CHAPTER 2
EXPERIMENTAL

2.1 Plant material.

The stems of *Gelonium multiflorum* were collected at Kosumpisai, Mahasarakham province in May 1998. The specimen of this plant was identified by associate professor Kosum Peeraman and deposited (BCU004089) at the herbarium of Department of Botany, Chulalongkorn University, Bangkok, Thailand.

2.2 Equipments.

2.2.1 Rotatory evaporator

The Eyela 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 spectormeter 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.

2.2.4 Melting point (m.p.)

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

2.2.5 Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS analysis was performed by a Fison Gas-Liquid Chromatography Model GC 8000-Fison Mass Spectrometer Model Trio 2000

2.2.6 Chromatotron equipment

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

2.3 Chemical Reagents

All solvents used in this research were a commercial grade and were purified by distillation prior to use, except solvents that were reagent grade. Various absorbents such Merck's silica gel 60, cat. no. 7734, 7749 and 7730 were used for Column Chromatography, Chromatotron and Preparative Thin Layer Chromatography (PTLC), respectively. Thin Layer Chromatography (TLC) was performed on precoated Merck's silica gel 60 F_{254} (0.25 mm-thick layer) to unite fractions containing the same components. The spots were visualized under iodine vapor and/or 10% H_2SO_4 in ethanol after being checked with UV lamp (254 or 365 nm).

Color Test and 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

1. 2,4 – Dinitrophenylhydrazine Reagent (DNPH)

This reagent was adapted from 2,4 – DNP reagent for the checking of aldehyde and ketone on TLC plate. It could be prepared by dissolving 0.1 g of 2,4 – DNP in 100 ml of methanol, followed by the addition of 1 ml of 36% HCl. The plate was dipped in this reagent and evaluated immediately in visible. Aldehyde or ketone would show a deep red spot.

2. Liebermann - Burchard Test

One milligram of the unknown was dissolved in chloroform, followed by a few drops of acetic anhydride. One drop of concentrated H_2SO_4 was added. If an unknown is a steroid, the color will gradually change from pink to permanent deep green. In case of a triterpenoid, the color would change to reddish pink.

2.4 Bioassay Procedures

2.4.1 A Microwell Cytotoxicity Assay using *Artemia salina.* (Brine shrimp) [34,35]

Brine shrimp test has been used as a bioassay for a variety of toxic substances and this method has also been applied to plant extracts in order to facilitate the isolation of biologically active compounds. This methodology offers a bioassay that can be rapid, simple and inexpensive.

General procedures for this bioassay were described:

Hatching the Shrimp.

Brine shrimp eggs were hatched in plastic box (13×8×4 cm) filled with artificial seawater (38 g of NaCl dissolved in 1L of deionized or distilled water). The box was divided into two unequal compartments linked with 2 mm diameter of holes. The eggs were put in the larger compartment which was darkened with aluminum foil while the smaller were illuminated and the box was kept at 20-29°C. After 24 hours, nauplii were collected by disposable pipette from the smaller compartment.

Sample Preparation

Four milligrams of pure compounds or plant extracts were dissolved in a small amount (80 µl) of the most soluble solvent. Artificial Seawater was then added to the test solution until the total volume was up to 4000 µl. Dissolution could be assisted by vigorous stirring to give solution I (1000 ppm). This stock solution were diluted to afford solution II (100 ppm) and solution III (10 ppm) , respectively. Finally, control solution was also prepared.

Bioassay

Five nauplii were transferred to each well of 24 well microplates by the disposable pipette, and tried to keep 100 µl of seawater. Six replications were made for each concentration. The whole plates were kept in the same condition as hatching. After 24 hours, a number of dead nauplii in each well were counted.

LC_{50} Determinations

LC_{50} values were then calculated by Probit analysis program. In cases where data were insufficient for this program, LC_{50} values were estimated by using logit transformation which did not provide confidence intervals.

2.4.2 The Inhibitory effect for tumor cell lines[36]

Preliminary screening of various crude extracts from the stems of *G.multiflorum* was carried out by scientists at Beijing Medical University, Beijing, China. There were 5 cell lines used in this method: Human Nasopharyngeal Carcinoma (KB), Human Gastric Carcinoma (BGC-823), Human Hepatocellular Carcinoma (Bel-7402), Human Colon Carcinoma (HCT-8) and Human Leukemia Carcinoma (HL-60). Inhibitory effect for all tumor cell lines except HL-60 (MTT assay) was evaluated using SRB assay.

