A fraction from Dojuksan 30% ethanol extract exerts its anti-inflammatory effects through Nrf2-dependent heme oxygenase-1 expression

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Abstract. Dojuksan is a traditional herbal medicine used in Korea and China to treat urinary diseases. In the present study, we aimed to examine the anti-inflammatory effects of an ethanol solvent extract of Dojuksan and a fraction (by bioassay-guided fractionation) derived from this extract, and to elucidate the specific mechanisms involved. The Dojuksan 30% ethanol extract (DEE) had a more significant and potent anti-inflammatory effect than the Dojuksan water extract (DWE). DEE markedly inhibited the production of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), nitric oxide (NO), prostaglandin E_2 (PGE_2), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), as well as nuclear factor-κB (NF-κB) binding activity. We found that the anti-inflammatory effects of DEE were mediated by the induction of nuclear factor E2-related factor 2 (Nrf2)-dependent heme oxygenase-1 (HO-1). To further explore the anti-inflammatory effects of DEE, we generated 6 different fractions of DEE. Of these, DEE-5 decreased the production of NO more significantly than the other fractions. DEE-5 also significantly decreased the expression of iNOS and COX-2, and the production of NO, PGE_2, TNF-α and IL-1β. In addition, DEE-5 also significantly increased HO-1 levels; HO-1 significanlty contributed to the inhibitory effects of DEE-5 on the production of pro-inflammatory mediators. In this study, we determined whether the choice of extraction solvent affects the biological activity of Dojuksan, a traditional herbal formula. Our findings demonstrate that DEE and a fraction derived from this extract exerts anti-inflammatory effects through Nrf2-dependent HO-1 expression, and that DEE may thus have greater potential therapeutic application than DWE.

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Introduction

Dojuksan is a traditional herbal prescription medicine used in Korea and China to treat urinary diseases which cause symptoms, such as yellowish-red urine, pain in the phallus, dysuria and stomatitis (1). Dojuksan is composed of 4 medicinal herbs: *Rehmanniae* radix, *Akebia* caulis, *Glycyrrhizae* radix and *Phyllostachys* folium. *Rehmanniae* radix (Scrophulariaceae) is the root of *Rehmannia glutinosa* Libosch. To date, there a number of studies have been conducted on *Rehmanniae* radix, including its effects on astrocytes (2), fatigue (3), wound healing (4), anemia (5) and nephropathy (6,7). The second ingredient, *Akebia* caulis (Lardizabalaceae), is the stem of *Akebia quinata* Decne and has been studied for its anti-nociceptive and anti-inflammatory effects (8), and for its cytotoxicity (9). The third ingredient, *Glycyrrhizae* radix (Leguminosae), is the root of *Glycyrrhiza glabra* L. or *Glycyrrhiza uralensis* Fisch. and it has been suggested that this reduces stress-induced anxiety (10), apoptosis (11), hepatitis C virus replication (12) and inflammation (13). It has also been suggested that it induces growth-hormone release (14) and acts as an antispasmodic (15). Finally, *Phyllostachys* folium (Gramineae) is the leaf of *Phyllostachys nigra* and has been shown to protect retinal ganglion cells (16), treat diabetes (17), enhance leukemic cell differentiation (18) and inhibit interleukin (IL)-12 (19).

Lipopolysaccharide (LPS) is a well-known and important pro-inflammatory factor that can cause endotoxemia, shock, and, eventually, multiple organ dysfunction syndromes (20). Stimulation with LPS can induce the expression of pro-inflammatory mediators in macrophages, such as inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, and chemokines (21). In addition, activated macrophages may secrete pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and IL-1β (22). Prostaglandin E_2 (PGE_2), which is synthesized by the COX enzyme, is the most abundant prostaglandin in the human body and plays many biological roles. PGE_2 is an important

mediator of inflammatory symptoms, including fever and pain. The COX enzyme exists in 2 forms: the constitutive COX-1 form and the inducible cyclooxygenase-2 (COX-2) form (23). It has a variety of effects on various biological processes, including inflammation, pain, tumorigenesis, vascular function, neuronal function, female reproduction, gastric mucosal health and kidney function (24,25). Nitric oxide (NO) is generated by nitric oxide synthases (NOSs), which catalyze the production of NO and L-citrulline from L-arginine in the presence of nicotinamide adenine dinucleotide phosphate (NADPH)-derived electrons and O_2. Compared with neuronal NOS (nNOS) and endothelial NOS (eNOS), iNOS is expressed in many cell types, including macrophages, neutrophils, dendritic cells, endothelial cells and epithelial cells. The NO produced by these reactions is harmful and plays the role of an effector, for example in macrophages (26,27).

Heme oxygenase-1 (HO-1) is the rate-limiting enzyme in the catabolism of heme, a process that leads to the formation of equimolar amounts of the bile pigment biliverdin, free iron and carbon monoxide (CO) (28,29). The degradation of heme is considered critical in cellular defense. It has been suggested that CO contributes significantly to the anti-inflammatory properties of HO-1 (30). In addition, it is well known that the nuclear translocation of activated nuclear factor E2-related factor 2 (Nrf2) is an important event upstream of HO-1 expression. Nrf2 is required for the expression of some inducible proteins, such as glutathione S-transferases, quinine reductase and HO-1 (31,32). In a previous study (33), Dojuksan was shown to inhibit inflammatory mediators in RAW264.7 cells. However, to the best of our knowledge, no study on the ethanol extracts or beneficial effects of a fraction from Dojuksan, and its specific anti-inflammatory mechanisms, has been undertaken thus far. In the present study, we investigated the effects of an ethanol solvent extract of Dojuksan and a fraction (by bioassay-guided fractionation) derived from this extract and investigated the anti-inflammatory mechanisms associated with Nrf2-dependent HO-1 expression. We used the murine macrophage-like cell line, RAW264.7, in order to examine the anti-inflammatory effects and mechanisms of action of Dojuksan extracted using different methods, such as 30% ethanol extract (DEE) and bioassay-guided fractionation (fraction DEE-5).

