CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Plant materials

Thirty-three plant species classified into 31 genera and 23 families (Table 2.1) were obtained commercially from herbal suppliers or collected from their natural habitats in Chiang Mai province, Thailand, and its neighboring provinces. In order to search for new repellents, selection of these plants was based on their abundance as well as the botanical and pharmacological information available in the literature (Lust 1974; Keys 1976; Pongboonrod 1976; Smitinand 1980; Huang 1993). The plant materials were identified scientifically and authenticated at Chiang Mai University (CMU) by Mr. James Franklin Maxwell, a botanist at the Herbarium, Department of Biology, Faculty of Science; and Ms. Wannaree Charoensup, a scientist at the Department of Pharmaceutical Science, Faculty of Pharmacy. A voucher specimen of each plant was deposited for future reference at the Department of Parasitology, Faculty of Medicine, CMU.

Table 2.1 Plant samples screened for repellent activity against female *Ae. aegypti*

Family/Species	Common name	Voucher number	Part used
Amaranthaceae			
Achyranthes aspera L.	Prickly chaff-flower	PARA-AC-003-St-Le/1	Stem & Leaf
Araceae			
Acorus calamus L.	Mytle grass	PARA-AC-001-Rh/1	Rhizome
Homalomena aromatica Schott.	Colla aromatica	PARA-HO-001-Rh/1	Rhizome
Avicenniaceae			
Avicennia marina (Forsk.) Vierh.	Olive mangrove	PARA-AV-001-St-Ba/1	Stem & Bark
Caricaceae			
Carica papaya L.	Papaya	PARA-CA-005-Se/1	Seed
Capparaceae			
Crataeva magna (Lour.) DC.	Crataeva	PARA-CR-001-St/1	Stem
Cleomeceae			
Cleoma viscosa L.	Wild spider flower	PARA-CL-002-St/1	Stem
Compositae			
Artemisia annua L.	Wormwood	PARA-AR-002-St-Le/2	Stem & Leaf
Atractylodes lancea (Thunb.) DC.	Atractylodes	PARA-AT-002-Rh/1	Rhizome
Tagetes erosus L.	Marigold	PARA-TA-002-Fl/1	Flower
Cucurbitaceae			
Cucurbita moschata Deene.	Pumpkin	PARA-CU-008-Se/1	Seed
Momordica charantia L.	Bitter cucumber	PARA-MO-001-Fr/1	Fruit
Euphorbiaceae			
Phyllanthus amarus Schum. & Thonn.	Tamalaki	PARA-PH-002-St-Le/1	Stem & Leaf
Fabaceae			
Pachyrhizus erosus (L.) Urb.	Jicama	PARA-PA-002-Se/1	Seed
Lauraceae			
Cinnamomum verum J. Presl.	Ceylon cinnamon	PARA-CI-007-Ba/1	Bark
Leguminosae			
Tamarindus indica L.	Tamarind	PARA-TA-001-Se/1	Seed
Malvaceae			
Abelmochus esculentus (L.) Moench.	Lady's finger	PARA-AB-001-Fr/1	Fruit
Menispermaceae			
Tinospora crispa (L.) Miers ex Hook.f. & Thomson	Bora phet	PARA-TI-001-St/1	Stem
Papilionaceae			
Butea monosperma (Lam.) Taub.	Flame of the forest	PARA-BU-001-Fl/1	Flower
Derris scandens (Roxb.) Benth.	Malay jewel vine	PARA-DE-001-St-Ba/1	Stem & Bark
Pedaliaceae			
Sesamum orientale L.	Sesame	PARA-SE-001-Se/1	Seed
Polygonaceae			
Polygonum odoratum Lour.	Vietnamese coriander	PARA-PO-001-St-Le/1	Stem & Leaf
Rutaceae			
Murraya paniculata (L.) Jack.	Orange Jasmine	PARA-MU-001-Le/1	Leaf
Scorphulariaceae			
Limnophila aromatica (Lam.) Merr.	Finger grass	PARA-LI-002-Wp/1	Whole plant
Solanaceae			
Solanum aculeatissimum Jacq.	Cockroach berry	PARA-SO-001-Fr/1	Fruit
Solanum indicum L.	Poison berry	PARA-SO-002-Fr/1	Fruit
Umbelliferae			
Angelica sinensis (Oliv.) Diels.	Dong quai	PARA-AN-003-Rh-Ro/1 PARA-AN-003-Rh-Ro/2	Rhizome & Root
Petroselinum crispum (Mill.) A.W. Hill	Garden parsley	PARA-PE-001-Fr/1	Fruit
Vitaceae			
Cissus quadrangularis L.	Veldt grape	PARA-CI-006-St-Le/1	Stem & Leaf
Zingiberaceae			
Curcuma zedoaria Roscoe.	Zedoary	PARA-CU-004-Rh/6	Rhizome
Curcuma xanthorrhiza Roxb.	Java ginger	PARA-CU-003-Rh/2	Rhizome
Dioscorea bulbifera L.	Shoebutton air potato	PARA-DI-001-Rh/1	Rhizome
Zingiber cassumunar Roxb.	Phlai	PARA-ZI-003-Rh/1	Rhizome

