

Anti-inflammatory and antioxidant activity of the traditional herbal formula Gwakhyangjeonggi-san via enhancement of heme oxygenase-1 expression in RAW264.7 macrophages

SOO-JIN JEONG^{1,2}, OHN-SOON KIM¹, SAE-ROM YOO³, CHANG-SEOB SEO³,
YEJI KIM^{3,4} and HYEUN-KYOO SHIN³

¹KM Convergence Research Division, Korea Institute of Oriental Medicine, Daejeon 34054;

²Korean Medicine Life Science, University of Science and Technology, Yuseong-gu, Daejeon 34113;

³K-herb Research Center, Korea Institute of Oriental Medicine, Daejeon, Chungcheong 34054;

⁴Mucosal Immunology Laboratory, Department of Convergence Medicine,
University of Ulsan College of Medicine/Asan Medical Center, Seoul 05505, Republic of Korea

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Abstract. Gwakhyangjeonggi-san (GHJGS) is a mixture of herbal plants, including *Agastache rugosa*, *Perilla frutescens*, *Angelica dahurica*, *Areca catechu*, *Poria cocos*, *Magnolia officinalis*, *Atractylodes macrocephala*, *Citrus reticulata*, *Pinellia ternata*, *Platycodon grandiflorum*, *Glycyrrhiza uralensis*, *Ziziphus jujuba* and *Zingiber officinale*. GHJGS has been used for treating diarrhea-predominant irritable bowel syndrome in traditional Korean medicine. In the present study, the anti-inflammatory and antioxidant effects of GHJGS were investigated using the RAW 264.7 murine macrophage cell line. GHJGS significantly reduced production of the proinflammatory cytokines, tumor necrosis factor- α , interleukin-6 and prostaglandin E₂ in lipopolysaccharide (LPS)-stimulated macrophages. GHJGS markedly suppressed LPS-induced phosphorylation of mitogen-activated protein kinases, whereas it had no effect on nuclear factor- κ B activation. Furthermore, GHJGS enhanced expression of heme oxygenase-1 and prevented the generation of reactive oxygen species in RAW 264.7 cells. These results indicate that GHJGS is a viable therapeutic agent against inflammation and oxidative stress-associated disorders.

Introduction

Gwakhyangjeonggi-san (GHJGS) is a traditional Korean herbal formula composed of the following 13 medicinal herbs, *Agastache rugosa*, *Perilla frutescens*, *Angelica dahurica*, *Areca catechu*, *Poria cocos*, *Magnolia officinalis*, *Atractylodes macrocephala*, *Citrus reticulata*, *Pinellia ternata*, *Platycodon grandiflorum*, *Glycyrrhiza uralensis*, *Ziziphus jujuba* and *Zingiber officinale*. It has been used for treating diarrhea-predominant irritable bowel syndrome (1). In addition, GHJGS has been identified as an effective treatment for allergies (2), respiratory (3) and cardiovascular (4) diseases, and bacterial infections (5). However, to the best of our knowledge, there have been no reports to date on the anti-inflammatory effect of GHJGS.

Inflammation is a protective response against various harmful stimuli, such as pathogens, damaged cells and irritants (6). This response is controlled by production of proinflammatory biomolecules (7,8). Overproduction of the proinflammatory cytokines, tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6), and the proinflammatory mediator, prostaglandin E₂ (PGE₂) may result in inflammatory disorders accompanied by fever, tissue destruction or pain (7,8). Therefore, targeting these proinflammatory cytokines or PGE₂ is considered to be a potential therapeutic approach for treating inflammatory disorders. Mitogen-activated protein kinase (MAPK) and/or nuclear factor- κ B (NF- κ B) signaling pathways are important in the regulation of inflammatory responses, including triggering the initiation of proinflammatory cytokine production (9). Additionally, previous studies have reported a link between anti-inflammatory and anti-oxidative regulation using various natural products through activation of heme oxygenase-1 (HO-1), an enzyme with antioxidant effects (10-12).

