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Short Communication

Anti-allergic activity of Thai medicinal plants used in longevity formulation

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Abstract

The ethanolic (EtOH) and water extracts of six plants including *Piper nigrum*, *Streblus asper, Cyperus rotundus*, *Tinospora crispa, Diospyros rhodocalyx* and *Albizia procera* used in Thai traditional longevity formulation, were examined for anti-allergic activity on antigen-induced β-hexosaminidase release from RBL-2H3 cells (rat-basophilic leukemia cell line), a tumor analog of mast cell. It was revealed that *Piper nigrum* (EtOH) extract exhibited the most potent activity with an IC_{50} value of 14.0 μg/ml, which was higher than that of ketotifen fumarate, a positive control (IC_{50} = 20.2 μg/ml). It was also found that the preparations of Piper-Diospyros (EtOH) and Piper-Tinospora (EtOH) in the ratio of 1 : 1 appreciably inhibited antigen-induced degranulation in RBL-2H3 cells with IC_{50} values of 23.5 and 26.7 μg/ml, respectively. The anti-allergic effects of these two preparations were higher than that of the original longevity formulation (IC_{50} = 66.6 μg/ml).

Keywords: Thai longevity herbal formulation, anti-allergic activity, RBL-2H3 cells

1. Introduction

Mast cells and basophils play a central role in allergic reactions mediated by immunoglobulin E (IgE) (Yamashita *et al.*, 2000). They are responsible for a variety of allergic disorders and immunoresponses to parasites (Lee *et al.*, 2004). Allergy is an immunological reaction to a foreign antigen (allergen) such as dust mites, pollen, cosmetics, food and mold spores that causes tissue inflammation and organ dysfunction (Nakatani *et al.*, 2002). The allergic mediators induce various physiological effects that cause allergic diseases (Lee *et al.*, 2004). The mediators such as histamine and serotonin are released from mast cells and basophils within minutes- the early phase reaction (Matsuda *et al.*, 2004a). When granules in mast cells or basophils are activated, an enzyme β-hexosaminidase stored in the secretory granules is released along with histamine. Thus, this enzyme activity is used as a marker of mast cell and basophil degranulation (Matsuda *et al.*, 2004b).

Recently, the use of herbal medicines has increased for the treatment of various diseases (Ikawati *et al.*, 2001). Many medicinal plants provide relief of symptoms comparable to modern medicines. Thai longevity formulation is a preparation consisting of Thai plants which exert health promotion activity and tonic effect, as well as immune balancing effect. In the present study, the water and ethanolic extracts of six Thai medicinal plants used in one longevity formulation were examined for their anti-allergic effects on antigen-induced β-hexosaminidase release from RBL-2H3 cells.

2. Material and Methods

2.1 Plant material

Six species of plants commonly used in Thai longevity formulation were tested for anti-allergic effect. They were *Piper nigrum* (fruit), *Streblus aspers* (seed), *Tinospora crispa* (stem), *Cyperus rotundus* (bulb), *Albizia procera* (stem) and

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Diospyros rhodocalyx (bark). These plant materials were bought from Thai traditional drug store, Hat Yai, Thailand. The voucher specimens are kept at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand. The voucher specimens are SKP146161401, SKP117190101, SKP114200301, SKP 060031801, SKP097.1011601, SKP067041801, respectively.

2.2 Preparation of plant extracts

The plant material was cut into small pieces and dried in an oven at 50-60°C. The dried materials (10 g) were extracted by reflux with water and 95% ethanol separately for 3 h. Each extract was further evaporated to dryness under reduced pressure to give the yield of water and ethanolic extracts as shown in Table 1. The extracts were then dissolved in DMSO as stock solutions (10 mg/ml) for bioassay.

2.3 Reagents

Minimum essential medium eagle (MEM) and anti-DNP IgE (Monoclonal Anti-DNP) were purchased from Sigma; fetal calf serum (FCS) was from Gibco; the dinitrophenylated bovine serum albumin (DNP-BSA) was prepared as described previously (Tada and Okumura, 1971). Other chemicals were from Wako; 24-well and 96-well microplates were from Sumitomo Bakelite Co., Ltd.

