CHAPTER III

EXPERIMENTAL

1. Source of Plant Material

The root of *Clausena guillauminii* Tanaka used in this study were collected from Srisakate, Thailand during May, 1984. Vouchers of the plant was identified by comparing with the herbarium that was deposited at the Bangkok Forest Herbarium, Royal Forest Department, Ministry of Agricultural and Cooperative, Bangken, Bangkok, Thailand.

2. General Techniques

2.1 Thin-Layer Chromatography (TLC)

The experimental details were summarised as following :-

a) Preparation of TLC plate

Technique	:	One way, ascending
Adsorbent	:	Silica gel 60 G (7731) 30 gm/60 ml of distilled water
Plate size	:	5 cm x 20 cm, 10 cm x 20 cm and 20 cm x 20 cm
Layer thickness	:	250 μ
Activation	:	Air dried for 15 minutes and then heating in an oven at 105℃ for 1 hour

b) Solvent Systems for TLC

System	Component	Ratio
1	Benzene	
2	Benzene : diethyl ether	5:1
3	Benzene : EtOAc : MeOH	18:2:0.5
4	Hexane : EtOAc	5:1
5	Hexane : EtOAc	1:1
6	Hexane : $CHCl_3$	5:1
7	Hexane : $CHCl_3$	1:2
8	$CHCl_3$	
9	Petroleum ether : diethyl ether	3:1
10	Benzene : $CHCl_3$	1:1

c) Detection of Compound on TLC plate

i) Methanolic potassium hydroxide. It is a 5% potassium hydroxide in methanol and is used for detection of coumarins, anthraquinone glycoside and their glycones.[73]

ii) Diazotized benzidine solution. A fushly prepare of equal volume of benzidine stock solution and 10% sodium nitrite solution. The solution can be kept for 2-3 hours after mixing and is used to detect the compound contains phenolic gorups (positive-red color).

Benzidine stock solution is prepared by mixing 5 gm of benzidine and 14 ml 36% hydrochloric acid was diluted to 1,000 ml with water and stored in refrigerator.

10% sodium nitrite is a solution in water.

iii) Iodine vapor. A few crystals of iodine is put in a closed vessel. The yellow spots was developed, for unsaturated organic compounds.

iv) Ferric chloride solution. A 1-5 gm of ferric chloride is dissolved in 0.5 N hydrochloric acid to make 100 ml. The solution give a red spot with hydroxamic acid, greenish blue with the compound contained phenolic group.

2.2 Column Chromatography (CC)

The experimental details were summarised as followed :-

Adsorbent	:	Silica gel 60 G (9385)
Solvent	:	Petroleum ether (b.p. 40-60), Diethyl ether, Benzene, $CHCl_3$
Size of Column	:	Diameter 5 cm and length 40 cm
Packing	:	120 gm silica gel 60 (9385) was added into the column in a slurry with potroleum ether. The column was tapped to make a uniform packing and the solvent level was not allowed to drain lower than the adsorbent material.
Elution	:	The packed column was loaded with the solution of the sample in appropiate solvent. The column was eluted with solvent using the gradient technique and the fractions were collected and monitered with TLC in order to combine the identical fraction.

2.3 Crystallization

The pure compounds was obtained by crystallization. The method of crystallization was performed as the following. The dried mixture compounds was dissolved in small amount of diethyl ether, a clear solution was added with dropwise of solvent (slightly dissolved in this compound) until a very sligt cloudness resulted. The solution was allowed in open air overnight or in the refrigerator. After botaining crystals, they were filtered, and washed with a few drops of solvent, and dried in open air. Recrystallization was sometime needed in order to obtain more pure compound.

2.4 Identification

a) Melting point

Melting point of the compounds were determined by Gallenkamp Melting point Apparatus.

b) Spectroscopy

i) Ultraviolet (UV) spectra

UV spectra were obtained by Shimadzu UV-VIS Recording Spectrophotometer UV-240 of the Scientific and Technological Research Equipment Center, Chulalongkorn University.

ii) Infrared (IR) spectra

IR spectra were obtained in potassium bromide disc by shimadzu IR 440 Spectrophotometer of the Scientific and Technological Research Equipment Centre, Chulalongkorn University.

iii) Nuclear Magnetic Resonance (NMR) Spectra

^{1}H NMR and ^{13}C NMR spectra were obtained from a Bruker WM-250, instrument in a deuterochloroform, using tetramethylsilane as the internal reference, in the Department of Chemistry, University of Arizona.

