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Effect of Pyeongwee-San (KMP6) on 2,4-dinitrofluorobenzene-induced atopic dermatitis-like skin lesions in NC/Nga mice

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ABSTRACT

Aims: Recently, some studies reported that digestive tract disease is closely associated with atopic dermatitis (AD). Pyeongwee-San (KMP6) is a Korean medicine, which has come onto the drugstore for the treatment of digestive tract disease. The aim of the present study was to examine whether KMP6 could suppress 2,4-dinitrofluorobenzene (DNFB)-induced AD-like skin lesions in NC/Nga mice.

Main methods: Mice were sensitized with DNFB by applying to shaved dorsal skin. At that time, the drugs or saline were orally administrated to DNFB-applied mice.

Key findings: The administration of KMP6 or glycyrrhizic acid (GL), a major component of KMP6, inhibited the scratching number in DNFB-induced AD model. The mRNA expressions of interleukin (IL)-4, interferon (IFN)- γ , tumor necrosis factor (TNF)- α , and CCR3 were upregulated by DNFB sensitization, but the upregulated mRNA expressions were significantly reduced by the administration of KMP6 or GL. In addition, the levels of IgE, histamine, and IL-4 were significantly reduced by the administration of KMP6 or GL in serum of DNFB-induced AD model. However, the level of IFN- γ in serum was significantly increased by KMP6 or GL. KMP6 or GL also significantly inhibited the numbers of inflammatory cells, mast cells, and protein level of IL-4 in lesions of DNFB-induced AD model. Finally, KMP6 or GL significantly decreased the productions of IL-4, IFN- γ , and TNF- α in anti-CD3 plus anti-CD28 antibody-stimulated splenocytes.

Significance: KMP6 showed anti-atopic potential in this setting; hence we suggest it as a potential prospect for anti-atopic agent besides being just a medicine for the stomach and bowels.

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Introduction

Atopic dermatitis (AD) is a chronically relapsing inflammatory skin disease with a clinical presentation of skin erythematous, lichenification by cutaneous hypersensitivity, and intense pruritus (Leung, 2000). AD has developed from complex interactions between genes and environment. Digestive tract disease has become a common problem and the blame goes to poor food habits. Niwa et al. (2004) asserted that ulcerative colitis is closely associated with AD. Oyama et al. (2001) reported that the elimination of indigenous microflora by antibiotic use during infancy may induce a shift in the Th1/Th2 balance toward Th2-dominant immunity. *Helicobacter pylori* infections were reported to play a role in the pathogenesis of a variety of skin diseases (Hernando-harder et al., 2009). Pyeongwee-San (KMP6) is a traditional

Korean medicine (TKM) that has been used as a basic prescription of digestive disorder. KMP6 has frequently been used for the treatment of AD in clinical care (Ko, 2005). However, the scientific investigation into anti-AD effect of KMP6 has not been reported. Glycyrrhizic acid (GL, C₄₂H₆₂O₁₆, Fig. 1) is a triterpenoid saponin glycoside and a representative constituent of *Glycyrrhiza uralensis* (Zhao et al., 2006). In ancient TKM book, it was written that *G. uralensis* has been used for the treatment of sore and ulcer (Heo, 1999). Triterpenoid saponins have a potential for the pharmaceutical industry as antibacterial, anti-inflammatory, and anti-leishmanial drugs (Lindner et al., 2010; Lambert et al., 2011). That GL belongs to triterpene saponin class underpinned our hypothesis that GL would have beneficial efficacy on AD model.

Th2 cytokines play important roles in the development of acute AD because the upregulation of IgE production has been extensively regulated with Th2 cytokines. The immune responses of Th1 cytokines are responsible for the pathogenesis of chronic AD or delayed-type allergic reactions (Tomimori et al., 2005). Delayed-type hypersensitivity reactions, such as, chemical contact allergies, are usually regarded as cell-mediated immune responses, which are frequently attributed to Th1 cells. Moreover, 2,4-dinitrofluorobenzene (DNFB)-induced AD is a T cell-mediated inflammatory

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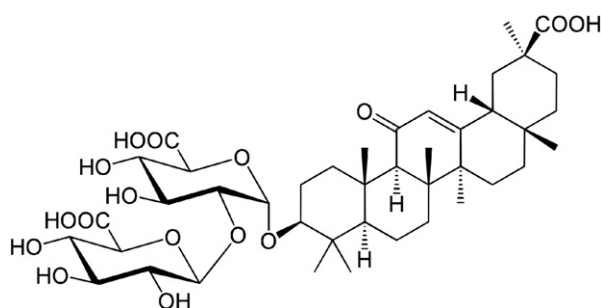


Fig. 1. Chemical structure of GL.

