CHAPTER II

EXPERIMENTS AND RESULTS

2.1 Plant Materials

Root bark of *Harrisonia perforata* Merr. were collected at Kladkaw district, Chonburi province, Thailand during the November 1992 and dried by sun light then milled to coarse powder. Specimen collection was no. T-16695.

2.2 Instruments and Equipments

2.2.1 Rotary Evaporator

The Eyela Model N-1 Rotary Evaporator was used for rapid removal of a large quantity of volatile solvent.

2.2.2 Fourier Transform Infrared Spectrophotometer (FT-IR)

The FT-IR spectra were recorded on a Perkin-Elmer Model 1760X Fourier Transform Infrared Spectrophotometer and calibrated with a polystyrene film. Solid samples were examined by incorporating the sample into a pellet of potassium bromide.

2.2.3 Melting Point Apparatus

Melting points were determined on a Fisher-John melting point apparatus and were uncorrected.

2.2.4 ^{1}H- and ^{13}C- Nuclear Magnetic Resonance Spectrometer

The ^{1}H-NMR and ^{13}C-NMR spectra were obtained by using a Bruker Model ACF 200 Spectrometer operated at 200.13 MHz. for ^{1}H and 50.32 MHz. for ^{13}C-nuclei. The chemical shifts (δ) were provided in ppm. down field from the TMS.

2.2.5 Mass Spectrometry (MS)

The mass spectra were obtained by a Fisons Instruments Mass spectrometer Model Trio 2000.

2.3 Chemical Reagents

2.3.1 All solvents used in this research were purified prior to use by distillation, except reagent grade solvents.

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

2.3.3 Merck's silica gel 60G Art. 7731 was used as adsorbent for quick column chromatography.

2.3.4 Merck's TLC aluminium sheets silica gel 60 F254 pre-coated 25 sheet 20 x 20 cm., layer 0.2 mm., was used for identifying the identical fractions.

2.4 Physical Separation Techniques

Separation of compounds from the extracts were performed with various procedures and techniques. Separation techniques used include :

- Quick Column Chromatography [20]

- Column Chromatography (CC) [21]

- Thin-Layer Chromatography [22, 23]

