Food Sci. Biotechnol. Vol. 18, No. 5, pp. 1063 ~ 1070 (2009)

Food Science and Biotechnology

ⓒ The Korean Society of Food Science and Technology

Anti-inflammatory Effect of *Dangyuja* (*Citrus grandis* Osbeck) Leaves in LPS-stimulated RAW 264.7 Cells

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Abstract *Dangyuja* (*Citrus grandis* Osbeck) is a native plant growing only on Jeju Island in Korea. In this study, anti-inflammatory effect of *dangyuja* leaves on a murine macrophage cell line was investigated. RAW 264.7 murine macrophage cells were stimulated with lipopolysaccharide (LPS, 1 μg/mL) to induce expression of pro-inflammatory markers [interleukin (IL)-6 and inducible nitric oxide synthase (iNOS)]. The crude extract (80% MeOH Ex.) and solvent fractions (hexane, $CHCl_3$, EtOAc, BuOH, and H_2O Ex.) were obtained from *dangyuja* leaves. The $CHCl_3$ fraction inhibited the nitric oxide (NO) and IL-6 production in a dose-dependent manner. Also, the $CHCl_3$ fraction inhibited mRNA expression and protein levels of iNOS in a dose-dependent manner. Furthermore, the $CHCl_3$ fraction inhibited LPS-induced nuclear factor (NF)-κB activation and phosphorylation of mitogen-activated protein kinases (MAPKs: ERK, JNK, and p38). These results suggest that *dangyuja* leaves may inhibit LPS-induced production of inflammatory markers by blocking NF-κB and MAPKs signaling in RAW 264.7 cells.

Key words: *dangyuja* (*Citrus grandis* Osbeck), inflammation, inflammatory marker, nuclear factor κB, mitogen-activated protein kinases (MAPKs)

Introduction

Dangyuja (*Citrus grandis* Osbeck) is a native plant growing only on Jeju Island in Korea. It consists of several component such as limonene, obacunone, nomiline, and naringin. Among them, limonoid has anticancer effects such as reducing proliferation of cancer cells (1). Moreover, the unripe fruit of *dangyuja* has been reported to have anti-inflammatory effects through suppression of the expression of inflammatory markers [interleukin (IL)-6, inducible nitric oxide synthase (iNOS), COX-2, TARC, and MDC] (2), and the unripe fruit and leaves of *dangyuja* reported the free radical scavenging activity of the anti-oxidant 1, 1-diphenyl-2-picrylhydrazyl (DPPH) (3,4). However, the leaves have not been reported anti-inflammatory effects.

Macrophages play an important role in immune responses to pathogen infection. The stimulation of macrophages with lipopolysaccharide (LPS) results in a number of functional responses including production of nitric oxide (NO) and cytokines increased (5,6). NO is a bioactive radical produced by iNOS and is an important mediator and effector molecule with various biological functions such as regulation of blood pressure, neurotransmission, antimicrobial defense, and immunomodulation (7-9). However, the overexpression of NO induces various harmful responses including tissue injury, septic shock, acute or chronic inflammation, and autoimmune disease (10,11). IL-6 is a key cytokine with pro-inflammatory functions, and it is induced by LPS (12,13).

LPS, a component of Gram-negative bacteria outer membranes, is a potent activator of the macrophage immune responses, such as nuclear factor (NF)-κB and mitogen-activated protein kinases (MAPKs) (14,15). NF-κB is a critical activator of genes involved in inflammation diseases, including IL-6 and iNOS (16, 17). NF-κB exists in the cytoplasm of unstimulated cells and is bound to the inhibitor κB (IκB). Phosphorylation of IκB leads to its degradation and the translocation of NF-κB to the nucleus where it activates transcription of target genes (18-20). The MAPKs family contains several subfamiles including the extracellular signal-regulated kinase (ERKs), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPKs), and p38 MAPkinase (21). These kinases are important in cell function including proliferation and apoptosis (22). The MAPKs have been implicated in the regulation of iNOS and IL-6 genes, MAPKs specific inhibitors are suppress iNOS expression in LPS-stimulated RAW 264.7 cells (23) and a p-38 inhibitor is suppresses IL-6 expression (24).

As we mentioned above, the unripe fruit of *dangyuja* have anti-inflammatory and antioxidant effects. Also, a recent study revealed that *dangyuja* leaves have antioxidant effects. However, the *dangyuja* leaves are still not out of the anti-inflammatory effect and their mechanism.

In the present study, thus, we investigated the anti-inflammatory activities of *dangyuja* leaves and their mechanisms. Therefore, we demonstrated that the *dangyuja* leaves inhibits the NO and IL-6 production by suppressing NF-κB and MAPKs in LPS-stimulated RAW 264.7 cells.

