Impact of the Stem Extract of Thevetia neriifolia on the Feeding Potential and Histological Architecture of the Midgut Epithelial Tissue of Early Fourth Instars of Helicoverpa armigera HÜbner

Authors: Mishra, Monika, Gupta, Kamal Kumar, and Kumar, Sarita

Source: International Journal of Insect Science, 7(1)

Published By: SAGE Publishing

URL: https://doi.org/10.1177/IJIS.S29127

Impact of the Stem Extract of *Thevetia neriifolia* on the Feeding Potential and Histological Architecture of the Midgut Epithelial Tissue of Early Fourth Instars of *Helicoverpa armigera* Hübner

Monika Mishra[1], Kamal Kumar Gupta[2] and Sarita Kumar[1]

[1]Department of Zoology, Acharya Narendra Dev College, University of Delhi, Kalkaji, New Delhi, India. [2]Department of Zoology, Deshbandhu College, University of Delhi, Kalkaji, New Delhi, India.

ABSTRACT: *Helicoverpa armigera* Hübner is one of the most important agricultural crop pests in the world causing heavy crop yield losses. The continued and indiscriminate use of synthetic insecticides in agriculture for their control has received wide public apprehension because of multifarious problems, including insecticide resistance, resurgence of pest species, environmental pollution, and toxic hazards to humans and nontarget organisms. These problems have necessitated the need to explore and develop alternative strategies using eco-friendly and biodegradable plant products. In view of this, the efficacy of *Thevetia neriifolia* methanol stem extract was evaluated against the early fourth instars of *H. armigera* as an antifeedant and stomach poison agent. Feeding of larvae with the diet containing 0.005%–5.0% extract resulted in 2.06%–37.35% antifeedant index; the diet with 5.0% extract caused 54.3% reduced consumption. The negative impact of extract on larval feeding resulted in 37.5%–77.7% starvation, causing adverse effects on the larval weight. Choice between control and experimental diet resulted in feeding preference of larvae for the control diet, leading to 7.3%–42.9% reduced consumption of extract-containing diet. The only exception was the diet with 0.005% extract, which could not cause any deterrence. The midgut histological architecture of *H. armigera* larvae fed with 0.005%–0.05% extract-containing diet with negligible antifeedant potential showed significant damage, shrinkage, and distortion and vacuolization of gut tissues and peritrophic membrane, causing the disintegration of epithelial, goblet, and regenerative cells; the damage increased with the increase in concentration. These changes in the gut caused negative impact on the digestion and absorption of food and thus nutritional deficiency in the larvae, which could probably affect their growth and development. This study reveal the appreciable stomach poison potential of *T. neriifolia* stem methanol extract against *H. armigera* larvae, which can be explored as an eco-friendly pest control strategy.

KEYWORDS: *Thevetia neriifolia, Helicoverpa armigera*, antifeedant, starvation, histopathology, midgut epithelium

CITATION: Mishra et al. Impact of the Stem Extract of *Thevetia neriifolia* on the Feeding Potential and Histological Architecture of the Midgut Epithelial Tissue of Early Fourth Instars of *Helicoverpa armigera* Hübner. *International Journal of Insect Science* 2015:7 53–60 doi:10.4137/IJIS.S29127.

TYPE: Original Research

RECEIVED: May 1, 2015. **RESUBMITTED:** September 7, 2015. **ACCEPTED FOR PUBLICATION:** September 7, 2015.

ACADEMIC EDITOR: Paul-Andre Calatayud, editorial board member

PEER REVIEW: Five peer reviewers contributed to the peer review report. Reviewers' reports totaled 2,129 words, excluding any confidential comments to the academic editor.

FUNDING: Funding was received through a research fellowship from the Council of Scientific and Industrial Research, New Delhi. The authors confirm that the funder had no influence over the study design, content of the article, or selection of this journal.

COMPETING INTERESTS: Authors disclose no potential conflicts of interest.

CORRESPONDENCE: saritakumar@andc.du.ac.in

Paper subject to independent expert blind peer review. All editorial decisions made by independent academic editor. Upon submission manuscript was subject to anti-plagiarism scanning. Prior to publication all authors have given signed confirmation of agreement to article publication and compliance with all applicable ethical and legal requirements, including the accuracy of author and contributor information, disclosure of competing interests and funding sources, compliance with ethical requirements relating to human and animal study participants, and compliance with any copyright requirements of third parties. This journal is a member of the Committee on Publication Ethics (COPE).

Published by Libertas Academica. Learn more about this journal.

Introduction

Helicoverpa armigera Hübner (Lepidoptera: Noctuidae), cotton bollworm, is one of the most important agricultural crop pests in the world and inhabits diverse ecological habitats, leading to heavy yield losses in a diverse range of crops. It is also widely distributed in India and attacks varied plant species, leading to substantial losses.[1] In the last few decades, the repeated and indiscriminate use of synthetic chemicals in the crop field of India has led to the resurgence and outbreak of bollworm with resistance to insecticides, elimination of existing natural enemies and environmental pollution. Reports are available that reveal the development of resistance in the pest to almost all the insecticides used for its control.[2] Environmental hazards posed by synthetic pesticides provide an impetus for investigations into more plant-based alternatives.[3]

