MATERIALS AND METHODS
To study the behaviour of the pest and also to carry out the bioassays subsequently, at different stages of life cycle (eggs, larvae, pupae and moths), an adequate supply of specimen of *Spodoptera litura* was required. Since collection of specimen from natural populations would lead to issues like variability in age of the specimen and densities of natural populations, this requirement was facilitated by continuous rearing of insects in the laboratory. As many as nine generations of the insect were maintained to get a homogenous population.

### 3.1. Insectary and Equipments required

The mass rearing of the insects was carried out in an insectary (7 x 9 sq feet) wherein optimum conditions of 24±2°C, 70±5% R.H., 12L: 12D (Light: Dark) were maintained at all stages of insect’s life cycle. The insectary was fumigated with 5% formalin bimonthly.

The equipments required for rearing were electronic balance, microwave, liquid blender, autoclave, polythene bags (autoclavable), steel racks, plastic trays, rearing vials (recycled amrutjan vials) of standard size: 4.5 x 3 cm³, oviposition containers (7 x 8 cm³), mating boxes (10 x 9 cm³), absorbent and non-absorbent cotton, forceps (blunt end), 10X Hand lens, watch-glass and camel hair brush.

### 3.2. Parental stock

A starter colony of *Spodoptera litura* was established from pupae (National Accession No. NBAII-MP-NOC-02) collected from National Bureau of Agriculturally Important Insects (NBAII), Hebbal, Bengaluru to obtain a disease-free, genetically efficient uniform parental stock.
3.3. **Mass rearing of insects**

The pupae obtained from NBAlII were sexed and maintained in separate adult eclosion boxes in a tray containing moistened pre-sterilized cloth. On emergence males and females were transferred to a smaller mating box lined with a sheet of paper along the walls which served as a substratum for the female to lay eggs. Cotton soaked in 10% (w/v) honey compounded with few drops of Vitamin E was provided as food source inside the mating box.

3.3.1. **Maintenance of eggs**

The paper containing egg patches were cut and placed in a high humidity environment till it hatched. The eggs were observed for hatching every day and once the eggs were hatched, the neonates were transferred to feed bottles containing 5 ml artificial diet.

3.3.2. **Maintenance of larvae**

The larvae were supported by an artificial diet. The diet was a modified composition of *Helicoverpa armigera* (Satyaprakash and Nagarakatti, 1974) compounded by Bavistin and ethyl alcohol with Chickpea (*Cicer arietinum*) flour as a base (Divakar, *et al.*, 2011). The neonates were reared in groups on diet in sterile petri plates and when the larvae reached 3rd instar, were transferred individually to the diet in rearing vial (5 x 3 cm²) using a camel hair brush and the vial’s mouth was plugged with sterile cotton wool. When the larvae reached the pre-pupa stage, the vials were inverted till they pupated to prevent burying of the pre-pupa into the diet.

3.3.3. **Preparation of artificial diet**

Artificial diet was prepared using the contents listed out in the Table 3. The contents of batch I were transferred to a domestic blender and ground thoroughly.
Table 3. Artificial Diet formulation

