



The ethyl acetate extract of *Cordyceps militaris* inhibits IgE-mediated allergic responses in mast cells and passive cutaneous anaphylaxis reaction in mice

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ABSTRACT

Ethnopharmacological relevance: *Cordyceps militaris* has been used as a traditional herbal medicine for treating allergy in East Asia.

Aim of the study: We investigated the anti-allergic efficacy of *Cordyceps militaris* and its mechanism of action.

Materials and methods: β -Hexosaminidase release of mast cells, a key parameter of degranulation, was evaluated. Anti-allergic potential of *Cordyceps militaris* was studied using passive cutaneous anaphylaxis (PCA) *in vivo*. The anti-allergic mechanism of *Cordyceps militaris* was investigated by immunoblotting analysis, RT-PCR and other biological approaches in mast cells.

Results: GSCM EtOAc extract (GSCME) inhibited antigen-induced degranulation with a IC50 value (28.5 μ g/ml) in RBL-2H3 cells and antigen-induced passive cutaneous anaphylaxis (PCA) response with a ED50 value (665 mg/kg) *in vivo*. The release of interleukin (IL-4) and tumor necrosis factor (TNF- α) were decreased by GSCME in RBL-2H3 cells. In order to elucidate the anti-allergic mechanisms of GSCME in mast cells, we examined the activated levels of signaling molecules. GSCME inhibited the phosphorylation Syk, ERK, p38 and JNK expression. Identified genistein, daidzein, genistein 7-O- β -D-glucoside 4''-O-methylate, genistein 4'-O- β -D-glucoside 4''-O-methylate, glycitein 7-O- β -D-glucoside 4''-O-methylate, daidzein 7-O- β -D-glucoside 4''-O-methylate and adenosine in GSCME, inhibited antigen-induced degranulation in RBL-2H3 cells.

Conclusions: Our study suggests that GSCME might be used as a therapeutic agent for allergic diseases.

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1. Introduction

The prevalence of type I allergic disease (IgE-mediated allergic disease) has been increased worldwide during the past two decades (Asher et al., 2006). Mast cells are the major effector cells in IgE-mediated allergic diseases, including atopic dermatitis, allergic rhinitis, asthma and eczema. The allergic cascade is initiated after allergens bound to IgE (Fc ϵ RI) receptors expressed on the surface of either mast cells or basophils. Upon antigen stimulation, cells release inflammatory mediators from granules such as histamine, leukotriene C4, β -hexosaminidase, proteases and prostaglandin D2, chemokines and cytokines (Brightling et al., 2003). Interleukin (IL-4) is necessary for IgE production, and promotes the switch from naïve T-cells to the allergic type Th2 cells (Hines, 2002). Tumor necrosis factor (TNF- α) is a major initiator of inflammation, and induces other cytokine production (Kumar et al., 2004). Activation of signaling pathways in antigen-stimulated mast cells depends on activated Src kinases that interact with Fc ϵ RI receptor and sub-

sequent activation of Syk and other downstream tyrosine kinases (Gilfillan and Tkaczyk, 2006). Mitogen-activated protein kinases (MAPKs) signaling cascades are involved in the degranulation and the regulation of gene expressions, including pro-inflammatory cytokines and chemokines (Gilfillan and Tkaczyk, 2006). Therefore, these kinases are major target molecules for screening anti-allergic drugs.

Current therapies for allergic diseases are largely based on allergen-specific immunotherapy, DNA vaccination, anti-histamine drugs and steroids treatments (Kaliner, 2009). Common small-molecule inhibitors to treat allergic diseases are mainly antagonists to leukotriens (LTs) or histamine receptors, but they produce undesirable side-effects, including drowsiness, dry mouth, chest congestion, and upset stomach (Oppenheimer and Casale, 2002). Recently, many practitioners and researchers are paying attention to traditional medicinal herbs and mushrooms as anti-allergic drugs that are known to modulate immune responses and neutralize allergic reactions without any adverse effects (Chan et al., 2008; Ellertsen and Hetland, 2009).

Cordyceps militaris (Family: Clavicipitaceae), a traditional medicine, has been widely used to treat inflammation, anemia, asthma, and cancer in East Asia (Kim et al., 2006; Shin et al., 2009).

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Recent studies have shown that the extracts of *Cordyceps militaris* has strong anti-inflammatory activity on the croton-oil induced ear edema in mice and are effective in treating asthma patient (Won and Park, 2005; Gao et al., 2009). Many nutritional compounds from soybeans have been reported to be effective in preventing various inflammatory diseases and cancers (Li et al., 2008). In this regard, we cultivated *Cordyceps militaris* on the germinated soybeans that might provide plentiful novel nutraceutical compounds that derive from the biologically effective components of the two materials (Choi et al., 2010). Recently, our group has reported that the methanol extracts of *Cordyceps militaris* grown on germinated soybeans (GSC) stimulated IL-8 production playing an important role in the activation of innate and adaptive immunity in A549 human pulmonary epithelial cells (Han et al., 2010). However, the evidence-based research into its anti-allergic activity of GSC is still in its infancy.

In this study, we investigated whether *Cordyceps militaris* grown on germinated soybean (GSC) could inhibit IgE-mediated allergic response and regulate the mechanisms involved in allergic events.

