CHAPTER III

EXPERIMENTAL

Source and authentication of plant material

The leaves of *Uncaria salaccensis* Bakh. f. nom provis were obtained from Khao-Yai, Nakorn-Rachasima, Thailand in September, 1976. The plant was identified by Miss Ampai Yongboonkird, Head of Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand.

Chemicals and solvents used

Acetic acid, glacial (May and Baker, laboratory grade)

Acetone (BDH Chemicals, analytical grade)

Ammonium hydroxide, strong solution (BDH Chemicals, analytical grade)

Chloroform B.P.C. (I.C.I.)

Diethyl ether, anaesthetic B.P. (Macfarlan Smith)

Ethyl acetate (Riedel, analytical grade)

Ethyl alcohol (The Government Pharmaceutical Organisation)

Ferric chloride crystals (Riedel, chem. pure)

Isopropyl alcohol (May and Baker, laboratory grade)

Methyl alcohol (Riedel, analytical grade)

Perchloric acid (Riedel, chem. pure)

Potassium iodide (Riedel, chem. pure)

Sodium sulphate, anhydrous (May and Baker, laboratory grade)

General techniques

1. The extraction of alkaloids

The dried coarsely powdered leaves were moistened with strong solution of ammonium hydroxide. It was then macerated with 95% ethyl alcohol for seven days and filtered. The filtrate was concentrated to syrupy mass under reduced pressure, mixed with glacial acetic acid and then poured into a large volume of warm distilled water to give about 5% acetic acid solution. The acidic filtrate was made alkaline with strong solution of ammonium hydroxide and extracted with chloroform. The combined chloroform extract was washed with distilled water, dried over anhydrous sodium sulphate and evaporated under reduced pressure to yield dry crude alkaloidal extract.

2. Thin layer chromatography (TLC)

Technique : one way, ascending

Adsorbents : silica gel G (E. Merck), 30 g/60 ml distilled water
aluminium oxide G (E. Merck), 70 g/85 ml distilled water

Plate size : 10 cm x 20 cm and 20 cm x 20 cm

Layer thickness : 250 μ

Activation : air dried for 15 minutes and then at 105°C for 1 hour

Solvent systems : a. silica gel G/chloroform : acetone (5:4)
b. silica gel G/chloroform : ethyl alcohol (95:5)

c. silica gel G/diethyl ether : ethyl acetate (1:1)

d. silica gel G/ethyl acetate : isopropyl alcohol : strong solution of ammonium hydroxide (100:2:1)

e. silica gel G/ethyl acetate : diethyl ether (8:2)

f. silica gel G/ ethyl acetate

g. silica gel G/diethyl ether

h. aluminium oxide G/chloroform : acetone (5:4)

i. aluminium oxide G/ethyl acetate

Distance : 15 cm

Temperature : laboratory temperature (20°-30°C)

Detection : chromogenic reagents employed were:-

a. Dragendorff's spray reagent

Solution A : bismuth subnitrate (850 mg), distilled water (40 ml) and acetic acid (10 ml)

Solution B : potassium iodide (8 g) and distilled water (20 ml)

Solution A and B, each of 5 ml, were mixed, 20 ml of glacial acetic acid and 70 ml of distilled water were added and used as spray reagent.

b. 0.2 M anhydrous ferric chlorode in 35% w/v perchloric acid spray reagent

Plate, after spraying was warmed gently with hot air stream from a hair dryer for 15 minutes.

3. Column chromatography

Adsorbents : silica gel 0.040-0.063 mm (E. Merck)

aluminium oxide (E. Merck)

Packing : adsorbent packed dry into the column

Addition of alkaloidal material

: alkaloidal material was dissolved in small volume of volatile solvent, mixed with small quantity of adsorbent, air dried and added onto the top of a dry column.

Solvents : a. anaesthetic diethyl ether

b. ethyl acetate

c. chloroform

d. ethyl alcohol

e. methyl alcohol

Collection of eluate : fractions of 20 ml were collected.

Examination of eluate : those fractions giving an orange-red colour with Dragendorff's spray reagent were examined by thin layer chromatography.

