CHAPTER III

EXPERIMENTAL

1. Source of Plant Material

The leaves of *Diospyros undulata* Wall. ex G. Don var. *cratericalyx* (Craib) Bakh. were collected from Chachoengsao province, Thailand in March, 1999. The plant was identified by comparison with herbarium specimens collected at the Royal Forest Department, Ministry of Agriculture and Co-operative, Bangkok, Thailand.

2. General Techniques

2.1 Chromatography

2.1.1 Analytical Thin-layer Chromatography (TLC)

Technique	:	One dimension, ascending
Adsorbent	:	Silica gel 60 F254 (E. Merck) precoated plate
Layer thickness	:	0.2 mm
Distance	:	7 cm
Temperature	:	Laboratory temperature (30-35 °C)
Detection	:	1). Visual detection under daylight 2). UV light at the wavelengths of 254 and 365 nm 3). Spraying with vanillin-sulphuric acid reagent (10% ethanolic sulphuric acid) and heating at 110 °C for 5-10 minutes

2.1.2 Column Chromatography

Packing method	:	Wet packing
Sample loading	:	The sample was dissolved in a small volume of organic solvent, then applied gently on the top of the column.

2.1.3 Gel Filtration Chromatography

Gel filter	:	Sephadex LH-20 (Pharmacia)

Packing method	:	The gel filter was suspended in the eluent and left standing to swell for 24 hours prior to use. It was then poured into the column and allowed to set tightly.
Sample loading	:	The sample was dissolved in a small volume of the eluent and applied onto the top of the column.

2.1 Spectroscopy

2.2.1 Ultraviolet (UV) Absorption Spectra

UV spectra (in methanol) were obtained on a Milton Roy Spectronic 3000 Array spectrometer (Pharmaceutical Research Equipment Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.2.2 Infrared (IR) Absorption Spectra

IR spectra (KBr disc and film) were obtained either on a Perkin Elmer FT-IR 2000 spectrometer or a Perkin Elmer FT-IR 1760X spectrometer (Scientific and Technological Research Equipment Center, Chulalongkorn University).

2.2.3 Mass Spectra (MS)

Electron Impact Mass Spectra (EIMS) were recorded on a Fison Micromass VG Platform II mass spectrometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.2.4 Proton and Carbon-13 Nuclear Magnetic Resonance (^{1}H and ^{13}C-NMR) Spectra

^{1}H-NMR (300 MHz) and ^{13}C-NMR (75 MHz) spectra were obtained with a Bruker Avance DPX-300 FT-NMR spectrometer (Faculty of Pharmaceutical Sciences, Chulalongkorn University).

^{1}H-NMR (500 MHz) spectra was obtained with a JEOL JMN-A 500 NMR spectrometer (Scientific and Technological Research Equipment Center, Chulalongkorn University). Deuterated chloroform ($CDCl_3$) was used as the solvent. Chemical shifts were reported in ppm scale using the chemical shift of the solvent as the reference signal.

