Research report

Anti-inflammatory compounds from *Rhinacanthus nasutus* leaves
(สารที่มีฤทธิ์ต้านการอักเสบ จากใบทองพันชั่ง)

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This report was funded by the university's budget

of Prince of Songkla University

The year 2007

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Abstract

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Abstract

Three naphthoquinone derivatives, rhinacanthin-C (**1**), -D (**2**) and -N (**3**) were isolated from the leaves of *Rhinacanthus nasutus* extract and were tested for anti-inflammatory activity. The result indicated that all three compounds possessed very potent anti-inflammatory activity against lipopolysaccharide (LPS)-induced nitric oxide release with IC_{50} values of 1.8, 6.2 and 3.0 μM, respectively. In addition, the effects of rhinacanthin-C, -D and -N on LPS-induced release of prostaglandin E_2 (PGE_2) and tumor necrosis factor (TNF-α) were also examined. It was found that rhinacanthin-C exhibited the most potent on PGE_2 release with an IC_{50} value of 10.4 μM, followed by rhinacanthin-D (IC_{50} = 14.4 μM) and rhinacanthin-N (IC_{50} = 52.1 μM), whereas those for TNF-α were inactive (IC_{50} > 100 μM). The mechanisms in transcriptional level of rhinacanthin-C were found to inhibit iNOS and COX-2 gene expressions in LPS-induced NO and PGE_2 releases from RAW264.7 cells as concentration-dependent manners.

The present study can be concluded that the rhinacanthin-C, -D and -N isolated from *R. nasutus* are responsible for anti-inflammatory effect through the inhibition of NO and PGE_2 releases. This finding may support the traditional use of *R. nasutus* leaves for treatment of inflammation and inflammatory-related diseases.

Keywords : RAW264.7 cells; iNOS; COX-2; *Rhinacanthus nasutus*, Acanthaceae

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บทคัดย่อ

สุภิญญา ติ๋วตระกูล พิมพ์พิมล ตันสกุล และ ภาคภูมิ พาณิชยูปการนันท์

สารที่มีฤทธิ์ต้านการอักเสบ จากใบทองพันชั่ง

การศึกษาฤทธิ์ต้านการอักเสบโดยการนำสารอนุพันธ์ของ naphthoquinone 3 ชนิด ได้แก่ rhinacanthin-C (**1**), -D (**2**) และ -N (**3**) ที่แยกได้จากใบทองพันชั่งมาทดสอบพบว่า สารทั้งสามชนิด มีฤทธิ์ต้านการอักเสบที่ดี ในการยับยั้งการหลั่ง nitric oxide (NO) ที่ถูกเหนี่ยวนำโดย lipopolysaccharide (LPS) โดยค่า IC_{50} ที่ได้มีค่า = 1.8, 6.2 และ 3.0 μM ตามลำดับ นอกจากนี้ยังมีการศึกษาฤทธิ์ยับยั้งการหลั่ง PGE_2 และ TNF-α ที่ถูกเหนี่ยวนำโดย LPS ของสารทั้งสามชนิดนี้ด้วย ผลการศึกษาพบว่า rhinacanthin-C มีฤทธิ์ยับยั้ง PGE_2 ดีที่สุด โดยให้ค่า IC_{50} = 10.4 μM รองลงมาได้แก่ rhinacanthin-D (IC_{50} = 14.4 μM) และ rhinacanthin-N (IC_{50} = 52.1 μM) อย่างไรก็ตามสารทั้งสามชนิดไม่มีผลยับยั้งการหลั่ง TNF-α (IC_{50} > 100 μM) กลไกการออกฤทธิ์ในระดับยีนของ rhinacanthin-C พบว่าสารชนิดนี้สามารถยับยั้ง iNOS และ COX-2 mRNA expression ได้ ในแบบ dose-dependent manner

ดังนั้นจึงสรุปได้ว่า rhinacanthin-C, -D และ -N ที่พบในใบทองพันชั่งมีฤทธิ์ต้านการอักเสบ โดยยับยั้งการหลั่ง NO และ PGE_2 การศึกษานี้จึงสนับสนุนการใช้ใบทองพันชั่งตามตำรายาไทย ในการรักษาโรคที่เกี่ยวข้องกับการอักเสบ

คำหลัก : RAW264.7 cells; iNOS; COX-2; *Rhinacanthus nasutus*, Acanthaceae

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Introduction

Rhinacanthus nasutus Kurz is one of the plants in the Acanthaceae family, locally known in Thai as Thong-pan-chung. The root and whole plant of *R. nasutus* have been used for treatment of Tinea versicolor, ringworm and skin diseases. The leaves have been used for treatment of abscess, haemorrhoid, fungal infection, skin diseases and cancers (Pengcharoen, 2002; Wutthithamavet, 1987; Farnswoth and Bunyapraphatsara, 1992).