2. Material and methods

2.1. Materials

Cordyceps militaris grown on germinated soybeans (Kucari 0903) (The Cell Activation Research Institute, Seoul, Korea), daidzein 7-O- β -D-glucoside 4'-O-methylate, glycitein 7-O- β -D-glucoside 4'-O-methylate, genistein 7-O- β -D-glucoside 4'-O-methylate, genistein 4'-O- β -D-glucoside 4'-O-methylate and adenosine used in this study that were purified as described in our previous study (Choi et al., 2010). Fetal bovine serum (Invitrogen, Carlsbad, CA), Penicillin (Invitrogen, Carlsbad, CA), Minimum essential medium, Eagle (Invitrogen, Carlsbad, CA), DNP-specific IgE (Sigma–Aldrich, St. Louis, MO), DNP-BSA (Sigma–Aldrich, St. Louis, MO), Piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) (Sigma–Aldrich, St. Louis, MO), Evans blue (Sigma–Aldrich, St. Louis, MO), Cetrazine (CZ, Sigma–Aldrich, St. Louis, MO), Src tyrosine kinase inhibitor (PP2) (Calbiochem, La Jolla, CA), anti-phospho-Syk (Cell Signaling Technology Inc., Danvers, MA) antibody, anti-phosphorylated linker for activation of T cells ((LAT) antibody (Cell Signaling Technology Inc., Danvers, MA)), anti-phospho-PKC α/β II (Cell Signaling Technology Inc., Danvers, MA) antibody, anti-phospho-p44/42 MAPK (Erk1/2) antibody (Cell Signaling Technology Inc., Danvers, MA), anti-phospho-SAPK/JNK antibody (Cell Signaling Technology Inc., Danvers, MA), anti-phospho-p38 antibody (Santa Cruz, CA) and anti- β -actin antibody (Santa Cruz, CA).

2.2. Preparation of extract of *Cordyceps militaris* grown on germinated soybean

Cordyceps militaris was grown on germinated soybeans (GSCs) as previously described (Han et al., 2010). An authenticated voucher specimen of *Cordyceps militaris* (CM, Kucari 0906) is deposited in the Herbarium at the College of Bioscience and Biotechnology, Konkuk University (Seoul, Korea). Briefly, mycelium of *Cordyceps militaris* (CM, Kucari 0906) was inoculated on germinated soybeans (*Glycine max* (L.) Merr), and was cultured at 20–25 °C for 4 weeks. The cultured material (1 kg) was ground and extracted with 80% MeOH (methanol extract of *Cordyceps militaris* grown on germinated soybeans (GSCM)) for 48 h under reflux. The total extract (178 g, yield 17.8%) were dissolved with water, after remove the insoluble solid by filtration, the liquid phase was extracted sequentially from non-polar to polar solvents by hexane, EtOAc, BuOH (1:10 (w/v) for all solvents), yielding four fractions. The liquid–liquid phase extraction was performed in Erlenmeyer flasks shaking and concentrated by a rotary evaporator to dryness, i.e.

hexane fraction (16 g, yield (w/w) 1.6%), EtOAc fraction (4.5 g, yield (w/w) 0.45%), BuOH fraction (8.25 g, yield (w/w) 0.825%) and water fraction (10.86 g, yield (w/w) 1.086%).

2.3. Cell culture

RBL-2H3 cells were obtained from ATCC (A Biological Resource Center, Manassas, VA). The RBL-2H3 were cultured in MEM supplemented with 15% FBS and 1% Penicillin. The cells were grown in 75 cm² culture flasks at 37 °C with 5% CO₂ in humidified atmospheric pressure.

2.4. Experimental animals

Male Balb/c mice (aged 4 weeks) were supplied from the Dae Han Experimental Animal Center (Eumsung, Korea). They were kept in cages with a temperature of (23 $\pm$ 2 °C) and a relative humidity of 55%. The animal study was performed in accordance with the institutional guidelines (The Institutional Animal Care and Use Committee (IACUC) at Konkuk University (Seoul, Korea)).

2.5. Cell viability assay

Cell cytotoxicity was measured using the Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan), as described previously (Park et al., 2010a). Cells (1 $\times$ 10⁴/ml) were treated with GSCME extract (10, 30, 100 and 300 μ g/ml) for 24 h. The cultures in 96-well plates were placed in 10 μ l of medium that contained CCK-8 and incubated for 2 h at 37 °C. The absorbance was measured with a microplate reader at 450 nm (Tecan, Männedorf, Switzerland).

2.6. β -Hexosaminidase secretion assay

As a marker of degranulation, the release of β -hexosaminidase was measured as previously described (Lee et al., 2008).

2.7. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the RBL-2H3 cells using RNA-Bee Reagent (Tel test, Friendwood, Texas) and reverse transcribed using Revertra Ace qPCR RT kit (Toyobo Biologics Inc., Osaka, Japan) as described previously (Lee et al., 2008). The polymerase chain reaction was performed at 94 °C for 2 min, at 94 °C for 30 s, at 55 °C for 30 s and at 68 °C for 1 min for 30 cycles. The following primers were used: rat TNF- α forward 5'-CACCACGCTCTTCTGTCTACTGAAC-3'; rat TNF- α reverse 5'-CCGGACTCCGTGATGTCTAAGTACT-3'; rat IL-4 forward 5'-ACCTTGCTGTACCTGTTC-3'; rat IL-4 reverse 5'-TTGTGAGCGTGGAATTC-3'; rat glyceraldehydes-3-phosphate dehydrogenase forward 5'-CTTACCACCATGGAGAAGGCTG-3'; rat glyceraldehydes-3-phosphate dehydrogenase reverse 5'-GACCACAGTCCATGCCATCACTG-3'.

