3. MATERIALS AND METHODS

To achieve the aims of the present study, work have been designed in two parts. Anti-mosquito as well as oviposition attractant activity of plant species were evaluate. Methodology applied in this study is described in different sections and overview of each method has been given through flowcharts. Both the work plan has been described in Figure 3.1.
STUDY PLAN

Collection and Identification of Plants

Work Plan A

Evaluation of plant for ANTI-MOSQUITO ACTIVITY


Preliminary screening of plant species for Larvicidal activity using Plant powder in different solvents

Solvent selected for further extraction using Soxhlet apparatus

Crude solvent extracts were evaluated for-

- Larvicidal
- Adulticidal
- Repellent
- Oviposition altering activity

Effective crude extract of plants was further selected for Bioassay guided isolation of effective compound

Crude extract was fractionated with series of solvents from non polar to polar solvents

Effective fraction was purified with repeated chromatographic Techniques along with bioactivity evaluation

Effective compound were identified using GC-MS analysis

Work Plan B

Evaluation of plant for OVIPOSITION ATTRACTANT ACTIVITY

1. Oryza Sativa
2. Cynodon dactylon

Optimization of fermentation period and concentration of infusion for oviposition attractant activity

Optimization of concentration of infusion for oviposition attractant activity

Effective infusion was selected for identification of compounds

Effective compound were identified using GC-MS analysis

Figure 3.1: Work plan of the study
3.1. COLLECTION AND IDENTIFICATION OF PLANT MATERIAL

Overview

**Literature search**
1. Plant reported for some insecticidal activity/ affect mosquito life cycle
2. Not much explored and has scope for future study
3. Regional availability

<table>
<thead>
<tr>
<th>Plants selected for study</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>Alstonia scholaris</em></td>
</tr>
<tr>
<td>2. <em>Callistemon viminalis</em></td>
</tr>
<tr>
<td>3. <em>Cassia tora</em></td>
</tr>
<tr>
<td>4. <em>Hyptis suaveolens</em></td>
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<tr>
<td>5. <em>Malvastrum Coromandelianum</em></td>
</tr>
<tr>
<td>6. <em>Prosopis juliflora</em></td>
</tr>
<tr>
<td>7. <em>Vernonia cinerea</em></td>
</tr>
<tr>
<td>8. <em>Catharanthus roseus</em>, Ornamental</td>
</tr>
<tr>
<td>9. <em>Oryza sativa</em></td>
</tr>
<tr>
<td>10. <em>Cynodon dactylon</em></td>
</tr>
</tbody>
</table>

**Sampling Material and Sampling strategies**

<table>
<thead>
<tr>
<th>Plant parts collected –</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>Alstonia scholaris</em></td>
</tr>
<tr>
<td>2. <em>Callistemon viminalis</em></td>
</tr>
<tr>
<td>3. <em>Prosopis juliflora</em></td>
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<tr>
<td>4. <em>Cassia tora</em></td>
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<td>5. <em>Hyptis suaveolens</em></td>
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<td>6. <em>Malvastrum Coromandelianum</em></td>
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<tr>
<td>7. <em>Vernonia cinerea</em></td>
</tr>
<tr>
<td>8. <em>Catharanthus roseus</em></td>
</tr>
<tr>
<td>9. <em>Oryza sativa</em></td>
</tr>
<tr>
<td>10. <em>Cynodon dactylon</em></td>
</tr>
<tr>
<td>1. DRDE Campus, Gwalior MP India</td>
</tr>
<tr>
<td>2. Morar Cantt Area, Gwalior MP India</td>
</tr>
<tr>
<td>3. Maharajpura, Gwalior MP India</td>
</tr>
<tr>
<td>4. University Campus, Gwalior MP India</td>
</tr>
<tr>
<td>5. New Collectorate area, Gwalior MP India</td>
</tr>
<tr>
<td>6. Defense colony, Gwalior MP India</td>
</tr>
</tbody>
</table>

**Identified plants evaluated for bioactivity against *Aedes albopictus* mosquito**

**Plants showing potential bioactivity confirmed by Authentication/Identification based on Microscopic/Anatomical Studies**
3.1.1. Collection of Plants

In the study period of 2011-2014, different parts of plant species were collected during September-November from different sites of district Gwalior, Madhya Pradesh, India (Table 3.1). Total 10 different plant species belong to 8 families were collected which included 2 trees, 5 weeds, 1 ornamental, 1 crop plant and 1 grass species (Figure 3.2). For all plant species only fresh plant parts were collected while for *Oryza sativa* and *Cynodon dactylon*, fresh aerial parts as well as senescent aerial parts were collected. Fresh parts were washed thoroughly and kept for morphological and anatomical identification.

