CHAPTER III

EXPERIMENTAL

1. Source and authentication of plant material

The leaves of Uncaria homomalla Miq. were collected from Samlan, Saraburi, during the fourth week of every month between May 1976 and June 1977. The plant materials were authenticated by comparison with the herbarium specimen of the Botany Section, Technical Division, Department of Agriculture Ministry of Agriculture and Cooperation, Thailand.

2. Chemicals and solvents used

2.1 Chemical: anhydrous sodium sulphate

2.2 Solvents: a. acetic acid, glacial

b. acetone

c. ammonium hydroxide, stronger solution

d. anaesthetic diethyl ether

e. chloroform B.P.

f. ethyl acetate

g. ethyl alcohol 95%

h. methyl alcohol

3. General techniques

3.1 Extraction of alkaloids

The total crude alkaloidal extract was obtained by maceration of dried coarsely powdered leaves with 95% ethyl alcohol. The filtered ethanolic extract was concentrated under reduced pressure, mixed with glacial acetic acid and poured into a large volume of warm distilled water to make a 5% acetic acid solution. The filtered acid solution was made alkaline (pH 8-9) with strong solution of ammonium hydroxide and extracted repeatedly with chloroform. The chloroform extract was washed with distilled water and dried over anhydrous sodium sulphate and evaporated to dryness under reduced pressure to yield dry crude alkaloidal extract.

3.2 Thin layer chromatography

Analytical

Technique:	one way, ascending
Adsorbent:	silica gel G (E.Merck), calcium sulphate binding 13%, 30 g/60 ml distilled water.
Plate size:	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. chloroform + acetone (5+4)
	b. chloroform + ethyl alcohol (95 + 5)
	c. ethyl acetate + ether (1+1)
	d. ethyl acetate + ether (9+1)

e. ethyl acetate + isopropyl alcohol + strong solution of ammonium hydroxide (100++22+1)1)

f. ethyl acetate + isopropyl alcohol + strong solution of ammonium hydroxide (80 + 15 + 5)

Distance: 10 cm

Laboratory temperature: 20 -30 C

Detection a. Dragendorff's spray reagent

b. 0.2 M anhydrous ferric chloride in 35% w/w perchloric acid spray reagent. Plates after spraying, is warm gently with hot air stream from a hair dryer for 15 minutes. The colour reaction indicates the nature of the alkaloids as shown below :-

Colour change	Type of alkaloid
a. green to brown	pentacyclic heteroyohimbine
b. transient green	pyridino-indolo-quinolizidinone
c. green to pink	pentacyclic oxindole

4.1 Extraction and examination of alkaloids from the leaves of Uncaria homomalla Miq. collected from the same plant at monthly intervals throughout the year

4.1 Extraction of alkaloids

The dried coarsely powdered leaves (100)g) were moistened with 10% ammonium hydroxide solution and allowed to stand overnight. It was then macerated with 95% ethyl alcohol (500 ml) for three days and filtered.

The marc was remacerated with another portion of ethyl alcohol (500 ml). 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 make about 5% acetic acid solution, and left to stand overnight. The filtered acid solution was made alkaline with strong solution of ammonium hydroxide and extracted with chloroform (3 X 100 ml). The combined chloroform extract was dried over anhydrous sodium sulphate and evaporated under reduced pressure to yield a dry crude alkaloid. Table 1. and Figure 26 page 145 show the quantities of crude alkaloids obtained.

TABLE I

Date	Crude alkaloid % (w/w)
1976 : May	1.54
June	0.83
July	0.18
August	0.41
September	0.27
October	0.26
November	0.55
December	0.54
1977 : January	0.41
February	0.55
March	0.41

Date	Crude alkaloid
April	0.79
May	0.99
June	1.20

The crude alkaloids from each month collection were examined by TLC comparing, both by hRf values and colours produced with spray reagents, with authentic alkaloids previously reported to be present in Uncaria species, especially those of Uncaria homomalla Miq.[10,25] Four pentacyclic oxindole alkaloids, viz isopteropodine, pteropodine, speciophylline and uncarine F were shown to be present in every sample. Also presented were two unidentified oxindoles with hRf values not corresponded to any of those previously reported and "base-line" oxindole alkaloid(s). Another unidentified oxindole was noticed from January to April, 1977. Only one heteroyohimbine alkaloid could be detected, i.e. tetrahydroalstonine in the samples collected from May to August, 1976 and also from April to June 1977. In addition, one pyridino-indoloquinolizidinone indole alkaloid, angustine, was detected in the samples collected in May 1976, July 1976 and from January to June 1977. These results are presented in Table 2 on page 105, and Figures 1-25 (pp. 120-144).

Table 2 Alkaloids in the leaves of Uncaria homomalla Miq. collected at monthly intervals.

