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Research Paper

Gamisasangja-tang suppresses pruritus and atopic skin inflammation in the NC/Nga murine model of atopic dermatitis

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ABSTRACT

Ethnopharmacological relevance: Gamisasangja-tang (GST) is a traditional herbal formula prescribed for patients with intractable pruritus in association with various inflammatory skin diseases. To evaluate the effects of GST on pruritic skin inflammation and investigate its cellular and molecular mechanisms.
Materials and methods: We orally administered GST to NC/Nga (NC) mice, an animal model of atopic dermatitis. Scratching frequency and the dermatitis index were evaluated, and histological examination was performed using hematoxylin and eosin and toluidine blue staining. The levels of interleukin (IL)-31 and T-helper cell type 2 (T_H2) cytokines were determined in both the dorsal skin and cultured splenocytes by real-time polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. The serum levels of chemokines and immunoglobulin E (IgE) were determined by ELISA. Changes in the inflammatory cell population were analyzed by a hemocytometer.
Results: GST significantly lowered scratching frequency and inhibited increases in dermatitis index, thickness of epidermis/dermis and infiltration of chemokine (C-C motif) receptor 3 $(CCR3)^+$ and cluster of differentiation $(CD)117^+$/FcεRIα (Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide)$^+$ cells in atopic skin. Both *IL*-31 mRNA expression and production were significantly reduced by GST, which was accomrease in the levels of *IL-4*, *IL-5*, and *IL-13*. Further, GST treatment suppressed the secretion of eotaxin, TARC (thymus and activation-regulated chemokine), IgE, and increases in the number of basophils and eosinophils in the blood.
Conclusion: GST may have potential as an effective treatment for pruritic skin disease such as atopic dermatitis.

1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by pruritic eczematous lesions. It is currently accepted that the development of atopic skin lesions is associated with skin barrier dysfunction and a skewed balance of T-helper type 2 (T_H2) cells in the immunological system (Berke et al., 2012). Pruritus is not only the most distressing symptom in patients with AD, but is also a major cause of skin barrier dysfunction (Kim et al., 2006). Cutaneous abnormalities such as dryness may initiate an itching sensation that leads to mechanical injury form scratching, and a recurrent itching-scratching cycle can worsen the disease by increasing the release of proinflammatory cytokines and chemokine production (Kabashima, 2013). This inflammatory process subsequently directs the

Abbreviations: ANOVA, analysis of variance; CCR3, chemokine (C-C motif) receptor 3; CD, cluster of differentiation; DNCB, 2,4-dinitrochlorobenzene; DNFB, 2,4-dinitrofluorobenzene; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3 phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; FcεRIα, Fc fragment of IgE, high affinity I, receptor for, alpha polypeptide; GST, Gamisasangja-tang; Dex, dexamethasone; IgE, immunoglobulin E; IL, interleukin; NC, NC/Nga; PBS, phosphate buffered saline; PE, phycoerythrin; PerCP, peridinin chlorophyll protein complex; PCR, polymerase chain reaction; ST, sasangja-tang; TARC, thymus and activation regulated chemokine; Th2, T-helper cell type 2

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recruitment of pathogenic leukocytes to the skin (Ley, 1996), and thus, itching directly contributes to the development of eczematous skin lesions. Recently, interleukin (IL)-31, originally identified as a T_H2 cytokine, has been proposed as a pruritogenic cytokine (Cornelissen et al., 2012). IL-31 transgenic mice develop spontaneous severe pruritus and defects in skin barrier formation with trans-epidermal water loss (Dillon et al., 2004). Indeed, skin biopsies of AD patients display increased *IL-31* mRNA expression compared to normal skin, and increased serum levels of IL-31 are correlated with itching and AD severity in human (Raap et al., 2008). Furthermore, the amplification of atopic skin inflammation has been associated with a T_H2 phenotype that includes T_H2 cytokine secretion, blood eosinophilia, and increased serum immunoglobulin E (IgE) (Akdis et al., 2000). It has been well known that increased levels of T_H2 cytokine expression, including IL-4, IL-5, and IL-13, are correlated with the severity of atopic dermatitis (Leung et al., 2004). IL-4 and IL-13, which are produced by both basophils and T_H2 cells, together direct IgE switching in B cells (Sallusto et al., 1998), and IL-5 promotes the survival and activation of eosinophils, resulting in eosinophilia and infiltration of the cells into skin (Simon et al., 2004). Chemokines such as eotaxin and thymus and activation-regulated chemokine (TARC), which is produced by T_H2 cells, keratinocytes, or endothelial cells, contribute to the infiltration of leukocytes including eosinophils, monocytes, and mast cells (Homey et al., 2006). The serum levels of IgE are also associated with the severity of AD (Matsuda et al., 1997), and the cross-linking of FCεRI (Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide) on the mast cell surface with IgE and antigens generates prompt release of various inflammatory mediators, leading to atopic skin inflammation, itching, and erythematous skin lesions (Novak et al., 2003).

