CHAPTER III

EXPERIMENTAL

1. Source and Authentication of Plant Materials

The Flowers of *Cassia spectabilis* DC. was collected from Saraburi Province, in October, 1988. The plant material was identified to be *Cassia spectabilis* DC. of family Caesalpiniaceae by comparison with herbarium specimen in the Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperative, Thailand.

2. General Techniques

2.1 Thin Layer Chromatography (TLC)

Technique : One way ascending, tank saturated

Plate size : 20 cm x 20 cm, 1o cm x 20 cm

Layer thickness : 25 μ m

Activation : Air dried for 15 minutes and then at 110°C for 1 hour.

Solvent system :

System	Component	Ratio
A	$CHCl_3$: EtOH : NH_3	20 : 1 : 0.5
B	$CHCl_3$: EtOH : NH_3	9 : 1 : 0.5

C	$CHCl_3$: MeOH : NH_3	50 : 1 : 0.5
D	$CHCl_3$: MeOH : NH_3	20 : 1 : 0.5
E	$CHCl_3$: MeOH : NH_3	9 : 1 : 0.5

Distance : 15 cm

Laboratory temperature : 25°C - 30°C

Authentic Samples : Cassine and iso-6-cassine were supplied by Assistant Professor Chaiyo Chaichantipyuth, staff of the Department of Pharmacognosy, Faculty of Pharmaceutical Science, Chulalongkorn University.

Detection :
1) UV light (254 nm)
2) Iodine vapour
3) Fluorescarmine solution (0.02% in Acetone)
4) Dragendorff's reagent

2.2 Column Chromatography (CC)

Adsorbent : Silica gel 230-400 mesh (E. Merck)

Packing of column: Wet-packing technique

Width of column : 10, 4.5, 2.5 cm

Length of column : 50, 40 cm

Solvent :
1) Chloroform
2) Methanol

3) Strong ammonia solution

Fraction Size : 100, 25, 10 ml

Examination of elute : Dragendorff's reagent and TLC monitoring

2.3 Melting Point Determination

All melting points were determined by Buchi melting point apparatus.

2.4 Ultraviolet Spectroscopy (UV)

UV spectra of all compounds were obtained in suitable concentration (in chloroform), with quartz cell, 1-cm width, using Shimadzu Double-Beam Spectrophotometer UV-180.

2.5 Infrared Spectroscopy (IR)

Infrared absorption spectra were obtained of Shimadzu Model IR 440 spectrophotometer, adsorption band were reported in wave number (cm^{-1})

2.6 Nuclear Magnetic Resonance Spectroscopy (NMR)

Proton magnatic resonance(^{1}H-NMR) spectra and Carbon-13 magnetic resonance (^{13}C-NMR) spectra were recorded at 90 MHz of Jeol FX 90Q. Tetramethylsilane (TMS) was using as an internal standard and chemical shift were reported on the ppm scale.

2.7 Mass Spectroscopy (MS)

These compounds were submitted for low resolution mass spectra study of Jeol mass spectrometer Model DX 300 at 70 eV.

UV, IR, NMR and MS apparatus are obtained from the Science and Technological Research Equipment Center, Chulalongkorn University.

3. Extraction and Purification

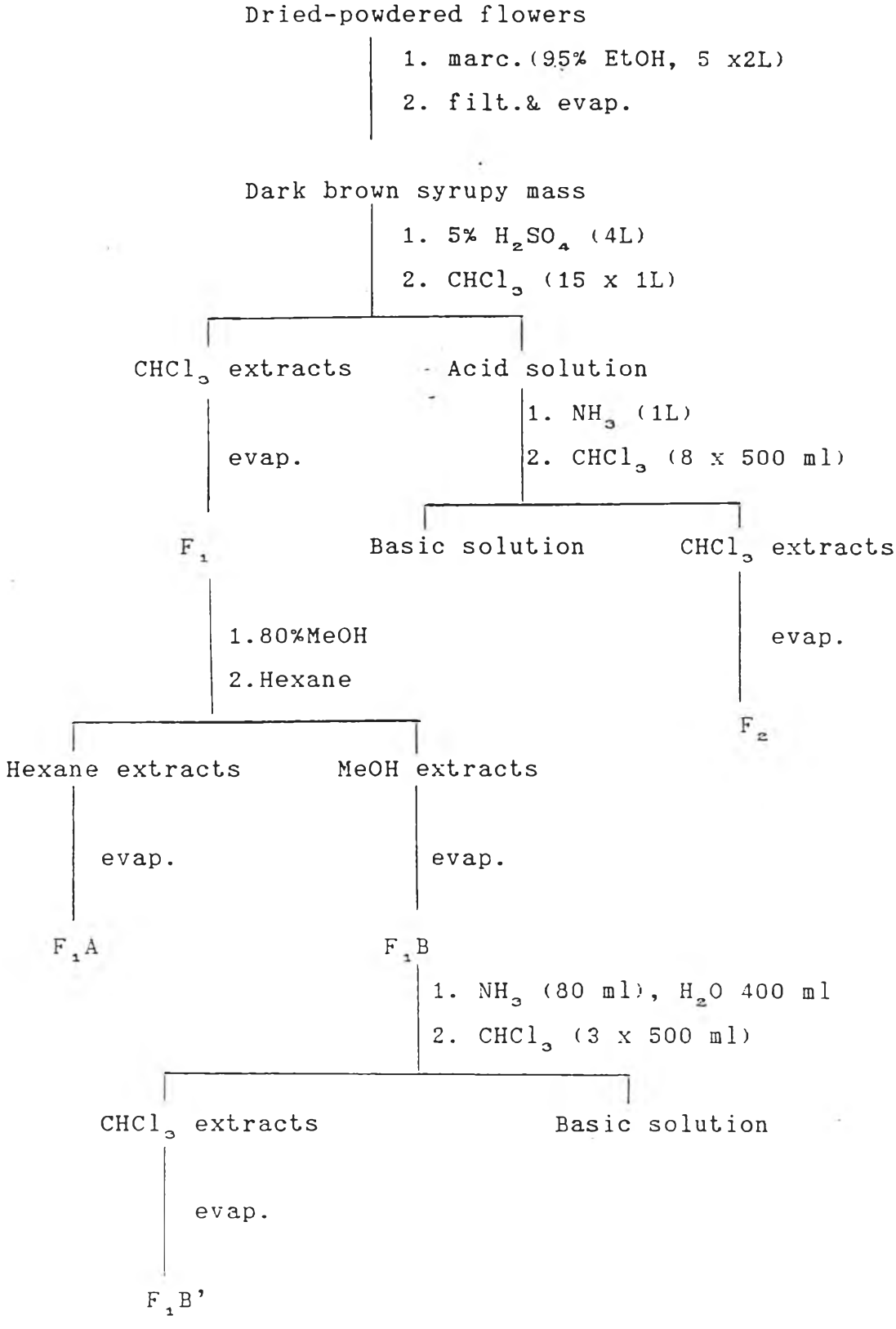
3.1 Extraction procedure

The dried coarsely powdered flowers (5.2 Kg) were macerated with 95% ethanol (5 x 2L) for five days, and filtered. The filtrated was concentrated under reduced pressure to dryness, and treated with 4L of 5% sulfuric acid . The acid solution was filtered, and extracted with chloroform (15 x1L). The combined chloroform extracts were evaporated, yielding 1.2 Kg of dark brown syrupy mass (F1). The aqueous solution was made to alkaline with 1L of strong ammonium hydroxide solution and extracted with chloroform(8 x 500 ml),after drying(Na_2SO_4 anhydrous) and evaporation, yielding 70 g of dark brown syrupy mass(F2).

The fraction F1 was dissolved with 80% methanol in water(1L) and extracted with hexane (5 x 1L), after drying and evaporation, yielding 800 g of dark yellow syrupy mass (F_1A). The methanol fraction was evaporated, made to alkaline with strong ammonium hydroxide solution(80 ml), diluted with 400 ml of water and extracted with chloroform (3 x 500 ml). The combined chloroform extracts, after drying, were evaporated to yield 150 g of dark brown syrupy mass (F_1B').

