

Anti-Inflammatory Activity of *Pinus koraiensis* Cone Bark Extracts Prepared by Micro-Wave Assisted Extraction

Sun-Ae Kang¹, Dong-Hee Kim², Shin-Hyub Hong³, Hye-Jin Park³, Na-Hyun Kim³, Dong-Hyun Ahn⁴, Bong-Jeun An⁵, Joong-Ho Kwon^{3,6}, and Young-Je Cho^{3,6}

¹Food Research Center, Quality Management Team, Hanhwa Hotel & Resort, Gyeonggi 17800, Korea

²Korea Promotion Institute for Traditional Medicine, Gyeongbuk 38540, Korea

³School of Food Science and Biotechnology and ⁶Food and Bio-Industry Research Institute, Kyungpook National University, Daegu 41566, Korea

⁴Department of Food Science & Technology, Pukyong National University, Busan 48513, Korea

⁵Department of Cosmeceutical Science, Daegu Hanny University, Gyeongbuk 38610, Korea

ABSTRACT: In this study, we compared the anti-inflammatory activity of *Pinus koraiensis* cone bark extracts prepared by conventional extraction and microwave-assisted extraction (MAE). Water extracts and 50% ethanol extracts prepared using MAE were applied to RAW 264.7 cell at 5, 10, 25, and 50 µg/mL of concentrations, and tested for cytotoxicity. The group treated with 50 µg/mL of 50% ethanol extracts showed toxicity. In order to investigate the inhibition of nitric oxide (NO) production in RAW 264.7 cells, extracts of water and ethanol were treated with 5, 10, and 25 µg/mL concentrations. The inhibitory activity of water and 50% ethanol extracts groups were determined as 40% and 60% at 25 µg/mL concentration, respectively. We found concentration dependent decreases on inducible NO synthase. The inhibitory effect against forming inflammatory cytokines, prostaglandin E₂, tumor necrosis factor- α , interleukin (IL)-6, and IL-1 β , was also superior in the 25 µg/mL treated group than the control group. According to these results, the water extracts and 50% ethanol extracts both inhibited inflammatory mediators by reducing the inflammatory response. Therefore, The MAE extracts of *P. koraiensis* cone bark can be developed as a functional ingredient with anti-inflammatory activity.

Keywords: anti-inflammation, extracts, micro-wave, *Pinus koraiensis*, cone bark

INTRODUCTION

The incidence of proliferative arthritis, shingles, rhinitis, and other chronic diseases has been increasing as a result of aging and dietary changes (1). Since there is a correlation between chronic inflammation and cancer and other diseases, there have been efforts to prevent these diseases by reducing inflammation with dietary interventions. Inflammation is one of the defense mechanisms against viral infections, and physical or chemical stimulation. In addition, it is a mechanism for regenerating or recovering damaged organs (2). During inflammatory responses, cells, such as macrophages, secrete inflammatory mediators including nitric oxide (NO), prostaglandin E₂ (PGE₂), tumor necrosis factor (TNF)- α , and interleukin (IL)-1 β (3). In macrophages, stimulation with cytokines, such as TNF- α , or lipopolysaccharide (LPS) activates nuclear factor- κ B, which is a transcription factor involved in inflam-

matory reactions. As a result, inducible NO synthase (iNOS) and cyclooxygenase (COX)-2 are expressed and produce excess NO and PGE₂ leading to inflammation (4). Synthetic anti-inflammatory drugs can be classified as steroids (hydrocortisone, prednisolon, and betamethasone) and non-steroids (aspirin, indomethacin, and ibuprofen). Most of these can cause side effects such as stomach, kidney, and heart disorders (5,6). Therefore, it is necessary to develop safer and more effective anti-inflammatory drugs from natural substances.

Pinus koraiensis Siebold is found in areas with cold climates in Korea, Russia, China, and Japan (7). *P. koraiensis* contains terpenoids, phenolics, tannins, and alkaloids (8). Terpinolene and borneol stimulate bile secretion thereby reducing cholesterol levels (9). *P. koraiensis* leaves contain gallic acid, protocatechuic acid, vanillic acid, syringic acid, *p*-coumaric acid, scopoletin, and catechin, among other substances (10,11). In addition, *P. koraiensis* contains

Received 8 April 2016; Accepted 22 June 2016; Published online 30 September 2016

Correspondence to Young-Je Cho, Tel: +82-53-950-7755, E-mail: yjcho@knu.ac.kr

Copyright © 2016 by The Korean Society of Food Science and Nutrition. All rights Reserved.

© This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

substances with anti-bacterial (12), insecticidal (13), and allelopathic effects. These chemical substances are used as skin irritants, anti-inflammatory drugs, laxatives, and flavor-retaining agents (14,15). *P. koraiensis* seeds, which can be obtained from the pine cone, contain fatty acids, amino acids, carbohydrates, and vitamins (16,17). Extracts from the bark of the plant have anti-cancer, -aging, -mutagenic, and other effects, which increase the economic value of the plant (18-20). Annually, 2,680 tons of *P. koraiensis* are harvested in Korea. Only the seeds of *P. koraiensis* are consumed and other by-products are discarded as waste. Therefore, research into an efficient recycling method is needed (21-24). *P. koraiensis* by-products contain many bioactive materials, and if recycled, its pollutant emissions will be reduced. In addition, recycling of waste resources will increase the value of *P. koraiensis*.

Therefore, we extracted compounds from *P. koraiensis* cone scales, which are normally discarded as agricultural waste. We determined that *P. koraiensis* cone scale extracts exerted an anti-inflammatory effect through the inhibition of iNOS and COX-2 activity, indicating that they can be used in functional food development.

MATERIALS AND METHODS

Samples

P. koraiensis cone scales were collected from by-products of *P. koraiensis* cones from the Gwacheon, Korea. The sample was dried in a dry oven at 50°C and ground to pass through a 40 mesh. Then, the samples were separated into particles, vacuum-packed, and stored at 4°C until use.