2.1.2 Human volunteers

Healthy human volunteers of both sexes (age 22-59 years old; weight 39-98 kg), without a history of the dermatologic disease or allergy to arthropod bites or repellent applications, were selected for investigating repellent efficacy. All volunteers were interviewed and informed of the objective and methodology of the study, probable discomforts from exposure to test substances and mosquito bites, and remedial arrangements before they provided their written informed consent to participate under protocol PAR-2556-01588 approved by the Research Ethics Committee of Faculty of Medicine, CMU. The volunteers also were advised to avoid alcohol and any fragrant products such as perfume, cologne, deodorant, and lotion during the entire study period.

2.1.3 Experimental animals

2.1.3.1 Mosquitoes

The free-mating laboratory populations of three mosquito vectors, including *Aedes aegypti*, *Anopheles minimus*, and *Culex quinquefasciatus* were used in this study. *Ae. aegypti* was established from field larvae collected from clean stagnant water at various breeding sites in Chiang Mai province in 1995 (Sutthanont et al. 2010). *An. minimus* sensu stricto (formerly *An. minimus* A) was obtained originally from the Office of Vector Borne Diseases Control, Department of Communicable Disease Control, Ministry of Public Health, Chiang Mai province in 1997 (Sanghong et al. 2015). *Cx. quinquefasciatus* (NIH strain) was obtained originally from the National Institute of Health, Ministry of Public Health, Nonthaburi province in 2015 (Sathantriphop et al. 2006). These mosquitoes were colonized and maintained continuously from the dates obtained for several generations in an insectary at the Department of Parasitology, Faculty of Medicine, CMU. Unfed female mosquitoes (5-7 days old) of these mosquito species were used for investigations on repellent efficacy. Prior to testing, female mosquitoes were starved by providing them only water for 12 h.

2.1.3.2 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 °C 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).

2.1.4 Chemicals

- Vanillin (EC No. 2044652: Sigma-Aldrich, France)
- Silicone grease (ACC Silicones Limited, England)
- Sodium sulphate anhydrous (CAS No. 7757-82-6: Fisher scientific, UK)
- *N*,*N*-diethyl-m-tolumide (DEET, EC No. 2051497: Sigma-Aldrich, China)
- Absolute ethanol (CAS No. 64-17-5, UN No. 1170: RCI-Labscan, Thailand)
- 95% Ethanol
- Methanol (UN No. 1230: Fisher scientific, UK)
- Hexane (EC No. 203-777-6: VWR International Ltd, UK)
- Acetone (EC No. 200-662-2: VWR International Ltd, UK)
- Glycerine
- Carbopol®940
- Triethanolamine (TEA)
- Sodium metabisulfite (SMS)
- Ethylenediamine tetraacetic acid (EDTA)
- Dork butterfly pea fragrance (A0403767)
- Tween® 20 (CAS No. 9005-64-5: Sigma-Aldrich, France)
- Sodium lauryl sulfate (SLS, CAS No. 151-21-3: VWR Chemicals, Belgium)
- Methylparaben
- Propylparaben

2.2 Methods

2.2.1 Mosquito test populations and rearing

A laboratory colony of three mosquito vectors, including *Ae. aegypti*, *An. minimus*, and *Cx. quinquefasciatus* 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 °C and 70-80% relative humidity under a photoperiod of 12:12 h (light/dark). The methods for mass rearing were slight modifications to the procedure described by Limsuwan et al. (1987). Rearing trays containing aquatic stages of mosquitoes were covered tightly at all times with a nylon screen in order to keep all of the colonies strictly isolated from each other. The unfed female mosquitoes (5-7 days old) were used for investigations of repellent activity.

