Andalin, an Insect Growth Regulator, as Reproductive Inhibitor for the Red Cotton Stainer, *Dysdercus koenigii* (F.) (Hemiptera: Pyrrhocoridae)

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**Abstract:** Effects of different concentrations (0.1%, 0.05%, 0.025%, 0.01%, 0.005% and 0.0025%) of Andalin (Flucycloxuron), an insect growth regulator, were observed on the reproductive system of the red cotton stainer, *Dysdercus koenigii* (Fabricius). At higher concentrations the fecundity of the emerged females of the treated nymphs was found to be nil whereas at lower concentrations reduction in the fecundity was statistically significant (P<0.01). Comparative anatomical and histological studies of the treated and untreated ovarioles revealed that there was inhibition in the development of ovarioles. The untreated *D. koenigii* showed normal meroistic-telotrophic ovarioles with large amount of yolk in the oocytes localized in the vitellarium portion. However andalin affected ovarioles showed disrupted structures of the germarium and vitellarium with either complete or partial damage of few or all oocytes. Ultrastructure, under transmission electron microscope, of the untreated ovarioles revealed accumulation of large amount of yolk in the oocytes whereas in case of Andalin treated ovarioles, the yolk was markedly reduced and acquired several vacuoles indicating the resorption of the yolk or reduction in its synthesis. The results demonstrated that Andalin causes rapid cessation of oviposition due to disruption of ovarian structure and inhibition of oocyte growth following topical treatment on 5th instar nymphs of *D. koenigii*.

**Key words:** Andalin · Flucycloxuron · *D. koenigii* · Ovarioles · Histology

**INTRODUCTION**

The red cotton bug, *Dysdercus koenigii* (Fabricius) is an important pest of cotton and okra. Although synthetic chemical pesticides can control it, the side effects are enormous. The use of conventional pesticides such as organochlorines, organophosphates and carbamates, has resulted in multifarious hazard problems like toxicity to humans, pollution, bioaccumulation, poisoning and teratogenic effects of residues and the development of pesticide resistance. In view of this, less hazardous options such as use of resistant cultivars, behaviour modifiers and insect growth regulators have gained prominence in agricultural pest management protocols [1]. The non-conventional insecticides such as plant-products, growth regulators, semio-chemicals and live pathogens can possibly cut down the use of pesticides. They are effective in small quantities, persistent, broadly toxic to pest insects and require little labor to apply them. After Wigglesworth’s pioneering studies performed during 1933-1940 on the control of insect growth by endocrine organs, Williams suggested that in addition to their theoretical interest, juvenile hormones could be accurately identified, then synthesized and used as insecticides [2]. Since then several commercial compounds called insect growth regulators or IGRs, have been used in insect pest control. The mode of action of IGRs is quite original since they are not stomach poisons and they do not exhibit neural-toxicity but they disrupt moulting process or cuticle formation. Because of environmental concerns related to the use of conventional insecticides, agrochemical research has focused on the discovery of more selective compounds which interfere with pest insect growth and development [3, 4].

The IGR are metabolic disruptors, molt inhibitors and behaviors modifiers of insects. Since the target site of action for these chemicals is specific and are known to disrupt only in certain species at certain times during the life cycle, these materials are thought to have fewer deleterious effects on the environment and also on non-target organisms [5]. IGRs may belong to selective insecticides and can be grouped according to their mode

of action as: chitin synthesis inhibitors and substances that interfere with the action of insect hormone (i.e. JHa, ecdysteroids) [6].

The mode of action of chitin synthesis inhibitors can be summarized as: 1) hormonal inhibition of a-ecdysone-metabolizing enzymes; 2) inhibition of DNA synthesis; 3) active metabolic formation, i.e. the production of an active and extremely potent metabolite of the Benzoylphenyl Urea compound itself; 4) inhibition of lipid-linked oligosaccharide intermediates; 5) inhibition of zymogen activation, in which BPU's may prevent the insect chitin synthetase zymogen; and 6) effects on regulatory mechanisms associated with the polymerization step in chitin synthesis [7].

