Chapter III

Materials and Methods

I. Preliminary screening for antibacterial activity of some Thai medicinal plants

A. Plant extracts

1. Plant materials

The sources of plant materials were mainly from medicinal herb shops in Bangkok and from Chulalongkorn campus. Most of them were authenticated by the staff of the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University. *Eleutherine palmifolia* (L.)Merr.,was authenticated by comparison with the herbarium specimens at the Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand.

2. Preparation for crude extracts

All of the plant materials mentioned above were dried in hot air oven at 50°C and grounded to coarsely powder. Each plant material (500 g) was macerated with 95% ethanol for 18 days ; 1.5 L on the frist 7 days , then 1 L on the second 6 days , and 650 ml on the last 5 days. The combined filtrate of each plant was concentrated under reduced pressure to give crude extract.

Table 6 Plant materials and their parts used

Thai name (Tem Smitinand, 1980)	Botanical name	Family	part used.
1. Makham kai (มะคำไก่)	*Putranjiva roxburghii* Wall.	Euphorbiaceae	leaf
2. Plao noi (เปล้าน้อย)	*Croton sublyratus* Kurz.	Euphorbiaceae	leaf
3. Jate-phang-khee (เจตพังคี)	*Croton crassifolius* Geisel.	Euphorbiaceae	root
4.Kaa faak (กาฝาก)	*Dendropthoe pentandra* Miq.	Loranthaceae	aerial part
5. Yaa-haeo-muu (หญ้าแห้วหมู)	*Cyperus rotundus* Linn.	Cyperaceae	bulb
6. Thao yaai mom (เท้ายายม่อม)	*Clerodendrum petasites* Moore	Verbenaceae	leaf
7. Pha kaa krong (ผกากรอง)	*Lantana camara* Linn.	Verbenaceae	aerial part
8. Ching chee (ชิงชี่)	*Capparis micracantha* DC.	Capparidaceae	root
9. Phanang nang (พนังนั่ง)	*Stephania glabra* (Roxb.) Miers.	Menispermaceae	tuber
10. Samo dee nguu (สมอดีงู)	*Terminalia citrina* Roxb. ex.Flem	Combretaceae	leaf
11. Waan hom daeng (ว่านหอมแดง)	*Eleutherine palmifolia* (L.) Merr.	Iridaceae	bulb

B. Screening of Antibacterial activity

The disc agar diffusion method was used (Bailey and Scott, 1974; Lorian, 1986).

1. Test Medium

Mueller Hinton Agar (Difco, control no. 779903) was used. The ingredients per litre were as follows :-

Beef, Infusion form	300.0 g
Casamino Acids, Technical	17.5 g
Starch	1.5 g
Bacto-Agar	17.0 g

To rehydrate the medium, suspended 38 g in 1,000 ml of cold purified water, USP., distilled or deionized water, and heat to boiling to dissolve the medium completely. Dispensed into flasks and sterilized in the autoclave for 15 minutes at 15 pound/inch^2 pressure (121°C). Avoided excessive heat during rehydration or sterilization. Final pH was 7.3±0.1

2. Preparation of discs

2.1 13.0 mm in diameter of antibiotic assay discs (Whatman Ltd. cat. no. 2017013) were sterilized by dry heat at 160°C for 2 hours.

2.2 Each dried crude extract from A.2 was dissolved to make the concentration of 200 mg/ml by 95% ethanol and was filled to the sterilized paper discs (from 2.1) to make 10 mg/disc by using 50 µl micropipette. (Transferpette®, Rudolf Brand GMBH & Co.) Allowed the paper discs to dry and kept at 4-8°C in the refrigerator.

2.3 Control paper discs absorbed with 95% ethanol were prepared.

The test was repeated three times.

3. Preparation of the inoculum

3.1 Test organisms; Five microorganisms which caused respiratory tract infection were used as follows :

- *Staphylococcus aureus* ATCC 25923
- *Streptococcus pyogenes* A 6/49
- *Pseudomonas aeruginosa* ATCC 27853
- *Klebsiella pneumoniae* ATCC 10031
- *Haemophilus influenzae* ,from Department of Microbiology, Chulalongkorn Hospital.