Nitric oxide (NO) is produced by the oxidation of L-arginine catalyzed by NO synthase (NOS). This inorganic free radical has been implicated in physiological and pathological processes, such as vasodilation, non-specific host defense and acute or chronic inflammation (Goldsby et al, 2002). NO acts as a host defense by damaging pathogenic DNA, and as a regulatory molecule with homeostatic activities (Kou and Schroder, 1995). In the NOS family, inducible NOS (iNOS) is particularly well known to be involved in the production of NO which causing inflammation and antimicrobial activity. However, excessive production of NO from iNOS can bind with other superoxide radicals and acts as a reactive radical which directly damages the function of normal cells (Moncada et al., 1991).

Cyclooxygenase-2 (COX-2) is an inducible enzyme catalyzing the conversion of arachidonic acid to prostaglandins. Recent studies have suggested that increased levels of prostaglandins and cyclooxygenase activity may play important roles in multiple epithelial cancers. COX-2-derived bioactive lipids, including prostaglandin E_2, are potent inflammatory mediators (Pan et al., 2006).

Since the leaves of *R. nasuthus* have been used in Thai traditional medicine for treatment of inflammation and the anti-inflammatory activity of the EtOAc extract fraction was potent with an IC_{50} value of 0.1 μg/ml in our preliminary experiment, the present study is therefore aimed to investigate anti-inflammatory effect of the active principles isolated from this plant extract on NO, PGE_2 and TNF-α releases as well as its mechanism using RAW264.7 cell model.

Materials and Methods

Reagents

Lipopolysaccharide (LPS, from *Escherichia coli*), RPMI-1640 medium, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), L-nitroarginine (L-NA), caffeic acid phenethylester (CAPE), indomethacin and phosphate buffer saline (PBS) were purchased from Sigma Aldrich (Sigma Aldrich, Missouri, USA). Fetal calf serum (FCS) was bought from Gibco (Invitrogen, California, USA). Penicillin-streptomycin was purchased from Invitrogen (Invitrogen, California, USA). 96-Well microplates were obtained from Nunc (Nunc, Birkrød, Denmark). ELISA test kits of PGE_2 and TNF-α were from R&D systems (R&D systems, Minnesota, USA). Other chemicals were from Sigma Aldrich (Sigma Aldrich, Missouri, USA).

Plant materials

R. nasutus leaves were collected from the Botanical garden at Narathiwat province, Thailand in 2006. 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 siliga gel using $CHCl_3$ to afford 8 fractions. Fraction 4 was then chromatographed further on sephadex LH-20 using methanol 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 siliga gel column was further purified on Sephadex LH-20 column eluting 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 ^{13}C-NMR spectral data with those reported (Sendl et al., 1996; Wu et al., 1998).

Anti-inflammatory activity assay

Inhibitory effects of compounds 1-3 on LPS-induced NO release from murine macrophage RAW264.7 cells

Inhibitory effect on NO production by murine macrophage-like RAW264.7 cells was evaluated using a modified method from that previously reported (Banskota et al., 2003). Briefly, the RAW264.7 cell line [(purchased from Cell Lines Service (CLS)] was cultured in RPMI medium supplemented with 0.1% sodium bicarbonate and 2 mM glutamine, penicillin G (100 units/ml), streptomycin (100 μg/ml) and 10% FCS. The cells were harvested with trypsin-EDTA and diluted to a suspension in a fresh medium. The cells were seeded in 96-well plates with 1 x 10^5 cells/well and allowed to adhere for 1 hr at 37°C in a humidified atmosphere containing 5% CO_2. After that the medium was replaced with a fresh medium containing 200 μg/ml of LPS together with the test samples at various concentrations and was then incubated for 48 h. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using Griess reagent. Cytotoxicity was determined using MTT colorimetric method. Briefly, after 48 h incubation with the test samples, MTT solution (10 μl, 5 mg/ml in PBS) was added to the wells. After 4 h incubation, the medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the formazan production in the cells. The optical density of the formazan solution was measured with a microplate reader at 570 nm. The test compounds were considered to be cytotoxic when the optical density of the sample-treated group was less than 80% of that in the control (vehicle-treated) group. L-NA (a non-specific NOS inhibitor), CAPE (an NF-κB inhibitor) and indomethacin (an NSAID) were used as positive controls. The stock solution of each test sample was dissolved in DMSO, and the solution was added to the medium RPMI (final DMSO is 1%). Inhibition (%) was calculated using the following equation and IC_{50} values were determined graphically (n = 4):

$$\text{Inhibition (\%)} = \frac{A - B}{A - C} \times 100$$

A-*C* : NO_2^- concentration (μM) [*A* : LPS (+), sample (-); *B* : LPS (+), sample(+); *C* : LPS (-), sample (-)].

