

CHAPTER II

EXPERIMENTAL AND RESULTS

2.1 Plant Materials

The leaves of *Excoecaria cochinchinensis* Lour. var. *viridis* Merr. or Lin Krabue were collected from Chulalongkon University , Bangkok , Thailand during the October, 1995. This specimen was identified by comparison with the herbarium No. 52675 at the Royal Forest Department of Thailand.

2.2 Equipments

2.2.1 Rotary Evaporator

Eyela rotary evaporator model N-1 was used for the rapid removal of large amounts of volatile solvents .

2.2.2 Fourie Transform - Infrared Spectrophotometer (FT-IR) and Infrared Spectrophotometer (IR)

The FT-IR were recorded on a Perkin-Elmer model 1760 x Fourie Transform Infrared Spectrophotometer and the IR spectra were recorded on a Perkin Elmer Infrared Spectrophotometer Model IR 718 . Solid samples were examined by incorporating the sample into a pellet of potassium bromide .

2.2.3 ^1H and ^{13}C -Nuclear magnetic Resonance Spectrometer

Routine ^1H -NMR and ^{13}C -NMR spectra were recorded on Bruker spectrometer Model ACF 200 operated at 200.13 MHz for ^1H and 50.26 MHz for ^{13}C -nuclei. The chemical shift (δ) in ppm were referenced to the signal from the residual proton in deuterated solvents. Assignments of carbon spectra were assisted by a Distortionless Enhancement by Polarization Transfer (DEPT) experiment. Specialized NMR experiments (COSY, NOESY, HMBC, HMQC, etc.) was performed on JEOL 500 MHz NMR spectrometer model JNM-A500 by Dr. Sathorn Suwan.

2.2.4 Gas Chromatography (GC)

The GC analysis was performed on a Shimadzu Gas Chromatograph GC-7AG

2.2.5 Gas Chromatograph - Mass Spectrometer (GC-MS)

The GC-MS analysis was performed on a Fison Gas-Liquid Chromatography Model GC 8000 coupled with a Fison Mass Spectrometer Model Trio-2000.

2.2.6 Melting Point Apparatus (m.p.)

The melting point were recorded on a Fisher - John melting point apparatus.

2.3 Solvents and Chromatographic Media

2.3.1 All solvents, except when they were reagent grade were purified by distillation.

2.3.2 Merck's silica gel 60 Art 7734.1000 (70-230 mesh ASTM) was used as adsorbents for column chromatography.

2.3.3 Merck's TLC aluminium sheets, silica gel 60 F254 pre-coated 25 sheet, 20 x 20 cm², layer thickness 0.2 mm was used for checking the fractions.

2.4 Physical Separation Techniques

2.4.1 Column Chromatography [17]

2.4.2 Thin-layer Chromatography (TLC) [18]

2.5 Extraction and Initial Fractionation .

The fresh leaves of *E. cochinchinensis* Lour. var. *viridis* Merr. (4300 g) were ground with 10 litres of methanol and soaked in methanol for 3 days at room temperature. The process was repeated for 3 times . The solution was filtered and the combined filtrate was evaporated to give the crude extract as reddish resinous material weighed 465.61 g (10.82 % wt . by wt. of the fresh leaves)

The crude extract was re-extracted by hexane until the solution was colorless . The filtered solution was concentrated under reduced pressure to afford the hexane crude extract as dark green oil of 40.80 g (Fraction I , 0.95% wt. by wt. of the fresh leaves) . After extraction with hexane , The residue was further processed by dichloromethane extraction , ethyl acetate extraction and methanol extraction, respectively in the same manner. The dichloromethane crude extract weighed 8.05 g (Fraction II, 0.19% wt. by wt. of the fresh leaves) . The ethyl acetate crude extract weighed 10.81 g and was a dark red oil (Fraction III, 0.25 wt. by wt. of the fresh leaves) . The methanol crude extract was a dark red oil weighed 378.52g (Fraction IV, 8.80 % wt. by wt. of the fresh leaves) .

