

Potential inhibitory effects of the traditional herbal prescription Hyangso-san against skin inflammation via inhibition of chemokine production and inactivation of STAT1 in HaCaT keratinocytes

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Abstract. Inflammatory skin disease are caused by multiple factors, including susceptibility genes, and immunologic and environmental factors, and are characterized by an increase in epidermal thickness and the infiltration of macrophages, keratinocytes, mast cells, eosinophils and other inflammatory cells. Keratinocytes may serve an important role in the pathogenesis of inflammatory skin diseases. The traditional herbal decoction Hyangso-san (HSS) has been used to treat symptoms of the common cold, including headache, pantalgia, fever and chills. However, to the best of our knowledge, there is no evidence regarding whether HSS has an effect on inflammatory skin diseases. The present study investigated the anti-skin inflammation activity of HSS using the HaCaT human keratinocyte cell line. The mRNA expression and production of inflammatory chemokines, including C-C motif chemokine ligand 22 (CCL22), CCL5, CCL17, and interleukin (IL)-8, was measured using reverse transcription polymerase chain reaction and ELISA analyses. Moreover, we evaluated the effect of HSS on signal transducer and activator of transcription 1 (STAT1) pathway in HaCaT cells. The cells were stimulated with tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) to induce an inflammatory reaction. In the TNF- α - and IFN- γ -stimulated cells, the production and expression of inflammatory chemokines were observed, including CCL22, CCL5, CCL17 and IL-8. In addition, stimulation with TNF- α and IFN- γ increased the phosphorylation and nuclear translocation of STAT1 in HaCaT cells. By contrast, HSS extract

treatment inhibited TNF- α - and IFN- γ -induced STAT1 activation. Results from the present study indicated that HSS exhibited inhibitory effects on TNF- α - and IFN- γ -mediated chemokine production and expression by targeting STAT1 in keratinocytes. Overall, the results indicated that HSS may be a potential candidate therapeutic drug for inflammatory skin diseases such as atopic dermatitis.

Introduction

Skin inflammation is the most common complaint in dermatologic diseases. Inflammatory skin diseases are divided into acute and chronic conditions (1). Acute skin inflammation is associated with occasional rashes, itching and skin redness, and may be caused by ultraviolet or ionizing radiation, allergens or chemical irritants. Chronic inflammatory skin diseases include atopic dermatitis (such as eczema), seborrheic dermatitis, psoriasis and rosacea. Chronic inflammatory skin diseases may lead to significant and serious disruption of skin immunity (2). The prevalence of atopic dermatitis in particular is gradually increasing and affected 230 million people worldwide in 2010 (3).

The type 2 T helper (Th2) cell-mediated inflammatory response is a crucial event in the development of inflammatory skin diseases (4,5). Th2 cells are attracted to the skin by inflammatory chemokines, including C-C motif chemokine ligand 5 (CCL5), CCL17, CCL22 and interleukin-8 (IL-8; also known as CXCL8) (6-9), that are synthesized by epidermal keratinocytes (10). Therefore, the inflammatory chemokines are considered important therapeutic targets for the treatment of inflammatory skin diseases. In addition, signal transducer and activator of transcription 1 (STAT1) has been reported to have pivotal roles in the regulation of chemokine production and inflammation (11). STAT1 is transactivated when keratinocytes are exposed to tumor necrosis factor- α (TNF- α) and/or interferon- γ (IFN- γ) (12).

Corticosteroids are the most commonly used medication to treat inflammatory skin diseases, as they are effective at controlling mild to moderate dermatitis with short-term

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use. However, long term administration of topical steroids may cause severe side effects, including skin atrophy, striae and telangiectasia (13). Thus, it is necessary to develop anti-inflammatory drugs with improved efficacy against skin inflammation, with fewer side effects, that may be used over longer periods of time. Recently, various studies have reported on the inhibitory effects of traditional herbal formulas on skin inflammation (14-18) and have indicated that herbal formulas may be valuable alternative medicines for treating skin inflammation diseases.

Hyangso-san (HSS) is a traditional herbal formula that has been used in Korea to treat symptoms of the common cold, including headache, pantalgia, fever and chills (19). HSS comprises seven herbal medicines, including *Cyperus Rhizoma*, *Perillae Herba*, *Atractylodes Rhizoma*, *Citri Unshii Pericarpium*, *Glycyrrhizae Radix et Rhizoma*, *Zingiberis Rhizoma Crudus* and *Allii Radix*. Previous studies have indicated antidepressant and anti-stress effects of HSS (20,21); however, to the best of our knowledge, the anti-inflammatory effects of HSS have not been examined. Therefore, the present study investigated the anti-inflammatory effects of HSS in skin using a TNF- α - and IFN- γ -stimulated HaCaT human keratinocyte cell line.

