

CHAPTER II

EXPERIMENTS

Plant Materials

The woods of *Croton oblongifolius* Roxb. were collected at Petchaboon province during October 1995. The specimen was compared with the herbarium No. 9607 in the Royal Forest Department, Bangkok, Thailand.

Instruments and Equipment

1. Rotary vacuum evaporator

The Eyela rotary vacuum evaporator type N was used for the rapid removal of large amounts of volatile solvents.

2. Melting point Apparatus

Melting points were obtained on a Fisher-John Melting point apparatus and was reported uncorrected.

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

FT-IR spectra were recorded on a Perkin-Elmer Model 1760X Fourier Transform-Infrared Spectrophotometer. Solid samples were examined by incorporating the sample into a pellet of potassium bromide. Liquid samples were prepared as thin film on a sodium chloride cell.

4. Gas Chromatography (GC)

GC analyses were performed on a Shimadzu Gas Chromatograph Model GC-7AG.

5. Mass Spectrometer (MS)

MS analyses were performed on a Fison Gas Chromatograph Model GC-8000, Fison Mass Spectrometer Model Trio-2000 at 70 eV.

6. Nuclear Magnetic Resonance Spectrometer (NMR)

The ^1H -NMR and ^{13}C -NMR spectra were obtained using Bruker NMR Spectrometer Model ACF200 operating at 200.13 MHz for ^1H and 50.32 MHz for ^{13}C nuclei and using Jeol NMR Spectrometer Model JNM-A500 operating at 500 MHz for ^1H nuclei.

Chemicals

1. All commercial grade solvents (hexane, dichloromethane, chloroform, ethyl acetate, methanol and acetone) were purified prior to use by distillation.
2. Merck's silica gel 60G 70-230 mesh ASTM and 230-400 mesh ASTM were used as adsorbents for normal column chromatography and flash column chromatography.
3. A mixture of Merck's silica gel 60G for thin-layer chromatography and silica gel 60GF₂₅₄ for thin-layer chromatography (ratio 10:1 wt. by wt.) were used as adsorbents for Preparative Thin Layer Chromatography.
4. Merck's precoated aluminium TLC sheets, silica gel 60 F₂₅₄, 20x20 cm², layer thickness 0.2 mm, was used for identifying the identical fractions.

5. Sephadex LH-20 was used as adsorbents for gel permeation chromatography.

Separation Techniques

1. Column Chromatography (20)

Glass columns with appropriate size and length were used. Ratio of crude to adsorbent is 1:20 by weight. Elution was carried out under medium pressure (flash column) or gravimetrically using a suitable solvents system.

2. Thin-Layer chromatography (21)

Merck's precoated aluminium TLC sheets, silica gel 60 F₂₅₄ sheets, layer thickness 0.2 mm was used and was developed using a suitable solvent system.

Extraction

The woods of *Croton oblongifolius* Roxb. (11.4 kg) were air-dried which reduced the weight to 8.00 kg. Then, they were ground and soaked in methanol 15 litres for 2 days at room temperature for 9 times. After each filtration and evaporation of the solvent under reduced pressure, the methanol crude extract was obtained as dark brown resinous material 182.00 g (equivalent to 2.27 % wt. by wt. of the air-dried woods).

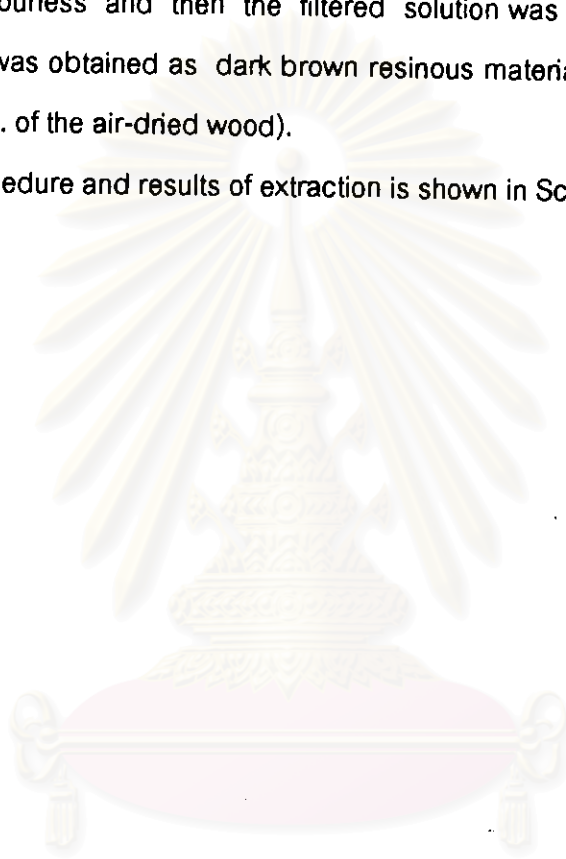
The methanol crude extract was re-extracted by hexane until the solution was colourless and then the filtered solution was evaporated. The hexane crude extract was obtained as yellowish-brown resinous material 18.90 g (equivalent to 0.24 % wt. by wt. of the air-dried woods).

