CHAPTER III

EXPERIMENTAL

1. Source and identification of plant material

The leaves of plant were collected from Sukhothai Province, Thailand in July, 1975.

Its herbarium was identified by Professor Tem Smitinand of the Royal Forest Department, Bangkok, as *Mitragyna brunonis* (Wall. ex G. Don) Craib and subsequently by Dr. C.E. Ridsdale of the Rijksherbarium, Leiden as *Mitragyna rotundifolia* (Roxb.) O. Kuntze, the accepted species of which the former is regarded as a synonym.

2. General techniques

2.1 Extraction of alkaloids

The dried coarsely powdered leaves were moistened with 10 % ammonium hydroxide solution, macerated with 95 % ethyl alcohol for seven days and filtered. The filtrate was concentrated to low bulk under reduced pressure, mixed with glacial acetic acid and poured into a large volume of warm distilled water to give about 5 % acetic acid solution. The 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.

2.2 Thin layer chromatography (TLC)

2.2.1 Analytical

Technique : one way, ascending.

Adsorbents : a) aluminium oxide G (E. Merck), calcium sulphate binder 10 %; 50 g / 60 ml distilled water.

b) silica gel G (E. Merck), calcium sulphate binder 13 %; 30 g / 60 ml distilled water.

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

Layer thickness : 250 µm.

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

Solvent system : a) aluminium oxide G / chloroform

b) silica gel G / chloroform : acetone = 5 : 4

c) silica gel G / chloroform : ethyl alcohol = 95 : 5

d) silica gel G / diethyl ether

e) silica gel G / diethyl ether : ethyl acetate = 1 : 1

Distance : 15 cm.

Laboratory temperature : 20° - 30°C.

Detection : a) Dragendorff's spray reagent

solution A : 0,85 g bismuth subnitrate is dissolved in a mixture of 10 ml glacial acetic acid and 40 ml distilled water.

solution B : 40 % solution of potassium iodide in water.

Stock solution : Equal volumes of A and B are mixed.

Spray reagent : 10 ml stock solution is mixed with 20 ml glacial acetic acid and water to make 100 ml solution.

b) 0.2 M ferric chloride in 35 % w/v perchloric acid spray reagent. Plate, after spraying, is warmed gently with a hot air stream from a hair dryer for 15 minutes and then placed in an oven at 90° - 110°C for 30 minutes.

2.2.2 Preparative thin layer chromatography

Technique : one way ascending, double development.

Adsorbent : silica gel G (E. Merck) : silica gel GF_{254} (E. Merck) = 2 : 1, 60 g / 120 ml distilled water.

Plate size : 20 cm x 20 cm.

Layer thickness : 500 µm.

Activation : air dried for 20 minutes and then at 105°C for 1 hour.

Solvent system : chloroform : ethyl alcohol = 95 : 5.

Distance : 18 cm.

Examination : under ultraviolet light (wavelength 254 nm).

2.3 Column chromatography

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

b) aluminium oxide, neutral (E. Merck).

Packing : adsorbents packed dry into the column.

Solvents : a) chloroform

b) diethyl ether, anaesthetic

c) ethyl alcohol, 95 %

d) methyl alcohol.

2.4 Melting point

Melting points were determined by heating stage microscope (Reichert). The value recorded were uncorrected.

2.5 Ultraviolet absorption spectra

Ultraviolet absorption spectra were obtained with Unicam SP 1800 recording spectrophotometer.

2.6 Infrared absorption spectra

Infrared absorption spectra were obtained with Perkin Elmer 283 and 421 grating spectrometer.

2.7 Nuclear magnetic resonance (N.M.R.) spectra

The N.M.R. spectra were obtained with an HA-100 instrument using deuterochloroform solution with tetramethylsilane (T.M.S.) as internal reference.

2.8 Mass spectrum

The mass spectrum was determined in the Department of Chemistry, University of Malaya, Kuala Lumper, Malaysia.

3. The isolation of alkaloids from the leaves of *Mitragyna brunonis* (Wall. ex G. Don) Craib

3.1 Extraction

The dried coarsely powdered leaves (3 kg) were moistened with 10 % ammonium hydroxide solution and allowed to stand overnight. The moist powder was macerated with 95 % ethyl alcohol (8 L) for seven days and filtered. The marc was remacerated with another portion of ethyl alcohol (6 L). The combined filtrate was concentrated under reduced pressure to yield syrupy residue, mixed with glacial acetic acid (150 ml) and then poured into a large volume of warm distilled water (2.8 L) to give about 5 % acetic acid solution and allowed to stand overnight. It was then filtered and the residue was washed with 5 % acetic acid solution. The filtrate was made alkaline with strong solution of ammonium hydroxide and extracted with chloroform (10 x 800 ml). The combined chloroform extract was washed with distilled water, dried over anhydrous sodium sulphate and evaporated under reduced pressure from which a yellowish brown crude base (21.5 g) was obtained.

