



Chemical Constituents Identified from Fruit Body of *Cordyceps bassiana* and Their Anti-Inflammatory Activity

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Abstract

Cordyceps bassiana is one of *Cordyceps* species with anti-oxidative, anti-cancer, anti-inflammatory, anti-diabetic, anti-obesity, anti-angiogenic, and anti-nociceptive activities. This mushroom has recently demonstrated to have an ability to reduce 2,4-dinitrofluorobenzene-induced atopic dermatitis symptoms in NC/Nga mice. In this study, we further examined phytochemical properties of this mushroom by column chromatography and HPLC analysis. By chromatographic separation and spectroscopic analysis, 8 compounds, such as 1,9-dimethylguanine (1), adenosine (2), uridine (3), nicotinamide (4), 3-methyluracil (5), 1,7-dimethylxanthine (6), nudifloric acid (7), and mannitol (8) were identified from 6 different fractions and 4 more subfractions. Through evaluation of their anti-inflammatory activities using reporter gene assay and mRNA analysis, compound 1 was found to block luciferase activity induced by NF- κ B and AP-1, suppress the mRNA levels of cyclooxygenase (COX)-2 and tumor necrosis factor (TNF)- α . Therefore, our data strongly suggests that compound 1 acts as one of major principles in *Cordyceps bassiana* with anti-inflammatory and anti-atopic dermatitis activities.

Key Words: *Cordyceps bassiana*, Phytochemical study, 1,9-dimethylguanine, NF- κ B, AP-1, Anti-inflammatory activity

INTRODUCTION

Cordyceps species are insect-born mushrooms that have been known to possess a variety of biological material. As such, the *Cordyceps* species has been used for a long time in Chinese and Korean traditional folk medicine as a tonic for longevity, endurance and vitality, and it has been traditionally used as a therapeutic remedy for various diseases, including eczema, skin diseases, chronic bronchitis, asthma, and tuberculosis (Ng and Wang, 2005; Zhou *et al.*, 2009). *Cordyceps bassiana* is known to be a teleomorph (sexually reproducing form) of *Beauveria bassiana*. *B. bassiana* is a ubiquitous fungal pathogen of insect and fungal biocontrol agents widely used against insects (Rehner *et al.*, 2011) and widely used as a medicine for the treatment of infantile convulsions, epilepsy, stroke, sore throat, and many sorts of wounds before being used for insect biocontrol (Madsen *et al.*, 2007). In contrast,

C. bassiana was described in China on a carpenterworm larva and connected to unequivocally *B. bassiana* (Rehner *et al.*, 2011). More recently, *C. bassiana* was found in Korea and the evidence of the teleomorph-anamorph connection in the *Cordyceps* specimen was provided, in addition to *in vitro* production of *C. bassiana* (Hyun *et al.*, 2013).

By systematic analysis of the pharmacological activities of the *Cordyceps* species to date, anti-oxidative, anti-cancer, anti-inflammatory, anti-diabetic, anti-obesity, anti-angiogenic, and anti-nociceptive activities have been demonstrated (Ng and Wang, 2005; Zhou *et al.*, 2009). Recently, extracts of the artificially cultivated *C. bassiana* have been reported to inhibit the production of nitric oxide, interleukin (IL)-12, and interferon (IFN)- γ in lipopolysaccharide (LPS)-stimulated macrophages, splenic lymphocytes (Byeon *et al.*, 2011a, 2011b), as well as ameliorating atopic dermatitis symptoms in 2,4-dinitrofluorobenzene-treated NC/Nga mice (Wu *et al.*, 2011). In spite

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of its valuable pharmacological activities, no reports on the chemical constituents of *C. bassiana* have been published to date. Simultaneously, we are trying to develop an anti-atopic dermatitis remedy with this species, and have also performed research to dissect the chemical constituents of *C. bassiana* in order to identify the active components. Herein, isolation, structure determination, and anti-inflammatory activities of the 8 major compounds from a water extract of *C. bassiana* fruit body displaying anti-atopic dermatitis activity are described.