In the present study, the anti-inflammatory and antioxidant activity of GHJGS was investigated using the murine macrophage cell line, RAW 264.7. The inflammatory reaction was induced by lipopolysaccharide (LPS) stimulation and the

Correspondence to: Dr Hyeun-Kyoo Shin, K-herb Research Center, Korea Institute of Oriental Medicine, 1672 Yuseong-daero, Yuseong-gu, Daejeon, Chungcheong 34054, Republic of Korea
E-mail: hkshin@kiom.re.kr

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production of TNF- α , IL-6, and PGE₂ was examined using enzyme-linked immunosorbent assays (ELISAs). In addition, the effects of GHJGS on activation of MAPK and NF- κ B signaling pathways, and the expression of HO-1 in RAW 264.7 cells were investigated.

Materials and methods

Plant materials. The 13 herbs that form GHJGS were purchased from Kwangmyungdang Medicinal Herbs (Ulsan, South Korea). The taxonomic classification of the 13 herbs was verified by Professor Je-Hyun Lee from Dongguk University (Gyeongju, South Korea). Voucher specimens (2012-KE32-1 to KE32-13) were deposited at the K-herb Research Center, Korea Institute of Oriental Medicine (Daejeon, South Korea).

Chemicals and reagents. Liquiritin, glycyrrhizin and 6-gingerol were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Hesperidin and rosmarinic acid were purchased from Acros Organics (Morris, NJ, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. The purity of each component was determined to be $\geq 98\%$ using high-performance liquid chromatography (HPLC) analysis. The chemical structures of the five marker compounds are presented in Fig. 1A. HPLC-grade reagents, methanol, acetonitrile and distilled water were obtained from J.T. Baker; Avanto Performance Materials (Phillipsburg, NJ, USA). Acetic acid was obtained from Merck KGaA (Darmstadt, Germany).

Preparation of GHJGS decoction. GHJGS was composed of 13 herbs (Table I; total weight, 5.0 kg, ~ 148.15 times the composition of a single dose) and extracted in distilled water at 100°C for 2 h under 98 kPa pressure using a COSMOS-660 electric extractor (KyungSeo Machine Co., Incheon, South Korea). The extracted solution was filtered using a standard sieve (no. 270; mesh size, 53 μ m; Chung Gye Sang Gong Sa, Seoul, Korea) and freeze-dried. The yield of the extract was 12.89% (644.5 g). The lyophilized GHJGS extract (40 mg) was dissolved in 50% methanol (20 ml) and mixed for quantitative analysis. The solution was filtered through a 0.2- μ m SmartPor GHP syringe filter (Woong Ki Science Co., Ltd., Seoul, South Korea) prior to being injected into a HPLC column.

Quantitative analysis of GHJGS. The quantitative determination was performed using a Prominence LC-20A series HPLC system (Shimadzu Corporation, Kyoto, Japan) consisting of a solvent delivery unit (LC-20AT), online degasser (DGU-20A3), column oven (CTO-20A), auto sample injector (SIL-20AC), and photodiode array (PDA) detector (SPD-M20A). Data were collected and processed using LC solution software (version 1.24; Shimadzu Corporation). A Gemini C18 column (250 mm x 4.6 mm; particle size, 5 μ m; Phenomenex, Inc., Torrance, CA, USA) was used for separation of the marker compounds and maintained at 40°C. The mobile phases consisted of 1.0% (v/v) acetic acid in distilled water (designated as A) and 1.0% (v/v) acetic acid in acetonitrile (designated as B). The gradient flow was as follows: 5-70% B for 0-40 min; 70-100% B for 40-50 min; 100% B for 50-55 min; and 100-5% B for 55-60 min. The analysis was conducted at 1.0 ml/min with PDA detection at 254 nm

(glycyrrhizin), 280 nm (liquiritin, hesperidin and 6-gingerol), and 330 nm (rosmarinic acid). The sample injection volume was 10 μ l.

Cell culture. The murine macrophage cell line, RAW 264.7, was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 5.5% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml; HyClone Laboratories, Inc., Logan, UT, USA), and streptomycin (100 μ g/ml; HyClone Laboratories, Inc.) in an incubator with 5% CO₂ at 37°C.

