CHAPTER II
EXPERIMENTAL

2.1 Plant Materials

The air-dried samples of *Achyranthes aspera* L. (stem), *Cleome viscosa* L. (leave), *Senna surattensis* (Burm.f.) (stem), *Piper aurantiacum* Miq. (seed), *Piper chaba* Hunt (fruit), *Piper cubeba* L.f. (fruit), *Piper nigrum* L. (seed), *Piper longum* L. (fruit), *Piper sarmentosum* Roxb. (fruit), *Mitragyna speciosa* (Roxb.) Korth (leave), *Paederia linearis* Hock.f. (leave), *Zingiber montanum* (Koenig) Link ex Dietr. (rhizome), *Zingiber zerumbet* (L.) Sm. (rhizome) (Tem, 2544) (Somsuk, 2546) were bought from Vechapong drug store, Bangkok in 2003. *Trigonostemon reidioides* (Kurz) Craib (leave) was obtained from Natural Products Research Unit, Thailand.

2.2 Chemicals

All solvents used in this research were purified prior to use by standard methodology except for those which were reagent grades. Other substances: Merck silica gel 60 Art. 7734.1000 (70-230 mesh ASTM 0.063-0.200 mm) was used as adsorbent for column chromatography, Merck silica gel 60 Art.7731.1000 (230-240 mesh ASTM 0.040-0.063 mm) was used as adsorbent for quick column chromatography and Merck TLC aluminum sheet, silica gel 60 F_{254} was used for identifying the fraction. Spots on the plate were observed under UV light or visualized by spraying with 10% H_2SO_4 in EtOH followed by heating at 100-110°C for 3 min. The following standard insecticides with purity of > 95%, namely, carbosulfan (98%) was purchased from Chem service Ltd.

2.3 Chemical Tests

2.3.1 Dragendroff Test

The sample was spotted on TLC plate and then developed in appropriate solvent system. After that, it was dipped into the Dragendroff's reagent. The positive results could obviously bring dark-orange spots suggesting the presence of the alkaloid nucleus.

2.4 Instruments and Equipment

2.4.1 Proton and Carbon-13 Nuclear Magnetic Resonance Spectrometer

The ^{1}H- and ^{13}C- NMR spectra were obtained in deuterated chloroform ($CDCl_3$) as an internal reference on Fourier Transform Nuclear Magnetic Resonance Spectrometer of Varian model Mercury plus 400.

2.4.2 Fourier Transform Infrared Spectrophotometer (FT-IR)

The FT-IR spectra were recorded on Nicolet impact 410 spectrophotometer. Spectra of samples were recorded as KBr pellets for solid samples.

2.4.3 High Pressure Liquid Chromatography (HPLC)

The HPLC analysis was carried out by using a Water model 600 controller and a Water model 2996 photodiode array detector. The column was Apollo C18 reverse phase (250 mm x 10 mm).

2.4.4 Plant Growth Chamber

CONTHERM controller model was used for control temperature, humidity and light.

2.4.5 Microapplicator

Burkard model: PAX-100-2 microsyringe HAMINTON 10 μL The microapplicator was used at Faculty of Science, Ramkhamhaeng University.

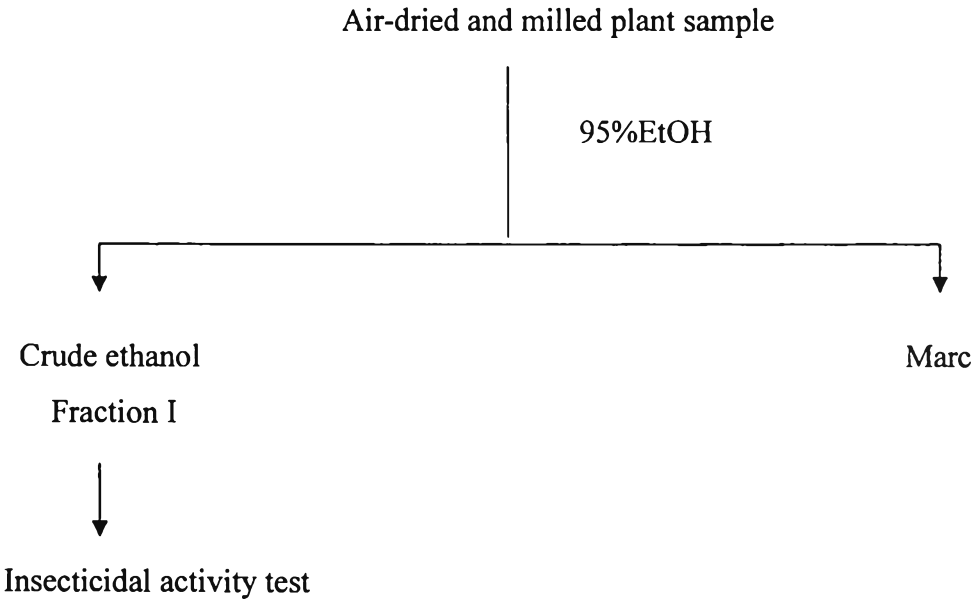
2.4.6 Melting Point (m.p.)

