CHAPTER III

EXPERIMENTAL

1. Source of Plant Materials

The flowering heads of *Xyris indica* Linn. were collected from Prachin Buri province, Thailand, during December, 1991. The plant material was authenticated by comparison with herbarium specimens at Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand. The voucher specimen of plant material has been deposited at the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

2. General Techniques

2.1 Thin-layer Chromatography (TLC)

Technique : one way, ascending

Adsorbent : Silica gel G (E.Merck) 30 gm/60 ml. of distilled water

Plate size : 10 cm x 20 cm and 20 x 20 cm

Layer thickness : 0.2 mm.

Activation : air dried for 15 minutes and then at 110 °c for 1 hour.

Solvent system : a) Petroleum ether : ethyl acetate (8:1)

b) Hexane : ethyl acetate (99:1)

c) Chloroform

d) Petroleum ether : ethyl acetate (5:1)

e) Benzene : ethyl acetate (15:1)

Distance : 15 cm.

Temperature : 25 - 30 °c

Detection :

a) Ultraviolet light. The compounds which contain unsaturated bonds became visible as quenching spots (254 nm) and coumarins as fluorescent blue to violet colored bands (365 nm) on TLC plate. For anthraquinones under the UV light gave orange-red colour and pink after spraying with alcoholic potash

b) Chromogenic agents

Liebermann-Burchard spray reagent (acetic anhydride-sulfuric acid)

Spray reagent :-

5 ml acetic anhydride were carefully mixed with 5 ml conc. sulfuric acid. This mixture was added cautiously to 50 ml. absolute ethanol.

Colours developed :-

Plate after spraying, was warmed in hot air oven. The colour reaction was violet or pink spot indicated the presence of steroidal compound.

2.2 Column Chromatography (CC)

Adsorbent : silica gel 0.040-0.063 mm (E.Merck)

Packing of column : Dry packing

Sample loading : A portion of crude extract was dissolved in a small amount of organic solvent and then, added slowly onto the top of a dry column.

Solvents : a) Petroleum ether : ethyl acetate (8:1)

b) Hexane : ethyl acetate (99:1)

c) Chloroform

d) Petroleum ether : ethyl acetate (5:1)

e) Benzene : ethyl acetate (15:1)

Temperature : 25 - 30 °c

Detection :

i) Liebermann-Burchard Test (For steroid or triterpenoid compound)

To dissolve 1-2 mg of a compound in 0.5 ml of chloroform, 2 drops of acetic anhydride were added and followed by 1 drop of conc. sulfuric acid. The colour changed from blue to green within a few minutes indicated the presence of steroid compound and pink-red indicated the presence of triterpenoid compound.

ii) Borntrager's Test

The fraction was extracted with chloroform. The chloroform layer was separated and shaken with potassium hydroxide solution. The alkaline layer became red when the anthraquinone was present.

2.3 Physical Constants

Melting point : melting point of the isolated compounds were determined by Gallenkamp Melting Point Apparatus and Yanaco micro-melting point apparatus.

2.4 Spectroscopy

2.4.1 Ultraviolet (UV) Absorption Spectra

Ultraviolet absorption spectra were measured on a Hitachi U-3400 Spectrophotometer.

2.4.2 Infrared (IR) Absorption Spectra

The infrared absorption spectra were obtained on a Hitachi 260-10 Infrared Spectrophotometer.

2.4.3 Nuclear Magnetic Resonance (NMR) Spectra.

The proton (^{1}H) and carbon-13 (^{13}C) NMR spectra were taken on a Jeol alpha FT NMR spectrometer 500 MHz with tetramethylsilane (TMS) as internal standards. The multiplicities for ^{13}C NMR spectra were determined by the Distortionless Enhancement by Polarization Transfer (DEPT), Heteronuclear Correlation Spectroscopy (HETCOR) and Heteronuclear Multiple Bond Connectivity (HMBC).

2.4.4 Mass Spectra

Electron impact MS (EI-MS) were measured at 70 eV with a Hitachi M-60 Mass Spectrometer (using a direct inlet system).

