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Antioxidant and Anti-Inflammatory Activities of *Sasa quelpaertensis* Leaf Extracts

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1. Introduction

Oxidative stress plays a critical role in the pathogenesis of inflammation (Winrow et al., 1993), which is a physiological response that protects the body from stimuli including infections and tissue injury. The magnitude of the inflammatory response is crucial, and insufficient responses result in immunodeficiency, which can lead to infection and cancer. Excessive responses cause morbidity and mortality from diseases such as rheumatoid arthritis, Crohn's disease, atherosclerosis, diabetes, Alzheimer's disease, multiple sclerosis, and cerebral and myocardial ischemia (Tracey, 2002). Inflammation is associated with a wide range of inflammatory mediators that initiate inflammatory responses, recruit and activate other cells to the site of inflammation, and subsequently resolve the inflammation (Gallin & Snyderman, 1999). The expression of pro-inflammatory mediators such as cytokines, chemokines, adhesion molecules, iNOS, and COX-2 involves nuclear factor- κ B (NF- κ B) (Baeuerle & Baltimore, 1996; Hayden & Ghosh, 2004). Mitogen-activated protein kinases (MAPKs) pathways are also reportedly stimulated by inflammatory mediators (Guha & Mackman, 2001).

The genus *Sasa* (Poaceae) is composed of perennial plants commonly known as bamboo grasses, and various *Sasa* species are widely distributed in Asian countries including China, Japan, Korea, and Russia (Okabe et al., 1975). *Sasa* leaves have been used in traditional medicine for their anti-inflammatory, antipyretic, and diuretic properties (Bae, 2000). Bamboo leaves have also been used in clinical settings to treat hypertension, cardiovascular disease, and cancer (Shibata et al., 1975).

Many recent studies have described the beneficial health effects of *Sasa* species leaves, which have been used as alternative medicines. *S. albomarginata* extract reportedly has anticancer properties (Shibata et al., 1979). Both lignin and polysaccharide preparation from *Sasa* species reportedly have antitumor properties (Suzuki et al., 1968; Yamafuji & Murakami, 1968). Two polysaccharide preparations (GK1 and GK2) from *S. kurilensis* was found to negatively affect the growth of Sarcoma-180 implanted in mice (Raidaru et al., 1997). Also, Sasa Health, an alkaline extract derived from *S. senanensis* leaves containing polysaccharides, chlorophyllin, lignin, and flavonoids, reportedly has a protective effect on

spontaneous mammary tumorigenesis (Tsunoda et al., 1998) and Her2/NeuN mammary tumorigenesis (Ren et al., 2004). Researchers have also recently reported that *S. senanensis* leaf extracts have antioxidant and immunostimulation-mediated antitumor properties (Kurokawa et al., 2006; Seki et al., 2008). Hagesewa et al. (2008) reported antioxidant C-glycosyl flavones in the leaves of *S. kurilensis* var. *gigantea*. Extract from *S. borealis* leaves reportedly improves chronic high glucose-induced endothelial apoptosis (Choi et al., 2008), as well as insulin resistance by modulating inflammatory cytokine secretion in high fat diet-induced obese C57/BL6J mice (Yang et al., 2010). Park et al. (2007) reported four antioxidant flavone glycosides (tricine-7-O- β -D-glucopyranoside, isoorientin, apigenin 6-C- β -D-xylopyranosyl-8-C- β -D-glucopyranoside, isoorientin 2-O- α -L-rhamnoside) from *S. borealis*. Two phenolic compounds, (-)-syringaresinol and tricetin, isolated from *S. borealis*, exhibited P-glycoprotein inhibitory properties in adrimamycin-resistant human breast cancer, MCF-7/ARD (Jeong et al., 2007).

S. quelpaertensis Nakai is another bamboo grass. It is a native Korean plant that grows only on Mt. Halla on Jeju Island, South Korea. This small bamboo grass has recently been the focus of much attention due to its potential biomass as well as its role as an invasive plant that inhibits the growth of other plants in the habitat on Jeju Island. Young leaves of *S. quelpaertensis* are used for a popular bamboo tea, but their beneficial health effects and the bioactive compounds contained in the plant have not yet been identified. Thus, systematic research about using its leaves as an industrial bio-resource is increasingly required. As a first step to evaluating the potential of *S. quelpaertensis* leaves as nutraceuticals, in this study, we investigated the anti-oxidative and anti-inflammatory activities of *S. quelpaertensis* leaf extract.

2. Materials and methods

2.1 Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin (PS) were obtained from Gibco-BRL (Grand Island, NY, USA). Antibody against inducible NOS (iNOS) was purchased from Calbiochem (San Diego, CA, USA), and antibody against cyclooxygenase-2 (COX-2) was obtained from Becton Dickinson (Mountain View, CA, USA). Anti-phospho-extracellular signal-regulated kinase (ERK1/2) was acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against ERK1/2, JNK1/2, phospho-JNK1/2, p38, and phospho-p38 were obtained from Cell Signaling Technology (Beverly, MA, USA). The lactate dehydrogenase (LDH) Cytotoxicity Detection Kit was purchased from Takara Shuzo Co. (Otsu, Shiga, Japan). Protein assay reagent was purchased from Bio-Rad Laboratories Inc. (Hercules, CA, USA). Trizol reagent was purchased from Molecular Research Center Inc. (Cincinnati, OH, USA), lipopolysaccharide (LPS) (*Escherichia coli* 026:B6) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). All other reagents were acquired from Sigma.

2.2 Preparation of extracts

S. quelpaertensis leaves were collected in October 2010, from Mt. Halla on Jeju Island, South Korea. A dried powder of the *S. quelpaertensis* leaves was extracted using 80% methanol

(MeOH) at room temperature for 48 h. This procedure was repeated twice. The combined extract was concentrated on a rotary evaporator under reduced pressure and freeze-dried to a powder. The dried extract was dissolved in water and then fractionated using the organic solvents *n*-hexane (hexane), ethyl acetate (EtOAc), and *n*-butanol (BuOH) at room temperature. Each fraction was concentrated on a rotary evaporator under reduced pressure and freeze-dried to a powder.

2.3 Measurement of antioxidant activities

2.3.1 DPPH radical scavenging assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was examined according to the method reported by Tateyama et al. (1997) with slight modifications. Briefly, the extracts were mixed with methanol and then added to 0.4 mM DPPH in methanol. After 20 min of incubation in the dark at room temperature, the reduction in the DPPH free radical was measured by absorbance, which was read using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA) at 517 nm. Trolox and BHA, a stable antioxidant, were used as a reference control and pure methanol was used as a control sample. Three replicates were made for each test sample. The radical scavenging activity of samples, expressed as percent inhibition, was calculated according to the following formula: % inhibition = $(A_0/AX)/A_0 \times 100$, where A_0 and AX are the absorbance values of the blank sample and the tested samples, respectively. The results were indicated as IC_{50} , which is the 50% inhibitory concentration of DPPH radical scavenging activity.