Gamisasangja-tang (GST) consists of six herbs including *Stemonae radix, Spirodelae herba, Cnidii fructus, Sophora flavescens root, Angelica gigas root, and Clematidis radix.* Sasangja-tang (ST), also called She-chuang-zi-tang, is described in the traditional herbal medicine textbook *Wai-ke-zheng-zong* and has been used for centuries in the treatment of inflammatory skin disease(Chen, 1983). ST was modified by adding *S. radix* and *S. herba* to formulate GST to improve therapeutic effects; because these two herbs were empirically recognized as an effective treatment for itching by Korean medical practitioners. Protostemonamide, stenonamine, and stenonine are stemona alkaloids, which are the main components from *S. radix* belonging to the Stemonaceae family (Yang et al., 2009). *Spirodela polyrrhiza* (Lemnaceae) herba had shown to contain several kinds of flavonoids such as vitexin, orientin, apigenin, and luteolin (Qiao et al., 2011; Seo et al., 2012). Torilin, torilolone, and osthol are the main active compounds found in *Cnidii monnieri* (Umbelliferae) fruit (Basnet et al., 2001; Oh et al., 2002). Matrine and oxymatirne are the most well-known bioactive alkaloids isolated from the root of *S. flavescens* (Leguminosae) family (Lin et al., 2011; Yang et al., 2014). The major constituents of *A. gigas* (Umbelliferae) root include wide variety decursinol derivatives such as decursin, decursidin, and decursinol (Kang and Kim, 2007). Saponin and clematernoside were discovered as chemical constituents from the root part of *Clematidis terniflora* (Ranunculaceae) (Kawata et al., 1998).

To evaluate the therapeutic effects of GST on atopic dermatitis-like skin lesions and to provide scientific evidence for its anti-pruritus effects, we utilized the NC/Nga (NC) mouse, a murine atopic dermatitis model. These animals develop clinical signs of AD that begin with scratching behavior followed by the onset of eczematous skin lesions (Matsuda et al., 1997). Here we report that oral administration of GST significantly suppresses itching, which could be mediated by the suppression of IL-31 expression. Further, GST treatment led to reductions in the thickness of the epidermis and dermis and in the degree of inflammatory cell infiltration. These changes could be mediated by the inhibitory effects of GST on various pathogenic factors such as IgE and TARC as well as T_H2 cytokines.

2. Materials and methods

2.1. Preparation of GST

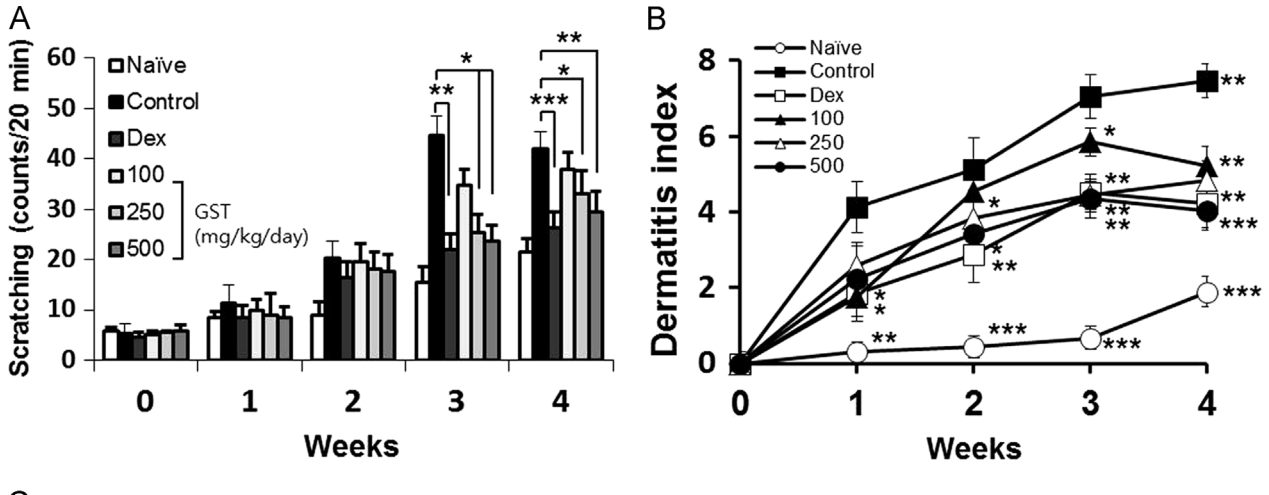
The herbs, *S. radix* (*Stemona sessilifolia* (Miq.) Miq.), *S. herba* (*S. polyrrhiza* (L.) Schleid.), *C. fructus* (*Cnidium monnieri* (L.) Cusson), *S. flavescens* root (*S. flavescens* Aiton), *A. gigas root* (*A. gigas* Nakai), and *C. radix* (*C. terniflora* var. *manshurica* (Rupr.) Ohwi) were purchased from Dong Kyung Pharm Co., Ltd. (Seoul, Korea). The herb samples were identified by Professor C.G. Son (College of Oriental Medicine, Daejeon University, Daejeon, Korea). Voucher specimens (No. 2014-004 to 009) of the collected herb samples were deposited in the herbarium, according to the procedure described by Park (Park et al., 2014b). GST was obtained by boiling the six herbs (1:1:1:1:0.5:0.5) in distilled water at 100 °C for 2 h. The boiled herbs were then filtered through a Whatman no. 2 filter (Maidstone, UK), concentrated under vacuum conditions, and freeze-dried. The yield of the dried extract was approximately 10.3%. The extract was stored at −80 °C and dissolved in phosphate-buffered saline (PBS) before use.

