

# Alleviation of atopic dermatitis-related symptoms by *Perilla frutescens* Britton

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**Abstract.** To ascertain whether an aqueous fraction of *Perilla frutescens* Britton (PfB/af) has advantageous anti-atopic dermatitis activity, we used a 2,4-dinitrofluorobenzene (DNFB)-induced animal model of atopic dermatitis symptoms to investigate the effects of the extract. We performed an ear swelling assay by comparing thickness of the DNFB-induced ear, and measured the numbers of eosinophils as well as total immune cells. We analyzed the expression levels of matrix metalloproteinase (MMP)-9, interleukin (IL)-31 and of the T-bet transcription factor. The results revealed that PfB/af (100 µg/ml) exhibited strong anti-atopic dermatitis activity, interceding drastic reduction (35%) of the immune response, as measured by the thickness of ear epidermis swelling, and resulting in decreased eosinophil levels (73.7%) in adjacent skin tissues. Collectively, the present results suggest that PfB/af has potential for mitigation of atopic dermatitis-like symptoms induced by DNFB in the mouse.

## Introduction

It is generally accepted that the daily consumption of plant-origin phytochemicals from vegetables, fruits, teas, or herb extracts may regulate the balance of redundant free-radical attacks (1-3). In the course of screening the active constituents of an immunomodulation from natural resources such as food and/or Oriental herb plants, we found that an aqueous fraction of *Perilla frutescens* Britton (PfB/af) scored as a good candidate by an *in vitro* assay (data not shown). *Perilla frutescens* is an ethnopharmacological plant in Korea and Japan, and its

leaf has been used as a food source such as MaeSilJangAhJji (in Korea) and Umeboshi (in Japan), which have anti-bacterial activities, and traditional medicine for colds, headaches and body aches, as a constituent of PaeDokSan in Korea. It has also been reported that the aqueous fraction of *Perilla frutescens* has potent anti-oxidant activity *in vitro* and *in vivo* when used as a hepatoprotective agent in an animal model (4,5).

On the other hand, atopic dermatitis is one of the foremost degenerative diseases, which presents as an unceasing inflammatory skin condition with a malfunctioning skin barrier (6). It is now well assumed that some proteases, including matrix metalloproteinases (MMPs), play a pivotal role in the immunohistological and clinical symptoms during the development of atopic dermatitis (7,8). With a few exceptions, classical skin without drastic damage does not exhibit MMP activities; therefore, the comparison of their enzyme activities is a vital index for the alleviation of an allergic reaction (9). However, there are only a few investigations on atopic-related disorders treated with a natural compounds/extracts, and it has been our main goal to investigate the biological effects of anti-atopic dermatitis activity by PfB/af.

In this study, we initially examined the ameliorating effects of PfB/af against 2,4-dinitrofluorobenzene (DNFB)-induced atopic dermatitis in mice. Various biochemical analyses, such as cytokine expressions, and phenotypic atopic symptoms were assessed in order to prove whether PfB/af has potential in alleviating the symptoms.

## Materials and methods

**Chemicals.** Interleukin (IL)-31, T-bet, and MMP-9 antibody were obtained from AnaSpec (no. 54582, Fremont, CA), Santa Cruz Biotechnology (no. 4B10, Santa Cruz, CA), and Cell Signaling (no. 3852, Danvers, MA), respectively. 2,4-Dinitrofluorobenzene (DNFB), formalin, hydrogen peroxide (all from Sigma-Aldrich, St. Louis, MO), and all other chemicals were commercially available.

**Animals and care.** C57BL/6 mice (6-7 weeks of age, male) were purchased from Samtaco Korea (Osan, Korea). They were housed in an air-conditioned animal room at a temperature of 22±1°C and with a humidity of 65±5%. All the procedures were performed in compliance with the Guiding Principles for the Care, and Use of Animals and with the in-house guidelines

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of the University (10), and the guidelines of the International Association for the Study of Pain Committee for Research and Ethical Issues (11). We strictly adhered to the rules and in-house guidelines for animal experiments. Animals were allowed to adapt to the laboratory atmosphere for at least 1 week prior to the experiments. The number of animals in each experimental group was limited to seven.

**Preparation and fractionation of samples.** The leaves of *Perilla frutescens* Britton were harvested from a farm of Song Kwang Mae Won Co., Chilgok, Korea between September and November, 2008. Leaves (100 g) were combined with a 3-fold volume of distilled water (200 ml), and extracted with an extractor (DWP-3800T; DaeWoong Co., Korea). The extracts were filtrated, lyophilized, and finally yielded a 1.3% aqueous extract (1.1 g/85 ml). The residue was then dissolved in appropriate buffers and adjusted to a concentration of 100 mg/ml, for further investigation (12). The fraction was used in various *in vitro* and *in vivo* assays. The voucher specimen of the plant has been deposited in the Lab of Food Enzyme Biotechnology, KNU.

