

## Extract of *Poncirus trifoliata* Fruit Reduces Elevated Serum IgE Concentrations in Chemically Induced Allergic Model Mice

Hyun Jung Park<sup>1†</sup>, Jong Bo Kim<sup>2†</sup>, In Song Han<sup>2</sup>, Jin Hoi Kim<sup>3</sup>, Bokyung Kim<sup>4</sup>, Sung-Ho Kang<sup>5</sup>,  
Ho-Sung Jung<sup>6</sup>, Sang Ho Moon<sup>1</sup>, Si Heung Sung<sup>1</sup>, and Hyuk Song<sup>1\*</sup>

<sup>1</sup>Department of Animal and Food Bioscience, College of Natural Science, Konkuk University, Chungju 380-701, Korea

<sup>2</sup>Major of Horticulture Science, College of Natural Science, Konkuk University, Chungju 380-701, Korea

<sup>3</sup>Major of Animal Biotechnology, College of Animal Biotechnology, Konkuk University, Seoul 143-701, Korea

<sup>4</sup>Department of Physiology, College of Medicine, Konkuk University, Chungju 380-701, Korea

<sup>5</sup>Department of Otolaryngology, School of Medicine, Konkuk University, Chungju 380-701, Korea

<sup>6</sup>Department of Emergency Medicine, School of Medicine, Konkuk University, Chungju 380-701, Korea

\*Corresponding author: lovelyhusband@kku.ac.kr

<sup>†</sup>These authors are contributed equally to this work.

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**Abstract.** The fruit of trifoliolate orange (*Poncirus trifoliata*) is widely used for treating allergies including allergenic dermatitis and inflammation in oriental medicine. Increased blood IgE concentration and histamine level following dermatitis, coughing and asthma are the most important clinical symptoms of allergic diseases. Decreases in the elevated serum IgE concentrations are essential in the treatment of allergy because increased IgE blood levels are initiated in allergenic immune disorders. This study was conducted to investigate the activities of an extract of trifoliolate orange fruit using 100% methanol followed by extraction with boiling water on the regulation of blood IgE concentration in the U266B1 human myeloma cell line and a 1-chloro-2,4-dinitrobenzene (DNCB) sensitized mouse model. The results revealed that the extracts of trifoliolate orange suppressed IgE production in U266B1 cells in a dose dependent manner with or without lipopolysaccharide (LPS) stimulation of the cells. In addition, chemically elevated blood IgE concentrations were dramatically decreased by oral administration, ventral injection, and topical application of the trifoliolate orange extracts (TOE). The results indicated that the fraction of TOE should contain an important compound of the fruit of trifoliolate orange, and TOE had the ability to reduce IgE concentrations in cultured cells and a chemically sensitized in vivo mouse model.

**Additional key words:** 1-chloro-2,4-dinitrobenzene (DNCB), ELISA, immunoglobulin E (IgE) concentration, trifoliolate orange extracts (TOE), U266B1 cells, Western blot

### Introduction

Possibility of therapeutic effects of plant extracts were widely studied in Korea (Al-Turki et al, 2008; Jung et al, 2009). Among many plant extracts, the fruits of Rutaceae (*Poncirus trifoliata* L.) have been widely used in oriental medicine as a remedy for allergic diseases. Recently, the applications of trifoliolate orange (*Poncirus trifoliata*) to several biological and medical treatments were reported. The aqueous extract of trifoliolate orange inhibited stem cell factor induced mast cell migration and decreased TNF- $\alpha$  and IL-6 secretion in rat peritoneal mast cells (Na et al., 2003). Additionally, cancer treatment studies have revealed that the extracts or compounds of trifoliolate orange have

anti-cancer activity in human hepatocellular carcinoma cells (Ha et al., 2004; Hong et al., 2008), colon cancer cells (Jayaprakasha et al., 2007), and human promyelocytic leukemia cells (Yi et al., 2004). Moreover, anti-oxidative effects of trifoliolate orange have also been reported (Arbona et al., 2008; Pokharel et al., 2006; Wu et al., 2006; Zhou et al., 2007).

Although very few scientific studies of the effects of trifoliolate orange on allergic diseases have been conducted, passive cutaneous anaphylaxis (PCA) in rat and histamine release from rat peritoneal mast cells induced by mouse anti-dinitrophenyl (DNP)-IgE were found to be significantly inhibited by the aqueous extract of the immature fruit of trifoliolate orange (Lee et al., 1997; Park et al., 2005). In

addition, production of serum IgE and active systemic anaphylaxis induced by immunization with ovalbumin, Bordetella pertussis toxin and aluminum hydroxide gel were inhibited by the aqueous extracts of trifoliate orange fruits (Kim et al., 1999).

