CHAPTER II

EXPERIMENTS AND RESULTS

2.1 Plant Materials

Stem bark of *Croton oblongifolius* Roxb. was collected from Pethchaboon province, Thailand in October 1995. This specimen was identical to the herbarium Voucher No. 9607 in the Royal Forest Department of Thailand.

2.2 Instruments and Equipment

2.2.1 Rotary Evaporator

Large amounts of volatile solvents were removed by the Eyela Model N-1 Rotary Evaporator.

2.2.2 Fourier Transform Infrared Spectrophotometer (FT-IR)

The FT-IR spectra were recorded as thin films for oils and as pellets of potassium bromide for solids on a Perkin-Elmer Model 1760X Fourier Transform Infrared Spectrophotometer and were calibrated with a polystyrene film.

2.2.3 Melting Point Apparatus

Melting points which are uncorrected were determined on a Fisher-John melting point apparatus.

2.2.4 ^{1}H- and ^{13}C-Nuclear Magnetic Resonance Spectrometer

500 MHz. spectra and specialized NMR experiments were recorded on a JNM 500 MHz. from JEOL, Japan. Routine ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker Model ACF 200 Spectrometer operated at 200.13 MHz. for ^{1}H and 50.32 MHz. for ^{13}C-nuclei. The chemical shifts are expressed in δ ppm. and were internally referenced to the residual protonated solvent (δ = 7.27 ppm. for $CHCl_3$, δ = 4.65 ppm. for D_2O).

2.2.5 Mass Spectrometry (MS)

The mass spectra were obtained in EI mode on a Fisons Instruments Mass Spectrometer Model Trio 2000.

2.3 Chemical Reagents

2.3.1 Merck's silica gel 60 Art. 7734.1000 (70-230 mesh ASTM) was used as an adsorbent for column chromatography.

2.3.2 Merck's TLC aluminium sheets silica gel 60 F254 pre-coated 25 sheet 20 x 20 cm. layer 0.2 mm. was used for identifying the identical fraction.

2.3.3 All solvents used in this research, except solvents that were of reagent grade were purified prior to use by distillation.

2.4 Physical Separation Techniques

Separation of the compounds from the crude extracts was carried out by various methods and techniques. The separation techniques used include :

- Extraction

- Column Chromatography [16]

- Thin-Layer Chromatography [16]

