CHAPTER 2

EXPERIMENT

2.1 Apparatus

Melting points were determined with Fisher-John Melting Point Apparatus and was uncorrected. Infrared spectra were recorded on NICOLET IMPACT 410 FT/IR spectrometer. EIMS were obtained on FISONS MS8000 mass spectrometer. NMR experiments were carried out with a JEOL JNM-A500 FT NMR spectrometer using TMS as an internal standard in some cases while ^{1}H-NMR spectra of certain synthetic furan analogues were recorded on Bruker AC-F 200 FT NMR spectrometer. Elemental analysis was performed on Perkin Elmer 24000 series II. Steroid and long chain hydrocarbon analysis was carried out by SHIMUDZU GC-7AG. Optical rotation was determined by Polax-D polarimeter. Centrifugal Thin Layer Chromatography was performed by using Chromatotron (model 7924, Harrison Research) on a silica gel plate of 2-mm thickness.

2.2 Chemicals

All solvents used in this research were a commercial grade and distilled prior used. Various absorbents such Silica gel 60 MERK, cat.no. 7734, 7749 and 7731 were used for column chromatography, chromatotron and Preparative Thin Layer Chromatography (PTLC), respectively. TLC was performed on precoated Merk silica gel 60 F_{254} (0.25 mm-thick layer). The spots were visualized under iodine vapor and/or 10%H_2SO_4 in ethanol after detecting with UV lamp (254 or 365 nm).

2.3 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.

2.3.1 2,4-Dinitrophenylhydrazine Reagent (DNPH)[14]

This reagent was adapted from 2,4-DNP reagent for the detection of aldehyde and ketone on TLC plate. It could be prepared by dissolving 0.1 g of 2,4-DNP in 100 ml of MeOH, 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 give a deep red spot.

2.3.2 Liebermann-Burchard Test[15]

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 Brine Shrimp Cytotoxic Lethality Test (BSCLT)

BSCLT was a main bioassay used to select the plant for this research and to evaluate toxicity of some compounds isolated from this plant. Although there were several methodologies, a convenient microwell method[16] was adapted for this experiment. General procedures for this bioassay were described in 2.4.1a-2.4.1d.

2.4.1a) Hatching the Shrimp

Brine shrimp eggs (*Artemia salina* Linn.) were hatched in an open shallow rectangular plastic box (13x18x4 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 ϕ holes. The eggs were sprinkled into the larger compartment which was darkened with aluminum foil while the smaller was illuminated with the 20-watt lamp, and the box was kept at 22-29 °C. After 24 hours, nauplii were collected by disposable pipette from the smaller compartment.

2.4.1b) Sample Preparation

Four milligrams of tested compound or plant extract was dissolved in a small amount (80 μl) of the most soluble solvent. Seawater was then added to the test solution until the total volume was 4000 μl. Dissolution could be assisted by vigorous stirring with shaker to afford solution I (1000 ppm). Serial dilution of this stock solution were made to obtain solution II (100 ppm) and solution III (10 ppm), respectively. Finally, control solution was also prepared.

2.4.1c) 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 covered plates were kept in the same condition as hatching. After 24 hours, numbers of dead nauplii in each well were counted under binocular microscope.

2.4.1d) LC_{50} Determinations

$LC_{50's}$ and 95% confidence intervals were calculated by probit analysis program. In cases where data were insufficient for this program, $LC_{50's}$ were estimated using logit transformation which does not provide confidence intervals.

2.4.2 Antibacterial Bioassay

This bioassay was performed by paper disc method[17],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.

2.4.3 The Inhibitory Effect for Tumor Cell Lines

Various crude extracts from the stems of *A. arborescens* were preliminary screened by using the MTT assay. Seven cell lines used in this method were Human Gastric Carcinoma (BGC-823), Human Hepatocellular Carcinoma (Bel-7402), Human Erytroleukemia Carcinoma (K562), Human Bladder Carcinoma (BIU), Human Nasopharyngeal (KB), Human Leukemia Carcinoma (HL-60) and Human Colon

Carcinoma (HCT-8). This experiment was performed by researchers at Beijing Medical University, Beijing, China.