Cytotoxicity assay. Cell viability assay was performed to determine the cytotoxicity of GHJGS using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Cells were plated onto a 96-well microplate at 3×10^3 cells/well and treated with 0, 15.625, 31.25, 62.5, 125, 250, 500 or 1,000 μ g/ml GHJGS for 24 h. Following incubation with CCK-8 reagent for 4 h, optical density (OD) at a wavelength of 450 nm was determined using a Benchmark plus microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell viability was calculated using the following equation: Cell viability (%) = mean OD_{GHJGS-treated cells} / mean OD_{untreated cells} x100

ELISAs for TNF- α , IL-6, and PGE₂. Cells were pretreated with 0, 250, 500 or 1,000 μ g/ml GHJGS for 4 h and stimulated with LPS (1 μ g/ml) for an additional 20 h. Production of TNF- α , IL-6 and PGE₂ in the culture supernatants was measured using commercial ELISA kits from R&D Systems, Inc. (Minneapolis, MN, USA), BD Biosciences (San Jose, CA, USA) and Cayman Chemical Company (Ann Arbor, MI, USA), respectively. Indomethacin (2.5 ng/ml; Sigma-Aldrich) was used as a positive control.

Western blotting. Whole cell extract (WCE) was prepared by suspending cells using the Mammalian Cell Lysis kit (Sigma-Aldrich) containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). Nuclear extract (NE) was isolated using NE-PER[®] Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Inc., Rockford, IL, USA) according to the manufacturer's protocol. The protein concentration was determined using the Bio-Rad Protein Assay kit II (Bio-Rad Laboratories, Inc.). Equal quantities of cell extract (30 μ g) were resolved by 4-20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad Laboratories, Inc.) at 100 v for 1 h and transferred to a polyvinylidene fluoride membrane (GE Healthcare Life Sciences, Piscataway, NJ, USA). The membrane was incubated with blocking solution [5% skimmed milk in Tris-buffered saline containing Tween-20 (TBST); DyneBio, Seongnam, Korea], followed by an overnight incubation at 4°C with the appropriate primary antibody, including rabbit polyclonal phosphorylated (p)-p38 MAPK (1:1,000 dilution; cat. no. 9211; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit polyclonal p-extracellular signal-regulated kinase (ERK; 1:1,000 dilution; cat. no. 9101; Cell Signaling Technology, Inc.), rabbit polyclonal p-c-Jun N-terminal kinase (JNK; 1:1,000

Table I. Composition of Gwakhyanjeonggi-san.

Latin name	Scientific name	Quantity (g)	Origin
Agastachis Herba	<i>Agastache rugosa</i> (Fisch. et Meyer) O. Kuntze	833.30	Andong, South Korea
Perillae Herba	<i>Perilla frutescens</i> var. <i>crispa</i> (Thunb.) H. Deane	555.56	Yeongcheon, South Korea
Angelicae Dahuricae Radix	<i>Angelica dahurica</i> Benth. et Hook. f.	277.78	Uljin, South Korea
Arecae Pericarpium	<i>Areca catechu</i> L.	277.78	China
Hoelen	<i>Poria cocos</i> F. A. Wolf	277.78	Pyeongchang, South Korea
Magnoliae Cortex	<i>Magnolia officinalis</i> Rehd. et E. H. Wils.	277.78	China
Atractylodis Rhizoma Alba	<i>Atractylodes macrocephala</i> Koidz.	277.78	China
Citri Unshius Pericarpium	<i>Citrus reticulata</i> Blanco	277.78	Jeju, South Korea
Pinelliae Tuber	<i>Pinellia ternata</i> Breit.	277.78	China
Platycodi Radix	<i>Platycodon grandiflorum</i> A. DC.	277.78	Andong, South Korea
Glycyrrhizae Radix et Rhizoma	<i>Glycyrrhiza uralensis</i> Fisch.	277.78	China
Zizyphi Fructus	<i>Ziziphus jujuba</i> var. <i>inermis</i> (Bunge) Rehder	555.56	Yeongcheon, South Korea
Zingiberis Rhizoma Crudus	<i>Zingiber officinale</i> Rosc.	555.56	Ulsan, South Korea
Total		5,000	

dilution; cat. no. 9251; Cell Signaling Technology, Inc.), mouse monoclonal HO-1 (1:1,000 dilution; cat. no. ab13248; Abcam, Boston, MA, USA), rabbit polyclonal NF- κ B p65 (1:1,000; cat. no. sc-372; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and mouse monoclonal β -actin (1:5,000; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.). The membranes were washed three times with TBST, and then incubated with polyclonal horseradish peroxidase-conjugated goat anti-mouse IgG (cat. no. 115-001-003; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA; 1:2,000 dilution) and goat anti-rabbit IgG (cat. no. 111-001-003; Jackson ImmunoResearch Laboratories, Inc.; 1:2,000 dilution) secondary antibodies for 1 h at room temperature. The membranes were washed three times with TBST, and developed using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.). Image capture was performed using Chemi-Doc (Bio-Rad Laboratories, Inc.).

