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Larvicidal, biological, and histopathological alterations induced by pomegranate peel extract, *Punica granatum* against *Culex pipiens* L. (Diptera: Culicidae)

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ABSTRACT

The insecticidal efficacy of agricultural waste pomegranate peel extract was determined according to the world health organization (WHO) against the *Culex pipiens* 3rd instar larvae. The mortality percentage of the treated larvae was the first indicator to evaluate the inhibition effect of the pomegranate peel extract. The median lethal concentrations (LC_{50}) were (95.63, 62.55, 54.47 and 44.35) ppm at different times post-treatment (24, 48, 72, 96 hours), respectively. The tested extract showed repellent against *Culex pipiens* females. Phytochemical screening was carried out to detect the effective chemical groups in the tested extract then the extract was analyzed by GC-MS and HPLC. The used extract caused a reduction in some biological aspects of *Culex pipiens*. Several forms of morphological malformations were detected and many aberrations have been induced. The activities of enzymes, as well as total proteins, carbohydrates, and lipids of Culex pipiens larvae, were significantly reduced compared to the untreated. Electrophoretic analysis of protein revealed inhibitory action of the tested extract on the protein contents and fraction patterns. Ultrastructure studies showed a drastic effect on some larval tissues in response to the pomegranate peel petroleum ether extract.

INTRODUCTION

Dipterous insects cause serious public health problems for both humans and animals **(Linthicum, 2012)**. In Egypt, *Culex pipiens* (Diptera: Culicidae), has been investigated and declared as a vector of several diseases **(El-Zayyat *et al.*, 2017)**. It transmits Rift valley fever virus **(Dodson *et al.*, 2017)**, Japanese encephalitis **(Chancey *et al.*, 2015)**, *Wuchereria bancrofti* accredited for human lymphatic filariasis transmission **(Joseph *et al.*, 2011)**, and West Nile virus **(Bassal *et al.*, 2017)**. *Culex pipiens* was incriminated as the filarial vector in Egypt **(El-Naggar *et al.*, 2017)** and has been recorded by all governorates without exception **(Abdel-Shafi *et al.*, 2016)**. Traditional insecticides were used to control mosquitoes **(Killeen *et al.*, 2017)** but introduced problems in the environment by leaving undesirable residues in food, toxicological implications to human health, increased cost and handling hazards **(Bonner and Alvanja, 2017)**. Flies have developed a certain level of resistance to most available insecticides

because of the random use of insecticides **(Acevedo *et al*., 2009)**. Accordingly, alternate strategies for pest control are needed, in which new compounds which are biodegradable, safe to humans, the environment, and non-target organisms.

Plant extracts and phyto-products are gaining special attention because of their relative safety, wide acceptance by consumers and their multipurpose functional uses **(Aleksic and Knezevic, 2014)**. They hold a promising effect, easily extractable, eco-friendly, biodegradable, low persistence in soil and have very low to no toxicity against mammals, vertebrates, birds, and fish **(Khater *et al*., 2011)**. Plant extracts with significant insecticidal activity have been considered as a new source of pesticides and insect growth regulators due to their fullness with bioactive and biodegradable chemicals **(Sosa *et al*., 2018)**.

Agricultural wastes are natural organic materials that are wasted, lost or disregarded **(FAO, 2011)**. Food and Agricultural Organization (FAO) recorded increased rates of food loss in Egypt **(Rutten and Kavallari, 2013)**. The pomegranate is an old fruit originated in the Middle East, tropical and subtropical regions which exhibits antioxidant, antiviral and anticancer activities **(Farag *et al*., 2015)**. The pomegranate peel extracts have been used successfully as pest and vector control agents **(Farag and Emam, 2016)**.

The current study investigates chromatographic isolation of certain chemical constituents of pomegranate peel extract using different chromatographic techniques. The toxicity of pomegranate peels extract on *Culex pipiens* mosquito laboratory conditions was performed. The mode of action of pomegranate peel extract on insect's main metabolites was studied using biochemical assays. The histopathological and ultra-structural changes of certain tissues in treated and untreated larvae to detect which cellular targets were affected.

MATERIALS AND METHODS

Maintenance of mosquito colony

Common house mosquito egg rafts, *Culex pipien*, were collected from the Research and Training Center on Vectors of Diseases (RTC), Faculty of Science, Ain Shams University. It was held at 27±2°C and relative humidity RH 75±5% in restricted laboratory environment for 16:8 photoperiods (light:dark). The egg rafts were put in enamel plates filled with distilled water. Freshly hatched larvae were fed on fish food (TetraMin, Germany), which was spread on the surface of the breeding bowl twice a day as a diet. Early third larval instars were used for toxicological studies.

Preparation of pomegranate peel extract

The waste of pomegranate peels was collected from food factories in Egypt. They were then washed in order to be cleaned and then left to dry under shade in the laboratory. The dried peels were cut into small pieces and ground in an electric grinder. One hundred grams of the resulting powdered materials of peels was exhaustively extracted with petroleum ether at 1:5 (w/v) for 72 hours at room temperature with occasional shaking to then be filtered using Whatman filter paper. The rest was re-extracted at least 2 times independently. All filtrates were collected and evaporated at 50°C by a rotary evaporator (Buchi, Lausanne, Switzerland) to remove solvent content. The powder extract was stored at -4C° in screw capped vials, until needed. Phytochemical screening of pomegranate peel extracts were assessed **(Selvaraj *et al*., 2014)**.

Larvicidal activity

The larvicidal effect was tested using the immersion procedure against 3rd instar larvae of *Cx. pipiens* **(WHO, 2005)**. The extract was used in six different concentrations (50, 100, 200, 300, 500, and 800 ppm). Sets of twenty five early third instar larvae of *Cx. pipiens* were transmitted by a plastic dropper into small disposable test cups, each adjusted to 100 ml of water in laboratory conditions. For each treatment, the experiment was performed using three replicates. Abnormal pupae were removed every day and kept in 70% ethanol. A drop of glycerin was added during photographing under binocular microscope. Mortality data was recorded in a probit regression line and calculated $LC_{50,}$ $LC_{90,}$ slope function and X^2 **(Finney, 1971)**.

Repellence action of the tested extract on females *Cx. Pipiens*

In order to test the repellent action of the pomegranate peel extract, standard cages (24×24×24cm) were used. For preparing different concentrations, the extract was dissolved in two millilitre distilled water by adding a drop of Triton X 100. The ventral surface of the pigeons' abdomens (5X6 cm) were wetted by one ml from each concentration and left for 10 minutes. The pigeons were placed in the cages for three hours with at least 20 female adult *Culex* starved for 5-7 day. Water was used as a negative control. While, commercial repellent 15 % DEET (N,N-diethyl-meta-toluamide) ($C_{12}H_{17}NO$) (Johnson Wax Egypt) was used as a positive control. In order to calculate mean repellence value, each repellence test was repeated three times. Post treatment, the repellence percentage was calculated according to the number of fed and unfed females by using Abbott formula **(Abbott, 1925)**. The repellency % = (% Y-% Z/100-Z %) × 100**,** where Y is the unfed females' percentage in treatment and Z is the unfed females' percentage in control.