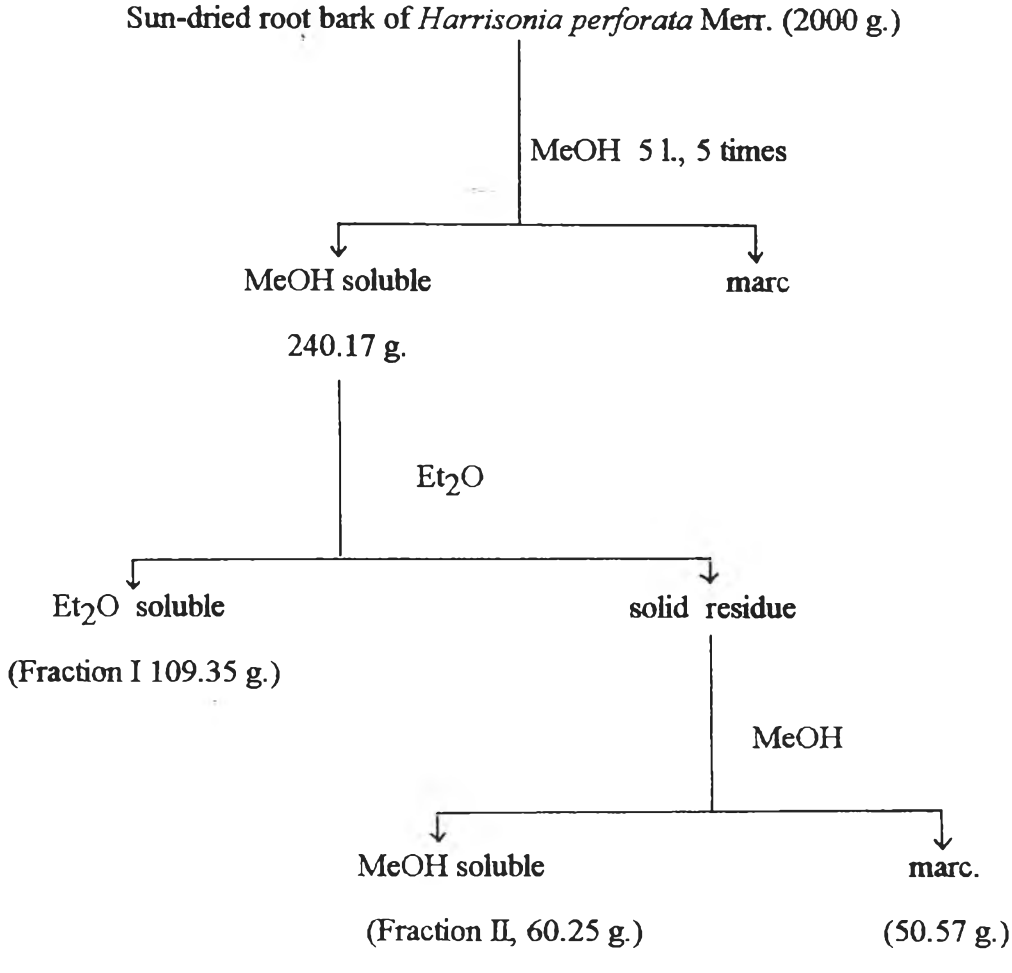
2.5 Extraction

The root bark of *Harrisonia perforata* Merr. (2000 g.) was separated and sun-dried for 5 days. The dried material was soaked at room temperature for 3-4 days before it was regrounded again. These grounding and soaking procedures were repeated 3 more times. After fractionation and solvent evaporation under reduced pressure, 240.17 g. (12.01 % wt. by wt.) of methanolic extract was obtained as a dark-red residue. This methanolic extract was repeatedly extracted by diethyl ether until the solution was clear and the filtered solution was evaporated to afford 109.35 g. (5.47 % wt. by wt.) of the crude (Fraction I) as a thick brown oil.

The diethyl ether insoluble part was resuspended in methanol repeatedly until the solution was clear. The methanol soluble part was collected and concentrated by rotatory evaporator under reduced pressure to afford 80.25 g. (4.01 % wt. by wt.) of methanolic extract (Fraction II) as a dark-red solid and 50.57 g. (2.53 % wt. by wt.) methanol insoluble part.

The extraction procedures are shown in Scheme 1.

Scheme 1 **Extraction of the root bark of *Harrisonia perforata* Merr.**



2.6 Isolation of the Chemical Constituents from the Root Bark of *Harrisonia perforata* Merr.

2.6.1 Fractionation of Fraction I

Quick column chromatography technique was used to separate the diethyl ether crude into various fractions.

Silica gel 60G Art. 7731 (adsorbent) was packed to the height of about 4 cm. (~300 g.). and 15.2 g. of diethyl ether crude was applied. The column was eluted with 200 ml. of hexane, hexane-diethyl ether, diethyl ether, and diethyl ether-methanol, respectively. Each eluent fraction, was then concentrated by rotary evaporator to a volume of about 50 ml.. Each fraction was analyzed by TLC. Fractions with identical TLC pattern were then combined and physical appearance of each combined fraction was determined. Fractions obtained from this procedure were presented in Table 4.

Table 4 Silica gel quick column chromatography of Fraction I

Eluent	Fraction No.	Remark
100 % hexane	1-2	-
	3-4	green-yellow oil (PA-1)
	5-8	yellow oil
5% Et_2O-hexane	9-16	yellow oil
	17-24	yellow needle solid in yellow oil
10% Et_2O-hexane	25-30	orange oil
	31-35	white ppt. in pale yellow oil (PA-2)
20% Et_2O-hexane	36-39	yellow oil
	40-45	white ppt. in yellow oil (PA-3)
30% Et_2O-hexane	46-52	yellow oil
	53-58	brown oil
	59-62	yellow oil
40% Et_2O-hexane	63-67	yellow oil
	68-70	orange oil
	71-78	white ppt. in pale yellow oil (PA-4)
	79-84	yellow oil
60% Et_2O-hexane	85-88	yellow oil
	89-94	white ppt. in pale yellow oil (PA-5)
	95-99	yellow needle solid in pale yellow oil
	100-108	yellow solid in pale yellow oil
	109-112	yellow oil
80% Et_2O-hexane	113-117	orange oil
100% Et_2O-hexane	118-122	red brown oil
5% MeOH-Et_2O	123-125	brown oil
10% MeOH-Et_2O	126-130	yellow oil
25% MeOH-Et_2O	131-135	yellow oil
50% MeOH-Et_2O	136-140	yellow oil

2.6.2 Fractionation of Fraction II

Quick column chromatography technique was used to separate 30.2 g. of the crude methanol into many fractions. Silica gel 60G Art. 7731 as adsorbent using 400 g. This column was initially eluted by 50% chloroform in n-hexane and collected about 600 $ml/_{fraction}$. Each fraction was concentrated to about 50 ml. They were further concentrated to 10 ml. with the water bath and checked by TLC plate. The fractions containing similar components were combined together. The results of the separation of the methanol extract by quick column chromatography was shown in Table 5.

