2. OBJECTIVES OF THE STUDY

The present study approaches the following objectives:

1. Screening of mosquito larvicidal properties of thirty ethnomedicinal plants from Mizoram.
2. Selective bioassay - mortality, lethal concentration ($LC_{50}$) and lethal time ($LT_{50}$) of certain plant extracts.
3. Estimation of nutrient reserves in treated mosquito larvae
4. To study the effect of plant extracts as insect growth regulators
5. To study adulticidal effect, repellency and oviposition deterrency of plant extracts against mosquitoes
6. Assessment of DNA damage in the treated larvae by RAPD – PCR tools
3. MATERIALS AND METHODS

3.1. Mosquito culture

Mosquito eggs and larvae were reared in plastic and enamel trays containing tap water. They were maintained, and all the experiments will be carried out, at 27±2°C and 75 –85% relative humidity under 14:10 light and dark cycles (WHO, 2005). *Aedes albopictus*, *Anopheles barbirostris* and *Culex quinquefasciatus* larvae were collected from various places to start the colony and were reared in plastic and enamel trays containing tap water (Plate 2: A). The mosquito species were identified with the help of standard keys (Das *et al.* 1990; Reuben *et al.* 1994; Rueda 2004). The laboratory colonies were maintained at 25-30°C and 80-90% relative humidity under a photoperiod of 14:10 h (light/dark) in the insectary of the Department of Zoology, Mizoram University, Aizawl, Mizoram. Pupae were transferred from the trays to a cup containing tap water (Plate 2: B) and were maintained in our insectary (45×45×40 cm) where adults emerged (Plate 2: C). Larvae were fed on dog biscuit, soya flour, brewers yeast or algae collected from ponds. The adults were provided with 10% sucrose and it was periodically blood-fed on restrained rats or chicken. Beakers with 50 ml of tap water lined with filter paper were kept inside the cage for oviposition.

3.2. Preparation of plant extracts

The air dried plant materials (leaves, bark, flowers) were powdered mechanically using commercial electrical stainless steel blender and extracted with petroleum ether, acetone, chloroform, methanol and water. To determine the efficacy of these chemical extracts, the air dried leaf powders and the solvent were placed in
conical flasks at the ratio of 1 mg: 1 ml. The conical flasks were covered with air tight seals and the mixture was then left to stand for 2-7 day. The flasks were shaken everyday for about one to three hour. The mixture was then filtered through filter paper. The solvent was evaporated off with the help of a water bath (Kotze and Eloff 2002). The concentrated extract was then placed in vials. The filtrate was considered as pure material and redissolved in absolute ethanol to 10% (w/v) standard formulation. By further dilutions with required amount of water, different ppm concentrations were prepared.

3.3. Larvicidal bioassay

Control experiments were conducted in parallel with each replicant. All the experiments were performed according to World Health Organization standard protocols (WHO 1981) with suitable modifications. Mortality counts were made after 24. Dead larvae were identified when they failed to move after probing with dropper. Percentage mortality is recorded from the average for the three replicates taken. The percentage mortality was calculated by using the formula:

\[
\text{Percentage of mortality} = \frac{\text{Number of dead insects}}{\text{Number of insects tested}} \times 100
\]

Controls with more than 20% mortality were discarded. When the mortality ranged from 5-20%, the corrected mortality was calculated by Abbott’s formula so as to remove the error, if any, on account of the mortality due to factors other than the toxic effect of the extract (Abbott, 1925). Initially, thirty III instar mosquito larvae in
three replicates were exposed to a particular concentration (500 ppm) of different plant extracts to find out the larvicidal activity.

### 3.3.1. Preliminary screening of larvicidal bioassay

In preliminary screening of larvicidal bioassay, third instar larvae of *Ae. albopictus*, *An. barbirostris* and *Cx. quinquefasciatus* were treated in 500 ppm of plant extracts. A total of 30 third instar larvae were exposed in three replicates of 10 larvae each. Control experiments were conducted in parallel with each replicant. Experiments were conducted at room temperature (28±2°C).

