

CHAPTER II

EXPERIMENTS AND RESULTS

Plant Materials

The branches of *Amoora gigantea* Pierre. were collected in Rayong province in February 1995. They were air-dried, minced to coarse powder and extracted by organic solvents.

Instruments and Equipments

1. Rotary Evaporator

Eyela type N-1 rotatory vacuum evaporator was used for the rapid removal of large amounts of volatile solvents.

2. Melting Points (m.p.)

The melting points were obtained on a Fisher-John apparatus.

3. Fourier Transform-Infrared Spectrophotometer (FT-IR)

The FT-IR spectra were recorded on a Perkin-Elmer Model 1760X Fourier Transform-Infrared Spectrophotometer.

4. Infrared Spectrophotometer (IR)

The IR spectra were recorded on a Perkin-Elmer Model IR 718. Solid samples were examined by incorporating the sample into a pellet of potassium bromide. Liquid samples were dropped on sodium chloride cell.

5. ^1H and ^{13}C - Nuclear Magnetic Resonance Spectrometer.

The ^1H -NMR and ^{13}C -NMR spectra were obtained by using a Bruker Model ACF 200 Spectrometer which operated at 200.13 MHz. for ^1H and 50.32 MHz. for ^{13}C nuclei. The chemical shifts were assigned with reference to the signal from the residual proton in deuterated solvent.

6. Mass Spectrometer (MS)

The mass spectra were obtained on Fison Mass Spectrometer Model Trio 2000.

7. Gas Liquid Chromatography (GLC)

The GLC analysis results were obtained from Shimadzu Gas-Liquid Chromatographic Model GC-7AG.

Chemical Substances

1. All solvents such as hexane, chloroform, ethylacetate and methanol were purified by distillation before use. Solvents of analytical grade were used in recrystallization.

2. Other substances.

- Merck's silica gel 60 Art. 7734 1000(70-230 mesh ASTM) was used as adsorbents for column chromatography.
- TLC aluminium sheets silica gel 60 F254 precoated sheets, 20x20 cm², 0.2 mm. thick were obtained from Merck.
- Sephadex LH-20 was used as adsorbents for column chromatography.

Physical Separation Techniques

1. Column Chromatography (CC)

Column Chromatography was presented in Reference No.5

2. Thin-Layer Chromatography (TLC)

Thin-Layer Chromatography was presented in Reference No.5

Extraction

The air-dried branches (5.1 kg.) of *Amoora gigantea* Pierre. were extracted by soaking in methanol for several days at room temperature. The soaking was repeated four times. After filtration and evaporation of the solvent under reduced pressure, the crude extract was obtained as a dark brown material (236.92 g, 4.65% wt. by wt. dried branch).

The crude extract was extracted by hexane until the solution was colorless. The filtered solution was evaporated under reduced pressure to afford a hexane crude extract, fraction I, as a green-yellow material (31.99 g. 0.63% wt. of dried branch)

The residue left after hexane extraction was reextracted by chloroform.

A sticky dark brown material was obtained as a chloroform crude extract, fraction II (40.85 g., 0.80% wt. by wt. of dried branch).

After extraction with chloroform, the crude extract was reextracted by ethylacetate. The ethyl acetate extract was evaporated and a yellow material was obtained as an ethylacetate crude extract, fraction III (8.75 g., 0.17% wt. by wt. of dried branch).

