CHAPTER III

EXPERIMENTAL

Source of Plant Material

The leaves of *Aglaia edulis* A. Gray was collected from Pa-la-uu Waterfall, Kangkrajan National Park, Prachuap Khiri Khan, Thailand, on May 5, 1994 in the late fruiting stage. It was identified by Dr. Thawatchai Suntisuk and compared with the voucher specimen BKF. No. 58415 at the Botany Section, Technical Division, Royal Forest Department , Ministry of Agriculture and Co-operative, Thailand.

The leaves were dried in an oven at low temperature (40-50 ^{o}C) and ground to powder in the Retsch KG type SK. 1 mill.

General Techniques

2.1 Chromatographic techniques

2.1.1 Thin-layer chromatography (TLC)

Technique : One way ascending

Stationary phase : Mixture of siliga gel 60G (No. 7731, E. Merck) and siliga gel 60 HF_{254}(No. 7739, E. Merck) (2:1)

Plate size : 5 X 20 cm.

10 X 20 cm.

20 X 20 cm.

Layer thickness : 0.25 mm.

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

Solvent systems : chloroform - ethanol (9.25 : 0.75)

chloroform - methanol (9.5 : 0.5)

benzene - methanol (9 : 1)

chloroform - methanol - water (7 : 3 : 0.4)

chloroform - methanol - water (8 : 2 : 0.2)

ethylacetate - acetone - water (9 : 1 : 0.4)

Distance : 15 cm.

Temperature : 28-35 °C (room temperature)

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

2) Dragendorff's reagent

2.1.2 Column Chromatography (CC)

Column : Flat bottom glass column (various diameter)

Stationary phase : Silica gel 60 (No. 9385, E. Merck)

Packing method : Wet packing

Technique : Short column chromatography

Long column chromatography

Solvent systems : chloroform - ethanol (9.63 : 0.38)

chloroform - methanol - water (7 : 3 : 0.4)

chloroform - methanol - water (8 : 2 : 0.2)

ethylacetate - acetone - water (9 : 1 : 0.4)

2.1.3 Gel filtration chromatography

Column	: Glass column 24 X 1/2 inches
Stationary phase	: Sephadex LH 20 (Pharmacia Biotech)
Packing method	: Wet packing
Solvent	: $CHCl_3$ - MeOH (1:1)

2.2 Spraying reagent

Dragendorff's spraying reagent :

Solution A : bismuth subnitrate (850 mg), distilled water (40 ml) and acetic acid (10 ml)

Solution B : potassium iodide (8 g) and distilled water (20 ml)

Solution A and B, each of 5 ml, were mixed, then 20 ml of glacial acetic acid and 70 ml of distilled water were added. The reagent was used as a general alkaloidal reagent giving orange spots as positive result.

2.3 Melting point

Melting point were determined on a Buchi capillary tube melting point apparatus.

2.4 Spectroscopy

2.4.1 Ultraviolet (UV) Absorption Spectra

The ultraviolet absorption spectra were obtained on a Milton Roy Spectronic 3000 Array. Methanol was employed as the solvent for all compounds.

2.4.2 Infrared (IR) Absorption Spectra

The infrared absorption spectra were obtained on a Perkin Elmer Model 1760 X USA infrared spectrophotometer. The absorption bands were reported in wave number (cm^{-1}). The materials were examined in KBr cells.

2.4.3 Mass Spectra (MS)

The electron impact mass spectra (eims) were obtained on a Fisons VG Trio 2000 quadrupole mass spectrometer operating at 20 eV. The high resolution electron impact mass spectra (hrms) were obtained on a Fisons VG Autospec mass spectrometer operating at 70 eV.

2.4.4 Proton and Carbon-13 Nuclear Magnetic Resonance (^{1}H- and ^{13}C-NMR) Spectra

^{1}H- and ^{13}C-NMR spectra were obtained on a JNM-A500 (Alpha series) 500 MHz NMR spectrometer. Chemical shifts were reported in ppm. scale,using deuteromethanol (CD_3OD) as operating solvent and TMS as reference internal standard.

