

Inhibitory effect of eupatilin and jaceosidin isolated from *Artemisia princeps* in IgE-induced hypersensitivity

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Abstract

To understand the antiallergic effect of *Artemisia princeps* (AP), which has been found to show inhibitory activity against degranulation and a passive cutaneous anaphylaxis (PCA) reaction, eupatilin and jaceosidin, as the active components, were isolated by degranulation–inhibitory activity-guided fractionation, with their antiallergic activity investigated. These isolated components potentially inhibited the release of β -hexosaminidase from RBL-2H3 cells induced by the IgE–antigen complex, with IC₅₀ values of 3.4 and 4.5 μ M, respectively. Eupatilin and jaceosidin potently inhibited the PCA reaction and scratching behaviors induced by IgE–antigen complex and compound 48/80, respectively. Orally administered jaceosidin more potently inhibited the PCA reaction than that of eupatilin, although the PCA reaction–inhibitory activity of intraperitoneally administered jaceosidin was nearly the same as that of eupatilin. Eupatilin and jaceosidin inhibited the gene expressions of TNF- α and IL-4 in RBL-2H3 cells stimulated by IgE–antigen complex. Eupatilin and jaceosidin inhibited the activation of NF- κ B. Based on these findings, eupatilin and jaceosidin may be useful for protection from the PCA and itching reactions, which are IgE-mediated representative skin allergic diseases.

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Keywords: *Artemisia princeps*; Eupatilin; Jaceosidin; Allergy; IgE; Hypersensitivity

1. Introduction

Mast cells and basophils are well-known as a critical participant in various biological processes, including allergic diseases [1–4]. These cells express IgE on their surface membrane receptors, with high affinity and

specificity. The interaction of multivalent antigens with surface-bound IgE initiates a series of biochemical events, which culminate in the release of histamine and the production of cytokines. These are then stored within numerous cytoplasmic granules, as biologically potent inflammatory mediators, and causes allergic diseases, such as anaphylaxis and rhinitis. Anti-histamines, steroids and immunosuppressants have been used against allergic diseases [5–7], but improvement of these diseases is too difficult. Therefore, traditional herbal medicines have been developed

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against allergic diseases, and their effectiveness has received increasing attention [8].

Artemisia princeps Pampanini (AP) and *Artemisia asiatica* Nakai (Family Asteraceae) are widely used in traditional medicine, and their pharmacologically active constituent is eupatilin, which has inhibitory effects on gastric ulcers, ulcerative colitis, naproxen-induced and ethanol-induced gastric damage, as well as in experimental animals [9–11]. It also induced apoptosis in human promyelocytic leukemia cells and cell cycle arrest in ras-transformed human mammary epithelial cells, and inhibited tyrosine kinase in activated guinea pig lung mast cells [12–14]. Recently, Kim et al. reported that *Artemisia asiatica* ameliorates airway inflammation of allergic asthma in mice, and our previous study also found that *Artemisia princeps* (AP) inhibited an in vivo passive cutaneous anaphylaxis (PCA) reaction during the screening program for the discovery of such compounds from natural products [15,16]. However, studies on the active component and its inhibitory mechanism remain to be thoroughly investigated.

Therefore, in the present study, anti-degranulation activity-guided fractionation of AP was performed, the active components isolated, and their antiallergic activity and inhibitory effect on mast cell cytokine biosynthesis investigated.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle medium (DMEM), anti-dinitrophenol (DNP)-IgE, DNP-human serum albumin (HSA), bovine serum albumin, Evans blue, disodium cromoglycate (DSCG), azelastine and dexamethasone were purchased from Sigma Co. (St Louis, MO, USA). TRI reagent was purchased from Molecular Research Center Inc. (Cincinnati, Ohio, USA).

2.2. Isolation of degranulation-inhibitory components from AP

AP, artificially cultured in GangHwa-Do, Korea, was collected, with a voucher specimen (KHU05067) deposited in the Laboratory of Natural Product Chemistry, Kyung Hee University, Korea. AP was extracted with 80% ethanol, evaporated in vacuo and suspended in water, and stepwise fractionated with ethyl acetate and butanol. The active compounds isolated from the degranulation-inhibitory fraction (ethyl acetate fraction) were isolated by silica gel column chromatography, and identified as eupatilin and jaceosidin (Fig. 1) through comparison of their spectroscopic data with those of previously reported in the literature [11,17,18].