2.3 Physical Properties

2.3.1 Melting Points

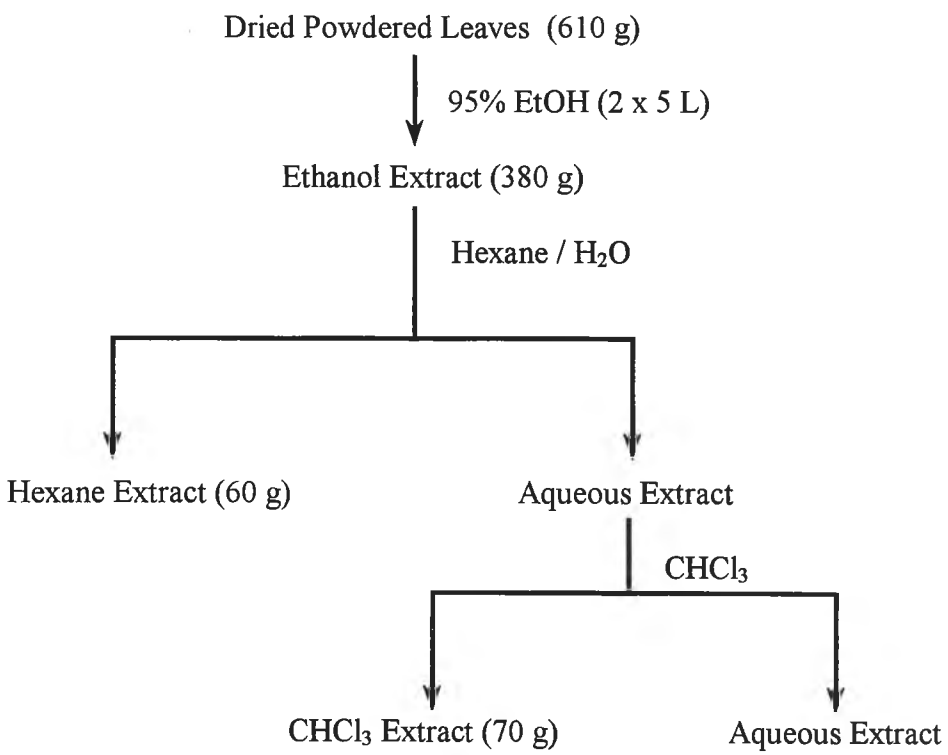
Melting points were obtained on a Fisher / Johns Melting Point Apparatus (Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University) and are uncorrected.

2.4 Solvents

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

3. Extraction Procedure

The dried leaves of *Diospyros undulata* Wall. ex G. Don var. *cratericalyx* (Craib) Bakh. (610 g) were chopped and blended into small pieces. They were extracted twice with 95% ethanol (5 L, 7 days each). The filtrates were pooled and evaporated under reduced pressure at temperature not exceeding 50 °C to yield the ethanol extract as a syrupy mass (380 g). The ethanol extract was diluted with water and partitioned with hexane (2 x 3 L) to give hexane extract (60 g). The ethanol layer was then extracted with chloroform (2 x 3 L). The chloroform extract was evaporated *in vacuo* to give a residue of 70 g.



Scheme 1 Extraction of *Diospyros undulata* Wall. ex G. Don var. *cratericalyx* leaves

4. Isolation Procedure

The hexane extract (60 g) was divided into 2 equal parts and subjected to two successive silica gel column chromatography, both using the same solvent mixture of chloroform - hexane (3 : 1) as the eluent. The extract was dissolved in a small volume of the eluent and applied to the top of a glass column (4.5 x 60 cm) already packed with a slurry of silica gel (400 g) in the eluting solvent. Fractions (40 ml each) were monitored by TLC, with chloroform – hexane (5 : 2) and hexane : acetone (5 : 1) as the developing solvent system. About 250 fractions were collected from each column and combined according to their TLC profiles into six major fractions (F001 – F006) as shown in Table 3. Both columns were then washed down with methanol and the eluate combined as fraction F007.

Table 3 Combined fractions from the hexane extract of *Diospyros undulata* Wall. ex G. Don var. *cratericalyx* (Craib) Bakh.

Code	Weight of combined fraction (g)
F001	4.50
F002	7.10
F003	10.25
F004	11.10
F005	8.48
F006	4.64
F007	4.33

4.1 Isolation of compound DU1

Fraction F002 was subjected to a silica gel column chromatography. The fraction (7.10 g) was dissolved in a small volume of chloroform-hexane (5 : 2) and applied to the top of a glass column (2.5 x 60 cm) already packed with a slurry of silica gel (250 g). This same solvent mixture was employed as the eluting solvent. The fraction volume collected was about 20 ml. All eluates were collected and combined according to their TLC patterns, using chloroform-hexane-acetone (8 : 2 : 1) as the developing solvent system. Fraction F002 was further separated into five fractions (F008-F012) as summarized in Table 4.

