CHAPTER 2

MATERIALS AND METHODS

2.1 Material

2.1.1 Plant materials

Eighteen plant materials used in this study are shown in Table 2.1. Plant materials were collected from various locations in Thailand; especially in Songkhla, and voucher specimens are deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University.

Table 2.1 Eighteen Thai medicinal plants

Sciencetific name	Herbarium No.	Part used	Collected location
Alpinia galanga (Zingiberaceae)	SKP 206 01 07 01	Rhizomes	Songkhla
Andrographis paniculata (Acanthaceae)	SKP 001 01 16 01	Leaves	Chiangrai
Azadirachta indica (Meliaceae)	SKP 112 01 09 01	Leaves	Songkhla
Boesenbergia pandurata (Zingiberaceae)	SKP 206 02 16 01	Rhizomes	Songkhla
Senna alata (Leguminosae)	SKP 097.1 03 01 01	Leaves	Songkhla
Centella asiatica (Umbelliferae)	SKP 199 03 01 01	Leaves	Songkhla
Cymbopogon citratus (Gramineae)	SKP 081 03 03 01	Stalks	Songkhla
Cinnamomum verum	SKP 096 03 22 01	Bark	Songkhla

(Lauraceae)			
Dioscorea membranacea (Dioscoreaceae)	SKP 062 04 13 01	Rhizomes	Songkhla

Sciencetific name	Herbarium No.	Part used	Collected location
Morus alba (Moraceae)	SKP 117 13 01 01	Leaves	Chiang-Mai
Ocimum americanum (Labiatae)	SKP 095 15 01 01	Leaves	Songkhla
Ocimum sanctum (Labiatae)	SKP 095 15 19 01	Leaves	Songkhla
Piper betle (Piperaceae)	SKP 146 16 02 01	Leaves	Songkhla
Plumbago zeylanica (Plumbaginaceae)	SKP 148 16 26 01	Roots	Songkhla
Punica granatum (Punicaceae)	SKP 158 16 07 01	Peel	Yunnan, China
Rhinacanthus nasutus (Acanthaceae)	SKP 001 18 14 01	Leaves	Songkhla
Syzygium aromaticum (Myrtaceae)	SKP 123 19 01 01	Flowers(buds)	Songkhla
Zingiber officinalis (Zingiberaceae)	SKP 206 26 15 01	Rhizomes	Songkhla

2.1.2 Microorganisms and media

Propionibacterium acnes (DMST 14916) was obtained from Department of Medical Science Center, Nonthaburi, Thailand. *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 14990) were obtained from Department of Microbiology, Faculty of Medicine and Thailand Institute of Scientific and Technological Research, respectively. Mueller-Hinton agar and Mueller-Hinton broth were purchased from Merck. *P.*

acnes was storaged in glycerol broth at -20 °C and cultured twice in Mueller-Hinton agar supplemented with 5% blood before used.

2.1.3 Chemicals

All solvents for general purposes were commercial grade and redistilled prior to use. The solvent for HPLC analysis was HPLC grade from Merck. The chemicals for formulation study were kindly provided from the Department of Pharmaceutical Technology and Department of Pharmacognosy and Pharmaceutical botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. Clindalin gel 1% (Union Drug Laboratories, Ltd.) was purchased from a drugstore. Silica gel precoated aluminium sheets, silica gel and Sephadex LH-20 were perchased from Merck, Germany. Standard tetracycline hydrochloride was kindly provided from Department of Medical Science Center, Nonthaburi. Standard tetracycline paper disc was purchased from Oxoid Ltd, England.

2.1.4 General instrumental equipment

^{1}H and ^{13}C NMR spectra were recorded on a Fourier Transform NMR Spectrometer 500 MHz, model UNITY INOVA, Varian.

IR spectra with neat technique were recorded on a Fourier Transform Infrared Spectrometer Model EQUINOX 55, Bruker.

Low resolution electron ionization mass spectrometry was recorded on a MAT 95 XL Mass Spectrometer, Thermofinnigan.

Quantitative determination of active substances was performed using HPLC Agilent series 1100.

pH of cream was measured with pH meter, Orion Model 410A

Viscosity was measured using viscometer, Brookfieled dial reading model RVT

Stability test was tested in Stability Chamber HI 150, Q. nic CO. LTD.