The residue after hexane extraction was re-extracted by dichloromethane until the solution was colourless and then the filtered solution was evaporated. The dichloromethane crude extract was obtained as brown resinous material 20.30 g (equivalent to 0.25 % wt. by wt. of the air-dried woods).

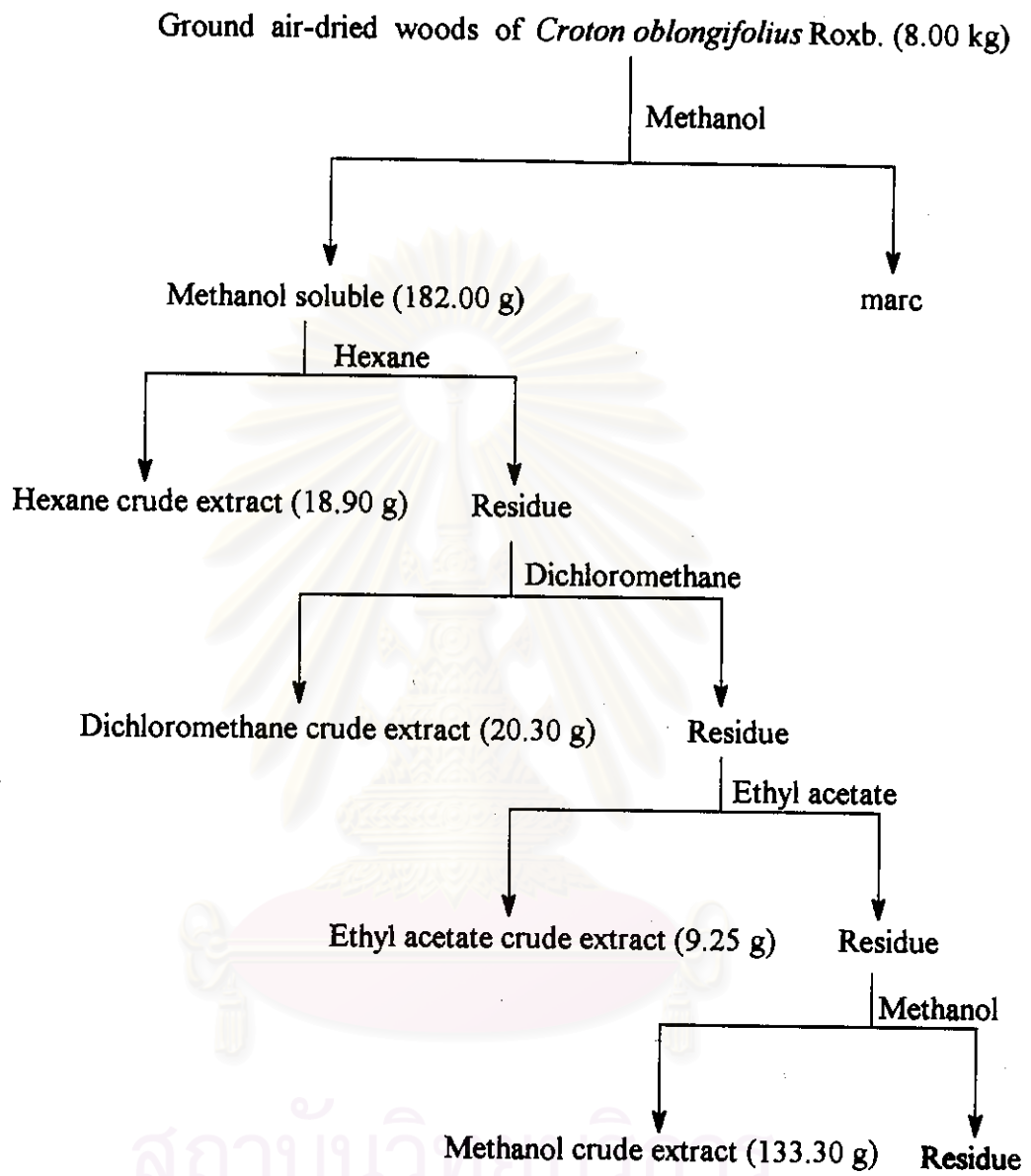
The residue after dichloromethane extraction was re-extracted by ethyl acetate until the solution was colourless and then the filtered solution was evaporated. The ethyl acetate crude extract was obtained as brown resinous material 9.25 g (equivalent to 0.12 % wt. by wt. of the air-dried woods).

