

A direct protein kinase B-targeted anti-inflammatory activity of cordycepin from artificially cultured fruit body of *Cordyceps militaris*

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ABSTRACT

Background: *Cordyceps militaris* is one of well-known medicinal mushrooms with anti-inflammatory, anti-cancer, anti-diabetic, and anti-obesity activities. **Objective:** The objective of the following study is to isolate chemical components from the ethanol extract (Cm-EE) from *Cordyceps militaris* and to evaluate their anti-inflammatory activities. **Materials and Methods:** Column chromatographic separation was performed and anti-inflammatory roles of these compounds were also examined by using NO production and protein kinase B (AKT) activity assays. **Results:** From Cm-EE, 13 constituents, including trehalose (1), cordycepin (2), 6-hydroxyethyladenosine (3), nicotinic amide (4), butyric acid (5), β -dimorphecolic acid (6), α -dimorphecolic acid (7), palmitic acid (8), linoleic acid (9), cordyceptide A (10), 4-(2-hydroxy-3-((9E,12E)-octadeca-9,12-dienoyloxy)propoxy)-2-(trimethylammonio)butanoate (11), 4-(2-hydroxy-3-(palmitoyloxy)propoxy)-2-(trimethylammonio)butanoate (12), and linoleic acid methyl ester (13) were isolated. Of these components, compound 2 displayed a significant inhibitory effect on NO production in lipopolysaccharide (LPS)-activated RAW264.7 cells. Furthermore, this compound strongly and directly suppressed the kinase activity of AKT, an essential signalling enzyme in LPS-induced NO production, by interacting with its ATP binding site. **Conclusion:** *C. militaris* could have anti-inflammatory activity mediated by cordycepin-induced suppression of AKT.

Key words: Anti-inflammatory activity, cordycepin, *Cordyceps militaris* (Clavicipitaceae), phytochemical study, protein kinase B

INTRODUCTION

Cordyceps militaris (Clavicipitaceae) is a type of medicinal mushroom with various beneficial activities such as increasing the vitality, stamina, and endurance of the body. More importantly, because of the curative effects of this mushroom on numerous diseases, such as eczema, skin diseases, chronic bronchitis, asthma, and tuberculosis, it has been prescribed for a long time

in many different countries such as China, Japan, and Korea. Through systematic research, a large amount of information on its chemical ingredients (which include cordycepin, militarin, 3,6-dihydroxydecanolide, cordycepilic acid, and cordyheptapeptide) and its wide range of pharmacological activities (including anti-inflammatory, anti-oxidative, anti-tumourigenic, anti-hyperglycaemic, anti-apoptotic, immunomodulatory, nephroprotective, and hepatoprotective effects) have been revealed.^[1-3]

In spite of the large number of beneficial roles of this mushroom, development of *C. militaris* as a nutraceutical remedy was limited due to restrictions in the harvested amount of this mushroom. However, many different culture methods aimed at increasing the cultivation amount of *C. militaris* have been designed. Our group has also tried to develop new cultivation methods with brown rice to produce artificially cultivated fruit bodies of this mushroom [Figure 1a]. Additionally, we have reported

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several pharmacological activities of this mushroom, including platelet aggregation and macrophage-mediated inflammatory reaction. Because phytochemical studies with artificially cultivated fruit bodies of *C. militaris* have not been fully carried out, isolation, structure determination, and anti-inflammatory activities of the compounds from a 70% ethanol extract (EE) of *C. militaris* fruit bodies are described in this study.

MATERIALS AND METHODS

Materials

Artificially cultivated *C. militaris* [Figure 1a] was purchased from Mush-Tech (Gangwon-do, Korea). *C. militaris* was identified by Prof. Jae Mo Sung (Kangwon National University, Chuncheon, Korea). A voucher specimen (Registration number: RDA-NHJ-312-Cm-EE) was deposited in the herbarium at National Institute of Horticultural and Herbal Science (RDA, Eumsung, Korea). Lipopolysaccharide (LPS, *Escherichia coli* 0111:B4), staurosporin (Stau: Purity >95%), and (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT), were purchased from Sigma Chemical Co. (St. Louis, MO). LY294002 (LY: Purity >95%) and Wortmannin (Wort: Purity >95%), PI3K inhibitors, were obtained from Calbiochem (La Jolla, CA). The luciferase construct containing binding promoters for NF- κ B was gift from Prof. Chung, Hae Young (Pusan National University, Pusan, Korea). Antibodies against inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Fetal bovine serum (FBS)

and RPMI 1640 were obtained from GIBCO (Grand Island, NY). RAW264.7 and HEK293 cells were purchased from ATCC (Rockville, MD). All of the other chemicals were purchased from Sigma. GFP-fused wild-type (WT) protein kinase B construct (GFP-Akt-WT) and its mutant form (GFP-Akt-C310A) were used as reported previously.^{14]}

General experimental procedures

Nuclear magnetic resonance (NMR) spectra were measured on a Varian UNITY INOVA 500 spectrometer or on a Bruker AMX 500 spectrometer operating at 500 MHz for ¹H-NMR and 125 MHz for ¹³C-NMR with chemical shifts given in ppm (δ). Electrospray ionization-tandem mass spectrometry (ESI-MS) was measured on an Agilent 1100 liquid chromatography/mass spectrometry (LC/MS) spectrometer with a Phenomenex Luna C18 analytical column (5 μ m, 4.6 \times 100 mm). The high performance liquid chromatography (HPLC) apparatus (Waters Alliance series 2795 system) was composed of a vacuum degasser, a quaternary pump, a photodiode array detector, an auto-injector, and a column compartment with a thermostat. Flash and open column chromatography was carried out on Merck silica gel 60 (70–230 mesh). Reversed-phase HPLC was performed on a Gilson 321 pump system using Phenomenex Luna C18 column (10 μ m, 10 \times 250 mm) with a flow rate of 4 ml/min or 2 ml/min.