Materials and methods

Preparation of Dojuksan extracts and MeOH fraction. The herbs that comprise Dojuksan were purchased from the University Oriental Herbal Drugstore (Iksan, Korea) in August 2011, and a voucher specimen was deposited at the Herbarium of the College of Pharmacy at Wonkwang University (Iksan, Korea). The Dojuksan water extract (DWE), and the 30% ethanol extract (DEE) were both deposited at the Standardized Material Bank for New Botanical Drugs (Wonkwang University). Dojuksan comprised *Rehmanniae* radix (8 g), *Akebia* caulis (8 g), *Glycyrrhizae* radix (8 g) and *Phyllostachys* folium (8 g). It (32 g) was extracted with either hot water or 30% ethanol (2 liters of each) for 2 h, and the extracts were concentrated *in vacuo* to obtain the 30% ethanol extract (NNMBS308). The 30% ethanol extract was subjected to C_{18}-functionalized silica gel flash column chromatography and eluted with a stepwise gradient of 0% (Fr. 1, DEE-1), 20% (Fr. 2, DEE-2), 40% (Fr. 3, DEE-3), 60% (Fr. 4, DEE-4), 80% (Fr. 5, DEE-5) and 100% (Fr. 6, DEE-6) (v/v) of MeOH in H_2O (4 ml each) (Fig. 4).

High performance liquid chromatography (HPLC). The solvents used for the extraction and flash column chromatography were reagent grade without further purification, whereas the solvents used for HPLC were of analytical grade. Flash column chromatography was performed using YMC octadecyl-functionalized silica gel (C_{18}). HPLC (YOUNGLIN-YL9100; Young Lin Instrument Co., Anyang, Korea) separation was performed using a Shiseido Capcell Pak C_{18} column (4.6x250 mm and 5 μm particle size; Shiseido Co., Ltd., Tokyo, Japan) with a flow rate of 0.7 ml/min, and an injection volume of 20 μl. The mobile phase was composed of (A) water and (B) acetonitrile, with an applied gradient of 5% B increasing to 100% B within 60 min. The column was cleaned with 100% B for 10 min, and the system was then equilibrated for 20 min under starting conditions. The detection wavelengths were adjusted to ELSD, 210 and 254 nm.

Chemicals and reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and all other tissue culture reagents used, were purchased from Gibco-BRL Co. (Grand Island, NY, USA) unless otherwise stated. Tin-protoporphyrin IX (SnPP), an inhibitor of HO activity, was obtained from Porphyrin Products (Logan, UT, USA). Lipofectamine™ 2000 was purchased from Invitrogen Life Technologies (Grand Island, NY, USA). Cobalt protophorphyrin IX (CoPP, Cat. no. C1900) and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated. RAW264.7 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Small interfering RNA (siRNA) against Nrf2 and primary antibodies, including those against HO-1 (SC-10789), Nrf2 (SC-722), COX-2 (SC-1745) and iNOS (SC-650), and appropriate secondary antibodies [anti-goat (SC-2768), anti-mouse (SC-2005) and anti-rabbit (SC-2004)] for western blot analysis, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Enzyme-linked immunosorbent assay (ELISA) kits for PGE_2, TNF-α and IL-1β were purchased from R&D Systems (Minneapolis, MN, USA). TLC silica gel 60 F254 aluminum plates were purchased from Merck (Darmstadt, Germany).

Cell culture and viability assay. Cell culture and viability were carried out as previously described (34). The RAW264.7 cells were maintained at $5x10^5$ cells/ml in DMEM supplemented with 10% heat-inactivated FBS, penicillin G (100 U/ml), streptomycin (100 mg/ml) and L-glutamine (2 mM), and were incubated at 37˚C in a humidified atmosphere containing 5% CO_2 and 95% air. The effects of various experimental modulations on cell viability were evaluated by determining mitochondrial reductase function with an assay based on the reduction of tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) into formazan crystals. The synthesis of formazan is proportional to the number of functional mitochondria in living cells. For the determination of cell viability, 50 mg/ml MTT solution were added to 1 ml cell suspension ($1x10^5$ cells/ml in 96-well plates) for 4 h. The formazan synthesized was dissolved in acidic 2-propanol, and the optical density was measured at 590 nm. The optical density of the formazan synthesized in the control

(untreated) cells was considered to be 100% cell viability. The majority of experiments were performed using incubation with LPS and samples, alone and in combination, unless stated otherwise. Pretreatment with samples was performed, beginning at 12 h before the incubation period, and then stimulation with LPS (1 μg/ml) was undertaken.

Determination of nitrite production, and PGE_2, TNF-α and IL-1β assays. The production of nitrite in the conditioned medium was determined using a method based on the Griess reaction, as described in a previous study (35). An aliquot (100 μl) of each supernatant was mixed with the same volume of Griess reagent [0.1% (w/v) *N*-(1-naphathyl)-ethylenediamine and 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid] for 10 min at room temperature. The absorbance of the final product was measured spectrophotometrically at 525 nm using an ELISA plate reader, and the nitrite concentration in the samples was determined from a standard curve of sodium nitrite made up in phenol red-free DMEM. The levels of PGE_2, TNF-α or IL-1β present in each sample were determined using a commercially available kit from R&D Systems (Abingdon, UK). The assay was performed according to the instructions provided by the manufacturer.

Western blot analysis. Western blot analysis was carried out as previously described (36). Western blot analysis was performed by lysing the cells in 20 mM Tris-HCl buffer (pH 7.4) containing a protease inhibitor mixture (0.1 mM PMSF, 5 mg/ml aprotinin, 5 mg/ml pepstatin A and 1 mg/ml chymostatin). The protein concentration was determined using a Lowry protein assay kit (P5626; Sigma Chemical Co.). An equal amount of protein for each sample was resolved by 12 or 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred onto a Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5%skimmed milk and sequentially incubated with primary antibody (Santa Cruz Biotechnology, Inc.) and horseradish peroxidase-conjugated secondary antibody, followed by ECL detection (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Preparation of cytosolic and nuclear fractions. Preparation of cytosolic and nuclear fractions was carried out as previously described (37). The cells were collected and washed with phosphate-buffered saline (PBS) and suspended in 200 μl lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF and a protease inhibitor cocktail]. The cells were allowed to swell on ice for 15 min; subsequently, 12.5 μl 10% NP-40 was added. The tubes were agitated on a vortex for 10 sec and then centrifuged for 5 min. The resulting supernatant represented the cytosolic extract. The nuclear pellets were resuspended in 50 μl ice-cold nuclear extraction buffer [20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF and a protease inhibitor cocktail] and incubated on ice for 1 h with intermittent vortexing. This nuclear extract was centrifuged for 10 min at 15,000 x g; the resulting supernatant represented the nuclear fraction.

