tao et al., 2017). These data suggest that *S. grosvenorii* extract may have therapeutic effects on pulmonary inflammatory diseases. *S. grosvenorii* fruit is extracted with water to produce mogroside, and the residue is generally discarded. *S. grosvenorii* residual extract (SGRE) is raw material available at a low cost, yet no studies have tested the effects of SGRE. Thus, this study evaluated the anti-inflammatory effects of SGRE on ovalbumin (OVA)-induced airway inflammation in a mouse model of asthma.

Materials and methods

Reagents

Mogroside V (purity, $\geq 97\%$), mogroside IV (purity, $\geq 97\%$), and mogroside III (purity, $\geq 97\%$) were purchased from Sigma-Aldrich (St Louis, MO, USA). The purity of the mogrosides were determined to be above 97% by HPLC analysis.

Preparation of *Siraitia grosvenorii* residual extract

Dried *Siraitia grosvenorii* fruits were purchased from Hunan Huacheng Biotech, Inc. (China), and the extract was deposited in the herbarium of the Herbal Medicine Research Division at the Korean Institute of Oriental Medicine. Dried *S. grosvenorii* fruits (200 g) were powdered to 1–5 mm size in a mill and reflux extracted twice with 2 l water for 3 h. After water extraction, the residual of *S. grosvenorii* was dried, and then the dried residual (50 g) was extracted once with 70% (v/v) ethanol solution by reflux. The residual extracts were filtered, concentrated, lyophilized, and stored at 4 °C. The yield of the dried extract from the crude material was 4.5% (w/w).

Animals

Seven-week-old male BALB/c mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in an air-conditioned room at a temperature of 21 ± 2 °C and humidity of $50 \pm 5\%$ under a 12 h:12 h light:dark cycle. Mice were fed a commercial diet and water ad libitum for one week before beginning the experiment. The committee for animal welfare at Daejeon University approved the experimental protocols used in this study. All animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication number 85–23, 1996 edition).

OVA sensitization and inhalation

According to a modified protocol described previously (Kim et al., 2012), we dissolved 50 μ l of 1 mg/ml albumin grade V (Sigma-Aldrich; St. Louis, MO, USA) and 80 μ l of 13 mg/ml aluminum hydroxide gel

(Alhydrogel, Sigma-Aldrich) in distilled water. For sensitization, all mice were immunized by intraperitoneal (*i.p.*) injection of alum-precipitated antigen (0.25 ml) containing 12.5 µg of OVA (Sigma-Aldrich) and 0.26 mg of aluminum hydroxide (Sigma-Aldrich) in phosphate-buffered saline (PBS) on three different days (on the first day of 0, 1, and 2 weeks before inhalational exposure), followed by intratracheal injection with 2 mg of OVA (100 µl in PBS) at 3 and 10 days after the first sensitization. Then, the mice were exposed to inhalation with 1% OVA in normal aerosolized saline using an ME-U12 ultrasonic nebulizer (Omron; Tokyo, Japan) for 30 min per day three days per week from day 21 to day 42. On day 45, mice were exposed to inhalation with 2% OVA in normal saline. Mice were divided into five groups with $n = 10$ per group as follows: (1) normal, (2) OVA-control, (3) 10 mg/kg OVA-Montelukast (Mon), (4) 200 mg/kg OVA-SGRE, and (5) 100 mg/kg OVA-SGRE. Montelukast was administered as a positive control (Sigma-Aldrich). Daily SGRE (200 and 100 mg/kg) and Mon (10 mg/kg) were administered orally for four weeks (days 21–46). One day after the last OVA exposure (2% OVA inhalation), we measured enhanced pause (Penh) to evaluate airway hyper-responsiveness. On day 47, mice were sacrificed, and serum, bronchoalveolar lavage fluid (BALF), and lung cell samples were collected for further molecular analyses. The experimental scheme is shown in Fig. 1A.

Determination of Penh

Airway hyper-responsiveness in mice was measured using a Buxco system (Biosystem XA; Data Sciences International, New Brighton, MN, USA) as previously described (Kim et al., 2014). Mice were aerosolized with OVA for 30 min/day, three days/week for three weeks. One day after the final inhalation, mice were given aerosolized saline, followed by increasing doses (6.25, 12.5, and 25 mg/ml) of aerosolized methacholine (Sigma-Aldrich). Airway reactivity was then monitored for 30 min, and respiratory curves were converted into Penh values.

Collection of BALF

BALF was obtained by washing the airway lamina via tracheal cannulation. BALF was collected via lung lavage and centrifuged at $400 \times g$ for 5 min at 4 °C. The supernatant was collected and stored at –20 °C for determination of cytokine levels. The cell pellets were resuspended in 1 ml PBS, and the total cell number was counted.

Sample collection and cell preparations

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood via Percoll density-gradient centrifugation. Single-cell suspensions from tissues and BALF were isolated in RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 20 mM HEPES, 50 µM 2-mercaptoethanol, and 2% fetal bovine serum (GIBCO; Grand Island, NY, USA). Briefly, the lung and mesenteric lymph node (MLN) tissues were removed from the mice, minced, and then incubated in PBS containing 1 mg/ml collagenase IV (Sigma) for 40 min at 37 °C. The cell suspension was filtered and then centrifuged for 20 min at 450 g. Between 2×10^3 and 4×10^3 cells (100 µl) from the cell pellet were spun onto glass slides via centrifuge at $400 \times g$ for 4 min using a cytospin centrifuge (Cellspin; Hanil, Seoul, Korea), and stained with Diff-Quick Stain Set solution (Baxter Healthcare; Miami, FL, USA). Differential cell counts were performed based on their microscopic morphology, and the cells were stained with various antibodies for flow cytometric analysis (FACS).

FACS

Cells from lung, MLN, and BALF were stained with the indicated antibodies in staining solution (PBS containing 1% FBS and 0.01%

NaN₃) for 10 min on ice. The stained cells were analyzed by two-color flow cytometry on a FACSCalibur using CellQuest software (BD Biosciences; Mountain View, CA, USA). All antibodies for FACS, including anti-CD3e, anti-CD4, anti-CD8, anti-CD19, anti-CD23, anti-CD69, anti-CD11b, anti-Gr-1, anti-CD23, and anti-B220, were purchased from BD PharMingen (San Diego, CA, USA). The number of absolute cells from each tissue was calculated as the percentage value of the total cell number.

Hematoxylin-eosin (H&E), Masson's trichrome (M-T), and Alcian blue-periodic acid Schiff (AB-PAS) staining

The lungs were removed and analyzed histologically. The tissue was fixed in 10% (v/v) neutral-buffered formalin, embedded in paraffin, and cut into 4 µm thick sections. The sections were stained with H&E to assess the infiltration of inflammatory cells, and with M-T (Sigma-Aldrich) to determine the collagen deposition. Other sections were stained with AB-PAS (Sigma-Aldrich) to determine the levels of goblet cell hyperplasia and mucus secretion. At least three different sections of each tissue were evaluated under light microscopy.

Enzyme-linked immunosorbent assay (ELISA)

OVA-specific IL-4 and IFN-γ production was detected in spleen cells suspended in RPMI 1640 medium supplemented with 2 mM L-glutamine and 5% FBS. The spleen cells were cultured for 48 h at a concentration of 1×10^5 cells/well in 96-well plates with or without 10 µg/ml of OVA in a humidified atmosphere of 5% CO₂ at 37 °C. The levels of IL-4, IL-5, IL-13, and IFN-γ in culture supernatants and BALF, and serum OVA-specific-IgE were measured using ELISA kits (R&D Systems; Minneapolis, MN, USA).

