

Full Length Research Paper

Anti-inflammatory activity of hot water extract of *Berberis koreana* in lipopolysaccharide-induced macrophage-like cells

Byung Hun Kim¹, Jaehwi Lee², Ting Shen¹, Jong Dai Kim¹ and Jae Youl Cho^{1*}

¹School of Bioscience and Biotechnology, and Institute of Bioscience and Biotechnology, Kangwon National University, Chuncheon 200-701, Korea.

²College of Pharmacy, Chung-Ang University, Seoul 156 -756, Korea.

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Berberis koreana has been known as enthoparmacologically valuable plant in Korea, China and Japan. This plant has been reported to display numerous pharmacological activities such as anti-oxidative, neuroprotective, and anti-cancer effects. Although the pharmacological potentials have been demonstrated, anti-inflammatory effect of this plant has not been fully elucidated yet. To evaluate its anti-inflammatory activity, macrophages activated by lipopolysaccharide (LPS) were employed and the production of inflammatory mediators was explored in terms of understanding its molecular inhibitory mechanism. Hot water extract from *B. koreana* (Bk-HWE) was able to suppress the production of NO and TNF- α production and up-regulation of surface levels of costimulatory molecules such as CD80 and CD86. The anti-inflammatory effect of Bk-HWE seemed to be due to the inhibition of MAPK activation and c-fos translocation, according to immunoblotting analysis. In addition, Bk-HWE strongly suppressed the cell-cell adhesion events induced by functional activation of adhesion molecules such as CD29 and CD43. Therefore, our results suggest that Bk-HWE can be applied as an anti-inflammatory herbal medicine. To prove this assumption, further *in vivo* efficacy test will be continued in the following project.

Key words: *Sorbus commixta*, macrophages, inflammatory mediators, AP-1 translocation, JNK activation.

INTRODUCTION

Macrophages play a critical role in managing inflammatory processes by secreting pro-inflammatory cytokines eg. tumor necrosis factor (TNF)- α and interleukin (IL)-1, and inflammatory molecules [eg. nitric oxide (NO) and oxygen species intermediates (ROS)] (Allam and Anders, 2008). The activation of macrophages is mediated by activation of pattern recognition receptors [eg. Toll like receptor (TLR)-4 and their ligands eg. lipopolysaccharide (LPS)] released from bacteria or virus (Lin and Yeh, 2005). The molecular interaction of these molecules triggers the inflammatory gene expression by mediating with NF- κ B and AP-1, redox sensitive transcription factors activated by radicals generated

(Pourazar et al., 2005). The activation of these transcription factors is mediated by sequential involvement of signaling complex composed of various protein kinases Src, Syk, phosphoinositide 3-kinase (PI3K), and Akt (protein kinase B) for NF- κ B translocation, and mitogen activated protein kinases (MAPKs) [such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38] for AP-1 translocation (Natarajan et al., 2002; Rahman et al., 2004). Inflammation is reported to be a serious cause in inducing various diseases such as cancer, autoimmune diseases, cardiovascular diseases, obesity, and diabetes. Therefore, it is considered that development of promising anti-inflammatory drugs without side effects could be valuable in terms of prevention or therapy of various inflammation-mediated diseases (Garcia-Lafuente et al., 2009).

Berberis koreana Palib (Berberidaceae), also known as "Korean barberry", is an endemic species found throughout Korea, Japan and China that have been used

*Corresponding author. E-mail: jaecho@kangwon.ac.kr. Tel: +82-33-250-6488. Fax: +82-33-241-6480.

as a traditional medicine against enteritis, fever, conjunctivitis, and sore throat (Kim et al., 2009). Numerous alkaloids such as benzyloisoquinoline and protoberberine derivatives and biphenyl derivatives such as aucuparin, methoxyaucuparin, and eriobofuran with medicinal importance have been isolated from this plant (Kim et al., 2009). It has been reported that an extract of *B. koreana* was reported to be neuroprotective against ischemic damage, and to exhibit cytotoxic and antioxidant activities (Kim et al., 2009; Yoo et al., 2008).

Nonetheless, anti-inflammatory effects of this plant and its molecular mechanism are still largely unelucidated, although it has been traditionally used for long time. In this study, therefore, we investigated the effect of hot water extract of *B. koreana* (Bk-HWE) on the modulation of LPS-induced inflammatory responses mediated by macrophage-like cells (RAW264.7 and U937 cells).

