



Antiallergic effect of *Trigonella foenum-graecum* L. extracts on allergic skin inflammation induced by trimellitic anhydride in BALB/c mice

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ABSTRACT

Ethnopharmacological relevance: Fenugreek (*Trigonella foenum-graecum* L.) has a wide variety of therapeutic properties for allergic and inflammatory diseases and is used as a traditional functional food, but its antiallergenic mechanism in these diseases is yet to be clearly elucidated.

Aim: In the present study, we investigated the antiallergic activity of fenugreek extract using trimellitic anhydride (TMA)-induced contact hypersensitivity (CHS) mice in vivo and ovalbumin (OVA)-immunized BALB/c mice *ex vivo* as represented model of T-helper (Th) 2-induced allergy.

Materials and methods: BALB/c mice were administered 250 mg/kg body weight (BW) of fenugreek extract for 7 days after sensitization and challenge treatment with 2–5% TMA. Ear thickness were noted, and the infiltration of eosinophils and mast cells was investigated by hematoxylin and eosin (H&E) and toluidine blue (TB) staining. The supernatants from homogenized ear and splenocytes were used for cytokine determination using ELISA. In addition, splenocytes from OVA-immunized BALB/c mice were treated with fenugreek extract *ex vivo*. The levels of cytokines present in the supernatants were determined by ELISA. The mRNA expression of T-box transcription factor 21 gene (T-bet), GATA-binding protein 3 (GATA-3), interferon (IFN)- γ , and interleukin (IL)-4 were evaluated by real-time RT-PCR.

Results: Fenugreek extract was found to reduce ear thickness as well as the infiltration of eosinophils and mast cells. In homogenized ear, the production of IL-4, IL-5, IL-13, and IL-1 β was suppressed. To determine the mechanism by which fenugreek extract inhibits allergic skin inflammation, detailed studies were conducted revealing that fenugreek extract prevented differentiation into Th2 cells in the splenocytes of OVA-induced allergic mice, resulting from suppressing the secretion of IL-4 and mRNA expression of GATA-3, an IL-4 transcription factor. In earlier phase, these extracts enhanced the secretion of IFN- γ , the mRNA expression of T-bet, an IFN- γ transcription factor, and the number of IFN- γ -producing CD4⁺ T cells.

Conclusions: These results indicate that fenugreek extract cures Th2-induced allergic skin inflammation by enhancing Th1 differentiation. These data suggest that fenugreek extracts may prove to be a useful therapeutic agent on allergic inflammatory diseases as traditional use as well as Th2-mediated allergic response.

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1. Introduction

Allergic dermatitis (AD) is characterized by allergic skin inflammation. Among the various types of AD, contact dermatitis is induced by an allergic response to a multitude of chemical substances brought on by environmental contamination. It is

important to consider the balance of Type 1T helper (Th1) cells/Type 2T helper (Th2) cells cytokines in patients with allergic diseases such as AD. Th1 cells secrete interferon (IFN)- γ and require T-box expressed in T cells (T-bet) for efficient immune responses against intracellular pathogens (Szabo et al., 2003) and tumors (Goedegebuure and Eberlein, 1995). Th2 cells produce interleukin (IL)-4 and require GATA-binding domain-3 (GATA-3) for immunity against helminths and other extracellular pathogens (Zheng and Flavell, 1997) or for the production of antibodies (Lee et al., 2000; Ting et al., 1996). IL-4 promotes the development of mast cells. Mast cells are recognized as key effector cells in immunoglobulin (Ig) E-associated Th2-type immune responses. Allergen-provoked mast cells trigger the release of allergic inflammatory mediators, including histamine via degranulation

Abbreviations: AD, allergic dermatitis; Ag, antigen; APC, antigen-presenting cell; CHS, contact hypersensitivity; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; HRP, horseradish peroxidase; IFN, interferon; Ig, immunoglobulin; IL, interleukin; mAb, monoclonal antibody; OVA, ovalbumin; TB, toluidine blue; Th, T-helper; TMA, trimellitic anhydride.

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(Galli et al., 2008). Some studies have demonstrated that IFN- γ suppresses the production of IL-4 from Th2 cells as well as the production of IgE by B cells (Finkelman et al., 1988; Gajewski and Fitch, 1988).

Several studies suggest the contribution of immunologic mechanisms to pathogenesis of AD. In the initiating phase of AD progression, Th2 cells producing IL-4, IL-5, and IL-13 play a critical role (Leung and Soter, 2001; Spergel et al., 1999). The development of dermatitis is thought to be caused primarily by the overproduction of Th2-mediated cytokines and IgE, as well as by the defective production of IFN- γ (Chen et al., 2004; Sandoval-López and Teran, 2001; Vestergaard et al., 2008). Many therapeutic trials have been conducted to evaluate agents that may modulate dermatitis, but prolonged drug use of such compounds has been found to cause a variety of side effects. Recently, natural herbs have been suggested to be potential alternative therapeutics for the treatment of dermatitis due to their proven safety and immune-regulatory effects. There are many studies on the use of natural herbs as improved therapies for the treatment of dermatitis (Koo and Arain, 1998; Kotani et al., 2000).

Fenugreek (*Trigonella foenum-graecum* L.) is a member of the legume family. Its seeds and leaves are used not only as food, but also as an ingredient in traditional medicine. Fenugreek is most commonly used as a spice in curries, seasoning blends, chutneys, pickles, and teas. The plant also has a wide variety of therapeutic properties and is used as a traditional functional food. Pharmacological studies have shown that the extract of this herbal plant exhibits immunomodulatory effect (Bin-Hafeez et al., 2003) as well as antidiabetic, antihypertensive, and cholesterol-lowering effects (Madar and Stark, 2002). In Ayurvedic and Unani systems of medicine, fenugreek is used for the treatment of allergic and inflammatory disease such as epilepsy, paralysis, gout, dropsy, chronic cough and piles (CCRUM). However, the inhibitory effect of fenugreek extract on Th2 cell-mediated allergic inflammatory response remains unclear.