<table>
<thead>
<tr>
<th>Batch</th>
<th>Ingredients</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Chickpea flour</td>
<td>105.00 g</td>
</tr>
<tr>
<td></td>
<td>Sorbic acid</td>
<td>1.00 g</td>
</tr>
<tr>
<td></td>
<td>Ascorbic acid</td>
<td>3.25 g</td>
</tr>
<tr>
<td></td>
<td>Streptomycin sulphate</td>
<td>0.25 g</td>
</tr>
<tr>
<td></td>
<td>Sterile water</td>
<td>400.00 ml</td>
</tr>
<tr>
<td>II</td>
<td>Agar-agar</td>
<td>12.50 g</td>
</tr>
<tr>
<td></td>
<td>Sterile water</td>
<td>400.00 ml</td>
</tr>
<tr>
<td>III</td>
<td>Bavistin</td>
<td>2.00 g</td>
</tr>
<tr>
<td></td>
<td>Formaldehyde 10%</td>
<td>2.00 ml</td>
</tr>
<tr>
<td>IV</td>
<td>Absolute alcohol</td>
<td>10 ml</td>
</tr>
<tr>
<td></td>
<td>Methyl p- hydroxy benzoate</td>
<td>2.00 g</td>
</tr>
<tr>
<td>V</td>
<td>Yeast (Brewer’s)</td>
<td>10.00 g</td>
</tr>
<tr>
<td></td>
<td>Multivitamin syrup</td>
<td>2.00 ml</td>
</tr>
<tr>
<td></td>
<td>Vitamin E capsules</td>
<td>2 nos.</td>
</tr>
</tbody>
</table>
Simultaneously agar-agar was melted in sterile water (batch II). The moulten liquid was transferred to the blender, and the contents were mixed for 5 min. The ingredients in batch III were added one after the other and blending was continued for 5 min by which time, the diet temperature was reduced to less than 70°C. Finally, the ingredients of batch IV and batch V were also added to the blender carefully and the contents were fully whirl mixed. The final volume of the diet was made up to one litre in sterilized water. Rearing vials were filled with 5 ml of diet while the diet was still semi-solid and after solidification the vials were stored in refrigerator in air tight polythene bags if not used immediately (Plate 1).

3.3.4. Maintenance of pupae
The pupae, which were burrowed in the diet, were removed carefully using sterile blunt forceps and sterilized with 0.2% sodium hypochlorite solution, thoroughly rinsed with distilled water; sexed using a 10X magnifying hand lens and placed separately on a filter paper in an eclosion box (10 x 9 cm²) and maintained in a humid environment till the emergence of adult moths.

3.3.5. Maintenance of adults
The adult moths were provided with 10% (w/v) honey compounded with few drops of Vitamin E as food source soon after their emergence till the completion of egg laying.

3.4. Life cycle and reproductive Behaviour
For studies on life cycle and normal reproductive behaviour, the larvae and the adults were kept under constant observation. The egg, larval and pupal period were noted. Morphology of different stages of S. litura was studied by observing under Binocular Microscope. The male and female moths were under constant observation from the time of emergence to study the calling, courtship, mating and egg laying
Plate 1. Mass rearing of insects

a. Transferring of larvae to diet
b. First two instars reared in groups
c. Diet dispensed in individual vials
d. Larvae reared individually in vials
behaviour. *Spodoptera litura* being nocturnal a red light was used to observe their behaviour during night times.

3.4.1. **Calling Behaviour**

The virgin females were individually released into a mating box. They were scored as calling if the ovipositor was visible and extruded, or has assumed the calling posture i.e., the female vibrates her wings at a 45° angle, assuming the ‘V’ position, with the ovipositor extended for as long as 15min. The onset of calling i.e., the time at which the female first called was noted.

3.4.2. **Courtship and Mating Behaviour**

To study this behaviour, one day old male and female moths were introduced into the mating box. Since these moths are nocturnal courtship and mating behaviour was observed across the scotophase on successive days till all the moths successfully mated. However, to record the peak period at which maximum number of females mated during a scotophase, number of moths mated at an interval of three hours was noted only on the 2nd scotophase.

3.4.3. **Egg laying/Oviposition Behaviour**

Oviposition behaviour can be described as the process of extrusion of the ovipositor accompanied by downward curvature of the abdomen and spotting of the substratum with deposition of the egg. The egg laying activity was observed in the females, irrespective of mating, during the scotophase. Peak oviposition was recorded in both virgins and mated females.

3.5. **Preparation of extracts**

The leaf and flower parts of *Calendula officinalis* L. were collected from plants procured by M/s Himalaya Herbs, Himachal Pradesh and sample authentication was
obtained from FRLHT, Bangalore. The leaf and flower parts were shade dried at room temperature and each of these samples was finely powdered in an electric blender to be used for extraction.

