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Research article

Histological effects of some essential oils combination on different tissues of the black cut worm larvae *Agrotis ipsilon* (Hufn.)

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Key words: Essential oils, Combination, Histological studies, Black cut worm, *Agrotis ipsilon.*

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Abstract

Plant essential oils and terpenes may be used as a spray or as a bait feeding trap for controlling *A ipsilon* as an alternative means of chemical pesticides in IPM programs. Oil combination promotes their effect and decrease insect buildup resistance because it contains many different terpenes, which have different modes of action. The combination of LC_{50} concentration of Garlic oil at (0.030%) and Mint oil (0.160%) in one artificial diet for the 3rd larval instar of *A.ipsilon* cleared great changes have been detected by light microscope in different larval tissues compared with the untreated control larvae. No pathological changes were detected on the fore gut, salivary gland or malpighian tubules, most of the histopathological changes were mostly localized in the mid gut, hind gut, fat body, integument and trachea. Different cells of the mid gut exhibited a swelling, appearance and microvilli showed complete disorders in many areas, increasing in goblet cells secretion with rupture of basement membrane, many vacuolation occurred in the cell cytoplasm. Tracheal tubes become narrow; Muscle fiber showed relaxation and disintegration of the fibrils, hind gut showed lyses in the intima layer, disintegration of some of the epithelial cells. Vacuolization of the fat bodies occurred. Detachment of the cuticle from epidermis in some areas. This article is the first record on the histopathological effects of the combination of natural essential oils (Garlic+ Mint) as a bio-insecticide on different tissues of *A. ipsilon* larvae and the data may contribute to a knowledge of the mode of action of this combination used as a bio-insecticide against the larvae of this insect pest. So the results suggested that the combination of the natural plant essential oils of garlic with mint in one spray solution may be used in IPM program against *A. ipsilon* larvae.

Introduction

The black cutworm, *A. ipsilon* is one of the most destructive insect pests attacking different field crops, such as cotton, soybean, corn, potatoes and tomatoes not only in Egypt but also in several countries throughout the year. Great losses occurred in yield due to *A. ipsilon* infestation especially at seedling stage [1]. The black cutworm control is currently based on heavy use of many insecticides, which damage the environment and/or pose a threat to public health via food residues, ground water or accidental exposure. The problems caused by pesticides and their residues have amplified the need for effective and biodegradable pesticides with great selectivity [2]. Because of the power of plant-insect interactions, the plant have well-developed a defense mechanism against herbivores and are excellent sources of new toxic substances for pests [3]. Plant essential oils and terpenes show wide and varied bioactivities against both agriculture pests and medically important insect species, ranging from toxicity with ovicidal, larvicidal, pupicidal and adulticidal activities to sublethal effects, including oviposition deterrence, antifeedant activity and repellent action as well as they may affect on biological parameters such as growth rate, life span and reproduction [4-6]. Accordingly, the use of plant essential oils or their constituents of terpenes can lead to the identification of new bio-insecticides. Sharaby *et al.* [7] recorded that Combination of some volatile oils and some terpenes increased their action and toxicity against *A. ipsilon*, the most effective mixture was garlic oil + Mint oil. Larvae that treated with emulsion of median lethal concentration (LC_{50}) essential oils cleared some biological differences in their development and caused malformation in larval and pupal stage. Jember *et al.* [8] mentioned the importance of oil

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combinations or their constituents of terpens in increasing their toxicity for controlling the stored grain pests and attributed that to variable constituents of each oil with variable side effects on the insect, this support the explanation that the oils differ in their toxicity according to their constituents. Labrano *et al.* [9] found it should be pointed that the synergistic effects of complex mixture such as essential oils are thought to be important in plant defense against herbivore predators. Plants usually present defenses as a set of compounds, thus, complex essential oils may be more efficient than individual pure compounds. Combination of Garlic at concentration of (0.03%) + Mint at (0.16%) oil increased their toxicities [7]. Insects have only a few cells that have to undergo division, a process known to be highly susceptible to damage by exposure to insecticides , some of these cells are placed in the epithelium of the mid gut, where they renew cells responsible for digestion and absorption of the products of digestion. Division of epithelial cells occurs as new cells grow in to replace old cells in a very metabolically active tissue. Several applications of essential oils, monoterpenoids and IGRS exhibited histopathological changes in the integument, mid gut and ovaries of insects [10-15]. Aim of the present research is to clear histological changes of different larval tissues to find out the mode of action of Garlic+ Mint essential oil combination on the life and development of the larval stage of *A. ipsilon* and considering this combination an alternatives bio-insecticides that may could be used through the integrated program of the insect.