Biological activities

Biological activities were done on 3rd instar larvae treated with sub-lethal concentrations of the petroleum ether extract of pomegranate peels after 48 hours of treatment and untreated one as control. The experiment was carried out and kept under the standard controlled conditions. The emerged adults were provided with cotton pads soaked with sugar solution (10%) and then allowed to feed on pigeon to acquire blood meal. All adults were left together for 2 days. Each female was then transferred individually into propylene tubes (4cm in diameter and 7cm depth) one third of which was filled with water and covered with muslin. Observations were made daily till the emergence of adults for egg laying. Also, daily observations were made until all larvae either pupated or emerged as adults.

Reproductive potential of resulted females

Emerging adult females were collected and held with normal adult males. For 3 days, adults were fed on a cotton piece soaked in 10% sugar solution. Adults were then starved for two days. On Day 5, the starving females were allowed to take a pigeon's blood meal to place their eggs on the clean water. The number of eggs in each raft of eggs was calculated with the use of binoculars. The eggs were separated into two groups (hatched and non-hatched) eggs according to **(Hassan *et al.*, 1996)**. By the apparent evidence of the existence of an embryo under a dissection microscope, non- embryonated eggs were further separated into embryonic and non-embryonic eggs. Hatched and non-hatched embryonated eggs were considered as fertilized, while non-hatched and non-embryonated eggs were considered as unfertilized ones **(Rak and Ishii, 1989)**.

The egg hatchability was calculated using the following equation **(El-Sheikh, 2002)**. Egg-hatchability % = A / B × 100, A: total eggs hatched, B: total egg laid. The percentage of sterility was determined by formula **(Toppozada et al., 1966)**.
Sterility percentage = 100 – [a×b /A×B] × 100, a is the number of laid eggs per female in treatment, b is the percentage of eggs hatched in the treatment, A is the number of laid eggs per female in control, and B is the percentage of eggs hatched in control.

Biochemical studies

For biochemical analysis, one hundred 3rd instar *Cx. pipiens* larvae untreated and treated by LC_{50} of pomegranate peels extracts for 48 hours were kept in distilled water under freezing conditions at -20˚C.

Quantitative analysis

The total protein content was measured by using the technique of Folin-Ciocalteu **(Lowry *et al.*, 1951)**. Total lipid was estimated quantitatively according to **(Knight *et al.*, 1972)** by using phospho vanillin reagent. Acid phosphatase activity was measured in untreated and treated tissue samples **(Laufer and Schin, 1971)**. The level of both transaminases AST (GOT) and ALT (GPT) was determined colorimetrically in untreated and treated tissue samples according to **(Reitman and Frankel, 1957)**. The activity of acetylcholinesterase (AChE) was measured in untreated and treated larval samples previously treated with LC_{50} of petroleum ether extract of pomegranate peels using the substrate acetylcholine bromide (AChBr) **(Simpson *et al.*, 1964)**. The activities of alpha esterases (α-esterases) and beta esterases (β-esterases) in both untreated and treated tissue samples were measured by using substrates α-naphthyl acetate or β-naphthyl acetate, respectively **(Van Asperen, I962)**. Chitinase activity was determined in untreated and treated tissue samples as described by **Boden *et al*. (1985)**.

Qualitative analysis

Treated and untreated 3rd instar *Cx. pipiens* larvae were homogenized using cold Teflon pestle as one gram of insect body in 1 ml buffer as described before **(Laemmli, 1970 and Studier, 1973)**. Non denaturing polyacrylamide gel electrophoresis was conducted to identify isozymes variations. The utilized isozymes were α and ß esterase. Isozymes were separated in 10% polyacrylamide electrophoresis.

Histopathological and ultrastuctural studies

Studies were demonstrated using JEOL JEM1011, transmission electron microscope (TEM) at the Center for Mycology and Biotechnology, Al-Azhar University. Untreated and treated larvae with LC_{50} of petroleum ether pomegranate peel extract were subjected to histopathological and ultrastructural studies. Treated larvae were prepared for ultrastructural studies **(Bowen and Ryder, 1976)**. Larvae were fixed in 3% glutaraldehyde prepared in 0.1M cacodylate buffer (PH 7.2) for an hour followed by an overnight wash in a fresh batch of the same buffer. Specimens were shortly washed in acetate buffer and incubated for an hour at 37˚C in medium of 5 tablets of P-nitrophenyl phosphate disodium salt, 25mg lead acetate and 25ml acetate buffer. Incubation step was stopped by further washing in cacodylate buffer before post fixing in osmium tetroxide followed by routine dehydration and embedding in araldite. The sections were cut on a Reichert-Jung Ultra-microtome. Semi and ultrathin sections of 0.5-1.0µ & 20-60mm were cut. Semi-thin sections were stained for 1-2 minutes in toluidine blue stain, washed under running tap water, dried and mounted in DPX.

Statistical analysis

Biological and biochemical data was expressed as a mean ± SE. Data between treated groups was analyzed using SPSS software package version 19. One way analysis of variance (ANOVA) was used to test the level of significance, followed by LSD post-hoc multiple comparisons **(Turner and Thayer, 2001)**. $P<0.05$ was considered statistically significant.

RESULTS

1. Petroleum ether extract of pomegranate peel

Qualitative analysis was done to determine the contents of the petroleum ether extract of pomegranate peel by GC/MS. Table (1) and Fig. (1) represent the chemical constituents of the extract, the retention time (RT), peak area, molecular weight and molecular formula of identified constituents, that led to identification of number of compounds.

Table 1. The main components identified by GC-MS in the petroleum ether extract of pomegranate peel.

Peak	RT	Area%	Name	Molecular formula	Molecular weight
1	3.80	2.66	Octane	C_8H_{18}	114.23
2	5.50	2.90	O-xylene	C_8H_{10}	106.16
3	9.39	2.15	Isooctane(ethenyloxy)-	$C_{10}H_{20}O$	156.26
4	11.44	3.30	Decane, 2-methyl	C_8H_{18}	114.23
5	12.58	4.81	Undecane, 2-methyl	$C_{12}H_{26}$	170.34
6	13.44	2.02	2 Tridecen-1-Ol,-(E)-	$C_{13}H_{26}O$	198.35
7	14.59	8.40	Undecane, 2methyl	$C_{12}H_{26}$	170.34
8	15.70	4.72	1-Iodo-2methylnonane	$C_{12}H_{25}I$	296.23
9	16.12	6.50	Decane	$C_{10}H_{22}$	142.29
10	17.47	2.93	1Heptanol, 2 propyl	$C_{10}H_{22}O$	158.28
11	17.60	5.18	Heptadecane, 2 methyl	$C_{18}H_{38}$	254.50
12	17.74	2.64	Naphthalene, 1,2, 3,4 tetrahydro5 methyl	$C_{11}H_{14}$	146.23
13	17.87	4.11	Dodecane, 2,6,11-trimethyl	$C_{15}H_{32}$	212.42
14	50.28	2.69	Octacosane	$C_{28}H_{58}$	394.77
15	57.11	44.99	Stigmastane-3,5 diene	$C_{29}H_{48}$	396.69

