

Uncovering the Molecular Mechanism of Anti-Allergic Activity of Silkworm Pupa-Grown *Cordyceps militaris* Fruit Body

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Abstract: *Cordyceps militaris* has been widely used as an herbal drug and tonic food in East Asia and has also been recently studied in the West because of its various pharmacological activities such as antitumoral, anti-inflammatory and immunomodulatory effects. In this study, we examined the molecular mechanism underlying the anti-allergic activity of ethanol extract prepared from silkworm pupa-cultivated *Cordyceps militaris* fruit bodies in activated mast cells. Our results showed that ethanol extract treatment significantly inhibited the release of β -hexosaminidase (a degranulation marker) and mRNA levels of tumor necrosis factor- α as well as interleukin-4 in RBL-2H3 cells. The cells were sensitized with 2,4-dinitrophenol specific IgE and then stimulated with human serum albumin conjugated with 2,4-dinitrophenol. Oral administration of 300 mg/kg ethanol extract significantly ameliorated IgE-induced allergic reaction in mice with passive cutaneous anaphylaxis. Western immunoblotting results demonstrated that ethanol extract incubation significantly inhibited Syk/PI3K/MEKK4/JNK/c-jun biochemical cascade in activated RBL-2H3 cells, which activated the expression of various allergic cytokines. In addition, it suppressed Erk activation and PLC γ evocation, which would respectively evoke the synthesis of lipid mediators and Ca²⁺ mobilization to induce degranulation in stimulated RBL-2H3 cells. A compound, identified as β -sitostenone, was shown to inhibit β -hexosaminidase secretion from activated mast cells. Our study demonstrated that ethanol extract contained the ingredients, which could inhibit immediate degranulation and *de novo* synthesis of allergic lipid mediators and cytokines in activated mast cells.

Keywords: *Cordyceps militaris*; Allergy; IgE Antibody; Passive Cutaneous Anaphylaxis (PCA); JNK.

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Introduction

An allergy is a type I hypersensitivity disorder mediated by an impaired immunological mechanism and can cause allergic diseases including atopic dermatitis, allergic rhinitis, asthma and atopic eczema (Galli and Tsai, 2012). An allergy is caused by the excessive activation of mast cells or basophils by a specific IgE antibody against an allergen. Allergens are normally harmless environmental substances, such as certain food, dust, mites and pollen. They evoke the production of antigen-specific IgE antibodies that bind to high affinity IgE receptors (FcεRI) on the surface of mast cells or basophils (Galli and Tsai, 2012). The cross-linkage of IgE molecules bound to FcεRI by allergens induces FcεRI aggregation and the immediate release of preformed inflammatory mediators from the granules via degranulation. These mediators include histamine, leukotriene C₄, β-hexosaminidase, chemokines and cytokines, which are responsible for allergic symptoms. IgE/allergen-activated mast cells also release lipid-derived mediators, such as prostaglandin D₂, leukotriene B₄ and cysteinyl leukotriene, in addition to various kinds of newly synthesized cytokines, chemokines and growth factors (Galli and Tsai, 2012). The aggregation of FcεRI by allergens causes Lyn to activate spleen tyrosine kinase (Syk) via phosphorylation. Activated Syk evokes the Ras/Raf1/Erk 1/2 pathway through LAT activation, which then modulates the synthesis of lipid mediators and cytokines. In addition, activated Syk can provoke the Rac/MEKK1/JNK pathway, which regulates *de novo* synthesis of cytokines. The activated Syk can also stimulate phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) to induce phospholipase C-γ (PLC-γ) via the direct phosphorylation or activation of B cell progenitor tyrosine kinase (BTK). This stimulated PLC-γ leads to either the release of Ca²⁺ from the endoplasmic reticulum of the cells or to an influx from outside to increase Ca²⁺ concentration in the cytoplasm. Both cause degranulation in mast cells (Galli and Tsai, 2012).

Cordyceps militaris (L.) Link is a popular traditional Chinese medicinal fungus and has been widely used as an herbal drug and a tonic food in East Asia. It has been studied for its various pharmacological activities. A variety of extracts isolated from *C. militaris* are shown to have various anticancer capabilities based upon various cell-based models such as breast cancer (Jin *et al.*, 2008), colon cancer (Mollah *et al.*, 2012) and lung cancer (Park *et al.*, 2009), and are examined to exhibit antimelanoma effectiveness in both cell and xenografted animal models (Ruma *et al.*, 2014). *C. militaris* extracts also have the efficacy in anti-allergy (Oh *et al.*, 2011), immunomodulatory (Han *et al.*, 2011), anti-inflammatory (Won and Park, 2005) and anti-asthma (Hsu *et al.*, 2008) cases. Recently, Hwang *et al.* (2016) reported that WIB801C, the cordycepin standardized extract of *C. militaris*, exhibits the alleviation ability of post-ischemia inflammation to improve the neurological deficit and survival rate of rats with cerebral ischemic stroke. They manifest a decreased infiltration of microglia as well as inflammatory cells, decreased infarct volume, cerebral edema and blood-brain barrier damages.

In this study, we examined the anti-allergic activity of silkworm pupa-cultured *C. militaris* fruit bodies respectively in allergen-stimulated RBL2H3 cells and in mice with passive cutaneous anaphylaxis (PCA). Our results showed that treatment of ethanol

extract of fruit bodies could suppress degranulation and *de novo* synthesis of IL-4 as well as TNF- α via the inhibition of Fc ϵ RI signaling events. Further animal studies demonstrated that administration of ethanol extract could alleviate the allergic response of PCA mice.