High Resolution Mass Spectra (HR-MS) were measured with a Hitachi RMU-7M Mass Spectrometer.

3. Extraction and Isolation

3.1 Extraction

The dried flowers of *Xyris indica* Linn. (1 kg) were extracted throughly by soxhlet apparatus, for two times (each 24 hours), with chloroform (2x10 L.) and filtered. After combination, the extracts were evaporated under reduced pressure to yield 50 grams of syrupy mass (crude XI).

3.2 Isolation

The crude chloroform extract (crude XI) was divided into 5 portions and each one was treated in the same manner. Each portion (approx. 10 g) was purified by silica gel column (2.5 x 15 cm) using Petroleum ether : ethyl acetate (8:1) as the eluent. Fifty milliliters fractions were collected and examined by thin layer chromatography (TLC). Those fractions of similar pattern were combined and evaporated to dryness as following :-

Fraction 3-19 afforded a residue A (3.247 g.)

Fraction 20-41 afforded a residue B (0.756 g.)

Fraction 42-58 afforded a residue C (0.611 g.)

Fraction 59-176 afforded a residue D (1.743 g.)

3.2.1 Residue A was rechromatographed on silica gel (2.5 x 20 cm) column using hexane : ethyl acetate (99:1) as eluent. Fraction 9-23 and 27-45 (50 ml each) were designated as XI-1 (974 mg, 0.097 %) and XI-2 (1.006 g, 0.10 %) respectively.

3.2.2 Residue B was rechromatographed on silica gel (2.5 x 26 cm) column using chloroform as eluent. Fraction 5-9 (50 ml each) was designated as XI-3 (77 mg, 0.0077 %).

3.2.3 Residue C was black syrupy mass with white crystals at the bottom. After washed several times with petroleum ether and filtered, white crystals were obtained. It was rechromatographed on silica gel (2.5 x 30 cm) column using petroleum ether : ethyl acetate (5:1) as eluent. Fraction 9-10 and 17-20 (50 ml each) were designated as XI-4 (98 mg, 0.0098 %) and XI-5 (218 mg, 0.022 %) respectively.

3.2.4 Residue D was rechromatographed on silica gel (2.5 x 20 cm) column using benzene : ethyl acetate (15:1) as eluent. Fraction 25-30 and 33-48 (50 ml each) were designated as XI-6 (431 mg, 0.043%) and XI-7 (98 mg, 0.0098 %) respectively.

4. Identification of the isolated compounds

The isolated compounds were characterized by the data of hRf values, melting points, ultraviolet absorption spetra, infrared absorption spectra, nuclear magnetic resonance spectra, mass spectra and compared with previously published data of known chemical compounds.

4.1 Identification of compound XI-1 as chrysazin

XI-1 was crystallized from n-hexane as dark orange plate. It was soluble in chloroform, hexane and petroleum ether. It gave a positive Borntrager's test. This reaction indicated that XI-1 might be an anthraquinone compound.

hRf value

The hRf values given are obtained with the following systems:-

a) silica gel G/petroleum ether : ethyl acetate (8:1) = 63

b) silica gel G/n-hexane : ethyl acetate (99:1) = 31

c) silica gel G/chloroform = 83

d) silica gel G/petroleum ether : ethyl acetate (5:1) = 55

e) silica gel G/benzene : ethyl acetate (15:1) = 75

The thin layer chromatograms of chrysazin (XI-1) are shown in figure 11-15, p.115-119.

Melting Point

188 °c (Uncorrected)

Molecular Weight

240 (Spectrometry)

UV Absorption Spectrum (EtOH) (Figure 16, p.120)

223, 250, 270, 280, 426 nm.

