# CHAPTER 2

# MATERIALS AND METHODS

## 1. MATERIALS

### 1.1 Plant materials

Fifteen plant species belonging to 11 families (Table 2.1) were selected for repellent screening on the basis of their volatile smell, abundance, and data available in the literature, including botanical information, pharmacological properties, and anti-mosquito activities (Sukumar et al., 1991; Shaalan et al., 2005; Amer and Mehlhorn, 2006; Maia and Moore, 2011; Patel et al., 2012; Lupi et al., 2013). The plant samples were collected from their natural habitats or purchased from a commercial herbal supplier in Chiang Mai and Lamphun provinces. Taxonomic identification of plant species was performed by James Franklin Maxwell, a botanist at the CMU Herbarium, Department of Biology, Faculty of Science, and Miss Wannaree Charoensup, a scientist at Department of Pharmaceutical Science, Faculty of Pharmacy, Chiang Mai University (CMU), Chiang Mai province, Thailand. A voucher specimen of each plant was deposited at the Department of Parasitology, Faculty of Medicine, CMU. Each plant sample was air-dried under shade at a prevailing temperature of about 30±5 ˚C for one week prior to preparing the plant products.

### 1.2 Human volunteers

Healthy human volunteers of both sexes (age 21-35 years old; weight 44-93 kg), who had no history of allergic reactions or dermatological disease to arthropod bites, stings, or repellent were recruited from the graduate students of CMU. All volunteers were interviewed and informed on the objective and methodology of the study, probable discomforts to subjects, and remedial arrangements, before signing an informed consent form under protocol PAR-11-808-EX 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.

**Table 2.1** List of the plants used for the repellent screening bioassay

| Family & Scientific name | English name | Thai name | Part used | Voucher No. |
|---|---|---|---|---|
| **Apiaceae (Labiatae)** | | | | |
| *Ocimum basilicum* L. | Sweet basil | โหระพา | Leaf | PARA-OC-001-Le/1 |
| *Ocimum americanum* L. | Hairy basil | แมงลัก | Seed | PARA-OC-002-Se/1 |
| **Asteraceae** | | | | |
| *Chromolaena odoratum* (L.) R.M. King & H.Rob. | Bitter bush | สาบเสือ | Stem & Leaf | PARA-CH-001-St-Le/1 |
| *Saussurea lappa* Clarke | Costus | โกฐกระดูก | Root | PARA-SA-001-Ro/1 |
| **Bixaceae** | | | | |
| *Bixa orellana* L. | Annatto | คำแสด | Seed | PARA-BI-001-Se/1 |
| **Fabaceae** | | | | |
| *Clitoria ternatea* L. | Blue pea | อัญชัน | Seed | PARA-CL-001-Se/1 |
| *Acacia concinna* (Willd.) DC. | Soap pod | ส้มป่อย | Pods | PARA-AC-001-Po/1 |
| **Poaceae** | | | | |
| *Vetiveria zizanioides* (L.) Nash | Vetiver | แฝกหอม | Rhizome & Root | PARA-VE-001-Rh-Ro/1 |
| **Polygonaceae** | | | | |
| *Rheum palmatum* L. | Rhubarb | โกฐน้ำเต้า | Root | PARA-RH-001-Ro/1 |
| **Rutaceae** | | | | |
| *Aegle marmelos* (L.) Correa ex Roxb. | Bael | มะตูม | Leaf | PARA-AE-001-Le/1 |
| | | | Fruit | PARA-AE-001-Fr/1 |
| **Saururaceae** | | | | |
| *Houttuynia cordata* Thunb. | Fish mint | คาวพลู | Leaf | PARA-HO-001-Le/1 |
| **Umbelliferae** | | | | |
| *Ligusticum sinense* Oliv. cv. Chuanxiong Horf | Chuanxiong rhizoma | โกฐหัวบัว | Rhizome | PARA-LI-001-Rh/1 |
| *Angelica dahurica* Fisch.ex Hoffm | Chinese angelica | โกฐสอ | Root | PARA-AN-001-Ro/1 |
| **Verbebaceae** | | | | |
| *Lantana camara* L. | Weeping lantana | ผกากรอง | Flower | PARA-LA-001-Fl/1 |
| **Zingiberacae** | | | | |
| *Zingiber zerumbet* (L.) Smith | Shampoo ginger | กระทือ | Flower | PARA-ZI-001-Fl/1 |
| | | | Rhizome | PARA-ZI-001-Rh/2 |