The residue after ethyl acetate extraction was re-extracted by methanol until the solution was colourless and then the filtered solution was evaporated. The methanol crude extract was obtained as dark brown resinous material 133.30 g (equivalent to 1.67 % wt. by wt. of the air-dried wood).

The procedure and results of extraction is shown in Scheme 1.



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Scheme 1 The Procedure of Extraction of the Woods of *Croton oblongifolius* Roxb.

Isolation

1. Separation of Hexane Crude Extract

Column chromatography technique was used for separating 18.90 g of hexane crude extract into various fractions. Silica gel 60G 70-230 mesh ASTM was used as adsorbent. The volume of eluting solvents used for each fraction was approximately 200 cm³. Each fraction was evaporated to about 30 cm³ and was transferred to a small flask, then analysed by TLC. Fractions with similar TLC were combined together. The combined fractions obtained from hexane crude extract are shown in Table 2.

Table 2 The Results of Separation of Hexane Crude Extract by Column Chromatography.

Fraction No.	Eluent (% by volume)	Remark
1-4	100% Hexane	yellow oil
5-8	100% Hexane	yellow oil
9-19	5+10% CHCl ₃ /Hexane	solid in yellow oil
20-22	20% CHCl ₃ /Hexane (Compound I)	solid in yellow oil
23-29	30% CHCl ₃ /Hexane	yellow oil
30-35	50% CHCl ₃ /Hexane	yellow oil
36-45	60% CHCl ₃ /Hexane	solid in orange oil
46-49	60% CHCl ₃ /Hexane	yellow oil
50-58	60% CHCl ₃ /Hexane (Compound II)	solid in yellow oil
59-62	80% CHCl ₃ /Hexane (compound III)	solid in yellow oil

Table 2 The Results of Separation of Hexane Crude Extract by Column Chromatography (continued).

Fraction No.	Eluent (% by volume)	Remark
63-68	90% CHCl ₃ /Hexane	solid in yellow oil
69-73	100% CHCl ₃ + 5% MeOH/CHCl ₃	solid in yellow oil
74	5% MeOH/CHCl ₃	brown oil
75-83	10% MeOH/CHCl ₃	brownish-yellow oil
84-89	20% MeOH/CHCl ₃	solid in yellow oil
90-94	30+50% MeOH/CHCl ₃	brown oil
95-108	100% MeOH	brown oil

2. Separation of Dichloromethane Crude Extract

Concentrated dichloromethane crude extract (20.30 g) was chromatographed on Silica gel 60G 70-230 mesh ASTM using column chromatography technique. The column was eluted with hexane, hexane-chloroform, chloroform, chloroform-ethyl acetate, ethyl acetate, ethyl acetate-methanol and methanol, respectively. The eluted fraction was collected about 200 cm³ and was evaporated to about 30 cm³, then analysed by TLC. Fractions with similar components were combined. The results of separation of dichloromethane crude extract are presented in Table 3.

Table 3 The Results of the Separation of Dichloromethane Crude Extract by Column Chromatography.

