

The Leaves of *Broussonetia kazinoki* Siebold Inhibit Atopic Dermatitis-Like Response on Mite Allergen-Treated Nc/Nga Mice

Hoyoung Lee^{1,†}, Hyekyung Ha^{2,†}, Jun Kyoung Lee², Sang-Joon Park³, Seung-II Jeong⁴ and Hyeun Kyoo Shin^{2,*}

¹Medical Research Division, ²Herbal Medicine Formulation Research Group, Korea Institute of Oriental Medicine, Daejeon 305-811,

³Department of Histology, College of Veterinary Medicine, Kyungpook National University, Daegu 702-701,

⁴Jeonju Biomaterials Institute, Jeonju 561-360, Republic of Korea

Abstract

Broussonetia kazinoki Siebold. (*B. kazinoki*) has long been used in the manufacture of paper in Asian countries. Although *B. kazinoki* leaves (BK) have been employed in dermatological therapy, use of BK has not been tested in patients with atopic dermatitis (AD). Using Nc/Nga mice, which are genetically predisposed to develop AD-like skin lesions, we confirmed the efficacy of BK in AD treatment. BK extract was applied topically to *Dermatophagoides farinae*-induced AD-like lesions in Nc/Nga mice, and the effects were assessed both clinically and by measuring skin thickness on the back and ears. We measured the effects of BK extract on plasma levels of IgE and IL-4. We also measured the ability of BK extract to inhibit the secretion of hTARC in HaCaT cells after stimulation by TNF- α and IFN- γ . We found that BK extract significantly reduced ear and dorsal skin thickness and the clinical signs of AD, as well as significantly down-regulating the plasma levels of IgE and IL-4 ($p < 0.01$ for each comparison). Moreover, 500 μ g/mL of BK extract inhibited hTARC secretion in HaCaT cells by activated TNF- α /IFN- γ by about 87%. These findings suggest that topical application of BK extract has excellent potential in the treatment of AD.

Key Words: *Broussonetia kazinoki* Siebold., Atopic dermatitis, *Dermatophagoides farinae*, TARC

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disorder associated with increased modernization and accompanying exposure to contaminants in food and the environment. Among the environmental factors associated with AD are exposure to allergens, bacteria, and dust. The number of AD patients, especially children, has increased rapidly, and AD has been found to be a risk factor for childhood asthma, affecting asthma occurrence, severity, and persistence (Buys, 2007). AD is likely related to early immunoglobulin E (IgE) production and later allergen/IgE reactivity (Bergmann *et al.*, 1998).

Among the clinical features of AD are pruritis, eczema, xerosis, and atypical vascular and keratosis pilaris. Topical corticosteroids are usually used to treat AD flare-ups (Gambichler *et al.*, 2005), although AD can also be treated by homeopathy, massage therapy, dietary restrictions, reduction of house mite dust, and herbal medicine. The use of herbal medicine may be preferred to steroids, because to the side effects of the latter.

Broussonetia kazinoki Siebold. (*B. kazinoki*) has long been

employed for paper manufacture in Asian countries, as well as a dermatological therapy in Korean traditional medicine to treat burns and acne. *B. kazinoki* extract has various biological activities, including the inhibition of tyrosinase (Hwang and Lee, 2007), antihyperglycemic and anti-diabetic effects (Cha *et al.*, 2008), and inhibition of nitric oxide over-production (Ryu *et al.*, 2003). Chemically, *B. kazinoki* leaves (BK) have been shown to contain flavonoids (Zhang *et al.*, 2001) whereas the branches contain alkaloids (Tsukamoto *et al.*, 2001). However, there were no results for the treatment of AD using BK extract.

AD patients commonly show overproduction of serum IgE. In addition, Th2 cytokines, such as interleukin (IL)-4, IL-5, and IL-13, have been reported to mediate the IgE isotype switch in B lymphocytes (Kang *et al.*, 2002). Mast cells are important in IgE-mediated allergic disorders. Upon activation, mast cells undergo degranulation and release a variety of biologically active substances, which play an important role in host defense and allergic reactions.

