CHAPTER II

EXPERIMENTAL

I. Source of Plant Materials

1. *Clausena harmandiana* Pierre

Clausena harmandiana Pierre of family Rutaceae is known in Thailand as Song faa dong (สองฟ้าดง). This plant is an unarmed shrub with few branches and has 1.5 meters in height. The leaves are 17 to 40 centimeters long with 5 to 9 leaflets. The leaflets are triangle, slight coriaceous, attenuate or cuneiform at the base and on the tip, variable in size (10-24 cm x 5.5-14.5 cm), and have crenulate margin. There are 6-11 pairs of lateral veins which are glabrous, sometimes bifurcate from the base, very prominent on lower surface and have a lot of visible glands. Petioles are cylindrical, glandular and pubescent. Petiolets have 0.5-1 centimeters in length (32).

The root bark of *Clausena harmandiana* was collected from Kalazinthu province in the northeast of Thailand in April 1982. Vouchers specimen of the plant was identified by comparing with the herbarium specimen that was deposited at the Botany Section, Botany and Weed Science Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangken, Bangkok, Thailand.

The root bark of *Clausena harmandiana* was dried in hot air oven at 50°C for 3 hours, then powdered with electric mill

and let through the sieve no 5.

2. *Micromelum minutum* Wight and Arn.

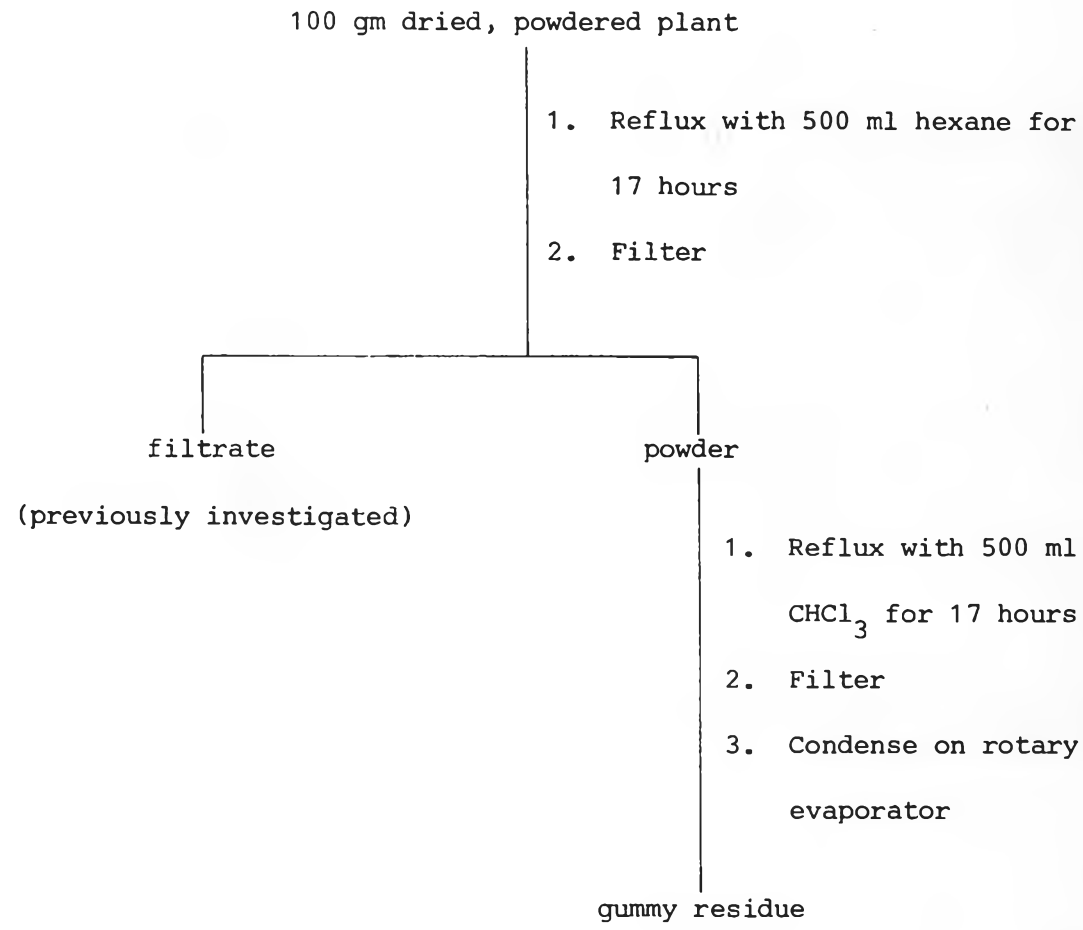
Micromelum minutum Wight and Arn. (Syn M. *pubescens* Blume) of family Rutaceae is known in Thailand as Mui-chang (หมุยช้าง), Hat-sa khun-Thai (หัศคุณไทย). It is a shrub or small tree with hairy branches and leaves. The leaves are 20 to 40 centimeters in length, with 9 to 12 leaflets on each side of the hairy rachis. The leaflets are variable in shape and size, ovate to broadly lanceolate, the terminal ones being longest and up to 15 centimeters in length. The flowers are fragrant, greenish yellow or white, and are borne in considerable numbers on compound inflorescences. The fruit is yellow when ripe, ovoid or oblong, and less than 1 centimeter in length (33).

