CHAPTER III

EXPERIMENTAL

1. Source of plant material

The whole plant of *Nepenthes thorelii* Lec. was collected from Chumporn province, Thailand in June, 1994. The plant material was authenticated by comparison with herbarium specimens in the Bangkok Herbarium, Botanical Sub-Division, Botany and Weed Science Division, Department of Agriculture, Ministry of Agriculture and Co-operatives.

2. General techniques

2.1 Thin-layer Chromatography (TLC)

Technique: One way, ascending

Adsorbent: Silica gel 60 F_{254} (E. Merck) precoated plate

Layer thickness: 0. 2 mm

Distance: 5 cm

Detection: Ultraviolet light at the wavelengths of 254 and 365 nm

2.2 Column Chromatography

Adsorbent: Silica gel 60 (No.7734) particle size 0.063-0.200 nm (E. Merck)

Silica gel 60 (No.9385) particle size 0.040-0.063 nm (E. Merck)

Packing method: Wet packing

Sample loading: The sample was dissolved in a small amount of an organic solvent then added gently on the column.

Examination of eluates: Fractions were examined by TLC observing under ultra-violet light at wavelengths of 254 and 365 nm. Fractions of similar pattern were combined.

2.3 Gel filtration chromatography

Gel filter: Sephadex LH 20 (Pharmacia)

Packing method: The gel was suspended in a mixture of $CHCl_3$ and MeOH (1:1) for 24 hours then packed in the column until settled tightly.

Sample loading: The sample was dissolved in a small amount of organic solvent then added gently on the column.

Examination of eluates: Fractions were examined by TLC using visual detection under ultra-violet light at wavelengths of 254 and 365 nm. Fractions of similar pattern were combinned.

2.4 Ion-exchange chromatography

Packing material: Dowex AX 8 200-400 mesh (Cl^--form) anion exchange

Packing method: The gel was suspended in purified water and then packed in a column. 1% NaOH solution was passed through the column until the eluate became basic. The column was washed with purified water until loss of basicity, and then purged with MeOH.

Sample loading: The sample was dissolved in MeOH then added on the column and eluted with MeOH.

2.5 Spectroscopy

2.5.1 Ultraviolet visible (UV) absorption spectra

UV spectra were obtained on a Milton Roy Spectronic 3000 Array spectrometer (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.5.2 Infrared (IR) absorption spectra

IR spectra were obtained from a Perkin Elmer FT-IR spectrometer 1760x (Scientific and Technological Reserch Equipment Center, Chulalongkorn University) in potassium bromide disc and as a solution in chloroform.

2.5.3 Mass spectra (MS)

Electron Impact Mass spectra (EIMS) were obtained with a Finnigan MAT Incos 50 mass spectrometer, operating at 70 eV (Department of Chemistry, Faculty of Science, Mahidol University) or a Kratos Profile mass spectrometer (Chemistry section, Scientific service division, Department of Science) or a Varian Saturn GC MS/MS (Scientific and Technological Research Equipment Center, Chulalongkorn University).

2.5.4 Proton and carbon-13 nuclear magnetic resonance ^{1}H and ^{13}C NMR spectra

500 MHz ^{1}H NMR spectra and 125 MHz ^{13}C NMR spectra were obtained with a JEOL JMN-A 500 spectrometer (Scientific and Technological Research Equipment Center, Chulalongkorn University).

Solvents for NMR spectra were acetone-d_6, chloroform-*d*, pyridine-d_5. Chemical shifts were reported in ppm scale using the chemical shift of tetramethylsilane (TMS) at 0 ppm as the reference signal.

2.6 Solvents

Throughout this work, all organic solvents were of commercial grade and were redistilled prior to use.

3. Extraction procedure

The dried coarsely powdered roots of *Nepenthes thorelii* Lec. (1.3 kg) were repeatedly macerated for four 3-day periods with 95% ethanol and then filtered. The filtrate from each maceration was evaporated under reduced pressure at temperature not exceeding 45 °C to yield 54.1 g of crude extract.