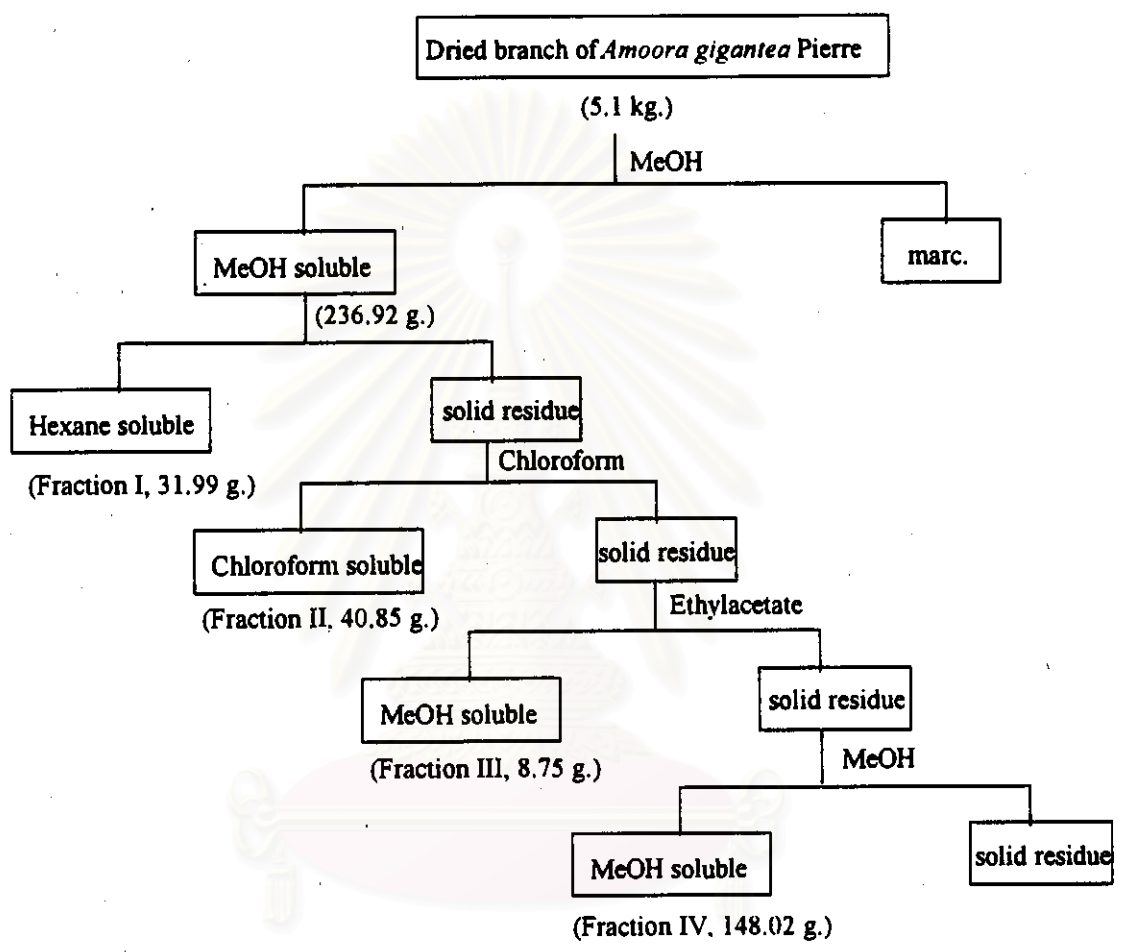
Finally, the crude extract of methanol was obtained as a dark brown material, fraction IV (148.02 g., 2.90% wt. by wt. of dried branch).

The procedure of the various extractions was shown in scheme I



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Scheme I Extraction of the branch of *Amoora gigantea* Pierre ex. Laness.



Isolation of the Chemical Constituents of the branch of *Amoora gigantea* Pierre ex. Laness.

1. Separation of Fraction I

Column chromatographic technique was used for separating 30.14 g. of the hexane crude extract. Silica gel (360 g.) was packed as adsorbent. The column was eluted stepwise from hexane, chloroform in hexane, methanol in chloroform and methanol, respectively. Eluting solvent for each fraction was collected about 800 ml., concentrated to a small volume and monitored by TLC. The fractions containing the same components were combined together. The results of the separation of fraction I by column chromatography are presented in Table 3.

