Butanol extracts of *Asparagus cochinchinensis* fermented with *Weissella cibaria* inhibit iNOS-mediated COX-2 induction pathway and inflammatory cytokines in LPS-stimulated RAW264.7 macrophage cells

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Abstract. Roots of *Asparagus cochinchinensis* have been widely used to treat fever, cough, kidney disease, breast cancer, inflammatory and brain disease, although the effects of their fermented products have not been assessed until now. To investigate the anti-inflammatory effects on macrophages of a butanol extract from *A. cochinchinensis* roots fermented with *Weissella cibaria* (BAW), alterations in the inducible nitric oxide synthase (iNOS)-mediated cyclooxygenase-2 (COX-2) induction pathway and inflammatory cytokine expression were measured in lipopolysaccharide (LPS)-activated RAW264.7 cells following pretreatment with BAW. Briefly, *W. cibaria* was selected from two bacterial strains for the fermentation of *A. cochinchinensis* roots based on its hyaluronidase inhibition and NO suppression rates. Following fermentation with *W. cibaria*, the level of various key components including total phenols and protodioscin were significantly enhanced in BAW. In addition, BAW exhibited high free radical scavenging activity (IC_{50}=31.62 μg/ml) and induced a decrease of intracellular ROS production in RAW264.7 cells following DCFH-DA staining. Significant suppressions in the expression level of important members of the iNOS-mediated COX-2 induction pathway and the phosphorylation of mitogen-activated protein kinase members were detected. The expressions of pro-inflammatory and anti-inflammatory cytokines were recovered in BAW pretreated RAW264.7 cells. Overall, these results suggest that BAW may suppress inflammatory responses through differential regulation of the iNOS-mediated COX-2 induction pathway and inflammatory cytokine expressions in LPS-activated RAW264.7 cells.

Introduction

Inflammation is a typical physiological response of an organism against various injurious insults including invading microorganisms, dust particles, altered self-cells, and transformed cancer cells (1). This response is usually classified into acute and chronic reactions according to the inflammatory process and cellular mechanisms (2). During an inflammatory response, a series of processes including dilation of venules and arterioles, enhanced blood vessel permeability, and blood flow with percolation of leukocytes into tissues have been detected in specific cells and tissues (3). Furthermore, inflammation could be associated with various chronic diseases including cancer, diabetes, obesity, autoimmune disease, inflammatory bowel disease, asthma, rheumatoid arthritis, and cardiovascular disease (2,4).

Meanwhile, several drug types including corticosteroids, NSAIDs, and biologics have been used to treat inflammatory diseases in humans, even though these drugs may have side effects and high cost. In particular, corticosteroids have long been used to treat rheumatoid arthritis, but they induce some serious side effects such as hypertension, Cushing's habitus, hyperglycemia, osteoporosis, and growth arrest (5). Therefore, natural products with anti-inflammatory activity have received a lot of attention as novel materials to overcome side effect problems (6). In particular, some natural products including *Actinidia argute* (7), *Ampelopsis grossedentata* (8), *Artemisia annua* Herba (9), *Cheilanthes albomarginata* (10) and *Grateloupia lanceolate* (11) have been shown to significantly reduce the expression of key inflammatory mediators.

Among these natural products, the anti-inflammatory activity of an *A. cochinchinensis* extract has been reported. The

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A. cochinchinesis extract significantly inhibited secretion of the pro-inflammatory cytokine, tumor necrosis factor (TNF)-α in LPS- and substance P-stimulated mouse astrocytes (12). In addition, aspacochinosides N, O, and P extracted from ethanol-treated *A. cochinchinesis* decreased NO concentration in LPS-stimulated BV-2 microglial cells. Moreover, an ethanol extract from *A. cochinchinesis* greatly decreased ectopic edema degree, ear thickness, cytokine secretion, and myelo-peroxidase activity, which are considered indicators of skin inflammation progression, in a skin inflammation-induced mouse model treated with 12-O-tetradecanoyl-phorbol-13-acetate (13). A crude aqueous extract of *A. cochinchinensis* effectively inhibits TNF-α-induced cytotoxicity (14), as well as increases the spleen index and SOD activity and decreases malondialdehyde in mice (15). A recent study reported inhibitory effects of *A. cochinchinensis* in allergic asthma-associated airway remodeling. The standardized herbal formula PM014, which includes the roots of *A. cochinchinensis*, efficiently inhibited the number of total cells, eosinophils, neutrophils, macrophages, and lymphocytes in the bronchoalveolar lavage fluid of cockroach allergen-induced mice (16). However, the underlying mechanism by which the fermented roots of *A. cochinchinensis* exert their anti-inflammatory effects in macrophages has not yet been clearly identified, even though the effects of fermented extracts were investigated in antigen-stimulated macrophages.

In this study, we investigated the fundamental mechanisms responsible for anti-inflammatory activities of BAW in LPS-induced RAW264.7 microphage cells. The results provide novel data indicating that BAW may be associated with suppression of various chronic inflammation-related diseases through the regulation of the iNOS-mediated COX-2 induction pathway and cytokine expression.

Materials and methods

Preparation of BAW and butanol extract from A. cochinchinensis roots fermented with Lactobacillus plantarum (BAL). The roots of *A. cochinchinensis* used in this study were collected from plantations in the Go-Chang area of Korea and dried in a drying machine (Ilshinbiobase, Dongducheon, Korea) at 60°C. Voucher specimens of *A. cochinchinensis* roots (WPC-14-003) were deposited in the functional materials bank of the PNU-Wellbeing RIS Center at Pusan National University. These samples were also identified by Dr. Shin Woo Cha at the Herbal Crop Research Division, National Institute of Horticultural & Herbal Science.

