TOXICITY, HISTOPATHOLOGICAL AND ULTRASTRUCTURAL EFFECTS OF *ARTEMISIA HERBA ALBA* EXTRACT IN THE MIDGUT OF *CULEX QUINQUEFASCIATUS* LARVAE (DIPTERA:CULICIDAE).

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ABSTRACT

Crude ethanolic extract from Artemisia herba alba,,was tested against the 3rd instar larvae of Culex quinquefasciatus .The larvae were exposed to serial concentrations (2.75 , 2.50 , 2.25 , 2.00 , 1.50 & 1.00 mg/l).The plant extract showed marked effects on larval mortality . Sequental observations using light and electron microscopes on effects of the LC_{50} value were made on gastric ceaca and midgut epithelial cells .Exposure times 6 , 12 , 24 and 48 hours were tested. The toxic effect of the extracts have a wide range of affecting degrees in the alimentary canal. The main alterations observed in gastric caecae and midgut were partial or total cell destruction, high cytoplasmatic vacuolization, disruption of rough endoplasmic reticulum ,cell hypertrophy and the epithelium did not maintain its monolayer appearance. At longer exposure period, the midgut epithelium cells from treated larvae appeared more elongate and swollen than cells observed from control larvae. The microvilli was degenerated. After 48 hours the changes were more obivous, with the apical region of the cell bulging into the gut lumen .The cells contained large cytoplasmic spaces ,cellular damage had increased ,with cells ruptured at the apical surface and leakage of cytoplasmic materials into the gut lumen. Observation of tissues sections from larvae treated for different exposure times revealed a wide and clear variations in the degree of damage occured. The present study provides a quite clear evidence regarding the mode of action of the A. herba alba extract used as bioinsecticidal for controlling mosquito larvae.

INTRODUCTION

Culex quinquefasciatus is a hematophagous insect , it is closely associated with human and is a vector of filariasis(Sarika *et al.*,2006).. Filariasis is caused by *Wucheria bancrofti* (Cobbald), a helminthic worm that lives in lymph glands and vessels that provokes edemas by lymph obstruction. It is responsible for the more severe clinical manifestations in the lower limbs (elephantiasis) and the scrotum (World Health Organization 1992, 1994). The high *Cx. quinquefasciatus* population density in the Cosmo tropical area has triggered several interventions by the public health authorities using wide synthetic insecticide application as the main means of combat and control. However, the defficiency of the organophosphate and carbamate insecticides (Bracco *et al.* 1999, Hemingway *et al.* 1985, Failloux *et al.* 1994), along with the need for safer methods regarding toxicity to man and generally the environment has stimulated the search for new means of control. Studies have proved the potentiality of some plants for use in *Cx. quinquefasciatus* control, such as *Agave americana* and *Kaempferia galanga* (Dharmshaktu *et al.* 1987, *Piper nigrum* (Chahad & Boof 1994), *Azadirachta indica, Rhazya sticta* and *Syzygium aromaticum* (Mishra *et al.* 1995, Su & Mulla 1998, Das *et al.* 1999, El-Hag *et al.* 1999), , Choochote *et al.* 1999, Pizarro *et al.* 1999), *Atriplex halimus* (El-Gougary 1998, Massoud & Labib 2000) and *Commiphora molmol* (Pitasawat *et al.* 1998). Investigation on the histopathological effect of plant extracts on mosquitoes have been reported by some authers (e.g.: Hammouda *et al.*,1996 ;Massoud and Labib,2000 and Arruda *et al.*,2003). The effect of plant extract was mainly localized in the midgut of the tested species. Assar and El-sobky (2003) demonstrated morphological lesions in the larval midgut of *Culex pipiens* which exhibited swollen cells, degenerated brush borders, cell vacuolization. *Artemisia herba alba* plant extracts are an alternative with potential for use.The objective of this study was to investigate the plant extract of *A.herba alba* to find an alternative successful method for *C. quinquefasciatus* control and describes the sequential changes in the alimentary canal of *C. quinquefasciatus* 3rd larval instar treated with $LC_{50's}$ of ethanolic extracts by different exposure time .

MATERIALS AND METHODS

Plants:

The tested plants were extracted by ethanol as solvent, which was evaporated by the rotary evaporator to obtain the crude extract, and stored in stock solution until use.

Mosquito larvae tested:

Laboratory reared strain of *C.quinquefasciatus* was obtained from the Faculty of Science King Saud University, originally collected from Riyadh region .The colony was maintained under laboratory conditions of 27± 2 C° and 70 ± 5 % R.H. The 3rd larvae instar were used through this investigation.

Study procedures:

The *C. quinquefasciatus* larvae used in the tests were reared in plastic basins with water from the public water supply, similar to the methodology used by Silva *et al.* (1998) in an acclimatized chamber with temperature of 28 ± 1°C , 80 ± 5%R.H. and 12 hrs photoperiod.

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Bioassay -

1- Susceptibility testes:

Ten mg from plant Extract was homogeneized using 1000 ml of distilled water, to obtain 10mg/l, then diluted in water to the appropriate concentration according to standard of(WHO ,2005) (1.00, 1.50, 2.00, 2.25, 2.50 & 2.75 mg/l) . Fourty larvae were then pipetted into each 20 ml volume(10 larvae in 4 replicates) . Mortality percentages were recorded after 24 and 48 expoure time, respectively. Larvae were considered dead or moribund if they stopped moving for a prolonged period even after gentle probing with a small spatula, as described by the Guidelines for laboratory and field testing of Mosquito larvicides, World Health Organization's technical report series (WHO/CDS/WHOPES/GCDPP/2005.13). Larvae maintained in distilled water were used as a control. The LC_{50} and LC_{90} were calculated using probit papers and ststistically analysed according to Finney's method (1952)by using SPSS

2- Histological studies:

Only live larvae were used. They were fixed in bouins solution (after 6, 12 , 24 and 48 hours from exposures)..After dehydration in a graded ethanol series, the material was embedded and cut with glass knives in a rotary microtome. The sections were stained with hematoxylin-eosin, examined, and photographed with an photomicroscope. For electron microscopic studies, the midgut was fixed in a solution containing 2.5% glutaraldehyed and 4% paraformaldehyed in 0.1 M phosphate buffer (pH 7.3), postfixed in 1% osmium tetroxide solution in the same buffer, dehydrated in a graded acetone solutions , and embedded .Ultra-thin sections were stained with uranyl acetate and lead citrate before examination.

