MATERIALS AND METHODS

REARING OF SPILOSOMA OBLIQUA

Insect Source

The culture of S. obliqua was established in the laboratory from the larvae collected from infested castor leaves from outskirts of Delhi (Seelampur and from the green belt having wildly grown castor plantation along the bank of Yamuna River in Timarpur region). The stock culture was maintained at the temperature 27 ± 2°C with 60-65% relative humidity, and 12:12 h L:D photoperiod (Fig. 2.4).

Adult Rearing

The larvae that collected from castor plants were reared on castor leaves in plastic containers of size (14.5 cm dia. X 17.5 cm ht.) until pupation and adult emergence. The adults, after eclosion were transferred to cubical oviposition cage made of plexiglass (20cm X 20cm X 20cm) (Fig. 2.5). Side walls of the cage were fitted with meshed window to provide aeration. The front wall of the cage had sliding door for handling and transferring of adults and for harvesting of eggs. Before transferring of adults for mating and oviposition, the lower surface of cage was lined with moist filter sheet. A castor leaf having its petiole dipped in water, contained reagent bottle so as to avoid wilting of leaf, was kept in cage. This provided stimulation and substrate to gravid females for oviposition. The cotton wool, soaked in mixture of 10% sugar and honey, contained in small cups were kept in the cage so as to provide food for adults. Five or six pairs of adults were released in this cage. The eggs laid by females on different substrates in oviposition cage were removed gently with the help of Camlin hair brush and kept in plastic container for hatching.

Egg Harvesting

Eggs were removed gently from castor leaves and walls of rearing cage with the help of Camlin hair brush No. 1. These eggs were sterilized by first rinsing in 0.2% sodium hypochlorite solution followed by 4% formalin. These eggs were then washed...
thoroughly in water and dried at room temperature, and placed in small circular, plastic containers (7 cm dia. X 6 cm. ht.) for emergence.

**Larval Rearing**

Freshly hatched larvae were transferred on fresh tender castor leaves kept in the plastic container (7 cm dia X 6 cm ht), lined with moist cotton and filter paper. Castor leaves were disinfected by dipping in KMnO₄ solution followed by washing in running tap water. These eggs were air dried at room temperature and stored in B.O.D. for emerging of eggs. The newly emerged larvae were transferred on castor, placed in plastic container having bottom lined with moist cotton. The presence of moist cotton ensures high relative humidity of 70±5% inside the container which is an essential requisite for the larval development of *S. obliqua*. Precautions were taken to remove moisture from the sidewalls of the container so as to avoid bacterial and fungal infections. The larvae were transferred daily on fresh castor leaves kept in clean containers till 3rd instar stage. After 3rd instar, the larvae were transferred to big container (14.5 cm dia. X 17.5 cm ht.); their number in the individual container was gradually reduced in successive instars to avoid the crowding. The last instar larvae when started excreting light pinkish fecal pellets and reducing its food intake, indicating preparation for cocoon formation. Such transitional stages of larvae are called as prepupae which ultimately stopped feeding and start forming a protective cover, the cocoon. The prepupae were allowed to remain in cocoon for 48h for successful transition into pupae and kept in separate container for pupation. The pupae were gently removed from their cocoons and sterilized with 0.05% sodium hypochlorite solution for 3 min. These sterilized pupae were washed thoroughly in running water, air dried at room temperature and kept for eclosion.

**PREPARATION OF EXTRACT**

Ripe fruits of *M. azedarach* were collected from trees in Delhi University campus in every year during February as this is the repining season of fruits (Fig. 2.3). Collected fruits were washed thoroughly in running water to remove dust and any infectious substances. These were shade dried for a day. Some of the fruits were used to obtain the extract and rest was kept for further use every.
Solvent Extraction

Based on previous studies in M. Phil. the methanolic extracts of *Melia azedarach* fruits were prepared. First the fruits were defatted with hexane, and then sequentially extracted in methanol with residues obtained after hexane extraction. At third step the methanol extract was dried in vacuum evaporator and again fractionated with acetone as mentioned below.