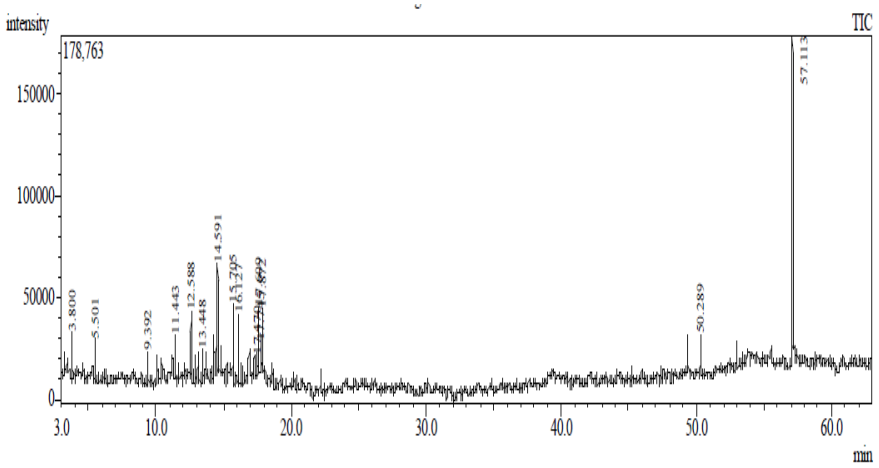

Figure 1. GC chromatogram of all compounds produced from GC-MS analysis of pomegranate peel extract.

2. Larvicidal bioassay

The larvicidal effect of different concentrations of the tested extract of pomegranate peel was evaluated against the freshly molted 3rd instar *Cx. pipiens* larvae (Table 2). The toxicity values varied according to the concentrations of the extract used and exposure time. The percentage mortality of larvae was increased with the increase of the concentrations and exposure time.

Table 2. Susceptibility of 3rd instars larvae *Culex pipiens* to pomegranate peels extract at different time intervals

Concentrations (ppm)	Percentage mortality (%) of pomegranate peels petroleum ether extract			
	24hrs.	48hrs.	72hrs.	96hrs.
untreated	0	0	0	0
50	21.33	44	46.66	53.33
100	53.33	65.33	74.66	84
200	78.66	82.66	94.66	96
300	94.66	96	98.66	100
500	96	98.66	100	100
800	100	100	100	100
LC_{25} (ppm)	25.0922	30.96	29.57	24.19
LC_{50} (ppm)	95.63	62.54	54.47	44.35
LC_{90} (ppm)	282.63	237.94	173.871	140.32
Slope± SE	2.72±0.22	2.20±0.20	2.54±0.27	2.56±0.26

3. Repellence effects of pomegranate peels extract against females *Cx. pipiens*

A variable degree of repellency at different concentrations of pomegranate peel petroleum ether extract and DEET (1.8mg/cm^2), against adults *Cx. pipiens* after four hours of treatment. The relative repellency was increased as the dose increased as shown in Table (3).

Table 3. Repellent effect of pomegranate peels extract against *Culex pipiens* females.

Tested extract	Dose (mg/cm$^{2)}$	% of fed	% of unfed	Repellency %
Pomegranate petroleum ether	6.67	0	100	100
	3.33	0	100	100
	1.67	6.3	93.7	93.1
	0.833	17.5	82.5	80.8
	0.417	30.6	69.4	66.5
DEET	1.8	0	100	100
Control	0.0	92	8	0

4. Effects of pomegranate peel extract on the biology of *Cx. pipiens*

The larvicidal activity, larval duration, pupation percentage, pupal duration, adult emergence percentage and growth index were shown in Table (4). A notable reduction in the percentage of adult emergence was detected with increasing the concentrations. No significant changes were observed in the mean developmental days at all concentrations as compared to untreated. The growth index for *Cx. pipiens* ranged between (15.2- 8.25) at concentrations (50-500) ppm compared to 15.38 for the untreated.

Table 4. Effect of petroleum ether extract of pomegranate peels on the biology of *Culex pipiens*

Concentration ppm	Larval mort. %	Mean Larval Period (days)±SE	Pupation %	Mean Pupal Period (days)±SE	Adult Emergence % (a)	Mean Development (days) (b)±SE	Growth Index (a/b)
Untreated	0.0 ^{a}	4.8±0.60 ^{a}	100.0 ^{a}	1.7±0.14^{a}	100 ^{a}	6.5±0.25 ^{a}	15.38
50	44 ^{b}	4.7±0.76 ^{a}	56 ^{b}	1.5±0.25 ^{a}	96 ^{b}	6.3±0.56 ^{a}	15.23
100	65 ^{c}	4.3±0.76 ^{b}	35 ^{c}	1.9±0.39^{a}	80 ^{c}	6.3±0.56 ^{a}	12.69
200	82 ^{d}	4.4±0.83 ^{b}	18 ^{d}	2±0.28^{a}	72 ^{d}	6.3±0.65 ^{a}	11.42
300	96 ^{e}	4.0±1.15 ^{b}	4 ^{e}	2.3±0.62 ^{a}	60 ^{e}	6.3±0.56 ^{a}	9.52
500	98 ^{e}	4.0±1 ^{b}	2 ^{f}	2.3±0.62 ^{a}	52 ^{f}	6.3±0.56 ^{a}	8.25
800	100 ^{f}	-	-	-	-	-	-

Numbers with the same letters are not significantly different in the same column.

5. Reproductive potential of resulted females

Data in Table (5) illustrates the statistical analysis of the reproductive potential of females resulted from treated larvae with sub lethal concentrations of petroleum ether pomegranate peel extract. There was a significant reduction in the number of egg laid by

female at different concentrations. In addition there was a noticeable increase in the percentage of sterility index for resulted females.

Table 5: Effect of petroleum ether extract of pomegranate peels on the fecundity, fertility and sterility index of *Culex pipiens* females.

Conc. ppm	No. of tested females	No. of eggs laid		No. of hatched eggs		No. of non –hatched eggs					Sterility index (S. I.) %)
		Total	Mean ±SE	Total	%	Total	Embryonated		Non-Embryonated		
							No	%	No	%	
Untreated	20	3893	194.65±54.84 [a]	3700	95.04	93	15	16.12	78	83.65	0.0
50	10	1917	191.7±4.40 [a]	1785	93.11	116	36	31.03	80	68.96	3.49
100	8	1385	173.125±10.03 [b]	1300	93.86	85	21	24.70	64	75.29	12.16
200	9	1500	166.66±11.66 [c]	1395	93.0	105	35	33.3	70	66.66	16.21
300	6	923	153.8±13.33 [d]	855	92.63	68	26	38.23	42	61.76	22.97
500	0	-	-	-	-	-	-	-	-	-	-

Numbers with different letters are significantly different in the same column.