Extraction and isolation

Dried and powdered fresh fruiting bodies of *C. militaris* (1.0 kg) were extracted with 70% EtOH to prepare the Cm-EE. The powder was then treated with 100% MeOH (three times, each with 10 l) using a reflux

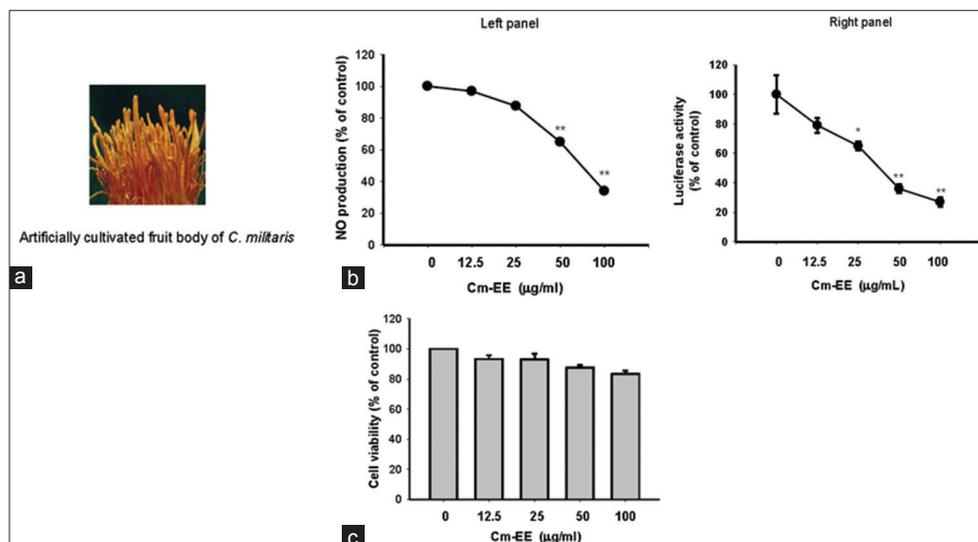


Figure 1: A photo of artificially cultivated fruit bodies of *Cordyceps militaris* and the anti-inflammatory activity of its ethanol extract (Cm-EE). (a) A photo of artificially cultivated fruit bodies of *C. militaris*. (b) The level of nitric oxide was determined by the Griess assay from culture supernatants of RAW264.7 cells treated with Cm-EE (0–100 μ g/ml) and lipopolysaccharide (1 μ g/ml) for 24 h. (c) Cell viability of RAW264.7 cells and HEK293 cells treated with Cm-EE was determined by an 3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole assay (** $P < 0.01$ compared to control)

apparatus that yielded a crude extracts (402 g) upon removal of the solvent *in vacuo*. The ethyl acetate layer (600 mg) was subjected to a C18 sepak chromatography eluting with step gradients of 20% (220 mg), 40% (130 mg), 60% (100 mg), 80% (60 mg), and 100% MeOH (40 mg) in H₂O, successively. The 20% MeOH fraction (220 mg) was separated by reversed-phase HPLC (Phenomenex Luna 10 μ m C18 (2), [10 \times 250 mm, 4 ml/min, UV 210 nm detection]) using a gradient solvent system from 10% to 100% acetonitrile over 60 min to afford compound 1 (t_R = 3 min, 5 mg) and compound 2 (t_R = 6.5 min, 3 mg). The 40% MeOH fraction (130 mg), contained the compound 3 and 4, was separated by reversed-phase HPLC (Phenomenex Luna 10 μ m C18 (2), [10 \times 250 mm, 4 ml/min, UV 210 nm detection]) using a gradient solvent system from 10% to 100% acetonitrile over 60 min. Compound 3 and 4 were finally purified by reversed-phase HPLC (Phenomenex Luna 10 μ m C18 (2) column [10 \times 250 mm, 2 ml/min, UV 254 nm detection]) with 10% methanol isocratic condition to yield 1.5 mg (t_R = 8 min) and 1 mg (t_R = 6 min), respectively. The methylene chloride crude extract (1.5 g) was fractionated on a silica gel (230–400 mesh) column and eluted with CH₂Cl₂-MeOH (100:1 \rightarrow 1:1, gradient system) to yield 10 fractions (MF1-MF10). The MF1 sub-fraction (400 mg) was purified by preparative C18 HPLC (10–100% acetonitrile in water for 60 min, 4 ml/min, 205 nm) to yield 13 (t_R = 55 min, 1.5 mg). The sub-fraction MF4 (250 mg) was purified by separation with preparative C18 HPLC (10–100% acetonitrile in water for 60 min, 4 ml/min, 210 nm) to yield 5 (2 mg), 8 (2.5 mg), 9 (5 mg), and 10 (2 mg) (t_R = 20 min, 57 min, 60 min, and 45 min, respectively). The compounds 6 and 7, which were isolated as *cis-trans* mixture (3 mg), were also isolated from sub-fraction M7 by preparative C18 HPLC (10–100% acetonitrile in water for 60 min, 4 ml/min, 240 nm). The MF10 sub-fraction (150 mg) was chromatographed on C18 sepak (230–400 mesh) column, and eluted with water and methanol (60% \rightarrow 100% methanol) to yield three fractions (MF1060, MF1080, and MF10100). The fraction MF10100 was purified by preparative C18 HPLC (10–100% acetonitrile in water for 60 min, 4 ml/min, 205 nm) to yield 11 (t_R = 45 min, 1.5 mg) and 12 (t_R = 47 min, 1.0 mg). The tested compounds were demonstrated to be pure as evidenced by NMR and HPLC analyses (purity \geq 95%).

