CHAPTER III

Experimental

Source of Plant Material

The stem of *Pycnarrhena lucida* (Teijsm. & Binn.)Miq. was collected from Krabi, Thailand, in July, 1994. It was identified by comparing with the description in the Flora of Thailand (Forman, 1988).

A voucher specimen of the plant material has been deposited in the Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

General Techniques

2.1 Thin-layer Chromatography (TLC)

Technique : One way ascending

Adsorbent : Mixture of siliga gel 60G (Number 7731, E. Merck) and siliga gel 60 HF_{254}(Number 7739, E. Merck) (in 2:1 ratio) 30 g in distilled water.

Plate size : 5 X 20 cm.

Layer thickness : 0.25 mm.

Activation : Air-dried for 15 minutes and then heated in hot air oven at 110 ^{o}C for 1 hour.

Solvent system : Various solvent systems depending on materials.

Distance : 15 cm.

Temperature : 28-35 ^{o}C

Detection : 1) UV light (254 and 366 nm.)

2) Dragendorff's reagent

2.2 Preparative Thin-layer Chromatography (PLC)

Technique : One way ascending

Adsorbent : Mixture of silica gel 60G (Number 7731, E. Merck) and silica gel 60 HF_{254}(Number 7739, E. Merck)(in 2:1 ratio) 30 g in distilled water.

Plate size : 20 X 20 cm.

Layer thickness : 0.5 mm.

Solvent system : $CHCl_3$:Hexane:Diethylamine (4:5:1)

Distance : 20 cm.

Temperature : 28-35 ^oC

Detection : UV light (254 and 366 nm.)

Substance recovering:

The scraped of zones were warmed with MeOH, and filtered. After removal of MeOH, the residues were taken in a mixture of $CHCl_3$:MeOH(1:1), and filtered. Again, removal of the solvent, the residues were taken in $CHCl_3$ and filtered.

2.3 Column Chromatography

2.3.1 Conventional column chromatography

Column sizes : The glass columns 1.25-5 cm. in diameter were used depending on the quantity of sample to be separated.

Adsorbent : Silica gel 60 (Number 9385, E. Merck)

Packing method : Wet packing

Solvent : Various solvent systems depending on materials

Addition of sample extract:

The extract was dissolved in a small volume of ethanol, triturated with sufficient quantity of silica gel, air-dried and then dried under the vacuum. The material was finely ground and put on the top of the column.

Examination of the eluates:

Fractions were examined by TLC using visual detection under ultraviolet light (254 and 366 nm) and Dragendorff's reagent.

2.3.2 Gel filtration chromatography

Column size : The glass column 1/2 inches in diameter

Adsorbent : Sephadex LH20 (Pharmacia Biotech)

Packing method : Wet packing

Solvent : $CHCl_3$:MeOH (1:1)

Addition of sample extract:

The extract was dissolved in a small volume of eluant and put on the top of the column.

Extraction of the eluates: Fractions were examined by TLC using visual detection under ultraviolet light at wavelengths of 254 and 366 nm.

2.4 Spraying reagent

The Dragendorff's reagent was used as a general alkaloidal detecting reagent which characterized the alkaloids by giving orange colour. The stock solution consisting of a mixture of bismuth oxynitrate 1.7g, glacial acetic acid 20 ml, distilled water 80 ml and 5% aqueous potassium iodide 100 ml.

The working solution was made by mixing 10 ml of stock solution with 20 ml glacial acetic acid and 70 ml distilled water.

2.5 Melting point

Melting point were determined on a GallenKamp Melting point Apparatus Model MFB 595.

2.6 Spectroscopy

2.6.1 Ultraviolet (UV) Absorption Spectra

The ultraviolet absorption spectra were obtained on a Milton Roy Sprotronic 3000 Ray. Methanol was employed as the solvent for all compounds.

2.6.2 Infrared (IR) Absorrtion Spectra

The infrared absorption spectra were 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.

2.6.3 Mass Spectra (MS)

The electron impact mass spectra (eims) were obtained by operating at 70 eV with a Fisons VG Trio 2000 quadrupole mass spectrometer.

