CHAPTER III

EXPERIMENTAL

1. Source of Plant Material

The plant used in this investigation was collected in March, 1982 from None-Mai-Daeng Village, Nakorn Rachasima province, Thailand. This plant was identified to be *Morinda talmyi* Pierre (synonym : *M. persicifolia* Williams var. *talmyi* Pitard) of the family Rubiaceae, Order Tubiflorales and it was authenticated by comparison with voucher specimens at the Royal Botanic Gardens, Kew, England.

2. Preliminary Screening for Anthraquinone from the Root of *Morinda talmyi* Pierre (Robinson, 1967)

Dried powdered roots (300 mg) were boiled with 20 ml of 10% hydrochloric acid for 20 minutes and filtered. The filtrate was extracted with chloroform. The chloroform layer was separated and shaken with potassium hydroxide solution. The alkaline layer became red when the anthraquinone is present.

3. General Technique

3.1 Thin Layer Chromatography (TLC)

The preparation of TLC plates for separation of the sample required silica gel G type 60 (E. Merck) containing 13% of calcium sulphate as binder. Thirty-five grams of silica gel G and 70 ml of distilled water were mixed to form slurry by shaking the

mixture for 2 minutes. The prepared slurry was applied to five clean glass plates 20 cm × 20 cm or ten clean glass plates 10 cm × 20 cm. The thickness of the layers was 0.25 cm. The chromatographic plates were air-dried, activated at 110 ℃ for one hour and allowed to cool before storage in a desicator.

The prepared samples were spotted on the plates at the interval of 1.5 cm between each spot. The spotted plates were chromatographed in the equilibrated chamber. The solvent systems used in this experiment were as follows:

solvent system number	solvent system
1	benzene : methanol (9:1)
2	benzene : chloroform (1:1)
3	chloroform : methanol (8.5:1.5)
4	chloroform : acetone (6:4)
5	n-butanol + acetone + water (4+5+1)
6	benzene : ethyl acetate (8.5:1.5)
7	methyl ethyl ketone + acetic acid + isopropanol + methanol (6+2+1+1)
8	methyl ethyl ketone + glacial acetic acid + 2-methyl propan-2-ol (6+2+2)
9	n-butanol + glacial acetic acid + diethyl ether + water (9+6+3+1)
10	n-butanol + glacial acetic acid + water (6+3+1)
11	chloroform + methanol + water (6+4+1)

The usual separation technique was one way ascending. The solvent front was usually 15 cm from the starting line. The

chromatographed plates were air dried at room temperature.

The samples were detected for anthraquinones under the UV light or by spraying with 5% alcoholic potash. The anthraquinones give orange-red under the UV light and pink after sprauing with alcoholic potash.

3.2 Column Chromatography (CC)

Column chromatography technique used in this investigation was "Short Column Chromatography" (Hunt and Rigby, 1967). The flat bottom glass columns of the diameters 5 cm and 10 cm were used. The adsorbent used, was silica gel 60 (230-400 mesh, E. merck). It was packed into the column by wet packing technique according to the method of Still (Still, 1978).

3.3 Melting Point

The melting points were determined on a Gallenkamp Melting Point and a Büchi Melting Point Apparatuses.

3.4 Ultraviolet (UV) Absorption Spectrometry

Ultraviolet absorption spectra were measured on a Shimadzu Double-Beam Spectrophotometer UV-180.

3.5 Infrared (IR) Absorption Spectrometry

Infrared absorption spectra were obtained on a Shimadzu Model IR 440 spectrophotometer, absorption bands were reported in wave number (cm^{-1}).

3.6 Nuclear Magnetic Resonance (NMR) Spectrometry

Proton nuclear magnetic resonance spectra were recorded at 90 MHz and 270 MHz on Jeol Fx 90 Q and Jeol Fx 270. Tetramethyl-

silane was used as an internal standard and chemical shifts were reported on the ppm scale.

3.7 Mass Spectrometry (MS)

The compounds were submitted for low resolution mass spectral study on a Jeol mass spectrometer, Model DX 300 at 70 eV.

4. Extraction and Isolation of Anthraquinones from the Roots of *Morinda talmyi* Pierre

4.1 Extraction and Isolation of Anthraquinone Aglycones

4.1.1 Extraction

Five hundreds grams of dried powdered root was hydrolyzed with 5 litres of acid alcohol (10% hydrochloric acid in 95% ethanol). The mixture was refluxed on the heating mantle for 8 hours and was filtered through Whatman filter paper No. 1. For completeness of extraction, the marc was reextracted for two more times. The combined filtrate was concentrated under reduced pressure to syrupy mass and was dissolved in 500 ml of hot distilled water. The solution was extracted with chloroform (500 ml each) several times until the extract gave no pink colour with 5% alcoholic potash. The combined chloroform extracts was concentrated under reduced pressure to about 500 ml. The concentrated chloroform extract was then partitioned with 5% aqueous sodium hydrogen carbonate solution (3 × 300 ml). The aqueous sodium hydrogen carbonate layers were combined to obtain aglycones (4.84 g) possessing free carbonyl group. The remaining chloroform layer was then extracted with 5% sodium hydroxide solution (6 × 400 ml). The combined sodium hydroxide extract (2.4 l) was acidified dropwised with concentrated hydrochloric acid until the

