CHAPTER II

MATERIAL AND METHODS.

2.1 Plant Materials

The air - dried whole plants of ***Euphorbia hirta*** Linn. (1,000 g), the air - dried leaves , stem and root of ***Euphorbia heterophylla*** Linn. (1,000 g , 1,000 g and 500 g) and the whole plant of ***Euphorbia thymifolia*** Linn.(1000 g) were collected from Nakhon Pathom , Thailand in June 1996.

2.2 General Procedures

Melting points were determined with a Fisher Johns melting point apparatus and are uncorrected. Thin layer chromatography (TLC) was performed on aluminium sheets precoated with silica gel (Merck Kieselgel 60 PF_{254}) (Randerath,1996). Column chromatography was preformed on silica gel (Merck Kieselgel 60 G) (Fessenden,1983).

The FT-IR spectra were recorded on Nicolet Model impact 410. Solid samples were incorporated to potassium bromide to form pellets. The ^{1}H-NMR and ^{13}C-NMR spectra were obtained from Fourier Transformed Nuclear Magnetic Resonance Spectrometer of a Bruker model AC-F 200 spectrometer.

2.3 Chemicals

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

Other substance : Merck's silica gel 60 Art. 7734 1000 (70-230 mesh ASTM) was used as adsorbents for column chromatography. Other chemicals, unless otherwise stated were of the highest purity available purchasing from Fluka chemical company and used without further purification.The spots were visualized with 10% H_2SO_4 in ethanol after detecting with UV lamp (254 or 365 nm).

2.4 Chemical Test

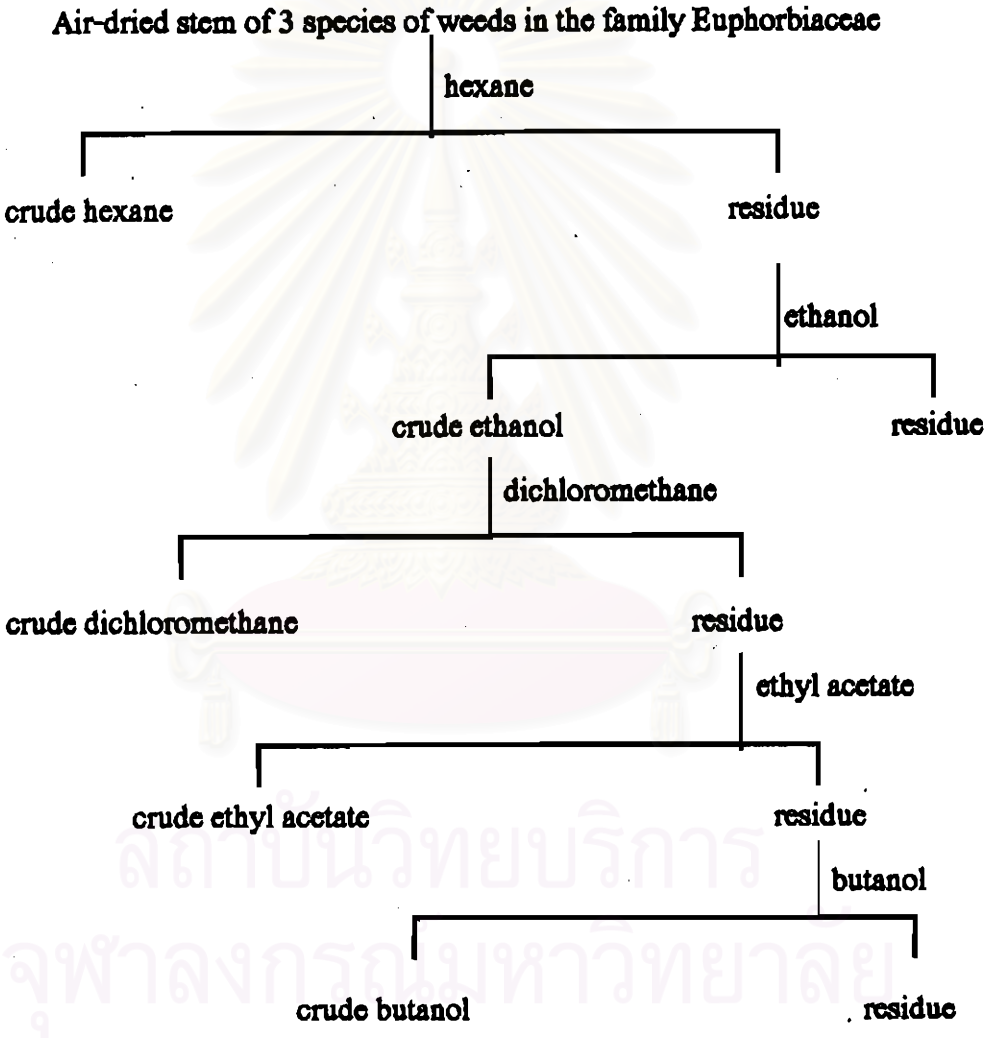
Liebermann Burchard's Test (Cook,1961)

