CHAPTER III

EXPERIMENTAL

1. Source of Plant Material

The root of *Sophora exigua* Craib were collected from Sanamchaikade, Chachoengsao province, Thailand. Authentication was achieved by comparison with the herbarium specimens at Forest Herbarium, Royal Forest Department, Ministry of Agriculture and Cooperative, Phahonyothin road, Bangkok, Thailand.

2. General Techniques

2.1 Thin Layer Chromatography (TLC)

Adsorbent : The TLC plates for routine work were

1) precoated TLC plates of silica gel 60 F_{254} (E.Merck)

2) precoated TLC plates of silica gel C-18 F 254 (E.Merck)

Layer Thickness : 250 µm

Technique : One way, ascending

Distance : 6.0 cm

Temperature : Laboratory temperature (30-35 C)

Detection : 1) Ultraviolets light wavelengths of 254 and 365 nm

2) Anisaldehyde-sulphuric acid spraying reagent (0.5% methanolic solution of anisaldehyde with 5 % sulphuric acid)

2.2 Column Chromatography

2.2.1 Quick Column Chromatography

Adsorbent : Silica gel 60 (No. 9385) particle size 0.040-0.063 mm (230-400 mesh ASTM)

Packing Method : Dry Packing

Sample Loading : The sample was dissolved in small amount of organic solvent, mixed with a small quantity of adsorbent , then dried, triturated and added gently on the top of the column.

Examination of eluate : fractions were examined by TLC using ultraviolet light(254 and 365 nm) , and followed by anisaldehyde-sulphuric acid spraying reagent.

2.2.2 Flash Chromatography

Adsorbent : silica gel 60(No. 9385) particle size 0.040-0.063 mm(230-400 mesh ASTM)

Packing Method : a)dry packing
b)wet packing

Sample Loading : a)The sample was dissolved in small amount of organic solvent, mixed with a small quantity of adsorbent, then dried, triturated and added gently on the top of the column.
b)The sample was dissolved in small amount of organic solvent, and slowly added on the top of the column.

Examination of Eluate : Fractions were examined by TLC using ultraviolet light (254 and 365 nm), and followed by anisaldehyde-sulphuric acid spraying reagent.

2.2.3 Medium Pressure Liquid Chromatography (MPLC)

Adsorbent : All column used were pre-packed column, pre- packed column LichroprepR RP-18 (0.040- 0.063) for reverse phase chromatography

Chromatographic Pump : Buchi 681 chromatographic pump

Saturation time : column was saturated with eluant prior to add the extract for 30 minutes.

Sample Loading : The extract was dissolved in a small volume of eluant, filtered through Toyopak^{R} C-18 filter, and loaded on the top of the column.

Examination of Eluates : Fractions were examined by TLC using ultraviolet light (254 and 365), and followed by anisaldehyde-sulphuric acid reagent)

2.3 Spectroscopy

2.3.1 Ultraviolet (UV) Absorption Spectra

The spectra were obtained on a Hitachi UV-220 A spectrometer (The Scientific and Technological Research Equipment Center, Chulalongkorn University). The samples were dissolved and adjusted the concentrations using methanol (garantee grade, E.Merck) as solvent.

2.3.2 Infrared (IR) Absorption Spectra

The spectra were obtained on a Shimadsu IR-440 infrared spectrometer (The Scientific and Technological Research Equipment Center, Chulalongkorn University), using potassium bromide disc to determined the spectra.

2.3.3 Mass Spectra(MS)

The electron impact mass spectra (eims) were determined on a Jeol FX 3000 (The Scientific and Technological Research Equipment Center, Chulalongkorn University) or Fisons VG Trio 2000 (Department of Chemistry, Faculty of Sciences, Chulalongkorn University) double focusing spectrometer which was operated at 70 ev with inlet temperature of 150-240°C).

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

The nmr spectra were obtained with a Jeol - A 500 spectrometer (500 MHz for 1H nmr and 125 MHz for 13C nmr) (The Scientific and Technological Research Equipment Center, Chulalongkorn University) or Bruker BZH-200 spectrometer (200 MHz for 1H nmr and 50 MHz for 13C nmr) (Department of Chemistry, Faculty of Sciences, Chulalongkorn University).