colour of the solution was turned from pink to yellow (to change anthraquinone salt to free anthraquinone). The yellow acid solution was extracted with chloroform. The chloroform extract was washed with distilled water, dried over anhydrous sodium sulphate and evaporated to dryness under reduced pressure to yield a yellow-brown semi-solid (13.6 g). Thin layer chromatograms showed the presence of nine anthraquinones.

4.1.2 Isolation

The brown semi-solid (13.6 g) was divided into five equal portions and each portion was subjected to silica gel column chromatography in the same manner. Each portion (approx. 2.7 g) was dissolved in 20 ml of eluting solvent, benzene : chloroform (3:1), and placed on top of a 10 cm diameter column of silica gel (300 g). The gradient of increasing elution polarity of the solvent mixture were applied. The various fractions (25 ml each) were examined and combined fractions (Table 1, p. 35) accordingly with the information obtained from the checked TLC (Fig. 12, p. 70)

The combined fraction A gave negative Bornträger test and was not futher investigated. The combined fraction B was shown by TLC to contain two spots of anthraquinones and was not further investigated.

Combined fraction C has only one spot in TLC. It was crystallized from benzene as yellow needles (718.2 mg) and was designated as Aq-1.

Combined fraction D was shown by TLC to contain two spots of anthraquinones, one of them was Aq-1. The combined

fraction D was rechromatographed in the same condition of the previous column and the additional amount of Aq-1 (89.5 mg) was obtained after crystallization. The total weight of Aq-1 was 807.7 mg. The remaining other anthraquinone fractions from this column were collected and combined.

Combined fraction E was shown by TLC to contain two spots of anthraquinones. This fraction was reseparated by column chromatography, the eluent was chloroform : methanol (99.75 : 0.25). Fractions 21-80 (25 ml each) containing pure anthraquinone was crystallized from absolute ethanol as orange thin needles (18.2 mg). It was designated as Aq-3. The first twenty fractions from this column chromatography were collected, they contained the small amount of Aq-3 and the other anthraquinone.

The rechromatographed fractions from the remaining combined fractions D and E were separated through a silica gel column (5.0 cm diameters, 150 g). This column was eluted with benzene : methanol (9:1). The first ten fractions (10 ml each) were shown by TLC to contain one spot of anthraquinone. It was crystallized from the mixture of 2-3 drops of acetone in 10 ml of benzene as yellow plate crystals (10.3 mg) and was designated as Aq-2. The remaining fraction gave an additional amount of Aq-3 (7.4 mg). The total weight of Aq-3 was 25.6 mg.

Combined fraction F was shown by TLC to contain several anthraquinones and was not further investigated due to its too small amount. The combined fraction G was the dark violet solution.

Table 1 Elution patterns of crude extracts

Tube No.	Solvent system	Volumn (litre)	Combined fraction	Remark
1-30	benzene : chloroform (3:1)	0.75	A	no residue
31-70	benzene : chloroform (3:1)	1.35	B	traces of two anthraquinones
71-110	benzene : chloroform (3:1)	1.00	C	one anthraquinone
111-140	benzene : chloroform (3:1)	0.75	D	mixture of two anthraquinones
141-210	benzene : chloroform (3:1)	1.75	E	mixture of two anthraquinones
211-240	benzene : chloroform (2:1)	0.50	F	mixture of three anthraquinones
241-270	benzene : chloroform (1:1)	0.50		
271-300	chloroform	0.50		
301-330	chloroform : methanol (1:1)	0.50	G	dark violet portions
331-360	methanol	0.50		

4.2 Extraction and Isolation of Anthraquinone Glycoside

Dried powdered root (100 g) of *Morinda talmyi* Pierre was macerated with 95% ethanol for 2-3 days and was filtered. The filtrate was concentrated until the yellow amorphous precipitated appeared, it was designated as Aq-4 (314.2 mg).

