Xanthii Fructus inhibits allergic response in the ovalbumin-sensitized mouse allergic rhinitis model

Nam-Gil Gwak, Eun-Young Kim, Bina Lee, Jae-Hyun Kim, Yong-Seok Im, Ka-Yeon Lee, Chang Jun-Kum, Ho-Seok Kim, Hyun-Joo Cho, Hyuk-Sang Jung, Youngjoo Sohn

Department of Anatomy, College of Korean Medicine, Institute of Korean Medicine, Kyung Hee University, Seoul, Korea

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INTRODUCTION

Allergic rhinitis (AR) is a common inflammatory disease characterized by inflammation of the nasal mucosa. AR can be divided into early and late phases. The early phase occurs within 5–30 min of exposure to antigens such as dust, mold, mites and animal dander. It is characterized by symptoms such as sneezing, itching, lacrimation and clear rhinorrhea. These responses are caused by the release of mast cell products such as histamine. Mast cells play a key role in the inflammatory process. Activated mast cells release pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin 6 (IL-6), IL-8 and IL-1β, and inflammatory factors including histamine, and serotonin. The late phase is characterized by the recruitment of other effector cells, notably T helper 2 (TH2) lymphocytes, eosinophils, and basophils, which cause late symptoms such as congestion, fatigue, malaise, and irritability, from 6 to 24 h postantigen exposure throughout the disease process. Eosinophils play an important role in the late phase of AR. Eosinophil numbers are increased in the nasal mucosa of patients with AR. They release pro-inflammatory mediators including eosinophil peroxidase, cysteinyl leukotrienes, cationin proteins and major basic protein. Currently, antihistamines, corticosteroids, and immunosuppressants are commonly used to treat AR. The use of these compounds is limited by undesirable side effects such as osteoporosis, hypertension, excessive sedation and disorder of glucose and lipid metabolism. These problems together with increasing...
global interest in safety and efficacy of natural drugs, has led to the search for anti-allergic agents among herbal medicines traditionally used in oriental clinics for the treatment of allergic diseases such as rhinitis, asthma and dermatitis.[16–20]

Xanthii Fructus (XF) is the dried mature fruit of xanthium sibiricum patrin. It has been used traditionally as herbal medicine to treat nasal obstruction, rhinitis of sinusitis with nasal discharge, nasal discharge, urticaria with itching, and rhinitis with muscular spasms by wind and dampness.[21] Huang et al. reported that the extract of XF exhibited various biological activities, such as antioxidant, antiinflammatory, and anti-inflammatory activities in the carrageenan-induced hind paw edema model.[22] An et al. reported that XF extract inhibits inflammatory responses in lipopolysaccharide-stimulated mouse peritoneal macrophages.[23] Song et al. determined that XF extract protects against cytokine-induced damage in pancreatic beta-cells through suppression of nuclear factor kappa-B (NF-κB) activation.[24] Zhong and Hong et al. each reported that XF inhibits the chronic airway inflammation in bronchial asthma and the expression of histamine and TNF-α in the mast cell-mediated allergic response.[25,26] However, the effect of XF on the treatment of AR has not been established.

We investigated the mechanism of XF effects by evaluating the frequency of sneezing behavior, serum cytokine levels, and caspase-1 levels and thickness of nasal septum tissue in an in vivo AR model induced by ovalbumin (OVA). The inhibitory mechanism of XF on the regulation of NF-κB was investigated by in vitro human mast cell-1 (HMC-1) activated by phorbol-myristate-acetate (PMA) plus A23187.

MATERIALS AND METHODS

Plant material

Xanthii Fructus was purchased from the Omni herb Inc. (Andong-si, Gyeongbuk, South Korea). Dried extract of XF was received from Hamsoa Pharmaceutical Co. R and D Center (Seoul, South Korea). 250 g of XF were extracted with 2,500 mL of boiling water (95–100°C) for 2 h, and filtered using filter paper No. 3. (Whatman, Maidstone, Kent, UK). The filtered extract was concentrated in a rotary evaporator at 60°C, and the concentrated extract was vacuum-dried (yield: 12.52 g, 5.01%).

