CHAPTER 2

Materials and methods

2.1 Plant materials

2.1.1 Source and authentication of the plant materials

The plants used in the work were collected from Chiang Mai, Chiang Rai and Lampang provinces, Thailand. The identity of the Lanna medicinal plants was verified by a taxonomist at Faculty of Pharmacy and the voucher specimens were kept in the Herbarium of Faculty of Pharmacy, Chiang Mai University.

2.1.2 Lists of medicinal plants used in the present study

Table 2.1 Lists of medicinal plants used in the present study

Scientific name	Part used	Voucher No.
Millingtonia hortensis	stem	004528
Caesalpinia sappan	heartwood	002276
Sindora siamensis	stem	003605
Celastrus paniculatus	stem	007361
Combretum deciduum	stem	005698
Combretum quadrangulare	stem	007346
Terminalia bellerica	stem	007198
Shorea obtusa	stem	007101
Erythroxylum cuneatum	stem	001391
Croton crassifolius	root	009378
Trigonostemon reidioides	root	023171

Table 2.1 Lists of medicinal plants used in the present study (continued)

Scientific name	Part used	Voucher No.
Leea indica	root, stem	003792
Leea rubra	root, stem	005978
Aganosma marginata	stem	003385
Derris scandens	stem	001965
Thunbergia laurifolia	stem	004895
Pterocarpus macrocarpus	stem	007385
Piper sp.	stem	023174
Ventilago denticulata	stem	023175
Ziziphus cambodiana	stem	023177
Ziziphus mauritiana	stem	023176
Ziziphus oenoplia	stem	006162
Anomianthus dulcis	stem	007575
Oxyceros horridus	stem	008465
Schleichera oleosa	stem	007275
Holoptelea integrifolia	stem	006963

2.2 Mahoog formula

Ya-Mud Mahoog formula used in this work was prepared by the traditional healers from San Pa Tong district, Chiang Mai, Thailand.

2.3 Bacterial strains

1) *Staphylococcus aureus* ATCC 25923
2) *Bacillus subtilis* ATCC 6633
3) *Escherichia coli* ATCC 25922
4) *Pseudomonas aeruginosa* ATCC 9027

2.4 Chemicals

1) Absolute ethanol	MercK
2) 2,2´-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)	Sigma-Aldrich
3) Chloramphenicol	Atlantic Laboratories Corp., Ltd.
4) Chloroform	RCI Labscan
5) Dimethyl sulfoxide	Sigma-Aldrich
6) 2,2-Diphenyl-1-picrylhydrazyl (DPPH)	Sigma-Aldrich
7) 95% Ethanol	The Liquor Distillery Organization
8) Ethyl acetate	RCI Labscan
9) Ferric chlorides (FeCl3.6H2O)	MercK
10) Ferrous sulphate (FeSO4, standard)	AnalaR
11) Gentamicin	Vesco Pharmaceutical Co., Ltd.
12) 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, standard (Trolox)	Sigma-Aldrich
13) Methanol	RCI Labscan
14) n-hexane	RCI Labscan
15) Potassium persulfate	Carlo Erba
16) Propylene glycol	Vidhyasom Co., Ltd.
17) Silica gel (cat. No. 7734 and 9385)	MercK
18) Tryptic soy agar	Difco
19) Tryptic soy broth	Difco
20) 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ)	Fluka

2.5 Instruments

1) Analytical balance	A&D model HM-200 Sartorius model ME
2) Autoclave	SANYO model MLS-3750
3) Condenser	
4) FT-IR Spectrophotometer	NEXUS-465, Thermo Nicolet Electron Corporation FT-IR

5) Heating mantle	Electronicmantle EM 0500/C MR1, ISOPAD U2/102
6) Laminar air-flow cabinet	
7) Mass spectrophotometer	JEOL GCmate spectrophotometer
8) Micropipet	Gilson MODEL PIPETMAN P20, P200, P1000
9) NMR Spectrometer	Bruker 400 Ultrashield
10) pH meter	Metrohm
11) Rotary evaporator	EYELA Rotary evaporator
12) Round bottom flask	
13) Soxhlet's apparatus	
14) Sterile Petri dish	Greiner bio-one
15) Temperature-controlled incubator	Memmert model BE 50
16) TLC plate (silica gel GF254) aluminium sheet	MercK
17) TLC tank	
18) Vortex mixer	SUPER-MIXER 1291
19) UV/VIS Spectrophotometer	Varian model Cary 1E Agilent 8453 UV-Visible

2.6 Extraction

The plant samples and Ya-Mud Mahoog were cut into small pieces, dried at 50 °C and then ground into powder. The sample powders were separately extracted with 95% ethanol by using soxhlet's apparatus and water by decoction. Each extract solution was filtered through Whatman filter paper No. 1 and concentrated by using a rotary evaporator for ethanol extract and freeze dryer for water extract.

