ULTRASTRUCTURE AND BIOCHEMICAL ALTERATIONS OF COTTON LEAFWORM, *SPODOPTERA LITTORALIS* TREATED WITH *IPOMEA CARNEA* EXTRACT

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Abstract

Ipomea carnea (Jacq.) is a wild grown and distributed plant in the Egyptian environment on banks and borders of streams and water courses along Nile river region. This study designed to evaluate the ultrastructural and biochemical changes in 4th larval instar of cotton leafworm as a result of application with the acetonic extract of this plant compared to traditional insecticide (chlorpyrifos). According to LC_{50} and LC_{90} values chlorpyrifos was the most effective that recorded (9.497 and91.126 ppm, respectively), followed by acetonic extract of *I. carnea* (24.622 and 164.947ppm.), respectively. Many ultrastructure changes in the treated larvae mid gut epithelial cells were recorded using LC_{50} of both tested treatments. These changes like nucleus elongation, strong agglomeration of chromatin, extreme dilation of endoplasmic reticulum, destruction of microvilli where apical cytoplasm was vesiculated. In addition, enormous changes in the structure of cuticle such as being edges and separation of endocuticle from epicuticle were also observed. Results showed that, *I. carnea* at LC_{25} and positive control (using acetone only) insignificant decrease the total soluble protein in the supernatant of the homogenated larvae than negative control (using water only). Whereas, *I. carnea* and chlorpyrifos at (LC_{50}) significantly increased the effect of total soluble protein than control. Additionally, all the treatments increased activities of AST and ALT in the supernatant of *S. littoralis* compared to negative control.

INTRODUCTION

Among more than 1300 insect species are recorded on cotton, the most important pest to have spread is the cotton leafworm, *Spodoptera littoralis* (Boisduval), which is found almost every where cotton is grown (Matthews and Tunstall, 1994). It is one of the most notorious and destructive phytophagous insect

pests in Egypt, not only to cotton, but also to other field crops and vegetables (Kandil *et al.*, 2003).

The massive application of pesticides resulted in build up of pest resistance to these poisons and adverse effects on environment, that include acute and chronic hazards to humans and non-target organisms, environmental pollution and upsetting the natural balance. Pest control strategies, especially those that are effective, cheap and environmentally non-hazardous are needed. Hence, crude plant extracts have played an important role in this aspect (Mahadevan, 1982).

Ipomea carnea belongs to Order: Solanales, Family: Convolvulaceae is popularly known as "Olleiq ek-kibber or Morning glory" for Nile Delta, Egypt, Al-Sadany (1998), it is a large diffuse shrub with milky juice. The flowers are pale rose, pink or light violet in lax, dichotomously branched axillaries and terminal, pedunculate cymes, fruits have a glabrous capsule, seed is silky (Cholich *et al.*, 2009). It has been reported in ancient system of medicine but did not gain much important and always remain in controversies. Otherwise, the fact is this plant had immense potential as anti-biotic activity, anti-oxidant, anti-microbial, wound healing properties, anti-inflammatory Sharma and Bachheti 2013. The present work was planned to experimentally assess the effect of acetonic extract of the aerial parts of *I. carnea* on ultrastructure and biochemical changes of 4th instar larvae of *S. littoralis*.

MATERIALS AND METHODS

1. Tested materials:

1.1. Preparation of *I. carnea* extract:

Plant has been defined according to the Herbarium (CAIM) – Flora and Phyto Taxonomy Researches, Horticultural Research Institute, Cairo, Egypt as *Ipomea carnea* spp. fistulosa from the Convolvulaceae family. Fresh aerial part of *I. carnea* Jacq (Convolvulaceae) were collected from local gardens of Abu Hammad, Sharquia, Egypt. The freshly collected aerial parts were spread to dry at normal room temperature for seven days in the shade. Upon drying, the aerial parts were pounded using mortar and pestle into smaller particles and then blended to powder and the powder was stored in airtight containers and kept under normal room temperature (28 ± 2°C) until required.

The powder of aerial part of *I. carnea* Jacq. was extracted at room temperature successively using acetone as a solvent depending on it is polarity. Two hundred grams (200gm) of powdered sample was soaked in 500 ml of acetone and put in1000 ml sterile conical flask. The flask was covered with cotton plug and then wrapped with aluminum foil and shaken every 3hr intervals for 72hr at room temperature, then the crude extract was filtered using muslin cloth and then Whatman no.1 filter paper. The filtrate was evaporated to dryness using rotary evaporator (Model 349/2, Corning Limited) maintained at 40°C and the dried substance was stored in refrigerator until further use.