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Table 3 The results of the separation of fraction I

Eluent	Fraction No.	Remark	Weight (g.)
100% Hexane	1-2	trace	1.29
10% CHCl ₃ /Hexane	3-5	light yellow oil	1.66
15% CHCl ₃ /Hexane	6-9	yellow oil	0.32
20% CHCl ₃ /Hexane	7-10	yellow oil	1.59
25% CHCl ₃ /Hexane	11-12	white solid in yellow oil	0.23
30% CHCl ₃ /Hexane	13-26	white solid in yellow oil	2.96
40% CHCl ₃ /Hexane	27-34	white solid in yellow oil	2.02
50% CHCl ₃ /Hexane	35-43	white solid in yellow oil	0.83
	44-50	yellow oil	0.14
60% CHCl ₃ /Hexane	51-53	yellow oil	0.67
70% CHCl ₃ /Hexane	54-65	yellowish-green oil	0.28
80% CHCl ₃ /Hexane	66-68	yellowish-green oil	0.08
90% CHCl ₃ /Hexane	69-71	yellowish-green oil	0.18
100% CHCl ₃	72-74	yellow oil	0.17
2% MeOH/CHCl ₃	75-81	yellow oil	3.37
5% MeOH/CHCl ₃	82-85	yellow oil	1.86
10% MeOH/CHCl ₃	86-87	light brown oil	0.56
20% MeOH/CHCl ₃	88-91	brown oil	0.89
40% MeOH/CHCl ₃	92-97	brown oil	0.73
60% MeOH/CHCl ₃	98-100	brown oil	0.25
80-100%MeOH/CHCl ₃	101-106	brown tar	2.86

2. Separation of Fraction II

Column chromatography was used for separation of 38.67 g. of the chloroform crude extract. Silica gel (480 g.) was packed as adsorbent. The separation was the same manner as the separation of fraction I

Table 4 The results for the separation of fraction II

Eluent	Fraction No.	Remark	Weight (g.)
100% Hexane	1-2	-	-
10-20% CHCl ₃ /Hexane	3-6	trace	0.01
35% CHCl ₃ /Hexane	7-10	yellow oil	0.06
60% CHCl ₃ /Hexane	11-16	yellow oil	0.91
70% CHCl ₃ /Hexane	17-21	yellow oil	0.26
80-90% CHCl ₃ /Hexane	22-23	yellow oil	0.25
	24-32	white solid in brown oil	0.63
100% CHCl ₃	33-43	dark brown tar	1.92
1% MeOH/CHCl ₃	44-46	brown oil	0.11
	47-50	dark brown tar	0.38
2% MeOH/CHCl ₃	51-56	solid in yellow oil	1.70
	57-59	brown oil	0.13
	60-61	green oil	5.21
	62-72	solid in green oil	5.05
5% MeOH/CHCl ₃	73-87	solid in green oil	4.85

Table 4 (continued)

Eluent	Fraction No.	Remark	Weight (g.)
10% MeOH/CHCl ₃	88-95	solid in green oil	0.80
20% MeOH/CHCl ₃	96-105	solid in green oil	1.41
	106-111	solid in green oil	0.45
30% MeOH/CHCl ₃	112-116	yellow oil	0.80
40% MeOH/CHCl ₃	117-119	brown oil	0.05
60% MeOH/CHCl ₃	120-123	brown oil	0.37
100% MeOH	124-126	brown oil	0.42

3. Separation of Fraction III

The ethyl acetate crude extract (8.75 g.) was separated by column chromatography silica gel (100 g.) was packed as adsorbent. The column was eluted with an increasing gradient of chloroform in hexane, methanol in chloroform and methanol. Each fraction was collected about 500 ml., concentrated and checked by TLC. The similar fractions were combined.

