Preparation of Standardized *Rhinacanthus nasutus* Leaf Extract by Green Extraction Methods and Evaluation of Antifungal Activity of Its Topical Solution against *Trichophyton rubrum*

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Thesis Title	Preparation of Standardized *Rhinacanthus nasutus* Leaf Extract by Green Extraction Methods and Evaluation of Antifungal Activity of Its Topical Solution against *Trichophyton rubrum*
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ABSTRACT

A simple and practical green extraction method was described for preparation of rhinacanthin-C enriched *Rhinacanthus nasutus* leaf extracts. The extraction conditions, including extraction methods, alternative solvents, and leaf powder to solvent ratios, were determined to increase the amount of rhinacanthin-C in the leaf extracts. Microwave assisted extraction (MAE), an innovative green extraction method was determined as a suitable extraction method for rhinacanthin-C by comparison with the conventional extraction method including maceration and heat assisted extraction (HAE) methods. Ethanol, propylene glycol, glycerol that are generally used in a preparation of topical solution were evaluated to be a suitable alternative green solvent for extraction of rhinacanthin-C from the dried powders of *R. nasutus* leaves. The content of rhinacanthin-C in the leaf extract was determined by a reversed phase HPLC method. The suitable extraction conditions for preparation of rhinacanthin-C enriched *R. nasutus* leaf extracts were: employing of a solution of 25% v/v glycerol in ethanol as solvent, with a leaf powder to solvent ratio of 15: 100 (g/mL), and using maceration method for 72 h. The obtained extract was brownish solution with the content of rhinacanthin-C up to 1.79 mg/mL. The leaf extracts of *R. nasutus* were therefore prepared using these extraction conditions and standardized to contain rhinacanthin-C content of not less than 1.7 mg/mL.

Three topical solutions containing the leaf extracts of *R. nasutus* as the active ingredient were formulated. The solutions were standardized to contain rhinacanthin-C at a concentration of 0.1% w/v. Thymol and menthol were constituted as

antioxidant and cooling agent, respectively, while water (10-20% v/v) and ethanol were used as vehicles. An adequate flavoring agent was also used. All three topical solutions containing the leaf extracts of *R. nasutus* had a brownish color with a pleasant smell. On the basis of HPLC analysis, all three topical solutions contained rhinacanthin-C contents of 0.1% w/v, and their appearances were not significantly different even after kept for a week. The formulation containing a minimum content of water (10% v/v) was selected for antifungal activity and stability evaluation.

Based on an agar well diffusion assay, the topical solutions containing the leaf extracts of *R. nasutus*, with its concentration equivalent to 0.1% w/v rhinacanthin-C showed significant inhibitory activity against *T. rubrum*, with an inhibition zone of 17.9 mm. In addition, rhinacanthin-C enriched *R. nasutus* leaf extract and the topical solution exhibited antifungal activity against *T. rubrum*, with MIC values of 8.6 and 28.5 mg/mL, at dilutions of 1:64 and 1:32, respectively.

Stability evaluations of rhinacanthin-C in rhinacanthin-C enriched *R. nasutus* leaf extracts as well as in the topical solution through a period of 4 months found that both rhinacanthin-C enriched *R. nasutus* leaf extracts and the topical solution were stable over a period of four months when stored in a well-closed container protected from light at 4 ± 2°C. In contrast, at 25 ± 2°C and accelerated conditions (45 ± 2°C, 75% relative humidity), rhinacanthin-C content decreased significantly after 8 weeks. However, the remained amount of rhinacanthin-C in the extracts and topical solutions were 92 and 86%, respectively after kept at 25 ± 2°C for 4 months. In addition, the remained amount of rhinacanthin-C in both extract and topical solution were 77% after kept in the accelerated conditions for 4 months. This implied that the content of rhinacanthin-C in the extracts and topical solutions may decrease by 23% after kept at room temperature for two years.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percentage
[bmim]Br	1-Butyl-3-methyl-1H-imidazol-3-ium bromide
±	Plus or minus
≤	Smaller than or equal to
≥	Greater than or equal to
°C	Degree celsius
µg/disc	Microgram per disc
µg/mL	Microgram per millilitre
µM	Micromolar
µm	Micrometer
ACD acidic pKa	Acid dissociation constant predicted by ACD /Percepta software
AlogP	Partition coefficient by atomic based prediction
cm	Centimeter
conc.	Concentration
DW	Dry weight
e.g.	Exempli gratia
etc.	Et cetera
g	Gram
h	Hour
HAE	Heat assisted extraction
HPLC	High performance liquid chromatography
IC_{50}	50% inhibitory concentration
log K	Partition coefficient
m	Meter
MAE	Microwave assisted extraction
mg/kg	Milligram per kilogram

mg/kg/d	Milligram per kilogram per day
mg/mL	Milligram per millilitre
MHz	Mega hertz
MIC	Minimum inhibitory concentration
min	Minutes
mL	Millilitre
mm	Millimeter
nm	Nanometer
PA	Pascal
PEG	Polyethylene glycol
pH	Power of hydrogen ion
ppm	Parts per million
q.s.	Quantum satis
rpm	Rotation per minute
RRn	Rhinacanthin rich
S.D.	Standard deviation
spores/mL	Spores per millilitre
SPSS	Statistical Package For Social Sciences
UAE	Ultrasound assisted extraction
v/v	Volume by volume
W	Watt
w/v	Weight by volume
α	Alpha
β	Beta

CHAPTER 1

INTRODUCTION

1.1 Background and rationale

Dermatophytes are a group of closely related fungi that invade keratinized tissue (skin, hair, and nails) to produce an infection, dermatophytosis, commonly referred to ringworm or tinea. Infection is generally cutaneous and restricted to the non-living cornified layers due to the inability of the fungi to penetrate the deeper tissues or organs of immunocompetent hosts (Weitzman and Summerbell, 1995). This could be the result of inhibition of fungal keratinases or the presence of nonspecific inhibitory factors in the serum (Gupta *et al.*, 1998). However, there may be severe and disseminated infection in immunocompromised patients, with the development of dermatophytic granulomas (Peres *et al.*, 2010). *Trichophyton rubrum* (causes between 80 and 90% of all chronic and recurrent infections) and *T. mentagrophytes* are the leader etiological fungi and are the most common cause of skin disease in tropical countries (Castro *et al.*, 2013). Skin infection causes lesions that are circular, erythematous and pruritic, due to direct action of the fungus or hypersensitivity reactions to the microorganism and/or its metabolic products (Peres *et al.*, 2010).

Tinea infections can be treated either topically or systemically, which will depend on the type of tinea infection, the severity of the infection, and the patient's preferences (Gupta and Tu, 2006). The treatment for dermatophytosis is long and expensive and lies within the two main antifungal drug families: the azoles and the allylamines (Weitzman and Summerbell, 1995; Gupta and Cooper, 2008; Peres *et al.*, 2010). There were reports of clinical resistance to oral treatment with terbinafine (Mukherjee *et al.*, 2003) and relapse to oral and/or topical treatment with terbinafine or itraconazole (Piraccini *et al.*, 2010). Thus, there is necessity for more effective management of these drugs and discovery of new broad-spectrum antifungal agents (Martinez-Rossi *et al.*, 2008).

Medicinal plants are important source of new drugs, new lead compounds, and new chemical entities (Balunas and Kinghorn, 2005). Due to their desired pharmacological activities, they are traditionally used as drugs, and some constituents of these plants are also developed into modern medicines (Ramawat and Mérillon, 2008). Nowadays, popularity and demand of herbal medicines have increased globally due to its accessibility and affordability for improvement of health condition of human beings as well as preventing and curing diseases (WHO, 2002; Mosihuzzaman and Choudhary, 2008; Bansal *et al.*, 2014).

In Thai traditional medicines, *Rhinacanthus nasutus* leaves and roots are widely used for various skin diseases (Sattar *et al.*, 2004; Panichayupakaranant *et al.*, 2009). *R. nasutus* is widely distributed in India, China, Thailand and Southeast Asia (Kodama *et al.*, 1993; Sendl *et al.*, 1996). Its root and stem extracts are used for the treatment of ringworm (Kodama *et al.*, 1993). In India, it is used as a poultice, in Thailand as a tincture either with alcohol or vinegar, and in Malay Peninsula as a paste prepared by compounding leaves with benzoin and sulphur. It was speculated that naphthoquinone and napthopyran derivatives in aqueous extract of *R. nasutus* exhibited a potent dose dependent antifungal activity against *Candida albicans* and *T. mentagrophytes* (Sattar *et al.*, 2004).

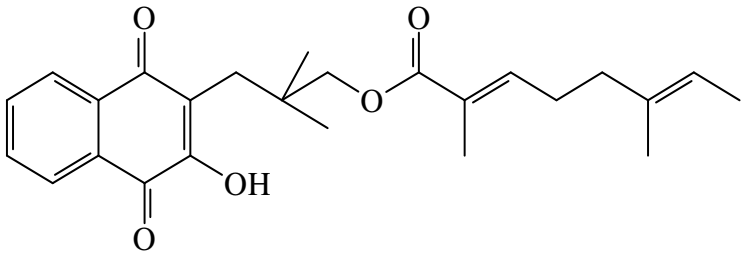

Figure 1 Chemical structure of rhinacanthin-C

It has been reported that rhinacanthin-C (Fig. 1) possessed potent antifungal, antibacterial, anti-inflammatory, anti-allergic and antiproliferative activities (Gotoh *et al.*, 2004; Panichayupakaranant *et al.*, 2009; Tewtrakul *et al.*, 2009b; Tewtrakul *et al.*, 2009a; Puttarak *et al.*, 2010). Rhinacanthin-C has been reported as the major and the most potent antifungal constituent of *R. nasutus* leaf extract (Puttarak *et al.*, 2010). In Thailand, "Tincture Thong-pan-chang" that is prepared from the fresh leaves of *R.*

nasutus macerated in 70% ethanol (10% w/v) has been approved in the List of Herbal Medicinal Products A.D. 2013 for the treatment of dermatophytosis (National Drug Committee, 2013).

But consistency in the plants constituents may vary according to differences in climate, cultivating and harvesting periods, post harvesting process, and storage (Bansal *et al.*, 2014). One of the regulatory approvals to ascertain consistent chemical profile and biological activity of future drug candidate reproducibility is by repetitive testing using different batches to control batch-to-batch variation and development of standard assay markers (Chawla *et al.*, 2013). Standardization can be based on identifying and quantifying an extract to a pure compound or concentrates of one or more as active constituents (Panichayupakaranant, 2011). Thus, standardization of *R. nasutus* extract on the basis of rhinacanthin-C content, the major potent antifungal constituent of *R. nasutus* leaf extract is prerequisite for the efficacy and safety against treatment of dermatophytosis.

The general methods for preparation of herbal extracts involve uses of organic solvents such as methanol, ethyl acetate, hexane, dichloromethane, chloroform and others. The organic solvents are problematic in the extraction/separation of biological active compounds from the herb because of their toxicity, volatility and flammability (Zhou *et al.*, 2011). Methods such as maceration, reflux, Soxhlet extraction, and others are commonly used to improve the kinetics of extraction of constituents. However, such methods are time and solvent consuming, and may have lower extraction efficiencies (Mosihuzzaman and Choudhary, 2008). Due to such disadvantages of using organic solvents for extraction of rhinacanthin-C, a more efficient approach of extraction is seemed necessary. Regarding the preparation of bioactive herbal extracts in industrial scale for pharmaceutical and cosmetic applications, green and efficient extraction processes that are free from toxic solvents are essential (Michel *et al.*, 2011).

Previous report suggested that moderate non polar solvents should be suitable for extraction of rhinacanthins from *R. nasutus* leaves (Charoonratana, 2007). Moreover, rhinacanthins were not stable in aqueous alcoholic solution, which was considered to be by hydrolysis (Puttarak *et al.*, 2010). Ethanol, propylene glycol and glycerol are semi polar solvents that have been used as co-solvents for increase

solubility of poorly soluble drugs and/or stabilization of degradable drugs in aqueous solutions (Soleymani *et al.*, 2013). Therefore, there is a potential for use of ethanol, propylene glycol and glycerol, which are generally used as the ingredients of a topical solution, as the alternative green solvents for preparation of the rhinacanthin-C extract with proper stability. These solvents are considered safe, so they can be directly used in the formulation. Thus, the step of solvent evaporation from the extract can be omitted, which may save time and minimize production cost.

A green extraction method, i.e. microwave assisted extraction (MAE) was determined for extraction of rhinacanthin-C compared with the conventional extraction method, i.e. maceration and reflux. The present studies therefore focused on preparation of rhinacanthin-C enriched *R. nasutus* leaf extracts from *R. nasutus* dried leaf powders using green solvents including ethanol, propylene glycol and glycerol as well as green extraction method. The obtained extracts are used for formulation of a topical antifungal solution containing 0.1% w/v rhinacanthin-C.

1.2 Objectives

1.2.1 To prepare a rhinacanthin-C enriched *R. nasutus* leaf extracts, standardized based on rhinacanthin-C content by a validated HPLC method and using an optimized green extraction method

1.2.2 To formulate a topical antifungal solution from the rhinacanthin-C enriched *R. nasutus* leaf extracts

1.2.3 To evaluate antifungal activities of the topical antifungal solution from the rhinacanthin-C enriched *R. nasutus* leaf extracts compared with a standard drug (1% Clotrimazole solution)

1.2.4 To evaluate stabilities of the rhinacanthin-C enriched *R. nasutus* leaf extracts and its topical antifungal solution

CHAPTER 2

LITERATURE REVIEW

2.1 Dermatophytes

Dermatophytes are a group of closely related fungi that invade keratinized tissue (skin, hair, and nails) to produce an infection, dermatophytosis, commonly referred to ringworm or tinea. Infection is generally cutaneous and restricted to the non-living cornified layers due to the inability of the fungi to penetrate the deeper tissues or organs of immunocompetent hosts (Weitzman and Summerbell, 1995). This could be the result of inhibition of fungal keratinases or the presence of nonspecific inhibitory factors in the serum (Gupta *et al.*, 1998b).

Some dermatophytes spread directly from one person to another (anthropophilic organisms) or transmitted to humans from soil where they live in (geophilic organisms), or from animal hosts to humans (zoophilic organisms). Transmission of dermatophytes also can occur indirectly from fomites, e.g. upholstery, hairbrushes, hats (Hainer, 2003). Dermatophytosis can be classified on the basis of conidial morphology and formation of conidia in the genera *Microsporum, Trichophyton* and *Epidermophyton* (Weitzman and Summerbell, 1995). There are many different species of dermatophytes that can cause infection in humans. Two of the most common types are *Trichophyton rubrum* and *Trichophyton tonsurans*, which are usually transmitted from person to person (Definition of Dermatophytes, 2012). The genera and their descriptions are shown in the Table 2.1.

Infections caused by dermatophytes (ringworm) have been traditionally named according to the anatomic locations involved by appending the Latin term designating the body site after the word tinea, i.e. tinea capitis (scalp, eyebrows, and eyelashes), tinea barbae (beard and moustache), tinea corporis (glabrous skin), tinea cruris (groin), tinea favosa (favus), tinea imbricata (ringworm caused by *T. concentricum*),

tinea manuum (hand), tinea pedis (feet), tinea unguium (nails) (Weitzman and Summerbell, 1995).

Table 2.1 Classification of dermatophytes on the basis of condial morphology and their description (Weitzman and Summerbell, 1995)

Genera	Macroconidia	Microconidia
Epidermophyton	• Broadly clavate with typically smooth, thin to moderately thick walls • One to nine septa, 20 to 60 by 4 to 13 mm in size They are usually abundant and borne singly or in clusters.	• Are absent
Microsporum	• Are characterized by the presence of rough walls which may be asperulate, echinulate, or verrucose • May have thin, moderately thick to thick walls • 1 to 15 septa and range in size from 6 to 160 by 6 to 25 mm.	• Sessile or stalked and clavate and usually arranged singly along the hyphae or in racemes as in *Microsporum racemosum*, a rare pathogen
Trichophyton	• When present, have smooth, usually thin walls and • One to 12 septa, are borne singly or in clusters, and may be elongate and pencil shaped, clavate, fusiform, or cylindrical. • They range in size from 8 to 86 by 4 to 14 mm.	• May be globose, pyriform or clavate, or sessile or stalked, • Are borne singly along the sides of the hyphae or in grape-like clusters.

