CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant material

Leaves of *R. nasutus* were collected from the Botanical Garden, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand in September 2006. They were authenticated at the Herbarium of the Southern Center of Traditional Medicine, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand, where herbarium specimen (Voucher No. 001 18 14) is kept.

3.1.2 Chemicals and reagents

3.1.2.1 For quantitative determination of rhinacanthins in the extract

Methanol, HPLC grade (Lab scan Asia co., Thailand)

Water was purified in a Milli-Q system (Millipore, Bedford, MA, USA)

Acetic acid, glacial AR grade (Lab scan Asia co., Thailand)

3.1.2.2 For extraction method and pre-purification method

Methanol, commercial grade (Lab scan Asia co., Thailand)

Ethyl acetate, commercial grade (Lab scan Asia co., Thailand)

Acetic acid, glacial AR grade (Lab scan Asia co., Thailand)

Amberlite® IRA-67 (SIGMA, USA)

3.1.2.3 For antifungal activity assay

Sabouraud Dextrose Agar (SDA) (Becton, Dickinson, France)

Sodium chloride, AR grade (Lab scan Asia co., Thailand)

Dimethyl sulfoxide (DMSO) AR grade (Riedel-de Haen, Germany)

T. rubrum, *T. mentagrophytes*, and *M. gypseum* were provided from Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University.

3.1.2.4 For establishment of the standard specification of extract

Methanol, AR grade (Lab scan Asia co., Thailand)

Ethyl acetate, AR grade (Lab scan Asia co., Thailand)

Ethanol, AR grade (Lab scan Asia co., Thailand)

Hexane, AR grade (Lab scan Asia co., Thailand)

Chloroform, AR grade (Lab scan Asia co., Thailand)

Dimethyl sulfoxide (DMSO) AR grade (Riedel-de Haen, Germany)

Acetic acid, glacial AR grade (Lab scan Asia co., Thailand)

n-Octanol AR grade (Riedel-de Haen, Germany)

Eosin Methylene Blue Agar (EMB) (Becton, Dickinson, France)

Sabouraud Dextrose Agar (SDA) (Becton, Dickinson, France)

Melted Plate Count Agar (PCA) (Becton, Dickinson, France)

Peptone (Becton, Dickinson, France)

Anhydrous *di*-sodium hydrogen orthophosphate (Anala R®, England)

Potassium *di*-hydrogen orthophosphate (M & B, England)

di-potassium hydrogen orthophosphate (M & B, England)

Standard rhinacanthin-D, -C, and -N were previously purified by Assoc. Prof. Dr. Pharkphoom Panichayupakaranant (Panichayupakaranant, 2002) and were identified by CHNO-elemental analysis. The CHNO-elemental analysis have been performed by CHNS-O analyzer.

3.1.3 Instrumentation

Instrument	Model	Company
UV-visible spectrophotometer	Genesis 5	Miltonroy, USA
Hot air over	DIN 12880-KI	Memmert, Germany
HPLC	Agilent 1100 series	Palo Alto, CA
HPLC column	ODS-80Ts	TSK-GEL™, Japan
pH meter	PHM 82	Radiometer, Denmark
Evaporator	N 1000	EYELA, Japan
Moisture analyzer	Satorious MA 100	Scientific Promotion, Germany
Muffle furnace	85P	Barkmey Division, U.S.A.
Vortex	G-560E	Scientific Industries, U.S.A.
Lamina air flow	HT-122	International Scientific Supply, Thailand
Hot plate	SLR	Schott Gerate, Germany
Centrifugal vacuum evaporator	AS 260	Global Medical Instrumentation, U.S.A.
Reciprocator	Heto RK 20-VS	Heto-Holten AIS, Denmark
CHNS-O analyzer	Flash 1112 series	CE instruments, Italy

3.2 Methods

3.2.1 Quantitative analysis of rhinacanthins in *R. nasutus* leaf extracts

3.2.1.1 Preparation of standard solutions

Standard rhinacanthin-C, -D, and -N were previously purified and identified (Panichayupakaranant, 2002). Separate stock solutions of the reference standards, rhinacanthin-C, -D, and -N were made in methanol. A working solution of the combined standards was subsequently prepared in methanol and diluted to provide series solution of rhinacanthin-C ranging from 12.6 - 201.0 µg/mL and rhinacanthin-D and -N ranging from 3.1 - 51.0 µg/mL, for use in constructing calibration curves for each of the target analytes.