Eupatilin; yellow amorphous powder; mp 226–228°C; IR (KBr, cm^{-1}) 3390, 3266, 1655, 1514; EI-MS m/z (70eV): 344 [M^+].

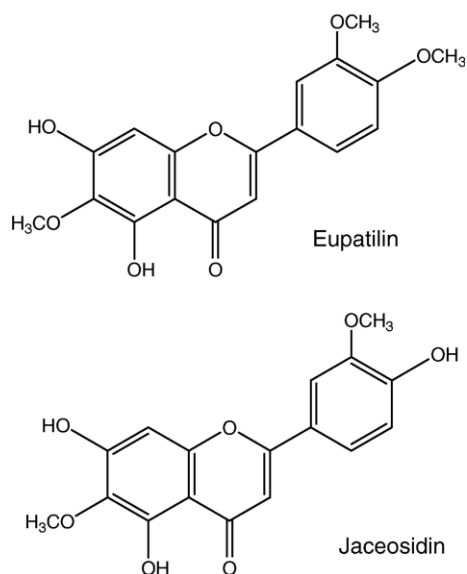


Fig. 1. The structures of eupatilin and jaceosidin isolated from *Artemisia princeps* Pampani.

Jaceosidin; yellow amorphous powder; mp 229–230°C; IR (KBr, cm^{-1}) 3360, 1662, 1610, 1515; EI-MS m/z (70eV): 330 [M^+].

2.3. Assay of RBL-2H3 cell degranulation by stimulation with IgE–DNA–HSA complex

The inhibitory activity of test ginsenosides against the release of β -hexosaminidase from RBL-2H3 cells was evaluated according to Choo et al. [19]. RBL-2H3 cells were grown in DMEM supplemented with 10% fetal bovine serum and L-glutamine and split every three days. Before experiment, the cells were dispensed into 24-well plates (5×10^5 cells per well), using the medium containing 0.5 $\mu\text{g}/\text{ml}$ mouse monoclonal IgE and were incubated overnight at 37°C in 5% CO_2 for sensitization of cells. The cells were washed with 500 μl of Siraganian buffer [pH 7.2, 119mM NaCl, 5mM KCl, 0.4mM MgCl_2 , 25mM piperazine- N,N' -bis(2-ethanesulfonic acid), 40mM NaOH] and incubated in 160 μl of Siraganian buffer containing 5.6mM glucose, 1mM CaCl_2 and 0.1% bovine serum albumin for additional 10min at 37°C. Then cells were exposed to 40 μl of test agents for 20min, followed by the treatment with 20 μl of antigen (DNP–HSA, 1 $\mu\text{g}/\text{ml}$) for 10min at 37°C to activate cells to evoke allergic reactions. The reaction was stopped by cooling in an ice bath for 10min. The reaction mixture was centrifuged at 800 $\times g$ for 10min and 25 μl aliquots of supernatant were transferred to 96-well plates and incubated with 25 μl of 1mM p-nitrophenyl-N-acetyl- β -D-glucosaminide for 1h at 37°C. The reaction was stopped by adding 200 μl of 0.1M $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, (pH 10.5). The absorbance was measured by ELISA reader at 405nm.

To evaluate the cytotoxic effect of tested agents against RBL-2H3 cells, the cells incubated overnight at 37°C in the

medium containing 0.5 µg/ml mouse monoclonal IgE were treated in various concentrations of test agents for 2 h and then their viabilities determined by MTT [3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay according to the method of Carmichael et al. [20].

2.4. Enzyme-linked immunosorbent assay (ELISA) and immunoblot

RBL-2H3 cells (5×10^5 cells), previously cultured in DMEM, were treated with 0.5 µg/ml mouse monoclonal IgE for sensitization of the cells. The cells (1.8 ml) were exposed to 0.2 ml of the test agents (dissolved in 0.5% dimethyl sulfoxide) for 4 h, followed by treatment with 0.2 ml of DNP-HSA (1 µg/ml) for 40 min at 37°C. The supernatant (50 µl) was transferred into 96-well ELISA plates, and the IL-4 and TNF-α concentrations then determined using commercial ELISA Kits (Pierce Biotechnology, Inc., Rockford, IL, USA) [21].

