CHAPTER III

EXPERIMENTAL

1. Source of Plant Material

The stem bark of *Croton roxburghii* N.P. Balakr was collected from Nahaew district, Loei province, Thailand.

The plant material was authenticated by comparison with the voucher specimen No. BKF 084729, deposited in the herbarium of the Royal Forest Department, Bangkan, Bangkok, Thailand.

2. General Techniques

2.1 Analytical Thin Layer Chromatography (TLC)

Technique : One dimension, ascending

Adsorbent : Silica gel 60 F_{254} precoated plate (E. Merck)

Layer thickness : 0.2 mm.

Developing distance: 6.0 cm.

Temperature : Laboratory room temperature (30-35 °C)

Detection : 1. Ultraviolet light at wavelength of 254 nm.

2. Iodine vapour

2.2 Column Chromatography

2.2.1 Conventional Column Chromatography

Adsorbent : 1. Silica gel 60 (No. 7734) (E. Merck)

Particle size 0.063- 0.200 nm. (70-230 mesh ASTM)

2. Silica gel 60 (No. 9385) (E. Merck)

Particle size 0.040- 0.063 nm. (230-400 mesh ASTM)

Packing method: Wet packing

Sample loading : The sample was dissolved in a small amount of eluent, then applied gently on top of the column.

Detection: Fractions were examined using TLC technique. In order to detect the compounds in each, the TLC plate was observed under UV light at wavelength of 254 nm and then exposed to iodine vapour.

2.2.2 Flash Column Chromatography

Adsorbent : 1. Silica gel 60 (No. 7734) (E.Merck)

Particle size 0.063- 0.200 nm. (70-230 mesh ASTM)

2. Silica gel 60 (No. 9385) (E.Merck)

Particle size 0.040- 0.063 nm. (230-400 mesh ASTM)

Packing method: Wet packing

Sample loading : The sample was dissolved in a small amount of eluent, then applied gently on top of the column.

Detection : Fractions were examined in the same manner as described in section 2.2.1

2.3 Spectroscopic Techniques

2.3.1 Ultraviolet (UV) Absorption Spectra

UV spectra were obtained on a Shimadzu UV-160A UV/VIS spectrophotometer at the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2.3.2 Circular Dichroism (CD) Absorption Spectra

CD spectra were obtained on a Jasco spectrophotometer at the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2.3.3 Infrared (IR) Absorption Spectra

IR spectra were recorded on a Perkin-Elmer FT-IR 1760X spectrometer at the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2.3.4 Mass Spectra (MS)

Electron Impact Mass Spectra (EIMS) of isolated compounds were obtained on a Micromass Platform II mass spectrometer at 70 eV. at the Department of Medicinal Organic Chemistry, Faculty of Pharmaceutical Sciences, Chiba University, Chiba, Japan.

2.3.3 Nuclear Magnetic Resonance (NMR) Spectra

^{1}H NMR spectra and ^{13}C NMR spectra of isolated compounds were recorded at 400 and 100 MHz respectively, on a JEOL JMN (Alpha series) Spectrometer at the Department of Medicinal Organic Chemistry, Faculty of Pharmaceutical Sciences, Chiba University, Chiba, Japan.

2D- NMR spectra of isolated compounds were recorded either on a JEOL JMN A500 (Alpha series) 500 MHz NMR Spectrometer at the Scientific and Technological Research Equipment Center, Chulalongkorn University.

Deuterated chloroform was used as the NMR solvent throughout this study. Spectral data were reported in ppm scale using the solvent chemical shift as the reference frequency.

2.4 Physical Property Measurement Apparatus

2.4.1 Melting Points

Melting points were determined on a Gallenkamp Melting Point Apparatus at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2.4.2 Optical Rotations

Optical rotations were measured on a Perkin-Elmer Polarimeter model 341 at the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2.4.3 Elemental components

Elemental components were analyzed on a Perkin-Elmer PE2400 Series II CHNS/O Analyzer (option CHN) at the Scientific and Technological Research Equipment Center, Chulalongkorn University, by the method of pyrolysis in high-purity oxygen (static-state oxidation) and quantitatively detected by thermal conductivity detector.

