CHAPTER III

EXPERIMENTS

3.1 Plant material.

Croton oblongifolius, the plant material, was collected from Amphoe Sai Yok Kanchanaburi province, Thailand in June, 1999. The plant specimen was identified by comparison against voucher specimen no. BKF 084729 deposited in the herbarium of the Royal Forest Department, Ministry of Agricultural and Cooperative, Thailand.

3.2 Instruments and equipments

1. Fourier Transform Infrared Spectrophotometre (FT-IR)

   The FT-IR spectra were recorded on a Nicolet Impact 410 Spectrophotometre. The KBr pellets were used to record spectra of solid sample and thinfilm on KBr cells for liquid samples.

2. Mass Spectrometry (MS)

   The mass spectra were recorded on a Fisons Instruments Mass Spectrometre Model Trio 2000 in EI mode at 70 eV .

3. Ultraviolet-Visible Spectrometry (UV-VIS)

The UV-VIS spectra were recorded on a Hewlett Packard 8452 A diode array spectrophotometre in chloroform.

4. ^{1}H and ^{13}C Nuclear Magnetic Resonance Spectrometre (NMR)

The ^{1}H and ^{13}C Nuclear Magnetic Resonance Spectra were recorded at 200.13 and 50.32 MH_z, respectively, on a Bruker Model AC-F200 Spectrometer and at 500.00 and 125.65 MH_z on a JEOL JNM-A500 spectrometre in $CDCl_3$. Chemical shifts are given in parts per million using residual protonated solvent as reference. COSY, NOESY, HMQC and HMBC experiments were performed on the JEOL JNM-A500 spectrometre.

5. Elemental Analysis (EA)

The EAs were measured on a Perkin Elmer PE2400 SERIES II (CHO/O ANALYSER)

6. Optical rotation

The Optical rotation were measured on a Perkin-Elmer 341 polarimeter in $CHCl_3$.

3.3 Chemical reagents

1. solvents

There are several solvents used in this research such as hexane, ethylacetate, and methanol. These solvents were commercial grade and were purified by distillation before use.

2. Other chemicals

2.1 Merck's silica gel 60 G Art. 7734 (70-230 mesh ASTM) were used as adsorbents for normal column chromatography and 9385 (230-400 mesh ASTM) for flash column chlomatography.

2.1 Merck's TLC aluminum sheet, silica gel 60 F_{254} precoated 25 sheets, 20×20 cm^2, layer 0.2 mm was used to identify the identical fractions.