### 3.3.2. Dose–response larvicidal bioassay (LC$_{50}$)

After screening of different plant extracts against the third instar larvae of *Ae. albopictus*, *An. barbirostris* and *C. quinquefasciatus*, plant extracts showing high mortality rate were selected for dose – response bioassay. Required concentrations of different plant extracts (concentrations of 12.5, 25, 50, 100, 200, 400, and 500 ppm) were prepared through the mixing up of stock extract with variable amounts of sterilized distilled water. Each of the earlier prepared concentrations of different extracts was transferred into the sterile glass beakers (500 ml capacity). For bioassay test, third instar larvae of *Ae. albopictus*, *An. barbirostris*, and *Cx. quinquefasciatus* were divided into respective groups in four batches of 25 numbers in 249 ml of water and individually added with 1.0 ml of different concentration (12.5, 25, 50, 100, 200, 400, and 500 ppm) of plant extract. No food was provided during the treatment. Mortality was recorded after 24 and 48 h of post-exposure (WHO 1981). Dead larvae were identified when they failed to move after probing with a needle in the siphon or
cervical region. The experiments were observed four times and conducted at 27±2°C and 80–90% relative humidity. The untreated control was set up with acetone solvent. The corrected mortality was calculated by Abbott’s formula (Abbott 1925).

3.3.3 Time response Larvicidal Bioassay (LT$_{50}$)

One milliliter of plant extract was added to 249 ml distilled water in a 500 ml plastic cup, which was shaken lightly to ensure a homogeneous test solution. Twenty five specimens each of third instar larval stages of *Ae. albopictus*, *An. barbirostris* and *Cx. quinquefasciatus* were divided into respective groups and placed in cups. No food was provided during the treatment. Lethal time was observed at the concentrations of 12.5, 25, 50,100, 200 and 400 ppm. Mortality was recorded at regular intervals of 1, 3, 5, 8, 15, 20, 24 and 48 h of post-exposure in each concentration. Larvae were considered dead if they were incapable of rising to the surface or did not show the characteristic dicing reaction when the water was disturbed (WHO 1981). The mean mortality number was recorded. Each experiment was performed in four replicates with a simultaneous control (1 ml 70% ethanol in 249 ml water). LT$_{50}$ values (lethal time for 50% mortality at a specific dose) were calculated using probit analysis. The mortality data was analyzed by Tukey’s multiple range test (Snedecor and Cochran 1989).

3.4. Estimation of nutrient reserves from treated and untreated mosquito larvae

A sublethal concentration was selected on the basis of dose response bioassay to observe the effect on nutrient reserves of freshly emerged III instar larvae of *A. albopictus*, *An. barbirostris* and *Cx. quinquefasciatus*. The bioassay experiment was
replicated thrice. Five mosquito larvae of the treated and untreated each mosquito species were placed separately in each microcentrifuge tube with 200 µl of sodium sulphate and crushed with a plastic pestle, and two x 0.8 ml volumes of chloroform–methanol (1:1) was used to wash the pestle. The tubes were vortexed and centrifuged at 3,000 rpm for 1 min. The supernatant was transferred to another microcentrifuge tube whereas pellet was retained for glycogen analysis. De-ionized water was mixed with the supernatant and centrifuged at 3,000 rpm for 1 min. Aqueous layer was separated for sugar analysis, and bottom portion was used for lipid analysis (van Handel 1985a).

3.4.1. Sugar analysis

The tubes were heated at 90°C until the solutions evaporated from the sample, and about 50–100 µl of solution is left in the sugar tubes. Anthrone (5 ml) was added to the the tubes, vortexed and heated for 20 min at 90°C. The absorbance was recorded at 625 nm.

3.4.2. Glycogen analysis

Anthrone (5 ml) was added to the precipitate of the tubes containing glycogen pellet, vortexed and heated for 20 min at 90°C. The absorbance was recorded at 625 nm.