2.2.1.1 *Aedes aegypti*

A laboratory colony of *Ae. aegypti*, which originates from specimens collected in Chiang Mai province, was colonized and maintained continuously in an insectary for several generations. Approximately 200 larvae were reared in a white plastic pan containing 2 L of tap water and fed on finely ground dog-biscuit. 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 netted cages (30×30×30 cm). After emerging, adults were fed with 10% sucrose and 10% v/v multivitamin syrup. 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. The filter paper with eggs was placed into trays filled with tap water for hatching. The larvae were hatch within 24 h after immersion and then transferred to a new rearing pan.

2.2.1.2 *Anopheles minimus*

A laboratory colony of *An. minimus*, which was obtained originally from the Vector Borne Disease Section, Office of Disease Prevention and Control No. 10, Chiang Mai, Thailand, was established and maintained continuously in an insectary for several generations. Approximately 300 larvae were reared in a white plastic tray containing 2 L of distilled water. Some water lettuces were added in the rearing tray as larval resting places. The larvae were fed on finely ground fish food consisting of 47.5% protein, 6.5% oil, 2.0% fiber, 10.5% ash, 6.0% moisture, and additives of vitamin A (29,770 IU/kg), D3 (1,860 IU/kg) and E (200 mg/kg) and L-ascorbyl-2-polyphosphate (138 mg/kg), lecithin, l-lysine monochlorhydrate, and citric acid. First and second instar larvae were fed twice per day, and this schedule was increased to 3-5 times daily after most of the larvae have reached third and fourth instars. After pupation, approximately 100 pupae were placed in a plastic cup (14.5 cm in diameter and 6 cm deep) containing 150 ml of distilled water and kept in a mosquito cage. The emerged adults were provided with both 10% sucrose and 10% multivitamin syrup solution, saturated in cotton wool coiled around a small piece of wood and placed in a small bottle. The bottle and food were changed every two days. Increased humidity for promoting adult survival was provided by covering the cage with a wet towel overlaid with a transparent plastic sheet. After blood feeding on rat, the engorged adult females were maintained for 4-5 days and/or until gravid in the insectariums. Then, they were placed in a black screen-topped oviposition plastic-cup containing 25 ml of filtered distilled water, with wet filter paper lining the inside. The eggs attached to the moist side of the filter paper and/or floating on the water's surface were rinsed and transferred to a white plastic tray containing 1,500 ml of filtered distilled water, with wet filter paper lining the inside. During the embryonation period, the eggs were exposed to a 40-watt artificial light instead of sunlight, for warming the eggs until hatching. After egg hatching, the first instar larvae were transferred daily from an ovipot to a new rearing pan.

2.2.1.3 *Culex quinquefasciatus*

A laboratory colony of *Cx. quinquefasciatus*, which was obtained originally from the National Institute of Health of Thailand (NIH strain), Department of Medical Sciences, Ministry of Public Health, Nonthaburi province. They were established and maintained continuously in an insectary for several generations. Approximately 300-400 larvae were reared in a plastic pan containing 2 L of distilled water mixed with hay-fermented water and fed daily on finely ground dog-biscuit. The mortality of larvae was minimized by supplying enough food and changing the rearing medium every 2 or 3 days. The scum on the surface of the rearing medium was removed with a strip of filter paper every day before the larvae are fed. After the larva has developed to pupae, the latter was picked up from the rearing pan with a pipette and put into plastic cups filled with distilled water. The cups with approximately 400 pupae were placed into the mosquito cage for emergence. The adult mosquitoes were fed on 10% sugar solution and 10% multivitamin syrup soaked in cotton wool, which were changed every other day. Seven days after emergence, the females were feed off rat overnight as a blood meal, which were offered once a week. The egg-collecting cups containing distilled water mixed with hay-fermented water were placed in the cages after each blood meal. Two or three egg rafts were transferred to each rearing pan, and larva hatching occurred about 30 h later.

2.2.2 Plant preparations

2.2.2.1 Preparation of crude plant extracts

Plant samples under investigation were shade-dried separately for 5-10 days in an open area with active ventilation and ambient temperature of about 30±5 °C, depending on which part of the plants would be used. Dried plant materials were pulverized to a coarse/fine powder before taking extractions, which were divided into two procedures: extracting by maceration with 95% ethanol to obtain ethanolic extracts, and isolating by steam distillation, thus yielding essential oils.

2.2.2.1.1 Solvent extraction

Half a kilogram of finely powdered material from each plant was extracted exhaustively at room temperature by maceration with 5 L of 95% ethanol. The extracted mixtures were filtered through cotton followed by Whatman No. 1 filter paper, concentrated in a rotary evaporator at 60 °C until the solvent has evaporated completely, and finally dried in a lyophilizer at -55 °C for complete removal of solvent and yield of dry ethanolic extracts.