Exposure of keratinocytes to tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) has been found to induce the expres-

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***Corresponding Author**

E-mail: hkshin@kiom.re.kr

Tel: +82-42-868-9464, Fax: +82-42-864-2120

[†]The first two authors contributed equally to this work.

sion of cytokines and chemokines that mediate the infiltration of monocytes/T cells into sites of inflammation in the skin (Grone, 2002). Thymus and activation-regulated chemokine, TARC/CCL17, is a member of the CC chemokine subfamily and is secreted by keratinocytes and monocyte-derived dendritic cells. Because TARC controls the migration of Th2 lymphocytes into sites of inflammation, TARC is considered to be mediator of inflammatory responses during the development of Th2-dominant inflammatory skin diseases such as AD.

To examine the effects of BK extract on AD, we investigated the effects of topically applied BK extract on AD-like skin lesions induced by an extract of the dust mite, *Dermatophagoides farinae* (*D. farinae*), in Nc/Nga mice, which are genetically predisposed to development of AD-like skin lesions. Furthermore, to gain insight into the molecular effects of BK extract, we investigated its effect on TNF- α /IFN- γ induced TARC secretion in the HaCaT, human keratinocyte cell line.

MATERIALS AND METHODS

Reagents

A Biostir-AD[®] (*D. farinae* extract) was purchased from Biostir Inc (Hyogo, Japan). The positive control was the immunosuppressive drug, 0.1% (w/v) tacrolimus (Protopic[®], Astellas, NY, USA). Human recombinant TNF- α , IFN- γ , and human TARC enzyme-linked immunosorbent assay (ELISA) kits were obtained from R&D Systems (Minneapolis, MN, USA). Forskolin and silymarin was purchased from Sigma-Aldrich Inc (St Louis, MO, USA). Mouse IgE and IL-4 ELISA kits were the products of Bethyl Laboratories Inc. (Montgomery, TX, USA) and Biosource (Nivelles, Belgium).

Plant material and extraction

Fresh leaves of *B. kazinoki* (BK) were donated by Chun-Yang Paper Corporation of Jeonbuk, Province, Korea, in July 2008. The plant material was identified by Professor Hongjun Kim, College of Oriental Medicine, Woosuk University, and a voucher specimen (JSI0862) was deposited at the Jeonju Biomaterials Institute. Air-dried BK (4.5 kg) was chopped into small pieces and extracted with 70% (v/v) ethanol (EtOH, 15 L) with reflux for 4 hours. The extracted solution was filtered and evaporated under reduced pressure to yield a 70% EtOH extract (512 g). The extract was thoroughly dried to completely remove solvent.

Phytochemical analysis

Flavonoids: The total flavonoids content was estimated as rutin equivalents in mg rutin/g dry weight. About 0.5 g of the BK was extracted with 70 mL of EtOH for 30 min in a boiling water bath and filtered through Whatman No. 1 filter paper. A 1.0 mL aliquot of each extract was mixed with 5 mL of 10% solution of aluminium chloride in EtOH in a 25 mL volumetric flask. Blanks were prepared as described above except aluminium chloride was replaced by EtOH. The absorption at 410 nm was measured after 30 min and compared to a rutin calibration curve.

Total phenolics: The total phenolic contents of the extracts was estimated as gallic acid equivalents, expressed as mg of gallic acid/g (dry wt) of extract, according to the Folin-Ciocalteu reagent method (Singleton *et al.*, 1999). Briefly, 1.0 g of BK extract filtered through Whatman No. 1 filter paper; 0.25

Table 1. Phytochemical analysis of *Broussonetia kazinoki* Siebold leaves (BK) extract

Constituent	mg/g BK extract
Flavonoids	24 \pm 0.7
Tannins	14 \pm 0.6
Total phenolics	22 \pm 0.3

The data are presented as mean \pm SEM (n=3).

mL was transferred to a 25.0 mL volumetric flask containing 6 mL of water, to which was added 1.25 mL of undiluted Folin-Ciocalteu reagent. After 1 min, 3.75 mL of 20% (w/v) aqueous Na₂CO₃ was added, and the volume was made to 25 mL with water. The controls contained all of the reaction reagents but not the extract. After incubation for 2 hr at 25°C, the absorbance at 760 nm was measured and compared to a gallic acid calibration curve.