Materials and Methods

Materials

Minimum essential medium and fetal bovine serum were purchased from GIBCO (Life technologies), Grand Island, NY, USA; Tris base, DTT, NaCl, PMSF, SDS, β -mercaptoethanol and bromophenol blue were acquired from aMResco, Solon, OH, USA; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, glucose, Na₂CO₃, EDTA and Triton X-100 were bought from Merck Millipore, Darmstadt, Germany; DMSO, DNP specific IgE antibody, DNP-HSA, Evans blue, NaHCO₃, p-nitrophenyl-N-acetyl- β -D-glucosaminide, and bovine serum albumin were purchased from Sigma-Aldrich, St. Louis, MO, USA; PIPES, MgCl₂·6H₂O and sucrose were from Avantor Performance Materials, Center Valley, PA, USA; KCl was from Scharlab, Sentmenat, Barcelona, Spain; DTT was from the USB corporation, Cleveland, OH, USA; Glycerol was from Kanto Chemical, Oregon, USA; SYBR Green master mix kit was from Bio-Genesis, Westminster, CO, USA; RNeasy Mini kit was from Qiagen, MD, USA; high-capacity cDNA reverse transcription kit was from Applied Biosystems, Waltham, MA, USA; PVDF membrane was from Stratagene, La Jolla, CA, USA; Chemiluminescence ECL detection system was from GE Healthcare Bio-Sciences AB, Uppsala, Sweden; ELISA kit for hexosaminidase B β was from Cloud-Clone Corp., Houston, TX; phospho-Syk (1: 1000), phospho-Erk (1: 2000) and phospho-JNK (1: 1000) antibodies were from Cell Signaling Technology, Danvers, MA, USA; phospho-PLC- γ (1: 1000) and phospho-Akt (1: 1000) antibodies were from Santa Cruz Biotechnology, Dallas, Texas, USA; phospho-LAT antibody (1: 1000) was from St John's Laboratory, London, United Kingdom; PI3K antibody (1: 1000) was from Epitomics, Burlingame, CA, USA; MEKK4 antibody (1: 1000) was from Upstate, Dublin, OH, USA and actin antibody (1: 5000) was from GeneTex, Irvine, CA, USA.

Preparation of the Ethanol Extract of *C. militaris* (L.) Link Fruit Body (CM-EE)

Silkworm pupa-cultivated *C. militaris* (L.) Link fruit bodies were purchased from Phytomedicine Biotechnology Co. LTD of Nan Pao Resins Chemical Co. LTD group as indicated in Supplemental Fig. S1. The Food Industry Research and Development Institute of Taiwan has identified the fungus strains obtained from Phytomedicine Biotechnology Co. LTD as *Cordyceps militaris* (L.) Link. *C. militaris* fruit bodies were ground into powder and then 50 g of resultant powder was mixed with 95% ethanol at a ratio of 1:10 (w/v) and soaked for 18 h at room temperature with shaking. After incubation, the suspension was

filtered through filter paper and then the filtrate was vacuum-dried to produce 13.16 g of brown syrup designated as CM-EE and stored at -80°C for future use. Cordycepin was measured by liquid chromatography tandem mass spectrometry provided by the service of ABM International Lab Inc., Taiwan.

Analysis of CM-EE by High Performance Liquid Chromatography (HPLC) and Gas Chromatography/Mass Spectrometry (GC/MS)

The HPLC system consisted of a Hitachi liquid chromatography equipped with one pump (Hitachi 5110), an auto sampler injector (ChromTech) and an UV photodiode array detection (Hitachi 5430). The chromatographic separation was performed with Inertsil[®] ODS-3 column (250 mm $\times$ 4.6 mm, 5 μm) (GL Sciences Inc., Tokyo, Japan) to dissect the ethanol extract. The extract was eluted at the flow rate of 0.4 ml/min for 165 min using the 0.1% (v/v) trifluoroacetic acid (TFA)/acetonitrile (ACN) gradient system (v/v) with ACN volume increased 5% every 5 min until reaching 80% of ACN and 20% of TFA. The injection volume was 20 μl . The detection wavelength was set at 260 nm for acquiring chromatograms.

The chemical composition of CM-EE was analyzed by GC/MS using a SHIMADZU QP2010 GC/MS with a DB-5 column (30 m, film 0.25 μm , ID 0.25 mm). The temperature of the column was programmed from 45°C to 270°C at $5^{\circ}\text{C}/\text{min}$ and the injector or detector temperature for the analysis was about 250°C . Helium was used as the carrier gas at a flow rate of 0.82 ml/min. The mass spectrometer was operated in its electron-impact ionization (EI) mode.

Identification of β -Sitosterone from Ethylacetate Layer of CM-EE

CM-EE was suspended in water and then partitioned with ethylacetate (EtOAc) at the ratio of 1:1. After EtOAc layer was vacuum dried, the EtOAc-soluble portion was fractionated using column chromatography packed with silica gel (230–400 mesh; Merck) and eluting with dichloromethane–diisopropyl ether (49:1) to afford four fractions. Among the acquired fractions, crystallized compounds T1 and T3 could be obtained from fractions 1 and 3, respectively. The ^1H NMR spectrum was measured using a Bruker AMX-400 spectrometer with TMS as the internal reference, and chemical shifts were expressed in δ (ppm). The compound T1 was a colorless powder with ^1H NMR (CDCl_3 , 400 MHz) as follows: 5.72 (1H, brs, H-4), 1.18 (3H, s, H-19), 0.91 (3H, d, $J = 6.5$ Hz, H-21), 0.86 (3H, t, $J = 7.2$ Hz, H-29), 0.83 (3H, d, $J = 7.2$ Hz, H-26), 0.81 (3H, d, $J = 6.9$ Hz, H-27), 0.71 (3H, s, H-18), and was further identified as β -sitosterone (Fig. 2B) through the comparison of analytical and spectroscopic data collected from experiments with those published findings.

EtOAc layers were also analyzed using HPLC. The HPLC system consisted of a Hitachi liquid chromatography equipped with one pump (Hitachi L-2130), an autosampler injector (Hitachi L-2200) and UV photodiode-array detection (Hitachi L-2450). The gradient elution was performed on a Inertsil ODS-3 column (250 mm $\times$ 4.6 mm, 5 μm , GL

Sciences) using water and acetonitrile as the mobile phase at a flow rate of 1 ml/min. The gradient (v/v) was formed as follows: 40% of acetonitrile to 90% in 30 min, to 100% at 40 min and finally 100% of acetonitrile at 75 min. The injection volume was 20 μ l. The detection wavelength was set at 210 nm for acquiring chromatograms. In addition, β -sitostenone was also detected in EtOAc layer using thin layer chromatography coated with silica gel 60 F254 (Merck).

Cell Line

Rat basophilic leukemia-2H3 (RBL-2H3) cells were purchased from American Type Culture Collection (ATCC) and cultured in minimum essential medium supplemented with 15% (v/v) fetal bovine serum (FBS) at 37°C with 5% CO₂ in a humidified environment. They have been used extensively to study Fc ϵ RI and the biochemical pathways for secretion in mast cells.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

A 96-well plate was seeded with 1×10^4 RBL-2H3 cells per well. After cultivation for the indicated time period, 20 μ l of MTT solution (5 mg/ml PBS) was added to each well and the plate was incubated at 37°C for 4 h. After medium removal, 200 μ l of DMSO was applied to each well and the plate was shaken gently for 5 min. The absorbance was measured at 540 nm. Quadruplicate wells were used for a specific time period.

