

RP-HPLC Analysis of Rhinacanthins in *Rhinacanthus nasutus*: Validation and Application for the Preparation of Rhinacanthin High-Yielding Extract

P. Panichayupakaranant^{1,*}, T. Charoonratana¹, and A. Sirikatitham²

¹Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla 90112, Thailand, ²Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla 90112, Thailand

Abstract

A reversed-phase high-performance liquid chromatographic method is described for the simultaneous determination of rhinacanthin-C, rhinacanthin-D, and rhinacanthin-N in *Rhinacanthus nasutus* leaves. The method involved the use of a TSK-gel ODS-80Ts column (5 μ m, 4.6 \times 150 mm i.d.) with the mixture of methanol and 5% aqueous acetic acid (80:20, v/v) as the mobile phase. The parameters of linearity, repeatability, accuracy, and specificity of the method were evaluated. The recovery of the method was 94.3–100.9%, and good linearity ($r^2 \geq 0.9999$) was obtained for all rhinacanthins. A high degree of specificity as well as repeatability and reproducibility (relative standard deviation values less than 5%) were also achieved. The limit of detection and quantification of all rhinacanthins were 0.75 and 3.0 μ g/mL, respectively. The solvents for extraction of rhinacanthins from *R. nasutus* leaves were examined in order to obtain the leaf extract with high rhinacanthin content. It was found that ethyl acetate was an appropriate solvent for rhinacanthin extraction. Fractionation of the ethyl acetate extract using a basic anion exchange resin (Amberlite IRA-67) eluted with 10% acetic acid in methanol afforded a rhinacanthin-rich extract (HRn). The total content of rhinacanthins was increased from 37.4% w/w to 77.5% w/w. The antifungal activities of HRn against *Trichophyton rubrum*, *T. mentagrophytes*, and *Microsporum gypseum* were also improved.

Introduction

Rhinacanthus nasutus (*R. nasutus*), a small shrub of the Acanthaceae family, has long been used in Thai traditional medicine for the treatment of tinea versicolor, ringworm, pruritic rash, abscess pain, and skin diseases (1). It has been reported that rhinacanthin-C, rhinacanthin-D, and rhinacanthin-N (Figure 1) isolated from *R. nasutus* possessed antifungal, cytotoxic, and antiviral activities (2–5).

One of the main problems of phytotherapy is the lack of a standardized method for herbal extracts. Using the herbal extracts, which are inadequately standardized, involves a considerable risk of distortion and produces a false negative overall result. For safety and efficiency, it is important to set up the standardization method of herbal extract. There is only one paper published using HPLC to determine rhinacanthin-C content in *R. nasutus* leaves (5). However, validation of the analytical procedure is not yet established. Therefore, there is a need to develop and validate a method to simultaneously quantify rhinacanthin-C, -D, and -N in *R. nasutus* leaves in order to be a valuable informative tool for quality control. In addition, extraction and fractionation methods were also developed to improve the rhinacanthin content in *R. nasutus* leaf extracts.

Experimentals

Plant material

Leaves of *R. nasutus* were collected in Demonstrated Botanical Garden (Narathiwat Province, Thailand). They were authenticated at the Herbarium of the Southern Center of Traditional Medicine, Faculty of Pharmaceutical Sciences (Prince of Songkla University, Thailand), where herbarium specimen (Voucher No. 001 18 14) is kept.

Standard solution

Standard rhinacanthin-C, -D, and -N were previously purified (4). Separate stock solutions of the standards, rhinacanthin-C, -D, and -N were made in methanol (Labscan Asia, Thailand). A working solution of the combined standards was subsequently prepared in methanol and diluted to provide series of rhinacanthin-C ranging from 12.6–201.0 μ g/mL and rhinacanthin-D and -N ranging from 3.1–51.0 μ g/mL for use in constructing calibration curves for each of the target analytes.

*Author to whom correspondence should be addressed: email pharkphoom.p@psu.ac.th.