### 1.3 Experimental animals

#### 1.3.1 Mosquitoes

Mosquito test populations in this study were composed of the free-mating laboratory *Aedes aegypti* and *Anopheles minimus*. A laboratory colony of *Ae. aegypti*, which originated from specimens collected in Chiang Mai province were colonized and maintained continuously for several generations in an insectary of the Department of Parasitology, Faculty of Medicine, CMU. The colony of *An. minimus* obtained originally from the Office of Vector Borne Diseases Control, Department of Communicable Disease Control, Ministry of Public Health, Chiang Mai province, were established and maintained in the insectary of 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 8-12 hr.

#### 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 hr light:dark period. Rats were used as a source of blood meal for female mosquitoes during egg production.

### 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 (EC No. 200-578-6: Darmstadt, Germany)
- 95% Ethanol
- Hexane

## 2. METHODS

### 2.1 Preparation of plant extracts

Plant samples were separated and shade dried at an ambient temperature. Dried plant materials were ground to a coarse/fine powder prior to extractions, which were divided into 2 procedures, solvent extraction and steam distillation.

#### 2.1.1 Solvent extraction

Finely ground materials of each plant were extracted with chemical solvents; 95% ethanol and hexane. Half a kilogram of dried powder from each plant was extracted successively three times by maceration with 5 liters of 95% ethanol or hexane at room temperature for 7 days. After vacuum filtration through a Bücher funnel, the solvent in combined filtrates was removed on a rotary evaporator at 60 ˚C (for 95% ethanol) and 40 ˚C (for hexane), until the solvent had evaporated completely. The residues were lyophilized to yield dry plant extracts. The extract of each plant was kept at -20 ˚C until required for the repellent screening bioassays.

#### 2.1.2 Steam distillation

Coarsely ground plant materials were extracted individually for volatile oil by steam distillation. 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 mixture was allowed to settle for 3-5 days, after which, the water (lower) layer was drawn off slowly until only the oil layer remains. The volatile oil was dried over anhydrous sodium sulfate ($Na_2SO_4$) and then collected and kept in a brown bottle at 4 ˚C until required for repellent screening tests.

### 2.2 Mosquito rearing

Laboratory colonies of *Ae. aegypti* and *An. minimus* used in this study were reared and maintained in the insectary of the Department of Parasitology, Faculty of Medicine, CMU, at a constant temperature of 27±2 ˚C and 70-80% relative humidity, and illuminated with a combination of natural daylight from a glass window and fluorescent lighting providing for approximately 12 hr a day. The methods for mass rearing were slight modifications of the procedure described by Limsuwan et al. (1987).

#### 2.2.1 *Aedes aegypti*

Approximately 200 larvae were reared in a plastic pan containing 2 liters of tap water and fed on finely ground dog-biscuit. The water was changed two or three times a week 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, with about 300 pupae, were placed into netted cages (30 x 30 x 30 cm). After emerging, adults were fed with 10% sucrose and 10% v/v multivitamin syrup solution. 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 use in the next rearing. The filter paper with eggs was placed into trays filled with tap water for hatching. The larvae were hatched within 24-48 hr after immersion and then transferred to a new rearing pan.

#### 2.2.2 *Anopheles minimus*

After blood feeding, the engorged adult females were maintained for 3-4 days and/or until gravid in the insectariums. Then, they were placed in a screen-topped oviposition plastic-cup containing 25 ml of filtered distilled water. Wet filter paper was lined inside the screen-topped cup, which was subsequently covered with a black plastic sheet. 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 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 white plastic tray containing 2,000 ml of distilled water, and 100 first instar larvae were reared in each tray, which placed with water lettuce. The first and second instar larvae were fed on finely ground fish food twice a day, and this schedule was increased to 3-5 times daily after most of the larvae had reached third and fourth instars.

After pupation, approximately 100 pupae were placed in a plastic cup containing 150 ml of distilled water and kept in a 30 x 30 x 30 cm cage. The emerged adults were provided with 10% sucrose and 10% multivitamin syrup solution. Increased humidity to promote the adults was provided by covering the cage with a wet towel overlaid with a transparent plastic sheet. Rats were used as a source of blood meal for egg producing females. The eggs were laid on filter paper soaked with water in egg collecting cups. The filter paper with eggs was placed into trays filled with distilled water for hatching. The larvae were hatched within 24-48 hr after immersion and then transferred to a new rearing pan.

