Standardized microwave extract of Sappan Lignum exerts anti-inflammatory effects through inhibition of NF-κB activation via regulation of heme oxygenase-1 expression

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Received June 2, 2018; Accepted December 18, 2018

DOI: 10.3892/mmr.2019.9811

Abstract. The extract of Sappan Lignum, the heartwood of *Caesalpinia sappan* L., has been used in medicine to improve blood circulation. Recently, the application of microwave extraction methods has been a major focus of research into the extraction of components from natural sources. In this experiment, we compared the anti-inflammatory effects of Sappan Lignum prepared by heat-70% EtOH extraction (CSE-H-70E) and microwave-70% EtOH extraction (CSE-MW-70E). High-performance liquid chromatography analysis was used to identify the compounds in these extracts. The heat-70% EtOH and microwave-70% EtOH extracts of Sappan Lignum had different chromatograms. CSE-MW-70E significantly inhibited the protein expression of iNOS and COX-2, PGE_2, TNF-α, and reduced NO and IL-1β production in macrophages exposed to LPS, whereas, only high concentrations of CSE-H-70E (20 μg/ml) resulted in any effects. Furthermore, CSE-MW-70E upregulated heme oxygenase-1 (HO-1) expression. In addition, the use of tin protoporphyrin, an inhibitor of HO-1, confirmed the inhibitory effects of CSE-MW-70E on pro-inflammatory mediators. These results suggested that the CSE-MW-70E-mediated upregulation of HO-1 played an important role in the anti-inflammatory effects of macrophages. Therefore, these findings showed that microwave extraction can be utilized to improve the extraction efficiency and biological activity of Sappan Lignum.

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Key words: Sappan Lignum, heated-extraction, microwave extraction, RAW264.7 macrophage, anti-inflammatory effects

Introduction

Prolonged inflammation can lead to many diseases, such as inflammatory bowel disease, arthritis, and septic shock syndrome. Excessive inflammatory responses can induce disease pathogenesis and may interrupt tissue functions (1). The exposure of immune cells and macrophages to specific agonists stimulates complex signaling cascades, which immediately generate the production of pro-inflammatory cytokines and chemokines, notably macrophage inflammatory protein-2 (MIP2), interleukin-6 (IL-6), nitric oxide (NO), tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX2), which play a vital part in the initiation of the inflammatory response (2-4).

Heme oxygenase-1 (HO-1) is a member of the heat shock protein (HSP) family; it also participates in heme catabolism as a rate-limiting enzyme, and this catabolism releases the three by-products including carbon monoxide (CO), free iron (Fe^{2+}), and biliverdin (5). Biliverdin reductase causes the conversion of biliverdin to bilirubin. The complete protective effects of HO-1 comprise the antioxidant effects of both biliverdin and bilirubin (6,7). HO-1 and its by-products play a significant role in the resolution stage of inflammation, in which macrophages are targeted (8). Previous experiments have determined that the expression of HO-1 prevents the production of chemokines and pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, in stimulated macrophages (9,10). Moreover, the upregulation of HO-1 expression mitigates the expression of the pro-inflammatory proteins COX-2 and iNOS, and consequently suppressed the production of COX-2-derived prostaglandin E_2 (PGE_2) and iNOS-derived NO production (11,12). Through the inactivation of nuclear factor-κB (NF-κB), HO-1 also prevents iNOS expression and NO production (13,14). Hence, a group of therapeutic agents have been found to upregulate the expression of HO-1 and utilize anti-inflammatory responses mediated by HO-1 initiation (13,15). NF-κB is an important aspect of the inflammation

response. NF-κB is stimulated by lipopolysaccharides (LPS) through the phosphorylation of inhibitor of κB-α (IκB-α). Subsequently, NF-κB translocates to the nucleus after detachment from IκB-α (16,17). After NF-κB reaches at the nucleus, this molecule attaches to defined sites on the DNA and coordinates the transcription of its target genes that are responsible for the production of pro-inflammatory mediators and cytokines (18).

Extraction is a vital stage in phytochemical processing for the introduction of new bioactive components from natural sources. The choice of a compatible extraction method is also vital for the standardization of natural herbal products, as it is applied for the isolation of desirable soluble components. Many types of extraction methods are popular, including conventional methods such as decoction, percolation, hot continuous extraction, and maceration. During the last three decades, an alternative technique known as microwave-assisted solvent extraction (MASE) has emerged as attractive (19). Microwaves are non-ionizing electromagnetic waves with a frequency of between 300 MHz and 300 GHz, positioned in the electromagnetic spectrum between infrared and X-ray radiation (20). Dipolar rotation and ionic conduction are the two important mechanisms that occur simultaneously in electric field heating (21). Hence, a significant advantage of modern MASE systems is solvent-free functionality. MASE also offers an environmentally friendly green extraction technique for essential oils and other volatile natural products.

