CHAPTER 3

MATERIALS AND METHODS

Materials and Equipments

1. Thai neem seed kernel, neem leaf, neem oil
2. Karate ®, Margosan-O ®, Neemix ®, Advantage ®
3. Laboratory apparatus (beakers, measuring cylinders, flasks, etc.)
4. Extracting solvents (acetone, methanol, distilled water)
5. Electric balance
6. Blender
7. Magnetic stirrer
8. Filter paper and linen cloth
9. Plastic boxes
10. Test cages (4 cm in diameter by 12.5 cm high)
11. Vial glass tube containing sugar solution or insecticide solution
12. Incubator
13. Thermometer and relative humidity meter (Hygrometer)
14. CO_2 gas tank
15. Microapplicator (type L.V. 65 Burkard)
16. High Performance Liquid Chromatrography (Shimadzu LC-6A)
17. Sprayer

18. 4 blue nylon cages (3 x 3 x 2 m^3)

19. Refrigerators

20. Experimental plants

- *Nymphaea nauchali* Burm.
- *Jatropha interrima* Jacq.
- *Portulaca grandiflora* Hook.

21. Plastic bags

22. Cooler bags

23. Sugar solution 50% w/w

Methods

1. Stocks experimental honey bee

Honey bee used in this study were *A. florea* and *A. cerana* . Small nests of *A. florea* which contain a population of about 3,000 - 5,000 bees per nest, and small hives of *A. cerana* which contain a population of about 5,000-10,000 bees per hive, were obtained from the Bee Biology Reserch Unit station, Tumbon Bangkanteak, Amphur Mae Klong, Changwat Samut-Songkhram. There were brought to the Bee Biology Reseach Unit, Chulalongkorn University, Bangkok, in small movable *A. florea* nest and small movable frame hives of *A. cerana* boxes.

2. Preparation of the experimental substance stock solutions

2.1 Cyhalothrin solution

This stock solution was prepared from Karate ® 2.5% w/v EC of the ICI Asiatic (Agriculture) Company Limited of Thailand. To prepare 1% concentration of cyhalothrin, 4 ml of Karate ® 2.5% EC were added to 10 ml of acetone to obtain a 1 % stock solotion. The resulting the 1% stock solution was then diluted further with acetone to the desired concentration.

2.2 Neem extract solution

Thai neem seeds and neem leaves were collected from Amphur Jakkarach, Chungwat Nakornrachasrima and Amphur Meang, Changwat Uttradit. These were prepared into four difference extracts as described in the following:

2.2.1 Alcohol neem-seed crude extract

One hundred grams of crushed Thai neem seeds were added to 300 ml of methanol, and the mixture was blended for 5 minutes in an electric blender. The blended mixture was left for 24 hours, before stirring for 1 minute and filtering through filter paper. The solvent was allowed to evaporate from the filtrate. The solid residue was dried to constant weight, and this extract was stored in a refrigerator as the stock of the extract until use. This stock of extract are then diluted with appropriate amounts of alcohol to desirable concentrations.

2.2.2 Neem-seed extract

A suspension of 10 grams of crushed Thai neem seeds in 100 ml of hexane was stirred occasionally at room temperature for 5 hours, and then filtered through filter paper. The defatted mare was then extracted with 100 ml of ethanol in the same way as the hexane extraction. The ethanol extracts were then concentrated and diluted with ethanol to the desired concentration.

2.2.3 Neem-leaf extract

Fresh leaves of neem were collected from local neem trees and dried to a constant weight in a shade. Air-dried leaves were then turned into extracts by blending for 5 minutes in an electric blender in the same manner as the neem-seed extract preparation described in section 2.2.1 above.

2.2.4 Neem oil and another commercial neem extract

Neem oil was diluted with hexane to a desired concentration, whereas other commercial neem extracts such as Margosan-o ® from Grace-Sierra Crop Protection CO., USA, Neemix ® from Peachpanthumachad CO., and Advantage ® from Nonkasert CO.,Thailand were diluted to desired concentrations with ethanol.

3. Quantitative determination of azadirachthin from neem extract

Quantity of azadirachthin from various neem extract was determined by using High Performance Liquid Chromatography(Appendix A).

4. Methodology in the study of toxicity of neem extract and cyhalothrin on *A. florea* and *A. cerana*

4.1 Contact toxicity by the topical application method

4.1.1. 30 worker bees of each species (*A. florea* and *A. cerena*) were removed from the colonies and placed into a plastic box(10x15x8 cm) To facilitate their transfer into the test cages, they are first immobilised by anaesthetizing with CO_2 . The experiments could then be carried out on the caged bees.

4.1.2. The anaesthetized bees were placed on a piece of filter paper and treated individually by applying 1 ul of each concentration of experimental solutions on the ventral surface of the thorax using an electric microapplicator.

