Toxicol. Res.
Vol. 27, No. 3, pp. 173-180 (2011)
Open Access http://dx.doi.org/10.5487/TR.2011.27.3.173

Official Journal of
Korean Society of Toxicology
Available online at http://www.toxmut.or.kr

The Inhibitory Effect of Premature *Citrus unshiu* Extract on Atopic Dermatitis *In Vitro* and *In Vivo*

Gyeoung-Jin Kang[1], Sang-Chul Han[1], Eun-Jou Yi[2], Hee-Kyoung Kang[1] and Eun-Sook Yoo[1]

[1]*Department of Pharmacology, School of Medicine,*
[2]*Department of Clothing & Textiles, College of Natural Sciences, Jeju National University, Jeju 690-756, Korea*

(Received July 1, 2011; Revised July 25, 2011; Accepted August 14, 2011)

Atopic dermatitis (AD) is a chronic, recurrent inflammatory skin disease that is associated with Th2 cell-mediated allergy. The process that leads to infiltration of inflammatory cells into an AD lesion is remarkably dependent on various chemokines, especially TARC (thymus and activation-regulated chemokine/CCL17) and MDC (macrophage-derived chemokine/CCL22). Serum levels of these chemokines are over-expressed in AD patients. *Citrus unshiu*, which is known as Satsuma mandarin, has anti-oxidative, anti-inflammation, and anti-microviral activity. Therefore, we investigated the effect of EtOH extract of premature *C. unshiu* on AD. We did this using a DNCB-induced AD mouse model. We also tried to confirm an inhibitory effect for premature *C. unshiu* on the expression of inflammatory chemokines in IFN-γ and TNF-α stimulated HaCaT human keratinocytes. We found that extract of premature *C. unshiu* reduced DNCB-induced symptoms such as hyperkeratosis, increased skin thickness, and infiltrated mast cells, in our AD-like animal model. The extract decreased levels of IFN-γ and IL-4 in ConA-stimulated splenocytes isolated from DNCB-treated mice. Also, extract of premature *C. unshiu* inhibited mRNA expression and protein production of TARC and MDC through the inhibition of STAT1 phosphorylation. These results suggest that *C. unshiu* has anti-atopic activity by regulating inflammatory chemokines such as TARC and MDC.

***Key words*:** DNCB, Atopic dermatitis, Hairless mouse, TARC/CCL17, MDC/CCL22, *Citrus unshiu*

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease that develops on an allergic and genetic background. It is characterized by pruritic and eczematous lesions, increased serum IgE levels, and the infiltration into lesioned skin of inflammatory cells such as Th2-type cells, eosinophils, mast cells, and macrophages (Abramovits, 2005; Bonness and Bieber, 2007). AD is assumed to be an aspect of the Th2 condition due to increases in Th2 cytokines such as IL-4 and IL-5 in acute phase skin lesions, and to be a mixed condition of Th1/Th2 in the chronic phase skin lesions (e.g., IFN-γ, IL-12, and GM-CSF). Moreover, it has been shown that changes in expression of various inflammatory chemokines produced by several cell types, including CCL1, CCL2, CCL5, CCL11, CCL13, CCL18, CCL20, CCL26, and CCL27, are similar to the AD phenotype (Imai *et al*., 1999).

Chemokines are a group of a small cytokines produced by various cell types and are divided into C, CC, CXC, and CX3C subfamilies based on NH_2-terminal cysteine-motifs. The main role of chemokines is regulation of dendritic cell (DC) trafficking and recruitment of inflammatory cells such as T cells, eosinophils, macrophages to sites of infection and inflammation (Pease and Williams, 2006). In particular, thymus-regulated and activation-regulated chemokines (TARC and CCL17) and macrophage-derived chemokines (MDC and CCL22) are produced by keratinocytes, dendritic cells, endothelial cells, and bronchial epithelial cells, and play important roles in the recruitment of Th2-type cells expressing CC chemokine receptor 4 (CCR4) on their surfaces. Previous studies showed that serum levels of these Th2-type chemokines are increased in patients with AD (Hijnen *et al*., 2004; Imai *et al*., 1999; Kakinuma *et al*., 2002; Leung *et al*., 2003).

Recently, many types of mouse models similar to our AD model have been developed. In one, the ears or the dorsal skin of a BALB/c mouse is repeatedly exposed to dinitrochlorobenzene (DNCB) or trinitrochloridebenzene (TNCB) (Kitagaki *et al*., 1995; Lee *et al*., 2010). Another is NC/Nga mice that is developed AD-like symptoms under conventional condition, but not specific pathogen-free (SPF) con-

Correspondence to: Eun-Sook Yoo, Department of Pharmacology, School of Medicine, Jeju National University, Jejudaehakno 66, Jeju 690-756, Korea
E-mail: eunsyoo@jejunu.ac.kr

ditions (Jin *et al*., 2009; Vestergaard *et al*., 1999). However, each mouse model has disadvantages. For example, in the first model, the ear surface area may be too small; or one has to shave or clip their fur prior to various assays which interferes with time-course studies due to hair re-growth. In contrast, the model of AD in which a hairless mouse is given repeated topical applications of TNCB is more similar to the features of human skin and is useful for doing non-invasive biophysical and histological studies (Matsumoto *et al*., 2004, 2005; Qi *et al*., 2009).

