CHAPTER III

Experimental

1. Sources of Plant Materials

The bark of *Fissistigma polyanthoides* (DC.) Merr. was collected from Nam Nao National Park, Petchaboon province, Thailand, in March 1993. The leaves of *Ochna integerrima* (Lour.) Merr. were collected from Sakaeraj Environmental Research Station, Nakhonratchasima province, Thailand, in April 1999. Authentication of the plant materials was done by comparison with herbarium specimens at the Royal Forest Department, Bangkok, Thailand.

2. General Techniques

2.1 Analytical Thin-Layer Chromatography (TLC)

Technique	:	One dimension, ascending
Adsorbent	:	Silica gel 60 F_{254} (E. Merck) precoated plate
Layer thickness	:	2 mm
Distance	:	6 cm
Temperature	:	Laboratory temperature (30-35°C)
Detection	:	1. Ultraviolet light at wavelengths of 254 and 365 nm
		2. Anisaldehyde and heat at 105°C for 10 min.

2.2 Column Cromatography

2.2.1 Vacuum Liquid Column Chromatography

Adsorbent	:	Silica gel 60 (No.7734) particle size 0.063-0.200 mm (70-230 mesh ASTM) (E. Merck)
Packing method	:	Dry packing
Sample loading	:	The sample was dissolved in a small amount of organic solvent, mixed with a small quantity of adsorbent, triturated, dried and then placed gently on top of the column.
Detection	:	Fractions were examined by TLC under UV light at the wavelengths of 254 and 365 nm

2.2.2 Flash Column Chromatography

Adsorbent	:	Silica gel 60 (No.7734) particle size 0.063-0.200 mm (70-230 mesh ASTM) (E. Merck)
Packing method	:	Wet packing
Sample loading	:	The sample was dissolved in a small amount of eluant and then applied gently on top of the column.
Detection	:	Fractions were examined by TLC under UV light at the wavelengths of 254 and 365 nm.

2.2.3 Gel Filtration Chromatography

Gel filter	:	Sephadex LH20 (Pharmacia)

Packing method : Gel filter was suspended in the eluant 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 eluant and applied on top of the column.

Detection : Fractions were examined by TLC under UV light at the wavelengths of 254 and 365 nm.

2.3 Spectroscopy

2.3.1 Ultraviolet (UV) Absorption Spectra

UV (in methanol) spectra were obtained on a Shimadzu UV-160A UV/vis spectrophotometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.3.2 Infrared (IR) Absorption Spectra

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

2.3.3 Mass Spectra

Fast atom bombardment mass spectra (FAB-MS) were measured with a JEOL JMS-D300 instrument (Japan). Electron impact mass spectra (EIMS) were measured on a Fison Micromass VG Platform II mass spectrometer (Pharmaceutical

Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

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

^{1}H NMR (300 MHz) , ^{13}C NMR (75 MHz), ^{1}H-^{1}H COSY, NOESY, HETCOR and COLOC spectra were obtained with a Bruker Avance DPX-300 FT-NMR spectrometer, (Faculty of Pharmaceutical Sciences, Chulalongkorn University). ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz) and HMBC spectra were recorded with a JEOL JMN-A 500 NMR spectrometer (Scientific and Technological Research Equipment Center, Chulalongkorn University).

Solvents for NMR spectra were deuterated dimethylsulfoxide (DMSO-d_6) and deuterated chloroform (chloroform-d). Chemical shifts were reported in ppm scale using the chemical shift of the solvent as the reference.

2.4 Physical Properties

2.4.1 Melting Points

Melting points were obtained on a Fisher/Johns melting point apparatus (Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.4.2 Optical Rotation

Optical rotations were measured on a Perkin Elmer 341 polarimeter (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.5 Solvents

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

3. Extraction and Isolation

3.1 *Fissistigma polyanthoides*

3.1.1 Extraction

The dried, finely powdered stem bark (2.4 Kg) of *F. polyanthoides* was marcerated with 95% ethanol three times (3x3 days) and filtered. After combination, the obtained ethanolic extract was evaporated to dryness under reduced pressure .

The ethanolic extract (613 g) was separated on a kieselguhr column, eluted with hexane (5×20L), chloroform (6×10L) and methanol (5×20L), respectively. Each fraction was evaporated under reduced pressure to yielded a hexane extract (17 g), a chloroform extract (36 g) and a methanol extract (60 g).

