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Article

Suppressive Effects of Korean Indigenous *Acanthopanax divaritacus* on the Allergic Inflammation

Seul-Ki Park, Jum-Ji Kim, Yu-Mi Jeon and Mi-Young Lee*

Department of Medical Biotechnology, SoonChunHyang University, Asan 336-600, Korea

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The water extracts of root, stem, and leaf from Korean indigenous *Acanthopanax divaritacus* were examined for their suppressive effects against allergic inflammations such as lipoxygenase activity, β-hexosaminidase release, inflammatory cytokine production, and serum IgE level. The root extract inhibited the release of β-hexosaminidase, a degranulation marker, from rat basophilic leukemia cells (RBL-2H3) much more potently than the stem and leaf extracts. The root extract also significantly reduced the expression of TNF-α and IL-1β in the RBL-2H3 cells challenged with antigen. Moreover, there was a significant fall in the serum IgE level by the treatment of the root extract. Taken together, the root extract could be the most potent inhibitor of allergic inflammation, suppressing β-hexosaminidase release and inflammatory cytokine expression, as well as reducing the rise of serum IgE level.

Key words: *Acanthopanax, anti-allergic inflammation, β-hexosaminidase, serum IgE*

About 20% of the human population are afflicted with allergic diseases such as atopic dermatitis, asthma, allergic rhinitis, and food allergy. Especially, patients of atopic dermatitis sensitized to the environmental and food allergens have been rapidly increasing in most countries. During the allergic responses, mast cells have been thought to play a major role in the development of a variety of physiological changes [Okunuki *et al.*, 2000]. Mast cell activations both by IgE-dependent and -independent stimuli, bring about the process of degranulation that results in the fusion of the cytoplasmic granules with the plasma membrane. This is accompanied by the fast external release of the granule-associated stored mediators, including histamine, serotonin, neutral proteases, chemotactic factors, and cytokines. Moreover, newly generated mediators, such as the products of arachidonic acid metabolism and an array of cytokines are released [Alber *et al.*, 1991]. Among the inflammatory substances released upon degranulation of mast cells, histamine remains the most potent vasoactive mediator implicated in the acute phase of the immediate hypersensitivity.

The β-hexosaminidase is located in the secretory granules of the mast cells, where histamine is stored, and is released along with the histamine when the mast cells are immunologically activated [Xu *et al.*, 2006]. Thus, β-hexosaminidase is designated as a degranulation marker, and the release of β-hexosaminidase has been used to determine the extent of degranulation and for the evaluation of anti-allergic activities [Hoffmann *et al.*, 1999].

Several natural products from tea, apple, and fagaceas were reported to inhibit the release of histamine from the RBL-2H3 cells after antigen stimulation [Kanda *et al.*, 1998; Chen *et al.*, 2000]. Especially, polyphenol strongly inhibited the release of histamine from the RBL-2H3 cells at the IgE sensitization stage and downstream from the antigen-stimulated stage. Epigallocatechin inhibited the histamine release from RBL-2H3 cells in response to antigen or the calcium-ionophore A23187, while epicatechin had little effect [Yamashita *et al.*, 2000]. These findings suggest that epigallocatechin prevents histamine release from the mast cells mainly by inhibiting tyrosine phosphorylation of proteins including $pp125^{FAK}$ [Yamashita *et al.*, 2000].

Acanthopanax sp., indigenous to Korea, is a typical oriental medicinal herb and has been used clinically as a tonic and prophylactic for the treatments of chronic bronchitis, hypertension, ischemic heart disease, and gastric ulcer, as well as rheumatism, diabetes, and cirrhosis [Yi *et al.*, 2001]. Four 3,4-seco-lupine-type triterpenoids, namely chiisanoside, chiisanogenin, 24-

*Corresponding author
Phone: +82-41-530-1355; Fax: +82-41-530-1355
E-mail: miyoung@sch.ac.kr

hydroxychiisanogenin, and 22α-hydroxychiisanogenin, have been isolated from the leaves of *A. divaricatus* var. *albeofructus* (Araliaceae) [Bae *et al.*, 2001]. Chiisanoside, the main component of *A.senticosus*, has been reported to have anti-hepatotoxic and antidiabetic activities and to affect the mitogen-induced proliferation of lymphocytes, among others [Bae *et al.*, 2001].

