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Herb Mixture Inhibits Proinflammatory Mediators Through the Suppression of JNK and P38 Activation in LPS-Stimulated Raw 264.7 Macrophages

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Abbreviations: DPPH: 2,2-Diphenyl-1-Picrylhydrazy; JNK: c-Jun Nterminalkinase; TNF-α: Tumornecrosisfactor-α; IL: Interleukin; NOS: Nitric Oxide synthase; LPS: Lipopolysaccharide; MIP: Macrophage, Inflammatory Protein; COX2: Cyclooxygenase2

ABSTRACT

Inflammation has been implicated as a pathophysiological feature underlying many chronic diseases. In the present study, we investigated the anti-inflammatory effect of new formulation of herb medicine, DG1102, composed by five different herbal ingredients such as *Taraxaci Herba*, *Rhei Rhizoma, Houttuynia cordata* Thunb. *Houttuyniae Herba*, *Eriobotryae Folium* which has been used for inflammatory diseases, in LPS-stimulated Raw 264.7 macrophages. DG1102 suppressed nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin (IL)-1α, IL-6, IL-10 and macrophage inflammatory protein production in the lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages, as well as inducible nitric oxide synthase and cyclooxygenase 2 expression. Furthermore, DG1102 suppressed the activation of p38 and c-Jun N-terminal kinase (JNK). Our results suggest that DG1102 has an immune-modulatory activity suppressing production of pro-inflammatory cytokines and chemokine via the suppression of JNK and p38 MAPK activation in LPS-stimulated RAW 264.7 macrophages. The present study provides the biological evidence of the efficacy of medicinal plants in the treatment of inflammatory diseases.

INTRODUCTION

Inflammation has been implicated as a pathophysiological feature underlying many chronic diseases. Inflammatory responses and clinical symptoms are controlled through cytokines, nitric oxide (NO) and lipid mediators including prostaglandins and leukotrienes produced by macrophages, neutrophils, and other inflammatory cells [1,2]. Although diverse mechanisms have been

proposed to account for inflammation, activated macrophages play a particularly important role in the mediation of inflammation via the generation of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), NO and prostaglandin E2 (PGE2) [3,4]. Macrophages are essential in releasing pro-inflammatory mediators in response to various pathogen microbes before initiating inflammatory cascade. Lipopolysaccharide (LPS), one of the pro-inflammatory stimuli as a major component of the outer membranes in Gram-negative bacteria, elicits strong immune responses. When macrophages are activated by LPS, the toll-like receptor (TLR)-4 signaling pathway, which involves the phosphorylation of mitogen-activated protein kinase (MAPK) and the activation of transcription factor nuclear factor-kappa B (NF-κB), is initiated [5]. This then induces the production of pro-inflammatory mediators such as inducible nitric oxide synthase (iNOS), NO, IL-6, IL-1β, TNF-α, cyclooxygenase-2 (COX-2), etc. [6,7].

Despite the differences in constituents and pharmacological activities of various species, the genus of Taraxacum has long been known to contain antioxidant property besides anti-inflammatory, anti-carcinogenic, anti-allergic, anti-hyperglycemic, anti-coagulatory and analgesic activities [8]. Taraxaci Herba (TH) (*Tarazacum mongolicum* Hand.-Mazz.) is derived from the dried whole plant and has been found to have an antiviral effect against herpes simplex virus by enhancing immunological response [9]. Especially, eudesmanolides rich in the ethyl acetate fraction of Taraxacum mongolicum suppressed production of NO in activated RAW 264.7 macrophages [10]. Because of its purgative, analgesic and anti-stroke activities, Rhei R*hizoma* (RR) with its primary constituent of stilbenes has traditionally been used as an ingredient in polyprescriptions. Rhaponticin found in the rhizome of *Rhei Rhizoma* as one of the stilbenes *was proven to have extensive anti-allergic and anti-thrombotic properties* [11]. *Houttuynia cordata* Thunb. (Saururaceae; HC) has been used in traditional medicine for treatment of suppuration, chronic bronchitis, pneumonia, otitis and cystitis [12]. *H. cordata* possesses a variety of pharmacological functions including ant-ioxidant, anti-inflammatory, and anti-allergy effects due to a number of phenolic components [13-15]. Although the whole plant of *H. cordata* is generally used as herbal medicine, more antioxidant activities as well as the total phenolic and flavonoid contents were observed in the leaf and flower than root and stem [16]. *Houttuyniae Herba* (HH), the aerial part of *H. cordata*, has been reported to have neuroprotective effects [17]. *Eriobotryae Folium* (EF), the dried leaves of *Eriobotryae japonica* (loquat), has been used for chronic bronchitis with known anti-oxidant and anti-inflammatory activities. Recently loquat tea extract was found to inhibit the production of pro-inflammatory factors by downregulating both MAPK and NF-κB pathways [18]. *Ampelopsis Radix* (AR), the root of *Ampelopsis japonica* (Thunb.) Makino, has been used as traditional medicine to treat pain and fever with resveratrol as one of main compounds [19]. Extract of *Ampelopsis Radix* protects dopaminergic neurons via antioxidant mechanisms in Parkinson's disease models [20].

In the present study, we investigated the anti-inflammatory effect of new formulation of herb medicine (named as DG1102), composed by five different herbal ingredients such as TH, RR, HH, EF and AR, in LPS-stimulated Raw 264.7 macrophages.