In this study, we reveal that the administration of fenugreek extract restrained the Th2 cell-mediated AD in a trimellitic anhydride (TMA)-induced contact hypersensitivity (CHS) model. Moreover, to determine the antiallergic inflammatory mechanisms of fenugreek extracts, we investigated the inhibitory effect of fenugreek extracts on Th2 differentiation. Our results strongly suggest that the mechanism underlying these effects on

OVA-induced Th2 differentiation was considered to promote Th1 differentiation by the production of IFN- γ , which is dependent on the mRNA expression of T-bet and IFN- γ .

2. Materials and methods

2.1. Sample preparation

The fenugreek used in this study was purchased from Kyungdong Oriental medicine market (Seoul, Korea), and identified by Professor Y. Bu, Department of Herbal Pharmacology, Kyung Hee University. The specimen (KFRI-SL-98) has been kept in functional materials research group, Korea Food Research Institute. The ground fenugreek seeds underwent reflux extraction twice in 70% ethanol using a Soxhlet apparatus. The ethanol extract was dried under a vacuum in a rotary evaporator. The concentrated extract was finally lyophilized, yielding a dried powder that was kept at 4 °C until needed. The dried ethanol extract (EtOH extract) was dissolved in saline (Sigma-Aldrich, St. Louis, MO) and DMSO prior to use. This fraction was used in all the *in vivo* and *ex vivo* mouse studies. TMA (Sigma-Aldrich, St. Louis, MO) was dissolved in an acetone/isopropyl myristate (4:1) solution. Prednisolone was purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Animals

Female BALB/c mice, weighing approximately 18–20 g, were purchased from OrientBio Inc. (Kyeonggi, Korea). The 4-week-old mice were housed in an air-conditioned room (23 ± 2 °C) with a 12 h light/dark cycle. They were allowed free access to food and tap water. All animal experiments were performed in accordance with the guidelines for the animal use and care of the Korea Food Research Institute.

2.3. Schedules for mice sensitization, challenges with TMA, and sample treatment *in vivo*

The induction of CHS using TMA was performed as previously described (Schneider et al., 2009). A schematic of the experimental procedure is shown in Fig. 1A. For the induction of CHS, mice

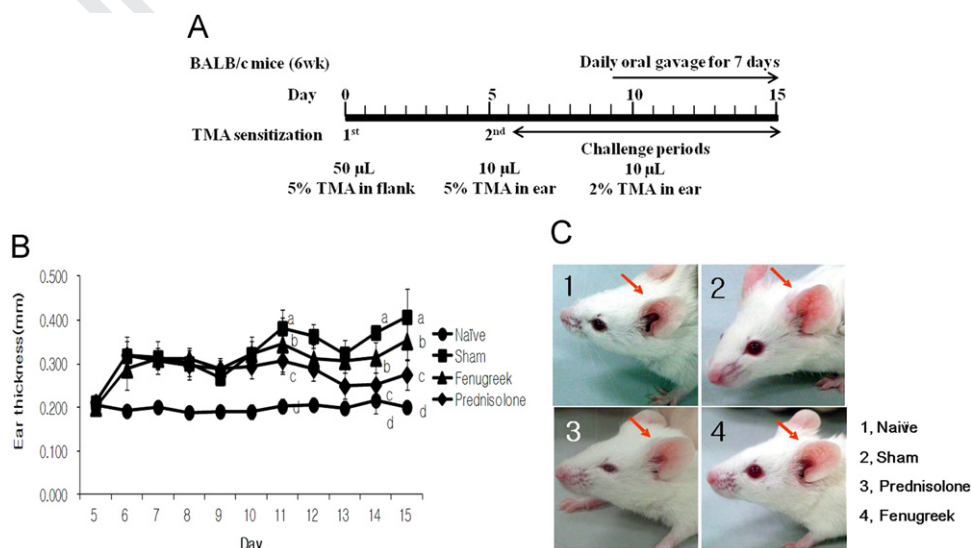


Fig. 1. Experimental protocol and inflammation parameter for trimellitic anhydride (TMA)-induced allergic response in mice. Experimental protocol (A). Ear thickness (B) and photographs (C) were taken after the last treatment. Data are shown as the mean $\pm$ SD. Data were analyzed using ANOVA followed by Tukey post hoc test. Data are statistically different ($P < 0.05$) among those columns with different symbols. The order of value is termed as order of alphabet.

were divided into four groups: naïve, sham, fenugreek, and prednisolone groups. Mice were sensitized with 50 μ L of 5% TMA in solvent on shaved flank skin on day 0. Challenges with 10 μ L of 5% TMA in solvent on the dorsum of both ears were performed on day 5. In the chronic model, animals received challenges on the ears with 10 μ L of 2% TMA in solvent on days 6–15. A solvent control group was exposed to acetone and isopropyl myristate (4:1, v/v) throughout the duration of the experiment. In the treatment groups, fenugreek extract (250 mg/kg body weight [BW]) and prednisolone (30 mg/kg BW), which served as a positive control, were administered orally 1 h before the challenges. Each sample treatment was performed on days 9–15. Body weight and water intake were measured daily. Ear thickness during the time course was determined with a custom-built micrometer (Schering AG, Germany). Ears were mechanically homogenized in 2 mL of PBS (Sigma-Aldrich, St. Louis, MO), centrifuged at 25,000g for 30 min at room temperature, and the supernatant was used for cytokine determination. Spleens were removed and incubated in the presence of 5 μ g/mL ConA for 72 h. The each cytokine production was analyzed using ELISA. Cell proliferation was measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