Table 1

The amount and composition of KMP6.

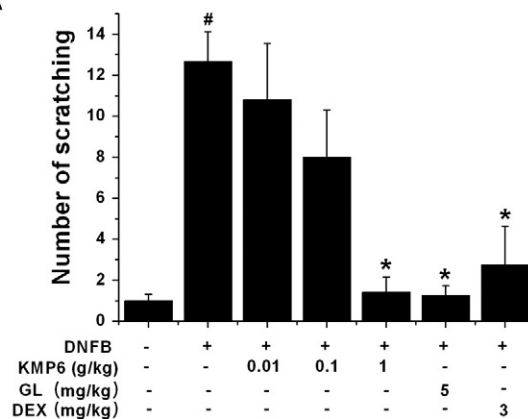
Family name	Scientific name	Plant part	Dose (g)	
Leguminosae	<i>Glycyrrhiza uralensis</i>	Radix	3.3	t1.4
Monocotyledoneae	<i>Zingiber officinale</i>	Rhizoma	3.3	t1.5
Rutaceae	<i>Citrus sunki</i>	Pericarpium	10	t1.6
Magnoliaceae	<i>Magnolia officinale</i>	Cortex	10	t1.7
Compositae	<i>Atractylodes japonica</i>	Rhizoma	13.3	t1.8
Rhamnaceae	<i>Zizyphus jujuba</i>	Fructus	6.7	t1.9
Total			46.6	t1.10

prepared by dissolving with ethanol and filtered through 0.22- μ m syringe filter.

Sensitization with DNFB

For active sensitization, 100 μ l 0.15% DNFB was topically applied to the shaved abdominal skin of NC/Nga mice ($n = 6$ per group). A week later, mice were sensitized with DNFB by applying to its shaved dorsal skin with 50 μ l 0.15% DNFB dissolved in acetone. At that time, the drugs or saline (control mice) were orally administrated 3 times a week to DNFB-applied mice according to a previous report (Moriya et al., 2009). The same volume of acetone was applied to the shaved dorsal skin and saline was orally administrated as an unchallenged control (vehicle mice). The DNFB challenge was repeated twice a week for 4 weeks. Spleen, serum, and dorsal skin samples were obtained 4 h after the last DNFB challenge. Mice were anesthetized with an intraperitoneal injection of mixture of zoletil and rompun. After anesthetization, blood was withdrawn from the heart of mice into syringes. Then, serum was prepared by centrifugation at 3000 rpm at 4 $^{\circ}$ C for 10 min.

A



B

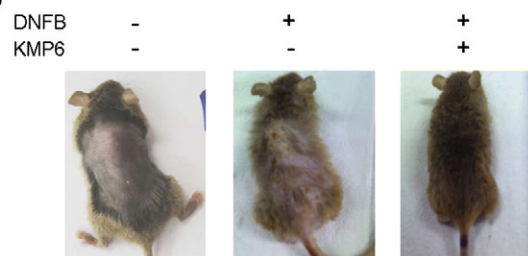


Fig. 2. Effect of KMP6 and GL on clinical symptom in DNFB-induced AD model. (A) The number of scratching was counted 4 h after the last DNFB challenge for 10 min. (B) Mice were taken pictures of the clinical features. Each datum represents the mean $\pm$ S.E.M. of three independent experiments. GL, glycyrrhizic acid; DEX, dexamethasone. * $p < 0.05$; significantly different from vehicle mice value. * $p < 0.05$; significantly different from the control mice (DNFB) value.

skin reaction that is believed to be associated with the activations of Th1 cells (Takeshita et al., 2004). Tumor necrosis factor (TNF)- α promotes inflammation and leukocyte infiltration and is an initiator of cytokine-related inflammation by stimulating cytokine production in other cell types (Karakurum et al., 1994). The chemokine receptor CCR3 is believed to play a major role in the development of allergic diseases such as asthma, atopic dermatitis, and allergic rhinitis (Willems and Ijzerman, 2010).

NC/Nga mice show very similar traits noted in AD, including increased IgE level, chronic dryness, and severe pruritis (Choi et al., 2010). Although NC/Nga mice spontaneously develop AD-like skin lesions under conventional conditions, the incidences of AD-like skin lesions are low. However, chemical haptens, such as DNFB induce allergic contact dermatitis in animals (Kim et al., 2011; Yuan et al., 2011).