Dermatophyte infections can be readily diagnosed based on the history, physical examination, and potassium hydroxide microscopy, Wood's lamp examination and fungal culture or histological examination. Potassium hydroxide microscopy aids in visualizing hyphae and confirming the diagnosis of dermatophyte infection (Hainer, 2003).

Trichophyton rubrum is anthropophilic organism, which is the most common pathogen of pedal and crural infections worldwide, often associated with tinea corporis and tinea unguium. Identification of *T. rubrum* can be frustrating due to variability of cultures of this species. The "downy" form, which is most commonly isolated in chronic tinea pedis and tinea corporis, is fluffy and white and has numerous aerial hyphae. The "granular" form manifests as a white colony that is flat, lacks aerial hyphae, and looks like sprinkles of sugar. The under surface of both forms is usually red. Some colonies of *T. rubrum* may appear indistinguishable from *T. mentagrophytes* (Rinaldi, 2000).

2.2 Current scenario and treatment of dermatophyte

Dermatophytes are among the most frequent and cosmopolitan infection, deteriorating quality of life of individuals in various age groups and leading to economic burden for treatment. WHO estimated about 25% of the world population to be affected by dermatophytes (Peres *et al.*, 2010). Despite advances in medicine, a retrospective analysis by Vena *et al.* (2012), showed increase in prevalence of some forms of dermatophytosis, with *T. rubrum* to be prevalent causative agent (64% of total cases) in last 35 years. These infections shows more extensive distribution and hostility among immunocompromised patients such as transplant recipients, patients with hematologic malignancies, and those receiving high dose chemotherapy (Lourdes *et al.*, 2014). However, these infections are rarely life-threatening (Elewski, 1998). Their incidences have been reported to be more frequent in communities with low socioeconomic status, with crowded living conditions, close proximity to animals, suboptimal hygienic condition and in tropical and subtropical regions with warm and humid environment, which is more suitable for dermatophytes. Moreover, globalization due to mass tourism and migration help for import and dissemination of such infections (Havlickova *et al.*, 2008).

The choice for treatment of tinea infections are either topically or systemically, which depend on the type of tinea infection, the severity of the infection, and the patient's preferences (Gupta and Tu, 2006). Additional factors that may influence the choice between equally effective therapies include tolerability, safety, compliance,

and cost (Fleece *et al.*, 2004). The oral antifungal medications has been associated with some potential for severe hepatic toxicity, rare serious skin events such as Stevens–Johnson syndrome, and possible drug–drug interactions due to metabolism through the cytochrome P-450 system (Gupta and Cooper, 2008). Topical therapy is usually preferred due to less potential for serious adverse effects. But when response of topical therapy is poor or infection involve a large skin area, systemic therapy is recommended (Gupta *et al.*, 1998a). For e.g.: Tinea capitis, tinea barbae and tinea unguium usually require systemic therapy (Weinstein and Berman, 2002). A combination of topical and systemic antifungal treatments or systemic treatments can increase the efficacy by acting in synergy, preventing drug resistance, or offering a broader spectrum of action against mixed infections. Techniques such as physical enhancers and chemical enhancers have been used to improve drug delivery for topical antifungal by increase in absorption (Gupta and Paquet, 2013). The available topical therapy for dermatophyte infections include different drugs from different categories like allylamines (naftifine, terbinafine), benzylamine (butenafine), imidazoles (clotrimazole, econazole, ketoconazole, miconazole, oxiconazole, sulconazole) and others (ciclopirox, tolnaftate) mainly for tinea corporis, tinea pedis, tinea curis, tinea manuum and tinea faciei. They are available in different dosage forms such as powder, gel, cream, lotion, shampoo or solution (Gupta *et al.*, 1998a).

But many of these treatment are not effective due to resistance, long duration of treatment, high relapse rate due to rapid drug clearance from skin (Hainer, 2003). For e.g.: patients who were mycologically negative with complete clinical cure for 12 months after completion of systemic treatment with itraconazole or terbinafine and on continuous use of an antifungal lacquer on the toenails, after cure of onychomycosis is unable to prevent relapse (Piraccini *et al.*, 2010).

Therefore, there is a need for development of a new generation of potent broad spectrum antifungals, with selective action against new targets in the fungal cells and without side effect in the host for effective control of dermatophytes (Martinez-Rossi *et al.*, 2008).

2.3 Botanical aspect of *R. nasutus*

Rhinacanthus nasutus (family Acanthaceae) is widely distributed in India, China, Thailand, Taiwan and Southeast Asia (Kodama *et al.*, 1993; Sendl *et al.*, 1996; Siripong *et al.*, 2006a). *R. nasutus* is commonly known as white crane and locally as Thong-pan-chung in Thailand, Nagamali in India and Yuthikaparni in Sanskrit (Rajasekaran *et al.*, 2009).

A

B

C

Figure 2.1 Flowers (A), Leaves (B), and entire plant (C) of *Rhinacanthus nasutus*

The plant is a slender, erect, branched, somewhat hairy shrub 1-2 m in height. The leaves are oblong, 4-10 cm in length, and narrowed and pointed at both ends. The inflorescence is a spreading, leafy, hairy panicle with the flowers usually in clusters. The calyx is green, hairy, and about 5 mm long. The corolla-tube is greenish, slender, cylindric, and about 2 cm long. The flowers is 2-lipped; the upper lip is white, erect, oblong or lance like, 2-toothed at the apex, and about 3 mm in both length and width;

and the lower lip is broadly obovate, 1.1-1.3 cm in both measurements, 3-lobed, and white, with a few, minute, brownish dots near the base. The fruit (capsule) is club-shaped and contains 4 seeds (Bukke *et al.*, 2011).

2.4 Ecology and propagation of *R. nasutus*

It is a shade loving perennial shrub. It has been reported that the temperature of 25 - 28°C is optimum whereas high temperature of 35°C and above is not favourable for growth of the plant (Das, 2006).

There is vigorous growth of the plant during rainy season but in summer the aerial part mostly dries up, though the root portion remains intact. Also, water logging and water stagnation for a period of 1-2 days can cause damage to the plant (Shams-Ghahfarokhi *et al.*, 2006).

2.5 Chemical constituents of *R. nasutus*

This plant contains various compounds, such as naphthoquinone, lignans, anthraquinones, triterpenoids, flavonoids and sterols. The studies of chemical compounds isolated from different parts of *R. nasutus* have been previously reported in many literatures.

Rhinacanthin-C has been reported to be the major and most potent antifungal compound in *R. nasutus* (Puttarak *et al.*, 2010). It is an anionic hydrophobic naphthoquinone ester with log K about 1.73 ± 0.154 (Siripong *et al.*, 2006b; Charoonratana, 2007; Panichayupakaranant *et al.*, 2009; Wongwanakul *et al.*, 2013). It is an acidic but electrically neutral molecule with molecular weight 410.5027 g/mol, molecular formula $C_{25}H_{30}O_5$, AlogP of 5.7 and ACD acidic pKa of 4.89 (ChEBI, 2014; ChEMBL, 2014).

List of the compounds found in *R. nasutus* are given in Table 2.2

2.6 Quantitative analysis of rhinacanthin

Quantitative analysis is an important routine procedure for quality control and standardization of herbal medicines. Quantitative analysis of rhinacanthin has been reported to be determined on the basis of spectrophotometric and HPLC method.

For spectrophotometric method, samples were dissolved and diluted in methanol and mixed with 20% Potassium hydroxide solution which were allowed to stand for 15 min. Then absorbance was determined at 468 nm with methanol as blank. The total rhinacanthins of each sample was calculated as rhinacanthin-C using the calibration curve of the authentic rhinacanthin-C (Panichayupakaranant *et al.*, 2006).

Rhinacanthin-C content was determined by HPLC which was performed on CAPCELL PAK C18 UG80 column (4.6 × 250 mm) with acetonitrile : double distilled water : trifluoroacetic acid (80:20:0.1, v/v) as mobile phase, at a flow rate of 1.0 mL/min. The injection volume was 10-15 µL and the quantification wavelength was set at 254 nm (Gotoh *et al.*, 2004).

Panichayupakaranant *et al.* (2009) described reverse phase HPLC method for the simultaneous determination of rhinacanthin-C, -D and –N which was performed isocratically on a TSK-gel ODS-80 Ts column (5 µm, 4.6 × 150 mm) at 25°C with a mixture of methanol and 5% aqueous acetic acid (80:20, v/v) as the mobile phase at a flow rate of 1.0 mL/min. The injection volume was 20 µL and the quantification wavelength was set at 254 nm.

Spectrophotometric is simple, direct and cost effective for single component determination. However, HPLC is useful for multi-component or complex formulations and extremely low potency dosage forms with wide dynamic linear range, improved specificity via separation and increased sensitivity (Ahuja and Dong, 2005).

2.7 Stability of rhinacanthin-C

The stability of rhinacanthin-C in rhinacanthins-rich *R. nasutus* (RRn) extract, standardized to contain total rhinacanthins not less than 70% w/w, through a period of 4 months was found to be stable when stored in a well closed container protected from light either at 4 ± 2°C or 30 ± 2°C but at accelerated condition at 45 ± 2°C and 75% relative humidity, the rhinacanthin-C content decreased significantly after 8 weeks. Rhinacanthin-C in the extract was not stable when exposed to light and significantly decreased after 1 week. In all these conditions, the physical appearance of the extract remained unchanged. It was cautioned for preparation of rhinacanthin-C in an aqueous solution because rhinacanthin-C content in the aqueous methanolic solution of the RRn extract was found to be significantly decreased after 1 week at pH 5.5 and after 1 week at pH at 7.0 and pH 8.0. The instability was suggested due to hydrolysis (Puttarak *et al.*, 2010).

2.8 Distribution of rhinacanthins in *R. nasutus*

Leaves, stems and roots of *R. nasutus*, determined for total rhinacanthin content collected at a different period of times, showed rhinacanthins markedly accumulated in the roots and leaves, but less accumulated in the stems. The maximum total rhinacanthins was obtained from the leaves and roots of the plants when harvested in July, before flower blossom (Panichayupakaranant *et al.*, 2006). This result is harmonious with the recommended part use of *R. nasutus* in traditional and primary health care systems (Farnsworth and Bunyapraphatsara, 1992).

Table 2.2 Chemical constituents of *Rhinacanthus nasutus*

Group of compounds	Name	Plant parts	References
Naphthoquinones	Rhinacanthin-A	Roots	(Wu *et al.*, 1988; Wu *et al.*, 1998a; Wu *et al.*, 1998b)
	Rhinacanthin-B	Roots	(Wu *et al.*, 1988; Wu *et al.*, 1998a; Wu *et al.*, 1998b)
	Rhinacanthin-C	Whole plant	(Sendl *et al.*, 1996; Wu *et al.*, 1998a; Wu *et al.*, 1998b)
	Rhinacanthin-D	Whole plant	(Sendl *et al.*, 1996; Wu *et al.*, 1998a; Wu *et al.*, 1998b)
	Rhinacanthin-G	Roots	(Wu *et al.*, 1998a; Wu *et al.*, 1998b)
	Rhinacanthin-H	Roots	(Wu *et al.*, 1998a; Wu *et al.*, 1998b)
	Rhinacanthin-I	Leaves and roots	(Wu *et al.*, 1998a; Wu *et al.*, 1998b)
	Rhinacanthin-J	Leaves and roots	(Wu *et al.*, 1998a; Wu *et al.*, 1998b)
	Rhinacanthin-K	Roots	(Wu *et al.*, 1998a; Wu *et al.*, 1998b)
	Rhinacanthin-L	Roots	(Wu *et al.*, 1998a; Wu *et al.*, 1998b)
	Rhinacanthin-M	Roots	(Wu *et al.*, 1998a; Wu *et al.*, 1998b)
	Rhinacanthin-N	Leaves and roots	(Wu *et al.*, 1998a; Wu *et al.*, 1998b)
	Rhinacanthin-O	Roots	(Wu *et al.*, 1998a; Wu *et al.*, 1998b)
	Rhinacanthin-P	Roots	(Wu *et al.*, 1998a; Wu *et al.*, 1998b)
	Rhinacanthin-Q	Roots	(Wu *et al.*, 1998b)
	Rhinacanthone	Leaves and roots	(Kodama *et al.*, 1993; Kuwahara *et al.*, 1995)
	Dehydro-α-lapachone	Roots	(Wu *et al.*, 1998a; Wu *et al.*, 1998b)
Lignans	Rhinacanthin-E	Aerial parts	(Kernan *et al.*, 1997)
	Rhinacanthin-F	Aerial parts	(Kernan *et al.*, 1997)
Benzenoids	*p*-hydroxyl-benzaldehyde	Roots	(Wu *et al.*, 1998b)
	Vanillic acid	Leaves and stems	(Wu *et al.*, 1995)
	Syringic acid	Leaves and stems	(Wu *et al.*, 1995)

Group of compounds	Name	Plant parts	References
	2-methoxy-4-propionylphenol	Leaves and stems	(Wu *et al.*, 1995)
	Methyl valinate	Roots	(Wu *et al.*, 1995)
	Syringaldehyde	Roots	(Wu *et al.*, 1995)
Anthraquinone	12-methyl anthraquinone	Leaves and stems	(Wu *et al.*, 1995)
Triterpenoids	β-amyrin	Roots	(Wu *et al.*, 1995)
	Glutinol	Roots	(Wu *et al.*, 1995)
	Lupeol	Roots	(Wu *et al.*, 1988; Wu *et al.*, 1995; Wu *et al.*, 1998b)
Flavonoids	Wogonin	Roots	(Wu *et al.*, 1998b)
	Oroxylin A	Roots	(Wu *et al.*, 1998b)
	Rutin	Flowers	(Subramanian and Nagarajan, 1981)
Sterols	Stigmasterol	Roots	(Wu *et al.*, 1988)
	β-sitosterol	Roots	(Wu *et al.*, 1988)
Chlorophyll	Methylpheophorbide-A	Leaves and stems	(Wu *et al.*, 1995)
Coumarins	(+)-praeruptorin	Roots	(Wu *et al.*, 1998b)
	Umbelliferone	Leaves and stems	(Wu *et al.*, 1995)
Amide	Allantoin	Roots	(Wu *et al.*, 1998b)
Carbohydrate	Methyl-α-D-galactopyranoside	Leaves and stems	(Wu *et al.*, 1995)
Quinol	4-acetonyl-3,5-dimethoxy-*p*-quinol	Leaves and stems	(Wu *et al.*, 1995)
Benzoquinone	2,6-dimethoxy benzoquinone	Leaves and stems	(Wu *et al.*, 1995)
Glycosides	Sitosterol-β-D-glucopyranoside	Leaves and stems	(Wu *et al.*, 1995)
	Stigmasterol-β-D-glucopyranoside	Leaves and stems	(Wu *et al.*, 1995)
	3,4-dimethylphenol-β-glucopyranoside	Leaves and stems	(Wu *et al.*, 1995)
	3,4,5-trimethylphenol-β-D-glucopyranoside	Leaves and stems	(Wu *et al.*, 1995)

2.9 Pharmacological and ethnomedical uses of *R. nasutus*

The pharmacological and ethnomedical uses of *R. nasutus* have been previously reported in many literatures as listed in Table 2.3.