2.2.2.1.2 Steam distillation

Isolation of essential oil was achieved by continuous steam distillation of the coarsely ground material of each plant at 100 °C for at least 3 h. The distillation apparatus 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 distilled water and 10-15 glass beads. The flask was heated to about 100 °C 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 volatile oil, was collected in a separating funnel. The oil layer was separated from the aqueous phase, dried over anhydrous sodium sulfate (Na_2SO_4), and collected in an amber-colored bottle. The percentage yield of each plant product was calculated according to the dry weight of the plant samples. Ethanolic extracts (EE) and essential oils (EO) derived from plant materials were kept at -20 °C and 4 °C, respectively, until required for experimental testing. Preparations of each plant, 25% EE dissolved in a proper vehicle, or pure EO was used in repellent bioassays.

2.2.2.2 Preparation of active extracts from the most effective plant

The most effective plant (*A. sinensis*) obtained from the screening repellent test was selected for isolating active extractants further by using solvents of varying polarity (hexane, acetone, methanol, and absolute ethanol) and different

extraction methods. All the chemicals and solvents used were of analytical grade. Half a kilogram of finely powdered material from *A. sinensis* was extracted exhaustively at room temperature by maceration with 5 L of hexane, acetone, methanol, and absolute ethanol. Each of the extracted mixtures was filtered through cotton followed by Whatman No. 1 filter paper, concentrated in a rotary evaporator at 45 °C, 40 °C, 50 °C, and 60 °C for hexane, acetone, methanol, and absolute ethanol, respectively until the solvent has evaporated completely. Finally, the liquid residues were dried in a lyophilizer at -55 °C to obtain dry plant extracts, which were *A. sinensis* hexane extracts (AHE), *A. sinensis* acetone extracts (AAE), *A. sinensis* methanolic extracts (AME), and *A. sinensis* ethanolic extracts (AEE). The isolated active extractants from *A. sinensis* were investigated for repellent activity and compared with the crude extract of the *A. sinensis* and determined for chemical composition.

2.2.2.3 Preparation of AHE-based repellent products

2.2.2.3.1 AHE-ethanol solution

AHE as well as DEET were prepared in absolute ethanol at various concentrations (5-25%) with and without 5% vanillin. Subsequently, the ethanol solutions of AHE and DEET (5-25% AHE-ES, AHEv-ES, DEET-ES, and DEETv-ES) were investigated for repellent activity against *Ae. aegypti* under laboratory conditions.

2.2.2.3.2 AHE-nanoemulsion

AHE-nanoemulsion comprised several ingredients, including AHE, fixative (vanillin), surfactant (tween 20), co-surfactant (glycerine), fragrance (dork butterfly pea), and deionized water. Briefly, coarse emulsion was prepared by mixing varied concentrations of AHE, surfactant:co-surfactant in ratio 5:1, and 5% vanillin in a screw capped flask (vortexed ~5 min), followed by the addition of dork butterfly pea. The final volume of this preparation was adjusted with the addition of deionized water (vortexed ~5 min). Then, coarse AHE emulsion was subjected to ultrasonic emulsification using a 40 kHz sonicator (Ultrasonics: DW-120DTN, USA) with a power output of 120 watts at 25 °C for 30 min to 2 h. The prepared 5-25% AHE nanoemulsion with and without 5% vanillin (5-25% AHE-NE and AHEv-NE) were

used for repellent investigation against *Ae. aegypti* under laboratory conditions. The AHEv-NE was selected for preparation of repellent gel products (10% AHEv-nanoemulsion gel).

2.2.2.3.3 10% AHEv-nanoemulsion gel

The systematic processes in preparations of 10% AHEv-nanoemulsion gel (10% AHEv-NEG) and 10% DEETv-nanoemulsion gel (10% DEETv-NEG) were similar, except for the active ingredient (AHEv-NE/DEETv-NE). The Carbopol®940 was used as a gelling agent in the gel formations. The 5% gelling agent was prepared by dissolving Carbopol®940 in deionized water (in a stainless bowl), mixing well by using a professional mixer (Princess: PW2028, Netherlands), and left overnight for gel setting. The 20% AHEv-NE was incorporated into the gel, followed by addition of deionized water. Then, humectant (glycerine), preservative (methylparaben and propylparaben), chelating agent (1% EDTA) and antioxidant (3% SMS) were added sequentially into the incorporated gel. Finally, TEA was added for pH adjustment. The formulation was stirred by mixer until well blend after each addition. The ingredient details of 10% AHEv-NEG and 10% DEETv-NEG are demonstrated in Table 2.2.