MATERIALS AND METHODS

Materials

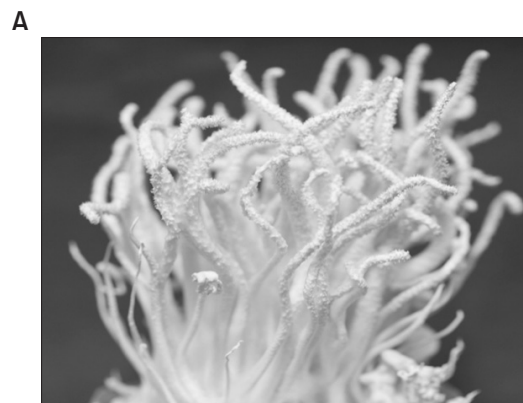
Artificially cultivated *Cordyceps bassiana* (*C. bassiana*) (Fig. 1A) were purchased from Mush-Tech (Gangwon, Korea), identified by Prof. Jae Mo Sung (Kangwon National University, Chuncheon, Korea). A voucher specimen (No. 1001) was deposited in the herbarium of Daewoong Pharmaceutical Co. Ltd. (Yongin, Korea). Lipopolysaccharide (LPS, *E. coli* 0111:B4) and (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bay 11-7082 (BAY), SB203580 (SB), and U0126 (U0) were obtained from Calbiochem (La Jolla, CA, USA). Luciferase constructs containing binding promoters for NF- κ B and AP-1 were gifts from Prof. Chung Hae Young (Pusan National University, Pusan, Korea) and Man Hee Rhee (Kyungpook National University, Daegu, Korea). Foetal bovine serum and RPMI 1640 were obtained from GIBCO BRL (Grand Island, NY, USA). RAW264.7 and HEK293 cells were purchased from ATCC (Rockville, MD, USA). All other chemicals were of Sigma grade. A powdered extract of *C. bassiana* was provided by Daewoong Pharmaceutical Co. Ltd (Yongin, Korea).

General experimental procedures

All NMR spectra were measured on an Agilent VNS-600 spectrometer or on a Bruker DPX 300 spectrometer operating at 600 or 300 MHz for $^1\text{H-NMR}$ and 150 or 75 MHz for $^{13}\text{C-NMR}$ in $\text{DMSO-}d_6$ using TMS as an internal standard. EIMS was measured on a Hewlett Packard model 5989B GC/MS spectrometer. HPLC (Agilent 1200 series system) composed of vacuum degasser, quaternary pump, diode array detector (DAD), manual injector, thermostatted column compartment using a Luna C18(2) 100A column (4.6 \times 250 mm 5 μm , Phenomenex) was used for isolation and purification of compounds. Silica gels (70-230 mesh, Merck, Darmstadt, Germany), Sephadex LH-20 (GE Healthcare, Uppsala, Sweden) were used for open column chromatography. TLC was performed on silica gel 60 F₂₅₄ (Merck).

Extraction and isolation

Powdered water extract of fruit bodies of *C. bassiana* (4.0 kg) was treated with MeOH (3 times, each with 10 liters) by using reflux apparatus for 3 h at 60°C that yielded a crude fraction (605 g) upon removal of the solvent *in vacuo*. The methanol fraction (165 g) was separated by silica gel column chromatography and eluted with CHCl_3 -MeOH mixtures of increasing polarity (100:1 \rightarrow 20:1) to afford 10 fractions (Fr. 1~10). Fraction 4 was purified by Sephadex LH-20 column chromatography using MeOH-Water (9:1) to produce compound 5. Fraction 6 was separated on a Sephadex LH-20 column chromatography



Artificially cultivated fruit body of *C. bassiana*

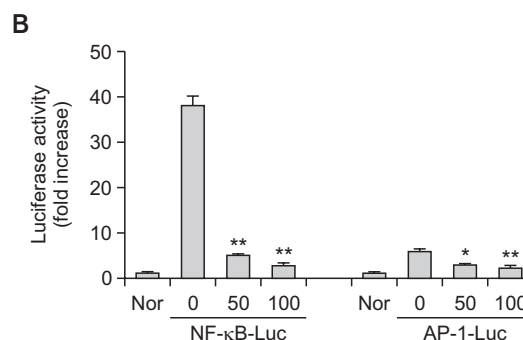


Fig. 1. A photo of an artificially cultivated fruit body of *Cordyceps bassiana* and the inhibitory activity of its water extract on AP-1 and NF- κ B-mediated luciferase. (A) A photo of an artificially cultivated fruit body of *Cordyceps bassiana*. (B) HEK293 cells co-transfected with plasmid constructs NF- κ B-Luc or AP-1-Luc (each 1 $\mu\text{g/ml}$) and β -gal (as a transfection control) were treated with the water extract of *Cordyceps bassiana* in the presence or absence of PMA (100 nM). Luciferase activity was measured using a luminometer. * $p < 0.05$ and ** $p < 0.01$ compared to control.