MATERIALS AND METHODS

Materials

Hot water extract of *B. koreana* (Bk-HWE) was prepared by conventional extraction method reported previously (Yoo et al., 2008). LPS and TNF- α were obtained from Sigma Chemical Co. (St. Louis, MO). U0126 and SP600125 were from Calbiochem (La Jolla, CA). RAW264.7 and HEK293 cells were purchased from ATCC (Rockville, MD). All other chemicals were of Sigma grade. Fibronectin was obtained with BD Bioscience (San Diego, CA). Anti-phospho or total antibodies to extracellular signal-regulated kinase (ERK), p38, c-Jun N-terminal kinase (JNK), p65 (NF- κ B), c-Jun, c-fos, β -actin and γ -tubulin were from Cell Signaling (Beverly, MA). Cell-cell adhesion-inducing antibodies to CD29 (MEM 101A, purified IgG1) and CD43 (161-46, ascites, IgG1) were used as reported previously (Cho et al., 2001).

Cell culture

RAW 264.7 and HEK293 cells obtained from American Type Culture Collection (Rockville, MD) 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% CO₂.

NO and TNF- α production

The inhibitory effect of Bk-HWE on NO and TNF- α production was determined as previously described (Lee et al., 2009). Bk-HWE solubilized with vehicle (100% DMSO) was diluted with RPMI1640. RAW264.7 cells (2×10^6 cells/ml) were incubated with LPS (1 μ g/ml) in the presence or absence of Bk-HWE for 6 (TNF- α) or 24 (PGE₂ and NO) h. Supernatants were assayed for NO and TNF- α contents using Griess reagent and TNF- α ELISA kits (Amersham, Little Chalfont, Buckinghamshire, UK).

Flow cytometry

Surface levels of CD18, CD29, CD69, CD80, CD82, and CD86 on U937 or RAW264.7 cells were determined by flow cytometric analysis as reported previously (Lee et al., 2008). Stained cells

were analyzed on a FACScan device (Becton-Dickinson, San Jose, Calif., USA).

MTT assay

Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously (Cho et al., 2000).

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), according to manufacturer's protocol. Semiquantitative RT reactions were conducted using MuLV reverse transcriptase as reported previously (Hong et al., 2003). The primers (Bioneer, Daejeon, Korea) were used as previously reported (Lee et al., 2006).

Preparation of cell lysates and nuclear fraction, and immunoblotting

RAW264.7 cells (5×10^6 cells/ml) were washed 3 times in cold PBS with 1 mM sodium orthovanadate and lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM ethyleneglycotetraacetic acid, 50 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1% Triton X-100, 10% glycerol, 10 μ g/ml aprotinin, 10 μ g/ml pepstatin, 1 mM benzimidazole, and 2 mM PMSF) for 30 min with rotation at 4°C. The lysates were clarified by centrifugation at 16,000 g for 10 min at 4°C and stored at -20°C until needed (Lee et al., 2009).

Nuclear lysates were prepared with a three-step procedure. After treatment, cells were collected with a rubber policeman, washed with 1 \times PBS, and lysed in 500 μ l of lysis buffer containing 50 mM KCl, 0.5% Nonidet P-40, 25 mM HEPES (pH 7.8), 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 20 μ g/ml aprotinin, and 100 μ M 1,4-dithiothreitol (DTT) on ice for 4 min. Cell lysates were then centrifuged at 14,000 rpm for 1 min in a microcentrifuge. In the second step, the pellet (the nuclei fraction) was washed once in washing buffer, which was the same as the lysis buffer without Nonidet P-40. In the final step, nuclei were treated with an extraction buffer containing 500 mM KCl, 10% glycerol, and several other reagents as in the lysis buffer. The nuclei/extraction buffer mixture was frozen at -80°C, and then thawed on ice and centrifuged at 14,000 rpm for 5 min. Supernatant was collected as nuclear extract.

Whole cell or nuclear lysates were then analyzed by immunoblotting. Proteins were separated on 10% SDS-polyacrylamide gels and transferred by electroblotting to polyvinylidene difluoride (PVDF) membrane. Membranes were blocked for 60 min in Tris-buffered saline containing 3% bovine serum albumin, 20 mM NaF, 2 mM EDTA, and 0.2% Tween 20 at room temperature. The membrane was incubated for 60 min with specific primary antibody at 4°C, washed 3 times with the same buffer, and incubated for an additional 60 min with HRP-conjugated secondary antibody. The total and phosphorylated levels of ERK, p38, JNK, I κ B α , γ -tubulin, and β -actin were visualized using the ECL system (Amersham, Little Chalfont, Buckinghamshire, UK).

