CHAPTER 2

Materials and methods

2.1 Materials

2.1.1 Plant materials

Seventeen plant species classified into 14 genera and 9 families (Table 2.1) were obtained commercially from herbal suppliers in Chiang Mai province, northern Thailand. These medicinal plants were selected from specific families with antimosquito properties such as larvicidal, adulticidal, and repellent activities, which have been reported in scientific journals (Sukumar et al., 1991; Shaalan et al., 2005; Amer and Mehlhorn, 2006; Lupi et al., 2013). All plant materials were identified by Mr. James Franklin Maxwell, a botanist at the Chiang Mai University (CMU) Herbarium, Department of Biology, Faculty of Science, CMU, Chiang Mai, Thailand, and Miss Wannaree Charoensup, a scientist at the Department of Pharmaceutical Science, Faculty of Pharmacy, CMU, Chiang Mai, Thailand. Voucher specimens of these plants were deposited for future reference at the Department of Parasitology, Faculty of Medicine, CMU.

Table 2.1 Seventeen plant species selected for preliminary larvicidal screening against the pyrethroid-susceptible strain of *Ae. aegypti*

Family/Species	Common name	Voucher number	Part used
Asteraceae			
Blumea lacera (Burm.f.) DC.	Blumea	PARA-BL-001-St-Le/1	Stem & Leaf
Cruciferae			
Raphanus sativus Linn. var.caudatus Alef	Radish	PARA-RA-001-Se/1	Seed
Lamiaceae			
Polygonum odoratum Lour.	Vietnamese coriander	PARA-PO-001-St-Le/1	Stem & Leaf
Myristicaceae			
Myristica fragrans Houtt.	Nutmeg	PARA-MY-001-Ma/2	Mace
Piperaceae			
Piper sarmentosum Roxb.	Wilde betel	PARA-PI-003-St-Le/3	Stem & Leaf
Scrophulariaceae			
Limnophila aromatica (Lamk.) Merr.	Finger grass	PARA-LI-002-Wp/1	Whole plant
Picrorhiza kurroa Royle & Benth.	Hellebore	PARA-PI-001-Rh/1	Rhizome
Solanaceae			
Solanum aculeatissimum Jacq.	Cockroach berry	PARA-SO-001-Fr/1	Fruit
Solanum indicum Linn.	Poison berry	PARA-SO-002-Fr/1	Fruit
Umbellifereae			
Coriandrum sativum Linn.	Coriander	PARA-CO-002-Fr/3	Fruit
Foeniculum vulgare Mill.	Fennel	PARA-FO-001-Fr/4	Fruit
Petroselinum crispum (Mill.) A.W. Hill	Parsley	PARA-PE-001-Fr/1	Fruit
Zingiberaceae			
Amomum uliginosum Koenig.	-	PARA-AM-002-Fr/3	Fruit
Curcuma aeruginosa Roxb.	Pink & Blue ginger	PARA-CU-001-Rh/2	Rhizome
Curcuma longa Linn.	Turmeric	PARA-CU-005-Rh/2	Rhizome
Kaempferia pandurata Roxb.	Fingerroot	PARA-KA-001-Rh/1	Rhizome
Kaempferia parviflora Wall. ex Baker	Black galingale	PARA-KA-002-Rh/1	Rhizome

