MATERIALS METHODS
MATERIALS AND METHODS

Colonization of *Aedes aegypti* collection of eggs

The egg of *Aedes aegypti* were collected from local (in and around Coimbatore, India) different breeding habitats with the help of a ‘O’ type brush. The egg were then brought to the laboratory and transferred to 18 X 13 X 4 cm Size enamel trays containing 500 ml water and kept for larval hatching. In which egg stage is of 1 day, larvae for 8 days and pupae for 2 days and adult stage for 15-20 days. The technique used for maintaining *Aedes aegypti* in laboratory are discussed below.

Rearing of immature stages

Freshly hatched larvae are transferred to white enamel basins containing 3-4” inches standing water. These trays are kept covered with wire screen lids to prevent any other mosquito, which may be leave in the colony room for laying eggs in the water. Brewer’s yeast powder mixed with an equal quantity (w:w) of ground dog biscuits is used in laboratory as a food for larvae. Approximately 1-2 of food, twice per days is enough for each rearing basin during the first two days. After which the quantity may be gradually increased. The larval basin are checked every day and if there is any scum formation on the surface of water it should be changed. While changing the water an ordinary cross wire sieve is used to hold the larvae. The larvae should be washed in clean water before transferring them to fresh basin. These basins must be thoroughly cleaned before and rinsed. Pupation commences at 5-6 days at ordinary room temperature.
PLATE - 1

Life cycle of *Aedes aegypti*
Aedes aegypti mosquitoes

- **EGG**: 2 days
- **LARVAL STAGE**: 5 - 7 days
- **PUPA**: 2 - 3 days
- **ADULT**: 2 - 3 weeks

2 days

5 - 7 days

2 - 3 days

ADULT

PUPA
Pupae

As soon as pupae are formed they should be removed with the pipette and transferred to bowl of water. The pupal bowl is then kept for emergence of adult. The emergence begins in 24-48 hours at room temperature.

Rearing of Adult Mosquito

The colony room is maintained at temperature approximately 28±2°C and relative humidity from 70-90%. The cage (90x90x90cm) used for holding about mosquito is made up of wire mesh screen, supported on wooden framework. With hardboard or plywood base and small window on in front side. To which is fixed a cloth sleeve through which food and water can be introduced.

The provide the females with necessary blood meal a rabbit held in small Restrainer cage is held in adult mosquito cage overnight. Glucose soaked cotton pads are usually kept in cage to provided the mosquito with the necessary additional nutritional requirement. A bowl of water is also kept in cage for the mosquito to lay eggs. The cage is kept clean; all the dead mosquitoes are removed every day. The wire screen is cleaned with hand brush and base with cloth or sponge soaked in hot water and cages are kept on any-protected tables.

Blood feeding of adult *Aedes aegypti* and egg laying

The adult female mosquitoes are allowed to feed on the blood of Rabbit (shaved on the dorsal side) for two days, to ensure adequate, blood feeding for five days. After blood feeding bowls were placed inside the cage for the adults eggs laying.
PLATE - II

Breeding habitats of *Aedes aegypti*
Preparation of *Bacillus sphaericus*

*Bacillus sphaericus* strain 2362 was supplied in power formulation containing approximately $1 \times 10^7$ spore mgm by H.T Dulamage. An aqueous suspension of the powder was prepared to the desired dosage level using distilled water.

The experiment was conducted in 180 ml alas jars in which 100ml of either primary or secondary sewer or bottled water was added. There were 100 jars set up from each type of water. The jars in each set were then divided into 3 groups: I, II and control.

Twenty larvae were added to the controls, all jars were inoculated with *Bacillus sphaericus* to a final concentration of $1 \times 10^{-5}$ sp.ml on day 0. the larvae in Group I died and the cadavers were left in the containers. On day 2,6,10,14,22 and 30,5 jars from each groups (3 jars from the controls) were selected and biosassy for the presence and level of *Bacillus sphaericus* using the following procedure.

