Chapter III

Experimental

Source and authentication of plant material

The bark of *Fissistigma polyanthoides* was collected from Nam Nao National Park, Petchaboon province, Thailand, in March 1993. It was authenticated by comparison with the herbarium specimen at Royal Forest Department, Bangkok, Thailand.

The voucher specimen of the plant material has been deposited in the Faculty of Pharmaceutical, Chulalongkorn University, Bangkok, Thailand.

General techniques

1. Thin-layer chromatography (TLC)

Technique: One way ascending

Adsorbent: Silica gel 60 G (Number 7731, E. Merck) : Silica gel 60 HF_{254} (Number 7739, E. Merck) (2 : 1)

Plate size: 5 × 20 cm., 10 × 20 cm., 20 × 20 cm.

Layer thickness: 0.25 mm.

Activation: Air dried for 15 minutes and then heat in hot air oven at 110° C for 1 hour.

Solvent system: Various solvent system depending on materials.

Distance: 15 cm.

Laboratory temperature: 28 - 35° C

Detection: 1) UV light (254 and 366 nm.).

2) Dragendorff's reagent.

2. **Preparative thin-layer chromatography**

Technique: One way ascending

Adsorbent: Silica gel 60 G (Number 7731, E. Merck) : Silica gel 60 HF_{254} (Number 7739, E. Merck) (2 : 1)

Plate size: 20 × 20 cm.

Layer thickness: 0.5 mm.

Solvent system: Chloroform : Acetone (4 : 1).

Distance: 20 cm.

Laboratory temperature: 28 - 35° C

Detection: UV light (254 and 366 nm.)

Substance recovering: The scraped off zones were warmed with a mixtures of chloroform : methanol (1 : 1) and filtered. After removal of the solvent, the residues were dissolved in

chloroform and filtered. The filtrate was left to crystallize.

3. Column chromatography (CC)

Column size: 2.5 × 10 cm.

Adsorbent: Silica gel 60 for column chromatography (Number 9385, E. Merck)

Packing method: Wet packing

Solvent: Hexane : Acetone (4 : 1)

Chloroform : Acetone (3 : 2)

Spectroscopy

1. Ultraviolet (UV) spectroscopy

Ultraviolet absorption spectra were determined on a Milton Roy Sprotronic 3000 Ray.

2. Infared (IR) absorption spectrophotometry

Infared absorption spectra were obtained on a Perkin Elmer Model 1760 X USA infared spectrophotometer. The absorption bands were reported in wave number (cm-1). The materials were examined in KBr. cell.

3. Mass spectroscopy (MS)

The electron impact mass spectra (eims) were obtained by operating at 70 eV with a Fisons VG Trio 2000.

4. Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H and ^{13}C-NMR spectra were obtained on a JNM-A 500 (Alpha series) 500 MHz NMR spectrometer. Deuterochloroform ($CDCl_3$) and acetone-d_6 were used as the solvents and TMS as the internal standard. The chemical shifts were reported on ppm scale.

Physical constants

1. Melting points

Melting points were determined on a Buchi melting point apparatus.

Extraction

The dried, finely powdered stem bark (2.4 Kg) of *Fissistigma polyanthoides* was marcerated in 95% ethanol (6 l.) three times for 3 day-periods and filtered. After combination, the ethanolic extracts was evaporated under reduced pressure to dryness.

The ethanolic extract (613 g.) was triturated with kieselguhr 1.28 Kg. It was then eluted with hexane 5 × 20 l., chloroform 6 × 10 l. and methanol 5 × 20 l. respectively. Each eluent was evaporated under reduced pressure to yielded hexane extract 17.2 g., chloroform extract 36.3 g. and methanol extract 60.1 g.