Sappan Lignum, the dried heartwood of *Caesalpinia sappan Linn*., has been used extensively in the Asia-Pacific region as a traditional herbal medicine. It has also been used as a natural red dye, conventional food and beverage ingredient, and as a traditional Chinese medicine component for analgesic and anti-inflammatory remedies or to promote menstruation and blood circulation (22). Studies of Sappan Lignum have reported various pharmacological effects, including anti-atherosclerotic, analgesic (23), antioxidant (24), hypoglycemic (25), anti-complementary (26), vasorelaxation (27), anti-inflammatory (28), spasmolytic activity (29), cytotoxic (30), muscle contractile (31), anticonvulsant (32), antiviral (33), and antibacterial (34) activities. The ethyl acetate (EtOAc) extract of Sappan Lignum exerted significant inhibitory effects on mitosis and growth-related signaling (35). In our previous study, we initially made each microwave-assisted solvent extract from twenty herbal plants. Later, we checked the anti-inflammatory effect of these twenty microwave-assisted solvent extracts, the microwave-70% EtOH extract of Sappan Lignum has good anti-inflammatory actions among all of the samples. Therefore, we compared the anti-inflammatory effects of Sappan Lignum extracted by using heat-70% EtOH extraction (CSE-H-70E) or microwave-70% EtOH extraction (CSE-MW-70E) in this investigation.

Materials and methods

Plant materials and extraction. The heartwood of *C. sappan* was purchased from Omni Herb, Daegu, Korea in March 2012. The voucher specimen (WK-2012-0301) was deposited at the Herbarium of the College of Pharmacy, Wonkwang University (Korea). The dried heartwood of *C. sappan* was cut and extracted in 70% EtOH by using heat or microwave extraction. The dried heartwood of *C. sappan* (30 g) was extracted in 70% EtOH (500 ml in a 1 L round flask) for 15 min at 80˚C by using a normal heat-extraction method. The heat-70% EtOH extraction (CSE-H-70E) was evaporated in vacuo to yield 240, 210, and 258 mg of residue (0.79±0.08% v/v; Table I). In addition, *C. sappan* (30 g) was extracted with 70% EtOH (500 ml in a 1 L round flask) by using microwave-extraction (three extractions at 2450 Hz and 700 W for 5 min). During the microwave extraction, 500 ml water was reacted as the coolant and the water was changed each time. Finally, the microwave-70% EtOH extraction (CSE-MW-70E) was evaporated in vacuo to yield 345, 297, and 375 mg of residue (1.13±0.13% v/v; Table I). Values are presented as mean ± standard deviation (mean ± SD).

Chemical and reagents. RPMI-1640, fetal bovine serum (FBS), and other cell culture reagents were purchased from Gibco-BRL Co. (Grand Island, NY, USA). All chemicals were obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, German). Antibodies to anti-iNOS, anti-COX-2, anti-HO-1, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture and viability assay. RAW264.7 cells were maintained at a density of $1x10^7$ cells/ml in RPMI-1640 medium supplemented with 10% FBS and 1% antibiotics and were incubated at 37˚C in a humidified atmosphere containing 5% CO_2. To determine the cell viability by using an MTT assay, the cells were maintained at $1x10^4$ cells/well and then treated with the extract. After incubation, the medium was removed from each well, 200 µl fresh medium was placed in each well, and the cells were incubated with 0.5 mg/ml MTT for 3 h (36).

High-performance liquid chromatography (HPLC). Chromatography was performed by using a YL-9100 series HPLC instrument (YoungLin, Korea). In all experiments, C-18 column (SHISEIDO CAPCELL PAK, 4.6x150 mm; 5 µm) was used as the stationary phase and the injection volume was 20 µl. Samples were prepared that contained 2 mg/ml CSE-H-70E and CSE-MW-70E. The mobile phase consisted of water plus 0.1% formic acid (A) and MeOH (B) eluted as a linear gradient of 0-50 min from 10% B to 100% B. The detection wavelength used was 254 nm and the flow rate was 0.7 ml/min.

Western blotting. The cells were harvested and pelleted by centrifugation at 14,000 x g for 3 min at 4˚C. The cells were washed with PBS and lysed in 20 mM Tris-HCl buffer (pH 7.4) containing a protease inhibitor mixture (0.1 mM phenylmethanesulfonyl fluoride, 5 mg/ml aprotinin, 5 mg/ml pepstatin A, and 1 mg/ml chymostatin). The protein concentration of the lysed samples was determined by using a Lowry protein assay kit (Sigma-Aldrich; Merck KGaA), and 30 µg of protein from each sample was resolved by 7.5 or 12% 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). Non-specific binding to the membrane was blocked by incubation in 5% skimmed milk and the membrane was sequentially processed by incubation with primary antibodies (Santa Cruz Biotechnology and Cell

Table I. Comparison between the extracted mass and percentage yield of CSE-H-70E and CSE-MW-70E.

Sample	Yield, mg			Yield, %
	Exp. 1	Exp. 2	Exp. 3	
CSE-H-70E	240	210	258	0.79±0.08
CSE-MW-70E	345	297	375	1.13±0.13

Data are presented as the mean ± standard deviation. n=3. Exp., experiment. CSE-H-70E, heat-70% EtOH extraction; CSE-MW-70E, microwave-70% EtOH extraction.

