CHAPTER II

EXPERIMENT

2.1 Plant material

The rhizomes of Waan Ma Lueang (*Curcuma* spp.) were collected in May 2001 from Sra Kaew Province, Thailand. The specimens were identified by Professor Puangpen Sirirugsa, Department of Biology, Faculty of Science, Prince of Songkla University at Hat Yai campus, Songkla Province, Thailand.

2.2 Equipments

2.2.1 ^{1}H and ^{13}C-Nuclear Magnetic Resonance Spectrometer

NMR spectra were recorded with a JEOL JNM-A 500 FT-NMR spectrometer and a BRUKER AV 500 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.

2.2.2 Fourier Tranform-Infrared Spectrophotometer (FT-IR)

The samples were analyzed in KBr pellet and NaCl cell. Infrared spectral data were recorded by using NICOLET IMPACT 410 FT/IR spectrophotometer.

2.2.3 Mass Spectrometer

EIMS was obtained on Mass Spectrometer Model VG Trio 2000

2.2.4 Melting Point Apparatus (m.p.)

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

2.2.5 Chromatotron Equipment

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

2.2.6 Rotary Evaporator

The BUCHI rotary evaporator was utilized to evaporate the large amount of all volatile organic solvents.

2.2.7 UV-Visible Spectrophotometer

UV-visible absorption spectra of the active compounds were recorded on UV-Vis Hewlett Packard 8452A diode array spectrophotometer.

2.2.8 pH Meter

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

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 as silica gel 60 Merck cat. No. 7734, 7749, and 9815 were used for open column chromatography, chromatotron, and flash column chromatography, respectively. Thin-layer chromatography (TLC) was performed on precoated Merck silica gel 60 F_{254} plates (0.25 mm. thick layer).

2.4 Dipping reagent

Ten percent of sulfuric acid in ethanol 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 DPPH radical scavenging activity

2.5.1.1 TLC autographic assay (Hostettmann et.al, 1997)

Using 2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH) radical as a TLC spray reagent was confirmed to be well suited for the screening of antioxidants in crude plant extracts. The assay involves spraying TLC plates with a 0.2% DPPH solution in methanol. The plates are considered 30 minutes after spraying. Active compounds occur as yellow spots on a purple background.

2.5.1.2 Spectrophotometric assay (Yen and Hsieh, 1997)

After isolation and purification, activities of pure compounds were quantified in this assay. Various concentrations of samples dissolved in methanolic solution (0.5 ml) were added to DPPH radical methanolic solution (0.2 mM, 1.0 ml). After 30 minutes incubation at room temperature in the dark, the absorbance was measured at 517 nm with a spectrophotometer. All tests were run in triplicate and averaged. The scavenging activity was evaluated from the decrease value of 517 nm absorption, which was calculated by the following equation. The activity was shown as IC_{50} values that denote the concentration of sample required scavenging 50% DPPH free radicals.

$$\boxed{\% \text{ Scavenging activity} = [\, 1 - A_{\text{Sample}} / A_{\text{blank}}] \times 100}$$

2.5.2 Xanthine oxidase-related activity

2.5.2.1 Assay for scavenging activity of $O_2^{-}\cdot$ by xanthine oxidase (Okamura. et.al, 2001)

Superoxide anion radical was generated from xanthine-xanthine oxidase method with a slight modification. The reaction mixture consisted of 0.1 M phosphate buffer (pH 8.0) containing 0.4 mM xanthine, 0.24 mM nitroblue tetrazolium, and 0.049 units of xanthine oxidase in a final volume of 1.0 ml. Samples at various concentrations in DMSO were added to the mixture (0.15 ml). After being incubated at 37 °C for 20 minutes, the reaction was terminated by addition of 0.05 ml of 69 mM sodium dodecyl sulfate. The absorbance of formazan produced was determined at 560 nm, and scavenging activity on $O_2^{-}\cdot$of each sample was estimated by the same equation as described before. The IC_{50} values were calculated from regression line.

$$\boxed{\% \text{ Scavenging activity} = [\, 1 - A_{\text{Sample}} / A_{\text{blank}}] \times 100}$$

