



Anti-allergic principles of *Rhinacanthus nasutus* leaves

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Abstract

Three naphthoquinone derivatives, rhinacanthin-C (1), -D (2) and -N (3) were isolated from the extract of *Rhinacanthus nasutus* Kurz leaves and were tested for anti-allergic effect. The result indicated that all three compounds possessed very potent anti-allergic activity against antigen-induced β -hexosaminidase release as a marker of degranulation in RBL-2H3 cells with IC_{50} values of 6.9, 8.9 and 6.4 μ M, respectively. In addition, the effects of rhinacanthin-C, -D and -N on antigen-induced release of TNF- α and IL-4 were also examined. It was found that rhinacanthin-C showed the most potent on antigen-induced TNF- α release with an IC_{50} value of 0.7 μ M, followed by rhinacanthin-D (IC_{50} = 3.8 μ M) and rhinacanthin-N (IC_{50} = 10.3 μ M), whereas those for IL-4 were rhinacanthin-D (IC_{50} = 5.4 μ M), rhinacanthin-C (IC_{50} = 7.0 μ M) and rhinacanthin-N (IC_{50} = 12.0 μ M), respectively. The mechanisms in the late phase reaction of rhinacanthin-C, -D and -N were found to inhibit TNF- α and IL-4 gene expression in antigen-induced TNF- α and IL-4 releases on from RBL-2H3 cells as dose-dependent manners.

The structure-activity trends of rhinacanthin-C, -D and -N on the inhibition of TNF- α release are as follow; substitution with octadienoic acid (rhinacanthin-C) conferred much higher activity than that of benzodioxo carboxylic acid ester (rhinacanthin-D) as well as naphthalene carboxylic acid ester (rhinacanthin-N). For the inhibition of IL-4 release, the substitution with octadienoic acid (rhinacanthin-C) and benzodioxo carboxylic acid ester (rhinacanthin-D) possessed the effect two-fold higher than that of naphthalene carboxylic acid ester (rhinacanthin-N).

As regards active constituents for anti-allergic activity of *R. nasutus*, rhinacanthin-C, -D and -N are responsible for anti-allergic effect of this plant on both the early phase and late phase reactions. The finding may support the traditional use of *R. nasutus* leaves for treatment of allergy and allergy-related diseases.

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Keywords: RBL-2H3 cells; Cytokine production; mRNA expression; *Rhinacanthus nasutus*; Acanthaceae

Introduction

Rhinacanthus nasutus Kurz is one of the plants in the Acanthaceae family, locally known in Thai as Thongpan-chung. The root and whole plant of *R. nasutus* have been used in Thai traditional medicine by topical application (alcoholic extract) for treatment of *Tinea versicolor*, ringworm, itching and skin diseases. The

leaves have been used for treatment of fungal infection, skin diseases, cancers and inflammation (Wutthithamavet 1997; Farnsworth and Bunyaphatsara 1992).

The allergy is an immune dysfunction, which is a serious health problem worldwide. Substances that cause allergic reaction are called allergens including food, pollen, dust mites, cosmetics, mold spores and animal hairs. Hypersensitivity type I, an allergic reaction, is an IgE-mediated immune response, resulting in histamine secretion from mast cells and blood basophils. The histamine causes smooth muscle contraction,

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increased vascular permeability and vasodilation. The early phase reaction of allergy occurs within minutes after allergen exposure, whereas the late phase reaction occurs hours later and involves in cytokines secretion such as TNF- α and IL-4 (Goldsby et al. 2002). Since, β -hexosaminidase is usually released along with histamine from mast cells or basophils, this enzyme is therefore used as the marker for mast cell degranulation in RBL-2H3 cell line (Cheong et al. 1998).

Since the leaves of *R. nasutus* have been used in Thai traditional medicine for treatment of skin diseases and the anti-allergic effect of the leaf extract (naphthoquinones-high yielding extract) was potent with an IC₅₀ value of 7.8 μ g/ml in our preliminary experiment, the present study therefore aimed to investigate anti-allergic effect of the active principles isolated from this plant on both the early phase and late phase reactions of hypersensitivity type 1 (allergic reaction).