Plants have been widely known to be gifted with the potential to produce an extensive range of phytochemicals, which protect them from phytophagous pests. Plant-derived phytochemicals have been used in the management of agricultural pests, though their production varies from plant to plant.[4] The selective though variable toxic potential of these chemicals against target pests, rapid degradability in the field, and low toxicity to beneficial organisms have made them appealing options for agricultural use. Certain parameters, such as age, location within, and response to the pest attack, can influence the production of phytochemicals and thus affect the insect feeding and oviposition on the plants.[5] Various plants, such as *Tephrosia vogelii* (family: Fabaceae) and *Solanum pseudocapsicum* (family: Solanaceae), have been reported to be efficient in the management of *H. armigera*.[6,7] The efficacy of certain plant extracts, such as larvicides,

ovicides, antifeedant, and growth regulators against *H. armigera*, has supported their use in the field.[8–10] The antifeedant activity of leaf aqueous extracts of 25 medicinal plants has been revealed against VI instars of *H. armigera* (Hübner).[11] The aqueous extracts of *Gnidia glauca* and *Toddalia asiatica* have also been evaluated for their antifeedant activity against *H. armigera*.[12] Abbaszadeh et al[13] reported that the extracts of Indian bhant tree, *Clerodendron infortunatum* L., could be used by the local farmers to control *H. armigera* populations due to the powerful antifeedant activity of clerodane diterpenoids present in the extracts. In addition to the impact of extracts on the feeding potential of *H. armigera*, it has been reported that feeding *H. armigera* larvae with plant extract-treated diet can cause alteration in the midgut tissues, suggesting their actions as stomach poisons.[14] Similar effects have been reported in other insects, such as *Spodoptera frugiperda*, fall armyworm, and *Anticarsia gemmatalis*, velvetbean caterpillar, when their immature stages were fed with diets treated with plant extracts or isolated phytochemicals.[15,16] The results of such feeding studies suggest that as the midgut region of insects is the principal site for the secretion of digestive enzymes, digestion of food, and absorption of nutrients, alteration in its morphology and histology can lead to considerable physiological changes affecting the growth and development of the insect pest.[17–19]

Thevetia neriifolia (family: Apocynaceae), commonly called as yellow oleander plant, is an evergreen tropical shrub or small tree that grows well in warm subtropical regions. The plant has been reported to contain cardiac glycosides and cardenolides, while its seed oil has been used to make paints with antifungal and antibacterial properties.[20] A number of phytochemicals, specifically cardiac glycosides, have been isolated from *T. neriifolia* with digitoxin and found to be the most potent.[21] Though the plant has been reported as toxic against rats,[22] the efficacy of the plant has not been explored much against insect pests. As *T. neriifolia* is commonly found in India, the objective of this study is to evaluate the effects of methanol extract, prepared from the stems of *T. neriifolia*, on the feeding behavior and the gut histology of *H. armigera*.

Materials and Methods

Rearing and maintenance of *H. armigera* under controlled conditions. The colony of *H. armigera* was maintained in the laboratory under the controlled conditions of 27 ± 1°C and 80 ± 5% relative humidity (RH) with a 12-hour:12-hour light/dark photoregime. Larvae of *H. armigera* were reared on artificial diet prepared using chickpea flour, casein, and yeast as the main components and certain necessary nutrients in traces and agar as the solidifying agents.[23]

The neonates of *H. armigera* were reared in groups for about seven days till they develop into third instars. Thereafter, to prevent cannibalism, individual larva was transferred to separate clean and sterilized Petri dishes (7 cm diameter) for further rearing. Pupae were sterilized with 0.5% sodium hypochlorite solution, washed with distilled water, and kept inside the plastic cages (15 × 15 cm) for adult emergence. Adults were fed on 10% sucrose solution that was kept in small vials in the cages. After a period of two days, 10 pairs of moths were selected, and each pair was released in a separate oviposition jar (2000 mL) covered with clean muslin cloth on the top for oviposition. The egg-laden cloth was replaced each day with the fresh cloth till the oviposition continued. The eggs collected were then allowed to hatch in a tight-lid box having tiny holes on the lid's surface.

Plant collection and preparation of extracts. The stems of the *T. neriifolia* plant were collected from the premises of Acharya Narendra Dev College, New Delhi, India. Healthy and disease-free stems were separated for assays and carefully washed with water. After drying under the shade at 27 ± 2°C for ~20 days, the stems were crushed, finely powdered, and sieved (150 µm) thoroughly. The fine powder of the stems thus obtained was weighed, and 200 g was extracted with 1000 mL of methanol for 24 hours at a temperature not exceeding the boiling point of the solvent (64.7°C) using Soxhlet apparatus. The crude extract (7.21 g) produced was concentrated using a Buchi-type rotator vacuum evaporator at 45°C under low pressure and stored in a refrigerator at 4°C as the stock solution for further use.

Preparation of extract-containing artificial diet. The artificial diet containing *T. neriifolia* stem methanol extract was prepared by replacing 1 mL of the 200 mL dry diet with that of the desired concentration of the extract. The diet was left undisturbed for the evaporation of solvent after which it was solidified using agar. The diet prepared by replacing 1 mL of the 200 mL with only solvent instead of the stem extract served as the negative control.

Antifeedant (no-choice) bioassay against early fourth instars of *H. armigera*. The antifeedant potential of *T. neriifolia* stem methanol extract was evaluated against the early IV instars of *H. armigera*, the early fourth instar of *H. armigera* being the most actively feeding and thus the most damaging stage. The larvae were provided with 1 g of the diet containing extracts at 0.005%, 0.01%, 0.05%, 0.1%, 0.5%, 1.0%, and 5.0% concentrations separately. The diet containing only solvent, instead of extract, served as the control diet. The diet, weighed using Shimadzu-BL220H electronic balance, was kept in the Petri plates lined with moist filter paper to avoid drying of the diet. For each concentration, 10 Petri plates were prepared.

A total of 10 IV instar larvae were selected, starved for four hours, and weighed. Individual larva was released at the center of separate Petri plates and kept in biochemical oxygen demand incubator under the controlled conditions of rearing. After a feeding period of 24 hours, the weight of the diet left in the Petri plates was measured. A total of five replicates were carried out for each assay, making a total of 50 larvae investigated at each concentration. The antifeedant index was calculated using the following formula[24]:

$$\text{Antifeedant index} = \frac{C - T}{C + T} \times 100$$

where C represents the weight of control diet (0.0% extract) consumed and T value indicates the weight of the extract-containing diet consumed by the early fourth instars. The results of % consumption of control diet and extract-containing diet were analyzed by one-way analysis of variance (ANOVA), and means were separated using Tukey's test for statistical significance considered for $P \leq 0.05$.