Production of extracts

To produce a water conventional extract (CE), 100 mL of distilled water was added to 5 g of dried *P. koraiensis* cone bark powder and boiled until the volume of the mixture was reduced to 50 mL. After cooling, the sample was extracted. To produce an ethanol CE, 50 mL of 50% ethanol was added to the sample and extracted for 24 h at room temperature. The extract was filtered through Whatman No. 1 filter paper prior to use. An open vessel type extractor (Mucrodigest unit, Prolabo, Paris, France) at 2,450 MHz was used for microwave-assisted extract (MAE). The extractor had a reflux condenser, programmable power (maximum 200 W), and a timer. To prepare the samples, 50 mL of water and 50% ethanol were added to 5 mg of *P. koraiensis* cone bark powder. Water and 50% ethanol extracts were subjected to 200 W microwave power for 6 and 4 min, respectively. Extracts were filtered through Whatman No. 1 filter paper prior to use.

Determination of phenolic compounds

The total phenolic content of each extract was determined using the Folin-Denis method (25). Briefly, 0.5 mL of 1 N Folin-Ciocalteu reagent was added to 1 mL of extract mixed with 5 mL of distilled water and 1 mL of 95% ethanol. Then, 1 mL of 5% Na₂CO₃ solution was added after 5 min. The optical density (OD) at 725 nm was determined within 1 h using a UV-visible spectrophotometer (OPTIZEN 3220UV, Mecasys Co., Ltd., Daejeon, Korea). The phenolic content was determined using a standard curve of gallic acid.

Determination of anti-inflammatory activity

To determine anti-inflammatory activity, hyaluronidase inhibition was measured using the Reissig method (26). The ability of hyaluronidase to produce glucozaxoline from *N*-acetyl-glucosamine formed from sodium-hyaluronic acid (HA) was determined by a colorimetric reaction with dimethylamine borane (DMAB). Hyaluronidase was dissolved in 0.1 M acetate buffer pH 3.5 to produce a 7,900 unit/mL solution. Then, 0.05 mL of hyaluronidase was added to 0.1 mL of sample solution and incubated for 20 min at 37°C, followed by the addition of 0.1 mL of 12.5 mM CaCl₂ and incubation for 20 min. HA (12 mg/mL in 0.1 M acetate buffer, pH 3.5) was added as a substrate and incubated for 40 min. Then, 0.1 mL of 0.4 N potassium tetraborate and 0.1 mL of 0.4 N NaOH solution were added, followed by a 3-min incubation at 37°C and complete cooling. The color reaction was developed for 20 min at 37°C after addition of 3 mL of DMAB. Inhibition of hyaluronidase activity was determined using the following formula after measuring OD₆₀₀. The inhibition rate (%) was expressed as (1 - absorbance of control/absorbance of sample) × 100.

Determination of cell toxicity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

RAW 264.7 cells, a mouse macrophage cell line, were purchased from the Korean Cell Line bank (Seoul, Korea). Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum albumin and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO₂, unless specified otherwise. Cell toxicity was determined as described previously (27). RAW 264.7 cells were seeded in a 96-well plate at a density of 5 × 10⁴ cells/mL. Then, 0.02 mL of extract at different concentrations were added, and the cells were incubated at 37°C for 24 h. The MTT solution (5 mg/mL, 0.02 mL/well) was added, and the culture medium was collected after 4 h. Culture media from each well were incubated with 0.15 mL of dimethyl sulfoxide at room temperature for 30 min and OD₅₄₀ was measured. Cell toxicity was determined by comparing the absorbance of treated and untreated cells using the following formula.

Control cells were treated with distilled water. Cell viability (%) was expressed as $(1 - \text{absorbance of control} / \text{absorbance of sample}) \times 100$.

Determination of NO levels

The NO level was determined by measuring the nitrite amount in the cell culture medium using the Griess reagent (Sigma, St. Louis, MO, USA). RAW 264.7 cells (2×10^6 cells/mL) were washed 2 times with phosphate-buffered saline (PBS) and cultured in serum-free medium for 12 h. Then, cells were stimulated with 1 μg LPS for 2 h followed by the addition of different concentrations of extracts. The control was stimulated with 1 μg LPS for 2 h without extracts. The supernatants were collected after 24 h and NO levels were determined by measuring OD₅₄₀ 10 min after the addition of the Griess reagent. Data are expressed relative to NO production in LPS-treated cells, which was set to 100%. NO (%) was expressed as $(1 - \text{absorbance of control} / \text{absorbance of sample}) \times 100$.

Determination of iNOS and COX-2 protein expression using the Western blot analysis

To determine iNOS protein expression, RAW 264.7 cells were cultured for 24 h in a 100- π tissue culture dish at 2×10^4 cells/mL. Then, cells were treated with different concentrations of extracts for 24 h, the medium was removed, and the cells were washed 2 times with PBS. One hundred microliters of cell suspension was added to the radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (complete Mini 1 tablet added to 10 mL RIPA buffer) followed by centrifugation at 12,000 rpm for 20 min at 4°C. The supernatant was collected, transferred to a new tube, and stored at -20°C for further use or protein determination using the Bradford assay. Proteins (20 μL) were separated on 10% sodium dodecyl sulfate polyacrylamide gels. Separated proteins were transferred to a polyvinylidene difluoride membrane using a semi-dry transfer cell apparatus (Bio-Rad Laboratories, Hercules, CA, USA) and incubated for 1 h with blocking buffer [5% skim milk in Tris-buffered saline containing Tween-20 (TBST)] at room temperature. The membrane was washed with TBST 3 times every 10 min and incubated overnight with primary antibodies against iNOS (1:1,000; BD Biosciences, San Jose, CA, USA), COX-2 (1:1,000; Cayman, Ann Arbor, MI, USA), and glyceraldehyde 3-phosphate dehydrogenase (1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4°C. The membrane was washed with TBST 3 times every 10 min and incubated for 2 h at room temperature with horseradish peroxidase (HRP)-conjugated mouse anti-rabbit IgG (1:1,000; Santa Cruz Biotechnology) and HRP-conjugated bovine anti-goat IgG (1:1,000; Santa Cruz Biotechnology). After washing 3 more times, mem-

branes were incubated with enhanced chemiluminescence reagent (Millipore, Bedford, MA, USA) in a dark-room and exposed to X-ray film. The intensity of each band was determined by using a Molecular Imager (Bio-Rad Laboratories) (28).