2.1.2 Experimental animals

2.1.2.1 Mosquitoes

The mosquito populations used in this study were the pyrethroid-susceptible and -resistant strains of *Aedes aegypti*, comprising Muang Chiang Mai-susceptible (MCM-S), Pang Mai Dang-resistant (PMD-R), and Upakut-resistant (UPK-R) strains. The MCM-S, PMD-R, and UPK-R strains were established from the field mosquitoes collected originally in Chiang Mai province at Muang Chiang Mai district in 1995 (Sutthanont et al., 2010), Ban Pang Mai Dang, Mae Tang district in 1997 (Prapanthadara et al., 2002), and Upakut Temple, Muang Chiang Mai district in 2006 (Plernsub et al., 2016), respectively. The PMD-R strain is homozygous for the C1534 allele, lacks S989P and V1016G, and is resistant to both DDT and permethrin, but susceptible to deltamethrin (Prapanthadara et al., 2002; Yanola et al., 2010; Somwang et al., 2011; Lumjuan et al., 2014). The UPK-R strain is homozygous for the S989P + G1016 alleles and resistant to DDT, permethrin, and deltamethrin (Plernsub et al., 2016). Mixed-function oxidases also was found to play a minor role in pyrethroid resistance in the PMD-R and UPK-R strains (Somwang et al., 2011; Plernsub et al., 2016). The PMD-R and UPK-R strains were reared under selection pressure by regular exposure to the WHO (WHO, 1998) discriminating dose (0.75% permethrin and 0.05% deltamethrin, respectively) in order to maintain the resistance level. The occurrence of the F1534C and V1016G mutations in these resistant strains also was checked regularly by allele-specific PCR (AS-PCR) methods (Yanola et al., 2011; Stenhouse et al., 2013). All test mosquitoes were maintained continually from their dates of collection in a climate-controlled insectary (25 ± 2 ºC, 80 ± 10% RH and 14:10 h light/dark photoperiod cycle) at the Department of Parasitology, Faculty of Medicine, CMU. From the laboratory reared *Ae. aegypti*, newly molted 4th instar larvae and non-blood-fed females (2-5 days old) were readily available for larvicidal and adulticidal bioassays, respectively.

2.1.2.2 Albino rats (*Ratus ratus*)

Male adult rats, age 6-8 weeks and weight 250-300 g, were obtained from the laboratory animal house, Faculty of Medicine, CMU. The animals were kept in an animal room where the temperature was maintained at 25-30 ºC in a 14:10 h light/dark period. Rats were used as a source of blood meal for female mosquitoes during egg

production. The blood feeding protocol has been approved by the Animal Ethics Committee, the Faculty of Medicine, CMU (Protocol Number 05/2558).

2.1.3 Chemicals

- Silicone grease (ACC Silicones Limited, England)
- Absolute ethanol (CAS No. 64-17-5, UN No. 1170: RCI-Labscan, Thailand)
- 95% Ethanol
- Methanol (UN No. 1230: Fisher scientific, UK)
- Acetone (EC No. 200-662-2: VWR International Ltd, UK)
- Sodium dodecyl sulfate (SDS, CAS No. 151-21-3: VWR Chemicals, Belgium)
- Dithiothreitol (DTT)
- Albumin standard (Thermo, USA)
- Bio Rad Protein Assay (CAT No. 500-0006: Bio-Rad Laboratories Limited, USA)
- Cytochrome c from bovine heart, C3131 (CAS No. 9007-43-6: Sigma-Aldrich Pte Limited, USA)
- Sodium acetate trihydrate
- Glacial Acetic Acid
- TMBZ (3,3′,5,5′-tetramethylbenzidine dihydrochloride hydrate powder) (CAS No. 207738-08-7: Sigma-Aldrich Pte Limited, USA)
- Hydrogen peroxide 30% (MERCK, Germany)
- Dipotassium hydrogen phosphate (K_2HPO_4)
- Potassium dihydrogen phosphate (KH_2PO_4)
- 1-chloro-2, 4-dinitrobenzene (minimum 98%) (Sigma-Aldrich Pte Ltd., Germany)
- L-glutathione reduced (minimum 98%) (Sigma-Aldrich Pte Ltd., Germany)
- 1% Triton X-100
- DTNB 5,5'-Dithiobis(2-nitrobenzoic acid) (CAS No. 69-78-3: Sigma-Aldrich Pte Limited, USA)

