Anti-inflammatory Actions of Herbal Formula Gyejibokryeong-Hwan Regulated by Inhibiting Chemokine Production and STAT1 Activation in HaCaT Cells

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Gyejibokryeong-hwan (GJBRH; Keishi-bukuryo-gan in Japan and Guizhi Fuling Wan in China) is a traditional herbal formula comprising five medicinal herbs and is used to treat climacteric syndrome. GJBRH has been shown to exhibit biological activity against diabetes, diabetic nephropathy, atherosclerosis, ischemia, and cancer. However, there is no scientific evidence of its activities against skin inflammation, including atopic dermatitis. We used the HaCaT human keratinocyte cell line to investigate the effects of GJBRH on skin inflammation. No significant cytotoxicity was observed in cells treated with GJBRH up to a concentration of 1000 µg/mL. Exposure to the proinflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) significantly increased HaCaT cell production of the following chemokines: macrophage-derived chemokine (MDC)/CCL22; regulated on activation, normal T-cell expressed and secreted (RANTES)/CCL5; and interleukin-8 (IL-8). In contrast, GJBRH significantly reduced the production of MDC, RANTES, and IL-8 compared with control cells stimulated with TNF-α and IFN-γ. Consistently, GJBRH suppressed the mRNA expression of MDC, RANTES, and IL-8 in TNF-α and IFN-γ-treated cells. Treatment with GJBRH markedly inhibited phosphorylation of signal transducer and activator of transcription 1 (STAT1) in HaCaT cells stimulated with TNF-α and IFN-γ. Our findings indicate that GJBRH impairs TNF-α and IFN-γ-mediated inflammatory chemokine production and STAT1 phosphorylation in keratinocytes. We suggest that GJBRH may be a potent therapeutic agent for inflammatory skin disorders.

Key words  Gyejibokryeong-hwan; skin inflammation; keratinocyte; chemokine; signal transducer and activator of transcription 1

Chemokines are a superfamily of small proteins secreted by various cell types that play a role in the immune cell infiltration into inflammatory or infectious sites.1,2) Skin inflammation is closely associated with the production of Th2 chemokines.3) When stimulated with the proinflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), skin keratinocytes produce chemokines, including macrophage-derived chemokine (MDC)/CCL22; regulated on activation, normal T-cell expressed and secreted (RANTES)/CCL5; and interleukin-8 (IL-8). These chemokines released from keratinocytes bind to and attract CCR4-positive Th2 cells into inflammatory tissues.4,5)

Signal transducer and activator of transcription (STAT) is a transcription factor protein family activated by Janus kinase (JAK) and is involved in various cellular processes, including growth, survival, proliferation, and differentiation. Among the members of the STAT family proteins, STAT1 plays a critical role in the regulation of primary inflammatory responses.6,7) In keratinocytes, STAT1 is a pivotal regulator of the IFN-γ and/or TNF-α-stimulated immune responses6,8) and eventually stimulate the production of chemokines, including TARC, MDC, and RANTES.13,14) Thus, STAT1 is considered an important molecule for studying inflammatory disorders of skin, including atopic dermatitis.

Gyejibokryeong-hwan (GJBRH; Keishi-bukuryo-gan in Japan and Guizhi Fuling Wan in China) is a traditional Korean herbal formula comprising the five medicinal herbs, *Citrus momomum cassia*, *Poria cocos*, *Paeonia suffruticosa*, *Pa. lactiflora*, and *Prunus persica*. GJBRH has been used in the treatment of patients with climacteric syndrome.15) Many groups have reported on the biological and pharmacological activities of GJBRH against diabetes,16) diabetic nephropathy,17) cardiovascular diseases,18) brain ischemia,19) and cancers.20,21) Interestingly, a recent paper reported that GJBRH inhibited the production of inflammatory cytokines by dermal endothelial cells.22) Based on the results of that paper, our present study aimed to investigate whether GJBRH has inhibitory effects on TNF-α and IFN-γ-induced Th2 chemokine production and to identify the underlying molecular mechanisms in HaCaT human keratinocyte cell line.

