Mahidol University Journal of Pharmaceutical Sciences 2006; 33(1-4): 15-22.

Original Article

Antibacterial Potential of *Rhinacanthus nasutus* against Clinically Isolated Bacteria from Thai Cancer Patients

P. Siripong,[1*] V. Wongseri,[2] S. Piyaviriyakul,[1] J. Yahaufai,[1]
R. Chanpai[1] and K. Kanokmedakul[3]

[1]Natural Products Research Section, Research Division, [2]Microbiology Section, Pathology Division, National Cancer Institute, Bangkok, [3]Department of Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand.

Abstract The antibacterial potential of fifteen extracts from roots, leaves and stems of *Rhinacanthus nasutus* (family Acanthaceae) was evaluated by the agar dilution method. Four quality control isolates of bacteria; *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 together with 68 clinically isolated bacteria from Thai cancer patients; coagulase positive staphylococci (12), coagulase negative staphylococci (10), β-hemolytic streptococci (16), enterococci (10), *Escherichia coli* (5), *Klebsiella* spp. (5), *Enterobacter* spp. (5) and *Pseudomonas aeruginosa* (5) were used as tested bacteria. The results demonstrated that *n*-hexane and chloroform extracts of roots and *n*-hexane extract of leaves showed potent antibacterial activity against Gram-positive bacteria, whereas the aqueous extracts of all parts, methanolic extracts of stems and leaves as well as 85% ethanolic extract of stems were inactive. None of the extracts showed activity against Gram-negative bacteria. Bioassay-directed fractionations of the active extracts led to the isolation of 3 main naphthoquinone esters which were classified by spectroscopic data as rhinacanthins-C, -N and -Q, respectively. Their antibacterial potential against the clinical and standard bacteria was then analyzed. Rhinacanthins-N, -Q and -C exhibited potent antibacterial activity against β-hemolytic streptococci, enterococci and staphylococci, with potencies comparable to those of gentamicin, an antibiotic drug. Among them, rhinacanthin-N was the most active compound with the MIC_{50} and MIC_{90} values of 4.9 and 9.76 μg/ml, respectively. Our findings suggest that it is of interest to further study the activity and usage of the *Rhinacanthus* plant as an antimicrobial drug for the treatment of infectious diseases in patients.

Keywords: Acanthaceae, antibacterial potential, cancer patients, clinically isolated bacteria, naphthoquinone esters, *Rhinacanthus nasutus*

INTRODUCTION

Infection is one of the main complications and often becomes life-threatening in cancer patients. The predisposing factors that make these patients prone to be susceptible to infection may be the alteration of the host immune system caused either by the neoplastic process itself or by the therapeutic process or both.[1,2] Treatment is necessary to combat any kind of infection in cancer patients, therefore drug resistance to the currently used antibiotics and the unwanted side effects are unavoidable.[3-4] Hence, natural products from plants which have been used as anti-infective agents in folkloric remedies might be an alternative way to solve this problem.[5]

[*]**Corresponding author:** Natural Products Research Section, Research Division, National Cancer Institute, Bangkok 10400, Thailand. Fax: (662) 3547025 ext 1414. Email: pongpun8@yahoo.com, pongpun@health.moph.go.th

Rhinacanthus nasutus Kurz. (family Acanthaceae) is a valuable plant which is widely distributed and cultivated in South China, Taiwan, India and also in Thailand. This plant has been used in folk remedies to treat various aliments such as ringworm and other fungal derived skin diseases, eczema, pulmonary tuberculosis, hepatitis, diabetes, hypertension and cancers.[6-11] In Thailand, its roots and leaves are ground in alcohol to be applied over infectious areas and other skin eruptions.[9,11] The *Rhinacanthus* plant is well known to be a rich source of flavonoids, steroids, triterpenoids, anthraquinones, lignans and especially naphthoquinone analogues.[7-14] Some bioactive naphthoquinone compounds from *R. nasutus* have been reported. Rhinacanthins (B-D, G-Q) show significant antiproliferative activity against cancer cells *in vitro*.[7,11,13-15] Liposomal rhinacanthins-C, -N and -Q showed antitumor activity against Meth-A sarcoma ascites cells in mice.[16] Rhinacanthone also showed potent activity against Dalton's lymphoma ascites in mice[17] as well as *in vitro* antifungal activity against *Pyricularie oryzae*, pathogenic fungus of rice.[8] Rhinacanthins-C and -D exhibited inhibitory effect in human cytomegarovirus (CMV).[9] Moreover, rhinacanthins-E and -F, two lignans, showed antiviral activity against influenza virus.[10] However, so far no scientific information on this plant has been found relating to its effect on microorganisms that were etiologic agents of infectious diseases in cancer patients.