Animals

Thirty-two male BALB/C mice (5 weeks old) were purchased from Nara biotech (Gangnam-gu, Seoul, Korea). The mice were allowed to acclimate to the laboratory environment for a week before testing. All experimental mice were 6 weeks old. All animals were housed under controlled light (12-h light: 12-h dark cycle), temperature (21–22°C) and relative humidity at approximately 50–60%. The mice had ad libitum access to food and water. Animal care and experimental procedures conformed to the “Guide for the Care and Use of Laboratory Animals” (Department of Health, Education, and Welfare, NIH publication #78–23, 1996) and were approved by Kyung Hee University Institutional Animal Care and Use Committee (KHUASP (SE)–13–006).

PREPARATION OF OVALBUMIN-INDUCED ALLERGIC RHINITIS MOUSE MODEL

The mice were divided into the following four groups; sham group (no sensitization and distilled water orally administered, n = 8), OVA group (OVA-sensitized and distilled water orally administered, n = 8), Cet group (OVA-sensitized and 10 mg/kg of cetirizine hydrochloride orally administered, n = 8), and XF group (OVA-sensitized and 8 mg/kg of XF water extract orally administered, n = 8).

The sensitization and challenge procedures were performed as described in Figure 1. Briefly, the animals of the OVA-sensitized group were sensitized intraperitoneally (i.p.) by the injection of 50 µg OVA (chicken egg albumin, sigma) in 2 mg of aluminum hydroxide (Alum, sigma) adjuvant, which was suspended in 200 µL of phosphate buffered saline (PBS; pH 7.4) on day 0, 7 and 14. The mice were challenged intranasally (i.n) from days 21 to 31, with 1 µg of OVA in 20 µL of PBS (both right and left nasal cavities alternately by pipette). The sham group was sensitized and challenged with PBS. After the last sensitization (day 14), the Sham, Cet (Korea Drug, Seoul, South Korea) and XF groups were administered orally distilled water, cetirizine and XF to the mice, respectively before 1 h of behavior test, once daily for 10 consecutive days during OVA challenge.

Evaluation of nasal symptom

The mice were challenged by OVA i.n 2 min before behavior test. Then, the animals were placed in observation...
cases and the time and number of sneezes were counted for 10 min by blinded observers for 10 consecutive days during the OVA challenge.

**Serological analysis**
Blood sample was collected via cardiac puncture after the last evaluation of nasal symptoms. Serum was obtained by centrifugation (2,000 rpm, 4°C) for 10 min. The concentration of histamine and OVA-specific immunoglobulin E (IgE) in sera were measured by an enzyme immunoassay (EIA, Oxford Biological research, MS) kit and enzyme-linked immunosorbent assay (ELISA, BD bioscience, San Diego, CA) kits. The levels of TNF-α, IL-1β, IL-5, IL-6, monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-2 were quantified using a mouse cytokine/chemokine magnetic bead panel kit (Millipore, Germany) according to the manufacturer’s instructions.

**Histological evaluation of nasal cavity**
The animals were sacrificed by overdose of pentobarbital sodium (HANLIM Pharm. Korea) at the completion of all experimentation. Heads were removed and after the lower jaw was discarded, they were fixed in 10% formalin for 2 days. The nasal tissues were separated from the skin, muscle and soft tissue, and decalcified in 10% EDTA buffer for 14 days. The tissue was rinsed with water and processed for dehydration through graded alcohol, and embedded in paraffin. The tissues of nasal mucosa were cut into 5 μm sections. The skin sections were stained with H and E. Nasal septum thickness was measured by blinded observers. Infiltrated eosinophils were counted in 8 high-power fields (×400) of the nasal septum.