Signaling Technology), horseradish peroxidase-conjugated secondary antibodies, and detected by the application of ECL (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Determination of nitrite as an indicator of NO production. The production of nitrite, a stable end-product of NO oxidation, was used for the measurement of iNOS activity. The concentration of nitrite in the conditioned medium was determined by a method based on the Griess reaction (37). The NO levels were determined from the concentration of nitrite as assessed by the Griess reaction. The supernatant (100 μl) was mixed with Griess reagent (100 μl), and then the absorbance at 525 nm was determined by using an ELISA plate reader from Bio-Rad.

PGE_2, IL-1β, IL-6, IL-12 and TNF-α assays and DNA binding activity of NF-κB. The culture medium was collected and the level of PGE_2, IL-1β, IL-6, IL-12 and TNF-α present in each sample was determined by using a commercially available kit from R&D Systems, Inc. The DNA-binding activity of NF-κB in nuclear extracts was measured by using the TransAM kit (Active Motif, Carlsbad, CA, USA). The assays, including PGE_2, IL-1β, IL-6, IL-12 and TNF-α production and NF-κB DNA-binding activity, were performed in accordance with the manufacturer's instructions.

Reverse transcription-polymerase chain reaction (PCR). Total RNA was isolated from the cells by using TRIzol (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's recommendations, and quantified spectrophotometrically at 260 nm. The following primer sequences were designed by using PrimerQuest (Integrated DNA Technologies, Cambridge, MA, USA): HO-1, forward, 5'-CTCTTGGCTGGCTTCCTT-3' and reverse, 5'-GGCTCCTTCCTCCTTTCC-3'; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward, 5'-ACTTTG GTATCGTGGAAGGACT-3' and reverse, 5'-GTAGAGGCA GGGATGATGTTCT-3'. The optimal conditions for PCR amplification of the cDNA were established by in accordance with the manufacturer's instructions. The data were analyzed by using StepOne software (Applied Biosystems, Carlsbad, CA, USA), and the cycle number at the linear amplification threshold (Ct) values for the endogenous control gene (GAPDH) and the target gene were recorded. The relative gene expression (target gene expression normalized to the expression of the endogenous control gene) was calculated by using the comparative Ct method ($2^{-\Delta\Delta Ct}$).

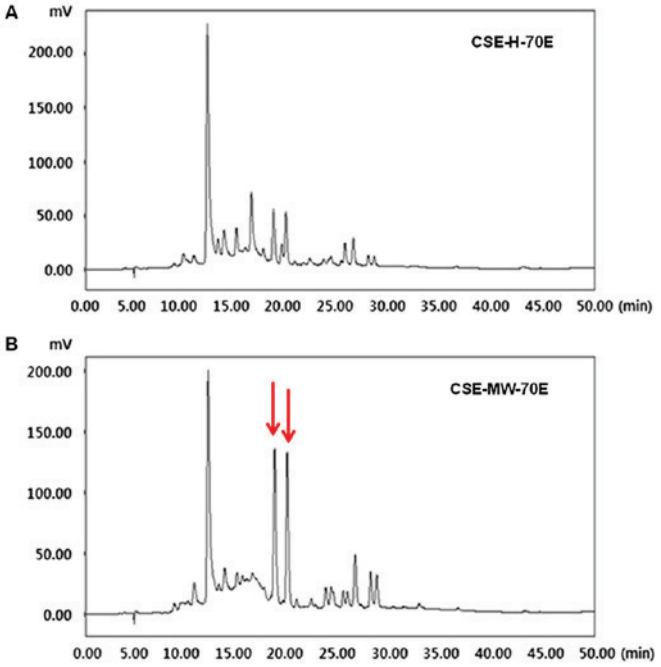


Figure 1. HPLC analysis demonstrated that there is a significant difference between the patterns of the two extracts. HPLC chromatograms of (A) CSE-H-70E and (B) CSE-MW-70E. HPLC, high-performance liquid chromatography; CSE-H-70E, heat-70% EtOH extraction; CSE-MW-70E, microwave-70% EtOH extraction.

Statistical analysis. The data are expressed as the mean ± standard deviation of three independent experiments. To compare between three groups, one-way analysis of variance followed by the Newman-Keuls post hoc test was used. Statistical analysis was computed using GraphPad Prism software, version 3.03 (GraphPad Software Inc., San Diego, CA, USA).

Results

HPLC analysis of CSE-H-70E and CSE-MW-70E. The HPLC analysis of CSE-H-70E and CSE-MW-70E was presented in the form of chromatograms of the detected reaction at 254 nm. As shown in Fig. 1A and B, the retention time of the main peak was 12.00 min for both fractions. However, in Fig. 1B, two identical peaks were observed at ~19-20 min for CSE-MW-70E.