Therefore, we investigated anti-AD effect of KMP6 and GL on DNFB-induced AD-like skin lesions in NC/Nga mice.

Materials and methods

Reagent

DNFB, dexamethasone (DEX), and *o*-phthalaldehyde (OPA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); IgE, IL-4, IFN- γ , and TNF- α antibodies from BD Pharmingen (Torreyana Road, San Diego, CA, USA); and GL from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

Animals

Male NC/Nga mice were obtained from Charles River Laboratories International, Inc. (Yokohama, Japan). The animals were maintained under conventional condition and the experiment was performed under approval from the animal care committee of Kyung Hee University [Protocol Number. KHUASP (SE)-10-016]. Mice were sacrificed with CO₂ inhalation.

Preparation of KMP6, GL, and DEX

KMP6 was provided by the Korea Medi Inc. (Seoul, Republic of Korea). For all experiments, KMP6 was extracted with distilled water (DW) at 80 $^{\circ}$ C for 3 h. The crude extracts were filtered and concentrated *in vacuo* at 60 $^{\circ}$ C. It was lyophilized and reduced to powder. The yield of dried extract from starting materials was about 21% (w/w). The KMP6 was dissolved in DW and filtered with 0.22 μ m syringe filter. KMP6 consists of 6 Korean herbal medicines as shown in Table 1. We have previously reported that the contents of hesperidin and GL in the KMP6 by HPLC analysis are about 5.26 and 2.60 mg/g (Jin et al., 2011). Hesperidin and GL were major compounds of KMP6. In this study, GL was used as a major compound. GL was prepared by dissolving with DW and filtered through 0.22- μ m syringe filter. DEX was used as a positive control (kim et al., 2009) and was

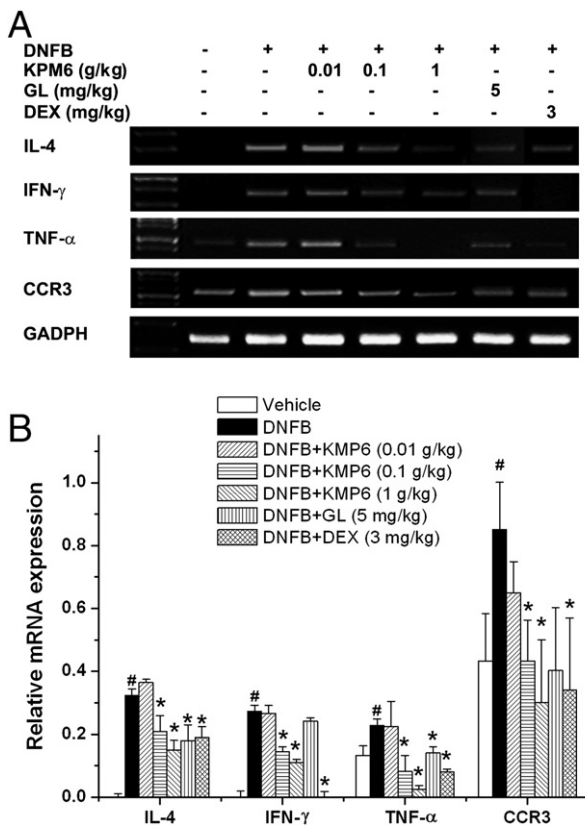


Fig. 3. Effect of KMP6 and GL on the mRNA expressions of cytokines and CCR3 in skin lesions of DNFB-induced AD model. The dorsal skins sensitized with DNFB were excised 4 h after the last DNFB challenge. (A) The mRNA expressions of IL-4, IFN- γ , TNF- α and CCR3 were measured by RT-PCR analysis. (B) The levels of mRNA expression were quantified by densitometry. Each datum represents the mean $\pm$ S.E.M. of three independent experiments. GL, glycyrrhizic acid; DEX, dexamethasone. # $p < 0.05$; significantly different from vehicle mice value. * $p < 0.05$; significantly different from the control mice (DNFB) value.