The fruits were crushed into powder using grinder, 100 g of crushed powder was dissolved in 500 ml of hexane in a glass beaker and stirred by magnetic stirrer for 1 h. This was left undisturbed for 12 h and then filtered through Whatman no.1 filter paper. Liquid extract was decanted in another beaker and 500 ml of hexane was further added to residue and shaken well, decanted and pooled with the 1st decanted aliquot. This process was repeated 3 times to obtain maximum extraction. The solid residue, so left, was air dried and extracted in methanol following an identical procedure discussed earlier. The extract so obtained was concentrated separately in a rotary evaporator at 35-40°C, under reduced pressure that yielded an oily and viscous dark-red and semi-solid residue called as methanol extract. This extract was washed with 500 ml of acetone and the residual extract of original methanol extract was again dissolved in methanol. The two extracts so obtained were dried separately in a rotary evaporator at 35-40°C, under reduced pressure. After that these were kept in small weighing bottles capped and preserved in refrigerator for further use. For the convenience these extracts were denoted as 1) AFME for acetone fraction of methanol extract, 2) MFME that was used for methanol fraction of methanol extract (Fig. 2.3, 2.4)

PREPARATION OF TREATMENT SOLUTION

*Acetone fraction of methanol extract (AFME)*

Five concentrations were made namely, 1000, 2000, 3000, 4000 and 5000 ppm. The concentrations were so made by taking respective amount say 10, 20, 30, 40 and 50 mg in 15 ml collecting bottles (Qualigen) and then each was dissolved in 1 ml of acetone solvent and after that 9 ml of distilled water was added to the bottles so as to make it 10 ml in each case.
**Methanol fraction of methanol extract (MFME)**

Test solutions of MFME were made in similar manner as described for acetone. Both extract either form methanol or acetone were prepared separately to avoid any confusion but in a similar manner. These extracts were freshly prepared on day of experiment from preserved extracts.

**Acetone/Methanol control solution**

Control solution was prepared by mixing 1 ml of either acetone or methanol in 9 ml of distilled water mixed with 0.5% Triton-X 100 as an emulsifier to make the volume of 10 ml.

**DIET PREPARATION**

In order to assess the effect of plant extract on larval growth, AFME and MFME were incorporated into artificial diet at different concentrations according to Akhtar and Isman (2004). Semisolid diet was prepared for each test solution separately while control diet was prepared by incorporating respective solvents only. Soybean and pea were powdered for the experimental diet and the floor of these seeds (in 50:50) were the main ingredients of this diet, and the important constituents were listed in the Table 2.1, treatment diets were prepared as follows.

For a diet (say 1 g) containing 1000 ppm concentration of test extract, 1 mg of the extract is required; accordingly 50 g of diet that was prepared for each of the extract by dissolving 50 mg of respective extract in 1 ml of carrier solvent (Acetone or Methanol). While for 2000 ppm 100 mg for extract was required and according to the increasing concentration increased amount of respective extract was dissolved.

The diet was divided in two parts, I part contained dry part of powdered diet and dissolved extract was pipetted on this in a glass beaker (100 ml, Borosil) and mixed thoroughly for proper mixing of extract as well as kept for some time until the fumes of the extract was evaporated, now the distilled water of known amount poured on to this and again mixing was done. II part of diet contained distilled water and Agar-agar. The agar was boiled for a few minutes and mixed in part I then poured gently in rectangular plastic box (16 X 10 X 2.5 cm) and allowed to cool at room temperature. These
containers were covered with lid and stored inside the refrigerator. Control diet was prepared similarly but it had only test solvents. Different bioassays were carried out on semisynthetic diets which are discussed below (Fig 2.7).

METHODS TO STUDY SURVIVAL AND DEVELOPMENT

Survivorship Bioassay

Neonate larvae (0-12h old) were used for this bioassay with both the extracts viz., MFMFE and AFME of *Melia* fruits. Fresh diet were cut into small strip like pieces of different concentrations of AFME and MFME individually, these were placed in circular plastic boxes (5 cm dia. X 5 cm ht.) covered with tissue paper so as to prevent excess moisture and escape of larvae. Neonate larvae were released on each test for 96 h. The neonate survival was observed after every 12 h interval from treatment. Each set of bioassay alongwith a control were replicated 5 times and each replicate consist of 10 neonate larvae.