Sample preparation

The leaves of *R. nasutus* were dried in a hot air oven at 50°C. The dried leaf powder of *R. nasutus* (100 mg) was extracted with ethyl acetate (Labscan Asia, Thailand) (20 mL) under reflux condition for 1 h. The extracts were filtered and then concentrated under reduced pressure. The sample was reconstituted and adjusted to 10 mL with methanol. Samples were analyzed immediately after extraction in order to avoid possible chemical alterations. The experiments were run in triplicate.

HPLC conditions

HPLC analysis was carried out using Agilent 1100 series equipped with photodiode-array detector (PDA) and autosampler (Palo Alto, CA). Separation was achieved isocratically at 25°C on a 150 mm × 4.6 mm i.d. TSK-gel ODS-80Ts column (Tosoh Bioscience, Tokyo, Japan). The mobile phase consisted of methanol and 5% aqueous acetic acid (80:20, v/v) and was pumped at a flow rate of 1 mL/min. The injection volume was 20 µL. The quantification wavelength was set at 254 nm.

Method validation

Calibration curves

Calibration curves were constructed on three consecutive days by analysis of a mixture containing each of the standard compounds at five concentrations and plotting peak areas against the concentration of each reference standard. The linearity of the detector response for the standards was assessed by means of linear regression.

Accuracy

Spiking an amount of the standard compounds before extraction assessed the accuracy of the assay. The amount of each analyte was determined in triplicate and percentage recoveries were then calculated.

Precision

Precision experiments were conducted for intra-day and inter-day analyses. The solution of one sample was used to achieve repeatability testing. The data of repeatability was the content of six injections done separately on the same day. The data used to calculate relative standard deviations (%RSD) of inter-day precision was the content of three samples analyzed in three days (three injections in succession each day).

Specificity

Peak identification was carried out using the standards and Photodiode-Array detector. The UV spectra were taken at various points of the peaks to check peak homogeneity.

Limits of detection and quantification

The limits of detection (LOD) and quantification (LOQ) were determined by means of serial dilution based on signal-to-noise ratios of 3:1 and 10:1, respectively.

Optimization of solvent for extraction

The dried powder (5 g) was extracted by maceration with various organic solvents, including chloroform, ethyl acetate,

dichloromethane, ethanol and methanol for three days (50 mL × 3). The pooled extracts of the same solvent were concentrated under reduced pressure. The residues were then weighed and the total rhinacanthin content was determined.

Fractionation of the ethyl acetate extract

An adequate volume of methanol (250 mL) was added into a 500 g Amberlite IRA-67 (Sigma, St. Louis, MO) and gently stirred. After it was allowed to stand for 15 min, the methanol was decanted and the slurry was washed twice with distilled water (2 × 250 mL), and then allowed to stand in methanol for a further 5–10 min. The treated resin was poured into a glass column (5 × 35 cm), and the excess methanol was drained. A portion of methanol (200 mL) was then added to settle the resin. The leaf extract of *R. nasutus* (5 g) was dissolved in methanol (200 mL) and filtered. The filtrate was then loaded on the anion exchange column, and the solution was allowed to pass through the column with a flow rate of 1.5 mL/min until finished. The column was then eluted with methanol until the green pigments were washed out. Rhinacanthins were then eluted with 10% acetic acid in methanol with a flow rate 2 mL/min. The eluent was evaporated to dryness under reduced pressure.

In vitro antifungal activity assay

The compounds were sterilized by filtration through a 0.45-µm membrane filter before testing. Ketoconazole and dimethyl sulfoxide (1%) were used as positive and negative controls, respectively. *Trichophyton rubrum*, *T. mentagrophytes*, and *Microsporum gypseum* were grown in Sabouraud dextrose agar slant (Becton, Dickinson, France). The selected colonies were mixed with sterile physiological saline, and the turbidity was adjusted by adding sterile physiological saline until a McFarland turbidity standard of 0.5 (10⁶ colony forming units per mL) was reached.

Minimum inhibitory concentration (MIC) was determined using the agar dilution method (6). The stock solution of the tested compounds was serially diluted with Sabouraud dextrose agar to give the final concentrations between 3.9 and 1,000 µg/mL. Suspension of the test dermatophytes (2 µL) was added to each plate and incubated at 30°C for 7 days. The lowest concentration that did not show any growth of dermatophytes was taken as the MIC.