2.2. Standardization of GST

To standardize GST, chlorogenic acid, vitexin, decursin, and torilin were used as surrogate markers and the content of each was quantified in GST samples (Ahn et al., 1996; Cho et al., 2008; Ge et al., 2007; Kim et al., 2010). Using high-performance liquid chromatography (HPLC), standardization of GST was carried out on a Waters system (Waters Corp., Milford, MA, USA), consisting of separation module (e2695) with a photodiode array detector (2998). UV absorbance was monitored from 200 to 500 nm. Qualitative analysis was carried out at 254 nm at a column temperature of 40 °C. Separation was carried out using an YMC-Triart C18 (250 × 4.6 mm; particle size, 5 μm; YMC Co. Ltd., Japan). The mobile phase was composed of 1% acetic acid in water (v/v, solvent A) and acetonitrile (solvent B). The flow rate was 1 mL/min. The gradient was 0.0 min, 90% A; 30.0 min, 60% A; 34.0 min, 25% A; 44.0 min, 10% A; 50.0 min, 10% A. Re-equilibration time was 10 min. The data shows a chromatogram obtained from a standard mixture and GST. The linearity detection of each compound was calculated from three concentrations. The content of each surrogate compound in GST and retention time was indicated (Supplementary data 1).

2.3. Animal experiments

Specific pathogen-free 6-week-old male NC/Nga (NC) mice were purchased from Central Lab Animal Inc. (Seoul, Korea). Animal experiments complied with the guidelines of the Daejeon University Animal Care and Use Committee (Written approval number DJUARB2013-006). The mice were acclimated for 1 week prior to the induction of AD. To induce AD-like skin inflammation, 0.2% 2,4-dinitrochlorobenzene (DNCB, Sigma-Aldrich, St. Louis, MO, USA) dissolved in an acetone and olive oil solution (3:1) was applied once a week on shaved dorsal skin. Treated mice were divided into five groups ($n = 10–12$ per group), and were orally treated with PBS, dexamethasone (Dex, 3 mg/kg/day, Sigma-Aldrich), or GST (100, 250, or 500 mg/kg/day) every day for 5 weeks. The Naïve group was treated with vehicle (acetone and olive oil) on the dorsal skin and was given oral doses of PBS. The severity of dermatitis was assessed once a week by three persons unaware of the identities of the groups, according to the method described by Leung (Leung et al., 1990). A total clinical index of dermatitis severity was defined as the sum of the individual scores graded as follows: 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for each of five signs and symptoms (erythema/hemorrhage,



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Sample	Conc (mpk)	CCR3+	CD117+/FCεRIα+
Naïve	-	4.7 ± 1.6*	4.3 ± 1.4*
Control	-	10.5 ± 1.8	7.8 ± 1.5
Dex	3	4.6 ± 0.5*	3.4 ± 0.6*
GST	100	9.0 ± 2.0	4.5 ± 1.5
	250	6.2 ± 1.9*	3.3 ± 0.9*
	500	5.3 ± 1.3*	2.3 ± 1.3**

Fig. 1. Effects of GST on the development of AD in NC mice. (A) Scratching frequency and (B) dermatitis index were assessed by the criteria described in Section 2. (C) Dorsal skin of NC mice was stained with hematoxylin and eosin or toluidine blue for detection of various inflammatory cells and mast cells, respectively. Cells in the dermis were observed using a visible-light microscope at a magnification of 200×. (D) FACS analysis of $CCR3^+$ and $CD117^+Fc\varepsilon RI\alpha^+$ cells from the skin of NC mice. ($n=10$, One-way ANOVA test $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. Control).

edema/hematoma, excoriation/erosion, itching/dryness, and lichenification [thickness of the skin]). The scratching frequency was measured by counting the number of times that mice scratched the body using the hind paws during a 20 min period.

