CHAPTER II
EXPERIMENTAL

Plant Material

The roots of *Trigonostemon reidioides* Craib. were bought from Vechapong Drug store. The voucher specimens (BKF No. 37530) have been lodged and were identified by comparing with those in the Herbarium of the Bangkok Forestry Department. These dried-roots were ground into small pieces.

General Procedure

Melting points were determined with a Fishers-Johns melting point apparatus and are uncorrected. Thin layer chromatography (TLC) was performed on aluminium sheets pre-coated with silica gel (Merck 's, Kieselgel 60 G) and quick column chromatography was proceeded on silica gel (5-40 μm small particle diameter).

The IR spectra were recorded on NICOLET IMPACT 410 FT/IR spectrophotometermeter. The ^{1}H and ^{13}C-NMR spectra including 2D-NMR were performed in deuterated chloroform (unless specified otherwise) with tetramethylsilane as an internal reference on Fourier Transformed Nuclear Magnetic Resonance Spectrometer of a Bruker, model AC-F 200 and a Jeol, model JNM-A 500. Mass spectrometry (MS) analysis was conducted on Fission instrument model Trio 2000.

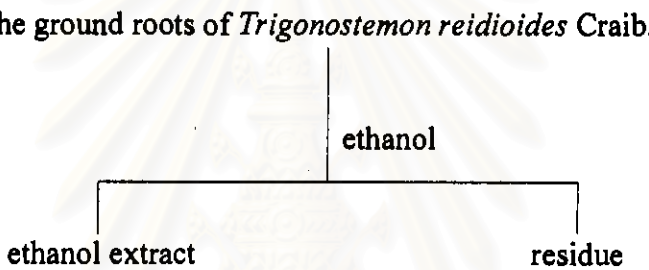
Chemicals

All solvents used in this research were purified prior to use by standard methodology except for those which were reagent grade. Merck 's silica gel 60 G Art 7734 (70-230 mesh) and silica gel 60 G 7731 were used as adsorbents for column chromatography and quick column chromatography, respectively.

Extraction for Preliminary Screening Test

The dried roots of *Trigonostemon reidioides* Craib. were ground into small pieces. The sample of approximately 1 kg was extracted by soaking in ethanol for a week at room temperature. The soaking was repeated for several times until the color of the extract was clear. The solution was filtered and the solvent was evaporated, an ethanolic crude extract was obtained. The general scheme of the extraction for preliminary screening tests is shown in Scheme 2.1

Scheme 2.1 Extraction procedure for the roots of *Trigonostemon reidioides* Craib .



Extraction and Initial Fractionation

The ground roots of *Trigonostemon reidioides* Craib. were extracted by the order of polarity of solvent with two different procedures.