Statistic analysis

The Student's *t*-test and a one-way 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

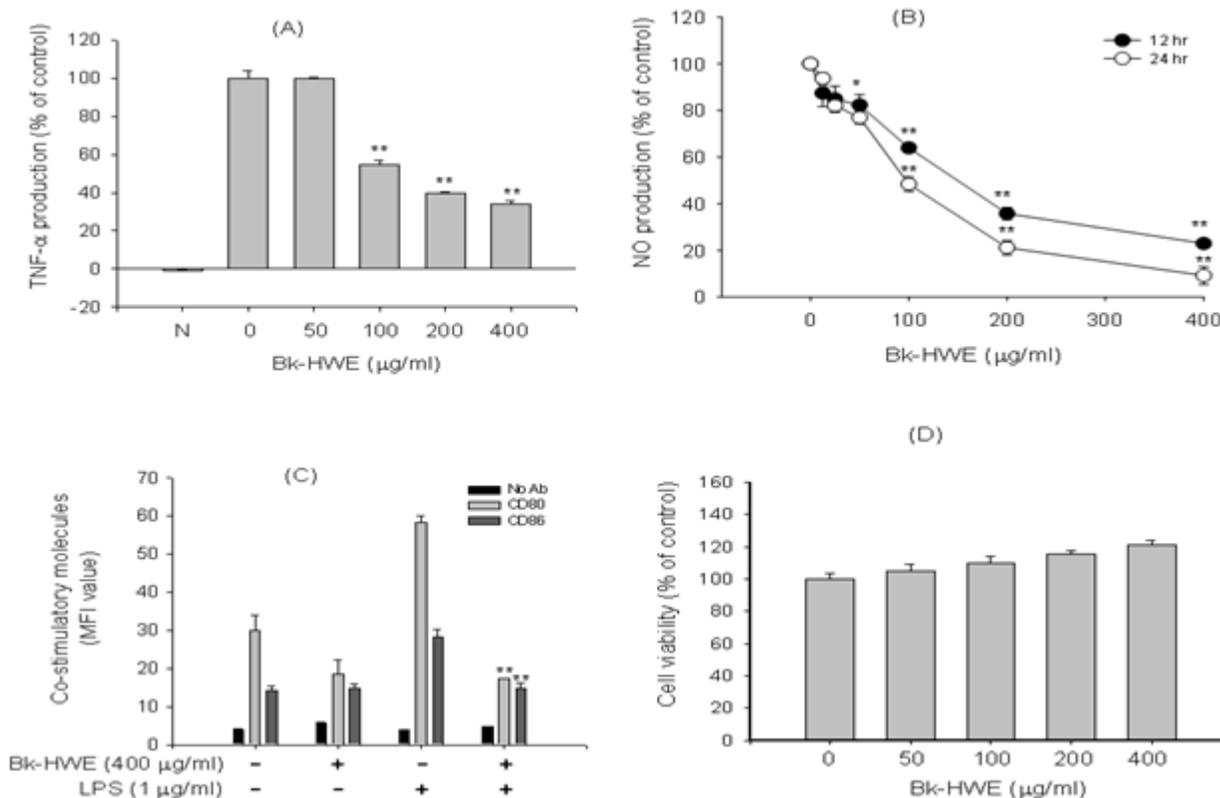


Figure 1. Effect of Bk-HWE on the production of NO and TNF- α and surface up-regulation of costimulatory molecules in LPS-activated RAW264.7 cells. (A, B, and C) RAW264.7 cells (2×10^6 cells/ml) were incubated with indicated concentrations of Bk-HWE in the presence of LPS (1 $\mu\text{g/ml}$) for 24 h (NO), 12 h (costimulatory molecules), and 6 h (TNF- α). NO (A) and TNF- α (B) levels in culture supernatant were determined by Griess assay and ELISA. (C) Surface levels of CD80 and CD86 were determined by flow cytometry. (D) The viability of RAW264.7 cells was determined under the same conditions by MTT assay after 24 h incubation. * $p < 0.05$ and ** $p < 0.01$ represent significant difference compared to LPS alone.

considered to be statistically significant.

RESULTS AND DISCUSSION

At present, inflammation is generally accepted to be a cause of numerous diseases such as cancer, diabetes, atherosclerosis, and obesity (Ferencik et al., 2007; Guzik et al., 2006). This point led us to developing novel anti-inflammatory drugs without side effects to prevent such diseases. In view of this, natural products traditionally used for long time are considered as attractive anti-inflammatory candidates. Our laboratory therefore has been tried to developing anti-inflammatory drugs using naturally-occurring compounds or plants for a decade.

Inhibitory effect of Bk-HWE was evaluated using LPS-activated macrophage-like (RAW264.7) cells under the production conditions of NO and TNF- α . Upon non-toxic concentrations (0 to 400 $\mu\text{g/ml}$) (Figure 1D), Bk-HWE strongly suppressed the production of TNF- α (Figure 1A) and NO (Figure 1B), and the surface up-regulation of

costimulatory molecules such as CD80 and CD86 (Figure 1C). The inhibition of TNF- α release occurred at both the transcriptional and translational levels, according to Figure 2. Thus, mRNA and protein levels of TNF- α were decreased significantly up to 40 - 70%. Interestingly, this extract also enhanced the mRNA expression of heme oxygenase (HO)-1, an anti-inflammatory gene (Immenschuh and Schroder 2006), indicating its involvement.

