CHAPTER III

EXPERIMENTAL

I. Source of plant material

Clausena harmandiana Pierre. of family Rutaceae is known in Thailand as Song faa Dong (สองฟ้าดง). This plant is an unarmed shrub with few branch, 1.5 m high, leaf 17-40 cm long; folioles 5-9, triangle, alternate, slight, coriaceous, lozenge (10-24 cm x 5.5-14.5 cm). Attenuate or cuneiform at the base and on the tip, crenulate margin, glabrous, lateral vein 6-11 pairs, sometimes bifurcate from the base and the veinlet very prominent in lower surface, a lot of glands very visible; petiolets 0.5-1 cm long; petiole cylindrical, glandular, pubescent.(75)

The root bark of *Clausena harmandiana* Pierre. was collected from Kalsinthy province in the northeast of Thailand in April, 1982. Vouchers of the plant was identified by comparing with the herbarium 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* Pierre. was dried in hot air oven at 50°C for 3 hours, then powdered them with electric mill and let through the sieve No. 5.

II. Extraction

The dried powdered root bark of *Clausena harmandiana* Pierre. (2.8 kg) was refluxed with 20 litres of Hexane for 17 hours, and then filtered. The filtrate was concentrated under vacumm evaporator to give a gummy residue (56 g).

III. Isolation

1. Thin-layer chromatography (TLC) - The experimental details were summarised as following :-

1.1 Preparation of TLC plate,

Technique	:	One way, ascending.
Adsorbents	:	Silica gel 60 GF 254 (E. Merck), 30 g/60 ml of distilled water.
Plate size	:	5 cm x 20 cm x 0.3 cm
Layer thickness	:	250µ
Activation	:	Air dried for 15 minutes and then at 105℃ for 1 hour.

1.2 Solvent systems for TLC. see table 8.

Table 8 TLC solvent systems

system	component	ratio
1	Chloroform	
2	Benzene : Chloroform	1:1
3.	Petroleum ether (bp 40-60°C) : Diethyl ether	5:1
4	Petroleum ether (bp 40-60°C) : Ethyl acetate	3:1
5	Hexane : Diethyl ether	1:1
6	Benzene	
7	Hexane : Diethyl ether	17:3
8	Benzene : Acetone	9:1
9	Benzene : acetone	9:3
10	Chloroform : Methanol	97:3

1.3 Detection of compounds on TLC plate.

1.3.1 Ultraviolet detection - Coumarins are fluoresced blue to green color on TLC plate. Two ultraviolet wavelengths used were :-

i. Short wavelength (254 nm)

ii. Long wavelength (365 nm)

1.3.2 Spraying reagents for TLC

i. Benzidine, diazotised[73] : for phenols.

Stock benzidine solution : 5 g benzidine and 14 ml 36% hydrochloric acid was diluted to 1000 ml with water and stored in refrigerator.

Nitrite solution : 10% solution of sodium nitrite in water .

(Prepared freshly before use) and was stored in refrigerator.

Spray reagent : 20 ml of the benzidine solution was mixed with 20 ml of the nitrite solution at 0°C, stirring continuously.

Note : The reagent can be kept 2-3 hours. The colour may appear very rapidly or after several hours, depending on the phenol. The red colour was developed rapidly for hydroxycoumarins and hydroxycarbazole alkaloid.

ii. Dragendorff's reagent[73] according to Munier and Macheboeuf : for alkaloids and other nitrogen-containing compounds.

Solution a : 0.85 g basic bismuth nitrate was dissolved in a mixture of 10 ml acetic acid and 40 ml water.

Solution b : A solution was made of 8 g potassium iodide in 20 ml water.

Stock solution : Equal volumes of a and b were mixed. This mixture can be stored for a long time in dark glass vessels.

Spray reagent : 1 ml stock solution was mixed with 2 ml acetic acid and 10 ml water before use.

Note : The carbazole alkaloide was developed orange color after spraying.

iii. Ferric chloride[73] : for phenols and hydroxamic acids.

Spray reagent : 1-5% solution of ferric chloride in 0.5 N hydrochloric acid.

Note : Hydroxamic acids yielded red spots, phenols were blue or greenish blue.

iv. Iodine vapor : for unsaturated organic compounds.

Reagent : A few crystals of Iodine in a closed vessel.