RESULTS:

1-Susceptibility testes:

Results of susceptibility tests for the extract of *A. herba alba* are presented in tables (1,2,3) & (Fig.1). *A. herba alba* extract caused mortality against larvae of *C. quinquefasciatus* at all tested concentrations. However, the susceptibility of the mosquito larvae was positively correlated to concentration tested, and the period of exposure. Thus, the LC_{50} was 1.81 mg/l after 24 hrs and the LC_{50} value decreased with the increase of exposure time to reach 0.99 mg/l after 48 hrs.

Table (1): Mortality percentages of treated 3rd instar larvae of *C.quinquefasciatus* with different concentrations of *A. herba alba* extract for 24 hours .

Conc. mg/l.	Tested larvae (N0)	Observed Mortality(%)	Expected mortality(%)	Liner log.(con.*10)	Liner probit
1.00	40	10.00	6.89	0.00	3.52
1.50	40	32.50	32.02	0.18	4.53
2.00	40	47.50	60.06	0.30	5.26
2.25	40	67.50	70.89	0.35	5.55
2.50	40	82.50	79.24	0.39	5.82
2.75	40	92.50	85.40	0.44	6.05
Control	40	0	0		

Table (2): Mortality percentages of 3rd instar larvae of *Cx.quinquefasciatus* after treated with different concentrations of *A. herba alba* extract for 48 hours .

Conc. mg/l.	tested larvae (No)	Observed Mortality(%)	Expected Mortality(%)	Liner log.(con.*10)	Liner probit
1.00	40	20.00	15.458	0	3.983
1.50	40	45.00	48.933	0.176	4.973
2.00	40	65.00	75.045	0.301	5.676
2.25	40	85.00	83.235	0.352	5.964
2.50	40	90.00	88.894	0.398	6.221
2.75	40	97.50	92.689	0.439	6.454
Control	40	0	0	0	0

Table (3): Insecticidal potency of *A.herba alba* extract on 3rd instar larvae of *Cx.quinquefasciatus* at different exposure periods.

Exposure Period (hrs)	50% confidence limits			Lc_{90} (mg/l)	95% confidence limits
	LC_{50}(mg/l)	Lower.....upper	slope±SE		Lower...upper
24	1.81	1.66.....1.94	5.78±0.72	3.01	2.71 .....3.53
48	0.99	0.84.....1.14	3.46±0.42	2.34	1.99 ... 2.92

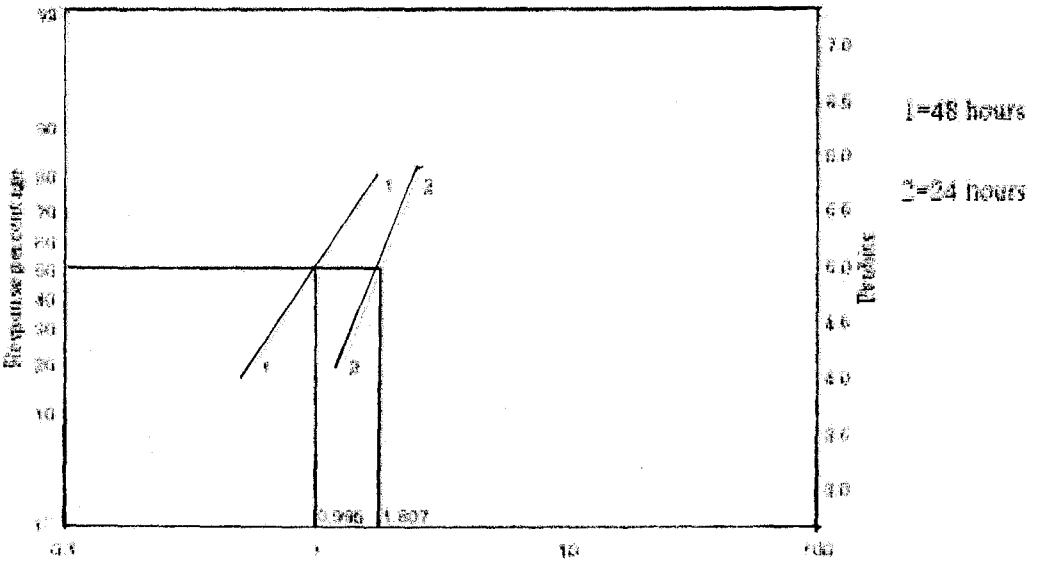

Fig. (1): Insecticidal potency of *A.herba alba* extracts on 3rd instar larvae of *Cx.quinquefasciatus* mosquitoes at different exposure periods.

2- Histological studies:

The alimentary canal of *C. quinquefasciatus* possesses a foregut, midgut and hindgut .In longitudinal sections of the thorax region, alimentary canal forms the special folding region that consists of posterior oesophagous , foregut and anterior midgut. These folding regions are followed by gastric caeca , which arise the balloon like , they are two in number in the longitudinal section (Fig. 2) , the present study only deals with the gastric caeca and midgut .A series of cross section through the thoracic and abdominal regions of untreated and treated 3rd larval instar were studied after (6,12,24&48 hrs) of exposure to the resulted LC_{50} of : *A. herba alba extract.*

A- Gastric ceaca

The non-treated gastric caeca cells are massive and have brush border (microvilli) occupying a large part of the thorax ,and it could be seen as 10-12 cells in the one caeca . Cells are large and massive with dark staining particles , the large nuclei occupy most of the cell (Fig.3).In treated larvae , cells of the gastric caeca with 1.81 mg/l of *A. herba alba* after 6 hours showed slight change in the cell (Fig.4). After 12 hrs the epithelium did not maintain its normal appearance. Some cells presenting an irregular structure brush border within 6-12 hrs, and became less conspicuous than normal , where they did not adhere to each other. In some parts they were elongated in shape, bulging from their free surface (Fig.5).The cells appeared swollen by a slight vacuolization after 24 hrs. (Fig.6).This vacuolization increased with increase number of secretion vesicles in the lumen after 48hrs. (Fig 7).

