CHAPTER III

EXPERIMENTAL

Instruments and Equipments

1. Fourier Transform-Infrared spectrophotometer (FT-IR)

IR spectra were recorded on a Nicolet Impact 410 Spectrophotometer. Spectra of solid samples were recorded as KBr pellets. Liquid samples were recorded as thin films on NaCl cell.

2. Mass Spectrometer (MS)

Low resolution mass spectra were obtained with a Fison Instruments Mass Spectrometer model Trio 2000 at 70 eV.

3. Nuclear Magnetic Resonance Spectrometer (NMR)

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

4. Optical Rotation

The optical rotation spectra were recorded on a Perkin-Elmener 341 polarimeter.

5. X-ray Diffractometer

The X-ray diffractometer were obtained on a SIEMEN SMART diffractometer at Department of Physics, Faculty of Science, Thammasart University.

Chemicals

All commercial grade solvents were distilled prior to use.

Silica gel (Merck Kiesel gel 60) and silica TLC plate (Silica gel $60F_{254}$) were purchased from Merck Company.

Plant Materials

The plant material of *Croton robustus* Kurz. used in this study was collected from amphur Nakhon Thai, Pitsanulok province, Thailand. The plant specimen was identified by comparison with the herbarium specimen No. 022771 in the Royal Forest Department, Ministry of Agriculture and Cooperation, Bangkok, Thailand.

Extraction and Isolation

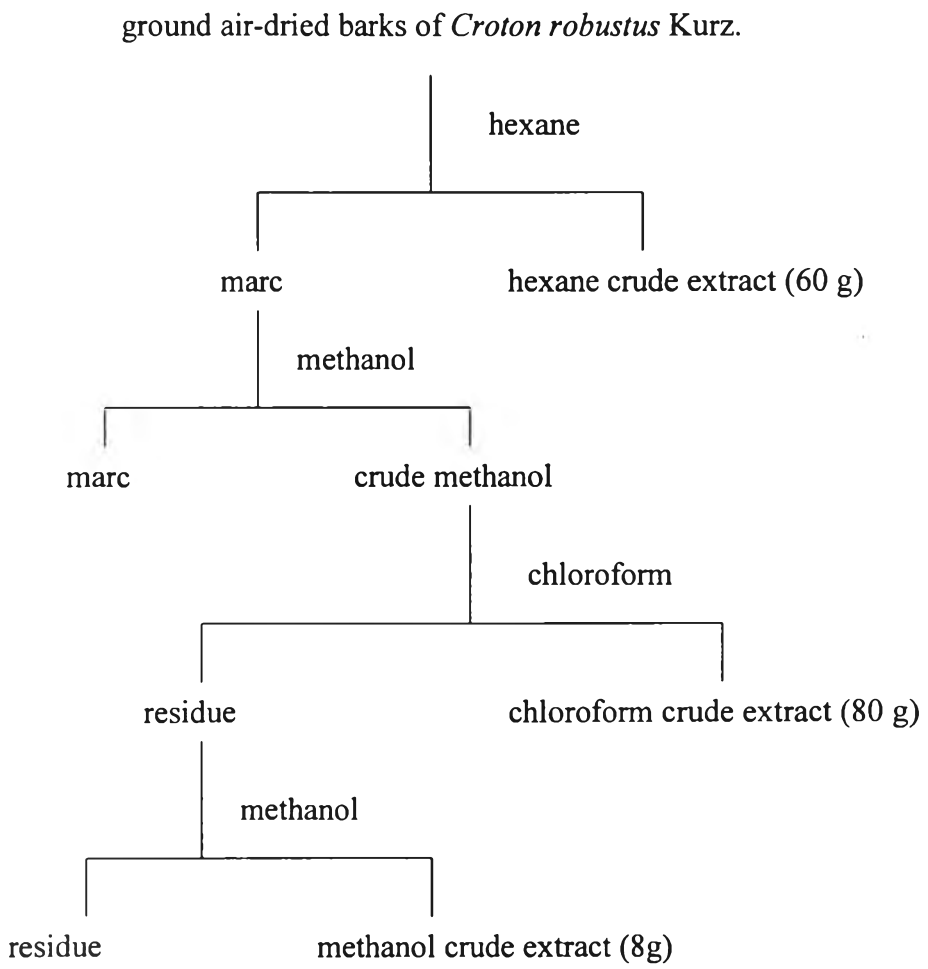
The powdered, sun-dried stem barks (6 Kg) of *Croton robustus* Kurz. was extracted with hexane. The hexane extract was filtered and evaporated under reduced pressure to obtain a dark yellow oil residue. Sun-dried stem barks were extracted again with methanol, then the methanol extract was filtered and evaporated to obtain a dark–red gummy residue, which was repeatedly extracted with hexane, chloroform, methanol respectively.