All test organisms were cultured overnight on Trypticase Soy Agar (TSA) slants (Difco, control no. 637441) at 37°C before testing. The ingredients per litre of TSA were as follows :-

Pancreatic Digest of Casein USP	15 g
Soy Bean Peptone	5 g
Sodium Chloride	5 g
Bacto- Agar	15 g

To rehydrate the medium, dissolved 40 g in 1,000 ml cold distilled water. Heated to boiling to dissolve the medium completely. Sterilized in the autoclave for 15 minutes at 15 pounds pressure (121°C).

For *S.pyogenes*, 3-5% whole sheep blood was added and chocolate agar with X-factor and V-factor was necessary for *H. influenzae* (Lorian, 1986).

3.2 Suspended the culture with a small volume of sterile normal saline solution and adjusted the inoculum

to match a 0.5 turbidity standard of Mc Farland No. 1 when comparing the tubes against a white background with a contrasting black line.

4. Preparation of test plates

Mueller Hinton Agar (MHA) was melted and allowed to cool at 45°C - 50°C in a water bath. Then 25 ml of the melted agar medium was dispensed into sterile glass petri dishes, with internal diameters of 9 cm, to yield a uniform depth of 4 mm. The agar was allowed to harden on a flat level surface. If, prior to use, moisture was presented on the agar surface, the plates should be placed in an incubator at 35°C with their lid slightly ajar in order to permit the evaporation of surface moisture. Evaporation was usually complete within 30 minutes.

As mentioned in 3.1, The blood agar plate was prepared to *S. pyogenes* and the chocolate agar plate with X-factor and V-factor was prepared for *H. influenzae*.

5. Inoculations of agar plates

A sterile cotton swab was dipped in each inoculum and the excess was removed by rotating the swab several times against the inside wall of the tube above the fluid level. The entire surface of the MHA plate was inoculated by streaking with the swab for 3 times and each time the plate was rotated 60°C. This would ensure an even distribution of inoculum.

6. Application of discs

As soon as possible and not later than 15 minutes after the inoculation of plates, the discs must be applied in order that diffusion and growth proceeded

simultaneously. In order to ensure complete contact of the discs to the agar surface, the discs had to be pressed down with slightly pressure.

Discs must be arranged at least 15 mm from the edge of the plate and apart from each other by a distance of 15-20 mm. The plates were left in room temperature for 30 minutes, then incubated at 35-37°C for 18 hours (overnight).

7. Test results

The zone diameters of inhibition were measured with a sliding calipers with an accuracy of nearest 0.1 mm. Faint growth or tiny colonies near the edge of the inhibition zones were ignored if they were presented.

Seven samples which showed high antibacterial activities or inhibited more test organisms were selected.

II. Extraction Procedure

A. Extraction Procedure

Each crude extract selected from I was mixed with small amount of purified, finely sand, then was dried and packed in the Soxhlet chamber. All these crude extracts were extracted with petroleum ether, chloroform and ethanol by using Soxhlet apparatus. The filtrate from each extract was evaporated under reduced pressure to dryness.

B. Determination of antibacterial activity.

1. Using the disc agar diffusion method as mentioned in I(B). The concentration of each extract from II (A) per disc was 10 mg and tested organisms were S. *aureus* ATCC 25923, S. *pyogenes* A6/49 and *H. influenzae*.

Three residues which showed high antibacterial activity were chosen for further study.

2. Three extracts from 1 were determined the efficacy of antibacterial activity by disc agar diffusion method. Each extract was diluted by a series of two fold dilutions to contain 46.875, 93.75, 187.50, 375.0, 750.0, 1,250.0, 2,500.0 and 5,000.0 µg/disc, using 95% ethanol as diluent.

III. Determination and isolation of antibacterial substances

According to the determination of antibacterial activity, the petroleum ether extract of *Eleutherine palmifolia* (L.) Merr. bulb in the least amount gave the first inhibition zone. The chromatographic techiques were done in order to isolate and identify (if possible) the active constituents.

