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A natural dye, Niram improves atopic dermatitis through down-regulation of TSLP

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ABSTRACT

Naju Jjok (*Polygonum tinctorium* Lour.) has been known to treat skin diseases in traditional Korean medicine. A natural textile dye, Niram made from Naju Jjok has traditionally been used to dye clothes. Thymic stromal lymphopoietin (TSLP) plays an important role in the development of atopic dermatitis (AD). Thus, we investigated that Niram might ameliorate AD through regulation of TSLP. Niram significantly inhibited the levels of TSLP through blockade of caspase-1/receptor-interacting protein 2 pathway in stimulated mast cells. Further, Niram ameliorated clinical symptoms in AD mouse. Niram significantly inhibited the infiltration of inflammatory cells in lesional skin. The levels of TSLP, caspase-1, IL-4, and IL-6 were inhibited in lesional skin applied topically with Niram. Niram significantly inhibited the serum levels of IgE and histamine in AD mouse. Finally, Niram significantly inhibited the levels of TSLP in polyriboinosinic polyribocytidylic acid-stimulated human keratinocyte HaCaT cells. These results establish Niram as a functional dye embracing the aspects of not only a traditional use but also a pharmacological effect.

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1. Introduction

Atopic dermatitis (AD) is an increasingly common inflammatory skin disorder that affects at least 15% of children and is characterized by cutaneous hyperreactivity to environmental triggers (Jin et al., 2009). The increase in the incidence of AD in developed countries has been related to familiar and environmental factors (Sebök et al., 2006).

There are many factors known to worsen the disease, including climatic factors and chemical or physical irritants (Ricci et al., 2004). Clothes can be a protective skin barrier against persistent scratching, allowing more rapid improvement of the eczematous lesions (Ricci et al., 2012). However, lymphomatoid dermatitis resulted from contact with textile dyes (Narganes et al., 2013). Contact urticaria resulted from a fluorescent dye in clothing (Sugiura et al., 2010).

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Chronic lesions of AD are characterized by diffuse epidermal hyperplasia and perivascular infiltration of mast cells (Kawakami et al., 2009). Mast cells have been understood as the key effector cell type in IgE-mediated immediate hypersensitivity and allergic disorders (Galli, 2000). The activated mast cells secrete various cytokines that are relevant in chronic skin inflammation (Harvima and Nilsson, 2011). The activated mast cells have highly expressed thymic stromal lymphopoietin (TSLP) and triggered allergic inflammation (Liu, 2006). Also, TSLP produced from keratinocytes aggravated a hyperreactive immune state in AD skin lesions (Kubo et al., 2014). TSLP has been linked to the pathogenesis of AD (Soumelis et al., 2002).

Caspase-1 is considered an important target to control inflammatory diseases (Cunha et al., 2010). Receptor interacting protein 2 (RIP2) is a caspase recruitment domain-containing kinase that interacts with caspase-1 and plays an important role in NF- κ B activation (Sarkar et al., 2006). Transgenic mice over-expressing caspase-1 displayed high serum levels of IgE and spontaneously develop chronic dermatitis (Nakano et al., 2003). The level of TSLP was increased through caspase-1 and RIP2 signal pathway in mast cells (Song et al., 2012).

Naju Jjok (*Polygonum tinctorium* Lour.) has been known to treat skin diseases in traditional Korean medicine. Niram made from Naju Jjok has traditionally been used as a natural textile dye in Korea. However, the protective effect of Niram on AD has not yet been clarified. Therefore, we investigated the inhibitory effect of Niram on phorbol myristate acetate plus calcium ionophore A23187 (PMACI)-stimulated human mast cell line (HMC-1) cells *in vitro*, 2,4-dinitrofluorobenzene (DNFB)-induced AD-like lesional dorsal skin of NC/Nga mice *in vivo*, and polyriboinosinic polyribocytidylic acid (poly(I:C))-stimulated human keratinocyte HaCaT cells *in vitro*.

2. Materials and methods

2.1. Reagents

We purchased Isocove's Modified Dulbecco's Medium (IMDM) and Dulbecco's Modified Eagle Medium (DMEM) from Gibco BRL (Grand Island, NY, USA); PMA, calcium ionophore A23187, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DNFB, and poly(I:C) from Sigma Chemical Co (St. Louis, MO, USA); TSLP antibody from R&D Systems (Minneapolis, MN, USA); RIP2, caspase-1, and actin antibodies from Santa Cruz Biotechnology (Santacruz, CA, USA); IL-4, IL-6, and IgE antibodies from BD Pharmingen (Torreyana Road, San Diego, CA, USA).

