CHAPTER II

EXPERIMENTS AND RESULTS

Plant Materials

The leaf of *Croton oblongifolius* Roxb. (Plao Yai) was collected at Wat Thamthepbundal, Pethchaboon provice, Thailand in October 1995. This specimen was compared with the forest herbarium (BKF) No. 9607 in the Royal Forest Department of Thailand.

Instruments and Equipments

1. Fourier Transform Infrared Spectrophotometer (FT-IR)

The FT-IR spectra were recorded on a Perkin-Elmer Model 1760X Fourier Transform Infrared Spectrophotometer. Solid samples were generally examined by incorporating the sample with potassium bromide (KBr) to form a pellet. Spectra of liquid samples were recorded as thinfilm on a sodium chloride (NaCl) cell.

2. ^{1}H- and ^{13}C-Nuclear Magnetic Resonance Spectrometer (NMR)

The ^{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 500 MHz. spectra and specialized NMR experiments were record on a JNM 500 MHz. The chemical shift was assigned in ppm unit and internally referenced with the residual protoated solvent. ($CDCl_3$, δ = 7.24 ppm.)

3. Gas Chromatograph (GC)

The chromatogram were recorded on a Shimadzu Gas Chromatograph Model GC-7AG. (pack column) and on a Fisons Instrument Model GC 8000 (capillary column).

4. Mass Spectrometry (MS)

The mass spectra were recorded on a Fisons Instrument Mass Spectrometer Model Trio 2000 in EI mode at 70 eV and on a Varian Saturn 4D in CI mode.

5. Melting Point Apparatus

The melting points were recorded on a Fisher-John melting point apparatus.

6. Rotary Evaporator

A large amounts of volatile solvents was removed by the Eyela type N-N rotary evaporator.

Chemical Reagents

1. Solvents

All solvents used in this research such as hexane, dichloromethane(CH_2Cl_2), chloroform($CHCl_3$), ethyl acetate(EtOAc) and methanol(MeOH) were commercial grade and were purified prior to use by distillation. The reagent grade solvents were used for recrystallization.

2. Other chemicals

2.1 Merck's silica gel 60 Art. 7734 (70-230 mesh ASTM) was used as adsorbent for column chromatography.

2.2 Sephadex LH-20 was used as a stationary phase for column chromatography.

2.3 Merck's silica gel 60G Art.7731 and $60GF_{254}$ Art.7730 were applied as adsorbent for preparative TLC.

2.4 Merck's TLC aluminium sheet, silica gel 60F 254 precoated 25 sheets, 20x20 cm^2., layer 0.2 mm. was used to identify the identical fractions.

Physical Separation Techniques

The following separation techniques were used.

1. Column Chromatography.(CC) [15,16,17]

2. Thin-Layer Chromatography.(TLC) [15,16,18]

3. Preparative Thin-Layer Chromatography.(Prep-TLC) [16,18]

Extraction

The fresh leaf of *Croton oblongifolius* Roxb. (12,000 g) was sun dried to dried leaf (1,800 g). It was powdered and soaked in hexane at room temperature. After for 7 days, the hexane solution was filtered and the filtrate evaporated under reduced pressure. The hexane extraction was repeated until the hexane solution was colourless. The hexane extract was obtained as a dark green oil 35.80 g.(Fraction I, 1.99%wt/wt of the dried leaf)

The residue,after hexane extraction, was extracted with CH_2Cl_2. The CH_2Cl_2 solution was filtered and evaporated the filtrate under reduced pressure. The CH_2Cl_2 extraction was repeated until the CH_2Cl_2 solution was colourless. The CH_2Cl_2 extract was obtained as a dark green oil 24.63 g.(Fraction II, 1.37%wt/wt of the dried leaf)

The residue,after CH_2Cl_2 extraction, was extracted with EtOAc. The EtOAc solution was filtered and the filtrate evaporated under reduced pressure. The EtOAc extraction was repeated until the EtOAc solution was colourless. The EtOAc extract was obtained as a dark brown oil 2.67 g.(Fraction III, 0.15%wt/wt of the dried leaf)