Inhibitory effects of compounds 1-3 on LPS-induced PGE_2 and TNF-α release from murine macrophage RAW264.7 cells

Briefly, the RAW264.7 cell line was cultured in RPMI medium supplemented with 0.1% sodium bicarbonate and 2 mM glutamine, penicillin G (100 units/ml), streptomycin (100 μg/ml) and 10% FCS. The cells were harvested with trypsin-EDTA and diluted to a suspension in a fresh medium. The cells were seeded in 96-well plates with 1.0 x 10^5 cells/well and allowed to adhere for 1 hr at 37°C in a humidified atmosphere containing 5% CO_2. After that the medium was replaced with a fresh medium containing 200 μg/ml of LPS together with the test samples at various concentrations and was then incubated for 48 h. The supernatant was transferred into 96 well ELISA plate and then PGE_2 and TNF-α concentrations were determined using commercial ELISA kits. The test samples were dissolved in DMSO, and the solution was added to RPMI. The inhibition on PGE_2 and TNF-α production was calculated and IC_{50} values were determined graphically.

Total RNA isolation and RT-PCR

In order to know the mechanism of action on cytokine release of rhinacanthin-C, the assays for mRNA expression of iNOS and COX-2 were carried out. The total RNA was isolated from RAW264.7 cells and was harvested after 20 h of incubation with samples in various concentrations (10, 30 μM) using the RNeasy Mini Kit (Qiagen Operon Co. Ltd., USA). 1 μg of 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 forward and reward primers for iNOS were 5′-ATCTGGATCAGGAACCTGAA-3′ and 5′-CCTTTTTTGCCCCATAGGAA-3′, respectively; the forward and reward primers for COX-2 were 5′-GGAGAGACTATCAAGATAGTGATC-3′ and 5′-ATGGTCAGTAGACTTTTACAGCTC-3′, respectively. The solution for cDNA synthesis consisted of RNA solution 11 μl, 5 x 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 (reverese 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 x 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 to make final volume of 100 μl. The condition for PCR was as follow; denaturation at 94 °C for 1 min, 98°C for 30 s, 55°C for 30 s and 74 °C for 1 min (30 cycles). The PCR products were then analyzed in 1.2 % agarose gel electrophoresis and visualized by SYBR safe 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 IC_{50} 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

The naphthoquinone derivatives, rhinacanthin-C (**1**), -D (**2**) and -N (**3**) were isolated from the leaves of *Rhinacanthus nasutus* extract (Fig. 1) and were tested for anti-inflammatory activity. It is indicated that all three compounds possessed very potent anti-inflammatory activity against LPS-induced NO release with IC_{50} values of 1.8, 6.2 and 3.0 μM, respectively (Table 1). Rhinacanthin-C (IC_{50} = 1.8 μM) and rhinacanthin-N (IC_{50} = 3.0 μM) possessed anti-inflammatory activity higher than that of CAPE (IC_{50} = 5.6 μM), an NF-κB inhibitor; whereas the activity of rhinacanthin-D (IC_{50} = 6.2 μM) was comparable to that of CAPE. These three compounds also exhibited higher activity than that of indomethacin (IC_{50} = 25.0 μM), a clinical used non-steroidal anti-inflammatory drug (NSAID) and higher than L-NA (NOS inhibitor, IC_{50} = 61.8 μM). In addition, the effects of rhinacanthin-C, -D and -N on LPS-induced release of PGE_2 and TNF-α were also examined. It was found that rhinacanthin-C exhibited the most potent on PGE_2 release with an IC_{50} value of 10.4 μM, followed by rhinacanthin-D (IC_{50} = 14.4 μM) and rhinacanthin-N (IC_{50} = 52.1 μM), whereas those for TNF-α were inactive (IC_{50} > 100 μM) (Table 2). The mechanisms in transcriptional level of rhinacanthin-C were found to inhibit iNOS and COX-2 gene expressions in LPS-induced NO and PGE_2 releases as concentration-

dependent manners (Fig. 2). Rhinacanthins have been revealed to possess several biological activities, such as anti-cancer effect (Wu et al., 1988); however, anti-inflammatory activity of these compounds have not been studied so far.

Regarding biological activity studies of *R. nasutus*, rhinacanthin-C 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 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 (Puntaree et al., 2005).

The present study can be concluded that rhinacanthin-C, -D and -N isolated from *R. nasutus* leaves are responsible for anti-inflammatory effect through the inhibition of NO and PGE_2 releases. This finding may support the traditional use of *R. nasutus* leaves for treatment of inflammation and inflammatory-related diseases.