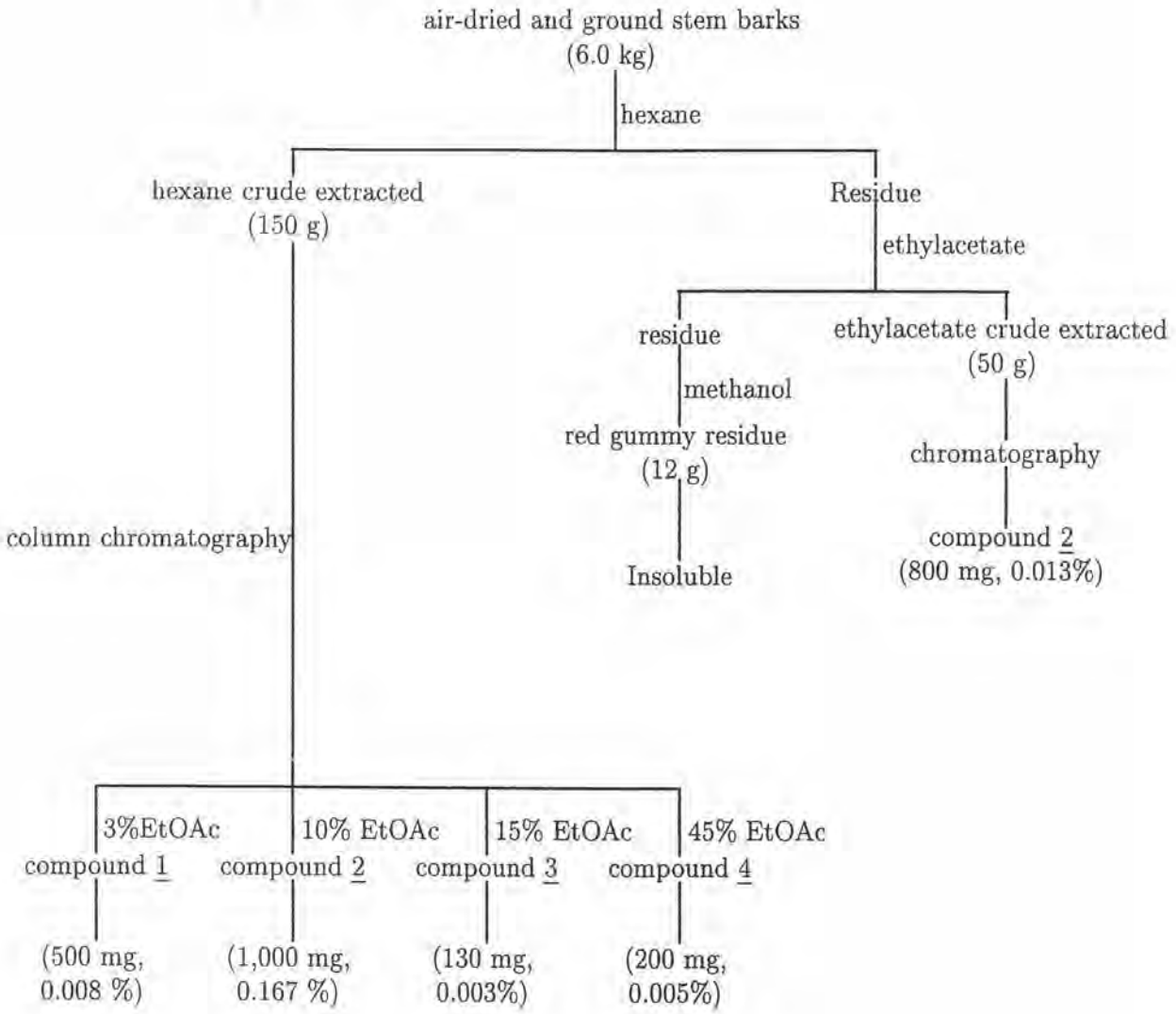
3.4 Extraction and isolation.

The air-dried and ground stem barks (6.0 kg.) of *C. oblongifolius* has been soaked in hexane 12 litres for a week at room temperature. The hexane extract was filtered and evaporated under reduced pressure to obtain 150 g. of dark-yellowish oily residue. The residue was soaked in ethylacetate 10 litres which was filterd and evaporated respectively. This procedure gave dark-yellowish oily residue.The residue was extracted with methanol to give dark-red gummy residue. All of crude extracts from stem barks of *C. oblongifolius* were assigned in Table 2. and the procedure of extraction were shown in scheme 1.

Table **2: The weight of crude extracted with various solvents from stem barks of** *C. oblongifolius* **Roxb.**

Solvent extract	Apperence	Weight (g)	% *wt/wt* of the dried stem barks
Hexane	yellowish green oil	150	2.50
Ethylacetate	dark-yellowish oil	50	0.83
Methanol	red gummy	12	0.20

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



3.5 Isolation of the chemical constituents from stem barks of *Croton oblongifolius* Roxb.

3.5.1 Separation of hexane crude extract.

The hexane crude extract (100 g) was factionated by column chromatography , using silica gel as adsorbent and hexane-ethylacetate as eluent in a stepwise fashion. The volume of each fraction was approximately 500 ml and each was evaporated to about 25 ml. The similar fractions were combined together according to the TLC pattern.

The result of separation was shown in Table 3.

3.5.2 Separation of ethylacetate crude extract.

Concentrated ethylacetate crude extract (50 g.) was separated on Silica gel column chromatographic technique. The colume was eluted with hexane, hexane-ethylacetate, ethylacetate, ethylacetate-methanol, respectively. The eluated fraction was collected at 250 ml and there was about 25 ml dark-yellowish oil residue left after evaporation. The similar fractions were combinded together after had been checked with TLC.

3.5.3 Separation of methanol crude extract.

The red gummy residue of the methanol crude extract (12 g.) was purified with column chromatography technique by using $CHCl_3$-MeOH (1:1) as eluent solvent.

Each fractions were collected 20 ml. and combined the similar fractions by using the basis of TLC analysis. There were a red gummy fractions with only small amount of material left after evaporation.

