



## CHAPTER III

### EXPERIMENTAL

#### Source of Plant Material

The stem bark of *Strychnos nux-blanda* A.W. Hill (Loganiaceae) used in this study were obtained from Ubon Ratchathani Province, Thailand in April 1989. The plant material was authenticated by comparison with the specimen at the Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences Chulalongkorn University. The stem barks were moistened with 95% ethanol immediately and grinded before maceration.

#### General Technique

##### 1. Thin-layer chromatography (TLC) for analytical

- Technique : one way, ascending
- Adsorbent : silica gel GF<sub>254</sub> (E.Merck) 30 g  
in 60 ml distilled water
- Plate sizes : 10 cm x 20 cm or 20 cm x 20 cm
- Layer thickness : 0.25 mm
- Activation : air-dried for 15 minutes and then  
heating in hot air oven at 110 °C  
for one hour.

- Solvent systems: 1) Chloroform : Ethyl acetate :  
Methanol: Ammonia (80:10:10:1)
- 2) Ethyl acetate : Isopropanol :  
Ammonia (80 : 15 : 5)
- 3) Chloroform : Methanol : Ammonia  
(90:10:1)
- 4) Ethyl acetate : Isopropanol :  
Ammonia (45:35:20)
- 5) Hexane : Chloroform ( 2:3 )

Distance : 10 cm

Laboratory Temperature : 25 - 30 °C

Development : The plate were developed in a chromatographic tank lined with filter paper.

Detection : 1) UV<sub>254</sub>  
2) Dragendorff's spray reagent  
3) Ferric chloride - perchloric acid  
spray reagent (heated at 110 °C for  
10 min)

## 2. Preparative Thin-Layer Chromatography (PLC)

Technique : one way ascending

Adsorbent : mixture of silica gel G (E. Merck) and  
silica gel GF<sub>254</sub> (E. Merck) (3 : 1)

Plates sizes : 20 cm x 20 cm and 20 cm x 40 cm

Layer thickness : 0.50 mm

Solvent systems : 1) Hexane : CHCl<sub>3</sub> (2 : 3)

2) CHCl<sub>3</sub> : Isopropanol : Hexane : DEA  
(10 : 15 : 15 : 3)

3) Hexane :  $\text{CHCl}_3$  : MeOH (5.5:11.5:3)

Distance : 18 cm

Laboratory Temperature : 25 - 30 °C

Development : The plates were developed in a chromatographic tank lined with filter paper

Detection : 1)  $\text{UV}_{254}$   
2) Dragendorff's spray reagent  
3) Ferric chloride - perchloric acid spray reagent

Substance Recovering : The scraped off zones were warmed with a mixture of  $\text{CHCl}_3$  : MeOH (1:1), and filtered. After removal of the solvent, the residues were taken up in  $\text{CHCl}_3$  and filtered.

Spray Reagents: 1) Dragendorff's spray reagent

The stock solution consisting of a mixture of bismuth oxynitrate 1.7 g, glacial acetic acid 20 ml, distilled water 80 ml and 5% aqueous potassium iodide 100 ml.

The working solution was made by mixing 10 ml of stock solution with 20 ml glacial acetic acid and 70 ml distilled water.

2) Ferric chloride - perchloric acid spray reagent

This reagent was made by mixing 1 ml

0.5 M ferric chloride solution with  
100 ml 35% aqueous perchloric  
acid solution

### 3. Column Chromatography

Column sizes : The glass column 3 - 10 cm in diameter  
depending on the quantity of sample  
Adsorbent : silica gel 0.040 - 0.063 mm (E. Merck)  
Packing method : wet packing  
Solvent : Several solvents were used for different  
materials

### 4. Medium Pressure Liquid Chromatography (MPLC)

Instrument : Medium Pressure Chromatography Buchi 681  
Column sized : The glass column 65 mm in diameter  
Adsorbent : Silica gel 0.015 - 0.040 mm (E. Merck for  
column chromatography)  
Packing : Dried packing  
Solvent : Hexane : Chloroform : MeOH (5.5 : 11.5 : 3)

### 5. Melting point

Melting points were determined on a Yanagimoto Micro  
Melting point apparatus.

### 6. Ultraviolet (UV) spectroscopy

Ultraviolet absorption spectra were determined on a  
Jasco Uvidec 650 double beam spectrophotometer.

#### 7. Infrared (IR) spectroscopy

Infrared absorption spectra were recorded as KBr disc on a Jasco Model 302 Spectrophotometer. The absorption bands were reported in wave number ( $\text{cm}^{-1}$ ).