Their different crude extracts of the stem barks of *C. robustus* are shown in Table 2.

Table 2 The various extract of the stem barks of *C. robustus*.

Solvent extract	Appearance	Weight (g)	% wt. by wt. of the dried stem bark
Hexane	dark-yellowed oil	60	1.00
Chloroform	dark-red oil	80	1.33
Methanol	dark-red gummy	8	0.13

The procedure and results of extraction are shown in Scheme 1.



Scheme 1 The procedure of extraction of the stem bark of *Croton robustus* Kurz.

Isolation of Crude Extract of *C. robustus*

-Separation of hexane crude extract.

The hexane crude extract was obtained as a dark-yellowed oil (60 g) after evaporation. The crude hexane extract (40 g) was fractionated by silica gel column chromatography using Merck's silica gel Art. 7734.1000 (70-230 mesh ASTM) as absorbent. The column was eluted with hexane-chloroform gradient in a stepwise fashion to give compound 1, 2 and 3 respectively.

-Separation of chloroform crude extract.

The chloroform crude extract (80 g) was separated on Silica gel 70-230 mesh ASTM using column chromatography technique. The column was eluted with hexane, hexane-ethyl acetate, ethyl acetate, ethyl acetate-methanol, respectively. From ^{1}H, ^{13}C-NMR [Fig. 44, 45] and TLC, the eluted fraction gave similar compound found in hexane crude extract.

-Separation of methanol crude extract.

The methanol crude extract was obtained as gummy residue (8g). It was dissolved in all solvent then crude methanol could not be purified.

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

Purification and properties of Compound 1

Compound 1 was eluted with 5% chloroform in hexane. Similar fractions were combined and the solvent was removed by rotary evaporation. It was further purified by column chromatography (Merck's silica gel Art. 1.09385.1000) the column was eluted with 5% chloroform. It is soluble in chloroform, ethyl acetate.

Compound 1 is a white solid (300 mg, 0.03 % wt. by wt. of hexane crude extract). mp. 128-131 °C. $[\alpha]_D^{20}$ –73.1°($CHCl_3$; *c*1.0).

This compound has not absorption peak in UV absorption.

FT-IR spectrum (Fig.17, Table 3) ν_{max} (cm^{-1}) : 3300-2400 (br), 1695 (h), 1460 (m) and 1265 (m)

^{1}H-NMR spectrum ($CDCl_3$, 500 MHz.)(Fig.18, Table 4)

δ (ppm) : 0.58(1H,dt), 0.78(1H,dd), 0.82(1H,dd), 0.88(3H,s), 0.99(1H,ddd), 1.0(1H,dd), 1.08(1H,br), 1.14(3H,s), 1.19(1H,m),1.21(3H,s),1.23(1H,d), 1.32(1H,dt), 1.39(1H,d), 1.45(1H,dt), 1.67(1H,ddd), 1.72(1H,ddd), 1.75(1H,dt), 1.84(1H,ddd), 1.87(1H,ddd) and 2.12(1H,ddd)

^{13}C-NMR spectrum ($CDCl_3$, 125 MHz.)(Fig.19, Table 4)