3.5.1. Extraction-Soxhletion

Soxhlet extraction of plant material was carried out as per the description of Martin Rathi et.al, (2005) as follows:

About 500g each of finely powdered plant material (shade dried flowers and leaves) was placed inside a thimble which was loaded into the main chamber of the Soxhlet extractor. The Soxhlet extractor was placed onto a flask containing the extraction solvent. The Soxhlet was then equipped with a condenser. The solvent was heated to reflux. The solvent vapour traveled up a distillation arm, and flooded into the chamber housing the thimble of solid. The condenser cooled the solvent vapour and dripped back down into the chamber housing the solid material. The chamber containing the solid material slowly filled with the warm solvent and the desired compound dissolved in the warm solvent. When the Soxhlet chamber was almost full, the chamber was automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. This cycle was allowed to repeat many times, over hours for each of the plant material. The extraction was carried out in different solvents (2.5 liters each) such as Petroleum ether (40° - 60°C B.P), Benzene, Chloroform, Methanol and Water, in the increasing order of their polarity, taken in succession. After extraction, the solvent was removed and evaporated on a water bath yielding the extracted compound. The non-soluble portion of the extracted solid remained in the thimble, and was discarded.

3.5.2. Preparation of stock solution and dilutions

The crude extract obtained was dissolved in minimal quantity of the respective solvent that served as a stock. Various dilutions (0.5%, 1%, 2.5%, 5%, and 10%) from
the stock were prepared in acetone and emulsified with few drops of sandovit. Respective concentrations of organic solvents in acetone emulsified with few drops of sandovit served as positive control.

3.5.3. Phytochemical screening of crude extracts based on biochemical tests
All the leaf and flower extracts were subjected to preliminary qualitative phytochemical screening in order to find out the components present in them. 0.5g of each extract was used for the following tests. Each of these tests distinctly answers only on the basis of a specific functional group present in the given extract hence different tests are used for confirmation of phytochemicals.

3.5.3.1. Detection of alkaloids
Extracts were dissolved individually in dilute hydrochloric acid and filtered. The filtrates were used to test for the presence of alkaloids.

- **Mayer’s Test**
  Filtrates were treated with Mayer’s reagent (saturated solution of Potassium Mercuric Iodide). Formation of a yellow cream precipitate indicates the presence of alkaloids.

- **Wagner’s test**
  Filtrates were treated with Wagner’s reagent (saturated solution of iodine in potassium iodide). Formation of brown/reddish brown precipitate indicates the presence of alkaloids.

- **Dragendorff’s test**
  Filtrates were treated with Dragendorff’s reagent (saturated solution of potassium bismuth iodide). Formation of red precipitate indicates the presence of alkaloids.

- **Hager’s test**
  Filtrates were treated with Hager’s reagent (saturated solution of picric acid solution). Formation of yellow colour precipitate indicates the presence of alkaloids.
3.5.3.2. Detection of carbohydrates

Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

➢ Molisch’s Test

Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube and 2 ml of Conc. Sulphuric acid was added carefully along the sides of the test tube. Formation of violet ring at the junction indicates the presence of Carbohydrates.

➢ Benedict’s test

Filtrates were treated with Benedict’s reagent and heated on water bath. Formation of orange red precipitate indicates the presence of reducing sugars.

➢ Fehling’s test:

Filtrates were hydrolysed with 1N Hydrochloric acid neutralized with alkali (NaOH) and heated with Fehlings’ A & B solutions. Formation of red precipitate indicates the presence of reducing sugars.

3.5.3.3. Detection of glycosides

Extracts were hydrolysed with dilute 1N Hydrochloric acid, and then subjected to test for glycosides.

➢ Modified Borntrager’s Test

Extracts were treated with Ferric chloride solution and heated on a boiling water bath for about 5 minutes. The mixture was cooled and shaken with an equal volume of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.