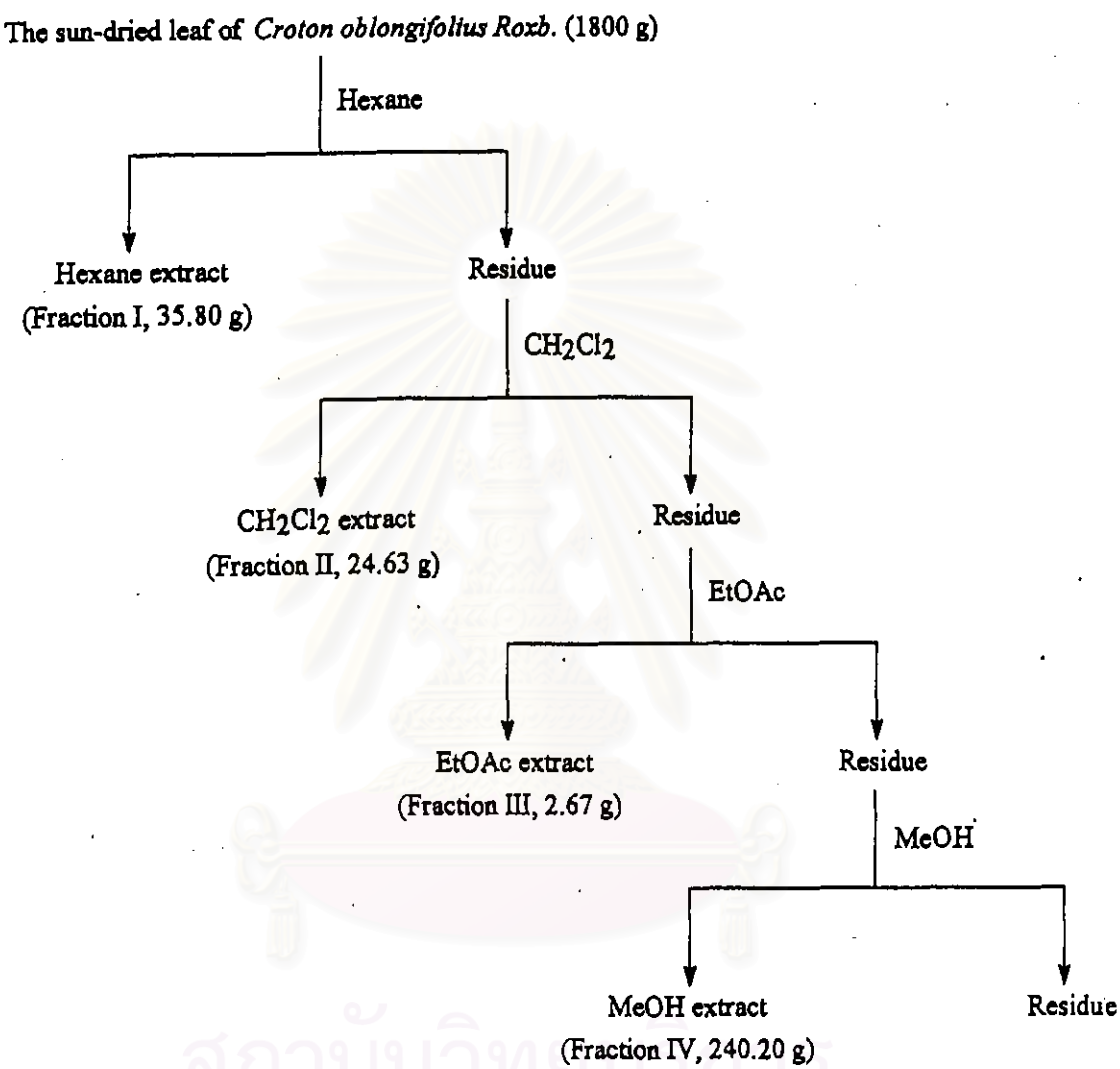
Finally, the residue,after EtOAc extraction, was extracted with methanol. The methanol solution was filtered and the filtrate evaporated under reduced pressure. The methanol extraction was repeated until the methanol solution was colourless. The methanol extract was obtained as a dark red oil 240.20 g.(Fraction IV, 13.34%wt/wt of the dried leaf)

Four different crude extracts of the leaf of *Croton oblongifolius* Roxb. are as shown in Table 2 and the extraction procedure are shown in Scheme 1.

Table 2 The various extracts of the leaf of *Croton oblongifolius* Roxb.

Fraction	Extract	Appearance	Weight (g)	%wt/wt of the dried leaf
I	Hexane	dark green oil	35.80	1.99
II	CH_2Cl_2	dark green oil	24.63	1.37
III	EtOAc	dark brown oil	2.67	0.15
IV	MeOH	dark red oil	240.20	13.34

Scheme 1 The extraction procedure of the leaf of *Croton oblongifolius* Roxb.



Isolation of the Chemical Constituents of the leaf of *Croton oblongifolius* Roxb.