DNA binding activity of nuclear factor-κB (NF-κB). As previously described (38), the DNA-binding activity of NF-κB in the nuclear extracts was measured using a TransAM kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, 30 μl complete binding buffer (DTT, herring sperm DNA and binding buffer AM3) was added to each well. The samples were nuclear extracts of RAW264.7 macrophages that had been stimulated for 30 min with LPS and specific concentrations of DEE. Subsequently, the samples were diluted in complete lysis buffer and added to each well (20 μg nuclear extract diluted in complete lysis buffer to a final volume of 20 μl). The plates were incubated for 1 h at room temperature with mild agitation (100 rpm on a rocking platform). After washing each well with wash buffer, 100 μl of diluted NF-κB antibody (1:1,000 dilution in 1X antibody-binding buffer) was added to each well, and the plates were then further incubated, as previously described, for 1 h. After washing each well with wash buffer, 100 μl diluted HRP-conjugated antibody (1:1,000 dilution in 1X antibody-binding buffer) was added to each well followed by incubation for 1 h, as before. Developing solution (100 μl) was added to each well and left to react for 5 min, followed by the addition of a stop solution to each well. Finally, the absorbance of each sample was read at 450 nm on a spectrophotometer (microplate reader model 680, serial no. 19590, Bio-Rad) within 5 min of reaction termination.

siRNA transfection of Nrf2. Cells were transiently transfected with Nrf2 siRNA (Santa Cruz Biotechnology, Inc.) for 6 h using Lipofectamine 2000™ (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and then incubated in fresh media containing 10% FBS for 24 h before further manipulation.

Statistical analysis. Data are expressed as the means ± SD of at least 3 independent experiments. To compare three or more groups, one-way analysis of variance (ANOVA) followed by a Newman-Keuls post hoc test was used. Statistical analysis was performed using GraphPad Prism software, version 3.03 (GraphPad Software Inc., San Diego, CA, USA).

Results

Inhibitory effects of DEE on the production of pro-inflammatory mediators and cytokines, and the expression of iNOS and COX-2 in LPS-stimulated RAW264.7 cells. We found that the Dojuksan 30% ethanol extract (DEE) was significantly more effective in terms of its anti-inflammatory effects than DWE (data not shown), and that it exerted its anti-inflammatory effects by inhibiting the expression of the pro-inflammatory enzymes, iNOS and COX-2, and suppressing the secretion of the pro-inflammatory cytokines, NO, PGE_2, TNF-α and IL-1β, through the inhibition of NF-κB activation. Since DEE had more potent anti-inflammatory effects, it was used in our subsequent experiments. The cytotoxicity of DEE and DEE-5 in the RAW264.7 cells was determined by MTT assay. We found that cell viability was not significantly decreased by either DEE or DEE-5 at concentrations of up to 400 μg/ml (data not shown). Subsequently, we pre-treated the RAW264.7 cells with either DEE at non-cytotoxic concentrations (50-400 μg/ml) for 12 h and measured the production of NO, PGE_2, TNF-α and IL-1β following stimulation with LPS (1 μg/ml) for 18 h. With the increasing concentration of DEE, NO production decreased in

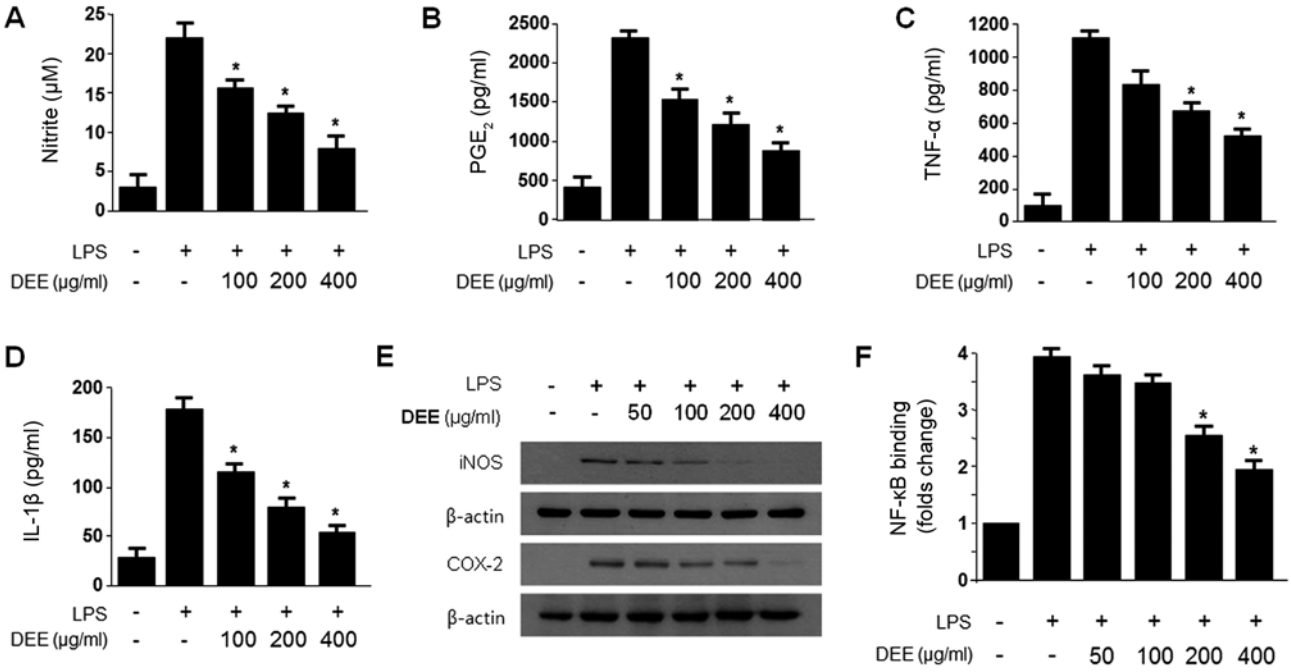

Figure 1. Effects of Dojuksan 30% ethanol extract (DEE) on the production of (A) nitrite, (B) prostaglandin E_2 (PGE_2), (C) tumor necrosis factor-α (TNF-α), and (D) interleukin-1β (IL-1β), and on the (E) protein expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in RAW264.7 cells stimulated with lipopolysaccharide (LPS). (A-E) The cells were pre-treated for 12 h with the indicated concentrations of DEE and then stimulated for 18 h with LPS (1 μg/ml). (F) The cells were pre-treated for 12 h with the indicated concentrations of DEE and then stimulated for 30 min with LPS (1 μg/ml). The concentrations of nitrite, PGE_2, TNF-α and IL-1β were determined as described in the Materials and methods. Western blot analysis was performed, and representative blots of 3 independent experiments are shown. A commercially available nuclear factor-κB (NF-κB) ELISA (active motif) was then used to test nuclear extracts and determine the degree of NF-κB binding. The data represent the means ± SD of 3 independent experiments. *p<0.05 compared with the group treated with LPS.

a dose-dependent manner until it reached a level close to that of the control cells (which were not treated with LPS) at 400 μg/ml. We observed a similar effect on the production of PGE_2, TNF-α and IL-1β in the cells treated with DEE (Fig. 1).

