Chapter III

Experimental

1. Source and authentication of plant material

The leaves of *Litsea cubeba* Pers. were collected in December, 1991 from the road-side forests in the hills of Amphor Pay, Mae Hong Son province, Thailand at the altitude of 1,180-1,300 m. above sea level. The plant material was identified by Dr. Thawatchai Santisuke, Royal Forest Department, Thailand. The voucher specimen (No. 079207) was deposited at the herbarium of the Royal Forest Department, Phahonyothin Road, Bangkok, Thailand.

2. General techniques

2.1 Thin Layer Chromatography (TLC)

Technique:	one way ascending, tank saturated
Adsorbent:	a) silica gel G (E.Merck): silica gel GF (E.Merck) 1:1
	b) Aluminium oxide (neutral) for TLC
Plate size:	10 x 20 cm., 20 x 20 cm.
Layer thickness:	0.25 mm.
Activation:	Air dried for 15 minutes and then heat at 110 °c for 45 minutes
Solvent system:	Hexane-Chloroform-Methanol
Detection:	Modified Dragendorff's spray reagent
	The alkaloids give orange or yellowish orange spots with the reagent.
Distance:	15 cm.
Temperature:	25-30° C

2.2 Column Chromatography (CC)

Column size: 2.5 x 40 cm., 5 x 50 cm.,

Adsorbent: a) Silica gel G 60 for column chromatography (E.Merck)

b) Aluminium oxide 90 active(nuetral) for column chromatography

Packing: Adsorbent packed wet into the column

Addition of alkaloidal material to column:

The portion containing alkaloids was dissolved in a small amount of chloroform-methanol (1:1), then mixed with a small quantity of the adsorbent, this mixture was dried, triturated and, then added to the top of the column.

Solvent: a) hexane-chloroform-methanol-ammonia solution (3:1:0.3:0.001)

b) hexane-chloroform-methanol-ammoniasolution (1:3:0.3:0.001)

c) hexane:chloroform-methanol (3:1:0.35)

d) chloroform-methanol (1:1)

c) ethyl acetate

d) methanol

2.3 Centrifugal Partition Chromatography (CPC)

Model: 8924 chromatotron

Adsorbent: Silica gel 60 GF254 (E.Merck) for TLC

Layer thickness: 2 mm.

Solvent system: chloroform-hexane-methanol (3:1:0.3)

Rotorpreparation: Silica gel 60 GF 60g was thoroughly mixed with 120 ml of distilled water, then poured on the rotor and was subsequently heated at 60-70° C for 3 hours. The excess sorbent was scraped out.

Sample loading: Solvent was pumped to the chromatotron until the sorbent was completely wetted. A further 5 min was allowed for equilibrium.

A portion of the sample, dissolved in a small amount of the eluateing solvent, then taken up through the pump to the chromatotron.

Flow rate: 6-8 ml/min

Fraction size: 10 ml .

3. Spectroscopy

3.1 Infrared (IR) Absorption Spectrophotometry

Infrared absorption spectrum was obtained on a Perkin Elmer Model 1760 X USA infrared spectrophotometer. The absorption bands were reported in wave number (cm^{-1}).The materials were examined in KBr cell.

3.2 Ultraviolet(UV) absorption spectra.

The ultraviolet absorption spectrum of compound Z-35 was obtained on Hitachi model 220 A double beam spectrophotometer, while that of compound D-47 was obtained on a Milton Roy Sprotronic 3000 Ray. Chloroform were employed as the solvent for both compounds.

3.3 Nuclear Magnetic Resonance (nmr) spectrometry

^{1}H and ^{13}C-NMR spectra were obtained on a JNM-A500 (Alpha series) 500 MHz NMR spectrometer. Deuterochloroform ($CDCl_3$) was used as the solvent. Solvent locked signals were used as standard. The chemical shifts were reported on ppm scale.

3.4 Mass Spectra

Electron Impact Mass Spectra were determined on a JEOL FX 3000 double focussing mass spectrometer operated at 70 eV with inlet temperature of 150-240° C.

All the Spectrometic measurements were performed at The Scientific and Technological Research Equipment Centre, Chulalongkorn University.

4. Physical constants

4.1 Melting points

Melting points were determined on a Buchi melting point apparatus.

4.2 Optical rotation

Optical rotations were determined by Qualarimeter model 241 NC (Perkin-Elmer).