Spectral and physicochemical data

Trehalose (1)

¹H NMR (500 MHz, D₂O) δ 5.02 (1H, d, J = 3.8 Hz, H-1), 3.47 (1H, dd, J = 10.0, 3.8 Hz, H-2), 3.64 (1H, m, H-3), 3.27 (1H, t, J = 9.5 Hz, H-4), 3.67 (1H, m, H-5), 3.67 (1H, m, H-6a), 3.58 (1H, dd, J = 12.0, 5.0 Hz, H-6b); ¹³C NMR (125 MHz, D₂O) δ 93.1 (C-1), 70.8 (C-2), 72.3 (C-3), 69.5 (C-4), 71.9 (C-5), 60.3 (C-6); ESIMS m/z 365 [M + Na]⁺.

Cordycepin (2)

¹H NMR (500 MHz, D₂O) δ 8.09 (1H, s, H-8), 7.97 (1H, s, H-2), 5.84 (1H, d, J = 2.2 Hz, H-1'), 4.62 (1H, m, H-2'), 4.44 (1H, m, H-4'), 3.74 (1H, dd, J = 12.7, 2.7 Hz, H-5'), 3.53 (1H, dd, J = 12.7, 4.5 Hz, H-5'), 2.12 (1H, ddd, J = 14.7, 8.9, 5.9 Hz, H-3'), 2.01 (1H, ddd, J = 13.8, 6.5, 3.2 Hz, H-3'); ¹³C NMR (125 MHz, D₂O) δ 155.3 (C-4), 152.3 (C-2), 149.0 (C-6), 139.6 (C-8), 119.6 (C-5), 90.7 (C-1'), 81.1 (C-4'), 74.8 (C-2'), 62.5 (C-5), 33.0 (C-3'); ESIMS m/z 252 [M + H]⁺.

N6-(2-Hydroxyethyl) adenosine (3)

Colourless gum; ¹H NMR (500 MHz, D₂O) δ 8.09 (1H, s, H-8), 8.04 (1H, s, H-2), 5.87 (1H, d, J = 5.6 Hz, H-1'), 4.61 (1H, t, J = 5.6 Hz, H-2'), 4.24 (1H, m, H-4'), 4.11 (1H, m, H-3'), 3.73 (1H, dd, J = 12.9, 3.6 Hz, H-5'), 3.67-3.65 (5H, m, H-5', H-9, H-10); ESIMS m/z 312 [M + H]⁺.

Nicotinic amide (4)

White powder; ¹H NMR (500 MHz, D₂O) δ 8.70 (1H, s, H-6), 8.49 (1H, d, J = 5.0 Hz, H-2), 8.01 (1H, d, J = 8.0 Hz, H-4), 7.37 (1H, m, H-3); ¹³C NMR (125 MHz, D₂O) δ 170.6 (C-7), 151.6 (C-6), 147.4 (C-2), 136.3 (C-4), 129.1 (C-5), 124.0 (C-3); ESIMS m/z 123 [M + H]⁺.

Butyric acid (5)

¹H NMR (500 MHz, CD₃OD) δ 2.27 (2H, t, J = 15.0 Hz, H-2), 1.63 (2H, dq, J = 15.0, 8.0 Hz, H-3), 0.96 (3H, t, J = 8.0 Hz, H-4); ¹³C NMR (125 MHz, CD₃OD) δ 176.1 (C-1), 35.4 (C-2), 18.0 (C-3), 12.6 (C-4).

β -Dimorphecolic acid (6)

¹H NMR (500 MHz, CDCl₃) δ 0.89 (3H, t, J = 7.0, H-18), 1.32 (14H, m, H-4, H-5, H-6, H-7, H-15, H-16, H-17), 1.52 (2H, m, H-8), 1.62 (2H, m, H-3), 2.16 (2H, m, H-14), 2.36 (2H, t, J = 7.5 Hz, H-2), 4.11 (1H, dd, J = 13.0, 6.5 Hz, H-9), 5.58 (1H, dd, J = 15.0, 7.0 Hz, H-13), 5.71 (1H, dd, J = 15.0, 7.0 Hz, H-10), 6.03 (1H, dd, J = 15.0, 10.0 Hz, H-12), 6.18 (1H, dd, J = 15.0, 10.0 Hz, H-11); ESIMS m/z 319 [M + Na]⁺.

α -Dimorphecolic acid (7)

Cis and *trans* form mixture, ESIMS m/z 319 [M + Na]⁺.