➢ Legal’s test

Extracts were treated with sodium nitroprusside in pyridine and methanolic alkali. Formation of pink to blood red colour indicates the presence of cardiac glycosides.
3.5.3.4. Detection of saponins

- **Foam test**

Small amount of extract was shaken with little quantity of water. If foam produced persists for ten minutes, it indicates the presence of saponins.

3.5.3.5. Detection of phytosterols/triterpenes

- **Salkowski’s Test**

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of Conc. sulphuric acid, shaken and allowed it to stand. Appearance of golden yellow colour indicates the presence of triterpenes.

- **Libermann Burchard’s test**

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Conc. sulphuric acid was added carefully along the sides of the test tube. Formation of brown ring at the junction indicates the presence of phytosterols.

- **Tshugajeu test**

Extracts were treated with chloroform and filtered. Excess of acetyl chloride and a pinch of Zinc chloride was added, kept aside for some time till the reaction was complete and then warmed on water bath. Appearance of eosin red colour indicates the presence of triterpenes.

3.5.3.6. Detection of oils & fats

- **Stain Test**

Small quantities of extracts were pressed between two filter papers. An oily stain on filter paper indicates the presence of fixed oil.
3.5.3.7. Detection of resins

- Acetone-water Test
  Extracts were treated with acetone. Small amount of water was added and shaken. Appearance of turbidity indicates the presence of resins.

3.5.3.8. Detection of phenols

- Ferric chloride Test
  Extracts were treated with few drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

3.5.3.9. Detection of tannins

- Gelatin Test
  To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

3.5.3.10. Detection of flavonoids

- Alkaline Reagent Test
  Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

- Lead acetate Test
  Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

- Shinoda Test
  To the alcoholic solution of extracts, a few fragments of magnesium ribbon and Conc.HCl was added. Appearance of magenta colour after few minutes indicates presence of flavonoids.
Zinc hydrochloric acid reduction Test

To the alcoholic solution of extracts, a pinch of Zinc dust and Conc. HCl was added. Appearance of magenta colour after few minutes indicates presence of flavonoids.

3.5.3.11. Detection of proteins

Xanthoproteic Test

The extracts were treated with few drops of concentrated Nitric acid solution. Formation of yellow colour indicates the presence of proteins.

Ninhydrin test

To the extract, 0.25% Ninhydrin reagent was added and boiled for few minutes. Formation of blue colour indicates the presence of amino acid.

Biuret Test

The extracts were treated with 1 ml of 10% sodium hydroxide solution and heated. To this a drop of 0.7% Copper sulphate solution was added. Formation of purplish violet colour indicates the presence of proteins.

3.5.3.12. Detection of diterpenes

Copper acetate Test

Extracts were dissolved in water and treated with few drops of copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes.

3.6. Studies on the effects of *Calendula officinalis* crude extracts on physiology of *Spodoptera litura*.

3.6.1. Effect on feeding of *Spodoptera litura* larvae

The dry part of the artificial diet was mixed with appropriate quantity of each of the diluted extract to get 2.5%, 5% and 10% concentrations, allowed to dry and later mixed with the wet part, properly mixed and dispensed into the bottles. Once the diet was solidified, it was weighed before introducing the pre-weighed 3rd instar, 4th
instar and 5th instar larvae starved for 4 hours. Thirty larvae for each trial were released individually in plastic containers and allowed to feed for a period of 48h. The weight of larvae, uneaten food and fecal material recorded after 48h of feeding were used for the evaluation of antifeedancy, nutritional and utilization indices.

3.6.1.1. Antifeedancy index

Antifeedancy index (AFI) was calculated using the formula

\[ AFI = \frac{[(C-T)/(C+T)] \times 100}{1} \]

where

C = Amount of diet consumed in control and T = Amount of diet consumed in treated.

3.6.1.2. Nutritional indices

Nutritional indices, such as Consumption index and Growth rate were calculated using the formulae CI = E/TA and GR = P/TA respectively.

3.6.1.3. Food utilization indices

Food utilization indices like Approximate digestibility, Efficiency of conversion of ingested food and Efficiency of conversion of digested food were calculated using the formulae AD = 100(E-F)/E, ECI = 100 P/E and ECD = 100 P/(E-F) respectively.