2.2. Preparation of Niram

Shin et al. (2012) indicated the crude Niram preparation. Briefly, Naju Jjok was dipped in water for 3 days. And then Naju Jjok was taken out of water. Lime was put in the water and the water was stirred until the color was changed into indigo blue. After indigo dye sank, supernatant which was changed clearly was drained. The indigo dye sunk like mud is named Niram. Niram was kindly provided from Naju city, Republic of Korea. We used liquid produced from this indigo dye. It was

lyophilized and reduced to powder. It was dissolved in DW and filtered with 0.22 μ m syringe filter.

2.3. Cells culture

HMC-1 cells were incubated in IMDM supplemented with 100 units/ml of penicillin, 100 μ g/ml of streptomycin, and 10% fetal bovine serum (FBS) at 37 °C in 5% CO₂ with 95% humidity. HaCaT cells were kindly provided by Prof. Sang-Hyun Kim (Kyungpook National University) and were cultured in DMEM supplemented with 100 units/ml of penicillin, 100 μ g/ml of streptomycin, and 10% FBS at 37 °C in 5% CO₂ with 95% humidity.

2.4. Cytokines assay

The levels of TSLP, IL-4, IL-6, and IgE were determined using a sandwich ELISA method according to the manufacturer's instructions (R&D Systems; BD Pharmingen).

2.5. Quantitative real time-polymerase chain reaction (PCR) analysis

Quantitative real time-PCR was performed using a SYBR Green master mix and the detection of mRNA was analyzed using an ABI StepOne real time-PCR System (Applied Biosystems, Foster City, CA, USA). Primer sequences for the reference gene GAPDH and gene TSLP were as follows: GAPDH (5' TCG ACA GTC AGC CGC ATC TTC TTT 3'; 5' ACC AAA TCC GTT GAC TCC GAC CTT 3'); TSLP (5' TAT GAG TGG GAC CAA AAG TAC CG 3'; 5' GGG ATT GAA GGT TAG GCT CTG G 3'). Typical profile times used were the initial step, 95 °C for 10 min followed by a second step at 95 °C for 10 s for 40 cycles with a melting curve analysis. The level of target mRNA was normalized to the level of the GAPDH and compared to the control. Data were analyzed using the $\Delta\Delta$ CT method.

2.6. MTT assay

Cell viability was measured by a MTT assay. HMC-1 cell (4×10^5) was treated with Niram for 2 h and stimulated with PMACI for 8 h and then harvested. HaCaT cells were (2×10^5) treated with Niram for 2 h and stimulated with poly(I:C) for 24 h and then harvested. The cell suspension containing MTT solution (5 mg/ml) was incubated at 37 °C for an additional 4 h. After washing the supernatant out, the insoluble formazan product was dissolved in dimethyl sulfoxide (DMSO). Then, the optical density of 96-well culture plate was determined using an ELISA method reader at 540 nm.

2.7. Western blot analysis

The stimulated cells were lysed and separated through 10% SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membranes and then the membranes were blocked and incubated with primary and secondary antibodies. Finally, the protein bands were visualized by an enhanced chemiluminescence assay according to the manufacturer's instructions (Amersham Co., Newark, NJ, USA).

2.8. Animals

Six-week-old male NC/Nga mice were obtained from Charles River Laboratories International, Inc. (Yokohama, Japan). And the animals were maintained under conventional condition and performed under approval from the animal care committee of Kyung Hee University [Protocol Number. KHUASP (SE)-11-009]. Mice were sacrificed with CO₂ inhalation.

2.9. Sensitization with DNFB

For active sensitization, 100 μ l 0.15% DNFB dissolved in acetone was topically applied to the shaved abdominal skin of NC/Nga mice. A week later, the shaved dorsal skin of NC/Nga mice was applied with 50 μ l 0.15% DNFB as a control group twice a week for 4 weeks (Wu et al., 2011). At that time, Niram (100 μ g/ml) was topically applied to shaved dorsal skin of DNFB-sensitized mice twice a week for 4 weeks. The same volume of acetone was applied to the shaved abdominal and dorsal skin and saline was topically applied as a vehicle group. The lesional dorsal skin and serum were obtained 4 h after the last DNFB sensitization on the basis of our previous study (Han et al., 2014b). After anesthetization, blood was withdrawn from the heart of mouse into syringes. And then, serum was prepared by centrifugation at 3400 rpm at 4 °C for 10 min.

2.10. Histological analysis

Formaldehyde (10%)-fixed dorsal skin samples were embedded in paraffin, cut into 4- μ m-thick sections. After dewaxing and dehydration, sections were stained with hematoxylin and eosin (H&E) to count the number of inflammatory cells and visualize epidermal thickness.

2.11. Clinical symptom of AD

Scratching behavioral test was evaluated by counting the number of scratching at 4 h after the last DNFB sensitization for 10 min. After counting, mice were anesthetized and taken pictures for observing clinical features.