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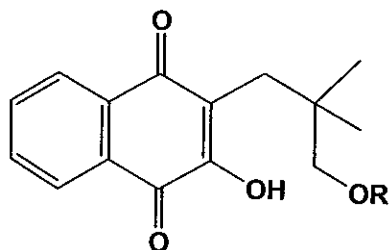

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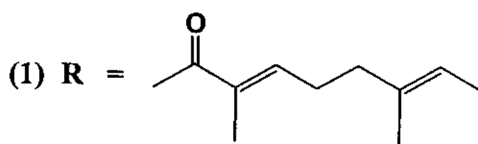

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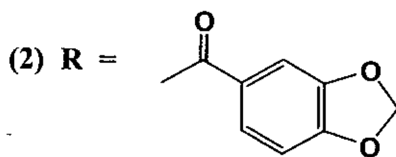

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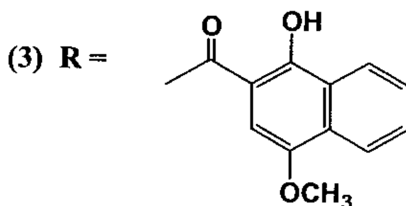

Figure 1. Chemical structures of rhinacanthin-C (1),-D (2) and -N (3) isolated from *R. nasutus*

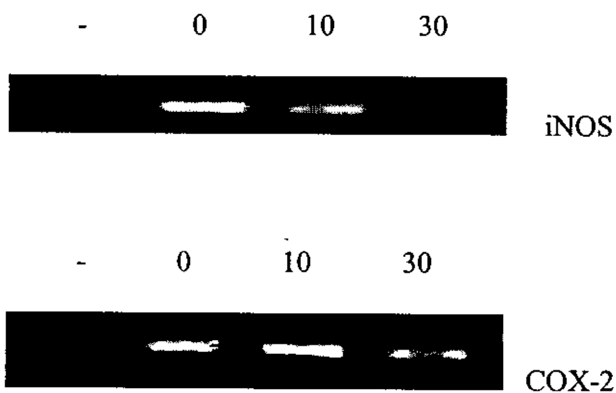

Figure 2. Effects of rhinacanthin-C (**1**) at various concentrations (0, 10, 30 μM) on mRNA expression of iNOS (580 bp) and COX-2 (860 bp) by LPS-induced NO and PGE_2 releases in RAW264.7 cells

(-) = LPS (-), sample (-); (0) = LPS (+), sample (-)

Table 1. Inhibition on NO production[a] of rhinacanthin-C, -D and -N isolated from *Rhinacanthus nasutus* leaves

Compounds	% Inhibition at various concentrations (μM)						IC_{50} (μM)
	0	1	3	10	30	100	
Rhinacanthin-C (1)	0.0 ± 8.0	32.1± 2.8	60.5 ± 4.0**	92.1 ± 1.2**	97.5 ± 1.8**	98.6 ± 1.7**	1.8
Rhinacanthin-D (2)	0.0 ± 8.0	-	22.3 ± 3.9	72.8 ± 3.7**	96.5 ± 1.5**	98.8 ± 3.7**	6.2
Rhinacanthin-N (3)	0.0 ± 9.8	13.0 ± 2.1	45.0 ± 5.9	94.0 ± 2.1**	99.0 ± 1.6**	98.4 ± 1.2[b]**	3.0
Indomethacin	0.0 ± 3.6	-	14.5 ± 2.7	30.2 ± 1.6**	47.6 ± 2.3**	80.3 ± 1.5**	25.0
L-Nitroarginine (L-NA)	0.0 ± 9.9	-	11.7 ± 4.6	20.2 ± 5.9	34.7 ± 1.8 *	71.6 ± 2.6**	61.8
Caffeic acid - phenethylester (CAPE)	0.0 ± 9.9	-	30.7 ± 3.2	68.6 ± 3.4[b]**	98.7 ±1.2[b]**	98.9 ± 2.1[b]**	5.6

[a]Each value represents mean ± S.E.M. of four determinations

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

[b]Cytotoxic effect was observed.

(-) = not tested

Table 2. Inhibition on PGE_2 (**2A**) and TNF-α release (**2B**) of rhinacanthin-C, -D and -N from RAW264.7 cells

2A

Compound	% Inhibition at various concentrations (μM) against PGE_2					IC_{50} (μM)
	0	3	10	30	100	
Rhinacanthin-C (**1**)	0.0 ± 2.5	25.0 ± 1.6*	53.5 ± 2.7**	62.8 ± 1.1**	98.8 ± 1.5**	10.4
Rhinacanthin-D (**2**)	0.0 ± 2.5	0.1 ± 1.9	51.3 ± 2.2**	69.2 ± 1.0**	98.5 ± 1.1**	14.4
Rhinacanthin-N (**3**)	0.0 ± 2.5	-0.3 ± 3.3	5.8 ± 2.1	36.4 ± 2.3**	94.5 ± 1.6**	52.1