2.7 Determination of antibacterial and antioxidant activities

The determination of antibacterial and antioxidant activities of the extracts and isolated compounds was performed by methods as follows:

2.7.1 Antibacterial assay

1) Agar diffusion method

Diameter of zone of inhibition was determined using agar well diffusion technique modified from Kirby-Bauer method[10]. A swab of the bacteria suspension containing 1×10^8 CFU/mL was spread on to sterile Petri dish containing Tryptic Soy Agar (TSA) media. Each extract was dissolved in propylene glycol to concentration 50 mg/mL and autoclaved at 121°C, 15 psi for 15 min. Wells were cut with sterile borer (6 mm) and 50 µl of the extracts were added into the wells. The plates were incubated at 37°C for 24 hours. The propylene glycol used as negative control while the standard chloramphenicol 10 mg/mL and gentamicin at 1 mg/mL were used as positive controls. Antibacterial activity was indicated by the presence of clear inhibition zone around the wells. Tests were done triplicatly.

2) Minimum inhibitory concentration (MIC)

The determination of MIC using microbroth dilution method was applied on extracts that already proved for their high efficacy against tested microorganisms. The extracts were dissolved in 50 % DMSO. The extracts were diluted by two-fold to obtain a concentration range 100–0.05 mg/mL with Tryptic Soy Broth (TSB) in the 96-well microplates. The microorganism suspension ($1x10^5$ CFU/mL) of 50 µl was added to the broth dilutions. These were incubated for 24 hours at 37ºC. MIC of each extract was taken as the lowest concentration that did not permit any turbidity of the tested microorganism.

3) Minimum bactericidal concentration (MBC)

The wells were used in the MIC studies that did not show any turbidity in the bacteria were determined for MBC. An aliquot of the suspension (0.02 mL) was spread onto TSA and incubated at 37°C for 24 hours. The MBC was the lowest concentration which the initial inoculums were killed as 99.9 % or more.

2.7.2 Antioxidant assay

1) ABTS free radical scavenging assay

The ABTS assay was performed using a modified method[151]. ABTS•+ stock solution was generated by oxidation of 7.0 mM ABTS with 2.45 mM potassium persulfate. The solution was protected from light and stored at room temperature for 12-16 hrs. The 2.0 mL of ABTS •+ working solution was mixed with 100 µl dilute extract, comparing it to Trolox. After 3 min incubation at room temperature, the color reaction was measured at 734 nm using a UV/VIS spectrophotometer. The results of ABTS assay were expressed as Trolox equivalent antioxidant capacity (TEAC). This index is defined as gram of standard is equivalent to 1.0 gram of the extract.

2) DPPH free radical scavenging assay

The DPPH free radical scavenging assay was done according to the method of Brand-Williams *et al*[152] with some modifications. The 2.1 milliliters of reaction mixture containing 2.0 mL DPPH solve in ethanol and 100 µl diluted extract. After an incubation in the dark at room temperature for 30 min, the absorbance was measured at 517 nm using a UV/VIS spectrophotometer. The results were calculated in terms of TEAC. This index is defined as gram of standard is equivalent to 1.0 gram of the extract.

3) Ferric Reducing Ability Power assay (FRAP)

The FRAP assay was done according to Benzie and Strain[20] with some modifications. The 3.1 milliliters of reaction mixture containing 3.0 mL FRAP reagent and 100 µl diluted extract. The mixtures were incubated in the dark for 4 min at 37°C. After an incubation, the absorbance was measured at 593 nm using a UV/VIS spectrophotometer. The results were calculated in terms of TEAC. This index is defined as gram of standard is equivalent to 1.0 gram of the extract.

2.8 Phytochemical screening test[153]

2.8.1 Test for alkaloids

Two mL of each extract placed into three separate test tubes.

Dragendroff's test: 2-3 drops of Dragendroff's reagent were added to sample solution.

Positive result: reddish brown precipitate

Mayer's test: 2-3 drops of Mayer's reagent were added to sample solution.

Positive result: turbid or white precipitate

Wagner's test: 2-3 drops of Wagner's reagent were added to sample solution.

Positive result: brown precipitate

2.8.2 Test for tannins

Two mL of each extract placed into two separate test tubes.

Ferric chloride test: 2-3 drops of 1 % Ferric chloride were added to the extract solution.

Positive result: bluish or greenish black color

Gelatin test: 2-3 drops of 1 % solution of gelatin in 10% sodium chloride were added to the extract solution.