Clinical symptom of AD

After the last DNFB challenge, clinical symptom was measured. Clinical symptoms were evaluated by counting the number of scratching for 10 min. After counting, mice were anesthetized and photographed for observing clinical features.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Using an easy-BLUE™ RNA extraction kit (iNtRON Biotech, Republic of Korea), total RNA was isolated from the dorsal skin in accordance with the manufacturer's instructions. PCR was performed with the following primers; IL-4 (5' ACG GAG ATG GAT GTG CCA AA 3'; 5' CGA GTA ATC CAT TTG CAT GA 3'); IFN- γ (5' TAC TGC CAC GGC ACA GTC ATT GAA 3'; 5' GCA GCG ACT CCT TTT CCG CTT CCT 3'); TNF- α (5' TAC AGG CTT GTC ACT CGA AT 3'; 5' ATG AGC ACA GAA AGC ATG AT 3'); CCR3 (5' TGC TAC TAT CAC CAG TAT CAT TAC C 3'; 5' GCT TGT TCT TTC CAT TTT CTC ACC 3'); and reference gene GAPDH (5' GGC ATG GAC TGT GGT CAT GA 3'; 5' TTC ACC ACC ATG GAG AAG GC 3') was used to verify that equal amounts of RNA were used for the reverse transcription and PCR amplification under different experimental conditions. The annealing temperature was 60 °C for TNF- α , 62 °C for IL-4 and IFN- γ , and 55 °C for CCR3.

Histamine assay

The histamine contents from sera were measured by the OPA spectrofluorometric procedure (Shore et al., 1959). The fluorescent

intensity was measured at 440 nm (excitation at 360 nm) in spectrofluorometer.

Enzyme-linked immunosorbent assay (ELISA)

The amounts of IgE, IL-4, and IFN- γ in the serum were determined using a sandwich ELISA method according to the manufacturer's specifications (BD Pharmingen). The cytokine production in a single cell from the spleen of normal NC/Nga mouse was assayed at a final concentration of 2.5×10^6 /ml and cultured in 24-well plates, either in a complete RPMI medium alone or with stimulation by immobilized anti-CD3 antibody (2 μ g/ml) and soluble anti-CD28 antibody (4 μ g/ml) or treatment with drugs.

Histological analysis

Histological analysis was performed as previously described (Jeong et al., 2011). In brief, Formaldehyde (10%)-fixed dorsal skin samples were embedded in paraffin, cut into 4- μ m-thick sections. After dewaxing and dehydration, sections were stained with hematoxylin and eosin (H&E) to visualize inflammatory cells and alcian blue and safranin O (A&S) to visualize mast cells. Additionally, tissue sections were immunostained for IL-4 before dewaxing and dehydration. Five sections were randomly coded and the numbers of inflammatory cells, mast cells, and IL-4 protein were counted by two blinded observers ($n = 6$ per group).

Statistical analysis

The results shown are a summary of the data from at least three experiments and are presented as the mean $\pm$ S.E.M. Statistical evaluation of the results was performed by an independent *t*-test and ANOVA with Tukey post hoc test. The results were considered significant at a value of $p < 0.05$.

Results

Effect of KMP6 and GL on clinical symptom in DNFB-induced AD model

To determine whether the administration of KMP6 and GL could inhibit the number of scratching in DNFB-induced AD model, we measured the number of scratching for 10 min after the last DNFB challenge. As shown in Fig. 2A, the scratching behavior was increased by DNFB sensitization. However, KMP6 (1 g/kg) and GL significantly decreased the scratching behavior ($p < 0.05$). DEX was used as a positive control (Yatsuzuka et al., 2007). DEX also significantly decreased the scratching behavior ($p < 0.05$). Mice in the DNFB group exhibited AD-like skin lesions, such as excoriation, scaling, and dryness. However, mice in the KMP6 group recovered and grew hair on the region challenged with DNFB (Fig. 2B). We have selected the doses of KMP6, DEX, and GL according to the previous reports (Oh et al., 2011; Kim et al., 2009; Ram et al., 2006).

Effect of KMP6 and GL on the mRNA expressions of cytokines and CCR3 in skin lesions of DNFB-induced AD model

To determine whether KMP6 and GL could inhibit mRNA expressions of cytokines and CCR3 in skin lesions of DNFB-induced AD model, we performed RT-PCR analysis. As shown in Fig. 3, the mRNA expressions of IL-4, IFN- γ , TNF- α , and CCR3 were upregulated by DNFB sensitization, but the upregulated mRNA expressions were significantly inhibited by KMP6 ($p < 0.05$). GL significantly inhibited the upregulated mRNA expressions of IL-4 and TNF- α ($p < 0.05$). Particularly, KMP6 (1 g/kg) inhibited the mRNA expression of TNF- α up to about 90%. DEX also significantly decreased the mRNA expressions of IL-4, IFN- γ , TNF- α , and CCR3 ($p < 0.05$).