with MeOH-Water to generate 8 subfractions (Fr.6-1~Fr.6-2). Fraction 6-5 was separated by silica gel column chromatography and eluted with CHCl_3 -acetone mixtures to afford 18 subfractions (Fr.6-5-1~Fr.6-5-18). Subfraction 6-5-6 was recrystallized from MeOH to afford compound 7. Fractions 8 and 9 were recrystallized from MeOH to afford compound 6 and compound 1. The remaining fraction 9 was separated using a Sephadex LH-20 column chromatography with MeOH-Water to generate 8 subfractions (Fr.9-1~Fr.9-8). Fraction 9-8 was recrystallized from MeOH to afford compound 2. Fraction 9-6 was further purified by semi-preparative reverse-phase HPLC on a Phenomenex Luna C18 column (250 \times 10.0 mm, 5 μm , Phenomenex) and eluted with Water-MeOH (flow rate, 2.0 ml/min; 5-20% MeOH 30 min; UV, 254 nm) to produce compound 3 (t_R =12.8 min) and compound 4 (t_R =18.3 min). Fraction 10 was recrystallized from MeOH to afford compound 8.

HPLC profiling of the extract

Metabolic profiling was performed on an Agilent 1200 series HPLC coupled to an Agilent DAD detector. A Luna C18 column (250 mm \times 4.6 mm i.d., 5 μm ; Phenomenex, Torrance, CA, USA) was used and the mobile phase consisted of water (A) and acetonitrile (B) at a flow rate of 0.9 ml/min, as reported

previously (Vo *et al.*, 2015). The gradient elution started at 20% B and increased linearly to 100% B in 30 min followed by an isocratic 100% for a further 15 min. The column temperature was set to 20°C. The detection wavelength was 210 nm. The retention times for each compound were 20.66 (1), 22.37 (2), 10.33 (3), 14.27 (4), 12.30 (5), 30.30 (6), and 21.64 (7) min.

Table 1. Primer sequences used in the semiquantitative RT-PCR analyses

Gene	Primer sequences
Semiquantitative PCR	
TNF- α	F 5'-TTGACCTCAGCGCTGAGTTG-3'
	R 5'-CCTGTAGCCACGTCGTAGC-3'
COX-2	F 5'-CACTACATCCTGACCCACTT-3'
	R 5'-ATGCTCCTGCTTGAGTATGT-3'
GAPDH	F 5'-CACTCACGGCAAATTCACGGCAC-3'
	R 5'-GACTCCACGACATACTCAGCAC-3'

Luciferase reporter gene activity assay

HEK293 cells (1×10^6 cells/ml) were transfected in 12-well plates with NF- κ B-Luc or AP-1-Luc (each 1 μ g/ml), as well as β -galactosidase (0.25 μ g/ml), using the PEI method, as reported previously (Chen *et al.*, 2014). After 24 h, the transfected cells were treated with DWJ503 in the presence or absence of PMA (100 nM) or TNF- α (10 ng/ml) for NF- κ B-Luc and AP-1-Luc assays, and the cells were harvested and lysed to determine luciferase activity 1 day later. Luciferase assays were performed using the Luciferase Assay System (Promega), as reported previously (Yang *et al.*, 2015). Luciferase activity was normalized to β -galactosidase activity.

mRNA analysis by semiquantitative reverse transcription-polymerase chain reaction

To determine cytokine mRNA expression levels, total RNA was isolated from LPS-treated RAW264.7 cells with TRIzol Reagent (GIBCO BRL), according to the manufacturer's instructions (Moon *et al.*, 2014). Total RNA was stored at -70°C until use. Semi-quantitative RT reactions were performed as reported previously (Lee *et al.*, 2014). The results were expressed as the ratio of the optimal density to the density of GAPDH. The primers (Bioneer, Daejeon, Korea) used are listed in Table 1.