Firstly, to prepare aqueous fractions of unfermented *A. cochinchinensis* (UnF), 20 g of freeze-dried *A. cochinchinensis* roots were ground to a powder and a hot water extract prepared with 1.2 l of deionized distilled water (dH₂0) for 2.5 h in a hot water extractor (DW-290, Daewoong, Kyunggi, Korea). After finishing aqueous extraction, samples were filtered through filter paper (Whatman No. 2, Whatman, Brentford, UK) and then evaporated by using a rotary vacuum evaporator (EYELA, N-1100 series, Tokyo, Japan). Finally, UnF was used for fermentation after freeze-drying. Yield from the hot water extraction process was 60.7%.

The two bacterial strains (*W. cibaria* and *Lactobacillus plantarum*) used in the fermentation process were provided by Professor Hong Joo Son, Department of Life Science and Environmental Biochemistry, Pusan National University. To prepare fermented products, UnF powder was dissolved with 1% (w/v) in dH₂O (pH 5.3), and the mixture sterilized at 121°C for 15 min. After cooling at room temperature, *W. cibaria* or *L. plantarum* precultivated in lactobacilli MRS broth (Difco Laboratories, Detroit, MI) until the final cell density approached 10^7 CFU/ml (OD600=0.1) were inoculated [5% (v/v)] to the UnF mixture. The mixture was then incubated in a shaking incubator (VS-8480; Vision Scientific, Bucheon, Korea) at 37°C at 200 rpm/min for 4.3 d. Fermented *A. cochinchinensis* products of *W. cibaria* (FPW) or *L. plantarum* (FPL) were obtained from the mixtures solution fermented with *W. cibaria* or *L. plantarum* by using centrifugation at 12,000 x g for 10 min.

To obtain the n-butanol fractions of UnF (BUnF), FPW (BAW) and FPL (BAL), an equal volume of butanol was firstly added to UnF, FPW or FPL. After vigorous mixing and incubating, the butanol phase was collected from each mixture by centrifugation at 12,000 x g for 10 min. Butanol extraction was repeated three times, after which all butanol phases were combined, evaporated under a rotary vacuum evaporator, freeze-dried, and stored at -20°C until use.

Hyaluronidase inhibition assay. The inhibition activity of hyaluronidase against BUnF, BAW, and BAL was investigated by using a colorimetric assay based on the modified Morgan-Elson method (17-19) which measures the amount of *N*-acetyl-D-glucosamine produced from sodium hyaluronate. Briefly, 12 µl of BUnF, BAW, or BAL were mixed with 12 µl of hyaluronidase (10 mg/ml) in 0.1 M sodium acetate buffer (pH 3.5), then incubated in a water bath at 37°C. After pre-incubation for 20 min, the sample and hyaluronidase solution mixture was added to 12 µl of 12.5 mM calcium chloride to activate the hyaluronidase and then incubated for 20 min. To the activated hyaluronidase solution, 24 µl of sodium hyaluronate (6 mg/ml) dissolved in 0.1 M sodium acetate buffer (pH 3.5) was added and incubated for an additional 40 min. Next, the solution was amended with 12 µl of 0.4 N NaOH and 0.4 M potassium tetraborate, then vortexed, boiled for 3 min, and placed on ice to terminate the hyaluronidase activity. Subsequently, 360 µl of DMAB solution (*p*-dimethylamino-benzaldehyde 4 g in a solution of acetic acid 350 ml mixed with 50 ml of 10 N HCl) was added, and the sample placed in a 37°C water bath for 20 min. The absorbance of each test tube at 540 nm was measured by using a microplate reader (Tecan Sunrise, Tecan, Hombrechtikon, Switzerland). Hyaluronidase inhibition activity was estimated by determining differences in the absorbance values of the sample solutions.

Analysis of bioactive compounds in BUnF and BAW. The concentration of three bioactive compounds including total phenols and crude saponins in BUnF and BAW were measured as previously described. The amount of total phenols in BAW was determined according to the Folin-Ciocalteu method (20). Briefly, BUnF and BAW (20 µl) was mixed with 100 µl of 0.2 N Folin-Ciocalteu reagent for 5 min, after which 300 µl of 20% sodium carbonate was added. Following incubation at room temperature for 2 h, the absorbance of the reaction mixture was measured at 765 nm. A calibration curve was generated with Gallic acid as the standard. Total phenolic content was

expressed in milligrams of gallic acid equivalents per gram of BAW.

The total crude saponins amount was detected as described previously (21). Briefly, BUnF and BAW dissolved in 30 ml dH$_2$O was repeatedly extracted with ethyl ether (50 ml) to remove lipid soluble substances. After collection of the aqueous layer, samples were further extracted with n-butanol (30 ml) three times. This layer was then concentrated by vacuum evaporation and lyophilization by using circulation extraction equipment (IKA Labortechnik, Seoul, Korea). Finally, total crude saponins was calculated by using the equation: crude saponin (mg/g)=A-B/S, where, A is the dry weight of the n-butanol layer (mg), B is the weight of the flask (mg), and S is the weight of the solid volume of the sample (g).