2.4. Histological observations

For histological analysis, the right ear was embedded in 10% formaldehyde and cut in 6- μ m-thick sections (Cryostat CM3050S, Leica Microsystems, Germany). The sections were stained with hematoxylin (Merck) and 0.5% eosin (Sigma; hematoxylin and eosin [H&E] staining) to investigate histological changes and cutaneous cell infiltration. For mast cell detection, acidified toluidine blue (TB) staining (Merck) was performed. Pictures were taken using an optical microscope (Axiocam MRC5; magnification, $\times$ 200) controlled by AxioVision software 4.72 (Carl Zeiss).

2.5. Sensitization and challenge with OVA and preparation of splenocyte cultures

All mice were sensitized with 20 μ g ovalbumin (OVA; Grade VI; Sigma-Aldrich, St. Louis, MO, USA) adsorbed in 2 mg/mL of Imject Alum (Pierce, Rockford, IL, USA) and administered by intraperitoneal (i.p.) injection on days 7 and 14 (Fig. 6A). Splenocytes were prepared by aseptically removing spleens from OVA-sensitized BALB/c mice. Homogenized single spleen cells were collected and treated by lysing the red blood cells (RBCs) with RBC-lysing buffer (Sigma-Aldrich). Splenocytes were adjusted to 5×10^6 cells/mL in RPMI medium using the trypan blue dye exclusion method. The splenocytes (200 μ L per well) were then cultured either in the absence or presence of OVA (100 μ g/mL/well). The plates were incubated at 37 °C for 0–72 h in a humidified incubator with 5% (v/v) CO₂ and 95% (v/v) air.

2.6. Measurement of cytokine levels using ELISA

A cytokine assay kit (BD PharMingen, San Diego, CA, USA) using antibodies for the measurement of cytokine levels released into the supernatant (IFN- γ , IL-1 β , IL-4, IL-5, IL-10, and IL-13) was used according to the manufacturer's instructions. Briefly, recovered supernatants and the standard solution were transferred to 96-well plates pre-coated with the appropriate monoclonal antibodies (mAbs) raised against each of the target cytokines, and then incubated at room temperature for 2 h. After thoroughly washing with the washing buffer included in the kit, a horseradish peroxidase (HRP)-conjugated secondary antibody was added to each well, and the incubation was continued at room

temperature for 2 h. After the removal of the secondary antibody, the substrate solution for the enzymatic reaction was added, and samples were then incubated for another 30 min in the dark. The reaction was terminated by the addition of stop solution, followed by the absorbance at 450 nm being measured in a microplate reader (Molecular Devices, Inc.). The IC₅₀ values were calculated from the reduction in the content of each cytokine by different concentrations of the test substance using linear regression analysis.

2.7. Measurement of cell proliferation using MTT assay

After splenocyte was incubated for 44 h in the presence of sample, 20 μ L of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 2 mg/mL) were added to each well. After 4 h in the initiation of culture with MTT, 100 μ L of 10% SDS was added and incubation was continued at 37 °C for 20 h in a humidified incubator with 5% (v/v) CO₂ and 95% (v/v) air. The absorbance at 570 nm was measured in a microplate reader (Molecular Devices, Inc.).

2.8. RNA isolation from skin and quantitative real-time RT-PCR analyses

RNA from spleen was extracted and purified using the RNeasy Mini Kit (Qiagen, Germany) and cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen, Germany). RT-PCR of the sample was performed in a Rotor-Gene Q 2plex System (Qiagen, Germany) under the following thermal conditions: 10 min at 95 °C, then 45 cycles of 10 s at 95 °C and 15 s at 60 °C. Using assays on demand from Qiagen, the following genes were analyzed: GAPDH as a housekeeping gene, GATA-3, IL-4, T-bet, and IFN- γ . The sequences of the primers used are provided in Table 1. The level of GAPDH was used to normalize the amounts of assayable RNA in each sample. The data are shown as the target gene's relative delta delta CT (ddCT) compared with that of the internal control. The quantity of gene in each sample was normalized to the quantity of GAPDH. "Fold-induction" of each mRNA species was calculated as follows: d threshold cycle (dCt)=(Ct of target mRNA) – (Ct of GAPDH); ddCt=(dCt of mRNA in target gene) – (dCt of mRNA in control gene); fold-induction=2^{-ddCt}.

2.9. Statistical analysis

Each result is expressed as the mean $\pm$ SD of triplicate determinations. Differences among the experimental data were assessed by ANOVA followed by Tukey post hoc test or Dunnett's multiple range tests. In Tukey post hoc test, data are statistically different ($P < 0.05$) among those columns with different symbols. The order of value is termed as order of alphabet. ND, not detected. In Dunnett's multiple range tests, statistical differences were presented as * $P < 0.05$, and ** $P < 0.01$.

Table 1
Sequences of primers used for real-time PCR.

