Inhibition of 2,4-dinitrofluorobenzene-induced atopic dermatitis by topical application of the butanol extract of *Cordyceps bassiana* in NC/Nga mice

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**A B S T R A C T**

*Ethnopharmacological significance:* The *Cordyceps* species are insect-borne mushrooms that have been ethnopharmacologically used for skin diseases such as eczema and dermatitis.

*Aim of the study:* In this study, we investigated the curative effects of the butanol fraction (CBBF) of *Cordyceps bassiana* on atopic dermatitis.

**Materials and methods:** Dermatitis was induced by repeated application of 2,4-dinitrofluorobenzene (DNFB) in NC/Nga mice. After a topical application of CBBF on the skin lesions, the dermatitis score, epidermal thickness, mast cell number, and interleukin (IL)-4 and interferon (IFN)-γ, as well as the levels of histamine and immunoglobulin E (IgE) in the serum, were measured. Moreover, effect of CBBF on histamine release was examined using RBL-2H3 under stimulation with 2,4-dinitrophenylated bovine serum albumin (DNP-BSA).

**Results:** CBBF inhibited atopic dermatitis symptoms and signs in the DNFB-treated NC/Nga mice. The suppressive activity of topically applied CBBF may be due to the dose-dependent blockade of a series of immunopathological events, including the release of histamine, the production of IgE, and the secretion of IL-4 and IFN-γ. However, this extract did not directly suppress the degranulation process, assessed by measuring β-hexosaminidase release.

**Conclusions:** Our results suggest that CBBF can be applied as an effective herbal remedy to treat atopic dermatitis.

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**1. Introduction**

Atopic dermatitis is a chronic inflammatory skin disease that presents with rash and severe itching. The prevalence of the disease, 10–20% in children and 1–3% in adults at the present time, is increasing (Julge et al., 2002; Kemp and Bjorksten, 2003). Although the pathophysiological mechanism of the disease is not clearly understood, the over-activation of immune cells, including macrophages, mast cells, and T-lymphocytes, infiltrating the skin lesions are regarded as the major cause (Uehara and Sawai, 1989; Cantani, 2001). It has been proposed that, depending on the disease stage, both Th2-type and Th1-type cells participate in the immuno-genesis of atopic dermatitis (Leung et al., 2004; Tomimori et al., 2005). Th2-type cells are reported to produce cytokines including interleukin-4 (IL-4), IL-5 and IL-10 (Forbes et al., 2010). These cytokines play a critical role in inducing the IgE isotype switch in B-lymphocytes, which is linked to the activation of mast cells to secrete inflammatory substances such as histamine (Ji, 2009). On the other hand, Th2 cytokines also stimulate eosinophils to secrete IL-12, which activates Th1 type cells and releases the interferon-γ (INF-γ) that is responsible for the severity of the skin disease (Wegmann, 2009).

The *Cordyceps* species are insect-born mushrooms that have been known to possess ethnopharmacologically active material in Korea, China and Japan (Ng and Wang, 2005). Thus, the mushroom has been used as a tonic for longevity, endurance and vitality, and it has been used as a therapeutic remedy for various diseases such as eczema, skin diseases, chronic bronchitis, asthma, and tuberculosis (Ng and Wang, 2005; Zhou et al., 2009). This mushroom has also been reported to display numerous pharmacologic activities,
Fig. 1. The effects of *Cordyceps bassiana* extracts on the pathological signs of atopic dermatitis in DNFB-treated NC/Nga mice. (A) Atopic dermatitis was induced by repeated application of DNFB on the dorsal skin twice a week for four weeks. The ethanol extract of *Cordyceps bassiana* (CBEE) or the butanol fraction of CBEE (CBBF) was homogenized in vaseline at a concentration of 5 mg/g, 10 mg/g and 20 mg/g, respectively, and 0.5 g of the homogenate was topically applied on the skin lesion twice a day for 12 days. The same amount of vehicle was applied on the skin lesion in the atopy control group. Dermatitis scores were then measured. (B) Epidermal thickness was also measured from tissue sections of the skin lesion, which were prepared from CBEE or CBBF-treated atopy mice. (C) Photos of the CBBF-treated atopic dermatitis mice and their tissue sections of skin lesion stained with hematoxylin-eosin were prepared with a digital camera. Data are presented as the mean ± S.E. of six mice. CBBF significantly and dose-dependently reduced the dermatitis score and the epidermal thickness. Original magnification ×100, Scale bar = 100 μm. **p < 0.01 indicates statistical significance compared to the atopy control group. NC: normal control, AC: atopy control.

