

The Fruits of *Cudrania tricuspidata* Suppress Development of Atopic Dermatitis in NC/Nga Mice

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The fruits of *Cudrania tricuspidata* are a medicinal herb in Korea, known for its antiatherosclerotic and anti-inflammatory effects. Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by the influx of lymphocytes into the dermis. Using an animal model of AD, we assessed whether *C. tricuspidata* suppresses the development of AD-like skin lesions. *Cudrania tricuspidata* was administered orally to NC/Nga mice with *Dermatophagoides-farinae*-induced AD-like lesions for 49 days. The effects of *C. tricuspidata* were assessed by measuring clinical symptoms, swelling of the skin on the back and ears, and plasma concentrations of mTARC (mouse thymus and activation regulated chemokine), histamine and immunoglobulin E (IgE). We found that *C. tricuspidata* (60 mg/kg/day) inhibited the development of AD-like skin lesions, reduced skin dermatitis scores and inhibited the histological changes induced by repeated application of *D. farinae*. In addition, *C. tricuspidata* inhibited the increases in plasma concentrations of mTARC, histamine and IgE induced by *D. farinae*. These findings indicate that *C. tricuspidata* inhibits the development of AD-like skin lesions induced by repeated applications of *D. farinae* in sensitized NC/Nga by suppressing plasma concentrations of mTARC, histamine and IgE. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: *Cudrania tricuspidata*; atopic dermatitis; *Dermatophagoides farinae*; mTARC; IgE; histamine

INTRODUCTION

Cudrania tricuspidata has long been used as a traditional medicine in Korea to treat pneumonia, phthisis, influenza and hyperpiesia, and its bark has been used to treat severe rash and paralysis. More recently, *C. tricuspidata* has been shown to reduce serum cholesterol concentrations and to inhibit inflammation (Kim *et al.*, 2000) and T-lymphocyte proliferation (Chang *et al.*, 2008) and to have antihypertensive effects (Kang *et al.*, 2002). Compounds; catecholic xanthone and flavonoid purified from the roots of *C. tricuspidata* were shown to have hepatoprotective and antiinflammatory effects (Park *et al.*, 2006), whereas compounds such as cudraflavone B, gericudranin E and gerontoxanthone A from the roots of *C. tricuspidata* were shown to have hepatic-protective effects (An *et al.*, 2006). Gancaonin A purified from the fruits *C. tricuspidata* inhibits the mouse brain monamine oxidase (Han *et al.*, 2005.).

Atopic dermatitis (AD) is a chronic inflammatory skin disorder, characterized by pruritus (Friedl *et al.*, 2001) and increased serum concentrations of IgE and histamine (Janothan and Linda, 1999), and peripheral blood eosinophilia. Histamine causes itching in humans (Hoffman *et al.*, 1975), and antihistamines have been regarded as primary antipruritic therapies (Wahlgren,

1992). Thymus and activation-regulated chemokine (TARC/CCL17), a member of the CC-chemokine superfamily, is produced by monocyte-derived dendritic cells (mdDCs), endothelial cells and keratinocytes. Serum concentrations of TARC and the expression of CC-chemokine receptor 4 have been shown to be significantly increased in AD patients (Stander and Steinhoff, 2002).

To assess the effects of *C. tricuspidata* on AD, we used an animal model in which AD-like lesions are induced in NC/Nga mice using *Dermatophagoides farinae* dust mite extract. We tested whether *C. tricuspidata* suppressed the sign of AD-like skin lesions in these animals.

MATERIALS AND METHODS

Plant material and extraction. The fruits of *C. tricuspidata* (100 g) were boiled under reflux with deionized water for 90 min (1 L). The extracts were filtered, concentrated using a rotary evaporator and lyophilized (yield 12.70%).