2.6.4 Proton and Carbon-13 Nuclear Magnetic Resonance (^{1}H- and ^{13}C-NMR) Spectra

^{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 signal were used as standard. The chemical shifts were reported on ppm scale.

2.7 Solvent

Throughout this work, all organic solvents were commercial grade and had to be redistillated prior to use.

Extraction

The dried coarsely powdered stems (0.5 Kg) were repeatedly marcerated for four times in ethanol (each, for 2 L, 2 days) and then filtered. The filtrate of each marceration was concentrated to remove ethanol under reduced pressure to yield 20 gm of dry crude extract (4% base on dried stem). TLC analysis of crude extract showed that at least four alkaloids were present with the addition of one compound which became blue under ultraviolet light.

Isolation Procedure

The crude extract (2g) was dissolved in a small volume of ethanol, triturated with sufficient quantity of silica gel, air-dried and then dried under the vacuum. The material was gently placed on top of the column of silica gel (150g,diameter 5cm.), repeated the same in 10 times. The column was eluted with chloroform:hexane:diethylamine (4:5:1) 4000 ml., and the fractional volume was about 20 ml. The eluates were examined by TLC using the same system as developing solvent, combined and evaporated to dryness under reduced pressure to give the results were summarized in table 5:

Table 5 The combined fractions from crude extract

Fraction	Number of eluates	Weight (g)
P-01	1-5	-
P-02	6-10	0.12
P-03	11-16	0.14
P-04	17-20	0.07
P-05	21-50	0.18
P-06	51-104	0.20
P-07	105-123	0.60
P-08	124-150	0.15
P-09	151-200	15

All fractions were examined by TLC. The fraction P-04 - P-08 contained some alkaloids and P-03 contained one compound which became blue under ultraviolet light. But fraction P-01, P-02 and P-09 have not been further studied.

The fraction P-03 (0.14g) was shown by TLC to contain one compound with a lot of dirty mass. This fraction was purified by the chromatographic technique using a column of Sephadex LH-20 with Chloroform:Methanol (1:1) as eluant. The eluates were collected fractions (20 ml each) of which mornitored by blue fluorescent under ultraviolet light.

One compound was crystallized from fraction 6-10. It yielded 10 mg and was named as CH05. This compound was identified as β-sitosterol.

The TLC chromatogram of fraction P-04 showed one alkaloid and another compound which fluoresce under ultraviolet light. This fraction (0.07g) was purified by the chromatographic technique using convention column of silica gel (50g, diameter 1.25 cm.) and eluted with chloroform:hexane:diethylamine (3:6:1). 20 ml.-fractions were collected. By TLC analysis, the liked fractions were combined to give the following portions:

P-04a (fraction 1-20) contained no substance

P-04b (fraction 21-30) contained one compound. It was the same compound that was formed in P-03, CH05(2 mg.), by mean of TLC analysis.

P-04c (fraction 30-50) contained mixture of alkaloid with other fluorescene substances.

Examination by TLC, fraction P-05 (0.18 g) contained at least 2 alkaloids. This fraction was further purified by conventional column chromatagraphy using silica gel (50g, diameter 1.25 cm.) and eluted with chloroform:hexane:diethylamine(4:5:1). 20 ml. of eluates were collected. After examining by TLC, the fractions were combined to give the following portions:

P-05a (fraction 1-28) contained no alkaloid.

P-05b (fraction 29-34) contained an alkaloid showing TLC pattern liked the combined P-04c. This fraction was separated for the compound named CH04 by preparative TLC technique using chloroform:hexane:diethylamine(4:5:1) as developing solvent. CH04 was separated and assigned as thalrugosine(206). (6.7 mg.)

P-05c (fraction 87-130) contained an alkaloid, via TLC analysis, together with other fluoresce substance. This fraction was purified by preparative TLC technique using chloroform:hexane:diethylamine(4:5:1) as developing solvent. CH03 was purified, recrystallized and identified as limacine (177). (3.9mg)

The fraction P-06 was recrystallized with benzene as pale yellow needles. It yielded 100 mg, was named as CH02. This compound was identified as berbamine (152).

The other pale yellow compound, from fraction P-08 was recrystallized from benzene. It yielded 50 mg named as CH01 which was identified as obamegine(193).