Materials and Methods

Reagents Dulbecco's modified Eagle's medium (DMEM)

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Received November 7, 2008; Revised April 28, 2009;
Accepted May 12, 2009

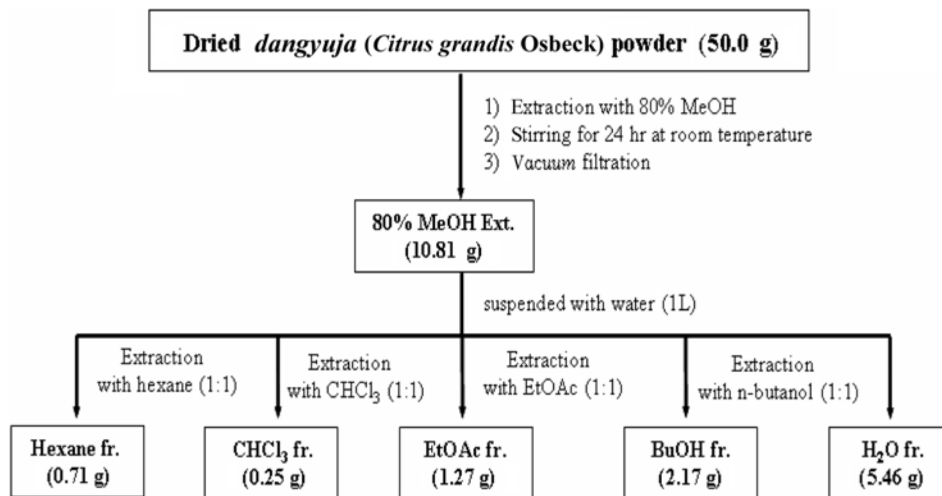

Fig. 1. Systemic purification using solvent partitioning from *dangyuja* leaves.

and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Lipopolysaccharide (LPS, *Escherichia coli* 0111:B4) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were analytical grade. The enzyme-linked immunosorbent assay (ELISA) kits for interleukin (IL)-6 were obtained from BD Biosciences (Mountain View, CA, USA). Antibody against inducible nitric oxide synthase (iNOS) was purchased from Calbiochem (San Diego, CA, USA) and antibodies against ERK, phospho-ERK, JNK, phospho-JNK, p-38, NF-κB (p65), and IκB-a were from Cell Signaling Technology (Beverly, MA, USA) and antibody against p-38 was purchased from BD Bioscience.

Preparation of plant fractions *Dangyuja* leaves were collected from Jeju Island on August 2005. The dried leaves (50.0 g) were extracted with 80% methanol (MeOH) twice at room temperature. The MeOH extract was suspended in distilled water and then partitioned sequentially with equal volumes of hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc), *n*-butanol, and H_2O (Fig. 1), as previously described (3).

Cell culture Murine macrophage RAW 264.7 cells were purchased from the Korean Cell Line Bank (Seoul, Korea). They were cultured in DMEM containing 2 mM glutamine, 10 mM HEPES, penicillin (100 units/mL), streptomycin (100 μg/mL), and 10% FBS. Cells were cultured at 37°C under 5% CO_2 in a humidified incubator.

MTT assay for cell viability Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (25,26). RAW 264.7 cells were cultured in 24-well plates for 18 hr, followed by treatment with LPS (1 μg/mL) in the presence of various concentrations (12.5, 25, 50, and 100 μg/mL) of the $CHCl_3$ fraction of *dangyuja* leaves. After 24 hr incubation, MTT was added to the medium for 4 hr. Finally, the supernatant was removed and the formazan crystals were dissolved in dimethyl sulfoxide (DMSO). Absorbance was measured at 540 nm. Percent of cells showing cytotoxicity was determined relative to the control group.

Nitric oxide (NO) assay NO accumulation was used as an indicator of NO production in the cell culture medium by the Griess reagent (27,28). The culture supernatant (100 μL) was mixed with same volume of Griess reagent [1% sulfanilamide and 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride in 5% phosphoric acid %] for 10 min, and absorbance was measured at 540 nm.

Measurement of IL-6 production The RAW 264.7 cells were cultured in 24-well plates for 18 hr, followed by treatment with LPS in the presence of various concentrations (12.5, 25 50, and 100 μg/mL) of the $CHCl_3$ fraction of *dangyuja* leaves. After 18 hr incubation, levels of IL-6 production in the culture supernatant were measured using ELISA kits (29).

