CHAPTER III

EXPERIMENTAL

1. Source of plant materials

The leaves and stem bark of Uncaria quadrangularis Geddes were obtained from Chumpawn, Thailand in October 1974. The plant materials were identified by Miss Ampai Yongboonkird.

2. General techniques

2.1 Thin layer chromatography (TLC)

The experimental details are summarized as follows :-

Analytical

Technique	:	One way, ascending TLC
Adsorbents	:	Aluminium oxide G (E. Merck) Calcium sulphate binding 10%, 70 g/80ml distilled water. Silica gel G (E. Merck), Calcium sulphate binding 13%, 30 g/60 ml distilled water.
Plate size	:	20 cm x 20 cm, 20 cm x 10 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/diethyl ether

b) Silica gel G/chloroform : acetone (5:4)

c) Aluminium oxide G/chloroform

Distance : 15 cm

Laboratory temperature : 20°- 30°C

Detection : a) Dragendorff's spray reagent

b) 0.2 M anhydrous 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. The colour reaction indicates the nature of the alkaloids shown below :-[46]

Colour changes	Type of Alkaloid
Oxindoles	
Greenish pink on warming turning to red	Closed E ring *allo* and *epiallo*
Orange on warming turning to red	Closed E ring *normal*

2.2 Column chromatography

Adsorbents:

a) Silica gel 0.05 - 0.2 mm (E. Merck)

b) Aluminium oxide, neutral (E. Merck)

Packing of column

a) Adsorbent packed dry into the column.

b) Adsorbent poured slowly as a suspension into the column.

Addition of alkaloidal material to column

a) Solution in small volumn of solvent carefully pipetted onto the top of a column holding the same solvent.

b) Solution in small volume of volatile solvent mixed with small quantity of adsorbent, dried and added to the top of a dry column.

Solvents

a) Anaesthetic diethyl ether (Macfarlan Smith Ltd., Edinburgh).

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

c) Methanol (E. Merck).

d) Ethyl alcohol 95% (The Government Pharmaceutical Organization).

e) Strong solution of Ammonium hydroxide (May and Baker).

f) Anhydrous sodium sulphate (May and Baker).

g) Absolute ethyl alcohol (E. Merck).

Authentic samples

a) Mitraphylline and isomitraphylline supplied by Assistant Professor Dr. Payom Tantivatana.

b) Pteropodine and isopteropodine supplied by Professor Dr. E.J.

Shellard Department of Pharmacy, University of London, Chelsea College London SW 3, England.

Collection of eluate

Fractions of 30 ml or more were collected manually.

Examination of eluate

Those fractions giving an orange-red colour with Dragendorff's spray reagent were examined by thin layer chromatography and liked fractions were combined and concentrated to dryness under reduced pressure.

2.3 Melting point

Determined by Buchi melting point apparatus.

2.4 Ultra-violet absorption spectra

Ultra-violet absorption spectra were determined in the Department of Science, Ministry of Industry by a Beckman-DK-2A Spectrophotometer.

2.5 Infra-red absorption spectra

Infra-red absorption spectra were determined in the Department of Science, Ministry of Industry by a Perkin-Elmer 421 Grating Spectrophotometer.

3. The isolation of alkaloids from Uncaria quadrangularis Geddes

3.1 The isolation of alkaloids from the leaves of Uncaria quadrangularis Geddes.

The dried coarsely powdered leaves (755 g) were moistened with 10% ammomium hydroxide solution and allowed to stand overnight. The powdered mixture was macerated with 95% ethyl alcohol (3.5 L) for three days and filtered. The marc was remacerated with another portion of ethyl alcohol (3 L). The combined filtrate was concentrated to syrupy mass under reduced pressure, mixed with glacial acetic acid then poured into a large volume of warm water to give about 5% acetic acid solution, and stand overnight. The filtered acid extract was made alkaline with strong solution of ammonium hydroxide and extracted with chloroform (6 x 500 ml). The combined chloroform extract was dried with anhydrous sodium sulphate and concentrated under reduced pressure to get a dry crude base (16.3 g).

Crude base (16.3 g) was divided into three portions. Each portion was dissolved in chloroform (5 ml), mixed with small amount of aluminium oxide, let the content to be air-dried and packed onto the top of dry aluminium oxide column (2.5 cm x 40 cm).

The column was eluted with chloroform, the eluate was collected and concentrated under reduced pressure to yield a purified crude base (12.0 g).

Thin layer chromatograms of the crude base obtained by the above method as shown in Fig. V, VI page 77, 78 indicated that at least two

alkaloids were present.

The crude base from the chloroform fractions was crystallized in absolute ethanol and dry diethyl ether yielding white needle crystals (4.5 g) designated as S_1 (Fig VII page 79). S_1 was subsequently identified as mitraphylline (m.p. 270°- 271°C).

The mother liquor from which S_1 was crystallized out , contained another the main alkaloid designated as S_2 and traces of S_1, when examined by thin layer chromatography. This mixture was evaporated to dryness under reduced pressure to yield a brown residue (7.2 g).