The alcohol extract was suspended in water (200 ml) and then partitioned with chloroform (5x1L). The chloroform fraction was evaporated to dryness under reduced pressure to give 18 g of brown syrupy mass. The syrupy mass was further partitioned between petroleum ether and methanol. The petroleum ether and

methanol fractions were separately evaporated to dryness under reduced pressure to yield dried residues of 9.70 g and 7.78 g, respectively.

The water extract was partitioned with butanol. Removal of the solvents yielded 3.50 g of a water extract and 17.80 g of a butanol extract.

4. Isolation procedure

4.1 Isolation of compound 18

The petroleum ether extract (9.70 g) was dissolved in a small amount of petroleum ether and added gently onto a silica gel column (2.5x20 cm). The eluents were used in the order as shown below :

Petroleum ether 200 ml

Petroleum ether : ethyl aceate 95:5 200 ml fractions 1-4

Petrolumn ether : ethyl aceate 90:10 200 ml fractions 5-8

Petroleum ether : ethyl aceate 85:15 200 ml fractions 9-12

Petrolumn ether : ethyl aceate 80:20 200 ml fractions 13-16

Petroleum ether : ethyl aceate 75:25 200 ml fractions 17-20

Petrolumn ether : ethyl aceate 70:30 200 ml fractions 21-24

Petroleum ether : ethyl aceate 65:35 200 ml fractions 25-28

Petrolumn ether : ethyl aceate 60:40 200 ml fractions 29-32

Petroleum ether : ethyl aceate 55:45 200 ml fractions 33-36

Petrolumn ether : ethyl aceate 50:50 200 ml fractions 37-40

Petroleum ether : ethyl aceate 45:55 200 ml fractions 41-44

Petrolumn ether : ethyl aceate 40:60 200 ml fractions 45-48

Table 3 The combined fractions from crude petroleum ether extract

Number of eluates	Fraction	Weight (g)
1 - 2	C - 1	0. 0177
3 - 10	C - 2	0. 0830
11 - 12	C - 3	0. 5011
13	C - 4	0. 2717
14 - 15	C - 5	3. 2220
16 - 17	C - 6	1. 9194
18 - 19	C - 7	0. 2448
20 - 21	C - 8	0.8800
22 - 35	C - 9	1. 3281
36 - 39	C - 10	0. 7897
40 - 41	C - 11	0. 1484
42 - 58	C - 12	0. 0578
59 - 63	C - 13	0. 0633
64 - 76	C - 14	0. 0526
77 - 81	C - 15	0.0538
82 - 92	C - 16	3. 3862

Petroleum ether : ethyl aceate 35:65 200 ml fractions 49-52

Petrolumn ether : ethyl aceate 30:70 200 ml fractions 53-56

Petroleum ether : ethyl aceate 25:75 200 ml fractions 57-60

Petrolumn ether : ethyl aceate 20:80 200 ml fractions 61-64

Petroleum ether : ethyl aceate 15:85 200 ml fractions 65-68

Petrolumn ether : ethyl aceate 10:90 200 ml fractions 69-72

Petroleum ether : ethyl aceate 5 :95 200 ml fractions 73-76

Ethyl aceate 100 200 ml fractions 77-80

Methanol : ethyl aceate 20:80 200 ml fractions 81-84

Methanol : ethyl aceate 60:40 200 ml fractions 85-88

Methanol : ethyl aceate 80:20 200 ml fractions 89-92

Methanol was used to wash the column until the eluates were diluted and clear compared to the previous ones. The eluates were examined by TLC using 20% ethyl acetate in petroleum ether and 70% ethyl acetate in petroleum ether as developing solvents. Fractions with similar chromatographic pattern were combined as shown in Table 3. Fraction C-5 showed one yellow spot on TLC. It was recrystallized from petroleum ether/acetone to give 3.2220 g (0.24% based on the dried weight of *N. thorelii*) of compound **18** as orange needles. This compound was identified as plumbagin (**18**).