3.2.1.2 Sample preparation

The leaves of *R. nasutus* were dried in hot air oven at 50 ^{o}C. The dried leaf powder of *R. nasutus* (100 mg) was extracted with ethyl acetate (20 mL) under reflux condition for an hour. The extracts were filtered and then concentrated under reduced pressure. The sample was reconstituted and adjusted to 10 mL with methanol. Samples were analyzed immediately after extraction in order to avoid possible chemical degradation. The experiments were in triplicate.

3.2.1.3 HPLC conditions

HPLC analysis was carried out using Agilent 1100 series equipped with Agilent 1100 series Photodiode-arrays detector (PDA) and autosampler. Data analysis was performed using Agilent software (Agilent, U.S.A). Separation was achieved isocratically at 25 ^{o}C on a TSK-gel ODS-80Ts column (150 mm x 4.6 mm i.d.). The mobile phase consisted of methanol and water (contaning 5 % of acetic acid) in ratio of 80:20 (v/v). The flow rate was 1 mL/min and the injection volume was 20 µL. The quantitative wavelength was set at 254 nm.

3.2.1.4 Method validation (Association of Official Analytical Chemists, 2002)

Calibration curve

Calibration curves were constructed on three consecutive days by analysis of a mixture containing each of the standard compounds at five concentrations and plotted peak area against the concentration of each reference standards. The linearity of the detector response for the standards was assessed by means of linear regression. The curves showed coefficient of correlation (r^2) $\geq$ 0.99 is the acceptable criteria.

Accuracy

The accuracy of the procedure was demonstrated by the recovery studies, which were carried out by fortify sample extract with the standard solutions. The amount of each analytes was determined in triplicate and percentage recoveries were then calculated. The percentage recoveries of the analytes need not to be 100 %, but the extent of recovery of an analytes should be consistent and precise.

Precision

Precision experiments were conducted for intraday and interday. The solution of one sample was used to achieve repeatability testing. The data used to calculate percentage relative standard deviation (%R.S.D.) of interday precision was the content of six samples analyzed in three days (six injections in succession each day). The data of repeatability was the content of six injections separately in the same day.

Specificity

The identification of the peaks was carried out using the standards and PDA. The UV spectra were taken at various points of the peaks to check the homogeneity of the peaks.

LOD and LOQ

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. LOD and LOQ were determined by means of serial dilution based on signal-to-noise ratios of 3:1 and 10:1, respectively.

3.2.2 Preparation of rhinacanthin high-yielding *R. nasutus* leaf extract

3.2.2.1 Extraction

The dried leaf powder of *R. nasutus* (2 kg) was extracted with ethyl acetate (8 L) by maceration for 4 days (x 3). The pooled extracts were dried *in vacuo* (Kongchai and Panichayupakaranat, 2002).

3.2.2.2 Fractionation

An adequate volume of methanol (500 mL) was added into 1,000 g anion exchange resin (Amberlite® IRA-67) and gently stirred. After allowed to stand for 15 min, the methanol was decanted and the slurry was washed twice with distilled water (2 x 500 mL), and then allowed to stand in methanol for a further 5-10 min. The treated resin was poured into a glass column (5 x 35 cm) and the excess methanol was drained. A portion of methanol (1,000 mL) was then added to settle the resin. The leaf extract of *R. nasutus* (50 g) was dissolved in methanol (3,000 mL) and filtered. The filtrate was then loaded on the anion exchange column and allowed the solution to pass through the column with a flow rate of 1.5 mL/min until finish. The column was then washed with methanol until the green color in the column was disappeared. Rhinacanthins were then eluted with 10 % acetic acid in methanol with a flow rate 2 mL/min. The eluent was evaporated to dryness *in vaccuo* (Kongchai and Panichayupakaranat, 2002).