Effect of KMP6 and GL on the level of IgE, histamine, IL-4, and IFN- γ in serum of DNFB-induced AD model

We investigated whether the levels of IgE, histamine, IL-4, and IFN- γ in serum of DNFB-induced AD model could be modulated by KMP6 or GL. The levels of IgE and histamine in serum were significantly reduced by KMP6 (Figs. 4A and B, $p < 0.05$). As shown in Fig. 4C, the level of IL-4 in serum was significantly reduced by KMP6 (1 g/kg, $p < 0.01$) or GL ($p < 0.05$). DEX also significantly decreased the levels of IgE, histamine, and IL-4. The level of IFN- γ was significantly reduced by DNFB sensitization. However, KMP6, GL, or DEX significantly increased the level of IFN- γ (Fig. 4D, $p < 0.05$).

Effect of KMP6 and GL on the infiltration of inflammatory cells and mast cells and the protein level of IL-4 in skin lesions of DNFB-induced AD model

Further, we analyzed the infiltration of inflammatory cells and mast cells and the protein level of IL-4 in skin lesions of DNFB-induced AD model. As shown in Fig. 5, the numbers of inflammatory cells and mast cells were significantly lower in KMP6 (1 protein level-administered mice compared to control mice in the skin lesions of DNFB-induced AD model ($p < 0.05$). Immunohistochemical analysis of the skin lesions of DNFB-induced AD model revealed that the protein level of IL-4 was significantly lower in KMP6 or GL-administered mice compared to control mice ($p < 0.05$). DEX also significantly decreased the number of inflammatory cells and mast cells and protein level of IL-4 ($p < 0.05$).

Effect of KMP6 and GL on the productions of cytokines in stimulated splenocytes

Finally, we examined whether KMP6 and GL could inhibit the productions of IL-4, IFN- γ , and TNF- α in anti-CD3 plus anti-CD 28

antibody-stimulated splenocytes isolated from normal NC/Nga mouse. As shown in Fig. 6, anti-CD3 plus anti-CD 28 antibodies increased the productions of IL-4, IFN- γ , and TNF- α . However, KMP6 or GL significantly decreased the productions of IL-4, IFN- γ , and TNF- α in stimulated splenocytes ($p < 0.05$). It has not shown the cytotoxicity at each dose of KMP6, GL, and DEX (data not shown). DEX also significantly decreased the productions of IL-4, IFN- γ , and TNF- α in stimulated splenocytes ($p < 0.05$).

Discussion

AD is one of the most common and severe skin diseases, which is characterized by eczematous skin lesion and IgE hyperproduction (Kang et al., 2006). The current regimen for the treatment of atopic dermatitis in human patients only temporally relieves the symptom of diseases. Unfortunately, topical corticosteroids induce various cutaneous adverse effects in locally applied sites (Furue et al., 2003). The prevalence of atopy is increased in challenge-proven nonsteroidal anti-inflammatory drug-intolerant patients (Sánchez-Borges and Capriles-Hulett, 2000). Hence, there is a need to develop new drugs with novel modes of action that do not produce considerable side effects. Natural product-based agents with good efficacy and lower risk of side effects offer promising treatment and prevention of inflammation-related conditions. It has been reported that natural immune modulators from herbal extracts or herbal derivatives may have a therapeutic effect on AD through various experimental and clinical investigations (Oku and Ishiguro, 2001; Park et al., 2007). Based on these data, we further investigated whether natural product-based prescription, KMP6 or its constituent, GL, could produce actual therapeutic effects on AD in NC/Nga mice. As described above, KMP6 consists of 6 different herbs. Mostly it contains hesperidin, GL, and liquiritin (Seo et al., 2011). Park et al. (2005) reported that hesperidin has an anti-AD effect. In this study, we found that

