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Anti-atopic dermatitis properties of *Cordyceps militaris* on TNFα/IFNγ-stimulated HaCaT cells and experimentally induced atopic dermatitis in mice

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[Purpose] This study evaluated the anti-atopic dermatitis (AD) properties of *Cordyceps militaris* (CM) aqueous extract in keratinocytes *in vitro* and *in vivo*. We investigated the nutraceutical composition of the CM extract, including its protein, carbohydrate, and selected phytochemical content.

[Methods] The expression of pathogenic cytokines in keratinocytes was assayed using an *in vitro* model. The CM extract downregulated extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 kinase expression in TNFα/IFNγ-stimulated HaCaT cells. We also established an *in vivo* AD model by repeatedly exposing the ears of mice to local *Dermatophagoides farinae* extract (DFE; house dust mite extract) and 2,4-dinitrochlorobenzene (DNCB). The epidermal and dermal ear thickness, mast cell infiltration, and serum immunoglobulin levels were measured following a 4-week oral administration of the CM extract.

[Results] Histopathological examination showed reduced epidermal/dermal thickness and mast cell infiltration in mouse ears. The CM extract also suppressed serum immunoglobulin levels and gene expression of T helper (Th)1/Th2 cytokines in mouse ear tissue.

[Conclusion] These results suggest that the CM extract may be useful for the treatment of AD-like skin lesions.

[Key words] atopic dermatitis, *Cordyceps militaris*, keratinocytes, nutraceutical composition, skin, Th1/Th2 cytokines

INTRODUCTION

Atopic dermatitis (AD) is a major chronic or relapsing inflammatory disease of the skin that often leads to asthma and other allergic disorders[1]. It is accompanied by pruritic and eczematous skin lesions[2]. Recently, the incidence of AD has rapidly increased and has become a major public health concern worldwide[3]. Although the pathogenesis of AD is not entirely understood, genetic susceptibility, the host's environment, skin barrier dysfunction, bacterial infection, and immunological factors appear to play a role in its development. The acute phase of AD is strictly related to increased T helper 2 (Th2) lymphocytes and basophils, while T helper 1 (Th1) cell-mediated AD lesions are found in chronic AD[2,4]. Furthermore, activated mast cells, increased immunoglobulin E (IgE), and increased levels of inflammatory cytokines are also found in AD skin lesions[5]. Although moisturizers and topical steroid therapy are commonly used for the treatment of AD, their ineffectiveness and side effects limit their use. Therefore, recent advancements in new treatment methods using herbal medicines with similar efficiency and reduced side effects are in progress[6].

The mushroom *Cordyceps militaris* (CM), an obligatory parasite that grows on insects or insect larvae, belongs to the family Clavicipitaceae and class Ascomycetes. CM, which is also an inhibitor of macrophage activation, has broad medical applications due to its varied pharmacological activities, including antioxidant, hypoglycemic, hypolipidemic, anti-inflammatory, antitumor, antibacterial, and antifungal activities[7]. Furthermore, it contains bioactive compounds, such as cordycepin (30-deoxyadenosine) and 20-deoxycoformycin[8-10]. CM extracts have demonstrated antioxidant, anti-inflammatory, and protective activities against acute hepatotoxicity in a murine macrophage model and in rats[11]. However, their activity on AD has not been demonstrated.

The present study, therefore, was designed to identify the nutraceutical composition of aqueous CM extracts and their anti-atopic dermatitis properties on tumor necrosis factor-α/interferon-γ (TNFα/IFNγ)-stimulated HaCaT cells and *Dermatophagoides farinae* extract/2,4-

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dinitrochlorobenzene (DFE/DNCB)-induced AD in BALB/c mice.

METHODS

Cordyceps militaris

CM was obtained from the National Institute of Horticultural and Herbal Science of Korea (Eumsung, Korea). CM specimens were lyophilized and subjected to a 2 h extraction in distilled water (DW) at 100 °C. The extract was filtered through a 0.25 µm pore filter and lyophilized in a freeze dryer for 5 days. TRIzol reagent used for RNA extraction was obtained from Invitrogen (Carlsbad, CA, USA). Primary antibodies and peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). All other reagents were of the highest commercially available grade.

Analysis of polysaccharides and monosaccharides

The total polysaccharide content of the extract was determined using the phenol-sulfuric acid method with D-glucose as a reference[12]. For polysaccharide analysis, 1 mg of protein-bound polysaccharide was hydrolyzed in 2 M trifluoroacetic acid (TFA) followed by evaporation. A high-pressure liquid chromatography (HPLC) system (Waters Corp., Milford, MA, USA) with a Sugar-Pak column (Millipore, Tokyo, Japan) and a differential refractive index (RI) detector (RID-6A) was used to detect monosaccharides at 80 °C.

