CHAPTER 2
EXPERIMENT

2.1 Plant material

The stems of *C. cochinchinensis* Lour were purchased from vetchapong-osot, a Thai medicinal plant shop, Bangkok, Thailand, in April, 1998. The specimen of this plant was deposited with a voucher number DKF105009 at the herbarium of Royal Forest Department, Bangkok, Thailand.

2.2 Equipments

2.2.1 Rotatory evaporator

The Buchi rotatory evaporator was used to evaporate the large amount of volatile solvents such as methanol, ethyl acetate, dichloromethane and hexane.

2.2.2 Fourier Transform-Infrared Spectrophotometer (FT-IR)

Infrared spectra were recorded on NICOLET IMPACT 410 FT-IR spectrometer.

2.2.3 ^{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 residual proton in deuterated solvent.

2.2.4 Melting point (m.p.)

The melting points were obtained on a Fishers-Johns melting point apparatus and are uncorrected.

2.2.5 UV-visible spectrometer

UV-visible absorbance was measured on UV-VIS Hewlett Packard 8452A diode array spectrophotometer.

2.2.6 Gas chromatography Mass spectrometer

EIMS were acquired by GC-MS Fisons Instruments VG TRIO 2000.

2.2.7 pH values

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

2.2.8 CHNS/O ANALYSIS

CHNS/O quantity was analysed by Perkin Elmer PE2400 series II: option CHN

2.3 Dipping Reagent

In addition to 10% H_2SO_4 in ethanol which was routinely used for detecting spots of compounds, the following reagents were used to detect certain functional groups or class of compounds.

2.4 Bioassay Procedures

2.4.1 Antibacterial Bioassay

This bioassay was performed by paper disc method, unless otherwise stated. The compounds were first tested with four susceptible bacteria: *Escherichia coli*, *Bacillus cereus*, *Staphylococus aureus* and *Staphylococus derby*. If some compounds show high inhibition, they will be further tested against more resistance bacteria. Stock solution was prepared by dissolving 10 mg. of test sample in 1000 µl of proper solvent. Thirty milliliters of stock solution were transferred by disposable pipette onto a disc. After 24 hours, diameter of clear zone was measured.

2.4.2 The Inhibitory Effect for Tumor Cell Lines

Some pure compounds from the stem of *C. cochinchinensis* were tested by using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The Human Nasopharyngal Carcinoma (KB cell lines) was used for this method.

2.4.3 Scavenging effects on DPPH radicals[20]

All samples (0.25 mM, 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 left for 30 min; the absorbance of the resulting solution was measured at 518 nm with a spectrophotometer. All tests and analyses were run in three replicate and averaged.

2.4.4 O_2^- Scavenging Activity[21]

The assay method for superoxide dismutase was modified from the method of Yoshikawa *et al.* Briefly, a reaction mixture containing 200 µM xanthine, 600 µM EDTA, 100 µM NBT, 0.01% bovine serum albumin, and 0.02 µU/ml xanthine oxidase in 33.3 mM sodium carbonate buffer (pH 10.2) was incubated for 30 min at room temperature. After incubation, The solution was mixed with 6 mM $CuCl_2$ to stop reaction. The formazan formation was monitored at 560 nm. In addition,