Materials and methods

Plant materials. HSS is comprised of seven herbal medicines, and each of the raw materials was purchased from Kwangmyungdang Medicinal Herbs (Ulsan, Korea) (Table I). All crude materials were analyzed by pharmacognosists Professor Je-Hyun Lee and Professor Young-Bae Seo (both from College of Oriental Medicine, Daejeon University, Daejeon, Korea). Voucher specimens (2012-KE44-1-7) have been deposited at the K-herb Research Center, Korean Institute of Oriental Medicine (Daejeon, Korea).

Chemicals and reagents. Liquiritin and glycyrrhizin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Liquiritin apioside and hesperidin were purchased from Shanghai Sunny Biotech Co., Ltd. (Shanghai, China). Rosmarinic acid and atractylenolide III were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and SK Biotek Co., Ltd. (Daejeon, Korea), respectively. Purities of the six reference standards, including liquiritin apioside, liquiritin, hesperidin, rosmarinic acid, glycyrrhizin and atractylenolide III, were >98.0% as determined by high-performance liquid chromatography (HPLC) analysis, as described below. HPLC-grade methanol, acetonitrile and water were purchased from Avantor (Center Valley, PA, USA). Reagent-grade formic acid was purchased from Sigma-Aldrich (Merck KGaA).

Preparation of HSS water extract. To prepare the HSS water decoction, the crude materials *Cyperus Rhizoma* (1,111 g), *Perillae Herba* (1,111 g), *Atractylodes Rhizoma* (833 g), *Citri Unshii Pericarpium* (556 g), *Glycyrrhizae Radix et Rhizoma* (278 g), *Zingiberis Rhizoma Crudus* (556 g) and *Allii Radix* (556 g) were mixed and extracted with 50 l of distilled water at 100°C for 2 h under pressure (98 kPa) using a COSMOS-660 Electric Vacuum Extractor (Kyungseo Machine Co., Incheon, Korea). The extracted solution was filtered using a standard sieve (no. 270, 53 μ m; Chung Gye Sang Gong Sa,

Seoul, Korea), and the filtered solution was subsequently freeze-dried to produce a powder using a PVTFD10RS vacuum freeze dryer. The amount of freeze-dried HSS powder obtained was 670.6 g (yield, 13.4%).

Cell culture. HaCaT human keratinocytes (CLS Cell Lines Service GmbH, Eppelheim, Germany) were incubated in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μ g/ml) (all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in an incubator containing 5% CO₂ at 37°C.

Cytotoxicity test. A cytotoxicity assay was performed using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's instructions. Briefly, HaCaT cells were seeded (1 \times 10³ cells/well) on a 96-well microplate and treated with various concentrations of HSS (125, 250, 500 or 1,000 μ g/ml) for 24 h in an incubator containing 5% CO₂ at 37°C. Control cells were treated with medium only. CCK-8 reagent (10 μ l) was added to each well and the mixture was incubated for 4 h in an incubator containing 5% CO₂ at 37°C. The absorbance was read at 450 nm using a Benchmark Plus Microplate Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell viability was calculated using the following equation: Cell viability (%) = (mean absorbance in HSS - treated cells/mean absorbance in untreated control cells) \times 100.

Measurement of chemokine production by ELISA. The production of chemokines was measured using commercial kits for CCL22, CCL5, CCL17 and IL-8 (R&D Systems, Inc., Minneapolis, MN, USA), according to the respective manufacturer's instructions. Briefly, HaCaT cells (1 \times 10⁶ cells/well) were cultured in 6-well plates for 18 h in an incubator containing 5% CO₂ at 37°C. After reaching confluency, the cells were washed and treated with or without HSS (125, 250 or 500 μ g/ml) in 1 ml of serum-free medium containing TNF- α and IFN- γ (each 10 ng/ml; R&D Systems Inc.) for 24 h. The culture supernatants were collected for 5 min at 16,000 \times g at 4°C, and subjected to ELISA for each chemokine. Silymarin (6.25, 12.5 or 25 μ g/ml; Sigma-Aldrich; Merck KGaA) was used as a positive control.

Reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR analyses for chemokine expression were performed as previously described (17). Briefly, HaCaT cells (1 \times 10⁶ cells/well) were cultured to 80-90% confluence in 6-well plates. When the cells reached confluence, they were washed and treated with HSS in 1 ml serum-free medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with TNF- α - and IFN- γ for 24 h. Total RNA (1 μ g) was isolated using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was synthesized using the iScript cDNA Synthesis kit and the PCR reaction (iScript™ Reverse Transcription Supermix)(both from Bio-Rad Laboratories, Inc.) was performed using the following gene-specific primers: CCL22 forward, 5'-AGGACAGAGCATGGCTCG CCTACAGA-3' and reverse, 5'-TAATGGCAGGGAGCT AGGGCTCCTGA-3'; CCL5 forward, 5'-CCCCGTGCCGAG

Table I. Herbal composition of Hyangso-san water extract.