2.5 Extraction

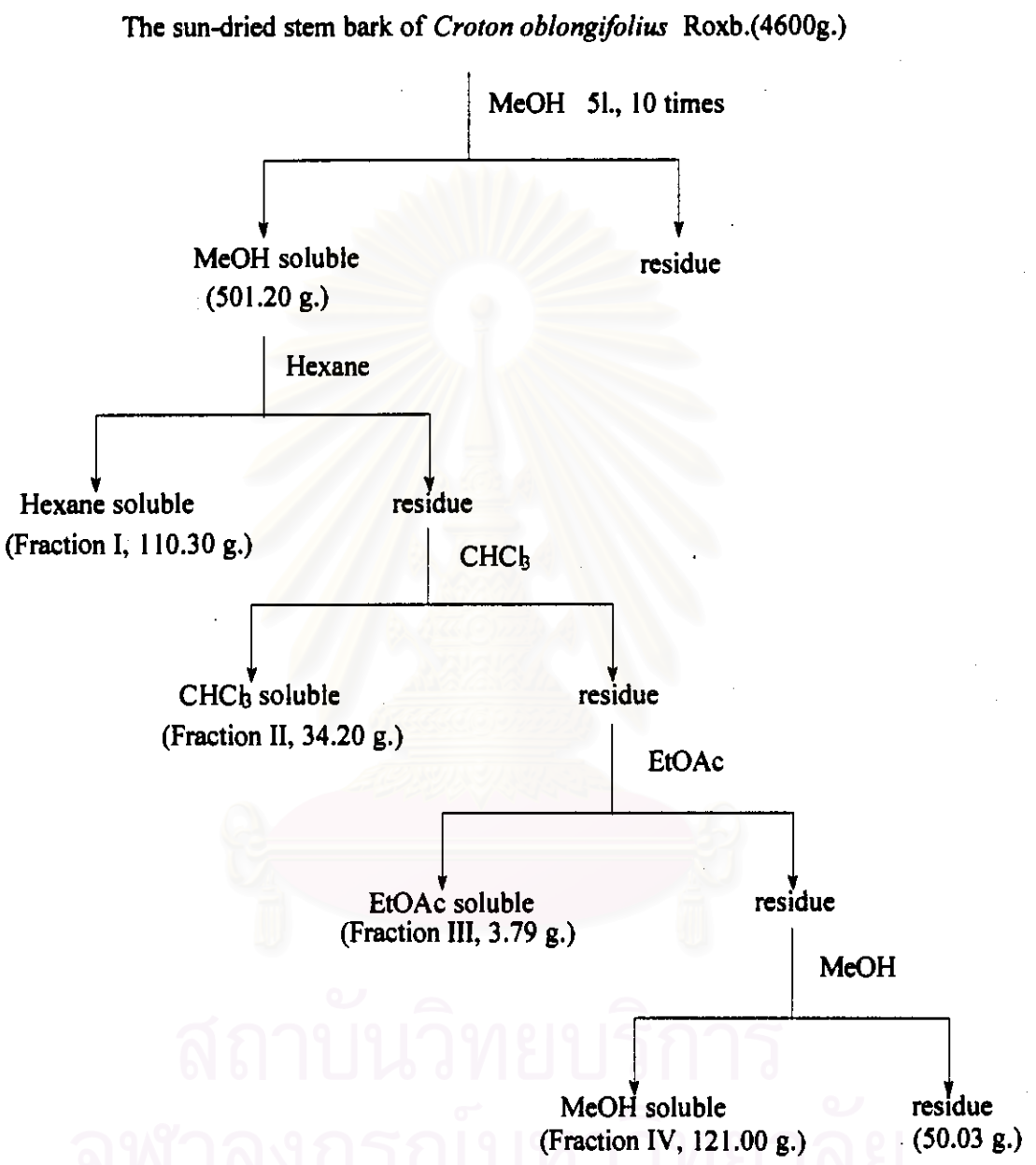
The powdered bark of *Croton oblongifolius* Roxb. (10,600 g.) was sun-dried for 7 days (4600 g.). After soaking in methanol at room temperature for 3-4 days, the methanol solution was filtered and evaporated under reduced pressure. The soaking procedure was repeated until the methanol solution was colourless. The combined methanolic extract was obtained as a dark-red gummy residue (501.2 g.). This was repeatedly extracted by hexane, chloroform, ethyl acetate and methanol respectively until the solutions were clear. After evaporating the filtered solutions, the hexane, chloroform, ethyl acetate and methanol extracts were obtained.

The weight of the extracts is shown in Table 1 and the extraction procedures are shown in Scheme 1.

Table 1 **The weight of the extracts**

Extract	Colour	Weight (g.)	% wt. by wt. of the fresh stem bark
Hexane	yellowish-green oil	110.30	1.04
$CHCl_3$	dark red oil	34.20	0.32
EtOAc	dark red oil	3.79	0.04
MeOH	dark reddish-brown oil	121.00	1.14

Scheme 1 **The extraction of the stem bark of *Croton oblongifolius* Roxb.**



2.6 Isolation of the Chemical Constituents from the stem bark of *Croton oblongifolius* Roxb.

2.6.1 Fractionation of Fraction I

The hexane extract (65.10 g.) was chromatographed on a column containing Silica gel 60 Act. 7734 as the adsorbent and was eluted with hexane , hexane-$CHCl_3$, $CHCl_3$ and $CHCl_3$-MeOH. Several 1000 ml. fractions were collected and concentrated to 20 ml.. Each fraction was monitored by TLC. The fractions containing similar components were combined. The results of the separation of the hexane extract by column chromatography were shown in Table 2.