Western blot analysis After incubation, the cells were washed twice with cold phosphate buffered saline (PBS). Whole cell lysates (30 μg for iNOS, p65 of NF-κB, IκB-a, and MAPKs) were separated by 10% sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and electrotransferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA USA). The membrane was incubated for 2 hr with Tris-Tween buffered saline (TTBS) containing 1% bovine serum albumin (BSA), and then incubated with a specific primary antibody at 4°C overnight. The membrane was washed 4 times with TTBS and incubated for 30 min with a peroxidase-conjugated secondary antibody at room temperature. Finally, the membrane was detected using the WEST-ZOL Western Blot Detection System (iNtRON Biotechnology, Gyeonggi, Korea).

RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR) iNOS and IL-6 mRNA expression was measured by RT-PCR. Total RNA was isolated using the Tri-Reagent (MRC, Cincinnati, OH, USA) method according to the manufacturer's instructions. RNA isolation was carried out in an RNase-free environment. The 4 μg of RNA was reverse-transcribed (RT) using MuLV reverse transcriptase (Promega, Madison, WI, USA), oligo $(dT)_{15}$ primer, dNTP (0.5 μM) and 1 U RNase

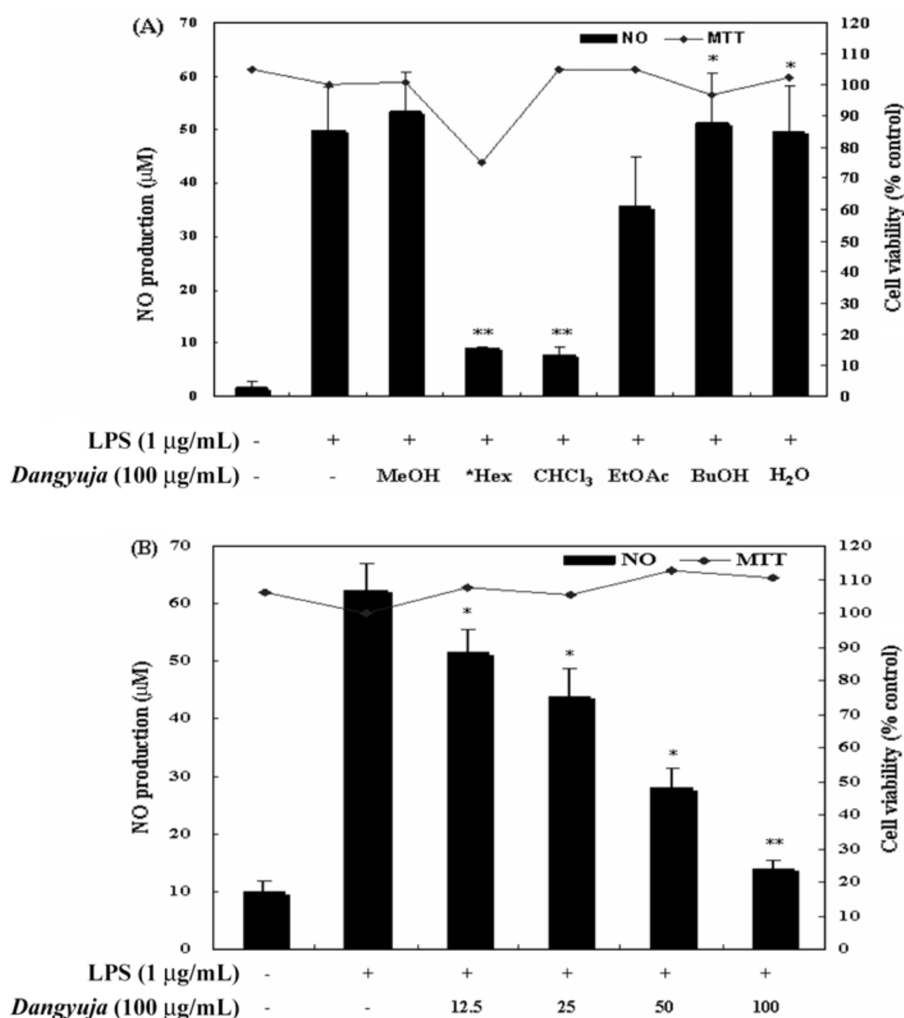

Fig. 2. Effect of crude extract and solvent fractions from *dangyuja* leaves on the nitric oxide (NO) production in RAW 264.7 cells. Production of NO was assayed from culture medium of cells stimulated with LPS in the presence of *dangyuja* leaves various fractions (A) and $CHCl_3$ fractions (B) for 24 hr. *Hex: cytotoxicity. Data represent the mean±SD of triplicate experiments. *p<0.05 **p<0.01 vs. LPS alone.

inhibitor. PCR analyses were performed with a DNA gene cycler (Bio-Rad), and the amplifications were performed for 30 cycles for β-actin, iNOS, and IL-6. The PCR products were electrophoresed on a 1.0% agarose gel and visualized by ethidium bromide (EtBr) staining with a gel documentation system (Gel Doc 2000; Life Science, Research, Hercules, CA, USA).

