

Evaluation of Anti-atopic Dermatitis Activity of *Hypsizigus marmoreus* Extract

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Hypsizigus marmoreus is an edible mushroom that is cultivated worldwide. In this study, we investigated antiinflammatory activities of *H. marmoreus* extract on atopic dermatitis (AD)-like symptoms. Ethanol extract of *H. marmoreus* (HMEE) was administrated in powder to BALB/c mice in which AD was induced by 1-chloro 2, 4, 6-trinitrobenzene [picryl-chloride, (PCL)]. The dermatitis severity score and the thickness of the epidermis were significantly decreased following daily intake of HMEE powder (1 g/kg/day) for 5 weeks compared with a PCL-treated group. The mRNA expression of proinflammatory cytokines, such as interleukin-1 beta (IL-1 β) and interferon-gamma (IFN- γ), was significantly attenuated in the dorsal skin of the HMEE-fed mouse group compared with the PCL-treated mouse group. In addition, in concanavalin A-stimulated and lipopolysaccharide-stimulated mouse splenocytes and macrophages, levels of IL-1 β and IFN- γ production were attenuated following the addition of HMEE. Interestingly, the administration of HMEE to mouse splenocytes stimulated the production of an antiinflammatory cytokine, IL-4. However, increases in the levels of proinflammatory cytokines and nitric oxide were attenuated by treating the mouse splenocytes, mouse macrophages, and Raw 264.7 cell line with HMEE. These results strongly suggest that HMEE exhibits anti-AD activity via the regulation of inflammatory responses. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: *Hypsizigus marmoreus*; atopic dermatitis; inflammation; cytokines; nitric oxide.

INTRODUCTION

Atopic dermatitis (AD) is an inflammatory skin disease associated with pruritus, long-term swelling, and redness (inflammation) of the skin (Bieber and Simon, 2011). Although there have been many clinical and experimental studies of AD, the pathophysiological mechanism underlying the condition has not been clearly identified (Jin *et al.*, 2009; Kabashima-Kubo *et al.*, 2012). Studies have suggested that several factors, including a genetic predisposition, immunological function, and the environment, may be involved in the pathogenesis of AD (Bieber, 2008; Leung *et al.*, 2004). Among these factors, innate immunity and adaptive immunity have been extensively studied to understand the pathophysiological mechanism of AD. Th2 cell-mediated alteration of interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-13 (IL-13) expression was reported in the acute phase of AD, and Th1 cell-mediated AD lesions were found in chronic AD (Grewe *et al.*, 1998; Hamid *et al.*, 1994). Tumor necrosis factor-alpha (TNF- α) was also reported to have an important role in acute phase AD (Yamada *et al.*, 1995), and increased expression of interferon-gamma (IFN- γ) was shown to contribute to

the pathogenesis of chronic AD (Vestergaard *et al.*, 1999). As the mechanism of AD in patients is still not clear, most anti-AD drugs are based on antiinflammatory or immunosuppressive effects. They generally contain steroids, antihistamines, and immunosuppressive agents, but many side effects inhibit their extensive use (Fardet *et al.*, 2007). For example, topical application of corticosteroids is the most effective treatment for AD, but long-term treatment with corticosteroids causes AD relapses and telangiectasia (Fardet *et al.*, 2007). Therefore, safer anti-AD drugs with greater therapeutic potency are needed.

Hypsizigus marmoreus is a popular edible mushroom and considered as a functional food in East Asia. *H. marmoreus* has been shown to exhibit various biological properties, including antitumor and antifungal activities. For example, Bao *et al.* (2011) reported that water-soluble extracts from *H. marmoreus* inhibit the growth of various cancer cell lines. Lam and Ng (2001) showed that hypsin, a ribosome-inactivating protein, has antifungal activity. Yoshino *et al.* (2008) found that *H. marmoreus* seems to have a preventive effect on mouse type IV allergy induced by oxazolone. In this study, we examined the effect of *H. marmoreus* on the development of picryl-chloride (PCL)-induced AD in mice. We evaluated the dermatitis score and cytokine levels in the dorsal skin of AD mice and performed a histological analysis of the epidermis and dermatitis. In addition, we investigated the regulatory function of *H. marmoreus* in inflammation using mouse splenocytes, mouse macrophages, and a Raw 264.7 cell line.

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MATERIALS AND METHODS

Preparation of *H. marmoreus* extracts (HMEE). *Hypsizigus marmoreus* was obtained from a local Nonghyup market (Seoul, Korea). Three hundred grams of *H. marmoreus* was crushed, lyophilized, and extracted with 85% ethanol (20 times volume for 4 h at 65 °C). The supernatant was concentrated using a rotary evaporator and lyophilized. The yield of the HMEE was 27.4% (w/w, dry weight 82.2 g).