Because transcriptional up-regulation of inflammatory mediators is mostly regulated by transcription factors (Hume et al., 2007), we next examined whether Bk-HWE was able to modulate the activation of transcription factors. To do this, NF- κB , representative redox-sensitive transcription factor activated in response to LPS (Adcock 1997), was first tested under Bk-HWE pre-treatment conditions. As Figure 3A shows, this extract did not block the phosphorylation of I $\kappa\text{B}\alpha$ and its degradation, an essential step for NF- κB (p65/p50) translocation (Baldwin, 1996), up to 400 $\mu\text{g/ml}$. In agreement, reporter gene assay done with a construct containing NF- κB

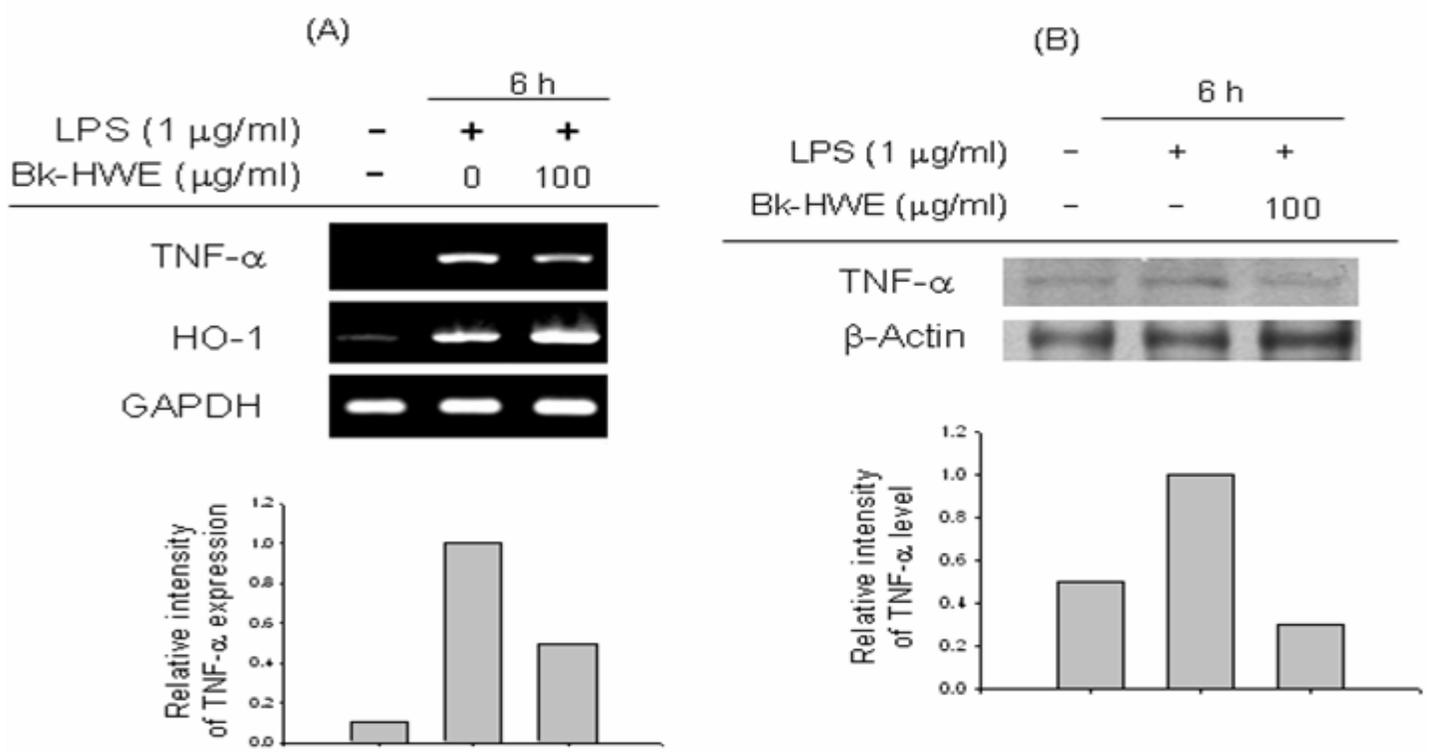


Figure 2. Effect of Bk-HWE on mRNA and protein levels of TNF-α and HO-1 in RAW264.7 cells under LPS stimulation. (A and B) RAW264.7 cells (5×10^6 cells/ml) were incubated with Bk-HWE in the presence of LPS (1 μg/ml) for 6 h. (A) The mRNA levels of TNF-α, HO-1, and GAPDH were determined by RT-PCR. (B) The protein levels of TNF-α and β-actin were examined by immunoblotting analysis. Relative intensity was determined by desitometric scanning.

