



Inhibitory Effects of Heartwood Extracts of *Broussonetia kazinoki* Sieb on the Development of Atopic Dermatitis in NC/Nga Mice

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We investigated the effects of a topically applied extract of the heartwood of *Broussonetia kazinoki* Sieb (*B. kazinoki*) on atopic dermatitis (AD)-like skin lesions induced by an extract of the house-dust mite *Dermatophagoides farina* in NC/Nga mice. We found that topically applied *B. kazinoki* extract suppressed the histological manifestations of AD-like skin lesions, and decreased the levels of plasma immunoglobulin E (IgE) and interleukin-4 (IL-4) in the mice. Moreover, *B. kazinoki* inhibited the induction of thymus-and-activation-regulated chemokine (TARC/CCL17), macrophage-derived chemokine (MDC/CCL22), and regulated-on-activation-normal T cell-expressed-and-secreted chemokine (RANTES/CCL5) in HaCaT cells activated by tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ). In conclusion, our results suggest that *B. kazinoki* extract has therapeutic advantages in the treatment of AD.

Key words: *Broussonetia kazinoki* Sieb; atopic dermatitis; NC/Nga mice; IgE; TARC

Atopic dermatitis (AD) is a chronic inflammatory skin disease accompanied by severe itching and eczematous lesions, caused by both genetic and environmental factors such as environmental pollutants and microbial infection.¹⁾ Chronic inflammation in AD is associated with elevated levels of serum immunoglobulin E (IgE) and eosinophilia in the tissues and peripheral blood, and frequently occurs in response to environmental allergens such as house-dust mites *Dermatophagoides pteronyssinus* and *Dermatophagoides farina*.^{2,3)}

Acute-phase AD is defined by the infiltration into skin lesions of T-helper 2-type cells (Th2) and augmented secretion of Th2-related cytokines, interleukin (IL)-4, IL-5, and IL-13.⁴⁾ Most patients with AD have reduced numbers of Th1 cells and low concentrations of Th1 cytokines such as interferon- γ (IFN- γ), which are capable of counteracting the Th2 response.⁵⁻⁷⁾

Th2 cells are chemoattracted by thymus-and-activation-regulated chemokine (TARC/CCL17) and

macrophage-derived chemokine (MDC/CCL22), both of which are specific ligands for CCR4.⁸⁻¹⁰⁾ TARC and MDC are thought to play important roles in the development of some skin diseases, such as AD. In many studies, serum levels of TARC and MDC have been found to be markedly elevated, and these increased chemokine levels are correlated with disease severity in patients with AD.^{11,12)} Regulated-on-activation-normal T cell-expressed-and-secreted chemokine (RANTES/CCL5) has been also found to be associated with AD.¹³⁾ The RANTES protein was detected at high levels in skin scales of AD patients, and dermal fibroblast were identified as a major source of this chemokine.¹⁴⁾

Although the pathogenesis of AD has not been fully elucidated, a Th1/Th2 imbalance is regarded as important in disease development. Immunosuppressive drugs such as glucocorticoids and tacrolimus have been found to be effective in treating AD, but long-term use causes adverse effects. The results of several studies indicate that AD patients can benefit from herbal therapy.¹⁵⁾

Broussonetia kazinoki Sieb has long been used in the manufacture of paper in Asian countries. In Korean traditional medicine, this plant is also used for dermatologic disease such as burns and acne. *Broussonetia kazinoki* Sieb extracts have been shown to have anti-diabetic and antihyperglycemic effects,¹⁶⁾ and to inhibit tyrosinase and melanin synthesis¹⁷⁾ and nitric oxide over-production.¹⁸⁾ Chemically, *B. kazinoki* leaves and branches have been found to contain flavonoids, including broussonol A, B, C, D, and E,¹⁹⁾ and alkaloids including broussonetines A, B, W, X, and J₁,²⁰⁾ respectively. To date, no biological effects of *Broussonetia kazinoki* Sieb extracts or its constituents have been reported for dermatologic disease.