**DNFB-induced animal model.** Mice were sensitized with DNFB and challenged as previously described (13). Briefly, the mice were soaked with 50  $\mu$ l of 0.5% DNFB solution onto their clipped abdominal skin for their first sensitization. A cutaneous reaction was evoked in the ear skin by repeated applications with 20  $\mu$ l of 0.2% DNFB solution. The DNFB challenge was repeated four times every three days for 2 weeks, starting at 5 days after the initial sensitization (Days 5, 8, 11 and 14). The vehicle mice were similarly treated with acetone without DNFB. The fraction was administered 8 times (Days 6, 7, 9, 10, 12, 13, 15 and 16) once a day for 2 weeks from the day after the first DNFB re-sensitization (Day 6), during the DNFB re-challenge.

**Immunohistochemistry.** Ear tissues were fixed for 24 h in a 10% neutral-buffered formalin solution and routinely processed as described elsewhere (12-14). In brief, paraffin sections were placed on Probe-On slides and incubated with methanol containing a 3% hydrogen peroxide solution, to inhibit endogenous peroxidase activity. The tissue sections were treated with a 10% normal goat serum for 1 h at room temperature, in order to block non-specific binding. The slides were subsequently incubated overnight at 4°C with rabbit anti-mouse MMP-9, IL-31 or T-bet antibodies.

**Statistical analysis.** Data are represented as the mean  $\pm$  standard deviation for the mean values. The statistical significance was determined by the Student's t-test or ANOVA test with independent means, by using a Microsoft Excel program (15). The critical level for significance was set at  $P < 0.05$ .

## Results and Discussion

*Perilla frutescens* was originally known as a food-friendly plant, because the leaf was used for anti-bacterial purposes against oral pathogenic bacteria (16). The plant's ripe fruit exhibits potential for ameliorating colds, coughs, insomnia, nerve system weakness and inflammation of various disor-

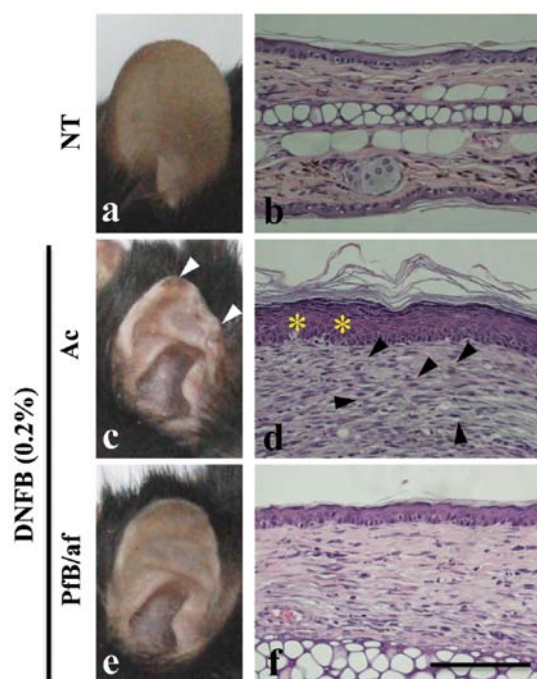


Figure 1. Effect of an aqueous fraction of *Perilla frutescens* Britton (PfB/af) on DNFB-induced ear swelling in C57BL/6 mice. A DNFB solution, an inducer of a chemically allergic reaction, was applied to a segment of clipped mouse abdominal skin. After initial sensitization, a cutaneous reaction was induced in the skin of the ear by repeated applications of 50  $\mu$ l amounts of 0.2% (w/v) DNFB solution on four occasions every 3 days for 2 weeks, beginning at 5 days after initial sensitization. Control vehicle mice were similarly treated with acetone (without DNFB). PfB/af was used at a concentration of 100  $\mu$ g/ml. (a, c and e) Clinical features of ear swelling as an index of the immune response, with (e) and without (c) PfB/af treatment after DNFB sensitization. (b, d and f) H&E staining exhibits the immune events in bulged tissues with (f) or without (d) sample treatment. DNFB-treated (d and f) and not treated groups (b). Asterisks indicates H&E-positive cells. Scale bar, 50  $\mu$ m.

ders (16-18). It has been reported that the constituents of the aqueous fraction of *Perilla frutescens* (PfB/af) exhibit *in vitro* antioxidant and free radical scavenging behaviors. They also include potent activities for antimicrobial, antitumor, antiviral, and anti-diabetic effects *in vitro* or *in vivo* (19-22). However, up to the present time, our knowledge has not gleaned sufficient data concerning its biological activities, and it has never been investigated whether or not PfB/af can be useful as an active whole food mixture in a DNFB-induced atopic dermatitis animal model.

