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Anti-influenza Effect of *Cordyceps militaris* through Immunomodulation in a DBA/2 Mouse Model

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The immune-modulatory as well as anti-influenza effects of *Cordyceps* extract were investigated using a DBA/2 mouse model. Three different concentrations of *Cordyceps* extract, red ginseng extract, or drinking water were orally administered to mice for seven days, and then the mice were intranasally infected with 2009 pandemic influenza H1N1 virus. Body weight changes and survival rate were measured daily post-infection. Plasma IL-12, TNF-α, and the frequency of natural killer (NK) cells were measured on day 4 post-infection. The DBA/2 strain was highly susceptible to H1N1 virus infection. We also found that *Cordyceps* extract had an anti-influenza effect that was associated with stable body weight and reduced mortality. The anti-viral effect of *Cordyceps* extract on influenza infection was mediated presumably by increased IL-12 expression and greater number of NK cells. However, high TNF-α expression after infection of H1N1 virus in mice not receiving treatment with *Cordyceps* extract suggested a two-sided effect of the extract on host immune regulation.

***Keywords*:** *Cordyceps* extract, influenza A virus, IL-12, NK cells

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Introduction

Influenza viruses cause acute infectious disease, which are usually transmitted through the air by coughing or sneezing. Influenza viruses invade the upper respiratory tract, and common symptoms include high fever, headache, muscle pain, and severe fatigue. However, influenza virus infection can lead to severe complications and even death when the infection progresses beyond the normal symptoms (Roxas and Jurenka, 2007). Influenza viruses cause seasonal epidemics around the world, resulting in about 250,000 to 500,000 annual deaths, increasing to millions in some pandemic areas (WHO, 2009). There are three types of viral strains: types A, B, and C. Types A and B can give rise to human influenza pandemics, but type A viruses are the most virulent human pathogens among the three influenza types (Klenk, 2008). Humans and birds are known to be the primary hosts for type A influenza, which can be subdivided into different serotypes based on the host antibody response to the specific viral antigens (Hay *et al*., 2001).

Cordyceps extract has been used as a traditional medicine for many years in Asian countries. Recently, many studies have tried to elucidate the pharmacological mechanism of Cordyceps, which include immune activation, anti-inflammatory, anti-cancer, and anti-viral effects. To date, four purified components from *Cordyceps* have been reported and they are cordycepin, cyclosporin, acetoxyscirpenol, and ergosterol peroxide (Tuli *et al*., 2013; Yue *et al*., 2013).

There are many species of *Cordyceps* mushrooms distributed in the world. Among them, *Cordyceps sinensis* and *Cordyceps militaris* have an extensive attention due to their potent activities for medicinal uses. Both of them are parasites on the larvae of caterpillars of moths. Traditional medicine generally involves both mushroom fruitbody and parasitized larvae. *Cordyceps sinensis* is the most well-known and expensive fungus, but it is rare and it cannot be easily cultured. However, *C. militaris* has been cultured successfully. The main active ingredients of *C. militaris* are cordycepin, cordycepic acid, sterols (ergosterol), nucleosides, and polysaccharides. A comparison study between *C. militaris* and *C. sinensis* found that *C. militaris* has higher content of cordycepin and cordycepic acid than *C. sinensis* (Zhou *et al*., 2009).

Ferrets and guinea pigs are widely used as animal models for influenza virus infections because they display similar clinical symptoms and transmission patterns to those of humans (Lowen *et al*., 2006; Bouvier and Lowen, 2010; Belser *et al*., 2011). However, the feasibility of using ferrets or guinea pigs for research studies on viral influenza infections has been complicated by the expense of housing these

animals and the lack of animal-specific reagents. Several inbred mouse strains have been used successfully as animal models for viral influenza infections including C57BL/6J, BALB/c, and DBA/2 (Maines *et al*., 2005; Salomon *et al*., 2006; Chen *et al*., 2008). Among them, the DBA/2 mouse strain was shown to be the most susceptible to influenza A (Boon *et al*., 2009; Kim *et al*., 2013).