2.12. Reverse transcription (RT)-PCR analysis

Using an easy-BLUE™ RNA extraction kit (iNtRON Biotech, Republic of Korea), we isolated the total RNA from dorsal skin samples in accordance with the manufacturer's specifications. We performed RT-PCR with the following primers: TSLP (5' TGC AAG TAC TAG TAC GGA TGG GG C 3'; 5' GGA CTT CTT GTG CCA TTT CCT GAG 3'); IL-4 (5' ACG GAG ATG GAT GTG CCA AA 3'; 5' CGA GTA ATC CAT TTG CAT GA 3'); IL-6 (5' CGG GAT CCA TGT TCC CTA CTT CAC AA 3'; 5' CCC AAG CTT CTA CGT TTG CC 3'). Reference gene GAPDH (5' GGC ATG GAC TGT GGT CAT GA 3'; 5' TTC ACC ACC ATG GAG AAG GC 3') was used to verify that equal amounts of RNA. The annealing temperature was 62 °C for TSLP and IL-4; 50 °C for IL-6; 60 °C for GAPDH. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide. The levels of each mRNA were normalized to the level of the GAPDH.

2.13. Analysis of confocal laser-scanning microscope

Dorsal skin samples were immediately fixed with 4% formaldehyde and embedded in paraffin. After dewaxing and dehydration, sections were blocked with FBS followed by 1 h of incubation and then stained with the following antibodies. Anti-rat TSLP (R&D Systems), FITC-conjugated anti-rat IgG (Sigma Chemical Co.), anti-rabbit c-Kit (Santa Cruz Biotechnology), and TRITC-conjugated anti-rabbit IgG (Sigma Chemical Co) were used to stain mast cells-derived TSLP. Anti-rabbit caspase-1 (Santa Cruz Biotechnology), FITC-conjugated anti-rabbit IgG (Santacruz Biotechnology), and PE-conjugated anti-mouse c-Kit antibodies (BD Pharmingen) were used to stain caspase-1 in mast cells. 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) containing mounting medium was used to counterstain the nuclei. The images of stained cells and specimens were randomly visualized using a confocal laser-scanning microscope.

2.14. Histamine assay

Histamine in the serum was measured by the addition of perchloric acid and centrifugation at 400 $\times$ g for 5 min at 4 °C. The histamine content was measured using the o-phthalaldehyde spectrofluorometric method described by Shore et al. (1959). The fluorescent intensity was measured at 440 nm (excitation at 360 nm) in spectrofluorometer.

2.15. HPLC analysis

Niram extract and indirubin were passed through a 0.2 μ m membrane. 20 μ l aliquots of the filtrate were injected into the HPLC. Synchronis C18 (150 mm $\times$ 2.1 mm; particle size 5 μ m) was used as an analytical column. The mobile phases were composed of solvent A (DIwater) and solvent B (acetonitrile) at the rate of 10:90. The analysis was carried out at a flow rate of 0.5 ml/min. The injection volume was 10 μ l. HPLC run for 55 min. The linearity of the peak area (y) vs. concentration (x, μ g/ml) curve was used to calculate the contents of indirubin in Niram.

2.16. Statistical analysis

The results shown *in vitro* and *in vivo* experiments are a summary of the data from at least-three experiments and are presented as the mean $\pm$ SEM. Statistical evaluation of the results was performed by an independent t-test and ANOVA with Tukey post hoc test. All statistical analyses were performed using SPSS v12.00 statistical analysis software (SPSS Inc.). The results were considered significant at a value of $p < 0.05$.

3. Results

3.1. Effect of Niram on the level of TSLP in stimulated HMC-1 cells

First, we investigated whether Niram could regulate the production and mRNA expression of TSLP in mast cells. Niram

