

CHAPTER III

EXPERIMENTAL

The study of chemical constituents in plant must proceed stepwise from selection and authentication of plant material, through collection, extraction, isolation, purification, and structure elucidation of the isolated compounds.

1. Source and Authentication of Plant Material

The stems of *Coscinium fenestratum* (Gaertn.) Colebr. were collected in November, 1990 from a liana growing in the garden in Amphor Makham, Chanthaburi, Thailand. The material was identified by Professor N.P. Nootboom of the Rijksherbarium, Hortus Botanicus, Lieden, The Netherland.

2. General Technique

2.1 Thin layer chromatography (TLC)

2.1.1 Analytical

Adsorbents : The TLC plates for routine work were precoated TLC Plates of Silica gel 60GF-254 (E.Merk) or Pre-coated TLC Plates of Aluminium oxide F-254 (Type E., E. Merck)

Layer thickness : 250 μ m.

Technique : One way, ascending, 6.5 cm.

Solvent system : (1) silica gel 60 GF-254/
chloroform: ethyl acetate: methanol (7:2:1)
(2) silica gel 60 GF-254/chloroform:
methanol (8:2)+1% of ammonium hydroxide
(3) silica gel 60 GF-254/
chloroform: methanol : acetone (7:2:1)
(5) silica gel 60 GF-254/
chloroform : methanol (9:1)
(6) silica gel 60 GF-254/chloroform :
methanol (8:2)+1% of diethylamine

Temperature : Laboratory temperature (20 -30 °C)

Detection : (1) Ultraviolet light at wavelength 360 nm.
(2) Dragendorff's spray reagent

Solution A:

Bismuth subnitrate (850 mg), distilled
water (40 ml), and acetic acid (10 ml)

Solution B:

Potassium iodide (8 gm) and distilled
water (20 ml)

Solution A and B, each of 5 ml, wer
mixed. Then 20 ml of glacial acetic
acid and 70 ml of distilled water were
added and used as a general alkaloidal
detecting reagent, the alkaloids give
orange spots as positive test.

2.1.2 Preparative

Adsorbent : Pre-Coated for preparative layer chromatography plates silica gel 60 GF-254 (E.Merk) were used:

Layer thickness : 1 mm.

Technique : one way, ascending, 15 cm (double development)

Application : As a continuous streak using a capillary tube

Solvent system : Chloroform:methanol : ethyl acetate (7:1:2)
+ 1 % of ammonium hydroxide

Temperature : Laboratory temperature (20-30°C)

Detection : The bands were visualized in ultraviolet light (254 nm), scraped off and the alkaloids, eluted from the silica gel by shaking with methanol : chloroform (8:2), filtered through sintered glass and evaporated to dryness.

2.2 Column chromatography

Adsorbents : 1. silica gel 60 GF-254 for thin-layer chromatography Art. 7730
2. silica gel 60 for column chromatography 9385 (230-240 mesh ASTM)

Packing : Adsorbent poured as a suspension into the column.

Addition of alkaloidal material

: Alkaloidal material was dissolved in small volume of volatile solvent and gently placed on top of the column.

Technique : Open column chromatography.

Short column chromatography.

Solvents : Chloroform, ethyl acetate, acetone, methanol,
ammonium hydroxide, diethylamine

Examination of eluate

: Fractions were examined by TLC using ultraviolet light at wavelength 360 nm. and followed with Dragendorff's spray reagent.

2.3 Physical constant

All melting points were determined on the Buchi 520 melting point apparatus. The values recorded are uncorrected.

2.4 Spectroscopy

2.4.1 Infrared absorption spectra were performed on a Shimadzu 440 and FT-IR. The materials were examined in potassium bromide disc. (The scientific and Technological Research Equipment Center, Chulalongkorn University).

2.4.2 Mass spectra were determined on a Jeol FX 3000 double focusing spectrometer for EI. Operating at 70 ev. with inlet temperature 150°C-240°C (The scientific and Technological Research Equipment Center, Chulalongkorn University).

