Materials and Methods
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(I). BREEDING AND MAINTENANCE OF STOCK CULTURE:

(A). *Dysdercus koenigii*: The adults and nymphs of red cotton bugs were collected from the okra field located at/near the Aligarh Muslim University campus and brought to the laboratory for present research work. These insects were kept in glass rearing jars measuring 20x15 cm containing 3 cm thick layer of damp sterilized sand at the bottom. These jars were placed in REMI’s Environmental Chamber maintained at 28 ± 1° C temperature and 70 - 80% relative humidity. The jars at the top were covered with a piece of muslin cloth tightly fixed by means of rubber band to stop the exit of an insect. The culture were fed on overnight soaked cotton seeds, which were changed on alternate days. When the eggs were laid, the adults were transferred to fresh rearing jars to ward-off any hindrance during egg-hatching. Over-crowding was avoided for proper growth of an insect.

(B). *Spodoptera litura*: Adults and larvae of cotton leaf-worm were collected from the Aligarh Muslim University campus. The larvae and adults were separately kept in rearing jars measuring 20 x 15 cm. The jars were covered with a piece of muslin cloth, tightly fixed by means of a rubber band. The temperature and humidity was maintained in REMI’s Environmental Chamber at 28 ± 1° C and 70 – 80 % RH. The adults were fed on 10 % honey solution. For this purpose, a piece of cotton was wrapped around a glass slide and it was soaked with fresh honey solution, the slide was obliquely placed against the jar.
Female moths oviposited eggs on the white paper strips which were placed in the jar. Newly hatched larvae were transferred to separate rearing jars and fed on fresh and tender castor leaves. The larval jars were cleaned daily to avoid any infection and were provided with fresh leaves. When the larvae reached to late 6th instar they were transferred to separate jars containing 3 - 4 inch thick layer of slightly damp sand for pupation. The larvae went 2 - 3 inch down in the sand and pupated. After two days of pupation, the pupae were taken out from the sand, cleaned with cotton and kept in separate jars for emergence. As soon as the moths emerged they were transferred to other rearing jars to maintain a large population of all stages at a controlled condition.

(II). SAMPLING OF EXPERIMENTAL INSECTS:

In the present work 4th instar nymph of Dysdercus koenigii were sorted out and maintained in separate jars. They moulted to 5th instar after 2-3 days and the moult was ascertained by observing the cast off exuviae and the head capsule. The newly moulted 5th instar nymph were then treated with different concentrations of the selected insecticides.

Similarly, the 5th instar larvae of Spodoptera litura were sorted out from the random culture and maintained in separate rearing jars. As soon as they moulted to 6th instar which can be visually ascertained by the size, head capsule and exuviae they were treated with varying concentrations of the selected insecticides.
(III). PREPARATION OF DIFFERENT CONCENTRATIONS OF CHEMICALS USED:

**ANDALIN (Flucycloxuron):** The chemical name of Andalin is 1-{{\alpha}-[(EZ)-4-chloro-\alpha-cyclopropylbenzylideneaminoxy]-p-tolyl}-3-(2,6-difluorobenzoyl) urea (ratio 50–80% (E)- and 50–20% (Z)- isomers).

Technical Andalin (1%) is obtained from Duphar B. V. (Weesp, Holland).

The **empirical formula** is C\textsubscript{25}H\textsubscript{20}ClF\textsubscript{2}N\textsubscript{3}O\textsubscript{3} and

**structural formula** is

\[\text{Diagram of structural formula}\]

\[(E)-\text{isomer}\]

\[(Z)-\text{isomer}\]
Six concentrations of Andalin viz., 0.1%, 0.05%, 0.025%, 0.01%, 0.005%, 0.0025% were prepared from 1% stock solution of Andalin. From the stock solution desired concentrations were prepared by serial dilution in acetone.

*Triticum vulgaris* lectin (Wheat germ agglutinin): *Triticum vulgaris* lectin is isolated from wheat germ. It has two subunits and a molecular weight of 43.2 kDa. The WGA lectin selectively binds to N-Acetyl glucosamine (GlcNAc) groups and to sialic acid.

*Triticum vulgaris* lectin is obtained from Sigma-Aldrich (USA).

Three concentrations of WGA viz., 0.5%, 0.25%, 0.125% were prepared in depolarized water. To make stock solution 2 mg of WGA were dissolved in 2 ml of solvent (depolarized water) to make 1% of the stock solution. From the stock solution, desired concentrations were prepared by serial dilution in acetone.