For the control group, the individuals were treated with the plain solvent of acetone for each of solution. This method was used to adjust for basline mortality .

After the treatment, the bees are transfered into test cages, at a rate of ten worker bees per cage. Thirty bees were used in the test groups for each concentration of each pesticidal solution and extract.

4.1.3. 50% sucrose solution was placed within each test cage to sustain the control and test bees during the test period. The test cages were stacked in an incubator set at 29.0 ± 1 °C and 60 % RH.

4.1.4 The mortality was assessed 24 hrs after treatment. Lack of

movement in response to prodding was used as the criterion for mortality. LD_{50} value of each solution was determined by probit analysis (Finney, 1971).

4.2 Oral toxicity by feeding methods

4.2.1. Honey bee workers of each species were obtained from the colonies and anaesthetized with CO_2 to facilitate handing and the tranfer into test cages. Thirty worker bees are transferred into each test cage and kept unfed for 1 hr.

4.2.2. The starved bees were then fed for 3 hrs by a feeder vial containing each concentration of the tested pesticide/extract. Normally, the bees consumed the treated syrup within 1 hr.

4.2.3. After the feeding period, the bees were fed 50% sucrose solution and placed in groups of ten in an incubator (at 29.0 ± 1 °C and 60% RH).

4.2.4 Mortality rates of the treated/control bees were determined 24 hrs. after treatment, using the criterion stated in section 4.1.4.

4.3 Data analysis

The calculation of the corrected percentage mortality was carried out by using Abbott's formula (Finney, 1971):

$$Pr = \frac{Po - Pc}{100 - Pc} \times 100$$

where Pr = % corrected mortality

Po = % observed mortality

Pc = % mortality in control group.

If % mortality in control group was more than 20% , the experiment had to repeated. A graph showing the relationship between the concentration of the tested pesticide/extract and the % corrected mortality was constructed for each pesticide solution/extract using Probit analysis (Finney, 1971). The LD_{50} for each pesticide solution/extract was determined from the graph.

Figure 3.1 : Neem extract.

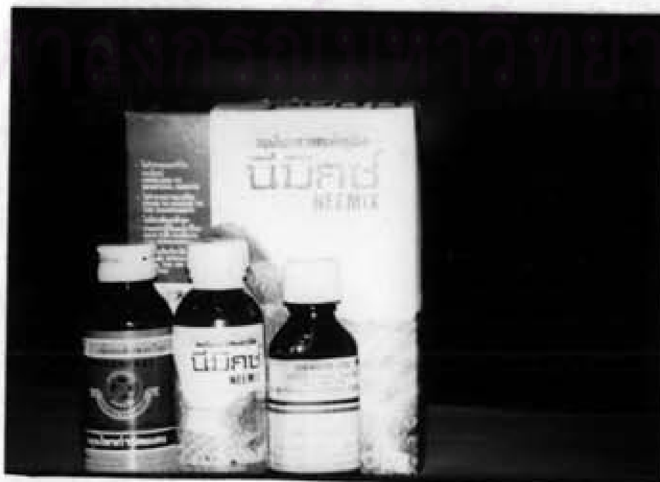

Figure 3.2 : Commercial neem extract.

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Figure 3.3 : Cyhalothrin (Karate ®)

Figure 3.4 : Electric microapplicator.

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Figure 3.5 : Test cages in the incubator.

5. Methodology in the study of the residual effects of neem extract and cyhalothrin in *Antigonon leptopus* plot on *A. florea* and *A. cerana*.

5.1 The trial site and experimental design

Antigonon leptopus was selected for studies on pesticide residual effects because it is highly attractive to bees and its flowers contain both pollen and nectar; it also produces many flowers in a relatively short time. During blooming period, plenty of *Antigonon leptopus* were found at the fence of Chitralada Royal Palace. They were divided into 3 plots, each about 4 m^2 in size. The plots were treated as follows :

Plot 1 : Sprayed with water.

Plot 2 : Sprayed with 1 % neem-seed extract.

Plot 3 : Sprayed with 0.002 % cyhalothrin.

Flower samples in each plot were collected 0, 1, 3, 6, 12, 24 and 48 hr after the treatment. For each sampling time, ten bouquets of flowers were collected per plot at random at different sites in the plot. Samples were taken immediately to the laboratory in a cooled plastic bag and assayed on the same day.

5.2 Study on the residual effect

5.2.1 For evaluation of the residual toxicity, the flowers in each sample were mixed gently and then processed in a blender.

5.2.2 Ten g of the blended flowers are then mixed in 30 ml of

solvent (ethanol for the plot treated with neem extract and acetone for the plot treated with cyhalothrin). The mixture was stirred at room temperature for several hours, and then filtered through a piece of filter paper.