Table 2.2 The ingredient details of 10% AHEv-NEG and 10% DEETv-NEG

Ingredients	10% AHEv-NEG	10% DEETv-NEG
20% AHEv-NE	7.50	-
20% DEETv-NE	-	7.50
5% Carbopol®940	3.30	3.30
Deionized water	2.69	2.69
Glycerine	0.45	0.45
1% EDTA	0.60	0.60
3% SMS	0.30	0.30
Preservative	0.02	0.02
TEA	0.15	0.15

2.2.3 Chemical analysis of *A. sinensis* extracts

The chemical profiles of *A. sinensis* extracts, including AEO, AHE, AAE, AME, and AEE were determined by gas chromatography/mass spectrometry (GC/MS) analysis at the Science and Technology Service Center, CMU. Conditions for chemical analysis were as follows:

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

  Inlet: 250 °C splitless

  Injection volume: 1.0 µl

  Split (Split ratio 10:1)

  Oven: 50 °C (0 min) → 5 °C/min → 300 °C (10 min)

  Total run time: 60 min

  Carrier: Helium 1.0 ml/min

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

  Detector temperature: 280 °C

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

  Scan parameters: 30-500 amu.

  MS Quadrupole: 150 °C

  MS Source: 230 °C

The GC/MS analysis of *A. sinensis* extracts was performed using a Agilent Hewlett Packard 7890A, equipped with a split-splitless injector and DB-5MS (30 m × 0.25 mm ID × 0.25 µm film thickness) columns, directly coupled to a quadrupole mass selective detector, Agilent MSD 5975C (EI). The total GC/MS running time was 60 min with the operating conditions programmed as follows: the oven temperature was 50-300 °C at 5 °C/min. The carrier gas was helium at a constant flow rate of 1.0 ml/min; and the temperature of the injector port and detector was 250 °C and 280 °C, respectively. Each plant sample (1.0 µl) was injected by splitting, with a split ratio of 10:1. Mass spectra were obtained at 70 eV and recorded in the range of 30-500 amu. The temperature of the interface and iron trap was 230 °C and 150 °C, respectively. The chemical constituents of each plant extract were identified by their retention time and computer matching with the Wiley 8N08 spectral library as well as comparing their

mass spectra with those of authentic samples. The relative percentage amount of each component was calculated by comparing its area of average peak to the total area.

2.2.4 Repellent bioassays

2.2.4.1 Laboratory repellent bioassay

2.2.4.1.1 Preliminary repellent screening

Crude plant extracts, including ethanolic extracts (EE) and essential oils (EO) were screened for repellent efficacy against the laboratory strain of *Ae. aegypti* by using the human-bait technique of the WHO (1996) standard method, with slight modifications. Repellency determinations were carried out in a 10×10×3 m room, at 27-30 °C, and relative humidity of 60-80%. The testing period was conducted between 08.00 to 16.00 h. Two hundred and fifty unfed female mosquitoes were selected at random and placed inside a standard mosquito cage (30×30×30 cm), which provide a density resting surface (or vertical resting surface per mosquito) of 14.4 (Gerberg et al. 1994). These mosquitoes were rested for 1 h before starting the experiment. Before application of the test samples, the arms of each volunteer were washed with water and the ventral part of the forearm covered by a plastic sleeve with a rectangular portion cut out (3×10 cm), thus exposing the treated area only. The hand was protected by a rubber glove. Approximately 0.1 ml of 25 g% plant extract dissolved in absolute ethanol or pure essential oil was applied smoothly onto a 30 cm^2 test area on one forearm of each volunteer. The other forearm was act as a control, and be treated with absolute ethanol by the same protocol as that for the test repellent. After air drying for 1 min, the control arm was put into a mosquito cage for 3 min in order to check mosquito bite activity. If at least 2 mosquitoes land on the control arm, the repellency test was performed by exposing the treated forearm in a similar manner. The control and test arms were switched alternately in order to test the readiness of the mosquitoes to bite and prevent any bias. The complete protection time was recorded after exposing the treated forearm for 3 min at 30-min intervals until either two bites occur in a single exposure period or one bite occurs in each of two consecutive exposure periods. After each experiment, the tested mosquitoes were discarded. Each test was duplicated on different days for each of two human volunteers (1 adult female, 1 adult male). No one

was tested more than 1 sample per day. Randomization was used to assign the order of tests and treatment of volunteers, who was blinded to the repellent applied. Skin irritation, hot sensation, and unpleasant odor from each experiment were recorded.