2B

Compound	% Inhibition at various concentrations (μM) against TNF-α					IC_{50} (μM)
	0	3	10	30	100	
Rhinacanthin-C (**1**)	0.0 ± 1.7	-	31.9 ± 3.6**	38.1 ± 2.8**	46.5 ± 1.7**	>100
Rhinacanthin-D (**2**)	0.0 ± 1.7	-	23.6 ± 2.2**	30.4 ± 1.8**	36.1 ± 0.8**	>100
Rhinacanthin-N (**3**)	0.0 ± 1.7	-	12.4 ± 0.7*	19.1 ± 0.5**	41.3 ± 1.3**	>100

Each value represents mean ± S.E.M. of four determinations.

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

Acknowledgements

The authors are grateful to the Prince of Songkla University, the Commission on Higher Education (CHE) and the Thailand Research Fund (TRF) for financial support. We also thank the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand, for providing laboratory facilities.

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Appendix

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Effects of rhinacanthins from *Rhinacanthus nasutus* on nitric oxide, prostaglandin E_2 and tumor necrosis factor-alpha releases using RAW264.7 macrophage cells

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Abstract

Three naphthoquinone derivatives, rhinacanthin-C (1), -D (2) and -N (3) were isolated from the leaves of *Rhinacanthus nasutus* extract and were tested for anti-inflammatory activity. The result indicated that all three compounds possessed very potent anti-inflammatory activity against lipopolysaccharide (LPS)-induced nitric oxide release with IC_{50} values of 1.8, 6.2 and 3.0 μM, respectively. In addition, the effects of rhinacanthin-C, -D and -N on LPS induced release of prostaglandin E_2 (PGE_2) and tumor necrosis factor (TNF-α) were also examined. It was found that rhinacanthin-C exhibited the most potent on PGE_2 release with an IC_{50} value of 10.4 μM, followed by rhinacanthin-D (IC_{50} = 14.4 μM) and rhinacanthin-N (IC_{50} = 52.1 μM), whereas those for TNF-α were inactive (IC_{50} > 100 μM). The mechanisms in transcriptional level of rhinacanthin-C were found to inhibit iNOS and COX-2 gene expressions in LPS-induced NO and PGE_2 releases from RAW264.7 cells in concentration-dependent manners.

Regarding active constituents for anti-inflammatory activity of *R. nasutus*, rhinacanthins are responsible for this effect through the inhibition of NO and PGE_2 releases. The finding may support the traditional use of *R. nasutus* leaves for treatment of the inflammatory-related diseases.
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Keywords: RAW264.7 cells; INOS; COX-2; *Rhinacanthus nasutus*; Acanthaceae

Introduction

Rhinacanthus nasutus Kurz is one of the plants in the Acanthaceae family, locally known in Thai as Thong-pan-chung. The root and whole plant of *R. nasutus* have been used for the treatment of Tinea versicolor, ringworm and skin diseases. The leaves have been used for treatment of abscess, haemorrhoid, fungal infection, skin diseases and cancers (Wutthithamavet 1997; Farnsworth and Bunyapraphattsara 1992).

Nitric oxide (NO) is one of the inflammatory mediators causing inflammation in many organs and it has potent antimicrobial activity (Goldsby et al. 2002). NO is produced by the oxidation of L-arginine catalyzed by NO synthase (NOS). This inorganic free radical has been implicated in physiological and pathological processes, such as vasodilation, non-specific host defense and acute or chronic inflammation. NO acts as a host defense by damaging pathogenic DNA, and as a regulatory molecule with homeostatic activities (Kou and Schroder 1995). In the NOS family, inducible NOS (iNOS) is particularly well known to be involved in the overproduction of NO in cells. NO can bind with other

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doi:10.1016/j.phymed.2008.12.022

Please cite this article as: Tewtrakul, S., et al., Effects of rhinacanthins from *Rhinacanthus nasutus* on nitric oxide, prostaglandin E_2 and tumor necrosis factor-alpha releases.... Phytomedicine (2009), doi:10.1016/j.phymed.2008.12.022

superoxide radicals and acts as a reactive radical which directly damages the function of normal cells (Moncada et al. 1991).

Cyclooxygenase-2 (COX-2) is an inducible enzyme catalyzing the conversion of arachidonic acid to prostaglandins. Recent studies have suggested that increased levels of prostaglandins and cyclooxygenase activity may play important roles in multiple epithelial cancers. COX-2-derived bioactive lipids, including prostaglandin E_2, are potent inflammatory mediators (Pan et al. 2006).

