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

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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 cytoxicity. 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 E_2, tumor necrosis factor-α, interleukin (IL)-6, and IL-1β, 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 E_2 (PGE_2), tumor necrosis factor (TNF)-α, and interleukin (IL)-1β (3). In macrophages, stimulation with cytokines, such as TNF-α, or lipopolysaccharide (LPS) activates nuclear factor-κB, 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 PGE_2 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

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 $50^{\circ}C$ and ground to pass through a 40 mesh. Then, the samples were separated into particles, vacuum-packed, and stored at $4^{\circ}C$ 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% Na_2CO_3 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 glucoxazoline 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 $37^{\circ}C$, followed by the addition of 0.1 mL of 12.5 mM $CaCl_2$ 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 $37^{\circ}C$ and complete cooling. The color reaction was developed for 20 min at $37^{\circ}C$ after addition of 3 mL of DMAB. Inhibition of hyaluronidase activity was determined using the following formula after measuring OD_{600}. The inhibition rate (%) was expressed as $(1-\text{absorbance of control/absorbance of sample}) \times 100$.

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 $37^{\circ}C$ in a humidified atmosphere containing 5% CO_2, 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 5×10^4 cells/mL. Then, 0.02 mL of extract at different concentrations were added, and the cells were incubated at $37^{\circ}C$ 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 OD_{540} 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 OD_{540} 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, membranes were incubated with enhanced chemiluminescence reagent (Millipore, Bedford, MA, USA) in a darkroom and exposed to X-ray film. The intensity of each band was determined by using a Molecular Imager (Bio-Rad Laboratories) (28).

Determination of PGE$_2$ 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 $18 \sim 24$ h, the cells were treated with 1 μg LPS and the supernatants were collected and stored at $-70°C$. PGE$_2$ levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Mineneapolis, 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 (B$_0$) 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 PGE$_2$ 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 OD_{450} was measured.

Determination of TNF-α levels

RAW 264.7 cells were cultured for $18 \sim 24$ 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'-tetramethylmenzidine substrate solution was added to each well, the plate was incubated for 30-min in the dark, and OD_{450} was measured.

Determination of IL-6 and IL-1β levels

RAW 264.7 cells were cultured for $18 \sim 24$ 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-1β 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-1β conjugate was added,

followed by a 2 h incubation with shaking. After washing 5 times with washing buffer, 100 μL of substrate solution was added, and the plate was incubated for 30 min in the dark. Then, 100 μL of stop solution was added to terminate the reaction and OD_{450} 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 anayze 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 β(1→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±2.06[a]	5.67±0.03[a]
	MAE	5.51±0.14[b]	9.84±0.05[b]
Hyaluronidase inhibition (%)	CE	13.55±1.41[a]	26.96±2.66[a]
	MAE	34.20±1.20[b]	42.06±0.03[b]