The major active constituents of the herb roots are eleutheroside, acanthoside, daucosterine, β-sitosterol, sesamine, and savinine. [Shan *et al.*, 1999]. These compounds have been reported to have diverse effects such as anticancer, immune activation, anti-inflammation, and anti-fever agents [Yoon *et al.*, 2002]. The root of *Acanthopanax* has also been reported to inhibit the cell-dependent anaphylaxis [Yi *et al.*, 2001]. However, it is still unclear how it regulates the anaphylaxis reaction and how effective it is, because the modes of action by the constituents of the plant have not yet been fully elucidated.

The suppressive effects of the extracts from the Korean indigenous *Acathopanax divaritacus* on the allergic inflammation in the rat basophilic leukemia cells were investigated in this study. The inhibitory effects on the ovalbumin-induced IgE in the sera were also examined.

Materials and Methods

Preparation of *Acathopanax* extract. The root, stem, and leaf of Korean indigenous *Acanthopanax divaricatus* var. *albeofructus* were provided by Susin Ogapy Co. Ltd (Cheonan-City, Chungnam, Korea). The water extract was prepared by decocting the dried herbs with boiling distilled water for 3 h. The decoction was filtered with membranes and lyophilized. The dried powder (2%) was dissolved in the sterile phosphate-buffered saline.

Lipoxygenase activity determination. The assay mixture contained 80 μM linoleic acid, a sufficient amount of soybean lipoxygenase and 20 μg/mL of the *A. divaricatus* var. *albeofructus* extract in 63 mM borate/NaOH buffer, pH 9.0. The reaction was started by the addition of the enzyme, and the increase in UV absorption at 234 nm was measured at 25°C for 1 min. One unit (U) of activity is the amount of enzyme required to catalyze the formation of 1 μmol of hydroperoxy linoleate/min under the assay conditions [Ulusu *et al.*, 2002].

MTT assay for cell viability. The MTT assay was used to determine the maximum concentration of the extract at which the cell viability is not affected. In brief, the cells were cultured in a 96-well plate (Corning Inc., Corning, NY) at a density of 5×10^3 cells per well. The cells were then treated with various concentrations of *A. divaricatus* root, stem, and leaf extracts, washed after 3 h, and treated with MTT. Plates were incubated in the dark for 4 h. After formazan formation, 100 μL DMSO was added, and the absorbance was measured at 570 nm using a microtiter plate reader. The determination of cell viability was calculated as [(absorbance of the drug-treated sample)/(control absorbance)] × 100.

RBL-2H3 cell culture. Rat basophilic leukemia cell line RBL-2H3 was maintained in RPMI-1640 medium (Gibco BRL, USA) with 10% fetal bovine serum at 37°C in 5% CO_2 in the air. The cell suspensions (5×10^5 cells) were sensitized with anti-DNP IgE (1 μg/mL) for 20 h. The cells were then harvested in the stationary phase and plated in a 24-well plate (10^5 cells/mL) with Tyrode's buffer (130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 10 mM HEPES, and 0.1% BSA, pH 7.4) for 30 min at 37°C in 5% CO_2. Triggering of the RBL-2H3 cells was induced by adding 20 μg/mL of DNP-BSA for 1 h in a humidified atmosphere at 37°C.