The expression of the pro-inflammatory enzyme, iNOS, plays a crucial role in immune-activated macrophages through the production of NO (26,27). In addition, prostaglandins are an important mediator of the symptoms of inflammation, including fever and pain. Inducible COX-2 is the major source of prostaglandins (24,25). Thus, in order to examine the effects of DEE on the expression of iNOS and COX-2, the RAW264.7 cells were challenged with LPS (1 μg/ml) in combination with the indicated concentrations of DEE, and the protein expression levels of iNOS and COX-2 were measured by western blot analysis (Fig. 1E). DEE decreased both iNOS and COX-2 protein expression in a concentration-dependent manner.

NF-κB is a key molecule in an important signaling pathway involved in inflammatory diseases, and it regulates iNOS and genes, such as COX-2 (39,40). Thus, to determine whether the NF-κB pathway is involved in the suppression of inflammatory responses by DEE, we measured NF-κB binding activity. DEE significantly decreased NF-κB binding activity in a concentration-dependent manner (Fig. 1D).

Effects of DEE on HO-1 expression are mediated by the nuclear translocation of Nrf2 in RAW264.7 cells. We examined whether HO-1 is the key player mediating the DEE-induced suppression of pro-inflammatory responses. The RAW264.7 cells were treated with the indicated concentrations of DEE for 12 h, or with 400 μg/ml of DEE for the indicated periods of time. We noted that DEE increased HO-1 expression in a concentration-dependent manner (Fig. 2A), and HO-1 expression began to increase 3 h following treatment with 400 μg/ml DEE (Fig. 2B). Nrf2 is as an indispensable regulator of the coordinated induction of phase II enzymes, including HO-1 (41). Therefore, we performed western blot analysis to determine whether treatment with DEE induces the nuclear translocation of Nrf2. When the cells were incubated with DEE for 0.5, 1.0 or 1.5 h at a concentration of 400 μg/ml, there was a gradual increase in the levels of nuclear Nrf2, with a concomitant decrease in the cytoplasmic levels (Fig. 2C). The role of Nrf2 in DEE-mediated HO-1 expression was confirmed using siRNA against Nrf2. The RAW264.7 cells were transiently transfected with Nrf2 siRNA, and then treated with 400 μg/ml DEE for 12 h. As shown in Fig. 2D, transient transfection with Nrf2 siRNA completely abolished DEE-induced HO-1 expression.

Effects of HO-1 expression on the inhibition of pro-inflammatory mediators, cytokines and NF-κB activity by DEE in LPS-stimulated RAW264.7 cells. To confirm the suppressive effects of HO-1 on pro-inflammatory mediators, cytokines and the NF-κB pathway, we used SnPP, which is a competitive inhibitor of HO activity. Previously, imidazole-dioxolane compounds have been shown to act as inhibitors of HO activity (42,43), as well as specific HO isoenzymes (44). In the present study, RAW264.7 cells were pre-treated with DEE (400 μg/ml) for 12 h in the absence or presence of SnPP (20 μM). The inhibitory effects of DEE on the LPS-induced production of NO, PGE_2, TNF-α and IL-1β, as well as NF-κB binding activity, were partially reversed by SnPP (Fig. 3).

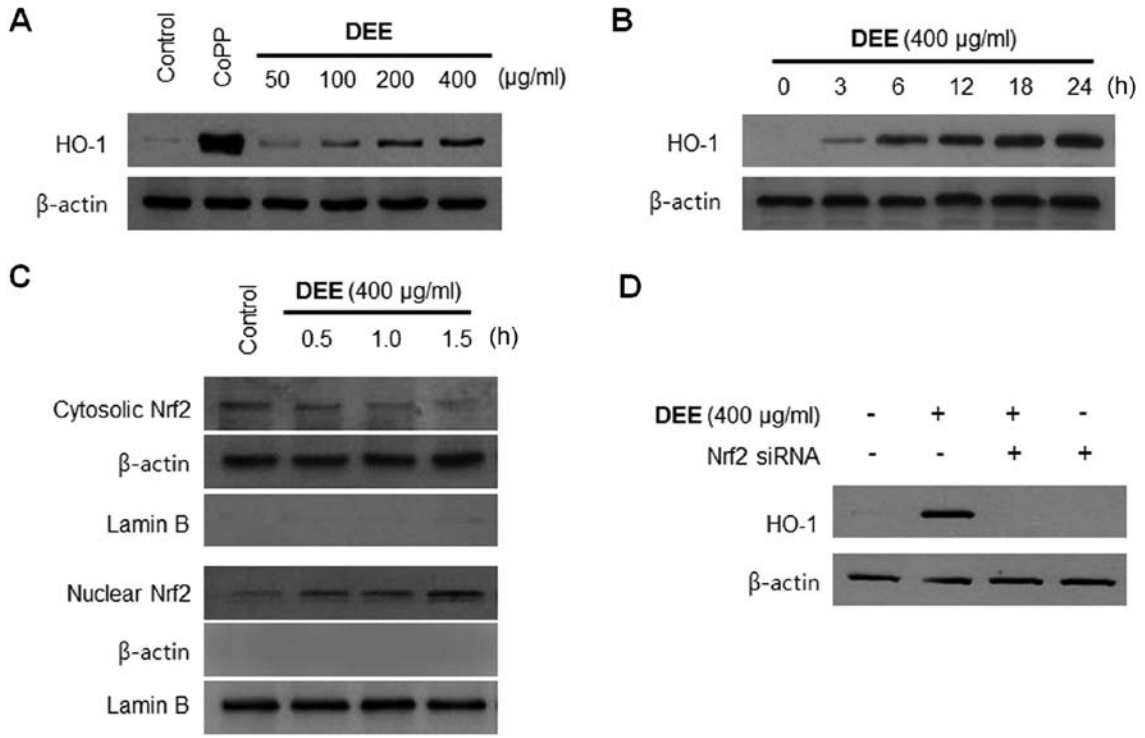

Figure 2. Effects of Dojuksan 30% ethanol extract (DEE) on heme oxygenase-1 (HO-1) expression and the nuclear translocation of nuclear factor E2-related factor 2 (Nrf2) in RAW264.7 cells. The cells were incubated for 12 h with (A) the indicated concentrations of DEE, or (B) for the indicated periods of time with 400 μg/ml DEE. (C) The cells were treated for 0.5-1 h with 400 μg/ml DEE. (D) RAW264.7 cells were transiently transfected with Nrf2 siRNA, and treated with 400 μg/ml DEE for 12 h. The positive control, the HO-1 inducer cobalt protoporphyrin IX (CoPP), increased the expression of HO-1 at 20 μM. Western blot analysis of HO-1 expression was performed as described in the Materials and methods, and representative blots of 3 independent experiments are shown.