MATERIALS AND METHODS

Chemicals and Reagents  Amygdalin and coumarin were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Albiflorin, paconiflorin, cinnamic acid, cinnamaldehyde, and paenol were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The purity of all standard compounds was ≥98.0%. The chemical structures of seven reference compounds are shown in Fig. 1. High-performance liquid chromatography (HPLC)-grade reagents, methanol, acetonitrile, and water were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Acetic acid was procured from Merck (Darmstadt, Germany).
Plant Materials  The five herbal medicines forming GJBRH were purchased from the Korean herbal medicine company, Kwangmyungdang Medicinal Herbs (Ulsan, Korea). The origin of these herbal medicines was taxonomically confirmed by Prof. Je Hyun Lee, Dongguk University, Gyeongju, Korea. A voucher specimen (2013-KE31-1–KE31-5) has been deposited at the Herbal Medicine Formulation Research Group, Korea Institute of Oriental Medicine.

Preparations of Standard and Sample Solutions  Standard stock solutions of seven compounds—amygdalin, albiflorin, paeoniflorin, coumarin, cinnamic acid, cinnamaldehyde, and paeonol—were dissolved in methanol at a concentration of 1.0 mg/mL and stored below 4°C. Working standard solutions were prepared by serial dilution of stock solutions with methanol.

As shown in Table 1, GJBRH comprising five herbal medicines was mixed (5.0 kg; 18.75 g × 266.7) and extracted in a 10-fold mass of water (50 L) at 100°C for 2 h under pressure (98 kPa) using an electric extractor (COSMOS-660; Kyungseo Machine Co., Incheon, Korea). The water extract was then filtered through a standard sieve (no. 270, 53 µm; Chung Gye Sang Gong Sa, Seoul, Korea) and the solution was evaporated to dryness and freeze dried to give a powder. The yield of GJBRH water extract was 11.8% (591.0 g). For HPLC analysis, 500 mg of lyophilized GJBRH water extract was dissolved in 20 mL of distilled water and then extracted by the sonicator for 10 min. The solution was filtered through a 0.2 µm syringe filter (Woongki Science, Seoul, Korea) before injection into the HPLC system.

HPLC Analysis of GJBRH Water Extract  Chromatographic analysis was performed for simultaneous determination of the GJBRH water extract using a Shimadzu Prominance LC-20A system (Shimadzu Co., Kyoto, Japan), which comprised a solvent-delivery unit (LC-20AT), an online degasser (DGU-20A1), a column oven (CTO-20A), a sample autoinjector (SIL-20AC), and a photodiode array (PDA) detector (SPD-M20A). The data processor used LCsolution software (Version 1.24). The seven analytes were separated on a Phenomenex Gemini C18 column (250×4.6 mm, 5 µm, Torrance, CA, U.S.A.) and maintained at 40°C. The mobile phases consisted of 1.0% (v/v) acetic acid in water (A) and 1.0% (v/v) acetic acid in acetonitrile (B). The gradient flow was as follows: 10–60% B for 0–30 min, 60–100% B for 30–40 min, 100% B for 40–45 min, 100–10% B for 45–50 min, and 100% B for 50–60 min. The flow rate was 1.0 mL/min, and the injection volume was 10 µL. The wavelength of the PDA was 190–400 nm and the detected wavelengths for quantitative analysis were monitored at 230 nm (albiflorin and paeoniflorin), 262 nm (amygdalin), and 280 nm (coumarin, cinnamic acid, cinnamaldehyde, and paeonol). All calibration curves were obtained by assessment of peak areas from standard solutions in the following concentration ranges: amygdalin, 3.52–225.00 µg/mL; albiflorin and coumarin, 0.39–50.00 µg/mL; paeoniflorin, 2.34–300.00 µg/mL; cinnamic acid, 0.08–10.00 µg/mL; cinnamaldehyde, 0.31–40.00 µg/mL; and paeonol, 0.78–100.00 µg/mL, respectively.

Cell Culture  HaCaT human keratinocytes were obtained from CLS Cell Lines Service GmbH (Eppelheim, Baden-Württemberg, Germany). The HaCaT cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco Inc., Grand Island, NY, U.S.A.), supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 µg/mL), and maintained at 37°C.

