

Analogous corticosteroids, 9A and EK100, derived from solid-state-cultured mycelium of *Antrodia camphorata* inhibit proinflammatory cytokine expression in macrophages

Shung-Te Kao^{a,b,1}, Yueh-Hsiung Kuo^{c,d,1}, Shulhn-Der Wang^e, Hong-Jye Hong^a, Li-Jen Lin^{a,*}

^a School of Chinese Medicine, College of Chinese Medicine, China Medical University, Taichung 40402, Taiwan

^b Department of Chinese Medicine, China Medical University Hospital, Taichung 40402, Taiwan

^c Department of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, China Medical University, Taichung 40402, Taiwan

^d Department of Biotechnology, Asia University, Taichung 41354, Taiwan

^e School of Post-Baccalaureate Chinese Medicine, College of Chinese Medicine, China Medical University, Taichung 40402, Taiwan

ARTICLE INFO

Keywords:

Antrodia camphorata
Solid-state-cultured mycelium
Chronic asthmatic mouse model
Dermatogoides pteronyssinus
Bronchoalveolar lavage fluid
MH-S cells

ABSTRACT

Antrodia camphorata mycelium is used in traditional Chinese medicine in Taiwan. The wild-type mycelium is rare and expensive, so a solid-state-cultured mycelium of *A. camphorata* (SCMAC) has been developed. Previous studies have found SCMAC to have anti-inflammatory effects. However, the immunomodulatory effects of SCMAC and of its active phytosterol compounds EK100 and 9A on asthma remain unknown. In this study, BALB/c mice were repeatedly exposed to *Dermatogoides pteronyssinus* (Der p) at 1-week intervals and were orally administered crude SCMAC extract before the Der p challenge. The mice were sacrificed 72 h after the last challenge to examine the airway remodeling, inflammation, and expression profiles of cytokines and various genes. Then, 30-μg/mL Der p-stimulated MH-S cells with 9A or EK100 were collected for real-time PCR analysis, and the effects of 9A and EK100 on macrophages were evaluated. The crude extract reduced Der p-induced airway hyperresponsiveness, total serum immunoglobulin E levels, and recruitment of inflammatory cells to the bronchoalveolar lavage fluid through cytokine downregulation and Th1/Th2/Th17 response modulation. Additionally, 9A and EK100 inhibited IL-1β and IL-6 expression in alveolar macrophages. These results indicate that the pharmacologically active compounds in a crude SCMAC extract exert synergistic effects on multiple targets to relieve asthma symptoms.

1. Introduction

Asthma is a major global public health problem that has increased in prevalence and severity in recent years [1]. Currently, as many as 5–10% of the population in developed countries has asthma, the treatment of which incurs a heavy socioeconomic burden [2].

The first-line treatment for persistent asthma is inhaled corticosteroid therapy, although other medications such as leukotriene receptor antagonists, anti-immunoglobulin (Ig) E, and antihistamines may also be used [3]. However, some patients who use inhaled corticosteroids continue to be symptomatic and in some cases develop serious side effects [4]. Various other medications exist, but they provide only temporary relief of inflammatory symptoms and cannot replace inhaled corticosteroids [5]. Thus, more effective therapies are urgently required.

A previous report demonstrated that up to 30% of adults and 60% of children with asthma use some form of complementary and alternative medicine to treat asthma [6]. Moreover, a series of publications have reported on the therapeutic mechanisms of the traditional Chinese medicines that have been found to be useful for treating allergic asthma [7]. One such agent, the mycelium of *Antrodia camphorata*, exerts immunomodulatory, anti-inflammatory, and antioxidant effects and has commonly been used in Taiwan to treat liver diseases, hypertension, and cancer [8]. However, the wild fruiting body of *A. camphorata* grows slowly and is expensive because the demand far exceeds the available supply [9]. Accordingly, a solid-state-cultured mycelium of *A. camphorata* (SCMAC) was developed and is commercially available from local companies [9]. SCMAC has similar anti-inflammatory effects as those of the wild fruiting body [10]. Moreover, it contains many secondary metabolites, such as lignans, sesquiterpenes, and phytosterols

* Corresponding author at: School of Chinese Medicine, College of Chinese Medicine, China Medical University, Taichung 404, Taiwan, Republic of China.

E-mail addresses: stkao@mail.cmu.edu.tw (S.-T. Kao), kuoyh@mail.cmu.edu.tw (Y.-H. Kuo), wsd917@mail.cmu.edu.tw (S.-D. Wang), hongjh@mail.cmu.edu.tw (H.-J. Hong), linljen@mail.cmu.edu.tw (L.-J. Lin).

¹ Equal contribution to this article.

[11]. Numerous reports have demonstrated the anti-inflammatory effects of phytosterols, which reduce the levels of proinflammatory cytokines in animals and humans [12]. However, no study has evaluated the immunomodulatory effects of SCMAC and its active phytosterol compounds on asthma.

Various pathophysiological mechanisms underlie the clinical presentation of asthma, including high serum IgE levels, airway hyperresponsiveness (AHR), eosinophilic recruitment, and airway inflammation and remodeling [13]. Furthermore, chronic allergic asthma is associated with the inappropriate secretion of cytokines from the Th1/Th2/Th17 cells and subsequent immune responses [14]. A fundamental feature of these cytokines and chemokines is their ability to direct immune cell trafficking, and MCP-1 recruits alveolar macrophages, together with resident cells, to form the first line of defense against infectious challenges in the lungs [15,16]. Alveolar macrophages can direct interactions with inhaled allergens and rapidly regulate the production of the proinflammatory cytokines IL-1 β and IL-6 [15].

Currently, more than 85% of asthma patients have been sensitized to allergens derived from *Deratogoides pteronyssinus* (Der p), the house dust mite. This study determined the immunomodulatory effects of a crude extract of SCMAC on a Der p-induced mouse model of chronic asthma and of the active phytosterol compounds EK100 and 9A on macrophages. In addition, this study examined whether the asthma prophylactic efficacy of this crude extract of SCMAC is associated with reduced leukocyte recruitment and molecular Th1/Th2/Th17 response modulation in the lungs.

2. Materials and methods

2.1. Mice and reagents

Specific pathogen-free male BALB/c mice aged 6–8 weeks (National Laboratory Animal Center, Republic of China) were used in these experiments. All animal care protocols and experimental treatments followed the guidelines of the National Science Council of the Republic of China and were conducted in agreement with the principles outlined by the Institutional Animal Care and Use Committee of China Medical University (No. 103-233-C). Lyophilized Der p (Allergon, Sweden) was extracted using ether and dialyzed against deionized water [16]. Dexamethason was purchased from Sigma.