Tannins: The total tannins content was estimated as tannin equivalents in mg tannin/g BK extract. Briefly, 2.0 g of BK was extracted with 250 mL of hot water for 30 min by refluxing. A 25 mL sample was mixed with 500 mL of water and 25 mL of indigo sulfuric acid solution, then titrated using 0.02 M KMnO₄.

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 \pm 2°C and 55 \pm 15% humidity. All procedures involving animals were conducted in accordance with the Guideline of the Institutional Animal Care and Use committee of the Korea Institute of Oriental Medicine. The approval number for the animal study was 09-100.

Sensitization

AD-like skin lesions were induced in 10-week-old male Nc/Nga mice using *D. farinae* extract as described by the manufacturer (Sumiyoshi *et al.*, 2003). The *D. farinae* extract was applied on twice per week for 4 weeks. Briefly, the hair on the upper back was shaved. For barrier disruption, 200 μ L of 10% (w/v) sodium dodecyl sulfate (SDS) was applied to the shaved dorsal skin and both surfaces of each ear until 2 times. After 2 times, 200 μ L of 4% (w/v) SDS was applied to the skin and ears before topical application of 50 mg *D. farinae* extract. At the start of the experiment, mice were randomized to one of four groups (n=5 each): untreated controls (70% EtOH; cont), *D. farinae* extract-treated (50 mg/mouse; *D. farinae*), *D. farinae* extract with Protopic[®]-treated (50 mg/mouse; Protopic), and *D. farinae* extract with BK extract-treated (10 mg/mouse/70% EtOH; BK). BK extract was dissolved in 70% EtOH. 70% EtOH was given to the animal in the vehicle control group.

Severity score

The severity of dermatitis was investigated macroscopically according to the scoring system as followed. The dorsal skin and ears skin lesions were scored by the following symptoms; erythema/ hemorrhage, edema, excoriation/erosion and scaling/dryness. The severity score was defined as the sum of individual scores, graded as 0 (no symptom), 1 (mild), 2 (moderate) and 3 (severe).