The present investigation was undertaken to evaluate the antibacterial potential of fifteen extracts from the roots, leaves and stems of *R. nasutus* against 68 isolates of Gram-positive and Gram-negative bacteria isolated from various specimens of Thai cancer patients. Furthermore, the antibacterial potential of three main naphthoquinone esters isolated from its active extracts was also examined.

MATERIAL AND METHODS

General Experimental Procedures

Melting points were determined on a Buchi 512 melting point apparatus and are uncorrected. ^{1}H and ^{13}CNMR spectra were recorded on a JEOL JNM-A 500 MHz spectrophotometer in $CDCl_3/CD_3OD$ using with TMS as internal standard. Mass spectra were measured on JEOL JMSAX 500/ JMSDX 303 operating at 70 eV. Silica gel (Merck type 60, 70-230 mesh) was used for column chromatography. Pre-coated silica gel plates (Merck, Kieselgel 60 F-254, 0.25 mm) were used for analytical TLC, detected under UV irradiation (254 and 356 nm) and by spraying with 10% sulphuric acid reagent followed by heating.

Plant Material

Rhinacanthus nasutus was collected in Prachinburi province, Thailand in 2000 and identified in comparison with an authentic herbarium specimen of this species. A voucher specimen (NCIP No. 0129) was deposited in the Herbarium of the Natural Products Research Section, Research Division, National Cancer Institute, Bangkok, Thailand.

Extraction of the Crude Extracts

Dried powder of each part of roots, leaves and stems (1 kg) of *R. nasutus* was extracted successively with methanol in a Soxhlet apparatus. The extracts were filtered, concentrated *in vacuo* and further partitioned with *n*-hexane, chloroform and methanol, affording part extracts of these solvents. From TLC chromatography, the pattern of naphthoquinone compounds of active *n*-hexane and chloroform extracts from the roots of *R. nasutus* were quite similar, so these active extracts were pooled and prepared for further isolation.

Aqueous and 85% alcoholic extracts of roots, leaves and stems were prepared by refluxing the drug powder of each part (200 g) for 2 hours with distilled water and macerating at room temperature for 48 hours. Each filtrate was concentrated *in vacuo* and lyophilized. The dried materials were kept in a freezer at -20°C until used.

Isolation of the Bioactive Naphthoquinone Esters from R. nasutus Roots.

Three main naphthoquinone esters were isolated from the roots of *R. nasutus*. Briefly, a portion of the chloroform extract (7.6 g)

was chromatographed on a silica gel column (200 g) and eluted with chloroform and methanol in order of increasing polarity. Fractions of 100 ml were collected to provide 5 fractions (A-E) and 3 middle fractions were used for further purification. Fraction B was chromatographed on silica gel and eluted with *n*-hexane and chloroform by increasing polarity, affording rhinacanthin-C as red oil (1.3 g). Fraction C was then chromatographed on silica gel, eluted with chloroform and methanol (19:1) and two compounds were isolated. Recrystallization of these compounds from *n*-hexane afforded rhinacanthin-C (0.69 g) and orange needles of rhinacanthin-N (0.112 g). Fraction D was rechromatographed on the silica gel column, eluted with ethyl acetate:methanol (4:1) and two compounds were obtained; rhinacanthin-N (0.62 g) and rhinacanthin-Q as yellow powder (0.58 g). The identity of the three active compounds was confirmed by spectroscopic data (UV, IR, ^{1}H- and ^{13}C-NMR, MS).[11,13,14,18]