such as anti-oxidative, anti-viral, anti-cancer, anti-fibrotic, anti-inflammatory, anti-nociceptive, anti-angiogenic, and anti-diabetic activities (Zhang, 1990; Kim et al., 2006; Zhou et al., 2009). *Cordyceps bassiana* Z.Z. Li, C.R. Li, B. Huang & M.Z. Fan is a mushroom, a species of the Genus Cordyceps, which also parasitizes in larvae of insects in the Order Lepidoptera (Obornik et al., 2001). No documentation on the biological activity of *Cordyceps bassiana* had been published until the fruit body of *Cordyceps bassiana* was artificially cultivated. Recently, it has been reported that the ethanol extract of the artificially cultivated *Cordyceps bassiana* inhibits the production of nitric oxide, IL-12 and IFN-γ in lipopolysaccharide (LPS)-stimulated macrophages and splenic lymphocytes (Byeon et al., in press-a), suggesting the possibility that *Cordyceps bassiana* may contain anti-inflammatory and atopic dermatitis regulatory substances.

Because few drugs (e.g., corticosteroids and anti-histamines) for the treatment of atopic dermatitis have been clinically available, numerous research groups have tried to develop safe and strong curative medications. Only a few new candidate materials from natural products have been proposed to be useful for treatment of the disease. These include an extract from *Astragalus membranaceous* Bunge (Lee et al., 2007), silymarin from *Silybum marianum* L. Gaertn (Kang et al., 2008), and fermented barley extract (Iguchi et al., 2009).

With the goal of developing a novel anti-atopic dermatitis remedy, this study aimed to evaluate the possibility that artificially cultivated *Cordyceps bassiana* exhibited anti-atopic dermatitis activity in vivo. For this purpose, 2,4-dinitrofluorobenzene (DNFB) was topically applied to the skin of NC/Nga mice to induce atopic dermatitis (Kitagaki et al., 1997; Yagi et al., 2002; Tomimori et al., 2005).

2. Materials and methods

2.1. Preparation of the butanol fraction from the fruit body of *Cordyceps bassiana*

Artificially cultivated *Cordyceps bassiana* was kindly donated by MushTech (Gangwon-do, Korea). Fresh fruiting bodies of *Cordyceps bassiana*, which were grown on brown rice, were dried at 50 °C and crushed in a blender. The crude powder was extracted with ethanol at 80 °C for 3 h. The ethanol extract of *Cordyceps bassiana* (CBEE) was concentrated in a rotary evaporator at 50 °C. The concentrated
extract was suspended in distilled water and then successively frac-
tionated with equal volumes of n-hexane followed by n-butanol. The n-butanol fraction of Cordyceps bassiana (CBBF) was evaporated and then freeze-dried after suspension in distilled water. Yield of CBBF was 4.8 ± 1.3% in three different extractions.

2.2. Induction of atopic dermatitis and treatment with CBBF in experimental animals

Animal use and relevant experimental procedures were approved by the Institutional Animal Care and Use Committee at Hallym University (Hallym 2009-36). Male NC/Nga mice, 6 weeks of age, were purchased from SLC (Shizuoka, Japan) and maintained under conventional conditions. Mice were housed in an air-conditioned room at 22 ± 2 °C and relative humidity of 55 ± 5% and were provided a laboratory diet and water. For induction of atopic dermatitis, 150 μl of 0.2% DNFB in acetone/olive oil (3:1) was repeatedly applied on the shaved skin of the dorsal area 2 times a week for 4 weeks (Tomimori et al., 2002). After the induction of atopic dermatitis, 0.5 g of CBBF, homogenized in vaseline at a concentration of 5, 10 and 20 mg/g, was topically applied on the skin lesion 2 times a day for 12 days. Vehicle alone was pasted...
on the skin lesion in the atopy control group. Neither extract in vehicle nor vehicle alone was applied on the skin in the normal control group. On the last day of the experiment, the severity of the skin lesion was scored by three people, and then blood was drawn by orbital puncture. After sacrifice by cervical dislocation, the skin sample at the size of $0.5 \times 1.0$ cm was taken from the lesion.

2.3. Determination of serum concentrations of IgE and histamine

Serum concentrations of IgE and histamine were determined with an IgE ELISA kit (Shibayagi, Japan) and histamine ELISA kit (Biochemical Research, MI, USA), respectively, according to the manufacturers’ instructions.

2.4. Histological observation of the skin lesion

The skin sample was fixed with 10% buffered formaldehyde solution and then embedded in paraffin. Sections of the skin at 4 $\mu$m thickness were stained with either hematoxylin–eosin to observe the pathological changes or toluidine blue to see mast cells infiltrating the lesion. The number of mast cells in 5 sites chosen at random was counted.