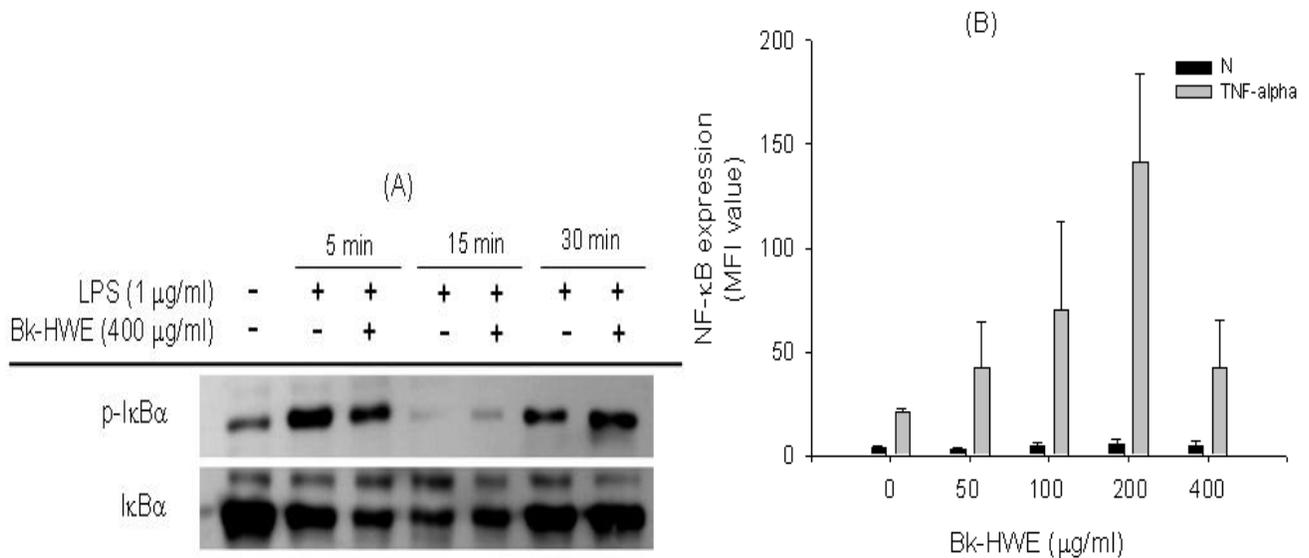


Figure 3. Effect of Bk-HWE on the upstream signaling pathway for transcriptional activation of NF-κB. (A) RAW264.7 cells (5×10^6 cells/ml) pre-treated with Bk-HWE for 1 h were stimulated with LPS (1 μg/ml) for indicated times. After immunoblotting, the levels of phospho- or total IκBα were identified by their antibodies. The results show one experiment out of three. (B) HEK293 cells co-transfected with the plasmid construct, NF-κB-Luc (1 μg/ml), and β-gal (as a transfection control) were treated with Bk-HWE in the presence or absence of TNF-α (20 ng/ml) for 18 h. Luciferase activity was determined by luminometry. Data represent mean ± SEM of three independent observations performed in triplicate.

