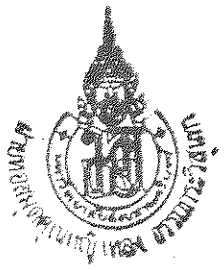

Formulation of Antifungal Cream from *Rhinacanthus nasutus* Leaf Extract

Napaporn Kongchai

Master of Pharmacy Thesis in Pharmaceutical Sciences
Prince of Songkla University
2004

Thesis Title	Formulation of Antifungal Cream from *Rhinacanthus nasutus* Leaf Extract
Author	Miss Napaporn Kongchai
Major Program	Pharmaceutical Sciences

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Graduate School

ชื่อวิทยานิพนธ์	การเตรียมครีมต้านเชื้อราจากสารสกัดใบทองพันชั่ง
ผู้เขียน	นางสาวนภาภรณ์ คงชัย
สาขาวิชา	เภสัชศาสตร์
ปีการศึกษา	2546

บทคัดย่อ

ทองพันชั่ง (*Rhinacanthus nasutus* (Linn.) Kurz) เป็นสมุนไพรที่ใช้สำหรับรักษาโรคผิวหนัง เช่น กลากเกลื้อน และผื่นคันมาเป็นเวลานาน ในการเตรียมครีมต้านเชื้อราจากสารสกัดใบทองพันชั่ง เพื่อให้ได้ตำรับยาที่มีความคงตัวที่สามารถควบคุมมาตรฐานสารสำคัญได้ จำเป็นต้องเตรียมสารสกัดให้มีความบริสุทธิ์ระดับหนึ่งก่อนนำไปใช้ในการเตรียมตำรับยา ในการศึกษานี้ได้ทำการทดลองหาวิธีวิเคราะห์ปริมาณสารสำคัญ และวิธีการเตรียมสารสกัดจากใบทองพันชั่งให้มีความบริสุทธิ์ในระดับหนึ่ง การใช้เทคนิค UV-vis spectrophotometry ร่วมกับเทคนิคการทำปฏิกิริยาเกิดสีสามารถวิเคราะห์ปริมาณ total rhinacanthins ในสารสกัดทองพันชั่ง โดยคำนวณในรูป rhinacanthin-C ได้ โดยพบว่าสารสกัดใบทองพันชั่งด้วย ethyl acetate มีปริมาณ total rhinacanthins เท่ากับ 33.05 $\pm$ 0.95 %w/w สารสกัดใบทองพันชั่งมีฤทธิ์ต้านเชื้อรา *Trichophyton rubrum, T. mentagrophytes* และ *Microsporum gypseum* โดยให้ค่า MIC เท่ากับ 125, 125 และ 500 µg/ml ตามลำดับ สามารถทำสารสกัดใบทองพันชั่งให้บริสุทธิ์ขึ้นโดยใช้ weakly basic anion exchange resin (Amberlite IRA-67) และใช้ methanol เป็นตัวล้าง และ10 % acetic acid in methanol เป็นตัวชะ พบว่าสามารถทำให้ rhinacanthins ในสารสกัดมีความเข้มข้นขึ้น และสามารถกำจัดสารอื่นที่ปนมาในสารสกัดออกไปอย่างเห็นได้ชัด ปริมาณ total rhinacanthins ในสารสกัดซึ่งผ่านการทำให้บริสุทธิ์ขึ้นแล้วเพิ่มขึ้นเป็น 64.05 $\pm$ 4.30 % w/w และเพิ่มฤทธิ์ต้านเชื้อ โดยมีค่า MIC ต่อเชื้อ *T. rubrum, T. mentagrophytes* และ *M. gypseum* เท่ากับ 36, 36 และ 288 µg/ml ตามลำดับ มีการเตรียมครีมต้านเชื้อรา 4 ตำรับ (Rx 1.1, Rx 1.2, Rx 2.1 และ Rx 2.2) โดยใช้สารสกัดจากใบทองพันชั่งที่ผ่านการทำให้บริสุทธิ์ขึ้นแล้วเป็นสารสำคัญ (ความเข้มข้น 1 %w/w และ 2 %w/w) และใช้ nonionic cream bases ที่ผ่านการคัดเลือกแล้ว 2 ชนิด พบว่าทุกตำรับมีลักษณะทางกายภาพและฤทธิ์ต้านเชื้อรา *T.*

rubrum, T. mentagrophytes และ *M. gypseum* เป็นที่น่าพอใจ อย่างไรก็ตามมีเพียงตำรับเดียวที่มีความคงตัวเป็นที่น่าพอใจคือตำรับ Rx 1.1

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Major program	Pharmaceutical Sciences
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Abstract

Rhinacanthus nasutus (Linn.) Kurz has long been used in the treatment of skin diseases such as tinea vesicolor, ringworm and pruritis. Topical antifungal creams were formulated using the leaf extract of this plant. In order to obtain a stable preparation that could be standardized for the active compounds, the crude extract need to be pre-purified prior to formulation. In this study, the methods for quantitative determination of the active compounds and pre-purification of *R. nasutus* leaf extract have been examined. UV-vis Spectrophotometry couples with a colorimetric technique was successfully used for the determination of total rhinacanthins, which was calculated as rhinacanthin-C. Based on this method, the content of total rhinacanthins in the ethyl acetate extract of *R. nasutus* leaves was 33.05 ± 0.95 %w/w. The leaf extract exhibited antifungal activity against *Trichophyton rubrum, T. mentagrophytes* and *Microsporum gypseum* with MIC values of 125, 125 and 500 μg/ml, respectively. Pre-purification of the ethyl acetate

extract was achieved using a basic anion exchange resin (Amberlite IRA-67) with methanol and 10% acetic acid in methanol as washing solvent and eluant, respectively. According to this pre-purification method, rhinacanthins were concentrated and some interference compounds were markedly excluded. The content of total rhinacanthins in the extract after pre-purification was increased to 64.05 ± 4.30 %w/w and the MIC values against *T. rubrum, T. mentagrophytes* and *M. gypseum* were improved to 36, 36 and 288 µg/ml, respectively. Four topical antifungal creams (Rx 1.1, Rx 1.2, Rx 2.1 and Rx 2.2) were formulated using the pre-purification extract of *R. nasutus* leaves as an active ingredient (1 %w/w and 2 % w/w) with two selected nonionic cream bases. All preparations showed satisfactory physical appearance and antifungal activities against *T. rubrum*, *T. mentagrophytes* and *M. gypseum*. However, the most suitable preparation that possessed satisfactory stability was Rx 1.1.

Acknowledgement

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Finally, none of this would have been possible without love and encouragement of my parents, my brother, my sisters and my dear friends for their kindness and continual support.

Napaporn Kongchai

Contents

Contents (continued)

Contents (continued)

List of Tables

List of Tables (continuted)

List of Figures

List of Abbreviations

°C	=	Degree celsius
cm	=	Centimeter
DMSO	=	Dimethyl sulfoxide
g	=	Gram
h	=	Hour
m	=	Meter
mg	=	Milligram
MIC	=	Minimum inhibitory concentration
min	=	Minutes
ml	=	Milliliter
mm	=	Millimeter
ng	=	Nanogram
nm	=	Nanometer
w/w	=	Weigh per weigh
µg	=	Microgram
µl	=	Microliter
µm	=	Micrometer

Chapter 1

Introduction

1.1 General

Over the last 10 years, the incidence of fungal infections has been increasing (Tortara *et al.*, 1998). There has been an increasing incidence of fungal infections due to a growth in immunocompromised population. The number of infections caused by fungi has increased considerably, including dermatophytosis. Dermatophytosis, commonly called ringworm or tinea, are fungal infections caused by dermatophytes (Mckane *et al*, 1996; Tortara *et al.*, 1998). Tinea infections are highly common skin disease and it is likely that the primary care physician will frequently treat affected patients. In USA, dermatophytosis is the second to an acne which is the most frequently reported skin disease (Weinstein *et al.*, 2002). In Thailand, superficial mycoses is the second to dermatitis from outpatient clinic reported by the Institute of Dermatology. Temperature and humidity especially in tropical countries are ideal. The growth of many microorganisms are supported by temperature leading to cause ringworm. The prevalence of ringworm has been increasing steadily over the past few years, and that it now constitutes a significant public health problem in many countries. Presently, many imported antifungal drugs have been used for the treatment of ringworm. This lead to an economic lost to

Thailand. Therefore, the evaluation of new antifungal drugs using locally available plant sources is necessary.

The imported drugs used for the treatment of this disease are expensive such as clotrimazole (Canesten®), ketoconazole (Nizoral®) and terbinafine (Lamisil®). In Thailand, there are many medicinal plants which exhibit antifungal activity and may be used for the treatment of infection diseases. In the past, many Thai herbs were used in traditional remedies for skin diseases. Thai medicinal plants which are currently recommended in primary health care for treatment of skin fungal infections are *Allium sativum* Linn., *Alpinia nigra* (Gaetn.) B.L. Burtt, *Cassia alata* Linn., *Piper betle* Linn. and *Rhinacanthus nasutus* (Linn.) Kurz. (สำนักงานคณะกรรมการการสาธารณสุขมูลฐาน, 2541). Except for *R. nasutus*, formulation data for all these herbs have been repcrted. Several studies have described the antifungal activity of *R. nasutus*, for example, the leaves have been reported to have antifungal activity against dermatophytes such as *Trichophyton rubrum*, *Epidermophyton floccosum* and *Microsporum gypseum* (Achararit *et al.*, 1984). Therefore, *R. nasutus* (Linn.) Kurz is an attractive plant to search for new antifungal drugs. Although many pharmacological studies of *R. nasutus* and its constituents have been published, the preparation of stable cream formulations and the quantitative analysis of active compound has not been studied.

As part of our interest in the formulation of an antifungal cream from the leaf extract of *R. nasutus*, we first aimed to develop a practical method for the

quantitative determination of active ingredients and then search for suitable techniques for pre-purification of crude leaf extracts prior to drug formulation. In addition, preparation of the antifungal cream from *R. nasutus* leaf extract and evaluation of its antifungal activity (*in vitro*) were performed.

Objectives

The objectives of this study were as follows:

1. To pre-purify the leaf extract of *R. nasutus* prior to drug formulation.
2. To determine rhinacanthins content in the *R. nasutus* leaf extract using UV-vis spectrophotometry.
3. To determine the antifungal activities of *R. nasutus*. leaf extract.
4. To prepare the antifungal cream from the leaf extract of *R. nasutus*, evaluate the stability and antifungal activity of the antifungal cream.

1.2 Literature review

1.2.1 *Rhinacanthus nasutus* (Linn.) Kurz

Botanical characteristics

Rhinacanthus nasutus (Linn.) Kurz, a small shrub of Acanthaceae family, is widely distributed in Southeast Asia, South China and India. In Thailand it is known as "Thong phan chang" or "Yaa man kai".

The characteristic features of *R. nasutus* (Figure 1.1) are described as follow. This plant is a small shrub, up to 1.5 m high; the stem is obtusely quadrangular, when young it is covered with fine up curved hairs. Leaves are simple opposite; elliptic or lanceolate; 4 - 6 by 2 - 3 cm entire; light green; shortly pubescent having acute base and apex. Flowers are white, in short clusters; densely pubescent. The calyx is divided into 5 deeply acute parted, light green, 5 - 6 mm long. The corolla-tube is about 2 cm, having brownish purple spots at the throat of the tube; bilabiate, upper lip erect, bifid, lower lip 3-lobed; 2 stamens , inserted in the throat; ovary 2-loculed. Capsule is loculicidally 2 valved (Farnsworth *et al.*, 1992).

