3. MATERIALS AND METHODS

3.1 COLLECTION OF PLANT MATERIALS:

Plant specimens were collected from four locations, namely Pallikaranai, Maduranthagam, Ramavaram and Pulicat lake representing four different habitats (Fig.1). Pallikaranai wetland is a freshwater swamp in the city of Chennai, India. It is situated adjacent to the Bay of Bengal, about 20 kilometres south of the city centre, and has a geographical area of 80 square kilometres. Pallikaranai marshland is the only surviving wetland ecosystem of the city and is among the few and last remaining natural wetlands of South India. The topography of the swamp is such that it always retains some storage, thus forming an aquatic ecosystem. The Ramavaram lake is about 7 Km from the Pallikaranai wetland and is a good source for ground water recharge. Maduranthagam lake is the second largest lake situated at Kancheepuram district, Tamil Nadu. The Pulicat lake is the second largest brackish water lake situated about 60 km from Chennai in the Tiruvallur district. The lake acts as a buffer to retain the accumulated flood water till the flood water is discharged gradually to the sea during the monsoon period and cyclones. A total of 24 specimens, six from each location were randomly uprooted for morphological, anatomical and physiochemical studies.

3.2 ALLOMETRIC STUDIES:

3.2.1 Morphological measurements

Measurements were made for eleven morphological characters of which six are vegetative and five reproductive. The following characters were chosen: length of
shoot, width of shoot base, number of leaves, length of leaf, width of leaf, thickness of leaf, length of male inflorescence, width of male inflorescence, length of female inflorescence, width of female inflorescence and gap between male and female inflorescence. The measurement from the base of the shoot to the base of the female inflorescence represented the length of the shoot. The first leaf below the female inflorescence was chosen for the measurement of the leaf characters. For measuring the width of the leaf, the middle portion of the leaf was selected. The thickness of the leaf was measured using hand-sectioned preparations under a WILD stereoscopic microscope. The microscope was calibrated before taking the measurements. The middle portion of the male and female inflorescence was used to measure the width.

3.2.2 Data analysis

All statistical analysis were performed using the PAleontological STatistics (PAST) software version 2.16. One-way ANOVA was carried out to test the significance of difference in mean and the Levene's test for homogeneity of variance. Pearson's correlation coefficient between pairs of variables was calculated after log transformation of the raw data. A hierarchical clustering routine analysis was conducted combining the significant morphological traits in order to visualize the difference in the morphological characters among the plants collected from the four locations. A dendrogram to illustrate the arrangement of the clusters produced by hierarchical clustering was developed using the unweighted pair-group average (UPGMA) and correlation statistics was used as the similarity measure. The scaling relationship between the morphological characters was studied by fitting the bivariate regression line. Three different algorithms were adopted namely, Ordinary Least
Square (OLS), Reduced Major Axis (RMA) and Major Axis (MA). Principal component analyses (PCA) was performed to elucidate the components accounting for as much as possible of the variance in the multivariate data. Since the variables were measured in different units, the correlation matrix was used in the PCA routine analysis. The 'Scree plot' which is a simple plot of eigenvalues, was drawn to indicate the number of significant components.

3.3 HISTOLOGICAL TECHNIQUES:

3.3.1 Sectioning

The paraffin embedded’ specimens were sectioned with the help of Rotary Microtome. The thickness of the sections was 10-12 µm. Dewaxing of the sections was by customary procedure (Johansen, 1940). The sections were stained with Toluidine blue as per the method published by O’Brien et al., (1964) since Toluidine blue is a polychromatic stain. The staining results were remarkably good; and some cytochemical reactions were also obtained. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc. Wherever necessary sections were also stained with safranin and fast-green and IKI (for starch).

3.3.2 Photomicrographs

Microscopic descriptions of tissues are supplemented with micrographs whenever necessary. Photographs of different magnifications were taken with Nikon labphotos 2 microscopic Unit. For normal observations bright field was used. For the study of crystals, starch grains and lignified cells, polarized light was employed. Since
these structures have birefringent property, under polarized light they appear bright against dark background. Magnifications of the figures are indicated by the scale-bars. Descriptive terms of the anatomical features are as given in the standard Anatomy books (Esau, 1964).

