CHAPTER III

EXPERIMENTAL

I. Source of Plant Materials

Leaves of *Annona squamosa* Linn. used in this study were collected from Muang District, Lopburi, Thailand in March 1985.

II. General Techniques

A. Thin Layer Chromatography

Technique : One way, ascending, tank saturated

Adsorbent : Silica gel G + Silica gel GF_{254} (E. Merck) (15 + 15) g./60 ml distilled water

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

Layer thickness : 250 μ

Activation : Air dried for 15 minutes and then at 110°C for 45 minutes

Solvent systems :
1) Ethyl acetate : Methanol (7:3)
2) Ethyl acetate : Methanol (9:1)
3) Chloroform + Acetone + Methanol (2+2+1)
4) Chloroform + Acetone + Methanol (4+6+1)
5) Benzene : Methanol (9:1)
6) Chloroform : Methanol (9:1)

7) Ether

8) Chloroform

Distance : 15 cm

Temperature : 25-30°C

Detection : 1) UV light of 366 nm

The alkaloids become visible as yellow fluorescent spots under UV light of 366 nm.

2) Modified Dragendorff's spray reagent

Solution A : Bismuth subnitrate (850 mg), distilled water (40 ml) and glacial acetic acid (10 ml)

Solution B : Potassium iodide (8 g) and distilled water (20 ml)

Solution A and B, 5 ml each, were mixed, 20 ml of glacial acetic acid and 70 ml of distilled water were added and used as spray reagent. The alkaloids give orange or yellowish orange spots with the reagent.

B. Column Chromatography

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

Adsorbent : Silica gel 0.040-0.200 mm (E. Merck)

Packing : Adsorbent packed wet into the column

Application of crude alkaloid to column :

1) Dry method (in case of partially soluble or insoluble material)

Crude alkaloid was dissolved in small amount of solvent, then, mixed with small quantity of adsorbent. Triturated until the solvent was evaporated, then added to the top of the column.

2) Wet method (in case of soluble material)

Crude alkaloid was dissolved in small amount of solvent, then cautiously pipetted into the top of the adsorbent without interfering the adsorbent front surface.

Solvents : 1) Acetone GR. Merck

2) Chloroform GR. Merck

3) Ethyl acetate GR. Merck

4) Methanol GR. Merck

Collection of eluate

: Fractions of 20 ml were collected.

Examination of eluate

: Those fractions giving an orange color with Modified Dragendorff's spray reagent were examined by Thin-layer chromatography.

C. Physical Constant

Melting point was determined in open capillaries by a Büchi 520 Melting Point Apparatus. The value recorded was uncorrected.

D. Spectroscopy

1) Ultraviolet absorption spectrum was measured in a Hewlett-Packard 8450 A UV/VIS spectrophotometer.

2) Infrared absorption spectrum was examined with a Perkin-Elmer 281 B Infrared spectrophotometer.

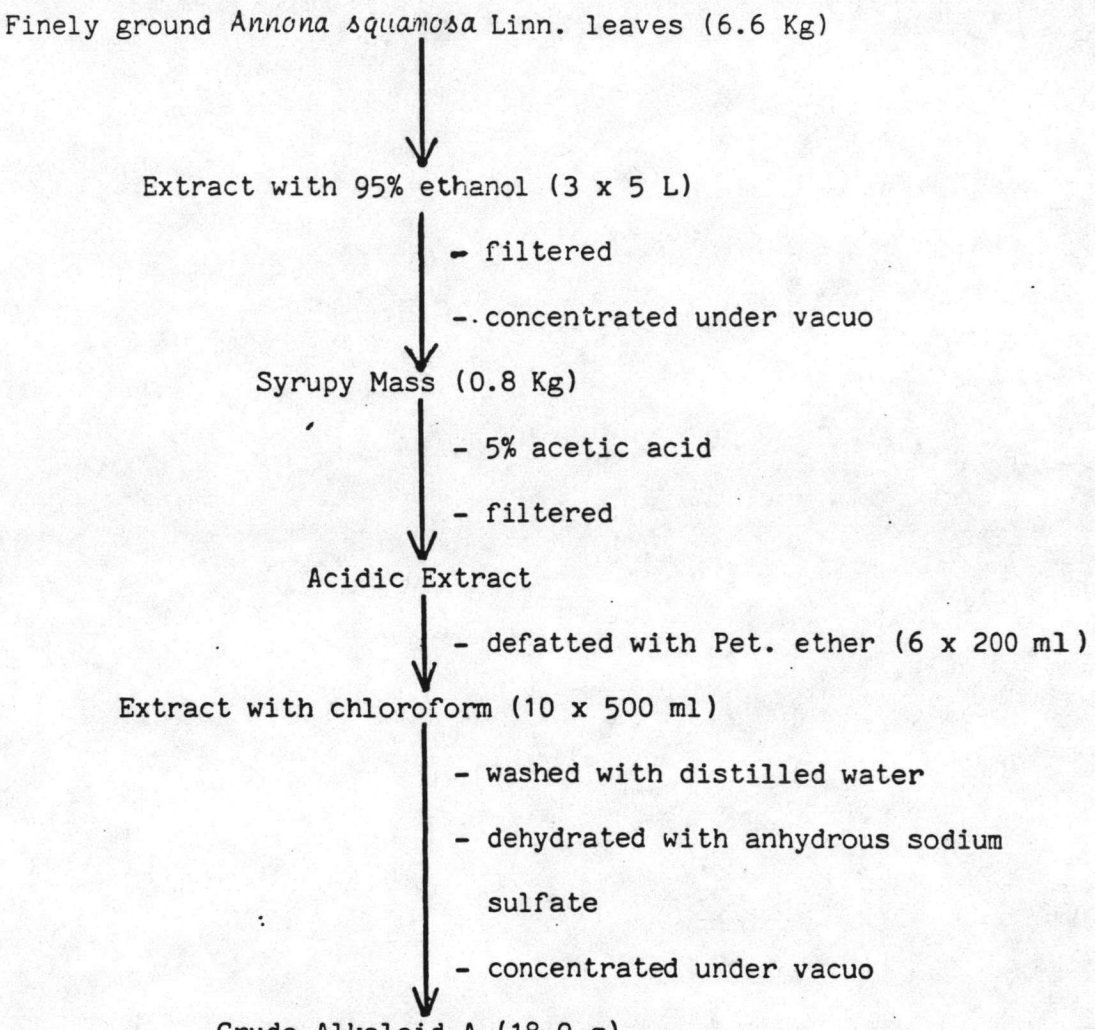
3) Nuclear magnetic resonance spectrum was obtained in $CDCl_3$ using a Bruker WP 200 MHz Supercon (FT) Spectrometer.