2.4. Histological examination of AD-like skin lesions

Small biopsies were obtained from the ear of the mice in each group ($n=8$–10/group) when they were sacrificed. Ear sections were fixed and embedded in paraffin, cut to a thickness of 4 μm, and stained with hematoxylin and eosin or toluidine blue for detection of infiltrated inflammatory cells or mast cells, respectively. Cells in the dermis were observed using a visible-light microscope at a magnification of 200× (Nikon, Japan).

2.5. Real-time PCR and RT-PCR

Total RNA was isolated by using the Trizol reagent (Invitrogen) and used for cDNA synthesis using the PrimeScript™ RT reagent kit (TaKaRa, Shiga, Japan). The mRNA of *IL-31* and glyceraldehyde-3

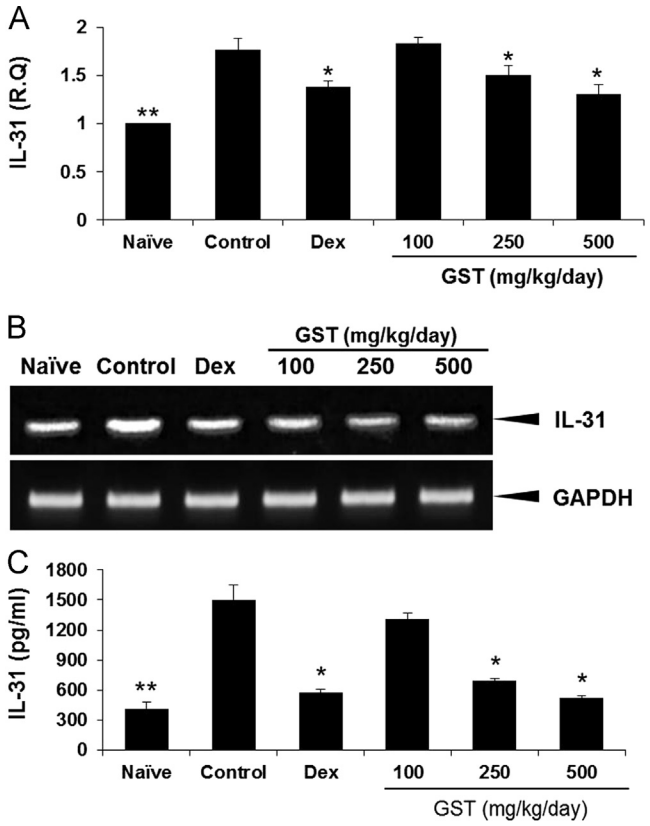

Fig. 2. Effects of GST on the IL-31 expression in skin and cultured splenocytes. Total RNAs were isolated and used for synthesis of cDNAs. Levels of *IL-31* mRNA were determined by (A) quantitative real-time PCR and (B) RT-PCR. (C) The isolated splenocytes were stimulated with anti-CD3 and anti-CD28 antibodies (5 μg/mL) for 72 h. Levels of IL-31 were determined by ELISA. The data represent the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ Control (One-way ANOVA).