B-Midgut:

The midgut untreated of *C. quinquefasciatus* larva showed a well-preserved layer of epithelial cells. The ovoid shaped nuclei are located in the center of the cells with clear or finely granular cytoplasm. Also, there was a variable number of small darkly staining cells located at the bases of columnar cells(the regenerative cells). (Fig.8).Long and regularly placed microvilli border in the midgut lumen (Fig.9). The midgut wall of the *C.quinquefasciatus* larvae consists of cellular peritrophic membrane that delimited the midgut lumen in ectoperitrophic and endoperitrophic spaces.Light microscopy revealed histological alterations in the midgut epithelium of treated larvae.It showed less stained cells detached from neighboring cells or from the basal lamina. The midgut of untreated larvae is exclusively composed of regular stained columnar cells.

The midgut of larvae exposed to 1.81 mg/l of *A.herba alba* extract showed some cells presenting on irregularly structured brush border (Fig.10). Gradually after 12hrs, the cells begin to be swollen by a slight vacuolization, the nuclei moved to margin of some cells , and columnar in shape with a globular structure bulging from their free surface (Fig.11,12).After 24hrs of exposure, the cell layer was irregular and increased in number , no continuously disposed brush border can be identified.Also,the nuclear chromatin in cells clumped into large irregular appearance and the structure changes occure in some of the epithelial cells. There were cell destruction and increase of subperitrophic space, which filled of some epithelial cells contents and detached of basement membrane in some places.(Fig.13).The epithelium was short and thick in shape. The peritrophic membrane was partially damaged. Some epithelium cells presented bubble shaped tips (Fig.13). Finally when 48hrs of exposure passed, the epithelial cells lost their normal appearance. The structural disorganization was evident, become elongated with destroyed tips. The space between the epithelial cells and peritrophic membrane decreased, (Fig.14) with irregularly and modified microvilli. Some midgut columnar cells extruded their cytoplasmic contents into the midgut lumen. This feature is confirmed by ultrastructure observation. From 24 to 48 hrs after exposure, structural changes occured in some of the epithelial cells showing increased in morphological changes of the epithelium. Most of the cells were vacuolated, with an increased number of secretion vesicles and an irregularly disposed brush border comparing with untreated (Fig.15,16). After 48 hrs, the pathological effects were observed in nearly all of the intestinal cells. The epithelium showed irregularly microvilli with bubble shape tips. Strong vacuolization of columnar cells and strong alterations of cytoplasmic structure and organelles were observed (Fig.17,18).

DISCUSSION

Today, the environmental safety of an insecticide is considered to be of paramount importance. An insecticide does not have to cause high mortality on target organisms in order to be acceptable (Kabaru and Gichia,2001). Phytochemicals may serves as suitable alternatives to synthetic insecticides now a days as they are relatively safe, inexpensive and are already available in many parts of the world. According to Bowers *et al.* (1995) the screening of locally available medicinal plants for mosquito control would generate local employment, reduce dependence on expensive imported chemicals and stimulate local efforts to enhance public health. Ethanolic suspensions of different concentrations of *A.hrba alba* extract were tested against the third instar larvae. conc.-response curves obtained after 24 hrs and 48 hrs of treatment were exponential, showing that the extract was very effective against *C. quinquefasciatus* larvae. The crude extracts of *A.herba alba* has been found to possess larvicidal activity against the mosquito *C. quinquefasciatus*. The biological activity of the plant extract might be due to the various compounds, these compounds may jointly or independently contribute to produce larvicidal toxicity against *C. quinquefasciatus* larvae. Studies on the larvicidal properties of *A.herba alba* extract for culicids are still vacant . However, studies with other plant extracts taking on the basis of their lethal effects when used, served as reference for the potentiality of *A.herba alba* extract for use in *C. quinquefasciatus* control. Pizzarro *et al* (1999) studied the activity of the saponine fraction of *Agave sisalana* and estimated the LC_{50} and LC_{90} against 3rd larval instar of *C. quinquefasciatus,* that were 183 and 408 ppm, respectively. These concentrations were much higher than those reported in the present study, but these authors suggested its use for control of this mosquito. The extract of *A.herba alba* is superior to various neem extracts, which were reported to be effective with LC_{50} values ranging from 55-65 ppm against mosquito larvae (Ascher and Meisner 1989). The median lethal concentrations (LC_{50}) of various parts of *Melia azederach* ranging from 30-40 ppm against larva of *Culex pipiens* (Al-Sharook *et al.*, 1991). The effect of various neem extracts and various parts of *Melia azederach* was slightly lower than that reported for the *A.herba alba* extract.

The insect midgut is the largest portion of the digestive tract, playing a major role in the absorption of nutrients, chemical and biological insecticides (Wigglesworth,1972). In our experiments, which were ended after 48 hrs of exposure to *A.herba alba* extract, *C. quinquefasciatus* larvae could survive when a short time of exposure was applied.. Histological alterations of some columnar cells of the midgut epithelium to the extract were observed during the exposure time tested. Nevertheless, after 6 hrs of toxin action well preserved