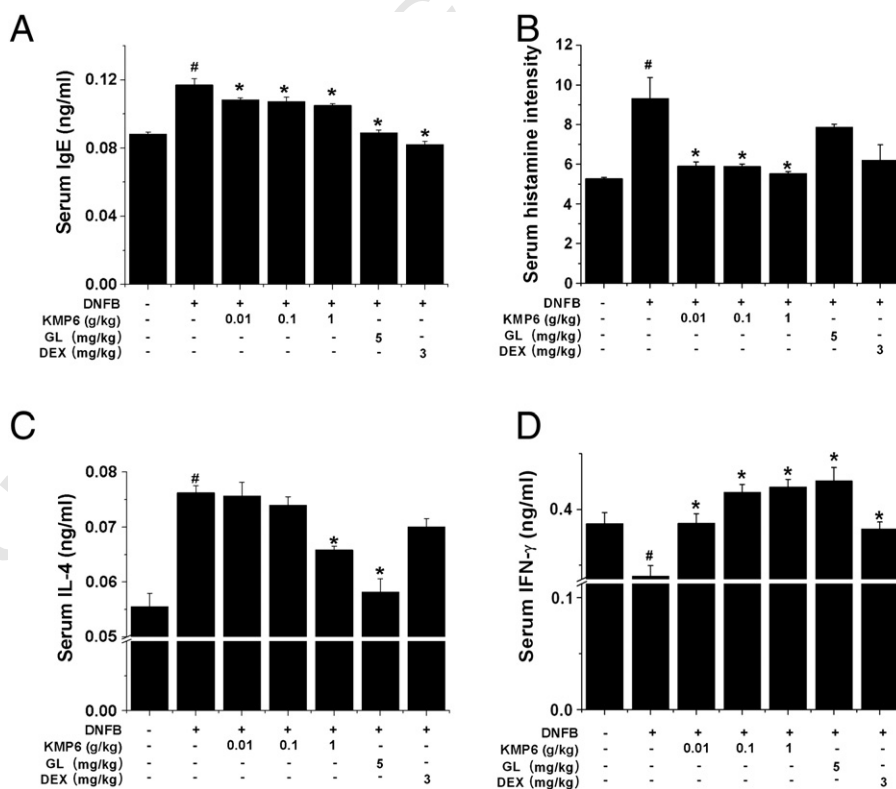


Fig. 4. Effect of KMP6 and GL on the level of IgE, histamine, IL-4, and IFN- γ in serum of DNFB-induced AD model. The serum was obtained 4 h after the last DNFB challenge. The levels of IgE (A), IL-4 (C), and IFN- γ (D) in serum were measured by ELISA analysis. (B) The intensity of histamine was assayed as described in Materials and methods. Each datum represents the mean $\pm$ S.E.M. of three independent experiments. GL, glycyrrhizic acid; DEX, dexamethasone. [#] $p < 0.05$; significantly different from vehicle mice value. ^{*} $p < 0.05$; significantly different from the control mice (DNFB) value.