2.4 Physical Constant

Melting points were determined by Gallenkamp melting point apparatus. The values recorded were uncorrected.

2.5 Solvents

Throughout this work, all organic solvent were commercial grade and had to be redistilled prior to use, excluding the solvents for MPLC were also filtered through filter paper.

2.6 Shinoda's Test

Shinoda's test carried out by adding magnesiun ribbon 1-2 pieces with concentrated hydrochloric acid to an ethanolic solution of samples. Positive tests gave red , magenta , violet , blue , pink , or yellow depended on the classes of flavonoid. The colors mentioned are only broad indications , because within a given class the color produced depends on the hydroxylation pattern and other substitution.

3. Bioactivity Determination

3.1 Brine Shrimp Lethality Activity Bioassay

This method, utilizing brine shrimp (*Artemia salina* Leach), was proposed as a simple bioassay for natural product research. It could detect a broad specstrum of pharmacological activities. In this work, the brine shrimp lethality activity bioassay was helpfully carried out by Dr. Colin C. Duke of Department of Pharmacy, The University of Sydney, Australia. The method was described as below (Meyer et al., 1982)

3.1.1 Hatching the Shrimp

Brine shrimp eggs were hatched in a shallow rectangular dish filled with artificial sea water. A plastic divider with several 2mm holes was joined in the dish to make two unequal compartments. The eggs (Ca 50 mg) were sprinkled in to the layer compartment which was darked, while the smaller compartment was illuminated. After 36 hours the phototropic nauplii were collected by pipette from the light side, having been separated by the divider from their shells.

3.1.2 Sample Preparation

Samples were prepared by dissolving 2 mg of sample in 2 ml of methanol (solution A), Solution B was prepared by diluting 0.05 ml of solution A to 0.5 ml with methanol. Appropriate amounts of solution (1500 µl A, and 100 µl B for 100, 10 µg/ml, respectively) were transfered to glass vial. The vials were allowed to dry in air overnight and, then added with 5 ml of artificial sea and deionized water (c=38 g/l). The control was prepared using only methanol

3.1.3 Method of Bioassay

Brine shrimps were transfer to each sample vial using a disposable pipette. A drop of dry yeast suspension (3 mg in 5 ml artificial sea water) was added as food to each vial. The vials were maintained under illumination. Survivors were counted after 24 hours, and the percent deaths at each dose and control were determined.

3.2 Red Blood Cell Ca^{2+}-ATPase and Mg^{2+}-ATPase Activity Assay

In This work, the RBC $Ca^{2+}$and Mg^{2+} ATPase activity was done by using erythrocyte membranes. The assay procedure was helpfully carried out by Dr. Colin C. Duke of Department of Pharmacy, The University of Sydney, Australia. The method of assay was briefly described as below.

3.2.1 Preparation of Erythrocyte Membranes

Calmodulin-depleted erythrocyte membranes were prepared by continuous filtration through a hollow fiber system, Ashahi plasma seperator as described by Price et al. Packed cells were obtained from the New South Wales Red Cross Transfusion, Sydney. The whole preparation was carried out at 4°C. 1 Unit of packed red cells was washed 3 times with isotonic buffer, containing 130 mM KCl, 20 mM Tris-HCl (pH 7.4), and the cells were collected by centrifugation at 4,000 RPM. The cells were hemolysed in a buffer containing 1 mM EDTA, 10 mM Tris-HCl (pH 7.4), 0.5 mM PMSF. The hemolyzate mixture was passed through the hollow fiber system until the membrane appeared white, and then washed with 10 mM potassium-Hepes (pH 7.4). The membranes were collected by centrifugation at 10,000 RPM for 20 min and resuspended in storage buffer containing 130 KCl, 2 mM dithiothreitol, 0.5 mM $MgCl_2$, 20 mM potassium-Hepes (pH 7.5). The membranes, with protein concentrations of 1.5-5.4 mg/ml, were stored at -80°C until required.