In the present study, we evaluated the protective potential of PfB/af on exposure to DNFB, which is well recognized to induce allergic dermatitis in mice skin. First, we assessed whether the PfB/af could be effective on an atopic condition that is induced by DNFB. We, therefore, carried out a histopathological analysis to compare the inner immune events with the DNFB-induced atopic lesions. We cut the ear tissues, sectioned them, and applied histochemical staining to evaluate the numbers of immune cells during DNFB-induced inflammation. Immunohistochemical analyses revealed that PfB/af has the potential of alleviating DNFB-induced atopic symptoms in mice. It was shown that DNFB re-sensitization (0.2%) brought on severe scarring and eczema on the ear skin (Fig. 1c and d, arrowheads), but the treatment of PfB/af (100  $\mu$ g/ml) led to a

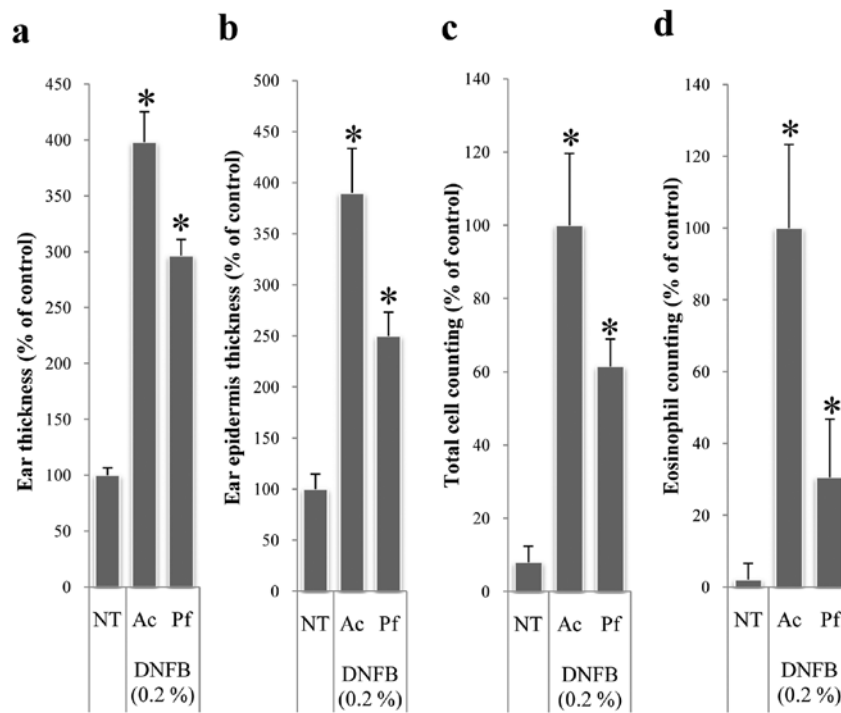


Figure 2. Comparison of atopic dermatitis-related phenotypic factors with or without application of an aqueous fraction of *Perilla frutescens* Britton (PfB/af). DNFB-treated process was carried out as described in Fig. 1. Cells were photographed under a phase contrast microscope (Nikon, TE-U1000, Tokyo, Japan). Data are expressed as means  $\pm$  standard deviations (n=5); \*P<0.05. Scale bar, 25  $\mu$ m; NT, not treated; Ac, DNFB alone dissolved in acetone; Pf, aqueous fraction of *Perilla frutescens* Britton.

recovery and alleviated the classical symptoms of atopic dermatitis (Fig. 1e and f), when compared to the control (Fig. 1a and b). To confirm the protective effects of the extract, we measured the ear swelling thickness (Fig. 2a and b). As shown in Figs. 1b and 2a, the ear thickness of control mice was  $167.7 \pm 19.7 \mu$ m, and DNFB treatment triggered ear swelling, exhibited by a 3.9-fold increase in swelling (Figs. 1d and 2a). However, the swelling was significantly decreased, to a 35% level in the ear epidermis thickness within the control group (Figs. 1f and 2b), by the application of PfB/af. This result indicates that the PfB/af treatment reduced the ear swelling thickness by regulating the dermal microenvironment.

