CHAPTER 2
EXPERIMENTAL

Plant Material

The dried heartwoods of *Mansonia gagei* Drumm were purchased from Vechapong drug store, Bangkok. The identity of this plant has been compared with a voucher specimen no.43281 at the herbarium of the Royal Foresty Department of Thailand.

General Procedure

Melting points were determined with a Fishers-Johns melting point apparatus and are uncorrected. Chromatotron equipment, Harrison Research Model 7924 T, was used for certain separations.[9] Thin layer chromatography (TLC)[10] was performed on aluminium sheets precoated with silica gel (Merck Kieselgel 60 PF_{254}). Column chromatography[11] was performed on silica gel (Merck Kieselgel 60 G) and flash chromatography[12] was performed on silica gel (40 μm average particle diameter).

The FT-IR spectra were recorded on a Fourier Transform Infrared Spectrophotometer model Impact 410: solid samples were incorporated in to potassium bromide to from a pellet. The ^{1}H-NMR and ^{13}C-NMR spectra including 2D-NMR were obtained with a Bruker model ACF 200 spectrometer and a Jeol, model JNM-A500 which operated at 200.13 MHz for ^{1}H and 50.32 MHz for ^{13}C nuclei and 500.00 MHz for ^{1}H and 125.00 MHz for ^{13}C nuclei, respectively. The chemical shifts were assigned by comparison with residue solvent protons. Gas chromatography analysis was carried out on a Shimudzu Gas Chromatography GC-7AG instrument equipped with a flame ionization detector with N_2 as a carrier gas. The column used for chromatography was OV-1. The mass spectra were obtained on Fisson Instrument model Trio 2000.

Ultraviolet-visible spectra were measured on Hewlett Packard diode array spectrophotometer. Medium Pressure Liquid Chromatography were performed on Buchi, model B-680 A.

Chemicals

All solvents used in this research were purified prior to use by standard methodology except for those which were reagent grade.

Chemical Reaction

Methylation[13]

The sample (0.2 g) was dissolved in dry acetone (20 mL) was treated with dimethyl sulfate (0.2 mL) and dry potassium carbonate (0.2 g) under reflux for 10 hr. After the reaction was completed, potassium carbonate was removed by filtration, and the filtrate was evaporated. The remaining material was then treated with dichloromethane and ammonia solution (1 mL). The organic layer was further washed with water (3 mL) and dried over anhydrous sodium sulphate. The dichloromethane layer was eventually filtered and evaporated. The methylated product was obtained upon purification using column chromatography.

Chemical Tests

Cardiac Glycoside Test[14]

About 5 mL of the methanol crude extract was treated with a 10% solution of lead acetate in water 40 mL. After heating for 15 minutes, the solution was cooled and filtered. The filtrate was extracted with dichloromethane (20 mL) three times. The

dichloromethane layer was dried over anhydrous Na_2SO_4 and the solution was evaporated to 1/10 of the original volume. The solution was divided into three parts.

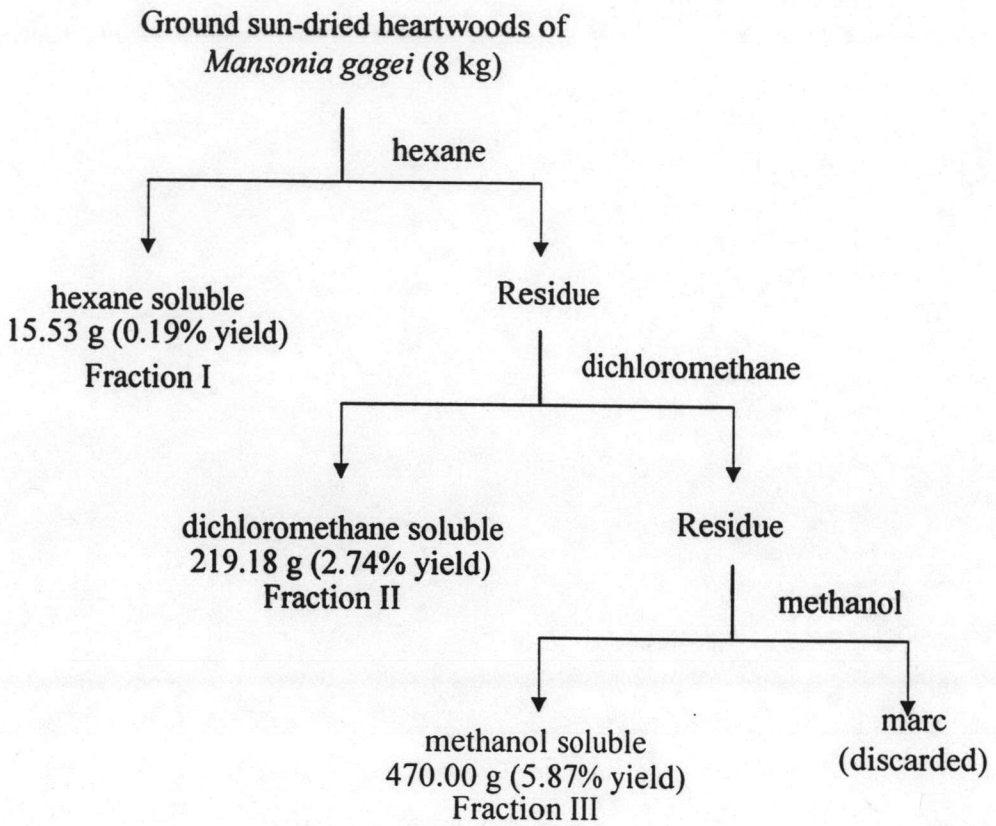
Liebermann-Burchard Reaction (steroid test): Dichloromethane solution in the first tube was evaporated almost to dryness and 3 drops of acetic anhydride were added with shaking. One drop of conc. H_2SO_4 was then added. The color change was observed immediately from pink $\rightarrow$ violet $\rightarrow$ blue $\rightarrow$ green.