Mean±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 μg/ mL), the cell viability decreased significantly as the concentration increased. Cell viability decreased to 12.48 % compared to the controls after treatment with 50 μg/ mL of *P. koraiensis* cone bark 50% ethanol extract. Treatment with 25 μg/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 μg/mL
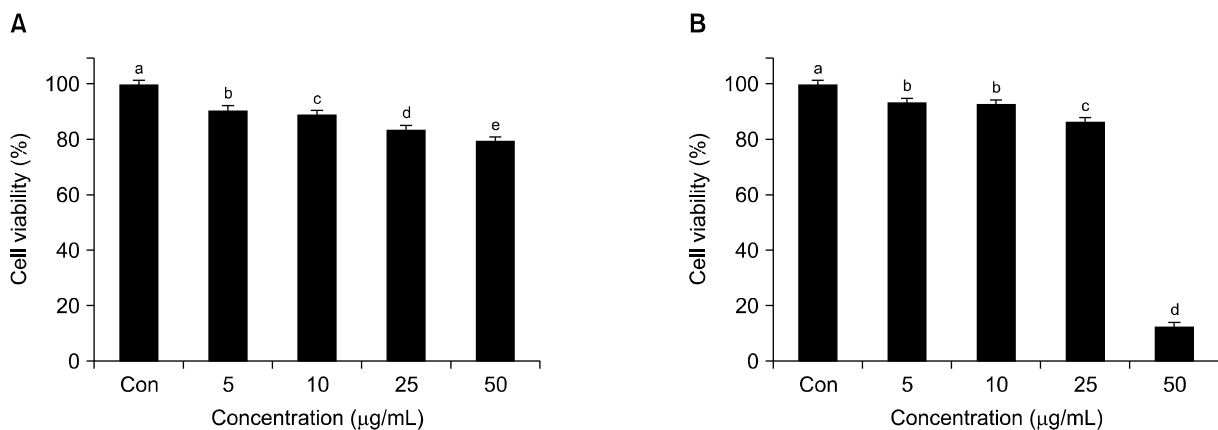
Fig. 1. Effect of *Pinus koraiensis* cone bark water extract (A) and ethanol extract (B) on RAW 264.7 cell viability. Different letters (a-e) above the bars indicate statistically different ($P<0.05$).
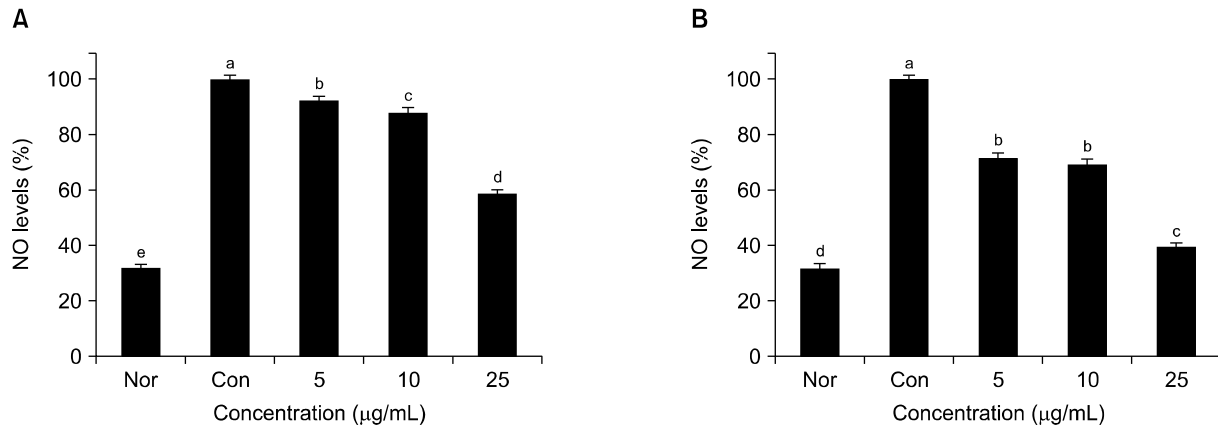
A

B

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).

