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Inhibition of cytokine expression by a butanol extract from *Cordyceps bassiana*

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Cordyceps species have been known since long as a multi-utility ethnomedicinal herbal in Korea, China and Japan. It has been reported to exhibit a number of properties such as anti-oxidative, anti-cancer, anti-inflammatory, anti-diabetic, and anti-obesity effects. In a previously conducted study, we had demonstrated that the ethanol extract of *Cordyceps bassiana* was able to suppress the production of interleukin (IL)-12 and interferon (IFN)-γ in macrophages and T lymphocytes. In this study, we were able to further explore the molecular basis of its inhibitory mechanism using a butanol fraction of this herbal (Cb-BF) preparation. Similarly, this fraction also blocked the expression of cytokines such as IL-12 and tumor necrosis factor (TNF)-α as well as the proliferation of splenic lymphocytes and their production of IFN-γ but not IL-4. Cb-BF suppressed the luciferase activities that are mediated by nuclear factor (NF)-κB, activator protein (AP)-1, and signal transducers and activators of transcription (STAT)-1. In agreement with this, these fractions diminished the translocation of the transcription factors into the nucleus. The study also demonstrated that the upstream signaling events for the activation of these factors such as spleen tyrosine kinase (Syk), janus kinase (JAK)-2, and extracellular signal-regulated kinase (ERK) were suppressed. Therefore, these results suggest that the butanol extract of *Cordyceps bassiana* may contain more than one active component capable of inhibiting the inflammatory signaling cascade and this can be considered as a potential candidate for treatment of diseases that require suppression of immune system.

1. Introduction

Cordyceps species are mushrooms ethopharmacologically known in Far East Asian countries such as Korea, China, and Japan. Preparation of the species *Cordyceps sinensis* and *Cordyceps militaris* have been traditionally prescribed as a tonic for longevity, endurance, and vitality (Zhou et al. 2009). Continuous scientific studies have found that these preparations are capable of exhibiting numerous pharmacological activities as anti-oxidative, anti-viral, anti-cancer, anti-fibrotic, anti-inflammatory, anti-nociceptive, anti-angiogenic, anti-diabetic, and anti-obesity drugs (Ng and Wang 2005; Zhou et al. 2009). Many species of *Cordyceps* (more than 600 types) have not yet been evaluated for their pharmacological activities. A preliminary study performed by us indicated that the biological activity varied between species and even between strains of the same species. *Cordyceps bassiana* is one of recently established species of *Cordyceps* intended for artificial cultivation (Fig. 1). Using an artificially prepared fruit body, we had tried to evaluate its therapeutic efficacy against animal atopic dermatitis models. This extract showed very promising curative activity

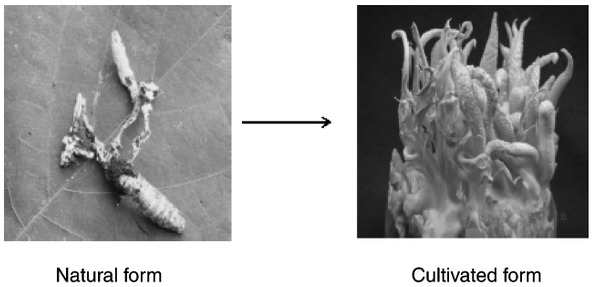


Fig. 1: Photos of natural and cultivated forms of *Cordyceps bassiana*

on 2,4-dinitro-1-fluorobenzene-induced dermatitis in NC/Nga mice (Wu et al., submitted). It has been demonstrated that the ethanolic extract of *Cordyceps bassiana* (Cb-EE) is able to suppress LPS-induced expression of IL-12, one of the important cytokines involved in the modulation of Th1 differentiation, in macrophages by blockade of p38 activation (Byeon et al. 2010). The production of cytokines in immune cells is critically controlled by the transcriptional activation mediated by var-