Determination of PGE₂ levels

RAW 264.7 cells were cultured in 12-well culture plates at a density of 5×10^4 cells/mL for 24 h. After 18~24 h, the cells were treated with 1 μg LPS and the supernatants were collected and stored at -70°C. PGE₂ levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA). Two hundred microliters of calibrator diluent RD5-56 was added to the non-specific binding (NSB) well, 150 μL to the zero standard (B₀) well, and 150 μL to other cells. Then, 50 μL of primary antibody solution was added to all wells, except for the NSB well, and the plate was covered and incubated for 1 h at room temperature with shaking. Fifty microliters of PGE₂ conjugate was added to all wells, followed by 2 h of incubation with shaking and 4 washes with washing buffer. Two hundred microliters of substrate solution was added after washing, and the plate was incubated at room temperature for 30 min in the dark. Then, 100 μL of the stop solution was added and OD₄₅₀ was measured.

Determination of TNF- α levels

RAW 264.7 cells were cultured for 18~24 h in 12-well plates at 5×10^4 cells/mL. Then, the cells were treated with 1 μg LPS and the supernatants were collected and stored at -70°C. TNF- α levels were determined using an ELISA kit (R&D Systems). Fifty microliters of each sample or standard were mixed with 50 μL of biotinylated antibody reagent, and the plate was covered and incubated for 2 h at room temperature. After 5 washes with washing buffer, 100 μL of HRP-streptavidin was added, followed by a 30 min incubation at room temperature and 5 washes with washing buffer. Then, 100 μL of 3,3',5,5'-tetramethylbenzidine substrate solution was added to each well, the plate was incubated for 30-min in the dark, and OD₄₅₀ was measured.

Determination of IL-6 and IL-1 β levels

RAW 264.7 cells were cultured for 18~24 h in 12-well plates at a density of 5×10^4 cells/mL, and the supernatants were collected and stored at -70°C. IL-6 and IL-1 β levels were determined using an ELISA kit (R&D Systems). Fifty microliters of assay diluent RD1-14 was added to a 96-well plate, followed by addition of 50 μL of standards, controls, or samples. The plate was covered and incubated for 2 h at room temperature with shaking and then washed 5 times with washing buffer. After washing, 100 μL of IL-6 or IL-1 β conjugate was added,

followed by a 2 h incubation with shaking. After washing 5 times with washing buffer, 100 μ L of substrate solution was added, and the plate was incubated for 30 min in the dark. Then, 100 μ L of stop solution was added to terminate the reaction and OD₄₅₀ was measured.

Statistical analysis

All tests were repeated 3 times and statistical analyses were performed using the SAS program (SAS Institute, Cary, NC, USA) (29). Duncan's multiple range tests were used to analyze the differences. Differences of $P < 0.05$ were considered to be significantly different.

RESULTS AND DISCUSSION

Comparison of the extraction rate for phenolic compounds with CE and MAE

To compare MAE, which has the advantage of short extraction time, and CE, we determined the total phenolic content of *P. koraiensis* cone bark water and 50% ethanol extracts. The extraction yields are shown in Table 1. The phenolic content in water and ethanol extracts of MAE were higher than that of CE. The elution rate of a microwave extract is high because when whole samples are exposed to microwave energy some targeted substances are partially heated and are therefore isolated as solvents (30).

Comparison of the anti-inflammatory activity of extracts prepared by CE and MAE

Hyaluronidase hydrolyzes the $\beta(1 \rightarrow 4)$ bond of glucuronic acid and *N*-acetyl-glucosamine in HA (33). HA or low molecular weight HA increases inflammation and fibrotic collagen deposition during wound healing, while inhibiting the phagocytic ability of macrophages. Inhibition of hyaluronidase leading to accumulation of the high molecular weight form of HA can be expected to have an

Table 1. Total phenolic contents and hyaluronidase inhibitory activity of extracts from *Pinus koraiensis* cone bark depending on different conditions of extraction

	Extraction type	Extraction solvent	
		Water	Ethanol
Total phenolic contents (mg/g)	CE	4.37 \pm 2.06 ^a	5.67 \pm 0.03 ^a
	MAE	5.51 \pm 0.14 ^b	9.84 \pm 0.05 ^b
Hyaluronidase inhibition (%)	CE	13.55 \pm 1.41 ^a	26.96 \pm 2.66 ^a
	MAE	34.20 \pm 1.20 ^b	42.06 \pm 0.03 ^b

Mean \pm standard deviation (n=6).

Means with different letters (a,b) within column are significantly different at $P < 0.05$ by Duncan's multiple range tests. CE, conventional extraction; MAE, microwave-assisted extraction.

anti-inflammatory effect (31). *P. koraiensis* cone bark water and 50% ethanol extracts prepared by MAE inhibited hyaluronidase activity by 34.20% and 42.06%, respectively, which was significantly higher than the inhibitory activity of extracts prepared by CE (Table 1). Therefore, MAE extracts were used for the experiments in this study (32).