2.2. Solid-state-cultured mycelium of *A. camphorata* (SCMAC) and preparation of crude SCMAC extract and phytosterols

Freeze-dried powdered SCMAC was provided by Well Shine Biotechnology Development Co. Ltd. (Taipei, Taiwan). This powder (3.0 kg) was extracted with MeOH (12 L) at room temperature (two 5-day steps), and the resulting extracts were evaporated to yield a solid residue that was mixed with H₂O to a total volume of 1 L. The chemical fingerprints of the mixture that were identified using high-performance liquid chromatography were described elsewhere [17]. This phase was extracted with EtOAc (three $\times$ 1 L), and the combined organic phase was evaporated to yield a solid residue (161.6 g), followed by chromatography on silica gel (elution: hexane and EtOAc solution). The 7,22-ergostadien-3 β -ol (9A) [18] and 7,9(11),22-ergostatrien-3 β -ol (EK100) [19] were extracted as reported. The elution yielded 9A (50 mg) (Fig. 1a) and EK100 (1.1 g) (Fig. 1b).

2.3. Effect of SCMAC extract on the Der p-induced model of chronic asthma

Twenty-four BALB/c mice were randomly separated into four groups. For mice in the Der p group, a chronic asthma model involving allergic airway inflammation and remodeling was induced by repeatedly challenging them intratracheally with Der p (1 mg/mL, 50 μ L) once per week for 5 weeks (six total doses). In the L (50 mg/kg) and H (250 mg/kg) groups, the mice received the indicated oral doses of the

crude SCMAC extract 30 min before the intratracheal Der p challenge. After 3 days, both groups received a second oral dose of the crude SCMAC extract. Naïve mice without Der p challenge and SCMAC treatment were included as controls in parallel experiments for comparison.

Three days after the final challenge, the mice were sacrificed through intraperitoneal injection of 200- μ g xylazine and 2-mg ketamine, as reported previously [17]. Serum samples were collected and stored at -80°C until further analysis. Bronchoalveolar lavage fluid (BALF) samples were collected and prepared for total leukocyte counts, differential counts, and cytokine assays.

2.4. Measurement of airway hyperresponsiveness

Airway resistance in the mice was measured using a single-chamber, whole-body plethysmograph (Buxco Electronics, Inc., Troy, NY, USA) according to the manufacturer's protocol. The enhanced pause (Penh) variable represents airway resistance. Briefly, mice were exposed to increasing doses of nebulized methacholine (0, 3.125, 6.25, 12.5, 25, and 50 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) for 3 min; Penh was measured over the subsequent 3-min period following the end of methacholine nebulization.

2.5. Measurement of total IgE, Der p-specific IgG1, and Der p-specific IgG_{2a/2b}

Total serum IgE levels were measured using an IgE-specific enzyme-linked immunosorbent assay (ELISA) kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. To evaluate Der p-specific antibodies, serum samples were added to ELISA plates coated with 2 μ g/mL Der p and incubated overnight at 4°C . After the plates were washed, biotinylated rat anti-mouse IgG1 or IgG_{2a/2b} mAb (2 mg/mL; BD Pharmingen) was added, followed by 1 h of incubation. Subsequently, the plates were washed with phosphate-buffered saline and incubated with a streptavidin-horseradish peroxidase (HRP) conjugate (1:1,000 dilution, BD Pharmingen). Finally, the plates were washed and developed using the TMB Microwell Peroxidase Substrate System (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Colorimetric reactions were measured at OD₄₅₀.

2.6. ELISA analysis of cytokine levels

IL-5, IL-6, and IL-12 levels were assayed using an ELISA Ready-SET-Go! Kit (eBioscience, San Diego, CA, USA), and IL-13, IFN- γ , and IL-1 β levels were assayed using an ELISA DuoSet! Kit (R&D Systems, Abingdon, UK). Cytokine concentrations were determined against standard concentrations according to the manufacturer's protocol.

2.7. Quantitative real-time polymerase chain reaction (RT-PCR)

Total RNA was prepared from lung tissue using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and converted to cDNA using the ImProm-II Reverse Transcription System kit (Promega Corp., Madison, WI, USA). Quantitative PCR was performed using the FastStart Universal SYBR Green Master kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. β -actin expression was used as a control. The following gene-specific primers (sense and antisense) were used: β -actin: forward, 5'-GGAAATCGTGCGTGACA-3' and reverse, 5'-CACA GGATTCCATACCCAAG-3'; MCP-1: forward, 5'-AGA AGGAATGGGTCCAGAC ATA-3' and reverse, 5'-TTAAGGCATCACAGT CCGAG-3'; IL-17: forward, 5'-CTC AACCGTTCACGTC-3' and reverse, 5'-CTCTCAGGCTCCCTCTT-3'; TLR4: forward, 5'-CAGCAGAGGAGAA AGCATC-3' and reverse, 5'-TTGGCAGCAATG GCTAC-3'; and TLR2: forward, 5'-GAGACCTATAGTGACTCCAG-3' and reverse, 5'-TGATGA TGACCCCAAGAC-3'; IL-6: forward, 5'-CTTCACAAGTCGGAG GCTTA-3' and reverse, 5'-TTGGTAGCATCCATCATTTCTT-3'; and IL-1 β :

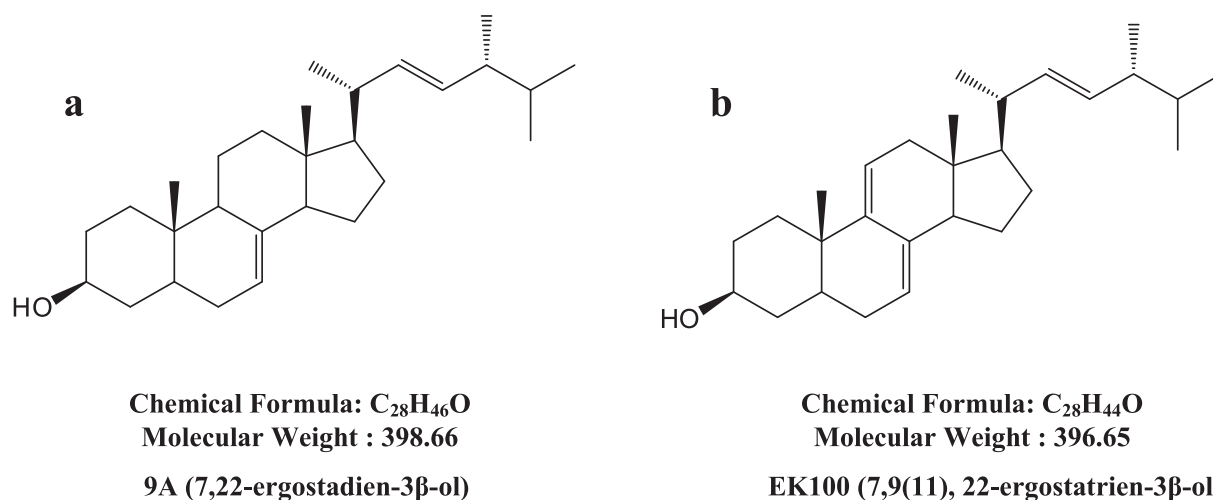


Fig. 1. Structures of the phytosterol compounds isolated from a solid-state-cultured mycelium of *Antrodia camphorata* (SCMAC). (a) 9A (b) EK100.

