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Influence of two new insecticides on toxicity, physiological and biological aspects of *Spodoptera littoralis* (Lepidoptera: Noctuidae)

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Abstract:

The Egyptian cotton leafworm *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), is a one of the major insects of cotton and other crops within its African tropical and subtropical countries. It has developed resistance to many conventional and nonconventional insecticides. The present investigation aimed to throw light on the efficiency of two novel insecticides, chlorfenapyr and metaflumizone on to lethal, biochemical parameters, and physiological aspects effects of chlorfenapyr and metaflumizone were determined by a leaf dipping technique bioassays to different two larval instars (2^{nd} and 4^{th}) *S. littoralis*. The 2^{nd} instar larvae were more susceptible than the older 4^{th} instars. Based on the LC_{50} values Chlorvenapyr is the most toxic to *S. littoralis* than the metaflumizone. The toxicity of chlorfenapyr was 1.55 fold with 2^{nd} than 4^{th} instar also, the toxicity of metaflumizone was 4.91 fold with 2^{nd} than 4^{th} instar. Biochemical studies indicated that treatment of 4^{th} instar larvae with LC50 of testing insecticides. Both of them reduced total carbohydrate contents. The total lipids decrease with metaflumizone but there was no significance with chlorfenapyr, the free amino acid was significantly lower with chlorfenapyr and metaflumizone. Both of them had no significant effect on Glutathione S-transferase (GST) activity in the larval homogenate. The histological examination of the mid gut of 4^{th} instar larvae of *S. littoralis* showed that all tested insecticides, lead to several damages occurred in all the cell layers in the midgut. Shrinkage of the epithelial layer cells was observed after the treatment with chlorfenapyr and the cytoplasm was vacuolated, and some of them lost their nuclei or appeared pyknotic. Changes were most noticeable in the columnar, regenerative, and caliciform cells. Larval treated by metaflumizone had a circular muscle layer and a longitudinal muscle layer seems to become thicker than normal and basement membrane partially separated, and lysis in the epithelial cells.

Introduction

Insects are one of the major menaces to agriculture, causing between 20 to 40 percent of global crop production loss annually. Each year, invasive insects cost the global economy around US$70 billion (FAO, 2019). The challenges of insect attacks

on crops have been noticed to be increased with climate change and global warming (IPPC, 2021). The Egyptian cotton leafworm *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) is one of the most devastating agricultural lepidopteran insects within its subtropical and tropical countries. *S. littoralis* cover more than 40 plant families, including about 90 host plant species of economic importance (Ghoneim, 2020). These caterpillars are very polyphagous, infesting decorative, industrial, and vegetable yields, resulting in hazardous economic losses (El-Sayed *et al.* , 2020).

The extensive use of conventional pesticides requires the application of several insecticides to control this pest; depending on extending and preserving the insecticide efficacy which is based on rotating numerous insecticides has resulted in several issues, including environmental pollution, the extinction of natural enemies, beneficial insect populations, and insect resistant to the certain insecticides (Mosallanejad and Smagghe, 2009). The control of this pest is focused on searching for new strategies with biological and ecological characteristics. One of these strategies, the use of new insecticides such as chlorfenapyr and metaflumizone insecticide. chlorfenapyr, which is one of the pyrrole insecticides classes, the biological action based on its transformation to another chemical. Oxidative removal of the N-methoxymethyl group of chlorfenapyr by mixed-function oxidases forming a toxic compound identified as CL 303268, which uncouples oxidative phosphorylation in the mitochondria, which cause disruption of the production of ATP and loss of energy, causing cell dysfunction cellular death, and ultimately organism mortality (EPA, 2001). This compound has low mammalian toxicity, also it is categorized according to WHO as a slightly hazardous insecticide because of it has a new mode of action (Tomlin, 2000). Metaflumizone classified in the new class of semicarbazone insecticides. Which is procured from pyrazoline (Takagi *et al*., 2007). Acts as a sodium channel blocker by binding selectively to the slow-deactivated status of the sodium channel, it produces a relaxed (Khakame *et al.*, 2013 and Hatem *et al.*, 2017).