Primer name	Sequence
GAPDH	sense, 5'-TGAACGGGAAGCTCACTGG-3' antisense, 5'-TCCACCACCCTGTTGGTGA-3'
GATA-3	sense, 5'-GAAGGCATCCAGACCCGAA AC-3' antisense, 5'-ACCATGGCGGTGACCATGC-3'
T-bet	sense 5'-TGCCCTGCAGTGCTTCAACA-3' antisense 5'-TGCCCCGCTTCCTCTCAACAA -3'
IFN- γ	sense, 5'-AGCGGCTGACTGAAGTCAAGATTGT AG-3' antisense, 5'-GTCGCTTCGTTGATCACA-3'
IL-4	sense, 5'-CGAAGAACCACCA GAGAGTGAGCT-3' antisense, 5'-GACTCATTCATGCTGCAGCTTATCG-3'

3. Theory/calculation

3.1. Theory

Fenugreek extracts are traditionally used as a treatment for allergic and inflammatory diseases such as epilepsy, paralysis and gout. However, the scientific proof for antiallergic effect of fenugreek extracts is not clear. We revealed the antiallergic effects of fenugreek extract in Th2-induced inflammatory dermatitis model.

3.2. Calculation

In the initiation phase of allergic dermatitis progression, Th2 cells producing IL-4, IL-5, and IL-13 play a critical role. The administration of fenugreek extract restrained the Th2 cell-mediated allergic inflammatory response in a TMA-induced CHS model. Moreover, our results strongly suggest that the mechanism underlying these effects on OVA-induced Th2 differentiation promoted Th1 differentiation through the production of IFN- γ , which is dependent on the mRNA expression of T-bet and IFN- γ . These results proved that fenugreek extracts may be useful agent in curing allergic inflammatory disease.

4. Results

4.1. Fenugreek extracts inhibit progression of dermatitis in TMA-induced CHS models

In the BALB/c model of TMA-induced CHS, mice are sensitized on flank skin and T-cell-dependent skin inflammation is induced on the ears by topical challenges with TMA. Thus, the severity of inflammation can be assessed using ear thickness measurements. In all the groups except the naïve group – the sham, fenugreek,

and prednisolone groups – a 5% TMA challenge on days 0 and 5 and subsequent daily low-dose challenges with 2% TMA for 10 days induced a significant increase in ear thickness from approximately 0.2 to 0.4 mm (Fig. 1). In the prednisolone group, which served as a positive control, an increase in ear thickness was significantly inhibited from day 11. Daily fenugreek gavage also led to an incremental decline in ear thickness from day 11 (Fig. 1).

4.2. Administration of fenugreek extract attenuates TMA-induced infiltration of inflammatory cells in the ear dermis

As shown in Fig. 2, the severity of CHS was evaluated by histopathological observations. In this study, we focused on the infiltration of eosinophils and mast cells in the dermis of the ears. TMA challenge resulted in the induction of significant infiltrations of eosinophils and mast cells into the dermis of the ears (Fig. 2, sham). The administration of fenugreek extract appeared to attenuate the infiltration of eosinophils and mast cells induced by TMA (Fig. 2, fenugreek extract).

The degree of infiltration was reflected by the epidermal thickness of the ear skin and the number of mast cells observed (Fig. 3). The administration of fenugreek extract significantly suppressed the increase in thickness of the epidermis induced by TMA and inhibited the number of mast cells recruited to the area; these ear tissue observations were also seen for prednisolone (Fig. 3).

4.3. Administration of fenugreek extract inhibits increases in inflammatory cytokine levels induced by TMA treatment in ear tissue and splenocytes

To investigate the inhibitory effects of fenugreek extract on the production of inflammatory cytokines in ear tissue, the levels of IL-4, IL-5, IL-13, IL-1 β , IFN- γ , and IL-10 were measured by ELISA (Fig. 4). The levels of Th-2-mediated cytokines, such as IL-4, IL-5,

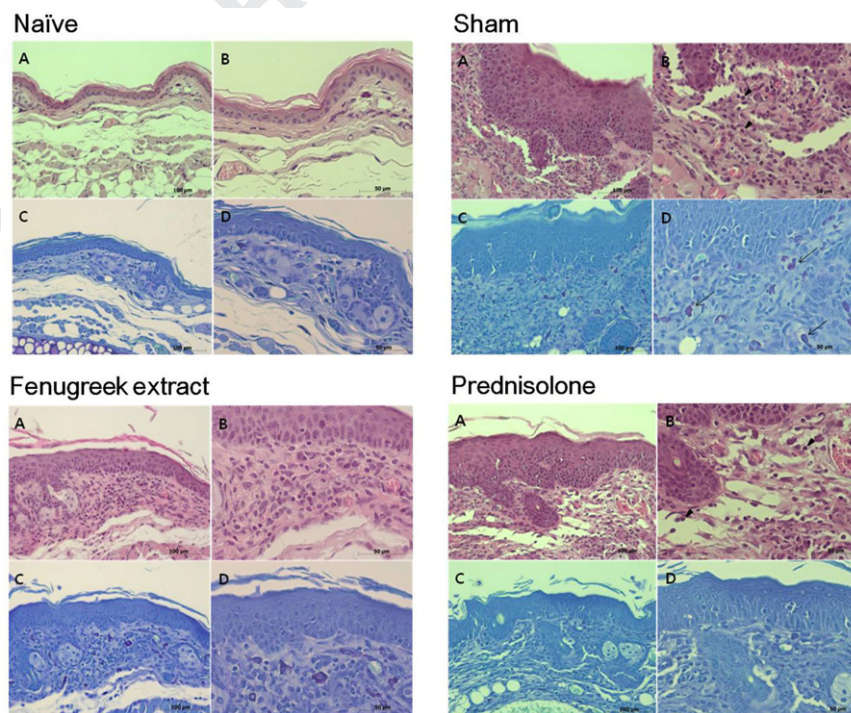


Fig. 2. Histological changes and cutaneous cell infiltration in ear skin after the oral administration of fenugreek extract in a chronic TMA-induced contact hypersensitivity (CHS) mouse model. BALB/c mouse skins obtained from the naïve, TMA (sham), fenugreek extract, and prednisolone groups were stained using hematoxylin and eosin (H&E), $\times 200$ (A), $\times 400$ (B), and toluidine blue (TB), $\times 200$ (C), $\times 400$ (D). The arrow heads and arrows in sham B and prednisolone B indicate eosinophils and mast cells, respectively.