Fraction No.	Eluent (% by volume)	Remark
1-5	100% Hexane	yellow oil
6-16	2+5% CHCl ₃ /Hexane	solid in yellow oil
17-25	10+20% CHCl ₃ /Hexane	solid in yellow oil
26-30	20% CHCl ₃ /Hexane	solid in brown oil
31-35	30% CHCl ₃ /Hexane	greenish-yellow oil
36-49	50% CHCl ₃ /Hexane	yellowish-orange oil
50-55	75% CHCl ₃ /Hexane (Compound II)	white solid in yellow oil
56-60	75% CHCl ₃ /Hexane	white solid in yellow oil
61-65	100% CHCl ₃	yellow oil
66-72	2% EtOAc/CHCl ₃ (Compound IV)	solid in yellow oil
73-76	5% EtOAc/CHCl ₃	solid in yellow oil
77-85	10% EtOAc/CHCl ₃	yellowish-brown oil
86-98	30+50% EtOAc/CHCl ₃	orange-brown oil
99-105	70% EtOAc/CHCl ₃	solid in brown oil
106-125	70% EtOAc/CHCl ₃ (Compound V)	solid in brown oil
126-141	100% EtOAc + 1% MeOH/EtOAc	brown oil
142-144	1% MeOH/EtOAc	yellowish-brown oil
145-150	1+5% MeOH/EtOAc	brown oil
151-158	50% MeOH/EtOAc	brown oil
159-165	100% MeOH	brown tar

2.1 The separation of the eluted fraction no. 66-72 (Table 3, pp.16, 102.6 mg)

The mixture of solids in yellow oil (102.6 mg) was separated by preparative thin-layer chromatography (PTLC). The size of the plate was 20.0x20.0 cm² and the layer thickness was 0.5 mm. The PTLC plate was developed with ethyl acetate and the compound visualized by UV illumination which showed the presence of two bands. The lower band was extracted from the adsorbent by ethyl acetate and the solution was evaporated. This substance was further purified by recrystallization from hexane/ethyl acetate to obtain 8.1 mg of Compound IV.

3. Separation of Ethyl Acetate Crude Extract

The ethyl acetate crude extract was concentrated to brown resinous material of 9.25 g. This was purified by silica gel column chromatography, eluting with hexane-chloroform, chloroform, chloroform-ethyl acetate, ethyl acetate, ethyl acetate-methanol and methanol. Each fraction was collected to about 100 cm³ and were analysed by TLC and similar fractions were combined. The results of separation were presented in Table 4.

Table 4 The Results of Separation of Ethyl Acetate Crude Extract by Column Chromatography.

Fraction No.	Eluent (% by volume)	Remark
1-8	80% CHCl ₃ /Hexane	yellow oil
9-15	80% CHCl ₃ /Hexane	orange-yellow oil
16-19	80% CHCl ₃ /Hexane	brown oil
20-21	90% CHCl ₃ /Hexane	yellow oil
22-30	90% CHCl ₃ /Hexane	solid in yellow oil
31-40	100% CHCl ₃	solid in yellow oil

Table 4 The Results of Separation of Ethyl Acetate Crude Extract by Column Chromatography (continued).

Fraction No.	Eluent (% by volume)	Remark
41-44	100% CHCl ₃	yellow oil
45-53	100% CHCl ₃	orange-yellow oil
54-68	100% CHCl ₃	yellowish-brown oil
69	1% EtOAc/CHCl ₃	solid in yellow oil
70-73	1% EtOAc/CHCl ₃	orange-yellow oil
74-82	1% EtOAc/CHCl ₃ (Compound IV)	solid in yellow oil
83-100	5% EtOAc/CHCl ₃	solid in yellow oil
101-109	10% EtOAc/CHCl ₃	orange-yellow oil
110-123	10% EtOAc/CHCl ₃	orange-yellow oil
124-134	10+20% EtOAc/CHCl ₃	yellow oil
135-146	20% EtOAc/CHCl ₃	orange-yellow oil
147-151	20% EtOAc/CHCl ₃	solid in orange-yellow oil
152-158	20% EtOAc/CHCl ₃	orange-yellow oil
159-168	20+40% EtOAc/CHCl ₃	brownish-yellow oil
169-182	40+60% EtOAc/CHCl ₃	brown oil
183-203	60% EtOAc/CHCl ₃	orange-brown oil
204-212	60+100% EtOAc/CHCl ₃ (Compound V)	solid in brown oil
213-227	100% EtOAc	brown oil
228-234	5% MeOH/EtOAc	brown oil
235-240	5% MeOH/EtOAc	dark brown oil
241-266	30+70% MeOH/EtOAc	dark brown oil
267-283	100% MeOH	dark brown oil