2.5 Solvent

The solvents used were redistilled from commercial grade solvent and analytical grade.

Phytochemical screening

Powdered leaves (100 g) were macerated with methanol (150 ml) over night. After the methanol extract was filtered, it was concentrated to syrupy mass on a water bath for further screening.

3.1 Screening for alkaloids

A small amout of the syrupy mass was dissolved in 5 ml of diluted HCl and filtered. The filtrate gave precipitate with Dragendorff's and Mayer's reagents, indicating the presence of alkaloids.

3.2 Screening for flavonoids

Cyanidin reaction (Shinoda's test) :

Small pieces of magnesium ribbon were added to the methanolic extract of the leaves, followed by the dropwise addition of concentrated HCl. Red color solution appeared indicating the presence of flavonoids (Farnsworth, 1966).

Extraction

Dried powdered leaves (2.7 kg) were macerated for 7 day periods three times with methanol (20, 15 and 10 l) and filtered. The methanolic extract was concentrated

under reduced pressure to give a residue (300 g) which was fractionated according to Figure 1.

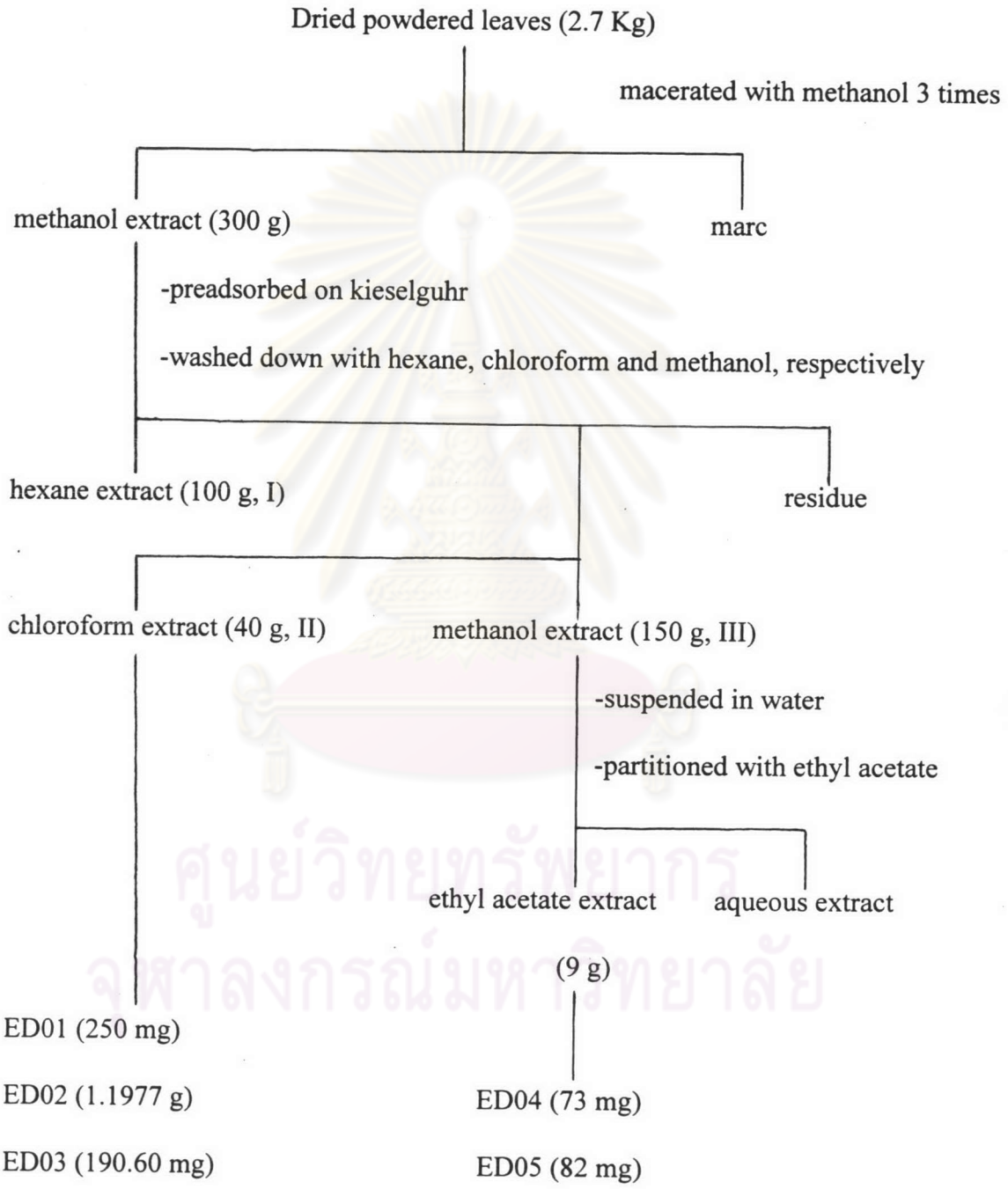

Figure 1 Extraction and scheme of *Aglaia edulis* A.Gray Leaves

The methanol extract was adsorbed on kieselguhr and put into a large cone percolator. It was then eluted with n-hexane until the eluate stopped giving positive result to Liebermann Burchard test. The eluate was evaporated to dryness to give 100 g of hexane extract (I). This extract tested negative for alkaloids and was not further investigated. The remaining kieselguhr mixture was air-dried and then exhaustively eluted with chloroform to give, on evaporation, 40 g of the chloroform extract (II) containing alkaloids. The air dried kieselguhr mixture was further eluted with methanol to give, on evaporation, 150 g of the methanol extract (III) containing crude flavonoids. The chloroform and methanol extracts were subjected to column chromatography for further purification.

Isolation Procedure

5.1 Fractionation of the chloroform extract

