Deer Bone Oil Extract Suppresses Lipopolysaccharide-Induced Inflammatory Responses in RAW264.7 Cells

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The aim of this study was to investigate the effect of deer bone oil extract (DBOE) on lipopolysaccharide (LPS)-induced inflammatory responses in RAW264.7 cells. DBOE was fractionated by liquid–liquid extraction to obtain two fractions: methanol fraction (DBO-M) and hexane fraction (DBO-H). TLC showed that DBO-M had relatively more hydrophilic lipid complexes, including unsaturated fatty acids, than DBOE and DBO-H. The relative compositions of tetradecenoyl carnitine, α-linoleic acid, and palmitoleic acid increased in the DBO-M fraction by 61, 38, and 32%, respectively, compared with DBOE. The concentration of sugar moieties was 3-fold higher in the DBO-M fraction than DBOE and DBO-H. DBO-M significantly decreased LPS-induced nitric oxide (NO) production in RAW264.7 cells in a dose-dependent manner. This DBO-M-mediated decrease in NO production was due to downregulation of mRNA and protein levels of inducible nitric oxide synthase (iNOS). In addition, mRNA expression of pro-inflammatory mediators, such as cyclooxygenase-2 (COX-2), interleukin (IL)-1β, and IL-12β, was suppressed by DBO-M. Our data showed that DBO-M, which has relatively higher sugar content than DBOE and DBO-H, could play an important role in suppressing inflammatory responses by controlling pro-inflammatory cytokines and mediators.

Key words deer bone oil extract (DBOE); RAW264.7; inducible nitric oxide synthase (iNOS); nitric oxide (NO); anti-inflammatory response

Inflammation is a normal physiological process that heals injury and fights pathogens. Although early responses of the body against harmful stimuli are beneficial, prolonged or chronic inflammation can be detrimental to the body, destroying healthy cells in the absence of any foreign pathogen and causing a wide range of disease conditions.1,2 Chronic inflammation has been linked with diverse diseases, such as cancer, diabetes, obesity, and heart disease.2–5 Chronic inflammatory responses are characterized by excessive or prolonged production of pro-inflammatory cytokines and mediators, such as nitric oxide (NO), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and inflammatory interleukins. Uncontrolled release or production of pro-inflammatory cytokines has been found in various diseases, including cancer and arthritis.6 All stages of cancer development have been associated with chronic and excessive production of pro-inflammatory cytokines from immune cells, such as macrophage and neutrophils, which stimulate cell proliferation, reduce apoptosis, and enhance angiogenesis.6 Autoimmune diseases, such as rheumatoid arthritis, have also been implicated in the dysregulation of pro-inflammatory cytokines.7 Controlling unregulated cytokines has been a target for the prevention and alleviation of chronic inflammatory responses. Generally, nonsteroidal anti-inflammatory drugs (NSAIDs) have been used to suppress the action of inflammatory mediators.8 However, NSAIDs have side effects, including gastric bleeding, kidney damage, and exacerbation of asthma. Therefore, the potential use of natural anti-inflammatory agents without any side effects has emerged as an alternative solution.9,10

In East Asia, deer bone has been widely used to relieve the symptoms of various diseases.11 Water extracts of deer bone or antler have various biological effects, including anti-aging, anti-inflammatory, anti-oxidant, and anti-ammemic activities.11–14 However, studies on the biological effects of these deer bone-derived lipids have not yet been investigated. Components of deer bone water extracts include uronic acids and sialic acid as well as components related to protection of cartilage, such as glucosamine, calcium, chondroitin sulfate, and collagen hydrolysate.13,15 Deer antler is also rich in a large number of lipid components, including neutral lipids, glycolipids, ganglioside, and phospholipids.15–18 In particular, pantocrine, an alcoholic extract of the lipid fraction from deer antler, has been used as a supplementary tonic agent for the enhancement of testosterone and growth factors in the body.19 In this study, we analyzed the lipid components of deer bone oil extract (DBOE) and examined the effect of the methanol fraction of DBOE (DBO-M) on inflammatory responses in RAW264.7 cells. The DBO-M fraction reduced inflammatory responses induced by lipopolysaccharide (LPS) by suppressing pro-inflammatory cytokines and mediators. Our study suggests the potential use of deer bone oil as an anti-inflammatory agent.