Table 2 **The results of the separation of Fraction I by column chromatography**

Eluent	Fraction No.	Remark
100% hexane	1-3	pale yellow oil
	4	pale yellow oil
	5-6	pale yellow oil
	7-8	pale yellow oil
5% $CHCl_3$-hexane	9-13	pale yellow oil
10% $CHCl_3$-hexane	14-18	pale yellow oil
20% $CHCl_3$-hexane	19-26	pale yellow oil
30% $CHCl_3$-hexane	27-29	orange oil
	30-33	orange oil
40% $CHCl_3$-hexane	34-36	orange oil
	37-38	orange oil
	39	pale yellow oil

Eluent	Fraction No.	Remark
	40-43	white solid (Compound 1)
50% $CHCl_3$-hexane	44-53	white solid (Compound 1)
60% $CHCl_3$-hexane	54-60	yellow oil
70% $CHCl_3$-hexane	61-65	white solid in yellow oil (Compound 2)
80% $CHCl_3$-hexane	66-70	white solid in yellow oil (Compound 2)
90% $CHCl_3$-hexane	71-74	white solid in yellow oil (Compound 2)
100%$CHCl_3$	75-83	white solid in orange oil (Compound 2)
5% MeOH-$CHCl_3$	84-90	green oil
10% MeOH-$CHCl_3$	91-93	orange oil
	94-95	orange oil
	96-98	orange oil
20% MeOH-$CHCl_3$	99-100	orange oil
40% MeOH-$CHCl_3$	101-104	orange oil

2.6.2 Fractionation of Fraction II

Concentrated chloroform extract (34.20 g.) was chromatographed on silica gel Art. 7734 column. The column was eluted with $CHCl_3$ and $CHCl_3$-MeOH . About 200 ml. of the eluted solution was collected for each solvents. Each fraction was evaporated, concentrated on water bath and checked by TLC. Similar fractions were combined. The results of the separation of Fraction II by column chromatography are presented in table 3.

Table 3 **The results of the separation of Fraction II by column chromatography**

Eluent	Fraction No.	Remark
100% $CHCl_3$	1-2	-
	3-5	yellow oil
2%MeOH-$CHCl_3$	6	white solid in green oil (Mixture 3)
	7-11	yellow oil
3% MeOH-$CHCl_3$	12-16	yellow oil
5% MeOH-$CHCl_3$	17-21	yellow oil
10% MeOH-$CHCl_3$	22	White solid in yellow oil (Mixture 4)
	23-27	yellow oil
20% MeOH-$CHCl_3$	28-31	orange oil
30% MeOH-$CHCl_3$	32-33	orange oil
50% MeOH-$CHCl_3$	34-38	orange oil
	39-40	orange oil

2.6.3 Fractionation of Fraction III

The concentrated ethyl acetate extract 3.79 g. was chromatographed on silica gel Art. 7734 column. The column was initially eluted with $CHCl_3$. About 40 ml. of each fraction was collected. The next steps were performed as above. The results of the separation of these fractions are shown in Table 4.

Table 4 **The results of the separation of the Fraction III by column chromatography**

Eluent	Fraction No.	Remark
100 % $CHCl_3$	1	-
	2-4	yellow oil
	5-7	yellow oil
	8-9	yellow oil
0.5% MeOH-$CHCl_3$	10	yellow oil
	11	white solid in green oil (Mixture 3)
	12-21	yellow oil
1% MeOH-$CHCl_3$	22-23	yellow oil
	24-31	yellow oil
2% MeOH-$CHCl_3$	32-33	yellow oil
	34-42	yellow oil
5% MeOH-$CHCl_3$	43-46	yellow oil
10% MeOH-$CHCl_3$	47-49	yellow oil
	50-53	yellow oil
	54-62	yellow oil
20% MeOH-$CHCl_3$	63-71	orange oil
40% MeOH-$CHCl_3$	72-80	orange oil

2.6.4 Fractionation of Fraction IV

The methanol extract (8.90 g.) was chromatographed on Sephadex LH-20 and eluted with $CHCl_3$/MeOH (1:1). Several 20 ml. fractions were collected. The similar fractions were combined. The results of the separation of Fraction IV by Sephadex LH-20 chromatography are presented in Table 5.