Infrared Absorption Spectrum (KBr) (Figure 17, p.121)

υ max (cm^{-1})

3453, 1675, 1626, 1600, 1568

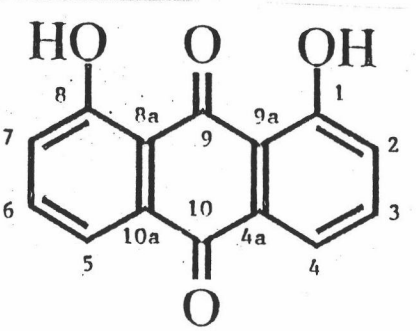
Proton NMR Spectrum (in $CDCl_3$, 500 MHz) (Figure 18-19, p.122-123)

Chemical shift (δ)	Proton	Multiplicity
11.98	1-OH	s
7.23	H-2	dd, J = 1.3, 7.8 Hz
7.62	H-3	dd, J = 7.8, 8.1 Hz
7.75	H-4	dd, J = 1.3, 7.8 Hz
7.75	H-5	dd, J = 1.3, 7.8 Hz
7.62	H-6	dd, J = 7.8, 8.1 Hz
7.23	H-7	dd, J = 1.3, 7.8 Hz
11.98	8-OH	s

Carbon-13 NMR Spectrum (in $CDCl_3$, 125.65 MHz) (Figure 20, p.124)

^{13}C-NMR chemical shifts of XI-1 (ppm) from TMS in $CDCl_3$

Carbon position	Chemical shifts (δ) ppm
1	162.50
2	124.60
3	137.23
4	120.01
4a	115.81
5	120.01
6	137.23
7	124.60
8	162.50
8a	133.55
9	193.03
9a	133.55
10	181.64
10a	115.81



XI-1

Chrysazin

Mass Spectrum (Figure 21, p.125)

m/z (% relative intensity)

240 (100), 223 (17), 212 (24), 211 (8), 184 (6), 138 (13), 128 (5), 102 (7), 63 (14)

4.2 Identification of compound XI-2 as 3-methoxy chrysazin.

XI-2 was crystallized from 25 % benzene in hexane as brilliant yellow needle. It was soluble in chloroform, hexane and petroleum ether. It gave a positive Borntrager's test. This reaction indicated that XI-2 might be an anthraquinone compound.

hRf value

The hRf values given are obtained with the following systems:-

a) silica gel G/petroleum ether : ethyl acetate (8:1) = 56

b) silica gel G/n-hexane : ethyl acetate (99:1) = 23

c) silica gel G/chloroform = 78

d) silica gel G/petroleum ether : ethyl acetate (5:1) = 49

e) silica gel G/benzene : ethyl acetate (15:1) = 69

The thin-layer chromatograms of 3-methoxy chrysazin (XI-2) are shown in figure 11-15, p.115-119.

Melting Point

180 - 183 °c (Uncorrected)

Molecular Weight

270 (Spectrometry)

UV Absorption Spectrum (EtOH) (Figure 22, p.126)

222, 245, 263, 280, 428 nm.

Infrared Absorption Spectrum (KBr) (Figure 23, p.127)

υ max (cm^{-1})

3442, 1675, 1627, 1609, 1595, 1576, 1560

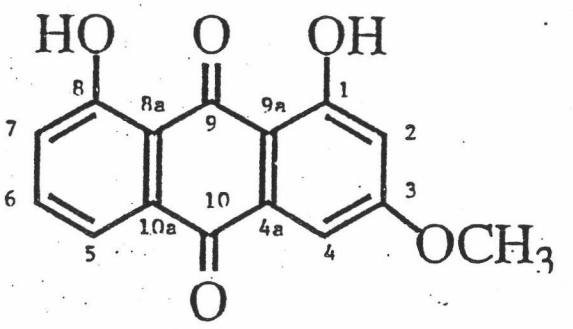
Proton NMR Spectrum (in $CDCl_3$, 500 MHz) (Figure 24-25, p.128-129)

Chemical shift (δ)	Proton	Multiplicity
12.19	1-OH	bs
6.61	H-2	d, J = 2.5 Hz
3.87	OCH_3	s
7.30	H-4	d, J = 2.5 Hz
7.73	H-5	dd, J = 7.6, 1.2 Hz
7.56	H-6	dd, J = 7.3, 2.5 Hz
7.21	H-7	dd, J = 7.6, 1.2 Hz
12.11	8-OH	bs

Mass Spectrum (Figure 26, p.130)

m/z (% relative intensity)