3.1.2. Identification of plants

*External Morphology*

Each specimen was morphologically identified with the help of available keys and online tools (Table 3.2). Plants were specifically identified on the basis of diagnostic characteristics. Morphological characters like shape, size, colour, and habitat etc. were also examined (Figure 3.3). In this connection help of SOS, Botany, Jiwaji University, Gwalior, was also taken. Voucher specimens were preserved in the laboratory of Defence Research and Development Establishment (DRDE), Gwalior.

*Anatomical study*

Among 10 plant species those were finally found to be most effective; were confirmed and authenticated by anatomical studies. Anatomy of Transverse sections of stem and leaf was studied according to the standard procedure (Khandelwal, 2008, Kokte et al., 2008). Fresh plant material (stem and leaf) were gently washed to remove any dust or dirt particles adhering to the plant tissues. All the specimens were fixed in
FAA solution (40% formalin, glacial acetic acid and 70% ethyl alcohol in the ratio 5:5:90 v/v). The materials were left in the fluid for three days, after which they were washed in water. Infiltration of the specimens was carried out by gradual addition of paraffin wax (melting point 58-60 °C) (Leica TP 1020, tissue Processor). The specimens were embedded into paraffin blocks for sectioning and longitudinal microtome sections (12 µm) were cut using Microm HM 360 Microtom. The resulting paraffin ribbons were stained according to johansen’s safranin and fast green staining method (Johansen, 1940) with some modifications (Figure 3.4). The slides, after staining with safranin, were dehydrated by employing a graded series of ethyl alcohol (30%, 50%, 70%, 90% and absolute alcohol; v/v). The specimens were stained with fast green in clove oil and xylol: alcohol (50:50), passed through 100% xylol and finally mounted in DPX (distyrene plasticizer and xylene) mount.
Figure 3.2: Collection of Plants

Figure 3.3: Sampling of Plants
Table 3.1: Different plants and plant parts collected from different sites of district Gwalior MP, India.

<table>
<thead>
<tr>
<th>Plant category</th>
<th>Plant Name (Common Name)</th>
<th>Family</th>
<th>Plant part collected</th>
<th>Collection sites in</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trees</td>
<td><em>Alstonia scholaris</em> (Satni, Devil’s Tree)</td>
<td>Apocynaceae</td>
<td>Leaves</td>
<td>DRDE Campus</td>
</tr>
<tr>
<td></td>
<td><em>Callistemon viminalis</em> (Cheel, Weeping bottlebrush)</td>
<td>Myrtaceae</td>
<td>Leaves</td>
<td>DRDE Campus</td>
</tr>
<tr>
<td>Weeds</td>
<td><em>Cassia tora</em> (Charota, Sickle-pod)</td>
<td>Caesalpiniaceae</td>
<td>Aerial Parts</td>
<td>New Collectorate Area</td>
</tr>
<tr>
<td></td>
<td><em>Hyptis suaveolens</em> (Wilaiti tulsi, Pignut)</td>
<td>Lamiaceae</td>
<td>Aerial Parts</td>
<td>New Collectorate Area</td>
</tr>
<tr>
<td></td>
<td><em>Malvestrum coromandelianum</em> (Kharenti, false mallow)</td>
<td>Malvaceae</td>
<td>Aerial Parts</td>
<td>DRDE Campus</td>
</tr>
<tr>
<td></td>
<td><em>Prospis juliflora</em> (Babool, Mesquite)</td>
<td>Fabaceae</td>
<td>Leaves</td>
<td>Morar Cantt Area</td>
</tr>
<tr>
<td></td>
<td><em>Vernonia cinerea</em> (Sahadevi, Little Iron weed)</td>
<td>Asteraceae</td>
<td>Whole plant</td>
<td>Jiwaji University Campus</td>
</tr>
<tr>
<td>Ornamental</td>
<td><em>Catharanthus roseus</em> (Sadabahaaar Madagascar periwinkle)</td>
<td>Apocynaceae</td>
<td>Aerial Parts</td>
<td>DRDE Campus</td>
</tr>
<tr>
<td>Crop plant</td>
<td><em>Oryza sativa</em> (Dhan, Asian Rice)</td>
<td>Poaceae</td>
<td>Dry Leaves</td>
<td>Maharajpura</td>
</tr>
<tr>
<td>Grass</td>
<td><em>Cynodon dactylon</em> (Doob, Bermuda grass)</td>
<td>Poaceae</td>
<td>Dry Leaves</td>
<td>DRDE Campus</td>
</tr>
</tbody>
</table>
Table 3.2: Plants morphologically identified with the help of following authorities.