β -Hexosaminidase Secretion Assay

A 24-well plate was seeded with 4×10^5 cells per well and RBL-2H3 cells were sensitized with 2 μ g/ml of 2,4-dinitro-phenol (DNP) specific IgE antibody overnight. Sensitized cells were then washed twice with PIPES buffer [25 mM PIPES, 119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM MgCl₂·6H₂O, 1% (w/v) BSA, pH 7.2] and treated in PIPES buffer with or without CM-EE (50 or 100 μ g/ml) for 30 min at 37°C. For the 1T or the 3T compound, 100 μ g/ml of each compound was used in the test. For inhibitor experiments, 10 μ M PP2 or 30 μ M SP600125 was added to replace CM-EE or combined with CM-EE. After incubation, cells were challenged with 100 ng/ml of human serum albumin conjugated with DNP (DNP-HSA) for 2 h at 37°C. After activation, 30 μ l of the supernatant was mixed with an equal volume of 2 mM substrate solution (p-nitrophenyl-N-acetyl- β -D-glucosaminide in 0.1 M citrate, pH 4.5) and incubated for 1 h at 37°C. The reaction was stopped by adding 250 μ l stop buffer (0.1 M Na₂CO₃/NaHCO₃, pH 10.0). The absorbance was measured with a microplate reader at 405 nm (BioTEK Instrument, Inc., Winooski, VT, USA) and analyzed with a Student's t-test (STATISTICA, StatSoft, Tulsa, OK, U.S.A.). Triplicate wells were exploited for each sample. Conditioned media of activated RBL2H3 cells were also implemented for ELISA kit for hexosaminidase β to quantify the enzyme according to the protocol suggested by the manufacturer. For western

immunoblotting, after appropriate CM-EE treatment and stimulation, cells were collected to detect proteins of interest.

Western Immunoblotting

After treatment, RBL2H3 cells were harvested and lysed in lysis buffer [10 mM Tris (pH 8.0), 0.32 M sucrose, 1% (v/v) Triton X-100, 5 mM EDTA, 2 mM DTT, and 1 mM PMSF]. After determining its protein concentration using Bio-Rad DC protein assay kit, equal volume of 2× sample buffer [0.1 M Tris (pH 6.8), 2% (w/v) SDS, 0.2% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, and 0.0016% (w/v) bromophenol blue] was combined with the protein lysate. Appropriate amounts of the lysates were separated by electrophoresis at 100 V with 10% (w/v) SDS-PAGE, and further transferred onto a PVDF membrane. After blocking for 1 h in 3% (w/v) bovine albumin serum (BSA) at room temperature, membranes were hybridized overnight at room temperature with the primary antibodies listed in “Materials”. The membranes were washed and probed with suitable secondary antibodies for 1 h at room temperature. Secondary antibodies binding on the membrane were detected by the chemiluminescence ECL detection system using Image-Quant LAS 4000 (Fujifilm super CCD area type chip) (GE Healthcare Life Sciences, Pittsburgh, PA, USA). The intensity of each protein band, normalized with actin expression level, was quantified by PDQUEST Quantity One software (Bio-Rad Laboratory, Hercules, CA) and analyzed with Student’s t-test (STATISTICA, StatSoft, Tulsa, OK, U.S.A.).

Reverse Transcriptase-Real Time Polymerase Chain Reaction (RT- Real Time PCR)

A 24-well plate was seeded with 4×10^5 RBL-2H3 cells per well and then cells were sensitized/challenged as described above. Total RNA was isolated from RBL2H3 cells, IgE-sensitized cells and mock, 50 µg/ml or 100 µg/ml CM-EE-treated IgE/DNP-HSA activated cells using a RNeasy Mini kit in accordance with the manufacturer’s suggestion. cDNA was synthesized by using a high-capacity cDNA reverse transcription kit following the manufacturer’s protocol. Real-time PCR assays to quantify mRNA abundance of rat tumor necrosis factor α (TNF-α), rat interleukin-4 (IL-4) and rat c-jun were performed with Applied Biosystems 7500 Real-Time PCR System using the following primers : rat TNF-α (forward, 5'-AACTCGAGTGACAAGCCCGTAG-3'; reverse, 5'-GTACCACCAGTTGG TTGT CTTTGA-3') and rat IL-4 (forward, 5'-ACCTTGCTGTCACCCTGTTC-3'; reverse, 5'-TTGTGAGCGTGGACTIONCATTC-3') and rat c-jun (forward, 5'-GCGATTG AAGA GTGGAAAGAAC-3'; reverse, 5'-TGGATGACGACTGAGAAGGAG-3'). Rat GAPDH primer (forward 5'-CCCCAATGTATCCGTTGTG-3'; reverse 5'-TAGCCC AGGATGCCCTTTAGT-3') was used as an internal control. 2 µl of cDNA was exploited in each real time PCR reaction using SYBR Green master mix kit with 25 µM of each primer. PCR amplifications were performed for one cycle at 50°C for 2 min and 95°C for 10 min, 50 cycles at 95°C for 15 s, 50°C for 10 s and 60°C for 40 s and at last one cycle at 95°C for 15 s, 50°C for 1 min and 95°C for 15 s. C_T Data were collected at the end of 50 cycles. Relative quantitative fold changes compared with control were calculated using

the comparative C_T method. C_T is the cycle number at which fluorescence intensity first exceeds the threshold level. $\Delta C_T = C_T$ (TNF- α or IL-4) - C_T (GAPDH). $\Delta\Delta C_T$ is ΔC_T (CM-EE-incubated activated RBL2H3) - ΔC_T (DMSO-treated activated RBL2H3). $2^{-[\Delta\Delta C_T]}$ was expressed as a fold difference of TNF- α or IL-4 gene expression of CM-EE-nurtured irritated RBL2H3 cells compared to that of DMSO-treated stimulated cells. $2^{-[\Delta\Delta C_T]}$ data were analyzed with Student's t-test (STATISTICA, StatSoft, Tulsa, OK, U.S.A.).