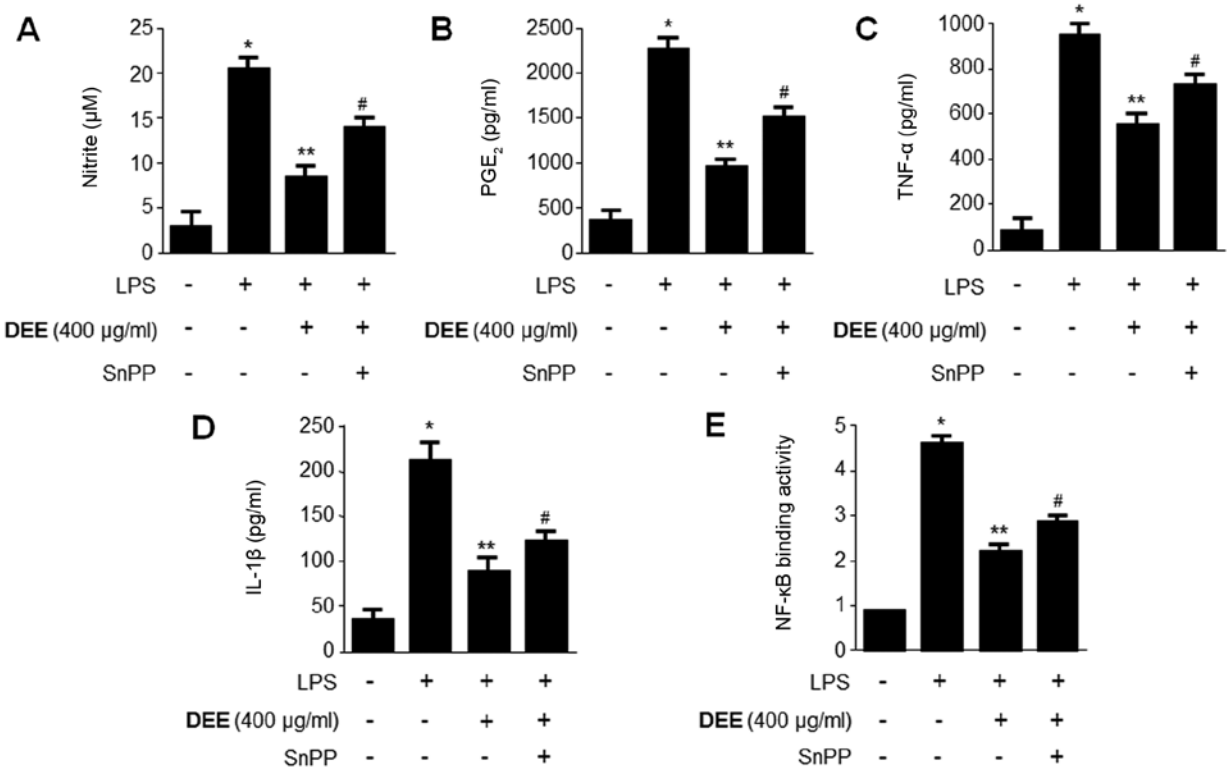

Figure 3. Effects of Tin-protoporphyrin IX (SnPP; HO-1 inhibitor) on Dojuksan 30% ethanol extract (DEE)-mediated inhibition of (A) nitrite, (B) prostaglandin E_2 (PGE_2), (C) tumor necrosis factor-α (TNF-α), and (D) interleukin-1β (IL-1β) production, and (E) nuclear factor-κB (NF-κB) DNA-binding activity in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. Cells were pre-treated with DEE (400 μg/ml) for 12 h in the presence or absence of SnPP (50 μM), and then stimulated with LPS (1 μg/ml) for 18 h. The concentrations of (A) nitrite, (B) PGE_2, (C) TNF-α, (D) IL-1β, and (E) NF-κB DNA-binding activity were determined as described in Materials and methods. Data shown represent the means of 3 experiments ± SD. *p<0.05 compared to the control group; **p<0.05 compared to the group treated with LPS alone; $^{\#}$p<0.05 compared to the group treated with DEE and LPS.

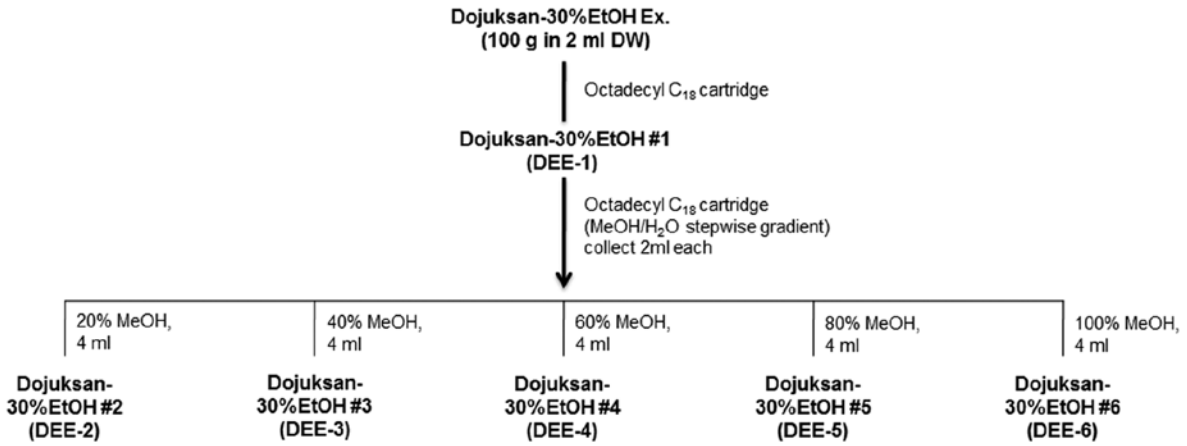

Figure 4. Fractions from 30% ethanol extract. The 30% ethanol extract was subjected to C_{18}-functionalized silica gel flash column chromatography and eluted with a stepwise gradient of 0% [Fr. 1, Dojuksan 30% ethanol extract (DEE)-1], 20% (Fr. 2, DEE-2), 40% (Fr. 3, DEE-3), 60% (Fr. 4, DEE-4), 80% (Fr. 5, DEE-5), and 100% (Fr. 6, DEE-6) (v/v) of MeOH in H_2O (4 ml each).