Therefore, in this study, we first confirmed whether DBA/2 mice are highly susceptible to disease following intranasal infection with 2009 pandemic H1N1 virus. In addition, we assessed whether the mortality of DBA/2 mice is correlated with other pathogenic appearances. Subsequently, the Cordyceps extract was evaluated for its anti-viral effect against 2009 pandemic H1N1 virus, and the mechanism underlying its anti-viral effect was then investigated in terms of host immune responses.

Materials and Methods

Preparation of *Cordyceps* extract from *Cordyceps militaris*

Cordyceps extract and red ginseng (RG) extract were provided by Dong-a Pharm. Co., LTD (Korea). Both extracts were diluted with distilled water for oral administration in the mouse experiments.

MDCK cells and influenza virus

Madin-Darby canine kidney (MDCK) cells and 2009 pandemic H1N1 virus [A/Korea/01/2009 (K/09)] were provided by Man-Seong Park (Hallym University, Chuncheon, Republic of Korea). MDCK cells were maintained in minimum essential medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, USA), 100 U/ml of penicillin and 100 μg/ml streptomycin at 37°C in a 5% CO_2 incubator. The virus was propagated in MDCK cells. Briefly, MDCK cells were seeded overnight in T75 flasks (2.5×10^6). The next day, cells were infected with H1N1 virus (2.5×10^4 pfu). At 48 h post-infection, the supernatant from infected cell cultures was harvested and centrifuged for 10 min at 3,000 rpm. Viral titer was measured using a cell-based plaque assay.

Animal experiments

DBA/2 mice (female, eight weeks old; Japan SLC, Inc., Japan) were adapted for one week before the experiments. All the animal experiments were conducted in accordance with the recommendations in the National Research Council's Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Animal Experiments Committee of Duksung Women's University (permit number: 2013-007-007).

Oral administration of *Cordyceps* extract and virus challenge

Two sets of animal experiments were organized. The first set of experiment was designed to investigate the effect of *Cordyceps* extract on host immune regulation. Mice were divided into four groups (Mock, RG extract 30 mg/kg, Cordyceps extract 30 mg/kg, and *Cordyceps* extract 300 mg/kg), each consisting of four animals, and were orally administered drinking water (Mock), RG extract (30 mg/kg), or *Cordyceps* extract (30 or 300 mg/kg). Whole blood was drawn on day 7 after treatment, and plasma cytokines (IL-12) and the percentage of NK cells were measured. The second set of experiment was designed to examine the anti-influenza effect of *Cordyceps* extract. Mice were divided into six groups (Mock, H1N1-infected, RG extract 30 mg/kg + H1N1, Cordyceps extract 30 mg/kg + H1N1, *Cordyceps* extract 100 mg/kg + H1N1, *Cordyceps* extract 300 mg/kg + H1N1), each including seven animals. Mice were orally administered drinking water, RG extract, or *Cordyceps* extract for seven days before virus challenge. Then, the mice were infected intranasally with influenza A virus (2009 H1N1; $5 \times 10^{3.38}$ pfu/animal). Body weight changes and survival rates were monitored daily post-infection. On days 4 and 11 post-infection, whole blood was drawn by cardiac puncture, and plasma cytokines (TNF-α and IL-12) were measured. The experimental design for anti-influenza effect of *Cordyceps* extract is shown in Fig. 1.

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from whole blood by density centrifugation as previously described (Cho, 2013) using Ficoll-Histopaque (Sigma Chemical Co., USA). Briefly, whole blood was gently layered on top of the Ficoll layer without disturbing the layering and centrifuged for 22 min at 2,200 rpm. Then, the plasma and buffy coat containing lymphocytes were isolated. Plasma was stored in a -80°C freezer for ELISA. The buffy coat was washed three times, and the cell pellet was counted for further experiments.