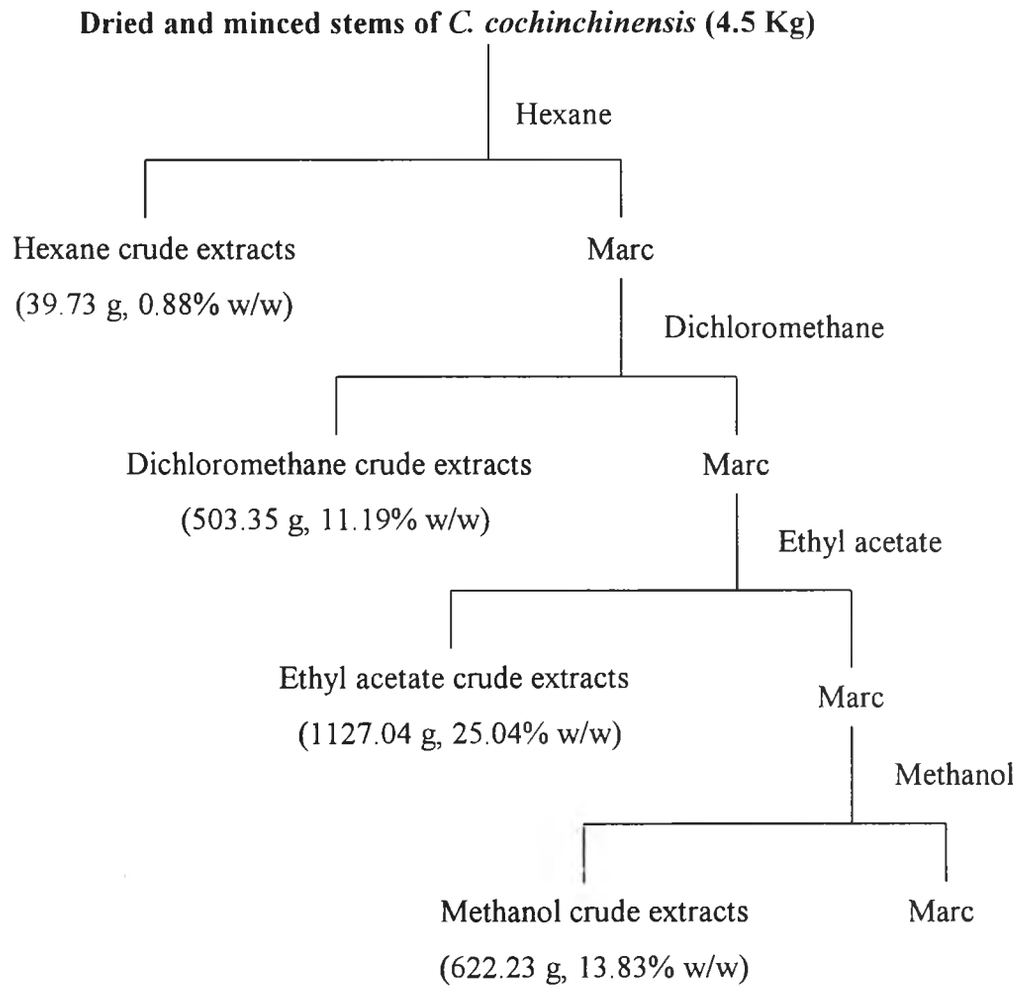
inhibitory effect of test samples on xanthine oxidase activity was examined to clarify whether the inhibition of the formazan formation was due to inhibition of xanthine oxidase.