Figure 1.1 *Rhinacanthus nasutus* (Linn.) Kurz

Ecology and propagation

R. nasutus is locally known and widely distributed in tropical countries. It is scattered along the edges of evergreen forests. *R. nasutus* plants are usually grown as ornamentals and require sandy and well-drained soil. They can be propagated by seeds or cuttings.

Ethnomedical use

R. nasutus has long been used in Thai traditional medicine for skin diseases such as pruritis, tinea versicolor, ringworm. The traditional recipes for treatment of ringworm are as follows (Farnsworth *et al.*, 1992).

- A tincture is prepared by soaking fresh leaves and roots in alcohol. Then it is applied over the infected area.
- The roots (6 - 7 roots) are pounded with match tips and vaseline then it is applied over the infected area.
- The roots are pounded with lemon and tamarind juices, then the mixture is applied over the infected area

Pounded roots mixed with vinegar or alcohol was applied on herpetic-like eruptions. For the same purpose, the leaves are applied with benzoin and sulphur in Malaysia. Indonesia: flowers and young leaves are rubbed with vinegar and lime to the skin (Wiart, 2000).

Chemical constitution study

The study of chemical compositions isolated from different parts of *R. nasutus* have been previously reported in many literatures. List of the compounds found in *R. nasutus* is given in Table 1.1. Structure of some compounds are given in Figure 1.2.

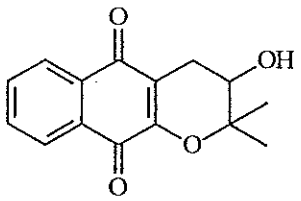
Table 1.1 The chemical constituents of *Rhinacanthus nasutus*

Chemicals	**Plant part**	**References**
Naphthoquinone		
rhinacanthin-A	root	Wu *et al.*, 1988; Singh *et al.*,1992; Wu *et al.*, 1998[a]; Wu, *et al.* 1998[b]
rhinacanthin-B	root and whole plant	Wu *et al.*, 1988; Sendl *et al.*, 1996; Wu *et al.*, 1998[a]; Wu *et al.*, 1998[b]
rhinacanthin-C	root and whole plant	Awai *et al.*, 1995; Sendl *et al.*, 1996; Wu *et al.*, 1998[a]; Wu *et al.*, 1998[b]
rhinacanthin-D	whole plant	Sendl *et al.*, 1996; Wu *et al.*, 1998[a]; Wu *et al.*, 1998[b]
rhinacanthin-G	root	Wu *et al.*, 1998[a]; Wu *et al.*, 1998[b]

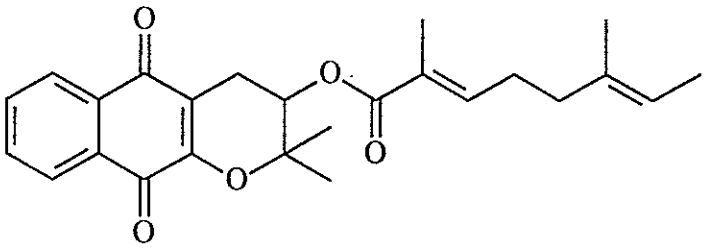
Chemicals	Plant part	References
rhinacanthin-H	root	Wu *et al.*, 1998[a]; Wu *et al.*, 1998[b]
rhinacanthin-I	root	Wu *et al.*, 1998[a]; Wu *et al.*, 1998[b]
rhinacanthin-J	root	Wu *et al.*, 1998[a]; Wu *et al.*, 1998[b]
rhinacanthin-K	root	Wu *et al.*, 1998[a]; Wu *et al.*, 1998[b]
rhinacanthin-L	root	Wu *et al.*, 1998[a]; Wu *et al.*, 1998[b]
rhinacanthin-M	root	Wu *et al.*, 1998[a]; Wu *et al.*, 1998[b]
rhinacanthin-N	root	Wu *et al.*, 1998[a]; Wu *et al.*, 1998[b]
rhinacanthin-O	root	Wu *et al.*, 1998[a]; Wu *et al.*, 1998[b]
rhinacanthin-P	root	Wu *et al.*, 1998[a]; Wu *et al.*, 1998[b]
rhinacanthin-Q	root	Wu *et al.*, 1998[a]

Chemicals	Plant part	References
3,4-dihydro-3,3-dimethyl-2H-naphtho-(1,2-B) pyran-5,6-dione	leaf and stem	Kodama *et al.*, 1993; Kuwahara *et al.*, 1995
3,4-dihydro-3,3-dimethyl-2H-naphtho-(2,3-B) pyran-5,10-dione	leaf and stem	Kodama *et al.*, 1993; Kuwahara *et al.*, 1995
Lignan		
rhinacanthin-E	whole plant	Kernan *et al.*, 1997
rhinacanthin-F	whole plant	Kernan *et al.*, 1997
Quinol		
4-acetonyl-3,5-dimethoxy-*p*-quinol	leaf and stem	Wu *et al.*, 1995
Anthraquinone		
2-methylanthraquinone	leaf and stem	Wu *et al.*, 1995
Benzenoid		
p-hydroxy-benzaldehyde	leaf and stem	Wu *et al.*, 1995
methyl-vanillate	leaf and stem	Wu *et al.*, 1995
syringaldehyde	leaf and stem	Wu *et al.*, 1995
2-methoxy-4-propionyl-phenol	leaf and stem	Wu *et al.*, 1995
Triterpenoid		
β-amyrin	leaf and stem	Wu *et al.*, 1995

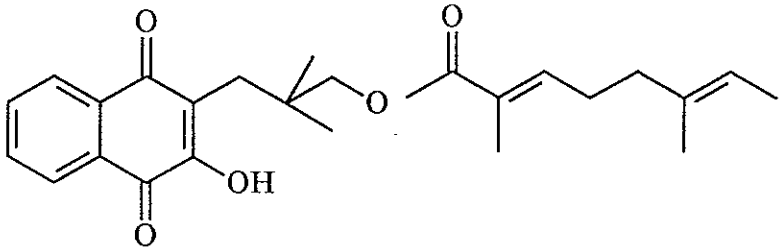
Chemicals	Plant part	References
glutinol	leaf and stem	Wu *et al.*, 1995
lupeol	leaf and stem	Wu *et al.*, 1995
Coumarin		
(+)-praeruptorin	leaf and stem	Wu *et al.*, 1995
umbelliferone	leaf and stem	Wu *et al.*, 1995
Flavonoid		
wogonin	root	Wu *et al.*, 1998[a]
oroxylin	root	Wu *et al.*, 1998[a]
Carbohydrate		
methyl-α-D-galactopyranoside	leaf and stem	Wu *et al.*, 1995
Amide		
allantoin	root	Wu *et al.*, 1998[a]
Chlorophyll		
methyl pheophorbide-A	leaf and stem	Wu *et al.*, 1995
Rutin		
quercetin-3-rutinoside	leaf and stem	Subramanian et *al.*, 1981;Wu *et al.*, 1995



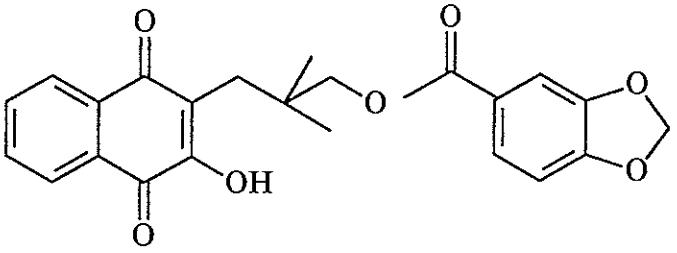
rhinacanthin-A



rhinacanthin-B

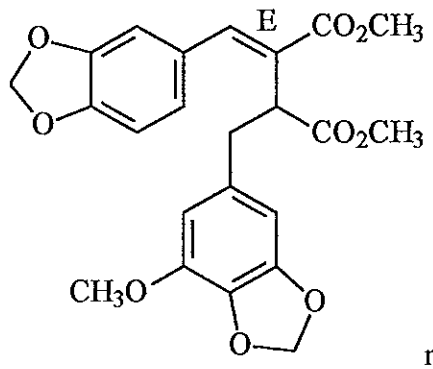


rhinacanthin-C

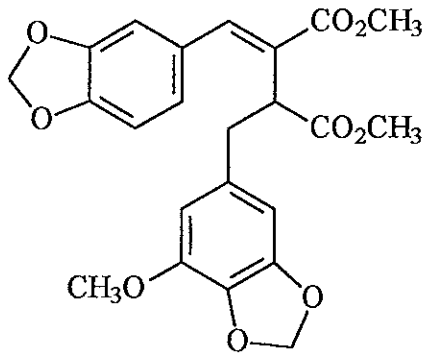


rhinacanthin-D

Figure 1.2 Structure of naphthoquinone found in *Rhinacanthus nasutus*

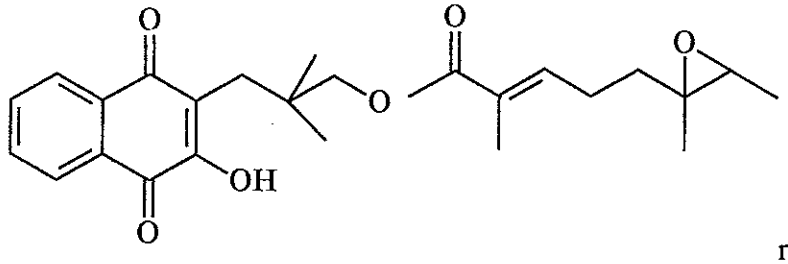


rhinacanthin-E

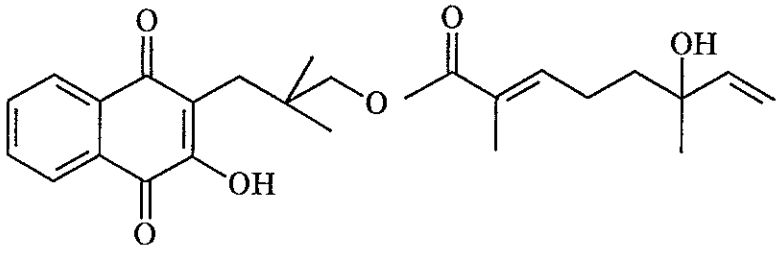


rhinacanthin-F

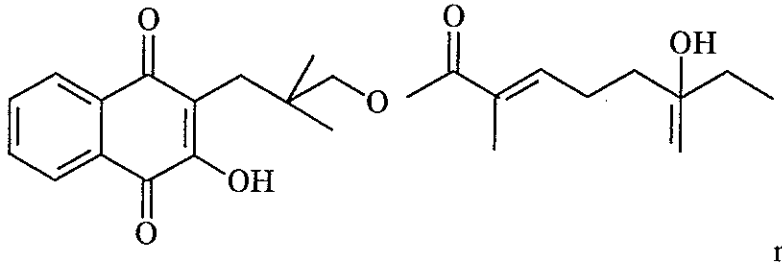
Figure 1.2 Structure of naphthoquinone found in *Rhinacanthus nasutus* (cont.)