3.2.2 Ca^{2+}-ATPase and Mg^{2+}-ATPase assay

The assay was done by the method described by Price et al. Calmodulin depleted erythrocyte membranes (0.071-0.098 mg/ml) were incubated at 37°C for one hour in a total volume of 0.4 ml of 65 mM KCl, 20 mM potassium-Hepes, 5 mM $MgCl_2$, 50 μM $CaCl_2$ (50.4 μM free Ca^{2+}), 0.1 μM calmodulin, 0.1 mM EGTA. The reaction was started by adding 2 mM ATP (pH 7.4) and the phosphate liberated to the medium was determined spectrometrically (Bu, R., 1980). The activity of the Mg^{2+}-ATPase (assayed in the absence of added $CaCl_2$) was subtracted from the total activity assayed in the presence of Ca^{2+}. Phenols were dissolved in dimethyl sulfoxide (DMSO), the final concentration of DMSO in the assay mixture was 2.5%. DMSO itself had no effect on ATPase activity. The solutions of phenols were added to the reaction buffer before adding ATP.

4.The Extraction

The dried, coarsely powdered roots of *Sophora exigua* Craib (3 Kg) were extracted throughly by percolation with 95 % ethanol (30 liters) for two days. The percolate was evaporated under reduced pressure to yield black-brown syrupy mass (440 g).

The crude extract dissolved in small volume of methanol triturated with silica gel (240 g). This mixture was dried under the vaccum.then, it was fractionated by the quick column chromatography technique using a sintered glass filter column of silica gel (700 g, 20x10 cm). The first eluant was hexane:chloroform (1:1). This eluant used until the eluates were diluted and then used chloroform as the second eluant until this eluates were diluted. Methanol, the last eluant, was use to wash the column until the eluates were diluted. These eluates (hexane:chloroform (1:1), chloroform, methanol) were evaporate under reduced pressure to yield 3 fractions as shown below.

1)Hexane:chloroform (1:1) fraction (50 g) : This fraction was called F-001.
2)Chloroform fraction (145 g) : This fraction was called F-002.
3)Methanol fraction (105 g) : This fraction was called F-003.

4.1 Isolation of Chemical Constituents from the Fraction F-001

The fraction F-001, yellow viscous oil, was fractionated by the column chromatographic technique, using a column of silica gel (250 g, 4.5x8 cm) . The sample was dissolved in small amount of chloroform, mixed with silica gel 60 (35 g), dried, triturated and added to the top of column. The column was eluted with chloroform:methanol (95:5, 9:1, 3:1, 1:1), then washed with methanol. Fifty ml fractions were collected and compared by TLC. The eluting solvents were altered to more polar systems when the difference pattern on TLC were not observed. Those fractions of similar TLC pattern were combined, concentrated and named from F-004 to F-016.

table 5 Information of the isolation of fraction F-001 by column chromatography.

Fraction number	Eluting solvent	combined fraction
	chloroform:methanol	
1-40	95:5	F-004
41-67	9:1	F-005
68-89	9:1	F-006
90-123	9:1	F-007
124-150	4:1	F-008
151-175	3:1	F-009
176-189	3:1	F-010
190-210	3:1	F-011
211-233	3:1	F-012
234-260	1:1	F-013
261-275	1:1	F-014
276-287	1:1	F-015
	methanol	F-016

4.1.1 Isolation of Chemical Constituent from the Fraction F-007

F-007 (6.20 g) was shown by TLC (silica gel GF_{254}/chloroform:hexane 8:2) to contain at least four chemical constituents which gave orange colour to anisaldehyde-sulphuric acid spraying reagent.

It was dissolved in small amount of chloroform:acetone (98:2) and added to the top of column (120 g, 4.6 x 45 cm). The column was eluted with chloform : acetone (98:2 and 95:5) and washed with methanol. Thirty-ml fractions were collected, examined by TLC and the similar fractions were combined, concentrated and named from F-017 to F-025.

Table 6 Information of the isolation of F-007 by column chromatography

Fraction number	eluting solvent	Combined fraction	remarks
	chloroform : acetone		
1-10	98:2	F-017	trace of substances
11-25	98:2	F-018	containing at least three substances (0.80g)
25-33	98:2	F-019	containing at least four substances (1.20g)
35-45	98:2	F-020	containing at least five substances (1.07g)
46-51	98:2	F-021	containing traces of substances (0.60 g)
52-60	95:5	F-021	containing at least three substances (0.47g)
61-79	95:5	F-023	containing at least three substances (0.58g)
80-91	95:5	F-024	containing traces of substances (0.35 g)
92-100	95:5	F-025	containing traces of substances (0.21 g)

The substance were detected under uv light at 254 and 365 nm.