MATERIALS AND METHODS

Reagents 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl methylene blue (DMMB), and phenol were purchased from Junsei (Tokyo, Japan), and sulfuric acid was from Showa (Tokyo, Japan). Solvents, including hexane, methanol, acetone, chloroform, and ether were purchased from Daejung Chemical & Materials (Gyonggi-do, Korea). Phosphatidyl choline, cholesterol, fatty acids mixture, and triglyceride were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, U.S.A.). Fetal bovine serum (FBS), penicillin, streptomycin, and Dulbecco’s modified Eagle’s medium (DMEM) with high glucose were purchased from Welgene

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(Fresh Media™, Daegu, Korea). iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies and secondary antibody were obtained from Cell Signaling Technology (Boston, MA, U.S.A.). Griess reagent and LPS (Escherichia coli, serotype 0111:04) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of the highest commercial grade available.

**Preparation and Analysis of Deer Bone Oil Extract**

Deer bone (*Cervus elaphus*) from adult male New Zealand elks (2 to 4 years old) was provided from Nongshim Co. (Seoul, Korea). The extraction procedure was performed as described by Lee et al. In brief, the deer bone was extracted two times with 6-fold distilled water (w/w) at 95–100°C for 4 h, cooked under pressure (120°C for 3 h, 15 psi), and then cooled. The lipid layer of the deer bone extract was obtained with an oil/water separator. The lipid extract was dehydrated by sodium sulfate and filtered with filter paper.

**Solvent Fractionation by Liquid–Liquid Extraction**

Deer bone oil was fractionated by liquid–liquid extraction to yield relatively hydrophilic and hydrophobic parts. Two immiscible solvents, hexane and methanol with a ratio of 2:1 (v/v), were used to separate each fraction. Deer bone oil was first dissolved in hexane to 10% (w/v) and transferred to a separating funnel. Then, the half volume of methanol was added to the solution, vigorously shaken, and stirred until the two separate layers appeared. Two solvent layers were separately collected, DBO-M and hexane fraction of DBO (DBO-H), and solvents were eliminated by high vacuum rotary evaporator. Two fractions were resuspended with the proper solvents for experimental use. The fractionation of DBOE is shown in Fig. 1.

**Lipid Analysis of Each Fraction by TLC**

Eight microliters of each sample (fractions) was dissolved in chloroform and applied on a 250 µm analytical grade silica gel 60F254 TLC plate (Merck, KGaA, Darmstadt, Germany). The samples were developed using the solvent system of hexane–diethyl ether–formic acid (40:10:1, v/v/v) for approximately 1 h. The spots were visualized by spraying the TLC plate with a 2,7-dichlorofluorescein solution (0.2% in 95% methanol). Separated lipids spots from samples were compared to those of standard lipids (phosphatidyl choline, cholesterol, fatty acids mixture, and triglyceride).

**Identification of Fatty Acid Composition by Gas Chromatography (GC)**

Fatty acid composition of whole lipid from deer bone oil and its two solvent fractions (DBO-M and DBO-H) were determined by GC. Samples were acid hydrolyzed and methylated with boron trifluoride (BF3) in methanol before GC analysis. The fatty acid methyl ester was subsequently analyzed by a Varian 3800 GC (Walnut Creek, CA, U.S.A.) equipped with a supelcowax 10 fused-silica capillary column (30 m × 0.32 mm i.d.; Supelco, Bellefonte, PA, U.S.A.) and flame-ionization detector. The column was held at 180°C for 1 min and then heated to 230°C for 34.33 min at a rate of 1.5°C/min. Helium was used as the carrier gas, with a total gas flow rate of 1.0 mL/min. The injector and detector temperatures were set at 240 and 250°C, respectively. The fatty acids were identified by comparison with the retention times of the standards. Heptadecanoic acid was used as an internal standard (IS).