6. Morphogenic abnormalities

Third instar larvae of *Cx. pipiens* treated with pomegranate peel petroleum ether extract induced some morphogenic abnormalities as 4th instar larvae surrounded by old exuvium in its posterior part, transparent body wall with non chitinized integument and abnormal shape of abdomen with collapsing of body wall comparing with healthy larvae in Figure (2). Beside, Larval pupal inter-mediate with larval head but the thorax has pupal structure and pupa lost its comma shape. Pupal-adult intermediate with adult head and thorax but enclosed by old cuticle of pupa (Figure 3). In addition, adult mosquito appeared with poorly developed wings and distended abdomen (Figure 4).

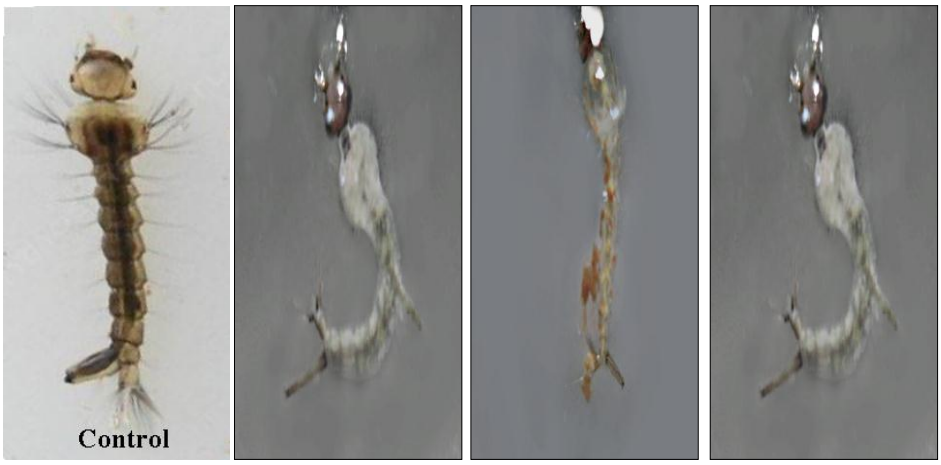

Figure 2. Larval deformities of *Culex pipiens* as a result of treatment of 3rd instar larvae with sub lethal concentrations of pomegranate peel extract compared to the control larva.

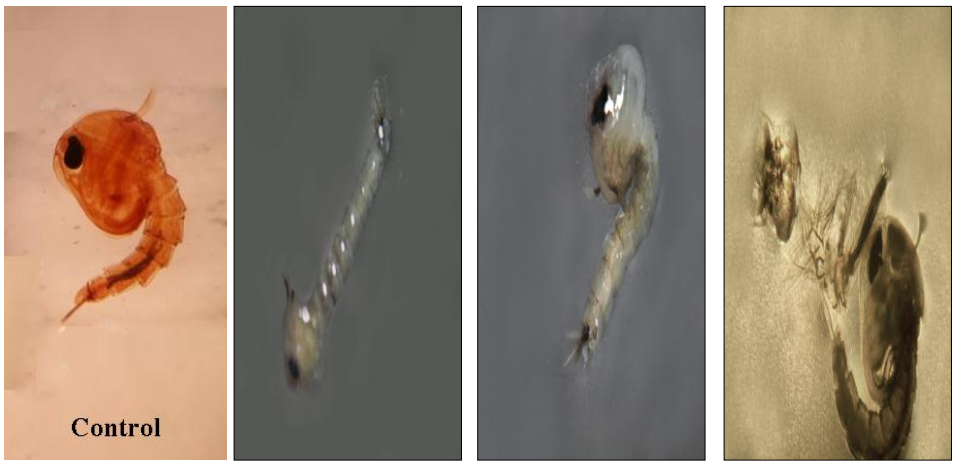

Figure 3. Pupal deformities of *Culex pipiens* as a result of treatment of 3rd instar larvae with sub lethal concentrations of pomegranate peel extract compared to the control pupa.

Figure 4. Adult deformities of *Culex pipiens* as a result of treatment of 3rd instar larvae with sub lethal concentrations of pomegranate peel extract compared to the normal adult.

7. Biochemical effects of pomegranate peel extract on *Cx. pipiens* larvae

7.1. Quantitative analysis:

The biochemical changes in the main body contents (carbohydrate, protein, and lipid) and also the activity of alkaline, acid phosphatases, GPT, GOT, acetylcholinesterase, α and ß-esterases, chitinase, and Glutathione S-transferases of the larvae of *Cx. pipiens* at 48 hours post treatment with LC_{50} of pomegranate peel petroleum ether extract are shown in Table (6). All of them showed significant reduction in contents and activity of treated larvae compared to untreated larvae.

Table 6. Quantitative analysis of different biochemical changes

	Untreated	Pomegranate peel petroleum ether extract	% Change
Total protein (mg/g.b.w)	22.6±0.57[a]	14.8±0.43[b]	-34.51
Total carbohydrates (mg/g.b.w)	10.1±0.43[a]	8.3±0.59[b]	-17.82
Total lipids (mg/g.b.w)	6.19±0.11[a]	3.9±0.36[b]	-36.99
Alkaline phosphatase ($\mu x10^3$ phenol/mg protein)	94±3.2[a]	32.6±3[b]	-65.31
Acid phosphatase ($\mu x10^3$ phenol/mg protein)	47.6±2.5[a]	17±1[b]	-64.28
GPT ($\mu X\ 10^3$/ mg protein)	29.1±2.9[a]	23.6 ± 1.6[b]	-18.90
GOT ($\mu X\ 10^3$/ mg protein)	311± 10.9[a]	72.6±4.9[b]	-76.65
Acetylcholinesterase (µg AChBr/ min/g)	3460±381[a]	524±75[b]	-84.85
α-esterases ± SE (µg α-naphthol/min/g)	105 ±7.6[a]	72 ±2[b]	-31.42
ß-esterases± SE (µg ß-naphthol /min/g)	10.13 ± 0.63[a]	7 ± 0.36[b]	-30.89
Chitinase (mg N acetyl glucose amine/ min/mg protein)	1870±125[a]	1436±78[b]	-23.20
Glutathione S-transferases (m mole sub. conjugated/min/mg protein)	2.04 ± 0.09[a]	1.33 ± 0.03[b]	-34.80

Numbers with different letters are significantly different in the same row.