**Immunohistochemistry**
Endogenous tissue peroxidases were blocked by incubation with 0.3% H₂O₂ in methanol for 10 min. The tissues were washed thrice with PBS and blocked for 1 h at RT with 10% normal serum in PBS. After washing thrice, the tissues were incubated for 18 h at 4°C with anti-caspase-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 1% bovine serum albumin (BSA, Sigma). The tissues were rinsed thrice and incubated for 1 h with peroxidase IgG (Jackson immuno research, West Grove, PA, USA). Tissues were washed with PBS, after which the signal was detected using 3,3-diaminobenzidine (DAB, Sigma, USA) mixed with NiCl₂·H₂O (Sigma, USA) for 20 min. The caspase-1 stained tissues were counterstained with methyl green and mounted in Canada balsam. The stained sections were examined by optical microscopy (Olymmpus, Tokyo, DP73).

**Cell culture**
Human mast cell-1 cells were kindly provided by Prof. Hyung Min Kim (Department of Pharmacology, Kyung Hee University, Republic of Korea). HMC-1 cells were grown in Iscove’s modified Dulbecco’s medium with 10% fetal bovine serum, 100 units/mL penicillin and 100 mg/mL streptomycin (Gibco-BRL, Grand Island, NY, USA) at 37°C in an atmosphere containing 5% CO₂ with 95% humidity. The cells were pretreated with various concentrations of XF extract (1, 10, and 100 μg/mL) for 1 h and then stimulated with 25 nM PMA plus 1 μM A23187 (PMACI). After 2 h of stimulation with PMACI, the cells were harvested.

**Cytotoxicity assay**
*In vitro* cell cytotoxicity was determined by the MTS Cell Proliferation Colorimetric Assay Kit (CellTiter 96 non-radioactive cell proliferation assay kit, Promega). HMC-1 cells were plated in triplicate in 96-well microplates at an initial concentration of 1 × 10⁵ cells per well. Approximately, 24 h after cell plating, the cells were cultured with various XF concentrations (1, 10 and 100 μg/mL) for 24 h, after which an MTS solution was added to each well. The plate was incubated under 5% CO₂ at 37°C for 2 h. Absorbance was measured on a microplate reader (VersaMax™ Tunable microplate reader, Molecular Devices Corporation, Sunnyvale, USA) at 490 nm.

**Western blot analysis**
After PMACI stimulation for 2 h, cells were harvested and washed thrice with cold PBS. Nuclear and cytoplasmic extracts were prepared using NE-PERT™ nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions. The protein concentrations of samples were determined by the BCA assay kit (Thermo Scientific Pierce, Rockford, IL). Thirty microgram of protein was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane (Whatman, United Kingdom). Each membrane was incubated for 1 h with 5% skim milk in TBS-T buffer (0.1 M Tris-HCl [pH 7.4], 0.9% NaCl, 0.1% Tween-20) to block nonspecific binding. The membranes were incubated with a primary antibody solution that blocked nonspecific binding. The membranes were incubated with diluted primary antibodies that recognized histone (1:1000, Santa cruz), p-IκBα and p-NF-κB (1:1000, Santa cruz) overnight at 4°C. After washing with TBST, each membrane was incubated with secondary antibodies (1:10,000, Jackson immuno research, West Grove, PA, USA) in 5% skim milk for 1 h at room temperature. After washing for 1 h, immune-complexes were detected using a chemiluminescence detection system (ECL, Amersham Pharmacia, Piscataway, NJ) according to the instructions of the manufacturer.

**Statistical analysis**
All values were expressed as the mean ± standard error. Statistical analysis was presented using the
Graph Pad PRISM Software (Graphpad Software Inc, CA, USA). Raw data were subjected to One-way ANOVA, followed by Dunnett’s multiple comparisons. A $P < 0.05$ was considered statistically significant.

**RESULTS**

**Effects of Xanthii Fructus on nasal symptom in allergic rhinitis mice**

The main symptoms of AR are sneezing, runny nose, itching, rhinorrhea, and/or nasal congestion.[27] We applied the OVA sensitized and challenged mouse model, an AR model, to study the potential *in vivo* anti-inflammatory effect of XF. We injected XF (i.p.) after sensitization to evaluate the anti-inflammatory effects; cetirizine (i.p.) was chosen as a positive control. The number of sneezing was present in the AR mouse after intranasal OVA challenge [Figure 2]. XF treatment showed anti-inflammatory activities as demonstrated by significantly less number of sneezing than that of the OVA sensitized mice ($P < 0.01$).