2.2 Methods

2.2.1 Preparation of plant extracts

Eighteen Thai medicinal plants, which have been traditionally used as antimicrobial or anti-inflammatory agents were examined for antibacterial activity against *P. acnes*.

Eighteen plants (Table 2.1) were washed with water, cut into small pieces and then dried in a hot air oven at 50° C for 48 hours and then powdered. The dried powders were macerated with ethyl acetate for 3 days (x2) and then with methanol for 3 days (x2). The solvents were removed under reduced pressure. Thirty-six plant extracts were obtained and their dried weights were recorded. All extracts were kept in a desiccator.

2.2.2 Evaluation of antibacterial activity against *Propionibacterium acnes*

2.2.2.1 Antibacterial activity screening

Disk diffusion method was used to test the susceptibility for antibacterial activity against *P. acnes*. This experiment was performed by the method of National Committee for Clinical Laboratory Standards with some modifications. *P. acnes* from glycerol broth was streaked and incubated in Mueller-Hinton agar (MHA) supplemented with 5% blood for 72 h under anaerobic condition. Then selected at least four well-isolated colonies of the same type from a culture agar plate, touched the top of each colony with a loop and transferred to a tube containing 0.85% sterile saline and adjusted the turbidity of the actively growing sterile saline to obtain a turbidity to yield 10^8 CFU/ml comparable to the 0.5 McFarland turbidity standard. A sterile cotton swab was dipped in the inoculum and the excess was removed by rotating the swab several times against the inside wall of the tube above the fluid level. The surface of MHA supplemented with 5% blood plate was inoculated by streaking the swab over the surface. Streaking was repeated three times, and each time the plate was rotated 60°. This ensured an even distribution of inoculum. A sterile paper disk (∅ 6 mm, Schleicher & Schuell) was impregnated with 36 plant extracts (10 μl) and the disk was applied with forceps and pressed down with slight pressure on the agar. The concentration of each plant extract was 500 mg/ml (5 mg/disk), dissolved in DMSO. Control disks were similarly prepared using DMSO as a negative control and standard tetracycline disc 30 μg/disk as a positive control. Inoculated plates were incubated at 37 °C for 72 h under anaerobic condition in an inverted position (Figure 2.1). The antibacterial

activity was expressed as the mean of inhibition diameter (mm, including the diameter of disk) (National Committee for Clinical Laboratory Standards, 1993; Lorian, 1996).

Figure 2.1 Anaerobic equipment

2.2.2.2 Minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations (MIC) was defined as the lowest concentration of the compound to inhibit the growth of microorganisms.

A modified microdilution method was used to determine MIC of the plant extracts that produced inhibition zones against *P. acnes*. MICs were determined by turbidity and Alamar blue assay methods.

Turbidity method

A sequential two-fold dilution method was used in MIC test. The plant extracts that showed inhibitory effect against *P. acnes* was dissolved in DMSO at the concentration of 100 mg/ml and diluted with Mueller-Hinton broth (MHB) to the concentration of 10 mg/ml. Tetracycline hydrochloride, a positive control, was diluted in sterile water to a concentration of 64 μg/ml and filtered through 0.45 micron sterile filter paper. The test was performed in 96-well plate. Two-fold dilutions were prepared directly in wells, as follows: 100 μl of the working solution of drug or plant extracts was added to well 1 of the dilution series. To each remaining well, 50 μl of MHB is added. With a sterile pipette, 50 μl was transferred from well 1 to well 2. After thorough mixing, 50 μl was transferred (with a separate pipette for this and each succeeding transfer) to well 3. This process was continued to the next to last tube, from which 50 μl was removed and discarded. The last well received no antimicrobial agent and served as a growth control. The stock solution of tetracycline hydrochloride was diluted with MHB to give the concentrations of 64 to 0.125 μg/ml (two-fold dilution serial). The stock solution of the plant extracts were diluted with MHB to give the concentration of 10000 to 19.5 μg/ml. The inoculum was prepared and adjusted, as noted in 2.2.2.1, to contain 10^8 CFU/ml, by adjusting the turbidity of saline culture to match the McFarland 0.5 standard. It was then further diluted 1:100 in MHB to contain 10^6 CFU/ml and 50 μl of the adjusted inoculum was added to each well. The final concentrations of tetracycline HCl and plant extracts were half of those initial dilution series because of the addition of an equal volume of inoculum in broth, 32 to 0.0625 μg/ml and 5000 to 9.75 μg/ml, respectively. The final concentration of *P. acnes* in each well was 5x 10^5 CFU/ml. The cultures were then incubated at 37 °C for 72 h under anaerobic condition. The lowest concentration that did not show any growth (turbidity was not observed) of *P. acnes* was taken as the MIC, and confirmed with colorimetric method using Alamar blue as an indicator.