2.4.3 Proton nuclear magnetic resonance (¹H-NMR) spectra were obtained with a Bruker BZH-200 (200MHz) spectrometer. Chemical shifts were reported in ppm scale, using Dimethyl sulfoxide-d₆ (DMSO-d₆) and deuteriochloroform (CDCl₃) as operating solvent. (Department of Chemistry, Faculty of Sciences, Chulalongkorn University).

2.4.4 ^{13}C - nuclear magnetic resonance (^{13}C -NMR) were obtained by the same spectrometer as the ^1H -NMR.

2.5 Solvents

Throughout the work, all organic solvents were redistilled before use.

2.6 Authentic alkaloids

2.6.1 Berberine authentic alkaloid is supplied by Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2.6.2 Crebanine authentic alkaloid is kindly supplied by Associate Professor Kalaya Pharadai.

3. The Extraction and Isolation of Alkaloids from the Stems of *Coscinium fenestratum* Colebr.

3.1 The extraction of alkaloids

The dried coarsely powdered stems (10.5 kg) were extracted with methanol (30 L.). It was allowed to macerate for four days and filtered. The marc was remacerated for two days with five successive portions of methanol (10 L-portion) and filtered. Testing for complete extraction was carried out with Dragendorff's spray reagent. The combined filtrate was concentrated to syrupy mass under reduced pressure to yield 957 gm of dry crude alkaloidal extract (9.1% based on dry stem). TLC analysis of crude alkaloidal extract showed that at least four

alkaloids were present with the addition of base-line alkaloid(s).

3.2 The isolation of alkaloids

Crude alkaloidal extract (15 gm) was dissolved in chloroform (20 ml) and gently placed on top of silica gel 9385 column (15 x 50 cm.) holding chloroform and have repeated the same in 3 times. The column was eluted with chloroform and then with chloroform containing increasing amounts of methanol and finally with methanol : chloroform = 9:1 until no traces of alkaloid could be detected. Fraction of 250 ml were collected and compared by TLC. Those fractions of similar alkaloidal pattern were combined and evaporated to dryness under reduced pressure to give the following fractions:

(1) fractions 1-25 containing no alkaloid assigned as fraction C-1 (10.521 gm)

(2) fractions 26-50 were combined and assigned as fraction C-2 (2.332 gm)

(3) fractions 51-70 were combined and assigned as fraction C-3 (7.052 gm)

(4) fractions 71-75 were combined and assigned as fraction C-4 (1.235 gm)

(5) fractions 76-82 were combined and assigned as fraction C-5 (12.530 gm)

(6) methanolic fractions were combined and assigned as fraction C-6 (12.120 gm), shown by TLC to contain traces of alkaloidal mixture and the base-line alkaloid(s). No

further study has been made.

3.2.1 Isolation of alkaloids from the fraction C-2

The fraction C-2 (2.332 gm) was shown by TLC to contain at least 2 alkaloids. It was dissolved in chloroform (7 ml) and gently placed on top of silica gel column (5 x 50 cm) holding chloroform. The column was eluted with chloroform : methanol (9:1) and then with chloroform containing increasing amounts of methanol, fractions of 50 ml were collected and by TLC analysis the liked fractions were combined to give the following portions

C-2a (fractions 1-15) containing no alkaloid.

C-2b (fractions 16-40) containing one alkaloid. It was assigned as Cf-1 (21 mg) and subsequently identified as tetrahydropalmatine.

C-2c (fractions 41-80) containing mixture of alkaloid Cf-1 and other alkaloid. (35 mg).

3.2.2 Isolation of alkaloids from the fraction C-3

The fraction C-3 (7.052 gm) was shown by TLC to contain mixture of at least 2 alkaloids. It was dissolved in chloroform : methanol : ethyl acetate (7:1:2) and gently placed on top of silica gel column (5 x 30 cm) holding chloroform : methanol : ethyl acetate (7:1:2). The column was eluted with this solvent system.