271 (65), 270 (100), 269 (24), 253 (6), 242 (12), 241 (36), 240 (22), 227 (11), 214 (4), 213 (8), 212 (14), 199 (11), 184 (14), 171 (15), 121 (11), 115 (12), 69 (12), 63 (11)



XI-2

3-Methoxychrysazin

4.3 Identification of compound XI-3 as xyridin A

XI-3 was crystallized from ethanol as yellow plate. It was soluble in chloroform, hexane and methanol.

hRf value

The hRf values given are obtained with the following systems:-

a) silica gel G/petroleum ether : ethyl acetate (8:1) = 33

b) silica gel G/n-hexane : ethyl acetate (99:1) = 15

c) silica gel G/chloroform = 57

d) silica gel G/petroleum ether : ethyl acetate (5:1) = 33

e) silica gel G/benzene : ethyl acetate (15:1) = 47

The thin-layer chromatograms of xyridin A (XI-3) are shown in figure 11-15, p.115-119.

Melting Point

68 - 70 °c (Uncorrected)

Molecular Weight

232 (Spectrometry)

UV Absorption Spectrum (MeOH) (Figure 27, p.131)

λ max (ε) = 203 (18337), 239 (39974), 282 (5892), 334 (4478)

Infrared Absorption Spectrum (KBr) (Figure 28, p.132)

υ max (cm^{-1})

3090, 2960, 2925, 2865, 1725, 1662, 1595, 1503, 1484, 1412, 1358, 1261, 1110, 1031, 1015, 981, 920, 848

Proton NMR Spectrum (in $CDCl_3$, 500 MHz) (Figure 29-30, p.133-134)

Chemical shift (δ) ppm	Proton	Multiplicity
6.09	H-4	s
6.66	H-5	s
7.51	H-8	s
6.03	$-O-CH_2-O-$	s
2.42	H-1′	t, J = 7.3 Hz
1.66	H-2′	tq, J = 7.3 Hz, 7.3 Hz
0.93	H-3′	t, J = 7.3 Hz

Carbon-13 NMR Spectrum (in $CDCl_3$, 125.65 MHz) (Figure 31, p.135)

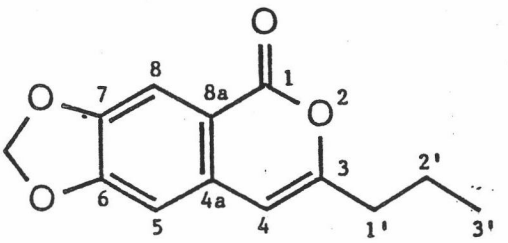
^{13}C-NMR chemical shifts of XI-3 (ppm) from TMS in $CDCl_3$

Carbon position	Chemical shifts (δ) ppm
1	162.63
3	156.99
4	102.96
4a	114.48
5	103.50
6	147.72
7	153.51
8	107.19
8a	135.19
$-O-CH_2-O-$	102.02
$1'$	35.16
$2'$	20.18
$3'$	13.40

Mass Spectrum (HREIMS) (Figure 36, p.140)

m/z (% relative intensity)

233 (16), 232 (100), 203 (41), 175 (23), 133 (10)



XI-3

Xyridin A

4.4 Identification of compound XI-4 as stigmasterol.

XI-4 was crystallized from petroleum ether as white needle. It was soluble in chloroform and hexane. It gave a violet colour with Liebermann-Burchard test. This colour reaction indicated that XI-4 might be a steroid compound.

hRf value

The hRf values given are obtained with the following systems:-

a) silica gel G/petroleum ether : ethyl acetate (8:1) = 26

b) silica gel G/n-hexane : ethyl acetate (99:1) = 13

c) silica gel G/chloroform = 36

d) silica gel G/petroleum ether : ethyl acetate (5:1) = 25

e) silica gel G/benzene : ethyl acetate (15:1) = 43

The thin-layer chromatograms of stigmasterol (XI-4) are shown in figure 11-15, p.115-119.