Note : Unsaturated organic compounds yielded yellow spots.

v. Sulfuric acid : for organic compounds.

Spray reagent : 10% sulfuric acid in water.

Note : Organic compounds yielded black spots after heat on a hot plate to dry.

2. Column chromatography (CC) - The experimental details were summarised as followed :-

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

Solvents : solvent A - Petroleum ether (bp 40-60℃) : Diethyl ether 5:1

solvent B - Diethyl ether.

Size of column : Diameter 1" and length 14 $\frac{1}{2}$"

Packing of column : 60 g of Adsorbent material was added into 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 adsorbent material.

Addition of gummy residue to column : To apply the sample, the plant extract was dissolved in a small amount of solvent which was used to pack the column, then a concentrated solution of plant extract was made, and applied directly to the top of the column by using a pipet.

Elution of the compounds : The gradient method was used to elute the compounds. The compounds were eluted with solvent A, 25 ml of each fraction was collected as F_1, F_2, F_3..., then followed by solvent B and the same amount of each fraction was collected. Each individual fraction was monitored by TLC in some solvent system and the identical fractions were combined. The combined fractions were evaporated on vacumm

evaporator to dryness and the crystallization was attempted.

3. Crystallization of the compounds - Crystallization was necessary in order to obtain pure compound or, if combined with another method to purify mixture of compounds.

The plant extracts or the dried combined fraction residue was dissolved in small amount of ether. To obtain a clear solution, filtration was sometimes necessary. Methanol was added dropwise to the clear solution until a very slight cloudness resulted (used methanol about 10 times of ether). The solution was allowed to evaporate slowly in open air until the small amount was obtained. This solution was placed in the refrigerator or, and open air and stored overnight. If no crystal appeared, the solution was evaporated under vacumm and the crystallization attempt was repeated Recrystallization was sometimes needed to reach a higher melting points.

After obtaining crystals, they were filtered under vacumm, washed with a few drops of solvent, and dried in open air or vacumm. The filtrate was then processed to obtain more crystal by repeating the above method.

4. Identification

4.1 Physical constant

Melting point - Melting point of the compounds were determined by Electrothermal Melting Point Apparatus in department

of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University

A few mg of sample was ground in agate mortar, and a finely powder was filled into capillary tube which was sealed at one end. The sample tube was put into the instrument and the apparatus was raised step by at a rate of 4-5℃ per minute and 1℃ per minute at the melting point range.

4.2 Spectroscopy

Ultraviolet spectra - Ultraviolet absorption spectra were recorded in methanol using Shimadzu Spectrophotometer UV-180 in Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Accurately weighed an amount of sample in 10 ml volumetric flask, then dissolved with methanol AR., adjusted to volumn with the same solvent. The dilution can be made in order to obtain a suitable concentration. This solution was used to prepare UV-spectrum and to measure the molar absorptivity.

Infrared spectra - Infrared absorption spectra were obtained in potassium bromide disc by Perkin-Elmer 283 Grating Infrared spectrophotometer in Faculty of Pharmaceutical Sciences, Chulalongkorn University.

A few mg of sample was ground with small amount of anhydrous potassium bromide in agate mortar. The homogenous mixture was transfered to a pellet maker. Applying 18,000-20,000 lb/sq.inch was enough to make a good pellet which can be used to

obtain a good IR spectrum.

NMR spectra - NMR spectra were determined in $CDCl_3$ using Jeol FX 90 Q (90 MHz) of The Scientific and Technological Research Equipment Centre, Chulalongkorn University.

10 mg of Sample was dissolved in 1-2 ml $CDCl_3$, filtered, transfered to a 5 mm NMR tube and the spectrum was obtained at room temperature. A technique of irradiation was used in order to assign proton chemical shift.

EIMS spectra - EIMS spectra were determined by using Jeol DX 300 double focusing Mass Spectrometer, of the Scientific and Technological Research Equipment Centre, Chulalongkorn University.

A few mcg of sample was introduced directly into the ionization chamber using sample probe. The sample was heated and the mass was scanned. The number of scan was selected and recorded as a mass spectrum.