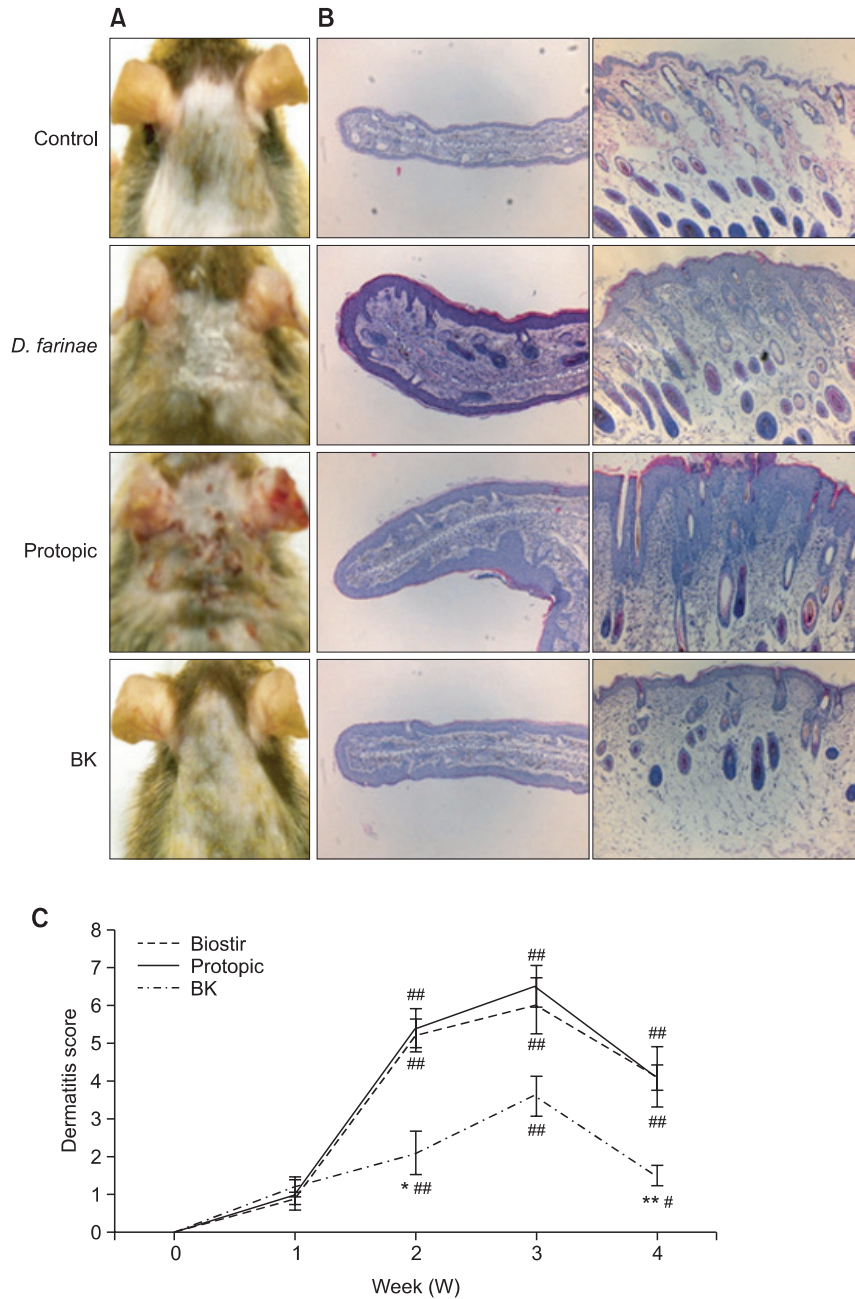


Fig. 1. Clinical and histological features on atopic dermatitis-like skin lesions topically treated with *Broussonetia kazinoki* Sieb leaves. (A) The group of untreated (cont), *D. farinae* plus vehicle-treated (*D. farinae*), *D. farinae* plus tacrolimus (Protopic®) treated (Protopic) and *D. farinae* plus *Broussonetia kazinoki* Sieb leaves (BK) treated Nc/Nga mice were excised (B) Clinical feature of ear and skin. (C) The dermatitis score on atopic dermatitis-like skin lesions (^{##} $p < 0.01$ compare with 0 week, ^{*} $p < 0.05$ compare with *D. farinae*, ^{**} $p < 0.01$).

Histopathology

After sacrifice, the dorsal skin and both ears of each mouse were fixed in 10% (v/v) natural buffered formalin for 24 hr at 4°C. Tissues were next embedded in paraffin, and thin-sectioned (4 μm thickness). Sections were stained with H&E solution (hematoxylin and eosin, Sigma-Aldrich Inc, MO, U.S.A.) and subsequently mounted under cover slips using Dako-mounting medium (Dakocytomation, Denmark, CA, USA). Photographs were captured using a photometric Quanax digital Camera and montages were assembled in adobe Photoshop.

To measure mast cell infiltration, skin sections were stained with toluidine blue and the numbers of mast cells in four randomly chosen sites were counted.

Cell culture

A human keratinocyte cell line, HaCaT, was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL., NY, U.S.A). The cells were seeded at densities of 1.0×10^3 cells/well in 96-well plates

