CHAPTER 2

EXPERIMENT

2.1 Plant material

The barks of *Carallia brachiata* Merr. were purchased from Vetchapong-osot, a Thai medicinal plant shop, Bangkok, Thailand, in April,1999. The specimen of this plant was compared with a voucher number BKF 127280 at the herbarium of Royal Forest Department, Ministry of Agriculture and Cooperative, Bangkok, Thailand.

2.2 Equipments

2.2.1 Rotary evaporator

The Buchi rotary evaporator was used to evaporate the large amount of all volatile organic solvents.

2.2.2 Melting point apparatus (m.p.)

Melting points were determined with Fisher-John Melting Point apparatus and the values were uncorrected.

2.2.3 Fourier Transform-Infrared Spectrophotometer (FT-IR)

Infrared spectra were recorded on NICOLET IMPACT 410 FT/IR spectrophotometer.

2.2.4 ^{1}H and ^{13}C – Nuclear Magnetic Resonance Spectrometer

NMR experiments were carried out with a JEOL JNM-A 500 FT-NMR spectrometer and a Bruker AC-F 200 FT-NMR spectrometer. The chemical shift in δ(ppm) was assigned with reference to the signal from the residual proton in deuterated solvent and using TMS as an internal standard in some cases.

2.2.5 Mass Spectrometer

EIMS was obtained on Mass Spectrometer Model VG Trio 2000 and FABMS was performed on JEOL JMS SX-102/SX-102, using *p*-nitrobenzyl alcohol as a metrix.

2.2.6 Chromatotron equipment

Chromatotron equipment on Harrison Research Model 7924 T was operated for certain separation.

2.2.7 UV-visible spectrometer

UV-visible absorbance were obtained on UV-VIS Hewlett Packard 8452 A diode array spectrophotometer.

2.2.8 pH meter

pH values were determined with 744 pH meter Ω Metrohm Ion analysis.

2.2.9 Gas Chromatography

Steroid analysis was carried out by SHIMADZU GC-7AG and performed at Scientific and Technology Research Equipment Center, Chulalongkorn University.

2.3 Chemicals

Most solvents used in this research were commercial grade and were distilled prior to use. For crystallization, reagent grade solvents were used. Absorbents such silica gel 60 Merck, cat. No. 7734, 7749, 7731 and 9815 were used for column chromatography, chromatotron, preparative thin layer chromatography (PTLC) and flash column chromatography, respectively. TLC was performed on precoated Merck silica gel 60 F_{254} plates (0.25 mm thick layer).

2.4 Dipping Reagent

10% Sulfuric acid in ethanol which was used for detecting spots of compounds, this reagent was also used to detect certain functional groups or class of compounds.

2.5 Bioassay Procedures

2.5.1 Scavenging effects on DPPH radicals

2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH) radical is a stable radical with a purple colour (λ_{max} 517 nm). Upon reduction by a scavenger, the extensive conjugation is disrupted and the compound turns yellow.

- TLC autographic assay[27]

After delveloping and drying, TLC plates were sprayed with a 0.2% DPPH in methanolic solution. The plates were examined 5 minutes after spraying. Active compounds appeared as yellow spots on a purple background.

- Spectrophotometric assay[28]

Sample at various concentration (0.5 ml) were added to a 1 ml methanolic solution of DPPH radical (final concentration of DPPH was 0.2 mM). The mixture was shaken vigorously and then left for 30 minutes ; the absorbance of the resulting solution was measured at 517 nm with a spectrophotometer. All tests and analyses were run in three replicates and averaged.

$$\% \text{ Radical scavenging} = (1 - A_{sample}/A_{blank}) \times 100$$

2.5.2 Superoxide dismutase(SOD) activity[29]

The assay for superoxide dismutase activity was performed by using the method of Okamura et al. with some modification. Superoxide anion radical was induced by the action of xanthine oxidase with xanthine as the substrate. The sample solutions (0.05 ml) were prepared at various concentrations in DMSO were added to the mixture (0.5 ml) consisting of 0.4 mM xanthine and 0.24 mM nitrobluetetrazolium (NBT) in 0.1 M phosphate buffer (pH 8.0). Xanthine oxidase (0.049 unit/ml) diluted in 0.1 M phosphate buffer (pH 8.0) 0.5 ml was added, followed by incubation at 37 °C for 20 min. The reaction was stopped by adding 2 ml of 69 mM sodium dodecyl sulfate (SDS) and the coloration of NBT was measured at 560 nm.