Microorganisms Tested

The bacteria used to assess the antibacterial properties of *R. nasutus* were four quality control isolates of bacteria; *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 together with 68 clinically isolated bacteria from various specimens, *e.g.* sputum, blood, wounds, vaginal swab of cancer patients at the National Cancer Institute, Bangkok, Thailand, which were isolated and identify according to the routine bacteriology laboratory. These bacteria were identified as: coagulase positive staphylococci (12 isolates), coagulase negative staphylococci (10 isolates), β-hemolytic streptococci (16 isolates), enterococci (10 isolates), *Escherichia coli* (5 isolates), Klebsiella spp. (5 isolates), Enterobacter spp. (5 isolates) and *Pseudomonas aeruginosa* (5 isolates). Both quality control strains and clinically isolates bacteria were grown in brain-heart infusion broth (BHI) at 37°C and maintained on Mueller-Hinton agar slant at 4°C until used.

Determination of Antibacterial Activity

Antibacterial activity of *R. nasutus* extracts and isolated compounds were determined by agar dilution method.[18] Briefly, all bacteria were grown on Mueller-Hinton agar and broth (Difco laboratories), excepts the β-hemolytic streptococci were grown and tested on Mueller-Hinton agar, supplemented with 5% sheep blood. Organic crude extracts and isolated compounds were dissolved in dimethyl sulfoxide (DMSO, E. Merck) and the aqueous extract was tested as aqueous solution. All dissolved extracts were then diluted two-fold to a final concentration ranging from 1-10,000 μg/ml in Mueller-Hinton agar. Gentamicin (M&H Manufactering Co., Ltd.) at the concentrations of 0.5-128 μg/ml, was used as a positive control agent. The inoculum was prepared from direct colony suspension equivalent to 0.5 McFarland turbidity standard and standardized to yield 0.5 colony forming units (CFU)/ml. The amount of 2.5 μl of each kind of bacteria was spotted onto the test plate as well as the solvent control plate (0.2% DMSO) and growth control plate (without drug or extract). All plates were incubated overnight at 37°C. Antibacterial activity was measured as the minimum inhibitory concentrations (MICs). Each assay was performed in triplicate and repeated twice.

Minimum inhibitory concentration (MIC) was measured by determining the smallest amount of the extract or pure compound needed to inhibit the growth of a test bacterium. A series of tubes were filled with 1 ml of liquid broth medium containing a series of two fold dilution of extracts (10-10,000 μg/ml) or pure compounds (1-1,000 μg/ml), test bacteria and solvent controls. After overnight incubation at 25°C, the tubes in which growth did not occur were noted. In the case of an MIC value of pure compounds lower than 200 μg/ml, the suspension was subcultured on Mueller Hinton agar to observe the growth of bacteria at 37°C for 24 hours.

RESULTS AND DISCUSSION

The results presented in Table 1 demonstrate that the *n*-hexane and chloroform extracts of roots and *n*-hexane extract of leaves showed remarkably potent antibacterial activity towards all isolates of Gram-positive bacteria; β-hemolytic streptococci, enterococci, staphylococci with the MICs of 39.06-1,250 μg/ml. The 85% ethanolic extracts of roots and leaves as well as chloroform extract of leaves showed moderate activity with the MICs of 1,250 -10,000 μg/ml, whereas *n*-hexane and chloroform extracts of stems showed selective inhibitory effects on β-hemolytic streptococci at the MICs of 2,500 and 5,000 μg/ml, respectively. However, the aqueous extracts of all parts, methanolic extracts of leaves and stems as well as 85% ethanolic extract of stems were inactive (MICs > 10,000 μg/ml). None of the extracts were active against the Gram-negative bacteria. Control experiments with solvent plates showed no activity. Therefore, it is clearly indicated that organic extracts of *n*-hexane and chloroform isolated from its roots had potent effects and greater than 85% ethanolic and aqueous extracts. These findings reveal that the polarity of the solvent seems to play an important role in exhibiting potential activity, suggesting that all of the bioactive compounds may be obtained through the organic extracts.