2.5 Solvents

Organic solvents used in extraction were of commercial grade. For column chromatography, solvents were redistilled prior to use.

3. Extraction and Isolation

3.1 Extraction of the stem bark

The dried, powdered stem bark of *Croton roxburghii* N.P. Balakr (2 kg.) was macerated twice with hexane (2 x 2 L), then with ethyl acetate (2 x 2 L) and acetone (2 x 2 L) successively, for three days each. The obtained extract was evaporated under reduced pressure at a temperature of approximately 40°C to give 213.7 g of hexane extract (10.69 % w/w) , 48.4 g of ethylacetate extract (2.42 % w/w) and 52.4 g of acetone extract (2.62 % w/w) (Scheme 1).

3.2 Isolation

3.2.1 Isolation of compound C-1

The crude hexane extract (10 g) was chromatographed on a conventional silica gel column (silica gel 60, No.7734, 100 g), eluted initially with hexane and increasing the polarity of the eluent by gradnally adding ethyl acetate to 100%, to yield 36 fractions of 50 ml each. The fractions that showed similar TLC patterns were combined together, then evaporated to give 6 major fractions. Compound C-1 (3.85 g), was obtained as crystals from fraction A-02. These crystals from fraction A-02 were further purified by recrystallization in ethyl acetate to yield compound C-1 (2.03 g , 2.16 %w/w) (Scheme 2).

Table 4. Combined fractions from column chromatography of the crude hexane extract (10 g)

Eluants	Fraction code	Number of fraction	Weight(g)
100% hexane	A-01	1-6	0.92
5% EtOAc in hexane	A-02	7-12	3.85***
10% EtOAc in hexane	A-03	13-18	0.86
20% EtOAc in hexane	A-04	19-24	1.53
40% EtOAc in hexane	A-05	25-30	0.81
100% EtOAc	A-06	31-36	1.94

