CHAPTER II

EXPERIMENTAL

1. Source of plant materials

Clausena cambodiana Guill., belonging to family Rutaceae, was called in Thailand as Samui hom (1). This plant is a small tree 5-8 m. high, branch glabrescent, leaf 15-20 cm. long, leaflet 5-11 cm., alternate, glabrate, almost equilateral at base, membranous, oval or oblong-lanceolate (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 is cylindrical long (5 mm.), petiole cylindric and pubescence. Inflorescence long equal leaf, terminate panicle, pubescent, pyramidal. Fruit, red-violet globose, diameter about 8 mm., strongly fragrance, pustular of oil grand(33).

The root barks of *Clausena cambodiana* Guill. were collected from Nakhon Si Thammarat province in the south of Thailand in May, 1985. The plant materials were identified by comparison with the herbarium specimen in the Botany section, Technical Division , Department of Agriculture, Ministry of Agriculture and Cooperative, Thailand.

2. Extraction

The root barks of *Clausena cambodiana* were dried in hot air oven at 65 c for 6 hours, and grinned with electric mill through sieve no. 7.

A 850 gram sample of dried power root bark was refluxed in certain time and certain amount of n-hexane as the following:

1. n-Hexane 5 liters refluxed for 5 hours then filtered.
2. n-Hexane 3.5 liters refluxed for 5 hours then filtered.
3. n-Hexane 3.5 liters refluxed for 5 hours then filter.

All filtrate were collected and concentrated under vacuum on rotary evaporator to yield a brown gummy residue(123 gm.).

3. Isolation purification and Crystallization

In the extract, analytical TLC showed major component of the compound therefor the major one can often be crystallized directly. The crystals obtained, if contaminated with another compounds, can be subjected to repeated the crystallization, or another chromatographic step.

For attempt of crystallization, the plant extracts were dissolved in small amount of ethanol. To obtain a clear solution, filtration was some time necessary. The clear solution was then placed in refrigerator and stored overnight. Recrystallization was

sometimes needed to read a higher purification. In some one recrystallization was done in other solvent for example chloroform. After obtaining crystals, the crystal were filtered under vacuum, washed with a few drop of solvent and dried in open air at room temperature. The filtrate, then was processed to obtain more crystals by repeating the above method.

Three obtained pure compounds (compound-1, compound-2, compound-3) were determined structure by spectroscopy technique for analysis of organic compound. The compound-2 was brought to study chemical reaction in next process. (27)

4. Chemical reaction of coumarins

Weigh 100 mg of compound-2 and dissolved in 50 ml absolute methanol, add 5 g potassium carbonate swhir gently. Reflux in water bath for 9 hours. Between reaction time, product will checked by TLC plate. After reaction complete, the reaction mixture was cooled and filtered. Add diluted hydrochoric acid to filtrate until precipitates (compound-4) are form. Filtered and wash precipitate with water and cool methanol two times (each time about 10 ml) and then dissolved the precipitate in hot methanol until completely dissolve, stand in room temperature for recrystalize of compound-2

5. Isolation and determination

5.1 Thin layer chromatography (TLC)

Obtaining crystals were checked the purity by technique of TLC. The TLC plates were prepared as the following:

Slurries of adsorbents (silica gel 60 G F254 (E.Merck) 25 gm and 50 ml of water) can be applied as thin layers to glass plates (five plates 20 x 20 cm) by spreading which STAHL's appicator " Model SII" for the preparation of layer 0.25 mm thick. After dried for 15 minutes, the plate were then activated in hot air oven at 120 C for 1 hour. The solution of samples, mostly in methanol, was applied on TLC plate and developed in solvent system below.

Solvent systems

System	Solvents	Ratio
1	Chloroform	
2	Benzene : Chloroform	1:1
3	Petroleum ether : Ether	5:1
4	Petroleum ether : Ether	3:1
5	n-Hexane : Ether	1:1
6	Benzene	
7	Benzene : Acetone	9:1
8	Benzene : Acetone	7:3
9	Chloroform : Ethyl acetate	3:1

10 Chloroform : Ethyl acetate 1:1

5.2 Detection of samples on TLC

5.2.1 10% Sulfuric acid in water.

Use by spray on plate .Organic compounds show black spots after heat on hot plate.

5.2.2 Iodine vapor.

A crystals of Iodine in closed vessel. For unsaturated compounds yielded yellow spot to brown.

5.2.3 UV determination.

Coumarin can fluoresced to give blue color spot on TLC plate. Two ultraviolet wavelengths used were used are

Short wavelength (254 nm)

Long wavelength (365nm)

5.2.4 Benzidine, diazotised

5.2.4.1 Stock benzidine solution:(Solvent) Prepared from 5g Benzidine and 14 ml 36% hydrochloric acid was diluted to 1000 ml with water and store in refrigerator.

5.2.4.2 Nitrite solution:(Solvent 2) 10% solution of sodium nitrite in water (prepared freshly before use) and stored in refrigerator. Mixed equal

amount of solvent 1 and solvent 2 at 0 C to form spray reagent. This reagent can develop red color rapidly when reacted with hydroxycoumarin and hydroxycarbazole alkaloid.

5.2.4.3 Ferric chloride

1-5% solution of ferric chloride in 0.5 N.hydrochloric acid. Ferric chloride reacts with phenoric compounds form blue color to greenish blue. (28)

6. Identification

6.1 Melting point.

The melting point of compounds were determine by Electrothermal melting point apparatus. Fine a few mg of compound in mortar, and filled into capillary tube which was sealed at one end. The capillary tube was put into melting point apparatus and operation was increase temperature 4-5 C per minute at the melting point range,the melting point range was observed. For the pure compound, the melting point range should not varies between 0.5-1.0 C

6.2 Infrared spectra

IR were determined in potassium bromide disk by

Infrared Spectophotometer, model IR-440, Shimadzu Ltd. Japan, of the Science and Technological Research Equipment Center, Chulalongkorn University.

A few mg of sample was ground combine with small amount of anhydrous potassium bromide. The homogeneous mixture brought to prepare pellet by a pellet maker. Apply 18,000-20,000 lb/sq.inch to make a pellet which can be used for determination of compound spectra.

6.3 Nuclear magnetic resonance.

NMR spectra The NMR spectra were determined in CDCl for using Nuclear Magnetic Resonance Spectrometer model FX-90Q (90 MHz) of The Scientific and Technological Research Equipment Center, Chulalongkorn University.

About 10 mg of sample was dissolved with suitable solvents (deuterated chloroform for sample 1,2,3 , compound-1 use pyridine-d5 as solvent in 5 mm NMR tube, the spectra was recorded on frequency of 90 MHz. The technique of irradiation was used in order to assign proton chemical shift for structure elucidation.

6.4 Mass spectra

MS were obtained on model JEOL DX-300 double focusing mass spectrometer at the Scientific and Technological Research Equipment center, Chulalongkorn University. A few mcg of compound-2

was introduced into the ionization chamber using sample probe. The sample was heated and scanned. The electron impact method was performed with all isolated compounds and product from reaction.