3.2.3 Evaluation of antifungal activity against dermatophytes (Lorian, 1996)

3.2.3.1 Preparation of sample

The stock solution of the rhinacanthin high-yielding *R. nasutus* leaf extract was prepared at the concentration of 100 mg/mL in DMSO. The sample solutions were prepared in the concentration between 0.393 and 100 mg/mL by serial two-fold dilution. The samples were

sterilized by filtration through a 0.45 μm membrane filter before testing. Clotrimazole and DMSO were used as positive and negative controls, respectively.

3.2.3.2 Preparation of test dermatophytes

Trichophyton mentagrophytes, *Trichophyton rubrum* and *Microsporum gypseum* were grown in Sabouraud Dextrose Agar slant. The selected colonies were mixed with sterile physiological saline and the turbidity was adjusted by adding sterile physiological saline until a McFarland turbidity standard of 0.5 (10^6 colony forming units per mL).

3.2.3.3 Determination of minimum inhibition concentration

Minimum inhibition concentration (MIC) was determined using agar dilution method (Lorian, 1996). The sample solutions were diluted with Sabouraud Dextrose Agar (1:100) to obtain the final concentrations between 3.93 and 1,000 μg/mL, and transferred into a 96-wells plate (150 μL/well). The tested dermatophytes were also diluted with Sabouraud Dextrose Broth (1:100) to achieve 10^4 cells/mL, and transferred into the 96-wells plate (2 μL/well). The plate was incubated at 30 ºC for 7 day. The lowest concentration that did not show any growth of dermatophytes was taken as the MIC.

3.2.4 Determination of the moisture content

(Subcommittee on the establishment of the Thai herbal pharmacopoeia, 1995)

Moisture content of the rhinacanthin high-yielding *R. nasutus* leaf extract was performed using loss on drying method. The extract was accurately weighed to 0.5 g in the pan of the Sartorius Moisture Analyzer and dried at 105 °C for 4-8 minutes until the weight was constant. The percentage loss on drying of the test sample was automatically recorded. The analyses were in triplicate.

3.2.5 Ash content

The total ash and acid insoluble ash contents of Thai Herbal Pharmacopoiea have been widely used as one of the indices to illustrate the quality as well as purity of herbal medicine (Subcommittee on the establishment of the Thai herbal pharmacopoeia, 1995).

3.2.5.1 Determination of the total ash content

The extract was accurately weighed to 0.5 g and placed in a tarred crucible, which was previously ignited, cooled and weighed. The sample was incinerated by gradually increasing the temperature not exceeding 450 °C in muffle furnace until free from carbon, then cooled and weighed. The percentage of the total ash was calculated with reference to the weight of the extract. The analyses were in triplicate.

3.2.5.2 Determination of the acid insoluble ash content

The residue from total ash would be used to determine acid insoluble ash content. The total ash was solved in 25 mL of 10 % HCl and boiled on hot plate for 5 minutes. Then the sample was filtrated with ashless paper no. 42. The paper was washed with distilled water until pH became neutral. Then, the paper was placed in a tarred crucible, which was previously ignited, cooled and weighed. The sample was incinerated by gradually increasing the temperature not exceeding 550 °C in muffle furnace until free from carbon, then cooled and weighed. The percentage of the acid insoluble ash was calculated with reference to the weight of the residue from total ash. The analyses were in triplicate.