MATERIALS and METHODS

Preparation of herbal extracts

All medicinal herbs used were purchased from Omni Herb (Seoul, Korea) and authenticated by Dr. D-I. Kim. Each voucher specimen was identified by Dr. S. D. Park (Department of Herbal Formula, College of Traditional Korean Medicine, Dongguk University) and deposited in the herbal formula. To prepare the extract of DG1102, a total of 115 g of the mixture containing five different herbal ingredients such as herba of *Tarazacum mongolicum* Hand.-Mazz. (*Taraxaci Herba*, TH, 40 g), rhizome of *Rheum officinale* Baillon *(Rhei Rhizoma*, RR, 20 g), herba of *Houttuynia cordata* Thunb. (*Houttuyniae Herba*, HH, 20 g), folium of *Eriobotrya japonica* (Thunb.) Lindl. (*Eriobotryae Folium*, EF, 20 g) and radix of *Ampelopsis japonica* (Thunb.) Makino (*Ampelopsis Radix*, AR, 15 g) was pulverized and extracted twice with 8 volume of distilled water at 100°C with reflux condenser for 3 h, then filtered with 50 μm filter and concentrated by vacuum evaporation at 60°C. The final yield from the whole procedure was approximately 19.88 g of dried mixture (average yield rate is 17.29%). The dried material was stored at -80°C until use. For cell culture works, each herbal plant was extracted separately with 30% ethanol and concentrated by vacuum evaporation at 60°C.

UPLC-ESI-MS analysis

In the current study, an ultraperformance liquid chromatography coupled with electrospray ionization mass spectrometry (UPLC-ESI-MS) method was applied for investigating the pharmacochemistry of DG1102. It is known that herbal effects are expressed through multicomponents with multitargets. Methyl alcohol and formic acid were high performance liquid chromatography (HPLC) grade and purchased from Duksan Pure Chemicals Co. (Ansan, South Korea). High purity nitrogen gas was provided by Shinyang Oxygen Co. (Seoul, South Korea). Other chemicals are of analytical grade. The 30 mg of DG1102 water extract was dissolved in 1 mL distilled water to yield a final concentration of 30 mg/mL and filtered through a 0.2 μm cellulose acetate syringe filter (Advantec, California, USA) before being injected for UPLC-ESI-MS analysis. The chromatographic separation of the components of DG1102 was performed on a Waters ACQUITY UPLC H-Class system (Waters Corp., Milford, MA, USA) fitted with Empower software. The PDA detector was recorded between 195~500 nm. The Brownlee SPP C18 column (3.0 × 100 mm, 2.7 cm) was selected for the UPLC study (PerkinElmer, USA). The monitoring wavelength was set to 280 nm. The mobile phase was comprised of acidified methyl alcohol with formic acid (0.1%, solvent A) and acidified water with formic acid (0.1%, solvent B). All solvents were filtered through a 0.2 um filter. The gradient program was 0-3 min, 1% of solvent A; 5 min, 10% of solvent A; 35 min, 40% of solvent A; 50-54 min, 100% of solvent A; 55 min, 1% solvent A, at a flow rate of 0.5 mL per min using a commercial splitter. The injection volume was 3 μL. AccuTOF® single-reflectron time-of-flight mass spectrometer was equipped with an ESI source (Electrospray

ionization, JEOL, USA) and was operated with Mass Centersystem version 1.3.7b (JEOL, USA). In the positive ion mode, the atmospheric pressure interface potentials were typically set to the following values: orifice 1=80 V and ring lens and orifice2=10, 5 V, respectively. The ion guide potential and detector voltage were set to 2000 V and 2300 V, respectively. ESI parameters were set as follows: needle electrode=2000 V, nitrogen gas was used as a nebulizer, desolvating and their flow rate were 1 and 3 L/min, desolvating chamber temperature=250°C, orifice 1 temperature=80°C. Mass scale calibration was accomplished with YOKUDELNA calibration kit (JEOL, Japan) for accurate mass measurements and calculations of the elemental composition. MS acquisition was set with a scan range of *m/z* 50 to 1500.

Cell culture and survival assay

The mouse macrophage cell line, Raw 264.7 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Welgene, Seoul, Korea), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Welgene, Seoul, Korea) in 5% CO_2 humidified atmosphere at 37°C. Cell survival was determined by MTT assay. Raw 264.7 cells were seeded in 24-well plates at density of 1×10^5 cells/well and incubated at 37°C for 24 h. Further incubation with serum-free DMEM was done at 37°C for 12 h. Starved cells were treated with various concentrations of herbal extract. Following incubation for 24 h or 48 h, 50 µl (5 mg/ml) of MTT solution was added and incubated for an additional 4 h. Then formazan crystals were dissolved by addition of dimethyl sulfoxide (DMSO). Following shaking incubation for 30 min, optical density of solubilized formazan crystals was examined by using spectrophotometer (Molecular Device, CA, USA) at 580 nm.

Immunoblotting

The cells were lysed with sampling buffer (2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.1 mg/ml bromophenol blue in Tris-HCl, pH 6.8) and heated at 100°C for 10 min. Twenty µg of cell lysates were electrophoresed by 10% SDS-PAGE and transferred onto nitrocellulose membrane. The blotted membrane was incubated with anti-phospho-JNK, anti-JNK, anti-phospho-p38, anti-p38, anti-TNF-α, anti-IL-6, anti-iNOS, anti-COX2 and anti-β actin (Cell Signaling Technology, Beverly, MA, USA), diluted in TBS-T containing 0.05% BSA, for overnight at 4°C. The bound antibodies were detected by horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG and the bands were visualized using the ECL system (Thermo Fisher Scienctific, USA). Band images were obtained by using Molecular Imager ChemiDoc XRS+ (Bio-Rad, Hercules, CA, USA) and band intensity was analyzed by Image Lab™ software version 2.0.1 (Bio-Rad).