The fractionation procedure using various solvent is shown in scheme 2.1 and the results of each extraction are presented in Table 2.1.

Scheme 2.1 Extraction of fresh leaves of *Excoecaria cochinchinensis* Lour.
var. *viridis* Merr.

fresh leaves of *Excoecaria cochinchinensis* Lour. var. *viridis* Merr.

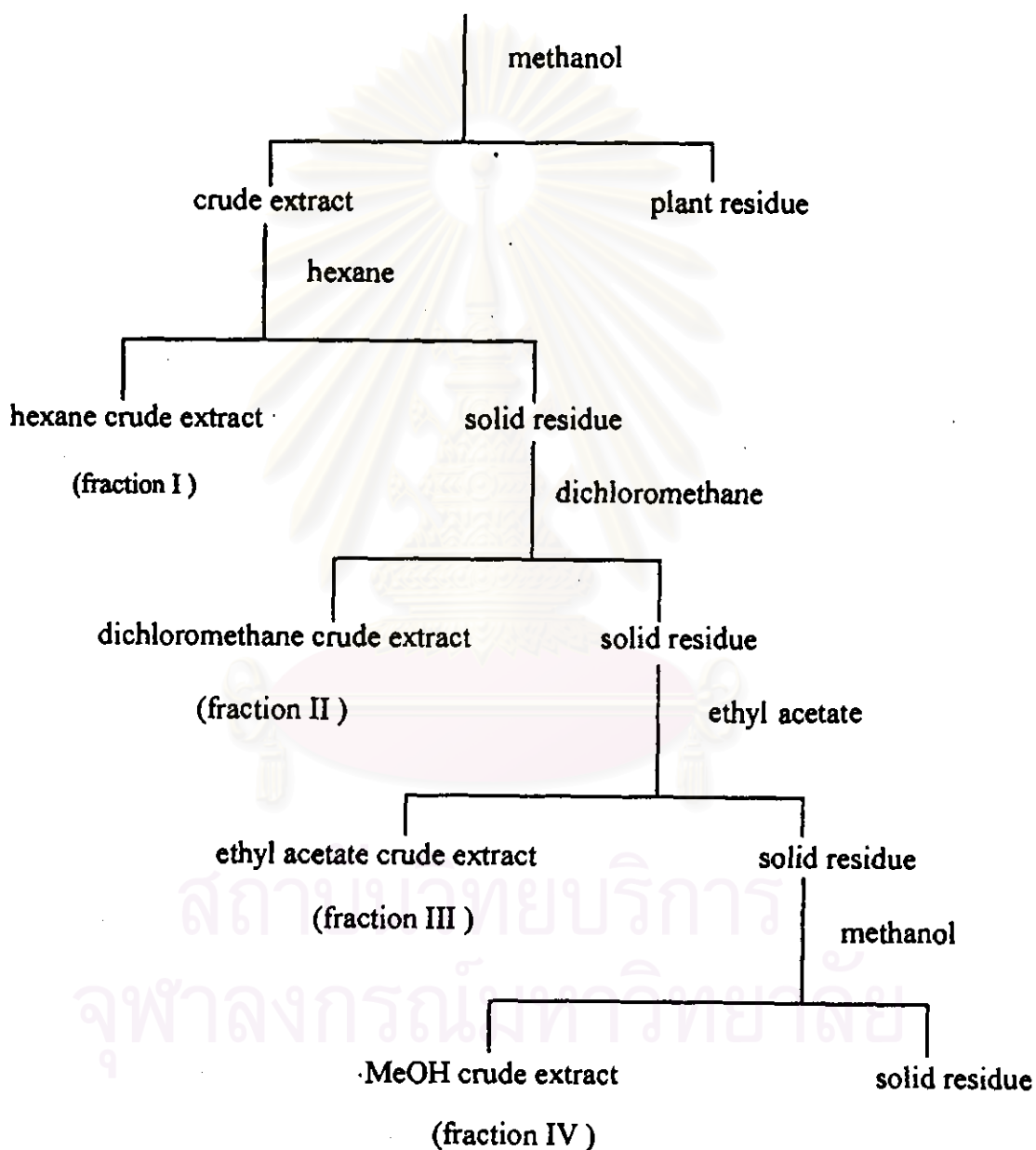


Table 2.1 The results of fresh leaves extraction of *E. cochinchinensis* Lour.*var. viridis* Merr.