Spectral Data of Isolated Compounds

5.1 Compound CH01

eims : *m/z* (% relative intensity); Figure 2

594(9), 402(6), 381(27), 367(10), 191(100), 174(17), 168(42)

uv : λ_{max} nm (ε), in ethanol; Figure 3

284(6357)

ir : ν cm^{-1}, KBr disc; Figure 4

3400, 1650, 1230

^{1}H-nmr : δ ppm, 500 MHz, in chloroform-d; Figure 5

2.29 (3H, s), 2.38(1H, dd, *J*=20.14, 4.88 Hz), 2.51(3H,s), 2.64(1H, dd,*J*=15.56,10.98 Hz), 2.8(4H,m), 2.9(2H,m), 3.25(1H, dd, *J*=23.19, 4.88 Hz), 3.26(1H, dd, *J*=14.34,10.68 Hz), 3.38 (1H, m), 3.62(1H, dd, *J*=10.68, 4.88Hz), 3.78 (3H,s), 3.91(3H,s), 4.01(1H, dd, *J*=10.98, 2.44 Hz), 6.06(1H,

s), 6.24(1H, d, *J*=1.83 Hz), 6.35(1H, s), 6.45(1H, dd, *J*=8.24, 2.14 Hz), 6.63 (1H, dd, *J*=7.93, 1.83 Hz), 6.74(1H, s), 6.81(1H, dd, *J*=8.24, 2.14 Hz), 6.76 (1H, d, *J*=7.93 Hz), 7.05(1H, dd, *J*=8.24, 2.14 Hz), 7.32(1H, dd, *J*=8.24, 2.14 Hz)

^{13}C-nmr : δ ppm, 125 MHz, in chloroform-d; Figure 6

23, 26, 38.2, 39.1, 42.4, 43, 43.9, 46, 56(2C), 61, 65, 107, 113, 114.5, 115,121.3, 123(4C), 124.3, 130.2, 130.5, 130.8, 132, 132.8, 135.6, 136.3, 143.4, 144.1, 147, 148, 149, 154

5.2 Compound CH02

eims : *m/z* (%relative intensity); Figure 15

608(11), 416(7), 395(28), 381(18), 192(26), 198(100), 175(52), 174(52)

uv : λ_{max} nm (ε), in ethanol; Figure 16

283 (7877)

ir : ν cm^{-1} , KBr disc; Figure 17

3450, 1650, 1225

^{1}H-nmr : δ ppm, 500 MHz , in chloroform-d; Figure 18

2.24(3H, s), 2.4(1H, brd), 2.56(3H, s), 2.59(1H, m), 2.8(4H, m), 2.9(2H, m) 3(1H, d, *J*=14.04 Hz), 3.12(3H, s), 3.25(2H, dd, *J*=12.81, 6.41 Hz), 3.4(1H, m), 3.57(3H, s), 3.74(3H, s), 5.98(1H, s), 6.27(1H, s), 6.43(2H, br), 6.52(1H, s), 6.62(1H, dd, *J*=8.24, 2.44 Hz), 6.74(1H, dd, *J*=8.24, 1.53 Hz), 6.81(1H, d, *J*=8.24 Hz), 7.09(1H, dd, *J*=8.24, 2.44 Hz), 7.25 (1H, dd, *J*=8.24, 2.44 Hz)

^{13}C-nmr : δ ppm, 125 MHz, in chloroform-d; Figure 20

25.5(3C), 37.7,38.5,42.7(2C), 45.9(2C), 55.6(2C), 60.4, 62.1, 63.4, 105.4, 111.2, 114.4, 115.2, 119.8, 120.7, 121.3, 121.6, 123.4, 127.5, 128.3, 128.7, 130.2, 132.2, 135.6(2C), 136.9, 143.5, 121.6, 147.4, 148.1, 149.8, 151.7, 153.7

