

Original article

Camellia japonica oil suppressed asthma occurrence via GATA-3 & IL-4 pathway and its effective and major component is oleic acid

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ABSTRACT

Background: In December 2016, WHO released a report stating that in 2015 there were 383,000 deaths caused by asthma and 235 million people suffering from asthma. As there are many adverse effects associated with the currently-used asthma drugs, new anti-asthmatic drugs need to be developed.

Purpose: In order to find new drug candidates with safe and low side effects, the anti-asthmatic function and mechanism of *C. japonica* oil were evaluated, and its active ingredients were analyzed for use in an ovalbumin asthma murine model.

Study design and methods: The study consisted of six groups: control; ovalbumin group; and dexamethasone group as a positive control; and 10, 100, and 500 mg/kg *C. japonica* oil treatment groups. In order to measure the anti-asthmatic effect of *C. japonica* oil, WBC and differential cell count in BALF, IgE in serum, morphological changes in pulmonary system, and gene and protein levels such as IFN- γ , IL-12p40, IL-4, IL-5, IL-6, TNF- α , and IL-6 were all evaluated.

Results: *C. japonica* oil had an anti-asthmatic effect and significantly controlled eosinophil in BALF, Th2-related factors such as GATA-3 that is Th2 cell transcription factor, IL-4, IL-5, and IL-13, and TNF- α in the lung. It also dose-dependently modulated inflammatory cells, T-bet, IL-12p40, and IL-6. Oleic acid was the major gradient (52.89%) in *C. japonica* oil and also had anti-asthmatic effects such as the downregulation of inflammatory cells, WBC, and eosinophil in BALF, IgE in serum, and morphological changes in the lung.

Conclusion: We concluded that *C. japonica* oil is a new anti-asthmatic drug candidate and that oleic acid is the major anti-asthmatic ingredient in *C. japonica* oil.

Introduction

The World Health Organization (WHO) estimated that there were 383,000 deaths caused by asthma in 2015, that 235 million people suffered from asthma worldwide, and that it was especially common in

the young and the old (2017). Asthma is pulmonary hyperresponsive-ness, a type I allergy, and the symptoms vary widely, from apnea to death (National Asthma Education and Prevention Program, N.A.E.a.P., 2002; Kay 2001). It is impossible to completely remedy asthma, and it is a major health problem (Slejek et al., 2014). There are many allergens

Abbreviations: *Camellia japonica*, *C. japonica*; Th, helper T; RT-PCR, real time polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; WHO, World Health Organization; IFN- γ , interferon gamma; TNF- α , tumor necrosis factor-alpha; IgE, immunoglobulin E; OVA, ovalbumin; DEX, dexamethasone; BALF, bronchoalveolar fluid; H&E, hematoxylin and eosin; PAS, periodic acid-schiff; PBS, phosphate-buffered saline; BM, basement membrane; cDNA, complementary deoxyribonucleic acid; RNA, ribonucleic acid; IF, immunofluorescence; IHC, immunohistochemistry; HPLC, high-performance liquid chromatography; SD, standard deviation; WBC, white blood cells

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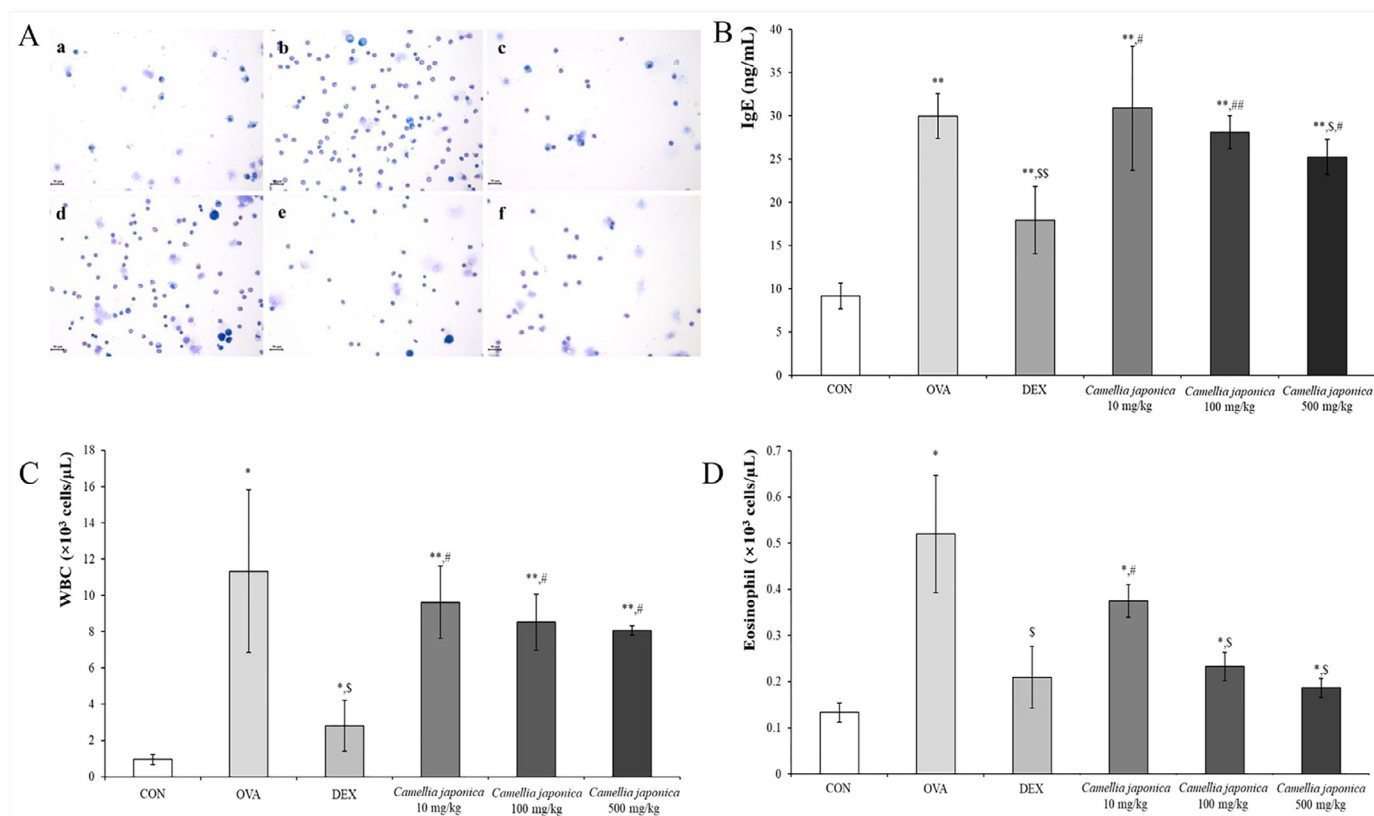


Fig. 1. *Camellia japonica* oil effectively controlled asthmatic factors such as inflammatory cells, eosinophil and IgE both in bronchioalveolar lavage fluid (BALF) and serum. (A) *C. japonica* oil significantly suppressed the number of inflammatory cells which were increased by ovalbumin treatment in BALF. (B) *C. japonica* oil dose-dependently downregulated the levels of IgE in serum. (C) Although there was no statistical significance *C. japonica* oil modulated the number of WBC in BALF. (D) The change of eosinophil number in asthma is very important and *C. japonica* oil significantly decreased the number of eosinophil in BALF. The level of eosinophil in 100 mg/kg *C. japonica* oil treatment group was similar to that in dexamethasone treatment group. a, vehicle control; b, asthma induction; c, dexamethasone; d, 10 mg/kg/day *C. japonica* oil; e, 100 mg/kg/day *C. japonica* oil; f, 500 mg/kg/day *C. japonica* oil. Each bar represents the mean $\pm$ SEM (n = 8). * p < 0.05 vs. control; ** p < 0.001 vs. control; \$ p < 0.05 vs. asthma induction; \$\$ p < 0.01 vs. asthma induction; # p < 0.05 vs. dexamethasone; ## p < 0.01 vs. dexamethasone. Scale Bar = 50 μ m. Magnification, $\times$ 400.

in the environment that can be classified into two categories: indoor allergens such as domestic mites, pet dander, etc., and outdoor allergens such as air pollution, chemicals, tobacco smoke, etc. (Plattes-Mills et al., 1997; Burge and Rogers, 2000).

The repetitive uptake of allergens can disturb the immune system, especially Th type 1 (Th1)/Th2 system, and induce asthma occurrence (National Asthma Education and Prevention Program, N.A.E.A.P., 2002; Kay 2001). Interferon gamma (IFN- γ) and interleukin 12 (IL-12) belong to Th1-related cytokine, Th2 cells release some cytokine such as IL-4, IL-5, IL-13, etc. and Th17 cell are very important cells for the modulation of Th1/Th2 balance and to the onset of asthma (Mosmann and Coffman, 1989; Singh et al., 2014). In most asthma patients, the levels of Th2-related cytokine such as IL-4, IL-5, and IL-13 are very high (Antczak et al., 2016; Foster et al., 1996; van der Pouw Kraan et al., 1998; Wenzel et al., 2007) and Th2-related cytokine is regulated by GATA-3, Th2 cell transcription factor (Yagi et al., 2011). Th1-related cytokines such as IFN- γ and IL-12p40 downregulate Th1-related cytokine levels, and T-bet is a Th1 cell transcription factor (Szabo et al., 2000; Zhu et al., 2012).