High performance liquid chromatography analysis. Analysis was performed using a Shimadzu LC-20A HPLC system (Shimadzu Co., Kyoto, Japan), consisting of a solvent delivery unit, an on-line degasser, a column oven, an autosampler and a photo-diode array (PDA) detector. The data processor employed LC Solution software (Version 1.24). The analytical column used was a Gemini C18 (250 × 4.6 mm, particle size 5 µm;

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Phenomenex, Torrance, CA, USA). The mobile phases were solvent A (1.0% acetic acid in H₂O) and solvent B (1.0% acetic acid in acetonitrile). The gradient flow was as follows: (A)/(B) = 80/20 (0 min) → (A)/(B) = 60/40 (10 min) → (A)/(B) = 30/70 (30 min; hold for 5 min) (A)/(B) = 80/20 (40 min). The column temperature was maintained at 40 °C. The analysis was carried out at a flow-rate of 1.0 mL/min at 360 nm and injection volume was 10 µL.

Chemicals and reagents. Kaempferol, quercetin and rutin were obtained from ChromaDex (Santa Ana, CA, USA). Pinocembrin was purchased from Fluka (St Louis, MO, USA). The purity of each compound was determined to be above 98% by HPLC analysis. HPLC-grade reagents, methanol, acetonitrile and water were obtained from J. T. Baker (Phillipsburg, NJ, USA). Other chemicals were of analytical grade.

Dermatophagoides farinae (Biostir-AD[®]), an ointment containing extracts of the house dust mite, was purchased from Biostir Inc. (Hyogo, Japan). Prednisolone (Sigma-Aldrich Inc, St Louis, MO, USA) was used as a positive control.

Preparation of standard and sample solution. Standard stock solutions of kaempferol, quercetin and rutin (all 1000 µg/mL) were prepared in methanol and held below 4 °C. Working standard solutions were prepared by serial dilution of stock solutions with methanol. The fruit of *C. tricuspidata* was extracted with distilled water three times by refluxing for 1 h and water solution was evaporated to dryness and freeze-dried (yield: 12.7%). The lyophilized extract was weighed (100 mg) into a 100 mL flask and methanol was added to the volumetric mark. The mixture was sonicated for 30 min at room temperature. After extraction, the mixture was filtered through a 0.2 µm syringe filter.

Animals. Male NC/Nga mice (8 weeks old), purchased from Central Laboratory Animal Inc. (Seoul, Korea), were housed in an air-conditioned room and maintained at 24 ± 2 °C and 55 ± 15% humidity. All procedures involving animals were conducted in accordance with the Guidelines of the Institutional Animal Care and Use Committee of the Korea Institute of Oriental Medicine (#09-033).

Sensitization. Atopic-dermatitis-like skin lesions were induced in 10-week-old male NC/Nga mice using Biostir-AD, as described by the manufacturer (Hirotsoshi *et al.*, 2004). Briefly, the hair on the upper back was shaved, and 200 µL of 4% (w/v) sodium dodecyl sulphate (SDS) was applied to the shaved dorsal skin and both surfaces of each ear for barrier disruption. After 2 h, 50 mg *D. farinae* was applied topically twice weekly for 7 weeks. *Cudrania tricuspidata* and prednisolone were each suspended in distilled water and administered orally to mice. At the start of the experiment, mice were randomized to one of four groups of five mice each: untreated controls (distilled water; control, p.o.), *D. farinae*-treated (50 mg/mouse), prednisolone-treated (3 mg/kg p.o.), and *C. tricuspidata*-treated (60 mg/kg p.o.).

Dermatitis score. We measured dermatitis scores in 10-week-old NC/Nga mice with no skin symptoms. The

severity of dermatitis was assessed macroscopically according to the Eczema Area and Severity Index (EASI) scoring system: 0, no symptoms; 1, mild symptoms; 2, moderate symptoms; and 3, severe symptoms. Dermatitis score was defined as the sum of scores for erythema/haemorrhage, oedema, excoriation/erosion and scaling/dryness (Hanifin *et al.*, 2001.).