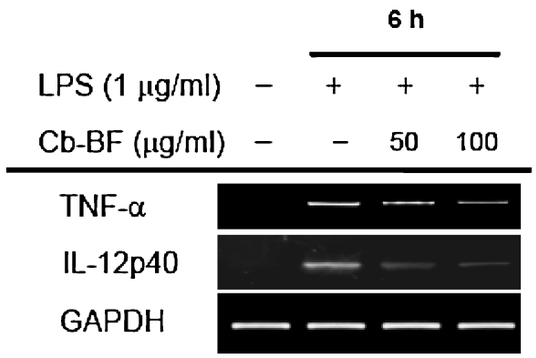
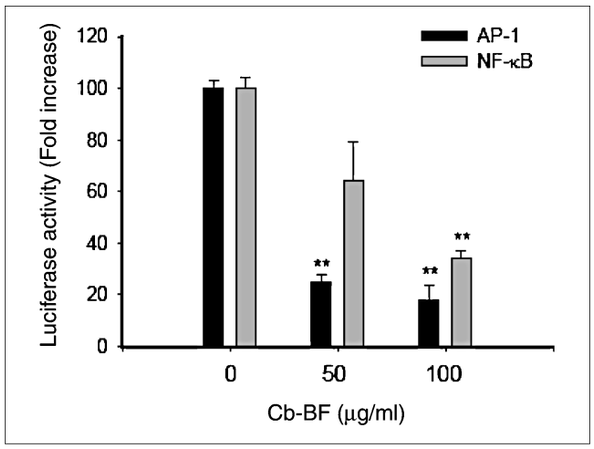
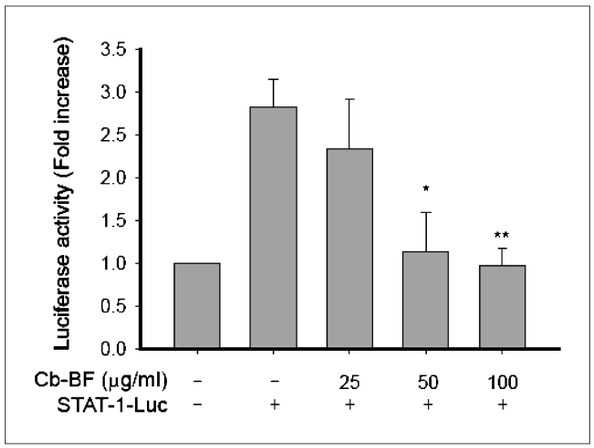


Fig. 2: Effect of Cb-BF on the mRNA expression of TNF-α and IL-12 in LPS-activated RAW264.7 cells. RAW264.7 cells (2×10^6 cells/ml) were incubated with Cb-BF in the presence of LPS (1 μg/ml) for 6 h. The mRNA levels of IL-12 and GAPDH were determined by RT-PCR



(A)



(B)

Fig. 3: Effect of Cb-BF on the luciferase activity mediated by NF-κB, AP-1 and STAT-1. (A and B) HEK293 cells co-transfected with one or two of plasmid constructs, STAT-1-Luc, NF-κB-Luc or AP-1-Luc (each 1 μg/ml) and β-gal (as a transfection control) were treated with Cb-BF in the presence or absence of PMA (100 nM, for AP-1-Luc), TNF-α (10 ng/ml, for NF-κB-Luc) or IFN-γ (10 ng/ml, for STAT-1-Luc) for 12 h. Luciferase activity was determined by luminometry. *: $p < 0.05$ and **: $p < 0.01$ represent significant difference compared to control group

ious transcription factors such as nuclear factor (NF)-κB, signal transducers and activators of transcription (STAT)-1, and activator protein (AP)-1 (Pourazar et al. 2005). The up-regulation of these transcription factors is an important step for the activation of a signaling cascade composed of various protein kinases [Src, Syk, phosphoinositide 3-kinase (PI3K), and Akt (protein kinase B)] for NF-κB translocation, mitogen activated protein kinases (MAPKs) [such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38] for AP-1 translocation, and Janus kinase (JAK)-2 for STAT-1 translocation (Natarajan et al. 2002; Rahman et al. 2004). Since excessive production of pro-inflammatory cytokines such as IL-12 and tumor necrosis factor (TNF)-α is known to play a major role in the etiology of various chronic immunological diseases such as atopic dermatitis and eczema, development of cytokine production modulatory drugs could be valuable in terms of prevention or therapy of these diseases (Garcia-Lafuente et al. 2009).