5. Characterization and Identification of Isolated Anthraquinones

Aq-1

Aq-1 was obtained as yellow needle crystals from benzene and gave positive Bornträger test. It was soluble in chloroform, benzene and ethanol. The structure of Aq-1 is shown in Fig. 9, p. 50.

solvent system number (p. 30)	hRf Value	
1	63.3	(Fig. 13, p. 71)
2	26.6	(Fig. 14, p. 72)
3	79.3	(Fig. 15, p. 73)

Melting Point

183.5 - 184.5 ℃

Ultraviolet and Visible Absorption Spectra (Fig. 24-25, pp. 82-83)

λ_{max} (EtOH) 210, 242, 275, 334, 411 nm

Infrared Absorption Spectrum (Fig. 26, p. 84)

potassium bromide disc, ν_{max} (cm^{-1})

3220 cm^{-1}	(hydroxyl group)
1670, 1630 cm^{-1}	(carbonyl group)
1600 cm^{-1}	(aromatic ring, C=C)
1400; 1370 cm^{-1}	(methyl group)
1100 cm^{-1}	(ether linkage)

Nuclear Magnetic Resonance Spectra (Fig. 27-28, pp. 85-86)

The assignment of the 90 MHz proton NMR spectrum ($CDCl_3$) was recorded in value (ppm) from tetramethylsilane (TMS).

Chemical Shift (δ)	Coupling Constant J	Multiplicity	Proton
1.26 - 1.42	7 Hz	t	3H (CH_3)
3.62 - 3.86	7 Hz	q	2H (CH_2)
4.95		s	2H (EtO-CH_2)
7.28		s	H (H_4)
7.70 - 7.81		m	2H ($H_{6,7}$)
8.20 - 8.31		m	2H ($H_{5,8}$)
9.6		broad s	H (-OH)

^{13}C NMR ($CDCl_3$) δ : 186.65(s), 181.94(s), 164.00(s), 161.62(s), 133.88(s), 133.34(s), 127.11(s), 126.57(s), 114.48(s), 109.55(s), 67.46(s), 66.81(s), 14.85(s)

Mass Spectrum (Fig. 29, p. 87)

m/z 298 (M^+, $C_{17}H_{14}O_5$, 8%), 269(7%), 254(25%), 253(24%), 252(100%), 224(14%), 196(32%), 168(17%), 140(10%), 139(21%), 115(7%), 77(8%)

Molecular Weight

298

Aq-2

Aq-2 was obtained as yellow plate crystals from the mixture of 2-3 drops of acetone in 10 ml of benzene and gave positive Bornträger test. It was soluble in chloroform, acetone, benzene and in ethanol.

The structure of Aq-2 is shown in (Fig. 10, p.51)

solvent system number (p. 30)	hRf Value	
1	54.6	(Fig. 13, p.71)
2	16.0	(Fig. 14, p.72)
3	73.3	(Fig. 15, p. 73)

Melting Point

231.0 - 232.0 ℃

Ultraviolrt and Visible Absorption Spectra (Fig. 30-31, pp. 88-89)

λ_{max} (EtOH) 208, 245, 279, 298, 411 nm

Infrared Absorption Spectrum (Fig. 32, p.90)

potassium bromide disc, ν_{max} (cm^{-1})

3395 cm^{-1}	(OH stretching)
1662, 1624 cm^{-1}	(carbonyl group)
1590 cm^{-1}	(aromatic ring, C=C)
1365 cm^{-1}	(methyl group)
1123 cm^{-1}	(ether linkage)

Nuclear Magnetic Resonance Spectrum (Fig. 33, p.91)

The assignment of the 270 MHz proton NMR spectrum ($CDCl_3$) was recorded in value (ppm) from tetramethylsilane (TMS).

Chemical Shift (δ)	Coupling Constant J	Multiplicity	Proton
3.577		s	3H ($-OCH_3$)
7.323		s	1H (H_4)
7.362		s	1H (H_1)
7.765 - 7.797		m	2H (H_6, H_7)
8.253 - 8.296		m	2H (H_5, H_8)
7.365		s	H (OH)

Mass Spectrum (Fig. 34, p. 92)

m/z 254 (M^+, $C_{15}H_{10}O_4$, 10%), 253(22%), 252(100%), 196(15%), 168(7%), 139(7%)

Molecular Weight

254

Aq-3

Aq-3 was obtained as orange thin needle crystals from absolute ethanol and gave positive Bornträger test. It was soluble in ethanol and in methanol and slightly soluble in chloroform. The structure of Aq-3 is shown in Fig. 11, p. 53)

solvent system number (p. 30)	hRf Value	
1	48.6	(Fig. 13, p. 71)
2	8.0	(Fig. 14, p. 72)
3	66.6	(Fig. 15, p. 73)

Melting Point

276.0 - 277.0 ℃

Ultraviolet and Visible Absorption Spectra (Fig. 35-37, pp. 93-95)

λ_{max} (EtOH) 219, 269, 297, 315, 411 nm

Infrared Absorption Spectrum (Fig. 38, p. 96)

potassium bromide disc, ν_{max} (cm^{-1})

3400 cm^{-1}	(OH stretching)
1650, 1620 cm^{-1}	(carbonyl group)
1590, 1570 cm^{-1}	(aromatic ring, C=C)
1030 cm^{-1}	(acetate stretching)
1360 cm^{-1}	(methyl group)

Nuclear Magnetic Resonance Spectrum (Fig. 39, p. 97)

The assignment of the 270 MHz proton NMR spectrum ($CDCl_3$) was recorded in value (ppm) from tetramethylsilane (TMS).