In order to treat asthma, inhaled combination therapy is used these days, and usually consists of corticosteroids and long-acting β_2 -agonist or leukotriene modifiers (Mishra et al., 2013). In asthma treatment, the most difficult things are that it is hard to perfectly cure, and that general medications have a lot of adverse effects such as growth suppression, eye problem, hypertension, hyperlipidemia, gastric ulcer, neurotoxicity, and so on (Wise 2014; Ciriaco et al., 2013). Efforts toward developing safe and effective anti-asthmatic drugs from natural

products have increased in recent years (Bang et al., 2015; Lee et al., 2017; Seo et al., 2016).

Camellia japonica (*C. japonica*) is from the *Theaceae* family, and recently, biological functions of it have been reported, such as an anti-oxidative effect and anti-migratory effect in breast cancer cells (Woo et al., 2017), anti-inflammatory and anti-ulcerative effect (Akanda and Park, 2017), blood pressure-lowering effect (Lim et al., 2017), anti-inflammatory and anti-oxidative effects on corneal epithelial cells (Lee et al., 2017), and so on. However, there is no report on the potential anti-asthmatic effects of *C. japonica* oil, and in the present study we investigated the anti-asthmatic effects and ingredients.

Materials and methods

Plant materials

C. japonica seeds were collected from the Wando Arboretum (Wando, Republic of Korea) and identified by Dr. Deuk-Sil Oh affiliated to the Wando Arboretum. Oil from *C. japonica* was provided by Dr. Deuk-Sil Oh and was used for further constituent analysis and *in vivo* experimentation.

Chemical profiling by GC–MS analysis

GC–MS analysis was conducted using a previously-reported method with slight modifications (Bae et al., 2014). Briefly, Agilent 7890 gas chromatograph system was used to analyze scanned organic compound.

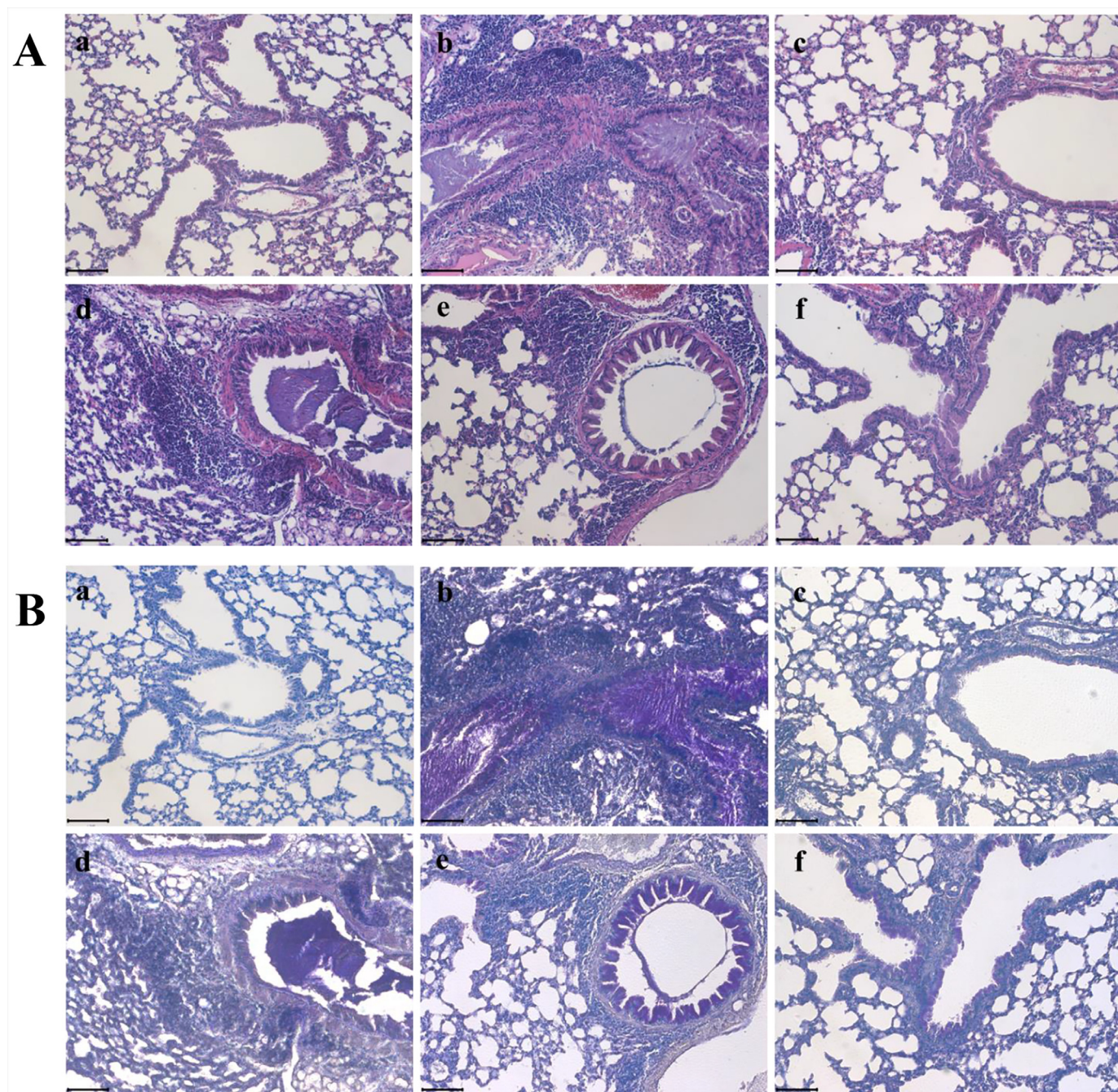


Fig. 2. *Camellia japonica* oil dose-dependently inhibited typical asthmatic changes in the lung. (A) The photos were obtained using with hematoxylin and eosin stain, ovalbumin treatment induced the histopathological changes such mucous hyper-secretion, eosinophil infiltration, bronchioalveolar epithelial cell hyperplasia, and goblet cell hyperplasia. *C. japonica* oil dramatically controlled ovalbumin-induced morphological changes in the lung in a dose-dependent manner. (B) In order to evaluate the change of mouse level in the bronchioalveolar space periodic acid-schiff (PA) staining was performed. Ovalbumin stimulated the hyper-secretion of mucous compared to both that in the control and that in the dexamethasone. However, *C. japonica* oil significantly inhibited the mucous secretion in a dose-dependent manner. a, vehicle control; b, asthma induction; c, dexamethasone; d, 10 mg/kg/day *C. japonica* oil; e, 100 mg/kg/day *C. japonica* oil; f, 500 mg/kg/day *C. japonica* oil. Scale Bar = 100 μ m. Magnification, $\times 200$.

It was coupled to a quadrupole Agilent 5975C electron ionization (70 eV) mass spectrometric detector (Agilent Technologies, Palo Alto, CA, USA) equipped with an Agilent HP-5MS fused silica capillary column (30 mm l. $\times$ 0.25 mm i.d., 0.25- μ m film thickness). GC–MS was tuned using perfluorotributylamine (PFTBA) with mass fragments of 69.0, 219.0, and 502.0 m/z under electron ionization (EI) conditions. The GC oven was heated as follows: isothermal at 65 $^{\circ}$ C for 10 min and 10 min $^{-1}$ to 300 with helium (He) as carrier gas. Transfer line was heated at 300 $^{\circ}$ C. The mass spectrometer was operated in a scan mode

of 50–550 amu. All mass spectra were compared to the data system library (NIST 2008).

Animal experiments

The animal study herein was conducted in the same way as our previous study (Lee et al., 2017) and according to Kianmehr et al. (2017) the asthma occurrence was evaluated. In order to evaluate the anti-asthmatic effects of *C. japonica* oil and oleic acid which were

Table 1
The quantitative score chart of histopathological changes in the lung.

	Mucous hypersecretion (0–3)	Epithelial cell hyperplasia (0–3)	Inflammatory cell infiltration (0–3)
CON	0.1 ± 0.35	0.4 ± 0.52	0.1 ± 0.35
OVA	2.9 ± 0.35**	2.8 ± 0.46**	2.9 ± 0.35**
DEX	0.3 ± 0.46 ^{§§}	0.6 ± 0.52 ^{§§}	0.6 ± 0.52 ^{§§}
<i>Camellia japonica</i> 10 mg/kg	2.8 ± 0.46 ^{***,##}	2.9 ± 0.35 ^{***,##}	2.6 ± 0.52 ^{***,##}
<i>Camellia japonica</i> 100 mg/kg	1.5 ± 0.76 ^{***,§§,##}	2.4 ± 0.52 ^{***,##}	1.9 ± 0.83 ^{***,§,##}
<i>Camellia japonica</i> 500 mg/kg	0.4 ± 0.52 ^{§§}	0.4 ± 0.52 ^{§§}	0.52 ± 0.53 ^{§§}

Each score explains the means ± standard deviation (N = 8).