The first procedure

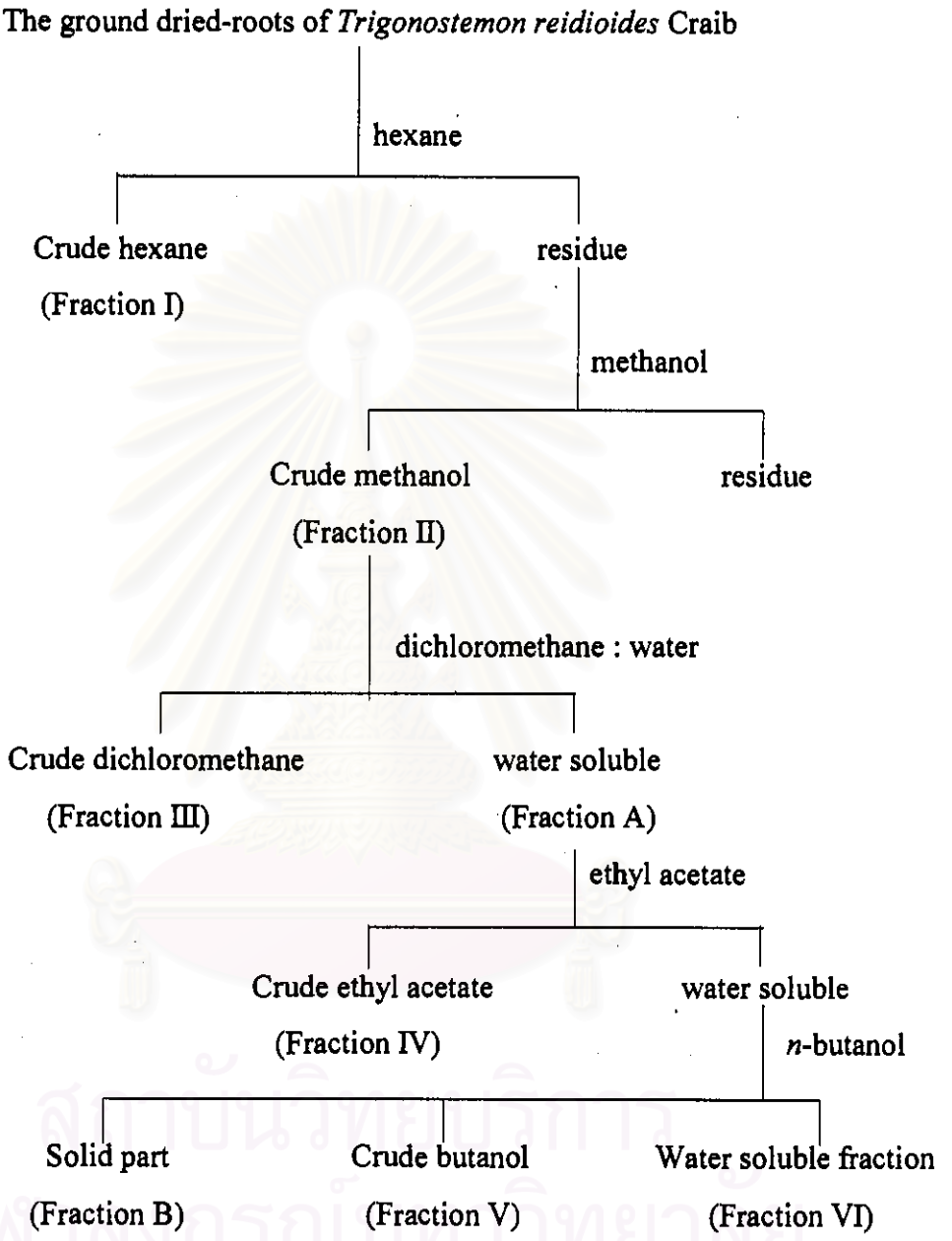
The ground roots of *Trigonostemon reidioides* Craib. (28.5 kg) were initially extracted with *n*-hexane by soaking at room temperature for 7 days. The soaking was repeated for 3 times. The solution was filtered and the solvent was evaporated yielding a hexane crude extract, 40.38 g (0.14% yield) (Fraction I). The plant residue was then extracted with methanol by using similar procedure to that of the extraction with hexane. After evporation of the solvent in *vacuo*, the methanolic extract 488.32 g (1.71% yield) (Fraction II) was obtained. The methanolic crude was equilibrated by partition between dichloromethne and water to afford a dichloromethane soluble fraction 57.06 g (0.2% yield) (Fraction III) and a water soluble fraction 221.55g (0.78% yield) (Fraction A). The Fraction A was further extracted with ethyl acetate and *n*-butanol, respectively by using

separatory funnel to gain an ethyl acetate soluble fraction 71.59 g (0.25% yield) (Fraction IV), *n*-butanolic soluble fraction 5.36 g (0.02% yield) (Fraction V), water soluble fraction 134.09 g (0.47% yield) (Fraction VI) and solid part (Fraction B), respectively.