Effects of CSE-H-70E and CSE-MW-70E on the expression of nitrite, PGE_2 production, and iNOS and COX-2 proteins in LPS-stimulated RAW264.7 cells. The primary indication of the anti-inflammatory effects of CSE-H-70E and CSE-MW-70E in LPS-induced RAW264.7 macrophages was assessed through the estimation of the concentrations of NO and PGE_2 and the expression of the iNOS and COX-2 proteins. We found that CSE-MW-70E was meaningfully more competent in terms of its anti-inflammatory effects than CSE-H-70E. RAW264.7 cells pretreated with CSE-MW-70E for 24 h exhibited lower iNOS expression (Fig. 2) and decreased PGE_2 production derived from COX-2 (Fig. 2). Similarly, CSE-MW-70E mitigated COX-2 expression and dose-dependently suppressed the production of NO (Fig. 2).

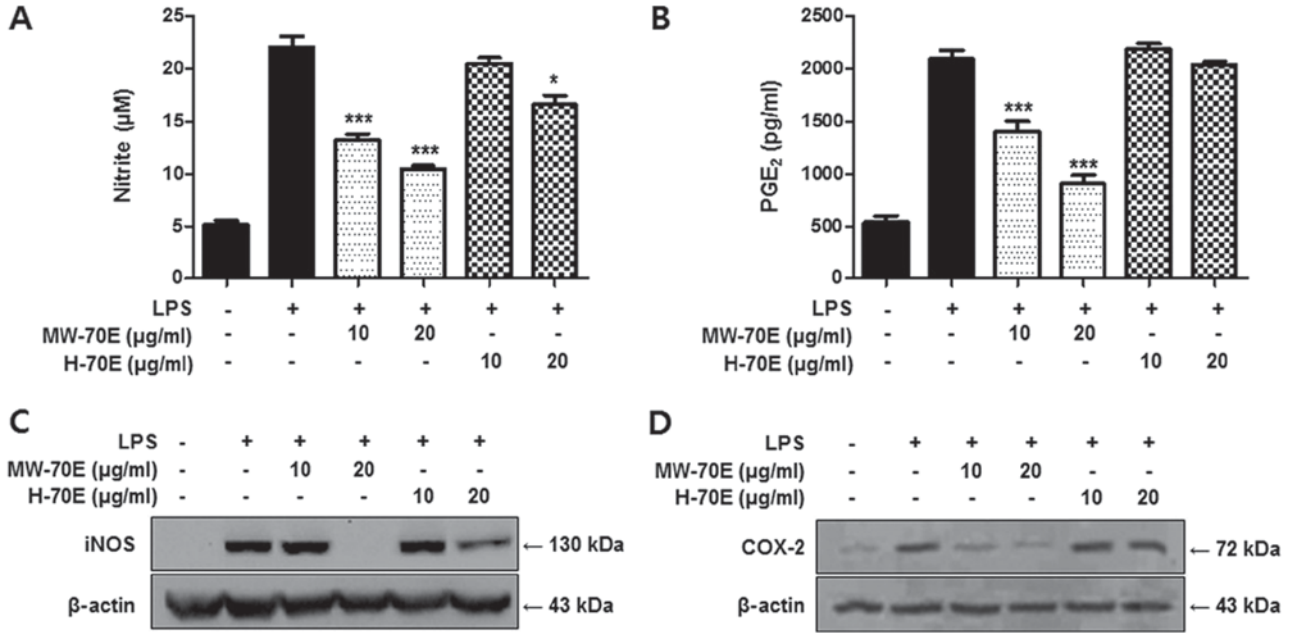


Figure 2. CSE-MW-70E suppresses the production of NO and PGE_2, and decreases iNOS and COX-2 protein expression. Effects of CSE-H-70E or CSE-MW-70E on (A) nitrite, (B) PGE_2, (C) iNOS and (D) COX-2 levels in RAW264.7 macrophages. Cells were pretreated for 3 h with indicated concentrations of Sappan Lignum extracts and stimulated 24 h with LPS (1 μg/m). Western blotting was performed; representative blots from three independent experiments are shown. The data are presented as the mean ± standard deviation of three experiments. *P<0.05, ***P<0.001 vs. the LPS-treated group. CSE-H-70E, heat-70% EtOH extraction; CSE-MW-70E, microwave-70% EtOH extraction; PGE_2, prostaglandin E_2; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; LPS, lipopolysaccharide.

Effects of CSE-H-70E and CSE-MW-70E on pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) and NF-κB binding activity in LPS-stimulated RAW264.7 cells. Further anti-inflammatory effects of CSE-H-70E and CSE-MW-70E were evaluated through the assessment of the TNF-α, IL-1β and IL-6 levels in LPS-stimulated RAW264.7 macrophages. Cells were pre-incubated with CSE-H-70E and CSE-MW-70E for 3 h and then treated with LPS for 24 h; CSE-MW-70E caused concentration-dependent decreases in IL-1β, IL-6 and TNF-α relative to CSE-H-70E (Fig. 3A-C). In addition, to confirm whether the NF-κB pathway was engaged in the suppression of inflammatory responses induced by CSE-H-70E and CSE-MW-70E, we measured NF-κB DNA binding activity in nuclear extracts from RAW264.7 cells that were stimulated by LPS for 1 h. This process resulted a relative 20-fold rise in the DNA binding activity of NF-κB, which was inhibited by CSE-MW-70E in a concentration-dependent manner (Fig. 3D).