A. General Techniques

1. Chromatographic Techniques

1.1 Thin Layer Chromatography (TLC)

Technique : one way, ascending, tank saturated

Adsorbent : Silica gel G and silica gel GF 254 (E. merck) : 15 g + 15 g in 60 ml distilled water

Plate sizes : 10 cm x 20 cm and 20 cm x 20 cm

Layer thickness : 250 µ

Activation : Air-dried for 15 minutes and then at 110°C for 1

hour

Solvent system : 1) chloroform
2) chloroform : hexane (8:2)
3) chloroform : petroleum ether (7:3)
4) chloroform : benzene (8:2)
5) benzene : acetone (9:1)
6) petroleum ether : ethyl acetate : chloroform (67:33:10)

Distance : 15 cm

Laboratory temperature : 28-35°C

Detection : UV light (254 nm and 366 nm); sprayed with 7% alcoholic potash then heated at 100°C, 10 minute

1.2 Pre-coated TLC aluminium sheet

Technique : one way, ascending, tank saturated

Adsorbent : aluminium sheet silica gel GF254 pre-coated (E. Merck)

Plate size : 5 x 10 cm

Layer thickness : 200 μ

Solvent system : 1) chloroform
2) chloroform : hexane (8:2)
3) chloroform : petroleum ether (7:3)

Distance : 8 cm

Laboratory temperature : 28-35°C

Detection : UV light (254 nm and 366 nm)

1.3 Column Chromatography (CC)

Column sizes : The glass column 2.5 x

45 cm, 5 x 60 cm and 10 x 40 cm (depending on the quantity of sample to be seperated)

Adsorbent : Silica gel 60, 230-400 mesh (E. Merck)

Packing : Adsorbent packed wet into the column (Smith, 1969)

Sample loading: The portion of crude extract was dissolved in small amount of organic solvent, added slowly to the top of the column

Solvent : 1) chloroform : hexane (8:2) (E.Merck)
2) chloroform (E.Merck)
3) methanol (E.Merck)

Collection of eluate : fractions of 25 ml were collected

2. Melting Points

Melting points were determined on the Buchi melting point apparatus. The values recorded were uncorrected.

3. Specific rotation

Specific rotation were determined by Polax (No. 45014) polarimeter in the concentration of 0.1% (g/ml) in chloroform.

4. Spectroscopy

4.1 Ultraviolet absorption

Spectra were determined by Hitachi 150-20 UV/VIS spectrophotometer.

4.2 Infrared absorption

Spectra were determined by Shimadzu IR-440 Infrared spectrophotometer.

4.3 Nuclear magnetic resonance

Spectra were determined by Varian XL-300 (300 MHz) spectrophotometer.

4.4 Mass spectroscopy

The low resolution mass spectra were obtained with a Varian MAT 112S instrument operating at 70 eV.

B. Determination of antibacterial substances by Thin Layer Chromatography (TLC) :

1. Dissolved the extract with small amount of organic solvent. The concentration to be applied to pre-coated TLC plate was one step higher than the concentration of the extract that could inhibit the test organisms (see in Table 14, p 66).

2. Pre-coated TLC aluminium sheet was developed in suitable solvent system. The detection was made under UV light (366 nm), then separated the sheet into five groups (Fig 2,p 104).

3. Direct assay was used by the same method as the disc agar diffusion method, but using the cut-TLC aluminium sheet instead of paper discs.

3.1 Test organism was *S. aureus* ATCC 25923.

3.2 Each group of the separated TLC aluminium sheet was applied onto the inoculated agar plate, pressed gently and left in room temperature for 1 hour, then incubated at 37°C for 18 hours.

4. The inhibition zone showed that the antibacterial

substance was in the third group of the TLC plate which gave the two black spots in UV light (366 nm).

C. Isolation of antibacterial substance from petroleum ether extract of *Eleutherine palmifolia* (L.) Merr. bulb

The crude extract (4.99 g) was packed on the silica gel (250 g) column. The column was eluted with chloroform : hexane (8:2), chloroform and methanol, respectively and 25 ml fractions were collected. Fractions were determined by TLC, combined the liked fractions together (Table 7).

Each combined fraction was concentrated under reduced pressure to dryness. Fraction C and E were rechromatographed by using silica gel as an adsorbent and chloroform:hexane (8:2), chloroform and methanol as an eluent. 10-15 ml fractions were collected and fractions were determined by TLC, combined the liked fractions together (Table 8 and Table 9).