3.1.2 Isolation

3.1.2.1 Isolation of Compound FP-1

The hexane extract (40 g) was dissolved in a small amount of methanol, triturated with silica gel 60 (No.7734) and dried under reduced pressure. It was then fractionated by vacuum liquid column chromatography using a sintered glass filter column of silica gel 60 (No.7734) (800 g). Elution was performed in a p' 'arity gradient manner with mixtures of hexane and ethyl acetate as the solvents (Table 3).

Table 3 Solvents in vaccuum liquid column chromatography of hexane extract of *Fissistigma polyanthoides*

Fraction	Percentage of ethyl acetate in hexane	Volume of solvent (ml)
1	20	500
2-4	25	1500
5-12	30	4000
13-16	50	2000
17-20	100	2000

The eluates were examined by TLC (SiO_2) using 40% ethyl acetate in hexane as developing solvent. Fractions with similar chromatographic pattern were combined to yield five fractions, as summarized in Table 4.

Table 4 Combination of fractions from vacuum liquid column chromatography of hexane extract from *Fissistigma polyanthoides*

Fraction	Weight (g)	Volume of solvent (ml)
1-4	0.264	2000
5-9	4.036	2500
10-12	0.278	1500
13-16	2.684	2000
17-20	3.147	2000

Fractions 10-12 were pooled and dried (280 mg). The obtained residue was divided into six portions, and each portion (approx. 50 mg) was separated by gel filtration (Sephadex LH20) with methanol as the eluant. The eluates were collected 10 ml per fraction and examined by TLC (SiO_2) using 40% ethyl acetate in hexane as the developing solvent. Fractions with similar chromatographic pattern were combined to yield compound FP-1 (R_f = 0.3, SiO_2, 40% EtOAc in hexane) (25 mg) and an unidentifiable product (1 mg). Compound FP-1 was subsequently identified as 5,8-dihydroxy-6,7-dimethoxyflavone (77).

3.2 *Ochna integerrima*

3.2.1 Extraction

The dried leaves of *Ochna integerrima* (1.8 Kg) were extracted with petroleum ether (3×20L), ethyl acetate (3×20L) and methanol(3×20L) to give a pet. ether extract (41g), an ethyl acetate extract (147 g) and a methanol extract (334 g).

3.2.2 Isolation

3.2.2.1 Initial Separation

The ethyl acetate extract (147 g) was divided into three portions:A (50 g), B (50 g) and C (47 g). Each was dissolved in a small amount of ethyl acetate, triturated with silica gel 60 (No.7734) and dried under vacuum, and then fractionated by vacuum liquid column chromatography using a sintered glass filter column of silica gel 60 (No.7734) (800 g). Elution was performed in a polarity gradient manner with hexane and ethyl acetate (Table 5).

Table 5 The percentage and volumes of solvents for vacuum liquid column chromatography of ethyl acetate extract of *Ochna integerrima*

Portion	Fraction	Percentage of ethyl acetate in hexane	Volume of solvent (ml)
A	1-2	40	1500
	3-16	60	7000
	17	80	500
	18-20	100	1500
	21	methanol	5000
B	1	0	500
	2	10	500
	3	20	500
	4	40	500

Table 5 The percentage and volumes of solvents for vacuum liquid column chromatography of ethyl acetate extract of *Ochna integerrima* (continued)

Portion	Fraction	Percentage of ethyl acetate in hexane	Volume of solvent (ml)
	5-21	60	8500
	22-23	80	1000
	24-28	100	2500
	29	methanol	5000
C	1	0	500
	2	10	500
	3	20	500
	4-6	40	1500
	7-9	50	1500
	10-27	60	8500
	28-29	80	1000
	30-33	100	2000
	34	methanol	5000

The eluates obtained from each column were examined by TLC (SiO_2) using 6% methanol in chloroform as the developing system. Fractions with similar chromatographic pattern were combined to yield 15 fractions, as shown in Table 6.