Table 2.3 The pharmacological and ethnomedical uses of *R. nasutus* (Siriwatanametanon *et al.*, 2010)

Parts used/extracts	Active compounds	Traditional uses/Ethnobotanical data	Biological activities	References
Fresh LV mixed with alcohol	N/A	An alcoholic solution was reported to be an excellent treatment for various skin conditions such as ringworm, severe eczema and *Tinea* infections (Thailand)	N/A	Saralamp et al. (2000), Suchawan (1989)
LV-EX	N/A	N/A	Antifungal activity against some dermatophytes; *Trichophyton* spp., and *Microsporum canis*	Darah and Jain (2001)
LV, EtOH-EX	N/A	In teas for treating colds, fever, refreshes the lungs. Relieves early stage of TB, headache from hypertension, reduces blood pressure, sore throat, constipation (Thailand)	Moderate antimicrobial activities against *Bacillus subtilis*, *Staphylococcus aureus* K147 methicillin-sensitive, *Escherichia coli* (wild), *Pseudomonas aeruginosa* 187 (wild)	Cheeptham and Towers (2002)
LV, EtAc-EX	Rhinacanthins-C, -D and -N	N/A	Potent antiallergic activity by inhibiting TNF-α and IL-4 gene expression in antigen-induced TNF-α and IL-4 releases on from RBL-2H3 cells	Tewtrakul et al. (2009a)

Parts used/extracts	Active compounds	Traditional uses/Ethnobotanical data	Biological activities	References
LV, EtAc-EX	Rhinacanthins-C, -D and -N	N/A	Anti-inflammatory activity against LPS-induced release of nitric oxide, PGE2 and TNF-α from RAW264.7 cells by inhibiting iNOS and COX-2 gene expressions	Tewtrakul et al. (2009b)
ST and LV, MeOH-EX	Naphthopyran derivatives, naphthoquinone derivatives		Antifungal activity against *Pyricularia oryzae*, the pathogen of rice blast disease	Kodama et al. (1993); Awai et al. (1995)
ST and LV, water and EtOH-Exs	N/A	The plant has been used in the treatment of mental disorders, inflammation, rheumatism, circulatory problems, asthma and bronchitis, epilepsy and immune system deficiencies	Modest increase in TNF-α expression but did not change iNOS	Punturee et al. (2004)
ST and LV, water and EtOH-Exs	N/A		Immunomodulatory activity	Punturee et al. (2005)
Aqueous EX of LV and EtOH-EX of RT	Rhinacanthin-C	In the treatment of hepatitis, diabetes, hypertension (South China and India) and skin diseases (Taiwan)	Antiproliferative activity	Gotoh et al. (2004)
LV and RT, soak in vinegar or alcohol, pounded with lemon or tamarind, or made into decoction	N/A	Treatment of skin disorders such as ringworm, eczema, scurf, skin infection e.g. herpes, antipyretic, anti-inflammatory and detoxicant. It is used against hypertension (Vietnam) and against cancers (Thailand)	N/A	de Padua et al. (1999), Farnsworth and Bunyapraphatsara (1992)
AP, EXed with 1:1 CH2Cl2-2-propanol	Rhinacanthin-C, rhinacanthin-D	RT and LV, pounded with vinegar or alcohol applied to herpes infections or other skin eruptions	Antiviral activity against cytomegalovirus (CMV)	Sendl et al. (1996)
AP	Rhinacanthins-E and -F	N/A	Antiviral activity against influenza type A	Kernan et al. (1997)

Parts used/extracts	Active compounds	Traditional uses/Ethnobotanical data	Biological activities	References
AP, hot percolation using PE	Rhinacanthone (3,4-dihydro-3,3-dimethyl-2H-naphtho-[1,2-B] pyran-5,6-dione)	N/A	Antitumour activity against Dalton's ascetic lymphoma in Swiss albino mice	Thirumurugan et al. (2000)
AP, MeOH-EX	N/A	N/A	Hepatoprotective effect from paracetamol induced-liver damage in rats	Suja et al. (2004)
AP, EtOH-EX	N/A	N/A	Analgesic activity in the acetic acid induced-writhing test	Karunambigai et al. (2005)
RT, MeOH-EX	Rhinacanthin derivatives	N/A	Antiplatelet aggregation and cytotoxicity activities in the P-388, A-549, HT-29 and HL-60 test systems	Wu et al. (1998)
RT, MeOH-EX	Rhinacanthin-N, rhinacanthin-Q, naphthoquinone esters	N/A	Cytotoxicities against human carcinoma cell lines (epidermoid carcinoma, HeLa, and HepG2) and vero cell line (African green monkey kidney cell)	Kongkathip et al. (2004)
RT, MeOH-EX	Rhinacanthins-C, -N and -Q	Used against cancers (Thailand)	Induction of apoptosis in human cervical carcinoma (HeLaS3) cells by associating with the activation of caspase-3 pathway	Siripong et al. (2006)

Abbreviations: AP – aerial parts, FL – flowers, LV – leaves, RB – root bark, RT – roots, SB – stem bark, ST – stem,WP – whole plant, EtAc – ethyl acetate, EtOH – ethanol, EX – extract/extraction, MeOH – methanol

2.10 Biological activity of *R. nasutus* and rhinacanthins

2.10.1 Antifungal activity

A report suggested that the napthopyran derivative from the leaves and stems of *R. nasutus* showed strong antifungal activity against *Pyricularia oryzae*, the pathogen of rice blast disease (Kodama *et al.*, 1993).

R. nasutus leaf extract showed antifungal against various species of dermatophytes *(Trichophyton mentagrophytes var. mentagrophytes, T. mentagrophytes var. interdigitale, T. rubrum, Microsporum canis and M. gypseum)* with minimum inhibitory concentration (MIC) reported at 13.6 mg/mL. The extract showed 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). The results suggested that the extract acted on the cell wall of the dermatophytes which subsequently leading to the formation of cytopathological and membrane structural degeneration and finally leading to cell lysis and death (Darah and Jain, 2001).

75% aqueous ethanolic *R. nasutus* leaves extract showed antifungal activity against *Candida tropicalis and C. parapsilosis* and showed a potent dose dependent activity against *C. albicans* and *T. mentagagrophytes*. The most potent dose of 5 mg/mL of the extract inhibited fungal growth of all the fungi tested (Sattar *et al.*, 2004).

Ethyl acetate and chloroform extract from *R. nasutus* leaves in dose of 20 mg/mL showed significant activity against *C. albicans* compared to ketoconazole (10 µg/disc) by disc diffusion method (Rajasekaran *et al.*, 2009).

According to a report, rhinacanthin-rich extract (HRn) that was standardized to contain not less than 70% rhinacanthins-C, -D and -N showed antifungal activity against *Trichophyton rubrum, T. mentagagrophytes, Microsporum gypeseum*. The MIC of HRn, rhinacanthin-C, -D and -N against *T. rubrum* were 7.5, 15.1, 31.2 and 62.5 µg/mL, respectively; MIC against *T. mentagagrophytes* were 31.2, 31.2, 31.2 and 250 µg/mL, respectively; and MIC against *M. gypeseum* were 125, 125, 125 and 250 µg/mL, respectively. HRn possessed better antifungal activity than the ethyl acetate extract and the activity was equal to that of rhinacanthin-C, the most potent

antifungal constituent of *R. nasutus* leaf extract which is suggested due to a synergistic effect of all the rhinacanthins (Panichayupakaranant *et al.*, 2009).

Formulations of nanoliposome containing rhinacanthin-C prepared by using modified ethanol injection method showed better antifungal activity when compared with pure rhinacanthin-C and ketoconazole after 4 and 7 days of incubation (MIC = 1.87 µg/mL both against *T. rubrum* and *Microsporum gypseum*) (Paosupap, 2011).

2.10.2 Antiviral activity

Rhinacanthin-C and rhinacanthin-D isolated from *R. nasutus* exhibited inhibitory activity against cytomegalovirus (CMV) with EC_{50} values of 0.02 and 0.22 µg/mL respectively (Sendl *et al.*, 1996).

Kernan *et al.* (1997), isolated and identified two lignans, rhinacanthin-E and rhinacanthin-F from the aerial parts of *R. nasutus* which showed significant antiviral activity against influenza virus type A with EC_{50} values of 7.4 and 3.1 µg/mL respectively.

Ethanolic extract of *R. nasutus* with non-toxic concentration tested on Vero cell showed the highest activity on HSV (Herpes simplex viruses) replication and also the highest anti-HSV-1 activity when treated after viral attachment to the cells by plaque reduction assay. Both aqueous and ethanolic extracts showed significant inhibition of HSV-1 and HSV-2 viral particles (Chaliewchalad *et al.*, 2013).

2.10.3 Antibacterial activity

Antibacterial activity evaluated by agar dilution method of 15 extracts from roots, leaves and stems of *R. nasutus* against four control isolates and 68 clinically isolated bacteria from Thai cancer patients showed that *n*-hexane and chloroform extracts of roots and *n*-hexane extract of leaves showed potent activity against Gram positive bacteria. The naphthoquinone esters rhinacanthin-C, -N and -Q isolated from the extract also exhibited potent bacteriostatic antibacterial activity against β-hemolytic streptococci, enterococci and staphylococci with potencies comparable to Gentamicin (Siripong *et al.*, 2006a).

Chloroform and ethyl acetate leaves extract of *R. nasutus* showed activity against *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumonia* (Rajasekaran *et al.*, 2009; Rao *et al.*, 2010).

Rhinacanthins-rich *R. nasutus* (RRn) extract exhibited potent bactericidal activity against *Streptococcus mutans* and potent bacteriostatic activity against *S. epidermidis*, *Propinibacterium acnes* and *Staphylococcus aureus.* RRn extract showed antibacterial activity almost equal to rhinacanthin-C which was reported may be due to synergistic effect of rhinacanthins (Puttarak *et al.*, 2010).

Anti-bacterial activity of different extracts of stem and leaves segments derived calli, aerial portions of mother plants and calli mediated shootlets were investigated against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Bacillus subtilis* and *Escherichia coli* (Antonisamy *et al.*, 2012).

2.10.4 Antiallergic activity

R. nasutus ethanol and water extracts showed significant increased proliferation and production of interleukin (IL)-2 and tumour necrosis factor (TNF)-α in human peripheral blood mononuclear cells (Punturee *et al.*, 2005).

Rhinacanthin-C, -D and -N possessed very potent anti-allergic activity against antigen induced β-hexosaminidase release as a marker of degranulation in RBL-2H3 cells (with IC_{50} of 6.9, 8.9 and 6.4 μM, respectively), effect on antigen induced TNF-α release (with IC_{50} of 0.7, 3.8 and 10.4 μM, respectively) and effect on antigen induced IL-4 release (with IC_{50} of 7.0, 5.4 and 12.0 μM, respectively) (Tewtrakul *et al.*, 2009a).

2.10.5 Anti-inflammatory activity

Rhinacanthin-C (IC_{50} of 1.8 μM), -D and -N isolated from ethyl acetate extract of the leaves of *R. nasutus* possessed very potent anti-inflammatory activity against lipopolysaccharide (LPS)-induced nitric oxide release. Rhinacanthin-C (IC_{50} of 10.4 μM) exhibited the most potent effect on LPS-induced prostaglandin E_2 release followed by rhinacanthin-D and rhinacanthin-N but they were inactive for tumor necrosis factor. The mechanisms in transcriptional level of rhinacanthin-C were found

to be inhibition of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) gene expression (Tewtrakul *et al.*, 2009b).

Standardized *R. nasutus* leaf extract showed similar anti-inflammatory activity as rhinacanthin-C at doses of 80, 160 and 320 mg/kg tested in male Wistar rats which were suggested due to synergistic activity of other rhinacanthins. Both showed dose dependent inhibition of edema formation with comparable inhibition as indomethacin (5 mg/kg) at dose of 320 mg/kg in carrageenan induced paw edema indicating inhibition of early inflammatory mediators. Both showed suppression of granuloma formation with a little lower inhibition than indomethacin (5 mg/kg) at dose of 80 mg/ kg in the cotton pellet induced granuloma in rats indicating suppression of the proliferative phase of inflammatory response (Bhusal *et al.*, 2014).

2.10.6 Analgesic activity

Standardized *R. nasutus* leaf extract and rhinacanthin-C were evaluated for analgesic activity at doses of 20, 40 and 80 mg/kg in male ICR mice. Both showed dose dependent inhibition with comparable inhibition as indomethacin (5 mg/kg) at dose of 80 mg/kg in acetic acid induced writhing indicating their antinociceptive activity. In formalin test, 80 mg/kg showed inhibition in early as well as late phase but 40 mg/kg showed inhibition only in late phase indicating dose dependent central effect. However, both were non effective in hot plate test (Bhusal *et al.*, 2014).

2.10.7 Cytotoxic and antitumor activity

The ethanol extract of root, aqueous extract of leaves of *R. nasutus* and chemically synthesized rhinacanthin-C were assed for *in vitro* antiproliferative activity against different cell lines. The *in vitro* antiproliferative activity of the ethanol extract of root of *R. nasutus* against different cell lines was suggested due to rhinacanthin-C, which was comparable with or slightly weaker than of 5-fluorouracil. The *in vivo* activity in sarcoma 180 bearing mice was seen after oral administration once daily at a dose of 500 mg/kg/d for 14 days which showed that the ethanol extract of root and aqueous extract of leaves with inhibition of 52.5% and 44.2% respectively (Gotoh *et al.*, 2004).

To solve the limited solubility of rhinacanthin-C, -N and -Q in aqueous medium, liposomal formulation of each were developed which showed strong antiproliferative activity against HeLaS3 cells with IC_{50} values of 32, 17, 70 µM; 19, 17, 52 µM and 2.7, 2.0 and 5.0 µM for the exposure time of 24, 48 and 72 h, respectively. Tumour growth was supressed in Meth-A sarcoma bearing BALB/c mice at dose of 5.0 mg/kg/d for 10 days with most significant activity by rhinacanthin-N (Siripong *et al.*, 2006b).

Aqueous extracts of both roots and stems of *R. nasutus* have been used traditionally for various cancers effectively. Siripong *et al.* (2008) demonstrated that the aqueous extracts of roots and stems, chloroform extract and rhinacanthin-C isolated from roots of the plant markedly suppressed tumour growth, number of tumour per rat and tumour incidence of the mammary gland carcinogenesis in the experiments rats induced by 7, 12-dimethylbenz[a]anthracene (DMBA).

Rhinacanthone inhibited proliferation of Human cervix cancer (HeLa) cells in a dose dependent manner and showed greater efficacy than β-lapachone (an anticancer drug with chemical structure related to rhinacanthone, used as positive control) with IC_{50} of 1.2 ± 0.1 to 5.5 ± 0.85 µM for 2-24 h exposure periods. It also displayed several apoptotic features, marked increase in the level of pro-apoptotic protein Bax and increased expression of apoptosis-inducing factor. The activity was demonstrated to be mediated primarily through the mitochondria dependent signalling pathway (Siripong *et al.*, 2009).

Water extract of roots of *R. nasutus* examined for chemopreventive potential against colonic neoplasm induced by azoxymethane combined with dextran sodium sulphate was given daily intragastrically to mice caused increase incidence and multiplicity of colonic tumours in mice fed at 100 and 500 mg/kg body weight, both in initiation and promotion phases. This suggests that *R. nasutus* extract may act as a tumour promoter or anticarcinogen depending upon the test species, initiating agent and exposure protocol (Kupradinun *et al.*, 2009).

R. nasutus ethyl acetate extract showed cytotoxicity against (HeLa) cells and the methanol extract showed specific cytotoxicity against the multidrug-resistant CEM/ADR5000 cells (Siriwatanametanon *et al.*, 2010).

2.10.8 Antioxidant activity

The methanolic extract of the aerial parts of *R. nasutus* significantly scavenged free radicals like superoxides, hydroxyl and lipid peroxides from the *in vitro* system (Suja *et al.*, 2004).

The methanolic extract of *R. nasutus* showed significant dose dependent DPPH (1, 1-diphenyl-2-picryl-hydrazyl) radical scavenging activity compared to standard antioxidant with IC_{50} of 34.4 µg/mL (Rao and Naidu, 2010b).

The ethanol extract of *R. nasutus* leaf and root showed antioxidant activity by free radical scavenging assay which confirmed the attenuation of the neuron cell death caused by glutamate and amyloid-β treatment in the mouse hippocampal cell line, HT-22 (Brimson *et al.*, 2012).

2.10.9 Anti-diabetic activity

200 mg/kg of *R. nasutus* leaf methanolic extract after oral doses administration over a period of 4 weeks significantly reduced blood glucose levels in Streptozotocin induced diabetic rats by 25-68% in a dose dependent manner with maximum effect after 15 days and which remained constant in third and fourth week (Rao and Naidu, 2010a).

2.10.10 Hypolipidemic activity

The methanolic extract of *R. nasutus* (200 mg/kg body weight) administered to streptozotocin induced diabetic male albino Wistar rats for 30 days, showed hypolipidemic activity by restoration of serum and hepatic cholesterol levels. The activity was suggested to be due to presence of glycosides in the extract (Rao *et al.*, 2011).

2.10.11 Hepatoprotective activity

The methanolic extract of the aerial parts of *R. nasutus* significantly showed hepatoprotective effect from paracetamol induced liver damage as evidenced from decreased level of serum enzymes and an almost normal architecture of the liver of *R. nasutus* treated rats (Suja *et al.*, 2004).