2.3.2 NO scavenging activity

Nitric oxide (NO) scavenging activity was measured using the method described by Feelisch and Stamler (1996) with slight modifications. Sodium nitroprusside (SNP) in an aqueous solution at physiological pH spontaneously generates nitric oxide, which can be measured using the Griess reagent system. The reaction solution containing 1 mM SNP in phosphate-buffered saline (PBS) (pH 7.4) was mixed with the extract, followed by incubation at room temperature for 3 h. Then, the reaction solution was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid). The absorbance at 540 nm was recorded using a microplate reader. The percentage of NO scavenging was measured through comparison with the absorbance values of the blank sample.

2.3.3 Superoxide radical scavenging assay

Superoxide (O_2^-) was generated using the enzymatic method. Briefly, the extracts were mixed with an equal volume of reaction reagent [2 mM Na_2 EDTA in phosphate buffer (50 mM KH_2PO_4/KOH , pH 7.4), 0.05 mM nitroblue tetrazolium chloride (NBT) in a buffer, and 1 mM hypoxanthine in 50 mM KOH]. The reaction was started by adding xanthine oxidase (XOD) in a buffer to the mixture (final XOD concentration was 0.05 U/ml). After 1 h of incubation at room temperature, scavenging activity was measured by absorbance, which was read using a microplate reader at 560 nm. Trolox and BHA, a stable antioxidant, were used as a reference control; SOD and allopurinol were used as a positive control. The NBT reduction (%) was calculated according to the following formula: % inhibition =

$(A_0/AX)/A_0 \times 100$, where A_0 and AX are the absorbance values of the blank sample and the tested samples, respectively.

2.3.4 Determination of XOD-inhibitory activity

XOD-inhibitory activity was measured by detecting uric acid formation according to the method described by Puig et al. (1989) with slight modifications. First, the extracts and reaction reagent [2 mM Na_2EDTA in a phosphate buffer (50 mM $\text{KH}_2\text{PO}_4/\text{KOH}$, pH 7.4) and 1 mM hypoxanthine in 50 mM KOH] were mixed in a 96-well microplate. The reaction was started by adding XOD in a buffer to the mixture (final XOD concentration was 0.05 U/ml). After 1 h of incubation at room temperature, uric acid production was measured by absorbance, which was read using a microplate reader at 295 nm. Trolox and BHA, a stable antioxidant, were used as a reference control. SOD and allopurinol were used as a positive control. Uric acid production was calculated according to the following formula: % inhibition = $(A_0/AX)/A_0 \times 100$, where A_0 and AX are the absorbance values of the blank sample and the tested samples, respectively.

2.4 Cell culture

The RAW 264.7 murine macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). Cells were cultured in 1% PS/DMEM containing 10% heat-inactivated FBS at 37°C in a 5% CO_2 incubator.

2.5 Cytotoxicity assay - MTT and LDH release assays

Cell viability and cytotoxicity were determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay and lactic dehydrogenase (LDH) release assay. Cells were seeded at a density of 5×10^4 cells/well into a 96-well flat-bottom cell culture plate in the presence or absence of the extracts. Mitochondrial enzyme activity, which is an indirect measure of the number of viable respiring cells, was determined using an MTT reagent after 40 h of treatment with methanol extract or its fractions. Absorbance was read using a microplate reader (Bio-Tek Instruments) at 595 nm. The effect of extracts on cell viability was evaluated as the relative absorbance compared to that of control cultures. LDH leakage is known to be a marker of damage to the cell membrane, and the LDH level was detected using the culture supernatants according to the LDH cytotoxicity detection kit instructions (Takara Shuzo Co.). Cytotoxicity was expressed as the percentage of released LDH (LDH released into the medium/maximal LDH release $\times 100$). Maximal LDH release was measured after lysis of the cells with 0.5% Triton X-100.

2.6 Measurement of NO and PGE_2 production

The amount of nitrite was determined using a colorimetric assay (Green et al., 1982). Briefly, 100 μl of cell culture medium was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid) and incubated at room temperature for 10 min. The absorbance at 540 nm was recorded using a microplate reader. Nitrite concentration was determined using extrapolation from a sodium nitrite standard curve. Concentration of prostaglandin E_2 (PGE_2) in the culture medium was

quantified by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions.

2.7 Measurement of iNOS enzyme activity

The activity of the iNOS enzyme in the cell lysate was measured as the L-arginine- and NADPH-dependent generation of nitrite. Briefly, 200 µg cell lysate was incubated for 180 min at room temperature in 100 µl of a reaction buffer containing 20 mM sodium phosphate buffer, 2 mM NADPH, 2 mM L-arginine, and 10 µM FAD at pH 6.7. The reaction was stopped by adding 10 U/ml LDH and 10 mM pyruvate. Next, the reaction mixture was incubated with an equal volume of Griess reagent. The absorbance at 540 nm was recorded using a microplate reader. Nitrite concentration was determined by extrapolation from a sodium nitrite standard curve.

2.8 Western blot analysis

Cells were washed twice with ice-cold PBS and collected. The cells were then treated with a lysis buffer [1× RIPA (Upstate Cell Signaling Solutions, Lake Placid, NY, USA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na₃VO₄, 1 mM NaF, 1 µg/ml aprotinin, 1 µg/ml pepstatin, and 1 µg/ml leupeptin] and incubated on ice for 1 h. Cell debris was removed by centrifugation and then protein concentration was determined using a Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Cell lysates were subjected to 7.5% or 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with a solution of 0.1% Tween 20/Tris-buffered saline containing 5% nonfat milk powder for 1 h at room temperature. After incubation overnight at 4°C with the indicated primary antibody, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunodetection was carried out using an ECL Western blotting detection reagent. The signal intensity of relative bands was determined using image acquisition and analysis software (LabWorks, Cambridge, UK).