Effect of *P. koraiensis* cone bark extracts prepared by MAE on RAW 264.7 cell viability

The cytotoxicity of *P. koraiensis* cone bark water and 50% ethanol extracts in RAW 264.7 was determined using the MTT assay. As shown in Fig. 1, when the cells were treated with *P. koraiensis* cone bark water and 50% ethanol extracts at different concentrations (5, 10, 25, or 50 μ g/mL), the cell viability decreased significantly as the concentration increased. Cell viability decreased to 12.48% compared to the controls after treatment with 50 μ g/mL of *P. koraiensis* cone bark 50% ethanol extract. Treatment with 25 μ g/mL of *P. koraiensis* cone bark water and 50% ethanol extracts significant decreased cell survival when compared with the control. Therefore, nitric oxide and cytokine levels were determined, and Western blot analyses were performed after treatment with 25 μ g/mL

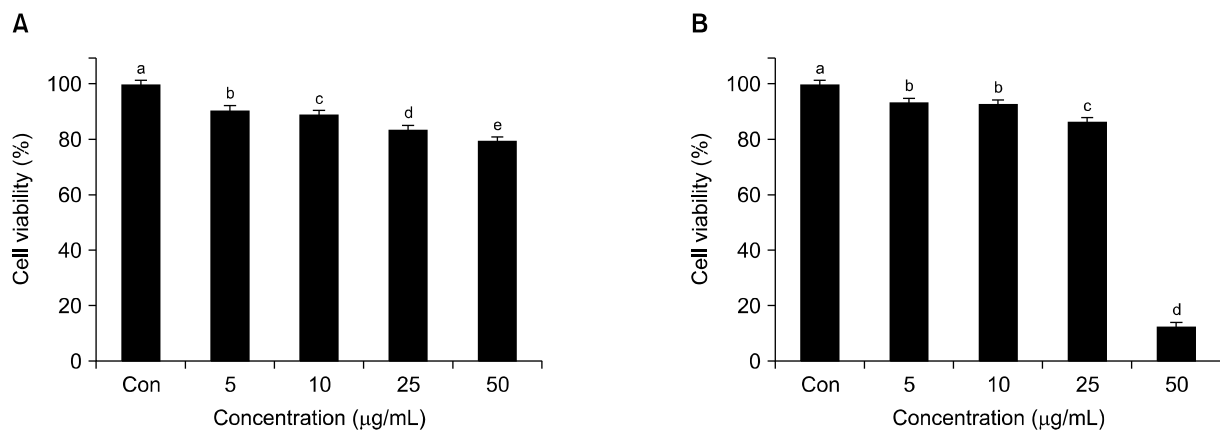


Fig. 1. Effect of *Pinus koraiensis* cone bark water extract (A) and ethanol extract (B) on RAW 264.7 cell viability. Different letters (a-e) above the bars indicate statistically different ($P < 0.05$).

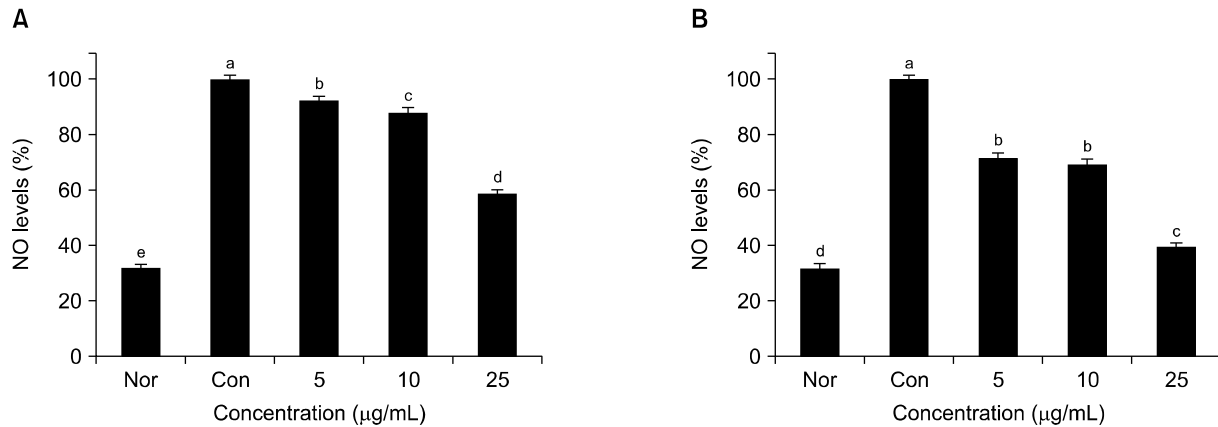


Fig. 2. Inhibition of nitric oxide (NO) production by *Pinus koraiensis* cone bark water extract (A) and ethanol extract (B) on RAW 264.7 cells. Different letters (a-e) above the bars indicate statistically different ($P < 0.05$).

of *P. koraiensis* extracts (32).

Inhibition of NO production

NO, which is produced from L-arginine by NOS, is involved in immune reactions, cell toxicity, neural transmission, and vasorelaxation. NO can exert its biological activity or induce cytotoxicity depending on its concentration (33). The results of NO production changes in RAW 264.7 cell treated with LPS and *P. koraiensis* cone bark water and 50% ethanol extracts at different concentrations (5, 10, and 25 µg/mL) are shown in Fig. 2. As the concentration of *P. koraiensis* cone bark water and 50% ethanol extracts increased, NO production rate decreased significantly. Treatment with 25 µg/mL of 50% ethanol extract decreased NO production by over 60% compared to the control of LPS-treated cells. These results indicate that the 50% ethanol extract was more effective at inhibiting NO production than the water extract. Kim et al. (34) reported that NO production was inhibited by up to 50% after treatment with 300 µg/mL of *Allium hookeri* root methanol extracts compared to the LPS-treated group. Therefore, *P. koraiensis* cone bark extracts at low concentrations can be used to reduce inflammation by inhibiting NO production (32).