Animals. Six-week-old male BALB/c mice were purchased from DBL (Daejeon, Korea). After 1 week of adaptation in a pathogen-free environment, the animals were housed under recommended conditions, with a temperature of 23 ± 2 °C, humidity of 50 ± 10%, and a 12-h light/dark cycle. The mice were provided with a diet based on AIN-93G (Table 1) and water *ad libitum*. After 1 week of adaptation, they were divided into three groups ($n=8$ per each group): normal (control), PCL, and PCL + HMEE. The normal group was sensitized with vehicle and fed a normal diet, and the PCL group was sensitized with PCL and fed a normal diet. The PCL + HMEE group was sensitized with PCL and fed a normal diet containing HMEE. The care and treatment of the animals conformed to the Sungkyunkwan University guidelines for the Ethical Treatment of Laboratory Animals.

AD mouse model. The BALB/c AD mouse model was used to confirm whether HMEE suppressed AD. The mouse has AD-like skin lesions induced by topical application of PCL (1-chloro 2, 4, 6-trinitrobenzene, Sigma, CAT. NO. 79874-1G-F). Hairs of the dorsal skin were shaved in all three groups. To produce AD-like lesions, the PCL and PCL + HMEE groups were sensitized with 100 µl of 5% PCL solution (dissolved in a 3:1 mixture of acetone and olive oil). Four days after sensitization, the dorsal skin was challenged with 120 µl of 1% PCL solution (once per week for 5 weeks). After

inducing AD, HMEE powder (1 g/kg/day) was added to the normal diet. The animals were sacrificed 35 days after the first application. The skin lesions were removed and subjected to histopathological examination and extraction of total RNA.

Histological analysis. Thirty-five days after the PCL application, the mice were sacrificed for histopathological assay and reverse transcriptase polymerase chain reaction (RT-PCR). The skin lesions were preserved in 10% buffered formaldehyde solution for tissue fixation, and the tissues were immersed in ethanol and a xylene gradient mixture for paraffin infiltration. The embedded tissues were sliced to a thickness of 5 µm for histological analysis. Tissue sections were stained with hematoxylin–eosin to observe the morphology of the skin lesions.

Clinical observation of AD. Evaluation of the severity of the dermatitis was performed according to a previously described method (Yamamoto *et al.*, 2007). Briefly, the severity of the dermatitis was measured once a week by evaluating the degree of erythema/hemorrhage, scarring/dryness, edema, and excoriation/erosion. These features were scored as 0 (none), 1 (mild, <20%), 2 (moderate, 20–60%), and 3 (severe, >60%). The dermatitis score was the sum of the individual scores.

Expression of cytokines in the dorsal skin area. At the end of the animal study, the dorsal skin was isolated, and total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RT-PCR was used for analyzing the expression of IL-1β and the level of IFN-γ in skin lesions. Briefly, 1 µg of total RNA template was mixed with 300 pmol of a random primer. Ultrapure water (Invitrogen, NY, USA) was added to give a total volume of 10 µl, which was heated at 65 °C for 5 min. To synthesize cDNA, 4 µl of 5X RTase Moloney Murine Leukemia Virus (M-MLV) buffer, 10 mM of deoxynucleotide (dNTP) mixture, RTase M-MLV (Takara, 2640A, Japan), and ultrapure water were added to a total volume of 20 µl, which was incubated at 37 °C for 50 min and heated 70 °C for 10 min. The cDNA was analyzed by a Thermal Cycler Dice (Takara, TP800, Japan) using a Power SYBR Premix Ex Taq kit (Takara, RP041A, Japan) and specific oligonucleotide primers, as shown in Table 2.

Cell culture of Raw 264.7 cell line. The mouse macrophage cell line, Raw 264.7, was obtained from American

Table 1. Composition of diets (unit: %)

Ingredients	Dietary groups	
	Normal + HMEE	Normal
Corn starch	39.8	39.8
Casein	20.0	20.0
Dextrinized corn starch	13.2	13.2
Sucrose	10.0	10.0
Mineral mixture ^a	3.5	3.5
Vitamin mixture ^b	1.0	1.0
L-cystine	0.3	0.3
Choline chloride	0.25	0.25
tert-Butylhydroquinone	0.0014	0.0014
Cellulose	5.0	5.0
HMEE	0.001	0.0
Soybean oil	7.0	7.0

HMEE, ethanol extract of *Hypsizigus marmoreus*.

^aAIN-93 mineral.

^bAIN-93 vitamin mixture.

Table 2. Oligonucleotide sequence of primers

Gene	Primer	Sequence
Mouse IFN-γ	Forward	5'-CGGCACAGTCATTGAAAGCCTA-3'
	Reverse	5'-GTTGCTGATGGCTGATTGTC-3'
Mouse IL-1β	Forward	5'-TCCAGGATGAGGACATGAGCAC-3'
	Reverse	5'-GAACGTCACACACCAGCAGGTTA-3'
β-actin	Forward	5'-CATCCGTAAAGACCTCTATGCCAAC-3'
	Reverse	5'-ATGGAGCCACCGATCCACA-3'

IFN-γ, interferon-gamma; IL-1, interleukin-1.