2.9 RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated using a Trizol reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. RNA concentration and purity were determined by measuring absorbance at 260 and 280 nm. A cDNA synthesis was performed using the ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA) with an oligo dT-15 primer, as recommended by the supplier. PCR analyses were then performed on aliquots of cDNA preparations using a thermal cycler. The reactions were carried out in a 25 µl volume containing (final concentration) 1 unit of Taq DNA polymerase, 0.2 mM dNTP, 10× reaction buffer, and 100 pmol primers (Table 1). The cycle number was optimized to ensure product accumulation in the exponential range. β-Actin was used as an internal control to normalize the RNA content of each sample. Amplification was initiated at 94°C for 5 min, followed by 18–27 cycles of denaturation at 94°C for 45 s, annealing at the appropriate primer-pair annealing temperature for 45 s, and extension at 72°C for 1 min, followed by a final extension of 10 min at 72°C. After amplification, portions of the PCR reactions were electrophoresed on 2% agarose gel and visualized using ethidium

bromide staining and a UV transilluminator (SLB Mylmaggr; UVP Inc., Upland, CA, USA). The signal intensity of relative bands was determined using image acquisition and analysis software (LabWorks).

Gene		Primer sequences
iNOS	Forward	5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3'
	Reverse	5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'
COX-2	Forward	5'-CACTACATCCTGACCCACTT-3'
	Reverse	5'-ATGCTCCTGCTTGAGTATGT-3'
TNF- α	Forward	5'-TTGACCTCAGCGCTGAGTTG-3'
	Reverse	5'-CCTGTAGCCCACGTCGTAGC-3'
IL-1 β	Forward	5'-CAGGATGAGGACATGAGCACC-3'
	Reverse	5'-CTCTGCAGACTCAAACCTCCAC-3'
IL-6	Forward	5'-GTACTCCAGAAGACCAGAGG-3'
	Reverse	5'-TGCTGGTGACAACCACGGCC-3'
β -Actin	Forward	5'-AGGCTGTGCTGTCCCTGTATGC-3'
	Reverse	5'-ACCCAAGAAGGAAGGCTGGAAA-3'

Table 1. The sequences of primers used in RT-PCR analysis.

2.10 Transient transfection and luciferase assay

Cells were seeded at a density of 5×10^3 cells/well into a 96-well flat-bottom cell culture plate and cultured for 18 h. RAW 264.7 cells were transiently transfected with or without a NF- κ B-promoted luciferase reporter gene plasmid pNF- κ B-Luc (Promega) and a Renilla luciferase reporter plasmid pRL-TK (Stratagene, La Jolla, CA, USA), to control for transfection efficiency using a FuGENE 6 transfection reagent (Roche, Indianapolis, IN, USA). At 24 h after the start of transfection, cells were incubated with LPS (100 ng/ml) in the presence or absence of the extracts for 24 h. Luciferase activity in the cell lysate was measured using a Dual-Luciferase Reporter Assay Kit (Promega) and a FLUOstar Optima (BMG Labtech, Offenbug, Germany). Luciferase activity was normalized to transfection efficiency, as monitored using a Renilla luciferase expression vector. The level of induced luciferase activity was determined as a ratio to the luciferase activity of unstimulated cells.

2.11 Statistical analysis

All experiments were conducted in triplicate, but only data from one representative trial are presented. Results are expressed as the mean \pm standard deviation (SD). Treatment effects were analyzed using a paired *t*-test.

3. Results

3.1 Antioxidant activities of *S. quelpaertensis* leaf extracts

Table 2 summarizes the antioxidant activities of *S. quelpaertensis* leaf methanol extract and its fractions, represented by IC₅₀. The IC₅₀ values for DPPH radical scavenging activity against MeOH extract, EtOAc, and BuOH fractions were 862.5, 288.9, and 166.4 μ g/ml, respectively.

DPPH radical scavenging activity of the BuOH fraction was more potent than that of other tested samples. For NO scavenging activity, the IC₅₀ value (259.4 µg/ml) was calculated only for the EtOAc fraction; other tested samples exhibited minimal NO scavenging activity. All tested samples (except the water fraction) exhibited XOD-inhibition activity and superoxide radical scavenging activity. XOD-inhibition activity occurred in the following (decreasing) order: EtOAc fraction > hexane fraction > MeOH extract > BuOH fraction. Superoxide radical scavenging activity occurred in the following (decreasing) order: EtOAc fraction > BuOH fraction > hexane fraction > MeOH extract > water fraction. The IC₅₀ values for the EtOAc fraction on XOD-inhibition activity and superoxide radical scavenging activity assay were 32.4 and 21.9 µg/ml, respectively. As shown in Table 2, overall antioxidant activity was more potent in the EtOAc fraction than in any other tested samples.

Sample	IC ₅₀ (µg/ml) ^{a)}			
	DPPH radical scavenging activity	Nitric oxide scavenging activity	Uric acid generation activity	Superoxide generation activity
Methanol extract	862.5 ± 6.4	*	352.9 ± 16.0	113.5 ± 13.4
Hexane fraction	*	*	238.4 ± 5.6	62.8 ± 4.3
EtOAc fraction	288.9 ± 12.7	259.4 ± 1.6	32.4 ± 1.6	21.9 ± 5.4
BuOH fraction	166.4 ± 9.4	*	473.5 ± 15.4	23.4 ± 5.9
Water fraction	*	*	-	305.2 ± 6.9
Trolox	3.49 ± 0.3	*	220.3 ± 12.3	32.5 ± 1.9
BHA ^{b)}	7.6 ± 0.2	*	801.7 ± 11.7	*
Allopurinol	N/A	N/A	1.33 ± 0.1	5.1 ± 0.1
SOD ^{c)}	N/A	N/A	*	4.9 ± 1.9

a) IC₅₀ values were calculated from regression lines using five different concentrations in triplicate experiments.

b) Butylated hydroxyl anisole

c) Superoxide dismutase

N/A: Not assay

*: Can't calculate the value of IC₅₀

-: < 5% xanthine oxidase inhibitory activity at maximum concentration used for assay.

Table 2. Antioxidant activities of the methanol extract and its various fractions from *Sasa queipaertensis* leaf.

Sample	TC ₅₀ ^{a)} (µg/ml)	IC ₅₀ ^{b)} (µg/ml)	Selectivity index ^{c)}
MeOH extract	>2000	1341.7 ± 4.9	>1.8
Hexane fraction	240.1 ± 19.8	175.0 ± 9.6	1.4
EtOAc fraction	465.2 ± 10.2	68.6 ± 0.1	6.8
BuOH fraction	>2000	>2000	-
Water fraction	>2000	>2000	-

a) TC₅₀ is the concentration producing 50% toxicity in RAW 264.7 cells.

b) IC₅₀ is the concentration producing 50% inhibition of NO production in RAW 264.7 cells.

c) Selectivity Index = TC₅₀ / IC₅₀.

Table 3. Cell toxicity and the effects of the methanol extract and its fractions of *Sasa queipaertensis* on LPS-induced NO production in RAW 264.7 cells.