Inhibition of iNOS and COX-2 protein expression

iNOS is not constitutively expressed; however, upon induction, it produces NO, which can cause inflammation through vasorelaxation, cell toxicity, and organ damage (35). To determine the effect of *P. koraiensis* cone bark water and 50% ethanol extracts on iNOS expression, we performed the Western blot analysis. RAW 264.7 cells activated with LPS were treated with 5, 10, or 25 µg/mL of *P. koraiensis* cone bark water and 50% ethanol extracts. As shown in Fig. 3A and 3B, treatment with 25 µg/mL of extract significantly decreased iNOS expression compared to the control of LPS-treated cells, which was associated with decreased NO production. *P. kor-*

aiensis cone bark water and 50% ethanol extracts inhibited iNOS expression by 50% relative to β-actin expression, which was used as a loading control (32). Kim et al. (36) reported that treatment with 20 µg/mL of *Paulownia coreana* leaf extract inhibited iNOS protein expression, thereby reducing inflammation. These results were in accordance with our study.

The COX family includes COX-1 and COX-2. COX-1, which is ubiquitously expressed, is involved in platelet formation, controlling blood flow in the kidney, and protecting the cell lining in the stomach. COX-2 is induced during inflammation, producing inflammatory mediators, such as PGE₂, which cause pain and fever (37). Therefore, we determined the effect of *P. koraiensis* cone bark water or 50% ethanol extract on COX-2 protein expression using Western blot analysis. As shown in Fig. 3C and 3D, treatment with *P. koraiensis* extracts inhibited COX-2 protein expression in a concentration-dependent manner. Treatment with 25 µg/mL of *P. koraiensis* cone bark water and 50% ethanol extracts inhibited COX-2 expression by 50% relative to β-actin expression. *P. koraiensis* cone bark extract reduced PGE₂ production by inhibiting COX-2 protein expression thereby exerting an anti-inflammatory effect. Therefore, the extract might be used to develop immunity enhancement therapies (32).

Inhibition of PGE₂ production

PGE is synthesized by COX from arachidonic acid. PGE₂ is a pro-inflammatory factor, which can cause erythema, edema, and pain (38). RAW 264.7 cells were treated with 5, 10, or 25 µg/mL of *P. koraiensis* cone bark water and 50% ethanol extracts to determine the effect on PGE₂ production (Fig. 4A and 4B). Treatment with the extracts inhibited PGE₂ production in a concentration-dependent manner as compared to LPS-treated cells. *P. koraiensis* cone bark water and 50% ethanol extracts at 25 µg/mL inhibited PGE₂ production by 30% and 50%, respectively. Noh et al. (39) reported that PGE₂ production was

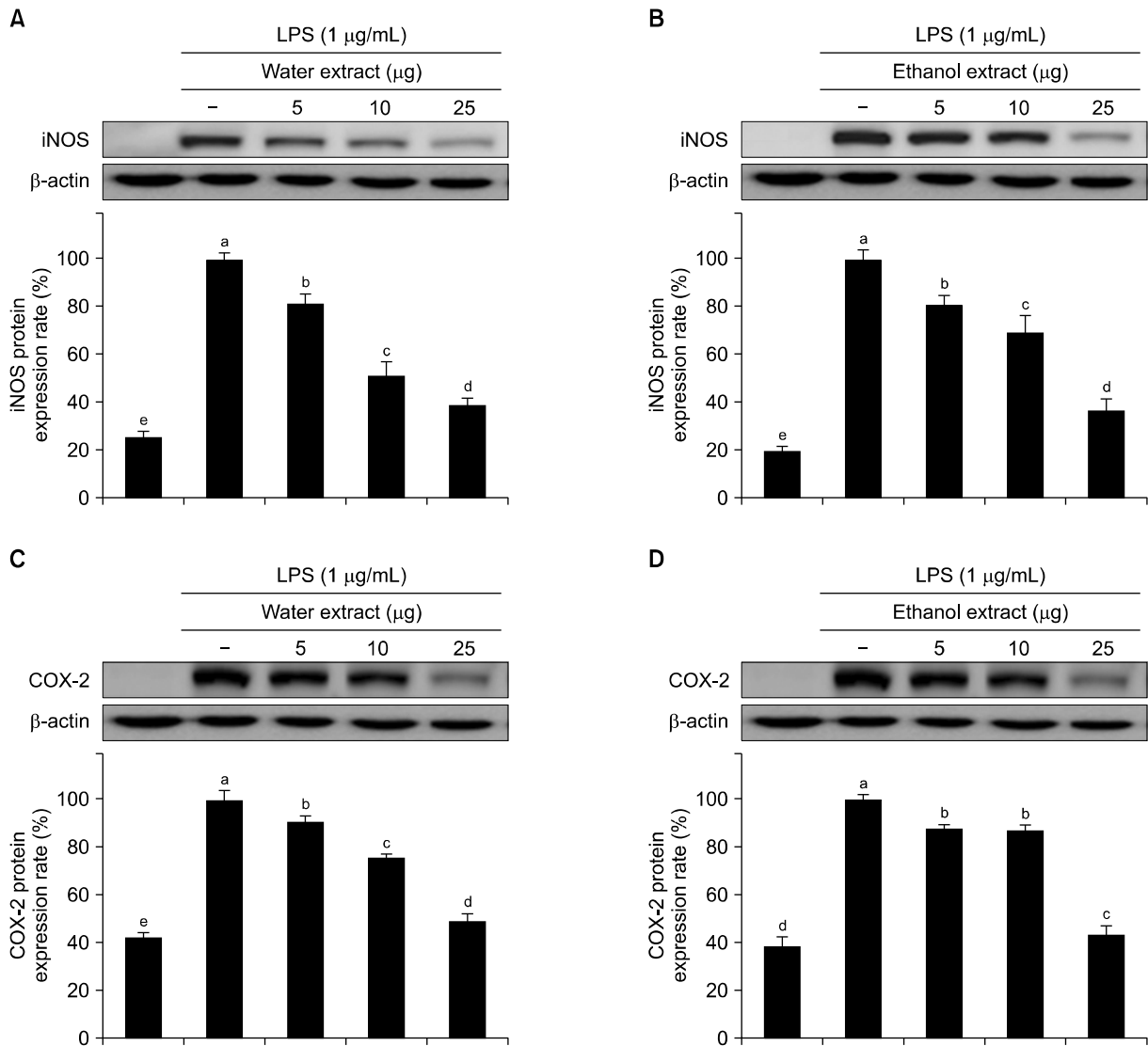


Fig. 3. Effect of *Pinus koraiensis* cone bark extracts on inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 protein expression in RAW 264.7. RAW 264.7 cells cultured in serum-free medium for 1 h were treated with 5, 10, or 25 μg/mL of *P. koraiensis* cone bark extracts for 24 h. iNOS expression was determined using the Western blot analysis. The histograms show the results of densitometric analysis of iNOS protein expression normalized to β-actin. *Pinus koraiensis* cone bark water extract (A) and ethanol extract (B) on iNOS, and water extract (C) and ethanol extract (D) on COX-2. Different letters (a-e) above the bars indicate statistically different ($P < 0.05$).

