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# Geumeunyoungji-tang Inhibits Serum IgE Level in Mouse Dermatitis Induced by 2,4-dinitrofluorobenzene

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Lonicerae Flos containing formulation, *Geumeumyoungji-tang* (GYT), is an herbal prescription prepared using 5 different herbal drugs, namely, Lonicerae Flos, Ganoderma, Lactucae Herba, Xanthii Fructus, and Smilacis Rhizoma. This study was focused on the investigation of the pharmacological effects of GYT on allergic reactions. As the first step of the study, GYT was administered BALB/c mice which were sensitized by 2,4-dinitrofluorobenzene (DNFB). As the result GYT ameliorated dermatitis provoked by DNFB. The serum IgE level of the DNFB sensitized-mouse was significantly decreased when GYT was administered. In order to confirm the moderating effect of this prescription on allergic reaction, GYT was pretreated to human mast cells (HMC-1) before they were stimulated by phorbol-12-myristate 13-acetate plus calcium ionophore A23187 (PMACI). GYT suppressed secretion of inflammatory cytokines, interleukin (IL)-6 and IL-8, from HMC-1. Additionally, pretreatment of GYT showed regulating effect on COX-2 expression. Collectively, these findings provide insights into the pharmacological actions of GYT as a potential agent for treatment of allergic dermatitis.

Key words : Lonicera japonica, Ganoderma, Mast cells, inflammatory cytokine, dermatitis

## Introduction

Atopic dermatitis (AD) is a chronic and relapsing inflammatory skin disease that affects approximately 20% children and 1-3% adults worldwide. Nowadays, in addition to genetic predisposition, environmental factors are the key causes that contribute to the increase in the number of AD incidences[1]. AD often develops with allergic symptoms of several disabilities or in medical conditions including allergic asthma and several ocular allergic diseases[2]. The causes of AD include allergic inflammation, which can be hereditary, or dysregulation of immune system[3]. The lesional skin of AD patients contains a higher number of mast cells as compared to non-lesional skin[4].

Allergic contact dermatitis (ACD) is a chronic skin disease. ACD is mediated by T cells caused by repeated exposure to contact allergens and other stimulus. ACD characterized by redness, papule, and vesicles, followed by scaling and dryness[5,6]. A widespread animal model of ACD, known as contact hypersensitivity, is the respense to haptens in mice. Haptens induce contact dermatitis immediately and evoke a local inflammation[7]. Contact dermatitis is reported to be related with IgE-mediated allergic responses. Mast cells were found at sites of ACD and play central roles in immediate-type allergic reactions[8].

Mast cells are broadly distributed in mammalian tissues, and these cells play an important role in a series of biological reactions. Mast cells generate a variety of cytokines, including IL-6 and IL-8, in response to different stimuli[4,9]. The release of these cytokines may be of prime importance in the development of a variety of inflammatory skin disorders[10]. Therefore, inhibition of the excretion of those cytokines from mast cells can aid in the development of a useful treatment strategy for allergic inflammatory diseases such as AD[11].

Cyclooxygenase (COX) is a critical enzyme in the synthesis of prostaglandin (PG) from arachidonic acid. COX has been involved in a number of physiological events, including the progression of inflammation, immunomodulation, and pain transmission[12]. Two COX isoenzymes have been identified. The first enzyme called

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COX-1 is a fundamental enzyme that is necessary for the formation of PGs that protect the stomach and kidneys from damage. The second enzyme called COX-2 is highly inducible by a number of stimuli, including cytokines, and is associated with inflammation. It has been suggested that the induction and regulation of COX-2 may be important in the pathophysiology of various inflammatory reactions[13]. Further, since COX-2 is normally expressed at very low levels and is rapidly induced by a variety of stimuli such as cytokines, growth factors, hormones, and carcinogens, COX-2 is believed to be responsible for mediating inflammation including AD through the production of prostaglandins.

As a result of our lasting efforts to find effective herbal prescriptions for AD with less adversary effect, Geumeumyoungji-tang (GYT) has been chosen as one of the candidate agents. GYT includes 5 different herbs, namely, Lonicerae Flos, Ganoderma, Lactucae Herba, Xanthii Fructus, and Smilacis Rhizoma. This study was designed to reveal the pharmacological effects of GYT thorough 2,4-dinitrofluorobenzene (DNFB)-induced AD model under in vivo conditions. Additionally, phorbol-12-myristate 13-acetate plus calcium ionophore A23187 (PMACI)-stimulated human mast cells (HMC-1) were used to evaluate the effects of GYT on the production of inflammatory cytokines and the expression of COX-2.