The present study investigated the effects of the heartwood of *B. kazinoki* on atopic dermatitis (AD)-like skin lesions induced by an extract of the house-dust mite *D. farina* in NC/Nga mice, which are genetically predisposed to develop AD-like skin lesions. To gain insight into the molecular biological effects of *B. kazinoki*

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extract on TARC, MDC, and RANTES production in a human keratinocyte cell line, HaCaT, was also examined to elucidate a possible mechanism of the anti-AD effect.

Materials and Methods

Animals. Forty male NC/Nga mice (8 weeks old) purchased from Central Laboratory Animals (Seoul, Korea), were housed in an air-conditioned room maintained at $24 \pm 2^\circ\text{C}$ at $55 \pm 15\%$ humidity. All procedures involving animals were conducted in accordance with the Guideline for Animal Experiments of the Korea Institute of Oriental Medicine.

Reagents and drugs. Biostir-AD, an ointment containing component of the house-dust mite *D. farina*, was purchased from Bostir (Hyogo, Japan). A 0.1% Tacrolimus (Protopic®; Astellas, New York), immunosuppressive agent was used as a positive control. Recombinant human tumor necrosis factor- α (TNF- α), recombinant human IFN- γ , and human TARC enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems (Minneapolis, Minnesota). Mouse IgE and mouse IL-4 ELISA kits were from Bethyl Laboratories (Montgomery, Texas) and Biosource (Nivelles, Belgium) respectively.

Plant materials and extraction. *B. kazinoki* was donated by the ChunYang Paper Corporation (Jeonju, Korea) in July 2008. The plant material was identified by Professor Hongjun Kim, College of Oriental Medicine, Woosuk University, and a plant specimen (JSI0861) was deposited at the Jeonju Biomaterials Institute. Dried *B. kazinoki* (5 kg) was crushed into a fine powder and extracted with EtOH (18 liters) under reflux for 4 h. The extracted solution was filtered and evaporated under reduced pressure to yield an ethanol extract powder (76 g), which was kept at 4°C and used in the present study.

Induction of atopic dermatitis. AD-like skin lesions were induced in male NC/Nga mice with Biostir-AD, as directed by the manufacturer. The animals were divided into four groups: untreated controls (Cont); Biostir-AD-treated (Biostir); Biostir-AD and Protopic®-treated (Protopic); and Biostir-AD and *B. kazinoki* extracts-treated (*B. kazinoki*), ($n = 5$ animals in each group). Briefly, the hair on the upper back was shaved with clippers. For barrier disruption, 150 μl of 4% w/v sodium dodecyl sulfate (SDS) was applied to the shaved back skin and both surfaces of each ear 3 h before topical application of 50 mg Biostir-AD. Biostir-AD (50 mg/mouse) and Protopic® (50 mg/mouse) were applied twice a week for 4 weeks.²¹ *B. kazinoki* extract was dissolved 70% ethanol and applied (10 mg/mouse) every day for 4 weeks. The start of treatment with Biostir-AD and *B. kazinoki* was same day. The mice were sacrificed under anesthesia with pentobarbital (200 μg /mouse, i.p.) on the day of the experiment (4 weeks after first application of Biostir). At autopsy, blood was collected from the posterior *vena cava*, and the back skin and each ear were excised for histopathological analysis.

Histopathology. After sacrifice, the back skins and ears of each mouse were fixed in 10% v/v natural buffered formalin for 24 h at 4°C . The Tissues were embedded in paraffin, and thin-sectioned (4 μm thickness). Sections were stained with H&E solution (hematoxylin and eosin, Sigma-Aldrich, St. Louis, MO), and subsequently mounted under cover slips using Dako-mounting medium (Dakocytomation, Denmark, CA). Photographs were taken using a photometric Quanax digital camera, and montages were assembled by 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-line 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 of penicillin, and 100 μg /ml streptomycin (Gibco, Grand Island). They were seeded at 1×10^6 cells/well into six-well plates until confluence was attained. The cells were washed with DMEM, and each well was

incubated with 1 ml DMEM containing TNF- α (10 ng/ml) plus IFN- γ (10 ng/ml) for 24 h. They were subsequently incubated with *B. kazinoki* extract (50, 100, or 250 μg /ml) for 24 h. As positive controls, cells were treated with forskolin (10 and 30 μM) and silymarin (20 and 50 μg /ml).