Herbal medicine	Scientific name	Family	Ratio (%)	Origin
Cyperi Rhizoma	<i>Cyperus rotundus</i> Linnaeus	Cyperaceae	22.2	Yeongcheon, Korea
Perillae Herba	<i>Perilla frutescens</i> Britton var. <i>acuta</i> Kudo	Lamiaceae	22.2	Namwon, Korea
Atractylodis Rhizoma	<i>Atractylodes chinensis</i> Koidzumi	Asteraceae	16.7	China
Citri Unshii Pericarpium	<i>Citrus unshiu</i> Markovich	Rutaceae	11.1	Jeju, Korea
Glycyrrhizae Radix et Rhizoma	<i>Glycyrrhiza uralensis</i> Fischer	Fabaceae	5.6	China
Zingiberis Rhizoma Crudus	<i>Zingiber officinale</i> Roscoe	Zingiberaceae	11.1	Ulsan, Korea
Allii Radix	<i>Allium fistulosum</i> Linnaeus	Amaryllidaceae	11.1	Hanam, Korea

ATCAAGGAGTATTT-3' and reverse, 5'-CGTCCAGCC TGGGGAAGGTTTT TGTA-3'; CCL17 forward, 5'-ACT GCTCCAGGGATGCCATCGTTTTT-3' and reverse, 5'-ACA AGGGGATGGGATCTCCCTCACTG-3'; IL-8 forward, 5'-GTGGCTCTCTTGGCAGCCTTCCTGAT-3' and reverse, 5'-TCTCCACAACCCTCTGCACCCAGTTT-3'; and β -actin forward, 5'-GTGATGGCATGGACTGTGGT-3' and reverse, 5'-AAGGGTCATCATCTCTGCCC-3'. The PCR conditions were as follows: Twenty-five cycles of predenaturation at 94°C for 5 min, denaturation at 94°C for 30 sec, annealing at 64°C for 1 min, extension at 72°C for 90 sec and final elongation at 72°C for 5 min.

Western blot analysis. HaCaT cells (1×10^6 cells/well) were stimulated with TNF- α and IFN- γ (10 ng/ml of each) with or without various concentrations of HSS (0, 125, 250 or 500 μ g/ml) for 30 min. Cells were lysed and protein fractions were extracted using the NE-PER Nuclear and Cytoplasmic Extraction reagents kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Protein concentrations were determined using a Bradford Protein Assay kit (Bio-Rad Laboratories, Inc.), according to the manufacturer's instructions. Equal amounts of nuclear extracts (30 μ g) were resolved by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were incubated with blocking buffer [5% skim milk in TBS containing 20% Tween-20 (TBST)] for 1 h at room temperature, followed by overnight incubation at 4°C with primary antibodies against STAT1 (1:1,000, ab3987, mouse), phosphorylated (p)-STAT1 (1:1,000, ab109461, rabbit) (both from Abcam, Cambridge, UK), or β -actin (1:1,000, 4967, rabbit; Cell Signaling Technology, Inc., Danvers, MA, USA). The membranes were washed three times with TBST and incubated with a horse-radish peroxidase-conjugated secondary antibody [1:3,000, cat. no. 111-035-003 (rabbit); cat. no. 115-035-003 (mouse); Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA] for 1 h at room temperature. The membranes were washed three times with TBST and developed using an Enhanced Chemiluminescence kit (Thermo Fisher Scientific, Inc.). Images of developed membranes were captured using a Chemi-Doc Gel Imaging System (Bio-Rad Laboratories, Inc.).

Immunofluorescence assay. HaCaT cells (1×10^6 cells/well) were seeded onto glass coverslips and incubated with TNF- α and IFN- γ (10 ng/ml of each) in the presence or absence of HSS (500 μ g/ml) for 30 min at 37°C. The cells were fixed in

4% paraformaldehyde and 100% acetone for 15 min at room temperature, and incubated in blocking solution (0.5% bovine serum albumin; Thermo Fisher Scientific, Inc.) for 30 min at room temperature. The slides were probed with anti-STAT1 antibody (diluted 1:500, no. 9172; Cell Signaling Technology, Inc.) by overnight incubation at 4°C, followed by incubation with Texas Red-conjugated anti-rabbit immunoglobulin G (diluted 1:500, A11037; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. The immunostained cells were mounted with medium containing DAPI (diluted 1:10,000, H-1200; Vector Laboratories, Inc., Burlingame, CA, USA) and visualized using an Olympus FluoView FV10i confocal microscope system (Olympus Corporation, Tokyo, Japan). Quantification of the effects was performed by measuring the density of HaCaT cells at x200 magnification using ImageJ 1.50i software (National Institutes of Health, Bethesda, MD, USA).