2.8. Immunoblotting

RBL-2H3 cells (1 $\times$ 10⁶/ml) were stimulated with 25 ng/ml DNP-BSA for 10 min. Cells were lysed in 100 μ l cell lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin, 1 mM PMSF) on ice for 10 min. Lysates were centrifuged at 14,000 $\times$ g for 10 min at 4 °C. The protein concentrations were determined using a BCA Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) as previously described (Park et al., 2010b). Then, 20 μ g aliquots of protein were subjected to electrophoresis on 4–15% gradient polyacrylamide gels and electrophoretically transferred onto polyvinylidene fluoride

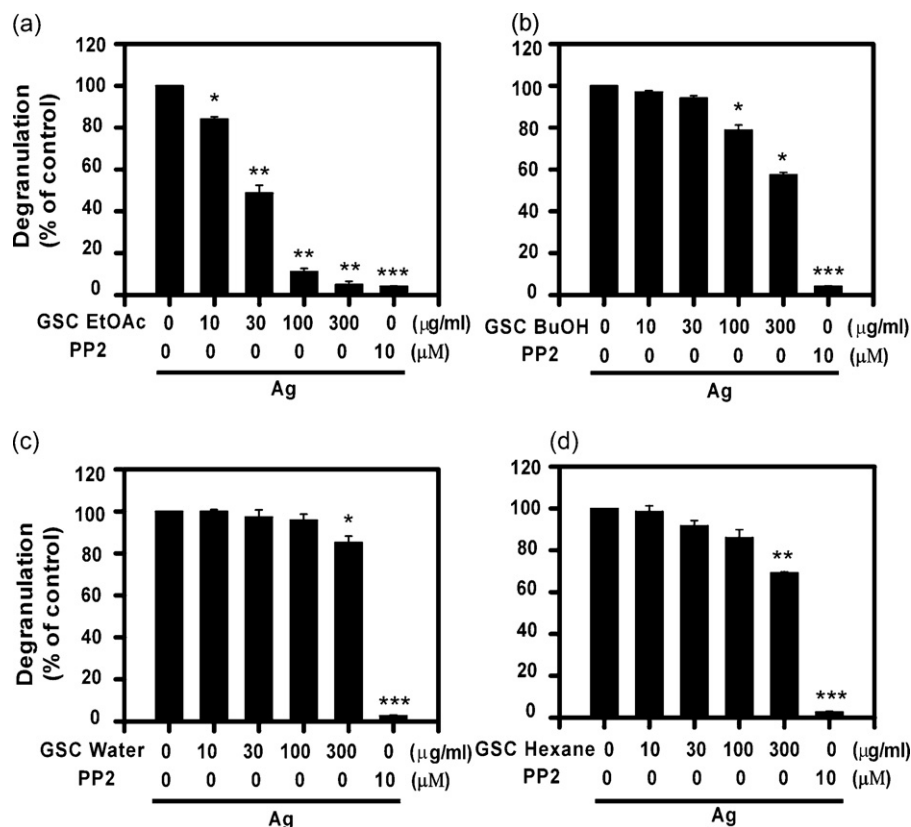


Fig. 1. Effect of GSC fractions on antigen-stimulated degranulation in RBL-2H3 cells. The degranulation was determined by measurement of β -hexosaminidase release in RBL-2H3 cells. The RBL-2H3 cells were treated with GSC fractions (a) EtOAc, (b) BuOH, (c) water, (d) hexane (10, 30, 100 and 300 μ g/ml) for 30 min and then stimulated for 10 min with antigen. PP2 is a general Src-family kinase inhibitor. Each value represents the mean $\pm$ SE of three experiments. * indicates significant differences from the control at $p < 0.05$.

membranes (Bio-Rad Laboratories, Berkeley, California). The membranes were incubated in 5% skim milk solution and then with antibodies against phospho-p44/42 (Erk1/2), phospho-SAPK/JNK, phospho-p38, phospho-PKC α/β II, phosphorylated linker for activation of T cells (LAT) and phospho-Syk. The membranes were washed and incubated horseradish peroxidase-conjugated secondary antibody for 1 hr. The protein bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.9. Passive cutaneous anaphylaxis (PCA)

Mice were intravenously injected with 250 μ g antigen (DNP-BSA) in 250 μ l PBS containing 4% Evans blue 24 h after the intradermal administration of a DNP-specific IgE (0.5 μ g) into the ear. To measure the activity of GSCME extract (100, 300, 1000 and 3000 mg/kg) or cetirizine (10 mg/kg, CZ), it was orally administered 1 h before Ag administration. The mice were euthanized 1 h after treatment with the Ag and the treated ear was excised in order to measure the amount of dye extravasated. The dye was extracted from the ear in 700 μ l formamide at 63 $^{\circ}$ C overnight, as previously described (Lee et al., 2008). The absorbance was measured with a microplate reader at 620 nm (Tecan, Männedorf, Switzerland).

2.10. Chemical derivatization and GC–MS analysis

The lyophilized sample extracts were derivatized in two steps to protect carbonyl functions (Choi et al., 2010). First, dried samples were dissolved in 100 μ l of a 20 mg/ml solution of methoxyamine hydrochloride in pyridine (Sigma, St. Louis, MO, USA) 60 $^{\circ}$ C for 60 min. Afterwards, to increase the volatility of polar

compounds, acidic protons were exchanged against trimethylsilyl group using 100 μ l *N*-methyl-*N*-trimethylsilyltrifluoro-acetamide (MSTFA, Sigma, St. Louis, MO, USA) at 70 $^{\circ}$ C for 60 min.