Passive Cutaneous Anaphylaxis (PCA)

The animal study was carried out in accordance with the institutional guidelines adhering to internationally accepted principles for laboratory animal use and care with admission number 104-4 [The Institutional Animal Care and Use Committee (IACUC) at Southern Taiwan University of Science and Technology (Tainan, Taiwan)]. Male Balb/c mice were supplied by the National Laboratory Animal Center, Taiwan. New arrival of male Balb/c mice (aged five weeks) were fostered in cages for one week with a temperature of $23 \pm 2^\circ\text{C}$ and a relative humidity of 55% for environmental accommodation prior to the experiment. Mice were divided into four subgroups as follows: anti-DNP IgE sensitized mice ($N = 7$), DNP-HSA challenged/anti-DNP IgE sensitized mice fed with virgin olive oil ($N = 4$), DNP-HSA/IgE mice administrated with 300 mg/ml ($N = 7$) or 500 mg/ml ($N = 7$) CM-EE. For DNP-HSA/IgE activated mice, BALB/c mice (aged six weeks) were intravenously injected with 250 μg DNP-HSA dissolved in 250 μl PBS containing 4% (w/v) Evans blue 24 h after the intradermal injection of 0.5 μg anti-DNP IgE into one ear per mouse. To measure the inhibitory activity in the animals, each activated mouse was orally fed with 300 mg/kg or 500 mg/kg CM-EE dissolved in virgin olive oil 1 h before DNP-HSA administration. Activated mice fed with virgin olive oil were deemed as vehicle control. Mice were euthanized 1 h after stimulation with allergen and irritated ears were excised in order to measure the amount of dye extravasated. The dye was extracted from the ear soaked in 700 μl formamide at 63°C overnight. The absorbance was measured with a microplate reader (BioTEK Instrument, Inc., Winooski, VT, USA) at 620 nm. Absorbance results were analyzed with the One Way Anova Tukey test (SigmaPlot version 12.0, San Jose, CA, U.S.A.). Mice sensitized only with IgE were challenged with PBS as DNP-HSA vehicle.

Results

Impact of CM-EE on the Degranulation From Antigen-Stimulated RBL-2H3 Cells

To investigate the anti-allergy activities of CM-EE, we first examined the inhibitory activity of antigen-stimulated RBL2H3 cells on degranulation. The secretion of β -hexosaminidase from activated RBL-2H3 into medium is seen as an index of degranulation (Oh *et al.*, 2011). The results of the β -hexosaminidase release assay showed that treatment of antigen-stimulated RBL-2H3 cells with 50 $\mu\text{g}/\text{ml}$ or 100 $\mu\text{g}/\text{ml}$ CM-EE could inhibit the release of

β -hexosaminidase via degranulation (Fig. 1A). Further results of the MTT assay showed that RBL-2H3 cells were not harmed by CM-EE treatment (Fig. 1C), indicating that the anti-allergic activity of CM-EE did not come from the inhibition of cell proliferation.

In order to get insight into the constituents present in CM-EE, first the typical and primary ingredient of *C. militaris* fruit body, cordycepin, was evaluated as described in “Materials and Methods” and 269 ppm cordycepin was detected in CM-EE (data not shown). Furthermore, HPLC analytic results of CM-EE demonstrated that most compounds in CM-EE were present in the medium polar area [retention time (RT) from 23 min to 70 min] while few compounds were located in hydrophobic region (RT from 150 min to 160 min and 179 min) (Fig. 2A). GC/MS was also used to evaluate the composition of CM-EE. As indicated in Table 1, the compounds present in CM-EE were fatty acids, esters, ketones etc., which were highly consistent with HPLC results.

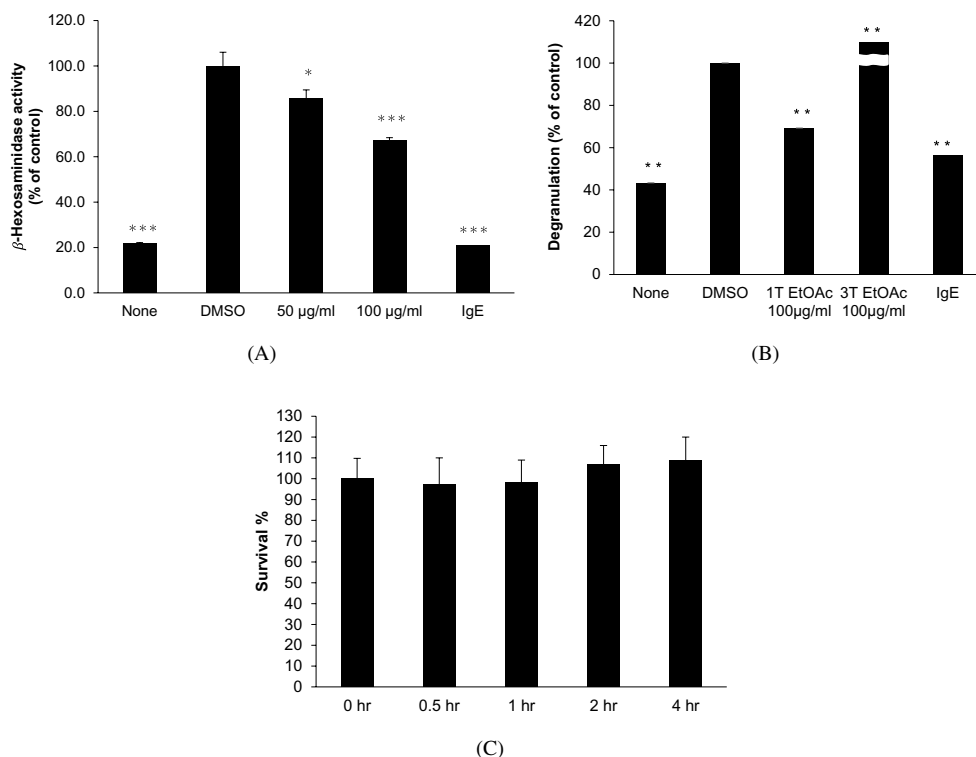


Figure 1. Effects of CM-EE or 1T as well as 3T compounds on β -hexosaminidase release from DNP-HSA/DNP specific IgE activated RBL2H3 cells. The β -hexosaminidase activity in conditioned medium was measured as described in “Materials and Methods” and was released from DNP-HSA stimulated/anti-DNP IgE sensitized RBL2H3 cells treated with various amounts of CM-EE (A) or 1T compound (B). The diagram was the typical result of three independent experiments except that two independent experiments were carried out in (B). (C) MTT assay of CM-EE treated activated RBL2H3 cells. MTT assay was carried out as indicated in “Materials and Methods.” Data were expressed as mean $\pm$ S.D. *, ** and *** represented $P < 0.05$ and $P < 0.01$ and $P < 0.001$ respectively as compared to DMSO control using Student’s t-test.