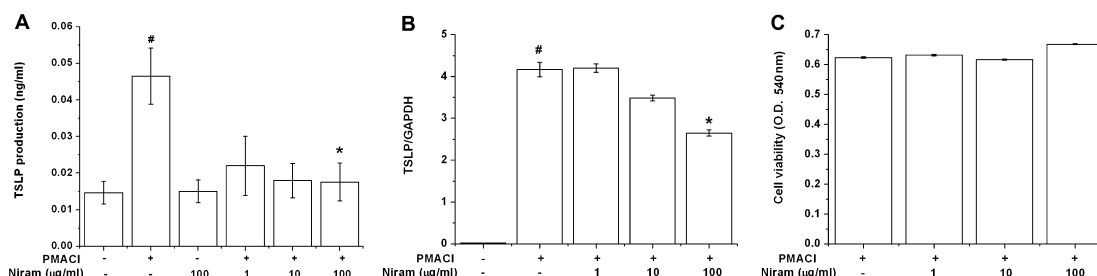


Fig. 1 – Niram inhibits the level of TSLP in stimulated HMC-1 cells. (A) HMC-1 cells (4×10^5) were pretreated with Niram (1, 10, and 100 $\mu\text{g/ml}$) for 2 h and stimulated with PMACI for 7 h. The production of TSLP was analyzed with the ELISA. (B) HMC-1 cells (1×10^6) were pretreated with Niram (1, 10, and 100 $\mu\text{g/ml}$) for 2 h and stimulated with PMACI for 5 h. The mRNA expression of TSLP was analyzed with the real time-PCR analysis. (C) Cell viability was analyzed with the MTT assay. Each datum represents the mean $\pm$ SEM of three independent experiments. [#] $p < 0.05$; significantly different from unstimulated cells. ^{*} $p < 0.05$; significantly different from PMACI-stimulated cells.

(100 $\mu\text{g/ml}$) significantly inhibited the production and mRNA expression of TSLP in PMACI-stimulated HMC-1 cells ($p < 0.05$; Fig. 1A and B). Niram (100 $\mu\text{g/ml}$) did not have influence on the production of TSLP in unstimulated HMC-1 cells. As shown in Fig. 1C, the cytotoxicity did not show at doses of 1, 10, and 100 $\mu\text{g/ml}$.

3.2. Effect of Niram on the expressions of caspase-1 and RIP2 in stimulated HMC-1 cells

Next, we investigated how Niram could regulate the level of TSLP mechanistically. Because Moon and Kim (2011) have reported that TSLP was regulated through caspase-1 signal pathway in mast cells, we analyzed the expressions of caspase-1 and RIP2 (an upstream activator of caspase-1) by Niram with Western blot analysis. Niram inhibited the activation of caspase-1 in PMACI-stimulated HMC-1 cells (Fig. 2). Niram also reduced the expression of RIP2 (Fig. 2).

3.3. Effect of Niram on clinical symptoms in DNFB-induced AD mouse model

We mimicked the traditional use of Niram as a textile dye and applied Niram (100 $\mu\text{g/ml}$) topically to DNFB-sensitized lesional dorsal skin. The conspicuous erythema and hemorrhage were present in the lesional dorsal skin; whereas the application with Niram (100 $\mu\text{g/ml}$) noticeably ameliorated these phenotypes in the lesional dorsal skin (Fig. 3A).

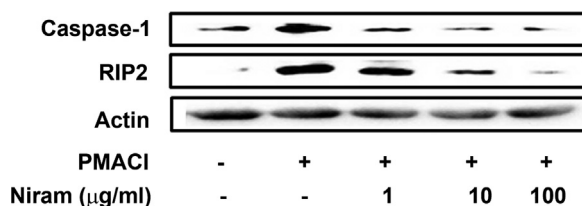


Fig. 2 – Niram regulates the expressions of caspase-1 and RIP2 in stimulated HMC-1 cells. HMC-1 cells (5×10^6) were pretreated with Niram (1, 10, and 100 $\mu\text{g/ml}$) for 2 h and stimulated with PMACI for 1 h. The expressions of caspase-1 and RIP2 were analyzed with Western blot analysis.

In addition, Niram significantly inhibited the infiltration of inflammatory cells and epidermis thickness in the lesional dorsal skin ($p < 0.05$; Fig. 3B). Niram significantly inhibited scratching behavior (Fig. 3C).

3.4. Effect of Niram on the level of TSLP and caspase-1 in DNFB-induced AD mouse model

Based on the regulatory effect of TSLP and caspase-1 by Niram *in vitro*, we investigated whether Niram also could regulate the level of TSLP and caspase-1 in the AD-like lesional dorsal skin. As shown in Fig. 4 A and B, the application with Niram inhibited the protein level and mRNA expression of TSLP in the lesional dorsal skin. Niram inhibited the expression of TSLP in mast cells from the lesional dorsal skin (Fig. 4C). Furthermore, Niram inhibited the mast cells-derived caspase-1 expression in the lesional dorsal skin (Fig. 4D).

3.5. Effect of Niram on the levels of AD-related factors in DNFB-induced AD mouse model

Finally, we investigated whether Niram could regulate AD-related factors in DNFB-induced AD mouse model. The mRNA expressions of IL-4 and IL-6 were inhibited in the lesional dorsal skin applied with Niram (Fig. 5A). Also, the protein levels of IL-4 and IL-6 were significantly inhibited by Niram ($p < 0.05$, Fig. 5B). In addition, Niram significantly inhibited the levels of serum IgE and histamine ($p < 0.05$, Fig. 5C).