<table>
<thead>
<tr>
<th>Name of tool</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taxonomy Laboratory</td>
<td>SOS Botany, Jiwaji University, Gwalior, MP</td>
</tr>
<tr>
<td>Flowers of India, Online</td>
<td><a href="http://www.flowersofindia.net/treeid/">http://www.flowersofindia.net/treeid/</a></td>
</tr>
<tr>
<td>USDA plants data, Online</td>
<td>Natural Resources Conservation Service</td>
</tr>
<tr>
<td>Online Plants data</td>
<td></td>
</tr>
<tr>
<td>Virtual Herbarium of Botanical survey of India</td>
<td><a href="http://bsilibraries.nic.in/homepage/index.asp">http://bsilibraries.nic.in/homepage/index.asp</a></td>
</tr>
<tr>
<td>India society of weed science, Online Weeds profile</td>
<td><a href="http://www.isws.in/invasive-plants-of-india.php">http://www.isws.in/invasive-plants-of-india.php</a></td>
</tr>
<tr>
<td>India biodiversity portal, Online plant profile</td>
<td><a href="http://indiabiodiversity.org/species/show/7455">http://indiabiodiversity.org/species/show/7455</a></td>
</tr>
</tbody>
</table>
List of Materials and Reagents used for anatomical study

- **Fixative solution**
  
  40% formalin, 5 vol  
  Glacial acetic acid 5 vol  
  70% ethyl alcohol 90 vol

- **Safranin O solution**
  
  Methyl cellosolve 2 vol  
  EtOH 100% 1 vol  
  DI 1 vol  
  Sodium acetate 1% (w/v)  
  Formalin (mordant) 2% (v/v)

- **Safranin O staining solution**
  
  Safranin O 1.0 % w/v in Safranin O solution

- **Fast Green solution**
  
  Methyl cellosolve 1 vol  
  Abs EtOH 1 vol  
  Methyl salicylate 1 vol

- **Fast Green FCF staining solution**
  
  Fast Green FCF 0.05% w/v in Fast green solution

3.1.3. Photographs and Photomicrographs

Photographs of different plants were taken using a Nikon digital camera. Photomicrographs of different magnifications were taken with the help of a Leica microscope. Magnifications of the figures are indicated by scale-bars.
**Johansen’s Staining Method**

Wax was removed from the sections with 2 times 10 min wash in 100% Xylene

Brought through a graded ETOH series from:
- 100% ETOH - 5 min
- 100% ETOH - 5 min
- 95% ETOH - 2 min
- 85% ETOH - 2 min
- 70% ETOH - 2 min

Stained 2 -24 hrs in the 1% w/v Safranin O solution: Let’s try for 2 hrs the first time

Rinsed the slides in ddH₂O for two times 5 minutes with gentle agitation

Dehydrated for 10 seconds in 95% ETOH plus 0.5% picric acid.

Washed for 10 seconds in 95% ETOH + four drops Ammonium hydroxide per 100 ml

Dipped about 10 seconds in 100% ETOH to finish dehydration

Counter stained for 10 sec. in Fast Green 0.05% w/v.

Excess Fast green was rinsed with clearing solution

Slides were washed in clearing solution by dipping for 10 seconds

Clearing solution was removed by dipping in the xylene plus 2-3 drops of 100% ETOH

Cleared in xylene (2 washes of 5-10 minutes each)

Mounted the coverslip with DPX.
3.2. REARING OF TEST INSECT

Overview

*Aedes albopictus* mosquito culture at Laboratory Conditions
27 ± 1°C temp.
70 ± 5 % relative humidity

Life cycle includes 4 stages

1. Eggs
2. Larvae
   - Early III instar larvae for Larvicidal Activity
   - Only 4-5 days old ♀
   - Non blood Fed ♀ For
     1. Adulticidal
     2. Repellent activity
   - Blood Fed ♀ For
     3. Oviposition altering activity
     4. Oviposition attractant activity
   - 1-2 days

3. Pupae
   - 8-10 days

4. Adult
   - 2-3 days
   - Evaluated for PLANT EXTRACTS

---

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3.2.1. Life Cycle of mosquito

The mosquito goes through four separate and distinct stages of its life cycle and they are as follows: Egg, Larva, pupa, and adult. Each of these stages can be easily recognized by their special appearance (Figure 3.5).

**Egg:** Eggs are laid one at a time and they float on the surface of the water. Most eggs hatch into larvae within 48 hours.

**Larva:** Mosquito larvae, commonly called "wrigglers", must live in water from 7 to 14 days depending on water temperature. Larvae must come to the surface at frequent intervals to obtain oxygen through a breathing tube called a siphon. Most larvae have siphon tubes for breathing and hang from the water surface. The larva feed on microorganisms and organic matter in the water. During growth, the larva molts (sheds its skin) four times. The stages between molts are called instars. At the 4th instar, the larva reaches a length of almost 1/2 inch. When the 4th instar larva molts it becomes a pupa.

**Pupa:** Mosquito pupae, commonly called "tumblers", must live in water from 1 to 4 days, depending upon species and temperature. The pupa is lighter than water and therefore floats at the surface. It takes oxygen through two breathing tubes called "trumpets". When it is disturbed it dives in a jerking, tumbling motion and then floats back to the surface. The pupa does not eat. The metamorphosis of the mosquito into an adult is completed within the pupal case. The adult mosquito splits the pupal case and emerges to the surface of the water where it rests until its body can dry and harden.