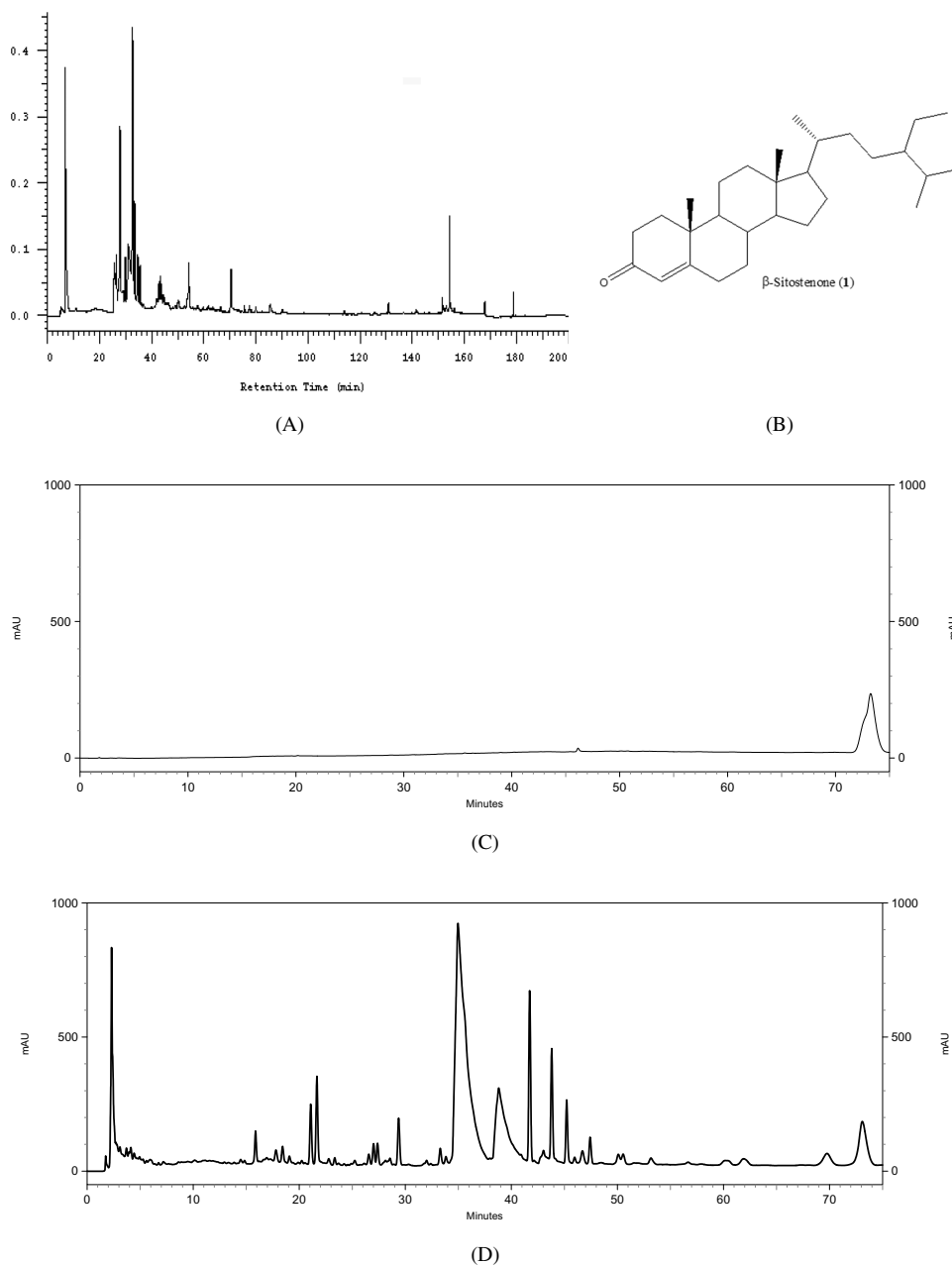


Figure 2. HPLC profiles of CM-EE and EtOAc layer. HPLC was carried out as described in “Materials and Methods.” (A) CM-EE. (B) The chemical structure of β -sitosterone. (C) β -sitosterone. (D) EtOAc layer.

Table 1. Compounds Present in CM-EE Identified by GC/MS

Constituents	Peak Area (%) ^a
6-Pentyl-5,6-dihydro-2H-pyran-2-one	0.61
3-Nitrobenzyl alcohol	6.01
Ethyl 9-hexadecenoate	0.30
Ethyl palmitate	9.29
Palmitic acid;	3.35
Stearic acid, ethyl ester	19.36
(9Z)-9,17-Octadecadienal	22.07
Cholesterol	4.05

^aTotally 21 peaks were separated in GC/MS. Only the compounds were listed, which have an identification score (SI) > 90.

Effects of CM-EE on the Secretion of Pro-Inflammatory Cytokines from IgE/Allergen Agitated RBL-2H3 Cells

TNF- α and IL-4 pro-inflammatory cytokines secreted from antigen-evoked mast cells via degranulation or *de novo* synthesis play key roles in allergic responses. Thus, we investigated whether CM-EE treatment could modulate TNF- α and IL-4 gene expression in DNP-IgE/DNP-HSA-activated RBL-2H3 cells. Results from a RT real time-PCR demonstrated that CM-EE nurturing could significantly decrease mRNA synthesis of TNF- α and IL-4 genes in irritated RBL-2H3 cells (Figs. 3A and 3B).

Impacts of CM-EE on the Fc ϵ RI-Triggered Signaling Pathway in IgE/Allergen Irritated RBL-2H3 Cells

Aggregation of Fc ϵ RI by allergen activates Syk to evoke a Ras/Raf1/Erk 1/2 pathway in order to increase the synthesis of lipid mediators and cytokines in mast cells. Activated Syk can also regulate *de novo* synthesis of various cytokines through the Rac1/MEKK4/JNK pathway. Moreover, activated Syk can augment Ca²⁺ concentration in the cytoplasm to stimulate degranulation via PI3K and PLC- γ (Galli and Tsai, 2012). Results from the signaling pathway investigation revealed that, in CM-EE-nurtured DNP-IgE/DNP-HSA activated RBL-2H3 cells, p-LAT expression was inhibited (Fig. 4A) and afterwards p-Syk level was decreased (Fig. 4B). In line with the above observation, diminished p-Syk amount inhibited the expression levels of PI3K, MEKK4 and p-JNK (Figs. 4C–4E). Furthermore, the amount of cytoplasmic (inactive form) and nuclear c-jun (the active form, a p-JNK immediate downstream effector) were also reduced (Fig. 4F), which suggested that c-jun expression as well as translocation might be interrupted and *de novo* synthesis of various cytokines was inhibited in DNP-HSA/IgE evoked RBL-2H3 cells treated with CM-EE. Further RT-real time PCR results offered the clue for decreased c-jun expression that c-jun mRNA synthesis was reduced in CM-EE-treated activated RBL-2H3 cells (Fig. 3C). In accordance with the finding that lower p-Syk level was found in treated agitated RBL-2H3 cells, the expression levels of p-PLC- γ and p-Erk were down-regulated

