CHAPTER III

EXPERIMENTAL

Source of Plant Material

The leaves of *Aglaia chittagonga* Miq. in the fruiting stage was collected from Chachoengsao Province, Thailand in March, 1996 . It was identified by Dr. Kongkanda Chayamarit and compared with the voucher specimen BKF No. 81683 at the Botany Section, Technical Division, Royal Forest Department, Ministry of Agriculture and Co-operative, Thailand.

The leaves were oven - dried at 40 - 50°C and then ground in the mill (Retsch KG type SK1).

Gerneral Techniques

1 Chromatographic Techniques

1.1 Thin-layer chromatography (TLC)

Technique	: one way ascending
Stationary phase	: TLC aluminium sheets silica gel 60F 254 layer thickness 0.2 mm
Solvent systems	: Hexane - Chloroform (4:1)
	: Hexane - Acetone (49:1), (9:1)
	: Chloroform - Methanol (24:1), (9:1)
Distance	: 5 cm
Temperature	: 28 – 35 °C (room temperature)
Detection	:1) UV light (254 and 365 nm)
	:2)10% sulfuric acid and heating at 110°C

1.2 Column Chromatography (CC)

Column	: Flat bottom glass column (various diameter)
Stationary phase	: Silica gel 60 (No. 9385, E. Merck) particle size

0.040 – 0.063 mm (230 – 400 mesh ASTM)

Packing method : Wet packing

Technique : Short column chromatography

: Long column chromatography

Solvent system : Hexane - Chloroform (4:1)

: Hexane - Acetone (49:1), (9:1)

: Chloroform - Methanol (9:1)

2 Spraying reagent : 10% Sulfuric acid heated at 110°C

The reagent was used as a universal chemical detection, giving colour spots as positive result.

: Liebermann Burchard test

The reagent consist of 3 drops of acetic anhydride and one drop of conc. H_2SO_4 The reagent was used as a terpenoid detection, giving purple spot as positive result.

3 Melting point

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

4 Spectroscopy

4.1 Infrared (IR) Absorption Spectra

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

4.2 Mass Spectra (MS)

The electron impact mass spectra (EIMS) were obtained on a Fisons VG Trio 2000 quadrupole mass spectrometer operating at 20 eV (Faculty of Pharmaceutical Sciences, Chulalongkorn University).

4.3 Proton and Carbon^{13} Nuclear Magnetic Resonance (^1H and ^{13}C NMR) spectra

^1H and ^{13}C NMR spectra were obtained on a JOEL JNM - A500

(Alpha series) 500 MHz NMR spectrometer (The Scientific and Technological Research Equipment Center, Chulalongkorn University) and a Bruker Avance DPX-300 300 MHz NMR spectrometer (Faculty of Pharmaceutical Sciences, Chulalongkorn University). The chemical shifts were reported in ppm scale, using deuterated chloroform ($CDCl_3$) as operating solvent and solvent peak was used as reference standard peak.

5 Solvent

The solvents used throughout this work were redistilled from commercial grade solvent and prior to use.

Phytochemical screening

Screening for terpenoids

Powder leaves (100g) were macerated with methanol (200ml) over night. After the methanol extract was filtered, an aliquot portion (24 ml) of the solution was evaporated to dryness on the steam bath. After the content were cooled to room temperature, it was washed with 10 ml of petrolium ether. The remaining residue was then extracted with 10 ml of chloroform. The chloroform extract was dried over anhydrous sodium sulfate and it was filtered into a clean evaporating disc, then evaporate to dryness. It was used for the Liebermann Burchard test. The sample, 3 drops of acetic anhydride, and 1 drop of conc. H_2SO_4 when mixed yielded a purple color indicating the presence of terpenoids.

Extraction

Dried powdered leaves (3 kg) were macerated for 7 day periods three times with methanol (15,10 and 10 L) and filtered. The methanolic extract was concentrated under reduced pressure and combined to give a residue (430g) which was further fractionated according to Figure 2.