$$\text{SOD activity } (\%) = (1 - A_{sample}/A_{blank}) \times 100$$

2.5.3 Xanthine Oxidase Inhibition Assay[8]

The assay was performed according to the method of Noro et al. A mixture consisting of xanthine oxidase (0.049 unit/ml, 0.04 ml), 0.1 M phosphate buffer (pH 7.5, 0.36 ml) and a test compound solution in 10% DMSO in H_2O (0.2 ml) was preincubated 10 min at 37 °C. Then xanthine solution (0.1 mM in phosphate buffer pH 7.5, 0.6 ml) was added and the mixture was incubated at 37 °C for 30 min. The enzyme reaction was terminated by adding 1 M HCl (0.2 ml). The absorbance of reaction mixture was measured at 290 nm. Xanthine oxidase inhibitory activity was expressed as (1 – A/B) x 100, where A and B are the activities of the enzyme without and with the test material, respectively.

2.5.4 Ferric thiocyanate assay[30]

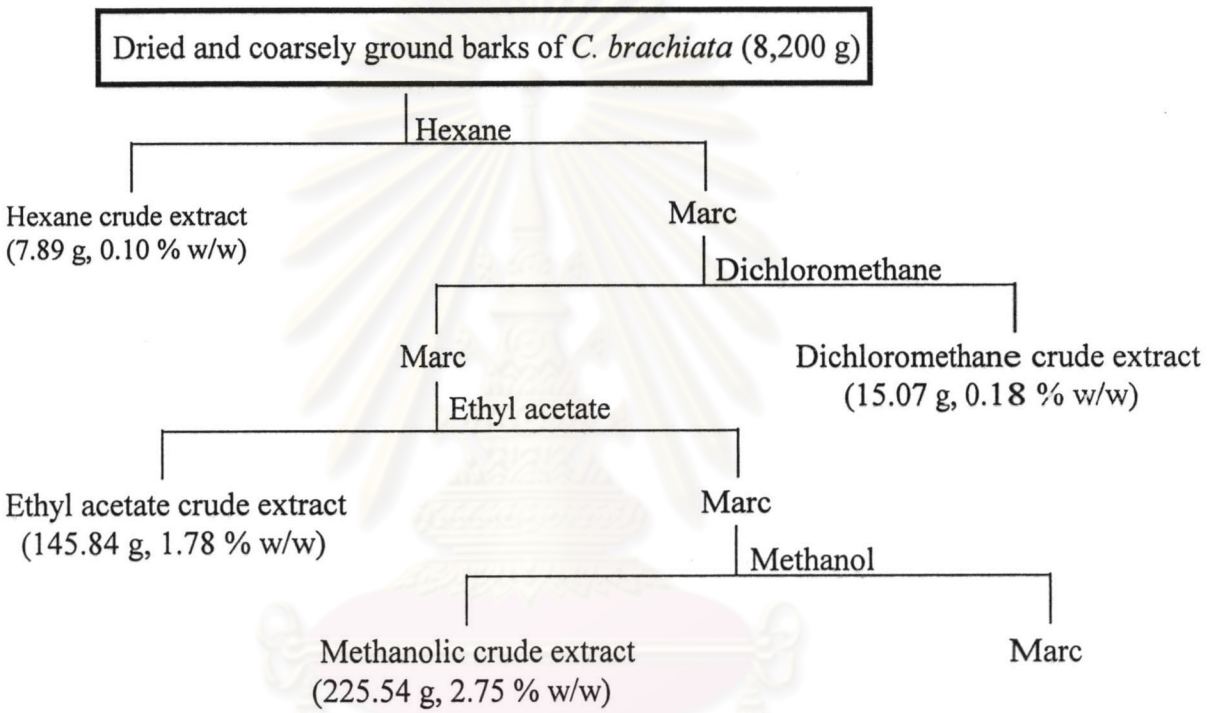
This assay was modified from the method of Asamari et al. First, linoleic acid pre-emulsion was prepared by mixing 1.5 ml of linoleic acid with 1.5 ml of emulsifier in 100 ml of 30% (v/v) ethanol. Sample in various concentration (0.5 ml) were added to 5 ml of pre-emulsion, and the final volume was adjusted to 12.5 ml with deionized water. The mixed solutions were incubated at 40 °C. The assay was conducted by adding 5 ml of 75% ethanol, 0.1 ml of ammonium thiocyanate solution (30% w/v) and 0.1 ml of ferrous chloride (0.1% w/v) to 0.1 ml of incubated mixture after 4 and 8 hours. The activity of sample was calculated as the absorbance difference at 500 nm between emulsions with and without sample. A negative value demonstrated as an antioxidant activity of sample.