2.5 Extraction

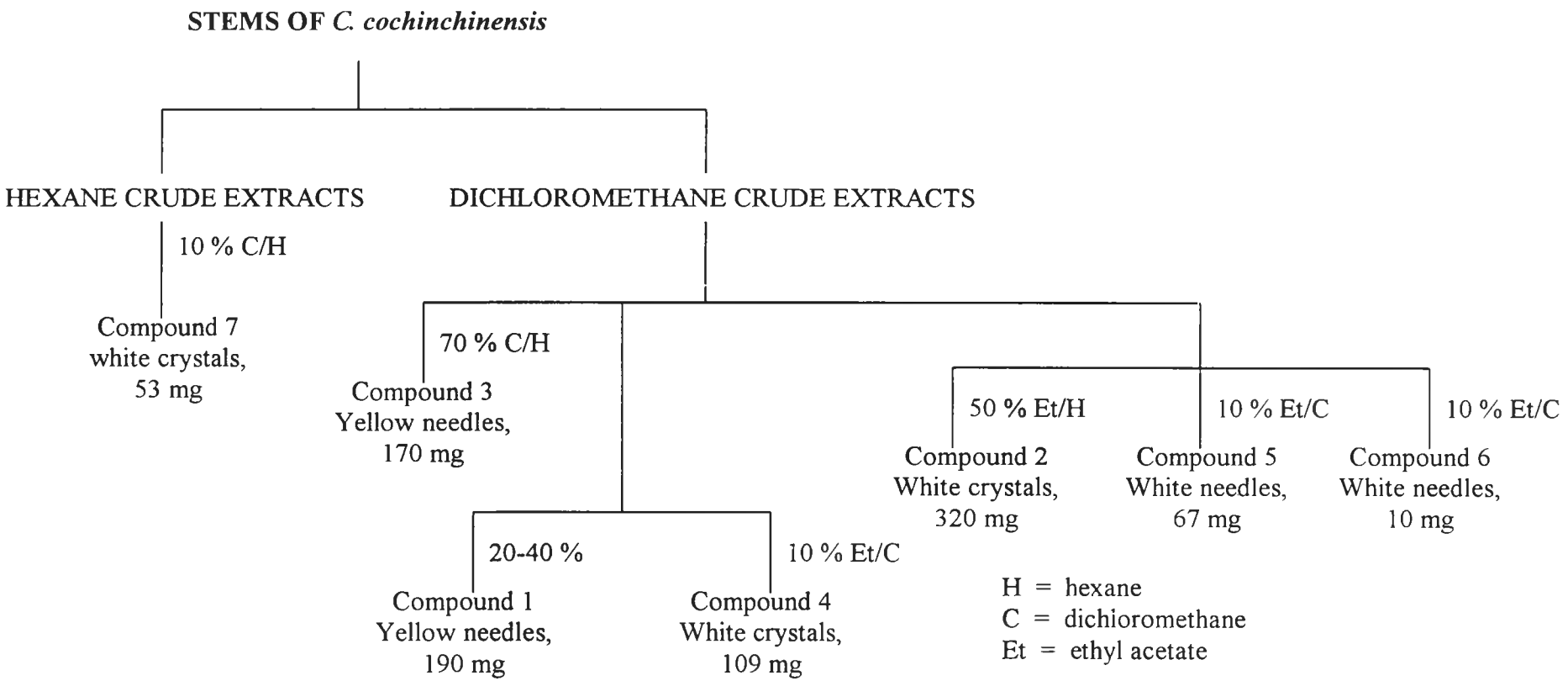
Four and a half kilograms of stems of *C. cochinchinensis* Lour were extracted with hexane three times. The solution of hexane was filtered and was evaporated by rotatory evaporator. The crude extract of hexane, 39.73 g, was obtained as a orange-brown oil. The residue after hexane extraction was then extracted with dichloromethane for three times, then the filtered solution was evaporated. The dichloromethane crude extract, 503.35 g, was obtained as a black-brown sticky. The residue after dichloromethane extraction was extracted by ethyl acetate three times. The solution of ethyl acetate was filtered and solvent was then removed by evaporator. The crude extract of ethyl acetate, 1127.04 g, was obtained as a black-brown sticky. The final residue was also extracted with methanol three times, then the solution was filtered and evaporated. The methanol crude extract, 622.23 g, was obtained as black-brown gum.

2.6 Separation and Purification

Hexane and dichloromethane crude extracts were separated by open column chromatography techniques. Silica gel number 7734 was packed in column chromatography. Crude extracts were mixed with silica gel to dryness before being added on the top of a column, and then the column was eluted with an increasing gradient of dichloromethane in hexane, ethyl acetate in dichloromethane and finally methanol in ethyl acetate. Every fraction was collected, concentrated to a small volume and then monitored by TLC in order to combine the fraction which had the same compounds. Each compound was further purified by column chromatography and recrystalization techniques. The separation of all of isolated compounds from hexane and dichloromethane crude extracts of stems of *C. cochinchinensis* were briefly summarized in **scheme 2.2** .



Scheme 2.1 The extraction procedure of the stems from *C. cochinchinensis*



Scheme 2.2 Flow chart showed the separation of stems from *C. cochinchinensis*