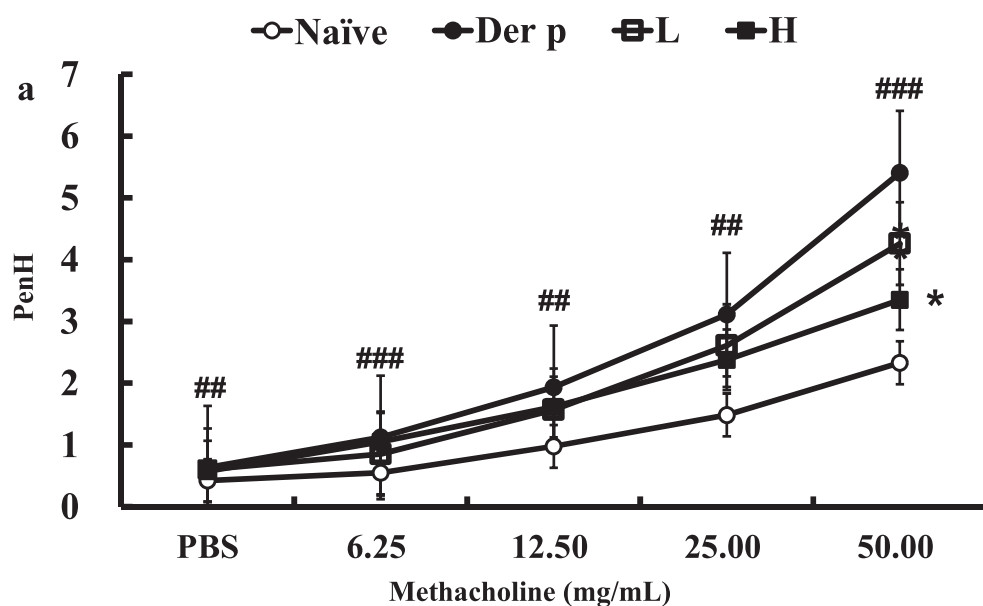
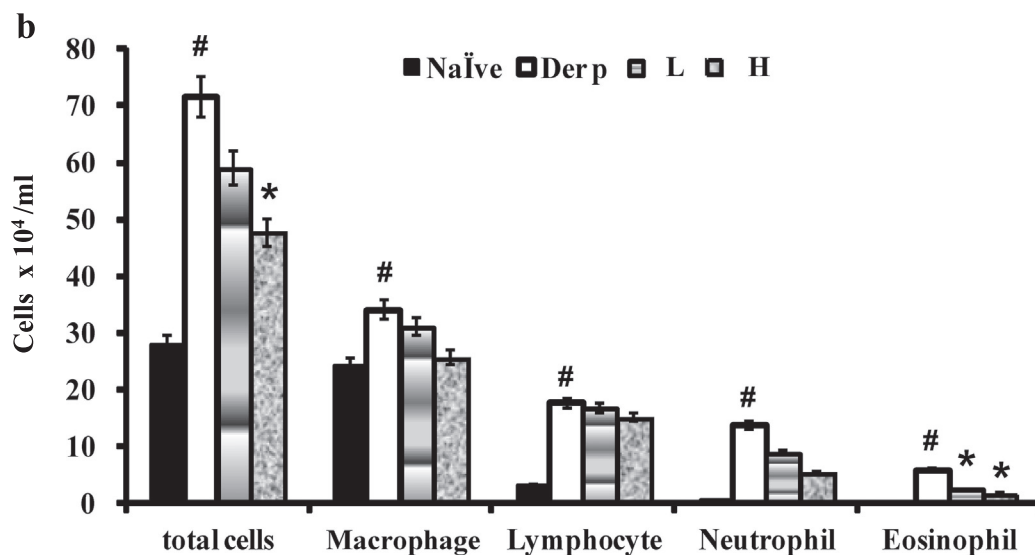


Fig. 2. Effects of a crude SCMAC extract on airway hyperresponsiveness, airway inflammation, and leukocyte infiltration during repeated intratracheal inoculations of *Dermatogoides pteronyssinus* (Der p; 1-week intervals). (a) Airway hyperresponsiveness was determined 2 days after the last Der p challenge. (b) The high-dose crude SCMAC extract (H) group exhibited significant reductions in the number of infiltrating total cells and eosinophils in the bronchoalveolar lavage fluid (BALF) at 72 h after the last Der p challenge. Values represent the means $\pm$ standard deviations of six mice. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ (vs. naïve group); * $P < 0.05$ (vs. nontreated and crude SCMAC extract-treated groups).