* $p < 0.05$ vs. control.

** $p < 0.001$ vs. control.

§ $p < 0.05$ vs. asthma induction.

§§ $p < 0.01$ vs. asthma induction.

$p < 0.05$ vs. dexamethasone.

$p < 0.01$ vs. dexamethasone.

isolated from *C. japonica* oil, a separate animal study was conducted. Two individual animal studies were consecutively conducted after confirmative study following animal study. For evaluation, the animal study of the anti-asthmatic effect of *C. japonica* oil and the confirmative study were done in succession. For each study, forty-eight female BALB/c mice were purchased from Samtako Korea (Osan, Korea) and divided into six groups according to treatment: (1) vehicle control (sterilized tap water), (2) ovalbumin (OVA)-induced asthma model, (3) 1 mg/kg/day dexamethasone with ovalbumin treatment, (4) 10 mg/kg/day *C. japonica* oil with ovalbumin treatment, (5) 100 mg/kg/day *C. japonica* oil with ovalbumin treatment, and (6) 500 mg/kg/day *C. japonica* oil with ovalbumin treatment. On days 1 and 8, all mice except for those used as the vehicle control were sensitized via intraperitoneal injections of 20 µg OVA (Sigma-Aldrich, St. Louis, MO, USA) and 1 mg aluminum hydroxide hydrate (Sigma-Aldrich) in 500 µl saline. From day 21 to day 25, the mice were challenged once daily with 5% OVA for 30 min using a nebulizer (3 ml/min, NE-U17, OMRON Co. Ltd., Kyoto, Japan). Over the same five-day period, the treatment groups were also treated once daily with oral doses of sterilized tap water, dexamethasone, 10 mg/kg *C. japonica* oil, (5) 100 mg/kg/day *C. japonica* oil, and (6) 500 mg/kg/day *C. japonica* oil 1 h prior to the OVA challenge. The mice in the vehicle control group were sensitized with OVA according to the same procedures as the other groups of mice (20 µg OVA and 1 mg aluminum hydroxide hydrate in 500 µl saline), after which they were exposed to saline and aluminum hydroxide hydrate by a nebulizer for five consecutive days. To evaluate the anti-asthmatic effect of oleic acid which is the major component in *C. japonica* oil, and which was supposed as the element for ameliorating ovalbumin-induced asthmatic symptoms in a murine model, a very similar animal study was done. From this study, we confirmed the level of oleic acid was 52.89% in *C. japonica* oil and based on the anti-asthmatic results of *C. japonica* oil 250 mg/kg/day oleic acid treatment was decided. The animals were divided into four groups according to treatment: (1) vehicle control, (2) OVA-induced asthma model, (3) 1 mg/kg/day dexamethasone with ovalbumin treatment, and (4) 250 mg/kg/day oleic acid with ovalbumin treatment. The last procedure was also conducted in the same as the animal study for *C. japonica* oil.

Ethics statement

All experiments were approved by the Institutional Animal Care and Use Committee at Jeonnam National University (Animal Study Approval No. CNU IACUC-YB-2018-38).

BALF and serum analysis

In the same way as in our previous study, BALF and serum analysis were conducted (Lee et al., 2017; Bang et al., 2015). One day after the

final treatment, the mice were anesthetized with intraperitoneal injections of 50 mg/kg Zoletin (Virbac, Fort Worth, TX, USA), and their tracheas were cannulated with disposable animal feeding needles. Lavages were performed with three 0.4 ml aliquots of cold phosphate-buffered saline (PBS). The BALF samples were collected and immediately centrifuged at 3000 rpm for 5 min at 4 °C (Sorvall Legend Micro 17R, Thermo Fisher Scientific, Inc. Marietta, OH, USA). The cell pellets were resuspended in PBS for total and differential cell counts. The number of total cells and differential cells were counted by the Hemavet Multispecies Hematology System (Drew Scientific Inc, Waterbury, CT, USA). Some resuspended cell pellets were stained by the Diff-Quick method (Skipper and DeStephano, 1989). The number of total cells was counted using a hemocytometer, and the number of eosinophils in BALF was counted on cytospin preparations that were stained using the Kwik-Diff staining set (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA).

The levels of IgE in the serum were measured using a specific mouse IgE enzyme-linked immunosorbent assay kit (BD bioscience, 555248, San Jose, CA, USA) according to the manufacturer's protocols.

Histopathological analysis

Histopathological analysis was conducted as in our previous study (Lee et al., 2017). Lung tissues were fixed in 10% (v/v) formaldehyde solution, dehydrated in a graded ethanol series (99.9%, 90%, 80%, and 70%), and embedded in paraffin. Paraffin-embedded lung tissues were then sectioned (4 µm) longitudinally and stained with hematoxylin and eosin. The sections were also stained with periodic acid schiff (PAS) for the semi-quantitative analysis of glycoproteins. The images were obtained using an Axioscope A1 (Carl Zeiss, Gottingen, Germany).

In order to clear the difference of histopathological changes among the groups we evaluated from 0 (none) to 3 (severe) on mucous hypersecretion, epithelial cell hyperplasia, and inflammatory cell infiltration.

Reverse transverse-poly chain reaction (RT-PCR) analysis

In order to evaluate changes in the cDNA levels of IFN-γ, IL-12p40, IL-4, IL-5, IL-13, TNF-α, and IL-6, which are related to asthma induction, RT-PCR analysis was conducted as in our previous study protocol (Lee et al., 2017). Total RNA was extracted from the lung using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA (100 ng) was used as a template for the reaction. Primers were synthesized for RT-PCR as follows: IFN-γ forward 5'-GGCCATCAGCAACAACATAAG-3', IFN-γ reverse 5'-GTTGACCTCAAACCTGGCAATAC-3'; IL-12p40 forward 5'-GGACCAAAGG GACTATGAGAAG-3', IL-12p40 reverse 5'-CTTCCAACGCCAGTTCA ATG-3'; IL-4 forward 5'-ACAGGAGAAGGGACGCCAT-3', IL-4 reverse