The protein of the collected cells was subjected to electrophoresis on 8–10% sodium dodecyl sulfate-polyacrylamide gel, as previously described [10,22], and then transferred to a nitrocellulose membrane. The TNF-α and IL-6 were assayed using anti-TNF-α and anti IL-6 antibodies. The MAPK (mitogen-activated protein kinase) p38, and its activated form in the cell fraction, lysed using 200 mM Hepes buffer (pH 7.3) containing 1% Triton X-100, 1 mM EDTA, 50 mM NaF, 2.5 mM p-nitrophenyl phosphate, 1 mM Na_3VO_4 , 10 µg/ml PMSF, 10 µg/ml leupeptin, and 10% glycerol, were determined using their corresponding antibodies [23]. The cytosolic IκBα, phospho-IκBα and nucleic p65 NF-κB were assayed using their corresponding antibodies, according to the previously reported method [19]. Immunodetection was carried out using an enhanced chemiluminescence detection kit.

2.5. Animals

Male ICR and BALB/c mice (20–25 g, 6 weeks old) were supplied from Orient Experimental Animal Breeding Center (Seoul, Korea). All animals were housed in wire cages at 20–22°C and 50 ± 10% humidity, fed standard laboratory chow (Orient Experimental Animal Breeding Center, Seoul, Korea) and allowed water ad libitum. All procedures relating to the animals and their care conformed to the international guidelines, Principles of Laboratory Animals Care, (NIH publication no. 85-23, revised 1985).

2.6. Assay of PCA reaction

An IgE-dependent cutaneous reaction was measured according to the previous method of Choo et al. [19]. Male ICR mice (25–30 g, 6 weeks old) were injected intradermally with 50 µl of anti-DNP IgE (10 µg) into each of two dorsal skin sites that had been shaved 48 h earlier. The sites were outlined with a water-insoluble red marker. Forty-eight hours later each mouse received an injection of 200 µl of 3% Evans blue PBS containing 200 µg of DNP-HSA via the tail vein.

Test agents dissolved in 1% carboxyl methyl cellulose were orally or intraperitoneally administered 1 h prior to DNP-HSA injection. Thirty min after DNP-HSA injection, the mice were sacrificed and their dorsal skins were removed for measurement of the pigment area. After extraction with 1 ml of 1.0 N KOH and 4 ml of a mixture of acetone and 0.6 N phosphoric acid (13:5), the amount of dye was determined colorimetrically (the absorbance at 620 nm).

2.7. Assay of scratching behavioral frequency

Male BALB/c mice (25–30 g, 6 weeks old) were placed into acrylic cages (22 × 22 × 24 cm) for approximately 10 min to allow for acclimation. The behavioral experiments were performed according to the method reported by Sugimoto et al. [24]. The samples were administered orally 1 h before administering the scratching agent. Compound 48/80 was dissolved in saline. The rostral part of the skin on the back of the

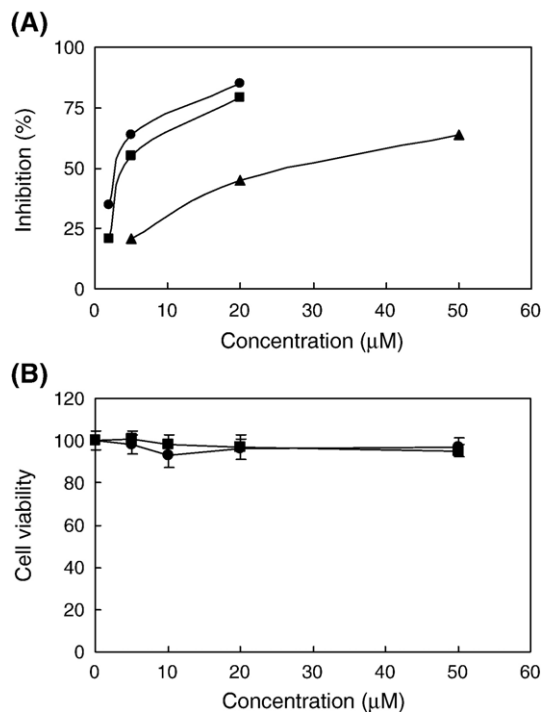


Fig. 2. The inhibitory effects of eupatilin and jaceosidin on the degranulation of RBL-2H3 cells. (A) Degranulation-inhibitory effect. RBL-2H3 cells (5×10^5 cells) were treated with 0.5 µg/ml mouse monoclonal IgE, exposed to 0.2 ml of the agents (closed circle, eupatilin; Square, jaceosidin; triangle, azelastine) for 20 min, followed by treatment with 0.2 ml dinitrophenol-human serum albumin for 10 min, with the β-hexosaminidase activity released due to degranulation then measured. (B) Cytotoxicity. RBL-2H3 cells incubated overnight in the medium containing 0.5 µg/ml mouse monoclonal IgE were treated in various concentrations of test agents (closed circle, eupatilin; Square, jaceosidin) for 2 h and their viabilities determined by MTT assay. Values represent the mean ± S.D. for triplicate experiments.