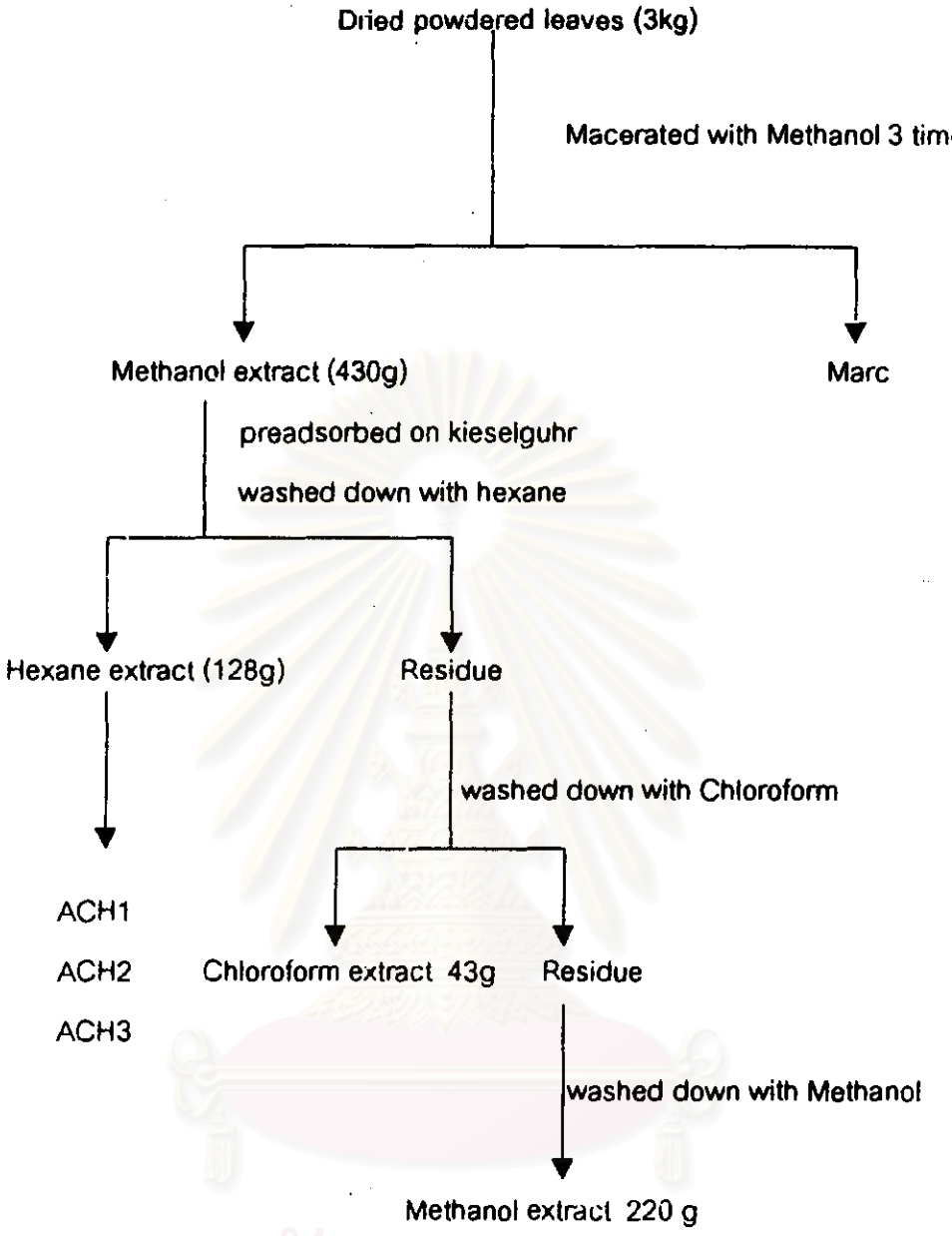

Figure 2. Extraction scheme of *Aglaia chittagonga* Miq. leaves

The residue 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 128 g of hexane extract. This extract tested positive for terpenoid and was subjected to column chromatography for further

purification. The remaining kieselguhr mixture was air-dried and then exhaustively eluted with chloroform to give, on evaporation 43 g of the chloroform extract. It was then subjected to column chromatography, no pure compound was isolated. The air-dried kieselguhr mixture was further eluted with methanol to give, on evaporation, 220 g of methanol extract.

