Lactobacilli-fermented Hwangryunhaedoktang has enhanced anti-inflammatory effects mediated by the suppression of MAPK signaling pathway in LPS-stimulated RAW 264.7 cells

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ABSTRACT

Background: Hwangryunhaedoktang (HR) has been traditionally used in oriental medicine as a drug for the treatment of melena, hemoptysis, and apoplexy. **Objective:** We investigated whether HR and lactobacilli-fermented HRs have an inhibitory effect on the production of proinflammatory mediators in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells. **Materials and Methods:** The investigation was focused on whether HR and fermented HRs could inhibit the production of prostaglandin (PG)E$_2$, nitric oxide (NO), tumor necrosis factor (TNF)-α, interleukin (IL)-6, cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS) and mitogen-activated protein kinases (MAPKs) in LPS-stimulated RAW 264.7 cells. **Results:** We found that HR weakly inhibited various inflammatory mediators induced by LPS. However, fermentation with lactobacilli significantly increased the inhibitory effect of HR on most of the inflammatory mediator expression. Furthermore, fermented HRs exerted a stronger inhibitory effect on MAPKs phosphorylation than that by non-fermented HR. **Conclusions:** These results suggest that lactobacilli-fermented HRs contains elevated potent anti-inflammatory activity that is mediated by inhibiting MAPKs pathway in macrophages.

Key words: Hwangryunhaedoktang and fermented Hwangryunhaedoktang, inflammatory cytokines, cyclooxygenase 2, inducible nitric oxide synthase, mitogen-activated protein kinases

INTRODUCTION

Hwangryunhaedoktang (HR) is a traditional herbal medication that consists of four herbs and is known to have remedial value for various diseases such as melena, hemoptysis, and apoplexy. These diseases were thought to be caused by "fire" or body heat according to the oriental medicine theory. According to oriental medicine, the body heat flows smoothly in the body and is then externally eliminated. Thus, disease development was thought to be a result of abnormal heat circulations in the body. HR has been widely used to treat these symptoms as a traditional herbal medication in East Asia.

Recent research has demonstrated that HR has various biologically active components.[1-4] The anti-inflammatory properties of the crude HR extract, such as the natural product and its herbal decoction, are of considerable research interest. A previous study demonstrated that the crude extract of the Lycium fruit has anti-inflammatory activity on LPS-stimulated RAW 264.7 cells.[5] Furthermore, another study revealed the anti-inflammatory effect of traditional Chinese herbal formula "Zuojin Pill" on RAW 264.7 macrophages.[6] However, whether HR has anti-inflammatory activity and whether the fermentation of HR affects its activity on macrophage cells is still unknown.

Inflammation is a complex biological process triggered by various stimuli such as pathogens, cell damage, or irritants.[7] Thus, inflammation can be initiated by physical injury, bacterial infection, or chemical stimulants such as endotoxins, and it is part of the immune response to remove the stimuli and is essential for the healing process. Inflammation is accompanied by the activation of macrophages, neutrophils, and lymphocytes. The activated

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macrophages are involved in the regulation of inflammation and the ensuing immune responses. Activated macrophages express inflammatory mediators such as PGE_2, NO, COX-2, iNOS and inflammatory cytokines, including TNF-α and IL-6.[8-10] These inflammatory mediators are essential for host survival and tissue repair.[8] In addition, deregulation of the inflammation process is related to various inflammatory diseases such as autoimmune diseases, inflammatory disorders, and infections.[11-13]

MAPK signaling pathways play an important role in delivering inflammatory signals into the cytoplasm and nucleus.[14] The cellular signals from MAPKs cascades lead to the activation of mediators that regulate cell growth, cell division, and cell differentiation.[15] There are 3 major groups of MAPKs: ERK 1/2, p38 and JNK MAPKs. The phosphorylation of MAPKs is known to induce NF-κB activation and thereby promotes the expression of inflammation-related genes such as COX-2 and iNOS,[16-18]

Several fermentation products such as yogurt, cheese, kimchi, and sausage are a part of the regular human diet. Moreover, our previous study showed that lactobacilli-fermented Oyaksungisan has enhanced anti-inflammatory effects.[19] Therefore, we investigated and compared the anti-inflammatory effects of HR and fermented HRs on macrophages by evaluating the expression of the inflammatory mediators.

MATERIALS AND METHODS

Materials and reagents

RPMI 1640, Fetal bovine serum (FBS), penicillin/streptomycin (P/S), LPS, dexamethasone ($\geq$98%), Bovine serum albumin (BSA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide (MTT) and ELISA antibody sets were obtained from Sigma (St. Louis, MO, USA), Hyclone (Logan, UT, USA) and BD Biosciences (San Jose, CA, USA). Various primary antibodies and horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA). RNA extraction kit was obtained from iNtRON Biotech (Daejeon, Korea). Oligonucleotide primers for TNF-α, IL-6, COX-2, iNOS and β-actin were synthesized from Bioneer (Daejeon, Korea). The standard compounds of the following 7 components_ geniposide, baicalin, palmatine, berberine, wogonoside, baicalein and wogonin_were purchased from Tianjin Pharma Tech (Tianjin, China), Sigma (St. Louis, MO, USA) and Korea Food and Drug Administration (KFDA; Cheongwon, Korea), respectively. The purities of marker compounds as determined by using high performance liquid chromatography (HPLC) were

higher than 98%. HPLC-grade methanol, acetonitrile and analytical-grade trifluoroacetic acid were purchased from J. T. Baker (Austin, TX, USA) and Sigma (St. Louis, MO, USA). Third distilled water was filtered through a 0.2-μm membrane filter (Advantec, Japan) before analysis.