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA from the lung was extracted using RNAzol B (Tel-Test; Austin, TX, USA) according to the manufacturer's instructions. cDNA was synthesized from 3 µg of total RNA using a ReverTra Ace-α cDNA Synthesis kit (Toyobo; Osaka, Japan). Gene expression was analyzed with a SYBR Green PCR Master Mix (Applied Biosystems; Grand Island, NY, USA) and 200 nM primers. The PCR was performed as follows: 2 min at 50 °C, 10 min at 94 °C, 40 cycles of 1 min at 94 °C, and 1 min at 60 °C. The cycle number at which the emission intensity of the sample rose above baseline was defined as the relative quantity (RQ) and was proportional to the target concentration. RT-PCR was performed using the Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems). The RQ in target gene expression relative to the control was normalized to the internal control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), using the $2^{-\Delta\Delta Ct}$ method. GAPDH (VIC dye-labeled) was selected using Assays-on-Demand Gene Expression Products (Applied Biosystems). The primer sequences were as follows: IL-13 (Accession number: NM_008355.3), sense 5'-ATGCC CAACAAAGCAGAGAC-3', antisense 5'-TGAGAGAACCAGGGAGC TGT-3'; TNF-α (NM_013693.3), sense 5'-GGCTTTCCGAATTCACCTGGA GCCT-3', antisense 5'-CCCCGGCCTTCCAATAAATACATTCTATA-3'; IL-17 (NM_010552.3), sense 5'-TCTCATCCAGCAAGAGATCC-3', antisense 5'-AGTTTGGGACCCCTTTACAC-3'; TARC/CCL17 (NM_011332.3), sense 5'-CCCATGAAGACCTTCACCTC-3', antisense 5'-ACTCTCGGCCT ACATTGGTG-3'; and MUC5AC (NM_010844.1) sense 5'-AGAATATCT TTCAGGACCCCTGCT-3', antisense 5'-ACACCAGTGCTGAGCATACT TTT-3'.

Western blotting

Extraction from the lung tissue samples of mice was performed using PRO-PREP protein extraction solution (Intron, Seoul, Korea). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto membranes. Membranes were blocked with 5% bovine serum albumin and then incubated with

primary antibodies against IL-13, TARC, MUC5AC, TNF- α , IL-17, and β -actin (abcam, Cambridge, UK) at 4 °C overnight. The membranes were then incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (abcam) for 1 h at room temperature. Membranes were treated with the enhanced chemiluminescence (ECL) detection reagent (Amersham Bioscience, Buckinghamshire, England), and protein bands were visualized using the Image Analyzer Las-1000 (Fujifilm, Tokyo, Japan). The relative band density was determined by densitometry with Image J software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

The results are presented as the mean $\pm$ standard error of the mean (SEM), and the differences were considered statistically significant for p values less than 0.05. The values were analyzed using one-way analysis of variance (ANOVA) followed by either Tukey's or Duncan's multiple comparison tests using SigmaPlot 13.0 software.

Results

Effect of SGRE on airway hyper-responsiveness

To evaluate the effect of SGRE on airway hyper-responsiveness, the Penh value was measured in mice the first day after the final inhalation. As shown in Fig. 1B, the Penh value was increased in OVA-induced asthmatic mice compared to normal mice (Fig. 1B). The Penh conversion to methacholine was significantly reduced in SGRE-treated (100 and 200 mg/kg) and Mon-treated mice relative to control mice sensitized with OVA.

Lung and trachea histology

Histological investigation of lung sections from OVA-challenged control mice revealed inflammatory changes, including peribronchial and perivascular infiltration of various inflammatory cells and fibrosis. Histological sections from SGRE and Mon-treated mice showed reduced airway inflammation characterized by decreased inflammatory cell infiltrate and fibrosis (Fig. 1C), however. Tissue from the trachea of OVA-challenged control mice exhibited induced inflammation and mucus hypersecretion due to goblet cell hyperplasia, thickened smooth muscle, and narrow lumen compared with normal mice (Fig. 1C). Together, these results indicate that SGRE inhibits histopathological changes in the lung and tracheal tissue of asthmatic mice.

Effect of SGRE on serum IgE levels

Given that elevated IgE production is closely associated with various inflammatory cells during the pathogenesis of allergic reactions, serum OVA-specific IgE levels were examined. OVA-specific IgE levels were markedly increased in the asthmatic control group compared with the normal group, and were significantly suppressed upon treatment with SGRE (200 mg/kg) and Mon (Fig. 1D).

Effect of SGRE in the blood of mice

Changes in the cell types present during airway inflammation, particularly eosinophils, provide important information about the development and pathogenesis of asthma. Inflammatory cells such as neutrophils, monocytes, eosinophils, and basophils were reduced in the blood of SGRE-treated mice (100 and 200 mg/kg; Table 1). Monocytes and basophils also were reduced in the blood of Mon-treated mice.

Table 1
Hematological parameters in mice.

	RBC ($\times 10^6/\mu\text{L}$)	PLT ($\times 10^3/\mu\text{L}$)	WBC ($\times 10^3/\mu\text{L}$)	WBC differential counting (%)		Monocytes	Eosinophils	Basophils	Large unstained cells
				Neutrophils	Lymphocytes				
BALB/c -Normal	9.45 $\pm$ 0.99	1023.75 $\pm$ 27.04	3.55 $\pm$ 0.33	15.83 $\pm$ 2.18	78.25 $\pm$ 1.67	2.28 $\pm$ 0.34	1.55 $\pm$ 0.21	1.15 $\pm$ 0.28	0.93 $\pm$ 0.18
OVA-Control	10.20 $\pm$ 0.14	1073.00 $\pm$ 79.86	4.46 $\pm$ 0.41	16.85 $\pm$ 1.03	75.35 $\pm$ 0.82	2.48 $\pm$ 0.15	3.53 $\pm$ 0.24##	1.10 $\pm$ 0.04	1.73 $\pm$ 0.73
OVA-Mon 10 mg/kg	10.35 $\pm$ 0.18	987.50 $\pm$ 32.06	4.02 $\pm$ 0.23	17.98 $\pm$ 0.97	75.33 $\pm$ 1.46	1.98 $\pm$ 0.13*	3.20 $\pm$ 0.41	0.58 $\pm$ 0.05**	0.98 $\pm$ 0.06
VA-SGRE 200 mg/kg	10.50 $\pm$ 0.10	1098.50 $\pm$ 27.04	3.67 $\pm$ 0.45	18.03 $\pm$ 1.88	76.53 $\pm$ 2.34	1.60 $\pm$ 0.19*	2.48 $\pm$ 0.41*	0.28 $\pm$ 0.08***	1.13 $\pm$ 0.28
OVA-SGRE 100 mg/kg	10.77 $\pm$ 0.04**	1137.30 $\pm$ 34.97	5.41 $\pm$ 0.59	14.25 $\pm$ 0.18*	81.13 $\pm$ 0.11***	1.35 $\pm$ 0.19**	2.05 $\pm$ 0.17***	0.23 $\pm$ 0.03***	1.05 $\pm$ 0.10

RBC, red blood cells; PLT, platelets; WBC, white blood cells; OVA, ovalbumin; Mon, Montelukast; SGRE, *S. grosvenori* residual extract. # p < 0.05, ## p < 0.01, and ### p < 0.001 indicate statistically significant differences between the normal and asthmatic control mice.

* p < 0.05.

** p < 0.01, and

*** p < 0.001 indicate statistically significant differences between the asthmatic control mice and the Montelukast (Mon)- or SGRE-treated mice.