In this study, we aimed to enhance the knowledge on the immunoregulatory role of *Cordyceps bassiana* during pro-inflammatory cytokine gene expression from macrophages and splenic lymphocytes. In particular, the approaches were focused on the understanding of inhibitory mechanisms by evaluation of signaling pathways for transcription factor activation and cytokine gene expression in macrophages mediated by a butanol extract of *Cordyceps bassiana* (Cb-BF).

2. Investigations, results and discussion

Previously, we found that Cb-EE was able to suppress IL-12 expression by suppressing the activation of p38 and subsequent translocation of AP-1 and CREB, without altering cell viability. In this study, the immunomodulatory mechanisms of Cb-BF prepared from Cb-EE have been examined under the activation events of macrophage and T cells such as cytokine production and proliferation.

As Fig. 2 shows, Cb-BF displayed strong inhibitory effects on cytokine (TNF-α and IL-12) expression as seen in crude ethanol extract (Byeon et al. 2010). To evaluate whether Cb-BF can suppress cytokine expression in a similar manner to Cb-EE, mechanistic approaches have tried to evaluate the involvement of transcription factors and their upstream signaling cascades. Similarly to previous reports, Cb-BF strongly blocked AP-1-mediated up-regulation of luciferase activity (Fig. 3A). Cb-BF also diminished both NF-κB- and STAT-1-mediated induction of luciferase activity (Fig. 3A and 3B), indicating that some components with STAT-1 and NF-κB inhibitory activities could be concentrated in this fraction. In agreement with these

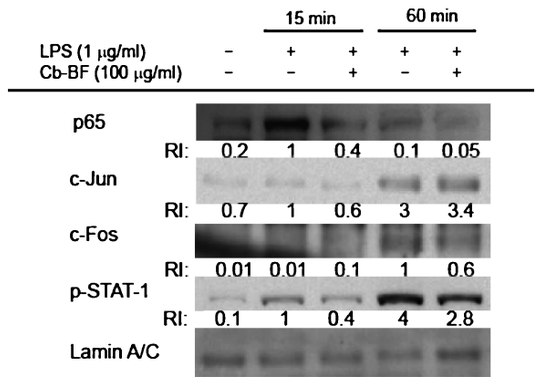


Fig. 4: Effect of Cb-BF on the nuclear translocation of NF-κB, AP-1, and STAT-1. RAW264.7 cells (5×10^6 cells/ml) pre-treated with Cb-BF for 1 h were stimulated in the absence or presence of LPS (1 μg/ml) for indicated times. After preparation of nuclear fraction, total or phospho-protein levels of p65, c-Jun, c-fos, STAT-1, and lamin A/C were determined by immunoblotting analysis with their total protein antibodies