Date	Isopteropodine	Pteropodine	Speciophylline	Uncarine F	Tetrahydroalstonine	Angustine	Unidentified oxindole 1	Unidentified oxindole 2	Unidentified oxindole 3	"base-line" alkaloid(s)
1976, May	+	+	+	+	+	+	+	+	-	+
June	+	+	+	+	+	-	+	+	-	+
July	+	+	+	+	+	+	+	+	-	+
August	+	+	+	+	+	-	+	+	-	+
September	+	+	+	+	-	-	+	+	-	+
October	+	+	+	+	-	-	+	+	-	+
November	+	+	+	+	-	-	+	+	-	+
December	+	+	+	+	-	-	+	+	-	+
1977, January	+	+	+	+	-	+	+	+	+	+
February	+	+	+	+	-	+	+	+	+	+
March	+	+	+	+	-	+	+	+	+	+
April	+	+	+	+	+	+	+	+	+	+
May	+	+	+	+	+	+	+	+	-	+
June	+	+	+	+	+	+	+	+	-	+

4.2 Quantitative examination of alkaloids

4.2.1 Standard solution of reference alkaloids

Standard solutions of authentic alkaloids in chloroform were prepared, concentration of 4 mg/ml were used for isopteropodine, pteropodine and uncarine F, while 2 mg/ml was used for speciophylline, depending on their sensitivities to the spray reagents. Accurate volumes of these standard solutions were applied to TLC plates via a Hamilton syrings. The volumes of 1 μl to 20 μl were applied to each plate, without damaging the surface of the thin layer. The solvent was evaporated by means of gentle stream of air. The solvent systems used for TLC were :-

a. chloroform + acetone (5+4)

b. ethyl acetate + ether (9+1)

The least μl of alkaloid spots which gave positive results with Dragendroff's spray reagent were recorded and sensitivity quantity X 10^{-6} g were calculated. The results are presented in Tables 3-6.

Table 3 Isopteropodine - concentration 4 mg/100ml

Solvent system	First performance µl	Second performance µl	Third performance µl	Mean µl	Sensitivity quantity x 10^{-6} g
chloroform + acetone (5+4)	10	9	9	9.33	0.36
ethyl acetate + ether	9	9	9	9	0.36

Table 4 Pteropodine - concentration 4 mg/100ml

Solvent system	First performance µl	Second performance µl	Third performance µl	Mean µl	Sensitivity quantity x 10^{-6} g
chloroform + acetone (5+4)	7	7	7	7	0.28
ethyl acetate + ether (9+1)	7	7	7	7	0.28

Table 5 Speciophylline - concentration 2 mg/100ml

Solvent system	First performance µl	Second performance µl	Third performance µl	Mean µl	Sensitivity quantity x 10^{-6} g
chloroform + acetone (5+4)	13	13	13	13	0.26
ethyl acetate + ether (9+1)	13	13	13	13	0.26

Table 6 Uncarine F - concentration 4 mg/100ml

Solvent system	First performance µl	Second performance µl	Third performance µl	Mean µl	Sensitivity quantity x 10^{-6} g
chloroform + acetone	19	19	19		0.76
ethyl acetate+ ether	18	19	19		0.75

4.2.2 Total alkaloids solutions

Total alkaloids were dissolved in chloroform to make concentrations of 0.01%, 0.02% 0.1% and 0.2% respectively. The volumes of 1 µl to 20 µl of each concentration were applied to TLC via Hamilton syringe.

For examination of isopteropodine and pteropodine, ethyl acetate + ether (9+1), was used as solvent while chloroform

chloroform + acetone (5+4) was used for that of speciophylline and uncarine F.

Percentages of the alkaloids presented were the following formula :-

$$\text{Percentage of alkaloid} = \frac{\text{S.Q. x 10}}{\text{C x S.V.}}$$

S.Q. = Sensitivity quantity in gram

S.V. = Sensitivity volume of total alkaloids solution in microlited

C = Concentration of total alkaloids solution

Quantity of alkaloids presented in the leaves of Uncaria homomalla Miq. collected at monthly intervals are shown in table 7 p. 110 and Figures 27-33 pp. 146-152

Table 7

Date	Isopteropodine %	Pteropodine %	Speciophylline %	Uncarine F %
1976, May	36.00	28.00	2.16	4.75
June	30.00	35.00	6.50	3.45
July	16.36	35.00	32.50	11.69
August	20.00	56.00	20.00	10.86
September	18.00	40.00	10.83	10.86
October	7.50	35.00	28.88	12.67
November	12.00	46.70	8.67	9.50
December	13.30	46.67	17.33	12.67
1977, January	30.00	23.33	3.71	4.00
February	22.50	35.00	4.33	4.22
March	20.00	28.00	6.50	5.07
April	30.00	23.33	4.33	3.16
May	36.00	35.00	2.88	4.77
June	25.70	20.00	4.33	4.47