Preparation of HR and fermented HRs

The HR is composed of Scutellaria root, Coptis rhizome, Phellodendron bark, Gardenia Fruit, which were purchased from Yeongcheon Oriental Herbal Market (Yeongcheon, Korea). All voucher specimens were deposited in the herbal bank of the KM-Based Herbal Drug Research Group, Korea Institute of Oriental Medicine. For extraction, the 4 medicinal herbs (250 g each) were placed in distilled water (10 L) and then heated for 3 h at 115°C in high pressure. Before beginning the fermentation process, the HR extract was cooled to room temperature, and the pH values of all HR samples were adjusted to 8.0. The HR extract was filtered to remove the plant debris and then used to prepare fermented HR-A by the addition of *Lactobacillus casei* (inoculum concentration: 1-5 × 10^8 colony-forming unit (CFU)/mL) and fermented HR-B by the addition of *L. confusus* (inoculum concentration: 1-5 × 10^8 CFU/mL). Pure cultures of both *L. casei* and *L. confusus* were obtained from the Korea Food Research Institute (Sungnam, Korea). The bacterial inoculum was prepared by inoculating strains in 50 mL of MRS broth (Difco™ Lactobacilli MRS Broth, Becton Dickinson, Franklin Lakes, NJ, USA) followed by an overnight incubation at 37°C. During the incubation period, the pH values of HR-A and HR-B decreased (<4.0) due to the acid production by the lactobacilli. The HR was fermented using lactobacilli at 37°C for 48 h and then lyophilized and stored in desiccators at 4°C. The freeze-dried powder was then dissolved in phosphate buffered saline, filtered and kept at 4°C. The yields of HR, HR-A and HR-B were 20.54%, 19.61% and 19.72%, respectively.

Cell culture and drug treatment

RAW 264.7 cells were obtained from the Korea Cell Line Bank (Seoul, Korea) and grown in complete RPMI 1640 medium. The cells were incubated in a humidified 5% CO_2 atmosphere at 37°C temperature. To stimulate the cells, LPS (200 ng/mL) was added[20,21] in the presence or absence of HR or fermented HRs (1, 10, 50 and 100 μg/mL) for the indicated periods.

MTT assay for cell viability

Cytotoxicity was analyzed using an MTT assay. HR or fermented HRs was added to cells and incubated for 48 hours at 37°C with 5% CO_2. 10 μL of MTT solution (5 mg/mL in PBS) was added and the cells were incubated for another 4 hours. The supernatant was then discarded and formazan was dissolved 100 μL of dimethyl sulfoxide (DMSO). The absorbance at 570nm was measured using

an ELISA reader (infinite M200, TECAN, Männedorf, Switzerland).[20,21]

Determination of PGE$_2$, TNF-α and IL-6 production

The inhibitory effect of HR and fermented HRs on the level of PGE$_2$, TNF-α and IL-6 produced by LPS stimulation was determined by an ELISA kit according to the manufacturer's instructions.

Measurement of no production

NO production was analyzed by measuring nitrite concentration in the supernatants. After preincubation of the RAW 264.7 cells for 18 hours, the cells were stimulated with LPS for 24 hours following pretreatment with HR or fermented HRs. The supernatant was mixed with a same volume of Griess reagent (0.1% naphthylethylenediamine dihydrochloride, 1% sulfanilamide and 2.5% phosphoric acid) and incubated at room temperature (RT) for 5 min. Nitrite concentrations of samples were quantified by reading at 570nm using an ELISA reader.[20-22]

RNA extraction and reverse transcription-polymerase chain reaction

Total cellular RNA was isolated using the easy-BLUE™ RNA extraction kit (iNtRON Biotech, Daejeon, Korea) according to the manufacturer's instruction. The total RNA (1 μg) was reserves transcribed into cDNA using RevoScript™ RT PreMix (iNtRON, Daejeon, Korea). The PCR primers used with mouse macrophage cDNA are listed in Table 1. The following PCR conditions were applied: TNF-α, IL-6, COX-2, iNOS and β-actin, 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 65ºC (TNF-α), 57ºC (IL-6), 50ºC (COX-2), 60ºC (iNOS) and 57ºC (β-actin) for 30 seconds, and extension at 72ºC for 1 min.[20,23,24] The PCR products were analyzed on EcoDye™ DNA Staining Solution (SolGent, Daejeon, Korea)-stained 1.5% agarose gels. The amount of mRNA was quantitated using i-MAX™ Gel Image Analysis System (Core Bio, Seoul, Korea).