3.4.3. Lipid analysis

Samples were heated at 90°C until all solutions were evaporated from the lipid. Sulphuric acid (200 µl) was added to the tubes containing lipid precipitate and
heated for 10 min at 90°C. 5 ml of vanillin–phosphoric acid reagent was added, vortexed and allowed to cool, and the absorbance was read at 525 nm (van Handel 1985b).

3.4.3. Protein analysis

Freshly emerged III instar larvae were collected from the experimental setup for the protein analysis. Five larvae were taken for each concentration and homogenized in 0.25 M sucrose solution in cold conditions. The homogenate was centrifuged at 12,000 rpm for 10–12 min, and the obtained supernatant was used to determine the total protein present in the sample (Lowry et al. 1951).

3.4.4. Standard curves

Known amounts of glucose and soybean oil (1 mg/ml) were prepared in deionized water and chloroform, respectively, as reported by van Handel (1985a,b). Glucose solution was prepared in amount of 25, 50, 100, 150 and 200 µg and brought to a volume of 5 ml with anthrone reagent, whereas soybean oil was prepared in amount of 50, 100, 200 and 400 µg and brought to a volume of 5 ml with vanillin–phosphoric acid reagent. Three replicates were prepared for each concentration; absorbance was read at 625 nm for glucose and 525 nm for soybean oil, and nutrient amount was calculated from the resulting linear regression equations.

3.5. Insect Growth Regulators

The insect growth regulators bioassay followed Sagar and Sehgal (1997) and the World Health Organization standard protocols (WHO 2005) with slight
modifications. Insect growth regulatory (IGR) activity of different plant extracts were tested against *Ae. albopictus*. Ten first instar larvae were introduced into 500ml enamel bowls containing 249 ml of water. Three different test concentrations 5 ppm, 10 ppm and 20 ppm were tested against *Ae. albopictus* and each test concentration were replicated four times. 250 ml of distilled water and distilled water with petroleum ether and Tween-20 served as control. Tween-20 was used as emulsifier in all the experimental media. The control experiments were run parallel with each replicate. Mortality of the larvae, pupae, larval pupal intermediate and adult mortality was recorded at regular intervals. Observation was continued in both treated and control bowls until the last immature pupates. Morphological abnormalities were also noted. The dead larvae and pupae removed daily and counted. The percentage emergences at different concentrations were recorded. Growth index was assessed by the following formula:

\[
\text{Developmental Period (DP)} = \frac{\text{No. of larvae moulted} \times \text{Days taken}}{\text{Total no. of larvae moulted from that instar}}
\]

\[
\text{Growth Index (GI)} = \frac{\text{% Survival in a particular stage}}{\text{Developmental period}}
\]

\[
\text{Adult Emergence IE(\%)} = 100 - \left( \frac{T \times 100}{C} \right)
\]

where \( T \) = percentage survival or emergence in treated batches \( C \) = percentage survival or emergence in the control.

The moribund and dead larvae in four replicates were combined and expressed as a percentage of larval mortality of each concentration. Dead larvae were
identified when they failed to move after probing with a needle in the siphon or cervical region. Moribund larvae were those incapable of rising to the surface (within a reasonable period of time) or showing the characteristic diving reaction when the water was disturbed. All surviving larvae were separately reared and maintained at 25-30°C and 80-90% relative humidity in the insectary. Pupation and adult emergence of these mosquitoes were recorded. The assays were terminated 3 day after the last control mosquito emerged.

The various dispersed effects that interfere with ecdysis was studied based on the characteristic toxic effects on mosquito larvae and were recorded according to the following eight criteria of Lapcharoen et al. (2005):

3.5.1. L (death as larvae). This category represents death during the larval stage with no evident initiation of pupation.