Table 5 **The results of the separation of Fraction IV by column chromatography**

Fraction No.	Remark
1-3	-
4-7	brown tar
8-15	colourless solid in red tar (Compound 5)
16-22	red tar

2.7 Purification and Properties of the Compounds Eluted from Column Chromatography

2.7.1 Purification and Properties of Compound 1

Compound 1 was isolated from 40%, 50% and 60% $CHCl_3$-hexane fraction No. 40-60 from silica gel column chromatography (Table 2).The obtained compound was recrystallized from hexane for several times to provide the colourless needle-like crystals (478.5 mg., 0.74% wt. by wt. of hexane crude) with a m.p. of 109-111 °C. R_f value was 0.13 in 100% chloroform system (SiO_2). It was soluble in chloroform, acetone, hot ethyl acetate, ether and hot methanol and slightly soluble in hexane.

ν_{max} (KBr, cm^{-1}) 3400-3000(br,s), 2980, 2900(s),2700-2600(br,m),1690 (s), 1640 (m),1480,1450(br,s), 1400, 1387, 1370(w), 1320(m), 1283(s), 1230(w), 1205 (m), 1170(w), 1139(m), 1119, 1100, 1055, 980(w), 950(m), 910, 879,859, 838, 823, 800, 756, 678, 650(w) (Fig. 1)

δ_H (200 MHz, $CDCl_3$) 5.90-6.03 (3H, m), 5.10 (1H, t), 2.70 (2H, q), 2.41 (4H, m), 2.33 (1H, m), 2.20 (2H, m), 2.15 (6H, m), 1.73 (3H, s), 1.54 (3H, s), 1.04 (6H, d) (Fig. 2)

δ_C (50.25 MHz, $CDCl_3$) 174.12 (s), 146.90 (s), 146.33 (d), 135.17 (s), 133.97 (s), 130.93 (s), 125.66 (d), 121.58 (d), 118.74 (d), 39.18 (t), 38.56 (t), 33.78 (d), 33.62 (t), 28.71 (t), 26.42 (t), 25.08 (t), 22.09 (2q), 17.00 (q), 15.78 (q) (Fig. 3-5)

m/z (EI) [M^+] 302, 152, 136, 121 and 93 (Fig. 6)

2.7.2 Purification and Properties of Compound 2

Compound 2 was obtained from hexane crude extract as colourless needle-like crystals . It was eluted by 70%, 80% and 90% $CHCl_3$-hexane in fractions 61-83 from silica gel column (Table 2). After recrystallization from hot methanol, 1.2517 g. (1.25% wt. by wt. of hexane extract) of Compound 2, 86-88 °C, was obtained. The R_f value was 0.25 in 100 % chloroform system (SiO_2).This compound was soluble in chloroform, hot ethyl acetate and hot methanol but not in hexane.

ν_{max} (KBr, cm^{-1}) 3080-3040(br,s), 2975,2940, 2880(s), 2660(m),1690(s), 1640 (m), 1580, 1560, 1545, 1525(w), 1510, 1460, 1425, 1410, 1390(m), 1368, 1320(w), 1265(s), 1220, 1200, 1180, 1165, 1115, 1085, 1068, 1028, 1010(m), 980, 965(w), 945, 920, 880, 820(m), 785(s), 760, 735, 715, 700, 660, 640 (m) (Fig. 10)

δ_H (200 MHz, $CDCl_3$) 7.33 (1H, s), 7.19 (1H, s), 6.85 (1H, s), 6.26 (1H, s), 2.16-2.50 (6H, m), 1.40-1.73 (8H, m), 1.26 (3H, s), 0.84 (3H, d), 0.76 (3H, s) (Fig. 11)