significantly decreased after treatment with 50 μg/mL of a green tea seed ethyl acetate fraction compared to the LPS-treated group. We have shown that treatment with low concentrations of *P. koraiensis* cone bark extracts can inhibit inflammatory factors by reducing PGE₂ production (32).

Inhibition of cytokine (TNF-α, IL-6, and IL-1β) production

TNF-α is secreted by macrophages and mast cells, and it is the main factor secreted in response to LPS stimulation (40). Therefore, to study the effect of *P. koraiensis* cone bark water and 50% ethanol extracts on LPS-induced TNF-α secretion, RAW 264.7 cells were treated with different concentrations of extracts (5, 10, or 25 μg/mL). As shown in Fig. 4C and 4D, treatment with *P. koraiensis* extracts inhibited TNF-α secretion in a concen-

tration-dependent manner compared with LPS-treated control cells. *P. koraiensis* water and 50% ethanol extracts (25 μg/mL) inhibited TNF-α secretion by 40% and 50%, respectively. Cho (41) reported that treatment with 100 μg/mL of *Acer palmatum* Thunb., *Lindera obtusiloba*, and *Ulmus davidiana* var. Japonica (Rehder) Nakai extracts inhibited TNF-α secretion by over 40%. Compared to these results, the inhibitory effects of *P. koraiensis* cone bark extracts were greater, which could reinforce immunity (32).

To determine the effect of *P. koraiensis* cone bark water and 50% ethanol extracts on IL-6 production, LPS-stimulated RAW 264.7 cells were treated with 5, 10, or 25 μg/mL of the extracts. Treatment with *P. koraiensis* cone bark extracts inhibited IL-6 production in a concentration-dependent manner compared to LPS-treated control cells (Fig. 4E and 4F). The water and 50% ethanol ex-