Effects of CSE-H-70E and CSE-MW-70E on heme oxygenase-1 expression in RAW264.7 macrophages. We subsequently examined the effects of CSE-H-70E and CSE-MW-70E on HO-1 expression in RAW264.7 macrophages. The cells were treated with different concentrations of CSE-H-70E and CSE-MW-70E (10-20 μg/ml) for 12 h. Our data showed that CSE-MW-70E and the positive control, CoPP, induced a dose-dependent increase in HO-1 protein (Fig. 4A) and HO-1 mRNA (Fig. 4B) expression.

Effects of SnPP on nitrite, PGE_2, IL-1β, IL-6, TNF-α and NF-κB DNA-binding activity in CSE-MW-70E pretreated LPS-stimulated RAW264.7 macrophages. To confirm the interaction between the induction of HO-1 and the anti-inflammatory activity of CSE-MW-70E, we studied the effects of tin protoporphyrin (SnPP). The cells were pretreated for 3 h with CSE-MW-70E (20 μg/ml) in the presence or absence of SnPP (50 μM) and activated with LPS (1 μg/ml) for 24 h (Fig. 5A-E) or 1 h (Fig. 5F). As shown in Fig. 6, pretreatment with SnPP partly prevented the suppressive effects of CSE-MW-70E on the production of pro-inflammatory mediators and cytokines in LPS-activated cells.

Discussion

Different extracts of *C. sappan* have been shown to have anti-inflammatory and anti-arthritic activities (38,39). The EtOAc extract of Sappan Lignum was found to exert significant inhibitory effects on mitosis and growth-related signaling (35). The anti-inflammatory effects of various compounds from the EtOAc extract of Sappan Lignum were recently investigated (40). 70% EtOH is generally one of the efficient extraction solvent conditions. Both polar and nonpolar compounds can be effectively extracted, especially for extracting major active ingredients such as glycoside, flavonoid, and polyphenols, etc. However, the anti-inflammatory effect of 70% EtOH Sappan Lignum extract prepared by heat extraction or microwave extraction was not previously elucidated.

MASE has become increasingly popular over the last few decades (19), as it was shown to be more efficient for most applications compared with traditional extraction methods (20). Microwaves increase decoction temperature as hot continuous extraction and maceration, it is also similar to normal heat extraction method. However, microwave extraction has

