CHAPTER III

RESEARCH METHODOLOGY

Materials and Instruments

1. Plant materials

The roots of *A. racemosus* were collected from Tak, Rayong, Kanchanaburi province, Thailand. The voucher specimens (Collection no. RKT 0001, RKT 0005, RKT 0008) were kept at Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok and at PBM herbarium, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand.

2. Microorganisms

Six microorganisms were use in this study. Of these, three bacterial species were *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* (laboratory strains, verified by biochemical test, Department of Microbiology, Faculty of Medical Sciences, Naresuan University. The fungi species used *C. albicans* (ATCC 90028), *M. furfur* (CBS 1878^T) and *M. globosa* (CBS 7966^T) was obtain from the Department of Microbiology and Parasitology, Faculty of Medical Sciences, Naresuan University.

3. Chemicals and reagents

Standard shatavarin IV was purchased from Natural remedies Pvt. Ltd. (India). Acetone, hexane, butanol, acetic acid, anisaldehyde R, sulfuric acid, chloroform, ethyl acetate, methanol (A.R. grade), were product from Labscan Asia Co., Ltd. (Thailand). Agar, sodium chloride, olive oil, ethanol 95%, methanol (commercial grade) were purchased from TTK Science Co., Ltd. (Thailand). Saboraund dextrose broth and trypticase soy broth were products from Himedia Laboratories Pvt. Ltd. (India). Dimethylsulfoxide (DMSO) and 1-Hydroxypyridine-2-thione zinc salt (Zinc pyrithione) and ketoconazole were purchased from Sigma Chemical Co. Ltd. (USA). 2,2-Diphenyl-1-picrylhydrazyl, Sterile water for injection was purchased from A.N.B. Laboratories Co., Ltd. (Thailand). Thin layer chromatography was product from Merck (Germany).

4. Instruments and equipments

All basic apparatus for preparation microorganism cultures such as autoclave (Tomy Seiko, Japan), laminar air flow (Heto-holten Dk 3450, Denmark), microscope (Olympus, Japan) and the incubator (New Brunswick Scientific, USA) were used. Turbidity of cells was measured with a microplate spectrophotometer (Perkin Elmer, USA). The equipment for preparations the extract were ultrasonic bath (Tran), hot air oven (Memmert GMbH Co. Ltd. Germany), freeze dryer (Thermo Electron Co., Ltd., USA), magnetic stirrer (Halikulgroup Co. Ltd., Thailand), rotary evaporator (BÜCHI Labortechnik, Switzerland) Analysis of saponin in *A. racemosus* extract was conducted by UV-VIS spectrophotometer (Varian, Inc. USA)

Extract Preparations

Preparation of plant extracts and quality control

The roots of *A. racemosus* were collected from Tak, Kanchanaburi, Rayong and Phetchabun province. The roots of *A. racemosus* were cut and primary air-dried in the shade at room temperature until dryness. Then they were dried in a hot air oven at 45 °C for 24 h to completely remove residual moisture, before milling into coarse powder. The powders were kept in air-tight bags until used. Two different extraction processes were performed as following:

1. Preparation of fractional extracts

The dried powdered roots of *A. racemosus* were first by macerated at room temperature with hexane (3 days). It was filtered and the filtrate was concentrated to dryness using a rotary evaporator to give the crude hexane extract (AR-H). Then, residue was macerated with 95% ethanol for 3 days. After that, it was filtered and the residue was extracted again using the same procedure. The filtrates were pooled and concentrated to dryness using a rotary evaporator to give the ethanolic extract (AR-E). Then, the marc was extracted by a decoction technique. The plant residue was put in 80 °C distilled water for 3 h. Half of the aqueous solution was collected and concentrated using freeze dried to give the aqueous extract-A (AR-A). Another half of the aqueous solution was mixed with acetone and the precipitation occurred. Both the precipitate and the solution were concentrated to dryness using a rotary evaporator and freeze dried to give the aqueous extract-B (AR-B) and the

sediment (AR-S), respectively. The scheme of extraction is shown in Figure 8. The yields of the extracts were calculated using percentage the following equation;