Experimental

The tested insects: The tested insects in this study were obtained from a standard laboratory culture reared on artificial diet as that described by [16] at a constant temperature of 25±5ºC and R.H 65±5% under a photo period of 14 light and 10 dark [17].

The tested oils: Garlic and Mint oils were obtained as a pure component from Sigma Co (St. Lous. Mo. AU.S.A).

Larval treatment: Third larval instar were obtained from the standard laboratory culture that reared on artificial diet, concentration of the LC_{50} of each of the two tested oils (0.030 for Garlic and 0.160% for Mint oils (that previously estimated by Sharaby A *et al.* [7] were mixed in one diet through its preparation (ml of oil/100ml diet), then the larvae left to fed on the treated diet in one combination for one day, the treated larvae after feeding were transferred for continues feeding on an untreated diet for another 7 days, each larva in a separate jar to prevent cannibalism habits. The remained still living larvae were obtained for the histological studies.

Histological studies: The remaining treated larvae after feeding for 1 day on the treated diet then on an untreated diet for another seven days were used for histological examinations; control groups that fed on untreated diet were also examined. Whole larvae were fixed in freshly prepared alcoholic Bouin's solution for 24 hrs. The specimens were washed with 70% ethyl alcohol and larvae preserved in 70% ethyl alcohol, then dehydrated in ascending alcoholic series (70, 80, 90, 96 and 100%) 1/2h for each and cleared in xylen for a few seconds, then specimens were infiltrated in three changes of hot paraffin wax each lasted 20 minutes (Xylol dish and three wax dishes wax I, wax II and wax III) in 50-52C° oven for 1/2h each, respectively. Embedding was made in hot paraffin wax by using standard plastic cups. The Paraffin blocks were so solidified by putting it in cold water. The blocks were then stuck to the holder of the rotary microtome; transverse sections were cut at five micron thickness. The ribbons of the sections were floated on slides coated by smear of egg albumin using drops of hot water. The preparations were kept on hot plate at 40°C to separate the wax ribbons. After complete evaporation of water in a drying oven (for at least 24h), the slides were dipped in xylol for 3-5 min and in a descending series of ethyl alcohol; each for 2 min (100, 90, 80 and 70%). Thereafter the slide was dipped in distilled water and stained in hamatoxylin for 30-45 min the slides were transferred to tape water for 2 min and then distilled water. The slide was then stained in 1% eosin as a counter stain for 5-10 sec and rapidly were rinsed in an ascending alcohol series (70, 80, 90 and 96%) and put in absolute alcohol for 10 min to remove any water residues and pass into two changes of xylol for 10-15 min each. Finally the slides mounted in Canada balsam and covered with a cover glass and were dried at 40°C for a day. The sections were examined and photographed by a light microscope.

Results and Discussion

Changes on the mid gut: The midgut consists of a unicellular layer (epithelium) resting upon a basement membrane. This membrane is surrounded externally by circular and then by longitudinal muscle fibers. The epithelium consists of columnar and goblet cells with clusters of small regenerative cells each of which contains a relatively large nucleus and strongly basophilic cytoplasm (Figure1A, C). The epithelium is also protected from the food particles by a detached sheath peritrophic membrane, surrounding lumen. Also as seen in figure 1 A & C is the control (normal) mid gut appears with the luminal surface of the epithelium which is provided with a striated border constituting long microvilli. Such microvilli protect inside the lumen and to increase the absorption surface of the cells as well as the space between them act as a kind of sieve.

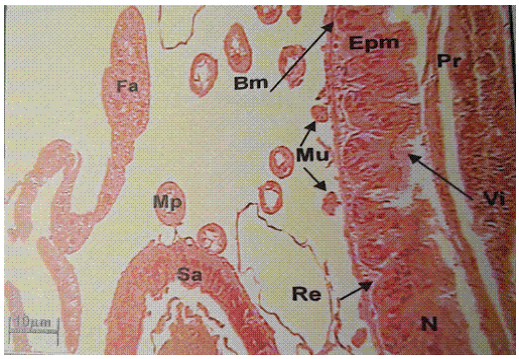


Figure 1, A. Cross section through larva abdominal segments in mid gut area, show different normal tissues.