Table 5 Silica gel quick column chromatography of Fraction II

Eluent	Fraction No.	Remark
50 % $CHCl_3$-hexane	1-3	yellow oil
75 % $CHCl_3$-hexane	4-8	yellow oil
$CHCl_3$	9-10	yellow oil
	11-15	brown-reddish oil
5 % MeOH-$CHCl_3$	16-20	brown-reddish oil
	21-24	yellow oil
10 % MeOH-$CHCl_3$	25-32	brown-reddish oil
20 % MeOH-$CHCl_3$	33-35	brown-reddish oil
	36-39	yellow oil
30 % MeOH-$CHCl_3$	40-41	yellow oil
	42-46	square solid in pale yellow oil
	47-51	yellow oil
40 % MeOH-$CHCl_3$	52-56	yellow oil
50 % MeOH-$CHCl_3$	57-63	brown tar
75 % MeOH-$CHCl_3$	64-70	brown tar
MeOH	71-80	brown tar

2.7 Purification and Properties of the Eluted Compounds by Column Chromatography

2.7.1 Purification and properties of PA-1

PA-1 was a mixture of green-yellowish oil (0.68 g.), which was obtained from the diethyl ether extract and eluted by 100 % hexane in fraction no. 3-4 from silica gel column chromatography. This oily mixture dissolved in hexane, diethyl ether, chloroform, ethanol and methanol.

FT-IR spectrum (neat) ν_{max} (cm^{-1}) 3078(w), 2960, 2929, 2863(s), 1759(s), 1672(s), 1547, 1453(m), 1377(s), 1188(m), 1028, 993(m), (Fig. 2)

The PA-1 was analyzed by GLC-MS spectrometer to identify its chemical constituents. GLC technique was performed on a DB-1 column. The conditions of GLC were as follows.

injection temperature	: 250 ^{o}C	oven temperature	: 70-260 ^{o}C
rate of heating	: 3 ^{o}C/min.	flow rate	: 50 cm^3/ min

The GC-MS spectrum was shown in Figure 3. Retention times of each component were 12.72(0.62 %), 16.09(0.38 %), 18.11(2.20 %), 18.42(2.96 %), 20.22(13.92 %), 21.48(1.90 %), 21.77(4.20 %), 22.21(1.83 %), 22.58(1.80 %), 22.71(0.95 %), 23.88(1.18 %), 25.26(2.92 %), 25.63(0.46 %), 25.88(1.82 %), 26.49(1.21 %), 27.00(2.33 %), 29.20(4.99 %), 33.36(0.92 %), 34.13(3.31 %), 35.40(10.37 %), 37.38(1.54 %), 41.06(12.78 %), 45.83(1.11 %), 46.16(4.98 %), 46.93(1.47 %), 47.08(7.30 %), and 50.91(1.82 %) min., respectively.

2.7.2 Purification and properties of PA-2

PA-2 was obtained from the diethyl ether part which was separated on the silica gel column with a mixture of *n*-hexane and diethyl ether as eluting solvent. The obtained compound was recrystallized from methanol several times to give bright white needle crystals 30 mg. (0.20 % wt. by wt. of diethyl ether crude) with m.p. 130-132 ^{o}C. The R_f value of this compound was 0.50 using 5 % methanol-chloroform as a developing solvent. It was solubled in chloroform, acetone, ethyl acetate, ether, hot methanol and slightly soluble in hexane. It gave a deep green color with Libermann Burchard's reagent.