groups of cells located at the base of the epithelium next to the basal membrane indicated that cell recovery was in progress. By increasing the exposure period, nearly all columnar-cells were affected and no preserved cell groups could be detected next to the basal membrane of the midgut. The mosquito larvae secrete a layer of non-cellular material which separates the food from the epithelial cells of the gut. This layer is called peritrophic matrix PM. The PM acts as a protective barrier against various chemical, physical and microbial food components (Peters 1992). In this study, larval mortality caused by methanol extract of *A.herba alba* appears to be related to disruption of PM structure and rupture of midgut cells.. The enlarged PM pores allow the passage of ingested food material to midgut epithelium (Harper et al. 1998, Wang & Granados 2000).Ultrastructural observations showed that the first cell damage due to the plant extract tested in the *C. quinquefasciatus* larvae midgut was related to brush border microvilli degeneration. As illustrated in Percy and Fast (1983) using purified *Bt.* crystal toxin (1 g/l) against silkworm larvae, the dissolution of cytoskeleton structures inside and at the basis of the microvilli were responsible for its decrease in size and further disappearance, when bubbles of cytoplasmic substances protrude into the midgut lumen as in *C. quiquefasciatus*. At this stage, before the cell death, the columnar cells appeared more elongated which detected by the light microscope. The use of *A.herba alba* originated vacuolization of the midgut epithelial cells as in the different experimental models in previous studies using *Bt.* endotoxin (Percy & Fast 1983, Charles & Barjac 1983, Rey et al. 1998), as also in *C. quinquefasciatus*. These vacuoles proceeded from enlarged rough endoplasmic cisterns that had lost their ribosomes (Percy & Fast 1983). The present article could be consider to be the first investigation concerned with the histopathological effects of *A.herba alba* extract in the gastric ceaca and midgut of *C. quinquefasciatus* larvae .Finally , the data obtained may contribute for better understanding the mode of action of this plant extract used as bioinsecticide against *C. quinquefasciatus* larvae. The findings of this present investigation revealed that the extract of *A.herba alba* possess remarkable larvicidal activity against mosquito, *C. quinquefasciatus*. So, further investigations are needed to elucidate this activity against other mosquito species .In addition, the active ingredient(s) of the extract responsible for larvicidal activity in *C. quinquefasciatus* should be identified and utilized, if possible, in preparing a commercial product / formulation to be used as a mosquitocidal

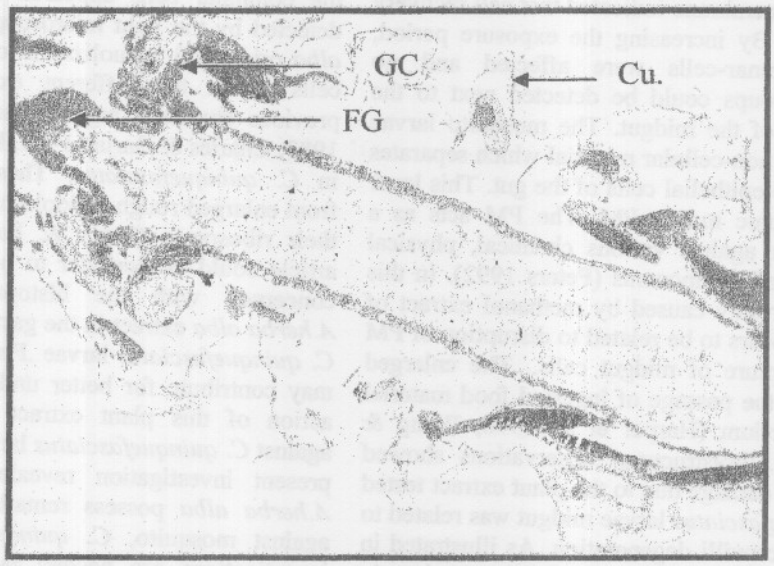

Fig. (2) : The *C. quinquefasciatus* larva midgut showing a single layered epithelium delimiting the lumen . Gastric caeca(GC),Cuticle(CU), forgut (FG) (X40)

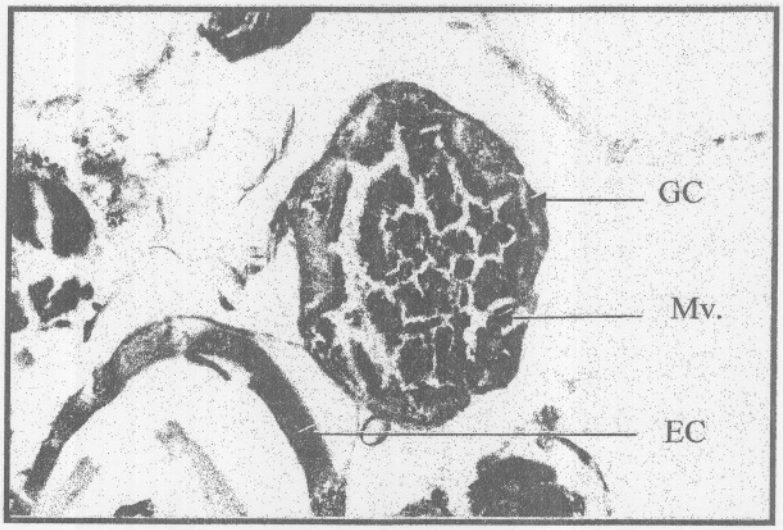

Fig. (3): Photomicrograph of cross section through the thoracic region of normal untreated *C. quinquefasciatus* 3rd instar larva showing the foregut-epithelia cells (EC) and the gastric caeca (GC) , Microvilli (Mv) (X200)

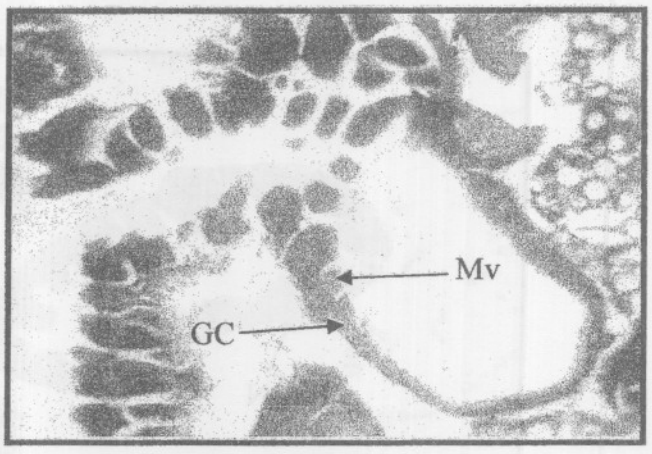

Fig. (4): Photograph of cross section through the gastric caeca of the 3rd instar larvae of *C. quinquefasciatus* treated with *A.herba alba* extract showing the effect after 6 hours of exposure (X400)