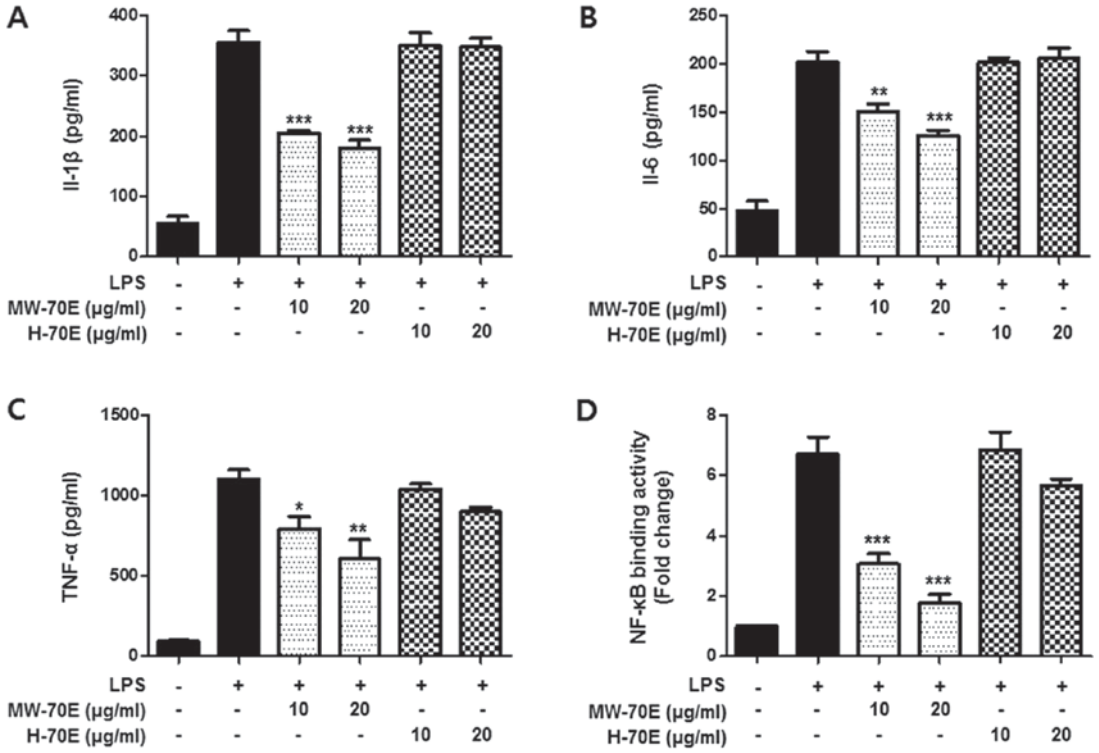


Figure 3. CSE-MW-70E suppresses LPS-induced production of IL-1 β, IL-6, TNF-α and NF-κB binding activity. Effects of CSE-H-70E or CSE-MW-70E on (A) IL-1β, (B) IL-6 and (C) TNF-α concentrations and (D) NF-κB binding activity in RAW264.7 macrophages. (A-C) The cells were pretreated for 3 h with the indicated concentrations of Sappan Lignum extracts and stimulated for 24 h with LPS (1 μg/ml). (D) The cells were pretreated for 3 h with the indicated concentrations of Sappan Lignum extracts and stimulated for 1 h with LPS (1 μg/ml). A commercially available NF-κB ELISA (Active Motif) kit was used to test the nuclear extracts and determine the degree of NF-κB binding. The data are presented as the mean ± standard deviation of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. the LPS-treated group. CSE-H-70E, heat-70% EtOH extraction; CSE-MW-70E, microwave-70% EtOH extraction; IL, interleukin; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; LPS, lipopolysaccharide.

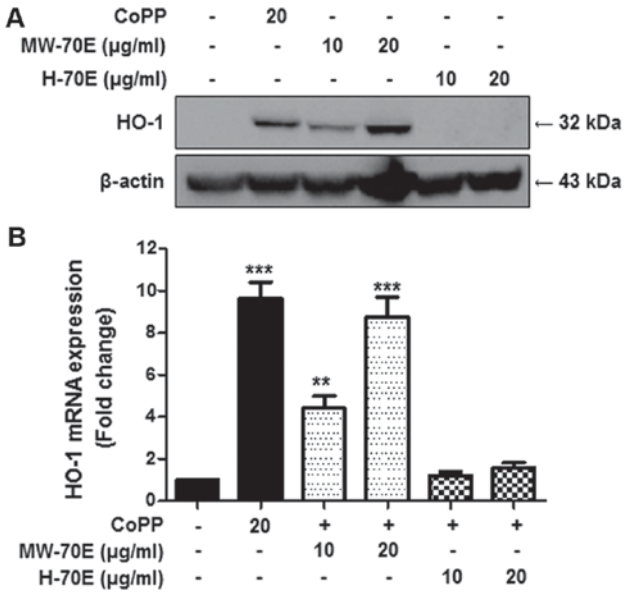


Figure 4. CSE-MW-70E increases the expression of HO-1 mRNA and protein. Inductive effects of CSE-H-70E or CSE-MW-70E on HO-1 expression in RAW 264.7 macrophages. The cells were pretreated for 12 h with indicated concentrations of Sappan Lignum extracts. (A) Western blotting results demonstrated concentration-dependent effects on HO-1 protein expression. (B) HO-1 mRNA expression was determined by reverse transcription-polymerase chain reaction. Representative blots from three independent experiments are presented. The data are presented as the mean ± standard deviation of three experiments. **P<0.01, ***P<0.001 vs. the control-treated group. CSE-H-70E, heat-70% EtOH extraction; CSE-MW-70E, microwave-70% EtOH extraction; HO-1, heme oxygenase-1; CoPP, cobalt protoporphyrin.

many advantages such as shorter extraction time, less solvent consumption, higher extraction rate, and better yield with lower energy consumption (41).

One of the complex biological responses of the body is inflammation, which causes adverse stimuli, such as pathogens or irritants that may disturb the function of cells (42). The response to inflammation is a complicated reaction of the immune system that is monitored by different inflammatory mediators and cytokines. Three isoforms of NOS are well-known: nNOS, eNOS, and iNOS (43). The production of NO in immune-activated macrophages is stimulated by iNOS, a crucial pro-inflammatory enzyme (44). In the current study, we observed that CSE-MW-70E dose-dependently suppressed the production of NO and PGE_2 and decreased iNOS and COX-2 protein expression (Fig. 2). The pro-inflammatory activities of cytokines, such as TNF-α, IL-1β, and IL-6, exert significant roles in the control of tumor progression and inflammation (45). In this study, we showed that the pretreatment of murine macrophages (RAW 264.7 cells) with CSE-MW-70E suppressed LPS-induced TNF-α, IL-6, and IL-1β production (Fig. 3). NF-κB is a fundamental molecule in a crucial signaling pathway associated with inflammatory diseases, and coordinates various pro-inflammatory genes and cytokines (46). We measured the NF-κB DNA-binding activity and found that CSE-MW-70E inhibited the increase in NF-κB DNA-binding activity in a dose-dependent manner in RAW264.7 cells stimulated with LPS to a greater extent than CSE-H-70E (Fig. 3). These findings suggested that the NF-κB pathway was a crucial