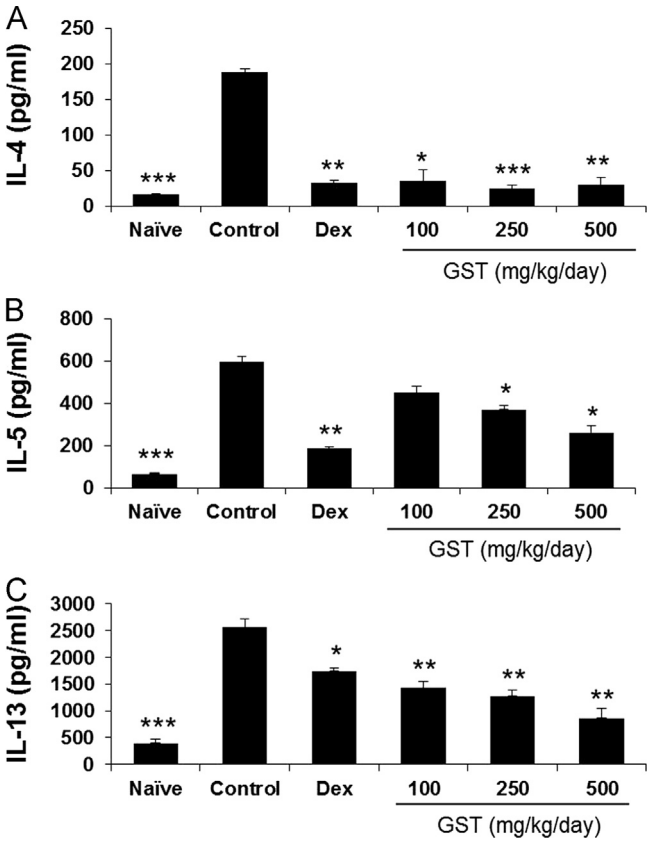

Fig. 3. Effects of GST on IL-4, IL-5, and IL-13 in cultured splenocytes. Splenocytes (2×10^6/mL) were stimulated with a plate-bound anti-CD3 and anti-CD28 antibody for 72 h. The levels of (A) IL-4, (B) IL-5, and (C) IL-13 in the cell culture supernatant were measured by ELISA. The data represent the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Control (One-way ANOVA).

phosphate dehydrogenase (*GAPDH*) was quantified using a 7500 real-time polymerase chain reaction (real-time PCR) system (Applied Biosystems, CA, U.S.A.) with Power SYBR® Green PCR Master Mix and TaqMan® Gene Expression Master Mix (Applied Biosystems). The sequences of the real-time PCR primers were: mouse *IL-31* forward; 5′-TCAGCAGACGAATCAATACAGC-3′, reverse; 5′-TCGCTCAACACTTTG ACTTTCT-3′ and mouse *GAPDH* primer set; Endogenous Control (VIC® / MGB Probe, Primer Limited) from Applied Biosystems (4352339E). To perform Reverse transcriptase-polymerase chain reaction (RT-PCR), the sequences of the mouse *IL-31* primers for real-time PCR were used. The sequences of the mouse *GAPDH* primers were forward; 5′-ACCACAGTCCATGCCAT-CAC-3′, reverse; 5′-TCCACCACCCTGTTGCTG TA-3′. The RT-PCR reaction was run for 35 cycles at 94 °C (20 s), 55 °C (30 s), and 72 °C (30 s) (Park et al., 2013).

2.6. Fluorescence-activated cell sorting (FACS)

The dorsal skin was minced and incubated in PBS containing 1 mg/mL collagenase IV (Sigma-Aldrich) and 2 mg/mL dispase (BD Biosciences) for 40 min at 37 °C. Cells were stained with phycoerythrin (PE)-, fluorescein isothiocyanate (FITC)-, or peridinin chlorophyll protein complex (PerCP)-conjugated antibodies specific for mouse chemokine (C–C motif) receptor 3 (CCR3)-PE, cluster of differentiation (CD)117-FITC, and FcεRIα-PerCP (BD Biosciences) in staining buffer (PBS containing 1% fetal bovine serum and 0.01% sodium azide) for 30 min on ice and analyzed by a FACScan analyzer using Cell-Quest software (BD Biosciences) (Park et al., 2013).

2.7. ELISA

Splenocytes (2×10^6/mL) were stimulated with a plate-bound anti-CD3 and anti-CD28 antibody (5 μg/mL, BD Biosciences, San Diego, CA, USA) for 72 h. The levels of mouse IL-4, IL-5, IL-13, and IL-31 in the supernatants of the suspensions were determined using enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences, San Diego, CA, USA or Uscn Life Science Inc., Wuhan, Hubei, PRC). The plasma levels of mouse IgE (Shibayagi, Gunma, Japan), eotaxin, and TARC (R&D Systems, Minneapolis, MN, USA) were measured using commercially available ELISA kits.

2.8. Analysis of total leukocytes, neutrophils, eosinophils, and basophils in peripheral blood

Blood was collected from individual groups of mice through heart puncture after the animals were sacrificed. The numbers of total leukocytes, neutrophils, eosinophils, and basophils in the heparinized blood were counted using a CELL-DYN® 3200 (Abbott Laboratories, Santa Clara, CA, USA).