2.4 Effects on the release of β-hexosaminidase from RBL-2H3 cells.

Inhibitory effects on the release of β-hexosaminidase in RBL-2H3 obtained from Cell Lines Service (CLS, Germany) were evaluated by a method reported previously (Matsuda *et al.*, 2004a,b). Briefly, RBL-2H3 cells in 24-well plates [$5x10^5$ cells/ml in MEM containing 10% FCS, penicillin (100 units/ml), streptomycin (100 µg/ml)] were sensitized with anti-DNP IgE (0.45 µg/ml). The cells were washed with Siraganian buffer [119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 25 mM piperazine-N,N2-bis(2-ethanesulfonic acid) (PIPES), and 40 mM NaOH, pH 7.2] supplemented with glucose, 1 mM $CaCl_2$, and 0.1% bovine serum albumin (BSA) (incubation buffer) and then incubated in 160 µl of the incubation buffer for 10 min at 37°C. After that, 20 µl of test sample solution was added to each well and incubated for 10 min, followed by an addition of 20 µl of antigen (DNP-BSA, final concentration was 10 µg/ml) at 37°C for 10 min to stimulate the cells to degranulate. The reaction was stopped by cooling in an ice bath for 10 min. The supernatant (50 µl) was transferred into 96-well plate and incubated with 50 µl of substrate (1 mM *p*-nitrophenyl-N-acetyl-β-D-glucosaminide) in 0.1 M citrate buffer (pH 4.5) at 37°C for 2.5 h. The reaction was stopped by adding 200 µl of stop solution (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0). The absorbance was measured with a microplate reader at 405 nm. The test sample was dissolved in dimethylsulfoxide (DMSO), and the solution was added to incubation buffer (final DMSO conc was 0.1%). The inhibition (%) of the release of β-hexosaminidase by the test samples was calculated by the following equation, and IC_{50} values were determined graphically:

$$\text{Inhibition (\%)} = [1 - (T - B - N) / (C - N)] \times 100$$

Control (C): DNP-BSA (+), test sample (–); test (T): DNP-BSA (+), test sample (+); blank (B): DNP-BSA (–), test sample (+); normal (N): DNP-BSA (–), test sample(–).

2.5 β-Hexosaminidase inhibitory activity

In order to clarify that the anti-allergic effects of samples are due to the inhibition on hexosaminidase release, but not from the inhibition of β-hexosaminidase activity, the following assay was then carried out.

The cell suspension (5×10^6 cells) in 10 ml of phosphate buffer saline (PBS) was sonicated. The solution was then centrifuged; and the supernatant was diluted with Siraganian buffer and adjusted to equal the enzyme activity of the degranulation tested above. The enzyme solution (45 µl) and test sample solution (5 µl) were transferred into a

Table 1. Medicinal plants used for anti-allergy activity test

Sample number	Botanical names	Family	Part-used	Yield (%w/w)	
				Water extract	Ethanolic extract
1.	*P. nigrum* Linn.	Piperaceae	Fruit	8.8	7.7
2.	*S. aspers* Lour.	Moraceae	Seed	20.3	4.8
3.	*T. crispa* Linn.	Menispermaceae	Stem bark	12.9	6.0
4.	*C. rotundus* Linn.	Cyperaceae	Bulb	11.3	19.8
5.	*A. procera* (Roxb.) Benth.	Leguminosae	Stem bark	18.2	12.7
6.	*D. rhodocalyx* Kurz.	Ebenaceae	Stem bark	11.7	11.7
7.	Longevity formulation[a]	-	-	18.7	6.7

[a]Longevity formulation is composed of six Thai plants (as shown in Table 1) in an equal amount in powder form.

Table 2. Inhibitory effects of Thai medicinal plants on the release of β-hexosaminidase in RBL-2H3 cells