7.2. Qualitative analysis of total protein:

Native polyacrylamide gel electrophoresis (PAGE) showed the general protein pattern in tissue of untreated and treated *Cx. pipiens* third larval instar with LC_{50} of pomegranate peel extract (Figure 5). The results showed that total number of bands in untreated samples was 9, while the total numbers of protein bands in treated samples with pomegranate peels extract were 7 bands. Treatments by pomegranate peels petroleum ether extract leads to disappearance of normal bands.

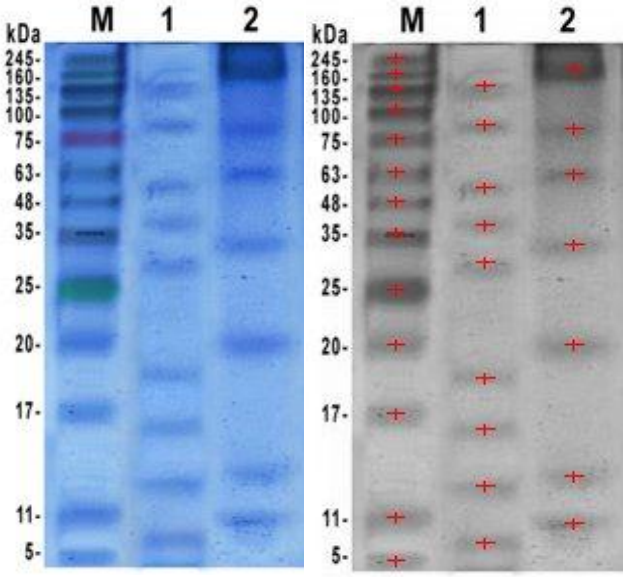

Figure 5. Electrophoretic native protein patterns of untreated and treated samples of *Culex pipiens* larvae. M: Marker, lane 1: Untreated samples, lane 2: Treated samples with pomegranate peels extract.

Fractionation protein, Sodium dodecyl sulfate (SDS-PAGE), revealed that the proteins of tissue of untreated and treated samples with pomegranate peels extracts were separated into 22 different bands according to their molecular weight values while in treated samples with pomegranate peels extract were 19 bands as shown in Figure (6). Treatments with pomegranate peels extract leads to disappearance of three normal bands.

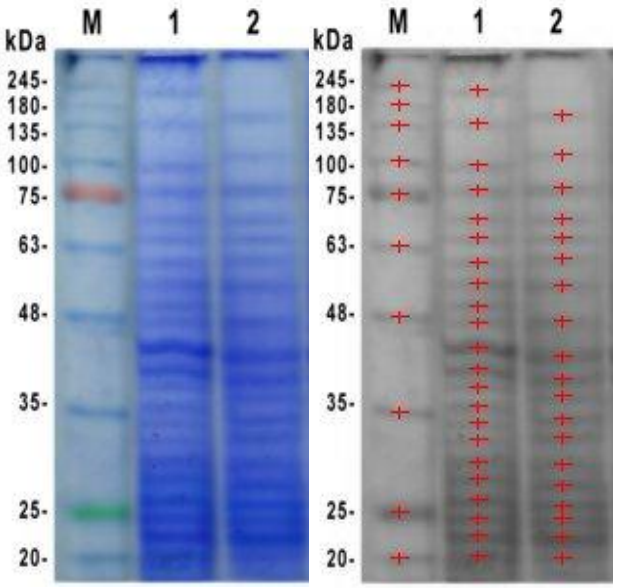

Figure 6. SDS-electrophoretic protein patterns of untreated and treated samples of *Cx. pipiens* larvae. M: Marker, lane 1: Untreated samples, lane 2: Treated samples with pomegranate peels extract.

Isozymes patterns: α-esterase patterns in samples of both untreated and treated samples of *Cx. pipiens* larvae using α-napthylacetate as a substrate were shown in Figure (7). The results showed that total number of bands in untreated samples were 11 bands while in treated samples with pomegranate peel extract were 10 bands. Treatment with pomegranate peels petroleum ether leads to disappearance of normal bands

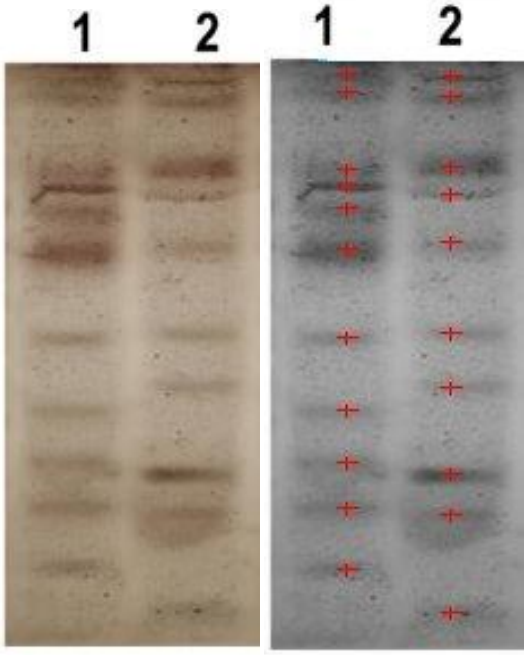

Figure 7. Electrophoretic α-esterase pattern of untreated and treated *Cx. pipiens* larvae by using α-naphthyl acetate as a substrate. 1: samples of untreated, 2: samples treated by pomegranate peel extract.

8. Ultra-structural studies

8.1. The integument

The integument of normal mosquito consists of three layers; the cuticle, the epidermal layer and the basement membrane (Fig. 8 A & Figs. 9 A, B). The cuticle is differentiated into inner endocuticle, outer exocuticle and epicuticle. The epicuticle is composed of thin non chitinous layer or cuticulin and an amorphous inner epicuticle. The procuticle consists of a series of laminar chitin fibers often with a helicoidal rotation to their orientation which gives rise to the lamellar pattern. The epidermis consists of a single layer of cells having oval nuclei containing plenty of chromatin and irregular nuclear membrane. In treated larvae with LC_{50} of pomegranate peel extract, great damage was observed in the cuticular layer, few fragmented epidermal cells, vacuolization of the hypodermis, the nuclei were degenerated or pycnotic. Damage and interruption in cell layer was noticed in the structure of the cuticle. A large space between endocuticle and epicuticle was observed. These dramatic changes caused abnormal state of insect life (Fig. 8 B & Figs. 9 C, D).