![Figure 2: The nasal sneezing symptom score of ovalbumin (OVA)-induced allergic rhinitis mouse. The time and number of nasal symptom were measured for 10 min after the last intranasal challenge. (a) The time of nasal sneezing. (b) The number of rubbing for each day. (c) Total number of sneezing for 10 days. (d) Total number of rubbing for 10 days. Data are expressed as the mean ± standard error. ++ $P < 0.01$, significantly different from the sham group. ** $P < 0.01$, significantly different from the OVA group.](image-url)
Effects of Xanthii Fructus on histamine, immunoglobulin E and ovalbumin-specific immunoglobulin E levels in serum of allergic rhinitis mice

Multiple i.p injections of OVA in BALB/c mice resulted in high serum levels of both total and OVA-specific IgE antibodies. Overexpression of IgE is a major characteristic of AR. We investigated the effect of XF on histamine, IgE and OVA-specific IgE using this model. Histamine, total and OVA-specific IgE levels in serum increased significantly ($P < 0.01$) in OVA sensitized mice compared to PBS-treated mice [Figure 3a-c]. The histamine release in blood was significantly decreased on treatment of mice with XF ($P < 0.05$, Figure 3a). Total and OVA-specific IgE levels were also significantly reduced ($P < 0.05$, Figure 3b and c).

Effects of Xanthii Fructus on cytokines in serum of allergic rhinitis mice

We estimated the concentration of TNF-α, IL-1β, IL-5, IL-6, MCP-1 and MIP-2 in serum of the AR mice to evaluate the anti-inflammatory effect of XF. As shown in Figure 4, the levels of cytokines were significantly increased in the OVA-sensitized mice, compared to the sham group. However, XF group significantly decreased TNF-α, IL-1β, IL-5, IL-6, MCP-1 and MIP-2 levels in serum compared with OVA group [Figure 4].

Histological change of nasal mucosa and infiltration of eosinophils

Figure 5 shows the infiltration of eosinophils in the nasal septum. According to the histological analysis,
OVA-sensitized mice ($P < 0.01$) showed severe infiltration of eosinophils in the entire region of lamina propria [Figure 5b]. Additionally, the nasal tissues were thickened as compared to PBS-treated mice ($P < 0.01$). While, XF treated mice reduced ($P < 0.01$) the infiltration of eosinophils [Figure 5b], they had marked reductions in lamina propria thickness [Figure 5].

**Effects of Xanthii Fructus on caspase-1 expression in nasal mucosa tissues**

Caspase-1, known as IL-1β-converting enzyme (IL-1 BCE or ICE), is a member of the caspase family of proteases. It is the main enzyme to process precursor IL-1β and IL-18 molecules into their active molecules in the extracellular compartment, thereby playing a role in regulating immune-mediated inflammation. The effect of XF on caspase-1 expression was evaluated in nasal mucosa tissue. As shown in Figure 6, caspase-1 was significantly increased after OVA sensitization ($P < 0.05$). XF inhibited OVA-induced increased caspase-1 expression ($P < 0.05$).

![Figure 5: Effects of Xanthii Fructus on the infiltration of eosinophils into nasal tissues of allergic rhinitis model.](image)

| Sham | OVA | OVA+Cet | OVA+XF |
|------|-----|---------|--------|
| ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) |

**Figure 5:** Effects of Xanthii Fructus on the infiltration of eosinophils into nasal tissues of allergic rhinitis model. (a-d) The nasal tissues were stained with hematoxylin and eosin ($\times$400). The arrows represent infiltration of eosinophils. (e) Eosinophil counts in the nasal mucosa of each group. (f) Thickness of nasal septum in nasal mucosa. (g) Images were captured and analyzed in nasal septum. The Columns and error bars represent the mean ± standard error. $^{*}P < 0.01$, significantly different from the sham group. $^{*}P < 0.05$ and $^{**}P < 0.01$, significantly different from the ovalbumin group.