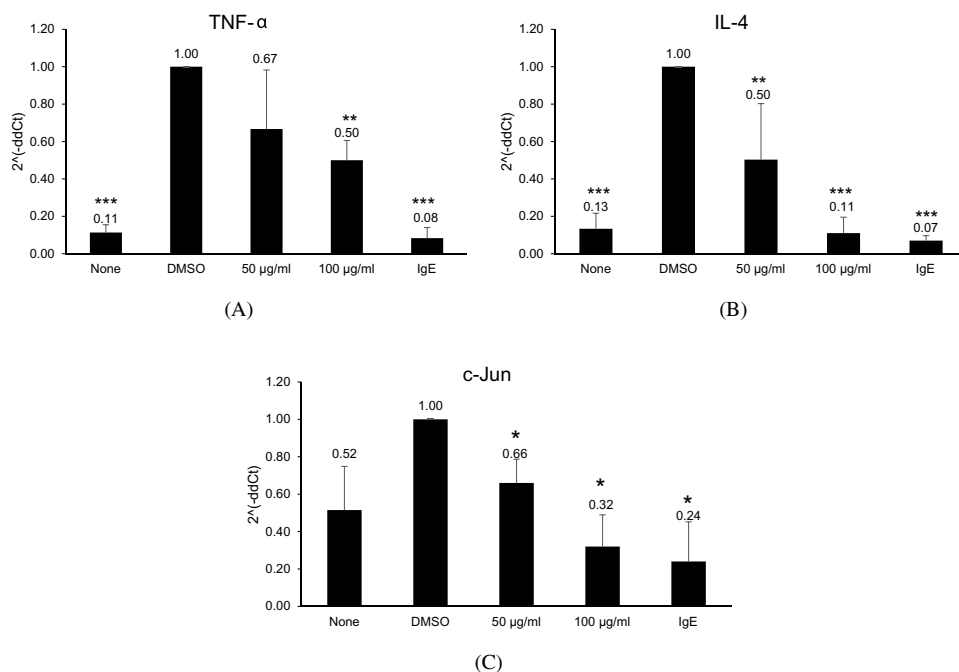


Figure 3. mRNA expression levels of TNF- α , IL-4 and c-jun in DNP-HSA/IgE stimulated RBL2H3 cells. mRNA was isolated from DNP-HSA/anti-DNP IgE irritated RBL2H3 cells treated with 50 μ g/ml or 100 μ g/ml CM-EE. Activated cells incubated with DMSO (DMSO) were recognized as vehicle control. Cells only sensitized IgE (IgE) and unstimulated/untreated cells (none) were also used as controls. RT real time PCR was performed as mentioned in “Materials and Methods.” (A) TNF- α , $N = 3$. (B) IL-4, $N = 3$. (C) c-jun, $N = 4$. Data were expressed as mean $\pm$ S.D. The number above each bar is the average number. *, ** and *** represented $P < 0.05$ and $P < 0.01$ and $P < 0.001$ respectively as compared to DMSO control using Student’s t-test.

(Figs. 4G and 4H), implicating that *de novo* synthesis of lipid mediators and degranulation were hindered. To further characterize the effects of CM-EE on p-JNK, both SP600125 (JNK inhibitor) and PP2 (the Src family kinase inhibitor) were exploited in allergen-stimulated RBL2H3 cells. As indicated in Fig. 4, SP600125 or PP2 treatment indeed diminished the corresponding kinases. However, there were no synergistic effects between CM-EE and SP600125 or CM-EE and PP2. It was possible that the CM-EE treatment triggered a different inhibitory pathway from that of SP600125 or PP2.

Influences of CM-EE on Passive Cutaneous Anaphylaxis Mice

The aforementioned results implied that CM-EE might inhibit the IgE-mediated allergic responses in RBL 2H3 cells by inhibiting degranulation and *de novo* synthesis of lipid mediators, cytokines and chemokines. Therefore, the PCA mouse model was used to further validate the results collected in RBL2H3 cells as described in “Materials and Methods.” Results gathered from the PCA experiment indicated that the administration of

300 mg/kg and 500 mg/kg could alleviate the allergic responses of mouse ears based upon the observation that the intensity of blue color in the ears of CM-EE fed PCA mice was less than that of un-treated PCA mice (Fig. 5), implicating that CM-EE have the ability to inhibit the IgE-evoked allergy in animals.