**Identification of Total Sugar Content of Each Fraction**

Total sugar content in whole lipid and its solvent fractions of deer bone oil was analyzed by the phenol–sulfuric acid method with external calibration curve of glucose. The absorbance was measured at 490 nm by microplate reader.

**Cell Culture and Treatment in RAW264.7 Cells**

RAW264.7 cells were purchased from Korean Cell Line Bank (KCLB, Seoul, Korea). Cells were maintained with 10% FBS and 1% penicillin streptomycin in DMEM at 37°C in humidified chamber with a 5% CO2 atmosphere. Cells were subcultured before they reached confluence, approximately 70% confluence, by gently scraping the attached cells. Cells were incubated with various concentrations of samples and then stimulated with LPS at 1 µg/mL for the indicated times.

**Cell Viability Assay**

RAW264.7 cells were seeded on 96-well plates (2.5 × 104 cells/mL) and preincubated for 24 h. Then, they were treated with DBO-M at various concentrations, followed by treatment with or without LPS 1 h later. After 20 h of DBO-M treatment, the media were removed, and cells were treated with 1 mg/mL of MTT reagent. Four hours later, cells were treated with 20% sodium dodecyl sulfate (SDS) in acidic isopropanol alcohol. After overnight incubation, the absorbance at 540 nm was determined using an enzyme-linked immunosorbent assay (ELISA) plate reader (Molecular Devices, LLC, Sunnyvale, CA, U.S.A.).

**NO Assay**

Briefly, RAW264.7 cells were seeded on 96-well plates (2.5 × 104 cells/mL) and incubated for 24 h. Then, they were treated with various concentrations of DBO-M and then incubated with or without 1 µg/mL of LPS for 20 h. The level of nitrite, a stable NO product, in the culture media was determined using Griess reagent. One hundred microliters of media was mixed with same volume of Griess reagent for 10 min. Absorbance was measured at 540 nm using an ELISA reader. Nitrite concentration was determined by a calibration curve using sodium nitrite.
Quantitative Real-Time Polymerase Chain Reaction (qPCR) Total RNA was extracted from cell cultures using TRIzol LS reagent (Invitrogen Corporation, Carlsbad, CA, U.S.A.) based on the manufacturer’s instructions. The amount of RNA was calculated as the ratio of absorbance at 260 nm/280 nm, using a microplate reader with a NanoQuant™ plate (Tecan, Männedorf, Switzerland). cDNA was synthesized from 1 µg of isolated RNA using oligo-dT and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. Genomic DNA (gDNA) was eliminated by using gDNA purification kit from Promega (Madison, WI, U.S.A.). SYBR® Green Real-Time Master Mix (Applied Biosystem, Life Technologies™, Waltham, MA, U.S.A.) was used for qPCR. The primers of target genes were obtained from Bioneer Inc. and are listed in Table 1. Using StepOnePlus™ Real-Time PCR System (Applied Biosystem, Life Technologies™), relative values of individual genes to that of the endogenous control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, were calculated to determine the mRNA expression level of each gene. The values are expressed in 2^−ΔΔCt. The PCR cycling condition was 2 min at 50°C, 10 min at 95°C, and 50 cycles of 15 s at 95°C and 1 min at 57.5°C.