Table 1

Inhibitory effects of eupatilin and jaceosidin on the passive cutaneous anaphylaxis reaction induced in mice by IgE

Agent	Dose (mg/kg)	Inhibition (%)	
		<i>p.o.</i>	<i>i.p.</i>
Eupatilin	5	–	48 ± 7 ^a
	10	26 ± 8 ^a	69 ± 12 ^b
	50	43 ± 7 ^b	– ^c
Jaceosidin	5	–	50 ± 12 ^a
	10	41 ± 8 ^b	67 ± 7 ^b
	50	59 ± 9 ^c	–
Azelastine	10	76 ± 6 ^d	87 ± 8 ^c

The passive cutaneous anaphylaxis reaction in mice was induced by an intradermal injection of anti-DNP–HSA. Tested agents were orally or intraperitoneally administered 60 min prior to the challenge with DNP–HSA antigen. The amounts of extravasated Evan blue in the dorsal skin (1 × 1 cm) of the control stimulated with the IgE–antigen complex and vehicle-treated groups were 25 ± 3 and 11 ± 2 μg, respectively. Values are expressed as the means ± S.D. (*n* = 5).

^{a,b,c,d}) Items with the same letter in each column were not significantly different.

^c) Not determined.

mice was clipped, and 50 μg/50 μl of compound 48/80 was injected intradermally into each mouse using a 29ga needle. The control mice received a saline injection in place of compound 48/80. Immediately after the intradermal injection, the mice (one animal/cage) were placed back into their cage and their scratching behaviors were recorded automatically using an 8-mm video camera (SV-K80, Samsung, Seoul, Korea). The frequency of scratching of the injection site by the hind paws was determined and compared with that of other sites, such as the ears. Each mouse was used for only one experiment. The mice generally scratched for 1 s, and a series of these behaviors was counted as a single scratching incident for 1 h.

2.8. Statistical analysis

All the data were expressed as the mean ± standard deviation, and statistical significance analyzed using one way ANOVA followed by the Student–Newman–Keuls test.

3. Results

3.1. Inhibition of eupatilin and jaceosidin on β-hexosaminidase release from RBL–2H3 cells

During the screening program to discover antiallergic agents from natural products, AP was found to show inhibitory activity against the degranulation and PCA reaction induced by the IgE–antigen complex. Therefore, degranulation–inhibitory activity-guided fractionation against AP was performed, with eupatilin and jaceosidin isolated as the active components. These isolated components potently inhibited the release of β-hexosaminidase from RBL–2H3 cells induced by the IgE–antigen complex (Fig. 2), with IC₅₀ values of 3.4 and

4.5 μM, respectively. Their inhibitory effects were more potent than that of azelastine, which has an IC₅₀ value of 32 μM. These agents at a concentration of less than 50 μM did not show the cytotoxicity against RBL–2H3 cells.

3.2. Inhibition of eupatilin and jaceosidin on PCA reaction

The inhibitory effects of eupatilin and jaceosidin on the PCA reaction induced in mice due to the IgE–antigen complex were investigated (Table 1). Orally and intraperitoneally administered eupatilin and jaceosidin potently inhibited the PCA reaction. Intraperitoneally administered eupatilin and jaceosidin showed more potent inhibition against the PCA reaction than when orally administered. When these components were orally administered, jaceosidin more potently inhibited the PCA reaction than eupatilin, but when intraperitoneally administered, the PCA reaction–inhibitory activity of jaceosidin was significantly different from that of eupatilin.

3.3. Inhibition of eupatilin and jaceosidin on scratching behaviors

The induced inhibitory activities of eupatilin and jaceosidin in compound 48/80 were also investigated on scratching behavioral mice (Table 2). Compound 48/80 was found to induce scratching behaviors. Eupatilin and jaceosidin potently inhibited the scratching behavior frequency, and at doses of 50 mg/kg inhibited the scratching behavior frequency by 31 and 48%, respectively. Their inhibitory activities against scratching behaviors were in proportion to their ability to prevent the PCA reaction.