Alamar blue assay method

The Alamar blue™ assay is designed to measure quantitatively the proliferation of various human and animal cell lines, bacteria and fungi. It incorporates a fluorometric/colorimetric growth indicator based on detection of metabolic activity. Specifically, the system incorporates an oxidation-reduction (REDOX) indicator that both fluoresces and change color in response to chemical reduction of growth medium resulting from cell growth.

Alamar blue solution was added into MHB with the ratio of MHB:Alamar blue was 10 ml : 100 μl, and added into each well and then incubated at 37 °C for 5-10 h. The color of the culture media that showed the growth of bacteria was pink, while that did not show any growth was still blue.

2.2.2.3 Minimum bactericidal concentration (MBC)

The minimum bactericidal concentrations (MBC) was defined as the lowest concentration of the compound to kill microorganisms.

The incubation mixtures that showed positive result of inhibitory effect were streaked on MHA supplemented with 5% blood and incubated at 37 °C for 72 h under anaerobic conditions. The lowest concentration that did not show any growth was taken as the MBC.

2.2.3 Evaluation of antibacterial activity against *Staphylococcus* spp.

The selected plant extract was subjected to antibacterial activity evaluation against *Staphylococcus aureus* and *S. epidermidis*.

2.2.3.1 Antibacterial activity screening

Disk diffusion method was used to test the susceptibility for antibacterial activity against *S. aureus* and *S. epidermidis*. This experiment was performed by the method of National Committee for Clinical Laboratory Standards with some modifications. *S. aureus* and *S. epidermidis* were streaked and incubated in MHA for 24 h under aerobic condition. Turbidity adjustment of the inoculum was described in 2.2.2.1. A sterile cotton swab was dipped in the inoculum. The surface of MHA plate was inoculated by streaking the swab over the surface. Streaking was repeated three times, and each time the plate was rotated 60°. A sterile paper disk (∅ 6 mm) was impregnated with the selected extract (10 μl) and the disk was applied with forceps and pressed down with slight pressure on the agar. The concentration of the selected extract was 200 mg/ml (2 mg/disk), dissolved in DMSO. Control disks were similarly prepared using DMSO as a negative control and standard tetracycline disc (30 μg/disk) as a positive control. Inoculated plates were incubated at 37 °C for 24 h under aerobic condition in an inverted position. The antibacterial activity was expressed as the mean of inhibition diameter (mm,

including the diameter of disk) (National Committee for Clinical Laboratory Standards, 1993; Lorian, 1996).

2.2.3.2 Minimum inhibitory concentration (MIC)

A broth microdilution assay was performed for the determination of the MIC (National Committee for Clinical Laboratory Standards, 1993). All tests were performed in MHB. A sequential two-fold dilution method was used in MIC test. The selected extract was diluted in DMSO with the concentration of 20 mg/ml and diluted with MHB to the concentration of 2 mg/ml. Tetracycline hydrochloride, a positive control, was diluted in sterile water to a concentration of 64 μg/ml and filtered through 0.45 micron sterile filter paper. The test was performed in 96-well plate. Two-fold dilution method was described in 2.2.2.2. The stock solution of tetracycline hydrochloride was diluted with MHB to give the concentrations of 64 to 0.125 μg/ml (two-fold dilution serial). The stock solution of the selected extract was diluted with MHB to give the concentration of 2000 to 3.9 μg/ml. The inoculum was prepared and adjusted, as described in 2.2.2.1, to contain 10^8 CFU/ml, by adjusting the turbidity of saline culture to match the McFarland 0.5 standard. It was then further diluted 1:100 in MHB to contain 10^6 CFU/ml and 50 μl of the adjusted inoculum was added to each well. The final concentrations of tetracycline HCl and selected extract were 32 to 0.0625 μg/ml and 1000 to 1.95 μg/ml, respectively. The final concentration of *S. aureus* and *S. epidermidis* in each well was 5x 10^5 CFU/ml. The cultures were then incubated at 37 $^{\circ}$C for 24 h under aerobic condition. The MIC was calculated as the highest dilution showing complete inhibition of the test strains.