*** = compound C-1

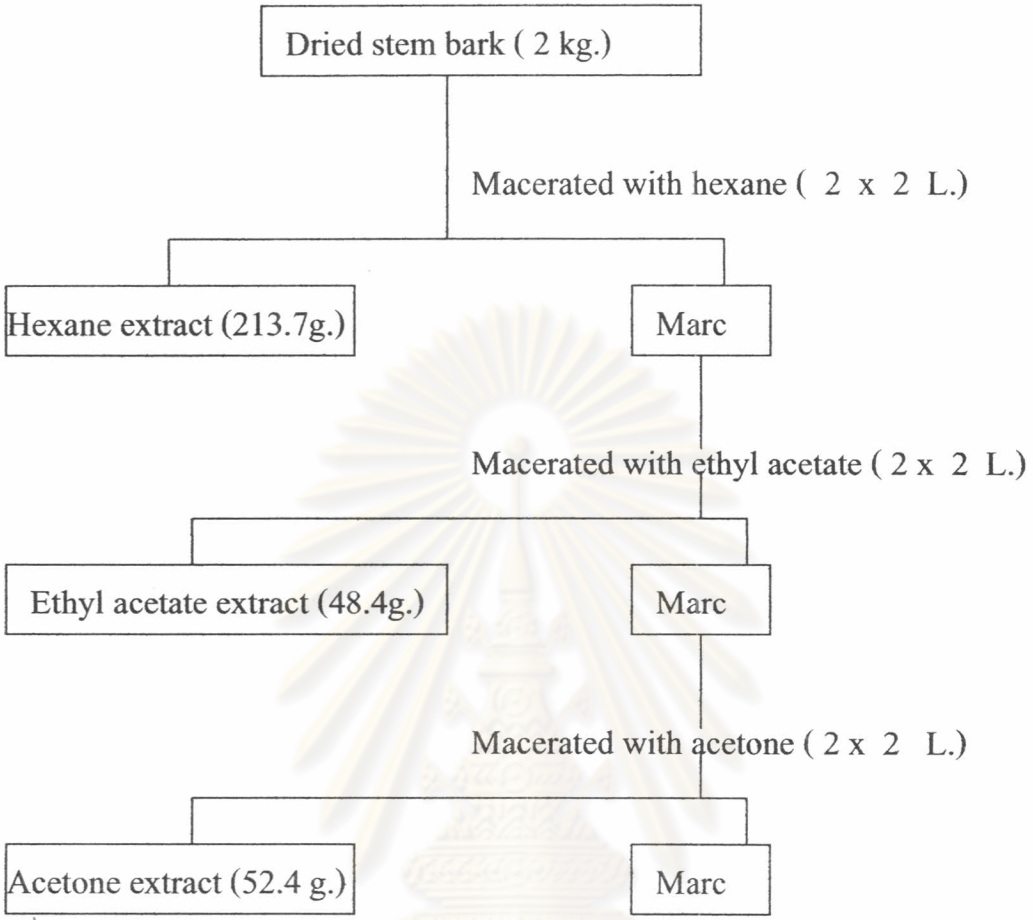
3.2.2 Isolation of compound C-2

Fraction A-04 (1.53 g) was further chromatographed on a silica gel 60 (No.7734) column and eluted with 5% ethyl acetate in hexane. Fractions (approximately 50 ml each) were collected and combined according to their TLC patterns to yield a total of seven fractions (B-01 - B-07), as shown in Table 5. Compound C-2 (1.25 g), was obtained as crystals from fraction B-05. These crystals from fraction B-05 were further purified by recrystallization in ethyl acetate to yield compound C-2 (1.02 g , 1.08 %w/w) (Scheme 2).

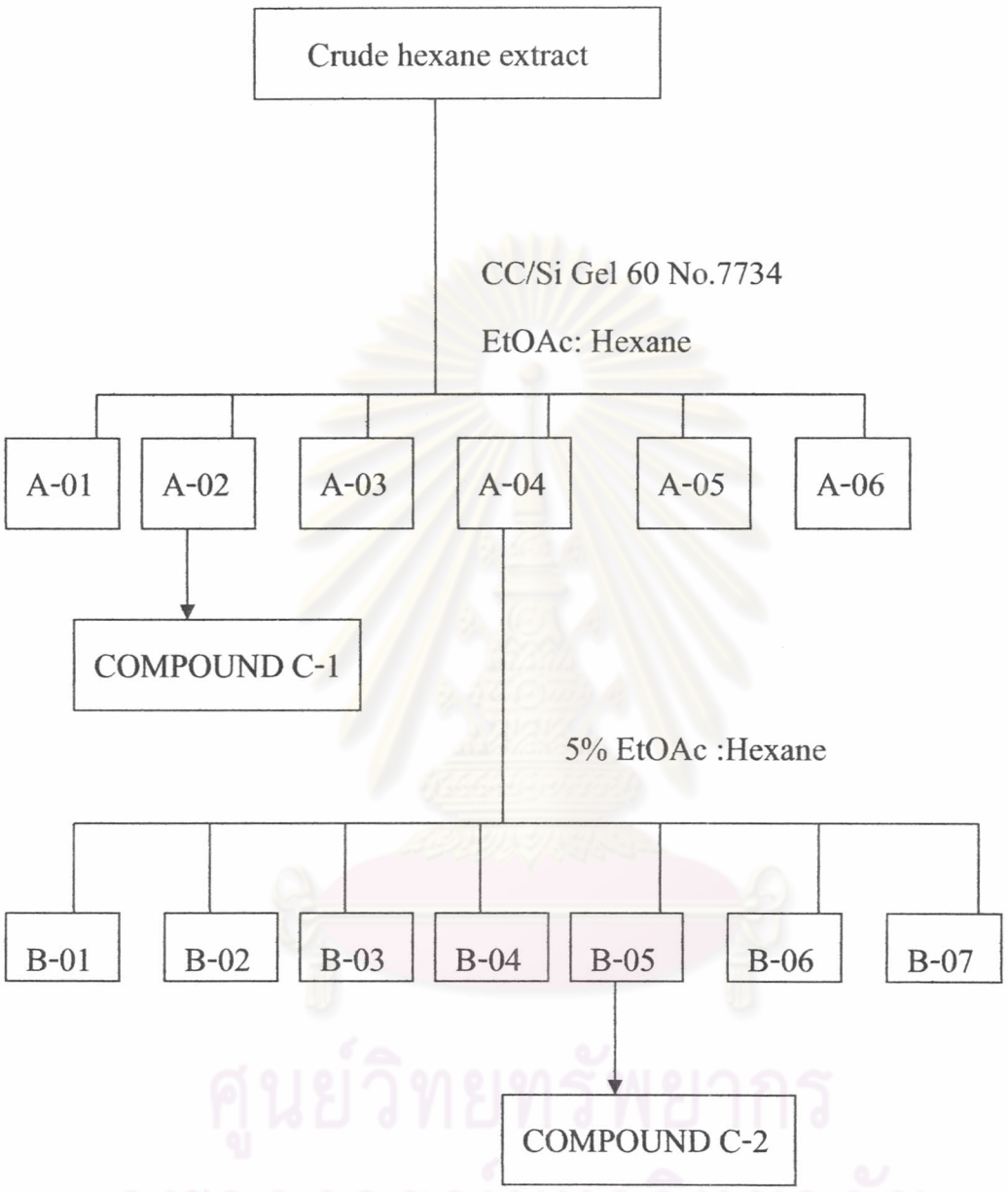
Table 5. Combined fractions obtained from column chromatography of fraction A-04

