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Scanning Electron Microscopy and Biochemical Studies on *Spodoptera littoralis* Larvae Following Treatments with Ethanolic Extract of *Taxodium distichum* and Lufenuron

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ABSTRACT

The efficacy of the *Taxodium distichum* (L.) ethanol extract fruits and the chitin synthesis inhibiting insecticide, lufenuron on 4th instar of larvae *Spodoptera littoralis* and determination of the possible damage induced to cuticle and head capsule were evaluated. The sublethal concentration LC_{50}s were (10490 & 1.41 ppm) for *T. distichum* ethanolic extract and lufenuron, respectively. The ethanolic extract of *T. distichum* was analyzed by (GC-MS). The major compounds were Ferruginol (16.96%), Di-(2-ethylhexyl) phthalate (10.03%), piperine (6.81%), 3alpha,12beta-Dihydroxy-5beta-cholan-2,4-oic acid methyl ester (4.53%), Didecyl phthalate (4.03%) and Octadecane,1-[2-(hexadecyloxy) ethoxy] (2.94%). The scanning electron microscope observations clearly revealed that the two compounds have approximately similar disrupting effects on external morphology of cuticle and head capsule, as disrupted dark zones in the cuticle with shrinking, double head capsule formation, dwarfed with indistinct structures of head capsule and some deformities noticed in the mouth parts of treated larvae. The variation in some carbohydrases, chitinase, proteinase and phenol oxidase enzymes activities could be attributed due to tested materials. The ethanolic extract of *T. distichum* fruits could be a promising agent for pest control programs.

Keywords: *Spodoptera littoralis*; *Taxodium distichum*; lufenuron; enzymes; scanning electron microscope.

INTRODUCTION

The Egyptian cotton leaf worm, *S. littoralis* (Lepidoptera: Noctuidae) has a national concern with a wild host range. Heavy infestation resulted in defoliation of the attacked plants causing sever loss of crop production Sakr and Roshdy (2015). The chemical control methods usually employed to control this insect has the ability to develop a quick resistance to the most conventional insecticides which resulted in search for new pesticides. It is very important to discover novel ecofriendly compounds to replace the hazard synthetic insecticides Wasu *et al.* (2013).

Due to the importance of cuticle to insect life this organ consider as a target organ for controlling *S. littoralis*. The cuticle affords support and protection through its rigidity and hardness. After ecdysis the cuticle is soft and flexible, but the outer part subsequently becomes hardened by a process known as tanning or sclerotization Ishaaya (1972). Some plant extracts that act as Insect growth regulators (IGRs) in inhibiting chitin synthesis of insect cuticle offer alternatives to conventional chemical larvicides. *Taxodium distichum* extracts are rich in monoterpenes, diterpenes, sesquiterpenes, flavonoids and glycosides, which have antiviral, cytotoxic, antitumor, anti-oxidant, anti-bacterial and antifungal activities (El Tantawy *et al.*, 1999; Ibrahim *et al*., 2006; Kusumoto *et al*., 2010 and Kushwaha *et al*., 2016). The insecticidal activity of *T. distichum* has not been investigated to date, the present work aim to identify the active constituents of ethanol extract of *T. distichum* fruit and evaluate its LC_{50} on the 4th larval instar and determining its external effects on cuticle and head capsule also studying some biochemical parameters related to its destructive action comparing these results by one of chitin synthesis inhibiting insecticide to prove that *T. distichum* could serve as potential natural insecticide for controlling *S. littoralis* larvae.

MATERIALS AND METHODS

1. Tested materials:

1. Lufenuron:

The chitin synthesis inhibiting insecticide used was: lufenuron (Match 5% EC). Lufenuron is a benzoylphenylurea derivative with the following chemical composition: N- [2,5-dichloro-4 (1, 1, 2, 3, 3, 3, hexa fluoropropoxy) phenylaminocarbonyl] -2, 6-difluorobenzamide (C17H8C12F8N2O3, MW551.15).

2. Extraction of *T. distichum*:

T. distichum fruits collected from El-orman garden Giza, Egypt. The whole body of fruits was dried under vacuum at 30 ºC until weight stabilized, then crashed into powder. About one kg of the powder was steeped at room temperature in ethanol, the pooled extracts was evaporated under vacuum at 50 ºC till dryness yield.

1. Identification of *T. distichum* ethanolic extract:

The ethanolic extract of *T. distichum* was analyzed at Regional Center of Mycology and Biotechnology, Al-Azhar University by gas chromatography–mass spectrometry (GC-MS). Thermo Scientific Trace 1310 Gas Chromatograph attached with ISO LT single quadrupole Mass Spectrometer.