Materials and methods

Reagents

Minimum Essential Medium Eagle (MEM) and anti-DNP-IgE (Monoclonal anti-DNP) were purchased from Sigma; fetal calf serum (FCS) was from Gibco; dinitrophenylated bovine serum albumin was prepared as described previously (Tada and Okumura 1971). Other chemicals were from Sigma. 24-well and 96-well plates were from Nunc.

Plant materials

R. nasutus leaves were collected from the Botanical garden at Narathiwat province, Thailand. The voucher specimen is SKP 0011814. The plant material was identified by Assoc. Prof. Dr. Pharkphoom Panichayupakaranant and the voucher specimen is kept at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand.

Preparation of the plant extract and isolation

Two hundred grams dried weight of *R. nasutus* leaves were ground and refluxed with ethyl acetate (EtOAc) for 1 h. The EtOAc extract was concentrated to dryness giving 9.6 g of the crude extract. The extract 5 g was chromatographed over silica gel using CHCl₃ as eluent to afford 8 fractions. Fraction 4 was then chromatographed further on sephadex LH-20 using methanol as eluent to yield three fractions. Fractions I and II were rechromatographed on the same sephadex LH-20 column and finally obtained rhinacanthin C (**1**, 450 mg) and rhinacanthin-N (**3**, 14 mg), respectively.

The fraction 6 from silica gel column was further purified on Sephadex LH-20 column eluted with methanol to yield 7 fractions. The fraction IV was purified on the same sephadex and afforded rhinacanthin-D (**2**, 25 mg). The structures of **1-3** were elucidated by comparing the ¹H and ¹³C-NMR spectral data with those reported (Sendl et al. 1996; Wu et al. 1998).

Anti-allergic activity assay

Inhibitory effects of compounds **1-3** on the release of β -hexosaminidase from RBL-2H3 cells

Inhibitory effects on the release of β -hexosaminidase from RBL-2H3 cells (purchased from ATCC) were evaluated by the following modified method (Matsuda et al. 2004). Briefly, RBL-2H3 cells were dispensed in 24-well plates at a concentration of 2×10^5 cells/well using Minimum Essential Medium Eagle (MEM) containing 10% fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (100 unit/ml) and anti-dinitrophenyl-immunoglobulin E (anti-DNP IgE) (0.45 μ g/ml), then incubated overnight at 37 °C in 5% CO₂ for sensitization of the cells. The cells were washed twice with 500 μ l of Siraganian buffer [119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM MgCl₂, 1 mM CaCl₂, 25 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES), 0.1% bovine serum albumin (BSA) and 40 mM NaOH, pH 7.2] and then incubated in 160 μ l of Siraganian buffer for an additional 10 min at 37 °C. After that, 20 μ l of test sample solution was added to each well and incubated for 10 min, followed by addition of 20 μ l of antigen (DNP-BSA, final concentration is 10 μ g/ml) at 37 °C for 20 min to stimulate the cells to degranulate. The supernatant was transferred into a 96-well plate and incubated with 50 μ l of substrate (1 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide) in 0.1 M citrate buffer (pH 4.5) at 37 °C for 1 h. The reaction was stopped by adding 200 μ l of stop solution (0.1 M Na₂CO₃/NaHCO₃, 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 Siraganian buffer (final DMSO concentration was 0.1%). The inhibition (%) of the release of β -hexosaminidase by the test samples was calculated by the following equation, and IC₅₀ 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 (–)

β-Hexosaminidase inhibitory activity

The following assay was carried out in order to clarify that the anti-allergic effects of samples were due to the inhibition of β-hexosaminidase release, and not from the inhibition of β-hexosaminidase activity.

The cell suspension (5×10^6 cells) in 10 ml of PBS (phosphate buffer saline) 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 96-well microplate and incubated with 50 μl of the substrate solution at 37 °C for 1 h. The reaction was stopped by adding 200 μl of the stop solution and the absorbance was measured using a microplate reader at 405 nm.