5.2.3 The filtrates were subsequently used for tests by the topical application method and the feeding methods in the same manner as toxicity studies described in sections 4.1 and 4.2 above.

5.2.4 Honey bee mortality was determined after 24 hr. The mortality rates were adjusted by using Abbott's formula.

6. Methodology in the study of repellant effects of neem extract and cyhalothrin on to honey bees in treated *Antigonon leptopus* plot.

The numbers of bees were counted foraging on flowers in 15 x 15 cm areas within 1 second in five different areas of each treatment plot during and after 5 minutes after spraying.

Treatment 1 : no spray (control group).

Treatmeat 2 : spray with 1.0 % neem extract.

Treatment 3 : spray with 0.002 % cyhalothrin.

Mean ($\bar{X}$) and standard deviation (SD) of the number of forageing bees were calculated.

Figure 3.6 : A fence of *Antigonon leptopus* at Chitralada Royal Palace.

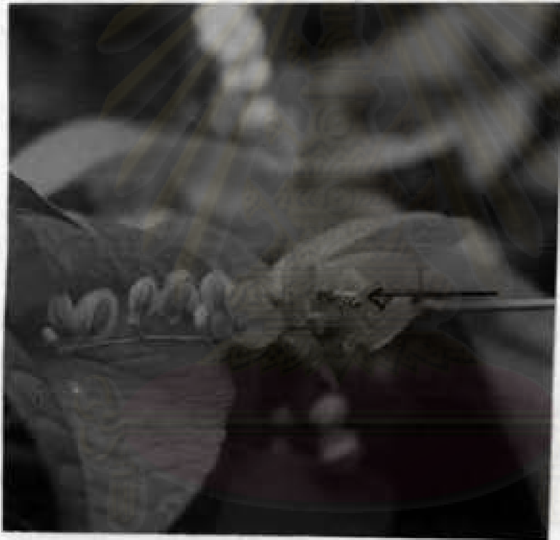

Figure 3.7 : *A. florea* on *Antigonon leptopus* flower.

Figure 3.8 : *A. cerana* on *Antigonon leptopus* flower.

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7. Methodology in a field trial to assess the effects of neem extract on *A. cerana*

7.1 The experimental cages

Four blue nylon cages ($3 \times 3 \times 2$ m^2) were built at Chitralada Royal Palace project. 10 flowerpots of *Nymphaea nouchali*, 25 flowerpots of *Jatropha integerrima* and 40 flowerpots of *Portulaca grandiflora* were introduced into each cage with water, nectar and pollen source to sustain the bees in the cages.

7.2 Description of the experimental bee hives

Small bee hives each containing five frames and about 5,000 to 10,000 adult bees of the same age as the queen were introduced into each cage two to three days prior to the application of the insecticide/extract.

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7.3 Application of neem-seed extract

cages 1,3 : control groups, sprayed with water every three days.

cages 2,4 : sprayed with neem extract 1 % every three days.

7.4 Climate observations

Air temperature (°C) and relative humidity (%RH) and the presence or absence of rainfall were recorded regularly every day at the trial sites during the study period. In addition, data on maximum

and minimum air temperature (°C) and rainfall (mm) were obtained from a meteological station in Chitralada Royal Palace.

7.5 Long-term hive development observations

The following parameters were recorded every 7 days during the study period. The study was carried in duplicate :

- foraging activity and bee behaviour.
- weight of each colony.
- nectar and pollen collection.
- hive population, adult, egg, larva, brood and overall condition.

Figure 3.9 :

Small movable frame hives of *A. cerana* in the experimental cages.

Figure 3.10 : A meteological station in Chitralada Royal Palace.

8. <u>Methodology in the study of the effect of neem extract on the larvae of *A. cerana*.</u>

Brood frames from the bee hives were taken to the laboratory where ten larvae aged 1 day,ten larvae aged 2 days, and ten larvae aged 3 days were treated with drops of 0.1 % neem extract. The larvae in the control groups were treated with drops of water. The brood frames were then taken back to bee hives.

After one week, the percentage of larvae which survived the treatment, and which become abnormal was recorded.

9. <u>Statistical analysis</u>

The data obtained from various from the experiments decribed

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above were analyzed statistically using the analysis of variance (completely randomized design), and the differences among treatments were compared using Duncan's multiple range test by SPSS-PC program.

10. Experimental places

10.1 Bee Biology Research Unit, Faculty of Science, Chulalongkorn University, Bangkok.

10.2 Bee Biology Unit, Tumbon Bangkanteck, Amphur Mae Klong, Changwat Samut-Songkhram.

10.3 Chitralada Royal Palace, Bangkok.

10.4 Interdepartment of Environmental Science, Chulalongkorn University, Bangkok.

10.5 Agricultural Toxic Substance Division , Bangkok.

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11. Experimental Duration

Experiments were conducted during June 1992 to November 1993.