The melting points were obtained on a Fisher-John apparatus and are uncorrected.

2.5 Extraction Procedure

2.5.1 The Extraction for Preliminary Screening Test

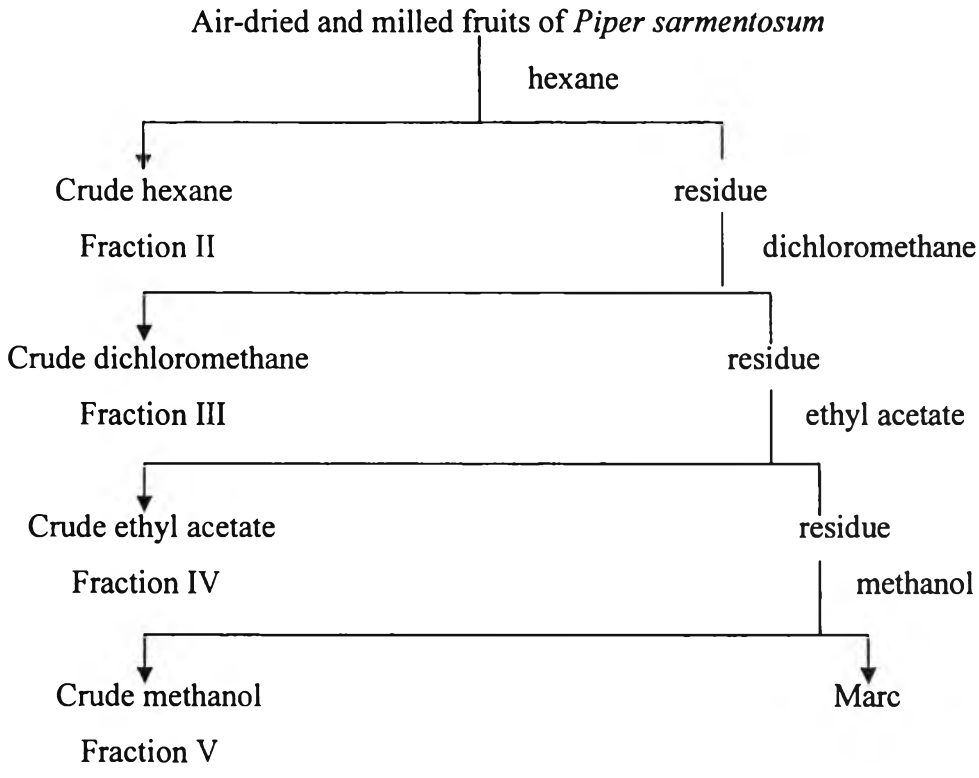
The air-dried and milled plant samples were extracted by soxhlet with 95%EtOH at 78°C until the color of extraction was clear. The EtOH extract was then evaporated using rotatory vaccum evaporator to almost dryness (Scheme 2.1). Each crude extract was subjected to insecticidal test against adult brown planthoppers by contact poison.



Scheme 2.1 The general extraction procedure for preliminary screening test of insecticidal activity.