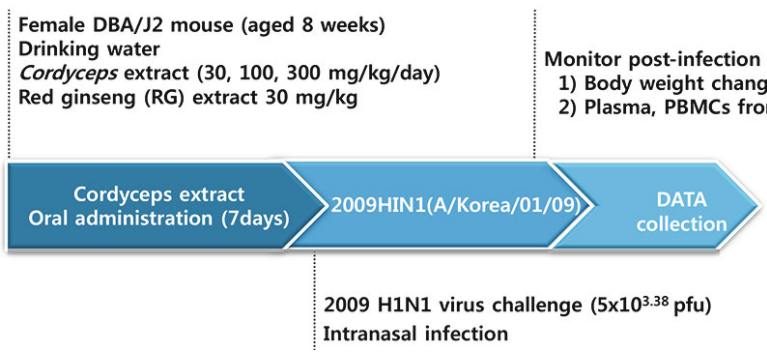

Fig. 1. Overview of the experimental design for evaluating the anti-influenza effect of *Cordyceps* extract. Drinking water (DW), RG extract, or *Cordyceps* extract were orally administered to mice for seven days prior to influenza A virus (2009 H1N1) infection. Viral challenge was performed by intranasal infection with $5 \times 10^{3.38}$ pfu/animal. Body weight changes and survival rate were monitored daily post-infection. Plasma cytokines (TNF-α and IL-12) and percentage of NK cells were measured immediately before infection and on days 4 and 11 post-infection.

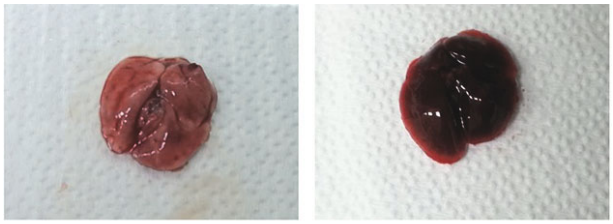

Fig. 2. Hyper-inflammatory lesions from an H1N1 virus-infected mouse. Lung lobes were collected from uninfected and H1N1-infected mice on day 11 post-infection.

Fluorescent antibody and cell surface antigen staining

APC-anti-Mouse CD49b (DX5) was purchased from eBioscience (USA). Cells were stained according to the manufacturer's instructions. Dead cells were excluded with 7-AAD. Briefly, PBMCs (1×10^6) were mixed with fluorescent antibodies and incubated at 4°C for 30 min in the dark. After staining, the cells were washed three times with staining buffer containing 1% BSA and 0.09% sodium azide in PBS. Cells were fixed with 2% paraformaldehyde (Sigma-Aldrich, USA). Events were acquired using the BD FACSCanto (Becton Dickinson, USA) and analyzed with FlowJo (Tree Star Inc., USA). Gating on live lymphoid cells was based on the forward and side scatter profiles as well as 7-AAD staining. The positivity of NK cells was defined using fluorescent anti-DX5 monoclonal antibody within the lymphocyte population. An unstained control was used to determine background levels of staining.

Assays for cytokines

Concentrations of TNF-α and IL-12 in plasma from whole blood were determined using an ELISA kit (BD Biosciences, USA) according to the manufacturer's instructions. Briefly, 96-well immunoassay plates (Greiner Bio One, Germany) were coated with 100 μl (per well) of capture antibody (anti-mouse TNF-α or anti-mouse IL-12p70) diluted in coating buffer and incubated at 4°C overnight. The next day, the plates were incubated with blocking reagents for 1 h at RT. Standard or sample (100 μl) was added to the appropriate wells. Plates were sealed and incubated for 2 h at RT. The amounts of TNF-α and IL-12 were measured after incubation with HRP-conjugated detection antibodies for 1 h at RT.

Statistical analysis

Parameters between the treatment groups were compared by ANOVA analysis. P values less than 0.05 were considered statistically significant.

Results

High susceptibility of DBA/2 mice to 2009 pandemic influenza H1N1 virus infection

DBA/2 mice are considered one of the best animal models for influenza infection because their morbidity is easily determined by body weight changes and survival rate (Kim *et al.*, 2013). In this study, we also found that DBA/2 mice were highly vulnerable to intranasal infection of influenza A virus. Figure 2 shows whole lungs from uninfected (left) and H1N1 virus-infected (right) mice. Uninfected lung lobes did not show any visible signs of external damage. However, on day 11 post-infection, lung lobes infected with H1N1 virus showed inflammatory lesions resulted from hemorrhagic pleural effusion and infiltration of cells.

Also, body weight was significantly decreased by day 5 post-infection (Fig. 3A, H1N1+water group) in the H1N1 virus-infected group compared to other groups, which clearly contributed to survival rate, as shown in Fig. 3B.