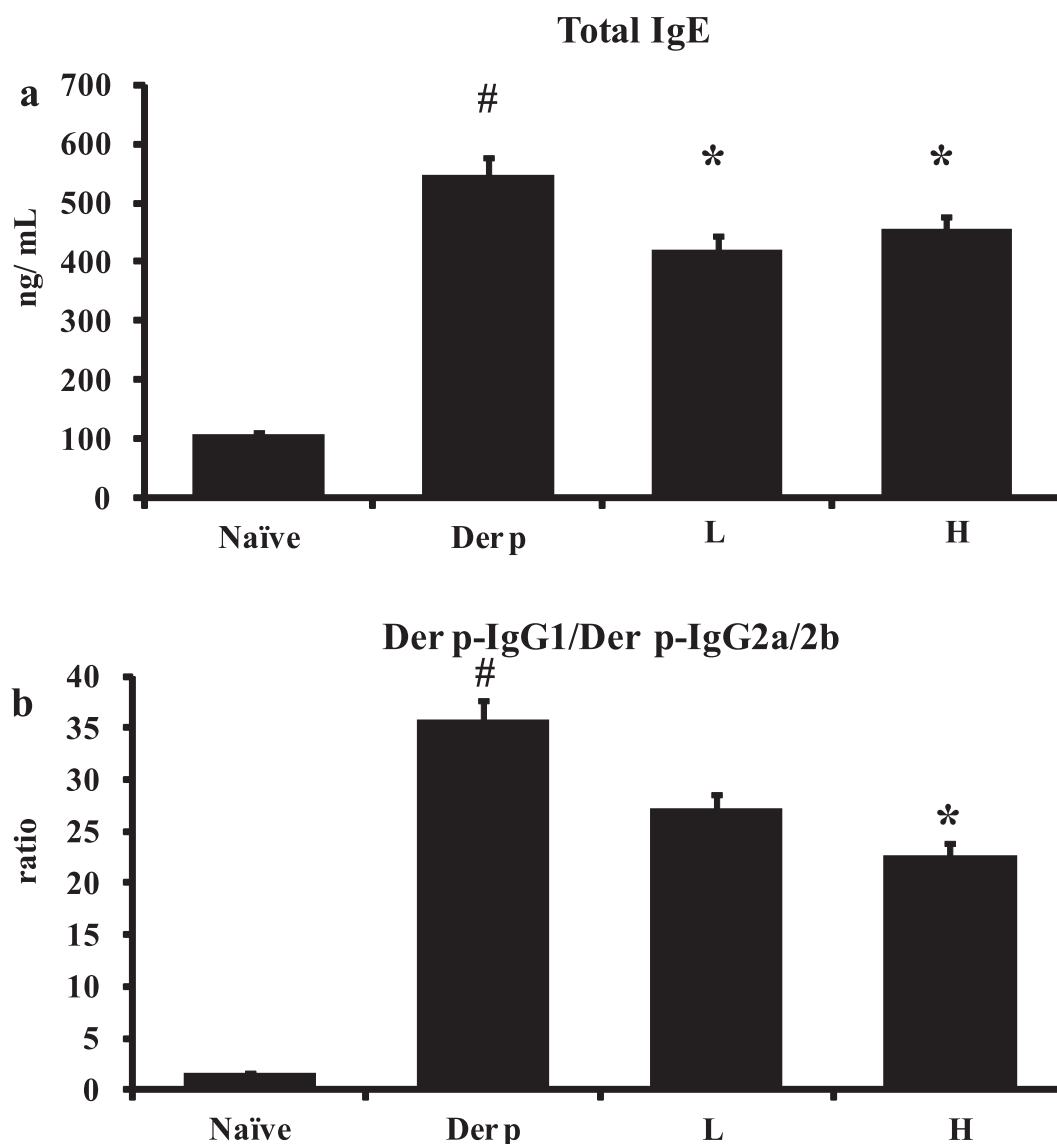


Fig. 3. Effect of a crude SCMAC extract on serum antibody levels in *Dermatoglyphes pteronyssinus* (Der p)-challenged mice. (a) Total IgE levels. (b). Der p-IgG1/Der p-IgG2a/2b ratios. Data are representative of the results of three separate experiments. [#] $P < 0.05$ (vs. naïve group); ^{*} $P < 0.05$ (vs. nontreated and crude SCMAC extract-treated groups).

forward, 5'-CTGTCCTGTGTAATGAAAGACG-3' and reverse, 5'-TGCTCTGTTGTGAGGT-3'.

2.8. Alveolar macrophage (MH-S) culture and gene expression analysis

The MH-S cell line was purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan) and cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, and 0.05 mM β -ME at 37 °C in a humidified atmosphere with 5% CO₂. Next, 1×10^6 cells/well were seeded in 6-well culture plates and pretreated/cotreated with 9A or EK100, pure compounds that had been extracted from the crude SCMAC extract and dissolved in DMSO, at various concentrations in the presence or absence of 30 μ g/mL Der p. The cultures were incubated for the indicated times, after which the cells were collected for reverse transcription and quantitative PCR.

2.9. Statistical analysis

All data are expressed as means $\pm$ standard deviations. Differences

between groups were evaluated using Student's *t* test. A *P* value < 0.05 was considered to indicate statistical significance.

3. Results

3.1. Crude SCMAC extract attenuates Der p-induced airway hyperresponsiveness and inflammation in an asthmatic mouse model

One function of a useful pharmacotherapeutic agent for asthma is the amelioration of AHR, which causes airway obstruction and then leading to cause of dyspnea, a major clinical symptom experienced by patients with asthma. In this study, we used a repeated Der p challenge experiment to test the effects of a crude extract of SCMAC on allergen-induced chronic AHR and inflammation in a mouse model. Mice in the Der p group exhibited markedly higher Penh values than the other groups did. Both groups treated with crude SCMAC extract exhibited a decrease in Penh values with the maximal dose of methacholine (50 mg/mL) relative to the Der p group (Fig. 2a). However, the H group exhibited a stronger relaxation effect than that of the L group in response to treatment with 50 mg/mL of methacholine.

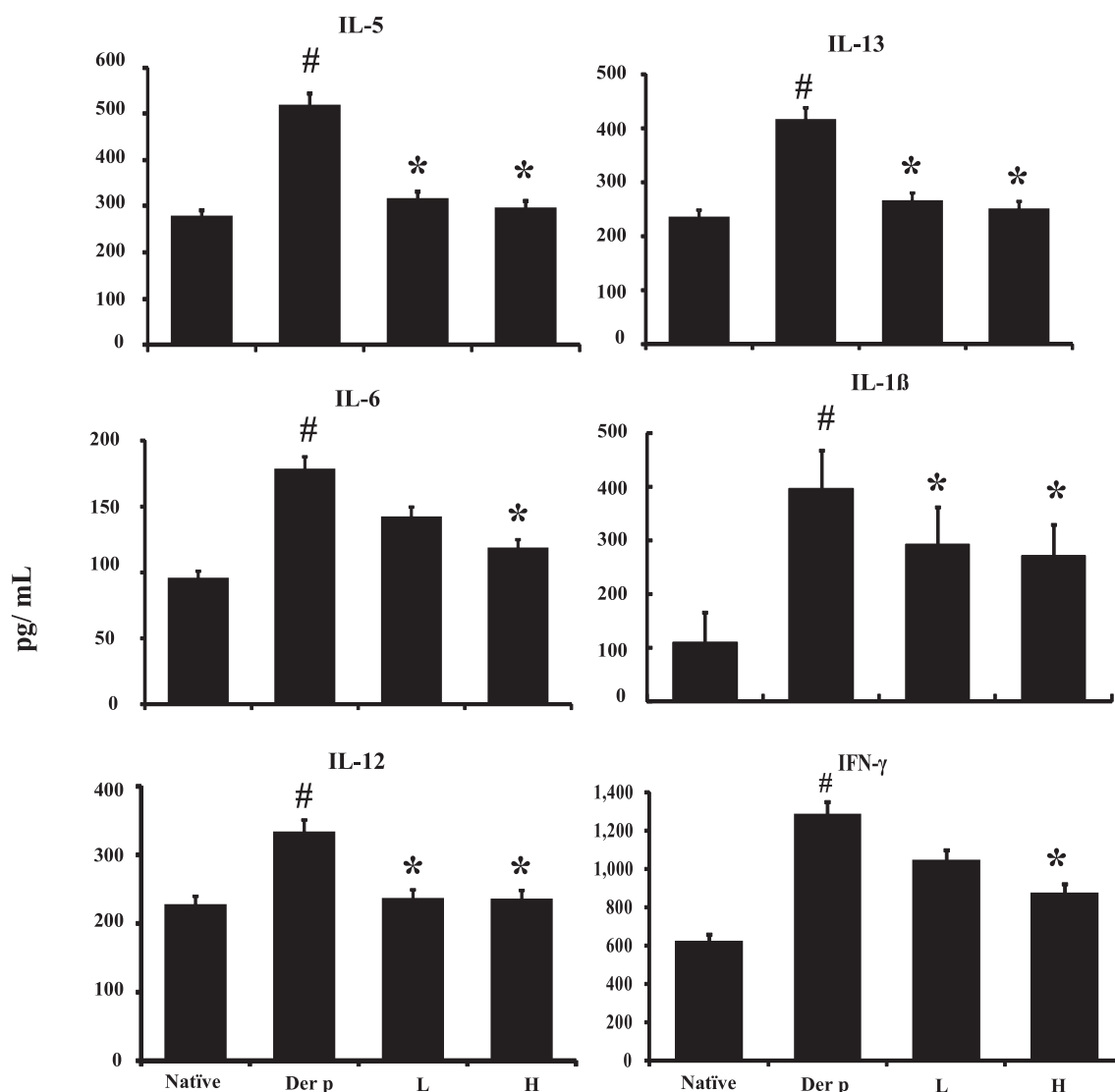


Fig. 4. Effects of a crude SCMAC extract on Th2 and Th1 cytokines in BALF. Treatment with a high dose of a crude extract of a SCMAC (H group) significantly reduced the expression levels of IL-5, IL-13, IL-6, IL-1 β , IL-12, and IFN- γ in the bronchoalveolar lavage fluid (BALF). [#] $P < 0.05$ (vs. naïve group); ^{*} $P < 0.05$ (vs. nontreated and crude SCMAC extract-treated groups).