Inhibitory effects on antigen-induced TNF-α and IL-4 release from RBL-2H3 cells

Inhibitory effects on the release of TNF-α and IL-4 from RBL-2H3 were evaluated by the method reported previously (Matsuda et al. 2004). RBL-2H3 cells (2×10^5 cells/well) were sensitized with anti-DNP-IgE as described above. The cells were washed with MEM containing 10% FCS, penicillin (100 units/ml) and streptomycin (100 μg/ml), and exchanged with 320 μL of fresh medium. Then 40 μl of test sample solution, and 40 μl of antigen (DNP-BSA, final concentration was 10 μg/ml) were added to each well and incubated at 37 °C for 4 h. The supernatant was transferred into 96 well ELISA plate and then TNF-α and IL-4 concentrations were determined using commercial ELISA kits. The test samples were dissolved in DMSO, and the solution was added to MEM (final DMSO was 0.1%). The inhibition on TNF-α and IL-4 production was calculated by the following equation, and IC₅₀ values were determined graphically:

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

Control (C): DNP-BSA (+), Test sample (–); Test (T): DNP-BSA (+), Test sample (+); Normal (N): DNP-BSA (–), Test sample (–)

Total RNA isolation and RT-PCR

In order to know the mechanism of action on cytokine releases of rhinacanthin-C, -D and -N, the assay for mRNA expression of TNF-α and IL-4 was carried out. The total RNA was isolated from RBL-2H3 cells harvested after 45 min of incubation with samples in various concentrations (3–100 μM) using the RNeasy Mini Kit (Qiagen Operon Co. Ltd., USA). The total RNA from each sample was used for cDNA synthesis

using first strand cDNA synthesis kit (Rever Tra Ace-α, TOYOBO Co., Ltd., Japan), followed by RT-PCR (Rever Tra Dash, TOYOBO Co., Ltd., Japan). The primers for TNF-α and IL-4 were used (forward primer for TNF-α : 5'-CGGAATTCGGCTCCCTCTCAT-CAGTTC-3' and its reverse primer: 5'-GCTCTA-GACCCTTGAAGAGAACCTGGGA-3'; forward primer for IL-4: 5'-TGATGGGYCTCAGCCCC-CACCTTGC-3' and its reverse primer: 5'-CTTTC-AGTGTGTGAGCGTGGACTC-3'. The solution for cDNA synthesis consisted of RNA solution 11 μl, 5 × RT buffer 4 μl, dNTP mixture (10 mM) 2 μl, RNase inhibitor (10 U/ μl) 1 μl, Oligo(dT)20 1 μl and Rever Tra Ace (reverse transcriptase enzyme) 1 μl for a 20 μl reaction. The condition for cDNA synthesis was as follow; 42 °C for 20 min, 99 °C for 5 min and 4 °C for 5 min. After that, 1/10 times (2 μl) of cDNA product was used further for PCR. The PCR mixture consisted of RT reaction mixture (cDNA product) 2 μl; sterilized water 85 μl, 10 × PCR buffer 10 μl, forward primer (10 pmol/ μl) 1 μl, reverse primer (10 pmol/ μl) 1 μl and KOD Dash (polymerase enzyme) 1 μl for final volume of 100 μl. The condition for PCR was as follows; denaturation at 94 °C for 1 min, 98 °C for 30 s, 60 °C for 30 s and 74 °C for 1 min (30 cycles). The PCR products were analyzed in 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining and UV irradiation.

Statistical analysis

The results were expressed as mean ± S.E.M of four determinations at each concentration for each sample. The IC₅₀ values were calculated using the Microsoft Excel program. Statistical significance was calculated by one-way analysis of variance (ANOVA), followed by Dunnett's test.