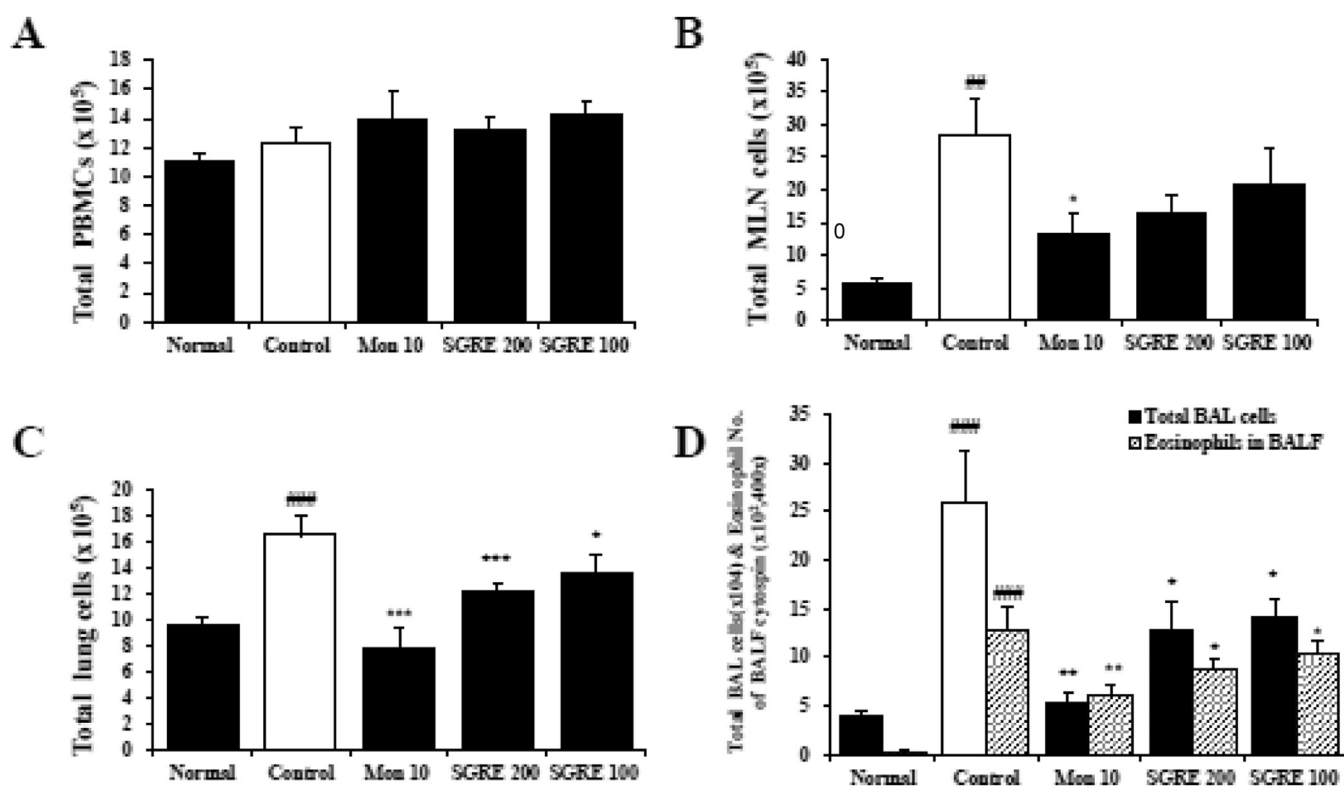


Fig. 2. The number of total peripheral blood mononuclear cells (PBMCs), lung, mesenteric lymph node (MLN), and bronchoalveolar lavage (BAL) cells in *Siraitia grosvenorii* residual extract (SGRE)-treated asthmatic mice. Total (A) PBMCs, (B) lung, (C) MLN, and (D) BAL cells and eosinophils in the BAL fluid (BALF) for each treatment group. The results are expressed as the mean $\pm$ standard error of the mean (SEM; $n = 10$ per group). # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ indicate statistically significant differences between the normal and asthmatic control mice. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ indicate statistically significant differences between the asthmatic control mice and the Montelukast (Mon)- or SGRE-treated mice.

Number of total PBMCs, lung, MLN, and BALF cells in SGRE-treated asthmatic mice

The total numbers of PBMCs and MLN cells were not decreased in SGRE-treated mice compared with control mice (Fig. 2A and B). The total numbers of lung and BALF cells, as well as the number of eosinophils in the BALF, were significantly decreased in SGRE (100 and 200 mg/kg) and Mon-treated mice, however, compared with control mice (Fig. 2C and D).

Effect of SGRE on the number of immune cell subtypes in the PBMCs, lung, MLN, and BALF

FACS was performed to evaluate the effect of SGRE on immune cell subtypes. The absolute numbers of CD19+ B cells and CD23+ /B220+ B cells in PBMCs were significantly decreased in the SGRE group compared with the control group (Fig. 3A). The absolute numbers of lymphocyte, neutrophils, monocytes, eosinophils, CD3e-, CD19-, CD4-, CD8-, B220-, CD23-, CD69-, CD11b-, and Gr-1-positive cells in the lung and BALF of OVA-challenged control mice were increased compared with normal mice (Fig. 3B and C). SGRE (200 mg/kg) and Mon treatment with OVA caused marked reduction in the numbers of lymphocyte, neutrophils, monocytes, eosinophils, CD3e+ /CD19+ cells (T cell/ B cell), CD4+ /CD69+ T cells, CD4+ /CD8+ T cells, and CD11b+ /Gr-1+ in the lung (Fig. 3B). SGRE (200 mg/kg) and Mon treatment with OVA also significantly decreased the number of lymphocytes, eosinophils, CD4+ /CD8+ T cells, and CD11b+ /Gr-1+ in BALF (Fig. 3C). The absolute numbers of CD3e+ /CD19+ cells and CD4+ /CD8+ cells in the MLN were increased in the OVA-challenged control group compared with the normal group, and these immune cells did not change in the MLN cells of SGRE-treated mice (Fig. 3D).

Effect of SGRE on cytokine production in the BALF and splenocytes

To determine whether SGRE influences cytokine secretion in the BALF and splenocytes, we measured IL-4, IL-5, IL-13, and IFN- γ levels by ELISA after the final OVA challenge. As shown in Fig. 4, SGRE (200 mg/kg) inhibited the IL-4, IL-5, and IL-13 levels in the BALF and splenocyte culture supernatants. In contrast, SGRE increased the IFN- γ levels in the BALF.

Effect of SGRE on mRNA and protein expression in the lung tissue of OVA-induced asthmatic mice

As shown in Figs. 5 and 6, IL-13, TARC, MUC5AC, TNF- α , and IL-17 expression was increased in the lung of OVA-challenged control mice. SGRE (200 mg/kg) and Mon treatments significantly reduced expression of these targets compared with control mice.

