Anti-allergic Effects of a Hot Water Extract of *Stephania tetrandra* S. Moore in RBL-2H3 Cells and an Allergic Rhinitis Mouse Model

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We investigated the anti-allergic activities of a hot water extract of *Stephania tetrandra* S. Moore using RBL-2H3 cells and an allergic rhinitis mouse model. The degranulation levels were significantly lower in the water-solvent fraction (WEx) and 99% ethanol fraction (99% EtEx) extracted from *S. tetrandra* than in the control. However, the cell viability of 99% EtEx was significantly lower than the control. In an ovalbumin allergic rhinitis mouse model, the frequency of sneezing after the fifth nasal ovalbumin (OVA) challenge in the groups intragastrically administered WEx and 99% EtEx decreased significantly compared to the control, although this decrease was lost following the sixth or seventh challenge. Plasma levels of OVA-specific immunoglobulin E in the WEx and 99% EtEx groups were significantly lower than the control at the experimental end point. Thus, *S. tetrandra* was proposed to be an effective anti-allergic natural medicine.

Keywords: anti-allergic compounds, *Stephania tetrandra* S. Moore, RBL-2H3 cells, allergic rhinitis mouse model

**Introduction**

The number of patients suffering from allergic diseases, such as allergic rhinitis (AR) and conjunctivitis, and allergies against pollen and food is increasing worldwide (Alessandro *et al.*., 2011). Allergic responses are classified into various types, such as type I (anaphylactic shock) and type IV (tuberculin reaction). In type I allergic responses, activation of the immunoglobulin E (IgE)-mediated FceRI receptor, known as the high-affinity IgE receptor, on the plasma membrane of mast and basophilic cells induces the release of β-hexosaminidase (β-hex), a common marker of degranulation, and various allergic mediators, including histamine, cytokines, prostaglandins, and leukotrienes (Amin, 2012). The cross-linked structure between IgE on FceRI and the allergen activates Lyn and Fyn, which are part of the Src family of non-receptor tyrosine kinases. Lyn activation induces phosphorylation of Syk kinase and Ca$^{2+}$ mobilization (Metcalfe *et al.*., 2009). The rat basophilic leukemia cell line, RBL-2H3, has been used to study IgE–FceRI interactions in the intracellular signaling for degranulation (Passante *et al.*., 2009). Moreover, RBL-2H3 cells are a useful tool for the *in vitro* screening of potential anti-allergic compounds. In AR models, BALB/c mice are sensitized with pollen, house dust, ovalbumin (OVA), etc. The AR mouse model induced by OVA is a useful tool for studying the effects of anti-allergic compounds *in vivo* because it exhibits high OVA-specific IgE levels in plasma with a short sensitization period.

The roots of *Stephania tetrandra* S. Moore have been widely used in multiple treatments (Ernesto *et al.*., 2007; Sun *et al.*., 2011; Chor *et al.*., 2009; Tsutsumi *et al.*., 2008). The main active...
components of *S. tetrandra* include cyclanoline (Cyc), tetrandrine (Tet), and fangchinoline (Fan). Cyc inhibits acetylcholinesterase (Inkaninan et al., 2006), while Tet and Fan are calcium channel blockers (Ernest et al., 2007). Previous study showed that Tet and Fan inhibit histamine release (Nakamura et al., 1992). Fan is an inhibitor of L-histidine decarboxylase (HDC), which is an enzyme involved in L-histamine synthesis (Adachi, 2006). The hot water extract of *S. tetrandra* suppressed the release of β-hex in RBL-2H3 cells and an OVA-induced AR mouse model.

We investigated the anti-allergic effects of a hot water extract of *S. tetrandra* using RBL-2H3 cells and an OVA-induced AR mouse model.

**Experimental**

**Sample preparation** *S. tetrandra* was purchased from the national crude drug market in the province of Anhui, China and powdered using a crusher (Retsch, Nissei Co., Tokyo, Japan). The powder was extracted in a 10-fold volume of methanol by sonication for 30 min. After sonication, the solution was mixed for 24 h on a rotary shaker (R-2, Nippon Medical & Chemical Instruments Co., Ltd, Osaka, Japan). Next, the solution was centrifuged for 15 min at 10,000 rpm and 4°C (Hi mac CR20G, Hitachi Ltd., Tokyo, Japan). The precipitate was dried at room temperature, and then extracted in a 20-fold volume of distilled water by sonication for 30 min. After sonication, the solution was mixed for 24 h with a magnetic stirrer and then incubated in a boiling-water bath for 1 h. After boiling, the solution was centrifuged for 30 min at 15,000 rpm and 4°C, and the supernatant was lyophilized (HWEx; hot water extract).