5. Screening for alkaloids

Powdered leaves (100g.) were macerated with methanol (200 ml.) overnight. After filtration, the methanolic extract (120 ml.) was evaporated to dryness over a steam bath. After the extract was cooled to room temperature, the residue was dissolved with 10 ml. of diluted HCl and filtered. The filtrate was subjected to the precipitation test with alkaloidal reagents. The orange precipitate formed with Dragendorff's reagent indicated the presence of alkaloid in the plant.

6. Extraction, seperation and isolation

6.1 Extraction

Finely ground leaves of *Litsea cubeba* Pers.(8 kg.) were boiled in 5% aqueous sulfuric acid (1kg./4 lt.) for 3 hours and filtered. The filtrate was extracted once with an equal amount of chloroform to reduce non alkaloid impurities. Then, the aqueous layer was made alkaline (pH 9-10) with strong ammonia solution and exhaustively extracted with chloroform (4 x 250 ml.). The combined chloroform extract was washed with a small amount of distilled water, dehydrated with anhydrous sodium sulfate, and concentrated under reduced pressure to give a crude alkaloid extract. The crude alkaloid extract was

once again subjected to the above-mentioned procedure to give crude alkaloid extract as a syrupy mass (5.62 g.).

6.2 Separation of the alkaloids

The crude extract (5.26 g.) was divided into 2 portions, each was subjected to silica gel column chromatography in the same manner. Each portion (approx. 2.6 g.) was dissolved in 4 ml. of chloroform and mixed with a small amount of silica gel. After the solvent was evaporated, the sample was added to the top of a glass column (5 x 30 cm.), already wet-packed with 70 g. of silica gel, using chloroform-hexane-methanol-ammonia solution (3:1:0.3:0.001) as solvent. The same system was utilized as eluting solvent, followed by methanol. Each 30 ml fractions were collected. Monitoring of all the fractions was routinely carried out by thin layer chromatograghy. Those of similar pattern on the thin layer chromatography were combined. The collective seperation profile are shown in Table 3.

Table 3 Major fractions from column chromatography of the crude extract (2.63 g).

Fraction number	Combined fraction	Remark
A	1-7	no alkaloid
B	8-13	alkaloid and impurity
C	14-15	no alkaloid
D	16-28	alkaloid and impurity
E(methanol)	29-50	no alkaloid

Fractions B and D were concentrated under reduced pressure to give 620 mg and 1.26 g. of crude extract, respectively.

6.2.1 Separation and isolation of an alkaloid from fraction B

An alkaloid from fraction B was isolated by column chromatography X,Y and Z successively, as follow;

Table 4 Column chromatography used in isolation of alkaloid from fraction B.

column	adsorbent	solvent	remark
column X 2 cm.diameter	Aluminiumoxide 20g	chloroform-methanol (1:1)	alk+impurity(320 mg) in fractions 16-18, each fraction was 10 ml.
column Y 2 cm.diameter	Silica G 60 15 g.	ethyl acetate	alk+impurity(96mg) in fraction 4-6, each fraction was 10 ml.
column Z 2 cm.diameter	Aluminium oxide 10 g.	ethyl acetate	alkaloid B in fractions 3-5 (24mg) in each fraction was 10 ml.

alk.=alkaloid

Fractions 3-5 contained only one alkaloid with the same Rf value. Therefore, they were combined and the residue was designated as Z-35 (24 mg.).

6.2.2 Separation and isolation of an alkaloid from fraction D

Fraction D (1.26 g.) was dissolved in a small amount of chloroform and mixed with a small quantity of aluminium oxide. After drying, the sample was packed onto the top of an aluminium oxide column(F), 100 g., 2.5 cm. diameter. The column was eluted with hexane-chloroform-methanol-ammonia solution (1:3:0.3:0.001 and 3:1:0.3:0.001) and, finally, the column was washed down with methanol. Each collected fraction was for 10 ml. The separation profile was displayed as Table 5.

Table 5 Column chromatography used in the separation of alkaloid from fraction D.

Fraction number	solvent system	remark
1-47	a	no alkaloid
48	b	no alkaloid
49-70	b	alkaloid+impurity(350 mg)
71-77	c	no alkaloid

Fractions 49-70 were combined and concentrated under reduced pressure. The alkaloid was isolated using centrifugal partition chromatography(G). Silica gel GF 60 (2 mm.thickness) was used as adsorbent. Ten fractions(10 ml.each) were eluted with chloroform-haxane-methanol (3:1:0.3). Fractions 4-7 contained only one alkaloid. They were combined and evaporated. Crystallization of the residue in methanol yielded alkaloid G-47 as pale pink plates (68 mg.).