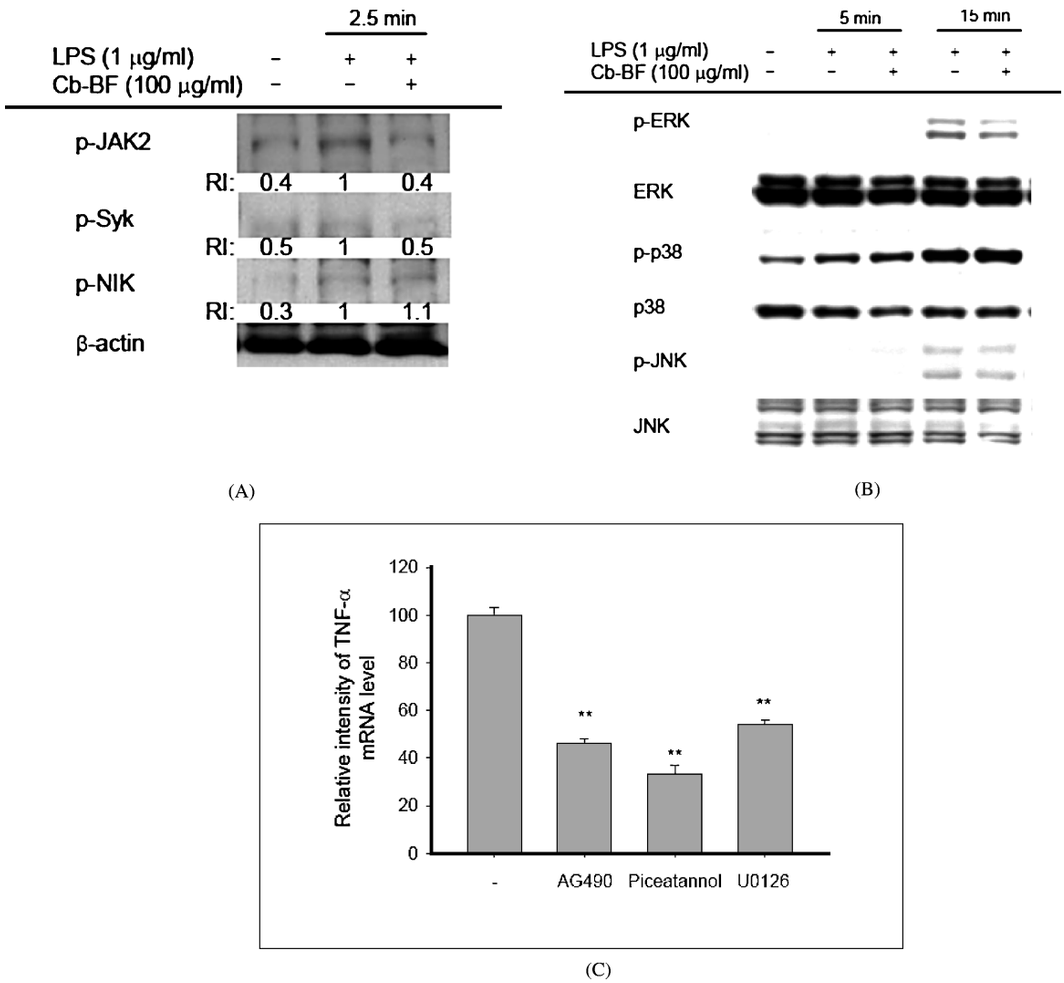


Fig. 5: Effect of Cb-BF on the upstream signaling for the activation of transcription factors. (A and B) RAW264.7 cells (5×10^6 cells/ml) pre-treated with Cb-BF for 1 h were stimulated in the absence or presence of LPS (1 μg/ml) for indicated times. After immunoblotting, the levels of phospho- or total JAK2, Syk, NIK, ERK, JNK, p38, and β-actin were identified by their antibodies. (C) RAW264.7 cells (2×10^6 cells/ml) were incubated with AG490, U0126, and piceatannol in the presence of LPS (1 μg/ml) for 6 h. The mRNA levels of TNF-α and GAPDH were determined by RT-PCR. **: $p < 0.01$ represents significant difference compared to control group

results, nuclear levels of p65, phospho-STAT-1, and STAT-1 were decreased by Cb-BF treatment (Fig. 4).

Since the activation signaling pathway of these transcriptional factors is well-studied, a representative signaling component of each factor was chosen to be tested. As shown in Fig. 5A, the phosphorylation of JAK2, a key enzyme for STAT-1 activation, Syk, an enzyme for NF-κB activation, but not NIK was interrupted by this fraction. Unlike the ethanol extract, Cb-BF did not block p38 phosphorylation, but suppressed the phosphorylation of ERK slightly at 15 min (Fig. 5B), suggesting that the active principles included in the butanol fraction were be different from those in the crude ethanol extract. Therefore, the inhibitory effect of Cb-BF on AP-1, NF-κB, and STAT-1 seems to be due to the blockade of JAK2, Syk, and ERK phosphorylation. Similarly, inhibitors (AG490, piceatannol, and U0126) of these enzymes showed inhibitory effects on the up-regulation of TNF-α gene expression (Fig. 5C), suggesting the involvement of these enzymes as the targets of Cb-BF pharmacology. Meanwhile, Cb-BF also strongly suppressed the proliferation of splenocytes induced by LPS, a B cell mitogen, and Con A, a T cell mitogen (Fig. 6A). In particular, this extract strongly inhibited the LPS-induced production of IFN-γ but not IL-4 (Fig. 6B and 6C), suggesting that the LPS-induced signaling pathway for cytokine production could be a more favorable target of this extract.