3.5.2. L (P) (larval cuticle with pupa inside). Death in this category has occurred at an early stage of pupation. The pupal abdomen can be seen to be withdrawn from the terminal part of the abdomen and the pupal respiratory trumpets are visible.

3.5.3. L-P (larvae with pupae partly emerged). At this stage the larval skin has been ruptured and the pupal body has partly emerged from the thoracic split. The abdomen has retracted to at least halfway along the larval abdominal skin and has adopted the characteristic pupal shape.

3.5.4. WP (white pupae). The pupae have completely escaped from the larval cuticle but have remained completely unmelanized except for eye pigment. The abdomen is held in an abnormally straight position.

3.5.5. BP (brown pupae). The pupae show some melanization.
3.5.6. P (A) (pupae with adult visible inside). In this category, most of the adult anatomy can be distinguished, but the pupal skin has not split. Unlike the previous categories, the dead insect normally floats, presumably because the internal air bubble is preserved.

3.5.7. P-A (pupae with adult beginning emergence). The adults have begun to escape from the pupal skin but are unable to free themselves completely. Sometimes the head and thorax are freed, but the abdomen remains enclosed. Occasionally, the whole body is nearly free except for the legs.

3.5.8. DA (death adult). This category is reserved for adults which have freed themselves completely from the pupal skin, but cannot escape from the water film.

When mortality in the control is over 20%, the tests would be discarded.

3.6. Adulticidal Bioassay

Three mosquito vectors, A. albopictus, An. barbirostris and Cx. quinquefasciatus mosquitoes were selected for the testing of adulticidal activities (WHO 1996). Appropriate concentrations (10000, 25000 and 50000 ppm) were dissolved in 2.5 ml of acetone and applied on Whatman no. 1 filter papers (size 12 x15 cm). Impregnated papers were left to dry at room temperature overnight prior to testing. Control papers were treated with acetone under similar conditions. Adulticidal activity was evaluated at three concentrations (10000, 25000 and 50000 ppm) with an untreated control.

Ten female mosquitoes (3-6 days old 10% glucose fed, blood starved) were collected and gently transferred into a plastic holding tube. The mosquitoes were allowed to acclimatize in the holding tube for 1 h and then exposed to test paper for 1
h. At the end of exposure period, the mosquitoes were transferred back to the holding tube and kept 24 h for recovery period. A pad of cotton soaked with 10 per cent glucose solution was placed on the mesh screen. Mortality of mosquitoes was determined at the end of 24 h recovery period. Per cent mortality was corrected by using of Abbott’s formula (Abbott 1925). Three observations were made for each concentration.

3.7. Repellent Bioassay

The duration of protection provided by plant extract was tested by means of arm-in-cage studies, in which volunteers insert their repellent-treated arms into a cage with a fixed number of unfed mosquitoes, and the elapsed time to the first bite is recorded (Fradin and Day 2002). Testing of repellent was conducted in a laboratory to reduce potential confounding variables (wind, speed, temperature, humidity, density of the mosquito population, the level of the mosquitoes’ hunger, and the species of the mosquitoes).

For each test, 10 disease-free, laboratory-reared *Ae. albopictus*, *An. barbirostris* and *Cx. quinquefasciatus* female mosquitoes separately that were between 5-10 days old were placed into separate laboratory cages measuring 30 cm by 22 cm by 22 cm. A batch of 10 mosquitoes that had not been exposed to the repellent being tested was used for each arm insertion. Mosquitoes were provided with a constant supply of 5 percent sucrose solution. Cages were placed in a laboratory at 27 ± 2°C, 80–90% RH and 14:10 light and dark cycles. Fifteen volunteers (5 men and 10 women) were used in the study.
The plant extracts were sequentially diluted to 5000, 10000 and 20000 ppm in absolute alcohol and tested on each subject. The repellent action was being tested three times on each subject. Most subjects only completed one test per day. The average time to completion of all three tests was 10 days. Before each test, the readiness of the mosquitoes to bite was confirmed by having subjects insert their untreated forearm into the test cage. Once subjects observed five mosquito landings on the untreated arm, they removed their arm from the cage and applied the repellent being tested from the elbow to the fingertips (Fradin and Day 2002).

