Chapter: 3
Materials And Methods

Introduction:

The Housefly, *Musca domestica* L. is well known cosmopolitan and household insect found almost all habitats in the world (Fenemore and Prakash, 1992). It belongs to order Diptera and family Muscidae. Housefly generally occurs in poultry farms, hog, horse stables, slaughter houses, kitchens etc. It is one of the important vectors for various disease causing organisms which causes diseases to humans as well as its domestic animals. It also cause nuisance(Khan and Ahmad, 2000).

*Musca domestica* L. produce an enzyme dehydrochlorinase that catalyzes the breakdown of DDT to HCl and nontoxic DDE which is main cause of resistance development (Romoser W. 1981).

These problems have encouraged scientists to search for alternative, which are safer and cheaper for selective control of housefly.

Since plant oils/extracts has been proved to be a good alternatives for conventional insecticides, we can use them to control flies. These plants have insecticidal properties; also blocks the spiracles of insects causing death. Some acts as poisons by interfering with the fatty acids and with their normal metabolism (Jenkins, 1964).
Material and methods:

A: Rearing Method:

Temperature maintained was 30±2°C and 55-75% R.H. Adults of *M.domestica* were reared in plastic jars of 35 cm height and 15 cm width, covered with muslin cloth. A cotton swab soaked in 5 gm of milk powder; 3 gm of yeast dissolved in 20 ml of water was offered to these adults as food and was changed after every 24 hours. This cotton swab also served as substrate for oviposition.

b) Adulticidal Assay:

This assay was carried out in 500 ml glass beakers. Filter paper was treated uniformly with the test oils and was placed inside the beaker lining the inner area including bottom surface. The beakers were covered with the muslin cloth. Ten 2-3 days old well fed adults were treated with various concentrations of plant oils and three replications done. Each experiment five times repeated. LC\textsubscript{50} was calculated using log probit analysis (Finney1971).

2: Insect Growth Regulatory Assay:

500 ml glass beakers containing 50 gm of rearing medium and the test oils corresponding to LC\textsubscript{20} dose was mixed thoroughly and moistened with 60 ml water was used for the assay. Parallel controls with 50 gm of the rearing medium along with carrier solvent and 60 ml water were carried out. 0-24 hour’s old eggs were released in each beaker. These larvae were allowed to develop in their respective containers till adult emergence. The development period required for each stage along with abnormalities in the emerging stages were recorded. For this experiment three to five replications done. Each experiment 5 times repeated.

3: Attraction/Repellent Assay:

This assay was carried out using double choice method. Twenty newly emerged adults were released in a cage (size 18”×18”) containing conical flasks. One flask contained 5% test oil in 10 ml of milk (diagnostic dose) while the other contained solvent
and 10 ml of milk to serve as control. Funnels (4'' diameter) were introduced in each flask to avoid the escape of the flies. Number of flies trapped in these flasks was counted after 24 hours. The results were expressed in terms of percentage attraction/repulsion. The percentage was determined by the number of adults trapped in each flask. For this experiment three to five replicates were taken and each experiment was repeated five times.

4: Oviposition attraction/deterrence:

Double choice method was used for this assay. 1-2 days old five males and five females were confined in a cage (size 18'' x 18''). Cotton swab soaked with 5% test oil and milk was offered to these flies. For control, cotton swab soaked with carrier solvent and milk was offered. After 24 hours egg count was taken. For this experiment three replications have done, six repetitions done for this experiment. Following formula was used to calculate % Oviposition deterrence. (Tare, 1995)

\[
\text{% Oviposition deterrence} = \frac{T - E}{T} \times 100
\]

\(T\) = Total number of eggs laid in both control and treated.
\(E\) = Number of eggs laid in treated.

d) Estimation of Total Protein contents:

Folin-Lowry method was followed for protein estimation (Plummer, 1988). Following reagents were prepared for this estimation.

i) Alkaline Solution:

50 ml of alkaline Sodium carbonate solution (20g/litre Na\textsubscript{2}CO\textsubscript{3} in 0.1 mol/liter NaOH) and 1 ml of Copper Sulphate - Sodium Potassium Tartarate solution (5 g/litre CuSO\textsubscript{4}.5H\textsubscript{2}O in 10 g/litre Na, K, tartarate. Prepared fresh by mixing solutions) were mixed together to get Alkaline solution.

ii) Standard Protein:
Albumin solution 0.2 mg/ml is prepared.

iii) **FolinCiocalteau Reagent (FCR):**

Commercially available reagent diluted in water (Using equal volume of water) on the day of use.