3.2.6 Determination of the microbial contamination

(British Pharmacopoeia commission, 2001)

3.2.6.1 Sample preparation

The extract was accurately weighed to 1 g and dissolved in 0.1 % (v/v) peptone water 9 mL, then adjusted to 10 mL with 0.1 % (v/v) peptone water. The stock solution was diluted with 0.1 % (v/v) peptone water to provide five concentrations ranging from 10^{-1}-10^{-5} g/mL.

3.2.6.2 Determination of aerobic bacteria contamination

Determination of aerobic bacteria contamination was performed by pour plate method. The sample solution (1 mL) as well as melted Plate Count Agar (15 mL to 20 mL) were added into Petri dishes (9 cm in diameter), and mixed until homogenously. At least two Petri dishes were prepared for each level of dilution. The plates were incubated at 37 ºC for 3 days. The plates corresponding to one dilution and showing the highest number of colonies less than 300 were selected. The number of colony-forming units per gram was calculated.

3.2.6.3 Determination of *Escherichia coli* contamination

Determination of *E. coli* contamination was performed by pour plate method. The sample solution (1 mL) as well as Eosin Methylene Blue Agar (15 mL to 20 mL) were added into Petri dishes (9 cm in diameter), and mixed until homogenously. At least two Petri dishes were prepared for each level of dilution. The plates were incubated at 37 ºC for 1 day. Growth of red, non-mucoid colonies of gram-negative rods indicates the possible presence of *E. coli*. The plates corresponding to one dilution and showing the highest number of colonies less than 300 were selected. The number of colony-forming units per gram was calculated.

3.2.6.4 Determination of fungi contamination

Determination of fungi contamination was performed by pour plate method. The sample solution (1 mL) as well as Sabouraud Dextrose Agar (15 mL to 20 mL) were added into Petri dishes (9 cm in diameter), and mixed until homogenously. At least two Petri dishes were prepared for each level of dilution. The plates were incubated at 20 - 25 ºC for 5 days. The plates corresponding to one dilution and showing the highest number of colonies less than 300 were selected. The number of colony-forming units per gram was calculated.

3.2.7 Determination of solubility

The extract was accurately weighed to 10 mg and place in a vessel of at least 100 ml capacity. The vessel was placed in a constant temperature device, maintained at a temperature of 25 ± 0.2 ºC. Various solvents (water, ethanol, methanol, ethyl acetate, chloroform and hexane) were examined by adding of the strength prescribed in the monograph by increments of 10 µL, shaking frequently and vigorously for 10 minutes. Record the volume of solvent added when a clear solution was obtained. If the solution was becomes cloudy or undissolved. The sample was continuously added until 10 mL. After addition of 10 mL of solvents, the sample or parts of it remained undissolved, the experiment had to be repeated in a 100 mL volumetric flask. At lower solubility, the time required to dissolve a substance can be considerably longer, at least 24 hours should be allowed (British Pharmacopoeia Commission, 2001; Organization for Economic Cooperation and Development, 1995).

Descriptive term of solubility and approximate volume of solvents required to completely dissolve a solute (in milliliters per gram of solute) are drawn as follow.

Solubility term	Volume of solvent required To dissolve 1 g of solute (mL)
Very soluble	less than 1
Freely soluble	from 1 to 10
Soluble	from 10 to 30
Sparingly soluble	from 30 to 100
Slightly soluble	from 100 to 1000
Very slightly soluble	from 1000 to 10,000
Practically insoluble	more than 10,000

The term partly soluble is used to describe a mixture of which only some of the components dissolve.

3.2.8 Partition coefficient

(Organization for Economic Cooperation and Development, 1995)

3.2.8.1 Pre-saturation of the solvents

Before a partition coefficient was determined, the phases of the solvent system were mutually saturated by shaking at the temperature of the experiment. It was practical to shake two large stock bottles of either *n*-octanol or water with a sufficient quantity of the other solvent for 24 hours on a mechanical shaker and then let them stand long enough to allow the phases to separate and to achieve a saturation state.