Samples volumes of 1 μ l were analyzed with a CP-3800 gas chromatograph coupled to a 4000 ion trap mass spectrometer equipped with a CP-8400 auto sampler (Varian, CA, USA). Derivatized samples were vaporized at 250 $^{\circ}$ C in standard split mode (1:25) and separated on a 30 m $\times$ 0.25 mm VF-1MS capillary column with 0.25 μ m coating equipped with an integrated 10 m guard column (Varian, CA, USA). The oven temperature was set at 100 $^{\circ}$ C, held for 2 min, then increased to 300 $^{\circ}$ C at 10 $^{\circ}$ C/min and held for 10 min. Helium carrier gas (purity > 99.999%) flow was adjusted to 1 ml/min. The interface and ion source temperature were set to 200 $^{\circ}$ C and electron impact ionization (70 eV) was utilized. The averaged scan was set to 3 μ scans and full scanning with a range of 50–1000 m/z . Metabolites were identified by comparison to the NIST 2005 database (version 2.0, FairCom Co., USA).

2.11. Statistical analyses

Results are expressed as mean $\pm$ standard error (SE). Student's *t*-test or One-way ANOVA/Dunnett's *t*-test was used to assess significance between controls and treatments. Statistical analysis was performed using SPSS, version 12 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effect of *Cordyceps militaris* (GSC) on degranulation from antigen-stimulated RBL-2H3 cells

To determine the anti-allergic activity of GSC extracts (BuOH, water, hexane, and EtOAc), we investigated their inhibitory

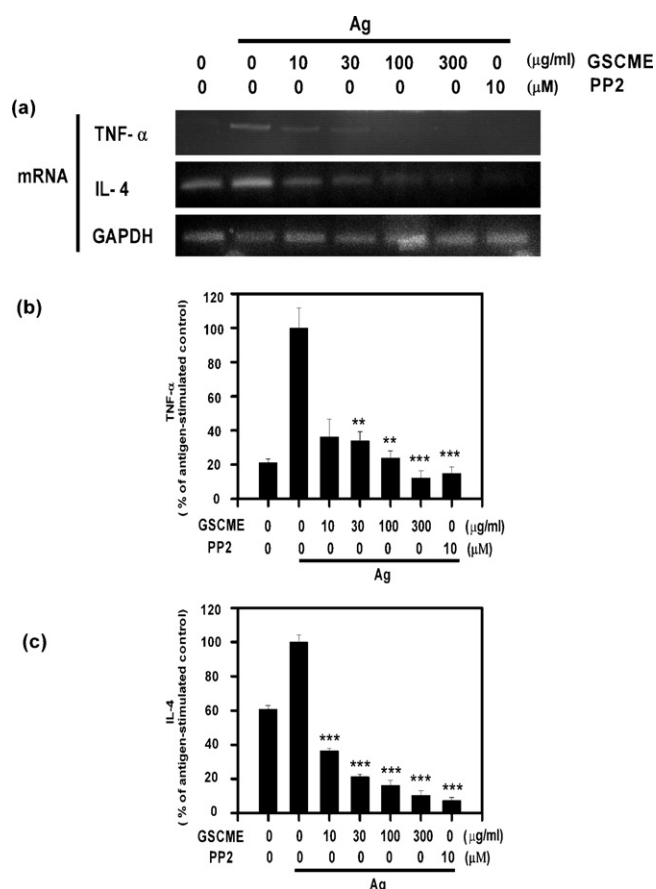


Fig. 2. GSCME reduced the production of pro-inflammatory cytokines in antigen-stimulated RBL-2H3 cells. (a) IL-4 or TNF- α mRNA was determined by quantitative RT-PCR. (b) Densitometric analysis of three blots (mean $\pm$ standard error of the mean) expressed as a percentage of values for corresponding antigen-stimulated groups (* p < 0.05; ** p < 0.01; *** p < 0.005).

effects on the degranulation of antigen-stimulated RBL-2H3 cells. β -Hexosaminidase release was determined as an index of degranulation (Shimoda et al., 2006). A CCK8 assay showed any of the fractions did not affect cell viability (data not shown). As shown in Fig. 1, β -hexosaminidase release was inhibited in the order of GSC EtOAc extract > GSC BuOH extract > GSC hexane extract > GSC water extract. Therefore, GSC EtOAc extract (GSCME) was chosen for further studies.

3.2. Effect of *Cordyceps militaris* EtOAc extract (GSCME) on antigen-stimulated pro-inflammatory cytokines in mast cells

Pro-inflammatory cytokines, IL-4 and TNF- α , are responsible for allergic responses (Theoharides and Kalogeromitros, 2006). We investigated whether GSCME could regulate gene expression of IL-4 and TNF- α in antigen-stimulated RBL-2H3 cells. GSCME significantly inhibited the antigen-induced IL-4 and TNF- α gene expression in a dose-dependent manner (10, 30, 100 and 300 μ g/ml) (Fig. 2).