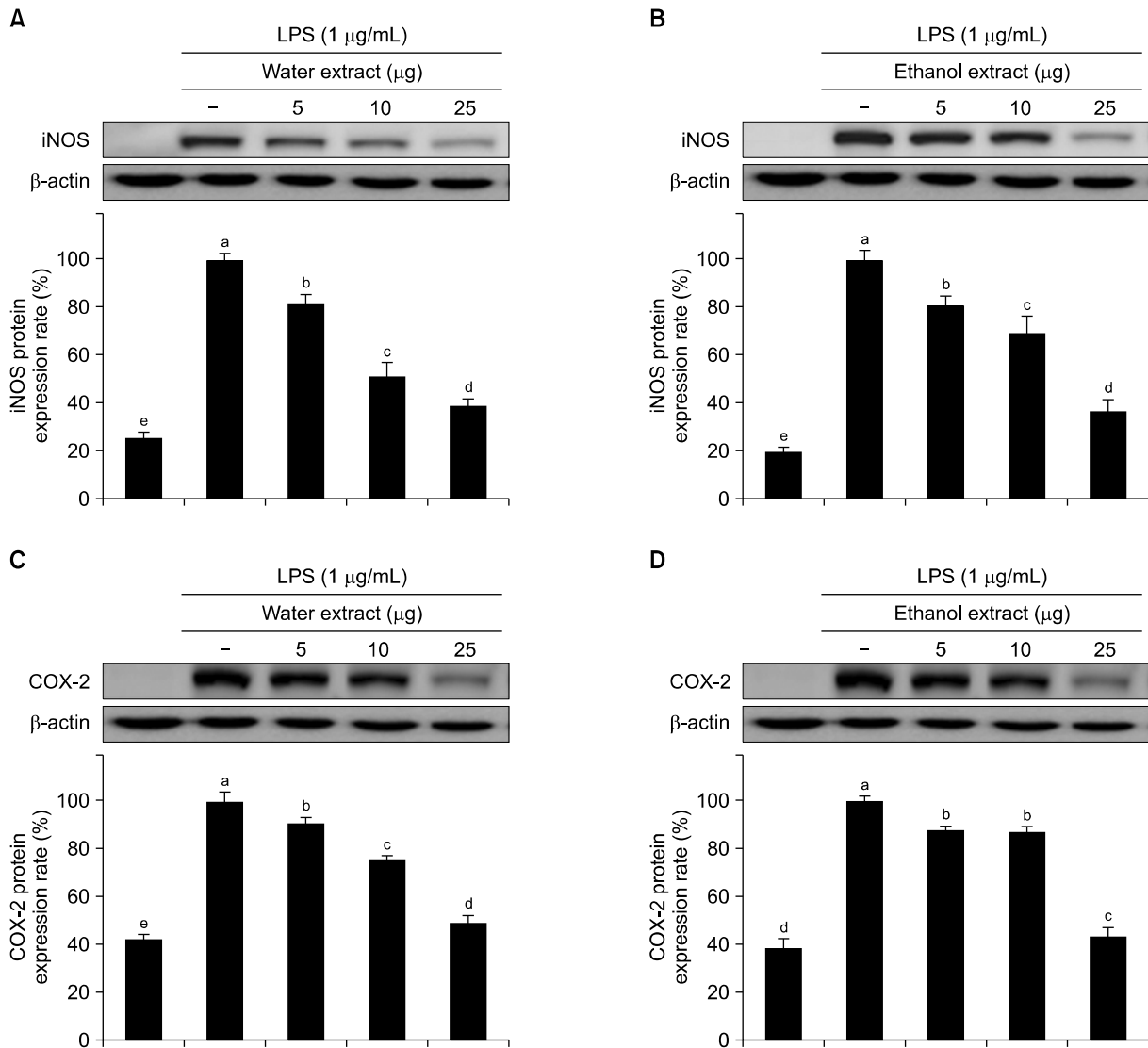### Inhibition of iNOS and COX-2 protein expression

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 PGE$_2$, 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 PGE$_2$ 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 PGE$_2$ production