Citrus unshiu is a kind of citrus fruit in the Rutaceae family. *C. unshiu* is composed of rind and sarcocarp and includes various bioactive substances such as essential oils, carotenoids, cellulose, pectin, limonoid, etc. In recent studies, it has been reported that flavonoids are also contained in *C. unshiu* extracts which have anti-oxidant, anti-cancer, and anti-inflammatory activity. Also, the flavonoid content changes during maturation of *C. unshiu* and is high in premature *C. unshiu* (Kang *et al*., 2005; Kim *et al*., 2009).

Here, we aimed to find the effect of premature *C. unshiu* on AD-like markers. We investigated the effects of premature *C. unshiu* extract on TARC and MDC, important markers of AD. Furthermore, we examined the effect of premature *C. unshiu* extract in a DNCB-challenged animal model.

MATERIALS AND METHODS

Cell culture and reagents. An immortalized human keratinocyte cell line, HaCaT, was cultured in DMEM supplemented with 10% FBS and 100 U/m*l* penicillin-streptomycin in a humidified CO_2 incubator. HaCaT keratinocytes were provided by Prof. Moon Je Cho (Department of Biochemistry, Jeju National University, Korea).

Recombinant human interferon-γ (hIFN-γ) and human tumor necrosis factor-α (hTNF-α) were obtained from GIBCO (Grand Island, NY). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and RPMI1640 were obtained from GIBCO. Dinitrochlorobenzene (DNCB) was purchased from the Tokyo chemical industry (Japan). Concanavalin A (ConA) was purchased from Sigma (St. Louis, MO). Enzyme-linked immunosorbent assay (ELISA) kits for human TARC and MDC or mouse IL-4 and IFN-γ were obtained from R&D Systems (St. Louis, MO). TARC/CCL17 and MDC/CCL22 primers were obtained from Bioneer (Korea). β-actin primers were purchased from Bionex (Korea). Antibodies against phospho-STAT1, STAT1, and β-actin were purchased from Cell Signaling (Beverly, MA), Becton Dickinson (San Diego, CA), and Sigma respectively. All other reagents were of reagent grade.

Preparation of C. unshiu extract. The dried powder (37.95 kg) of *C. unshiu* was extracted with 80% ethanol (475 *l*) for 4 hr at 60°C and the extract was incrassated by rotary evaporator for 3 hr. To remove the ethanol, water (20 *l*) and extract were mixed and incrassated again. Subsequently, extracted liquid was filtered through filter paper and frozen on a freezing tray for 48 hr. Freeze drying for 60 hr afforded a perfectly dried extract (7.95 kg) of *C. unshiu* which was dissolved in EtOH for experiments.

Experimental animals and DNCB application. Female, 7-week-old SKH1-hairless mice (25 ± 2 g) were purchased from Orient Bio (Korea) and were maintained for 1 week before the start of any experiments. These mice were housed in a conventional animal room under controlled temperature (23 ± 1°C), humidity (60 ± 10%) and light (light on from 08:00 to 20:00 hr). They were fed a standard laboratory diet. Water was given ad libitum. Mice were divided into four groups (normal, induction, positive control, and CU groups; n = 5/group). The whole dorsal skins of 15 mice were sensitized to DNCB by application of 100 μ*l* of 1% DNCB in acetone (day −7). Seven days later, 100 μ*l* of 0.5% DNCB was applied to the dorsal skin (day 0). The application was repeated every 2 days for up to 36 days. EtOH extract of premature *C. unshiu* (CU) was applied at 2-day intervals from day 16. For a positive control group, HYDCORT cream (Green Cross, Korea) containing 2 mg/g hydrocortisone valerate was used. This animal study was approved by the Animal Care and Use Committee at Jeju National University.

Measurement of Skin-fold thickness and histological analysis. Skin-fold thickness was measured with a Digital Thickness Gauge (Mitutoyo, Japan) by pulling up the skin from shoulder to hip. Mice were sacrificed at the end of the experiment. The skin biopsies were fixed with 10% buffered formalin and then embedded in paraffin. Paraffin sections (3 μm each) were stained with hematoxylin and eosin solution for detecting skin histological feature and various inflammatory cells and toluidine blue solution for detecting mast cells.

Spleen cell culture. Mice from each group were sacrificed by cervical dislocation and their spleens were removed aseptically. To produce single-cell suspensions, spleens were forced through wire gauzes (70 μm pore size) using the plunger from a 5 m*l* syringe. Red blood cells (RBC) were removed using RBC lysis buffer. After washing, the splenocytes were seeded (5.0×10^6 cells/m*l*) using trypan blue assays and incubated in the presence of concanavalin A (2 μg/m*l*) without any drugs for 6 days. Following incubation, the supernatants were collected to determine levels of IL-4 and IFN-γ.

Cell viability assay. Cell viability was determained using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assays. Briefly, HaCaT cells were stimulated with IFN-γ and TNF-α in the absence or presence of extract

of premature *C. unshiu*. After incubating for 24 hr, cells were treated with 20 μ*l* MTT for 4 hr. The formazan precipitate was dissolved in 200 μ*l* of dimethyl sulphoxide (DMSO) for 30 min, and the absorbance of the contents of each well was measured at 540 nm using a microplate reader.

ELISA. Production of IL-4, IFN-γ, TARC and MDC proteins in the supernatant were measured with ELISA kits according to the manufacturer's instructions.