4. Physical constant

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

5. Spectroscopy

a. Ultraviolet absorption spectra were obtained with a Beckman DK-2A Spectrophotometer.

b. Infrared absorption spectra were obtained with a Perkin Elmer 421 Grating Spectrophotometer.

c. Nuclear magnetic resonance (NMR) spectra were obtained with an HA-100 instrument in deuterochloroform using tetramethylsilane (T.M.S.) as internal reference.

d. Mass spectra were determined on an AEI MS 902 spectrometer at 70 eV with inlet temperature between 220°-240°C.

The extraction and isolation of alkaloids from the leaves of *Uncaria salaccensis* Bakh. f. nom provis

1. The extraction of alkaloids

The dried coarsely powdered leaves (650 g) were moistened with strong solution of ammonium hydroxide and allowed to stand overnight. It was then macerated with 95% ethyl alcohol (3 L) for seven days and filtered. The marc was shaken with portions of 95% ethyl alcohol (3x1.5 L) and filtered. The combined filtrate was concentrated to syrupy mass under reduced pressure, mixed with glacial acetic acid (100 ml) then poured into a large volume of warm distilled water to give about 5% acetic acid solution (2 L), well shaken and left to stand overnight. The filtered acidic solution was made alkaline with strong solution of ammonium hydroxide and extracted with chloroform (16x200 ml). The combined chloroform extract was washed with distilled water, dried over anhydrous sodium sulphate and evaporated under reduced pressure to yield dry crude alkaloidal extract (7.5 g).

Thin layer chromatograms of this crude alkaloidal extract, shown in Figures I-VII pages 143-149, indicated that at least six alkaloids were present with the addition of base-line alkaloid(s).

2. The isolation of alkaloids

Crude alkaloidal extract was divided into seven portions. Each portion (about 1 g) was dissolved in chloroform (5 ml) and mixed with small amount of silica gel G. The content was air dried and packed onto the top of dry silica gel G column (2.5 cm x 40 cm). The column was eluted with anaesthetic diethyl ether (1.5 L) and methyl alcohol (300 ml). Twenty ml fractions of the eluate were collected until no traces of alkaloid could be detected in the last fraction. The ethereal fractions were examined by TLC and the liked fractions from all seven columns were combined. It was washed, dried and evaporated to dryness under reduced pressure to give the following fractions:-

a. first portion (700 ml)-containing no alkaloid.

b. second portion (560 ml)-containing two oxindole alkaloids, yielding 850 mg of Fraction A.

c. third portion (1.4 L)-containing no alkaloid.

d. fourth portion (840 ml)-containing other two alkaloids with hRf values lower than those of the alkaloids in Fraction A, one being indole and the other oxindole, yielding 1.22 g of Fraction B.

e. fifth portion (700 ml)-shown to be a mixture of Fraction B and two other alkaloids with lower hRf values. No further isolation has been made.

f. sixth portion (420 ml)-containing the two alkaloids with hRf values lower than those of the alkaloids in Fraction B, one being indole and the other oxindole, yielding 665 mg of Fraction C.

TLC of Fractions A, B and C are shown in Figures VIII-X pages 150-152.

Fraction A has been subjected to column chromatography both of silica gel G and aluminium oxide G, and with various solvents but separation of the two alkaloids was not successful. TLC using many solvent systems on both silica gel G and aluminium oxide G plates were also tried without any adequate separation of the two to be able to use preparative TLC.

The combined methanol fraction was shown by TLC to contained base-line alkaloid(s). No further attempt to isolate the individual alkaloids has been made.

1. The isolation of the alkaloids in Fraction B

Fraction B was divided into five portions. Each portion (about 240 mg) was dissolved in chloroform (3 ml), mixed with small amount of silica gel G, air dried and packed onto the top of dry silica gel G column (1.5 cm x 30 cm). The column was eluated with ethyl acetate (650 ml). Twenty ml fractions were collected until last fraction showed no traces of alkaloid. The fractions were examined by TLC and the liked fractions from all five columns were combined to give the following portions:-

a. first portion (750 ml)-containing no alkaloid.

b. second portion (200 ml) - shown by TLC (Figures XI-XIV pages 153-156) to contain one oxindole alkaloid. It was washed, dried and evaporated under reduced pressure to dryness. Small volume of anaesthetic diethyl ether was added and white needle crystals (80 mg) were obtained, designated as O_1 which was subsequently identified as uncarine B.

c. third portion (1.5 L)-containing no alkaloid.

d. fourth portion (500 ml)-shown by TLC (Figures XI-XIV pages 153-156) to contain one indole alkaloid with hRf values lower than those of O_1. It was also washed, dried and evaporated under reduced pressure to dryness yielding pale yellow amorphous alkaloid (351 mg) designated as I_1 which was subsequently identified as 19-epi-3-isoajmalicine.