#### 8. Nuclear Magnetic Resonances (NMR) Spectroscopy

The  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra were obtained with Nuclear Magnetic Resonance spectrometer Model FX 900 (JEOL) at 90 MHz.

#### 9. Mass spectroscopy

The low resolution mass spectra were obtained on a Mass spectrometer Model DX 300 (JEOL). A direct inlet system operating at 70 eV with the temperature range between 150 to 300 was used.

#### 10. Solvent

The solvents of commercial grade were redistilled before used.

#### 11. Reference Compound

The reference compounds, strychnine 54 and brucine 55 were available in the laboratory of the Department of Pharmaceutical Botany, the Faculty of Pharmaceutical Sciences, Chulalongkorn University .

## Isolation of Chemical Substances from *Strychnos nux-blanda*

A.W. Hill stem bark

### 1. Extraction

Chopped fresh stem barks (12.2 kg.) were moistened with concentrated ammonia solution for one night and after that macerated with chloroform three times for three days-period (35 L, 20 L and 20 L). The chloroform extracts were combined, and evaporated under reduced pressure to give a dark viscous mass (32.33 g equal to 0.27%). The viscous mass (32.33 g) was dissolved in 250 ml of chloroform and extract with 6 quantities each of 250 ml of diluted sulfuric acid. Then made the combined extracts alkaline with concentrated ammonia and extract with chloroform gave crude alkaloids

### 2. Isolation of chemical compounds

#### 2.1 Column chromatography

The crude alkaloids was subjected to silica gel column chromatography, using chloroform : methanol (19:1) as eluent. Six hundred twenty fractions (15 ml, each) were collected and the column was washed with methanol those of similar fractions when examined on thin-layer chromatography were combined and evaporated.

Combined fraction 1 - 482 (3.93 g) showed the presence of 4 spots on checked TLC in system Hexane :  $\text{CHCl}_3$  2:3 and Ferric chloride-Perchloric acid as a spray reagent this fraction

was designated as fraction P.

Combined fraction 483 - 539 (10 - 17 gm) showed the presence of 4 spot on checked TLC in system  $\text{CHCl}_3$  : Isopropanal : Hexane : DEA (10: 15 : 15 : 3) and Ferric chloride - Perchloric acid as a spray reagent. This fraction was designated as fraction C.

Combined fraction 540 - 620 ( 9.74 g) showed the presence of 5 spot on checked TLC in system Hexane :  $\text{CHCl}_3$  : MeOH (5.5 : 11.5 : 3) and Ferric chloride - Perchloric acid as a spray reagent. This fraction was designated as fraction PC.

## 2.2 Preparative Thin-Layer Chromatography

The fraction P (3.93 g) was separated by preparative thin-layer chromatography using solvent system (1, page 90) to give 4 zones of substances ( $P_1$ ,  $P_2$ ,  $P_3$  and  $P_4$ ).

$P_1$  was crystalized in acetone to yield 0.1399 g of crystal.

$P_2$  was crystalized in ehtanol and recrystalized in methanol to yield 0.0044 g of crystal.

$P_3$  was crystalized in ethanol and recrystalized in methanol to yield 0.0051 g of crystal.

$P_4$  was crystalized in chloroform - ethanol and wash with ethanol to yield 0.0119 g of crystal.

$P_1, P_2, P_3$  and  $P_4$  were non-alkaloidal substances, each of which in very small quantities.

The fraction C (10.17 g) was separated by preparative thin-layer chromatography using solvent system (2 ,page 90) to give 4 zones of substances ( $C_1$ ,  $C_2$ ,  $C_3$  and  $C_4$ ).

$C_1, C_2, C_3$  and  $C_4$  were non-alkaloidal substances, each of which in very small quantities.

### 2.3 Medium Pressure Liquid Chromatography (MPLC)

The fraction PC (9.74 g.) was separated by MPLC to gives 5 fractions of substances. ( $PC_1$ ,  $PC_2$ ,  $PC_3$ ,  $PC_4$  and  $PC_5$ )

$PC_1$  was too small amount to separate.

$PC_2$  was separated by sublimation and give the sublimated substance  $PC_2$ .

$PC_3$  was purified by Preparative thin-layer chromatography using solvent system (3, page 90), and eluted out with chloroform. Crystallized in the mixture of chloroform and methanol to give prismatic crystals of  $PC_3$  .