Diflubenzuron, a benzoylphenlyurea was the first chitin synthesis inhibitor to be introduced as a novel insecticides. Diflubenzuron has been considered a potent compound against lepidopterous larvae of common cutworm, Spodoptera litura and Cydia pomonella [8].

The proposed study is aimed to evaluate the effectiveness of an insect growth regulator andalin (Flucycloxuron) as a reproductive inhibitor against D. koenigii by the toxicity caused during ovarian development with the help of histological studies both by light and transmission electron microscopy.

MATERIALS AND METHODS

Breeding and Maintenance of 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 containing a thick layer of damp sterilized sand at the bottom and the jars were placed in Environmental Chamber (REMI’s make) maintained at 28±1°C temperature and 70-80% relative humidity. The insects 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 development of an insect.

Sampling of Experimental Insects: In the present work 4th instar nymph of D. koenigii were sorted out and maintained in separate jars. They moulted to 5th instar nymphs after 2-3 days and the moulted 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.

2 µl of each concentrations i.e. 0.1%, 0.05%, 0.025%, 0.01%, 0.005% and 0.0025% with respective strength of Andalin (Flucycloxuron) 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 nymphs of D. koenigii.

Anatomical Preparation of an Ovary: The ovaries were dissected out from treated female bugs of required age. Each pair of ovaries was then dehydrated in alcohol series 30%, 50%, 70% for 5 min. Ovaries were then stained in eosin followed by 80% and 90% alcohol for 5 min each and finally in 100% alcohol for 15 min. They were then kept in xylene for less than 1 min and transferred to glass slide for mounting in DPX. For comparison, the slides of ovaries of untreated/solvent treated females were also prepared.

Histological Preparation of Ovariole for Light Microscopy: The ovarioles were fixed in Bouin’s solution for 14-16 hrs, transferred to 70% alcohol and dehydrated in alcohol series 70%, 80%, 90% for half an hr each and in xylene and paraffin wax (1:1) and then in paraffin wax only for 30 min. An ovariole was 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 straighten the creases, they were processed in xylene (2 changes) for 30 min each, followed by an alcohol series 30%, 50%, 70%, 90% for 5 min each and in 100% for an hr, followed by 100% alcohol and xylene solution (1:1) for 15 min. Incubation was done at 60°C in xylene and paraffin wax (1:1) and then in paraffin wax only for 30 min. An ovariole was then stained in paraffin wax whose 5 µm microtome sections were cut into a rolling ribbon.

The slides were then stained in Ehrlich’s haematoxylin for 2-3 min and rinsed with tap water. Again the slides were stained with eosin for 10 min, 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.
Histological Preparation of Ovarioles for Transmission Electron Microscopy: The ovariole were primarily fixed for 2-4 hrs 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 hrs (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 for 5 min in distilled water followed by upgrade dehydration in 50%, 70%, 90%, 95%, 100% ethanol (4 changes) for 15 min each. The sample was then kept in propylene oxide (2 changes) for 10 min, followed by propylene oxide:resin (1:1 mixture) for 1-2 hrs. 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 were then transferred to a drop of water on the slide using a steel loop, the slides were dried by slide warmer 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 were at 60-90 nm thick (silver-yellow color) (Ultra-microtome-Model-UC6, Reichet) and the sections were collected onto grids. The sections were dried overnight before staining and finally the grids were stained with uranyl acetate for 15 min and lead acetate for 1-2 hrs. 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 were then transferred to a drop of water on the slide using a steel loop, the slides were dried by slide warmer 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 were at 60-90 nm thick (silver-yellow color) (Ultra-microtome-Model-UC6, Reichet) and the sections were collected onto grids. The sections were dried overnight before staining and finally the grids were stained with uranyl acetate for 15 min and lead acetate for 5 min and observed under transmission electron microscope (Model-Morgagni, 268D, Fei, Netherland).