Reactive oxygen species (ROS) staining. To examine the generation of ROS, the ROS-ID™ Total ROS Detection kit (Enzo Life Sciences, Inc., Plymouth Meeting, PA, USA) was used. The effect of GHJGS on ROS generation was examined by immunofluorescence staining. Cells were plated on μ -Dishes 35 mm (Ibidi, Aarhus, Denmark), treated with GHJGS (1,000 μ g/ml) and LPS (1 μ g/ml) for 30 min, and fixed in 4% paraformaldehyde (Sigma-Aldrich) and 100% acetone (Sigma-Aldrich). The ROS detection solution was loaded to the cells and incubated at room temperature for 1 h. Following the addition of a mounting medium (Vector Laboratories Inc., Burlingame, CA, USA), the stained cells were visualized using a FLUOVIEW FV10i confocal microscope (Olympus Corporation, Tokyo, Japan).

Statistical analysis. The data are expressed as the mean \pm standard error of the mean. Data were analyzed using one-way analysis of variance and Dunnett's multiple comparisons test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Quantitative determination of the five marker compounds in GHJGS. The novel HPLC-PDA method was applied for simultaneous quantification of the five marker compounds in GHJGS. The typical chromatogram patterns for standard compounds and the GHJGS decoction are presented in Fig. 1B and C. The retention times of the liquiritin, hesperidin, rosmarinic acid, glycyrrhizin and 6-gingerol were ~17.35, 19.35, 20.66, 33.39, and 33.88 min, respectively. The concentrations of the five components were between 1.18 and 3.16 mg/g (Table II).

GHJGS inhibits production of TNF- α , IL-6 and PGE₂ in LPS-stimulated RAW 264.7 cells. Cytotoxicity of GHJGS was evaluated using RAW 264.7 cells. Cells were treated with serial dilutions of GHJGS for 24 h. Fig. 2 demonstrates that no cytotoxic effect was observed up to 1,000 μ g/ml GHJGS treatment. For the subsequent assays, cell treatment with GHJGS was performed in the nontoxic concentration range (250-1,000 μ g/ml).

GHJGS reduces the levels of TNF- α , IL-6 and PGE₂ in LPS-stimulated RAW 264.7 cells. To examine the anti-inflammatory effect of GHJGS, production of TNF- α and IL-6 was assessed in LPS-stimulated RAW 264.7 cells. LPS stimulation significantly increased levels of TNF- α and IL-6 in RAW 264.7 cells, compared with untreated controls. By contrast, GHJGS treatment significantly reduced LPS-induced production of TNF- α and IL-6 ($P < 0.01$; Fig. 3A and B). The quantity of PGE₂ was also determined and indomethacin served as a positive control. The level of PGE₂ was significantly increased in cells treated with LPS alone. By contrast, GHJGS treatment significantly reduced PGE₂ production by LPS stimulation ($P < 0.01$; Fig. 3C).

GHJGS suppresses phosphorylation of MAPK family proteins in LPS-stimulated RAW 264.7 cells. As demonstrated by

Table II. Concentration of the five marker compounds in Gwakhyangjeonggi-san by high-performance liquid chromatography.

Compound	Concentration			Source
	Mean (mg/g; n=3)	SD	RSD (%)	
Liquiritin	1.18	0.01	1.08	<i>Glycyrrhiza uralensis</i>
Hesperidin	3.16	0.01	0.27	<i>Camellia reticulata</i>
Rosmarinic acid	1.45	0.01	0.74	<i>Agastache rugosa</i> and <i>Perilla frutescens</i>
Glycyrrhizin	3.03	0.02	0.58	<i>Glycyrrhiza uralensis</i>
6-Gingerol	1.35	0.02	1.13	<i>Zingiber officinale</i>

SD, standard deviation; RSD, relative standard deviation.

