CHAPTER III

EXPERIMENTAL

1. SOURCE OF PLANT MATERIAL

The heartwood of Artocarpus lakoocha Roxb. was obtained from Phukae Botanical Garden, Saraburi province, Thailand in May, 1993.

2. GENERAL TECHNIQUE

2.1 Analytical Thin-layer Chromatography (TLC)

Technique : One way, ascending

Adsorbent : Silica gel 60 F254 (E.Merck) precoated plate

Layer thickness : 0.2 mm.

Sovent system : various solvent systems depending on material

Distance : 6.5 cm.

Temperature : Room temperature (28-35°C)

Detection : 1. Visual detection under Ultraviolet light at the wavelength of 254 nm and 366 nm.

2. Iodine vapore generated from iodine crystal that bind with unsaturated organic compound an present as colour spot

3. Libermann-Burchard colour reaction was frequent used for the detection of sterols

2.2 Column Chromatography (CC)

Adsorbent : Silica gel 60 (number 7734) particle size 0.063-0.200 nm. (70-230 mesh ASTM)

Packing method : Wet packing

Sample loading : Dissolved sample with small amount of organic solvent then added small amount of silica, triturated and allow to dry, and added gently on the top of column.

Solvent : Various solvent systems depending on materials.

Column size : The glass column 3/4-2 inches in diameter were used depending on the quantity of sample to be separated.

2.3 Spectroscopy

2.3.1 Ultraviolet (UV) Absorption Spectra

The spectra were obtained by Milton Roy Spectronic 3000 array. (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University). Methanol was used as solvent.

2.3.2 Infrared (IR) Absorption Spectra

The spectra were obtained by a Shimatsu IR 440 infrared Spectrometer (The Scientific and Technological Reserch Equipment Center, Chulalongkorn University) in KBr disc.

2.3.3 Mass Spectra (MS)

The Electron Impact Mass Spectra (EIMS) gerenrated by a Fisons VG Trio

2000 quadrupole mass spectrometer (Department of Sciences, Chulalongkorn University) and by JEOL JMS-DX300 Mass spectrometer (The Scientific and Technological Rearch Equipment Center, Chulalongkorn University)

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

The 500 MHz ^1H NMR spectra and 125 MHz ^{13}C NMR spectra were obtained by a JEOL JMN-A 500 Spectrometer (The Scientific and Technological Rearch Equipment Center, Chulalongkorn university).

The DMSO-d_6, acetone-d_6, methanol-d_4, chloroform-d_1 and mixture of chloroform-d_1 and DMSO-d_6 were used as operating solvent. Chemical shifts were reported in ppm (δ) scale and tetramethylsilane (TMS) was used as standard reference.

2.4 Crystallization Technique

The impure crystalline compounds were purified by crystallization from suitable solvent. The Crystallization process consisted of:-

1. Dissolved impure compounds with suitable small amount of solvent and warmed untill all crystals were dissolved.
2. The hot solution was filtrated.
3. Allowed the solution cool down to room temperature.
4. If it did not crystallize out, put the second solvent that the compound was less dissolved into the solution.
5. Separated the crystal by vacuum filtration.

Checked the purity of crystal by TLC and melting point. Recrystallization could be used if the compound was not pure.

2.5 Melting point

Melting point was obtained by a Buchi glasscapillary apparatus (made in Switzerland).