Tentative identification of the main components in SGRE by UPLC-qToF MS

The main components from SGRE that exhibited anti-asthmatic effects were investigated by ultra-performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC-qToF MS), which serves as one of the best analytical techniques for simultaneous identification of complicated phytochemicals, owing to its high-resolution electrospray ionization mass spectrometry (HRESI-MS) function. As presented in Fig. 7, a complete chromatographic separation of phytochemicals was reached within 12 min under base peak ion (BPI) in negative mode. The three phytochemicals were tentatively identified by MS analysis as the following known phytochemicals: mogrosin V (1), mogrosin IV (2), and mogrosin III (3), which are well-known major components of SGRE. These compounds were characterized using

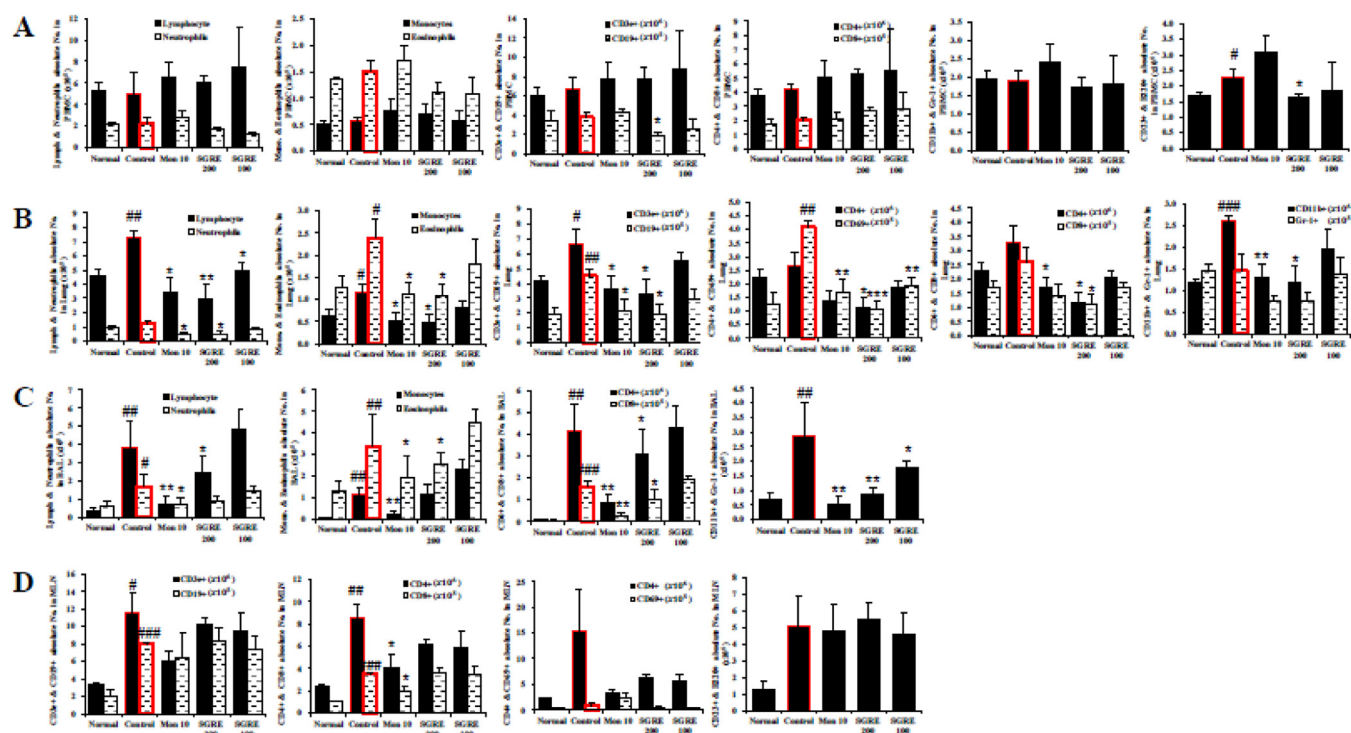


Fig. 3. The quantification of immune cell subtypes by flow cytometric (FACS) analysis in *Siraitia grosvenorii* residual extract (SGRE)-treated ovalbumin (OVA)-induced asthmatic mice. The absolute number of immune cells in the (A) peripheral blood mononuclear cells (PBMNCs), (B) lung, (C) bronchoalveolar lavage (BAL), and (D) mesenteric lymph node (MLN) of SGRE-treated OVA-induced asthmatic mice. The results are expressed as the mean $\pm$ standard error of the mean (SEM; $n = 10$ mice per group). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ indicate statistically significant differences between the asthmatic control group and drug-treated groups. WBC: white blood cell.

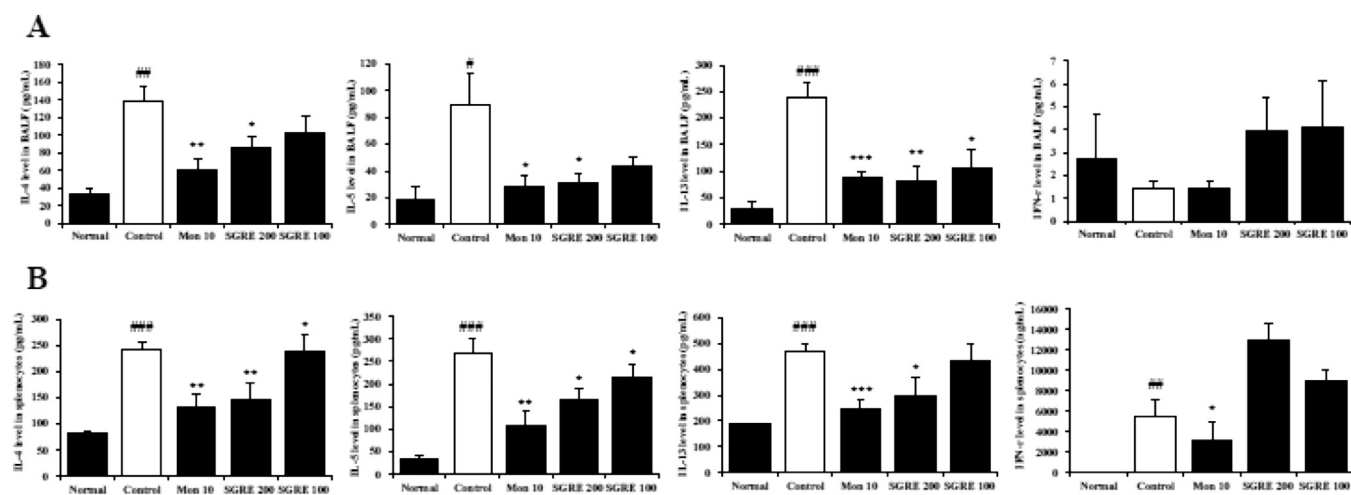


Fig. 4. The effect of *Siraitia grosvenorii* residual extract (SGRE) on cytokine production in bronchoalveolar lavage fluid (BALF) and splenocytes. BALF production of (A) IL-4, (B) IL-5, and (C) IL-13. Splenocyte production of (D) IL-4 and (E) IFN- γ . The results are expressed as the mean $\pm$ standard error of the mean (SEM; $n = 10$ mice per group). # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ indicate statistically significant differences between the normal and asthmatic control mice. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ indicate statistically significant differences between the asthmatic control mice and the Montelukast (Mon)- or SGRE-treated mice.

spectroscopic data, including retention times, mass spectral data of molecular ions, and elemental composition compared with the published literature (Supplementary Materials).

Discussion

The fruit of *S. grosvenorii* (Luo Han Guo) is a famous traditional herb used in the treatment of dry cough, sore throat, extreme thirst, and constipation in China. No studies have investigated the effect of SGRE in allergic lung inflammation, however. In this study, we demonstrated

that SGRE reduced lung inflammation in an OVA-induced asthma mouse model and exerted its anti-asthmatic effect via the inhibition of Th2 and Th17 cytokines and the increase of Th1 cytokine.