The best plant sample (*A. sinensis*) established from the repellent screening test, was selected for isolating active extractions by using solvents of increasing polarity, including hexane, acetone, methanol, and absolute ethanol and further determination of repellent activity.

2.2.4.1.2 Repellent investigation of active extracts from the most effective plant (*A. sinensis*)

The active extractants of the most effective plant (*A. sinensis*), including AHE, AAE, AME, and AEE were tested against *Ae. aegypti* with the procedure modified from the WHO standard method (WHO 1996), as described previously, and then compared with EO and EE of *A. sinensis* for repellent activity. The most effective extract of *A. sinensis* (AHE) was the candidate for preparations of AHE-based repellent products, repellent investigation under laboratory and field situations, and determination of potential skin irritant as well as biological stability.

2.2.4.1.3 Repellent investigation of AHE-based repellent products

2.2.4.1.3.1 AHE-ethanol solution

Ethanol solutions of AHE and DEET, including 5-25% AHE-ES, AHEv-ES, DEET-ES, and DEETv-ES were investigated for repellent activity against *Ae. aegypti*, with the procedure modified from the WHO standard method (WHO 1996), as described previously. The best repellent formulation was chosen as a candidate for field study (Field I).

2.2.4.1.3.2 AHE-nanoemulsion

The 5-25% AHE-NE and AHEv-NE were tested against *Ae. aegypti* with the procedure modified from the WHO standard method (WHO 1996), as described previously, and then compared with AHE-ES and AHEv-ES for repellent activity.

2.2.4.1.3.3 10% AHEv-nanoemulsion gel

The 10% AHEv-nanoemulsion gel (10% AHEv-NEG) and 10% DEETv-nanoemulsion gel (10% DEETv-NEG) were evaluated for repellency against three mosquito vectors, including *Ae. aegypti*, *An. minimus*, and *Cx. quinquefasciatus*, with the protocol modified from the WHO standard method (WHO 1996), as described previously, with more volunteers of either sex (6 humans: 3 adult females and 3 adult males). As each mosquito species has preferences in biting time; night-biting *An. minimus* and *Cx. quinquefasciatus* was tested from 18.00 to 08.00 h, while *Ae. aegypti*, the day biter, was tested between 06.00 to 18.00 h. This product was determined also for repellency under field conditions (Field II), biological stability, and potential skin irritant.

2.2.4.2 Field repellent bioassay

AHE-based repellent products were tested for their repellency against a natural mosquito population in low-risk areas from vector-borne diseases. Field repellent investigations were divided into 2 parts, including Field I and Field II, which performed at the same residential site in a suburban area of Sunpesua subdistrict, Muang district, Chiang Mai province (18° 83' 26" N, 09° 00' 15" E) in hot and rainy seasons of 2013 and 2016, respectively. This area of approximately 300 human habitations with trees, shrubs, grass, ponds, and abundant breeding places such as ditches and sewage effluents, produced large populations of mosquitoes. While almost all inhabitants lived in houses with mosquito screened doors and windows, domestic animals in this area such as dogs, cats, and poultry, had no special protection from insect bites, and they possibly served as blood sources for mosquitoes. Despite the rarity of vector-transmitted diseases in the area, documents based on a mosquito collection conducted in August 2003 by Tuetun et al. (2004) confirms that this location is suitable as a test site, due to its large and varied populations of mosquitoes, such as *Aedes gardnerii* (35.1%), *Culex tritaeniorhynchus* (29.2%), *Culex vishnui* (19.4%), *Aedes lineatopennis* (5.1%), *Armigeres subalbatus* (3.8%), and *Mansonia uniformis* (2.3%), which were abundant enough for repellency assessment.

2.2.4.2.1 Field I

2.2.4.2.1.1 Preliminary survey

After getting the permission from the owner of this private land, the preliminary human-baited-trap surveys were undertaken 3 times during the hot season from late March to April 2013 in order to determine a suitable time for collecting mosquitoes. Pilot collections were performed for approximately 180-200 min, split into nine or ten 20-min periods between 17.00 and 21.00 h, in order that each of the three volunteers exposed to mosquitoes in natural field conditions received nine or ten biting collections. Mosquitoes landing on the exposed lower legs of volunteers were captured before they imbibed blood by trained collectors, with the aid of a mouth aspirator and small flashlight. Mosquitoes collected from each volunteer in each period were placed into a screen-topped cup individually marked with the date, time of collection, and collecting site number. All collected mosquitoes were subsequently counted and identified into species under a stereomicroscope using the taxonomic keys of Tanaka et al. (1979) and Rattanarithikul and Panthusiri (1994).