Chromatographic conditions. Quantitative analysis of the components in HSS was performed using a Shimadzu Prominence LC-20A series HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a two LC-20AT pumps, a DGU-20A3 on-line degasser, a CTO-20A column oven, a SIL-20A auto sample injector and a SPD-M20A photodiode array (PDA) detector. Chromatographic data were recorded and processed using Lab Solution software version 5.54 SP3 (Shimadzu Corporation). Separation of the major components of HSS was achieved on a Phenomenex Gemini C18 column (250x4.6 mm, 5 μ m; Phenomenex, Inc., Torrance, CA, USA) and the column oven temperature was maintained at 40°C. The mobile phases consisted of 1.0% (v/v) distilled water-acetonitrile, which both contained 0.1% (v/v) formic acid. The gradient conditions for efficient separation of the major components was as follows: 5-60% B (30 min), 60-100% B (10 min), held at 100% B (5 min), 100-5% B (5 min) and held at 5% B for 10 min. The flow rate and injection volume were 1 ml/min and 10 μ l.

Statistical analysis. All experiments were repeated at least three times and data are presented as the mean \pm standard error of the mean. One-way analysis of variance was used to detect significant differences between the control and HSS-treatment groups using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA) followed by Dunnett's post hoc multiple comparison test. $P < 0.05$ was considered to indicate a statistically significant difference.

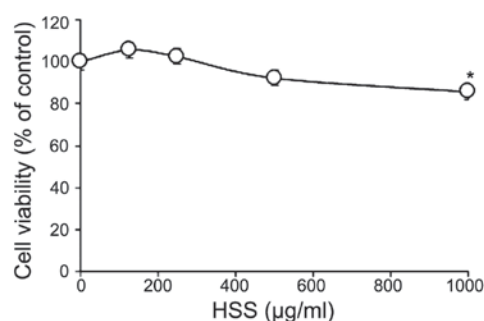


Figure 1. Cytotoxicity of the HSS extract in HaCaT cells. Cells were seeded into 96-well plates and treated with various concentrations of HSS water extract (0, 125, 250, 500 or 1,000 $\mu\text{g/ml}$) for 24 h. Cell viability was assessed using a Cell Counting Kit-8 assay. Data are presented as the mean \pm standard error of the mean of three independent experiments. * $P < 0.05$ vs. non-treated cells. HSS, Hyangso-san.