Since the leaves of *R. nasuthus* have been used in Thai traditional medicine for treatment of inflammation and the anti-inflammatory activity of the EtOAc extract was potent with an IC_{50} value of 0.1 μg/ml in our preliminary experiment. The present study is therefore aimed to investigate the anti-inflammatory effect of the active principles isolated from this plant extract on NO, PGE_2 and TNF-α releases as well as its mechanism using RAW264.7 cell model.

Materials and methods

Reagents

Lipopolysaccharide (LPS, from *Escherichia coli*), RPMI-1640 medium, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), L-nitroarginine (L-NA), caffeic acid phenethylester (CAPE), indomethacin and phosphate buffer saline (PBS) were purchased from Sigma Aldrich (Sigma Aldrich, Missouri, USA). Fetal calf serum (FCS) was bought from Gibco (Invitrogen, California, USA). Penicillin–streptomycin was purchased from Invitrogen (Invitrogen, California, USA). 96-well microplates were obtained from Nunc (Nunc, Birkrød, Denmark). ELISA test kits of PGE_2 and TNF-α were from R&D systems (R&D systems, Minnesota, USA). Other chemicals were from Sigma Aldrich (Sigma Aldrich, Missouri, USA).

Plant materials

R. nasutus leaves were collected from the Botanical garden at Narathiwat province, Thailand in 2006. The voucher specimen is SKP 0011814. The plant material was identified by Associate 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 $CHCl_3$ to afford 8 fractions. Fraction 4 was then chromatographed further on sephadex LH-20 using methanol to yield three fractions. Fractions I and II were rechromatographed on the same sephadex LH-20 column and finally rhinacanthin-C (**1**, 450 mg) and rhinacanthin-N (**3**, 14 mg), respectively, are obtained. Fraction 6 from siliga gel column was further purified on Sephadex LH-20 column eluting 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 ^{13}C-NMR spectral data with those reported (Sendl et al. 1996; Wu et al. 1998).

Anti-inflammatory activity assay

Inhibitory effects of compounds 1–3 on the release of NO from RAW264.7 cells

Inhibitory effect on NO production by murine macrophage-like RAW264.7 cells was evaluated using a modified method from that previously reported (Banskota et al. 2003). Briefly, the RAW264.7 cell line [purchased from Cell Lines Service (CLS)] was cultured in RPMI medium supplemented with 0.1% sodium bicarbonate and 2 mM glutamine, penicillin G (100 units/ml), streptomycin (100 μg/ml) and 10% FCS. The cells were harvested with trypsin–EDTA and diluted to suspension in a fresh medium. The cells were seeded in 96-well plates with 1×10^5 cells/well and allowed to adhere for 1 h at 37 °C in a humidified atmosphere containing 5% CO_2. After that the medium was replaced with a fresh medium containing 200 μg/ml of LPS together with the test samples at various concentrations and was then incubated for 48 h. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent. Cytotoxicity was determined using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) colorimetric method. Briefly, after 48 h incubation with the test samples, MTT solution (10 μl, 5 mg/ml in PBS) was added to the wells. After 4 h incubation, the medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the formazan production in the cells. The optical density of the formazan solution was measured with a microplate reader at 570 nm. The test compounds were considered to be cytotoxic when the optical density of the sample-treated group was less than 80% of that in the control (vehicle-treated) group. L-NA and caffeic acid phenethylester (CAPE) were used as positive controls. The stock solution of each test sample was dissolved in DMSO, and the solution was added to the medium RPMI (final

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DMSO is 1%). Inhibition (%) was calculated using the following equation and IC_{50} values were determined graphically ($n = 4$):

$$\text{Inhibition } (\%) = \frac{A-B}{A-C} \times 100$$

$A-C$: NO_2^- concentration (μM) [A: LPS (+), sample (−); B: LPS (+), sample(+) and C: LPS (−), sample (−)].

Inhibitory effects of compounds 1–3 on LPS-induced PGE_2 and TNF-α release from RAW264.7 cells

Briefly, the RAW264.7 cell line was cultured in RPMI medium supplemented with 0.1% sodium bicarbonate and 2 mM glutamine, penicillin G (100 units/ml), streptomycin (100 μg/ml) and 10% FCS. The cells were harvested with trypsin–EDTA and diluted to a suspension in a fresh medium. The cells were seeded in 96-well plates with 1.0×10^5 cells/well and allowed to adhere for 1 h at 37 °C in a humidified atmosphere containing 5% CO_2. After that the medium was replaced with a fresh medium containing 200 μg/ml of LPS together with the test samples at various concentrations and was then incubated for 48 h. The supernatant was transferred into 96-well ELISA plate and then PGE_2 and TNF-α concentrations were determined using commercial ELISA kits. The test samples were dissolved in DMSO, and the solution was added to RPMI. The inhibition on PGE_2 and TNF-α production was calculated and IC_{50} values were determined graphically.