Palmitic acid (8)

¹H NMR (500 MHz, CDCl₃) δ 2.36 (2H, t, J = 7.5 Hz, H-2), 1.64 (2H, dt, J = 15.0, 7.5 Hz, H-15), 1.38-1.22 (24H, m, H-3,4,5,6,7,8,9,10,11,12,13,14), 0.89 (3H, t, J = 7.0 Hz, H-16); ¹³C NMR (125 MHz, CDCl₃) δ 180.0 (C-1), 34.0 (C-2), 31.9 (C-14), 29.6 (C-6, 7, 8, 9, 10, 11, 12), 29.3 (C-5), 29.2 (C-13), 29.0 (C-4), 24.7 (C-3), 22.7 (C-15), 14.1 (C-16).

Linoleic acid (9)

Colourless oil; ¹H NMR (500 MHz, CDCl₃) δ 5.44-5.25 (4H, m, H-9, 10, 12, 13), 2.78 (2H, m, H-11), 2.35 (2H, m, H-2), 2.06 (4H, m, H-8, 14), 1.64 (2H, m, H-17), 1.44-1.22 (14H, m, H-3,4,5,6,7,15,16), 0.90 (3H, m, H-18); ¹³C NMR (125 MHz, CDCl₃) δ 180.6 (C-1), 130.1 (C-9), 129.9 (C-13), 128.0 (C-10), 127.8 (C-12), 34.1 (C-2), 31.5 (C-16), 29.5 (C-6), 29.4 (C-15), 29.1 (C-7), 29.0 (C-5), 29.0 (C-4), 27.2 (C-14), 27.1 (C-8), 25.6 (C-11), 24.6 (C-3), 22.6 (C-17), 14.1 (C-18).

Cordyceptide (10)

ESIMS m/z 488 [M + H]⁺.

Glycerfatty acid (11)

ESIMS m/z 498 [M + H]⁺.

Glycerfatty acid (12)

ESIMS m/z 474 [M + H]⁺.

Linoleic acid methyl ester (13)

¹H NMR (500 MHz, CDCl₃) δ 5.44-5.30 (4H, m, H-9, 10, 12, 13), 3.67 (3H, s, -OCH₃), 2.78 (2H, m, H-11), 2.35 (2H, m, H-2), 2.05 (4H, m, H-8, 14), 1.62 (2H, m, H-3), 1.41-1.26 (14H, m, H-4,5,6,7,15,16,17), 0.89 (3H, m, H-18); ¹³C NMR (125 MHz, CDCl₃) δ 174.4 (C-1), 130.2 (C-9), 130.3 (C-13), 128.0 (C-10), 127.8 (C-12), 51.4 (-OCH₃), 34.1 (C-2), 31.5 (C-16), 29.6 (C-6), 29.4 (C-15), 29.1 (C-7), 29.1 (C-5), 29.1 (C-4), 27.2 (C-14), 27.2 (C-8), 25.6 (C-11), 24.9 (C-3), 22.6 (C-17), 14.1 (C-18).

Cell culture

RAW264.7 and HEK293 cells were cultured with RPMI 1640 medium supplemented with 10% heat-inactivated FBS, glutamine, and antibiotics (penicillin and streptomycin) at 37°C in a 5% CO₂ atmosphere. For each experiment, the cells were detached with a scraper. Examination of cell densities of 2 × 10⁶ cells/ml revealed that the proportion of dead cells was consistently < 1% using Trypan blue dye exclusion as the criteria for viability.

Nitric oxide and PGE₂ production

After pre-incubation of RAW264.7 cells (1 × 10⁶ cells/ml) for 18 h, the cells were pre-treated with Cm-EE and its chemical ingredients for 30 min. The cells were then further incubated with LPS (1 μg/ml) for 24 h. The inhibitory effects of Cm-EE and its chemical ingredients on NO and PGE₂ production were determined by analyzing NO and PGE₂ levels using the Griess reagent and an EIA kit, as described previously.^[5,6]

mRNA analysis by semi-quantitative reverse transcriptase-polymerase chain reaction

To evaluate cytokine mRNA expression levels, total RNA was isolated from the LPS-treated RAW264.7 cells

with TRIzol Reagent (Gibco BRL), according to the manufacturer's instructions. The total RNA was stored at -70°C until use. Semi-quantitative reverse transcriptase (RT) reactions were conducted as reported previously.^[7,8] The primers (Bioneer, Seoul, Korea) used are indicated in Table 1.

Cell viability test

After pre-incubation of RAW264.7 cells (1 × 10⁶ cells/ml) for 18 h, Cm-EE and its chemical ingredients were added to the cells and incubated for 24 h. The cytotoxic effects of Cm-EE and its chemical ingredients were then evaluated by a conventional MTT assay, as reported previously.^[9] Three hours prior to culture termination, 10 μL of a MTT solution (10 mg/ml in phosphate buffered saline, pH 7.4; PBS) was added, and the cells were continuously cultured until termination of the experiment. The incubation was halted by the addition of 15% sodium dodecyl sulfate into each well to solubilize the produced formazan.^[10] The absorbance at 570 nm (OD₅₇₀) was measured using a SpectraMax 250 microplate reader (BioTex, Bad Friedrichshall, Germany).

