CHAPTER II

EXPERIMENTAL

1. Source of Plant Materials

The plant used in this investigation was collected in August 1987 from Nongkai province,Thailand.This plant was identified to be *Erycibe subspicata* Wall. in the family Convolvulaceae, order Tubiflorae. It was authenticated by comparison with herbarium specimens at the Botany section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand.

2. General Techniques

2.1 Chromatographic Techniques

2.1.1 Thin-Layer Chromatography(TLC)

Technique	: One way ascending
Adsorbent	: Silica gel G (E Merck) 30 g in 60 ml distilled water
Plate sizes	: 10 cm x 20 cm or 20 cm x 20 cm
Layer thickness	: 0.25 mm
Activation	: Air-dried for 15 minutes and then heated at 110°C
Solvent system	: 1) $CHCl_3$:MeOH (9:2) 2) MeOH:Acetone:$CHCl_3$:Benzene (12.5:25:30:20)
Distance	: 15 cm

Laboratory temp. : 28° - 35° C

Development : Lined filter paper in a chromatographic tank.

Detection : 1) 5% Alcoholic potash and UV light (265 and 366 nm).

2) 50% Sulphuric acid in Ethanol.

2.2 Spraying Reagents

1) Alcoholic Potash

This reagent was used as a general coumarin detecting reagent which characterized the coumarins by illuminating under UV light.

The working solution was made by dissolving 5 g. of Potassium Hydroxide pellets in 100 ml of 95% ethanol.

2) 50% Sulphuric Acid in Ethanol

This reagent was made by mixing 50 ml concentrated sulphuric acid with 50 ml of 95% ethanol.

The reagent gave the pink to black colour depending on the nature of the compounds.The colour would developed after spraying and heating the chromatographic plates at 110° C for 5 minutes.

2.3 Column Chromatography (CC)

Column sizes : The glass columns 3 - 10 cm diameter (depending on the quantity of sample to be separated)

Absorbent : 1) Silica gel 60 (E Merck)

2) Sephadex LH-20 (Sigma),

Bead size = 25 - 100 μm.

Packing Method : Wet packing

Solvent : Several solvents were used for different materials and the details were demonstrated later.

2.4 Melting Point

Melting points were determined on the Gallenkamp Melting Point apparatus model MFB 595.

The melting points are uncorrected.

2.5 Spectroscopy

2.5.1 Infrared (IR) Spectroscopy

Infrared absorption spectra were recorded as KBr disc on a Perkin Elmer Model 283 Spectrophotometer.The absorption bands were reported in wave number (cm^{-1})

2.5.2 Nuclear Magnetic Resornance (NMR) Spectroscopy

The proton (^{1}H) and Carbon 13 (^{13}C) spectra were obtained with 2 different instruments:

a) Nuclear Magnetic Resonance Spectrometer Model GX 270 (JEOL) at 270 MHz for ^{1}H and ^{13}C NMR of ES-1.

b) Nuclear Magnetic Resonance Spectrometer Model FX 90 (JEOL) at 90 MHz for ^{1}H NMR and ^{13}C NMR for ES-2.

Tetramethylsilane was used as an internal standard (0.00 ppm).

2.6 Mass Spectroscopy

The low resolution mass spectra were obtained on a Mass Spectrometer Model DX 300 (JEOL).A direct inlet system operating at 70 eV with the temperature range between 150° to 300° C was used.

2.7 Lyophilization

The pure solution was dried by Termovac Lyophilizer.

2.8 Solvent

The solvents of commercial grade were redistilled before used.

3. Extraction Procedure

The air-dried and ground stems of *Erycibe subspicata* Wall.(11 kg) were macerated with methanol (5 l) for 5 days and was filtered through Whatman filter paper No.1. For completeness of extraction, the marc was reextracted for two more times. The combined methanolic extract was evaporated in vacuo to dryness. The residue (172.0 g) was washed with Benzene (1000 ml) and rewashed with Chloroform (1000 ml). The Benzene and Chloroform filtrates were evaporated under reduced pressure to dark greenish viscous mass. The Benzene residue was designated as residue BE (9.10 g). The Chloroform residue was designated as residue CE (10.12 g). The Benzene and Chloroform insoluble portion,ie.,

methanol residue, was designated as residue ME (152.50 g).

The BE and CE fractions were combined after TLC examination revealed their identical profiles (BE + CE = 19.22 g).

4. Isolation of chemical substances from *Erycibe subspicata* Wall.

4.1 Isolation of the ES-1

The combined non-polar (14.54 g) fraction was chromatographed over silica gel 60 (1000 g) and then eluted with MeOH:$CHCl_3$ (2:9).

Fractions (40 ml each) were collected and combined according to the results of the TLC. check as shown in table I. The fraction 24 - 38 showed a similar pattern on TLC,hence they were combined. The combined fraction was evaporated in vacuo to dryness (650 mg) and was further purified with the aid of Sephadex LH-20 which was eluted with pure methanol. Fractions (25 ml each) were collected and combined according to the results of the TLC check. The fraction 20-25 showed one relatively identical fluorescense spot appeared, hence they were combined. The combined fraction was crystallised in methanol yielding colourless needle crystal (100 mg) and was designated as ES-1.ES-1 gave blue fluorescent spot under the UV.light at 254 and 366 nm after the plate had been sprayed by 5 % alcoholic potash.