Table 5 The results of the separation of fraction III

Eluent	Fraction No.	Remark	Weight (g.)
50% CHCl ₃ /Hexane	1-5	-	-
60% CHCl ₃ /Hexane	6-10	-	-
70% CHCl ₃ /Hexane	11-17	trace	0.01
80% CHCl ₃ /Hexane	18-22	yellow oil	0.09
90% CHCl ₃ /Hexane	23-27	yellow oil	0.05
100% CHCl ₃	28-37	yellow oil	0.08
1% MeOH/CHCl ₃	38-54	green oil	0.27
2% MeOH/CHCl ₃	55-70	yellow oil	1.08
5% MeOH/CHCl ₃	71-93	solid in yellow oil	3.16
10% MeOH/CHCl ₃	94-105	solid in yellow oil	2.81
20% MeOH/CHCl ₃	106-115	yellow oil	0.18
30% MeOH/CHCl ₃	116-125	yellow oil	0.21
50% MeOH/CHCl ₃	126-131	brown oil	0.04
80% MeOH/CHCl ₃	132-133	brown tar	0.03
100% MeOH	134-135	brown tar	0.06

4. Separation of Fraction IV

The methanol crude extract (80.51 g.) was separated by using 900 g. of silica gel as adsorbent. The separation was the same manner as the ethyl acetate crude extract excepted that each fraction was collected about 800 ml. The results of separation of Fraction IV are presented in Table 6

Table 6 The results of the separation of Fraction IV

Eluent	Fraction No.	Remark	Weight (g.)
50% CHCl ₃ /Hexane	1-4	yellow oil	0.06
60-80% CHCl ₃ /Hexane	5-16	yellow oil	0.10
90% CHCl ₃ /Hexane	17-20	yellow oil	0.52
100% CHCl ₃	21-28	yellow oil	0.73
2% MeOH/CHCl ₃	29-30	yellow oil	0.64
	31-34	green oil	0.15
	35-37	yellow oil	0.16
5% MeOH/CHCl ₃	38-48	yellow oil	1.05
10% MeOH/CHCl ₃	49-50	solid in yellow oil	0.04
	51-52	brown oil	0.54
	53-54	solid in brown oil	0.90
15% MeOH/CHCl ₃	55-72	brown oil	1.02
	73-80	brown oil	1.28
	81-87	yellow oil	2.38

Table 6 (continued)

Eluent	Fraction No.	Remark	Weight (g.)
20% MeOH/CHCl ₃	88-102	solid in yellow oil	7.70
25% MeOH/CHCl ₃	103-105	solid in yellow oil	9.51
30% MeOH/CHCl ₃	116-118	solid in yellow oil	3.72
	119-123	brown oil	2.52
40% MeOH/CHCl ₃	124-135	solid in brown oil	9.92
50% MeOH/CHCl ₃	136-141	brown oil	8.69
60% MeOH/CHCl ₃	142-143	brown tar	1.90
80% MeOH/CHCl ₃	144-145	brown tar	1.79
100% MeOH	146-147	brown tar	1.65

5. Separation of Fraction IV by using sephadex as adsorbent

The methanol crude extract (10.53 g.) dissolved with 50% MeOH in chloroform. After it was filtered and evaporated, the methanol crude (3.82 g.) was obtained. Concentrated methanol crude (1.08 g.) was chromatographed on a sephadex LH-20 column packed in 50% MeOH/CHCl₃ (approximately 37 cm.). It was eluted with 50% MeOH in chloroform. After the eluent was collected (approximately 50 ml.) for each fraction, it was concentrated and monitored by thin-layer chromatography. The results of separation are shown in Table 7.

Table 7 The results of the separation of Fraction IV by using sephadex as adsorbent.

Eluent	Fraction No.	Remark	Weight (g.)
100% EtOAc	1	brown tar	0.1551
	2	brown tar	0.5167
	3	solid in yellow oil	0.4433
	4	yellow oil	0.0688
	5	yellow oil	0.0079
	6	yellow oil	0.0054
	7	yellow oil	0.0073
	8	yellow oil	0.0071
	9	yellow oil	0.0137
	10	yellow oil	0.0131
	11	solid in yellow oil	0.0179
	12	solid in yellow oil	0.0218
	13	yellow oil	0.0168
	14	yellow oil	0.0044
	15	yellow oil	0.0039
	16	yellow oil	0.0013
	17	yellow oil	0.0002
	18	orange oil	0.0025
	19	orange oil	0.0008
100% MeOH	20-25	yellow oil	0.0909

Purification and properties of the eluted substances.