2. The isolation of the alkaloids in Fraction C

Fraction C was divided into three portions. Each portion (about 220 mg) was dissolved in chloroform (3 ml), mixed with small amount of silica gel G, air dried, and packed onto the top of dry silica gel G column (1.5 cm x 30 cm). The column was eluated with ethyl acetate (800 ml). Twenty ml fractions were collected, until no traces of alkaloid could be detected in the last fraction. The fractions were examined by TLC and the liked fractions from all three columns were combined to give the following portions:-

a. first portion (750 ml)-containing no alkaloid.

b. second portion (150 ml)-shown by TLC (Figures XI-XIV pages 153-156) to contain one oxindole alkaloid. It was washed, dried and evaporated under reduced pressure to dryness. White needle crystals (60 mg) were crystallised out upon the addition of small volume of anaesthetic diethyl ether, designated as O_2 which was subsequently identified as mitraphylline.

c. third portion (900 ml)-containing no alkaloid.

d. fourth portion (360 ml) - shown by TLC (Figures XI-XIV pages 153-156) to contain one indole alkaloid. It was also washed, dried and evaporated under reduced pressure to dryness yielding an amorphous creamy coloured alkaloid (445 mg), designated as I_2 which was subsequently identified as 3-isoajmalicine.

Identification of the isolated alkaloids

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

The hRf values given are those obtained with the following solvent systems:-

a. silica gel G / chloroform : acetone (5:4)

b. silica gel G / chloroform : ethyl alcohol (95:5)

c. silica gel G / diethyl ether : ethyl acetate (1:1)

d. silica gel G / ethyl acetate : isopropyl alcohol : strong solution of ammonium hydroxide (100:2:1)

1. Identification of O_1 as uncarine B

O_1 was obtained as white needle crystals from anaesthetic diethyl ether. It was soluble in ethyl acetate, chloroform and ethyl alcohol and insoluble in light petroleum.

hRf values

a. 53 b. 55 c. 29 d. 37

Melting point

197°C

Molecular weight

368 (mass spectrometry)

Ultraviolet absorption spectrum (Ethyl alcohol)

λ_{max} 241 nm

λ_{min} 226 nm

shoulder at 280 nm

Infrared absorption spectrum (Potassium bromide)

ν_{max} 3260 (imino), 1720 (ester carbonyl), 1700 (oxindole carbonyl), 1615 (double bond), 1100 (ether), 740 (benzene ring) cm^{-1}

NMR spectrum in deuterochloroform at 100 MHz in δ values (ppm) from tetramethylsilane (T.M.S.)

δ	1.33	(3H, d, J 7 Hz)	C(19)-CH_3
	3.58	(3H, s)	-OCH_3
	3.82	(1H, m, partly hidden)	C(19)-H
	6.75-7.45	(4H, m)	aromatic-H
	7.49	(1H, s)	olefinic
	8.50	(1H, s)	imino

Mass spectrum

m/e (%) 368(M^+, 85), 351(6), 337(8), 223(100), 222(13), 208(16), 146(6), 145(7), 144(8), 130(15), 69(35)

O_1 is identical in hRf values, melting point, ultraviolet, infrared, NMR and mass spectra with authentic sample of uncarine B from *Uncaria attenuata* Korth. spp. *attenuata* Korth. (Phillipson and Hemingway, 1975). It is therefore concluded that O_1 is uncarine B.

2. Identification of O_2 as mitraphylline

O_2 was obtained as white needle crystals from anaesthetic diethyl ether. It was soluble in ethyl acetate, chloroform and ethyl alcohol, and insoluble in light petroleum.

hRf values

a. 45 b. 49 c. 22 d. 31

Melting point

270°C

Molecular weight

368 (mass spectrometry)

Ultraviolet absorption spectrum (Ethyl alcohol)

λ_{max} 240 nm

λ_{min} 225 nm

shoulder at 280 nm

Infrared absorption spectrum (Potassium bromide)

ν_{max} 3260 (imino), 1720 (ester carbonyl), 1700 (oxindole carbonyl), 1615 (double bond), 1100 (ether), 750 (benzene ring) cm^{-1}

NMR spectrum in deuterochloroform at 100 MHz in δ values (ppm) from tetramethylsilane (T.M.S.)