### 2.3 Laboratory repellent bioassay

#### 2.3.1 Screening for the repellent activity of plant samples

Plant products, including essential oil, ethanolic extract, and hexane extract of each plant species were screened for repellent efficacy against laboratory-reared *Ae. aegypti* females by using the human-bait technique of the WHO (1996) standard method, with slight modifications. Repellency determinations were carried out in a 10 x 10 x 6 m room, at 27-30 ˚C and relative humidity of 60-80%. The testing period was run between 06.00 to 18.00 hr, because *Ae. aegypti* is usually a day-biter.

Two hundred and fifty starved female mosquitoes were chosen at random and placed inside a standard mosquito cage (30 x 30 x 30 cm), and rested for 1 hr before starting the experiment. Each volunteer's ventral part of the forearm was covered by a plastic sleeve with a rectangular portion cut out (3 x 10 cm), thus exposing the treated area only. The hand was protected by a rubber glove. Approximately 0.1 ml of undiluted essential oil, 25% solvent (ethanol/hexane) extracts, or 25% DEET solution was applied evenly onto a 30 $cm^2$ test site on one forearm of each volunteer. The other forearm, acting as a control, was treated with solvent by the same protocol as that for

the test repellent. After air drying for 1 min, the control arm was inserted into an experimental cage for 3 min in order to make comparative checks and determine how active the mosquitoes are to bite. 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 interchanged regularly to test for 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 occurred in a single exposure period or one bite occurred 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

#### 2.3.2 Investigation on repellent activity of the most effective sample

The best repellent sample, with the longest-lasting protection time, established from the laboratory screening was selected as candidates for further repellent investigations against two target mosquitoes, *Ae. aegypti* and *An. minimus*, with more volunteers of either sex (6 humans: 3 females and 3 males) under the standard cage conditions. The selected plant sample was prepared in suitable solvent with and without 5% vanillin and evaluated for repellency in comparison to the standard repellent, DEET. The control was treated with 5% vanillin in ethanol. The experiments were conducted inside a standard cage (30 x 30 x 30 cm) with the procedure modified from the WHO standard method (WHO, 1996), as described previously. The timing of the test periods depends on whether the target mosquitoes are day or night-biters. Day-biting *Ae. aegypti* was tested from 06.00 to 18.00 hr, while night-biting *An. minimus* was tested between 18.00 to 09.00 hr.

### 2.4 Field repellent bioassay

The best repellent samples of each group, plant and DEET products, established from the laboratory repellent bioassays were candidates tested for their repellency

against a natural population of mosquito at the low-risk areas of vector-borne diseases in Chiang Mai province. The field repellent study conducted at minimal risk in human use procedure (Choochote et al., 1999; Ansari et al., 2000) was divided into 2 steps, preliminary survey and field experiment.

### 2.4.1 Preliminary survey for the field experimental areas

Preliminary surveys using a human-baited trap were performed to locate the experimental areas and times for collecting mosquitoes. A suitable area should consist of human habitations, trees, shrubs, grass, and ponds, which are capable of harboring a large and wide range of mosquito populations that are sufficient for repellent investigation. The collections were performed for 180 min, split into nine 20-min periods between 17.30 and 21.30 hr, in order that nine biting collections were made on each volunteer exposed to natural field populations of mosquito. Mosquitoes that landed on each volunteer were captured by trained collectors, with the help of a mouth aspirator and flashlight, and placed into separate cups for subsequent counting and identification under a stereomicroscope using the taxonomic keys of Tanaka et al. (1979) and Rattanarithikul and Panthusiri (1994).

### 2.4.2 Field experimental procedure

For each field trial, two groups of volunteers each comprising two testers and one control, were employed as baits. Of the three volunteers in each group, two were applied topically with 2-ml aliquots of repellent samples (plant and DEET ethanolic solutions), as uniformly as possible, on both lower legs from the base of the knee to the ankle. The remaining control volunteer was treated with 5% vanillin in ethanol. Each volunteer was directed to wear a jacket with hood, gloves, shoes with socks, and long trousers rolled up to the knee, thus exposing only the lower legs to blood-seeking mosquitoes.