The fraction of 50 ml were collected and examined by TLC, the similar fractions were combined to give the following portions:

C-3a (fractions 1-15) containing no alkaloid.

C-3b (fractions 16-30) containing mixture of 2 alkaloids and one of them is Cf-1. This portion was rechromatogram by treated with chloroform : methanol (8:2) + 1 % ammonium hydroxide. Yielding Cf-2 and subsequently identified as berberine.

C-3c (fractions 31-60) containing mixture of 2 alkaloids One of them is berberine.

3.2.3 Isolation of alkaloids from the fraction C-4

The fraction C-4 (1.235 gm) was shown by TLC to contain mixture of two alkaloids. One of them liked berberine and the other one is overlapped the former. It was then dissolved in chloroform : methanol (8:2) and gently placed on top of silica gel column (3.5 x 45 cm) holding chloroform : methanol (8:2) and was eluted with chloroform : methanol (8:2) with 1% of diethylamine. Berberine was isolated and weighed 150 mg.

3.2.4 Isolation of alkaloids from the fraction C-5

The fraction C-5 (12.530 gm) was shown by TLC to contain at least 2 alkaloids including the base-line alkaloid(s). It was then dissolved in chloroform : methanol (8:2) and gently placed on top of silica gel column (7 x 45 cm) holding chloroform : methanol (8:2). The column was eluted with chloroform : methanol (8:2) adding 1% ammonium hydroxide. The volumes of eluting solvents used were 3000 ml and yielding 2 alkaloids and traces of other

alkaloids. The alkaloid was separated by preparative TLC plates using chloroform : methanol : ethyl acetate (7:1:2) + 1% of ammonium hydroxide as developing solvent. Cf-3 (42 mg) and Cf-4 (54 mg) were separated and assigned as crebanine and jatrorrhizine, respectively.

4. Reduction of Quaternary Protoberberine Alkaloids

A small amount of quaternary alkaloid was dissolved in methanol and NaBH_4 was added until the reduction was completed (followed by TLC). The solution was taken to dryness and then dissolved in chloroform and filtered. The chloroform solution was taken to dryness.

5. Identification and Characterization of the Isolated Alkaloids

The isolated alkaloids were identified by comparison of the R_f values, melting points, infrared, nuclear magnetic resonance, and mass spectra with authentic samples of published values, as indicated.

The R_f values given in table 5 are those obtained with the following solvent systems:

- (1) silica gel 60 GF-254/chloroform : methanol (9:1)
- (2) silica gel 60 GF-254/chloroform : methanol : ethyl acetate (7:2:1)
- (3) silica gel 60 GF-254/chloroform : acetone (6:4)
- (4) silica gel 60 GF-254/n-hexane : methanol (7:3)

(5) silica gel 60 GF-254/chloroform : methanol :
acetone (5:3:2)

5.1 Identification of Cf-1 as tetrahydropalmatine

Cf-1 was obtained as pale yellow feather-like crystals from chloroform - methanol. It is soluble in chloroform.

Melting point : 150-152 °C

Molecular weight : 355 (mass spectrometry)

hRf value : see Table 5 (page 203)

Infrared absorption spectrum : (KBr) (Figure 8, page 229)

$\bar{\nu}_{\max}$ (cm⁻¹) : 1050, 1420, 1580, 2700

Mass spectrum : (EI) (Figure 11, page 234)

m/e (% , relative intensity)

356[(M+1), 23], 355(M⁺, 100), 354(65), 340(9),

324(18), 192(11), 190(29), 164(92),

149(63), 121(12)

Proton Magnetic Resonance : (CDCl₃, 200 MHz)

(Figure 9 , page 232)