2. Rearing technique of *S. littoralis*:

A laboratory (susceptible) strain of *S. littoralis* was reared away from any insecticidal contamination for ten generations at the division of Cotton Leafworm, Branch of Plant Protection Research Institute at Zagazig, Sharqia Governorate under constant conditions 27±1 °C and 70±5% R.H. to provide insects used in the present investigation according to El-Defrawi *et al.* (1964).

3. Bioassay:

Serial successive concentrations of each compound prepared using distilled water. Disks (9cm.diameter) of castor bean leaves were dipped in the tested concentrations for 10 seconds, left to dry and offered to larvae, which starved for 4-6 hours before treatment Merdan (1986). Larvae were placed into glass jars (5 pounds); each treatment was replicated 5 times (10 larvae per each). Control disks were dipped in distilled water only. The larvae were allowed to feed on treated disks for 48 hr. then on untreated ones. Mortality

percentages were recorded after 72 hr. and corrected according to Abbott's formula (1925).

4. Sample Preparation for Scanning Electron Microscopy:

Scanning electron microscopy (JEOL-JSM-5500 LV) high vacuum mode was used to illustrate the external morphology of the *S. littoralis* larvae at Regional Center of Mycology and Biotechnology, Al-Azhar University. Specimens were fixed with 2.5% glutaraldehyde. and dehydrated in a series of alcohol solutions (30%, 50%, 70%, 95%) for 10 minutes each and finally 100% 5 minutes, 3 times using automatic tissue processor (Leica EM TP), then dried using CO2 critical point drier (Tousimis Audosamdri-815). They were critical point dried and sputter coated with a gold coater (SPI-Module).

5. Biochemical determinations:

Samples of the 4th instar larvae of *S. littoralis* were collected after 72 hrs of treatments with LC50s of *T. distichum* ethanolic extract and Lufenuron untreated larvae were used as control. Samples were homogenized in distilled water using Teflon homogenizer. The homogenates were centrifuged at 500 r.p.m for 20 minutes at 5ºC. Supernatants were kept in a deep freezer at -20 ºC till use. Three replicates were used for determination.

1. Carbohydrases enzymes activity:

The determination of carbohydrases enzymes; invertase, amylase and trehalase activities were based on the digestion of sucrose, starch and trehalose, respectively, according to the method of (Ishaaya and Swirski, 1976).

2. Chitinase enzyme activity:

Chitinase activity was recorded using 3,5-dinitrosalicylic acid reagent to determine the free aldehydic groups of hexoamines liberated on chitin digestion according to the method of Ishaaya and Casida (1974).

3. Phenol oxidase enzyme activity:

Phenol oxidase activity was determined according to a modification of Ishaaya *et al.* (1971), adding catechol solution initiated enzyme reaction.

4. Protease enzyme activity:

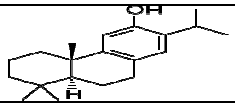
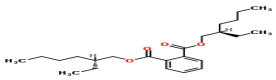
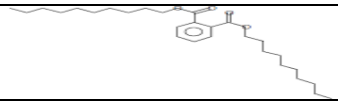
Protease enzyme activity was measured by the increase in free amino acids split from substrate protein (albumin), during one hour incubation at 30 ^{0}C according to Tachell *et al*. (1972).

RESULTS AND DISSCUSION

Identification of *T. distichum* ethanolic extract:

Data in Table (1) and Fig. (1) illustrates GC/MS analyses of *T. distichum* ethanolic extract. Six compounds belonging to alkaloids, terpenoids, steroids, and phthalate groups were identified with ferruginol (16.96%) as the major one, while the other five compounds were Di-(2-ethylhexyl) phthalate (10.03%), piperine (6.81%), 3alpha,12beta-Dihydroxy-5beta-cholan-24-oic acid methyl ester (4.53%), Didecyl phthalate (4.03%) and Octadecane,1-[2-(hexadecyloxy) ethoxy] (2.94%). In the present study, the composition of the *T. distichum* ethanolic extract differs from that obtained from *T. distichum* essential oils reported in literature of Ogunwande *et al.* (2007) The essential oils analyzed by gas (GC-MS) and the main compounds were a-pinene (60.5%) and thujopsene (17.6%) from the fruits and thujopsene (27.7%), pimara- 8(14),15-diene (13.1%), widdrol (12.8%), and b-caryophyllene (11.4%) from the leaves. El Tantawy *et al.* (1999) the chemical composition of the essential oil of the fruit of *T. disttchum* was monoterpenoid Flamini *et al.* (2000) oil of the feminine cones of *T. distichurn* contains monoterpene hydrocarbons, while branch oils contained more oxygenated monoterpenes and sesquiterpene hydrocarbons.