2.11 Standardization of herbal medicine

A herbal medicine is a plant or part of a plant or unpurified plant extracts containing variety of naturally occurring biochemical, usually working synergistically which is valued for its medicinal or therapeutic value (Kunle *et al.*, 2012).It is estimated by World Health Organization (WHO), that about 80% of the world population still uses herbs and other traditional medicines for fulfilling the primary health care needs (Chawla *et al.*, 2013). Herbal medicines are becoming globally popular due to accessibility, affordability, and because of high pharmacological activity with low toxicity for improving human health as well as prevention and cure of diseases (WHO, 2002; Bansal *et al.*, 2014). Herbal medicines usually do not possess an immediate or strong pharmacological action so they are not used for emergency treatment but are frequently used to treat chronic diseases (Calixto, 2000).

Herbal medicine contains many compounds which even in low concentrations may be important for quality, safety and efficacy (Bansal *et al.*, 2014). But various factors such as genetic, cultural, environmental (temperature, light exposure, water availability, nutrients), harvesting (age and part of the plant, method and time of collection), batch to batch variations, processing (size reduction, drying), storage, transportation, extraction methods significantly affect the herbal drugs. These factors affect the bio-efficacy, reproducible therapeutic effect, standardization of herbal drug and restrict its commercial utility (Calixto, 2000; Kunle *et al.*, 2012; Chawla *et al.*, 2013). For e.g., the results of clinical trials for Ginger (*Zingiber officinale*), which is widely used for treating pregnancy related nausea and vomiting, has been contradictory (Panichayupakaranant, 2011). Only few well controlled placebo controlled trials have been reported to prove the safety and efficacy which may be due to lack of standardization and quality control of the herbal drugs in clinical trials (Calixto, 2000). Moreover, herbal preparations are usually considered as safe but some biologically active substances could be toxic with undesirable side effects. For e.g., most cytotoxic anticancer plant derived drugs, digitalis, the pyrrolizidine alkaloids, ephedrine, phorbol esters, etc. (Calixto, 2000; Kunle *et al.*, 2012). The problem in herbal medicine is mainly due to misidentification, lack of standardization, contamination, substitution, deliberate adulteration, incorrect preparation and/or

dosage and inappropriate or incomplete labelling and/or advertising (Panichayupakaranant, 2011). A high quality herbal medicine can be obtained only by ensuring that correct plant material, or mixture of plant materials, or their extracts, exactly as stated on the product label, free from contamination with other undeclared plant materials, and free from pesticides, radioactive particles, heavy metals, microbes and mycotoxins (WHO, 2007b). Therefore, along with the quality control during plant selection, harvesting, processing, extraction and concentration, standardization of herbal drug to ensure its safety, efficacy and potency for human use is prerequisite.

Standardization is defined as the establishment of reproducible pharmaceutical quality by adjusting the herbal drug preparation to a defined content of a constituent or group of substances with known therapeutic activity which assures quality, safety and efficacy (Mosihuzzaman and Choudhary, 2008; Panichayupakaranant, 2011; Chawla *et al.*, 2013). It is also stated that if constituents with known therapeutic activities are known, the active ingredients should be standardized to contain a defined amount of this/these constituents(s) (WHO, 2007a).

One of the approaches is to ascertain specified amount of active ingredients as a pure compound. For e.g.: 30-80% of silymarin or silybin in Milk Thistle (*Silybum mariannnum*), 30-70% of kavalctones in Kava (*Piper methysticum*), curcumin, capsaicin, asiaticoside, etc. (Panichayupakaranant, 2011; Chawla *et al.*, 2013).

Some herbal medicines are greatly concentrated in order to improve their therapeutic efficacy (Calixto, 2000). So, the second approach is to concentrate and ascertain one or more constituents as active constituent-enriched extracts during which there may be displacement and lack of other constituents and may be even more effective than the originally presumed active constituents. For e.g., the antifungal activity of rhinacanthin-rich *R. nasutus* extract against *Trichophyton rubrum* (MIC 7.5 µg/mL) is higher than its major and most potent active constituent, rhinacanthin-C (MIC 15.1 µg/mL) (Panichayupakaranant *et al.*, 2009; Panichayupakaranant, 2011). Also, standardized powder extract of Ginkgo biloba (50:1) which means that 50 parts of raw material were processed to yield 1 part of standardized powder extract is an example. During such process some secondary metabolites which may produce undesirable side effects may also be removed (Calixto, 2000).

In many cases, the active constituents responsible for pharmacological action are unknown (Calixto, 2000). So, the next approach uses marker compounds for standardization which are biochemical constituents characteristic of the plant but not indicators of therapeutic activity (For e.g.: echinoside of Echinacea angustifolia or parthenolide for Feverfee (Panichayupakaranant, 2011; Kunle *et al.*, 2012; Chawla *et al.*, 2013).

However, it is difficult to ensure constituents in the final product for safety and efficacy, due to the complex nature, inherent variability of the constituents of plant based drugs and because many of them are unknown or only partly explained (Kunle *et al.*, 2012).The expenditure for quality control, standardization and clinical testing is quite expensive but the scientific approach will increase acceptance and confidence in the marketed product by the public and clinicians (Panichayupakaranant, 2011). The recent advances in purification, isolation and structure elucidation of natural products have created a scope to establish appropriate strategies for the analysis of quality and standardization of herbal preparations (Calixto, 2000). The major challenge is to formulate a simple, affordable and reliable standardization method or protocol which can ensure scientific and clinical validation of botanicals and herbal preparations (Mosihuzzaman and Choudhary, 2008). Moreover, the regulatory mechanism, pharmacopeia and monographs and their harmonization must be established in order to define basic criteria for evaluating the quality, safety and efficacy of herbal medicines.

2.12 Green extraction

The extraction of natural products by traditional extraction processes such as maceration, steam or hydro distillation, pressing, decoction, infusion, percolation and Soxhlet extraction causes loss of time, energy and large amount of organic solvents with very low yield (Lu *et al.*, 2011).

The extraction of natural products usually uses organic solvents which are problematic because they are flammable, volatile and toxic and cause environment pollution and greenhouse effect (Zhou *et al.*, 2011). Moreover, there are regulations to show the absence of risk during extraction.

Therefore, 6 principles of green extraction for discovery and design of extraction processes which will reduce energy consumption, allow use of alternative solvents and renewable natural products, and ensure a safe and high quality extract/ product has been reported as mentioned below (Chemat *et al.*, 2012).

Principle 1: Innovation by selection of varieties and use of renewable plant resources.

Principle 2: Use of alternative solvents and principally water or agro-solvents.

Principle 3: Reduce energy consumption by energy recovery and using innovative technologies.

Principle 4: Production of co-products instead of waste to include the bio- and agro refining industry.

Principle 5: Reduce unit operations and favour safe, robust and controlled processes.

Principle 6: Aim for a non-denatured and biodegradable extract without contaminants.

Industries are looking for green and efficient extraction processes that are safe, economic, and environmental friendly and free from toxic solvents (Michel *et al.*, 2011; Chemat *et al.*, 2012). Ionic liquids such as [bmim]Br solution consisting of ions which are liquids at ambient temperature, non-flammable, negligible vapour pressure, high thermal stability, low chemical reactivity and with a wide range of miscibility have been used as green solvents to replace conventional volatile organic solvents (Lu *et al.*, 2011; Zhang *et al.*, 2014). Solvents such as 2-methyltetrahydrofuran (2-MeTHF) and cyclopentyl methyl ether (CPME) which can minimize solvent waste and improve laboratory safety are mentioned as environmental friendly green solvents (LLC, 2014). The non-ionic surfactant (Triton X-100) was reported to be used as alternative solvent enhancing extraction of ginsenosides from ginseng roots in pressurized liquid extraction (Choi *et al.*, 2003). Ethanol, propylene glycol and glycerol which are generally used as the ingredients of a topical solution can be potential green solvents. Ethanol is the most common bio-solvent which is flammable and potentially explosive but generally used as it is easily available in high purity, low price and completely biodegradable. Combination of 50% v/v ethanol in water has been reported as green solvent (Desgrouas *et al.*, 2014). Moreover, water has also

been used as green solvent with optimal extraction for polyphenols compared to organic solvents and, for different classes of chemical compounds like flavonoid, organic acid and alkaloid (Tan *et al.*, 2011; Vuong *et al.*, 2013). Dielectric constant (polarity) of water can be manipulated by application of temperature making it suitable for extraction of polar, moderately polar and non-polar compounds (Mustafa and Turner, 2011). Major drawback of traditional extraction methods is the subsequent concentration and clean-up of the extracts (Mustafa and Turner, 2011). But the uses of green solvents reduce unit operation to eliminate residual traces of undesirable solvents which leads to reduction in cost, time and energy. Moreover, solvent free extraction has been reported for extraction of essential oil from fresh or moistened dried plant materials (Li *et al.*, 2012). Besides extraction efficiency, economic, safety and sustainability aspects for selection of solvent should also be considered (Mustafa and Turner, 2011).

Various modern techniques like microwave, ultrasound, supercritical fluid extraction and pressurized liquid extraction have been used as green extraction techniques which considerably reduce extraction time and energy. Recently patent has been filed for claiming high extraction efficiency, simple operation steps, environment friendly and industrial scale method of supercritical extraction of rhinacanthin form *R. nasutus* with liquid CO_2 which is dissolved in ethanol, separated by alumina resin column followed by extraction and adding recovering reagent to obtain rhinacanthin (Dongfeng, 2012). Other examples include, MAE as green extraction method, PEG and ionic aqueous solution as green solvent for extraction of different compounds like flavone, coumarin, polyphenolic compounds and essential oil from medicinal plants (Du *et al.*, 2009; Zhou *et al.*, 2011; Li *et al.*, 2012), UAE as green extraction method, sunflower oil as green solvent for carotenoids (Li *et al.*, 2013), supercritical fluid extraction for D-limonene and rosemary oleoresin using CO_2 as green solvent (Ibáñez *et al.*, 1999; McKenzie *et al.*, 2004), pressurized liquid extraction for phenolic compounds, lignans, carotenoids, oils, lipids, essential oils and other nutraceuticals from foods and herbal plants (Mustafa and Turner, 2011).

Thus, green extraction has a great prospect in extraction of secondary active metabolites from plant.

2.13 Microwave assisted extraction (MAE)

MAE is an advanced extraction system employing microwave energy for various active compounds from plant materials (Chan *et al.*, 2015). The accelerated extraction of secondary metabolites by MAE is due to the interaction of polar components with microwave, causing heating by ionic conduction and dipole rotation. MAE usually performed from few minutes up to half an hour (Chan *et al.*, 2011). The resistance of the solution to the flow of ions results in friction and therefore, heating of the solution (Mandal *et al.*, 2009). Heating rate has an important role in extraction efficiency which is determined by two parameters: i) Dielectric constant which defines the polarizability of the molecule to an electric field and it is a measure of the ability of a material to store electromagnetic radiation; ii) Dielectric loss factor which is the measure of the efficiency in which the absorbed microwave energy can be converted into heat, inside a material when an electric field is applied. From these two parameters, dissipation factor is defined. The efficiency of the microwave heating depends on the dissipation factor of the material, which measures the ability of the sample to absorb microwave energy and dissipate that energy in the form of heat (Hemwimon *et al.*, 2007). The increase in content might be due to destruction of plant matrix, as evident by Scanning Electron Micrographs, by microwave energy generating molecular movement and heating causing the analyte to diffuse out and dissolve in the solvent (Chan *et al.*, 2011; Tatke and Jaiswal, 2011).

The major components of microwave assembly includes: i) Magnetron, which is the microwave generator; ii) A wave guide, which direct the propagation of microwave from the source to the microwave cavity; iii) Applicator, where the sample holder along with the sample is placed; iv) Circulator which regulated the movement of microwaves only in forward direction (Tatke and Jaiswal, 2011).

MAE is generally classified as closed system and open system. In closed system, the extractions are carried out in a sealed vessel with high pressure, controlled below the working pressure of the vessel, and high temperature, regulated above the normal boiling point of extraction solvent. High pressure and temperature allows fast and efficient extraction with different mode of microwave radiations. In open system, the extractions are carried out at more mild conditions (under atmospheric conditions)

with higher sample throughput and more solvent can be added to the system. It is more suitable in terms of safety and for thermo-labile compounds (Chan *et al.*, 2011).

The mode of operation determines the performance of MAE which includes constant power heating, intermittent power heating and constant temperature heating. Microwave in constant power MAE is provided with heating at specific power to extraction system and suitable to extract thermally stable active compounds, in intermittent power MAE is provided for pulsed heating at certain power and suitable for thermo-labile compounds while in constant temperature MAE with controlled extraction temperature and suitable for highly degradable active compounds (Chan *et al.*, 2015).

More efficient MAE techniques have been developed to overcome poor extraction yield due to thermal degradation and oxidation which includes nitrogen protected, vacuum, ultrasonic, dynamic and solvent free MAE (Chan *et al.*, 2011). The various factors affecting MAE are properties of solvent, solvent to feed ratio, time of extraction, cycle of extraction, microwave power, extraction temperature and characteristics of matrix. Optimization strategies have to be applied to obtain efficient working condition (Chan *et al.*, 2011; Tatke and Jaiswal, 2011).

MAE has been reported to be the simplest, rapid, economical and effective technique compared to traditional extraction techniques such as Soxhlet extraction, heat reflux extraction, ultrasonic assisted extraction and maceration (Sun and Liu, 2008; Mandal *et al.*, 2009; Chan *et al.*, 2011). Some of the drawbacks related with MAE are requirements of additional clean up step to remove solvent from sample matrices, low selectivity and the restriction to polar solvent. When use of non-polar solvents cannot be avoided, the problem with non-polar solvents can be overcome by adding modifiers into them to enhance the microwave absorbing capacity of the solvent (Chan *et al.*, 2011).

CHAPTER 3

MATERIALS AND METHODS

3.1 Plant material

The fresh aerial parts of *R. nasutus* were collected from the botanical gardens of Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Thailand during June, 2014. A voucher specimen (Voucher no. 001 18 14) was identified by Associate Professor Pharkphoom Panichayupakaranant, and has been deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. They were washed with tap water and the leaves were separated by hand picking. The leaves were dried at 50°C for 24 h in a hot air oven, and then ground to fine powder using a grinder, and passed through the sieve number 45. The dried leaf powders were kept in a well-closed container protected from light.

3.2 Chemicals and microorganism

3.2.1 For extraction

- Ethanol, commercial grade (High Science Ltd. Partnership, Thailand)
- Propylene glycol B.P. (Vidhyasom Co. Ltd., Thailand)
- Glycerol U.S.P. (Vidhyasom Co. Ltd., Thailand)

3.2.2 For formulation of topical solution containing rhinacanthin-C enriched extract

- Thymol (Srichand United Dispensary Co. Ltd., Thailand)
- Menthol (Vidhyasom Co. Ltd., Thailand)
- Fragrance (Daily Defence) (PC Drug Center Co. Ltd., Thailand)

3.2.3 For quantitative determination of rhinacanthin-C in the extract

- Acetic acid, glacial, HPLC grade (RCI Labscan Ltd., Thailand)
- Methanol, HPLC grade (RCI Labscan Ltd., Thailand)
- Water purified in Direct-Q® 5UV-R & Direct-Q UV-R water - purification system, (Merck KGA)
- Standard rhinacanthin-C was previously purified from the crude ethyl acetate extract of *R. nasutus* leaves (Panichayupakaranant *et al.*, 2009).

3.2.4 For antifungal activity assay

- Agar granulated (Becton, Dikinson and Co., France)
- Sabouraud Dextrose Broth (Becton, Dikinson and Co., France)
- Sodium chloride, 99% (RCI Labscan Ltd., Thailand)
- Candix®, 1% Clotrimazole solution (Public Pharmaceutical Lab. Co. Ltd., Thailand)
- *T. rubrum* were provided from DMST culture collection, National Institute of Health, Department of Medical Science, Thailand

3.3 Instrumentations

Instrument	Model	Company
Hot air oven	-	-
Grinder	Blender 8011BU HGB2WT	High Science Ltd. Part, USA
Balance (4 digit)	AL 204	Mettler Toledo, Switzerland
Shaker	3020	GFL
Water bath	LWB-222 A	Daihan Lab Tech., Korea
Fume hood	Flexlab®	Official Equipment Mfg. Co. Ltd.
Microwave	MS2127CW	LG Electronics Inc., Thailand
Vacuum pump	WJ-20	Sibata, Japan
pH meter	Five Easy Plus FEP20	Mettler Toledo, Switzerland
Balance (5 digits)	ABS135-S	Mettler Toledo, Switzerland
HPLC	Agilent 1100 series	Agilent, USA
HPLC column	TSK-gel ODS-80 Ts	Tosho Bioscience, Japan
0.45 microporous membrane	Nylon	Agela Technology
Measuring cylinder 1000 mL	Witeg Germany diffico	Duran, Germany
Autoclave	HICLAVE™ HVE-50	Hirayama, Japan
Refrigerator	NR-A18N1	Panasonic
Spectrophotometer	Genesys	Thermo Fisher Scientific, USA
Cuvette	-	-
Laminar air flow cabinet	ISOCIDE™ 5237	ESCO
Cotton swabs	-	-
96 well plate	-	Thermo Fisher Scientific, Denmark
200 µL tip	HCE-1111 Tip	Hyscon plastic
1000 µL tip	-	Ligand Scientific Co. Ltd, Thailand
Micropipette 20, 200, 1000 µL	Pipetman	Gilson, France
Incubator	-	Memmert, Germany
Vernier calliper	-	-

3.4 Determination of suitable solvent and extraction method for rhinacanthin-C

The dried powders of *R. nasutus* leaves were separately extracted using maceration and heat assisted extraction using a single solvent including ethanol, propylene glycol and glycerol.