3.4. Inhibition of eupatilin and jaceosidin on cytokine production of RBL–2H3 cells

The inhibitory effects of eupatilin and jaceosidin against protein expression of IL-4 and TNF-α were also measured in RBL–2H3 cells stimulated by the IgE–antigen complex using ELISA analysis (Fig. 3). Eupatilin and Jaceosidin, at a concentration of 20 μM, inhibited the expressions of these

Table 2

Inhibitory effects of eupatilin and jaceosidin on the compound 48/80-induced mouse scratching behaviors in mice

Agent	Dose (mg/kg)	Inhibition (%)
Eupatilin	10	16 ± 8 ^a
	50	31 ± 6 ^b
Jaceosidin	10	23 ± 9 ^a
	50	48 ± 8 ^c
Azelastine	10	47 ± 3 ^d

Scratching behaviors in mice were induced by an intradermal injection of compound 48/80. Tested agents were orally administered 60 min prior to the challenge with compound 48/80.

Values are expressed as mean ± S.D. (*n* = 5).

^{a,b,c,d}) Items with the same letter in each column were not significantly different.

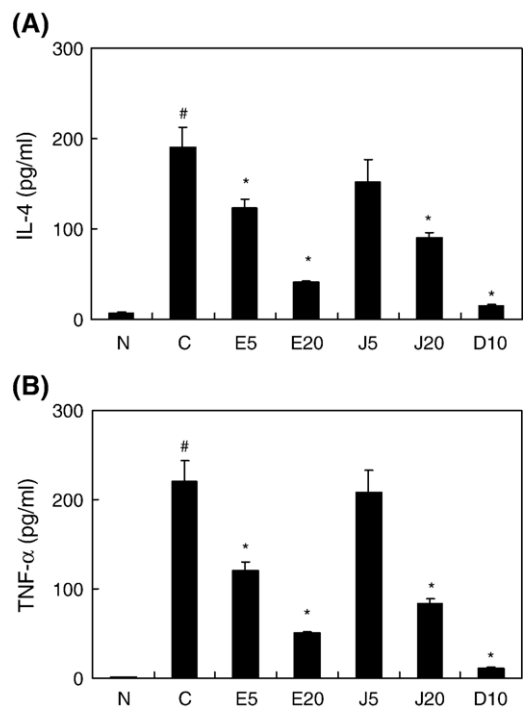


Fig. 3. The effects of eupatilin and jaceosidin on the levels of expression of IL-4 and TNF- α of RBL-2H3 cells induced by IgE. RBL-2H3 cells (5×10^5 cells) were treated with 0.5 μ g/ml mouse monoclonal IgE, exposed to 0.2 ml of the agents (C, vehicle alone; E5, 5 μ M eupatilin; E20, 20 μ M eupatilin; J5, 5 μ M jaceosidin; J20, 20 μ M jaceosidin; D10, 10 μ M dexamethasone) for 4 h, followed by treatment with 0.2 ml dinitrophenol-human serum albumin (DNP-HSA, 1 μ g/ml) for 4 h at 37 $^{\circ}$ C and the levels of IL-4 (A) and TNF- α (B) were measured by ELISA. Normal (N) was treated with vehicle alone instead of an agent and IgE-antigen. Values represent the mean $\pm$ S.D. for triplicate experiments. [#]Significantly different from the normal control group ([#] $P < 0.05$). ^{*}Significantly different from the control group (^{*} $P < 0.05$).

cytokines by 73 and 72%, and 51 and 63%, respectively. Eupatilin and jaceosidin inhibited the mRNA expressions of TNF- α , IL-4 and IL-6. The inhibitory effects of eupatilin against these protein expressions was more potent than those of jaceosidin.

3.5. Effect of eupatilin and jaceosidin on activation of NF- κ B

To understand the inhibitory mechanism of proinflammatory cytokines in RBL-2H3 cells stimulated by the IgE-antigen, the effects of eupatilin and jaceosidin on the activation of NF- κ B were investigated (Fig. 4). The treatment of the IgE-antigen complex to RBL-2H3 cells also activated p-I κ B α and NF- κ B in the cytosolic and nuclei fractions, respectively, thus, the I κ B was reduced. However, the treatment of eupatilin and jaceosidin attenuated the NF- κ B activation induced by the IgE-antigen complex. The inhibitory effect of eupatilin was slightly more potent than that of jaceosidin.