The chloroform extract (40 g, II) was dissolved in glacial acetic acid (30 ml). The acid solution was diluted with distilled water (300 ml) until complete precipitation of chlorophyll and some other impurities had occurred. The precipitate was separated by vacuum filtration. The clear acid filtrate was basified with 25 % ammonium hydroxide solution to approximately pH 10 and exhaustively extracted with chloroform to give 8 g of crude alkaloid residue. It was then subjected to silica gel short column chromatography, using chloroform - ethanol (9.63 : 0.37) as the eluent. Fractions (30 ml each) were consequently collected and examined by TLC, using chloroform - ethanol (9.25 : 0.75) as developing solvent. Those with similar pattern

on TLC plates were combined and evaporated to dryness to give six major fractions (Table 5).

Table 5 Combined fractions from short column chromatography of the chloroform extract

Fraction	Number of eluates	Weight (g)
A-01	1-9	0.15
A-02	10-17	0.35
A-03	18-29	0.30
A-04	30-40	1.40
A-05	41-65	0.25
A-06	66-80	0.50

Fraction A-01 was not further studied and was later discarded.

Fraction A-02 showed traces of alkaloids and some other compounds on TLC plates. It was rechromatographed on a silica gel column using benzene - ethyl acetate - ethanol (9 : 0.5 : 0.5) as the eluent. No pure compound was obtained due to large amount of impurities with very minute amount of alkaloids presented in this fraction.

Fraction A-03, A-04 and A-05 each showed one alkaloid positive spot on TLC plates using chloroform - ethanol (9.25 : 0.75) as developing solvent at hRf values of 29.10, 36.40 and 43.60, respectively. They were crystallized/ recrystallized in

methanol to yield ED01 as colorless needles (250 mg, 0.0095 %), ED02 as colorless needles (1.1977 g, 0.0444 %) and ED03 as pale orange needles (190.60 mg, 0.0071 %), respectively.

Fraction A-06 showed traces of alkaloids and some other compounds on TLC plates. It was rechromatographed on a silica gel short column using chloroform - ethanol (9.5 : 0.5) as the eluent. No pure compound was obtained due to large amount of impurities with very minute amount of alkaloids presented in the fraction.