SGRE significantly reduced the levels of Th2 cytokines IL-4, IL-5, and IL-13 in BALF and splenocyte culture supernatants and also reduced serum OVA-specific IgE levels. SGRE also decreased IL-13 and IL-17 mRNA levels in the lung. In contrast, the level of Th1 cytokine IFN- γ in the BALF was increased by SGRE. SGRE significantly decreased the absolute number of lymphocyte, neutrophils, monocytes, eosinophils, CD3e+ T cells, CD19+ B cells, CD4+ /CD69+ T cells, CD4+ /CD8+ T

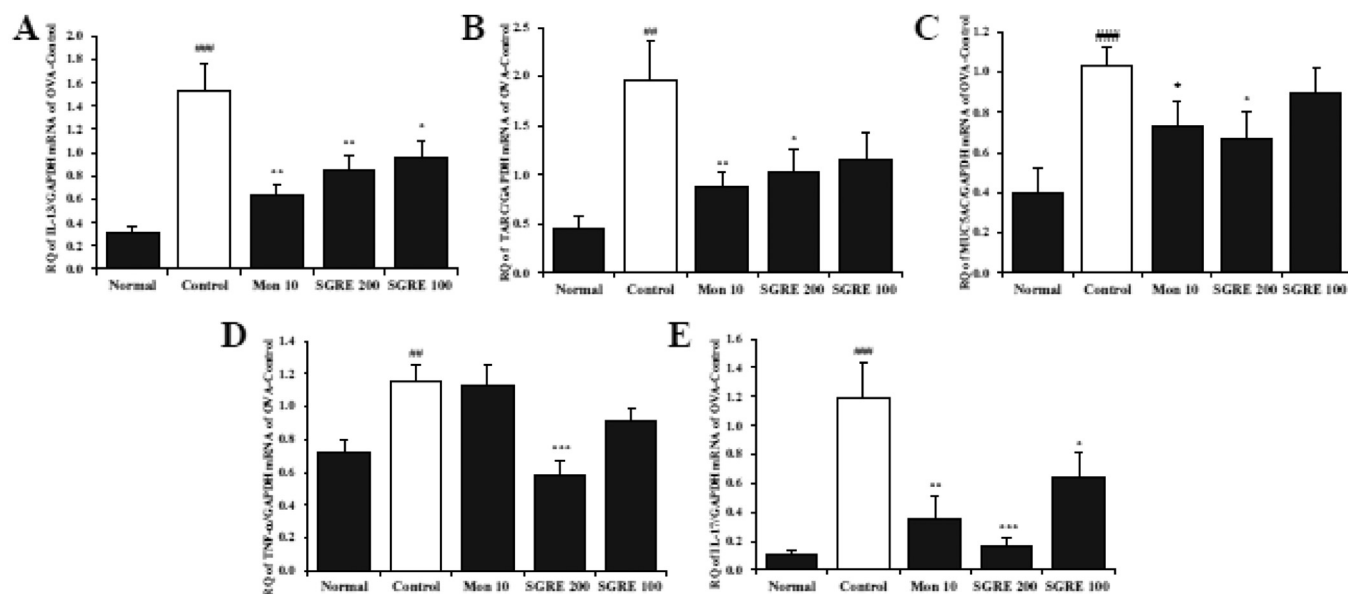


Fig. 5. The effect of *Siraitia grosvenorii* residual extract (SGRE) on the mRNA expression of (A) IL-13, (B) TARC, (C) MUC5AC, (D) TNF- α , and (E) IL-17 in the lung tissue of ovalbumin (OVA)-induced asthmatic mice. The results are expressed as the mean $\pm$ standard error of the mean (SEM; $n = 10$ mice per group). $\#p < 0.05$, $\# \# p < 0.01$, and $\# \# \# p < 0.001$ indicate statistically significant differences between the normal and asthmatic control mice. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ indicate statistically significant differences between the asthmatic control mice and the Montelukast (Mon)- or SGRE-treated mice.

cells, and CD11b+/Gr-1+ cells in the lung, and the number of lymphocyte, eosinophils, CD4+/CD8+ T cells, and CD11b+/Gr-1+ in the BALF. In addition, the absolute numbers of CD19+ B cells and CD23+/B220+ B cells in the PBMCs were significantly decreased by SGRE treatment. CD4+ and CD8+ T cells are dominant-activated T cell

subtypes in asthma and contribute to airway hyper-responsiveness, airway eosinophilia, and allergic inflammation via the production of Th2 cytokines (Schaller et al., 2005; Raemdonck et al., 2016). CD4+ T cells promote the exacerbation of chronic allergic asthma by producing IL-17 (Wang et al., 2010). CD11b is a cell surface molecule that is

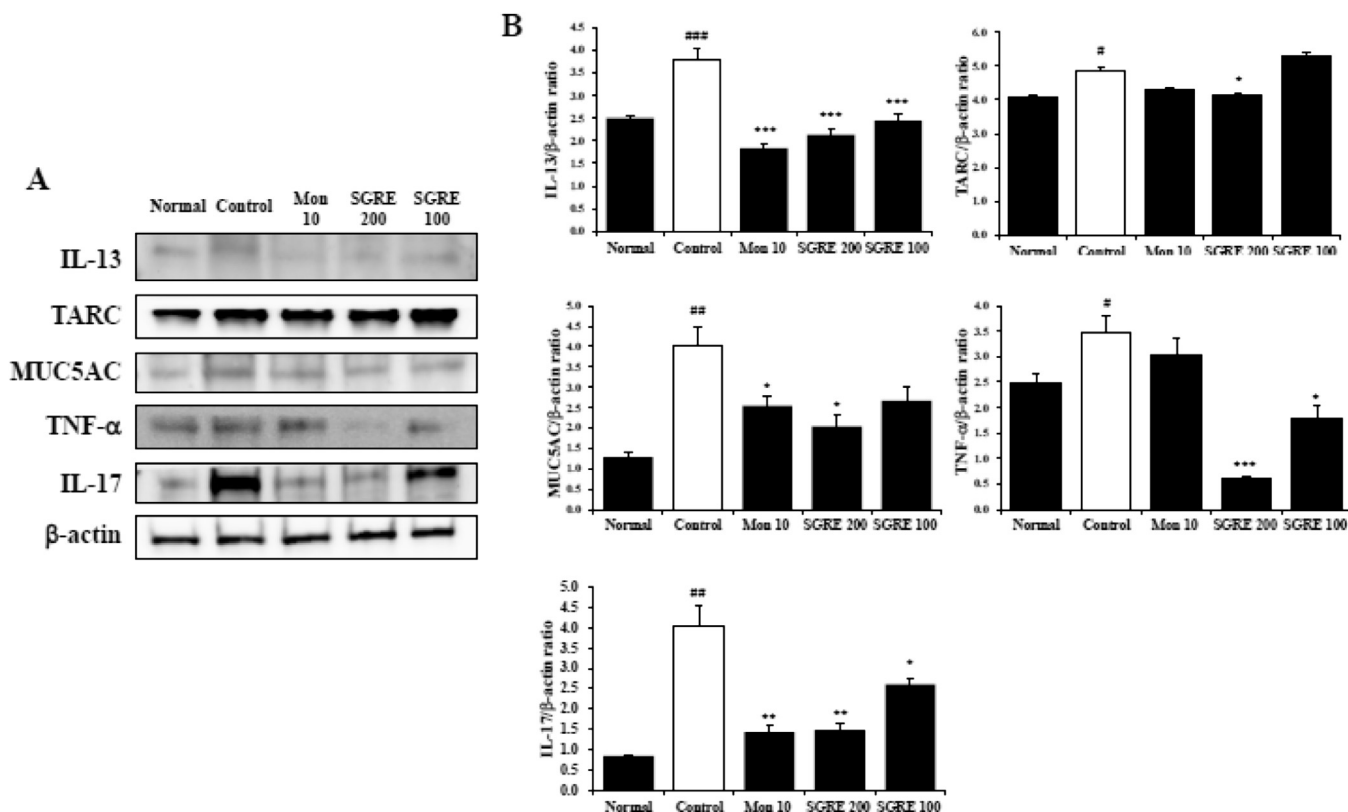


Fig. 6. The effect of *Siraitia grosvenorii* residual extract (SGRE) on the (A) protein expression of IL-13, TARC, MUC5AC, TNF- α , and IL-17 in the lung tissue. (B) Quantitative analysis of protein bands was performed with the ImageJ program. The results are expressed as the mean $\pm$ standard error of the mean (SEM; $n = 10$ mice per group). $\#p < 0.05$, $\# \# p < 0.01$, and $\# \# \# p < 0.001$ indicate statistically significant differences between the normal and asthmatic control mice. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ indicate statistically significant differences between the asthmatic control mice and the Montelukast (Mon)- or SGRE-treated mice.