The immunoreactivity of IL-4 and INF-$\gamma$ in the skin lesion was stained according to the method reported previously (Yagi et al., 2002). The primary antibodies for IL-4 (11B11) and INF-$\gamma$ (XMG1.2) were purchased from Biolegend (CA, USA), and the biotinylated secondary antibody (PK-6104) was from Vector Laboratories (Burlingame, CA, USA). After incubation in Vectastain ABC
reagents, we treated RBL-2H3 cells with DNP-labeled IgE (Byeon et al., 2009). RBL-2H3 cells (2 × 10^5 cells/well) pretreated with CBEE or azelastine (Sigma, St Louis, MO) were sen-
sitized with 1 μg/ml anti-DNP-IgE for 60 min at 37 °C. IgE-sensitized cells were washed with PBS. The IgE-sensitized cells were stimu-
lated with 4 μg/ml of 2,4-dinitrophenylated bovine serum albumin (DNP-BSA) or BSA in PBS at 37 °C for 30 min. Degranulation was
assessed by measuring β-hexosaminidase release. Briefly, 40 μl of
supernatant or cell lysates and 100 μl of 2 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide (in 0.4 M citrate and 0.2 M phosphate
buffer, pH 4.5) were added to each well of a 96-well plate, and
color was developed for 30 min at 37 °C. The enzyme reaction was
terminated by adding 200 μl of 0.2 M glycine–NaOH at pH 10.7.
The absorbance at 405 nm was measured using the SpectraMAX 250 microplate reader.

2.5. Hexosaminidase secretion assay

The RBL-2H3 cells were grown in DMEM supplemented with
10% FBS in a 5% CO2-containing atmosphere as described pre-
viously (Byeon et al., 2009). RBL-2H3 cells (2 × 10^5 cells/well) were
pretreated with CBEE directly modulated histamine release from activated mast
cells, we treated RBL-2H3 cells with DNP-labeled IgE (Byeon et al.,
2009), and their degranulation events were continuously followed.
As Fig. 2D shows, we found no inhibition of degranulation by mea-
suring β-hexosaminidase release, which is required for histamine release
(Enoki et al., 2004), implying that CBEE cannot directly block
histamine release from mast cells but can modulate the upstream
pathway for mast cell activation.

2.6. Statistical analysis

All results were illustrated as the mean±SE. The data were
analyzed using the Student’s t-test. Differences were considered
significant when the P value was less than 0.05.

3. Results and discussion

Because the Cordyceps species has been ethnopharmaceutically
used for a long time and Cordyceps bassiana, one of the species
(Ng and Wang, 2005), has been reported to display in vitro a modula-
tory effect on the production of cytokines and inflammatory
mediators critically involved in the pathophysiology of atopic der-
matitis (Byeon et al., in press-a), the in vivo curative effects of
extracts prepared from artificially cultivated Cordyceps bassiana
was elucidated in this study.

Fig. 1 shows that CBEE but not CBBF was able to exhibit anti-
atopic dermatitis activity. Thus, CBEE very strongly suppressed the
DNFB-induced atopic dermatitis measured by the dermatitis
score in a dose-dependent manner (Fig. 1A, right panel). A sim-
ilar inhibitory effect was seen in the measurement of epidermal
thickness. Namely, the DNFB-induced increase of epidermal thick-
ness was dose-dependently inhibited by CBEE treatment by up to
75% (Fig. 2B). Indeed, the inhibitory activity was clearly observed in
the photos of DNFB-treated NC/Nga mice (Fig. 1C). To understand
its inhibitory mechanism, we chose a representative symptom-
inducing substance such as histamine (Hammerberg et al., 2001)
to test whether CBEE was capable of suppressing the release of this
molecule. Interestingly, this fraction inhibited the enhanced level
of histamine induced by DNFB by 50% (Fig. 2A). Furthermore, CBEE
significantly suppressed the numbers of mast cells infiltrating in
the skin lesions of the atopic dermatitis mice by up to 40% (Fig. 2B
and C), suggesting that the activation and migration of mast cells
may be a target immunopharmacology of CBEE. To show whether
CBEE directly modulated histamine release from activated mast
cells, we treated RBL-2H3 cells with DNP-labeled IgE (Byeon et al.,
2009), and their degranulation events were continuously followed.
As Fig. 2D shows, we found no inhibition of degranulation by mea-
suring β-hexosaminidase release, which is required for histamine release
(Enoki et al., 2004), implying that CBEE cannot directly block
histamine release from mast cells but can modulate the upstream
pathway for mast cell activation. It has been known that mast cell activation and their histamine
release are tightly modulated by IgE from B cells (Dvorak et al.,
1985). Therefore, we measured the serum IgE levels in the DNFB-
treated mice. As shown in Fig. 3A, DNFB enhanced the level of
serum IgE by up to 5-fold. Expectedly, CBEE but not CBBF strongly
and dose-dependently suppressed IgE production stimulated by
DNFB treatment. Since IgE production is managed by B cells stimu-
lated with IL-4 (Oettgen, 2000), the level of IL-4 in skin lesions
was also checked. Fig. 3B shows that DNFB caused an increase in
IL-4-positive Th2 cells, while CBEE treatment dramatically down-
regulated the levels of those cells. In agreement with these results,
the extract also suppressed the level of IFN-γ-positive Th1 cells
in the skin lesions, suggesting that CBEE can effectively block the
production of IL-4 and IFN-γ from both Th1 and Th2 cells. Further
inhibitory mechanisms will be explored in the future to explain
how CBEE suppresses IL-4 production.

