CHAPTER II

EXPERIMENTAL

2.1 Plant Material

C. fenestratum (Gaertn.) Colebr. was collected in October 2000 from Tha Khaek province, Laos. A voucher specimen is deposited in the herbarium of the Royal Foresty Department of Thailand (BKF No. 60811)

2.2 General Procedure

Silica gel Merck Kieselgel 60, no. 7734, 7731 were used for column chromatography and quick column chromatography, respectively. TLC analyses were carried out with Whatman precoated silica gel (Merck Kieselgel 60 PF_{254}) and spots on the plate were observed under UV light or visualized by spraying with 10% H_2SO_4 in ethanol followed by heating or by applying anisaldehyde to TLC plates and dried. Melting points were determined with a Fisher-Johns and Electrothermal melting point apparatus and are uncorrected.

NMR spectra were recorded in $CDCl_3$, DMSO-d_6 and pyridine-d_5 on Bruker ACF 200, AV-500 and a Jeol JNM-A500 spectrometer using tetramethylsilane (TMS) as an internal reference in the ^1H and ^{13}C measurement and standard Bruker pulse sequence for two-dimensional experiments. Assignment of the ^{13}C-NMR chemical shift was made with the aids of DEPT spectra. The FT-IR spectra were recorded on a Fourier Transform Infrared Spectrophotometer model Impact 410 (KBr pellets). The mass spectra were obtained on Fission Instrument model Trio 2000 operating at 70 eV ionization voltages.

2.3 Chemicals Reagants

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

2.4 Chemical Tests

2.4.1 Steroid and Triterpenoid Test

Liebermann-Burchard's Reaction[22]

A sample (2-3 mg) was dissolved in dry chloroform 0.5 mL and a few drops of acetic anhydride was added, shaking solution and followed by one drop of concentrate sulfuric acid. After a couple minutes, the color change was observed. The deep green indicated the presence of steroidal ring system while the purple color suggested a triterpenoid skeleton.

2.4.2 Alkaloid Test

Dragendroff's reagent[23]

Add a few drops of Dragendroff's reagent into the sample solution. The orange precipitate suggested the presence of alkaloid nucleus.

2.5 Chemical Reactions

2.5.1 Reduction of Compound 2[13, 14]

Compound 2 (100 mg) was dissolved in methanol. Upon addition of a few grain of sodium borohydride the solution became decolorized. After 10 min the methanol was evaporated under reduced pressure. The residue was dissolved in chloroform and filtered. The chloroform solution was taken to dryness, Compound 2A was obtained.

2.5.2 Preparation of Berberine Salt

Berberine phosphate[24]

Compound 2 (500 mg) was suspended in acetone and excess of 20% phosphoric acid was added. A yellow precipitate was collected and washed with cold methanol. After recrystallized many times with a mixture of methanol and water, a yellow solid was obtained. Yield: 350 mg (70%), R_f value of 0.68 (20% methanol in dichloromethane).

Berberine sulphate [24]

Compound 2 (500 mg) was dissolved in 20% acetic acid and heated at 50 °C for 30 min. The decoction was filtered and evaporated to a syrupy liquid. After dissolved a syrupy liquid with boiling water, methanol and a small amount of 20% sulfuric acid were added. A yellow needle crystal was collected. Yield: 430 mg (86%), R_f value of 0.65 (20% methanol in dichloromethane).

Berberine carbonate[24]