4.2 Isolation of compound 19

Fraction C-12 (0.0578 g) was dissolved in a small amount of petroleum ether and acetone mixture and loaded on a silical gel-60 column (2x52 cm). The column was eluted with mixtures of petroleum ether : ethyl acetate in a polarity gradient manner. Twenty-ml fractions were collected and combined after examination with TLC, using 20% acetone in petroleum ether. Fractions 28-38 gave one spot on TLC. They were combined and further purified by a siliga gel 60 column, using 25% acetone in petroleum ether as the eluent. Ten-ml fractions were collected and combined after examination with TLC using 30% acetone in petroleum ether as the developing solvent. Compound **19** was obtained as orange crystals from the column, after removal of the solvent. The yield was 0.0070 g (0.00053% based on the dried weight of *N. thorelii*). This compound was identified as droserone (**19**).

4.3 Isolation of compound 102

Fraction C-9 (1.3281 g) was divided into 2 portions. Each portion was fractionated by column chromatography using a column of silica gel 60 (2.5x52 cm) with $CHCl_3$: ethyl acetate gradient elution. Twenty-ml fractions were collected. The eluates were examined by TLC, and fractions showing the same pattern were combined.

Fractions 30-32 showed one spot (other fractions showed another single spot which was later purified according to the procedure described in section 4.3). This was further purified by preparative thin-layer chromatography technique

using silica gel 60 F_{254} (E. Merck) precoated plate (20x10 cm), petroleum ether : ethyl acetate (85:15) as the developing solvent. Compound **102** was obtained as a yellow semi-solid from this method. The yield was 0.0143 g (0.0011% based on the dried weight of *N. thorelii*). This compound was identified as isoshinanolone (**102**).

4.4 Isolation of compound 103

The other fractions (21-28), appearing homogeneous on TLC of C-9 was purified using a silica gel column (2x52 cm) with chloroform : ethyl acetate gradient elution. Twenty-ml fractions were collected and combined after examination with TLC, using 20% ethyl acetate in petroleum ether. Fractions 25-60 (0.1192 g) were combined and further purified by preparative TLC technique with silica gel 60 F_{254} (E. Merck) precoated plates (20x10 cm), using 20% ethyl acetate in petroleum ether as the developing solvent. Compound **103** was obtained by this method as a white powder (0.0250 g, 0.0019% based on the dried weight of *N. thorelii*). The structure of this compound was established as octadecyl caffeate (**103**).

4.5 Isolation of compound 104

Fraction C-6 (1.9194 g) was dissolved in a small amount of chloroform. It was fractionated by a column of sephadex LH 20 (1x80 cm). Twenty-ml fractions were collected and combined after examination with TLC using 10% acetone in petroleum ether and 5% ethyl acetate in toluene as the developing solvents. Fractions 5-7 (0.0111 g) was further purified by preparative TLC using

5% ethyl acetate in toluene as the developing solvent. This method gave compound **104** as an orange solid compound (0.0020 g, 0.00015% based on the dried weight of *N. thorelii*). This compound was identified as 2-methylnaphthazarin (**104**).

5. Chemical transformations of plumbagin (18)

5.1 Acetylation of plumbagin (18)

Plumbagin (18) 100 mg was dissolved in 1 ml of pyridine and 2 ml acetic anhydride was added. The mixture was stirred overnight and then extracted with chloroform (3x10 ml). The chloroform fraction was dried over sodium sulphate (Na_2SO_4). After evaporation to dryness, it gave compound **105** (0.076 g, 76% based on plumbagin weight).

5.2 Methylation of plumbagin (18)

To a solution of plumbagin (18) (0.750 g) in chloroform (10 ml), silver (I) oxide (3 g) and iodomethane (15 ml) were added. The mixture was stirred overnight at the room temperature. The reaction mixture was filtered and the filtrate was evaporated under reduced pressure to give 5-methoxyplumbagin (**106**) as yellow needles (0.737 g, 97% based on plumbagin weight).

5.3 Hydroxylation of 5-methoxyplumbagin (106) at C-3

5.3.1 Epoxidation of 5-methoxyplumbagin (106)

Compound **106** (5-methoxyplumbagin) 0.700 g was dissolved in 10 ml of methanol and warmed. Then 7 ml of hydrogen peroxide in sodium bicarbonate solution (5% w/v) was added. The reaction mixture was then extracted

with $CHCl_3$ (7x10 ml). The chloroform extract was evaporated to yield a white powder of 5-methoxyplumbagin-2,3-epoxide (**107**), (0.517 g, 73% based on the weight of starting material).