2.5.2 The Extraction for Separation

The air-dried and milled fruits of *Piper sarmentosum* 6.2 kg were extracted with hexane by soxhlet for 6-7 days until the color of extract was clear. The hexane extract was evaporated using rotatory evaporator to almost dryness. The plant residue was further extracted with CH_2Cl_2 by the same fashion until the color of extract was clear. Then the CH_2Cl_2 extract was evaporated. The plant residue was further extracted with EtOAc by the same procedure until the color of extract was clear. After being concentrated, the EtOAc extract was obtained. The extraction of the residue was further proceeded by MeOH giving the MeOH extract. The extraction procedure is presented in Scheme 2.2.



Scheme 2.2 The extraction procedure

2.6 Bioassay Experiments

2.6.1 Biological Materials

The bioassay experiments were conducted during January 2004 to September 2005. The insects were reared under natural conditions in Thailand.

Adult of brown planthoppers (F_1) from Chinat population were obtained from Rice Research Institute, Department of Agricultural, Ministry of Agriculture and Cooperatives. Wantana *et al.*, 1990 reported that among brown planthoppers from 17 provinces of Thailand, those from Chinat, Khonkhaen, Pichit and Pattalung could resist insecticides in the class of carbamate, organophosphate and synthetic pyrethroid while those from Phrae could not resist to insecticides. The adult of brown planthoppers were reared into wooden cage (30 x 60 x 40 cm) (Figure 2.1) and mass reared on rice division (RD) 7 after two days transplanting to another new pots of rice plants.

2.6.2 Insecticidal Activity Tests

2.6.2.1 Contact poison

Topical application (Busvine, 1971) was used to test against adult brown planthoppers. The method was test mode of action of the compound. The adult brown planthoppers were kept into the standard condition 25°C, 70% of humidity for 24 h. After that adult brown planthoppers were anesthetized with CO_2 for 40-45 seconds at flow rate of 2.5 mL/sec (Heinrichs *et al.*, 1981). By the use of microapplicator and microsyring (Figure 2.2), 0.2 μL of insecticide solution was treated on prothorax female adult brown planthoppers. They were transferred into a glass tube (2.7 Ø x 20 high cm) in the presence of rice division (RD) 7- 10 adults: 1 tube, 4 replications were performed for each model. The first one was untreated and the second one was rinsed by acetone. The treated adult brown planthoppers were kept at 25°C, 70% of humidity under long-day (16L/8D) condition. The numbers of dead insects were counted after being treat for 24, 48 and 72 h.

2.6.2.2 Systemic poison

Paraffin method (Nagata, 1981) and (Henrich and Valencia, 1981) has a simple method for test systemic poison. In the first section, the fifth instars of brown planthopper were kept under the standard conditions: 25 °C, 70 % of humidity for 24 h. Brown planthoppers were taken into the plastic cup container covering with 0.05 inch of paraffin films: 10 brown planthoppers a cup. The test solution was prepared for 3 concentrations: 5 15 and 30% (wt/wt) of crude extract adjusted with 2% of sucrose. In addition, and 0.2% DMSO in solution was also dropped of 2 mL solution on to the top film. The treated insects were kept at 25°C and 70% relative humidity under long-day condition. The numbers of dead insects were counted after being treated for 24, 48 and 72 h. In the second section, the adult brown planthoppers were required for the test.

In this research, both adult brown planthoppers and nymp fifth stars (Wantana *et al.*, 1990) were performed using Parafilm method while only adult brown planthoppers were taken in Topical application method.