Table 3: **The result from column chromatography of hexane crude extract.**

Fraction No.	Appearance of solution	Combined fractions	Compounds
1	clear	A1	
2			
3			
4			
5	light yellowish	A2	light yellowish oil
6			
7			
8			
9			
10	yellowish	A3	compound 1
11			
12			
13			
14		A4	yellowish oil
15			
16			
17			
18			
19			
20		A5	compound 2
21			
22			
23	light yellowish		
24			
25			
26		A6	

Fraction No.	Appearance of solution	Combined fractions	Compounds
27			
28			compound 3
29	light yellowish	A6	
30			
31			
32			
33			
34	light green		
35			
36		A7	light green oil
37			
38			
39			
40	dark green		
41			
42		A8	dark green oil
43			
44			
45			
46	light yellowish		
47		A9	compound 4
48			
49			
50			
51			
52			
53	dark yellowish	A10	dark brown oil
54			
55			
56			
57			
58	light brown	A11	
59			light brown oil
60			

Fraction No.	Appearance of solution	Combined fractions	Compounds
61	light brown	A11	
62			
63	dark brown	A12	dark brown oil
64			
65			
66			
67	light yellowish	A13	light yellowish oil
68			
69			
70			
71			
72			

3.6 Purification and properties of the compounds eluted from column chromatography of hexane crude extract.

3.6.1 Purification and properties of compound 1

Compound 1 was eluted with 3 % ethylacetate in hexane fraction on silica gel column chromatography. The solvent was removed by rotary evaporation and the compound was recrystallized from haxane for several times to provide the colorless rectangle-like crystals (500 mg, 0.33 % wt by wt of hexane crude) with melting point 96-98 °C. This compound is soluble in hexane, chloroform and ethylacetate.

Compound 1 was colorless crystals (500 mg), $[\alpha]_D^{20}$ -37.9 ($CHCl_3$, c 1.0), R_f ; 0.48 in 10 % ethylacetate in hexane system (SiO_2).

FT-IR spectrum (KBr), λ_{max} (cm^{-1}) : 3200 (br), 2971, 2930 and 2873 (s), 1634 (s) (Fig. **10**)

^{1}H-NMR spectrum ($CDCl_3$, 200 MHz) δ (ppm) 6.01 (1H, dd), 4.91 (2H, d), 2.21 (1H, dd), 1.76 (1H, dt), 1.60-1.68 (3H, m), 1.36-1.56 (5H, m), 1.24-1.30 (4H, m), 1.22 (3H, s), 1.13 (3H, s), 0.95 (1H,dd), 0.86 (4H, s), 0.78 (3H, s), 0.72 (3H,s). (Fig. **11**)

^{13}C-NMR spectrum ($CDCl_3$, 200 MHz) δ (ppm) 147.7 (d), 109.5 (t), 76.0 (s), 73.2 (s), 58.5 (d), 56.4 (d), 43.1 (t), 42.1 (t), 39.3 (t), 37.3 (q), 36.5 (s), 34.8 (t), 33.3 (q), 33.2 (s), 29.6 (t), 23.9 (q), 21.2 (q), 19.8 (t), 18.6 (t), 15.8 (q) (Fig. **12**)

EI MS spectrum $m/z(70\ eV)$: 308 [M^+], 293, 290, 199, 81, 43 [18]

3.6.2 Purification and properties of compound 2

The compound 2 was eluted with 10 % ethylacetate in hexane. Similar to compound 1, the solvent in compound 2 fraction was removed by rotary evaporation and recrystalized by using ethylacetate to provide the hexagon-like crystals (1.00 g., 0.67 % wt by wt of hexane crude and 0.167 % yield from starting material) with melting point 171-172 °C. This compound is soluble in ethylacetate and methanol.

Compound 2 was colorless rectangular crystals, $[\alpha]_D^{20}$ -50.2 ($CHCl_3$, c 1.0), R_f ; 0.30 in 5 % methanol in chloroform system (SiO_2).