Immunoblotting analysis

HEK293 cells (5 × 10⁶ cells/ml) were lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1% Triton X-100, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM benzimidazole, and 2 mM hydrogen peroxide) for 30 min rotating at 4°C. The lysates were clarified by centrifugation at 16,000 × g for 10 min at 4°C. The soluble cell lysates were immunoblotted, and the total level of AKT was visualised as previously reported.^[11]

Immunoprecipitation and *in vitro* kinase assay for protein kinase B

HEK 293 cells were placed on ice and extracted with lysis buffer as reported previously.^[4] Lysates

Table 1: Primer sequences used in the RT-PCR analysis

Gene		Primer sequences
IL-6	F	5'-GTA CTCCAGAAGACCAGAGG-3'
	R	5'-TGCTGGTGACAACCACGGCC-3'
iNOS	F	5'-CCCTTCCGAAGTTTCTGGCAGCAG-3'
	R	5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'
COX-2	F	5'-CACTACATCCTGACCCACTT-3'
	R	5'-ATGCTCCTGCTTGAGTATGT-3'
TNF-α	F	5'-TTGACCTCAGCGCTGAGTTG-3'
	R	5'-CCTGTAGCCACGTCGTAGC-3'
GAPDH	F	5'-CACTCACGGCAAATCAACGGCAC-3'
	R	5'-GACTCCACGACATACTCAGCAC-3'

IL-6: Interleukin-6; TNF-I: Tumor necrosis factor; COX-2: Cyclooxygenase-2; iNOS: Inducible nitric acid synthase; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; RT-PCR: Reverse transcriptase-polymerase chain reaction

prepared from GFP-AKT-WT or GFP-AKT-C310A overexpressing cells was immunoprecipitated with anti-GFP monoclonal antibody immobilized on protein G-Sepharose (Amersham Biosciences). The kinase activity of the immunoprecipitated beads was measured with peptide substrate, Suntide (RRKDGATMKTFCGTPE), as reported previously.^[4] The kinase activity of purified AKT1 enzyme was evaluated by the kinase profiler service from Millipore (Billerica MA) as reported previously.^[4]

Statistical analysis

All of the data except Figures 1a and 2 are presented as the means \pm standard mean of the error of three different experiments performed with three samples. For the statistical comparisons, these results were analyzed using an ANOVA/Scheffe's *post hoc* test and a Kruskal–Wallis/Mann–Whitney test. A $P < 0.05$ was considered to be

statistically significant. All of the statistical tests were performed using SPSS (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

After establishing a cultivation method to greatly increase production of *C. militaris* [Figure 1a], we sought to determine the pharmacological activities of the mushroom for later application to various purposes, such as development of functional food products, cosmetics, and new druggable compounds.^[12] A 70% EE of *C. militaris* (Cm-EE) was able to strongly inhibit the production of nitric oxide (NO) from LPS-treated RAW264.7 cells up to 100 $\mu\text{g}/\text{mL}$ [Figure 1b, left panel] without altering cell viability [Figure 1c]. Similarly, Cm-EE dose-dependently suppressed luciferase activity mediated by nuclear factor (NF)- κB [Figure 1b, right panel], a representative