Where,

A = Mean weight of animal during the period T,
E = Weight of food eaten,
F = Weight of feces produced,
P = Weight gain of insect and
T = Duration of experimental period (on fresh weight basis) (Waldbauer, 1968).

3.6.2. Biochemical profile of digestive enzymes and their substrates

The bioassays were performed with 4th and 5th instar larvae of Spodoptera litura using 10% concentration of extracts of Calendula officinalis leaves and flower compounded in the artificial diet. Positive control was prepared with 10% organic solvents in
acetone and the feed of untreated diet served as negative control. The freshly moulted 4th and 5th instar larvae were starved for 4 h and then fed with treated and untreated diet for 48 hours after which the larvae were sacrificed to carry out various biochemical tests.

3.6.2.1. Dissections

The larvae (4th instar and 5th instar) were frozen at -21°C and the entire digestive tract was dissected out in ice-cold insect Ringer’s solution. The Malphigian tubules, adhering tissues and gut contents were removed. The midgut tissue and midgut content were taken separately, dried on a tissue paper and weighed and used for enzyme assays and substrate estimations.

3.6.2.2. Preparation of Enzyme extracts for Biochemical Analysis

The extracts of haemolymph, Midgut and Midgut-content were prepared from the larvae using standard procedures as follows.

3.6.2.2.1. Collection of Haemolymph (HL)

Haemolymph samples of control and treated fourth instar and fifth instar larvae of S. litura were collected by puncturing the proleg and the exudates were drawn into insect Ringer’s solution containing a few crystals of sodium thio-sulphate to prevent melanization. The haemolymph sample (2% homogenate) was centrifuged at 10,000rpm for 30min at 4°C and used as the enzyme source.

3.6.2.2.2. Extraction of Midgut tissue (MG)

The method used to prepare the biochemical source was that of Applebaum and Applebaum et al, (1961). The gut was homogenized for 3min at 4°C in ice-cold citrate-phosphate buffer (pH 6.8) to get 2% homogenate using a tissue grinder. The homogenized gut was re-suspended in ice cold buffer and centrifuged at 5000rpm for 15min and the supernatant was used as the source for enzyme assays.
3.6.2.3. Midgut-Content (MGC)

The midgut-content was separated from the midgut tissue and homogenized with ice-cold citrate-phosphate buffer (pH 6.8) to obtain 2% homogenate using a tissue grinder. Homogenized sample was re-suspended in ice cold buffer and centrifuged at 5000rpm for 15min and the supernatant was used as the source for enzyme assays.

3.6.2.3. Biochemical Assays

Quantification of various substrates and enzyme activity was carried out using standard procedures in haemolymph, midgut tissue and midgut content of treated as well as untreated larvae. The substrates measured were total sugar (Dubois et al., 1956), total protein (Lowry et al., 1951), lipid content (Handel et al., 1985). The specific activity of the digestive enzymes protease (Snell and Snell, 1949), amylase (Bernfield 1955) and lipase (Cherry and Crandall, 1932) were assayed (Appendix I, II, III).

3.6.3. Study of crude extracts on growth and development of S. litura larvae

The effect of crude extracts on the growth and development of the pest was studied by treating the larvae in different modes of application.

3.6.3.1. Oral route

Freshly moulted 3rd instar larvae were introduced into the diet incorporated with 10% concentration of all the extracts and allowed to complete the life cycle.

3.6.3.2. Topical application

Freshly moulted 3rd instar larvae were topically applied with 10% concentration, for 5 seconds, of all the extracts and introduced into the untreated artificial diet and allowed to complete the life cycle.
In both the cases individual events such as the duration of larval period, pupal period, malformed larva or pupae, adult longevity, oviposition and percentage egg hatchability were recorded. Dead larvae if any were removed every 24h.