All the bioassays conducted, were kept in BOD incubator at 27±1°C temperature and 70±5% relative humidity and 12:12 h L:D photoperiod.

Development Bioassay

Development and survival of *S. obliqua* were studied by using semisynthetic diets incorporated with different test extracts and their concentrations were 100, 200, 300, 400, 500, 1000, 2000, 3000, 4000 and 5000 ppm.

For this bioassay, newly emerged 1\textsuperscript{st} instar larvae were taken as a batches of 10 per replicate and their were five replicates for freshly prepared diets of particular extract i.e. AFME or MFME, in the individual transparent plastic box (5cm x 5cm) (Fig2.7) and the diets pieces were similar as described earlier. The box was covered with tissue paper, and loosely screwed with lid so as to maintain proper moisture on one hand prevent escape of larvae on the other. These larvae were reared up to 2\textsuperscript{nd} instar in this box. Thereafter thinning was done and the single larva was confined in individual container having the test/control diets. These larvae were transferred into clean container after every two day and provided with fresh diet. The larvae were observed and recorded daily.
at a fixed time of day for their mortality, and moulting into next instar. If any dead larvae were found, the container was discarded. In the prepupal stage larvae were transferred to new boxes as they do a purging behaviour so a very small or no diet is supplied at that time to avoid excess moisture. The larvae after completing development make a cocoon and within that they pupated.

The pupae were collected and rinsed with 0.2% formaldehyde solution, washed thoroughly with distilled water and kept in transparent plastic boxes (5 cm x 5 cm), lined with tissue paper. These pupae were separated sex wise, and placed into containers, and inspected regularly for the adult emergence.

The parameters studied for development and survivorship were (i) larval period, (ii) larval survival, (iii) Total developmental period, (iv) % pupation, (v) pupal period, (vi) pupal survival, (vii). adult emergence and (viii) Sex ratio. Deformities in pupae or adults were also recorded. The “growth index value” was calculated by dividing the percentage of insects completing development during larval stage and average larval period (Mohamed et al., 2007). The “developmental index value” was calculated as ratio between percentages of neonate larvae completing development up to adult, and the period required to do so as per the method followed by Tamhanker et al., (1992). The “sex ratio” was calculated as number of females or males emerged, divided by total number of males and females emerged (Greenberg et al., 2001):

\[
\% \text{ Sex ratio} = \frac{\text{No. of female or male emerged}}{\text{Total no. of male + female emerged}} \times 100
\]

**METHODS TO STUDY FEEDING BEHAVIOUR**

**Antifeedant Bioassay**

The antifeeding potential of the AFME and MFME was tested by a conventional leaf disc method (Kubo and Nakanishi, 1977; Abdelgaleil and Nakatani, 2003) against 4\textsuperscript{th} instar larvae of *S. obliqua*. Freshly excised leaf of Castor, (*Ricinus communis* L.) was trimmed to the size of 5 cm diameter. Now a single leaf disc was immersed for 15s in the
respective concentrations of the extracts. Whereas, control discs were immersed in control solution. These leaf discs were kept under fan for 5 min. at room temperature so as to evaporate surface liquid. The treated discs were kept in the middle of a Petri dish (9 cm diameter), having its floor lined with moist filter paper to avoid dryness of leaf discs (Fig 2.8). One freshly moulted 4th instar larvae were placed on the disc in each Petri dish each replicate consist of 10 larvae. Thee replicates of each concentration were carried out. After 24 h the larvae were removed. The leaf area consumed by larvae was traced on graph paper and measured (Singh and Singh, 1993; Sarma and Kalita, 2001) the number of fecal pellets was also counted for the antifeedant activity of extract treatment.

