CHAPTER III

EXPERIMENTAL

1. Source of Plant Materials

The stems of *Myriopteron extensum* Schum. (Periplocaceae) were collected from Pakthongchai, Nakorn-Ratchasima province, Thailand in May, 1987 and authenticated by comparison with the herbarium specimens at Botany Section , Technical Division , Department of Agriculture , Ministry of Agriculture and Cooperatives, Thailand.

2. General Techniques

2.1 Thin-Layer Chromatography (TLC)

Technique	:	One way, ascending
Adsorbent	:	Silica gel GF_{254} (E Merck), 30 g/60 ml of distilled water
Plate size	:	5 x 20 cm, 10 x 20 cm, 20 x 20 cm
Layer thickness	:	0.25 mm
Activation	:	Air-dried for 15 minutes and then at 110 C for 1 hour
Solvent system	:	a) Chloroform : Methanol (98:2)
		b) Chloroform : Acetone (9:1)
		c) Hexane : Ethyl Acetate (4:1)
		d) Hexane : Acetone (1:1)
		e) Petroleum Ether : Acetone (7:3)
Distance	:	15 cm

Laboratory tempemperature : 25 - 30°C

Detection on chromatographic plate:

a) Ultraviolet light at wavelength 254 nm

Most compounds which contain unsaturated bonds become visible as quenching spots under UV light at 254 nm.

b) Iodine vapour

Compounds containing unsaturated bonds gave brown spots with iodine vapour.

c) Vanillin - HCl spraying reagent

(5% Vanillin in ethanol with concentrated hydrochloric acid in the ratio 4:1, just prior to use.)

Red or purplish-red spots are produced by catechins and proanthocyanidins immediately on spraying and warming (hairdryer), and by flavanones and dihydroflavonols more slowly.

2.2 Column Chromatography (CC)

Absorbent : Silica gel 60 (0.04 - 0.063 mm) (E Merck)

Packing of column : Dry packing

Sample loading : A portion of crude extract was dissolved in a small amount of volatile solvent, mixed with a small quantity of adsorbent, air dried, triturated and add to the top of a column.

Solvent : a) Increasing 10% (0-90%) chloroform in hexane

b) Increasing 5% (0-15%) methanol in chloroform

Examination of eluate: Fractions were examined by thin layer chromatography using UV light and iodine vapour. Those fractions of similar patterns were combined together.

2.3 Physical constant

2.3.1 Melting Point

Melting point was determined on the Gallenkamp Melting Point Apparatus with digital thermometer Model MFB-595 (uncorrected).

2.4 Spectroscopy

2.4.1 Ultraviolet (UV) - Visible Absorption Spectroscopy

The UV absorption spectrum was obtained with a Hitachi U 3400 spectrophotometer.

2.4.2 Infrared (IR) Absorption Spectroscopy

The IR absorption spectrum was obtained with a Shimadzu model IR 440 spectrophotometer.

2.4.3 Nuclear Magnetic Resornance (NMR) Spectroscopy

a) Proton (^{1}H) spectra were obtained by operating at 90, 400 and 500 MHz with a Jeol FX-90 Spectrometer , a Jeol GSX-400 Spectrometer and a Jeol GSX-500 spectrometer respectively.

b) Carbon (^{13}C) NMR spectrum was obtained by operating at 100 MHz with a Jeol GSX-400 spectrometer.

c) The homonuclear COSY spectrum was obtained by operating at 400 MHz with a Jeol GSX-400 spectrometer.

d) The heteronuclear HETCOR spectrum was obtained by operating at 100 MHz with a Jeol GSX-400 spectrometer.

e) The selective INEPT spectra were obtained by operating at 100 MHz with a Jeol GSX-400 spectrometer.

f) The two dimension NOESY sprectrum was obtained by operating at 400 MHz with a Jeol GSX-400 spectrometer.

All spectra were taken by using Tetramethylsilane (TMS) as an internal standard and Deuterochloroform as a solvent. Chemical shifts were reported as δ value (ppm).

2.4.4 Mass Spectroscopy (MS)

The mass spectrum was recorded on a mass spectrometer Model DX 300 (Jeol) operating at 70 eV.

3. Extraction and Isolation of Chemical Substance from the Stems of *Myriopteron extensum* Schum.

3.1 Extraction

The dried,coarsely powdered stems of *Myriopteron extensum* Schum. (600 g) were extracted by percolation with 95% ethanol (10 liters). The percolate was evaporated under reduced pressure to yielded a residue (79 g). The residue was treated with water (3 liters), followed by extraction with hexane (500 x 3 ml) . The combined hexane extract was dried with anhydrous sodium sulfate and evaporated under reduced pressure to dryness to give a crude hexane extract (14.2 g). The aqueous layer was then extracted with chloroform (500 x 3 ml) . The combined chloroform extract was dried by the same process as the hexane extract to give a crude chloroform extract (20.8 g).