Type Culture Collection (Manassas, VA, USA). Raw 264.7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (WelGENE Inc., LM 001-05) supplemented with 10% fetal bovine serum (FBS) (HyClone, SH30919.03), penicillin (100 U/ml), and streptomycin (100 µg/ml). A total of 10^6 cells of Raw 264.7 cells was used to seed a 12-well plate. After incubation for 24 h, for detecting nitric oxide (NO) production, the culture media were changed to serum-free DMEM containing 1 µg/ml of lipopolysaccharide (LPS) (Sigma, St. Louis, MO, USA) and 0.1 ng/ml of IFN- γ and were incubated for an additional 24 h. For measuring the IL-6 and TNF- α expression, the culture media were changed to serum-free DMEM containing LPS (10 ng/ml) and IFN- γ (0.1 ng/ml) and were incubated for an additional 24 h. The culture media were collected and used for measuring the amount of NO, IL-6, and TNF- α by using an NO assay kit (abcam, ab65328), and OptEIL kits (PharMingen, San Diego, CA, USA, IL-6:555240, TNF- α :555268) according to the manufacturer's protocol.

Isolation of mouse splenocytes and cellular response. Six-week-old male BALB/c mice purchased from DBL (Daejeon, Korea) were sacrificed, and their spleens were isolated. The spleen was placed in ice-cold, 4 °C phosphate buffered saline (PBS) and gently ground between frosted slides to produce a single-cell suspension. The suspension was centrifuged at $300 \times g$ for 10 min, and the pellet was resuspended in red blood cell lysis buffer (Sigma, St. Louis, MO, USA). The remaining cells were washed with PBS and centrifuged at a concentration of $300 \times g$ for 10 min. After the number of cells was counted, they were resuspended in Roswell Park Memorial Institute medium (RPMI) 1640 and plated to a 12-well plate at a concentration of 10^6 cells/ml. Mouse splenocytes were activated with 2.5 µg/ml concanavalin A (ConA) for 48 h (Sigma, St. Louis, MO, USA).

Preparation of mouse peritoneal macrophages. Thioglycollate-elicited peritoneal macrophages were isolated from the BALB/c mice according to previously published method (Miles *et al.*, 2000). Briefly, 5 ml of sterile 3% Fluid Thioglycollate Medium (DIFCO, Franklin Lakes, NJ, USA) was intraperitoneally injected. Three days later, the mice were sacrificed by cervical dislocation, and peritoneal exudate cells were isolated by peritoneal lavage and centrifuged and then resuspended in Red Blood Cell (RBC) lysis buffer for erythrocyte lysis. Finally, the cells were plated to a 12-well plate at a concentration of 10^6 cells/ml with RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 1% penicillin–streptomycin, 10 mM HEPES, and 50 µM of β -mercaptoethanol. Mouse peritoneal macrophages were activated with 10 ng/ml of LPS for 48 h (Sigma, St. Louis, MO, USA). Cell counting using trypan blue exclusion revealed >98% viability.

Enzyme-linked immunosorbent assay (ELISA). The concentrations of IL-2, IFN- γ , IL-6, IL-1 β , IL-4, and TNF- α were measured using OptEIA ELISA kits (PharMingen, San Diego, CA, USA). A standard ELISA procedure was performed according to the manufacturer's instructions. Briefly, a 96-well plate was

coated overnight with a capture antibody for a specific cytokine, followed by washing and blocking the plate. Diluted samples and standards (provided in the kit) were then added and incubated in room temperature. An extensive wash was applied before secondary antibody and enzyme conjugates were added. The plates then went through another round of incubation and extensive washing. Developing reagent was then added to the plate for 15 min. Color development was stopped during linear increases in the utilization of the substrate by adding 0.5 M of sulphuric acid to disrupt enzymatic activity. The optical density (OD) value was measured with a ELx800 Absorbance Microplate Reader (BioTek Instruments Inc., Winooski, VT, USA).

Statistical analysis. The results are presented as mean $\pm$ standard deviation (SD), and the data were analyzed by one-way analysis of variation (ANOVA) using the XLSTAT program (Addinsoft, NY, USA). A Student–Newman–Keuls test was then conducted. *P* values of 0.05 were considered significant.