Real-time reverse transcription-polymerase chain reaction

The real-time RT-PCR oligonucleotide primers used with mouse macrophage cDNA are listed in Table 2. The reactions were setup in duplicates with 20-μL total volume: 0.3 μM final concentrations of each primer, 10μL of FastStart Universal SYBR Green Master (ROX, Roche), and 2μL of template DNA. The following PCR conditions were applied: TNF-α, IL-6, COX-2, iNOS and β-actin, 40 cycles of 94°C for 15 s, 60°C for 1 min.[25] The amplification and analyses were performed using an ECO™ Illumina Real-Time PCR system. Samples were compared using the relative CT method. The fold-increase or -decrease in gene expression was determined relative to a blank control after normalization to β-actin gene using 2$^{-\Delta\Delta CT}$.[25]

Table 1: Primers used for RT-PCR

Target gene	Primer sequence
TNF-α	F: 5'-AGCCCACGTCGTAGCAAACCACCAA-3'
	R: 5'-AACACCCATTCCCTTCACAGAGCAAT-3'
IL-6	F: 5'-CATGTTCTCTGGGAAATCGTGG-3'
	R: 5'-AACGCACTAGGTTTGCCGAGTA-3'
COX-2	F: 5'-ACTCACTCAGTTTGTTGAGTCATTC-3'
	R: 5'-TTTGATTAGTACTGTAGGGTTAATG-3'
iNOS	F: 5'-CCTCCTCCACCCTACCAAGT-3'
	R: 5'-CACCCAAAGTGCTTCAGTCA-3'
β-actin	F: 5'-TGGAATCCTGTGGCATCCATGAAA-3'
	R: 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'

F: Forward; R: Reverse; RT-PCR: Reverse transcription-polymerase chain reaction

Table 2: Primers used for real-time RT-PCR

Target gene	primer sequence
TNF-α	F: 5'-TTCTGTCTACTGAACTTCGGGGT GATCGGTCC-3'
	R: 5'-GTATGAGATAGCAAATCGGCTGA CGGTGTGGG-3'
IL-6	F: 5'-TCCAGTTGCCTTCTTGGGAC-3'
	R: 5'-GTGTAATTAAGCCTCCGACTTG-3'
COX-2	F: 5'-TGAGTACCGCAAACGCTTCTC-3'
	R: 5'-TGGACGAGGTTTTTCCACCAG-3'
iNOS	F: 5'-GGCAGCCTGTGAGACCTTTG-3'
	R: 5'-GCATTGGAAGTGAAGCGTTTC-3'
β-actin	F: 5'-AGAGGGAAATCGTGCGTGAC-3'
	R: 5'-CAATAGTGATGACCTGGCCGT-3'

F: Forward; R: Reverse; RT-PCR: Reverse transcription-polymerase chain reaction

Western blot analysis

The RAW 264.7 cells were pretreated with HR or fermented HRs. After 30 min, LPS was added to the cells and then incubated for the indicated periods at 37°C. After PBS washing, the cells were resuspended in protein lysis buffer (PRO-PERP, iNtRON, Sungnam, Korea). The protein concentration in the supernatant was determined through Bradford's methods using the Bio-Rad protein assay buffer (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were separated by 8-12% SDS-PAGE and transferred to nitrocellulose membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 3% BSA in Tris-buffered saline with 0.1% Tween 20 (TBS-T) and incubated overnight at 4°C with each primary antibodies. The membrane was then washed and incubated with HRP-conjugated secondary antibodies.[5] The specific proteins were visualized using enhanced chemiluminescence (ECL) (Millipore, Billerica, MA, USA) and quantitated by Davinch-chemi™ Chemiluminescence Imaging System CAS-400SM (Core Bio, Seoul, Korea).

Preparation of standard solutions and samples

The standard solution of seven geniposide, baicalin, palmatine, berberine, wogonoside, baicalein and wogonin

were prepared by dissolving 2 mg of each compound in 10 mL of methanol and adjusting the concentration to 200 µg/mL. To prepare analytical samples, HR and fermented HRs powder was accurately weighed and dissolved with pure methanol at a concentration of 50 mg/mL. Prior to analysis, sample preparation was filtered through a 0.2 µm PVDF membrane filter.

HPLC analysis condition

The experiments were performed with Waters HPLC system equipped with a pump, auto sampler, column oven, photodiode array UV/VIS detector (Waters HPLC system, USA) and Empower software program was used to data acquisition and process. The chromatographic columns used in this experiment are commercially available; obtained from Phenomenex Luna C_{18} (4.6 × 250 mm, 5µm, Daejeon, Korea). The column oven temperature was kept at 40°c. The injection volume was 10 µL and the flow rate of the mobile phase was 1.0mL/min. The wavelength of the UV detector was set at 254nm. The mobile phase composed of acetonitrile 100% (A) water containing 0.1% trifluoroacetic acid (B). The run time was 60 min and the mobile phase program was the step gradient elution as follows: Time 0-10 min A-B (10-90), 10-25 min (20-80), 25-33 min (30-70), 33-43 min (33-67), 43-50 min (40-60) and 50-60 min (44-56). The chromatographic conditions were presented in Table 3.

Statistical analysis

The results are expressed as mean ± SD values for the number of experiments. Statistical significance was compared each treated group with the negative control and determined by one-way ANOVA test. Each experiment was repeated at least three times to yield comparable results. Values with $P < 0.05$ and $P < 0.01$ were considered significant.