2.6 Solvent

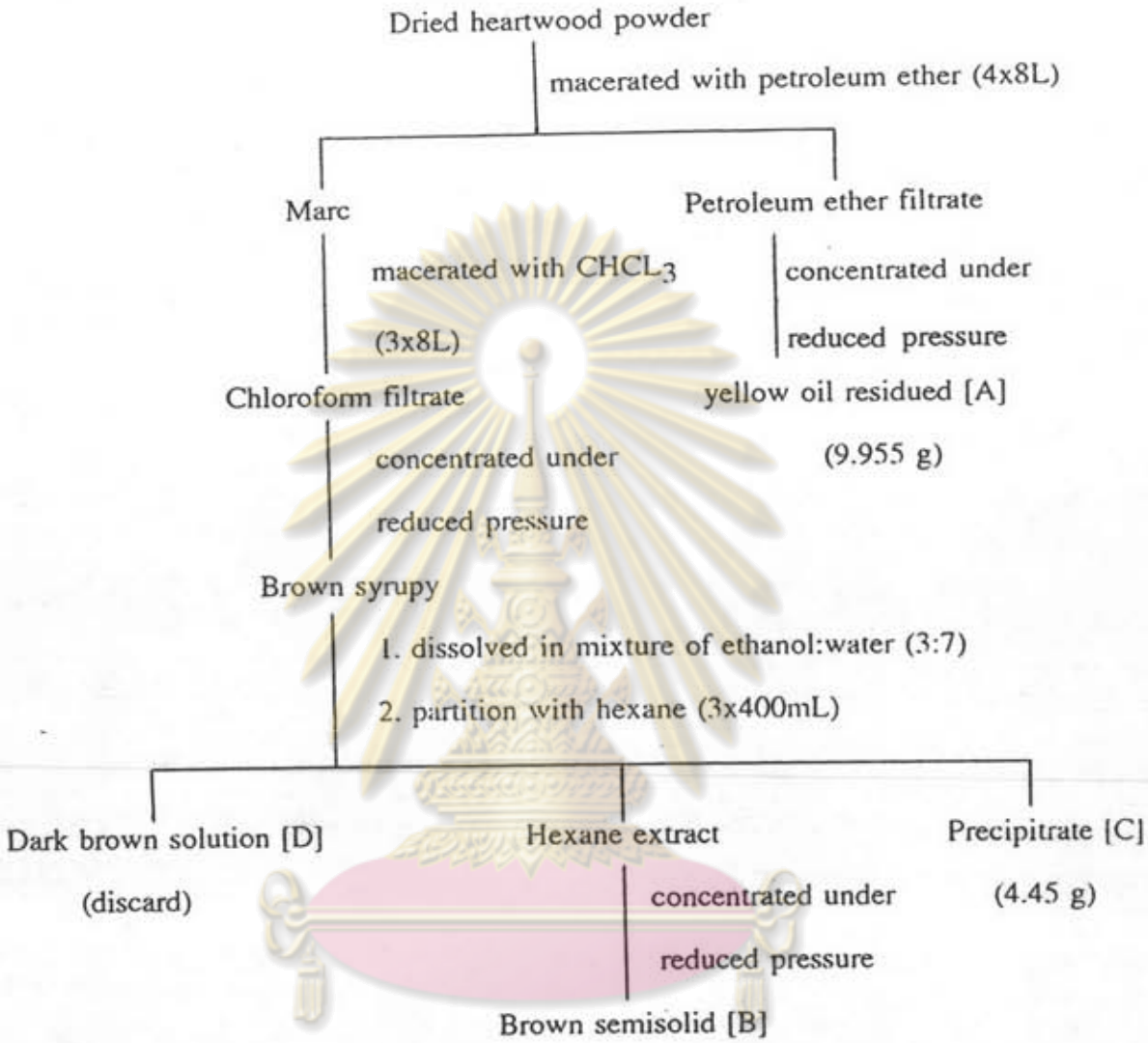
All solvents for chromatography were commercial grade and had to be redistillated prior to used. Solvents for crystallization were analytical grade.

3. EXTRACTION

The dried heartwood of Artocarpus lakoocha Roxb. was cut to a small pieces, air dried at room temperature for one week and then ground it in to powder. The 1.5 Kg powder was marcerated for four times in 8 liters redistilled petroleum ether ,each time for three days. The collected filtrate was concentrated under reduced pressure at temperature not over 60°C, giving the 9.955g. yellow oil residue [Fraction A].

The marc was repeatedly marcerated for three times in 8 liters chloroform, each time for three days. The collected chloroform was concentrated under reduced pressure at temperature not over 60°C, giving the 23.010g. brown syrupy residue. The chloroform residue was dissloved in 400 mL mixture of ethanol:water (3:7) and then partition with each 400 mL hexane for three times. During the partition the precipitate was form and was filtrated to give Fraction C (4.45g.). The hexane extract was concentrated and evaporated under reduced pressure at temperature not over 60°C, thus giving the 3.289g. brown semisolid residue, [Fraction B].