I. Group : Jars with cadavers

a. Cadavers were removed, ground-up with a mortar and pestle and diluted with 100 ml of tap water. This was called the stock solution. Ten-fold serial dilutions were prepared from this solution to final dilutions of $1 \times 10^{-5}$.

b. After removal of the cadavers, the water remaining in the jars was diluted in 10 fold serial dilutions to a final concentrations of $1 \times 10^{-3}$.
Breeding habitats (un used tyres) *Aedes aegypti*
II. Group: Jars without cadavers

a. The water in jars was treated in the same manner as "b" above.

III. Ten additional control jars containing the tap water used in the bioassay were set up along with the 3 jars from each water type.

IV. Twenty larvae were inoculated to each of the test and control jars and the survivors remaining after 72 hrs were rabbit pellets was added to all containers after 24 hrs as food source.

V. Statistical Analysis: All Proportions were transformed using the arcsine square root transformation (2 x arcsin [sqrt(proportion)]. Analysis of variance was conducted using Duncan's multiple range test to distinguish between the sample means (P = 0.05).

VI. Bottled spring water was added periodically to all of the stock solutions to maintain water volume.

A second series of experiments were conducted in a screenhouse where natural light was reduced by approximately 50%. These experiments used plastic dish tubes, 27 x 34 x 13 cm, containing No. 2 Monterey white sand and 3 - 4 cm of water. The water had been allowed to stand for several days prior to the experiments. The tubes were divided into 7 groups. I, II, III, IV, V, VI and controls. Groups I contained 5 tubes and had 100 larvae added before the addition of the pathogen. Groups II-VI contained 3 tubs each and were inoculated with the pathogen without larvae. All tubes were inoculated with Bacillus sphaericus strain 2362 to final concentration of $1 \times 10^{-5}$ sp/ml,
PLATE -IV
Breeding habitats of *Aedes aegypti*

a. Flower Pot
b. Mud Pot
c. Broken Plastic Pot
with the exception of 4 tubes which were used for controls. Larvae which died in Group I were left in the tubes. After pathogen inoculation, 100 larvae were added 4 times to the various tubes over a 20 – days period in a sequence.

The number of survivors remaining after 48 hrs was recorded and the mean percent mortality was determined for each of the groups. This experiment was conducted concurrently under, conditions of shade and not shade where light intensity averaged < 2 and 2800 ft candles, respectively, at 1300 hr from July 9 to 15.$^5$ Bacillus sphaericus total protein including active ingredient 5% W/W) 10% w/w power fermentation solids 10 W/W inert ingredient 40% W/W non ionic surfactant 0.2% W/W food grade preservative 0.3% UV portectant 0.1% and water 71.4%. Total 100% w/w active specifically against mosquito larvae. The required concentration ranging from were prepared by diluting with distilled water.

Collection of Plant Materials

Azadirachta indica was collected Maruthamalai hills, Western ghats, Southern India, Coimbatore. The plants were identified at BSI (Botanical Survey of India) and the specimens were deposited at Zoology Department, Bharathiar University, Coimbatore, India.

Preparation of plant extracts

Azadirachta indica leaves were washed with tap water and shade dried at room temperature. The dried plant materials were powdered by an electrical blender. From the powder 200 g of the plant material were extracted with 2.5 litres of organic solvents (Petroleum either, ethanol, benzene, chloroform and Methanal) for 8 hrs in Soxhlet apparatus (Vogel, 1978) The crude plant extracts were evaporated to dryness in rotary vacuum evaporator.
Breeding habitats of *Aedes aegypti*

a and b. cistern

c. Grinding stone
Preparation of required plant extract concentration

One gram of the plant residue was dissolved in 10ml of acetone (stock solution) considered as 1% stock solution. From this stock solution different concentrations were prepared ranging from 2 to 10% respectively.