**Adult:** The newly emerged adult rests on the surface of the water for a short time to allow itself to dry and all its parts to harden. Also, the wings have to spread out and
dry properly before it can fly. On average, a female mosquito will live 2-3 weeks, but the male’s lifespan is shorter. Within their lifetime both adult male and female mosquitoes will feed on nectar and plant fluids. It is only the females that require a blood meal. The majority of species require this blood meal as a protein source for egg development.

3.2.2. Mass colonization of Test Insect- *Aedes albopictus* in laboratory

Experiments for the screening of bioactivity of plant extracts were performed using third instar larvae and adult mosquitoes of *Ae. albopictus*. Different stages of mosquito were taken from cyclic colony of *Ae. albopictus* mosquito maintained in DRDE laboratory under standard rearing conditions of 27±2°C room temperature and 70±10% relative humidity (Figure 3.6). Adult mosquitoes were reared in wooden cages (75 × 65 × 65 cm) and 10% sugar solution *ad libitum* dispensed through a cotton wick in a Petri dish were provided as food. A rabbit with under belly shaved was made available to provide source for blood meal twice in a week. Moist filter paper was kept in a beaker inside the cage for collecting eggs. The eggs were collected and transferred to enamel bowls (2.5 L) containing approximately two litres of dechlorinated water, for the rearing of larvae. Larvae were provided with yeast tablets as the source of food. After completion of the four larval stages (4 molts), the pupae were collected and kept in breeding cages for the emergence into adults.
Figure 3.5: Life Cycle of *Ae. albopictus* mosquito
Figure 3.6: Rearing of *Ae. albopictus* mosquito in Laboratory
Work Plan A

Evaluation of Anti-mosquito activity of Plants
Section 3A.1
SCREENING OF PLANT EXTRACTS FOR BIOACTIVITY AGAINST AEDES ALBOPICTUS

Overview

1. Alstonia scholaris (Leaves)
2. Callistemon viminalis (Leaves)
3. Cassia tora (Aerial parts)
4. Catharanthus roseus (Aerial parts)
5. Hyptis suaveolens (Aerial parts)
6. Malvastrum Coromandelianum (Aerial parts)
7. Prosopis juliflora (Leaves)
8. Vernonia cinerea (Aerial parts)

Plant material
Powdered

Powder dissolved in various Solvents
1. Water
2. Isopropanol
3. Methanol
4. Acetone
5. DMSO

Larval Bioassay for screening of effective solution (solvent + plant powder) (High throughput screening method)

Effective plant material in suitable solvent selected for SOLVENT EXTRACTION
Using Soxhlet apparatus

Solvent extracts of Different concentrations And bioactivity against different stages of Ae. albopictus mosquito

Larval stage
Adult stage

LARVICIDAL
REPELLENT
ADULTICIDAL
OVIPOSITION BEHAVIOR

Effective Solvent extract/infusion was selected for further Analysis
3A.1.1. Preparation of dried plant material

Freshly collected plant parts were chopped into small pieces and shade-dried at room temperature to prevent photolysis and thermal degradation. After 6-10 weeks, the dried material was ground into coarse powder in an electric pulverizer. Fine plant powder was obtained by passing it through 40 mesh sieve. The powders were stored in an airtight glass container until further use.

3A.1.2. Preliminary Screening for Larvicidal activity of plant powder

In order to determine the Larvicidal activity of plants and to save the time and resources, a High throughput screening method was applied to validate the effective solvent for further extraction of plant materials in which the plant powder was dissolved in different solvents (according to polarity) and this whole solution was used to test the Larvicidal efficacy (Figure 3.7). Primarily all the plants were screened for larvicidal activity. Only effective powder solutions were further extracted in their respective solvent.

Development of High throughput screening method for selecting effective solvent extract as Larvicidal

Aerial parts of all plants were powdered to fine particles of mesh size 250 μm approximately. Powdered material was weighed in 0.02, 0.04, 0.06, 0.08, 0.1 g quantity and soaked in 1 ml of each solvent; isopropanol, methanol, acetone, dimethyl-sulfoxide and water (in separate vials for each concentration and replication), at 25°C with occasional shaking. After 24 h, the entire 1 ml solution in each vial was used as
such (without filtering) for larval bioassay to get the final concentration of leaf powder as 0.2, 0.4, 0.6, 0.8, 1 g/l in water.

**Larval bioassay**

Early III instar larvae of *Ae. albopictus* were used for larvicidal bioassay as per WHO procedure with some modifications (WHO, 1981). Experiments were carried out in four batches with control. A total of 20 larvae were released in glass beakers (250 ml) containing 99 ml tap water. Different doses of leaf powders in solvents (1 ml) were added in each beaker containing larvae and the controls were treated with 1 ml of solvents. Larval mortality was recorded after 24 h of the treatment.