High performance liquid chromatography (HPLC) analysis. Protodioscin, a saponin, was identified in BUnF and BAW by using an ILC 3000 HPLC system (Interface Engineering Co. Ltd., Seoul, Korea) equipped with a Corona® CAD® detector (ESA Biosciences, Inc., Chelmsford, MA, USA). Chromatographic separation was performed on a CapCell PAK MG C18 (4.6x250 mm, particle size 5 μm; Shiseido Co., Ltd., Tokyo, Japan). The mobile phase consisted of solvent A (dH$_2$O) and solvent B (acetonitrile) prepared by using a gradient elution program: 0-25 min with 30-90% solvent B and 25-40 min with 90% solvent B. A flow rate of 1.0 ml/min was used for sample analysis. The nebulizer gas was compressed nitrogen. Gas flow rate and gas pressure were maintained at 1.53 l/min and 35±2 psi, respectively. The output signal of the detector was recorded by using Clarity™ chromatography software (DataApex, Prague, Czech Republic).

Free radical scavenging activity. The scavenging activity of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical against BAW was measured as previously described (22). Briefly, each sample (100 μl) in eight different concentrations of BAW (7.8 to 1,000 μg/ml) was mixed with 100 μl of 0.1 mM DPPH (Sigma-Aldrich Co., St. Louis, MO, USA) in 95% ethanol solution or 100 μl of 95% ethanol solution, then incubated for 30 min at room temperature. Next, the absorbance of the reaction mixture was measured at 517 nm by using a Versa-max plate reader (Molecular Devices, Sunnyvale, CA, USA). The DPPH radical scavenging activity of the BAW was expressed as the percent decrease in absorbance relative to that in the control. The IC$_{50}$ value is defined as the concentration of substrate that produces a 50% loss in DPPH activity.

Cell viability assay. The RAW264.7 cell line used in this study consists of macrophage cells originating from the Abelson murine leukemia virus-induced tumor provided by the Korean Cell Line Bank (Seoul, Korea). The RAW264.7 cells were grown with Dulbecco's Modified Eagle's Medium (DMEM, Thermo Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS, S001-01, Welgene, Gyeongsan-si, Korea), L-glutamine, penicillin, and streptomycin (Thermo Scientific) in a humidified incubator at 37˚C under 5% CO$_2$ and 95% air. Cell viability was determined by using tetrazolium compound 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich Co.). To determine cell viability, RAW264.7 cells were seeded at a density of 5x10^4 cells/0.2 ml and grown for 24 h in a 37˚C incubator. When the cells attained 70-80% confluence, they were either untreated (No) or treated with DMSO (Vehicle), or pretreated with 100 μg/ml (BAWLo) or 200 μg/ml (BAWHi) of BAW dissolved in DMSO. Following incubation for 24 h, the supernatants were discarded, after which 0.2 ml of fresh MEM media and 50 μl of MTT solution [2 mg/ml in phosphate buffered saline (PBS)] were added to each well. The cells were then incubated at 37˚C for 4 h. Formazan precipitate was dissolved in DMSO, after which the absorbance at 570 nm was read directly in the wells by using a Molecular Devices VERSA max Plate reader (Sunnyvale, CA, USA). The morphological features of RAW264.7 cells in each treated group were observed by using a microscope (Leica Microsystems, Switzerland).

NO concentration analysis. NO concentration in the culture supernatant of RAW264.7 cells was measured by using Griess reagent [1% sulfanilamide, 5% phosphoric acid, 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride; Sigma-Aldrich Co.] as described previously (23). Briefly, RAW264.7 cells were treated with Vehicle or BAW (100 or 200 μg/ml) for 2 h followed by LPS (1 μg/ml) for 24 h. Following collection of the supernatant, each sample (100 μl) was mixed with the same volume of Griess reagent and incubated at room temperature for 10 min. The absorbance was read at 540 nm by using a VersaMax microplate reader (Molecular Devices).

Analysis of intracellular ROS level. Intracellular ROS levels in RAW264.7 cells were measured by staining with 2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich Co.), which is a cell permeable, nonfluorescent agent that can be deacetylated by intracellular esterases to form nonfluorescent DCFH. In the presence of ROS, DCFH is converted to highly fluorescent DCF intracellularly. Briefly, RAW364.7 cells were seeded at 5x10^5 cells/2 ml in 6-well plates, then grown with two different concentrations of BAW for 2 h in a 37˚C incubator. After washing once with 1x PBS, the cells were incubated with 1 μg/ml of LPS for another 24 h. Next, cells were incubated with 25 μM DCFH-DA for 30 min at 37˚C. Finally, the cells were washed twice with PBS, after which the green fluorescence was observed at 200x magnification via fluorescence microscopy (Eclipse TX100, Nikon, Tokyo, Japan).

