CHAPTER III

EXPERIMENT

1. Source of plant materials

Clausena cambodiana Guill; of family Rutaceae is known in Thailand as Samui hom (39) . This plant is a small tree 5-8 m high, branch glabrescent, leaf 15-20 cm. long, leaflet 5-11, alternate, glabrate, almost equilateral at base, membranous, oval or oblonglanceolate (9-15 cm. x 3-5 cm.), cuneate at base, cuspidate at apex, lateral nerve 6-9 pairs, likly vein, clear visible and prominent in lower surface. The petiolet cylindric and pubescence. Inflorescence long equal leaf, terminate panicle, pubescent, pyramidal. Fruit, red-fragrance, pustular of oil grand (40).

The root bark of Clausena cambodiana Guill was collected from Nakhon Si Thammarat province in the Southern of Thailand in August. 1988. this plant was identified by comparison with the herbarium specimen in the Botany section, Techinical Devision, Department of Agriculture; Ministry of Agriculture and cooperative, Bangken, Bangkok, Thailand.

The root bark of Clausena cambodiana Guill. was dried in hot air oven at 50 C for 24 hours and then ground into the powder by electric mill and pass along sieve No 7.

2. General techniques

1. Thin layer chromatography (TLC)

The experimental details are summarised as follows :-

Analytical

Technique : One way, ascending

Adsorbents : Silica gel G (E.merk),calcium sulphate binder 13%, 30 g/ 60 ml of distilled water.

Plate size : 20 cm x 5 cm.

Layer thickness : 250 μ

Activation : Air dried for 15 minutes and then at 105 °C for 1 hour

Distance : 15 cm.

Laboratory temperature : 25°- 30 °C

Solvent system

System	Component	Ratio
1	Chloroform	
2	Petroleum ether (b.p. 40-60 ^C):Diethyl ether	5:1
3	Petroleum ether (b.p. 40-60 ^C):Ethyl acetate	1:1
4	Hexane : Ethyl acetate	3:1
5	Chloroform : Methanol	44:2
6	Benzene : Chloroform	1:1
7	Benzene : Acetone	9:1
8	Hexane : Diethyl ether	9:1

Detection of compound on TLC plate.

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

1. Short wavelength (254 nm)
2. Long wavelength (365 nm)

Spraying reagent for TLC

1. Benzidine,diazotised (41):- for phenol.

stock benzidine solution : 5 g benzidine and 14 ml 30% hydrochloric acid was diluted to 1000 ml with water

Nitrite solution : 10 % solution of sodium nitrite in water prepared freshly before use

Spray reagent : 20 ml of the benzidine solution are 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

4.Ferric chloride (41) : for phenol and hydroxamic acid

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

Note : Hydroxaminc acid yeild red spots,phenol blue or greenish.

3. Iodine vapor (41) : for unsaturated organic compounds.

Reagent : Iodine crystal

Note : The chromatogram is introduced into a closed vessel on the floor of which some crystals of Iodine have been placed. Iodine vapous is more quickly generated through gently warming the vessel. Many organic compound yeild brown spots.

2. Column chromatography

Column size : 1.5 inch x 24 inch,1 inch x 14 1/2 inch 0.8 inch x 10 inch

Absorbent : Siliga gel 0.040-0.063 mm. (E. merk)

Packing : Adsorbent packed wet into the column.

Addition of material to column : The portion of crude extract was dissolved in a small

amount of solvent which was used to paek the column,and appied directly to the top of the column by using a pipet

Solvent : Petroleum ether (Commercial grade)

: Diethyl ether (E. merk)

: Hexane (E. merk)

: Ethyl acetate (E. merk)

: Chloroform (E. merk)

: Acetone (E. merk)

3. Crystallization of the compound

Crystallization was another way for purification of compound. This method was simple and effective

The technique involved dissolving the material in hot solvent (or solvent mixture) and cooled the solution slowly. The dissolved material has a decreased solubility at lower temperature and will precipitate from the solution on cooling crystal growth relatively slow and selective

The dried combined fraction residue (which were separated from column) was dissolved in small amount of ethanol. To obtain a clear solution. Filtration was sometime necessary. The solution was standed to evaporate in open air untill the small amount was obtained. this

solution was placed in the refrigerator or open air and stored overnight.