3.4 PHYTOCHEMICAL TECHNIQUES:

3.4.1 Proximate analysis

The entire plant was uprooted from the Pallikaranai marsh land, washed thoroughly with water and shade dried in the laboratory. The rhizome, leaf and inflorescence were separated and chopped into fine pieces and then powdered in a mixi. The following quantitative parameters were carried out using standard methods (African Pharmacopoeia, 1986; British Pharmacopoeia, 1988).

a. Moisture content/water loss on drying:

The powdered material of rhizome, leaf and inflorescence of *T. angustifolia* (2.0 g) was weighed into a clean crucible of known weight. After oven drying at 115°C for 5 hrs the crucible was cooled and weighed to determine weight loss in the powdered material. The average percentage weight loss, with reference to the air dried powdered drug was determined for three replicates.

b. Total ash determination:

The crucibles were washed thoroughly, dried in hot oven at 100°C, cooled in a desiccator and weighed. A 2.0 g portion of each of the samples was weighed into the crucible and put in the furnace. Heating was started gradually until temperature of 600°C was reached. This temperature was maintained for 6 h. The crucible was then
put inside a desiccator and cooled. After cooling the sample was reweighed and the percentage ash calculated.

\[
\text{Percentage of ash} = \frac{W-Z}{N} \times 100
\]

Where, \( W \) = weight of the crucible and ash; \( Z \) = weight of empty crucible; \( N \) = weight of the sample.

c. **Water soluble ash value determination:**

The crucible with the total ash was transferred into a beaker containing 25 ml of distilled water. The beaker and its contents were boiled for 5 min and filtered through an ashless filter paper (Whatman). The filter paper containing the residue was folded and placed in a weighed crucible. The crucible was then heated in the muffle furnace, until the filter paper was completely ashed. The crucible and its content were cooled and weighed and the final weight noted. The weight of the residue was then calculated by subtracting the constant weight of the second crucible and its ash. This is the water insoluble ash. The weight of the water soluble ash was obtained by subtracting the weight of the water insoluble ash from the total ash. The weight of the water soluble ash divided by the initial weight of the crude plant material and the value was multiplied by 100 to obtain the water soluble ash value.

d. **Acid insoluble ash value determination**

The water insoluble ash obtained from the above procedure was transferred into a beaker containing 25 ml of diluted HCl. The beaker and its contents were boiled for 5 min and then filtered through an ashless filter paper (Whatmann). The washings were then passed through the filter paper in a manner as to allow the collection of the
residue at the tip of the cone of the filter paper. The weight of the clean and heated porcelain crucible was accurately determined. The filter paper with the residue was folded with a small cone and transferred into the crucible. The crucible was gently heated until the filter paper was completely ashed, and then heated strongly for a few minutes. The crucible and its contents were cooled, weighed and the final weight was noted. The weight of the residue (ash) was then calculated. This was done by subtracting the constant weight of the crucible and ash. The weight of the ash divided by the initial weight of the plant material and multiplied by a hundred was taken as the acid insoluble ash value.

e. Extractive value

Extraction of shade dried powdered plant materials (50g) was carried out. Dried plant materials like rhizome, leaves and inflorescence were coarsely powdered and subjected separately to successive solvent extraction by a process of continuous extraction (soxhlation). The extraction was done with different solvents in their increasing order of polarity such as Chloroform, Ethyl acetate, Methanol and Water. Each time the marc was dried and later extracted with other solvents. All the extract were concentrated by distilling the solvent in a rotary vacuum evaporator and evaporated to dryness and stored in desiccators. The dried extract was weighed and dissolved in respective solvents and stored in cold temperature for further use. For each extract, the yield was calculated in percentage on the basis of dry weight of the whole herbs used (50 g) and the quantity of dry mass obtained after extraction (w/w).
3.4.2 Preliminary screening of phytochemicals

Different tests were performed for establishing the chemical profile of the extract. The following chemical tests for various phyto-constituents in the chloroform, ethyl acetate, methanol and water extracts were carried out as described below:

a. Detection of Alkaloids (Wagner, 1993):

To a few ml of plant extract, few drops of Wagner’s reagent was added along the side of the tube. A reddish-brown precipitate confirms the test as positive.