The HWEx was dissolved in a 20-fold volume of distilled water. The solution was fractionated using a glass column (φ 9.4 × 250 mm) packed with 20 g of Diaion® HP-20 (Mitsubishi Chemical Co., Tokyo, Japan) and eluted with a gradient of polar solvents [distilled water to 99% ethanol (EtOH) (100:0, 80:20, 50:50, 0:100)]. Four fractions were obtained (WEx, 20% EtEx, 50% EtEx, 50% EtEx (Cont), and 10% fetal calf serum (Corning, Inc., Corning, NY, USA) at 37°C in a humidified atmosphere of 5% CO₂.

**β-Hex and MTT assays with RBL-2H3 cells** We used β-hex as an index of degranulation. The assay was performed using a previously published method (Asano et al., 2011; Isoda et al., 2012). RBL-2H3 cells were seeded in 96-well plates at a density of 1.0 × 10⁴ cells/mL in 100 µL of medium. The cells were incubated and sensitized for 16 – 18 h at 37°C and 5% CO₂ with 0.3-µg/mL mouse monoclonal anti-dinitrophenyl (DNP) IgE (Sigma-Aldrich, St. Louis, MO, USA). The cells were washed twice with a releasing mixture (RM; 116.9 mM NaCl, 5.4 mM KCl, 25 mM HEPES, 2.0 mM CaCl₂, and 1.0 mg/mL BSA, pH 7.7) to eliminate free IgE. The cells were incubated at 37°C for 10 min in 140 μL/well of RM containing the samples. A 10-µL aliquot of PBS was used as a negative control (Cont), and 10 µL of 3.0 mM ketotifen fumarate (Keto; LKT Laboratories, Inc., St. Paul, MN, USA) was used as a positive control. Next, 10 µL/well of 4.0 µg/mL DNP-BSA (EMD Bioscience, Inc., San Diego, CA, USA) in PBS was added and incubated at 37°C for 50 min. The plates were put on ice to terminate the reaction, and then 70 µL of the supernatants was transferred to another 96-well plate. Next, 70 µL of the substrate solution (2.5 mM p-nitrophenyl 2-acetamido 2-deoxy-β-D-glucopyranoside (Nacalai Tesque, Inc.) in 100 mM citrate buffer, pH 4.5) was added to the supernatants, and the plates were incubated at 37°C for 30 min. Finally, 100 µL/well of stop buffer (2.0 M glycine buffer, pH 10.4) was added, and the absorbance at 403 nm was measured.
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405 nm was determined using a microplate reader (SUNRise Thermo RC-R, TECAN Ltd., Männedorf, Switzerland).

Cell viability was measured using a MTT assay. After the supernatant from the β-hex assay was removed and washed, 100 µL of 0.5 mg/mL MTT dissolved in medium without phenol red was added to the wells and incubated for 1 h at 37°C and 5% CO2. After incubation, the medium was removed. After 200 µL of dimethyl sulfoxide was added to the wells, the absorbance at 570 nm (650 nm reference absorbance) was determined using a microplate reader (Infinite M200, TECAN Ltd., Männedorf, Switzerland).

AR mouse model and treatment Twenty 6-week-old BALB/c female mice were purchased from Clea Japan (Tokyo, Japan). The animal care and treatment conformed to the guidelines for the ethical treatment of laboratory animals established by Mukogawa Women’s University (FSN-01-2015-02-A). Animals were housed at 22°C and 60% humidity under a 12-h light (08:00 – 20:00) – dark (20:00 – 08:00) cycle.

We employed the AR mouse model for this study (Mo et al., 2011). All mice were sensitized with an intraperitoneal (i.p.) injection of 100 µL of 1 mg/mL OVA (grade V, Sigma-Aldrich) in saline and 100 µL of aluminum hydroxide gel (Injef™ Alum Adjuvant, Thermo Fisher Scientific Inc., Waltham, MA, USA) on days –21 and –14. On day –7, the boosted sensitization was completed with an i.p. injection of 50 µg of OVA. On day –1, we collected mouse plasma, and OVA-specific IgE levels in the plasma were determined in order to confirm OVA sensitization. Seven days after the booster (day 1), the sensitized mice were divided into four groups of five mice each (Control group, Low-WEx treatment group, High-WEx treatment, and 99% EtEx treatment group). After general sensitization, mice were given an intranasal (i.n.) injection of 500 µg of OVA per 10 µL on days 1 to 7 (i.n. challenge). In addition, selected mice were treated by intragastric (i.g.) administration (Control group: 100 µL saline, Low-WEx group: 100 µL of 6 mg/mL WEx, High-WEx group: 100 µL of 12 mg/mL WEx, and 99% EtEx group: 100 µL of 0.5 mg/mL 99% EtEx) 30 min prior to the i.n. challenge. One mouse each in the control and High-WEx groups died during the i.n. challenge. We recorded sneezing frequencies for 15 min after the i.n. challenge. At the experimental end point (day 10: Control group and Low-WEx group, day 11: High-WEx group and 99% EtEx group), mice were euthanized with isoflurane to obtain whole blood and dorsal skin samples.