Chemical Shift (δ)	Coupling Constant J	Multiplicity	Proton
2.29		s	3H ($-CH_3$)
2.50		s	DMSO
7.22 - 7.26	8 Hz	d,d	H ($H_{7 \text{ or } 6}$)
7.47 - 7.48	2 Hz	d	H ($H_{5 \text{ or } 8}$)
7.57 - 7.66	8 Hz	2 sets of doublets	2H (H_{3-4})
8.09 - 8.13	8 Hz	d	H ($H_{8 \text{ or } 5}$)
13.01		s	H (-OH)

Mass Spectrum (Fig. 40, p. 98)

m/z 298 (M^+, $C_{16}H_{10}O_6$, 0.07%), 254(100%), 253(17%), 226(12%), 197(14%), 169(4%), 139(3%)

Molecular Weight

298

Aq-4

Aq-4 was obtained as yellow needle crystals from ethanol and gave positive Bornträger test. It was soluble in ethanol and in methanol, was slightly soluble in chloroform and in acetone. The structure of Aq-4 is shown in Fig. 8, p. 49

solvent system number (p. 30)	hRf Value	
5	46.6	(Fig. 16, p. 74)
7	73.6	(Fig. 17, p. 75)

Melting Point

176.5 - 177.5 ℃

Ultraviolet and Visible Absorption Spectra (Fig. 41-44, pp. 99-103)

λ_{max} (MeOH) 315, 341, 348, 400, 422 nm

-$AlCl_3$ added, gave bathochromic shift : 315 → 336 nm

-$AlCl_3$ and NaOAc added, gave bathochromic shift : 241 → 358 nm

Infrared Absorption Spectrum (Fig. 45. p. 103)

potassium bromide disc, ν_{max} (cm^{-1})

3380 cm^{-1} (OH stretching)

1670, 1630 cm^{-1} (carbonyl group)

1600 cm^{-1} (aromatic ring, C=C)

1100 cm^{-1} (ether linkage)

Nuclear Magnetic Resonance Spectra (Fig. 46-48, pp. 104-105)

Proton NMR spectrum of Aq-4 (in DMSO) was recorded in value (ppm) from tetramethylsilane (TMS).

Chemical Shift (δ)	Coupling Constant J	Multiplicity	Proton
2.97 - 3.11		m	H (C-6')
3.34 - 3.39		m	H (C-4')
3.7		m	sugar -OH
3.94		m	H (C-3')
4.15		m	H (C-5')
4.62		q	CH_2OH
5.12		m	H (C-1')
7.49		s	H (H_4)
7.94 - 7.97		m	2H ($H_{6,7}$)
8.18 - 8.26		m	2H ($H_{5,8}$)

^{13}C NMR (DMSO, $CDCl_3$) δ : 186.82(s), 181.18(s), 161.78(s), 136.69(s), 135.5(s), 132.64(s), 126.40(s), 123.50(s), 111.18(s), 106.36(s) 100.83(s), 75.69(s), 73.2(s), 69.41(s), 67.94(s), 50.98(s)

Mass Spectrum (Fig. 49, p. 107)

m/z 448 (M^+, $C_{21}H_{20}O_{11}$), 270(100%), 254(51%), 197(8%), 168(6%), 139(12%)

Molecular Weight

448

Hydrolysis of Aq-4

Fifty milligram of Aq-4 was suspended in 25 ml of water and heated on a water bath for 15 minutes, cooled and filtered. Ten millilitre of 25% hydrochloric acid was added to the filtrate and heated for 15 minutes. The hydrolyzed solution was extracted with ether for three times. Ether extract gave positive Bornträger test while the aqueous layer gave positive Molisch test. This indicated that Aq-4 was a glycoside. The aglycone was identified as lucidin by comparing with authentic lucidin by TLC in solvent system numbers 1 and 6 (p.30). They were detected by spraying with alcoholic potash (Fig. 18-19, pp. 76-77). The sugar was designated as sugar-X and identified as glucose by HPLC and by TLC (Fig. 20-23, pp. 78-81) comparing with authentic sugar in solvent system numbers 8 to 11 p.30.

Detection of sugar-X and reference sugars

Spray reagents

a) anisaldehyde - sulfuric acid spray reagent

b) α-naphthol - sulfuric-acid spray reagent

heating at 110 ℃ for 15 minutes after spraying