Extraction of total RNA and RT-PCR. Total RNA was isolated using easy-Blue™ Total RNA Extraction kit (iNtRON Biotechnology, Korea) according to the manufacturer's instructions. Reverse transcription was performed using a First-Strand cDNA Synthesis kit (Promega). Briefly, total RNA (1 μg) was incubated with oligo$(dT)_{18}$ primer at 70°C for 5 min and cooled on ice for 5 min. After addition of the RT premix, reactions were incubated at 42°C for 60 min. Reactions were terminated by raising the temperature to 70°C for 15 min. The PCR reaction was conducted using i-Taq™ DNA polymerase (iNtRON Biotechnology) with the appropriate sense and antisense primers for TARC, MDC, and β-actin. The primers used in this experiment are shown in Table 1. PCR was performed using a C1000 (Bio-Rad, Hercules, CA) and thermal cycling conditions were set to 94°C for 30 sec, annealing at 55~60°C for 30 sec, and extending at 72°C for 2 min, repeated 30~35 times, and followed by incubation at 72°C for 10 min. The reaction products were visualized by electrophoresis on a 1.2% agarose gel (Promega) and UV light illumination after staining with ethidium bromide. The relative intensity was analyzed using Quantity One software version 4.2.1.

SDS-PAGE and western blot analysis. HaCaT cells were washed twice with ice-cold PBS and then disrupted in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 % Nonident P-40, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 25 μg/m*l* leupeptin] on ice for 30 min. The cell lysates were centrifuged at 15,000 rpm for 15 min at 4°C and supernatants were used for western blotting. Total protein concentration of each sample was quantified by the Bio-Rad assay method (Bio-Rad, Hercules, CA). Extracts containing 30 μg of protein were separated on an 8% sodium dodecylsulfate (SDS) polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with blocking buffer (5% skim milk in TTBS) for 2 hr at room temperature, followed by incubation with rabbit anti-p-STAT1 (1 : 1000), mouse anti-STAT1 (1 : 1000), and mouse anti-β-actin (1 : 2500) antibodies overnight at 4°C. All antibodies were diluted in 1% BSA in TTBS buffer. After washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG and anti-rabbit IgG diluted 1 : 5000 for 1 hr at RT. The result was visualized with a western blot detection system (iNtRON Biotechnology, Korea) according to the manufacturer's instructions.

Statistical analysis. Quantity One version 4.2.1 and Image-Pro plus version 4.5 software were used to transform images into numerical values. Student's t-test and two-way analysis of variance were used to determine the statistical significance of differences between values for the experimental and control groups. Data represent the mean ± standard error.

RESULTS

Extract of premature C. unshiu reduced symptoms in DNCB-challenged hairless mice. We identified the effect of premature *C. unshiu* using DNCB-challenged AD-like mice. To the naked eye, 3% *C. unshiu* application resulted, on day 36, in a reduction of symptoms such as wrinkles and redness in the dorsal skin of DNCB-treated mice (Fig. 1A~D). Histopathological analysis was done using H&E staining and toluidine blue staining on day 36. In the Normal group, no histopathological change was observed. In the DNCB-treated group, there was marked hyperkeratosis and epidermal hyperplasia. DNCB-induced histological abnormalities were reduced in DNCB-treated mice given 3% *C. unshiu* (Fig. 1E~H, I~L). Also, the skin-fold thickness of the CU group was significantly reduced compared to the Induction group (Fig. 2).

Extract of premature C. unshiu reduced the production of Th1 and Th2 cytokines by splenocytes from DNCB-challenged mice. Next, we analyzed the effect of premature *C. unshiu* on the production of cytokines induced

Table 1. The sequence of primers and fragment sizes of the investigated genes in RT-PCR analysis

Gene		Primer sequences	Fragment siz (bp)
TARC	Sense	5'-ATGGCCCCACTGAAGATGCT-3'	351
	Antisense	5'-TGAACACCAACGGTGGAGGT-3'	
MDC	Sense	5'-GCATGGCTCGCCTACAGACT-3'	497
	Antisense	5'-GCAGGGAGGGAGGCAGAGGA-3'	
β-actin	Sense	5'-ATGGGTCAGAAGGATT-CCTATG-3'	588
	Antisense	5'-CAGCTCGTAGCTC-TTCTCCA-3'	