An antifeedant index (AI) was calculated using the formula as

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

Where C is for leaf area consumed in control and T is for leaf area consumed by the insect in treatment (Isman et al., 1990). Whereas, number of fecal pellets was calculated by using the formula, (Bentley et al., 1984 and Shukla et al., 2000).

\[ AI = 1 - \frac{\text{No. of fecal pellets in treatment}}{\text{No. of Fecal pellets in control}} \times 100 \]

**Food Preference Bioassay**

Food preference by the larvae was assayed by using diet block. Freshly moulted 6th instar larvae were selected for this bioassay. The larvae prior to moulting (when they stop feeding) at late 5th instar stage were taken out from rearing jar and kept in small circular plastic boxes (5 cm dia. X 5 cm ht.), and provided with distilled water soaked cotton. They were kept overnight and in next morning their fresh weight were taken before bioassay. Freshly prepared diet of approximately 1000±10 mg weighed on Mettler Toledo balance (Model PG503-S, sensitivity 0.001g) of both control and treatment was taken. One treated and one control diet block was placed in a plastic Petri box (10 cm dia X 3.5 cm ht) at a distance of approximately 5 cm, thereafter single larvae was released in the middle of the box and allowed to feed for 24 h (Fig. 2.8). Each Petri box was covered by its lid and all the setup was kept in BOD at the same conditions as done in previous
experiments. After 24 h the larvae were removed from the diet and the weights of larvae, final diets as well as fecal pellets were recorded. The food preference was calculated as:

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

where, C is for wt of food consumed in control and T is for wt of food consumed by the insect in treatment (Isman et al., 1990).

**Food Consumption and Nutritional Indices**

**Food Consumption**

The growth and development after food consumption for AFME and MFME incorporated in semisynthetic diet were studied in 6th instar larvae. The larvae were reared on castor leaves up to the late penultimate instar, which were identified by the presence a rupture bulge behind the head capsule. The larvae prior to moulting were transferred in individual plastic boxes (5 cm dia. X 5 cm ht.) and supplied with wet cotton swab and left overnight for moulting. The Petri dishes were inspected the next morning and active larvae, which moulted successfully, were selected for bioassays.

The larvae were again starved for 2 hours before the bioassay, provided with water on cotton swab, so as to bring them in identical physiological condition. All the tests for food consumption of larvae were conducted in last instar as they consume maximum amount of food in this instar, so one can easily conclude the maximum deleterious effect of desired inhibiting compound. Moreover, the consumption, growth and food utilization were measured for last instar larvae in 48 h by using a standard gravimetric technique, given by Waldbauer (1968).

Semi-synthetic diets containing extracts of test plant species were prepared as discussed earlier. The basic ingredients of these diets were same, except AFME or MFME and control solvent which was incorporated in diet. AFME and MFME extract were supplied in five concentrations that were 1000, 2000, 3000, 4000 and 5000 ppm.

Diet pieces of equal size and shape (Cubes) were taken out from the diet container with help of the spatula. These pieces were weighed individually which gave initial fresh weight (IFWD) of the diet. The diet pieces were kept in individual Plastic boxes (5 cm dia. X 5 cm ht), and numbered.
The final instar larvae, selected and prepared for the bioassay, were grouped into replicates of 10 each. Each larva was weighed individually on the balance (sensitivity 0.001g), which gave initial fresh weight (IFWL). Larva was released individually into the box containing the test/control diets and numbered. Same amount of fresh diets were kept in Petri dishes without larvae under the same conditions to estimate the loss of moisture for calculating the corrected weight of ingested food.

All the plastic boxes containing experimental larvae were kept inside the BOD incubator. Each larva was allowed to feed for 48 h, after which they were taken out and weighted individually that gave their final weight (FFWL). The unconsumed test diet and faecal matter were also removed and weighed separately (FFW). Weights of control diet pieces were also taken which gave their final weights of diets (FFWD). Weight gain by individual larva after 48 h of feeding was calculated by subtracting the initial weight of larva at the beginning of the experiment from the final fresh weight at the end.

The nutritional indices based on fresh weights were calculated as follows:

**Consumption Index (CI)** = weight of ingested food/ [feeding period X average weight of insect]

**Growth Rate (GR)** = weight gain during the feeding period/ [feeding period X mean body weight of larva during the feeding period].