3.6. Effect of Niram on the level of TSLP in stimulated HaCaT cells

Finally, we investigated whether Niram also could regulate the production and mRNA expression of TSLP in HaCaT cells. Niram (100 $\mu\text{g/ml}$) significantly inhibited the production of TSLP in poly(I:C)-stimulated HaCaT cells ($p < 0.05$; Fig. 6A). The mRNA level of TSLP was elevated, peaking 2 h following exposure to poly(I:C) in HaCaT cells ($p < 0.05$; Fig. 6B). And Niram (100 $\mu\text{g/ml}$) significantly inhibited the mRNA expression of TSLP 2 h after exposure to poly(I:C) ($p < 0.05$; Fig. 6C). The cytotoxicity did not show at all doses of Niram in stimulated HaCaT cells (Fig. 6D).

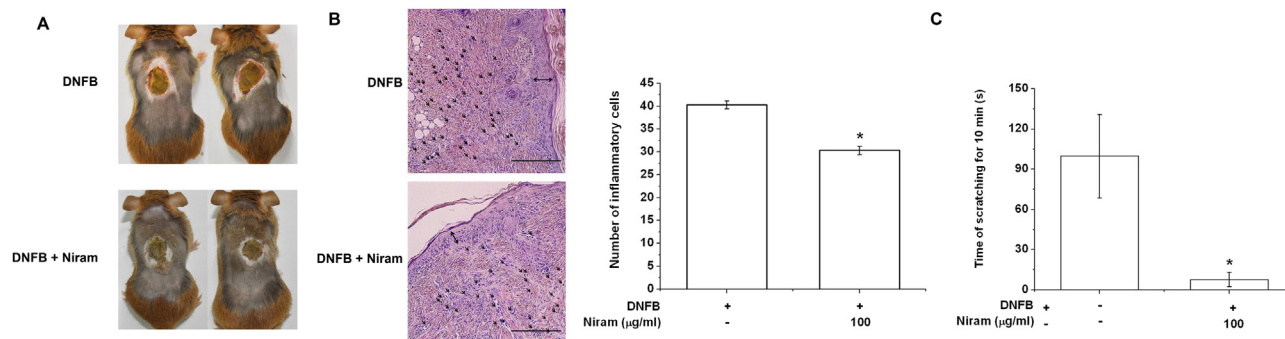


Fig. 3 – Niram ameliorates clinical symptoms in DNFB-induced AD mouse model. (A) The clinical features were observed 4 h after the last DNFB sensitization. **(B)** Histological analysis of the lesional dorsal skin was examined by H&E staining for inflammatory cells and epidermis thickness. The inflammatory cells were counted from five tissue sections ($1.5 \times 10^3 \mu\text{m}^2$) selected randomly. The inflammatory cells were indicated by arrows. Representative photomicrographs were examined at $400\times$ magnification. **(C)** The time of scratching behavior was measured at 4 h after the last DNFB sensitization for 10 min. * $p < 0.05$; significantly different from DNFB-sensitized group (control). $n = 3$.

3.7. HPLC analysis of indirubin from Niram

Finally, the indication of potential single compound was determined from Niram. We analyzed the content of indirubin from Niram through HPLC. Indirubin has reported to be a main compound from Niram (Shin et al., 2012). The retention time of the indirubin was approximately 4 min (Fig. 7). Indirubin from Niram was included about 42.3%.

4. Discussion

We determined an inhibitory effect of Naju Jjok which is the main ingredient of Niram on AD in previous study (Han et al., 2014a). And Chu and Kim (2014) reported that the number of mast cells was decreased in 2,4-dinitrochlorobenzene-induced AD-like lesional dorsal skin of BALB/c mice. Furthermore, the present study provides the additional and specific evidence that Niram has an inhibitory effect on AD by regulating the levels of TSLP in mast cells. Niram inhibited the production and mRNA expression of TSLP through the blockade of caspase-1 and RIP2 signal pathway in stimulated human mast cells. In addition, Niram inhibited the levels of TSLP, IL-4, IL-6, and caspase-1 in the lesional dorsal skin of AD mouse model. Niram inhibited the levels of serum IgE and histamine in AD mouse model. Also, Niram inhibited the levels of TSLP in stimulated keratinocytes.