4.4.1.1 Isolation of Compound SE-1 from Fraction F-018

The TLC chromatogram of fraction F-018 showed that there was one major compound which was quenching under ultraviolet light (254) and became orange when sprayed with anisaldehyde-sulphuric acid spraying reagent. The isolation for this major compound was carried out by a two-step process. Fraction F-018 (800 mg) was isolated using a column of silica gel (60 g,2x30 cm) eluted with 10 % petroleum ether in chloroform. Fifty ml fractions were collected based on the TLC patterns. The fractions of the major compound after being examined by TLC, were combined and isolated using a column of silica gel (35 g,1.6x30 cm) eluted with 10 % methanol in benzene. Ten-ml fractions were collected based on the TLC patterns. The major compound was separated out and combined (Fig.9). The major compound was obtained as a yellow viscous oil and was named as SE-1. It yielded 30 mg (0.06 % of F-001), was identified as exiguaflavanone B (65).

4.2 Isolation of Chemical Constituent from Fraction F-002

The fraction F-002 (145 g), black-brown syrupy mass, was fractionated by the column chromatographic technique, using a column of silica gel (1.4 kg, 10x85 cm). The sample was dissolved in small amount of chloroform, mixed with a silica gel 60 (260 g), dried ,triturated and added to the top of column. The column is eluted with chloroform:hexane (1:1), chloroform, 15 % acetone in chloroform to 50 % acetone in chloroform, respectively, then washed with methanol. Thousand ml fractions were collected and compared by TLC. Those fractions of similar TLC pattern were combined, concentrated and named from F-031 to F-041.

Table 7 Information of the Isolation of fraction F-002 by column chromatography.

Fraction number	Eluting Solvent	Combined Fraction
	chloroform:hexane	
1-3	50:50	F-031
4-11	50:50	F-032
12-17	chloroform	F-033
18-22	chloroform	F-034
23-28	chloroform	F-035
	chloroform:acetone	
29-36	85:15	F-036
37-40	85:15	F-037
41-43	70:30	F-038
44-45	70:30	F-039
46-48	60:40	F-040
49-52	50:50	F-041

4.2.1 Isolation of Compound SE-2 and SE-3 from Fraction F-040

The white amorphous compound (9.5 g) was crystallized from the fraction F-040. The TLC chromatograms of this compound showed one spot in many solvent systems but it showed two spot in reversed phase TLC chromatograms.

The white amorphous compound from fraction F-040 was divided to 1 g for isolation. The isolation of this compound used the MPLC reversed phase column (Lichoprep^{R} RP-18 column , 2x30cm). The MPLC pump was operated using 70 % acetonitrile in water as an eluant (operating pressure 1-2 bar, flow rate 3-4 ml/min). Ten-ml fractions were collected and examined. Those fractions of similar TLC pattern were combined to give following fractions (Fig 10).

1) fraction 2-6 afforded SE-2 (200 mg)

2) fraction 13-15 afforded SE-3 (90 mg)

The flavonoids SE-2 (200 mg) was isolated from the fraction 2-6. It was concentrated to small volume and obtained white neddles which were purified by recrytallization. It was identified as exiguaflavanone A (66)

The flavonoid SE-3 (90 mg) was isolated from the fractions 13-15. It was concentrated to small volume and obtained as white needles which were purified by recrystallization. It was identified as kushenol A (67).

4.2.2 Isolation of Compound SE-4 from Fraction F-041

The fraction F-041 (5 g) was shown by TLC (silica gel GF_{254} / chloroform : hexane , 7:3) to contain at least three chemical constituents which gave orange colour to anisaldehyde-sulphuric acid spraying reagent.
It was dissolved in small amount of chloroform, mixed with silica gel 60 (4 g) , dried, triturtated and added to the top of column (150 g, 4 x 35 cm) The column is eluted with 20 % acetone in hexane. Fifty-ml fractions were collected based on the TLC pattern. The fractions of the major compound after examined by TLC, were combined and isolated using a column silica gel (60 g, 2 x 30 cm) eluted with 10 % ethyl acetate in hexane. Thirty-ml fraction were collected based on the TLC patterns (Fig. 10). The major compound was separated out and combined.