Statistics: The fecundity results are presented as Mean±SE. The significance difference was estimated using student t-test at 1% level.

RESULTS

Effects on Fecundity and Hatchability: At 0.1% Andalin concentration only 4% adult emerged which possessed malformed wings and legs and died within 24 hours of emergence that is why no mating occurred and hence no fecundity. In case with 0.05% and 0.025% Andalin treatment, normal adult emergence was only 30% and 47.37% respectively. In the former dose (0.05%) the apparently normal adults thus formed could not survive more than 24-48 hrs and during this period no mating occurred and ultimately no oviposition. Whereas, in the later concentration (0.025%) though normal adults mated but they failed to lay eggs and died within 4-5 days. Therefore, no fecundity was recorded in the females emerged from 5th instar nymphs treated with 0.1%, 0.05% and 0.025% Andalin. At lower concentrations of Andalin i.e. 0.01%, 0.005% and 0.0025%, an average of 27.75±1.25, 38.74±0.85 and 47.75±1.70 number of eggs were laid as compared to control and untreated females which laid an average of 132.25±0.85 and 144.25±1.49 eggs respectively (Table 1). The reduction in the fecundity was statistically significant at 0.01% (t=8.2929, P<0.01), 0.005% (t= 10.4641, P<0.01) & 0.0025% Andalin (t=8.1319, P<0.01). The average number of eggs laid by the females emerged from 0.01% Andalin treated 5th instar nymphs dropped to more than quarter (approximately) of the control and about one-fifth of the untreated. The females emerged from the lowest concentration (0.0025%) of Andalin treated 5th instar nymphs also showed more than half and one-third decrement in the average number of eggs laid as compared to controlled and untreated fecundity respectively.

Effects of Andalin on Female Reproductive System and Development of Ovarioles of D. Koenigii: The reproductive system of D. koenigii consists of a pair of ovaries. Each ovary is composed of seven separate egg-tubes or ovarioles (Ov) which open into lateral oviduct (LO). The lateral oviduct of each side join to form median oviduct (MO) which open posteriorly into a genital chamber. The genital chamber develops to form bursa copulatrix for reception of the male genitalia. A single ovariole is divided into 4 distinct region (i) Terminal filament (TF): which is a thread-like apical prolongation of the peritoneal layer (ii) Germarium (Gr): forms the apex of an ovariole, below the terminal filament and housing anteriorly the nurse cells and posteriorly the young oocytes. (iii) Vitellarium (Vt): constitutes the major portion of an ovariole and composed of a series of oocytes in their follicular sheaths, which become progressively large towards the posterior end and (iv) Pedicel (Pd): in which the mature eggs are lodged before passing into the lateral oviduct (Fig 1 a & b).

The ovarioles of the female emerged from different concentrations (0.01%, 0.005% and 0.0025%) of Andalin treated 5th instar nymphs of D. koenigii showed several

Table 1: Fecundity and fertility of females Dysdercus koenigii, emerged from treated 5th instar nymphs with Andalin.

<table>
<thead>
<tr>
<th>Concentrations (%)</th>
<th>Number of eggs laid (Mean±SE)</th>
<th>Number of eggs hatched (Mean±SE)</th>
<th>% Hatching</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>0.05</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>0.025</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>0.01</td>
<td>27.75±1.25</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>0.005</td>
<td>38.75±0.85</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>0.0025</td>
<td>47.75±1.70</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>Control (Acetone)</td>
<td>132.25±0.85</td>
<td>127.50±2.50</td>
<td>96.41</td>
</tr>
<tr>
<td>Untreated</td>
<td>144.25±1.49</td>
<td>141.50±1.26</td>
<td>98.09</td>
</tr>
</tbody>
</table>
disrupted structures. The germarium and vitellarium portion showed either complete or partial damage with resorption of few or all oocytes. (Fig 2, 3 & 4).