FT-IR spectrum (KBr), λ_{max} (cm^{-1}) : 2940, 2909 and 2868(m), 1737(s), 1204(m) and 1025(s) (Fig. **14**)

^{1}H-NMR spectrum ($CDCl_3$, 200 MHz) δ (ppm) 7.37(1H, d), 7.35(1H, t), 6.37(1H, d), 5.31(1H, dd), 5.26(1H, s), 5.11(1H, d), 4.48(1H, ddd), 3.69(3H, s), 2.83(1H, dd), 2.28-2.40(2H, m), 2.23(1H, dd), 2.13(1H, dd), 2.02(1H, dt), 1.95(1H, m), 1.84(1H, m), 1.70(2H, m), 1.57(2H, m), 1.43(1H, ddd), 1.34(1H, m), and 0.93(3H, d) (Fig. **15**)

^{13}C-NMR spectrum ($CDCl_3$, 200 MHz) δ (ppm) 170.2, 143.4(d), 139.3(d),127.2(s), 108.6(d), 104.5, 100.7, 75.7(d), 74.9, 54.0(d), 51.6 (OMe), 50.3(s), 44.3(s), 38.9(d), 38.6(t), 37.4(d), 31.5(t), 30.5(t), 26.5(t), 20.2(t) and 16.9(q) (Fig. **17**)

EI MS spectrum $m/z(70\ eV)$: 374 [M^+], 328, 248, 234, 176, 163 and 94 (Fig. **15**)

3.6.3 Purification and properties of compound 3

The compound 3 was isolated from 15 % ethylacetate in hexane fraction and purification by recrystalized in methanol to provide 0.13 g ofcolorless rectangular crystal.(0.87 % yield from hexane crude and 0.003 % yield from starting materia) Its melting is point 189 °C. This compound is soluble in chloroform, ethylacetate and medium soluble in methanol.

Compound 3 was colorless rectangular crystals (130 mg), $[\alpha]_D^{20}$ +91.8 ($CHCl_3$, *c* 1.0), R_f ; 0.40 in 5 % methanol in chloroform system (SiO_2), UV ($CHCl_3$) λ_{max} 242 sh (log ϵ 3.81), Found C 65.88; H 5.88; O 28.24 % Calc. C 65.98; H 5.98; O 28.04 %

FT-IR spectrum (KBr), λ_{max} (cm^{-1}) : 3500 and 3100 (m), 2955), 2906 and 2877 (m), 1731(s) and 1030(s) (Fig. **19**)

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz) δ (ppm) 7.85 (2H, dd), 7.53 (1H, dt), 7.42 (2H, t), 7.33 (2H, t), 6.52 (1H, s), 6.26 (1H, dd), 5.28 (1H, s), 5.01 (1H, t), 4.15 (1H, s), 3.78 (3H, s), 3.20 (1H, m), 3.08 (1H, ddd), 2.57 (1H, ddd), 2.24-2.21 (5H, m), 1.80-1.60 (3H, m), 1.24 (1H, m), and 0.99 (3H, s) (Fig. **21**)

^{13}C-NMR spectrum ($CDCl_3$, 400 MHz) δ (ppm) 202.3 (s), 171.6 (s), 165.1 (s), 143.7 (d), 138.7 (d), 133.2 (d), 129.8 (d), 129.8 (d), 129.6 (s), 128.9 (s), 128.3(s), 128.3(s), 108.3 (d), 98.5 (d), 93.5 (d), 80.9 (s), 69.0 (d), 53.9 (q), 46.3 (s), 44.9 (s), 38.3 (d), 37.6 (t), 36.9 (d), 35.8 (t), 29.3 (t), 28.8 (t), 24.3 (t), and 16.8 (q) (Fig. **22**)

EI MS spectrum m/z (70 eV) : 510 [M^+], 388, 360, 283, 122, 105, 94, and 77 (Fig. **20**)

3.6.4 Purification and properties of compound 4

From the crude hexane extract, the 45 % ethylacetate in hexane fraction was fractionated with hexane-ethylacetate solvent system. The compound 4 was obtained in 50 % ethylacetate in hexane fraction. It was recrystallized in 40 % ethylacetate in hextane solvent to obtain 0.20 g white solid (0.13 % yield from hexane crude and 0.005 % yield from starting material) and its melting point is 72-73 °C. The compound 4 was soluble in chloroform, ethylacetate, methanol and ethanol. The R_f value was 0.40 in 5 % methanol in chloroform system. (SiO_2)

Compound 4 was white solid (200 mg), $[\alpha]_D^{20}$ -223.1 ($CHCl_3$, c 1.0), R_f ; 0.40 in 5 % methanol in chloroform system. (SiO_2) .