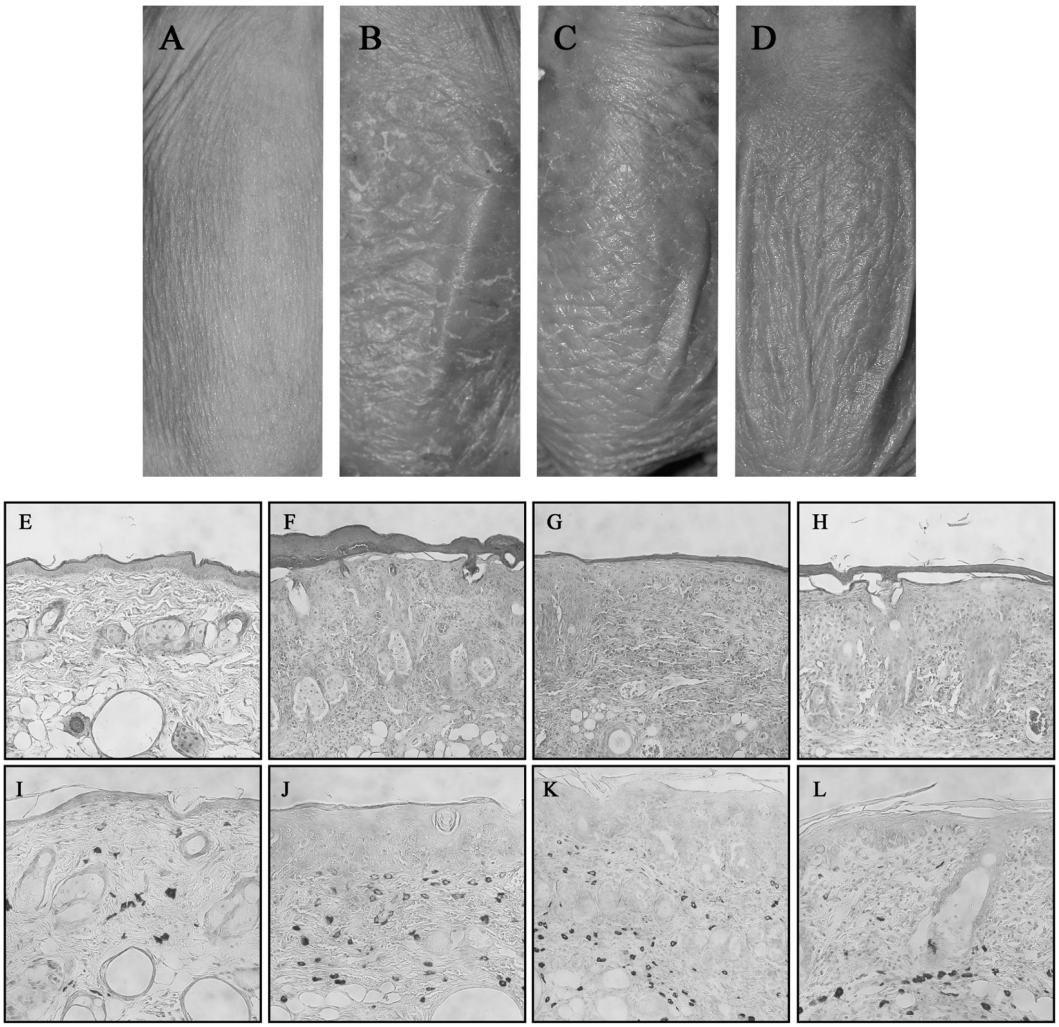

Fig. 1. Direct visual observations of skin surface features (A~D), histological features of skin tissue stained with hematoxylin and eosin (×200) (E~H) or toluidine blue (×200) (I~L) in DNCB-challenged hairless mice (Day 36) treated with *Citrus unshiu* (CU). A, E, I: Normal; B, F, J: Induction; C, G, K: Positive cont.; D, H, L: 3% CU.

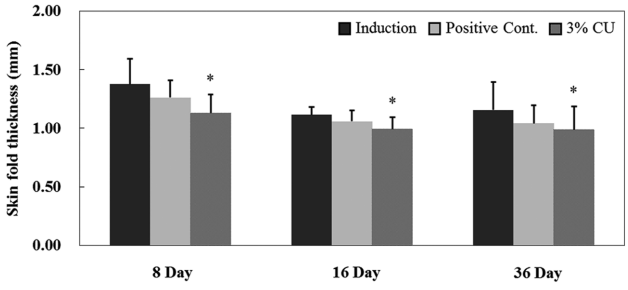

Fig. 2. Effect of premature *Citrus unshiu* on skin-fold thickness in DNCB-challenged hairless mice. Each bar graph represents mean ± SD for five mice. *, $P < 0.05$.

by modulation of the Th1 and Th2 cytokine milieu in splenocytes isolated from DNCB-challenged mice. In the presence of ConA, levels of IL-4 (22.4 ± 1.4 pg/m*l*) and IFN-γ (227.6 ± 15.8 pg/m*l*) was increased in splenocytes from the Induction group. However, EtOH extract of premature *C. unshiu* reduced the production of IL-4 (2.64 ± 0.9 pg/m*l*) and IFN-γ (no detection). HYDCORT cream, which was used as a positive control, also reduced levels of both cytokines, although the decrease in IL-4 by HYDCORT cream was not statistically significant (Fig. 3).

Extract of premature C. unshiu inhibited expression of mRNA and protein for TARC and MDC in IFN-γ and TNF-α-stimulated HaCaT cells. To identify the effect of premature *C. unshiu* on cell viability in IFN-γ and TNF-α stimulated HaCaT human keratinocytes, cell viability was examined using MTT assay. As shown in Fig. 4, premature *C. unshiu* extract had no effect on the viability of HaCaT cells. EtOH extract of premature *C. unshiu* did inhibit mRNA levels of TARC and MDC (Fig. 5 & 6). Treatment with

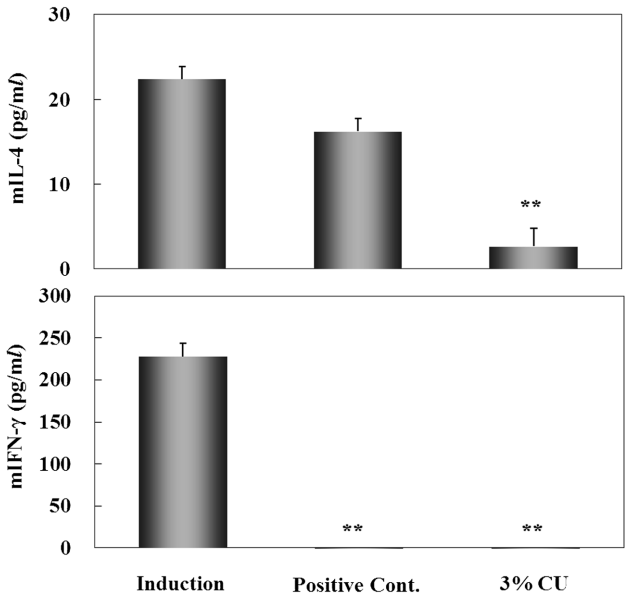

Fig. 3. Effect of premature *Citrus unshiu* on mouse IL-4 or IFN-γ production by ConA-stimulated splenocytes from each experimental group. Mouse splenocytes were isolated from spleens of each group. Cells (5.0×10^5 cells/m*l*) were then pre-incubated for 24 hr and stimulated with ConA (2 μg/m*l*) for 6 days. Mouse IL-4 and IFN-γ production from splenocytes were determined using ELISA. **, $P < 0.005$.