Food preference (choice) bioassay against early fourth instars of *H. armigera*. The food preference assay was also carried out against early fourth instars of *H. armigera* by providing them a feeding choice between control diet and a diet containing stem methanol extract of *T. neriifolia*. Each Petri plate was divided into two halves: one half with 1 g of control diet and the other half with 1 g of experimental diet.

Individual early fourth instars of *H. armigera* was released in the center of each Petri plate and allowed to feed for 24 hours. The side of diet was changed after every six hours to negate positional error. After 24 hours, weight of each larva and the diets remaining in the Petri dish were recorded for all the dishes. For each assay, five replicates, each with 10 larvae, were carried out. Percent consumption of each diet was calculated as follows[25]:

$$\text{\% consumption of control diet} = \frac{\text{Amount of control diet consumed}}{\text{Consumed amount of control diet + extract-containing diet}} \times 100$$

$$\text{\% consumption of extract – containing diet} = \frac{\text{Amount of extract – containing diet consumed}}{\text{Consumed amount of control diet + extract-containing diet}} \times 100$$

The preference or choice index was estimated by dividing % consumption of experimental diet by % consumption of control food. The values >1 indicate the preference for extract-containing diet, whereas the values <1 indicate the preference for control diet. The antifeedant index was estimated as described in the choice assay.

The % diet consumption in control at each concentration was analyzed by one-way ANOVA, and means were separated using Tukey's test for statistical significance considered for $P \leq 0.05$.

Starvation index assay. A total of 50 larvae of IV instars of *H. armigera* were selected and weighed. The larvae were starved for 24 hours and reweighed. The weights of these larvae were compared with the initial and final weights of each larva during no-choice bioassay carried out at 0.005%, 0.01%, 0.05%, 0.1%, 0.5%, 1.0%, and 5.0%. Percent starvation for each assay was calculated according to the following formula:

$$\text{Percent starvation} = \frac{C - E}{C - S} \times 100$$

where E is the mean weight gain of larvae fed on experimental diet at each assayed concentration within 24 hours, C represents the mean weight gain of larvae fed on control diet within 24 hours, and S is the mean weight gain of starved control larvae within 24 hours.

Impact of the extract-treated diet on midgut epithelium. The impact of the *T. neriifolia* stem methanol extract as stomach poison against IV instars of *H. armigera* was investigated by evaluating the changes in their gut epithelium. The extract concentrations with antifeedant potential, 0.1%, 0.5%, 1.0%, and 5.0%, were rejected for the assay. The evaluation was carried out at 0.005%, 0.008%, 0.01%, 0.02%, and 0.05%. A total of 10 larvae fed with diet containing selected concentration of extract and 10 larvae fed with control diet were selected randomly and sacrificed. The alimentary canal was exposed, and the midgut was separated. After fixation in the Bouin's solution, standard histological techniques were used to cut midgut sections using a microtome. The thickness of the sections was preset between 4 and 6 µm. The sections were carefully transferred on a clean slide and stained with hematoxylin and counterstained with 70% eosin. The sections were observed under light microscope and photographed using a Canon PowerShot SX50 HS digital camera.

Results

The results of the antifeedant potential of the methanol stem extract of *T. neriifolia* investigated against *H. armigera* larvae presented in Tables 1 and 2 reveal the moderate antifeedant efficacy of the extract. The antifeedant assay carried out with diet containing 0.005%–5.0% stem methanol extract of *T. neriifolia* revealed the antifeedant index ranging from 2.06 to 37.35 (Table 1). The diet added with 0.005%, 0.01%, and 0.05% extract did not show significant antifeedant potential ($P > 0.05$), causing only 4.1%–20.1% reduced diet consumption. On the other hand, the larvae exhibited significant reduced consumption of 54.3% ($P < 0.0001$), as compared to the consumption of the control diet, when diet was added with 5% stem methanol extract of *T. neriifolia*.

When the early fourth instars of *H. armigera* were given a feeding choice between control diet and the diet containing *T. neriifolia* stem methanol extract, the larvae exhibited appreciable feeding preference over control diet. The only exception observed was with the diet containing 0.005% extract that could not deter feeding in the larvae ($P > 0.05$). Furthermore, the addition of diet with 0.05% extract caused only 7.3% reduction in diet consumption ($P > 0.05$) as compared to the control diet indicating insignificant antifeedant potential (Table 2). In contrast, the diet containing 0.5%–5.0% *T. neriifolia* stem methanol extract reduced feeding considerably from 10.9% ($P < 0.05$) to 42.9% ($P < 0.0001$). Our results show that at higher concentrations, *T. neriifolia* methanol stem

Table 1. Antifeedant no-choice assay against early IV instars of *Helicoverpa armigera* when fed on 1 g artificial diet containing stem methanol extract of *Thevetia neriifolia*.

CONCENTRATION OF EXTRACT (%)	FINAL WEIGHT OF DIET (g) MEAN ± SE	DIET INGESTED (g) MEAN ± SE	ANTIFEEDENT INDEX
Control	0.578 ± 0.025a	0.421 ± 0.0246a	–
0.005	0.596 ± 0.011a	0.404 ± 0.011a	1.20 ± 0.26a
0.01	0.624 ± 0.002a	0.376 ± 0.002a	2.06 ± 0.89a,b
0.05	0.664 ± 0.042a,b	0.336 ± 0.042a,b	5.64 ± 1.04a,b
0.1	0.666 ± 0.009b	0.334 ± 0.009b	11.37 ± 1.36b
0.5	0.667 ± 0.017b	0.333 ± 0.017b	11.54 ± 2.48b
1.0	0.677 ± 0.010b	0.323 ± 0.010b	11.81 ± 1.15b
5.0	0.808 ± 0.008b	0.192 ± 0.008c	13.17 ± 2.06b

Notes: Mean ± SE, calculated for five replicates, each replicate with 10 larvae. Figures in each column followed by different letters are significantly different ($P < 0.05$, one way ANOVA followed by Tukey's all pair wise multiple comparison test).

extract showed higher antifeedant potential during choice bioassay in comparison to the no-choice bioassay, whereas at lower concentrations, the extract exhibited more antifeedancy during no-choice assays than that in choice assays.