3.1 The separation of the eluted fraction no. 74-82 (Table 4, pp.17-18, 240.1 mg)

The eluted fractions no. 74-82 contained a white precipitate in yellow oil (240.1 mg). The combined fractions were rechromatographed on a silica gel column. The fractions were initially eluted by 100% CHCl_3 , then with chloroform-methanol and methanol, 20 cm³ of each fraction being collected. Each fraction was analysed by TLC and similar fractions were combined. The results of the separation are shown in Table 5.

Table 5 The Results of Separation of the Eluted Fraction No.74-82 by Silica Gel Column Chromatography.

Fraction No.	Eluent (% by volume)	Remark
1-5	100% CHCl_3	yellow oil
6-8	100% CHCl_3	yellow oil
9-17	100% CHCl_3	white solid in yellow oil
18-24	100% CHCl_3 (Compound IV)	white solid
25	100% CHCl_3	white solid in yellow oil
26-34	100% CHCl_3	yellow oil
35-48	100% CHCl_3	yellow oil
49-58	1+5% MeOH/ CHCl_3	yellow oil
59-75	5+10% MeOH/ CHCl_3	brownish-yellow oil
76-90	30+50% MeOH/ CHCl_3	brownish-yellow oil
91-95	100% MeOH	brownish-yellow oil

4. Separation of Methanol Crude Extract

Methanol crude extract was concentrated to dark brown resinous material (133.30 g). This crude was separated into two parts by solvents, 50 % methanol/ethyl acetate and 100 % methanol. The crude obtained from 50 % methanol/ethyl acetate was purified by sephadex column chromatography. Eluting solvent was 50 % methanol/ethyl acetate. Each fraction is about 30 cm³ and was analysed by TLC and fractions with similar components were combined. The results of separation of 50 % methanol/ethyl acetate crude part are presented in Table 6. The crude obtained from methanol was purified by sephadex column chromatography. Eluting solvent was 100 % methanol. Each fraction is about 30 cm³ and was analysed by TLC and fractions with similar components were combined. The results of separation of 100% methanol crude part are presented in Table 7.

Table 6 The Results of Separation of 50 % Ethyl Acetate/Methanol Crude Part by Column Chromatography.

Fraction No.	Eluent (% by volume)	Remark
1-9	50 % MeOH/EtOAc	yellowish-brown oil
10-13	50 % MeOH/EtOAc	brown oil
14-17	50 % MeOH/EtOAc	yellowish-brown oil
18-32	50 % MeOH/EtOAc	yellowish-brown oil
33-44	50 % MeOH/EtOAc	yellow oil
45-53	50 % MeOH/EtOAc	yellowish-brown oil
54-59	50 % MeOH/EtOAc	yellowish-brown oil
60-62	50 % MeOH/EtOAc	yellow oil
63-72	50 % MeOH/EtOAc	yellowish-brown oil
73-88	50 % MeOH/EtOAc	yellow oil
89-95	50 % MeOH/EtOAc	trace

Table 7 The Results of Separation of 100 % Methanol Crude Part by Column Chromatography.

Fraction No.	Eluent (% by volume)	Remark
1-3	100 % MeOH	dark brown oil
4-6	100 % MeOH	brown oil
7-8	100 % MeOH	brown oil
9-20	100 % MeOH	yellowish-brown oil
21-24	100 % MeOH	brown oil
25-36	100 % MeOH	dark brown oil
37-49	100 % MeOH	brown oil
50-66	100 % MeOH	brown oil
67-80	100 % MeOH	yellowish-brown oil
81-91	100 % MeOH	brown tar
92-108	100% MeOH	dark brown tar

Purification and Properties of Substances from Column Chromatography.