Many studies were managed to define the basic metaflumizone toxicity and there are cross-resistance between indoxacarb and metaflumizone, both of them sodium channel blocker (Shen *et al*., 2020). Therefore, this study makes a major contribution to research on the effects of chlorfenapyr and metaflumizone on *S. littoralis* larvae. (a) Lethal effects of both insecticides, (b) The lethal effects of chlorfenapyr and metaflumizone as a biological control agent on some biochemical parameters such as carbohydrates, total lipids, free amino acids, and GST enzyme activity in 4^{th} larval instars of *S. littoralis*, and (c) Histological changes of the treated midgut of 4^{th} larvae were also determinate.

Materials and methods

1. Insects:

A laboratory colony of *S. littoralis* continuously was reared reared in the laboratory in the plant protection Research Institute. Newly hatched larvae were transferred to clean glass jars covered with muslin held in position with rubber bands. They were fed on fresh castor bean leaves, *(Ricinus communis* L.). Moths were fed on a 10% sugar solution soaked a piece of cotton tissue, *Nerium Oleander* L. as an oviposition site. The insects were maintained at 25 $\pm$ 2°C and 65 $\pm$ 5% RH. and a photoperiod of 16:8 hrs. (L: D). Rearing of insects was conducted

following the technique described by EL-Defrawi *et al*. (1964). As larva reached the 2nd and 4th instars, they were used in the experiments described below.

2. Leaf dip bioassay:

To assess the activity of different concentrations of chlorfenapyr and metaflumizone was prepared in water. The larvae used in the experiments were freshly 2nd and 4th instar larvae by using the leaf dipping bioassay technique. Fresh castor bean leaves were immersed for 1 minute in each concentration. Then let to dry at laboratory temperature. Each concentration was replicated three times and each replicate contained 30 larvae. Castor bean leaves treated with distilled water were fed to control larvae. Mortality was recorded after five days of treatment. mortality percentage was corrected using (Abbott's formula, 1925). The adjusted mortality percentage of each compound was calculated statistically using (Finney, 1971) from which the corresponding concentration probit lines (Ld-P lines) 50% and 95% mortality, slope values of tested compounds were also determined**.**

3. Biochemical studies:

3.1. Preparation of insects for analysis:

Preparation of sample biochemical analysis, the 4th instar larvae of *s. littoralis* after five days of all LC_{50} treatments and control by (Amin, 1998). The starved larvae were placed in clean glass jars. The starved larvae were homogenized in distilled water (50 mg/1ml) at 8000 r.p.m. supernatants, When kept at 5°C until analysis, enzyme extracts can be stored for at least one week without losing significant activity.

3.2. Free amino acids:

Total amino acids were measured using the ninhydrin reagent in a colorimetric manner, as reported by Lee and Takahashi (1966). A 1 ml sample is added to a 1.9 ml ninhydrin-citrate buffer-glycerol combination, which includes 0.5 ml of 1 percent ninhydrin solution in 0.5 M citrate buffer (pH 5.5); 0.2 ml of 0.5 M citrate buffer (pH 5.5); and 1.2 ml glycerol. The mixture was cooked for 10 minutes in a boiling water bath before cooling in a tap water bath. At 570 nm, the developed colour was measured. The amino acids were measured in µg alanine each body weight.

3.3. Total carbohydrates:

The phenol-sulphuric acid reaction was used to determine total carbohydrates in the acid extract of the sample (Dubois *et al*., 1956 and Crompton and Birt, 1967). Homogenization of the sample (1 gm) in 0.3N HClO4 (5 ml) at 0 °C for 1 minute, for another 10 minutes, the homogenate was maintained on ice. Insoluble debris was removed by centrifugation at 2000 rpm for 3 minutes, then redispersion and centrifugation twice in ice-cold HClO4 (5ml). The acid extract was made up of the three supernatant. In a colorimetric tube, 100 microliters of acid extract were added to 0.5 ml of phenol (20 percent w/v). Then, with shaking, 5 mL of strong sulfuric acid was added. The total carbohydrate content is calculated as g glucose per g of fresh weight.

3.4.Total lipids:

Total lipids were determined using the phosphovanillin reagent (Knight *et al*., 1972), which was created by dissolving 0.6 gm pure vanillin in 10 ml ethanol and diluting to 100 ml with distilled water. After that, 400 mL of concentrated phosphoric acid was added. 250 l of the sample was mixed with 5 ml of concentrated sulphuric acid in a test tube and heated in a boiling water bath for 10 minutes. The digest was added to the phosphovanillin reagent after cooling to room temperature (6 ml). The produced

colour was measured at 525 nm against the reagent blank after 45 minutes. The optical density of the samples was compared to a reference standard, and the findings were reported in mg lipids/ ml hemolymph.