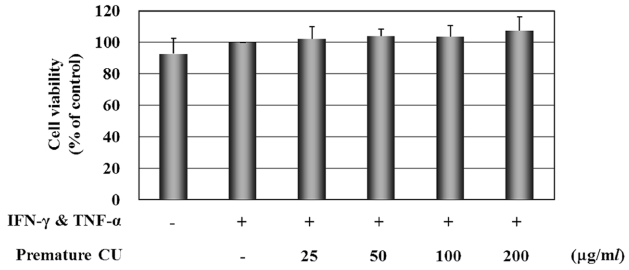

Fig. 4. Effect of premature *Citrus unshiu* on cell viability in IFN-γ and TNF-α stimulated HaCaT human keratinocytes. HaCaT cells (3.0×10^5 cells/m*l*) were pre-incubated for 18 hr. Cells were then stimulated with IFN-γ (10 ng/m*l*) and TNF-α (10 ng/m*l*) in the presence of premature CU (25, 50, 100, or 200 μg/m*l*) for 24 hr. Cell viability was analyzed by MTT assay. The error bars indicate standard deviation. Each measurement was performed in triplicate.

EtOH extract of premature *C. unshiu* dose-dependently inhibited the induction of TARC and MDC protein (Fig. 7) in supernatants from cultured HaCaT cells that had been stimulated with IFN-γ and TNF-α for 24 hr.

Extract of premature C. unshiu reduced TARC and MDC expression through inhibition of STAT1 phosphorylation in IFN-γ and TNF-α-stimulated HaCaT cells. Various cytokines act through signaling pathway of their receptors.

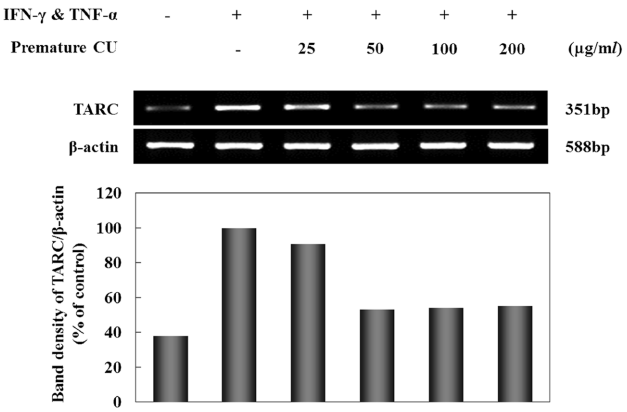

Fig. 5. Effect of premature *Citrus unshiu* on mRNA expression of TARC in HaCaT human keratinocytes. HaCaT cells (5.0×10^5 cells/m*l*) were pre-incubated for 18 hr. mRNA expression of the TARC was determined from the 18 hr culture of cells stimulated by IFN-γ (10 ng/m*l*) and TNF-α (10 ng/m*l*) in the presence of premature CU in a dose-dependent manner (25, 50, 100, or 200 μg/m*l*).

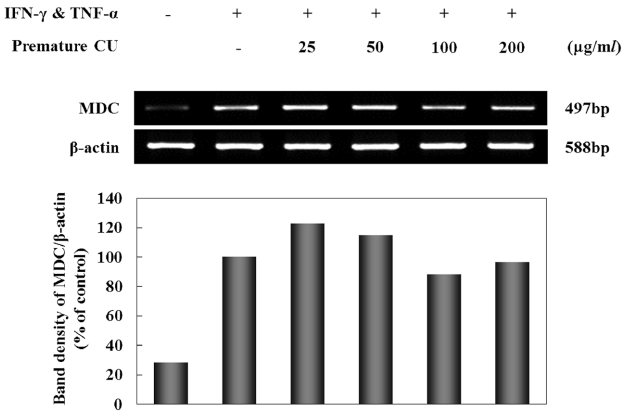

Fig. 6. Effect of premature *Citrus unshiu* on mRNA expression of MDC in HaCaT human keratinocytes. HaCaT cells (5.0×10^5 cells/m*l*) were pre-incubated for 18 hr. mRNA expression of the MDC was determined from the 18 hr culture of cells stimulated by IFN-γ (10 ng/m*l*) and TNF-α (10 ng/m*l*) in the presence of premature CU in a dose-dependent manner (25, 50, 100, or 200 μg/m*l*).

IFN-γ stimulates the activation of its receptor and the phosphorylation of Jaks that are combined with receptor. Subsequently, STAT1 phosphorylated by Jaks is translocated from cytosol to nucleus and acts as a transcription factor. TARC and MDC production in IFN-γ and/or TNF-α-stimulated HaCaT cells was increased by activation of Jak-STAT signaling (Ju *et al*., 2009; Qi *et al*., 2009, 2011). Therefore, we examined whether EtOH extract of premature *C. unshiu* inhibited phosphorylation of STAT1 in HaCaT cells. We found that IFN-γ and TNF-α-stimulation induced the phosphorylation of STAT1 in HaCaT cells after 30 min; EtOH