β-hexosaminidase assay. Enzyme assays were performed on the cell supernatants. After stimulation by DNP-BSA, the cells were spun at 5,000 × g for 1 min, and the supernatants were collected and chilled on ice. Fifty microliters of each sample was incubated with 50 μL of 1 mM γ-nitrophenyl-N-β-D-glucosaminide (Sigma, USA) dissolved in 0.1 M citrate buffer (pH 5) in a 96-well microtiter plate at 37°C for 1 h. The reaction was stopped by adding 200 μL/well of 0.1 M carbonate buffer (pH 10.5). The plate was then read at 405 nm in an ELISA reader. Results were expressed as percentage of the total release minus the spontaneous release, and analyses were performed in triplicates. All values are the means ± SE of the samples from three independent experiments, each triplicated.

Cytokine determination. Expressions of TNF-α and IL-1β were measured by a modified enzyme-linked immunosorbent assay (ELISA). The ELISA was performed by coating the 96-well plates with the murine monoclonal antibody having specificity for TNF-α or IL-1β. The coated plates were washed with the phosphate-buffered saline containing 0.05% Tween-20. All reagents used in this study were incubated for 2 h at 37°C. Assay plates were exposed to the biotinylated 2nd antibody, avidine peroxidase, and ABTS substrate solution containing 30% H_2O_2. The plate was read at 405 nm in an ELISA reader, and the cytokine level was measured by comparing with that of the standard.

Measurement of ovalbumin-induced IgE. BALB/c male mice were obtained from the Orient Bio Co. Ltd (Seoul, Korea). Mice, 7 weeks old and weighing 18-19 g, were acclimatized for 2 days under 25°C ± 2 and normal day/night cycle before starting the experiment. They were sensitized with 0.2 mL of the normal saline containing 100 mg ovalbumin (OVA) (Sigma, St. Louis, MO) adsorbed

in 20 mg aluminum hydroxide intraperitonially (i.p.) on days 0 and 14. Seven days after the final i.p. injection, the mice were given the sample extract orally at a dose of 0.5 mL (2%) /26 g body weight after 30 min of OVA (150 μg/100 μL) inhalation for 3 days (from day 21 to 23). On day 24, all mice were sacrificed, and the OVA-specific IgE levels in the sera were measured by the enzyme-linked immunosorbent assay (ELISA). The results are expressed in ng/mL of serum.

Results and Discussion

The inhibitory effects of aqueous extracts of *A. divaricatus* habitating in Korea on the lipoxygenase activity were examined (Fig. 1). Lipoxygenases were found to be involved in the formation of a group of biologically active lipids known as leukotrienes, which have important roles in the inflammation and allergic responses [Ulusu *et al.*, 2002]. Lipoxygenase activities decreased approximately 25% by the treatment of root, stem or leaf extract (20 μg/mL each) in dose-dependent manners (data not shown). The results indicated that the root, stem, and leaf extracts have similar inhibitory effects on the lipoxygenase activity.

Rat basophilic leukemia RBL-2H3 cells are the mucosal mast cell type that is very useful in the examination of IgE-mediated degranulation [Jeong *et al.*, 2002]. The cells express Fc ∈ RI, a heterotetrameric receptor that consists of one IgE-binding α subunit, one β subunit, and two disulfide-bonded γ subunits [Alber *et al.*, 1991]. Stimulation of RBL-2H3 cells with IgE and specific antigen has been reported to mimic the mast cell activation by allergens under the physiological conditions. Therefore, to determine whether *A. divaricatus* extract can modulate Ag-induced β-hexosaminidase release, IgE stimulated RBL-2H3 cells were treated with the extracts, and then challenged with DNP-BSA. Figure 2 shows the degranulating effects as measured by assaying the β-hexosaminidase release from the allergen-sensitized RBL-2H3 cells. About 35% of the β-hexosaminidase was induced and released after the DNP-BSA challenge as compared to the control. Upon the treatment of 625 μg/mL of the root extract, β-hexosaminidase release was reduced by 24% as compared to the DNP-BSA sensitized cell. Equal amounts of the stem and leaf extracts (625 μg/mL each) showed 12 and 18.6% inhibitory effects, respectively, on the β-hexosaminidase release from the DNP-BSA sensitized cell. The results showed that *A. divaricatus* extract notably inhibited the inflammation-mediating β-hexosaminidase release. Especially, the root was the most potent inhibitor of β-hexosaminidase release. It has been reported that β-hexosaminidase is