Vascular permeability We measured vascular permeability according to a previously published method (Goto et al., 2009). Mice were intravenously (i.v.) injected with 200 µL of 1.5% FITC-albumin (Sigma-Aldrich) under isoflurane anesthesia, and then intradermally injected at 4 points (test: 2 points, 5 µM OVA; control: 2 points, Tyrode’s solution) in the shaved dorsal skin. Sample solutions (100 µL) were administered by oral gavage 30 min prior to the i.v. injection. Thirty min after the i.v. injection, mice were euthanized with isoflurane to obtain dorsal skin samples and plasma. The fluorescence intensity of the extract from the dorsal skin and plasma was measured automatically using a fluorescence plate reader (Infinite M200, TECAN Ltd.) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The vascular permeability (10 µL plasma equivalent) was calculated as follows: (higher test fluorescence value–higher control fluorescence value)/(plasma fluorescence value).

Measurement of OVA-specific IgE levels in the plasma We measured the levels of OVA-specific IgE using a capture enzyme-linked immunosorbent assay with modifications (Strouse et al., 1991). Plasma samples were collected at day –1 and the end point. The anti-mouse IgE antibody was purchased from Bethyl Lab, Inc. (Montgomery, TX, USA), and streptavidin-HRP was purchased from Abcam (Cambridge, UK). The biotinylation of OVA was completed with a biotinylation kit (Sulfo-OSu, Dojindo Laboratories, Kumamoto, Japan). The levels of OVA-specific IgE were expressed as absorbance values.

Statistical analysis Statistically significant differences in the β-hex assay, MTT assay, vascular permeability, and absorbance of OVA-specific IgE were determined using a one-way ANOVA followed by Dunnett’s multiple comparison test. Statistically significant differences in sneezing frequency were determined using a repeated two-way ANOVA followed by Bonferroni’s multiple comparison test. P values less than 0.05 were considered significant. GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA) was used for all analyses.

Results Fractionation and analysis We subjected HWEx to HP-20 column chromatography using a stepwise EtOH elution method and obtained five fractions: non-adsorbed extract, WEx, 20% EtEx, 50% EtEx, and 99% EtEx. The weights of the extracted fractions from 1 g of HWEx were 480 mg (non-adsorbed extract), 320 mg (WEx), 130 mg (20% EtEx), 50 mg (50% EtEx), and 6 mg (99% EtEx).

We further analyzed the four fractions (WEx, 20% EtEx, 50% EtEx, and 99% EtEx) using HPLC. Figure 1A shows a typical chromatogram for the Tet reference. The elution time of Tet was approximately 28.5 min (Fig. 1A). Figure 1B-1E shows the chromatograms (Fig. 1A). Tet and Fan were not present in the chromatograms (Fig. 1B-1E). Based on the chromatograms (Fig. 1B-1E), Tet and Fan were not present in WEx and 20% EtEx.

HPLC analysis determined that WEx exhibited an absorbance peak at 280 nm; however, it was lower than the other fractions. Diaion™ HP-20 mainly captures peptides greater than 1 kDa in size, proteins, and polyphenols. Next, we analyzed the presence of peptides and proteins in WEx. In the CB-X assay, absorbance at 595 nm was not observed for WEx (Fig. 2A); however, absorbance...
at 562 nm in the BCA assay was detected in WEx (Fig. 2A). Moreover, WEx showed two spots on the TLC plate after the ninhydrin reaction (Fig. 2B). No positive spots were detected after the plates were sprayed with Dragendorff’s reagent and 1% FeCl₃ (data not shown). The data from Figs. 1 and 2 suggest that WEx contained peptides, while 20% EtEx contained Cyc, 50% EtEx contained Cyc, Fan, and Tet, and 99% EtEx contained Fan and Tet.