Western Blot Cells were lysed by lysis buffer (pH 7.4) including phenylmethylsulfonyl fluoride (PMSF), benzamidine, aprotinin, leupeptin, pepstatin, sodium orthovanadate, sodium fluoride, sodium pyrophosphate, and β-glycerophosphate. The cell lysate was centrifuged at 14000 rpm for 10 min at 4°C, and the supernatant was collected. The protein content was determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Rockford, IL, U.S.A.). Thirty micrograms of protein were resolved on 10–12% SDS polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked with 3% skim milk in Tris buffered saline (TBS)/Tween 20 buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20) for 2 h at 4°C. Membranes were then incubated overnight at 4°C with primary antibodies against iNOS or GAPDH, followed by incubation with horseradish peroxidase-conjugated secondary antibody in 3% skim milk in TBS buffer for 2 h at room temperature. The membranes were washed with TBS/Tween 20 buffer and treated with a chemiluminescence reagent (Pierce™ ECL Plus detection kit, Thermo Fisher Scientific) according to the manufacturer’s instructions. The bands were visualized by Gel Doc™ and analyzed by Image Lab™ software (Bio-Rad, Hercules, CA, U.S.A.). The relative protein expression of iNOS was expressed as the ratio to that of the endogenous control protein, GAPDH.

Statistical Analysis The results are presented as the mean±standard deviation (S.D.) or standard error (S.E.). Differences among the groups were analyzed using one-way ANOVA with Tukey’s multiple range tests. Statistic values of p<0.05 were considered significantly significant. All of the statistical analyses were performed using the Statistical Package for Social Sciences (SPSS, IBM® SPSS® Statistics 21, Armonk, NY, U.S.A.).

RESULTS

Solvent Fractionation by Liquid–Liquid Extraction DBOE has poor solubility in dimethyl sulfoxide (DMSO), a common solvent for dissolving hydrophobic materials in cell culture experiments. The low solubility of DBOE hampered investigation of the anti-inflammatory effects of DBOE in this study. Accordingly, DBOE was fractionated using solvents with differential hydrophobicity. We fractionated DBOE into two fractions by liquid–liquid extraction using methanol and hexane. DBO-M and DBO-H were obtained and used to determine cell viability and NO production in RAW264.7 cells. The yield of DBO-M was approximately 3–4%. Fractionation of DBO-M is shown in Fig. 1. DBO-M fraction was solubilized in DMSO, a vehicle solvent in this study, with a yellowish liquid state, unlike DBO-H fraction, which is beige solid state in room temperature (25°C) (Fig. 1S).

Effects of DBO-M on RAW264.7 Cell Viability The effect of DBO-M on cell viability was determined. DBO-M was nontoxic to the RAW264.7 cells at the indicated range of concentrations (0–800 µg/mL) (Fig. 2). Cell viability tended to increase at concentrations greater than 400 µg/mL. In addition, DBO-M showed no morphological alterations in the cells, while LPS (only)-treated cells were shown to be activated with morphological changes. However, such morphological difference in LPS-only-treated group was not observed in DBO-M-pretreated group (Fig. 2S). These results indicated that DBO-M was not cytotoxic at any given concentration, and suppressed the LPS-induced activation of macrophage.

Effects of DBO-M on NO Production in LPS-Activated RAW264.7 Cells We examined the effect of DBO-M on LPS-induced NO production, a mediator of the inflammatory response. DBO-M alone (without LPS) did not induce significant NO production up to 800 µg/mL, the concentration shown to be safe for cell viability. DBO-M inhibited LPS-induced NO production in RAW264.7 cells from concentra-

Table 1. The Sequences of the Target Gene Primer Pairs Used in qPCR

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Reference sequences</th>
<th>Primer sequence (5’→3’)</th>
<th>Product size (bp)</th>
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<tr>
<td>iNOS (Nos2)</td>
<td>NM_010927.3</td>
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<td></td>
<td></td>
<td>R: ACCACTCTGACTTGGGATGC</td>
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<td>COX-2 (Ptg2)</td>
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<td></td>
<td></td>
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<tr>
<td></td>
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<tr>
<td>IL-12β</td>
<td>NM_008352.2</td>
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<tr>
<td></td>
<td></td>
<td>R: ACACATTTGGGCGTAGGAAACA</td>
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F and R mean forward and reverse primer, respectively.
sections of 250 µg/mL and higher (Fig. 3). A high concentration of DBO-M (800 µg/mL) decreased NO production by approximately 60% (Fig. 3). Thus, DBO-M effectively suppressed LPS-induced NO production, suggesting an anti-inflammatory effect of DBO-M. The effect of DBO-H was also tested on LPS-induced NO production. Due to the poor solubility of DBO-H, a suspension of DBO-H in DMSO was used. DBO-H did not suppress NO production (Fig. 3S).