Table 1. Chemical composition of ethanolic extract of *T. distichum* using GC-MS analysis.

RT	Compound name	Compound formula	Compound structure	Area%
45.16	Ferruginol	$C_{20}H_{30}O$		16.96
48.06	Di-(2-ethylhexyl)phthalate	$C_{24}H_{38}O_4$		10.03
35.07	Piperine	$C_{17}H_{19}NO_3$		6.81
43.93	3alpha,12beta-Dihydroxy-5beta-cholan-24-oic acid Methyl ester	$C_{25}H_{42}O_4$		4.53
53.39	Didecyl phthalate	C28H46O4		4.03
50.28	Octadecane, 1-[2-(hexadecyloxy)ethoxy]	C36H74O2		2.94

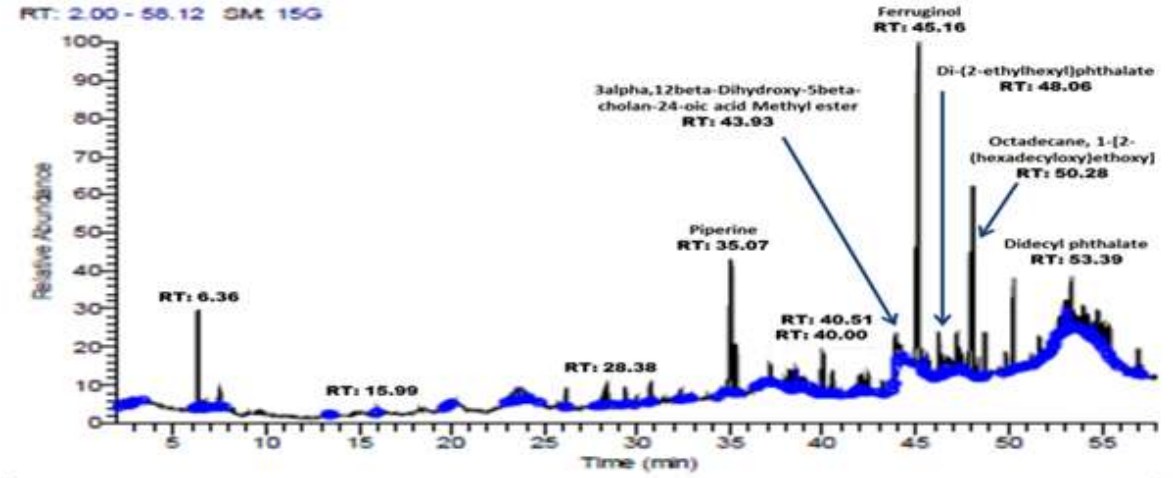


Fig. 1. GC/MS chromatogram of ethanolic extract of *T. distichum* fruits.

2. Susceptibility of *S. littoralis* 4th instar larvae to ethanolic extract of *T. distichum* and lufenuron:

The toxic effects were listed in table (2). LC_{50}s and LC_{90}s values were (1.41 & 10490 ppm) and (28.48 & 27890 ppm) to lufenuron and *T. distichum* ethanolic extract, respectively for 4th instar after 72 hours of treatment.

Table 2. Toxicity of *T. distichum* ethanolic extract and lufenuron on the 4thinstar larvae of *S. littoralis*.

Treatments	LC_{50} ppm (Lower-Upper)	LC_{90} ppm (Lower - Upper)	Slope
T. distichum	10490 (8280 – 11910)	27890 (22230 - 47100)	3.007±0.631
Lufenuron	1.41 (0.93 - 2.40)	28.48 (19.56 – 51.69)	1.90± 0.40

This study expected that, the toxicity of the *T. distichum* ethanolic extract may be related to the presence of terpenoid and alkaliods compounds, terpenoids have different methyl and hydroxyl groups located at different position furthermore alkaloids are alkali like, nitrogen-containing organic constituents many are poisonous due to their potent biological activities. Kushwaha *et al.* (2016) reported that, ethanolic extract of aerial parts of *T. distichum* plant possesses potent antifilarial activity these findings indicate that labdane diterpenoid molecules may provide valuable leads for design and development of new macrofilaricidal agent. Torres *et al.* (2003) mentioned that, methanol extract from the bark of *Yucca periculosa* had growth regulatory activity against *S. frugiperda*. These compounds could be involved in interference of sclerotization and moulting. Sakr and Roshdy (2015) demonstrated the potential insecticidal action of the aerial parts of *Hyptis brevipes* methanol extract toward *S. littoralis* larvae. The extract disrupted the normal growth and development of the treated larvae. This plant is known to produce a range of terpinoids, flavonoids and pyrons. Karimzadeh *et al.* (2007) Effects of five chitin synthesis inhibitors, diflubenzuron, cyromazine, lufenuron, hexaflumuron and triflumuron, on 2nd instars of *Leptinotarsa decemlineata.* lufenuron and hexaflumuron seem to be more potent.