To a solution of the sample to be tested (2-3 mg) in dried chloroform (0.5 ml.) was added a few drops of acetic anhydride, followed by one drop of concentrated sulfuric acid. If an unknown was steroid, the color would gradually change from pink to deep green. In case of an unknown was triterpenoid, the color would change to reddish pink.

2.5 General Extraction for Preliminary Study

A plant sample was air-dried and milled. Each specimen was then soaked in hexane for 3 days at room temperature. The soaking procedure was repeated three times or until the color of the extract was clear and then soaked in 95% ethanol for 3 days at room temperature. The soaking procedure was repeated until the color of extract was

clear. The extract was evaporated by using rotary vacuum evaporator to almost dryness, and then partition ethanol extract with dichloromethane, ethyl acetate and butanol. These extracts were evaporated by using rotary evaporator to almost dryness. The extract procedure is showed in Scheme 2.1.



Scheme 2.1 Extraction for Preliminary test

2.6 Biological Test

2.6.1 Inhibition of Rice Growth Bioassay Experiments.

Preliminary test.

2.6.1a) Sample Preparation (Zungsonthiporn,1993)

The crude extract of hexane, ethanol, dichloromethane, ethyl acetate and butanol from **2.5** was diluted with the solvent that was used for extraction to contain 1.0, 0.5 and 0.1 g for 3 ml9which were equivalent to the extracts from 1.0, 0.5 and 0.1 g of *E. heterophylla*, respectively). Three ml of each concentration was poured in glass tubes (∅ 30 mm.×120 mm.) which contained 1.5 g of cellulose powder(Toyo, type D), and 3 tubes were used for each concentration. The same amounts of each solvents were added instead of extraction as control. All the tubes were dried up *in vacuo* oven at 50°C overnight. Then cellulose powder was stirred well-mixed, 4.2 ml of distilled water was added to each tube.

2.6.1b) Preparation of rice for testing

Soaking rice (*Oryza sativa* cv. RD 23) in distillation water for 12 hours. Then germinated for 20 hours. The seedling that showed 1-2 mm.length of radical were selected.

2.6.1c) Bioassay

Six seedlings(1-2 mm. Length of radical) were transplanted in each tube. Then the tubes were sealed with transparent vinyl film and kept in growth chamber at 30oC, 24 hours daylight. Lengths of the root and the second leaf sheath were recorded 7 days after treatment. Compared with control and calculated in percentation of inhibition by

$$\text{inhibition (\%)} = \frac{\text{A-B} \times 100}{\text{A}}$$

Where A is the average root length and leaf sheath height of control and B is the average root length and leaf sheath height of the treated in each concentration.