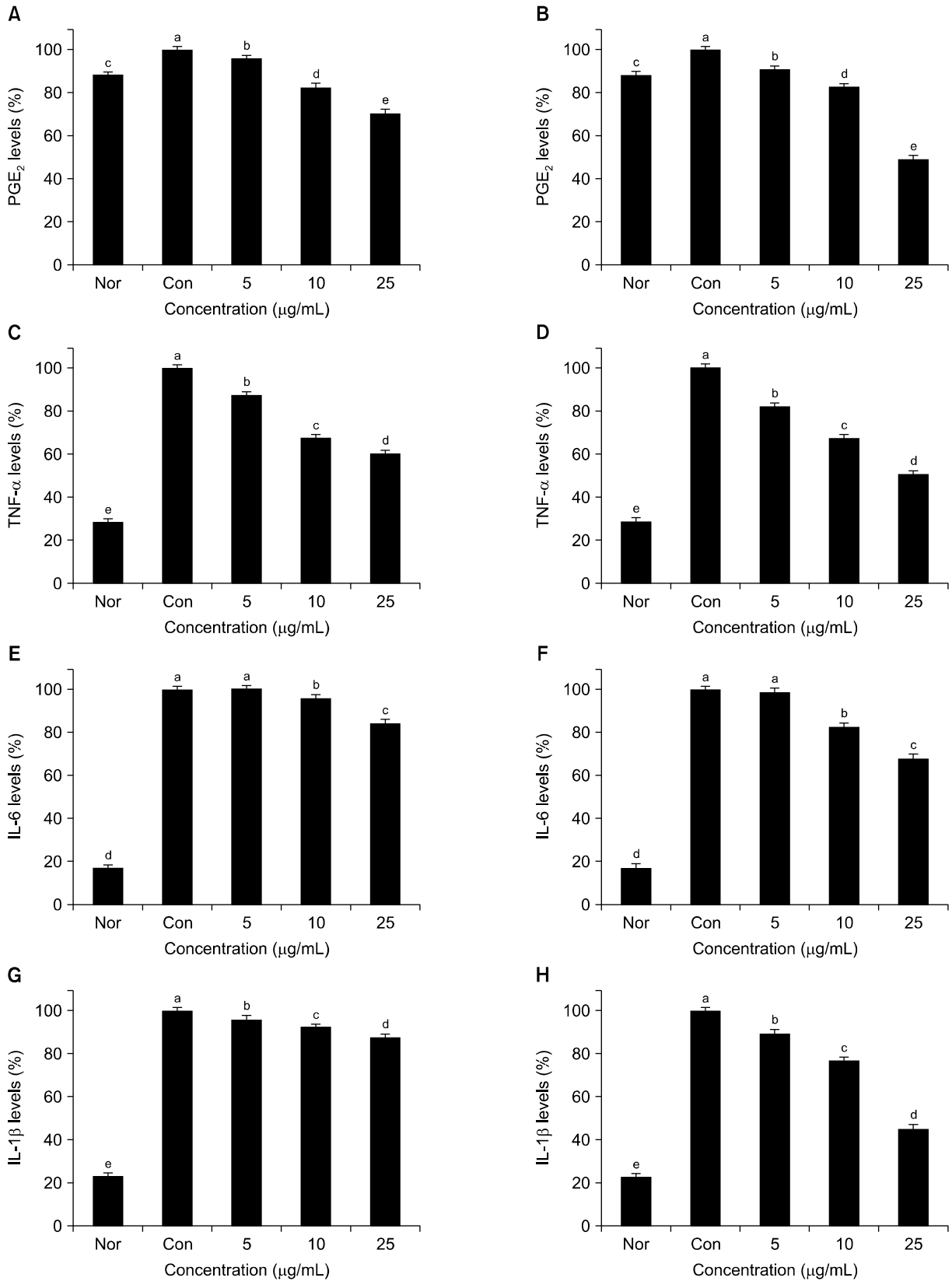


Fig. 4. Inhibition rate of extracts from *Pinus koraiensis* corn bark. RAW 264.7 cells were incubated with various concentration (5, 10, and 25 µg/mL) of *Pinus koraiensis* extracts for 1 h and then treated with 1 µg/mL of lipopolysaccharide for 24 h. (A) prostaglandin E₂ (PGE₂), (C) tumor necrosis factor (TNF)-α, (E) interleukin (IL)-6, and (G) IL-1β in water extracts, (B) PGE₂, (D) TNF-α, (F) IL-6, and (H) IL-1β in ethanol extracts. Different letters (a-e) above the bars indicate statistically different ($P < 0.05$).

tracts at 25 µg/mL inhibited IL-6 production by 20% and 30%, respectively (32). Compared to the 15% inhibition of IL-6 production by 25 µg/mL of *Acanthopanax henryi* Harms root bark extract (42), *P. koraiensis* cone bark extracts showed a greater anti-inflammatory effect. Therefore, *P. koraiensis* cone bark extracts can be used as anti-inflammatory agents.

IL-1β is a pro-inflammatory cytokine, which is secreted by monocytes, macrophages, B cells, dendritic cells, endothelial cells, neutrophils, and hepatocytes, and is involved in different immunological mechanisms along with TNF-α, IL-2, and IL-6. IL-1β promotes T cell activation, B cell maturation, and NK cell activity (43). To investigate whether *P. koraiensis* cone bark water and 50% ethanol extracts could inhibit IL-1β production, LPS-stimulated RAW 264.7 cells were treated with 5, 10, or 25 µg/mL of extracts. Compared to LPS-treated control cells, the *P. koraiensis* cone bark extracts inhibited IL-1β production in a concentration-dependent manner. Treatment with 25 µg/mL of water and 50% ethanol extracts inhibited IL-1β production by 10% and 50%, respectively (Fig. 4G and 4H) (32).

These results indicate that the inhibitory effect on PGE₂, TNF-α, IL-1β, and IL-6 production increased as the cells were treated with higher concentrations of *P. koraiensis* cone bark extracts. In addition, the 50% ethanol extract showed a higher inhibitory effect on cytokine production than the water extract.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

REFERENCES

- Hyun EA. 2004. Anti-inflammatory effect of *Salvia officinalis* L. extract. *MS Thesis*. Jeju National University, Jeju, Korea.
- Zamora R, Vodovotz Y, Billiar TR. 2000. Inducible nitric oxide synthase and inflammatory diseases. *Mol Med* 6: 347-373.
- Guha M, Mackman N. 2001. LPS induction of gene expression in human monocytes. *Cell Signal* 13: 85-94.
- Nishida T, Yabe Y, Fu HY, Hayashi Y, Asahi K, Eguchi H, Tsuji S, Tsujii M, Hayashi N, Kawano S. 2007. Geranylgeranylacetone induces cyclooxygenase-2 expression in cultured rat gastric epithelial cells through NF-κB. *Dig Dis Sci* 52: 1890-1896.
- Dagne JM, Hanson J, Supuran C, Pratico D. 2006. Coxibs and cardiovascular side-effects: from light to shadow. *Curr Pharm Des* 12: 917-975.
- Makins R, Ballinger A. 2003. Gastrointestinal side effects of drugs. *Expert Opin Drug Saf* 2: 421-429.
- Kong WS. 2004. Species composition and distribution of native Korean conifers. *J Korean Geographical Soc* 39: 528-543.
- Hong WT, Go GM, Lee JG, Jang HJ, Kwag JJ. 2002. Volatile compounds of pine needle (*Pinus rigida* Miller) extracts. *J Kor Soc Tob Sci* 24: 53-59.
- Kim YK, Chung KN, Ishi H, Muraki S. 1986. Volatile components of pinenut. *Korean J Food Sci Technol* 18: 105-109.
- Bae BH, Kim YO. 2003. Effect of leaf aqueous extracts from some gymnosperm plant on the seed germination, seedling growth and transplant of *Hibiscus syriacus* varieties. *Korean J Ecol* 26: 39-47.
- Cuyckens F, Claeys M. 2004. Mass spectrometry in the structural analysis of flavonoids. *J Mass Spectrom* 39: 1-15.
- Cimanga K, Kambu K, Tona L, Apers S, De Bruyne T, Hermans N, Totté J, Pieters L, Vlietinck AJ. 2002. Correlation between chemical composition and antibacterial activity of essential oils of some aromatic medicinal plants growing in the Democratic Republic of Congo. *J Ethnopharmacol* 79: 213-220.
- Baričević D, Milevoj L, Borštnik JB. 2001. Insecticidal effect of oregano (*Origanum vulgare* L. ssp. *hirtum* Letswaart) on bean weevil (*Acanthoscelides obtectus* Say). *Int J Hortic Sci Technol* 7: 84-88.
- Lim YS, Park KN, Bae MJ, Lee SH. 2001. Antimicrobial effects of *Pinus densiflora* Sieb. et Zucc. extract on pathogenic microorganisms. *Korean J Postharvest Sci Technol* 8: 462-468.
- Yang JK, Kang BK, Kim TH, Hong SC, Seo WT, Choi MS. 2002. Efficient extraction methods and analysis of essential oil from softwood leaves. *Korean J Biotechnol Bioeng* 17: 357-364.
- Yoon TH. 1987. Fatty acid composition of total lipids from seeds of *Pinus koraiensis*. *J Korean Soc Food Nutr* 16: 93-97.
- Han SS, Hwang BH. 1990. Analysis of amino acid, fatty acid, and vitamin in Korean pine (*Pinus koreaiensis*) seeds. *Jour Korean For Soc* 79: 345-351.
- Asset G, Staels B, Wolff RL, Baugé E, Madj Z, Fruchart JC, Dallongeville J. 1999. Effects of *Pinus pinaster* and *Pinus koraiensis* seed oil supplementation on lipoprotein metabolism in the rat. *Lipids* 34: 39-44.
- Bagchi D, Bagchi M, Stohs SJ, Das DK, Ray SD, Kuszynski CA, Joshi SS, Pruess HG. 2000. Free radicals and grape seed proanthocyanidin extract: importance in human health and disease prevention. *Toxicology* 148: 187-197.
- Li K, Li Q, Li J, Zhang T, Han Z, Gao D, Zheng F. 2007. Antitumor activity of the procyanidins from *Pinus koraiensis* bark on mice bearing U14 cervical cancer. *Yakugaku Zasshi* 127: 1145-1151.
- Lee HJ, Do JR, Kwon JH, Kim HK. 2011. Physiological properties of oak mistletoe (*Loranthus yadoriki*) extracts by microwave extraction condition. *Korean J Food Preserv* 18: 72-78.
- Lee EJ, Kim JS, Kim HK, Kwon JH. 2011. Prediction of the optimum conditions for microwave-assisted extraction of the total phenolic content and antioxidative and nitrite-scavenging abilities of grape seed. *Korean J Food Preserv* 18: 546-551.
- Lopez-Avila V, Young R, Beckert WF. 1994. Microwave-assisted extraction of organic compounds from standard reference soils and sediments. *Anal Chem* 66: 1097-1106.
- Jocelyn Paré JR, Bélanger JMR, Stafford SS. 1994. Microwave-assisted process (MAPTM): a new tool for the analytical laboratory. *Trends Anal Chem* 13: 176-184.
- Folin O, Denis W. 1912. On phosphotungstic-phosphomolybdic compounds as color reagents. *J Biol Chem* 12: 239-243.
- Reissig JL, Strominger JL, Leloir LF. 1995. A modified colorimetric method for the estimation of N-acetylamino sugars. *J Biol Chem* 270: 959-966.
- Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. 1987. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res* 47: 936-942.
- Cho YJ, An BJ. 2008. Anti-inflammatory effect of extracts from *Cheongmoknosang* (*Morus alba* L.) in lipopolysaccharide-stimulated RAW cells. *J Korean Soc Appl Biol Chem* 51: 44-48.