3.5. Glutathione S-transferase (GST) activity :

Glutathione S-transferase (GST) catalyses the conjugation of reduced glutathione (GSH) with 1-chloro 2.4-dinitrobenzene (CDNB) via the –SH group of glutathione . The conjugate, S-(2.4-dinitrophenyl)-L-glutathione, was identified using the technique described by Habig *et al*. (1974). 1 ml potassium salt of phosphate buffer (pH 6.5), 100 l GSH, and 200 l larval homogenate made up the reaction mixture. The reaction began with the addition of 25 µl of CDNB solution as the substrate. Both GSH and CDNB concentrations were changed to 5m M and 1m M, respectively. For 5 minutes, using a molar extinction coefficient of 9.6/mM/cm, the increase in absorbance at 340 nm was measured against a blank containing everything but the enzyme to estimate the nanomole substrate conjugated/min/larva.

4. Histopathological studies:

The effect of LC_{50S} of chlorfenapyr and metaflumizone on the histological structure of the mid-gut of the 4th instar S. *littoralis* larvae was determined. After 5 days of treatment, 10 larvae of each treated and control group were dissected under a binocular microscope. The separated mid-gut was transferred into alcoholic Bouin's solution was used as a fixative, for dehydration and removal of the yellow color of Bouin's solution the larvae were rinsed in a series of ethanol solutions. They were transferred first into 50% ethyl alcohol for 2 hrs at 40 °C (Two changes) then left for 24 hrs. Then the larvae passed through a series of alcoholic treatments each for 2 hrs at room temperature starting with 80% , followed by 90%, 96%, and ending with 100%. After dehydration, the larvae were placed in a solution of amy1 acetate and colloidal to clear the tissues. Treated with soft wax stated by placing them in vials containing equal portions of fresh amy1 acetate solution and soft paraffin wax and leaving them for 24 hrs. at 50 °C. Serial longitudinal sections at 6 microns were made by microtome and mounted on clean slides using Mayer's albumin. Sections were mounted on glass slides and stained with haematoxylene and counterstained in alcoholic Eosin and prepared for observation and photomicroscope (Humason and Freeman, 1979).

5. Statistical analysis:

The findings of biochemical measurements were aggregated from triplicate determinations in all studies with three replicates (Insect homogenates). Using Costat (2007) statistical software, the findings were evaluated using one–way analysis of variance (ANOVA) (Cohort software, Berkeley). The Tukey's HSD test was used to compare means when the ANOVA data were significant < (P 0.05).

Results and discussion

1. Leaf dip bioassay:

The bioassay to the toxicity of chlorfenapyr and metaflumizone on 2nd and 4th instars *S. littoralis* larvae demonstrated that 2nd instar larvae were more susceptible than the 4th instar. As evidenced by Table (1) LC_{50} and LC_{95} values were 35.90 and 148.14 ppm for 2nd instar larvae and 55.62 and 621.01 ppm for 4th instar larvae, respectively. The slope values were 4.21 ± 0.38 and 4.92 ± 0.53 and 2.77 ± 0.36 and 3.31 ± 0.32 in treating 2nd instar larvae, there was higher homogeneity than in treating 4th instar larvae for the corresponding instar larvae. Based on the LC_{50} values chlorvenapyr is the most toxic to *S. littoralis* than the

metaflumizone. The toxicity of chlorfenapyr was 1.55 fold with 2nd than 4th instar also, the toxicity of metaflumizone was 4.91 fold with 2nd than 4th instar. This result correspondingly, chlorpyrifos and spinosad insecticides were acting as the same effect on *Prodenia litura* (F.) (Lepidoptera : Noctuidae) treated larvae (Singh and Sohi, 2008).

Table (1): Relative toxicity of chlorfenapyr and metaflumizone against *Spodoptera littoralis* 2nd and 4th instar larvae on the basis of LC_{50} values.