Next, we compared the effects of *S. quelpaertensis* leaf extract on LPS-induced NO production in RAW 264.7 cells (see Table 3). Among the tested samples, the EtOAc fraction had the highest selectivity index (6.8), indicating it had potent anti-inflammatory properties. Thus, we focused on the anti-inflammatory effects of the EtOAc fraction using LPS-stimulated RAW 264.7 cells.

3.2 EtOAc fraction inhibits NO production and iNOS expression

The effects of the EtOAc fraction on NO production in LPS-stimulated RAW 264.7 cells were investigated by measuring the amount of nitrite released into the culture medium using a

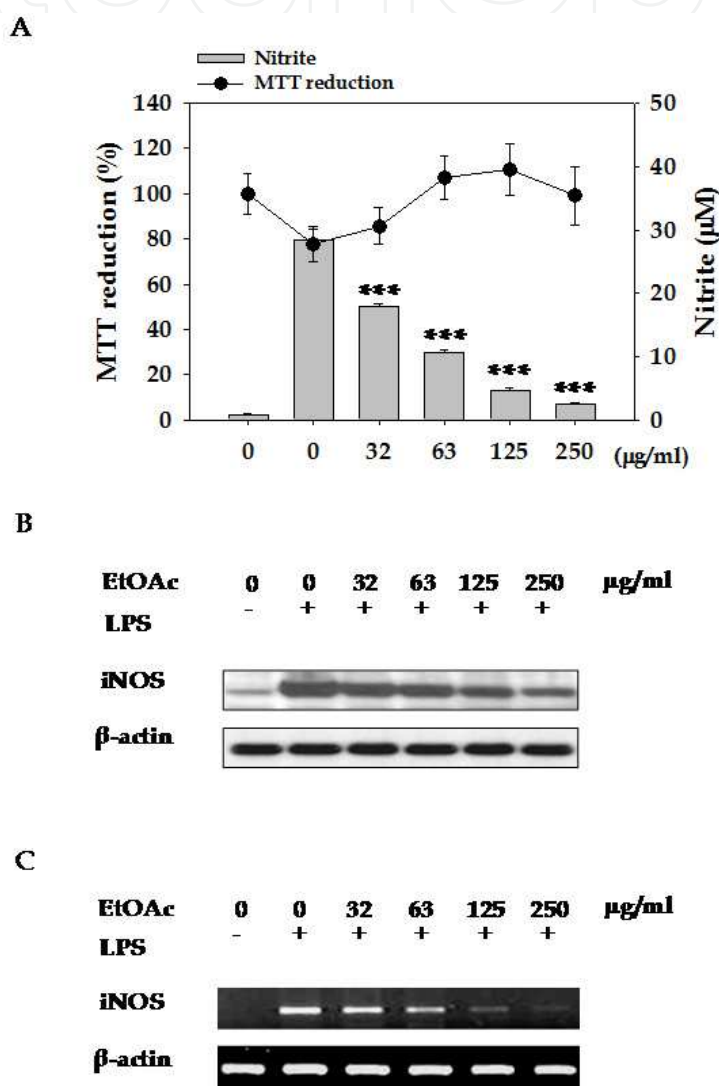


Fig. 1. Effect of EtOAc fraction on the NO production, iNOS protein and iNOS mRNA expression level in LPS-stimulated RAW 264.7 cells. (A) Cells were treated with LPS (100 ng/ml) alone or LPS plus the indicated concentrations of EtOAc fraction for 24 h. *** $P < 0.001$ vs LPS alone-treated cells. Data are expressed in area density as the mean \pm SD for three independent experiments. (B) Cells were treated with LPS (100 ng/ml) alone or LPS plus the indicated concentrations ($\mu\text{g/ml}$) of EtOAc fraction for 24 h. (C) Cells were co-treated with LPS (100 ng/ml) or LPS with EtOAc fraction at the indicated concentration for 6h. Total RNA was subjected to RT-PCR.

Griess reagent system. As shown in Fig. 1A, NO levels increased remarkably with LPS, up to 30 μM , but co-treatment with the EtOAc fraction significantly decreased NO levels in a dose-dependent manner. In addition, the EtOAc fraction did not affect the viability of RAW 264.7 cells at concentrations from 32 to 250 $\mu\text{g}/\text{ml}$.

To investigate whether the inhibition of NO production by the EtOAc fraction was a result of inhibition of the corresponding gene expression, we analyzed the expression of iNOS protein and mRNA using Western blotting and RT-PCR. LPS increased the levels of cellular iNOS protein and mRNA at 24 h and 6 h after treatment, respectively. However, co-treatment of the EtOAc fraction (32, 63, 125 and 250 $\mu\text{g}/\text{ml}$) with LPS decreased both LPS-induced protein (Fig. 1B) and iNOS mRNA (Fig. 1C) in a dose-dependent manner.

We also measured iNOS enzymic activity to investigate whether the EtOAc fraction affected enzymic activity. As shown in Fig. 2, EtOAc treatment (32, 63, 125 and 250 $\mu\text{g}/\text{ml}$) did not affect iNOS protein enzyme activity. However, iNOS enzyme activity was inhibited by 1400 W, which was used as the positive control (data not shown). These results confirmed that the EtOAc fraction inhibited NO production in LPS-stimulated RAW 264.7 cells through the regulation of iNOS gene expression.

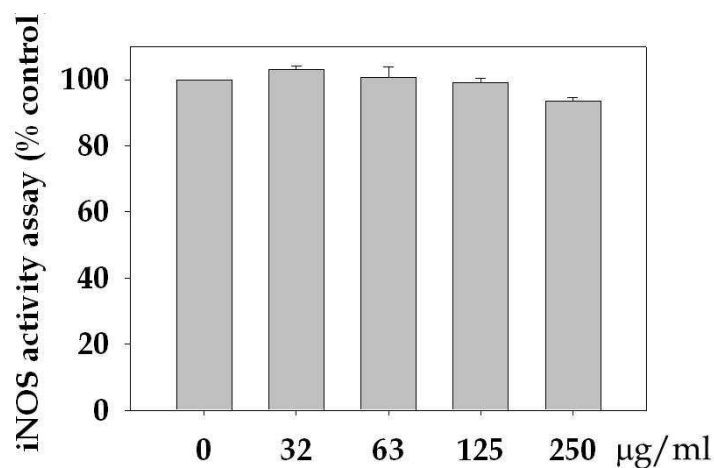


Fig. 2. Effect of EtOAc fraction on iNOS enzyme activity. The iNOS activity was measured as using L-arginine as substrate and NADPH-dependent generation of nitrite, the stable oxidation products of NO. The assay was performed by incubating with 200 μg of the cytosol protein from LPS-stimulated cells, in the absence or presence of EtOAc fraction, for 180 min at room temperature in 200 μl reaction buffer containing 20 mM Tris-HCl, pH 8.0, 2 mM NADPH, 2 mM L-arginine, 10 μM FAD. NO_3^- was reduced to NO_2^- by incubation at 37°C for 15 min with 0.1 U/ml nitrate reductase, 0.1 mM NADPH, 5 μM FAD.