The physiopathology of allergic immune responses is complex and has been shown to be influenced by various factors, including genetic susceptibility, route of exposure, allergen dose, and, in some cases, the structural characteristics of the allergen (Akdis, 2006). Patients with allergy have peripheral blood eosinophils and increased serum IgE concentrations. Generally, immediate hypersensitivity reactions are called atopic and often induce higher levels of IgE in blood and more IgE receptors in mast cells. The over-expression of IgE as an immune response is generally believed to be responsible for the pathogenesis of atopic symptoms such as asthma, eczema, hay fever and urticaria (Heyman, 1990; Kahlert et al., 1992). Allergic immune response requires sensitization and development of specific immune response toward the allergen. During sensitization to allergens, priming of allergen-specific CD41 T<sub>H</sub>2 cells results in the production of T<sub>H</sub>2 cytokines (such as IL-4 and IL-13), which are responsible for class switching to the heavy chain for IgE production by B cells, mucus production, and activation of endothelial cells for T<sub>H</sub>2 cell and eosinophil migration to tissues (Larche et al., 2006; Romagnani, 2004). This immunological event can result in increased production of IgE in serum, which plays an important role in allergy hyperresponsiveness (Burrows et al., 1989; Sears et al., 1991).

Studies of allergy using animal models have been limited because the allergic disease occurs via various routes. Generally, allergic model animals are generated via genetic mutations and/or treated with chemical allergens. Exposure of mice to chemical allergens results in the stimulation of specific immune responses characterized by T lymphocyte activation with the synthesis and secretion of cytokines and antibodies (Dearman and Kimber, 1991). Low-molecular-weight chemical sensitizers are haptens that form complete antigens by combining with proteins (Dearman et al., 1991). These chemical sensitizers are often classified as skin sensitizers or respiratory sensitizers. Chemicals that are known to cause respiratory allergy include isocyanates [toluene di-isocyanate (TDI)], acid anhydrides [trimellitic anhydride (TMA)], reactive dyes, platinum salts, and glutaraldehyde. 1-Chloro-2,4-dinitrobenzene (DNCB) and oxazolone are typical dermal sensitizers known to induce mainly contact dermatitis (Gerberick et al., 2004). DNCB induced an increase in serum total IgE content, and that the response was dependent on the concentration of DNCB (Potter and Wederbrand, 1995). Specifically, the increases in total IgE content were 2.5, 3.5, and 4-fold in 1–3% DNCB-treated

mice, suggesting that DNCB could be an optimal sensitizer for increased blood IgE concentrations.

In the present study, the effect of trifoliate orange extracts on the regulation of IgE concentration was determined in vitro cultured IgE producing cells and DNCB treated in vivo mice model with an elevated IgE concentrations to elucidate the possible applications of trifoliate orange for treatment of allergy.

## Materials and Methods

### Extraction and Fractionation of Trifoliate Orange

Aqueous extracts were prepared by boiling 1 g of dried trifoliate orange in 8 mL of water at 100°C for 35 minutes, and the aqueous extracts were then filtered. Next, the extracts were collected and freeze dried to give a powder, which was subsequently dissolved in 1 × PBS (phosphate bicarbonate saline) to give a final concentration of 2 mg·mL<sup>-1</sup> (w/v) and stored at -20°C until use.

Ion-exchange chromatography was used for further separation of the trifoliate orange extracts. Specifically, aqueous extracts of trifoliate orange were applied to a column containing Diaion HP-20 (Samyang Ltd., Daejeon, Korea) for adhesion/binding. The aqueous extracts bound column was then washed with pure water three times, after which 100% methanol was added to separate the extracts by solvent added dependent. The extracts in methanol were then concentrated by evaporation to eliminate the solvents, after which they were dried into powder. This powder, trifoliate orange extract (TOE), was dissolved in 1 × PBS to give a final concentration of 2 mg·mL<sup>-1</sup> (w/v) and stored at -20°C until use.