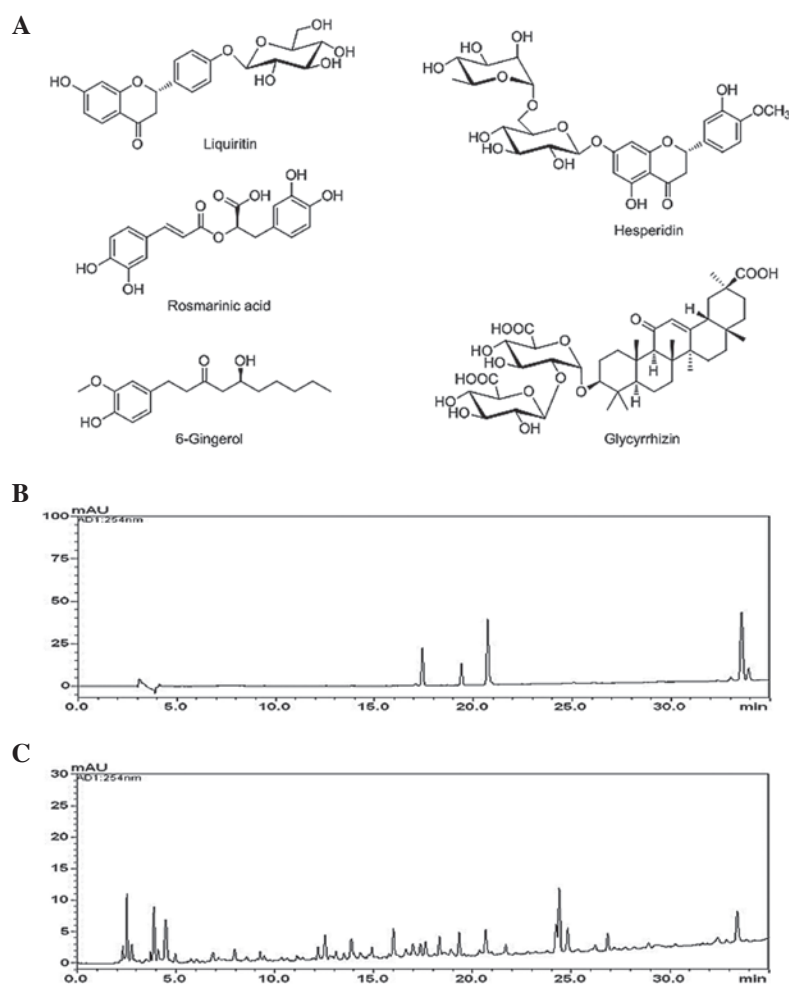


Figure 1. Chemical structures of (A) the five marker compounds, liquiritin, hesperidin, rosmarinic acid, 6-gingerol and glycyrrhizin. High-performance liquid chromatography chromatograms of the (B) standard mixture and (C) Gwakhyangjeonggi-san extract at 254 nm.

Fig. 4A, LPS markedly enhanced phosphorylation of p38 MAPK, ERK and JNK in RAW 264.7 cells, compared with untreated controls. LPS-induced activation of MAPKs was inhibited when cells were pretreated with GHJGS. NF- κ B p65 expression in the nucleus of RAW 264.7 cells was also analyzed. LPS increased the expression level of NF- κ B p65, compared with that of untreated controls. When cells were exposed to LPS and GHJGS, the GHJGS exerted no

influence on NF- κ B p65 in the LPS-stimulated RAW 264.7 cells (Fig. 4B).

GHJGS induces HO-1 expression and blocks LPS-induced ROS generation in RAW 264.7 cells. Cells distinctly stained with green fluorescence were observed in LPS-stimulated cells compared with the undifferentiated control (Fig. 5A). By contrast, GHJGS treatment blocked LPS-mediated ROS

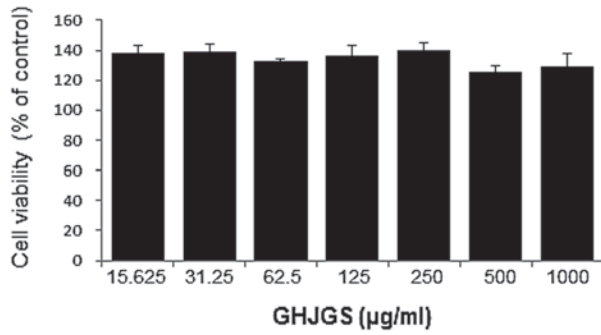


Figure 2. Cytotoxic effect of GHJGS on RAW 264.7 cells. Cells were plated onto 96-well microplates and treated with various concentrations of GHJGS for 24 h. Cell viability (%) was assessed using a Cell Counting Kit-8 assay. The values are expressed as the mean \pm standard error of three independent experiments. GHJGS, Gwakhyangjeonggi-san.