RESULTS

Dietary HMEE improved AD-like skin lesions in the PCL-treated BALB/c mice

Repeated applications of PCL on the mouse dorsal skin induced AD-like symptoms. The histological analysis showed the development of AD-like skin symptoms, such as erythema, hemorrhage, edema, and erosion/excoriation in the PCL-treated group. The skin severity score in the PCL+HMEE group was significantly suppressed ($p < 0.05$) compared with the PCL group (Fig. 1C). The HMEE group did not show a significant weight change compared with the control group (data not shown). The histological examination showed ameliorated hypertrophy in the PCL+HMEE mice group compared with the PCL-treated group. The thickness of the epidermis in the PCL-treated group (277 ± 58.3 µm) was significantly reduced compared with that of the HMEE group (116.6 ± 21.6 µm) (Fig. 1A and 1B). Analysis of the expression levels of proinflammatory cytokines in the dorsal skin of the three groups showed that dietary HMEE significantly inhibited elevated levels of IFN- λ compared with the PCL-treated group ($p < 0.05$). In the PCL-treated group, the expression of IL-1 β was strongly upregulated when compared with that of the normal group (Fig. 2A). Interestingly, dietary HMEE decreased elevated levels of IL-1 β to levels comparable with those of the normal group ($p < 0.05$).

HMEE attenuated the synthesis of inflammation-related cytokines in Raw 264.7 cells

As an inhibitory effect of HMEE on the expression of proinflammatory cytokines was detected in the AD-like mouse model (Fig. 1), to clearly identify the effect of HMEE on AD, we explored the influence of HMEE on TNF- α and IL-6 production in Raw 264.7 cells exposed to inflammatory stimulation. The synthesis

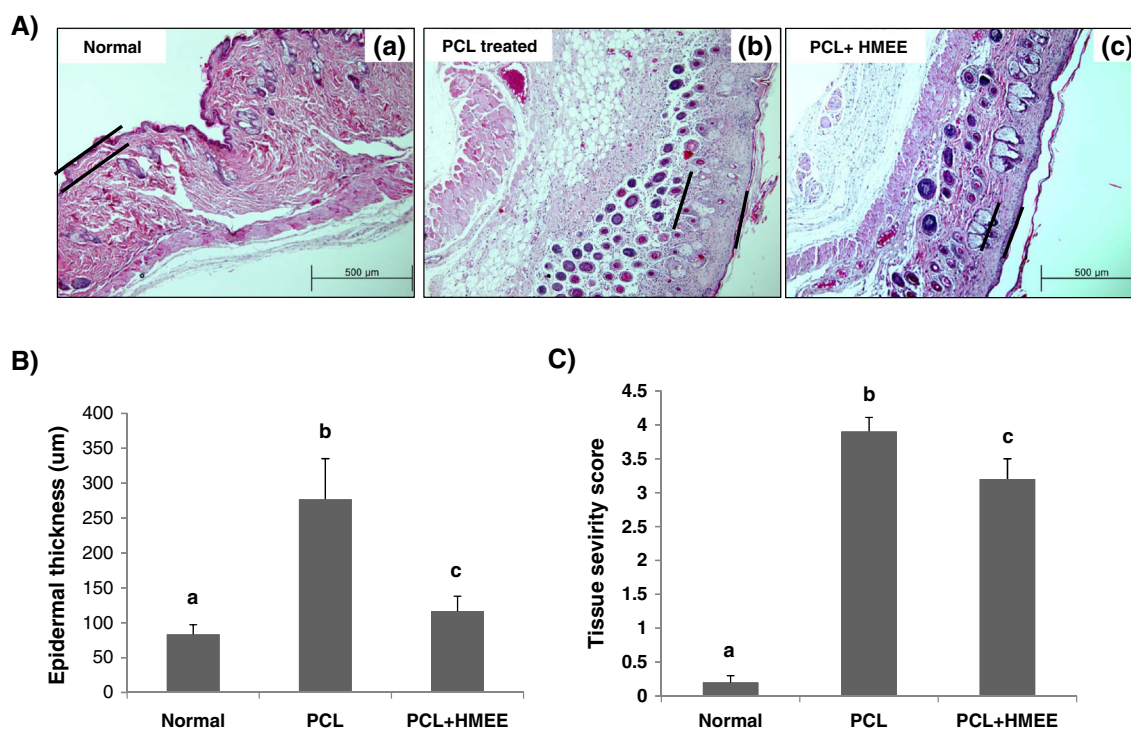


Figure 1. Administration of HMEE attenuated the thickness of the epidermis and the skin severity score. (A) Representative histopathological features of skin lesions: (a) no treatment, (b) PCL treatment, and (c) PCL + HMEE (PCL treated with oral administration of HMEE). (B) The thickness of the epidermis was measured between the lines and calculated. (C) The skin severity score was significantly decreased in the PCL + HMEE mouse group compared with the PCL-treated mouse group. The data were analyzed by ANOVA, and Student–Newman–Keuls test was then conducted. Data are presented as the means $\pm$ SD ($n = 8$). The different letters indicate a statistically significant difference ($p < 0.05$).