3.3 EtOAc fraction inhibits PGE_2 production and COX-2 expression

PGE_2 is the major metabolite produced by COX-2 at inflammation sites. Therefore, we examined the effects of the EtOAc fraction on PGE_2 production and COX-2 expression. PGE_2 production increased remarkably with LPS (7,800 pg/ml), but co-treatment with the EtOAc fraction significantly decreased PGE_2 production in a dose-dependent manner (Fig. 3A).

To investigate whether the inhibition of PGE_2 production by the EtOAc fraction was due to inhibition of the corresponding gene expression, we analyzed the expression of COX-2

protein and mRNA using Western blotting and RT-PCR. As shown in Fig. 3B, COX-2 protein levels increased in response to LPS treatment, but co-treatment of the EtOAc fraction (32, 63, 125 and 250 $\mu\text{g}/\text{ml}$) with LPS decreased COX-2 protein levels in a dose-dependent manner. In addition, RT-PCR analysis revealed that the expression of COX-2 mRNA was correlated with its protein levels (Fig. 3C). These results indicate that the inhibitory effect of the EtOAc fraction on PGE₂ production involved regulation of COX-2 gene expression.

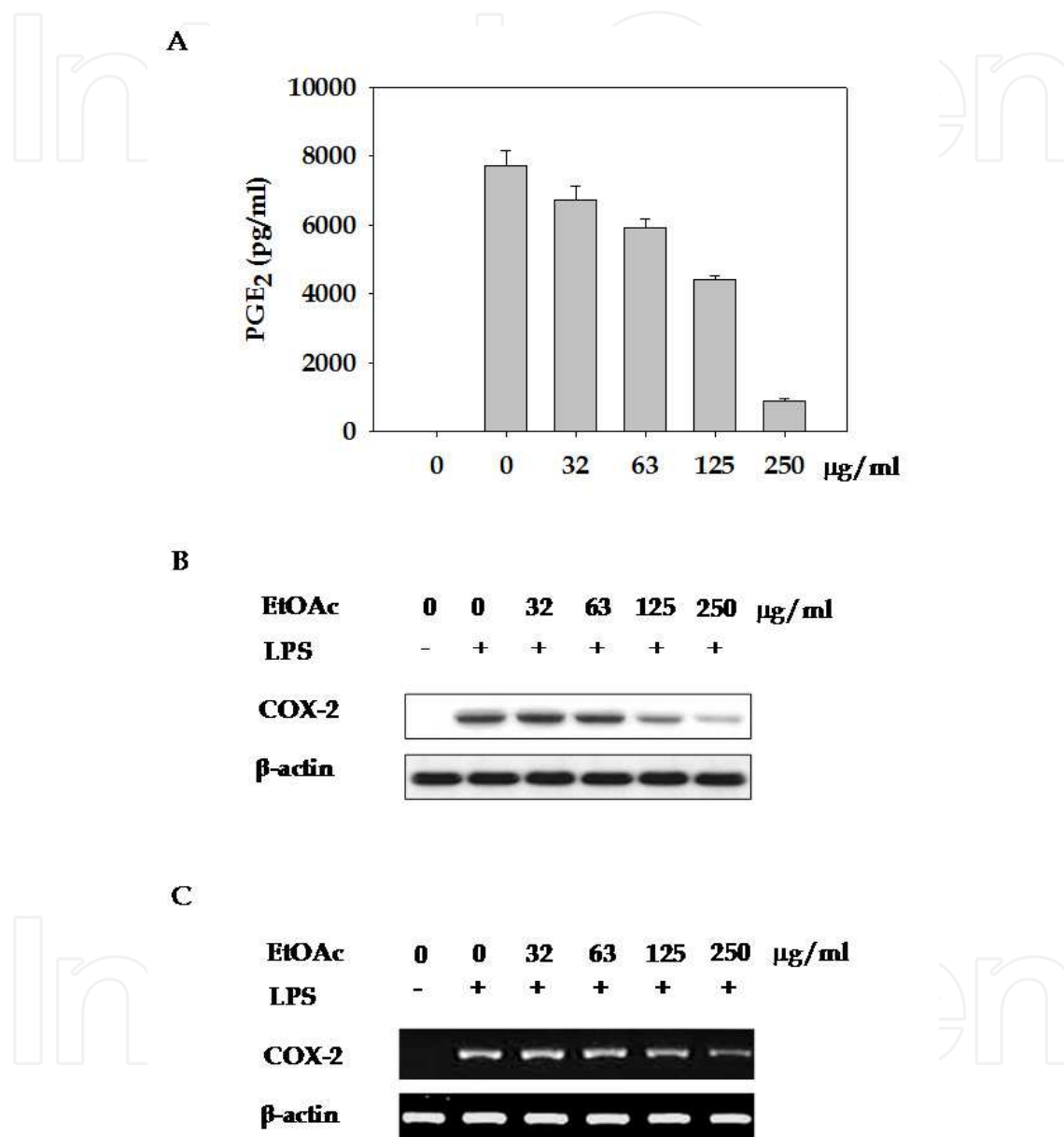


Fig. 3. Effect of EtOAc fraction on the PGE₂ production, COX-2 protein and mRNA expression level in LPS-stimulated RAW 264.7 cells. (A) Cells were treated with LPS (100 ng/ml) alone or LPS plus the indicated concentrations of EtOAc fraction for 24 h. *** $P < 0.001$ vs LPS alone-treated cells. Data are expressed in area density as the mean \pm SD for three independent experiments. (B) Cells were treated with LPS (100 ng/ml) alone or LPS plus the indicated concentrations ($\mu\text{g}/\text{ml}$) of EtOAc fraction for 24 h. (C) Cells were co-treated with LPS (100 ng/ml) or LPS with EtOAc fraction at the indicated concentration for 6 h. Total RNA was subjected to RT-PCR.

3.4 EtOAc fraction inhibits pro-inflammatory cytokine mRNA expression

Pro-inflammatory cytokines, such as IL-1 β , IL-6, and TNF- α , are known to affect LPS-induced macrophage activation during immune responses. Therefore, we investigated the effects of the EtOAc fraction on the expression of TNF- α , IL-1 β , and IL-6 mRNA in LPS-stimulated RAW 264.7 cells using RT-PCR analysis. The mRNA levels of these cytokines increased at 6 h (IL-1 β and IL-6) or 4 h (TNF- α) after LPS treatment. However, co-treatment with the indicated concentrations of EtOAc fraction significantly decreased LPS-induced TNF- α , IL-1 β , and IL-6 mRNA levels in a dose-dependent manner (Fig. 4); at a EtOAc fraction concentration of 125 μ g/ml, mRNA levels of IL-1 β , IL-6, and TNF- α decreased by 55%, 60%, and 45%, respectively (Fig. 4B).