4) Mass spectrum was recorded in a Kratos MS 9/50 instrument.

III. Extraction and isolation of alkaloids from the leaves of *Annona squamosa* Linn.

A. Extraction of crude alkaloids

The oven dried (40-50°C), finely ground leaves (6.6 Kg) were exhaustively extracted with 95% ethanol (3 x 5 L). The ethanolic extract was filtered and then concentrated in vacuo to a dark green viscous mass (0.8 Kg). The resulting syrupy mass was dissolved in glacial acetic acid (200 ml) and then poured into warm distilled water to give about 5% acetic acid solution. The insoluble materials were removed by filtration through Kieselguhr layer. The acidic filtrate was defatted with petroleum ether (6 x 200 ml) and extracted with chloroform (10 x 500 ml). The completeness of extraction was controlled with Modified Dragendorff's reagent. The combined chloroform extract was washed with distilled water and dehydrated by anhydrous sodium sulfate, then concentrated under reduced pressure to give a crude alkaloid A (18.9 g). The crude alkaloid A contained at least seven alkaloids (two main alkaloids and five minor alkaloids) as indicated by TLC on silica gel plates. (Fig. I and II pages 80, 81)



Scheme XI Outline of extraction process

B. Isolation of alkaloids

The crude alkaloid A (18.9 g) was divided into 3 portions, 6.3 g each, and treated in the same manner. Dissolved each portion in chloroform (15 ml), mixed with small amount of silica gel. Triturated until the solvent had been evaporated, then added to the top of the silica gel column (5 cm x 50 cm) which had been already wet packed by using ethyl acetate as solvent.

The column was eluted with ethyl acetate : methanol (9:1) and 20 ml fractions were collected until no traces of alkaloid could be detected. Fractions were examined by TLC. The identical fractions were combined together and concentrated to dryness under vacuo.

Because only traces of alkaloids were detected, the first three combined fractions were discarded. The fourth combined fraction composed of one main alkaloid (designated N_1). By means of re-column chromatography, using silica gel/chloroform + acetone + methanol (4+6+1) system, the only one yellow band appeared in the column was eluted. As a result, N_1 was purified. When it was dissolved in chloroform : methanol (95:5) and allowed to stand in the dark for 24 hours, transparent glistening yellowish-brown tabular crystals were obtained. They were washed with ether. After drying the alkaloid N_1 in desiccator overnight, it yielded 20 mg of alkaloid N_1.

C. Identification of alkaloid N_1 as Lanuginosine

N_1 crystalized in chloroform : methanol (95:5) as yellowish-brown tabular crystals.

Its solubility is as follows :

Solubility	Solvents
very soluble	methanol
soluble	chloroform, ethyl acetate
very slightly soluble	ether

1. hRf values

The hRf values were obtained from the solvent systems as follows :

Ether	=	0
Chloroform	=	8
Benzene : Methanol (9:1)	=	33
Ethyl acetate : Methanol (9:1)	=	45
Chloroform + Acetone + Methanol (4+6+1)	=	59

(hRf = Ratio between the distances of the spot on chromatogram and the solvent front, multipled by 100)

The thin layer chromatograms of alkaloid N_1 are shown in Fig. III-VII, pages 82-86.

2. Melting Point

270.0°C (decomposed)

3. Molecular Weight

305 (mass spectrometry)

4. Ultraviolet Absorption Spectrum

λ_{max}^{MeOH} nm : 246, 273, and 315

λ_{max}^{MeOH} + HCl nm : 258, 283, and 334

5. Infrared Absorption Spectrum

ν_{max}^{KBr} cm^{-1} : 2900, 2820, 1655 (conjugated C=O), 1600, 1448, 1282, 1095, and 990 ($CH_2<^{O-}_{O-}$)

6. Nuclear Magnetic Resonance Spectrum (200 MHz, $CDCl_3$ and TMS as internal standard)

δ (ppm)	3.99 (3H, s)	OCH_3
	6.35 (2H, s)	$CH_2<^{O-}_{O-}$
	7.14 (1H, s)	C_3-H
	7.30 (1H, dd, J = 9, 2.8)	C_{10}-H
	7.77 (1H, d, J = 5)	C_4-H
	8.02 (1H, d, J = 2.8)	C_8-H
	8.55 (1H, d, J = 9)	C_{11}-H
	8.88 (1H, d, J = 5)	C_5-H

7. Mass Spectrum

m/e (% rel.int.) 305 (M^+, 100),

275 ($M^+ - CH_2O$, 30)

The UV, IR, NMR and Mass spectra of N_1 are identical with data from the literature of lanuginosine isolated from *Annona squamosa* Linn. in all aspects (4,101,102). It is therefore concluded that N_1 is lanuginosine.