RESULTS

Effect of HR and fermented HRs on PGE_2 and NO production by LPS stimulation

We first evaluated the cytotoxicity of HR and fermented HRs using MTT assay to determine the optimal concentration that would be effective for inflammation-treatment with minimum toxicity. As shown in Figure 1a, HR and fermented HRs had little cytotoxicity on RAW 264.7 macrophages for HR concentrations that were lower than 100 µg/mL. To compare the anti-inflammatory activity of HR and fermented HRs, we investigated the inhibitory effects of HR and fermented HRs on LPS-induced PGE_2 and NO production. The cells were pretreated with HR or fermented HRs before LPS stimulation and then PGE_2, and NO levels in the supernatant were measured. We used 10 µM dexamethasone as a positive control for comparing the inhibitory effects of HR or fermented HRs. As shown in Figure 1b, HR and fermented HRs showed strong inhibitory effect on PGE_2 expression at concentration of 10 µg/mL. However, fermented HRs exerted strong PGE_2 repression at concentrations of 50 µg/mL and 100 µg/mL, compared with that by non-fermented HR. Next, we measured NO production after LPS stimulation in the presence of either HR or fermented HRs. Both HR and fermented HRs showed a dose-dependent inhibitory effect on NO generation. However, fermented HRs exerted greater suppression on NO production than that by non-fermented HR [Figure 1c]. Thus, HR fermentation leads to greater suppression of PGE_2 and NO generation in LPS-stimulated macrophages.

Fermentation by lactobacillus increases the inhibitory effect of HR on inflammatory cytokine production in macrophages

Next, we investigated the effect of HR and fermented HRs on the expression of TNF-α and IL-6 by using ELISA, RT-PCR and real-time RT-PCR analysis. The macrophages were first incubated with HR or fermented HRs at various concentrations, then stimulated with LPS to induce TNF-α and IL-6 production. As shown in Figure 2a, TNF-α suppression was significantly greater by fermented HRs (100 µg/mL) than by non-fermented HR (100 µg/mL). Consistent with the ELISA data, fermented HRs showed a stronger suppressive effect on TNF-α mRNA expression than that by non-fermented HR [Figure 2b and c]. In particular, HR-A showed a significantly enhanced inhibitory effect compared with that by HR at an identical dosage. However, fermented HRs exerted a little elevated suppressive effect on IL-6 cytokine production [Figure 3a] and mRNA expression [Figure 3b and c].
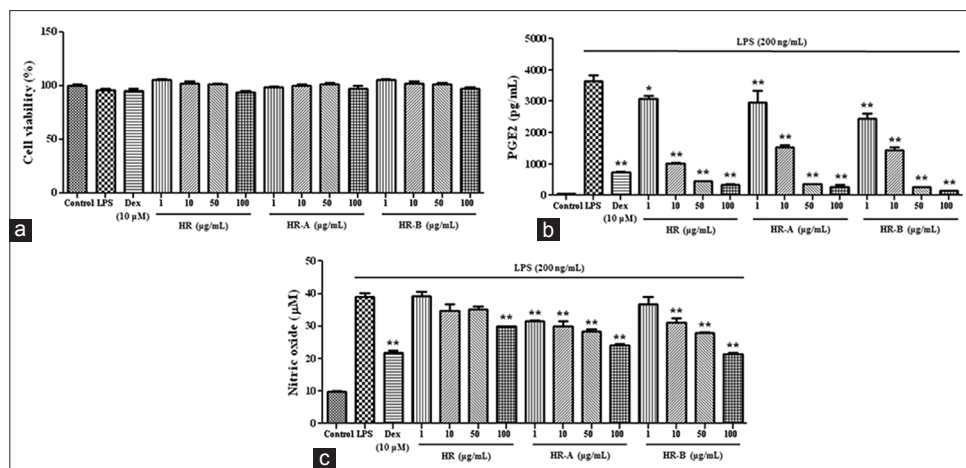
Table 3: HPLC operating conditions for analysis of HR and fermented HRs

Item	Condition	
RP Column	Phenomenex Luna C_{18} (250 x 4.6 mm x 5 µm)	
Injection volume	10 µL	
Flow rate	1.0 mL/min	
Column oven temp.	40°C	
UV wavelength	254 nm	
Mobile phase/Time (min)	**Solvent composition**	
	A (%)	**B (%)**
0	10	90
10	10	90
25	20	80
33	30	70
43	33	67
50	40	60
60	44	56

A: Acetonitrile (ACN); B: Water containing o.1% Trifluoroacetic acid (TFA); HPLC:High performance liquid chromatography; HR: Hwangryunhaedoktang