3. Scanning electron microscope studies:

Detailed examination of morphological features of *S. littoralis* larvae were detected by Scanning electron microscope requires comparisons between untreated larvae and those previously treated with LC_{50}s of ethanolic extract of *T. distichum* and others whom treated with lufenuron. The tegumentary ultrastructure of normal untreated larvae recorded smooth body without any processes the head, thoracic segments and abdominal segments are normally differentiated (Fig.2a&b).

Changes in the external outer body surface in samples subjected to *T. distichum* treatment showed high alterations in body shape and cuticle surface of the larvae (Fig.2c). The larvae may lose its cylindrical form (Fig.2c-e) due to dehydrations of body (Fig.2d). The whole size of these larvae become very small as compared with untreated ones (Fig.e&g), disrupted dark zones in the cuticle with shrinking also observed (Fig.2f), ventral view for abnormal larvae ensuring the latter harmful effects of the botanical extract (Fig. 2g) moreover deformations in anal prolegs (crochets) which are responsible for attachment to the substrate noticed in (Fig. 2h).

Data in figure (3) discussed general external comparative morphology between normal larvae (Fig.3a&b) and abnormal larvae treated with lufenuron (Fig.3c-h). Apparently emissions of old exuviate adhered to the cuticle of the larvae is clearly shown in incapability for shedding the old cuticle.

Scanning electron microscope observations showed remarked similarity between *T. distichurn* extract and lufenuron in their effect on the external morphology of cuticle and head capsule due to the presence of terpenoid compounds like feruginol and phthalate compounds as Di-(2-ethylhexyl) phthalate and Didecyl phthalate in *T. distichurn* extracts while, terpen group in their structure and activities have characteristics resembling those of

IGRs compounds (Passino *et al.*, 1999 and Belles *et al*., 2005). Steroid compounds found in the extract may reflect some internal physiological actions on treated stage as reported by Hussein (2002) Sharma *et al.* (2006) the incapability to shed the old cuticle is caused by the inhibition of release of the eclosion hormone which prevent normal ecdysis. Moreover phthalate compounds play cytotoxic effect because it causes apoptosis in cells and consider as bioactive compound due to its potent larvicidal activity Khatiwora *et al.* (2013).

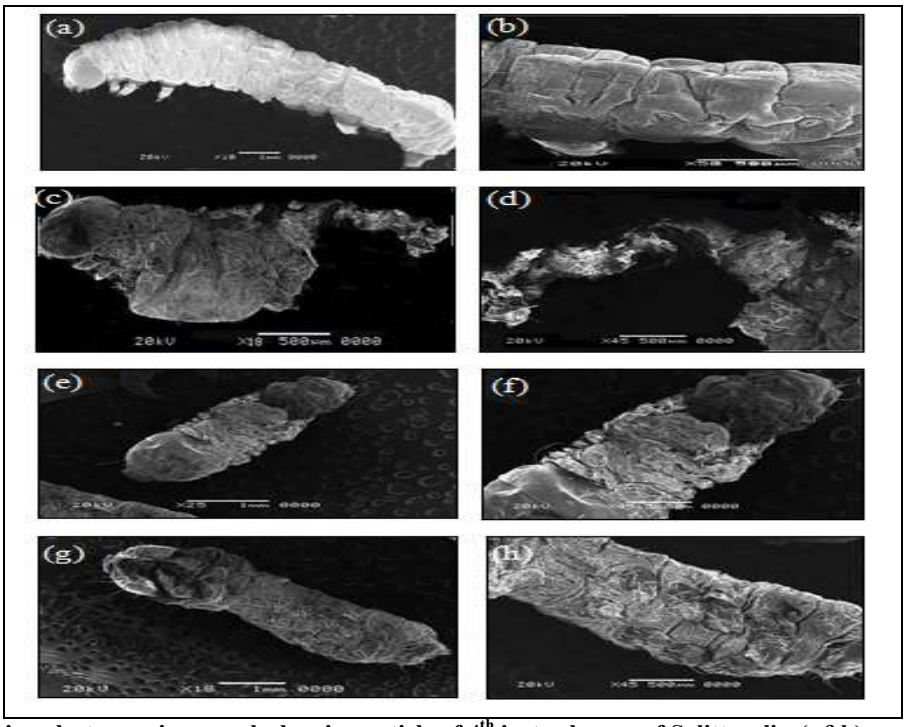


Fig. 2. Scanning electron micrograph showing cuticle of 4th instar larvae of S. littoralis, (a&b) normal cuticle. (c-h) abnormal cuticle of larvae treated with ethanolic extract of *T. distcum*.