β-Hex and MTT assays  We examined the inhibitory effects of HWEx fractions on the degranulation of RBL-2H3 cells in vitro. Among these fractions, WEx and 99% EtEx showed a concentration-dependent decrease in degranulation (data not shown). The degranulation for 5 mg/mL WEx in a volume of 5 μL was significantly lower than the control (Fig. 3A). The addition of 5 μL WEx showed the minimum degranulation of 49.8% (Fig. 3A). This finding suggests that WEx contains anti-allergic compounds. The level of degranulation for 0.2 mg/mL 99% EtEx in a volume of 5 μL was significantly lower than the control (Fig. 3B). However, the cell viability after the addition of 5 μL of 99% EtEx was lower than the control (Fig. 3C). The degranulation percentage and cell viability after treatment with 99% EtEx showed a positive correlation ($R^2 = 0.979$). Thus, it was suggested that WEx and 99% EtEx contained anti-allergic compounds. However, these results also imply that the decrease in degranulation may be caused by the cytotoxicity of 99% EtEx, not by its anti-allergic effect.

AR mouse model  We examined the anti-allergy effects of WEx and 99% EtEx in AR mice. The sneezing frequency after the fifth i.n. challenge (day 5) was significantly lower in all treatment groups than the control (Fig. 4A and 4B). However, the reduction in sneezing frequency was lost on days 6 and 7, and the vascular permeability remained unchanged for all groups (Table 1). The absorbance of OVA-specific IgE in the plasma of mice at the experimental end point was significantly lower in all treatment groups than the control (Table 1). From these results, it was concluded that oral administration of WEx and 99% EtEx...
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Attenuated the induction of allergic reaction in AR mice by repeated intranasal challenges. Thus, it was suggested that WEx and 99% EtEx exerted anti-allergic effects *in vivo*.

**Discussion**

*S. tetrandra* contains strong biologically active substances such as Tet and Fan. The hot water extract of *S. tetrandra* suppressed the release of β-hex in RBL-2H3 cells (Asano *et al.*, 2011). However, Tet and Fan are only slightly soluble in water. Therefore, we speculated that *S. tetrandra* contained water-soluble anti-allergic compounds.

The main active components of *S. tetrandra* are alkaloids. Alkaloids constitute the largest group of nitrogen-containing secondary metabolites in plants (Ernesto *et al.*, 2007). Most alkaloids in plants bind organic acids and can be extracted with a weak acidic solution (e.g., acetic acid in water, ethanol, or methanol). Tet, Fan, and Cyc can be extracted from *S. tetrandra* by methanol (Huang *et al.*, 2006). We removed Tet and Fan from *S. tetrandra* by methanol processing because these compounds inhibit histamine release (Nakamura *et al.*, 1992). However, we could not remove all traces of Tet, Fan, and Cyc from the *S. tetrandra* powder. Next, we subjected the HWEx to Diaion® HP-20 open column chromatography. Diaion® HP-20 mainly captures peptides greater than 1 kDa in size, proteins and polyphenols. The absorbance of WEx at 280 nm was nearly undetectable with HPLC. Our results suggest that WEx did not contain polyphenols, while 20% EtEx contained Cyc, 50% EtEx contained Cyc, Fan and Tet, and 99% EtEx contained Fan and Tet. The extraction characteristics of WEx included low solubility in alcohol and high stability during heat treatment; in addition, WEx could be captured by Diaion® HP-20. WEx reacted to BCA and ninhydrin, but not CB-X (Fig. 2). These data suggest that WEx contains peptides. Moreover, we developed a simplified separation method for hot water extracts of *S. tetrandra*.

Tet and Fan are calcium channel blockers and inhibit histamine release (Ernesto *et al.*, 2007; Nakamura *et al.*, 1992). However, anti-allergic components of *S. tetrandra* besides Tet and Fan have not been studied. The sneezing frequency at day 5 and the absorbance of OVA-specific IgE in the plasma of mice at the end point were significantly lower in the WEx treatment groups than the control. However, the reduction in sneezing frequency of the WEx groups at day 5 was lost at days 6 and 7, since WEx exhibited modest anti-allergic effects *in vitro*, with a maximum inhibition of approximately 50%. Here, we determined that WEx has mild anti-allergic effects *in vitro* and *in vivo*. Moreover, WEx exhibited very low toxicity. Anti-allergic peptides have been previously reported, including KVPEDRV-Y-EELNI-Y-SAT-Y-SELEDPG (Sekiyama, 2016), LDAVNR and MMLDF (Vo *et al.*, 2014), PFNQGTFAS (Ko *et al.*, 2016), and peptides derived from casein (Tanaka *et al.*, 2012). Thus, we speculate that WEx peptides may exert anti-allergic effects, and that WEx contains safe and water-soluble anti-allergic compounds.