% yield = [dried extract weight (g) / plant material sample weight (g)] x 100

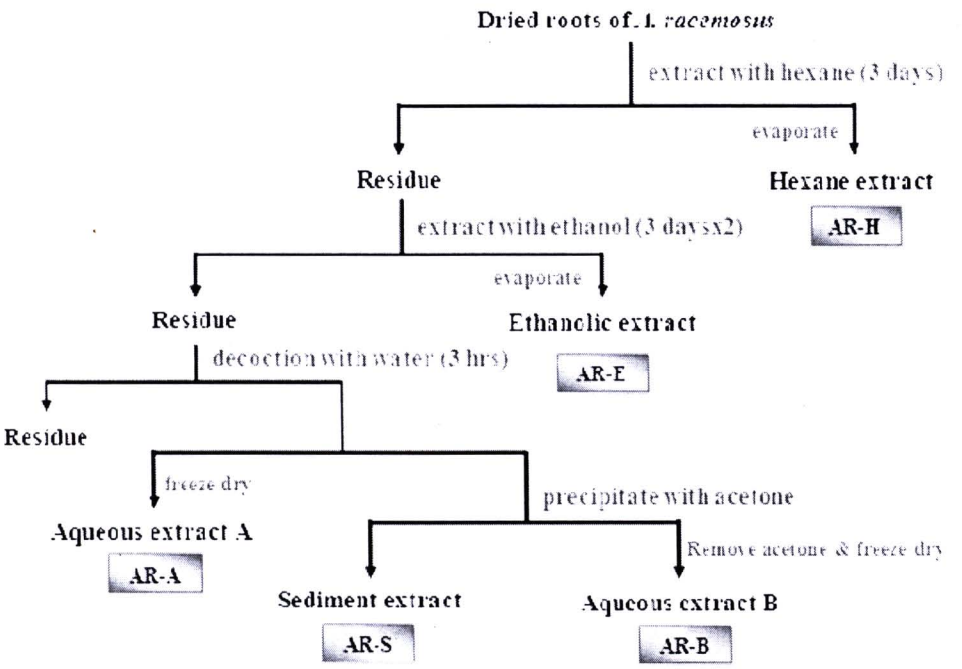

Figure 8 Preparation of fractional extracts

2. Preparation of saponin enriched extract

The dried powdered roots of *A. racemosus* (150 g) were percolated with acetone (500 ml) for 10 minutes and then macerated with methanol (3 x 500 ml) at ambient temperature. The methanolic extract was concentrated under reduced pressure. The concentrated extract was then, precipitated with acetone (1500 ml). The precipitate was dissolved in distilled water (25 ml) and partitioned with n-butanol (400ml). The organic layer was collected and the solvent was removed under reduced pressure to obtain a dark-brown residue (AR-En).

Analyses of chemical constituents in *A. racemosus*

1. Thin layer chromatography (TLC)

One-hundred milligrams of the extract were dissolved in 1 mL of methanol. The solution was applied on the TLC sheet and developed with a mobile

phase. The mobile phase system used in this study was ethyl acetate : methanol : water (75:15:10). The TLC profiles were observed under UV illumination (254 and 366nm). After that the TLC plate will be sprayed with detection reagent, vanillin-sulphuric acid reagent or anisaldehyde sulphuric reagent. The TLC plate was heated and observed in visible light.

2. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

LC-MS/MS was used for quantitative analysis of a saponin glycoside, shatavarin IV in *A. racemosus* extract. The separation was performed using liquid chromatograph (phenomenex Luna 5u C8 (2) 100A 5µm 150x4.6 mm.). The mobile phase was a gradient elution of 0.1% formic acid in water with methanol (Table 2). Injection volume was 20 µl.