With further separation of active extracts from roots of *R. nasutus* using bioassay-guided fractionation, three main naphthoquinone esters were isolated and identified as rhinacanthins-C, -N and -Q (Figure 1). These structures were elucidated by means of spectroscopic data.[9,11,13,14,19] The antibacterial potentials of these compounds were then analyzed. As shown in Table 2, rhinacanthins-N, -Q and -C exhibited potent antibacterial activity against β-hemolytic streptococci, enterococci and staphylococci with MIC_{50} and MIC_{90} values in the range of 4.9-39.06 and 9.76-625 μg/ml, respectively. Interestingly, rhinacanthin-N was the most active compound, with MIC_{50} and MIC_{90} values of 4.9 and 9.76 μg/ml, respectively, and had greater potency than gentamicin, an antibiotic drug. None of the isolated compounds were effective against Gram-negative bacteria (MICs > 1,000 μg/ml). However, the present study also suggested that three main rhinacanthins possesses only bacteriostatic and not a bactericidal activity, since the growth of these bacteria could still be detected after subculture. To our knowledge, this is the first report of antibacterial naphthoquinone esters isolated from *R. nasutus* and their efficacy against clinical bacteria isolated from various specimens of cancer patients.

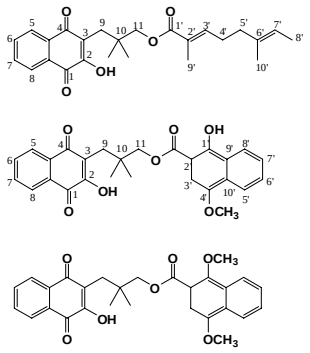

Figure 1. Structures of the naphthoquinone esters isolated from *R. nasutus* roots.

From the above results, *Rhinacanthus* extracts and three main naphthoquinone esters were shown to be effective towards isolates of Gram-positive bacteria. β-hemolytic streptococci appeared to be the most sensitive microorganism tested. However, none of the extracts were active against any isolates of Gram-negative bacteria. It is therefore theorized that Gram-positive bacteria are more susceptible than Gram-negative bacteria due to the differences in their cell wall structure. This action may be attributed to Gram-positive bacteria lacking the outer membrane but having a much thicker wall of the peptidoglycan, which is not an effective permeability barrier. The drugs enable the barriers to be overcome more in Gram-positive cell walls than in Gram-negative ones.[20,21]

By comparison, with the structure-activity relationship of three active naphthoquinone esters (rhinacanthins-C, -N and -Q), we found

Table 1. Antimicrobial activity of crude extracts from *R. nasutus* against clinically isolated and standard bacteria

Microorganisms	Minimal Inhibitory Concentrations (MICs, μg/ml)														
	Roots					Leaves					Stems				
	Hexane	Chloroform	Methanol	85% Ethanol	Aqueous	Hexane	Chloroform	Methanol	85% Ethanol	Aqueous	Hexane	Chloroform	Methanol	85% Ethanol	Aqueous
Clinically isolated bacteria															
Gram positive															
Coagulase positive staphylococci (12)	78.12	312.5	5,000	1,250	-	1,250	5,000	-	1,250	-	10,000	10,000	-	-	-
Coagulase negative staphylococci (10)	312.5	625	5,000	1,250	-	1,250	5,000	-	1,250	-	10,000	10,000	-	-	-
β-Hemolytic streptococci (16)	39.06	78.12	2,500	1,250	-	312.5	5,000	-	1,250	-	2,500	50,000	-	-	-
Enterococi (10)	39.06	78.12	2,500	1,250	-	78.12	5,000	-	1,000	-	10,000	10,000	-	-	-
Gram negative															
Escherichia coli (5)	-	-	-	-	-	-		-	-	-	-	-	-	-	-
Pseudomonas aeruginosa (5)	-	-	-	-	-	-		-	-	-	-	-	-	-	-
Enterobacter spp. (5)	-	-	-	-	-	-		-	-	-	-	-	-	-	-
Klebsiella spp. (5)	-	-	-	-	-	-		-	-	-	-	-	-	-	-
Standard bacteria															
Staphylococcus aureus ATCC 29213	156.25	312.5	5,000	1,250	-	5,000	10,000	-	5,000	-	10,000	10,000	-	-	-
Enterococcus faecali ATCC 29212	78.12	156.25	5,000	1,250	-	5,000	5,000	-	5,000	-	5,000	10,000	-	-	-
Escherichia coli ATCC 25922	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa ATCC 27853	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