Maceration

The dried powders of *R. nasutus* leaves (1.0 g) were separately extracted with ethanol, propylene glycol and glycerol (20 mL) in 125 mL flask at the room temperature on a shaker at 125 rpm for 24 h. The extracts were filtered and volumes were recorded. The extracts were kept in well-closed containers protected from light until determination of rhinacanthin-C content by HPLC.

Heat assisted extraction (HAE)

The dried powders of *R. nasutus* leaves (1.0 g) were put in 50 mL test tubes and separately extracted with ethanol, propylene glycol and glycerol (20 mL) at 80°C, for 1 h (under reflux conditions for ethanol). The extracts were filtered and volumes were recorded. The extracts were kept in well-closed containers protected from light until determination of rhinacanthin-C content by HPLC.

3.5 Determination of suitable solvent systems for extraction of rhinacanthin-C

The dried powders of *R. nasutus* leaves (1.0 g) was separately extracted by a combination of two solvents, including propylene glycol in alcohol and glycerol in alcohol at 25, 50 and 75% v/v (20 mL) respectively, using maceration method on a shaker at 125 rpm for 24 h. The extracts were filtered, and volumes were recorded. The extracts were kept in well-closed containers protected from light until determination of rhinacanthin-C content by HPLC.

3.6 Optimization of leaf powder to solvent ratio for extraction of rhinacanthin-C

The dried powders of *R. nasutus* leaves (5, 10, and 15 g) were separately extracted with 25% v/v glycerol in ethanol (100 mL) using maceration method on a shaker at 125 rpm for 72 h. The extracts were filtered, and volumes were recorded. The extracts were kept in well-closed containers protected from light until determination of rhinacanthin-C content by HPLC.

3.7 Comparison of maceration and microwave assisted extraction

The dried powders of *R. nasutus* leaves were separately extracted by maceration and microwave assisted extraction.

Maceration

The dried powders of *R. nasutus* leaves (15 and 90 g) were separately extracted with 25% v/v glycerol in ethanol (100 and 600 mL respectively) using maceration method on a shaker at 125 rpm, at the room temperature for 72 h, with plant powder to solvent ratio of 15: 100 (g/mL). The extracts were filtered, and volumes were recorded. The extracts were kept in well-closed containers protected from light until determination of rhinacanthin-C content by HPLC.

Microwave assisted extraction (MAE)

The dried powders of *R. nasutus* leaves (15 and 90 g) were separately extracted with 25% v/v glycerol in ethanol (100 and 600 mL respectively) by MAE, with plant powder to solvent ratio of 15: 100 (g/mL). Extraction was carried out in a household microwave apparatus (MS2127CW; LG Electronics Inc., Thailand) with microwave frequency of 2450 MHz, and power of 600 W. The extraction times were: 1 and 2 min for 100 mL and, 7 and 8 min 30 s for 600 mL. The extracts were filtered, and volumes were recorded. The extracts were kept in well-closed containers protected from light until determination of rhinacanthin-C content by HPLC.

3.8 Preparation of topical solution containing rhinacanthin-C enriched extract

3.8.1 Preparation of rhinacanthin-C enriched *R. nasutus* leaf extract

The dried powders of *R. nasutus* leaves were extracted by the optimized extraction conditions: 25% v/v glycerol in ethanol (400 mL) with plant powder to solvent ratio of 15: 100 (g/mL) using the maceration method for 72 h. The extracts were filtered, and volumes were recorded. The extracts were kept in well-closed containers protected from light until determination of rhinacanthin-C content by HPLC.

3.8.2 Formulation of topical solution containing rhinacanthin-C enriched extract

On the basis of the MIC value of rhinacanthin-C (15.1 µg/mL) against *T. rubrum* from the previous study (Panichayupakaranant *et al.*, 2009), the equivalent volume of rhinacanthin-C enriched *R. nasutus* leaf extract was used as active in formulation of the antifungal topical solution, and standardized to contain rhinacanthin-C at a concentration of 0.1% w/v (the concentration of rhinacanthin-C is about 100 times higher than the MIC value of rhinacanthin-C). Thymol and menthol were separately dissolved in about 10 mL of ethanol and added to the extract. Then water was added with sufficient quantity of daily defence. Finally volume was made up to 100 mL with ethanol. Excipients used in the formulations are shown in Table 3.1. The solutions were prepared and kept in well-closed containers protected from light until determination of rhinacanthin-C content by HPLC.

Table 3.1 Formulation of topical solution containing rhinacanthin-C enriched extract

Ingredient	Function	Formulations		
		Rx1	Rx2	Rx3
Rhinacanthin-C enriched leaf extract (mL)	Active	Volume equivalent to 0.1% rhinacanthin-C		
Thymol (g)	Antioxidant	0.02	0.02	0.02
Menthol (g)	Odour enhancer	0.1	0.1	0.1
Water (mL)	Vehicle	10	15	20
Daily defence (mL)	Fragrance	q.s.		
Ethanol (mL) q.s. to	Vehicle	100 mL		

3.9 Preparation of 10% Thong-pan-chang tincture

Fresh leaves of *R. nasutus* (10 g) were separately blended with 70% ethanol (100 mL) and macerated on a shaker at 125 rpm for 3 days (National Drug Committee, 2013). The tinctures were filtered, and kept in well-closed containers protected from light until determination of rhinacanthin-C content by HPLC.

3.10 Quantitative analysis of rhinacanthin-C

HPLC analysis was carried out on an Agilent 1100 series with Agilent 1100 series Photodiode Array Detector (PDA) and auto sampler. The chromatographic data were recorded and processed with the Agilent Software (Agilent, USA).

The samples (0.20 mL) were reconstituted with methanol and the volume were adjusted to 5 mL, and then filtered through a 0.45 µm microporous membrane for subsequent HPLC analysis. Chromatographic separation of rhinacanthin-C was performed isocratically on a TSK-gel ODS-80 Ts column (5 µm, 4.6 × 150 mm) at 25°C with a mixture of methanol and 5% aqueous acetic acid (80:20, v/v) as the mobile phase at a flow rate of 1.0 mL/min. The injection volume was 20 µL and the quantification wavelength was set at 254 nm (Panichayupakaranant *et al.*, 2009). The experiments were performed in triplicate.

The calibration curve was established from the standard rhinacanthin-C at the concentrations between 25 - 400 µg/mL by plotting peak areas against concentrations.

3.11 *In vitro* antifungal activity assay

Antifungal activity of rhinacanthin-C enriched *R. nasutus* leaf extracts, topical solution containing rhinacanthin-C enriched extract, Thong-pan-chang tincture and the standard drug (1% Clotrimazole solution) were determined against *T. rubrum* using the agar well diffusion and agar microdilution methods.

3.11.1 Preparation of culture media

T. rubrum was grown on Sabouraud dextrose agar (SDA). SDA was prepared by dissolving Sabouraud dextrose broth (30 g) and agar (15 g) in 1000 mL of distilled water and then boiling. Melted SDA was filled in each tube for slant agar, which was sterilized by autoclave at 121°C, 15 PA for 15 min. After that, the tubes were cooled to obtain slant agar and stored at 4°C until use.

3.11.2 Tested fungi-dermatophytes preparation

T. rubrum was grown aerobically on slant SDA and incubated in an incubator at 37°C for 10 days before use. The fungus was used for the preparation of inocula.

3.11.3 Preparation of the spore suspension

T. rubrum was grown on slant SDA for 10 days, after which, spores was harvested from sporulating colonies and suspended in normal saline solution sterile (0.85% NaCl w/v). The normal saline solution was transferred through funnel with cotton filter to collect spore suspension only. The concentration of spores in suspension was adjusted to match the turbidity of 0.5 McFarland standards. It was determined by transferring spore suspension (1 mL) to a cuvette (1.5 mL) and measuring turbidity by using a spectrophotometer at wave length 530 nm to give values in a range of 0.12 - 0.15 corresponding to 1~5 $\times$ 10^6 spores/mL. The suspension was diluted with Sabouraud dextrose broth to prepare final inoculums suspension (1.0×10^4 spores/mL) (Lee and Chee, 2010).

3.11.4 Agar well diffusion assay

Antifungal activity of the rhinacanthin-C enriched *R. nasutus* leaf extract, topical solution containing rhinacanthin-C enriched extract and Thong-pan-chang tincture were determined using the agar well diffusion method (Al-Zoreky, 2009) with some modifications. Briefly, 20 mL of the culture medium (Sabouraud dextrose agar) was poured in each 90 mm diameter sterilized petri plate, and then plates were kept for 5 min for drying the surface of agar. 100 µL of the fungal suspension (1~5 × 10^6 spores/mL) was spread onto the surface plate containing cooled culture medium. 6 mm diameter wells were punched in the agar using a sterilized stainless steel borer. Each well was filled with 80 µL of the sample to be tested. Control wells, containing SDA (growth control), rhinacanthin-C solution (400 µg/mL) and 1% clotrimazole topical solution (positive control) and vehicles of each sample solution (negative control) for the tested fungi, were also run parallel. Plates were incubated at 30°C for 72 h. Inhibition zones (well diameter) around the well were measured. Antimicrobial activity was expressed as the diameter of the zone of inhibition for the respective drug against test microorganism. Each test was done in triplicate and the values were reported as mean values of three experiments.

3.11.5 Agar microdilution method

The minimum inhibitory concentration (MIC) of the rhinacanthin-C enriched *R. nasutus* leaf extract, topical solution containing rhinacanthin-C enriched extract and Thong-pan-chang tincture were evaluated by the agar microdilution method (Panichayupakaranant *et al.*, 2009) with some modifications. 1% Clotrimazole topical solution (9 – 0.03 mg/mL) (1 mL) was used as positive and vehicles of each sample solution (1 mL) as negative control. The sample solutions (1 mL/ tube) were diluted with Sabouraud Dextrose Agar by two fold dilutions to obtain the final concentrations. The last tube with no sample served as a growth control. 150µL of each two fold concentration were transferred in 96 well plate and was inoculated with 2 µL of spore suspension of *T. rubrum* (5.0 × 10^4 spores/mL), and then incubated at 30°C for 7 days. The lowest concentration showing no visible growth was determined as minimum inhibitory concentration (MIC).

3.12 Stability tests

Stability evaluation of rhinacanthin-C enriched *R. nasutus* leaf extract and topical solution containing rhinacanthin-C enriched extract were performed in various conditions using the method previously described (Puttarak *et al.*, 2010). The physical appearances including clarity, precipitation and colour change of the samples were assessed by visual observations under normal light and pH of the solution was measured by a pH meter.

3.12.1 Effect of temperature on stability

The samples (5 mL) were kept in well-closed containers, protected from light and stored at 4 ± 2°C and room temperature 25 ± 2°C for 4 months. An aliquot of each sample was taken for quantitative analysis of rhinacanthin-C using the HPLC method at 0, 2, 4, 6, 8, 12 and 16 weeks. The physical appearances and pH were also determined. The experiments were performed in triplicate.

3.12.2 Effect of accelerated conditions for stability

The samples (5 mL) were kept in well-closed containers, protected from light and stored in a stability chamber at 45°C, 75% humidity for 4 months. An aliquot of each sample was taken for quantitative analysis of rhinacanthin-C using the HPLC method at 0, 2, 4, 6, 8, 12 and 16 weeks. The physical appearances and pH were also determined. The experiments were performed in triplicate.

3.13 Statistical analysis

Values were expressed as a mean ± S.D. Data was subjected to analysis of variance (ANOVA), and the means was then compared with one-way ANOVA, using Tukey's test for multiple comparison. The term significant has been used to denote the differences for which $P < 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Quantitative analysis of rhinacanthin-C

The HPLC method described previously by (Panichayupakaranant *et al.*, 2009) was used for quantitative analysis of rhinacanthin-C (Figure 4.1). The method was reported to show good reproducibility and reliability with both intraday and interday precision lower than 5%.

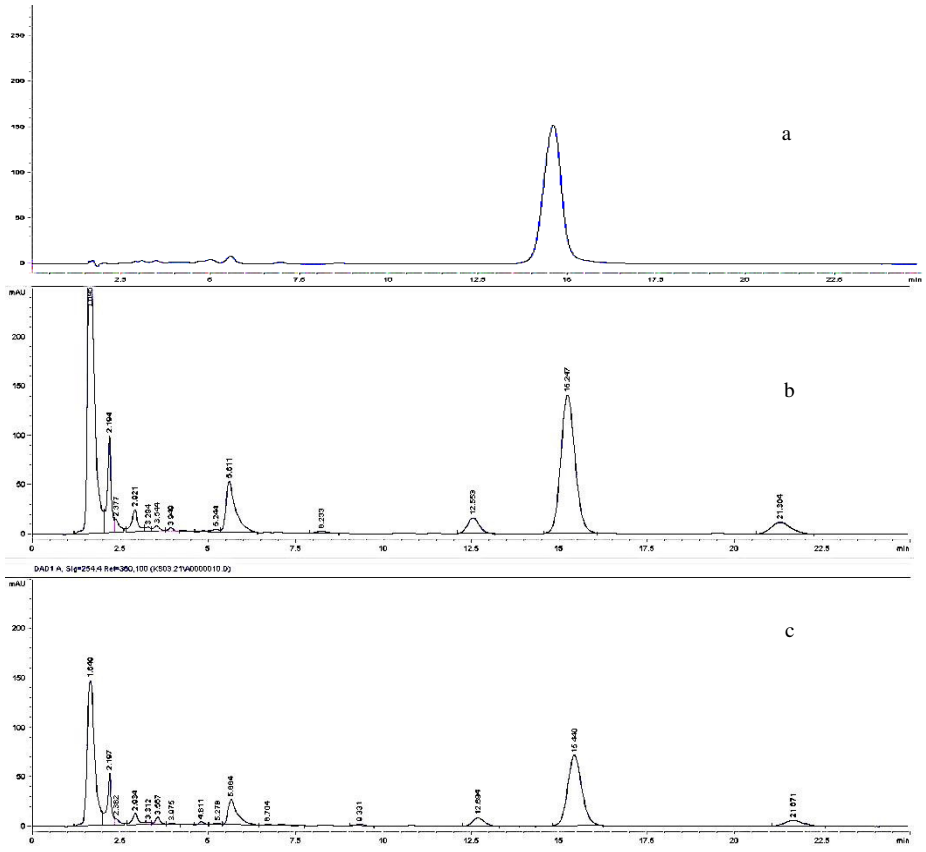

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Figure 4.1 HPLC chromatogram: (a) Standard rhinacanthin-C (200 µg/mL); (b) Rhinacanthin-C enriched extract; (c) Topical solution containing rhinacanthin-C enriched extract

Specificity of the method as reported, evaluated by UV-absorption spectra compared with standard, taken at three points of the peaks showed homogeneity of spectra. The method was also reported to be very sensitive with limit of detection and quantification of 0.75 and 3.0 µg/mL, respectively.

A calibration curve was established from the standard rhinacanthin-C at the concentrations between 25 - 400 µg/mL by plotting peak areas against concentrations (Table 4.1). The calibration curve exhibited linearity over the evaluated range, with the linear equation of Y = 51.303X + 185.6 (r^2 = 0.9997) (Figure 4.2).