Western blot analysis. Total homogenate of RAW264.7 cells obtained by using Pro-Prep Protein Extraction Solution (iNtRON Biotechnology, Seongnam, Korea) was centrifuged at 13,000 rpm/min for 5 min, then the concentration of total protein was quantified by using a SMARTTM BCA protein assay kit (Thermo Scientific) for western blotting. Briefly, the proteins were separated by 4-20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for 2 h, after which resolved proteins were transferred to nitrocellulose membranes for 2 h at 40 V. Each membrane was then incubated overnight separately at 4˚C with the following primary antibodies: SAPK/JNK antibody (Cell Signaling Technology, Danvers, MA, USA), p-SAPK/JNK (Thr183/Tyr185) antibody (Cell Signaling Technology), ERK1 (K-23) antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA), p-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (Cell Signaling Technology), p38 MAPK antibody (Cell Signaling Technology),

p-p38 MAP Kinase (Thr180/Tyr182) antibody (Cell Signaling Technology), and anti-actin antibody (Sigma-Aldrich Co.). Next, the membranes were washed with washing buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 0.05% Tween 20) and incubated with 1:1,000 diluted horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) at room temperature for 1 h. Finally, membrane blots were developed by using Amersham ECL Select western blotting detection reagent (GE Healthcare, Little Chalfont, UK). The chemiluminescence signals that originated from specific bands were detected by using FluorChemi®FC2 (Alpha Innotech, San Leandro, CA, USA).

Enzyme-linked immunosorbent assay (ELISA) for IL-6 cytokine. The concentration of cytokine IL-6 in the culture supernatant of RAW264.7 cells treated with BAW was determined by using an IL-6 ELISA kit (Biolegend, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, RAW264.7 cells were treated with two different concentrations of BAW (100 or 200 μg/ml) for 2 h, followed by 1 μg/ml of LPS for 24 h. After collection of the supernatant, 100 ml of serial dilutions of the culture supernatant were added to a 96-well plate coated with anti-IL-6 antibody and then incubated for 2 h at room temperature. After five washes with wash solution (PBS, 0.05% and Tween-20, pH 7.4), 100 μl of avidin-horseradish peroxidase solution was added to each well, and the plates were allowed to develop at 37°C for 2 h. After five washes with wash solution, the plate was maintained at 37°C for 30 min to react with 100 μl of substrate solution. The reaction was then stopped by the addition of 100 μl of blocking solution, after which the absorbance at 450 nm was read with a VersaMax microplate reader (Molecular Devices).

RT-PCR analysis for cytokine gene expression. The mRNA levels of iNOS, COX-2, TNF-α, IL-1β, and IL-6 were measured by RT-PCR as previously described (24). First, total RNA molecules were purified by removing media from a subset group of cells and then homogenizing the cells in RNAzol CS104 (Tel-Test Inc., Friendswood, USA). The isolated RNA was then quantified by using UV spectroscopy. Expressions of the target genes were assessed by using RT-PCR with 5 μg of total RNA from cells of each group. Next, 500 ng of oligo-dT primer (Invitrogen, Carlsbad, CA, USA) were annealed at 70°C for 10 min. The complementary DNA (cDNA), which was used as the template for further amplification, was synthesized by the addition of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and deoxythymidine triphosphate (dTTP) with 200 units of reverse transcriptase (Superscript II, Invitrogen, 200 U/μl). Next, 10 pmol of the sense and antisense primers were added, and the reaction mixture was subjected to 28-32 cycles of amplification. Amplification was conducted in a Perkin-Elmer Thermal Cycler and used the following cycle: 30 sec at 94°C, 30 sec at 62°C, and 45 sec at 72°C. The primer sequences for target gene expression identification were as follows: iNOS, sense primer: 5'-CACTTGGAGTTC ACCCAGT-3', anti-sense primer: 5'-ACCACTCGTACTTGG GATGC-3'; COX-2, sense primer: 5'-CAGGTCATTGGTGGA GAGGTGTATC-3', anti-sense primer: 5'-CCAGGAGGATGG

AGTTGTTGTAGAG-3'; TNF-α, sense primer: 5'-CCTGTA GCCCACGTCGTAGC-3', anti-sense primer: 5'-TTGACC TCAGCGCTGACTTG-3'; IL-1β, sense primer: 5'-GCACAT CAACAAGAGCTTCAGGCAG-3', anti-sense primer: 5'-GCT GCTTGTGAGGTGCTGATGTAC-3'; and IL-6, sense primer: 5'-TTGGGACTGATGTTGTTGACA-3', anti-sense primer: 5'-TCATCGCTGTTGATACAATCAGA-3'. The experiment was repeated three times, and all samples were analyzed in triplicate. The final RT-PCR products were separated on 1-2% agarose gel and then visualized by ethidium bromide staining. The densities of specific bands were quantified by using the Kodak Electrophoresis Documentation and Analysis System 120 (Eastman Kodak, Rochester, NY).

Statistical analysis. One-way ANOVA was used to identify significant differences between No-treated and LPS-treated groups (SPSS for Windows, Release 10.10, Standard Version, Chicago, IL, USA). Differences in the responses of the Vehicle+LPS-treated group and the BAW+LPS-treated groups were evaluated by using a post hoc test (SPSS for Windows, Release 10.10, Standard Version). All values are reported as the mean ± standard deviation (SD) and a P-value of <0.05 was considered significant.

Results

Selection of bacterial strain for optimal fermentation. To enhance the anti-inflammatory activity of *A. cochinchinensis* roots, fermentation technique was firstly applied to these roots using two bacterial strains (*W. cibaria* and *L. plantarum)*. Anti-inflammatory activity and toxicity were measured in the two final products (BAW and BAL) obtained from fermented *A. cochinchinensis* roots using butanol extraction. As shown Table I, a significance alternation was detected in hyaluronidase inhibition rate and suppression rate of NO concentration. Hyaluronidase inhibition rate was dramatically enhanced after fermentation although this level was slightly higher with 18-21% in BAW than BAL. A similar pattern to that observed for the hyaluronidase inhibition rate was also observed in the suppression rate of NO concentration. After fermentation, the suppression rate of NO concentration was increased regardless of their concentration. But, the increase of these level was greater with 57-64% in BAW than BAL. Meanwhile, any significance differences were not detected in cell viability of group treated with BUnF, BAW and BAL. Therefore, the present results suggest that the fermentation with *W. cibaria* can successfully change from the roots of *A. cochinchinensis* to the fermented products with high anti-inflammatory activity and non-toxicity. Also, above results showed that BAW can be considered as the better candidate for suppressing the inflammatory response.