The control mice have a classical ear thickness and their clinical observations were identified without any abnormalities (Fig. 1a and b). When the DNFB treatment enlarged the inflammation and broadened their thickness by expressing clear scars and eczema (Fig. 1c and d), the evident inflammatory stained cells increased in number (arrowheads in Fig. 1d). On the contrary, the expressions of inflammation-related cells were markedly reduced by the treatment of PfB/af (100  $\mu$ g/ml), which is shown in the H&E staining (magnification, x400; Fig. 1e and f). Total cell counting revealed that the inflammatory cells, by induction of DNFB, marked a 16.6-fold increase in number, whereas that of the PfB/af-treated group was decreased to 58.4% of the control (Fig. 2c). The numbers of eosinophils were  $10.6 \pm 2.3$  cells/block in the DNFB alone group, and  $3.0 \pm 1.5$  cells/block in the PfB/af-treated group (Fig. 2d). This 73.7% of decrease in the level of eosinophil numbers may be associated with the active constituents of PfB/af with inhibitory activity (Fig. 2d).

The balance between MMPs is largely responsible for the remodelling of skin tissues (9). Various pathological conditions

are caused by the unbalanced relationship between MMPs and their inhibitors around the skin's boundary. In both conditions, it appears that the erosion or eczema of skin epithelium is associated with an overexpression of MMP-1, -3, -8, -9 and/or -13 (9,10). In particular, there are certain data that MMP-8 and MMP-9 play mostly a pivotal role in skin remodeling and re-construction during a chemically-induced inflammatory response. Therefore, we examined whether PfB/af affects MMP activity by immunohistochemistry. Fig. 3g-i, illustrates the effects of the DNFB-induced atopic trigger on the expression of MMP-9. Interestingly, the MMP-9 expression levels of the PfB/af treatment (100  $\mu$ g/ml) were markedly reduced as compared with that of the DNFB-induced group (compare h and i), suggesting that PfB/af can inhibit DNFB-induced skin erosion (Fig. 4c).

It is now recognized that IL-31, preferentially produced by T helper type 2 cells, is over-expressed with severe pruritus, alopecia, and skin lesions (23,24). Furthermore, the IL-31 receptor expression was increased in diseased tissues derived from an animal model of allergic dermatitis and airway hypersensitivity (25). The above facts led us to examine whether the presence of PfB/af can ameliorate skin lesions. As shown in Fig. 3a-c, we clearly witnessed a decrease of IL-31 positive cells, which was scored approximately at 19.9% of DNFB alone (Fig. 4a). On the other hand, T-bet exhibited reverse effects. Convincing data can be obtained from T-bet positive cells (Fig. 3d-f). We have already made a stable cell line harboring T-bet promoter genes, in order to select some hits to activate the promoter gene, resulting in a regulated balance between Th1 and Th2 cells (26). Positively in our intention, within the T-bet promoter assay, PfB/af-induced a 1.5-fold increase in the gene expression at 300  $\mu$ g/ml (data not shown). Moreover, the T-bet protein expression was also