3.3. Effect of *Cordyceps militaris* extract (GSCME) on the activation of Syk, LAT and PKC kinases in antigen-stimulated RBL-2H3 cells

It is well documented with reports that Fc ϵ RI signaling events, including Syk, LAT and PKC, regulate degranulation and pro-inflammatory cytokine production in mast cells (Lorentz et al., 2003). To unravel the anti-allergic mechanism of GSC, we checked

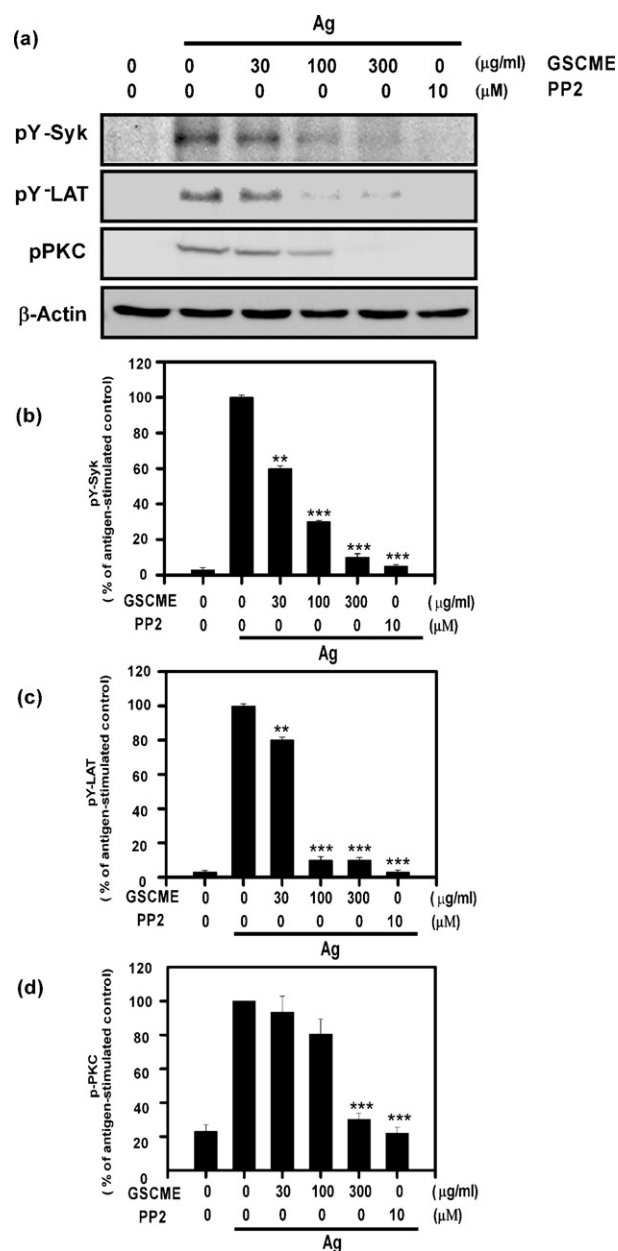


Fig. 3. GSCME inhibited the activation of Syk, LAT and PKC protein in RBL-2H3 cells. (a) Cell lysates were prepared as described in Section 2. The levels of phosphorylated Syk, phosphorylated LAT, and phosphorylated PKC were measured by immunoblotting analysis. PP2 is a general Src-family kinase inhibitor. (b–d) Densitometric analysis of three immunoblots (mean $\pm$ standard error of the mean) expressed as a percentage of values for corresponding antigen-stimulated groups (* p < 0.05; ** p < 0.01; *** p < 0.005).

whether GSC could affect the activation of Syk and LAT (Beaven and Metzger, 1993). As shown in Fig. 3, GSCME attenuated activation of Syk and LAT kinases in a dose-dependent manner. Activated PKC is known to involve in Fc ϵ RI dependent degranulation (Gilfillan and Tkaczyn, 2006). The phosphorylation of PKC was inhibited by GSCME in a dose-dependent manner (Fig. 3).

3.4. Effect of *Cordyceps militaris* extract (GSCME) on the phosphorylation of MAPKs in antigen-stimulated RBL-2H3 cells

Many studies reported that activated MAP kinases (ERK1/2, p38 and JNK) involve in the production of TNF- α and IL-4