## Materials and Methods

1. Reagents

Phorbol-12-myristate 13-acetate (PMA), calcium ionophore A23187 (A23187), avidin peroxidase, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and DNFB were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Iscove's Modified Dulbecco's Medium (IMDM) was purchased from Gibco BRL (Grand Island, NY). Fetal bovine serum (FBS) was purchased from JR Scientific, Inc. (Woodland, CA, USA). Anti-human IL-6/IL-8, recombinant IL-6/IL-8, biotinylated IL-6/IL-8, anti-mouse IgE, recombinant IgE, and biotinylated IgE were purchased from Pharmingen (San Diego, CA, USA). COX-2, histone, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

2. Herbal medicines

Lonicerae Flos, Ganoderma, Lactucae Herba, Xanthii Fructus, and Smilacis Rhizoma were purchased from Daehak Oriental Pharmacy (Iksan, Republic of Korea). These were listed in Table 1.

Table 1. The composition of Geumeumyoungji-tang(GYT)

| The Ratio of the Component in GYT | Ratio |
|---|---|
| Dried bud (Lonicerae Flos) of *Lonicerae japonica* Thumb. | 15 g |
| Dried fruit body(Ganoderma) of *Ganoderma lucidum* Krast | 15 g |
| Dried whole plant (Lactucae Herba) of *Lactuca indica* L. | 10 g |
| Dried Fruit (Xanthii Fructus) of *Xanthium strumarium* L | 10 g |
| Dried Rhizome (Smilacis Rhizoma) of Smilax glabra Roxb. | 10 g |

3. Animals

The original stock of male BALB/c mice (age, 5 weeks; weight, 19-20 g) was purchased from Da-Mool Science (Daejeon, Korea). The animals were maintained at the College of Pharmacy, Wonkwang University. About 6 to 7 animals were housed per cage in a laminar air-flow room maintained at a temperature of 22 ± 1℃ and relative humidity of 55 ± 1% throughout the study. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care according to the Wonkwang University guidelines.

4. Preparation of GYT

An extract of GYT was prepared by decocting 600g of the dried prescription of herbs with distilled water for about 3 h. This process was repeated to achieve requited amount of sample. The decoction was filtered, lyophilized, and maintained at 4℃. The yield of GYT in the dried extract prepared from the starting material was about 3%. The sample was dissolved in distilled water and then filtered through a 0.22-μm syringe filter.

5. DNFB-induced dermatitis

Experiments were conducted according to a previously described protocol[14]. The dorsal skin of the BALB/c mice was shaved and treated with a depilatory before the experiment. The mice were sensitized by applying a 100 μl solution of 0.15% DNFB in acetone–olive oil (3:1) or vehicle to the dorsal skin twice per week for 5 weeks. After 4 weeks, GYT (1 g/kg) was orally administered for 1 week.

6. Cell culture

A human mast cell line, HMC-1, was obtained from the Korea Research Institute of Bioscience and Biotechnology (Republic of Korea) and grown in IMDM medium supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated FBS at 37 in 5% $CO_2$.

7. MTT assay

To test the cell viability after treatment with different

concentrations of GYT, the MTT colorimetric assay was performed. Briefly, HMC-1 cells ($5\times10^5$cells/well)were incubated for 8h after stimulation in the presence or absence of GYT . After the addition of MTT solution (500 µg/ml), the cells were incubated at 37 for 4 h. The crystallized MTT (formazan) was dissolved in dimethyl sulfoxide, and the absorbance was measured at 540 nm.