### Treatment of U266 Cell Lines with TOE

The human U266B1 multiple myeloma cell line was cultured at 37°C under 5% CO<sub>2</sub>. RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, and 100 U·mL<sup>-1</sup> of penicillin/streptomycin was used to maintain the cell culture. In addition, 4 µg·mL<sup>-1</sup> of lipopolysaccharide (LPS) was added to 1 × 10<sup>6</sup> cells·mL<sup>-1</sup> of cultured U266B1 cells to increase the concentration of IgE secretion, after which 1 µg·mL<sup>-1</sup> to 4 mg·mL<sup>-1</sup> of TOE was added to the LPS treated U266B1 cells to regulate the secreted IgE level. The U266B1 cells were then incubated for seven days in a CO<sub>2</sub> incubator, after which the culture medium was collected to determine the concentrations of IgE. Additionally, 4 µg·mL<sup>-1</sup> of LPS was added to stimulate the IgE production in U266B1 cells, and the level of IgE secretion was then assessed after seven days of TOE treatment using an IgE ELISA kit (Bethyl Laboratory Inc., Montgomery, TX).

## Animal

BALB/c mice (Central lab Inc., Umsung, Korea) aged 5 weeks were housed in a conventional temperature- and humidity-controlled room and provided with standard laboratory food and water.

## Preparation of DNCB Sensitized and IgE Elevated Mouse Model

Five week old male BALB/c mice were used for production of a serum IgE elevated model animal. Briefly, the fur on the backs of the mice was removed one day prior to the treatment with 1-chloro-2,4-dinitrobenzene (DNCB) (Sigma, St. Louis, MO). The first treatment consisted of 200  $\mu$ L of 1% DNCB solutions (DNCB dissolved in 3:1 acetone:olive oil), while a second treatment with 0.1% DNCB was applied four days after the first treatment. Additional treatments with 0.1% DNCB were then conducted once every three days for four weeks.

## Treatment of IgE Elevated Mouse Model with TOE

TOE, separated by 100% methanol after extraction with water, was applied to mice with elevated IgE levels in response to DNCB treatment for two weeks. Five groups of mice (20 mice per group) were used for the study. Group 1 was normal healthy mice that were not treated with any chemicals or extracts, group 2 was treated with DNCB for six weeks, group 3 was treated with oral dose of 1% (w/v) TOE dissolved in water once every three days for four weeks after two weeks of treatment with DNCB, group 4 was injected 1% (w/v) TOE dissolved in water (pH 7.0) to the peritoneal cavity once every three days for four weeks after two weeks of treatment with DNCB, and group 5 was applied 2% (w/v) TOE dissolved in acetone to the skin once every three days for four weeks after two weeks of treatment with DNCB. During the TOE treatment period, 0.1% (w/v) DNCB was administered at the same time as the TOE treatment.

## Serum Preparation for Immunoassays

Total blood samples were collected from the tail vein for the experimental groups twice a week for six weeks for IgE ELISA. A total of 60–100  $\mu$ L of blood was collected into heparin containing tubes and centrifuged at 1200g for 20 min to separate the plasma. For immunoblotting of the serum IgE, approximately 1 mL of blood was collected by direct heart puncture from the each group of mice. The blood was allowed to clot and the serum was collected and centrifuged at 1200x g for 20 min. The supernatant was then separated and stored at  $-20^{\circ}\text{C}$ .

## IgE ELISA

The IgE concentrations in the total blood samples were measured using a mouse IgE ELISA Quantitation kit (Bethyl Laboratory Inc., Montgomery, TX). 1  $\mu$ g of the anti-mouse IgE (Bethyl Laboratory Inc., Montgomery, TX) was diluted with 100  $\mu$ L of coating buffer and incubated for one hour on 96 well plates. The non-binding excessive antibody was then aspirated and the plates were washed three times using washing solution. Next, 200  $\mu$ L of blocking solution was added, and the samples were incubated for 30 min, 100  $\mu$ L of sample diluents were mixed with the samples, which were then incubated for an additional hour. The samples were then washed five times with washing solutions (provided by manufacturer). Horse radish peroxidase (HRP)-conjugated secondary antibody (Bethyl Laboratory Inc., Montgomery, TX) was added, and the samples were incubated for one hour at room temperature. HRP solution was then removed, and the samples were washed five times with washing solutions. 100  $\mu$ L per well of TMB solution (Bethyl Laboratory Inc., Montgomery, TX) was added and then incubated for 20 min at room temperature. Finally, 100  $\mu$ L of  $\text{H}_2\text{SO}_4$  was added, and the IgE concentrations were determined based on the absorbance at 450 nm using a SUNRISE-BASIC TECAN micro-plate reader (Tecan Austria, Grodlig, Austria).