5.3 Compound CH03

eims : *m/z* (%relative intensity); Figure 28

609(7), 608(6), 381(32), 367(15), 191(100), 174(23), 168(28)

uv : λ_{max} nm (ε), in ethanol; Figure 29

284(8765)

ir : ν cm^{-1}, KBr disc; Figure 30

3450, 1645, 1240

^{1}H-nmr : δ ppm, 500 MHz, in chloroform-d; Figure 31

2.46(1H, dd, *J*=15.96, 4.28 Hz), 2.57(1H, d, *J*=13.74 Hz), 2.65(3H, s), 2.8(3H, m), 2.9(4H, m), 3.30(1H, dd, *J*=12.51, 5.49 Hz), 3.43(1H,m), 3.53(1H,m), 3.78(4H, overlaping), 3.93(4H, overlaping0, 6.05(1H,s), 6.30(1H,s), 6.32(1H, dd, *J*=8.24, 2.13 Hz), 6.52(1H, s), 6.56(1H, d, *J*=1.84Hz), 6.81(1H, dd, *J*=8.24, 2.13 Hz), 6.84(1H, d, *J*=8.24 Hz), 6.90 (1H, dd, *J*=8.24, 1.84 Hz), 7.13(1H, dd, *J*=8.24, 2.13Hz), 7.33(1H, dd, *J*=8.24, 2.13 Hz)

^{13}C-nmr : δ ppm, 125 MHz, in chloroform-d; Figure 32

21.8, 25, 38, 42, 42.5(2C), 44.9, 45.1, 56.1(2C), 61.2, 63.8, 105, 111.8, 112.5, 116, 120.8, 121(2C), 123(3C), 127.6, 128, 130, 132.3, 135(3C), 142.5, 143, 145.7, 146.7, 148.4, 149, 154

5.4 Compound CH04

eims : *m/z* (%relative intensity); Figure 40

608(9), 381(33), 367(17), 191(100), 174(26), 168(25)

uv : λ_{max} nm (ε), in ethanol; Figure 41

284(8734)

ir : ν cm^{-1} , KBr disc; Figure 42

3450, 1645, 1240

^{1}H-nmr : δ ppm, 500 MHz, in chloroform-d; Figure 42

2.32(3H, s), 2.35(1H, dd, *J*=16.48, 6.8 Hz), 2.51(3H, s), 2.64(1H, dd,

J=14.65, 10.68), 2.8(4H, m), 2.9(3H, m), 3.23(2H, m), 3.38(1H, m), 3.61(1H, dd, *J*=11.29, 4.88Hz), 3.78(3H, s), 3.94(6H, s)4.01(1H, dd, *J*=10.68, 2.14 Hz), 121.5(1H, s), 6.29(1H, d, *J*=1.83 Hz), 6.35 (1H, s), 6.47(1H, dd, *J*=8.24, 2.14 Hz), 6.69(1H, dd, *J*=8.24, 1.83 Hz), 6.75 (1H, S), 6.80(1H, d, *J*=8.24 Hz), 6.68(1H, dd, *J*=8.24, 2.14 Hz), 7.06 (1H, dd, *J*=8.24, 2.14 Hz), 7.31(1H, dd, *J*=8.24, 2.14Hz)

^{13}C-nmr : δ ppm, 125 MHz, in chloroform-d; Figure 45

21.3, 25.9, 38.1, 39.3, 42.1, 43.3, 44, 46, 56(3C0, 60.2, 65.2, 107.9, 111.5, 112.5, 115, 121.5, 122, 122.8, 123, 124.4, 130, 130.8, 131, 132, 133.6, 135.6, 136.5, 143.6, 144.3, 146.8, 147, 149.2, 150.3, 154.5

5.5 Compound CH05

eims : *m/z* (%relative intensity); Figure 52

414(10), 396(5), 381(3), 329(8), 273(8), 255(24), 231(6), 213(28), 173(7), 147(30)

ir : ν cm^{-1} , KBr disc; Figure 53

3500-3200, 3000-2800, 1640, 1470, 1390, 1090

^{1}H-nmr : δ ppm, 500 MHz, in chloroform-d; Figure 54

0.68-1.01, 3.52(m), 5.35(m)

^{13}C-nmr : δ ppm, 125 MHz, in chloroform-d; Figure 55

11.9, 12, 18.8, 19, 19.4, 19.8, 21, 23, 24.3, 26.1, 28.3, 29.1, 31.7, 32(2C), 33.9, 36.2, 36.4, 37.2, 39.8, 42.3(2C), 45.9, 50.1, 56, 56.8, 72, 122, 140.5