1. Purification and properties of Compound I

The white solid in yellow oil was obtained in fraction no. 20-22 from the hexane crude extract (20 % CHCl₃/Hexane) (Table 2). It was further purified by recrystallization from hexane for several times to give Compound I as a white solid 24.1 mg (0.13 % wt. by wt. of hexane crude extract), mp. 57-58 °C. This solid was soluble in hexane, dichloromethane and chloroform and insoluble in ethyl acetate, methanol and acetone.

FT-IR spectrum (KBr) (Fig. 6) ν_{max} (cm⁻¹): 2925(s), 2860(s), 1470(m), 1380(w), 805(w) and 725(w)

Gas chromatogram (Fig. 7) (conditions: column OV-1, column temp. 250°C, injection temp. 290°C, carrier gas N₂ 50 ml/min) The chromatogram showed seven peaks at retention times 6.26, 8.01, 10.22, 13.30, 17.12, 22.19 and 28.59 min, respectively.

2. Purification and properties of Compound II

This substance was obtained from hexane crude extract in fraction no. 50-58 (60 % CHCl₃/hexane) (Table 2) and was also obtained from dichloromethane crude extract in fraction no. 50-55 (75 % CHCl₃/hexane) (Table 3) as solid in yellow oil which was recrystallized from a mixture of hexane and chloroform for several times to give white needle like crystals (998.2 mg, equivalent to 847.2 mg, 4.48 % wt. by wt. of hexane crude extract and equivalent to 151.0 mg, 0.74 % wt. by wt. of dichloromethane crude extract), mp. 152-154 °C and R_f value was 0.55 (silica gel, 5 % MeOH in CHCl₃). It was soluble in hot hexane, dichloromethane and chloroform, slightly soluble in ethyl acetate and methanol.

FT-IR spectrum (KBr) (Fig. 9) ν_{\max} (cm⁻¹) : 3100-3700(s), 2960(m), 2868(m), 1635(m), 1459(w), 1379(w), 1262(m), 1024-1092(m) and 807(m)

¹H-NMR spectrum (CDCl₃, 200 MHz) (Fig. 10) δ (ppm) : 0.66-2.26(m, 47H), 3.50 (m, 1H), 5.10(m, 1H) and 5.35(d, 1H)

¹³C-NMR spectrum (CDCl₃, 50 MHz) (Fig. 11) δ (ppm) : 11.85-56.86, 71.73(d), 121.67(d), 129.26(d), 138.31(d) and 140.75(s)

Gas chromatogram (Fig. 12) (conditions : column OV-1, column temp. 255 °C, injection temp. 290 °C, carrier gas N₂ 40 ml/min), chromatogram showed three peaks at retention times 19.13, 20.30 and 23.25 min, respectively.

Mass spectrum (Fig. 13) m/e (% relative intensity) : 414(27), 412(53), 400(23), 396(13), 382(11), 329(9), 300(29), 273(17), 271(44), 255(47), 213(30), 159(46), 105(74), 83(99) and 55(100)

3. Purification and properties of Compound III

Compound III was obtained from hexane crude extract in fractions no. 59-62 (80 % CHCl₃/hexane) (Table 2) as a mixture of white solid and yellow oil. It was further purified by recrystallization from hexane/chloroform for several times to give a white amorphous solid, 29.9 mg (0.16 % wt. by wt. of hexane crude extract). The R_f value was 0.77 (silica gel, 10 % MeOH/CHCl₃) and melting point was 73-75 °C. This compound was soluble in dichloromethane, chloroform, ethyl acetate and methanol.

FT-IR spectrum (KBr) (Fig. 14) ν_{\max} (cm⁻¹): 2800-3600(s), 2919(s), 2851(s), 1715 (m), 1471(m), 1262(w) and 721(w)

¹H-NMR spectrum (CDCl₃, 200 MHz) (Fig. 15) δ (ppm) : 0.86(t, 3H), 1.24(s), 1.61(m, 2H) and 2.33(t, 2H)

¹³C-NMR spectrum (CDCl₃, 50 MHz) (Fig. 16) δ (ppm) : 14.10(q), 22.68-29.69, 31.92(t), 33.94(t) and 179.49(s)

Gas chromatogram (Fig. 17) (conditions : column OV-1, column temp. 240 °C, injection temp. 290 °C, carrier gas N₂ 40 ml/min) The chromatogram showed 8 peaks at retention times 2.19, 3.53, 4.57, 5.93, 7.75, 10.09, 13.22 and 17.33 min, respectively.