1.2.Traditional insecticide: Dursban® (48% EC).

Common name: Chlorpyrifos.

Chemical name: O, O - diethyl O- (3, 5, 6–trichloro-2-pyridinyl) phosphorothioate.

Rate: 1 liter / feddan.

Basic product: Dow AgroSciences.

2. Rearing technique of *S. littoralis*:

S. littoralis strain used in this study is a laboratory susceptible strain reared in the Plant Protection Research Institute at Zagazig, Sharquia Governorate according to El-Defrawi *et al.*, 1964. The culture was maintained under optimum conditions (27 °C ± 2 and 70±5 R.H) and reared on fresh castor bean leaves until the 4th larval instar which used in this study.

3.Toxic effect of tested toxicants against 4th larval instar of *S. littoralis*:

The efficiency of the acetone extract of *I. carnea* and chlorpyrifos were assessed against 4th instar larvae. Serial successive concentrations of the extract were used 3, 5 and 8% (w/v). As for chlorpyrifos, the serial concentrations starting with the recommended concentration, all the used concentrations of chlorpyrifos and control were prepared using distilled water. Castor bean leaves were dipped for 15 seconds in each concentration then left to dry. The treated leaves were offered to newly molted 4th instar larvae for 48 hr then replaced by untreated leaves for 24 hr. Mortality percentages were recorded after 72 hr. and corrected according to natural mortality (Abbott, 1925). To estimate the LC_{50} values, the corrected mortality percentages were subjected to probit analysis according to Finney (1952).

4. Ultrastructure studies of *S. littoralis* using acetonic extract of *I. carnea* and chlorpyrifos:

Ultra structure sections were made for the epithelial cells of midgut and cuticle muscles for 4th instars larvae after three days of treating with LC_{50} of acetonic extract of ***I. carnea*** and after one day with LC_{50} of chlorpyrifos according to its mode of action, untreated larvae were used as control. Stained sections were examined with a JEOL 1010 Transmission Electron Microscope at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University.

5. Biochemical tests:

5.1.Preparation of samples for biochemical assay:

Larval samples used for biochemical assays were collected after three days of treatment the 4th instar larvae with LC_{25}, LC_{50} of acetonic extract of *I. carnea* and after one day with LC_{50} Dursban (chlorpyrifos) according to its mode of action, untreated larvae were used as control.

Samples were homogenized in distilled water using a Teflon homogenizer. The homogenates were centrifuged at 500 r.p.m for 20 minutes at 5ºC. The supernatants were immediately assayed to determine total soluble protein and transaminase enzymes (AST & ALT).

5.2. Determination of total soluble protein:

Colorimetric determination of total soluble protein in total homogenate *S. littoralis* larvae were carried out as described by Gornall *et al.*, (1949).

5.3. Transaminase enzymes:

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzyme activities were determined calorimetrically according to the method of Reitman and Frankle (1957).

6. Statistical analysis:

The significance of the main effects was determined by analysis of variance (ANOVA). The significance of various treatments was evaluated by Duncan's multiple range test ($p < 0.05$) (Snedecor and Cochran 1980). Data were subjected to statistical analyses using a software package CoStat® Statistical Software (2005) a product of Cohort Software, Monterey, California.

RESULTS AND DISCUSSION

1. Toxic effects of tested toxicants against 4th instar larvae of *S. littoralis:*

According to LC_{50} and LC_{90} values chlorpyrifos was the most effective that recorded (9.49 and 91.12 ppm, respectively), followed by acetone extract (24.62 and 164.94 ppm.), Table 1.

Table 1. Susceptibility of the fourth instar larvae of *S. littoralis* to different tested treatments.

Treatments	LC_{50} ppm (Lower - Upper)	LC_{90} ppm (Lower - Upper)	Slop	Toxicity index
Chlorpyrifos	9.49 (7.80 – 11.39)	91.12 (69.41 – 137.49)	1.305	100
Acetone Ex.	24.62 (13.65 – 37.15)	164.94 (121.52 – 205.76)	1.552	38.57

Toxicity Index based on LC_{50}.