MICs = minimal inhibition concentration; crude extracts > 10,000 μg/ml; isolated compounds > 1,000 μg/ml.
(-) = no inhibitory effects observed.

Table 2. Antimicrobial activity of isolated compounds from *R. nasutus* against clinically isolated and standard bacteria

Microorganisms	Minimal Inhibitory Concentrations (MICs , μg/ml)							
	RN-C		RN-N		RN-Q		Gentamicin	
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Clinically isolated bacteria								
Gram positive								
Coagulase positive staphylococci (12)	19.53	39.06	4.9	9.76	19.53	78.12	2	128
Coagulase negative staphylococci (10)	19.53	39.06	4.9	9.76	19.53	312.5	32	128
β-Hemolytic streptococci (16)	39.06	39.06	4.9	9.76	9.76	9.76	8	16
Enterococci (10)	39.06	39.06	4.9	9.76	9.76	625	64	128
Gram negative								
Escherichia coli (5)	-	-	-	-	-	-	4	64
Pseudomonas aeruginosa (5)	-	-	-	-	-	-	4	128
Enterobacter spp. (5)	-	-	-	-	-	-	2	64
Klebsiella spp. (5)	-	-	-	-	-	-	1	2
Standard bacteria								
Staphylococcus aureus ATCC 29213	19.53		4.9		39.06		1	
Enterococcus faecalis ATCC 29212	19.53		4.9		9.76		16	
Escherichia coli ATCC 25922	-		-		-		2	
Pseudomonas aeruginosa ATCC 27853	-			-		-	2	

MICs = minimal inhibition concentration; crude extracts > 10,000 μg/ml; isolated compounds > 1,000 μg/ml.
(-) = no inhibitory effects observed; RN-C = rhinacanthin-C; RN-N = rhinacanthin-N, RN-Q = rhinacanthin-Q

that the MICs of Gram-positive bacteria had greater susceptibility to rhinacanthin-N than rhinacanthins-Q and -C (Table 2). Therefore, these data also confirm that the presence of a C-2 hydroxyl group on the naphthoquinone ring, two methyl substituents on the C-10 of propyl chain and naphthoate ester containing a hydroxyl group at C-1' and a methoxy group at C-4' on the naphthalene ring relate to increasing growth inhibitory effect to these bacteria. Additionally, the naphthoate ester moiety in the 1,4 naphthoquinone structure might contribute antibacterial efficacy in comparison with aliphatic esters. These findings are in agreement with our previous observations.[11,15,16,19]

CONCLUSION

Our findings demonstrate for the first time that rhinacanthins-N, -Q and -C isolated from roots of *R. nasutus* have potent antibacterial efficacy against Gram-positive clinically isolated bacteria from various specimens of Thai cancer patients with potencies comparable to those of gentamicin, an antibiotic drug. Rhinacanthin-N was the most effective drug. Interestingly, this observation confirms the evidence in the Thai folk remedies of the use of the *Rhinacanthus* plant for the treatment of infectious diseases in patients. Moreover, the usage of its root and leaf parts is more selective for infectious therapy than the stem.

ACKNOWLEDGEMENTS

Financial support was by the Thai Government Grant of the National Cancer Institute, Ministry of Public Health, Bangkok, Thailand. The authors are grateful to Assistant Professor Chongrak Permmomgkol, Mahidol University, for her kind suggestions.