3.2 Isolation of Chemical Substance

The chloroform extract (20.8 g) was divided into 4 portions. Each portion (5.2 g) was chromatographed over a silica gel column. Mixture of hexane and chloroform along with chloroform and methanol in various ratio (see table 3.1) were used as eluents. Fractions of 25 ml were collected and examined by thin layer chromatography (TLC). Those

fractions of similar patterns were combined together as shown in table 3.1.

Table 3.1

Fraction	Eluent component	Remarks
1-23	hexane : chloroform (100:0, 90:10,...to 30:70)	colourless to pale-yellow soln.
24-40	hexane : chloroform (20:80, 10:90) chloroform : methanol (100:0, 95:5)	yellow solution
41-50	chloroform : methanol (90:10, 85:15, 0:100)	reddish brown to brown solution

The fractions 24-40 gave interesting spots on TLC after treated with vanillin-HCl spraying reagent. The combined fraction was evaporated under reduced pressure to dryness to afford a crude mixture (2.7 g) which was rechromatographed over a silica gel column. The same eluents as above were used. The fractions which showed that interesting spot as a major spot on TLC were combined together and then evaporated to dryness, dissolved in small amount of chloroform and hexane was added dropwise to yield yellow needle crystals (1.39 g), designated as ME-1.

4. Identification of the Isolated Compound

ME-1 was obtained as yellow needle crystals. It is soluble in diethyl ether, ethyl acetate and chloroform.

4.1 hRf Value

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

a) Chloroform:methanol (98:2) = 45

b) Chloroform:acetone (9:1) = 48

c) Hexane:ethyl acetate (4:1) = 28

d) Hexane:acetone (1:1) = 64

e) Petroleum ether:acetone (7:3) = 43

(Figures 3.1-3.5, page 88-92)

4.2 Colour Reaction

a) Shinoda test

A few crystals of ME-1 were dissolved in two drops of ethanol, added with Mg powder and then a drop of 5 M. HCl . View against a white background . ME-1 gave a red colour with this test.

b) Reaction with alcoholic $FeCl_3$

ME-1 gave a dark green colour with alcoholic $FeCl_3$. Treatment of ME -1 with formic acid gave compounds which still responded to this reaction.

4.3 Melting Point

121 -123 °C

4.4 Molecular weight

406

4.5 Ultraviolet (UV) - Visible Absorption Spectrum

225 sh, 268 sh, 275, 300 sh, 314 and 364 nm

(Figure 3.6, page 93)

4.6 Infrared (IR) Absorption Spectrum

3250,2980, 2925, 1650, 1620, 1600 cm

(Figure 3.7, page 94)

4.7 Nuclear Magnetic Resonance (NMR) spectra

a) Proton (^{1}H) NMR Spectrum (in $CDCl_3$, 500 MHz)

(Figure 3.8, page 95) See table 3.2

b) Carbon-13 (C^{13}) NMR Spectrum (in $CDCl_3$, 400 MHz)

(Figure 3.15,page 102) See table 3.3

Table 3.2 ^{1}H-NMR assignment of ME-1

H Position	δ (ppm)	Multiplicity	J (Hz)
2	5.33	dd	12.7, 3.0
3-cis	2.79	dd	17.0, 3.0
3-trans	3.03	dd	17.0,12.7
5-OH	12.24	s	
2',6'	7.33 (2H)	d	8.5
3',5'	6.87 (2H)	d	8.5
4' OH	4.85	s	
2"-CH_3	1.43 (3H)	s	
	1.45 (3H)	s	
3"	5.50	d	10.1
4"	6.63	d	10.1
1"'	3.20 (2H)	d	7.4
2"'	5.14	t	7.4
3"' -CH_3	1.65 (6H)	s	

Table 3.3 ^{13}C-NMR Assignment of ME-1

Carbon position	Chemical shift (δ ,ppm)
2	78.52
3	43.23
4	196.51
5	155.86
6	102.66*
7	159.36*
8	108.65
9	159.90*
10	102.84*
1'	131.05
2'	127.72
3'	115.52
4'	156.58
5'	115.52
6'	127.72
2"	78.14
3"	126.00
4"	115.65
2"- CH_3	28.30
2"- CH_3	28.40
1"'	21.48
2"'	122.48
3"'	131.11
3"' - CH_3 (E-CH_3)	17.84
3"' - CH_3 (Z-CH_3)	25.82

* This value may be interchanged.