2.2.4.2.1.2 Field repellent bioassay

Field experimental trials were divided into two groups of volunteers, with each comprising two treated volunteers and a control individual. Each volunteer was protected by a jacket with hood, shoes with socks, gloves, and long trousers rolled up to the knee, thus exposing only the lower part of the leg. Accordingly, subjects deployed for collecting the mosquitoes that land on each exposed area were covered completely with outdoor clothes, gloves, and head mask to avoid attracting the mosquitoes. Of the three participants in each group, the two treated volunteers were given 2-ml aliquots of test samples [25% AHE with 5% vanillin (25% AHEv-ES) and 25% DEET with 5% vanillin (25% DEETv-ES) ethanolic solutions] topically and spread as evenly as possible on both lower legs from the base of the knee to the ankle. An equal volume of 5% vanillin in ethanol was applied in a similar manner on the lower legs of each control. After the treatment, all of the volunteers were instructed not to moisten, wash or dry their exposed leg until after the study time. Application of cosmetics, fragrances, and body care products was avoided on the day of the assay. In order to capture the mosquitoes, the two groups of volunteers were situated at least 20

m away from each other. The two repellent testers and one ethanol-treated control in each group sat on chairs about 5 m away from each other, while a mosquito collector sat opposite each volunteer. Exposure time was 180 min divided into nine 20-min periods between 18.00 and 21.30 h, in order that nine mosquito collections could be made on each subject. Mosquitoes landing on the exposed lower legs of treated and control volunteers were mouth aspirated by the collectors before the insects could imbibe any blood. After each 20-min period, the volunteers were moved to a new site at least 20 m from the last one, where they waited for 2 min before starting the next capture. The mosquitoes captured from each individual at each site were kept separately in labeled cups for counting and identifying under a stereomicroscope, using the taxonomic keys of Tanaka et al. (1979) and Rattanarithikul and Panthusiri (1994). Data from the field assessments were analyzed to determine the number and species of mosquitoes collected during the exposure period, as well as the collecting rate and percentage repellency provided by the test samples, as compared with the control. During the course of the study, volunteers, collectors, and their positions were rotated randomly in order to prevent bias from any variations such as position and personal differences, which included the number of mosquitoes, catching ability, skin absorption and persistence of repellent, as well as attractiveness to the mosquitoes.

2.2.4.2.2 Field II

2.2.4.2.2.1 Preliminary survey

The preliminary human-baited-trap surveys were undertaken 2 times during the rainy season in June 2016 in order to determine a suitable time for collecting mosquitoes, with the procedure as described in 2.2.4.2.1.1 Pilot collections were performed for approximately 220-240 min, split into eleven or twelve 20-min periods between 17.00 and 21.30 h, in order that each of the four volunteers exposed to mosquitoes in natural field conditions received eleven or twelve biting collections.

2.2.4.2.2.2 Field repellent bioassay

Nanoemulsion-developed repellent gel products of AHE and DEET were investigated for their repellency against a natural mosquito population, with slight modifications from the procedure of the Field I, as described previously. The

study was conducted 12 nights (June to July), on two groups of volunteers, with each comprising two treated volunteers and a control individual. Of the three participants in each group, the two treated volunteers were given 2 g of test samples [10% AHEv-NEG and 10% DEETv-NEG] topically and spread as evenly as possible on both lower legs from the base of the knee to the ankle. An equal volume of 5% vanillin in ethanol was applied in a similar manner on the lower legs of each control. Application of the products to all volunteers began at 16.00 and ended at 16.15 h. Volunteers were then allowed to attend to their usual affairs until 18.00 h, but they were not allowed to moisten, wash or dry their exposed leg. In order to capture the mosquitoes, the two groups of volunteers were situated at least 20 m away from each other. The distance between two repellent testers and one 5% vanillin in ethanol-treated control in each group sat on chairs about 10 m away from each other, while a mosquito collector sat opposite each volunteer. Exposure time was 220 min divided into eleven 20-min periods between 18.00 and 22.00 h, in order that eleven mosquito collections could be made on each subject. Mosquitoes landing on the exposed lower legs of treated and control volunteers were mouth aspirated by the collectors before the insects could imbibe any blood. After each 20-min period, the volunteers were moved to a new site at least 20 m from the last one, where they waited for 2 min before starting the next capture. At each site, mosquitoes landing on the legs of treated and control volunteers were captured. The mosquitoes captured from each individual at each site were kept separately in labeled cups for counting and identifying under a stereomicroscope, using the taxonomic keys of Tanaka et al. (1979) and Rattanarithikul and Panthusiri (1994). Data from the field assessments were analyzed to determine the number and species of mosquitoes collected during the exposure period, as well as the collecting rate and percentage repellency provided by the test samples, as compared with the control.