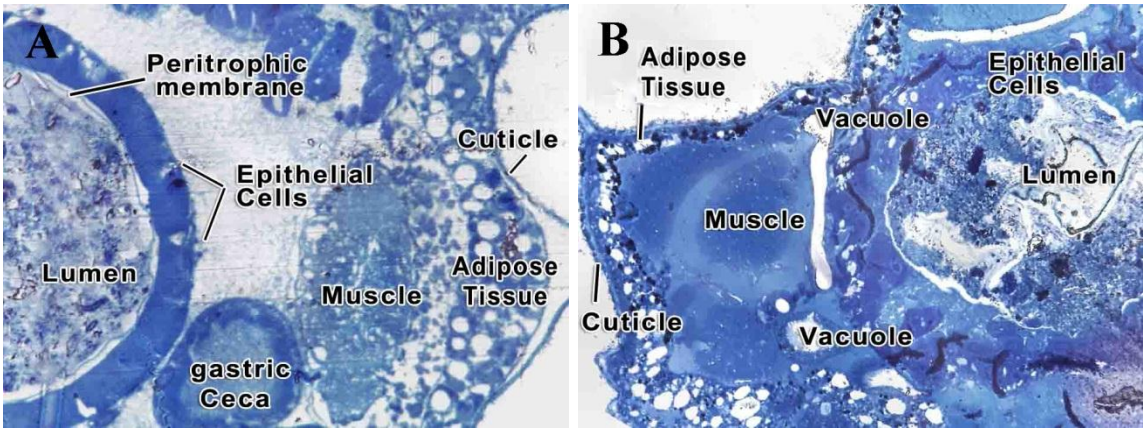

Figure 8. Semi-thin section in larvae of *Cx. pipiens* cuticle showing normal cuticle (A) and Treated larvae with pomegranate peel extract (B). (X=400)

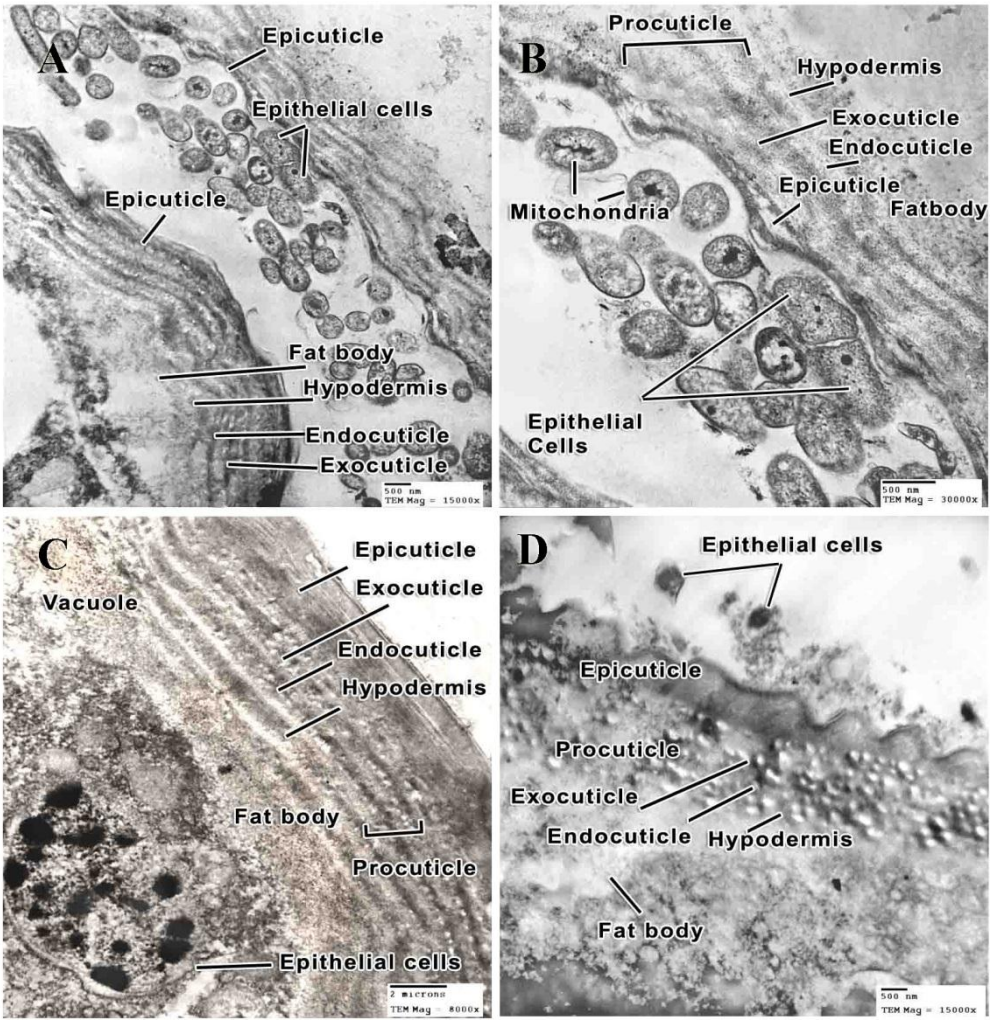

Figure 9. Electro micrograph of normal cuticle of *Cx. pipiens* larvae (A) & (B), while (C) & (D) showing the cuticle of treated larvae with pomegranate peel extract.

8.2. Skeletal muscle

The normal muscles of the skeletons consist of elongated, contractile fibers that lie at the point of insertion parallel to each other. Sometimes they are incredibly various (Figure 10 A). The muscles tend to consist of striated fibres; any fibre is composed of parallel fibrillas or sarcostyles, which are more or less filled with glycogen in a nuclear plasma or sarcoplasm.

The fibrils are tiny threads with slight noticeable differentiation. Larvae treated with LC_{50} pomegranate peel extract revealed a vacuolization, disorganized muscles, and disappearance of the sarcoplasmic reticulum 48 hours after treatment. Shrinkage, reduction and disorganization in fibrils size were noticed compared to the untreated larvae. Disappearance of mitochondria and destruction of the nucleus with condensed chromatin were observed (Figure 10 B).

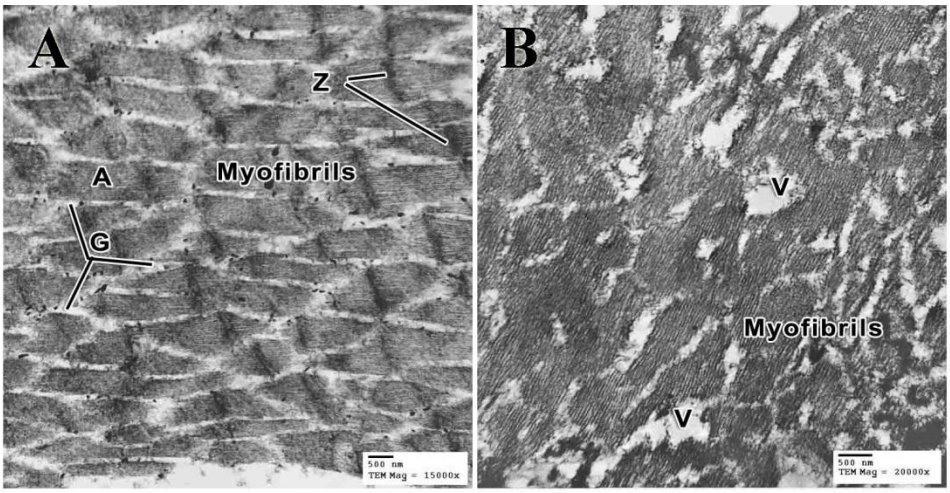

Figure 10. Electron micrograph of skeletal (striated) muscle fibrils in untreated *Cx. pipiens* 3rd instar larvae (A) while (B) treated larvae with pomegranate peel extract.