Table 6 Combination of fractions from vacuum liquid column chromatography of ethyl acetate extract of *Ochna integerrima*

Combined fractions	Fractions	Total Weight (g)	Volume of solvent (ml)
P-I	1-4 A	1.824	2000
P-II	6-9 B	1.209	2000
P-lII	4-8 C	0.810	2500
P-IV	5-7 A	0.315	1500
P-V	10-15 B	1.184	3000
P-VI	9-13 C	1.158	2500
P-VII	8-16 A	0.700	4500
P-VIII	16-23 B	1.054	4000
P-lX	14-27 C	1.978	7000
P-X	17-20 A	1.346	2000
P-XI	24-28 B	8.793	2500
P-XII	28-33 C	8.368	3000
P-XIII	21 A	41.14	5000
P-XIIII	29 B	34.38	5000
P-XV	34 C	29.18	5000

For fractions P-I (1.824 g), P-II (1.209 g) and P-VI (1.158 g), each was fractionated on a column using silica gel 60 (No.7734) (50 g) as the adsorbent, and elution was performed in a polarity gradient manner with chloroform and methanol (Table 7).

Table 7 The percentage and volumes of solvents for column silica gel 60 of P-I, P-II and P-VI

Portion	Fraction	Percentage of methanol in chloroform	Volume of solvent (ml)
P-I	1-5	0	125
	6-10	1	125
	11-15	2	125
	16-20	3	125
	21-36	4	625
	37-41	5	250
	42-52	6	500
	53	7	250
	54	8	200
	55	9	200
	56	10	200
	57	20	200
	58	40	200
P-II	1-3	1	150
	4-6	2	150
	7-9	3	150
	10-30	4	1000
	31-32	5	100
	33-34	6	100

Table 7 The percentage and volumes of solvents for column silica gel 60 of P-I, P-II and P-VI (continued)

Portion	Fraction	Percentage of methanol in chloroform	Volume of solvent (ml)
	35-36	7	100
	37-38	8	100
	39-40	9	100
	41-42	10	100
	43-44	20	100
	45-50	40	300
	51-52	60	100
P-VI	1-3	1	150
	4-6	2	150
	7-9	3	150
	10-30	4	1000
	31-32	5	100
	33-34	6	100
	35-36	7	100
	37-38	8	100
	39-40	9	100
	41-42	10	100
	43-44	20	100
	45-46	40	100
	47-50	60	200

The eluates obtained from each column were examined by TLC (SiO_2) using 6% methanol in chloroform as the developing system. Fractions with similar chromatographic pattern were combined to yield 5 fractions, as shown in Table 8.

Table 8 Combination of fractions from column chromatography of P-I, P-II and P-VI

Combined fractions	Fractions	Total Weight (g)	Volume of solvent (ml)
F-I	33-36 P-I 11-14 P-II 11-13 P-VI	1.225	550
F-II	37-41 P-I 15-18 P-II 14-16 P-VI	0.810	600
F-III	42-47 P-I 19-20 P-II 17-19 P-VI	0.513	550
F-IV	48-55 P-I 21-30 P-II 20-31 P-VI	0.500	1500
F-V	56-58 P-I 31-46 P-II 32-45 P-VI	0.500	1650

3.2.2.2 Isolation of Compound OC-1

Fraction P-XIII (1 g) was fractionated on a column using silica gel 60 (No.7734) (50 g) as the adsorbent. Elution was performed in a polarity gradient manner with chloroform and methanol. Fifty-two fractions, approximately of 25 ml, were collected. The eluates were examined by TLC (SiO_2) using 15% methanol in chloroform as the developing solvent. Fractions showing similar chromatographic pattern were combined. The TLC chromatogram of fractions 20-23 showed only one spot under UV light at 254 nm, R_f 0.13 (silica gel, 15% methanol in chloroform). These fractions were combined and evaporated under reduced pressure to give 413 mg of compound OC-1 as pale yellow needles (2.78% based on dried weight of leaves). It was later identified as 6-γ,γ-dimethylallyl taxifolin 7-*O*-β-D-glucoside (77).

3.2.2.3 Isolation of Compound OC-2

The combined fractions 19-20 of P-II gave a yellow powder after removal of the organic solvent. The powder was purified by washing with methanol. The TLC chromatogram of this purified powder showed only one spot under UV light at 254 nm, R_f 0.13 (silica gel, 6% methanol in chloroform). It was assigned as OC-2 (27 mg) (0.0015% based on dried weight of leaves) and later identified as 2″,3″-dihydroochnaflavone (80).