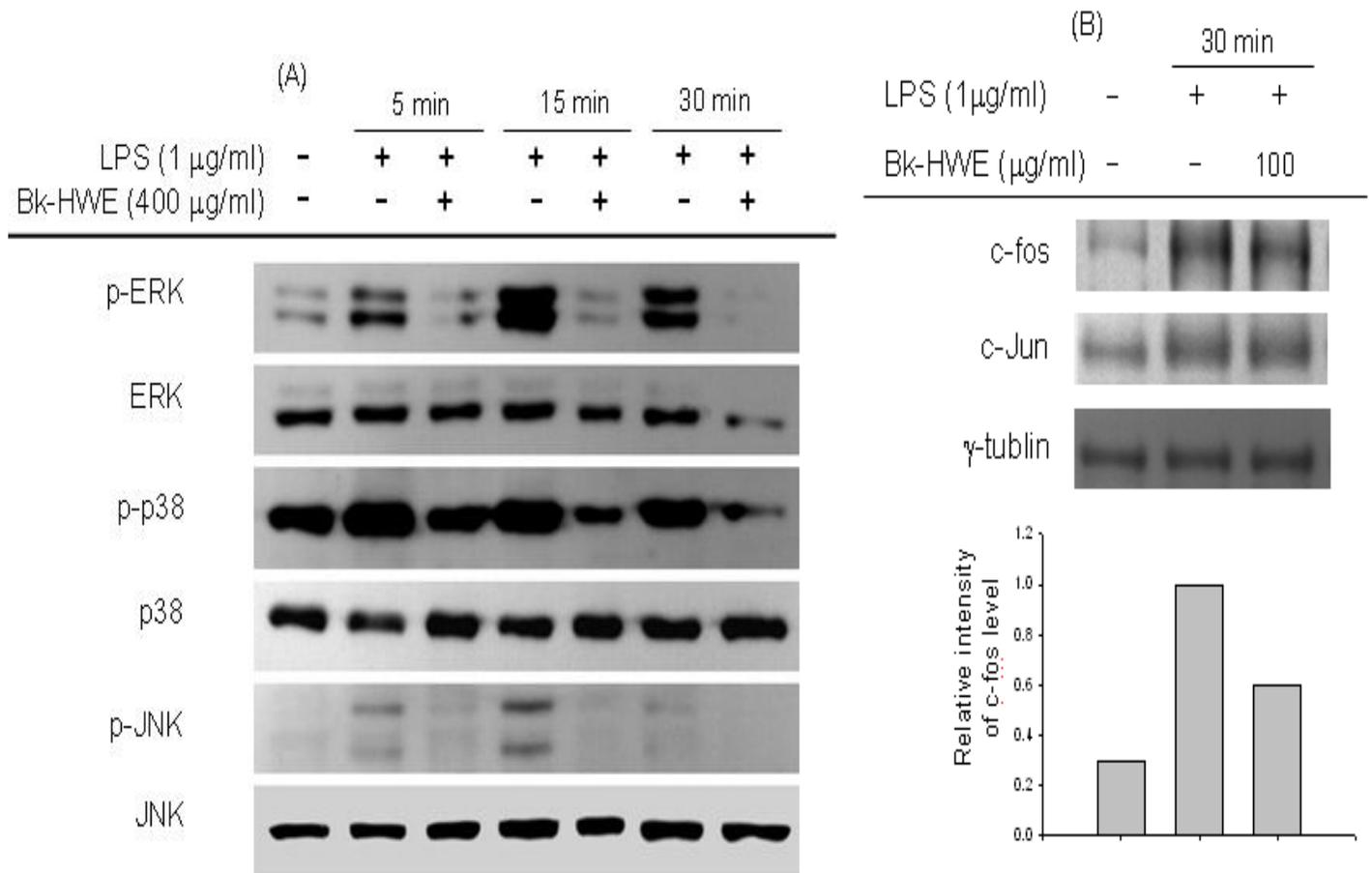


Figure 4. Effect of Bk-HWE on the upstream signaling pathway for transcriptional activation of AP-1. (A) RAW264.7 cells (5×10^6 cells/ml) pretreated with Bk-HWE (300 $\mu\text{g/ml}$) were stimulated with LPS (1 $\mu\text{g/ml}$) for indicated times. After immunoblotting, the levels of phospho- or total ERK, p38, and JNK were identified by their antibodies. (B) RAW264.7 cells (5×10^6 cells/ml) were incubated with Bk-HWE in the presence or absence of LPS (1 $\mu\text{g/ml}$) for 30 min. Then, nuclear fraction was obtained from the cells, as described in Materials and Methods. The nuclear levels of c-fos, c-Jun, and γ -tubulin were determined by immunoblotting analysis. The results show one representative experiment of three. Relative intensity was determined by densitometric scanning.

binding sequences showed similar non-inhibitory pattern. Thus, there was no inhibition of luciferase activity by Bt-HWE up to 400 $\mu\text{g/ml}$ (Figure 3B). Furthermore, the activation of upstream signaling kinase, PI3K, involved in IKK/I α B α phosphorylation, was not blocked by Bt-HWE exposure (data not shown). Therefore, these results suggest that NF- κ B and its upstream signaling pathway is not a direct target of Bk-HWE.

Since AP-1 (c-Jun/c-fos) is also major transcription factor managing LPS-induced inflammatory responses (Dokter et al., 1993), the involvement of this molecules and its upstream signaling pathway was examined by measuring MAPK phosphorylation and AP-1 translocation levels (Pang et al., 2009). Intriguingly, Bk-HWE strongly suppressed the phosphorylation of ERK, p38, and JNK without altering total levels of these enzymes (Figure 4A). In agreement, translocation level of c-fos, but not c-Jun was significantly suppressed by this extract up to 40% (Figure 4B). These results therefore suggest that MAPK

is a prime target of Bk-HWE with anti-inflammatory function.

As other major roles of macrophages, adhesion and phagocytic uptake are of well characterized events seen in inflammatory process (Virgin and Levine, 2009; Yusuf-Makagiansar et al., 2002). Therefore, finally we examined whether Bk-HWE is able to modulate these pro-inflammatory events. 400 $\mu\text{g/ml}$ of Bk-HWE strongly suppressed CD29 (β 1-integrins) - and CD43-mediated cell-cell adhesion (Figure 5), while phagocytosis was not inhibited (Figure 6). However, this extract did not suppress the adhesion between U937 cell and fibronectin, an extracellular matrix protein, acting as a ligand of CD29, indicating that cell-cell adhesion seems to be more sensitive target event rather than cell-extracellular matrix interaction.

Since the phosphorylation of MAPK was strongly suppressed by Bk-HWE, the involvement of these enzymes was finally examined with specific inhibitors.