1. Purification and properties of mixture I

The yellow oil was obtained 1.66 g. (5.51% wt./wt. of the hexane crude extract) from fraction no.3-5 in the hexane crude extract separation (10% CHCl_3 in hexane). This oily mixture was soluble in hexane, dichloromethane, and chloroform but slightly soluble in ethyl acetate, acetone and methanol.

FT-IR spectrum (neat), ν_{max} (cm^{-1}) : 2927, 2855(s), 1740(s), 1459(m), 1378(m), 1171(m) and 725(m) (Fig.3)

$^1\text{H-NMR}$ spectrum (CDCl_3), δ (ppm.) : 0.84(t), 1.29(s), 1.58(m), 2.19(t) and 3.58(s) (Fig.4)

$^{13}\text{C-NMR}$ spectrum (CDCl_3), δ (ppm.) : 13.72, 22.50, 24.81, 27.54, 29.03-29.78, 32.05, 33.83, 51.50, 121.52-131.16 and 173.95 (Fig.5)

The mixture I was analyzed by GC-MS spectrometer to identified its chemical constituents. Gas chromatography (condition ; column DB-1, column temp. 90-310°C, injection temp. 250°C, carrier gas He) showed retention times at 6.34, 6.45, 7.74, 8.46, 8.57, 9.81, 10.69, 11.19, 12.23, 13.85, 15.09, 16.68, 16.69, 20.3, 23.19 and 25.51 min., respectively. (Fig.6)

2. Purification and properties of compound II

The amorphous bright solid in yellow oil was obtained from fraction no.11-12 in the hexane crude extract separation (25% CHCl₃ in hexane). It was purified by recrystallization from hot methanol to obtain bright solid (30.27 mg., 0.10% wt./wt. of the hexane crude extract). The melting point was 76-77°C and the R_f value was 0.7 (silica gel : CHCl₃). It was soluble in dichloromethane, chloroform and ethyl acetate but slightly soluble in ethanol and methanol. This compound was tested with Liebermann-Burchard reaction and showed a purple colour.

FT-IR spectrum (KBr), ν_{\max} (cm⁻¹) : 3070(s), 2947, 2875(s), 1705(s), 1641(w), 1452 (m), 1381(m), 881 and 836(w) (Fig.11)

¹H-NMR spectrum (CDCl₃), δ (ppm.) : 0.88(s), 0.95(s), 1.02(s), 1.04(s), 1.08(s), 1.62(s), 1.69(s), 0.98-2.54(m), 4.75 and 5.13(m) (Fig.12)

¹³C-NMR spectrum (CDCl₃), δ (ppm.) : 15.34, 15.82, 16.03, 17.69, 19.65, 21.00, 21.89, 24.96, 25.67, 26.74, 27.05, 28.86, 31.33, 34.10, 34.74, 34.85, 36.91, 39.95, 40.36, 45.40, 47.39, 47.73, 49.39, 50.28, 55.37, 100.11, 107.57, 124.41, 131.44, 152.58 and 218.12 (Fig.13)

DEPT-135 ¹³C-NMR spectrum (CDCl₃), δ (ppm.) : (Fig.14)

CH₃, CH signals (up phase) 15.34, 15.82, 16.03, 17.69, 21.00, 25.67, 26.74, 45.40, 47.73, 50.28, 55.37 and 124.41

CH₂ signals (down phase) 19.65, 21.89, 24.96, 27.05, 28.86, 31.33, 34.10, 34.74, 34.85, 39.95 and 107.57

DEPT-90 ¹³C-NMR spectrum (CDCl₃), δ (ppm.) : 45.40, 47.73, 50.28, 55.37, 124.41

Mass spectrum (m/e) : 424, 205, 109, 189 and 95 (Fig.15)

3. Purification and properties of mixture III

The white solid in yellow oil was obtained from fraction no.13-18 in the hexane crude extract separation (30% CHCl₃ in hexane). It was purified by recrystallization with hot hexane to obtain the colorless needle crystal 0.98 g. (3.25% wt./wt. of the hexane crude extract). The melting point was 131-132°C and the R_f value was 0.6 (silica gel : 5% MeOH in CHCl₃). It was soluble in dichloromethane, chloroform and ethyl acetate but slightly soluble in ethanol and methanol. This mixture was tested with Liebermann-Burchard reaction and showed a green colour.