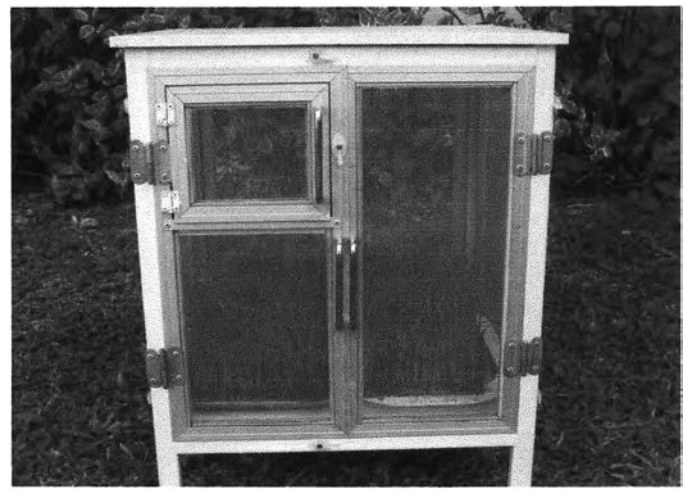


Figure 2.1 Mass rearing of brown planthopper

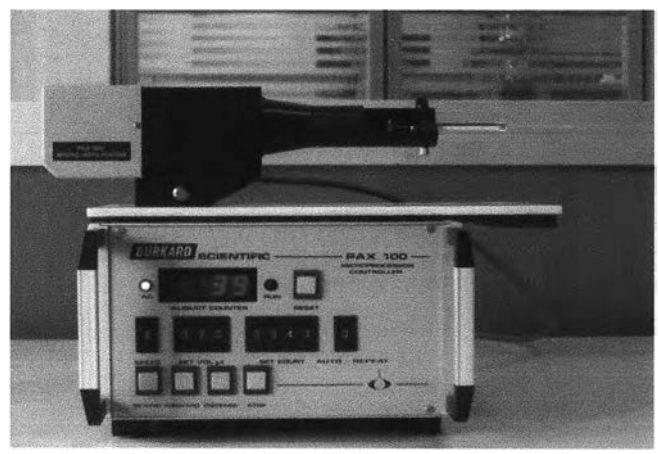


Figure 2.2 Microapplicator Burkard model PAX-100-2

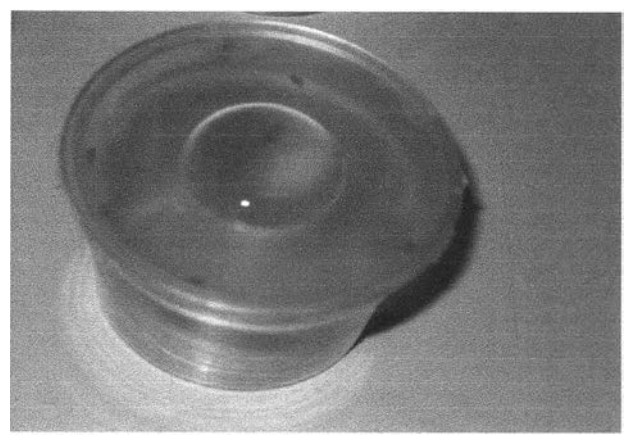


Figure 2.3 Parafilm method

2.6.3 Data analysis

The experiment was recorded the number of dead insects after being treat for 24, 48 and 72 h and reading were subjected to Abbot's formula revealing the result of %mortality.

$$\text{Percentage of mortality} = \frac{\text{dead insect}}{\text{Total insect treat}} \times 100$$

$$\text{Abbot formula} = \frac{X - Y}{X} \times 100$$

where;

X = % untreated insect control

Y = % untreated insect in treated

Data from each experiment was compared for differences between treatments and control. The mortality was recorded for 24 h after treatment, and the lethal concentration (LC_{50}) of the insecticides was determined by 95% fiduncial limits Probit analysis program using a computer program (Finney, 1971) and the lethal concentration (LD_{50}) was calculated by varied the size of the insect and purpose of comparison, is expressed in μg/g body weight of the insect. LC_{50}'s < 100 ppm was considered as highly toxic, those with LC_{50}'s ranging from 100 to 1,000 ppm as moderate toxic and those with LC_{50}'s > 1,000 ppm as non-toxic (Charles *et al.*, 1988)

$$LD_{50} = \frac{LC_{50}\ (\mu g/mL) \times 0.2 \times 1/1{,}000\ mL/insect}{\text{Body weight (g)}}$$

2.7 Molecular Docking Calculations

The docking calculations were performed using the AutoDock 3.0 software (Morris *et al.*, 1998) an automated molecular docking program. The Lamarckian genetic algorithm in combination with a rapid grid-based energy evaluation method was used to search for a suitable docked structure. A grid map of dimensions 80 x 80 x 80 $Å^3$ with a grid spacing of 0.375 Å was selected. 100 docking runs were carried out for each compound. The structure of acetylcholinesterase was taken from the Protein Data Bank, code 1QON, (Barril *et al.*, 2001). The Kollman method was used

to calculate its atomic charges. For the ligands, their structures were geometry optimized at HF/3-21G level of theory using Gaussian98 software (Frisch *et al.*, 2001) and the Gasteiger atomic charges were assigned.