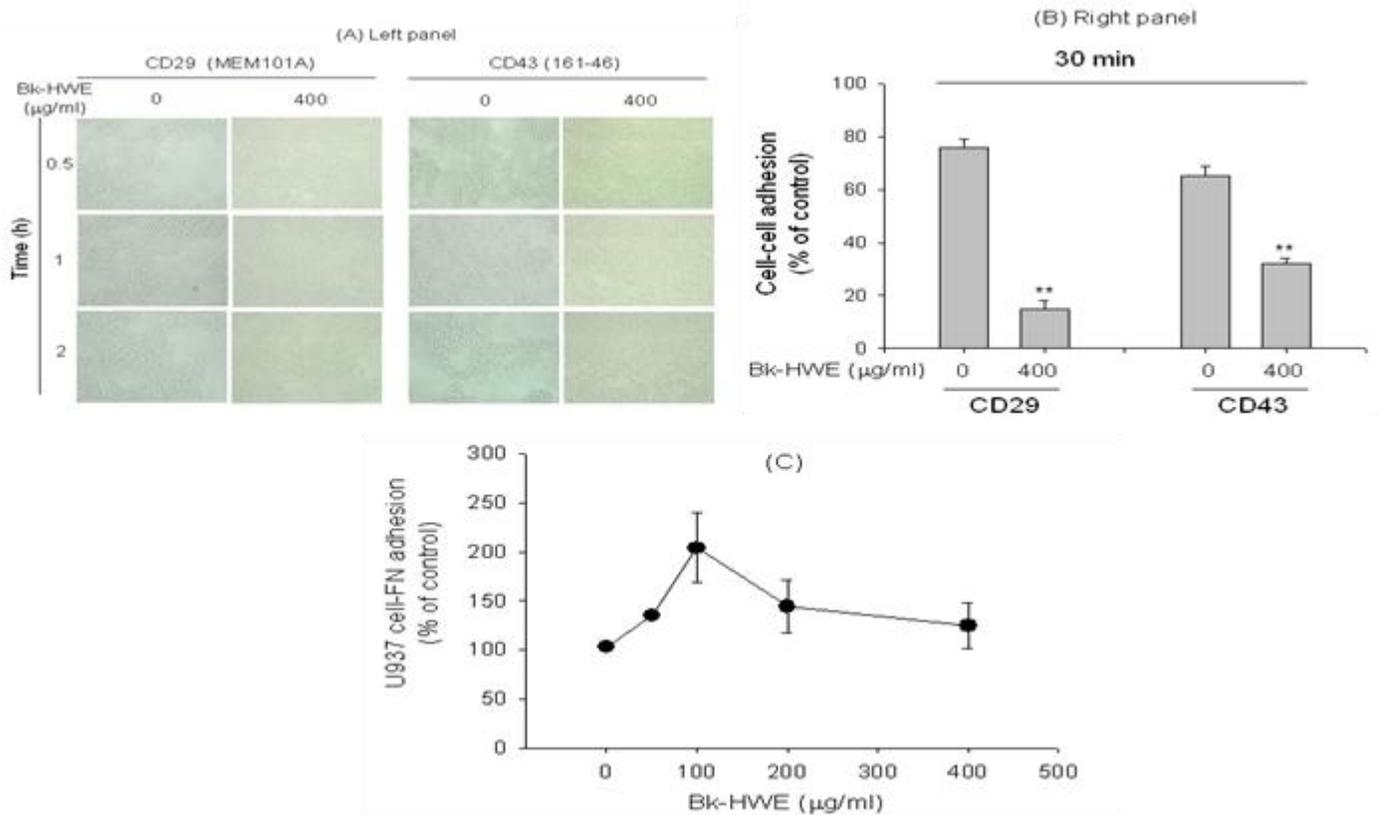


Figure 5. Effect of Bk-HWE on cell-cell adhesion and cell-fibronectin (FN) adhesion. (A left and right panels) U937 cells pretreated with Bk-HWE for 1 h were incubated in the presence or absence of anti-CD29 antibody MEM101A (1 µg/ml) or anti-CD43 antibody 161-46 (1 µg/ml). (A left panel) Images of the cells in culture were obtained using an inverted phase contrast microscope attached to a video camera. (A right panel) Quantitative analysis of cell-cell clusters was assessed using a quantitative cell-cell adhesion assay as described in Materials and methods. (B) U937 cells pretreated with Bk-HWE for 30 min were seeded on fibronectin (50 µg/ml)-coated plates and further incubated for 3 h. The numbers of attached cells were determined by crystal violet assay, as described in Materials and Methods. **p < 0.01 represents significant difference compared to control group.