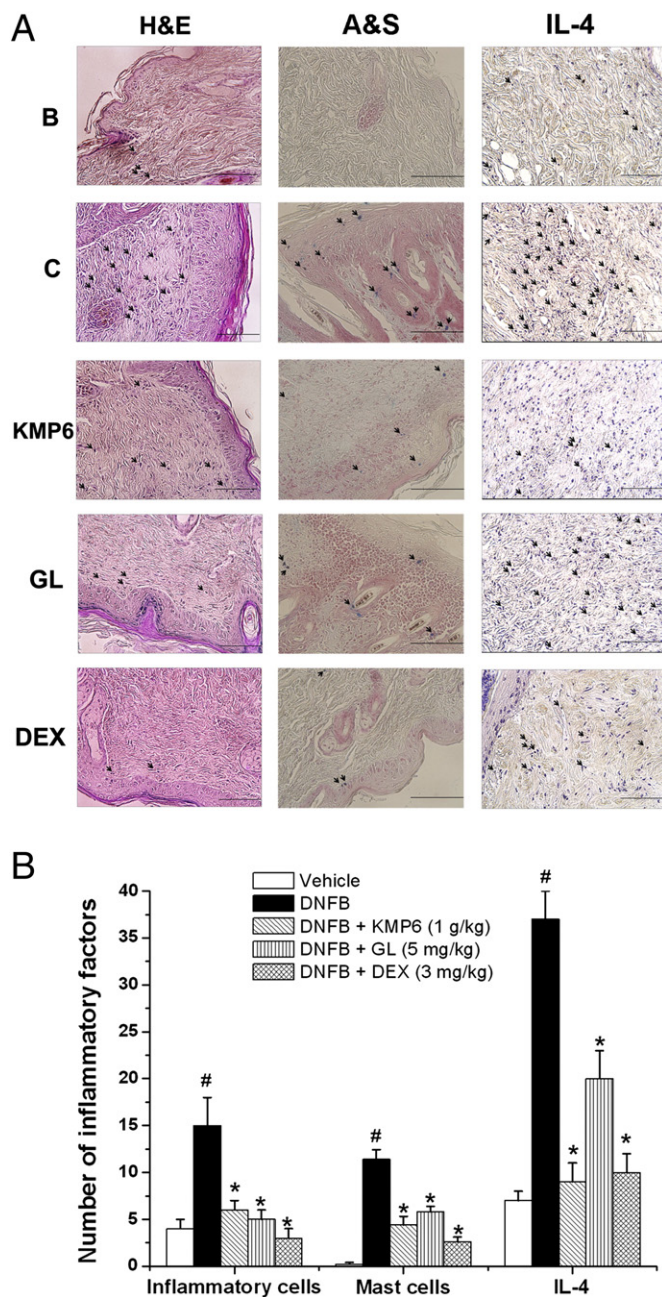


Fig. 5. Effect of KMP6 and GL on the infiltration of inflammatory cells, mast cells, and the protein level of IL-4 in skin lesions of DNFB-induced AD model. The dorsal skins sensitized with DNFB were excised 4 h after the last DNFB challenge. (A) Histological analysis of dorsal skin sections were examined by H&E staining for inflammatory cells and A&S staining for mast cells and immunohistochemistry for IL-4. The inflammatory cells, A&S positive mast cells, and IL-4 protein were indicated by arrows. Representative photomicrographs were examined at 400 $\times$ magnification. Scale bar: 100 μ m (B) Five tissue sections ($1.5 \times 10^3 \mu\text{m}^2$) per mouse were randomly selected and counted. Each datum represents the mean $\pm$ S.E.M. of three independent experiments. B, vehicle mice; C, control mice (DNFB); GL, glycyrrhizic acid; DEX, dexamethasone. [#] $p < 0.05$; significantly different from vehicle mice value. ^{*} $p < 0.05$; significantly different from the control mice (DNFB) value.