The crude base (21.5 g) was divided into three portions. Each portion was dissolved in chloroform (3 ml), mixed with small amount of aluminium oxide, air dried and packed onto the top of dry aluminium

oxide column (2.5 cm x 30 cm).

The column was eluted with chloroform (400 ml), until no traces of alkaloid could be detected, then washed with methyl alcohol (200 ml). The chloroform collected was concentrated under reduced pressure to yield a purified crude base designated as C (12 g) (Figs. XIX - XX). The methanol eluate was concentrated under reduced pressure to yield a brownish mass. TLC showed traces of alkaloids with low hR_f values and also 'base-line' alkaloid(s). No further study of this fraction has been made.

3.2 Isolation of alkaloids

The purified crude base (C) was dissolved in ether and divided into two portions :-

a) EI (5 g), the ether insoluble fraction, and

b) ES (7 g), the ether soluble fraction.

TLC indicated them to contain at least three and six alkaloids, respectively (Figs. XXI - XXIV).

3.2.1 Separation of alkaloids in EI

EI was divided into ten portions, each of 0.5 g. Each portion was dissolves in small volume of chloroform (2 ml) and mixed with small amount of silica gel. The content was air dried and packed onto the top of dry silica gel column. The column was eluted with 5 % ethyl alcohol in chloroform (350 ml) followed by methyl alcohol (150 ml), 15 ml portions being collected. TLC monitoring of all ten portions

allowed bulking of like portions to give the following fractions :-

1. 5 % ethyl alcohol in chloroform (60 ml) - containing no alkaloid.
2. 5 % ethyl alcohol in chloroform (75 ml) - a mixture of at least three alkaloidal spots, TS_3, TS_4 and TS_5. It was evaporated to dryness under reduced pressure to give the alkaloidal fraction EI_1 (2.8 g) (Fig. XXV).
3. 5 % ethyl alcohol in chloroform (45 ml) - composed of TS_4 and TS_5, it was evaporated to dryness under reduced pressure to give the alkaloidal fraction EI_2 (0.2 g) (Fig. XXV).
4. 5 % ethyl alcohol in chloroform (170 ml) - composed of only one alkaloid, it was evaporated to dryness under reduced pressure and recrystallised from absolute ethyl alcohol to yield white, rosette crystals of TS_5 (0.15 g), which was subsequently identified as ciliaphylline (Figs. XXV - XXVII).
5. methyl alcohol (150 ml) - composed of traces of TS_5 and 'base-line' alkaloid(s). It was evaporated to dryness under reduced pressure. No further study had been made.

The EI_2 (0.2 g) was divided into 30 batches (approximately 6 mg) and each dissolved in ethyl alcohol (1 ml), applied as a narrow band on preparative TLC plates, double developed in chloroform : ethyl alcohol = 95 : 5 and observed under UV light as a guide to the alkaloidal bands. The bands corresponded to TS_4 and TS_5 were scraped

off, extracted with methyl alcohol, filtrated and evaporated under reduced pressure, dissolved in chloroform, filtered and evaporated to dryness under reduced pressure. By this method the yellow solid TS_4 (50 mg) and TS_5 (60 mg) were obtained and after recrystallised in absolute ethyl alcohol, TS_4 (45 mg) and TS_5 (50 mg) were obtained as rosette crystals. TS_4 was subsequently identified as specionoxeine (Figs. XXV - XXVII).

3.2.2 Separation of alkaloids in ES

The ES fraction was divided into 10 portions of 0.5 g, each was dissolved in chloroform (2 ml) and mixed with small amount of silica gel, air dried and packed onto the top of dry silica gel column. It was eluted with diethyl ether (150 ml) and 5 % ethyl alcohol in chloroform (300 ml) respectively, 15 ml portions being collected. TLC monitoring allowed bulking of like portions to give the following fractions :-

1. diethyl ether (60 ml) - containing no alkaloid.
2. diethyl ether (90 ml) - shown by TLC to contain two alkaloids, TS_1 and TS_2. The eluate was evaporated to dryness under reduced pressure to give alkaloidal fraction ES_1 (0.5 g) (Fig. XXVIII).
3. 5 % ethyl alcohol in chloroform (75 ml) - containing no alkaloid.
4. 5 % ethyl alcohol in chloroform (120 ml) - shown by TLC to contain three oxindole alkaloids, TS_3, TS_4 and TS_5. The eluate was evaporated to dryness under reduced pressure to

give alkaloidal fraction ES_2 (0.9 g) (Fig. XXVIII).

5. 5 % ethyl alcohol in chloroform (105 ml) - shown by TLC to contain three alkaloids, i.e. an oxindole, TS_6 and two indole alkaloids, TS_7 and TS_8. The eluate was evaporated to dryness under reduced pressure to give alkaloidal fraction ES_3 (0.1 g) (Fig. XXVIII).