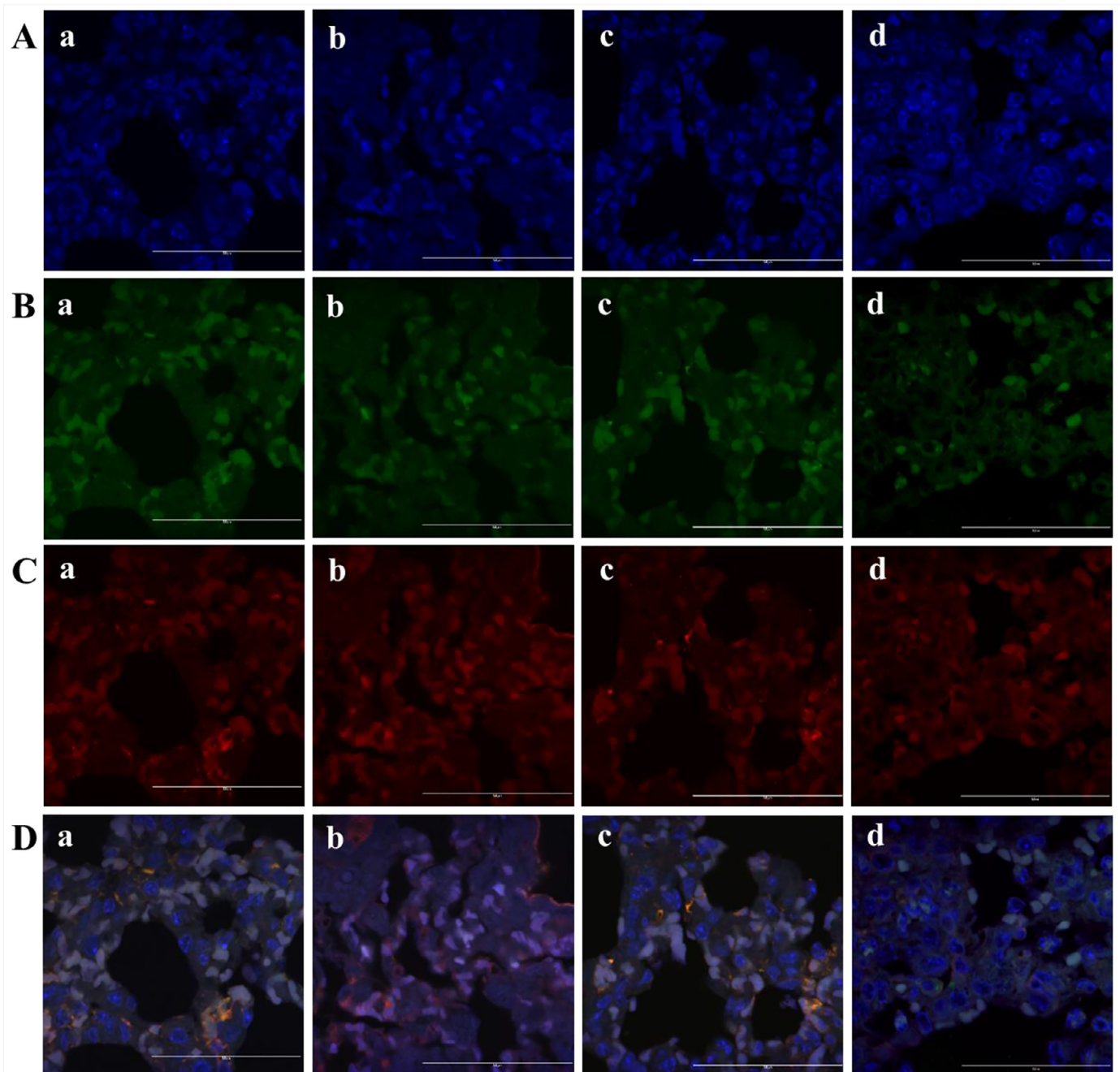


Fig. 3. *C. japonica* oil suppressed not only the expression of Th1 cell transcription factor, T-bet but also that of Th2 cell transcription factor, GATA-3. (A) The blue spots were nuclei in the cells for counter-staining. *C. japonica* oil perfectly inhibited both (B) the activation of helper 1 T cell transcription factor, T-bet and (C) that of helper 2 T cell transcription factor, GATA-3. (D) The photos meant the merge of nucleus (blue spots), T-bet (green spots), and GATA-3 (red spots). a, vehicle control; b, asthma induction; c, dexamethasone; d, 10 mg/kg/day *C. japonica* oil; e, 100 mg/kg/day *C. japonica* oil; f, 500 mg/kg/day *C. japonica* oil. Scale Bar = 50 μ m. Magnification, $\times$ 1000.

5'-GAAGCCCTACAGACGAGCTCA-3'; IL-5 forward 5'-TGCATCAGGGTCTCAAGTATTC-3', IL-5 reverse 5'-GGATGCTAAGTTGGGTATGT-3'; IL-13 forward 5'-CAGCCCTCAGCCATGAAATA-3', IL-13 reverse 5'-CTTGAGTGTGAACAGGCCATTCT-3'; IL-6 forward 5'-GATAAGCTGGAGT CACAGAAGG-3', IL-6 reverse 5'-TTGCCGAGTAGATCTCAAAGTG-3'; TNF- α forward 5'-CTGAGTTCTGCAAAGGGAGAG-3', TNF- α reverse 5'-CCTCAGGGAAGAATCTGGAAG-3'; GAPDH forward 5'-GTGGAGTCATACTGAACATGTAG-3', and GAPDH reverse 5'-AATGGTGAAGGTCGTGTG-3'. The RT-PCR cycles consisted of denaturation at 95 $^{\circ}$ C for 5 s and annealing/extension at 65 $^{\circ}$ C for 30 s for 40 cycles. The results were obtained using an Axioscope A1 (Carl Zeiss, Gottingen, Germany).

Immunofluorescent analysis

In order to find the localizations of Th1 cell transcription factor, T-bet, and Th2 cell transcription factor, GATA-3 immunofluorescent analysis was conducted, and only four groups were measured: control, OVA, dexamethasone with ovalbumin treatment, and 1500 mg/kg macmoondong-tang with ovalbumin treatment. Prior to the antibody binding step, the same materials for immunohistochemical analysis but rabbit anti-mouse T-bet (Biorbyt, orb7075, Cambridge, UK) or goat anti-mouse GATA-3 (OriGene, TA305795, Rockville, MD, USA) were used as primary antibodies for 1 h at room temperature. The slides were

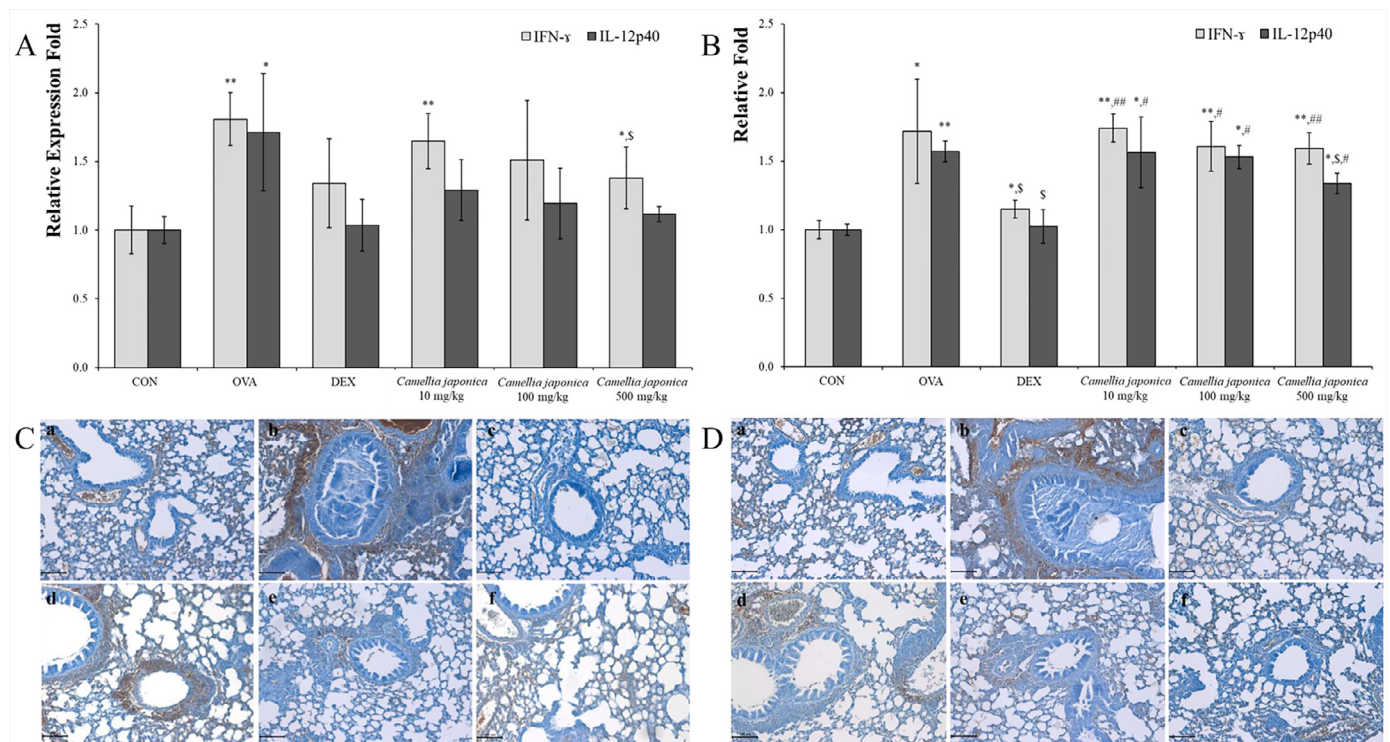


Fig. 4. *C. japonica* oil dose-dependently down-regulated the expression of IL-12p40 protein. (A) *C. japonica* oil little affected the genes' changes of Th1-related cytokine such as IFN- γ and IL-12p40. However, *C. japonica* oil modulated the protein levels of same cytokine. (B) In the quantitative point of view, there was no statistical significance in the change of IFN- γ but (C) a slight difference was shown among *C. japonica* oil-treated groups in the immunohistochemical staining. (D) Compared to the change of IFN- γ *C. japonica* oil dose-dependently suppressed the level of IL-12p40 expression and (D) the qualitative change of IL-12p40 was same to the quantitative change of that. a, vehicle control; b, asthma induction; c, dexamethasone; d, 10 mg/kg/day *C. japonica* oil; e, 100 mg/kg/day *C. japonica* oil; f, 500 mg/kg/day *C. japonica* oil. Each bar represents the mean $\pm$ SEM (n = 8). * p < 0.05 vs. control; ** p < 0.001 vs. control; ^s p < 0.05 vs. asthma induction; ^{ss} p < 0.01 vs. asthma induction; # p < 0.05 vs. dexamethasone; ## p < 0.01 vs. dexamethasone. Scale Bar = 100 μ m. Magnification, $\times$ 200.

incubated for 2 h with FITC-conjugated anti-rabbit IgG (Jackson ImmunoResearch, 315-095-003, West Grove, PA, USA) or Alexa Fluor 555-conjugated anti-goat IgG (ThermoFisher Scientific, A-21127, Waltham, MA, USA) and the cells were counterstained with DAPI (ThermoFisher Scientific, 62249, Waltham, MA, USA). The images were obtained using a K1-Fluo confocal microscope (Nanoscope System, Daejeon, South Korea).