Measurement of DPPH radical scavenging activity

Herbal extract at various concentrations of 1-1000 µg/mL was mixed with 0.2 mM DPPH (2,2-diphenyl-1-picrylhydrazyl) ethanol solution (1:1). After incubation at room temperature in the dark for 30 min, the absorbance of the mixture was determined at 517 nm using spectrophotometer (VersaMax microplate reader; Molecular Device, Sunnyvale, CA, USA). Moreover, the DPPH radical scavenging activities of extract were expressed as half maximal inhibiting concentration (IC_{50}) which is defined as the concentration of required to scavenge 50% of DPPH radicals. IC_{50} was calculated using Calcusyn software (Biosoft, Cambridge, UK).

Measurement of NO production

The nitrite concentration in the culture medium was measured as an indicator of NO production by the Griess reaction system (Promega, WI, USA). Raw 264.7 cells (5×10^4 cells/well) in 96-well plates were cultured for 24 h. The cells were then treated with the samples listed above plus LPS (1 µg/mL) or LPS alone for 20 h. The supernatant was mixed with the same volume of Griess reagent and incubated at room temperature for 5 min. The concentration of nitrite was determined by measuring the absorbance by using spectrophotometer (Molecular Device, CA, USA) at 540 nm.

RNA extraction and quantitative real-time polymerase chain reaction (PCR)

Raw 264.7 cells (5 × 105 cells/well in six-well plates) were treated with herbal extracts (25, 50, 100, or 200 µg/mL) plus LPS (1 µg/mL) for 16 h. To evaluate the expression levels of cytokine mRNA, total cellular RNA was extracted with Trizol (Invitrogen, Life Technologies, USA) according to the manufacturer's instructions. Total RNA (1 µg) was mixed with RNA to cDNA EcoDry Premix (Clontech Laboatories, Inc. CA, USA) containing oligo-dT primers and water to a final volume of 20 µL and incubated at 42°C for 60 min. Real-time PCR was performed by using the Rotor Gene Q system (Qiagen, Hilden, Germany) and a reaction mixture that consisted of SYBR Green 2 × PCR Master Mix, cDNA template, and forward and reverse primers. The PCR protocol consisted of 40 cycles of denaturation at 95°C for 15 sec, followed by 55°C for 30 sec to allow for extension and amplification of the target sequence. The expression levels of each cytokine mRNA was normalized to that of actin via the $2^{-\Delta\Delta CT}$ method. The primer sequences employed in this study are shown in **Table 1**. After finishing real-time PCR, PCR products were electrophoresed in agarose gel and bands were visualized by using SYBR® Safe DNA gel stain (Invitrogen, Life Technologies, USA). Band images were obtained by using Molecular Imager ChemiDoc XRS+ (Bio-Rad, Hercules, CA, USA).

Statistics

All statistical analyses were conducted with SPSS (ver. 21, Somers, NY, USA). All the values were expressed as means ± SEM. The data were analyzed by one-way ANOVA, and then differences among means were analysed using the Dunnett's test or Tukey-Kramer's multiple comparison test. Differences were considered significant at $P < 0.05$.

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Table 1. The primer sequences.

Gene symbol	Gene accession no.	Primer sequences (F-3')
Actin	NM_001101	F:gtc ggc cgc tct agg cac caa R: ctc fit gat gtc acg cac gat ttc
IL-10	NM_000572	F: ttg agt ctg gac tcc agg acc tag aca R: ggc agc caa aca ata cac cat tcc cag agg
IL-6	NM_000600	F: atg aag ttc ctc tct gca aga gac t R: cac tag gn tgc cga gta gat ctc
IL-lα	NM_000575	F: atg gcc aaa gtt cct gac ttg ttt R: cct tca gca aca cgg gct ggt c
MIP1α	NM_002983	F: cca ctg ccc ttg ctg ttc ttc tct R: cag gca ttc agt tcc agg tca gtg
MIP2	NM_002089	F: tgc cgg ctc ctc agt gc R:tta gcc ttg cct ttg ttc agt atc
TNF-α	NM 000594	F: atg agc aca gaa agc atg atc cgc R: cca aag tag acc tgc ccg gac tc

RESULTS

Establishment of UPLC profile

In order to establish the standard chromatogram for the DG1102, we adopted UPLC system. Gradient elution system is the best way of choice to obtain entire chromatographic profile of the phytochemicals from natural resources. The identification of major peaks of chromatogram was performed by UPLC-ESI-MS study.

Identification of phytochemicals by UPLC-ESI-MS

The retention time, observed mass, mass difference and proposed compounds of six peaks are listed in **Table 2.**

Table 2. The observed and calculated mass numbers of UPLC peaks of DG1102 water extract.