5.3.2 Treatment of compound 107 with concentrated sulfuric acid

Compound **107** (0.411 g) was treated with 1 ml of conc sulfuric acid, until the white powder turned red. It was then cooled in ice, and purified water was added until yellow precipitates occurred. Collection of the precipitates by filtration yielded 3-hydroxy-5-methoxy-plumbagin (**108**) (0.242 g, 58.88% based on the weight of starting material).

5.3.3 Acetylation of 3-hydroxy-5-methoxyplumbagin (108)

Compound **108** (39 mg) was dissolved in 0.5 ml of pyridine. One ml of acetic anhydride was added and the solution was stirred over night. The reaction mixture was then extracted with chloroform (3x10 ml) and dried over Na_2SO_4. Evaporation of the solvent gave compound **109** (0.019 g, 48.71% based on the weight of starting material).

5.3.4 Methylation of 3-hydroxy-5-methoxyplumbagin (108)

To the solution of compound **108** (0.070 g) in $CHCl_3$ (10 ml) was added silver (I) oxide 400 mg and methyl iodide 10 ml. The mixture was stirred overnight. After a usual workup, two products were detected and then separated by preparative TLC technique using petroleum ether : ethyl acetate (70:30) as the developing solvent to give compound **110** (0.012 g, 17.14% based on the starting

material weight) and compound **111** (0.007 g, 10% based on the starting material weight).

5.4 Halogenation of 5-methoxyplumbagin (106) at C-3

5.4.1 Preparation of compound 107

This reaction was same as the epoxidation of 5-methoxy-plumbagin to give compound **107.**

5.4.2 Chlorination of compound 107

Compound **107** (70 mg) was dissolved in 5% HCl in methanol, and was refluxed in steam-bath for 2 hours. The acid solution was neutralized by ion exchange chromatography. A TLC chromatogram suggested three products. Separation of these compounds was performed by preparative TLC to give compound **112** (0.012 g, 17% based on the weight of starting material). Compound **110** (0.010g, 14.28% based on starting the material weight) and compound **108** (0.0142 g, 20.28% based on the starting material weight).

6. Characterization of isolated compounds and chemical transformation products

6.1 Characterization of compound 18

Compound **18** was obtained as orange crystals. It was soluble in chloroform.

EIMS : ***m/z*** (% relative intensity)

188 (100.00), 173 (25.11), 160 (24.80), 145 (5.10),

131 (44.99), 120 (22.64), 114 (4.04), 103 (9.42),

92 (26.45), 77 (12.26), 74 (5.78), 63 (25.46), 51 (10.78)

IR : ν cm^{-1}, KBr disc

1664, 1455, 1364, 1259

UV : λ_{max} nm (log ε), in methanol

209 (4.50), 264 (4.06), 418 (3.58)

^{1}H NMR : δ ppm, 500 MHz, in chloroform-*d*

See Table 4

^{13}C NMR : δ ppm, 125 MHz, in chloroform-*d*

See Table 4

6.2 Characterization of compound 19

Compound **19** was obtained as orange crystals. It was soluble in chlorofrom.

EIMS : ***m/z*** (% relative intensity)

204 (52.70), 158 (19.22), 147 (61.36), 130 (45.34),

121 (48.28), 97 (8.95), 91 (47.63), 83 (21.45),

75 (34.80), 69 (26.02), 63 (79.25), 55 (81.29)

IR : ν cm^{-1}, KBr disc

3485, 1626, 1450, 1100

UV : λ_{max} nm (log ε), in methanol

228 (4.70), 279 (4.68), 401 (4.15)

^{1}H NMR : δ ppm, 500 MHz, in chloroform-*d*

See Table 5

^{13}C NMR : δ ppm, 125 MHz, in chloroform-*d*

See Table 5

6.3 Characterization of compound 102

Compound **102** was obtained as a yellow semi-solid. It was soluble in chloroform.