- ASCHI (acetylthiocholine iodide) (CAS No. 1866-15-5: Sigma-Aldrich Pte Limited, USA)
- *α*-naphthyl acetate (CAS No. 830-81-9: Sigma-Aldrich Pte Limited, Switzerland)
- *β*-naphthyl acetate (CAS No. 1523-11-1: Sigma-Aldrich Pte Limited, USA)
- *α*-naphthol (CAS No. 90-15-3: Sigma-Aldrich Pte Limited, USA)
- *β*-naphthol (CAS No. 135-19-3: Sigma-Aldrich Pte Limited, USA)
- Fast Blue B Salt-Dye content (CAS No. 14263-94-6: Sigma-Aldrich Pte Limited, USA)
- Tris (hydroxymethyl) aminomethane (tris)
- Hidrochloric acid
- *p*-nitrophenyl phosphate (CAS No. 4264-83-9: Sigma-Aldrich Pte Limited, USA)
- *p*-nitrophenol (CAS No. 100-02-7: Sigma-Aldrich Pte Limited, Singapore)
- Sodium hydroxide
- Permethrin (CAS No. 52645-53-1: Sigma-Aldrich Pte Limited, USA)
- Deltamethrin (CAS No. 52918-63-5: Sigma-Aldrich Pte Limited, USA)
- Temephos (CAS No. 3383-96-8: Sigma-Aldrich Pte Limited, USA)

2.2 Methods

2.2.1 Mosquito rearing

A laboratory colony of pyrethroid susceptible and resistant strains of *Ae. aegypti*, including Muang Chiang Mai-susceptible (MCM-S), Pang Mai Dang-resistant (PMD-R), and Upakut-resistant (UPK-R) were reared and maintained continually in the insectary of the Department of Parasitology, Faculty of Medicine, CMU without exposure to any pathogens or insecticides at a constant temperature of 27 ± 2 ºC and 80 ± 10% RH under a photoperiod of 14:10 h (light/dark). The methods for mass rearing were slight modifications of the procedure described by Limsuwan et al. (1987).

Approximately 200 larvae were reared in a white plastic pan containing 2 L of tap water, and fed on finely ground dog-biscuit twice daily. Each plastic pan containing aquatic stages of mosquitoes were covered tightly at all times with a nylon screen in order to keep all mosquito colonies strictly isolated from each other. The water was changed two or three times weekly in order to avoid scum forming. After pupation, the pupae were removed from the rearing pans and transferred to plastic cups containing distilled water. The cups containing about 300 pupae were placed into the netted cages (30 × 30 × 30 cm). After emerging, adults were fed with 10% aqueous sucrose and 10% multivitamin syrup solution made available continuously on soaked cotton pads. Rats were used as a source of blood meal for females in egg production. The eggs were laid on filter paper soaked with water in an egg collecting cup. They were kept for 3-4 days to air-dry before being used in the next rearing. From laboratory-reared *Ae. aegypti*, newly molted fourth instar larvae and non-blood-fed females (2-5 days old) were readily available for larvicidal and adulticidal bioassays, respectively.

2.2.2 Plant preparation

2.2.2.1 Preparation of crude plant extracts

The plant materials identified were air-dried separately at ambient temperature (21-35 ºC) under indoor conditions for one week before preparing the plant products. After grinding, the resulting material of each plant was subjected to extractions, which were divided into 2 procedures: isolating by steam distillation at 100 ºC for at least 3 h, thus obtaining essential oils; and extracting by maceration with 95% ethanol, which yielded ethanolic extracts.

2.2.2.1.1 Steam distillation

Coarsely ground dry material of each plant was extracted individually for essential oil by steam distillation. The distillation apparatus was consist of an electromantle, a round-bottomed distillation flask, an extraction column, a condenser, and cool ace. Two hundred and fifty grams of each plant material was placed in the extraction column connected to a distillation flask containing about 1,600 ml of distillation water and 10-15 glass beads. The flask was heated to about 100 ºC and allowed to boil until the distillation is complete. The vapor generated in the flask was ventilated

to the extraction column, which contained the plant material. After passing through the extraction column, steam was condensed by cool water passing from the cool ace through the coil in the condenser. The liquid formed, together with essential oil, was collected in a separating funnel. The mixture was allowed to settle for 3-5 days, after which, the essential oils obtained were separated from the aqueous phase. The oil was dried over anhydrous sodium sulfate (Na_2SO_4) and then collected and kept in a light-protected bottle at 4 ºC until required for investigation of insecticidal activity.

2.2.2.1.2 Solvent extraction

Finely ground plant materials were extracted with 95% ethanol. Half a kilogram of dried powder from each plant was extracted successively three times by maceration with 5 L of 95% ethanol at room temperature for 5-7 days. After vacuum filtration through a Bücher funnel, the combined filtrates were concentrated to dryness by rotary evaporation at 60 ºC, and lyophilized at -55 ºC for complete removal of solvent. The extract of each plant was kept at -20 ºC until required for investigation of insecticidal activity.