The brown residue (7.2 g) was divided in three equal portions. Each portion was dissolved in small amount of anaesthetic diethyl ether and poured onto the top of silica gel column (2.5 cm x 30 cm) holding the same solvent.

Elution of the columns began with anaesthetic diethyl ether (600 ml) followed by chloroform (210 ml) and methanol (200 ml) respectively.

The eluates were evaporated under reduced pressure to dryness yielding alkaloidal base (5.0 g) from the diethyl ether fraction, but the chloroform and methanol fractions contained only traces of alkaloids.

The alkaloidal base (5.0 g) from the diethyl ether fraction was divided into equal portions. Each portion was dissolved in a small volume of chloroform and mixed with aluminium oxide, air dried and packed onto the top of aluminium oxide column. The column was eluted with chloroform (260 ml) until no traces of alkaloid could be detected in the last fraction and then with methanol (150 ml). Chlorophyll and some impurities were discarded by this treatment. The chloroform eluate was collected

and concentrated under reduced pressure to yield pale yellow alkaloidal base S_2 (3.7 g).

The purification of S_2 (700 mg) was made by dissolving in small amount of chloroform and mixed with aluminium oxide, air dried and packed onto the top of aluminium oxide column, (2.5 cm x 30 cm). The column was eluted with chloroform to give three fractions (3 x 30 ml) and then with methanol.

The chloroform eluates (fractions 1 and 2) were combined and evaporated under reduced pressure to dryness yielding an amorphous creamy colour alkaloid S_2 (230 mg).

The last fraction of chloroform was evaporated under reduced pressure to dryness yielding pale yellow amorphous alkaloid S_2 (162 mg).

S_2 was subsequently identified as isomitraphylline as shown in Fig. VII page 79 (m.p. 117°C). It was not found possible to crystallise this alkaloid.

3.2 The isolation of alkaloids from the stem bark of Uncaria quadrangularis Geddes.

Dried powdered bark (3.6 kg) were moistened with 10% solution of ammonium hydroxide and allowed to stand overnight. The mixture was macerated with ethyl acetate (11 L) for three days and then filtered. The marc was remacerated with ethyl acetate (6 L) for three days and then filtered. The filtrates were combined and concentrated under reduced pressure to low bulk (500 ml) and extracted with 2% sulphuric acid (10 x 500 ml). The combined acid extract was made alkaline with strong

solution of ammonium hydroxide and extracted with chloroform (8 x 500 ml). The combined chloroform extract was evaporated under reduced pressure to yield 55 g crude alkaloidal base.

A portion of the above crude alkaloidal base (6 g) was dissolved in a small volume of chloroform and mixed with aluminium oxide, air dried and packed onto the top of three aluminium oxide column (2.5 cm x 30 cm). Each column was eluted with chloroform (150 ml) until no traces of alkaloid could be detected in the last fraction and then with methanol (50 ml). Chlorophyll and some impurities were discarded by this treatment.

The combined chloroform eluate was evaporated under reduced pressure to dryness yielding crude base (723 mg).

TLC examination of this crude base indicated at least two major alkaloids and traces of oxindole alkaloids (Fig. IX page 81).

The methanolic eluate contained only traces of base line oxindole alkaloids.

Crude base (723 mg) from the combined chloroform eluate was crystallised in absolute ethanol and dry diethyl ether yielding white crystals (618 mg). TLC examination of this material indicated the presence of two alkaloids (S_3 and S_4). The R_F values of these two alkaloids are very close to each other and cannot be seperated by column chromatography. They correspond to authentic pteropodine and isopteropodine.

The mixed crystals of two alkaloids were divided into two portions. Each portion was dissolved in benzene (15 ml) and extracted with 0.2 N acetic acid (15 x 10 ml). Each 10 ml portion of acetic acid extract was

re-extracted with benzene (2 x 10 ml).

The benzene fractions were examined by TLC and the liked fractions were combined to give :-

(i) the (6 x 10 ml) fractions, containing one alkaloid only. After washing and drying over anhydrous sodium sulphate, this combined extract was evaporated under reduced pressure to yield a white amorphous powder (S_3). After crystalisation from absolute ethanol, S_3 was obtained as white crystals and subsequently identified as pteropodine (103.3 mg).

(ii) the (12 x 10 ml) fractions, containing two alkaloids (S_3 and S_4). After washing and drying over anhydrous sodium sulphate, this combined extract was evaporated under reduced pressure yielding white powder of two alkaloids (312 mg).

(iii) the (12 x 10 ml) fractions, containing one alkaloid only, After washing and drying over anhydrous sodium sulphate, this combined extract was evaporated under reduced pressure, yielding an amorphous pale cream colour powder (S_4). The amorphous powder was crystallised from absolute ethanol to yield white prismatic crystals (32.6 mg). The crystals were subsequently identified as isopteropodine (m.p. 199°C).

