CHAPTER II

EXPERIMENTS

2.1 General experimental procedures

All solvents were distilled prior use. Melting points were determined on a Fischer-Johns melting point apparatus and are reported uncorrected. The optical rotation was determined on a Perkin-Elmer 341 polarimeter. UV-VIS spectra were recorded on a Milton-Roy Spectronic 3000 Array UV-VIS spectrophotometer. IR spectra were obtained on a Nicolet Impact 410 Spectrophotometer. Spectra of solid samples were recorded as KBr pellets and liquid samples were recorded as thin films (KBr cells). Low-resolution mass spectra were obtained with a Fisons Instruments Mass Spectrometer model Trio 2000 at 70 eV. ^{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. COSY, NOESY, HMQC and HMBC experiments were performed on the JOEL JNM-A500 Spectrometer. Elemental analyses were measured on a Perkin Elmer PE2400 SERIES II (CHN/ O ANALYSER). Silica gel (Merck Kieselgel 60 and silica TLC plates (Si gel 60 F_{254}) were purchased from Merck Company.

2.2 Chemical Reagents

2.2.1 Solvents

All solvents used in this research such as hexane (C_6H_{14}), 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.

2.2.2 Other chemicals

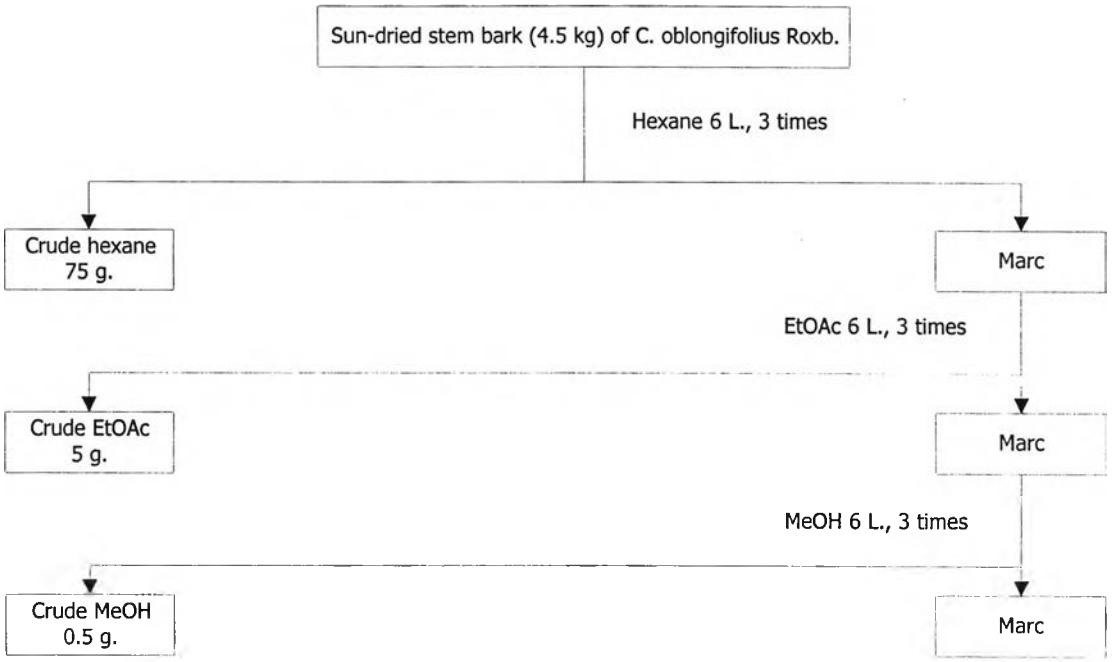
- Merck's silica gel 60 Art. 1.07734.1000 (70-230 mesh ASTM) was used as adsorbent for column chromatography.
- Merck's silica gel 60 Art. 1.09385.1000 (230-400 mesh ASTM) was used as adsorbent for flash column chromatography.
- Merck's silica gel 60G Art. 1.07731.1000 and $60GF_{254}$ Art. 1.07730.1000 were applied as adsorbent for preparative TLC.
- 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.
- Sephadex LH-20 was used as a stationary phase for column chromatography. The gel filter was dispersed in the eluting solvent and left standing for gel initiation for about 24 hr. before use.