2.2.3 Investigation of the insecticidal activity of plant products

2.2.3.1 Preliminary screening for larvicidal activity

Essential oil and/or ethanolic extract of each plant were screened at a high concentration of 100 ppm, in order to check for larvicidal activity against pyrethroid-susceptible *Ae. aegypti*, MCM-S strain, under laboratory conditions by using a slightly modified version of the WHO standard protocol (WHO, 1981). Each plant product was dissolved in a suitable solvent such as ethanol, acetone, or dimethyl sulfoxide (DMSO) for preparing the required dosage of test solution. Four batches of 25 fourth instar larvae of the MCM-S strain were taken for bioassay tests in 249 ml of distilled water and 1.0 ml of the desired plant extract concentration. Control and the untreated groups were maintained in solvent-distilled water and distilled water only, respectively. No food was provided to the larvae during the experiment. Bioassays were carried out at 25 ± 2 ºC and 80 ± 10% RH. Larval mortality was assessed after 24 h of treatment. The larvae were considered dead if they were incapable of moving or failed to respond when probed with a blunt dissecting needle. Moribund larvae were those that showed discoloration,

unnatural positions, tremors, incoordination, or rigor as well as a characteristic diving reaction when the water was disturbed. The moribund and dead larvae in each test were combined and expressed as mortality percentage, and corrected for control mortality using Abbott's formula (Abbott, 1925). The promising plant products that elicited mortality rates $\geqslant$ 90% were subjected to a dose-response larvicidal bioassay.

2.2.3.2 Dose-response larvicidal bioassay

A series of five to six concentrations of each test sample was prepared in the range of 30 to 100 ppm by diluting in proper solvent. Dose-response bioassays were conducted on the MCM-S strain, according to the screening protocol (WHO, 1981) described previously, by using four batches of 25 larvae, with a final total of 100 larvae for each concentration. Each bioassay was repeated four times with mosquitoes from different rearing batches. The mortality percentage after 24 h was reported from the average of four replicates. The most effective extract was a candidate for determining antimosquito property, and comparing efficacy with synthetic chemicals against larvae and adults of both pyrethroid-susceptible and -resistant strains of *Ae. aegypti*.

2.2.3.3 Antimosquito bioassays of plant products and conventional synthetic insecticides

The potential of the most effective plant for killing larvae and adults of three laboratory *Ae. aegypti* strains (MCM-S, PMD-R, and UPK-R) were investigated and compared with that of synthetic organophosphates (temephos) and pyrethroids (permethrin and deltamethrin). Plant extract and the synthetic chemicals were diluted serially in appropriate solvents in order to prepare a graded series of test concentrations.

2.2.3.3.1 Larvicidal bioassay

Larvicidal bioassays were conducted according to the modified versions of WHO guidelines (WHO, 1981), as described previously.

2.2.3.3.2 Adulticidal bioassay

The topical application technique, as modified from the WHO standard method (WHO, 1996), was used for assessing the adulticidal activity of the test

samples, as follows. A group of 25 non-blood-fed females (2-5 days old), which were derived from each strain of *Ae. aegypti*, was treated at each concentration, with at least four different concentrations of each test sample. After being anesthetized briefly with carbon dioxide (CO_2), the mosquitoes were weighed and kept immobile over dry filter paper on a refrigerating plate for subsequent treatment with test solutions. The treatment was performed with the aid of a stereomicroscope. A 0.1-µl droplet of test solution was applied topically to the dorsal thorax (pronotum) of the immobilized female by using Hamilton's digital syringe (700 series MICROLITER™, Hamilton Company, USA). The filter paper was replaced after each treatment of test solution to prevent the mosquitoes from being exposed to other test samples. The control group was treated with solvent in a similar manner to that for the groups treated with test materials. Every bioassay was carried out at 25 ± 2 ºC and 80 ± 10% RH, and replicated four times with mosquitoes from different rearing batches. After treatment, the females in each group were observed and then kept separately in plastic cups, supplied with 10% sucrose plus 10% multivitamin syrup, and maintained for 24 h before scoring the mortality. The average percentage of mortality for the four replicates was recorded and corrected using Abbott's formula (Abbott, 1925).