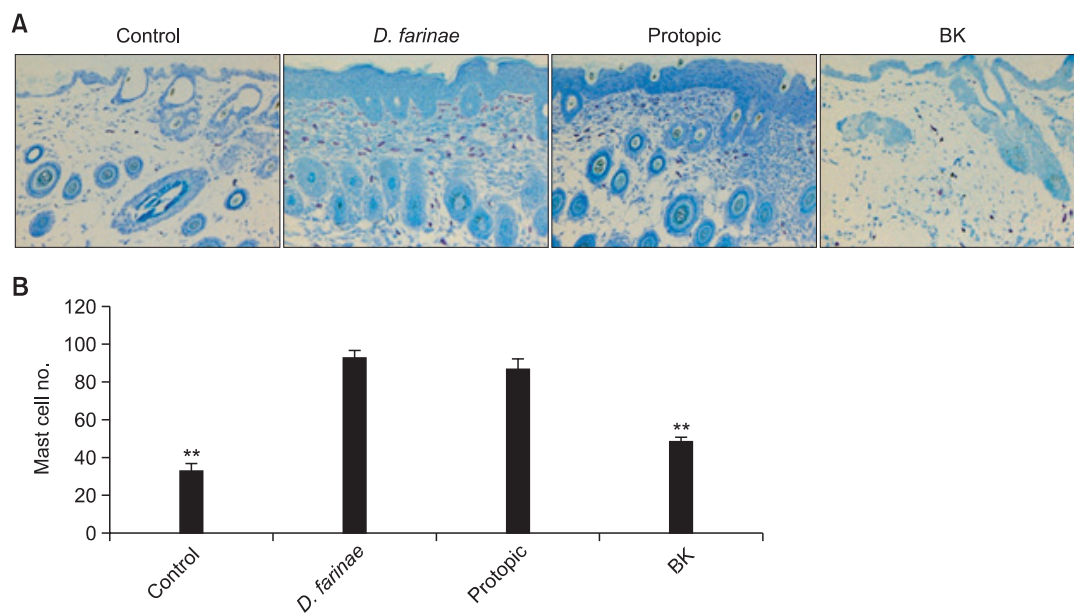


Fig. 2. Infiltration of mast cells into the skin on atopic dermatitis-induced Nc/Nga mice. The group of untreated (cont), *D. fariniae* plus vehicle-untreated (*D. fariniae*), *D. fariniae* plus tacrolimus-treated (Protopic) and *D. fariniae* plus *Broussonetia kazinoki* Sieb leaves (BK) treated Nc/Nga mice were excised (A) The dorsal skin sections were stained with toluidine blue. (B) The number of mast cells was counted (** $p < 0.01$ compare with *D. fariniae*).

for the cytotoxicity assay. The cells were then incubated with different concentrations of BK extract (200, 500, and 1000 $\mu\text{g}/\text{mL}$), forskolin (10 and 30 μM), and silymarin (20 and 50 $\mu\text{g}/\text{mL}$) for 24 hr. The vehicle control was 1% DMSO. After treatment, 10 μL of Cell Counting Kit-8 reagent (CCK-8, Dojindo, Japan) was added to each well and the plates were incubated for 4 hr. The absorbance was measured at 450 nm using a microplate reader (BenchmarkPlus, Bio-Rad Laboratories Inc., U.S.A.) and the percentages of viable cells were calculated. BK extracts in the range of 200-1000 $\mu\text{g}/\text{mL}$ did not cause cytotoxicity in HaCaT cells (data not shown) and non-cytotoxic concentrations of BK extracts were used for the subsequent experiments.

To measure the level of TARC, cells were seeded at 1×10^6 cells/well in 6-well plates until they attained confluence. The cells were washed and incubated with 1 mL serum-free medium containing TNF- α (10 ng/mL)/IFN- γ (10 ng/mL) (Yamamoto *et al.*, 2007), with BK extract (200, 500, and 1000 $\mu\text{g}/\text{mL}$) for 24 hr. This study used the forskolin (10 and 30 μM), inhibitors of cAMP, and silymarin (20 and 50 $\mu\text{g}/\text{mL}$) as the positive controls.

ELISA

Blood samples were drawn from mice. Plasma was separated by centrifugation at $10,000 \times g$ for 10 min at 4°C , and stored at -80°C . Plasma IgE and IL-4 levels in Nc/Nga mice and TARC concentrations in the supernatants of HaCaT cells were measured by ELISA, according to the manufacturer's instructions.

Statistical analysis

Data are reported as means \pm SEM and compared by ANOVA and Bonferroni multiple comparison method (SYSTAT 8.0 SPSS Inc. U.S.A.). A p -value < 0.05 was defined as statistically

significant.

RESULTS

Phytochemical analysis

The phytochemical profiles of BK extract are shown in Table 1. BK extract was rich in flavonoids and tannins (Table 1).