PGE is synthesized by COX from arachidonic acid. PGE$_2$ 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 PGE$_2$ production (Fig. 4A and 4B). Treatment with the extracts inhibited PGE$_2$ 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 PGE$_2$ production by 30% and 50%, respectively. Noh et al. (39) reported that PGE$_2$ 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 PGE_2 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-
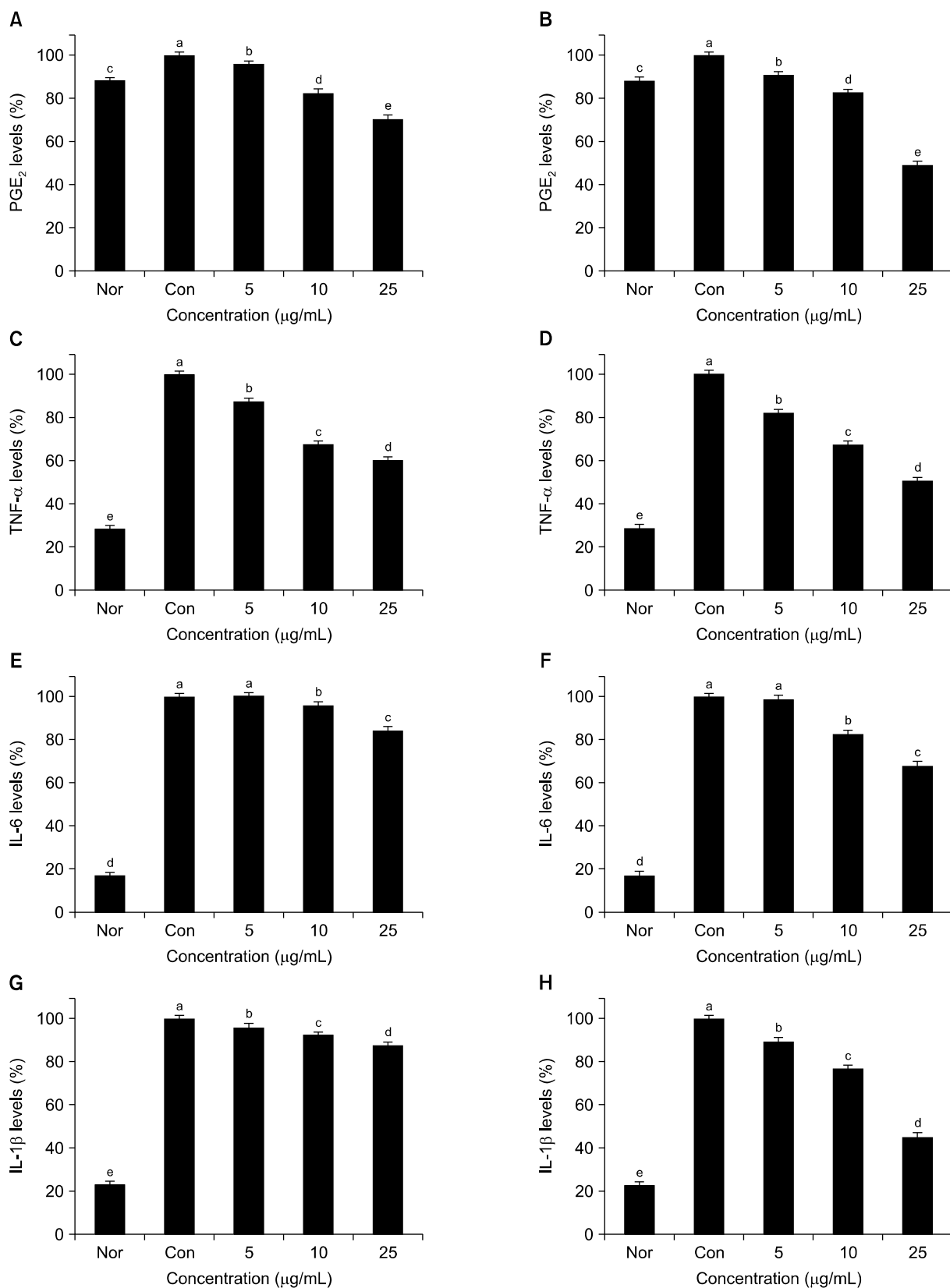
Fig. 4. Inhibition rate of extracts from *Pinus koraiensis* corn bark. RAW 264.7 cells were incubated with various concentration (5, 10, and 25 μg/mL) of *Pinus koraiensis* extracts for 1 h and then treated with 1 μg/mL of lipopolysaccharide for 24 h. (A) prostaglandin E_2 (PGE_2), (C) tumor necrosis factor (TNF)-α, (E) interleukin (IL)-6, and (G) IL-1β in water extracts, (B) PGE_2, (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 PGE$_2$, 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.

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