Preparation of Wagner’s reagent:

1.27g of Iodine and 2g of Potassium iodide was dissolved in 5 ml of water and made up to 100ml with distilled water.

b. Detection of Reducing Sugars:

The extracts were shaken with distilled water and filtered. Then boiled with few drops of Fehling’s solution A and B for a few minutes. An orange red precipitate indicates the presence of reducing sugars.

c. Detection of Glycosides:

Borntrager’s test (Evans, 1997):

2 ml of plant extract was hydrolysed with concentrated Hydrochloric acid for 2 hrs on a water bath. To the hydrolysate, 3ml of Chloroform was added and shaken
well. The chloroform layer was separated and 10% ammonia solution was added to it. Formation of the pink colour indicated the presence of glycosides.

d. Detection of Phenolic compounds:

   **Ferric chloride test (Mace, 1963):**

   To the 2 ml of extract, 5 ml of distilled water was added. To this, few drops of neutral 5% ferric chloride solution was added. A dark green colour indicated the presence of phenolic compounds.

e. Detection of Tannins:

Five ml of the extract and a few drops of 1% lead acetate were added. Formation of an yellow precipitate, indicates the presence of tannins.

f. Detection of Flavonoids:

To one ml of the extract, a few drops of dilute sodium hydroxide was added. An intense yellow colour produced in the plant extract, which become colourless on addition of a few drops of dilute acid indicates the presence of flavonoids (Kumar *et al.*, 2009).

g. Detection of steroids:

   One ml of the extracts was dissolved in 10 ml of chloroform and equal volume of concentrated sulphuric acid was added along the sides of the test tube. The upper layer turns red and sulphuric acid layer showed yellow with green fluorescence. This indicated the presence of steroids (Kumar *et al.*, 2009).
h. Cardiac glycosides:

Keller-kilian test:

To 2 ml extract, 1 ml of glacial acetic acid, Ferric chloride and conc. Sulphuric acid was added. Green blue color indicated the presence of cardiac glycosides (Jigna et al., 2007).

3.4.3 Gas chromatography-mass spectrometry analysis

The Gas Chromatography/Mass Spectrometry (GC/MS) instrument separates chemical mixtures (the GC component) and identifies the components at a molecular level (the MS component). The GC works on the principle that a mixture will separate into individual substances when heated. The heated gases are carried through a column with an inert gas (such as helium). As the separated substances emerge from the column opening, they flow into the MS. Mass spectrometry identifies compounds by the mass of the analyte molecule. A “library” of known mass spectra, covering several thousand compounds, is stored on a computer.

The Chloroform, Ethyl acetate, and Methanol extracts of the rhizome, leaf and inflorescence of *T. angustifolia* were analysed using Agilent technologies 6890 GC-MS instrument. An inlet of 0.75 mm I.D., which improves the GC resolution, was used. The carrier gas was helium (1 mL.min⁻¹) and the injector temperature was 250°C. The analytes were separated on a HP-5MS 30 m × 0.32 mm × 0.25 mm column (Supelco, Inc., Bellefonte, USA), kept at 50 °C for 2 minutes and then ramped to 240 °C at 4 °C/min and held at the final temperature for 10 minutes. The transfer
line was kept at 250 °C and the ion source was held at 230 °C. Mass spectra were measured at 70 eV and collected at the rate of 1 scan/second over an m/z range of 35 to 400. Chromatographic retention indices of separated compounds were calculated relative to a C8-C22 n-alkanes mixture.

 Constituents were identified by comparing their mass spectra to those in NIST database and with mass spectra of authentic standards. In many compounds, the identities were confirmed by their relative retention indices with authentic standards. Mass spectra from the literature were also compared.

3.5 MOLECULAR CHARACTERIZATION:

3.5.1 Genomic DNA isolation

A slightly modified method of Doyle and Doyle (1990) was employed. Fresh-leaf tissue (0.2 g) was ground in a 1.5-ml centrifuge tube with a micropestle and preheated freshly prepared 800 μl of CTAB extraction buffer (0.1 M Tris-Cl (pH 9.5), 20 mM EDTA (pH 8), 1.4 M NaCl, CTAB (2%, w/v), b-mercaptoethanol (1%, v/v) ) was immediately added to the tube. The tube was incubated at 65°C for 35-45 min, with inversion during incubation. An equal volume of chloroform: isoamyl alcohol (24:1, v/v) was added and then the tubes were inverted 8-10 times and centrifuged at 13,000 rpm for 15 min. The supernatant was placed in a new centrifuge tube and an equal volume of absolute ice-cold isopropanol was added. The tubes were centrifuged at 13,000 rpm for 10 min. The supernatant was discarded and the pellet was washed with 70% (v/v) ethanol. The pellet was air-dried at room temperature and then dissolved in 20 μL TE buffer. The DNA samples were stored at -20°C until further
use. The purity of the DNA extracted was checked by recording the absorbance of the sample at 260nm and 280 nm. The extracted samples was analyzed in 0.8 % agarose gel electrophoresis stained with Ethidium bromide.