2.4.4 Plant Growth Inhibition Test[18]

Three plants used in this experiment were *Oryza sativa* Linn. var. RD.23 (rice), *Echinochloa crus-galli Beauv.* (Yah Khao Nok) and *Mimosa pigra* Linn. (giant sensitive plant or in Thai is "Maiyarap Yak") seedlings of each plant were cultured in cellulose powder which was readily mixed with solution of tested compound. The controlled seedlings were also prepared in the same method. Seven days after cultivation, the length of root and shoot of both treatment and controlled plants was measured. Percent of growth inhibition could be calculated from the following formula.

$$\% \text{ Growth Inhibition} = [1-(T/C)] \times 100\%$$

where "T" and "C" are root or shoot length of treated and controlled seedling, respectively.

2.5 Source of Plant Material

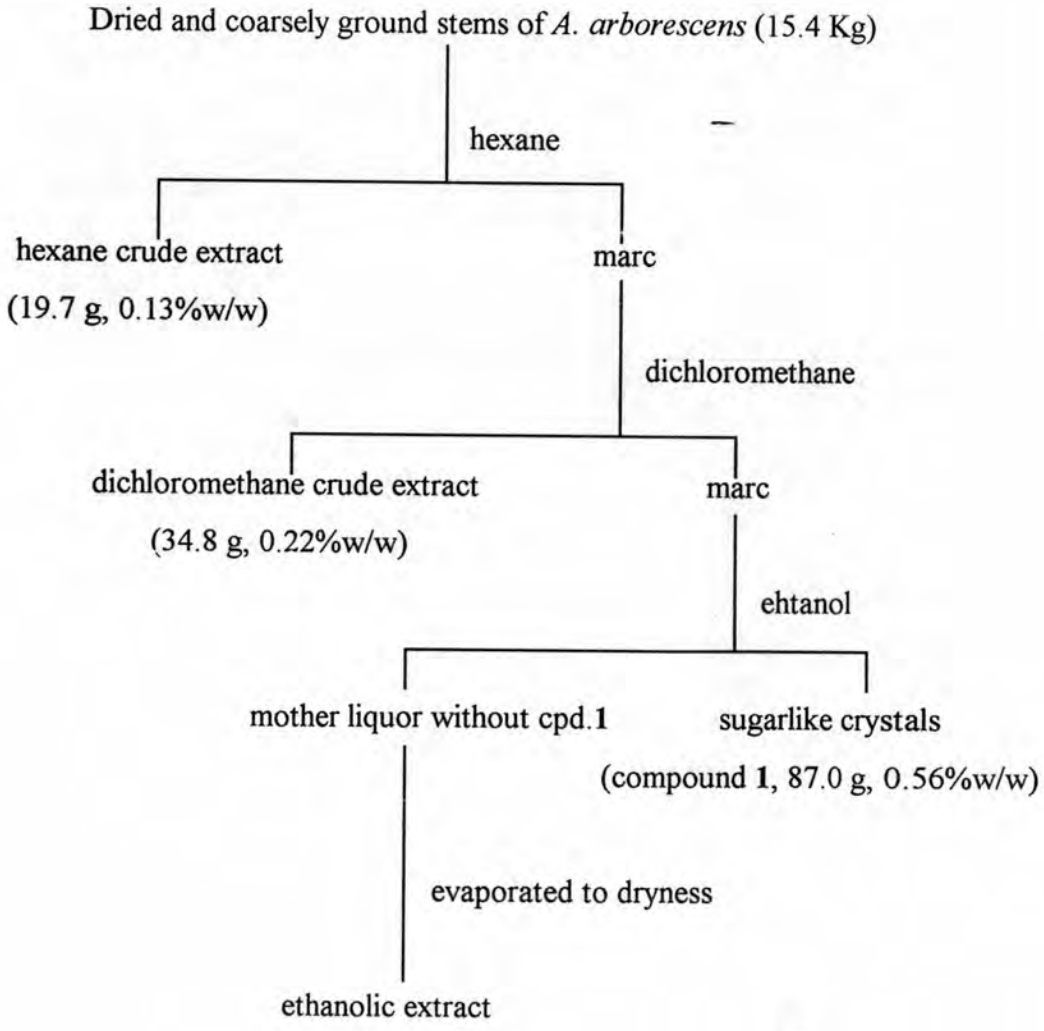
The stems of *A. arborescens* were bought from Chao-Krom-Per, a Thai medicinal plant shop, Bangkok, Thailand, in March, 1996. The specimen of this plant was compared with a voucher number BKF 46699 by the botanist of the Royal Forest Department, Ministry of Agriculture and Cooperative, Bangkok, Thailand.