DISCUSSION

Several studies indicated that plant extracts represent a possible alternative to traditional chemical insecticides **(Overgaard *et al.*, 2014 and Pavela, 2016)**. Phytochemical analysis for petroleum ether extract of pomegranate peel detected the presence of sterols, quiniens and terpenoids in moderate amounts, tannins, saponins, flavonoids, phenols in low amounts, and absence of both alkaloids and resins. Gas Chromatographic analysis (GC) indicated that steroid group was the dominant chemical group for the tested extract. Results showed a relationship between sterols contents and its toxicity. As revealed from the obtained results, pomegranate peel petroleum ether extract caused 50% mortality of *Cx. pipiens* larvae at concentration 62.54 ppm. In agreement with the present results, the efficiency of agricultural waste extracts against *Cx. pipiens* and other insects has been detected by several authors **(Helmy *et al.*, 2012; Masotti *et al.*, 2012 and Hlila *et al.*, 2017)**. The present findings agreed with **Das *et al.* (2018)** who reported that petroleum ether extract exhibited the highest larvicidal activity against 3rd instar larvae of *Cx. quinquefasciatus* than the remaining checked extracts. **Koide *et al.* (1998)** reported that the astringent properties of tannins included in the peel fruit was the main reason for the

toxicity caused by pomegranate. The chemical composition of phenolic moieties in the phenolic compound tends to be related to the insecticide activity of pomegranate. The aromatic nucleus of these compounds is attached to a polar function group (OH).

Extract of pomegranate peels showed repellent activity against female *C. pipiens*. Our findings revealed that toxic active ingredients contained in the tested extract acting primarily through contact application. The repellent activity of plant extracts can be triggered by the complexity of their components' chemical composition **(Kumar *et al.*, 2008)**. **Mohammad (2013)** proved that the ethanol extracts of the pomegranate fruit peel have a high repellent effect (86.7 %) for *Triboleum confusum* after two hours of exposure to a concentration of 2.5%. **El-Sheikh *et al.* (2016)** investigated repellence and biting deterrence effect on plant extract that evoked 100%, was the petroleum ether extract on both *Cx. pipiens* and *Aedes aegypti* compared with 100% repellency for commercial formulation, (DEET). Also, our results agreed with Jayapriya and **Shoba (2015)** that proved that, the petroleum ether extract had strong repellent action against mosquitos as it provided 100% protection against *Culex quinquefasiatus* and *A. aegypti*.

From present results, lethal and sub-lethal treatments of the tested insecticides had adverse effects on biological activities of *Cx. pipiens*. The decline in pupation and adult *Cx. pipiens* was attributed to plant extract treatment close to the evidence collected from **Farag and Emam (2016)**. The fact that toxin blocked the maturity of the imaginal disc which was primordial for adult integumentary structures in endopterygote insects may contribute to a reduction in the rate of adult emergence **(Suh *et al.*, 2000)** or owed to distortion of chitin in adults **(Abo-El-Mahasen *et al.*, 2010)** or might be related to two or more of the following issues: unsaturated fatty acids, accelerating the process of melanization and hardening larvae; thus adults couldn't free themselves from the pupal exuvia; insufficient pressure in the ptilinum and hardening of opercular suture. The reduction in larval duration of *Cx. pipiens* following treatment with both pomegranate extract agrees with **(Sosa *et al.*, 2018)**. Such reduction may be due to the accelerated molting process, where these compounds are capable to disrupt the endocuticle deposition during the building up of a new cuticle **(Abdalla and Sammour, 1992)**. The prolongation of pupal duration of *Cx. pipiens* following treatment with pomegranate extract is similar to data obtained **(Granados-Echegoyen *et al.*, 2014)**. The number of eggs laid per female mosquito of *Cx. pipiens* together with the percentage of egg-hatch were decreased due to the treatment. These findings are in accordance with the results of **Peres *et al.* (2017)**. The reduction in both fecundity and fertility is attributed to a sterilization of females or inability of sperms of males to be transferred to the females during copulation **(Ismail, 1980)**. Also, the egg maturation in culicinae species is under hormonal control **(Laemmli, 1970)**. This may indicate that the used plant extracts interfere with the hormonal system of *Cx. pipiens*. The tested agricultural waste extracts used in the present study showed different morphogenetic abnormalities in the larval, pupal and adult stages. Similar results were also obtained against the same insect species **(Yu *et al.*, 2015)**.

Biochemical studies were carried out in an attempt to disclose the effect of median lethal concentration (LC_{50}) of the tested agricultural waste extract (pomegranate peels) on *Cx. pipiens* larvae. In general, an obvious significant reduction in protein levels

was observed. The drop in the protein contents of the tissue samples in the present work in all treatments might be due to protein binding with foreign compounds as the tested agricultural waste extracts or may be due to mobilization of amino acids during plant extracts stress to meet the energy or might be due to the destructive effects on some of the cerebral neurosecretory cells of the brain responsible for secretion of the protein of the treated larval instars of *Cx. pipiens.* Similar findings were obtained by **Senthikumar *et al.* (2009)** who found that protein levels were decreased in the *Anopheles stephensi* larvae treated with certain plant extracts as a result of interfere with the process of natural protein synthesis. Carbohydrates are of vital importance since they can be utilized by the insect body for production of energy or conversion to lipids. Total carbohydrates are a major component necessary for growth and development **(Visser *et al.*, 2017)**. So the activity change as a result of treatment with tested agricultural wastes and in general disturbance in carbohydrate metabolism as expressed by reduction of carbohydrate levels could be resulted from inhibition of chitin synthesis **(Salem *et al.*, 1997)**. The obtained results induced highly significant inhibition in carbohydrate levels with the tested extract. The present results agreed with **Sharma *et al.* (2011)** who observed decrease in the carbohydrate of culicine larvae treated with *Artemisia annua* petroleum ether extract and *Azadirachta indica* methanolic extract. The essential structural component of cell membrane and cuticle is lipids **(Pesch *et al.*, 2016)**. They provided a good source of energy supply. They facilitated water conservation by creating an impermeable cuticular membrane and supplying metabolic water after oxidation **(Visser *et al.*, 2017)**. The obtained data declared that the tested agricultural waste extract caused a highly significant reduction in lipid contents as compared with untreated mosquito larvae. The great reduction in total lipids might be due to its conversion to protein **(Abdel-Aziz, 2000)**. The present findings were in harmony with **Peres *et al.* (2017)** who noticed reduction in lipid contents of *Plutella xylostella* treated with aqueous extracts of *Alibertria* spp. The lysosomal marker enzyme, which is active in guts, is known as acid phosphatase **(Shelomi, 2017)**. Brush border membrane marker enzyme, alkaline phosphatase, was particularly active in tissue with active membrane transport (Qari *et al.*, 2017). So it could be used as a parameter for determining antifeedant activity **(Abdel-Aziz, 2000)**. The obtained data clearly showed a highly significant inhibition in the acid phosphatase and alkaline phosphatase after treatment with the tested extracts. This decrease in enzyme activity might be due to a strong inhibition of ecdysone which is accompanied by a decrease in the number of lysosomes. It leads to a decrease in acid phosphatase levels **(Hassan, 2002)**. It was cleared from results that treatment of *Cx. pipiens* larvae with the tested agricultural waste extract caused significant reduction in the transaminases activity. These changes were in line with **Ghoneim *et al.* (2014)** who studied the biochemical effects of ethanolic, n-butanol and petroleum ether pomegranate peel extracts on transaminases enzymes (GOT and GPT) activity in some tissues of *Schistocerca gregaria*. He demonstrated a significant reduction on GOT activity in fat bodies of treated nymphs. Acetylcholinesterase is a serine esterase in the α/β hydrolase fold enzyme family **(Lenfant *et al.*, 2013)** that terminated nerve impulses at cholinergic synapses by breaking down the neurotransmitter acetylcholine. The obtained results recorded highly significant reduction in AChE affected by pomegranate peel petroleum ether extract. The present results were in tune with **(Ghoneim, 2015)** who found a potent inhibitory activity in AChE of *Schistocerca gregaria* treated with pomegranate peel