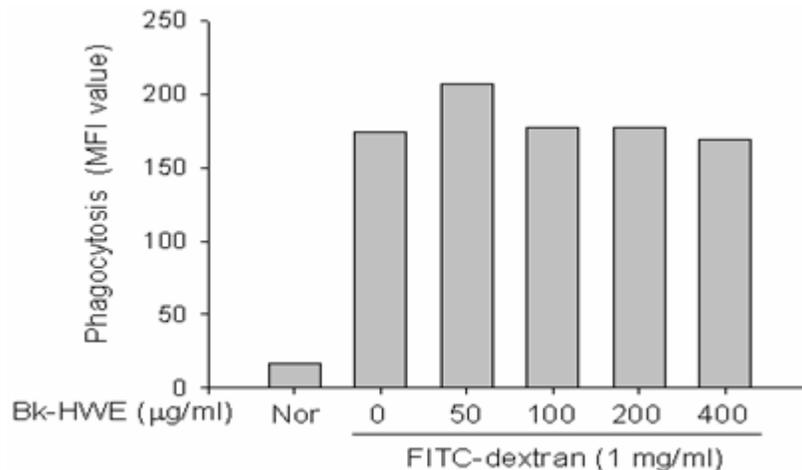


Figure 6. Effect of Bk-HWE on the phagocytic uptake of FITC-labeled dextran. RAW264.7 cells (1×10^6) were incubated with Bk-HWE in the presence or absence of 1 mg/ml of FITC-labeled dextran for 30 min. The uptake level of dextran was determined by flow cytometric analysis as described in materials and methods.

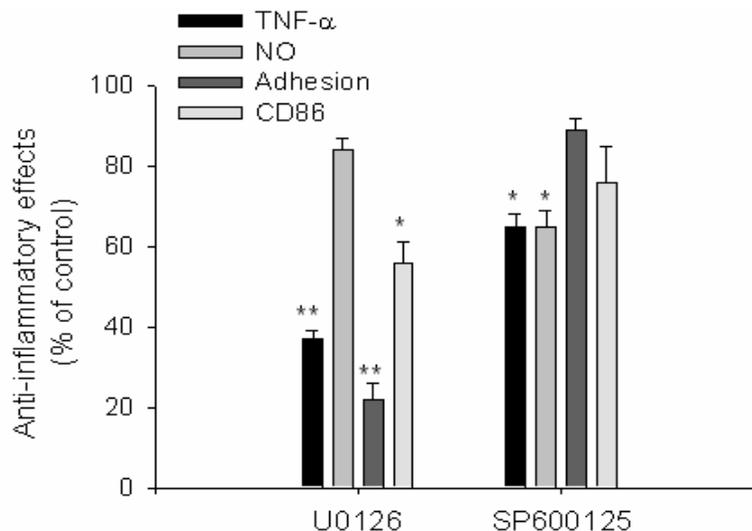


Figure 7. Effect of MAPK inhibitors (U0126 and SP600125) on the production of TNF- α and NO, CD29-mediated cell-cell adhesion, and the up-regulation of surface CD86. RAW264.7 cells (2×10^6 cells/ml) were incubated with indicated concentrations of Bk-HWE in the presence of LPS (1 μ g/ml) for 24 h (NO), 12 h (CD86 expression), and 6 h (TNF- α). NO and TNF- α levels in culture supernatant were determined by EIA, and ELISA. CD29-mediated cell-cell adhesion was done with U937 cells pretreated with Bk-HWE in the presence of MEM101A (1 μ g/ml) for 1 h. * $p < 0.05$ and ** $p < 0.01$ represent significant difference compared to control group.

Interestingly, U0126, an ERK inhibitor, was found to significantly suppress the production of TNF- α , CD29-mediated adhesion, and the surface up-regulation of CD86, while SP600125, a JNK inhibitor, significantly inhibited the release of TNF- α and NO (Figure 7). In addition, it has been reported that MAPK inhibitors block the production of inflammatory mediators (Berenbaum et al., 2003; O'Sullivan et al., 2009).

So far, various alkaloids such as benzylisoquinoline and protoberberine derivatives and biphenyl derivatives such as aucuparin, methoxyaucuparin, and eriobofuran have been isolated from this plant. Although some of alkaloids such as berberin have been reported to exhibit anti-inflammatory effects (Wang et al., 2008), whether these isolated compounds block the inhibitory effects on the production of inflammatory mediators, expression of costimulatory molecules, and cell-cell adhesion should be examined using isolated compounds from Bk-HWE. To identify or isolate active compounds from this plant, gas chromatographic and preparative chromatographic analyses will be followed. Other biological efficacy tests will be also continued in the next experiments.

Conclusion

In this study, we found that hot water extract from *B. koreana* was able to suppress the production of NO and TNF- α and up-regulation of surface levels of

costimulatory molecules such as CD80 and CD86. The anti-inflammatory effect of Bk-HWE seemed to be due to the inhibition of MAPK activation and AP-1 (c-fos) translocation, according to immunoblotting analysis. In addition, Bk-HWE strongly suppressed the cell-cell adhesion events induced by functional activation of adhesion molecules such as CD29 and CD43. Therefore, our results suggest that Bk-HWE can be applied as an anti-inflammatory herbal medicine. To prove this assumption, further *in vivo* efficacy test will be continued in the next project.

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