2.6 Extraction

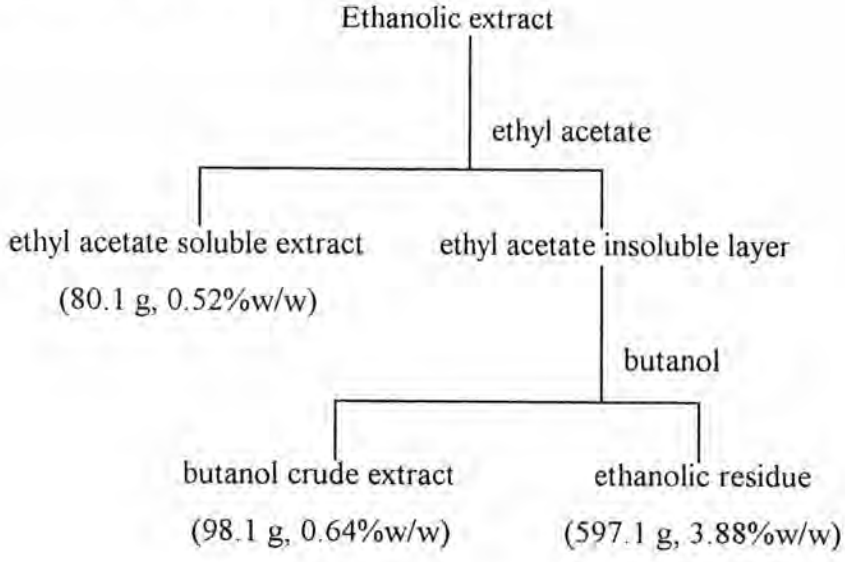
Dried and coarsely ground of *A. arborescens* stems (15.4 Kg) were first defatted with hexane before soaking in dichloromethane and ethanol, respectively. The first two extracts were evaporated under reduced pressure to dryness, yielding hexane crude extract (19.7 g) and dichloromethane residue (34.8 g). As for ethanolic extract, it was also evaporated *in vacuo* to give the concentrated one. After standing the extract overnight, sugarlike crystal (compound **1**) was obtained. Later, the mother liquor was concentrated to afford compound **1**. This procedure was repeated until no

crystal deposit. The dry ethanolic extract without compound **1** was partitioned using ethyl acetate and then butanol to give ethyl acetate extract (80.1 g), butanol extract (98.1g) and ethanolic residue (597.1 g). The procedures and results of the extraction were summarized in scheme 2.1 and 2.2 .

Scheme 2.1 The extraction procedure of the stem of *A. arborescens*



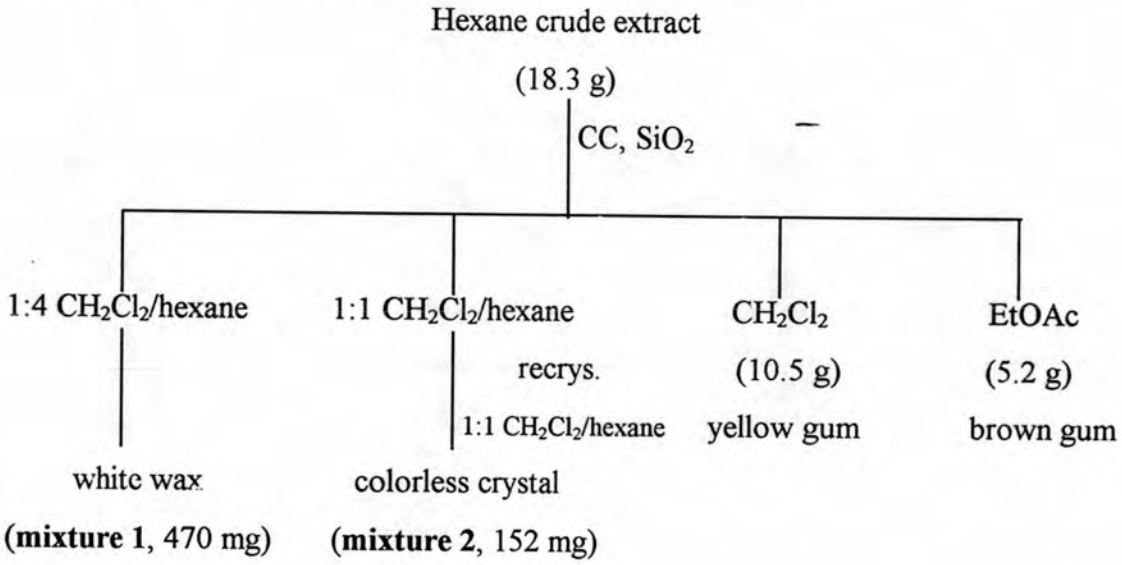
Scheme 2.2 Further extraction of the ethanolic extract