Peak No.	Rt (min)	Theoretical mass[M+Na]$^+$	observed mass [M+Na]+	Mass difference (mmu)	Identification
1	11.60	355.03790	335.04032	2.42	trans-Caftaric acid
2	14.24	291.08686	291.08695	0.08	(+)-Catechin
3	15.30	355.10291	355.10437	1.47	3-O-Caffeoylquinic acid or 4-O-Caffeoylquinic acid
4	20.59	413.12124	413.12368	2.44	Resveratroloside
5	23.21	497.06959	497.07331	3.71	Chicoric acid
6	29.20	469.07468	469.08095	6.27	Rhein 8-glucoside

From the mass spectra, the protonated molecules were the most abundant ion for peak 2, peak 3, and peak 4 **(Figures 1 and 2).** The sodium adduct ion with the sodium adduct dimer ion were most abundant ion for peak 1, peak 6 and peak 5 **(Figure 2).** Six major phytochemicals in DG1102 are as follows: *trans*-caftaric acid and chicoric acid from TH, resveratroloside and rhein 8-glucoside from RR, (+)-catechin from RR or HH and 3-*O*-caffeoylquinic acid or 4-*O*-caffeoylquinic acid from EF. These six compounds composed of the DG1102 were identified by comparing both UV and MS spectrum **(Figures 1 and 2).**

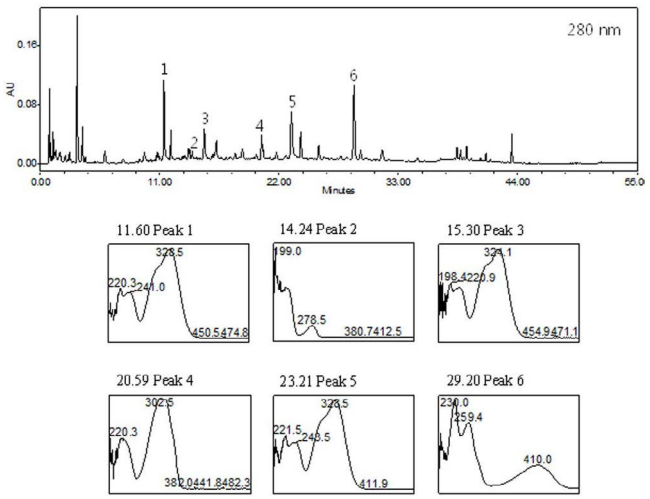

Figure 1. UPLC chromatogram of the DG1102 water extract and UV-visible absorption spectra of six peaks.

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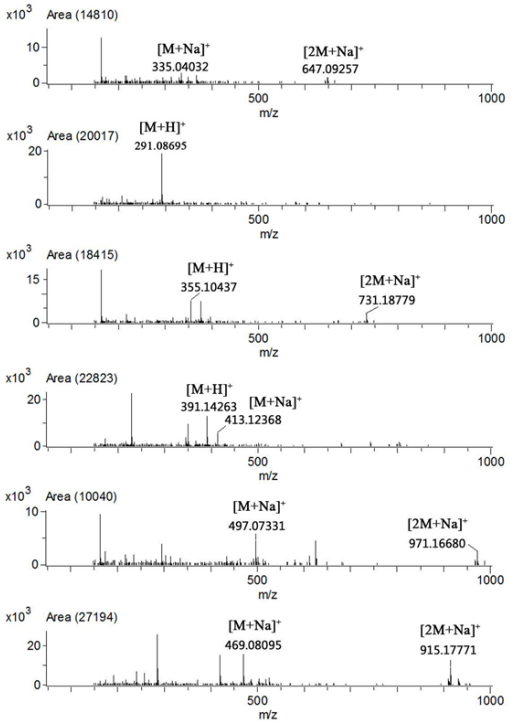
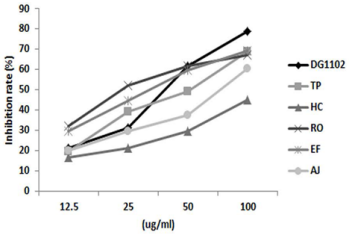


Figure 2. ESI-MS spectra of six peaks from DG1102 water extract.

Radical scavenging activities of DG1102 and its components.

To examine the antioxidant capacities of extract of DG1102 and its single compositions, we performed DPPH free radical scavenging activity assay. DG1102 and its single compositions exhibited dose-dependently increased activities. Among them, DG1102 at 100 ug/ml exhibited highest scavenging activity (78.81 ± 0.52%) than that of other single components in DPPH **(Figure 3A).** The half maximal inhibitory concentration (IC_{50}) was calculated and compared with that of a positive control, ascorbic acid. DPPH IC_{50} for DG1102, TH, RR, HH, EF and AR were 37.4, 52.3, 23.4, 125.5, 27.5 and 75.6 µg/ml, respectively **(Figure 3B).**

A.



B.

GROUP	NAME	EC_{50} (ug/ml)
Control	Ascorbic acid	2.6
Mixture	DG1102	37.4
Single	Taraxaci Herba (TH)	52.3
	Rhei Rhizoma (RR)	23.4
	Houttuyniae Herba (HH)	125.5
	Eriobotryae Follium (EF)	27.5
	Ampelopsis Radix (AR)	75.6

Figure 3. Radical scavenging activities of herbal extracts on DPPH-radicals. (A) The reaction mixture contained 60 µM of DPPH and 6.2, 12.5, 25, 50, or 100 µg/mL of herbal extracts. (B) IC_{50} of herbal extracts on radical scavenging activities.