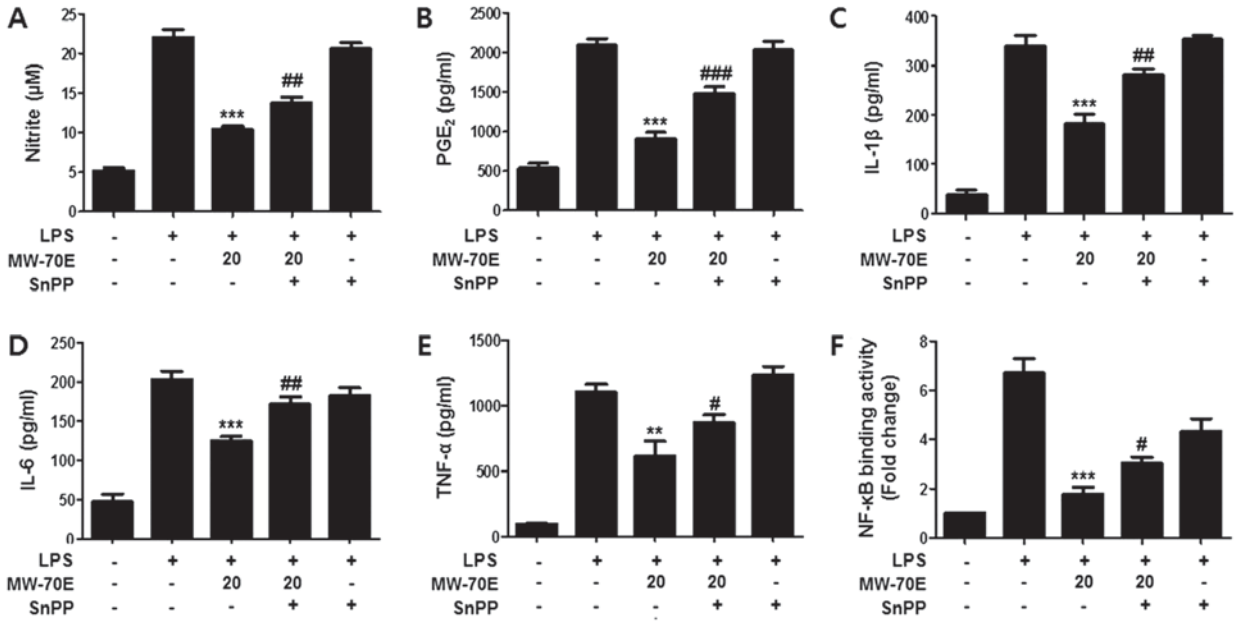


Figure 5. Pretreatment with SnPP partly reverses the suppressive effects of CSE-MW-70E against the production of pro-inflammatory mediators, cytokines and NF-κB DNA binding activity. Effects of SnPP on (A) nitrite, (B) PGE_2, (C) IL-1β, (D) IL-6, (E) TNF-α and (F) NF-κB DNA-binding activity in CSE-MW-70E pretreated LPS-stimulated RAW264.7 macrophages. The cells were pretreated for 3 h with Sappan Lignum extracts (20 μg/ml) in the presence or absence of SnPP (50 μM) and stimulated with LPS (1 μg/ml) for (A-E) 24 h or 1 h (F). The data are presented as the mean ± standard deviation of three experiments. $^{**}P<0.01$, $^{***}P<0.001$ vs. with the group treated with LPS alone; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. the group treated with CSE-MW-70E and LPS. SnPP, tin protoporphyrin; PGE_2, prostaglandin E_2; IL, interleukin; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; CSE-MW-70E, microwave-70% EtOH extraction; LPS, lipopolysaccharide.

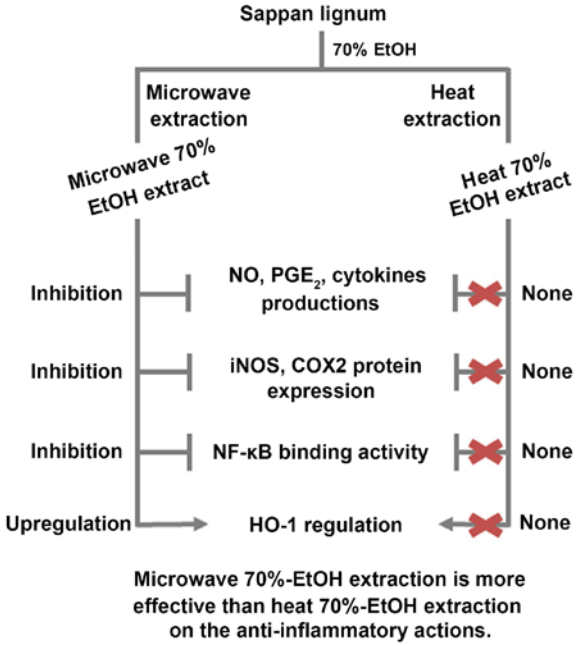


Figure 6. Extraction method influences the biological activity of Sappan Lignum. Scheme comparing the anti-inflammatory effects of microwave 70%-EtOH extract and heat 70%-EtOH extract. Microwave 70%-EtOH extraction was more effective than heat 70%-EtOH extraction with regard to the anti-inflammatory effects. Our results suggested that microwave extraction can be utilized for the improvement of the extraction efficiency and biological activity of Sappan Lignum. NO, nitric oxide; PGE_2, prostaglandin E_2; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; NF-κB, nuclear factor-κB; HO-1, heme oxygenase-1.