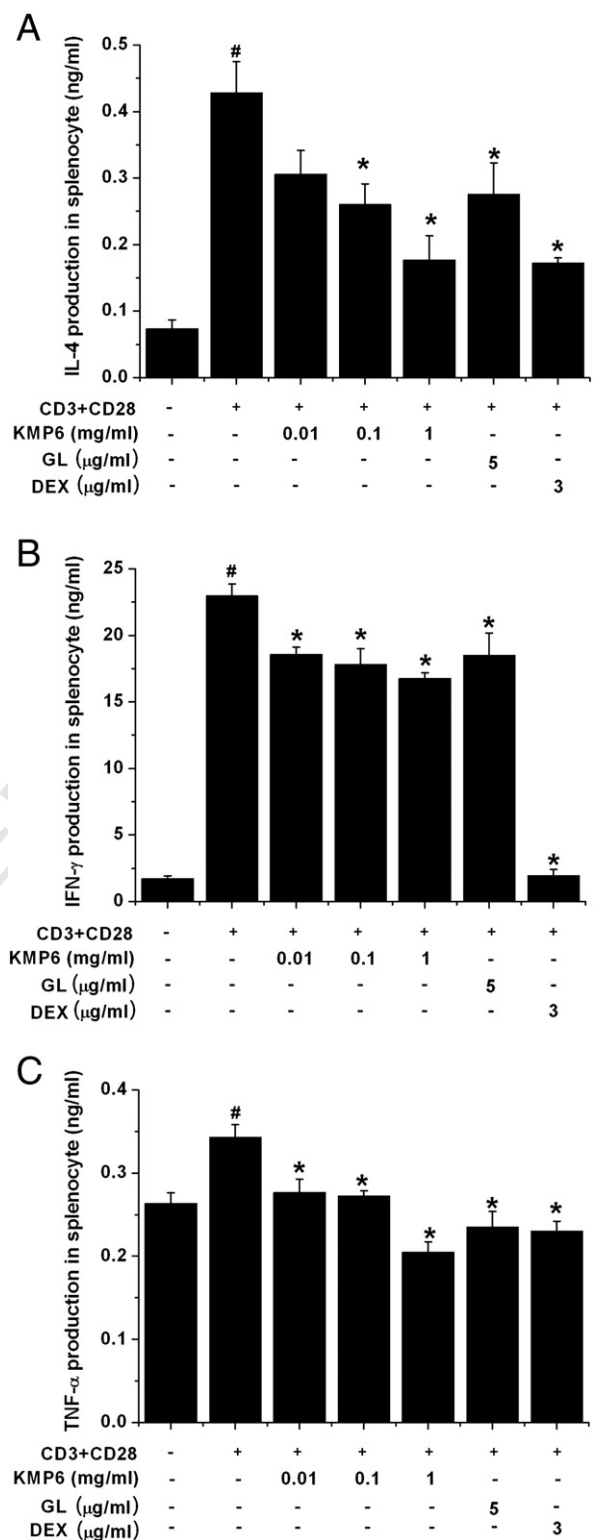


Fig. 6. Effect of KMP6 and GL on the productions of cytokines in stimulated splenocytes. Splenocytes were stimulated with immobilized anti-CD3 and soluble anti-CD28 antibodies and treated with KMP6, GL, or DEX. Culture supernatants were thereafter processed for the cytokine measurements as described in the Materials and methods. Each datum represents the mean $\pm$ S.E.M. of three independent experiments. GL, glycyrrhizic acid; DEX, dexamethasone. [#] $p < 0.05$; significantly different from unstimulated cells value. ^{*} $p < 0.05$; significantly different from the anti-CD3 plus anti-CD28-stimulated cells value.

skin diseases. It is well known that scratching causes destruction of skin barriers, resulting in worsening of the skin lesion and more itching (Inagaki et al., 2000). Histamine is the only mediator characterized for

inducing itch in humans. Oxatomide, an anti-allergic agent, has been applied to the treatment of itch, and its efficacy is reported for patients with chronic urticaria and atopic dermatitis (Peremans et al., 1981). Similar to oxatomide, we showed that KMP6 or GL significantly inhibited the number of scratching and the level of histamine in serum of DNFB-induced AD model. Therefore, we can deduce that KMP6 or GL inhibits histamine-induced scratching behavior.

Although a contact sensitizer primarily visualizes a Th1 response, repeated exposure to the agent results in a shift from the Th1 response to Th2 response or to mixed responses of Th1 and Th2. DNFB increased the levels of Th2 cytokines, while it decreased the levels Th1 cytokines in serum (Kim et al., 2011). On the other hand, the expressions of both Th1 and Th2-type cytokines were significantly induced in murine skin after repeated epicutaneous exposures to ovalbumin (Wang et al., 2007). DNFB challenge increased mRNA expressions of IL-4 and IFN- γ in the skin lesions (Inagaki et al., 2006). We also found that DNFB challenge decreased the level of serum IFN- γ , however, it increased the levels of serum IgE and IL-4. DNFB challenge increased mRNA expressions of IL-4 and IFN- γ in the skin lesions. Our model showed the different results of the levels of Th1/Th2 cytokines between serum and skin lesion. Thus, further study is necessary to clarify the imbalance of Th1/Th2 in AD-like skin lesions induced by DNFB challenge.

TNF- α is key mediator of the cutaneous inflammatory response. CCR3 is expressed on the cell surface of eosinophils, a subset of Th2 lymphocytes, basophils, and mast cells and CCR3 as a therapeutic target does play a role in allergic diseases. Previous reports suggested that the inhibition of proinflammatory TNF- α and CCR3 might be beneficial for the treatment of allergic contact dermatitis (Willems and Ijzerman, 2010; Yuan et al., 2010). Consistent with previous research, we found that KMP6 or GL significantly reduced the mRNA expressions of TNF- α and CCR3 elevated in skin lesions of DNFB-induced AD model. Therefore, we can speculate that KMP6 or GL exerts beneficial anti-AD effect.

The CD3 T cell co-receptor is a protein complex and generates an activation signal in T lymphocytes. CD28 is one of the molecules expressed on T cells that provide co-stimulatory signals, which are required for T cell activation. Naïve T cells from spleen were stimulated with anti-CD3 plus anti-CD28 (Ci et al., 2010). Co-stimulation is well known to be required for priming conventional T cells in response to the specific antigen through the recognition by T cell receptor (TCR). Therefore, the effect of CD28-mediated co-stimulation together with CD3-mediated TCR signaling was examined on the primary and secondary cytokine productions (Iyoda et al., 2010). In this study, KMP6 or GL significantly decreased the productions of IL-4, IFN- γ , and TNF- α in anti-CD3 plus anti-CD28-stimulated splenocytes. These data suggest that the anti-inflammatory effects of KMP6 or GL are mediated at least partially through a suppressive action on T lymphocytes.

In conclusion, the present study demonstrated that administration of KMP6 or GL suppressed the development of AD-like skin lesions in NC/Nga mice. We believe that KMP6 may be a new potential medicine to treat for AD.

Conclusion

KMP6 or GL suppressed the development of AD-like skin lesions in NC/Nga mice.

Conflict of interest statement

There is no conflict of interest.

Acknowledgements

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