The mechanism by which CBEE is able to suppress both IL-4
and IFN-γ production was not elucidated in this study. CBEE can
strongly suppress the production of IL-12, a Th1 differentiation-
stimulatory cytokine, from LPS-activated macrophages (Byeon
et al., in press-a), and the LPS-induced IFN-γ production in spleno-
cytes was remarkably diminished by CBEE (Byeon et al., in press-b),
leading us to consider the important role of antigen presenting cells
such as macrophages or dendritic cells that control the IL-12 level.
Furthermore, we also demonstrated that the inhibition of IL-12 pro-
duction by CBEE was Syk-, Src- and JAK-dependent (Byeon et al.,
in press-b). These data indicate that the inhibition of IFN-γ and
Th1 differentiation may be due to the suppression of IL-12 release
from antigen presenting cells through a block on upstream kinases.
Nonetheless, the mechanism by which CBEE is capable of reducing
IL-4 production has not yet been explained. Since IL-4 production
from normal splenocytes during concanavalin A exposure was not
blocked by CBEE (Byeon et al., in press-b), it may be assumed that
the CBEE-mediated inhibition of IL-4 production might occur dur-
ing the initial stage of Th2 differentiation that is managed by a
balance of transcription factors such as T-bet and GATA-3 (Afkarian
et al., 2002; Cho et al., 2003). Otherwise, the indirect modulation of
tumor growth factor-β and IL-10-producing regulatory T cell
(Treg) activity (Wilczynski et al., 2008) might be considered another
potential mechanism of CBEE. Therefore, detailed study of relevant
mechanisms will be pursued in future projects.

What is the major component of CBFF with anti-atopic der-
matitis property has not been elucidated yet. The HPLC profile of
CBFF indicated that cordycepin, a major active component found
in Cordyceps militaris, was not included in the butanol fraction
(Byeon et al., in press-b). It has been reported that water sol-
able nucleosides and their derivatives such as cordycepic acid,
3′-amino-3′-deoxyadenosine, and homocitrullyl aminoadenosine,
have been identified as active principles in Cordyceps species (Das
et al., in press; Paterson, 2008). In addition, ophiocordin, ergostrol,
and cordyheptapeptide with anti-fungal activity were also isolated from
Cordyceps species (Huang et al., 2003). Since these com-
ponents can be extracted in the butanol layer, a possibility that these
can act as anti-atopic dermatitis principles should be tested with
exact phytochemical study.

In conclusion, this study demonstrated that the butanol extract of
Cordyceps bassiana was able to inhibit atopic dermatitis symp-
toms in DNFB-treated NC/Nga mice. The suppressive activity of
topically applied CBFF may be due to the interruption of a series
of immunopathological events, including the release of histamine,
the production of IgE, and the secretion of IL-4 and IFN-γ. Topical
application of CBFF at doses employed in the present study seems
to be safe because CBFF, given orally, showed wide margin (from
2.5 g/kg to 10 g/kg) of safety in acute toxicity in ICR mice (Park et al.,
2008). In addition, ophiocordin, ergostrol, and cordyheptapeptide with anti-fungal
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2.5 g/kg to 10 g/kg) of safety in acute toxicity in ICR mice (Park et al.,
2008). Furthermore, Bombyx corpus, a silkworm died of muscar-
dine disease, has been used to treat eczematous skin eruptions long
Cordyceps bassiana of Korea. The causative agent of *Bombyx mori* is *Beauveria bassiana* (Bals.) Vuill. that is the same fungus as that of *Cordyceps bassiana*. Therefore, our results suggest that CBBF can be applied as an herbal remedy to treat atopic dermatitis.

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