Enzyme-linked immunoassay (ELISA) analysis

To analyze the levels of IFN- γ , IL-12p40, IL-4, IL-5, IL-6, and TNF- α in lung tissue, OptEIA mouse ELISAs were purchased from BD Biosciences. The IL-13 levels were assessed using the AbFrontier Cymax mouse ELISA kit (AbFrontier, Seoul, Korea). All assays were conducted according to the manufacturers' guidelines. All lung samples were prepared by a lysis buffer made with a protease inhibitor cocktail and an RIPA buffer (Thermo Fisher Scientific). Aliquots of lung tissue from all groups were weighed and homogenized with lysis buffer. They were then centrifuged at 8200 rpm for 15 min, and the supernatants were harvested and measured using a microplate reader (EZ Read 400, Biochrom, Cambourne, UK).

Immunohistochemical analysis

Immunohistochemical analysis was conducted like in our previous study (Lee et al., 2017). Deparaffinized tissue sections were treated with 3% hydrogen peroxide in methanol for 10 min in order to remove endogenous peroxidase. Antigen retrieval was carried out with sodium citrate buffer (0.1 M) using the hot plate method. The slides were incubated with normal serum in order to block nonspecific binding, then incubated for 1 h at 4 °C with primary antibodies (diluted 1:100 to

1:200) such as rat anti-mouse IFN- γ monoclonal (Santa Cruz, sc-74104), rat anti-mouse IL-12p40 monoclonal (Santa Cruz, sc-57258), rat anti-mouse IL-4 monoclonal (Santa Cruz, sc-73318), rabbit anti-mouse IL-5 polyclonal (Santa Cruz, sc-7887), goat anti-mouse IL-13 polyclonal (Santa Cruz, sc-1776), goat anti-mouse IL-6 polyclonal (Santa Cruz, sc-1265), and rabbit anti-mouse TNF- α polyclonal (BioVision, 3053R-100, Milpitas, CA, USA). The slides were incubated with biotinylated pan-specific secondary antibody for 10 min and then reacted with streptavidin peroxidase complex for 5 min (Vector Laboratories Universal Quick Kit, Burlingame, Canada). Signals were detected using the 3,3'-diaminobenzidine tetrahydrochloride substrate chromogen solution, and the cells were counterstained with Mayer's hematoxylin. The cells were finally imaged using an Axioscope A1 (Carl Zeiss).

Statistical analysis

Results are expressed as mean $\pm$ standard deviation (SD). Group differences were evaluated by one-way analysis of variance, followed by Dunnett's multiple comparison test. Significance was considered at p < 0.01 or p < 0.05.

Results

C. japonica oil significantly suppressed inflammatory cells and IgE level

The inflammatory cells were increased more by ovalbumin treatment (Fig. 1A.b, $\times$ 400) than those in the control group in BALF (Fig. 1A.a, $\times$ 400). *C. japonica* oil dose-dependently decreased the number of inflammatory cells that were up-regulated by ovalbumin treatment (Fig. 1A.d–A.f, $\times$ 400). In the same pattern as that in the change of inflammatory cells, *C. japonica* oil effectively suppressed the

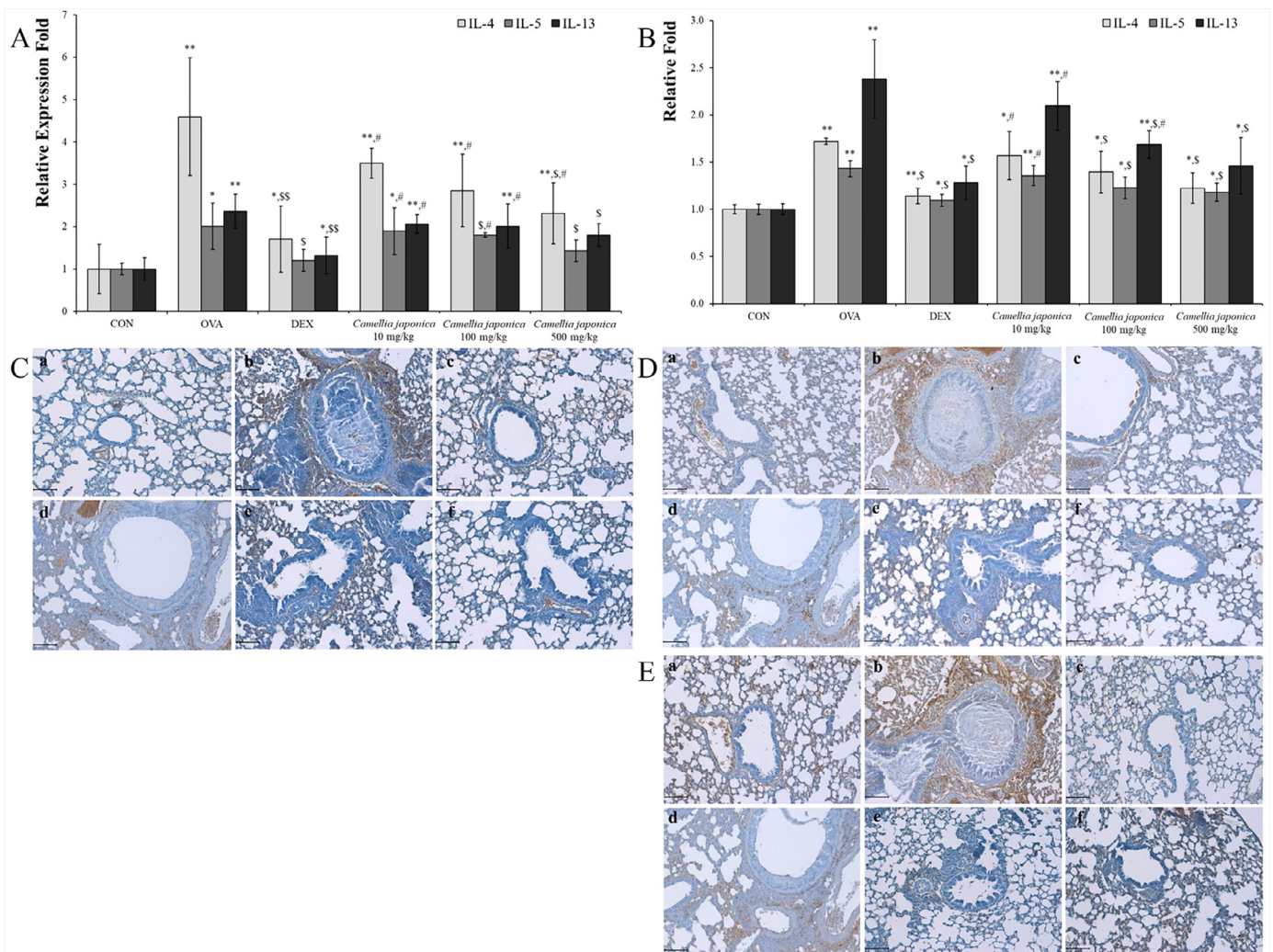


Fig. 5. *C. japonica* oil dramatically inhibited not only cDNA levels but also protein expressions in all Th2-related cytokine such as IL-4, IL-5, and IL-13. (A) *C. japonica* oil significantly and dose-dependently suppressed the genes' levels of Th2-related cytokine such as IL-4, IL-5 and IL-13 and especially the IL-5 level decreased from 100 mg/kg *C. japonica* oil treatment. *C. japonica* oil using with 100 mg/kg treatment perfectly controlled Th2-related cytokine such as IL-4, IL-5, and IL-13, not only (B) in the quantitative point of view, but also (C-E) in the qualitative point of view. a, vehicle control; b, asthma induction; c, dexamethasone; d, 10 mg/kg/day *C. japonica* oil; e, 100 mg/kg/day *C. japonica* oil; f, 500 mg/kg/day *C. japonica* oil. Each bar represents the mean $\pm$ SEM (n = 8). * p < 0.05 vs. control; ** p < 0.001 vs. control; * p < 0.05 vs. asthma induction; ** p < 0.01 vs. asthma induction; # p < 0.05 vs. dexamethasone. Scale Bar = 100 μ m. Magnification, $\times$ 200.

level of IgE in serum (Fig. 1.B., p < 0.001 to p < 0.05). The surge of eosinophil in asthma patients is one of the important indices (Uhm et al., 2012) and the number and the levels of WBC and eosinophil in BALF were also measured (Figs. 1.C. and D., p < 0.05). The increments of WBC and eosinophil were observed in the ovalbumin treatment group but dexamethasone effectively controlled the levels of WBC and eosinophil (p < 0.001 to p < 0.05). In particular, 100 mg/kg and 500 mg/kg *C. japonica* oil treatment significantly suppressed the level of eosinophil like that in the dexamethasone treatment group (p < 0.05).