Fraction code	Weight (g)
B-01	-
B-02	0.01
B-03	0.02
B-04	0.23
B-05***	1.25
B-06	0.01
B-07	-

*** = compound C-2



Scheme 1: Extraction of the stem bark of *Croton roxburghii* N.P. Balakr.



Scheme 2: Isolation of the hexane extract of *Croton roxburghii* N.P. Balakr.

Physical and Spectral Data of the Isolated Compounds

1. Compound C-1

Compound 1 was obtained as colourless prism crystals (2.03 g)

Elemental analysis: C:H:O = 70.98: 27.42: 1.60

Melting point : 147 – 148 °C

$[\alpha]^{20}_{D}$: - 26 °[$CHCl_3$; c 0.50]

UV : λ_{max} nm (log ε), in MeOH; Figure 10.

227 (4.02)

CD : 342.6, -260.6 ; Figure 11

IR : ν_{max} cm^{-1}, KBr disc; Figure 12.

3025, 3075, 2960, 2930, 2860, 1655, 1615, 994, 913, 825

EIMS : *m/z* (% relative intensity); Figure 13.

302 [M^+] (2), 287 (100), 269 (5), 204 (13), 189 (15).

^{1}H-NMR : δ ppm, 400 MHz, in $CDCl_3$; Figure 14.

7.10 (1H, d, *J*=10.0 Hz), 5.89 (1H, dd, *J* = 16.0, 10.0 Hz)

5.86 (1H, d, *J*=10.0 Hz), 5.15 (1H, dd, *J* =18.0, 2.0 Hz), 4.94

(1H, dd, *J* =10.0, 2.0 Hz), 1.92 (1H, m), 1.90 (1H, m), 1.84

(1H, m), 1.78 (1H, m), 1.72(1H, m), 1.70 (2H, m), 1.62 (1H,

m), 1.53 (1H, m), 1.52 (1H, m), 1.38 (3H, s) 1.32(3H, s),

1.16 (3H, s), 1.08 (3H, s), 1.05 (3H, s).

^{13}C -NMR : δ ppm, 100 MHz, in $CDCl_3$; Figure 15.

205.1 (s), 157.6 (d), 147.4 (d), 125.8 (d), 110.7 (t), 75.0 (s), 73.7 (s), 53.2 (d), 49.9 (d), 44.6 (t), 42.3 (s), 39.4 (s), 35.4 (t), 28.6 (q), 27.6 (q), 25.6 (q), 21.3 (q), 20.2 (t), 18.6 (q), 15.5(t).