δ_C (50.25 MHz, $CDCl_3$) 172.81 (s), 142.65 (d), 141.54 (s), 140.19 (d), 138.28 (d), 125.45 (s), 110.93 (d), 46.71 (d), 38.81 (s), 38.62 (t), 37.60 (s), 36.26 (d), 35.80 (t), 27.51 (t), 27.30 (t), 20.54 (q), 18.36 (q), 18.21 (t), 17.48 (t), 16.03 (q) (Fig. 12-14)

m/z (EI) [M^+] 316, 299, 221, 203. 125, 96, 81 (Fig. 15)

2.7.3 Purification and Properties of Mixture 3

Mixture 3 (15 mg., 0.04% wt. by wt. of chloroform crude) was obtained as colourless needle crystals from column chromatography of fraction No. 6 from the chloroform extract (Table 3) and fraction No. 11 from the ethyl acetate (Table 4). The R_f value was 0.58 in 5% methanol in chloroform system, m.p. 130-132 °C. This mixture was recrystallized from hexane.

ν_{max} (KBr, cm^{-1}) 3589-3271(br,s), 2868,2936, 2959(s), 1645(w), 1463(m), 1379(m), 1058(m), 960(m), 802(w) (Fig. 19)

m/z (EI) $[M^+]$ 414, 412, 400, 396, 394, 255, 213 (Fig. 20)

The GLC analysis was performed on a 2% OV1. The conditions of GLC were shown as follows.

injection temperature : 290 °C oven temperature : 255 °C

flow rate of N_2 : 40 ml./min. column length : 2 m.

The GLC analysis data of mixture of standard steroids, campesterol, stigmasterol and β-sitosterol, showed three compounds at retention times 18.74, 19.59 and 22.26 respectively (Fig. 21) and the data of Mixture 3 showed three compounds at 18.96, 19.80 and 22.41 min. respectively. (Fig 22).

2.7.4 Purification and Properties of Mixture 4

After recryatallization from hot ethanol, the Mixture 4 was obtained as a white amorphous solid, (1.03 g., 3.01% wt. by wt. of chloroform crude), m.p. 255 °C (decompose).The R_f value was 0.23 in 10% methanol in chloroform system. It was isolated from 10% MeOH-$CHCl_3$ in fraction No. 22 (Table 3). This Mixture 4 was soluble in hot ethanol but insoluble in hexane, chloroforom, ethyl acetate and methanol.

ν_{max} (KBr, cm^{-1}) 3340(br,s), 2860, 2940 and 2960(s), 1650(w), 1470(m), 1380 (m), 1025-1080(br,s), 890(w) (Fig. 23)

δ_H (200 MHz, $CDCl_3$) 0.63-2.69 (m), 2.88-3.55 (m), 3.69 (m), 4.25 (d), 4.51 (t), 4.93 (d), 5.15 (m), 5.35 (d) (Fig. 24)

δ_C (50.25 MHz, $CDCl_3$) 140.41, 138.01, 128.79, 121.19, 100.75, 76.86, 76.73, 73.43, 70.05, 61.05,56.15, 55.32, 50.58, 49.58, 45.10, 41.83, 41.72, 36.81, 36.20, 35.47, 31.38, 29.25, 28.66, 23.86, 22.57, 21.10, 20.94, 20.57, 19.70, 19.09, 18.91, 18.83, 18.60, 12.12, 11.83, 11.77, 11.66 (Fig. 25)

m/z (EI) $[M^+]$ 414, 412, 400, 396, 394, 255, 213 (Fig. 26)

2.7.5 Purification and Properties of Compound 5

Compound 5, 15 mg., was isolated from methanol crude extract by column chromatography (Table 5). It was colourless crystals, m.p. above 300 °C. This compound was soluble in H_2O but insoluble in hexane, chloroform, acetone,methanol and ethyl acetate.

Compound 5 was dissolved in water and then it was tested for inorganic ions. The test results of Compound 5 are shown in Table 6.

Table 6 **The results of the reaction of Compound 5**

Reaction	Observation
Flame Test	purple-red colour
$Na_3[Co(NO_2)_6]$	yellow precipitate which was insoluble in dil. acetic acid
$AgNO_3$	white precipitate which was soluble in ammonia, which, after adding dil. HNO_3, the white precipitate re-appeared