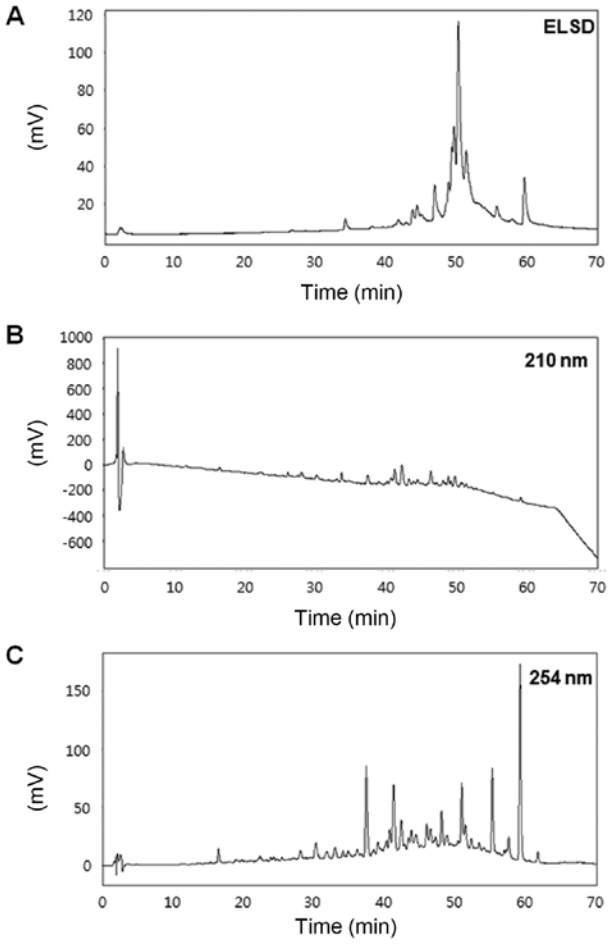

Figure 5. The HPLC chromatograms of DEE-5. HPLC chromatograms of DEE-5 were obtained using (A) an ELSD detector and (B) a UV/Vis detector operated at 210 nm or (C) 254 nm.

Comparison of Dojuksan fractions by HPLC. To further explore the differences in the anti-inflammatory effects of DEE, we generated 6 different fractions of DEE using a C_{18} cartridge with a stepwise elution of MeOH in H_2O (Fig. 4), as the HPLC chromatograms of DEE showed unclear patterns (data not shown). We then performed a comparative analysis of the HPLC chromatograms of each of the 6 fractions of DEE (Fig. 5). Data from the HPLC analysis of DEE-5 was obtained in the form of chromatograms by monitoring detector responses at ELSD, 210 and 254 nm (Fig. 5). Of the 6 fractions, DEE-5 induced the greatest decrease in NO production in the LPS-stimulated RAW264.7 cells (data not shown). Therefore, we further examined the anti-inflammatory effects of the Dojuksan ethanol extract using DEE-5.

Effects of DEE-5 on the inhibition of pro-inflammatory mediators and cytokines in LPS-stimulated RAW264.7 cells. To confirm the inhibitory effects of DEE-5 on pro-inflammatory mediators and cytokines, we pre-treated the RAW264.7 cells with DEE-5 (100 or 200 μg/ml) for 12 h and then measured the protein expression of iNOS and COX-2, as well as the production of NO, PGE_2, TNF-α and IL-1β following stimulation with LPS (1 μg/ml) for 18 h. DEE-5 significantly decreased the protein expression of iNOS and COX-2, and the production of NO, PGE_2, TNF-α and IL-1β (Fig. 6).

Effects of DEE-5 on HO-1 expression and the inhibition of pro-inflammatory mediators and cytokines in RAW264.7 cells. We also examined whether DEE-5 is able to induce the expression of HO-1. The RAW264.7 cells were treated with the indicated concentrations of DEE-5 for 12 h, and we observed a significant increase in HO-1 expression at a concentration of 100-200 μM (Fig. 7A). The role of Nrf2 in DEE-5-mediated HO-1 expression was confirmed using siRNA against Nrf2. The RAW264.7 cells were transiently transfected with Nrf2 siRNA, and then treated with 200 μg/ml DEE-5 for 12 h. As shown in Fig. 7B, transient transfection with Nrf2 siRNA completely abolished DEE-5-induced HO-1 expression. To confirm the suppressive effects of HO-1 on pro-inflammatory mediators and cytokines, we used SnPP, which is a competitive inhibitor of HO activity. The RAW264.7 cells were pre-treated with DEE-5 (200 μg/ml) for 12 h in the absence or presence of SnPP. The inhibitory effects of DEE-5 on LPS-stimulated NO, PGE_2, TNF-α, and IL-1β production were partially reversed by SnPP (Fig. 7C-F).