Detection of IgE, IL-4, and TARC. Blood samples were collected from abdominal aorta into K₂-EDTA-treated tubes, and plasma was separated by centrifugation at $3,000 \times g$ for 10 min at 4°C and stored at -80°C . We measured the plasma levels of IgE and IL-4. The TARC level in the supernatants of the HaCaT cells was also measured with ELISA kits (R&D System, Boston, MA), according to the manufacturer's instructions. The absorbance was determined by using a microplate reader.

Gene expression (RT-PCR). After cell culture as described above for 24 h, total RNA was isolated using TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA). cDNA was synthesized from total RNA (2 μg) using an iScript Select cDNA Synthesis kit (Bio-Rad, Hercules, CA) following the manufacturer's instructions. For PCR amplification, we used specific primers for TARC (forward, 5'-actgctccaggatgccatcggtttt-3'; reverse, 5'-acaaggggaggggatccctcactg-3'), MDC (forward, 5'-aggacagagcatggctgcctacaga-3'; reverse, 5'-taatggcagggaggtaggctcctga-3'), RANTES (forward, 5'-ccccgtgccacatcaaggagtattt-3'; reverse, 5'-cgtccagcctgggaagggttttga-3'), and hGAPDH (forward, 5'-aagggtcatcatctctgcc-3'; reverse, 5'-gtgatggcatggactgtgtg-3'), which were expected to yield fragments of 270 bp, 362 bp, 313 bp, and 204 bp respectively.

Statistical analysis. Data are reported as means $\pm$ SEMs, and were compared by ANOVA and the Bonferroni multiple comparison method. A p value < 0.05 was defined as statistically significant.

Results

Histological analysis

Photographs showed that *B. kazinoki* effectively treated AD-like skin lesion induced by Biostir-AD on NC/Nga mice. The *B. kazinoki* group showed broader recovery from AD-like lesions and hair growth on the dorsal skin (Fig. 1). Histological examination of the skin lesions showed no abnormal changes in the control group, whereas the Biostir-AD and Protopic® groups showed significant inflammatory changes, including hemorrhage, hypertrophy, and hyperkeratosis of the epidermis. Such changes were significantly less severe in the *B. kazinoki* group. We also observed the infiltration of many inflammatory cells, including lymphocytes, and mast cells into the dermis and epidermis of the Biostir-AD group, but this was much less severe in the *B. kazinoki* group (Fig. 2A).

To investigate this phenomenon further, we measured the effect of topically applied *B. kazinoki* extract on the infiltration of mast cells by staining with toluidine blue. The Biostir-AD group showed increased infiltration of mast cells. This was significantly suppressed by topical application of the *B. kazinoki* extract, but not by the immunosuppressive drug Protopic® (Fig. 2B).

Effects of *B. kazinoki* on plasma levels of IgE and IL-4

When we measured plasma IgE and IL-4 concentrations in the experimental mice, we found that Biostir-AD (288.07 ± 23.19 ng/ml) increased the levels of IgE as compared with the control group (191.06 ± 46.9 ng/ml), whereas in the mice treated with the *B. kazinoki* extract (240.61 ± 25.80 ng/ml), the IgE level was reduced less than Biostir-AD (Fig. 3A). As for the IL-4 level, the *B. kazinoki* group (6.67 ± 0.62