Present studies revealed that the diet containing 0.005% and 0.05% *T. neriifolia* stem methanol extract did not show significant antifeedant potential ($P < 0.05$) in the larvae. Thus, the extracts in the range of 0.005%–0.05% were selected to evaluate their effects on the growth and development of larvae.

When the early fourth instars of *H. armigera* were starved for 24 hours, a weight decrease of 13% was observed, while feeding the larvae with the control diet resulted in 354% increase in their weight. However, when the larval weight was recorded after they were fed with extract-containing diet for 24 hours, a significant reduction in the larval weight, resulting in 37.5%–77.7% starvation, was observed. The larval weight further decreased with increase in the concentration of stem methanol extract in the diet ($P < 0.05$) (Table 3).

The study of the midgut tissues of *H. armigera* larvae fed on control diet and a diet with extracts showed remarkable changes in the epithelium amplifying with the increase in the concentration of the extract. The larvae fed on the control diet exhibited large, elongated, and columnar epithelial cells (ECs) of uniform sizes surrounding the wide gut

Table 2. Food preference (choice) assay against early IV instars of *Helicoverpa armigera* when fed on 1 g artificial diet containing stem methanol extract of *Thevetia neriifolia*.

CONCENTRATION OF EXTRACT (%)	ASSAY	FINAL DIET WEIGHT (g) MEAN ± SE*	DIET INGESTED (g) MEAN ± SE*	% RELATIVE CONSUMPTION	CHOICE/ PREFERENCE INDEX	ANTIFEEDENT INDEX#
0.005	Treated	0.567 ± 0.0454a	0.433 ± 0.0454a	50.92	1.03	–1.80 ± 0.57a
	Control	0.583 ± 0.0407a	0.417 ± 0.0407a	49.05		
0.01	Treated	0.682 ± 0.0312a	0.318 ± 0.0312b	48.25	0.93	3.49 ± 0.92a
	Control	0.659 ± 0.0378a	0.341 ± 0.0378b	51.75		
0.05	Treated	0.809 ± 0.0370c	0.191 ± 0.0370c	46.35	0.86	7.28 ± 1.16a
	Control	0.779 ± 0.0130c	0.221 ± 0.0130c	53.64		
0.1	Treated	0.733 ± 0.0261d	0.267 ± 0.0261d	46.11	0.86	7.77 ± 1.23a,b
	Control	0.688 ± 0.0158d	0.312 ± 0.0158d	53.88		
0.5	Treated	0.821 ± 0.0165e	0.179 ± 0.0165e	44.52	0.80	10.94 ± 1.09b
	Control	0.777 ± 0.0146e_1	0.223 ± 0.0146e_1	55.47		
1.0	Treated	0.703 ± 0.0147f	0.297 ± 0.0147f	38.17	0.62	23.65 ± 2.36c
	Control	0.519 ± 0.0086f_1	0.481 ± 0.0086f_1	61.82		
5.0	Treated	0.909 ± 0.0041g	0.091 ± 0.004g	28.52	0.40	42.94 ± 2.14d
	Control	0.772 ± 0.0166g_1	0.228 ± 0.0166g_1	71.47		

Notes: Mean ± SE, calculated for five replicates, each replicate with 10 larvae. *Means followed by the same letter for a specific concentration regimen are not significantly different ($P < 0.05$, Student's t-test). #Figure in each column followed by different letters are significantly different ($P < 0.05$, one way ANOVA followed by Tukey's all pair wise multiple comparison test).

Table 3. Starvation Index of early IV instars of *Helicoverpa armigera* when fed on 1 g artificial diet containing stem methanol extract of *Thevetia neriifolia*.

CONCENTRATION OF EXTRACT (%)	INITIAL WEIGHT (g/LARVA) MEAN ± SE	FINAL WEIGHT (g/LARVA) MEAN ± SE	DIFFERENCE IN MEAN WEIGHT (g/LARVA)	% CHANGE IN WEIGHT	STARVATION INDEX
Control	0.0625 ± 0.0018a	0.2834 ± 0.0036a	0.2209 ± 0.0036a	(+) 353.52	–
Starved	0.0634 ± 0.0043a	0.0538 ± 0.0041b	0.0096 ± 0.0027b	(–) 13.07	91.67 ± 3.74a
0.005	0.0689 ± 0.0030a	0.2033 ± 0.0090c	0.1344 ± 0.0103c	(+) 194.92	37.52 ± 2.04b
0.01	0.0672 ± 0.0065a	0.1957 ± 0.0088c,d	0.1285 ± 0.0235c,d	(+) 191.22	40.08 ± 5.89b,c
0.05	0.0664 ± 0.0018a	0.1899 ± 0.0107d	0.1234 ± 0.0105d	(+) 185.78	42.29 ± 1.85c
0.1	0.0696 ± 0.0025a	0.1692 ± 0.0064e	0.0933 ± 0.0062e	(+) 134.05	55.35 ± 4.32d
0.5	0.0647 ± 0.0061a	0.1388 ± 0.0112f	0.0741 ± 0.0049f	(+) 114.52	63.68 ± 2.94e
1.0	0.0622 ± 0.0025a	0.1080 ± 0.0035g	0.0458 ± 0.0028g	(+) 73.63	75.96 ± 5.85f
5.0	0.0671 ± 0.0028a	0.1089 ± 0.0023g	0.0418 ± 0.0033g	(+) 62.32	77.70 ± 1.38f

Notes: Mean ± SE, calculated for five replicates, each replicate with 10 larvae. Figures in each column followed by different letters are significantly different ($P < 0.05$, one way ANOVA followed by Tukey's all pair wise multiple comparison test).

lumen (GL) that was lined by peritrophic membrane (PM). Each EC was distinctly nucleated and had smooth and complete base (Fig. 1A and B). The epithelial layer was in close contact with the basement membrane (BM) with negligible space in between (Fig. 1B). Groups of large spongy digestive mucous secreting goblet cells (GCs) were observed at the bases of columnar cells with their inner ends projecting into the GL. Smaller basal cells, regenerative cells (RCs), were found in the form of patches at the bases of ECs (Fig. 1A).