Larval toxicity text of plant extract and Bacillus sphaericus

A laboratory colony of Aedes aegypti larvae were used for the larvicidal activity. Twenty five number of first, second, third and fourth instar larvae were kept in 500 ml glass beaker containing 249 ml of dechlorinated water and 1 ml of desired concentration of plant extract and BS were added. Larval food was given for the test larvae. At each tested concentration 2 to 5 trials were made and each trial consisted of three replicates. The control was set up by mixing 1 ml acetone with 249 ml of dechlorinated water. In this BS toxicity the larvae exposed to dechlorinated water without acetone served as control. The control mortalities were corrected by using abbott’s formula (Abbott’s, 1925)

\[
\text{Corrected mortality} = \frac{\text{Observed mortality } - \text{Observed mortality in control in treatment}}{100 - \text{Control mortality}} \times 100
\]

Percentage mortality = \frac{\text{Number of dead larvae}}{\text{Number of introduced larvae}} \times 100

\( \text{LC}_{50} \) \( \text{LC}_{90} \) were calculated from toxicity data by using probit anylisis (Finney, 1971).
PLATE -VI

Colony maintenance of Aedes aegypti

a and b. larval breeding trays

c. Adult barad cage.
Pupal Toxicity test

A laboratory colony of *Aedes aegypti* pupa were used for pupicidal activity. Twenty numbers of freshly emerged pupae were kept in 500 ml glass beaker containing 249 ml of dechlorinated water and 1 ml of desired plant extract concentrations was added. Five replicates were set for each concentration and control was setup by mixing 1 ml of acetone with 249 ml of dechlorinated water. The control morality was corrected by abbott’s formula (Abbott’s, 1925).

Corrected mortality = Observed mortality in treatment –
Observed mortality in control
------------------------------------------------------------- x 100
100 – Control mortality

Percentage mortality = Number of dead pupae
----------------------------------------------- x 100
Number of pupae introduced

$\text{LC}_{50}$ $\text{LC}_{90}$ were calculated from toxicity data by using probit analysis (Finney, 1971)

Laboratory evaluation of insect growth regulatory activity against mosquito larvae

Laboratory evaluation of IGR bioassay against mosquito larvae *Aedes aegypti* was based on the method developed by Sacher (1971). Freshly prepared solutions of BS and NSKE were dissolved in water. The dilutions of test solutions were prepared to give required concentrations of 0.5 to 0.25 $\mu$g/ml BS and 20 to 320 ppm to NSKE.
PLATE -VII

a. Blood source for adult mosquito (shaved rabbit)
b. Cage for mosquito maintenance
c. Ovipositional Tray
Six glass beakers of 500 ml were emerged in series and were marked to identify that concentrations. To each glass beakers of 200 ml of tap water was added. The marked test concentrations of IGR was applied to the water in the beaker. Twenty five late third instars larvae from standard laboratory colony were fed yeast powder and observed under continuous exposure to detect juvenile effect. The water level was initially marked and continuously maintained by the adding of the water. Larval mortality, number of larvae pupating and the number of adults emerging were recorded. Percentage inhabitations caused by IGR was determined on the basis of emergence of adults from the tested larvae. Control consist of untreated larvae and larvae were exposed to the water. Controls were kept in five replicates. The emergence inhibition concentration (E150 and E190) was derived from the experimental data through probit analysis (Finney 1971).

**Fecundity Studies**

The fecundity experiments were conducted by taking an equal number of male and female *Aedes aegypti* which had emerged from the control and treated sets. They were settled in the cages of 30 x 30 cms dimension individually of each concentration. Three days after the blood meal eggs were collected daily from the small plastic bowls containing water kept in ovitrap in the cages. The fecundity was calculated by the number of eggs layed in ovitrap divided by number of females let to mate (The death of adults in the experiments was also considered).