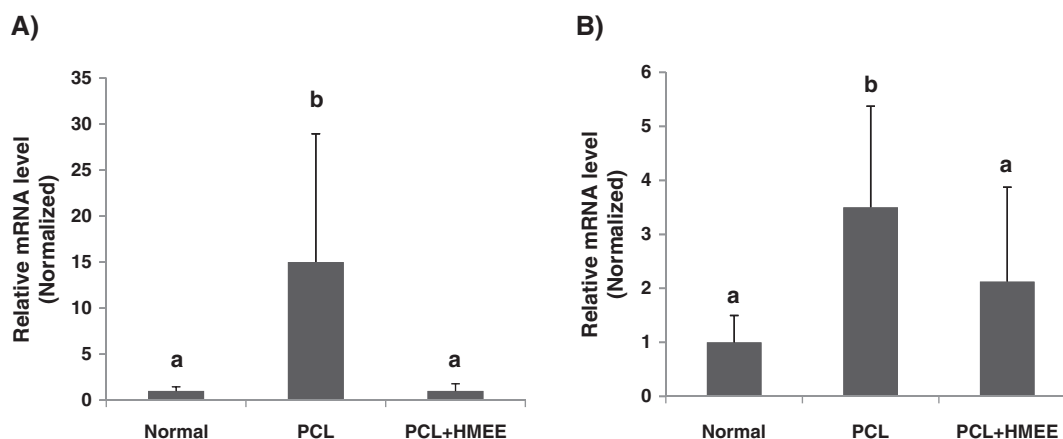


Figure 2. The expression of proinflammatory cytokines IL-1 β and IFN- γ was reduced in skin lesions in the PCL + HMEE group. The relative level of expression of IL-1 β (A) and IFN- γ (B) in the mouse skin, normalized to beta actin, was determined by qRT-PCR. Increased levels of IL-1 β and IFN- γ in the PCL-treated mouse group were significantly suppressed in the PCL + HMEE mouse group. The data were analyzed by ANOVA, and Student–Newman–Keuls test was then conducted. Data are presented as the means $\pm$ SD ($n = 3$). The different letters indicate a statistically significant difference ($p < 0.05$).

of TNF- α was dramatically inhibited by the HMEE treatment of Raw 264.7 cells (Fig. 3A). About 52% of TNF- α production was attenuated by the addition of 10 μ g/ml of HMEE. TNF- α production was further inhibited when the concentration of HMEE was increased. The synthesis of IL-6 was also inhibited by the HMEE treatment of Raw 274.7 cells (Fig. 3B). Although there was no significant change in the production of IL-6 when the concentration of HMEE was 10 μ g/ml, increasing the concentration of HMEE to 50 μ g/ml resulted in significant differences in IL-6 production (1.48 ± 0.03 ng/ml) compared with a negative control (2.16 ± 0.14 ng/ml) stimulated by LPS and IFN- γ only.

HMEE inhibited the synthesis of proinflammatory cytokines and stimulated the production of antiinflammatory cytokines

To further investigate the immune regulatory function of HMEE on the synthesis of inflammation-related cytokines, splenocytes and murine peritoneal macrophages were isolated and exposed to the inflammatory stimulators ConA and LPS, respectively. The synthesis of proinflammatory cytokines, including IFN- γ and IL-2, was dramatically inhibited by adding HMEE to the splenocytes (Fig. 4A and 4B). Similar to IFN- γ , HMEE administration significantly inhibited LPS-induced