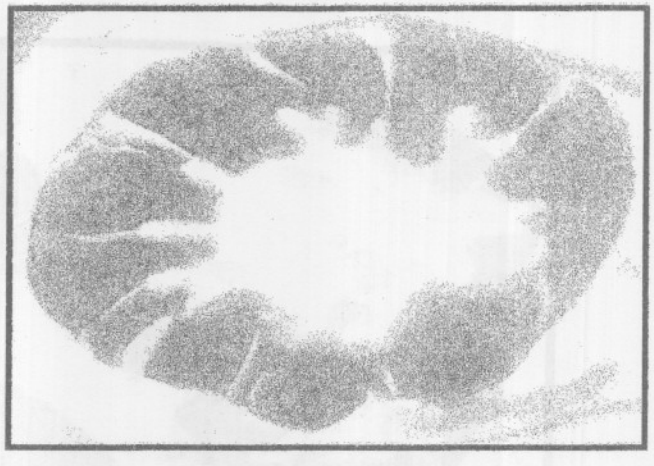

Fig. (5): Photograph of cross section through the gastric caeca of the 3rd instar larvae of *C. quinquefasciatus* treated with *A.herba alba* extract showing the effect after 12 hours of exposure (X400)

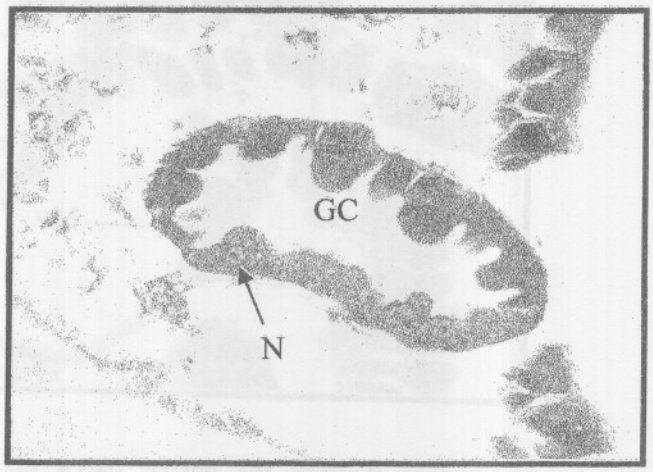

Fig. (6): Photograph of cross section through the gastric caeca of the 3rd instar larvae of *C. quinquefasciatus* treated with *A.herba alba* extract showing the effect after 24 hours of exposure (X400)

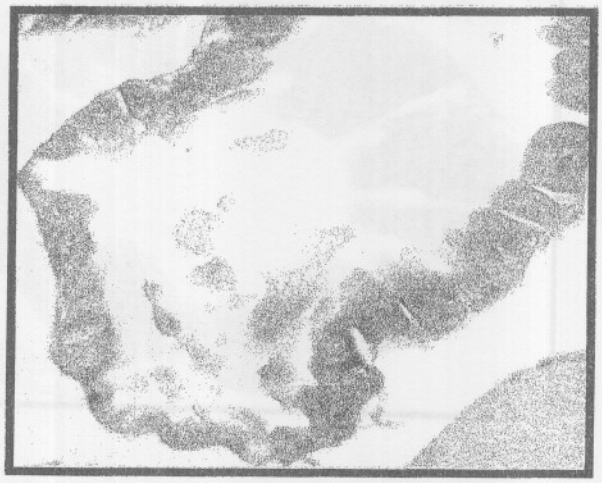

Fig. (7): Photograph of cross section through the gastric caeca of the 3rd instar larvae of *C. quinquefasciatus* treated with *A.herba alba* extract showing the effect after 48 hours of exposure (X400)

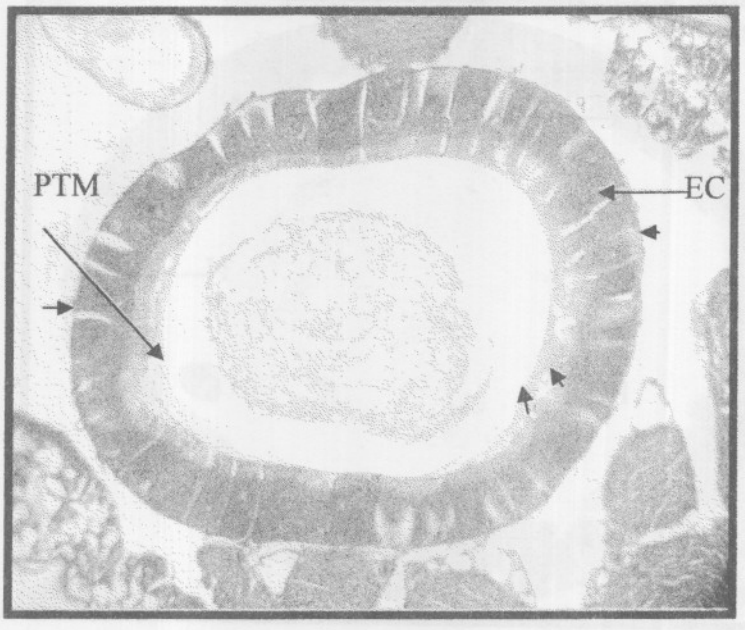

Fig. (8): Photograph of cross section through the midgut of untreated *3rd* instar larvae of *C. quinquefasciatus* showing the epithelial cells (EC), Perirophic membrane (PTM). Regenerative cells (arrows) (X400)