Anti-influenza effect of *Cordyceps* extract in a DBA/2 mouse model

We utilized the DBA/2 mouse model to investigate the antiviral effect of *Cordyceps* extract against influenza A virus. Mice were divided into six groups, and *Cordyceps* extract (30 mg/kg, 100 mg/kg, or 300 mg/kg), RG xtract (30 mg/kg), and drinking water were orally administered to mice for seven days prior to influenza A virus (2009 H1N1) infection. Virus challenge was performed by intranasal infection with $5 \times 10^{3.38}$ pfu/animal. Body weights immediately before virus challenge were set at 100% for each group. Body weight changes and survival rates were monitored daily until the experimental end point. As shown in Fig. 3A, the H1N1-infected group that received neither RG nor *Cordyceps* extract showed a significant decrease in body weight by day 5 post-infection. Moreover, the reduction in body weight correlated with high mortality, as shown in Fig. 3B.

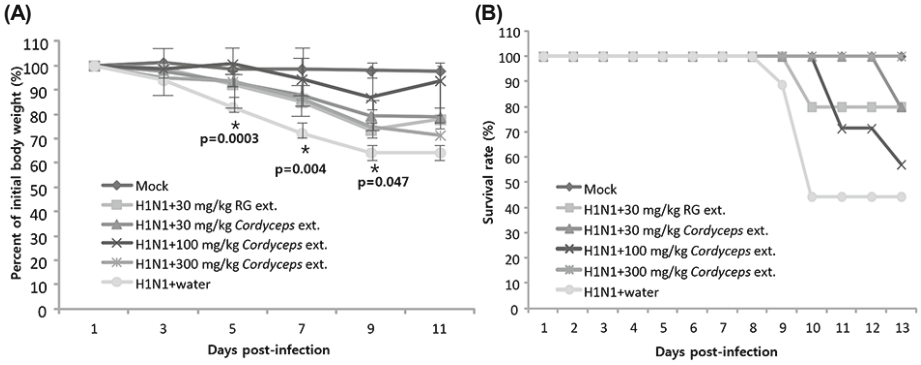

Fig. 3. Anti-influenza effect of *Cordyceps* extract in a DBA/2 mouse model. Six groups of mice were orally administered *Cordyceps* extract (30, 100, or 300 mg/kg), RG extract (30 mg/kg), or drinking water (DW) for seven days prior to viral challenge ($5 \times 10^{3.38}$ pfu). Body weights immediately before challenge were set at 100% in each group. Body weight changes (A) and survival rate (B) were monitored daily until the experimental end point.

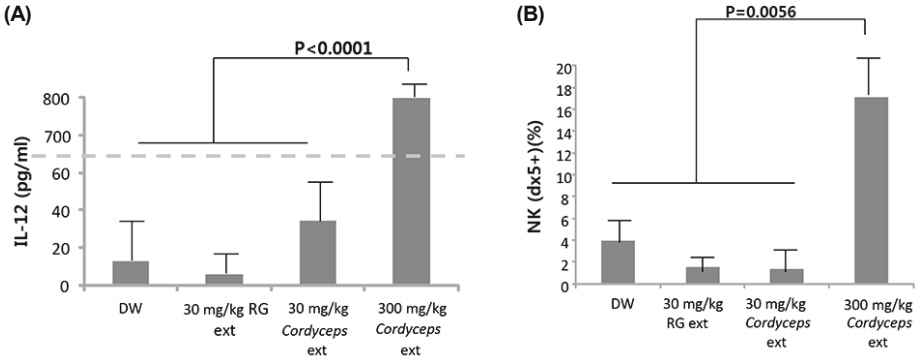

Fig. 4. Immune activation of *Cordyceps* extract through IL-12 and natural killer cells. Mice were orally administered drinking water (DW), RG extract, or *Cordyceps* extract for seven days. (A) IL-12 in plasma from whole blood. (B) Percentage of natural killer (NK) cells in PBMCs isolated from whole blood.

