Chapter III

Experimental

Source of Plant Materials

The leaves of *Ageratum conyzoides* Linn. were obtained from the rambutan orchard, Chantaburi province, Thailand in September 1984. The plant materials were authenticated by comparison with the herbarium specimens in the Botanical Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand and The Royal Botanic Garden, Kew, England.

Preliminary Phytochemical Screening

Screening for Coumarins

Four leaves of plant were chopped and placed in a test tube, 5-10 drops of water added and the material was briefly crushed with a glass rod. The tube was then firmly corked, with a filter paper moistened with 10% dilute sodium hydroxide solution suspend inside and heated in a boiling water bath for several minutes, the paper removed and exposed to ultraviolet (UV) light. A yellow-green fluorescence appeared within a few minutes indicating the presence of coumarins (Farnsworth, 1966).

It should be noted that this procedure is applicable only to coumarin and related volatile compounds. Without alkali, some coumarin derivatives fluoresce when they are exposed to UV light.

The colour produced under UV light vary from compound to

compound, e.g. coumarin itself fluoresces yellow-green whereas scopoletin and umbelliferone fluoresce blue (Dean, 1963).

Screening for Flavonoids (Cyanidin test)

The leaf materials were macerated with 95% ethanol overnight. The plant extract was evaporated to syrupy residue and allowed to cool to room temperature. It was defatted several times with petroleum ether until the last extract was colourless. The defatted residue was dissolved in 80% ethanol and filtered. The filtrate was submitted to cyanidin test by adding a small piece of magnesium ribbon, followed by dropwise addition of concentrated HCL. Weak red colour was obtained indicating positive test (Farnsworth, 1966).

General Techniques

1. Thin Layer Chromatography (TLC)

Technique	:	one way, ascending
Adsorbent	:	silica gel G (E. Merck) calcium sulphate binder 13%, 35 g/70 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/heated at 110 ℃ for 1 hour
Solvent system	:	1) chloroform : acetone (9:1) 2) 10% acetone in diethyl ether
Distance	:	15cm
Temperature	:	25-30 ℃

Detection : 5% potassium hydroxide in 95% ethanol/ UV light

2. Column Chromatography (CC).

2.1 Column Chromatography for Separation of Coumarin

Column size : diameter 10 cm

Adsorbent : silica gel 60, 230-400 mesh (E. Merck)

Packing : wet packing technique (Edwards, 1969)

Solvent : 1) benzene
2) chloroform
3) acetone

Fraction size : 25 ml

Examination of eluate : TLC monitoring

2.2 Column Chromatography for Separation of Flavones

Column size : diameter 5 cm

Adsorbent : silica gel 60, 230-400 mesh (E. Merck)

Packing : wet packing technique (Edwards, 1969)

Solvent : 1) petroleum ether (35-60 ℃)
2) diethyl ether
3) acetone

Fraction size : 20 ml

Examination of eluate : TLC monitoring

3. Preparative Thin Layer Chromatography

Technique : one way, ascending

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

Plate size : 20 cm x 40 cm

Layer thickness	:	250 μ
Activation	:	air dried for 15 minutes/heated at 110 ℃ for 1 hour
Solvent system	:	diethyl ether
Distance	:	37 cm
Temperature	:	25-30 ℃
Collection of flavone	:	chloroform elution from scraped-off zones
Examination of substance	:	long wave UV light

4. Physical Constant

Melting points (MP) were determined by Gallenkamp melting point apparatus.

5. Spectroscopy

5.1 UV absorption spectra were obtained on Shimadzu double beam spectrophotometer UV-180.

5.2 Infrared (IR) absorption spectra were obtained in potassium bromide disc on Shimadzu IR 440 spectrophotometer, absorption bands were reported in wave numbers (cm^{-1}).

5.3 Nuclear magnetic resonance (NMR) spectra were obtained on Nicolet NMC 200 (200 MHz) and on NMR Jeol Fx 90Q, instruments. Tetramethylsilane (T.M.S.) was used as an internal standard and chemical shifts were reported on the ppm scale.