**3A.1.3. Solvent extraction of plant material using SOXHLET apparatus**

Dried plant powder was separately subjected to solvent extraction with Soxhlet apparatus (Make-Borosil) (Figure 3.8). Which solvent has to be used for extraction is already been decided in the High throughput screening method hence only those solvents which were effective, taken for further solvent extraction. Plant material was extracted according to the requirement in batches and for each batch 200 gm of powdered material in 500 ml solvent was extracted for 8 h at boiling point range 50–80 ºC. The extracts were filtered through a Buchner funnel with Whatman filter paper number 1. Crude extracts were dried using rotary evaporator, stored in glass vials. Stock solutions of 50 mg/ml for each plant extracts were prepared (as per WHO 2005 norms with slight modifications) in their respective solvents.
Figure 3.7: Scheme of High throughput screening method for selecting effective solvent extract as Larvicidal
Effective Solvents selected for further extraction:

1. A. scholaris  Isopropanol
2. C. viminalis  Isopropanol
3. C. tora    Acetone
4. C. roseus  Acetone
5. H. suaveolens  Acetone
6. M. Coromandelianum  Acetone
7. P. juliflora  Methanol.
8. V. cinerea  Acetone

Soxhlet extraction

Filtered with Whatman filter paper number 1

Crude extracts were dried using rotary evaporator

Stock solutions were prepared (50 mg/ml) and then diluted (as per WHO 2005 norms) with water to make final concentration of - 25, 50, 100, 200, 400 mg/L.

Evaluated for different bioactivity – Larvicidal, Repellent, Adulticidal, Oviposition altering activity

Figure 3.8: Solvent extractions procedure of plants using Soxhlet apparatus
3A.1.4. Evaluation of bioactivity of crude extracts against different stages of mosquito

*Larvicidal Bioassay*

Test solutions of five concentrations- 25, 50, 100, 200, 400 mg/L (Final concentrations), were prepared by diluting the stock solution with distilled water. Bioassay for the larvicidal activity was carried out as per WHO (2005) procedure (Figure 3.9). A total of 20 early third instar larvae of *Ae. albopictus* were introduced in glass beakers (250ml) containing 99 ml tap water. One ml of test solutions of different concentrations was introduced in beakers for treatment along with control. Four replicates were set up for each test concentration and larval mortality was recorded after 24h.

*Repellent Bioassay*

For the evaluation of repellent activity modified repellent testing method was applied (Debboun and Wagman, 2004). The stock solutions of the extracts were diluted with acetone, to obtain test solutions of 1.0, 2.5, and 5.0 mg/cm² was prepared separately (Amerasan *et al.*, 2012). For repellent experiment, 200 laboratory reared blood-starved adult female (4-5 days old) were placed into separate laboratory cages (60×60×45 cm) (Figure. 3.10). Before each test, the hand of a human subject were washed with unscented neutral soap, thoroughly rinsed, and allowed to dry for 10 min before extract application. Total 200 µl volume of extracts were applied on the external surface of the fist of human hand over an area of about 180 cm². Control Palm was treated with acetone. Tests were conducted during 10:00 hr to 17:00 hr in the light room and in the laboratory temperature 27 ± 2 °C and 70 ±5 % RH was maintained for
experiment. The control and treated Palms were introduced simultaneously into the
cage. The number of bites was counted over 5 min, in every 30 min up to 210 minutes.
Number of biting mosquitoes was counted during exposure up to 5 min. During the
exposure; engorged females were considered as biting mosquitoes. Protection time
was recorded as the time onwards the first observation till the confirmed bite was
obtained. The experiments were conducted four times in separate cages and in each
replicate different volunteer were used to nullify any effect of skin differences on
repellency.
Figure 3.9: Larval bioassay

Figure 3.10: Repellent Testing
**Adulticidal Bioassay**

Sugar-fed adult female mosquitoes (5 to 6 days old) were used for the evaluation of adulticidal activity of plant extracts. Three concentrations- 100, 200 and 400 mg/L of test solution of each plant extract were prepared by diluting the stock solution with distilled water. The diluted plant extracts were impregnated on filter papers (140× 120 mm). A blank paper consisting of only ethanol was used as control. The papers were left to dry at room temperature to let the ethanol evaporate overnight. Impregnated papers were prepared fresh prior to testing. The bioassay was conducted in an experimental kit consisting of two cylindrical plastic tubes both measuring125×44 mm following the method of WHO (1981) (Figure 3.11). One tube served to expose the mosquitoes to the plant extract and another tube was used to hold the mosquitoes before and after the exposure periods. The impregnated papers were rolled and placed in the exposure tube. Each tube was closed at one end with a 16-mesh wire screen. Sucrose-fed and blood-starved 20 females were released into the tube, and the mortality effects of then extracts were observed every 10 min for a 1-h exposure period. After exposure mosquitoes were placed in the untreated holding tubes. Cotton pads soaked in 10 % sugar solution were placed in the tube during the holding period for 24 h. Mortality of the mosquitoes was recorded after 24 h. The above procedure was carried out in triplicate for plant extract of each concentration.