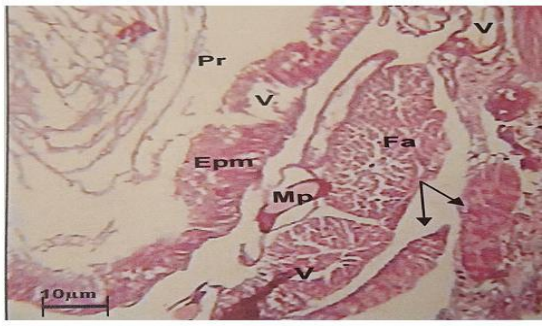


Figure 1, B. Cross section through larva abdominal segments in mid gut area, show different damaged tissues after treatment.

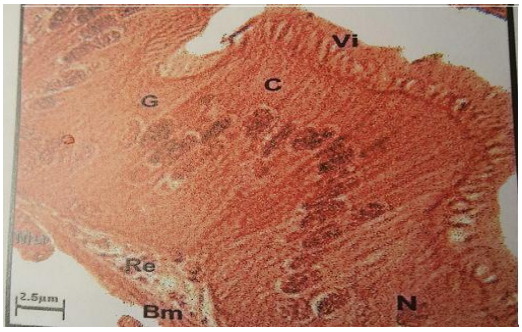


Figure 1, C. Normal midgut epithelial cells, show normal columnar, regenerative cells, goblet cells, will developed microvilli, and basement membrane.

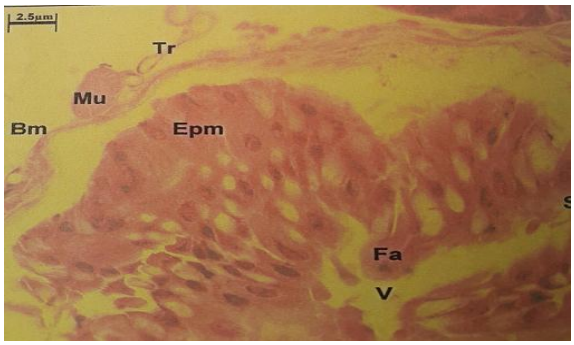


Figure 1, D. Section through damaged and vacuolated midgut, epithelial cells detached from the basement membrane showing many vacuoles.

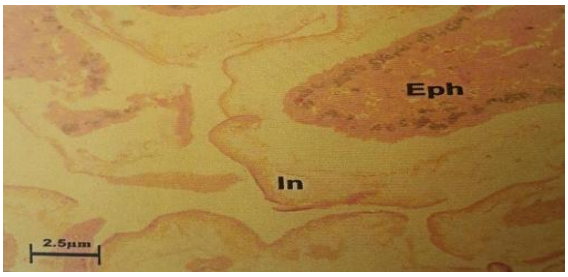


Figure 2, A. Section through normal hindgut, show Intema and epithelial cells of the hindgut.

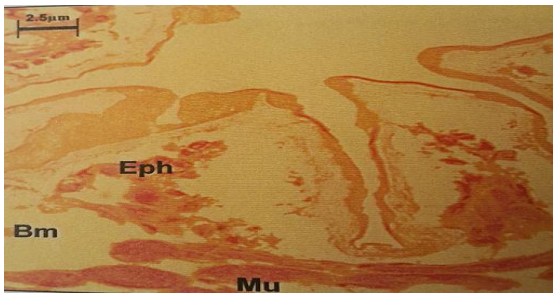


Figure 2, B. Section through treated hindgut, show destructive intema and damaged the epithelial cells.