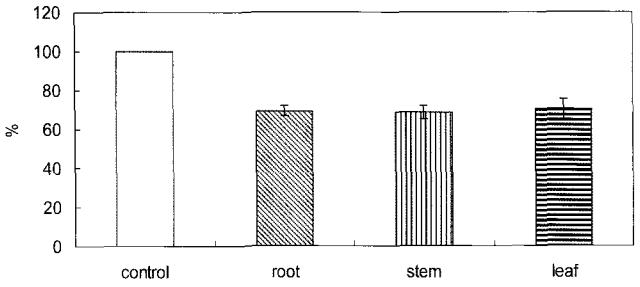

Fig. 1. Inhibition of the lipoxygenase activity by the root, stem, and leaf extracts of the Korean indigenous *Acanthopanax divaricatus*.

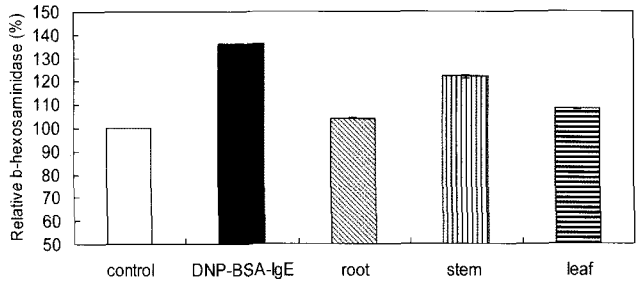

Fig. 2. Inhibition of the β-hexosaminidase release from RBL-2H3 cells by the extracts of root, stem, and leaf of the Korean indigenous *Acanthopanax divaricatus*.

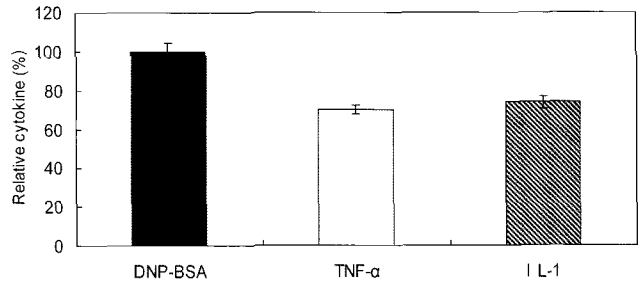

Fig. 3. Inhibition of the expressions of TNF-α and IL-1β in RBL-2H3 cells by the root extract of the Korean indigenous *Acanthopanax divaricatus*.

located in the secretory granules of mast cells, where histamine is stored, and is released along with histamine when mast cells are immunologically activated. Therefore, the inhibitory effects on the histamine release and β-hexosaminidase from the rat peritoneal mast cells were regarded as the anti-allergic activity [Cox. 1967]. This result suggests that *A. divaricatus* root extract would exhibit strong anti-allergenic activity as an inhibitor of the β-hexosaminidase release.

The RBL-2H3 cells were pretreated with 625 μg/mL of the root extract for 30 min prior to the antigenic stimulation to investigate whether *A. divaricatus* extract can modulate Ag-induced TNF-α and IL-1β production (Fig. 3). The cultural supernatant of RBL-2H3 cell was assayed for determination of the TNF-α and IL-1β