Histopathology. Mice were sacrificed 50 days after *D. farinae* sensitization. The back skin and one ear of each mouse were fixed in 10% (v/v) natural buffered formalin for 24 h at 4 °C. Tissue samples were embedded in paraffin and thin-sectioned (4 µm thickness), and the sections were stained with haematoxylin and eosin solution (Sigma-Aldrich) and mounted under cover slips using Dako-mounting medium (Dakocytomation, Denmark, CA). The stained sections were photographed using a photometric Quantix digital camera and montages were assembled using Adobe Photoshop.

Plasma concentrations of mTARC, histamine and IgE. Blood samples were drawn from mice, and plasma was separated by centrifugation at 10000 × g for 10 min at 4 °C and stored at -80 °C. Plasma concentrations of mTARC (R&D Systems, USA), histamine (Oxford Bio-medical Research, USA) and IgE (Bethyl Laboratories Inc., USA) were measured by enzyme linked immunosorbent assay (ELISA), according to the manufacturer's instructions.

Statistical analysis. Data were reported as means ± SEMs and compared by ANOVA and the Bonferroni multiple comparison method. A *p* value < 0.05 was defined as statistically significant. All statistical analyses were performed using the SYSTAT[®] 8.0 program (SYSTAT Inc., Evanston, IL, USA).

RESULTS

High performance liquid chromatography analysis

The HPLC chromatogram was obtained using a HPLC-PDA detector. Using optimized chromatography conditions, three compounds were eluted before 20 min in sample analysis by using mobile phases consisting of solvent A (1.0% acetic acid in H₂O) and solvent B (1.0% acetic acid in acetonitrile). The HPLC chromatogram of the standard solution and *C. tricuspidata* extract is shown in Fig. 1. The retention times of the compounds in standard solution were 7.3 min (rutin), 14.3 min (quercetin) and 17.2 min (kaempferol). However, quercetin and kaempferol were not detected in *C. tricuspidata* extract (Fig. 1).

Dermatitis score in NC/Nga mice

Dermatophagoides-farinae-induced AD-like lesions in NC/Nga mice, with dermatitis EASI scores that increased over time (Fig. 2A). In prednisolone-treated mice, symptoms increased until day 28 but decreased thereafter. Oral administration of *C. tricuspidata* 60 mg/kg inhibited the development of AD-like lesions, with significant differences observed from day 28 to