8. Assay of cytokines

IL-6 and IL-8 secretion was evaluated by a modified enzyme-linked immunosorbent assay (ELISA). In this assay, 100-ml aliquots of anti-human IL-6 and IL-8 monoclonal Abs were introduced in each well of the 96-well plates containing 1.0 mg/ml PBS (pH 7.4) and incubated overnight at 4℃. After additional washes, 100 ml of cell medium or IL-6 and IL-8 standards were added to the wells and the plates were incubated at 37℃ for 2 h. Subsequently, the wells were washed, followed by the addition of 0.2 mg/ml biotinylated anti-human IL-6 and IL-8 and incubation at 37℃ for 2 h. The wells were washed after incubation and then avidin-peroxidase was added; the plates were then incubated for 30 min at 37℃. The wells were washed again and ABTS substrate was added. Color development was measured at 405 nm using an automated microplate ELISA reader. A standard curve was obtained for each assay plate using serial dilutions of IL-6 and IL-8.

9. Western blot analysis

HMC-1 cells ($5\times10^6$ cells/well) were stimulated with PMACI (PMA; 50 nM, A23187; 1 µM). The stimulated cells were rinsed twice with PBS and then lysed in lysis buffer for 1 h. Cell lysates were centrifuged at 15,000 g for 5 min at 4℃ and supernatants were obtained. The supernatants were mixed with 2× sodium dodecyl sulfate (SDS) sample buffer, boiled for 5 min, and then separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The membranes were blocked with 10% skim milk for 1 h, washed with PBST/0.1% Tween 20, and incubated for 1 h with primary antibodies. After 3 washes in PBST/0.1% Tween 20 for 30 min, the membranes were incubated for 30 min with secondary antibodies. Subsequently, the membranes were washed 3 times in PBST/0.1% Tween 20 for 30 min, and protein bands were visualized by using an enhanced chemiluminesence detection system according to the recommended procedure (Amersham Corp. Newark). The quantity of protein was evaluated by performing a bicinchoninic acid (BCA) protein assay (Sigma. st. Louis, MO, USA).

10. Statistical analysis

The experimental results are shown as a summary of data from at least 3 experiments and presented as mean ± standard error of mean (S.E.M). Statistical evaluation of the results was performed by the independent t-test. For all experiments, $P < 0.05$ was considered significant. Statistical analyses were performed using SPSS Inc. (IBM software, New York, USA)

# Results

1. The effect of GYT on DNFB-induced dermatitis

DNFB was administered to BALB/c mice for the evaluation of the regulatory effects of GYT in in vivo AD model. The mice were sensitized by the application of DNFB to the dorsal skin twice per week for a total period of 5 weeks. Cutaneous application of DNFB was repeated to induce atopic dermatitis in BALB/c mice(n=4). As shown in Fig. 1A, treatment of mice with GYT for 1 week contributed to improve AD to a significant extent. Serum IgE levels were measured by enzyme immunosorbent assay (ELISA) to evaluate the effects on blood IgE change in the animal. DNFB exposure caused the increase of IgE levels; in contrast, GYT treatment significantly reduced the IgE levels(Fig. 1B).

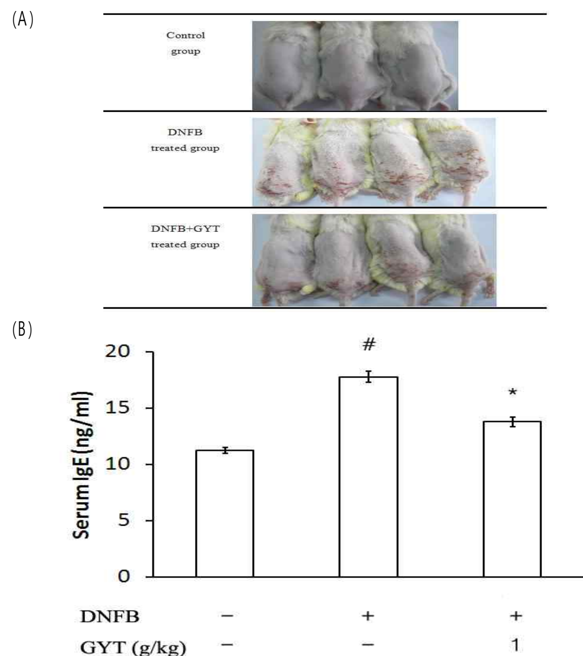


**Fig. 1. The effect of GYT on the DNFB-induced dermatitis and serum IgE level.** (A) BALB/c mice (n = 5) were sensitized with 100 µl of 0.15% DNFB in acetone – olive oil (3:1) or with a vehicle. They were applied to the dorsal skin of experimental mice twice each week for a total period of 5 weeks. GYT (1 g/kg) was orally administered after 4 weeks. (B) Blood samples were collected and the levels of serum IgE in the indicated groups were measured by ELISA. Each data represents the mean ± S.E.M. of 3 independent experiments. #$P < 0.05$, significantly different from vehicle *$P < 0.05$, significantly different from.