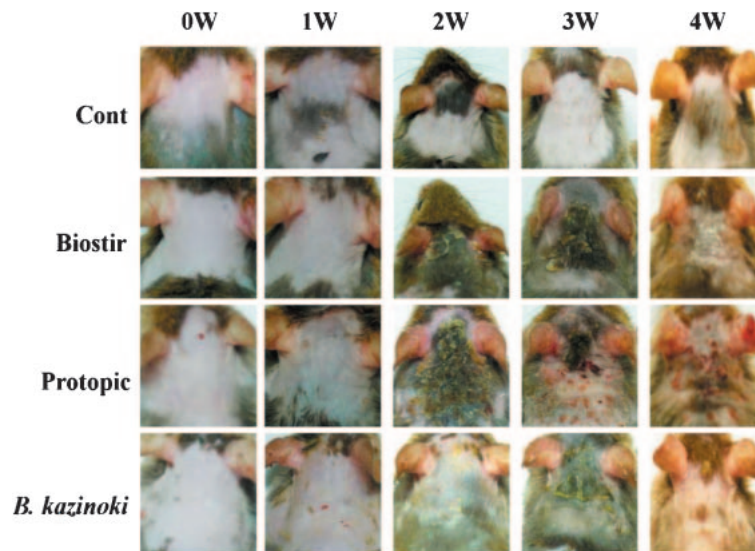


Fig. 1. Clinical Features of AD-Like Skin Lesions Topically Treated with Heartwood of *Broussonetia kazinoki* Sieb (*B. kazinoki*).

The photographs obtained from untreated (Cont), Biostir-AD plus vehicle-treated (Biostir), Biostir-AD plus Protopic®-treated (Protopic), and Biostir-AD plus *B. kazinoki* treated (*B. kazinoki*) NC/Nga mice. The features were monitored once a week during 4 weeks.

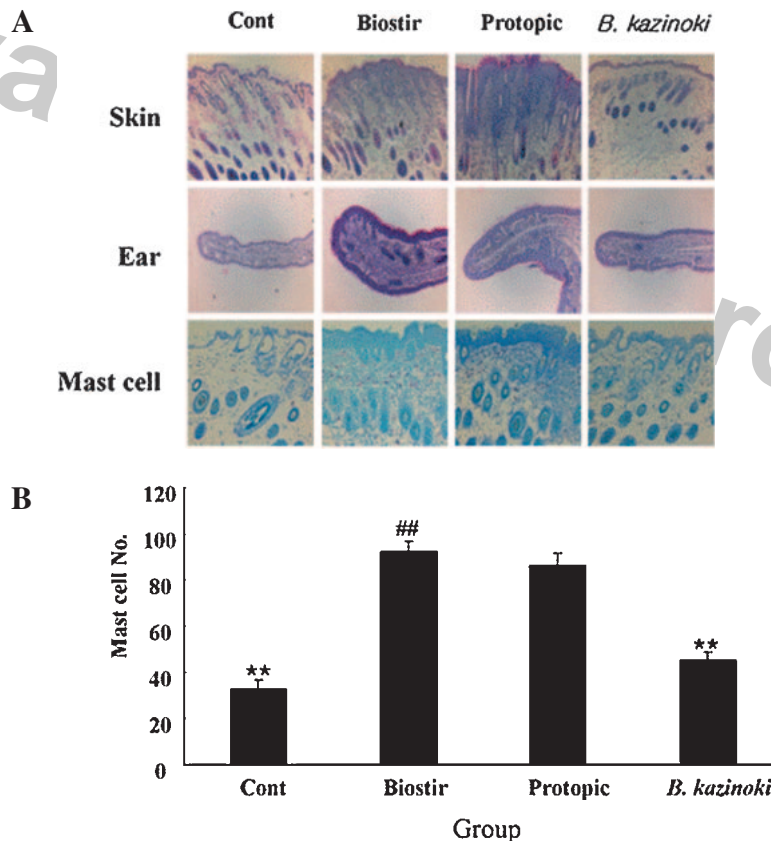


Fig. 2. Effects of Heartwood of *Broussonetia kazinoki* Sieb (*B. kazinoki*) on Biostir-AD Induced Histological Features and Infiltration of Mast Cells into the Skin of NC/Nga Mice.