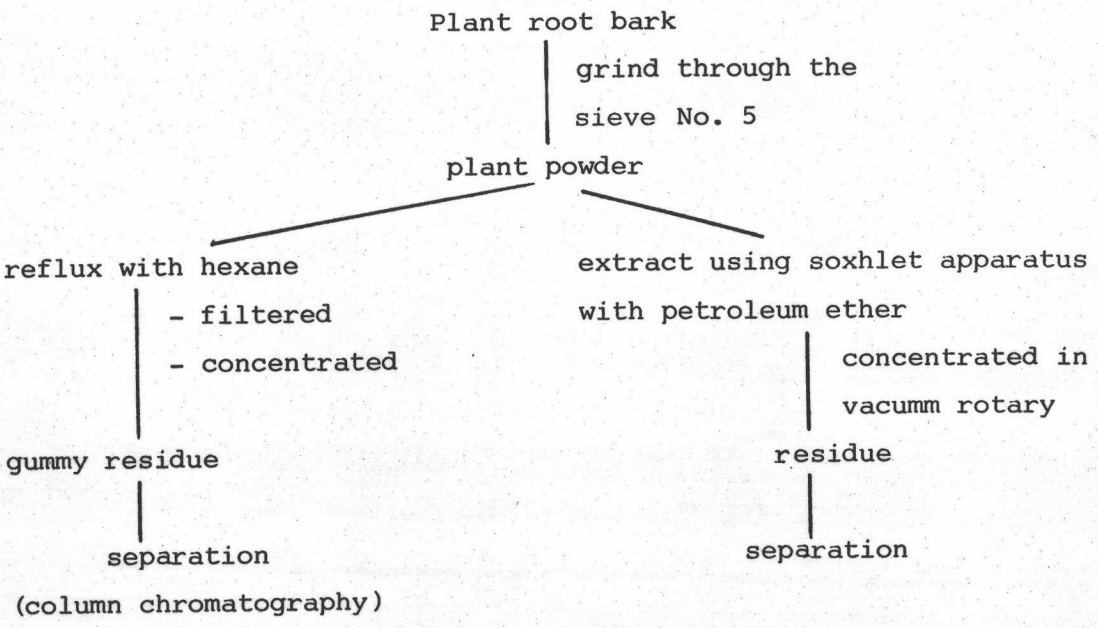
IV. Extraction and isolation of *Clausena harmandiana* Pierre. root bark.

The roots of *Clausena harmandiana* Pierre were obtained from Kalasinthy province, peeled out the bark of root, cut the bark into piece, and dried at 50°C for 3 hours and pulverized through the sieve No. 5.

The powdered plant was divided into two portions, each portion

about 100 g was tried to extract with hexane and petroleum ether (bp. 40-60℃) as showed in the scheme I.

The hexane extracted and petroleum ether extracted were concentrated under vacumm on rotary evaporator to yield the residue about 1 g respectively. On silica gel TLC in solvent system 3 indicated that the hexane extracted contained large amount and more compounds than petroleum ether extracted. Therefore the method using hexane as extracting solvent was selected to use in the extraction of this plant.



Scheme I Method of extraction.

A powdered plant (2.8 kg) was extracted by reflux with hexane (20 litres) for 17 hours. The hexane extract was filtered through the filter paper and condensed under vacumm on rotary evaporator to yield a gummy residue (56 g). The gummy residue (5 g) was processed to separate by column chromatography. A gradient method was selected to elute the column. The adsorbent material (60 g silica gel G 60, 230-400 mesh ASTM) was packed into a 1 x 14 $\frac{1}{2}$" glass column with solvent A. The sample was dissolved in solvent A and applied directly to the top of the column. Development was made with solvent A and 25 ml of each fraction was collected as F_1, F_2,....to F_{43}, then followed by solvent B and the same amount of each fraction was collected as F_{44}, F_{45},....to F_{54}.

Each individual fraction was monitored on TLC in solvent system 3 and the identical fractions were combined. The combined fraction F_{12}-F_{17}, F_{18}-F_{20}, F_{22}-F_{29}, F_{39}-F_{41}, F_{46} and F_{47}-F_{48} were condensed under vacumm on rotary evaporator. The crystallization of each combined fraction was attempted in methanol and diethyl ether. Recrystallization was made in some fractions to obtain a pure compound. All crystallized compounds were subjected to identified by spectroscopic method (UV, IR, NMR, EIMS) and color reactions with spraying reagent on TLC plate (benzidine and ferric chloride spraying reagent give positive test for phenolic compounds but dragendorff's reagent gives positive for alkaloid and nitrogen containing compounds).