1. Separation of Fraction I

The hexane extract (Fraction I, 30.0 g) was separated by column chromatography. The column was packed with silica gel 60 Act.7734 and the crude extract, which was adsorbed on silica gel, was added to the top of column. The column was eluted with hexane, hexane-$CHCl_3$, $CHCl_3$ and $CHCl_3$-MeOH. Each fraction was concentrated to a small volume, about 20 mL, and checked by TLC. The fractions containing similar components were combined. The results of separation of Fraction I by column chromatography were presented in Table 3.

Table 3 The results of separation of Fraction I by column chromatography

Eluents	Fraction No.	Remarks
100% Hexane	1	white solid (Mixture 1)
	2-7	nothing
10% $CHCl_3$ in hexane	8-11	yellow oil
20% $CHCl_3$ in hexane	12-13	yellow oil
30% $CHCl_3$ in hexane	14-18	yellow oil
	19-27	yellow oil (Compound 2)
	28-29	yellow oil
40% $CHCl_3$ in hexane	30-33	white solid in yellow oil (Mixture 3)
50% $CHCl_3$ in hexane	34-41	yellow oil
60% $CHCl_3$ in hexane	42-51	yellow oil
	52-53	white solid in yellow oil (Mixture 4)
70% $CHCl_3$ in hexane	54-57	yellow oil
80% $CHCl_3$ in hexane	58-70	yellow oil
90% $CHCl_3$ in hexane	71-73	yellow oil
100% $CHCl_3$	74-90	yellow oil
	91-104	brown oil
	105-109	yellow oil
1% MeOH in $CHCl_3$	110-130	yellow oil
5% MeOH in $CHCl_3$	131-132	yellow oil
20% MeOH in $CHCl_3$	133-138	yellow oil
40% MeOH in $CHCl_3$	139-144	yellow oil
100% MeOH	145-146	yellow oil

2. Separation of Fraction II

The CH_2Cl_2 extract (Fraction II, 22.0 g) was separated by column chromatography. The column was packed with silica gel 60 Act.7734 and the crude extract, which was adsorbed on silica gel, was added to the top of column. The

column was eluted with hexane, hexane-$CHCl_3$, $CHCl_3$, $CHCl_3$-EtOAc, EtOAc, EtOAc-MeOH and MeOH. Each fraction was concentrated to a small volume, about 20 mL, and checked by TLC. The fractions containing similar components were combined. The results of separation of Fraction II by column chromatography were presented in Table 4.

Table 4 The results of separation of Fraction II by column chromatography

Eluents	Fraction No.	Remarks
100% Hexane	1-3	nothing
20% $CHCl_3$ in hexane	4-8	yellow oil
40% $CHCl_3$ in hexane	9-11	yellow oil
60% $CHCl_3$ in hexane	12-23	yellow oil
80% $CHCl_3$ in hexane	24-30	colorless solid in yellow oil (Compound 5)
100% $CHCl_3$	31-33	yellow oil
	34-38	green oil
5% EtOAc in $CHCl_3$	39-43	dark green tar
10% EtOAc in $CHCl_3$	44-46	dark green tar
20% EtOAc in $CHCl_3$	47-50	dark green tar
50% EtOAc in $CHCl_3$	51-55	dark green tar
70% EtOAc in $CHCl_3$	56-60	dark green tar
90% EtOAc in $CHCl_3$	61-63	dark brown tar
100% EtOAc	64-67	dark brown tar
5% MeOH in EtOAc	68-71	dark brown tar
10% MeOH in EtOAc	72-75	dark brown tar
20% MeOH in EtOAc	76-79	dark brown tar
50% MeOH in EtOAc	80-83	brown oil
80% MeOH in EtOAc	84-87	brown oil
100% MeOH	88-92	yellow oil

3. Separation of Fraction III

The EtOAc extract (Fraction III, 2.4 g) was separated by column chromatography. The column was packed with silica gel 60 Act.7734 and the crude extract, which was adsorbed on silica gel, was added to the top of column. The column was eluted with $CHCl_3$, $CHCl_3$-EtOAc, EtOAc, EtOAc-MeOH and MeOH. Each fraction was concentrated to a small volume, about 20 mL, and checked by TLC. The fractions containing similar components were combined. The results of separation of Fraction III by column chromatography were presented in Table 5.