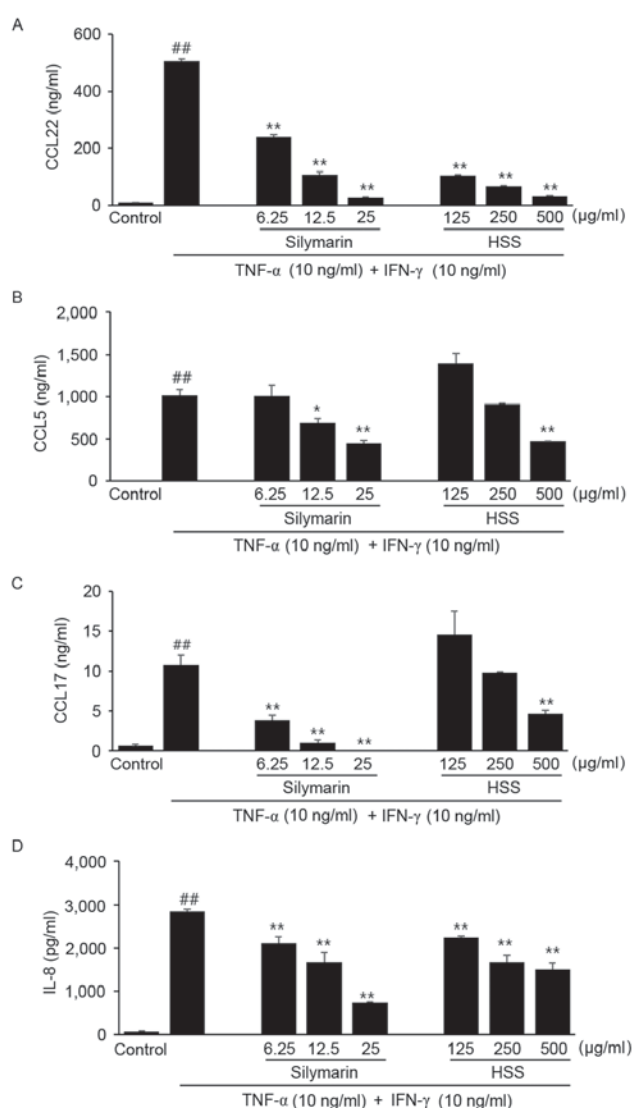


Figure 2. Effects of HSS on the production of chemokines in TNF- α - and IFN- γ -stimulated HaCaT cells. Production of (A) CCL22, (B) CCL5, (C) CCL17 and (D) IL-8 was measured using the culture supernatant from cells co-treated with HSS extract (125, 250 or 500 $\mu\text{g/ml}$), and TNF- α and IFN- γ (10 ng/ml each) for 24 h. Silymarin (6.25, 12.5 or 25 $\mu\text{g/ml}$) was used as a positive control. Data are presented as the mean \pm standard error of the mean of three independent experiments. ## $P < 0.01$ vs. control cells; * $P < 0.05$ and ** $P < 0.01$ vs. TNF- α - and IFN- γ -treated cells. CCL, C-C motif chemokine ligand; HSS, Hyangso-san; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

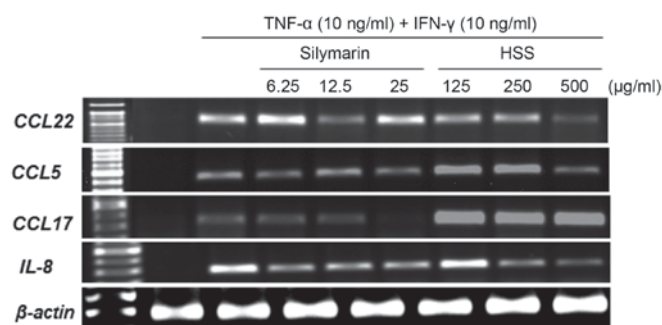


Figure 3. Effects of HSS on chemokine mRNA expression levels in TNF- α - and IFN- γ -stimulated HaCaT cells. Reverse transcription-polymerase chain reaction was performed to determine the mRNA levels of CCL22, CCL5, CCL17 and IL-8. β -actin was used as a housekeeping control gene. CCL, C-C motif chemokine ligand; HSS, Hyangso-san; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

Results

Cytotoxic effects of HSS on HaCaT human keratinocyte cells. To determine the nontoxic concentrations of the HSS water extract in HaCaT cells, a cytotoxicity assay was performed. Cells were treated with various concentrations of HSS extract (0, 125, 250, 500 or 1,000 $\mu\text{g/ml}$) for 24 h. As demonstrated in Fig. 1, cell viability was maintained at $>90\%$ for up to 500 $\mu\text{g/ml}$ ($92.27 \pm 0.80\%$ at 500 $\mu\text{g/ml}$) but was reduced to $85.69 \pm 1.69\%$ at 1,000 $\mu\text{g/ml}$. These results indicated that HSS is nontoxic to the cells at physiologically relevant concentrations (≤ 500 $\mu\text{g/ml}$). Subsequent assays were performed in the range of nontoxic concentrations.

Inhibitory effects of HSS on chemokine protein production in TNF- α - and IFN- γ -stimulated HaCaT cells. To investigate whether HSS extract has inhibitory effects on inflammatory reactions in keratinocytes, ELISAs were performed to measure the production of inflammatory chemokines. Inflammatory reactions were induced by stimulating HaCaT cells with TNF- α and IFN- γ , and concurrently treating the cells with or without various concentrations of HSS extract (0, 125, 250 or 500 $\mu\text{g/ml}$) for 24 h. Treatment with TNF- α and IFN- γ significantly increased the expression levels of CCL22, CCL5, CCL17 and IL-8 in HaCaT cells compared with untreated control cells (Fig. 2). However, HSS treatment significantly reduced the TNF- α - and IFN- γ -mediated increase in CCL22 and IL-8 expression, in a dose-dependent manner (Fig. 2A and D). The inhibitory effects of HSS on CCL5 and CCL17 production were observed only at 500 $\mu\text{g/ml}$, and not at the lower concentrations (Fig. 2B and C). A positive control, silymarin evoked a significant decrease in TNF- α - and IFN- γ -stimulated chemokine production. The inhibitory effects of HSS were similar to those of silymarin.