To determine whether reduced inflammation was associated with AHR suppression, we measured the cellular contents of BALF collected from the treated mice. Fig. 2b demonstrates the lack of detectable eosinophils in the BALF of naïve mice. By contrast, markedly elevated numbers of total cells and eosinophils were detected in the BALF of Der p-treated mice. However, mice that received oral doses of crude SCMAC extract had significantly lower total cell and eosinophil counts in the BALF than those in the Der p group did.

3.2. Effects of a crude extract of SCMAC on serum total IgE and the ratio of Der p-specific IgG1/IgG_{2a/2b} antibodies

A high level of total serum IgE is a characteristic finding in asthma patients. Accordingly, we measured the serum total IgE, Der p-specific IgG1, and Der p-specific IgG_{2a/2b} antibody levels to evaluate the influence of treatment with a crude SCMAC extract on the humoral immune response. Mice in the Der p group exhibited a significant increase in the levels of serum total IgE compared with naïve mice.

Among chronic Der p-challenged mice, which received two doses of crude SCMAC extract, the serum total IgE concentrations were significantly decreased compared with the Der p group mice (Fig. 3a). Moreover, the Der p-specific IgG1/IgG_{2a/2b} ratio was markedly lower in

H group mice than in Der p group mice (Fig. 3b).

3.3. Crude SCMAC extract significantly reduced the levels of proinflammatory, Th2, and Th1 cytokines in BALF

Helper T (Th) cell inflammatory responses play an important role in the pathogenesis of asthma. We determined the effects of our crude SCMAC extract on Th cell cytokine levels in BALF. Mice treated with the crude extract exhibited significant, dose-dependent attenuation of Der p-induced IL-5, IL-13, IL-6, IL-1 β , IL-12, and IFN- γ in the BALF compared with the Der p group mice (Fig. 4).

3.4. Crude extract of SCMAC decreased the expression of Der p-induced immune response genes in the total lung tissues

The Der p group mice exhibited the highest levels of MCP-1, IL-17, TLR2, and TLR4 mRNA expression in lung tissues. Dose-dependent patterns were observed in the L and H groups. In addition, the expression levels of these genes were significantly decreased in the H group compared with the Der p group (Fig. 5).

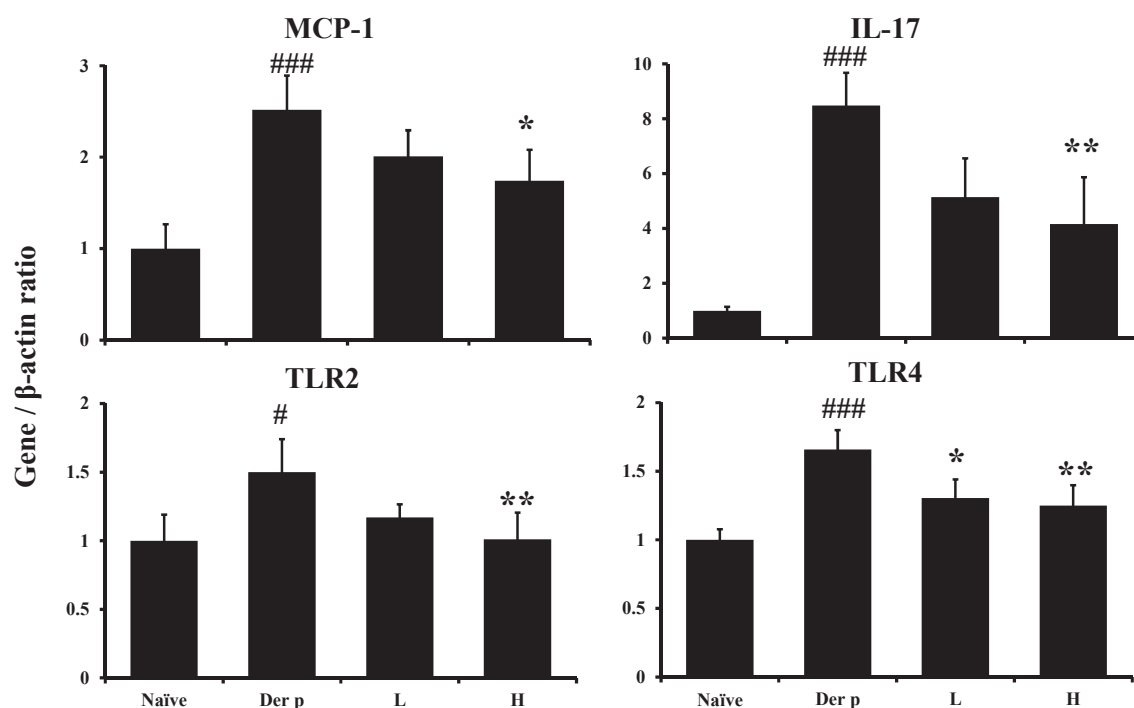


Fig. 5. Effects of a crude SCMAC extract on Der p-induced immune response genes in the total lung. Real-time PCR analysis revealed significant suppression of gene expression in lung tissues following treatment with a crude extract of a SCMAC. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ (vs. naïve group); * $P < 0.05$, ** $P < 0.01$ (vs. nontreated and crude SCMAC extract-treated groups).

3.5. Effects of 9A and EK100, isolated from the crude SCMAC extract, on Der p-induced immune response gene expression in MH-S alveolar macrophages

Airway macrophages play a pivotal role in amplifying inflammatory responses by secreting proinflammatory cytokines to stimulate epithelial cells, which results in the recruitment of inflammatory cells, and phytosterols inhibit proinflammatory cytokines. We speculated that the crude SCMAC extract reduced the number of total cells in BALF (Fig. 2b), which might be related to the immunological activity of their phytosterols. Therefore, we determined the anti-inflammatory effects of two phytosterols, EK100 and 9A, on macrophages.