Table 5 The results of separation of Fraction III by column chromatography

Eluents	Fraction No.	Remarks
100% $CHCl_3$	1-4	nothing
5% EtOAc in $CHCl_3$	5-7	greenish-brown oil
10% EtOAc in $CHCl_3$	8-10	greenish-brown oil
20% EtOAc in $CHCl_3$	11-13	greenish-brown oil
50% EtOAc in $CHCl_3$	14-16	brown tar
70% EtOAc in $CHCl_3$	17-19	brown tar
90% EtOAc in $CHCl_3$	20-22	brown tar
100% EtOAc	23-25	brown tar
1% MeOH in EtOAc	26-28	brown tar
5% MeOH in EtOAc	29-31	brown tar
10% MeOH in EtOAc	32-34	solid in brown oil
20% MeOH in EtOAc	35-37	solid in brown oil
40% MeOH in EtOAc	38-40	brown oil
60% MeOH in EtOAc	41-43	yellow oil
80% MeOH in EtOAc	44-47	brown oil
100% MeOH	48-53	brown oil
	54-56	yellow oil

4. Separation of Fraction IV

The methanol extract (Fraction IV, 10.0 g) was chromatographed on Sephadex LH-20 and eluted with $CHCl_3$-MeOH (1:1). Each 20 mL fraction was collected and checked by TLC. The fractions containing similar components were combined. The results of separation of Fraction IV by column chromatography were shown in Table 6

Table 6 The results of separation of Fraction IV by column chromatography

Fraction No.	Remarks
1-2	nothing
3-8	brown tar
9-16	brownish-yellow oil
17-20	colourless solid in yellow oil (Compound 6)
21-26	yellow oil

Purification and Properties of the Eluted Compounds by Column Chromatography.

1. Purification and Properties of Mixture 1

A white amorphous solid , Mixture 1 , was obtained after column chromatography from fraction no.1 of the hexane crude extract . It was eluted from the column with hexane. It was recrystallized from hexane, yielding 0.32 g (1.1% wt/wt of hexane crude), m.p. 58-60°C. This Mixture was soluble in hexane, dichloromethane and chloroform. The R_f value was 0.6 (stationary phase: silica, solvent system: hexane).

FT-IR (KBr): υ max($cm.^{-1}$) 2918 and 2849 (s, C-H stretching), 1473 and 1463 (m, C-H bending), 721 (w, C-H rocking). (Fig. 3)

GC : (condition; column 2% OV-1, column temp. 250°C, injection temp. 290 °C, carrier gas N_2 50 mL/min. and FID detector) The chromatogram showed 7 peaks at retention times 6.37, 8.19, 10.58, 13.63, 17.69, 22.78 and 29.41 min respectively. (Fig. 5)

2. Purification and Properties of Compound 2

A pale yellow oil, Compound 2 , was obtained after column chromatography from fractions no.19-27 of the hexane crude extract . It was eluted from the column with 30% $CHCl_3$ in hexane. Compound 2 was further purified by preparative TLC in 50%$CHCl_3$/hexane, yielding 0.03 g (0.1% wt/wt of hexane crude). This compound was soluble in dichloromethane and chloroform. The R_f value was 0.42 (stationary phase: silica, solvent system: 50%$CHCl_3$ in hexane).

FT-IR (neat): υ max($cm.^{-1}$) 2953, 2928 and 2870 (s, C-H stretching), 1718 (m, C=O stretching), 1461 and 1378 (m, C-H bending). (Fig.7)

EI-MS: Mass spectrum showed the important fragmentation ion peaks at m/e 268, 250, 210, 179, 165, 137, 123 and 109. (Fig.8)

CI-MS: Mass spectrum showed the important ion peak at m/e 269. (Fig.10)

3. Purification and Properties of Mixture 3

A white amorphous solid , Mixture 3 , was obtained after the column chromatography from fractions no.30-33 of the hexane crude extract . It was eluted from the column with 40% $CHCl_3$ in hexane. It was recrystallized from hot hexane, yielding 0.15 g (0.50% wt/wt of hexane crude), m.p. 85-87°C. This Mixture was soluble in hot hexane, dichloromethane and chloroform. The R_f value was 0.65 (stationary phase: silica, solvent system: $CHCl_3$).

FT-IR (KBr): υ max($cm.^{-1}$) 3504-3230 (br, O-H stretching), 2919 and 2849 (s, C-H stretching), 1473 and 1463 (m, C-H bending), 1065 (w, C-O stretching), 722 (w, C-H rocking). (Fig.11)

GC : (condition; column 2% OV-1, column temp. 250°C, injection temp. 290 °C, carrier gas N_2 50 mL/min. and FID detector) The chromatogram showed 5 peaks at retention times 0.79, 1.05, 1.52, 2.37 and 3.78 min respectively. (Fig.13)

4. Purification and Properties of Mixture 4

Bright white needle like crystal , Mixture 4 , was obtained after column chromatography from fractions no.52-53 of the hexane crude extract . It was eluted from the column with 80% $CHCl_3$ in hexane, yielding 0.26 g (0.87%wt/wt of hexane crude), m.p. 139-141°C. This Mixture was soluble in hot hexane, dichloromethane and chloroform. The R_f value was 0.65 (stationary phase: silica, solvent system: 50%$CHCl_3$ in hexane).