**Oviposition Bioassay**

Four concentrations- 50, 100, 200 and 400 mg/L of test solution of each plant extract were prepared by diluting the stock solution with distilled water for the treatment. Oviposition behavior of mosquitoes, in four replicates for each
concentration was tested using dual choice oviposition bioassay according to Xue et al., (2003) method with few modifications (Figure 3.12). A total of 20 gravid females were released in a cage of size 60x60x45 cm and two white plastic bowl of 500 ml capacity were filled with 99 ml tap water, one as control and another as treatment bowl and the filter paper strip of size 30x 5cm was placed inside each bowl for oviposition. Treatment bowl contained test solution and control bowl was treated with simple water. Thirty minutes after treatment, bowls were placed in cages in diagonal position approx. Fifteen inches apart from each other. Egg laying in treated as well as control bowls were observed after 24h of treatment.
Figure 3.11: Adulticidal Bioassay using WHO kit
Figure 3.12: Bioassay for evaluation of Oviposition response of *Ae. albopictus* gravid females towards crude plant extracts
3A.1.5. Statistical Analysis-

*Larvicidal activity* - To determine the lethal concentration for each plant species, data were analyzed by Probit analysis (Finney, 1971) using POLO PC software. The corrected mortality was estimated by Abbott’s formula (1925) and the LC$_{50}$ values were calculated.

*Oviposition activity* - Oviposition activity Index (OAI) was estimated using (Karmer and Mulla 1979), OAI= (Nt-Nc)/ (Nt+Nc), where Nt is number of eggs laid in treatment, Nc-No. of eggs laid in control bowls for both oviposition deterrent and attractant bioassays. OAI of +0.3 and above are considered as attractants, while those with an OAI of -0.3 and below are considered as repellents.

*Repellent Activity* - The percentage protection was calculated by using the following formula (Fradin and Day, 2002).

Percent Protection = (Nc - Nt/ Nc)*100,

Where Nc = No. of bites received by control,  Nt= No. of bites received by treatment.
Section A.2

ISOLATION AND IDENTIFICATION OF ACTIVE COMPOUNDS

Overview

<table>
<thead>
<tr>
<th>Fractionation of solvent extracts (Liquid- Liquid extraction) using separating funnel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larvicidal Bioassay for each fraction</td>
</tr>
<tr>
<td>Effective solvent taken for Chromatography (TLC)</td>
</tr>
<tr>
<td>Column Chromatography for purification of fraction</td>
</tr>
<tr>
<td>Similar Fractions were identified using TLC and pooled</td>
</tr>
<tr>
<td>Larvicidal Bioassay for each fraction</td>
</tr>
<tr>
<td>Effective fraction was taken for further Column Chromatography for separation of subfractions</td>
</tr>
<tr>
<td>Larvicidal Bioassay for each subfraction</td>
</tr>
<tr>
<td>Effective subfraction was purified by repeated chromatography and Larvicidal bioassays</td>
</tr>
</tbody>
</table>

Identification of compounds present in subfraction supposed to be responsible for Bioactivity against mosquito collectively or alone
3A.2.1. Liquid –Liquid Extraction of solvent extract

Extraction partitioning was done by following the method described by Kupchan and Tsou, (1973) with some modifications (Figure 3.13). Different fractions were separated with the help of separating Funnel (Figure 3.14). 5 gram of crude extract was dissolved in 90% methanol in water. This solution was further fractionated with different solvents of increasing polarity using including n hexane, petroleum ether, chloroform and ethanol according to the scheme given in figure 3.13. All the fractions were dried using rotary evaporator and kept in air tight containers for further analysis.

Larval Bioassay of fractions

Stock solution of the concentration 10 mg/ml of each dried fraction was prepared by dissolving in respective solvents. Test solutions of four concentrations-100, 50, 25, 10 mg/L (Final concentrations), were prepared by diluting the stock solution with distilled water. Bioassay for the larvicidal activity was carried out as described in section 3A.1.4.
Figure 3.13: Scheme of liquid-liquid extraction/ fraction partitioning method

(Modified Kupcan method)
Figure 3.14: Liquid–Liquid partitioning of crude extract, using Separating Funnel.
3A.2.2. Thin layer chromatography for effective fraction

Sample was loaded on TLC plates pre-coated with silica gel (Merck, TLC silica gel 60 F254) on aluminum plate. These plates were developed using the different combinations (1:9, 2:8, 3:7, 4:6, 1:1, 6:4, 7:3, 8:2 and 9:1) of solvents system ethyl acetate and hexane in the at ambient temperature of 20 ± 1°C. The plates were developed as per conventional one dimensional ascending method (Figure 3.15). A strip of the precoated silica gel was cut out. A spot of the sample was applied on the plate about 1.0 cm from the edge using glass capillaries. The strip was lowered into a small chromatographic jar containing the solvent system. The jar was covered with a glass lid. The solvent was allowed to ascend until the solvent front was about ¾ of the length of the strip. The strip was removed and dried by a hot air dryer and then sprayed with freshly prepared iodine reagent to detect the bands/spots. The spots were identified both in the UV light and visible light and the images were collected in visible light.