![Figure 6: Effect of Xanthii Fructus on caspase-1 expression in the nasal mucosa of mice with allergic rhinitis.](image)

| Sham | OVA | OVA+Cet | OVA+XF |
|------|-----|---------|--------|
| ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) |

**Figure 6:** Effect of Xanthii Fructus on caspase-1 expression in the nasal mucosa of mice with allergic rhinitis. (a-d) Nasal mucosa tissues were processed for IHC staining for caspase-1. The photographs are representative images taken at a magnification, $\times$100. (e) Caspase-1 expression in nasal mucosal sections was quantified using Image J. (f) Images were captured and analyzed in nasal septum. The Columns and error bars represent the mean ± standard error. $^{*}P < 0.05$, significantly different from the sham group. $^{*}P < 0.05$ and $^{**}P < 0.01$, significantly different from the ovalbumin group.
Effects of Xanthii Fructus on PMACI-induced nuclear factor kappa-B activation and inhibitor of nuclear factor kappa B-alpha degradation in human mast cell-1

We measured NF-κB activation and IkB-α degradation in HMC-1 mast cells treated with XF by Western blotting. The results showed that XF concentration-dependently inhibited IkB-α degradation. XF at a concentration of 100 µg/mL significantly (P < 0.05) decreased IkB-α degradation compared with activated HMC-1. In addition, NF-κB activation was dose-dependently inhibited by XF; NF-κB activation was significantly (P < 0.01) reduced at a concentration of 100 µg/mL compared to activated HMC-1 [Figure 7].

DISCUSSION

The present study showed that XF alleviated the AR symptom, reduced the infiltration of eosinophils and thickness of nasal septum in the nasal mucosa tissue of AR mice. XF reduced the expression of histamine, OVA-specific IgE, pro-inflammatory cytokines (TNF-α, IL-1β, IL-5, IL-6) and chemokines (MCP-1, MIP-2). In addition, XF significantly inhibited the phosphorylation of NF-κB and inhibitor of nuclear factor kappa B-α (IkB-α) in HMC-1. AR is an IgE-mediated inflammatory disease of the nasal mucosa.

Allergic rhinitis is clinically characterized by symptoms such as sneezing, nasal rubbing, rhinorrhea, and nasal congestion. The search for the treatment of AR has led to the establishment of the animal model that demonstrates similar symptoms of AR.[30,31] We showed that repeated topical intranasal OVA application caused typical AR symptoms. The pathology of AR is characterized by the production of IgE and inflammatory mediators including histamine, leukotrienes, and prostaglandins.[32] OVA induces a typical IgE-mediated systemic immune response. After the establishment of the systemic IgE-mediated response, continuous exposure to the same allergen at a specific site may lead to the development of TH2-dominant local allergic reactions, such as skin or nasal symptoms.[33,34] XF strongly reduced rhinitis symptoms, serum levels of histamine, IgE and OVA-specific IgE. Our results suggested that XF had an anti-allergic effect by preventing the production of allergic mediators including IgE and histamine.

Cetirizine is a major metabolite of hydroxyzine, and a racemic selective H1 receptor inverse agonist used in the treatment of allergies, angioedema, and urticaria.[35] Cetirizine reportedly reduces nasal inflammatory infiltration in children with AR and cytokine production in in vitro studies.[36] Cetirizine was used as a positive control drug. The inhibitory effects of XF on nasal symptoms, serum levels of histamine and IgE were similar to those of cetirizine.

Allergic diseases are mediated by the expansion of the TH2-cell subset of T cells, together with isotype switching of B cells to generate specific IgE antibodies for common environmental allergens.[37] IgE-sensitized mast cells in the