Table 2 Gradient elution of the mobile phase of the LC-MS/MS analyses

Time(min)	Flow rate (ul/ml)	0.1%formic acid	MeOH
0	600	50	50
5	600	10	90
12	600	10	90
12.1	600	50	50
15	600	50	50

The liquid chromatograph was coupled to a mass spectrometer with a turbo electrospray ion source (API 4000 triple quadrupole (MDS-SCIEX, Toronto, Canada) and was used in negative ionization mode. The multiple reaction monitoring (MRM) pair monitored for determination quantity of shatavarin IV was *m/e* 886/578. Analyst 1.3.2 software (Sciex) was used for data registration and calibration. The MS condition is shown in Table 3.

Table 3 MS condition (MRM) for the analyses of shatavarin IV

Q1(amu)	Q3(amu)	Time(msec)	CE(volts)	CXP(volts)
886.4	578.4	100	-78	-23
886.4	724.4	100	-99	-29

Note: Declustering potential(DP) = -100

Collision gas(CAD) = 2

Curtain gas(CUR) = 20

Ion source gas 1(GS1) = 55

Ion source gas 2(GS2) = 45

Ion spray voltage(IS) = -4500

Temp = 400

3. Competitive ELISA

The saponin equivalent to shatavarin IV in the extracts of *A. racemosus* were analyzed by an enzyme-linked immunosorbent assay (ELISA) using monoclonal antibody (MAb) against shatavarin IV.

MAb against shatavarin IV was obtained as described before (Reamyindee et al., 2011). The ELISA procedure was as following. Shatavarin IV-HSA conjugated was diluted in 0.05 M carbonate buffer (pH 9.6), coated in 96-well microplates and kept at 37 ◦C for 1 h. After three times washing with 0.05% Tween 20 phosphate buffer saline (TPBS), the plate was treated with 300 µl of 0.2% gelatin in phosphate buffer saline to reduce nonspecific adsorption. After that, 50 µl of various concentrations of shatavarin IV or sample in 20% methanol were incubated with 50 µL of the MAb solution for 1 h. The plate was washed 3 times with TPBS and then, the antibody was combined with 100 µl of a 1:1000 diluted solution of peroxidase conjugated goat IgG fraction to mouse IgG Fc and kept for 1 h. After washing the plate 3 times with TPBS, 100 µl of the aforementioned substrate solution was added and incubated for 15 min. The plate was measured at 405 nm by a microplate reader. The calibration curve was constructed from the serial concentrations of shatavarin IV

and their absorbance measured via ELISA. The range of linearity was selected. The amount of saponin equivalent to shatavarin IV was calculated from the equation obtained from the calibration curve.

Antimicrobial activity determination

1. Preparation of microbial cultures

A single colony of pure bacterial and fungi cultures were preincubated at 37°C 24 h for *E. coli*, *Ps. aeruginosa*, *S. aureus*, *C. albicans* and 48 h for *M. furfur* and *M. globosa* on agar media by using the streaking method. (Trypticase Soy agar: TSA for bacterial, Saboraund dextrose agar: SDA for *C. albicans* and SDA with 1% olive oil for *M. furfur and M. globosa*) (Appendix B). Microbial colonies were collected, suspended in a NaCl solution (0.85%) and adjusted to the bacterial suspension of approximately 10^8 cell/ml and the fungi suspension of approximately 10^6 cell/ml by using heamacytometer (Appendix C.). This culture was used for disc diffusion test. The fungi suspension of approximately 10^4 cell/ml was used for broth microdilution method and checkerboard synergy test.

2. Disc diffusion method

The disc diffusion test was performed to screen the antifungal activity of the *A. racemosus* extracts modified from the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2007). The inoculums suspension was prepared as in 4.1 of each microbial species then transferred on the entire surface of agar plate using sterile cotton swab. Pieces of paper disc (Whatman AA discs, 6 mm diameter) were aseptically placed on agar surface. Ten µl of the extracts solution (100 mg/ml) immediately added to the discs (final concentration of the extracts in each disc was 1 mg). Negative control plates received only respective solvent and zinc pyrithione and ketoconazole at concentration 10 mg/ml will be used as positive control. Triple copies of the prototypes were prepared for each experimental set. The diameter of inhibition zones around the discs was measured after incubation at 37°C 48 h.