Melting Point

169 °c (Uncorrected)

Molecular Weight

412 (Spectrometry)

Infrared Absorption Spectrum (KBr) (Figure 37, p.141)

υ max (cm^{-1})

3433, 2936, 2867, 1625, 1462, 1381, 1060 and 970

Proton NMR Spectrum (in $CDCl_3$, 500 MHz) (Figure 38-39, p.142-143)

Chemical shift (δ) ppm	Proton	Multiplicity
0.68	Me-18	s
0.78	Me-29	t
0.82	Me-26,27	d
0.92	Me-21	d
1.01	Me-19	s
3.5	OH-3	m
4.98	H-22	dd (J = 15.3,8.5 Hz)
5.14	H-23	dd (J = 15.3,8.5 Hz)
5.33	H-6	br d

Carbon-13 NMR Spectrum (in $CDCl_3$, 125.65 MHz) (Figure 40-41,p.144-145)

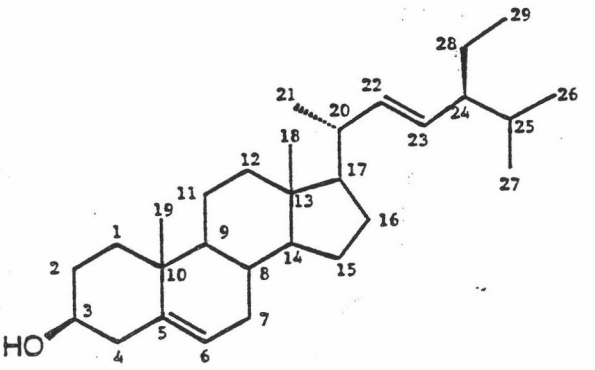
^{13}C-NMR chemical shifts of XI-4 (ppm) from TMS in $CDCl_3$

Carbon position	Chemical shifts (δ) ppm	Carbon position	Chemical shifts (δ) ppm
1	37.23	16	28.9
2	31.61	17	55.92
3	71.77	18	12.02
4	42.19	19	19.37
5	140.73	20	40.47
6	121.69	21	21.06
7	31.87	22	138.30
8	31.87	23	129.24
9	50.13	24	51.21
10	36.48	25	31.87
11	21.06	26	21.19
12	39.65	27	18.96
13	42.25	28	25.38
14	56.84	29	12.23
15	24.34		

Mass Spectrum (Figure 42, p.146)

m/z (% relative intensity)

412 (23), 394 (3), 379 (2), 271 (33), 253 (7), 229 (8), 211 (2), 159 (26), 147 (23), 135 (18), 119 (11), 105 (40), 97 (47), 83 (100), 81 (73), 67 (34)



XI-4

Stigmasterol

4.5 Identification of compound XI-5 as α -spinasterol.

XI-5 was crystallized from petroleum ether as white needle. It was soluble in chloroform and hexane. It gave a pink colour with Liebermann-Burchard test. This colour reaction indicated that XI-5 might be a steroid compound.

hRf value

The hRf values given are obtained with the following systems:-

a) silica gel G/petroleum ether : ethyl acetate (8:1) = 23

b) silica gel G/n-hexane : ethyl acetate (99:1) = 9

c) silica gel G/chloroform = 31

d) silica gel G/petroleum ether : ethyl acetate (5:1) = 19

e) silica gel G/benzene : ethyl acetate (15:1) = 37

The thin-layer chromatograms of α -spinasterol (XI-5) are shown in figure 11-15, p.115-119.

Melting Point

170 °c (Uncorrected)

Molecular Weight

412 (Spectrometry)

Infrared Absorption Spectrum (KBr) (Figure 43, p.147)

υ max (cm^{-1})

3436, 2957, 2870, 1664, 1447, 1382, 1041 and 971

Proton NMR Spectrum (in $CDCl_3$,500 MHz) (Figure 44-45, p.148-149)

Chemical shift (δ) ppm	Proton	Multiplicity
0.77	Me-18	s
0.78	Me-29	t
0.89	Me-26,27	d
0.91	Me-21	d
1.01	Me-19	s
3.5	OH-3	m
5.01	H-23	dd (J = 15.4,8.5 Hz)
5.13	H-22	dd (J = 15.2,8.5 Hz)
5.13	H-7	br d