Fraction	Remark	weight (g)	% wt. by wt. of fresh leaves
crude extract	reddish stricky liquid	465.61	10.82
I	dark green oil	40.80	0.95
II	dark green oil	8.05	0.19
III	dark red oil	10.81	0.25
IV	dark red oil	378.52	8.80

2.6 Isolation of the chemical constituents of *Excoecaria cochinchinensis* Lour.*var. viridis* Merr**2.6.1 Separation of Fraction I**

Hexane crude extract (fraction I, 40.0 g) was chromatographed on silica gel Art. 7734 that was used for separate fraction I into small fractions according to their polarities. The column was initially eluted with hexane and then with an increased gradient of dichloromethane in hexane, then an increased gradient of methanol in dichloromethane. The eluted solution was collected approximately 700 ml for each fractions and was evaporated to about 20 ml by rotary evaporater. Each one was monitored by using TLC and the similar fractions were combined. The results of the separation of fraction I are presented in Table 2.2 .

Table 2.2 . The results of the separation of Fraction I

Eluent	Fration No.	Remark
100% Hexane	1-5	yellow oil
10% CH ₂ Cl ₂ -Hexane	6-10	yellow oil
10-20% CH ₂ Cl ₂ -Hexane	11-15	yellow oil
20% CH ₂ Cl ₂ -Hexane	16-17	pale yellow oil
30% CH ₂ Cl ₂ -Hexane	18-23	yellow oil
40% CH ₂ Cl ₂ -Hexane	24-29	yellow-orange oil
40% CH ₂ Cl ₂ -Hexane	30-39	needle solid in orange oil
50% CH ₂ Cl ₂ -Hexane	40-49	needle solid in orange oil
50% CH ₂ Cl ₂ -Hexane	50-56	solid in orange oil
60% CH ₂ Cl ₂ -Hexane	57-63	reddish-orange oil
60% CH ₂ Cl ₂ -Hexane	64-78	dark green oil
70% CH ₂ Cl ₂ -Hexane	79-94	solid in dark green oil
80% CH ₂ Cl ₂ -Hexane	95-120	solid in dark green oil
80% CH ₂ Cl ₂ -Hexane	121-137	dark green oil
90% CH ₂ Cl ₂ -Hexane	138-165	brownish-green oil
100% CH ₂ Cl ₂	166-197	solid in dark green oil
100% CH ₂ Cl ₂	198-209	brownish-green oil
5% MeOH-CH ₂ Cl ₂	210-214	greenish-yellow oil
5-10% MeOH-CH ₂ Cl ₂	215-232	solid in yellow oil
10% MeOH-CH ₂ Cl ₂	233-236	brown oil
30% MeOH-CH ₂ Cl ₂	237-240	brown oil

2.6.2 Separation of Fraction II

Dichloromethane crude extract (Fraction II, 7.0 g) was chromatographed on silica gel Art 7734 using a column chromatography. The chromatographic separation conditions were the same as described in the separation of Fraction I. The results of separation of Fraction II are presented in Table 2.3.