C. japonica oil effectively prevented the asthma typical changes in pulmonary system morphology which are caused by ovalbumin

In order to evaluate the blocking effect against ovalbumin-induced morphological changes, H&E staining was conducted (Fig. 2.A., $\times$ 200, and Table 1.). In the ovalbumin treatment group, asthma typical changes in the pulmonary system were observed as mucous hypersecretion in the bronchioalveolar region, epithelial cell hyperplasia, inflammatory cells infiltration near the bronchioalveolar duct and vessel, and so on (Fig. 2.A.b, $\times$ 200) compared to that in the control group (Fig. 2.A.a, $\times$ 200). Dexamethasone which recently is used as anti-

asthmatic drug effectively controlled the pulmonary changes (Fig. 2.A.c, $\times$ 200) and *C. japonica* oil dose-dependently prevented the ovalbumin-induced morphological changes (Fig. 2.A.c–A.f, $\times$ 200). In asthma patients, one of the most severe symptoms is respiratory difficulty, which is caused by the narrowness of bronchioalveolar ducts and the major reason for that is mucous hypersecretion. In order to evaluate the level of mucous in the bronchioalveolar region, PA staining was conducted. *C. japonica* oil dose-dependently suppressed mucous secretion (Fig. 2.B.d–B.f, $\times$ 200) and especially in 500 mg/kg *C. japonica* oil treatment group (Fig. 2.B.f, $\times$ 200) the level of mucous was similar to that in dexamethasone treatment group (Fig. 2.B.e, $\times$ 200).

C. japonica oil controlled both the activations of Th1 cell transcription factor, T-bet and Th2 cell transcription factor, GATA-3

T-bet is Th1 cell transcription factor and is modulated by IFN- γ with positive feedback (Zhu et al., 2012). Th2 cell transcription factor, GATA-3 is regulated by IL-4 with positive feedback (Yagi et al., 2011). In order to evaluate the relation of Th1-/Th2-related factors' changes the translocation of T-bet and GATA-3 using with immunofluorescent assay was measured (Fig. 3., $\times$ 1000). In the control group, T-bet and

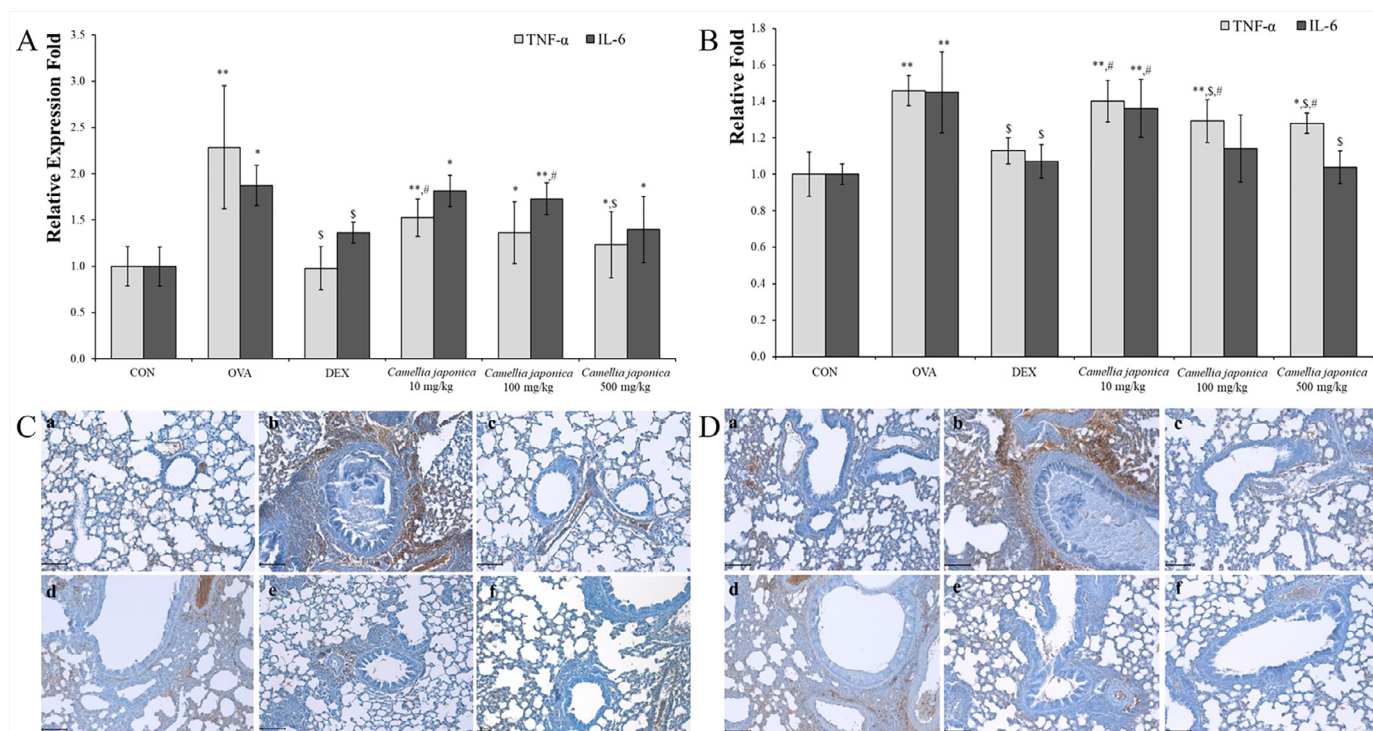


Fig. 6. *Camellia japonica* oil reduced the level of Th17-related cytokine such as TNF- α and IL-6. (A) *C. japonica* oil reduced the relative expression gene level of Th17-related cytokines such as TNF- α and IL-6 and especially dose-dependently suppressed the gene level of TNF- α . (B) *C. japonica* oil controlled the levels of TNF- α and IL-6 in a dose-dependent manner. (C) According to the *C. japonica* oil treatment the change of TNF- α expression in the lung was shown. (D) Based on the immunohistochemical study the change of expression level of IL-6 was similar to the that of ELISA method about IL-6. a, vehicle control; b, asthma induction; c, dexamethasone; d, 10 mg/kg/day *C. japonica* oil; e, 100 mg/kg/day *C. japonica* oil; f, 500 mg/kg/day *C. japonica* oil. Each bar represents the mean $\pm$ SEM (n = 8). * p < 0.05 vs. control; ** p < 0.001 vs. control; ^s p < 0.05 vs. asthma induction; [#] p < 0.05 vs. dexamethasone. Scale Bar = 100 μ m. Magnification, $\times$ 200.

GATA-3 were located in the cytoplasm (Fig. 3.D.a, $\times$ 1000), but in the ovalbumin treatment group they were in the nucleus, ready to act as transcription factors (Fig. 3.D.b, $\times$ 1000). Therefore, ovalbumin stimulated the translocation of T-bet and GATA-3 from cytoplasm to nucleus for their activation. As shown in Fig. 3.D.d, *C. japonica* oil inhibited the translocation of T-bet and GATA-3 from cytoplasm to nucleus like the result in the dexamethasone treatment group (Fig. 3.D.c, $\times$ 1000).

C. japonica oil dose-dependently suppressed the expression of IL-12p40 in the lung

In order to analyze the modulation effect on Th1-related cytokine by *C. japonica* oil, we evaluated the gene and protein levels of IFN- γ and IL-12 (Fig. 4.). The expressions of gene and protein of IFN- γ were not effectively suppressed by *C. japonica* oil treatment (Fig. 4.A.–C., $\times$ 200, p < 0.001 to p < 0.05). However, the protein level of IL-12p40 was dose-dependently controlled by *C. japonica* oil (Fig. 4.B. and D., $\times$ 200, p < 0.001 to p < 0.05), but its gene level was not (Fig. 4.A.).

C. japonica oil dramatically down-regulated the levels of Th2-related cytokine such as IL-4, IL-5, and IL-13

There have been many reports that Th2-related cytokine plays an important role in various hyper-responsiveness such as asthma (Busse, 2001; Wills-Karp, 1999). In most asthma patients, the levels of Th2-related cytokines such as IL-4, IL-5, and IL-13 are highly elevated from normal functioning. In order to measure the changes in Th2-related cytokines levels by *C. japonica* oil treatment, both gene level and protein level were analyzed (Fig. 5.). The IL-4 gene level significantly increased by ovalbumin treatment but *C. japonica* oil effectively and dose-dependently suppressed its level (Fig. 5.A., p < 0.001 to p < 0.05).