Table 4.1 Calibration curve data of rhinacanthin-C

Concentration (µg/mL)	Peak area (Mean ± S.D., n=3)
400	20,605 ± 33.78
200	10,623 ± 74.06
100	5,440 ± 45.01
50	2,681 ± 9.01
25	1,341 ± 6.00

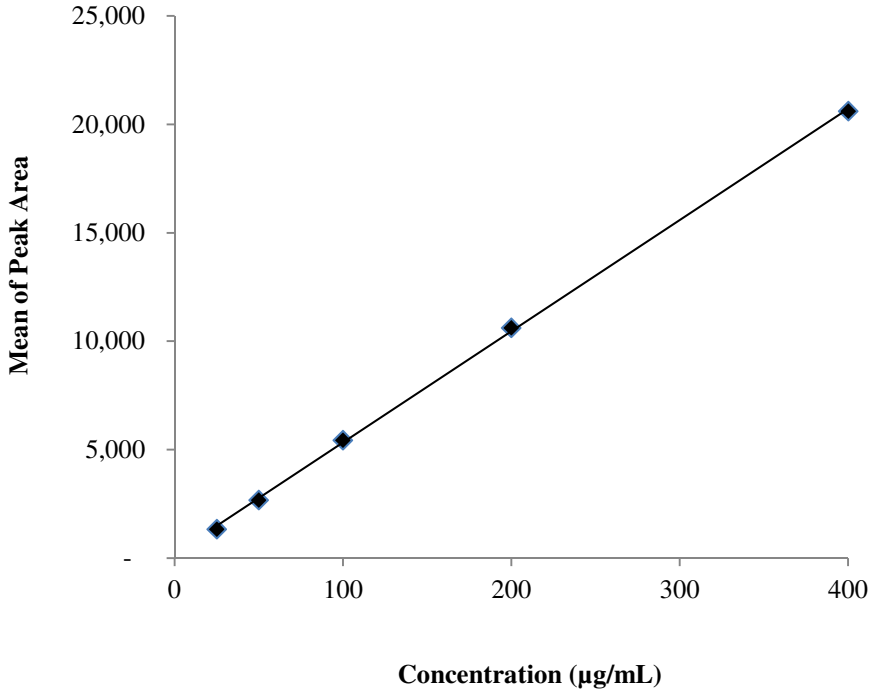

Figure 4.2 Calibration curve of rhinacanthin-C

4.2 Establishment of suitable solvent and extraction method for rhinacanthin-C

Several factors such as selectivity, density, toxicity, volatility, reactivity, physical hazards and miscibility with aqueous media has to be considered while choosing a solvent to extract active components from the matrix (Prabu and Suriyaprakash, 2012). Non polar solvent, ethyl acetate has been suggested to be the suitable for extraction of rhinacanthins from *R. nasutus* leaf (Panichayupakaranant *et al.*, 2009). However, non-polar solvents including, hexane, carbon tetrachloride, benzene, chloroform, dioxane and ethyl acetate cannot not be used for industrial application due to their inherent toxicity and volatility.

Therefore, three green solvents, i.e. ethanol, propylene glycol, and glycerol which are generally used as co-solvents for increase in solubility of poorly water-soluble drugs and/or stabilization of degradable drugs in aqueous solutions and are usually constituted in topical solutions were evaluated as a suitable solvent for extraction of rhinacanthin-C from *R. nasutus* dried leaf powders. Among these solvents, ethanol gave the extracts that contained the highest concentrations of rhinacanthin-C, with the lowest yields of the extracts for both maceration and heat assisted extraction (HAE) (Table 4.2).

Table 4.2 Determination of suitable extraction solvent and method for rhinacanthin-C

Extraction methods	Solvents	Volume of the extracts (mL)	Rhinacanthin-C conc. (mg/mL)	Total rhinacanthin-C content (mg)
Maceration	Ethanol	13.3 ± 0.66^{a}	0.69 ± 0.03^{a}	9.17 ± 0.80^{a}
	Propylene Glycol	18.2 ± 0.29^{b}	0.30 ± 0.00^{b}	5.49 ± 0.14^{b}
	Glycerol	17.8 ± 0.50^{b}	0.05 ± 0.00^{c}	1.00 ± 0.03^{c}
HAE	Ethanol	14.0 ± 0.50^{a}	0.73 ± 0.04^{a}	10.17 ± 0.21^{d}
	Propylene Glycol	17.5 ± 0.43^{b}	0.37 ± 0.01^{d}	6.46 ± 0.23^{b}
	Glycerol	17.8 ± 0.29^{b}	0.07 ± 0.00^{c}	1.20 ± 0.05^{c}

The results were expressed as Mean ± S.D. of three determinations for each sample. Means followed by the same letter in the same column are not significantly different according to one-way analysis of variance (ANOVA), followed by Tukey's HSD test. The term significant has been used to denote the differences for which $P < 0.05$.

The lower yields of the ethanol extracts may be due to evaporation of ethanol during the extraction process. However, ethanol gave the highest calculated total content of rhinacanthin-C when compared to the other two solvents. The lower extractability of propylene glycol and glycerol may be due to their higher polarity and viscosity properties.

The results from this study also indicated that HAE slightly accelerated the extraction of rhinacanthin-C from *R. nasutus* dried leaf powders, but the yield of the extracts as well as concentrations of rhinacanthin-C were not significantly different when compared to those of maceration method. Generally, HAE is a high energy consumption method, but less time consuming. It has been reported that a yield of naphthoquinones usually depends on temperature used for extraction, and some naphthoquinones are heat labile e.g. juglone, whose yield was significantly decreased when increased temperature for extraction (Babula *et al.*, 2009). Chatkrapunt (2004) reported a lower total rhinacanthin content of *R. nasutus* leaf extract when the period of extraction was increased. This indicated that rhinacanthins has less stability in a high temperature. Maceration method, which is a simple and unheated method, was therefore selected for further studies.

Interestingly, the extracts obtain from ethanol and propylene glycol had a green color, while those from glycerol had a reddish-brown color (Figure 4.3). The green color indicated the presence of chlorophylls (Xynos *et al.*, 2012). Chlorophylls were not extracted by glycerol due to a high polarity property of glycerol. Aesthetic limitation like undesired color is one of the challenges with formulation of natural products (Othmer, 2012). Brown colored extract may be more favourable aesthetically for formulation of topical solution than the green colored extracts. Recently, it has been reported that an ion exchange chromatographic method using Amberlite® IRA-67 column was used to exclude interference compounds including chlorophylls and other pigments. This method was capable of increasing the total rhinacanthin content in *R. nasutus* extracts up to 77.5 % w/w (Panichayupakaranant *et al.*, 2009). However, the method was multistep process, and organic solvents were required.

It has been reported that use of solvent mixtures can enhance the extraction yields, one solvent improving solubility and other enhancing desorption (Mustafa and Turner, 2011). Extractions of *R. nasutus* dried powder using combinations of ethanol and propylene glycol or ethanol and glycerol may improve extraction yield of rhinacanthin-C as well as decrease the content of chlorophylls.

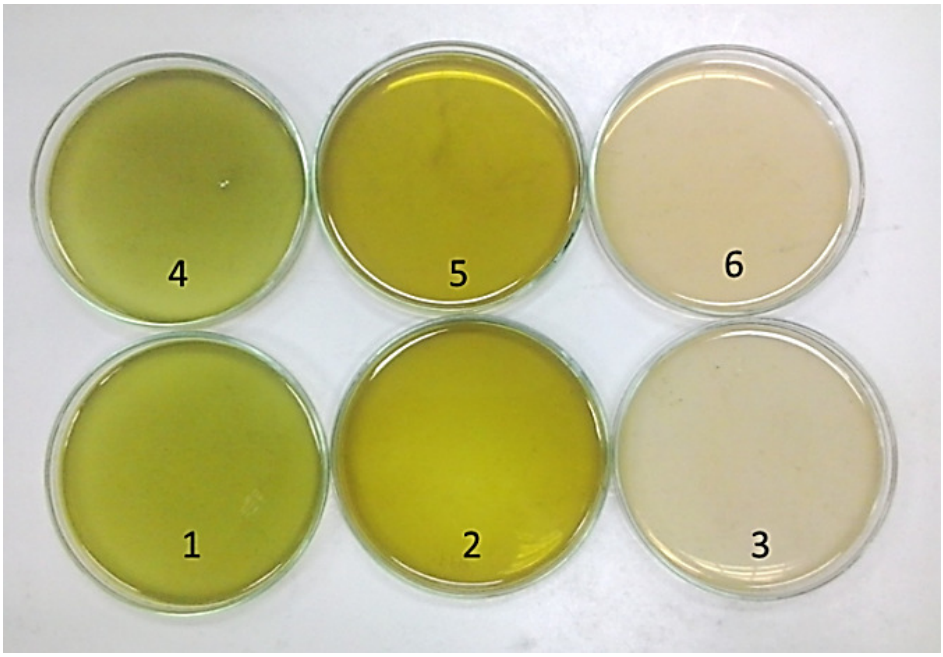

Figure 4.3 Colour of the extracts: (1) propylene glycol; (2) ethanol; (3) glycerol obtained from maceration; and (4) propylene glycol; (5) ethanol; (6) glycerol obtained from HAE.

4.3 Determination of co-solvent systems for extraction of rhinacanthin-C

The extraction of pharmacologically active compounds from herbal plants is one of the most critical steps in natural products research (Xynos *et al.*, 2012). The combination of solvents at different ratios gave extracts with slight color variation, which may be due to influence of pH (Zhou *et al.*, 2012) or selectivity of solvents for chlorophyll.

Ionic liquids, ultra-pure water and combination of ethanol with water, etc. have been reported to be used as green solvents for extraction (Desgrouas *et al.*, 2014; Zhang *et al.*, 2014). Combination of co-solvents are used to yield a polarity of solvent similar to drug substance which can deliver optimum solubilizing capacity (Williams *et al.*, 2011). In this study, 25, 50 and 75% v/v propylene glycol in ethanol as well as 25, 50 and 75% v/v glycerol at in ethanol were determined for extraction of rhinacanthin-C from *R. nasutus* dried leaf powders in order to increase rhinacanthin-C content of the extracts.

The results revealed that 25% v/v propylene glycol in ethanol as well as 25 and 50% v/v glycerol in ethanol gave the highest content of total rhinacanthin-C, which were not significantly different (Table 4.3). Highly concentrated extract allows use of small amount of the extract in the final formulation, which is aesthetically beneficial (Dayan and Kromidas, 2011). However, combination of propylene glycol with ethanol gave green coloured extract which were aesthetically unacceptable (Figure 4.4). Therefore, 25% v/v glycerol in ethanol that gave significantly high rhinacanthin-C concentration of 0.84 mg/mL, with brown colored extract was selected as a suitable co-solvent for extraction of rhinacanthin-C.

Table 4.3 Comparison of binary solvents system: propylene glycol in ethanol and glycerol in ethanol at different percentage for rhinacanthin-C extraction

Solvent in ethanol (%)		Volume of the extracts (mL)	Rhinacanthin-C conc. (mg/mL)	Total rhinacanthin-C content (mg)
Propylene glycol	75	16.2 ± 0.29^{a}	0.47 ± 0.03^{a}	7.63 ± 0.42^{a}
	50	13.7 ± 0.29^{b}	0.55 ± 0.01^{b}	7.57 ± 0.23^{a}
	25	12.1 ± 0.29^{cd}	0.76 ± 0.02^{c}	9.29 ± 0.15^{b}
Glycerol	75	16.2 ± 0.29^{a}	0.49 ± 0.01^{a}	7.86 ± 0.23^{a}
	50	12.8 ± 0.76^{bc}	0.71 ± 0.03^{c}	9.10 ± 0.25^{b}
	25	11.5 ± 0.50^{d}	0.84 ± 0.01^{d}	9.68 ± 0.33^{b}

The results were expressed as Mean ± S.D. of three determinations for each sample. Means followed by the same letter in the same column are not significantly different according to one-way analysis of variance (ANOVA), followed by Tukey's HSD test. The term significant has been used to denote the differences for which $P < 0.05$.

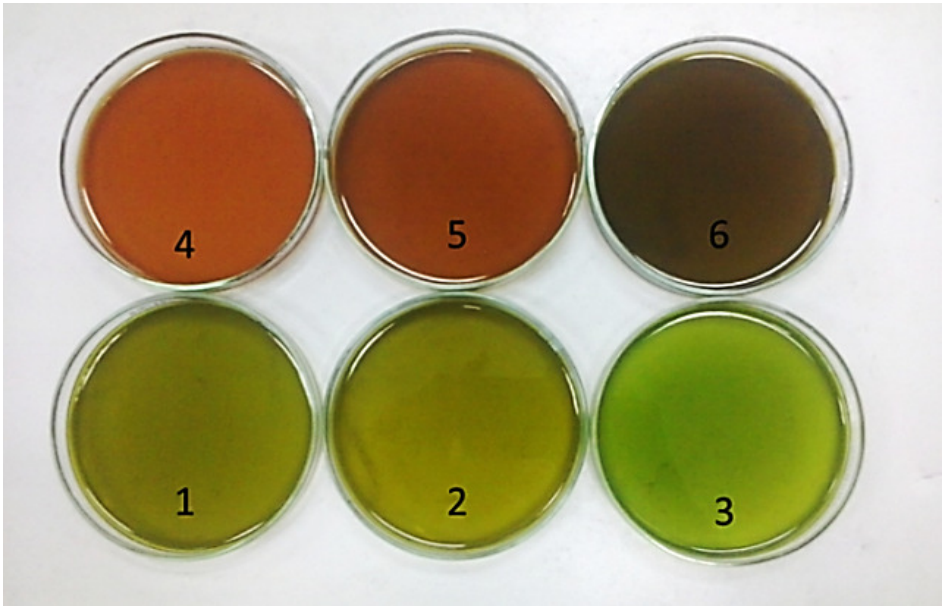

Figure 4.4 Colors of *R. nasutus* extracts obtained from co-solvents: (1) 75%; (2) 50%; (3) 25% v/v propylene glycol in ethanol; (4) 75%; (5) 50%; (6) 25% v/v glycerol in ethanol, respectively.

4.4 Determination of a suitable leaf powder to solvent ratio for extraction of rhinacanthin-C

The rhinacanthin-C extract prepared from 25% v/v glycerol in ethanol was ready to use without solvent evaporation process. Thus, the concentration of rhinacanthin-C should be high enough for formulation of a topical solution, in order to obtain a sufficient active ingredient concentration for a therapeutic application. Generally, an increase in ratios of leaf powder to solvent resulted in an increased concentrations of active compounds. A higher powder to solvent ratio leads to a larger concentration difference, and then favouring mass transfer and increasing extraction efficiency (Lu *et al.*, 2011). The suitable leaf powder to solvent ratio was therefore determined to obtain a sufficient concentration of rhinacanthin-C.

In this study, volume of the solvent was fixed at 100 mL, and the amounts of leaf powders were varied as 5, 10 and 15 g. It was found that an increase in leaf powders to solvent ratios resulted in an increased concentration of rhinacanthin-C, with a decrease in the yields of extracts (Table 4.4). Considering the concentration of rhinacanthin-C obtained, the suitable leaf powder to solvent ratio should be 15 g/100 mL, which gave 3.1 and 2.2-fold higher concentration of rhinacanthin-C than those of 5 g/100 mL, and 10 g/100 mL, respectively. In addition, its calculated total contents of rhinacanthin-C were also 2.7 and 2.0-fold higher than those of 5 g/100 mL, and 10 g/100 mL, respectively.

Table 4.4 Determination of a suitable leaf powder to solvent ratio for extraction of rhinacanthin-C

Powder/solvent (g/100 mL)	Volume of the extracts (mL)	Rhinacanthin-C conc. (mg/mL)	Total rhinacanthin-C content (mg)
5	73.0 ± 1.73[a]	0.65 ± 0.04[a]	47.71 ± 2.07[a]
10	69.0 ± 1.00[b]	0.92 ± 0.03[b]	63.87 ± 2.14[b]
15	64.7 ± 1.53[c]	2.02 ± 0.04[c]	130.36 ± 2.23[c]

The results were expressed as Mean ± S.D. of three determinations for each sample. Means followed by the same letter in the same column are not significantly different according to one-way analysis of variance (ANOVA), followed by Tukey's HSD test. The term significant has been used to denote the differences for which $P < 0.05$.

Considering economic efficiency and saving cost of solvent, the ratio of 15 g/100 mL was more suitable for preparation of rhinacanthin-C enriched *R. nasutus* leaf extract. The obtained extracts were in a dark brown color (Figure 4.5).