3.6.4. Study of crude extracts effect on physiology of *Spodoptera litura* moths.

Two-day old female moths were smeared with 5μl of 5% concentration of all the flower and leaf extracts at the intersegmental region in the abdomen and allowed for mating. Number of eggs laid by treated and control moths were counted. Percentage reduction in oviposition and percentage hatchability of the eggs were determined as mentioned in sections 3.6.1.2 and 3.7.1 respectively.

3.6.5. Study of crude extracts effect on physiology of *Spodoptera litura* eggs.

Freshly laid single egg patch/mass was dipped in different concentrations (0.5%-5%) of all the extracts and positive control (acetone) and negative control (distilled water) for 5 seconds, air dried and observed for egg hatchability. The number of eggs hatched in control and treatments were recorded and the percentage of ovicidal activity was calculated.

Ovicidal activity (OA) is expressed in terms of Percentage egg hatchability calculated using the formula (No. of eggs hatched/No. of eggs laid) x 100

3.7. Phytochemical analysis of effective crude extract by GC and MS

The effective crude extract giving significant activity in an overall perspective was chosen for GC-MS analysis.

Gas Chromatographic analysis and Mass Spectroscopic analysis of effective crude extract was carried out on a Thermo GC-Trace Ultra Version:5.0, Thermo MS DSQ II system and gas chromatograph interfaced to a mass spectrometer (GC-MS)
instrument employing the following conditions: column DB 35-MS Capillary Standard Non-polar column (30 Mts, ID: 0.25 mm, Film: 0.25μM), operating in electron impact mode at 70eV; helium (99.99%) was used as carrier gas at a constant flow of 1ml/min and an injection volume of 1μl was employed. The oven temperature was programmed from 50°C (isothermal for 2min), with an increase of 10°C/min, to 200°C, then 5°C/min to 280°C ending with a 9min isothermal at 280°C. Mass spectra were taken at 70eV; a scan interval of 0.5 seconds. Total GC running time was 35.91min.

3.8. Isolation of bioactive compounds

3.8.1. Fractionation of effective crude extract
The chloroform leaf extracts of Calendula were subjected to fractionation using several solvent systems till very good separation into bands was achieved. In order to separate out specific active compounds, the crude extract was subjected to TLC (precoated Merck®Silica gel 60 F254 chromatographic plates of 1 mm thickness) using individual solvent systems like hexane, petroleum ether, chloroform, acetone, methanol, and combination of solvents like chloroform : petroleum ether (4:1), benzene : acetone (9:1), chloroform : methanol (99:1), benzene : methanol (9:1), benzene : ether (2:3), petroleum ether : chloroform : ethyl acetate (2:2:1), benzene : acetone : ethyl acetate : methanol (4:1:0.5:0.5), benzene :acetone : ethyl acetate : methanol (3:1:1:1), benzene : acetone : ethyl acetate : methanol (4:1:0.5:0.5:2), benzene : acetone : ethyl acetate (4:1:0.5), and benzene : acetone : ethyl acetate : methanol : water (4:1:1:1:2). The best suitable solvent system which gave distinct separation was chosen for collection of the active fraction and scale up.

3.8.2. Preparative Thin Layer Chromatography
About 100g of silica gel G was mixed with 220ml of distilled water, stirred for 30min in a mechanical stirrer. Exactly 50ml of the slurry was measured, poured on
glass plate (20 x 20 cm) and spread uniformly to get the TLC plate with thickness 10 mm. Then the plates were allowed to settle down under room temperature, for about half an hour, then in a hot air oven for 1 hr at 105°C. The plates were allowed to cool and the chloroform leaf extract (10 ml) was applied as a streak with capillary tube on to the plate and allowed to dry. This plate was placed in the TLC chamber which was saturated with mobile phase for two hours. Mobile phase was determined by trial and error method. Benzene: acetone: ethyl acetate: methanol: water in the ratio of 4:1:1:1:2 served as a developing solvent mixture. The plate was run till the bands got separated distinctly. The chromatogram developed was observed under the visible light as well as the UV light. Thick and darker bands were marked and scraped off from the plate along with silica gel. A number of plates were developed in the same manner in order to harvest larger quantities. Each of the bands were dissolved in chloroform and methanol and centrifuged at 3000 rpm for 10 minutes, later the supernatant was decanted and dried at room temperature. The residues thus collected were dissolved in acetone and used for bioassays.