MH-S cells were pretreated for 1 h with EK100 or 9A. Subsequent analysis revealed marked, dose-dependent reductions in the expression of IL-6 and IL-1 β mRNA with both compounds (compared with Der p treatment alone). Similar results were obtained with cotreatment. However, 9A exhibited stronger inhibitory activity than EK100 did (Fig. 6).

4. Discussion

In the present study, we demonstrated that the oral administration of a crude SCMAC extract led to reductions in AHR, total serum IgE levels, and the total number of inflammatory cells in the BALF of mice subjected to Der p-induced asthma. Moreover, we provided the first experimental evidence that two pure compounds extracted from SCMAC—9A and EK100—reduce the expression of IL-6 and IL-1 β in an alveolar macrophage cell line.

TLR2 and TLR4 are expressed on the surfaces of epithelial cells, macrophages, and dendritic cells and serve as primary mediators in the recognition of exogenous molecules and the initiation of innate immune responses, which results in the release of IL-1 β and IL-6 [20–22]. Moreover, TLR2 and TLR4 play pivotal roles in inflammatory responses and asthma onset. For example, TLR2 activation is associated with a strong, Th2-based immune response and an imbalance of the Th1/Th2 ratio in asthma [21]. Furthermore, augmented TLR2 and TLR4

expressions have been detected in deceased patients with allergic asthma [21]. Therefore, the signaling cascades associated with TLR2 and TLR4 are attractive targets for therapeutic intervention [23], and various plant-based compounds have been used to alleviate allergic asthma symptoms through TLR4 cascade attenuation [21]. In our study, the crude SCMAC extract reduced the expression of both TLR2 and TLR4 (Fig. 5), resulting in a reduction in abnormal immune responses.

Cytokines and chemokines recruit and activate the cells necessary for the pathomechanism of asthma [24–26]. For example, IL-13 activates airway epithelial cells and promotes the expression of proinflammatory mediators in airway smooth muscle cells [25]. IL-5 not only stimulates eosinophil production and release from the bone marrow and maturation in the blood, but also promotes the accumulation and survival of eosinophils in areas of inflammation in the lungs [14,27,28]. Therefore, IL-5 suppression might reduce the pathophysiological processes of asthma that are mediated by eosinophils [29]. IL-6 modulates the influx of CD4⁺ T lymphocytes into the airways and the subsequent fates of these cells [30]. In a recent paper, IL-6 was shown to promote Th2 and Th17 differentiation while inhibiting Th1 differentiation [30]. In addition, IL-17 plays a crucial role in controlling the influx of neutrophils into the airways [31], controlling bronchial hyperresponsiveness through direct effects on bronchial smooth muscle cell contraction, and contributing to airway remodeling through goblet cell hyperplasia [22,31]. We further observed that MCP-1 is involved in the recruitment of monocytes [16].

In the present study, we demonstrated that our crude SCMAC extract reduced the total cell and eosinophil counts in BALF following Der p treatment. We speculated that our crude SCMAC extract reduced cell recruitment to the lungs by modulating the abilities of early-arriving cells to produce cytokines and chemokines. Th1, Th2, and Th17 immune responses are known to cause inappropriate responses such as autoimmune disease and eosinophil- and neutrophil-predominant asthma, respectively [32]. We used the expression levels of Th2, Th1, and Th17 cytokines in naïve group as baseline to compare the levels of Th2, Th1, and Th17 cytokines between naïve group, Der p group and SCMAC extract group. Those cytokines expression levels in Der p group

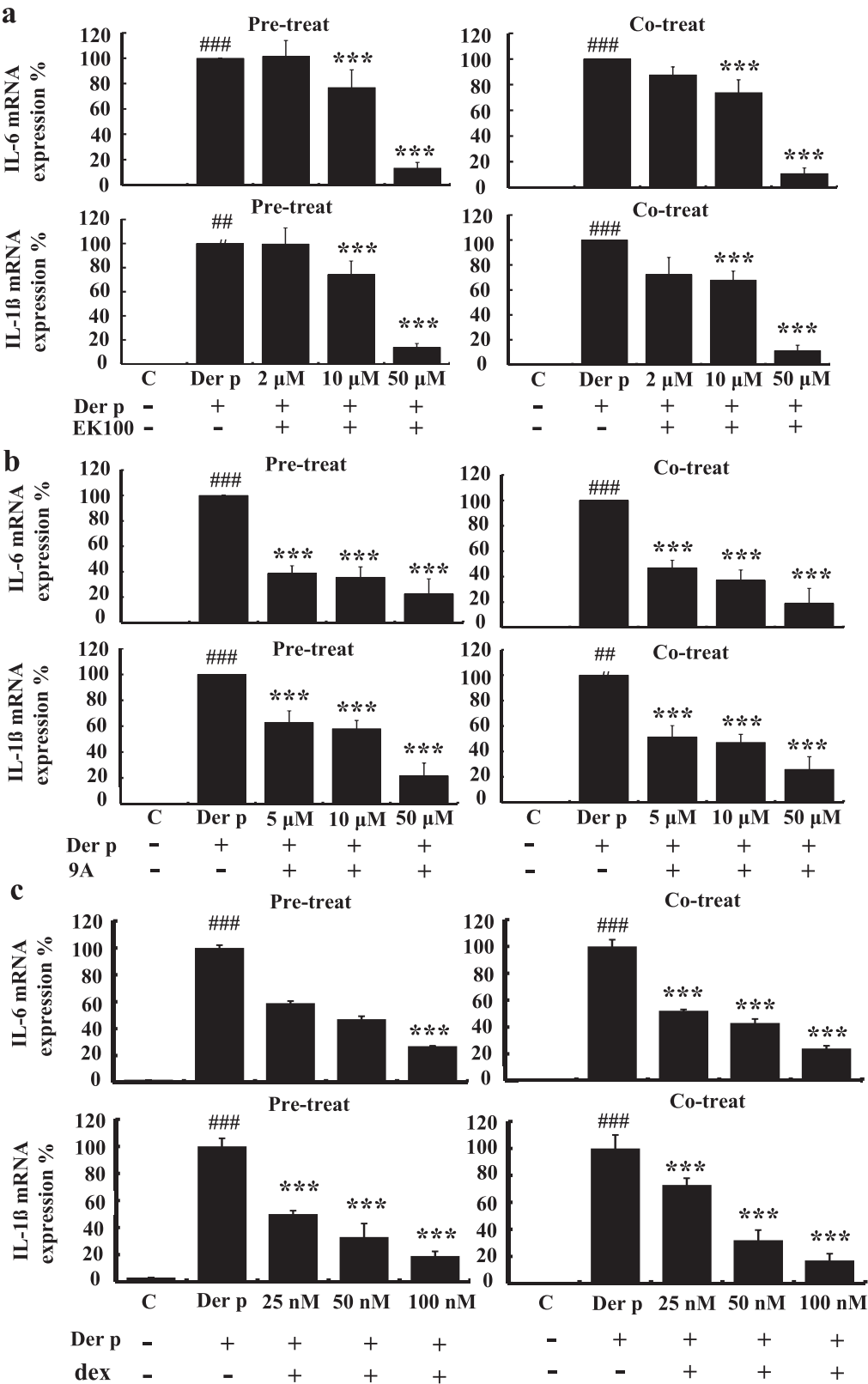


Fig. 6. Treatment with phytosterol suppresses IL-6 and IL-1 β gene expression in the MH-S alveolar macrophage cell line. (a) Der p-stimulated MH-S cells were treated with EK100 pretreatment or cotreatment. (b) Der p-stimulated MH-S cells were treated with 9A pretreatment or cotreatment. (c) Der p-stimulated MH-S cells were treated with dexamethasone pretreatment or cotreatment. Data were determined using real-time PCR. ### $P < 0.001$ (vs. control group); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (vs. Der p group and treated groups).