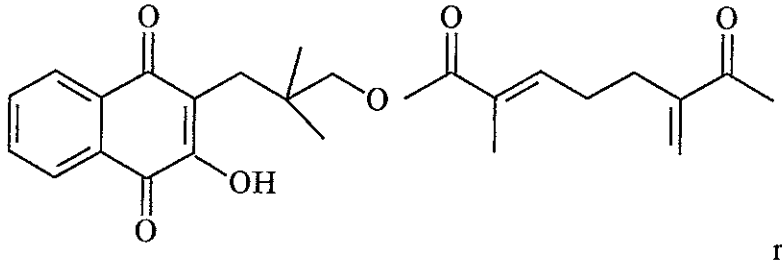
rhinacanthin-G



rhinacanthin-H

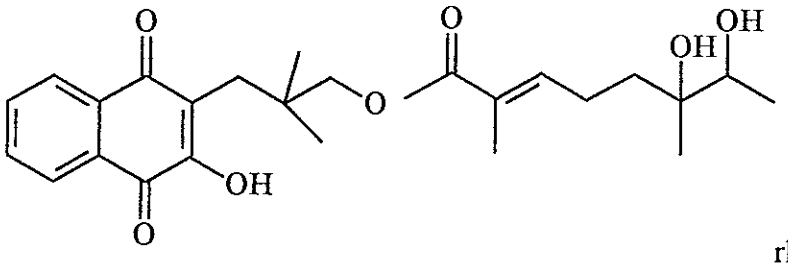


rhinacanthin-I

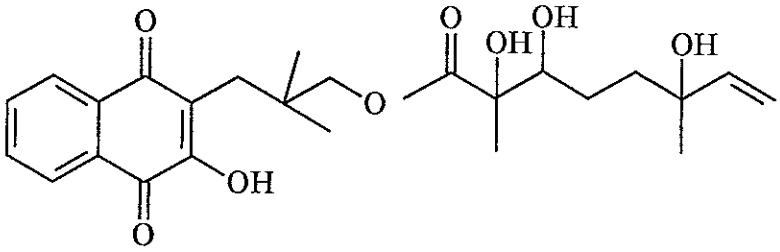


rhinacanthin-J

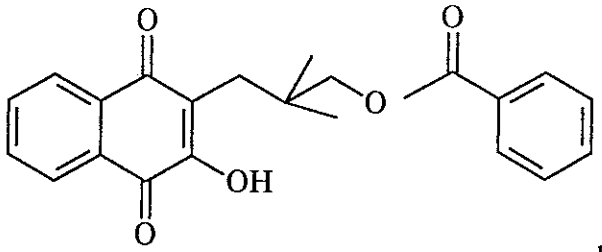
Figure1.2 Structure of naphthoquinone found in *Rhinacanthus nasutus* (cont.)



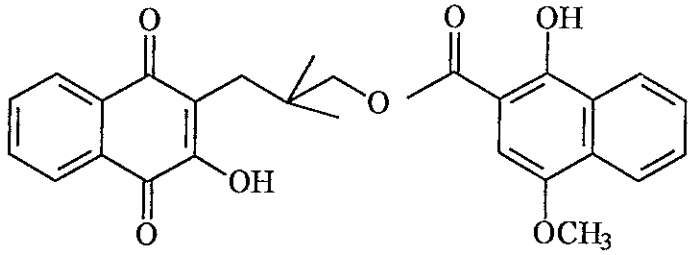
rhinacanthin-K



rhinacanthin-L

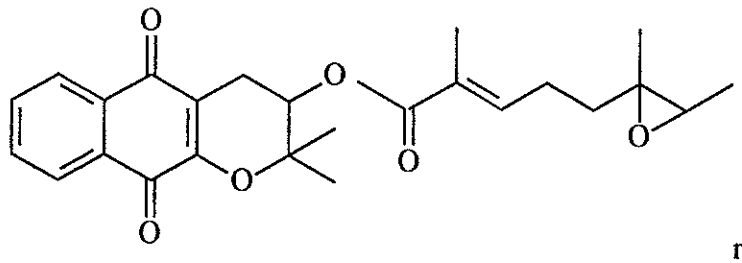


rhinacanthin-M

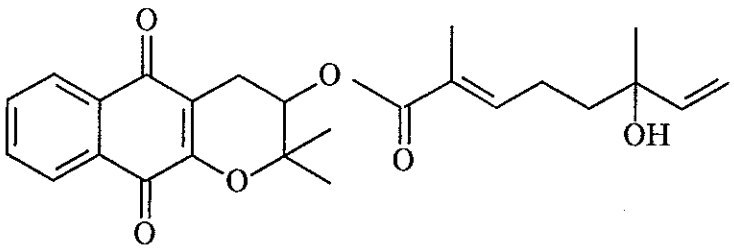


rhinacanthin-N

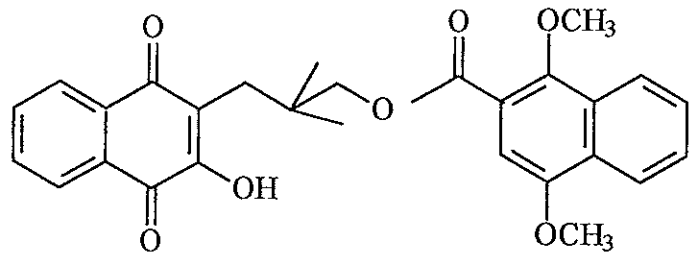
Figure 1.2 Structure of naphthoquinone found in *Rhinacanthus nasutus* (cont.)



rhinacanthin-O



rhinacanthin-P



rhinacanthin-Q

Figure 1.2 Structure of naphthoquinone found in *Rhinacanthus nasutus* (cont.)

Biological Activities

It has been reported that the extract of *Rhinacanthus nasutus* and the compounds isolated from this plant exhibited interesting biological activities include:

Antifungal activity

It has been reported that the extracts from *Rhinacanthus nasutus* possessed an antifungal activity against *Microsporum gypseum*, *Trichophyton rubrum*, *Epidermophyton floccosum*, *Candida albicans*, and *Cryptococcus neoformans* *Saccharomyces* spp. (Achararit *et al.*, 1983; Achararit *et al.*, 1984). It has been demonstrated that the water extract of *R. nasutus* leaves exhibited the lowest antifungal activity, while the chloroform and 95% ethanol extracts showed similarly inhibitory activity against filamentous fungi In addition, it has been reported that the methanol, dichloromethane and hexane extracts of *R. nasutus* leaves possessed antifungal activities against *E. floccosum*, *M. gypseum*, *T. mentagrophytes* and *T. rubrum* (Farnsworth *et al.*, 1992).

The minimum inhibitory concentration (MIC) values of the leaf extract against *T. mentagrophytes* var. *mentagrophytes*, *T. mentagrophytes* var. *interdigitate*, *T. rubrum*, *Microsporum gypseum* and *M. canis* was reported at 13.6 mg/ml. The leaf extract exhibited fungistatic activity at lower concentration (<13.6 mg/ml or below the MIC value) and fungicidal activity at higher concentration (>13.6 mg/ml or above the MIC value). Moreover, it has been suggested that the extract of *R. nasutus* leaves acted on the cell wall of the dermatophytes which subsequently leading to the

formation of cytopathol and membrane structural degeneration and finally leading to cell lysis and death. (Darah *et al.*, 2001).

In addition, rhinacanthone has been demonstrated to be an antifungal active compound, which exhibited inhibitory action on spore germination of *Pyricularia oryzae* (Kuwahara, *et al.*, 1995).

Antibacterial activity

It has been reported that the leaf and stem extract of *R. nasutus* exhibited inhibitory activity against oral *Streptococcus* spp. (22 isolates), which were isolated from dental plaque of 25 patients. It was found that the MIC of the extract was 3.8 ng/ml (Apisariyakul *et al.*, 1991).

Antiviral activity

It has been reported that the naphthoquinone esters, rhinacanthin-C and rhinacanthin-D, which were isolated from the aerial parts of *Rhinacanthus nasutus* exhibited an antiviral activity against human cytomegalovirus, with the IC_{50} values of 0.02 and 0.22 μg/ml, respectively. However, both of them were not active against influenza virus type A, herpes simplex virus type 2 and respiratory syncytial virus (Sendl *et al.*, 1996). In addition, the lignans, rhinacanthin-E and rhinacanthin-F, which were isolated from aerial parts from *R. nasutus* were shown to active against influenza virus type A, with EC_{50} values of 7.4 μg/ml and 3.1 μg/ml respectively (Kernan *et al.*, 1997).

Cytotoxic activity

It has been reported that the methanolic extract of the root of *Rhinacanthus nasutus* showed significant cytotoxic activity in the human mouth carcinoma epidermoid (KB cell). In addition, rhinacanthin-B, which were isolated from the root of *R. nasutus* was demonstrated to the active compound (ED_{50} = 3.0 μg/ml) on the human mouth carcinoma epidermoid (KB cell) (Wu *et al.*, 1988).

The naphthoquinones and flavonoid including rhinacanthin-A, -B, -C, -D, -G, -H, -I, -K, -M, -N, -Q and wogonin, which were isolated from the root of *R. nasutus* showed significant cytotoxicity activity on P-338 (lymphocytic leukemia cell), A-549 (human lung carcinoma), HT-29 (human colon adenocarcinoma) and HL-60 (human leukemia, promyelocytic) cells with the ED_{50} values as showed in Table 1.2 (Wu *et al.*, 1998[b]).

Table 1.2 Cytotoxic activity of naphthoquinones and flavonoids isolated from the root of *Rhinacanthus nasutus*

Compound	Cell lines ED_{50} (μg/ml)				
	KB	P-388	A-549	HT-29	HL-60
rhinacanthin-A	6.75	0.72	3.06	2.17	1.16
rhinacanthin-B	8.01	0.35	6.50	3.01	2.57
rhinacanthin-C	6.26	0.26	0.35	0.68	0.68
rhinacanthin-D	25.0	3.79	8.26	8.89	11.8
rhinacanthin-G	4.45	0.14	0.75	0.57	1.14
rhinacanthin-H	23.8	6.43	9.97	11.5	8.87
rhinacanthin-I	13.2	4.88	7.18	6.30	5.12
rhinacanthin-K	17.3	3.17	16.4	7.75	6.81
rhinacanthin-M	19.2	3.95	8.90	10.1	19.9
rhinacanthin-N	4.80	0.71	1.97	2.67	1.38
rhinacanthin-Q	>50	0.61	3.61	7.60	8.90
wogonin	4.46	1.70	4.14	3.35	4.66

KB = human mouth carcinoma epidermoid

P-338 = lymphocytic leukemia cell

A-549 = human lung carcinoma

HT-29 = human colon adenocarcinoma

HL-60 = human leukemia, promyelocytic

Antitumor activity

The antitumour activity of rhinacanthone against Dalton's ascitic lymphoma (DAL) in mice has been reported (Thirumugann *et al.*, 2000). A significant enhancement of mean survival time of tumor bearing mice and peritoneal cell count in normal mice was observed with respect to the control group.

Hypotensive activity

The extract obtained from hot water maceration (decogtion) from *R. nasutus* leaf extract were studied in anesthetized rats for their pharmacological action. The hypotension action of *R. nasutus* extract were found to be increased with correlation to the amount of the extract (วรรณดี แต้โสตถิกุล, 2528).

Antioxidant activity

Food, cosmetics and pharmaceuticals containing extracts of *Rhinacanthus nasutus* are reported to have antioxidant activity. The mechanism is to remove superoxide from the human body. Cosmetic containing the extracts might be useful to reduce aging and hair loss (Wiart *et al.*, 2000).

Antiplatelet activity

The antiplatelet aggregation the naphthoquinones, which were isolated from the root of *Rhinacanthus nasutus* including rhinacanthin-A, -B, -C, -G, -H, -I, -K, -M and -Q has been reported. These compounds demonstrated 36-100% inhibition of

rabbit platelet aggregation induced by arachidonic acid (100mM). Rhinacanthin-A, -B and -C (10 μg/ml) showed 72-100% inhibition of the rabbit platelet aggregation induced by collagen, while rhinacanthin-B (2 ng/ml) inhibited platelet aggregation induced by platelet activation factor (Wu *et al.*, 1998[b]).

Insect attractant and signalling properties

An ether extract of *Rhinacanthus nasutus* from roots exhibited the properties of an insect attractant and signalling to male Mediterranean fruit flies but showed equivocal results on *Aspiculurus tetraptera*, both male and female melon flies and oriental fruit flies (*Dacus dorsalis*) (Keiser *et al.*, 1975).