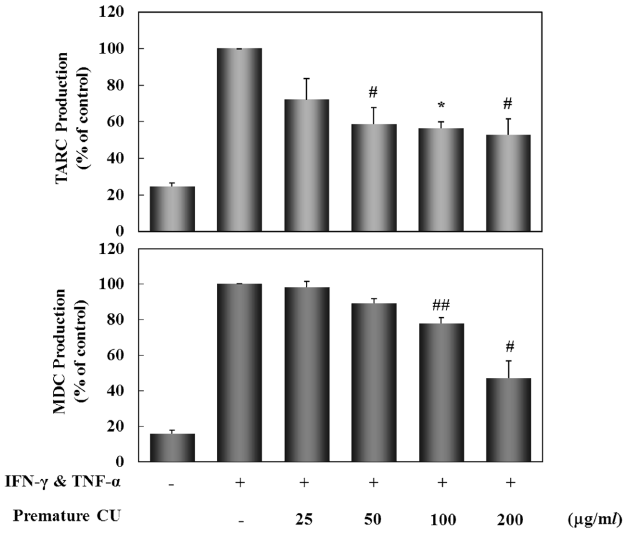

Fig. 7. Effect of premature *Citrus unshiu* on IFN-γ and TNF-α induced TARC and MDC protein productions in HaCaT human keratinocytes. HaCaT cells (3.0×10^5 cells/ml) were pre-incubated for 18 hr and TARC and MDC expression were determined from the culture supernatant of cells stimulated by IFN-γ (10 ng/ml) and TNF-α (10 ng/ml) with the indicated concentrations of premature CU (25, 50, 100, or 200 μg/ml) for 24 hr. Protein levels of TARC and MDC were measured by ELISA. The error bars indicate standard deviation. #, $P < 0.05$; ##, $P < 0.01$; *, $P < 0.005$.

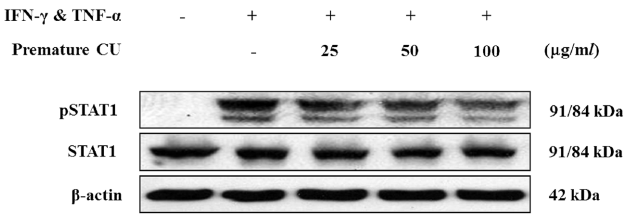

Fig. 8. Effect of premature *Citrus unshiu* on the phosphorylation of STAT1 by IFN-γ and TNF-α in HaCaT human keratinocytes. HaCaT cells (5×10^5 cells/ml) were pre-incubated for 18 hr. The cells were pre-treated with premature CU over a range of doses (25, 50, 100 μg/ml) for 2 hr and stimulated by IFN-γ (10 ng/ml) and TNF-α (10 ng/ml) for 30 min. Levels of phosphorylated or total proteins (pSTAT1 & STAT1) were determined using Western blotting.

extract of premature *C. unshiu* dose-dependently inhibited STAT1 phosphorylation (Fig. 8).

DISCUSSION

Extract of premature *C. unshiu* not only inhibited the production of TARC and MDC in IFN-γ and TNF-α-stimulated HaCaT keratinocytes, but also, it reduced symptoms in our animal model of AD. *C. unshiu* is one of the most popular fruits in the world. The dried peel has been used in medicinal preparations in oriental medicine. There are many studies that show that components of *C. unshiu* are changed according to harvest date and that the premature fruit contains a higher percentage of flavonoids than the mature fruit (Kang *et al*., 2005; Kim *et al*., 2009). Premature *C. unshiu* contains flavonoids such as naringin, hesperidin, neohesperidin, narirutin, nobiletin, etc., that are known to have anti-inflammation, anti-oxidant, anti-diabetes, and anti-allergy effects *in vitro* and *in vivo* (Choi *et al*., 2007; Harasstani *et al*., 2010; Itoh *et al*., 2009; Lee *et al*., 2010; Murakami *et al*., 2000; Sakata *et al*., 2003).

Of various animal models, TNCB and DNCB-stimulated mice show diverse symptoms that are similar to those of AD, such as skin infiltration of inflammatory cells, and increases in mast cell numbers and in serum IgE levels (Kitagaki *et al*., 1995; Matsumoto *et al*., 2004; Qi *et al*., 2009). Therefore, we used the DNCB-challenged hairless mice model to identify the effects of *C. unshiu in vivo*. To the naked eye, symptoms like hyperkeratosis, redness, and increases in skin thickness were reduced in DNCB-treated mice by treatment with premature *C. unshiu*. Furthermore, histological observation indicated that *C. unshiu* decreased the hyperkeratosis and the infiltration of inflammatory cells into the dermis of lesional skin. In general, depending on the disease phase of AD, the cytokine milieu is known to change during the acute phase, including increases in Th2 cell cytokines such as IL-4, IL-5 and IL-13; and during the chronic phase to Th1/0 cell cytokines such as IFN-γ, IL-12 and GM-CSF (Bonness and Bieber, 2007). Hence, we identified the effect of premature *C. unshiu* extract on production of IL-4 and IFN-γ in splenocytes stimulated with ConA, a T cell specific antigen. As Fig. 3 shows, productions of both cytokines were inhibited by extract of premature *C. unshiu.* This shows that premature *C. unshiu* may reduce AD symptoms by direct control of T cell activation.

AD is an inflammatory skin disease and is distinguished by infiltration of numerous inflammatory cells in to the site of inflammation (Abramovits, 2005; Bonness and Bieber, 2007). Several chemokines have been reported to participate in leukocyte recruitment to the lesional skin. Two of them, TARC and MDC, which are chemokines specific for Th2 cells, were increased in the serum of AD patients (Hijnen *et al*., 2004; Kakinuma *et al*., 2002; Leung *et al*., 2003; Nakazato *et al*., 2008). Therefore, we investigated the inhibitory effects of *C. unshiu* extract on levels of TARC and MDC in the IFN-γ and TNF-α-stimulated HaCaT keratinocytes. Premature *C. unshiu* suppressed mRNA expression and protein production of TARC and MDC (Fig. 5~7). Premature *C. unshiu* may therefore regulate the recruitment of Th2-type cells into lesions of AD by suppressing production of inflammatory chemokines related to AD.