The stem bark of *Micromelum minutum* was collected from Nakornsrithammarat province in the southern part of Thailand in May 1984. Vouchers specimen of the plant was identified at the Royal Forest Department, Ministry of Agriculture and Cooperatives, Bangkok, Thailand.

II. Extraction

1. *Clausena harmandiana* Pierre

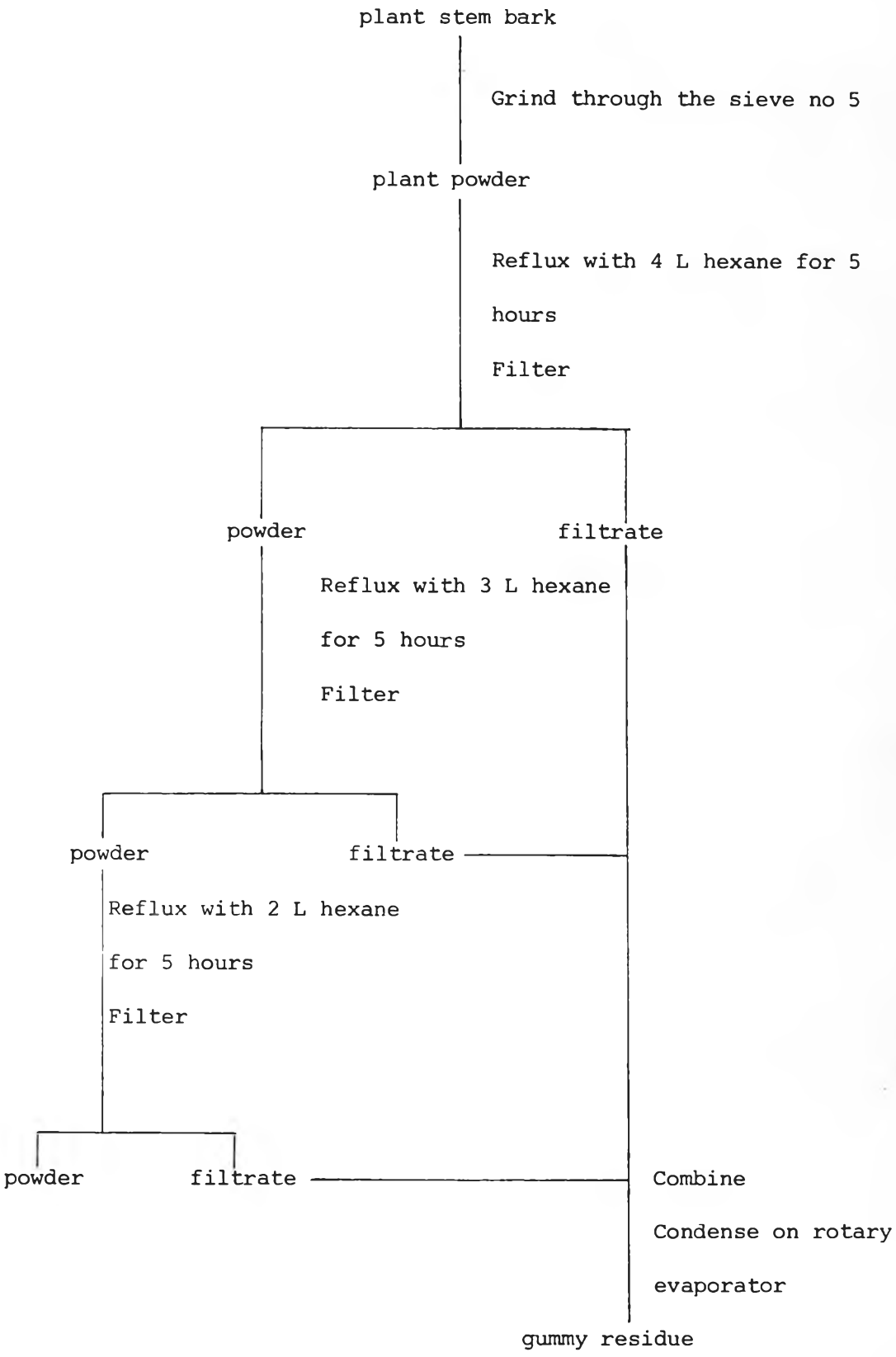
A 100 gm dried, powdered root bark of *Clausena harmandiana* was refluxed with 500 ml n-hexane for 17 hours. The hexane extract was filtered through the filter paper and the powder was allowed to dry at room temperature. The dry powder was returned to the flask and 500 ml Chloroform was added. The content was refluxed for 17 hours and filtered. The filtrate was concentrated under vacuum on rotary evaporator to yield a gummy residue (5 gm) (scheme 1)