3.8.3. Bioassays with TLC fractions

3.8.3.1. Eggs
The fractions thus collected by preparative TLC were used to test for ovicidal activity. The freshly laid eggs; 1-day old and 2-day old eggs were dipped in 0.1% Conc. of each fraction for 3 seconds and air dried and observed for hatchability.

3.8.3.2. Adults
10μl each of the fractions (0.1% Conc.) was topically applied to the abdomen at intersegmental region of 1-day old female moths and were allowed to mate; post mating, the number of eggs laid and percentage egg hatchability were noted. Parallel to this an untreated set of moths without smearing was also maintained and oviposition and percentage egg hatchability was noted in these moths also.
3.8.4. Purification of the fractions

After conducting the bioassays, the active fraction giving significant activity was subjected to further purification using different solvents such as hexane, chloroform and methanol. The active fraction was mixed with the solvent (1:50 w/v) and continuously shaken for about 15-20 minutes on a shaker and allowed to stand for 10 minutes. This mixture was then filtered and the filtrate was decanted into a petri dish and allowed to dry. The resulted residues were subjected for further analysis.

3.8.5. UV-Visible Spectrophotometric analysis of TLC fractions

The TLC fractions were subjected to UV spectral analysis in Systronics Double Beam UV-VIS Spectrophotometer: 2201. The readings of absorbance maxima at a range of wavelengths (100-999 nm) were taken under the scan mode.

3.8.6. Infra red (IR) Spectral analysis of TLC fractions

The IR spectral analysis was carried out in Nicolet 380 Series FTIR instrument. The sample was loaded at the resolution of 4.0 and optical velocity of 0.6329 and the aperture was 100, 00.

3.8.7. Molecular weight determination of TLC fractions by Liquid Chromatography and Mass spectrometric (LC-MS) analysis

The TLC fractions were subjected to Liquid Chromatography using HPLC: 2010A, Shimadzu, Japan with Xcalibur software under the following experimental conditions:

Column: Thermo PGC C18 (Reverse Phase); Length (mm): 150 I.D. (mm): 2.1; Particle Size (μm): 3; Detection: UV at 254nm-Channel B. Detector details: HPLC PDA / UV detector; temperature: Ambient; Injection Volume of 5μl. Eluent: Solvents- Methanol: water 90:10, Flow rate: 0.2ml / min; Run Time (min): 60.00.
Mass Spectrometric Experimental Conditions were as follows. Probe/source voltage: 4.5kV; Sheath gas flow (arbitrary units): 40.00; Auxiliary/Sweep gas flow (arbitrary units): 20.00; Source type: ESI (Electro Spray Ionization); Capillary Temp (C): 275; Capillary voltage (V): 16.00; Nebulization gas flow: Helium at 1mL/min approx; Helium in the mass analyzer cavity is maintained at 0.1Pa (10⁻³).

3.8.8. Structural elucidation of TLC fractions by H-NMR and ¹³C-NMR studies.

Nuclear Magnetic Resonance spectra viz., H-NMR and ¹³C-NMR using DMSO-D₆-CDCl₃ mixture and CDCl₃ solvents respectively were obtained on a Bruker Avance Series, 400 MHz.

3.9. Statistical analysis

All the experiments were conducted in triplicates and repeated thrice. The data of all the three trials were expressed as the Mean values with Standard Deviation (SD) for each measurement. The data were also analyzed by one-way analysis of variance (one-way ANOVA). Tukey’s Multiple Range Test was used for determination of significance of difference (p < 0.05). Analysis was performed with SPSS 11.0 (SPSS, Inc., Chicago, IL).