Juvenile hormone activity

An ether extract of *Rhinacanthus nasutus* roots, at a dose of 500.0 μg per animal exhibited juvenile hormone activity on *Oncopeltus fasciatus*, but no activity was observed at a dose of 250.0 μg per animal (Jacobson *et al.*, 1975).

1.2.2 Dermatophytes

Dermatophytes are fungi that infect skin, hair, and nails due to their ability to utilize keratin (Gupta *et al.*, 1998). The organisms colonize the keratin tissues, inflammation is then caused by host response to metabolic by-products. Occasionally the organisms invade the subcutaneous tissues, resulting in kerion development. The dermatophytes consist of three genera:

***Epidermophyton* spp.** The macroconidia are broadly clavate with typically smooth, thin to moderately thick walls and one to nine septa, with 20 – 60 x 4 - 13 μm in size. They are usually abundant and borne singly or in clusters. Microconidia are absent. This genus has only two known species to date, and only *E. floccosum* is pathogenic.

***Microsporum* spp.** Macroconidia are characterized by the presence of rough walls which may be asperulate, echinulate, or verrucose (Weitzman, 1995). There are 19 described species but only 9 are involved in human or animal infections.

***Trichophyton* spp.** Macroconidia, which have smooth, usually thin walls and one to 12 septa, are borne singly or in clusters. They may be elongate and pencil shaped, clavate, fusiform, or cylindrical. Their size are in the range of 8 - 86 x 4 - 14 μm. Microconidia, usually more abundant than macroconidia, may be globose, pyriform or clavate, or sessile or stalked, and borne singly along the sides of the hyphae or in grape-like clusters (Weitzman,1995). There are 22 species, most of them causing infections in humans or animals.

1.2.3 Dermatophyte infections

Dermatophytes invade the stratum corneum or keratinized structure derived from the epidermis, causing skin lesions, hair and nail infections (Duek *et al.*, 2004). Dermatophyte infections commonly known as ringworm because the appearance of the lesions led to the erroneous belief that the infected skin harbored worms beneath its surface. Ringworm infections are named "tinea" followed by a second word that designates the infected site. For example, tinea capitis is ringworm of scalp; tinea

corporis is ringworm of the body; tinea pedis is the disease popularly known as athlete's foot; tinea cruris is jock itch; and tinea unguium is ringworm of the nails (McKane *et al.*, 1996). Tinea pedis is the most common type of dermatophyte infection in the US and the rest of the world. Tinea capitis is one of the most common infections in children (Weinstein, 2002). *T. rubrum* is the most common cause of tinea corporis, tinea cruis, tinea pedis, and nail infection worldwide (Wilson, 2001; Weinstein, 2002).

1.2.4 Antifungal agents

The development of antifungal agents has lagged behind that of antibacterial agents. This is a consequence of the cellular structure of the organisms involved. Bacteria are prokaryotic and hence offer numerous structural and metabolic targets that differ from those of the human host. Fungi, in contrast, are eukaryotes, and consequently most agents toxic to fungi are also toxic to the host (Prescott *et al.*, 2003). Despite these limitations, numerous advances have been made in developing new antifungal agents and in understanding the existing ones.

The antifungal agents can be grouped by structure and mechanism of action. The two principal pharmacologic groups are azoles and the allylamines. Polyenes (amphotericin B) and nystatin are not discussed in this article because this group of compounds is not effective in the treatment of dermatophyte infections. Other agents that do not fit into the two main groups are tolnaftate, haloprogin, ciclopirox and butenafine.

Azoles: Fluconazole, itraconazole and ketoconazole inhibit cytochrome P_{450}-dependent enzymes (particularly C14-demethylase) involved in the biosynthesis of ergosterol, which is required for fungal cell membrane structure and function.

Allylamines: Allylamines (naftifine, terbinafine) inhibit ergosterol biosynthesis at the level of squalene epoxidase.

Most tinea corporis, cruris, and pedis infection canbe treated with topical formulations (Weinstein *et al.*, 2002). For the effective therapy of tinea capitis, an oral antifungal is generally necessary. Generally the topical agent is available as a cream, sometimes for use intravaginally. Less commonly, the formulation may be in the form of a powder, lacquer, spray, gel or solution. For the treatment of tinea corporis, tinea cruris tinea versicolor and cutaneous candidosis, once or twice daily application may be required, the most common duration of therapy being 2 to 4 weeks. For tinea pedis the most common treatment duration is 4 to 6 weeks (Gupta *et al.*, 1998).

Table 1.3 Common Antifungal Medications and Their Forms

(Weinstein *et al.*, 2002)

Agent	Rx or OTC	Sol or Spray	Lotion	Cream	Gel Ointment	Powder
Tolnaftate	OTC	Yes	Yes	Yes	Yes	No
Haloprogin	Rx	Yes	No	Yes	No	No
Ciclopirox	Rx	Lacquer	Yes	Yes	No	No
Clotrimazole	OTC	Yes	Yes	Yes	No	No
Miconazole	OTC	Yes	Yes	Yes	No	Yes
Ketoconazole	Rx	Shampoo	No	Yes	No	No
Sulconazole	Rx	No	No	Yes	No	No
Oxiconazole	Rx	No	Yes	Yes	No	No
Econazole	Rx	No	No	Yes	No	No
Naftifine	Rx	No	No	Yes	Yes	No
Terbinafine	Rx	Yes	No	Yes	No	No

Rx = Prescription; OTC = Over the counter

The incidence of fungal infections, including resistant infection, has increased during the last 10 years. Although the prevalence of drug resistance in fungi is below that observed in bacteria, many mycologists, consider that selective pressure will, over time, lead to more widespread resistance (Hudson, 2001). Drug resistance to the polyene antifungal is almost always primary (intrinsic) resistance rather than secondary (acquried) resistance. The resistance to antifungal agents can originate from a too low intracellular drug content, the consequence of an impaired uptake or

of over expression of drug efflux pumps, resistance can also originate from ampification of the gene coding for the target enzyme, from changes at the target sites, change in the ergosterol level, from differnces in the nature of the accumulating sterols or from a decreased activation of the antifungal agents. Thus, the fungus has several mechanisms to escape from the effects of antifungal agents (Vanden, 1992). Just as with antibacterial drugs, overuse of antifungal agents leads to increase in drug resistance (Prescott *et al.*, 2003). Ideally, an antifungal agent will provide clinical and mycologic cure, symptomatic relief, and low relapse rate, along with ease of use (Weinstein *et al.*, 2002).

1.2.5 Thai medicinal plants with antifungal activity

Medicinal plants have played an important role in the medicinal system of Thai people in the past. Medicinal plants are currently being promoted through the primary health care system in Thailand. There are many medicinal plants which exhibit antifungal activity. We are interested in antifungal activity of medicinal plants in primary health care (2541) and these are described below:

Allium sativum Linn. is a medicinal plant of the Alliaceae family. The common name is Garlic. Water extract, juice or fresh garlic exhibited antifungal and antiyeast activities against fungi or yeast causing leucorrhoea, or ringworm (Ploddee *et al.*, 1987). Antifungal activity of garlic is more prominent than antibacterial as evidenced by showing fungicidal activity, but only bacteriostatic activity. Garlic showed its efficacy in dermatophytosis therapy in rabbits infected with *M. canis*

(Presad *et al.*, 1982). Antifungal activity was found in bulbs more than leaves and stems (Barone *et al.*, 1977). One compound reaponsible for antifungal activity is allicin. Allicin exhibits antifungal activity against not only fungi pathogenic to man but also to plants (Durbin *et al.*, 1971). Ajoene was later found as another antifungal agent from garlic. Ajoene exhibits 95% inhibition of *candida aibicans* and *Aspergillus niger* at 5 μg/ml and 20 μg/ml respectively (Farnsworth *et al.*, 1992).

Alpinia galanga (Linn.) Sw. is a medicinal plant in the family Zingiberaceae. It was reported that alcohol and chloroform extracts possessed antifungal activity against *M. gypseum, T. rubrum, E. floccosum, C. albicans, Cryptococcus neoformans* and *Saccharomyces sp.* Low inhibitory action was shown by the water extract. No activity was found in fresh juices (Achararit *et al.*, 1984). In another study, petroleum ether, chloroform and 95% alcohol extracts were found to exhibit antifungal activities against *T. rubrum* and *M. gypseum* (Limsrimanee *et al.*, 1992).

Cassia alata Linn. is from the Leguminosae family. It has been reported that preparations of leaves can be used for treatment of skin diseases. A 5% water extract of *C. alata* leaves exhibited antifungal activity against *T. mentagrophytes* (Mulchandani *et al.*, 1975). A 50% alcohol extract of the aerial parts (25 μg/ml) showed the same activity (Dhawan *et al.*, 1977). Apart from these fungi, the extracts also showed antifungal activity against *M. canis, C. albicans* and *Aspergillus niger.* The chloroform, ether, alcohol and water extracts of leaves were investigated for antifungal activity and were found to be effective against *T. rubrum, T. mentagrophytes, E. floccosum* and *M. gypseum,* but not effective against *C.*

albicans, A. fumigatus and *Penicillium puberulum*. In a clinical trial using an alcohol extract prepared as a cream preparation at a concentration of 20% for treatment of *Pityriasis versicolor* patients, it was reported that 100% of patients were cured (Farnsworth *et al.*, 1992).

Piper betle Linn. is a plant in the Piperaceae family. Petroleum ether, ether, chloroform and 95% alcohol extracts of betel leaves were found to be effective against *T. mentagrophytes* and *T. rubrum* . Best results were obtained with the ether extract (Bunyaratanakornkij *et al.*, 1997). The ether extract of betel leaves was subsequently formulated into several dosage forms for external uses; ointment, cream and paste using various kinds of bases. It was found that at a concentration of 2%, all dosage forms were effective against the stated bacteria and fungi (Farnsworth *et al.*, 1992).

Chapter 2

Experimental

2.1 Material

2.1.1 Plant material

The leaves of *Rhinacanthus nasutus* (Linn.) Kurz from 1 year old plant were collected from the Demonstrated Botanical Garden, Muang, Narathiwat province, Thailand in August 2002.

2.1.2 Chemicals

All solvents for general purposes were commercial grade and were re-distilled prior to use. The chemicals for preparation of the formulation were kindly provided from the Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University. The standard rhinacanthin-C was previously purified by Assistant Professor Dr. Pharkphoom Panichayupakaranant (Panichayupakaranant *et al.*, 2000). Clotrimazole cream 1% (Canesten® Bayer), Miconazole cream 2% (Thai Government Pharmaceutical Organization, Thailand) and Ketoconazole cream 2% (Nizoral® Janseen-Cilag) were purchased from a drug store. Sabouraud Dextrose Agar was from Difco, France.

Silica gel precoated aluminium sheets was from Merck, Germany. Amberlite IRA67 and Amberlite IRA 400 were from Sigma, USA.

2.1.3 Dermatophytes

Trichophyton rubrum, T. mentagrophytes and Microsporum gypseum were kindly provided from Department of Microbiology, Faculty of Sciences, Prince of Songkla University.

2.2 Methods

2.2.1 Preparation of *Rhinacanthus nasutus* leaf extracts

2.2.1.1 Searching for a suitable organic solvent for extraction

The leaves of *R. nasutus* were dried in a hot air oven at 50 °C for 48 hours. The dried leaves were then ground into a coarsely powder. 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 x 3). The pooled extracts of the same solvent were concentrated *in vaccuo* to dryness. The residues were then weighed and determined the total rhinacanthin content.