2.5.2.2 Assay for inhibitory activity against xanthine oxidase (Kweon et.al, 2001)

For studying of xanthine oxidase inhibitory activity, the arise in the absorbance at 290 nm due to uric acid production was measured in the absence of nitroblue tetrazolium. Allopurinol, which is a drug for gout treatment, was used as a standard for this assay. The inhibitory activity was shown as percent inhibition, which was estimated from the following equation. The IC_{50} values were determined from regression line.

$$\% \text{ Inhibition} = [\, 1 - A_{\text{Sample}} / A_{\text{blank}}] \times 100$$

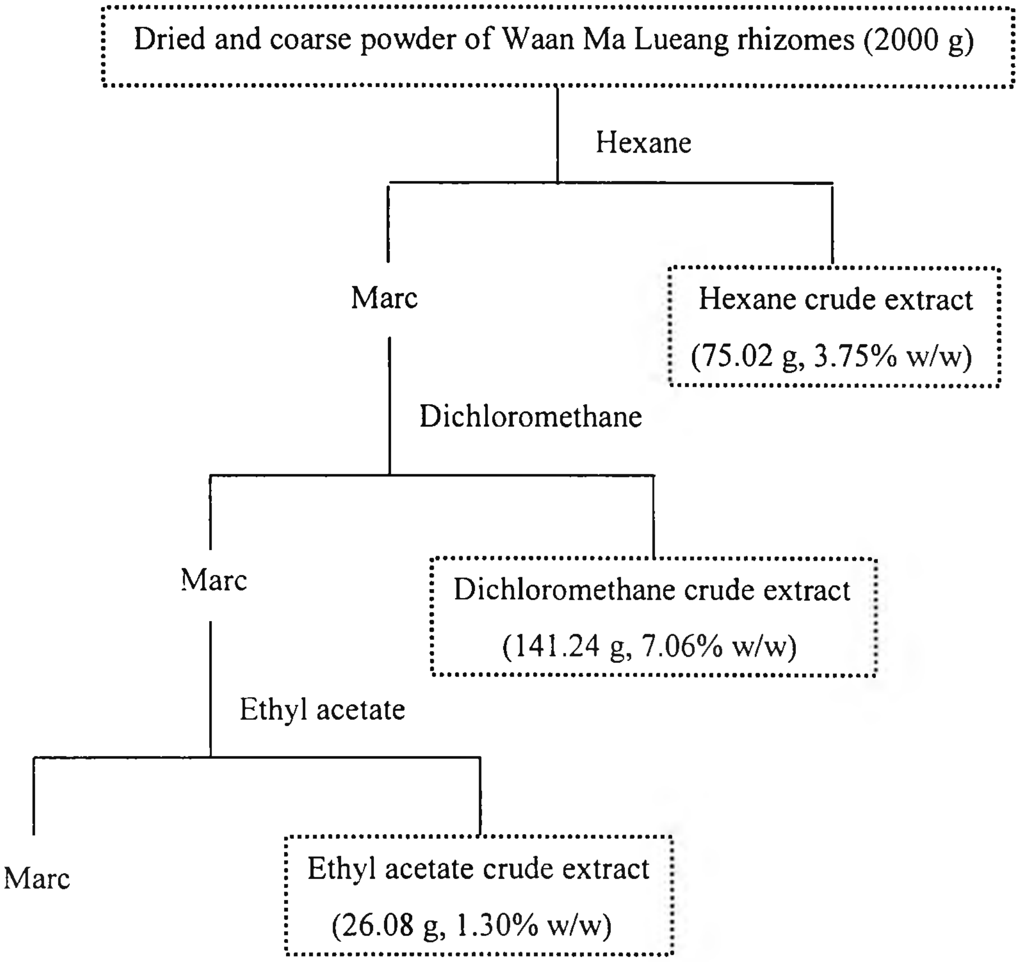
2.5.3 Ferric thiocyanate assay (Wijewickreme et.al, 1999)