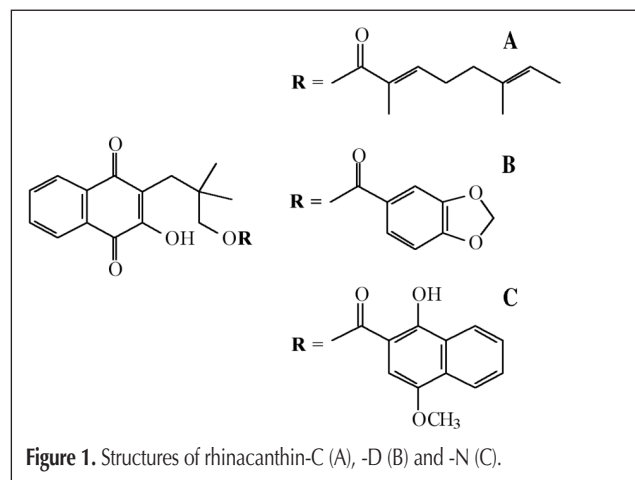


Figure 1. Structures of rhinacanthin-C (A), -D (B) and -N (C).

Results and Discussion

We examined the optimal conditions for the simultaneous quantitative determination of rhinacanthin-C, -D, and -N in *R. nasutus* leaf extract using the isocratic reverse-phase HPLC system. As all the three compounds have absorption at 254 nm, this wavelength was used for quantification. Mixtures of methanol and 5% aqueous acetic acid were examined as the mobile phase and its composition was optimized. The ratio of methanol to 5% aqueous acetic acid required for obtaining a

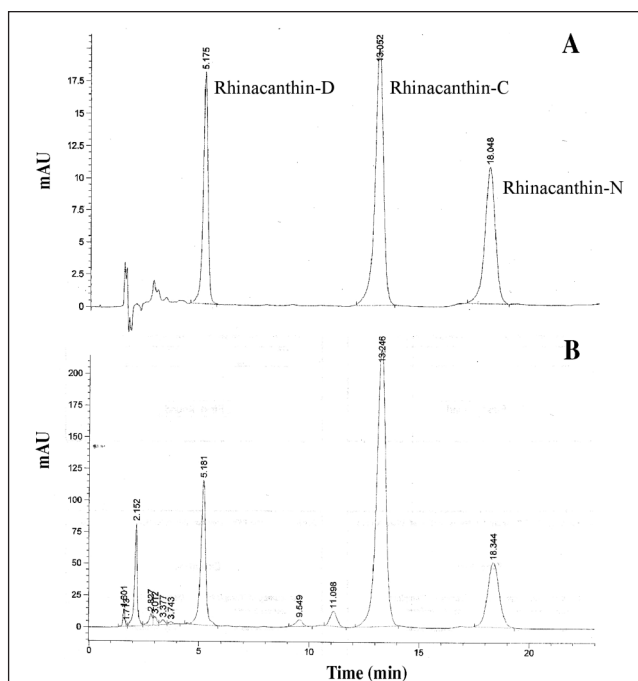


Figure 2. HPLC-chromatograms of the authentic rhinacanthin-C, -D and -N, (A); and *R. nasutus* leaf extract (B).

Table I. Rhinacanthin Content in *R. nasutus* Leaf Extract

Compounds	Rhinacanthin content (% w/w \pm SD*)
Rhinacanthin-C	1.91 \pm 0.044
Rhinacanthin-D	0.16 \pm 0.008
Rhinacanthin-N	0.07 \pm 0.001

*SD = standard deviation

Table II. Linear Ranges and Correlation Coefficients (r^2) of Calibration Curves

Compounds	$y = ax + b$ linear model*	r^2	Concentration (μ g/mL)
Rhinacanthin-C	$y = 40.569x + 35.263$	1.0	12.6 – 201.0
Rhinacanthin-D	$y = 79.615x - 4.168$	1.0	3.1 – 51.0
Rhinacanthin-N	$y = 112.810x + 18.292$	0.9999	3.1 – 51.0

* y = peak area; x = concentration (μ g/mL)

good resolution of the rhinacanthins was 80:20 v/v. All three compounds were eluted within 20 min with satisfactory resolution (Figure 2). On the basis of the HPLC analysis, rhinacanthin-C was a major rhinacanthin with a content of 1.9% w/w. Rhinacanthin-D and -N were only minor constituents (Table I). The simultaneous quantitative determination of rhinacanthin-C, -D, and -N is the advantage to this relatively simple and fast method. The previously reported HPLC method was only for determination of rhinacanthin-C, and validation of the analytical procedure is not yet established (5).