2.2.3.3 Minimum bactericidal concentration (MBC)

Referring to the results of the MIC assay, the wells showing complete absence of growth were identified and the incubation mixtures of each well were streaked on agar plate (MHA) and incubated at previously mentioned times and temperatures. The complete absence of growth was considered as the minimum bactericidal concentration.

2.2.4 Bioassay-guided isolation of *Alpinia galanga* extract

2.2.4.1 Preparation of *Alpinia galanga* extract

A. galanga dried powder (980 g) was macerated with ethyl acetate for 3 days (3700 ml x 3). The pooled extract was concentrated *in vaccuo* to dryness. The dried weight was then recorded.

2.2.4.2 Isolation of the active compound

The ethyl acetate extract of *A. galanga* rhizome (44.1 g) was triturated with silica gel (1:1) and loaded into column (13 cm-diameter). The column was then eluted with hexane (500 ml/fraction) with the aid of vacuum pump until the eluent was colorless. The solvent was then changed to chloroform, ethyl acetate, and methanol, respectively, and elution was done in the same manner. The collected fractions were pooled with the aid of their thin layer chromatographic (TLC) chromatograms and subjected to evaluation of antibacterial activity against *P. acnes* as described in 2.2.2.2.

The pooled active fraction (fraction 2) was further purified by Sephadex LH-20 gelfiltration chromatography eluted with methanol (20 ml/fraction). The collected fractions were pooled with the aid of their TLC chromatograms and subjected to evaluation of antibacterial activity against *P. acnes* as described in 2.2.2.2

The pooled active fraction (fraction I) obtained from Sephadex LH-20 gelfiltration chromatography was further purified by silica gel colum chromatography eluted with a mixture of chloroform and hexane (1:1, 30 ml/fraction). An oily liquid; AP1 (696 mg) was obtained from the pooled active fraction (fraction C). The active compound isolated from *A. galanga* extract was identified by ^{1}H and ^{13}C, NMR, IR and mass spectrometry.

2.2.5 Quantitative determination of 1′-actoxychavicol acetate (1′-ACA)

1′-ACA isolated from *A. galanga* as previously described (2.2.4) was used as the authentic compound for quantitative determination of 1′-acetoxychavicol acetate in the *A. galanga* extract. The calibration curve of 1′-acetoxychavicol acetate was established from the authentic compound at the concentration range between 0.0625 to 20 mg/ml. The conditions of HPLC were described below. All samples were analyzed in triplicate.

HPLC conditions:

Chromatographic column : TSK-GEL® ODS-80TS 4.6 mm x 150 mm

Mobile phase : H_2O : MeOH (gradient elution)

(Figure 2.2 and Table 2.2)

Flow rate : 1 ml/min

Run time : 32 min

Detector : UV 226 nm (photodiode array)

Injection volume : 10 μl

Table 2.2 Gradient profile of the mobile phase (H_2O : MeOH)