This assay was slightly modified. The linoleic acid emulsion was prepared by vortex mixing 3.0 ml of linoleic acid with 3.0 ml of sodium dodecyl sulfate (SDS) as emulsifier and 200 ml of 30% (v/v) ethanol. Each sample at various concentrations in ethanolic solution (0.5 ml) was mixed with 5 ml of emulsion and the final volume of the mixture was adjusted to 12.5 ml. The reaction mixture was incubated in a conical flask at 40 °C in the dark. Aliquots of 0.05 ml were taken at eight hours during incubation and tested for lipid peroxidation products. The assay was carried out by adding 2.5 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 0.05 ml of sample. After the mixture was left for 3 minutes, the absorbance of the reaction mixture was measured at 500 nm. The activity was revealed as percent inhibition that was examined from the following equation. The IC_{50} values that denote the concentration of sample required scavenging 50% peroxyl radicals were calculated from regression line.

$$\% \text{ Inhibition} = [\, 1 - A_{\text{Sample}} / A_{\text{blank}}] \times 100$$

2.6 Extraction

The rhizomes of Waan Ma Lueang (*Curcuma* spp.) were sun dried for a week. Two kilograms of dried rhizomes were ground into powder and exhaustively extracted by maceration at room temperature with hexane, dichloromethane, and ethyl acetate thrice for each solvent. Extracts of each solvent were filtrated and evaporated to dryness in vacumm. The extraction afforded 75.02 g of hexane crude extract, 141.24 g of dichloromethane crude extract, and 26.08 g of ethyl acetate crude extract, respectively. The procedure of the extraction was summarized in Scheme 2.1.