Table I

Elution pattern of the silica gel column for ES-1

Fraction	UV.light and then sprayed with 50% H_2SO_4 in EtOH
1-5	Chlorophyll
6-14	"
15-23	Chlorophyll & 2 Black spots
24-38	Fluorescent spot & purple spots
39-42	Long tail purple spots
44-58	Nothing

4.2 Isolation of ES-2

The methanol extract (5.0 g) was subjected to quick column chromatography eluting with MeOH:Acetone:$CHCl_3$:Benzene (12.5:25:30:20). Fifty fractions (30 ml) were collected and compared by TLC as shown in table II. The fraction 13-15 gave single spot on the TLC and the spot gave blue fluorescent spot under the UV.light at 254 and 366 nm after sprayed with 5 % alcoholic potash. They were combined and crystallised in methanol yielding white amorphous (98 mg) and it was designated as ES-2.

ME residue was further investigated by using the aid of gel filtration as it seems that the other spots could not be isolated by simple column chromatography.

Table II

Elution pattern of the silica gel column for ES-2

Fraction	UV.light & then sprayed with 50% H_2SO_4 in EtOH
1-5	Nothing
6-10	2 Black spots
11-12	2 Black spots + 1 Fluorescent spot
13-15	1 Fluorescent spot
16-20	1 Fluorescent spot + 2 purple spots
21-25	3 purple spots
25-35	3-4 purple spots
35-40	"
41-50	Residue (wash with MeOH)

5.Characterization of Isolated Compounds

The compounds were characterized by studies on its melting points and infrared,nuclear magnetic resonance,and mass spectra.

ES-1 was obtained as colourless crystalline needles.It is slightly soluble in water or cold alcohol; soluble in hot water or hot glacial acetic acid;moderately soluble in chloroform;practically insoluble in benzene.

hRf values

a. MeOH:Acetone:$CHCl_3$:Benzene = 57 (see Figure 1 page 71)
(12.5:25:30:20)

b. MeOH:Dichloroethane = 73 (see Figure 2 page 72)
(2:9)

c. MeOH:Benzene = 53 (see Figure 3 page 73)
(15:85)

d. $CHCl_3$:MeOH = 80 (See Figure 4 page 74)
(9:3)

Infrared absorption spectrum (KBr disc) (see Figure 7 page 77).

ν_{max} (cm^{-1}): 3350, 3040, 3000, 2950, 1710, 1585, 1515, 1295, 1140, 1010, 921, 862, 820, 730.

Melting point

198° - 199° C

Molecular weight

192 a.m.u.(Mass spectrometry)

Mass spectrum

m/z (% rel. int) (see Figure 11 page 81)

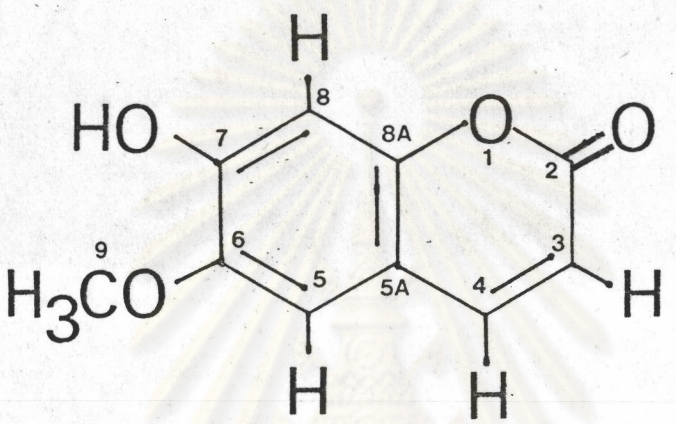
192 (M^+, 100, $C_{10}H_8O_4$), 177 (13.63), 164 (43.64),149 (67.43), 135 (1.48), 121 (20.46), 105 (3.24), 92 (3.35), 79 (21.98), 69 (36.38), 65 (11.87), 51 (29.47), 39 (10.97).

Proton nuclear magnetic resonance spectrum (see Figure 5, page 75) (90MHz, $CDCl_3$)

Chemical shifts (δ) ppm.	Proton	Multiplicity
6.182	1H	singlet
3.989	3H	singlet
6.264	1H	doublet .
6.848	1H	singlet
6.952	1H	singlet
7.646	1H	doublet

Carbon-13 nuclear magnetic resonance spectrum (see Figure 9,page 79) ($CDCl_3$)

Chemical shift (δ) ppm.	Multiplicity
161.2	singlet
152.0	singlet
149.5	singlet
144.288	doublet
144.281	singlet
113.890	doublet
112.187	singlet
111.768	doublet
99.976	doublet
56.868	quartet



SCOPOLETIN

ES-1 was identified as Scopoletin

ES-2 was obtained as white amorphous compound.It is soluble in water,alcohol and practically insoluble in chloroform and ether.