Table 4 Combined fractions from F002

Code	Number of eluates	Weight of combined fraction (g)
F008	1-9	0.12
F009	10-21	1.47
F010	22-37	1.31
F011	38-48	0.96
F012	49-56	0.60

Both fractions F009 and F010 appeared to contain the same major component. They were combined and purified by recrystallization in methanol to give compound DU1 as white powder (2.70 g).

4.2 Isolation of compound DU2

Fraction F003 (10.25 g) was separated by column chromatography using a column (2.5 x 60 cm) of silica gel (400 g) with chloroform - hexane (4 : 1) as the eluent. The fraction (10.25 g) was dissolved in a small volume of the eluent and loaded onto the top of the column. The volume of each collected fraction was approximately 15 ml. Monitored by TLC examination with chloroform-hexane-acetone (2 : 2 : 1) as the developing solvent system, thirty-three fractions were collected and combined. Finally, the column was washed down with methanol. Therefore, fraction F003 was further fractionated into 4 major portions as summarized in Table 5.

Table 5 Combined fractions from F003

Code	Number of eluates	Weight of combined fraction (g)
F013	1-14	3.62
F014	15-24	0.99
F015	25-33	0.56
F016	Methanol eluted	1.50

Fraction F013 was further purified by recrystallization in methanol to give a pure compound which appeared as white powder (3.61 g) and was later shown to be identical with compound DU1. Another fraction, F015, was also purified by recrystallization in acetone to give compound DU2 as colorless needles (45.0 mg).

4.3 Isolation of compound DU3

The components of fraction F004 were separated by silica gel column chromatography. This fraction (11.10 g) was dissolved in a small volume of the eluent [chloroform-hexane (5: 1)] and applied to the top of the silica gel column (400 g, 2.5 x 60 cm). Seventy-six 20-ml fractions were eluted and combined into 5 major fractions (F017-F021). The column was then washed down with methanol to give fraction F022. These combined fractions are summarized in Table 6.

Table 6 Combined fractions from F004

Code	Number of eluates	Weight of combined fraction (g)
F017	1-14	0.87
F018	15-31	1.91
F019	32-45	2.14
F020	46-65	1.88
F021	66-76	0.95
F022	Methanol eluted	2.25

After recrystallization in methanol, fractions F017-F019 all yielded the previously isolated compound DU1 with the combined weight of 4.91 g. On the other hand, fraction F020 was purified by recrystallization in hexane to afford compound DU3 as red needles (37.0 mg).

4.4 Isolation of compound DU4

Fraction F005 (8.48 g) was subjected to silica gel column chromatography (300 g, 2 x 60 cm), using chloroform-hexane (1 : 1) as the mobile phase. Fifty-seven fractions of about 10 ml each were collected and combined according to their TLC profiles in chloroform-hexane (3 : 1). Methanol was then used to wash down the column. Four major fractions (F028-F031) were therefore collected as summarized in Table 7.

Table 7 Combined fractions from F005

Code	Number of eluates	Weight of combined fraction (g)
F028	1-9	1.21
F029	10-22	1.54
F030	23-57	1.46
F031	Methanol eluted	3.48

Fraction F028 (1.21 g) was purified by recrystallizing in benzene - hexane (1 : 1) to yield compound DU4 as red needles (56.7 mg). In addition, compound DU1 (2.91 g) was further collected upon recrystallization of fractions F029 and F030 in methanol.

4.5 Isolation of compound DU5

Fraction F006 (4.64 g) was separated by a silica gel column (100 g, 2 x 60 cm) with chloroform-hexane (5 : 1) as the mobile phase. The fractional volume was about 15 ml each. Toluene-ethyl acetate (4 : 1) was used as the developing solvent system for TLC monitoring of these fractions. Seventy-seven fractions were collected and then combined into 5 major ones (F032-F036) as summarized in Table 8.

Table 8 Combined fractions from F006

Code	Number of eluates	Weight of combined fraction (g)
F032	1-18	1.11
F033	19-31	0.17
F034	32-49	1.31
F035	50-63	0.10
F036	64-77	1.61

Fractions F034 and F035, which displayed an identical yellow spot on TLC, were combined and recrystallized in hexane to yield compound DU5 (1.41 g) as orange needles.