Transient transfection and luciferase assay Cells were cotransfected with 50 μg of an NF-κB promotor luciferase reporter gene plasmid (Panomics, Redwood City, CA, USA) and 10 ng of a Renilla luciferase reporter plasmid (Promega), which served as the internal standard, using the TransFast™ transfection reagent (Promega). After 24 hr, cells were incubated with LPS (1 μg/mL) in the presence or absence of the $CHCl_3$ fraction of *dangyuja* leaves. After 15 hr incubation, luciferase activity in the cell lysate was determined using Dual-luciferase Reporter assay kits (Promega). Luciferase activity was normalized to the transfection efficiency as monitored by Renilla luciferase expression. The level of luciferase activity was determined as a ratio compared to cells with no stimulation.

Statistical analysis Results are expressed as mean± standard error (SE) of at least triplicate experiments. Student's *t*-test was used to assess the statistical significance of differences. A *p*-values of less then 0.05 were considered statistically significant.

Results and Discussion

Effect of *dangyuja* leaves on the NO production in LPS-stimulated RAW 264.7 cells To assess whether the tested fractions of *dangyuja* affected cell viability, RAW 264.7 cells were incubated with LPS (1 μg/mL) in the presence of fraction of *dangyuja* leaves (100 μg/mL). Only the hexane fraction was cytotoxic (Fig. 2A).

In order to investigate the effect of *dangyuja* leaves on the NO production, we measured NO concentrations in the culture medium. We incubated RAW 264.7 cells with LPS (1 μg/mL) in the presence of a crude extract of *dangyuja* or of solvent fractions of *dangyuja* leaves (100 μg/mL). The $CHCl_3$ fraction markedly inhibited NO production (Fig. 2A). The inhibition was dose-dependent (Fig. 2B). Previous studies reported that NO is increased by expression of