2. Compound C-2

Compound 2 was obtained as long prism crystal (1.02 g)

Elemental analysis: C:H:O = 71.88: 27.16:0.96

Melting point : 141 - 142 °C

$[\alpha]^{20}_{D}$: - 32.4 °[$CHCl_3$; c 0.50]

UV : λ_{max} nm (log ε), in MeOH ;Figure 20.

228 (3.98)

CD : 342.6, -260.8 ; Figure 21.

IR : ν_{max} cm^{-1}, KBr disc; Figure 22.

3521, 3086, 2925, 1660, 1463, 1156, 1082, 992, 915, 828

EIMS : *m/z* (% relative intensity); Figure 23.

318 [M^+] (2), 300 (9), 248 (52), 205 (93), 189 (!00).

^{1}H-NMR : δ ppm, 400 MHz, in $CDCl_3$; Figure 24.

7.13 (1H, d, *J* =10.0 Hz), 5.88 (1H, d, *J* = 10.0 Hz), 5.81(1H, dd, *J* = 18.0, 12.0 Hz), 5.44 (1H, dd, *J* =18.0, 2.5 Hz), 5.26 (1H, dd, *J* =12.0, 2.5 Hz), 3.80 (1H, q, *J* = 2.5 Hz), 2.09(1H, *J* =10.0, 6.0 Hz), 1.98 (1H, m), 1.94 (1H, m), 1.92 (1H, m), 1.85 (1H, dd, *J* =10.0, 5.0 Hz), 1.72 (1H, dd, *J* = 13, 3 Hz),

1.58 (1H, m), 1.52 (1H, m), 1.39 (3H, s), 1.38 (3H, s), 1.16 (3H, s), 1.09 (3H, s), 1.04 (3H, s).

^{13}C -NMR : δ ppm, 100 MHz, in $CDCl_3$; Figure 25.

205.0 (s), 157.3 (d), 142.5 (d), 125.9 (d), 115.8 (t), 76.7 (s), 75.3 (s), 70.0 (d), 53.2 (d), 44.6 (s), 43.5 (d), 41.5 (t), 38.7 (s), 27.6 (q), 27.4 (q), 25.3 (q), 22.9 (t), 21.2 (q), 20.1 (t),18.9(q).

4. Biological activity test

4.1 Cytotoxicity test

Cytotoxicity test was carried out at the Institute of Biotechnology and Genetic Engineering. Bioassay of cytotoxic activity against human tumor cell culture *in vitro* was performed by the MTT (3-(4,5-dimethylthaizol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method [Carmichael *et al.*, 1987; Twentyman and Luscombe, 1987]. In principle, the viable cell number/well is directly proportional to the production of formazan which, following solubilization, can be measured spectrophotometrically.

The human tumor cell line was harvested from exponential-phase maintenance culture (T-75 cm^2 flask), counted by trypan blue exclusion, and dispensed within replicate 96-well culture plates in 100 μl volumes using a repeating pipette. Following a 24-h incubation at 37°C, with 5%CO_2 , 100% relative humidity and 100 μl of culture medium. Culture medium containing sample was dispensed within appropriate wells (control group, N=6; each sample treatment group, N=3). Peripheral wells of each plate (lacking cells) were utilized for sample blank (N=2) and medium/tetrazolium reagent blank (N=6) " background" determinations. Culture plates were then incubated for 4 days prior to the addition of tetrazolium reagent. MTT stock solution was prepared as follows: 5 mg MTT/ml PBS was steriled and filtered 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 prewarmed 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 h depending upon individual cell line requirements. Following incubation, cell monolayers and formazan were inspected microscopically: culture plates containing suspension lines or any detached cells were centrifuged at low speed for 5 min. All 10-20 μl of culture medium supernatant was removed from wells by slow aspiration through a blunt 18-guage needle and replaced with 150 μl of DMSO using a pipette. Following through formazan solubilization, the absorbance of each well was measured using a microculture 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 subtraction of mean "background" absorbance.

Samples were also tested for cytotoxic activity towards 6 cancer cell lines, including HS27 (fibroblast), HEP-G2 (hepatoma), SW 620 (colon), Chago (lung), Kato-3 (gastric) and BT 474 (breast), following the experimental method for bioassay of cytotoxic activity.