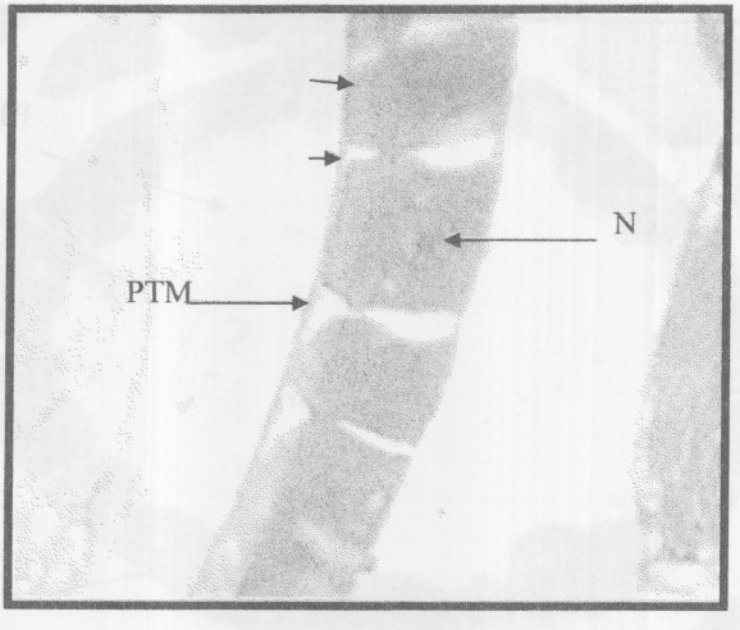

Fig. (9): The continuous brush border (arrows) characterizes the midgut epithelium in untreated *C. quinquefasciatus*3rd instar larvae. peritrophic membrane (PTM) nucleus (N),) (X1000)

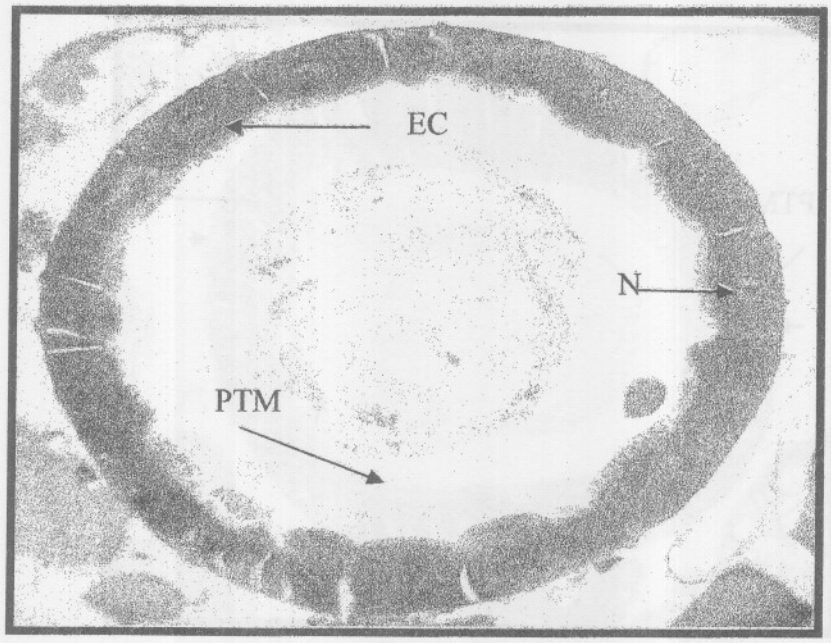

Fig. (10): Photograph of cross section through the midgut of the 3rd instar larvae of *C. quinquefasciatus* treated with *A.herba alba extract* showing the effect after 6 hours of exposure on the epithelial cells (EC), Peritrophic membrane(PTM)and nucleus(N)(X400)

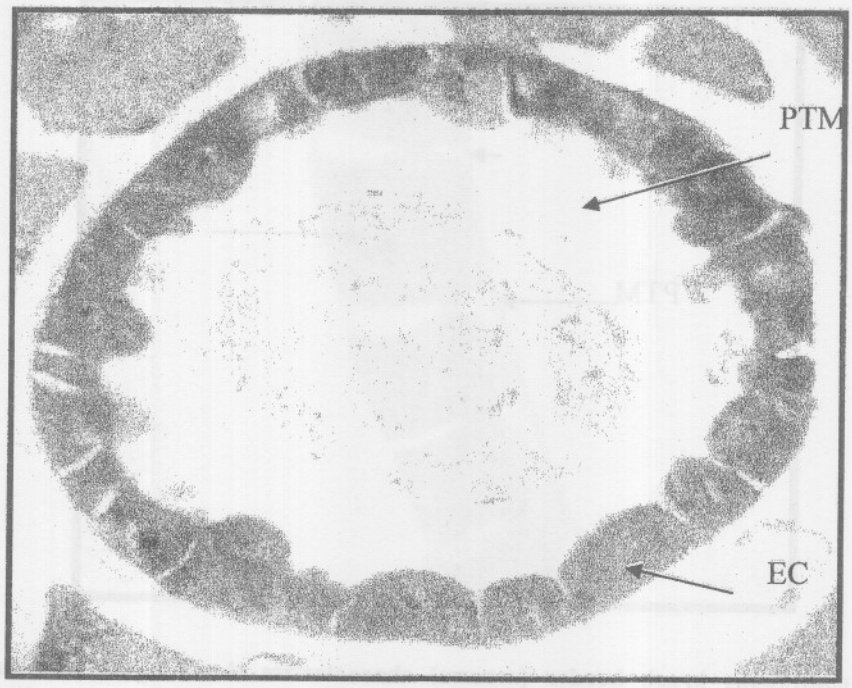

Fig. (11): Photograph of cross section through the midgut of 3rd instar larvae of *C. quinquefasciatus* treated with *A.herba alba* extract showing the effect after 12 hours of exposure on the epithelial cells (EC)and Peritrophic membrane(PTM)(X400)