**Approximate digestibility (AD)** = [weight of ingested food – weight of faeces/ weight of Ingested food] X 100.

**Efficiency of conversion of ingested food to body substance (ECI)**

= [weight gain by larva/weight of ingested food] X 100

**Efficiency of conversion of digested food to body substance (ECD)**

= [Wt. gain by larva/ weight of ingested food - weight of faeces] X 100

Growth rate and percent growth inhibition were calculated (Waldbaur, 1968 and Shukla et al, 2000).
METHODS TO STUDY ORIENTATION BEHAVIOUR

Oviposition Behaviour

Five pairs of freshly emerged males and females were released into the oviposition chamber and provided food as described below. These insects start mating after the emergence from pupa. The procedure of experiment is discussed below.

Test Chamber

The ovipositional responses of moths were conducted in transparent rectangular oviposition cage made up of plaxiglass. For No-choice test the cubical cage with the dimensions of 20 x 20 x 20 cm cage was used, whereas for choice test, a larger (45 x 20 x 20 cm) cage was taken (Fig 2.12). The cage used for single choice was divided into three sectors by discontinuous markings, which constituted a middle sector of 25 cm and two end sectors of control/treatment of 10 cm. The cage has a well defined ventilation window as its side walls on each side, had round window of 10 cm dia., covered with fine nylon net (app. 40 mesh/cm). The front wall of the test chamber had a door (15 x 15 cm) in the middle, fitted with nylon net sleeve to facilitate the supply of test and food material as well as handling of moths. The bottom of cage was lined with filter paper. The food was provided in plastic stopper (here the term was used as diet cup) of 1 cm dia., having cotton soaked with 10% honey solution, These cups were placed near the test plants kept at each side of the cage. Similarly for no-choice test the cubical chamber were used and there were no sector as described for single choice test. Castor leaves were used for oviposition assays and its procedure is mentioned below.

Choice bioassay

Freshly excised twigs containing leaves of small to medium size (app. 20 cm dia.) so as to keep the approximately same surface area of desired leaves for every time. The twig of the leaf was wrapped with cotton and dipped in water which is kept in reagent bottle (120 ml) so as to stop wilting of leaf. The mouth of reagent bottle was again plugged tightly with cotton, which in turn was wrapped with aluminum foil. In case of choice test, the leaves were coated the freshly prepared treatment solution on both the
abaxial as well as adaxial surfaces with the camlin hair painting brush no. 6.00, and control leaf was coated with a mixture of control solution (solvent and water) alone. Leaf twigs of castor plants of both control and treatment were placed opposite to each other near the window walls inside the oviposition cage (45 x 20 x 20 cm).

**No choice bioassay**

The ovipositional response of females in no-choice arena towards the extract/control was evaluated in the laboratory by coating the leaves with respective treatment or control solution alone, after that the leaves were air dried and kept individually (either treated or control leaf twig) in the centre of respective oviposition cage (20 x 20 x 20 cm). These cages were marked as treatment or control on outside also.

Five pairs of moths (5 female and 5 male), were released at 19.00 h in the oviposition cage. In the next morning moths were removed from the cage and number of eggs laid on leaves, and elsewhere in the oviposition cage was counted. Each female was used only once, and each test was replicated 5 times, having five pairs of insects. The ovipositional response of *S. obliqua* for test materials was compared on the basis of,

(i) Proportion of total number of eggs laid on the leaf surface that was calculated as

\[
\text{No. of eggs laid on the leaf} \div \text{Total no. of eggs laid} \times 100
\]

(ii) Relative suitability of leaves coated with different test materials that was calculated on the basis of ovipositional preference index (OPI)

\[
\text{OPI} = \frac{\text{No. of eggs laid on side (A) - No. of eggs laid on side (B)}}{\text{No. of eggs laid on side (A) + No. of eggs laid on side (B)}} \times 100
\]