Kedde's Reaction (α , β-unsaturated lactone test): Dichloromethane solution in the second test tube was evaporated almost to dryness and then about 10 mL of Kedde's reagent and 2-3 drops of 1 M NaOH were added. The positive test, a blue or violet/pink color, was observed.

Keller-Kiliani Reaction (deoxy sugar test): Dichloromethane solution in the third test tube was added about 3 mL of 5% $FeCl_3$. After shaking, conc. H_2SO_4 was poured along the side of the test tube. The positive test exhibiting a brown ring at the junction between two layers and the color in the upper layer was observed.

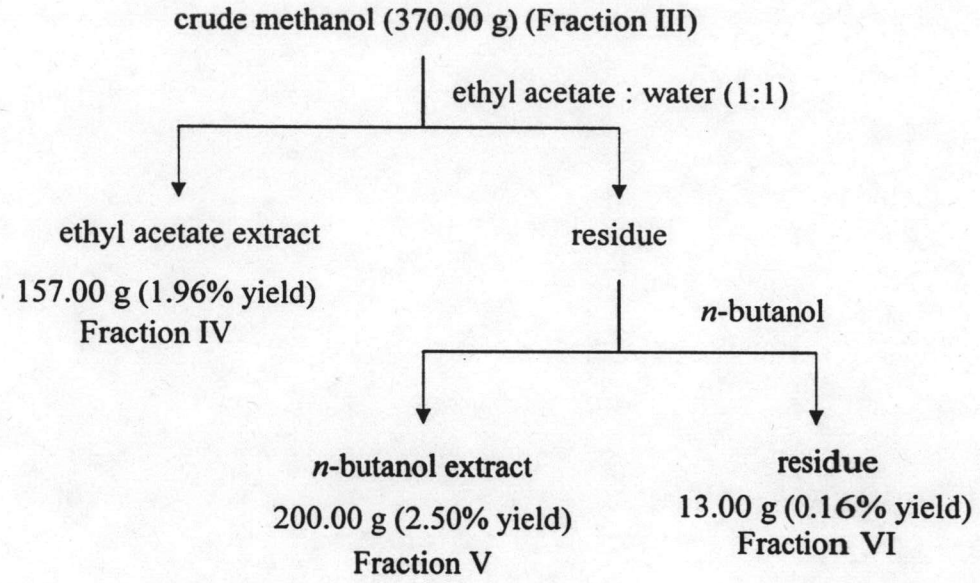
Extraction

Eight kilograms of dried heartwoods of *Mansonia gagei* were extracted with hexane at room temperature for 4 days. The solution was filtered and the solvent was removed by rotarory evaporator. The residue was reextracted with hexane for two more times. The hexane crude extract (Fraction I), 15.53 g (0.19% yield of the dried wood), was obtained as a dark-brown oil. The residue left after hexane extraction was soaked with dichloromethane until the solution was colorless and then the filtered solution was evaporated. The crude dichloromethane extract (Fraction II), 219.18 g (2.74% yield of the dried wood), was obtained as a brown oil. The residue left after dichloromethane extraction was further extracted with methanol until the extracted solution was colorless. The methanol extract was evaporated and a dark-black material (Fraction III), 470.00 g (5.87% yield of the dried wood), was obtained. The extraction procedure is shown in Scheme 2.1.



Scheme 2.1 The extraction procedure

The methanol crude extract (370.00 g) was partitioned between ethyl acetate and water in a 1:1 ratio to give the ethyl acetate soluble fraction (Fraction IV), 157.00 g (1.96% yield of the dried wood), and the water soluble fraction. The latter was then extracted with *n*-butanol to afford the *n*-butanol extract (Fraction V), 200.00 g (2.50% yield of the dried wood), and water soluble fraction (Fraction VI), 13.00 g (0.16% yield of the dried wood). The scheme for further extraction is shown in Scheme 2.2.



Scheme 2.2 The further extraction procedure of methanol crude extract

Bioassay Experiments

As mentioned earlier, one of the goals of this research was to search for an active principle from *Mansonia gagei* which could possibly use for medicinal purposes, particularly as an anticancer agent. The following bioassay experiments were therefore performed.

Brine Shrimp (*Artemia salina* Linnaeus) Toxicity Test[15]

The assay ic begun 36 to 48 h after sowing of the cysts (i.e., with larvae that are 18 h old). Multiwelled culture plates can be used for the bioassay, although any clear glass container with flat bottoms (for exaple, small beakers or glass vials) will do. Five nauplii are collected, using a Pasteur pipette, from the hatching dish and are transferred to a well, using the seawater 100 μL. The concentration of sample were 10, 100 and 1000 μg/mL). The tests solution are added, and the time is noted, thereby requiring 30 nauplii for each concentration of test sample. A parallel series of tests with the standard ethanol solution and blank control are always conducted.

Anticell Line Tests[16]

As a collaborative research between Natural Products Research Unit, Department of Chemistry, Chulalongkorn University and Beijing Medical School, Republic of China, this biological test was kindly conducted in China.