Samples	Inhibition (%) at various concentration (μg/ml)					IC_{50} (μg/ml)	Enzyme inhibition at 100 μg/ml
	0	3	10	30	100		
Piper nigrum (EtOH)	0.0±6.1	-14.4±8.6	62.6±6.5**	81.3±4.4**	95.8±1.0**	14.0	16.3
P. nigrum (H_2O)	0.0±4.4	–	–	22.4±6.6	64.6±0.2**	65.8	16.6
Streblus aspers (EtOH)	0.0±7.0	–	-40.7±5.9	2.4±8.9	60.2±7.1**	82.2	10.4
S. aspers (H_2O)	0.0±7.0	–	–	–	38.2±7.1*	>100	-
Tinospora crispa (EtOH)	0.0±6.0	–	-11.9±7.6	16.0±2.1	44.2±2.9**	>100	-
T. crispa (H_2O)	0.0±7.0	–	18.9±5.4	33.0±4.9*	65.2±5.0**	83.0	17.7
Cyperus rotundus (EtOH)	0.0±2.5	-7.7±2.1	2.2±3.2	19.1±1.7**	43.7±2.9**	>100	-
C. rotundus (H_2O)	0.0±5.1	–	-3.5±8.3	31.1±6.2	63.6±4.3**	61.0	12.6
Diospyros rhodocalyx (EtOH)	0.0±6.7	–	-3.7±6.5	4.2±7.4	38.4±5.8*	>100	-
D. rhodocalyx (H_2O)	0.0±6.6	–	-2.3±12.4	36.5±9.5	84.9±3.4**	40.8	18.6
Albizia procera (EtOH)	0.0±5.6	–	–	–	-95.8±11.3**	>100	-
A. procera (H_2O)	0.0±5.6	–	–	–	-25.2±10.2	>100	-
Logevity herbal formulation (EtOH)	0.0±3.3	-1.1±2.3	10.1±3.5	21.5±5.7	43.5±5.9*	>100	-
Logevity herbal formulation (H_2O)	0.0±5.5	–	-5.0±5.0	23.3±5.4	63.5±2.5**	66.6	18.2
Ketotifen fumarate	0.0±6.9	–	12.8±0.5	38.3±3.2**	68.2±1.5**	47.5μM (20.2 μg/ml)	15.8

Each value represents the mean ± SEM. Significantly different from control, $*p < 0.05$, $**p < 0.01$.

96-well microplate and incubated with 50 μl of the substrate solution at 37°C for 2.5 h. The reaction was stopped by adding 200 μl of the stop solution. The absorbance was measured using a microplate reader at 405 nm.

2.6 Statistics

Values are expressed as mean ± SEM. One-way analysis of variance (ANOVA) followed by Dunnett's test was used for statistical analysis. A difference was considered significant at $*p<0.05$, $**p<0.01$.

3. Results and Discussions

3.1 Inhibitory effects of crude extracts on the release of β-hexosaminidase from RBL-2H3 cells

Longevity herbal formulation, together with water and ethanolic extracts of six plants were determined for their anti-allergic effect. As shown in Table 2, *P. nigrum* (EtOH) exhibited the most potent activity with an IC_{50} value of 14.0 μg/ml, followed by *D. rhodocalyx* (H_2O, 40.8 μg/ml), *C. rotundus* (H_2O, 61.0 μg/ml), *P. nigrum* (H_2O, 65.8 μg/ml), longevity herbal formulation (H_2O, 66.6 μg/ml), *S. aspers* (EtOH, 82.2 μg/ml) and *T. crispa* (H_2O, 83.0 μg/ml), whereas others were inactive ($IC_{50} > 100$ μg/ml). It was indicated that anti-allergic effect of *P. nigrum* (EtOH, IC_{50} = 14.0 μg/ml) was higher than that of ketotifen fumarate, a positive control (IC_{50} = 20.2 μg/ml). The crude extracts were also examined on the enzyme activity of β-hexosaminidase. As a result, they showed weak inhibition against this enzyme activity at 100 μg/ml (Table 2). The result indicated that these extracts inhibited the antigen-induced degranulation but not substantially affected the activity of β-hexosaminidase.

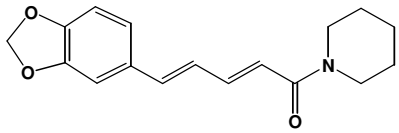

Figure 1. Chemical structure of piperine isolated from *P. nigrum* fruits

Since *P. nigrum* extract appreciably exhibited anti-allergic activity, it is suggested that this plant may contain some active compounds showing anti-allergic activity. Piperine (Figure 1), a major pungent substance in the fruit of the black pepper was tested for its anti-allergic effect. It was shown that piperine possessed IC_{50} value of 16 μg/ml, which was more potent than that of ketotifen fumarate, a positive control (IC_{50} = 20.2 μg/ml) and was comparable to that of *P. nigrum* extract (IC_{50} = 14.0 μg/ml)(Table 3). It is implied that piperine may responsible for anti-allergic activity of *P. nigrum*.