Time (min)	% MeOH
0-10	40
10-20	55
20-27	55
27-32	40

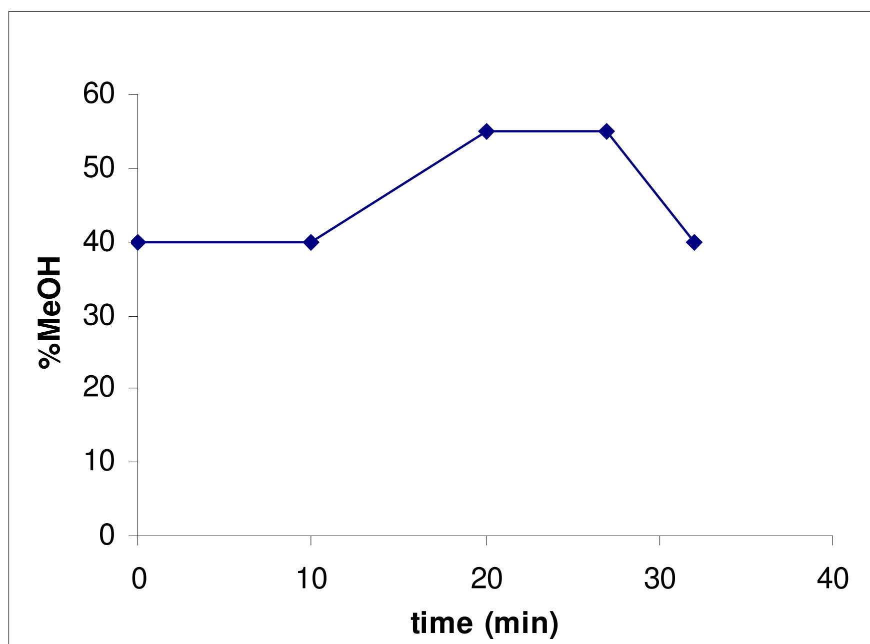

Figure 2.2 Gradient profile of the mobile phase (H_2O : MeOH)

2.2.6 Preparation of *A. galanga* extract

Dried powder of *A. galanga* (0.8 kg) was refluxed with hexane for 1 h (2.5 L x2). The extract was then filtered through filter paper. The solvent was removed under reduced pressure. The obtained oily extract was filtered through filter paper.

2.2.7 Solubility study

To approximately 10 mg of the sample in a glass-stoppered 10 ml graduated cylinder, increasing volume of propylene glycol, glycerine, mineral oil, ethanol or water at room temperature were added beginning with 10 μl. After each addition of the indicated amount of solvent, the mixture was shaken vigorously for 10 min and was visually checked for any undissolved parts of the sample. Then, the sample was continuously added until 10 ml. If, 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 solubilities the time required to dissolve a substance can be considerably longer (at least 24 h should be allowed). The approximate solubility was given in the Table 2.3 according to the volume of added solvent in which complete dissolution of the sample occurs.

In the statements of solubility, the term used has the following significance referred to a temperature 25 $^{\circ}$C in Table 2.3 (British Pharmacopoeia, 2001)

Table 2.3 Solubility criteria of the extract in various cosmetic solvents

Descriptive term	Approximate volume of solvent in milliliters per gram of solute
Very miscible	Less than 1
Freely miscible	From 1 to 10
Miscible	From 10 to 30
Sparingly miscible	From 30 to 100
Slightly miscible	From 100 to 1000
Very slightly miscible	From 1000 to 10,000
Practically immiscible	More than 10,000

2.2.8 Effect of light on stability of *A. galanga* extract

The extract was kept in tight containers at 25±2°C for 10-week period. Three vials of the sample were stored in transparent glass vials and exposed to light from a fluorescent lamp (30 W) and the other three vials were stored and protected from light. The content of 1′-ACA was evaluated fortnightly. Twenty mg of the samples was weighed and adjusted to 10 ml with methanol. The color of the samples was observed and the content of 1′-ACA was analyzed by HPLC. The experiment was carried out in triplicate.

2.2.9 Effect of temperature on stability of *A. galanga* extract

All samples were kept in tight containers and protected from light during a 10-week period, under three different temperature conditions 45±2°C, 30±2°C (room temperature) and 25±2°C with 75% relative humidity (RH). The content of 1′-ACA was evaluated fortnightly. Twenty mg of the samples was weighed and adjusted to 10 ml with methanol. The color of the samples was observed and the content of 1′-ACA was analyzed by HPLC. The experiment was carried out in triplicate.