Insecticides	larval instars	LC 50 (%) [a] (ppm)	95% Confidence limits (%) Lower	Upper	Slope [b] b ± S. E	X [c]	Relative Potency[d]
Chlorfenapyr	2nd	35.90	33.19	38.75	4.21±0.38	4.39	1.55
	4th	55.62	49.76	65.02	2.77±0.36	6.44	1
Metaflumizone	2 nd	148.14	138.19	156.96	4.92±0.53	5.86	4.91
	4 th	621.01	566.75	692.21	3.31±0.32	4.79	1

2. Biochemical studies:

The results of biochemical analysis of *S. littoralis* 4th instar larvae treated with LC_{50} of chlorfenapyr and metaflumizone compared were shown in (Table 2). There was a significant difference between chlorfenapyr and metaflumizone and control in total carbohydrate contents, both of them reduced total carbohydrate contents. Also, the total lipids decrease with metaflumizone it was (4.05±0.15 mg/g.b.wt) but there was no significant defferent between chlorfenapyr and untreated (5.9±0.13 and 5.7±0.26 mg/g.b.wt, respectively). In addition, the free amino acid was significantly lower in a chlorfenapyr and metaflumizone treated larvae comported with control (1.71±0.052, 1.92±0.025, and 2.22±0.102 mg alanine/g. b. wt., respectively.

Chlorfenapyr decreased the carbohydrate and free amino acid contents significantly compared with the control. All macronutrients include carbohydrates, proteins, and lipids that supply energy for insects to maintain their vital processes, these macronutrients can be affected by exhibits factors such as insecticides. These study findings suggest that chlorfenapyr can influence carbohydrate reduction, which can be explained by the high quantity of energy required for all body processes. This defect could be caused by chlorfenapyr's action, which is an uncoupler of oxidative phosphorylation in the mitochondria. As a result, ATP generation is disrupted, and energy is lost (Zhao *et al*., 2018). Trehalose is an insect's major blood sugar that can successfully prevent protein denaturation and function loss in stress conditions (Jain and Roy, 2009).

Data obtained in the present work disclosed a significant reduction in the carbohydrate, Total lipids, and free amino acid contents of *S. littoralis* 4th instar larvae treated with LC_{50} of metaflumizon. This finding was also reported by Hatem *et al*. (2017), metaflumizone caused a reduction in total carbohydrates and total protein in treating *S. littoralis* larvae. Antifeedant impact of the investigated substances was demonstrated by a considerable decrease in free amino acids and carbohydrate, indicating that the treated larvae with tested compounds which were unable to digest the diet and the reduction in lipids may be due to the larvae use it as an energy in case of starvation due to temporary stop feeding as a result of treatment (Abd-El-Aziz *et al*., 2020; Abd-El-Aziz and Salama, 2020). No significant differences in the activities of GST in larvae exposed to LC_{50} of both insecticides compared with control. Metaflumizone, like indoxacarb, is a

voltage-dependent sodium channel blocker that preferentially targets voltage-gated sodium channels in the slow inactivated state by binding to or near the local aesthetic receptor within the sodium channel pore (Tian *et al*., 2014).

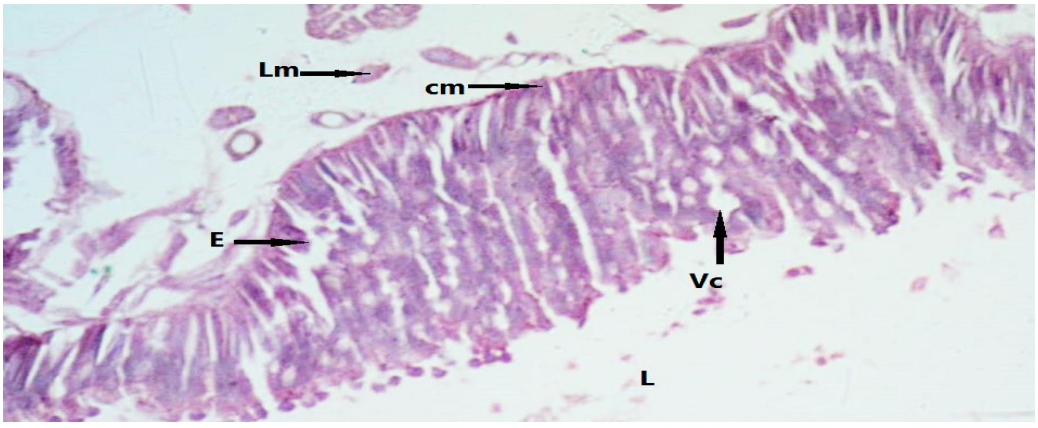
Our results were in agreement with Zhao *et al*. (2018), after exposing *Bradysia odoriphaga* Yang et Zhang (Diptera: Sciaridae) larvae with chlorfenapyr for 24 hrs., the activities of GST were assessed. The LC_{20} and LC_{50} GST activities were not significantly different from the control.GST activities were not significantly different between susceptible and resistant *Tetranychus urticae* Koch (Acari: Tetranychidae) strains to chlorfenapyr (Van Leeuwen *et al*., 2006). The GST had no obvious effect on metaflumizone in resistant and susceptible strains of *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) and *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) (Shen *et al*., 2020 and Tian *et al*., 2014). On the other hand, many results reflect the important role of GST in the metaflumizone accordance with Hatem *et al*. (2017) and Yalcin *et al*. (2015), which confirmed an increased enzyme activity due to metaflumizone treatment. These results are useful for formulating chlorfenapyr and metaflumizone insecticides management strategies to control *S. littoralis* in the field.