D. fariniae-induced AD-like skin lesions in Nc/Nga mice

Photographs taken once weekly for 4 weeks showed the progressive development of skin lesions induced by *D. fariniae* (50 mg/mouse) in Nc/Nga animals. *D. fariniae* group showed symptoms of AD after 1 week. Over time, chronic signs of inflammation appeared, including erythema, induration/population, excoriation, and lichenification. Protopic group did not develop symptoms or lesions for 2 weeks. At 3 weeks, however, these groups showed sudden development of AD-like lesions. Repeated topical application of BK significantly decreased the thickness of both the epidermis (the outermost layer of skin) and the dermis, the layer of skin beneath the epidermis (Fig. 1A, B). The severity score of dermatitis was evaluated on a week for 4 weeks. The control group Nc/Nga mice did not show any clinical symptoms of dermatitis such as erythema/hemorrhage, edema, excoriation/erosion and scaling/dryness. BK treatment significantly reduced the skin severity score of AD-like lesions induced Nc/Nga mice as compared to the Protopic group (Fig. 1C).

Mast cell infiltration into the dorsal skin of Nc/Nga mice

When we assessed the effect of *D. fariniae* and BK groups on the infiltration of mast cell, an important effector cell involved in AD, into the dorsal skin, we found that treatment with *D. fariniae* increased mast cell infiltration (Fig. 2). This was

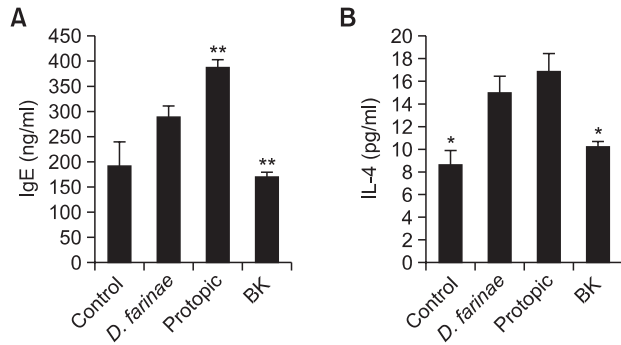


Fig. 3. Plasma levels of IL-4 and IgE in atopic dermatitis-induced Nc/Nga mice. The plasma samples were collected from untreated (Cont), *D. farinae* plus vehicle-treated (*D. farinae*), *D. farinae* plus tacrolimus-treated (Protopic) and *D. farinae* plus *Broussonetia kazinoki* Sieb leaves (BK) treated Nc/Nga mice. The plasma concentrations of IgE (A) and IL-4 (B) were measured by ELISA (* $p < 0.05$ compare with *D. farinae*, ** $p < 0.01$).

significantly suppressed by treatment with BK, but not with Protopic.

The levels of IL-4 and IgE in plasma

When we assessed the effect of *D. farinae* and BK group on the plasma levels of IgE and IL-4 in Nc/Nga mice, we found that *D. farinae* group significantly increased IgE level compared to the untreated control group ($288.07 \pm 23.19 \mu\text{g/mL}$ vs. $191.06 \pm 16.73 \mu\text{g/mL}$). BK group decreased the serum IgE level to $169.10 \pm 10.82 \mu\text{g/mL}$, below that of untreated group. Protopic group had no effect on the *D. farinae*-mediated increase in IgE level (Fig. 3A). *D. farinae* group also increased the plasma level of IL-4, but this rise was inhibited 32% by BK (Fig. 3B).