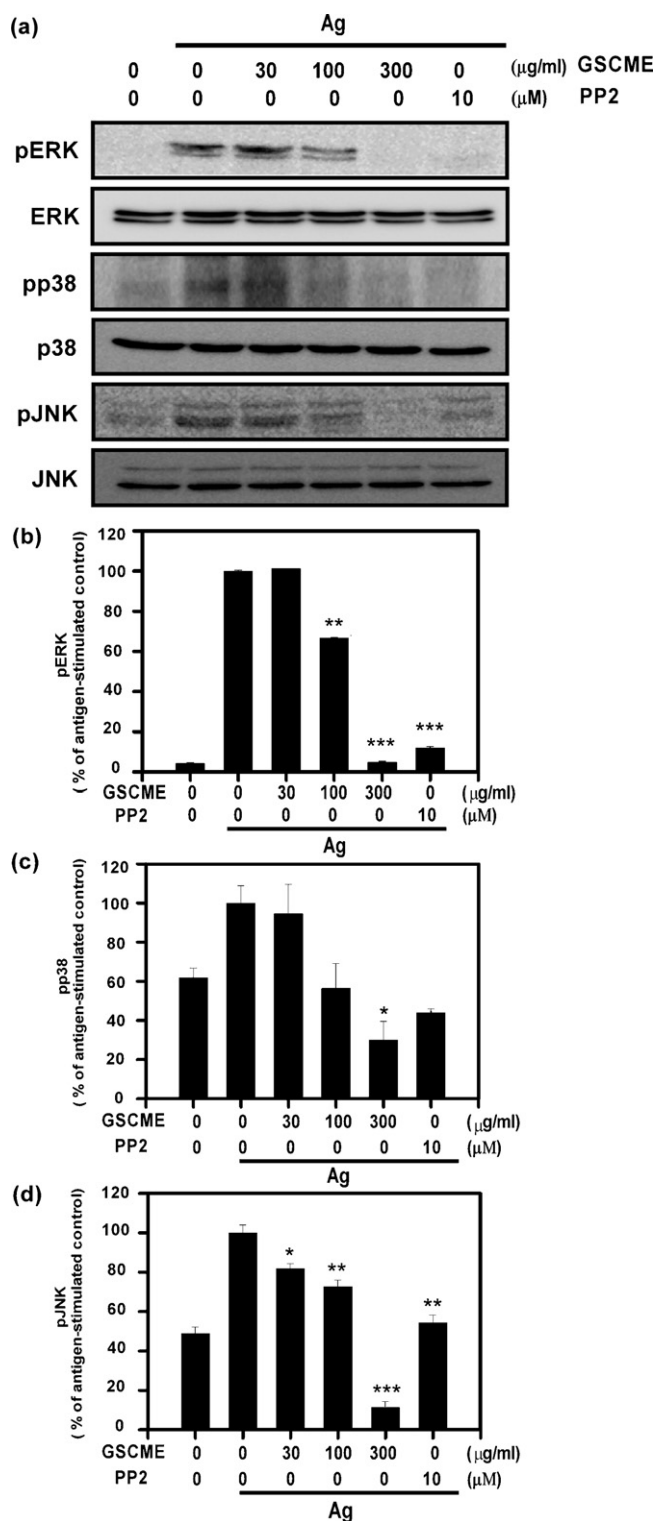


Fig. 4. GSCME inhibited the phosphorylation of MARK protein in RBL-2H3 cell. (a) Cell lysates were prepared as described in Section 2. The levels of phosphorylated MAPK protein were measured by immunoblotting analysis. PP2 is a general Src-family kinase inhibitor. (b–d) Densitometric analysis of three immunoblots (mean $\pm$ standard error of the mean) expressed as a percentage of values for corresponding antigen-stimulated groups (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$).

(Frossi et al., 2007). GSCME suppressed the activation of ERK1/2 and p38 protein (Fig. 4). Slight decrease of phosphorylated JNK was observed in GSCME treated group (Fig. 4).

3.5. Effect of *Cordyceps militaris* extract (GSCME) on passive cutaneous anaphylaxis

Degranulation of mast cells plays a key role in the early phase allergic reaction (Theoharides and Kalogeromitros, 2006). Passive cutaneous anaphylaxis (PCA) is one of the most frequently used experimental models in evaluating anti-allergic drugs (Lee et al., 2008). We examined the anti-allergic effect of GSCME by evaluating the cutaneous extravasation of albumin-bound Evans blue dye. The PCA reaction was suppressed by GSCME extract in a dose-dependent manner (Fig. 5). The inhibitory effect of GSCME extract (1000 mg/kg) was near to that of 10 mg/kg cetirizine (CZ), a typical anti-histamine drug.

3.6. Identified compounds of *Cordyceps militaris* extract (GSCME)

In order to identify the active compounds of GSCME, we performed GC–MS analysis, and compared with our secondary metabolite MS/MS spectrum library (Choi et al., 2010). In GC–MS analysis, biologically active compounds of GSCME were demonstrated that the stearic acid ($t_R = 16.49$ min) and cordycepin ($t_R = 18.98$ min) using NIST library as summarized in Table 1.

3.7. Inhibitory effect of active compounds of *Cordyceps militaris* EtOAc extract (GSCME) on degranulation in antigen-stimulated RBL-2H3 cells

We evaluated whether active compounds from GSCME could inhibit degranulation in antigen-stimulated RBL-2H3 cells. Daidzein, genistein, daidzein 7-O- β -D-glucoside 4''-O-methylate, glycitein 7-O- β -D-glucoside 4''-O-methylate, genistein 7-O- β -D-glucoside 4''-O-methylate, and genistein 4'-O- β -D-glucoside 4''-O-methylate were detected as major compounds of GSCME (Choi et al., 2010). Novel isoflavonoids, daidzein, and genistein significantly suppressed the release of β -Hexosaminidase in antigen-stimulated RBL-2H3 cells (Table 2). Adenosine slightly inhibited the release of β -Hexosaminidase (Table 2). However, cordycepin did not inhibit degranulation in antigen-stimulated RBL-2H3 cells.

4. Discussion

Type I allergic (IgE-mediated allergic disease) patient population has been significantly increased in many developed countries (Asher et al., 2006). Although many regimens have been developed by adopting novel and innovative approaches, current therapies failed to cure allergic diseases (Oppenheimer and Casale, 2002). Therefore, the active medicinal compounds or extracts from traditional herbal medicines come into spotlight due to their pharmacological efficacies with low adverse effects. *Cordyceps militaris* (Korean name, Dong-Choong-Ha-Cho), a medicinal fungus, has been widely used to treat asthma, bronchitis, hyperlipidemia, cancer and hepatic cirrhosis in East Asia (Paterson, 2008; Gao et al., 2009). However, the anti-allergic efficacies and mechanisms of *Cordyceps militaris* have not been reported yet. In this study, we investigated that *Cordyceps militaris* grown on germinated soybeans (GSC) exerted anti-allergic activities.