EIMS : ***m/z*** (% relative intensity)

192 (78.87), 177 (13.69), 163 (8.92), 150 (35.65), 131 (13.41), 121 (100.00), 115 (6.76), 103 (5.71), 93 (14.61), 83 (19.50), 77 (11.00), 65 (23.83), 55 (2.83)

IR : ν cm^{-1}, KBr disc

3101, 1638, 1455, 1343, 1243

UV : λ_{max} nm (log ε), in methanol

214 (3.99), 258 (3.80), 332 (3.33)

^{1}H NMR : δ ppm, 500 MHz, in chloroform-*d*

See Table 6

^{13}C NMR : δ ppm, 125 MHz, in chloroform-*d*

See Table 6

6.4 Characterization of compound 103

Compound **103** was obtained as a white solid. It was soluble in pyridine and acetone.

EIMS : ***m/z*** (% relative intensity)

432 (11.20), 252 (0.70), 224 (1.30), 180 (41.60), 163 (27.60), 111 (10.90), 97 (22.30), 83 (43.40), 69 (52.50), 55 (73.40), 43 (100.00)

IR : ν cm^{-1}, KBr disc

3500, 2990, 2800, 1700, 1610, 1230, 1190

^{1}H NMR : δ ppm, 500 MHz, in acetone-d_6

See Table 7

^{13}C NMR : δ ppm, 125 MHz, in pyridine-d_5

See Table 7

6.5 Characterization of compound 104

Compound **104** was obtained as an orange solid. It was soluble in chloroform.

EIMS : ***m/z*** (% relative intensity)

204 (93.55), 189 (82.26), 175 (17.74), 161 (75.81), 156 (38.71), 147 (38.71), 141 (27.42), 133 (53.23), 128 (17.74), 119 (70.97), 105 (100.00), 91 (87.10), 81 (46.77), 77 (41.94), 67 (22.58), 61 (9.68), 55(25.81)

IR : ν cm^{-1}, KBr disc

2922, 1640, 1230

^{1}H NMR : δ ppm, 500 MHz, in chloroform-*d*

See Table 8

^{13}C NMR : δ ppm, 125 MHz, in chloroform-*d*

See Table 8

6.6 Characterization of compound 105

Compound **105** was obtained as yellow crystals. It was soluble in chloroform.

EIMS : ***m/z*** **(% relative intensity)**

230 (2.00), 188 (100.00), 173 (10.90), 160 (18.80), 145 (1.20),
120 (8.50), 103 (3.40), 92 (7.30), 85 (1.10), 77 (6.60), 63 (8.30),
51 (4.00), 43 (58.70)

IR : ν cm^{-1}, KBr disc

1790, 1650, 1580, 1400, 1250, 1000

^{1}H NMR : δ ppm, 400 MHz, in chloroform-*d*

See Table 9

^{13}C NMR : δ ppm, 100 MHz, in chloroform-*d*

See Table 9

6.7 Characterization of compound 106

Compound **106** was obtained as yellow crystals. It was soluble in chlorofrom.

EIMS : ***m/z*** **(% relative intensity)**

202 (100.00), 189 (8.00), 173 (22.40), 156 (15.30), 145 (12.20),
128 (14.00), 115 (22.00), 104 (26.20), 88 (13.70), 76 (42.10),
69 (12.10), 55 (17.00), 39 (26.30), 32 (10.90)

IR : ν cm^{-1}, KBr disc

2900, 1660, 1590, 1430-1500, 1300, 1220

^{1}H NMR : δ ppm, 400 MHz, in chloroform-*d*

See Table 10

^{13}C NMR : δ ppm, 100 MHz, in chloroform-*d*

See Table 10

6.8 Characterization of compound 107

Compound **107** was obtained as a white powder. It was soluble in chloroform.