2.2.4 Determination of mosquitocidal actions of the most effective plant

In assessing toxic action of plant substance, the behavioral, physical, and biochemical responses in the treated larvae and adults of both pyrethroid susceptible and resistant strains of *Ae. aegypti*, including MCM-S, PMD-R, and UPK-R strains were investigated.

2.2.4.1 Behavioral response observation

Batches of 25 newly fourth instar larvae and adult mosquitoes were treated with LC_{99} and LD_{99} of plant preparations, respectively. The control group of larvae were received solvent-distilled water, while that of adults were treated with ethanol or acetone. Behavioral symptoms in treated mosquitoes compared to those of the controls were observed, recorded, and photographed immediately and at frequent time intervals by using a digital camera. During the treatment period, no food was offered. Mortality rates were assessed 24 h later and the treated mosquitoes were examined further for

physical changes. The treatment bioassays were replicated four times with mosquitoes from different rearing batches.

2.2.4.2 Physical change observation

Physical changes of treated mosquitoes were examined in both macroscopic and microscopic aspects. After behavioral change observations the treated specimens of either dead or live mosquitoes were subjected to macroscopic observations. Morphological changes of the whole body such as skin color, body shrinkage, and body lesions were recorded and photographed, after which, the mosquito specimens were studied further for morphological changes under light and scanning electron microscopes.

2.2.4.2.1 Light microscopic (LM) study

The mosquito larvae of three strains that exposed with discriminating concentration (LC_{99}) of the most effective plant for 4 h were fixed in 2.5% glutaraldehyde, mounted with euparal on a microscopic slide, and arranged in anatomical position, which is head down and dorsal side up. Morphological changes in treated specimens were compared to those of the controls, including body segments (head, thorax, and abdomen) and other organs such as eyes, antennae, mouth brushes, setae, saddle, siphon, and anal gills, and scrutinized and photographed under a light microscope. The adults exposed with discriminating dose (LD_{99}) of the most effective plant for 12, 24, and 36 h were observed under light microscope. Morphological alterations of treated adults, including color, shape, sizes, and other abnormal changes such as body shrinkage and lesions were compared with those of the controls, and recorded and photographed.

2.2.4.2.2 Scanning electron microscopic (SEM) study

The test specimens exposed to the most effective plant at LC_{99} for 4 h (larvae) and LD_{99} for 12, 24, and 36 h (adults) were processed under the scanning electron microscopic (SEM) examinations as follows:

The treated larvae were placed in 1.5 ml tubes with 2.5% glutaraldehyde fixative. After the fixing process, larval specimens were washed in 0.1 M sodium phosphate buffer solution pH 7.2 (10 min, with two changes) and post-fixed in 1% osmium tetroxide solution in 0.1 M sodium phosphate buffer pH 7.2. The specimens were

washed again in 0.1 M sodium phosphate buffer solution pH 7.2 (10 min, with two changes), and dehydrated in graded series of ethanol 70%, 80%, 85% (3 h), 90% (3 h, with two changes), and followed by absolute ethanol (3 h, with two changes). The treated adults were not passed through fixing process, but they were dehydrated in graded series of ethanol 70%, 80%, 85% (1 h), 90% (1 h, with two changes), and followed by absolute ethanol (1 h, with two changes). Subsequently, the specimens were critical point dried using CO_2, attached to aluminum stubs with double-stick tape, and coated with gold in a sputter-coating apparatus before being viewed with a JEOLJSM-6610LV scanning electron microscope (JEOL: Japan). Morphological images were captured and determinations were carried out based on these records.