There are no reports on the anti-allergic effects of Tet and Fan *in vivo*. Based on our HPLC results (Fig. 1), the main compounds in the 99% EtEx were Tet and Fan. AR symptoms and OVA-specific IgE plasma levels were suppressed after treatment with 99% EtEx. Various allergic mediators, which are released from mast and basophilic cells during type 1 allergic reactions, activate helper T cells (Amin, 2012). Furthermore, the activated helper T cells induce IgE class switching of B cells. The augmented B cells, through a series of allergic reactions, produce the allergen-specific IgE. Thus, we suggest that the OVA-specific IgE levels in the
plasma of the 99% EtEx group did not increase because the sustained oral administration of 99% EtEx inhibited allergic reaction by repeated i.n. challenge. Furthermore, Tet and Fan may have anti-allergic effects in vivo.

In conclusion, the hot water extract of *S. tetrandra* contains compounds with anti-allergic effects. Tet is a strong calcium channel blocker, while Fan is a calcium channel blocker and HDC inhibitor. The WEx fraction has low toxicity and anti-allergic effects. Moreover, WEx may be a viable option for the treatment of allergic diseases because of its low toxicity and high availability. Thus, *S. tetrandra* may be an effective anti-allergic medicine in light of the synergistic anti-allergic effects of WEx and 99% EtEx. Further study is needed to confirm whether the powder of raw *S. tetrandra* can be used to treat allergic diseases in humans.

**Reference**


Nakamura, K., Tsuchiya, S., Sugimoto, Y., Sugimura, Y., and Yamada, Y.
The sneezing frequency over 15 min after an OVA challenge in the AR mouse model (A) and the statistical results (B).

The values are expressed as mean ± SE. n = 4 – 5. Mice were treated via intragastric administration (Cont group: 100 μL saline, Low-WEx group: 100 μL of 6 mg/mL WEx, High-WEx group: 100 μL of 12 mg/mL WEx, and 99% EtEx group: 100 μL of 0.5 mg/mL 99% EtEx) 30 min prior to the intranasal challenge. The sneezing frequency was recorded during the 15 min after an intranasal injection of 500 μg OVA per 10 μL. The statistically significant differences in sneezing frequency were determined using a repeated two-way ANOVA followed by Bonferroni’s multiple comparison test. * \( p < 0.05 \), and ** \( p < 0.01 \) vs. cont (in the each group), and † \( p < 0.05 \), and †† \( p < 0.01 \) vs. day 1 (in the each group).

Table 1. Absorbance of OVA-specific IgE at day -1 and the end point, and the vascular permeability at the end point.\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>Cont</th>
<th>Low-WEx</th>
<th>High-WEx</th>
<th>99% EtEx</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVA-specific IgE (day -1)</td>
<td>0.548 ± 0.061</td>
<td>0.438 ± 0.063</td>
<td>0.457 ± 0.030</td>
<td>0.469 ± 0.039</td>
</tr>
<tr>
<td>OVA-specific IgE (end point)</td>
<td>0.792 ± 0.043</td>
<td>0.458 ± 0.078</td>
<td>0.533 ± 0.063</td>
<td>0.541 ± 0.061</td>
</tr>
</tbody>
</table>

Vascular permeability (10 μL plasma equivalent)

<table>
<thead>
<tr>
<th></th>
<th>Cont</th>
<th>Low-WEx</th>
<th>High-WEx</th>
<th>99% EtEx</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.366 ± 0.044</td>
<td>0.489 ± 0.049</td>
<td>0.439 ± 0.124</td>
<td>0.395 ± 0.068</td>
<td>0.712</td>
</tr>
</tbody>
</table>

\(^1\)Values are shown as mean ± SE, n = 4 – 5.

\(^2\)OVA-specific IgE was measured using capture ELISA. The diluted ratio of plasma was 1:40, and the HRP reaction time was for 10 mins at room temperature.

\(^3\)The vascular permeability (10 μL plasma equivalent) was calculated as follows: (higher test fluorescence value – higher control fluorescence value)/(plasma fluorescence value).

\(^4\)Statistical significance was determined using one-way ANOVA followed by Dunnett’s multiple comparison test. * \( p < 0.05 \), and ** \( p < 0.01 \) vs. cont.