2.9. Statistical analysis

All data are expressed as mean ± standard deviation (SD) and represent one of three independent experiments. One-way analysis of variance (ANOVA) was performed using SPSS software (IBM-SPSS Inc., Chicago, IL) was used to analyze the differences between the groups. Duncan's multiple comparison tests were used to compare the mean values of the treatments. Differences with $P < 0.05$ were considered statistically significant. One-way ANOVA

was used to analyze the interaction effect between the groups for which the parameters were found to be significantly different at the indicated time (see Fig. 2).

3. Results

3.1. Effects of GST on the development of atopic dermatitis

Since GST has been traditionally used for the treatment of itching skin, we first examined whether GST had anti-pruritic effects in DNCB-sensitized NC mice. NC mice were orally treated with GST (100, 250, or 500 mg/kg/day), Dex (3 mg/kg/day), or PBS (Control, 100 μL/mouse/day) daily for 5 weeks. As shown in Fig. 1A, NC mice gradually developed itching symptoms over time, and reached a peak at 3 weeks after dermatitis induction. Animals in the Control group scratched approximately 50 times within 20 min at the 4-week time point, while Naïve mice scratched just 15 times. The GST-treated mice showed significantly reduced scratching frequency compared with those of the Control group, and were comparable to mice treated with Dex, a well-known anti-inflammatory drug. To assess the efficacy of GST on AD skin lesions, we also evaluated the dermatitis index. As expected, the Control group gradually developed inflammatory skin disease (Fig. 1B). The AD skin symptoms such as dryness, erythema, and eczema worsened with time, and reached a peak at 3 weeks. However, GST treatment significantly decreased the dermatitis index, resulting in less edema, erythema, and hemorrhage in a dose-dependent manner (Fig. 1B). Histological analysis at the end of the experiment demonstrated that the skin lesions of Control mice reflected AD, a marked thickening of the epidermis and dermis, accompanied by increased infiltration of inflammatory cells into the skin tissue (Fig. 1C). However, GST inhibited the pathologic changes including hyperkeratosis and hemorrhage at a dose of 250 mg/kg (Fig. 1C), resulting in a histological presentation very similar to that of the Naïve group. Toluidine blue staining indicated that GST suppressed DNCB-induced infiltration of mast cells into skin (Fig. 1C). We also investigated the immune cell types by labeling with cell-specific markers. Following GST treatment, the number of $CCR3^+$ cells, classified into eosinophil and monocytes, in the dorsal skin decreased to almost normal levels (Fig. 1D). There was a significant increase in the percentage of mast cells ($CD117^+FcεRIα^+$ cells) in DNCB-induced atopic dermatitis mice from 4.3 ± 1.4% to 7.8 ± 1.5% (Fig. 1D). GST treatment significantly reduced the mast cell population in dorsal skin to 3.3 ± 0.9% (250 mg/kg) and 2.3 ± 1.3% (500 mg/kg), levels that were lower than that of the Naïve group. These data suggest that GST treatment suppress the development of AD-like skin lesions by inhibiting itching and reducing the infiltration of inflammatory cells in atopic skin disease.

Table 1
Effects of GST on eotaxin and TARC production in peripheral blood.

Group	Concentration (mg/kg of body weight)	Chemokine (pg/ml)	
		Eotaxin	TARC
Naïve	–	902.1 ± 33.6**	8.1 ± 2.6**
Control	–	1886.6 ± 62.0	59.9 ± 3.2
Dex	3	912.5 ± 190.0*	24.8 ± 1.2*
GST	100	1603.7 ± 115.4	33.5 ± 2.0*
	250	1523.8 ± 112.9	21.1 ± 5.3*
	500	1083.3 ± 109.9*	15.4 ± 0.2**

Blood plasmas were isolated from each group of mouse at 4 weeks after dermatitis induction. The plasma levels of eotaxin and TARC were determined using ELISA. The data represent the mean ± SD of triplicate determinations. $n = 10$, Dex, dexamethasone; GST, gagamsasangja-tang.

* $P < 0.05$ vs. control (One-way ANOVA).
** $P < 0.01$ vs. control (One-way ANOVA).

Table 2
Effects of GST on IgE in blood plasma.

Group	Concentration (mg/kg of body weight)	IgE (ng/ml)
Naïve	–	87.8 ± 31.8***
Control	–	6744.3 ± 228.3
Dex	3	3403.6 ± 248.0**
GST	100	3591.8 ± 359.0**
	250	3141.6 ± 122.6*
	500	2048.8 ± 344.3**

Blood plasmas were isolated from each group of mouse at 4 weeks after dermatitis induction. The plasma level of IgE was determined using ELISA. The data represent the mean ± SD of triplicate determinations. $n = 10$, Dex, dexamethasone; GST, gamisasangja-tang.