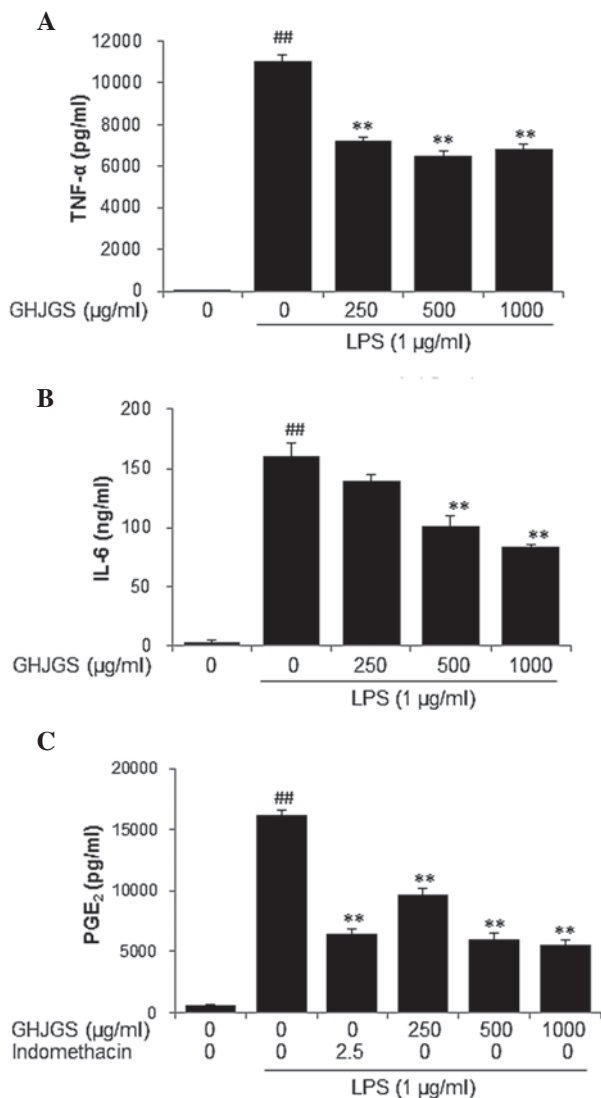


Figure 3. Effects of GHJGS on production of TNF- α , IL-6 and PGE₂ in LPS-stimulated RAW 264.7 cells. Levels of (A) TNF- α , (B) IL-6 and (C) PGE₂ were estimated by enzyme-linked immunosorbent assays. Cells were pretreated with a range of different GHJGS concentrations for 4 h and exposed to LPS for an additional 20 h. The bar graphs represent the mean \pm standard error of three independent experiments. ##P<0.01 vs. vehicle control cells; **P<0.01 vs. LPS-treated cells. TNF- α , tumor necrosis factor- α ; GHJGS, Gwakhyangjeonggi-san; LPS, lipopolysaccharide; IL-6, interleukin-6; PGE₂, prostaglandin E₂.

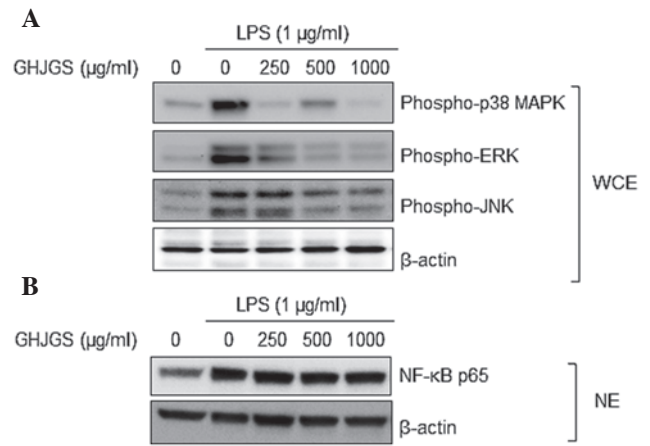


Figure 4. Effect of GHJGS on the activation of MAPKs and NF- κ B in LPS-stimulated RAW 264.7 cells. (A) WCEs were prepared and subjected to western blotting to detect phosphorylation of p38 MAPK, ERK and JNK. (B) NEs were isolated and subjected to western blotting for NF- κ B p65 detection. GHJGS, Gwakhyangjeonggi-san; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; NF- κ B, nuclear factor- κ B; WCE, whole cell extract; NE, nuclear extract.