Immune activation by *Cordyceps* extract through IL-12 and natural killer cells

We investigated whether the anti-influenza effect of Cordyceps extract might be associated with stimulation of innate immune responses. Four groups of mice were individually administered 30 mg/kg or 300 mg/kg of *Cordyceps* extract, 30 mg/kg of RG, or drinking water. On day 7, whole blood was drawn by cardiac puncture. IL-12 production and the percentage of NK cells were measured. Figure 4A shows that oral administration of 300 mg/kg *Cordyceps* extract significantly upregulated IL-12 expression compared to the other groups (300 mg/kg *Cordyceps* extract, 758.33 pg/ml; 30 mg/kg *Cordyceps* extract, 34.20 pg/ml; and 30 mg/kg RG, 5.63 pg/ml). In addition, IL-12 production in the group receiving 30 mg/kg *Cordyceps* extract was higher than that of the group receiving 30 mg/kg RG, which suggests that *Cordyceps* extract has better immune-enhancing effects compared to RG. Interestingly, the expression of IL-12 shown in Fig. 4A strongly correlated with the increased frequency of natural killer cells shown in Fig. 4B (300 mg/kg *Cordyceps* extract, 17.1%; 30 mg/kg *Cordyceps* extract, 1.5%; and 30 mg/kg RG, 1.4%).

Increased TNF-α production from H1N1 virus-infected mice without prior treatment with *Cordyceps* extract

The observed immune-enhancing effect of IL-12 by prior treatment with *Cordyceps* extract led us to investigate changes in the expression of other cytokines after H1N1 virus infection. As shown in Fig. 5A, TNF-α expression in the group infected with H1N1 virus but not treated with *Cordyceps* extract was increased compared to the other groups (H1N1 + water, 98.17 pg/ml; H1N1 + 30 mg/kg RG, 26.7 pg/ml; H1N1 + 30 mg/kg *Cordyceps* extract, 0.73 pg/ml; H1N1 + 300 mg/kg *Cordyceps* extract, 8.7 pg/ml). TNF-α is a representative pro-inflammatory cytokine that is usually detectable in the early stages of inflammation. However, TNF-α is also known to cause considerable amounts of tissue damage during severe inflammation, which may be related to the lung damage shown in Fig. 2. In addition, significantly lower amounts of TNF-α were observed in infected mice receiving prior treatment with *Cordyceps* extract. In case of IL-12 (Fig. 5B), the cytokine production was higher in *Cordyceps* extract-treated infected groups (30 mg/kg, 300 mg/kg) compared to untreated infected group without any statistical significance.

Discussion

The results of Fig. 1 show that DBA/2 mice were highly susceptible to influenza A virus (2009 H1N1) infection, which was strongly associated with mortality and decreased body weight. However, influenza viruses are not fatal in all mouse strains. In some strains, viral adaptation increases susceptibility. Studies by Boon *et al.* showed that the DBA/2 strain is more susceptible to H5N1 virus infection than the C57BL/6 strain, and influenza B virus infection without prior viral adaptation is lethal in the DBA/2 strain (Boon *et al.*, 2009, 2010; Pica *et al.*, 2011). Recently, Kim *et al.* (2013) reported that the DBA/2 strain is more susceptible to H1N1 virus infection than the BALB/c strain. We found that the high susceptibility of the DBA/2 strain to influenza viral infection correlated with visibly damaged lesions in lung lobes from H1N1 virus-infected animal.

Cordyceps extract has been reported to have an inhibitory

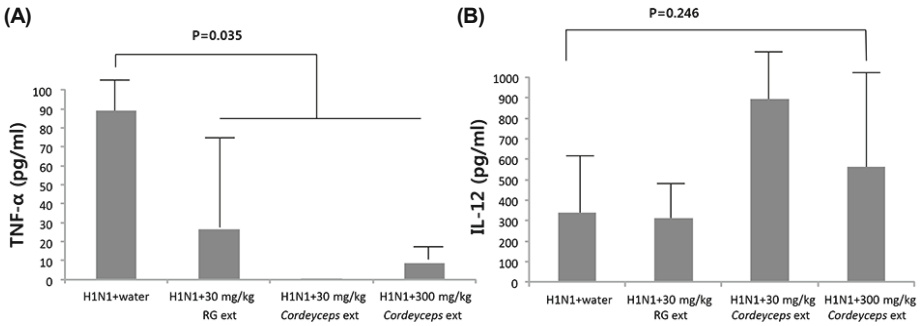

Fig. 5. Increased TNF-α production from H1N1 virus-infected mice in the absence of *Cordyceps* extract treatment. Mice were orally administered drinking water (DW), RG extract, or *Cordyceps* extract for seven days and then infected with 2009 H1N1 virus. Plasma was collected on day 4 post-infection. TNF-α (A) and IL-12 (B) in plasma.