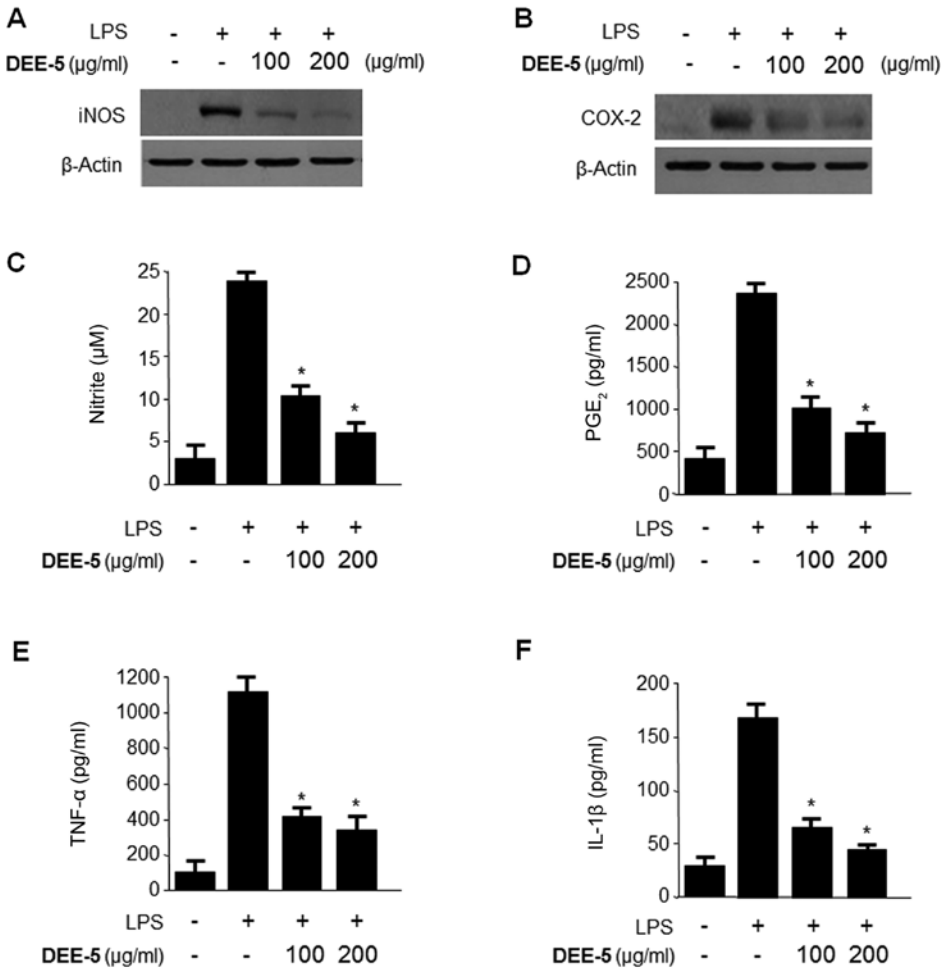

Figure 6. Effects of Dojuksan 30% ethanol extract (DEE)-5 on the expression of (A) inducible nitric oxide synthase (iNOS) and (B) cyclooxygenase-2 (COX-2), and the production of (C) nitrite, (D) prostaglandin E_2 (PGE_2), (E) tumor necrosis factor-α (TNF-α), and (F) interleukin-1β (IL-1β) in RAW264.7 cells stimulated with lipopolysaccharide (LPS). The cells were pre-treated for 12 h with the indicated concentrations of DEE-5 and then stimulated for 18 h with LPS (1 μg/ml). Western blot analysis was performed, as described in the Materials and methods, and representative blots of 3 independent experiments are shown. The concentrations of nitrite, PGE_2, TNF-α and IL-1β were determined. The data represent the means ± SD of 3 independent experiments. *$p<0.05$ compared with the group treated with LPS.

Discussion

Dojuksan is a traditional herbal prescription medicine used in Korea and China to treat urinary diseases which cause symptoms, such as yellowish-red urine, pain in the phallus, dysuria and stomatitis (1). In a previous study (33), water extracts of Dojuksan inhibited inflammatory mediators in RAW264.7 cells. To date, and to the best of our knowledge, however, there have been few studies on Dojuksan, and no comparisons of the biological efficacy of Dojuksan extracts or its anti-inflammatory mechanisms have been made. In addition, our data suggested that DEE exerted a significantly more prominent anti-inflammatory effect than DWE (data not shown). Based on these findings, in the present study, we suggest a novel approach to understanding and improving the therapeutic effects of this traditional herbal formula.

Inflammation is a part of the complex biological response of the body to harmful stimuli, such as pathogens or irritants that have the capacity to cause cell damage (45). Inflammation is a protective attempt to remove injurious stimuli and to initiate the healing process by the organism. Various symptoms are associated with inflammation, including flares, fever, swelling, itching and functional disorders caused by the induction of various pro-inflammatory mediators (46). It is well known that NO exerts broad physiological and pathological effects on a number of tissues, including immune cells. There are three well-known isoforms of NOS: nNOS, eNOS and iNOS (47). In lymphoid tissues, iNOS is the principal isoform of NOS. Macrophages appear to be a major source of iNOS (26,47,48). iNOS differs from other NOS isoforms, since it is not constitutively present, but is instead induced by cytokines, such as interferon-γ (IFN-γ) and TNF-α, or other immunological stimuli, including LPS. Prostaglandin endoperoxide H synthase and COX convert arachidonic acid (AA) to prostaglandin endoperoxide H2. Synthesized PGH_2 is converted to prostaglandins (PGD_2, PGE_2 and $PGF_{2\alpha}$), prostacyclin (PGI_2), or thromboxane A2 by tissue-specific isomerases (49). In the present study, we noted that DEE decreased iNOS and COX-2 protein expression and the production of pro-inflammatory cytokines, namely NO, PGE_2 TNF-α and IL-1β, in a concentration-dependent manner. Moreover, to examine whether NF-κB DNA-binding activity, a key signaling pathway leading to pro-inflammatory