The skin and ear tissues of untreated (Cont), Biostir-AD plus vehicle-treated (Biostir), Biostir-AD plus Protopic®-treated (Protopic), and Biostir-AD plus *B. kazinoki* treated (*B. kazinoki*) NC/Nga mice were excised, fixed with 10% formaldehyde, and embedded in paraffin, and thin sections were made. The histological features of the skin and ear lesions were stained H&E (hematoxylin and eosin) and the back skin sections were stained with toluidine blue (A). The number of mast cells was counted (B). (## $p < 0.01$ as compared with the control group; ** $p < 0.01$ as compared with Biostir group)

pg/ml) decreased significantly as compared with the Biostir-AD treatment group (15.00 ± 1.45 pg/ml) (Fig. 3B). In contrast, Protopic® had no effect on the IgE or the IL-4 concentration.

Inhibitory effect of B. kazinoki on TARC production in HaCaT cells

TNF- α plus IFN- γ treated HaCaT cells at 24 h strongly upregulated TARC, by about 5-fold (to 63.10 ± 4.64 μ g/ml) as compared with the control,

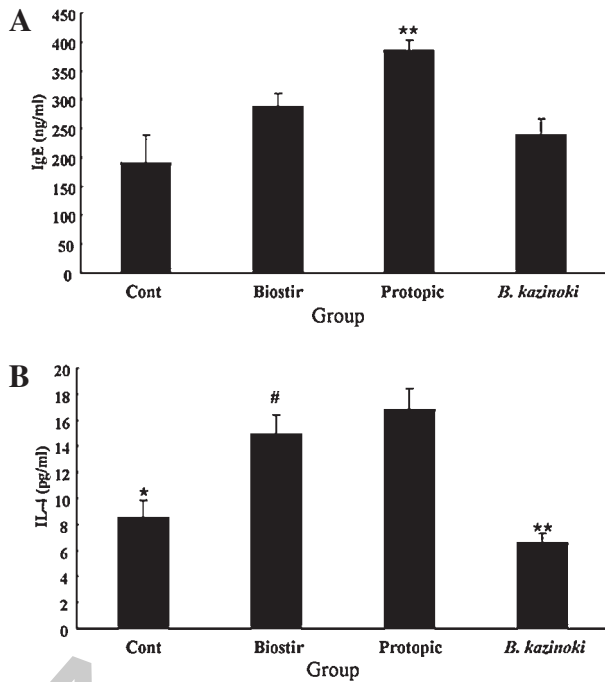


Fig. 3. Plasma Levels of IgE and IL-4 in the NC/Nga Mice.

Plasma samples were obtained from the untreated (Cont), Biostir-AD plus vehicle-treated (Biostir), Biostir-AD plus Protopic®-treated (Protopic), and Biostir-AD plus the heartwood of *Broussonetia kazinoki* Sieb treated (*B. kazinoki*) groups of NC/Nga mice. The plasma concentrations of IgE (A) and IL-4 (B) were measured by ELISA. (* $p < 0.05$ compare with control group; * $p < 0.05$, ** $p < 0.01$ as compared with Biostir group)

untreated cells ($12.78 \pm 0.80 \mu\text{g/ml}$) (Fig. 4A). The positive controls, silymarin and forskolin, inhibited TARC production in a concentration-dependent manner, as did *B. kazinoki* extract (50 $\mu\text{g/ml}$ extract, $62.36 \pm 2.61 \mu\text{g/ml}$, 100 $\mu\text{g/ml}$ extract, $50.06 \pm 1.89 \mu\text{g/ml}$, 250 $\mu\text{g/ml}$ extract, $16.11 \pm 0.35 \mu\text{g/ml}$).