2.2.10 Preliminary formulation study and stability test

2.2.10.1 Formulation of cream bases

Five different cream bases were prepared. The compositions of five cream bases are shown in Table 2.4. All cream bases were prepared by beaker method. The ingredients in oil and water phases were accurately weighed and placed into two separated beakers. The oil phase ingredients were melted together in a water bath to 75 °C. Meanwhile, the water phase ingredients were warmed at 75 °C. Then the aqueous solution was slowly added, with constant stirring, to the oil phase mixture. The mixture was then slowly cooled with continuous stirring until the mixture was congealed at room temperature. The obtained cream base was packed in tight container and kept at room temperature.

2.2.10.2 Formulation of *A. galanga* cream

The hexane extract of *A. galanga* was levigated with the selected cream base to make a smooth paste and added cream base by geometric dilution method until *A. galanga* cream is mixed completely. *A. galanga* cream was formulated with two different concentrations of *A. galanga* extract, 1% and 2% w/w.

Table 2.4 The ingredients of the cream bases

Ingredients (g)	Formulation				
	Rx1	Rx2	Rx3	Rx4	Rx5
Cetyl alcohol	2	2	5	-	-
Stearyl alcohol	1	-	-	1	2
Glyceryl monostearate	2	0.4	4	1	4
Cetomacrogol 1000	3	-	-	-	-
Soft paraffin	3	-	-	-	-
Mineral oil	5	10	-	-	3

Glycerine	5	2	-	-	-
Propylene glycol	-	2	-	5	-
Stearic acid	-	2	3	1	-
Isopropyl palmitate	-	1	-	3	-
Isopropyl myristate	-	-	4	-	-
Triethanolamine	-	1	-	1	-
Potassium hydroxide	-	-	0.2	-	-
Petrolatum	-	-	5	2	-
Carbomer 934, 2% aqueous	-	-	-	32	-
Polysorbate 20	-	-	-	1.5	-
Dimethicone	-	-	-	2	-
Silicone oil	-	-	-	-	3
Polawax®	-	-	-	-	3
Veegum	-	-	-	-	1
Xanthan gum	-	-	-	-	0.5
Uniphen p-23	-	-	-	-	0.3
Paraben conc.	1	1	1	1	-
Purified water qs to	100	100	100	100	100

2.2.10.3 Stability test

The heating-cooling cycle method (Rieger, 1979) was used for the stability test of the five cream bases. The cream bases were kept alternately at 4°C (48 h) and 45°C (48 h) for 8 cycles. The physical appearances of the cream base such as color, smoothness and phase separation were observed before and after testing. The data were used to select the best cream base for preparing the *A. galanga* cream. In the case of the *A. galanga* cream, the physical properties including viscosity and pH were measured before, after heating-cooling cycle test and at room tmperature after 30 days. In addition, the content of 1′- acetoxychavicol acetate in *A. galanga* cream was analyzed by HPLC.

2.2.10.4 Quantitative determination of 1′-acetoxychavicol acetate in the

A. galanga cream

A. galanga cream (0.1 g) was dissolved in methanol (10 ml) and sonicated for 30 minutes. The mixture was adjusted to 10 ml with methanol and put in the refrigerator for 15 minutes. The mixture was then centrifuged at 4°C, 4500 rpm for 10 minutes. The supernatant was filtered through membrane filter (0.45 micron) and subjected to quantitative determination of 1′-ACA using HPLC as described in section 2.2.5.

2.2.11 Evaluation of antibacterial activity against _P. acnes_

Antibacterial activity of *A. galanga* cream against *P. acnes* was evaluated by means of the agar diffusion method (Charnock *et al*, 2004). *P. acnes* was mixed into the agar before pouring. For the surface inoculation technique, a suspension of bacteria in physiological salt water with a density equivalent to McFarland 0.5 was used. When included in the agar, 250 μl of suspension was used per 24.75 ml of growth medium (Mueller-Hinton agar). Typically 25 ml of bacteria-inoculated growth medium was poured into an empty 9 cm plated and allowed to set. Equidistant wells were established in the agar using steriled borer apparatus (5 mm diameter). The *A. galanga* creams were introduced into the appropriate well and then incubated at 37°C for 72 h under anaerobic condition. Inhibition zone diameters were measured. Clindamycin 1% gel (Clindalin gel) was used as positive control and the cream base was used as a negative control. The assay was performed in triplicate.

2.2.12 Statistical analysis

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