2.6.2 Brine shrimp Cytotoxicity Lethality Test.

A method utilizing brine shrimp (***Artemia salina*** Linn.) is proposed as a simple bioassay for natural product research. (Olis ***et.al.***, 1993) The procedure determines LC50 values in μg/ml. Of active compounds and extracts in brine shrimp medium. Although there were several methodologies, a convenient microwell method (Harborne,1973) was adapted for this experiment, caused this method is rapid, inexpensive and convenient to guide phytochemical screening and fractionation. (Meyer ***et.al.***, 1983)

2.6.2a) Hatching the Shrimp (Colegate and Molyneux,1987)

Brine shrimp eggs were hatched in a shallow rectangular dish (13×18 cm) filled with artificial sea water was prepared by dissolving about 38 g of a commercial sodium chloride in 1 liter of distilled water. The shallow rectangular dish had two unequal compartments and had two little pores that made two parts joined. The eggs (***ca.*** 30 mg) were sprinkled into the larger compartment which was darkened, while the smaller compartment was illuminated. After 24 hours the phototropic nauplii were collected by pipette from the light side, having been separated by the divided from their shells.

2.6.2b) Preparation of test solution

Sample were prepared by dissolving 4 mg of interested compound in 80 μL of solvent and added artificial sea water to make 4,000 μL (Solution A). Solution B was prepared by diluting 400 μL of Solution A with 3,600 μL of artificial sea water. Then diluting 400 μL of Solution B with 3,600 μL of artificial sea water to obtain Solution C. Concentration of Solutions A, B and C were 1,000, 100 and 10 μg/mL, respectively.

Control solution was prepared by using 80 μL of solvent and make the volume of the solution to be 4,000 μL by using artificial sea water.