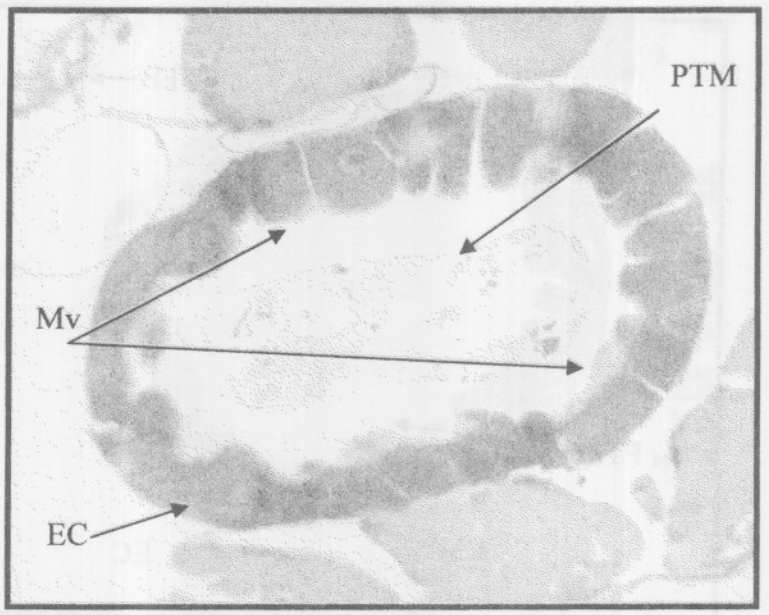

Fig. (12): Photograph of cross section through the midgut of the 3rd instar larvae of *C. quinquefasciatus* treated with *A.herba alba extract* showing the effect after 12 hours of exposure on the epithelial cells (EC)and Peritrophic membrane(PTM)(X400)

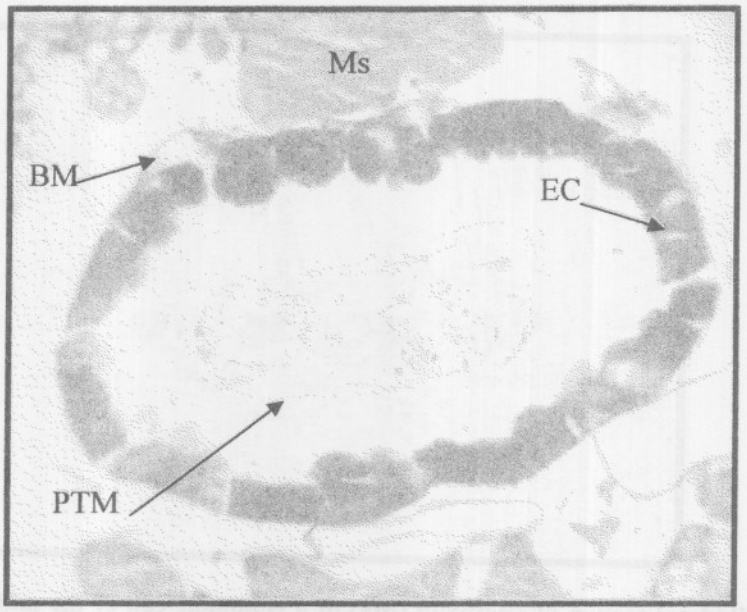

Fig. (13): Photograph of cross section through the midgut of the 3rd instar larvae of *C. quinquefasciatus* treated with *A.herba alba extract* showing the effect after 24 hours of exposure on the epithelial cells (EC) and Peritrophic membrane(PTM)(X400)

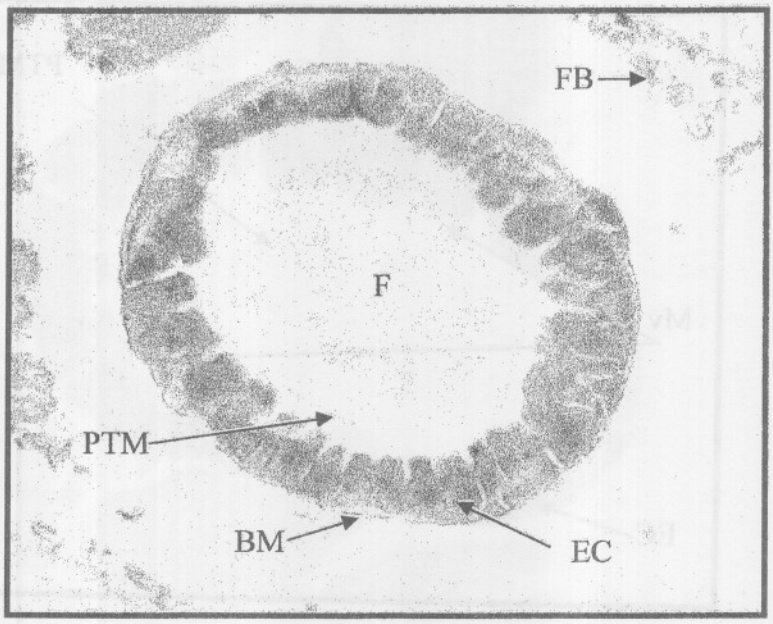

Fig. (14): Photograph of cross section through the midgut of the 3rd instar larvae of *C. quinquefasciatus* treated with *A.herba alba extract* showing the effect after 48 hours of exposure on the epithelial cells (EC) and Peritrophic membrane(PTM)(X400)

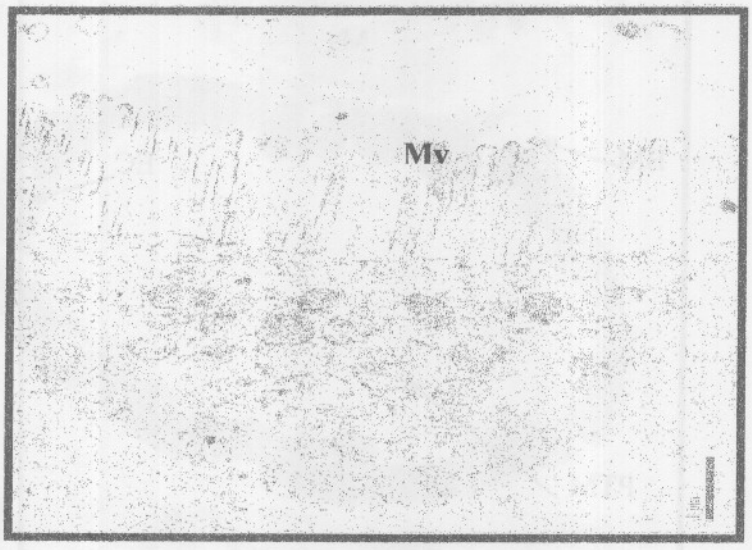

Fig. (15): Longitudinal section of microvilli (Mv) of a columnar cell of the midgut in a control larvae of *C. quinquefasciatus*. Transmission electron micrograph.