5.2 Fractionation of the methanol extract

The methanol extract (150 g, III) was suspended in water and partitioned with ethyl acetate. The ethyl acetate fraction was concentrated under reduced pressure to give 9 g of ethyl acetate extract. The extract gave positive result for flavonoids (Shinoda's test) and was subjected to silica gel column chromatography, using chloroform - methanol - water (7 : 3 : 0.4) as the eluent. Thirty six fractions (30 ml each) were collected and the column was then washed down eluted with methanol.

The initial 17 fractions tested negative test flavonoids and were discard. Fractions No.18-36 gave at least two flavonoid positive spots under UV light. They were combined and evaporated to give 4.7 g residue. Purification of the residue was made by gel filtration technique on Sephadex LH-20 column using chloroform - methanol (1 : 1) as the mobile phase. The process was repeated three times, then the purified residue (1.4 g) was subjected to silica gel column chromatography, using

ethyl acetate - acetone - water (9 :1 : 0.4) as the eluent. Forty six fractions (30 ml each) were collected. Fractions No.16-29 showed 2 flavonoid spots on TLC plates under UV light. They were evaporated to give 0.37 g of extract which was purified by silica gel column chromatography using chloroform - methanol - water (8 : 2 : 0.2) as the mobile phase. Thirty fractions (30 ml each) were collected. Yellow needles 150 mg crystallized from fractions 19-26. These were collected and recrystallized in methanol to give 73 mg of ED04 (0.0027 %). Fractions No. 21-46 showed another flavonoid spot on TLC plates. It was crystallized/recrytallized in methanol to yield 82 mg of yellow amorphous Powder designated as ED05, subsequently identified by spectroscopic techniques as a mixture of flavonoids and were not further investigated.

Characterization of isolated compounds

6.1 Compound ED01

Appearance : colorless needles

Solubility : slightly soluble in methanol

hRf value : a) 43.60 (chloroform - ethanol (9.25 : 0.75))

b) 40.00 (chloroform - methanol (9.5 : 0.5))

c) 23.00 (benzene - methanol (9 : 1))

Melting Point : 162-163 °C

Spectral Data : a) EIMS (*m/z* (% relative intensity) ; Figure 3, page 137)

324(20.73), 233(54.88), 91(100)

b) HRMS

324.4081 (calculated 324.1838)

c) UV (λ_{max} nm (ε), in methanol ; Figure 4, page 138)

222(3370)

d) IR (ν cm^{-1}, KBr disc ; Figure 5, page 139)

3250, 3067, 1660, 1630, 1567, 767, 683, 617,550

e) ^{1}H-NMR (δ ppm, 500 MHz, in CD_3OD; Figure 6, page 140)

1.47(4H, *m*), 3.15(4H, *m*), 3.46(2H, s), 7.21(1H, *m*),

7.26(2H, *m*), 7.28(2H, *m*)

f) ^{13}C-NMR (δ ppm, 125 MHz, in CD_3OD ; Figure 8, page 142)

27.6(x2), 40.1(x2), 43.8, 127.8, 129.5(x2),

129.9(x2),136.9, 173.9

5.2 Compound ED02

Appearance : colorless needles

Solubility : slightly soluble in methanol

hRf value : a) 36.40 (chloroform - ethanol (9.25 : 0.75))

b) 35.00 (chloroform - methanol (9.5 : 0.5))

c) 20.00 (benzene - methanol (9 : 1))

Melting Point : 140-141 °C

Spectral Data : a) EIMS (*m/z* (% relative intensity) ; Figure 15, page 149)

306(6.51), 291(2.96), 215(10.37), 101(100), 91(53.66)

b) HRMS

306.3213 (calculated 306.1402)

c)UV (λ_{max} nm (ε), in methanol ; Figure16, page 150)

210(8262), 222(8996), 271(24908)

d) IR (ν cm^{-1}, KBr disc ; Figure 17, page 151)

3260, 3125, 2933, 2867, 1680, 1640, 1600, 1550,

1350, 1283, 967, 867, 750,717, 650,550

e) ^{1}H-NMR (δ ppm, 500 MHz, in CD_3OD ; Figure 18, page 152)