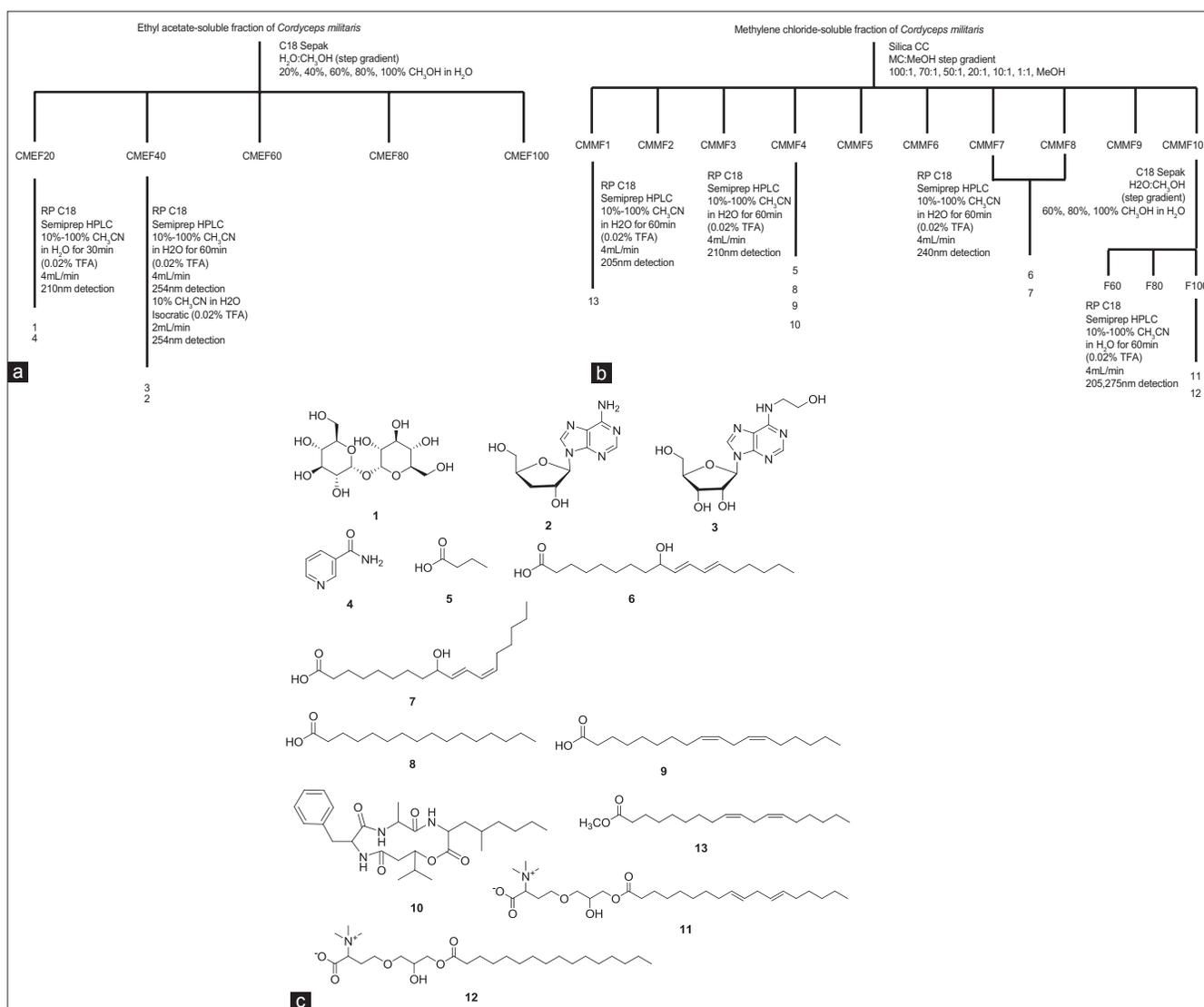


Figure 2: Phytochemical analysis of cultivated fruit bodies of *Cordyceps militaris*. (a) Isolation of ingredients from the ethyl acetate layer of Cm-EE. (b) Isolation of ingredients from the methylene chloride layer of Cm-EE. (c) Chemical structures of purified compounds (1–13)

inflammation-regulatory transcription factor.^[13] These results led us to employ column chromatography and HPLC to further investigate what types of compounds from the EE of *C. militaris* can attenuate inflammation [Figure 2a and b]. As we expected, we isolated 13 different compounds from

6 fractions [Figure 2a and b]. Through spectroscopic analysis, these compounds [Figure 2c] were identified as trehalose (1), cordycepin (2), 6-hydroxyethyladenosine (3), nicotinic amide (4), butyric acid (5), β -dimorphecolic acid (6), α -dimorphecolic acid (7), palmitic acid (8), linoleic

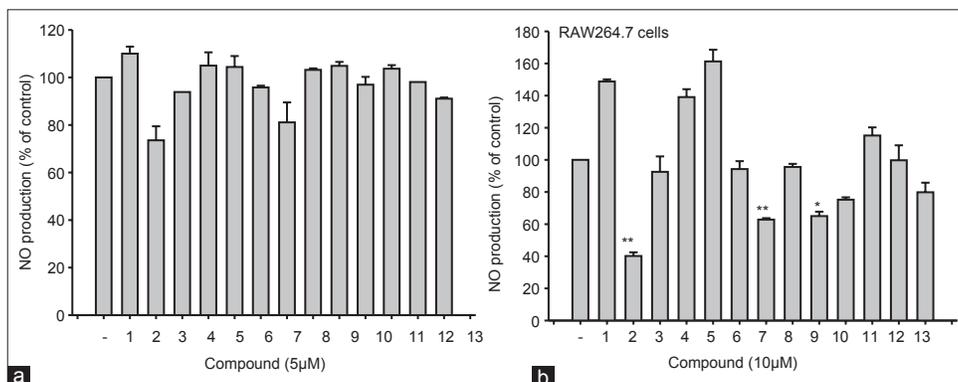


Figure 3: Inhibitory effects of compounds (1–13) on the production of nitric oxide (NO) in lipopolysaccharide (LPS)-treated RAW264.7 cells. (a and b) Level of NO was determined by the Griess assay from culture supernatants of RAW264.7 cells treated with compounds (1–13) and LPS (1 g/ml) for 24 h (a: Right panel)