Results and discussion

Three naphthoquinone derivatives, rhinacanthin-C (1), -D (2) and -N (3) were isolated from the leaves of *R. nasutus* extract (Fig. 1) and were tested for anti-allergic effect. The result indicated that all three compounds possessed very potent anti-allergic activity against antigen-induced β-hexosaminidase release as a marker of degranulation in RBL-2H3 cells with an IC₅₀ value of 6.9, 8.9 and 6.4 μM, respectively (Table 1) without any cytotoxic effect on non-sensitized cells. They also exhibited higher anti-allergic effects than that of ketotifen fumarate (IC₅₀ = 47.5 μM), a clinical used drug. A kind of naphthoquinone, lawsone methyl ether, was reported to exhibit inhibitory effect on cell degranulation in RBL-2H3 cells with an IC₅₀ value of 26.7 μM (Reanmongkol et al. 2003). The result

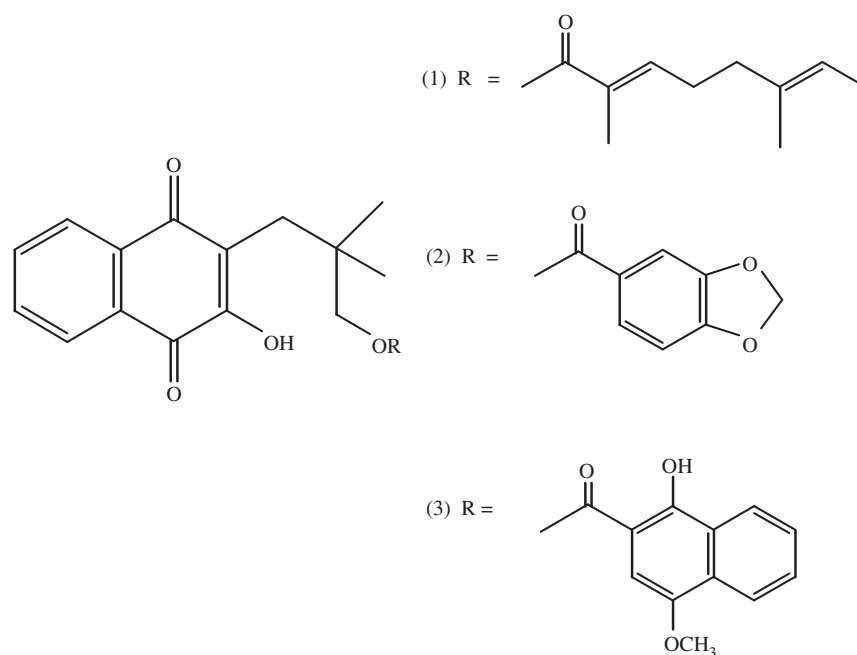


Fig. 1. Structures of rhinacanthin-C (1), rhinacanthin-D (2) and rhinacanthin-N (3) isolated from *Rhinacanthus nasutus*.

Table 1. Anti-allergic activities of rhinacanthin-C, -D and -N from *Rhinacanthus nasutus* leaves^a on antigen-induced β -hexosaminidase release from RBL-2H3 cells.

Compound	% Inhibition at various concentrations (μ M)					IC ₅₀ (μ M)	Enzyme inhibition at 100 μ M
	0 μ M	3 μ M	10 μ M	30 μ M	100 μ M		
Rhinacanthin-C (1)	0.0 $\pm$ 7.1	21.5 $\pm$ 2.5	67.7 $\pm$ 1.8**	90.3 $\pm$ 0.5**	99.3 $\pm$ 0.3**	6.9	21.4
Rhinacanthin-D (2)	0.0 $\pm$ 8.3	8.6 $\pm$ 4.4	60.4 $\pm$ 3.2**	88.9 $\pm$ 2.4**	105.0 $\pm$ 0.9**	8.9	18.5
Rhinacanthin-N (3)	0.0 $\pm$ 3.4	18.6 $\pm$ 4.4	74.8 $\pm$ 1.1**	100.4 $\pm$ 0.5**	101.2 $\pm$ 0.8**	6.4	19.2
Ketotifen fumarate	0.0 $\pm$ 5.9	–	12.8 $\pm$ 0.5	38.3 $\pm$ 3.2**	68.2 $\pm$ 1.5**	47.5	15.8

Statistical significance, * p < 0.05, ** p < 0.01.