Table 7 Informations of *Eleutherine palmifolia* (L.) Merr. extract column chromatographic isolation

Fraction number	Solvent	Combined fraction	Remarks under UV light
1-4	$CHCl_3$:hexane (8:2)	A	2 orange spots (O_1, O_2), 1 yellow spot (Y) and 1 blue spot (B)
5-10	$CHCl_3$:hexane (8:2)	B	Blue spot (B) and first black spot (EP_1)
11-15	$CHCl_3$:hexane (8:2)	C	2 black spots (EP_1+EP_2)
16	$CHCl_3$:hexane (8:2)	D	The second black spot (EP_2)
17-20	$CHCl_3$:hexane (8:2)	E	The second black spot (EP_2, and yellow-green (YG)
21-25	$CHCl_3$	F	Long tail fluorescent spots (F)
26-35	Methanol	G	Residue (R)

Remark : black spots under UV light were yellow spots in visible light and when sprayed with 7% alcoholic potash, heated at 100°C for 10 minutes, the spots became pink colour.

: $CHCl_3$ = chloroform

Table 8 Informations of Fraction C column chromatographic isolation

Fraction number	solvent	Remarks under UV light
1-2	$CHCl_3$:hexane (8:2)	First black spot (EP_1, with a very pale blue spot(B)
3-9	$CHCl_3$:hexane (8:2)	2 black spots (EP_1 + EP_2)
10-11	$CHCl_3$:hexane (8:2)	Second black spot (EP_2)
12-20	$CHCl_3$	Residue

Table 9 Informations of Fraction E column chromatographic isolation

Fraction number	solvent	Remarks under UV light
1-2	chloroform:hexane(8:2)	Second black spot (EP_2)
3-10	chloroform:hexane(8:2)	Second black spot (EP_2, and yellow-green spot (YG)
11-18	chloroform	Yellow-green spot (YG) and a little bit long tail spot (F)
19-28	methanol	Residue

The second black spot under UV light from Fraction C and E was isolated and when determined on TLC, it was the same second black spot as Fraction D, so they were combined. The combined fraction was concentrated to small volume and crystallised in hexane, yielding yellow needle crystal (155.5 mg) and was designated as EP_2.

The combined fraction of the first black spot under UV light (EP_1) and impurity from Fraction B and C were concentrated to syrupy mass. The isolation and purification might be done in further study.

D. Characterization of isolated compound

The characteristics of the compound included melting point, specific rotation, and spectroscopic data such as; ultraviolet, infrared, nuclear magnetic resonance, and mass spectra were performed.

IV. Laboratory evaluation of antibacterial activity of EP_2 to isolated pathogenic organism from patients

For laboratory evaluation of antibacterial activity of EP_2, 10 kgs of *Eleutherine palmifolia* (L.) Merr. bulb were macerated with 95% ethanol and the procedure of extraction and isolation was repeated as mentioned before in II(A) and III(C) to receive the yellow needle crystal (EP_2).

One hundred of two pathogenic organisms, *S. aureus* and *S. pyogenes*, were used. The organisms were derived from specimens of the patients from Chulalongkorn Hospital and Siriraj Hospital.

A. Antimicrobial susceptibility Test

Pathogenic organism from clinical specimens were tested for their susceptibilities to five standard antibiotic discs by the disc agar diffusion method as described in I(B).

1. Discs

The standard antibiotic discs tested were the following (Bailey and Scott, 1974; Lennette, 1985)

- Cephalothin 30 µg/disc (BBL) lot.no.907579
- Clindamycin 2 µg/disc (Difco) lot.no.760799
- Erythromycin 15 µg/disc (BBL) lot.no.907532
- Penicillin 10 units/disc (BBL) lot.no.903617
- Tetracycline 30 µg/disc (BBL) lot.no.904556

The test was three times repeated .

2. Interpretation of the test results

The diameters of inhibition zone were measured with a sliding calipers to the nearest 0.1 mm, reading to the point of complete inhibition as judged by the unaided eye from the underside of the plate. The zone diameter interpretation standards of WHO (World Health Organization) (1977; quoted in Lorian, 1986) was used as shown in the Table 10.