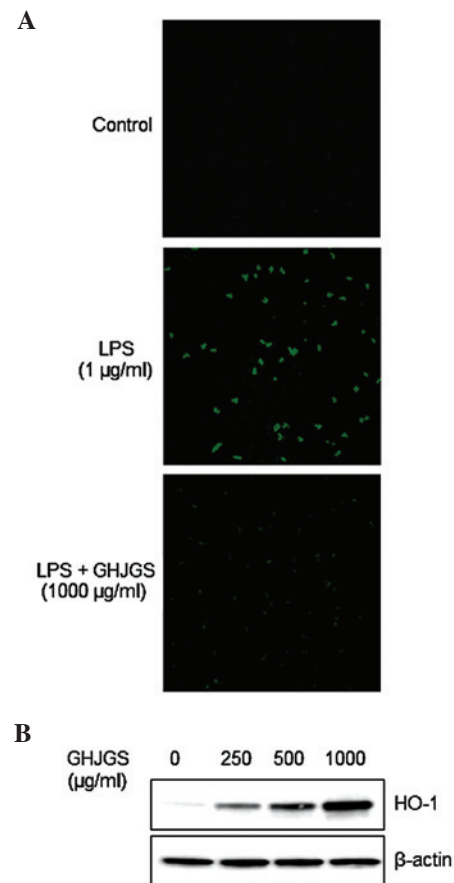


Figure 5. Effects of GHJGS on ROS generation and HO-1 expression in RAW 264.7 cells. (A) Cells were pretreated with GHJGS (1,000 μ g/ml) for 4 h and stimulated with LPS (1 μ g/ml) for 30 min. Cells were fixed in 4% paraformaldehyde, stained with Oxidative Stress Detection Reagent and visualized under an Olympus FLUOVIEW FV10i confocal microscope ($\times 100$). GHJGS reduced LPS-mediated ROS generation stained with green fluorescence. (B) Cells were treated with 250, 500 or 1,000 μ g/ml GHJGS for 24 h. Whole cell extracts were prepared and subjected to western blotting for HO-1. LPS, lipopolysaccharide; GHJGS, Gwakhyangjeonggi-san; HO-1, heme oxygenase-1; ROS, reactive oxygen species.

generation (Fig. 5A). Additionally, GHJGS induced HO-1 expression in a dose-dependent manner (Fig. 5B).

Discussion

Although the global market share for synthetic therapeutic agents is gradually increasing annually, various negative features, including toxicity and severe side effects, may limit their therapeutic efficacies and result in reduced quality of life (13,14). To overcome the issues associated with synthetic therapeutic agents, natural products (including herbal medicines) have been considered as a valuable source for establishing novel remedies for numerous years (15,16). Therefore, the present study aimed to determine the anti-inflammatory and antioxidant effects of the herbal formula, GHJGS using *in vitro* experimental models. GHJGS suppressed LPS-stimulated production of TNF- α , IL-6 and PGE₂, and inhibited LPS-mediated phosphorylation of MAPKs. GHJGS increased the scavenging activity of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) and di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium radicals, and reduced low-density lipoprotein oxidation (data not shown). In addition, GHJGS enhanced the level of HO-1 expression and reduced the LPS-induced generation of ROS in RAW 264.7 macrophages.