The midgut of the larvae fed on the diet with 0.005% and 0.008% stem methanol extracts of *T. neriifolia* showed slight but distinct shrinkage and distortion of the absorptive ECs coupled with altered shape and structure (Fig. 2A and B). The GCs and RCs were not distinctly visible, but the coherent sheet of epithelial layer was found to be in close contact with the BM. The increased concentration of the extract in the diet resulted in further decreased absorptive surface area and increased GL (Fig. 2C). More severe results were obtained when larvae were fed on the diet with 0.02% extract, ECs showing increased disruption with augmented vacuolization (Fig. 2D). Detachment of the epithelial layer from the BM resulted in the appearance of the large space detached area (DA).

When the larvae of *H. armigera* were fed on the diet added with 0.05% extract, the ECs were nonuniform, shrunken, and strongly vacuolated with complete disappearance of intercellular lines (Fig. 2E). The most conspicuous observation was the complete loss of PM and wide separation and exfoliation of the midgut epithelium from its BM, resulting in its complete destruction (Fig. 2F).

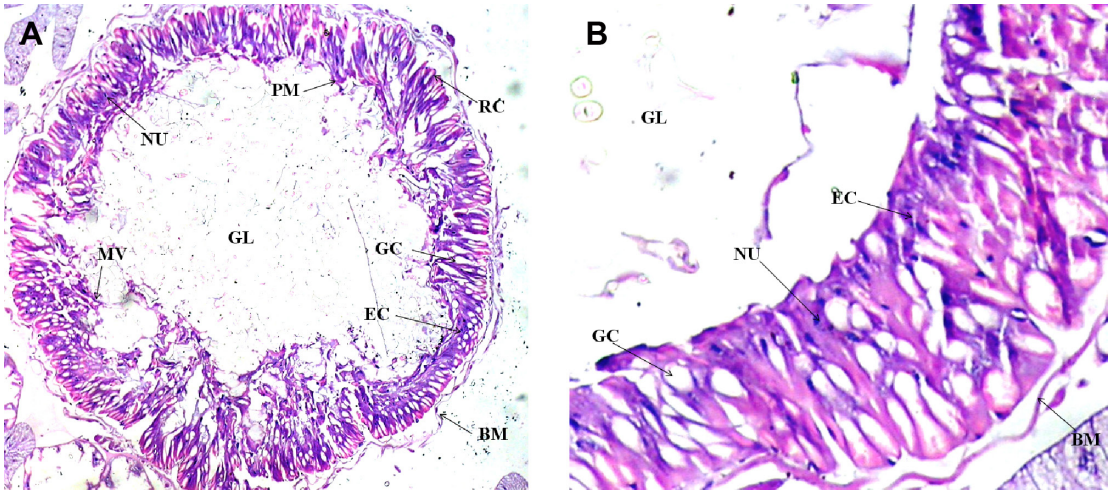

Figure 1. (**A**) T.S. of the midgut of IV instars of *Helicoverpa armigera* fed on control diet (40X); (**B**) Enlarged view of the midgut section.
Abbreviations: BM, Basement membrane; EC, Epithelial cell; GC, Goblet cell; GL, Gut lumen; MV, Microvilli; NU, Nucleus; PM, Peritrophic membrane; RC, Regenerative cell.