In the present investigation, chlorpyrifos was tested against 4th instar larvae of *S. littoralis* as a reference standard for the activity of plant extract. The highly toxic effect of chlorpyrifos suggests that insecticide is highly effective, similarly, Gaaboub *et al.*, (2012) found that chlorpyrifos proved as the most effective insecticide against 4th instar larvae of *S. littoralis* comparing to other insecticides and plant extracts.

2. Ultrastructure studies of *S. littoralis*:

Ultrastructural studies showed that, acetonic extract of *I. carnea* and chlorpyrifos caused pathological changes in the mid gut epithelial cells in 4th larval instar of *S. littoralis*.

The mid gut epithelial cells in control characterized by ovoid nuclei (N) located toward the base, mitochondria (MI) are elongated and numerous and are oriented longitudinally, while the endoplasmic reticulum (ER) forms a continuous network of canaliculated and fenestrated sacules, the Golgi apparatus is well developed (Fig. 1a) Each cell has a striated or brush border formed by closely parallel microvilli (MV). (Fig. 2a).

The treatments with acetonic extract of *I. carnea* shows many forms of disorganization like accumulation of chromatin and elongation of nucleus, appearance of highly condensed mitochondria at first then swelled with the disappearance of internal cristae around the nucleus, very large fat vacuole (Fig. 1b). In addition to dilation of endoplasmic reticulum (ER) (Fig. 1d), enlargement and deformation of the basal enfolding in microvilli (MV) were recorded in (Fig. 2b).

Similar observations were reported by Shoukry (1996) showed that, the volatile constituents of chamomile, *Matricaria chamomilla* L. flowers and Jasmaine, *Clerodendrom inerme* G. leaves recorded ultrastructral effects on *Musca domestica* L. appearance of large vacuoles and considerable swelling in the mitochondrial bodies. Partial to complete loss of mitochondrial cristae was also detected. Sharma *et al.,* (2003) recorded that, 0.15 % azadirachtin caused vacuolization in the cytoplasm, degeneration of organelles and destruction of the plasma membrane according to the ultrastructure studies on *S. litura* larvae.

Rharrabe *et al.,* (2007) studied the cytotoxicity of harmaline on the mid gut epithelial cells of *Plodia interpunctella* by electron microscopy resulting in marked vacuolization of the cytoplasm, appearance of numerous autophagic vesicles and lysosomic structures, fragmentation of rough endoplasmic reticulum cisternae, disruption of microvilli, rupture of the plasma membrane leading to shedding of the cytoplasm contents into the mid gut lumen Sabry (2009) found that the mid gut epithelial cell of the 4^{th} larval instars of *S. littoralis* showed extreme dilation in endoplasmic reticulum after lup20(29)-en-3-ol-acetate and stigmast-en-3-ol administration, many large vacuoles appear in the cytoplasm the presence of numerous small sized electron dense mitochondria large and small vacuoles and the microvilli.