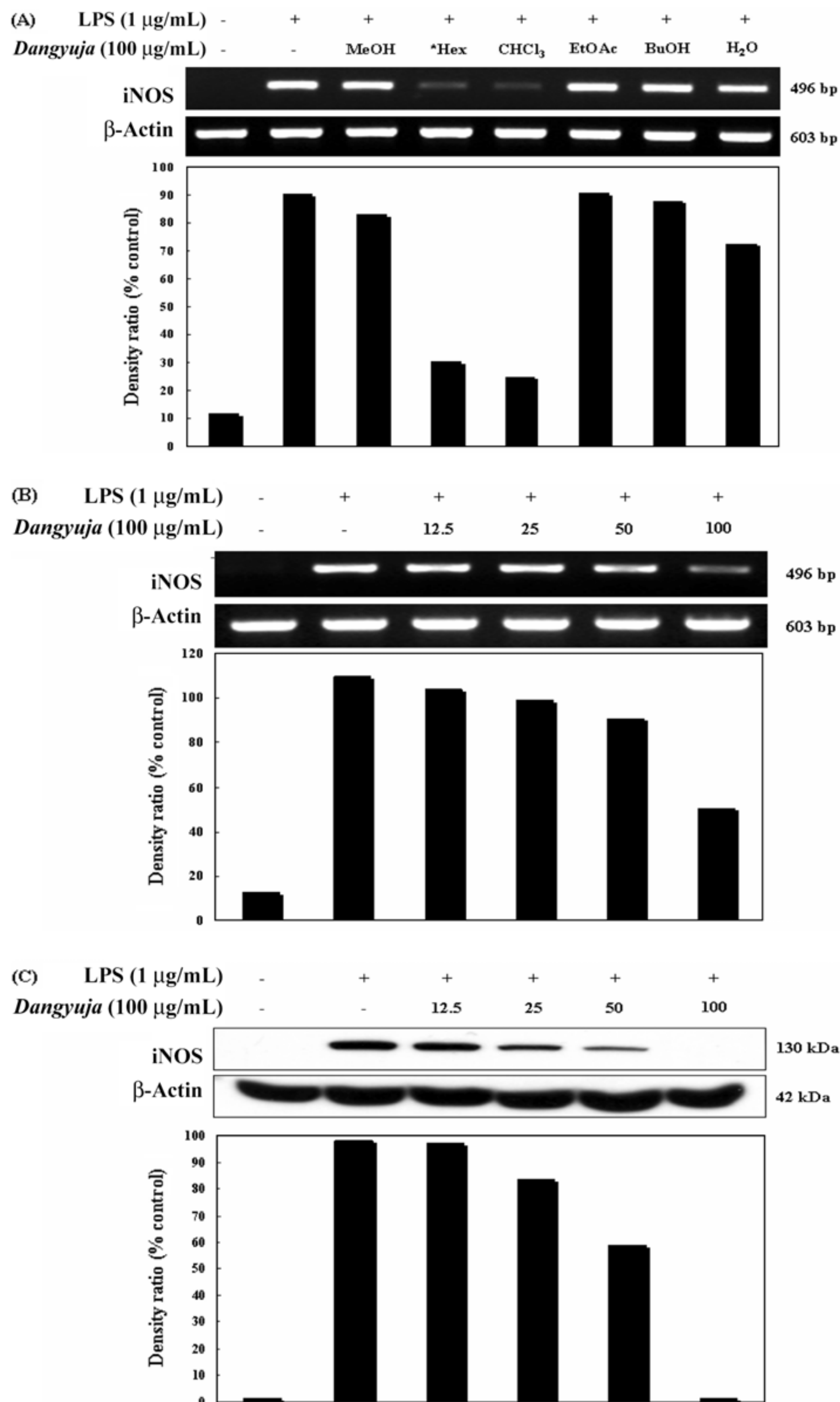

Fig. 3. Effect of crude extract and solvent fractions from *dangyuja* leaves on the mRNA expression and protein level of iNOS in LPS-stimulated RAW 264.7 cells. Cells were stimulated with LPS in the presence of *dangyuja* leaves various fractions (A) and $CHCl_3$ fractions (B, C) for 24 hr (A and B by RT-PCR, C by Western-blotting). The β-actin was a loading control.

iNOS (30). To explain the mechanism of inhibition of NO production, we investigated inhibition of iNOS gene expression. The $CHCl_3$ fraction inhibited iNOS mRNA expression and reduced iNOS protein levels in a dose-dependent manner (Fig. 3A, 3B, and 3C). Thus, the $CHCl_3$ fraction of *dangyuja* leaves may inhibit NO production by inhibiting protein and mRNA expression of iNOS without cell cytotoxicity.