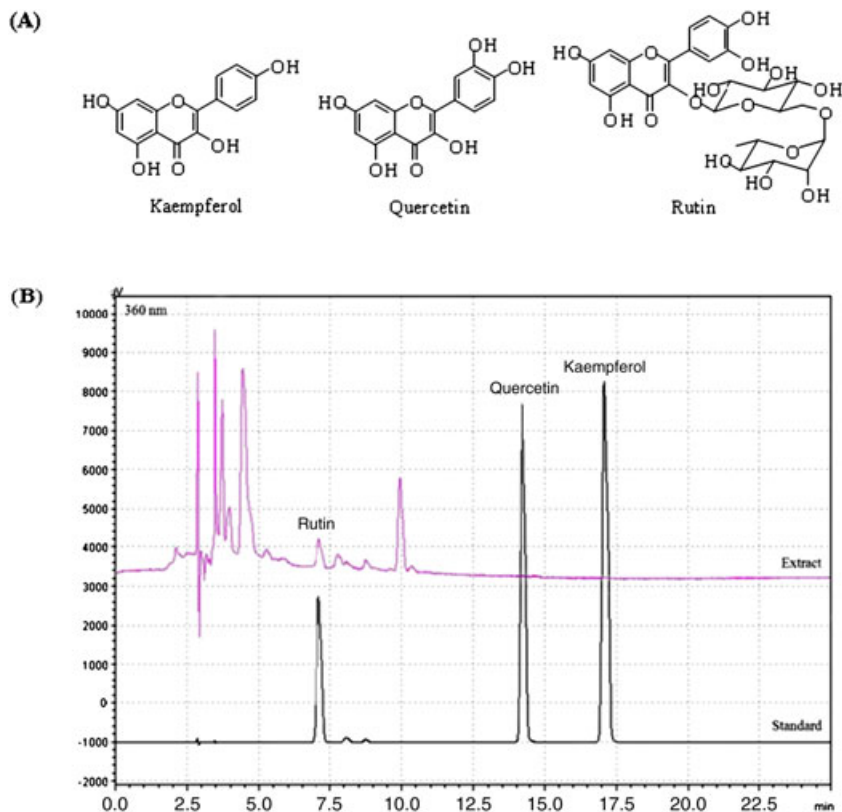


Figure 1. Structures and HPLC chromatograms of flavonoids. (A) Chemical structure of *Cudrania tricuspidata*, (B) HPLC chromatogram of *C. tricuspidata* extract and standard solution at 360 nm.

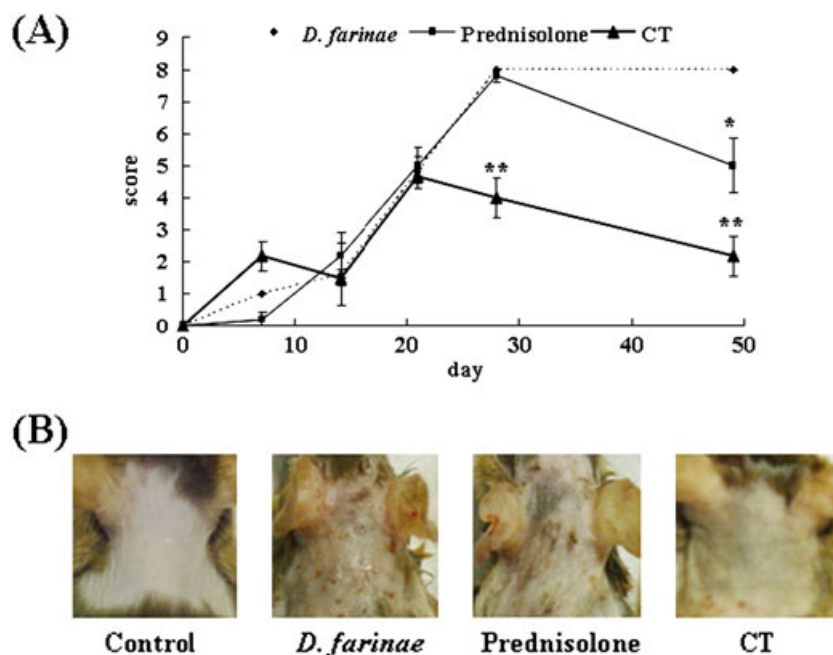


Figure 2. Severity of dermatitis and macroscopic skin features in the back and ears. (A) Effect of consecutive administration of the fruits of *Cudrania tricuspidata* and prednisolone on dermatitis score of *Dermatophagoides farinae*-induced atopic dermatitis (AD)-like lesions in the back and ears. *Cudrania tricuspidata* and prednisolone were administered once daily for 49 days. The dermatitis score was evaluated by the procedures described in the Materials and Methods section (mean $\pm$ SEM ($n = 5$); * $p < 0.05$, ** $p < 0.01$ compare with *D. farinae*-induced group). (B) Representative features of the effect of consecutive administration of *C. tricuspidata* and prednisolone on *D. farinae*-induced AD-like lesions in the back and ears. The features show the back and ears on day 50 after sensitization. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr

day 49 ($p < 0.01$). Representative photographs 50 days after sensitization are shown in Fig. 2B. No symptoms were observed in the control group, including erythema/haemorrhage, oedema, excoriation/erosion and scaling/dryness.