Change of key components in A. cochinchinensis after fermentation with W. cibaria. To identify the key components altered by fermentation, the concentration of several key components were analyzed in BUnF and BAW. As shown in Fig. 1A, BUnF contained significantly different concentrations of total phenolic compounds (1.82 mg/g) and total crude saponins (150 mg/g). After fermentation, the total phenolic and crude saponin concentrations in BAW were significantly

Table I. Hyaluronidase inhibition rate, suppression rate of NO concentration and cell viability of butanol extracts of *A. cochinchinensis* fermented with *W. cibaria* and *L. platarum*.

Category	Type of *A. cochinchinensis*	Dose	BAW (*Weissella cibaria*)	BAL (*Lactobacillus plantarum*)
Hyaluronidase inhibition rate (%)	BUnF	Low	ND	
		High	4.14±0.3	
	BF	Low	26.95±3.5[a,b]	22.43±2.7[a]
		High	28.35±2.9[a,b]	23.96±3.4[a]
Suppression rate of NO concentration (%)	BUnF	Low	7.7±1.1	
		High	13.7±1.6	
	BF	Low	22.7±2.8[a,b]	14.2±1.6
		High	23.1±3.1[a,b]	14.3±1.8
Cell viability (%)	BUnF	Low	92±11	
		High	97±12	
	BF	Low	103±11	100±9
		High	98±8	98±10

BUnF, Butanol extracts of unfermented *A. cochinchinensis*; BF, Butanol extracts of fermented *A. cochinchinensis*; BAW, Butanol extracts of *A. cochinchinensis* fermented with *W. cibaria* for 4.3 days; BAL, Butanol extracts of *A. cochinchinensis* fermented with *L. plantarium* for 4.3 days; Low, Low concentration of extracts; High, High concentration of extracts; ND, Not detected. [a]P<0.05 compared to the No treated group. [b]P<0.05 compared to the BAL treated group.
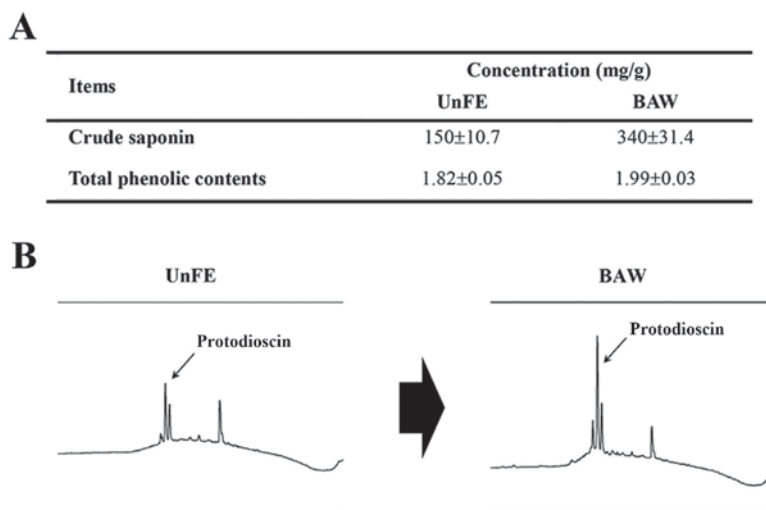

Figure 1. Change in components composition before and after fermentation. (A) Concentrations of two key components were measured under the conditions described in Materials and methods. (B) Chromatograms of protodioscin were obtained by performing high performance liquid chromatography of BAW. The peak height/area reflects the concentration of protodioscin in BAW.

increased with 9.3, 126.6%, respectively. Furthermore, the protodioscin concentration, detected as a sharp specific peak in the HPLC curve, was also higher in BAW (Fig. 1B). These results demonstrate that concentrations of several key components that associated with high anti-oxidative capacity can be increased in the extracts of roots of *A. cochinchinensis* after fermentation with *W. cibaria*.