Defining the linearity, accuracy, intra-day and inter-day precision, and specificity validated the HPLC method. Linearity was evaluated using standard samples over five calibration points with six measurements for each calibration points. Rhinacanthin-C, -D, and -N exhibited good linearity over the evaluated ranges with correlation coefficients of 1.0000, 1.0000, and 0.9999, respectively (Table II).

The precision of the method was assessed by determining %RSD of intra-day and inter-day analysis. The method was shown to be reproducible and reliable with both intra-day and inter-day precision being lower than 5% (Table III).

Accuracy of method was evaluated by analyzing *R. nasutus* leaf extracts spiked with a known concentration of the standards. Prior to spiking, the background levels of rhinacanthin-C, -D, and -N in the extracts were determined so as to calculate actual recoveries. Mean recoveries in the range of 94–100 % were observed for all compounds (Table III).

Specificity of the method was evaluated using UV-absorption spectra produced by PDA. The spectra were taken at three points of the peaks. When they were compared with the standard, homogeneity of spectra of all peaks was found. Finally, it was discovered that the HPLC method was very sensitive for all rhinacanthins with LOD and LOQ of 0.75 and 3.0 μ g/mL, respectively.

Table III. Repeatability, Reproducibility, and Recoveries of Rhinacanthin-C, -D, -N from *R. nasutus* Leaf Extract

Compounds	RSD* (%)		% Recovery (Mean \pm SD)
	Intra-day ($n = 6$)	Inter-day ($n = 3$)	
Rhinacanthin-C	0.24	2.32	100.9 \pm 0.19
Rhinacanthin-D	2.59	4.94	95.6 \pm 2.50
Rhinacanthin-N	0.84	1.68	94.3 \pm 0.33

* RSD = relative standard deviation

Table IV. Yield and Total Rhinacanthin Content in *R. nasutus* Leaf Extracts

Solvents	Yield (%w/w \pm SD)	Total Rhinacanthin Content (%w/w \pm SD)
Dichloromethane	3.7 \pm 0.12	11.7 \pm 0.25
Chloroform	4.9 \pm 0.22	14.3 \pm 0.14
Ethyl acetate	4.8 \pm 0.16	33.0 \pm 0.16
Ethanol	11.5 \pm 0.28	5.4 \pm 0.24
Methanol	18.4 \pm 0.43	6.7 \pm 0.31

Among the solvents that were used for extraction of rhinacanthins from *R. nasutus* leaves, methanol gave the highest yield of the crude extract (Table IV). Unfortunately, the obtained methanol extract gives a rather low content of total rhinacanthin. In contrast, ethyl acetate gave a rather low yield of the crude extract but with the highest content of total rhinacanthin. This indicates that ethyl acetate is a suitable extraction solvent.

Ion exchange chromatography was developed to improve the rhinacanthin concentration in the extract as well as to diminish the interference compounds from the extract. Anion exchange resin is widely used for separation or pre-purification of many compounds. For example, a separation of lactic acid would require a strong basic anion exchange resin, Amberlite IRA-400 (7), and weak anion exchanger, Amberlite IRA-92 (8). Rhinacanthins are anion compounds that can be enriched by anion exchange resins. It was found that the Amberlite IRA-67 column was capable of improving the rhinacanthin content in the extract. The content of total rhinacanthins in HRn was increased to 77.5% w/w (Table V). The interference compounds including chlorophyll and other pigments were also markedly excluded. The weak basic anion exchanger, Amberlite IRA-67,

was therefore suitable for preparation of HRn.