Positive result: white precipitate

2.8.3 Test for coumarins

1 g of each extract was added into test tubes. The test tube was covered with filter paper moistened with 20 % Sodium hydroxide, then heated on water bath for 5-10 min. The filter paper was examined under UV light.
Positive result: intense fluorescence

2.8.4 Test for anthraquinones

Borntrager's test: About 1 g of each extract was added 5 mL of chloroform in a test tube and shaken for 5 min. The mixture was filtered and the filtrate shaken with 5 mL of 10 % ammonia solution.
Positive result: pink violet or red color in the ammonia layer (lower layer)

2.8.5 Test for saponins

Froth test: To 2 mL of each extract was added 6 mL of water in a test tube. The mixture was vigorously shaken for 1 min.
Positive result: persistent froth (observe for 30 min)

2.8.6 Test for flavonoids

Shinoda's test: To 5 mL of each extract was added with a few pieces of magnesium ribbon. Then, 2-3 drops were added.
Positive result: pink or red color

2.8.7 Test for steroids and terpenoids

Liebermann Burchard's test: To about 1 g of each extract in test tube was added acetic anhydride 2 mL and shaken well. One mL of concentrated sulphuric acid carefully added from sides of the test tube.
Positive result: A reddish violet ring presence at the junction of the two layers

- Steroids: a bluish green color in the upper layer
- Terpenoids: a reddish color in the upper layer

2.8.8 Test for cardiac glycoside

Kedde's test: Two mL of each extract was evaporated to dryness on a water bath. Then, 0.5 mL of Kedde's reagent (3,5-dinitrobenzoic acid) and 2-3 drops of 1 N NaOH was then added into cooled, dried extract.

Positive result: violet color

2.8.9 Test for anthocyanins

Two mL of each extract was added to 2mL of 2 N HCl and ammonia.

Positive result: appearance of pink red turned to blue violet

2.9 Isolation and purification of chemical constituents by using bioassay-guided isolation

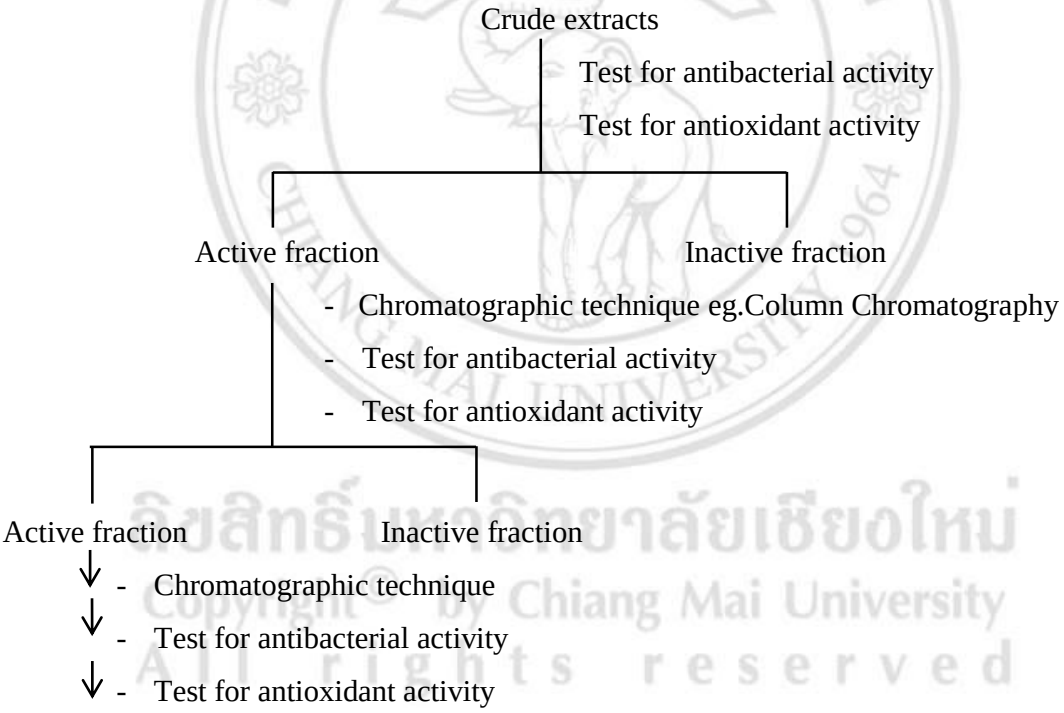

Figure 2.1 Isolation and purification by using bioassay-guided isolation

2.10 Structural identification

2.10.1 Mass spectra (MS): The electron impact mass spectra (EIMS) of the isolated compounds were measured by a JEOL GCmate spectrometer.

2.10.2 Ultraviolet absorption spectra (UV): The UV-spectra were measured by the UV-VIS spectrophotometer model Agilent 8453 Diode Array.

2.10.3 Infrared absorption spectra (IR): The IR-spectra were obtained by the Thermo Nicolet Nexus 470 FT IR Spectrophotometer. The isolated compounds were examined as micro KBr discs.

2.10.4 Proton and Carbon nuclear magnetic resonance spectra ^{1}H-NMR and ^{13}C-NMR: The ^{1}H-NMR and ^{13}C-NMR spectra were measured with a Bruker 400 Ultrashield™ spectrometer; 400 MHz for ^{1}H-NMR and 100 MHz ^{13}C-NMR in deuterated chloroform ($CDCl_3$). The chemical shifts were recorded in ppm with reference to TMS signal.