Carbon-13 NMR Spectrum (in $CDCl_3$, 125.65 MHz) (Figure 46-47,p.150-151)

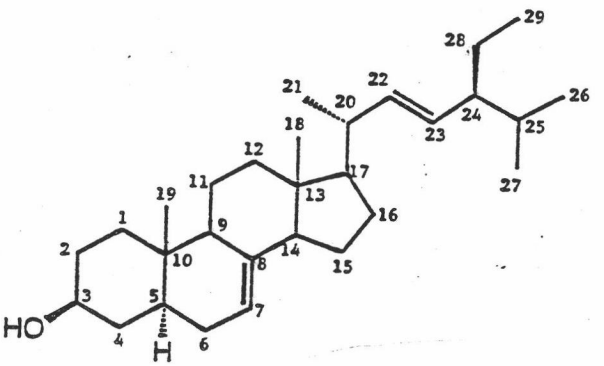
^{13}C-NMR chemical shifts of XI-5 (ppm) from TMS in $CDCl_3$

Carbon position	Chemical shifts (δ) ppm	Carbon position	Chemical shifts (δ) ppm
1	37.16	16	28.48
2	31.48	17	55.93
3	71.05	18	12.04
4	37.99	19	13.02
5	40.28	20	40.79
6	29.65	21	21.06
7	117.46	22	138.14
8	139.55	23	129.47
9	49.47	24	51.25
10	34.23	25	31.86
11	21.55	26	21.36
12	39.48	27	18.99
13	43.29	28	25.38
14	55.13	29	12.22
15	23.01		

Mass Spectrum (Figure 48, p.152)

m/z (% relative intensity)

414 (11), 412 (9), 397 (4), 369 (3), 300 (4), 272 (8), 271 (11), 255 (14), 213 (8), 181 (9), 147 (10), 133 (9), 121 (11), 107 (16), 95 (13), 81 (28), 79 (18), 69 (14), 55 (11)



XI-5

α -Spinasterol

4.6 Identification of compound XI-6 as xyridin B

XI-6 was crystallized from hexane as a colourless plate. It was soluble in chloroform, ethyl acetate and methanol.

hRf value

The hRf values given are obtained with the following systems:-

a) silica gel G/petroleum ether : ethyl acetate (8:1) = 13

b) silica gel G/n-hexane : ethyl acetate (99:1) = 5

c) silica gel G/chloroform = 10

d) silica gel G/petroleum ether : ethyl acetate (5:1) = 11

e) silica gel G/benzene : ethyl acetate (15:1) = 22

The thin-layer chromatograms of xyridin B (XI-6) are shown in figure 11-15, p.115-119.

Melting Point

199 - 200 °c (Uncorrected)

Molecular Weight

246 (Spectrometry)

UV Absorption Spectrum (MeOH) (Figure 49, p.153)

λ max (ε) = 227 (12238), 262 (36808), 333 (14293)

Infrared Absorption Spectrum (KBr) (Figure 50, p.154)

υ max (cm^{-1})

3080, 3055, 2980, 2925, 1733, 1695, 1632, 1613, 1505, 1490, 1412, 1346, 1275, 1243, 1192, 1648, 1015, 943, 908

Proton NMR Spectrum (in $CDCl_3$, 500 MHz) (Figure 51-52, p.155-156)

Chemical shift (δ) ppm	Proton	Multiplicity
7.24	H-4	s
6.92	H-5	s
7.62	H-8	s
6.11	$-O-CH_2-O-$	s
2.92	H-2′	q (J = 7.3 Hz)
1.14	Me-3′	t (J = 7.3 Hz)

Carbon-13 NMR Spectrum (in $CDCl_3$, 125.65 MHz) (Figure 53, p.157)