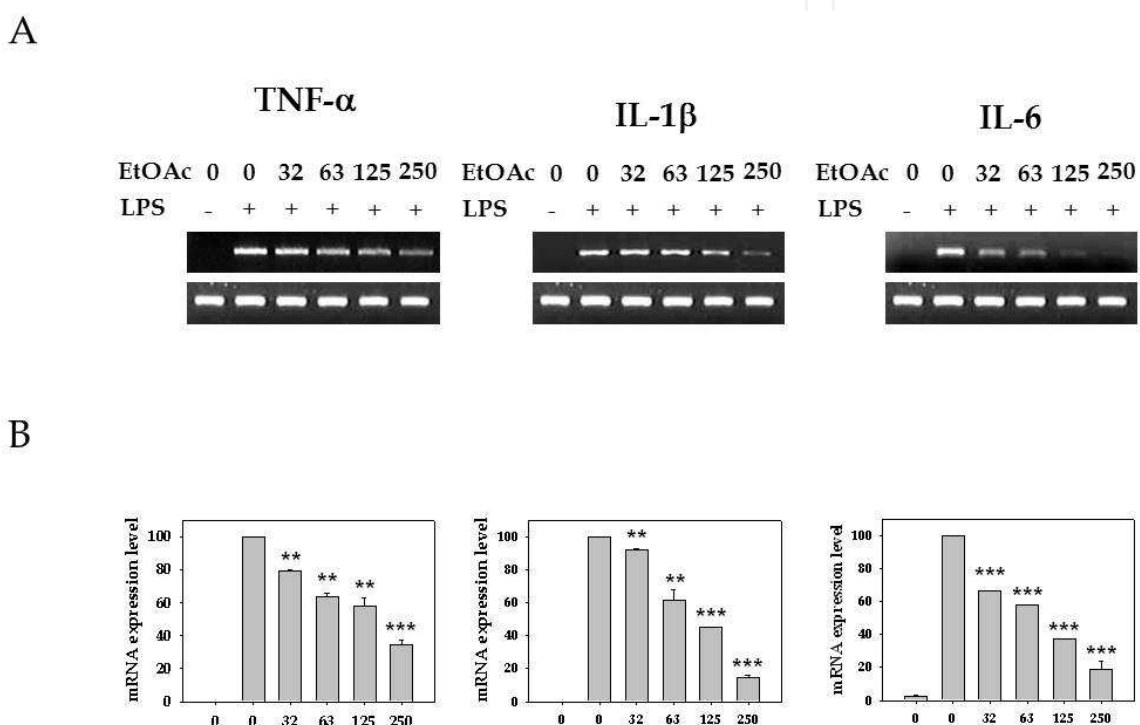


Fig. 4. Effect of EtOAc fraction on TNF- α , IL-1 β and IL-6 mRNAs expression level in LPS-stimulated RAW 264.7 cells. (A) RT-PCR analysis of TNF- α , IL-1 β and IL-6 mRNA expression using total RNA extracted RAW 264.7 macrophages stimulated with LPS (100 ng/ml) alone or LPS plus the indicated concentrations (μ g/ml) of EtOAc fraction for 6 h (TNF- α for 4h). (B) Quantification of TNF- α , IL-1 β , and IL-6 mRNA expression was performed by densitometric analysis of the RT-PCR products. The relative level was calculated as the ratio of pro-inflammatory mRNA expression to β -actin mRNA expression. *** $P < 0.001$, ** $P < 0.01$ vs LPS alone-treated cells.

3.5 EtOAc fraction suppresses NF- κ B transcriptional activation

NF- κ B regulated the expression of pro-inflammatory cytokines, iNOS, and COX-2. Therefore, we investigated the effect of the EtOAc fraction on NF- κ B activation using a transient transfection assay with a NF- κ B-promoted luciferase reporter gene plasmid (pNF- κ B-Luc) in RAW 264.7 cells. The treatment group of EtOAc fraction alone exhibited activity similar to the unstimulated control group, confirming that the EtOAc fraction had no effect

on NF- κ B activation in the cells. However, LPS treatment (100 ng/ml, 24 h) increased luciferase activity 12-fold compared to the unstimulated control group. The EtOAc fraction significantly decreased LPS-induced luciferase activity in a dose-dependent manner (Fig. 5). At a concentration of 125 μ g/ml, the EtOAc fraction decreased luciferase activity by approximately 70% compared to the LPS-stimulated control group. This finding suggests that the EtOAc fraction exerts anti-inflammatory effects by suppressing the NF- κ B activation pathway.

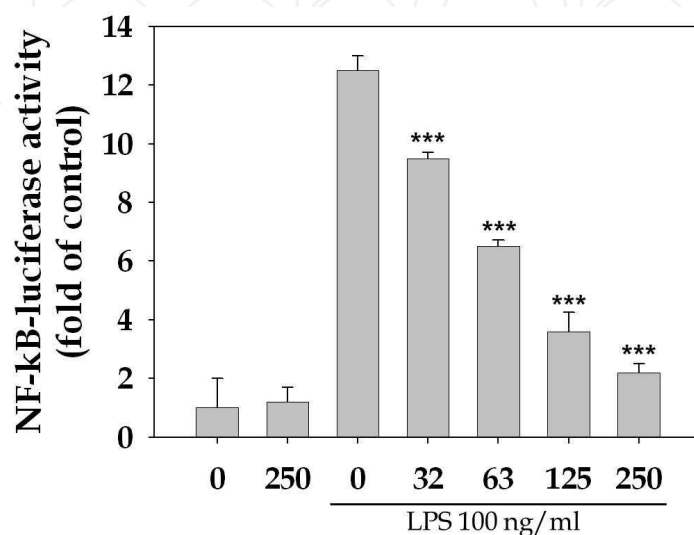


Fig. 5. Effect of EtOAc fraction on LPS-induced NF- κ B transcriptional activation. Cells were treated with LPS (100 ng/ml) alone or LPS plus the indicated concentrations (μ g/ml) of EtOAc fraction for 24 h. NF- κ B activation detected by luciferase reporter assays.