The chloroform extract (36.3 g.) was acidified with 10% aqueous sulfuric acid (3 l.) and extracted with chloroform (4 × 0.5 l.). The aqueous layer was basified (pH 9-10) with strong ammonium hydroxide solution and exhaustively extracted with chloroform (8 × 0.5 l.). The combined chloroform extract was washed with a small amount of distilled water, dehydrated with anhydrous sodium sulfate, and concentrated under reduced pressure to give a crude alkaloid extract (1.7 g.).

Crude hexane extract (17.72 g.), crude alkaloid extract (1.7 g.) and crude methanol extract (60.1 g.) were further subjected to column chromatographic separation.

Separation and isolation

1. **Separation and isolation of chemical substance from crude hexane extract**

The crude hexane extract (17.72 g.) was divided into 9 portions and each one (approx. 2 g.) was treated in the same manner. Each portion was chromatographed over silica gel 60 (100 g.) glass column (ϕ 2″). and then eluted with hexane-acetone (4:1), followed by methanol. Each 20 ml. fraction was collected and combined according to the similar pattern on the thin layer chromatography. The collective fractions are separated as shown in Table 2.

Table 2 The collective fractions from column chromatography of the crude hexane extract (2 g)

Fraction number	Combined fraction	Color of eluate
A	1 - 21	pale-yellow to golden-yellow
B	22 - 35	gold
C	36 - 50	yellow
D	51 - 63	pale - green
E	64 - 78	pale - yellow

The fraction B and D showed interesting spots on TLC, visible under UV light.

The fraction B was concentrated under reduced pressure and allowed to stand for crystallization to give orange crystals. It was recrystallized in chloroform : hexane (1:1) to yeild yellow-gold prism crystals (0.2352 g.), designated as HEX-A.

The fraction D was concentrated under reduced pressure to give pale-green crystals. It was recrystallized in chloroform : hexane (1:1) to yield pale-green needle crystals (0.0628 g.), designated as HEX-B.

2. Separation and isolation of alkaloid from crude chloroform extract

The crude chloroform extract (1.7 g.) was chromatographed over siliga gel 60 (100 g.) glass column (ϕ 2″). The mixture of chloroform and acetone (3:2) was used as eluent. Fractions of 20 ml. were collected and examined by thin layer chromatography (TLC). Those fractions exhibited similar pattern were combined together as shown in Table 3.