Down regulation of TARC, MDC, and RANTES mRNA expression by *B. kazinoki*

We also tested the effects of *B. kazinoki* extract on the mRNA expression of TARC, MDC, and RANTES in HaCaT cells. We found that the levels of expression of these three transcripts markedly increased in the cells treated with TNF- α plus IFN- γ , but they were reduced by silymarin and forskolin, in a concentration-dependent manner. In addition, *B. kazinoki* extract concentration-dependently and significantly inhibited the expression of TARC, MDC, and RANTES mRNA (Fig. 4B).

Discussion

We investigated the effects of *B. kazinoki* on the development of AD-like skin lesions in NC/Nga mice, an animal model of human AD. Skin lesions clinically and histologically very similar to human AD, with increased numbers of eosinophils, mast cells, macrophages, and CD4+ T cells, develop spontaneously in NC/Nga mice, and there is a marked elevation in the plasma level of IgE, but not when the mice are raised under specific pathogen-free conditions.^{22,23} We found that repeated topical application of Biostir-AD induced AD in NC/Nga mice, as shown by the induction of

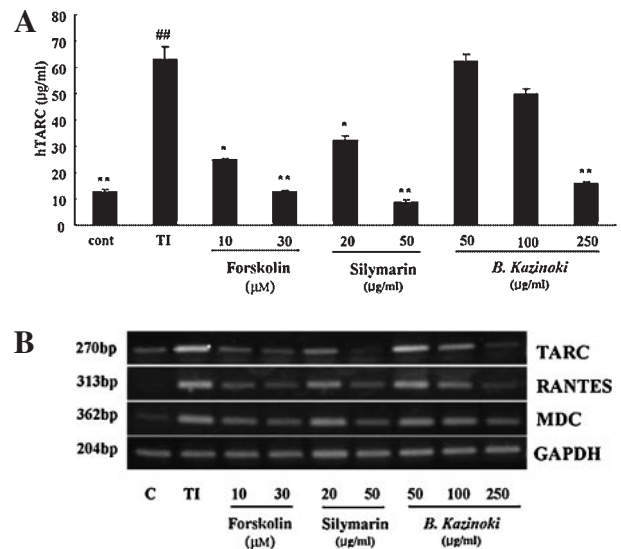


Fig. 4. Inhibitory Effects of Heartwood of *Broussonetia kazinoki* Sieb (*B. kazinoki*) on TNF- α Plus IFN- γ -Treated HaCaT Cells.

HaCaT cells were incubated in a medium containing *B. kazinoki* and were treated with TNF- α plus IFN- γ (TI) (10 ng/ml, each) for 24 h. *B. kazinoki* significantly inhibited the production of TARC in the TI-treated HaCaT cells. The concentration of TARC in the supernatant was determined by TARC-specific ELISA assay (** $p < 0.01$ as compared with the control group; * $p < 0.05$, ** $p < 0.01$ as compared with the TI group) (A). TARC, MDC, and RANTES mRNA expression levels were determined by RT-PCR analysis. *B. kazinoki* also suppressed the mRNA expression of TARC, MDC, and RANTES. The cells were treated with forskolin (10, 30 μM), silymarin (20, 50 $\mu\text{g/ml}$), and *B. kazinoki* (50, 100, 250 $\mu\text{g/ml}$) (B).

inflammation, hemorrhage, hypertrophy, and hyperkeratosis in eczematous skin lesions and by a significant increase in plasma levels of IgE and IL-4.

All the mice treated with Biostir-AD exhibited AD-like skin lesions, characterized by erythema, hemorrhage, excoriation, scaling, and dryness, whereas topically applied *B. kazinoki* extract reduced these symptoms. The severity of skin lesions was markedly reduced by treatment with *B. kazinoki* extract, but not by the immunosuppressive drug Protopic® (tacrolimus). We found that 0.1% w/v Protopic® did not have a significant effect on AD lesions in the NC/Nga mice.