Total RNA isolation and RT-PCR

In order to know the mechanism of action on cytokine release of rhinacanthin-C, the assays for mRNA expression of iNOS and COX-2 were carried out. The total RNA was isolated from RAW264.7 cells and was harvested after 20 h of incubation with samples in various concentrations (10 and 30 μM) using the RNeasy Mini Kit (Qiagen Operon Co. Ltd., USA). One microgram of 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 iNOS and COX-2 were used (forward primer for iNOS: 5′-ATCTGGATCAGGAACCTGAA-3′ and its reverse primer: 5′-CCTTTTTTGCCCCATAGGAA-3′; forward primer for COX-2: 5′-GGAGAGACTATCAAGATAGTGATC-3′ and its reverse primer: 5′-ATGGTCAGTAGACTTTTACAGCTC-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 follows: 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, 55 °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 SYBR safe 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 IC_{50} 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

The naphthoquinone derivatives, rhinacanthin-C (**1**), -D (**2**) and -N (**3**) were isolated from the leaves of *Rhinacanthus nasutus* extract (Fig. 1) and were tested for anti-inflammatory activity. It is indicated that all the three compounds possessed very potent anti-inflammatory activity against LPS-induced NO release with IC_{50} values of 1.8, 6.2 and 3.0 μM, respectively (Table 1). Rhinacanthin-C ($IC_{50} = 1.8$ μM) and rhinacanthin-N ($IC_{50} = 3.0$ μM) possessed anti-inflammatory activity higher than that of CAPE ($IC_{50} = 5.6$ μM), an NF-κB inhibitor; whereas the activity of rhinacanthin-D ($IC_{50} = 6.2$ μM) was comparable to that of CAPE. These three compounds also exhibited higher activity than that of indomethacin ($IC_{50} = 25.0$ μM), a clinically used non-steroidal anti-inflammatory drug (NSAID) and higher than L-NA (NOS inhibitor, $IC_{50} = 61.8$ μM). In addition, the effects of rhinacanthin-C, -D and -N on LPS-induced release of PGE_2 and TNF-α were also examined. It was found that rhinacanthin-C exhibited the most potent PGE_2 release with an IC_{50} value of 10.4 μM, followed by rhinacanthin-D ($IC_{50} = 14.4$ μM) and rhinacanthin-N ($IC_{50} = 52.1$ μM), whereas those for TNF-α were inactive ($IC_{50} > 100$ μM) (Table 2). The mechanisms in transcriptional level of rhinacanthin-C were found to inhibit iNOS and COX-2 gene expressions in LPS-induced NO and PGE_2 releases in concentration-dependent manners (Fig. 2). Rhinacanthins have been revealed to possess several biological activities, such as anti-cancer effect (Wu et al. 1988);

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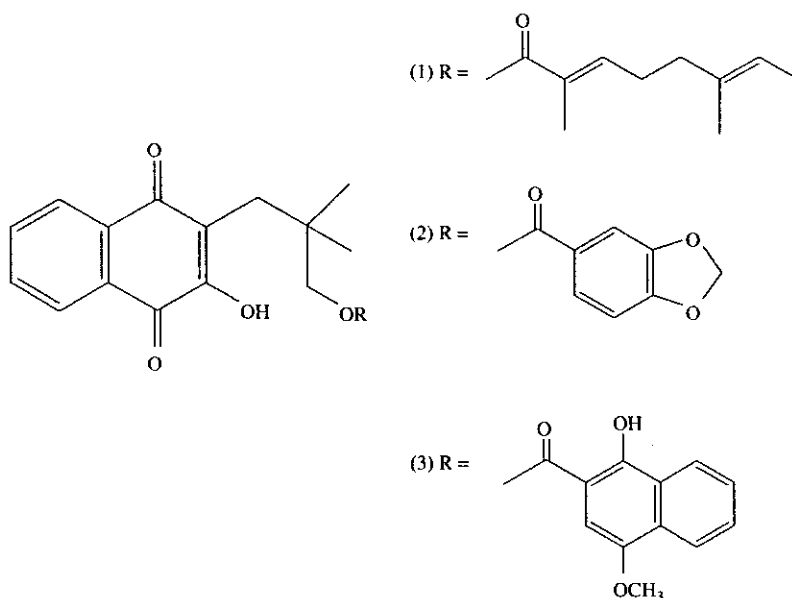

Fig. 1. Chemical structures of rhinacanthin-C (**1**), -D (**2**) and -N (**3**) isolated from *R. nasutus*.

Table 1. Inhibition on NO production[a] of rhinacanthin-C, -D and -N isolated from *Rhinacanthus nasutus* leaves.