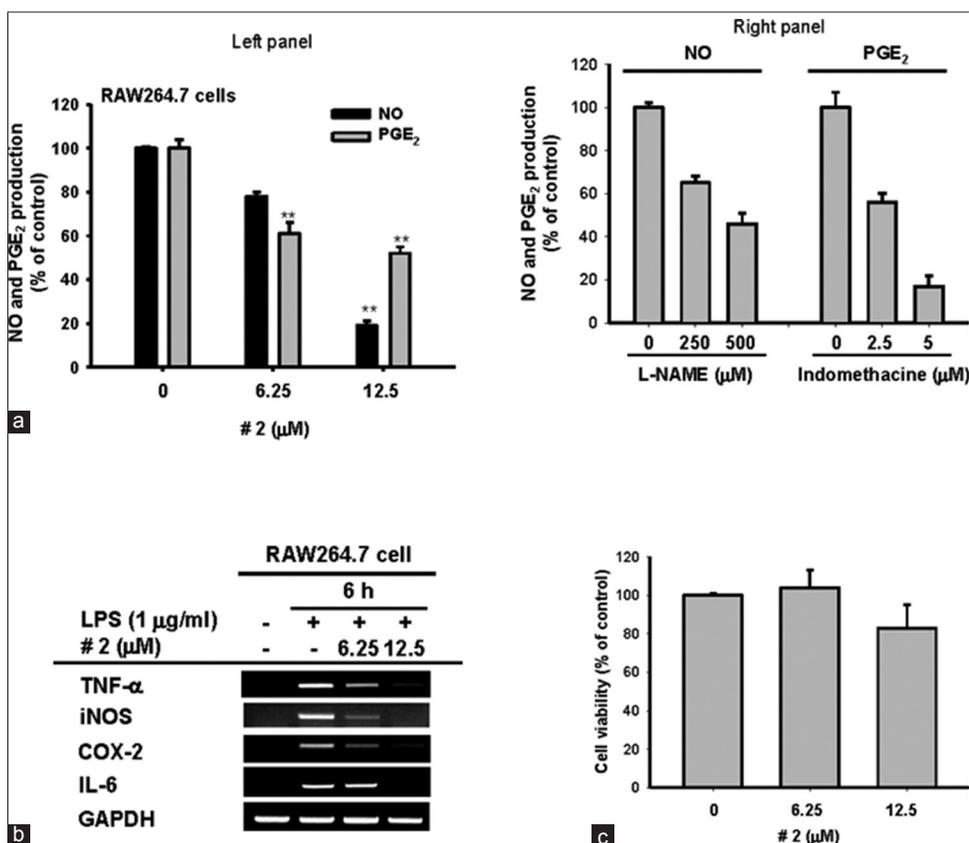


Figure 4: Inhibitory effects of compound 2 on the production of nitric oxide (NO) and PGE₂, the expression of inflammatory genes, and the viability of RAW264.7 cells. (a: Left panel and right panel) Levels of NO and PGE₂ were determined by the Griess assay, ELISA, and EIA from the culture supernatants of RAW264.7 cells treated with compound 2 (0–12.5 μM), standard compounds (L-NAME and indomethacine), and lipopolysaccharide (LPS) (1 μg/ml) for 24 h. (b) The mRNA levels of inducible nitric oxide synthase, cyclooxygenase-2, tumor necrosis factor- α , and interleukin-6 were determined by reverse transcriptase-polymerase chain reaction using RAW264.7 cells treated with compound 2 (0–12.5 μM) and LPS (1 μg/ml) for 24 h. (c) Cell viability of RAW264.7 cells treated with compound 2 (0–12.5 μM) was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole assay. All data are presented as the mean \pm standard error of the mean of three different experiments performed with three samples (* P < 0.05 and ** P < 0.01 compared to control or normal group)

acid (9), cordyceptide A (10), 4-(2-hydroxy-3-((9E,12E)-octadeca-9,12-dienoyloxy) propoxy)-2-(trimethylammonio) butanoate (11), 4-(2-hydroxy-3-(palmitoyloxy) propoxy)-2-(trimethylammonio) butanoate (12), and linoleic acid methyl ester (13) by comparison with their previously published spectroscopic data.^[14-20] Of these compounds, cordyceptide A (10), 4-(2-hydroxy-3-((9E,12E)-octadeca-9,12-dienoyloxy) propoxy)-2-(trimethylammonio) butanoate (11), and 4-(2-hydroxy-3-(palmitoyloxy) propoxy)-2-(trimethylammonio) butanoate (12) were isolated for the first time from the *C. militaris*, indicating that these compounds could potentially serve as useful chemotaxonomic markers for this mushroom.

To confirm which of these components possess anti-inflammatory activity, the suppressive activities of the 13 compounds on the production of NO and PGE₂ and the expression of inflammatory genes such as iNOS, COX-2, tumor necrosis factor (TNF)- α , and interleukin (IL)-6 were examined in LPS-treated RAW264.7 cells.^[21-24] We also evaluated the molecular target of the main active component using immunoprecipitated and purified enzyme.

Of 13 compounds, compound 2 showed the highest NO inhibitory activity at 5 and 10 μ M, while other compounds (7 and 9) also significantly decreased NO production [Figure 3a and b]. Furthermore, compound 2 dose-dependently suppressed the release of NO and PGE₂ [Figure 4a, left panel] and the expression of various inflammatory genes such as TNF- α , iNOS, COX-2, and IL-6 [Figure 4b], without altering the viability of RAW264.7 cells [Figure 4c], implying that the inhibitory activity of compound 2 is not due to its nonspecific pharmacological action. Meanwhile, standard compounds (L-NAME and indomethacine) diminished the release of NO and PGE₂ [Figure 4a, right panel], indicating that our experimental conditions were well established.

It has been reported that compound 2 (cordycepin) is capable of suppressing the phosphorylation of AKT,^[25] an essential step for inducing inflammatory signaling for NF- κ B activation,^[4] in LPS-treated RAW264.7 cells. Therefore, we examined whether this compound can directly suppress the enzyme activity of AKT using an *in vitro* kinase assay. As we expected, compound 2 blocked immunoprecipitated AKT enzyme activity up to 85%, similar to the effect of hydroquinone [Figure 5a],