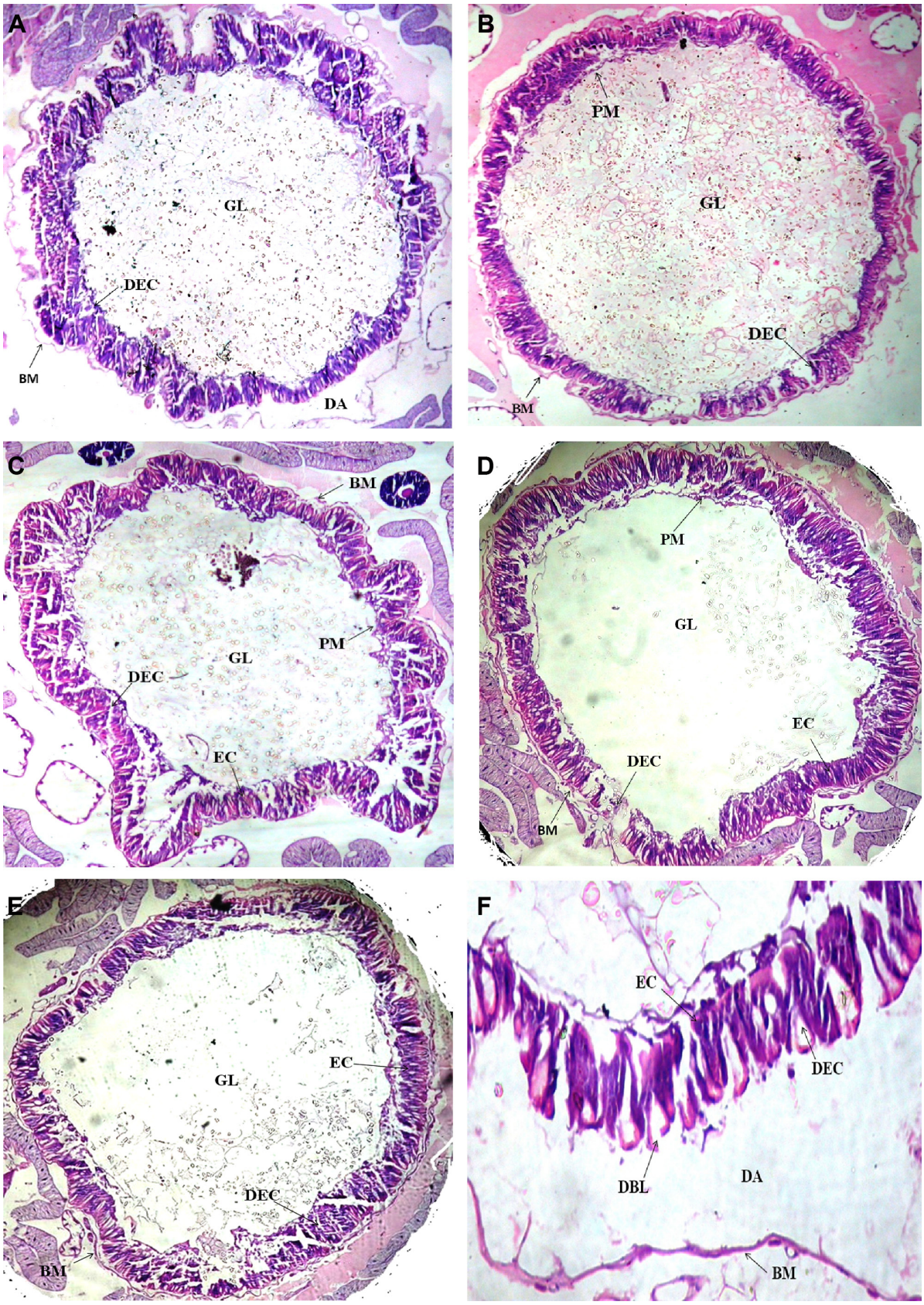

Figure 2. T.S. of the midgut of IV instars of *Helicoverpa armigera* fed on the diet treated with *Thevetia neriifolia* stem methanol extract (40X) at (**A**) 0.005%; (**B**) 0.008%; (**C**) 0.01%; (**D**) 0.02% and (**E**) 0.05%; (**F**) Enlarged view of the midgut section of the larva fed on 0.05% *Thevetia neriifolia* stem methanol extract.

Abbreviations: BM, Basement membrane; DA, Detached area; DBL, Disappeared boundary lines; DEC, Disintegrated epithelial cell; EC, Epithelial cell; GC, Goblet cell; GL, Gut lumen; PM, Peritrophic membrane.

Discussion

Natural products in insect pest management programs are gaining recognition in recent years due to environmental pollution, pest resistance and resurgence, and undesirable effects to the nontarget organisms caused by unsystematic use of synthetic pesticides. Several plant extracts or isolated active compounds have been shown to possess antifeedant,[11] growth-inhibitory,[26] and toxic effects[27] against a number of economically important insects, such as *H. armigera* and *Spodoptera littoralis*.[28,29]

T. neriifolia, commonly called as yellow oleander, has been reported to contain a number of toxic principles in all parts, the kernels being the most toxic in comparison to the other parts.[22] Antifeedant assay carried out with the diet containing 0.005%–5.0% stem methanol extract of *T. neriifolia* revealed the antifeedant index ranging from 2.06 to 37.35. On the other hand, feeding deterrence in *H. armigera* was found to be 100% when fed on 1.0% *Crataegus crenulata* and *Xylosma longifolium*.[30] Nevertheless, aqueous extracts of *Calotropis procera* and *Datura stramonium* have displayed about 90% feeding protection against *H. armigera*,[31] while varied antifeedant potentials of leaf aqueous extracts of 25 medicinal plants have been reported against VI instars of *H. armigera*.[11] The antifeedant activity of acetone, chloroform, ethyl acetate, hexane, and methanol extracts prepared from the peel, leaf, and flowers of *Citrus sinensis*, *Ocimum canum*, *Ocimum sanctum*, and *Rhinacanthus nasutus* has also been confirmed against the fourth instars of *H. armigera*.[32] Likewise, 0.2% and 0.5% petroleum ether extracts of *Parthenium hysterophorus* have been found to exhibit 100% feeding deterrence in *H. armigera*.[33] Our studies on choice bioassays also revealed feeding preference of *H. armigera* larvae for control diet than the diet containing *T. neriifolia* extracts. Nevertheless, the addition of 0.005% *T. neriifolia* extract could not deter larval feeding and the addition of 0.05% extract caused only 7.3% reduced consumption. However, feeding on 5.0% ethyl acetate crude extracts of *Syzygium lineare* leaves resulted in significant antifeedant activity of 79.4% against the fourth instars of *Spodoptera litura*.[34]

Feeding on experimental diet resulted in significant reduction in the weight of *H. armigera* larvae, which decreased drastically with the increase in the concentration of extract. The larvae exhibited 37.5%–77.7% starvation after feeding with 0.005%–0.05% methanol stem extract-containing diet. These results are in agreement with that of Barakat[35] who showed increased percent starvation in the larvae of *S. littoralis* when provided with castor leaf discs treated with increased concentration of acetone extract of *Casimiroa edulis* (Rutaceae) leaves. They observed 3.2% starvation on treatment with 10.4 mg/cm^2 extract, while the highest tested concentration of 332.8 mg/cm^2 extract resulted in 75.2% starvation. Similarly, 354.50–396.66 mg reduced weight in III instars of *H. armigera* was reported when fed with seed extracts of *Pongamia pinnata*.[36]

It is well known that the insects' digestive system is one of the main physiochemical barriers against many toxins and pathogenic agents. Hence, the toxins taken by insects during feeding that overcome these barriers should damage the cellular architecture.[14] The gut is the main organ responsible for digestion, assimilation, and absorption of food; any anomaly in the gut region could affect the growth and development of the insect pest and its survival. Our results showed damaged absorptive ECs, atrophied nuclei, and distorted epithelium of the larvae when fed with extract-containing diet. Similar results of completely dystrophied midgut tissues with shrunken columnar ECs and atrophied nuclei have been reported in *H. armigera* when fed with diet containing ethanolic leaf extracts of *Lantana camara*.[14] The histopathological effects of *Artemisia monosperma* extract have also been revealed on the midgut of *S. littoralis*, resulting in the destruction of midgut epithelium.[37] Comparable effects were reported on the midgut cells of *Schistocerca gregaria* and *Locusta migratoria* when fed with azadirachtin, causing various pathological changes, including slow necrosis, destruction of midgut cells, and damaged circular and longitudinal muscles with separation from BM.[38] The histopathological disturbances in the midgut cells of *S. littoralis* with vacuolization and destruction of nuclear content, degenerated columnar ECs, and their detachment from the BM have been elucidated when fed on castor leaves dipped in crude extracts of *Azadirachta indica* and *Citrullus colocynthis*.[39]