Figure 1: Effect of Hwangryunhaedoktang (HR) and fermented HRs on (a) cell viability and LPS-induced (b) PGE2, (c) NO production in RAW 264.7 cells
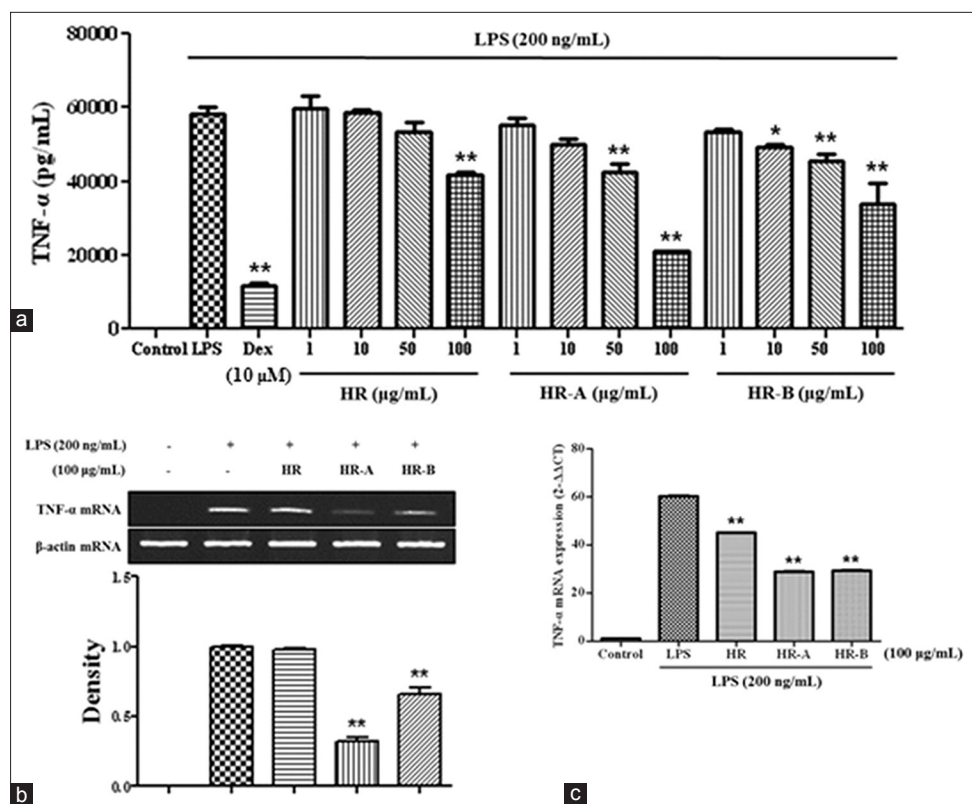

Figure 2: Effects of Hwangryunhaedoktang (HR) and fermented HRs on TNF-α (a) cytokine and (b, c) mRNA expression

Inhibitory effect of HR and fermented HRs on LPS-Induced COX-2 and iNOS expression

To investigate whether the inhibitory effects of HR and fermented HRs on PGE_2 and NO production are related to the PGE_2/NO synthesis enzyme repression, we examined the effect of HR and fermented HRs on COX-2 and iNOS expression. The fermented HR-B had a remarkably potent inhibitory effect on COX-2 protein and mRNA expression and was stronger than that of HR [Figure 4a and b]. Similarly, HR-B had a stronger inhibitory effect on iNOS protein and mRNA expression than that by HR [Figure 4c and d].

Enhancement of Inhibitory effect of HR by fermentation on phosphorylation of MAPKs in LPS-stimulated RAW 264.7 Cells

The effect of HR and fermented HRs on the activity of 3 MAPKs was assessed using Western blot analysis. The HR imposed a weak inhibitory effect on MAPKs phosphorylation. However, fermented HR-A and B significantly suppressed LPS-induced ERK and JNK MAPKs phosphorylation [Figure 5a and c]. Although, both HR and fermented HRs exerted a little affect the p38 MAPK activity [Figure 5b]. The levels of nonphosphorylated
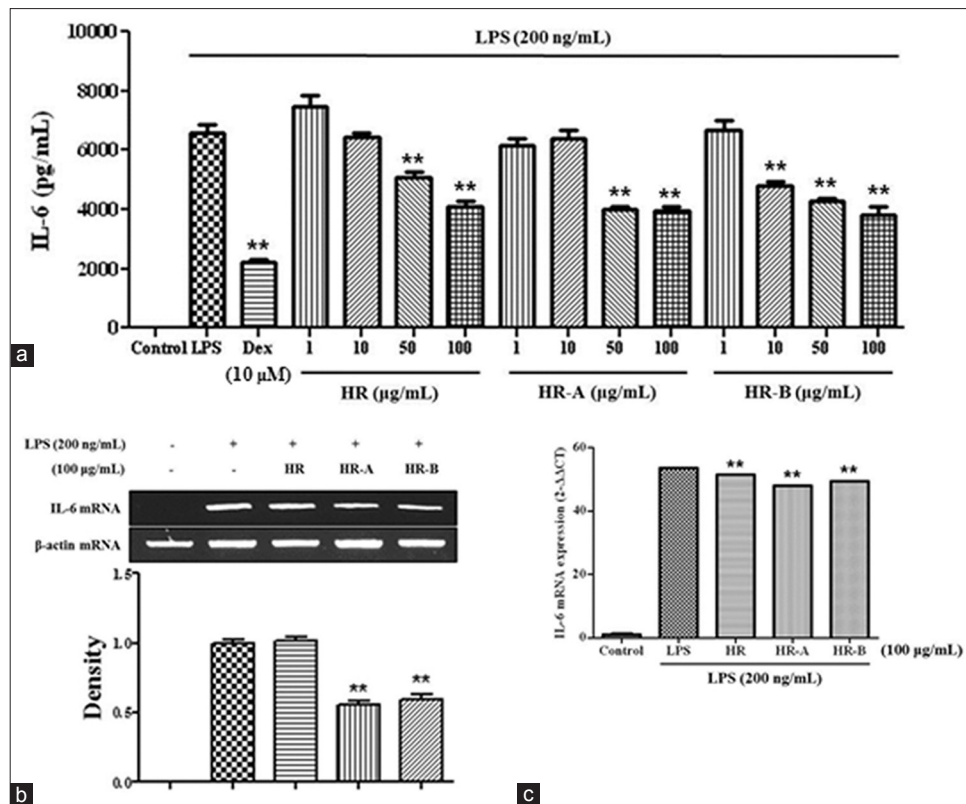
Figure 3: Effects of HR and fermented HRs on IL-6 (a) cytokine and (b, c) mRNA expression
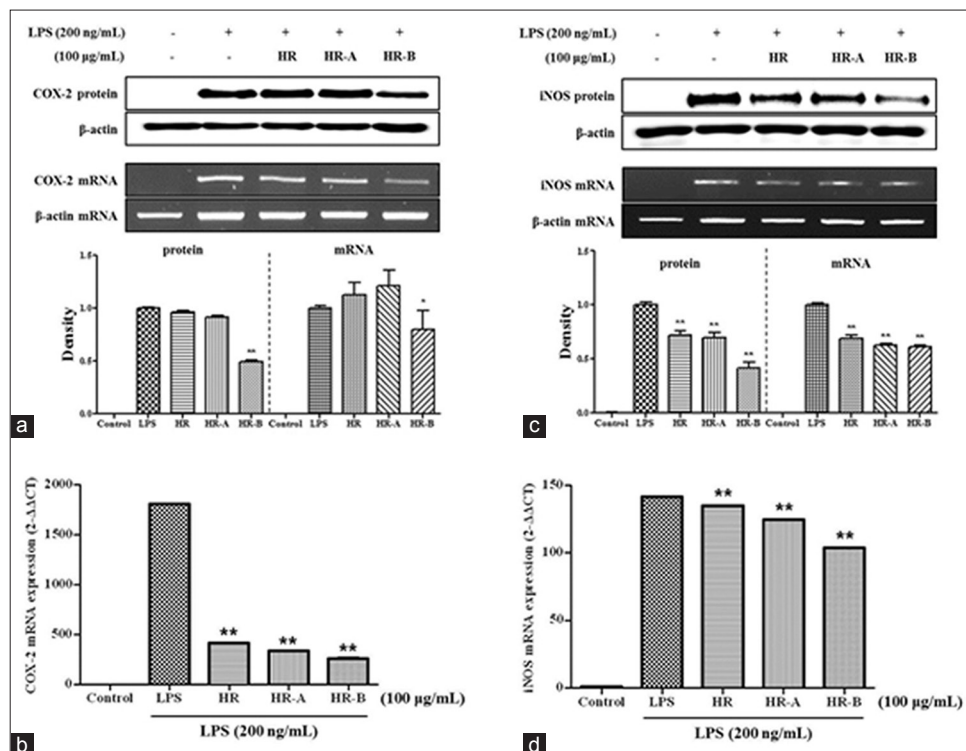