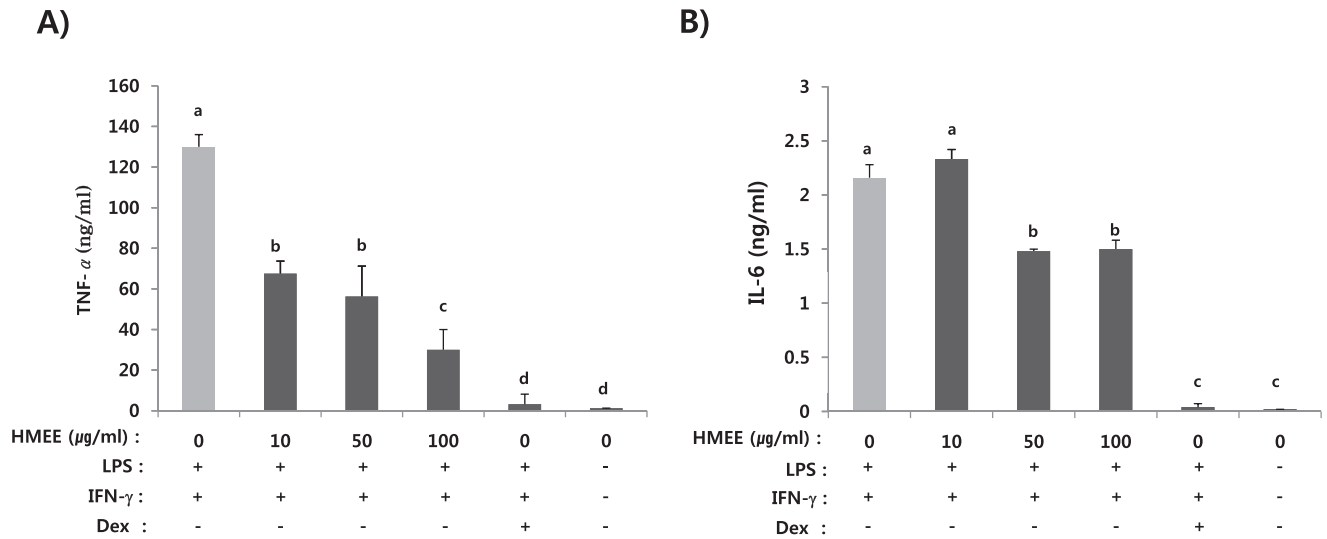


Figure 3. Inhibitory effect of HME on TNF- α and IL-6 production in Raw 264.7 cells. The amount of both (A) TNF- α and (B) IL-6 from supernatant of Raw 264.7 cells was measured using an ELISA kit. The expression of TNF- α was inhibited dose dependently by the addition of HME, and the expression of IL-6 was significantly attenuated at a dose of 50 μ g/ml of HME. LPS-stimulated (10 ng/ml) and IFN- γ -stimulated (0.1 ng/ml) production of TNF- α and IL-6 was decreased to almost basal levels by the addition of Dex (dexamethasone, 1 μ g/ml) (lane 5). Normal media without treatment with LPS (10 ng/ml) and IFN- γ (0.1 ng/ml) were used as a control (lane 1). The data were analyzed by ANOVA, and Student–Newman–Keuls test was then conducted. Data are presented as the means $\pm$ SD ($n = 3$). The different letters indicate a statistically significant difference ($p < 0.05$).