Table (2): Amounts of total carbohydrates, yotal lipids, free amino acids, and the activity of GST enzyme in *S. littoralis* 4th instar larvae treated with LC_{50} of chlorfenapyr and metaflumizone compared with untreated control larvae (Mean ± SE).

Insecticides	Total carbohydrates (mg/g.b. wt)	Total lipids (mg/g.b. wt)	Free amino acids (mg alanine/g.b. wt)	GST (mmole sub. Conj./min/g.b. wt)
Chlorfenapyr	23.13±0.58b	5.9 ±0.31a	1.71±0.052a	21.23±0.14a
Metaflumizone	25.55±0.45b	4.05 ±0.15b	1.92±0.025a	21.2±0.10a
Control	33.63±1.06a	5.7 ±0.26a	2.22±0.102b	22.13±0.57a

3. Histopathological studies:

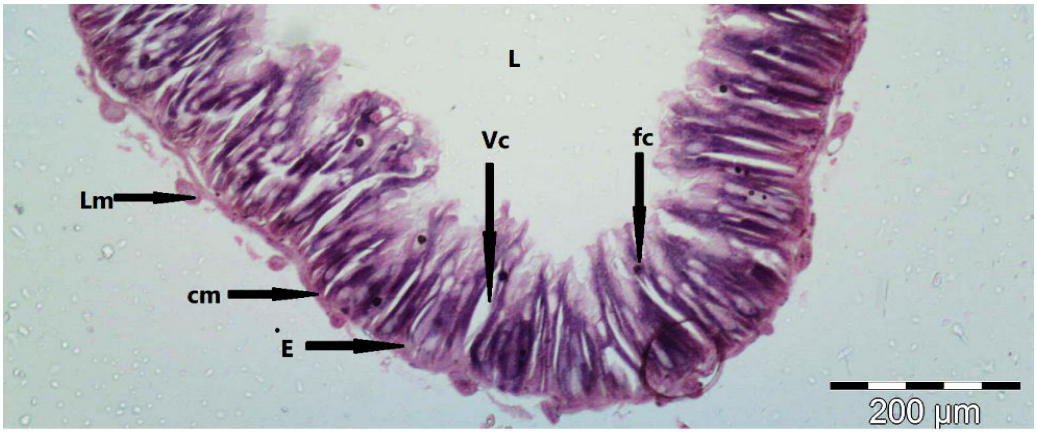
Histological studies of the mid-gut of the treated larvae with LC_{50} show that the insect muscles were affected by the tested compound. Normal structural features of the mid-gut of the 4th larval instar of *S. littoralis* are illustrated in Figure (1).



Figuer (1): Transverse section of control mid-gut of the 4th instar larva of *Spodoptera littoralis* cm = circular muscle; E= epithelial layer; L= lumen; lm = longitudinal muscle; vs = vesicles (Scale bar = 200µm).