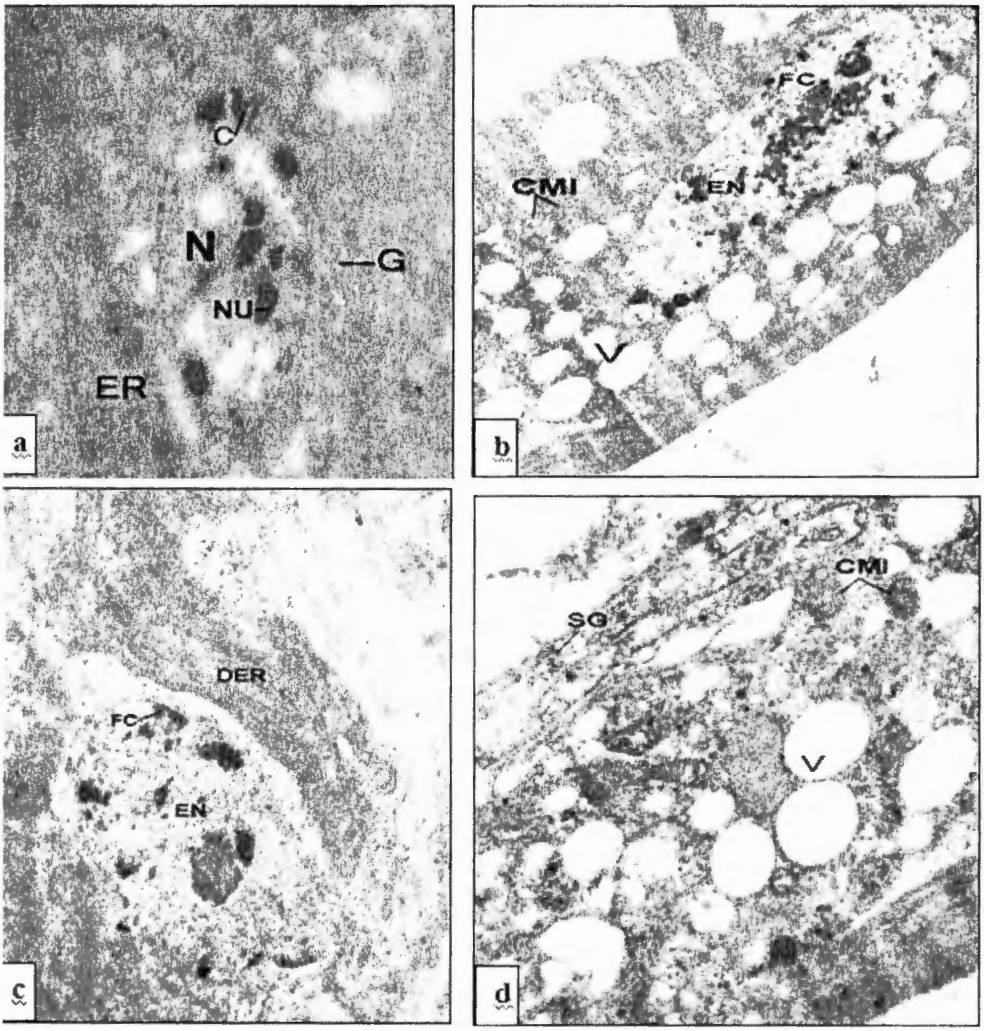

Fig. (1): Electron Micrograph (EM.) illustrating transeverse section in the midgut epithelial cell of the 4th larval instar of *S. littoralis*.

[a]: Normal epithelial cell. [8000X].

[b & c]: Treated with acetonic extract of *I. carnea.* [12000X]

[d]: Treated with chlorpyrifos. [20000X]

Chromatin (C). Condensed Mitochondria (CMI). Dilated Endoplasmic Reticulum (DER). Elongated Nucleus (EN). Endoplasmic Reticulum (ER). Fragmented Chromatin (FC). Golgi Apparatus (G). Nucleus (N). Secretory Granules (SG).Vacuoles (V).