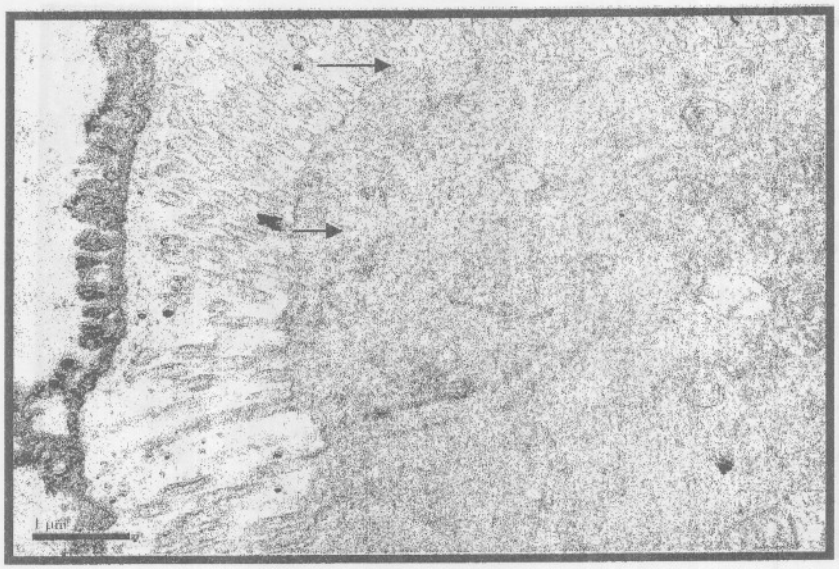

Fig. (16): Longitudinal section of microvilli of a columnar cell of the midgut in a treated larvae of *C. quinquefasciatus* after 24 hr. Transmission electron micrograph. Microvilli (arrow)

Fig. (17): Longitudinal section of microvilli of a columnar cell of the midgut in a treated larvae of *C. quinquefasciatus* after 48Transmission electron micrograph.Microvilli (arrow)

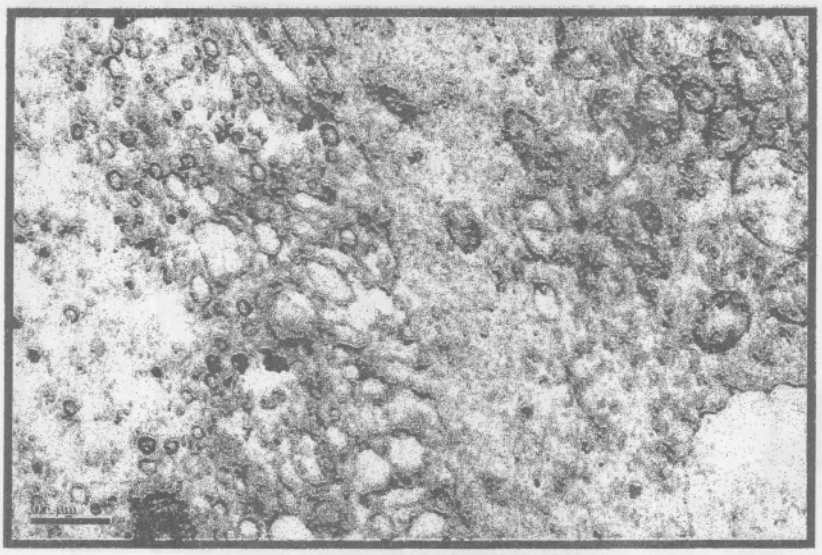

Fig. (18): Longitudinal section of microvilli of a columnar cell of the midgut in a treated larvae of *C. quinquefasciatus* after 48 .Transmission electron micrograph. Note the strong vacuolization of columnar cells and strong alterations of cytoplasmic structure and organelles.

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الملخص العربى

التأثيرات السمية والنسيجية المرضية لمستخلص نبات الشيح *Artemisia herba alba* على المعي المتوسط ليرقات بعوضة *Culex quinquefasciatus*

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اختبر المستخلص الايثانولي لنبات *Artemisia herba alba* ضد يرقات العمر الثالث لبعوضة *Culex quinquefasciatus* تم تعريض اليرقات لسلسلة من التركيزات (٢,٧٥ ، ٢,٥٠ ، ٢,٢٥ ، ٢,٠٠ ، ١,٥٠ ، ١,٠٠ ملجم / لتر). أظهر المستخلص النباتي تأثيرات واضحة على نسب الموت. تمت متابعة التغيرات النسيجية لتأثير قيم (LC_{50}) على الردوب الكبدية والمعي المتوسط باستخدام المجهر الضوئي بعد (٦،١٢،٢٤،٤٨ ساعة) وباستخدام المجهر الالكتروني بعد ٢٤،٤٨ ساعة.

وأظهر التأثير السمي للمستخلص النباتي مدى واسعا من درجات التأثر في القناة الهضمية. والتغيرات الأساسية التي تمت ملاحظتها في الردوب الكبدية والمعي المتوسط كانت تحطم جزئي أو كلي للخلية ، فجوات سيتوبلازمية، تحطم في الشبكة الاندوبلازمية ، تضخم خلوي ، وفقد الطلائية لمظهرها الأحادي الطبقة. وبزيادة مدة التعرض يصبح مظهر الخلايا الطلائية أكثر استطالة وانتفاخا عن مثيلتها في حالة اليرقات غير المعاملة وتتحلل الخملات. وتصبح التغيرات بعد ٤٨ ساعة أكثر شدة باندفاع قمم الخلايا المنتفخة الى داخل تجويف المعي واحتواء الخلايا على فراغات سيتوبلازمية كبيرة ، ويزداد الضرر الخلوي باندفاع مكونات الخلية وخروجها الى تجويف المعي. أظهرت القطاعات النسيجية من اليرقات المعاملة في فترات زمنية مختلفة درجات واسعة من الضرر. وبناء على الفعالية التي أظهرها المستخلص الايثانولي لنبات *A.hera alba* فان الدراسة الحالية تقدم دليلاً جيداً لاستخدامه كمبيد حيوي ضد يرقات البعوض.