



CHAPTER III

EXPERIMENT

1. SOURCE OF PLANT MATERIALS

Micromelum minutum Wight & Arn. (Syn *M. pubescens* Blume) of Family Rutaceae is known in Thailand as Mui-chang, Hat-sakhun-thai. 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 (42).

The root bark of *Micromelum minutum* was collected from Surathani province in the southern part of Thailand in 1989. This plant was identified by comparison with the herbarium specimen in the Royal Forest Department, Ministry of Agriculture and Cooperative, Bangkok, Thailand.

The root bark of *Micromelum minutum* was dried in hot air oven at 50°C for 24 hours and then ground into the powder by electric mill and passed along sieve No. 7.

2. GENERAL TECHNIQUES

1. THIN LAYER CHROMATOGRAPHY (TLC)

The experimental details are summarised as following :-

ANALYTICAL TLC

- Technique : One way, Ascending
- Adsorbents : Silica gel GF₂₅₄ (E. Merk),
30 g/60 ml of distilled water.
- Plate size : 20 cm x 20 cm, 20 cm x 10 cm,
20 cm x 5 cm
- Layer thickness : 250 μ
- Activation : Air dried for 15 minutes and
then dried at 105°C for 30 minutes
- Distance : 15 cm
- Laboratory temperature : 25° - 30°C

Solvent systems

System	Component	Ratio
1	Hexane : Ethyl acetate	1 : 1
2	Benzene : Acetone	9 : 1
3	Chloroform : Acetone	4 : 1
4	Chloroform : Methanol	49 : 1
5	Chloroform : Ethyl Acetate	1 : 1
6	Petroleum Ether (b.p. 40-60°C) : Ethyl Acetate	2 : 3
7	Hexane : Acetone	1 : 1
8	Acetone : Methanol	3 : 2

DETECTION OF COMPOUND ON TLC PLATE

ULTRAVIOLET DETECTION : - Coumarins are fluoresced blue to green color on TLC plate under UV light. Two ultraviolet wavelengths used were

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

SPRAYING REAGENT FOR TLC

1. BENZIDINE, DIAZOTISED (43) : 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 stirring continuously.

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

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2. FERRIC CHLORIDE (43) : for phenol and hydroxamic acid

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

Note : Hydroxamic acid yield red spots, phenol yield blue or greenish blue.

3. IODINE VAPOUR (43) : for unsaturated organic compounds

Reagent : Iodine crystal

Note : The chromatogram is introduced into a closed vessel containing some crystal of iodine. Iodine vapour generated bind to the spot of organic compounds presented as brown spots usually.

2. COLUMN CHROMATOGRAPHY

Column size : 1 inch x 14 1/2 inch, 1.5 inch x 24 inch, 5 inch x 12 inch

Adsorbent : Silica gel G60 230-400 mesh astm (E. Merk)

- Packing : Adsorbent was packed as wet method into the column.
- Sampling loading : The portion of crude extract was dissolved in a small amount of eluting solvent and applied directly to the top of the column.
- Solvent : Hexane (E. Merk)
Ethyl acetate (E. Merk)
Chloroform (E. Merk)
Methanol (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 crystallize slowly and selectively from the cooling solution.

The dried combined fraction residue (which were separated from column) was dissolved in small amount of diethyl ether. Filtration was sometimes necessary in order to obtain a clear solution. The clear solution was standed to evaporate in open air until the small amount of crystal was obtained. Then the solution was placed in the

refrigerator or open air stored overnight to yield more crystals.

If no crystal was formed. The solution was evaporated under vacuum to dryness and the crystallization was repeated with other solvent. After obtaining crystals, the crystals were filtered under vacuum, washed with a few drops of solvent, and dried in open air or under vacuum. Recrystallization was sometimes needed to obtain a more purified 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 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 raised step by at a rate of 4-5°C per minute and 1°C per minute at the melting point range.

4.2 SPECTROSCOPY

ULTRAVIOLET SPECTRA - Ultraviolet absorption spectra were recorded in methanol using Hitachi 100-60 in The Scientific and Technological Research Equipment Center, Chulalongkorn University.

Accurately weighed an amount of sample in 10 ml volumetric flask, then dissolved with methanol, adjusted to volume with the same solvent. The dilute can be made in order to obtain a suitable concentration. This solution was used to obtain the UV spectrum.

INFRARED SPECTRA - Infrared absorption spectra were obtained in potassium bromide disc. By Shimadzu IR-440. Infrared Spectrophotometer of the Scientific 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 homogeneous mixture was transferred 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 90Q (90 MHz) of the Scientific and Technological Research Equipment Center, Chulalongkorn

University and using Jeol (500 MHz) of ICI Agrochemicals Company, Bracknell, Jealous Hill, England.

10-50 mg of sample was dissolved in 1-2 ml CDCl_3 , filtered, transferred 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 high resolution NMR, the spectra were obtained by 500 MHz NMR. Proton and Carbon-13 NMR were performed with differential pulse program COSY, DEPT, INEPT and NOESY also were used in order to assign the exact carbon position in the structure of isolated compound.