After the application of the repellent, subjects were instructed not to rub, touch, or wet the treated arm. Subjects were provided with a standardized log sheet to ensure accurate documentation of the duration of exposure and the time of the first bite. The elapsed time to the first bite was then calculated and recorded as the “complete-protection time” for that subject in that particular test (Fig. 3).

### 3.8. Oviposition Deterrent Test

The oviposition deterrent test was performed using the method of Xue et al. (2001). Fifteen gravid females of *Ae. albopictus* (10 days old, 4 days after blood feeding) were transferred to each mosquito cage (45 × 38 × 38 cm). 10% sucrose solution was given as food supplement. Serial dilutions of leaf extract were made in ethanol. Enamel bowls containing 100 ml of rainwater would be treated with leaf extract to obtain test solutions of 5, 10 and 20 ppm. Two enamel bowls holding 100 ml of rainwater were placed in opposite corners of each cage, one treated with the test material, and the other with a solvent control that contained 1% ethanol. The positions of the bowls were alternated between the different replicates so as to nullify
any effect of position on oviposition. Five replicates for each concentration were run, with cages placed side by side for each bioassay. After 24 h, the number of eggs laid in treated and control bowls would be recorded.

The percent effective repellency for each leaf extract concentration was calculated using the following formula

\[
ER(\%) = \frac{NC - NT}{NC} \times 100 \%
\]

Where \( ER \) = percent effective repellency; \( NC \) = number of eggs in control; and \( NT \) = number of eggs in treatment.

The oviposition experiments were expressed as mean number of eggs and oviposition activity index (OAI), which was calculated using the following formula.

\[
OAI = \frac{NT-NS}{NT+NS}
\]

Where \( NT \) = total number of eggs in the test solution and \( NS \) = total number of eggs in the control solution. Oviposition active index of +0.3 and above are considered as attractants, while those with – 0.3 and below are considered as repellents (Kramer and Mulla 1979). Positive values indicate that more eggs were deposited in the test cups than in the control cups and that the test solutions were attractive. Conversely, negative values indicate that more eggs were deposited in the control cups than in the test cups and that the test solutions were a deterrent.
3.9 RAPD-PCR profiling of treated mosquito larvae

3.9.1 DNA Extraction

DNA from mosquito larvae was extracted by the method of Ballinger-Crabtree et al. (1992) with slight modifications. Ethanol preserved specimens were ground in 200 µl lysis buffer (100 mM Tris–HCl, pH 8.0; 1% sodium dodecyl sulphate; 50 mM NaCl; 50 mM EDTA), and the mixture was treated with 5 µl of proteinase K (20 mg/ml) for 16 h at 37°C. The suspension was extracted twice with equal volume of phenol–chloroform, and DNA was extracted by the addition of 0.2 volumes of 5 M NaCl and 2.0 volumes of ethanol at room temperature. The mixture was incubated overnight at −20°C and spun at 12,000 rpm for 10 min to get pellet which was resuspended in 100 µl of sterilized distilled water and stored at 4°C. DNA concentrations were determined by spectrophotometric analysis.

3.9.2 RAPD-PCR amplification

RAPD amplification was done with a 15 µl PCR mix, containing 1x PCR buffer 1.5 µl, MgCl2 (1 mM) 0.6 µl, dNTP (0.2 mM) 0.3 µl, BSA (0.533µl/ml) 0.8 µl, primers (MA-09, MA-12 and MA-26 – Table 8) 0.3 µl, 1.5 unit of Taq (0.3µl) and filled up with sterile deionized water to the final volume. 1µl of extracted DNA was also added in each PCR tube. Three primers were randomly selected for RAPD analysis. The reaction mixture was given a short spin for thoroughly mixing of the cocktail components. PCR tubes were loaded on to a thermal cycler. The PCR programme included an initial denaturation step at 94°C for 4 mins followed by 45 cycles with 94°C for 1 min for DNA denaturation, annealing as mentioned with each primer, extension at 72°C for 2 minutes and final extension at 72°C for 10 minutes were carried out (Table 9).
3.9.3. Agarose gel Electrophoresis