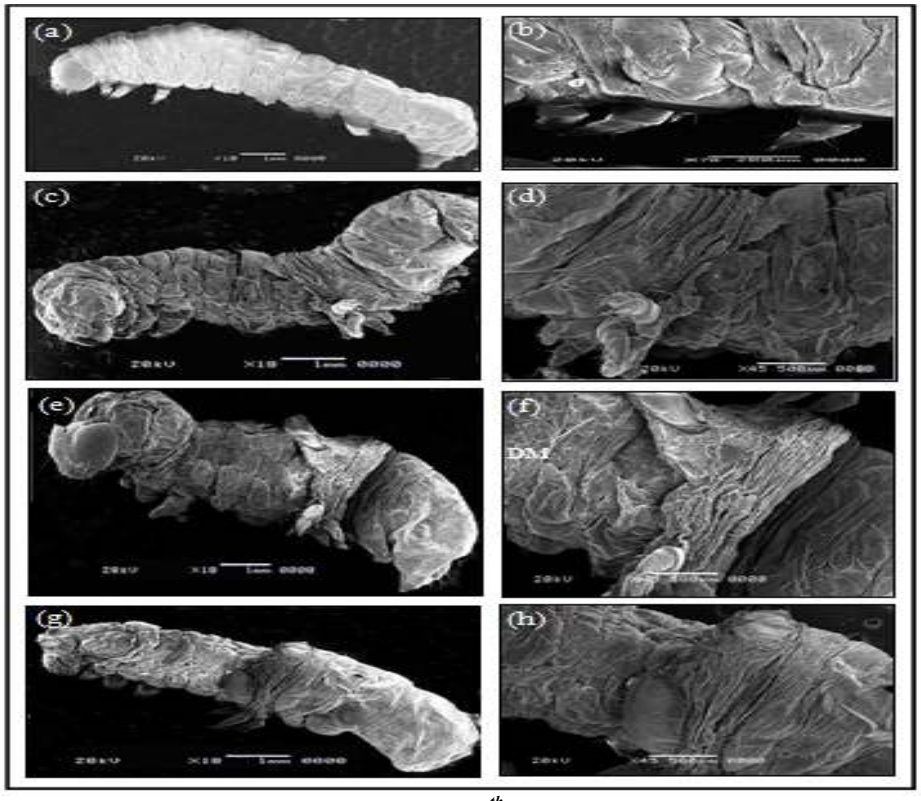


Fig. 3. Scanning electron micrograph showing cuticle of 4th instar larvae of *S. littoralis,* (a&b) normal cuticle. (c-h) abnormal cuticle of larvae treated with lufenuron.