Histologically, the lesions in the Biostir-AD group showed degeneration of the basal layer and infiltration of inflammatory cells, such as mast cells, into the skin. Mast cells are key effector cells involved in the development of various allergic diseases, including AD. We found that topical application of *B. kazinoki* significantly suppressed the infiltration of mast cells induced by Biostir-AD treatment, providing further evidence that topical application of this extract inhibits house-dust mite induced AD-like skin lesions in NC/Nga mice, suggesting that the *B. kazinoki* extract is a promising topical agent for AD patients.

Balance between the Th1 and Th2 responses is regarded as important for efficient immune system function, imbalances leading to various immune-related diseases, such as autoimmune and allergic diseases.²⁴ AD patients show a Th2-dominant phenotype, with increased expression of Th2 cytokines such as IL-4, IL-5, and IL-13.²⁵ As for the NC/Nga mice, the constitutive and enhanced Jak3 phosphorylation in B

cells was attributed to the high sensitivity of CD40L and IL-4 signaling.²⁶⁾ In general, IgE synthesis by B cells is primarily regulated by Th2 cytokines such as IL-4 and IL-13. We tested the effects of a topically applied *B. kazinoki* extract on the plasma concentrations of IgE and IL-4 in Biostir-AD-treated NC/Nga mice. We found that the extract significantly inhibited the plasma IgE and IL-4 level in the Biostir-AD induced AD-like NC/Nga mice. IL-4 is also a mast-cell growth factor. Mast cells have long been recognized as effector cells in allergy and asthma. Mast-cell responses are characterized by the production of vasoactive mediators, proteases, lipid-derived factors, and cytokines.²³⁾ The numbers of mast cells were reduced in the skin of the mice in the *B. kazinoki* group as compared with the Biostir group.

The chemokines TARC, MDC, and RANTES are thought to play important roles in the development of certain skin diseases such as AD.^{9–14)} Hence we tested the effects of the *B. kazinoki* extract on the secretion of TARC in TNF- α plus IFN- γ treated HaCaT cells and on the production of specific mRNA-encoding of TARC, MDC, and RANTES. Forskolin and silymarin were used positive controls. Forskolin, a cAMP inhibitor was previously found to reduce TARC and MDC in HaCaT cell supernatant,²⁷⁾ and silymarin was earlier found to suppress *Dermatophagoides pteronyssinus*-induced AD-like skin lesions in NC/Nga mice.²⁸⁾ *B. kazinoki* significantly inhibited TARC production on TNF- α plus IFN- γ -treated HaCaT cells, in a dose-dependent manner. The TNF- α plus IFN- γ -treated HaCaT cells also showed increased production of TARC, MDC, and RANTES mRNAs, as compared with the unstimulated cells, but this increased synthesis was dose-dependently suppressed by the *B. kazinoki* extract, as well as by silymarin and forskolin. These findings suggest that the *B. kazinoki* extract suppresses AD-like skin lesions by inhibiting the production of TARC, MDC, and RANTES. Since the adenylyl cyclase-cAMP system inhibits the production of TARC and MDC by suppressing NF- κ B through the p38 MAPK pathway, it is necessary to assay NF- κ B and p38 in these cells to determine whether the activity of *B. kazinoki* is associated with the adenylyl cyclase-cAMP system.

In conclusion, we found that topically applied *B. kazinoki* extract inhibited the development of AD-like skin lesions in NC/Nga mice. The extract also significantly inhibited plasma IgE and IL-4 levels. In the TNF- α plus IFN- γ -treated HaCaT cells, we found that the extract reduced TARC protein production and the expression of mRNA encoding the Th2-chemokines TARC, and MDC and the Th1-chemokine RANTES. These chemokines are related to infiltration of mast cells in the skin region. The *B. kazinoki* extract might act by modulating the Th1/Th2 imbalance in AD, perhaps by selectively inhibiting Th2 cell responses. It might prove by a new anti-inflammatory treatment for AD. We suggest that the heartwood extract from *B. kazinoki* suppresses IgE production in B cell and mast cell infiltration by IL-4 and chemokines via inhibition of Th2-cell activation by allergens.

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