If no crystal was formed. It needed to repeat crystallization in other solvent. Recrystallization was sometimes needed to reach a more purifid crystal.

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 $^{\circ}$C per minute and 1 $^{\circ}$C per minute at the melting point range.

4.2 Spectroscopy

Infrared spectra - Infrared absorption spetra were obtained in potassium bromide disc by. Shimaszu IR-440. Infrared Spectrophotometer of the science and Technological Research Equipment Center, 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.

10-50 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.

For C-13 NMR ,the off resonance; proton noise decouping and long-range coupling (Gated decouping) were performed

MS spectra - EIMS spectra were determined by using Jeol FX 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.

3. Extraction

The dried powdered root bark of Clausena cambodiana Guill. (2.2 kg) was refluxed with 5 liters of hexane for 6 hours to remove the nonpolar substance and follow by reflux with 5 liters of chloroform for 6 hours (three time) as in figure 18 The chloroform filtrate was concentrated under vacuum evaporator to give a gummy residue (156 gm.)

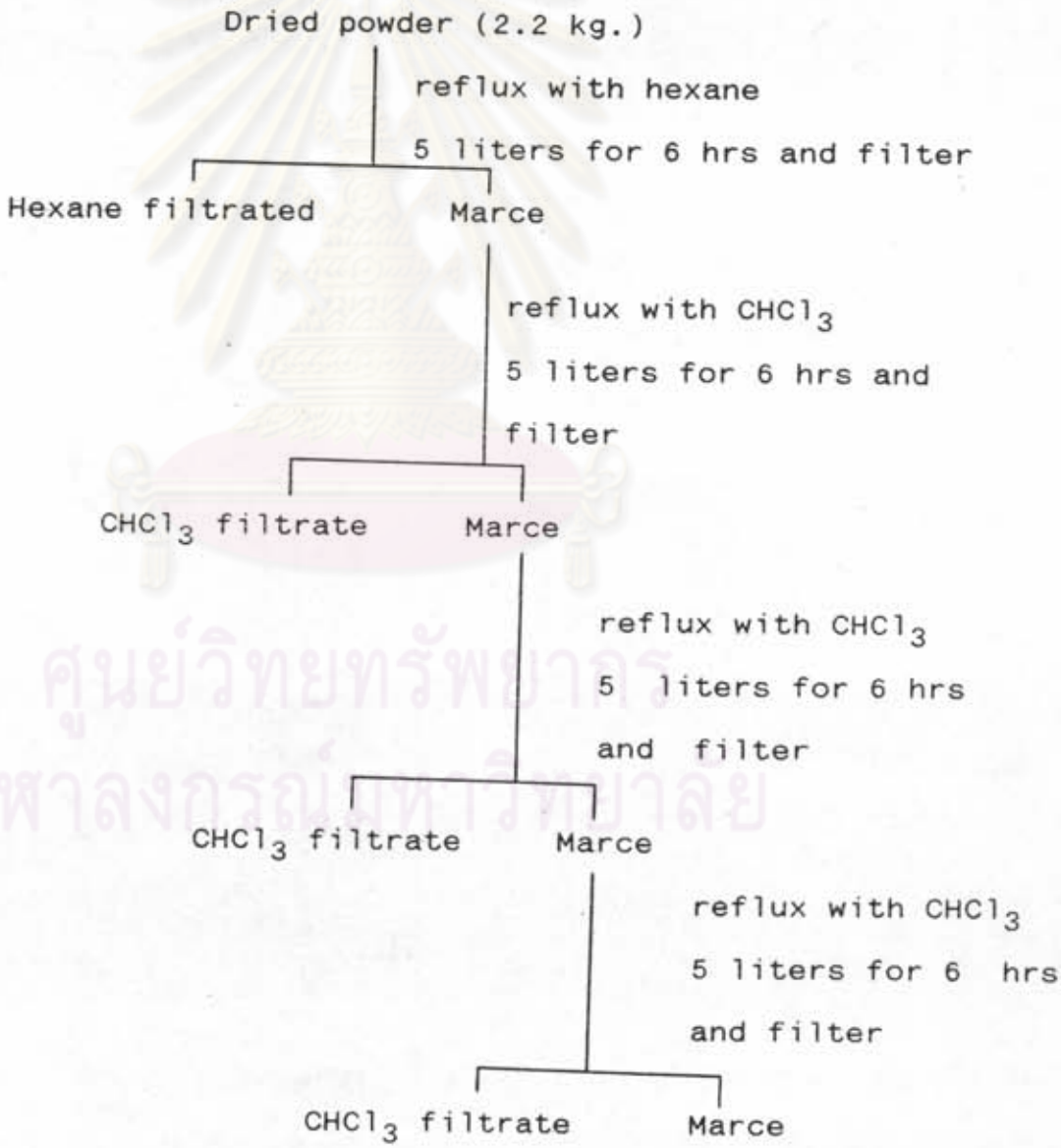

Figure.18 Method of extraction

4. Isolation

The combined chloroform extract remaining after filtered through the filter paper were concentrated under reduce pressure to give a gummy residue (156 gm.)

The gummy residue (40 gm.) was placed on a column which made of glass with a sintered glass frit. (The diameter of column was 10 inchs. and 5 inchs hight) and packed with silica gel (silica gel G 60 230-400 mesh ASTM) follow the method of Vacuum Liquid Chromatography.(34) The elution procedure was outlined in Figure.19

The volume of eluent in each fraction from quick column was 100 ml. (20 fractions) each fraction was monitored on TLC