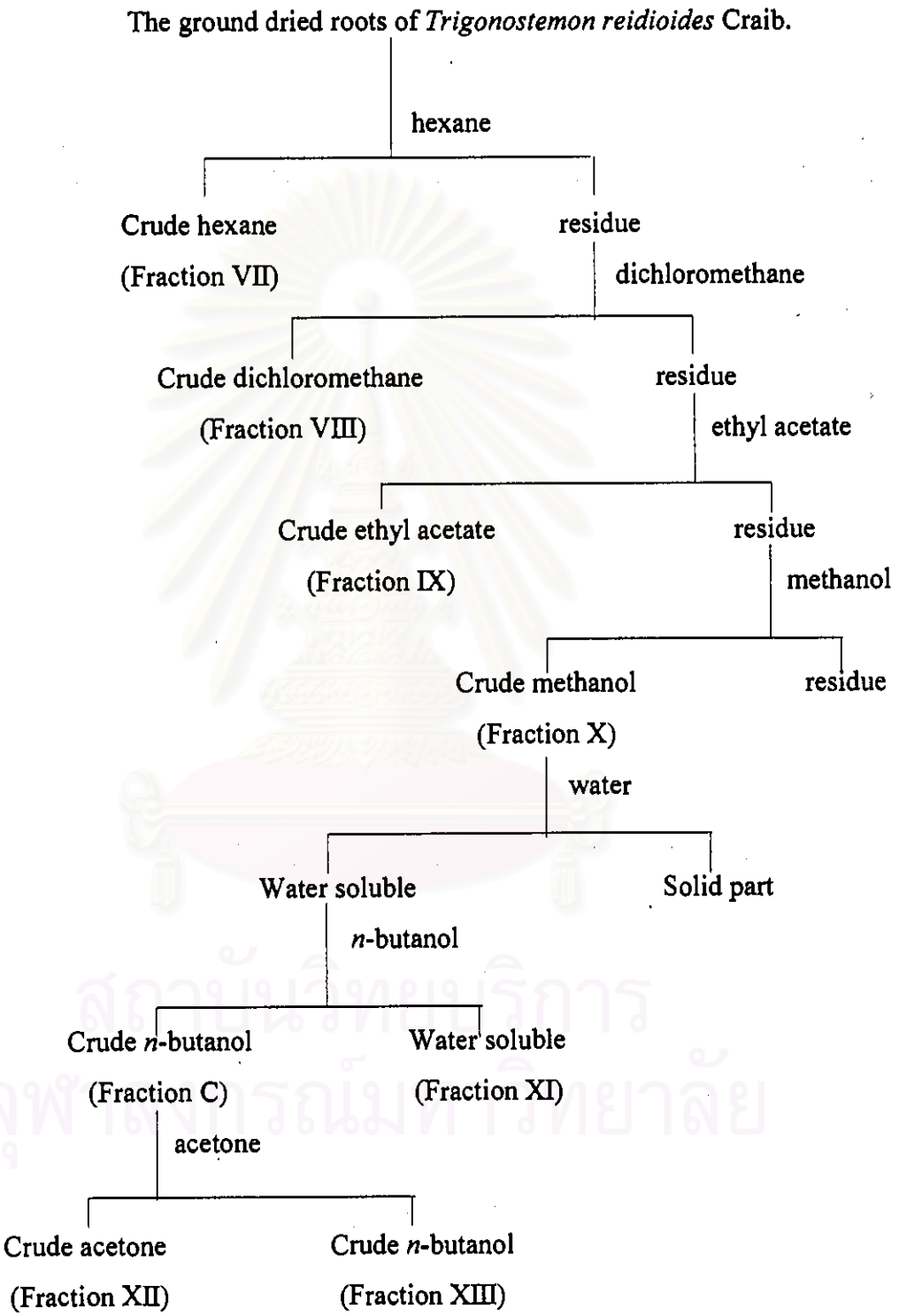
The second procedure

This extraction procedure was carried out using similar fashion to that described earlier by soaking the plant material (15.0 kg) in *n*-hexane, dichloromethane, ethyl acetate and methanol, respectively. Four crudes namely *n*-hexane crude Fraction VII (29.85 g, 0.19% yield), dichloromethane crude (Fraction VIII, 32.26 g, 0.21% yield), ethyl acetate (Fraction IX, 21.35 g, 0.14% yield) and methanol crude (Fraction X, 240.81 g, 1.61% yield) were obtained. Fraction X was further extracted by stirred with water given the water soluble part and solid part. The water soluble part was extracted continue with *n*-butanol by using separatory funnel to gain butanolic soluble fraction (Fraction C, 7.1 g, 0.05% yield) and water soluble fraction (Fraction XI, 88.73 g, 0.59% yield). The butanolic fraction (Fraction C) was further extracted with acetone to yield an acetone crude extract (Fraction XII, 3.91 g, 0.03% yield) and a remianing *n*-butanolic crude (Fraction XIII, 2.70 g, 0.02% yield). The general schemes for extraction and initial fractionation of the roots of *Trigonostemon reidioides* Craib. by two procedures are shown in Schemes 2.2 and 2.3.

Scheme 2.2 Extraction and fractionation by the first procedure



Scheme 2.3 Extraction and initial fractionation by the second procedure



Synthesis

The hydrolysis of compound A

Dissolved compound A 100 mg with dichloromethane in the 100 mL round bottom, added 10% ethanolic NaOH 100 mL and stirred with together by refluxing on oil bath for 24 hours, investigated the reaction was completed by thin layer chromatography compared with starting material. After reaction was completed put the solution in the distilled water 100 mL and acidified with diluted hydrochloric acid, the product was yielding and filtered by suction and purified by crystallization with dichloromethane-methanol in several times, the compound **A1** was obtained.

The methylation of compound A with diazomethane [29]

Dissolved 50 mg of the compound A in a little anhydrous ether, cooled in ice and added the etherael solution of diazomethane in the small portions until the solution was to be pale yellow. And added diazomethane to over excess by observation the pale yellow color of the solution existed for the presence, tested the reaction was completed by thin layer chromatography compared with starting material. After the reaction was completed, evaporated to removed the solvent out and the white solid was recieved. Purification the compound by crystallization with dichloromethane-meyhanol in several times, the compound **A2** was obtained.