* $P < 0.05$ vs. control (One-way ANOVA).

3.2. Effects of GST on IL-31 expression

To provide additional evidence for the anti-pruritic effects of GST at a molecular level, we measured the expression of IL-31, a pruritogenic cytokine in atopic dermatitis (Sonkoly et al., 2006). The level of *IL-31* mRNA in dorsal skin was increased by DNCB treatment by approximately 2-fold compared with Naïve mice. GST treatment reduced *IL-31* expression in a dose-dependent manner to a level similar to that of the Dex group at 500 mg/kg (Fig. 2A). This result was confirmed by semi-quantitative RT-PCR (Fig. 2B). We also measured the secretion of IL-31 protein in the culture supernatant of splenocytes activated by CD3/CD28 antibodies. The Control group showed a roughly 3-fold increase in IL-31 production compared to the Naïve group (Fig. 2C). However, splenocytes

Table 3
Effects of GST on blood cell population.

Sample	Concentration (mg/kg body weight)	Cell number (10^5 cells/ml)			
		Total leukocyte	Eosinophil	Basophil	Neutrophil
Naïve	–	31.0 ± 1.1**	0.1 ± 0.01***	0.1 ± 0.01***	5.0 ± 1.0*
Control	–	46.1 ± 0.8	2.5 ± 0.1	1.0 ± 0.01	15.2 ± 0.8
Dex	3	37.3 ± 0.7**	0.1 ± 0.01***	0.2 ± 0.07*	12.45 ± 0.5*
GST	100	42.9 ± 3.7	1.0 ± 0.2*	0.5 ± 0.2	12.3 ± 2.5*
	250	40.4 ± 0.7**	0.2 ± 0.01***	0.4 ± 0.1	12.45 ± 1.6*
	500	39.2 ± 0.5*	0.3 ± 0.07***	0.2 ± 0.07*	9.8 ± 0.7*

Blood samples were isolated from each group of mice at the age of 11 weeks. The numbers of total leukocytes, eosinophils, basophils, and neutrophils in the heparinized blood were counted using a hemocytometer. Values are expressed as means ± SEM for 10 animals.

* $P < 0.05$ vs. Control (One-way ANOVA).
** $P < 0.01$ vs. Control (One-way ANOVA).
*** $P < 0.001$ vs. Control (One-way ANOVA).

** $P < 0.01$ vs. control (One-way ANOVA).

of GST-treated mice showed decreased levels of the protein in a dose-dependent manner, which was comparable with the Dex group (Fig. 2C). These results suggested that GST might suppress scratching frequency through the inhibition of IL-31 expression.

3.3. Effects of GST on T_H2 cytokine expression

As shown in Fig. 3, splenocytes from the Control group produced higher levels of IL-4, IL-5, and IL-13 than those from the Naïve group. However, treatment with GST greatly decreased the production of IL-4, IL-5, and IL-13 in a dose-dependent manner, to a level similar to the Dex group. These results suggested that GST might inhibit atopic skin inflammation by inhibition of T_H2 cytokine expression.

3.4. Effects of GST on chemokine secretion

Atopic skin inflammation is also associated with expression of eotaxin as well as TARC (CCL17), which is known to be induced by IL-31 (Bilsborough et al., 2006; Schofield and Calhoun, 2011). Thus, we examined whether GST affected the plasma levels of chemokines including eotaxin and TARC. As shown in Table 1, the levels of eotaxin and TARC were highly increased in the Control group compared with the Naïve group by 2- and 7-fold, respectively. However, GST treatment decreased the levels in a dose-dependent manner; at the dose of 500 mg/kg, the TARC level was lower than that in the Dex-treated group. The effect of GST on the eotaxin level was also statistically significant at the 500 mg/kg dose. These data suggest that GST might inhibit atopic skin inflammation and itching by suppression of chemokine production.

3.5. Effects of GST on IgE

Next, we assessed the plasma level of IgE, which is an inflammatory mediator in AD. The concentration of IgE in the plasma of Control group mice was highly increased to the milligram scale at 4 weeks, while the Naïve group showed nanogram scale levels. Treatment with GST (500 mg/kg/day) or Dex lowered the IgE concentration by approximately 70% and 50%, respectively (Table 2). These data suggest that GST might suppress atopic dermatitis like skin lesions by the inhibition of IgE production.