EIMS : ***m/z*** (% relative intensity)

218 (100.00), 203 (32.20), 189 (28.80), 175 (43.90), 161 (33.90), 147 (22.90), 135 (35.10), 119 (29.50), 91 (20.60), 76 (60.90), 63 (32.40), 50 (29.10), 43 (91.80), 32 (17.80)

IR : ν cm^{-1}, KBr disc

2947, 2922, 1690, 1588, 1451, 1344-1255

^{1}H NMR : δ ppm, 500 MHz, in chloroform-*d*

See Table 11

^{13}C NMR : δ ppm, 125 MHz, in chloroform-*d*

See table 11

6.9 Characterization of compound 108

Compound **108** was obtained as a yellow powder. It was soluble in chloroform.

EIMS : ***m/z*** (% relative intensity)

218 (100.00), 200 (7.30), 189 (10.20), 172 (16.30), 147 (13.90), 131 (14.10), 105 (12.80), 91 (12.20), 84 (18.40), 76 (29.30), 65 (15.30), 55 (29.80), 43 (35.40), 32 (50.10)

IR : ν cm^{-1}, KBr disc

3255, 2811, 1658, 1636, 1538, 1202

^{1}H NMR : δ ppm, 400 MHz, in chloroform-*d*

See Table 12

^{13}C NMR : δ ppm, 125 MHz, in chloroform-*d*

See Table 12

6.10 Characterization of compound 109

Compound **109** was obtained as a yellow powder. It was soluble in chloroform.

EIMS : ***m/z*** (% relative intensity)

260 (2.20), 218 (85.00), 200 (12.20), 172 (12.30), 161 (9.40), 131 (6.40), 91 (6.30), 76 (15.20), 63 (9.20), 55 (14.30), 43 (100.00), 32 (6.70)

IR : ν cm^{-1}, KBr disc

3454, 1663, 1276, 1165,

^{1}H NMR : δ ppm, 500 MHz, in chloroform-*d*

See Table 13

^{13}C NMR : δ ppm, 50 MHz, in chloroform-*d*

See table 13

6.11 Characterization of compound 110

Compound **110** was obtained as a yellow powder. It was soluble in chloroform.

EIMS : ***m/z*** (% relative intensity)

232 (100.00), 217 (41.90), 202 (13.10), 189 (23.00), 173 (21.70),

159 (12.40), 145 (9.80), 135 (16.20), 115 (19.50), 91 (11.90),

76 (42.80), 63 (22.60), 55 (19.50), 39 (26.40)

IR : ν cm^{-1} ,KBr disc

3471, 2970-2847, 1690, 1584, 1256

^{1}H NMR : δ ppm, 400 MHz, in chloroform-*d*

See Table 14

^{13}C NMR : δ ppm, 125 MHz, in chloroform-*d*

See Table 14

6.12 Characterization of compound 111

Compound **111** was obtained as a white powder. It was soluble in chloroform.

HRFAB-MS : ***m/z*** (% relative intensity)

265.1088 $[M+H]^{+}$ (28.78), 205.10 (7.81), 187.10 (2.50), 94.10 (2.86)

EIMS : ***m/z*** (% relative intensity)

264, 232 (0.10), 205 (100.00), 190 (8.90), 175 (2.30), 144 (2.70),

115 (2.60), 91 (3.80), 77 (3.70), 63 (2.30), 51 (2.30), 45 (6.35),

32 (1.40)

IR : ν cm^{-1}, in chloroform

3443, 2961-2931, 1726, 1272, 1004

^{1}H NMR : δ ppm, 500 MHz, in chloroform-*d*

See Table 15

^{13}C NMR : δ ppm, 125 MHz, in chloroform-*d*,

See Table 15

6.13 Characterization of compound 112

Compound **112** was obtained as a yellow powder. It was soluble in chloroform.

EIMS : ***m/z*** (% relative intensity)

238 (35.52), 236 (100.00), 219 (4.00), 201 (27.75), 186 (7.63), 171 (47.74), 158 (9.29), 143 (69.89), 130 (11.42), 115 (89.65), 102 (32.01), 91 (15.86), 76 (56.88), 63 (35.30), 50 (23.52)

IR : ν cm^{-1}, KBr disc

3400, 2800, 1656, 1585, 1321, 1275, 1055

^{1}H NMR : δ ppm, 400 MHz, in chloroform-*d*

See Table 16

^{13}C NMR : δ ppm, 125 MHz, in chloroform-*d*

See Table 16