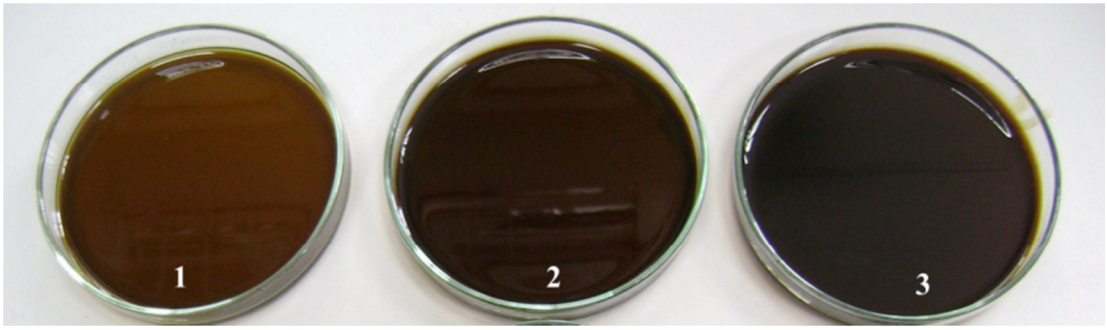

Figure 4.5 Colors of *R. nasutus* extracts obtained from leaf powder to solvent ratios of: (1) 5 g/100 mL; (2) 10 g/100 mL; (3) 5 g/100 mL.

4.5 Evaluation of microwave assisted extraction (MAE) as an alternative extraction method

MAE has been reported to be a green extraction method that can shorten extraction time, reduce solvent consumption, and increase extraction yields when compared to the conventional extraction methods e.g. maceration, percolation, Soxhlet, heat reflux and ultrasonic assisted extraction (Chan *et al.*, 2011; Tan *et al.*, 2011; Desgrouas *et al.*, 2014). The extraction efficiency of maceration using 25% v/v glycerol in ethanol as solvent, and leaf powder to solvent ratio of 15 g/100 mL, was compared with MAE for preparation of rhinacanthin-C enriched *R. nasutus* extracts. MAE conditions used in this study were set to reach two extraction temperatures of 60 and 70°C by varying the extraction time to be 1 and 2 min, respectively using a microwave power of 600 W. The results revealed that MAE (1 min or 60°C) produced *R. nasutus* extracts with the same content of rhinacanthin-C compared to maceration method (Table 4.5). However, MAE could markedly shorten the extraction time. In addition, an increased extraction time (2 min) or temperature (70°C) for MAE could not increase the content of rhinacanthin-C in the extracts. The optimized temperature for extraction of rhinacanthin-C using MAE was therefore set at 60°C.

Table 4.5 Comparison of maceration and microwave assisted extraction for 100 mL scale

Extraction Method	Volume of the extracts (mL)	Rhinacanthin-C conc. (mg/mL)	Total rhinacanthin-C content (mg)
Maceration (72 h)	63.3 ± 0.58^{a}	1.99 ± 0.05^{a}	126.15 ± 3.93^{a}
MAE (1 min)	61.0 ± 1.00^{b}	1.91 ± 0.09^{a}	116.55 ± 5.55^{ab}
MAE (2 min)	61.0 ± 0.00^{b}	1.84 ± 0.04^{a}	112.42 ± 2.65^{b}

The results were expressed as Mean ± S.D. of three determinations for each sample. Means followed by the same letter in the same column are not significantly different according to one-way analysis of variance (ANOVA), followed by Tukey's HSD test. The term significant has been used to denote the differences for which $P < 0.05$.

However, when increase the extraction scale to 600 mL, the time for maceration was 72 h, while those for MAE were 7 min (55°C) and 8 min 30 sec (75°C). The time difference in extraction for scale-100 and scale-600 mL of extraction solvents due to

energy density required per mass for a given unit of time. Amongst these conditions, MAE for 8 min 30 sec significantly increased rhinacanthin-C content up to 3.15 mg/mL, but the yield of extraction was decreased (Table 4.6).

Table 4.6 Comparison of maceration and microwave assisted extraction for 600 mL scale

Extraction Method	Volume of the extracts (mL)	Rhinacanthin-C conc. (mg/mL)	Total rhinacanthin-C content (mg)
Maceration (72 h)	387.3 ± 2.52^{a}	1.79 ± 0.02^{a}	691.63 ± 8.63^{a}
MAE (7 min)	375.0 ± 8.66^{a}	2.72 ± 0.11^{b}	1018.39 ± 23.11^{b}
MAE (8 min 30 sec)	335.0 ± 13.22^{b}	3.15 ± 0.13^{c}	1055.09 ± 26.28^{b}

The results were expressed as Mean ± S.D. of three determinations for each sample. Means followed by the same letter in the same column are not significantly different according to one-way analysis of variance (ANOVA), followed by Tukey's HSD test. The term significant has been used to denote the differences for which $P < 0.05$.

The increase in rhinacanthin-C content might be due to destruction of plant matrix by microwave energy generating molecular movement and heating causing the analyte to diffuse out and rapid loss in extraction solvent due to evaporation forming concentrated extract (Mandal *et al.*, 2009; Chan *et al.*, 2011). Generally, the elevated temperatures decreases the viscosity and surface tension of solvents that could increase diffusivity of the solvent into the internal parts of the plant matrix and thus enhance desorption and partition of components from the active sites of the plant matrix into the extraction solvent (Chan *et al.*, 2011; Lu *et al.*, 2011; Tan *et al.*, 2011). Considering the calculated total content of rhinacanthin-C, both MAE conditions gave the same level of total rhinacanthin-C contents, which were higher than the maceration method. This implied that MAE may be used as an alternative green extraction method for preparation of rhinacanthin-C enriched *R. nasutus* leaf extracts.

The MAE was carried out in household microwave oven due to its easy availability and low cost. However, it lacks process monitoring system for temperature and pressure. Thus, further increase in extraction efficiency may be obtained by using commercial extractor (Chan *et al.*, 2011).

4.6 Preparation of topical solution containing rhinacanthin-C enriched extract

Rhinacanthin-C enriched *R. nasutus* leaf extract was prepared by the extraction of dried leaf powder of *R. nasutus* (60 g) with 25% v/v glycerol in ethanol (400 mL) using maceration for 72 h. On the basis of HPLC analysis, the obtained extract (265 mL) contained rhinacanthin-C content of 1.79 ± 0.03 mg/mL. In order to formulate topical solution containing rhinacanthin-C enriched extract (100 mL), the equivalent volume of the rhinacanthin-C enriched *R. nasutus* leaf extract used in the formula was 56 mL as shown in the calculation below. In this study, three topical solutions containing the leaf extracts of *R. nasutus* that equivalent to 0.1% rhinacanthin-C were formulated as shown in Table 4.7.

Calculation of equivalent volume of the rhinacanthin-C enriched *R. nasutus* leaf extract for formulation of topical solution containing rhinacanthin-C enriched extract:

1.79 mg	rhinacanthin-C is contained in	1 mL of extract.
i.e. 1.00 mg	rhinacanthin-C is contained in	0.56 mL of extract.
i.e. 100.00 mg	rhinacanthin-C is contained in	56 mL of extract.
i.e. 0.1 g	rhinacanthin-C is contained in	56 mL of extract.

All three topical solutions had a dark brownish color (Figure 4.6) with pleasant smell. Their rhinacanthin-C content analyzed by HPLC was shown in Table 4.8.

Table 4.7 Three formulas of topical solution containing rhinacanthin-C enriched extract

Ingredient	Function	Formulations		
		Rx 1	Rx 2	Rx 3
Rhinacanthin-C enriched leaf extract (mL)	Active constituent	56.0	56.0	56.0
Thymol (mg)	Antioxidant	20.0	20.0	20.0
Menthol (mg)	Odour enhancer	100.0	100.0	100.0
Water (mL)	Vehicle	10	15	20
Daily defence (mL)	Fragrance	q.s.		
Ethanol (mL) q.s. to	Vehicle	100 mL		

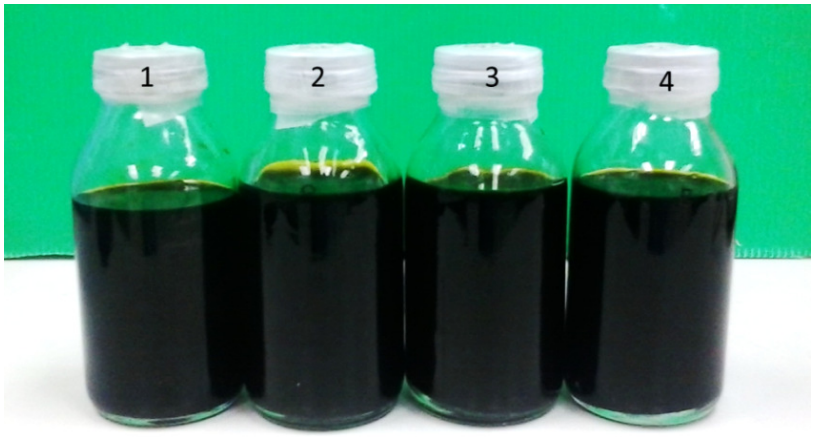

Figure 4.6 Rhinacanthin-C enriched *R. nasutus* leaf extract (1); and topical solution containing rhinacanthin-C enriched extract: Rx 1 (2); Rx 2 (3); Rx 3 (4)

Table 4.8 Rhinacanthin-C content in topical solution containing rhinacanthin-C enriched extract

Formula	Rhinacanthin-C concentration* (mg/mL) (Mean ± S.D., n=3)
Rx 1	1.00 ± 0.02
Rx 2	1.01 ± 0.02
Rx 3	1.00 ± 0.01

* Analyzed by HPLC

All three topical solutions contained rhinacanthin-C contents of 0.1% w/v and they were not found to be significantly different between the formulations even after kept for a week, and also no precipitation was observed. This may be a preliminary indication of their uniformity. Among the three formulations, Rx 1 with the lowest content of water (10% v/v) was selected, considering a hydrophobicity of rhinacanthin-C, for further determination of its antifungal activity and stability.

4.7 Preparation of 10% Thong-pan-chang tincture

It has been reported that a selected medicinal plants for development of herbal medicine based on traditional medicines should be prepared by mimic their traditional uses (Sasidharan *et al.*, 2011). Therefore, Thong-pan-chang tinctures were approved in the List of Herbal Medicinal Products A.D. 2013 (National Drug Committee, 2013).

Thong-pan-chang tincture prepared from 100% v/v ethanol had a significantly higher content of rhinacanthin-C than that from 70% v/v ethanol (Table 4.9) due to a hydrophobicity of rhinacanthin-C. However, the tincture prepared from 70% v/v ethanol appeared to be more aesthetically acceptable than in 100% ethanol due to its lesser content of chlorophylls (Figure 4.7).

Table 4.9 Rhinacanthin-C content in Thong pang chang tincture

Solvents used for preparation of tincture	Volume of the extracts (mL)	Rhinacanthin-C conc. (mg/mL)
100% v/v Ethanol	91.3 ± 1.53[a]	0.15 ± 0.01[a]
70% v/v Ethanol	91.7 ± 2.08[a]	0.12 ± 0.00[b]

The results were expressed as Mean ± S.D. of three determinations for each sample. Means followed by the same letter in the same column are not significantly different according to one-way analysis of variance (ANOVA), followed by Tukey's HSD test. The term significant has been used to denote the differences for which $P < 0.05$.

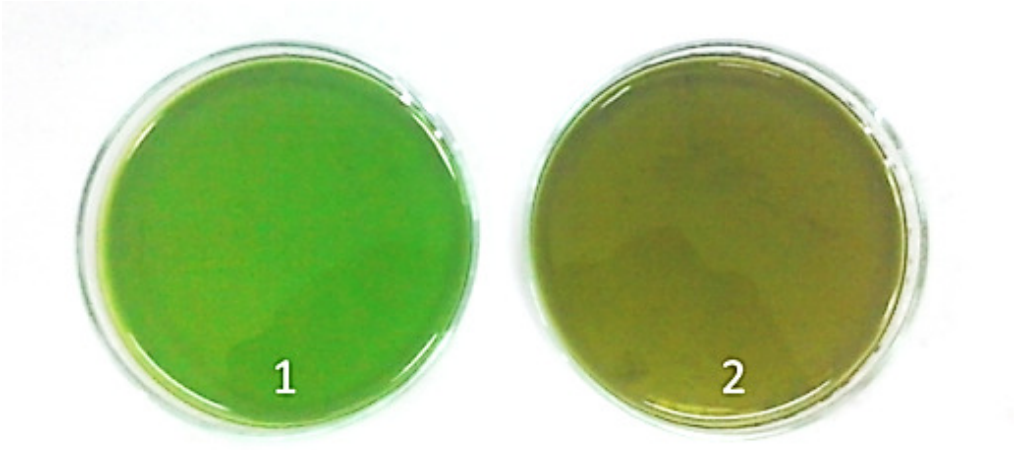

Figure 4.7 Color of tincture extracted by: (1) 100% ethanol; (2) 70% ethanol

Based on the traditional preparation of Thong-pan-chang tincture, the levels of the antifungal active compound, rhinacanthin-C (0.12 – 0.15 mg/mL) were rather low. The concentrations of rhinacanthin-C were only 8 to 10 times higher than the reported MIC value of rhinacanthin-C against *T. rubrum* (15.1 µg/mL). Generally, a concentration of active ingredient in a formulated topical preparation should be about 100 times higher than its MIC or IC_{50} value (Barel *et al.*, 2005). The rhinacanthin-C concentration in Thong-pan-chang tincture was therefore insufficient for its antifungal efficacy. Moreover, the odors of Thong-pan-chang tincture were not pleasant. In addition, a high concentration of ethanol in the preparation may cause dehydration of skin or skin irritation when use for a long period.

4.8 *In vitro* antifungal activity against *T. rubrum*

The standardized rhinacanthin-C enriched *R. nasutus* leaf extract, topical solution containing rhinacanthin-C enriched extract, Thong-pan-chang tincture, rhinacanthin-C solution (400 µg/mL) and the standard drug (1% Clotrimazole solution) were determined for antifungal activity against *T. rubrum*.

On the basis of antifungal activity screening using the agar well diffusion method (Al-Zoreky, 2009) with some modification, all tested samples exhibited antifungal activity against *T. rubrum* at the given concentrations of rhinacanthin-C, with their diameters of inhibition zone was much greater than those of their own controls (Table 4.10). Among the samples from *R. nasutus*, rhinacanthin-C enriched extract exhibited the highest inhibition zone (21.4 mm). The diameters of the inhibition zone should be related to their rhinacanthin-C concentrations. Accordingly, Thong-pan-chang tincture exhibited the lowest inhibition zone diameter (Figure 4.8).