Plant material

The stem bark sample of *C. oblongifolius* used in this study was collected from Amphoe Pranburi, Prachuabkhirikhan Province, Thailand in June 1999. The botanical identification of the plant was determined by comparing with the voucher specimen No. 084729 in the herbarium collection of the Royal Forest Department of Thailand.

2.3 Extraction and Isolation

The powdered, sun-dried stem bark (4.5 kg) of *C. oblongifolius* was extracted with hexane 6 L for 3 times. The hexane extract was filtered and evaporated in vacuo to obtain a yellowish-green oil (75 g). The remaining portion of the powdered stem bark was re-extracted with MeOH. The marc was repeatedly re-extracted with hexane, chloroform, ethyl acetate and methanol crude respectively. The extraction procedures are shown in Scheme 1.



Scheme 1. The extraction procedures of stem bark of *C. oblongifolius* Roxb.

2.4 Separation of hexane crude extract

The hexane extrac (75 g.) was subjected to column chromatography (silica gel, 950 g.) using elutents of increasing polarity from hexane to ethylacetate. The results from the separation of hexane crude extract were tabulated in Table 1.

Table 1. The results from separation of hexane crude extract

Eluents	Fraction No.	Appearance	Weight (g.)
100% Hexane	1-4	Colorless liquid	6.2
100% Hexane	5-8	Yellow liquid	3.3
10% EtOAc in Hexane	9-24	Yellow viscous liquid (Containing compound 1)	10.3
20% EtOAc in Hexane	25-36	Yellow viscous liquid (Containing compound 2)	8.4
30% EtOAc in Hexane	37-46	Yellow viscous liquid (Containing compound 3)	5.6
50% EtOAc in Hexane	47-56	Yellow viscous liquid	4.4
70% EtOAc in Hexane	57-63	Yellow viscous liquid	3.2
100% EtOAc in Hexane	64-70	Brown viscous liquid	0.7
1% MeOH in EtOAc	71-74	Brown viscous liquid	5.6
5% MeOH in EtOAc	75-79	Dark brown gummy	4.3
10% MeOH in EtOAc	80-87	Dark brown gummy	2.6

Biological evaluation. Bioassay of cytotoxic activity against human tumor cell culture in vitro was performed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] colorimetric method [14, 16, 17].

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

2.5.1 Purification and properties of Compound 1

Compound 1 was eluted with pure hexane. Similar fractions were combined and the solvent was removed by rotary evaporation. In order to remove the non-acid part, 100 ml diethyl ether was first added to the combined crude fractions in a separating funnel. Next 50 ml of 10% NaOH was added then shook and the layer of NaOH was separated and acidified with 5% HCl. The acid part was precipitated and washed with water 2-3 times after being purified by column chromatography (Merck's silica gel Art. 1.09385.1000). It is soluble in hexane, dichloromethane, chloroform, ethyl acetate, diethyl ether, and methanol.

2.5.2 Purification and properties of Compound 2

Compound 2 was in an organic solvent layer (diethyl ether) after being separated from the acid part and washed by water until neutral. Then the solvent was removed by rotary evaporation and the non-acid crude was purified by column chromatography (Merck's silica gel Art. 1.09385.1000). This compound is soluble in dichloromethane, chloroform, ethyl acetate, diethyl ether, and methanol.

2.5.3 Purification and Properties of Compound 3

Compound 3 was eluted with 5% ethyl acetate in hexane. Similar fractions were combined and the solvent removed by rotary evaporation and further purified by column chromatography (Merck's silica gel Art. 1.09385.1000). This compound is soluble in dichloromethane, chloroform, ethyl acetate, diethyl ether, and methanol.

2.6 Derivative of Compound 2

2.6.1 Coupling of compound 2 with 2,4-dinitrophenylhydrazine

Compound 2 (1 g, 3.497 mmol) was coupled with 2,4-DNP in MeOH under acid conditions described earlier [17,18]. The reaction product was purified with 25% ethyl acetate in hexane to give Compound 2a as an orange-red crystaline (670 mg, 67% yield).