The amplification products were analyzed by electrophoresis (Sambrook et al. 1989). Along with the PCR amplified products, 100 bp DNA ladders as standard marker were subjected to electrophoresis in 1.5% agarose gel in TAE buffer and stained with ethidium bromide. Molecular size of the marker was 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp. The amplified pattern was visualized on a UV transilluminator and documented. Polymorphism was evidenced as the presence and / or absence of DNA fragments between the samples. The RAPD profiles of the treated insects were evaluated on 1.5% agarose gel run for 30 min. at 120 volts.

3.10. Statistical analysis

Log probit for the larvicidal efficacy of plant extracts were calculated by following Finney (1971) method to generate regression equation, LC$_{50}$, and LT$_{50}$ values (95% class intervals) with upper and lower fiducial limits, slope and chi square values. Results with P<0.05 were considered to be statistically significant. Completely randomized three-way factorial ANOVA and correlation coefficient were carried out using mosquito species, period of bioassay and different concentrations as variables to find the significance between the above parameters and mortality. Results of biochemical profile of nutrient reserves and primary metabolites studies were analysed statistically and Student’s t test was used to analyse mean difference between control and treated groups. Data collected on adulticidal and repellent action were subjected to ANOVA and F value, critical difference (CD) and coefficient of variation (CV%) of mean (P < 0.05) and Tukey’s multiple range test (P < 0.05) were used for taking statistical decisions (Snedecor and Cochran 1989).
Fig. 3 Study design for repellent test

Initial Test (Performed by each subject)*  2nd and 3rd Test (Performed by each subject)*

*If at any time during testing, mosquitoes were seen to land on the skin but not bite (a sign of imminent failure of the repellent), then the interval between insertions was decreased to five minutes until the first bite was confirmed.
<table>
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<th>Annealing Tm °C/Sec</th>
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<tbody>
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<td>Primer MA-09</td>
<td>GACGGATCAG</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>Primer MA-12</td>
<td>ACCGCGAAGG</td>
<td>34</td>
</tr>
<tr>
<td>3</td>
<td>Primer MA-26</td>
<td>GACGTGGTGA</td>
<td>32</td>
</tr>
</tbody>
</table>

Table 9. RAPD-PCR reaction conditions

<table>
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<th>Profile</th>
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<th>No of cycles</th>
</tr>
</thead>
<tbody>
<tr>
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<td>94°C 2min</td>
<td>Initial denaturation</td>
</tr>
<tr>
<td>2</td>
<td>94°C 1min</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>36°C 1min</td>
<td>45cycles</td>
</tr>
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<td>4</td>
<td>72°C 30sec</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>72°C 10min</td>
<td>Final extension</td>
</tr>
<tr>
<td>6</td>
<td>4°C</td>
<td>Hold</td>
</tr>
</tbody>
</table>
PLATE 1: Mosquito Vectors Used In The Present Study

*Aedes albopictus*

*Anopheles barbirostris*

*Culex quinquefasciatus*
PLATE 2: MOSQUITO CULTURE

A. Mosquito larvae in a tray

B. Mosquito pupae about to emerge in a culture plastic container

C. Adult culture cage
PLATE 3 Plants tested for mosquitocidal properties

Acacia gagaena

Alstonia scholaris

Antidesma acidum

Blumea lanceolaria

Brugmansia suaveolens

Centella asiatica

Clerodendron colebrookanum

Croton caudatus
Polygonum plebeium

Securinega virosa

Syzygium aromaticum

Tagetes erecta

Thespesia lampas

Tithonia diversifolia