Analysis of protein and amino acid content

The protein concentration of the extract was determined using the Bradford method[13]. Bovine serum albumin (BSA) (0.1-1.0 mg mL^{-1}) was used to construct a standard calibration curve. For the analysis of amino acids, the extract was hydrolyzed in 6 N hydrochloric acid in vacuum-sealed tubes at 110 °C for 24 h. The amino acid content of the extract was determined using an amino acid analyzer (L-8900, Hitachi High-Technologies Corp., Tokyo, Japan) with post-column derivatization with ninhydrin. The amino acid analyzer conditions were as follows: ion exchange column (4.6 × 60 mm), injection volume of 20 µL, UV detector, VIS1: 570 nm, and VIS2: 440 nm. An amino acid standard solution (016-08641, Wako, Osaka, Japan) was used for identification and quantification of amino acid content.

Phytochemical content

The total polyphenolic (TP) content of the extract was determined using a recently developed Fast Blue BB (FBBB) method[14]. First, the aqueous extract was diluted with deionized water (DW). The TP analysis consisted of adding 0.1 mL of 0.1% FBBB diazonium dye to 1 mL of the diluted sample, followed by the addition of 0.1 mL of 5% NaOH. After a 90-min reaction time, the absorbance was measured at 420 nm using a UV/Vis spectrophotometer (Shimadzu UV-1650 PC, Shimadzu, Tokyo, Japan). The standard curve was generated using 15 - 250 µg mL^{-1} gallic acid. The TP content was expressed as gallic acid equivalents (GAE) per g extract in DW.

The flavonoid content of the extract was determined using a colorimetric method described by Jia et al.[15] with modifications introduced by Barros et al.[16]. The extract (250 µL) was mixed with 1.25 mL of Milli-Q water (MQ) and 75 µL of 5% $NaNO_2$ solution. After 5 min, 150 µL of 10% $AlCl_3$ solution was added, and 6 min later, 500 µL of 1 M NaOH and 275 µL of MQ were added to the mixture, which was then thoroughly mixed. The intensity of the pink color of the solution was measured at 510 nm using a UV/Vis spectrophotometer (Shimadzu UV-1650 PC, Shimadzu, Tokyo, Japan). (+)-Catechin was used to generate the standard curve (0.022 - 0.34 mM). The flavonoid content was expressed as mg of (+)-catechin equivalents (CEs) per g of extract.

The β-carotene and lycopene contents of the extract were determined according to a method used in a previous study[17]. The dried extract (100 mg) was vigorously shaken with 10 mL of an acetone-hexane mixture (4:6) for 1 min and filtered through Whatman No. 4 filter paper. The absorbance of the filtrate was measured at 453, 505, and 663 nm. The amounts of β-carotene and lycopene were calculated using the following equations:

$$\text{Lycopene (mg per 100 mL)} = -0.0458_{A663} + 0.372_{A505} - 0.0806_{A453}$$

$$\beta\text{-carotene (mg per 100 mL)} = 0.216_{A663} - 0.304_{A505} + 0.452_{A453}$$

The results of these analyses were expressed as µg of carotenoid per g^{-1} of extract.

Fourier-transform infrared (FT-IR) spectroscopy

FT-IR spectra were obtained using an Agilent Cary 630 spectrophotometer (Agilent Technologies, Wilmington, DE, USA) equipped with a single bounce diamond ATR module for potassium bromide (KBr) and the MicroLab and Resolutions Pro Software. FT-IR spectra were obtained in transmission mode in the 350-6,300 cm^{-1} range, at a resolution of 2 cm^{-1}.

Cell culture

HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 10 µg/100 mL penicillin/streptomycin in a 5% CO_2 atmosphere at 37 °C. For the migration assay, HaCaT cells were stimulated with TNFα (10 ng/mL) and INFγ (10 ng/mL). After 6 h of stimulation, cells were harvested, and total RNA and protein were isolated.

Animals

Eight week-old female BALB/c mice were purchased from Samtako BioKorea Co., Ltd. (Osan, Republic of Korea) and housed under specific pathogen-free conditions. All experiments were approved by the Institutional Animal Care and Use Committee of Konkuk University (Case # KU14011).