3.2.8.2 Test substance

The stock solution of the rhinacanthin high-yielding *R. nasutus* leaf extract was prepared in *n*-octanol that pre-saturated with water to produce 3 concentrations of the extract. The concentration of this stock solution should be precisely determined before it is employed in the determination of the partition coefficient. This solution should be stored under conditions which ensure its stability. Triplicate test vessels containing the required, accurately measured amounts of the two solvents together with the necessary quantity of the stock solution should be prepared for each of the test conditions. The *n*-octanol phase should be measured by volume. The test vessels were placed in a reciprocator and rotating quickly through 360° about its transverse axis so that any trapped air rises through the two phases. Experience has shown that 50 such rotations are usually sufficient for the establishment of the partition equilibrium. To be certain, 100 rotations in five minutes are recommended. For the determination of the partition coefficient, only the concentration in *n*-octanol was determined with the concentration in the aqueous phase calculated by difference. An aliquot of *n*-octanol phase was taken, reconstituted with methanol, and analyzed by HPLC. The total rhinacanthin content in both phases should be calculated and compared with the rhinacanthin content originally introduced by this equation.

$$K = \frac{C_{n-\text{octanol}}}{C_{\text{water}}}$$

K	=	Partition coefficient
$C_{\text{n-octanol}}$	=	Concentration of rhinacanthins in *n*-octanol phase (µg/mL)
C_{water}	=	Concentration of rhinacanthins in water phase (µg/mL)

3.2.9 Stability test

3.2.9.1 Effect of photo on stability of the extract

The rhinacanthin high-yielding *R. nasutus* leaf extracts were weighed to 100 mg and kept in well-closed containers. The extracts were then stored at room temperature (30 ± 2 ºC) and under exposed to light (36-watt fluorescent lamp and 40 cm distance from the containers). An aliquot of each sample was taken at 0, 1, 2, 3, 4, 6, 8, 12, and 16 weeks and subjected to quantitative analysis of the rhinacanthins using HPLC. The experiments were in triplicate.

3.2.9.2 Effect of temperature on stability of the extract

The rhinacanthin high-yielding *R. nasutus* leaf extracts were weighed to 100 mg and kept in well-closed containers, protected from light. The extracts were then stored at 4 ± 2 ºC and room temperature (30 ± 2 ºC). Prepare for each temperature at three replicates. An aliquot of each sample was taken at 0, 1, 2, 3, 4, 6, 8, 12, and 16 weeks and subjected to quantitative analysis of the rhinacanthins using HPLC. The experiments were in triplicate.

3.2.9.3 Effect of accelerated conditions for stability of the extract

The rhinacanthin high-yielding *R. nasutus* leaf extracts were weighed to 100 mg and kept in well-closed containers, protected from light. The extracts were then stored in a stability chamber at 45 ºC, 75 % humidity for 4 month. An aliquot of each sample was taken at 0, 1, 2, 3, 4, 6, 8, 12, and 16 weeks and subjected to quantitative analysis of the rhinacanthins using HPLC. The experiments were in triplicate.

3.2.9.4 Effect of pH on stability of the extract

The rhinacanthin high-yielding *R. nasutus* leaf extracts were accurately weighed to 10 mg and dissolved in the solution of methanol and phosphate buffer solution pH 5.5, 7.0, and 8.0 in the ratio of 1: 1. The sample solutions were kept in well-closed containers, protected from light and stored at room temperature (30 ± 2 ºC) for 4 month. An aliquot of each sample was taken at 0, 1, 2, 3, 4, 6, 8, 12, and 16 weeks and subjected to quantitative analysis of the rhinacanthins using HPLC. The experiments were in triplicate.

3.2.10 Statistic

Values are expressed as mean ± S.D. Data were analyzed by student *t*-test. The level of statistical significance was taken at $P < 0.05$.