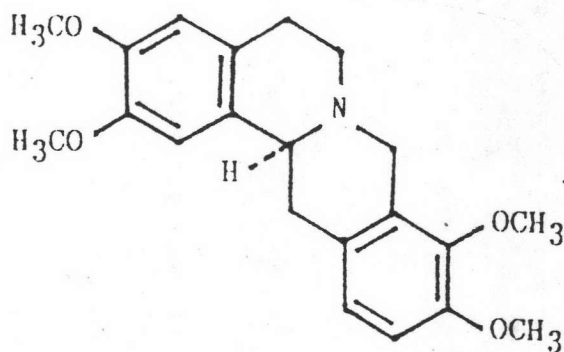
<u>Chemical shift</u> δ (ppm)	<u>Proton</u>	<u>Multiplicity</u>
6.89	C(12)-H	1H, d (J = 8 Hz)
6.79	C(11)-H	1H, d (J = 8 Hz)
6.74	C(1)-H	1H, s
6.63	C(4)-H	1H, s
4.25	C(8)-H _{eq}	1H, d (J = 15.8 Hz)
3.90	C(10)-OCH ₃	3H, s
3.88	C(9)-OCH ₃	3H, s
3.86	C(2),C(3)-OCH ₃	6H, s
3.55	C(8)-H _{ax}	1H, d (J = 15.8 Hz)

¹³C-NMR spectrum : (CDCl₃, 200 MHz)

(Figure 10 , page 233)

(Table 6 , page 204)

These data are in agreement with the published values of tetrahydropalmatine (Verpoorte *et al*, 1981; Ruangrunsi, 1986; Hussain *et al*, 1989). It is therefore concluded that Cf-1 is tetrahydropalmatine.



Tetrahydropalmatine

5.2 Identification of Cf-2 as berberine

Cf-2 was obtained as yellow needle crystals. It was soluble in methanol.

Melting point : 218-220 °C

Molecular weight : 336 (Mass spectrum)

hRf : see Table 5 (page 203)

Infrared absorption spectrum : (KBr) (Figure 14, page 237)

$\bar{\nu}_{\max}$ (cm⁻¹) : 1050, 1480, 1600, 3400

Mass spectrum : (EI) (Figure 13, page 236)

m/e (% , relative intensity)

339(M+1), 174(45), 164(90), 149(100), 77(50)

Proton Magnetic Resonance : (DMSO-d₆, 200 MHz)

(Figure 12, page 235)

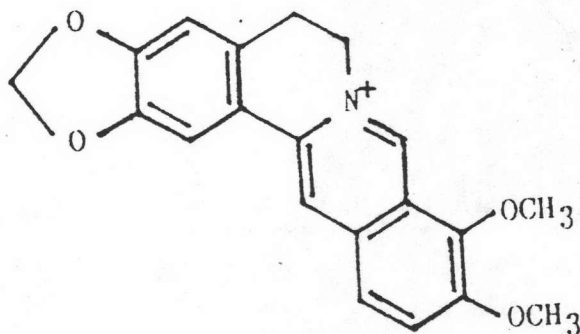
<u>Chemical shift</u> (ppm)	<u>Proton</u>	<u>Multiplicity</u>
9.91	C(8)-H	1 H, s
8.96	C(13)-H	1 H, s
8.22	C(11)-H	1 H, d(J = 9 Hz)
8.01	C(12)-H	1 H, d(J = 9 Hz)
7.81	C(1)-H	1 H, s
7.10	C(4)-H	1 H, s
6.19	C(2,3)-(OCH ₂ O)	2 H, s
4.95	C(6)-2H	2 H, t(J = 6 Hz)
4.11	C(9)-OCH ₃	3 H, s
4.08	C(10)-OCH ₃	3 H, s
3.22	C(5)-2H	2 H, t(J = 6 Hz)

^{13}C -NMR spectrum : (DMSO- d_6 , 200 MHz)

(Figure 15, page 238)

(Table 6 , page 204)

Cf-2 is identical in hRf values and melting point with authentic sample of berberine sulfate and also confirmed by IR and ^1H -NMR spectra (Siwon, 1980; Blasko, *et al*, 1988). ^{13}C -NMR spectrum and the reduction product of Cf-2 is also identical in ^1H -NMR and mass spectra with tetrahydroberberine (Siwon, 1980; Ohiri *et al*, 1983). It is therefore concluded that Cf-2 is berberine.