Cytotoxicity Assay  Cell viability was assessed using a Cell Counting Kit-8 assay (CCK-8 from Dojindo, Kumamoto, Japan) according to the manufacturer’s instructions. HaCaT cells (1 × 10³ cells/well) were incubated in 96-well plates with various concentrations of GJBRH extract for 24 h. The CCK-8 reagent was added to each well, the plates were incubated for an additional 4 h, and the absorbance was measured at 450 nm using a Benchmark Plus microplate reader (Bio-Rad Labora-
The percentage of viable cells was calculated using the following equation: cell viability (%) = \[\frac{\text{mean absorbance in test wells}}{\text{mean absorbance in control wells}}\] \times 100.

**Enzyme-Linked Immunosorbent Assay (ELISA) for Chemokine Production**

HaCaT cells \((1 \times 10^6 \text{ cells/well})\) were cultured in 6-well plates. After reaching a confluent state, the cells were washed and treated with GJBRH in 1 mL of serum-free medium that contained TNF-α and IFN-γ (each 10 ng/mL; R&D Systems Inc., Minneapolis, MN, U.S.A.) for 24 h. The production of chemokines (MDC, RANTES, and IL-8) was quantified in the supernatant using an ELISA kit (R&D Systems Inc.).

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

The total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen,
Carlsbad, CA, U.S.A.). One microgram of total RNA was converted to cDNA with an iScript cDNA Synthesis kit (Bio-Rad, Laboratories), that contained the oligo-dT primers, and diethyl pyrocarbonate-treated water was added to produce a final volume of 20 µL, before incubation at 42°C for 30 min. The PCR amplification used gene-specific primers for MDC (forward, 5’-AGG ACA GAG CAT GGC TCG CCT ACA GA-3’; reverse, 5’-TAA TGG CAG GGA GCT AGG GCT CCT GA-3’), RANTES (forward, 5’-CCC CGT GCC GAG ATC AAG GAG TAT TT-3’; reverse, 5’-CGT CCA GCC TGG GGA AGG TTT TTG TA-3’), IL-8 (forward, 5’-GTG CCT TCT TTG GCA GCC TTC CTG AT-3’; reverse, 5’-TCT CCA CAA CCC TCT GCA CCC AGT TT-3’), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward, 5’-GTT ATG GCA TGG ACT GTG GT-3’; reverse, 5’-AAG GGT CAT CAT CTC TGC CC-3’). The RT-PCR reaction mixture comprised 1 µL of cDNA and 1.56 µL of µTag PCR master mix, which contained 1.5 mM MgCl2, 0.1 µM of each forward and reverse primer, and 7.44 µL of water in a final volume of 10 µL. The PCR reaction comprised 25 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 1 min, and extension at 72°C for 1 min 30 s for all of the chemokines and IL-8, and 25 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 1 min, and extension at 72°C for 1 min 30 s for GAPDH. The relative expression levels of MDC, RANTES, and IL-8 were adjusted based on the expression of GAPDH as a control.

**Western Blotting** The cells were treated with a nontoxic concentration of GJBRH for 1 h, and then incubated in the presence of TNF-α and IFN-γ for 24 h. The cells were collected by centrifugation, washed twice with PBS, and suspended in extraction lysis buffer (Sigma-Aldrich) containing protease inhibitors. The protein concentration was determined using a protein assay reagent (Bio-Rad Laboratories). Equal amounts of nuclear extract (30 µg) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was incubated with blocking solution (5% skim milk), followed by overnight incubation at 4°C with the appropriate primary antibody. The following primary antibodies and dilutions were used: anti-β-actin (1:1000 dilution; Cell Signaling Technology, Danvers, MA, U.S.A.), anti-STAT1, and anti-phospho-STAT1 (1:1000 dilution; Abcam, Cambridge, U.K.). The membranes were washed three times with Tris-buffered saline containing Tween 20 (TBST), and then incubated with a 1:3000 dilution of a horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch, PA, U.S.A.) for 1 h at room temperature. The membranes were again washed three times with TBST, and then developed using an enhanced chemiluminescence (ECL) kit (Thermo Scientific, Rockford, IL, U.S.A.). Image capture was performed using Chemi-Doc (Bio-Rad Laboratories).

**Immunofluorescence Assay** Cells were seeded onto glass coverslips and incubated with TNF-α and IFN-γ (each 10 ng/mL) in the absence or presence of GJBRH (500 µg/mL) for 30 min. The cells were fixed in 4% paraformaldehyde and 100% acetone, blocked in 0.5% bovine serum albumin, and incubated with anti-STAT1 antibody (Cell Signaling Technology) for 1 h at room temperature. Then, fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin G (IgG) and diethyl pyrocarbonate-treated water was added to produce a final volume of 20 µL, before incubation at 42°C for 30 min.

