CHAPTER 2

EXPERIMENT

2.1 Plant material

The dried stems of *D. cochinchinensis* Pierre were purchased from vetchapong-osot, a Thai medicinal plant shop, Bangkok, Thailand, in April, 1998. The specimen of this plant was compared by specialist of Royal Forest Department with a voucher number BKF97028 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 ppm was assigned with reference to the residual proton in deuterated solvent.

2.2.4 Melting point apparatus

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

2.2.5 UV-visible spectrophotometer

UV-visible absorbance was measured on a 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 meter

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

2.2.8 X-ray diffractometer

X-ray data collection performed at room temperature with Bruker Axs AMART diffractometer equipped with CCD area detector using graphite-monochromated Mo Kα radiation

2.2.9 Chromatotron

Model 7924T, Harrison Research, Ser. No W34 Patented, Made in U.S.A

2.3 Dipping Reagent

In addition to 10% H_2SO_4 in ethanol which was routinely used for detecting spots of compounds.

2.4 Bioassay Procedures

2.4.1 The Inhibitory Effect for Tumor Cell Lines [17]

Some pure compounds from the stem of *D. 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. This assay was performed at Natural Product Research Section, National Cancer Institute, Thailand.

2.4.2 Scavenging effects on DPPH radicals [18]

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.

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

2.4.3 *O_2^- Scavenging Activity* [19]

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 was added to the mixture (0.5 ml) consisting of 0.4 mM xanthine and 0.24 mM nitro blue tetrazolium (NBT) in 0.1 M phosphate buffer (pH 8.0). Xanthine oxidase (0.049 unit/ml, 0.5 ml) diluted in 0.1 M phosphate buffer (pH 8.0) was added followed by the 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.

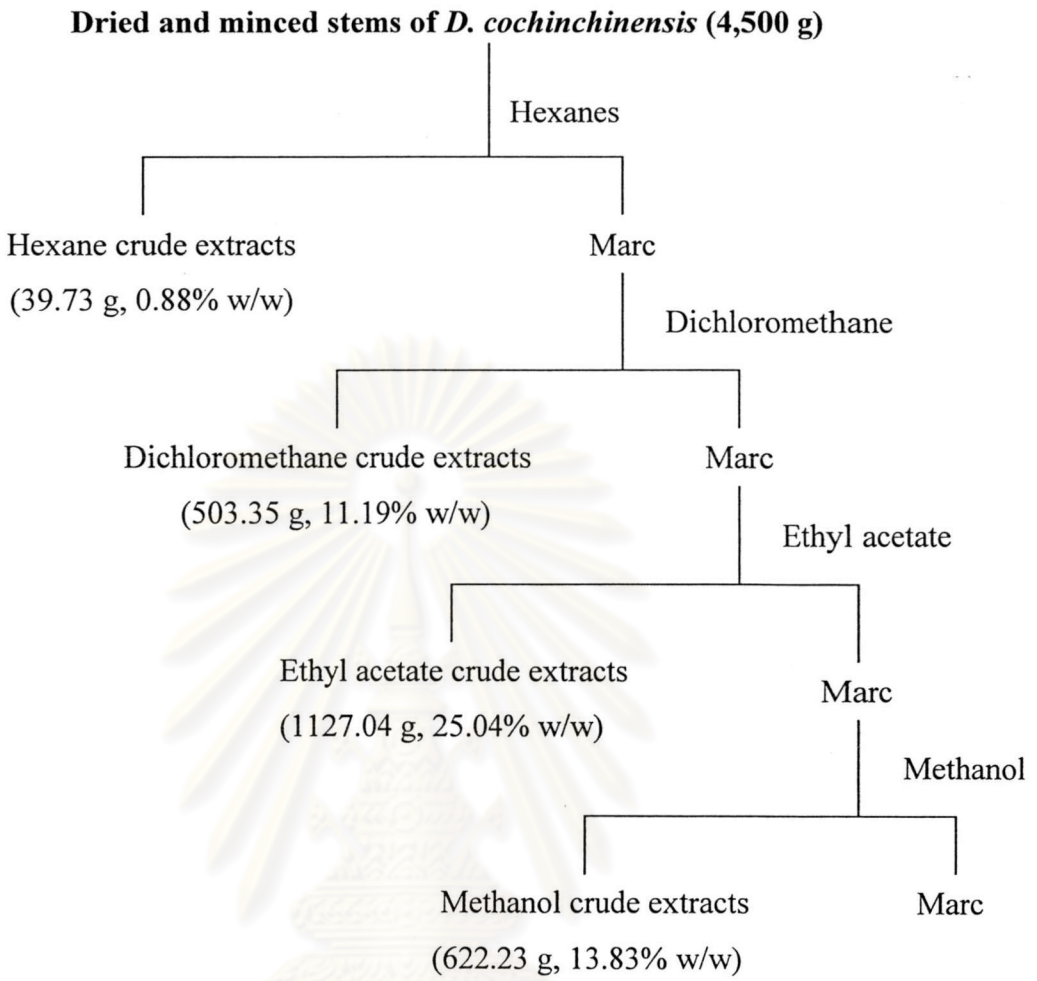
$$\text{SOD activity (\%)} = (1 - A_{sample}/A_{blank}) \times 100$$

2.5 Extraction

Four thousand and five hundred grams of stems of *D. cochinchinensis* Lour were extracted with hexanes three times. The solution of hexanes was filtered and then evaporated by rotatory evaporator. The crude extract of hexanes, 39.73 g, was yielded as an orange-brown oil. The residue after hexane extraction was then extracted three times with dichloromethane, The filtered solution was evaporated. The dichloromethane crude extract, 503.35 g, was yielded as a black-brown sticky oil. The residue after dichloromethane extraction was extracted three times by ethyl acetate. The solution of ethyl acetate was filtered and the solvent was then removed by evaporation. The crude extract of ethyl acetate, 1127.04 g, was yielded as a black-brown sticky oil. The final residue was also extracted three times with methanol, then the solution was filtered and evaporated. The methanol crude extract, 622.23 g, was yielded as black-brown gum.

2.6 Separation and Purification

Ethyl acetate crude extract was 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. The fractions which contain the same compounds were combined. Each compound was further purified by column chromatography and recrystallization techniques.



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