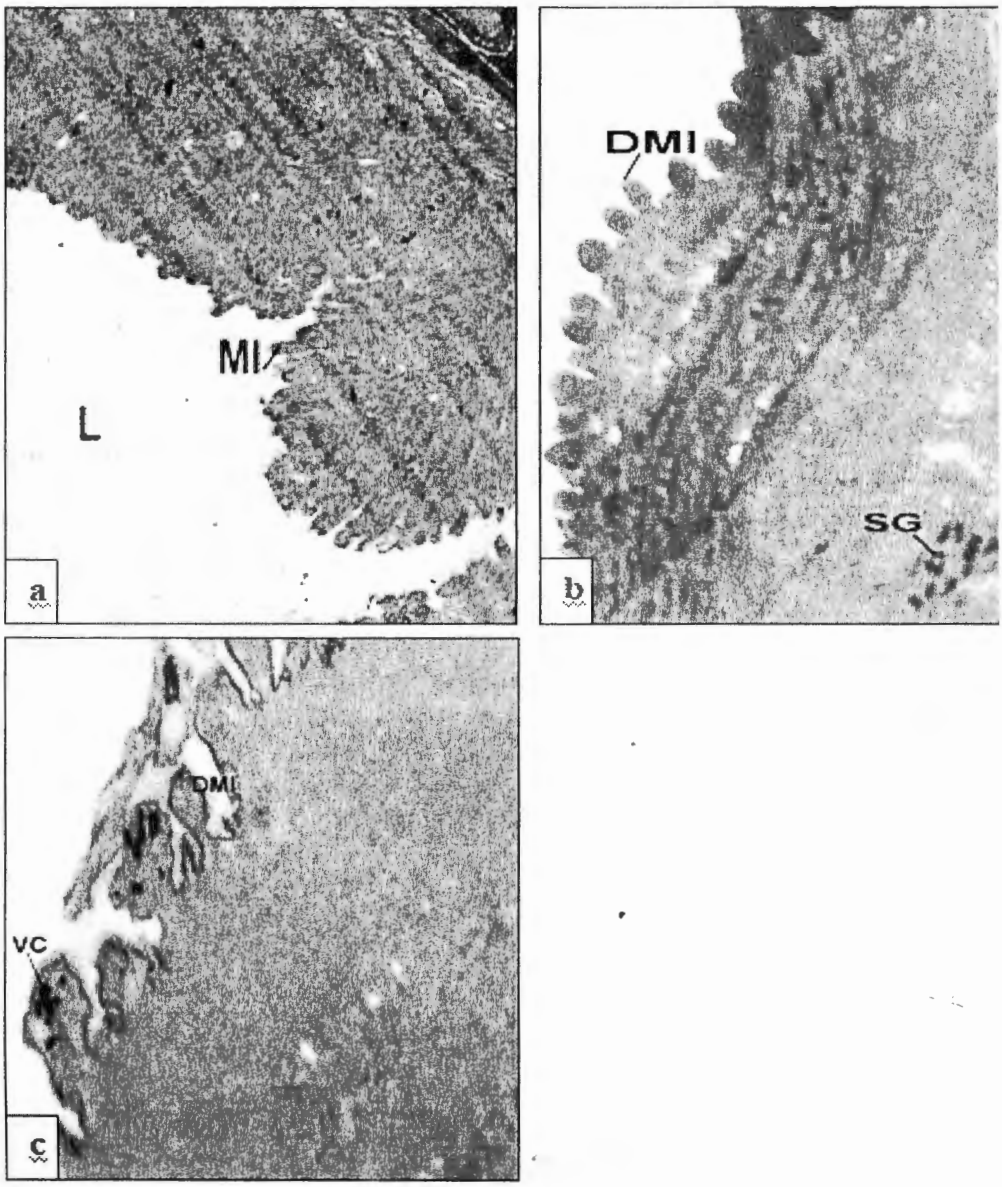

Fig. (2): Electron Micrograph (EM.) illustrating transeverse section in the microvilli of the midgut epithelial cell of the 4th larval instar of *S. littoralis*.

[a]: Normal microvilli [4000X]

[b]: Treated with acetonic extract of *I. carnea* [4000X].

[c]: Treated with chlorpyrifos [4000X].

Degenerated Microvilli (DMI). Lumin (L). Microvilli. Secretory Granules. (SG). Vesiculated Cytoplasm (VC).

The treatment with chlorpyrifos showing strong agglomeration of nucleus chromatin, condensation of mitochondria while mitochondria coalescence together forming large bodies (Fig. 1c). In addition, destruction of microvilli, the apical microvilli were disorganized and the apical cytoplasm was vesiculated (Fig. 2c).

These observations were mentioned by Sherif and Ali (1997) proved that, LD_{50} of dichlorvos rupture the plasma membranes of the fat body cells after extensive vacuolization and the cytoplasmic content almost disappeared. The chromatin material darkened and become concentrated in the middle of the cells. Younes *et al.*, (2000) studied the histopathological effects of malathioin, cypermethin and dimilin on the tissues of the mid gut and integument of 5th instar larvae of the lesser cotton leaf worm, *S. exigua*. They found the mid gut elongation of epithelial cells, vacuolation fading of cell boundaries, chromatin clumping or lysis and degeneration of cells. Adamski *et al.*, (2005) and Kalender *et al.*, (2005) exposure of *Tenebrio molitor* larvae and *T. pityocampa* larvae to the insecticides fenitrothion and endosulfan, respectively caused alterations in the ultrastructure of the fat body cells and mid gut cells. The nuclei, endoplasmic reticulum and mitochondria revealed numerous prominent malformations: invaginations and swelling of the nuclear envelope Adamski (2007) examined the toxic effect of carbamate insecticide carbaryl on *S. exigua* larvae. Nuclear condensation and many abnormalities in mitochondria were observed.

On the other hand, all treatments causing damage in cell layer with interruption and the cuticle space decrease with big change in the structure of it shown in its different appearance from control one (Fig. 3a) the surface of cuticle being edges, separate of endocuticle from epicuticle (Fig. 3b-c).