**Method:**

A mixture of 5 ml alkaline solution and 1 ml test solution were homogenized and kept at room temperature for more than 10 min. Followed by quick addition and mixing of FCR(0.5 ml). 30 minutes later extinction read against appropriate blank at 750 nm.

Estimation of protein was done using standard curve.

**Introduction:**

*Phthorimaea operculella* Zeller commonly called as Potato tuber moth (PTM)is a cosmopolitan important potato pest belonging to order Lepidoptera. The infection is limited to the family Solanacaea. Potato, tobacco, eggplant and occasionally tamarillo leaves are commonly attacked by PTM and are of economic importance.

Both foliage and tubers suffer extensive damage due to larval attack of *P. operculella*. In case of total foliage destruction, the larvae abandon it and search for tubers. Foliar infestation results in destruction of the plant. Larvae enter through eyes of tubers and make a network of tunnels by feeding on them. Such tubers identified by excreta of larvae at the tunnel entry. During summer there if high infestation leads to severe damage.

Conventional synthetic insecticides causes several problems even though they have undoubted pest control efficiency (Parmar and Devkumar1993, Edwards 1973). Some of these are known to cause intrinsic high toxicities. Due to their persistence nature they cause continuing, long term and wide spread pollution of the environment. These have an affinity for fatty tissue, resulting in accumulation there and leading to the phenomenon of bio-magnifications in the ecological food chains and pyramids (Smith and Van den Bosch 1967).
Several plant oils with pest control potential have been studied in order to develop new strategy for the control of *P. operculella* (Kroschel and Koch 1999), still there is enormous scope to explore various plant products as selective control agents for *P. operculella*.

This chapter deals with the study of effects of the test oils on various bioactivities against *P. operculella*.

**Material and Method:**

**A: Rearing Method:**

Potato Tuber Moth culture was obtained from Entomology, National Chemical Laboratory, Pune. PTM colony was maintained at 30±2°C and 55-75% R.H. Plastic jars covered with black muslin cloth was used for rearing adults. Black muslin cloth was used as preferred substrate for oviposition. 25 % honey solution and pricked potato tubers were given to adults and larvae respectively. Infested potatoes were kept on sterilized soil, which was subsequently used for cocoon formation by larvae. Pupae were kept in separate containers for adult emergence.

**B: Bioassays:**

1: Toxicity:

a) Larvicidal Assay:

Small jars of 50 ml capacity covered with muslin cloth were used to perform toxicity assays. A filter paper was placed in each jar lining inner area including bottom surface. A filter paper was treated uniformly with test oil concentrations. Only carrier solvent i.e. acetone was used for control. Twenty prepupal larvae were introduced in each jar. Each experiment was repeated ten times with eight to ten replicates. After 24 hours mortality count was taken. Log-probit analysis (Finney 1971) was used to calculate LC<sub>50</sub>. 
b) Adulticidal Assay:

Appropriate jars (40cm × 15cm) covered with muslin cloth were used to carry out assays. The adulticidal effects of test oils were studied by using Residual application method. Different concentrations of test oils were uniformly applied on filter paper and placed in the jars lining the inner area including the bottom surface. Twenty adults were exposed to different doses of oils for 24 hours to observe mortality. Each experiment was repeated ten times with eight to ten replicates. After 24 hours mortality count was taken. Log-probit analysis (Finney 1971) was used to calculate LC\textsubscript{50}.

2. Insect Growth Regulator Assay:

Separate trays (Sized 18" × 12" × 6") containing sterilized soil were used to carry out assays. LC\textsubscript{20} concentration of test oils were applied on pricked potatoes and introduced in the trays. The muslin cloth with 30 eggs which were about hatch was kept on the treated potatoes to ensure easy entry of the newly hatched larvae. The period required for the development of first instar larvae to adult emergence was recorded. The period required for development of the in between stages up to the prepupal instar was not recorded, as they spend this entire period inside the potatoes. The resulting development and growth period along with abnormalities in the emerging stages were monitored and recorded. Assays were repeated five.