Table 2.3 The results of the separation of Fraction II

Eluent	Fraction No.	Remark
20% CH ₂ Cl ₂ -Hexane	1-6	pale yellow oil
30% CH ₂ Cl ₂ -Hexane	7-13	yellow oil
40% CH ₂ Cl ₂ -Hexane	14-21	yellow oil
50% CH ₂ Cl ₂ -Hexane	22-43	yellow oil
60% CH ₂ Cl ₂ -Hexane	44-50	yellow oil
70% CH ₂ Cl ₂ -Hexane	51-57	yellow oil
80% CH ₂ Cl ₂ -Hexane	58-63	yellow oil
80-85%CH ₂ Cl ₂ -Hexane	64-89	brown oil
85% CH ₂ Cl ₂ -Hexane	90-104	brown oil
90% CH ₂ Cl ₂ -Hexane	105-124	solid in brown oil
95% CH ₂ Cl ₂ -Hexane	125-132	solid in brown oil
95% CH ₂ Cl ₂ -Hexane	133-156	solid in reddish-brown oil
100% CH ₂ Cl ₂ -Hexane	157-164	solid in reddish-brown oil
100% CH ₂ Cl ₂ -Hexane	165-185	reddish-brown oil

Table 2.3 (continued)

Eluent	Fraction No.	Remark
3% MeOH-CH ₂ Cl ₂	186-203	dark green oil
3% MeOH-CH ₂ Cl ₂	204-210	brown oil
5% MeOH-CH ₂ Cl ₂	211-214	solid in brown oil
10% MeOH-CH ₂ Cl ₂	215-220	solid in brown oil
20% MeOH-CH ₂ Cl ₂	221-236	brown oil
40% MeOH-CH ₂ Cl ₂	237-250	brown oil

2.6.3 Separation of Fraction III

Ethyl acetate crude extract (Fraction III, 10g) was chromatographed on silica gel (Art. 7734) column. The column was initially eluted by 10% ethyl acetate in hexane. The polarity of eluent was gradually increased from ethyl acetate in hexane to ethyl acetate and then to methanol in ethyl acetate. The eluted fractions were collected to approximately 200 mL for each fraction and the solvent was, then, evaporated to approximately 20 mL. Each fraction was monitored by TLC and fractions with similar composition were combined together. The results of separation of Fraction III are presented in Table 2.4.

Table 2.4 The results of separation of Fraction III

Eluent	Fraction No.	Remark
10% EtOAc-hexane	1-2	-
10% EtOAc-hexane	3-11	yellow oil
20% EtOAc-hexane	12-15	yellow oil
30% EtOAc-hexane	16-22	brown oil
30% EtOAc-hexane	23-32	solid in brownish-yellow oil
30% EtOAc-hexane	33-37	solid in yellow oil
40% EtOAc-hexane	38-47	solid in dark green oil
50% EtOAc-hexane	48-57	solid in brown oil
60% EtOAc-hexane	58-74	solid in brown oil
70% EtOAc-hexane	75-80	dark brown oil
80% EtOAc-hexane	81-86	dark brown oil
100% EtOAc-hexane	87-96	dark brown oil
5% MeOH-EtOAc	97-102	brown oil
10-40% MeOH-EtOAc	103-116	brown oil

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2.6.4 Separation of Fraction IV

Methanol crude extract (Fraction IV, 1.0 g) was chromatographed on Sephadex LH 20 column. The column was eluted with 50% methanol in dichloromethane. The eluted fractions were collected approximately 30 mL each and checked by TLC. The fraction with similar composition were combined together. The results of separation of Fraction IV are presented in Table 2.5 .

Table 2.5 The results of separation of Fraction IV

Fraction No.	Remark
1	-
2	dark brown oil
3	dark brown oil
4-6	solid in reddish-brown oil
7-9	brown oil
10	solid in pale brown oil
11-14	brown oil
15-16	brown oil

2.7 Purification and properties of the eluted compounds

2.7.1 Purification and properties of Mixture I

Mixture I was obtained as white solid in green oil from Fraction I in fraction No. 95-120 which were eluted from silica gel with 80% dichloromethane in hexane (Table 2.2). Recrystallization from dichloromethane afforded Mixture I as white amorphous solid, 47 mg (0.11×10^{-2} % wt. by wt. of fresh leaves), m.p. 72-73 °C. This mixture I was soluble in dichloromethane and insoluble in hexane .