2.4.3 Antioxidant Activity

2.4.3A Antioxidant Test [37]

Reduction of 2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) radical. TLC autographic assay: after developing and drying, TLC plates were sprayed with a 0.2 % DPPH solution in methanol. The plates were determined at 30 min after spraying. Active compounds appeared as yellow spots against a purple background.

Bleaching of β- carotene: TLC autographic assay: after developing and drying, TLC plates were sprayed with a β- carotene solution in chloroform (0.2 mg/ml). The plates were exposed to 254 nm UV light for 20 min before examination.β-carotene underwent bleaching except in places where antioxidative substances prevented the degradation. Active compounds appeared as orange spots against a white background.

2.4.3B Antioxidant Test [38]

Reactive oxygen species (ROS) have been considered to play some important roles in the carcinogenesis process including tumor promotion.

The inhibitory effects on O^{-}_2 formation in the XA/XOD of rosmarinic acid which is mainly responsible for the potent superoxide scavenging activity of *Perilla frutescens* . Analytical instruments used were as follows: HPLC.

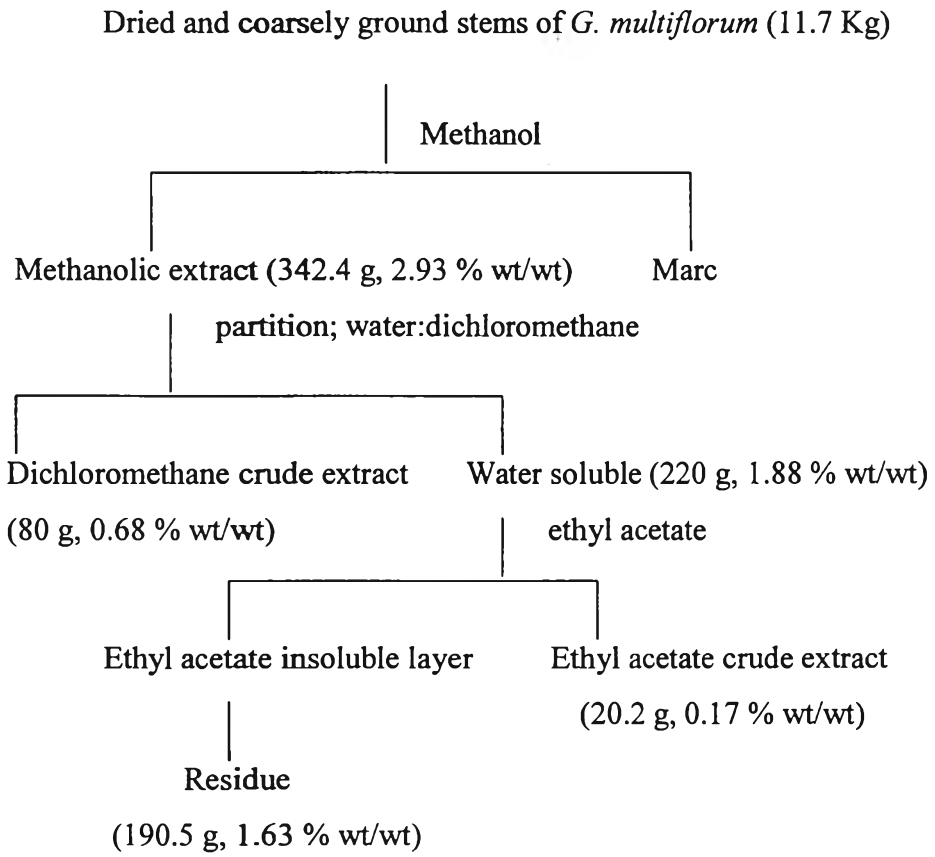
O^{-}_2 Scavenging Test in the XA/XOD system. O^{-}_2 scavenging activity was measured by an XA/XOD system using SOD Test Wako. In this system, O^{-}_2 scavenging activity was estimated by measuring the nitrobluetetrazolium (NBT) reduction and XOD inhibitory activities as previously reported. O^{-}_2 scavenging (SOS) activity is given by the following equation:

SOS activity (%) = NBT reduction inhibitory activity (%) – XOD inhibitory activity (%)

The value of 50% inhibition concentration (IC_{50}) was determined for the comparison with the inhibitory activities.