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Induction of AD lesions in the ear

AD was induced in the mice by repeated local DFE (house dust mite extract) and DNCB exposure on the ears, as previously described[18]. For AD induction, the mice were divided into four groups (control, AD-only, AD + CM extract: 250 mg/kg/day, and AD + CM extract: 500 mg/kg/day). The surfaces of both earlobes were stripped five times using surgical tape (Nichiban, Tokyo, Japan). After stripping, 20 µl of 1% DNCB was painted on each ear, followed 4 days later with 20 µl of DFE (10 mg/mL). DNCB or DFE treatment was administered alternately once per week for 4 weeks. The animals received the CM extract (250 or 500 mg/kg/day orally) throughout the 4 weeks of AD induction.

Ear thickness was measured 24 h after DNCB or DFE application by using a dial thickness gauge (ID-C1012XBS, Mitutoyo Corp., Kawasaki, Japan). At days 14 and 28, blood samples were collected using orbital puncture. Plasma samples were prepared from the blood samples and stored at −70 °C for further analysis. After blood collection, the ears were removed and used for histopathological analysis. Serum immunoglobulin (IgE) and IgG2a levels were measured at days 14 and 28 after the first induction by using an IgE enzyme-linked immunosorbent assay kit (Bethyl Laboratories, Inc., Montgomery, TX, USA) according to the manufacturer's instructions.

Histological observations

The excised ears were fixed in 4% paraformaldehyde for 16 h and embedded in paraffin. Subsequently, thin (6 µm) ear sections were stained with hematoxylin and eosin (H&E), and the epidermal and dermal thickness were measured under a microscope. Microphotographic images were obtained at a magnification of 100 ×. To measure mast cell infiltration, skin sections were stained with toluidine blue, after which the number of mast cells was counted in five randomly chosen fields of view.

Analysis of mRNA expression

For the reverse transcription polymerase chain reaction (RT-PCR), the total cellular RNA was isolated from the ear tissue of the mice in each treatment group using TRIzol reagent according to the manufacturer's protocol[18]. The first-strand complementary DNA (cDNA) was synthesized using Superscript II reverse transcriptase (Invitrogen). The conditions used for RT-PCR were similar to those previously described in related studies[18]. Quantitative PCR (qPCR) was performed in triplicate by using 12.5 µL of SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan) and 2 µL of cDNA as a template in a final volume of 25 µL. Prior to being subjected to 40 cycles of amplification, the mixture was incubated for 15 min at 95 °C. The denaturation was performed for 30 s at 95 °C, annealing was performed in a transitional temperature range from 58 °C to 62 °C, with an increase of 0.5 °C per cycle, and the extension was performed for 30 s at 72 °C with fluorescence detection at 72 °C after each cycle. After the final cycle, melting-point analyses of all the samples were performed within a temperature range from 65 °C to 95 °C, with continuous fluorescence detection. Target gene mRNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels using the following formula:

$$\text{Relative mRNA expression} = 2^{-(\text{Ct of target gene} - \text{Ct of GAPDH})},$$

where *Ct* is the threshold cycle value. In each sample, the expression level of the analyzed gene was normalized to that of GAPDH and presented as a relative mRNA level.

Statistical analysis

Statistical analysis was carried out using SAS statistical software (Release 9.1.3, SAS Institute, Cary, NC, USA). Multiple group data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple range test. All results were expressed as the mean ± standard deviation of comparative fold differences. Data are representative of three independent experiments. The threshold for statistical significance was set at $P<0.05$.

RESULTS

Partial chemical characterization of CM

Confirmatory results of the nutritional value of the CM extract are shown in Table 1. The total carbohydrate and

Table 1. Carbohydrate, monosaccharide, total protein, amino acid, and selected phytochemical content in the aqueous extract of *Cordyceps militaris* (CM)

Constituents		Content
Total carbohydrates (µg/g)		419.21±1.91
Monosaccharides (µg/mL)	Arabinose	0.41 ± 0.03
	Galactose	3.26 ± 0.22
	Glucose	264.01 ± 4.10
	Mannose	6.36 ± 1.29
	Xylose	3.51 ± 0.82
Total protein (µg/g)		338.92 ± 1.91
Amino acids (g/100 g)	Asp	2.8737
	Thr	1.3488
	Ser	1.0166
	Glu	3.1316
	Pro	1.3925
	Gly	0.9519
	Ala	1.2848
	Val	1.1854
	Ile	0.4527
	Leu	0.8631
	Tyr	1.2787
	Phe	0.4766
	Lys	2.2942
	His	0.6461
	Arg	1.2327
	Cys	0.4193
	Met	1.2897
Phytochemicals	Polyphenols (mg/g)	19.79 ± 1.02
	Flavonoids (mg/g)	275.52 ± 11.01
	β-Carotene (µg/g)	24.51 ± 0.25
	Lycopene (µg/g)	3.42 ± 0.09