had higher than naïve group, but in SCMAC extract group those cytokines expression were reduced to close baseline. Therefore, we believe that our crude SCMAC extract could restore the critical Th1/Th2/Th17

balance by simultaneously reducing the levels of Th2 (IL-5 and IL-13), Th1 (IL-12 and IFN- γ), and Th17 cytokines (IL-17) (Figs. 4 and 5). Moreover, we speculated that levels of IL-5, IL-13, and IL-12 were

reduced close to those in naïve group treated with high doses of SMAC extract. The treatment could significantly reduce the influx of inflammation cells into BALF, in particular the eosinophils (Fig. 2b).

Asthma patients tend to have elevated levels of total and allergen-specific IgE compared with healthy individuals, and the degree of IgE elevation is associated with the incidence or severity of allergic asthma. IgE plays a role in asthma and is an important mediator in both the early and last phases of allergic response [33]. Therefore, the development of an immunological drug that interferes with IgE synthesis or inhibits its actions is a favorable approach. Omalizumab, an anti-IgE antibody, was approved solely for the clinical treatment of allergic disease in the USA and Europe [34,35]. Omalizumab could reduce the levels of AHR and airway remodeling [34,36]. Hence, we speculate that our crude SCMAC extract exhibits a similar function to omalizumab with respect to the abrogation of AHR (Fig. 2a) by a significant decrease in serum total IgE levels (Fig. 3a). We further observed that treatment with our crude extract led to a reduced Der p-IgG1/Der p-IgG2a/2b ratio (Fig. 3b).

The National Heart, Lung, and Blood Institute Expert Panel Report 3 recommends treatment with inhaled corticosteroids for asthma patients. Inhaled corticosteroids bind the glucocorticoid response element (GRE) of the glucocorticoid receptor (GR); this complex (GRE/GR) subsequently inhibits the transcriptional activity of several genes that encode proinflammatory proteins, including IL-1 β and IL-6 [38,39]. However, airway macrophages are primary responders to extracellular allergens and might secrete “first-wave” cytokines such as IL-1 β and IL-6 [24,40,41], thus inducing epithelial cells to release a second wave of cytokines to recruit inflammatory cells (e.g., eosinophils) and amplify inflammatory responses [24]. In our study, the two phytosterol compounds purified from our crude extract (EK100 and 9A) shared structural features with corticosteroids (Fig. 1). Because treatment with EK100 and 9A reduced the expression of IL-1 β and IL-6 in macrophages in a dose-dependent manner (Fig. 6), we speculated that these two compounds might function similarly to dexamethason. Moreover, SCMAC may contain other compounds that could regulate different cell types in the lung; we will evaluate this in the future.

In conclusion, in a Der p-induced asthma mouse model, our crude SCMAC extract reduced the expression of cytokines such as IL-6, IL-1 β , IL-5, and IL-13, possibly by suppressing TLR2 and TLR4 expression, leading to reduced inflammatory cell recruitment and activation in the lung. Our crude SCMAC extract also significantly inhibited the production of total IgE while reducing its action associated with allergic asthma pathogenesis. These findings suggest that this crude SCMAC extract could be used to alleviate asthma symptoms such as AHR and inflammatory cell inflammation in the lungs. We speculate that this crude extract likely contains many pharmacologically active compounds that exhibit synergistic effects on multiple targets, thus relieving allergic asthma symptoms. EK100 and 9A, which were purified from the crude extract, were found to inhibit IL-6 and IL-1 β expression in macrophages, similar to the action of corticosteroids. Therefore, the isolation and identification of active compounds that contribute to the anti-inflammatory and AHR inhibitory effects of this crude extract will improve understanding of the mechanisms underlying the effects of SCMAC on asthma symptoms.

5. Notes

The authors declare no competing financial interests.

Acknowledgments

This work was supported by grants from the National Science Council, Taiwan; the Republic of China (MOST 103-2320-B-039-018) and the China Medical University under the Aim for Top University Plan of the Ministry of Education, Taiwan (CHM106-5-2), and Taiwan Ministry of Health and Welfare Clinical Trial Center, Taiwan

(MOHW106-TDU-B-212-113004).