^aEach value represents mean $\pm$ S.E.M. of four determinations.

indicated that rhinacanthin-C (1, IC₅₀ = 6.9 μ M), -D (2, IC₅₀ = 8.9 μ M) and -N (3, IC₅₀ = 6.4 μ M) whose structures have long chain substituents exhibited much higher anti-allergic activity than lawsone methyl ether. Compounds 1–3 were also examined on the enzyme activity of β -hexosaminidase. As a result, they showed weak inhibition against this enzyme activity at 100 μ M (Table 1). The result indicated that these compounds inhibited the antigen-induced degranulation but not substantially affected the activity of β -hexosaminidase.

Moreover, the effects of rhinacanthin-C, -D and -N on antigen-induced release of TNF- α and IL-4 in the late phase reaction were also examined. It was found that rhinacanthin-C (1) showed the most potent on antigen-induced TNF- α release with an IC₅₀ value of 0.7 μ M, followed by rhinacanthin-D (2, IC₅₀ = 3.8 μ M) and rhinacanthin-N (3, IC₅₀ = 10.3 μ M), whereas those for IL-4 were rhinacanthin-D (2, IC₅₀ = 5.4 μ M),

rhinacanthin-C (1, IC₅₀ = 7.0 μ M) and rhinacanthin-N (3, IC₅₀ = 12.0 μ M), respectively (Table 2). Cromolyn sodium, a Ca²⁺ influx blocker, is used as a positive control. Its IC₅₀ values against TNF- α and IL-4 releases were found to be 14.1 and 11.2 μ M, respectively. The result indicated that rhinacanthin derivatives possessed higher effects against TNF- α and IL-4 releases than that of cromolyn sodium, except for rhinacanthin-N that exhibited comparable effect to this drug. The mechanisms in the late phase reaction of rhinacanthin-C, -D and -N were found to inhibit TNF- α and IL-4 mRNA expression on antigen-induced TNF- α and IL-4 releases in RBL-2H3 cell line model as dose-dependent manners (Figs. 2 and 3).

Our group also studied on total rhinacanthins content (rhinacanthin-C, -D and -N) from the ethyl acetate fraction of *R. nasutus* leaves using the high performance liquid chromatography (HPLC) technique, the result

Table 2. Inhibitory effects of rhinacanthin-C, -D and -N from *Rhinacanthus nasutus* on antigen-induced TNF- α (A) and IL-4 (B) releases from RBL-2H3 cells.

A							
Compound	% Inhibition at various concentrations (μ M) for TNF- α						IC ₅₀ (μ M)
	0 μ M	0.3 μ M	1 μ M	3 μ M	10 μ M	30 μ M	
Rhinacanthin-C (1)	0.0 $\pm$ 3.8	21.1 $\pm$ 2.8	60.3 $\pm$ 3.0**	93.3 $\pm$ 0.5**	93.5 $\pm$ 3.0**	99.6 $\pm$ 4.2**	0.7
Rhinacanthin-D (2)	0.0 $\pm$ 3.6	–	21.4 $\pm$ 2.5**	55.7 $\pm$ 4.8**	70.1 $\pm$ 3.1**	73.5 $\pm$ 2.7**	3.8
Rhinacanthin-N (3)	0.0 $\pm$ 4.5	–	–	37.8 $\pm$ 3.6**	45.0 $\pm$ 5.0**	92.1 $\pm$ 4.3**	10.3
Cromolyn sodium	0.0 $\pm$ 7.2	–	–	0.9 $\pm$ 4.2	41.5 $\pm$ 3.6**	76.8 $\pm$ 4.1**	14.1

B							
Compound	% Inhibition at various concentrations (μ M) for IL-4						IC ₅₀ (μ M)
	0 μ M	1 μ M	3 μ M	10 μ M	30 μ M	100 μ M	
Rhinacanthin-C (1)	0.0 $\pm$ 0.1	–	39.9 $\pm$ 0.3**	49.3 $\pm$ 2.2**	74.7 $\pm$ 1.2**	100.4 $\pm$ 0.1**	7.0
Rhinacanthin-D (2)	0.0 $\pm$ 0.5	–	34.3 $\pm$ 1.8**	69.8 $\pm$ 0.9**	78.0 $\pm$ 0.8**	99.5 $\pm$ 0.1**	5.4
Rhinacanthin-N (3)	0.0 $\pm$ 3.8	–	–2.4 $\pm$ 3.2	61.0 $\pm$ 1.7**	83.1 $\pm$ 0.0**	99.8 $\pm$ 0.1**	12.0
Cromolyn sodium	0.0 $\pm$ 4.6	–	12.9 $\pm$ 2.8	51.5 $\pm$ 1.9**	77.2 $\pm$ 2.3**	101.2 $\pm$ 1.2**	11.2