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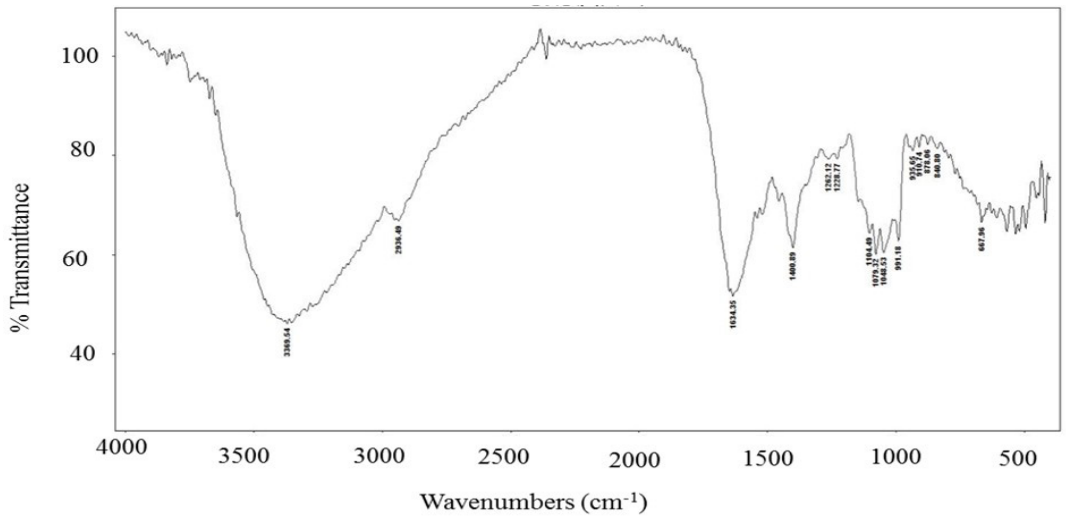

Figure 1. FT-IR spectrum of the crude aqueous extract of *Cordyceps militaris* (CM).

monosaccharide composition of the CM aqueous extract was determined in our series of experiments. The aqueous CM extract contained 419.21 ± 1.91 μg/g of total carbohydrate. The monosaccharide constituents of the extract were primarily glucose (264.01 ± 3.22 μg/mL) and mannose (6.36 ± 1.29 μg/mL) (Table 1). Furthermore, our results demonstrated that the CM aqueous extract was rich in amino acids. It contained 338.92 ± 1.91 μg/g of total protein with the primary amino acid being glutamic acid (3.1316 g/100 g) as shown in Table 1. The results of the phytochemical screening are provided in Table 1. Polyphenols were the primary constituents of the extract (19.79 ± 1.02 mg/g), followed by flavonoids (275.52 ± 11.01 μg/g). The contents of β-carotene and lycopene were 24.51 ± 0.25 μg/g and 3.42 ± 0.09 μg/g, respectively.

The FT-IR spectrum of the CM extract is shown in Figure 1. The bands at 3369 cm^{-1} indicate hydroxyl stretching in hydrogen bonds and N–H vibrations. The band at 2936 cm^{-1} was attributed to the C–H stretching vibration, whereas the band at 1634 cm^{-1} indicated asymmetric vibration of the carboxylic group. The peak at 1400 cm^{-1} was associated with the typical stretching frequencies of the OH groups of phenolic compounds. Peaks at 1104, 1079, and 1048 cm^{-1} were associated with the C–O–C linkages of sugars remaining in the extract[25]. Our data are consistent with those of other researchers who reported that the bands at 3,405 and 2,930 cm^{-1} were attributed to OH and C–H bond stretching, respectively, while bands at 1,420 and 1,366 cm^{-1} arose from the bending modes of CH_2, CH, and OH[26]. Intense overlapping bands in the region of 800-1300 cm^{-1} correspond to CO, CC stretching, and COH bending modes.