2.2.4.3 Biochemical change observation

2.2.4.3.1 Determination of lethal threshold time for mortality of larvae and adults

Groups of 25 newly hatched fourth instar larvae and adults were treated with the median lethal values, including LC_{50} and LD_{50} of the most effective plant, respectively. The control group of larvae was received solvent-distilled water, whereas that of the adults was exposed to acetone. The bioassays were replicated four times with mosquitoes from different rearing batches. The lethal threshold time points for larvae and adults were determined after recording the percentage mortality until 24 and 48 h, respectively. The earliest time point demonstrating 20-50% mortality of the test organisms after exposure to a specific concentration/dose of plant substance was considered as the threshold time for the lethal effect. Therefore, biochemical constituents of the test mosquitoes compared with those of the control and untreated groups were investigated after exposing the mosquitoes to respective substances at the lethal threshold time. These treated specimens were then subjected for preparation of whole body homogenates for the analysis of biochemical compositions. Bioassays were carried out at 25 ± 2 °C and 80 ± 10% RH.

2.2.4.3.2 Preparation of whole body homogenates

After exposure, the live larvae were washed in deionized water to remove the test solutions, except in adults, and the adhering water was completely

removed from the body surface with tissue paper. The larvae were then placed in 1.5 ml tubes, and stored at -80 ºC until whole body enzyme analysis was performed. Fourth instar larvae or adults in each group of treated, untreated, and control specimens were pooled and homogenized in desired homogenization buffer for each biochemical assay. The homogenates were centrifuged at 10,000 × g, 4 °C for 20 min and the clear supernatants were used immediately for determining total protein content and activity of enzymes, including glutathione S-transferase (GSTs), esterases (α- and β-esterases), Mixed-function oxidase (MFO), acid and alkaline phosphatases (ACP and ALK), and acetylcholinesterase (AChE).

2.2.4.3.3 Determination of protein concentrations

Total protein content was determined according to the method of Bradford et al. (1976) with some minor modifications. Two hundred microliters of Bio Rad protein reagent solution (1:4 dilution) were added to 10 µl of the crude homogenate. The mixtures were incubated in a 96-well microtiter plate. Homogenate buffer was used as a blank. The reaction was read at 595 nm after 5 min at room temperature. Protein concentrations in mg/ml were calculated from a standard curve of bovine serum albumin (0-0.5 mg/ml).

2.2.4.3.4 Determination of enzyme activity

2.2.4.3.4.1 Glutathione S-transferases (GSTs)

GSTs activity was determined as described by Habig et al. (1974) with minor modifications. Ten live fourth instar larvae (0, 3, 6, and 24 h) or adults (0, 24, and 48 h) in each group of treated, untreated, and control specimens were pooled and homogenized in 200 µl of homogenization buffer (0.1 M potassium phosphate buffer, pH 6.5). Two hundred microliters of GSH/CDNB working solution (10 mM reduced glutathione prepared in 0.1 M potassium phosphate buffer, pH 6.5 and 3 mM chlorodinitrobenzene diluted in methanol) were added to 10 µl of homogenate. The mixtures were incubated in a 96-well microtiter plate. Ten microliters of homogenization buffer was used as a blank. Enzyme rates were measured at 340 nm for 5 min. The GSTs activity was reported as µmol CDNB conjugated/min/mg protein, using published

extinction coefficients corrected for the path length. The enzyme activity was calculated following the formula:

$$\text{Activity} = \frac{(\text{mOD of test-mOD of blank}) \times \text{enzyme dilution}}{1000 \times \text{light path of 96 well plate} \times \varepsilon^{\text{of product}} \times \text{protein concentration}}$$

Enzyme dilution = 21

Light path of 96 well plate = 0.6 cm

(ε) or extinction coefficient of GS-DNB conjugated at 340 nm = 9.6 $mM^{-1} \cdot cm^{-1}$ (Habig et al., 1974)

Protein concentration = the concentration of sample from protein determination assay