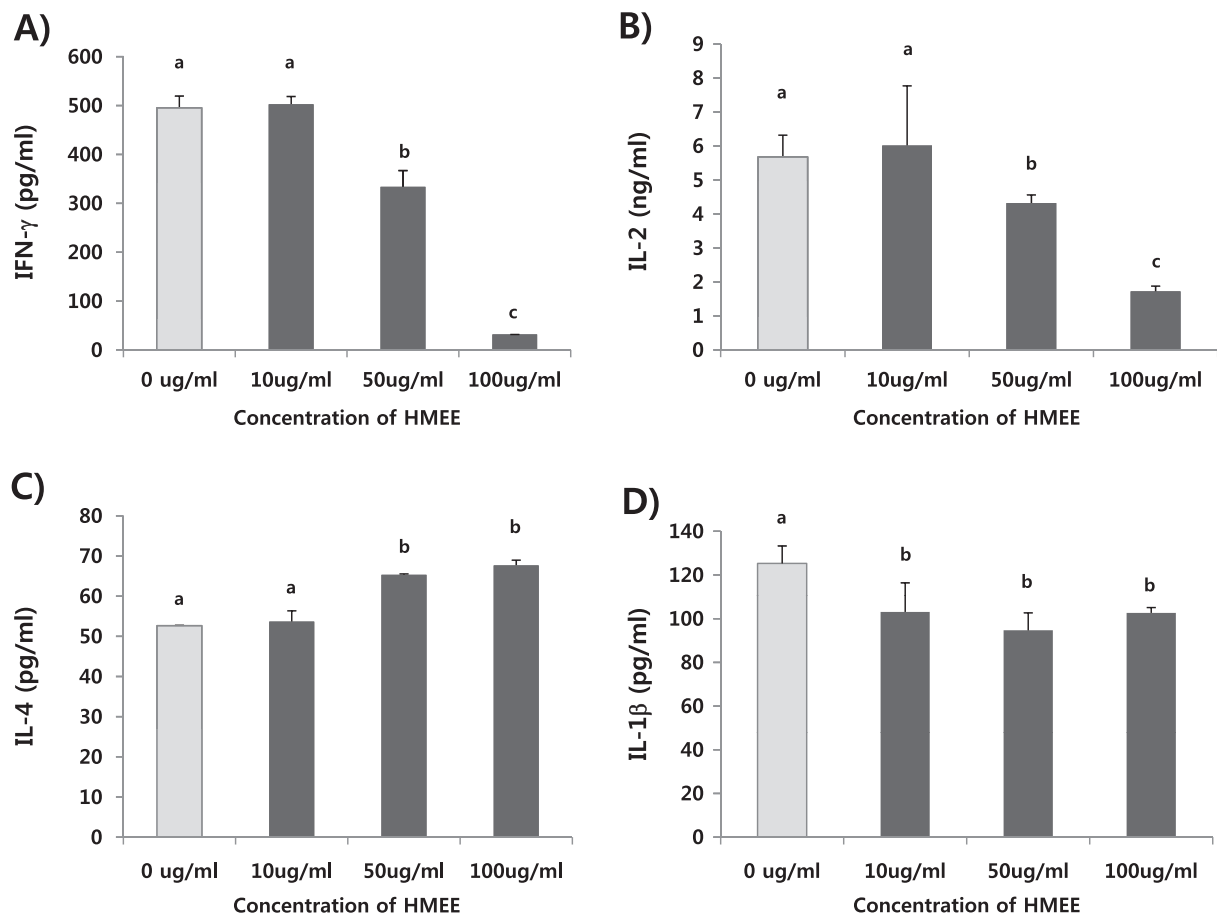


Figure 4. Effect of HME on the production of proinflammatory and antiinflammatory cytokines. ConA-stimulated (2.5 μ g/ml) mouse splenocytes (A–C) and LPS-stimulated (10 ng/ml) macrophages (D) were used to investigate the effect of HME on the production of IFN- γ (A), IL-2 (B), IL-4 (C), and IL-1 β (D). Various concentrations of HME (0 ~ 100 μ g/ml) were added to culture media treated for 48 h, and an ELISA assay was performed to detect the amount of IFN- γ (A), IL-2 (B), IL-4 (C), and IL-1 β (D) in the culture media. Data are presented as the mean $\pm$ SD ($n = 3$). The data were analyzed by ANOVA, and Student–Newman–Keuls test was then conducted. The different letters indicate a statistically significant difference ($p < 0.05$). Culture media without HME treatment (control) were used for the detection of each cytokine.

synthesis of IL-1 β in murine peritoneal macrophages (Fig. 4D). Interestingly, the production of IL-4, which is an antiinflammatory cytokine, was significantly increased when ConA-stimulated splenocytes were supplemented with HMEE (Fig. 4C).

NO synthesis was attenuated by HMEE in Raw 264.7 cells

As AD is considered an inflammatory dermatological disease, we tested whether HMEE inhibited the NO production, which is a well-known inflammation-related factor. Stimulation by LPS and IFN- γ induced NO production ($87.5 \pm 3.75 \mu\text{M}$) in Raw 264.7 cells, and NO production was significantly attenuated by adding $1 \mu\text{g/ml}$ of HMEE to $72.22 \pm 2.40 \mu\text{M}$. In addition, almost 50.5% amount of NO production was decreased when the amount of HMEE was increased to a concentration of $100 \mu\text{g/ml}$ (Fig. 5).

DISCUSSION

This study demonstrated anti-AD effects of HMEE using an *in vivo* AD mouse model. The results revealed that HMEE is involved in immune responses through regulating the synthesis of cytokines and NO. Although diverse biological activities of *H. marmoreus* have been reported (Sato *et al.*, 2011; Wong *et al.*, 2008), this is the first trial to investigate the anti-AD activity of HMEE using an animal model. The clinical tissue severity score (Fig. 1C) was improved, and the thickness of the epidermis was reduced (Fig. 1B) in the HMEE + PCL AD-like mouse model. In addition, the administration of HMEE reduced the levels of proinflammatory

cytokines, such as IL-1 β and IFN- γ , in dorsal skin lesions of AD (Fig. 2). Cytokines are well-known regulators of the host response to infection, immune responses, inflammation, and trauma. They are divided into proinflammatory and antiinflammatory cytokines (Dinarello, 2000; Opal and DePalo, 2000). Inflammation worsens when levels of the proinflammatory cytokines IL-1, IL-2, IL-6, TNF- α and IFN- γ are increased. Antiinflammatory cytokines, such as IL-4 and IL-13, attenuate the activities of proinflammatory cytokines (Opal and DePalo, 2000). Although the exact pathological role of cytokines in the development of skin lesions of AD is not clearly understood, recent studies have suggested possible pathogenic roles of cytokines in AD. In one study, chronic AD patients showed antigen-reactive skin infiltrating T cells, which secreted IFN- γ (Tomimori *et al.*, 2005), and thymus and activation-regulated cytokine was highly expressed in the epidermis of AD-like skin lesions of NC/Nga mice induced by IFN- γ (Vestergaard *et al.*, 1999). Other studies reported that levels of IL-1 β correspond with the severity of AD (Nutan *et al.*, 2012) and that IL-1 β neutralizing drugs seem to suppress autoinflammatory diseases, including AD (Krause *et al.*, 2012). Similarly, in our study, the expression of IFN- γ and IL-1 β was strongly increased in the dorsal skin of the PCL-induced AD-like mouse model, and dietary HMEE significantly decreased the expression of IFN- γ and IL-1 β (Fig. 2). To elucidate the roles of HMEE in regulating the synthesis of cytokines, we examined whether HMEE regulates mitogen-stimulated cytokine production in immune cells, such as splenocytes and mouse macrophages. HMEE treatment up to a concentration of $100 \mu\text{g/ml}$ did not show cytotoxicity in splenocytes (data not shown), but ConA-stimulated IFN- γ expression and LPS-stimulated IL-1 β were significantly attenuated by adding HMEE to splenocytes and murine macrophages, respectively. Furthermore, HMEE inhibited the synthesis of TNF- α , IL-6, and IL-2, all of which are well-