Bioassay Experiment

The insect antifeedant activities employing Greater wax moth larvae (*Galleria mellonella* Linn.) were conducted.

Preparation of Greater Wax Moth *Galleria mellonella* Linn. [30]

Larvae: Greater Wax Moth larvae were obtained from the damage combs of Bee Research Unit of Chulalongkorn University, Tambol Bangkhantak, Amphur Muang, Samutsongkharam province, Thailand. The wax moth adult larvae were reared on artificial diet and life stages were kept in environmental chambers 27°C at Bee Research Unit, Department of Biology, Chulalongkorn University. The larvae food were modified according to those described by Dutky *et al.*, 1962. The mixtures were consisted of ceresol : bee pollen (3:1) and honey : distilled water : glycerol (1:1.4:1.1).

Insect Antifeedant Test [31]

General Procedure

The larve food consists of ceresol and bee pollen (approximately 3:1) and honey, mixed together (amount of honey about 10 mL in ceresol 3 g and bee pollen 1 g). Two grams of food were put in a square bowl, folded aluminium foil of size 3×3 cm^2. The food bowls were weighed before use. The plant crude extract solutions of various concentrations (percentage wt. by wt. of food) were dripped into food bowl, labeled as "test". The control food was prepared by dripping the same solvent used to dissolve the crude extract or an appropriate solvent. The solvent was allowed to evaporate from each food bowl by air drying for 3 hours. After that each bowl was reweighed and then placed pair-wise (test + control) in a plastic box. Ten larvaes of the same size, 0.75 cm length, were chosen and put in the same box. It was kept in the incubator at temperature 35 - 36°C. After 2 days, the larvaes were counted and both food bowls were weighed to determine the weight loss from tested food and controlled food. Insect antifeedant activity was expressed as a % T/C value, where :

$$\% \text{ T/C} = (1 - \text{weight loss of tested sample} / \text{weight loss of controlled sample}) \times 100$$

- T/C value of 100 % represents total inhibition of insect feeding activity