Lipid Analysis of Each Fraction by TLC To estimate lipid components of DBOE, fractions from DBOE were analyzed by TLC. Spots on TLC revealed that DBOE and DBO-H contained more triglyceride (TG) but less free fatty acid, cholesterol, and phospholipids than DBO-M. DBO-M had relatively more hydrophilic lipid complexes, which were located between free fatty acid and phospholipids, in comparison to DBOE and DBO-H (Fig. 4A). These results suggested that the relatively hydrophilic components in DBO-M afforded its increased solubility in DMSO.

Fatty Acid Composition of Each Fraction Fatty acid composition of each fraction was analyzed by GC (Table 2). DBO-M exhibited a higher compositional level of unsaturated fatty acid than DBOE and DBO-H, while the composition of saturated fatty acid, such as stearic acid, was significantly reduced in DBO-M. Compositions of tetradecenoyl carnitine, α-linoleic acid, and palmitoleic acid were increased 61, 38, and 32%, respectively, in DBO-M compared with DBOE. DBO-M was significantly richer in unsaturated fatty acid and short chain fatty acid contributed to the higher solubility and lower melting point of DBO-M.

Total Sugar Content of Each Fraction To investigate sugar levels of DBOE and its two fractions, total sugar content was measured by the phenol–sulfuric acid method. The concentration of sugar moieties in DBO-M was significantly higher than DBO-H (Fig. 4B). The DBO-M fraction contained...
3-fold higher levels of sugar than DBOE. These results suggest that the higher level of sugar content in DBO-M compared to DBOE could contribute to the enhanced solubility of DBO-M in DMSO and that DBO-M is composed of higher amounts of mixed lipids containing sugar, such as glycolipids, than DBOE.

**Inhibitory Effects of DBO-M on Gene Expressions of Interleukin (IL)-1β, IL-12β, and COX-2 in LPS-Activated RAW264.7 Cells**

To investigate the effects of DBO-M on regulation of several important pro-inflammatory cytokines in RAW264.7 cells, mRNA expression levels of IL-1β and IL-12β were analyzed with real-time PCR in DBO-M treated cells. The expression of IL-1β was greatly increased by LPS (Fig. 5A). The LPS-induced increase in IL-1β was significantly decreased by DBO-M treatment. However, the effect of DBO-M was not dose-dependent. High dose of DBO-M (800 µg/mL) reduced IL-1β mRNA expression by 65% compared with the control group (Fig. 5A). Transcriptional expression of IL-12β, another proinflammatory cytokine, was effectively reduced by DBO-M treatment. mRNA level of IL-12β was decreased by over 70% with DBO-M (800 µg/mL) treatment compared with control (Fig. 5B). DBO-M also inhibited LPS-induced expression of COX-2, an important enzyme responsible for inflammation and pain. DBO-M suppressed LPS-induced expression of COX-2 by over 60% (Fig. 5C). These results showed that DBO-M effectively suppresses LPS-induced expression of pro-inflammatory cytokines and mediators. Our data suggest that DBO-M can be used to control inflammatory responses.