Antioxidative capacity of BAW. To investigate the antioxidative capacity of BAW, alterations in DPPH scavenging activity and the inhibitory activity of intracellular ROS production were measured in RAW264.7 cells treated with BAW. The scavenging activity against the DPPH radical was gradually enhanced with the increase in BAW concentration (7.8 and 1,000 μg/ml), and the IC$_{50}$ value for BAW was 31.62 μg/ml (Fig. 2A). In addition, the intracellular level of ROS was notably enhanced after LPS treatment. However, the ROS level markedly decreased with depending on BAW concentration increase in LPS+BAWLo and LPS+BAWHi treated group (Fig. 2B). These results indicate that BAW may have a high anti-oxidative capacity that is obtained via suppression of intracellular ROS production and DPPH radical scavenging activity.
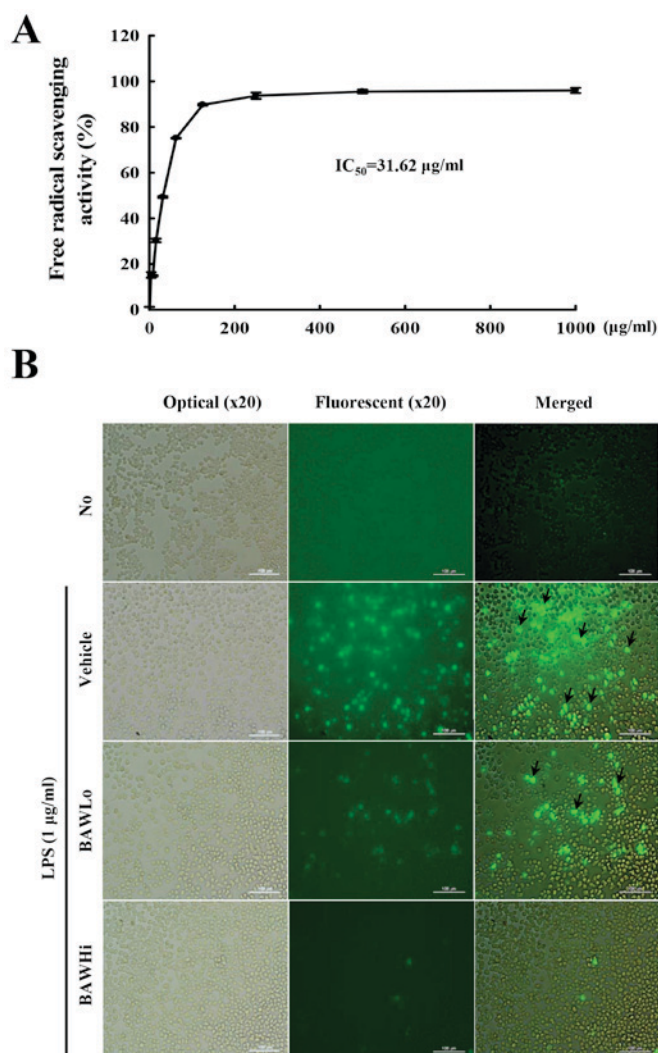
Figure 2. Antioxidant activity of BAW. (A) DPPH radical scavenging activity was assayed in a mixture containing different concentrations of BAW. IC_{50} reflects the substrate concentration required to produce a 50% reduction in DPPH activity. (B) Determination of intracellular ROS production. After DCFH-DA treatment, green fluorescence in subset groups of cells was observed at 200x magnification using a fluorescence microscope (Eclipse TX100, Nikon, Tokyo, Japan). Arrows indicate cells stained with DCFH-DA.
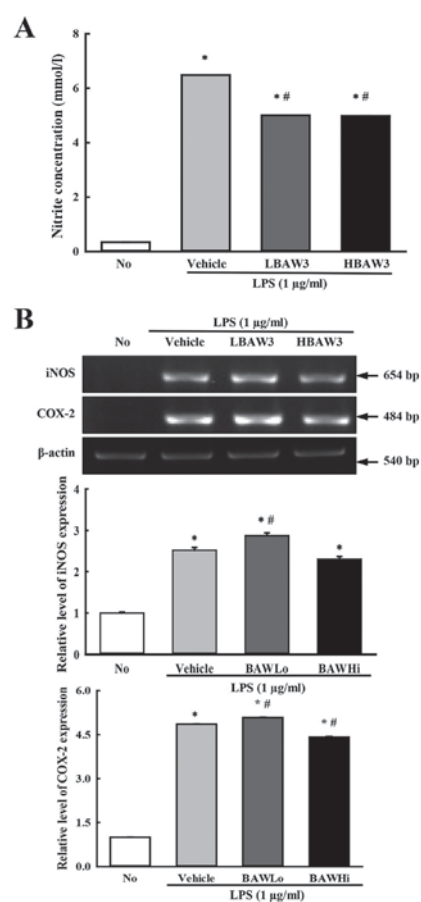

Figure 3. Determination of NO concentration as well as COX-2 and iNOS expressions. (A) NO concentration. The level of NO was determined by using a NO assay kit and supernatant collected from LPS-activated RAW264.7 cells treated with different concentrations of BAW. Triplicate trials per group were evaluated by NO assay. (B) RT-PCR analysis. Changes in the transcript levels of COX-2 and iNOS were examined by RT-PCR in the No-, Vehicle+LPS- and BAW+LPS-treated groups using specific primers. The data shown represent the means ± SD of three replicates. *P<0.05 relative to the No-treated group. #P<0.05 relative to the Vehicle+LPS-treated group.

Suppression effects on the iNOS-mediated COX-2 induction pathway. The signaling pathway for iNOS-mediated COX-2 induction has a key role during the inflammation process (25). Thus, we investigated the suppressive effects of BAW on the potential iNOS-mediated COX-2 induction pathway in LPS-activated Raw264.7 cells after BAW pretreatment. Firstly, we observed that the enhanced NO concentration after LPS treatment was dramatically decreased in the BAW-treated group (Fig. 3A). Also, the levels of the iNOS and COX-2 gene transcripts were higher in the Vehicle+LPS-treated group than that in the No-treated group. However, these levels were slightly decreased in BAWHi+LPS-treated Raw264.7 cells (Fig. 3B). These results suggest that BAW pretreatment can recover the signaling pathway for iNOS-mediated COX-2 induction during the inflammatory process.