3.2.2.4 Isolation of Compound OC-3

Fraction F-I (1.225 g) gave a yellow amorphous powder after removal of the solvent. The TLC chromatogram of this powder showed two spots under UV light at 254 nm. This powder was fractionated on a column using

sephadex LH20 with methanol as the eluent. Twenty-one fractions, approximately of 20 ml, were collected. The eluates were examined by TLC (SiO_2) using 6% methanol in chloroform as the developing solvent. Fractions 5-12, showing only one spot under UV light at 254 nm with R_f 0.38, were combined and evaporated under reduced pressure to give 36 mg of compound OC-3 as a yellow amorphous powder (0.002% based on dried weight of leaves). It was later identified as 2″,3″-dihydroochnaflavone 7″-*O*-methyl ether (**81**).

4. Methylation of Pure Compounds

4.1 Methylation of OC-2

To a solution of OC-2 (2 mg) in MeOH (2 ml), Ag_2O (20 mg) and CH_3I (2 ml) were added. The reaction mixture was stirred at room temperature for 6 hours. After a usual work-up and purification by column chromatography (SiO_2, $CHCl_3$), a reaction product was obtained and assigned as OC-2-Me (2 mg).

4.2 Methylation of OC-3

To a solution of OC-3 (5 mg) in dimethylformamide (DMF) (5 ml), Ag_2O (40 mg) and CH_3I (3 ml) were added. The reaction mixture was stirred at room temperature for 6 hours. After a usual work-up and purification by column chromatography (SiO_2, $CHCl_3$), a reaction product was obtained and assigned as OC-3-Me (5 mg).

5. Physical and Spectral data of Isolated Compounds and Chemical Reaction Products

5.1 Compound FP-1

Compound FP-1 was obtained as yellow crystals (25 mg). It was soluble in chloroform.

EIMS : *m/z* (% relative intensity); Figure 5

314 (M^+, 100), 299 (97), 281 (27), 197 (32), 169 (23), 102 (25), 77 (22), 69 (44)

^{1}H NMR : δ_{ppm}, 300 MHz, in $CDCl_3$; see Figure 6 and Table 9

^{13}C NMR : δ_{ppm}, 75 MHz, in $CDCl_3$; see Figure 7 and Table 9

5.2 Compound OC-1

Compound OC-1 was obtained as yellow crystals (413 mg). It was soluble in methanol.

Melting Point : 170-172°C

$[\alpha]^{20}_D$: -6.57° (*c* 0.502 g/100 ml, in methanol)

UV : λ_{max} nm (log ε), in methanol ; Figure 18

346 (3.57), 289 (4.31), 207 (4.55)

IR : ν_{max} cm^{-1}, KBr disc ; Figure 19

3413 (br), 2921, 1636, 1583, 1445, 1286, 1176, 1101, 986

FAB-MS : *m/z* (% relative intensity) ; Figure 20

535 ([M+H]$^+$, 8), 373 (10), 317 (24), 155 (60), 135 (35), 119 (100), 103 (43), 85 (59), 77 (11), 57 (14)

^{1}H NMR : δ_{ppm}, 300 MHz, in DMSO-d_6 ; see Figure 21 and Table 11

^{13}C NMR : δ_{ppm}, 75 MHz, in DMSO-d_6 ; see Figure 22 and Table 11

5.3 Compound OC-2

Compound OC-2 was obtained as a yellow powder (27 mg). It was soluble in methanol.

Melting Point : >300°C

$[\alpha]^{20}_D$: +6.76° (*c* 0.207 g/100 ml, in methanol)

UV : λ_{max} nm (log ε), in methanol ; Figure 34

334 (4.33), 289 (4.43), 209 (4.78)

IR : ν_{max} cm^{-1}, KBr disc ; Figure 35

3250 (br), 1648, 1616, 1500, 1435, 1353, 1304, 1261, 1161, 1089, 1032, 838, 758, 737

FAB-MS : *m/z* (% relative intensity) ; Figure 36

541 ([M+H]$^+$, 9), 309 (12), 155 (61), 135 (42), 119 (100), 103 (46), 85 (57), 77 (10), 57 (14)