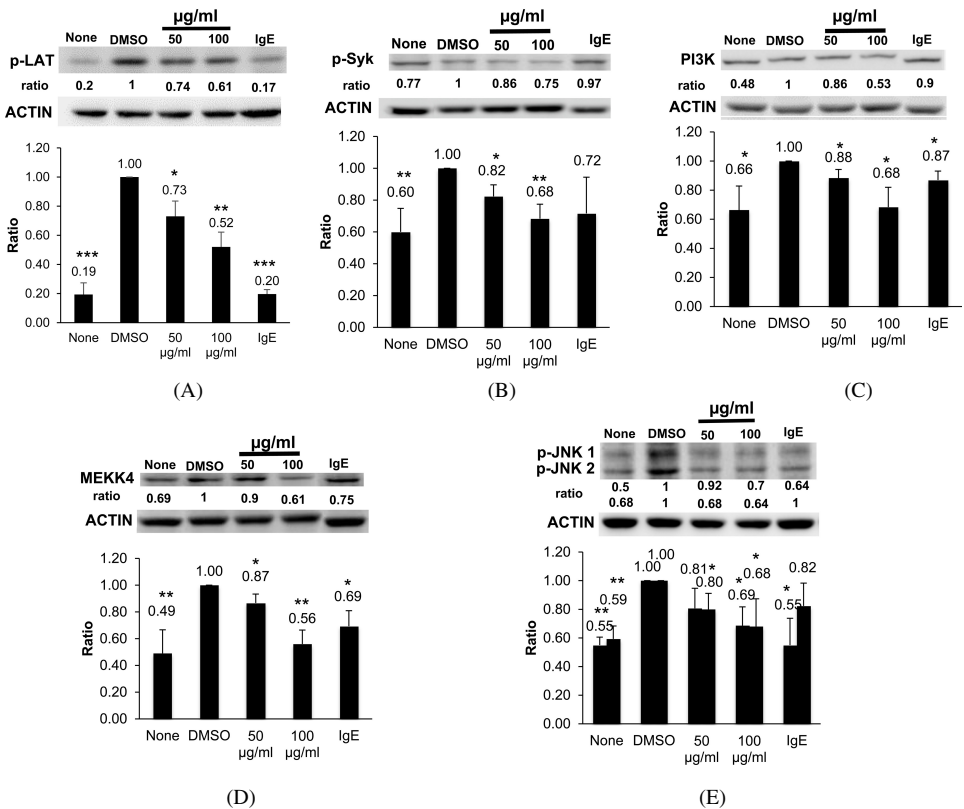


Figure 4. Impacts of CM-EE on DNP-HSA/IgE-evoked biochemical cascades in RBL2H3 cells. RBL2H3 cells were stimulated with DNP-HSA/anti-DNP IgE and incubated with various concentrations of CM-EE as indicated as described in “Materials and Methods.” Then the expression levels of p-LAT, p-Syk, PI3K, MEKK4, p-JNK, c-jun, p-PLC γ and p-Erk in cell lysates were measured respectively as described in “Materials and Methods” using western immunoblotting with the antibodies against p-LAT (A), p-Syk (B), PI3K (C), MEKK4 (D), p-JNK (E), c-jun (F), p-PLC γ (G), p-Erk (H), p-JNK in the presence of 30 μ M SP600125 plus 100 μ g/ml CM-EE (I) and p-Syk in the presence of 10 μ M PP2 as well as 100 μ g/ml CM-EE (J). The blot in each diagram is the representative result of at least three independent experiments. The protein expression ratio (mean $\pm$ S.D.) is expressed as the ratio of normalized intensity (observed protein/actin) at each concentration divided by that at DMSO. The number above each bar is average number. SP600125 is a broad-spectrum JNK inhibitor and PP2 is an inhibitor for Src family kinases. *, ** and *** represent $P < 0.05$ and $P < 0.01$ and $P < 0.001$ respectively as compared to DMSO control using Student’s t-test.

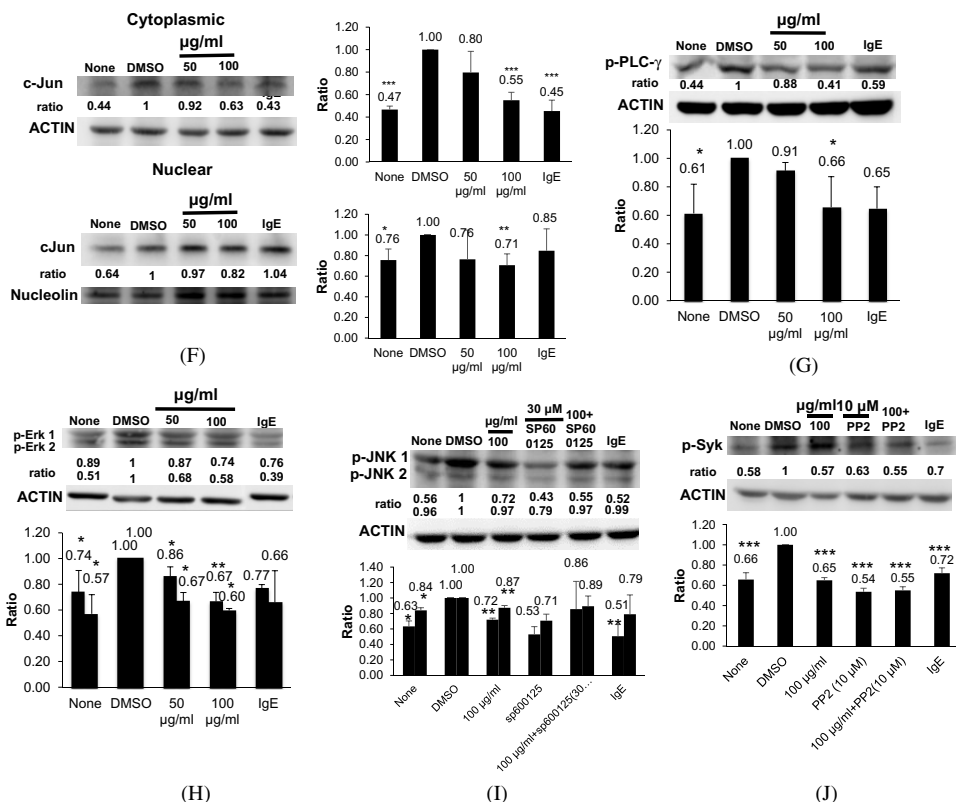


Figure 4. (Continued)

Identification of Active Anti-Allergy Ingredients Present in CM-EE

In order to identify the active anti-allergy components present in CM-EE, CM-EE was partitioned with EtOAc. HPLC profile of EtOAc layer demonstrated that there was a single peak shown at 73.093th min, highly similar to RT of β -sitosterone peak (Figs. 2C and 2D). TLC analyses of EtOAc layer also showed that a single spot was well separated from a broad spot (data not shown). Based upon the HPLC results, we tried to isolate the compounds present at about RT 73th min and therefore EtOAc layer was further divided into four fractions by column chromatography as described in “Materials and Methods.” Crystallized compounds T1 and T3 could be acquired from fractions 1 and 3, respectively. Further identification indicated that compound T1 was recognized as β -sitosterone (Fig. 2B) while compound T3 remained a mixture. Compound T3 probably existed in the range of RT from 35th to 50th min. A β -hexosaminidase secretion assay was performed to measure whether compounds T1 and T3 conducted inhibitory activity toward degranulation. As indicated in Fig. 1B, compound T1 possessed the suppressive effectiveness against