The scheme of extraction was shown below



scheme 5

4. ISOLATION OF CHEMICAL COMPOUND

4.1 The Isolation of AA-4 and AA-5.2

Fraction A (9.955 g) was divided into portions (4.9g and 5.0g) and each portion was separated on column chromatography.

The column was eluted with different solvent and 100 mL of fraction was collected. The eluent were used as below.

Hexane		500mL	Fraction 1 - 5
Hexane : Chloroform	(9:1)	1000mL	" 6 - 15
Hexane : Chloroform	(7:3)	2000mL	" 16 - 35
Hexane : Chloroform	(4:6)	2000mL	" 36 - 55
Chloroform		1000mL	" 56 - 65
Chloroform : Ethylacetate	(8:2)	500mL	" 66 - 70
Chloroform : Ethylacetate	(6:4)	500mL	" 71 - 75
Ethylacetate		500mL	" 76 - 80

Ethanol was used for washing column untill the eluate was clear. Each fraction was examined by TLC using hexane: chloroform (7:3) and hexane: petroleum ether : chloroform: ethylacetate (4: 1: 3: 2) as developing solvent.

The fraction, giving the same chromatographic pattern, were combined and concentrated to gived a portion as shown in table 4

Table 4 The combined portions from Fraction A

Fractions	Portions	Weight (g)
1-15	A-1	0.077
16-30	A-2	0.143
31-47	A-3	0.085
48-52	A-4	0.210
53-80	A-5	0.345
ethanol eluated	A-6	0.075

Fraction 48-52 (A-4), the yellow crystal was precipitated. It was recrystallized with 95% ethanol and 58mg of white plate crystal (AA-4) was obtained, Which was identified as β-sitosterol.

Fraction 53-80 (A-5) was performed for further isolation by column chromatography. The eluant was mixture of hexane : petroleum ether : chloroform : ethylacetate (5:4:1:2) and fractions based on the colour bands (appoximate 25 mL) were collected. Each fraction was determined by TLC using hexane : petroleum ether : chloroform : ethylacetate (4:1:3:2) as developing solvent. The fraction which showed the same pattern of chromatogram were combined, concentrated and assigned as shown in table 5.

Fraction 1-10 (A-5.1) was crystallized in 95% ethanol and obtained white plate crystal (14 mg), it was identified as AA-4. The combination of AA-4 gave 72 mg. The total yield of AA-4 was 4.8×10^{-3}% based on dry weight of A. lakoocha Roxb. powder.

Table 5 The combined portions from fraction A-5

Fractions	Portions	weight (g)
1-10	A-5.1	0.054
11-16	A-5.2	0.106
17-25	A-5.3	0.057

Fraction 11-16 (A-5.2) was crystallized in 95% ethanol and obtained yellow crystal (22 mg). This compound was identified to be cycloartocarpin (AA-5.2).

4.2 Isolation of BB-2 and BB-3.3

Fraction B was separated by column chromatography with chromatographic condition the same as portion A-5. The combined portion was shown in table 6.

Table 6 The combined portions of fraction B.

Fractions	Portions	Weight (g)
1-6	B-1	0.092
7-15	B-2	0.132
16-26	B-3	0.416

Fraction 7-15 (B-2) was crystallized in 95 % ethanol and yellow crystal (94 mg) was obtained. This compound was identified to be AA-5.2. Because most of AA-5.2 was obtained in portion B-2 then AA-5.2 was renamed to BB-2.

Fraction 16-26 (B-3) was perform for further isolation by column chromatography. The column was eluted with different solvents and 100 mL of fraction was collected. The eluent was used as below.