Similar to the result of gene level *C. japonica* oil significantly controlled the protein expression of IL-4 which increased by ovalbumin treatment (Fig. 5.B. and C., $\times$ 200, p < 0.001 to p < 0.05). *C. japonica* oil suppressed not only the levels of gene and protein of IL-5 (Fig. 5.B. and D., $\times$ 200, p < 0.001 to p < 0.05) but also those of IL-13 (Fig. 5.B. and E., $\times$ 200, p < 0.001 to p < 0.05). In particular, 100 mg/kg and 500 mg/kg *C. japonica* oil treatment inhibited all of the protein expressions of Th2-related cytokines (Fig. 5.B., p < 0.001 to p < 0.05).

C. japonica oil dose-dependently suppressed the protein level of Th17-related cytokine, TNF- α

Th17 cells may regulate Th1 cells and Th2 cells, and then play an important role in hyperresponsiveness such as in the case of asthma (Zhao et al., 2013). We evaluated the gene and protein levels of Th17-related cytokines such as TNF- α and IL-6 (Fig. 6.). Although *C. japonica* oil did not effectively suppress the gene and protein levels of IL-6 it could control those of TNF- α with statistical significance (Fig. 6.A. and B., p < 0.001 to p < 0.05). As shown in Fig. 6.D. ($\times$ 200), *C. japonica* oil significantly inhibited the expression of TNF- α , which was upregulated by ovalbumin treatment (p < 0.001 to p < 0.05).

Identification of active constituents in *C. japonica*

Typical GC–MS chromatograms of phytochemical contents and their retention times are shown in Fig. 7. Major compounds [i.e., oleic acid (52.89%, Fig. 7.A.), 6-octadecenoic acid (9.12%, Fig. 7.B.), 9,12-octadecadienoic acid (3.32%, Fig. 7.C.), 1-mono-oleoylglycerol (2.68%, Fig. 7.D.), β -amyryn (2.15%, Fig. 7.E.), and octadecanoic acid (2.13%, Fig. 7.F.)] were identified by GC–MS analysis. As was shown in Fig. 7.G. we got the result of total ion chromatogram of *C. japonica*.

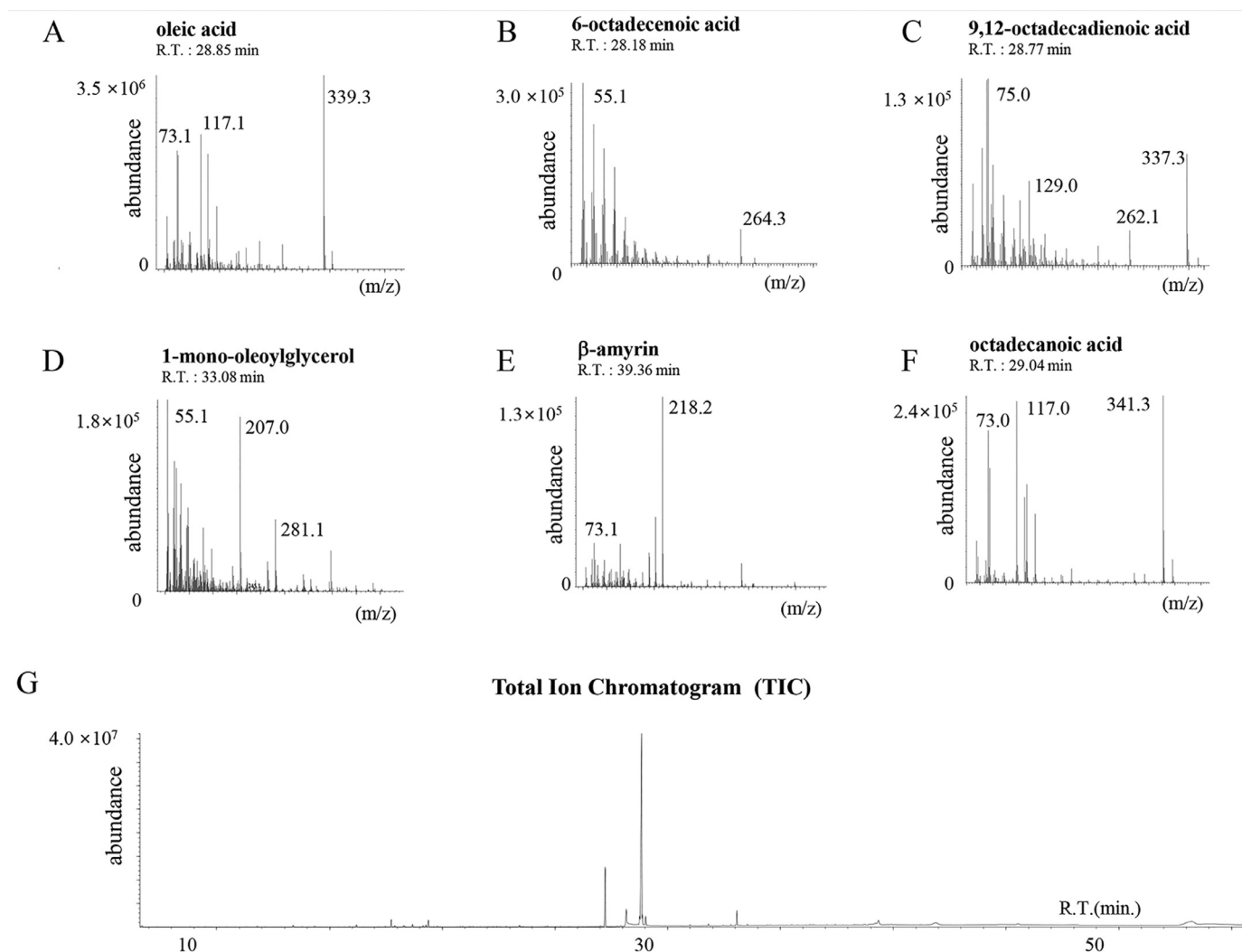


Fig. 7. GC chromatogram of *Camellia japonica* oil (A) Octadecanoic acid, (B) 1-monooleoylglycerol, (C) b-Amyrin, (D) 6-Octadecenoic acid, (E) Oleic acid, (F) 9,12-Octadecadienoic acid, (G) Total Ion Chromatogram (TIC).

Oleic acid was the major component in C. japonica oil and significantly suppressed typical asthmatic changes

In order to evaluate the anti-asthmatic effect of oleic acid, which is the major component in *C. japonica* oil using with asthma murine model, we additionally conducted the animal experiment. We also evaluate the typical asthmatic changes such as inflammatory cell population, WBC, and eosinophil in BALF, IgE level in serum and morphological changes in the lung (Fig. 8.). As the level of oleic acid was 52.89% in *C. japonica* oil 250 mg/kg, oleic acid treatment, which might be similar to the maximum dose of *C. japonica* oil, of 500 mg/kg was used for this study. The inflammatory cell proliferation which was caused by ovalbumin treatment (Fig. 8.A.b, $\times 400$) was decreased by oleic acid treatment (Fig. 8.A.d, $\times 400$). The results of WBC and eosinophil were very similar to those of inflammatory cells in BALF (Fig. 8.B., $p < 0.05$). Oleic acid significantly suppressed the level of IgE in the serum (Fig. 8.C., $p < 0.001$ to $p < 0.05$). In order to evaluate the pulmonary morphological changes, H&E staining and PAS staining were conducted (Fig. 8.D. and E., $\times 200$). Oleic acid effectively prevented the asthma typical changes such as mucous secretion, inflammatory infiltration, epithelial cell hyperplasia, and so on. In particular, the suppression level of asthma treated by oleic acid occurrence was similar to that by dexamethasone.

Discussion

Immunological tissue damage is classified as one of four categories (Todd et al., 2015). Type I is immediate hypersensitivity and is mediated with IgE. Type II relates with cell or membrane reaction and mediates with IgG, IgA, or IgM. Type III and type IV mediate immune complex and cell, respectively. Asthma is involved in Type I immunological tissue damage and mediated with IgE (Ariza et al., 2014). Asthma stimulators, which are called allergens, are categorized into two groups: indoor allergens such as house-dust-mite, pet dander, cockroach, etc. (Plattes-Mills et al., 1997), and outdoor allergens such as pollen, soy dust, fungi, arthropods, etc. (Burge and Rogers, 2000). These days in particular, fine dust might be one of the outdoor allergens behind asthma occurrence (Yonhap New Agency, 2017).

