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Note

Anti-allergic Actions of the Leaves of *Castanea crenata* and Isolation of an Active Component Responsible for the Inhibition of Mast Cell Degranulation

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The anti-allergic actions of the leaves of *Castanea crenata* (Fagaceae) were studied. The water extract demonstrated potent anti-allergic actions in *in vivo* and *in vitro* experiments. The oral or intraperitoneal administration of the extract (100 or 200 mg/kg) caused a significant inhibition of the 48 hr-PCA (up to 90%) and the vascular permeability induced by histamine or serotonin in rats (about 80%). The anaphylactic release of β-hexosaminidase from RBL-2H3 cells was also significantly inhibited by the extract in a dose-dependent manner with an IC_{50} value of 230 μg/ml. The activity-guided fractionation of the extract, based on the determination of inhibitory effect upon the release of β-hexosaminidase, led to the isolation of quercetin as an active principle responsible for the inhibition of degranulation.

Key words : Allergy, *Castanea crenata*, Quercetin, 48 hr-PCA, Degranulation, β-Hexosaminidase

INTRODUCTION

The plant *Castanea crenata* (Fagaceae) is a fruit tree widely cultivated in Asian countries (sweet chestnut or Japanese chestnut) and the decoction of the leaves had been applied for the treatment of whoop-cough and lacquer poisoning in folk medicine (Chiej, 1984). These applications strongly implied an anti-allergic effect of this plant. Therefore, we have investigated the anti-allergic actions of the water extract of the leaves of *C. crenata* in *in vivo* experiments *i.e.* the inhibitory effect on the 48 hr-PCA (passive cutaneous anaphylaxis) and on the enhanced vascular permeability induced by histamine or serotonin in rats. We also isolated an active principle responsible for the inhibition of the mast cell degranulation by the bioassay-guided fractionation.

MATERIALS AND METHODS

Plant material

The plant material was collected in October, 1995 from Chonnam Province, Korea. The plant was identified by the herbarium of the Faculty of Pharmacy, Chonnam National University, where a voucher specimen (herbarium No. 9512) has been deposited.

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Animals

Male Sprague-Dawley rats weighing 180 to 250 g were purchased from Korea Laboratory Animal Center (Taejeon, Korea). Animals were maintained at 22±2°C on a 12 h light and 12 h dark schedule (8:00 a.m.~8:00 p.m.), and they were freely accessible to food and water.

Antigens and antibodies

DNP-BSA (2,4-dinitrophenylated bovine serum albumin) and DNP-specific rat monoclonal IgE were kindly provided by Dr. Koda (College of Pharmacy, Gifu University, Japan). DNP-specific mouse monoclonal IgE was obtained from the hybridoma.

48 hr passive cutaneous anaphylaxis in rats

The inhibitory effect on the 48 hr-PCA reaction was assessed according to the method of Ovary (1958) with some modifications (Kim *et al.*, 1996). The water extract of the leaves of *C. crenata* was dissolved in saline and administered intraperitoneally to rats 1 hr prior to challenge with antigen at the dose of 200 mg/kg. In case of oral treatment, the water extract was

administered at the dose of 100 or 200 mg/kg twice per day for two days. An additional treatment was carried out on the following day, 2 h prior to challenge with antigen. Exactly the same experimental protocol was employed for the control group except that saline was administered instead of the water extract.

Experiments on the vascular permeability changes induced by histamine and serotonin

Effects of the extract of the leaves of *C. crenata* on the mediator-induced vascular permeability change were tested by the method of Katayama *et al.* (1978). Saline (150 μl), histamine (5 μg/ml), and serotonin (1 μg/ml) were injected to the rats intradermally on the shaved back skin under ether anesthesia, followed by intravenous injection of 1 ml of 1% Evans Blue in saline. The same experimental protocol was used as in 48 hr-PCA experiment.

Measurement of histamine release from rat peritoneal mast cells

Histamine release from rat peritoneal mast cells was determined according to the method of Foreman and Mongar (1972) with a slight modification (Kim *et al.*, 1996).

Measurement of β-hexosaminidase release from RBL-2H3 cells

The release of β-hexosaminidase from RBL-2H3 cells was evaluated according to the Cheong *et al.* (1998a, 1998b).