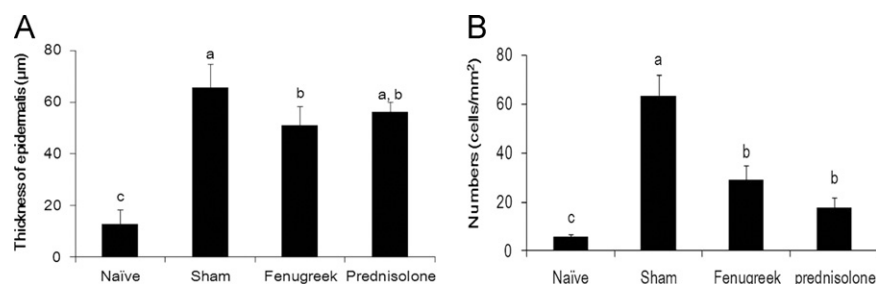


Fig. 3. Effect of fenugreek extract on allergic ear responses in the TMA-induced contact hypersensitivity (CHS) reaction mouse model. An image analysis program was used to measure the epidermal thickness of ear skin (A) and mast cell densities in the dermis (B) in sections stained with H&E and TB ($\times 200$ magnification), respectively. Values are presented as mean $\pm$ SD ($n=16$). Data were analyzed using ANOVA followed by Tukey post hoc test. Data are statistically different ($P < 0.05$) among those columns with different symbols. The order of value is termed as order of alphabet.

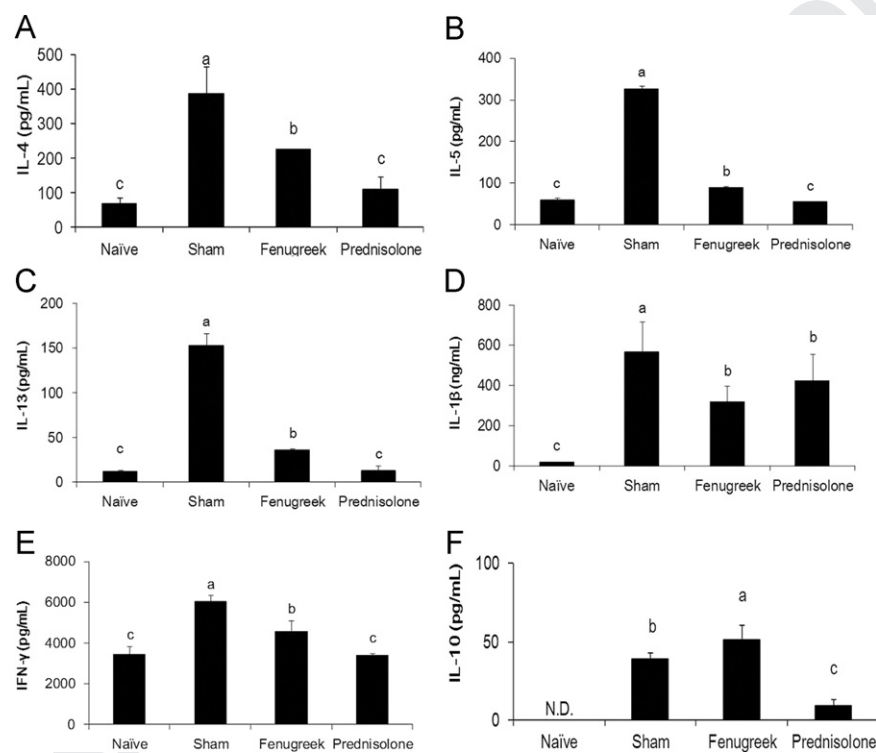


Fig. 4. Effect of fenugreek extract on the production of cytokines in ears of TMA-induced CHS model mice. The levels of Th2 cytokines IL-4 (A), IL-5 (B), and IL-13 (C), inflammatory cytokine IL-1β (D), and anti-inflammatory cytokines IFN-γ (E) and IL-10 (F) in the ears of mice were measured by ELISA. Values are presented as mean $\pm$ SD ($n=16$). Data were analyzed using ANOVA followed by Tukey post hoc test. Data are statistically different ($P < 0.05$) among those columns with different symbols. The order of value is termed as order of alphabet.

and IL-13, and inflammatory cytokine IL-1β, were found to be highly augmented in ear tissue of sham group. However, the administration of fenugreek extract lowered the levels of IL-4, IL-5, IL-13, IL-1β, and IFN-γ. The administration of prednisolone also inhibited the levels of all 3 Th2 cytokines, as well as IL-1β and IFN-γ. However, the level of IL-10 was significantly elevated as a result of the administration of fenugreek extract, but not that of prednisolone (Fig. 4).

When splenocytes isolated from TMA-induced CHS mice were cultured in the presence of ConA, the levels of IL-4 were increased in sham group (Fig. 5). However, the administration of fenugreek extract as well as prednisolone was found to lower the level of IL-4. No significant difference in cell proliferation was observed between sham group and fenugreek administration group as assessed by an MTT assay (Fig. 5).

These results indicate that fenugreek extract contains an activity that may negatively modulate Th2 polarization in TMA-induced CHS, eventually resulting in a decrease in the level of Th2

cytokines as well as inflammatory cytokines and an increase in the anti-inflammatory cytokine IL-10 in ear lesion.