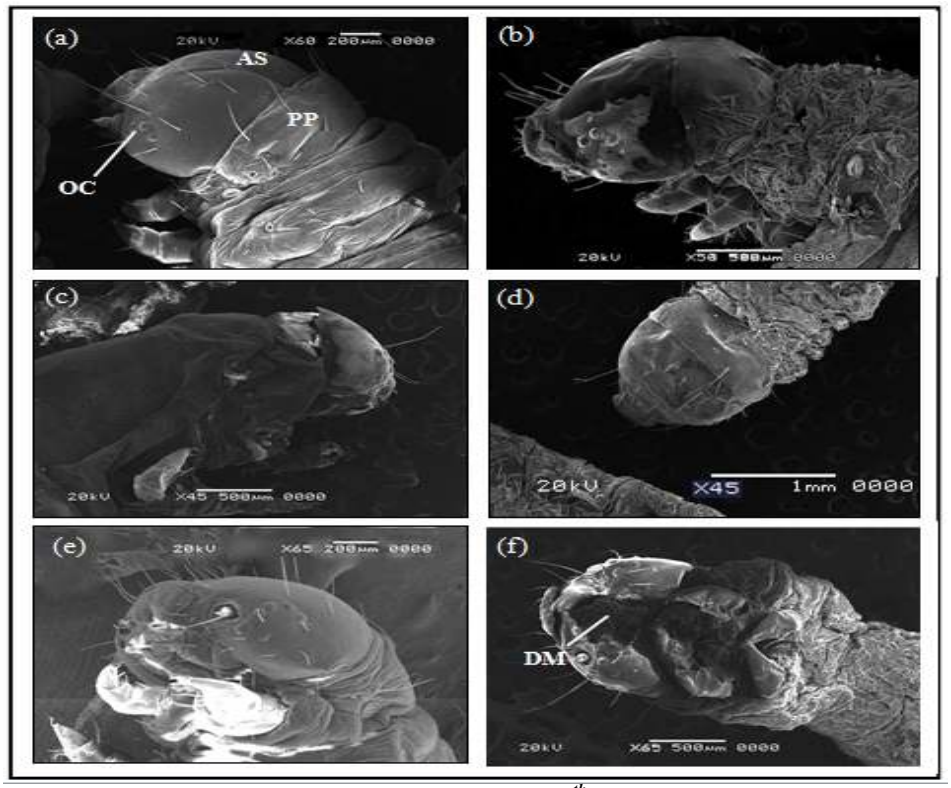


Fig. 4. Scanning electron micrograph showing head capsule of 4th instar larvae of *S. littoralis*, (a) normal head capsule. (b-d) abnormal head capsule of larvae treated with ethanolic extract of *T. distcum*. (e) normal mouth parts. (f) deformed mouth parts of larvae treated with ethanolic extract of *T. distcum*. AS, adfrontal suture; DM, deformed mouth parts and PP, prothoracic plate.

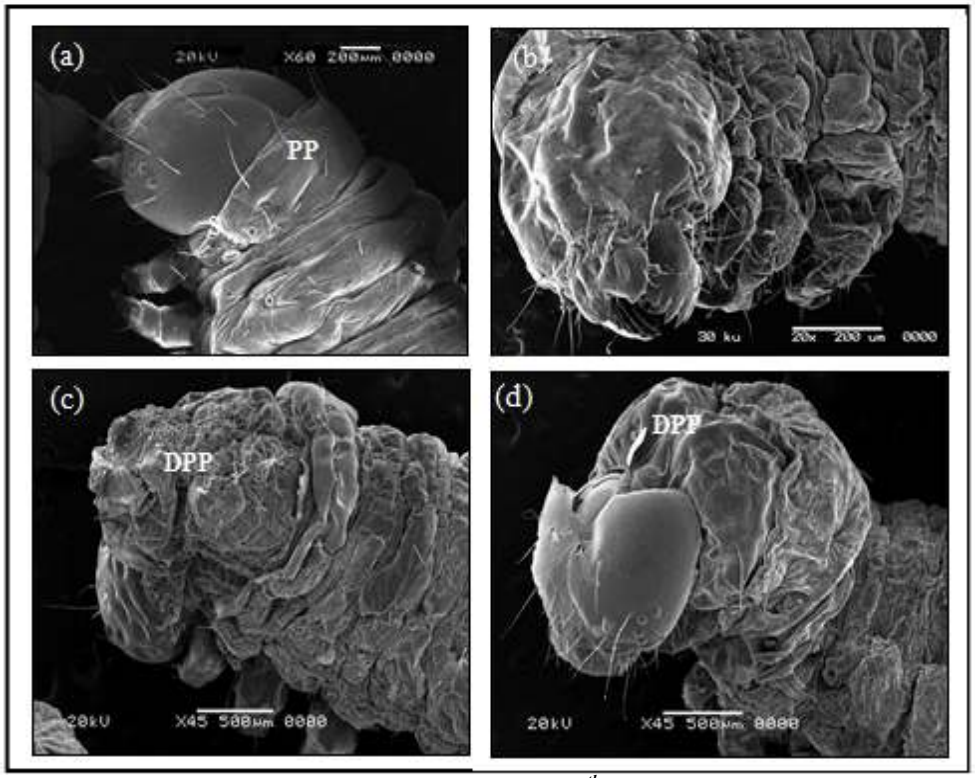


Fig. 5. Scanning electron micrograph showing head capsule of 4th instar larvae of *S. littoralis*, (a) normal head capsule. (b-d) abnormal head capsule of larvae treated with lufenuron. DPP, deformed prothoracic plate; PP, prothoracic plate

The application of *T. distichurn* extract symptoms resembling those found by Wasu *et al.* (2013) treatments of *S. frugiperda* with methanolic extracts of *Parthenium hysterophorus* and *Ageratina adenophora* causes many deformities like larva with abdominal distension, Larva with point of necrosis in integument and other with incomplete molting. Summarwar *et al.* (2016) stated that leaf and seed extracts of *Azadirachta indica*, *Catharanthus roseus* and *Ocimum sanctum* induced morphological abnormalities on *S. litura* larvae, body became dark and spotted losing its usual characteristic pattern. It became thin and stretched and ruptured at various regions. Many researchers found that IGRs caused disrupting activities for lufenuron Dean *et al.* (1999) mentioned that the treatment of adult *Ctenocephalides felis* with lufenuron inhibited endocuticle formation by producing abnormal endocuticle consisting of protein globules embedded in an amorphous chitin matrix, mortality of treated fleas was the result of a weakened endocuticle and the corresponding decrease in resiliency of the cuticle to expansion. Karimzadeh *et al.* (2007) noticed stretched intersegmental membranes and partial molting in *L. decemlineata* larvae treated with diflubenzuron, lufenuron, triflumuron and hexaflumuron.