Statistical significance, * p < 0.05, ** p < 0.01.^aEach value represents mean $\pm$ S.E.M. of four determinations.

**Fig. 2.** Effects of rhinacanthin-C (1), rhinacanthin-D (2) and rhinacanthin-N (3) at various concentrations (3–100 μ M) on mRNA expression of TNF- α by antigen-induced cytokine production in RBL-2H3 cells. (–) = LPS (–), sample (+); (0) = LPS (+), sample (–)

showed that the total rhinacanthins was found to be 77.5% w/w. Among these rhinacanthins, the amount of rhinacanthin-C was the highest (66.4%), followed by rhinacanthin-D (7.5%) and rhinacanthin-N (3.6%), respectively (Panichayupakaranant et al. 2009).

The structure-activity trends of rhinacanthin-C, -D and -N on the inhibition of TNF- α release are as follow; substitution with octadienoic acid (rhinacanthin-C) conferred much higher activity than that of benzodioxo carboxylic acid ester (rhinacanthin-D) as well as naphthalene carboxylic acid ester (rhinacanthin-N). For the inhibition of IL-4 release, the substitution with

octadienoic acid (rhinacanthin-C) and benzodioxo carboxylic acid ester (rhinacanthin-D) possessed the effect two-fold higher than that of naphthalene carboxylic acid ester (rhinacanthin-N).

Regarding biological activity studies of *R. nasutus*, rhinacanthin-C, it was reported to possess antiproliferative effect *in vitro* and its activity was comparable to that of 5-FU, a clinical used anti-cancer drug (Gotoh et al. 2004). Rhinacanthin-C, -D and -Q isolated from the roots of *R. nasutus* induced apoptosis of human cervical carcinoma HeLaS3 cells. The liposomal formulations of these three compounds showed strong antiproliferative

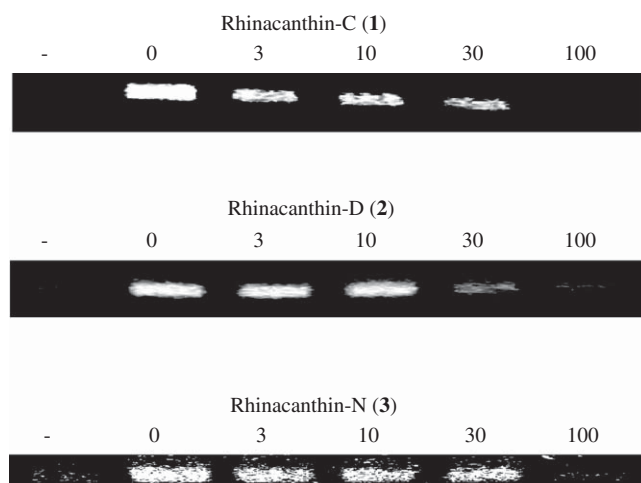


Fig. 3. Effects of rhinacanthin-C (1), rhinacanthin-D (2) and rhinacanthin-N (3) at various concentrations (3–100 μM) on mRNA expression of IL-4 by antigen-induced cytokine production in RBL-2H3 cells. (-) = LPS (-), sample (-); (0) = LPS (+), sample (-)

activity against HeLaS3 cells and these liposomes also suppressed the tumor growth in Meth-A sarcoma-bearing BALB/c mice (Siripong et al. 2006). Moreover, *R. nasutus* extract has been reported to possess immunomodulatory activity on both non-specific cellular and humoral immune responses (Punturee et al. 2005).