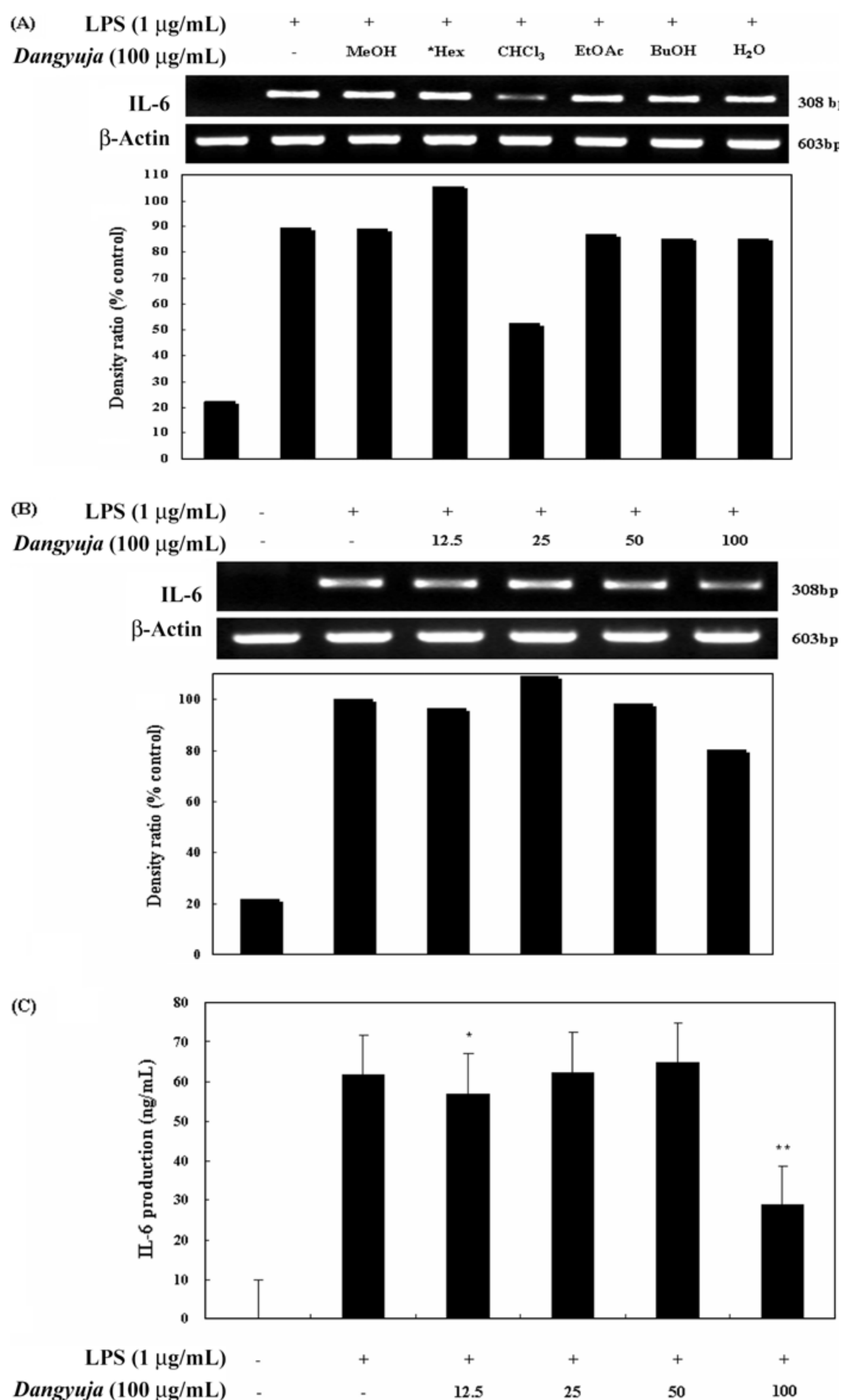

Fig. 4. Effect of crude extract and solvent fractions from *dangyuja* leaves on the mRNA expression and production level of IL-6 in LPS-stimulated RAW 264.7 cells. Cells were stimulated with LPS in the presence of *dangyuja* leaves various fractions (A) and $CHCl_3$ fractions (B, C) for 24 hr (A and B by RT-PCR, C by ELISA method). Data represent the mean±SD of triplicate experiments. *p<0.05, **p<0.01 vs. LPS alone. *Hex: cytotoxicity

Effect of *dangyuja* leaves on IL-6 production in LPS-stimulated RAW 264.7 cells IL-6 plays a pivotal role in controlling the immune system and in communication among mammalian cells (31). Recently, several studies showed that various plant suppress gene expression of cytokines such as IL-6 in RAW 264.7 cells (32,33). Thus, we examined the effects of the $CHCl_3$ fraction on IL-6 production in LPS-stimulated RAW 264.7 cells using the ELISA and RT-PCR. The $CHCl_3$ fraction inhibited IL-6 production and IL-6 mRNA expression in LPS stimulated RAW 264.7 cells (Fig.