**Fig. 3. Cytotoxicity of GJBRH Extract in HaCaT Cells**

Cells were seeded into 96-well plates and treated with various concentrations (0, 62.5, 125, 250, 500, or 1000 µg/mL) of GJBRH extract for 24 h. Cell viability was assessed using a CCK-8 assay. The values are expressed as mean±S.D. of three independent experiments.

**Fig. 4. Effects of GJBRH Extract on Production of Chemokines in TNF-α and IFN-γ-Stimulated HaCaT Cells**

Productions of MDC (A), RANTES (B), and IL-8 (C) were measured using the culture supernatant from cells co-treated with GJBRH extract (125, 250, or 500 µg/mL), and TNF-α and IFN-γ (each 10 ng/mL) for 24 h. Silymarin (6.25, 12.5, or 25 µg/mL) was used as a positive control for atopic dermatitis. Values are expressed as mean±S.E.M. of three independent experiments. *p<0.01 vs. vehicle control cells; **p<0.01 vs. TNF-α and IFN-γ-treated cells.
antibody (Invitrogen) was used as a secondary antibody. The immunostained cells were mounted with medium containing 4'-6-diamidino-2-phenylindole (DAPI) and visualized by use of Olympus FLUOVIEW FV10i confocal microscope.

Statistical Analyses The data were expressed as the mean±S.E.M. All of the experiments were performed at least three times. One-way ANOVA was used to detect significant differences between the control and treatment groups. Dunnett’s test was used for multiple comparisons. The differences were considered significant at p<0.05.

RESULTS

Quantitative Analysis of Seven Marker Components in GJBRH We obtained satisfactory HPLC chromatograms using two mobile phase systems with a gradient elution. Simultaneous determination of seven compounds for quality control was achieved by PDA detection from 190 to 400 nm, based on the peak areas. Using optimized chromatography conditions, all components were separated before 30 min, and a representative HPLC chromatogram of GJBRH water extract is shown in Fig. 2. The calibration curves of all compounds showed good linearity, with an \( r^2 \geq 0.9999 \) for eight different concentration ranges. These data showed that the calibration curve was linear. The limit of detection (LOD) and limit of quantification (LOQ) data were obtained under the chromatographic conditions using signal-to-noise (S/N) ratios of 3 and 10, respectively. The LODs and LOQs were 0.02–2.11 \( \mu \text{g/mL} \) and 0.06–7.04 \( \mu \text{g/mL} \), respectively. These data are shown in Table 2. The retention times of amygdalin, albiflorin, paeoniflorin, coumarin, cinnamic acid, cinnamaldehyde, and paeonol were 9.60, 14.80, 15.55, 19.56, 22.08, 24.53, and 26.37 min, respectively. The amounts of the seven marker compounds were in the range 0.54–46.62 mg/g and are summarized in Table 3.

Cytotoxic Effect of GJBRH Extract against HaCaT Cells To evaluate the cytotoxic effect of GJBRH extract

Table 2. Regression Equations, Linearity and Correlation Coefficient, LOD, and LOQ for Seven Compounds of GJBRH

<table>
<thead>
<tr>
<th>Compound</th>
<th>Linear range (( \mu \text{g/mL} ))</th>
<th>Regression equation(^a)</th>
<th>Correlation coefficient ((r^2))</th>
<th>LOD(^b) (( \mu \text{g/mL} ))</th>
<th>LOQ(^c) (( \mu \text{g/mL} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amygdalin</td>
<td>3.52–225.00</td>
<td>( y=308.43x+48.26 )</td>
<td>1.0000</td>
<td>2.11</td>
<td>7.04</td>
</tr>
<tr>
<td>Albiflorin</td>
<td>0.39–50.00</td>
<td>( y=9522.33x-1496.19 )</td>
<td>1.0000</td>
<td>0.16</td>
<td>0.52</td>
</tr>
<tr>
<td>Paeoniflorin</td>
<td>2.34–300.00</td>
<td>( y=8911.69x-6277.17 )</td>
<td>1.0000</td>
<td>0.47</td>
<td>1.56</td>
</tr>
<tr>
<td>Coumarin</td>
<td>0.39–50.00</td>
<td>( y=48149.55x+2606.00 )</td>
<td>1.0000</td>
<td>0.04</td>
<td>0.13</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>0.08–40.00</td>
<td>( y=89229.20x+812.34 )</td>
<td>1.0000</td>
<td>0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>0.31–40.00</td>
<td>( y=112104.36x+4133.03 )</td>
<td>1.0000</td>
<td>0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>Paeonol</td>
<td>0.78–100.00</td>
<td>( y=63861.00x+23842.04 )</td>
<td>0.9999</td>
<td>0.03</td>
<td>0.10</td>
</tr>
</tbody>
</table>