2.2.1.2 Preparation of ethyl acetate leaf extract

Dried powder of *R. nasutus* leaves (3000 g) were macerated with ethyl acetate for three days (15000 ml x 3). The pooled extract was concentrated *in*

vaccuo to dryness. The residue was then weighed and determined the total rhinacanthin content.

2.2.2 Optimization of the conditions for quantitative determination of total rhinacanthin by spectrophotometric method

Quantitative determination of total rhinacanthins was on the basis of Borntrager reaction couple with spectrophotometry. The amount of total rhinacanthins was calculated as rhinacanthin-C.

2.2.2.1 Preparation of rhinacanthin-C standard solution

Rhinacanthin-C was accurately weighed about 5 mg, and dissolved in methanol and adjusted to a final concentration of rhinacanthin-C 0.1 mg/ml.

2.2.2.2 Optimization of sample/reagent ratio

A standard solution of rhinacanthin-C was mixed with the reagent (20% KOH in methanol) in various ratio, including 1:1, 2:1, 3:1, 4:1, 5:1, 6:1 and 7:1 ml/ml. After allowed to stand at the room temperature for 30 minutes, the mixtures were then measured absorption spectrum (Spectro UV–vis RS Labomed, USA) between 200 to 600 nm, using methanol as a blank. Wavelength at which maximal absorbance (λ_{max}) was obtained at 486 nm, and the ratio of the mixture that contributed a highest absorbance at 486 nm was selected for all further studies.

2.2.2.3 Optimization of the reaction time

The time course of the reaction between rhinacanthin-C and the reagent was investigated. The experiment was performed using the suitable ratio obtained from the experiment in section 2.2.2.2 and varied the duration after mixing, that were 0, 5, 15, 30 and 60 min. The absorbance then measured at λ_{max} 486 nm. A suitable reaction time was selected for all further studies.

2.2.3 Quantitative determination of total rhinacanthins

The total rhinacanthin content, calculated as rhinacanthin-C was determined on the basis to spectrophotometric method as follows:

2.2.3.1 Establishment of a calibration curve of rhinacanthin-C

A stock solution of the authentic rhinacanthin-C was prepared by dissolving rhinacanthin-C in methanol to give a concentration of rhinacanthin-C 0.1 mg/ml. Adequate portions the stock solution were diluted with methanol to give standard solutions of 6.25, 12.5, 25 and 50 μg/ml. The standard solutions (4 ml) were mixed with 20% KOH solution (1 ml), allowed for 15 minutes and then determined the absorbance of each solution at 486 nm, using methanol as blank. The absorbance versus the final concentration was plotted to get the calibration curve and the regression equation.

2.2.3.2 Determination of total rhinacanthins in the extracts

The extract of *R. nasutus* leaves (5 mg) was accurately weighed and dissolved in methanol (25 ml). The solution was filtered through Whatman® paper No.1, if necessary. The sample solutios (4 ml) was mixed with 20% KOH solution (1 ml), allowed to stand for 15 minutes and then determined the absorbance at 486 nm, using methanol as blank. The total rhinacanthins content was calculated as rhinacanthin-C using the calibration curve of the authentic rhinacanthin-C. The analyses in all experiments were performed in triplicate.

2.2.3.3 Assay validation

A stock solution of crude extract (0.05 mg/ml) was prepared. Validation of the assay was accomplished using spiked method. The solution was spiked with standard rhinacanthin-C at various concentrations, including 7.98, 15.94 and 39.60 μg/ml. Then the absorbance of each solution was determined using stock solution of crude extract as blank. Accuracy of the assay was calculated from the percentage of recovery and the percentage of coefficient of variation. The assay was done in triplicate.

2.2.4 Pre-purification of the leaf extract

Ion exchange chromatography was developed for pre-purification of *R. nasutus* leaf extract in order to obtain a high yield of rhinacanthins crude extract for use in further formulation preparation. The following methods were used.

2.2.4.1 Preparation of anion exchange resin

An adequate volume of methanol (250 ml) was added into 500 g anion exchange resin (Amberlite IRA-67 or Amberlite IRA-400) and gently stirred for a few minutes. After allowed to stand for 15 min, the methanol was decanted and the slurry was washed twice with distilled water (2 x 250 ml), and then allowed to stand in methanol for a further 5-10 min. The treated resin was slowly poured into a glass column (5 x 35 cm). The excess methanol was drained. A portion of methanol (200 ml) was then added to settle the resin. The column was then ready for sample loading.

2.2.4.2 Sample loading and separation

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 allowed the solution to pass through the column with a flow rate of 1.5 ml/min until finish. The column was then washed with methanol (washing solvent) until the green color in the column was disappeared. After that 10% acetic acid in methanol was used to elute the sample from the column with a flow rate 1 ml/min. The collected eluent was then evaporated to dryness under vacuum at 40°C.

2.2.4.3 TLC finger-print of the extract

Thin layer chromatography (TLC) was used for establishment of TLC-fingerprint of the leaf extract before and after pre-purification. Siliga gel F254 and a mixture of chloroform: ethyl acetate (39: 1) were used as stationary and mobile

phase respectively. Rhinacanthin-C was used as a marker. The chromatogram was detected under UV at wavelength 254 nm.

2.2.5 Evaluation of antifungal activity

2.2.5.1 *In vitro* Antifungal activity assay

Preparation of sample

The extract was dissolved in DMSO to give a concentration of 1 g/ml. This solution was further diluted to obtained sample solutions with concentrations of 62.5, 125, 250 and 500 mg/ml.

Preparation of test dermatophytes

Trichrophyton rubrum, T. mentagrophytes and *Microsporum gypseum* were grown in Sabouraud dextrose agar (SDA) slant. The colonies from this agar were suspended in steriled 0.9% normal saline solution (NSS) and the turbidity was adjusted equivalent to a 0.5 McFarland standard before use.

Antifungal activity screening

Disc diffusion method was used to test the susceptibility of the fungi to the leaf extract of *R. nasutus*. Paper discs containing 20 µl of each sample solution were placed on the test plates. Each concentration was studied in triplicate. DMSO was used as negative controls. The plates were incubated at 30 °C for 3-5 days. The diameter of an inhibition zone surrounding each disc was measured.

2.2.5.2 Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) was determined using agar dilution method (Lorian, 1996). The stock solution of the extract was diluted with Sabouraud dextrose agar to give the final concentrations of 0.0625, 0.125, 0.25, 0.5 and 1 mg/ml. Suspension of the test dermatophyte (3 µl) was added to each plate and incubated at 30 °C for 3-5 day. The lowest concentration that did not show any growth of dermatophytes was taken as the MIC.

2.2.6 Formulation and stability test

2.2.6.1 Formulation of cream base

Five different cream bases were prepared. The compositions of the five cream bases are shown in Table 2.1. All cream bases were prepared by fusion method. The oil and water phases were accurately weighed and placed into two separate containers. The oil phase ingredients were melted together in a stem bath to about 70 - 75 °C. Meanwhile, the water phase ingredients were melted together at 75 °C. Then the aqueous solution is slowly added, with constant stirring, to the oil phase mixture. The mixture was then slowly cooled with the stirring continued until the mixture was congealed at room temperature. The obtained cream base was packed in tight containers and kept in a cool place until use.

Table 2.1 The ingredients of the cream bases.

Ingredients	Content (g)				
	Rx1	Rx2	Rx3	Rx4	Rx5
Cetyl alcohol	2	-	5	2	4
Stearyl alcohol	1	7	5	2	4
Glyceryl monostearate	2	5	2	2	-
Cremophor A6	-	-	-	-	3
Tween 80	-	2	-	-	-
Spermaceti	-	3	-	-	-
Cetomacrogol 1000	3	-	3	3	-
Soft paraffin	3	-	2	3	-
Mineral oil	5	5	5	5	10
Glycerin	5	-	5	5	5
Sorbitol solution	-	10	-	-	-
Paraben concentrate	1	1	1	1	1
Purified water to	100	100	100	100	100

2.2.6.2 Formulation of *R. nasutus* cream

The pre-purified leaf extract of *R. nasutus* was incorporated to the best cream base which was selected from section 2.2.6.1 by the geometric dilution method to form a smooth cream. *R. nasutus* cream was formulated with two different concentration of the pre-purified leaf extract, 1% and 2% w/w.

2.2.6.3 Stability test

The freeze-thaw cycle method (Prommobol *et al.*, 1997) was used for the stability test of the five cream bases. The cream bases were kept alternately at 4°C (24 h) and 45°C (24 h) for 5 cycles. The physical appearances of the cream base such as color, smoothness and phase separation were observed before and after testing. The data was used to select the best cream base for preparing the *R. nasutus* leaf extract cream. In the case of the *R. nasutus* cream, the physical properties including viscosity and pH were measured by a viscometer (Brookfield model DV-III, Brookfield Engineering, INC.) and a digital pH meter (pH/Conductivity meter model 20, Denver Instrument Co., Ltd.), respectively. In addition, the content of rhinacanthins in *R. nasutus* cream was analyzed. *R. nasutus* cream was kept at the room temperature and determined rhinacanthin content every one month, for a three-months period, and compared with the content of rhinacanthins in *R. nasutus* cream at initial time.

2.2.6.4 Quantitative determination of rhinacanthins in the *R. nasutus* cream

R. nasutus cream (0.2g) was shaken with 25 ml of methanol for 5 minutes and then centrifuged for 10 minutes. The volume of the supernatant was adjusted to 25 ml with methanol, and subjected to quantitative determination of rhinacanthins in the same method as described in section 2.2.3.2.

The statistic Package for Social Science (SPSS for windows) was used to analyze data. The data was analyzed and compared by the paired t-test. The level of statistical significance was taken at p-value of less than 0.05.

2.2.7 Evaluation of antifungal activity of the *R. nasutus* cream

Agar diffusion method (Lorain, 1996) was used for the evaluation of antifungal activity of the *R. nasutus* cream. The inoculated suspensions (1 ml) were added into molten and cooled SDA (24 ml). These were mixed and poured into 9 cm plates and allowed to set. Equidistant wells were established in the agar using steriled borer apparatus (5 mm diameter). The *R. nasutus* creams were introduced into the appropriate well and then incubated at 30°C for 3 - 5 days. Inhibition zone diameters were measured. Clotrimazole1%, miconazole 2% and ketoconazole 2% cream were used as positive control and the cream base was used as a blank. The assay was performed in triplicate.

The statistic Package for Social Science (SPSS for windows) was used to analyze data. An analysis of variance (ANOVA) was used to compare the different effect of varies inhibition zone of each formulations. The p-value of less than 0.05 ($p < 0.05$) was considered to be statistically significant difference.

Chapter 3

Results and Discussions

3.1 Quantitative determination of rhinacanthins

A method for quantitative determination of total rhinacanthins calculated as rhinacanthin-C has been developed using a UV-vis spectrophotometry with colorimetric method. The colorimetric technique used in the assay is on the basic of Borntrager reaction, with some modification. Borntrager reaction is the specific reaction for quinone compound (Evans, 2002), including naphthoquinone and anthraquinone. Quinone gives intense bathochromic shifts in alkali medium. Plumbagin (5-hydroxynaphthoquinone), for example, has λ_{max} at 220, 226 and 418 nm; in alkali medium the visible maximum of plumbagin is at 526 nm (Vickery *et al.*, 1981). This method has been approved for the quantitative determination of total anthraquinone glycoside in anthraquinone glycoside containing herbs, e.g. *Senna alata* (Thai Herbal Pharmacopoeia Subcommitte, 1998), *Senna angustifolia, Rheum plamatum* (British Pharmacopoeia Commission, 1993). An appropriate wavelength for measuring the absorbance had first selected. In this study, the solution of 20 % KOH in methanol was used as a reagent for the colorimetric reaction to shift the wavelength of maximum absorption of rhinacanthin-C from 282 to 486 nm (Figure 3.1 and Figure 3.2). The maximum absorption wavelength (λ_{max}) at 486 nm was

therefore chosen for performing quantitative determination of total rhinacanthins calculated as rhinacanthin-C for the subsequent studies.