δ	1.11	(3H, d, J 6 Hz)	C(19)-CH_3
	3.59	(3H, s)	-OCH_3
	4.41	(1H, q, J 3,6 Hz)	C(19)-H
	6.85-7.50	(4H, m)	aromatic-H
	7.44	(1H, s)	olefinic

Mass spectrum

m/e (%) 368(M^+, 40), 351(3), 337(4), 223(100), 222(13), 208(11), 146(6), 145(10), 144(6), 130(11), 69(27)

O_2 is identical in hRf values, melting point, ultraviolet, infrared, NMR and mass spectra with authentic sample of mitraphylline from *Mitragyna hirsuta* Havil. (Shellard, Tantivatana and Beckett, 1967). It is therefore concluded that O_2 is mitraphylline.

3. Identification of I_1 as 19-epi-3-isoajmalicine

I_1 was obtained as pale yellow amorphous solid. It was soluble in ether, chloroform and ethyl alcohol and insoluble in light petroleum.

hRf values

a. 29 b. 51 c. 16 d. 21

Melting point

150°C

Molecular weight

352 (mass spectrometry)

Ultraviolet absorption spectrum (Ethyl alcohol)

λ_{max} 223, 273, 282, 290 nm

λ_{min} 213 nm

Infrared absorption spectrum (Potassium bromide)

ν_{max} 3420 (imino), 1685 (ester carbonyl), 1618 (double bond) cm^{-1}

NMR spectrum in deuterochloroform at 100 MHz in δ values (ppm) from tetramethylsilane (T.M.S.)

δ	1.28	(3H, d, J 6 Hz)	C(19)-CH_3
	3.72	(1H, m)	C(19)-H
	4.56	(1H, m)	C(3)-H β
	7.0-7.5	(4H, m)	aromatic-H
	7.44	(1H, s)	olefinic
	8.15	(1H, s)	imino

Mass spectrum

m/e (%) 352(M^+, 100), 351(75), 337(3), 265(2), 251(1), 225(4), 223(4), 222(7), 184(38), 170(22), 169(19), 156(75)

I_1 is identical in hRf values, melting point, ultraviolet, infrared, NMR and mass spectra with authentic sample of 19-epi-3-isoajmalicine from *Uncaria attenuata* Korth. ssp. *bulusanensis* Ridsd. (Phillipson and Hemingway, 1975). It is therefore concluded that I_1 is 19-epi-3-isoajmalicine.

4. Identification of I_2 as 3-isoajmalicine

I_2 was obtained as amorphous creamy colour solid. It was soluble in ether, chloroform and ethyl alcohol and insoluble in light petroleum.

hRf values

a. 19 b. 43 c. 10 d. 13

Melting point

107°C

Molecular weight

352 (mass spectrometry)

Ultraviolet absorption spectrum (Ethyl alcohol)

λ_{max} 222, 280, 290, nm

λ_{min} 213 nm

Infrared absorption spectrum (Potassium bromide)

ν_{max} 3500 (imino), 1680 (ester carbonyl),
1610 (double bond) cm^{-1}

NMR spectrum in deuterochloroform at 100 MHz in δ values (ppm) from tetramethylsilane (T.M.S.)

δ	0.94	(3H, d, J 6 Hz)	C(19)-CH_3
	3.73	(3H, s)	-OCH_3
	4.35	(1H, q, J 2,6 Hz)	C(19)-H
	4.55	(1H, m)	C(3)-H β
	7.0-7.5	(4H, m)	aromatic-H
	7.51	(1H, s)	olefinic
	8.33	(2H, s)	imino

Mass spectrum

m/e (%) 352(M^+, 100), 351(74), 337(4), 265(2), 251(1), 225(6), 223(4), 222(5), 209(8), 184(52), 170(10), 169(11), 156(89)

I_2 is identical in hRf values, melting point, ultraviolet, infrared, NMR and mass spectra with authentic sample of 3-isoajmalicine from *Mitragyna parvifolia* (Roxb.) Korth. (Shellard, Phillipson and Gupta, 1968 b). It is therefore concluded that I_2 is 3-isoajmalicine.