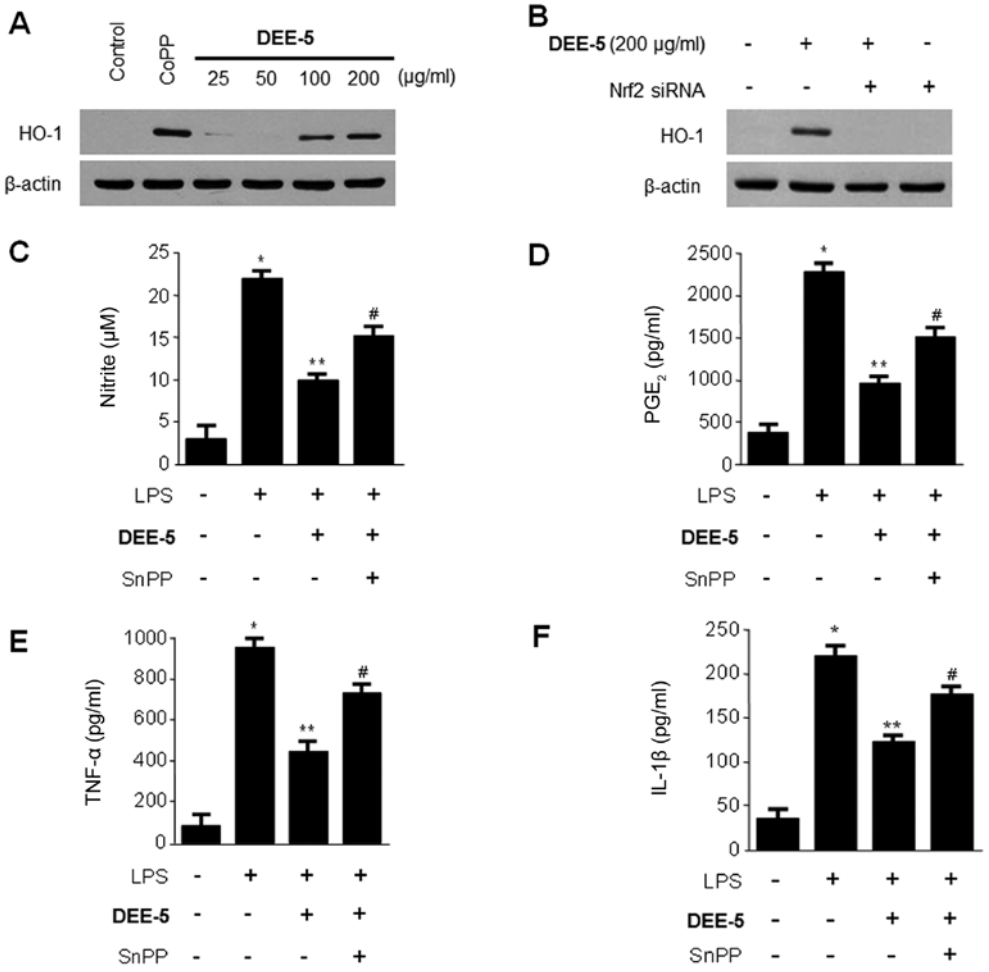

Figure 7. Effects of Dojuksan 30% ethanol extract (DEE)-5 on (A and B) heme oxygenase-1 (HO-1) expression and (C) effects of Tin-protoporphyrin IX (SnPP; HO-1 inhibitor) on the DEE-5-mediated inhibition of nitrite, (D) prostaglandin E_2 (PGE_2), (E) tumor necrosis factor-α (TNF-α), and (F) interleukin-1β (IL-1β) production in RAW264.7 cells. (A) The cells were incubated for 12 h with the indicated concentrations of DEE-5. The positive control, the HO-1 inducer cobalt protoporphyrin IX (CoPP), increased the expression of HO-1 at 20 μM. (B) RAW264.7 cells were transiently transfected with nuclear factor E2-related factor 2 (Nrf2) siRNA, and then treated with 200 μg/ml DEE-5 for 12 h. Western blot analysis of HO-1 expression was performed as described in the Materials and methods, and representative blots of three independent experiments are shown. Cells were pretreated with DEE-5 (200 μg/ml) for 12 h in the presence or absence of SnPP (50 μM), and then stimulated with lipopolysaccharide (LPS) (1 μg/ml) for 18 h. The concentrations of (C) nitrite, (D) PGE_2, (E) TNF-α, (F) IL-1β were determined as described in Materials and methods. Data shown represent the mean values of 3 experiments ± SD. $^{*}p<0.05$ compared to the control group; $^{**}p<0.05$ compared to the group treated with LPS alone; $^{\#}p<0.05$ compared to the group treated with DEE-5 and LPS.

gene expression, is an upstream target for the inhibitory effects of DEE, we also examined the appearance of NF-κB DNA-binding activity. We noted that DEE inhibited the increase in NF-κB DNA-binding activity in RAW264.7 cells stimulated with LPS in a dose-dependent manner. In addition, as mentioned above, we found that DEE exerted a significantly more potent anti-inflammatory effect than DWE, and exerted its anti-inflammatory effects by inhibiting the expression of the pro-inflammatory enzymes, iNOS and COX-2, and suppressing the secretion of the pro-inflammatory cytokines, NO, PGE_2, TNF-α and IL-1β, via the inhibition of NF-κB activation (data not shown). Therefore, we only used DEE in all our subsequent experiments.

Along with examining its antioxidant effects, recent studies have also demonstrated the anti-inflammatory effects of HO-1 reaction in a number of inflammatory models (37,50). The anti-inflammatory effects of HO-1 are mediated by inhibiting the production of pro-inflammatory cytokines and chemokines, such as TNF-α, IL-1β and IL-6, in activated macrophages (51). HO-1 and its product, carbon monoxide, can also suppress the expression of pro-inflammatory COX-2 and iNOS, thereby reducing COX-2-drived PGE_2 and iNOS-derived NO production (52). Therefore, we suggest that regulating the degree of generation of oxidative stress and macrophage activation via the upregulation of HO-1 is an important aspect to be considered when formulating a strategy for treating inflammatory diseases. To determine whether HO-1, which is involved in both antioxidant and anti-inflammatory activities, is the key player in the DEE-mediated suppression of pro-inflammatory responses, we treated RAW264.7 cells with DEE and examined both tje concentration- and time-dependent effects. The expression of HO-1 increased as the concentration of DEE increased, and began to increase 3 h following treatment with 400 μg/ml DEE. To confirm the suppressive effects of HO-1 on pro-inflammatory mediators, we examined the effects of SnPP, which is a competitive inhibitor of HO-1 activity. The suppressive effects of DEE on LPS-induced NO, PGE_2, TNF-α and IL-1β production were partially reversed by SnPP. These

results indicate that the inhibition of the pro-inflammatory mediators resulted, at least partially, from a stimulatory effect of DEE on HO-1. In addition, it has previously been noted that Nrf2 is an indispensable regulator of the coordinated induction of phase II enzyme genes, including HO-1 (41). Under normal homeostatic conditions, Nrf2 is retained in the cytoplasm via its interaction with Keap1 and is degraded by the proteasome. Under oxidative conditions, or as a result of specific stimuli, Nrf2 is released from Keap1, translocates to the nucleus, forms a heterodimer with small Maf proteins, and recognizes and binds to a cis-acting antioxidant response element (ARE), where it eventually recruits the transcriptional machinery, including RNA polymerase II, necessary for the transcription of its target genes (31,32,52). In the present study, there was a gradual increase in Nrf2 levels in the nuclear fractions of the DEE-treated RAW264.7 cells, whereas the cytoplasmic levels were concomitantly decreased. This supports the hypothesis that Nrf2-mediated HO-1 expression contributes to the inhibitory effects of DEE on the production of pro-inflammatory mediators via the NF-κB pathway.

To further clarify the effects of Dojuksan, we also examined potential differences in the effects of DWE and DEE and examined whether the anti-inflammatory efficacy of the subfractions differed. We therefore generated 6 different fractions from DEE, and chromatographic analysis led to additional experiments using the 80% MeOH fractions of DEE-5. Of the fractions, DEE-5 was responsible for the most significantly reduced NO production in the LPS-stimulated RAW264.7 cells (data not shown). Therefore, we further examined the anti-inflammatory effects of Dojuksan extracts using DEE-5. DEE-5 markedly suppressed the protein expression of iNOS and COX-2, and the production of NO, PGE_2, TNF-α and IL-1β. Furthermore, DEE-5 induced HO-1 protein expression, and the suppressive effects of DEE-5 on LPS-induced NO, PGE_2, TNF-α and IL-1β production were partially reversed by SnPP. These findings support the hypothesis that the induction of HO-1 contributes to the inhibitory effects of DEE-5 on the production of pro-inflammatory mediators.

In this study, we determined that the choice of extraction solvent affects the biological activity of Dojuksan, a traditional herbal formula. In conclusion, our results indicated that the Dojuksan 30% ethanol extract, and its fraction, exerted its anti-inflammatory effects through Nrf2-mediated HO-1 expression, and that DEE likely has greater potential therapeutic applications than the water extract. We also suggest that the use of different extraction solvents or bioassay-guided fractionation is an option for improving the therapeutic efficacy of this traditional herbal formula.

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