Figures (4a&5a) concerning the normal head capsule with two large compound eyes separated by adfrontal suture which help in ecdysiast and six eye spots or stemmata, the capsule followed by prothoracic plate and the first thoracic segment which has distinct spiracle responsible for respiration process. All affected individuals due to extract treatments showed clear signs of deformed head capsule with dark satin color (Fig.4b) apolysis, indicating the induction of premature larval moulting because of an ecdysteroidal activity; Showed also morphological changes such as double head capsule formation, (Fig.4c) dwarfed with indistinct structures head capsule (Fig. 4d) some deformities noticed in the mouth parts of treated larvae (Fig.4f) as compared with control (Fig.4e). Many destructive forms of head capsule also observed in larvae treated with lufenuron (Fig. 5b-d) the prothoracic plate the spiracles showed highly shrinking (Fig. 5d-e).

Similar observations were reported by Arivoli and Samuel (2013) the treated larvae *S. litura* were unabigusly smaller that its control counterpart and several deformities in head size and body length Karimzadeh *et al.* (2007) stated that inability in casting the old head capsule occurred this was followed by death of *Leptinotarsa decemlineata* larvae. Abderrahim and Rehimi (2014) shows that some mosquitoes *Culiseta morsitans* treated by methoxyfenozide progressed further in the moulting process and synthesized new cuticle, but never success to detach the old head capsule and ecdysis presumably.

4. Biochemical studies:

Different enzymes activities related to cuticle synthesis of 4th larval instar of *S. littoralis* showed many changes after treatments with LC_{50}s of ethanolic extract of *T. distichum* and lufenuron comparing to control, results are tabulated in Table (3).

Table 3. Effect of Lufenuron and *T. distichum* ethanolic extract on some biochemical parameters of 4th instar larvae of *S. littoralis*.

Treatments	Invertase	Trehalase	Amylase	Phenole oxidase	Protease	Chitinase
T. distichum	29.45	14.46	37.10	25.33	72.10	778.00
Lufenuron	104.51	206.59	40.15	34.64	41.25	236.20
Control	19.41	55.49	71.35	43.78	50.8	720.80

1. Charbohydrates hydrolyzing enzymes:

Invertase activity recorded (29.45) while trehalase and amylase activity decreased by (14.46 & 37.10) with *T. distichum* treatment. In case of lufenuron treatment invertase and amylase showed an increase in their activities percentages by (104.51 & 206.59), respectively where amylase recorded remarked decrease by (40.15) when compared by control enzymes activities percentages (19.41), (55.49) and(71.35) for invertase trehalase and amaylase, respectively.

Trehalase is one of the most important carbohydrolases which plays an important role in energy supply to an insect it catalyzes the hydrolysis of trehalose into two glucose molecules for internal supply for chitin synthesis; its elevation was observed in case of lufenuron treatments. The decrease in the trehalase activity in larvae treated with *T. distichum* could be attributed to decreased metabolic hydrolysis of trehalose to release glucose. Tatun *et al.* (2014) determined inhibitory activity of methanolic extracts of latex obtained from three species of plant *Morus alba*, *Artocarpus heterophyllus* and *Ficus benjamina,* against trehalase in the red flour beetle, *Tribolium castaneum*. Anwar and Abd El-Mageed (2005) evaluated an inhibition in the amylase and invertase activities for both laboratory and field strain of 2nd and 4th instars larvae of *S. littoralis* to six IGRs (Diflubenzuron, Tebufenozide, Hexaflumuron, Flufenoxuron, Chlorfluazuron and Lufenuron). They stated that generally insect amylases are capable of hydrolyzing starch, amylopectin and solubilized amylase.

2. Phenol oxidase enzyme:

All treatments showed certain reduction in Phenol oxidase enzyme activities percentages by (25.33 & 34.64) for *T. distichum* and lufenuron, respectively comparable to control (43.78). The decrease in Phenoleoxidase activity causes disturbance in the formation of external body cuticle and formation of dark spots this is in harmony with Ashida and Yamazaki (1990) some types of phenoloxidase are involved in wound healing and sclerotization of the cuticle. (Ashida and Brey, 1995; Kouakou *et al*., 2009) Phenoleoxidase inter in the synthesis of melanin, a pigment found in the cuticle. Ishaaya (1972) Isolated larval phenoloxidase from the Egyptian cotton worm, *S. littoralis* Boisd., and determined its optimum conditions for reaction in order to use this system for detecting compounds able to inhibit this enzyme.