### 3.5.2 Qualitative and quantitative determination of DNA by spectrophotometric method

**Principle:**

A solution of nucleic acids strongly absorbs UV with an absorbance maximum of 260nm and proteins at 280nm which is linearly related with the concentration of DNA and RNA and the amount of contamination in the solution. The intense absorption is primarily due to the presence of aromatic rings in the purine and pyrimidine. The concentration of nucleic acid in a solution can be calculated if one knows the value of $A_{260}$ of the solution. A solution of double-stranded DNA at a concentration of 50ug/ml in a 1cm quartz cuvette will give $A_{260}$ reading of 1. A solution of single-stranded DNA/RNA that has $A_{260}$ of 1 in a cuvette with a 1cm path length has a concentration of 40ug/ml. Proteins are usually the major contaminants in nucleic acids extract and these have absorption maximum at 280nm. The ration of absorbance at 260 and 280nm hence provides a clear idea about the extent of contamination in the preparation. A ratio between 1.8 and 1.9 is indicative of fairly pure DNA preparation. But values less than 1.8 signify the presence of proteins as impurities. The values greater than 1.9 signify the presence of organic solvent in the DNA preparations. A ratio of 1.8 determines the pure DNA preparation.
Procedure:

The spectrophotometer and the UV lamp was switched on. The wavelength was set at 260nm and 280nm. The instrument was set at zero absorbance with T.E buffer or sterile water as blank. 5 or 7ul of the sample was taken in a quartz cuvette and made up to 3ml with TE buffer or sterile water. Absorbance of the solution was read. The concentration of DNA in the sample was calculated using the given formula:

Concentration of dsDNA = $A_{260} \times 50\mu g \times$ dilution factor

Purity of the DNA

$A_{260} : A_{280}$ ratio = $A_{260} / A_{280}$

= 1.8: pure DNA

= 1.7 – 1.9; fairly pure DNA (acceptable ratio for PCR)

= less than 1.8; presence of proteins.

= greater than 1.8; presence of organic solvent

3.5.3 Visualization of extracted DNA through Agarose Gel Electrophoresis

Principle:

For the majority of DNA samples, electrophoretic separation is carried out in agarose gels. This is because DNA molecules and their fragments are considerably larger than proteins; therefore larger size agarose gels are required. Under an electric field, any given fragment of DNA should move towards the anode with the same mobility. This is due to the charge per unit length owing to the phosphate groups.
Separation on agarose gels is achieved because of resistance to their movement caused by the gel matrix. Thus the largest molecules will have difficulty moving, whereas the smallest molecules will be relatively unhindered. Consequently the mobility of DNA molecules during gel electrophoresis will depend on size. Gel concentrations must be chosen based on the molecules to be separated such as for plasmid molecules – 1%; genomic DNA – 0.8% and RNA – 1.5%, mitochondrial DNA – 0.8% and amplified samples at 1.5% was used.

**Procedure:**

1 gm agarose was weighed and mixed with 100ml 1X TAE buffer. To this a pinch of ethidium bromide was added and this mixture was heated to 100°C. The heated solution was made to cool and was loaded on to the castic plate assembly to form gel. After loading this gel mixture, to form wells for loading sample a comb was placed. Once the gel is totally polymerized, the comb was removed and the sample was loaded and was subjected to run under constant electric field (50v). This gel assembly was placed in a buffer. The separated DNA fragments were visualized using UV-transilluminator.

**3.5.4 Polymerase Chain Reaction (PCR)**

**Principle:**

PCR is an invitro method of enzymatic synthesis of specific DNA sequence developed by Kary Mullis in 1987. It is a very simple and inexpensive technology for characterizing, analyzing, synthesizing, a specific DNA or RNA from virtually, any living organism, plant, animal, virus or bacteria. It exploits the natural function of
polymerase present in all living things to copy genetic material or to perform molecular photocopy. PCR consists of three steps:

1. **Denaturation**: during this step, the two strands melts, open to form single stranded DNA and all enzymatic reaction stoops. This is generally carried out at 92 to 96°C.

2. **Annealing**: annealing of primer to each original strand for new strand synthesis is carried out between 40–60°C.