**Egg hatchability Test**

The eggs were placed in the enamel tray for hatching. The percentage of hatchability was calculated as number experimented by number of eggs hatched.
PLATE-VIII

a and c. colony maintenance

b. Adult feeding source for male (Glucose soaked cotton)
Number of eggs hatched

Hatchability (%) = \[ \frac{\text{Number of eggs hatched}}{\text{Number of eggs tested}} \times 100 \]

**Mosquito Longevity Test**

The adult longevity of male and female of *Aedes aegypti* was also recorded. This was calculated by the number of days lived by the adult. The emergency day and mortal days of the adults were recorded and the means were calculated to give the mean longevity in days.

**Field Trial**

**Entomological study**

*Aedes aegypti* mosquito survey was carried out in Coimbatore District. A demographic mapping of the study was prepared and house to be examined in each survey were marked.

**Larval Survey**

Larval survey need torchlight ladle, dipper, tea strainer, pencil and Chalk and plastic containers.

In each larval survey 100 houses in each area was searched both inside and outside for breeding places of *Aedes aegypti* using single larval technique Sheppard *et al.*, (1969). Each household was examined every water container inside the house on the patio and garden. A flashlight was used indoors to see into the containers. Note all those containers with water and those with *Aedes aegypti* larvae or pupae. If detailed information on the relative importance of container types is required, the form should have separate column for each type (e.g., drums, tanks and tyres). For quick surveys where only the House
PLATE IX

a. Egg of *Aedes aegypti*

b. and c. Larval stages of *Aedes aegypti*
Index is required, the search continuous only until the first breeding site is found.

If identification of the species from each positive container is required, one fourth-stage larva or, in its absence, one third-stage larvae or one pupa, is placed in a small vial in 70% ethanol. Next a label, written in pencil and containing the date, province, municipality, locality, house number, kind of receptacle, and name of worker is inserted into the vial. Pasting labels to the outside the vial or attaching them to the outside with a rubber band is not recommended because these labels tend to get lost.

At the end of each day sum up the result of the findings at the bottom of form. The larvae are inspected in the laboratory under a stereoscopic microscope (at least 20 magnification) and when necessary corrections are made to the field form to rectify any error in identification of species.

Standard larval Indices: The commonly used larval indices are defined below:

1. **House Index or percentage of houses infested**
   \[
   \text{House Index} = \frac{\text{Number of infested houses}}{\text{Number of inspected houses}} \times 100
   \]

2. **Container Index or percentage of Containers infested**
   \[
   \text{Container Index} = \frac{\text{Number of infested containers}}{\text{Number of inspected containers with water}} \times 100
   \]
PLATE - IX

a. Egg of *Aedes aegypti*
b. and c. Larval stages of *Aedes aegypti*
3. Breatheau Index or number of infested containers
   Number of infested containers
   \[ \text{Number of inspected house} \]
   \[ \times 100 \]

In campaigns against *Aedes aegypti*, the larval house Index is the most frequently used and best understood index. The Container Index indicates the relative preference for larval breeding for each kind of receptacle. The Breteau Index combines the other two indices and gives a better measure of total larval production per house. The Breteau Index by container type measures the relative importance of each type in larval positivity.

Other indices that may be used as part of larval survey include the following:

1. Pupal Density Index, which give the number per positive container and which is a useful method to estimate adult production; and

2. Larval Density Index, which shows the number of larvae, by stage, for each positive container, and which is usually used only for special studies.

**Adult Surveys**

Adult surveys show the various species present and their relative abundance. These data and information on reproductive habits can be used to conduct and effective search for larval breeding sites.