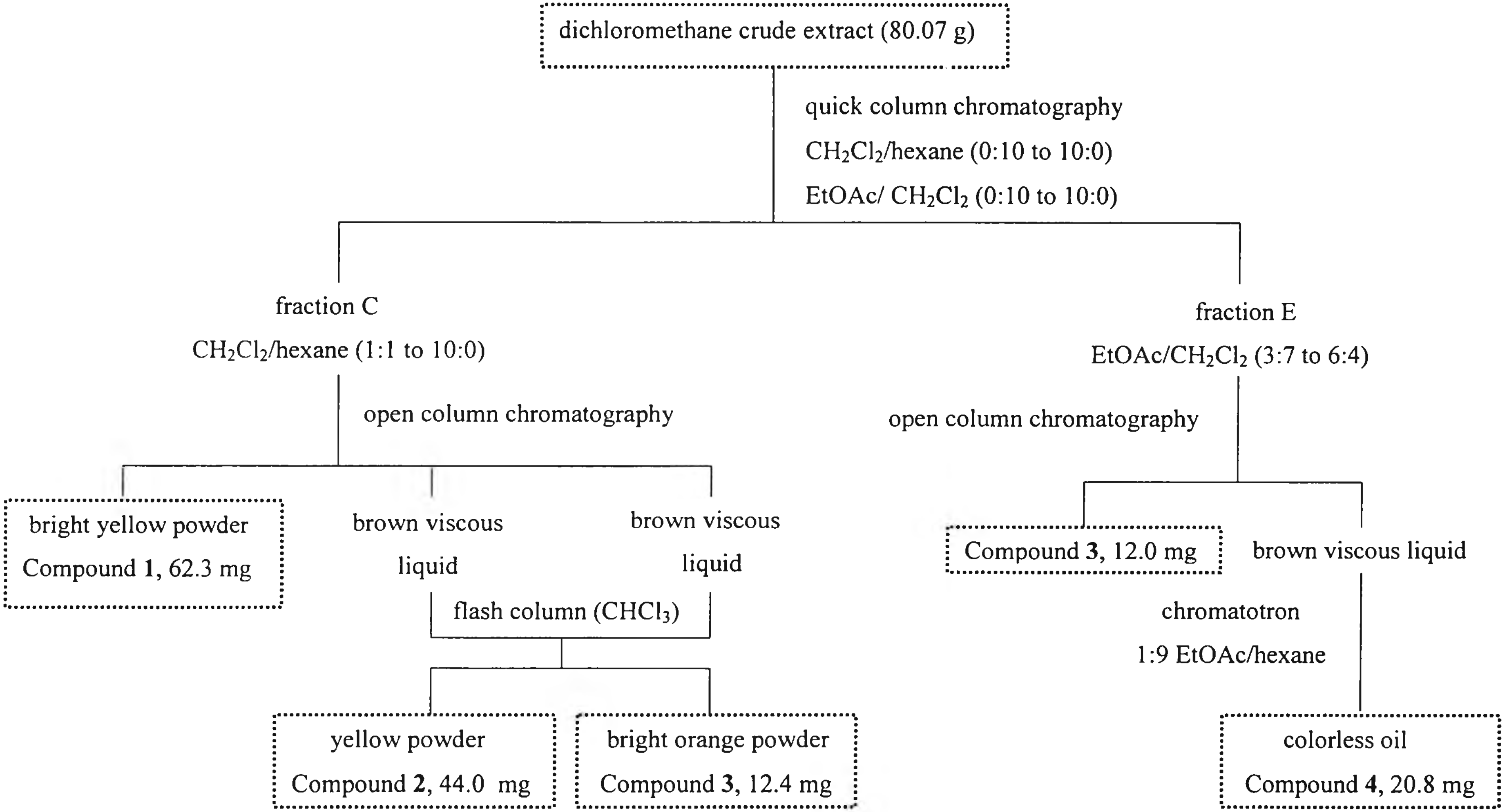


Scheme 2.1 The extraction procedure of the rhizomes of Waan Ma Lueang

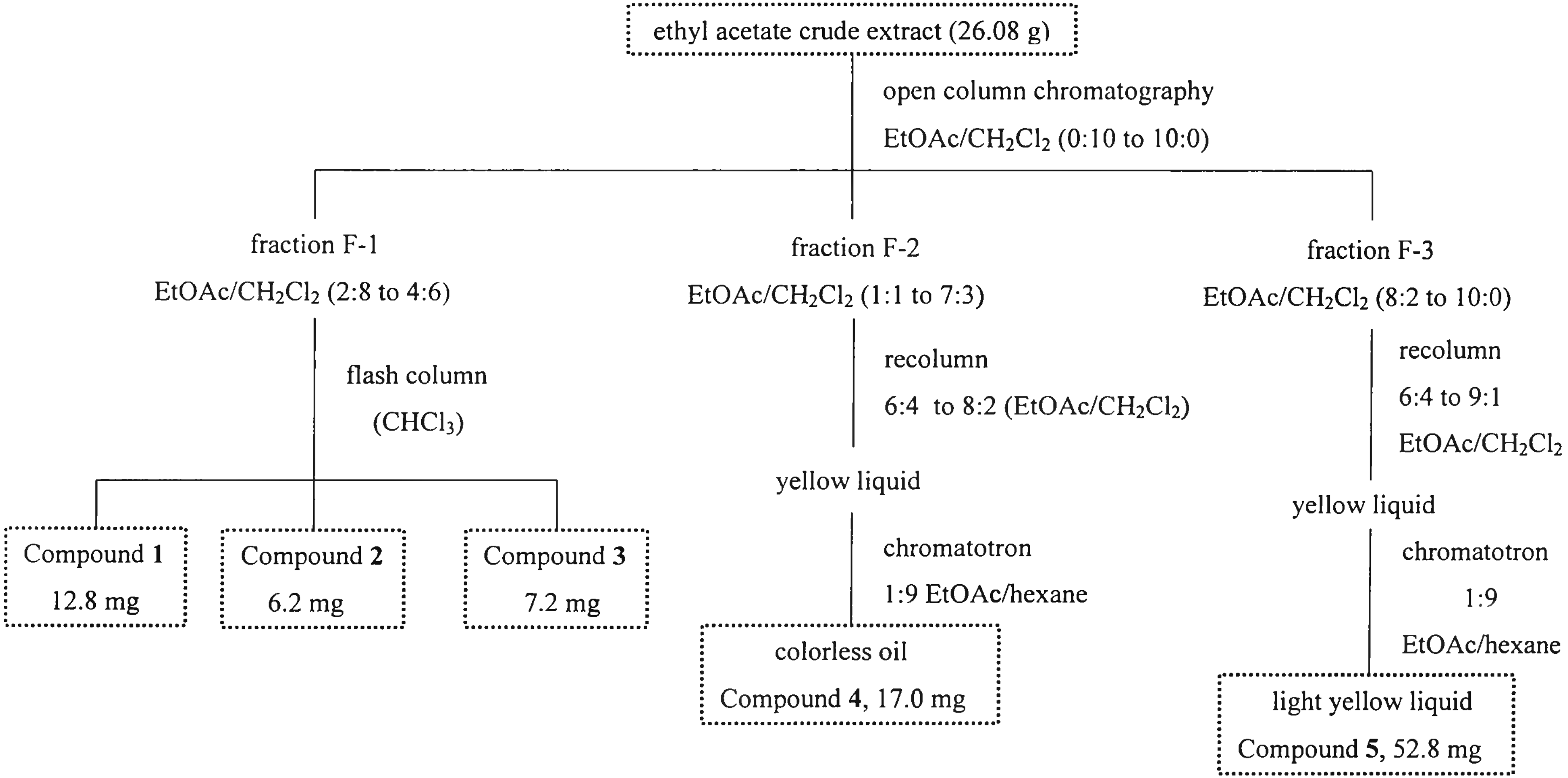
2.7 Separation and purification

The dichloromethane crude extract (80.07 g) was fractionated by silica gel quick column chromatography eluting with a gradient of CH_2Cl_2/hexane (0:10 to 10:0) and EtOAc/ CH_2Cl_2 (0:10 to 10:0). Nineteen fractions were collected and pooled on the basis of their TLC profiles to yield 6 fractions. Antioxidant activity in TLC autographic assay for DPPH radical scavenging effect showed two active fractions: fractions C and E. Fraction C, eluted with CH_2Cl_2/hexane (1:1 to 10:0), was rechromatographed on a silica gel open column to furnish compound **1** (62.3 mg). The remaining part of this fraction was further separated by flash column chromatography eluting with $CHCl_3$ to give compound **2** (44.0 mg) and compound **3** (12.4 mg). While fraction E, eluted with EtOAc/CH_2Cl_2 (3:7 to 6:4), was rechromatographed on the silica gel open column to provide compound **3** (12.0 mg). The abiding of this fraction was fractionated by radial chromatographic technique (chromatotron) eluting with EtOAc/hexane (1:9) to contribute compound **4** (20.8 mg). The isolation procedure was briefly summarized in Scheme 2.2.