The histological longitudinal section of an ovariole of normal D. koenigii showed meristic-telotrophic ovariole which is surrounded by an epithelial sheath (ES) (Fig 5a) and an inner thin elastic membrane called tunica propria which covers whole of the ovarioles and the terminal filament. Meristic-telotrophic ovarioles are characterized by the presence of trophic tissue as well as oogonia and oocytes in the distal germarium and each oocyte is connected to the germarium by a cytoplasmic nutritive cord which extends to the trophic core (TC) (Fig 5a). Proximal to the trophic tissue (germarium) is the vitellarium which consists of the oocytes (Oc) and the prefollicular tissues (PFT). The normal germarium of an ovariole of D. koenigii contains prefollicular tissue and the stem line oogonium. The oogonium develops into an oocyte and as the oocytes passes down the ovariole they enlarge. The oocyte (Oc) leaving the germarium clothed by the prefollicular tissue which forms the follicular epithelium (FE) (Fig 5 b). Oocyte growth continues and the follicular epithelium keeps pace by cell division and the cells become cuboid or columnar. During previtellogenesis, the nutritive cord breaks and the follicle cells (FC) form a complete layer around the oocyte. The cytoplasm of the oocytes contain few microtubules, small Golgi complexes and profuse network of endoplasmic reticulum and many free ribosomes. Finally, as the process of vitellogenesis (vitellarium) proceeds, deposition of yolk in the oocytes occurs, (Fig 5c) in the more proximal parts of the ovariole which results in increase in size of the oocytes and then the follicle cells become stretched over the oocyte as a flattened, squamous epithelium. Numerous yolk spheres (YS), lipid droplets and vacuoles are present in the mature oocytes of the normal ovariole of D. koenigii (Fig 1).

The ovarioles of females of D. koenigii emerged from treated 5th instar nymphs with varying concentrations of Andalin (0.01%, 0.005% and 0.0025%), showed alteration in structure of the ovariole (Fig 6, 7 & 8). The epithelium sheath (ES) of an ovariole was found to be disrupted, (Fig 6a, 7a & 8a) and was almost reduced to a thin membrane without any distinct cells. The epithelium lost its cytological organization and intercellular spaces can be seen in the follicular epithelium. Occasional breakage of the follicular epithelium (FE) was observed in the longitudinal section of the ovariole of female adults of treated 5th instars nymphs, (Fig 6a, 7a & 8a). Also the uniform deposition of the yolk in the oocytes was not
Fig. 4: Shows ovarioles of the female emerged from 0.0025% Andalin treated 5th instar nymphs of *D. koenigii*

Fig. 5: Shows normal L.S. of (a) ovariole of *D. koenigii* at 50X, oocyte at (b) 100X & (c) 400X

Fig. 6: Shows L.S. of ovariole of the female emerged from 0.01% Andalin treated 5th instar nymphs of *D. koenigii* at (a) 100X & (b) 400X
Fig. 7: Shows L.S. of ovariole of the female emerged from 0.005% Andalin treated 5th instar nymphs of *D. koenigii* at (a) 100X & (b) 400X

Fig. 8: Shows L.S. of ovariole of the female emerged from 0.0025% Andalin treated 5th instar nymphs of *D. koenigii* at (a) 100X & (b) 400X

Fig. 9: Shows electron micrograph of oocyte of an ovariole of normal female of *D. koenigii* at 1.5 kX
seen and clumping of the yolk spheres (YS) was observed as compared to normal (control) ovarioles. The yolk was markedly shrunk and acquired several vacuoles indicating the resopion of the yolk or the reduction in its synthesis and deposition due to Andalin treatment (Fig 6b, 7b & 8b). Ultrastructure under transmission electron microscope (Fig 9) of the control ovariole revealed accumulation of large amount of yolk in the oocytes of a meroistic-telotrophic ovariole of D. koenigii. The yolk was in the form of small and large spheres uniformly distributed in the oocytes. Also the yolk spheres were densely packed in the oocytes of an ovariole. Whereas in case of ovarioles of the female of treated nymphs with different concentrations (0.01% and 0.0025%) of Andalin, the disruption of the yolk in the oocytes was seen. Moreover, there were apparent reduction in the amount of the yolk and even the mature oocytes showed the formation of prominent gaps or vacuoles like structure (Fig 10 & 11).