Antimicrobial activity

The concentration of the aqueous extract of DG1102 and single component was calculated as 1 mg/ml. To examine antimicrobial activity of each extract, *S. epidermidis* bacterial strain, reported as a skin pathogen, was screened for sensitivity against the extract.

Initial screening of each extract for antimicrobial activity on *S. epidermidis* was carried out via Kirby-Bauer disc diffusion assay **(Table 3).**

All the extracts were found to be sensitive (zone of inhibition>10 mm) with maximum zone of inhibition obtained in herbal mixture, DG1102 (13.5 mm).

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Table 3. Results of the antimicrobial activity of each herbal extracts in agar diffusion method.

Group	Name	Zone of inhibition for aqueous extract (mm)
Mixture	DG1102	13.5
Single	Taraxaci Herba (TH)	11
	Rhei Rhizoma (RR)	12
	Houttuyniae Herba (HH)	11
	Eriobotryae Follium (EF)	11.5
	Ampelopsis Radix (AR)	10.5

Effects of DG1102 and its components on cell viability and measurement of NO production in LPS-induced Raw 264.7 cells

Figure 4A shows the results of cytotoxicity testing of each herbal extract in Raw 264.7 macrophages by means of MTT assay. DG1102 and its components had no cytotoxic effect on cells up to 100 μg/ml **(Figure 4A).** Accordingly, the dose of DG1102 and its components was used at 25 or 50 μg/ml in the subsequent experiments. Figure 4B shows the cell viability at 50 μg/ml of herbal extracts and co-treatment of LPS for 20 h. LPS had cytotoxicity at concentration of 0.5 μg/ml showing 68.64±3.3 % cell viability compared with control. Co-treatment of 0.5 μg/ml LPS to the medium containing herbal extract did improve the cell viability for DG1102 and RR. The effect of herbal extracts on LPS-induced NO formation in Raw 264.7 macrophages was determined and results are shown in Figure 4C. Incubation of the cells with DG1102, TH, RR and EF led to inhibition of LPS-induced NO production by 150.00±1.6 %, 202.27±6.2 %, 179.19±5.1 % and 179.19±5.1 %, respectively.

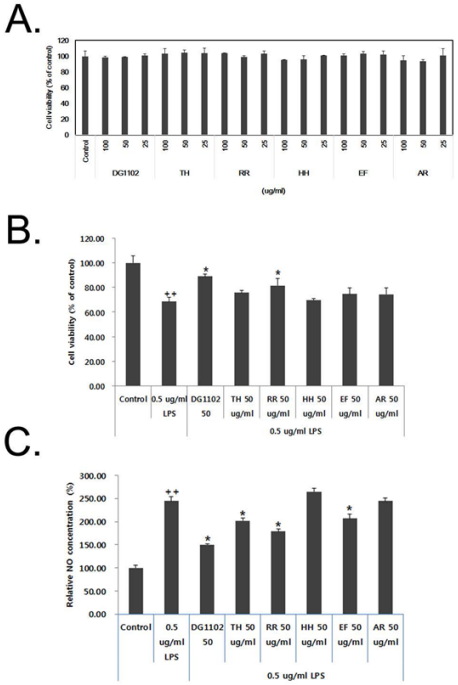

Figure 4. Anti-inflammatory effects of herbal extracts on Raw 264.7 cells. (A) Macrophage cells, Raw 264.7 were exposed to different concentration of herbal extracts. Cell viability was measured by MTT assay at 24 h after herbal extracts treatment. Data, expressed as percentage of control (Con), are the mean ± SEM of three separate experiments. (B) Cells were seeded in 24 well plates and were stimulated with 0.5 ug/ml of LPS plus herbal extracts for 24 hour in serum reduced media. After that, cell viability was measured by MTT assay at 24 h after herbal extracts treatment. Data, expressed as percentage of control are the mean ± SEM of three separate experiments. ++p<0.01 vs. control; *p<0.05 vs. LPS; **p<0.01 vs. LPS. (C) Cells were seeded in 24 well plates and were stimulated with 0.5 ug/ml of LPS plus herbal extracts for 48 hour in serum reduced media. After that, supernatant of cells were mixed with Griess reagent. Data, expressed as percentage of control are the mean ± SEM of three separate experiments. ++p<0.01 vs. control; *p<0.05 vs. LPS; **p<0.01 vs. LPS.

Effects of DG1102 and its components on TNF-α and IL-6 production in LPS-induced Raw 264.7 cells

Figure 5 demonstrates the role of herbal extracts in the effect of TNF-α production in LPS-challenged macrophage cells. All herbal extracts used for this assay suppressed LPS-induced TNF-α production at 24 hrs after LPS exposure. Among them, DG1102 showed highest inhibition on the production of TNF-α. To examine the dose dependency of DG1102, 25 or 50 ug/ml of DG1102 was co-treated on the LPS-challenged macrophage cells for 24 hrs. As shown in **Figure 6A,** DG1102 significantly suppressed secretion of TNF-α and IL-6 dose dependently.

Effects of DG1102 on expression of iNOS and COX2 in LPS-induced Raw 264.7 cells

Next, we examined whether the inhibitory effect of DG1102 on the induction of iNOS and COX2 which are related with the

NO production. **Figure 6B** demonstrates that 0.5 μg/ml LPS significantly induced iNOS and COX2 expression. Co-treatment of DG1102 significantly reduced the expression of both proteins in LPS-induced macrophage cells. These data suggest that DG1102 inhibits LPS-induced NO production via the inhibition of iNOS and COX2 expression in Raw 264.7 macrophage cells.