*** $P < 0.001$ vs LPS alone-treated cells.

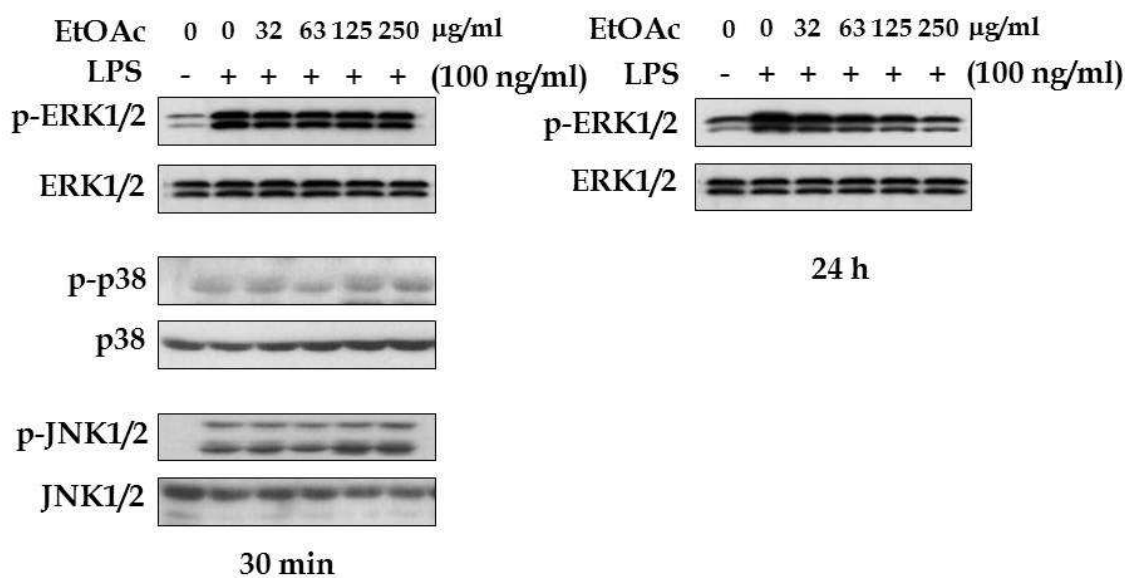


Fig. 6. Effect of EtOAc fraction on the phosphorylation of ERK1/2, p38, and JNK in LPS-stimulated RAW 264.7 cells. Cells were treated with LPS (100 ng/ml) alone or LPS plus the indicated concentrations (μ g/ml) of EtOAc fraction for 30 min or 24 h. The protein levels were determined by Western blotting.

3.6 EtOAc fraction suppresses the phosphorylation of ERK1/2

Because some MAPKs are known to be stimulated by inflammatory mediators, we also investigated how the EtOAc fraction affected three MAPKs (ERK1/2, JNK1/2, and p38 MAPK) in LPS-stimulated RAW 264.7 macrophages. The phosphorylations of these three kinases were detected after cells were subjected to 30 min of LPS treatment. The activation of the three MAPKs by LPS treatment did not decrease with co-treatment of the EtOAc fraction, but the EtOAc fraction suppressed the phosphorylation of ERK1/2 at 24 h after LPS treatment (Fig. 6).

4. Discussion

In the human body, oxidative stress is associated with many diseases. Therefore, researchers are currently intensely focused on identifying antioxidant agents in plants that may protect against oxidative stress, including the *Sasa* species already used in alternative medicines (Jensen et al., 2008; Nakajima et al., 2003; Sood et al., 2009). This study evaluated the potential of using *S. quelpaertensis* leaf in nutraceuticals. As the first step toward identifying phytochemicals with beneficial health effects from *S. quelpaertensis* leaf extracts, we evaluated *in vitro* antioxidant capacities such as DPPH radical scavenging activity, NO scavenging activity, XOD-inhibitory activity, and superoxide radical scavenging activity.

Among the various fractions, the *n*-butanol soluble fraction exhibited the strongest DPPH radical scavenging activity ($IC_{50} = 166.4 \mu\text{g/ml}$). This result was consistent with the results of Park et al. (2007), who also found significant DPPH radical scavenging activity in the *n*-butanol soluble fraction among *n*-hexane, EtOAc, and aqueous extracts from *S. borealis*. However, the EtOAc fraction was the most potent in nitric oxide scavenging activity ($IC_{50} = 259.4 \mu\text{g/ml}$), superoxide scavenging activity ($IC_{50} = 21.9 \mu\text{g/ml}$), and xanthine oxidase inhibitory activity ($IC_{50} = 32.4 \mu\text{g/ml}$), suggesting its potential as an antioxidant agent.

Antioxidants such as vitamins C reportedly exhibit anti-inflammatory activity via suppression of NF- κ B activation (Calfee-Mason et al., 2002; Muñoz et al., 1997). Therefore, we further investigated the anti-inflammatory potential of the EtOAc fraction, which exhibited the strongest antioxidant potential among various solvent fractions using the RAW 264.7 cell line.

NO is an essential bio-regulatory molecule within the nervous, immune, and cardiovascular systems (Bredt & Snyder, 1990; Gold et al., 1990; Palmer et al., 1987). However, increased levels of NO derived from iNOS can result in the formation of peroxynitrite after reaction with oxygen free radicals during inflammatory responses (Posadas et al., 2000). In the RAW 264.7 cell, NO production is closely associated with COX-2 expression (Salvemini et al., 1995), which produces PGE_2 and induces an inflammatory reaction (Bennett et al., 1977a, 1980b, 1982c; Rigas et al., 1993). iNOS and COX-2 are key enzymes regulating the production of NO and PGE_2 , central mediators of inflammation (Posadas et al., 2000; Tsatsanis et al., 2006).

This study demonstrated that the EtOAc fraction inhibited NO production and iNOS expression, but it had no effect on iNOS enzyme activity. Additionally, PGE_2 production and COX-2 expression were attenuated by the EtOAc fraction in a dose-dependent manner. These results suggest that the EtOAc fraction from *S. quelpaertensis* leaves reduced NO and PGE_2 production via transcriptional regulation of iNOS and COX-2 genes.

Pro-inflammatory cytokines, such as IL-1 β , IL-6, and TNF- α , interact with each other (Pålsson-McDermott & O'Neill, 2004) and affect LPS-induced macrophage activation during immune responses (Conti et al., 2004). Moreover, IL-1 β , IL-6, and TNF- α secretion increases in patients with some inflammatory diseases, such as ulcerative colitis and Crohn's disease (Reinecker et al., 1993); these cytokines play an important role in particle-induced inflammation in the lung (Driscoll, 2000; Mansour & Levitz, 2002; Yucesoy et al., 2002). TNF- α is the main mediator of the LPS reaction and is involved in innate immune reactions and chronic inflammatory reactions (Lee et al., 2003). IL-1 β is associated with T-cell activation, B-cell maturation, and NK cell activation (Delgado et al., 2003). Therefore, effective regulation of inflammatory mediators is essential (Driscoll, 2000; Hotamisligil, 2008; Hummasti & Hotamisligil, 2010). In this study, the EtOAc fraction significantly decreased LPS-induced TNF- α , IL-1 β , and IL-6 mRNA levels. This finding suggests that the EtOAc fraction exerts a beneficial health effect by inhibiting the production of many inflammatory mediators.