According to a peak profile obtained with HPLC analysis, cordycepin (retention time: 6.75 min), a major active compound isolated from *Cordyceps* spp., did not seem to be included in the butanol fraction of *Cordyceps bassiana* (Fig. 7). Cb-EE and Cb-BF exhibited a pharmacologically distinct inhibitory mechanism in terms of the activated levels of transcription factors and target enzymes suggesting that the active principles of each fraction could be different according to fractionation methods. The HPLC profile of Cb-BF was distinct from that of Cb-EE (data not shown). Thus, there is a need for attempts to isolate the active compounds, such as the yellow colored compounds, found as major dye compound in Cb-BF (data not shown).

In conclusion, this study demonstrated that the butanol extract of *Cordyceps bassiana* is able to inhibit the expression of cytokines like IL-12 and TNF-α, at the transcriptional levels. The suppressive activity of Cb-BF may be due to the interruption of NF-κB, AP-1, and STAT-1 translocation, according to immunoblotting analysis done with nuclear fraction and luciferase assay. Cb-BF clearly demonstrated its ability to diminish the phosphorylation of JAK2, a prime signal involved in STAT-1 translocation, Syk, a prime signal for NF-κB activation, and ERK, an important signaling enzyme for AP-1 translocation. Furthermore, this extract suppressed the production of IFN-γ but not IL-4. Therefore, our results suggest that Cb-BF can be regarded as a cytokine pro-