Compounds	% Inhibition at various concentrations (μM)						IC_{50} (μM)
	0	1	3	10	30	100	
Rhinacanthin-C (**1**)	0.0 ± 8.0	32.1 ± 2.8	$60.5 \pm 4.0^{**}$	$92.1 \pm 1.2^{**}$	$97.5 \pm 1.8^{**}$	$98.6 \pm 1.7^{**}$	1.8
Rhinacanthin-D (**2**)	0.0 ± 8.0	–	22.3 ± 3.9	$72.8 \pm 3.7^{**}$	$96.5 \pm 1.5^{**}$	$98.8 \pm 3.7^{**}$	6.2
Rhinacanthin-N (**3**)	0.0 ± 9.8	13.0 ± 2.1	45.0 ± 5.9	$94.0 \pm 2.1^{**}$	$99.0 \pm 1.6^{**}$	$98.4 \pm 1.2^{b,**}$	3.0
Indomethacin	0.0 ± 3.6	–	14.5 ± 2.7	$30.2 \pm 1.6^{**}$	$47.6 \pm 2.3^{**}$	$80.3 \pm 1.5^{**}$	25.0
L-Nitroarginine (L-NA)	0.0 ± 9.9	–	11.7 ± 4.6	20.2 ± 5.9	$34.7 \pm 1.8^{*}$	$71.6 \pm 2.6^{**}$	61.8
Caffeic acid-phenethylester (CAPE)	0.0 ± 9.9	–	30.7 ± 3.2	$68.6 \pm 3.4^{b,**}$	$98.7 \pm 1.2^{b,**}$	$98.9 \pm 2.1^{b,**}$	5.6

*Statistical significance $p < 0.05$.
**Statistical significance $p < 0.01$.
[a]Each value represents mean ± S.E.M. of four determinations.
[b]Cytotoxic effect was observed.

however, anti-inflammatory activity of these compounds have not been studied so far.

Regarding biological activity studies of *R. nasutus*, rhinacanthin-C was reported to possess antiproliferative effect *in vitro* and its activity was comparable to that of 5-FU, a clinically 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 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).

From the present study it can be concluded that the rhinacanthins from *R. nasutus* are responsible for anti-inflammatory effect through the inhibition of NO and PGE_2 release. This finding may support the traditional use of *R. nasutus* leaves for treatment of inflammation and inflammatory-related diseases.

Acknowledgements

The authors are grateful to the Prince of Songkla University, the Commission on Higher Education (CHE) and the Thailand Research Fund (TRF) for

Table 2. Inhibition on PGE_2 (**2A**) and TNF-α release (**2B**) of rhinacanthin-C, -D and -N from RAW264.7 cells.

Compound	0	3	10	30	100	IC_{50} (μM)
(A) % Inhibition at various concentrations (μM) against PGE_2						
Rhinacanthin-C (**1**)	0.0±2.5	25.0±1.6*	53.5±2.7**	62.8±1.1**	98.8±1.5**	10.4
Rhinacanthin-D (**2**)	0.0±2.5	0.1±1.9	51.3±2.2**	69.2±1.0**	98.5±1.1**	14.4
Rhinacanthin-N (**3**)	0.0±2.5	−0.3±3.3	5.8±2.1	36.4±2.3**	94.5±1.6**	52.1
(B) % Inhibition at various concentrations (μM) against TNF-α						
Rhinacanthin-C (**1**)	0.0±1.7	–	31.9±3.6**	38.1±2.8**	46.5±1.7**	>100
Rhinacanthin-D (**2**)	0.0±1.7	–	23.6±2.2**	30.4±1.8**	36.1±0.8**	>100
Rhinacanthin-N (**3**)	0.0±1.7	–	12.4±0.7*	19.1±0.5**	41.3±1.3**	>100

Each value represents mean±S.E.M. of four determinations.
*Statistical significance $p<0.05$.
**Statistical significance $p<0.01$.

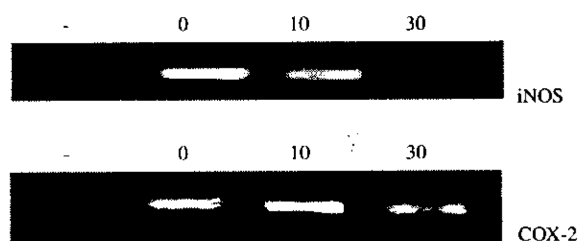

Fig. 2. Effects of rhinacanthin-C at various concentrations (0, 10 and 30 μM) on mRNA expression of iNOS (580 bp) and COX-2 (860 bp) by LPS-induced NO and PGE_2 releases in RAW264.7 cells (−) = LPS (−), sample (−); (0) = LPS (+), sample (−).

financial support. We also thank the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand, and the International Foundation for Science (IFS), for providing laboratory facilities.

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