Inhibitory effect of CM on inflammatory stress in stimulated keratinocytes

The ability of CM to block the effects of the pro-inflammatory mediators TNFα (10 ng/mL) and IFNγ (10 ng/mL) in HaCaT keratinocytes was assessed by using RT-PCR (Figure 2A). The CM extract reduced the mRNA expression levels of TNFα, chemokine C-C motif ligand 17 (CCL17), interleukin-1β (IL-1β), and interleukin-6 (IL-6) following TNFα/IFNγ-induction in a dose-dependent (0.25, 0.5, and 1.0 mg/mL) manner. This indicated that the CM extract could inhibit chemoattractant and pro-inflammatory cytokine production.

Intracellular signaling induced by CM was investigated by examining the phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun NH2-terminal kinases (JNKs), and p38 in TNFα/IFNγ-induced HaCaT cells. The expression levels of phosphorylated ERK1/2 and p38 were increased in cells treated with TNFα/IFNγ. Furthermore, pretreatment with CM extract suppressed the phosphorylation of ERK1/2 and p38 in TNFα/IFNγ-stimulated HaCaT cells (Figures 2B, 2C). However, neither the CM extract nor the positive control suppressed JNK activation (Figure 2D), suggesting that the CM extract only blocked the activation of ERK1/2 and p38 MAP kinase. Moreover, the inhibition of AD by the CM extract was mediated through inhibition of the overexpression of pro-inflammatory cytokine-related mRNA associated with p38 and ERK1/2 but not with JNK activation, in HaCaT cells.

CM reduced AD symptoms in a murine model

After orally administering the CM extract to mice, ear thickness was examined (Figure 3). While the ear thickness and AD lesions significantly increased following the application of DFE and DNCB, the CM extract led to a reduction in DFE/DNCB-induced ear thickness (Figure 3A, 3B). Thickening of the epidermis and dermis was also observed using a microscope, and a significantly reduced epidermal and dermal thickness was observed in a dose-dependent manner in the CM extract-treated group than in the AD control group (Figures 3C, 3D).

Allergic responses associated with mast cells originate in myeloid stem cells. Therefore, a histological analysis of atopic ears was performed to examine its correlation with the visual evaluation of AD symptoms. Excised ears from

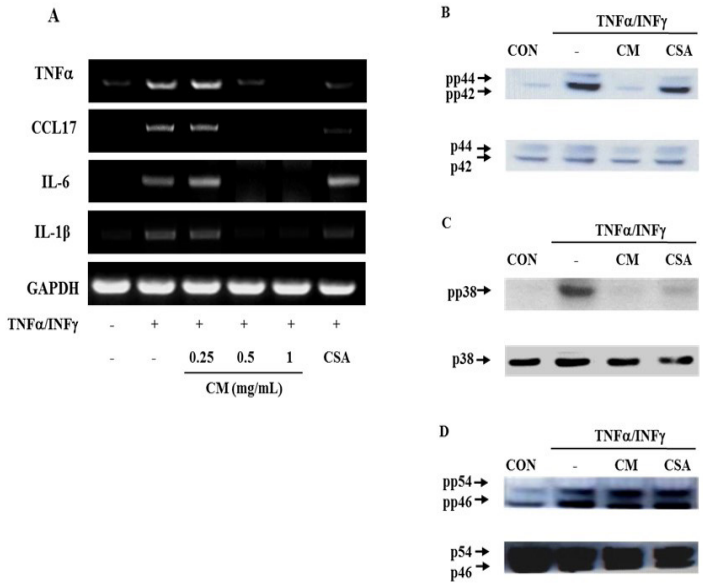

Figure 2. (A) Gene expression levels of inflammatory cytokines (TNFα, CCL17, IL-1β, and IL-6) measured by conventional RT-PCR. Total protein profiles after 6 hours of stimulation measured by phosphorylation of (B) ERK1/2, (C) p38, and (D) JNK for each treatment group. The results were similar in three independent experiments. CON, control; CM, *Cordyceps militaris*; CSA, cyclosporin.