The necessary equipment for adult collection includes a flashlight, and aspirator tube, a small net, cardboard containers with mesh tops, and forms attached to a clip board. Mosquitoes restings or flying inside the houses were
PLATE -XI

a. Adult of *Aedes aegypti*
b. Neem seed kernel extract
c. Life cycle of *Aedes aegypti* and *Bacillus sphaericus*
collected in the morning for 15 minutes per house using a mouth aspirator and flash light. In each area per survey three insect collectors spent 2 hours each (Ten men hours per area) and the average number of adults per man hour (PMD) was estimated. Aedes species landing on human volunteers (from whom informed consent was obtained) were collected in the morning and late afternoon for 30 minutes per voluntary and density was expressed as females and PMD.

**Resting Collections**

Resting collections are conducted by searching for adult mosquitoes in bedroom and in other rooms in houses, garages and outbuildings, mosquitoes may also be collected from yards, cemeteries, tyres and Junkyards. The adults are captured with small vials, hand sweep nets or aspirators. A small net may be used to flush the mosquitoes from under furniture and then capture them in flight. However, if information capture the mosquitoes directly from the surface.

\[
\text{No. of Adult landing} \times 60
\]

\[
\text{No. of Insect Collector}
\]

Mosquitoes usually rest in shaded places and dark corners on mosquito nets and under tables, chairs or beds. *Aedes aegypti* can be found resting throughout the day so there is no restriction on time in each house, e.g., about 15 to 60 minutes. This process allows collections density to be expressed per house and per man-hour. The captured mosquitoes must be identified by species and sex. Collections stations can be selected at random, or they can be located at predefined sites.
PLATE - XII

Field trial at Urban area by use of neem seed kernel extract and *Bacillus sphaericus*
Adult indices could be expressed in a manner similar to the larval indices:

1. Adult House Index = Percentage of houses infested,
2. Adult Room Index = Percentage of rooms infested, and
3. Adult Breteau Index = Number of infested rooms per 100 houses.

The Adult House Index is usually similar to the larval house Index, although this depends on the amount of time spent searching for the adults.

If the captured adult mosquitoes are countered, then the number of adults per 100 houses, the number per 100 rooms, and the number collected per hour can also be computed.

For the field trial the quantity of plant residues required (Based on Laboratory LC values) for each treatment was determined by calculating the total surface area of the water in each habit. The required quantities of NSKE and BS were mixed thoroughly with water in a bucket with constant agitation. Tepol was used as emulsifying agent (0.5%). Field application of the NSKE and BS were done with the help of knapsack sprayer and steer pump. Uniformly on the surface of the water in each habit. Dipper sampling and counting of larvae monitored the larval density before 24 hours, 1st week, 2nd week, 3rd week and after the treatment. A separate sample was taken to determine the species composition of each larval habitat. Twelve trials were conducted for BS and NSKE alone and combined treatment. The percentage of reduction was calculated by the following formula.
PLATE -XIV

a. Treatment with neem seed kernel extract
b. Treatment with *Bacillus sphäricus*
c. Treatment with neem seed kernel extract and *Bacillus sphäricus*
\[ = 100 - \left( \frac{C_1}{T_1} \times \frac{T_2}{C_2} \right) \]

where \( C_1 \) and \( T_1 \) are the pre-treatment density and \( T_2 \) and \( C_2 \) are the post-treatment density of III+IV inter larval per dip in the control and treated habitats (Mulla 1987; Ansari and Razdan 1999)

**Statistical Analysis**

All data were subjected to analysis of Variance (ANOVA) and the means were separated using Duncan's multiple range test (DMRT) (Alder and Rosler, 1977). LC\textsubscript{50} and LC\textsubscript{90} values and their 95% confidence limits were estimated by getting a probit regression model to the observed relationship between percentage mortality of larvae and logarithmic concentration of the substance. Separate probit model were fitted for each larval stage. The goodness of fit of the model was tested using Chi-Square test. An \( \alpha \) value of less than 0.05 was considered as a significant departure of the model from observations. In case of significant departure a heterogeneity factor was used to calculate the 90% confidence limit for LC\textsubscript{50} and LC\textsubscript{90}. All analysis were carried out using SPS Software version 9.0.