FT-IR spectrum (KBr) ν_{max} (cm^{-1}) 3443-3341(br,s), 2959, 2937, 2868(s), 1641(m), 1464(s), 1381(s)and 1059, 1023(s) and 802(s). (Fig. 31)

^{1}H-NMR spectrum ($CDCl_3$) δ (ppm.) 0.68-1.98, 2.26, 3.50, 5.09, 5.35 (Fig. 32)

^{13}C-NMR spectrum ($CDCl_3$) δ (ppm.) 11.35-57.13, 71.91, 71.51, 121.61, 129.21, 138.22, 140.68 (Fig. 33)

EI MS spectrum m/e 414.0, 412.0 and 400 (calcd. for $C_{29}H_{50}O$, $C_{29}H_{48}O$ and $C_{28}H_{48}O$, respectively) 396.0, 381, 329.0, 303.0, 273.0, 255.0, 210 (Fig. 34)

The GLC analysis (Fig. 35) indicated three peaks on the chromatogram at retention times shown in Table 6..

Table 6 The retention times of various peaks from the gas chromatogram of PA-2 compare with steroid standards.

Compound	Retention time (mins)	Peak area
choresterol	5.73	
campesterol	6.79	
stigmasterol	7.27	
β-sitosterol	7.94	
Compound(II)	6.9	10.1 %
	7.38	46.6 %
	8.14	43.3 %

(column DB-1, col. temp. 290 ^{o}C, inj. temp. 250 ^{o}C, Mass detector , He flow rate 50 cm^3/sec.)

2.7.3 Purification and properties of PA-3

PA-3 was isolated from diethyl ether part which was separated on the silica gel column with a mixture of *n*-hexane and diethyl ether as eluting solvent. The obtained compound was recrystallized from 50 % methanol-chloroform for several times to give a white amorphous solid 40 mg. (0.26 % wt. by wt. of diethyl ether crude) with m.p. 153-154 ^{o}C. It was solubled in chloroform, acetone, ethyl acetate, ether, methanol.

FT-IR spectrum (KBr) ν_{max} (cm^{-1}) 3446(br,s), 3152, 3076(w), 2986, 2953, 2903(m), 1742, 1718(s), 1631(m), 1267, 1211, 1185(s) and 876(s). (Fig. 36)

^{1}H-NMR spectrum ($CDCl_3$) δ (ppm.) 1.16, 1.19, 1.28, 1.37, 1.50, 1.56, 1.65-1.95, 3.00, 3.61, 3.79, 4.29, 5.06, 5.67, 5.79, 5.98, 6.02, 6.33, 7.40, 7.42 (Fig. 37)

^{13}C-NMR spectrum ($CDCl_3$) δ (ppm.) 14.6, 15.1, 17.2, 18.2, 24.0, 26.2, 27.3, 39.4, 46.7, 49.5, 49.8, 51.9, 57.2, 68.5, 78.3, 80.8, 88.5, 108.2, 109.8, 120.9, 123.0, 141.0, 142.9, 153.8, 166.6, 167.7, 216.6 (Fig. 38)

DEPT-135 ^{13}C-NMR ($CDCl_3$) δ (ppm.) (Fig. 39)

CH_3, CH signal (up phase) 14.6, 17.2, 18.2, 24.0, 27.3, 46.7, 51.9, 57.2, 68.5, 78.3, 109.8, 123.0, 141.0, 142.9, 153.8

CH_2 signal (down phase) 15.1, 26.2

DEPT-90 ^{13}C-NMR ($CDCl_3$) δ (ppm.) (Fig. 39)

CH signal 49.8, 57.2, 78.3, 109.8, 123.0, 141.0, 142.9, 153.8

CH-correlation, COSY and NOESY (Fig. 40, 41, 42)

CI MS spectrum m/e 516, 498, 470 (Fig. 43)

EI MS spectrum m/e 516, 498, 472, 467, 430, 398, 393, 375, 347, 306, 291, 259, 313, 287, 273, 255 (Fig. 44)

2.7.4 Purification and properties of PA-4

PA-4 was collected from the diethyl ether crude in fraction no. 71-78 which was eluted by 40 % diethyl ether in hexane by column chromatography. The precipitate was purified by recrystallization from methanol. The white amorphous solid was designated as PA4 33.5 g. (0.22 % wt. by wt. of diethyl ether crude) with m.p. 292-295 ^{o}C. Rf value was 0.40 in 20 % methanol in chloroform system. This compound was soluble in methanol and ethanol and slightly soluble in chloroform , diethyl ether but insoluble in hexane. It gave positive test (green-blue colour) to Liebermann-Burchard's reagent.