Figure 3.15: Thin layer chromatography in glass chamber
3A.2.3. Column Chromatography

Chloroform fraction of *V. cinerea* was subjected to column chromatography to separate it into its component fractions (Figure 3.16). Silica gel (60-120 mesh) was used as the stationary phase while hexane –ethyl acetate solvent combinations of increasing polarity were used as the mobile phase. The wet packing method was used in preparing the silica gel column (Length 500mm, Bore 25 mm). Firstly column was washed with hexane and dried before packing. In the setting up of column, drain opening at the lower part of the glass column was stocked with glass wool to prevent the draining of silica gel with solvent. The slurry prepared by mixing of silica gel and hexane was poured down carefully into the column. The tap of the glass column was left open to allow free flow of solvent into a conical flask below. Column was packed with silica gel approximately 2/3 rd of column length. After the packing process, the tap was locked and column was allowed 24 h to stabilize. The sample was prepared in a ceramic mortar by adsorbing 2.0 g of the extract to 10 g of silica gel in hexane and dried on a hot plate. The dry powder was allowed to cool and then gently layered on top of the column. The column tap was opened to allow the eluent to flow at the rate of 1 ml/ minute. Elution of the extract was done with the following ratios of solvent combinations sequentially- n-hexane: ethyl acetate 100:0, 80:20, 60:40, 40: 60, and 20: 80. To prevent the disturbance of top most layer of extract, a measured volume (500 ml) of each solvent combination was sprayed uniformly by the sides of the glass into the column each time. All the fraction were collected and concentrated in a rotovaporator and the mass of the different fractions was determined.
Figure 3.16: Column Chromatography
3A.2.4. Bioassay guided purification of effective fraction

Components of the fractions were identified by analyzing TLC profile of all fractions (Procedure described previously under the sub heading Thin layer Chromatography). Fractions of similar spots were pooled. All the pooled fraction were tested for larvicidal activity. Only effective fraction was selected for further purification. Components present in effective fraction were separated with the help of repeated Column Chromatography process for four times. Subfractions collected, pooled and tested for larvicidal activity for achieving minimum number of active compounds in the subfractions (Figure 3.17).
Figure 3.17: Bioassay guided purification of effective fraction

Chloroform fraction

Column Chromatography

Collection and pooling of fraction using TLC (14 fraction F1……A14)

Larvicidal bioassay of pooled fraction (at the conc. 5, 10, 20, 50 ppm)

Column chromatography of Effective fraction (F4)

Collection and pooling of Sub fraction using TLC (sub fractions F4.1…..F4.8)

Larvicidal bioassay of pooled subfraction (at the conc. 1, 5, 10, 25 ppm)

Column chromatography of Effective sub fraction (F4.3)

Collection and pooling of Sub fraction using TLC (sub fractions F4.31…..F4.35)

Larvicidal bioassay of pooled subfraction (at the conc. 0.5, 1, 5, 10 ppm)

Column chromatography of Effective sub fraction (F4.32)

Collection and pooling of Sub fraction using TLC (sub fractions F4.321…..F4.323)

Larvicidal bioassay of pooled subfraction (at the conc. 0.5, 1, 5, 10 ppm)

Number of compound identified in subfraction supposed to be responsible for larvicidal activity
3A.2.5. Comparative Larvicidal efficacy of final effective Fraction (F4.32) with synthetic insecticide (Temephos)

Market formulation of Temephos (Abate) was used for larvicidal bioassay. Temephos 50% (SC) was purchased from Bayer Crop Sciences Ltd. (Mumbai, India). Stock solution of concentration 1mg/ml was prepared in acetone for both Temephos and plant fraction. Stock solution was diluted in water to prepare final concentrations as- 0.025, 0.05, 0.1, 0.25, 0.5 and 1 mg/L. Susceptibility assay of mosquito larvae were carried out according to WHO method described in previous section.