FT-IR spectrum (Kbr), ν_{\max} (cm⁻¹) : 3608-3219(s), 2958-2870(s), 1640(m), 1465, 1381(s), 1058(s), 970, 960(m) and 839 and 802(m) (Fig.16)

¹H-NMR spectrum (CDCl₃), δ (ppm.) : 0.66-2.26(m), 3.55(m), 5.10(m) and 5.34(d) (Fig.17)

¹³C-NMR spectrum (CDCl₃), δ (ppm.) : 11.85, 11.97, 12.25, 19.02, 19.39, 19.82, 21.07, 21.21, 23.05, 24.30, 25.41, 26.04, 28.24, 28.92, 29.69, 31.64, 31.89, 33.70, 33.93, 36.14, 36.50, 37.24, 39.67, 39.76, 40.50, 42.29, 45.81, 50.12, 51.13, 55.93, 56.04, 56.75, 56.85, 71.79, 121.71, 129.25, 138.32 and 140.74 (Fig.18)

GC chromatography (condition ; column 2%OV-1, column temp. 255°C, injection temp. 290 °C, carrier gas N₂ 40.0 ml/min.) showed retention times at 19.93, 21.01 and 23.82 min., respectively. (Fig.19, 20)

Mass spectrum (m/e) : 414, 412 and 400 (Fig.21)

4. Purification and properties of mixture IV

The white solid in yellow oil, mixture IV, was obtained from fraction no.27-34 in the hexane crude extract separation (40% CHCl₃ in hexane). It was purified by recrystallization with hot methanol to obtain the white solid 0.27 g. (0.90% wt./wt. of the hexane crude extract). The melting point was 75-77°C and the R_f value was 0.7 (silica gel : 5% MeOH in CHCl₃). It was soluble in dichloromethane, chloroform and ethyl acetate but slightly soluble in ethanol and methanol.

FT-IR spectrum (KBr), ν_{\max} (cm⁻¹) : 3629-3010(s), 2918, 2850(s), 1706(m), 1464, 1377(s) and 724(m) (Fig.22)

¹H-NMR spectrum (CDCl₃), δ (ppm.) : 0.86(t), 1.23(s) and 2.33(t) (Fig.23)

¹³C-NMR spectrum (CDCl₃), δ (ppm.) : 14.14, 22.70, 24.68, 29.70, 31.93, 33.95 and 179.53 (Fig.24)

This mixture was converted to methyl ester and analyzed by GC-MS technique. Gas chromatography (condition ; column DB-1, column temp. 90-310°C, injection temp. 250°C, carrier gas He) showed retention times at 16.65, 17.77, 20.34, 21.85, 23.22, 24.44, 25.56, 26.56 and 27.51 min. (Fig.26)

5. Purification and properties of mixture V

The white solid in green oil, compound V, was obtained from fraction no.77-78 in the chloroform crude extract separation (5% MeOH in CHCl_3) and from fraction no. 72-93 in the ethylacetate crude extract separation (5% MeOH in CHCl_3). It was purified by recrystallization with hot ethanol to obtain the white solid 1.15 g. The melting point was 270-275°C and the R_f value was 0.6 (silica gel : 20% MeOH in CHCl_3). It was insoluble in dichloromethane, chloroform, ethyl acetate and acetone.