nRf value

a.MeOH:Acetone:$CHCl_3$:Benzene = 40 (see Figure 1 page 71)
(12.5:25:30:20)

b. MeOH:Dichloroethane = 23 (see Figure 2 page 72)
(2:9)

c. MeOH:Benzene = 13 (see Figure 3 page 73)
(15:85)

d. $CHCl_3$:MeOH = 37 (See Figure 4 page 74)
(9:3)

Melting point

210°- 211°C

Molecular weight

354 a.m.u. (mass spectrometry)

Mass spectrum

m/z (% rel. int) (see figure 12 page 82)

354 (M^+, 0.13, $C_{16}H_{20}O_5$), 336 (0.10), 235 (0.24), 192 (Base peak, 100), 177 (32.81), 164 (17.94), 149 (23.18),121 (10.21), 69 (20.16), 60 (16.15),44 (21.00).

Infrared absorption spectrum (KBr disc)(See Figure 8,page 78)

ν_{max} (cm^{-1}): 3500, 3200, 2950, 1728, 1705, 1620, 1575, 1520,1420, 1395, 1280, 1250, 1080, 1040.

Proton NMR. spectrum (in DMSO,90 MHz) (see Figure 6 page 76)

Chemical shift(δ) ppm	Proton	Multiplicity
7.806	1H	doublet
7.111	1H	singlet
7.029	1H	singlet
6.300	1H	doublet
3.879	3H	singlet
4.822 - 5.02	1H	broad doublet

Carbon-13 NMR.spectrum (in DMSO,$CDCl_3$,90 MHz)

(see figure 10 page 80)

Chemical shift (δ) ppm	Multiplicity
160.789	Singlet
150.225	"
149.250	"
146.542	"
143.562	Doublet
113.712	"
112.74	"
104.725	"
109.540	Singlet
56.395	Quartet
100.872	Doublet
78.065	"
77.000	"
75.194	"
69.993	"
61.325	Triplet

O - Acetylation

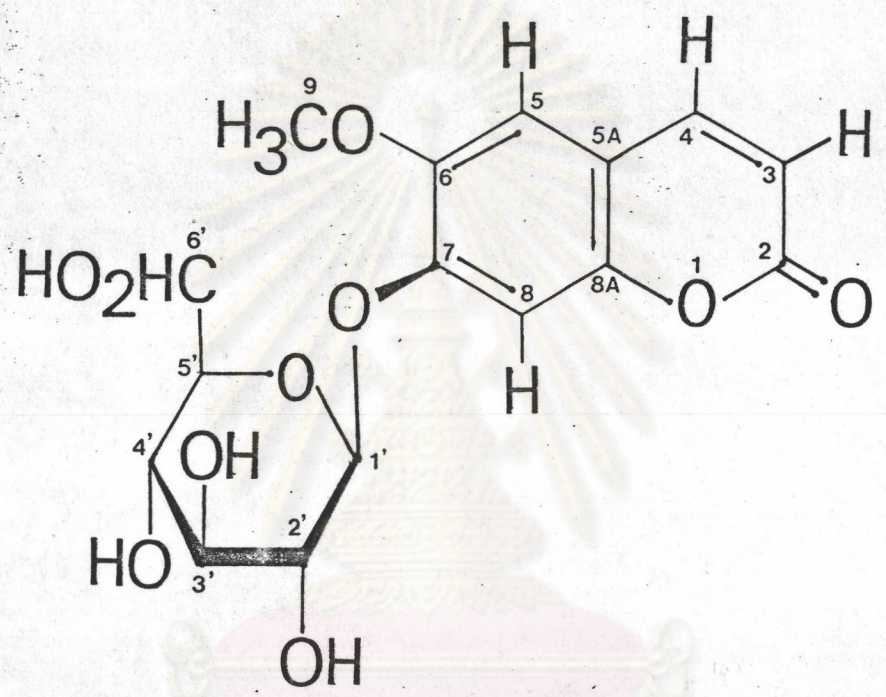
The sample was dissolved in a small amount of dry pyridine and mixed with a few drops of acetic anhydride. The solution was left for 24 hours at room temperature. The acetylated solution was basified with sodium bicarbonate to remove excess acetic anhydride. The basified solution was then extracted with chloroform. The chloroform layer was washed with water, dried over anhydrous sodium sulphate and evaporated under reduced pressure.

Hydrolysis of ES-2

ES-2 (50 mg) was stirred with 10% H_2SO_4 at 80°C for 24 hours. The reaction mixture was cooled and extracted with chloroform. Removal of the solvent from the chloroform layer afforded colourless needles (20 mg) crystallizing from ethanol.

The aqueous layer was neutralized through the addition of $NaHCO_3$ and was evaporated to near dryness. The residue was shown to contain D-glucose by paper chromatography with n-butanol-pyridine-water (9:5:4, Rf 0.14) and by TLC on cellulose with n-butanol-acetic acid-water (6:2:2, Rf 0.40) (Munechisa Arisawa et al.).

ES-2 was identified as scopolin (scopoletin glucoside).



SCOPOLIN