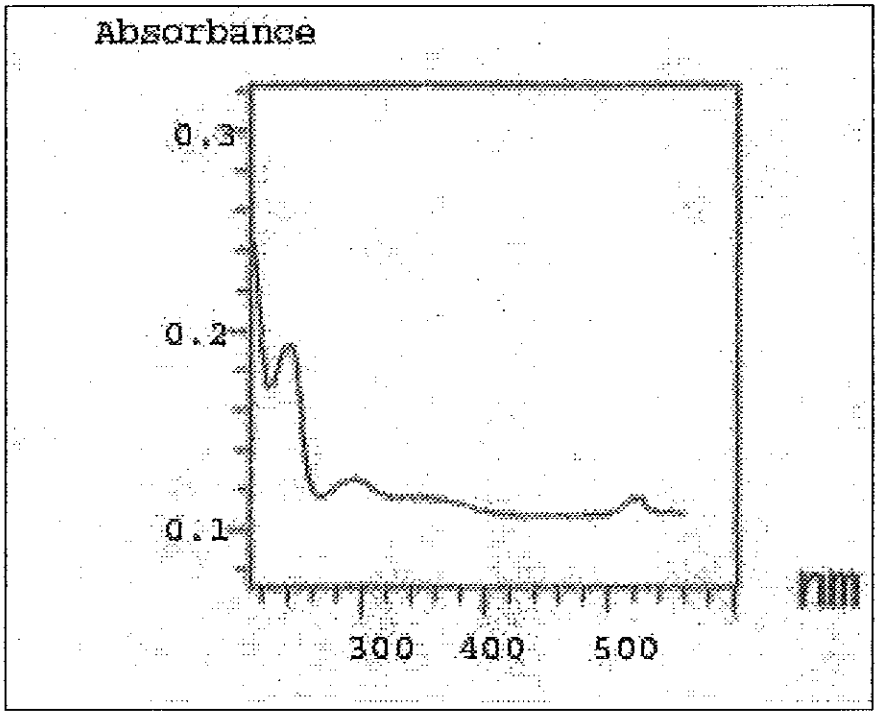


Figure 3.1 Absorption spectrum of rhinacanthin-C solution

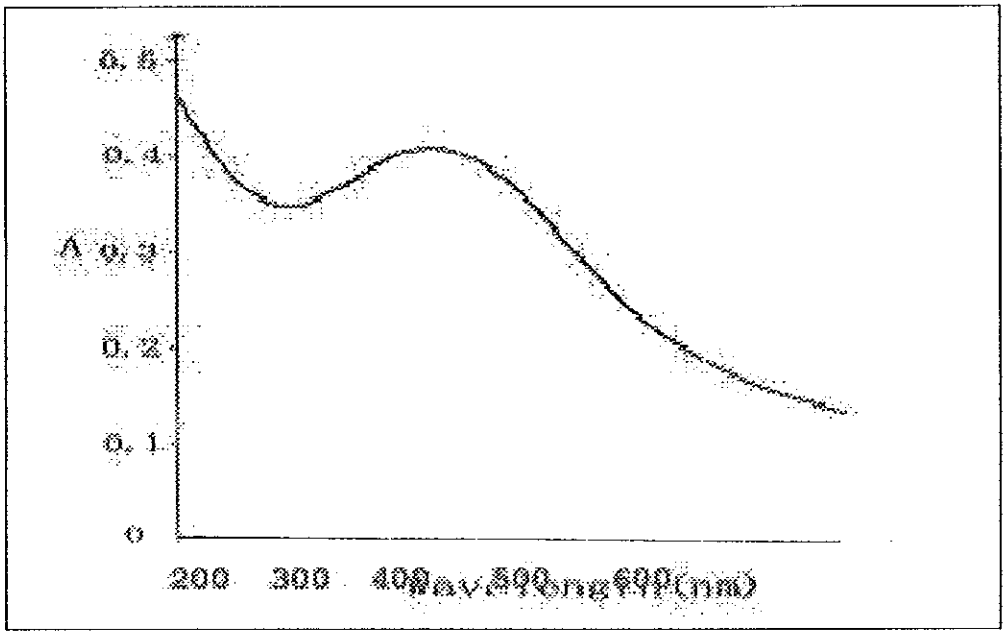


Figure 3.2 Absorption spectrum of mixture of rhinacanthin-C solution and 20% KOH

The factors affecting this assay reaction including sample/reagent ratio and time course of the chemical reaction were studied. Variation of sample/reagent ratio found that the increasing of the sample concentration the higher absorbance at 486 nm was observed at the ratio of 6: 1. However, the sample/reagent ratio of 4:1 gave an appropriate absorbance and reaction volume for the determination (Figure 3.3). The sample/reagent ratio of 4:1 was therefore chosen and used for the further determination.

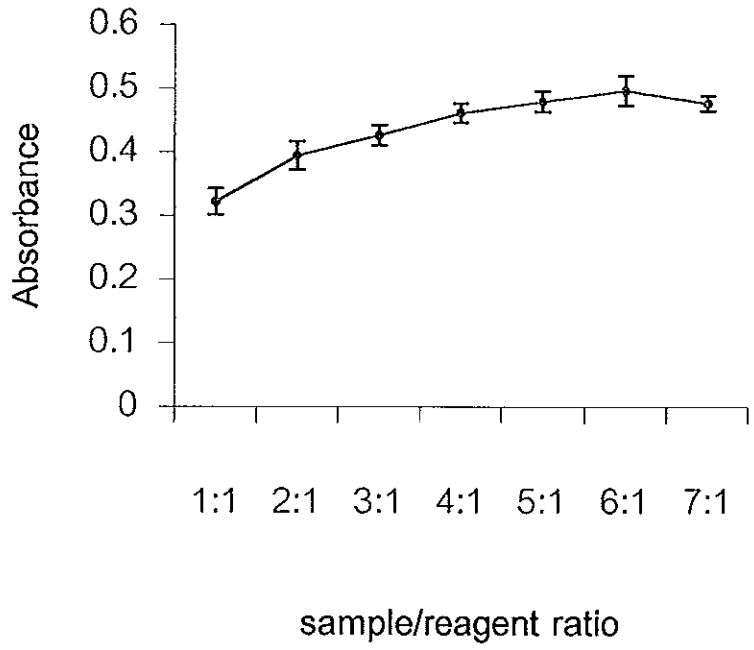


Figure 3.3 Effect of the sample/reagent ratio on the absorbance at 486 nm

The duration time of the reaction is an essential factor for the experiment. The study on the time course of the reaction found that an increasing of the reaction time a higher absorbance at 486 nm was observed. The absorbance seemed to be constant after reaction time of 15 minutes (Figure 3.4). Thus, 15 minutes was sufficient for reaching its maximum absorbance. Therefore, in this assay, after mixing of the sample with 20% KOH solutions, the reaction mixtures were therefore allowed to stand for at least 15 minutes before subjected to measure the absorbance at 486 nm.

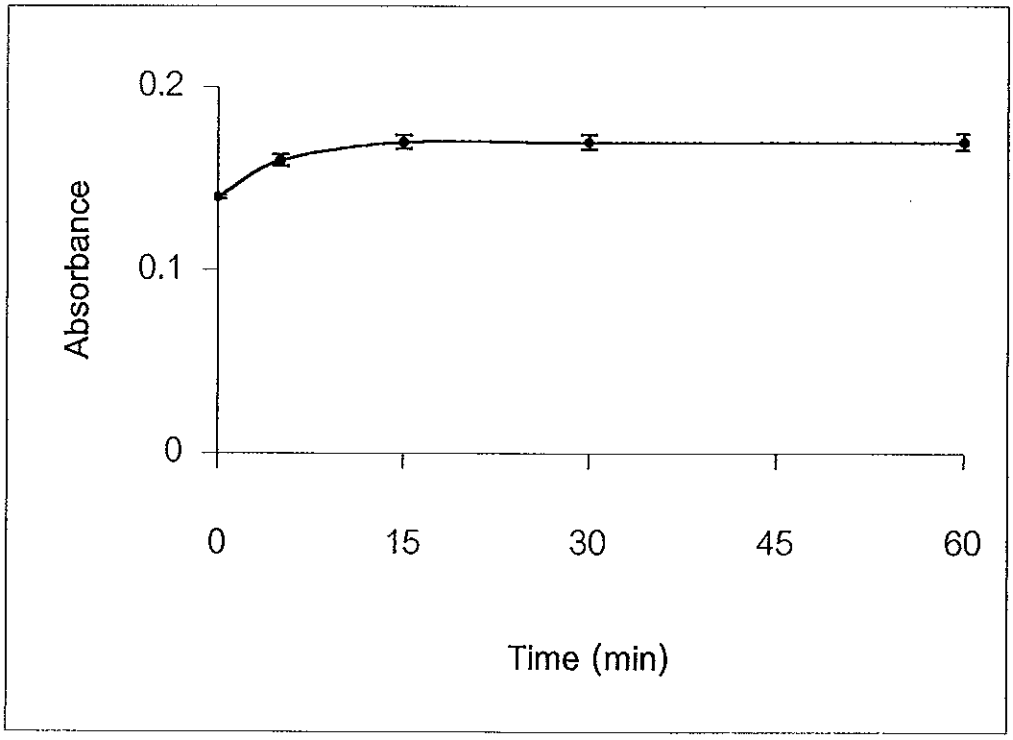


Figure 3.4 Effect of the reaction time on the absorbance at 486 nm

The content of total rhinacanthins in the leaf extract of *Rhinacanthus nasutus* was calculated as rhinacanthin-C, using the calibration curve of rhinacanthin-C. The calibration curve was established by the method as described in section 2.2.3.1 The calibration curve of the standard rhinacanthin-C was observed to be linear in the range from 19.6 – 78.4 μg/ml with r^2 value of 0.9982 -0.9997. The equation of Y= 0.0042X + 0.0845 was fit to the calibration curve (Figure 3.5).

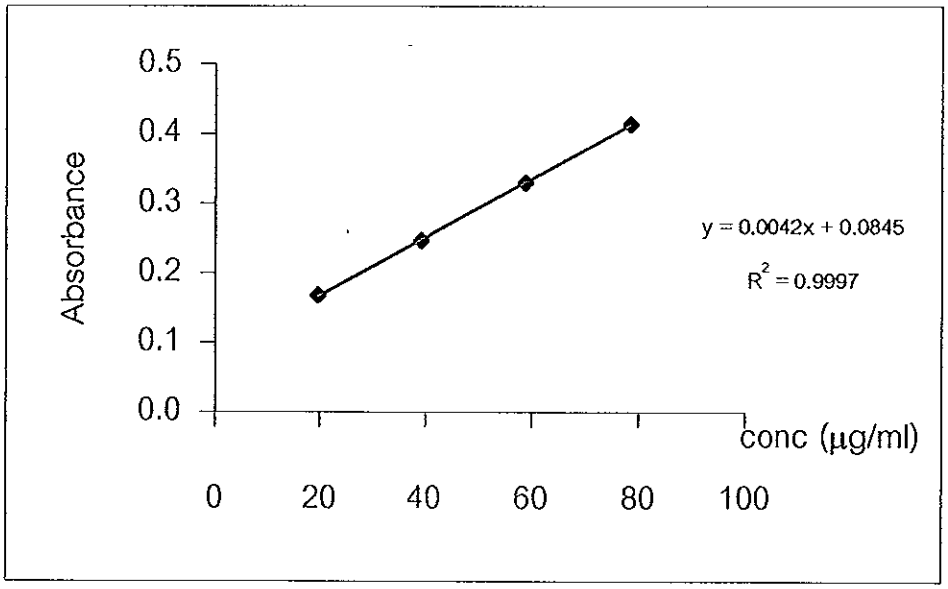


Figure 3.5 Calibration curve of rhinacanthin-C (n = 3)

Validation of the method was accomplished using spiking technique. Various concentrations of rhinacanthin-C were added in the stock solution of crude extract and determined the concentration of the recovered rhinacanthin-C. Table 3.1 assessed the validity of the method with regard to accuracy and precision. Precision and accuracy of this method was assessed from the percentage of coefficient of variation (C.V.) and the percentage of recovery, respectively (Table 3.1). Patel and co-worker reported that the acceptable range of C.V. not exceed 8% for varietal trials (Patel *et al.*, 2001). The results from Table 3.1 show the result from an accuracy and precision validation which indicated that the established method can be applied for the quantitative determination of total rhinacanthins.