Isolation Procedure

The hexane extract (30g) was subjected to silica gel short column chromatography using 2.5% acetone in hexane as the eluent. Seventy-five 50-ml fractions were collected and combined according to their TLC pattern into 4 major fractions. (Table 4)

Table 4 Combined fractions from column chromatography of the hexane extract

Fraction	Number of eluates	Weight (g)
A1	1-6	1.5
A2	7-63	1.5
A3	64-75	0.5
A4	washed down with methanol	19 g

Fraction A2 was recrystallized in methanol to yield ACH1 as white needle (620 mg, 2.06%). Fractions A1 and A3 showed no interesting spot on TLC and therefore were not further investigated. The column was washed down with methanol and, after evaporation, gave 19g of residue (A4). The fraction A4 was subjected to another silica gel column chromatography using 9.5% acetone in hexane as the eluent. Sixty-seven 30-ml fractions were collected and combined into 5 major fractions (B1-B5) (Table5).

Table5 Combined fractions from column chromatography of fraction A4

Fraction	Number of eluates	Weight (g)
B1	1–9	0.5
B2	10 – 21	0.05
B3	22 – 26	0.05
B4	27 – 35	0.05
B5	36 – 67	0.4

Fraction B1 showed traces of ACH1 in the majority of impurities and was later discarded.

Fractions B2 and B5 showed no terpenoid spot on TLC plate and were not further investigated.

Fractions B3 showed one terpenoid positive spot on TLC plates. It was recrystallized in methanol to yield ACH2 as white needles,(20 mg,0.067%).

Fraction B4 showed one terpenoid positive spot on TLC plates. It was recrystallized in methanol to ACH3 as white needles, (20mg, 0.067%).

Characterization of Isolated Compounds

Compound ACH1

Appearance : white needles

Solubility : Soluble in methanol, chloroform, and acetone

hRf value : 52 (hexane-acetone, 4:1)

: 34 (hexane-acetone, 9:1)

: 22 (hexane-chloroform, 2:3)

Melting Point : 215-216°C

Spectral Data

a) EIMS *m/z* (% relative intensity) ; (Figure 9)

427 (18), 426 (50), 411(17), 408 (11), 394 (2), 393 (3), 383 (2), 365 (5),

344 (1), 339 (1), 317 (2), 316 (5), 315 (5), 299 (3), 297 (3), 273 (2), 272 (3), 257 (16), 247 (5), 234 (18), 219 (18), 218 (50), 207 (40), 203 (30), 191 (20), 189 (48), 187 (13), 175 (30), 163 (20), 161 (33), 149 (40), 147 (52), 136 (50), 135 (92), 123 (60), 121 (82), 109 (100), 107 (90), 95 (82), 93 (60), 91 (22), 81 (60), 79 (30), 69 (82), 68 (98), 57 (50), 55 (75)

b) IR(ν_{max} cm^{-1} (KBr disc) (Figure 8)

3464, 2943, 2863, 1700, 1640, 1454, 1381, 1042, 882

c) ^{1}H NMR (δ ppm,300 MHz, $CDCl_3$) (Figure 10-11)

0.65 (1H,d *J* =4Hz),0.7 (3H, s), 0.75 (3H, s), 0.8 (3H,s), 0.9 (3H,s), 0.95 (6H,s), 1.0 (3H,s), 1.9 (1H, m), 2.4 (1H, m), 3.2 (1H, dd *J*=5, 2 Hz), 4.5 (1H,s), 4.7 (1H,s)

d) ^{13}C NMR (δ ppm, 75MHz, $CDCl_3$) (Figure12-13)

14.7 (q), 15.5(q), 16.1 (q), 16.3 (q), 18.1 (q) , 18.5 (t), 19.4 (q), 21.0 (t), 25.2 (t), 27.5 (t),27.6 (t), 28.1 (q), 29.9 (t), 34.4 (t), 35.6 (t), 37.3 (s), 38.1 (d),38.8 (t), 38.9 (q), 40.1 (t), 40.9 (s),42.9 (s), 43.1 (s) , 48.1 (d), 48.4 (d), 50.5 (d),55.3 (d), 79.0 (d), 109.2 (t), 150.8 (s)

Compound ACH2

Appearance : White needles

Solubility : Soluble in chloroform, acetone, and methanol

hRf : 64 (chloroform – acetone ,9:1)

: 37(hexane - acetone ,4:1)

: 16 (hexane – acetone ,9:1)

Melting Point : 144 - 145°C

Spectral Data

a) EIMS *m/z* (% relative intensity), (Figure 15)