To investigate whether alteration of iNOS and NO level accompanies a change in the MAP kinase pathway, the phosphorylation levels of ERK, JNK and p38 were measured in LPS-stimulated RAW 264.7 cells treated with BAW. As shown in Fig. 4, the phosphorylation levels of the three members were higher after LPS activation with various ratios than they were after No treatment. However, the levels of only ERK and JNK were significantly decreased after BAW pretreatment, while the level of p38 was continuously increased with depending on BAW concentration (Fig. 4). Therefore, the present data suggest that the suppression of iNOS and NO induced by BAW treatment may exert it regulatory activity by controlling the ERK and JNK in MAP kinase pathway.

Suppression of the expression of inflammatory cytokines. To investigate alteration of the expression of pro-inflammatory and anti-inflammatory cytokines during an anti-inflammatory response induced with BAW treatment, the transcript levels of TNF-α, IL-1β, and IL-6 were measured by performing RT-PCR of LPS-activated Raw264.7 cells. Among the three cytokines, IL-1β and IL-6 showed a suppression pattern in the BAW+LPS-treated group. In contrast, the TNF-α transcript maintained a constant level (Fig. 5A and B). Furthermore, the concentration of IL-6 in the culture supernatant of LPS-activated Raw264.7 cells was measured by ELISA in
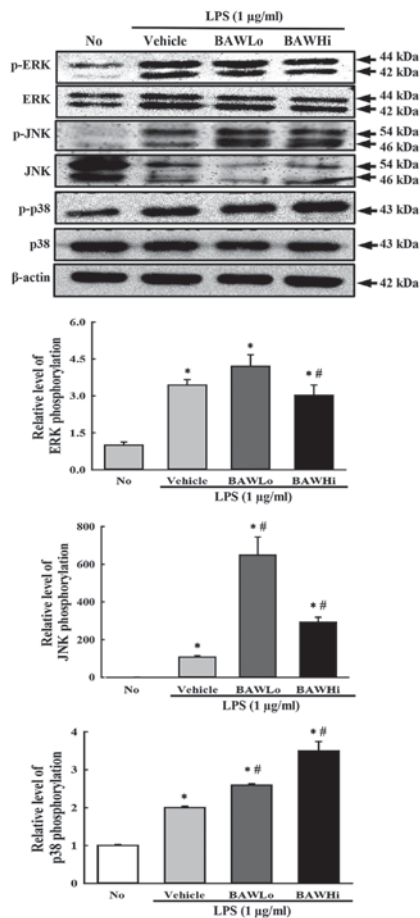
Figure 4. Expression of three members of the MAP kinase signaling pathway. Nitrocellulose membranes containing 30 μg of total protein from RAW264.7 cells were incubated with antibodies specific to p-ERK, ERK, p-JNK, JNK, p38, p-p38, and β-actin, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG. After the intensity of each band was determined by using an imaging densitometer, the relative levels of the six proteins were calculated based on the intensity of actin protein. The data represent the means ± SD of three replicates. *P<0.05 compared to the No-treated group. #P<0.05 compared to the Vehicle+LPS-treated group.
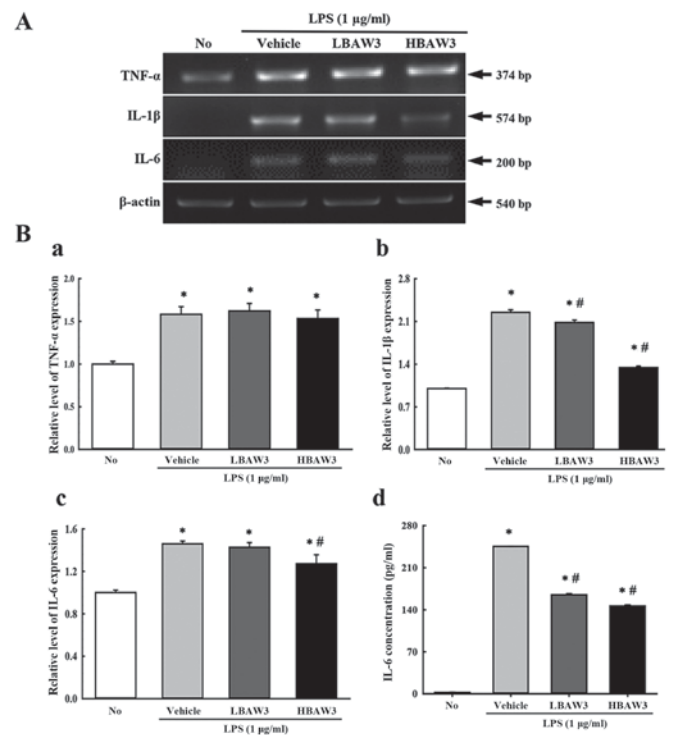

Figure 5. Measurement of pro-inflammatory and anti-inflammatory cytokines. The mRNA levels of the TNF-α (A and Ba), IL-1β (A and Bb), and IL-6 (A and Bc) genes were examined by RT-PCR in the No-, Vehicle+LPS-, BAWLo+LPS-, and BAWHi+LPS-treated group using specific primers. (Bd) IL-6 concentration in the culture supernatant. After collection of culture supernatant from RAW264.7 cells co-treated with BAW+LPS, IL-6 concentrations were measured by using a IL-6 ELISA kit that could detect IL-6 at 9.3 pg/ml. The data shown represent the means ± SD of three replicates. *P<0.05 relative to the No-treated group. #P<0.05 relative to the Vehicle+LPS-treated group.

order to confirm the RT-PCR results. The IL-6 concentrations in the culture supernatant completely reflected the results for the IL-6 transcripts, although a few differences were observed in the BAWLo+LPS-treated group (Fig. 5B). Therefore, these results indicate that BAW pretreatment suppresses the enhancement of pro-inflammatory cytokine expressions that were induced by LPS treatment in RAW264.7 cells.