2.2.4.3.4.2 Esterases (α- and β-esterases)

The esterase assays were performed following the procedure of van Asperen (1962) with minor modifications. Two fourth instar larvae (0, 3, 6, and 24 h) or adults (0, 24, and 48 h) in each group of treated, untreated, and control specimens were pooled and homogenized in 1 ml of homogenization buffer (20 mM potassium phosphate buffer, pH 7.2). The homogenates were diluted with the homogenization buffer in the ratios of 1:4 before measuring. Two hundred microliters of α or β -naphthyl acetate (NA) solution (200 µl of 30 mM α or β -NA in ethanol in 20 ml of 20 mM potassium phosphate buffer, pH 7.2) were added to 20 µl of homogenates. The mixtures were incubated in a 96-well microtiter plate for 30 min at room temperature before the addition of 50 µl of Fast blue B stain solution (22.5 mg Fast blue in 2.25 ml distilled water, then 5.25 ml of 5% sodium lauryl sulphate diluted in distilled water) were added to stop the enzymatic reaction. Replicate blanks contained 20 µl of homogenization buffer, 200 µl of substrate solution and 50 µl of stop solution. Enzyme was read at 570 nm as an end point. Esterase activities were calculated from α or β -naphthyl acetate standard curve. The results were reported as nmol of the α or β -naphthol released/min/mg protein. The activity was calculated following the formula:

Activity = nmol of α or β- naphthol released/30 min/mg protein (mg/ml)

2.2.4.3.4.3 Mixed-function oxidases (MFO)

Ten fourth instar larvae (0, 3, 6, and 24 h) or adults (0, 24, and 48 h) in each group of treated, untreated, and control specimens were pooled and homogenized in 700 µl of homogenization buffer (0.25 M sodium acetate buffer, pH 5.0). The total amount of mixed-function oxidases in mosquitoes was titrated using the haem-peroxidase assay followed by Brogdon et al. (1997) with slight modifications. One hundred microliters of homogenates were incubated with 225 µl of substrate mixture [200 µl of 6.3 mM TMBZ solution (0.01 g of 3,3′,5,5′-tetramethylbenzidine in 5 ml of absolute methanol mixed with 15 ml of 0.25 M sodium acetate buffer, pH 5.0, prepared fresh daily)] and 25 µl of 3% H_2O_2 in a 96-well microtiter plate. Replicate blanks contained 100 µl of homogenate buffer, 225 µl of substrate mixture. The reactions were incubated for 5 min at room temperature before reading at 630 nm using microplate reader (Spectra MR, DYNEX technologies) and the mixed-function oxidase values were compared with known concentrations of cytochrome c from horse heart type VI (0-1.4 ng/µl) and reported as nmol of cytochrome c equivalent units/mg protein. The enzyme activity was calculated following the formula:

$$\text{Activity} = \frac{\text{nmol of cytochrome c}}{\text{mg protein}}$$

2.2.4.3.4.4 Acid and alkaline phosphatases (ACP and ALK)

The levels of ACP and ALK were measured following the procedure of Asakura (1978) with slight modifications. Ten fourth instar larvae (0, 3, 6, and 24 h) or adults (0, 24, and 48 h) in each group of treated, untreated, and control specimens were pooled. These specimens were homogenized in 500 µl of homogenization buffer. While 50 mM sodium acetate buffer, pH 4.0 was used as homogenization buffer for determining ACP activity, 50 mM Tris-HCl buffer, pH of 8.0, with 1 mM DL-dithiothreitol was used for ALK determination. The acid phosphatase activity was analyzed by mixing 10 µl of homogenates with 200 µl of substrate mixtures containing 25 mM sodium acetate buffer, pH 4.0 and 6.25 mM *p*-nitrophenyl phosphate in a 96-well microtiter plate. After 15 min incubation at 37 ºC, the enzymatic reactions were terminated by adding 50 µl of 0.5 N NaOH. The absorbance was read at 405 nm. Absorbance levels for each sample were compared with standard curve of known concentrations of *p*-nitrophenol (0.0625-4 µg/µl). The acid phosphatase activity was

reported as nmol of the *p*-nitrophenol released/min/mg protein. The activity was calculated following the formula:

Activity = nmol of *p*-nitrophenol released/15 min/mg protein (mg/ml)