2.5.5 The Inhibitory Effect for Tumer Cell Lines[31]

Some pure compounds from the barks of *C. brachiata* were tested with KB cell lines (Human Nasopharyngal Carcinoma) by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. This assay was investigated by Natural Products Research Section, National Cancer Institute, Thailand.

2.6 Extraction

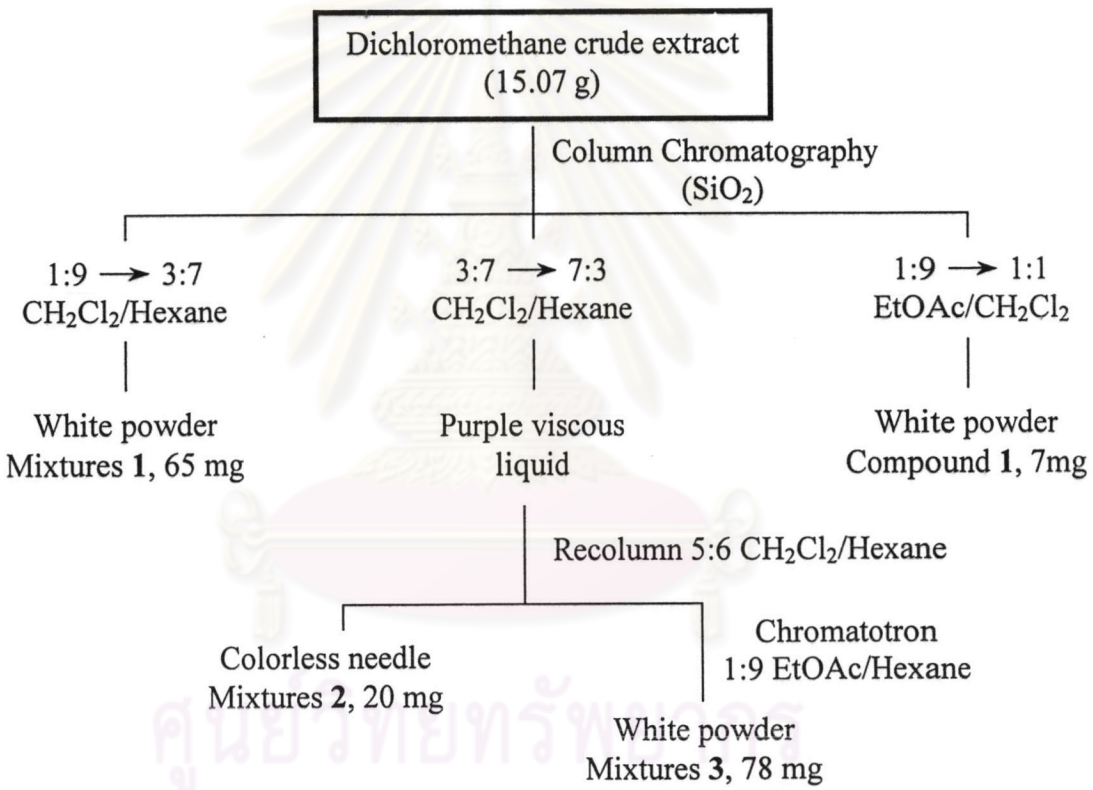
The dried barks of *C. brachiata* (8,200 g) were ground to a coarse powder and then defatted with hexane before extracted with organic solvents, dichloromethane, ethyl acetate and methanol, respectively. The extracts were concentrated under reduced pressure, yielding dichloromethane crude extract 15.07 g, ethyl acetate crude extract 145.84 g and 225.54 g from methanolic crude extract. The procedures and results of the extractions were summarized in **Scheme 2.1**