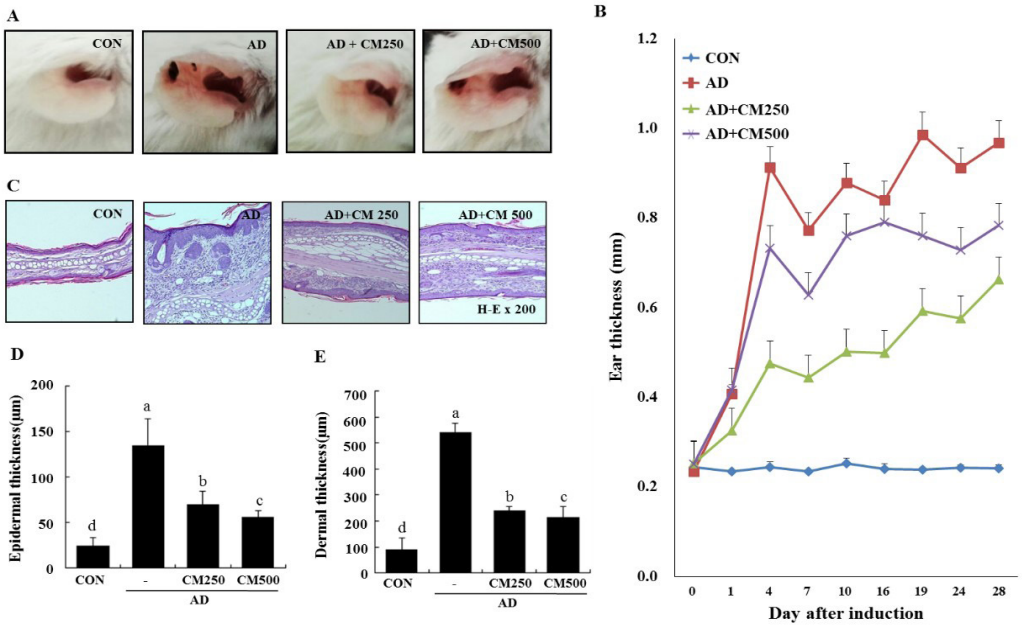

Figure 3. (A) Photographs of the ears of mice from each group on day 28. (B) Ear thickness was measured with a dial thickness gauge every 3 days after 2,4-dinitrochlorobenzene (DNCB) or *Dermatophagoides farinae* extract (DFE) application. From (C) microphotographs of hematoxylin and eosin-stained-tissue, (D) epidermal and (E) dermal thickness were measured. Data is presented as the mean ± SD of triplicate determinations. Means with different letters (a-d) within a graph are significantly different from each other at $P<0.05$. The pictures shown are representative of each group (n = 3-6). The original magnification was 200×. CON: control; AD: atopic dermatitis; CM250, *Cordyceps militaris* 250 mg/kg/day; CM500, *Cordyceps militaris* 500 mg/kg/day.

each group were stained with toluidine blue to identify infiltration by mast cells. Compared with the AD control group, the CM extract-treated group showed a significantly reduced number of infiltrated immune cells, such as mast cells ($P<0.05$) (Figures 4A, 4B).

The CM extract-treated mice had significantly reduced total IgE and IgG2a levels in comparison with those of the DFE/DNCB-treated mice ($P<0.05$) (Figure 4C, 4D). These data suggest that the potential therapeutic effect of the CM extract in AD progression is related to the downregulation of serum Ig levels.

To further explain the fundamental mechanism underlying the effects of the CM extract, the mRNA expression levels of AD-related pathogenic cytokines were examined

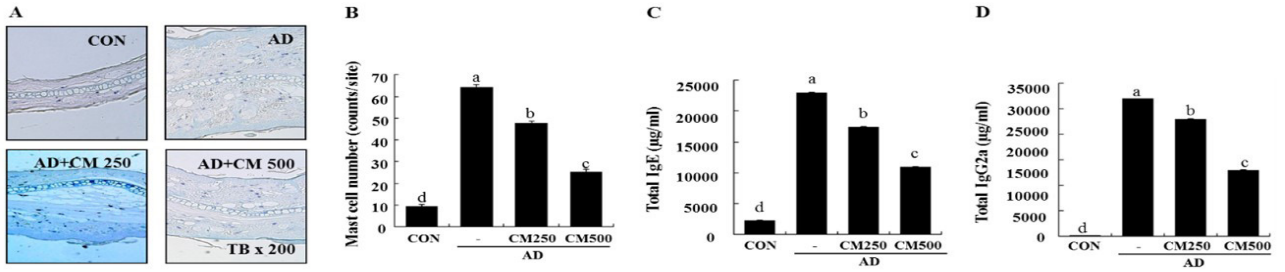

Figure 4. Histopathological and serum analysis following treatment with CM extract and control groups of mast cell infiltration and immunoglobulin concentration. (A) toluidine blue staining, (B) The number of infiltrated mast cells (B). On day 28, (C) plasma IgE and (D) IgG2a levels quantified by enzyme-linked immunosorbent assay. Data is presented as the mean ± SD of triplicate determination. Means with different letters (a-d) within a graph are significantly different from each other at $P<0.05$. Individual microphotographs are representative of each group (n = 3-6). CON, control; AD: atopic dermatitis; CM250, *Cordyceps militaris* 250 mg/kg/day; CM500, *Cordyceps militaris* 500 mg/kg.