## IgE Western Blot Analysis

Blood IgE levels from seven groups of mice were assessed by anti-IgE immunoblotting. To obtain blood immunoglobulin without serum albumin contamination, a blood protein separation kit (Yu-il biotech, Chung-ju, Korea) was used to precipitate whole blood proteins and elute serum containing immunoglobulins. The serum immunoglobulins were then separated by SDS-PAGE in 10% Tris-glycine gels and transferred electrophoretically to polyvinylidene fluoride membranes. The membrane was then incubated in TBS-T (20 mM Tris-HCL, 137 mM NaCl, pH 7.6 supplemented with 0.1% Tween) containing 3% bovine serum albumin (BSA) for one hour at room temperature. After rinsing in TBS-T, the membrane was incubated with a HRP conjugated anti-mouse IgE antibody (Bethyl Laboratory Inc., Montgomery, TX) for one hour at room temperature. The membrane was washed three times in TBS-T. The immunoreactive proteins were then detected using enhanced chemiluminescence (Amersham, Buckinghamshire, UK) and Hyperfilm<sup>TM</sup> ECL<sup>TM</sup> (Amersham). The film was processed in developer and fixer solutions to visualize the protein bands.

## Statistical Analysis

The SPSS statistical package ver. 15.0 for Windows

(IBM corp, Somers, NY) was utilized for data analysis. All data were expressed as the mean and standard deviation. The values of IgE concentration following treatment of LPS and TOE were compared by a paired t-test. The null hypothesis was rejected when the probability was less than 0.05.

## Results and Discussion

To evaluate the influence of TOE on IgE production in U266B1 cells, different concentrations (1, 10, 100, 1,000, 2,000, and 4,000  $\mu\text{g}\cdot\text{mL}^{-1}$ ) of TOE were added to the untreated U266B1 cells. Treatment of normal U266B1 cells with TOE revealed a decrease in IgE production in a dose dependent manner, with IgE production being diminished in response to treatment with 100  $\mu\text{g}\cdot\text{mL}^{-1}$  TOE, and almost completely attenuated in response to treatment with 4  $\text{mg}\cdot\text{mL}^{-1}$  TOE (Fig. 1). 4  $\mu\text{g}\cdot\text{mL}^{-1}$  of LPS was added to stimulate the secretion of IgE in cultured media, and IgE production by treatment with LPS revealed significantly higher IgE production than in normal U266B1 cells (Fig. 1). Co-treatment of U266B1 cells with LPS and TOE revealed a significant decrease in response to 10  $\mu\text{g}\cdot\text{mL}^{-1}$ , 1,000  $\mu\text{g}\cdot\text{mL}^{-1}$ , 2,000  $\mu\text{g}\cdot\text{mL}^{-1}$ , and 4,000  $\mu\text{g}\cdot\text{mL}^{-1}$  TOE (Fig. 1). Moreover, almost no IgE secretion was detected after treatment of cultured cells with 4  $\text{mg}\cdot\text{mL}^{-1}$  TOE.

In accordance with our data, similar observations of *Poncirus trifoliata* fruit extract inhibiting IgE production in U266 human IgE-bearing cells have been reported (Kim et

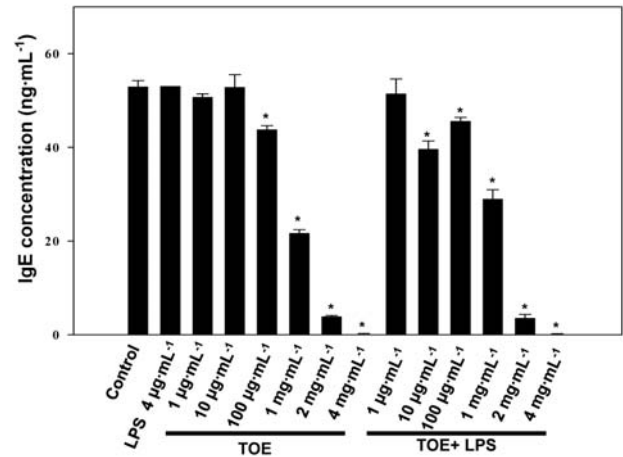


**Fig. 1.** Effect of TOE on the IgE production of U266B1 cells. Six different concentrations (1, 10, 100  $\mu\text{g}\cdot\text{mL}^{-1}$  and 1, 2, 4  $\text{mg}\cdot\text{mL}^{-1}$ ) of TOE were added to  $1 \times 10^5$  cultured U266B1 cells. The U266B1 cells were then incubated for seven days in a  $\text{CO}_2$  incubator. The culture medium was collected to analyze and identify the concentrations of IgE. Four  $\mu\text{g}\cdot\text{mL}^{-1}$  of LPS was added to stimulate IgE production in U266B1 cells. The results shown are the means  $\pm$  SD of four independent experiments. Statistical significances compared with control were indicated by asterisk (\*) in  $P < 0.05$ .