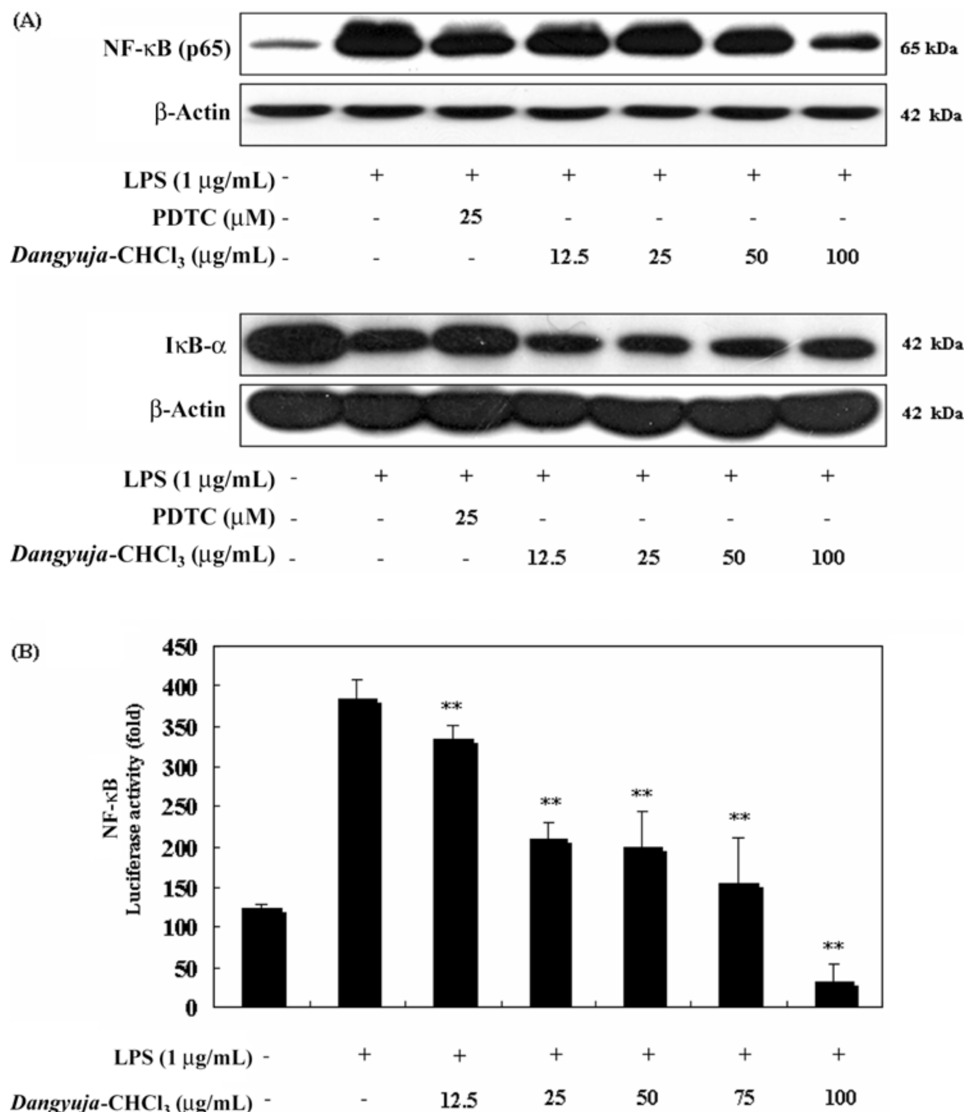

Fig. 5. Effect of $CHCl_3$ fraction from *dangyuja* leaves on the activation of NF-κB without affecting IκB-α degradation in LPS stimulated RAW 264.7 cells. (A) Cells were stimulated with LPS in the presence of *dangyuja* leaves $CHCl_3$ fraction or PDTC. β-Actin was a loading control. (B) Cells were transiently cotransfected with NF-kB promoted luciferase reporter plasmid (pNF-κB-Luc) and Renilla luciferase reporter plasmid (pRL-null) as internal control for 24 hr, and then treated with LPS in the presence of *dangyuja* leaves $CHCl_3$ fraction for 15 hr. The luciferase activity was measured and data were normalized by Renilla luciferase expression vector. Data represent the mean±SD of triplicate experiments. *$p<0.05$, **$p<0.01$ compared with LPS alone.

4). These results showed that the $CHCl_3$ fraction inhibits IL-6 production in LPS stimulated RAW 264.7 cells.

Effect of the $CHCl_3$ fraction on NF-κB activation in LPS-stimulated RAW 264.7 cells The expression of iNOS and IL-6 in murine macrophages has been shown to be dependent on NF-κB activity and p65 is the major component of NF-κB when activated by LPS in macrophages (34). Thus, we examined levels of p65 in cytoplasmic extracts by Western blotting. The $CHCl_3$ fraction of *dangyuja* leaves inhibited LPS-induced NF-κB-p65 expression without affecting IκB degradation (Fig. 5A). Also, we examined the effect of the $CHCl_3$ fraction of *dangyuja* leaves on NF-κB activation. Luciferase activity assays showed that the $CHCl_3$ fraction inhibited NF-κB activation in a dose-dependent manner (Fig. 5B). These results showed that the $CHCl_3$ fraction inhibits activity of NF-κB without affecting IκBα degradation in LPS stimulated RAW 264.7 cells.

Effect of the $CHCl_3$ fraction on phosphorylation of MAP kinase in LPS-stimulated RAW 264.7 cells There are 3 families of MAPKs (ERK, JNK, and p38 MAPK) that induce activation of macrophages. These kinases are important mediators involved in production of NO and pro-inflammatory cytokines (IL-6) (35,36). Also, JNK-dominant negative mutant murine macrophages significantly reduced IL-6 expression (37).

In order to determine the mechanism of inhibition of NO and IL-6 expression, we investigated the effect of *dangyuja* leaves on the phosphorylation of these 3 MAP kinases. LPS induced a strong and transient increase in phospho-JNK, which peaked at 20-30 min and declined by 60 min.