2. The effect of GYT on PMA plus A23187-stimulated cell viability

To investigate cell viability by GYT, the author examined MTT assay. When HMC-1 cells were pre-treated with various concentrations of GYT for 30 min and then stimulated with PMA plus A23187 for 8 h, the cell viability was not decreased. As shown Fig. 2, GYT had not cytotoxicity on HMC-1 cells.

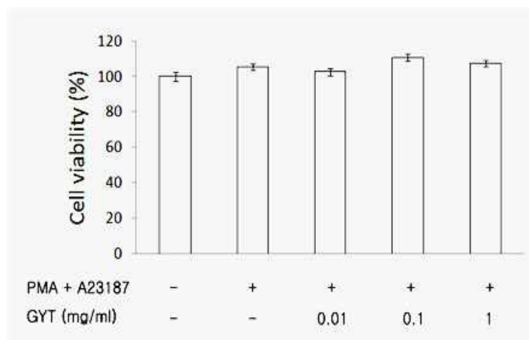


**Fig. 2. the effect of GYT on cell viability.** HMC-1 cells (5x10^{5}cells/well)were pre-treated with various concentrations of GYT for 30 min and then stimulated with the PMA(50 nM) plus A23187(1 μM). The supernatant was used to measure by MTT assay. Values are the mean ± S.E.M of duplicate determinations from three independent experiments.

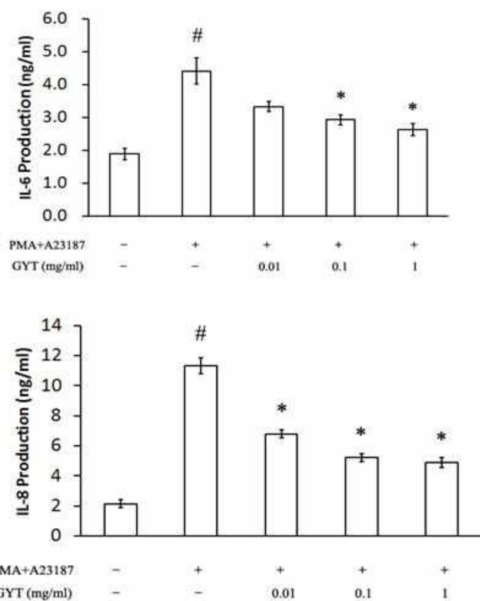


**Fig. 3. The effect of GYT on the production of inflammatory cytokines in PMACI-stimulated HMC-1 cells.** HMC-1 cells (5×10^{5}cells/well)were pre treated with GYT for 30 min and then stimulated with PMACI for 8 h. The levels of inflammatory cytokines (IL-6 and IL-8) in the cell supernatant were measured using ELISA. Three independent experiments were performed, and the values are the mean ± S.E.M. of duplicate experiments. #P < 0.05, significantly different from unstimulated cells. *P < 0.05, significantly different from PMACI-stimulated cells.

3. The effect of GYT on the production of inflammatory cytokines in PMACI-stimulated HMC-1 cells

To determine the molecular mechanism of GYT, we used the HMC-1 cell line in this study. Whether GYT modulated PMACI-induced IL-6 and IL-8 production was determined by pretreating the cells with various concentrations (0.01-1 mg/ml) of GYT for 30 min, 8 h before PMACI stimulation. The IL-6 and IL-8 levels in culture supernatants were determined by ELISA. As shown in Fig. 3, the PMACI-induced production of IL-6 and IL-8 was inhibited due to pretreatment with GYT. No GYT-mediated cytotoxicity was observed in the cells.

4. The effects of GYT on COX-2 expression in PMACI -stimulated HMC-1 cells

Western blot analysis was performed to determine the effect of GYT on PMACI-induced COX-2 expression. The cells were pretreated for 1 h with GYT and then treated for 24 h with PMACI. As shown in Figure. 4, PMACI enhanced the levels of COX-2 expression relative to that observed in unstimulated cells. However, PMACI inhibited the increase in COX-2 levels.