(IV). APPLICATION OF INSECTICIDES:

**ANDALIN (Flucyloxuron):** 2 μl of each dilution with respective strength of Andalin (Flucyloxuron) was topically applied with the help of micropipette on the dorsal surface of pro and meso-thoracic region on one day old individual nymphs of 5th instar of *Dysdercus koenigii* and one day old individual larvae of 6th instar of *Spodoptera litura*.

*Triticum vulgaris* lectin: 2 μl of each dilution were applied topically with the help of micropipette on the dorsal surface of pro and meso-thoracic regions on one day old...
individual nymphs of 5\textsuperscript{th} instar of *Dysdercus koenigii* and one day old individual larvae of 6\textsuperscript{th} instar of *Spodoptera litura*.

Similarly, nymphs and larvae were treated with 2 \textmu l acetone solution to serve as parallel control both in case of Andalin (Flucycloxuron) as well as *Triticum vulgaris* lectin (Wheat Germ Agglutinin).

**(V). METHODS FOR ESTIMATING MOULTING, METAMORPHOSIS, FECUNDITY AND HATCHABILITY:**

After applying each dose of the Chitin synthesis inhibitor (Andalin) and Wheat germ agglutinin (*Triticum vulgaris* lectin) topically, nymphal and larval mortality after 24 hours, abnormality regarding moulting and metamorphosis were recorded. Parallel control in acetone both in case of Andalin and *Triticum vulgaris* lectin treated insects of the corresponding instars and age was also maintained for comparision.

The females bugs which were emerged after different dose treatment were paired with untreated males of the corresponding age. Each pair were maintained in controlled condition in separate rearing jars and was also provided with proper food i.e. soaked cotton seeds in case of bugs. The number of eggs laid by each female were recorded and then the eggs were transferred in separate rearing jars having damp sterilized sand at the bottom. Parallel control was also kept consisting males and females emerged from the
control nymphs and larvae. The hatched and unhatched eggs were counted and percent hatchability was recorded.

(VI). METHOD FOR ANATOMICAL PREPARATION OF AN OVARY:

The ovaries were dissected out from treated female bugs of required age. Each pair of ovaries were then dehydrated in alcohol series 30%, 50%, 70% for 5 min. Ovaries were then stained in eosine (5 minutes) followed by 80% and 90% alcohol for 5 min each and finally in 100% alcohol for 15 min. They are then kept in xylene for less than 1 min and transferred to glass slide for mounting in DPX. Parallel control ovary slide was also prepared for comparison.

(VII). METHOD FOR HISTOLOGICAL PREPARATION OF OVARIOLE FOR LIGHT MICROSCOPY:

From the ovaries of the treated bugs the ovarioles were dissected out and fixed in Bouin’s solution for 14-16 hours, washed with distilled water and dehydrated in alcohol series 30%, 50%, 70%, 80%, 90% for half an hour each and in 100% for an hour, followed by 100% alcohol and xylene solution (1:1) for 15 mints. Incubation was done at 60°C in xylene and paraffin wax (1:1), and then in paraffin wax only for 30 min. An ovariole is then embedded in paraffin wax whose 5 μm microtome sections were cut into a rolling ribbon. The ribbon was placed on the glass slide which was lubricated by glycerine and
albumin solution (1:1). The slides containing section were warmed slightly to straightened the creases, they were possessed in Xylene (2 changes) for 30 min each, followed by an alcohol series of 100% (2 changes), 90%, 70%, 50% and 30% for 5 min each. The slides were then stained in Ehlich's haematoxylin for 2-3 min and rinsed with tap water. Again the slides were stained with Eosine for 10 minutes, rinsed with water and started with an upgrade dehydration alcohol series 30%, 50%, 70%, 90% for 5 min each. The slides were then kept in 100% alcohol (2 changes) for 10 min, followed by Xylene (2 changes) for 10 min each, finally the slides were mounted with DPX and observed under compound light microscope. The histological preparation of control ovariole was also done.