NF- κ B is an inducible eukaryotic transcription factor that can regulate the expression of numerous genes involved in proliferation, apoptosis, and the immediate-early steps of inflammatory and immune responses (Place et al., 2003). NF- κ B activation in response to pro-inflammatory stimuli involves the degradation of inhibitor κ B (I κ B) by the I κ B kinase (IKK) complex. NF- κ B is subsequently released, translocates into the nucleus, and initiates expression of pro-inflammatory mediators such as cytokines, chemokines, adhesion molecules, iNOS, and COX-2 (Baeuerle & Baltimore, 1996; Hayden & Ghosh, 2004). Most anti-inflammatory drugs have been shown to suppress the expression of these genes by inhibiting the NF- κ B activation pathway (Gilroy et al., 2004). Researchers have recently been working to identify an anti-inflammatory agent to suppress the NF- κ B activation pathway (Le et al., 2009; Reddy & Reddanna, 2009). Thus, an NF- κ B inhibitor may be useful in the development of therapeutic drugs to control the inflammation associated with human diseases in a clinical environment.

The other major extracellular signal transduction pathway stimulated by inflammatory mediators is the MAPK pathway (Guha & Mackman, 2001). Three major MAPK pathways are a highly conserved family of protein serine/threonine kinases and include the ERK1/2, the c-Jun NH₂-terminal kinase (JNK1/2), and the p38 mitogen-activated kinase (p38). These kinases can trigger the nuclear accumulation and activity of various transcription factors, such as NF- κ B, ATF2, Elk1, c-fos, and c-jun, which can modulate cytokine and inflammatory mediator expression (Aga et al., 2004; Herlaar & Brown, 1999).

LPS produces inflammatory mediators by activating the NF- κ B and MAPK pathways, and then induces inflammation in macrophages (Aga et al., 2004; Guha & Mackman, 2001; Zhang & Ghosh, 2000). On the basis of the inhibitory effect of the EtOAc fraction on the production of inflammatory mediators such as iNOS, COX-2, IL-1 β , IL-6, and TNF- α , we examined the effect of the EtOAc fraction on NF- κ B and MAPK activation in LPS-stimulated RAW 264.7 cells. The EtOAc fraction reduced the transcriptional activities of NF- κ B, as well as the delayed phosphorylation of the ERK1/2 in LPS-stimulated RAW 264.7 cells. Taken together, these results suggest that the EtOAc fraction from *S. quelpaertensis* leaves exhibited at least some anti-inflammatory properties by suppressing NF- κ B transcriptional activity and delaying ERK1/2 activation in LPS-stimulated RAW 264.7 cells.

Plants of the genus *Sasa* are known to biosynthesize various compounds such as triterpenoids, flavonoids, phenylpropanoids, and flavonolignans (Lee et al., 2007; Sultana &

Lee, 2009). In previous research, we demonstrated that a hot water extract of *S. quelpaertensis* leaves exhibited moderate anti-inflammatory activities in LPS-stimulated RAW 264.7 cells (Hwang et al., 2007). An ethanol/water extract of bamboo leaf mainly contains flavones, glycosides, phenolic acids, coumarin lactones, anthraquinones, and amino acids (Lu et al., 2005; Zhang & Ding, 1996). Thus, the moderate anti-inflammatory activity of the hot water extract may be due to water-soluble phytochemicals. The EtOAc fraction of *S. quelpaertensis* leaves, which contains mainly lipid-soluble compounds, has exhibited potent anti-proliferative effects via inducing apoptosis on human leukemia HL-60 cells (Jang et al., 2008). However, the bioactive compounds contained in the plant have not yet been identified (Sultana & Lee, 2010). We confirmed that the EtOAc extract contains various compounds, including tricetin 7-*O*- β -D-glucopyranoside, two phenylpropanoids, *p*-hydroxy benzaldehyde, and *p*-coumaric acid, as we investigated the relationships between various compounds and their antioxidant or anti-inflammatory properties.

5. Conclusion

As an initial step to evaluate the beneficial health effects of *S. quelpaertensis*, we investigated the antioxidant activity and anti-inflammatory activity of *S. quelpaertensis* leaf extract. Among the various fractions, the *n*-butanol soluble fraction exhibited the strongest DPPH radical scavenging activity ($IC_{50} = 166.4 \mu\text{g/ml}$). The EtOAc soluble fraction had the strongest inhibitory effect in the NO scavenging activity, superoxide scavenging activity, and xanthine oxidase inhibitory activity assay (IC_{50} values were 259, 21.9, and 32.4 $\mu\text{g/ml}$, respectively). Next, we investigated the anti-inflammatory properties of the EtOAc fraction in LPS-stimulated RAW 274.7 cells. The EtOAc fraction inhibited production of NO, PGE_2 , iNOS, and COX-2 in a dose-dependent manner. Additionally, pro-inflammatory cytokines, such as IL-1 β , IL-6, and TNF- α , decreased after co-treatment with the EtOAc fraction compared to the LPS-treated group. These results indicate that the EtOAc fraction exhibits anti-inflammatory properties via the inhibition of many inflammatory mediators. Finally, on the basis of the inhibitory effect of the EtOAc fraction on inflammatory mediators, we examined how the EtOAc fraction affected the LPS-induced activation of the NF- κ B and MAPK pathways. The EtOAc fraction inhibited the phosphorylation of ERK1/2 and the transactivation of NF- κ B, suggesting that the EtOAc fraction suppresses the production of pro-inflammatory mediators via the inhibition of NF- κ B transactivation and ERK 1/2 phosphorylation. Taken together, these results indicate that *S. quelpaertensis* leaf has potential for use as an antioxidant and anti-inflammatory agent.

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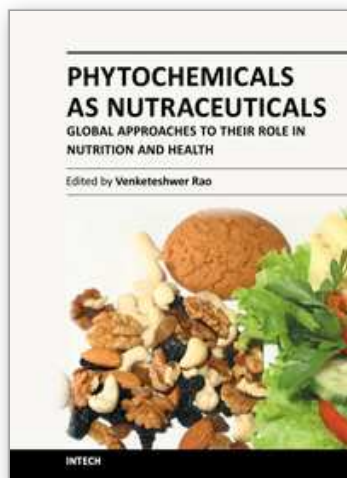
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