2.5 Extraction

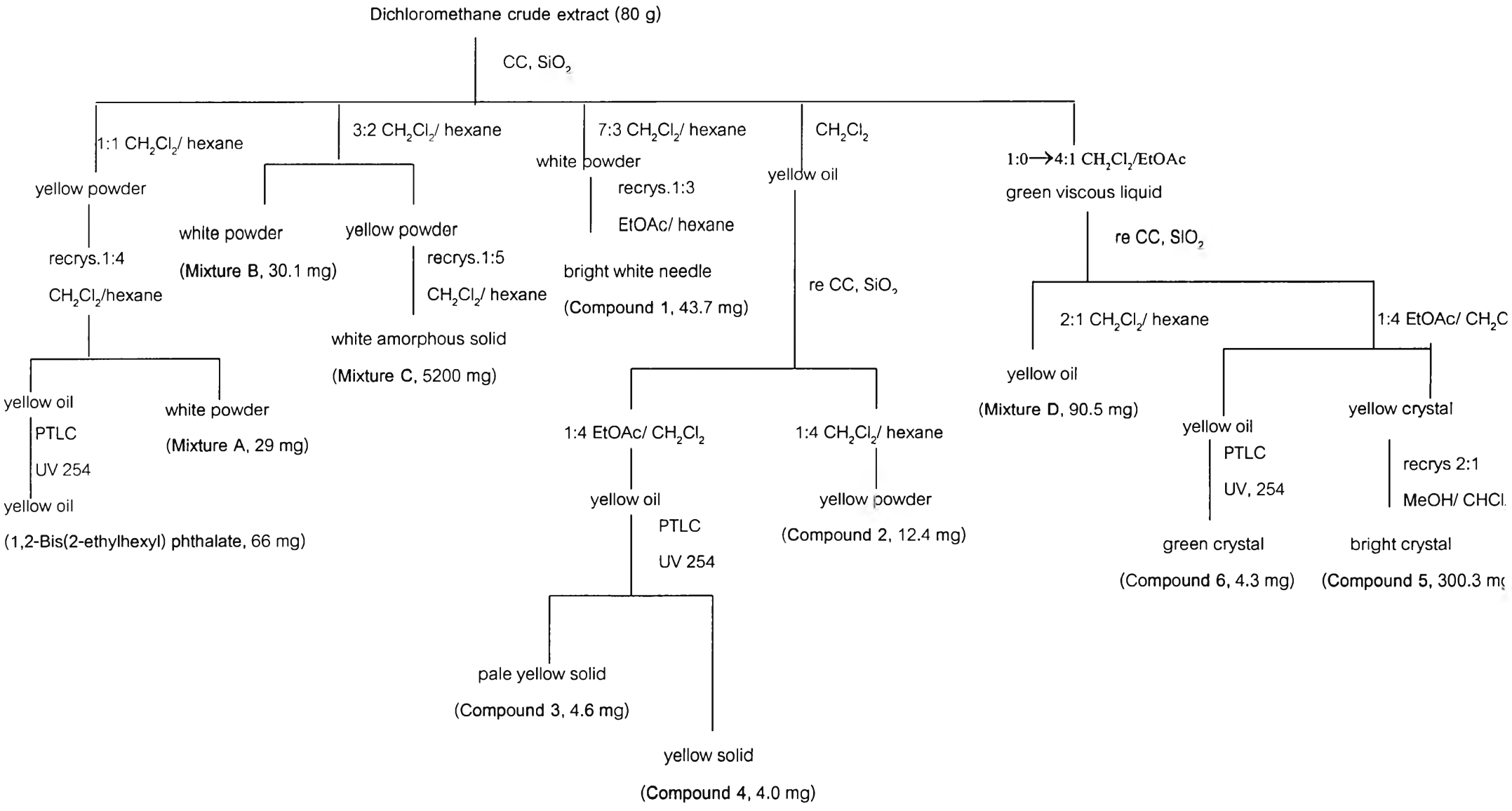
The oven-dried (60°C) stems of *G. multiflrum* (11.7 Kg) were minced and then extracted with the organic solvents. The plant was soaked in methanol for about 5-6 days several times until the solvent became clear. It was then filtered and the solvent was evaporated under reduced pressure to dryness, yielding methanolic crude extract (342.4 g). The methanolic extract was partitioned between water and dichloromethane. The dichloromethane extract was evaporated to dryness, yielding dichloromethane crude extract (80 g) while water residue (220 g) was further extracted with ethyl acetate to afford ethyl acetate extract (20.2 g) and residue (190.5 g). The procedures and results of the extraction were summarized in **scheme 2.1.**