3. Protease enzyme:

The response of larval Protease enzyme activity to *T. distichum* treatment produced elevation by (72.10) on the other hand lufenuron reduces its activity to (41.25) while that of control recorded (50.80). On contrary Khosravi *et al.* (2011) found that, protease

activity in *Glyphodes pyloalis* larvae was reduced using *Artemisia annua* methanol extract also Khosravi and Sendi (2013) noticed reduction in protease activity in *G. pyloalis* caused by Azadirachtin.

4. Chitinase enzyme:

Only *T. distichum* treatment causes an increase in Chitinase enzyme by (778.00) than control (720.80) and in contrary lufenuron decrease its activity to (236.20). The mode of action of lufenuron as chitin synthesis inhibitor actually comes from its interference with the activity some important enzymes. Many outhers came with similar observation like Sabry and Khedr (2014) Treatment of 4th larval instar of *S. littoralis* with tebubenzuron caused decrease in chitinase and increase in protease, phenoloxidase and trehalase activities, while the activity of chitinase was significantly elevated using tebufenozide and teflubenzuron. Assar *et al.* (2016) Recorded pronounced increase in activity of chitinase, Phenoloxidase, carbohydrates hydrolyzing enzymes against *S. littoralis* larvae after application of some insect growth regulators and bioinsecticides.

CONCLUSION

The ethanolic extract of *Taxodium* distichum fruits and the chitin synthesis inhibiting insecticide; lufenuron on 4th instar of *S. littoralis* larvae induced remarked damage on cuticle and head capsule also, caused disturbance in vital enzymes which retard the development of *S. littoralis* larvae. So the two compounds are successful methods in pest control programs.

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دراسة تاثير المستخلص الكحولي لنبات *تكسوديوم دستيكم* ومنظم النمو لوفينورون علي يرقات دودة ورق القطن بالاستعانة بالميكروسكوب الالكتروني الماسح وبعض الدراسات البيوكميائية.

هند محمد صبرى

معهد بحوث وقاية النباتات – الدقى – جيزة - مصر

تم تقييم فعالية كل من مستخلص الايثانول لثمار اشجار التاكسوديوم و مبيد لوفينورون المثبط لتخليق الكيتين ، على يرقات العمرالرابع لدودة ورق القطن وتحديد الضرر المحتمل الناجم علي الجليد وكبسولة الرأس.حيث كانت التركيزات المميته لنصف التعداد باستخدام مستخلص الايثانول لثمار التاكسوديوم و مبيد لوفينورون كانت ١٠٤٩٠ و١.٤١ جزء في المليون علي التوالي .وتم تحليل مستخلص الايثانول لثمار التاكسوديوم بواسطه جهاز التحليل الكروماتوجرافي الغازي المقترن بجهاز قياس طيف الكتله وكانت المركبات الرئيسية فيروجينول (١٦.٩٦٪)، دي - (٢-إيثيل هيكسيل) فثالات (١٠.٠٣٪)، بيبرين (٦.٨١٪)، إستر ميثيل -٣ الفا، 12بيتا-ديهيدروكسي-٥بيتاكولان-٢٤،-أويك (٤.٥٣٪)، ديديسيل فثالات (٤.٠٣٪) و ١- [٢- هيكساديسيلوكسي) إيثوكسي] أوكتاديكان (٢.٩٤٪). وكشف الفحص بالميكروسكوب الإلكتروني الماسح بوضوح أن كلا المركبين المختبرين لهما تقريبا آثار مماثلة على التشكل الخارجي للجليد وكبسولة الرأس تمثلت في وجود مناطق داكنه متجعدة مع تقلص الجليد ، تكوين كبسولة الرأس المزدوجه ,التقزم المصحوب بعدم وضوح كبسولة الرأس كما لوحظ بعض التشوهات في أجزاء الفم في اليرقات المعالجة.وعلاوة على ذلك، يعزي الاضطراب في نشاط بعض الإنزيمات مثل الانزيمات المحلله للكربوهيدرات،وكل من انزيمات الكيتيناز والبروتييز والفينول أوكسيديز الي تاثير المواد المختبرة. و عليه يمكن اعتبار المستخلص الإيثانولي لثمار لثمار التاكسوديوم عنصر واعدا في برامج مكافحة التكامله الأفات.