The major compound was obtained as amorphous powder. It yielded 20 mg (0.01 % of F-002) , was named as SE-4, and was identified as 5,7,2',4',6' pentahydroxy-8-lavandulylflavanone (68).

5. Characterization of Isolated Compounds

5.1 Compound SE-1

EIMS ; m/z (relative intensity) ; Figure 11
438 (4), 315 (65) , 297 (100) , 179 (55) ,123 (4) ,69 (9)

UV ; λ_{max} nm (ε) , in methanol ; Figure 12
289 (4249) , 340 (876)
; λ_{max} nm (ε) , in methanol with $AlCl_3$/HCl; Figure 12
312 (6263) , 390 (1314)

IR ; ν cm^{-1} , NaCl plates , Figure 13
3455, 2928, 1635

^1H NMR ; δ ppm, 500 MHz, in deuterated chloroform ; Figure 14
1.46 (3H, singlet) , 1.58 (3H, s), 1.63 (3H,s), 2.01 (1H, dt, *J* = 20 , 6.7 Hz) , 2.02 (1H, *J* = 20 , 8.3 Hz) , 2.24 (1H, tt , *J* = 8.3 , 6.1) , 2.55 (1H, dd *J* = 14, 6.1 Hz) , 2.65 (1H , dd , *J* = 14 . 8.3 Hz) , 2.83 (1H, dd, *J* = 17.4,2.8 Hz) , 3.22 (1H, dd, *J* =14.8,17.4Hz), 3.84 (3H, s) , 4.48 (1H , brd , *J* = 1.1 Hz), 4.64 (1H, p , *J* = 1.1 Hz) , 4.99 (1H, brt, *J* = 7 Hz), 5.95 (1H, dd,*J* = 14.8, 2.8 Hz) , 6.15 (1H , s) , 6.24 (2H, s) , 6.45 (2H, d, *J* = 8.3 Hz) , 7.09 (1H, t, *J* = 8.3 Hz), 12.25 (1-OH ,s)

^{13}C NMR ; δ ppm, 125 MHz, in deuterated chloroform ; Figure 16
17.71, 18.80, 25.60, 26.75, 31.48, 41.27, 55.82, 75.54, 93.54, 102.99, 108.92 (2 signals) , 109.41, 110.53, 110.93, 122.95, 130.03, 131.99, 148.02, 154.59 (2 signals) ,157.96, 163.05, 166.05,196.90

5.2 Compound SE-2

EIMS ; m/z (relative intensity) ; Figure 21
424 (3) , 407 (2) , 301 (56) , 288 (100) , 219 (9) , 165 (62) , 136 (8) , 123 (15) , 69 (8)

UV ; λ_{max} nm (ε) , in methanol ; Figure 22
291 (15155) , 342 (2756)
; λ_{max} nm (ε) , in methanol with $AlCl_3$/HCl; Figure 22
314 (20966) , 390 (3180)

IR ; ν cm^{-1} , KBr disc , Figure 23
3550, 3180, 1650

^{1}H NMR ; δ ppm, 500 MHz, in deuterated acetone ; Figure 24
1.48 (3H, singlet) , 1.54 (3H, s), 1.61 (3H,s), 2.06 (2H, m) , 2.53 (1H, dd J = 17, 14 Hz) , 4.55 (2H, m) , 4.96 (1H, brt, J = 5.5 Hz), 6.02 (1H, s) , 6.15 (1H , dd, J = 14, 4 Hz) , 6.98 (2H, d, 9 Hz) , 7.03 (1H, t, J = 9 Hz), 12.26 (1-OH ,s)

^{13}C NMR ; δ ppm, 50 MHz, in deuterated acetone ; Figure 26
17.84, 19.07, 25.83, 27.73, 31.93, 40.65, 47.85, 73.76, 96.29, 103.27, 107.88, 108.45 (2 signals) , 110.89, 111.12, 124.46, 130.80, 131.59, 149.17, 157.69 (2 signals) , 162.25 , 163.14, 165.07, 198.72

melting point ; 169-170 °C (uncorrected)