Histological examination

In contrast to the normal control group, which showed no abnormal changes in the back skin and ears, repeated challenge with *D. farinae* caused prominent

inflammatory changes, such as erosion, hyperplasia of the epidermis and dermis, and infiltration of cells. Treatment with both 3 mg/kg prednisolone and 60 mg/kg *C. tricuspidata* significantly inhibited the development of inflammatory signs in the back skin and ears (Fig. 3A and B). We confirmed that the *C. tricuspidata* suppressed the mast cell infiltration in back skin (Fig. 3C).

Plasma concentrations of mTARC, histamine and IgE

To assess the mechanisms underlying the induction of AD in these mice, we measured plasma concentrations of mTARC, histamine and IgE. We found that plasma concentrations of mTARC were significantly lower in *C. tricuspidata*- (6.77 ± 1.59 pg/mL) and prednisolone (6.09 ± 0.12 pg/mL) -treated mice than in *D. farinae*-treated mice (16.89 ± 3.44 pg/mL) ($p < 0.05$ each) (Fig. 4A). Treatment with *C. tricuspidata* (44.39 ± 3.87 nM) and prednisolone (37.52 ± 2.07 nM) also suppressed plasma histamine concentrations compared with *D. farinae*-treated mice (59.70 ± 0.92 nM) (Fig. 4B). We also found that plasma concentrations of IgE were significantly lower in mice treated with *C. tricuspidata* (335.99 ± 21.36 ng/mL) and prednisolone (321.95 ± 15.93 ng/mL) than in mice treated with *D. farinae* alone (522.66 ± 28.53 ng/mL) (Fig. 4C).

DISCUSSION

We have shown here that *C. tricuspidata* significantly suppresses the generation of AD-like skin lesions induced by *D. farinae* (Biostir-AD[®]) in NC/Nga mice. Atopic-dermatitis-like skin lesions in NC/Nga mice highly resemble those of human AD, showing various

grades of signs and symptoms including erythema and haemorrhage, followed by oedema, superficial erosion, deep excoriation, alopecia and dryness in the skin (Matsuda *et al.*, 1997.). In addition, NC/Nga mice with AD-like skin lesions show IgE hyperproduction in serum, severe itching behavior and invasion of inflammatory cells into the skin (Maekawa *et al.*, 2002.).

In a previous study we compared AD-like lesions and IgE plasma concentrations in mice treated with 1-chloro-2,4-dinitrobenzene (DNCB) and *D. farinae* extract. Although the severity of symptoms was greater in mice treated with DNCB, the concentrations of IgE and mTARC were lower than in mice treated with *D. farinae* extract. We therefore used *D. farinae*-sensitized NC/Nga mice as a model for AD.

We found that histological changes were greater with *C. tricuspidata* than with prednisolone, as shown by the thickness of the back skin and ears and EASI score, and that the plasma concentrations of mTARC, histamine and IgE were lower in *C. tricuspidata*- than in prednisolone-treated mice, indicating that application of *C. tricuspidata* had a greater effect on the generation of AD-like lesions than did prednisolone.

Thymus and activation-regulated chemokine may be involved in conditions other than AD-like skin lesions and allergic diseases (Sugawara *et al.*, 2002). For example, platelets of AD patients have been reported to contain TARC (Fujisawa *et al.*, 2002). Although the role of TARC in platelets remains unknown, it may be related to chemokines in skin (Darbonne *et al.*, 1991). Indeed, we found that *D. farinae* extract increased the plasma concentration of TARC, whereas prednisolone and *C. tricuspidata* reduced the concentration of this chemokine.