Scheme 1 Extraction of *Clausena harmandiana*

2. *Micromelum minutum*

A 500 gm dried, powdered stem bark of *Micromelum minutum* was refluxed with 4 L n-hexane for 5 hours. The extract was filtered through filter paper. The powder was refluxed again with 3 L and 2 L n-hexane for 5 hours of each. The filtrate was combined and the solvent was removed under vacuum on rotary evaporator to give a syrupy residue 12 gm (scheme 2).



Scheme 2 Extraction of *Micromelum minutum*

III Isolation

1. Adsorption column chromatography

In case the plant extracts were to be separated by adsorption column chromatography, first the plant extracts were tested to determine the best solvent systems. To determine which solvent is good for separation by adsorption column chromatography, the R_f value of the desired compound was measured and put into the formula :

$$K = \frac{1 - R_f}{R_f}$$

where R_f = R_f values of the compounds which one would like to separate.

In a good separation, K should have values between 2.5 - 8.0 which means that the desired compounds can be eluted by the amount of solvent 2.5-8.0 times the column volume. This information also can be used to decide how many fractions should be collected and what volume of fractions are needed. If the gradient method was applied for the separation by column adsorption chromatography, the solvents will begin with non-polar ones, usually benzene. The increasing polarity of solvents was done by increasing chloroform and then methanol concentrations. The most polar solvents used for final washing of the column was methanol. The change of solvents was decided by visualization on the column under uv light, by the color of bands which developed down the column, or by testing the column eluants with spraying reagents.

The amount of adsorbant material used was 100-150 times the weight of the plant extracts. The adsorbant material was added to the column in a slurry with the solvent which was used first for

elution. The slurry was poured into the column which contained the same solvent. The column was tapped to make a uniform packing, and the solvent level was not allowed to drain lower than the adsorbant material. To apply the sample two methods were used : (1) If the plant extracts would dissolve in a small amount of the solvent which was used to pack the column, then a concentrated solution of the plant extract was made and applied directly to the top of the column using a pipet. (2) If the plant extracts would not dissolve in the solvent which was used to pack the column, then some solvents like methanol, chloroform, ethanol or a mixture of these were used; these solutions of plant extracts were transferred to a mortar and dried onto a small amount of the adsorbant material; the dried material was transferred to the top of the column and developed with appropiate solvents (34).

2. Thin-layer chromatography (tlc)

All fractions obtained from adsorption chromatography were subjected to thin-layer chromatography (tlc), and the fractions that showed the same pattern were combined. Tlc can be used for checking the purity of compounds.

Preparation of tlc plates : The suspension for five plates (20 x 20 cm) was prepared by shaking 30 gm silica gel 60 G F_{254} (E. Merck) and 60 ml of distilled water for 30 seconds, and was then spread on the plates with an applicator to a thickness of 0.25 mm. After air dried for 15 minutes, the plates were then activated at 120°C for 1 hour.

Useful developing solvent were listed in table 6. After

the chromatograms were developed, the dried plates were visualized by placing under short and long wave uv light. Any zones of fluorescence or quenching were marked, and then the plates were sprayed with spraying reagents which were listed in table 7. The plates after spraying with Fluram were viewed under long wave uv light; the conjugates formed between fluorescamine and primary amines appeared green to yellow while secondary amine conjugates appeared dark blue. The reaction is very fast at room temperature and at pH greater than 7 (35). In order to differentiate between phenolic and non-phenolic compounds, tetrazotized benzidine (TZB) was used as the spraying reagent. Most of the alkaloids and other nitrogen containing compounds give a positive reaction (orange color) with Dragendorff's reagent. The Liebermann-Burchard reagent is frequently used for the detection of sterols. It is characterized by the color sequence red → violet → blue → blue green (36). In some cases iodine vapor can be used for visualization of organic compounds.