3: Attractant/Repellent Assay:

This assay was carried out using Double choice method. Thirty newly emerged adults were released in a cage (size 20"×20") containing two conical flasks. One flask contained carrier solvent and honey solution while other

contained 5% test oil in honey solution. Funnels (5"diameter) were introduced in each flask to avoid the escape of the moths. Number of moths trapped in these flasks was counted for 24 hours. The results were expressed in terms of percentage
attraction/repulsion. The number of adults trapped in each flask was used to determine percentage. Each assay was carried out ten times and for each set five replicates was taken.

4: Oviposition Attraction / Deterrent Assay:

Twenty pairs of 24 hours old adults were released in plastic jars. These were covered with muslin cloth treated with 5 mg/cm² dose of test oils. The muslin cloth is preferred substrate for oviposition. Egg count was recorded after 24 hours and % inhibition of eggs laying capacity was determined by the following formula (Tare 1995).

\[
\text{% Oviposition Deterrence} = \frac{T - E}{T} \times 100
\]

T = Total number of eggs laid in both control and treated.
E= Number of eggs laid in treated.

5: Biochemical procedures:

4\textsuperscript{th} instar larvae were used for all biochemical assays. Larvae were treated with LC\textsubscript{20} test oil concentration for 24 hours which were then harvested and mixed in 20 ml of phosphate buffer of pH 6.8 and centrifuged at 8000 rpm in Ultra Centrifuge (Remi-CM12) for 10 minutes at 4\textdegree C. Larvae weighing about 100 mg were used to obtain supernatant which was used for biochemical assays.

Estimation of Total Protein content:

Folin-Lowry method was followed for protein estimation (Plummer, 1988). Following reagents were prepared for this estimation.

i) Alkaline Solution:

50 ml of alkaline Sodium carbonate solution (20g/litre Na\textsubscript{2}CO\textsubscript{3} in 0.1 mol/liter NaOH) and 1 ml of Copper Sulphate - Sodium Potassium Tartarate solution (5 g/litre
CuSO$_4$.5H$_2$O in 10 g/litre Na, K, tartarate. Prepared fresh by mixing solutions) were mixed together to get Alkaline solution.

**ii) Standard Protein:**

Albumin solution 0.2 mg/ml is prepared.

**iii) FolinCiocalteau Reagent (FCR):**

Commercially available reagent diluted in water (with equal volume) on the day of use.

**Method:**

5 ml alkaline solution and 1 ml test solution were evenly mixed and kept at room temperature for 10 minutes or more, followed by quick addition of 0.5 ml FCR. 30 minutes later extinction read against appropriate blank at 750 nm.

Estimation of protein was done using standard curve.

**Introduction:**

Various problems caused by synthetic pesticides have forced on the need for development of ecofriendly pest/vector control programs. These also known to affect non-target organisms. Such as soil microbes; honey bees or other animals like fish,earthworm that plays critical role in an ecosystem (cope, 1971).

It leads to pest resurgence of which the carabid predators of the cabbage root fly is a classic example (weight et al 1960). It obvious that when pesticide is applied to pest, the non-target organisms also suffer slowly with them (Moore 1967). It also results in bio-magnifications since these are non- biodegradable (Gustafson 1993).

From above it is evidence that pesticides/insecticides affect drastically on non-target organizing .To overcome all such effects a new natural, safer, molecules must be discovered.
It concludes that it was important to study the effect of selected medicinal plants on non-target organizers; which were examined for their insect control potential in the present work.

Soil bacteria are selected as a representative of non-target organizers.

**Material and Methods:**

A.R. grade acetone was used to dissolve all test oils (200 mg/ml). Serial dilutions were made as per requirement for microbiological analysis.

Soil samples were collected at random, minimum eight, from field and mixed thoroughly to make a composite sample and serial dilutions were made as per the requirement for microbiological analysis.

**a) Effect of selected plant oils on soil bacteria:**

For culturing the bacteria agar plates were prepared. Petri plates were inoculated with serially diluted soil sample along with the LC\textsubscript{50} concentrations of the test oils. The plates were incubated at 37\textdegree C for 48 hours. These plates were observed for the colonies of microorganisms. The colony count was recorded. For control the same procedure was done without adding the test oils and colony count was recorded. Each assay was carried out eight times and for each set five replicates were taken and recorded data were subjected statistical analysis.