Isolation of active component for the inhibition of degranulation

The activity-guided fractination method was used (Oh *et al.*, 1998). The water extract (20 g) was dissolved in 1 l of distilled water and applied to octadecylsilica (ODS) gel column (Merck, 2.5×30 cm), which was eluted with 1 l of water (F1, 13.9 g), 1 l of 20% MeOH (F2, 4.0 g) and finally with MeOH (F3, 1.3 g). Each fraction was evaluated for the inhibitory effect on the β-hexosaminidase release from the RBL-2H3 cells. The EC_{50} values (μg/ml) of each fraction were >2,000 (F1), 300 (F2) and 80 (F3), whereas that of water extract from powdered sample was *ca* 230. Then, the F3 fraction was applied to the ODS column and it was divided into five sub-fractions *i.e.*, F31 (0.2 g), F32 (0.18 g), F33 (0.1 g), F34 (0.14 g), and F35 (0.36 g) using MeOH in water as eluent by gradient manner (F31; water, F32; 20% MeOH, F33; 30% MeOH, F34; 40% MeOH and F35; MeOH). Among these sub-fractions, only the F35 fraction exhibited a significant inhibition of the hexosaminidase release (EC_{50} value, *ca* 40 μg/ml). The sub-fraction F35 was further fractionated by ODS column chromatography to give 40 μg of active component. The *Rf* value was 0.5 in ODS-TLC using 70% MeOH as developing solvent, and the EC_{50} value for the inhibition of hexosaminidase release was *ca* 1 μg/ml. It was a yellow crystal of needle type in MeOH and identified as quercetin in direct comparison with the authentic sample by spectral analyses (IR, Mass, UV, ^{1}H-NMR, ^{13}C-NMR, DEPT-NMR).

Statistics

Student's *t* tests were used for the statistical analysis.

RESULTS AND DISCUSSION

Effects of the extract on 48 hr-PCA and on the mediator-induced vascular permeability

Anti-allergic activity of drugs can be assessed by determining their effect on the changes of vascular permeability caused by antigen-antibody reaction (PCA, Ovary, 1958) or by chemical mediators such as histamine or serotonin (Owen *et al.*, 1984).

The water extract was tested for the effects on PCA and mediator-induced increase of vascualr permeability. Ketotifen, a reference anti-allergic agent (Craps *et al.*, 1978; von Wichert, 1980), inhbited the PCA reaction and the enhancement of vascular permeability by histamine. However, serotonin-induced change of vascular permeability was less markedly and inconsistently affected by ketotifen (Table I). The water extract from the leaves of *C. crenata* inhibited the PCA reaction regardless of the route of administration, oral or intraperitoneal. Especially, intraperitoneal administration (200 mg/kg) of the extract caused a remarkable inhibition (up to 90%) of PCA reaction, and also resulted in a significant reduction of vascular permeability enhanced by histamine or serotonin (Table I). Meanwhile, in the case of oral administration (100 and 200 mg/kg). the water extract also exhibited a remarkable inhibition of PCA reaction, but failed to reverse mediators-induced change of vascular permeability.

These results suggest that the extract of *C. crenata* might contain more than two distinct anti-allergic components. The active component acting on mediator-induced change of vascular permeability may not be readily absorbed in gastrointestinal tract or metabolically modified in the process of absorption through gastrointestinal tract.

Isolation of an active component responsible for the inhibition of mast cells degranulation

During allergic reaction, crosslinking of IgEs by antigen causes degranulation of mast cells to release chemical mediators such as histamine and serotonin that play

Table I. Effects of the water extract on the 48 hr-PCA and on the vascular permeability enhanced by histamine and serotonin in rats

Group	Dose (mg/kg)	Route of administration	% of Inhibition		
			PCA	Histamine	5-HT
Ketotifen	5	i.p.	75.0±8.0**	93.2±10.0**	42.8±8.9**
		oral	84.9±6.9**	82.0±8.1**	32.1±38.8
Quercetin	10	i.p.	11.9±2.1	16.7±3.2	no effect
C. crenata water extract	200	i.p.	90.0±13.3**	82.6±17.7**	80.2±13.7**
	100	oral	66.9±19.6*	12.1±30.5	10.1±46.7
	200	oral	68.0±2.5**	not measured	not measured

The extract in saline or control (saline) was injected intraperitoneally (200 mg/kg) 1 h prior to challenge with antigen. In oral administration, the animals were administered (100 or 200 mg/kg) for 2 days with an additional treatment on the next day, 2 h prior to the challenge with antigen. The positive control drug, ketotifen, was administered (5 mg/kg) with the same schedule as for the extract. PCA and vascular permeability experiments were conducted for every experimental animal (PCA, histamine, and serotonin). The amount of dye (ng/spot) extravasated for the control group was estimated to be 74.0±10.8 (PCA), 15.3±3.1 (histamine) and 8.7±1.1 (serotonin). The significance of difference and the % inhibition for each test were determined by comparing the absolute amount of dye for each treatment group with that of control group (n=7 for each group).
*: $p<0.05$, significance of difference compared with control group of each test.
**: $p<0.01$, significance of difference compared with control group of each test.

important roles in the pathogenesis of allergic and inflammatory disorders. Therefore, the inhibitory effect of drugs on the histamine release from rat peritoneal mast cells was often rendered to be the anti-allergic activity (Cox, 1967). The β-hexosaminidase is located in the secretory granules of mast cells where histamine is stored, and is released along with histamine when mast cells are immunologically activated (Cheong *et al.*, 1998a; Schwartz *et al.*, 1981). Therefore, β-hexosaminidase is designated as 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 (Fischer *et al.*, 1995). Thus, we employed this bioassay system to evaluate the inhibitory effect on degranulation event *in vitro*.