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

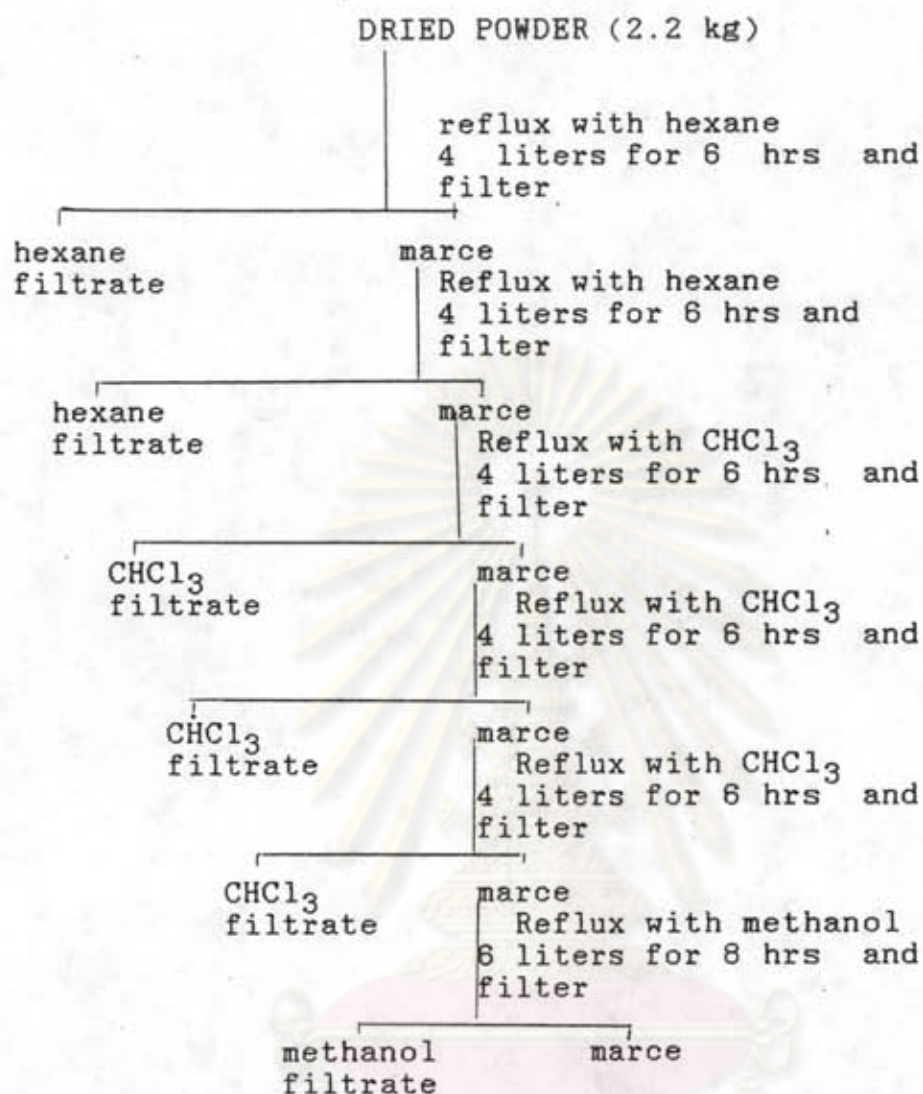
A few mcg of sample was introduced directly into the ionization chamber using sample probe. The sample was heated and the mass was scanned. The number of scan was selected and recorded as a mass spectrum.

3. EXTRACTION

The dried powdered root bark of *Micromelum minutum* Wight & Arn. (1.2 kg) was refluxed with the following solvents : hexane (4 l x 2, each for 6 hours), chloroform (4 l x 3, each for 6 hours), and methanol (6 l for 8 hours). The scheme of extraction was shown. The hexane, chloroform and methanol filtrate were concentrated separately under vacuum evaporator to give hexane (18 g), chloroform (31 g) and methanol gummy residue (95 g) respectively.



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The Scheme of Extraction

4. ISOLATION

The combined hexane filtrate was concentrated under reduce pressure to give a gummy residue. (18 g) The hexane gummy residue (10 g) was chromatographed on a fractional column chromatography (column size 1.5" x 24") that packed with silica gel (silica gel G 230 - 400 mesh astm). Elute with hexane : ethyl acetate (6 : 1). The

volume of eluent in each fraction was 25 ml. At last osthol was recrystallized from F₂₂ - F₃₀. (0.324 g).

The combined CHCl₃ filtrate was concentrated under reduced pressure by rotary evaporator to give a gummy residue (31 g). The gummy residue (10 g) was chromatographed over column chromatography with silica gel (size 1.5" x 24") and elute with hexane : ethyl acetate (3 : 2). Compound II was separated from the column (0.183 g).

The combined methanol filtrate was concentrated under reduced pressure by rotary evaporator to give a gummy residue (95 g). The gummy residue (50 g) was chromatographed over column chromatography packed with silica gel (size 5" x 12") and eluted with hexane : ethyl acetate (1 : 10). The volume of eluent in each fraction was 50 ml. The combined fraction F₂₁₋₂₉ were concentrated under reduced pressure and rechromatographed over column chromatography (size 1" x 14 1/2") packed with silica gel and elute with chloroform : methanol (98 : 1). Compound III was separated from column (0.012 g).

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