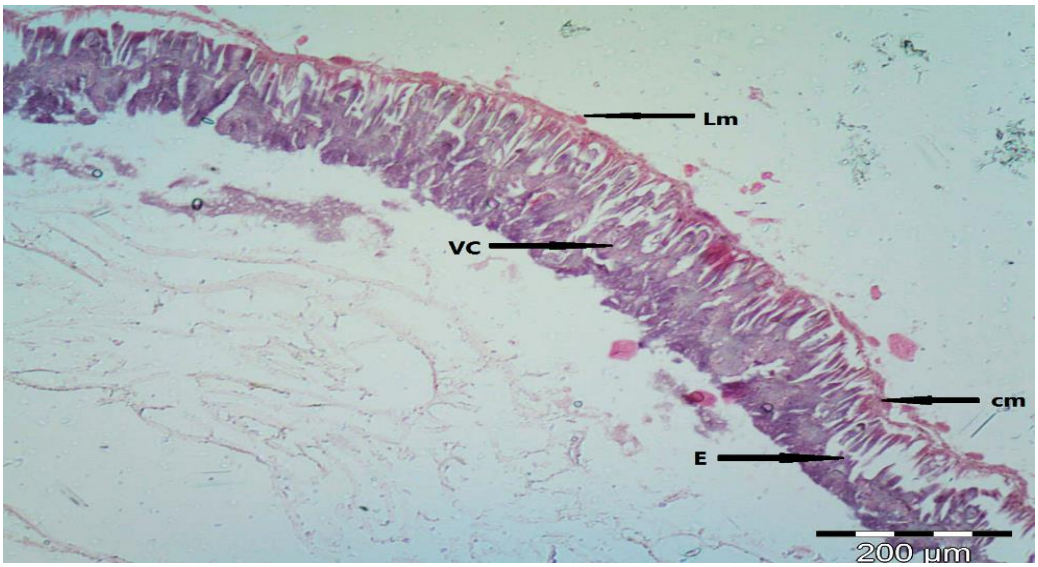
The mid-gut epithelium is a simple epithelium composed of three main cell types, columnar epithelial cells, regenerative cells, and goblet cells supported by a basement membrane which is surrounded from inside to the outside by a circular muscle layer and a longitudinal muscle layer, which is surrounded from inside to the outside by a circular muscle layer and a longitudinal muscle layer. Larvae treated with chlorfenapyr showed induced several irreversible histopathological changes in the mid-gut of larvae the epithelial cells' cytoplasm was vacuolated, and some of them lost their nuclei or appeared pyknotic. Changes were most noticeable in the columnar, regenerative, and caliciform cells. These cells' apical surfaces were damaged.

The empty apical vesicles indicate that certain epithelial cells have lost their ability to secrete. Shrinkage of the epithelial layer cells was observed after the treatment with chlorfenapyr (Figure 2).



Figuer (2): Transverse section in the mid-gut of 4th larval instar of *Spodoptera littoralis*, treated with LC_{50} of Chlorfenapyr fc= cells with pyknotic nuclei.

The changes histopathological were observed in the mid-gut of 4th larval instar of *S. littoralis*, treated by metaflumizone. The circular muscle layer and a longitudinal muscle layer seem to become thicker than normal and the basement membrane is partially separated. Lysis in the epithelial cells (Figure 3).



Figuer (3): Transverse section in the mid-gut of 4th larval instar of *Spodoptera littoralis*, treated with LC_{50} of metaflumizone.

Similar observations were mentioned by many authors, Youssef (2006) found pyriproxyfen and abamectin caused defects in columnar epithelium-cells and stretching the lead to peritrophic membrane tearing. Saleh *et al.* (2021) found lufenuron, diflubenzuron, emamectin benzoate Chlorantraniliprole, methoxyfenozid, and spinosad destruction of both goblet and columnar cells in the mid-gut. The basement membrane was separated from the epithelial layer, which was disordered and accompanied by an increased vacuolization. Basement and peritrophic membranes are separated (Hanan *et al.*, 2020). The midgut of *S. littoralis* was treated with *Hyptis brevipes* dichloromethane extract, which caused the peritrophic membrane to be detached and the midgut lumen was packed with pycknotic-nuclei-epithelial cells. Extensive destruction of and indoxacarb on the 2nd and 4th larval instar of the *S. littoralis* larvae exhibit destruction of the mid-gut muscle layers, disorganization in the epithelial cells, separation of the peritrophic membrane, and occurrence of visualizations and detachment of the basement membrane**.**
the epithelium with cells lacking nuclei in the midgut (Sakr, 2014).

Histomorphological disturbances in the mid-gut of 4th instar larvae *S. littoralis* treated with the tested compounds were vacuolation, lysis, and necrosis of the epithelial cells and destruction of epithelial cells and their boundaries. The present histopathological destruction caused by the investigated chlorfenapyr and metaflumizone insecticides may suggest that any of these insecticides are capable of causing a disturbance in the function and gradual death of an insect when entering into tissues in adequate amounts.

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