The defect of the skin barrier has played an important role in the pathogenesis of AD causing the development of eczematous lesions after exposure to repeated irritants (Ricci et al., 2004). Mast cells play an important role in triggering the skin inflammation (Harvima and Nilsson, 2011). Disrupted skin barrier of patients with atopic eczema facilitated contact between mast cells in the skin and environmental triggers of the disease (Ribbing et al., 2011). Mast cells generated from patients with atopic eczema have enhanced levels of granule mediators such as histamine and IL-6 (Ribbing et al., 2011). Also, keratinocytes are involved in skin inflammation through the regulation of expression of chemokines and cytokines (Giustizieri et al., 2001). TSLP is a general biomarker for

skin-barrier defects and the expression of TSLP is sustained as long as barrier defects persist (Demehri et al., 2008). TSLP was overproduced in keratinocytes of human AD skin lesions (Soumelis et al., 2002). Furthermore, Demehri et al. (2009) have reported that the skin-derived TSLP was sufficient to trigger the atopic march, sensitizing the lung airways to inhaled allergens. Blockade of signal pathway of TSLP inhibited TSLP-driven Th2 inflammatory cell infiltration, cytokine secretion, and IgE production (Seshasayee et al., 2007). In this study, Niram inhibited the production and mRNA expression of TSLP in stimulated human mast cells. Niram inhibited the mRNA expression and protein expression of TSLP in the lesional dorsal skin of AD. Also, Niram decreased the expression of mast cells-derived TSLP in the lesional dorsal skin. In addition, Niram inhibited the production and mRNA expression of TSLP in stimulated human keratinocytes. Thus, we imply that Niram might prevent AD aggravated by increased TSLP.

TSLP was regulated through caspase-1 signal pathway in mast cells (Moon and Kim, 2011). Caspase-1 transgenic mice overexpressing caspase-1 spontaneously suffered from chronic dermatitis and had a significant increase in the serum levels of histamine and IgE (Murakami et al., 2006; Yamanaka et al., 2000). And infiltration of mast cells was markedly elevated in lesions of caspase-1 transgenic mice (Murakami et al., 2006). The level of RIP2 protein was up-regulated in allergic airway inflammation. The deficiency of RIP2 reduced the levels of IL-4 and serum IgE, infiltration of inflammatory cells, and mRNA expression of TSLP in mouse asthma model (Goh et al., 2013). In this study, Niram inhibited the expressions of caspase-1 and RIP2 in stimulated human mast cells. Niram inhibited the expression of mast cells-derived caspase-1 in the lesional dorsal skin. Thus, we imply that Niram has an anti-AD effect by regulating TSLP through the blockade of RIP2 and caspase-1 in mast cells.

Cytokine imbalance could initiate inflammation in patients with AD, thus emphasizing the important role of cytokines in AD (Gharagozlou et al., 2013). The overproduction of cytokines such as IL-4 and IL-6 could activate B cell to produce IgE and cause atopic manifestations (Novak et al., 2002). Overproduction of IL-4 has been reported in lesional from patients