Great effect were found in the development and morphology of the larvae treated with a combination of the median lethal concentration of both Garlic and Mint essential oil, few numbers of larvae remained alive reached 4th larval they were in small size with slow motion cleared symptoms of death , while the control untreated larvae reached 6th larval stage. Histological studies cleared no changes was observed on fore gut, malpighian tubules and salivary glands. Most histopathological changes were detected in mid gut, hind gut, fat bodies, outer and inner cuticular layer and on some of trachea that connected with the hind and in muscular layers. The most affected tissue was the mid gut epithelium when compared with the untreated mid gut. The epithelium possessed deeply stained nuclei, the regenerative cells were not pronounced and could not been identified in some areas at the base of the epithelial cells due to the sever destruction of the epithelium, goblet cells increased their secretion (Figure 1 B, D), as those in the control (Figure 1 A, C) . Epithelial cells of the treated larvae were destroyed, large vacuoles were found between the epithelium and the muscular layer, regenerative cells were separated from each other at the base of the epithelium and the muscular layer became shrinkage than of the control, great destruction in the epithelium, large and many vacuoles were present in the cells cytoplasm, irregular and disappear cell boundaries, damage in villi and decreased its size and the muscular layer was broken in many places.), apical swelling into the gut lumen, reducing intercellular contacts with the neighboring cells and degeneration of the nuclei and brush border (Figure 1 D). In comparing with the control that shows normal intercellular contact along the whole lateral plasma membrane, normal nuclei, a well-developed brush border, and normal adhesive basement membrane as observed in

(Figure 1 A & C). Our results agreed with the results of Abdullah MAR [18], who noticed histopathological lesion, dissolved of nuclei and destroyed of epithelial cells of *Rhynchophorus ferrugineus* larvae poisoned by *Buxus chinensis* oil and precocene II. The results also are in agreement with Sharaby A *et al.* [13] who observed Sever effects on the alimentary canal and fat bodies of 1st nymphal instar of *Heteracris littoralis* after treatment with LC_{50} concentration of three essential oils (garlic, mint and eucalyptus), these effects comprise destruction of epithelial cells, microvilli and the peritrophic membrane were curled and rupture than those of control treatment. Our studies cleared separation of the epithelial cells from the basement membrane with damage of the peritrophic membrane leading to the mixing of the gut contents with the haemolymph caused the larval mortality. The microvilli enhanced the rate of absorption [19], the swollen and elongation protruded of villi into its lumen as a bulbous eversion was a result of enzymatic activity of the epithelial cells [20]. These results are in agreement with findings of Shoukry IF *et al.,* Sabry HM, Ibrahim A A. [21-23].

Changes on the hind gut: Colon consists of a layer of epithelial cells with a big nucleus based on basement membrane and covered at their internal side with cuticular layer called intema and surrounded with muscular layer (Figure 2A) . Histopathological changes cleared damage in some places of the epithelial cells and partially separation on the intema layer, great disturbance in the epithelial nucleus, vacuolization and shrinkage and rupture in the basal basement membrane in some aeries (Figure 2 B). These observation are agreed with that mentioned with Armah CN *et al* [24] on *Schistocerca gregaria* and Sharaby A *et al.* [13] on the grasshopper *Heteracris littoralis* who recorded a partial loss of cellular integrates which reached even the muscular bases leading to a loss of integrity of the basement membrane of the cells and there were partial changes including the intima layer that lined the cells was affected to some extents.

Changes on the fat body: The untreated larvae show a visceral and parietal fat body made up of cellular clusters, which often take the form of cellular cord (Figure 3 & 5 A). The cells appear rectangular or irregular cells surrounded by a membranous sheath with core and cytoplasm filled with many lipid droplets (Figure 5 B). The treated fat cells became destruction separation from each other, noticeable vacuolization, destruction of the membranous sheath (Figure 4 B). This tissue loss is often accompanied by cellular excretion causing a loss of the cellular content. Chaibe, I *et al.* [25] speculated that the destruction of the cellular structure is probably due to the membrane disruption followed by the rupture of the membrane and the discharge of the cellular contents and that provokes cell death. The same authors revealed a cytotoxic effect of Certum perqui saponins on the fat body of *S.littoralis* larvae, the cells of this tissue decreased in size and became more colored by loss their cytoplasmic content, they concluded that saponins interact with membrane cholesterol, this cause a membrane destabilization and that leading to the cell death.

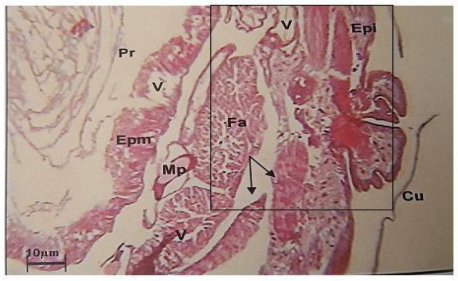


Figure 3. Section through normal visceral and parietal fat tissues and muscular layer.