**Inhibitory Effect of DBO-M on iNOS Expression in LPS-Activated RAW264.7 Cells**

DBO-M treatment effectively reduced LPS-induced increase in iNOS mRNA at all given concentrations, although the effect was not dose dependent (Fig. 6A). iNOS expression was reduced approximately 55% by DBO-M (800 µg/mL). This DBO-M-mediated downregulation of iNOS was also observed at the protein level. DBO-M treatment decreased iNOS protein levels in a dose-dependent manner. A high dose (800 µg/mL) of DBO-M caused a 90% reduction in iNOS protein expression (Figs. 6B, C). This result showed that DBO-M-mediated reduction of iNOS protein level was due to the downregulation of DBO-M mRNA expression. These data on the iNOS expression correlated with data on NO production, which was decreased by DBO-M treatment as well (Fig. 3). These results indicated that LPS-induced NO production was due to the upregulation of iNOS and that DBO-M-mediated downregulation of iNOS was responsible for the reduction of NO production. Therefore, our data shows that DBO-M is an effective suppressor of inflammatory responses caused by upregulation of inflammatory cytokines or mediators.

**DISCUSSION**

In this study, we investigated the anti-inflammatory effect of DBOE with fractional analysis. Numerous studies have
been performed on the pharmacological effects of deer bone and antler, 11–14) but most studies were executed using water extraction of deer bone, which produces a large amount of oil remnants that are not well studied. Here, we examined the biological effects of oil fractions from deer bone extraction and performed compositional analysis of the lipids. Deer bone oil is insoluble in DMSO because of its hydrophobicity, making it difficult to perform experiments. Therefore, liquid–liquid fractionation of the sample was performed, yielding a fraction (DBO-M) that was more hydrophilic and soluble in DMSO.

DBO-M was in liquid form at room temperature, while DBO-E needs heating process (50–60°C) to be liquid state. The content of unsaturated fatty acids and sugars in DBO-M was higher than the other fractions (DBO-H and DBO-E) (Table 2, Fig. 4), which contributed to it increased solubility and lower melting point.

Although deer bone-derived active substances for therapeutic effects have not been identified, several studies have reported the presence of complex lipids, such as glycolipids and phospholipids, as the active substances in deer antler. 16,17,21) TLC analysis in current study revealed that the DBO-M fraction possessed complex lipids containing a much more hydrophilic moiety than the DBO-H fraction, which had no anti-inflammatory effect (Fig. 4). Previously, another study using the same solvent system as ours showed the same positions of phospholipids and glycolipids in TLC analysis, suggesting that those complex lipids in our study likely include phospholipids and glycolipids. 22) Several studies have suggested a relationship between ganglioside, a biological molecule that is composed of glycosphingolipids and sialic acid, and the pharmacological effect of deer bone. 12,17,21) Specifically, gangliosides are regulators of local and systemic inflammation and have anti-inflammatory effects. 23,24) Therefore, DBOE-derived anti-inflammatory substances are considered a kind of glycolipid such as ganglioside, but further detailed analysis is needed to identify the active substances.

Our data also showed that DBO-M fraction significantly decreased the production of NO (Fig. 3), a mediator of inflammatory responses, in RAW264.7 cells. In contrast, DBO-H, a fraction with higher hydrophobicity than DBO-M, did not inhibit NO production (Fig. 3). These data indicated that molecules containing a relatively hydrophilic moiety of deer bone oil are inhibitory in inflammatory responses. These data were correlated with the higher sugar content in DBO-M (Fig. 4). Indeed, the DBO-H fraction was difficult to dissolve in DMSO, a vehicle solvent in cell treatment, and a suspension of DBO-H was applied to the cells for the NO assay. However, DBO-H in suspension becomes clear in the cell culture medium, suggesting DBO-H is dissolved in the medium when it is treated to the cells. DBO-M inhibited NO production, implying that DBO-M is a regulator of inflammatory responses. Normal levels of NO are involved in vasodilation, but excessive production of NO in abnormal conditions leads to inflammation. 25,26) In osteoarthritis patients, levels of NO are high in their cartilage even in the absence of inflammatory stimuli, such as IL-1β and LPS. 25,26) In addition, the synthesis of proteoglycan and collagen, components of healthy and elastic skin, are known to be inhibited by NO. 26) In our study, the decrease of NO synthesis with DBO-M treatment was due to the downregulation of iNOS, a pro-inflammatory enzyme responsible for NO production. DBO-M effectively suppressed LPS-induced upregulation of iNOS at both the mRNA and protein
level (Fig. 6). Increase of NO production has been shown to stimulate the expression of COX-2, another pro-inflammatory biomarker.\textsuperscript{27} COX-2, which is associated with the conversion of arachidonic acid to prostaglandin and eicosanoids, is clinically important as a major target of NSAIDs, like aspirin.\textsuperscript{28,29} However, these synthetic drugs are linked with undesirable side effects, such as intestinal bleeding, swelling of tissue, and allergies.\textsuperscript{5,10} DBO-M effectively suppressed COX-2 expression at the mRNA level (Fig. 5), suggesting that deer bone oil can be used as a natural source to suppress the COX-2-associated inflammatory responses.