References

- [1] H. Hammad, B.N. Lambrecht, Dendritic cells and airway epithelial cells at the interface between innate and adaptive immune responses, *Allergy* 66 (2011) 579–587.
- [2] S.E. Wenzel, Asthma phenotypes: the evolution from clinical to molecular approaches, *Nat. Med.* 18 (2012) 716–725.
- [3] S.T. Holgate, R. Djukanović, T. Casale, J. Bousquet, Anti-immunoglobulin E treatment with omalizumab in allergic diseases: an update on anti-inflammatory activity and clinical efficacy, *Clin. Exp. Allergy* 35 (2005) 408–416.
- [4] X.M. Li, C.K. Huang, T.F. Zhang, et al., The Chinese herbal medicine formula MSSM-002 suppresses allergic airway hyperreactivity and modulates T_H1/T_H2 responses in a murine model of allergic asthma, *J. Allergy Clin. Immunol.* 106 (2000) 660–668.
- [5] J. Bousquet, C. Bachert, G.W. Canonica, et al., Unmet needs in severe chronic upper airway disease (SCUAD), *J. Allergy Clin. Immunol.* 124 (2009) 428–433.
- [6] C.A. Slader, H.K. Reddel, C.R. Jenkins, et al., Complementary and alternative medicine use in asthma: who is using what? *Respirology* 11 (2006) 373–387.
- [7] X.M. Li, L. Brown, Efficacy and mechanisms of action of traditional Chinese medicines for treating asthma and allergy, *J. Allergy Clin. Immunol.* 123 (2009) 297–306.
- [8] P.Y. Yue, Y.Y. Wong, T.Y. Chan, et al., Review of biological and pharmacological activities of the endemic Taiwanese bitter medicinal mushroom *Antrodia camphorata* (M. Zang et C. H. Su) Sh. H. Wu et al higher Basidiomycetes, *Int. J. Med. Mushrooms* 14 (2012) 241–256.
- [9] S.C. Chien, M.L. Chen, H.T. Kuo, et al., Anti-inflammatory activities of new succinic and maleic derivatives from the fruiting body of *Antrodia camphorata*, *J. Agric. Food Chem.* 56 (2008) 7017–7022.
- [10] D.Z. Liu, H.J. Liang, C.H. Chen, et al., Comparative anti-inflammatory characterization of wild fruiting body, liquid-state fermentation, and solid-state culture of *Taiwanofungus camphoratus* in microglia and the mechanism of its action, *J. Ethnopharmacol.* 113 (2007) 45–53.
- [11] M. Geethangili, Y.M. Tzeng, Review of pharmacological effects of *antrodia camphorata* and its bioactive compounds, *Evid. Based Complement Alternat. Med.* 2011 (2011) 212641.
- [12] R.A. Othman, M.H. Moghadasian, Beyond cholesterol-lowering effects of plant sterols: clinical and experimental evidence of anti-inflammatory properties, *Nutr. Rev.* 69 (2011) 371–382.
- [13] S.K. Jung, J. Ra, J. Seo, et al., An Angiotensin I converting enzyme polymorphism is associated with clinical phenotype when using differentiation-syndrome to categorize Korean bronchial asthma patients, *Evid. Based Complement Alternat. Med.* 2011 (2011) 498138.
- [14] S.P. Commins, L. Borish, J.W. Steinke, Immunologic messenger molecules: cytokines, interferons, and chemokines, *J. Allergy Clin. Immunol.* 125 (2010) S53–S72.
- [15] C.F. Liu, Y.L. Chen, W.T. Chang, et al., Mite allergen induces nitric oxide production in alveolar macrophage cell lines via CD14/toll-like receptor 4, and is inhibited by surfactant protein D, *Clin. Exp. Allergy* 35 (2005) 1615–1624.
- [16] L.J. Lin, C.C. Lin, S.D. Wang, et al., The immunomodulatory effect of You-Gui-Wan on dermatogoides-pteronyssinus-Induced Asthma, *Evid. Based Complement Alternat. Med.* 2012 (2012) 476060.
- [17] Y.T. Tung, T.C. Tsai, Y.H. Kuo, et al., Comparison of solid-state-cultured and wood-cultured *Antrodia camphorata* in anti-inflammatory effects using NF-kappaB/luciferase inducible transgenic mice, *Phytomedicine* 21 (2014) 1708–1716.
- [18] Y. Takaishi, Y. Murakami, T. Ohashi, et al., Three triterpenes from *Astragalus hygrometricus*, *Phytochemistry* 26 (1987) 4.
- [19] G.J. Huang, S.S. Huang, S.S. Lin, et al., Analgesic effects and the mechanisms of anti-inflammation of ergostatrien-3beta-ol from *Antrodia camphorata* submerged whole broth in mice, *J. Agric. Food Chem.* 58 (2010) 7445–7452.
- [20] Z. Dong, L. Xiong, W. Zhang, et al., Holding the inflammatory system in check: TLRs and their targeted therapy in asthma, *Med. Inflamm.* 2016 (2016) 2180417.
- [21] L. Zuo, K. Lucas, C.A. Fortuna, et al., Molecular regulation of toll-like receptors in asthma and COPD, *Front. Physiol.* 6 (2015) 312.
- [22] J.P. McAleer, J.K. Kolls, Directing traffic: IL-17 and IL-22 coordinate pulmonary immune defense, *Immunol. Rev.* 260 (2014) 129–144.
- [23] P. Cristofaro, S.M. Opal, Role of toll-like receptors in infection and immunity: clinical implications, *Drugs* 66 (2006) 15–29.
- [24] Y. He, S. Peng, W. Xiong, et al., Association between polymorphism of interleukin-1 beta and interleukin-1 receptor antagonist gene and asthma risk: a meta-analysis, *Sci. World J.* 2015 (2015) 685684.
- [25] J.M. Schuh, K. Bleasle, S.L. Kunkel, C.M. Hogaboam, Chemokines and cytokines: axis and allies in asthma and allergy, *Cytokine Growth Factor Rev.* 14 (2003) 503–510.
- [26] K.F. Chung, P.J. Barnes, Cytokines in asthma, *Thorax* 54 (1999) 825–857.
- [27] M.E. Rothenberg, S.P. Hogan, The eosinophil, *Annu. Rev. Immunol.* 24 (2006) 147–174.
- [28] P.S. Foster, A.W. Mould, M. Yang, et al., Elemental signals regulating eosinophil accumulation in the lung, *Immunol. Rev.* 179 (2001) 173–181.
- [29] R.T. Venkatesha, E. Berla Thangam, A.K. Zaidi, H. Ali, Distinct regulation of C3a-induced MCP-1/CCL2 and RANTES/CCL5 production in human mast cells by extracellular signal regulated kinase and PI3 kinase, *Mol. Immunol.* 42 (2005) 581–587.
- [30] M. Rincon, C.G. Irvin, Role of IL-6 in asthma and other inflammatory pulmonary diseases, *Int. J. Biol. Sci.* 8 (2012) 1281–1290.
- [31] M.L. Manni, K.M. Robinson, J.F. Alcorn, A tale of two cytokines: IL-17 and IL-22 in asthma and infection, *Expert Rev. Respir. Med.* 8 (2014) 25–42.

- [32] G. Pelaia, A. Vatrella, M.T. Busceti, et al., Cellular mechanisms underlying eosinophilic and neutrophilic airway inflammation in asthma, *Med. Inflamm.* 2015 (2015) 879783.
- [33] K. Samitas, V. Delimpoura, E. Zervas, M. Gaga, Anti-IgE treatment, airway inflammation and remodelling in severe allergic asthma: current knowledge and future perspectives, *Eur. Respir. Rev.* 24 (2015) 594–601.
- [34] L.K. James, The cloning and expression of human monoclonal antibodies: implications for allergen immunotherapy, *Curr. Allergy Asthma Rep.* 16 (2016) 15.
- [35] K.F. Chung, Asthma phenotyping: a necessity for improved therapeutic precision and new targeted therapies, *J. Intern. Med.* 279 (2016) 192–204.
- [36] J.Y. Kang, J.W. Kim, J.S. Kim, et al., Inhibitory effects of anti-immunoglobulin E antibodies on airway remodeling in a murine model of chronic asthma, *J. Asthma* 47 (2010) 374–380.
- [37] J.L. Trevor, J.S. Deshane, Refractory asthma: mechanisms, targets, and therapy, *Allergy* 69 (2014) 817–827.
- [38] G. Pelaia, A. Vatrella, G. Cuda, et al., Molecular mechanisms of corticosteroid actions in chronic inflammatory airway diseases, *Life Sci.* 72 (2003) 1549–1561.
- [39] K. Pappas, A.I. Papaioannou, K. Kostikas, N. Tzanakis, The role of macrophages in obstructive airways disease: chronic obstructive pulmonary disease and asthma, *Cytokine* 64 (2013) 613–625.
- [40] C.F. Liu, D. Drocourt, G. Puze, J.Y. Wang, et al., Innate immune response of alveolar macrophage to house dust mite allergen is mediated through TLR2/-4 co-activation, *PLoS One* 8 (2013) e75983.