5.4 The Mass spectra (MS) were obtained on Jeol JMS-DX300 in ionization voltage 70 ev. and ionization current 300 μA.

Extraction and Isolation

1. Extraction of Crude Coumarins and Some Flavonoids

The chopped fresh leaves of *Ageratum conyzoides* Linn. (4.4 kg) were macerated with chloroform for 2 days and filtered. The filtrate was evaporated under reduced pressure to dryness. The dried residue was dissolved in 95% ethanol (500 ml). After 4% aqueous lead acetate solution (500 ml) was added, it was allowed to stand overnight for complete precipitation. The precipitate was then removed by filtration through kieselguhr. The filtrate was exhaustively extracted with chloroform. The combined chloroform extract was dried over anhydrous sodium sulphate and evaporated under reduced pressure to dryness (28.34 g).

2. The Isolation of Coumarin

The crude extract (28.34 g) was divided into 5 equal portions (approx. 5.6 g), each portion was separated by silica gel (250 g) column in the same manner.

The column was eluated with solvents and various fractions were collected and combined (Table 6) in accordance with the information obtained from TLC (Figure 15, page 104).

Table 6. Column Chromatography Information of Crude Extract.

Fraction number	Solvent	Combined fraction	Remark
	Benzene : Chloroform		
2 - 14	8.5 : 1.5	A	no coumarin
15 - 72	8.5 : 1.5	B	mainly coumarin
73 - 91	8.5 : 1.5	C	mixture of coumarin and flavonoids
92 - 95	1 : 1	C	mixture of coumarin and flavonoids
96	Acetone	C	mixture of coumarin and flavonoids

Combined fraction B was concentrated under reduced pressure to dryness. Crystallization of the residue in hexane yielded colourless long rod crystals (3.93 g), designated as Co_1.

Combined fraction C contained two main flavonoids and trace of coumarin, was evaporated to syrupy mass (4.95 g) under reduced pressure.

3. The Isolation of Flavonoids

Fraction C (4.95 g) was divided into 3 equal portions (approx. 1.6 g), each portion was separated by silica gel (40 g) column in the same manner. The column was eluated with solvents and the various fractions were collected and combined (Table 7) in accordance with the information obtained from TLC (Figure 16, page 105).

Table 7. Column Chromatography Information of Fraction C

Fraction number	Solvent	Combined fraction	Remark
	Petroleum Ether : Diethyl Ether		
1 - 30	3 : 2	Ca	trace of Co_1
31 - 37	2 : 3	Cb	trace of Co_1 and trace of impurity
38 - 55	2 : 3	Cc	trace of Co_1 and trace of impurity
56 - 61	2 : 3	Cd	flavonoid, trace of Co_1 and impurity
62 - 64	1 : 4	Ce	flavonoid, trace of Co_1 and impurity
65 - 69	1 : 4	Cf	two flavonoids and trace of impurity
70 - 76	1 : 4	Cf	two flavonoids
77 - 94	Diethyl Ether	Cf	two flavonoids
95	Acetone	Cg	trace of two flavonoids and some impurities

Conbined fraction Cf was evaporated to dryness under reduced pressure. Recrystallization of the material in acetone yielded colourless crystalline plates (22 mg), designated as fl_1.

The mother liquor was concentrated under reduced pressure to give syrupy mass (328 mg) , designated as fraction Y. It was subjected to preparative TLC on silica gel using diethyl ether as a solvent. After double developments, 4 bands of separation were obtained (Figure 17, page 106). The substance of second band was recovered from the scraped-off zones by chloroform. The chloroform extract was evaporated to dryness under reduced pressure. Crystallization of residue in hexane/acetone yielded colourless crystalline prisms, designated as fl_2 (71 mg).

Characterization of Isolated Compounds

1. Co_1

Co_1 was obtained as colourless long rod prisms from hexane, soluble in carbon tetrachloride, benzene, dichloromethane, chloroform, diethyl ether, acetone, methanol and ethanol.

hRf Values

a) 81 in diethyl ether (Figure 18, page 107)

b) 74 in petroleum ether : diethyl ether (1:2) (Figure 19, page 108)

c) 27 in benzene : chloroform (7:3) (Figure 20, page 109)

d) 48 in dichloromethane (Figure 21, page 110)

e) 76 in chloroform : acetone (9:1) (Figure 22, page 111)

Melting Point

66-67 ℃

UV Absorption Spectra

λ_{max} (MeOH) 317 nm (Figure 28, page 117)

λ_{max} (MeOH + 10% NaOH) 317 nm

IR Absorption Spectrum (Figure 29, page 118)

ν_{max} (KBr) 1709, 1601, 1564, 1450, 1178, 1120 and 755 cm^{-1}

^{1}HNMR Spectrum (Figure 30, page 119)