FT-IR spectrum (KBr), ν_{max} (cm^{-1}) : 3655-3149(s), 3026(w), 2960, 2934(s), 1640(s), 1465, 1379(s), 1163-1027(s), 892(w), 840 and 800(w) (Fig.36)

$^1\text{H-NMR}$ spectrum (DMSO), δ (ppm.) : 0.55-2.40(m), 2.75-3.55(m), 3.62(m), 4.20(d), 4.45(t), 5.10(m), and 5.29(m) (Fig.37)

$^{13}\text{C-NMR}$ spectrum (DMSO), δ (ppm.) : 11.64, 11.75, 18.59, 18.90, 19.07, 19.68, 20.58, 22.58, 23.85, 25.40, 27.78, 28.66, 29.23, 31.38, 33.32, 35.48, 36.18, 36.82, 40.72, 41.82, 45.11, 49.58, 55.41, 56.16, 61.04, 70.02, 73.42, 76.72, 76.90, 100.79, 121.17, 128.80, 138.02 and 140.41 (Fig.38)

Mass spectrum : the dominant fragmentation ion peaks at (m/e) 414, 412 and 400.

(Fig.39)

6. Purification and properties of compound VI

The white solid in brown oil, compound VI, was obtained from fraction no. 110-111 in the methanol crude extract separation (25% MeOH in CHCl_3). It was purified by recrystallization with hot ethanol to obtain the colorless crystal 0.11 g. (0.14% wt./wt. of the MeOH crude extract). The melting point was 192°C and the R_f value was 0.4 (silica gel : 20% MeOH in CHCl_3). It was insoluble in dichloromethane, chloroform, ethyl acetate and acetone.

FT-IR spectrum (KBr), ν_{max} (cm^{-1}) : 3530-3014(s), 2994, 2982(m), 1476, 1461, 1386 (m) and 1161-1006(s) (Fig.40)

$^1\text{H-NMR}$ spectrum (DMSO), δ (ppm.) : 3.53-3.56(m), 4.41(q), 4.49(d), 4.77(q), 5.06 (d), 5.17(t)(Fig.41)

$^{13}\text{C-NMR}$ spectrum (DMSO), δ (ppm.) : 61.98, 62.09, 69.77, 71.58, 72.78, 74.22, 76.94, 82.51, 96.82 and 103.98 (Fig.42)

Mass spectrum (m/e) : 293, 221, 163, 145 and 127 (Fig.43)

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7. Purification and properties of compound VII

The white solid in brown oil, compound VII, was obtained from fraction no. 116-117 in the methanol crude extract separation (30% MeOH in CHCl_3). It was purified by recrystallization with hot ethanol to obtain the colorless needle crystal 0.65 g. (0.81% wt./wt. of the MeOH crude extract). The melting point was 223-224°C and the R_f value was 0.2 (silica gel : 20% MeOH in CHCl_3). It was insoluble in dichloromethane, chloroform, ethyl acetate and acetone.

FT-IR spectrum (KBr), ν_{\max} (cm^{-1}) : 3500-3010(s), 2920, 2845(m), 1455, 1417(m), 1370(m) and 1171-1000(s) (Fig.44)

$^1\text{H-NMR}$ spectrum (DMSO), δ (ppm.) : 2.49-4.65(Fig.45)

$^{13}\text{C-NMR}$ spectrum (DMSO), δ (ppm.) : 71.22, 72.41, 72.83 and 75.10 (Fig.46)

Mass spectrum (m/e) : 144, 126, 109, 102, 89, 71 and 60 (Fig.47)

8. Purification and properties of compound VIII

The white needle crystal in brown oil, compound VIII, was obtained from fraction no. in the methanol crude extract separation (40% MeOH in CHCl_3) and from fraction no. 11-12 in the methanol crude extract separation which used sephadex as adsorbent (50% MeOH in CHCl_3). After washing the brown oil, colorless needle crystals were obtained 2.75 g. (3.37% wt./wt of the MeOH crude extract). This compound was reacted with sodiumcobaltinitrite and then obtained the yellow precipitate. The flame test gave purple-red colour. When the solution was reacted with AgNO_3 , it gave white precipitate that was soluble in ammonia solution.