2.2.4.3.4.5 Acetylcholinesterase (AChE)

The activities of AChE were measured according to the method of Ellman et al. (1961) with some minor modifications. Five fourth instar larvae (0, 3, 6, and 24 h) or adults (0, 24, and 48 h) in each group of treated, untreated, and control specimens were pooled and homogenized in 250 µl of homogenization buffer (0.1 M potassium phosphate buffer, pH 7.0 with 1 µM DL-dithiothreitol and 1% Triton X-100). The reaction mixture contained 25 µl of homogenates, 155 µl of 0.65 mM dithiobis 2-nitrobenzoic acid (DTNB) solution, and 25 µl of 10 mM acetylthiocholine iodide (ASCHI) as substrate. The reactions were monitored continuously at 405 nm for 5 min in a microliter plate reader. The AChE activity was reported as µmoles of the acetylthiocholine hydrolyzed/min/mg protein, using published extinction coefficients corrected for the path length. The enzyme activity was calculated following the formula:

$$\text{Activity} = \frac{(\text{mOD of test-mOD of blank}) \times \text{enzyme dilution}}{1000 \times \text{light path of 96 well plate} \times \varepsilon^{\text{of product}} \times \text{protein concentration}}$$

Enzyme dilution = 8.2

Light path of 96 well plate = 0.6 cm

(Ɛ) or extinction coefficient of ACT hydrolysed at 405 nm = 13.6 $mM^{-1} \cdot cm^{-1}$ (Ellman et al., 1961)

Protein concentration = the concentration of sample from protein determination assay

2.2.5 Gas chromatography-mass spectrometry (GC-MS) analysis

The chemical profile of the most effective plant product was determined by GC-MS analysis at the Science and Technology Service Center, CMU. Equipment and conditions for chemical analysis were as follows:

- **Gas chromatography: GC 7890A Agilent Technology**

  Inlet: 250 ºC splitless

Injection volume: 0.2 µl

Split (Split ratio 100:1)

Oven: 50 ºC (4 min)→10ºC/min→250 ºC

Total run time: 20.0 min

Carrier: Helium 1.0 ml/min

Column: DB-5MS 30 m × 0.25 mm ID × 0.25 µm film thickness

Detector temperature: 250 and 280 ºC

- **Mass spectrometer detector: MSD 5975C (EI) Agilent Technology**

  Scan parameters: 50-550 amu.

  Scan rate: 2.91 scans/s

  MS Quadrupole: 150 ºC

  MS Source: 230 ºC

The GC/MS analysis of the most effective plant product was performed using a Hewlett-Packard GC 7890A Agilent Technology interfaced to a single quadrupole mass selective detector, MSD 5975C (EI) Agilent Technology. The column was a DB-5MS (30 m × 0.25 mm ID × 0.25 µm film thickness). The total GC-MS running time was 20 min. Helium was the carrier gas, set at a constant flow rate of 1.0 ml/min. The column temperature program was increased by 10 ºC/min between 50 and 250 ºC. The diluted sample (1/10 %v/v, in CH_2Cl_2) of 0.2 µl was injected manually in a split mode, with a 100:1 split ratio. The injector and detector temperatures were performed at 250 and 280 ºC, respectively. The mass spectra were operated in the electron ionization (EI) mode at 70 eV. Data were acquired over a range of 50-550 amu with a scan rate of 2.91 scans/s. The retention times were determined in relation to a homologous series of *n*-alkanes (C_8-C_{40}) under the same operating conditions. Chemical components of the plant product were identified based on the calculated KI by comparing their mass spectra with the GC-MS (NIST 2008 and Wiley 8NO8) spectral libraries. The relative concentration of each compound was quantified according to the peak area integrated by the analysis program.

2.2.6 Statistical analysis

Data for antimosquito performances were analyzed by using LdP Line software to calculate probit analysis according to Finney (1971). The lethal values of 50, 95, and 99% in larvae (LC_{50}, LC_{95}, and LC_{99}, respectively) and adults (LD_{50}, LD_{95}, and LD_{99}, respectively) as well as the corresponding 95% confidence intervals (95% CI) were obtained and used to measure larvicidal and adulticidal efficacy, respectively. The lethal values of each treatment were considered as significantly different from one another when their 95% CI did not overlap. Each experiment in the biochemical bioassays were performed with triplicate of six or ten biological samples from different preparations. One-way ANOVA followed by Tukey's HSD test (IBM SPSS statistics 24) was used for comparing the means of enzyme activity among three strains of mosquitoes ($P < 0.05$). Mann-Whitney *U* test was used for comparing the means of enzyme level between control and treated groups ($P < 0.05$). The independent sample *t*-test was used to determine the mean differences between untreated and control groups of all enzymes and time point in the three strains of mosquitoes.