These results are in harmony with the finding of Sabry (2009) and Rawi *et al.*, (2011) that histopathological changes appearing in the integument of the 4th instar larvae *S. littoralis* treated with lup 20 (29)-en-3-ol-acetate and stigmast-en-3-ol and LC_{10} and LC_{25} extract of *C. locynthis,* respectively showed cytoplasm granulation, completely destroyed epidermal cells, fat accumulation, detaching and folding of epithelium cells. Separation of epidermis from cuticle and distortion of muscle fibers. Hassan (2009) showed that histological examinations of 6th larval instar cuticle (after treatment of fourth larval instar by LC_{50} of methoxyfenozide) showed destruction in the cuticle layers, fissures in the endocuticle and irregular distribution of the hypodermal cells. While indoxacarb and spinetoram treatments

showed slight effect in the cuticle layers as compared to methoxyfenozide. Alkhalaf (2013) estimated the effect of botanical extracts on the cuticle of the 3rd larval instar of *Cx. quienqufasciatus*, by using the LC_{50} of the methanol extract of *Matricharia chamomella* for periods of 6, 12, 24 and 48 hours. The ultrastructure studies on the cuticle indicated that the larvae were affected according the time elapse

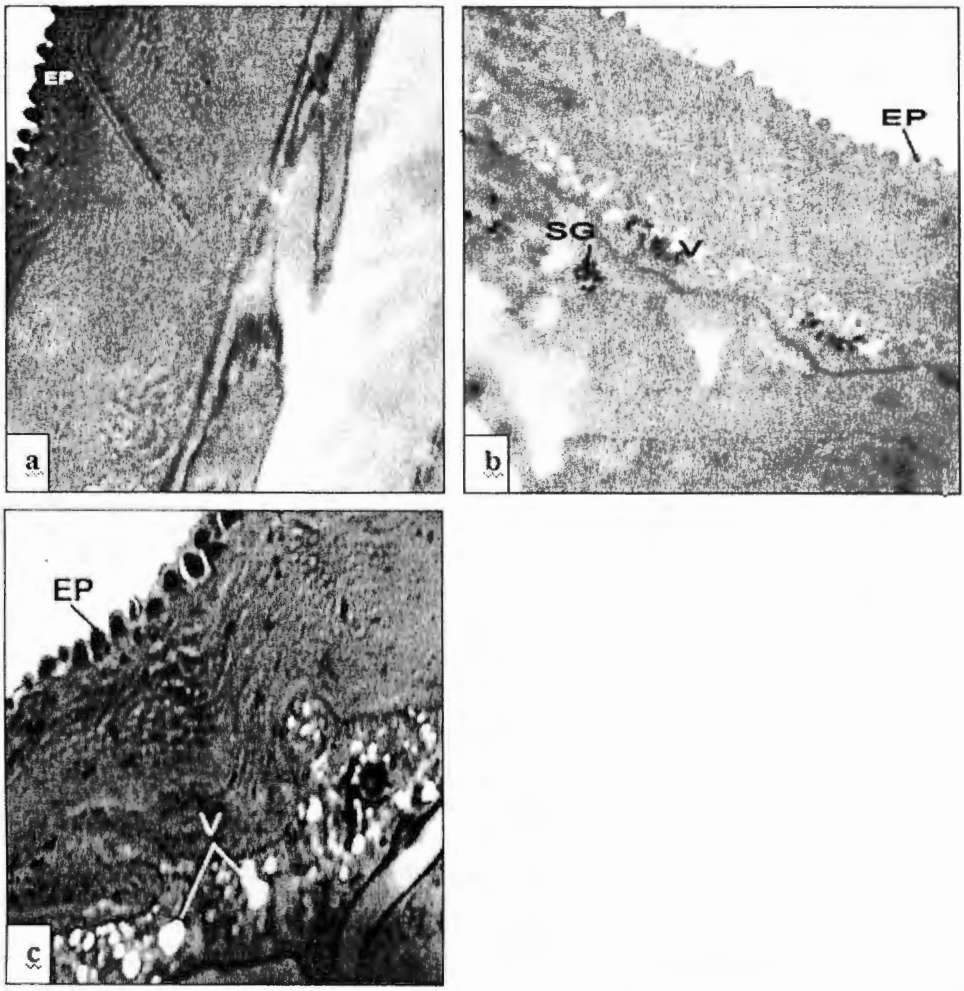

Fig. (3): Electron Micrograph (EM.) illustrating transeverse section in the cuticle of the 4th larval instar of *S. littoralis*.

[a]: Normal microvilli [4000X]

[b]: Treated with acetonic extract of *I. carnea* [4000X].