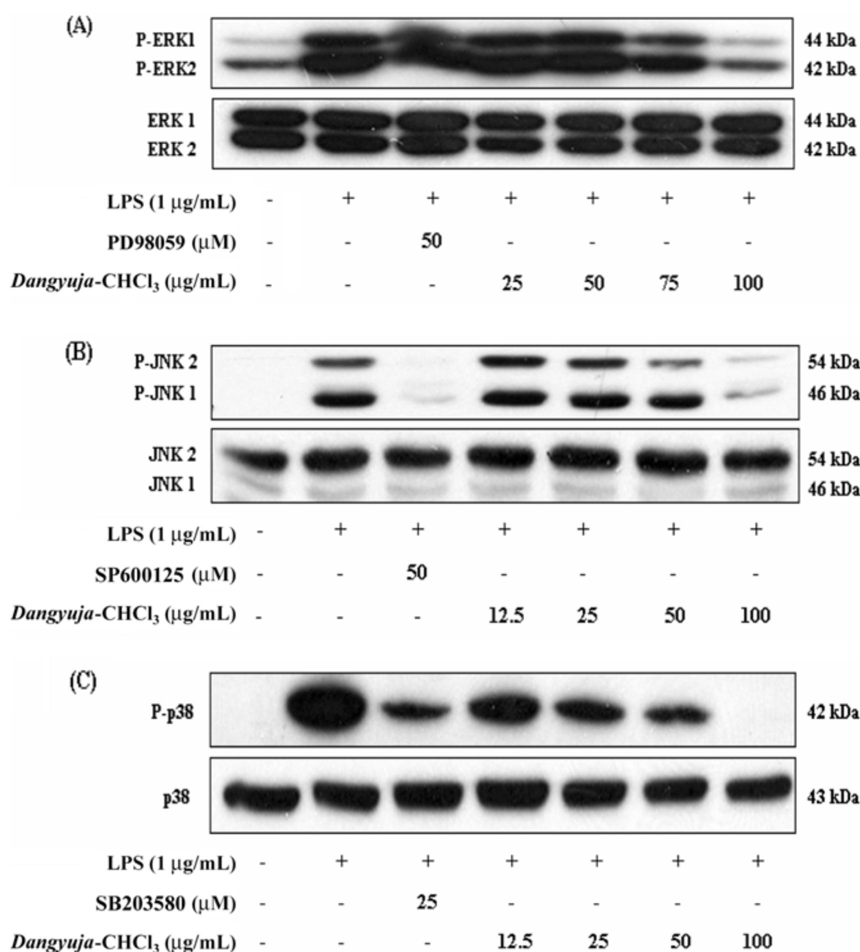

Fig. 6. Effects of $CHCl_3$ fraction from *dangyuja* leaves on the activation of MAPKs in LPS stimulated RAW 264.7 cells. (A) Cells were stimulated with LPS in the presence of *dangyuja* leaves $CHCl_3$ fractions or PD98059 for 15 min. (B) Cells were stimulated with LPS in the presence of *dangyuja* leaves $CHCl_3$ fractions or SP600125 for 30 min. (C) Cells were stimulated with LPS in the presence of *dangyuja* leaves $CHCl_3$ fraction or SB203580 for 15 min. Whole-cell lysate (25 μg) were prepared and the protein level was subjected to 10% SDS-PAGE, and expression of p-ERK, ERK, p-JNK, p-p38, and p38 were determined by Western blotting.

Also, phospho-p38 and phospho-ERK peaked at 15 min. Treatment with the $CHCl_3$ fraction blocked LPS-induced p-JNK, p-p38, and p-ERK activation (Fig. 6). The inhibitory effect on MAPKs of the $CHCl_3$ fraction was comparable to the effects of SP600125, SB203580, and PD98059, which are JNK, p38, and ERK inhibitors, respectively. These results indicated that the $CHCl_3$ fraction is able to attenuate the expression of proinflammatory genes (iNOS, IL-6) via a blockade of MAPKs phosphorylation in LPS-stimulated RAW 264.7 cells.

In conclusion, we demonstrated that the $CHCl_3$ fraction of *dangyuja* leaves markedly inhibits the expression of macrophage-mediated inflammation factors such as NO and IL-6 without cell cytotoxicity via a blockade of NF-κB activation and MAPKs (ERK1/2, JNK, and p38) phosphorylation in LPS-stimulated RAW 264.7 cells. Moreover, the high performance liquid chromatography (HPLC) analysis of the $CHCl_3$ fraction of *dangyuja* leaves revealed a nobiletin (data not shown). Nobiletin contributes to pharmacological activities such as anti-cancer (38), anti-inflammation, and antioxidant effects (39), and the peel of Citrus fruit were detected nobiletin. These properties may provide anti-inflammatory effect of *dangyuja* leaves that caused by nobiletin action.

Acknowledgments

This study was supported by a 2007 Technology Development Program of the Ministry of Agriculture and Forestry, Republic of Korea and the Brain Korea 21 program of the Korea Research Foundation.

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