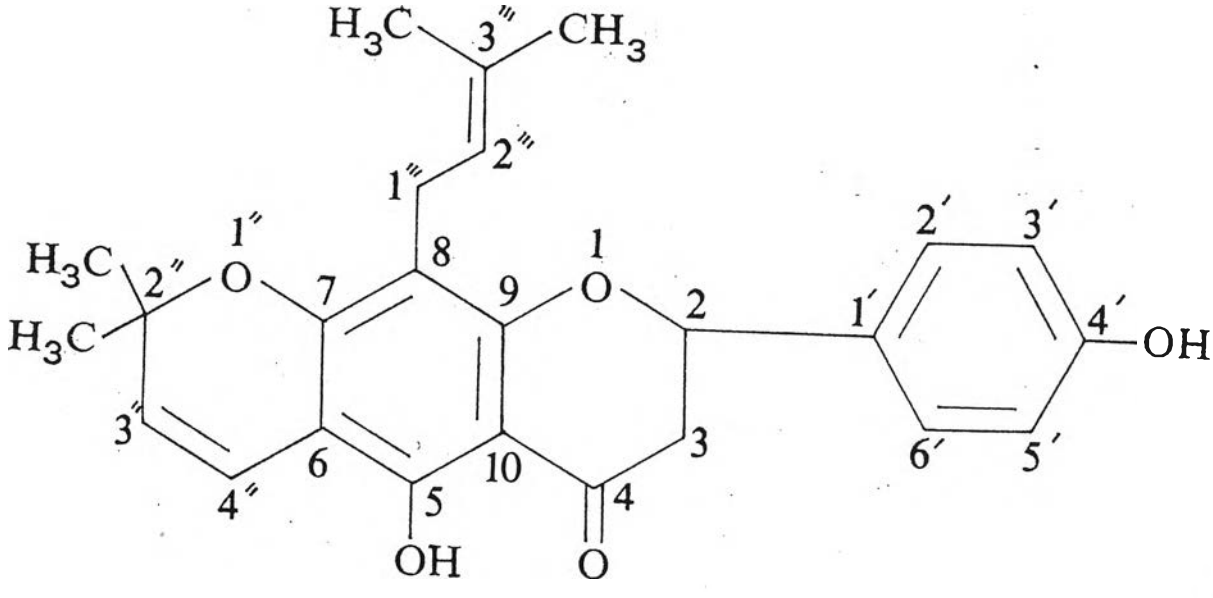
4.8 Mass Spectrum (EIMS)

m/z (% relative intensity)

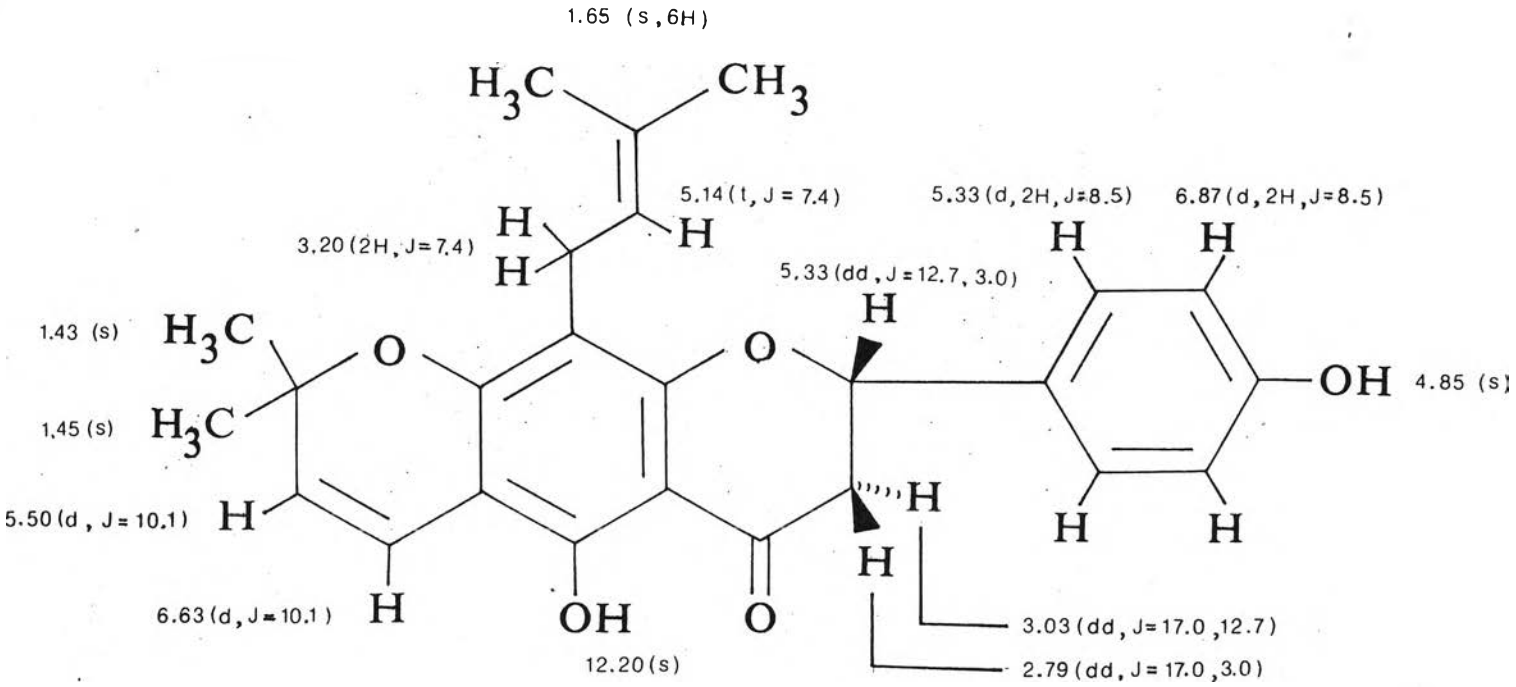
406 (M^+, 67.73), 391(100), 363(7.49), 351(7.60), 335 (7.18), 285 (7.99), 271 (35.45),243 (17.95), 215 (59.10), and 120 (4.23).