Our studies suggest that the toxic effects of *T. neriifolia* methanol stem extracts on the midgut tissues of *H. armigera* are concentration dependent with the most damage observed at a diet with 0.05% extract and the highest concentration tested. The atrophied RCs, fading of cell boundaries, and degradation of PM indicate the increased entry of plant toxins into the midgut, leading to the damage of ECs. It further suggests that toxins present in the *T. neriifolia* stem methanol extracts had destructed the RCs, which thus could not replace the damaged epithelium. PONNEEM has been reported to cause disintegration of peritoneal membrane in the midgut region of the IV instars of *H. armigera* with the dilution of circular and longitudinal muscles, suggesting the inability of midgut to push the food into the hindgut.[40]

Our studies showed that though the stem methanol extract of *T. neriifolia* exhibited moderate antifeedant potential against the early fourth instars of *H. armigera*, they led to remarkable damage to the larval midgut, thus causing appreciable nutritional deficiency in the larvae that could probably affect their growth and development. This suggests the possible use of *T. neriifolia* stem methanol extracts in the field against *H. armigera* larvae as an eco-friendly product. Further investigations are, however, needed to explore the potential of extract as growth-inhibitory and growth-arresting agent against *H. armigera* in the fields for formulating preparations with enhanced potency and stability.

Acknowledgments

The authors are highly grateful to the Council of Scientific and Industrial Research, New Delhi, for providing research

fellowship to carry out the present investigations. Thanks are extended to Dr. Savithri Singh, Principal, Acharya Narendra Dev College, for providing infrastructure and research facilities.

Author Contributions

Conceived and designed the experiments: MM, KKG, and SK. Analyzed the data: MM. Wrote the first draft of the manuscript: MM. Contributed to the writing of the manuscript: KKG and SK. Agreed with manuscript results and conclusions: KKG and SK. Jointly developed the structure and arguments for the paper: SK and MM. Made critical revisions and approved the final version: SK. All authors reviewed and approved the final manuscript.