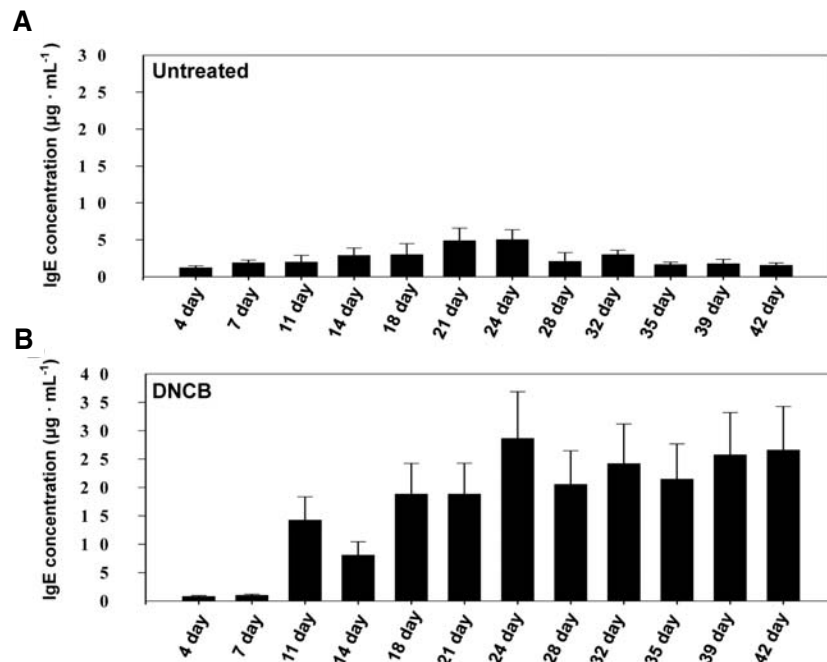


**Fig. 2.** Changes in blood IgE concentrations between untreated normal and DNCB treated mice over 42 days as determined by ELISA. **(A)** Blood samples from normal untreated mice were collected every three to four days for 42 days, after which the blood IgE concentration was assessed by ELISA. **(B)** Blood samples from DNCB treated mice were collected every three to four days for 42 days, and the blood IgE concentration was assessed by ELISA. Treatment with DNCB was conducted as described in materials and methods section. The results shown are the means  $\pm$  SD of 20 independent experiments.

al., 1999). These previous findings and the results of the present study indicated that the effect of trifoliate orange extract on IgE production was not transitory, but rather a physiologically significant event.

Serum IgE concentrations of normal and DNCB treated mice during the experimental period (42 days) were evaluated by ELISA. The IgE concentrations of the normal untreated mice (group 1) were maintained continuously at the basal level for the entire period (Fig. 2A), whereas the IgE level of the DNCB treated mice (group 2) showed an increase from the day 11 to a maximum of  $30 \mu\text{g}\cdot\text{mL}^{-1}$  on the day 24. It was maintained at greater than  $20 \mu\text{g}\cdot\text{mL}^{-1}$  for the remainder of the study (Fig. 2B). Comparison of the blood IgE level of both the untreated and DNCB exposed mice was also conducted by Western blot. Based on the ELISA data, the IgE intensity of untreated mice was slightly up-regulated from day 0 to the 14<sup>th</sup> day, after which it decreased (Fig. 3A, upper panel). Conversely, the IgE intensity in DNCB exposed mice showed a continuous increase from the seventh day to the end of the experimental period (Fig. 3B, upper panel). Therefore, DNCB successfully increased the IgE concentrations in mouse serum, and the IgE elevated mouse model was useful for evaluating the anti-allergic effects of TOE and other treated materials.

DNCB is a typical dermal sensitizer that selectively induces

the response of T-helper 1 cytokines such as IFN- $\gamma$  and IL-12 (Dearman et al., 2002). DNCB also induces an allergic dermatitis with increased blood IgE levels in experimental models. DNCB treated mice have often been used to screen for anti-allergic materials. Specifically, they have been used to evaluate the use of *Bambusae caulis* in liquamen, an extract of the bamboo stem, for the treatment of DNCB induced AD-like skin lesions in hairless mice with diminished elevated serum IgE (Qi et al., 2009). In this respect, the use of DNCB treated mice as an allergy model animal is physiologically and biologically relevant.