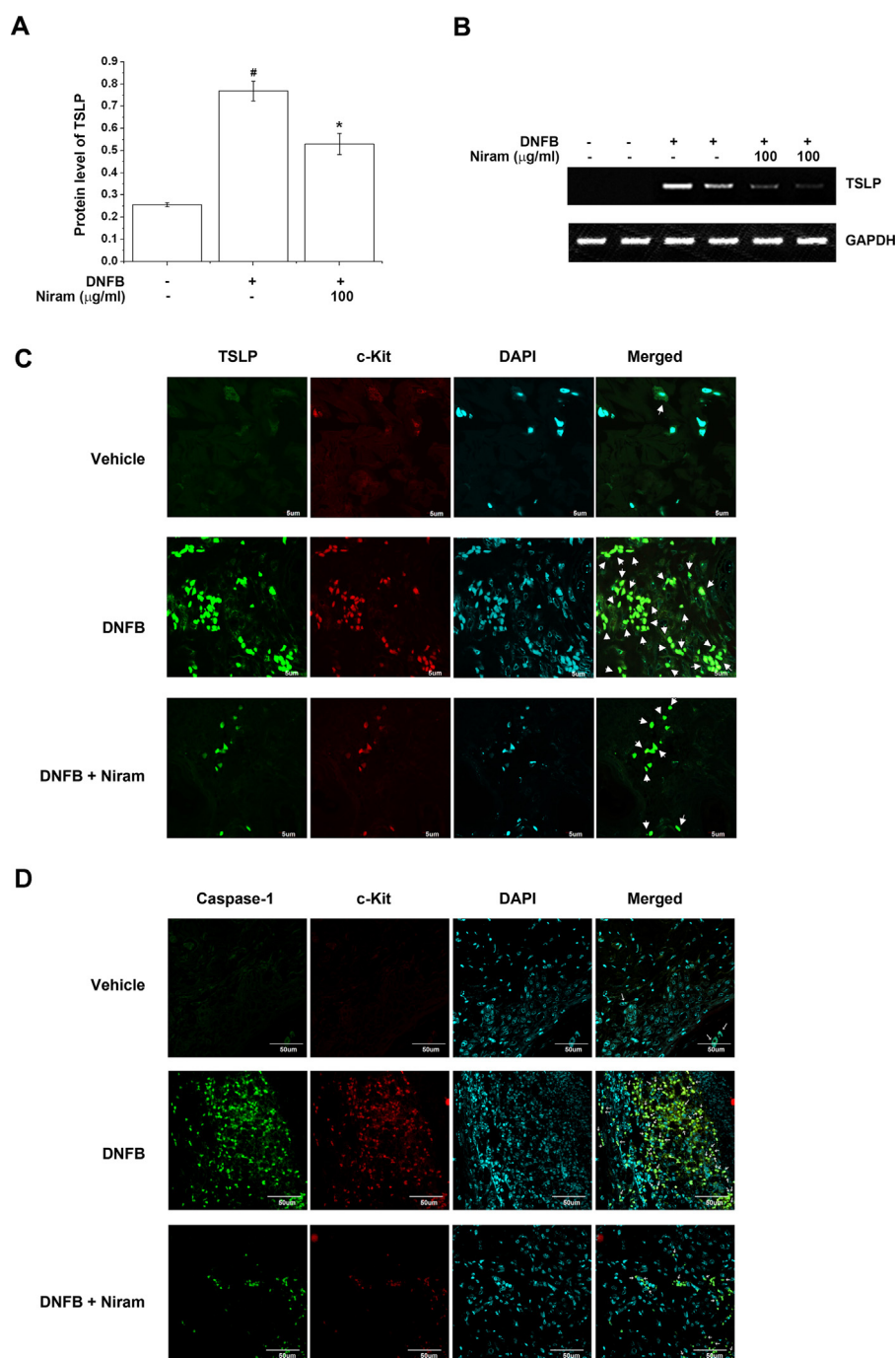


Fig. 4 – Niram regulates the levels of TSLP and caspase-1 in AD mouse model. (A) The protein expression of TSLP from the lesional dorsal skin homogenates was analyzed with ELISA. **(B)** The mRNA expression of TSLP was analyzed with RT-PCR analysis. **(C)** The TSLP⁺ (FITC) and c-Kit⁺ (TRITC) cells in the DNFB-sensitized skin lesions were examined with a confocal laser-scanning microscope. Mast cells are identified as c-Kit⁺ cells. The arrows in merged images show the colocalization of mast cells and TSLP. **(D)** The caspase-1⁺ (FITC) and c-Kit⁺ (PE) cells in the DNFB-sensitized skin lesions were examined with a confocal laser-scanning microscope. The arrows in merged images show the colocalization of mast cells and caspase-1. [#] $p < 0.05$; significantly different from vehicle group. ^{*} $p < 0.05$; significantly different from DNFB-sensitized group (control). $n = 3$.

with AD (Jung et al., 2003). Peripheral blood T cells derived from patients with AD spontaneously produced more IL-6 than that from T cells from normal subjects (Toshitani et al., 1993). The level of IgE is associated with severity of AD and

contributed by abnormality of skin barrier, a key feature of AD (Liu et al., 2011). TSLP induced the histamine release from mast cells through signaling cascade (Ito et al., 2012). All effects of histamine were decreased by caspase-1 inhibitor,

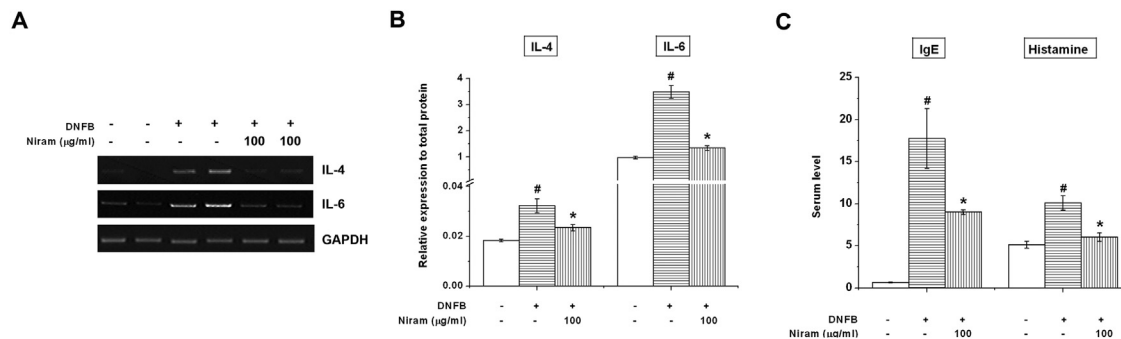


Fig. 5 – Inhibitory effect of Niram on the levels of AD-related factors in AD mouse model. (A) The mRNA expressions of IL-4 and IL-6 were analyzed with RT-PCR analysis. **(B)** The protein expressions of IL-4 and IL-6 from the lesional dorsal skin homogenates were analyzed with ELISA. **(C)** The level of serum IgE was analyzed with ELISA. The intensity of serum histamine was assayed as described in materials and methods section. [#] $p < 0.05$; significantly different from vehicle group. ^{*} $p < 0.05$; significantly different from DNFB-sensitized group (control). $n = 3$.