Figure 4: Effects of HR and fermented HRs on (a, b) COX-2 and (c, d) iNOS expression

MAPKs remained unchanged after LPS and drug treatment. These data indicate that fermentation by lactobacilli augments the suppressive effect of HR on LPS-induced ERK and JNK MAPK phosphorylation.

HPLC analysis of HR and fermented HRs

A comparison of bioconversion constituents were simultaneously identified analysis from HR and fermented HRs. We characterized the 7 marker constituents in HR and fermented HRs by HPLC-DAD analysis. The optimum HPLC analysis conditions for the separation and quantification of the 7 constituents were determined by

changing the solvent compositions of the mobile phase [Table 4]. Figure 6 shows a HPLC-DAD chromatogram of the markers of HR and fermented HRs at optimized HPLC conditions. All major compounds of HR and fermented HRs [Figure 7] were baseline-resolved. The 7 compounds were characterized by comparing the HPLC retention times (t_R) and the UV spectra maximum absorptions of the target peaks in the HR with those of the standards: Geniposide (1), baicalin (2), palmatine (3), berberine (4), wogonoside (5), baicalein (6), and wogonin (7). Thus, HPLC was used to separate geniposide (1, t_R 17.51 min), baicalin (2, t_R 36.10 min), palmatine (3, t_R 39.59 min), berberine (4, t_R 39.77 min), wogonoside (5, t_R 41.19 min), baicalein (6, t_R 48.44 min), and wogonin (7, t_R 58.50 min), respectively. After fermentation, berberin (4) and baicalein (6) were remarkably elevated in the fermented HR-A and HR-B. In addition, baicalin (2) was selectively increased in the HR-B. Moreover, the baicalein (6) in fermented HR-B was 6-times higher than in the non-fermented HR. Therefore, HR fermented by lactobacilli can affect the content of several compounds will be improves its absorption for develop the bioactivity.
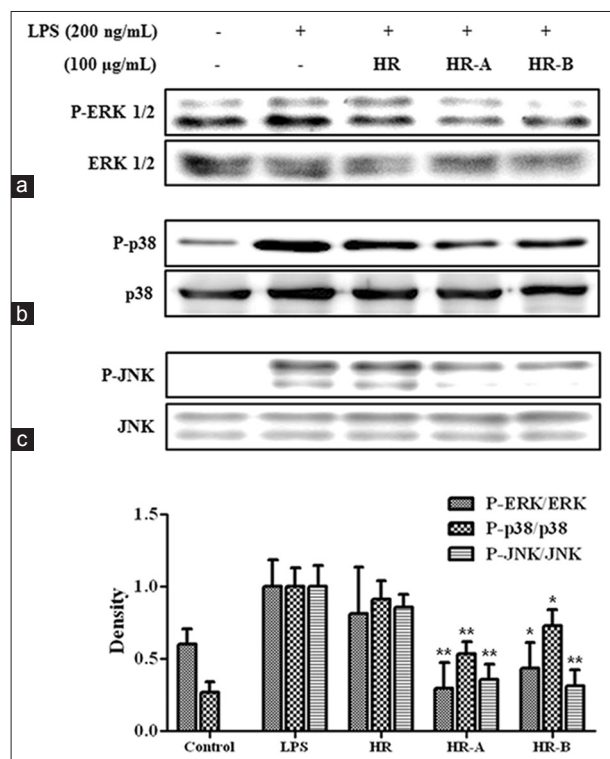
DISCUSSION