FT-IR (KBr): υ max($cm.^{-1}$) 3525-3198 (br, O-H stretching), 2940 and 2869 (s, C-H stretching), 1645 (w, C=C stretching), 1462 and 1380 (m, C-H bending), 1061 (w, C-O stretching), 960 and 802 (w, C-H bending).(Fig.15)

GC : (condition; column 2% OV-1, column temp. 250°C, injection temp. 290 °C, carrier gas N_2 50 mL/min. and FID detector) The chromatogram showed 2 peaks at retention times 18.84 and 21.46 min respectively.(Fig.17)

EI-MS: Mass spectrum showed the important fragmentation ion peaks at m/e 414 ($C_{29}H_{50}O$) and 412 ($C_{29}H_{48}O$) and other fragmentation ion peaks at m/e 396, 394, 275, 273, 255 and 213.(Fig.18)

5. Purification and Properties of Compound 5

Colourless needle like crystal, Compound 5, was obtained after column chromatography from fractions no.24-30 of the CH_2Cl_2 crude extract .It was eluted from the column with 80%$CHCl_3$ in hexane. yielding 0.19 g (0.86%wt/wt of CH_2Cl_2 crude), m.p.127-129°C. This compound was good soluble in chloroform, hot ethyl acetate, hot methanol and slightly soluble in hexane. The R_f value was 0.5 (stationary phase: silica, solvent system: EtOAc).

FT-IR (KBr), υ max($cm.^{-1}$) showed the absorption peak at 3400-3050 (br, O-H stretching), 2956 and 2929 (s, C-H stretching), 1684 (s, C=O stretching), 1635 (m, C=C stretching), 1424 and 1381 (m, C-H bending) and 1281 (m, C-O stretching). (Fig.19)

^{1}H-NMR: The ^{1}H-NMR spectrum ($CDCl_3$) showed the chemical shift (δ) at 1.05(6H,d), 1.68(3H,s), 1.71(3H,s), 2.21(8H,m), 2.38,(5H,m), 5.14(1H,t), 5.91(1H,d), 6.01(1H,d) and 6.89(1H,t) ppm. (Fig.20)

^{13}C-NMR: The ^{13}C-NMR spectrum ($CDCl_3$) showed the chemical shift (δ) at 17.38, 17.96, 22.09, 24.70, 26.74, 29.14, 30.54, 34.56, 37.71, 38.54, 118.61, 120.02, 127.77, 132.08, 134.76, 135.55, 145.70, 146.49 and 173.49 ppm.(Fig.21)

DEPT-90 ^{13}C-NMR spectrum ($CDCl_3$) showed the chemical shift (δ) of CH signal at 34.56, 118.61, 120.02, 127.77 and 145.70 ppm. (Fig.22)

DEPT-135 ^{13}C-NMR spectrum ($CDCl_3$) showed the chemical shift (δ) of CH and CH_3 signal (up phase) at 17.38, 17.96, 22.09(x2), 34.56, 118.61, 120.02, 127.77 and 145.70 ppm. and showed the chemical shift (δ) of CH_2 signal (down phase) at 24.70, 26.74, 29.14, 30.54, 37.71, and 38.54 ppm. (Fig.22)

EI-MS: Mass spectrum showed the molecular ion peak at m/e 302 ($C_{20}H_{30}O_2$) and other fragmentation ion peaks at m/e 152, 136, 121 and 93.(Fig.23).

6. Purification and Properties of Compound 6

The colourless crystal, Compound 6 , was obtained from the fraction no.17-20 of the methanol crude extract separation by column chromatography. It was eluted from the Sephadex LH-20 column with 50%$CHCl_3$ in methanol, yielding 0.02 g (0.2% wt/wt of methanol crude), m.p. above 300°C. This compound was very soluble in water but insoluble in hexane, dichloromethane, chloroform, acetone, ethyl acetate and methanol.

Compound 6 was soluble in water and then it was tested for inorganic ions. [19] The results of the inorganic ion tests of Compound 6 ware shown in Table 7.

Table 7 The results of the inorganic ion tests of Compound 6

Reaction	Observation
Flame Test	purple-red flame
Sodium cobaltinitrite	yellow precipitate
Silver nitrate	white precipitate which was soluble in ammonia and precipitated back on acidification with nitric acid