extract. Also, the results showed reduction in enzyme activity of Glutathion S-transferase (GST) following the treatment of tested extracts. The suppression of detoxification enzyme indicated that this enzyme showed no role in detoxification of tested extracts and increased the susceptibility of *Cx. pipiens* to the tested extracts as stated by Abd El-aziz and **El-Sayed (2009)** when they tested six essential oils extracted from garlic against *Tribolium confusum* adults and larvae at sixth instar.

In the present study, the patterns of native protein in larval tissue samples revealed different amounts of protein bands in untreated and treated larval samples of *Cx. pipiens* according to their molecular weight. Immune responses, reproduction, and metabolic processes influenced by these proteins **(Al-Qahtani *et al.*, 2012)**. According to this study, native, fractionation and isozymes protein patterns revealed that, there were common bands; these proteins might be characteristics for *Cx. pipiens* larvae. Changes in the protein pattern might be due to toxicity of the tested extracts. These results agreed with those of *Phylosami aricini*, in which the amount of electrophoretically separated protein bands decreased from 11 to 6 on the seventh day after treatment **(Poonia, 1979)**. The untreated and the treated 3rd larval instar of *Cx. pipiens* were subjected to SDS-PAGE protein electrophoresis. There were obvious variations between treated and untreated larvae in protein patterns. In contrast to the untreated samples, treatment with plant extract caused normal bands to vanish and abnormal bands to appear. Other experimental findings such as decreased pupation, pupal weight, and adult emergence could be explained by the absence of many protein bands in treated samples, which could be attributed to the toxic action of tested agricultural waste extracts, which inhibited the synthesis and expression of protein. On the other hand, the appearance of new protein bands may be attributed to an increase in protein synthesis. These results were in accordance with that found by **Bakr *et al.* (2002)** who tested the effect of four plant extracts on native protein and SDS-separated protein obtained from the haemolymph of treated larvae of *S. littoralis*. Esterases are a very broad class of enzymes, all of which can break an ester bond with the aid of a water molecule. The majority of these enzymes hydrolyzed endogenous substances and were essential in metabolism **(Jackson *et al.*, 2013)**. In the present work, great differences in number of zones of esterase activity and in substrate specificity between treated and untreated samples were noticed. Also, the tested agricultural waste extract decreased the activity of esterase (intensity of bands). These events would finally lead to the observed failure in metamorphosis, which was characterized by the evaluation of unhealthy adults. The present results coincided with that found by **El-Bermawy (2004)** who analyzed esterases from body extracts of 6th larval instar and newly formed pupa of *S. littoralis* treated by different botanical extracts. The author stated that, any unusual increase or decrease in the activities of the enzymes in treated samples might be interpreted, on the molecular level, to depression or mutation of the regulating genes responsible for biosynthesis of polypeptide chains building these enzymes.

In studying the effect of pomegranate peel petroleum ether extract against *Cx. Pipiens* larvae, alterations in the structure of the integument and muscles were found. Similar observations were noticed by **Younes *et al.* (1999)** when they treated *S. littoralis* larvae with plant extracts of both *Brassica tournefortii* and *Zygophyllum coccineum* and observed degeneration of the cuticle and detachment of the epidermal cell from each other. **Khalaf *et al.* (2009)** observed detachment of cuticle from hypodermis,

disintegration in the hypodermis and destruction of the basement membrane when *Synthesiomyia nudiseta* larvae treated with certain volatile oils. The histopathological effect of pomegranate peel petroleum ether extract on muscles was ranged between slight degeneration by the occurrence of fissures, to complete destruction of the whole tissue. The appearance of fissures and the breaking down of muscles into small parts were attributed to the destruction of the sarcolemma. Similar observations were noticed by **Bakr *et al.* (2007)** on *Shistocerca gregaria* treated with rice bran extract. The deformities in midgut tissues may be due to the presence of saponins and phenolic compounds present in petroleum ether extract of pomegranate peels. Saponins are natural glycosides with a wide range of pharmacological properties, including cytotoxic activity. Most saponins' cytotoxic effect was attributable to their ability to induce apoptosis in living cells. The general cytotoxicity of saponins is dependent on their plasma membrane toxicity and that the membrane toxicity might be caused by the loss of cholesterol molecules from the cell membrane **(Podolak *et al.*, 2010)**. This is suggested to explain the alteration patterns observed in the midgut epithelium of the treated larvae. Plants synthesize phenolic compounds through the pentose phosphate and phenylpropanoid pathways as secondary metabolites. After penetrating the cell, phenols undergo active transformation, primarily due to the involvement of oxidases within cytochrome P450. Transformation processes often lead to a rapid increase in toxicity through the formation of electrophilic metabolites that can bind and damage DNA or enzymes in the cell. The cytotoxic effects of phenolic compounds mainly depend on their reactivity. Phenols undergo radical reactions causing lipid peroxidation in the cell membrane and damage to the endoplasmic reticulum, mitochondria, and nucleus membranes, as well as their biochemical components such as enzymes and nucleic acids **(Randhir *et al.*, 2004; Michałowicz and Duda, 2006)**.

CONCLUSION

We concluded that the tested agricultural waste extract of pomegranate peels showed pronouncing insecticidal activities against mosquitoes in terms of both concentrations and time. It could be used in pest control management strategies as alternatives to traditional insecticides in controlling insect pest to decrease costs and impact of pesticides on the environment and positively affect human health.

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