REFERENCES

1. Kranthi KR, Kranthi NR. Modeling adaptability of cotton boll worm *Helicoverpa armigera* (Hübner) to *Bt* cotton in India. *Curr Sci*. 2004;87:1096–1107.
2. Kranthi KR, Jadhav DR, Kranthi S, Wanjari RR, Ali S, Russell D. Insecticide resistance in five major insect pests of cotton in India. *Crop Prot*. 2002;21:449–460.
3. Subashini HD, Malarvannan S, Pillai RR. *Dodonaea angustifolia*—a potential biopesticide against *Helicoverpa armigera*. *Curr Sci*. 2004;86:26–28.
4. Ahmad M. Insecticide resistance mechanisms and their management in *Helicoverpa armigera* (Hübner)—a review. *J Agric Res*. 2007;45:319–335.
5. Ramya S, Rajasekaran C, Kalaivani T, Sundararajan G, Jayakumararaj R. Biopesticidal effect of leaf extracts of *Catharanthus roseus* L. (G) Don. on the larvae of gram pod borer—*Helicoverpa armigera* (Hübner). *Ethnobot Leaflets*. 2008; 12:1096–1101.
6. Wambua LM, Deng AL, Ogendo JO, Owuoche J, Bett PK. Toxic, antifeedant and repellent activity of aqueous crude extracts of *Tephrosia vogelii* Hook on the larval stages of *Helicoverpa armigera* Hübner. *Baraton Interdiscip Res J*. 2011;1:19–29.
7. Jeyasankar A, Premalatha S, Kuppusamy E. Biological activities of *Solanum pseudocapsicum* (Solanaceae) against cotton bollworm, *Helicoverpa armigera* Hübner and armyworm, *Spodoptera litura* Fabricius (Lepidotera: Noctuidae). *Asian Pac J Trop Biomed*. 2012;2:981–986.
8. Chopra RN, Badhwar R, Ghosh S. *Poisonous Plants of India*. New Delhi: ICAR; 1994.
9. Jaglan MS, Khokhar KS, Malik MS, Singh R. Evaluation of neem (*Azadirachta indica* A. Juss) extracts against American bollworm, *Helicoverpa armigera* (Hübner). *J Agric Food Chem*. 1997;45:3262–3268.
10. Koul O, Jain MP, Sharma VK. Growth inhibitory and antifeedant activity of extracts from *Melia dubia* to *Spodoptera litura* and *Helicoverpa armigera* larvae. *Ind J Exp Biol*. 2000;38:63–68.
11. Ramya S, Jayakumararaj R. Antifeedant activity of selected ethno-botanicals used by tribals of Vattal Hills on *Helicoverpa armigera* (Hübner). *J Pharm Res*. 2009;2:1414–1418.
12. Sundararajan G, Kumuthakalavalli R. Antifeedant activity of aqueous extract of *Gnidia glauca* Gilg. and *Toddalia asiatica* Lam. on the gram pod borer, *Helicoverpa armigera* (Hübner). *J Environ Biol*. 2001;22:11–14.
13. Abbaszadeh G, Srivastava C, Walia S. Insecticidal and antifeedant activities of clerodane diterpenoids isolated from the Indian bhant tree, *Clerodendron infortunatum*, against the cotton bollworm, *Helicoverpa armigera*. *J Insect Sci*. 2014; 14(29):1–13.
14. Prasad A, Roy SB. Histoarchitechtural alterations in the midgut tissues of fourth instar larvae of gram pod borer, *Helicoverpa armigera* (Hub) fed with leaf extract of plant *Lantana camara* (L). *Int J Pharma Biosci*. 2011;2:613–620.
15. Corzo FL, Gilabert M, Alcaide MFA, Bardón A. Toxicity of *Porella chilensis* sesqui- and diterpenoids against larvae of the corn pest *Spodoptera frugiperda* (J.E. Smith) (Lepidotera: Noctuidae). *Neotrop Entomol*. 2012;41:414–419.
16. de Almeida GD, Zanuncio JC, Senthil-Nathan S, et al. Cytotoxicity in the midgut and fat body of *Anticarsia gemmatalis* (Lepidoptera: Geometridae) larvae exerted by neem seeds extract. *Invertebr Surv*. 2014;11:79–86.
17. Mordue AJ, Nisbet AJ. Azadirachtin from the neem tree *Azadirachta indica*: its action against insects. *Anais da Sociedade Entomológica do Brasil*. 2000;29: 615–632.
18. Pauchet Y, Muck A, Svatos A, Heckel DG, Preiss S. Mapping the larval midgut lumen proteome of *Helicoverpa armigera*, a generalist herbivorous insect. *J Proteome Res*. 2008;7:1629–1639.
19. De Sousa ME, Wanderley-Teixeira V, Teixeira AA, de Siqueira HA, Santos FA, Alves LC. Ultrastructure of the *Alabama argillacea* (Hübner) (Lepidoptera: Noctuidae) midgut. *Micron*. 2009;40:743–749.
20. Singh K, Agarwal KK, Mishra V, Mubbin SU, Shukla A. A review on *Thevetia peruviana*. *Int Res J Pharm*. 2012;3:74–77.
21. Gulati A, Jain SK, Srivastava PS. Experimental studies on *Thevetia neriifolia* Juss—a review. *Ind J Chem*. 2000;39(B):808–812.
22. Pahwa R, Chatterjee VC. The toxicity of yellow oleander (*Thevetia neriifolia* juss) seed kernels to rats. *Vet Human Toxicol*. 1990;32:561–564.
23. Singh AK, Rembold H. Maintenance of the cotton bollworm, *Heliothis armigera* Hübner (Lepidoptera: Noctuidae) in laboratory culture—rearing on semi-synthetic diet. *Ins Sci Appl*. 1992;13:333–338.
24. Ladhari A, Laarif A, Omezzine F, Haouala R. Effect of the extracts of the spiderflower, *Cleome arabica*, on feeding and survival of larvae of the cotton leafworm, *Spodoptera littoralis*. *J Insect Sci*. 2013;13:61.
25. Ting A, Ma X, Hanson FE. Induction of feeding preference in larvae of patch butterfly. *Acta Zool Acad Scientiarum Hungaricae*. 2002;48:281–295.
26. Martinez SS, Van-Emden HF. Growth disruption, abnormalities and mortality of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) caused by azadirachtin. *Neotrop Entomol*. 2001;30:113–125.
27. Abdel-Aziz NF. *Chemical composition and insecticidal activity studies on some wild plant extracts against Spodoptera littoralis (Boisd.)* [Ph.D. thesis]. Egypt: Cairo University; 2007.
28. Brem B, Seger C, Pacher T, Hofer O, Vajrodaya S, Greger H. Feeding deterrence and contact toxicity of Stemona alkaloids—a source of potent natural insecticides. *J Agric Food Chem*. 2002;50:6383–6388.
29. Stavri M, Ford CHJ, Bucar F, et al. Bioactive constituents of *Artemisia monosperma*. *Phytochemistry*. 2005;66:233–239.
30. Choudhary RK, Veda OP, Mandloi KC. Use of neem, *Azadirachta indica* and garlic, *Allium sativum* in management of bollworms, *Helicoverpa armigera* in cotton. In: Proceedings of 88th Session of Indian Science Congress Association; New Delhi, India; 2001:40–42.
31. Dodia DA, Patel IS, Pathak AR. Antifeedant properties of some indigenous plant extracts against larvae of *Helicoverpa armigera*. *Pestology*. 1995;19:21–22.
32. Kamaraj C, Rahuman AA, Bagavan A. Screening for antifeedant and larvicidal activity of plant extracts against *Helicoverpa armigera* (Hübner), *Sylepta derogata* (F.) and *Anopheles stephensi* (Liston). *Parasitol Res*. 2008;103:1361–1368.
33. Janardhan RS, Chitra KC, Kameswara RP, Subramaniyam RK. Antifeedant and insecticidal properties of certain plant extracts against *Helicoverpa armigera*. *J Ins Sci*. 1999;5:163–164.
34. Jeyasankar A, Raja N, Ignacimuthu S. Antifeedant and growth inhibitory activities of *Syzygium lineare* Wall (Myrtaceae) against *Spodoptera litura* Fab (Lepidoptera: Noctuidae). *Curr Res J Biol Sci*. 2010;2:173–177.
35. Barakat DA. Insecticidal and antifeedant activities and chemical composition of *Casimiroa edulis* La Llave & Lex (Rutaceae) leaf extract and its fractions against *Spodoptera littoralis* larvae. *Aust J Basic Appl Sci*. 2011;5:693–703.
36. Reena SR, Singh R. *Helicoverpa armigera* biology as affected by *Pongamia pinnata* seed extracts and fractions. *Ind J Entomol*. 2007;69:63–70.
37. Adel MM, El-Hawary FM, Abdel-Aziz NF, Sammour EA. Some physiological, biochemical and histopathological effects of *Artemisia monosperma* against the cotton leaf worm, *Spodoptera littoralis*. *Arch Phytopathol Plant Prot*. 2010;43: 1098–1110.
38. Nasiruddin M, Mordue LAJ. The effect of azadirachtin on the midgut histology of the locusts, *Schistocerca gregaria* and *Locusta migratoria*. *Tissue cell*. 1993; 25(6):875–884.
39. Rawi SM, Bakry FA, Al-Hazm MA. Biochemical and histopathological effect of formulated and non-formulated plant extract on *Spodoptera littoralis*. *Int J Plant Sci*. 2011;2:107–118.
40. Packiam SM, Baskar K, Ignacimuthu S. Insecticidal and histopathological effects of botanical formulations against *Helicoverpa armigera* (Hub.) (Lepidoptera: Noctuidae). *Int J Agric Technol*. 2013;9:553–563.