The water extract of the leaves of *C. crenata* significantly inhibited the anaphylactic histamine release from rat peritoneal mast cells in a dose-dependent manner at concentrations as low as 30 μg/ml (data not shown). This suggested that the water extract of *C. crenata* contained anti-allergic component (s) which inhibited the degranulation of mast cells. The activity-guided fractionation of the extract, based on the inhibitory effect upon the release of β-hexosaminidase, led to the isolation of quercetin as an active principle responsible for the inhibition of degranulation. The content of quercetin in the extract was calculated to be ca 0.2%. The inhibitory effect of the crude extract and quercetin upon the

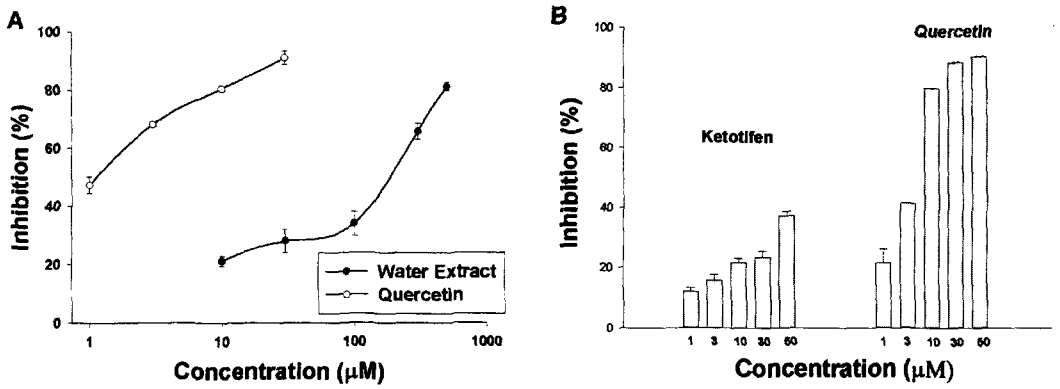

Fig. 1. Dose-response relationship for the inhibitory effects of water extract and quercetin on β-hexosaminidase release from RBL-2H3 cells. (A) The inhibitory effect of water extract and quercetin was examined as follow; quercetin was dissolved in 100% DMSO and the final concentration of DMSO was adjusted to 0.5%. To exclude the effects of DMSO itself on the degranulation of RBL-2H3 cells, 0.5% DMSO was also added to the control group. Each data point represents mean±S.E.M. of triplicate determinations. (B) The inhibitory effect of quercetin was compared with that of ketotifen. % of inhibition=(Treated-Blank-Spontaneous)/(Control-Blank-Spontaneous). Control : normal allergen-IgE response was evoked with test material not added; Treated : normal allergen-IgE response was evoked with test material added; Blank : only test material and substrate were added into ELISA plate. Spontaneous : allergen-IgE response was not evoked with test material not added.

release of β-hexosaminidase from cultured RBL-2H3 cells is summarized in Fig. 1A. The calculated values of IC_{50} for quercetin and water extract were ca 1.1 μg/ml and 230 μg/ml, respectively. When the inhibitory activity of quercetin on the degranulation of RBL-2H3 cells was compared with that of ketotifen (Fig. 1B), a clinically used anti-allergic drug, quercetin (IC_{50}, 3 μM) was much more potent than ketotifen (IC_{50}, 100 μM).

Next, the *in vivo* anti-allergic activity of quercetin was studied (Table I). The intraperitoneal administration of quercetin at the dose of 10 mg/kg, equivalent to, in the amount of quercetin, 500 mg/kg of the water extract, did not exhibit a significant inhibition of the PCA reaction and the vascular permeability changes induced by histamine or serotonin. In contrast, an intraperitoneal administration of the water extract at the dose of 200 mg/kg significantly inhibited both PCA reaction and mediators-induced vascular permeability (Table 1). Thus, it is suggested that the inhibitory effect of the extract on PCA reaction *in vivo* might be contributed by (an) unidentified component (s) other than quercetin which showed an inhibitory effect on the degranulation of mast cells *in vitro*. Therefore, the characterization of genuine active components responsible for the whole anti-allergic action of the extract in *in vivo* experiment, and the detailed mechanism of the anti-allergic action of the extract remain to be further investigated.

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