Fermentation, a decomposition process mediated by microbes such as lactic acid bacteria, creates low-molecular-weight substances such as aglycone from glycoside. Recently, we demonstrated that fermentation by lactic acid bacteria improves biological activity of oyaksungisan, an oriental herbal medicine.[19] Several studies have investigated the anti-inflammatory effects of naturally
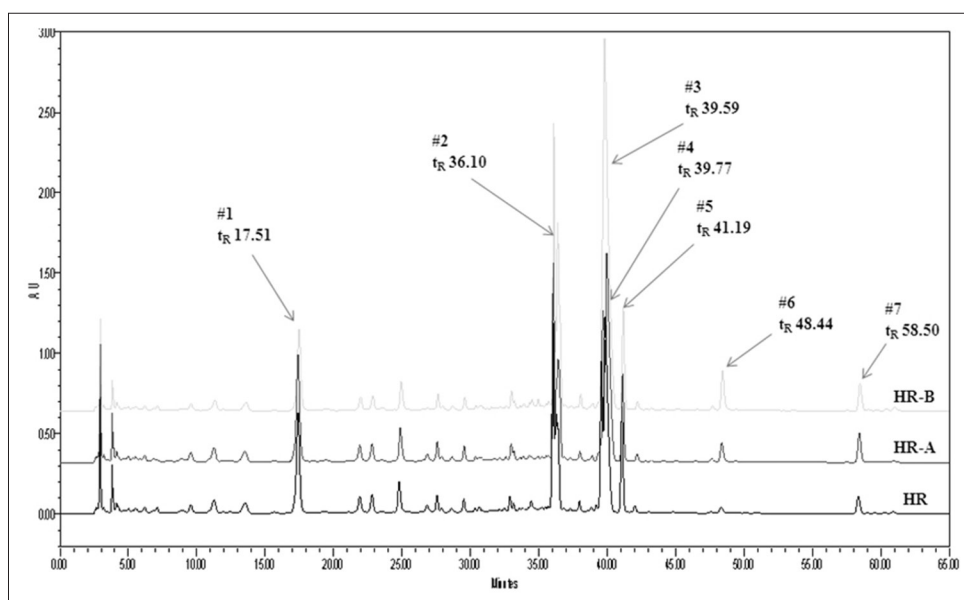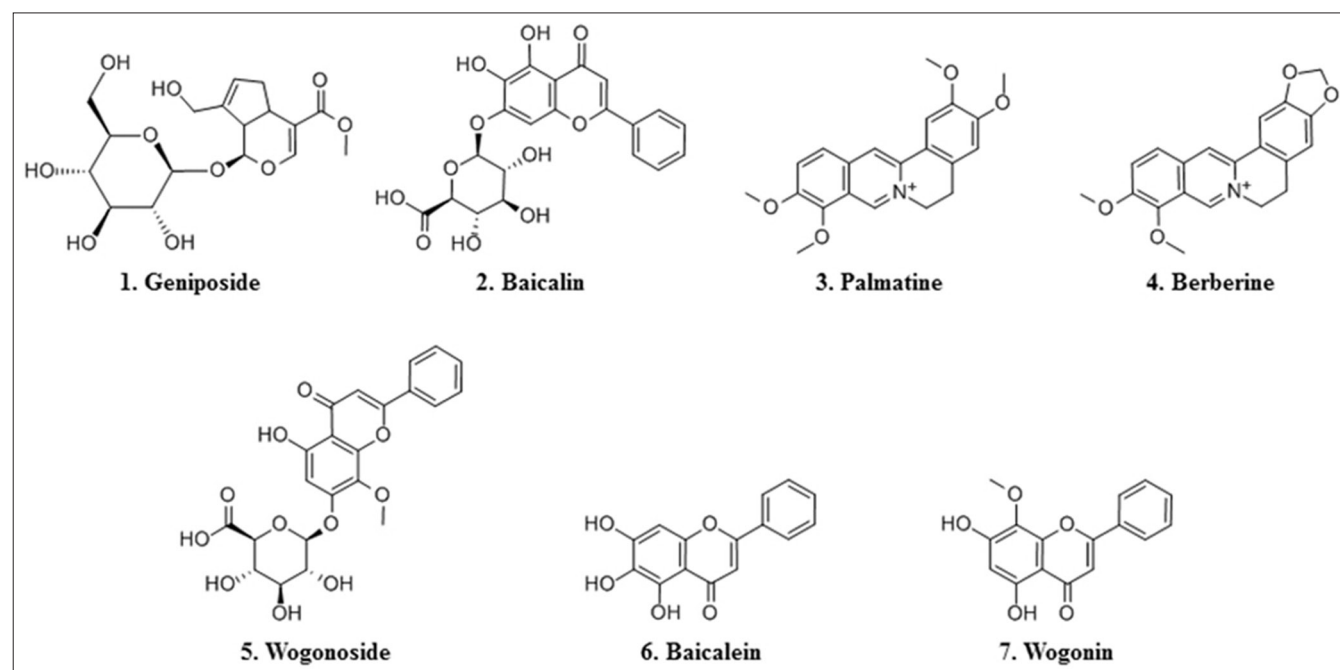
Figure 5: Effects of HR and fermented HRs on the (a-c) phosphorylation of MAPKs in LPS-stimulated macrophages