4.4. Effects of fenugreek extract on the production of Th1 and Th2 cytokines by splenocytes from OVA-sensitized mice *ex vivo*

The results described above suggest that the administration of fenugreek extract may downregulate the production of Th2-mediated cytokines *in vivo*, resulting in the inhibition of allergic skin inflammation. Therefore, we next examined how fenugreek extract could inhibit Th2 polarization *ex vivo*. The levels of IL-4 and IFN-γ in splenocytes were determined using the OVA-induced Th2 allergic mouse as an *ex vivo* model. Mice were immunized by OVA (20 μg) and Alum (2 mg) twice on days 7 and 14. On day 21, isolated splenocytes from each mouse were incubated in the presence of OVA and the samples for 72 h; the cultured supernatants were collected to measure the levels of cytokines (Fig. 6A). Splenocytes cultured in the presence of fenugreek

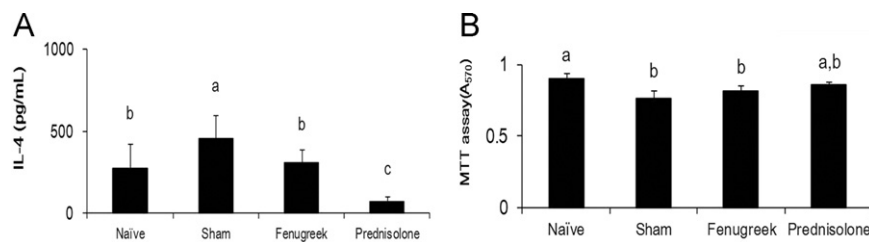


Fig. 5. Inhibitory effect of fenugreek extract on the production of Th2 cytokines in the splenocytes of TMA-induced CHS model mice. Splenocytes isolated from TMA-induced CHS mice were cultured in the presence of 5 µg/mL ConA. Secreted IL-4 (A) was quantified by ELISA after 72 h of culture and the cell in the presence of 5 µg/mL ConA. proliferation was analyzed by MTT assay, as described in the Materials and methods section. Values are presented as mean ± SD ($n=16$). Data were analyzed using ANOVA followed by Tukey post hoc test. Data are statistically different ($P < 0.05$) among those columns with different symbols. The order of value is termed as order of alphabet.

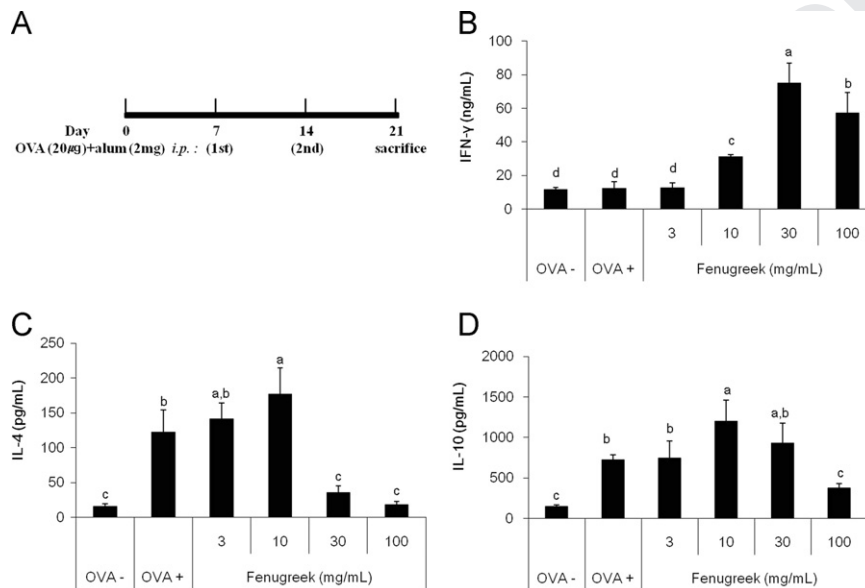


Fig. 6. Increased IFN-γ and decreased IL-4 secretion in splenocytes from OVA-induced allergic mice in the presence of fenugreek extract. The experimental protocol for the OVA-induced allergic response in mice is shown in (A). Secreted IFN-γ (B) and IL-4 (C) per cells were quantified by ELISA after cells were cultured for 72 h with splenocytes from OVA-induced allergic mice in the presence of 3–100 M fenugreek extract, as described in the Materials and methods section. Values are presented as mean ± SD ($n=3$). Data were analyzed using ANOVA followed by Tukey post hoc test. Data are statistically different ($P < 0.05$) among those columns with different symbols. The order of value is termed as order of alphabet.

extract produced significantly higher levels of IFN-γ and lower levels of IL-4 than control cells for 72 h (Fig. 6B and C). Moreover, similar to the findings in the *in vivo* model, IL-10 production was increased, but not in a dose-dependent manner (Fig. 6D).

The inhibitory activities of Th2 polarization were measured as a ratio of IC₅₀ with IL-4 versus IFN-γ, and the IC₅₀ values were calculated and compared (Table 2). The ratio of IC₅₀ for IL-4 versus IFN-γ was 2.5 in splenocytes cultured with fenugreek extract. This ratio was almost 11- to 17-fold lower than that of turmeric extract or green tea extract (Table 2). Furthermore, FACS analysis showed that treatment with fenugreek extract slightly increased the proportion of IFN-γ⁺ CD4⁺ cells by almost 1.3-fold versus OVA treatment (data not shown). These results firmly indicate that fenugreek extract may augment Th1-skewing through the up-regulation of IFN-γ production and the cell population.