It is well-known that activated mast cells play a major role in the type I hypersensitive allergic reaction by initiating and orchestrating immune responses through degranulation and release of cytokines and chemokines (Theoharides and Kalogeromitros, 2006). We used rat mast cell line RBL-2H3, expressing functional Fc ϵ R1 to test the anti-allergic activities of *Cordyceps militaris*. We showed that GSCME inhibited the degranulation mostly among the GSC fractions in antigen-stimulated RBL-2H3 cells without affecting RBL-2H3 cell viability. Released histamine is responsible for

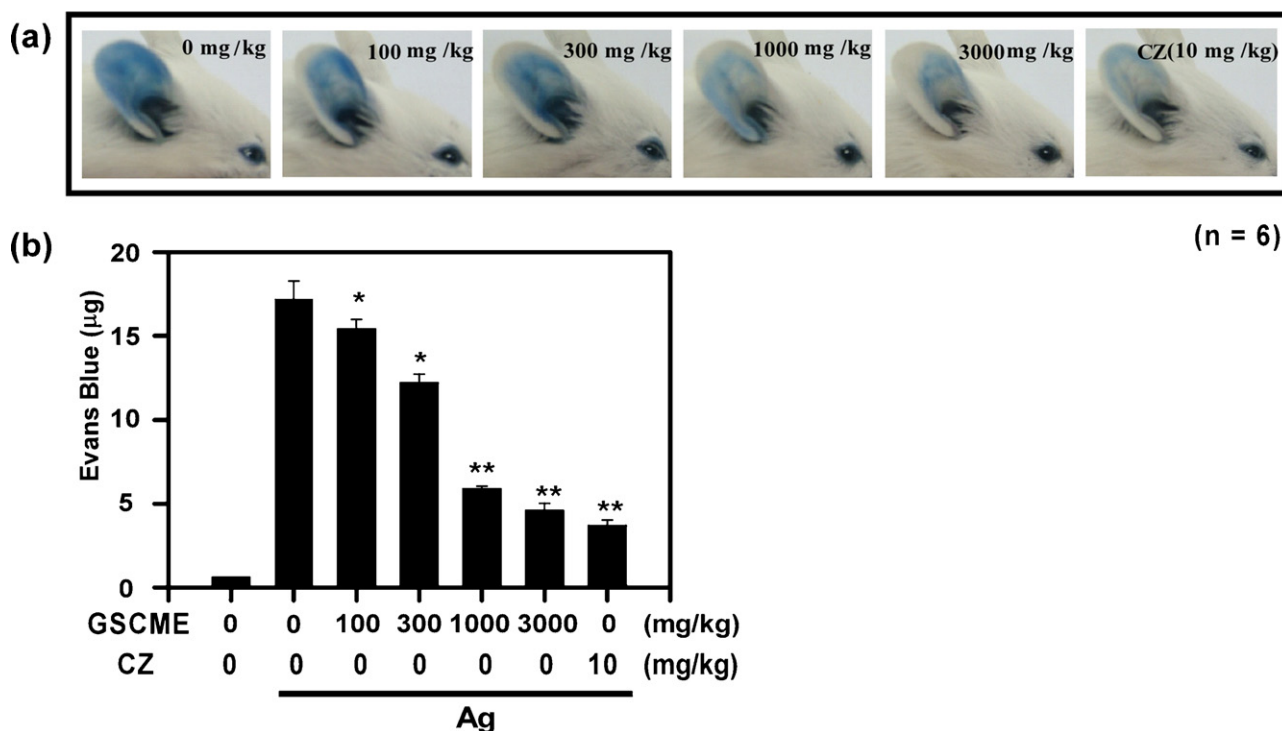


Fig. 5. Effect of GSCME on IgE-mediated passive cutaneous anaphylaxis (PCA) murine models. (a) The anti-allergic effect was assessed using PCA mouse model. To measure the activity of GSCME extract (100, 300, 1000 and 3000 mg/kg) or cetrazine (10 mg/kg, CZ) it was orally administered 1 h before antigen stimulation ($n=6$). (b) The dye was extracted from the ear and the amount was measured by absorbance. (c) The values are expressed as % of inhibition. The asterisks indicate significant difference from antigen-stimulated controls without GSCME (* $p < 0.01$; ** $p < 0.005$). CZ (cetrazine, 10 mg/kg) was used as a typical anti-histamine reference drug.

the increasing vascular permeability in PCA reaction (Inagaki et al., 1984). GSCME significantly inhibited anti-DNP IgE-mediated PCA reaction in accordance with the cellular data.

It is believed that mast cell activation is triggered by IgE-mediated antigen via the high-affinity receptor IgE. Following the receptor aggregation, the subsequent activation of Syk, LAT and other downstream tyrosine kinases occurs (Olivera et al., 2006). Therefore, Syk and LAT are attractive targets to develop allergic disease drugs. GSCME inhibited both the activation of Syk and LAT. MAP kinases, including ERK1/2, p38 MAP, and JNK kinases, are major target signaling molecules for screening anti-allergic drugs (Duan and Wong, 2006). They are downstream signaling molecules of Syk/LAT pathway, which involve in the generation of pro-inflammatory cytokines (Duan and Wong, 2006). GSCME inhibited activation of MAP kinases in a dose-dependent manner. Activated mast cells produce pro-inflammatory cytokines such as TNF- α and IL-4 (Middleton and Drzewiecki, 1984; Galli et al., 2005). These pro-inflammatory cytokines involve in allergic inflammation, mast cell activation and leukocyte infiltration and act as initiator cytokines that could stimulate other cytokine production (Jeong et al., 2002). TNF- α is responsible for allergic inflammation (Hide et al., 1997). IL-4 is necessary for Th2 response development and promotes B cell switching to IgE production (Kalesnikoff and Galli, 2008). GSCME significantly suppressed inflammatory cytokines such as TNF- α and IL-4 gene expression in mast cells.