**DISCUSSION**

The deleterious effects of conventional insecticides such as organochlorines, organophosphates, carbamates etc. on both health and environment has led to alternative control measures by the use of non-conventional insecticides. The toxicity of insecticides to humans and wildlife resulted in much public concern and prompted the use of more target-specific chemicals [12]. Insect growth regulators are bio-rational compounds that are species-specific and highly selective in action [13] and act by disrupting the normal development of several species of insects [14]. Thus, it becomes inevitable to find out the most effective chemicals against particular species of insect pest which do not adversely affect the environment and are also bio-degradable.

In the present investigation, three concentrations (0.01%, 0.005% and 0.0025%) of Andalin (Flucycloxuron) were tested topically on the 5th instar nymphs of D. koenigii which emerged as apparent normal adults but showed poor development of ovaries which reduced fecundity and inhibition in egg-hatching. Histological studies of the ovarioles of the females emerged from the treated nymphs showed inhibition in development of female reproductive system. There was disintegration of the follicular epithelium and poor deposition of the yolk in the majority of oocytes of the ovarioles of females emerged from the treated 5th instar nymphs. Several past studies have reported gonadal inhibition after application of a variety of insect control agents viz. chemosterilant, IGRs, botanicals and insecticides. A chitin synthesis inhibitor, benzoylphenyl urea, diflubenzuron (DFB), was found to retard ovarian development of grasshopper, Oxya japonica causing an increase in the percentage of terminal oocyte resorption [15], DFB also disturbed growth and development of oocytes in mealworms, Tenebrio molitor, it was suggested that diflubenzuron interfered with the vitellogenesis via biochemical processes [16]. DFB action on ovarian development and hemolymphatic edysteroid levels were also evaluated in females of Schistocerca gregaria [17]. Chlorfluazuron also showed inhibition in the ovarian development and oogenesis in common cutworm Spodoptera litura at sublethal doses [18]. The reduction in oocytes number,
the ovaries weight, the size and the volume of the basal oocyte during sexual maturation, after treatment with Flucycloxuron were also observed [19]. These morphogenetic abnormalities in the reproductive system where reduction in the number of oocytes, inhibition of vitellogenesis and deformation of germarium due to effect of benzyolphenyl urea in the previous work correlated with the present work where some alterations resulting disruption of the oocytes by Andalin treated *D. koenigii* were observed.

The presence of vacuoles within oocytes and shrinkage or degeneration of yolk in the gonad tissue may be attributed to an increase in the osmotic pressure of the plasma membrane of the oocytes which may lead to water loss or dehydration that cause the occurrence of dominant vacuoles [20]. Reproductive inhibitory effects of bisfluron were evaluated against lace bug, *Corythucha ciliate* [21]. Sterility and retardation of oocyte growth by penfluron was reported in soapnut bug, *Leptocoris coimbatorensis* (Gross) [22], who suggested that during early vitellogenic stages disorganisation of oocytes started and ovarioles became smaller than control ovarioles and by late vitellogenic stages the oocytes got resorbed in all ovarioles. The present findings regarding the inhibition of vitellogenesis in oocytes by Andalin treatment on *D. koenigii* are in agreement with the above mentioned studies. Hence, it is concluded that the present chemical andalin produced adverse gonadotropic effects which included cessation of ovisposition due to disruption of ovarian structures and inhibition of oocyte growth by topical treatment on 5th instar nymphs of *D. koenigii*. Thus andalin can be recommended for use as an effective reproductive inhibitor or insect growth regulator in IPM modules against the pest insects.

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**REFERENCES**