effect on various types of viruses including human immunodeficiency virus (HIV), herpes virus (HSV), hepatitis C virus (HCV), and influenza virus. Wang *et al.* (2012) found that cordysobin from *Cordyceps sobolifera* had an *in vitro* antiviral effect through inhibition of HIV-1 reverse transcriptase. RG extract is also traditionally known to have multiple biological activities including anti-inflammatory, antioxidant, anti-tumor, and immune-modulatory action (Keum *et al.*, 2000; Block and Mead, 2003). Recently, the anti-viral effect of RG extract has been reported in influenza virus A (H1N1) infection study (Kim *et al.*, 2011). However, the association between anti-viral effect of RG and immunological parameters has not been reported. Therefore, we included RG extract treatment group in our study to compare with *Cordyceps* extract.

The Ohta group showed that extracts from *C. militaris* reduce viral titers in lungs from H1N1-infected BALB/c mice. Moreover, they also showed a correlation between the recovery of mice and increased expression of TNF-α and IFN-γ by treatment with *Cordyceps* extract (Ohta *et al.*, 2007). In the present study, the results shown in Fig. 3 are in agreement with the Ohta group. An anti-viral effect was observed in the group treated with *Cordyceps* extract in terms of less body weight changes and reduced mortality. We used body weight change as a primary marker for morbidity because body weight loss has been reported to be an optimal indicator of ultimate mortality in influenza virus-infected mouse model (Trammell and Toth, 2011). Figure 3A shows 100 mg/kg *Cordyceps* extract group had less reduction in body weight change compared to that of 30 mg/kg group. However, in Fig. 3B, mice survival rate from 100 mg/kg *Cordyceps* extract group is worse than that from 30 mg/kg *Cordyceps* extract group. We speculate that there is some discrepancy between body weight change and survival rate even though survival rate is well known to be reflected by body weight change.

In addition, we also found that oral administration with *Cordyceps* extract-stimulated innate immunity through IL-12 expression and the activation of natural killer cells. IL-12 is an immune activating cytokine that primarily activates natural killer cells to fight virus-infected host cells. Therefore, we suggest that orally administered *Cordyceps* extract (specifically 300 mg/kg) upregulated IL-12 production and stimulated natural killer cells; thus, preparing the host immune system to fight viral infection. The immune-enhancing effect of *Cordyceps* extract has been reported in several studies. Lee and Hong (2011) showed that polysaccharides isolated from *C. militaris* promote NO, ROS, TNF-α, and phagocytic uptake in mouse peritoneal and RAW264.7 macrophages. Wang *et al.* (2013) also demonstrated immune-enhancing activities of *C. militaris* polysaccharides through lymphocyte proliferation and production of IFN-γ and IL-4 in chickens. A study by Song *et al.* (2013) reported that exopolysaccharides from *C. sinensis* increase expression of MHC II, CD40, CD80, and CD86 of dendritic cell sarcoma cells to enhance antigen presentation.

TNF-α is known to have dual roles in host immune responses. In the early stages of infection, TNF-α acts as a pro-inflammatory cytokine; however, it can also cause severe tissue damage in chronic stages of infection. Therefore, we speculate that the hyper-inflammatory lung lesions shown in Fig. 2 might be associated with overproduction of TNF-α by H1N1 virus infection. Interestingly, the group treated with *Cordyceps* extract showed lower amounts of TNF-α compared to the untreated group, suggesting that *Cordyceps* extract might have an immune modulatory effect on over-activation of host immune responses. Li *et al.* (2009) found that *C. sinensis* extract acts as a stimulatory factor for maturation of dendritic cells and induces proinflmmatory cytokines in naive dendritic cells. However, *C. sinensis* extract was also shown to suppress LPS-induced inflammatory responses by decreasing the expression of costimulatory molecues (Li *et al.*, 2009). The two-sided activity of *Cordyceps* extract was also observed in our study. *Cordyceps* extract had an immune-enhancing effect in uninfected, healthy animals and an immune-inhibitory effect in H1N1 virus-infected animals. Therefore, we suggest that *Cordyceps* extract has a Yin-Yang balancing effect on host immune regulation.

Acknowledgements

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