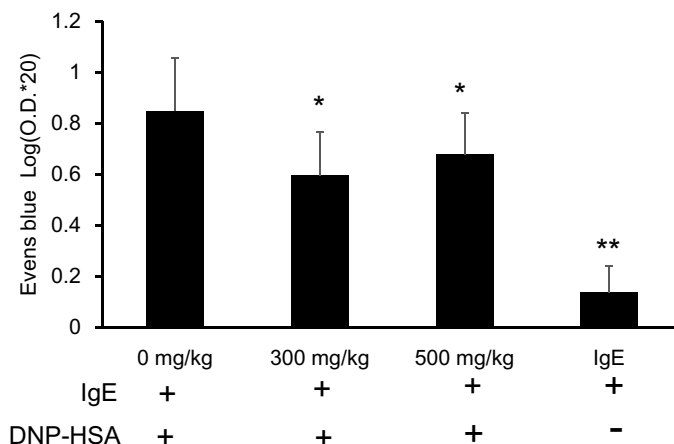


Figure 5. Attenuation of passive cutaneous anaphylaxis by CM-EE administration in mice. PCA induced in mice was treated as described in "Materials and Methods." DNP-HSA/IgE stimulated mice fed with olive oil were deemed as 0 mg/ml (vehicle control). Quantitative results are expressed as mean $\pm$ S.D. * and ** stand for $P < 0.05$ and $P < 0.01$ respectively as compared to 0 mg/ml control using the Tukey test of One Way Anova.

degranulation while compound T3 increased β -hexosaminidase secretion, suggesting that β -sitosterone probably was one of the anti-allergy ingredients present in CM-EE. However, more studies are required to recognize β -sitosterone as an anti-allergy compound and to find other anti-allergy components.

Discussion

Incidences of allergies (the type I hypersensitivity disorder) are steadily increasing in many developed countries. Current common therapies for allergic diseases are mainly small-molecule antagonists to leukotrienes or histamine receptors and steroid treatment (Harvima *et al.*, 2014). However, these traditional ways of treatment will lead to uncomfortable side effects including drowsiness, dry mouth and upset stomachs. Nowadays, adverse-effect-free anti-allergic cures produced from medicinal compounds or extracts isolated from traditional medicinal herbs or medicinal fungi are gaining the spotlight. Previous studies have showed that *Cordyceps* spp. possess anti-allergy efficacy (Gao *et al.*, 2009; Oh *et al.*, 2011). Thus *C. militaris* fruit bodies may be one of the ideal candidates for anti-allergic therapy. In this study, silkworm pupa-cultured *C. militaris* fruit bodies were used for examining the anti-allergy efficacy. Today *C. militaris* products made from pupa-grown *C. militaris* fruit bodies are rarely seen on the market worldwide since most of the *C. militaris*-related products are produced from grains or soybean-cultivated fruit bodies.

Mast cells are the primary effector cells that participate in IgE-mediated allergic responses by secreting inflammatory mediators and cytokines. Allergens on mast cells crosslink Fc ϵ RI and thereby initiate multiple signaling pathways that result in the degranulation and *de novo* synthesis of allergic mediators and cytokines. Both of the latter provoke the release of allergic mediators and cytokines from activated mast cells (Galli and

Tsai, 2012). Our results demonstrated that CM-EE nurturing could suppress degranulation (β -hexosaminidase release) and inhibit LAT activation in DNP-HSA/IgE stimulated RBL2H3 cells. LAT attenuation in turn diminished Syk. Further, we found that the decreased Syk activity attributing to CM-EE treatment could block PI3K/MEKK4/JNK/c-jun cascade and also dwindle Erk activation. Consistent with reduced JNK activity, our results showed that nuclear c-jun expression level was reduced at the same time. However, surprisingly, the cytoplasmic c-jun amount level was also decreased. Reduced mRNA synthesis in extract-incubated stimulated RBL-2H3 cells explains why CM-EE treatment decreased the cytoplasmic c-jun expression amount. Further investigation is required to uncover the mechanism of the CM-EE-triggered reduced expression of c-jun mRNA in the future. The aforementioned findings suggested that CM-EE incubation might interfere with the translocation of active c-jun into the nucleus through the blockage of the JNK pathway. It might also prevent active c-fos from moving into the nucleus via the inhibition of the Erk pathway. Nuclear c-fos would be associated with nuclear c-jun to form the AP-1 transcription factor, which initiates *de novo* synthesis of cytokines/chemokines in allergen-activated mast cells. Taken together, CM-EE treatment might reduce *de novo* synthesis of cytokines/chemokines and lipid mediators in activated RBL-2H3 cells. In accordance with the above findings, mRNA levels of two essential allergic cytokines, IL-4 and TNF α , were decreased in CM-EE nurtured DNP-HSA/IgE stimulated RBL-2H3 cells.

A previous study has demonstrated that the ethylacetate (EtOAc) fraction of the methanol extract of germinated soybean grown *Cordyceps militaris* fruit bodies (GSCM) can inhibit both the β -hexosaminidase release and the signaling pathway evoked by Fc ϵ R cross-linking. EtOAc extract also can alleviate passive cutaneous anaphylaxis in mice (Oh *et al.*, 2011). The above results imply that the constituents present in EtOAc extract possess the anti-allergic effectiveness. Therefore, genistein 4'-O- β -D-glucoside 4''-O-methylate, an anti-allergic compound, is further isolated from the methanol extract of GSCM (Park *et al.*, 2012). The inhibitory activity of this methyl-glycoside on β -hexosaminidase release is stronger than genistein or genistin, both of which are major isoflavones in most soy products and can modulate immune responses. It also can inhibit the expression of IL-4 as well as TNF- α and Fc ϵ R-evoked biochemical cascade. In contrast to the above-mentioned studies, we found that the ethanol extract of pupa-cultured *C. militaris* fruit bodies had the anti-allergic efficacy *in vitro* as well as *in vivo* and no further partition was required to recognize the anti-allergic fraction. Similar to the above results, a triterpenoid, β -sitostenone, was purified from C-EE, which appeared as a single peak in HPLC profile of CM-EE. Only few documented findings demonstrate the anti-allergic effectiveness of β -sitostenone. Among them, a previous documented study indicated that two triterpenoids, β -sitosterol and β -sitostenone isolated from *Leucosidea sericea* (Rosaceae), exhibited anti-inflammatory activity via inhibitions of COX-1 and COX-2 (Nair *et al.*, 2012). The aforementioned results imply that β -sitostenone might be one of the active ingredients in CM-EE.

Our study demonstrated that CM-EE contained the ingredients, which could inhibit immediate degranulation and *de novo* synthesis of allergic lipid mediators and cytokines in IgE-triggered allergic mast cells.

Acknowledgments

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Supplemental Figure



Figure S1. Silkworm pupa-cultivated *C. militaris* (L.) Link fruit bodies.