The structure of compound was confirmed by ^1H NMR in $CDCl_3$ and DMSO-d_6 using Jeol FX 90 Q (90 MHz) in The Scientific and Technological Research Equipment Center, Chulalongkorn University.

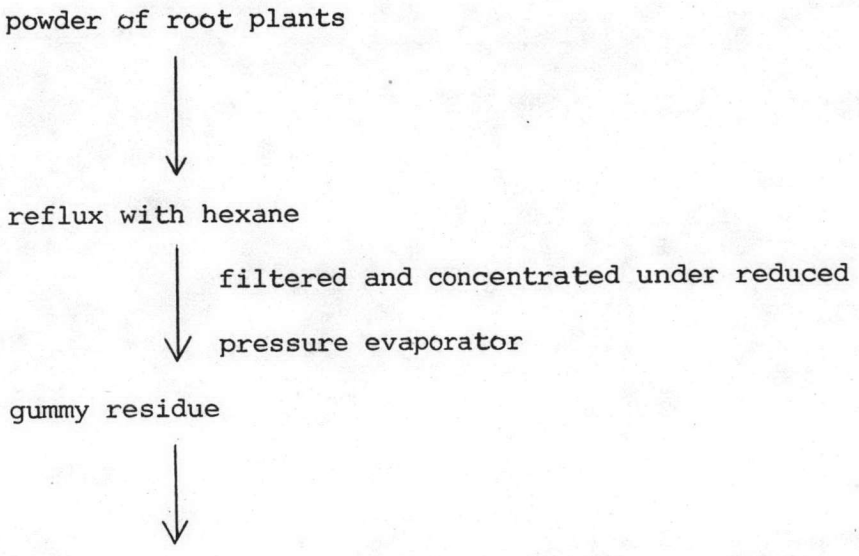
iv) Election impact mass spectra (EIMS)

EMIS were obtained from Jeol model JMS-DX 300 double focusing Mass Spectrometer in ionization voltage 70 eV and ionization current 300 A, in Scientific and Technological Research Equipment Center, Chulalongkorn University.

3. Extraction and Isolation

3.1 Extraction

The dried powder root of *Clausena guillauminii* Tanaka (2 kg) was extracted by reflux with hexane (18 litres) for 3 hours. The hexane extract was filtered through the filter paper condensed under reduced pressure on rotary evaporator to yield a gummy residue (18.8 gm)



3.2 Isolation

The position of gummy residue (10 gm) of the hexane extract was processed to separate by column chromatography. The extract was dissolved with a small amount of mixture of petroleum ether and a few drops of diethyl ether. The resulting solution was applied directly to the top of the column by using a pipet. Development was made with mixtured of petroleum ether and diethyl ether in a ratio of as petroleum ether : diethyl ether 5:1, 2:1, 1:2 and diethyl ether, respectively. A fractions was collected and monitoried with TLC, solvent system 9 (Fig. 14). The identical fractions were combined and the five major combined fractions were assigned to be

Fractions 16-18 (a)

Fractions 30-38 (b)

Fractions 40-47 (c)

Fractions (67-73) (d)

Fractions (81-82) (e)

Fractions (a), (b), (d) and (e) were condensed under reduced pressure on rotary evaporator until a small amount solution was obtained. Crystallization was attempted of each fraction. The obtaining crystal filtered, washed with a few drops of solvent and recrystallization was necessary in order to obtain a pure compound. The crystal obtained from fraction a, b, d and e were assigned to be compound I, II, IV and V respectively. Fraction (c) was showed by Thin Layer Chromatography (system 10) (Fig. 15) to contain two major spot. It was evaporated under reduced pressure to dryness and then was rechromatographed in the same manner using benzene and benzene : chloroform (1:1) as eluent. The fractions were collected and the

identical fraction were combined. Two combined fractions which showed to contain a major compound were subjected to crystallization and the obtaining crystals were filtered and the recrystallization was performed in order to obtained more pure compound. Two compounds, III and VI were obtained from the above method of seperation.

4. Identification

The identification of isolated compounds were basically compared to the authentic compound which were obtained from the isolation of *Clausena harmandiana* Pierre in our laboratory. The comparison, mostly, were on the Rf value, melting point, chemical reactions on TLC plate (detection of spot) UV and IR spectroscopic data. Some of them were identified by using NMR and MS spectroscopic data. A special method, such a solvent effect, APT (attacked proton test) were also performed in order to confirmed the structure of compounds.