The symptoms in ovalbumin murine asthma model are very similar to those in asthma patients and the pathogenic mechanism was supposed to have an imbalance relationship between the Th1 cell and Th2 cell (Lee, et al., 2017). Th1 cells could produce many cytokines such as IL-12, IFN- γ , etc., and Th2 cells could produce several cytokines such as IL-4, IL-5, IL-13, and so on (Mosmann and Coffman, 1989). Th1 cell transcription factor is T-bet, which could make IFN- γ with positive feedback (Szabo et al., 2000; Zhu et al., 2012) and GATA-3, Th2 cell transcription factor is controlled by IL-4 (Yagi et al., 2011). IL-12p40 is one of the important factors that can control Th1-related cytokine and

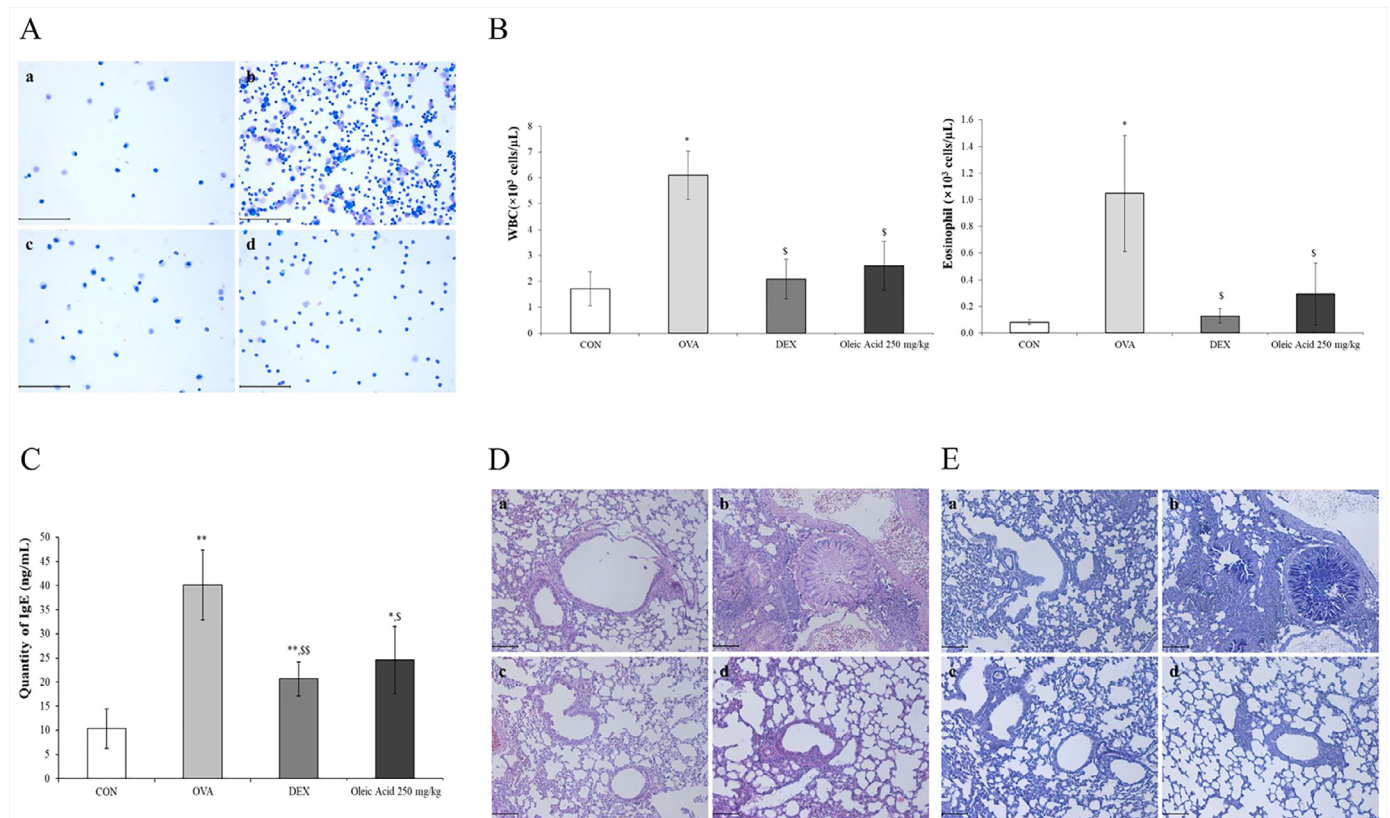


Fig. 8. Oleic acid had anti-asthmatic effect in ovalbumin-induced asthma animals as similar to the results of *Camellia japonica* oil. (A) Oleic acid which was isolated from *C. japonica* oil significantly inhibited the level of inflammatory cells in BALF. Magnification, × 400 (B) The decreased levels of WBC and eosinophil by oleic acid were similar to the changes of them by dexamethasone. (C) Oleic acid inhibited the level of IgE which was induced by ovalbumin as similar to that by dexamethasone. (D) Oleic acid controlled the typical asthmatic change of lung such as mucous hyper-secretion, bronchioalveolar epithelial layer hyperplasia, inflammatory cells infiltration near the bronchioalveola and vessel, and so on. Magnification, × 200. (E) Oleic acid decreased the mucous secretion similar to the level of that by dexamethasone. Magnification, × 200; a, vehicle control; b, asthma induction; c, dexamethasone; d, 10 mg/kg/day *C. japonica* oil; e, 100 mg/kg/day *C. japonica* oil; f, 500 mg/kg/day *C. japonica* oil. Each bar represents the mean ± SEM (n = 8). **p* < 0.05 vs. control; ***p* < 0.001 vs. control; §*p* < 0.05 vs. asthma induction; §§*p* < 0.01 vs. asthma induction. Scale Bar = 100 μm.

induce IFN- γ releasing and is related to asthma (Hamaza et al., 2010). *C. japonica* oil more effectively modulated the change of IL-12p40 than IFN- γ (Fig. 4.B.). There have been many reports that in asthma patients, Th2-related cytokines, such as IL-4, IL-5, IL-13, etc., were much higher than those in healthy people (Antczak et al., 2016; Foster et al., 1996; van der Pouw Kraan et al., 1998; Wenzel et al., 2007). IL-4 plays an important role in asthma, as induces the production of eosinophil and neutrophil via mast cell stimulation, produces Th2-related cytokine via GATA-3 activation, stimulates IgE production via B cell activation, excessively secretes mucous, and so on (Larche et al., 2003; Lee and In 2006). IL-5 stimulates the survival and proliferation of eosinophil (Kouro and Takatsu 2009) and finally relates the increment of eosinophil in BALF and eosinophil infiltration in the lung. The asthma-related functions of IL-13 are very similar to those of IL-4; it stimulates IgE, eosinophil, and basophil production, induces mucous hypersecretion (Larche et al., 2003; Platts-Mills, 2001). However, the differential functions between IL-13 and IL-4 are to induce airway hyperresponsiveness (AHR) and airway remodeling such as inflammation, fibrosis, obstruction, etc. in the lung (Zhu et al., 1999). Although *C. japonica* oil suppressed both Th1-related cytokines, IL-12 and Th2-related ones, IL-4, IL-5, and IL-13, it effectively controlled much more the Th1-related one than the Th2-related cytokine (Figs. 4. and 5.). The gene and protein levels of IL-4, IL-5, and IL-13 in 100 and 500 mg/kg *C. japonica* oil treatment groups were similar to those in dexamethasone treatment group only except the results of IL-13 (Figs. 5.A. and B.). Th17 cell is one of the very important factors to modulate asthma occurrence (Singh et al., 2014), chemo-attracts neutrophil and eosinophil

(Lukacs et al., 1995) and stimulates eosinophil's cell death via oxidative stress in respiratory epithelial cells (Slungaard et al., 1990). *C. japonica* oil effectively inhibited the TNF- α releasing (Fig. 6.A. to C.).

Asthma is an incurable pulmonary disease (Slejko et al., 2014), and the one way to control it is to relieve its symptoms. In the present, corticosteroid hormone has been broadly used for the suppression of that by inhalation method, but has many adverse effects: children's growth inhibition, central nervous disorder, cataract and glaucoma, hyper-lipidemia, peptic ulcer, and so on (Wise, 2014; Ciriaco et al., 2013). As inhaled medication for asthma treatment has many side effects, many efforts have recently been carried out to find the anti-asthmatic drug candidates which have relatively lower adverse effects.

β -Amyrin, has been shown to exert anti-inflammatory effect on dimethyl nitrosamine-induced hepatic fibrosis in male rats (Thirupathi et al., 2017). Oleic acid is a naturally occurring fatty acid found in animal fats and vegetable oils. It has several biological effects such as reducing low-density lipoprotein and increasing high-density lipoprotein, reducing blood pressure (Peyrat-Maillard et al., 2003), and anti-inflammation (Erdinest et al., 2015). Although there have been reports that a low concentration of oleic acid exacerbates LPS-induced cell death and inflammation in human alveolar epithelial cells (Kucukgul and Erdogan, 2017), the relation of oleic acid and asthma occurrence or prevention remains unclear. Chung et al., (2015) reported that oleic acid suppressed peanut and cashew nut-related IgE increment. In the present study we clearly elucidated that oleic acid suppressed typical asthmatic changes such as inflammatory cell proliferation, the increase of WBC and eosinophil in BALF, IgE surge in

serum, and morphological changes in the lung (Fig. 8).

From these results, we concluded that although further study is needed for the mode of action, the major anti-asthmatic ingredient in *C. japonica* oil was oleic acid, and *C. japonica* oil is a promising candidate for asthma preventive protocol.

Conflict of interest

The authors declare no competing financial interest.

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