\(^a\) \( y \): peak area (mAU) of compounds; \( x \): concentration (\( \mu \text{g/mL} \)) of compounds. \(^b\) LOD = 3× signal-to-noise ratio. \(^c\) LOQ = 10× signal-to-noise ratio.

Table 3. Amounts of Seven Components in the GJBRH by HPLC (\( n=3 \))

<table>
<thead>
<tr>
<th>Component</th>
<th>Mean (mg/g)</th>
<th>S.D.</th>
<th>RSD (%)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amygdalin</td>
<td>17.75</td>
<td>0.46</td>
<td>2.60</td>
<td>Pr. persica</td>
</tr>
<tr>
<td>Albiflorin</td>
<td>2.32</td>
<td>0.02</td>
<td>0.83</td>
<td>Pa. lactiflora</td>
</tr>
<tr>
<td>Paeoniflorin</td>
<td>46.62</td>
<td>0.27</td>
<td>0.58</td>
<td>Pa. lactiflora</td>
</tr>
<tr>
<td>Coumarin</td>
<td>2.68</td>
<td>0.00</td>
<td>0.12</td>
<td>C. cassia</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>0.54</td>
<td>0.00</td>
<td>0.31</td>
<td>C. cassia</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>4.28</td>
<td>0.01</td>
<td>0.26</td>
<td>C. cassia</td>
</tr>
<tr>
<td>Paeonol</td>
<td>8.76</td>
<td>0.01</td>
<td>0.09</td>
<td>Pa. suffruticosa</td>
</tr>
</tbody>
</table>

Fig. 5. Effects of GJBRH Extract on Production of Chemokines in TNF-\( \alpha \) or IFN-\( \gamma \)-Stimulated HaCaT Cells

Productions of MDC (A), RANTES (B), and IL-8 (C) were measured using the culture supernatant from cells co-treated with GJBRH extract (500\( \mu \text{g/mL} \)), and either TNF-\( \alpha \) or IFN-\( \gamma \) (each 10ng/mL) for 24h. Values are expressed as mean±S.E.M. of three independent experiments. \(* p<0.01 \) and \(** p<0.001 \) vs. vehicle control cells; \(* p<0.05 \) and \(*** p<0.001 \) vs. TNF-\( \alpha \) and IFN-\( \gamma \)-treated cells or IFN-\( \gamma \)-treated cells.
on HaCaT cells, a cytotoxicity test was performed using the CCK-8 assay. GJBRH extract did not produce any significant cytotoxic effects for 24 h at concentrations ranging from 62.5 to 1000 µg/mL (Fig. 3). Thus, we used the GJBRH extract at 125, 250, or 500 µg/mL in the subsequent experiments.

Inhibitory Effects of GJBRH Extract on Chemokine Production in TNF-α and IFN-γ-Stimulated HaCaT Cells

The effects of the GJBRH extract on MDC, RANTES, and IL-8 production were assessed in TNF-α and IFN-γ-stimulated HaCaT cells. As shown in Fig. 4, treatment with TNF-α and IFN-γ significantly increased production of all chemokines by HaCaT cells compared with the untreated control. By contrast, GJBRH extract significantly reduced TNF-α and IFN-γ-induced production of the chemokines in a dose-dependent manner.