Discussion

Fermentation techniques have been widely applied in the production of various high value substances because fermentation can provide economic and environmental advantages to individuals and industry (26). Moreover, many products produced by fermentation show therapeutic effects against several inflammatory diseases (27-29). In an effort to identify novel fermentation-derived candidates for the treatment of inflammatory disease, we investigated the anti-inflammatory effects of BAW in LPS-activated RAW264.7 cells. Our results demonstrated that BAW with its high anti-oxidative activity could significantly suppress the inflammatory response in

LPS-activated RAW 264.7 macrophages through regulation of iNOS-mediated Cox-2 induction pathway and inflammatory cytokines expression. Thus, BAW may be considered a potential anti-inflammatory drug for use in the treatment of various chronic inflammatory diseases.

The roots of *A. cochinchinesis* have long been considered a therapeutic drug for various disease because their anti-inflammatory, diuretic, antiseptic, antitussive, antibacterial, nervine, sialogoue, antipyretic, and stomachic effects. In addition, they are used in combination with other natural products to treat the lungs, spleen, immune system, and aging (15,30). Furthermore, the above functions of *A. cochinchinesis* can be associated with some functional compounds including β-sitosterol (31), daucosterol (32), n-ethatriacontanoic acid (33), palmitic acid (34), 9-heptacosylene (35), smilagenin (36), diosgenin (37), sarsasapogenin-3-O-β-D-glucoside feeding grapes imidacloprid (38), 5-methoxy methyl furfural, yame sapogenin, diosgenin-3-O-β-D imidacloprid feeding glucose glycosides (39,40), aspacochioside D (41), iso-agatharesinoside (42) and seven steroidal saponins (43). Concentration enhancement of the above compounds has been an important issue in several recent studies. However, a fermentation technique has not been applied previously to *A. cochinchinesis* roots as a method to increase the concentration of functional compounds and produce novel beneficial compounds. In this study, the roots of *A. cochinchinesis* were fermented with *W. cibaria* and *L. plantarum* for 4.3 days. After fermentation,

the hyaluronidase inhibition rate and NO suppression rate enhancement was greater in the fermentation product from *W. cibaria* than it was in that fermented with *L. plantarum*. Furthermore, the concentration of total phenols and crude saponins were significantly increased by 9.3 and 126.6% in BAW, and an increased protodioscin peak was detected. The results, therefore, indicate that the functional compounds in the roots of *A. cochinchinesis* may be successfully enhanced by fermenting the roots with *W. cibaria*.

Extracts from many fermented natural products can effectively suppress an LPS-stimulated inflammatory response. The production of pro-inflammatory mediators such as NO, iNOS, COX-2, TNF-α, and IL-6 is inhibited by the treatment of Oyaksungisan (OY) and Sipjeondaebotang (SJ) fermented by *Lactobacillus* (28,44), fermented *Rhizoma coptidis* (29), and three fermented herbs (Rhizome *Atractylodes macrocephalae*, Massa Medicata Fermentata, and Dolichoris Semen) (45) in LPS-stimulated RAW264.7 cells. Also, a fermented barley extract can effectively suppress oxidative stress in LPS-induced inflammation, while fermented red ginseng extract and fermented soybean extract can inhibit the production of NO in the same cells (30,46,47). Similar inhibitory effects on the production of pro-inflammatory mediators were observed in our BAW-based study, although the inhibition rate and solvent used for extraction were different. Furthermore, BAW pretreatment suppressed the level of NO, iNOS, COX-2, IL-6, IL-1β, and TNF-α in LPS-activated macrophage cells. However, additional research is needed to elucidate the key factors within BAW that control the regulation of pro-inflammatory mediators.

The iNOS and COX-2 proteins can be considered key factors when investigating the therapeutic effects of anti-inflammatory drugs because their expression can be induced by a variety of pro-inflammatory stimuli such as LPS and TNF-α in many disease conditions (48,49). Furthermore, NO production induced by iNOS expression is regulated via COX-2 expression through the MAPK signaling pathway, which has a critical role in the regulation of cell growth and differentiation, as well as in the control of cellular responses to cytokines and stresses (50,51). Some fermented natural products induce significant alteration of the iNOS-mediated COX-2 induction pathway in RAW264.7 cells. After the treatment of fermented OY extracts (27), fermented SJ extract (27), fermented soybean extract (47), and fermented RG extract (46), the levels of NO and COX-2 were significantly decreased in RAW264.7 cells. In the present study, our results for the iNOS-mediated COX-2 induction pathway obtained from BAW-treated RAW264.7 cells were very similar to those of previous studies, although some differences in the suppression of MAP kinases were observed. These differences might be due to factors such as the innate composition of, and the key molecules within, the herbal medicine.

Taken together, the results of the present study indicate that the antioxidant and anti-inflammatory activity of the roots of *A. cochinchinesis* can be enhanced by fermentation with *W. cibaria*. In addition, the results provide novel evidence that BAW may suppress inflammatory responses through the regulation of iNOS-mediated COX-2 induction pathway and inflammatory cytokine expression.

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