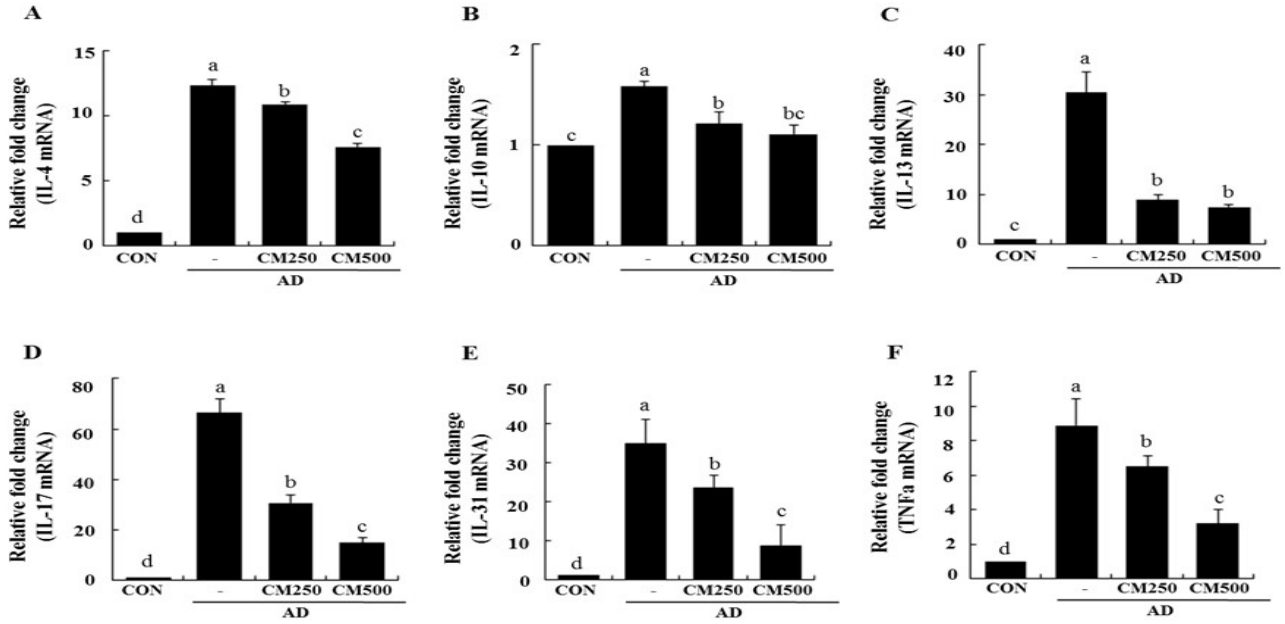

Figure 5. Effect of *Cordyceps militaris* (CM) extract on the expression of various pathogenic factors including (A) IL-4, (B) IL-10, (C) IL-13, (D) IL-17, (E) IL-31, and (F) TNFα in the ear tissue. Data is presented as the mean ± SD of triplicate determination. Means with different letters (a-d) within a graph are significantly different from each other at $P<0.05$. CON, control; AD: atopic dermatitis; CM250, *Cordyceps militaris* 250 mg/kg/day; CM500, *Cordyceps militaris* 500 mg/kg/day.

in ear tissues by using qPCR. All cytokine-related mRNAs that were tested were upregulated in ear tissues from the AD group. The CM extract significantly reduced Th2 cytokines and Th1-related cytokine TNFα in ear tissue in a dose-dependent manner (Figure 5).

DISCUSSION

Edible mushrooms are an attractive source of many different nutraceuticals, such as polysaccharides, polyphenols, peptides, lycopenes, and carotenoids[19]. CM extracts are a rich source of proteins, carbohydrates, and selected phytochemicals. A previously published report identified four different exopolysaccharides with molecular masses ranging from 50 kDa to 2260 kDa from a newly isolated Cordyceps species, *Cordyceps militaris* NG3[20]. Furthermore, another research group isolated four polysaccharides from CM[21], which interrupted the progression of hepatocellular carcinoma while also demonstrating anti-inflammatory properties in rodents[22]. Therefore, considering the pharmacological activity of CM, the determination of the carbohydrate content of the CM extract is a meaningful scientific advancement. The identified polyphenolic compounds include several different subclasses including flavonoids and phenolic acids that display a large diversity of structures and functions. Mushroom fruiting bodies are reportedly rich in polyphenols and carotenoids, and possess a variety of pharmacological activities, such as antioxidant and anti-inflammatory activities, in biological systems[23]. To further our understanding of the pharmacological activity of CM and its polyphenol and flavonoid content, the main compounds contributing to the

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antioxidant properties of mushrooms as well as the levels of two carotenoids (β-carotene and lycopene) were determined. Our data are consistent with those of another study that also determined the polyphenol, β-carotene, and lycopene content of dried, edible mushrooms[24].