ν_{\max} (KBr, cm^{-1}) 3500-2400 (w,b), 2930 (s), 2860 (s), 1715 (m), 1470 (m) ; GC-MS of methyl esters prepared from reactions of Mixture I with CH_2N_2 in ether [19] showed peaks at retention time 23.15 min ($m/z = 354, \text{M}^+$), 24.36 min ($m/z = 368, \text{M}^+$), 25.52 min ($m/z = 382, \text{M}^+$), 26.51 min ($m/z = 396, \text{M}^+$), 27.49 min ($m/z = 410, \text{M}^+$), 28.39 min ($m/z = 424, \text{M}^+$), 29.25 min ($m/z = 438, \text{M}^+$). (Condition of GC : column DB-1 HT 30m x 0.25mm id , injection port temperature 250 °C , carrier gas Helium with flow rate of 0.8 ml/min, temperature program 60 °C hold for 1 min, EI-MS 70eV)

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2.7.2 Purification and properties of Compound II

Compound II was obtained as white needle-like solid in orange oil from fractions 40-49 (FractionI) which was eluted from silica gel column with 50% dichloromethane in hexane. It was purified by recrystallization from hot hexane to afford compound II as colorless needles, 1.09 g (2.53×10^{-2} % wt. by wt. of fresh leaves), m.p. 136-137 °C and R_f value 0.72 (SiO₂ : 5% MeOH in CH₂Cl₂). This compound was soluble in dichloromethane , slightly soluble in hexane, ethyl acetate and insoluble in methanol. Compound II gave green color with Liebermann-Burchard's reagent.

ν_{\max} (KBr, cm⁻¹) 3690-3080 (s, br), 2940(s), 2860 (s), 1640 (w), 1460(m), 1380 (m), 1060 (w) , 800 (w) ; m/z (EI) 414 (M⁺,67%), 396(28), 381(23), 329(38), 303(47), 273(26), 255(33), 231(24), 213(44), 145(66), 107(69), 95(67), 81(79), 55 (100) ; δ_H (200 MHz, CDCl₃) 0.66-2.26 (m) , 3.47 (m) , 5.32 (d, $J = 5.1$ Hz) ; δ_C (50.26 MHz, CDCl₃) 11.84, 11.97, 18.76, 19.01, 19.38, 19.81, 21.07, 23.04, 24.29, 26.03, 28.24, 29.12, 31.63, 31.89, 33.92, 36.13, 36.48, 37.23, 39.75, 42.27, 45.81, 50.11, 56.03, 56.75, 71.79, 121.72, 140.74

GLC analysis of Compound II showed one peak at retention time at 25.76min (Condition of GLC analysis : column OV -1 , column temperature 250 °C, injection temperature 290 °C , N₂ flow rate 50 mL/min) . Mixture of standard steroid : campesterol, stigmasterol and β -sitosterol showed peaks at retention time 21.52, 22.57 and 25.66 min, respectively.

2.7.3 Purification and properties of Compound III

Compound III was obtained as solid in yellow oil from Fraction I in fraction No. 215-232 which was eluted by 5-10% methanol in dichloromethane and was obtained from Fraction II in fraction No. 211-220 eluted by the same eluent. It was purified by recrystallization from hot ethanol to afford compound III as white amorphous solid, 0.37 g (0.86×10^{-2} % wt. by wt. of fresh leaves). The melting point was 255-257 °C and the R_f value was 0.43 (SiO₂ : 10% methanol in dichloromethane). This compound was slightly soluble in methanol and insoluble in hexane.