4.6 Isolation of compound DU6

An amount (10.0 g) of the chloroform extract was applied to a silica gel column (300 g, 4.5 x 60 cm) and eluted with a mixture of chloroform-acetone (5 :1). Sixty-four fractions (40 ml each) were collected and monitored by TLC, then combined into 4 major fractions (F037-F040) as shown in Table 9. Methanol was then used to wash down the column to give the final fraction, F041.

Table 9 Combined fractions from the chloroform extract

Code	Number of eluates	Weight of combined fraction (g)
F037	1-10	3.31
F038	11-33	1.55
F039	34-46	1.36
F040	47-64	1.58
F041	Methanol eluted	2.13

Fractions F037 and F038 , which showed similar major spot on the TLC, were combined and subjected to another silica gel column chromatography (150 g, 2 x 60 cm) with chloroform-acetone (5 : 1) as the mobile phase. Forty-nine 30-ml fractions were collected and combined according to their TLC patterns into 3 major fractions (F042-F044). Fractions F042 and F043, when combined and recrystallized in acetone, yielded compound DU6 as white crystalline solid (4.70 g).

5. Physical and Spectra Data of Isolated Compounds

5.1 Compound DU1

White powder from methanol. Soluble in chloroform.

m. p. : 215-216 °C

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

427 (82), 412 (28), 394 (7), 384 (1), 370 (3), 357 (1), 344 (2), 315 (10), 299 (3), 272 (2), 257 (5), 247 (5), 235 (5), 218 (47), 207 (62), 189 (53), 175 (17), 147 (37), 135 (72), 121 (74), 107 (74), 93 (64), 81 (81), 67 (79) and 55 (100)

(Figure 2, page 80)

IR ν_{max}, KBr disc, cm^{-1} :

3441, 2943, 2871, 1637, 1455, 1382, 1067, 1042, 945 and 883

(Figure 3, page 81)

^{1}H-NMR (δ ppm, 300 MHz, $CDCl_3$) :

4.70 (1H, *s*) , 4.50 (1H, *s*), 3.20 (1H, *dd J* = 5, 2 Hz), 2.40 (1H, *m*), 1.90 (1H, *m*), 1.00 (3H, *s*), 0.95 (6H, *s*), 0.90 (3H, *s*), 0.80 (3H, *s*), 0.70 (3H, *s*), 0.75 (3H, *s*), 0.65 (1H, *d, J* = 4Hz)

(Figure 4a-4b, page 82-83)

^{13}C-NMR (δ ppm, 75 MHz, $CDCl_3$) :

150.8 (*s*), 109.2 (*t*), 79.0 (*d*), 55.4 (*d*), 50.6 (*d*), 48.4 (*d*), 48.1 (*q*), 43.1 (*s*), 43.0 (*s*), 41.0 (*s*), 40.1 (*t*), 39.0 (*s*), 38.9 (*t*), 38.2 (*d*), 37.3 (*s*), 35.7 (*t*), 34.5 (*t*), 30.0 (*t*), 28.1 (*q*), 27.6 (*t*), 27.6 (*t*), 25.4 (*t*), 21.1 (*t*), 19.5 (*q*), 18.5 (*t*), 18.2 (*q*), 16.3 (*q*), 16.2 (*q*),15.5 (*q*), 14.7 (*q*)

(Figure 5a-5b, page 84-85)

5.2 Compound DU2

Colorless needles. Soluble in chloroform.

m. p. : 243-245 °C

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

426 (19), 411 (6), 341 (5), 302 (10), 287 (5), 273 (20), 257 (5), 246 (25), 232 (12), 218 (28), 205 (38), 191 (18), 179 (26), 163 (32), 149 (21), 137 (21), 123 (55), 109 (66), 95 (81), 81 (76), 69 (100) and 55 (100)

(Figure 7, page 91)

IR ν_{max}, KBr disc, cm^{-1}:

3441, 2933, 2869, 1715, 1632, 1457, 1390, 1112, 1077 and 920

(Figure 8, page 92)

^{1}H-NMR (δ ppm, 300 MHz, $CDCl_3$)

2.39 ; 2.29 (H-2), 2.24 (H-4), 1.96 ; 1.67 (H-1), 1.54 (H-10), 1.54 (H-19), 1.48 ; 1.33 (H-15), 1.30 ; 1.45 (H-11), 1.40 ; 1.34 (H-12), 1.33 ; 1.47 (H-7), 1.28 ; 1.75 (H-6), 1.37 ; 1.55 (H-16), 1.37 (H-8), 1.36 ; 1.20 (H-19), 1.28 ; 1.47 (H-21), 0.94 : 1.53 (H-22)

(Figure 9a-9b, page 93-94)

^{13}C-NMR (δ ppm, 75 MHz, $CDCl_3$)

213.2 (*s*), 59.5 (*s*), 58.2 (*s*), 53.1 (*s*), 42.8 (*d*), 42.1 (*d*), 41.5 (*t*), 41.3 (*t*), 39.7 (*s*), 39.2 (*d*), 38.3 (*d*), 37.4 (*d*), 36.0 (*t*), 35.6 (*t*), 35.3 (*t*), 35.0 (*q*), 32.7 (*d*), 32.4 (*t*), 32.1 (*q*), 31.7 (*q*), 30.5 (*t*), 30.0 (*s*), 28.1 (*s*), 22.3 (*t*), 20.2 (*q*), 18.6 (*q*), 18.2 (*t*), 17.9 (*q*), 14.6 (*q*), 6.8 (*q*)

(Figure 10a-10b, page 95-96)

5.3 Compound DU3

Red needles from hexane. Soluble in chloroform.

m. p. : 194-195 °C

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

374 (100), 346 (6), 331 (38), 317 (21), 303 (68), 278 (29), 250 (48), 221 (6), 205 (3), 189 (5), 165 (21), 176 (5), 165 (21), 139 (26), 125 (7), 83 (97), 69 (15) and 55 (11)

(Figure 14, page 103)

UV λ_{max} (MeOH), nm (log ε) :

264, 436 nm (log ε 4.42, 3.87) (Figure 15, page 104)

IR ν_{max}, KBr disc, cm^{-1} :

1656 (sh), 1647, 1614, 1455, 1360, 1320, 1216, 1026, 900 and 782

(Figure 16, page 105)

^{1}H-NMR (δ ppm, 300 MHz, $CDCl_3$)

12.36 (2H, *s*), 7.19 (2H, *d*, $J = 9$), 7.10 (2H, *d*, $J = 9$), 6.76 (2H, *q*, $J = 1.5$), 1.99 (6H, d $J = 1.5$)

(Figure 17a-17b, page 106-107)

^{13}C-NMR (δ ppm, 75 MHz, $CDCl_3$)

190.47 (*s*) 185.17 (*s*), 161.31 (*s*), 150.00 (*d*), 137.94 (*s*), 135.53 (*s*), 134.91 (*d*), 128.14 (*s*), 124.30 (*d*), 115.43 (*s*), 16.64 (*q*)

(Figure 18, page 108)

5.4 Compound DU4

Red needles from benzene-hexane. Soluble in chloroform.

m. p. : 260-261 °C

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

374 (100), 357 (8), 346 (6), 331 (10), 318 (2), 303 (4), 289 (3), 278 (11), 261 (2), 250 (2), 233 (2), 221 (3), 202 (2), 189 (4), 176 (4), 165 (15), 153 (8), 139 (9), 126 (8), 115 (5) and 83 (35)

(Figure 24, page 119)

UV λ_{max} (MeOH), nm (log ε) :

212, 250, 285, 420 nm (log ε 3.97, 3.63, 3.32, 3.09)

(Figure 25, page 120)

IR ν_{max}, KBr disc, cm^{-1} :

3600 (br), 1600, 1500, 1200, 1150, 975 and 825

(Figure 26, page 121)