The residual acetic acid extracts were combined, made alkaline with strong solution of ammonium hydroxide and extracted with chloroform (4 x 40 ml). The chloroform extract was washed and dried over anhydrous sodium sulphate. The chloroform was evaporated under reduced pressure to yield a pale cream colour powder, which contained one alkaloid. Crystallisation from absolute ethanol yielded white crystals of S_3 (109.4 mg), subsequently identified as pteropodine (m.p. 216°C).

4. Identification of isolated alkaloids.

4.1 Identification of S_1 as mitraphylline

S_1 was obtained as white needle crystals from absolute ethanol (m.p. 270°- 271°C). The alkaloid was soluble with difficulty in ethanol and chloroform and was only slightly soluble in diethyl ether.

Thin layer chromatography

R_f value on Silica gel G/chloroform : acetone (5:4) = 0.64 (Fig. VII page 79).

R_f value on Silica gel G/diethyl ether = 0.08 (Fig. VIII page 80).

Ultraviolet absorption spectrum (Ethanol) (Fig. XIV page 86)

λ_{max}	243	nm
λ_{min}	224	nm

Infrared absorption spectrum (KBr) (Fig. XVIII page 90).

ν_{max}	3250 cm^{-1}	(imino)
	1700 cm^{-1}	(ester and oxindole carbonyl)
	1620 cm^{-1}	(double bond)
	1100 cm^{-1}	(ether)

S_1 is identical in m.p., ultraviolet and infrared absorption spectra, R_f values and colour reactions with the authentic sample of

mitraphylline from Mitragyna javanica Koord. and Valeton.[54] It is therefore concluded that S_1 is mitraphylline.

4.2 Identification of S_2 as isomitraphylline

S_2 was obtained as amorphous cream colour solid, m.p. 117°C. The alkaloid was readily soluble in ethanol, chloroform and diethyl ether, but insoluble in light petroleum.

Thin layer chromatography

R_f value on Silica gel G/chloroform : acetone (5:4) = 0.71 (Fig. VII page 79).

R_f value on Silica gel G/diethyl ether = 0.38 (Fig. VIII page 80).

Ultraviolet absorption spectrum (Ethanol) (Fig. XV page 87)

λ_{max}	241	nm
λ_{min}	225	nm

Infrared absorption spectrum (KBr) (Fig. XIX page 91)

ν_{max}	3200	cm^{-1}	(imino)
	1700	cm^{-1}	(ester and oxindole carbonyl)
	1610	cm^{-1}	(double bond)
	1095	cm^{-1}	(ether)

S_2 is identical in m.p., ultraviolet and infrared absorption spectra, R_f values and colour reactions with the authentic sample of isomitraphylline from Mitragyna javanica Koord. and Valeton.[54] It is therefore concluded that S_2 is isomitraphylline.

4.3 Identification of S_3 as pteropodine

S_3 was obtained as white crystals from absolute ethanol (m.p. 216°C). The alkaloid was soluble in chloroform, ethanol and diethyl ether.

Thin layer chromatography

R_f value on Silica gel G/diethyl ether (developing twice) = 0.45 (Fig. XIII page 85).

Ultraviolet absorption spectrum (Ethanol) (Fig. XVI page 88)

λ_{max}	245	nm
λ_{min}	225	nm

Infrared absorption spectrum (KBr) (Fig. XX page 92)

ν_{max}	3200	cm^{-1}	(imino)
	1700	cm^{-1}	(ester and oxindole carbonyl)
	1620	cm^{-1}	(double bond)
	1080	cm^{-1}	(ester)

S_3 is identical in m.p., ultraviolet and infrared absorption spectra, R_f value and colour reactions with the authentic sample of pteropodine isolated from the leaves of Mitragyna parvifolia (Roxb.) Korth. obtained from Maharashtra State.[34] It is therefore concluded that S_3 is pteropodine.

4.4 Identification of S_4 as isopteropodine

S_4 was obtained as white prismatic crystals from absolute ethanol (m.p. 199°C). The alkaloid was soluble in chloroform, ethanol and diethyl ether.

Thin layer chromatography

R_f value on Silica gel G/diethyl ether (developing twice) = 0.51 (Fig. XIII page 85).

Ultraviolet absorption spectrum (Ethanol) (Fig. XVII page 89)

λ_{max}	246	nm
λ_{min}	227	nm

Infrared absorption spectrum (KBr) (Fig. XXI page 93)

ν_{max}	3200	cm^{-1}	(imino)
	1700	cm^{-1}	(ester and oxindole carbonyl)
	1620	cm^{-1}	(double bond)
	1080	cm^{-1}	(ester)

S_4 is identical in m.p., ultraviolet and infrared absorption spectra, R_f value and colour reactions with the authentic sample of isopteropodine isolated from Mitragyna parvifolia (Roxb.) Korth. obtained from Maharashtra State.[34] It is therefore concluded that S_4 is isopteropodine.