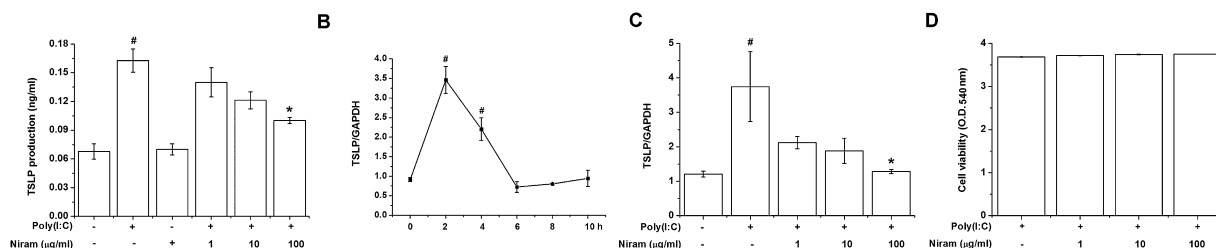


Fig. 6 – Niram inhibits the level of TSLP in stimulated HaCaT cells. (A) HaCaT cells (2×10^5) were pretreated with Niram (1, 10, and 100 µg/ml) for 2 h and stimulated with poly(I:C) 10 µg/ml for 24 h. The production of TSLP was analyzed with the ELISA. **(B)** HaCaT cells (1×10^6) were stimulated with poly(I:C) 10 µg/ml for 10 h. The mRNA expression of TSLP was analyzed with the real time-PCR analysis. **(C)** HaCaT cells (1×10^6) were pretreated with Niram (1, 10, and 100 µg/ml) for 2 h and stimulated with poly(I:C) for 2 h. The mRNA expression of TSLP was analyzed with the real time-PCR analysis. **(D)** Cell viability was analyzed with the MTT assay. Each datum represents the mean $\pm$ SEM of three independent experiments. [#] $p < 0.05$; significantly different from unstimulated cells. ^{*} $p < 0.05$; significantly different from poly(I:C)-stimulated cells.

indicating that histamine action is dependent on cytokines mediated by caspase-1 (Nishibori et al., 2001). In this study, Niram inhibited the levels of IL-4 and IL-6 in the lesional dorsal skin and serum IgE and histamine. Thus, we postulate that Niram might inhibit the clinical symptoms through down-regulating the levels of predominant mediators of AD, such as IL-4, IL-6, IgE, and histamine.

Naju Jjok has been reported to have effects of anti-oxidant, anti-cancer, anti-inflammation, etc. (Janget al., 2012; Kim et al., 2010). Indirubin is an active compound of Naju Jjok (Kim et al., 2010). Indirubin inhibited inflammatory reactions in delayed-type hypersensitivity (Kunikata et al., 2000). Indirubin also inhibited allergic contact dermatitis through regulating Th

cell-mediated immune responses (Kim et al., 2013). Also, we indicated indirubin as a potential compound of Niram made from Naju Jjok through HPLC chromatographic analysis. Thus, we imply that indirubin, an active compound of Niram, might regulate the level of TSLP and inhibit AD in this study.

Taken together, Niram inhibited the levels of TSLP in stimulated mast cells, keratinocytes, and AD mouse model. Senti et al. (2006) have reported that antimicrobial silk clothing showed potential to become an effective treatment of AD. Similarly, we propose that clothes dyed with Niram could also be functional clothes to prevent skin disorders in every life. Furthermore, Niram might provide the beneficial effect as clothes for AD patients.

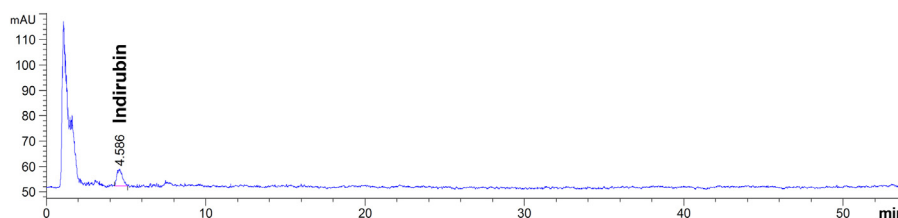


Fig. 7 – Representative HPLC chromatogram of Niram. The HPLC fraction was monitored by recording the UV absorbance at 230 nm.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The [Transparency document](#) associated with this article can be found in the online version.

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