TARC production by BK in HaCaT cells

As TARC may play a role in the development of AD, we tested the effects of BK extract on TARC secretion. We found that incubation of HaCaT cells with $\text{TNF-}\alpha/\text{IFN-}\gamma$ increased the production of TARC about five-fold ($63.10 \pm 4.64 \text{ ng/mL}$) compared with control ($12.78 \pm 0.80 \text{ ng/mL}$), untreated HaCaT cells. In $\text{TNF-}\alpha/\text{IFN-}\gamma$ stimulated HaCaT cells, we found that 10 and 30 μM forskolin inhibited hTARC production by 75% ($12.83 \pm 0.18 \text{ ng/mL}$) and 99%, respectively, whereas 20 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$ of silymarin inhibited hTARC production by about 61% ($32.36 \pm 1.73 \text{ ng/mL}$) and 107% ($9.51 \pm 0.63 \text{ ng/mL}$) (Fig. 4). In addition, BK extract, at concentrations of 200, 500, and 1000 $\mu\text{g/mL}$, inhibited hTARC production by 36% ($45.13 \pm 1.67 \text{ ng/mL}$), 87% ($19.47 \pm 2.44 \text{ ng/mL}$), and 84% ($20.69 \pm 0.41 \text{ ng/mL}$), respectively (Fig. 4).

DISCUSSION

B. kazinoki has been used in the manufacture of paper, especially because of the whitening effect afforded by the material, and to treat skin disorders. BK has been shown to have anti-melanogenesis and antihyperglycemic effects. We have not been able to confirm the compound profiling by HPLC because there is no a known-marker compound of BK. Many medicinal plants contain phenolic compounds including tannins and flavonoids which have anti-inflammatory and antioxidant

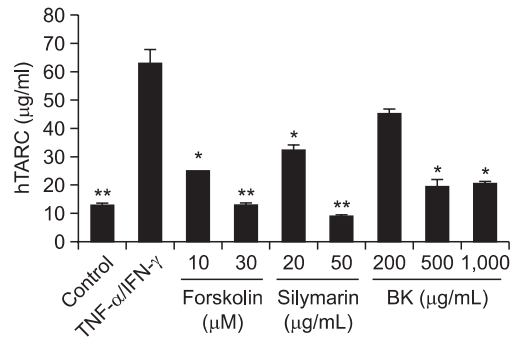


Fig. 4. The hTARC production on $\text{TNF-}\alpha/\text{IFN-}\gamma$ stimulated HaCaT cells. The HaCaT cells were incubated in medium containing *Broussonetia kazinoki* Sieb leaves (BK) and treated with $\text{TNF-}\alpha/\text{IFN-}\gamma$ (TI) (10 ng/mL, each) for 24 hr. The concentration of hTARC in the medium was determined using ELISA (* $p < 0.05$ compare with TI group, ** $p < 0.01$).

effects (Diaz *et al.*, 2012). Therefore, the contents of tannins, flavonoids and total phenolic compounds were determined in BK. Flavonoids has anti-inflammatory hepatocyte protective and antioxidative effects (Romano *et al.*, 2013). Tannin has various efficacies of anthelmintic effect (Athanasidou *et al.*, 2001), anti-oxidative stress (Fedeli *et al.*, 2004), and immunopotentiating effects (Nakashima *et al.*, 1992). The BK extract was applied to *in vitro* and *in vivo* models for AD-like lesion because flavonoids and tannins have anti-inflammatory and anti-oxidant effect.

In the present study, we assessed the effect of BK on *D. farinae*-induced AD-like skin lesions in Nc/Nga mice. The Nc/Nga mouse has been used as a model of human AD (Aioi *et al.*, 2001). Following treatment with *D. farinae*, Nc/Nga mice show hyperproduction of serum IgE, severe itching behavior, and invasion of inflammatory cells into the skin (Matsuda *et al.*, 1997). Inflammatory responses on destructive skin barrier, which are induced by allergens including house dust mite. In allergic inflammatory conditions, such as AD and asthma, systemic IgE level was increased. The increased IgE stimulates mast cell activation and thereby activated mast cells result in the release of mediators including histamine and cytokines (IL-4 and IL-13).