Inhibitory effects of HSS on chemokine mRNA expression in TNF- α - and IFN- γ -stimulated HaCaT cells. To confirm anti-inflammatory effects of HSS on HaCaT cells, RT-PCR was performed to assess the mRNA expression of the inflammatory chemokines CCL22, CCL17, CCL5 and IL-8. Consistent with the ELISA results aforementioned, HSS extract suppressed the mRNA expression levels of CCL22

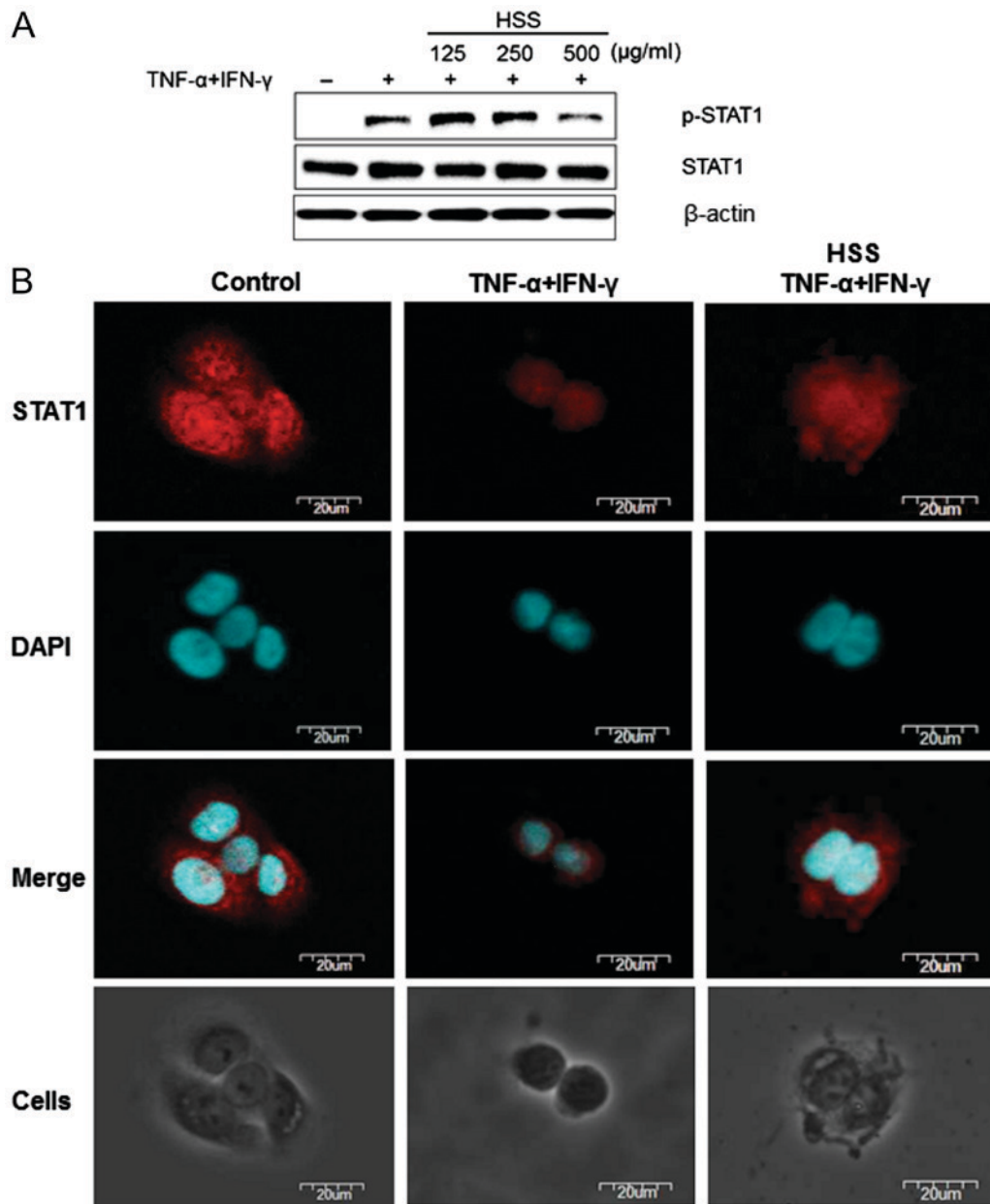


Figure 4. Effects of HSS on STAT1 activation in TNF- α - and IFN- γ -stimulated HaCaT cells. (A) Cells were treated with TNF- α and IFN- γ (10 ng/ml each) in the absence or presence of HSS (125, 250 or 500 μ g/ml) for 30 min. Cell lysates were prepared and subjected to immunoblotting with anti-p-STAT1 and anti-STAT1 antibodies. (B) Cellular localization of STAT1 was analyzed by confocal microscopy. Cells were treated with TNF- α and IFN- γ (10 ng/ml each) in the absence or presence of HSS (500 μ g/ml) for 30 min on glass coverslips and incubated with anti-STAT1 primary antibody and Texas Red-conjugated secondary antibody. Immunostained cells were mounted with medium containing DAPI and visualized using an Olympus FluoView FV10i confocal microscope. HSS, Hyangso-san; IFN, interferon; p, phosphorylated; STAT1, signal transducer and activator of transcription 1; TNF, tumor necrosis factor.

and IL-8 in TNF- α - and IFN- γ -treated HaCaT cells. In addition, the inhibitory effect of CCL5 expression was observed at a concentration of 500 μ g/ml in TNF- α - and IFN- γ -treated HaCaT cells (Fig. 3). However, the inhibitory effect on CCL17 expression by HSS was not affected. Silymarin was used as a positive control (22) for the production and expression of the chemokines, and it evoked a decrease in TNF- α - and IFN- γ -stimulated chemokine production (Fig. 3).

Effects of HSS on the activation of STAT1 in TNF- α - and IFN- γ -stimulated HaCaT cells. The transcription factor STAT1 serves an important role in the regulation of skin inflammation and is associated with chemokine production

in keratinocytes that have been treated with inflammatory stimuli (18,23). In the present study, TNF- α and IFN- γ stimulation notably increased the expression levels of p-STAT1 compared with the untreated HaCaT control cells (Fig. 4A). Cells co-treated with HSS extract exhibited a reduction in the TNF- α - and IFN- γ -induced p-STAT1 expression at a dose of 500 μ g/ml, but not at lower concentrations (125 and 250 μ g/ml; Fig. 4A). To further confirm the involvement of STAT1 in the anti-inflammatory activity of HSS, immunocytochemical analysis was performed. HSS extract (500 μ g/ml) co-treatment reversed the TNF- α - and IFN- γ -mediated decrease in the cytoplasmic expression of STAT1 in HaCaT cells (Fig. 4B).

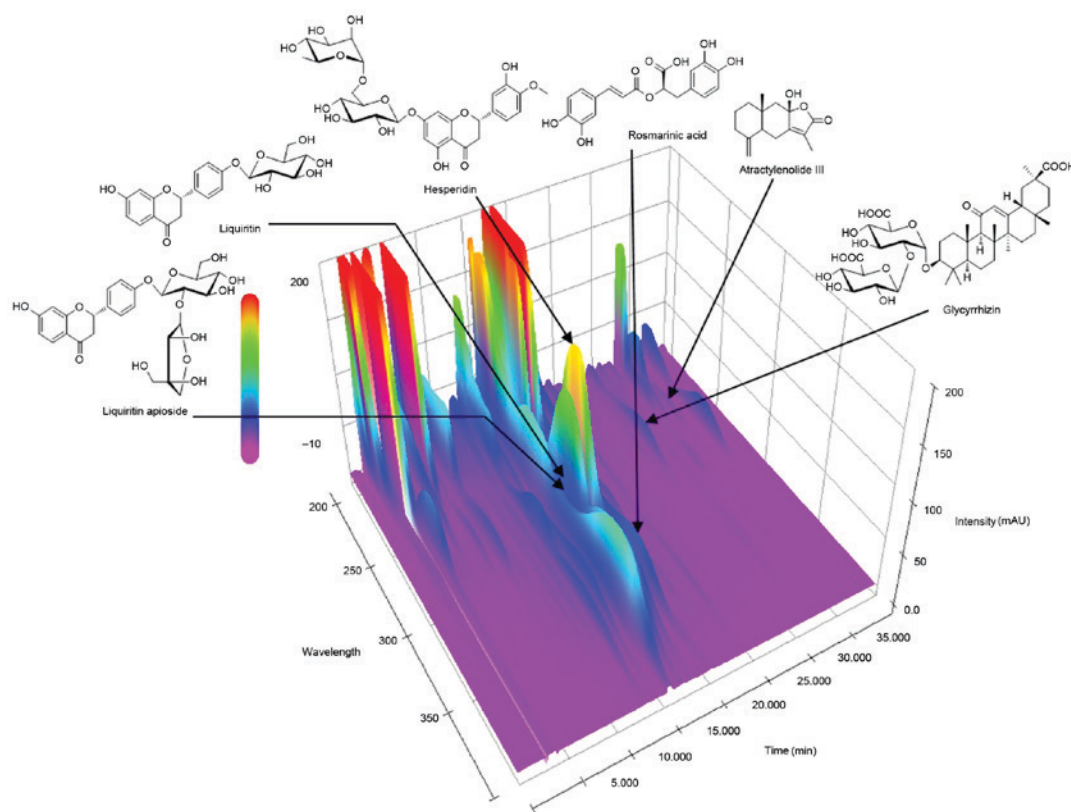


Figure 5. Three-dimensional chromatogram of Hyangso-san sample analyzed by HPLC-photodiode array. HPLC, high-performance liquid chromatography; mAU, milli-absorbance units.