ν_{\max} (KBr, cm⁻¹) 3400 (m, br), 2940 (s), 2860 (s), 1650 (m), 1465 (m), 1380 (m), 1055 (m), 1020 (w), 800 (w); m/z (EI) 414(M⁺, 24%), 396(42), 381(17), 329(11), 303(19), 273(11), 255(21), 231(10), 213(25), 145(56), 107(44), 95(52), 81(54), 55(100); δ_H (200 MHz, DMSO-d₆) 0.66-2.50 (m), 2.94-3.69 (m), 4.25 (d, J= 7.6), 5.32 (d, J= 5.1); δ_C (50.26 MHz, DMSO-d₆) 11.65, 11.77, 18.63, 19.04, 19.61, 20.63, 22.76, 23.83, 25.87, 27.69, 28.96, 29.32, 31.40, 31.52, 35.44, 36.26, 36.88, 38.48-41.01 (overlapped with the solvent peak), 41.92, 45.34, 49.75, 55.61, 56.25, 61.39, 70.37, 73.58, 76.70, 76.93, 77.18, 100.98, 121.02, 140.61 .

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2.7.4 Purification and properties of Compound IV

Compound IV was obtained as dark green solid in dark green oil from Fraction I in fraction No. 166-194 and from Fraction II in fraction No. 105-132 by elution from silica gel with 100% dichloromethane and 90-95% dichloromethane in hexane, respectively. After washing the dark green oil with acetone, the solid was recrystallized from dichloromethane in hexane to afford Compound IV as dark green solid, 8 mg (0.02×10^{-2} % wt. by wt. of fresh leaves), m.p. 145-147 °C, R_f value 0.30 (SiO₂ : 30% ethyl acetate in hexane). The Compound IV gave red fluorescence when irradiated by long-wave (304 nm) UV light. It was soluble in dichloromethane, slightly soluble in acetone, methanol and insoluble in hexane.

ν_{\max} (KBr, cm⁻¹) 3445(br, m), 3110(w), 2980(s), 930(s), 2850(s), 1740(s), 1730(s), 1700(s), 1640(m), 1550(m), 1460(m), 1500(m), 1300(m), 1200(m), 1160(s); δ_H (500 MHz, CDCl₃) 9.46(1H, s), 9.32(1H, s), 8.54(1H, s), 7.94(1H, dd, J=11.6, 18.0), 6.24(1H, s), 6.26(1H, dd, J=0.9, 11.7), 6.15(1H, dd, J=0.9, 11.6), 4.45(1H, m), 4.20(1H, m), 3.87(3H, s), 3.66(3H, s), 3.62(2H, m), 3.56(3H, s), 3.37(3H, s), 3.17(3H, s), 2.62(1H, m), 2.50(1H, m), 2.31(1H, m), 2.23(1H, m), 1.80(3H, d, J=7.6), 1.66(3H, t, J=7.6), 0.50(1H, br s), -1.66(1H, s); δ_C (125.6 MHz, CDCl₃) 189.6s, 173.3s, 172.2s, 169.6s, 161.2s, 155.6s, 151.0s, 149.7s, 145.2s, 142.1s, 137.9s, 136.5s, 136.2s, 136.1s, 131.8s, 129.0s, 128.9d, 128.9s, 122.8t, 105.2s, 104.4d, 97.5d, 93.1d, 64.7d, 52.8q, 51.7q, 51.1d, 31.0t, 29.9t, 23.1q, 19.4t, 17.4q, 12.1q, 12.1q, 11.2q.

2.7.5 Purification and properties of Compound V

Compound V was obtained as yellow solid in yellow brown oil from Fraction III in fraction No. 27-32 (Table 2.4) by elution from silica gel with 30 % ethyl acetate in hexane. It was purified by recrystallization from dichloromethane and methanol to afford Compound V as a pale yellow powder , 42 mg (0.10x10⁻²% wt. by wt. of fresh leaves) . It had a melting point of 264-266 °C and R_f value of 0.51 (SiO₂ : 10% methanol in dichloromethane) . This compound was soluble in methanol, ethyl acetate and acetone and slightly soluble in dichloromethane .