We found that the application of BK extract reduced skin and ear thickness and significantly ameliorated the clinical signs provoked by *D. farinae*, including erythema, edema, scaling, and excoriation. Histologically, mast cell infiltration into the dorsal skin was reduced by treatment of BK extract. BK extract application significantly suppressed AD such lesions in Nc/Nga mice but 0.1% (w/v) tacrolimus (Protopic®) application had no effect on AD lesions in our experimental model. Immunosuppressive drugs are very effective in the treatment of AD. Tacrolimus and CsA bind calcineurin, resulting in inhibition of NF-AT nuclear translation (Sandoval-Lopez and Teran, 2001). Hanifin *et al.* reported that the most common application site adverse events of tacrolimus were pruritus, skin burning (eg, burning or warmth sensation, stinging), and skin infection (which included all cutaneous infections not otherwise specified, eg, bacterial infections, molluscum, and pyoderma) (Hanifin *et al.*, 2005). So it was adverse effect which Protopic® increased the mast cell infiltration, IgE and IL-4.

Mast cells are known to play a major role in allergic dis-

eases such as atopic dermatitis and asthma and so on. Mast cell receives numerous stimulatory and inhibitory factors for activation resulting in enhancing or counteracting effects. The responses to allergens, infections, and wounds each produce a different set of factors that can affect mast cells. Type I hypersensitivity reactions are mediated by IgE and factors produced primarily from Th2 responses (Chang and Shiung, 2006). Thus, mast cells and basophiles express high-affinity IgE Fc receptor (FcεRI), which is almost entirely bound by IgE. Also, Th2 cells are controlled in turn activate B cell via the release of cytokines such as IL-4 and IL-13 critical for development of plasma cell that will start the production of allergen specific IgE (Romagnani, 2007). In this result, BK extracts decreased the mast cell compared to *D. farina* treated group. Also BK extract application decreased the plasma levels of IgE and IL-4.

Many studies have shown that TARC actively participates in the pathogenesis of AD in Nc/Nga mice (Saeki and Tamaki, 2006) and that TNF- α /IFN- γ synergistically induces TARC production by human keratinocyte and HaCaT cells. So we hypothesized that BK suppressed the production of TARC induced in HaCaT cells by TNF- α /IFN- γ .

AD has been associated with elevation of cAMP, as cAMP hydrolytic activity is increased in patients with allergic diseases such as AD, asthma, and allergic rhinitis (Grewe *et al.*, 1982). Salpietro *et al.* reported that the measurement of cAMP level is useful to diagnostic and therapy of AD patient (Salpietro DC *et al.*, 1998). Therefore we selected forskolin which is an activator of cAMP as a positive control. The another positive control, silymarin suppress *D. farinae* induced AD-like skin lesions in Nc/Nga mice (Kang *et al.*, 2008) and reported the effectiveness of treatment of allergic rhinitis (Bakhshae *et al.*, 2011).

The adenylyl cyclase-cAMP system may reduce infiltration of Th2 cells into skin lesions by suppressing the binding of TARC/macrophage-derived chemokine (MDC/CCL22) to CCR4, thereby decreasing disease severity in patients with skin inflammation involving Th2 type chemokines (Qi *et al.*, 2009). The forskolin and silymarin were used the positive control in this study.

TARC functions as a selective chemoattractant and assists in the recruitment and migration of Th2 cells, which express CCR4. Treatment with anti-TARC antibody would therefore suppress the development of AD, suggesting that TARC may be an important mediator of AD. The effects of BK extract on the clinical and histological signs of AD and on the plasma levels of IgE and IL-4 might be associated with the BK extract-induced inhibition of TARC production in HaCaT cells. We found that BK extract dose-dependently inhibited TNF- α /IFN- γ -induced TARC production by HaCaT cells. The positive controls, forskolin and silymarin, also inhibited TARC production, suggesting that the BK extract inhibition of TARC production may be associated with a mechanism involving cAMP. We will investigate the function of cAMP that associate the NF- κ B and p38 MAPK pathway. We supposed that BK extract may therefore be an important new anti-inflammatory drug for the treatment of Th2-skewed inflammation.

In conclusion, BK extract was successful in suppressing AD-like lesions induced in Nc/Nga mice by *D. farina* which IgE and IL-4 were inhibited by reducing mast cells. Also they inhibited TARC production by TNF- α /IFN- γ -treated HaCaT cells. Topical BK extract was effective in treating AD-like skin lesions

in Nc/Nga mice. Topical application of BK may therefore be a novel approach to the treatment of AD.

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