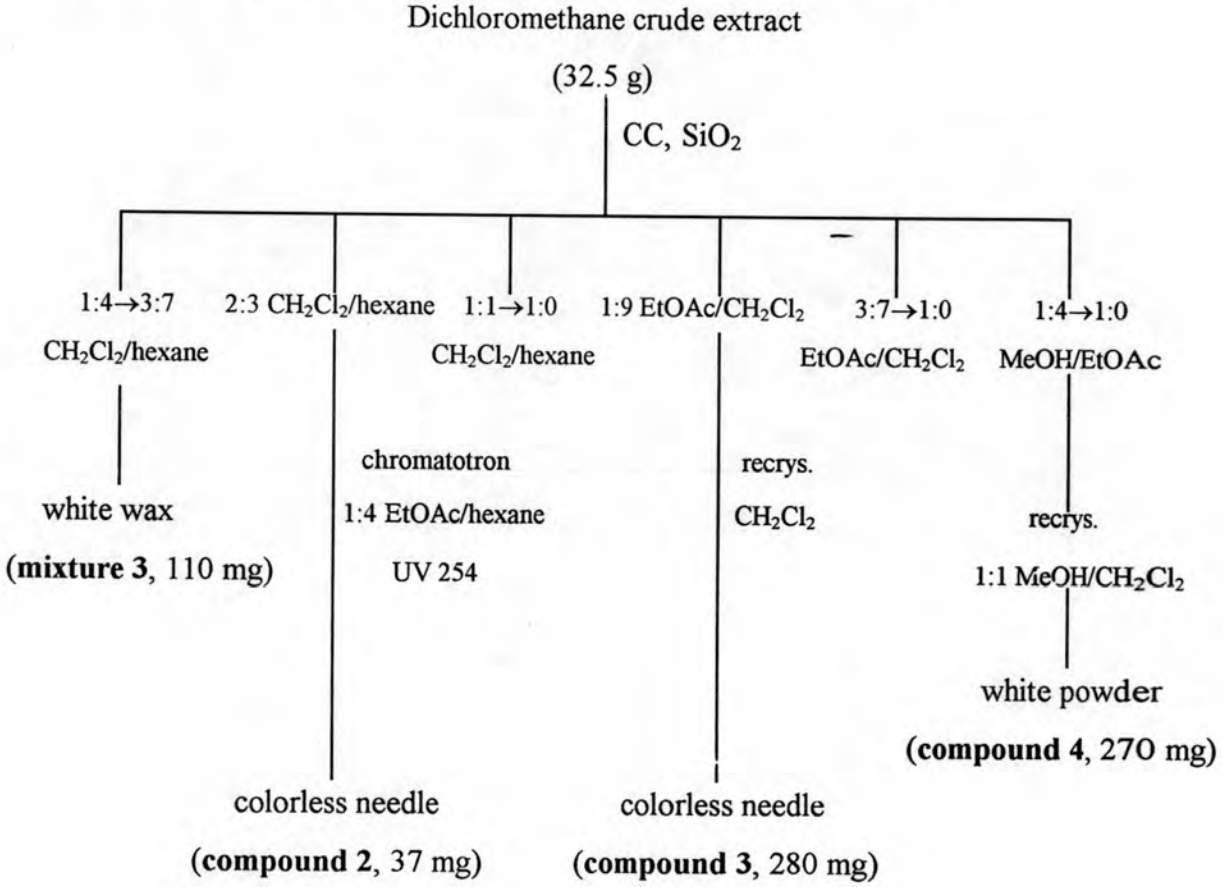
2.7 Separation and Purification

Hexane, dichloromethane and ethyl acetate crude extracts were separated by open column chromatography techniques. The extracts were dissolved in a small amount of a suitable solvent and mixed with silica gel (1:1) to afford the extract paste. The paste was evaporated to dryness under reduced pressure before being subjected on the top of a column. The column was eluted by increasing polarity of solvents. 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. The isolations of the mixtures and compounds from hexane, dichloromethane and ethyl acetate extracts of *A. arborescens* stems were briefly summarized in scheme 2.3-2.5, respectively.

Scheme 2.3 Isolation procedure of the hexane crude extract



Scheme 2.4 Isolation procedure of the dichloromethane crude extract



Scheme 2.5 Isolation procedure of the ethyl acetate crude extract