2.2.5 Evaluating potential skin irritation from AHE-based repellent products

A human 4 h patch test (Basketter et al. 1997) using the commercially available patch; Hill Top 25 chamber (Hill Top Research, Cincinnati, OH), were performed for evaluating skin irritant from AHE-based repellent products, including 25% AHE-ES and 10% AHEv-NEG. Thirty healthy male and female volunteers, aged 22-59 years, who show no signs of dermatological diseases, were recruited as participants in this experiment. The patch test procedure involves applying 0.2 ml/0.2 g

of test products on a Hill Top Chamber containing a Webril pad to the skin of human volunteers. The patch embedded with repellent products were placed for up to 4 h on the upper inner arm of the volunteers. Absolute ethanol and a 20% aqueous sodium lauryl sulfate (20% SLS) were used as negative and positive references, respectively. After the 4 h period of exposure for testing the solution, the patch were removed and sites of application gently wiped with wet gauze before washing with water. The applied sites were examined and scored at 24, 48 and 72 h after patch removal. Response to irritation was measured by using the four point scale (Table 2.3) of increasing severity by Basketter et al. (1997).

Table 2.3 Assessment of reactions

Grading	Description of response
(0)	No reaction
(+)	Weakly positive reaction (usually characterized by mild erythema or dryness across most of the treatment site)
(++)	Moderately positive reaction (usually distinct erythema possibly spreading beyond the treatment site)
(+++)	Strongly positive reaction (strong, often spreading erythema with edema)

A volunteer with a '+' or greater reaction at any of the assessments was considered to have a positive skin irritant reaction.

2.2.6 Testing the physical and biological stability of AHE-based repellent products

Prior to the introduction of any newly developed product as a promising repellent, it is necessary to investigate its physical and biological stability. There are at least two conventional procedures to test the physical and biological stability of any products, i.e., the temperature-time (keeping at conditions that vary in temperature and time storage) and/or the rapid (heating and cooling) methods. In this study, AHE and 10% AHEv-NEG samples were selected for investigation of their biological stability, as follows.

2.2.6.1 Temperature-time method

The experiment was set to determine physical characteristics and biological stability (persistence of repellent activity) of AHE and 10% AHEv-NEG samples after being kept in conditions that vary in temperature and time storage. AHE

samples were kept at various temperatures (4 °C, ambient temperature: AT, and 45 °C) for different durations (1, 3, and 6 months), and 10% AHEv-NEG samples were kept at the same temperature with AHE (4 °C, AT, and 45 °C) for 1, 2, 3, and 6 months. After determining physical characteristics, the stored AHE and 10% AHEv-NEG samples were tested for repellent activity against *Ae. aegypti*, following the standard method (WHO 1996), and compared with the fresh preparation.

2.2.6.2 Heating and cooling method

Samples of 10% AHEv-NEG were determined for physical characteristics and repellent activity against *Ae. aegypti* after they had been kept under a heating (45 °C, 48 h) and cooling (4 °C, 48 h) cycle for at least 4 cycles, and compared with the fresh preparation.

2.2.7 Statistical analysis

The median complete-protection time in the laboratory bioassays was used as a standard repellency criterion of the test samples against *Ae. aegypti*. Differences in significance were analyzed by a Kruskal-Wallis one-way ANOVA using SPSS 17.0 software. Statistically significant results were considered at $P < 0.05$. The effect of vanillin in prolonging the protection time of the test repellents was analyzed using the Mann-Whitney U Test. In analyzing field data, the total number of mosquitoes collected during each exposure was log-transformed before the mean and standard errors (SE) were analyzed. The Kruskal-Wallis one-way ANOVA also was used to determine the significance of difference between the controls and those volunteers treated at the critical level of 0.05. Percentage repellency (% Repellency) in the field trials was calculated by the following formula (Sharma and Ansari 1994; Yap et al. 1998).

$$\% \text{ Repellency} = \frac{C - T}{C} \times 100$$

where, C is the number of mosquitoes collected from the lower legs of the controls and T is the number collected from the treated legs.