Berberine

5.3 Identification of Cf-3 as crebanine

Cf-3 obtained as pale brownish needle crystals from chloroform : methanol (7:3). It is soluble in chloroform.

Melting point : 115-119 °C

Molecular weight : 339 (Mass spectrum)

hRf value : see Table 5 (page 203)

Infrared absorption spectrum : (KBr) (Figure 21, page 244)

$\bar{\nu}_{\max}$ (cm⁻¹) : 1050, 1430, 1520, 1680

Mass spectrum : (EI) (Figure 22, page 245)

m/e (% , relative intensity)

339(M⁺, 61), 338(100), 324(9), 323(9), 322(3),

308(8), 296(28), 281(8), 265(15), 162(7)

Proton Magnetic Resonance : (DMSO-d₆, 200 MHz)

(Figure 16, page 239)

<u>Chemical shift(ppm)δ</u>	<u>Proton</u>	<u>Multiplicity</u>
7.72	C(11)-H	1H, d (J = 8.7 Hz)
7.02	C(10)-H	1H, d (J = 8.7 Hz)
6.62	C(3)-H	1H, s
6.12	C(1,2) (O-CH ₂ -O)	1H, s
5.95		1H, s
*3.84	C(8)-OCH ₃	3H, s
*3.72	C(9)-OCH ₃	3H, s
3.53	C(6a)-H	1H, dd (J = 14.7 Hz, 4.3 Hz)
3.05	C(7)-H _{ax}	1H, dd (J = 10 Hz, 4.0 Hz)
2.82	C(4)-CH ₂	2H, m
2.50	N-CH ₃	3H, s
2.37	C(5)-CH ₂	2H, m
2.10	C(7)-H _{eq}	1H, dd (J = 7.0 Hz, 4.0 Hz)

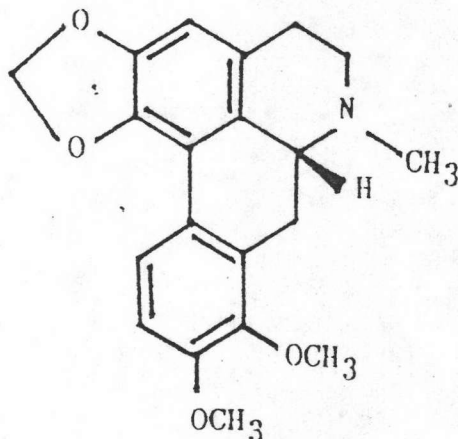
Note* : Assignments may be interchanged

^{13}C -NMR spectra of Cf-3 (DMSO- d_6 , 200 MHz)

(Figure 18., page 241)

<u>Carbon</u>	<u>Chemical shift δ(ppm)</u>
C (1)	142.0
C (1a)	126.0
C (1b)	129.0
C (2)	145.0
C (3)	106.5
C (3a)	127.0
C (4)	28.0
C (5)	52.0
C (6) - NCH_3	43.5
C (6a)	60.0
C (7)	26.5
C (7a)	115.5
C (8)	146.0
C (8) - OCH_3	61.0
C (9)	152.0
C (9) - OCH_3	55.0
C (10)	111.0
C (11)	123.0
C (11a)	123.5
C (1,2) - OCH_2O -	100.0

Cf-3 is identical in melting point, hRf values and spectrum data (UV, IR, Mass, NMR) values with authentic sample of crebanine obtained from *Stephania vanosa* Spreng. (Pharadai, k. et al, 1981) It is therefore concluded that Cf-3 is crebanine.



Crebanine

5.4 Identification of Cf-4 as jatrorrhizine

Cf-4 was obtained as pale brownish needle crystals from methanol. It is soluble in methanol.