474 (1), 456 (3), 442 (1), 441 (1), 427 (2), 409 (4), 334 (4), 315 (4), 297 (3), 255 (2), 175 (19), 159 (17), 147(24), 135 (30), 121(35),109 (51), 95 (64), 73 (100), 67(31), 55 (60)

b) IR(ν_{max} cm^{-1} (KBr disc) ; (Figure 16)

3470, 3350, 2944, 2863, 1462, 1377, 1059

c) ^{1}H NMR (δ ppm, 500 MHz, $CDCl_3$) (Figure 17-22)

0.31 (1H,d,*J*=4Hz), 0.53 (1H,d,*J*=4Hz), 0.78 (3H,s), 0.86 (3H,d,*J*=7Hz), 0.87(3H,s) 0.94 (6H,s), 1.07 (3H,s), 1.11 (3H,s), 1.24 (2H,dd,*J*= 2.7,11.6Hz),1.28 (2H,dd,*J*=12.8,4.6Hz), 1.5 (1H,dd,*J*=12, 4.9Hz), 2.44 (1H, brs), 3.2 (3H,s), 3.34 (1H,dd,*J*=10,1.8Hz)

d) ^{13}C-NMR(δ ppm,125MHz, $CDCl_3$) (Figure23)

14.0 (q), 18.1 (q), 18.4 (q), 18.8 (q), 19.3 (q), 19.9 (t), 20.8 (q) 21.1 (t), 25.4 (t), 26.0 (t), 26.1 (s), 26.5 (t), 28.1 (t), 28.3 (t), 29.9 (t), 30.4 (t), 32 (t),32.9(t), 33.7 (t), 35.5 (t), 36.4 (d), 40.5 (s), 45.3 (s), 47.1 (d), 48.0 (d),48.8 (s), 49.0(q), 52.4 (d), 77.6 (s), 77.6 (d), 78.8 (d)

Compound ACH3

Appearance : White needles

Solubility : Soluble in chloroform, acetone, and methanol

hRf value : 62 (chloroform-acetone, 9:1)

: 34 (hexane-acetone, 4:1)

: 14 (hexane-acetone, 9:1)

Melting Point : 170-171°C

Spectral Data

a) EIMS *m/z* (% relative intensity) ;(Figure 40)

460 (1), 443 (3), 442 (5), 428 (2), 427 (2), 410 (2), 395 (5), 334 (4), 301 (5), 201 (5),159 (26.), 147 (32),133 (38), 121(49), 109 (60), 107 (64), 95 (72), 93 (62), 81 (60), 73 (100), 69 (58), 67 (52), 55 (76)

b) IR ν_{max} cm^{-1} (KBr disc) (Figure 41)

3402, 2925, 2871, 1458, 1403, 1377, 1062, 1001

c)^{1}H NMR (δ ppm,500 MHz $CDCl_3$) (Figure42-47)

0.12 (1H,d,*J*= 4 Hz),0.36 (1H,d,*J*= 4 Hz), 0.56 (1H,ddd,*J*=12.8,11.7,3 Hz),

0.865 (3H, d,*J* =6.4Hz), 0.87 (3H, s), 0.94 (3H,s), 0.95 (3H,d,*J* = 6.1 Hz), 1.01 (1H,ddd, *J*= 18.5,10.2,3.8 Hz), 1.07 (3H,s), 1.1 (3H,s), 1.65 (1H,ddd,*J* =13.1,7.8, 4.4 Hz), 1.8 (2H, tdd, *J* =12.7, 4.3,3 Hz), 2.44 (1H,brs) 3.18 (1H,dd, *J*=10,5.2 Hz), 3.2(3H,s), 3.34 (1H, brd ,*J*=9.4Hz)

c) ^{13}C NMR (δ ppm,125 MHz, $CDCl_3$) (Figure 48)

14.4 (q), 17.8 (q), 18.4 (q), 18.8 (q), 19.1 (q), 20.8 (q), 23.5 (s), 24.6 (t), 25.2 (t), 26.9(t), 27.3 (t), 28.1 (t), 28.3 (t), 29.5 (s), 30.8 (t), 32.9 (t), 33.7 (t), 34.8 (t), 35.3 (t), 36.4 (d), 43.3 (d), 44.6 (d), 45.3 (s), 46.9 (d), 48.9 (s), 49.0 (q), 52.3 (d), 76.6 (d), 77.58 (d), 77.63 (s)