Figure 6: The HPLC analysis chromatograms of Hwangryunhaedoktang (HR), HR-A and HR-B

Table 4: Data for quantitative analysis of useful compounds of HR and fermented HRs

	Geniposide	Baicalin	Palmatine	Berberine	Wogonoside	Baicalein	Woogonin
HR							
#1	21,762,424	17,850,604	7,775,416	13,946,891	14,727,056	985,007	3,596,744
#2	21,929,636	17,604,047	7,833,367	14,257,931	14,907,064	1,022,008	3,889,902
Average	21,846,030.0	17,727,326	7,804,392	14,102,411	14,817,060	1,003,508	3,743,323
SD	118,236.74	174,342	40,978	219,938	127,285	26,164	207,294
RSD (%)	0.54	0.98	0.53	1.56	0.86	2.61	5.54
HR-A							
#1	23,618,508	15,958,487	6,829,786	14,972,473	12,226,171	3,481,201	6,538,765
#2	23,855,571	16,374,559	6,414,706	14,693,419	12,311,102	3,507,082	6,600,002
Average	23,737,039.5	16,166,523	6,622,246	14,832,946	12,268,637	3,494,142	6,569,384
SD	167,628.85	294,207	293,506	197,321	60,055	18,301	43,301
RSD (%)	0.71	1.82	4.43	1.33	0.49	0.52	0.66
HR-B							
#1	17,866,316	21,988,325	3,409,986	28,568,095	14,291,977	7,005,094	5,953,424
#2	17,714,332	22,041,064	2,845,845	28,804,245	14,199,516	7,021,316	6,017,227
Average	17,790,324.0	22,014,695	3,127,916	28,686,170	14,245,747	7,013,205	5,985,326
SD	107,468.92	37,292	398,908	166,983	65,380	11,471	45,116
RSD (%)	0.60	0.17	12.75	0.58	0.46	0.16	0.75

HR: Hwangryunhaedoktang #1, 2: Peak area (mAU)

Figure 7: Chemical structures of 7 marker constituents of Hwangryunhaedoktang (HR), HR-A and HR-B

occurring plant products. Therefore, we examined whether fermentation improves the anti-inflammatory effect of HR.

The cytotoxicity of drugs needs to be evaluated before developing it for therapeutic applications. Therefore, we examined the effect of HR and fermented HRs on the viability of RAW 264.7 cells. Fermented HRs and HR did not show cytotoxicity at concentrations below 100 µg/mL. Macrophages showed elevated production of PGE_2 and NO after LPS stimulation. In particular,

NO production has been closely related to autoimmune- and inflammation-related diseases.[26,27] In the present study, we found that HR fermented by lactobacilli significantly repressed PGE_2 and NO production as compared to that by HR in RAW 264.7 cells. Moreover, fermentation enhanced the inhibitory effect of HR on the expression of PGE_2 and NO synthesizing enzymes, COX-2 and iNOS. These results suggest that fermentation by lactobacilli directly increases the anti-inflammatory effects.

Since MAPK signaling plays an important role in iNOS and proinflammatory cytokines expression,[28,29] we also investigated the inhibitory effects of HR and fermented HRs on the phosphorylation of MAPKs induced by LPS stimulation of RAW 264.7 cells. Our results showed that non-fermented HR (100μg/mL) partially inhibited all MAPKs. However, fermented HRs significantly suppressed LPS-induced ERK and JNK MAPK phosphorylation.

In addition, HPLC data indicated that fermentation of HR by lactobacilli increased levels of baicalin, berberin and baicalein. A previous study reported that baicalin and baicalein inhibited LPS-induced iNOS and COX-2 gene expression in macrophages.[30] Berberine was also shown to suppress the proinflammatory responses of RAW 264.7 cells through AMPK activation.[31] These data suggest that enhanced anti-inflammatory activity of fermented HRs is related to the increased levels of active components such as baicalin, berberin and baicalein.

Recent studies show that many natural products such as herbal decoctions contain various biological activities including anti-inflammatory effects.[19,25] Therefore, on the basis of the previous studies on natural products, we investigated the anti-inflammatory effect of HR, which is widely used in traditional Korean medicine. Furthermore, we compared the anti-inflammatory activities of non-fermented HR and HR fermented by lactobacilli. Taken together, our results suggest that HR and fermented HRs can inhibit the production of proinflammatory mediators in LPS-stimulated RAW 264.7 cells and that fermentation significantly enhances the anti-inflammatory effect by inhibiting proinflammatory mediators production, including PGE_2, NO, TNF-α and IL-6 by repressing COX-2 and iNOS production. Consistent with these results, the inflammation related MAPKs activities were also significantly inhibited by fermented HRs.

CONCLUSION

In conclusion, fermentation by lactobacilli remarkably increases the anti-inflammatory activity of HR without cytotoxicity in macrophages. Thus, additional *in vivo* studies are needed for the development of fermented HRs as a new anti-inflammatory herbal medicine.

ACKNOWLEDGEMENTS

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