Petroleum ether : Chloroform	(4:6)	2000mL	Fraction	1-20
Petroleum ether : Chloroform	(3:7)	1000mL	"	21-30
Chloroform		500mL	"	31-35
Chloroform : Ethylacetate	(8:2)	1000mL	"	36-45
Chloroform : Ethylacetate	(1:1)	1500mL	"	46-60

Ethylacetate was used for washing column untill the eluate was clear. The eluate collected based on the colour bands and was examined by TLC using hexane: petroleum ether: chloroform : ethylacetate (4:1:3:2:) as developing solvent. The fractions, giving the same chromatographic pattern, were combined and concentrated to gave portions as shown in table 7.

Fraction 31-39 (B-3.2) was crystallized and was identified to be BB-2 (51 mg). The combination of BB-2 gave 177 mg. The total yield of BB-2 was 11.8 x 10^{-3} % based on dry weight of A. lakoocha Roxb. powder

Table 7 The combined portions of fraction B-3

Fractions	Portions	Weight (g)
B-3.1	1-30	0.089
B-3.2	31-39	0.051
B-3.3	40-the last fraction	0.137

B-3.3 portion was brown crystals, after crystallization in benzene, giving brown needle 37 mg (BB-3.3). The yield of BB-3.3 was 2.47x10^{-3}% based on dry weight of A. lakoocha Roxb. powder. This crystal was identified to be 2,4-dihydroxybenzaldehyde.

4.3 Isolation of CC-1

Fraction C was dissloved in 40 mL of ethanol. This solution was poured into 200 mL of water. The grey needle crystal was precipitated and collected them by filtration. The crystal was crystallized in 95% ethanol. The pure crystal was yellow needle that named CC-1 (2.477 g). The yield of CC-1 was 1.63x10^{-1}% based on dry

weight of heartwood of A. lakoocha Roxb. powder.

5. CHEMICAL REACTION (Acetylation)

Twenty five miligrams of AA-4 was dissloved in 10 mL of benzene. Then added 3mL of acetic anhydride and warmed in hot water for 45 minutes. After the solution cooled down, partition with 25 mL of water for 3 times. The benzene part was collected and benzene was removed under reduced pressure . The white residue was given, crystallized it in ethanol. The 11 mg white plate crystal (AA-4Ac) was obtained.

6. PREPARATION OF SHIFT REAGENTS

Sodium Methoxide (NaOMe)

Approximately 2.5 g of metallic sodium was cut into small portions and added cautiously to 100 mL of AR methanol.

Aluminium chloride ($AlCl_3$)

About 5 g of fresh, dry, $AlCl_3$ was added cautiously to 100 mL of AR methanol.

Hydrochloric acid (HCl)

Concentrated reagent grade HCl (50 mL) was added to 100 mL of distilled water.

7. CHARACTERIZATION OF ISOLATED COMPOUND

7.1 Characterization of AA-4

AA-4 was crystallized in ethanol as white plate compound. It was soluble in

ethanol, chloroform, hexane.

EIMS ; m/z (relative intensity) ; Figure 5

414 (21), 412 (3), 396 (18), 394 (4), 329 (30),

273 (9), 145 (43), 43 (100)

IR ; υ cm^{-1} ,KBr disc ; Figure 2

3600-3100, 2938, 2868, 1643, 1465, 1379, 1068, 950, 802

^{13}C NMR; δ ppm, 125 MHz ; in Chloroform-d_1 ; Figure 4

11.85, 11.97, 18.77, 19.03, 19.38, 19.78, 21.08, 23.07,

24.29, 25.38, 26.11, 28.23, 29.18, 31.65, 31.91, 33.96,

36.14, 36.50, 37.25, 39.78, 42.30, 42.32, 45.85, 50.14,

51.23, 56.07, 56.77, 71.79, 121.69, 129.28, 138.29,

140.76

mp ; 136-137° C

7.2 Characterization of AA-4Ac

AA-4Ac was crystallized in ethanol as white plate compound. It was soluble in ethanol, chloroform, hexane.

IR ; υ cm^{-1}, KBr disc ; Figure 3

2939, 2907, 2870, 1732, 1465, 1371, 1250, 1040, 803, 609

mp ; 124-125° C

7.3 Characterization of BB-2

BB-2 was crystallized in ethanol as yellow needle compound. It was soluble in dimethylsulfoxide, partial soluble in chloroform, ethylacetate.