3. **Extension**: the polymerase adds dNTPs complementary to the template strand at the 3’end of the primers. Since both the stands are copied in the PCR there is an exponential increase in the number of copies of the required gene.

These 3 steps are repeated for about 20 to 30 times in an automated thermal cycler, which heat and colds the reaction mixture in the tube in a very short time. This result in exponential increase accumulation of the specific DNA fragments.

**Procedure:**

- 100ng of DNA was used for molecular based detection of genes.

- The PCR reaction was performed for 20µl.

- The PCR tubes were placed in thermocycler and the reaction was carried out.
3.5.5 Amplification of matK gene

PCR was carried out in an Eppendorf Personnel Master Cycler (Germany) at 4°C. The PCR conditions were 94°C for 3 minutes (Initial denaturation), 94°C for 30 seconds (Denaturation), 47°C for 1 minute (primer annealing), 72°C for 1 minute 20 seconds (extension), and further 72°C 7 minutes for final extension. The run had 40 cycles. The primers used for amplification were forward matK F 5’-CGATCTATTCAATTATTTTC-3’ and reverse matK R 5’-TCTAGCACACGAAAGTCGAAGT-3’. This amplified product was sequenced using the ABI PRISM 3730XL Analyzer.

PCR set up for gene amplification -Standardization

<table>
<thead>
<tr>
<th>Components</th>
<th>Stock concentration</th>
<th>Final concentration</th>
<th>Volume for 20 µl setup</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milli Q water</td>
<td>-</td>
<td>-</td>
<td>9.6µl</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>2mM</td>
<td>0.2mM</td>
<td>2 µl</td>
</tr>
<tr>
<td>MgCl2</td>
<td>25mM</td>
<td>1.5mM</td>
<td>1.2µl</td>
</tr>
<tr>
<td>Taq buffer</td>
<td>10X</td>
<td>1X</td>
<td>2 µl</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>3µM</td>
<td>0.3µM</td>
<td>2 µl</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>3µM</td>
<td>0.3µM</td>
<td>2 µl</td>
</tr>
<tr>
<td>DNA Template</td>
<td>100ng/µl</td>
<td>100ng</td>
<td>1 µl</td>
</tr>
<tr>
<td>Taq DNA Polymerase</td>
<td>5U/µl</td>
<td>1U</td>
<td>0.2 µl</td>
</tr>
</tbody>
</table>
**PCR Reaction Conditions:**

Initial denaturation : 94°C – 3min

Denaturation : 94°C – 1min

Annealing : 47°C – 1min

Extension : 72°C – 1min 20sec

Final extension : 72°C – 7min

Hold : 4°C

Total number of cycles 40

This amplified product was further used and sequenced using the ABI sequencer instrument

### 3.5.6 Restricted Fragment Length Polymorphism (RFLP)

The amplified matK gene was digested with BamHI restriction endonuclease. BamHI binds at the recognition sequence 5'-GGATCC-3' , and cleaves these sequences just after the 5'-guanine on each strand. The digested product was separated and visualized through agarose gel electrophoresis.

### 3.5.7 matK gene sequence analysis and phylogenetic reconstruction

A Blastn similarity search was conducted using the sequenced matK gene of *T. angustifolia* as the query sequence. The nucleotide collection database was searched with the organism key as Poales, taxid 38820. The programme was
optimized for highly similar sequences (megablast). The top sequences producing significant alignments were selected. These nucleotide sequences were aligned using the ClustalW option present in the MEGA5 software (Tamura et al., 2011). After computing the alignment, the data menu was opened and the active data was explored for analysis of various sites such as conserved sites, parsimonious informative sites, variable sites etc., using the highlight section of the sequence data explorer window of the MEGA tool. The statistics of the nucleotide composition was analysed and automatically exported to Microsoft Excel 2007. Further, the aligned sequences were used to find the Best DNA model and to compute the pair-wise distance in order to estimate the evolutionary divergence between the sequences. To construct Phylogenetic trees, the Maximum Likelihood method and the Neighbor-Joining method were employed and the test of phylogeny had 500 bootstrap replication.