2.6.2c) Bioassay procedure

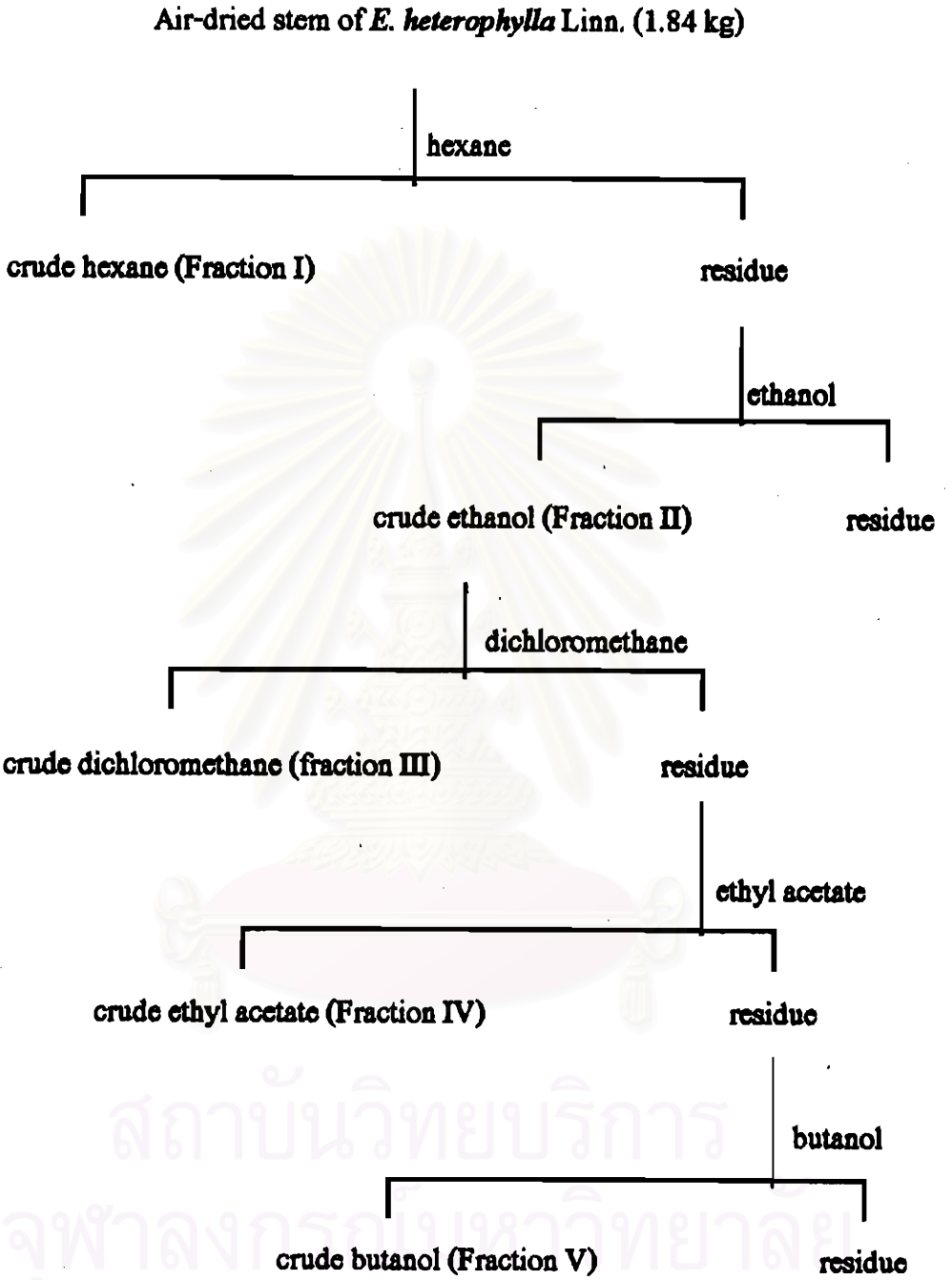
Five brine shrimps in 100 μL artificial sea water were transferred to each well containg 400 μL of a tested solution. Each compound with 3 concentrations (Solution A,B and C) was tested with six replications for each solution. All tests had control solution. The plates were covered and incubated at 22-29 °C for 24 hours. Plates were then examined under a binocular microscope and the numbers of dead (non-motile) nauplii in each wellwere counted. LC_{50} values were then calculated by Probit analysis, which showed in μg/mL in brine shrimp medium.

2.6.3 The Inhibitory Effect for Carcinoma Cell lines

Several crude extracts from E. hirta, E. thymifolia and E. heterophylla were preliminary screened by using the MTT assay (Wiboonphan,1996). This method used eight Carcinoma Cell lines; Human Gastric Carcinoma (BGC-823), Human Hepatocellular Carcinoma (Bel-7402), Human Nasopharyngeal (KB), Human Leukemia Carcinoma (HL-60), Proliferation of Mouse (B) Lymphocyte, Proliferation of Mouse (T) Lymphocyte, Human Colon Carcinoma (HCT-8) and Human Erythroleukemia Carcinoma (K-562). These experiment were performed by researchers at Beijing Medical School, Beijing, China.

2.7 Extraction

The air dried stems of *E. heterophylla* Linn. (1.84 kg) were minced to coarse powder. The plant initially extracted with n-hexane by soaking for 4 - 5 days at room temperature. The soaking procedure was repeated for three times. The solution was filtered and evaporated yielding a hexane crude extract as viscous green liquid (Fraction I) 34.6 g. The plant residue was then extracted with 95% ethanol by using the same fashion as that described for using hexane as a solvent, giving an ethanolic extract as black liquid (Fraction II) 170.59 g. the ethanolic crude extract was further extracted with dichloromethane (FractionIII) 40.53 g., ethyl acetate (Fraction IV) 25.48 g and butanol (Fraction V) 20.15 g. The extraction procedure of the stem of *E. heterophylla* Linn. is depicted as showed in Scheme 2.2



Scheme 2.2 Extraction and fractionation of *E. heterophylla* L.