Melting point : 240-245 °C

Molecular weight : 338 (Mass spectrum)

hRf value : see Table 5 (page 203)

Infrared absorption spectrum : (KBr) (Figure 25, page 248)

$\bar{\nu}_{\max}$ (cm⁻¹) : 1050, 3450

Mass spectrum : (EI) (Figure 29, page 252)

m/e (% , relative intensity)

338(M⁺,4), 337(15), 336(13), 320(12), 307(5),

164(1), 52(29), 50(100), 44(20)

Proton Magnetic Resonance : (DMSO-d₆, 200 MHz)

(Figure 23 , page 246)

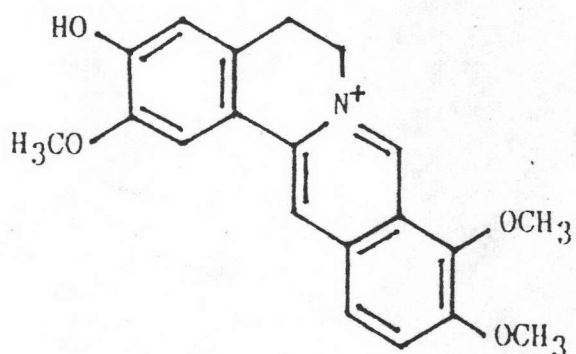
<u>Chemical shift(ppm)δ</u>	<u>Proton</u>	<u>Multiplicity</u>
9.68	C(8)-H	1H, s
8.73	C(13)-H	1H, s
8.10	C(11)-H	1H, d (J = 9 Hz)
7.91	C(12)-H	1H, d (J = 9 Hz)
7.46	C(1)-H	1H, s
6.62	C(4)-H	1H, s
4.81	C(6)-CH ₂	2H, t (J = 4 Hz)
4.07	C(9)-OCH ₃	3H, s
4.04	C(10)-OCH ₃	3H, s
3.86	C(2)-OCH ₃	3H, s
3.05	C(5)-CH ₂	2H, t (J = 4 Hz)

¹³C-NMR spectrum : (DMSO-d₆ , 200 MHz)

(Figure 24 , page247)

(Table 6 , page204)

These data are in agreement with the published values of jatrorrhizine (Siwon *et al*, 1980; Hussain *et al*, 1989). And the ¹H-NMR of reduction product of Cf-4 (figure...,page...) is identical with tetrahydrojatrorrhizine (Ohiri *et al*, 1983). It is therefore concluded that Cf-4 is jatrorrhizine.



Jatrorrhizine

Table 5 hRf values of the isolated alkaloids

Alkaloids	Solvent system				
	(1)	(2)	(3)	(4)	(5)
Cf-1	75	83	68	72	80
Cf-2	35	50	55	30	45
Cf-3	60	68	72	75	72
Cf-4	30	30	25	25	30

Note

$$\text{hRf} = \frac{\text{distance of spot center from start point}}{\text{distance of solvent front from start point}} \times 100$$

Table 6 ^{13}C -NMR spectra of Cf-1, Cf-2, Cf-4

Carbon	Chemical shift δ (ppm)		
	Cf-1	Cf-2	Cf-4
C (1)	109.4	105.5	109.2
C (2)	147.2	147.7	149.3
C (3)	147.2	149.8	144.5
C (4)	111.7	108.5	115.5
C (4a)	126.4	130.7	133.9
C (5)	28.6	26.4	26.2
C (6)	50.9	55.2	56.0
C (8)	53.4	145.5	143.4
C (8a)	127.7	121.4	118.0
C (9)	149.9	143.7	149.4
C (10)	144.4	150.4	143.4
C (11)	111.2	126.7	123.1
C (12)	123.7	123.6	126.8
C (12a)	128.3	132.9	129.5
C (13)	35.7	120.3	118.0
C (13a)	58.9	137.5	139.5
C (13b)	129.7	120.5	120.7
2,3-OCH ₂ O	-	102.1	-
2-OCH ₃	55.5	-	57.1
3-OCH ₃	55.8	-	-
9-OCH ₃	59.6	62.0	61.8
10-OCH ₃	55.5	57.1	55.5