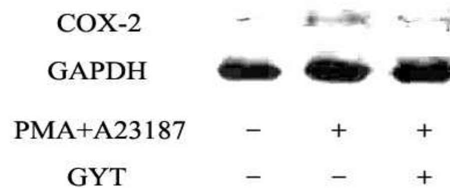


**Fig. 4. The effect of GYT on COX-2 expression in PMACI-stimulated HMC-1 cells.** Cells were pretreated with GYT (1 mg/ml) for 30 min and then stimulated with PMACI for 24 h. The COX-2 levels in protein extracts were determined by western blot analysis.

## Discussion

GYT is prepared by using a combination of anti-inflammatory or anti-allergic herbs used in the traditional medicine. According to a previous study, each medicinal herb has different effects. Flower of *L. japonica* (*Caprifoliaceae*), which is known as an anti-inflammatory agent in Korea since ancient times, is widely used for treating upper respiratory tract infections, diabetes mellitus, and rheumatoid arthritis[15,16]. The extract of *G. lucidum* has been shown to possess anti-allergic activity, which is mainly attributed to its inhibitory effect on histamine release from mast cells[17,18]. Additionally, polysaccharides from *G. lucidum* have been reported to possess promising immune modulating effects. Recent studies have suggested that water extracts of *L. indica* effectively scavenged free radicals and reduced oxidative stress in human promyelocytic leukemia (HL)-60 and macrophage cell lines[19]. Methanol extracts of *L. indica* effectively decrease the serum levels of total cholesterol and low-density lipoprotein (LDL) cholesterol[20]. The semen of *X. strumarium* has been used to

treat bacterial infections, diabetes, inflammatory diseases like rhinitis, empyema, and rheumatoid arthritis in Orient[21]. S. glabra Roxb. has been used in folk medicine for its good anti-inflammatory, antivenereal, and detoxifying activities[22]. The findings of this study show that GYT inhibited DNFB-induced allergic reactions under in vivo conditions. Additionally, GYT inhibited the production of IL-6 and IL-8 and the expression of COX-2 in PMACI-stimulated HMC-1.

In this study, we evaluated the effects of GYT on DNFB-induced allergic reactions *in vivo*. The findings of this study revealed that GYT significantly reduced DNFB-induced dermatitis. Additionally, GYT reduced IgE levels in serum, which was mediated by DNFB. These results suggest that GYT exerts a potential effect on anti-allergic responses by regulating the IgE levels as shown in Fig. 1B.

Mast cells are ubiquitous in the body and play an important role in allergic reactions because they secrete numerous vasoactive molecules and cytokines[23]. When activated, mast cells can produce histamine as well as a wide variety of other inflammatory mediators such as eicosanoids, proteoglycans, proteases, and several proinflammatory and chemotactic cytokines such IL-6 and IL-8[24]. In this study, we showed that GYT inhibited the secretion of IL-6 and IL-8 in PMACI-simulated HMC-1 cells. These results indicate that GYT exerts an anti-inflammatory effect via the regulation of inflammatory cytokine production. COX-2 activates mast cells during allergic inflammation and in the event of a hypersensitive response. COX-2 is an inducible enzyme found at low concentrations in healthy tissues; however, its expression is upregulated in response to tissue damage during inflammation[25]. In this study, we found that the production of COX-2 was effectively inhibited by treatment of HMC-1 cells with GYT. Our results suggest that GYT may exert a beneficial effect on anti-inflammatory reactions.

Histamine is involved in the induction of itching and edema in pathological skin conditions[26]. Histamine can be released by some inflammatory cytokines in mast cells[27]. IL-8 is a potent neutrophil chemotactic and activating factor and expressed IL-8 lead to immune function disorder contained with histamine release[28]. It is suggested that GYT might play roles in suppressing histamine release through inhibition IL-8 production.

In Conclusion, GYT can regulate allergic responses in vivo, such as DNFB-induced skin inflammation. Additionally, we showed that the anti-inflammatory activities of GYT could be attributed, at least in part, to the inhibition of proinflammatory cytokine production (IL-6 and IL-8) and expression of COX-2. These results provide experimental evidence that suggests the usefulness of GYT in the treatment of allergic inflammatory diseases.

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