4. Discussion

Azelastine, a representative antiallergic medicine [7], does not exhibit an H1-receptor antagonistic effect, but does decrease degranulation (mediator release from mast cells and basophils). In the present study, azelastine was also found to inhibit chemical mediator release from RBL-2H3 cells. Eupatilin and jaceosidin, which were isolated from *Artemisia* species as well as *Saussurea* species [9,11,17,18,25], also potently inhibited the degranulation of RBL-2H3 cells.

Mast cells produce histamine, as well as proinflammatory cytokines, especially TNF- α , IL-4 and IL-6 [26]. These cytokines have a critical biological role in the allergic reaction. Mast cells are a principal source of TNF- α in human dermis, and degradation of mast cells in the dermal endothelium is abrogated by the inhibition of the action of TNF- α [27]. IL-6 produced from mast

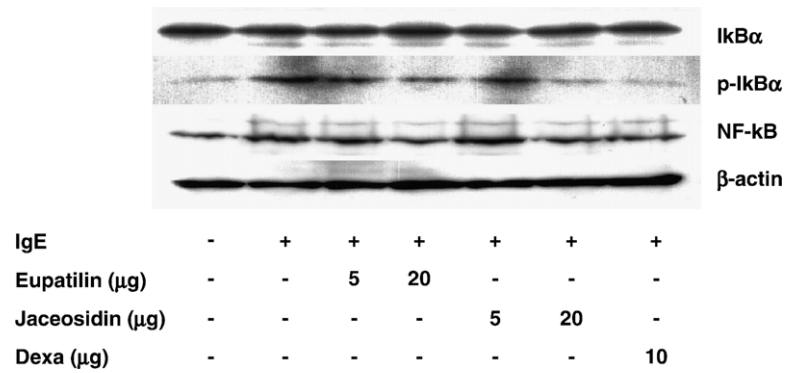


Fig. 4. The inhibitory effects of eupatilin and jaceosidin on the NF- κ B activation. RBL-2H3 cells were stimulated by IgE, and the treated with eupatilin and jaceosidin, and then stimulated with the antigen (DNP-HSA). The cells were collected 1 h after the antigen treatment. The cell extract was analyzed for I κ B α and p-I κ B α in cytosolic fraction and NF- κ B in nuclei fraction using immunoblotting.

cells, when locally accumulated, is associated with a PCA reaction [28]. The inhibition of proinflammatory cytokine expression from mast cells must be one of the key indicators of reduced allergic symptoms. In the present study, eupatilin and jaceosidin were found to inhibit the degranulation and gene expressions of TNF- α , IL-4 and IL-6 in IgE-induced RBL-2H3 cells. However, the inhibitory potencies of eupatilin and jaceosidin against the degranulation was not identical to those for IL-4 and TNF- α expression. These results suggest that the inhibitory mechanism of the components against the expression of IL-4 and TNF- α cytokines may be independent on the inhibition of degranulation. Eupatilin and jaceosidin also inhibited the in vivo mouse PCA reaction induced by the IgE-antigen complex and the scratching behaviors induced by compound 48/80. These results suggest the allergic effects of eupatilin and jaceosidin may be due to the inhibition of degranulation and cytokine biosynthesis. Neither the in vitro antiallergic effect and anti-degranulation activity of eupatilin and jaceosidin nor the in vivo anti-allergic effects, such as anti-scratching behavior and anti-PCA reaction activities, of intraperitoneally administered eupatilin and jaceosidin were significantly different. However, the in vivo anti-allergic effects of orally administered eupatilin were less potent than those of jaceosidin. These findings may have been due to the difference in their absorptions from the intestine into blood, their metabolisms by intestinal microflora and/or unknown mechanisms.

To understand the inhibitory mechanism of eupatilin and jaceosidin against the PCA reaction and proinflammatory cytokine expression, the effect of eupatilin on the NF- κ B activation was examined. The NF- κ B activation is an important signaling pathway in immune responses [22,26]. The activation of NF- κ B is essential for the expression of the proinflammatory cytokines. The IgE-antigen complex was found to activate NF- κ B in RBL-2H3 cells. Eupatilin and jaceosidin inhibited the activation of NF- κ B, which is known to increase the productions of TNF- α and IL-6. These results demonstrate that eupatilin and jaceosidin can attenuate the activation of NF- κ B and down-regulate the productions of TNF- α and IL-6.

Based on these findings, eupatilin and jaceosidin may be useful in the protecting against PCA reaction and itching reactions, which are representative IgE-mediated skin allergic diseases.

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