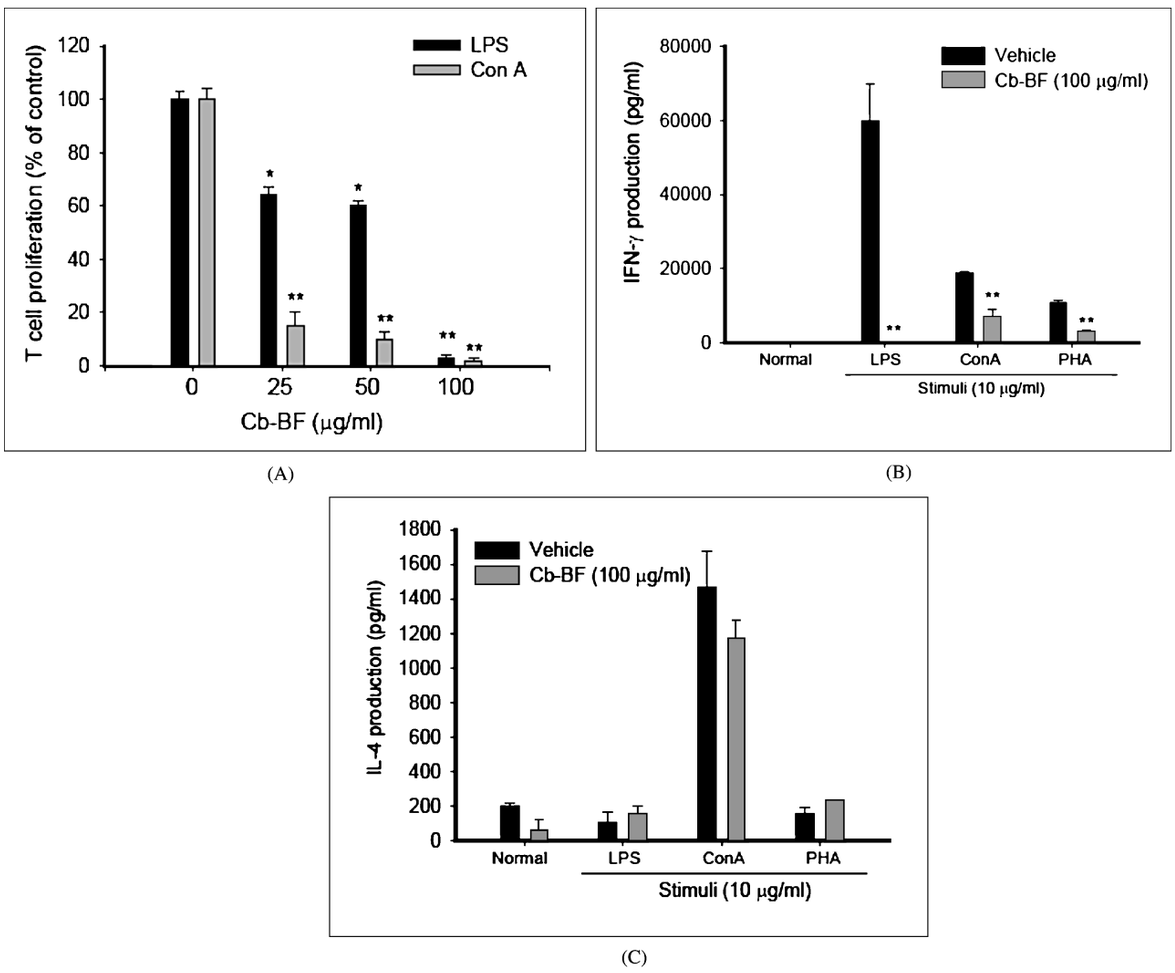


Fig. 6: Effect of Cb-BF on the proliferation of splenic lymphocytes and the production of IFN-γ and IL-4 in LPS-, Con A-, and PHA-treated splenocytes. (A) The proliferation of splenocytes (2×10^6 cells/ml) pretreated with Cb-BF in the presence or absence of LPS (10 μg/ml) or Con A (10 μg/ml) was determined by MTT assay. (B and C) Splenocytes were incubated with Cb-BF in the presence or absence of LPS (10 μg/ml), PHA (10 μg/ml), or Con A (10 μg/ml) for 48 h. The levels of IFN−γ and IL-4 in culture supernatant were determined by ELISA. *: $p < 0.05$ and **: $p < 0.01$ represent significant difference compared to control group

duction regulatory prepration modulating various inflammatory processes. Further *in vivo* efficacy tests exploring its potential therapeutic use against relevant immunological diseases such as chronic atopic dermatitis will be reported.

3. Experimental

3.1. Materials

Lipopolysaccharide (LPS), cordycepin, concanavalin A (Con A), phytohemaglutinin A (PHA), tumor necrosis factor (TNF)-α, forskolin, and phorbol-12-myristate-13-acetate (PMA) were obtained from Sigma Chemical Co. (St. Louis, MO). U0126, piceatannol, and AG490 were from Calbiochem (La Jolla, CA). *Cordyceps bassiana* was identified by Prof. Jae Mo Sung (Kangwon National University, Chuncheon, Korea). A voucher specimen of this (number 278-Cb-1) was deposited in the herbarium of our laboratory. RAW264.7 and HEK293 cells were purchased from ATCC (Rockville, MD, USA). All other chemicals were of reagent grade. Anti-phospho or total antibodies to JAK2, Syk, NIK, ERK, p38, JNK, p65 (NF-κB), c-Jun, c-fos, STAT-1, β-actin, and lamin A/C were from Cell Signaling (Beverly, MA, USA).

3.2. Preparation and characteristic of Cb-BF

The fruiting bodies were dried at 50 °C and crushed in a blender and the crude powder was extracted with ethanol at 70 °C for 3 h. The extracts were evaporated at 60 °C under pressure and resuspended in distilled water. The ethanolic extract (yield, 22%), suspended in water was successively extracted with equal volumes of *n*-hexane, *n*-butanol and ethyl acetate. The fractions were evaporated on a rotary evaporator under reduced pressure. The concentrated butanol fraction was again dried using a freeze-dryer to give a solid *n*-butanol sub-fraction (final yield, 7%). Phytochemical characteristics of Cb-BF were identified by HPLC analysis; the system was equipped with KNAUER (Wellchrom HPLC-pump, K-1001, Wellchrom fast scanning spectrophotometer K-2600, and 4 channel degasser K-500). Elution solvents were distilled water and acetonitrile. The gradient step of the solvent was "water to acetonitrile 1%/min" and (Vydac C18) Column was used.

3.3. Cell culture

RAW264.7 and HEK293 cells were cultured with RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY), glutamine and antibiotics (penicillin and streptomycin) at 37 °C with 5% CO_2.

3.4. IL-4 and IFN-γ production

Splenocytes were prepared from the spleen of BALB/C mice sacrificed by cervical dislocation under sterile conditions, using the method described previously (Cho 2008). Splenocytes (5×10^6 cells/ml) were incubated with T cell or B cell mitogens [PHA (10 μg/ml) or LPS (10 μg/ml)] in the presence of Cb-BF for 48 h (Cho et al. 2000). Supernatants were assayed for the content of IL-4 and IFN-γ using ELISA kits (Amersham, Little Chalfont, Buckinghamshire, UK).