FT-IR spectrum (KBr) ν_{max} (cm^{-1}) 3475(br,s), 2946(br,s), 1682(s), 1540, 1457, 1436(s), 1375(s),1232, 1201, 1169, 1143(m), 1011(m), 743(m). (Fig. 45)

^{1}H-NMR spectrum ($CDCl_3$) δ (ppm.) 0.56, 0.70, 0.72, 0.74, 0.76, 0.80, 0.97, 1.04-2.99 (Fig. 46)

^{13}C-NMR spectrum ($CDCl_3$ + DMSO) δ (ppm.) 15.3, 17.1, 18.8, 19.4, 20.2, 21.7, 24.7, 27.1, 27.5, 27.7, 29.4, 29.8, 30.1, 30.5, 30.8, 32.3, 34.1, 34.8, 36.6, 36.9, 37.1, 38.4, 39.6, 41.4, 44.4, 50.2, 78.0, 133.5, 133.7, 180.4 (Fig. 47)

DEPT-135 ^{13}C-NMR ($CDCl_3$ + DMSO) δ (ppm.) (Fig. 48)

CH_2, CH signal (up phase) 15.3, 17.1, 19.4, 21.7, 27.7, 30.8, 32.3, 44.4, 50.2, 78.0

CH_2 signal (down phase) 18.8, 20.2, 24.7, 27.1, 27.5, 29.4, 39.8, 30.1, 34.1, 34.8, 36.6

DEPT-90 ^{13}C-NMR ($CDCl_3$+ DMSO) δ (ppm.) (Fig. 48)

CH signal 44.4, 50.2, 78.0

COSY (Fig. 49)

CI MS spectrum m/e 456, 438 (Fig. 50)

EI MS spectrum m/e 456, 441, 423, 410, 395, 377, 331, 259, 247, 229, 273, 255 (Fig. 51)

2.7.5 Purification and properties of PA-5

PA-5 was obtained from diethyl ether part in fraction no. 89-99 which was eluted by 60 % diethyl ether in hexane by column chromatography. After recrystallization from ethanol for several times, the white amorphous solid was obtained (125.7 mg., 0.83 % wt. by wt. of diethyl ether crude). Its melting point was 222-223°C and R_f value was 0.35 in 30 % methanol in chloroform system. This compound was readily soluble in chloroform, ethyl acetate, hot methanol and hot ethanol, but slightly soluble in hexane, ethanol, methanol and ether.

FT-IR spectrum (KBr) ν_{max} (cm^{-1}) 3658-3423(br,m), 3210(m), 3134(m), 3041(m), 2974, 2950, 2889(s), 1745, 1700(br,s) 1626(m), 1536, 1506, 1455, 1422(m), 1394, 1372(s), 1343(m) 1283, 1221, 1164(s), 1118, 1071, 1027, 993(s), 917, 894(s), 875(s), 822, 803(s) (Fig. 52)

^{1}H-NMR spectrum ($CDCl_3$) δ (ppm.) 1.10, 1.22, 1.43, 1.49, 1.86, 2.10, 2.26, 2.58, 2.97, 3.63, 5.44, 5.93, 6.34, 6.50, 7.38, (Fig. 53)

^{13}C-NMR spectrum ($CDCl_3$) δ (ppm.) 16.4, 16.9, 19.4, 21.0, 26.7, 31.9, 32.7, 37.4, 39.8, 43.1, 49.1, 52.9, 53.2, 57.2, 65.0, 77.9, 83.9, 109.7, 120.0, 122.9, 140.9, 143.1, 156.7, 166.6, 166.8 and 207.4 (Fig. 54)

DEPT-135 ^{13}C-NMR ($CDCl_3$) δ (ppm.) (Fig. 55)

CH_3, CH signal (up phase) 16.4, 16.9, 21.0, 26.7, 31.9, 49.1, 53.2, 57.2, 77.9, 109.7, 122.9, 140.9, 143.1, 156.7

CH_2 signal (down phase) 19.4, 32.7, 39.8

DEPT-90 ^{13}C-NMR ($CDCl_3$) δ (ppm.) (Fig. 55)

CH signal 49.1, 53.2, 57.2, 77.9, 109.7, 122.9, 140.9, 143.1, 156.7

CH-correlation and COSY (Fig. 56, 57)

CI MS spectrum m/e 454 (Fig. 58)

EI MS spectrum m/e 454, 439, 397, 375, 331, 313, 287, 273, 255 (Fig. 59)