5.3 Compound SE-3

EIMS ; m/z (relative intensity) ; Figure 28
408 (12) , 393 (20) , 288 (3), 285 (100) , 267 (78) , 219 (10) , 165 (66) , 123 (7) , 120 (4) , 19 (7)

UV ; λ_{max} nm (ε) , in methanol ; Figure 29
291 (11057) , 342 (2244)
; λ_{max} nm (ε) , in methanol with $AlCl_3$/HCl; Figure 29
315 (15198) , 390 (2448)

IR ; ν cm^{-1} , KBr disc , Figure 30
3389, 3377, 1600

^{1}H NMR ; δ ppm, 500 MHz, in deuterated acetone ; Figure 31
1.47 (3H, d , J = 1.2 Hz) , 1.56 (3H, d , J = 1.2 Hz), 1.66 (3H,dd, J = 1, 1 Hz), 2.07 (2H, m) , 2.54 (1H, m) , 2.90 (1H, dd, J = 17, 3 Hz) , 3.30 (1H, dd, J =17,14 Hz) , 4.57 (1H , brd , J = 1 Hz), 4.61 (1H, p , J = 1 Hz) , 5.00 (1H, triplets of heptet , J = 6.7 , 1.2 Hz), 5.74 (1H, dd, J = 14, 3 Hz) , 6.05 (1H , s) , 6.95 (1H, dd, J = 7.5, 1 Hz) , 6.96 (1H, ddd, J = 7.5, 7.5, 1 Hz) , 7.22 (1H, ddd , J = 7.5, 7.5, 1.8Hz), 7.62 (1H, dd , J = 7.5 , 1.8 Hz), 12.16 (1-OH ,s)

^{13}C NMR ; δ ppm, 50 MHz, in deuterated acetone ; Figure 33
17.84, 19.20, 25.81, 27.80, 31.87, 42.78, 47.85, 75.46, 96.38, 103.22, 107.92, 111.21, 116.17, 120.68, 124.40, 126.77, 127.39, 130.00, 131.70, 149.14, 154.63, 162.79, 163.08, 165.41, 197.79

melting point ; 173-174 °C (uncorrected)

5.4 Compound SE-4

EIMS ; m/z (relative intensity) ; Figure 35
440 (4), 422 (3) , 317 (28) , 299 (100) , 165 (38) ,
69 (23) , 123 (12)

UV ; λ_{max} nm (ε) , in methanol ; Figure 36
289 (4249) , 340 (876)
; λ_{max} nm (ε) , in methanol with $AlCl_3$/HCl; Figure 36
312 (6263) , 390 (1314)

IR ; ν cm^{-1} , KBr disc , Figure 37

^1H NMR ; δ ppm, 500 MHz, in deuterated acetone ; Figure 38
1.56 (3H, d , J = 1.2 Hz) , 1.62 (3H, d , J = 1.2 Hz), 1.71 (3H,dd , J = 1.2 , 1.1 Hz), 2.08 (2H, m) , 2.59 (1H, m) , 2.47 (1H, dd, J = 18, 3 Hz) , 2.68 (2H, m) , 3.93 (1H, dd, J = 18, 14, Hz) , 4.57 (1H , brd, J = 1.2 Hz), 4.61 (1H, dq , J = 1.3 , 1.2 Hz) , 5.05 (1H, triplets of heptet , J = 7 , 1.2 Hz), 5.07 (1H, s), 5.90 (1H, dd, J = 14, 3 Hz) , 6.03 (2H , s) , 8.33 (1-0H, s), 8.49 (2-OH,s), 12.64 (1-OH ,s)

^{13}C NMR ; δ ppm, 50 MHz, in deuterated acetone ; Figure 41
17.93, 18.94, 25.89, 27.42, 32.15, 41.15, 47.59, 76.44, 95.14, 96.12 (2 signals), 102.94, 103.93, 108.19, 111.26, 124.56, 131.54, 149.10, 158.65, (2 signals), 162.80, 163.06, 163.11, 165.12

melting point ; 170-172 °C (uncorrected)