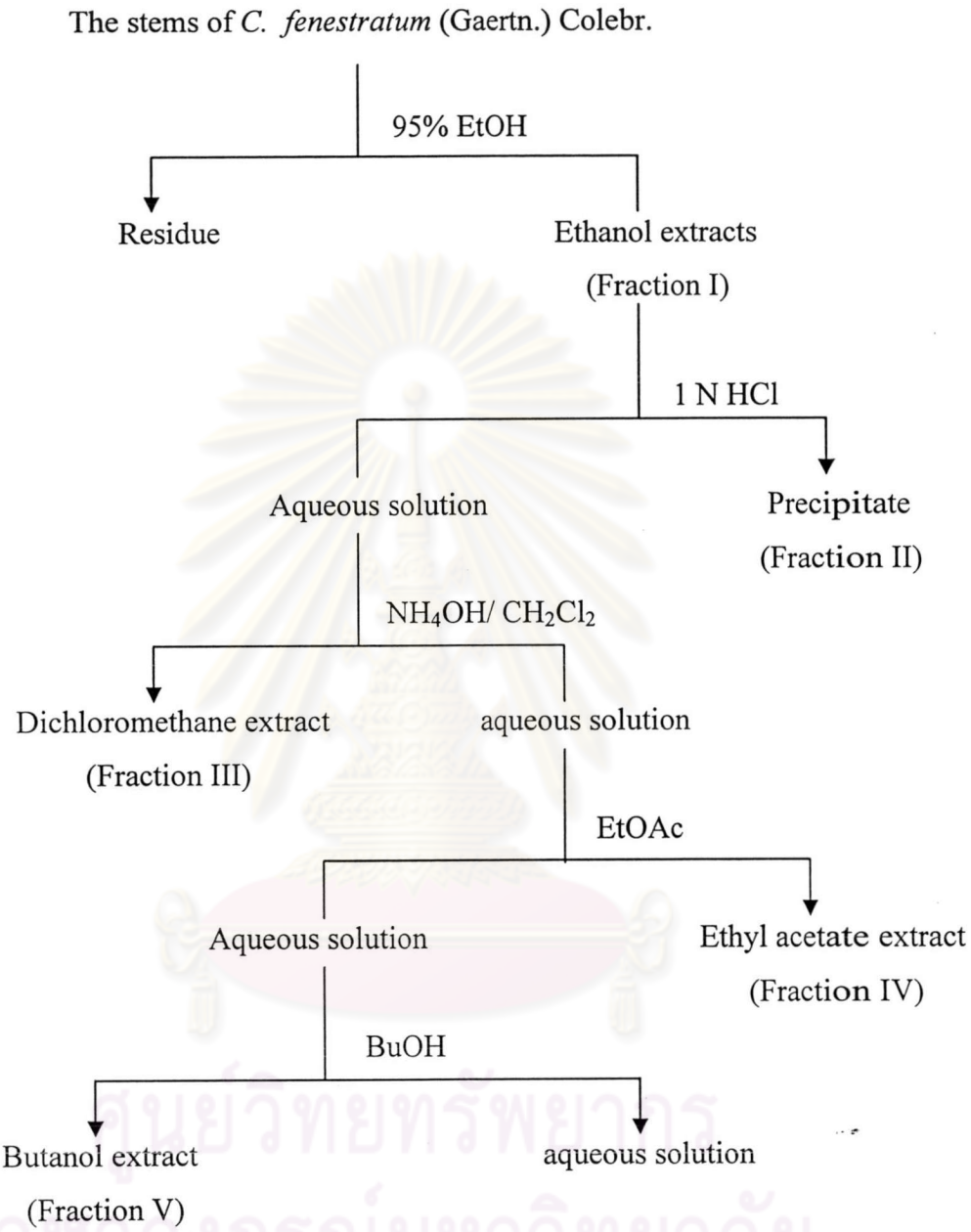
Compound 2 (500 mg) was dissolved in methanol and carbon dioxide was passed into a concentrated alcoholic solution. After salt formed completely, a yellow precipitate was collected and washed with cold water and dried. Yield: 400 mg (80%), R_f value of 0.70 (20% methanol in dichloromethane).

Berberine picrate[25]

Compound 2 (200 mg) was dissolved in 50% acetic acid and saturated solution of picric acid was added. An orange yellow solid was collected, washed with water and dried. Yield: 175 mg (88%), R_f value of 0.48 (8% methanol in dichloromethane).

2.6 Extraction Procedure

Ground sun-dried stem (2.6 kg) was successively percolated with 95% ethanol for four days at room temperature and then filtered solution was removed under vacuum giving ethanol extract. This process was repeated for three times. The obtained ethanolic crude (Fraction I) was treated with 1 N hydrochloric acid and precipitate fraction (Fraction II) was obtained. Ammonium hydroxide was added to aqueous solution, which was then extracted with dichloromethane, ethyl acetate and butanol using liquid-liquid extractor and evaporated to furnish dichloromethane (Fraction III), ethyl acetate (Fraction IV) and butanol (Fraction V) crude extracts. The extraction procedure is exhibited in Scheme 2.1



Scheme 2.1 The general extraction procedure

2.7 Bioassay Experiment

In the search for biologically active compounds from *C. Fenestratum* (Gaertn.) Colebr., the bioactivities of crude extracts and pure compounds were assayed using the following bioassay experiments.

2.7.1 Brine Shrimp Cytotoxicity Lethality Test (BSCLT) [26, 27]

The samples were assayed at 10, 100, 1,000 μg/mL in DMSO and by using 10 second-star larvae of the brine shrimp *Artemia salina* Linn. in triplicate. The brine shrimp were observed periodically over 24-h period. After 24 h contact, the number of surviving organisms was recorded and the LC_{50} was evaluated by the calculation using the probit statistical analysis program.

2.7.2 Plant Growth Regulation Assay [28]

Pure compound was prepared for the concentration of 10, 100 and 1000 μg/mL in an appropriate solvent. Three milliliters were taken and poured into petri dishes (diameter 90 mm) that contained a filter paper disc. Each concentration was performed three replications. A control was an equal amount of the solvent used to prepare the tested solution. Thirty seedlings of tested weed were transplanted in each petri dish. Then, the petri dishes were covered with transparent film and kept in a growth chamber at 30°C for 24 h daylight. After seven days, five seedlings were randomly selected to measure the length of root and shoot. Each experiment was repeated for three replications. The inhibition effect of the substance was calculated with the formula:

$$\% \text{ Inhibition} = [(A-B)/A] \times 100$$

Where "A" was the means of root length or height of secondary leaf sheath in control.

"B" was the means of root length or height of leaf sheath in control.

Growth inhibition of 100 % indicated a complete inhibitory effect.

2.7.3 Antipathogenic Fugal Assay [29]

This biological assay was kindly performed by C. Thammakasadsri, a master student majoring in Biotechnology, Faculty of Science, Chulalongkorn University.

Using the agar medium assay was carried out for this bioassay. Each experiment was performed for three replications. The results were expressed as the percentage of inhibition, which had relative to the inhibitory effect of substance to pathogenic fungi.

In addition, anti HSV-1, cytotoxicity, anticancer, antimalarial, antifungal and antituberculosis activity tests were conducted at Bioassay Laboratory, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency Building, Thailand.