As regards active constituents for anti-allergic activity of *R. nasutus*, rhinacanthin-C, -D and -N are responsible for anti-allergic effect on both the early phase and late phase reactions. Furthermore, the inhibition on cytokine releases (TNF- α and IL-4) of these compounds clearly demonstrates anti-allergic potential of this plant through the inhibition of TNF- α and IL-4 gene expression in RBL-2H3 cells. This finding may support the traditional use of *R. nasutus* leaves for treatment of allergy and allergy-related diseases.

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References

- Cheong, H., Choi, E.J., Yoo, G.S., Kim, K.M., Ryu, S.Y., 1998. Desacetylmaticarin, an anti-allergic compound from *Taraxacum platycarpum*. *Planta Medica* 64, 577–578.
- Farnsworth, N.R., Bunyaphratharsa, N., 1992. Prachachon Co., Ltd., Bangkok, pp. 72–73.
- Goldsby, R.A., Kindt, T.J., Osborne, B.A., Kubly, J., 2002. Immunology, fifth ed. W.H. Freeman and Company, New York.
- Gotoh, A., Sakaeda, T., Kimura, T., Shirakawa, T., Wada, Y., Wada, A., Kimachi, T., Takemoto, Y., Iida, A., Seigo, I., Hirai, M., Tomita, H., Okamura, N., Nakamura, T., Okumura, K., 2004. Antiproliferative activity of *Rhinacanthus nasutus* (L.) Kurz extracts and the active moiety, rhinacanthin C. *Biological and Pharmaceutical Bulletin* 27 (7), 1070–1074.
- Matsuda, H., Tewtrakul, S., Morikawa, T., Nakamura, A., Yoshikawa, M., 2004. Anti-allergic principles from Thai zedoary: structural requirements of curcuminoids for inhibition of degranulation and effect on the release of TNF- α and IL-4 in RBL-2H3 cells. *Bioorganic and Medicinal Chemistry* 12, 5891–5898.
- Panichayupakaranant, P., Charoonratana, T., Sirikatitham, A., 2009. RP-HPLC analysis of rhinacanthins in *Rhinacanthus nasutus*: validation and application for the preparation of rhinacanthin high-yielding extract. *Journal of Chromatographic Science*, in press.
- Punturee, K., Wild Christopher, P., Kasinrerk, W., Vinitketkumnuen, U., 2005. Immunomodulatory activities of *Centella asiatica* and *Rhinacanthus nasutus* extracts. *Asian Pacific Journal of Cancer Prevention* 6 (3), 396–400.
- Reanmongkol, W., Subhadhirasakul, S., Panichayupakaranant, P., Kim, K.-M., 2003. Anti-allergic and anti-oxidant activities of some compounds from Thai medicinal plants. *Pharmaceutical Biology* 41 (8), 592–597.
- Sendl, A., Chen, J.L., Jolad, S.D., Stoddart, C., Rozhon, E., Kernann, M., 1996. Two new naphthoquinones with antiviral activity from *Rhinacanthus nasutus*. *Journal of Natural Products* 59 (8), 808–811.
- Siripong, P., Yahuafai, J., Shimizu, K., Ichikawa, K., Yanezawa, S., Asai, T., Kanokmedakul, K., Ruchirawat, S., Oku, N., 2006. Antitumor activity of liposomal naphthoquinone esters isolated from Thai medicinal plant: *Rhinacanthus nasutus* Kurz. *Biological and Pharmaceutical Bulletin* 29 (11), 2279–2283.
- Tada, T., Okumura, K., 1971. Regulation of homocytotropic antibody formation in the rat. I. Feed-back regulation of passively administered antibody. *Journal of Immunology* 106 (4), 1002–1011.
- Wu, T.S., Hsu, H.C., Wu, P.L., Teng, C.M., Wu, Y.C., 1998. Naphthoquinone esters from the root of *Rhinacanthus nasutus*. *Chemical and Pharmaceutical Bulletin* 46 (3), 413–418.
- Wutthithamavet, W., 1997. Thai Traditional Medicine, revised ed. Odean Store Press, Bangkok, p. 314.