Fig. 6. Effect of GJBRH Extract on Chemokine Expressions in TNF-α and IFN-γ-Stimulated HaCaT Cells

(A) RT-PCR was performed to determine the mRNA expression levels of MDC, RANTES, and IL-8. (B–D) The intensities of the PCR bands for MDC (B), RANTES (C), and IL-8 (D). Values are expressed as mean±S.E.M. of three independent experiments. ## p<0.01 vs. vehicle control cells; * , ** p<0.05 and p<0.01 vs. TNF-α and IFN-γ-treated cells.
manner (Fig. 4). Similarly, silymarin, a positive control for atopic dermatitis, had a dose-dependent inhibitory effect on chemokine production.

To examine whether GJBRH inhibits chemokine production only in co-stimulation of TNF-α and IFN-γ, HaCaT cells were treated with either TNF-α or IFN-γ in the absence or presence of GJBRH. As shown in Fig. 5, IFN-γ stimulated production of MDC, but not RANTES and IL-8, and GJBRH significantly reduced IFN-γ-stimulated MDC generation. TNF-α stimulated IL-8, but not MDC and RANTES in HaCaT cells, but GJBRH had no effect on TNF-α-stimulated IL-8 production in HaCaT cells.

Inhibitory Effects of GJBRH Extract on mRNA Expression of Chemokines in TNF-α and IFN-γ-Stimulated HaCaT Cells  To confirm the inhibitory effects of GJBRH on chemokine expressions, we analyzed the mRNA expression of the chemokines in TNF-α and IFN-γ-stimulated HaCaT cells. As shown in Fig. 6, the mRNA levels of MDC, RANTES, and
IL-8 increased markedly after TNF-α and IFN-γ stimulation of the cells. Expressions of MDC, RANTES, and IL-8 was significantly reduced at the concentrations of 250 and 500 μg/mL GJBRH.

**Effect of GJBRH Extract on STAT1 Phosphorylation in TNF-α and IFN-γ-Stimulated HaCaT Cells** Next, we used Western blot analysis to investigate whether GJBRH could modulate phosphorylation of STAT1 in TNF-α and IFN-γ-stimulated HaCaT cells. TNF-α and IFN-γ stimulation clearly increased STAT1 phosphorylation compared with unstimulated HaCaT cells. By contrast, treatment with GJBRH extract reduced STAT1 phosphorylation induced by TNF-α and IFN-γ. Treatment with silymarin reduced STAT1 phosphorylation compared with HaCaT cells stimulated with only TNF-α and IFN-γ (Figs. 7A, B). Furthermore, immunostaining was performed to detect cellular localization of STAT1 in TNF-α and IFN-γ-treated HaCaT cells with or without GJBRH. As shown in Fig. 7C, STAT1 is clearly localized in the nucleus in the TNF-α and IFN-γ-treated cells. By contrast, the nuclear localization of STAT1 was blocked and localized in the cytoplasm by GJBRH treatment in the cells.

**DISCUSSION**

GJBRH comprises five herbs *C. cassia*, *Po. cocus*, *Sa. fruticosus*, *Pa. lactiflora*, and *Pr. persica* in 1:1:1:1:1 proportions. The main constituents of each herbal medicine are as follows: coumarin (e.g. coumarin, cinnamic acid, and cinnamaldehyde) from *C. cassia*,24-25 triterpenoids (e.g. pachymyic acid and dehydropachymyic acid) from *Po. cocus*,26,27 phenols (e.g. paenol) from *Sa. fruticosus*,28 monoterpenoids (e.g. alboliflorin and paenolflorin) from *Pa. lactiflora*,28 and cyanoglucoisides (e.g. amydalgin) from *Pr. persica*.28 We tried to analyze seven of these compounds using HPLC–PDA: coumarin, cinnamic acid, and cinnamaldehyde (*C. cassia*); paenol (*Pa. safruticosus*); alboliflorin and paenolflorin (*Pa. lactiflora*); and amydalgin (*Pr. persica*). The established HPLC–PDA method was applied for simultaneous analysis of the seven compounds in GJBRH extract. Consequently, paenolflorin (46.62 mg/g), a marker compound of *G. jasminoides*, was detected as the main components in this sample. The results of HPLC–PDA analyses will be helpful for improving the quality control of GJBRH.

Among the five herbal components, *C. cassia* and *Po. cocus* have been reported to have inhibitory effects on atopic dermatitis-like skin disease.29,30 However, the effects of GJBRH on skin diseases have not been reported. In the present study, we found that the traditional herbal prescription GJBRH inhibited the production and expression of proinflammatory chemokines by targeting STAT1 in TNF-α and IFN-γ-stimulated keratinocytes.