HPLC analysis of the six marker compounds in HSS water extract. The above-described HPLC-PDA method was used for the quantitative analysis of the six marker compounds in the HSS extract. The retention times of liquiritin apioside, liquiritin, hesperidin, rosmarinic acid, glycyrrhizin and atractylenolide III were 16.23, 16.63, 18.33, 19.80, 29.86 and 34.03 min, respectively (Fig. 5). The concentrations of the six marker compounds were calculated using calibration curves of each reference standard; the concentrations of liquiritin apioside, liquiritin, hesperidin, rosmarinic acid, glycyrrhizin and atractylenolide III in the HSS extract were determined to be 1.26, 1.82, 5.40, 1.29, 1.70 and 0.56 mg/g, respectively.

Discussion

Keratinocytes serve a major role in the regulation of acute and chronic skin inflammation by means of chemokine production (24). Therefore, activated keratinocytes are valuable as an *in vitro* model for the investigation of inflammatory skin diseases. A number of previous studies have reported that TNF- α and IFN- γ stimulation of keratinocytes leads to the generation of specific inflammatory chemokines, including CCL22, CCL17, CCL5 and IL-8 (25-27). To investigate whether HSS has inhibitory effects on skin inflammation, a HaCaT human keratinocyte cell line was used and stimulated with TNF- α and IFN- γ to induce an inflammatory reaction. Consistent with the results of previous studies, the present study demonstrated that TNF- α and IFN- γ significantly increased the production and expression of CCL22, CCL17, CCL5 and IL-8 compared with unstimulated cells. Inhibition of CCL17

expression by HSS was not affected by mRNA levels; treatment with HSS extract decreased the expression levels of the chemokines released into the culture supernatants as well as their respective mRNA expression in TNF- α - and IFN- γ -stimulated HaCaT cells, particularly at a dose of 500 μ g/ml, which indicated a potential anti-inflammatory effect of HSS extract in the skin.

STAT1 is a member of the STAT protein family and has a crucial role in the IFN/cytokine-signaling pathways (28). The binding of IFN- γ to the IFN- γ receptor rapidly induces the phosphorylation of Janus tyrosine kinase 1 (JAK1) and JAK2, which subsequently leads to the activation of STAT1 (29). These events ultimately stimulate the production of inflammatory chemokines (26,27,30); therefore, STAT1 is considered as a therapeutic target for the development of anti-inflammatory agents for skin diseases. The results of the present study demonstrated that treatment with HSS extract (at a dose of 500 μ g/ml only) suppressed the expression levels of p-STAT1 in TNF- α - and IFN- γ -stimulated HaCaT cells. Furthermore, HSS treatment reversed the decreased expression of STAT1 that was induced by TNF- α and IFN- γ stimulation in HaCaT cells, which suggested the involvement of STAT1 in the inhibition of inflammatory cytokine production in keratinocytes by HSS.

Notably, the present study conducted a quantitative HPLC-PDA analysis of HSS marker compounds. The principal compounds of the HSS water extract have previously been reported to be the following: The sesquiterpenoid nootkatone from *Cyperus Rhizoma* (31); the phenylpropanoid rosmarinic acid and the flavonoid luteolin from *Perillae Herba* (32,33);

the sesquiterpenoids atractylenolide I and III from *Atractylodes Rhizoma* (34); the flavonoid hesperidin from *Citri Unshii Pericarpium* (35); the triterpenoid saponin glycyrrhizin and the flavonoids liquiritin and liquiritigenin from *Glycyrrhizae Radix et Rhizoma* (36); the phenol 6-gingerol from *Zingiberis Rhizoma Crudus* (37); the phenylpropanoid ferulic acid and the flavonoid kaempferol from *Allii Radix* (38). Simultaneous analysis of six compounds (liquiritin apioside, liquiritin, hesperidin, rosmarinic acid, glycyrrhizin and atractylenolide III) in the HSS sample demonstrated that hesperidin was detected as the major compound (5.40 mg/g).

In conclusion, the results of the present study demonstrated that the Korean traditional herbal formula HSS water extract exhibits anti-inflammatory effects in HaCaT keratinocyte cells and may function by suppressing chemokine production and expression. In addition, the transcription factor STAT1 may contribute to the HSS-mediated inhibition of the inflammatory response in keratinocytes. Further investigation is required to determine the *in vivo* activity of HSS using a specific animal model, such as one for atopic dermatitis, and to identify the compound in HSS that may be responsible for the anti-inflammatory action.

Acknowledgements

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