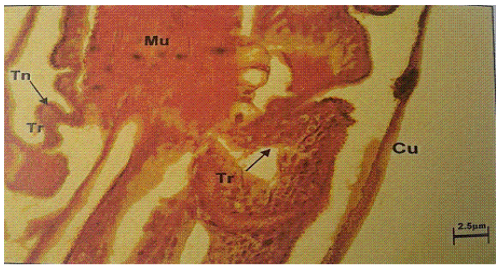


Figure 4, A. Section through normal trachea

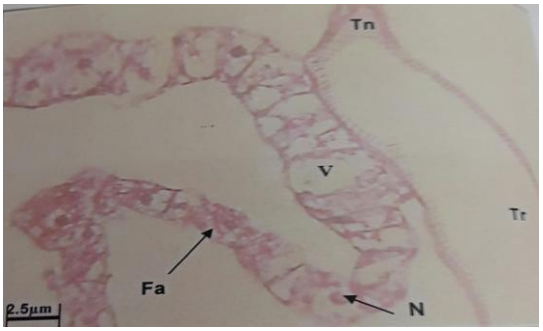


Figure 4, B. Section through degenerated trachea and ruptured fat tissues.

Changes on the Integument: The larvae have a soft thin cuticle over most of their body (Figure 5 A). The cuticle consists of a row of epithelial cells lying resting on connective tissue or basement membrane followed by an inner layer (endocuticle) then an outer layer (exocuticle).
Histological changes of the oil combination on the cuticle (Figure 1B, 5B & C) cleared detachment of the cuticle from epidermis and undistinguishable the cuticular layers, separation occurred between different layers of the cuticle. The endocuticle was not properly attached to the epidermis, the appearance of vacuoles between cuticle and hypoderms, the endocuticle was reduced. These findings revealed that the treatment with oil combination (garlic+ mint) has a

specific mode of action which interferes with the rate if chitin deposition in the endocuticle. The newly formed cuticle become less rigid and therefore it cannot withstands the internal pressure during ecdysis or give sufficient support to the muscles involved in ecdysis and thus resulting in an inability to cast the exuviae and finally in death as mentioned by Chaibe *et al.* [25]. Our results in agreement with Al-Dahafer *et al.* [12] that most larvae of *A. ipsilon* were died during ecdysis after treatment with the oil combination of garlic and mint, and at LC_{50} concentration the compound induced developmental retardation and few deformities , in this view it responsible to a little extent the juvenile hormone analogues. Porter *et al.* [27] reported a relationship between the chitin content and activity of the chitin synthetase enzyme in the final larval instar and pupa of the lepidopterous insect *Prodenia eridania*. A correlation was observed between changes in chitin concentration and activity of this enzyme. From these data, it seems that the oil combination interferes with the activity of chitin synthesis in the cuticle in such way that the daily rate of chitin deposition and its growth are reduced or retarded. Vinson *et al.* [28] recorded that chitin deposition was inhibited in locust that fed on Dimilin which resulted in wrecking the cuticle and thus its rigidity was reduced to half of that of the normal cuticle.

Changes on muscles: There are skeletal muscles attached to the body wall, and visceral muscles surrounded the alimentary canal and ducts. The muscles are striated, muscle cells are massed into muscle fibers into functional unit. Skeletal muscles attached to the body wall with attachments fibers (Figure 1 A, 3, 5 A), where they can move different parts of the body. The mascular fiber has many cells with a plasma membrane and outer sheath or sarcolimma. Visceral muscles produce peristalsis as demonstrated in the digestive system. Nerve impulsis are required to initiate muscle contraction. These muscles are also known as neurogenic or synchronous muscles. The changes on the different kinds of muscular system (Figure 1 D, 2 B) was breaking down of the muscles into small parts are attributed to the destruction of the sarcolemma, disintegration and partially rupture on the muscles fiber and cells leading to defect and disorder the epithelial layer where the muscles lost their function. The results are in agreement with those obtained by Sabry, Ibrahim, Abdullah, Sharaby A *et al.* [22,23,18,13] they observed sever effects on the alimentary canal, fat bodies and muscles of *H. littoralis* after treatment with combination of garlic, mint and eucalyptus essential oils, these effects comprise destruction of muscles, epithelial cells and the peritrophic membrane were curled and ruptured than those of control group. Ranjini *et al.* [11] mentioned that a concentration of 3% front leaf extract of *Clerodendrum infortunatum* and *Eupatorium odoratum* effects on the muscles of the sixth instar larvae of *Orthaga exvinacea* causing separation in the muscle fibers in some areas of the larval body .