3.6 ALLELOPATHIC STUDIES:

3.6.1 Collection of plant material and preparation of extracts

Plant samples were uprooted from the marshlands at Pallikaranai, Chennai, Tamil Nadu, India, and brought to the laboratory for species authentication using the herbarium available in the Department of Botany, Guru Nanak College. Care was taken to select only mature plants that had inflorescence since the chemistry of plant species changes with age. The plants were washed thoroughly in running tap water to clear off the adhering soil matter. The rhizome, leaves and inflorescence were then separated, cut into small pieces and weighed separately. Aqueous extracts of these plant parts were prepared using de-ionized water. One gram of plant material was ground in 10 ml of deionized water and left for 12 hrs at room temperature. It was
then filtered using a double layered muslin cloth and the filtrate centrifuged at 5000
rpm for 5 minutes. The supernatant was made up to a final volume of 100ml (20% w/v) and this represented the stock solution. The various extracts were diluted with
deionized water appropriately to obtain 25, 50, 75 and 100% (v/w) concentration of
test extracts. (Maria et al., 2002).

3.6.2 Selection of test seeds and experimental protocol

In the search for potential herbicides derived from plants, the purpose is to
demonstrate allelopathic activity and to determine the activity range of the resultant
allelochemical herbicide with respect to necessary dose and target. In such
experiments, sensitive plant species such as lettuce or tomato are normally selected.
Black gram, *Vigna mungo* (L) Hepper was selected for this study as it is one of the
most widely used pulse crop in India, very rich in phosphoric acid, and the size of the
seeds make it convenient for various types of experimental work. Certified seeds of *V.
mungo* were obtained from agricultural agencies at Chengalpattu, Tamil Nadu. The
seeds were surface sterilized with 70% alcohol for 2 minutes and rinsed with distilled
water several times. Ten seeds for each treatment were spread in a petridish lined with
double layered filter paper and 10 ml of test solution (25%, 50%, 75%, and 100% v/v
of aqueous extracts of rhizome, leaf, and inflorescence) was gently added to the
petridish and allowed to be soaked by the filter paper. Thereafter, the seeds were
moistened with water whenever necessary till the end of the study period. Ten ml of
deionized water was used for the control sample. The petridishes were incubated at
room temperature (28°C±2°C) for 7 days in a germination rack illuminated with
fluorescent light during day time till the end of the experiment. Each treatment had
three replicates. The percentage of seed germination was calculated at the end of the 4th day after treatment. The protrusion of radical through seed coat was taken as the criterion for germination. On the 7th day after treatment, allelopathic parameters such as germination percentage, root and shoot lengths and wet and dry weights of the root and shoots were recorded.

3.7 INSECTICIDAL PROPERTIES:

3.7.1 Preparation of extracts

Extracts of the leaf, rhizome and inflorescence were prepared by maceration method using four different solvents in sequential order of increasing polarity such as Chloroform, Ethyl acetate, Methanol and Water. Fifty gram of the plant material was macerated for 3 hrs with 100ml of Chloroform and then shaken continuously in an electronic shaker for 24 hours. The macerated slurry was filtered through a Whatman No.1 filter paper and transferred into a volumetric flask. The volume was made up to 100 ml with respective solvent to form the primary stock solution of the extract (Deepthy et al., 2010). The pulp obtained from the filtration process was weighed accurately and then subjected to sequential extraction in ethyl acetate, followed by methanol and finally with water. Each extract obtained was made up to 100ml using the respective solvent.

3.7.2 Preparation of Artificial diet:

Artificial diet was used for the mass rearing of Helicoverpa armigera in laboratory condition (Gupta et al., 2004; Bilal Haider et al., 2007; Nimbalkar et al., 2009). The diet ingredients for the preparation of 500ml of diet consisted of chickpea flour (53.5 g), distilled water (412.5 ml), agar-agar (7.5 g), dried yeast granules
(12.2 g), casein (7.5 g), ascorbic acid (3.0 g), methyl-p-hydroxybenzoate (1.0 g), sorbic acid (0.6 g), formaldehyde 40% (0.5 ml), ABDEC drops (multivitamin solution, 0.75 ml). All the ingredients were weighed separately and the dry ingredients were mixed together in distilled water. The mixture was boiled to nearly 60°C. The remaining wet ingredients were then added to the mixture with thorough mixing for 10 minutes. The freshly prepared diet was cooled to room temperature and served to insects.