NC/Nga mice with AD-like skin lesions have been shown to have increased serum concentrations of IgE

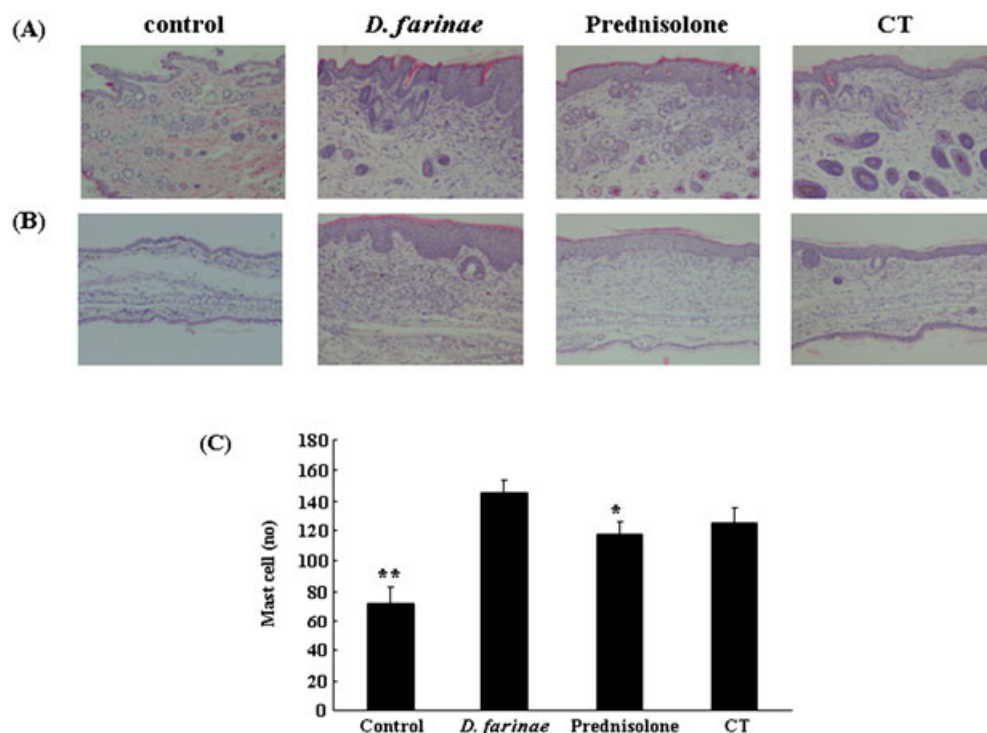


Figure 3. Histological features on atopic dermatitis (AD)-like lesion administrated with the fruits of *Cudrania tricuspidata*. *Dermatophagoides farinae* application on back and ears in NC/Nga mice for 49 days induced the AD-like skin lesions: (A) Back skin, (B) ears (haematoxylin and eosin) and (C) mast cell counts (mean ± SEM ($n = 5$)): * $p < 0.05$, ** $p < 0.01$ compare with *D. farinae*-induced group). This figure is available in colour online at wileyonlinelibrary.com/journal/ptr