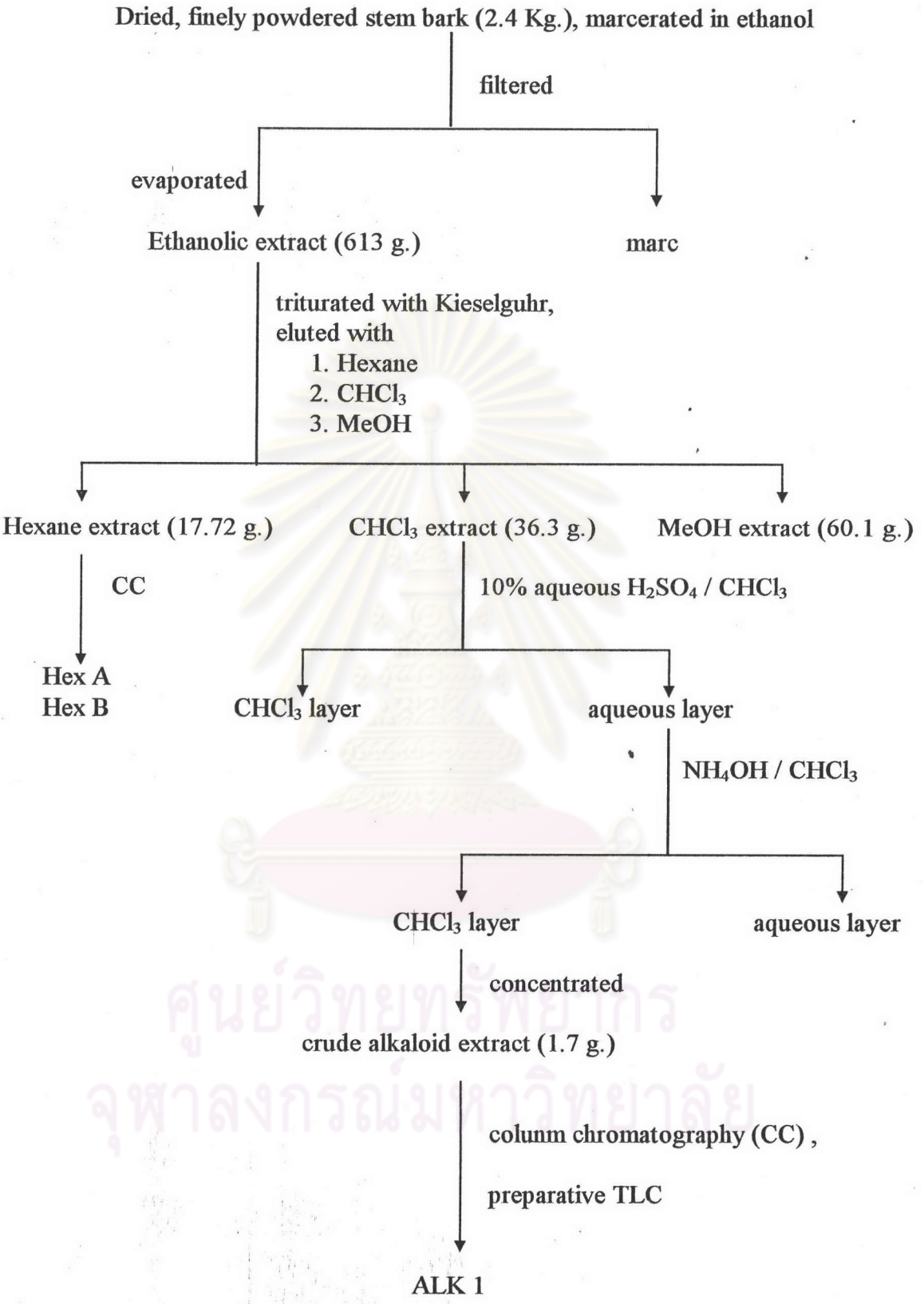
Table 3 The collective fractions from column chromatography of the crude chloroform extract (1.7 g)

Fraction number	Combined fraction	Color of eluate
F	1 - 8	yellow to brown
G	9	yellow
H	10 - 14	yellow
I	15 - 40	yellow

The fraction 9 was evaporated under reduced pressure and allowed to stand for crystallization to give white crystals (0.0493 g.). Preparative TLC was utilized in order to isolate each component of the small quantity of residue available.

Preparative TLC

The fraction 9 (0.0493 g.) was subjected to the preparative thin-layer chromatography using chloroform : acetone (4:1) as mobile phase. The chromatogram gave two zones of substances fluoresced under short wave ultraviolet light. The lower zone was washed from the adsorbent and purified by crystallization in chloroform to give white needle crystals. (0.0036 g.), designated as ALK 1.



Scheme 1 Extraction and Separation of *Fissistigma polyanthoides* stem bark

Characterization of the isolated compounds

1. Characterization of compound HEX-A

Physical properties

melting point : 123 - 126 °C

hRf value : 51 (in hexane : acetone = 3 : 2)

87 (in chloroform : methanol = 9 : 1)

83 (in dichloroethane : ethylacetate = 3 : 2)

Spectral data

EIMS ; *m/z* (% relative intensity); Figure 8, page 83.

332(47.5, M^+), 301(9), 227(100), 212(17.7), 211(20.1),

200(16.2), 197(25.4), 185(12.3), 105(6.2) 103(8.1), 91(93.1)

77(13.8) and 69(27)

UV ; λ_{max} nm (log ε) in methanol; Figure 10, page 85

365 (3.94), 284 (3.83)

IR ; ν cm^{-1}, KBr disc; Figure 9, page 84.