3.7.3 Rearing of *Helicoverpa armigera*:

Larvae of *Helicoverpa armigera* were collected from the okra field at Thenpakkam village, Chenkalpattu, Kanchipuram district. The insects were reared in the laboratory condition (26°C ± 2°C, 57-65% RH) separately on freshly prepared artificial diet in plastic cups (3x1 1/2 inches) closed with aluminum foil. The diet was changed at an interval of two days up to third instar stage and daily for later instars. The pupae were kept in folds of tissue paper in a petridish placed inside a plastic container covered with plastic netted lid. The adults that emerged from the pupae were released into oviposition chambers for egg laying and provided with 10% of honey solution mixed with Multivitamin drops in a cotton swab was placed as feed for the adults. Castor leaves were kept inside the cages to facilitate egg laying. Egg masses were collected, kept in Petri dishes and newly hatched larvae were maintained on the host leaves. The newly hatched first instar larvae were transferred safely using thin paint brush to the freshly prepared artificial diet. First instar larvae were reared in groups until the third instar, after which they were separated into individual petridishes to prevent cannibalism (Fig.2).
3.7.4 Quantal dose response and Toxicity studies through Topical application of plant extract:

a. Preparation of experimental concentrations:

Stock solution was prepared by dissolving 5 mg extract in 10 ml of solvent. This solution was used for making further dilutions. The different extract concentrations such as 1%, 3%, and 5% were used for further study following trial runs with various concentrations of the extract.

b. Method of treatment

Thirty six number of third instar larva of *H.armigera* were selected for each set of treatment. Six numbers of glass beakers of 250 ml capacity were taken and labeled for different concentrations in addition to one for the control. In case of control, respective solvent was added in place of extract. Insects were dipped into the solution for two minutes and then transferred back in the rearing medium. Each experiment was conducted in triplicates along with the control group. Mortality of insects following exposure was recorded after 24h. The LC$_{50}$ value was determined after fitting the regression equation using the log dose and probit values. The probit values were obtained from the appendix table in Heong *et al.*, (2011).

c. Correction for control mortality—the Abbott formula

In bioassays, it is common to expect a proportion of the insects in the control batches to die during the experiment due to natural causes or the control treatment with the solvent. To correct for this, the Abbott formula is often used and is usually in the form
\[
P = \frac{Po - Pc}{100 - Pc} \times 100
\]

where \( P \) is the corrected mortality, \( Po \) is the observed mortality, and \( Pc \) is the control mortality, all expressed in percentages.

### 3.7.5 Feeding Bioassay for determining toxicity of extracts:

The Bioassay was carried out using diet incorporation method as per Gujar et al., (2000). Third instar larvae of uniform size and age were taken from the mass culture maintained in the laboratory. 2 ml, 6 ml and 10 ml of plant extract from the stock was taken and mixed with 100 ml of freshly prepared artificial diet and mixed well so as to obtain of 1, 3 and 5% concentration of test extract. For each treatment, 10 larvae starved for 3 hrs were singly introduced in separate containers containing 6g of feed. Six replicates were maintained for each treatment. Feed without the plant extract and with the solvents such as 1%, 3% and 5% of Chloroform, Ethyl acetate, Methanol and Water served as controls. All the experiments were conducted at 26°C ± 2°C, 57-65% RH. After 24hrs of feeding, the weight of left over feed, faeces and larvae were recorded carefully. Data on the percentage of larval mortality, Feeding deterrence index and metabolic rate were estimated as follows:

**Percentage larval mortality** was calculated after a period of 24h using Abbott’s formula (Abbotts, 1925).

\[
\frac{T - C}{100 - C} \times 100
\]

Where, \( T \)- Percentage mortality in treated,

\( C \)- Percentage mortality in Control.
**Feeding deterrence index** for each treatment was calculated using the formula of Ben Jannet *et al.*, (2000).

\[
\frac{C - T}{C + T} \times 100
\]

Where, \(C\) – Consumption in Control, \(T\) - Consumption in Treated.

### 3.7.6 Nutritional indices:

The following formulae were used according to Waldbauer (1968) and Slansky and Scriber (1982) to calculate CR (consumption rate), GR (growth rate) and ECI (efficiency of conversion of ingested food):

\[
\text{CR} = \frac{\text{Weight of food eaten}}{\text{Duration of Experiment (Days)}}
\]

\[
\text{GR} = \frac{\text{Weight Gain}}{\text{Duration of Experiment (Days)}}
\]

\[
\text{ECI} = \frac{\text{Weight Gain}}{\text{Weight of food eaten}} \times 100
\]

All the indices were calculated on the dry weight basis. To find the dry weights, the feed, faeces, and larvae (freeze killed larvae, 10 specimens for each) were weighed, oven-dried (48 hours at 60° C), and then re-weighed to establish a percentage of their dry weight.