^{1}H NMR : δ_{ppm}, 300 MHz, in DMSO-d_6 ; see Figure 37 and Table 12

^{13}C NMR : δ_{ppm}, 75 MHz, in DMSO-d_6 ; see Figure 38 and Table 12

5.4 Compound OC-2-Me (87)

EIMS : *m/z* (% relative intensity) ; Figure 50

610 ([M]$^+$, 0.6), 331(19), 182(21), 170(100), 155(10), 140(17), 128(31), 124(37), 110(86), 98(36), 82(40), 70(58)

^{1}H NMR : δ_{ppm}, 300 MHz, in $CDCl_3$; Figure 49

δ 2.78 (1H, dd, *J* = 17.1, 3.1 Hz, H-3″), δ 3.04 (1H, dd, *J* = 17.1, 12.8 Hz, H-3″), δ 3.79 (3H, s, 7″-OCH_3), δ 3.88 (3H, s, 5″-OCH_3), δ 3.89 (3H, s, 7-OCH_3), δ 3.90 (3H, s, 4′-OCH_3), δ 3.93 (3H, s, 5-OCH_3), δ 5.38 (1H, dd, *J* = 12.8, 3.1 Hz, H-2″), δ 6.07 (1H, d, *J* = 2.2 Hz, H-6″), δ 6.14 (1H, d, *J* = 2.2 Hz, H-8″), δ 6.34 (1H, d, *J* = 2.1 Hz, H-6), δ 6.51 (1H, d, *J* = 2.1 Hz, H-8), δ 6.55 (1H, s, H-3), δ 6.92 (2H, d, *J* = 8.9 Hz, H-3‴ and H-5‴), δ 7.10 (1H, d, *J* = 8.5 Hz, H-5′), δ 7.40 (2H, d, *J* = 8.9 Hz, H-2‴ and H-6‴), δ 7.53 (1H, d, *J* = 2.5 Hz, H-2′), δ 7.71 (1H, dd, *J* = 8.5, 2.5 Hz, H-6′)

5.5 Compound OC-3

Compound OC-3 was obtained as a yellow powder (36 mg). It was soluble in chloroform : methanol (3:2).

Melting Point : 180-182°C

$[\alpha]^{20}_D$: +7.69° (*c* 0.195 g/100 ml, in methanol)

UV : λ_{max} nm (log ε), in methanol ; Figure 52

333 (4.45), 288 (4.56), 211 (4.85)

IR : ν_{max} cm^{-1}, KBr disc ; Figure 53

3401 (br), 1651, 1619, 1507, 1437, 1352, 1300, 1161, 1088, 1031, 839, 772, 743

FAB-MS : *m/z* (% relative intensity) ; Figure 54

555 ([M+H]$^+$, 8), 309 (18), 155 (67), 135 (35), 119 (100), 103 (42), 85 (56), 77 (10), 57 (13)

^{1}H NMR : δ_{ppm}, 300 MHz, in DMSO-d_6 ; see Figure 55 and Table 13

^{13}C NMR : δ_{ppm}, 75 MHz, in DMSO-d_6 ; see Figure 56 and Table 13

5.6 Compound OC-3-Me (87)

^{1}H NMR : δ_{ppm}, 300 MHz, in $CDCl_3$; Figure 66

δ 2.78 (1H, dd, J = 16.5, 2.9 Hz, H-3″), δ 3.02 (1H, dd, J = 16.4, 13.2 Hz, H-3″), δ 3.80 (3H, s, 7″-OCH_3), δ 3.88 (3H, s, 5″-OCH_3), δ 3.89 (3H, s, 7-OCH_3), δ 3.90 (3H, s, 4′-OCH_3), δ 3.93 (3H, s, 5-OCH_3), δ 5.38 (1H, dd, J = 13.1, 2.5 Hz, H-2″), δ 6.08 (1H, d, J = 1.9 Hz, H-6″), δ 6.14 (1H, d, J = 2.1 Hz, H-8″), δ 6.35 (1H, d, J = 1.8 Hz, H-6), δ 6.51 (1H, d, J = 1.9 Hz, H-8), δ 6.60 (1H, s, H-3), δ 6.98 (2H, d, J = 8.6 Hz, H-3‴ and H-5‴), δ 7.10 (1H, d, J = 8.7 Hz, H-5′), δ 7.40 (2H, d, J = 8.5 Hz, H-2‴ and H-6‴), δ 7.54 (1H, d, J = 2.0 Hz, H-2′), δ 7.69 (1H, dd, J = 8.6, 2.0 Hz, H-6′)