4.5. Effects of fenugreek extract on transcription factors involved in CD4⁺ T cell differentiation

To examine the molecular mechanisms underlying the anti-allergic activities of fenugreek extract, we investigated whether fenugreek extract directly or indirectly regulates cellular transcription factors related to the Th1 and Th2 pathways. It is well known that the balance between Th1 and Th2 cells is determined

Table 2

The IC₅₀ values for IL-4 and IFN-γ and their ratio. Each ratio is presented as the mean of triplicate determinations in three trials.

Sample extract	IC ₅₀ for IL-4 (µg/ml)	IC ₅₀ for IFN-γ (µg/ml)	Ratio of IFN-γ/IL-4
Turmeric	148	22	0.15
Green tea	80.3	18.3	0.23
Fenugreek	15.4	40	2.5

by the interactions of transcription factor expression as well as cytokine production (Szabo et al., 2000; Zheng and Flavell, 1997). Splenocytes isolated from OVA-sensitized mice were cultured for 0–24 h in the presence of fenugreek extract, and the mRNA levels of several transcription factors were determined using quantitative real-time PCR (Fig. 7). Splenocytes treated with fenugreek extract enhanced the mRNA expression level of T-bet by almost 6-fold, whereas that of GATA-3 was suppressed by approximately 5-fold. IFN-γ and IL-4 mRNA expression also showed the same tendency as that observed for T-bet and GATA-3, respectively. Moreover, the enhancing activity of T-bet expression was the greatest at 6 h and the suppressing activity of GATA-3 expression was strongest at 24 h (Fig. 7). These results indicate that fenugreek extract may suppress the differentiation to Th2 by skewing

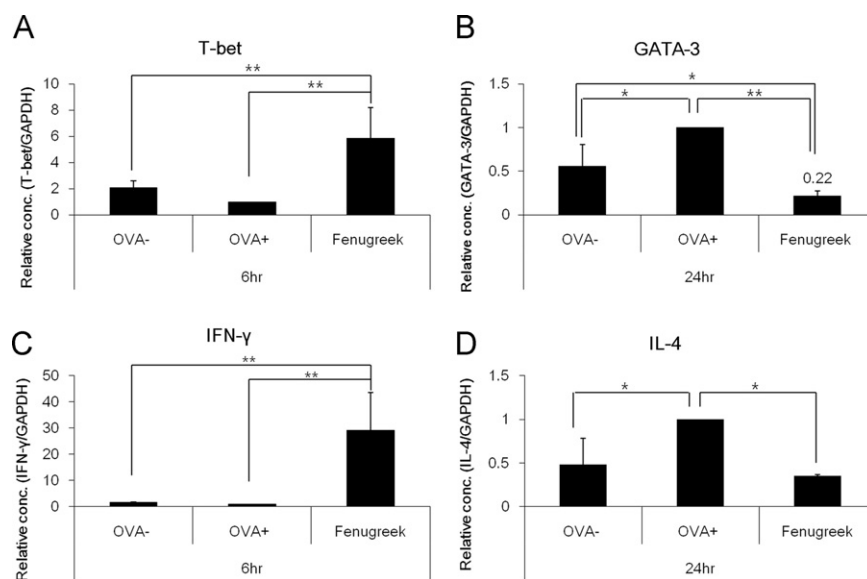


Fig. 7. Effect of fenugreek extract on the expression of transcription factors involved in T cell differentiation. The splenocytes of OVA-induced allergic mice were cultured in the presence of 30 mg/mL fenugreek extract and total RNA was isolated. Quantitative real-time PCR was performed as described in the Materials and methods section. Data are presented as the mean $\pm$ SD of triplicate determinations and were analyzed using ANOVA followed by Dunnett's multiple range tests. * $P < 0.05$ vs. OVA control; ** $P < 0.01$.

the differentiation to Th1 via an increase in the mRNA levels of T-bet and IFN- γ .

5. Discussion

In this study, we investigated the effect of fenugreek extract on TMA-induced CHS-like skin lesions and OVA-induced allergic responses in a BALB/c mouse model. The biological activities of fenugreek observed in this study strongly indicate that this extract demonstrates potent antiallergic effects resulting from the inhibition of Th2 polarization and the enhancement of Th1 skewing.

This study is the first to demonstrate that the administration of fenugreek extract inhibits the over-activation of the Th2 pathway in the TMA-induced CHS mouse model. In mice, TMA is routinely used to trigger T-cell-dependent CHS reactions and Th2-polarized chronic skin inflammation. A TMA-induced response was elicited, including the infiltration of eosinophils and T-cells, as well as the increased production of mast cells and Th2 cytokines. Thus, the severity of inflammation could easily be evaluated using ear thickness measurements (Dearman et al., 2002; Sailstad et al., 2003; Schneider et al., 2009). In this study, the administration of fenugreek extract not only inhibited TMA-induced ear thickness (Fig. 1), but also attenuated the infiltration of eosinophils and mast cells in the inflamed ear (Figs. 2 and 3). These skin inflammations are known to result from the production of Th2 cytokines, which are among the dominant mediators initiating the progression of allergic reactions (Schneider et al., 2009). Our results indicate that the administration of fenugreek extract inhibited the production of IL-4, IL-5, and IL-13 in the ears of TMA-induced CHS mice (Fig. 4), and also suppressed the production of IL-4 in splenocytes (Fig. 5). Furthermore, the administration of fenugreek extract lowered the production of pro-inflammatory cytokine IL-1 β and increased the production of anti-inflammatory cytokine IL-10 (Fig. 4). These results show that the administration of fenugreek extract constricted TMA-induced dermal inflammation, and that the biological basis of the inhibitory effects observed appears to involve modulating the balance of Th2 cells in the immune system.