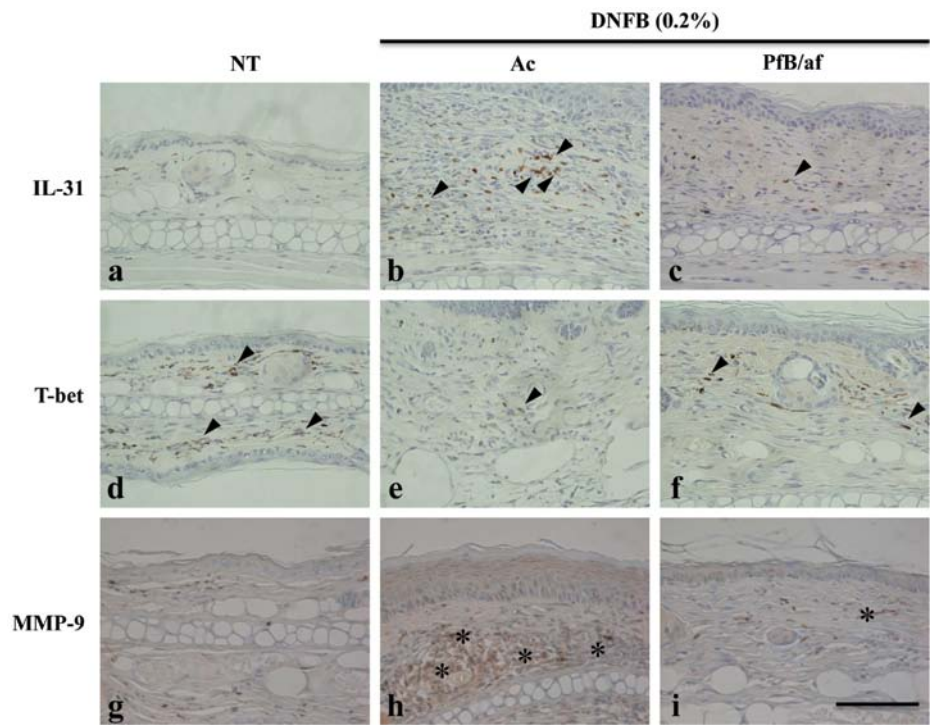


Figure 3. Comparison of atopic dermatitis-related biomarker expression with or without application of an aqueous fraction of *Perilla frutescens* Britton (PfB/af). The expression levels of IL-31, and T-bet, or MMP-9 positive cells in DNFB-treated mice, either exposed to PfB/af (c, f and i) or not (a, b, d, e, g and h), were assayed by immunohistochemical analysis as described in ‘Materials and methods’. Arrowheads indicate cells expressing the designated proteins (b, c, d, e and f). Data shown are representative of the features of each group of 7 mice. Scale bar, 25  $\mu$ m.

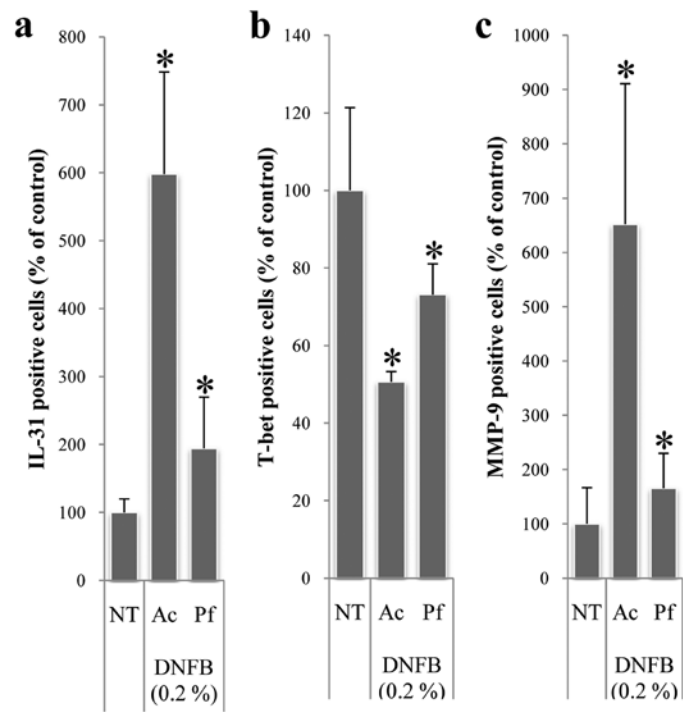


Figure 4. Analysis of atopic dermatitis-related biomarker expressions with or without application of an aqueous fraction of *Perilla frutescens* Britton (PfB/af). The measurement of IL-31 (a), and T-bet (b), or MMP-9 (c) responsive cells in DNFB-treated mice or not was assayed as described in ‘Materials and methods’. Data are the means  $\pm$  standard deviations of three independent areas of Fig. 3 data. \*P<0.05. NT, not treated; Ac, DNFB alone dissolved in acetone; Pf, aqueous fraction of *Perilla frutescens* Britton.

augmented with a 1.4-fold increase from the PfB/af treatment (Figs. 3d-f and 4b). These results indicate that PfB/af regulated the biomarkers regarding atopic dermatitis by balancing Th1

and Th2 cells. The T-bet gene is a unique transcription factor which is expressed during Th1 and Th2 cell differentiation, that was identified from asthmatic symptoms in children (27,28).



Therefore, it is probable that anti-asthmatic compounds/extracts from *Helianthus annuus*, *Camellia sinensis*, *Angelica archangelica*, or *Spinacia oleracea* may exhibit potential in mitigating atopic effects in mice (10,25,28,29).

In conclusion, we proved that PfB/af inhibited DNFB-induced atopic inflammation by alleviating MMP-9 and IL-31, but also augmenting T-bet activity. The present data show that PfB/af has potential in controlling the atopic molecular markers by balancing the Th cell subsets. If we can efficiently modulate the anti-atopic activity by controlling the atopic-related molecular marker(s), a precise preventive medication as a whole food against atopic dermatitis could be adopted.

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