$PC_4$  was separated by Preparative thin-layer chromatography using solvent system (3, page 90) to give 3 substances ( $PC_{4-1}$ ,  $PC_{4-2}$  and  $PC_{4-3}$ ).  $PC_{4-2}$  and  $PC_{4-3}$  are too small to separate.  $PC_{4-1}$  crystallized in the mixture of acetone-water to give crystals .

$PC_5$  was too small amount to separate.

### Characterization of the isolated alkaloid

Two alkaloids were isolated from the bark of *S.nux-blanda* A.W. Hill. The chemical and physical characteristic



properties of the individual alkaloids are described as follows :

### PC<sub>3</sub>

The base crystallized from chloroform-methanol as colorless crystal (72 mg = 0.0006%) and gave pink colored spot with the ferric chloride-perchloric acid spray reagent.

TLC system (hRf) 1(59) 2(53) 3(83) 4(96)

Melting point 267-268 °C (uncorrected)

UV (ethanol) (see figure 13 , page 123 )

max(nm) 254, 278, 288

IR (KBr disc) (see figure 14, page 124 ) .

max(cm<sup>-1</sup>) 1660, 1600, 1480, 1390, 1100, 760

<sup>1</sup>H NMR (see figure 15, page 125 )

Chemical Shift (ppm)	Proton	Multiplicity
8.09	1 H; H-12	d(J=8.45 Hz)
7.27-7.15	3 H; H-9, H-10	
	H-11	m
5.89	1 H; H-19	t (ill-defined)
4.28	1 H; H-17	
4.14-4.07	2 H; H-18A ,	
	H-18B	m

$^{13}\text{C}$  NMR (see figure 16, page 126 )

Carbon	Chemical shift (ppm)
C-2	60.24
C-3	60.13
C-5	50.38
C-6	42.61
C-7	52.01
C-8	132.78
C-9	122.27
C-10	124.22
C-11	128.56
C-12	116.26
C-13	142.26
C-14	26.87
C-15	31.64
C-16	48.27
C-17	77.63
C-18	64.63
C-19	127.20
C-20	140.58
C-21	52.71
C-22	169.30
C-23	42.53

MS (EIMS, 200 °C) (see figure 17, page 127 )

m/z 334 (M<sup>+</sup> C<sub>21</sub>H<sub>22</sub>N<sub>2</sub>O<sub>2</sub>, 100% )

335(24),	334(100),	319(5),
306(5),	305(4),	172(4),
167(8),	163(9),	162(20),
161(15),	144(14),	143(14),
136(9),	130(15),	121(12),
120(22),	107(19)	

**PC<sub>4</sub>-1**

The base crystallized from acetone-water as aggregate of prisms (98 mg = 0.0008%) and gave a yellow colored spot with the ferric chloride-perchloric acid reagent.

TLC system (hRf) 1(52) 2(35) 3(81) 4(95)

Melting point 179 °C (uncorrected)

UV (ethanol) (see figure 18, page 128)

max 264, 301

IR (KBr Disc) (see figure 19, page 129 )

max (cm<sup>-1</sup>) 1650, 1500, 1460, 1450,  
1400, 1290, 1280, 1110,  
850, 750

<sup>1</sup>H NMR (in CDCl<sub>3</sub> at 90 MHz) (see figure 20, page 130 )

Chemical Shift (ppm)	Proton	Multiplicity
7.81	1 H; H-12	s
6.67	1 H; H-9	s
5.89	1 H; H-19	t (ill-defined)
4.29	1 H; H-17	m
4.14-4.07	2 H; H-18A , H-18B	m
3.90	3 H; OCH <sub>3</sub>	s
3.86	3 H; OCH <sub>3</sub>	s

$^{13}\text{C}$  NMR (see figure 21, page 131 )

Carbon	Chemical Shift (ppm)
C-2	60.40
C-3	59.97
C-5	50.22
C-6	42.42
C-7	51.95
C-8	123.52
C-9	105.64
C-10	146.27
C-11	149.30
C-12	101.09
C-13	152.34
C-14	26.87
C-15	31.58
C-16	48.32
C-17	77.79
C-18	64.63
C-19	127.26
C-20	140.64
C-21	52.71
C-22	168.97
C-23	
2 x $\text{OCH}_3$	52.23, 56.50

MS EIMS, 150 °C (see figure 22, page 132 )

m/z 394 ( $M^+$ ,  $C_{23}H_{26}N_2O_4$  100%)

394(100), 393(4), 380(5),

379(20), 204(3), 203(6),

197(7), 190(4), 162(5),

161(3), 146(2), 138(3),

136(4), 134(4), 122(3),

121(4), 120(7), 108(4),

107(7)