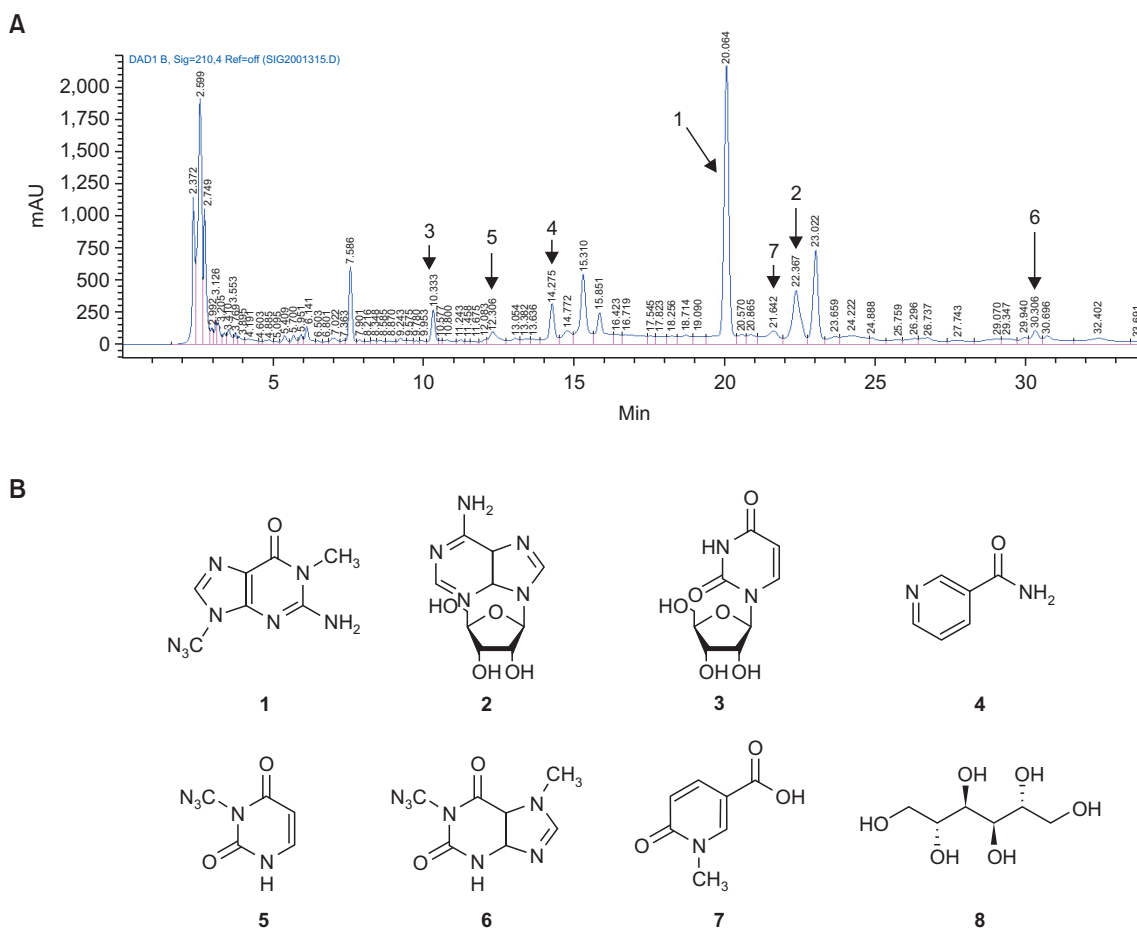


Fig. 2. Identification and isolation of ingredients from the methanol layer of *Cordyceps bassiana* water extract. (A) HPLC chromatogram of *C. bassiana* water extract (254 nm). (B) Chemical structures of purified compounds 1-8.