Table 3.1 Assay validation of the UV-vis spectrophotometric method for quantitative determination of total rhinacanthins (n=3)

Amount spiked (μg/ml)	**Amount recovered (Mean ± S.D.)**	**% Recovery**	**% C.V.**
7.98	7.50 ± 0.23	94.29	3.07
15.94	15.30 ± 0.56	96.06	3.66
39.60	36.60 ± 0.61	92.51	1.67

3.2 Preparation of *Rhinacanthus nasutus* leaf extract

A search for a suitable organic solvent for extraction were performed by using dried powder of *R. nasutus* leaves to macerate with various organic solvents for three days. The percentage yields and total rhinacanthins that calculated as rhinacanthin-C of each crude extract was determined. The percentage yield of crude extract and the total rhinacanthins content are shown in Table 3.2. It was found that among the organic solvent used for extraction, methanol gave the highest yield of the crude extract. Unfortunately, the obtained methanol extract gives a low content of total rhinacanthins. In contrast, ethyl acetate gave the lowest yield of the crude extract but contain the highest content of total rhinacanthins. Ethyl acetate is therefore a suitable organic solvent for the extraction process, which can concentrate rhinacanthins into the obtained crude extract. Result from rhinacanthins could be best dissolved in ethyl acetate.

Table 3.2 Percentage yield of crude extract from *R. nasutus* leaves

Solvent for extraction	**Yield of crude extract (%w/w)**	**Total rhinacanthins (%w/w)**
Dichloromethane	3.72 ± 0.12	11.70 ± 0.25
Chloroform	4.87 ± 0.22	14.31 ± 0.14
Ethyl acetate	4.84 ± 0.16	33.05 ± 0.16
Ethanol	11.49 ± 0.28	5.44 ± 0.24
Methanol	18.45 ± 0.43	6.75 ± 0.31

3.3 Pre-purification of crude extract

Ion exchange chromatography was developed for pre-purification of the crude ethyl acetate extract in order to obtain a concentrated extract, using for the further formulation and to diminish the interference compounds, which may affecting a stability of the preparation.

A very large number of anions have been successfully separated by anion chromatography. Polarizable anions have an affinity for the ion exchange stationary phase (Fritz *et al.*, 2000). Anion exchange resin is widely used for separation or pre-purification of many compounds, for example, a separation of lactic acid using strongly basic anion exchange resin, Amberlite IRA-400 (Cao *et al.*, 2002) and weak anion exchanger, Amberlite IRA-92 (Tong *et al.*, 2004). Rhinacanthins were also a group of anion compound. Thus, they should be separated with anion exchange resins.

To study the pre-purification process of the crude ethyl acetate extract of *Rhinacanthus nasutus* leaves, two basic anion exchange resins including weakly basic anion exchange resin and a strongly basic anion exchange resin were performed. Amberlite IRA-67 is a weakly basic anion exchanger, while Amberlite IRA-400 is a strongly basic anion exchanger. The pre-purification was conducted by two resins in the same manner and compared the efficiency of resins on concentration of rhinacanthins together with diminution of some interference compounds. It was found that the pre-purification step using weakly basic anion exchange resin gave higher percentage yield of the extract and higher content of total

rhinacanthins than that pre-purified by the strongly basic anion exchange resins (Table 3.3 and Figure 3.6). This may be due to a higher ionic force of the strongly basic anion exchange resin that can strongly bind with rhinacanthins and hardly elute by 10% acetic acid in methanol. The interference compounds including chlorophyll and other pigments were also markedly exclude from the pre-purified extract by Amberlite IRA-67 (Figure 3.6). According to this pre-purification, rhinacanthins were concentrated. The content of total rhinacanthins in the extract after pre-purification was increased to 64.05 ± 4.30 %w/w (Table 3.3). Amberlite IRA-67 was also the suitable resins for the deacidification of the passion fruit juice because of the low consumption of regenerants and the high yield (Vera *et al.*, 2003). The weakly basic anion exchanger, Amberlite IRA-67 was therefore selected and used for pre-purification crude extract of *R. nasutus* leaves prior to formulation.

Table 3.3 Total rhinacanthins content in the ethyl acetate extract before and after pre-purification

Ethyl acetate extract	**Yield (% w/w)**	**Total rhinacanthins %w/w (Mean ± S.D.)**
Before pre-purification	23.27 ± 6.11	33.05 ± 0.95
After pre-purification	25.43 ± 5.27	64.05 ± 4.30

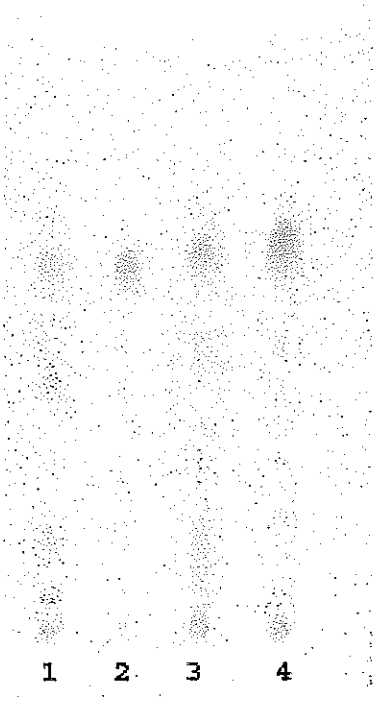


Figure 3.6 TLC chromatogram of *R. nasutus* leaf extract before and after pre-purification. (mobile phase: Chloroform: ethyl acetate, 39:1)

1 = before pre-purification

2 = rhinacanthin-C

3 = after pre-purification with Amberlite IRA-67

4 = after pre-purification with Amberlite IRA-400

3.4 Evaluation of the antifungal activity

Antifungal activity was assayed against *Trichophyton rubrum*, *T. mentagrophytes* and *Microsporum gypseum*. The antifungal activity of the ethyl acetate extract after pre-purification was evaluated and compared with those of the ethyl acetate extract before pre-purification and the standard rhinacanthin-C. It has been reported that rhinacanthin-C against *Candida albicans* and antibacterial against *Staphylococcus aureus* (Panichayupakaranant *et al.*, 2000). However, there is no report on the antifungal activity of rhinacanthins against *T. rubrum*, *T. mentagrophytes* and *M. gypseum*. The result showed that antifungal activity of the extract after pre-purification against *Trichophyton* and *Microsporum* was one times better than that of the crude ethyl acetate extract (Table 3.4). The standard rhinacanthins, rhinacanthin-C exhibited the best antifungal activity against all test fungi. The antifungal activity of the extract after pre-purification was closed to that of the standard rhinacanthin-C. The result from Table 3.4 showed that ethyl acetate extract after per-purification has more concentration of rhinacanthins than extract before pre-purification. Thus, the extract of *R. nasutus* leaves after pre-purification was therefore effective enough for the further formulation of topical antifungal cream.

Table 3.4 Antifungal activity of rhinacanthin-C, the ethyl acetate extract before and after pre-purification against *Trichophyton rubrum*, *T. mentagrophytes* and *Microsporum gypseum*

Sample	**MIC (μg/ml)**		
	T. rubrum	*T. mentagrophytes*	*M. gypseum*
Extract (before pre-purification)	125	125	500
Extract (after pre-purification)	62.50	62.50	250
rhinacanthin-C	31.25	31.25	125

3.5 Formulation *Rhinacantus nasutus* leaf extract cream

The stability of cream base was tested by freeze-thaw cycling method as described in section 2.2.6.3. An observation of physical appearance such as color, smoothness and phase separation after freeze-thaw cycle let's to the selection of cream base Rx 1 and Rx 2 (Table3.5 and Figure 3.7) for the further formulation. In addition a viscosity of cream base Rx 1 and Rx 2 did not significantly change after the test.

Table 3.5 Physical stability of cream base before and after freeze-thaw cycle

Physical properties	**Before Freeze-Thaw cycle**					**After Freeze-Thaw cycle**				
	Rx1	**Rx2**	**Rx3**	**Rx4**	**Rx5**	**Rx1**	**Rx2**	**Rx3**	**Rx4**	**Rx5**
Color	w	w	w	w	w	w	w	w	ow	ow
Smoothness	S	S	S	S	S	S	S	S	R	R
Phase separation	No	No	No	No	No	No	No	No	No	No

w = white, ow = off-white, S = soft, R = rough

Rx 1 = cream base Rx 1 (Table 2.1)

Rx 2 = cream base Rx 2 (Table 2.1)

Rx 3 = cream base Rx 3 (Table 2.1)

Rx 4 = cream base Rx 4 (Table 2.1)

Rx 5 = cream base Rx 5 (Table 2.1)

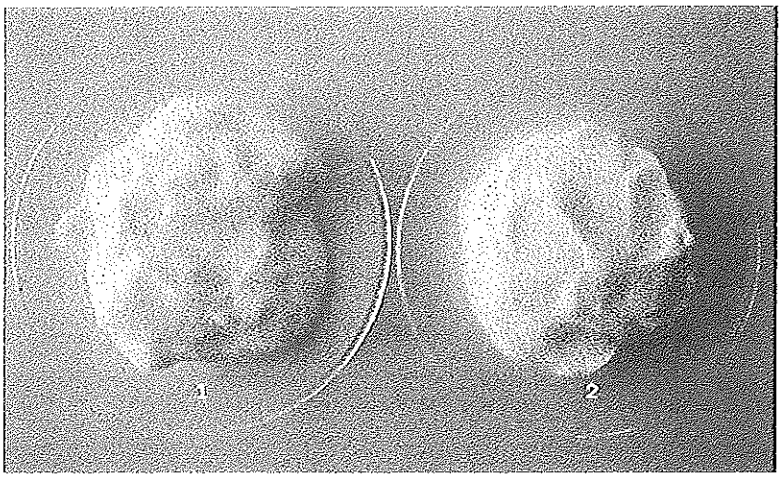


Figure 3.7 Cream base Rx 1 and Rx 2 after freeze-thaw cycle

The antifungal creams were prepared from pre-purification extract of *R. nasutus* at concentration of 1% and 2% w/w, which equivalent to the concentration of total rhinacanthins 0.64 % w/w and 1.28 %w/w, respectively. The pre-purification extract was added into the two selected cream bases, Rx 1 and Rx 2. Thus, four preparations were obtained (Rx 1.1, Rx 1.2, Rx2.1 and Rx 2.2). When the extract was incorporated into the cream bases, the color of the cream bases was changed from white to green. The tone of green color is depending on the concentration of the extract in the preparation (Figure 3.8). All four preparations have good appearance.

3.6 Stability test

Viscosity and pH of four preparations of *R. nasutus* were compared before and after freeze-thaw cycle. After freeze-thaw cycle, it was found that the viscosity and pH of all preparations was slightly increased (Table 3.6). In addition, all preparations still showed satisfied physical appearances after freeze-thaw cycle (Figure 3.8).