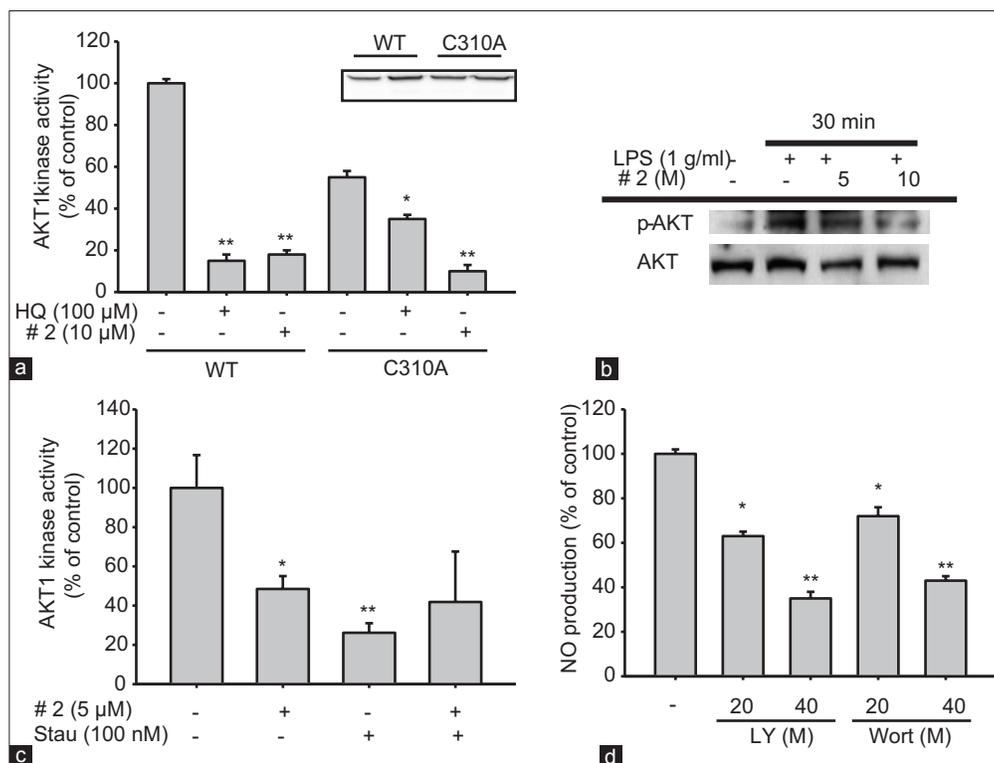


Figure 5: Inhibitory effect of compound 2 on protein kinase B (AKT) kinase activity. (a and c) Direct inhibition of AKT kinase activity by compound 2 or staurosporin (Stau) was measured using immunoprecipitated or purified AKT (WT or mutant [C310A]). The level of AKT transfected was measured by immunoblotting analysis. (b) After immunoblotting, levels of phospho- or total AKT in whole lysates from lipopolysaccharide (LPS)-activated RAW264.7 cells pretreated with compound 2 (5 and 10 μ M) were identified using specific antibodies. (d) Level of nitric oxide was determined by the Griess assay from the culture supernatants of RAW264.7 cells treated with standard compounds (LY294002 [LY] and wortmannin [Wort]), and LPS (1 μ g/ml) for 24 h. All of the data are presented as the mean \pm standard error of the mean of three different experiments performed with three samples * P < 0.05 and ** P < 0.01 compared to control or normal

a powerful thiolation-inducing benzene metabolite with chemical reactivity.^[4] In agreement with a previous report,^[25] compound 2 also diminished p-AKT levels [Figure 5b]. Unlike hydroquinone, this compound still maintained its suppressive activity in the kinase assay performed with mutant Akt (Akt-C310A) with alanine at the cysteine-310 residue [Figure 5a], which is one of the allosteric sites affecting its kinase activity.^[4] This indicates that compound 2 is not able to bind to this site. Instead, co-treatment of compound 2 with staurosporin, a competitive inhibitor to the ATP binding site, indicated the possibility that cordycepin can also bind to the ATP binding site, since no additive or synergistic inhibitory activity was exhibited toward purified AKT even with co-treatment of these two compounds [Figure 5c]. The importance of AKT in the NO production process in LPS-treated RAW264.7 cells was also confirmed by treatment with PI3K/AKT inhibitors LY294002 (LY) and wortmannin (Wort). These compounds clearly diminished the level of NO produced by LPS exposure in RAW264.7 cells [Figure 5d]. Therefore, these results strongly suggest that compound 2 is an active component in Cm-EE with anti-inflammatory effects associated with direct suppression of AKT.

Cordycepin (compound 2) is well-known major component identified in *C. militaris*, which has been received much attention because of its broad-spectrum biological activities, such as anti-cancer, antioxidant and anti-inflammatory activities.^[26-28] In fact, in our study, compound 2 was also revealed to inhibit inflammatory responses through the blockade of AKT [Figure 5]. Even though the possibility that cordycepin can suppress AKT pathway by measuring its simple phosphorylation level,^[25,29] there was no direct evidence to prove this possibility. However, we apparently verified in detail that cordycepin can directly suppress AKT activity [Figure 5a]. Thus, cordycepin blocked the AKT kinase activity by competing with ATP to ATP binding domain of AKT, indicating direct AKT regulatory role of cordycepin. Because AKT is a critical molecule in numerous inflammatory responses,^[4] our data seem to open further application of cordycepin for the development of various functional products, such as, cosmetics, functional foods and new drugs to ameliorate various inflammation-based symptoms.

CONCLUSION

Thirteen compounds were identified from the EE of *C. militaris*. Using phytochemical and pharmacological studies, compound 2 was found to be a major anti-inflammatory compound in *C. militaris* with direct AKT inhibitory property. Therefore, further investigation will be conducted to evaluate its potential application using

various *in vivo* inflammatory disease models such as gastritis, colitis, arthritis, and hepatitis, mediated by AKT.

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