1.55(4H, *m*), 2.33(3H, s), 3.18(2H, *m*), 3.21(2H, *m*),

3.47(2H, s), 5.79(1H, d, *J*=14.7 Hz), 7.22(2H, *m*),

7.27(2H, *m*), 7.29(2H, *m*), 7.53(1H, dd, *J*=14.7 Hz)

f) ^{13}C-NMR (δ ppm, 125 MHz, in CD_3OD ; Figure 20, page 155)

14.3, 27.7, 27.8, 39.9, 40.1, 43.9, 116.7, 127.8,

129.5(x2), 130.0(x2), 137.0, 143.4, 167.3, 174.0

5.3 Compound ED03

Appearance : pale orange needles

Solubility : slightly soluble in methanol

hRf value : a) 29.10 (chloroform - ethanol (9.25 : 0.75))

b) 25.00 (chloroform - methanol (9.5 : 0.5))

c) 23.00 (benzene - methanol (9 : 1))

Melting Point : 164-165 °C

Spectral Data : a) EIMS (*m/z* (% relative intensity) ; Figure 26, page 164)

288(1.78), 273(3.05), 241(6.10), 101(100)

b) HRMS

288.267 (calculated 288.0966)

c) UV (λ_{max} nm (ε), in methanol ; Figure 27, page 165)

203(4931), 230(9769), 271(39537)

d) IR (ν cm^{-1}, KBr disc ; Figure 28, page 166)

3310, 3117, 2933, 1640, 1600 ,1567, 1350, 1267, 1200, 1016, 967, 850, 683

e) ^{1}H-NMR (δ ppm, 500 MHz, in CD_3OD ; Figure 29, page167)

1.55(4H, *m*), 2.33(4H, *m*), 2.52(3H, *s*), 5.80(1H, *d*, *J*=14.7 Hz), 7.53 (1H, *d*, *J*=14.7 Hz)

f) ^{13}C-NMR (δ ppm, 125 MHz, in CD_3OD ; Figure 31, page 170)

14.37(x2), 27.94(x4), 40.03(x4), 116.86(x2), 143.45(x2), 167.42(x2)

5.4 Compound ED04

Appearance : yellow needles

Solubility : slightly soluble in methanol

hRf value : a) 41.50 (chloroform - methanol - water (7 : 3 : 0.4))

b) 31.00 (chloroform - methanol - water (8 : 2 : 0.2))

c) 25.00 (ethyl acetate - acetone - water (9 : 1 : 0.4))

Melting Point : 196-197 °C

Spectral Data : a) EIMS (*m/z* (% relative intensity) ; Figure 37, page 176)

302(100), 274(6.71), 153(4.27), 137(4.88)

b) UV (λ_{max} nm (ε), in methanol ; Figure 38)

220(35616), 256(45965), 349(34765 ,

c) IR (ν cm^{-1}, KBr disc ; Figure 41, page 180)

3381, 1680, 1647, 1200, 1150, 950, 820

d) ^{1}H-NMR (δ ppm, 500 MHz, in CD_3OD ; Figure 42, page 181)

0.9 (3H, *d*, *J*=6.10 Hz), 3.35 (1H, *t*, *J*=9.5 Hz),

3.42 (1H, *dq*, *J*=6.1, 9.5 Hz), 3.76 (1H, *dd*, *J*=3.36, 9.5 Hz),

4.23 (1H, *dd*, *J*=1.7, 3.4 Hz), 5.35 (1H, *d*, *J*=1.7 Hz),

6.20 (1H, *d*, *J* = 2.1 Hz), 6.37 ((1H, *d*, *J* = 2.1 Hz),

6.91 (1H, *d*, *J*=8.2 Hz), 7.31 (1H,*dd*, *J*=2.1, 8.2 Hz),

6.34 (1H, *d*, *J*=2.1 Hz)

e) ^{13}C-NMR (δ ppm, 125 MHz, in CD_3OD ; Figure 44, page 183)

0.9, 71.8, 71.9, 72.0, 73.2, 94.7, 99.7, 103.4, 105.8, 116.3,116.9,

122.8,122.9,136.1,146.3,149.7,158.4,159.2,163.1,165.2