IFN-γ acts through IFNGR1 and R2 and then activates JAK/STAT, ERK, p38 MAPK, and NF-κB pathways (Gough

et al., 2008). Ju *et al*. found that TARC production from IFN-γ and TNF-α stimulated HaCaT keratinocytes was decreased by treatment with Jak/STAT and NF-κB inhibitors. Qi *et al*. found that TARC and MDC production in IFN-γ stimulated HaCaT keratinocytes were inhibited by Jak inhibitor I, SB203580, and Bay11-7082, inhibitors of Jak1/2, p38 or NF-κB, respectively. The Janus family of tyrosine kinases (Jaks) is an integral factor in IFN-γ activated signaling cascades and regulates tyrosine phosphorylation of STAT proteins. STAT protein translocation to the nucleus and binding to IFN-γ activated sequences (GAS) is a crucial component of IFN-γ signaling (Gough *et al*., 2008; Saha *et al*., 2010). Therefore, we examined the effects of premature *C. unshiu* on the phosphorylation of STAT1 by stimulation of IFN-γ and TNF-α. Extract of premature *C. unshiu* inhibited STAT1 phosphorylation after IFN-γ and TNF-α treatment at 30 min in a dose-dependent manner. However, further investigation is needed to determine whether the inhibitory effect of premature *C. unshiu* on STAT1 phosphorylation occurs through inhibition of Jak1/2 or through effects on other pathway components such as ERK, p38, and NF-κB. Also, we need to explore the effect of premature *C. unshiu* on suppressor of cytokine signaling proteins 1 (SOCS1) that is known to be a negative regulator and is an important component of the IFN-γ signaling pathway (Yoshimura *et al*., 2007).

In summary, premature *C. unshiu* moderates symptoms in DNCB-treated mice and suppresses the expression of TARC and MDC in HaCaT keratinocytes by suppressing STAT1 activation. The biological effects of premature *C. unshiu* confirmed in this study indicate that an extract of premature *C. unshiu* or a preparation of its components might be useful as an anti-atopic agent.

REFERENCES

Abramovits, W. (2005). Atopic dermatitis. *J. Am. Acad. Dermatol*., **53**, S86-93.

Bonness, S. and Bieber, T. (2007). Molecular basis of atopic dermatitis. *Curr. Opin. Allergy Clin. Immunol*., **7**, 382-386.

Choi, I.Y., Kim, S.J., Jeong, H.J., Park, S.H., Song, Y.S., Lee, J.H., Kang, T.H., Park, J.H., Hwang, G.S., Lee, E.J., Hong, S.H., Kim, H.M. and Um, J.Y. (2007). Hesperidin inhibits expression of hypoxia inducible factor-1 alpha and inflammatory cytokine production from mast cells. *Mol. Cell Biochem*., **305**, 153-161.

Gough, D.J., Levy, D.E., Johnstone, R.W. and Clarke, C.J. (2008). IFNgamma signaling-does it mean JAK-STAT? *Cytokine. Growth Factor Rev*., **19**, 383-394.

Harasstani, O.A., Moin, S., Tham, C.L., Liew, C.Y., Ismail, N., Rajajendram, R., Harith, H.H., Zakaria, Z.A., Mohamad, A.S., Sulaiman, M.R. and Israf, D.A. (2010). Flavonoid combinations cause synergistic inhibition of proinflammatory mediator secretion from lipopolysaccharide-induced RAW 264.7 cells. *Inflamm. Res*., **59**, 711-721.

Hijnen, D., De Bruin-Weller, M., Oosting, B., Lebre, C., De Jong, E., Bruijnzeel-Koomen, C. and Knol, E. (2004). Serum thymus and activation-regulated chemokine (TARC) and cutaneous T cell- attracting chemokine (CTACK) levels in allergic diseases: TARC and CTACK are disease-specific markers for atopic dermatitis. *J. Allergy Clin. Immunol*., **113**, 334-340.

Imai, T., Nagira, M., Takagi, S., Kakizaki, M., Nishimura, M., Wang, J., Gray, P.W., Matsushima, K. and Yoshie, O. (1999). Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *Int. Immunol*., **11**, 81-88.

Itoh, K., Masuda, M., Naruto, S., Murata, K. and Matsuda, H. (2009). Antiallergic activity of unripe Citrus hassaku fruits extract and its flavanone glycosides on chemical substance-induced dermatitis in mice. *J. Nat. Med*., **63**, 443-450.

Jin, H., He, R., Oyoshi, M. and Geha, R.S. (2009). Animal models of atopic dermatitis. *J. Invest. Dermatol*., **129**, 31-40.

Ju, S.M., Song, H.Y., Lee, S.J., Seo, W.Y., Sin, D.H., Goh, A.R., Kang, Y.H., Kang, I.J., Won, M.H., Yi, J.S., Kwon, D.J., Bae, Y.S., Choi, S.Y. and Park, J. (2009). Suppression of thymus- and activation-regulated chemokine (TARC/CCL17) production by 1,2,3,4,6-penta-O-galloyl-beta-D-glucose via blockade of NF-kappaB and STAT1 activation in the HaCaT cells. *Biochem. Biophys. Res. Commun*., **387**, 115-120.