The antifungal activity of HRn against *T. rubrum*, *T. mentagrophytes*, and *M. gypseum* was evaluated and compared with those of the ethyl acetate extract and standard rhinacanthins. The result showed that antifungal activity of HRn was better than that of the ethyl acetate extract (Table VI). The antifungal activity of HRn was equal to that of rhinacanthin-C. This may be due to a synergistic effect of all the three rhinacanthins on antifungal activity. Thus, HRn was suitable for further study of the formulation of a topical antifungal cream.

Acknowledgements

The authors wish to thank National Research Council of Thailand and the Graduate school, Prince of Songkla University, for support in the form of research grant.

References

1. N.R. Fransworth and N. Bunyapraphatsara. *Thai medicinal plants: Recommended for primary health care system*, Prachachon, Bangkok, 1992, pp. 216–217.
2. A. Sendl, J. L. Chen, S.D. Jolad, C. Stoddart, E. Rozhon, and M. Kernan. Two new naphthoquinones with antiviral activity from *Rhinacanthus nasutus*. *J. Nat. Prod.* **59**: 808–811 (1996).
3. T.S. Wu, H.C. Hsu, P.L. Wu, C.M. Teng, and C.Y. Wu. Rhinacanthin Q, A naphthoquinone from *Rhinacanthus nasutus* and its biological activity. *Phytochemistry* **40**: 2001–2003 (1998).
4. P. Panichayupakaranant, S. Yuenyongsawad, and N. Kongchai. Antifungal and antitumor activities of rhinacanthins from *Rhinacanthus nasutus* (L.) Kurz leaves. *The 3rd International Symposium on Natural Drugs*. Naples, Italy, October 2–4 2003, pp. 56.
5. A. Gotoh, T. Sakaeda, T. Kimura, T. Shirakawa, Y. Wada, A. Wada, T. Kimachi, Y. Takemoto, A. Iida, S. Iwakawa, M. Hirai, H. Tomita, N. Okamura, T. Nakamura, and K. Okumura. Antiproliferative activity of *Rhinacanthus nasutus* (L.) Kurz extracts and the active moiety, rhinacanthin C. *Biol. Pharm. Bull.* **27**: 1070–1074 (2004).
6. V. Lorian. *Antibiotic in laboratory medicine* 4th ed. Williams & Wilkins, Baltimore, MD, 1996, pp. 28–49.
7. X. Cao, H.S. Yun, and Y.M. Koo. Recovery of L-(+)-lactic acid by anion exchange resin Amberlite IRA-400. *Biochem. Eng. J.* **11**: 189–196 (2002).
8. W.Y. Tong, X.Y. Fu, S.M. Lee, J. Yu, J.W. Liu, D.Z. Wei, and Y.M. Koo. Purification of L-(+)-lactic acid from fermentation broth with paper sludge as a cellulosic feedstock using weak anion exchanger Amberlite IRA-92. *Biochem. Eng. J.* **18**: 89–96 (2004).

Manuscript received September 19, 2007;
Revision received March 10, 2008.

Table V. Total Rhinacanthin Content in the Ethyl Acetate and HRn Extracts of *R. nasutus* Leaves

Compounds	Rhinacanthin Content (% w/w; Mean \pm RSD)	
	Ethyl Acetate Extract	HRn Extract
Rhinacanthin-C	34.5 \pm 1.0	66.4 \pm 13.05
Rhinacanthin-D	1.8 \pm 4.4	7.5 \pm 1.37
Rhinacanthin-N	1.1 \pm 0.5	3.6 \pm 0.57
Total Rhinacanthin	37.4	77.5

Table VI. Antifungal Activity of Rhinacanthins, Ethyl Acetate Extract and HRn Extract Against *M. gypseum*, *T. mentagrophytes*, and *T. rubrum*

Extracts/Compounds	MIC* (μ g/mL)		
	<i>M. gypseum</i>	<i>T. mentagrophytes</i>	<i>T. rubrum</i>
EtOAc extract	500.0	62.5	31.2
HRn extract	125.0	31.2	7.5
Rhinacanthin-C	125.0	31.2	15.1
Rhinacanthin-D	125.0	31.2	31.2
Rhinacanthin-N	250.0	250.0	62.5
Ketoconazole [†]	8.0	8.0	0.5

* MIC = minimum inhibitory concentration
[†] positive control