Expression of iNOS and COX-2, which are representative inflammatory markers, are upregulated by pro-inflammatory cytokines, such as IL-1β and IL-12β, in various immune cell types.\textsuperscript{25,30} IL-1β is usually produced by macrophages, and particularly, IL-1β-induced COX-2 induction in the central nervous system is known to be responsible for inflammatory pain hypersensitivity.\textsuperscript{23} IL-12β is linked to autoimmune disease with induction of the T helper cell immune response.\textsuperscript{22} In addition, IL-12β seems to be an important mediator for perpetuating inflammation and successive death in the diseased area.\textsuperscript{21} Our data showed that DBO-M significantly inhibited mRNA expressions of these inflammatory cytokines, suggesting that DBO-M regulates the expression of cytokines to suppress the inflammatory response. These data are supported by other studies showing improvement of inflammation conditions with suppression of inflammatory cytokines.\textsuperscript{13,34,35} In addition, a recent study reported that deer bone extract activated macrophages to alleviate neutropenia, which is characterized by a low neutrophil number and an increased level of cytokines (IL-6 and tumor necrosis factor alpha (TNF-α) in mouse neutropenia model system).\textsuperscript{36} Collectively, deer bone-derived samples seem to have beneficial effects on inflammation, as shown in our study. However, this study did not address signaling pathways involved in DBO-M-mediated inhibition of inflammatory responses. Inflammatory cytokines and mediators, including iNOS and COX-2, have been shown to be controlled by nuclear factor kappa B (NF-κB), a transcription factor responsible for the synthesis of inflammatory substances.\textsuperscript{37} Translocation of NF-κB into the nucleus increases its binding to the genes of inflammatory cytokines, thereby promoting their expression.\textsuperscript{38} Activation of NF-κB is controlled by upstream signaling pathways like the Toll-like receptor (TLR) system.\textsuperscript{38} Chronic stimulation of TLR leads to the activation of inflammatory genes via NF-κB activation.\textsuperscript{39} Li et al. showed that chloroform extract of deer antler inhibited osteoclast differentiation by receptor activator of NF-κB ligand (RANKL)-stimulated mouse bone marrow.\textsuperscript{40} Based on these data, DBO-M may mediate its anti-inflammatory effects by inhibiting NF-κB signaling. Future efforts should focus on determining the detailed mechanistic action of deer bone oil in anti-inflammation, including the role of the TLR and NF-κB pathways.

In conclusion, DBO-M fraction of deer bone oil is enriched with unsaturated fatty acid and sugars, which can contribute to the solubility and the formation of active components in DBOE. DBO-M effectively inhibited inflammatory responses by suppressing inflammatory cytokines and mediators. Our study suggests that deer bone oil can be used as a natural anti-inflammatory agent.

\textbf{Conflict of Interest} The authors declare no conflict of interest.

\textbf{Supplementary Materials} The online version of this article contains supplementary materials.

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18. Ge R, Pu H. Effects of ginsenosides and pantocrine on the repro-