The extraction procedure of the flowers of *Cassia spectabils* DC.is shown in scheme 5.

Scheme 5. THe Extraction Procedure of the Flowers of *Cassia spectabilis* DC.



3.2 Isolation

The combined F1B' and F2 (220g) was devided into ten equal portion and each portion was subjected to column chromatography in the same manner. Each portion (appoximate 22.0 g) was dissolved in 20 ml of chloroform and placed on the top of 10 cm diameter column of silica gel (1 kg). The gradient of increasing elution polarity of the solvent mixture was applied from chloroform to chloroform+methanol until methanol. The various fractions (100 ml each) were examined and combined (table 3) according to the information obtained from the check TLC (figure 3).

The combined fraction A was yellow mass, gave negative to Dragendoff's test and was not further investigated.

The combined fraction B was chlorophyll with few traces of alkaloids and was not further investigated.

The combined fraction C was shown by TLC to contain spot of L-1. This fraction was devided to 3 equal portion and placed on silica gel column (100 g, diameter 4.5 cm), using chloroform : methanol (50:1) as eluent. Each 25 ml of elute was collected, evaporated and compared by TLC. Fraction 14-16 were combined , evaporated to dryness and designated as L-1 (600 mg, 11.5 mg%).

The combined fraction D was shown by TLC to contain spot of L-2. This fraction was devided to 5 equal portion and rechromatographed, twicely, same manner as the combined fraction C. Fraction 23-25 was designated as L-2 (200 mg, 3.8 mg%).

The combined fraction E was shown by TLC to contain spot of L-3, L-4 and L-5. This fraction was devided to five equal portions and rechromatographed on silica gel column(30 g,diameter 2.5 cm),using chloroform: methanol : strong ammonia solution (20:1:0.5) as eluent. Each 10 ml of elute was collected, evaporated to dryness and compared by TLC :

1) fraction 15-16 was designated as L-5
2) fraction 16-23 was designated as L-3
3) fraction 24-26 was designated as L-4

The combined fraction 15-16 was evaporated to dryness, yielding clear oily mass of L-5(80 mg, 1.5 mg%). The combined fraction 16-23 were recrysallized in hexane to yield white needle crystals of L-3 (300 mg, 5.8 mg%). And the combined fraction 24-26 was evaporated to dryness, yielding clear oil of L-4 (150 mg, 2.9 mg%).

The combined fraction F was shown by TLC to contain small amount of alkaloid and was not further investigated .

The combined fraction G was dark brown mass of resin.

Table 4 Elution pattern of combined fraction F_1B'and F_2

Flask No.	Solvent system	Volume(L)	Combined fraction	Remark
1-2	chloroform	0.2	A	yellow mass
3-6	chloroform	0.4	B	chlorophyll with traces of alkaloids
7-10	chloroform : methanol (50:1)	0.4	C	one alkaloid mainly
10-15	chloroform : methanol (50:1)	0.6	D	one alkaloid mainly
15-23	chloroform : methanol (20:1)	0.9	E	mixture of alkaloids
24-30	chloroform : methanol (20:1)	0.7	F	trace of few alkaloids
31-50	methanol	2.0	G	dark brown mass

4. Hydrolysis of L-1

L-1 was weighted about 50 mg,dissolved with 10 ml of methanol. Sodium bicarbonate solution (5%wt/vol) was added into solution of L-1, boiled in steamed bath for one hour and extracted with chloroform (3 x 10 ml). The combined chloroform extracts were evaporated to yield D-1. Basic aqeous solution was treated with hydrochloric acid until solution was acid, extracted with chloroform (3 x 5 ml) and evaporated to yield unknown benzoic acid.

5. Acetylation of cassine and iso-6-cassine

About 50 mg of cassine and 100 mg of iso-6-cassine was dissolved with 1 ml of pyridine. Each solution was added 2 ml of acetic anhydride, stirred for four hours, extracted with chloroform (3 x 10 ml) and washed the extracts with distilled water to get rid of excess acetic anhydride and pyridine. The combined extract of acetylation product of cassine, after evaporation to dryness, is Ac-1 and of iso-6-cassine is Ac-2.