3. Minimum inhibitory concentration (MIC) determination

The MICs of the antifungal agents against the various fungi were determined by the broth microdilution method based on the M27-A broth microdilution reference procedure of the National Committee for Clinical Laboratory

Standards (NCCLS). Yeasts were prepared as already mentioned for the paper disc method. The crude extracts from extraction with different solvents (AR-1 to AR-5) were serially diluted with 10% dimethyl sulfoxide (DMSO) to the concentrations of 200 - 0.39 mg/ml. The AR-En was prepared to a series of concentration ranging from 25 to 0.05 mg/ml in 10 % DMSO. Ketoconazole and zinc pyrithione were similarly diluted in the same solvent to generate a series of concentrations ranging from 80 to 0.156 μg/ml per testing well. After shaking, 100 μl of the antifungal agent solutions was added to the wells of 96-well plates. The suspension of each organism was adjusted to $0.5x10^4$ – 10^4 CFU/ml, added to the individual wells at 100 μl/well ($0.5x10^3$–$2.5x10^3$ CFU/well), and cultivated at 37°C for 48 hours. The MIC was defined as the lowest concentration that completely inhibited visible fungal growth in the wells after incubation. MIC values was determined in duplicate and retested if the values differed. Each organism was also cultured with a control solution containing DMSO at levels equivalent to those in the test compound solutions to certify that they did not affect fungal growth. The tests were performed in triplicate to confirm the values.

The minimum fungicidal concentration (MFC) of the extracts was determined by plating 80 μl samples from every clear well of MIC assay plate with growth inhibition into freshly prepared agar plate. The plates were then incubated at 37°C (48 h for *C. albicans, M. furfur* and *M. globosa*). The MFC was recorded as the lowest concentration that did not permit any visible fungal colony growth on the agar plate after the period of incubation.

4. Synergistic effect testing (checkerboard synergy testing)

AR-E and the AR-En extracts from Rayong were tested for synergistic effect with ketoconazole (KTC) and zinc pyrithione (ZPT). Eight serial two-fold dilutions of the tested extracts and the drugs were prepared with the same solvents used in the MIC tests. Fifty microliter aliquots of each extract was added to the wells of 96-well plates in a vertical orientation, and 20 μl aliquots of each ketoconazole or zince pyrithione dilution was added in a horizontal orientation so that the plate contained various concentration combinations of the two compounds. A 100 ml suspension of tested fungi ($0.5x10^4$ – 10^4 CFU/ml) was added to each well and incubated at 37°C (48 h for *M. furfur* and *M. globosa*). Fractional inhibitory

concentrations (FICs) were calculated as the MIC of the combination of the extract and antifungal agent (KTZ or ZPT) divided by the MIC of the extract or antifungal agent alone. The FIC index was calculated by adding both FICs and interpreted as a synergistic effect when it was ≤ 0.5, as additive or indifferent when it was > 0.5 to 4.0, and as antagonistic when it was > 4.0. The concentration combinations of the two compounds are shown in Figure 9.