**Egg Hatchability**

The effect of *Melia* extracts on the hatchability of eggs was tasted by applying extracts directly on the egg surface. Overnight laid eggs were removed from leaf surface, and divided into two groups. One group was treated with AFME of *M. azedarach* at 5 concentrations i.e., 1000, 2000, 3000, 4000 and 5000 ppm, Whereas, other group was
treated with MFME. There were 5 replicates in each group and each replicate was consisted of 25 eggs. 1 ml of the extracts of desired concentration of treatment solution was applied on the eggs and in control eggs were treated with 1 ml of respective solvent alone. The solutions were allowed to evaporate, and each group was kept in separate plastic container (5h x 5d cm) lined with filter paper. These containers were kept in B.O.D. under maintained conditions at the temperature 27 ± 2°C with 70- 75% relative humidity and 12:12 h L:D photoperiod, for the emergence of larva. The number of larva hatched out from each group was counted. The % hatchability was calculated as:

Percent hatchability: \( \frac{\text{No of larvae hatched}}{\text{Total no. of eggs treated}} \times 100 \)

Larval Orientation

Petri Dish Bioassay with Neonate Larvae

To investigate the orientation response of neonate larvae to various concentrations of *M. azedarach*, overnight hatched neonate larvae were selected for bioassay. The larvae were first released on wet cotton, placed in a petri dish for 10 minutes, so as to bring them in identical physiological condition. The active larvae were selected by putting the Petri dish below the light source and those larvae moving actively were taken for the test.

The orientation experiment with neonate larvae was conducted in a glass Petri dish (9 cm dia. and 1.5 cm ht). This Petri dish was marked with a line at the middle to divide into two equal sectors i.e., C (control) and T (treatment). Now individual leaf discs of 2 cm diameter were cut from freshly excised castor leaf and dipped in the respective concentrations of the extracts for 15 s, whereas, the control discs were dipped in control solution. These leaf discs were kept under electric fan at room temperature for 5 minutes so as to evaporate surface liquid. The treated discs as well as control leaf disc were kept at opposite ends in their respective sectors of Petri dish having its floor lined with moist filter paper to avoid dryness of leaf discs. 10 neonate larvae were placed in the middle of the Petri dish with the help of brush and the petri dish was covered with lid. All Petri dishes were transferred in a plastic tray and kept in B.O.D. Thee replicates of each concentration were carried out. After 1 h duration, the larvae were observed on the
respected leaf discs and their numbers were counted. The bioassay was repeated three times with 5 replicates of each concentration.

**Preference index (I)** was calculated using the formula as

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

Where, C is number of larvae present on control leaf disc and T is number of larvae present on treatment.

**STATISTICAL ANALYSIS**

Data for the responses of the insects under different conditions were subjected to one-way and two-way Analysis of Variance (ANOVA). Means were compared by Tukey’s test to analyze the significant difference between control and different concentrations also among different concentrations and between different extracts as well. While, the data under no-choice and single choice conditions were analyzed by chi-square test for goodness of fit or by t-test. The statistical analysis of various responses of *S. obliqua* was performed on computer software statistical program Sigma Stat 2.0 (Jandel Scientific, 1995).
Fig. 1.1 Distribution Map of *S. obliqua*

Source: [http://www.nic.funet.fi/pub](http://www.nic.funet.fi/pub)
Fig. 2.1: *Spilosoma obliqua* Adult

Male

Female
Fig. 2.2: Spilosoma obliqua Larvae
Fig. 2.3: *Melia azedarach*: Plant and Fruits
Fig. 2.4: Rearing Jars for Larvae

Fig. 2.5: Oviposition Cage
Dried fruits Powder

\[ \text{Defatted with Hexane for 12 h} \]

\[ \text{Decanted 3 times} \]

Residual powder was kept in Methanol for 12 h

\[ \text{Decanted 3 times} \]

\[ \text{Evaporate} \]

Dried extract was washed with Acetone for 4 h

Residual extract was re-dissolved in Methanol

\[ \text{Evaporate} \]

\[ \text{Stored at 4ºC} \]

Fig. 2.6: Preparation of Extract
Fig. 2.7: Semisynthetic Diet for Larvae
Fig. 2.8: Experimental Set up for Feeding Bioassay
Fig. 2.9 *Spilosoma obliqua* Larve

(a) Normal Larva

(b) Larval-pupal intermoult
Fig. 2.10 *Spilosoma obliqua* Pupae
Fig. 2.11 *Spilosoma obliqua* Adults
Fig. 2.12 Oviposition Cage