[c]: Treated with chlorpyrifos [4000X].

Epicuticle (EP). Secretory Granules. (SG). Vacuoles (V).

3. Biochemical response of *S. littoralis* to acetonic extract of *I. carnea* and chlorpyrifos:

The changes in enzymatic activities (Transaminase enzymes) and level of total soluble protein of 4th instar larvae of *S. littoralis* as responses of treatment with LC_{25} and LC_{50} of the acetonic extract of *I. carnea* after 72hr and chlorpyrifos at concentration (LC_{50}) after 24hr of treatment according to its mode of action, therefore, two controls (negative control (-ve) using water only) were inspected after 24 and 72hr post treatments. Additionally, another control was used acetone only as a solvent (positive control (+ve) after 72hr post treatment to study the effect of acetone solely. Data were expressed as percentages in the activity as relative to its –ve controls.

3.1. Total soluble protein:

The changes in the level of total soluble protein in the supernatant of the homogenated larvae caused insignificant decrease as affected by LC_{25} of *I. carnea* and +ve control that gave (98.58 and 92.31%), relative to (–ve) control (100%). On the other hand, LC_{50} of both chlorpyrifos and *I. carnea* showed remarkable significant increase (140.90 and 127.27 %), respectively. P= 0.0000 Table (2).

3.2. Transaminase enzymes (AST and ALT):

Data represented in Table (2), show the changes in aspartate Aminotransferase (AST) or Glutamic Oxaloacetic Transaminase (GOT) and Alanin Aminotransferase (ALT) or Glutamic Pyruvic Transaminase (GPT) activities of the supernatant cotton leafworm larvae as a result of tested treatment.

Data indicate that all the treatments caused increase in AST activities as compared to (–ve) controls that ranged between an insignificant slight increase 104.47 and 104.94% for control (+ve) and LC_{50} of *I. carnea* respectively to the higher significant increase that recorded 123.91 and 216.30% for LC_{25} of *I. carnea* and LC_{50} of chlorpyrifos, respectively. The statistical analysis showed significant difference among the treatments, P=0.0000.

With regard to ALT activity, all the treatments caused elevation in the ALT activity with the exception of control (+ve) That gave slight insignificant decrease as compared to (-ve) controls (99.18 %), Table (2). Chlorpyrifos (LC_{50}) recorded the highest significant increase 386.12% followed descendingly by LC_{25} and LC_{50} of *I. carnea* (129.60 and 128.16%, respectively), P=0.0000.

Table 2. Changes in the activities of transaminase enzymes and total soluble protein in *S. littoralis* larvae after treatment with *I. carnea* extract and chlorpyrifos.

Treatments	Total soluble protein (gm/100ml)		Transaminase enzymes(AST) mg/L		Transaminase enzymes (ALT) mg/L	
	Concentration	%	Activity	%	Activity	%
LC_{25} of *I. carnea*	1.8436± 0.0603 b	98.58	0.2410± 0.0177 c	123.91	0.1896± 0.0007b	129.60
LC_{50} of *I. carnea*	2.3800± 0.0399 a	127.27	0.2041± 0.0125 d	104.94	0.1875± 0.0047b	128.16
(+ve) control after72hr.	1.7263± 0.0607bc	92.31	0.2032± 0.0075 d	104.47	0.1451± 0.0081 c	99.18
(-ve) control after 72hr.	1.8701± 0.0292b	100.00	0.1945± 0.0030 d	100.00	0.1463± 0.0080 c	100.00
LC_{50} of chlorpyrifos	2.2427± 0.1108 a	140.90	0.7350± 0.0104 a	216.30	0.5062± 0.0139 a	386.12
(-ve) control after 24hr	1.5917± 0.0107 c	100.00	0.3398± 0.0040 b	100.00	0.1311± 0.0003 c	100.00
$L.S.D_{0.05}$	0.1873		0.0326		0.0232	
P	0.0000***		0.0000***		0.0000***	

Data expressed aş Meanı ± Stander Error (S.E.).

Means under each variety sharing the same letter are not significantly different at $P< 0.05$.

*** mean $P\leq0.01$: Highly significant effect.

(-ve) control: using water only. (+ve) control: using Acetone.