To identify the effects of TOE on changes in the blood IgE concentration level, 1% (w/v) TOE was administered to DNCB exposed mice by three different methods, oral injection (group 3), ventral injection (group 4), and topical application (group 5). All DNCB exposed mice used in these experiments showed an increased blood IgE concentration from the day 11 to 18 after treatment with DNCB, with a maximum of  $17 \mu\text{g}\cdot\text{mL}^{-1}$  being observed (Fig. 4). These elevated blood IgE concentrations rapidly decreased to  $3.5 \mu\text{g}\cdot\text{mL}^{-1}$  in response to oral administration of TOE from day 21, and were maintained at normal levels in untreated mice. Moreover, no additional increase in blood IgE concentration was observed after day 21 to the end of experiment (Fig. 4A). Immunoblotting also showed an in-

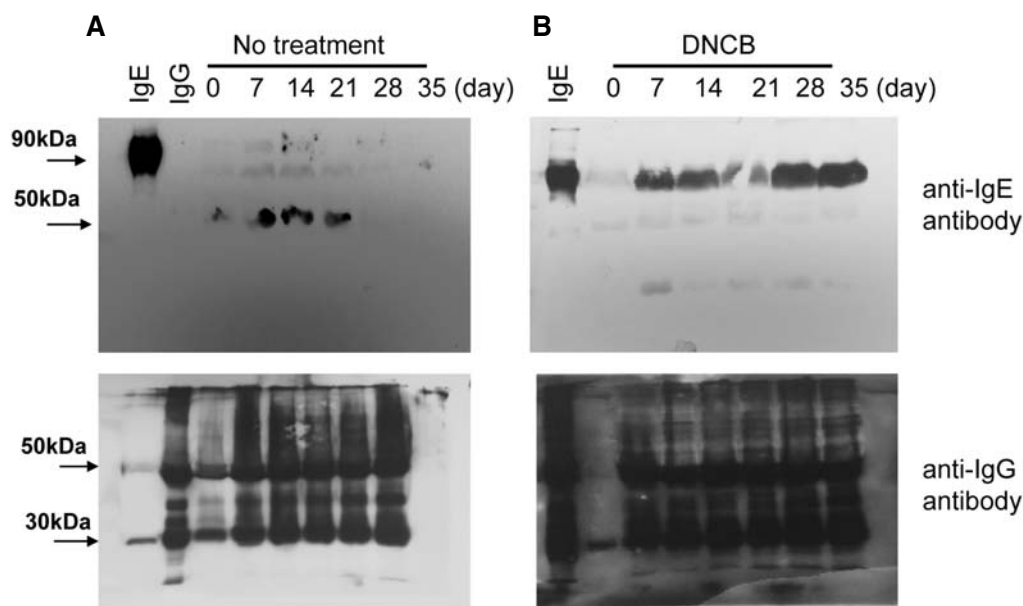


**Fig. 3.** Changes in blood IgE concentrations between untreated normal (A) and DNCB treated (B) mice over 42 days as determined by anti-IgE immunoblot assay. (A) Blood samples from normal untreated mice were collected every seven days for 35 days (five weeks), and the blood IgE concentration was assessed by Western blot using anti-IgE antibody (upper panel). (B) Blood samples from DNCB treated mice were collected every seven days for 35 days (five weeks), and the blood IgE concentration was assessed by Western blot using anti-IgE antibody (upper panel). As a protein loading control, anti-IgG immunoblot was conducted using the same membrane used for anti-IgE immunoblot after it was stripped (lower panels). This experiment was performed at least three times, and consistent results were obtained.