The skin immune reaction is important for host defense against pathogenic microbes, and disruption of the skin’s immune system can mediate inflammatory skin diseases.31 Among the several cell types involved in skin inflammation, keratinocytes appear to act as primary inducers and targets of the immunological responses.32 Keratinocytes produce proinflammatory mediators, and chemokines in particular.33 It has been reported that the chemokines MDC, RANTES, and IL-8 are responsible for the initiation of skin inflammation, including atopic dermatitis.3-42 Consistent with the results of previous reports, our findings also showed that stimulation with TNF-α and IFN-γ markedly increased the production and mRNA expression of MDC, RANTES, and IL-8 in HaCaT cells. By contrast, GJBRH treatment reduced both the secretion and mRNA expression of these chemokines compared with the TNF-α and IFN-γ-treated control (Figs. 4, 6). Inhibitory effects of GJBRH was not revealed in the treatment with either TNF-α or IFN-γ (Fig. 5), implying that GJBRH inhibition of inflammatory chemokine production is determined by depending on stimuli.

STAT1 and nuclear factor kappa B (NF-κB) are important transcriptional molecules activated by various inflammatory stimuli such as TNF-α and IFN-γ. Studies have shown that STAT and/or NF-κB activation are mediated by TNF-α and/or IFN-γ in keratinocytes.9-12 Because the promoters of MDC, and RANTES contain STAT- and NF-κB binding sites,34,35 it is possible that chemokine production can be regulated via activation of the STAT1 and NF-κB pathways. We found that TNF-α and IFN-γ treatment increased the level of phospho-STAT1 in HaCaT cells, whereas treatment with GJBRH extract markedly reduced this phosphorylation of STAT1 (Figs. 7A, B). Consistently, immunofluorescence staining with anti-STAT1 antibody showed that GJBRH treatment the nuclear translocation of STAT1 (Fig. 6C). We also examined whether GJBRH could influence NF-κB activation in HaCaT cells. GJBRH had no significant effect on NF-κB compared with TNF-α and IFN-γ-treated cells (data not shown).

Finally, we investigated if inhibitory effects of GJBRH on chemokine production is associated with STAT1 inactivation in TNF-α and IFN-γ-stimulated HaCaT cells. We treated with a specific STAT1 inhibitor ‘fludarabine’36 with GJBRH in TNF-α and IFN-γ-stimulated cells. Fludarabine treatment did not prevent GJBRH inhibition of chemokine production (data not shown), indicating that anti-inflammatory activity of GJBRH in keratinocytes are regulated by two or more distinct pathways such as chemokine- or STAT1-related signaling.

Several recent studies have reported the anti-inflammatory effects of herbal medicines in experimental models. Thongkard et al. reported that Thai native herb extracts exerted protective effects against UVB toxicity in human keratinocytes.37 Wen et al. reported that Pueraaria Radix stimulated hyaluronic acid production in normal human epidermal keratinocytes, indicating its potential as a new cosmetic ingredient in moisturizers and as an anti-aging agent.38 Tse et al. suggested that the anti-proliferative action of Radix Rubiae on human epidermal keratinocytes may contribute to develop an herb-based topical agent for psoriasis treatment.39 We also reported that *Saussurea lappa* alleviates inflammatory chemokine production in HaCaT cells and house dust mite-induced atopic-like dermatitis in Nc/Nga mice.40 These results suggest that herbal medicines could be developed as valuable medications with less toxicity and side effects for various skin diseases. In particular, our present study provides the scientific evidences for effect of herbal formula on skin inflammatory diseases. Since herbal formula consist of multiple herbs that contain multi-bioactive compounds, they can target multiple signaling pathways related to various diseases. Thus, herbal formula will be considered as useful agents for new drug development compared with synthetic chemical drugs in the future.

In conclusion, our findings demonstrate that GJBRH inhibits expression of inflammatory Th2 chemokines in kerati-
necytes. The molecular mechanisms responsible for anti-skin inflammation is related to the STAT1 pathway. Additional studies will be necessary to confirm the inhibitory effects of GJBRH in skin diseases using a specific animal model such as topical dermatitis.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES


