CHAPTER III

EXPERIMENTAL

A. *Cassia timoriensis* DC. leaves.

1. Source and anthentication of plant materials.

The young leaves of ***Cassia timoriensis*** DC. were obtained from Erawan National Park area, Kanchanaburi Province, Thailand, in October 1977. The plant materials were identified by comparision with the herbarium specimens of the Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperatives.

2. Solvents and chemicals used.

a) Acetic acid, glacial

b) Ammonium hydroxide, strong solution.

c) Benzene

d) Butanol

e) chloroform

f) Methanol

3. General techniques.

3.1 Thin layer chromatography (TLC)

Technique : One way, ascending, tank saturated.

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

Plate size	:	20 cm x 20 cm, 20 cm x 10 cm
Layer thickness	:	0.25 mm
Activation	:	Air dried for 15 minutes and then heated at 105°C for 1 hour.
Solvent systems	:	a) Silica gel G/Chloroform, methanol 9+1 b) Silica gel G/Chloroform, methanol 6+4 c) Silica gel G/Butanol, acetic acid, water 4+1+1 d) Silica gel G/Benzene, methanol 9+1
Distance	:	15 cm
Laboratory temperature	:	24°-30°C
Authentic sample	:	Barakol supplied by Mr. Chaiyo Chaichantipyuth, graduate student of the Department of Pharmacognosy, Chulalongkorn University, Faculty of Pharmaceutical Sciences.
Detection	:	a) Ultraviolet light b) Dragendorff's spraying reagent, (Solution A: 0.85 g bismuth subnitrate is dissolved in a mixture of 10 ml glacial acetic acid and 40 ml of water, solution B: 8 g potassium iodide in 20 ml of water. The spraying reagent is the mixture of 5 ml of solution A, 5 ml of solution B, 20 ml of glacial acetic acid, and 70 ml of water).

3.2 Column chromatography

Adsorbent	:	Silica gel 0.040-0.063 mm (E. Merck).
Packing of column	:	Adsorbent packed dry into the column.
Width of column	:	2.5 cm
Length of column	:	40 cm

Addition of crude extract to the column:

Crude extract was dissolved in a small volume of chloroform, mixed with small amount of the adsorbent, air dried and added to the top of the dry column.

Solvents	:	a) Chloroform b) Chloroform, methanol 9+1
Collection of eluate	:	Fractions of 25 ml were collected manually.
Examination of eluate	:	Those fractions were separately examined by thin layer chromatography, the like fractions giving greenish yellow fluorescence under ultraviolet light, were combined and concentrated to dryness under reduced pressure.

3.3 Melting point determination.

The melting point was determined by using Büchi melting point apparatus.

3.4 Ultraviolet absorption spectrum.

Ultraviolet absorption spectrum was recorded in ethanol using a Beckman-DK-2A spectrophotometer.

3.5 Infrared absorption spectrum.

Infrared absorption spectrum was determined in Nujol (pure liquid paraffin) using Perkin-Elmer 421 Grating spectrophotometer.

3.6 Nuclear magnetic resonance (NMR) spectrum.

NMR spectrum was obtained in deuterochloroform using Perkin-Elmer R12 instrument.

4. The isolation of barakol from the leaf-extract of *Cassia timoriensis* DC.

The small pieces of fresh young leaves (4 kg) were boiled with 2% acetic acid (15 L) for one hour and filtered. The extraction was repeated by boiling with another portion of 2% acetic acid (10 L). The combined acid filtrate was made alkaline with strong solution of ammonium hydroxide and extracted with chloroform (50 x 300 ml) in portions until they were exausted. The combined chloroform extract was washed with distilled water (7 x 500 ml) and concentrated under reduced pressure to about 500 ml. The concentrated solution (500 ml) was extracted with several portions of 5% acetic acid (200 ml each) until a colourless extract was obtained. The strong solution of ammonium hydroxide was added dropwise to neutralise the acidic solution. Upon cooling in the refrigerator,

greenish yellow needle crystals (1.4 g) were obtained. Thin layer chromatogram showed the presence of one main compound and two minors. (Figures 8-9, pp. 80-81)

The crystals (1.4 g) were divided into 3 portions, each portion (about 0.46 g) was dissolved in chloroform (5 ml), mixed with small amount of silica gel, air dried, and added to the top of a dry silica gel column (2.5 cm x 40 cm). The column was eluted with chloroform, the mixture of chloroform, methanol 9+1, and washed with methanol. Fractions of 25 ml were collected, examined by TLC and combined, giving the following fractions:-

Fraction	Solvent	Volume of eluate (ml)
1-2	chloroform	50
3-5	chloroform, methanol 9+1	75
6-11	chloroform, methanol 9+1	150
12-34	chloroform, methanol 9+1	575
35	methanol	250

The fractions 6-11 (150 ml) contain one main compound designated as B_1.

The combined eluate was evaporated to dryness under reduced pressure to yield a dark green solid, then dissolved in a small volume of methanol (3 ml). The distilled water was added dropwise and greenish yellow needle crystals were obtained. The crystals

were shown by TLC to contain almost pure main compound (0.53 g). The purification of crystals were undertaken as mentioned above (by column chromatography), yielding pure greenish yellow needle crystals of B_1 (0.23 g), decompose at 164°-165°C, and was subsequently identified as barakol. Four kilograms of fresh young leaves of *Cassia timoriensis* DC. yielded about 0.7 g of pure barakol (17.5 mg%)

5. Identification of B_1 as barakol.

B_1 was obtained as greenish yellow needles, m.p. 164°-165°C (decomposed). It is soluble in acetone, ethanol and methanol.

hRf values

Silica gel G/chloroform, methanol 9+1 = 33 (Figure 10, p. 82)

Silica gel G/chloroform, methanol 6+4 = 77 (Figure 11, p. 83)

Silica gel G/butanol, acetic acid, water 4+1+1 = 9 (Figure 12, p. 84)

Silica gel G/benzene, methanol 9+1 = 9 (Figure 13, p. 85)

Ultraviolet absorption spectrum in ethanol (Figure 24, p. 96)

λ_{max} 239 (ε 204812) nm

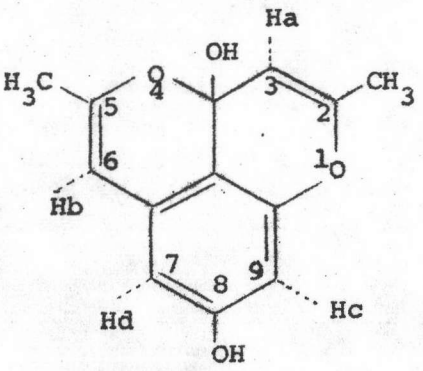
Infrared absorption spectrum in Nujol (pure liquid paraffin) (Figure 26, p. 98)

ν_{max} 3,400 (broad OH), 1680 (C=C-O), 1635 (C=C-Ar), 1600, 1570 and 1465 (aromatic C-H stretching) cm^{-1}

NMR-spectrum in deutero chloroform ($CDCl_3$) (τ values) with tetramethylsilane (TMS) as internal standard. (Figure 29, p. 101)

τ values 3.49 (d, AB system, J_{cd} = 1.7 Hz, 1H, Hd), 3.61 (d, AB system, J_{cd} = 1.7 Hz, 1H, Hc), 3.62 (broad s, allylic coupling with methyl protons, 1H, Hb), 3.89 (broad s, allylic coupling with methyl protons, 1H, Ha), 7.55, 7.66 (2 x s, 6H, two methyl protons)

According to the melting point, the ultraviolet, infrared, and nuclear magnetic resonance spectra,[119,121] B_1 was identified as barakol.



Barakol

B. *Cassia grandis* L. leaves

1. Source and authentication of plant material.

The leaves of *Cassia grandis* L. were obtained from Sampran District, Nakornpratom Province, Thailand, in March 1977. The plant materials were identified by comparision with the herbarium specimens of the Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperatives.

2. Solvents and chemicals used.

a) Acetic acid, glacial

b) Acetone

c) Ammonium hydroxide, strong solution

d) Benzene

e) Butanol

f) Chloroform

g) Di-isopropyl ether

h) Ethyl acetate

i) Ethanol 95%

j) Hydrochloric acid

k) Methanol

l) Petroleum ether b.p. 40°-60°C

m) Sodium bicarbonate

n) Sodium hydroxide

o) Sodium sulphate, anhydrous

3. General Techniques.

3.1 Thin layer chromatography. (TLC)

Technique	:	One way, ascending
Adsorbent	:	Silica gel G (E. Merck), 13% calcium sulphate binder, 30 g/60 ml distilled water.
Plate size	:	20 cm x 20 cm, 20 cm x 10 cm.
Layer thickness	:	0.25 mm
Activation	:	Air dried for 15 minutes and then heated at 105°C for 1 hour

Solvent systems : a) Silica gel G/Benzene, methanol 9+1

b) Silica gel G/Chloroform, methanol 6+4

c) Silica gel G/Di-isopropyl ether

d) Silica gel G/Benzene, ethyl acetate, acetic acid 75+24+1

e) Silica gel G/Petroleum ether b.p. 40°-60°C, ethyl acetate, acetic acid 45+5+3

f) Silica gel G/Butanol, acetic acid, water 4+1+1

g) Silica gel G/Acetone, Chloroform 5+95

h) Silica gel G/Benzene, ethyl acetate 4+1

i) Silica gel G/Chloroform, methanol 9+1

Distance : 15 cm

Laboratory temperature : 24°-30°C

Detection of anthraquinones : a) Ultraviolet light, they fluoresce orange-red.

b) NH_3 vapour, they develop pink colour immediately after contact.

c) After spraying with 5% alcoholic potash, they give pink colour

d) They develop pink colour after spraying with 0.5% magnesium acetate in methanol (identification of compounds containing two *meta* hydroxyls.

3.2 Column chromatography.

Adsorbent : Silica gel 0.040-0.063 mm (E. Merck)

Packing of column : Adsorbent packed dry into the column.

Addition of anthraquinone material to the column :

The mixture of anthraquinones was dissolved in small volume of chloroform, mixed with small amount of adsorbent, air dried and then added to the top of the dry column.

Solvents : a) Benzene

b) Benzene, methanol 9+1

Collection of eluate : Fractions of 25 ml were collected manually.

Examination of eluate : Those fractions were separately examined by thin layer chromatography, the like fractions giving pink colour after contact with ammonia vapour were combined and evaporated to dryness under reduced pressure.

3.3 Melting point determination.

The melting point was determined using Reichert heating stage microscope.

3.4 Ultraviolet absorption spectrum.

Ultraviolet absorption spectrum was recorded in ethanol using a Beckman-DK-2A spectrophotometer.

3.5 Infrared absorption spectra.

Infrared absorption spectra were recorded in Potassium bromide disc using Perkin Elmer 421 Grating spectrophotometer.

3.6 Nuclear magnetic resonance (NMR) spectra.

NMR spectra were obtained in deutero dimethyl sulphoxide (DMSO-d_6) using Perkin Elmer R12 instrument.

3.7 Mass spectrum.

Mass spectrum was obtained by using DS-50S mass spectrometer.

4. The test and isolation of anthraquinone from the leaves of *Cassia grandis* L.

4.1 Test for anthraquinones.

Air dried powdered leaves (5 g) were boiled with dilute hydrochloric acid (25 ml) for 10 minutes and filtered. The filtrate was cooled and extracted with benzene (10 ml), the benzene layer was separated and shaken with ammonium hydroxide solution. The pink colour in the aqueous layer indicates the presence of anthraquinones.

4.2 Isolation of anthraquinone.

The dried coarsely powdered leaves (3 kg) were refluxed with 70% ethanol (5 L) for 3 hours and filtered. The marc was re-extracted exhaustively by repeating reflux and filtered. The combined filtrate was concentrated under reduced pressure until no trace of ethanol left, acidified with glacial acetic acid (150 ml)

and refluxed again for 4 hours to hydrolyse anthraquinones and their corresponding glycosides to free aglycones. The solution of free aglycones was filtered through kieselguhr with which chlorophyll and some other impurities were removed. The acidic filtrate was extracted with portions of chloroform until the last extraction gave no pink colour with ammonium hydroxide solution. For complete extraction, five liters of chloroform were used. The combined chloroform extract was concentrated under reduced pressure to about 500 ml, then extracted with 5% sodium bicarbonate solution (3 x 300 ml) to separate aglycones with free carboxyl groups. The chloroform layer was extracted with 5% sodium hydroxide solution (4 x 200 ml). Concentrated hydrochloric acid was added dropwise to the combined sodium hydroxide extract until the colour of solution changed from pink to yellow. The yellow acidic solution was then extracted with benzene (4 x 250 ml). The combined benzene extract was washed with distilled water, dried over anhydrous sodium sulphate and concentrated under reduced pressure to yield a brown syrupy mass of crude anthraquinones (1.22 g). TLC showed the presence of one main anthraquinone and three minors (Figure 14, p. 86)

Crude anthraquinones (1.22 g) were divided into 4 portions, each portion was dissolved in chloroform (5 ml), mixed with small amount of silica gel, air dried, and added to the top of a dry silica gel column (2.5 cm x 40 cm). The column was eluted with benzene, the mixture of benzene and methanol 9+1, and washed with

methanol. Fractions of 25 ml were collected, examined by TLC and combined, giving the following fractions:-

Fraction	Solvent	Volume of eluate (ml)
1-2	benzene	50
3-9	benzene, methanol 9+1	175
10-15	benzene, methanol 9+1	150
16-35	benzene, methanol 9+1	500
36	methanol	270

The fractions 10-15 (150 ml) contained one main anthraquinone designated as A_1. The combined eluate was concentrated under reduced pressure to about 30 ml and let to stand over night, the orange-yellow needle crystals of A_1 (54 mg) were obtained. The crystals were washed through the suction with a small volume of benzene, and dried in a vacuum desiccator. After concentration, mother liquor yielded a second crop of yellow needle crystals of A_1 (15 mg). A_1 was subsequently identified as aloe-emodin, m.p. 223°-225°C. Three kilograms of dried powdered leaves of *Cassia grandis* L. yielded about 230 mg (8 mg%) of pure aloe-emodin.

4.3 Acetate formation of anthraquinone.

A_1 (75 mg) was dissolved in acetic anhydride (15 ml) and 3 drops of pyridine were added. The mixture was refluxed for 6 hours and then poured into ice water (25 ml) with vigorous stirring about

10 minutes to complete acetylation of anthraquinone. The pale yellow needle crystals were obtained, filtered, washed thoroughly with distilled water and dried, yielded 90 mg of pale yellow acetate derivative of anthraquinone which is readily soluble in methanol, ethanol and chloroform, m.p. 160°-161°C.

5. Identification of A_1 as aloe-emodin.

A_1 was obtained as orange-yellow needle crystals (m.p. 223°-225°C). It is soluble in benzene, chloroform, ethanol and methanol.

hRf values

a) Silica gel G/Benzene, methanol 9+1: A_1 = 61, A_1-acetate = 85 (Figure 15, p. 87)

b) Silica gel G/chloroform, methanol 6+4: A_1= 78, A_1-acetate = 81 (Figure 16, p. 88)

c) Silica gel G/Di-isopropyl ether = 37 (Figure 17, p. 89)

d) Silica gel G/Benzene, ethyl acetate, acetic acid 75+24+1 = 45 (Figure 18, p. 90)

e) Silica gel G/Petroleum ether b.p. 40°-60°C, ethyl acetate, acetic acid 45+5+3 = 10 (Figure 19, p. 91)

f) Silica gel G/Butanol, acetic acid, water 4+1+1 = 71 (Figure 20, p. 92)

g) Silica gel G/Benzene, ethyl acetate 4+1 = 26 (Figure 21, p. 93)

h) Silica gel G/Acetone, chloroform 5+95 = 31 (Figure 22, p. 94)

i) Silica gel G/Chloroform, methanol 9+1 = 75 (Figure 23, p. 95)

Ultraviolet absorption spectrum in ethanol. (Figure 25, p. 97)

λ_{max} 226 (ε = 127406), 255 (ε = 67500), 287 (ε = 31219) nm

Infrared absorption spectrum in KBr disc. (Figure 27, p. 99)

ν_{max} 3340 (broad hydroxyl group), 1680 (C=O free), 1630 (C=O chelated), 1600, 1575, 1470 (aromatic C-H stretching) cm^{-1}

Infrared absorption spectrum of acetate derivative in KBr disc. (Figure 28, p. 100)

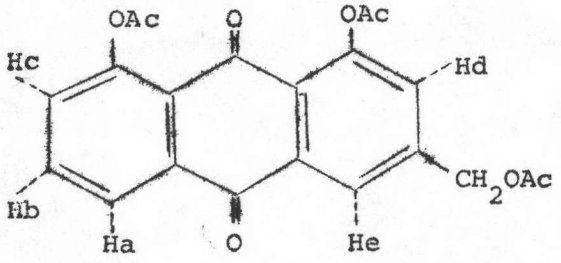
ν_{max} 1775 (carbonyl group of $-CH_2-O-\overset{O}{\overset{\|}{C}}-CH_3$), 1472 (carbonyl group of $\emptyset-O-\overset{O}{\overset{\|}{C}}-CH_3$), 1675 (C=O free), 1620, 1600 (aromatic C-H stretching) cm^{-1}

NMR-spectrum in deutero dimethyl sulphoxide (DMSO-d_6) (τ values) with tetramethylsilane (TMS) as internal standard. (Figure 30, p. 102)

τ (values) 5.35 (s, 2H, $\underline{CH}_2$), 2.8-2.1 (m, 5H, aromatic protons)

NMR-spectrum of acetate derivative in deuterochloroform ($CDCl_3$) (τ values) with tetramethylsilane as internal standard. (Figure 31, p. 103)

τ (values) 7.82 (s, 3H, $C-O-\overset{O}{\overset{\|}{C}}-\underline{CH}_3$), 7.55 (s, 6H, $2Ar-O-\overset{O}{\overset{\|}{C}}-\underline{CH}_3$), 4.72 (s, 2H, $\underline{CH}_2$), 2.55 (d, J_{de} = 1.6 Hz, 1H, Hd), 2.50 (dd, J_{bc} = 7.5 Hz, J_{ac} = 1.6 Hz, 1H, Hc), 2.15 (t, J = 7.5 Hz, 1H, Hb), 1.75 (d, J_{de} = 1.6 Hz, 1H, He), 1.70 (dd, J_{ab} = 7.5 Hz, J_{ac} = 1.6 Hz, 1H, Ha)



Aloe-emodin triacetate

Mass spectrum (Figure 32, p. 104)

m/e (%) 270 (M^+, 100) 286 (6.4), 242 (14.8), 241 (80.5), 224 (7.5), 213 (8.9), 168 (7.0), 139 (13.3), 128 (6.0) 127 (6.5), 121 (16.6)

According to the melting point, the ultraviolet, infrared and mass spectra of A_1 which were identical with those of aloe-emodin obtained from *Cassia garrettiana* Craib,[29,124] and from *Rumex* spp.,[102] also the NMR spectra of A_1 and A_1-acetate which were similar to those of aloe-emodin obtained from *Cassia garrettiana* Craib,[29] it was concluded that A_1 was aloe-emodin.