3434, 2936, 1605, 1495, 1457, 1436, 1412, 1390

^{1}H NMR ; δ ppm, 500 MHz in acetone-d_6; Figure 12, page 87.

2.99(2H, dd, J = 7.6 Hz), 3.80(3H, s), 3.87(3H, s),

3.98 (3H, s), 3.41(2H, dd, J = 7.6, 8.6 Hz), 7.27 (4H, m),

7.15(1H, m)

^{13}C NMR ; δ ppm, 125 MHz in $CDCl_3$; Figure 15, page 90.

30.98, 45.85, 60.87, 61.07, 61.41, 111.39, 126.69, 129.18,

129.24, 136.07, 137.52, 142.50, 144.95, 148.79, 150.91,

208

2. Characterization of compound HEX-B

Physical properties

melting point : 116 °C

hRf value : 39 (in hexane : acetone = 3 : 2)

79 (in chloroform : methanol = 9 : 1)

67 (in dichloroethane : ethylacetate = 3 : 2)

Spectral data

EIMS ; *m/z* (% relative intensity); Figure 21, page 96

330 (22.5, M^+), 227(9), 226 (99), 211(99), 183 (27), 127(13),

104 (10), 103 (17) 91(4.2), 77(16) and 69(31)

UV ; λ_{max} nm (log ε), in methanol; Figure 23, page 98.

358 (5.07), 280 (4.96)

IR : ν cm^{-1}, KBr disc; Figure 22, page 97.

3402, 2966, 2943, 2838, 1680, 1595, 1488, 1461, 1426

^{1}H NMR ; δ ppm, 500 MHz in $CDCl_3$; Figure 26, page 101.

2.87(1H, dd, *J* = 17.0, 3.05 Hz), 3.02(1H, dd, *J* =13.0 ,17.0 Hz)

3.86(3H,s), 3.92(3H, s), 4.10(3H, s)

5.43(1H, dd, *J* = 13.0, 3.05 Hz) 7.38(1H, m), 7.43(2H, m)

7.48(2H, m)

^{13}C NMR δ ppm 125 MHz in $CDCl_3$; Figure 28, page 103.

45.79, 61.25, 61.43, 61.75, 79.28, 110.87, 125.89, 128.55,

128.73, 136.96, 137.63, 138.74, 142.29, 147.26, 189.87

3. Characterization of compound ALK-1

Physical properties

melting point : 172 °C

hRf value : 25 (in hexane : acetone = 3:2)

32 (in chloroform : methanol = 9:1)

31 (in dichloroethane : ethylacetate = 3:2)

Spectral data

EIMS ; *m/z* (%relative intensity); Figure 36, page 111.

357(97.5, M^+), 356(66.9), 342(12.5), 326(21.3), 208(100), 206(20.1), 150(17.5), 149(24.4), 135(29.4), 91(5), 77(7.5)

IR ; ν cm^{-1}, KBr disc; Figure 37, page 112.

2996, 2936, 2039, 2751, 2685, 2616, 2349, 1725

^{1}H NMR ; δ ppm ;500 MHz in $CDCl_3$; Figure 38, page 113.

2.59(1H,ddd, J = 11, 10, 5.5 Hz), 2.83(3H, m), 3.24(2H, m, J = 9.76, 3.36 Hz), 3.58(2H, dd, J = 15.56, 11, 3.6 Hz), 3.82(3H, s), 3.88(3H, s), 3.90(3H, s), 4.22(1H,d, J = 15.56 Hz), 6.80(1H, d, J = 8.39 Hz) 6.83(1H, d, J = 8.39 Hz)

^{13}C NMR ; δ ppm; 125 MHz in $CDCl_3$; Figure 44, page 119.

23.02, 36.10, 50.97, 53.93, 55.92, 60.69, 100.62, 114.05, 114.66, 124.87, 127.03, 127.87, 133.55, 133.68, 143.16, 146.36, 146.50, 150.50