To effectively prove the mechanism by which fenugreek extract regulates the balance of Th cells, we used an OVA-induced allergic response BALB/c mouse model. Mice sensitized with OVA/alum intraperitoneally can exhibit abnormal development of the Th2 pathway in the presence of OVA *in vitro*. The overproduction of IL-4 from OVA-sensitized splenocytes was suppressed in the presence of fenugreek extract (Fig. 6). Moreover, the IC₅₀ ratio calculated for Th1/Th2 cytokines confirmed that the suppressing effect of fenugreek extract was much stronger than that of turmeric extract or green tea extract (Table 2). Interestingly, this ratio indicates that fenugreek extract is a very powerful inhibitor of Th2 polarization in allergic responses, given that turmeric (Kim et al., 2005) and green tea (Fujimura et al., 2001, 2005) have already been shown to have potent antiallergic effects. The effect of fenugreek extract on the expression of transcription factors, such as GATA-3 and T-bet, was examined to elucidate the mechanism of Th2 inhibition at the molecular level (Fig. 7). T-bet, a Th1-specific transcription factor, was reported to be involved in the development of Th1 cells by inducing the synthesis of IFN- γ , and also, to negatively regulate the Th2 pathways (Lighvani et al., 2001; Szabo et al., 2000, 2003). The modulation of Th1 and Th2 pathways is thought to be one of the potential therapeutic strategies for developing immune regulation (Carter and Dutton, 1996; Finkelman et al., 1988; Gajewski and Fitch, 1988; Koo and Arain, 1998; Lee et al., 2000; Leung and Soter, 2001; Spergel et al., 1999). Our data demonstrate that fenugreek extract up-regulated the mRNA expression of T-bet, and eventually increased IFN- γ mRNA expression (Fig. 7A and C) and cytokine production in the splenocytes of OVA-induced allergic mice (Fig. 6B). In addition, the population of IFN- γ -producing CD4⁺ T cell increased in the presence of fenugreek extract (data not shown). Notably, treatment with fenugreek extract showed the enhancing effect of mRNA expression related to Th1 (6 h) earlier than the suppressing effect of mRNA related to Th2 (24 h) (Fig. 7). These findings strongly suggest that the T-bet expression-dependent enhancing effect of fenugreek extract on Th1 differentiation lead to the inhibition of Th2 differentiation.

It might also need to analyze the mRNA expression patterns of cytokine transcriptional factor such as T-bet and GATA-3 in TMA-

induced CHS mouse model. However, it has little information for mRNA expression of T cell differentiation in TMA-induced mouse dermatitis model because of unknown mechanism of pathology. Recently, Kosaka et al. studied that GATA-3 increased by sensitization and challenge of TMA in lung-associated lymph node of mice, but not in ear lesion or splenocyte, moreover T-bet expression was not studied (Fukuyama et al., 2010). To did propose solutions, we investigated the effect of oral administration of fenugreek extract on mRNA expression (T-bet and GATA-3) in OVA-induced allergic mice model that the mechanism was well-known relatively. As a result, we revealed that oral administration of fenugreek extract decreased OVA-induced GATA-3 expression and increased T-bet expression (data not shown). On the basis of these results, it was expected that the mRNA expression of cytokine transcriptional factor in T cell differentiation would show the same pattern in TMA-induced Th2-polarized chronic skin inflammation by oral administration of fenugreek extract.

Based on the various effects observed in both the *in vivo* and *ex vivo* systems, fenugreek extract is thought to contain different active compounds. The main chemical constituents of fenugreek extract are polysaccharides, saponins, flavonoid, fiber, trigonelline and choline (Jayaweera, 1981; Yoshikawa et al., 1997). Polysaccharides were described as immunomodulator in many studies that stimulate the function of macrophage, enhance natural killer cell activity, and exhibit anticancer effects and immunostimulating activities (Choi et al. 2005; Kim et al. 2007; Yoon et al., 2008). Some studies reported that saponin has immunostimulatory effect on the immune function of lymphocyte or the immune responses to vaccine immunization (Liu et al., 1995; Zhai et al. 2011). Some of the constituents such as flavonoid also might be having antioxidant effect. They have been reported to possess immunomodulatory activity (De La Fuente and Victor, 2000; Devasagayam and Sainis, 2002). However, it is crucial to identify active compounds in an herbal plant extract and confirm same activity as identified single compound without toxicity. Given that fenugreek has generally been administrated as a form of complex extracts, the observed antiallergic effects and safety may also result from the interaction of active compounds in the extracts. Our data demonstrate that these extracts are safe, even at high concentrations. Oral treatment with fenugreek extract (250 mg/kg/d BW) for 2 weeks showed no significant side effects in mice. It was proven that these extracts might be a safe and natural therapy for allergic and inflammatory diseases such as epilepsy (Billiau et al., 2005), paralysis (Mitchison, 1968) and gout (Harkavy, 1949), as shown by the biological data on the T cell polarization-induced allergic activities of fenugreek extract in this study. However, to further examine the exact mechanism underlying these activities, additional studies are necessary to isolate single compounds in fenugreek extract and to confirm the safety of the isolated useful compounds.

6. Conclusions

We have demonstrated that fenugreek extract suppresses allergic skin inflammation in a TMA-induced CHS mouse model, and exerts its effect by negatively regulating a skewed Th2 response through Th1 polarization that is dependent on T-bet expression in an OVA-induced allergic mouse model. These results proved that fenugreek extracts may be an useful agent in curing allergic inflammatory disease such as epilepsy, paralysis and gout as traditional use. Furthermore, it was emphasized that fenugreek extract has potent potential as a pharmacotherapeutic agent and as a potential source of therapies for preventive medicine for Th2-polarized allergic inflammatory responses.

Uncited reference

Yoshie et al. (1999).

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