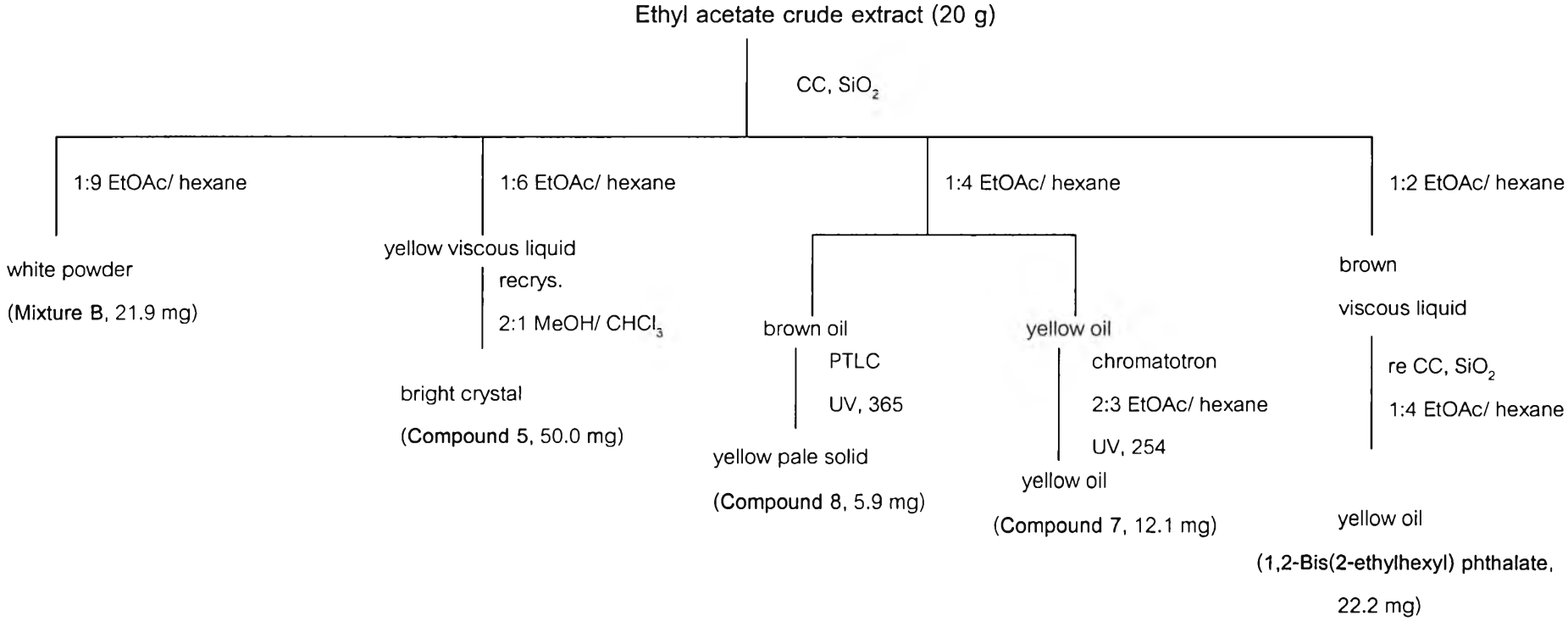
Scheme 2.1 The Extraction procedure of the stems from *G.multiflorum* A.Juss.

2.6 Separation and Purification

Dichloromethane and ethyl acetate crude extracts were separated by open column chromatography techniques. After the column was packed with silica gel, the extracts were dissolved in a small amount of a suitable solvent and mixed with silica gel (1:1) to dryness before being added on the top of a column. The column was eluted with an increasing gradient of dichloromethane in hexane and then methanol in ethyl acetate. Each fraction (about 500 ml) was collected, concentrated to a small volume and then checked by TLC in order to combine the fractions which had the same components. The fractions which contained UV active components were further purified by proper methods such as chromatotron, PTLC or crystalization, etc. The isolation of the mixtures and compounds from dichloromethane and ethyl acetate extracts of stems from *G. multiflorum* was 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