target for the action of CSE-MW-70E in the suppression of the induction of pro-inflammatory enzymes, mediators, and cytokines. The anti-inflammatory effects of the reaction of HO-1 were also recently demonstrated in a number of inflammatory models, along with its antioxidant effects (47,48). HO-1 exerts cytoprotective effects through its anti-inflammatory, anti-proliferative, and antiapoptotic activities, which are mediated by relatively few heme products containing CO, biliverdin, and free iron (Fe^{2+}). CO restrains pro-inflammatory responses and boosts anti-inflammatory responses in macrophages. By minimizing the expression of iNOS and COX-2, HO-1 and CO can also suppress the production of NO and PGE_2 (49). To evaluate whether the initiation of HO-1 plays a significant role in the CSE-H-70E and CSE-MW-70E, derived suppression of the pro-inflammatory responses, RAW264.7 cells were pretreated with CSE-H-70E and CSE-MW-70E, and the expression of HO-1 mRNA and protein was evaluated. We observed that pretreatment with CSE-MW-70E for 12 h significantly increased the expression of HO-1 mRNA and protein (Fig. 4).

As SnPP is a competitive inhibitor of the HO-1 response, we tested its influence on the suppressive effects of CSE-MW-70E on the production of pro-inflammatory mediators and cytokines in LPS-stimulated cells to confirm the interaction between HO-1 induction and the anti-inflammatory activity of CSE-MW-70E. Macrophages were pretreated with CSE-MW-70E (20 μg/ml) for 3 h in the presence or absence of SnPP (50 μM) and then activated by LPS. As shown in Fig. 5, pretreatment with SnPP partly reversed the suppressive effects of CSE-MW-70E against the production of proinflammatory mediators, cytokines, and NF-κB DNA binding activity in the cells. These findings provided further evidence that the anti-inflammatory activity of CSE-MW-70E was linked to the effects on the HO-1 expression pathway. The present study is not enough to validate the anti-inflammatory effects

of *Caesalpinia sappan* L., because major compounds have not yet identified and not conducted *in vivo* study. However, this study was focused and designed to investigate the difference between extraction efficiency and the active ingredient according to the extraction method.

In conclusion, it was found that the extraction method influenced the biological activity of Sappan Lignum. Our results showed that two types of Sappan Lignum 70% ethanol extract (CSE-H-70E and CSE-MW-70E) induced anti-inflammatory responses that were associated with their inhibitory effects against NF-κB pathway activation in LPS-stimulated cells. CSE-MW-70E has a greater potential for therapeutic applications than the CSE-H-70E. In addition, it was proven that the anti-inflammatory activity of CSE-MW-70E was correlated with its ability to HO-1 mRNA and protein expression (Fig. 6). In this manuscript we tried to compare the extraction efficiency and evaluation of biological activities of these two extracts of Sappan Lignum and. Thus, HPLC analysis showed there is a significant difference between these two extracts pattern at ~20 min (Fig. 1). Our bioassay results also showed that the anti-inflammatory effects are excellent due to the difference of these patterns, that might be active components. These results suggested that 70% ethanol-microwave extraction of Sappan Lignum (CSE-MW-70E) may be a favorable therapeutic agent and should be investigated further to establish its potential as a remedy for a range of inflammatory diseases. Further detailed investigation into the anti-inflammatory effects of CSE-MW-70E in *in vitro* and *in vivo* models of inflammatory diseases would assist in the development of its therapeutic potential. Safety of *Caesalpinia sappan* L. should be discussed in order to develop as a medicine. In our experiments, cell viability assay was assessed, and subsequently *in vitro* assay model was evaluated within a non-toxic concentration range. However, in order to evaluate the toxicity of *Caesalpinia sappan* L., it is necessary to conduct the clinical test through *in vivo* model assay. Therefore, in further study, we will conduct an anti-inflammatory mechanism experiment on *in vivo* model to confirm the possibility of development as a medicine.

Acknowledgements

Not applicable.

Funding

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (grant nos. 2015R1C1A1A02036465 and NRF-2018R1C1B6001913).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MDAC, MC and WK participated in the acquisition, analysis and interpretation of the data, and contributed to the writing of the manuscript. HL participated in the acquisition, analysis and interpretation of the data. SCK, HO and ERW designed the study and contributed to the writing of the manuscript. YCK and DSL designed the study, participated in the acquisition, analysis, and interpretation of the data, and contributed to the writing of the manuscript. All authors read approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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