3.6. Effects of GST on blood cell population

We further investigated the composition of the blood cell population following GST treatment. As shown in Table 3, total leukocytes in the Control group were significantly increased compared with to the Naïve group, but GST treatment significantly reduced the number. In addition, the numbers of eosinophils, basophils, and neutrophils were considerably increased to 25-, 10-, and 3-fold, respectively, though still below the level of eosinophilia. GST treatment reduced granulocytes levels in a dose-dependent manner, resulting in reductions of approximately 88%, 80%, and 36% compared to Control group, respectively. These data indicated that GST might inhibit atopic skin inflammation by reduction of the number of inflammatory leukocytes.

4. Discussion

Pruritus is a representative feature of AD, and triggers a vicious cycle of barrier dysfunction and skin inflammation, leading to greatly diminished quality of life. It is known that histamine and acetylcholine provoke itching sensation by direct binding to itching receptors (Stander and Steinhoff, 2002) and several other mediators including neuropeptides, proteases, and kinins, which cause pruritus in AD (Buddenkotte and Steinhoff, 2010). However, non-sedative anti-histamines show only limited antipruritic effects (Darsow et al., 2011) and novel therapeutics targeting the mediators have not been established yet.

In this study, we observed that GST, an herbal formula prescribed for the treatment of itching skin, potently suppressed scratching frequency and atopic skin inflammation. It is notable that GST has inhibitory effects on both pruritus and IL-31 expression. Recently, IL-31, an IL-6 family cytokine, has been recognized as a pruritogenic cytokine. IL-31 overexpression in lymphocytes resulted in severe pruritus and atopic skin inflammation characterized by erythematous lesions, hyperkeratosis, inflammatory cell infiltration, and an increased number of mast cells (Bilsborough et al., 2006). In humans, IL-31 is significantly upregulated in pruritic skin diseases including AD and prurigo nodularis, but not in non-pruritic (psoriatic) forms of chronic skin inflammation (Raap et al., 2012). IL-31 is preferentially expressed by T_H2 cells (Neis et al., 2006) and transfers cell signals through a heterodimeric receptor composed of the IL-31 receptor A (IL-31RA) and the oncostatin M receptor (OSMR), which is expressed in monocytes, dendritic cells, and keratinocytes (Nobbe et al., 2012), and is abundant in the dorsal root ganglia (Cevikbas et al., 2014). The activation of these receptors induces AD-associated chemokines such as TARC (CCL17) in human keratinocytes, and may provoke pruritus, leading to atopic skin inflammation (Dillon et al., 2004). We demonstrated that GST treatment significantly lowered *IL-31* transcripts in dorsal skin as well as IL-31 production in activated splenocytes, and furthermore that GST treatment inhibited TARC production. These results indicate that the suppressive effects of GST on itching and the infiltration of inflammatory cells might be mediated in part by inhibition of IL-31 expression. In addition, GST inhibited the expression of factors known to contribute to AD pathology including T_H2 cytokines. Increases in the numbers of inflammatory cells including eosinophils and basophils and high production of IgE were also suppressed by GST. These effects might lead to reduced thickening of the epidermis and dermis, and infiltration of eosinophils ($CCR3^+$) and mast cells ($CD117^+Fc\varepsilon RI\alpha^+$) into skin as well as fewer erythematous skin lesions. Additionally, the previous dose (single and repeated) toxicity studies (Lee et al., 2014; Han et al., 2013; Kim et al., 2014) revealed GST is safe. Current immunosuppressive therapeutics such as steroids and cyclosporine have considerable side effects such as nausea, abdominal discomfort, hypertension, hypothalamic-pituitary-adrenal axis dysregulation, and renal impairment (Madan and Griffiths, 2007). Therefore, our study indicates that GST has potential applications as a safe anti-pruritic and anti-inflammatory therapeutic for the treatment of atopic skin diseases.

It has reported that several herbs found in GST have inhibitory effects on skin inflammation. Extracts from *S. flavescens* and *C. fructus* showed inhibitory effects on 2,4-dinitrofluorobenzene (DNFB)-induced contact dermatitis (Kim et al., 2012; Matsuda et al., 2002). *S. radix*, *S. herba*, and *C. fructus* have suppressive effects on AD-like skin lesions in NC/Nga mice (Park et al., 2014a). However, to our knowledge there has been no study that the constituting herbs for GST have suppressive effects on IL-31 expression. Recently we determined that torilin and osthol, the two components of GST from *C. fructus*, have inhibitory effects on IL-31 production in activated mast cells and splenocytes activated by CD3/CD28 (data not shown). Therefore, our next study will focus on examining each herb's effects on IL-31 expression and on identifying the active compounds.

Since there are limited drugs with low side effects and effective inhibition of itching, well-controlled clinical studies are warranted to demonstrate the beneficial effects of GST on human AD.

Author disclosure statement

No competing financial interests exist.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2015.02.040.

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