^{1}HNMR (90 MHz, $CDCl_3$) δ 6.42 (d, *J* 9, 1H), 7.27 (s, 1H), 7.36 (s, 1H), 7.45 (s, 1H), 7.53 (s, 1H), 7.71 (d, *J* 9, 1H) ppm

Mass Spectrum (Figure 31, page 120)

m/z 146 (M^+, $C_9H_6O_2$, 100%), 118 (100%), 90 (49%), 89 (38%), 64 (11%), 63 (30%), 62 (12%)

Molecular Weight

146

2. fl_1

fl_1 was obtained as colourless crystalline plates from acetone. It was soluble in dichloromethane, chloroform and ethyl acetate.

hRf Values

a) 57 in petroleum ether, chloroform, acetone (1+8+1) (Figure 23, page 112)

b) 61 in benzene, diethyl ether, methanol (8+1+1) (Figure 24, page 113)

c) 38 in cyclohexane : ethyl acetate (1:1) (Figure 25, page 114)

d) 71 in 5% acetone in diethyl ether (Figure 26, page 115)

e) 51 in petroleum ether : ethyl acetate (1:2) (Figure 27, page116)

Melting Point

188-189 °C

UV Absorption Spectra

λ_{max} (MeOH) 334 nm (Figure 32, page 121)

λ_{max} (MeOH + 10% NaOH) 334-5 nm

λ_{max} (MeOH + NaOAc) 334-5 nm

λ_{max} (MeOH + $AlCl_3$) 334-5 nm

λ_{max} (MeOH + H_3BO_3) 334-5 nm

IR Absorption Spectrum (Figure 33, page 122)

ν_{max} (KBr) 2950, 1630, 1510, 1115, 1100 and 1040 cm^{-1}

^{1}HNMR Spectrum (Figure 34-36, page 123-125)

^{1}HNMR (200 MHz, $CDCl_3$) δ 3.949 (s, 3H), 3.952 (s, 3H), 3.987 (s, 3H), 4.016 (s, 3H), 4.105 (s, 3H), 6.088 (s, 2H), 6.568 (s, 1H), 7.103 (s, 1H), 7.149 (s, 1H) ppm

Mass Spectrum (Figure 37, page 126)

m/z 416 (M^+, $C_{21}H_{20}O_9$, 57%), 401 (100%), 358 (13%), 241 (0.5%), 240 (0.8%), 225 (13%), 200 (8%), 176 (18%), 83 (42%), 75 (16%), 53 (18%), 15 (78%)

Molecular Weight

416

3. fl_2

fl_2 was obtained as colourless crystalline prisms from hexane/acetone. It was soluble in carbon tetrachloride, dichloromethane chloroform, ethyl acetate, acetone and ethanol.

hRf Values

a) 52 in petroleum ether, chloroform, acetone (1+8+1) (Figure 23, page 112)

b) 52 in benzene, diethyl ether, methanol (8+1+1)
(Figure 24, page 113)

c) 30 in cyclohexane : ethyl acetate (1:1)
(Figure 25, page 114)

d) 59 in 5% acetone in diethyl ether (Figure 26, page 115)

e) 37 in petroleum ether : ethyl acetate (1:2)
(Figure 27, page 116)

Melting Point

112-113 ℃

UV Absorption Spectra

λ_{max} (MeOH) 327-8 nm (Figure 38, page 127)

λ_{max} (MeOH + 10% NaOH) 327-8 nm

λ_{max} (MeOH + NaOAc) 327-8 nm

λ_{max} (MeOH + $AlCl_3$) 327-8 nm

λ_{max} (MeOH + H_3BO_3) 327-8 nm

IR Absorption Spectrum (Figure 39, page 128)

ν_{max} (KBr) 2960, 2860, 1645, 1595, 1510, 1130, 1015, 835 and 720 cm^{-1}

^{1}HNMR Spectrum (Figure 40, page 129)

^{1}HNMR (200 MHz, $CDCl_3$) δ 3.93 (s, 3H), 3.96 (s, 12H), 4.03 (s, 3H), 4.11 (s, 3H), 6.64 (s, 1H), 7.17 (s, 2H) ppm

Mass Spectrum (Figure 41, page 130)

m/z 432 (M^+, $C_{22}H_{24}O_9$, 77%), 417 (100%), 356 (11%), 241 (0.8%), 240 (0.9%), 225 (12%), 197 (25%), 195 (5%), 192 (7%), 83 (20%), 15 (19%)

Molecular Weight

432