Protein is among the most important compounds. The decrease of total protein in treated larvae may reflect the decreased in the activity of various enzymes (El-Kordy *et al.*,1995). Extensive work has been carried out in order to determine how various toxic agents affect protein synthesis. A diminution in the rate of ATP synthesis and inhibition of RNA synthesis are also the main causes of decreased total protein content (Nabih *et al.*, 1990), or may be due to degradation of protein to release energy and conversion of protein to amino acids. These results are

supported by the works of (Essa, 2003) when observed reduction in the level of total soluble protein in *S. littoralis* larvae treated with different plant extracts. However, El-Sheakh *et al.*, (1990) noticed an increase in the total soluble protein after treating 4th instar larvae of *S. littoralis* with LC_{50} of insecticides; osbic, cyanox and soybean phytoalexins (plant extract).

AST and ALT in insect are the most active transaminase enzymes (Carbetree and Newssholom, 1970). The amino transaminase enzyme. Especially ALT is one of the components of oxidative metabolism of protein (Bursell, 1963). Elevation of AST and ALT after exposure to different plant extracts in *S. littoralis* larvae has been investigated by (Mohsen, 1999).

The greeter and continuous release of AST might be due to the necessity of enhancing domination of aspartic acid for the process of gluconeogensis especially under conditions of impaired carbohydrate metabolism.

CONCLUSION

In the light of the present finding, it could be concluded that acetonic extract of *I. carnea* showed biochemical and histological effects against *S. littoralis* larvae. Future studies are necessary to evaluate the mode of action and cost-efficiency of *I. carnea* extracts on wide range of pests as alternative compound in pest control. Such knowledge may help in the control strategies of *S. littoralis*.

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التغيرات فى التركيب الدقيق و البيوكيميائى لدودة ورق القطن المعاملة بمستخلص نبات إيبوميا كارنيا

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نبات ايبوميا كارنيا هو احد النباتات التى تنتشر وتنمو بشكل برى فى البيئة المصرية على حواف الترع و المجارى المائية بطول نهر النيل و قد صممت هذ الدراسة لمعرفة التغيرات البيوكيميائية و التركيب الدقيق لمعى و جليد يرقات العمر الرابع لدودة ورق القطن نتيجة المعاملة بالمستخلص الأسيتونى لهذا النبات مقارنة بالمبيد التقليدى كلوربيريفوس و المستخدم فى مكافحة هذه الآفة وكانت النتائج كالتالى:

طبقا لقيم التركيزات القاتلة لنسبة (٥٠ و ٩٠٪) من اليرقات كان كلوربيروفوس هو الأكثر كفاءة مسجلا٩,٤٩٧ و ٩١,١٢٦ جزء فى المليون على الترتيب متبوعا بالمستخلص الأسيتونى لنبات ايبوميا الذى سجل ٢٤,٦٢٢ و ١٦٤,٩٤٧ جزء فى المليون على الترتيب.

عند دراسة التركيب الدقيق للقطاعات العرضية فى الخلايا الطلائية المبطنة للمعى الأوسط حدث تكثف للمادة الكروماتينية و استطالة شديدة لكل من النواة و الشبكة الإندوبلازمية ، كما تهتكت أهداب المعى و ظهرت العديد من الحويصلات فى السيتوبلازم وذلك بالمعاملة بالتركيز القاتل لنسبة ٥٠٪ من اليرقات لكلا المعاملتين. و لم يقتصر تأثير المعاملات سابقة الذكر على محتويات الخلية فقط و لكن تسببت فى تلف شديد فى منطقة الجليد حيث تغير الجليد السطحى و انفصلت طبقتى الجليد الداخلية و الخارجية عن بعضهما باستخدام التركيز القاتل لنصف عدد الأفراد فى كلا المعاملتين .

كما أظهرت النتائج حدوث نقص غير معنوى في البروتين الكلي الذائب في اليرقات المعاملة بكل من التركيز القاتل من مستخلص ايبوميا لنسبه ٢٥٪ من اليرقات وكذلك المقارنة الموجبة (بإستخدام الأسيتون فقط) عن المقارنة السالبة بإستخدام الماء فقط. بينما أحدث التركيز القاتل لنسبة ٥٠% من اليرقات لكلا من الايبوميا كلوربيريفوس زيادة معنوية في البروتين الكلي الذائب عن يرقات المقارنة السالبة و الموجبة، وبصفة عامه سببت كل المعاملات المستخدمة حدوث زيادة في نشاط الإنزيمين الناقلين للبروتين عن المقارنة السالبة.