δ (ppm) : 184.5(s), 57.0(d), 52.8(d), 50.3(t), 43.7(s), 40.7(s), 39.4(t), 39.2(t), 38.9(s), 37.8(t), 33.1(t), 28.8(q), 24.3(d), 22.4(s), 21.7(t), 20.5(d), 20.5(q), 19.7(t), 18.7(t) and 12.4(q)

m/z (EI) (Fig.21) : 302[M^+], 287(30), 260(12), 257(10), 246(80), 231(30)

Purification and properties of Compound 2

Compound 2 was eluted with 15% chloroform in hexane. Similar fractions were combined and the solvent was removed by rotary evaporation. It was further purified by column chromatography (Merck's silica gel Art.1.09385.1000) the column was eluted with 15% chloroform in hexane. This compound is soluble in chloroform, ethyl acetate and methanol.

Compound 2 was transparent oil (60 mg, 0.03 %wt. by wt. of hexane crude extract). $[\alpha]_D^{20}$ – 128.9°($CHCl_3$; *c*1.0).

FT-IR spectrum (Fig.26, Table 7) ν_{max} (cm^{-1}) : 3400-2400(br), 1697(h), 1446 (m), 1373(m) and 1257(m)

^{1}H-NMR spectrum ($CDCl_3$, 200 MHz.)(Fig.27)

δ (ppm) : 5.0-6.2(5H), 1.5-2.5(m), 1.82(3H,s), 1.62(3H,s) and 0.80-0.84(9H)

^{13}C-NMR spectrum ($CDCl_3$, 50 MHz.)(Fig.28, Table 8)

δ (ppm) : 173.3(s), 147.6(d), 135.1(s), 131.3(d), 130.9(s), 130.4(d), 128.8(s), 127.9(d), 125.7(d), 47.8(d), 38.8(t), 32.7(t), 32.1(d), 28.9(t), 26.2(t), 25.8(t), 20.9(q), 19.9(q), 19.3(q) and 14.4(q)

m/z (EIMS) (Fig.30) : 302[M^+], 259(22), 213(10), 119(22), 107(22), 105(85), 93(80)

Purification and properties of Compound 3

Compound 3 was eluted with 50% chloroform in hexane. Similar fractions were combined and the solvent was removed by rotary evaporation and purified by column chromatography (Merck's silica gel Art. 1.09385.10000). It's soluble in ethyl acetate, chloroform.

Compound 3 is a white solid. (40 mg, 0.0016 %wt. by wt.) mp. = 130-131 °C $[\alpha]_D^{20}$ –42.2° ($CHCl_3$; *c*1.0).

FT-IR spectrum (Fig.31, Table 9) ν_{max} (cm^{-1}) : 3450(h), 2930(s), 2858(s), 1640 (m), 1440(m), 1370(m) and 1035(m)

^{1}H-NMR spectrum ($CDCl_3$, 500 MHz.)(Fig.32, Table 10)

δ (ppm) : 0.54(1H,dt), 0.72(1H,dd), 0.78(1H,dd), 0.86(1H,dd), 0.88(1H,m), 0.89 (3H,s), 0.91(3H,s), 1.08(1H,m), 1.1(3H,s), 1.12(1H,m), 1.13(1H,m), 1.21(1H,d), 1.28 (1H,dd), 1.31(1H,m), 1.35(1H,d), 1.4(1H,dd), 1.48(1H,m), 1.54(1H,m), 1.61(1H,ddd), 1.73(1H,ddt), 1.85(1H,ddd), 2.0(1H,d), 3.39(1H,dd) and 3.69(1H,d)

^{13}C-NMR spectrum ($CDCl_3$, 125 MHz.)(Fig.33, Table 10)

δ (ppm) : 65.6(t), 56.8(d), 53.4(d), 50.3(t), 40.7(s), 39.4(t), 39.3(t), 38.3(s), 38.2(s), 35.7(t), 33.4(t), 26.8(q), 24.2(d), 22.4(s), 20.6(d), 20.5(q), 20.3(t), 19.9(t), 17.8(t) and 15.1(q)

m/z (EIMS) (Fig.35) : 288[M^+], 273(20), 271(5), 257(80), 217(30)

Purification and properties of modification of Compound 1

Methylation of Compound 1

The Compound 1 (200 mg, 0.66mmol) was methylate with diazomethane in ether to give Compound 1a. as a white solid (209.27 mg, 0.66 mmol, 100%) after recystalization from MeOH. mp. 98-100°C $[\alpha]_D^{20}$ –73.8° ($CHCl_3$; *c*1);
This compound has not absorption peak in UV absorption.