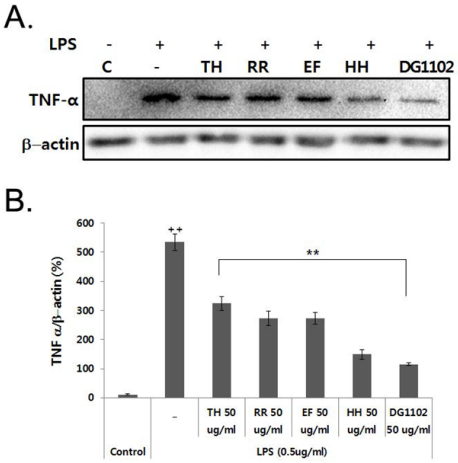

Figure 5. The effects of herbal extracts on LPS-induced TNF-α in Raw 264.7 cells. (A) Cells were seeded in 6 well plates and were stimulated with 0.5 ug/ml of LPS and herbal extracts for 24 hour. After that, whole cell lysates were electrophoresed in SDS-PAGE and analyzed by immunoblotting with anti-TNF-α or β-actin antibody. (B) The intensity of TNF-α and β-actin bands were quantitated by densitometric analysis, and the amounts of TNF-α were normalized versus β-actin. The data represent the means ± SEM of three independent experiments. C: Unstimulated cells. ++p<0.01 vs. control; **p<0.01 vs. LPS.

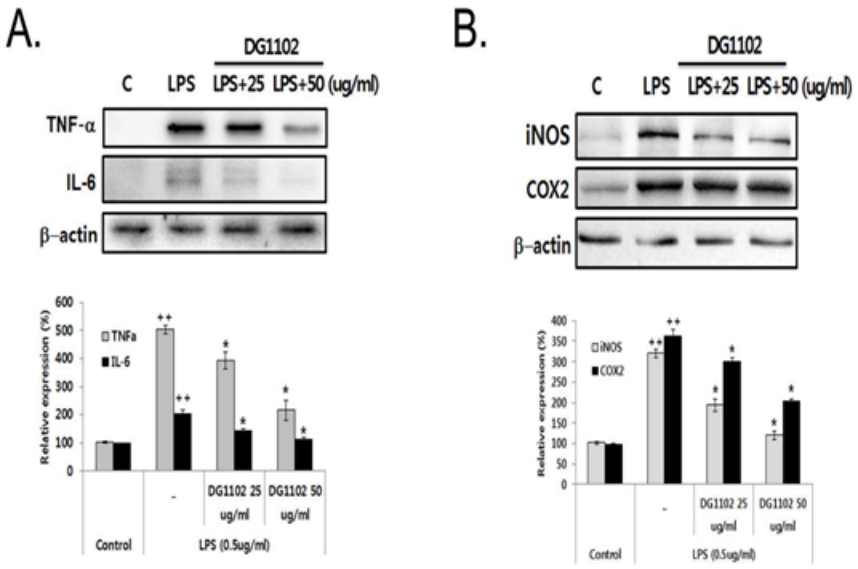

Figure 6. The effects of DG1102 on LPS-induced TNF-α, IL-6, iNOS, and COX2 in Raw 264.7 cells. Cells were seeded in 6 well plates and were stimulated with 0.5 ug/ml of LPS and 25 or 50 ug/ml of DG1102 for 24 hour. (A) After that, cell supernatants were electrophoresed in SDS-PAGE and analyzed by immunoblotting with anti-TNF-α, IL-6 or β-actin antibody. The intensity of TNF-α, IL-6 and β-actin bands were quantitated by densitometry analysis, and TNF-α and IL-6 were normalized versus β-actin. (B) Total cell lysates were electrophoresed in SDS-PAGE and analyzed by immunoblotting with anti-iNOS, COX2 or β-actin antibody. The intensity of iNOS, COX2 and β-actin bands were quantitated by densitometry analysis, and iNOS and COX2 were normalized versus β-actin. The data represent the means ± SEM of three independent experiments. C: Unstimulated cells. ++p<0.01 vs. control; *p<0.05 vs. LPS; **p<0.01 vs. LPS.

Effects of DG1102 and its components on JNK and p38 phosphorylation in LPS-induced Raw 264.7 cells

In order to determine the precise mechanisms operating in the herbal extract treatments, we measured the phosphorylations of two MAPK proteins such as JNK and p38 using Western blot analysis as shown in **Figure 7.**

0.5 μg/ml LPS significantly increased the phosphorylation of JNK and p38. Co-treatment of herbal extracts significantly reduced the phosphorylation of JNK and p38 in LPS-induced macrophage cells. These data suggest that herbal extracts inhibit LPS-induced NO and TNF-α production via the inhibition of JNK and p38 phosphorylation in Raw 264.7 macrophage cells.