3A.2.6. GC – MS analysis

The GC – MS analysis was carried out using Agilent gas chromatograph (2880A) equipped with 30m x 320 μm x 0.25 μm of capillary column and coupled to Agilent mass selective detector in the electron impact mode (Ionization energy: 70 eV) (Figure 3.18). Helium gas was used as the carrier gas at constant flow rate 2 ml/min and an injection volume of 1 μl was employed (Split ratio of 100:1) injector temperature 250°C; ion-source temperature 260°C, capillary: 30 m X 230μm, film thickness 0.25 μm, average velocity 38.051 cm/s, pressure 0.68 psi, purge flow 3 ml/min, purge time 0.20 min. The oven temperature was programmed from 50 to 325°C (isothermal for 2 min) with an increase of 5°C/min to 160°C, then 20°C/min to 250°C, equilibration time 1 min, ramp 5°C/min, ending with a 30 min isothermal at 290°C. Total GC running time was 20 minutes. The volume of injected specimen was of 1μl of diluted fraction (1%). The constituents of extract fraction were identified in comparison with their retention indices and computer matching with the PBM libraries.
Figure 3.18: Gas Chromatography/ Mass Spectrometry (GC/MS)
Work Plan B
Evaluation of Oviposition attractant activity of Plants
Section B.1

OVIPOSITION ATTRACTANT ACTIVITY OF PLANT INFUSION

Overview

1. Oryza sativa
   (Senescent Leaves)
2. Cynodon dactylon
   (Senescent Leaves)

INFUSION was prepared by keeping Plant material for
1. 3 days
2. 7 days
3. 10 days

Oviposition Bioassay for Optimization of infusion time period and concentration

Evaluation of OVIPOSITION ATTRACTANT Activity
3B.1.1. Preparation of plant infusion

Mature dropped leaves of *Cynodon dactylon* (Bermuda grass) and *Oryza sativa* (Rice) were collected. The leaves were cut in to small pieces of 1-2 cm and infusion was prepared by adding 1g of these dry and chopped leaves in 100 ml tap water. This infusion was kept in cotton plugged Eleynmayer flask for three different time periods (separate flask for each time period)- 3 days, 7 days and 10 days at 27± 2°C temperature. Different aged infusions were filtered and stock solution of 1g/100ml was prepared for ovitrap bioassays. Tap water was also incubated for the same time period and used as the control for all experiments.

3B.1.2. Optimization of infusion time period and concentration for oviposition attractancy

In order to find effective attractant infusion, the time period and concentration of infusion was optimized. In preliminary experiments 3 days, 7 days and 10 days old bamboo infusions were studied for oviposition response towards gravid females. For each replication twenty gravid females were released in the cage of size 60x60x45 cm. Two containers, one of treated with bamboo infusion (100 ml) and other filled with tap water only as control (100 ml) were placed in the cage. Stock solution (1g/100ml) of filtered infusion was diluted to 1, 5 and 10% with water and total volume of 100 ml was prepared for treatment. Containers were placed in cages in diagonal position 15 inches apart from each other. Egg laying in treated as well as control containers were monitored after 48 hrs.
**Oviposition attractant activity of selected infusion**- Based on the pre-screening results, experiments with 7 days old infusion of *O. sativa* was continued at 5 and 10% dilutions assuming this incubation time period of higher efficacy. Stock solution (1g/100 ml) of filtered infusion was diluted to 5 and 10% with distilled water to prepare final concentration. Four replications were performed and for each replication twenty gravid females were released in the cage of size 60x60x45 cm (Figure 3.19). Two white plastic bowl of 500 ml capacity were used, one as control and another as treatment bowl and the filter paper strip of size 30x 5cm was placed inside each bowl for oviposition. Treatment bowl contained infusion (5% and 10%) and control bowl was treated with incubated water. Containers were placed in cages in diagonal position 15 inches apart from each other. Egg laying in treated as well as control containers were monitored after 48 hrs.
Figure 3.19: Bioassay for evaluation of Oviposition response of *Ae. albopictus* gavid females towards plant infusion

**Plant material (Bermuda grass and Rice leaves)**

**Leaf Infusion**

**Dual cage bioassay**
Section B.2
IDENTIFICATION OF BIOACTIVE COMPOUNDS

Overview

Effective infusion was selected

Sample preparation for GC-MS analysis
(Liquid- Liquid Extraction with dichloromethane)

GC-MS Analysis of infusion
3B.2.1. Liquid –Liquid Extraction of Plant infusion

For the analysis of chemical compounds present in plant infusion, it was extracted with dichloromethane. 100 ml infusion was mixed with equal amount of dichloromethane and the aqueous layer and dichloromethane layer was separated using separating funnel. The process was repeated thrice with aqueous layer. Finally dichloromethane extract was collected and concentrated in rotary evaporator. After that extract was carried out for GC-MS analysis.

3B.2.2. GC- MS analysis

Seven days old *O. sativa* infusion was found to be effective as oviposition attractant against gravid females of *Ae. albopictus*. This infusion was subjected to GC-MS analysis for the identification of bioactive components. Methodology for the analysis has been described in section 3A.2.6.