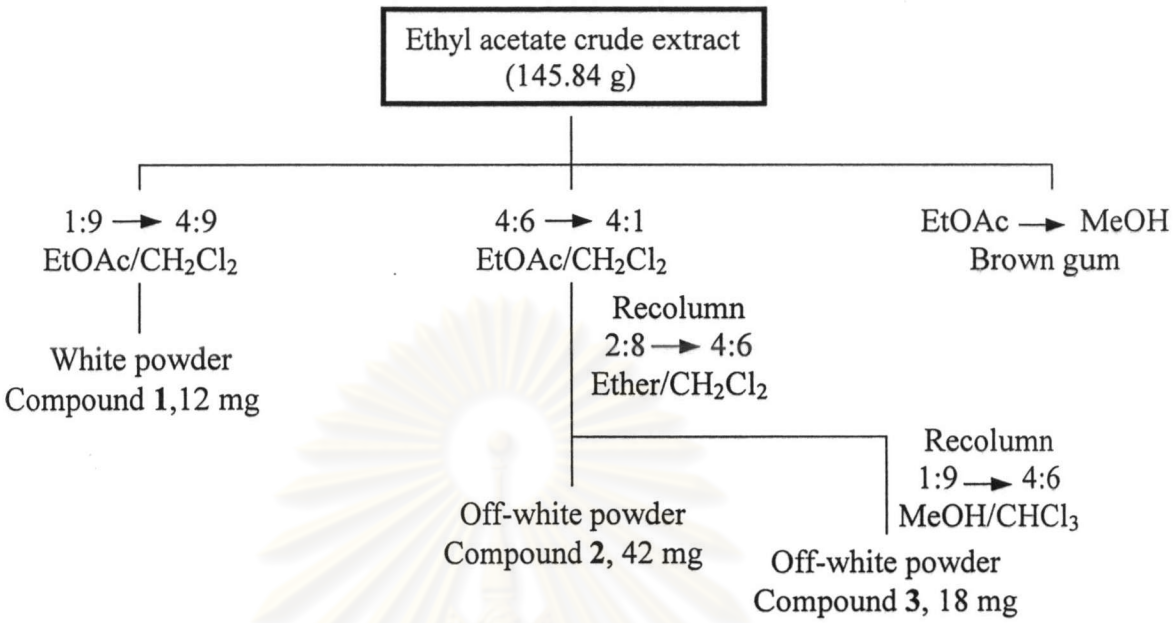
Scheme 2.1 The extraction procedure of the barks of *C. brachiata*

2.7 Separation and Purification

Dichloromethane and ethyl acetate crude extracts were fractioned by open column chromatography on silica gel. This column was eluted by suitable solvent system, depend on polarity of each compound. All fractions were monitored by TLC on silica gel and the fractions with the same components were combined. Each fraction was further purified with proper techniques such as flash column chromatography, chromatotron, PTLC etc. The isolation of the mixtures and compounds from dichloromethane and ethyl acetate extracts of *C. brachiata* barks were briefly summarized in **Scheme 2.2 and 2.3**, respectively.



Scheme 2.2 Isolation procedure of the dichloromethane crude extract



Scheme 2.3 Isolation procedure of the ethyl acetate crude extract