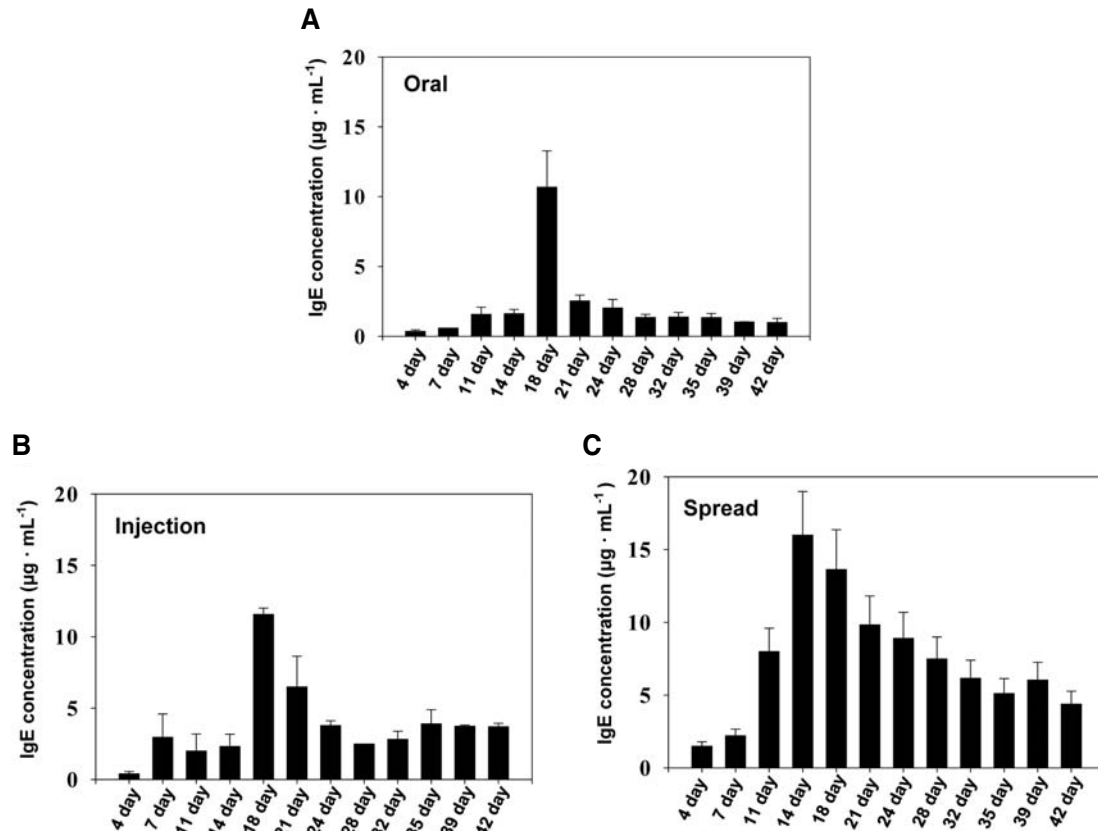


**Fig. 4.** Effect of TOE on the blood IgE concentrations in the DNCB sensitized mouse model. (A) ELISA analysis of blood samples from DNCB sensitized mice treated with TOE. 1% (w/v) TOE was administered orally (A), by ventral injection (B), and topically (C) for 28 days after two weeks of treatment with DNCB. Blood samples were collected every three to four days, and the blood IgE concentration was assessed by ELISA. During the TOE treatment period, 0.1% (w/v) DNCB was also applied at the same time as the TOE treatment. The results are expressed as the means  $\pm$  SD of 20 independent experiments.

crease in IgE intensity at day 14 in response to DNCB exposure, but a dramatic decrease in response to TOE treatment from day 21 to 35 (Fig. 5A).

Ventral injection of TOE to DNCB exposed mice resulted in a decrease in the elevated blood IgE concentration at day 21 from 12.3 to 7.4  $\mu\text{g} \cdot \text{mL}^{-1}$ , and further decreases were detected after day 24 (Fig. 4B). The decreased level of blood IgE in response to the ventral injection of TOE was the same as IgE concentration in normal mice. Western immunoblot of blood IgE from mice that received ventral injection of TOE also showed a similar decrease in blood IgE, with the intensity of blood IgE peaking at day 14, decreasing at 21 and returning to normal after day 28 (Fig. 5B).

Topical application of TOE to DNCB exposed mice also induced a decrease in blood IgE concentrations at day 21, but the response of IgE was not similar to that observed in response to orally or ventrally administered TOE. As shown in figure 5C, the elevated serum IgE concentrations did not rapidly decrease to normal levels, but gradually decreased from day 18 to 32 and were then maintained at normal

levels after day 35 (Fig. 4C). This pattern of decrease was also observed upon Western blot, which revealed an increased IgE level at day 7 and 14, followed by a gradual decrease (Fig. 5C).

In support of our findings, *Poncirus trifoliata* fruit extract inhibited the active systemic anaphylaxis and IgE production in mice sensitized with ovalbumin, *Bordetella pertussis* toxin and aluminum hydroxide gel (Kim et al., 1999). These data and the results of the present study revealed the same inhibition of production of IgE in mouse serum, although we used topical application of the chemical sensitizer, DNCB, to increase the serum IgE concentrations.