The biological effects of cytokines on keratinocytes demonstrate the fact that keratinocytes are direct targets of specific cytokines involved in skin inflammation[27]. Furthermore, pro-inflammatory cytokines, such as the interleukin (IL) family cytokines, are important regulators of epidermal functions, as their receptors have been detected in keratinocytes[28]. In addition, IFNγ is associated with the maintenance of chronic AD by increasing the expression of chemoattractants CCL17 (TARC) and CCL22 (MDC), which are related to the production of effector T cells at the inflamed site[29]. The CM extract reduced TNFα, CCL17, IL-1β, and IL-6 mRNA expression against TNFα/IFNγ-induction, suggesting that it inhibits chemoattractant and pro-inflammatory cytokine production.

Specific intracellular processes are involved in the recognition and response to extracellular stimuli, such as the signaling cascade resulting in activation of mitogen-activated protein kinases (MAPKs). The most widely studied groups of MAPKs are the ERK1/2, JNKs, and p38 kinases, which play a significant role in chronic inflammatory disease processes and skin homeostasis[30]. In the present study, the CM extract only blocked the activation of ERK1/2 and p38 MAP kinase but did not affect the activation of JNK in HaCaT cells.

In order to evaluate the immunomodulatory activity of the CM extract *in vivo*, the extract was orally administered to AD-induced mice. Experimental AD was induced on both earlobes of BALB/c mice by alternative weekly painting of DNCB and mite extract for 4 weeks. DFE and DNCB application substantially increased ear thickness and the appearance of AD lesions; however, the CM extract-treated group showed significantly reduced epidermal and dermal thickness compared to the AD control group.

AD is associated with elevated serum IgE levels and sensitization to a variety of environmental allergens[31]. The cross-linking of IgE and the allergen activates mast cells at the sites of allergic reactions, which leads to the release of chemical mediators, including histamine and cytokines[32]. Furthermore, AD is associated with the typical Th2 type immune response, which shows elevated total IgE serum levels and Th2 type cytokine expression[33]. Elevated total IgE serum levels are a strong risk factor for AD in children from birth to six years of age, and are associated with persistent eczema in adults[34]. In contrast, higher total IgG levels have been linked with the Th1 response[25]. To determine whether the CM extract exerts its effects primarily via the Th1 or Th2 response, the serum levels of IgE (total and DFE-specific) and IgG2a were measured in each group following treatment completion. The results of this study suggest that the therapeutic effects of the CM extract in AD are related to the downregulation of serum Ig levels.

Previous reports studying the beneficial effects of the Cordyceps species in various inflammatory diseases include studies of ethanolic extracts of cultured fruiting bodies and mycelia of CM, which exhibited anti-inflammatory activity in the croton oil-induced ear edema test in mice[35].Other studies using treatment with water extract of *C. sinensis* reported down-regulation of inflammation-related genes in the rat kidney[36]. Finally, a CM hot water extract inhibited lipopolysaccharide (LPS)-stimulated nitric oxide (NO) production, and TNFα and IL-6 release in RAW 264.7 cells[37]. Our results are consistent with these previous reports. Furthermore, mushroom extracts suppressed the overexpression of Th2 and Th1-related cytokines[38]. Thus, our results demonstrating that CM extract inhibits the expression of Th1 and Th2 cytokines are consistent with those of previous reports. These data support the therapeutic use of CM extract when treating both the acute and chronic stages of AD.

This study identified the nutraceutical composition and therapeutic effects of the aqueous extract of CM against AD *in vitro* and *in vivo*. CM extract inhibited TNF-α, CCL17, IL-1β, and IL-6 mRNA production and reduced ERK1/2 and p38 expression in HaCaT keratinocytes. Oral administration of the CM extract decreased the severity of AD, including histopathological signs, and significantly reduced the production of Ig and expression of pathogenic cytokines in DFE/DNCB-induced BALB/c mice. Therefore, our results indicate that the aqueous extract of CM could be utilized as an effective complementary alternative medicine to prevent or treat atopic dermatitis symptoms.

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