EIMS ; m/z (relative intensity) ; Figure 22

44 (14.90), 55 (2.97), 69 (3.53), 162 (7.05), 202

(3.58), 335 (100),378 (24.8), 379 (35.86), 391 (32.22)

419 (4.24), 434; M^+(49.27)

UV ; λ max nm. (log ε), in Methanol ; Figure 6

259(4.029), 292(4.122), 368(4.041)

IR ; υ cm^{-1}, KBr disc ; Figure 8

3401, 2939, 2861, 1651, 1623, 1553, 1481, 1210, 855

^1H NMR; δ ppm, 500 MHz, in mixture of chloroform-d_1 and DMSO-d_6; Figure 9

1.1 (6H, dd, J= 6.8, 1.5 Hz), 1.6 (3H, d, J= 1.2 Hz),

1.9 (3H, d, J= 1.2 Hz), 2.47 (H, m, J= 7, 6.8, 1.1 Hz),

3.94 (3H, s), 5.48 (H, dt, J= 9.2, 1.2 Hz),

6.22 (H, d, J= 9.2 Hz), 6.45 (H, d, J= 2.1 Hz)

6.48 (H, s), 6.55 (H, dd, J= 16, 1.1),

6.57 (H, dd, J= 8.5, 2.1 Hz), 6.7 (H, dd, J= 16, 7 Hz),

7.63 (H, d, J= 8.5 Hz), 9.83 (H, s)

^{13}C NMR ; δ ppm, 125 MHz in mixture of Chloroform-d_1 and DMSO-d_6. Figure 13

18.32, 22.38, 25.57, 32.75, 55.68, 69.27, 89.45,

104.11, 105.20, 107.04, 109.03, 109.38, 109.87, 115.40,

120.89, 124.82, 138.26, 142.06, 154.88, 155.46, 157.73,

158.68, 162.05, 162.92, 178.14

mp ; 259-261° C

7.4 Characterization of B.B-3.3

B.B-3.3 was crystallized in benzene as brown needle. It was soluble in methanol, DMSO , less soluble in chloroform.

EIMS ; m/z (relative intensity); figure 32

51 (6), 109 (8), 110 (2), 120 (4), 137 (100), 138 (85)

UV ; λ max nm. (log ε) ; in Methanol ; Figure 24

231 (3.895), 279 (4.074), 314 (3.775)

IR ; υ cm^{-1}, KBr disc ; Figure 23

3150-2400, 1632, 1581, 1480, 1230, 806

^{1}H NMR; δ ppm, 500 MHz, in Methanol-d_4 ; Figure 25

9.69 (H, s), 7.48 (H, d, J= 8.56 Hz),

6.45 (H, dd, J= 8.56, 2.14 Hz),

6.28 (H, d, J= 2.14 Hz)

^{13}C NMR ; δ ppm, 125 MHz in Methanol-d_4 ; Figure 29

195.47, 167.28, 165.6, 136.81, 116.23, 109.94, 103.30

mp ; 133-134° C

7.5 Characterization of CC-1

CC-1 was crystallized in ethanol as yellow plate compound. It was soluble in ethanol, methanol, acetone, ethylacetate.

IR ; υ cm^{-1} ,KBr disc ;Figure 33

3500-3000,1660-1590,1500-1450,1200-1100,980-960,872,720-670

^{1}H NMR; δ ppm, 500 MHz, in acetone-d_6 ;Figure 34

6.24 (1H, t, J=2.1 Hz), 6.38 (1H, dd, J= 2.4, 8.3 Hz),

6.44 (1H, d, J=2.4 Hz), 6.52 (2H, d, J= 2.1 Hz)

6.88 (1H, d, J=16.8 Hz), 7.33 (1H, d, J= 16.8 Hz)

7.39 (1H, d, J= 8.3 Hz), 8.22 (2H, s), 8.42 (1H, s),

8.60 (1H, s)

mp ; 203° C