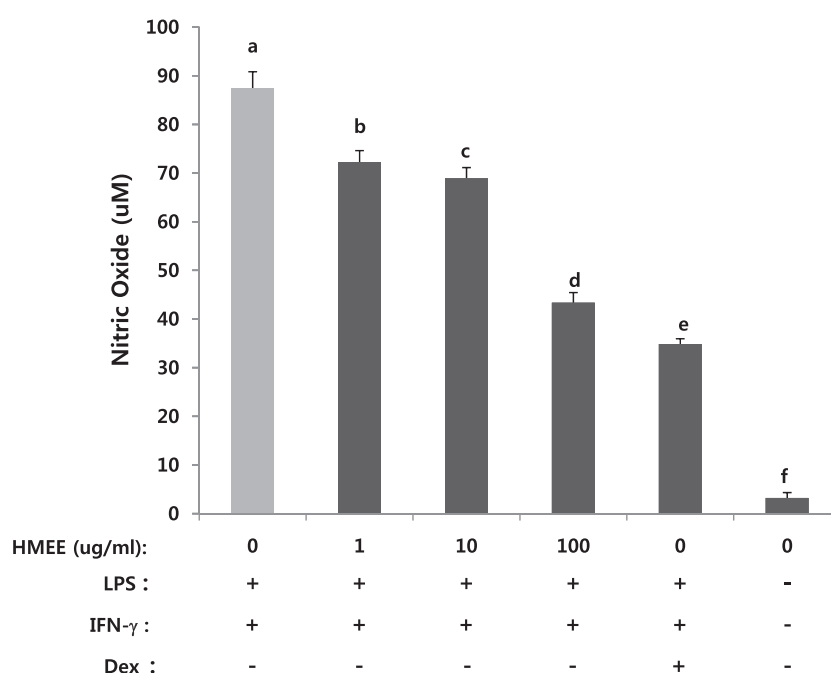


Figure 5. Effect of HMEE on NO production in Raw 264.7 cells. The expression of NO was determined using an NO ELISA kit. LPS-stimulated ($1 \mu\text{g/ml}$) and IFN- γ -stimulated (0.1 ng/ml) NO production was dose dependently inhibited by the addition of HMEE to the Raw 264.7 cells. NO production in Dex (dexamethasone)-treated ($1 \mu\text{g/ml}$) cells decreased dramatically (lane 5). The data were analyzed by ANOVA, and Student–Newman–Keuls test was then conducted. Data are presented as the mean $\pm$ SD ($n = 3$). The different letters indicate a statistically significant difference ($p < 0.05$).

known proinflammatory cytokines. Taken together, these data indicate that HMEE inhibits the synthesis of proinflammatory cytokines and that it exerts a curative effect on AD-like symptoms in PCL-treated mice.

Atopic dermatitis is an inflammatory skin disease. Therefore, regulating the expression of both proinflammatory and antiinflammatory components is important to control AD. Among the antiinflammatory cytokines, IL-4 is well known to inhibit the synthesis of LPS-induced proinflammatory cytokines (Opal and DePalo, 2000). IL-4 was reported to suppress monocyte-derived proinflammatory cytokines, such as IL-1, TNF- α , and IL-6 (Brown and Hura, 1997; te Velde *et al.*, 1990; Wang *et al.*, 1995). It was also reported to have a suppressive effect on macrophage-derived NO production (Vannier *et al.*, 1992). Interestingly, our study showed that HMEE administration increased the ConA-stimulated IL-4 production in mouse splenocytes (Fig. 4C). These results suggest that HMEE may control inflammatory diseases such as AD by attenuating the expression of proinflammatory cytokines and upregulating the expression of the antiinflammatory cytokine IL-4.

To elucidate other immune regulatory roles of HMEE, we measured the production of NO with and without HMEE treatment in Raw 264.7 murine macrophage cells. Macrophages respond to invading pathogens by releasing proinflammatory cytokines and mediators, such as TNF families and NO. Although the detailed

mechanism of how the edible mushroom extract regulates NO production is not yet known, edible mushrooms seem to contain ingredients that control NO production. For example, the mushroom *Neolentinus lepideus* was shown to inhibit NO production (Li *et al.*, 2013), and the edible oyster mushroom was reported to have antiinflammatory activity and to inhibit LPS-induced NO production (Jedinak *et al.*, 2011). In our study, the administration of HMEE to Raw 264.7 cells showed dose-dependent inhibition of LPS-induced NO production (Fig. 5). Taken together, our results are the first to demonstrate that HMEE is effective in inhibiting the AD-like phenotype *in vivo* through regulating the inflammation response. Although a single compound with anti-AD activity needs to be identified, these results suggest that HMEE is a good candidate for developing anti-AD and antiinflammation drugs.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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