FT-IR spectrum (Fig.40) ν_{max} (cm^{-1}) : 2950-2850(br), 1731(s), 1465(m), 1444 (m), 1240(m), 1200(m) and 1150(m)

^{1}H-NMR spectrum ($CDCl_3$, 200 MHz.)(Fig.41)
δ (ppm) : 3.6(3H,s), 2.2-5.5(m), 1.2(3H,s), 1.1(3H,s) and 0.8(3H,s)

^{13}C-NMR spectrum ($CDCl_3$, 50 MHz.)(Fig.41, Table 13)
δ (ppm) : 178.0(s), 57.0(d), 52.7(d), 51.0(s), 50.3(t), 43.7(s), 40.7(s), 39.5(t), 39.2(t), 38.6(s), 38.1(t), 33.1(t), 28.7(q), 24.2(d), 22.4(s), 21.8(t), 20.5(d), 20.5(q), 19.7(t), 18.8(t) and 12.3(q)

m/z (EIMS) (Fig.42) : 316[M^+], 301(20), 284(5), 260(60), 257(50), 256(20), 241(50), 201(15)

The crystal experimental data of compound 1a were given in Table 14, 15, 16 and 17, respectively.

Reduction of compound 1a

Reduction of Compound 1a methyl ester (200 mg, 0.63mmol) in 10 ml of anhydrous diethyl ether was added slowly from a dropping funnel into a stirred solution of lithium aluminum hydride (120.27 mg, 0.38 mmol) in 20 ml of anhydrous diethyl ether in a 50 ml round-bottom flask previously flushed with nitrogen. After the addition was completed, the reaction mixture was stirred for 12 hours at room temperature. The reaction was stopped and worked up as usual manner. The organic layer was concentrated by rotary evaporation and purified by column chromatography (Merck's silica gel Art. 1.09385.1000) and eluted with 50% chloroform in hexane to

obtain Compound 3 as a white solid (150 mg, 0.66mmol, 78.65%). The spectral data (^{1}H and ^{13}C)[Fig 43] of this compound was identical to that of natural material.

X-ray Diffraction

X-ray Diffraction Experiment

A colorless crystal of Compound 1a was recrystallized from methanol. All data were collected at room temperature using graphite monochromated Mo Kα radiation (lamda = 0.71069 A°) on siemens SMART CCD diffractometer. The data were corrected for Lorentz and polarization effects. The crystal experimental data of compound 1a were given in Table 14.

The structure was solved by direct methods using SHELXS-97 and refined by full matrix least-square on F^2 using SHELXLS-97 with anisotropic thermal parameters for all the non-hydrogen atoms. All the hydrogen atoms were found in difference Fourier maps and were included in refinement. The fraction coordinates of non-hydrogen atom and selected bond distances and angles of compound 1a were listed in Table 15, 16 and 17, respectively.

Biological assay

Cytotoxicity Test [15-16]

Bioassay of cytotoxic activity against towards 6 tumor cell lines, which were Hs 27 (fibroblast), Kato-3 (gastric), BT 474 (breast), Chago (lung), SW 620 (colon) and HEP-G2 (hepatoma) culture *in vitro* was performed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method [15-16]. In principle, the viable cell number / well is directly proportional to the production of formazan, which follow solubilization, can be measured spectrophotometrically.

Samples were also tested for cytotoxic activity towards 6 tumor cell lines which were harvested from exponential-phase maintenance cultures (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, 5% CO_2, 100% relative humidity, 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 additions 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 (50µl) 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 (± 1 SD) absorbance units and / or percentage of control absorbance (± 1 SD%) following subtraction of mean "background" absorbance.