In addition, possible mechanism of the inhibitory effect of trifoliata orange extract on IgE production has been suggested by studies of anti-allergic inflammatory actions of the fruits of trifoliata orange. The expression of pro-inflammatory cytokines including tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-6 were effectively decreased in response to treatment of the human mast cell line with trifoliata orange, and activation of NF- $\kappa$ B was also attenuated (Shin et al., 2006). Moreover, 21-methylmelianodiol isolated

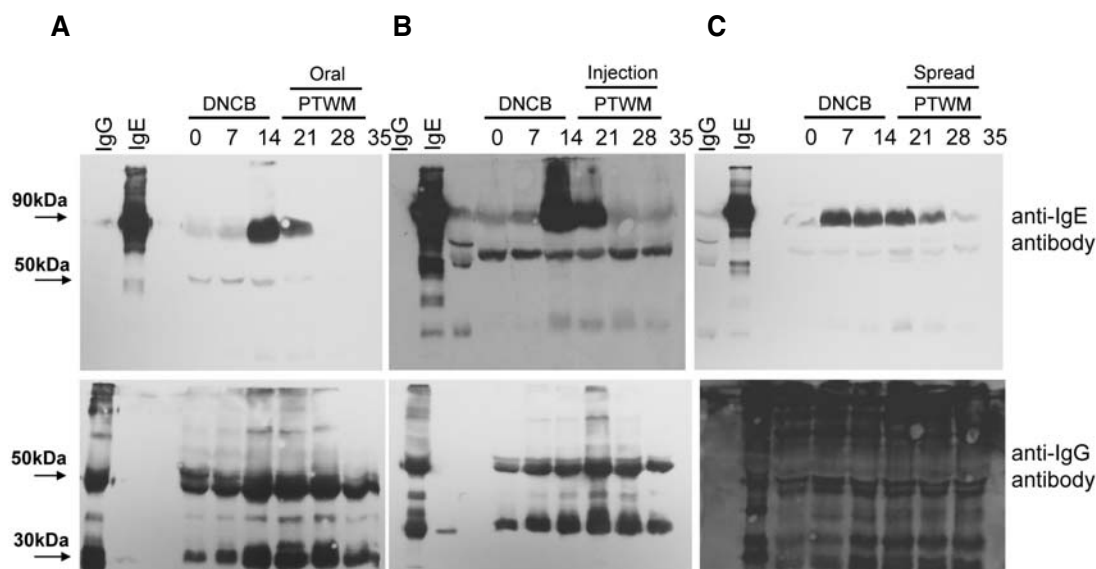


**Fig. 5.** Changes in the blood IgE concentrations in response to TOE treatment of DNCB sensitized mice. Western blot analysis of the blood IgE concentrations in a DNCB sensitized mouse model with TOE treatment. 1% (w/v) TOE was administered orally (A), via ventral injection (B), and topically (C) for 21 days after two weeks of treatment with DNCB. Blood samples were collected every seven days, and the blood IgE concentration was assessed by Western blot using anti-IgE antibody (upper panel). As a protein loading control, anti-IgG immunoblot was conducted using the same membrane used for anti-IgE immunoblot after it was stripped (lower panels). During the TOE treatment period, 0.1% (w/v) DNCB was also administered at the same time as the TOE treatment. This experiment was performed at least three times, and consistent results were obtained.

from the fruits of trifoliate orange effectively inhibited nitric oxide production, cyclooxygenase-2, TNF- $\alpha$  and IL-1 $\beta$  in macrophage cells (Zhou et al., 2007). These findings support the results of our present study, because the increase of blood IgE concentration resulted in increased histamine and inflammatory cytokines from mast cells, which in turn caused an inflammation event. Therefore, the inhibitory effects of isolated materials or extracts from trifoliate orange on the inflammatory action of mast cells and inflammation related cytokine production indicated that the extract of trifoliate orange inhibited IgE production and had the ability to regulate cytokines related to allergy and inflammation.

In this study, we used the fruit extract of *Poncirus trifoliata* to identify changes in the blood IgE concentration in IgE over producing mice. The results revealed that the extracts of *Poncirus trifoliata* dramatically decreased the elevated blood IgE concentration. Our findings suggest that the extracts of *poncirus trifoliata* help regulate the blood IgE concentrations. Therefore, the biological activities of TOE observed in this study strongly suggested that the extract of trifoliate orange would be an excellent candidate for potent and unique anti-allergic agents.

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