(Figure 3.18, page 105)

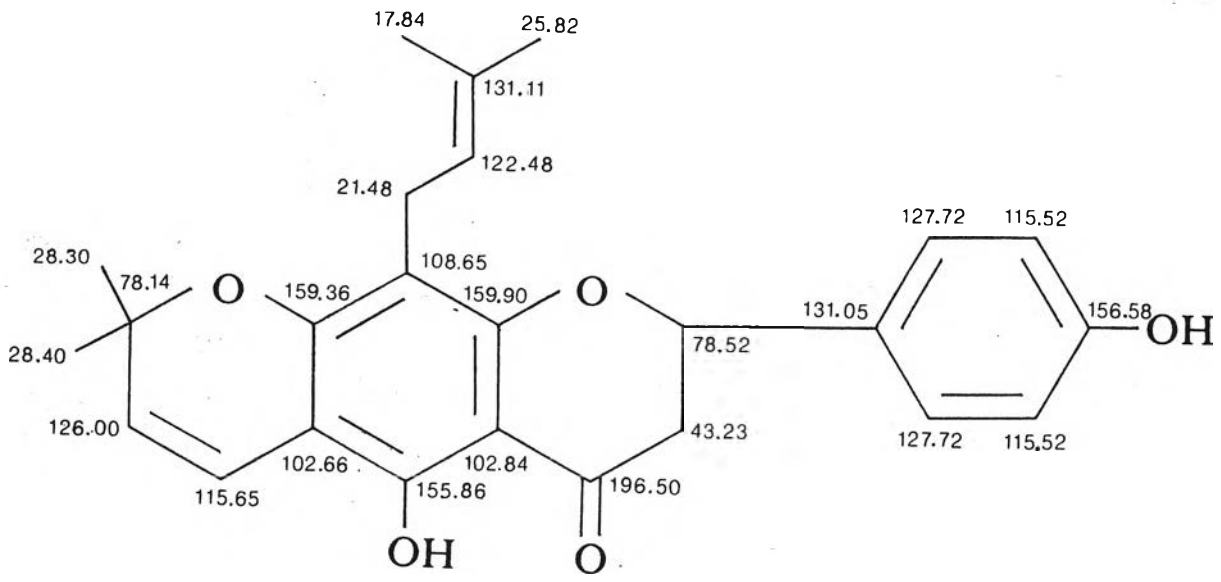
These data are in agreement with the published values of lupinifolin, the known flavonoid isolated from *Tephrosia lupinifolia* Burch (DC) of family Leguminosae (Smalberger, Vleggar and Weber,1974). It is therefore concluded that ME-1 is lupinifolin.



LUPINIFOLIN



^{1}H-NMR assignment of ME-1



^{13}C-NMR assignment of ME-1

5. Identification of the sugar in the aqueous portion

5.1 Osazone formation

Place the sample in a test tube, add 0.4 g of phenylhydrazine hydrochloride, 0.6 g of crystalline sodium acetate, and 4 ml of distilled water. Stopper the test-tube with one-holed cork, and place in a beaker of boiling water. It is necessary to shake the tube occasionally to avoid supersaturation. After 20 minutes, remove the tube from the hot water bath and set aside to cool. Pour a small amount of the liquid and crystals on a watch glass. Tip the watch glass from side to side to spread out the crystals, and absorb some of the mother liquor with a piece of filter paper, taking care not to crush or break up the clumps of crystals.

5.2 Infrared spectroscopy

Infrared absorption spectrum was recorded on a Shimadzu model IR 440 spectrophotometer.