Table 6 Tlc Solvent System

System	Components	Ratio
A	benzene : acetone	9:1
B	chloroform : methanol	97:3
C	chloroform : ethyl acetate	9:1
D	chloroform : benzene	1:1
E	chloroform	1
F	ether : petroleum ether	3:2

Table 7 Tlc Spray Reagents

Reagent	Composition	Reference
Fluram	Fluorescamine (4-phenylspir (furan-2(3H), 1-phthalan)-3,3'-dione), 0.02% in acetone	35
Tetrazotized benzidine (TZB)	Equal volumes of solution 1 and 2. Solution 1 was 10% aqueous sodium nitrite. Solution 2 was prepared by triturating 5 gm of benzidine with 15 ml of 12 N HCl and dissolving the resulting suspension in 980 ml of water	37
Modified Dragendorff's	Equal volumes of solution 1 and 2. Solution 1 was 40% aqueous potassium iodide. Solution 2 was prepared by dissolving 8.5 gm basic bismuth subnitrate in 400 ml of water and 100 ml of glacial acetic acid	37
Lieberman-Burchard	5 ml acetic anhydride are carefully mixed under cooling with 5 ml conc. sulfuric acid; this mixture is added cautiously to 50 ml absolute methanol with cooling.	37
Iodine vapor	A few crystals of iodine in a closed vessel.	37

3. Crystallization

Crystallization was necessary in order to obtain pure compound or combined with another method to purify mixture of compounds.

In the plant extracts or the combined fractions from column chromatography, analytical tlc showed major and minor components. Therefore, the major components can often be crystallized directly while the mother liquors were used for further separation. The crystals obtained, if contaminated with another compounds, can be subjected to repeated crystallizations, or rechromatographic step for complete purification.

For attempted crystallization, the plant extracts were dissolved in small amounts of chloroform. To obtain a clear solution, filtration was sometimes necessary. Acetone was added dropwise to the clear solution until a very slight cloudiness resulted. The solution was allowed to evaporate slowly in open air until the small amount was obtained. This solution was placed in the refrigerator or open air and stored overnight. If no crystals appeared, the solution was evaporated under vacuum to dryness and then kept in a vacuum desiccator under vacuum overnight, and the crystallization attempt was repeated. After obtaining crystals, the crystals were filtered under vaccum, washed with a few drops of solvent, and dried in open air or vacuum. The filtrate was processed to obtain more crystal by repeating the obove method.

IV Physical and spectral methods for identification

A convenient preliminary identification of crystallized compounds was analytical tlc. Co-chromatography of an isolated compound with refercence compound should be performed in at least five different solvent systems.

A melting point was also used to identify the isolated compounds; this was determined on Electrothermal Melting Point Apparatus in Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University. A few mg of sample was ground in mortar, and a finely powder was filled into capillary tube which was sealed at one end. The sample tube was put into the melting point apparatus which was heated comparatively rapidly until the temperature was within 15℃ of the melting point of the substance, and then slowly and regularly at the rate of about 2℃ per minute until the compound melted completely. The temperature at which the substance commenced to liquefy and the temperature at which the solid disappeared, the melting point range, were observed. The melting point can indicated the purity of compounds. For a pure compound, the melting point range should not exceed 0.5-1℃ (38).

Ultraviolet (uv) spectra were used to establish the chromophores of isolated compounds. All uv spectra were obtained in methanol with a 1- cm cell using Shimadzu Spectrophotometer UV-180 in Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University. A sample was accurately weighed and transferred into 10 ml volumetric flask,

dissolved with methanol, adjusted to volume with the same solvent. The dilution could be made in order to obtain a suitable concentration. This solution was used for preparing uv-spectrum and measuring the molar absorptivity.

Infrared spectra (ir) were taken in KBr pellets on a Perkin-Elmer 283 Grating Infrared Spectrophotometer in Faculty of Pharmaceutical Sciences, Chulalongkorn University. A sample was ground with small amount of anhydrous potassium bromide in motar. The homogeneous mixture was transferred to a pellet maker. Applying 18,000-20,000 $lb/inch^2$ was enough to make a good pellet which could be obtained a good ir spectrum.

Nuclear magnetic resonance (nmr) spectroscopy has become one of the most important methods in elucidating structure of compounds. Deuterated chloroform ($CDCl_3$) is a good solvent for many compounds. Hexadeuterodimethyl sulphoxide (DMSO-d_6) is a good solvent for more polar compounds which cannot dissolve in $CDCl_3$. The main disadvantage of DMSO-d_6 as a solvent for ^{1}H-nmr study is that its own signals (due to partly deuterated DMSO contaminants), and the signals of the H_2O it so readily absorbs, may obscure compound signals in the 2-4 ppm region (39). The ^{1}H-nmr spectra were recorded in 15 percent (w/v) solutions on Jeol FX 90 Q NMR spectrometer with a frequency of 90 MHz at the Scientific and Technological Research Equipment Center, Chulalongkorn University. Deuterated chloroform was the solvent for compound no 1, 3, 4 and 5. Hexadeuterodimethyl sulphoxide was used as solvent for compound no. 2.; and its solvent effect (40,41) was also used for structure analysis for compound no.1.

Mass spectra were obtained on a Jeol DX 300 double focusing Mass spectrometer at the Scientific and Technological Research Equipment Center, Chulalongkorn University. The electron impact (ei) method was performed with all isolated compounds.