Table 10 Zone diameter interpretative standards

Antimicrobial agent	Disc content	Zone diameter, nearest whole mm		
		Resistant	Intermediate	Susceptible
Cephalothin	30 µg	≤ 14	15-17	≥ 18
Clindamycin	2 µg	≤ 14	15-16	≥ 17
Erythromycin	15 µg	≤ 13	14-17	≥ 18
Penicillin when testing staphylococci	10 units	≤ 20	21-28	≥ 29
When testing other microorganisms	10 units	≤ 11	12-21	≥ 22
Tetracycline	30 µg	≤ 14	15-18	≥ 19

(WHO, 1977; quoted in Lorian, 1986)

B. Determination of Minimal Inhibitory Concentration (MIC) of EP_2

The agar dilution method was used (Barry, 1976; Lorian, 1986). The materials and methods were described as followed :-

1. Test Medium

MHA (see I(B), p 43) was freshly prepared and sterilized at 121°C under 15 pounds per square inches for 15 minutes and allowed to cool to 45-50°C in a water bath. The 18 ml of it was transferred to each sterile screw-capped tube (Pyrex ®)

2. Preparation of the antimicrobial dilutions

The diluent of EP_2 was acetone (A.R.grade)

and the antimicrobial dilutions were prepared as followed :-

2.1 0.100 g of EP_2 crystal were weighed and dissolved in acetone to make 100.0 ml solution in a volumetric flask. The final concentration of the solution would be 1,000 µg/ml, designatd as solution 1.

2.2 The solution 1 was further diluted with acetone to make a serial dilution of EP_2 in a range from 100 µg/ml to 700 µg/ml as followed, using aseptic technique.

No. of solution	Proportion of the solution to acetone	Final concentration (µg/ml)
2	35 ml of solution 1 + 15 ml of acetone	700
3	12 ml of solution 1 + 8 ml of acetone	600
4	25 ml of solution 1 + 25 ml of acetone	500
5	20 ml of solution 1 + 30 ml of acetone	400
6	12 ml of solution 4 + 8 ml of acetone	300
7	10 ml of solution 5 + 10 ml of acetone	200
8	2 ml of solution 1 + 18 ml of acetone	100

Dilutions of antimicrobial agent were prepared at a concentration 10 times that desired in the final test.

3. Preparation of test plates

2 ml of each EP_2 dilution was added to 18 ml of the melted agar medium in sterile screw-capped tube. The tubes were mixed thoroughly but gently and the agar was poured into the sterile 90 mm petri dishes. For S. *pyogenes*, 3-5% sheep blood would be added immediately after the antimicrobial agent and just before mixing and pouring (Lorian, 1986). The plates were then set aside on a flat, horizontal

surface and allowed to harden undisturbed.

The agar plates would give the final EP_2 concentration of 10.0, 20.0, 30.0, 40.0, 50.0, 60.0 and 70.0 µg/ml. The control plate with diluent but without EP_2 and the control plate without both EP_2 and diluent were prepared for visible growth of test organisms.

4. Preparation of the inoculum

50 isolates of *S. aureus* were separately inoculated in TSA slant tubes and incubated overnight at 37°C. For *S. pyogenes*, the blood agar slants were used. Each culture was suspended by a small volume of normal saline solution and the turbidity of inoculum was standardized to match a 0.5 Mc Farland No. 1 standard. For proper adjustment of turbidity, it was necessary to prepare a white background with contrasting black lines and a standard light source.

5. Inoculation and Incubation of test plates

The inoculum-replicating apparatus (Barry, 1976) was used. About 25 standardized bacterial suspensions were transferred to the appropiate wells in each seed plate containing 28 reservoirs, the control strain of *S. aureus* ATCC 25923 (when testing *S. aureus* strains)or *S.pyogenes* A 6/49 (when testing *S. pyogenes* strains) and sterile normal saline solution were placed to the other 2 wells in every plate.

An aluminium replicating device(the multipoint inoculator apparatus) was dipped into the wells of inoculum in the seed plate and then the inoculum suspension were spotted onto the previously dried surface of each antimicrobic-containing plate, by touching the end of the inoculators on

the agar surface. The inoculated plates were allowed to stand undisturbed until the spot of inoculum had absorbed completely, the plate were then inverted and allowed to incubate at 37°C for 16-20 hours.

6. Reading of test results

The agar dilution plates were examined for growth, after incubation. First, the control plates without EP_2 but diluent, or without both EP_2 and diluent, were checked to make sure that each test strain was capable for providing adequate growth. Then the remaining plates were examined to determine the minimal concentration of drug required for inhibition of growth. In reading the end points, a faint haze of growth or growth of a single colony was disregarded, the MIC was defined as the lowest concentration which completely inhibited growth (Lorian, 1984).