FT-IR spectrum (KBr), λ_{max} (cm^{-1}) : 3421 (br), 2950, 2925 and 2879 (s), 1465 (m), and 1090 (m). (Fig. **28**)

^{1}H-NMR spectrum ($CDCl_3$, 200 MHz) δ (ppm) 6.31(1H, dd), 5.52(1H, dd), 5.02(1H, dd), 4.86(2H, d), 3.48(1H, dd), 2.36(1H, dd), 2.16(1H, dd), 1.81(1H, dd), 1.73(4H, d), 1.60(2H, m), 1.40(2H, m), 1.35(2H, m), 1.26(1H, dd), 1.10(3H, s), 1.00(1H, dd), 0.85(4H, s), 0.81(3H, s), and 0.77(3H, s) (Fig. **30**)

^{13}C-NMR spectrum ($CDCl_3$, 200 MHz) δ (ppm) 141.5(d), 135.5(d), 132.7(s), 110.6(t), 80.3(d), 78.1(s), 76.4(s), 60.2(d), 53.6(d), 41.6(t), 39.8(t), 39.2(s), 33.5(q), 27.6(t), 23.5(t), 21.6(q), 18.5(t), 17.9(q), 15.6(q), and 11.9(q) (Fig. **31**)

EI MS spectrum $m/z(70\ eV)$: 306 [M^+], 288, 207,189, and 150 (Fig. **29**)

3.7 Purification and properties of the compounds eluted from column chromatography of ethylacetate crude extract.

The spectral data [^1H and ^{13}C NMR spectra] of ethylacetate crude extract were similar to those of hexane crude extract. Purification by column chromatography give compound 2 (0.80 g, 0.013 %)

3.8 Biological evaluation

Samples were also tested for cytotoxic activity towards 6 cell lines, which were HS 27 (fibroblast), HEP-G2 (hepatoma), SW 620 (colon), CHAGO (lung), KATO-3 (gastric) and BT 474 (breast) following the experimental method of bioassay of cytotoxic activity using by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method. [28]

3.9 Biological assay

3.9.1 Cytotoxicity test

Bioassay of cytotoxic activity against 6 cell lines, which contain P388 (leukemia), SW 620 (colon), KATO (gastric), BT 474 (breast), HEP-62 (hepatoma), HS 27 (fibroblast), *in vitro* was performed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphyltetrazolium bromide) colorimetric method. [28] In principle, the viable cell number/well is directly proportional to the formazan production, that following

solubilization, can be measured spectrophotometrically.

The 6 cell lines were harvested from exponential-phase maintenance cultures (T-75 cm^2 flask; Falcon Plastics 3023), counted by trypan blue exclusion, and dispensed within replicate 96-well culture plates (Falcon Plastics 3075) in 100-(μl) volumes using a repeating pipette. Following a day (24 hrs) incubation at 37 °C, 5% CO_2, 100% relative humidity, 100 μl of culture medium, the culture medium, containing the sample, was dispensed within appropiate wells. (control group, N=6, each sample treatment group, N=3) Peripheral wells of each plate (lacking cells) were used for sample blank (N=2) and medium/tetrazolium reagent blank (n=6) "background" determination. Culture plates were directly incubated for 4 days prior to the tetrazolium reagent additions. MTT stock solution was prepared as followes: 5 mg MTT/ml PBS was sterile and filterd with 0.45 μm filtered units. MTT working solution was prepared just prior to culture application by diluting MTT stock solution 1:5 (v/v) in pre-warmed standard culture medium. MTT working solution (50 μl) was added to each culture well resulting in 50 μg MTT/250 μl total medium volume) and cultures were incubated at 37 °C for 4 to 24 hrs depending upon the individual cell line requirement. Incubation cell monolayers and formazan were then inspected muscopically: culture plates containing suspension lines or any detached cells have been centrifuged at low speed for 5 minutes. All 10-20 μl of culture medium supernatant was removed from wells by slow inspiration through a blunt 18-gauge needle and replaced with 150 μl of DMSO using a pipette. As the formazan solubilization, the measurement of each well absorbance has been investigated by using a multiculture plate reader at 540 nm (single wavelength, calibration factor = 1.00)

Cell line growth and growth inhibition were expressed in terms of mean (pm 1 SD) absorbance units and /or percentage of control absorbance (pm 1 SD%) following by subtraction of mean "background" absorbance.