Ethyl acetate crude extract (26.08 g) was subjected to silica gel open column chromatography eluting with EtOAc/CH_2Cl_2 (0:10 to 10:0) to yield three active fractions: F-1, F-2, and F-3. Fraction F-1, eluted with EtOAc/CH_2Cl_2 (2:8 to 4:6), was further isolated on flash column chromatography eluting with $CHCl_3$ to give compound **1** (12.8 mg), compound **2** (6.2 mg), and compound **3** (7.2 mg). From fraction F-2, eluted with EtOAc/CH_2Cl_2 (1:1 to 7:3), was rechromatographed on silica gel column eluting with a gradient of EtOAc/CH_2Cl_2 (6:4 to 8:2). Yellow liquid was obtained and then further purified with chromatotron technique eluting with EtOAc/hexane (1:9) to furnish compound **4** (17.0 mg). Fraction F-3 was column chromatographed on silica gel column. Elution was performed by EtOAc/CH_2Cl_2 (6:4 to 9:1) to give yellow liquid. From this remaining part, it was further purified by chromatotron technique eluting with EtOAc/hexane (1:9) to provide compound **5** (52.8 mg). The isolation procedure was concisely abstracted in Scheme 2.3.



Scheme 2.2 The isolation procedure of dichloromethane crude extract



Scheme 2.3 The isolation procedure of ethyl acetate crude extract