Table I presents the relative quantities and origin of the 13 herbs that form GHJGS. To improve quality control of GHJGS, simultaneous analysis of the marker compounds in GHJGS were conducted using the HPLC-PDA method. The primary active ingredients of each herb are as follows: Phenylpropanoids (e.g. rosmarinic acid) from *A. rugosa* (17), phenylpropanoids (e.g. rosmarinic acid) and flavonoids (e.g. luteolin) from *P. frutescens* (18,19), coumarins (e.g. imperatorin) from *A. dahurica* (20), coumarins (e.g. catechin) from *A. catechu* (21), triterpenoids (e.g. pachymic acid) from *P. cocos* (22), lignans (e.g. magnolol) from *M. officinalis* (23), sesquiterpenoids (e.g. atractylenolide I) from *A. macrocephala* (24), flavonoids (e.g. hesperidin) from *C. reticulata* (25), phenolic acid (e.g. homogentisic acid) and phenolic aldehyde (e.g. 3,4-dihydroxybenzaldehyde) from *P. ternate* (26), triterpenoids (e.g. platycodin D) from *P. grandiflorum* (27), triterpene saponin (e.g. glycyrrhizin) and flavonoids (e.g. liquiritin and liquiritigenin) from *G. uralensis* (28), flavonoids (e.g. spinosin and 6'''-feruloylspinosin) from *Z. jujube* (29), and phenols (e.g. 6-gingerol) from *Z. officinale* (30). Among those components, five compounds were investigated, including liquiritin and glycyrrhizin (*G. uralensis*), hesperidin (*C. reticulata*), rosmarinic acid (*A. rugosa* and *P. frutescens*), and 6-gingerol (*Z. officinale*) using HPLC-PDA. Consequently, hesperidin (3.16 mg/g) and glycyrrhizin (3.03 mg/g), marker compounds of *C. reticulata* and *G. uralensis*, respectively, were identified as the predominant components.

The inhibitory effects of GHJGS on inflammatory response were evaluated using the murine macrophage cell line, RAW 264.7. Macrophages are involved in the initiation, maintenance and resolution of inflammation (31,32), and thus considered to be useful for inflammation-associated studies. Activated macrophages stimulate production of proinflammatory cytokines, such as TNF- α and IL-6 during pathological conditions of inflammatory disease (33). Significant increases

in the production of TNF- α and IL-6 in LPS-stimulated RAW 264.7 cells were observed in the current study. GHJGS significantly inhibited TNF- α and IL-6 production induced by LPS treatment. PGE₂, a proinflammatory mediator, is produced through inflammatory stimulation of cyclooxygenase-2 (34). LPS stimulation significantly increased the level of PGE₂, whereas GHJGS treatment markedly reduced LPS-mediated PGE₂ production in RAW 264.7 cells. These results indicate the anti-inflammatory properties of GHJGS.

Production of proinflammatory factors, including TNF- α , IL-6, and PGE₂, is regulated by numerous intracellular signaling pathways at the transcription and post-transcription level (9). Inflammatory stimuli, such as LPS, activate MAPK and/or NF- κ B signaling pathways associated with inflammatory cytokine production (35-37). In the present study, LPS stimulation markedly enhanced the levels of p-p38 MAPK, p-ERK and p-JNK, and nuclear expression levels of NF- κ B p65 in RAW 264.7 cells. By contrast, GHJGS suppressed LPS-induced phosphorylation of p38 MAPK, ERK and JNK. However, NF- κ B activation was not altered by GHJGS in LPS-treated RAW 264.7 cells.

Inflammation is associated with oxidative stress. During inflammation, ROS generation is a critical event in the elimination of pathogens (38), and induces production of proinflammatory cytokines and molecules (32,39). Thus, the present study examined whether GHJGS has an inhibitory effect on ROS generation and determined that GHJGS suppressed LPS-induced ROS generation in macrophages. HO-1, a stress-inducible and redox-sensitive enzyme, is important during the inflammatory response (40). HO-1 negatively regulates production of proinflammatory cytokines, such as TNF- α , IL-1 β and IL-6 in activated macrophages (41). Thus, HO-1 is a potential molecular target against inflammation and oxidative stress (42). The current study identified that GHJGS increased the expression of HO-1 in a dose-dependent manner.

In conclusion, the findings of the present study demonstrate that GHJGS inhibits LPS-stimulated production of proinflammatory biomarkers, TNF- α , IL-6 and PGE₂ through suppression of the MAPK signaling pathway. Furthermore, GHJGS inhibited ROS generation and enhanced HO-1 expression. Overall, these findings confirm the anti-inflammatory and antioxidant actions of GHJGS, thus presenting it as a potential candidate for targeting inflammatory diseases and oxidative stress-associated diseases.

Acknowledgements

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