29. Statistical Analysis System Institute. 2001. SAS users guide. Version 8.1. Cary, NC, USA.
30. Kim HK, Kwon YJ, Kim KH, Jeong YH. 2000. Changes of total polyphenol content and electron donating ability of *Aster glehni* extracts with different microwave-assisted extraction conditions. *Korean J Food Sci Technol* 32: 1022-1028.
31. Park GH, Lee JY, Kim DH, Cho YJ, An BJ. 2011. Anti-oxidant and antiinflammatory effects of *Rosa multiflora* root. *J Life Sci* 21: 1120-1126.
32. Kang SA. 2013. Anti-inflammatory effect of *Pinus koraiensis* Zieb et Eucc cone peel extracts. *MS Thesis*. Kyungpook National University, Daegu, Korea.
33. Kim JY, Jung KS, Jeong HG. 2004. Suppressive effects of the kahweol and cafestol on cyclooxygenase-2 expression in macrophages. *FEBS Lett* 569: 321-326.
34. Kim CH, Lee MA, Kim TW, Jang JY, Kim HJ. 2012. Anti-inflammatory effect of *Allium hookeri* root methanol extract in LPS-induced RAW264.7 cells. *J Korean Soc Food Sci Nutr* 41: 1645-1648.
35. Ryu JH, Ahn H, Kim JY, Kim YK. 2003. Inhibitory activity of plant extracts on nitric oxide synthesis in LPS-activated macrophages. *Phytother Res* 17: 485-489.
36. Kim NK, Kim MH, Yoon CS, Choi SW. 2006. Studies on the anti-inflammatory activity of *Paulownia coreana* Uyeki leaf extract. *J Soc Cosmet Scientists Korea* 32: 241-247.
37. Liao CH, Sang S, Liang YC, Ho CT, Lin JK. 2004. Suppression of inducible nitric oxide synthase and cyclooxygenase-2 in downregulating nuclear factor-kappa B pathway by garcinol. *Mol Carcinog* 41: 140-149.
38. Harris SG, Padilla J, Koumas L, Ray D, Phipps RP. 2002. Prostaglandins as modulators of immunity. *Trends Immunol* 23: 144-150.
39. Noh KH, Jang JH, Min KH, Chinzorig R, Lee MO, Song YS. 2011. Suppressive effect of green tea seed coat ethyl acetate fraction on inflammation and its mechanism in RAW264.7 macrophage cell. *J Korean Soc Food Sci Nutr* 40: 625-634.
40. Lee AK, Sung SH, Kim YC, Kim SG. 2003. Inhibition of lipopolysaccharide-inducible nitric oxide synthase, TNF- α and COX-2 expression by sauchinone effects on I- κ B α phosphorylation, C/EBP and AP-1 activation. *Br J Pharmacol* 139: 11-20.
41. Cho JY. 2007. Inhibitory effect of extracts from woody plants on tumor necrosis factor- α production in lipopolysaccharide-stimulated RAW264.7 cells. *Korean J Medicinal Crop Sci* 15: 271-275.
42. Kim JH. 2010. The pharmacognostical studies on the root barks of *Acanthopanax henryi* Hamrs. *MS Thesis*. Kyung Hee University, Seoul, Korea.
43. Byun BH. 2005. Effects of *Inonotus obliquus* ethanol extract on cytokine expression in RAW 264.7 cell. *Kor J Herbology* 20: 55-60.