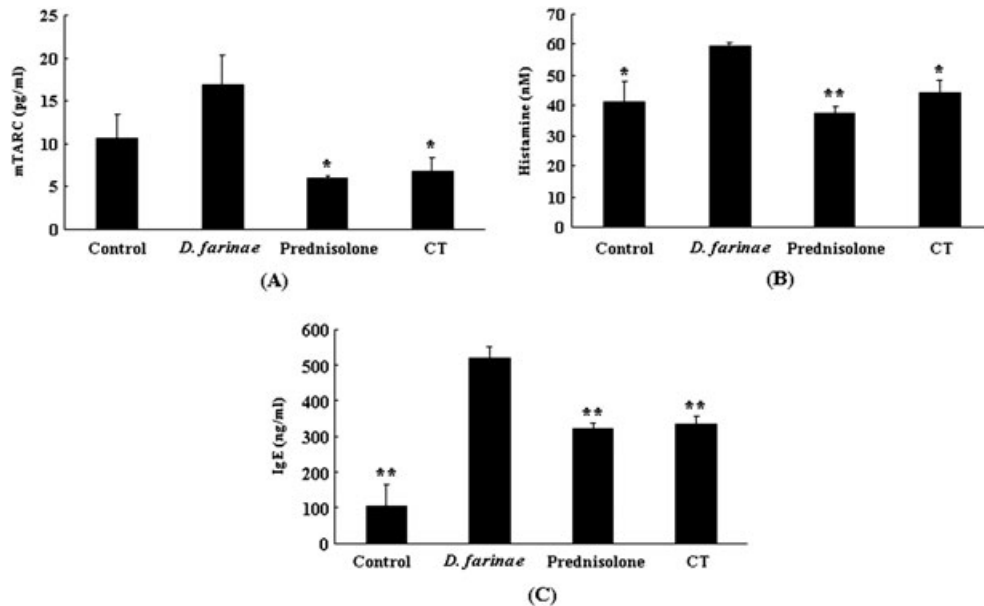


Figure 4. Effect of *Cudrania tricuspidata* on *Dermatophagoides farinae* atopic dermatitis increase in plasma level of IL-4, histamine and IgE in NC/Nga mice. The plasma samples were untreated: control, *D. farinae* plus vehicle treated; *D. farinae* plus prednisolone treated; prednisolone and *D. farinae* plus *C. tricuspidata* treated. The plasma concentrations of (A) mTARC, (B) histamine and (C) IgE were measured by enzyme linked immunosorbent assay (mean $\pm$ SEM ($n = 5$): * $p < 0.05$, ** $p < 0.01$ compare with *D. farinae* group).

(Aioi *et al.*, 2001). IgE synthesis in B cells is primarily regulated by cytokines, including T-helper cell 2 (Th2)-derived cytokines such as IL-4 and IL-13, IgE class-switching factors (which up-regulated IgE synthesis) and Th1-derived cytokines (especially IFN- γ), all of which are strong inhibitors of IgE synthesis (Ramahnani, 1994).

The Th1/Th2 paradigm has been applied to explain the pathogenesis of immune diseases, including AD. In general, AD patients show increased serum levels of Th2 cytokines, such as IL-4, IL-5 and IL-13 and an increased serum level of IgE. IL-4 also has been shown to inhibit IFN- γ synthesis and stimulate B lymphocytes to synthesize IgE (Vercelli *et al.*, 1990). Scratching precipitated by pruritus causes trauma and further insult to the already compromised stratum corneum and induces keratinocytes to release a variety of proinflammatory cytokines (William, 2005). We suggested that the inhibition of IgE and mTARC is caused by *C. Tricuspidata*, since *C. tricuspidata* regulated Th2 cytokines such as IL-4.

Histamine, serotonin and substance P are regarded as mediators that cause itching in humans (Greaves and Wall, 1996.). Treatment with antihistamines, steroids and immunosuppressants has been shown to reduce this scratching and itching behavior. We used the corticosteroid prednisolone as our positive control. Indeed, we found that both prednisolone and *C. tricuspidata* reduced plasma histamine concentrations in AD-induced NC/Nga mice.

We performed analysis for components such as kaempferol, quercetin and rutin of *C. tricuspidata* fruits using HPLC-PDA. However, kaempferol and quercetin were not detected in *C. tricuspidata*. Only rutin was detected. Therefore, we must be performed to identification for major peak at retention time of 10.0 min and activity test about these compounds. *Cudrania tricuspidata* has many flavonoids but the major compound has not been investigated. We are therefore investigating the isolation of compounds and analysis by HPLC and NMR. A major compound of atopic dermatitis has not yet been found, but examination of such a compound is necessary.

In conclusion, we have demonstrated that oral administration of *C. tricuspidata* inhibited the development of AD-like lesions in NC/Nga mice. Our results indicate that this inhibition may be due to the ability of *C. tricuspidata* to suppress histamine concentrations and the Th2 cell response.

Acknowledgement

This research was supported by a grant from Korea Research Council of Fundamental Science and Technology (KRCF), No. kiom-2009-s2, Korea.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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