4. Purification and Properties of Compound IV

Compound IV was obtained as the colourless needles (18.3 mg) and mp. 206-208 °C (equivalent to 8.1 mg = 0.04 % wt. by wt. of dichloromethane crude extract and equivalent to 10.2 mg = 0.11 % wt. by wt. of ethyl acetate crude extract). It was obtained from the separation by preparative TLC technique of fraction no. 66-72 of the dichloromethane crude extract (25 % ethyl acetate/chloroform) (Table 3) and re-chromatographed from ethyl acetate crude extract of fraction no. 74-82 (Table 4). The R_f value was 0.17 (silica gel, 1% MeOH/CHCl₃). This compound was soluble in dichloromethane, chloroform, ethyl acetate and methanol and insoluble in hexane.

FT-IR spectrum (KBr) (Fig. 19) $\nu_{\max}(\text{cm}^{-1})$: 3000-3600(m), 2959(s), 2927(s), 2852(m), 1714(s), 1611(m), 1568(s), 1509(m), 1458(m), 1294(m), 1263(s), 1166(m), 1141(m), 1102(s), 1021(s), 922(m), 862(m) and 804(s)

$^1\text{H-NMR}$ (CDCl_3 , 200 MHz) (Fig. 20) δ (ppm) : 3.93(s, 3H), 6.19(d, 1H), 6.82(s, 1H) 6.90(s, 1H) and 7.54(d, 1H)

$^{13}\text{C-NMR}$ spectrum (CDCl_3 , 50 MHz) (Fig. 21) δ (ppm) : 56.48(q), 103.25(d), 107.84(d), 111.52(s), 113.43(d), 142.88(d), 144.12(s), 149.90(s), 150.46(s) and 161.00(s)

Mass spectrum (Fig. 24) m/e (% relative intensity) : 192(100), 177(58), 164(22), 149(49), 125(13), 121(22), 85(20), 83(26), 79(17) and 69(29)

5. Purification and Properties of Compound V

This compound was obtained from the column chromatography of the dichloromethane crude extract in fractions no. 106-125 (70 % ethyl acetate/chloroform) (Table 3) and from the ethyl acetate crude extract in fractions no. 204-212 (60 % ethyl acetate/chloroform and 100 % ethyl acetate) (Table 4). This compound was purified by recrystallization from chloroform/methanol for several times to yield a white amorphous solid, 203.8 mg (154.1 mg = 0.82 % wt. by wt. of dichloromethane crude extract and 49.7 mg = 0.24 % wt. by wt. of ethyl acetate crude extract). This solid had mp. 271-273 °C and R_f value was 0.16 (silica gel, 10 % MeOH/ CHCl_3). Compound V was slightly soluble in chloroform, ethyl acetate and methanol and was insoluble in hexane.

FT-IR spectrum (KBr) (Fig. 26) $\nu_{\max}(\text{cm}^{-1})$: 3100-3600(s), 2936(s), 2868(s), 1651(w), 1462(m), 1371(m), 1261(m), 1025-1074(s) and 804(m)

$^1\text{H-NMR}$ spectrum (DMSO, 200 MHz) (Fig. 27) δ (ppm) : 0.64-2.51(m), 2.90(m), 3.05(m), 3.36-3.74(m), 4.04(s, 1H), 4.20(d, 1H), 4.87(d, 1H), 5.07(m, 1H) and 5.32(d, 1H)

$^{13}\text{C-NMR}$ spectrum (DMSO, 50 MHz) (Fig. 28) δ (ppm) : 11.64-56.25, 61.04(t), 70.03(d), 73.41(d), 76.71(d), 76.91(d), 100.78(d), 121.14(d), 128.79(d), 138.02(d) and 140.42(s)

Mass spectrum (Fig. 29) m/e (% relative intensity) : 414(17), 412(19), 400(8), 396(81), 394(94), 382(30), 351(10), 300(10), 255(49), 213(21), 159(29), 145(40), 95(64), 83(100), 69(74) and 55(73)



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