Figure 7: Effects of Xanthii Fructus (XF) on PMACI-induced p-IκB-α and p-NF-κB. (a) HMC-1 viability after 24 h of treatment with XF. To investigate the mechanism of XF, cells were treated with various concentrations of XF for 1 h followed by PMACI stimulation for 2 h. (b) Nuclear p-IκB and p-NF-κB were reduced on treatment with XF following PMACI stimulation (lanes 3, 4 and 5). (c) The protein ratio of p-IκB-α/actin, (d) p-NF-κB/histone. Columns and error bars represent the mean ± standard error. *P < 0.05, significantly different from PMACI (-). **P < 0.05 and ***P < 0.01, significantly different from PMACI (+). NF-κB: Nuclear factor kappa-B, IκB-α: Inhibitor of nuclear factor kappa B-alpha.
allergic response degranulate, releasing both pre-formed and newly synthesized mediators including histamine, cysteinyl leukotrienes and cytokines (TNF-α, IL-1 β, IL-5, IL-6).\[^{[38,39]}\] TNF-α is known to play an important role in allergic inflammation and is required for both the production of TH2 cytokines and the migration of TH2 cells to the sites of allergic inflammation.\[^{[40]}\] High levels of IL-1 β are mostly found after challenge in the nasal secretions of patients with AR. Secreted IL-1 β, activates T lymphocytes and endothelial cells and leads to a further release of cytokines.\[^{[41]}\] IL-5 has been identified as a central mediator of eosinophilic inflammation and in the etiology of asthma and allergic disease.\[^{[42,43]}\] IL-6, a multifunctional proinflammatory cytokine, could theoretically also be involved in the development of allergic disorders. Several of these studies demonstrated a temporal relationship between the elevated local IL-6 and the development of nasal secretions and symptoms.\[^{[44]}\] Histamine releasing factors (HRFs) are defined as cytokine-like molecules that induce basophil and/or mast cell secretion of histamine in the absence of a particular antigen or anti-IgE antibody. Chemokines such as MCP-1 and MIP-2 are thought to play an important role in the allergic inflammatory process. MCP-1 is known to activate basophils and eosinophils.\[^{[45]}\] Recently, MCP-1 was found at increased levels in the bronchoalveolar lavage fluid of patients with active asthma.\[^{[46]}\] XF treatment significantly decreased the level of TNF-α, IL-1β, IL-5, IL-6, MCP-1, and MIP-2. These results demonstrated that XF inhibited IgE production by downregulating pro-inflammatory cytokines in AR.

Allergic rhinitis mainly causes infiltration of eosinophils in the nasal mucosa. Eosinophils move from the blood vessels to the inflammatory site on inflammatory stimulation that is a role of antigen presenting cells in inflammatory reactions.\[^{[47]}\] The reduction of eosinophils means a weakening of the inflammatory response. Caspase-1 is a member of the cysteine-aspartic acid protease (caspase) family. Activated caspase-1 controls the maturation of cytokines of the IL-1 family and plays a role in regulating immune disease.\[^{[48]}\] The infiltration of eosinophils and the thickness of lamina propria in nasal septum were remarkably reduced by XF. XF also strongly reduced the expression of caspase-1. These results demonstrated that XF could inhibit the allergic immune response of the AR model by reducing the infiltration of eosinophils and the expression of caspase-1 in nasal mucosa tissue.

Human mast cell-1 cell line is useful for studying cytokine activation pathways.\[^{[49]}\] Expression of pro-inflammatory cytokines is dependent on the activation of transcription factor NF-kB.\[^{[50]}\] Activation of NF-kB requires phosphorylation and proteolytic degradation of the inhibitory protein I-κB, an endogenous inhibitor that binds to NF-kB in the cytoplasm.\[^{[51]}\] Inhibition of NF-kB is an anti-inflammatory strategy in AR. XF significantly inhibited the phosphorylation of NF-kB and IκB-α in HMC-1. These data demonstrated that XF could regulate the expression of the proinflammatory cytokines by attenuating the activation of NF-kB.

**CONCLUSIONS**

In this study, our results demonstrated that XF alleviated rhinitis symptoms by inhibiting the release of histamine, OVA-specific IgE and reducing the infiltration of eosinophils. XF had anti-inflammatory activity via suppression of TNF-α, IL-1β, IL-5 and IL-6 production. In addition, XF significantly inhibited the phosphorylation of NF-kB and IκB-α in HMC-1. These results suggested that XF might be an alternative drug for the treatment of AR.

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