Kakinuma, T., Nakamura, K., Wakugawa, M., Mitsui, H., Tada, Y., Saeki, H., Torii, H., Komine, M., Asahina, A. and Tamaki, K. (2002). Serum macrophage-derived chemokine (MDC) levels are closely related with the disease activity of atopic dermatitis. *Clin. Exp. Immunol*., **127**, 270-273.

Kang, Y.J., Yang, M.H., Ko, W.J., Park, S.R. and Lee, B.G. (2005). Studies on the major components and antioxidative properties of whole fruit powder and juice prepared from premature mandarin orange. *Korean J. Food Sci. Technol*., **37**, 783-788.

Kim, Y.D., Mahinda, S., Koh, K.S., Jeon, Y.J. and Kim, S.H. (2009). Reactive Oxygen Species Scavenging Activity of Jeju Native Citrus Peel during Maturation. *J. Korean Soc. Food Sci. Nutr.*, **38**, 462-469.

Kitagaki, H., Fujisawa, S., Watanabe, K., Hayakawa, K. and Shiohara, T. (1995). Immediate-type hypersensitivity response followed by a late reaction is induced by repeated epicutaneous application of contact sensitizing agents in mice. *J. Invest. Dermatol*., **105**, 749-755.

Lee, E.J., Ji, G.E. and Sung, M.K. (2010). Quercetin and kaempferol suppress immunoglobulin E-mediated allergic inflammation in RBL-2H3 and Caco-2 cells. *Inflamm. Res*., **59**, 847-854.

Lee, S.H., Heo, Y. and Kim, Y.C. (2010). Effect of German chamomile oil application on alleviating atopic dermatitis-like immune alterations in mice. *J. Vet. Sci*., **11**, 35-41.

Leung, T.F., Ma, K.C., Hon, K.L., Lam, C.W., Wan, H., Li, C.Y. and Chan, I.H. (2003). Serum concentration of macrophage-derived chemokine may be a useful inflammatory marker for assessing severity of atopic dermatitis in infants and young children. *Pediatr. Allergy Immunol*., **14**, 296-301.

Matsumoto, K., Mizukoshi, K., Oyobikawa, M., Ohshima, H., Sakai, Y. and Tagami, H. (2005). Objective evaluation of the efficacy of daily topical applications of cosmetics bases using the hairless mouse model of atopic dermatitis. *Skin Res. Technol*., **11**, 209-217.

Matsumoto, K., Mizukoshi, K., Oyobikawa, M., Ohshima, H. and

Tagami, H. (2004). Establishment of an atopic dermatitis-like skin model in a hairless mouse by repeated elicitation of contact hypersensitivity that enables to conduct functional analyses of the stratum corneum with various non-invasive biophysical instruments. *Skin Res. Technol*., **10**, 122-129.

Murakami, A., Nakamura, Y., Torikai, K., Tanaka, T., Koshiba, T., Koshimizu, K., Kuwahara, S., Takahashi, Y., Ogawa, K., Yano, M., Tokuda, H., Nishino, H., Mimaki, Y., Sashida, Y., Kitanaka, S. and Ohigashi, H. (2000). Inhibitory effect of citrus nobiletin on phorbol ester-induced skin inflammation, oxidative stress, and tumor promotion in mice. *Cancer Res*., **60**, 5059-5066.

Nakazato, J., Kishida, M., Kuroiwa, R., Fujiwara, J., Shimoda, M. and Shinomiya, N. (2008). Serum levels of Th2 chemokines, CCL17, CCL22, and CCL27, were the important markers of severity in infantile atopic dermatitis. *Pediatr. Allergy Immunol*., **19**, 605-613.

Pease, J.E. and Williams, T.J. (2006). Chemokines and their receptors in allergic disease. *J. Allergy Clin. Immunol*., **118**, 305-318.

Qi, X.F., Kim, D.H., Yoon, Y.S., Li, J.H., Jin, D., Deung, Y.K. and Lee, K.J. (2009). Effects of Bambusae caulis in Liquamen on the development of atopic dermatitis-like skin lesions in hairless mice. *J. Ethnopharmacol*., **123**, 195-200.

Qi, X.F., Teng, Y.C., Yoon, Y.S., Kim, D.H., Cai, D.Q. and Lee, K.J. (2011). Reactive oxygen species are involved in the IFN-gamma-stimulated production of Th2 chemokines in HaCaT keratinocytes. *J. Cell Physiol*., **226**, 58-65.

Saha, B., Jyothi Prasanna, S., Chandrasekar, B. and Nandi, D. (2010). Gene modulation and immunoregulatory roles of interferon gamma. *Cytokine*., **50**, 1-14.

Sakata, K., Hirose, Y., Qiao, Z., Tanaka, T. and Mori, H. (2003). Inhibition of inducible isoforms of cyclooxygenase and nitric oxide synthase by flavonoid hesperidin in mouse macrophage cell line. *Cancer Lett*., **199**, 139-145.

Vestergaard, C., Yoneyama, H., Murai, M., Nakamura, K., Tamaki, K., Terashima, Y., Imai, T., Yoshie, O., Irimura, T., Mizutani, H. and Matsushima, K. (1999). Overproduction of Th2-specific chemokines in NC/Nga mice exhibiting atopic dermatitis-like lesions. *J. Clin. Invest*., **104**, 1097-1105.

Yoshimura, A., Naka, T. and Kubo, M. (2007). SOCS proteins, cytokine signalling and immune regulation. *Nat. Rev. Immunol*., **7**, 454-465.