Effects of DG1102 on cytokine mRNA expression in LPS-induced Raw 264.7 cells

As it is well known that cytokines are produced by activated macrophages, we next examined whether DG1102 influences

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LPS-induced cytokine mRNA expressions as shown in **Figure 8.**

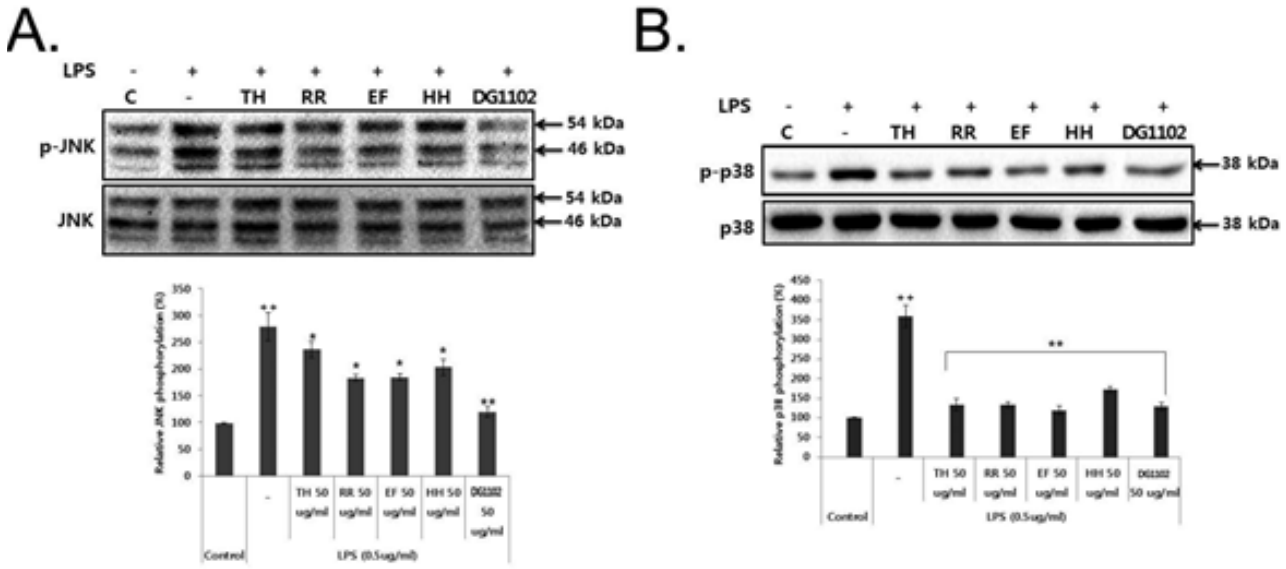

Figure 7. The effects of herbal extracts on LPS-induced JNK or p38 phosphorylation in Raw 264.7 cells. (A) Cells were seeded in 6 well plates and were stimulated with 0.5 ug/ml of LPS and herbal extracts for 1 hour. After that, whole cell lysates were electrophoresed in SDS-PAGE and analyzed by immunoblotting with anti-JNK or anti-phospho-JNK antibody. The intensity of each bands were quantitated by densitometry analysis, and the amounts of phosphorylated JNK were normalized versus JNK. (B) Cells were seeded in 6 well plates and were stimulated with 0.5 ug/ml of LPS and herbal extracts for 1 hour. After that, whole cell lysates were electrophoresed in SDS-PAGE and analyzed by immunoblotting with anti-p38 or anti-phospho-p38 antibody. The intensity of each bands were quantitated by densitometry analysis, and the amounts of phosphorylated p38 were normalized versus p38. The data represent the means ± SEM of three independent experiments. C: Unstimulated cells. ++p<0.01 vs. control; *p<0.05 vs. LPS; **p<0.01 vs. LPS.

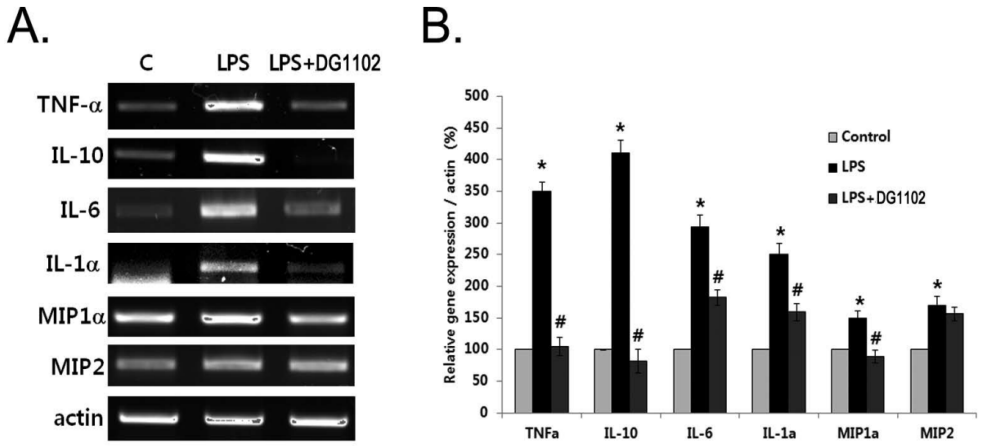

Figure 8. The effects of herbal extracts on LPS-induced cytokines induction in Raw 264.7 cells. (A) Cells were seeded in 6 well plates and were stimulated with 0.5 ug/ml of LPS and herbal extracts for 24 hour. After that, total RNA was extracted and cDNA was reverse transcribed with poly A primer. The cDNA was used in the PCR reaction with each primer. After finishing real time PCR, PCR products were electrophoresed in agarose gel and bans were visualized (B) Quantitative amounts of real-time PCR product were analyzed and the amounts of each product were normalized versus actin. The data represent the means ± SEM of three independent experiments. C: Unstimulated cells. *p<0.05 vs. control, #p<0.05 vs. LPS.