The combined fraction F_3-F_6 were concentrated under reduce pressure on rotary evaporator and rechromatograph on a fractional column chromatography that packed with silica gel (column size 1.5 x 24"). Elute with Petroleum ether : diethyl ether (1 : 1). At last with recrystallization. We obtained crystal of Clausenidin (2.12 gm.) and Dentation (0.8 gm).

Combined fraction of F_8-F_9 were concentrated under reduced pressure and rechromatograph by fractional column chromatography paked with silica gel (size 1 x 14 1/2") Eluted with Hexane : Eythyl acetate (3 : 1). We could separated Clausarin and Xanthoxyletin from the column and purified by recrystallization yeild 0.120 gm of Clausarin

POLARITY STAGES

Fraction No.	% of solvent		
	Hexane	Chloroform	Methanol
1	100	0	
2	98	2	
3	95	5	
4	90	10	
5	85	15	
6	80	20	
7	75	25	
8	65	35	
9	55	45	
10	50	50	
11	0	100	0
12		95	5
13		90	10
14		85	15
15		80	20
16		75	25
17		65	35
18		55	45
19		50	50
20		30	70

Figure.19 The elution sequence of vacuum liquid chromatography.

and 0.060 gm of Xanthoxyletin.

Combined fraction of F_{15}-F_{17} were concentrated under reduce p[ressure and rechromatograph by fractional column chromatography (size 1 x 14 1/2") packed with silica gel. Elute with $CHCl_3$: Acetone (44 : 2). Nordentatin was separated from column and recrystallized in Ethanol yeild 0.040 gm of crystal.

5. Epoxidation of Clausenidin

Clausenidin (280 mg) was disolved in $CHCl_3$ (5 ml) and add solution of m-chloroperbenzoic acid (183 mg in $CHCl_3$ 5 ml), stirred in the ice bath (0-4 ^ C) for 2 hrs and then allowed the reaction mixture to stand overnight at room temperature.

The reaction mixture was concentrated under reduced pressure and separated the product by column chromatography (size 0.8 x 10") packed with silica gel (25 g.). The solvent system was Petroleum ether : diethyl ether (1 : 1) we could separated the product (100 mg) and purified by recrystallization.