7. Characterization of the alkaloids Z-35 and G-47.

The isolated compounds were characterized according to their spectroscopic and physical data.

7.1 Characterization of compound Z-35

7.1.1 Physical properties

melting point : 151-157°C

optical rotation: $[\alpha]^{25}$ -512° (c 1.4 mg/ml, methanol)

hRf value : 0.60 (chloroform-methanol, 17:3)

0.53 (toluene-methanol, 3:2)

0.40 (ethyl acetate-methanol, 1:1)

0.43 (chloroform-hexane-methanol, 3:1:0.2)

7.1.2 Spectral data

eims ; *m/z* (relative intensity); Figure 3, page 124

325 (93.0, M^+), 324 (100), 310 (36), 282 (36.4), 267 (15.9) and 250 (20.3)

uv ; λ max nm (ε), in chloroform; Figure 4, page 125

310 (15437), 260 (17875)

ir ; ν cm^{-1} , KBr disc; Figure 5, page 126

3400, 2900, 1640, 1450, 1270, 1030, 800

^{1}H nmr ; δ ppm, 500 MHz in $CDCl_3$; Figure 6, page 127

2.19 (1 H, m), 2.32 (1 H, m), 2.58 (3 H, s), 2.64 (1 H, ddd, *J*=4, 14.1, 14.1 Hz), 3.00 (1 H, dd, *J*= 4, 14.1 Hz), 3.14(1 H, dd, *J*=5.5, 10.7 Hz), 3.17 (1 H, ddd, *J*=1.2, 4, 13.6 Hz), 3.91 (3 H, s), 4.18(1 H, dd, *J*=8,8), 5.95 (1 H, d, *J*=1.5 Hz), 5.97 (1H, d, 1.5 Hz), 6.55 (1 H, s), 6.75(1 H, s), 7.93 (1 H, s)

^{13}C nmr ; δ ppm, 125 MHz in $CDCl_3$; Figure 7, page 129
27.7, 34.6, 43.3, 53.1, 56.2, 62.2, 100.8, 108.3, 108.8, 109.1, 119.5, 123.5, 125.7, 140.8, 145.9, 146.1, 146.3.

7.2 Characterization of compound G-47

7.2.1 physical properties

melting point: 121-122° C

optical rotation: $[\alpha]^{25}$ +11° (c 13.2 mg/ml, in $CDCl_3$)

hRf value: 0.70 (chloroform-methanol , 17:3)
0.62 (toluene-methanol, 3:2)
0.69 (ethyl acetate-methanol, 1:1)
0.57 (chloroform-hexane-methanol, 3:1:0.2)

7.2.2 spectral data

eims ; *m/z* (relative intensity); Figure 12, page 133
327 (90.6, M^+), 326 (100), 312 (29), 296 (12), 284 (36.9), 269 (17.9) and 253 (10.9)

uv ; λ max nm^{-1} (ε), KBr disc; Figure 13, page 134
241(3720) and 313.7(4560)

ir ; ν cm^{-1}, KBr disc; Figure 14, page 135
3200-3500, 2900

^1H nmr ; δ ppm, 500 MHz in $CDCl_3$; Figure 15, page 136
2.51(1H, dt, *J*=3.7,12.7 Hz), 2.53 (3H, s), 2.55 (1H, dt, *J*=0.92, 13.8 Hz), 2.63 (1 H, dd, *J*=3.4,15.8), 2.98 (1 H, dd, *J*=4.23, 13.8 Hz), 3.02 (1H, d, *J*=3.7), 3.04(1 H, m), 3.14(1 H, dd *J*=6.1, 12.7, 15.9 Hz), 3.93 (3 H, s), 3.91 (3 H, s), 6.54 (1 H, s), 6.82 (1 H, s), 8.01(1 H,s)

^{13}C nmr ; δ ppm, 125 MHz in $CDCl_3$; Figure 16, page 137

28.9, 34.2, 44.0, 53.5, 56.0, 56.1, 62.6, 108.7, 111.6, 113.9, 119.7, 124.0, 124.2, 127.3, 129.8, 140.5, 144.5, 144.9, 145.7.