Changes on the trachea: The tracheal tube is contain ridge -like circumferential rings (taenidia) winds spirally through the membranous wall to prevent its collapse and keeping it open against the internal pressure of the body fluids. This design gives tracheal tubes the ability to flex and stretch without developing kinks that might restrict air flow (Figure 4A). In the treated larvae, the observed changes in the tracheal tube indicated partial rupture in the taenidia (Figure 1D, 4B) torsion in the tube and decreasing in their lumen, collapsible the tracheal lumen in some areas. The ventilation process disrupts the oxygen movement from the atmosphere up the trachea tissues and tracheoles through body insect and the passage of carbon dioxide in the opposite direction. Damage of the attached muscles also disrupts the ventilation process, thus maintaining a sluggish circulation. Digestive food was disrupted.

From the foregoing studies, it appears that the combination of median lethal concentration of (Garlic + Mint) essential oils induced no direct toxic effect as in conventional insecticides. The compound showed similarity in some aspects to juvenile hormone analogues since it caused developmental retardation and induced deformities. The compound showed a specific mode of action connected with inhibition of ecdysis. This is due to a reduction in the rate of endocuticular deposition and chitin that has been confirmed histopathologically.

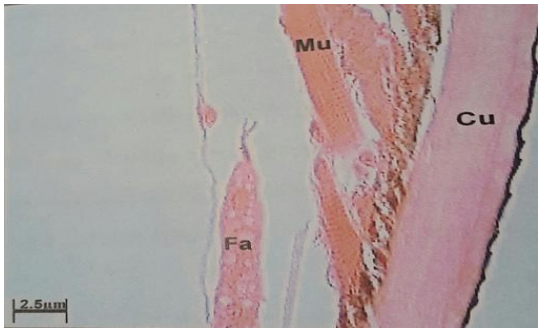


Figure 5, A. Section through normal larval integument. Show the intact cuticle, parietal fat tissues and muscles.



Figure 5, B. Section through larval body after treatment, show damaged of the parietal and visceral fat tissues, and, exo and endo cuticle.

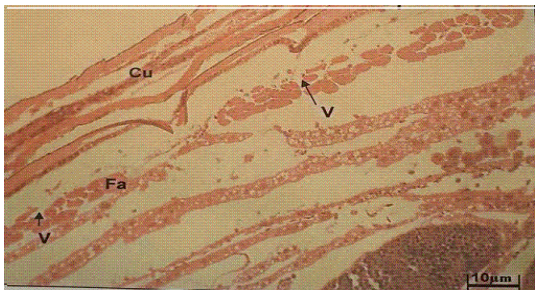


Figure 5, C. Magnified section through larval integument after treatment, show damaged and separation of different layer of the integument.

Figures abbreviations: Pritrophic membrane (Pr), Epithelial cells of the midgut (Epm), Villi (Vi), Portion from salivary gland (Sa), Malpighian tubuli (Mp), Basement membrane (Bm), Regenerative cells (Re), Muscles (Mu), Fat tissues (Fa), Nucleus (N), Goblet cells (G), Collamnar cellc. Cuticle (Cu), Epicuticle (Epi), Vacuole (V), Colon epethelium (Eph). Secrition droplets (Se), Trachea (Tr), Intema (In), Taenid (Tn), Salivary gland (Sa).

Conclusion

Combination of LC_{50} concentration of Garlic oil at (0.030% and Mint oil at (0.160%) in one artificial diet for the 3rd larval instar of *A. ipsilon* cleared great histopathological changes have been detected by light microscope in different larval tissues compared with the untreated control larvae. The compound showed similarity in some aspects to juvenile hormone analogues. So the results suggested that the combination of the natural plant essential oils of garlic with mint in one spray solution may be used in IPM program against *A. ipsilon* larvae as a biorational insecticide.

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