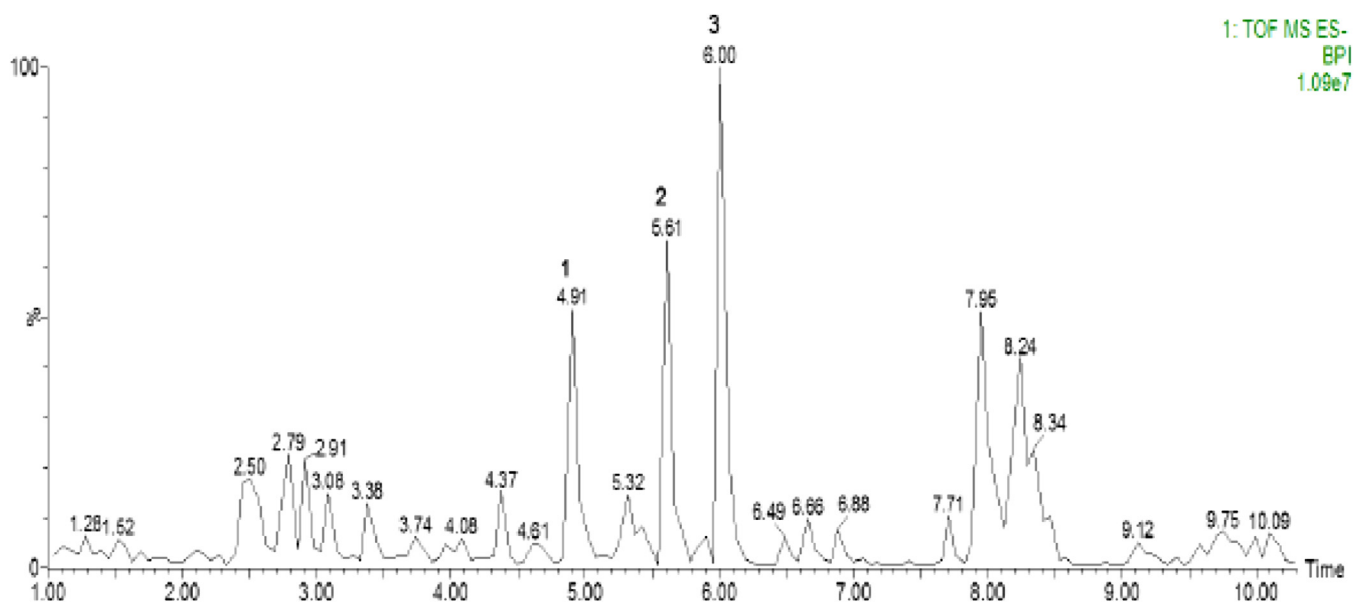


Fig. 7. Representative ultra-performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC-QToF MS) base peak intensity (BPI) chromatogram of 70% ethanol extract from *Siraitia grosvenorii* residual extract (SGRE).

highly expressed in eosinophils (Yachie et al., 1993), and Gr-1 is a granulocytic marker that is related to the differentiation and maturation of granulocytes (Fleming et al., 1993). Increased eosinophils in the lung and BALF, therefore, may constitute a substantial portion of the CD11b+/Gr-1+ cell population (Kim et al., 2011). CD23 (Fc ϵ RII) is a low-affinity receptor for IgE that is expressed in various cell types, including B cells, T cells, monocytes, dendritic cells, and neutrophils, and the CD23 surface density on antigen-presenting B cells is associated with IgE production as well as T cell activation (Selb et al., 2017). CD19 is a B cell-specific cell surface molecule involved in B cell development, and CD19 expression in B cells promotes IL-4, IL-13, and IL-17 production (Yanaba et al., 2013). Decreases in these immune cell subtypes in the lung and BALF of SGRE-treated OVA mice therefore may contribute to the inhibition of allergic lung inflammation via the decrease in cytokines (IL-4, IL-5, IL-13, and IL-17) and serum IgE levels.

In this study, SGRE decreased TARC, MUC5AC, and TNF- α expression in OVA-induced asthmatic mice. TARC/CCL17, as a selective chemoattractant of Th2 cells, regulates T cell and eosinophil infiltration into the lung and bronchial hyper-responsiveness (Berin, 2002). MUC5AC is a major respiratory mucin produced by the bronchial epithelium and submucosal glands, and its increase contributes to airway mucus hypersecretion in asthma (Chen et al., 2015). Anti-TNF- α reduces mucous cell hyperplasia by decreasing airway MUC5AC gene expression in a mouse model of asthma (Busse et al., 2009). Thus, decreases in TARC, MUC5AC, and TNF- α expression in the lung by SGRE in asthmatic mice may contribute to improvements in asthma via the inhibition of airway mucus hypersecretion and infiltration of inflammatory cells into the lung.

According to the tentative identification of phytochemicals, mogroside V (1), mogroside IV (2), and mogroside III (3) are the main components of SGRE. As previously reported, mogroside V has been shown to prevent airway inflammation induced by LPS in a mouse model of acute lung injury. These results suggest that *S. grosvenorii* extract containing mogroside V may have therapeutic effects on pulmonary inflammatory diseases. The residue of *S. grosvenorii* fruit after water extraction is generally discarded. SGRE is raw material available at a low cost, yet no studies have tested the inflammatory effects of SGRE. In our previous studies, SGRE exerted the stronger anti-inflammatory effects than *S. grosvenorii* fruit extract in mast cells and macrophages (data not shown). Additionally, our quantitative analysis identified mogroside V and 11-oxo-mogroside V in *S. grosvenorii*

extract (data not shown). This difference in anti-inflammatory action might be attributed to their chemical difference in SGRE and *S. grosvenorii* extract. However, mogroside II and III, which are members of the triterpene glycoside family, have not been reported to have effects against airway inflammation or related pulmonary diseases. Further investigation into the effects of these compounds on airway inflammation is therefore required.

Conclusion

These results indicate that SGRE exerts anti-asthmatic effects via the inhibition of Th2 and Th17 cytokines and the increase of Th1 cytokine, suggesting that SGRE may be a potential therapeutic agent for allergic lung inflammation.

Conflict of interest

The authors declare no conflict of interests.

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.phymed.2019.152835](https://doi.org/10.1016/j.phymed.2019.152835).