For mosquito captures, the two groups of volunteers were situated at least 20 m apart from each other. Two repellent testers with one ethanol-treated control subject from each group sat on chairs spaced 5 m apart, with both legs exposed for 180 min divided into nine 20-min periods between 17.30 and 21.30 hr, so that nine mosquito collections were made on each volunteer. Mosquitoes landing on the exposed lower legs

of treated and control volunteers were captured before they imbibed any blood by experienced mosquito collectors, using a mouth aspirator and flashlights. The mosquitoes caught from each individual at each site were kept separately in labeled cups for counting and identifying later. After each 20-min period, the volunteers were moved to a new site at least 20 m from the last one and stayed for 2 min before starting the next capture.

During the course of the study, volunteers, collectors, and their positions were randomly rotated 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, and attractiveness to mosquitoes. All the mosquitoes collected were counted and identified 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 colleted during the exposure period, the collecting rate, and the percentage repellency provided by the test samples, as compared with the control.

**2.5 Testing the physical and biological stability of the most effective plant sample**

For highly effective and practical application, any mosquito repellent needs to maintain its physical property and bioactivity for an appropriate period of time. The best repellent of plant sample was investigated for its physical and biological stability. The experiment was set to determine physical changes and repellent activity persistence of the plant product after being kept in conditions that vary in temperature and time storage. The test for biological stability was followed a modification of the WHO standard method (WHO, 1996), as described previously. For this step, samples of the most effective product were kept at various temperatures (4 ˚C, ambient temperature, and 45 ˚C) for different durations (1, 2, and 3 months). They were observed, tested, and compared to earlier results of fresh preparation for physical characteristics and protection times against the target mosquitoes. The repellent test was conducted two times on each of 6 human volunteers (3 females and 3 males).

### 2.6 Chemical analysis of the most effective plant extract

Qualitative analysis of the chemical constituents of the best repellent sample was analyzed by gas chromatography coupled to mass spectrometry (GC/MS) at the Central Laboratory (Thailand) Co.,Ltd. (CLT), Chiang Mai branch. Equipment and conditions for chemical analysis were as follows:

- **Gas chromatography: GC 6890 Agilent technologies (Germany)**

  Inlet temperature: 250 ˚C

  Inlet split: 1.0 µl of 20 mg/ml, split ratio 10:1

  Oven: 40 ˚C hold 5.0 min, 5 ˚C/min to 280 ˚C hold 7.0 min

  Carrier: Helium gas (flow rate: 1.0 ml/min)

  Column: DB-5MS (30 m x 0.25 mm x 0.25 µm film thickness)

- **Mass spectrometer detector: MSD 5973 Agilent technologies (USA)**

  : MS Quadrupole temperature 150 ˚C

  : MS Source temperature 230 ˚C

The GC/MS analysis of plant product was performed using a Hewlett-Packard 6890 gas chromatograph (Agilent technologies) equipped with a split-splitless injector and DB-5MS (30 m x 0.25 mm x 0.25 µm film thickness) columns directly coupled to a quadrupole mass selective detector, MSD 5973 (Agilent technologies). The injector temperature was set at 250 ˚C and the oven temperature was initially at 40 ˚C and hold 5 min, then programmed to 280 ˚C at the rate of 5 ˚C/min and held at 280 ˚C for 7 min. Helium was used as the carrier gas with a flow rate of 1.0 ml/min. The sample (1.0 µl) was injected neat with split ratio of 10:1. The mass spectrometer (MSD 5973) was operated in the electron impact (EI) mode at 70eV. The ion source temperature and the quadrupole temperature were set at 230 ˚C and 150 ˚C, respectively. The oil components were identified by comparison with standards, by spiking, and on the basis of their mass spectral fragmentation using the Wiley 7N librarie. Percentage of the identified compound was computed from a Total ion chromatogram (TIC).

**2.7 Statistical analysis**

The median complete-protection time was used as a standard measure of the repellent efficacy of the plant extract against *Ae. aegypti* and *An. minimus* in the laboratory. Differences in significance were determined by comparing the range of protection time of each plant. The effects of vanillin in prolonging the protection time of the repellents were analyzed using the Mann-Whitney U Test. In the field repellent bioassays, the total number of mosquitoes collected during each exposure was log-transformed before the mean and S.E. is analyzed. A Kruskal-Wallis one-way ANOVA were used to determine the significant difference between the controls and volunteers treated at the critical level of 0.05, by using the SPSS program. Percentage repellency (% Repellency) in the field study was analyzed 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 legs of the control and T is the number collected from the treated legs.