In most TLC solvent systems, it appeared that there was one single alkaloid, TS_2, in the fraction ES_1. TS_2 was shown to have hR_f values on several solvent systems and also colour developed with ferric chloride in perchloric acid spray reagent correspond to those of authentic isorhynchophylline. Only with silica gel G / chloroform : ethyl alcohol = 95 : 5 that TS_1 was revealed as an oxindole alkaloid with hR_f values higher, and present in relatively much smaller amount, than those of TS_2. The attempt to separate TS_1 from TS_2 in fraction ES_1 by column chromatography had been carried out but was not successful. Hence, TS_2 could only partly be identified as isorhynchophylline by its hR_f values and colour with spray reagent as it still could not be obtained pure.

Fraction ES_2 was shown to be similar to Fraction EI. No further attempt to separate individual alkaloid has been made.

Fraction ES_3 showed one open E ring oxindole alkaloid, TS_6, with two indole alkaloids, TS_7 and TS_8. TS_6 could partly be identified as rhynchociline by comparing hR_f values and colour developed with ferric chloride in perchloric acid spray reagent. Both TS_7 and TS_8 were present in too small the quantities to be isolated out and identified.

4. Identification of the isolated alkaloids

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

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

a) Sg / chloroform : ethyl alcohol = 95 : 5

b) Sg / chloroform : acetone = 5 : 4

c) Sg / diethyl ether : ethyl acetate = 1 : 1

4.1 Identification of TS_4 as specionoxeine

TS_4 was obtained as white rosette crystals from absolute ethyl alcohol. It was soluble in chloroform, ethyl alcohol and methyl alcohol, and insoluble in petroleum ether.

hR_f values

a) 34 b) 44 c) 19

Melting point

233°C

Molecular weight

412 (mass spectrometry)

Ultraviolet absorption spectrum (Ethyl alcohol)

λ_{max} 221, 243, 292 nm

Infrared absorption spectrum (Potassium bromide)

ν_{max} 3460 (imino),
1715 (ester and oxindole carbonyl),
1615, 1650 (double bond),
925, 950 (vinyl) cm^{-1}

N.M.R. spectrum in deuterochloroform at 90 MHz in δ value (ppm) from tetramethylsilane (T.M.S.)

δ	3.62	three-proton singlet	$-OCH_3$ (ester)
	3.70	three-proton singlet	$-OCH_3$ (vinyl)
	3.86	three-proton singlet	$-OCH_3$ (aromatic)
	4.95	two-proton multiplet	C(19) - $CH=CH_2$
	5.55	one-proton multiplet	
	6.55	three-proton multiplet	aromatic - H
	7.25	one-proton singlet	olefinic
	8.70	one-proton singlet	imino

Mass spectrum

m/e (%) 412 (M^+, 100), 239 (16), 224 (6), 210 (1), 208 (4), 189 (7), 176 (6), 175 (7), 174 (10), 160 (6), 69 (41).

TS_4 is identified in hR_f values, melting point, ultraviolet, infrared, N.M.R. and mass spectra with authentic sample of specionoxeine from *Mitragyna speciosa* Korth. (Shellard, Houghton and Resha, 1978b). It is therefore concluded that TS_4 is specionoxeine.

4.2 Identification of TS_5 as ciliaphylline

TS_5 was obtained as white rosette crystals from absolute ethyl alcohol. It was soluble in chloroform, ethyl alcohol and methyl alcohol, and insoluble in petroleum ether.

hR_f values

a) 23 b) 32 c) 14

Melting point

230°C

Ultraviolet absorption spectrum (Ethyl alcohol)

λ_{max} 221, 243, 293 nm

Infrared absorption spectrum (Potassium bromide)

ν_{max} 2925, 3100 (imino),
1710, 1735 (ester and oxindole carbonyl),
1610, 1635 (double bond) cm^{-1}

N.M.R. spectrum in deuterochloroform at 60 MHz in δ value (ppm) from tetramethylsilane (T.M.S.)

δ	0.86	three-proton triplet	$-CH_2CH_3$
	3.61	three-proton singlet	$-OCH_3$ (ester)
	3.69	three-proton singlet	$-OCH_3$ (vinyl)
	3.86	three-proton singlet	$-OCH_3$ (aromatic)
	6.53-6.64	three-proton multiplet	aromatic - H
	7.19	one-proton singlet	olefinic
	8.78	one-proton singlet	imino

TS_5 is identical in hR_f values, melting point, ultraviolet, infrared and N.M.R. spectra with authentic sample of ciliaphylline from *Mitragyna tubulosa* (Arn.) Havil. (Rungsiyakul, 1973; Shellard and Rungsiyakul, 1973). It is therefore concluded that TS_5 is ciliaphylline.