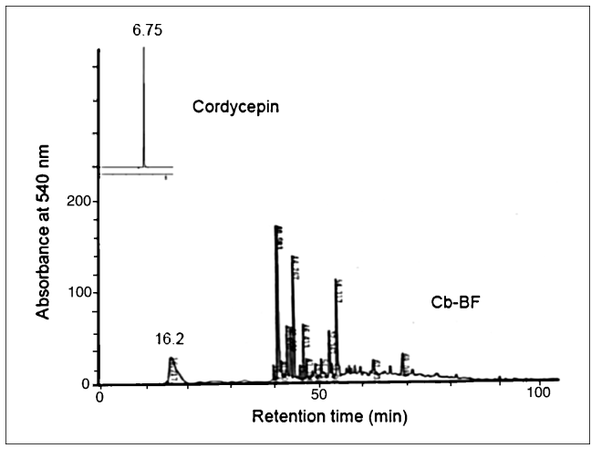


Fig. 7: Characteristic of the butanol fraction from *Cordyceps bassiana* (Cb-BF). Cb-BF and cordycepin was analyzed by high performance liquid chromatography (HPLC), equipped with KNAUER. Elution solvents were distilled water and acetonitrile. The gradient step of the solvent was "water to acetonitrile 1%/min" and (Vydac C18) Column was used

3.5. MTT assay

Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously (Kong et al. 2009).

3.6. Luciferase reporter gene activity assay

HEK293 cells (1×10^6 cells/ml) were co-transfected with one or two of plasmid constructs containing NF-κB-Luc or activator protein (AP)-1-Luc as well as β-galactosidase using the calcium phosphate method in a 12-well plate, as reported previously (Kim et al. 2010b). The cells were then further incubated in the absence or presence of TNF-α (20 ng/ml) or PMA (0.1 μM) and finally harvested for experiments 48 h after transfection. Luciferase assays were performed using the Luciferase Assay System (Promega, Madison WI, USA) (Jeon et al. 2009; Kim et al. 2010a).

3.7. Extraction of total RNA and semiquantitative RT-PCR amplification

The total RNA from the LPS treated-RAW264.7 cells was prepared by adding TRIzol Reagent (Gibco BRL), as per the manufacturer's protocol. Semiquantitative RT reactions were conducted using MuLV reverse transcriptase as per the method reported previously (Hong et al. 2003; Sun et al. 2010). The primers (Bioneer, Daejeon, Korea) for IL-12, TNF-α, and GAPDH detection were used as previously reported (Lee et al. 2006).

3.8. Preparation of cell lysate and immunoblotting

For total protein extraction: RAW 264.7 cells were harvested, washed with cold PBS and 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 and 10 μg/ml pepstatin, 1 mM benzimidine and 2 mM phenylmethane sulphonylfluoride) for 30 min rotation at 4 °C. Lysates were clarified by centrifugation at 16,000 × g for 10 min at 4 °C (Kim et al. 2009). For nuclear protein extraction: Nuclear proteins were obtained by three steps. After the treatment, cells were harvested and lysed in 500 μl of lysis buffer (50 mM KCl, 0.5% Nonidet P-40, 25 mM HEPES, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 20 μg/ml aprotinin and 100 μM 1,4-dithiothreitol) on ice for 4 min. Cells lysates were centrifuged at 14,000 rpm for 1 min at 4 °C. In the second step, the pellet was washed with the wash buffer, which was the same as the lysis buffer excluding Nonidet P-40. In the final step, the nuclei were incubated with an extraction buffer (500 mM KCl, 10% glycerol, 10 mM HEPES, 300 mM NaCl, 0.1 mM 1,4-dithiothreitol, 0.1 mM PMSF, 2 μg/ml leupeptin and 2 μg/ml aprotinin) and centrifuged at 19,000 *g* for 5 min. Supernatant was collected as nuclear protein extract. Soluble cell lysates were immunoblotted and phospho-ERK levels were visualized as previously reported (Lee et al. 2008).

3.9. Statistic analysis

The Student's *t*-test and one–way analysis of variance (ANOVA) were used to determine the statistical significance between values of the various experimental and control groups. *P* values of 0.05 or less were considered to be statistically significant.

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