CHAPTER III

EXPERIMENTAL

1. Source of plant material

The leaves of *Cassia garrettiana* Craib were collected from Sam Lan National Park, Saraburi Province, Thailand in June 1975. The leaves were dried and identified by comparison with the herbarium specimen in the Department of Pharmacognosy, Chulalongkorn University Faculty of Pharmaceutical Sciences.

2. General techniques

2.1 Thin layer chromatography

Technique : One way, ascending

Adsorbent : Silica gel G (E. Merck) 13% calcium sulphate binding, 30 g/60 ml distilled water.

Plate size : 20 cm x 20 cm, 20 cm x 10 cm.

Layer thickness : 250 μ

Activation : air dried for 15 minutes and then 1 hour at 105°C

Solvent system : Benzene, Methyl alcohol 9+1

: Benzene, Ethyl acetate, Acetic acid 75+24+1

: Petroleum ether b.p. 40°-70°C, Ethyl

acetate, Acetic acid 45+5+3

: Di-iso-propyl ether

: Chloroform, Methyl alcohol 6+4

Distance : 15 cm

Laboratory temperature : 20°-30°C

Detection : Ultraviolet light

: NH_3 vapour

: 5% Alcoholic potash

: 0.5% Magnesium acetate in Methyl alcohol

2.2 Column chromatography

Adsorbent

Silica gel (Woelem)

Packing of column

Adsorbents packed dry into the column.

Addition of anthraquinone material to the column

Solution in small volume of volatile solvent mixed with small quantity of adsorbent, air dried and added to the top of a dry column.

Solvents and Chemicals

a. Benzene (Shell)

b. Chloroform (I.C.I.)

c. Ethyl acetate (May and Baker)

d. Glacial acetic acid (BHD Chemical Ltd.)

e. Hydrochloric acid (Riedel)

f. Methyl alcohol (Riedel)

g. Petroleum ether b.p. 30°-50°C (Carlo erba)

h. Petroleum ether b.p. 40°-70°C (Carlo erba)

i. Ethyl alcohol 95% (The Government Pharmaceutical Organization)

j. Sodium hydroxide (May and Baker)

k. Sodium bicarbonate (I.C.I.)

l. Anhydrous sodium sulphate (May and Baker)

Collection of eluate :

Fractions of 20 ml or more were collected manually.

Examination of eluate :

Those fractions giving an orange colour with 5% alcoholic potash spray reagent were examined by thin layer chromatography, like fractions were combined and concentrated to dryness under reduced pressure.

2.3 Melting point determination.

Determined by means of Buchi melting point apparatus.

2.4 Ultraviolet absorption spectrum.

Ultraviolet absorption spectrum was determined with a Unicam SP 800 in Mahidol University Faculty of Science.

2.5 Infrared absorption spectra.

Infrared spectra were determined in Mahidol University Faculty of Science, by a Perkin Elmer 421 Grating Spectrometer.

2.6 Nuclear magnetic resonance (NMR) spectra.

NMR spectra were determined in Mahidol University Faculty of

Science, by a Varian A-60 D spectrometer using tetramethylsilane (TMS) as an internal standard, chemical shifts are δ valued in parts per million down field from TMS.

3. The isolation of anthraquinone from the leaves of *Cassia garrettiana* Craib

3.1 Test for anthraquinones :-

Dried powdered leaves (250 mg) were boiled with 10 ml of water and 5 ml of concentrated hydrochloric acid for 15 minutes and filtered. The filtrate was cooled and extracted with ether. The ether layer was separated and shaken with ammonium hydroxide solution. The ammonium hydroxide layer became red if anthraquinone was present.

3.2 Isolation of anthraquinone :-

The dried coarsely powdered leaves (8 Kg) were refluxed with 3% acid alcohol (97 ml of 70% ethyl alcohol and 3 ml of glacial acetic acid) about 4 hours. The purpose of this step was to liberate the free anthraquinones and their corresponding glycosides from their magnesium, potassium, or sodium salts which are present in the plants. Filtered and re-extracted the marc exhaustively with 3% acid alcohol. The combined filtrate was then evaporated under reduced pressure to syrupy mass (16 g).

The syrupy mass (16 g) was dissolved in hot 3% acetic acid (5 L) and then filtered through kieselguhr from which chlorophyll and some other impurities were removed. The filtrate was extracted with chloroform until the last extraction gave no pink colour with 5% alcoholic

potash. For complete extraction, chloroform (4 L) was required. The combined chloroform solution was concentrated under reduced pressure to about 400 ml residue. The residue (400 ml) was extracted with 5% sodium bicarbonate solution (2 x 400 ml) for extraction of aglycones which have free carboxyl groups. The residue (400 ml) was then extracted with 5% sodium hydroxide solution (4 x 200 ml). The combined sodium hydroxide extract (800 ml) was added dropwise with concentrated hydrochloric acid until the colour of the solution was turned from pink to yellow. By means of this process, anthraquinone salt was changed into free anthraquinone. The yellow acid solution was extracted with chloroform (3 x 300 ml). The combined chloroform extract was washed with distilled water, dried over anhydrous sodium sulphate and evaporated to dryness under reduced pressure to yield a brown semi-solid (2.05 g). Thin layer chromatography showed the presence of at least 3 anthraquinones (Fig. 1,2 P. 48,49).

The brown semi-solid (2.05 g) was divided into 8 equal portions and each portion was treated in the same manner. The brown semi-solid (approx. 0.26 g) was dissolved in chloroform (6 ml), mixed with silica gel (5 g), air dried, added to the top of a dry silica gel column (2.5 cm x 22 cm) and eluted with benzene, methyl alcohol 9+1 (500 ml). The 25 ml fractions were collected and like fractions determined by TLC were combined. The combined eluate was evaporated to dryness under reduced pressure to yield a yellow solid (1.46 g). This solid was shown to contain only one anthraquinone by TLC (Fig. 3 p.50). It was dissolved in benzene, methyl alcohol 9+1 (35 ml) and petroleum ether b.p.

30°-50°C (5 ml) was added dropwise and yielded yellow needle crystals of I_1 (1.15 g). Two recrystallisations from the same mixture of solvents as mentioned above yielded pure yellow needle crystals of I_1 (0.9 g) m.p. 220°-222°C, I_1 was subsequently identified as aloe-emodin.

3.3 Acetate derivative of anthraquinone

I_1 (70 mg) was dissolved in acetic anhydride (10 ml) and 3 drops of pyridine were added. The mixture was refluxed for 6 hours and then poured into 30 ml of ice water with vigorous stirring. Stirring was continued until excess acetic anhydride was completely hydrolysed. Precipitates (130 mg) were removed by filtration, washed throughly with distilled water, and purified by crystallisation. The crystals are yellowish green needles which are readily soluble in methyl alcohol, ethyl alcohol, and chloroform.

4. Characterisation of I_1 as aloe-emodin

I_1 was obtained as yellow needles (m.p. 220°-222°C). It was slightly soluble in ethyl alcohol and methyl alcohol.

Thin layer chromatography

hR_f value on silica gel G/ petroleum ether b.p. 40°-70°C, ethyl acetate, acetic acid 45+5+3 = 17 (Fig. 3, p.50)

hR_f value on silica gel G/methyl alcohol, benzene 1+9 = 48 (Fig. 4, p.51)

hR_f value on silica gel G/di-iso-propyl ether = 64 (Fig. 5, P.52)

hR_f value on silica gel G/chloroform, methyl alcohol 6+4 = 76 (Fig. 6, p.53)

Ultraviolet absorption spectrum of I_1 in ethyl alcohol (Fig.7, p.54)

λ_{max} 245.5 nm (log ε = 4.31), 272.8 nm (log ε = 4.16), 430.7 nm (log ε = 4.03)

Ultraviolet absorption spectrum of authentic aloe-emodin isolated from *Rumex sp.* in methyl alcohol[75]

λ_{max} 225 nm (log ε = 4.59), 279 nm (log ε = 4.03), 430 nm (log ε = 4.03)

Infrared absorption spectrum of I_1 in Nujol (pure liquid paraffin) (Fig. 8, p.55)

ν_{max} 3,300 cm^{-1} (broad hydroxyl group), 1,670 cm^{-1} (free carbonyl group), 1,630 cm^{-1} (chelated carbonyl group), 1,570 cm^{-1} (C = C)

Infrared absorption spectrum of authentic aloe-emodin isolated from *Rumex sp.* in KBr.[75]

ν_{max} 3,400 cm^{-1} (hydroxyl group), 1,670 cm^{-1} (free carbonyl group), 1,630 cm^{-1} (chelated carbonyl group), 1,570 cm^{-1} (C = C)

Infrared absorption spectrum of acetate derivative of anthraquinone in Nujol (pure liquid paraffin) (Fig. 9, p.56)

ν_{max} 1,775 cm^{-1} (carbonyl group $-CH_2-O-\overset{O}{\overset{\|}{C}}-CH_3$), 1,750 cm^{-1} (carbonyl group of $\phi-O-\overset{O}{\overset{\|}{C}}-CH_3$), 1,600 cm^{-1} (C = C)

NMR spectrum in Deutero dimethyl suphoxide (DMSO) of I_1 in ppm (δ values) from TMS (Fig. 10, p.57)

I_1 is slightly soluble in DMSO the spectrum can not be determined.

NMR spectrum in Deuterochloroform ($CDCL_3$) of acetate derivative of anthraquinone in ppm (δ values) from TMS (Fig. 11, p.58)

δ 2.20 ppm (sharp singlet, 3H, $-CH_2-O-\overset{O}{\overset{\|}{C}}-CH_3$)

δ 2.49 ppm (sharp singlet, 6H, $2\phi-O-\overset{O}{\overset{\|}{C}}-CH_3$)

δ 5.30 ppm (sharp singlet, 2H, $\phi-CH_2-\overset{O}{\overset{\|}{C}}-CH_3$)

δ 7.50-8.55 ppm (multiplet, 5H, aromatic protons)