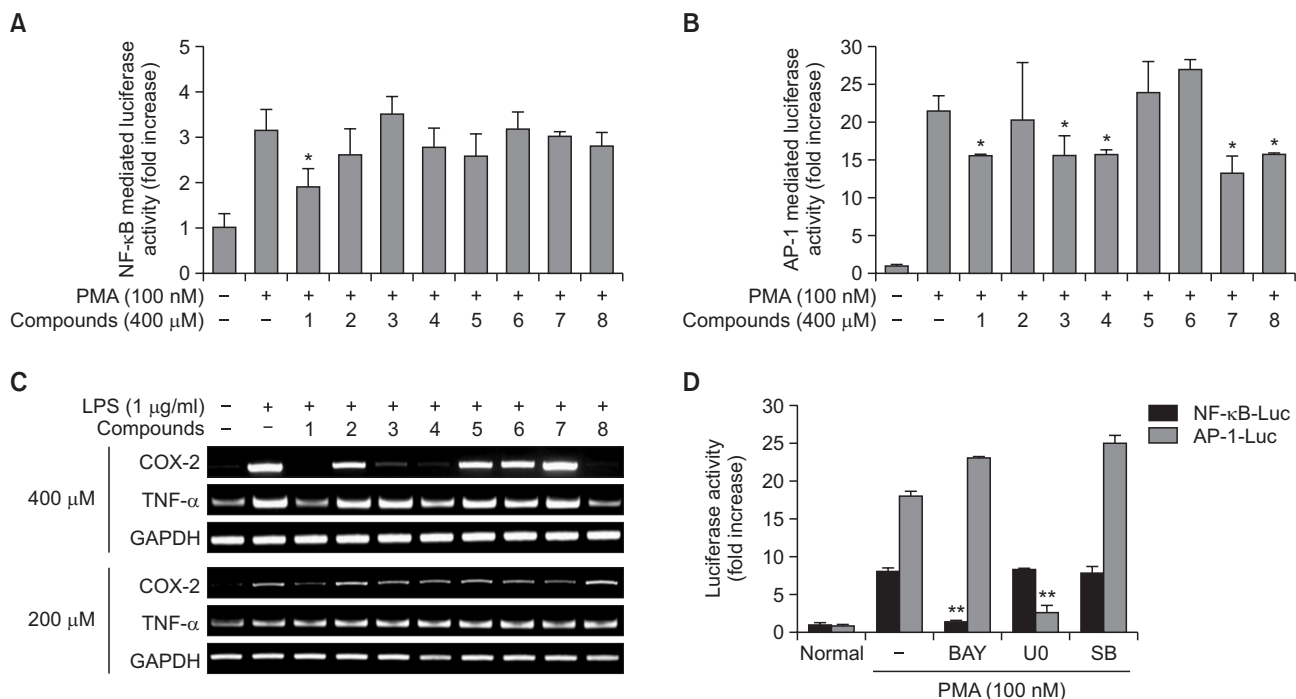


Fig. 3. The inhibitory effects of compounds 1-8 on the activation of NF-κB and AP-1, and the mRNA expression of pro-inflammatory genes. (A, B, D) HEK293 cells co-transfected with plasmid constructs NF-κB-Luc or AP-1-Luc (each 1 μg/ml) and β-gal (as a transfection control) were treated with compounds 1-8 or standard compounds [Bay 11-7082 (BAY), U0126 (UO), and SB203580 (SB)] in the presence or absence of PMA (100 nM). Luciferase activity was measured using a luminometer. (C) The mRNA levels of COX-2 and TNF-α were determined by semiquantitative PCR. **p*<0.05 and ***p*<0.01 compared to control.

Statistical analysis

All data are presented as the mean ± SEM of 3 different experiments performed with 4 samples (Fig. 1B, 3A, 3B, 3D) for *in vitro* experiments. For statistical comparisons, these results were analyzed using ANOVA/Scheffe’s post hoc test and a Kruskal–Wallis/Mann–Whitney test. A *p*-value of <0.05 was considered statistically significant. All statistical tests were performed using SPSS (SPSS Inc., Chicago, IL, USA). Similar experimental data were also observed by an additional independent set of *in vitro* experiments performed with the same numbers of samples.

RESULTS

Isolation and identification of chemical constituents

Since the total water extract of *C. bassiana* (Cb-WE) displayed strong inhibitory activities of transcriptional activation mediated by NF-κB and AP-1 (Fig. 1B), we further explored the compounds that can act as an anti-inflammatory principle in *C. bassiana* using column chromatography and HPLC. Indeed, as Supplementary Fig. 1, Fig. 2A show, we could separate 7 compounds from 6 different fractions and 4 more sub-fractions. Compound 8 was not detected under UV spectrum light due to the absence of a chromophore. Through column chromatographic separation, after all, 8 compounds were isolated as 1,9-dimethylguanine (1), adenosine (2), uridine (3), nicotinamide, 3-methyluracil (5), 1,7-dimethylxanthine (6), nudifloric acid (7), and mannitol (8) (Fig. 2B).