(VIII). METHOD FOR HISTOLOGICAL PREPARATION OF OVARIOLE FOR TRANSMISSION ELECTRON MICROSCOPY (TEM):

The ovariole dissected out from control female and each of the treated female were primarily fixed for 2 - 4 hours at 4°C in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH = 7.4). After that the ovariole was kept in phosphate buffer saline (PBS) (pH = 7.4) at 4°C for 2 hours (or overnight). The secondary fixation of the ovariole was done in 1% osmium tetroxide in distilled water for 1 hr at room temperature and then washed 2 x 5 min in distilled water followed by upgrade dehydration in 50%, 70%, 90%, 95%, 100% ethanol (4 changes) for 15 minutes each. The sample is then kept in propylene oxide (2 changes) for 10 min, followed by propylene oxide : resin (1:1 mixture) for 1 - 2 hours.
The sample is infiltrated with a resin before being placed in an embedding mould, which was then polymerised in an oven at 60° C. 0.5 - 1.0 μm sections were cut and the sections are then transferred to a drop of water on the slide using a steel loop, the slides are dried by slide warmer or a lamp and then stained in Toluidine blue for 2-5 min. The sections were observed under microscope for precise location to cut for ultrathin sections. Ultrathin sections are cut at 60-90 nm thick (silver-yellow color) (Ultra-microtome – Model – UC6, Reichet) and the sections are collected onto grids. The sections are dried overnight before staining and finally the grids are stained with uranyl acetate for 15 minutes and lead acetate for 5 minutes and observed under transmission electron microscope (Model – morgagni, 268D, Fei, Netherland, Sophisticated Analytical Instrument Facility for Electron Microscopy, Department of Anatomy, AIIMS, New Delhi, INDIA)

(IX). STATISTICAL ANALYSIS:

The data obtained was statistically analyzed by the application of the following methods and formulae.

Mean: It is obtained by summing up all the observations and dividing it by the total number of observations.

\[
\text{Mean} (\overline{X}) = \left( \frac{\sum X}{N} \right)
\]

where, \( X = \) sum of the observations

\( N = \) number of observations
**Standard Deviation:** is used as a measure of dispersion and defined as “Root mean square deviation from mean”.

\[
\text{Standard deviation (SD)} = \sqrt{\frac{\sum (X - \overline{X})^2}{N}}
\]

where, \(X = \text{value of variables}\)
\[\overline{X} = \text{arithematic mean}\]
\(N = \text{number of observations}\)

**Standard Error:** It is the ratio of standard deviation of the sample divided by the square root of the total number of observations.

\[
\text{Standard error (SE)} = \frac{SD}{\sqrt{N}}
\]

where, \(SD = \text{standard deviation}\)
\(N = \text{number of observations}\)

**Regression:** The tendency to remain towards central position is called regression. In order to draw a relationship, observations of two variables are plotted in the form of dots in a scatter diagram. A straight line is drawn which will approach as close as possible to all these points in the graph.

The main objective of regression analysis is to predict the values of one variable using the known value of the other. The existence of relationship between the independent
variable X and the dependent variable Y can be expressed in a mathematical form known as the regression equation.

**Regression equation of X on Y:**

\[ X = a + b \cdot y \]

**Regression equation of Y on X:**

\[ Y = a + b \cdot x \]

where, \( x \) = value of variable

\( y \) = value of variable

\( a \) = constant

\( b \) = constant

**Test of Significance:** For test of significance the following formula was applied.

\[ t = \frac{M_1 - M_2}{\sqrt{\frac{SD_1^2}{n_1} + \frac{SD_2^2}{n_2}}} \]

where, \( t \) = significant value

\( M_1 \) = mean value of first set of observations

\( M_2 \) = mean value of second set of observations

\( SD_1 \) = SD of first set of observations

\( SD_2 \) = SD of second set of observations

\( n_1 \) = Number of observations of first set

\( n_2 \) = Number of observations of second set

The calculated ‘t’ value was compared with the tabulated ‘t’ value at 1% level. If the former value is higher than the later value, the data is significant otherwise insignificant.

The tabulated value of ‘t’ at 1% level is 4.541.
**Probit analysis:** It is commonly used in toxicology to determine the relative toxicity of chemicals to living organisms. This is done by testing the response of an organism under various concentrations and then comparing the concentrations at which one encounters a response. The response is always binomial (e.g. death/no death) and the relationship between the response and the various concentrations is always sigmoid. Most common outcome of a dose-response experiment in which probit analysis is used is the Lc50 and Lc90.

*All statisticals were performed by using MS-excel and SPSS (17.0 version).*