Next, we focused in identifying active compounds from GSCME that might regulate allergic reactions. Recently, our

group has reported identified novel isoflavonoid compounds from GSC, which is likely to be produced as metabolites after cultivating *Cordyceps militaris* on germinated soybeans (Han et al., 2010). However, anti-allergic activities of these compounds have not been studied yet. Many studies have shown that non-selective tyrosine kinase inhibitor, genistein suppressed antigen-induced activation of tyrosine kinase and degranulation (Wong et al., 1997). We identified genistein 7-O- β -D-glucoside 4''-O-methylate, genistein 4'-O- β -D-glucoside 4''-O-methylate, glycitein 7-O- β -D-glucoside 4''-O-methylate, daidzein 7-O- β -D-glucoside 4''-O-methylate, genistein, daidzein, cordycepin and adenosine in GSCME, using the HPLC analysis. The backbone structures of novel isoflavonoids from GSCME were similar to these of genistein or daidzein. Notably, novel isoflavonoids, genistein and daidzein from GSC significantly inhibited degranulation in antigen-stimulated RBL-2H3 cells. Cordycepin was reported to be the major component of *Cordyceps militaris* which exerted anti-inflammatory activity (Won and Park, 2005). However, we observed cordycepin did not inhibit degranulation in antigen-stimulated RBL-2H3 cells. It is likely that novel isoflavonoids confers the anti-allergic activities of GSCME. Further experiments need to be done to clarify the anti-allergic mechanism of these identified compounds.

In conclusion, GSCME suppressed degranulation and the release of pro-inflammatory cytokines in antigen-stimulated RBL-2H3 cells. PCA reaction was blocked by GSCME. GSCME inhibited the activation of Syk, LAT, PKC and MAPKs (Fig. 6). Identified novel isoflavonoids (genistein 7-O- β -D-glucoside 4''-O-

Table 1
Identification of GSCME extract metabolites by GC–MS.

No.	Name	t_R (min)	MS fragment ions (m/z)	Derivatized ^a
1	Stearic acid	16.49	356, 341, 201, 132, 117, 73, 55	TMS($\times 1$)
2	Cordycepin	18.98	469, 452, 280, 260, 207, 192, 155, 73	TMS($\times 3$)

^a Number of hydrogen atoms derivatized.

Table 2

Effect of identified compounds in GSCME extract on antigen-stimulated degranulation in RBL-2H3 cells.

Sample	IC ₅₀ (μg/ml) ^a
Genistein	26.3 ± 7.7** ^b
Daidzein	28.9 ± 3.6**
Genistein 7-O-β-D-glucoside 4''-O-Methylate	45.5 ± 5.6*
Genistein 4'-O-β-D-glucoside 4''-O-methylate	39.3 ± 14.2*
Glycitein 7-O-β-D-glucoside 4''-O-methylate	36.3 ± 2.1*
Daidzein 7-O-β-D-glucoside 4''-O-methylate	44.5 ± 7.3*
Adenosine	76.1 ± 2.4
Cordycepin	>100
Stearic acid	>100

Degranulation was determined by measuring the amount of secreted β-hexosaminidase. Cells were incubated with genistein, daidzein, genistein 7-O-β-D-glucoside 4''-O-methylate, genistein 4'-O-β-D-glucoside 4''-O-methylate, glycitein 7-O-β-D-glucoside 4''-O-methylate, daidzein 7-O-β-D-glucoside 4''-O-methylate, adenosine, cordycepin and stearic acid for 30 min and then stimulated for 10 min with antigen.

^a IC₅₀ (μg/ml), 50%-inhibitory concentration (IC₅₀) of GSCME extract on antigen (DNP-BSA) in RBL-2H3 cells.

^b Each value represents the mean ± SE of three experiments. One-way ANOVA was used for comparisons of multiple group means followed by Dunnett's *t*-test (**p* < 0.05 vs. control; ***p* < 0.01 vs. control).

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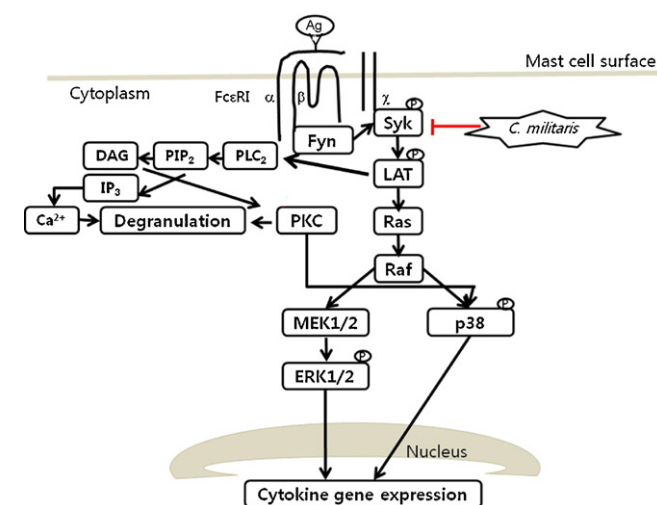


Fig. 6. GSCME suppressed IgE-mediated allergic responses. The main pathways activated by the IgE receptors (FcεRI) were affected by GSCME. GSCME can suppress degranulation through inhibiting activation of Syk, LAT and PKC. Downregulation of activated p38 kinase and ERK by GSCME treatment regulated the expression of pro-inflammatory cytokine genes involved in the allergic response in mast cells.

methylate, genistein 4'-O-β-D-glucoside 4''-O-methylate, glycitein 7-O-β-D-glucoside 4''-O-methylate, daidzein 7-O-β-D-glucoside 4''-O-methylate, genistein, daidzein and adenosine from GSCME inhibited degranulation in antigen-stimulated RBL-2H3 cells. Our findings suggest that *Cordyceps militaris* grown on germinated soybeans might be applied as a therapeutic agent for preventing or treating IgE-mediated allergic diseases.

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