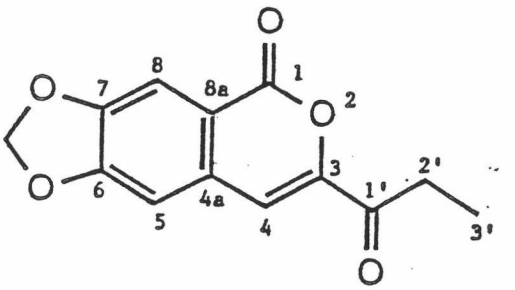
^{13}C-NMR chemical shifts of XI-6 (ppm) from TMS in $CDCl_3$

Carbon position	Chemical shifts (δ) ppm
1	160.42
3	148.68
4	108.52
4a	118.39
5	106.32
6	150.34
7	153.86
8	108.09
8a	132.57
$-O-CH_2-O-$	102.71
$1'$	194.91
$2'$	31.63
$3'$	7.33

Mass Spectrum (HREIMS) (Figure 58, p.162)

m/z (% relative intensity)

247 (15), 246 (100), 189 (92), 161 (37), 133 (28)



XI-6

Xyridin B

4.7 Identification of compound XI-7 as 1,3,8-trihydroxy-9,10-anthraquinone.

XI-7 was crystallized from methanol as orange cluster. It was soluble in ethyl acetate, methanol and ethanol.

hRf value

The hRf values given are obtained with the following systems:-

a) silica gel G/petroleum ether : ethyl acetate (8:1) = 9

b) silica gel G/n-hexane : ethyl acetate (99:1) = 3

c) silica gel G/chloroform = 6

d) silica gel G/petroleum ether : ethyl acetate (5:1) = 7

e) silica gel G/benzene : ethyl acetate (15:1) = 13

The thin-layer chromatograms of 1,3,8-trihydroxy-9,10-anthraquinone are shown in figure 11-15, p.115-119.

Melting Point

282°c (Uncorrected)

Molecular Weight

256 (Spectrometry)

UV Absorption Spectrum (MeOH) (Figure 59, p.163)

λ max (ε) = 218 (29119), 245 (16443), 266 (16512), 284 (18534)

Infrared Absorption Spectrum (KBr) (Figure 60, p.164)

υ max (cm^{-1})

3385, 1675, 1621, 1578, 1477, 1217 and 1022

Proton NMR Spectrum (in DMSO-d_6, 500 MHz) (Figure 61-62,p.165-166)

Chemical shift (δ) ppm	Proton	Multiplicity
12.03	1-OH	s
6.58	H-2	d, J = 2.5 Hz
-	3-OH	-
7.11	H-4	d, J = 2.5 Hz
7.65	H-5	dd, J = 8.5,1.2 Hz
7.74	H-6	dd, J = 8.5,1.2 Hz
7.33	H-7	dd, J = 8.5,1.2 Hz
12.07	8-OH	s

Carbon-13 NMR Spectrum (in DMSO-d_6, 125.65 MHz) (Figure 63,p.167)

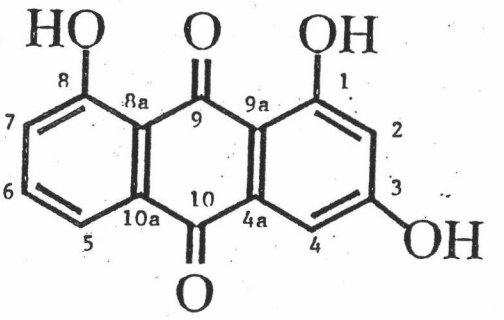
^{13}C-NMR chemical shifts of XI-7 (ppm) in DMSO-d_6

Carbon position	Chemical shifts (δ) ppm
1	164.53
2	107.94
3	165.76
4	108.84
4a	109.07
5	119.29
6	136.76
7	124.47
8	161.24
8a	115.60
9	190.07
9a	135.13
10	181.29
10a	133.12

Mass Spectrum (Figure 67, p.171)

m/z (% relative intensity)

256 (100), 255 (2), 239 (4), 229 (3), 228 (20), 200 (14), 171 (7), 155 (2), 154 (4), 126 (4), 115 (6), 114 (4), 69 (6), 63 (4), 57 (4)



XI-7

1,3,8-trihydroxy-9,10-anthraquinone