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SUPPLEMENTARY MATERIAL

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① Instrument

details	Instrument
UPLC(UPLC-PDA)	[Waters] AQUITY™ Ultra Performance LC
Mass	[Waters] Xevo Q-TOF MS

② Solvents

details	Solvent
Solvent A	0.1% Formic acid/D.W.
Solvent B	0.1% Formic acid/ACN

③ Method - Gradient

Time(min)	Flow (mL/min)	%A	%B
(Initial)	0.400	85	15
1.00	0.400	85	15
9.00	0.400	60	40
10.00	0.400	10	90
12.00	0.400	0	100

④ Mass Conditions

details	
Desolvation gas	N ₂
Desolvation flow rate	800 L/h
Desolvation temperature	350 °C
Source temperature	110 °C
Capillary voltage	2300 V
Cone voltage	50 V
Scan mode	Negative
m/z range	100-1500 Da

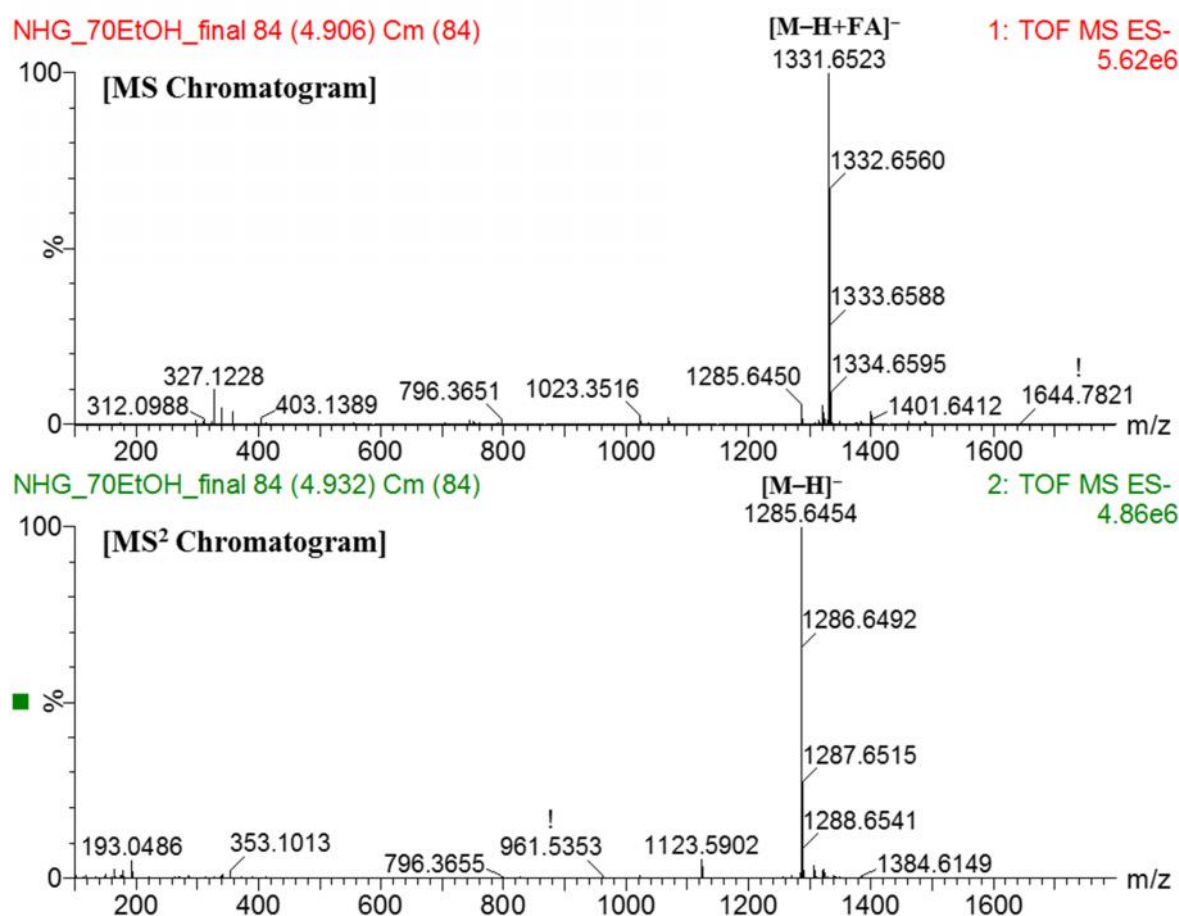
⑤ Column

ACQUITY UPLC® BEH C18 1.7 µm 2.1 x 100mm Column

⑥ Sample Concentration : extract: 2 mg/mL

⑦ Injection Volume : 2 µL

Fig. S1. Ultra-performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC-qTof MS) profile conditions.



[Elemental Composition: high-resolution electrospray ionization mass spectrometry (HRESI MS)]

Single Mass Analysis

Tolerance = 4.0 PPM / DBE: min = -1.5, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

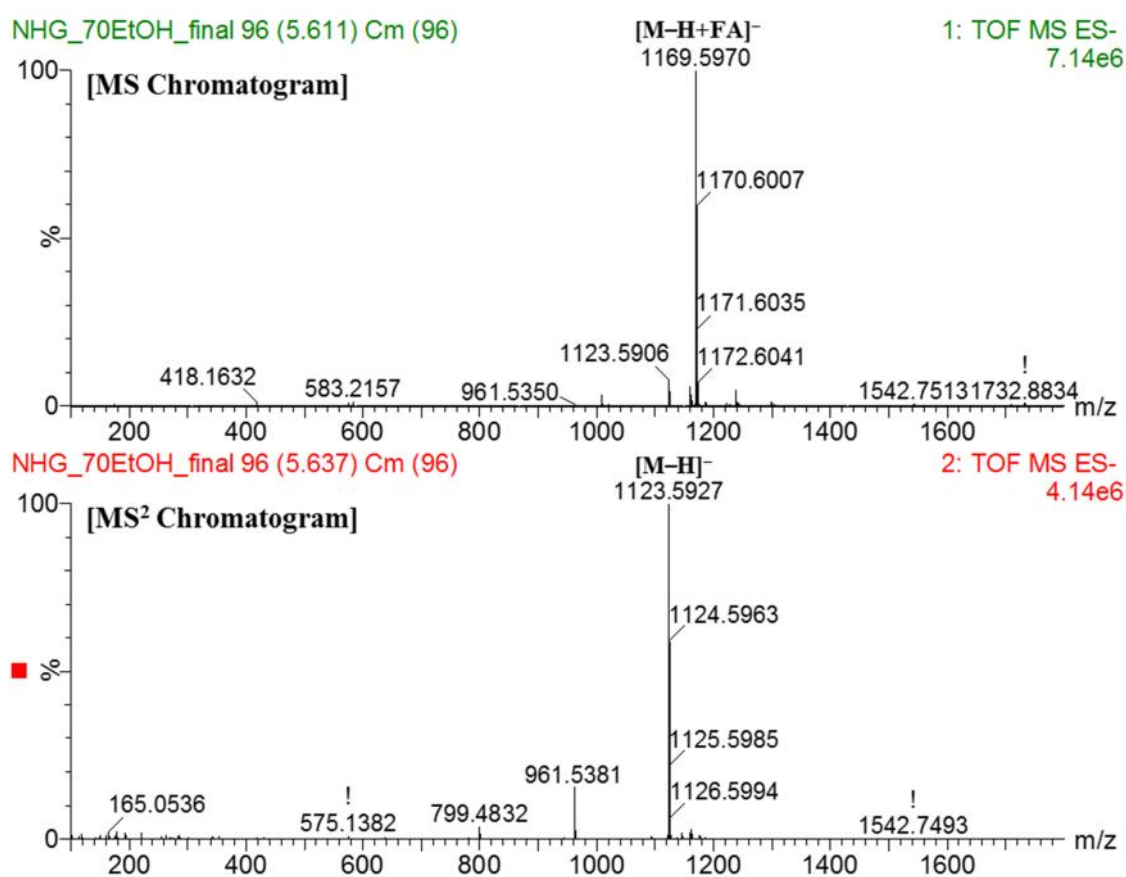
760 formula(e) evaluated with 3 results within limits (up to 50 best isotopic matches for each mass)

Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	O
1331.6523	1331.6483	4.0	3.0	10.5	C ₆₁ H ₁₀₃ O ₃₁	517.6	0.052	94.91	61	103	31
	1331.6542	-1.9	-1.4	1.5	C ₅₄ H ₁₀₇ O ₃₆	520.5	2.991	5.02	54	107	36
	1331.6518	0.5	0.4	32.5	C ₇₉ H ₉₅ O ₁₈	524.9	7.369	0.06	79	95	18

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	O
1285.6454	1285.6429	2.5	1.9	10.5	C ₆₀ H ₁₀₁ O ₂₉	601.5	0.008	99.16	60	101	29
	1285.6487	-3.3	-2.6	1.5	C ₅₃ H ₁₀₅ O ₃₄	606.3	4.810	0.82	53	105	34
	1285.6464	-1.0	-0.8	32.5	C ₇₈ H ₉₃ O ₁₆	610.0	8.460	0.02	78	93	16
	1285.6405	4.9	3.8	41.5	C ₈₅ H ₈₉ O ₁₁	613.8	12.262	0.00	85	89	11

Fig. S2. Tentative identification of peak 1 (mogroside V).



[Elemental Composition: high-resolution electrospray ionization mass spectrometry (HRESI MS)]

Single Mass Analysis

Tolerance = 4.0 PPM / DBE: min = -1.5, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

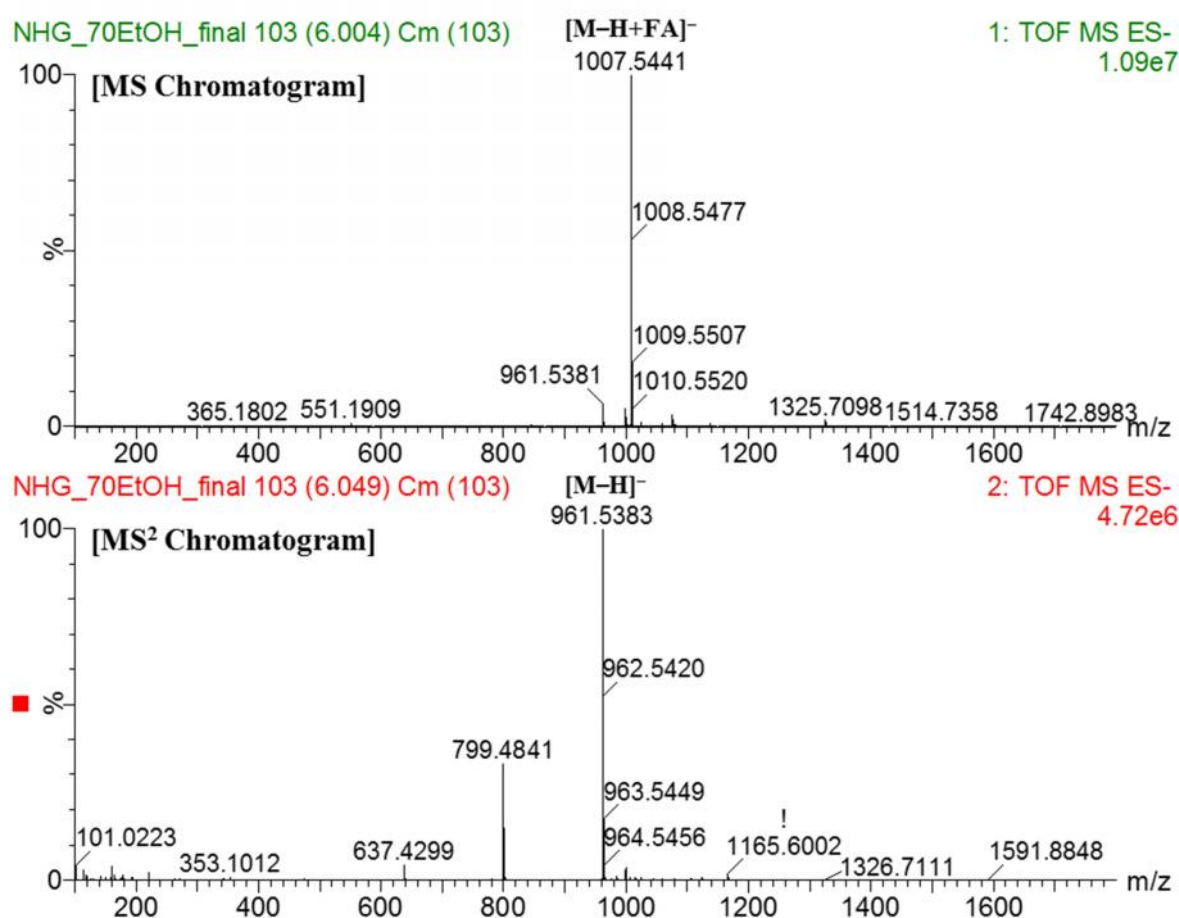
Monoisotopic Mass, Even Electron Ions

585 formula(e) evaluated with 4 results within limits (up to 50 best isotopic matches for each mass)

Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	O
1169.5970	1169.5955	1.5	1.3	9.5	C55 H93 O26	610.0	0.008	99.24	55	93	26
	1169.6014	-4.4	-3.8	0.5	C48 H97 O31	614.9	4.906	0.74	48	97	31
	1169.5990	-2.0	-1.7	31.5	C73 H85 O13	618.4	8.385	0.02	73	85	13
	1169.5931	3.9	3.3	40.5	C80 H81 O8	621.2	11.233	0.00	80	81	8
1123.5927	1123.5900	2.7	2.4	9.5	C54 H91 O24	658.0	0.005	99.48	54	91	24
	1123.5959	-3.2	-2.8	0.5	C47 H95 O29	663.3	5.303	0.50	47	95	29
	1123.5935	-0.8	-0.7	31.5	C72 H83 O11	666.6	8.565	0.02	72	83	11

Fig. S3. Tentative identification of peak 2 (mogroside IV).



[Elemental Composition: high-resolution electrospray ionization mass spectrometry (HRESI MS)]

Single Mass Analysis

Tolerance = 4.0 PPM / DBE: min = -1.5, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

453 formula(e) evaluated with 3 results within limits (up to 50 best isotopic matches for each mass)

Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	O
1007.5441	1007.5427	1.4	1.4	8.5	C ₄₉ H ₈₃ O ₂₁	694.6	0.000	99.99	49	83	21
	1007.5462	-2.1	-2.1	30.5	C ₆₇ H ₇₅ O ₈	703.7	9.091	0.01	67	75	8
	1007.5403	3.8	3.8	39.5	C ₇₄ H ₇₁ O ₃	706.7	12.084	0.00	74	71	3

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	O
961.5383	961.5372	1.1	1.1	8.5	C ₄₈ H ₈₁ O ₁₉	663.6	0.000	100.00	48	81	19
	961.5407	-2.4	-2.5	30.5	C ₆₆ H ₇₃ O ₆	674.2	10.644	0.00	66	73	6
	961.5348	3.5	3.6	39.5	C ₇₃ H ₆₉ O	676.7	13.129	0.00	73	69	1

Fig. S4. Tentative identification of peak 3 (mogroside III).