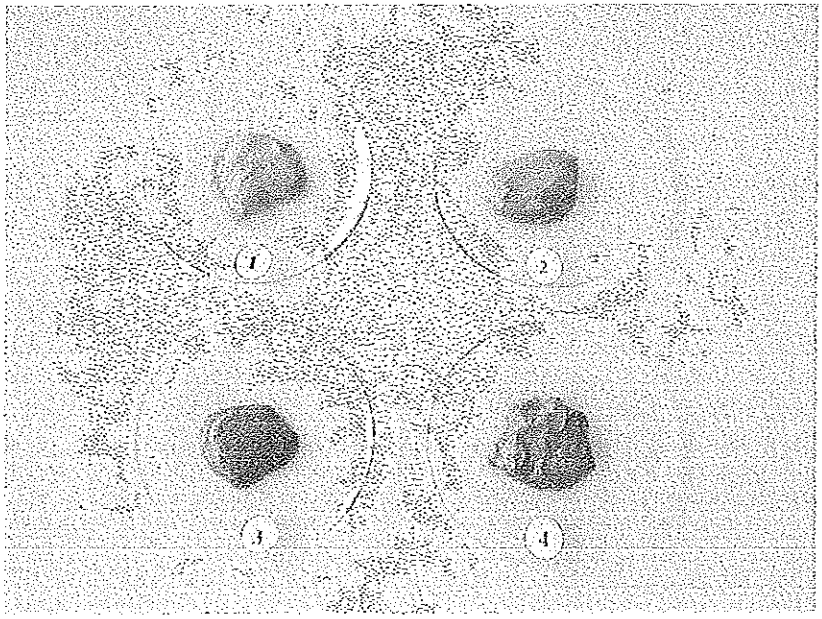


Figure 3.8 *R. nasutus* cream after freeze-thaw cycle

1 = Rx 1.1 (1% w/w extract of *R. nasutus* in cream base Rx 1)

2 = Rx 1.2 (1% w/w extract of *R. nasutus* in cream base Rx 2)

3 = Rx 2.1 (2% w/w extract of *R. nasutus* in cream base Rx 1)

4 = Rx 2.2 (2% w/w extract of *R. nasutus* in cream base Rx 2)

Table 3.6 Viscosity and pH of *R. nasutus* creams before and after freeze-thaw cycle

Physical Property	**Before Freeze-Thaw cycle**				**After Freeze-Thaw cycle**			
	Rx1.1	**Rx1.2**	**Rx2.1**	**Rx2.2**	**Rx1.1**	**Rx1.2**	**Rx2.1**	**Rx2.2**
Color	Pale green	Pale green	Dark green	Dark green	Pale green	Pale green	Dark green	Dark green
Smoothness	S	S	S	S	S	R	R	R
Phase separation	No	No	No	No	No	No	No	No
Viscosity (cps) (Mean ± S.D.)	2910.0 ± 10.0	2952.7 ± 9.3	3722.0 ± 8.2	3645.3 ± 5.5	3866.7 ± 7.6	3515.7 ± 4.0	3652.0 ± 7.2	4230.7 ± 5.1
pH	3.20	4.12	3.37	4.60	3.54	4.15	4.20	4.50

S = soft, R = rough

Rx 1.1 = 1% w/w extract of *R. nasutus* in cream base Rx 1

Rx 1.2 = 1% w/w extract of *R. nasutus* in cream base Rx 2

Rx 2.1 = 2% w/w extract of *R. nasutus* in cream base Rx 1

Rx 2.2 = 2% w/w extract of *R. nasutus* in cream base Rx 2

The stability of rhinacanthins in the preparation was also evaluated. The preparations were separately kept at room temperature (30 ± 2 °C) and 45 °C, and the total rhinacanthins content in the preparations was determined monthly for the three-month period. The antifungal creams were extract with methanol and determined the total rhinacanthins according the method described in section 2.2.6.4.

Table 3.7 Total rhinacanthin content in four preparations of *R. nasutus* creams kept at room temperature (30 ± 2 °C) and 45 °C for three-months period.

Storage (months)	% Total rhinacanthins (w/w)							
	Room temperature				45 °C			
	Rx1.1	Rx1.2	Rx2.1	Rx2.2	Rx1.1	Rx1.2	Rx2.1	Rx2.2
0	100.59 ±0.91	103.29 ±1.20	103.24 ±1.82	101.85 ±3.02	100.59 ±0.91	103.29 ±1.20	103.24 ±1.82	101.85 ±3.02
1	98.98 ±0.78	98.34 ±1.12	99.11 ±1.54	98.26 ±0.34	96.22 ±1.45	96.62* ±1.19	97.16* ±2.57	94.74* ±1.16
2	98.66 ±0.56	99.49 ±1.44	94.68* ±2.83	86.47* ±2.39	88.18* ±2.99	84.75* ±3.33	87.51* ±4.22	82.49* ±1.44
3	96.20 ±1.32	84.58* ±2.15	94.13* ±2.26	89.25* ±1.07	82.61* ±1.86	80.48* ±0.06	85.82* ±1.64	83.63* ±2.79

* significance at $p<0.05$

Rx 1.1 = 1% w/w extract of *R. nasutus* in cream base Rx 1

Rx 1.2 = 1% w/w extract of *R. nasutus* in cream base Rx 2

Rx 2.1 = 2% w/w extract of *R. nasutus* in cream base Rx 1

Rx 2.2 = 2% w/w extract of *R. nasutus* in cream base Rx 2

The result showed that when the creams were stored at the room temperature, the total rhinacanthin content in most preparations significantly decreased after one (Rx 2.1 and Rx 2.2) or two months (Rx 1.2) of storage (Table 3.7). There is only one preparation (Rx 1.1) that the total rhinacanthins content was not significantly decrease within three-month period ($p<0.05$).

When the creams were stored at 45 °C, it was found that the total rhinacanthins content in most preparations (Rx 1.2, 2.1 and 2.2) significantly decreased within one month storage. There is only one preparation (Rx 1.1) that the total rhinacanthins content was significantly decrease after two months storage. These results were similary with the report by Pitakvongsaporn who revealed that turmeric cream which storage at 40 °C and 50 °C for 4 months turned to dark orange color, oil phase and water phase were obviously separated (Pitakvongsaporn, 2000). As commonly known that most creams and ointments must be stored at temperature below 30 °C to prevent the softening and even the liquefying of the base (Ansel *et al.*, 1995)

The result indicates that the preparation Rx 1.1 is better stabilize rhinacanthin compounds than the others preparation. In addition, *R. nasutus* cream should be kept in cool temperature or at least at room temperature along storage.

3.7 Test for antifungal activity of *R. nasutus* cream

The antifungal activities of *R. nasutus* cream were tested by agar diffusion method. It was found that all preparations possessed satisfactory antifungal activity

against *Trichophyton rubrum, T. mentagrophytes* and *M. gypseum* (Table 3.8). The ranges of inhibition zone diameters (mm) among *R. nasutus* creams were not significantly difference ($p<0.05$) (Table 3.8). It implies that, the antifungal activity of *R. nasutus* extract at the concentration 1 %w/w (total rhinacanthins = 0.64 %w/w) is not different to that of 2 %w/w (total rhinacanthins = 1.28 %w/w. However, the diameters of the inhibition zone of all preparations were lower than those of the all standard topical antifungal creams, including 1 % clotrimazole cream, 2 % miconazole cream and 2 % ketoconazole cream. This may be the potency of *R. nasutus* extract (MIC = 36 μg/ml for *Trichophyton* and 288 μg/ml for *Microsporum*) is lower than those of clotrimazole (MIC = 0.01-10 μg/ml for *Trichophyton* and 0.1-2 for *Microsporum*), miconazole (MIC =0.8-1μg/ml for *Trichophyton* and μg/ml for *Microsporum*) and ketoconazole (MIC = 0.5-1.3 μg/ml for *Trichophyton* and 0.002-0.004 μg/ml for *Microsporum*) (Lorain, 1996; Karaca *et al.*, 2004).

Table 3.8 Inhibition zone of *R. nasutus* creams compared with the commercially available topical antifungal creams (clotrimazole cream, miconazole cream and ketoconazole cream) against *Trichophyton rubrum*, *T. mentagrophytes* and *Microsporum gypseum*

Preparations	**Inhibition zone diameters (mm, Mean ± S.D.*)**		
	T. rubrum	***T. mentagrophytes***	***M. gypseum***
Rx 1.1	18.20 ± 1.20	15.20 ± 1.51	10.20 ± 0.20
Rx 1.2	16.60 ± 1.05	15.10 ± 0.66	10.30 ± 0.20
Rx 2.1	16.17 ± 1.00	16.57 ± 1.06	10.90 ± 0.36
Rx 2.2	19.97 ± 0.95	16.93 ± 0.67	10.30 ± 0.10
Clotrimazole cream (1%)	39.13 ± 3.38	48.90 ± 2.44	40.23 ± 1.08
Miconazole cream (2%)	36.40 ± 3.72	33.43 ± 3.77	31.10 ± 0.95
Ketoconazole cream (2%)	35.03 ± 2.78	40.03 ± 1.05	32.57 ± 1.99

*Mean ± S.D. (n= 3)

Adjustment for multiple comparisons: ANOVA

Rx 1.1 = 1% w/w extract of *R. nasutus* in cream base Rx 1

Rx 1.2 = 1% w/w extract of *R. nasutus* in cream base Rx 2

Rx 2.1 = 2% w/w extract of *R .nasutus* in cream base Rx 1

Rx 2.2 = 2% w/w extract of *R. nasutus* in cream base Rx 2

Analysis of variance (ANOVA) data (Table 3.8) of inhibition zone diameters between four preparation of *R. nasutus* cream revealed that no significant difference was found for the antifungal activity against *T. rubrum, T. mentagrophytes* and *M. gypseum*.

In addition, a factor that also affecting the diameter of inhibition zone is a diffusion ability of either rhinacanthins or cream bases into agar. The effect of the relationship between rhinacanthins diffusion and concentration, solubility of rhinacanthins in agar and the effect of a chemical component in agar are also affect to diffuse cream into agar. The influence of results suggested that the antifungal preparation Rx 1.1 (1 %w/w *R. nasutus* extract) is the most suitable formula for the topical antifungal dosage form.

Chapter 4

Conclusions

From this research work the following conclusions can be drawn:

1. The ethyl acetate extract of *Rhinacanthus nasutus* contained high content of total rhinacanthins (33.05 $\pm$ 0.95 %w/w) and exhibited significant antifungal activity against *Trichophyton rubrum*, *T. mentagrophytes* and *Microsporum gypseum*.

2. UV-vis spectrophotometry couple with a colorimetric technique was established for determination of total rhinacanthins, calculated as rhinacanthin-C in *R. nasutus* leaf extract.

3. The pre-purification step is necessary for concentration of rhinacanthins and removal of interference compounds in the ethyl acetate extract of *R. nasutus* leaves prior to formulating.

4. The pre-purification step was achieved using a basic anion exchange resin (Amberlite IRA-67) with methanol and 10% acetic acid in methanol as washing solvent and eluant, respectively.

5. The antifungal creams prepared from the extract of *R. nasutus* was established and showed satisfactory physical appearance and antifungal activity against *T.*

rubrum, *T. mentagrophytes* and *M.* gypseum. However, the most suitable preparation that possessed satisfactory stability is Rx 1.1. The formulation is as follows;

R. nasutus leaf extract	1g
Cetyl alcohol	2g
Stearyl alcohol	1g
Glyceryl monostearate	2g
Cetomacrogol 1000	3g
Soft paraffin	3g
Mineral oil	5g
Glycerin	5g
Paraben concentrate	1g
Purified water to	100g

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