3.2 Inhibitory effects of selected crude extracts (1:1 ratio) on β-hexosaminase release from RBL-2H3 cells

The plant extracts showing high anti-allergic activity (*P. nigrum*, P; *Diospyros rhodocalyx*, D; *Tinospora crispa*, T; *Cyperus rotundus*, C) were prepared in the ratio of 1 : 1 and were determined for their anti-allergic effect. As shown in Table 4, it was found that the preparations of Piper-

Table 3. Inhibitory effects of *P. nigrum* and piperine on the release of β-hexosaminidase

Samples	Inhibition (%) at various concentrations (μg/ml)					IC_{50} (μg/ml)	Enzyme inhibition at 100 μg/ml
	0	3	10	30	100		
P. nigrum (EtOH)	0.0±6.1	-14.4±8.6	62.6±6.5**	81.3±4.4**	95.8±1.0**	14.0	16.3
Piperine	0.0±4.9	-2.8±13.6	34.1±5.8*	90.1±6.3**	88.1±3.6**	16.0	16.6
Ketotifen fumarate	0.0±6.9	–	12.8±0.5	38.3±3.2**	68.2±1.5**	47.5μM (20.2 μg/ml)	15.8

Each value represents the mean ± SEM. Significantly different from control, $*p < 0.05$, $**p < 0.01$.

Table 4. Inhibitory effects of Thai herbal preparations in ratio of 1 : 1 on the release of β-hexosaminidase in RBL-2H3 cells

Samples	Inhibition (%) at various concentrations (μg/ml)					IC_{50} (μg/ml)	Enzyme inhibition at 100 μg/ml
	0	3	10	30	100		
PN-E + CR-W (PC-EW)	0.0±5.5	-10.7±5.9	11.4±3.2	60.4±7.1**	86.2±6.9**	27.2	13.5
PN-E + TC-W (PT-EW)	0.0±1.4	-2.0±0.9	4.9±1.0	32.4±2.0**	71.7±1.3**	49.7	12.9
PN-E + TC-E (PT-E)	0.0±11.9	–	12.8±11.8	52.2±9.8*	103.2±0.9**	26.7	12.6
PN-E + DR-W (PD-EW)	0.0±4.7	-4.9±1.7	9.2±3.3	40.1±4.8**	97.1±0.4**	32.1	18.8
PN-E + DR-E (PD-E)	0.0±5.7	-3.3±1.5	14.3±4.6	66.5±2.7**	100.5±0.4**	23.5	16.9
Ketotifen fumarate	0.0±6.9	–	12.8±0.5	38.3±3.2**	68.2±1.5**	47.5μM (20.2 μg/ml)	15.8

Each value represents the mean ± SEM. Significantly different from control, $*p < 0.05$, $**p < 0.01$.
PN-E : *Piper nigrum* (EtOH)
CR-W : *Cyperus rotundus* (H_2O)
TC-W : *Tinospora crispa* (H_2O)
TC-E : *Tinospora crispa* (EtOH)
DR-W : *Diospyros rhodocalyx* (H_2O)
DR-E : *Diospyros rhodocalyx* (EtOH)

Diospyros (EtOH = PD-E) and Piper-Tinospora (EtOH = PT-E) in the ratio of 1 : 1 significantly inhibited antigen-induced degranulation in RBL-2H3 cells with IC_{50} values of 23.5 and 26.7 μg/ml, respectively, whereas PC-EW (IC_{50} = 27.2 μg/ml), PT-EW (IC_{50} = 49.7 μg/ml) and PD-EW (IC_{50} = 32.1 μg/ml) possessed appreciable anti-allergic activity. The anti-allergic effects of PD-E and PT-E were higher than that of the original longevity formulation itself (IC_{50} = 66.6 μg/ml) and comparable to that of ketotifen fumarate (IC_{50} = 20.2 μg/ml).

From the present study, it is concluded that some Thai medicinal plants traditionally used in longevity formulation possessed marked anti-allergic effects. The results support traditional use of these plants for longevity and provide insight for the discovery of pharmaceutical products for treatment of allergy.

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