^{1}H-NMR (δ ppm, 300 MHz, $CDCl_3$)

6.83 (d, *J*=1.1 Hz) , 7.24 (2H,br s), 7.71 (2H, br s), 2.21 (6H, d, *J*= 1.1Hz)

(Figure 27a-27b, page 122-123)

^{13}C-NMR (δ ppm, 75 MHz, $CDCl_3$)

190.45 (*s*), 184.53 (*s*), 158.90 (*s*), 149.81 (*s*), 137.65 (*d*), 135.52 (*d*), 131.94 (*s*), 131.23 (*s*), 118.73 (*d*), 115.33 (*s*), 16.50 (*q*),

(Figure 28, page 124)

5.5 Compound DU5

Orange needles from hexane. Soluble in chloroform.

m. p. : 75-76 °C

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

160 (20), 145 (6), 131 (74), 120 (24), 103 (32), 92 (78), 77 (45), 63 (100)

(Figure 33, page 134)

UV λ_{max} (MeOH), nm (log ε) :

210 (4.53), 255 (4.07), 267 (4.08) and 424 (3.67)

(Figure 34, page 135)

IR ν_{max}, KBr disc, cm^{-1} :

1660 and 1640

(Figure 35, page 136)

^{1}H-NMR (δ ppm, 300 MHz, $CDCl_3$)

11.73 (1H, *s*), 7.47 (1H, *dd*), 7.39 (1H, *dd*), 7.07 (1H, *dd*), 6.66 (2H, *q*), 2.13 (3H, *d J* = 1.5 Hz)

(Figure 36a-36b, page 137-138)

^{13}C-NMR (δ ppm, 75 MHz, $CDCl_3$) :

190.23 (*s*), 184.74 (*s*), 161.12 (*d*), 149.57 (*s*), 136.07 (*d*), 135.41 (*d*), 131.99 (*s*), 124.13 (*d*), 119.25 (*d*), 115.07 (*s*), 16.52 (*q*)

(Figure 37, page 139)

5.6 Compound DU6

White needles from acetone. Soluble in chloroform.

m. p. : 261 °C

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

443 (14), 411 (32), 393 (6), 386 (4), 299 (1), 288 (6), 273 (3), 257 (5), 234 (20), 220 (10), 203 (39), 189 (55), 175 (33), 161 (22), 147 (26), 135 (32), 119 (36), 105 (50), 93 (79), 81 (100), 67 (100) and 55 (100)

(Figure 41, page 149)

IR ν_{max} , KBR disc, cm^{-1} :

3449, 2932, 2869, 1716, 1632, 1457, 1389, 1077, 1051 and 984

(Figure 42, page 150)

^{1}H-NMR (δ ppm, 300 MHz, $CDCl_3$) :

6.82 (d, *J*=1.0 Hz), 7.63 (1H, *J*=7.4, 2.1 Hz), 7.61 (1H, *J*=7.4 Hz), 7.24 (1H, *J*=7.4, 2.1 Hz), 2.20 (3H, d, *J*=1.0 Hz), 12.01 (1H, s)

(Figure 43a-43b, page 151-152)

^{13}C-NMR (δ ppm, 75 MHz, $CDCl_3$)

150.27 (*s*), 109.59 (*t*), 78.97 (*d*), 60.56 (*t*), 55.29 (*d*), 50.42 (*d*), 48.78 (*d*), 47.83 (*s*), 47.83 (*d*), 42.76 (*s*), 40.96 (*s*), 38.93 (*s*), 38.75 (*t*), 37.36 (*s*), 37.22 (*d*), 34.29 (*t*), 34.05 (*t*), 29.82 (*t*), 29.25 (*t*), 28.07 (*q*), 27.46 (*t*), 27.12 (*t*), 25.29 (*t*), 20.93 (*t*), 19.20 (*q*), 18.41 (*t*), 16.24 (*q*), 16.09 (*q*), 15.49 (*q*), 14.88 (*q*)

(Figure 44a-44b, page 153-154)