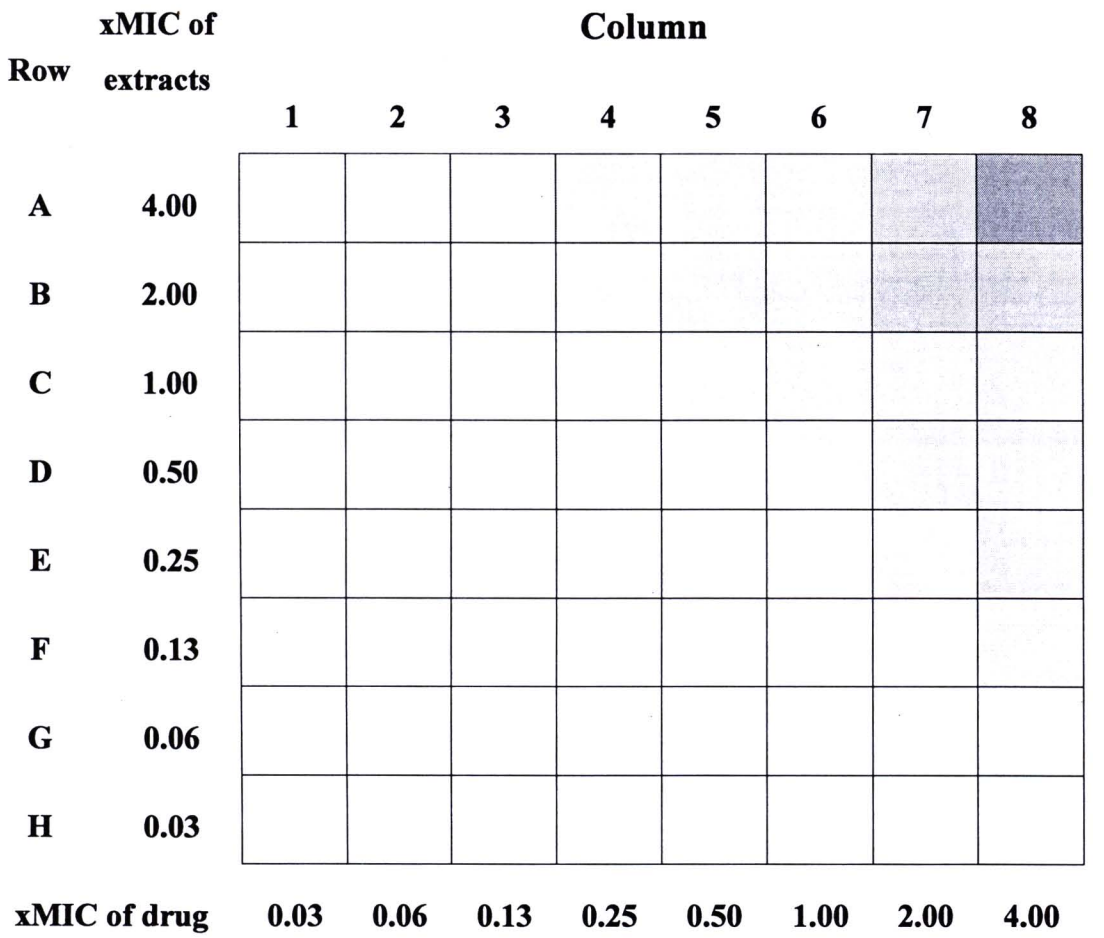

Figure 9 Schematic of the concentration the extract and antifungal agent using checkerboard synergy testing method on 96-wells plate

The grey shade indicates the concentration. The darker zone means that the concentration is higher than clear zone.

Fractional inhibiting concentrations (FICs) and FIC indices (FICI) were calculated using the following formula;

$$\text{FIC of } A.racemosus \text{ extract} = \frac{\text{MIC of extract in combination with the antifungal agent}}{\text{MIC of the extract alone}}$$

$$\text{FIC of antifungal agent} = \frac{\text{MIC of antifungal agent in combination with the extract}}{\text{MIC of the antifungal agent alone}}$$

FICI = FIC of the extracts + FIC of antifungal agent.

Stability study

Stability test

AR-E was kept at 50 °C in hot air oven for 30 days. All samples were stored in amber glass containers. Changing in % saponin equivalent to shatavarin IV was measured using competitive ELISA. The antifungal activity was determined by both microdilution method for find the MIC and MFC value after the extracts was kept for 30 days at 50 °C and compared with that of first day as the control. Each sample was tested in triplicated.

Statistical analysis

The data were expressed as the mean with a standard deviation (SD). Statistical data were analyzed by paired-sample *t* test. The significance criterion for the correlation measurements was set at 0.05.