REFERENCES

1. Armstrong D. Infectious complications in cancer patients treated with chemical immunosuppressive agents. *Transplant Proc* 1973; 5: 1245-8.
2. Ketchel SJ, Rodriguez V. Acute infections in cancer patients. *Semin Oncol* 1978; 5: 169-79.
3. Meyers JD, Thomas ED. Infectious complicating bone marrow transplantation. In: Robin RH, Yong LS, eds. Clinical Approaches to Infection in the Comprised Host. New York: Plenum, 1981; 507-51.
4. Wade JC, Schimpft SC. Infections in patients with suppressed cellular immunity. In: Klastersky J, Staquet MJ, eds. Medical Complication in Cancer Patients. New York: Raven, 1981: 273-90.
5. Cowan MM. Plant products as antibacterial agents. *Clinic Microbial Rev* 1999; 12: 564-82.
6. Rojanapo W, Tepsuwan A, Siripong P. Mutagenicity and antimutagenicity of Thai medicinal plants. *Basic Life Sci* 1990; 52: 447-52.
7. Wu T-S, Tien H-J, Yeh M-Y, *et al.* Isolation and cytotoxicity of rhinacanthin-A and -B, two naphthoquinones from *Rhinacanthus nasutus. Phytochemistry* 1988; 27: 3787-8.
8. Kodama O, Ichikawa H, Akatsuka T, *et al.* Isolation and identification of an antifungal naphthopyran derivative from *Rhinacanthus nasutus. J Nat Prod* 1993; 56: 292-4.
9. Sendl A, Chen JL, Jolad SD, *et al.* Two new naphthoquinones with antiviral activity from *Rhinacanthus nasutus. J Nat Prod* 1996; 59: 808-11.
10. Kernan MR, Sendl A, Chen JL, *et al*. Two new lignans with activity against influenza virus from the medicinal plants, *Rhinacanthus nasutus. J Nat Prod* 1997; 68: 635-7.
11. Siripong P, Kanokmedakul K, Piyaviriyagul S, *et al.* Antiproliferative naphthoquinone esters from *Rhinacanthus nasutus* Kurz. roots on various cancer cells. *J Trad Med* 2006; 23: 166-72.
12. Wu TS, Yang CC, Wu PL, *et al.* A quinol and steroids from the leaves and stems of *Rhinacanthus nasutus*. *Phytochemistry* 1993; 40: 1247-9.
13. Wu TS, Hsu MC, Wu PL, *et al.* Naphthoquinone esters from the root of *Rhinacanthus nasutus. Chem Pharm Bull* 1998; 46: 413-8.
14. Wu TS, Hsu HC, Wu PL, *et al*. Rhinacanthin Q, a naphthoquinone from *Rhinacanthus nasutus* and its biological activity. *Phytochemistry* 1998; 49: 2001-3.
15. Siripong P, Yahuafai J, Shimizu K, *et al.* Induction of apoptosis in tumor cells by three naphthoquinone esters isolated from Thai medicinal plants: *Rhinacanthus nasutus* Kurz. *Biol Pharm Bull* 2006; 29: 2070-6.

16. Siripong P, Yahuafai J, Shimizu K, *et al.* Antitumor activity of liposomal naphthoquinone esters isolated from Thai medicinal plant: *Rhinacanthus nasutus* Kurz. *Biol Pharm Bull* 2006; 29: 2079-83.
17. Thirumurugan RS, Kavimani S, Srivastava RS. Antitumor activity of rhinacanthone against Dalton ascitic lymphoma. *Biol Pharm Bull* 2000; 23: 1438-40.
18. NCCLS. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically: Approved Standard, 5th ed. NCCLS documents M7-A5. NCCLS: Wayne, PA, USA, 2000.
19. Kongkathip K, Luangkamin S, Kongkathip B, *et al.* Synthesis of novel rhinacanthins and related anticancer naphthoquinone esters. *J Med Chem* 2004; 47: 4427-38.
20. Adwan K, Abu-Hason N. Gentamicin resistance in clinical strains of Enterobacteriaceae associated with reduced gentamicin uptake. *Folia Microbial* 1998; 43: 438-40.
21. Tortora GJ, Funke BR, Case CL. Microbiology: an Introduction. Benjamin Cummings, San Francisco, 2001: 88.