Effect of chemical constituents on inflammatory responses

Continuously, we evaluated the anti-inflammatory activities of 8 compounds using the same reporter gene assay and RT-PCR analysis of inflammatory genes. As Fig. 3 shows that compound 1 blocked luciferase activity induced by NF-κB activation, whilst compounds 1, 3, 4, 7, and 8 significantly suppressed AP-1-mediated luciferase activity, suggesting that these compounds may have an anti-inflammatory function. To confirm these activities, we next examined whether these compounds are able to modulate the expression of TNF-α and COX-2. At 200 μM, compound 1 clearly suppressed the mRNA levels of COX-2, whereas compounds 1, 3, 4, and 8 at 400 μM strongly suppressed COX-2 expression and compounds 1, 4, and 8 diminished the mRNA expression of TNF-α.

DISCUSSION

After establishing a technology to artificially cultivate the fruit bodies of *C. bassiana*, our group has focused on exploring the pharmacological activity of this mushroom and have attempted to expand the medical and nutraceutical applications of *C. bassiana* (Wu *et al.*, 2011). Because Cb-WE strongly inhibited transcriptional activation triggered by both NF-κB and AP-1 (Fig. 1B), representative redox-sensitive transcription factors inducing inflammatory gene expression (Lee *et al.*, 2011; Gil *et al.*, 2015), anti-inflammatory principle in *C. bassiana* was further isolated using column chromatography and HPLC. By this work, in fact, 8 compounds could be identified, including 1,9-dimethylguanine (1) (Sigel *et al.*, 2000), adenos-

ine (2) (Ciuffreda *et al.*, 2007), uridine (3) (Bagno *et al.*, 2008), nicotinamide (Jung *et al.*, 2012), 3-methyluracil (5) (Wong *et al.*, 2002), 1,7-dimethylxanthine (6) (Jiang *et al.*, 1998), nudifloric acid (7) (Yuyama and Suzuki, 1991), and mannitol (8) (Bock and Pedersen, 1983), respectively, based on their spectroscopic data (Fig. 2B). As mentioned earlier, chemical investigation of *C. bassiana* has never been previously carried out. Thus, compounds 1-8, which were isolated in this study, are all report for the first time from *C. bassiana*, despite the fact that their nucleic acids and glycosides were reported to be isolated from the *Cordyceps* species and adenosine (2), uridine (3), nicotinamide (4), 1,7-dimethylxanthine (6), and mannitol (8) have been already reported from various *Cordyceps* species. In this study, 1,9-dimethylguanine (1), 3-methyluracil (5), and nudifloric acid (7) were isolated for the first time from the *Cordyceps* species. According to our results, the occurrence of 1,9-dimethylguanine (1), 3-methyluracil (5), and nudifloric acid (7) in the *Cordyceps bassiana* may possibly serve as useful chemotaxonomic markers for species of this genus.

We next investigated the anti-inflammatory activities of the isolated constituents at the transcriptional levels. As Fig. 3 indicates, compound 1 was found to suppress NF- κ B-mediated luciferase activity, whereas others [compounds 1, 3, 4, 7, and 8] targeted to block AP-1-mediated luciferase activity, implying that these compounds may have an anti-inflammatory function. This possibility was also confirmed by testing the levels of TNF- α and COX-2 genes, representative inflammatory genes mediated by the activation of NF- κ B and AP-1 (Yu *et al.*, 2012). At 200 μ M, compound 1 was found to inhibit the mRNA levels of COX-2, but at 400 μ M, compounds 1, 3, 4, and 8 inhibited COX-2 expression and compounds 1, 4, and 8 downregulated TNF- α expression. Based on our data, these results strongly suggest that compound 1 seems to have potential activities acting as a major principle with anti-inflammatory activity. It has been previously reported that *C. bassiana* has the ability to ameliorate anti-atopic dermatitis symptoms (Wu *et al.*, 2011) and to suppress NF- κ B/AP-1-dependent cytokine production (Byeon *et al.*, 2011a, 2011b). Therefore, whether the compound 1 can be further developed as anti-atopic dermatitis drug with anti-inflammatory activity will be further elucidated.

Conclusively, we have isolated 8 compounds, 1,9-dimethylguanine (1), adenosine (2), uridine (3), nicotinamide (4), 3-methyluracil (5), 1,7-dimethylxanthine (6), nudifloric acid (7), and mannitol (8) from the water extract of *Cordyceps bassiana*. By evaluation of their anti-inflammatory activities, compound 1 has been revealed to be a major player in the action of *Cordyceps bassiana*. Therefore, further investigation of the immunopharmacological activities of this compound will be continued in our future projects.

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