ν_{\max} (KBr, cm⁻¹) 3432 (s,b), 3030 (m) , 1656 (m), 1616 (s), 1571 (w), 1508 (m), 1383 (m), 1307 (m), 1178 (m), 1012 (w) ; δ_{H} (200 MHz, CD₃COCD₃) 6.24 (d,J= 2.0) , 6.51 (d,J= 2.0) , 7.02 (d,J= 9.0) , 8.11 (d,J= 9.0) , 9.13 (br), 9.75 (br) , 12.16 (s) ; δ_{C} (50.26 MHz, CD₃COCD₃) 94.43d, 99.11d, 104.09s, 116.28d (2C), 123.25s, 130.41d (2C), 136.60s, 146.98s, 157.72s, 160.15s, 162.26s, 164.96s, 176.56s ; m/z (EI) 286(M⁺, 100 %), 257(23), 229(19), 213(12), 153(14), 121 (49), 69(17) .

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2.7.6 Purification and properties of Compound VI

Compound VI was obtained as white solid in brown oil from Fraction III in fraction No. 58-74 (Table 2.4) by elution from silica gel with 60% ethyl acetate in hexane. The brown oil was removed by washing with ethyl acetate. The remaining solid was recrystallized from methanol to afford compound VI as white needle-like crystals, 87 mg (0.20×10^{-2} % wt. by wt. of fresh leaves) . It had a melting point of 237-238 °C (dec.) and R_f value was 0.63 (SiO₂ : methanol). This compound dissolved in water, methanol, ethyl acetate and acetone and insoluble in dichloromethane.

ν_{\max} (KBr, cm⁻¹) 3496-2538 (s, br), 3013 (s), 1651 (s), 1613 (s), 1542 (m), 1427 (s), 1386 (m), 1320 (s), 1269 (s), 1221 (s), 1100(w), 1040 (s) ; δ_H (200 MHz, CD₃COCD₃) 7.14 (s) ; δ_C (50.26 MHz, CD₃COCD₃) 167.90s, 145.93s (2C), 138.63s, 121.92s, 110.03s ; m/z (EI) 170(M⁺, 18 %), 153(14), 126(100), 108(24), 97(16), 80(31), 52(38) .

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2.7.7 Purification and properties of Compound VII

Compound VII was obtained as white solid in brown solution from Fraction IV in fraction No. 4-6 (Table 2.5) by elution from Sephadex LH 20 with 50% methanol in dichloromethane. The solid was purified by recrystallization from hot ethanol to afford compound VII as a white crystal, 20 mg (0.04×10^{-2} % wt. by wt. of fresh leaves). It had a melting point of 239-240 °C. Compound VII was soluble in water and insoluble in ether and others common organic solvents.

ν_{\max} (KBr, cm^{-1}) 3450(s, br), 2966(w), 1628(w), 1421(w), 1135(m), 1092(m), 1019(m); δ_{H} (200 MHz, DMSO- d_6) 4.61 (d, $J = 2.4$), 4.40 ($d, J = 2.4$), 4.28 ($d, J = 5.6$), 3.63 (s), 3.41 (s), 3.27(m); δ_{C} (50.26 MHz, DMSO- d_6) 73.10s (2C), 72.15s (2C), 70.68s (2C); m/z (EI) 180(M^+ , %), 144(2), 115(2), 102(18), 73(100), 60(27), 44(15).

2.7.8 Purification and properties of Compound VIII

Compound VIII was obtained as white solid in pale brown oil from Fraction IV in fraction No.10 (Table 2.5) by elution from Sephadex LH 20 with 50% methanol in dichloromethane. The solid was purified by recrystallization from water and methanol to afford compound VIII as white crystals, 12 mg (0.03×10^{-2} % wt. by wt. of fresh leaves) and melting point was over 300 °C. Compound VIII was soluble in water and insoluble in organic solvents. It gave yellow precipitate with sodium cobaltinitrite ($\text{Na}_3\text{Co}(\text{NO}_2)_6$), flame test gave purple-red color and with AgNO_3 test gave white precipitate.