0.5 μg/ml LPS significantly increased mRNA expression of TNF-α, IL-10, IL-6, IL-1α, MIP-1α and MIP-2. Co-treatment of DG1102 significantly reduced the mRNA expression of TNF-α, IL-10, IL-6, IL-1α and MIP-1α. However, reduction of MIP-2 mRNA expression was inhibited by DG1102 treatment in LPS-induced Raw 264.7 macrophage cells.

DISCUSSION

The present study demonstrates the anti-inflammatory of herbal mixture, DG1102 on LPS-stimulated RAW 264.7 cells and the molecular mechanisms involved in terms of TNF-α, IL-6, iNOS, COX-2, and NO production. These result showed that DG1102 was more efficacious than the other single component at suppressing the LPS-induced release of NO and TNF-α. Moreover, DG1102 suppressed the LPS-induced phosphorylation of JNK and p38 in RAW 264.7 cells.

In the present study, we investigated the anti-inflammatory effect of new formulation of herb medicine, DG1102 composed by five different herbal ingredients and we compared the efficacy of DG1102 with single components of DG1102. Five ingredients of

DG1102 have been used as a traditional medicine for the treatment of many inflammatory diseases because of their antioxidant potential. In this study, we also examined the anti-oxidative effect of single or mixture extract by using DPPH radical scavenging activity **(Figure 3).** Although DG1102 and its single compositions exhibited dose-dependently increased activities, DG1102 at 100 ug/ml exhibited highest scavenging activity than that of other single components in DPPH. Moreover, in the LPS-induced macrophages, DG1102 showed the highest suppressive effect of TNF-α production, suggesting that the effect of the composite herb medicine might be better than a single one.

In the present study, we found six major phytochemicals in DG1102: *trans*-caftaric acid and chicoric acid from TH, resveratroloside and rhein 8-glucoside from RR, (+)-catechin from RR or HH and 3-*O*-caffeoylquinic acid or 4-*O*-caffeoylquinic acid from EF **(Table 2).** Among them, three phytochemicals have been reported showing anti-inflammatory effect; (+)-catechin reduces NO production in LPS-stimulated RAW 264.7 cells, chicoric acid strengthens the anti-inflammatory activity of luteolin through NF-κB attenuation in LPS stimulated RAW 264.7 cells and 3-*O*-caffeoylquinic prevents NF-κB nuclear accumulation, induced by LPS, in N9 cells [21-23]. Although in this study, we did not examine the NF-κB activation by DG1102 treatment in the LPS-induced RAW 264.7 cells, these suggest that these components of DG1102 may be synergistically effective on the NO and TNF-α production through inhibition of NF-κB in LPS stimulated RAW 264.7 cells.

The expression of many pro-inflammatory genes by LPS is mediated by the MAPK pathway [24]. JNK and p38 MAPK are major MAPK subfamily members which are activated in LPS-stimulated RAW 264.7 cells **(Figure 7).**

DG1102 and its components significantly suppressed LPS-induced phosphorylation of JNK or p38 MAPK. Previous studies have reported that the JNK activity is involved in iNOS expression in LPS-stimulated RAW264.7 macrophages and extracellular stimuli of the p38 MAP kinase pathway include a variety of cytokines (IL-1α, IL-2, IL-7, IL-17, IL-18, TGF-β, and TNF-α) [25,26]. DG1102 significantly inhibited LPS-induced iNOS expression and subsequent NO production in RAW 264.7 cells. In addition, DG1102 suppressed the expression of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, in the LPS-stimulated RAW 264.7 cells. Taken together, these results suggest that the suppression of LPS-induced iNOS expression and reduced cytokine production by DG1102 is mediated by the inhibition of JNK and p38 activation.

LPS-stimulated macrophages up-regulates various pro-inflammatory mediators such as cytokines/chemokines, NO, and PGE_2 [27]. Dysregulated production of pro-inflammatory mediators leads to many pathologic states, including cancer and diabetes [28,29]. Therefore, modulation of pro-inflammatory mediators, such as iNOS, COX2 and NO, provide possible targets for the development of therapeutics against various inflammatory diseases. We observed that DG1102 significantly suppressed LPS-induced iNOS and COX2 expression and in RAW 264.7 cells **(Figure 6B).** These results suggest that DG1102 has a modulatory activity towards the inflammation process.

MIP-1α has pro-inflammatory properties and has been shown to activate mast cells and basophils, to be chemotactic for T cells and monocytes, and to induce an oxidative burst in neutrophils [30]. MIP-1α, and related chemokines, act as signal transducers in inflammatory injury, and perform important regulatory functions [31]. In the present study, DG1102 significantly suppressed LPS-stimulated MIP-1α induction but not MIP2, suggesting that DG1102 may regulate macrophage inactivation through different pathway. In fact, in the ethanol-fed rats, MIP-1 α is increased in serum but not MIP-2 [32], suggesting that two chemokines may be regulated different pathway.

CONCLUSIONS

In conclusion, we provide a molecular basis that DG1102 has more strong immune-modulatory activity suppressing production of pro-inflammatory cytokines and chemokine via the suppression of JNK and p38 MAPK activation than single component in LPS-stimulated RAW 264.7 macrophages. Although more data are required to explain the exact mechanism and in vivo effect of DG1102 on the inflammatory system, our study nonetheless provides the biological evidence of the synergistic efficacy of plants mixture in the treatment of inflammatory diseases.

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DECLARATION OF INTEREST

There is no conflict of interest to declare.

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