Table 4.10 Antifungal activity screening using the agar well diffusion method

Sample	Concentration of rhinacanthin-C (µg/well)	Inhibition zone (mm) (Mean ± S.D.)
Rhinacanthin-C enriched leaf extract	143.04	21.4 ± 1.65
Rhinacanthin-C enriched leaf extract [c]	-	6.3 ± 0.31
Topical solution containing rhinacanthin-C enriched extract	80.24	17.9 ± 1.68
Topical solution containing rhinacanthin-C enriched extract [c]	-	6.6 ± 0.45
Thong-pan-chang tincture	11.84	12.2 ± 2.28
Thong-pan-chang tincture [c]	-	8.2 ± 0.85
Rhinacanthin-C solution	32	16.1 ± 2.11
1% Clotrimazole solution	-	68.7 ± 1.25

The results were expressed as Mean ± S.D. of three determinations for each sample.
[c] = Solvent control of respective samples
Diameter of the well = 4.3 ± 0.15 mm

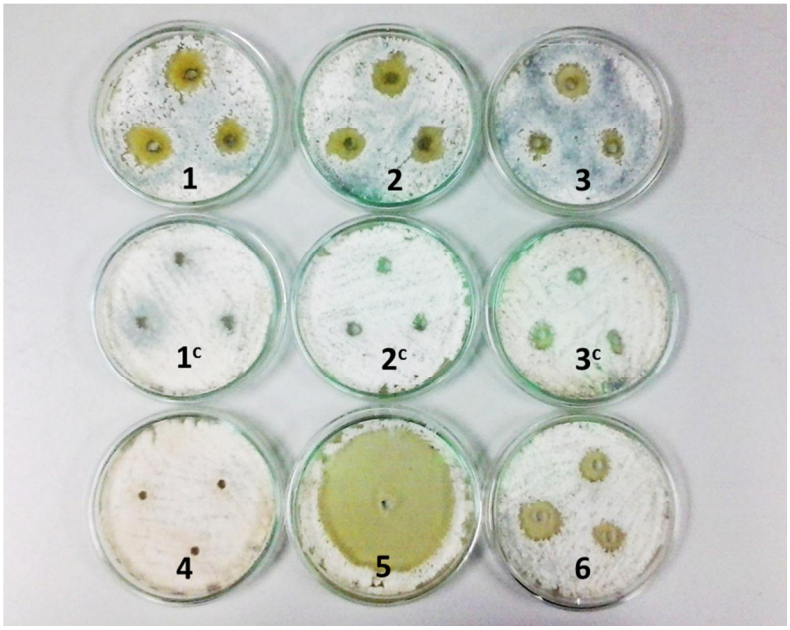

Figure 4.8 Inhibition zones exerted by: (1) Rhinacanthin-C enriched leaf extract; (2) Topical solution containing rhinacanthin-C enriched extract; (3) Thong-pan-chang tincture; (4) Diameter of the well; (5) 1% Clotrimazole solution; (6) Rhinacanthin-C solution; against *T. rubrum* by well diffusion assay

[c] = Solvent control of respective samples

The minimum inhibitory concentration (MIC) of the rhinacanthin-C enriched *R. nasutus* leaf extract, the topical solution containing rhinacanthin-C enriched extract and the tincture against *T. rubrum* were evaluated using the agar dilution method (Panichayupakaranant *et al.*, 2009) with some modifications. The rhinacanthin-C enriched leaf extract was diluted with Sabouraud Dextrose Agar to contain rhinacanthin-C concentration of 1.0 mg/mL. The topical solution contained rhinacanthin-C concentration of 1.0 mg/mL, while Thong-pan-chang tincture used in this study, contained rhinacanthin-C concentration of 0.52 mg/mL.

Amongst these, rhinacanthin-C enriched extracts exhibited the strongest antifungal activity against *T. rubrum*, with MIC value of 8.67 mg/mL at dilution of 1:64 (Table 4.11). Topical solution containing rhinacanthin-C enriched extract showed similar activity as 0.1% rhinacanthin-C solution, with MIC value of 28.5 and 25.1 mg/mL, both at dilution of 1:32, respectively. However, Thong-pan-chang tincture showed the lowest antifungal activity with MIC value of 52.1 mg/mL at dilution of 1:16. The result showed that antifungal activity of rhinacanthin-C enriched leaf extract was better than topical solution and the tincture which can be correlated with high rhinacanthin-C content in the extract.

Thymol and menthol have been reported to show antimicrobial effect (Xu *et al.*, 2008; Kamatou *et al.*, 2013). Thymol has been reported to show antifungal activity against *T. rubrum* at MIC value around 166.25 µg/mL (Danielli *et al.*, 2013). However, the highest concentration of thymol in the topical solution for antifungal assay was 100 µg/mL. Menthol has been reported to be used for treating nail infection at concentration of 2 to 10% (Mckenzie, 2002) but in formulation it was used only at concentration of only 0.1%. Moreover, vehicles of each sample solution (negative control), did not show any activity at the tested concentrations, indicating no contribution in antifungal activity from these compounds.

Table 4.11 Antifungal activity of rhinacanthin-C enriched leaf extract and preparations against *T. rubrum*

Sample	MIC*	Rhinacanthin-C equivalent content (μg/mL)
Rhinacanthin-C enriched leaf extract	1:64	15.6
Topical solution containing rhinacanthin-C enriched extract	1:32	31.2
Thong-pan-chang tincture	1:16	31.2
0.1% rhinacanthin-C solution	1:32	31.2
1% Clotrimazole solution#	1:6400	-

* MIC in terms of dilution of samples

Positive control

In general, antimicrobial activities of naphthoquinone such as rhinacanthin-C, has been reported due to their several mechanism of actions, including inhibition of electron transport, uncoupling of oxidative phosphorylation, intercalation of biomolecules, and production of reactive oxygen radicals by redox cycling under aerobic conditions (Babula *et al.*, 2009; Eilenberg *et al.*, 2010).

4.9 Stability tests

It has been reported that rhinacanthin-C that is constituted in a dried rhinacanthins-rich *R. nasutus* extract was stable when kept in a well-closed container protected from light and stored either under 4 ± 2°C or 30 ± 2°C (Puttarak *et al.*, 2010). However, rhinacanthin-C was not stable when the extract was prepared as an aqueous alcoholic solution. It has been reported that the compounds with ester functional group are susceptible to hydrolytic degradation (Florence and Attwood, 2011). Thus, instability of rhinacanthin-C in the solution should be considered as its hydrolysis. Moreover, rhinacanthin-C structure also contains a hydroxyl group directly bonded to an aromatic ring, which is prone to oxidation (Troy *et al.*, 2006). The approach to replace some or all of the water in the system with a solvent such as alcohol or propylene glycol to prevent hydrolysis of compounds has been suggested (Liu, 2000; Florence and Attwood, 2011).

In this study, rhinacanthin-C enriched *R. nasutus* leaf extract (650 mL) was prepared by the maceration method previously described, and standardized to contain rhinacanthin-C at a concentration of 1.79 ± 0.02 mg/mL. The topical solution containing 1% w/v rhinacanthin-C (300 mL) was then prepared from the obtained extract. On the basis of HPLC analysis, the topical solution contained a rhinacanthin-C content of 1.05 ± 0.02 mg/mL.

The effect of temperature on the stability of rhinacanthin-C enriched *R. nasutus* leaf extract and topical solution containing rhinacanthin-C enriched extract were examined under two temperatures; 4 ± 2°C and 25 ± 2°C, and protected from light over a period of 16 weeks. It was found that physical appearances of both rhinacanthin-C enriched extracts and the topical solutions were not changed, while their pH were slightly decreased over a period of 16 weeks in both conditions. The content of rhinacanthin-C in rhinacanthin-C enriched extracts and the topical solution was not significantly decreased over a period of 16 weeks when stored in a well-closed container protected from light at 4 ± 2 °C (Table 4.12, Table 4.13). In contrast, the rhinacanthin-C content decreased significantly after 8 weeks when kept under 25 ± 2 °C, protected from light. However, the remained levels of rhinacanthin-C in the

rhinacanthin-C enriched extracts and topical solutions were 92 and 86%, respectively after kept under 25 ± 2 °C for 16 weeks.

Moreover, the effect of accelerated conditions on the stability of the rhinacanthin-C enriched *R. nasutus* extract and topical solution containing rhinacanthin-C enriched extract were examined in a stability chamber at 45°C, 75% relative humidity over a period of 16 weeks. The results showed that physical appearances of both rhinacanthin-C enriched extracts and the topical solutions were not changed, while their pH were slightly decreased over a period of 16 weeks. The levels of rhinacanthin-C in both extract and topical solution significantly decreased and dropped below 90% after kept for 8 weeks (Table 4.12, Table 4.13). In addition, the remained levels of rhinacanthin-C in both extracts and topical solutions were 77% after kept in the accelerated conditions over a period of 16 weeks. This implied that the content of rhinacanthin-C in the extracts and topical solutions may decrease by 23% after kept at room temperature for two years.

Table 4.12 Effect of temperature and accelerated conditions on rhinacanthin-C content and pH of rhinacanthin-C enriched *R. nasutus* leaf extract

Weeks	Rhinacanthin-C content (% remaining; Mean ± S.D.)			pH		
	4 ± 2°C	25 ± 2°C	45 ± 2°C, 75% RH	4 ± 2°C	25 ± 2°C	45 ± 2°C 75% RH
0	99.98 ± 1.31^{a}	99.98 ± 1.31^{ab}	99.98 ± 1.31^{a}	6.22± 0.02^{a}	6.22 ± 0.02^{a}	6.22 ± 0.02^{a}
2	100.75 ± 1.99^{a}	101.27 ± 2.30^{a}	99.55 ± 2.66^{a}	6.11 ± 0.02^{b}	6.11 ± 0.02^{b}	6.11 ± 0.02^{b}
4	101.87 ± 2.56^{a}	101.27 ± 1.28^{a}	98.08 ± 2.60^{a}	6.23 ± 0.02^{a}	6.18 ± 0.02^{c}	6.12 ± 0.02^{b}
8	101.68 ± 2.30^{a}	94.77 ± 1.10^{bc}	83.91 ± 2.78^{b}	6.23 ± 0.02^{a}	6.21 ± 0.01^{ac}	6.15 ± 0.01^{b}
12	98.06 ± 0.73^{a}	92.35 ± 2.92^{c}	81.61 ± 3.25^{b}	6.11 ± 0.01^{b}	6.10 ± 0.01^{b}	6.00 ± 0.01^{c}
16	97.93 ± 4.14^{a}	91.90 ± 2.59^{c}	77.34 ± 2.05^{b}	6.10 ± 0.03 ^{b}	6.09 ± 0.01 ^{b}	5.97 ± 0.01^{c}

The results were expressed as Mean ± S.D. of three determinations for each sample. Means followed by the same letter in the same column are not significantly different according to one-way analysis of variance (ANOVA), followed by Tukey's HSD test. The term significant has been used to denote the differences for which $P < 0.05$.

For chemical entity, like rhinacanthin-C whose hydrolytic degradation is catalysed by hydrogen or hydroxyl ion, the influence of buffer components and ionization of drug should be considered for the determination of the pH of optimum stability (Gad, 2008). The buffer independent pH rate profile can be obtained by measurement of reaction rate at a series of buffer concentration at each pH extrapolated to zero buffer concentration, which is essential for maximum stability of the drugs in solution (Attwood and Florence, 2012). Further improvement in stability may be achieved by preparation of solution at pH of optimum stability with suitable buffer, suppressing the ionization of rhinacanthin-C in aqueous phase and preventing the hydrolysis (Pandit, 2007; Gad, 2008).

It has been reported that nanoliposome containing rhinacanthin-C could improve stability of rhinacanthin-C more than 80% for up to 3 months at pH 4.0 and 6.6 when compared with the solution of rhinacanthin-C at the same pH (Paosupap, 2011). The improved stability has been suggested due to the protection of drugs by isolating them from degrading enzymes.

Table 4.13 Effect of temperature and accelerated conditions on rhinacanthin-C content and pH of topical solution containing rhinacanthin-C enriched extract

Weeks	Rhinacanthin-C content (% remaining; Mean ± S.D.)			pH		
	4 ± 2°C	25 ± 2°C	45 ± 2°C, 75% RH	4 ± 2°C	25 ± 2°C	45 ± 2°C 75% RH
0	99.94 ± 2.13^{a}	99.94 ± 2.13^{a}	99.94 ± 2.13^{a}	6.35 ± 0.02^{a}	6.35 ± 0.02^{a}	6.35 ± 0.02^{a}
2	100.76 ± 2.72^{a}	100.51 ± 0.72^{a}	97.08 ± 1.36^{a}	6.28 ± 0.02^{b}	6.26 ± 0.02^{bc}	6.22 ± 0.02^{b}
4	100.31 ± 4.20^{a}	98.67 ± 3.18^{ab}	93.49 ± 1.87^{ab}	6.32 ± 0.01^{ab}	6.28 ± 0.01^{b}	6.18 ± 0.01^{b}
8	100.38 ± 0.97^{a}	93.04 ± 1.00^{b}	88.72 ± 4.43^{b}	6.30 ± 0.02^{b}	6.27 ± 0.02^{bc}	6.22 ± 0.05^{b}
12	99.08 ± 2.78^{a}	92.72 ± 2.52^{b}	88.37 ± 3.31^{b}	6.30 ± 0.01^{b}	6.26 ± 0.01^{bc}	6.07 ± 0.01^{c}
16	93.07 ± 4.51^{a}	86.05 ± 2.39^{c}	77.34 ± 3.77^{c}	6.31 ± 0.01^{ab}	6.23 ± 0.01^{c}	6.02 ± 0.07^{c}

The results were expressed as Mean ± S.D. of three determinations for each sample. Means followed by the same letter in the same column are not significantly different according to one-way analysis of variance (ANOVA), followed by Tukey's HSD test. The term significant has been used to denote the differences for which $P < 0.05$.

Limitation of the study

In the present study, rhinacanthin-C enriched *R. nasutus* leaf extract was prepared by 25% v/v glycerol in ethanol, with a leaf powder to solvent ratio of 15: 100 (g/mL), with maceration as green extraction method at 125 rpm for 72 h. This was used for formulation of topical solution containing rhinacanthin-C enriched extract, antifungal activity assay and stability tests. The selection of maceration over MAE was based on comparison of maceration and MAE experiment for 100 mL solvent, which showed no significant difference in the rhinacanthin-C content in between the processes and moreover content decreased when extracted by MAE for 2 min.

However, when the comparison between maceration and MAE was done with 600 mL of solvent, power 600 Watt for 8 min 30 s gave significantly high rhinacanthin-C content up to 3.15 mg/mL. The experiment provided clear evidence that MAE is capable of increasing in the chemical constituents and decrease time of extraction. The use of maceration as green extraction method is the limitation of this study. Therefore, it is recommended to use MAE as green extraction method for preparation of rhinacanthin-C enriched *R. nasutus* leaf extract for future studies.

CHAPTER 5

CONCLUSION

5.1 Solution of 25% v/v glycerol in ethanol was selected as an alternative green solvent for extraction of rhinacanthin-C from *R. nasutus* dried leaf powders. The suitable extraction conditions were extraction by maceration for 72 h, and using a dried leaf powder to solvent ratio of 15:100 (g/mL). These extraction conditions produced rhinacanthin-C enriched *R. nasutus* leaf extracts, with rhinacanthin-C content up to 1.79 mg/mL.

5.2 An innovative green extraction method, microwave assisted extraction or MAE (power of 600 Watt, for 8 min 30 s) could be used alternatively to the maceration method for preparation of rhinacanthin-C enriched *R. nasutus* leaf extracts. The MAE significantly increased rhinacanthin-C content up to 3.15 mg/mL, and markedly reduced the extraction time.

5.3 The rhinacanthin-C enriched *R. nasutus* leaf extracts were standardized to contain rhinacanthin-C content of not less than 1.7 mg/mL, and directly used for formulation of 0.1% w/v rhinacanthin-C topical solutions without solvent evaporation process prior to formulation.

5.4 Three formulations (Rx 1 to Rx 3) of topical solution containing rhinacanthin-C enriched extract were prepared from rhinacanthin-C enriched *R. nasutus* leaf extracts. All preparations were brown coloured solutions with a pleasant odor. The formula Rx 1 that contained the lowest water content (10% v/v) was selected for further stability evaluation.

5.5 On the basis of agar well diffusion assay, topical solution containing rhinacanthin-C enriched extract showed satisfactory antifungal activity against *T. rubrum* with inhibition zone of 17.90 mm. In addition, based on agar dilution method, the standardized rhinacanthin-C enriched *R. nasutus* leaf extracts and topical solution containing rhinacanthin-C enriched extract possessed antifungal activity against *T. rubrum* with MIC values of 8.6 and 28.5 mg/mL, at dilutions of 1:64 and 1:32, respectively.

5.6 Stability evaluations of rhinacanthin-C in the standardized rhinacanthin-C enriched *R. nasutus* leaf extracts and topical solution containing rhinacanthin-C enriched extract through a period of 4 months found that both of them were stable over a period of four months when stored in well-closed containers protected from light at 4°C. In contrast, at 25°C and accelerated conditions (45°C, 75% RH), rhinacanthin-C content in both extracts and topical solutions decreased significantly after 8 weeks. However, the remained amount of rhinacanthin-C in the extracts and topical solutions were 92 and 86%, respectively after kept at 25 ± 2 °C for 4 months. In addition, the remained amount of rhinacanthin-C in both extracts and topical solutions were 77% after kept in the accelerated conditions for 4 months. This implied that the content of rhinacanthin-C in the extracts and topical solutions may decrease by 23% after kept at room temperature for two years.

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APPENDIX

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List of Publication and Proceeding

Shakya, K. and Panichayupakaranant, P. (2014). Preparation of Rhinacanthin-C Extract from *Rhinacanthus nasutus* Leaves Using Green Extraction Concept. The 3rd Current Drug Development International Conference, Ao Nang Beach Resort, Ao Nang, Krabi, Thailand.

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