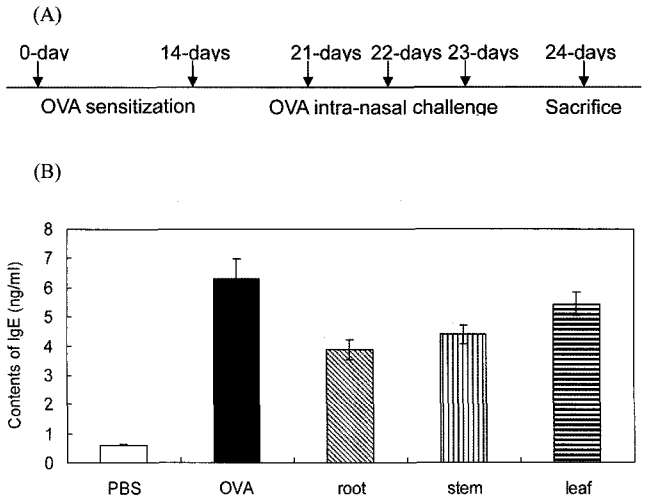

Fig. 4. (A) Experimental protocol for the protective effect of water extract of the Korean indigenous *Acanthopanax divaricatus*. (B)Inhibition of the rise in the serum IgE level by the extracts of root, stem, and leaf of the Korean indigenous *Acanthopanax divaricatus*.

protein levels. TNF-α production decreased about 30% by the treatment of the root extract in the antigen-stimulated cell. IL-1 production also decreased about 26% by the treatment of the Acanthopanax root extract. Cell cytotoxicity was not found at all concentrations used in the MTT assay (data not shown). These results suggest that *A. divaricatus* root extract exhibit anti-inflammatory effect by modulating TNF-α and IL-1 production. TNF-α has been thought to be an initiator of the cytokine-related inflammatory states by stimulating cytokine production. TNF-α itself is well known to promote inflammation, leukocyte infiltration, and tissue fibrosis [Kang *et al.*, 1996]. IL-1 has been reported to be involved in the inflammation and immune regulation by stimulating CD4+ T cell proliferation, inducing cytotoxicity of thymocyte, and increasing IL-6 and TNF-α productions [Kang *et al.*, 1998].

To investigate the effect of Korean indigenous *A. divaricatus* extract on the serum IgE levels, the OVA-specific IgE levels in mice treated with vehicle or the extract (root, stem or leaf) were examined (Fig 4). The IgE levels increased approximately 6.5 times in the OVA-challenged mice as compared to the saline-challenged mice. The oral administration of the extract during the OVA-challenged period significantly prevented the rise in the serum IgE levels. When mice were first OVA-challenged and then treated with the extract, a significant drop in the IgE levels in the serum was observed. The root extract reduced the serum IgE level by 40% as compared to the saline-challenged mice. Approximately 30 and 25% of serum IgE levels were also inhibited by the stem and leaf extracts, respectively. These results indicate that the root extract would be a stronger inhibitor of the serum IgE production than the extracts of stem and leaf in *A. divaricatus*.

The activation of the mast cell and basophils has been suggested to lead to the IL-4 production, which favors the differentiation of T-helper cells into TH2 cells, which, in turn, leads to the B-cell activiation for an IgE response [Ram *et al.*, 2004]. The stimulations on the mast cells and basophils also induce synthesis and secretion of the cytokines, including TNF-α, IL-6, granulocyte-macrophage CSF, IL-8, IL-13, and leukemia inhibitory factor with proinflammatory and immune regulatory properties, via the NF-κB activation [Kim *et al.*, 1999]. Phospholipase A2 inhibitor such as *para*-bromophenacyl bromide was reported to switch the TH2 response towards the TH1-dominated response, which inhibits IgE production, and thereby reduces the allergen-induced inflammation, bronchoconstriction [Ram *et al.*, 2004]. In conclusion, our study demonstrated that *A. divaricatus* extracts could alleviate the IgE-mediating secretion of β-hexosaminidase from the mast cell, reduce the IgE levels in mice serum, and modulate the TNF-α and IL-1 productions.

Therefore, *A. divaricatus*, especially its root extract, might be useful in the development of agents for modulating allergic inflammations including atopic dermatitis. However, it has not yet been clarified that the inhibitory effect of the extract on the allergic inflammation could be contributed by the unidentified component, which showed an inhibitory effect on the degranulation of mast cells and on the prevention of the rise in the serum IgE level. Therefore, the characterization of genuine, active components responsible for the whole anti-allergic inflammatory action of the extract, and the detailed mechanism of the anti-allergic action remains to be further investigated.

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