TOXICOLOGICAL, BIOCHEMICAL AND BIOLOGICAL EFFECTS OF POMEGRANATE (*PUNICA GRANATUM*) PEEL EXTRACTS AGAINST DIFFERENT LARVAL INSTARS OF COTTON LEAFWORM, *SPODOPTERA LITTORALIS* (BOISD.)

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ABSTRACT

Investigation of green pesticides is the most important method to maintain sustainable agriculture and avoid health and environmental hazards resulting from the excessive use of chemical pesticides against controlling cotton leafworm *Spodoptera littoralis*. Therefore, the present study evaluated the toxic effects of four different pomegranate peel extracts against 2nd and 4th instar larvae of cotton leafworm during different exposure periods (24, 48, 72 and 96 hours). Ethanolic pomegranate peel extract (EPPE) resulted in the highest toxic effect with LC_{50} values (0.0014 and 0.0022 %) after 96hrs against 2nd and 4th instar larvae. While those values of Hexane (HPPE), Methanol (MPPE) and Acetone (APPE) pomegranate peel extracts were (0.0019 and 0.0042 %), (0.002 and 0.0043 %), (0.0022 and 0.0045 %), respectively. Pretreatment with LC_{50} of the most potent extract (EPPE) after 48 hours of exposure resulted in biological changes represented by a significant decrease in the percentage of pupation (30.94% and 41.4%) of 2nd and 4th instar larvae, respectively, compared to the control (95% and 84.2%). EPPE treatment highly affected the sex ratio (number of emerged males was greater than females). EPPE treatment induced a highly significant decrease in total protein and soluble carbohydrate content in both the 2nd and 4th instar. The total lipid content increased weakly in the 2nd instar but no significant difference in processing was recorded in the 4th instar when compared to the control. The present study suggested using pomegranate peel extract in integrated pest management programs of *S. littoralis* especially against early larval instar.

Keywords: *Spodoptera littoralis;* Pomegranate peel extract; insecticidal activity; biological changes; biochemical studies.

INTRODUCTION

Cotton leafworm is one of the most destructive pests in Egypt. Despite being known as a key polyphagous pest, it also attacks more than 29 hosts from other vegetable crops and more than 60 different cultivates (Shoaib et al., 2014). The use of chemical pesticides is a very efficient method for the maintenance of crop yield stability but most of them are highly toxic and their residues can accumulate in the soil for several decades (Mansour & Aly, 2015).

Excessive use of chemical pesticides resulted in resistance problems, especially in controlling cotton leafworm. Accordingly, there was an urgent need to develop a set of practices to serve in preventing or delaying the development of pesticide resistance which was referred to as Insect resistance management (IRM) (Agi et al., 2001). Using transgenic plants represents one of the most effective methods in IRM programs to solve resistance problems, especially with economic crops. Since this method is very expensive for growers, scientists have designed many types of research to develop more economical methods such as investigating natural pesticides derived from plants (Ramalho et al., 2014). The efficacy of these botanical pesticides has been referred to as the secondary metabolites which represent the primary defense of plants against phytophagous pests (Hatem et al., 2011). They represent an eco-friendly control method that is nontoxic to mammals, non-phytotoxic and can be degraded after a short time so there is no need for the pre-harvest interval (Souto et al., 2021) and they could be applied alone or as a part of pest management programs (Campion et al., 1977).

Using plant biopesticides is one of the most important steps towards the green revolution. Future environmental risks of pesticides are expected to be affected by two variables: climate change and pesticide management. Climate change will affect patterns of pesticide exposure, while pesticide management can lead to changes in pesticide doses. These changes can be influenced by the spread of agricultural pests or the implementation of environmental protection policies like the "farm to fork" strategy. (Martínez-Megías et al., 2023).

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Pomegranate peels are considered agro-industrial wastes and they have proved efficacy as antimicrobial agents for controlling plant and food-borne pathogens and fungal and bacterial field diseases (Eldiasty et al., 2014). Many researchers have proven the efficacy of pomegranate peels against many pests such as rice weevils, house dust mites and mosquitoes (Ben Hamouda et al., 2014; Jung, 2015; Eldiasty et al., 2014; Farag & Emam, 2016).

The scope of the present study was to investigate the toxicological, biological and biochemical activity of different pomegranate peel extracts against cotton leafworm to obtain a new economic botanical agent that could be used in control management programs in the future.

MATERIALS AND METHODS

Preparation of pomegranate peel extracts

Pomegranate peels were extracted using a low-heat extraction method by the Soxhlet apparatus. Pomegranate ripe fruits were washed, and fresh peels were removed, shade dried and crushed using an electric blender and kept at 4ºC till extracted. Four different peel extracts were prepared using four different solvents (Ethanol, Methanol, Acetone and Hexane). The method of Freedman et al. (1979) was adopted with a minor modification. Samples of 100 g of plant material were inserted in the Soxhlet thimble and solvents were added at a rate of 3 ml/g plant material for 8 hrs. extraction period. The solvent was evaporated to dryness under vacuum using a rotavapor with a water bath adjusted to 40°C. The crude residues were then weighed to estimate their yield percentages and kept in a deep freezer (-4ºC) until used.

The percent yield of prepared extracts was computed according to the following equation:

$$\% \text{ Yield} = \frac{\text{Weight of crude extract}}{\text{Weight of plant powder}} \text{ X } 100$$

Rearing of *Spodoptera Littoralis* (Boisd.)

Spodoptera littoralis (Boisd.), the cotton leafworm used in this study, was obtained from Syngenta Company, Qaha, Qalyubia Governorate. Insects were maintained for 10

generations without exposure to chemicals, insecticidal and /or microbial pressure about once a year at the Physiology Research Department, Plant Protection Research Institute (PPRI), Dokki, Giza, Egypt. It was reared on castor oil (*Ricinus Communis*) leaves at 25 ±2°C and 65 ±5% R.H. with the same feed and photoperiod (Eldefrawi et al., 1964). All tests were performed on 2nd and 4th instar larvae of *S. littoralis.*

Dipping technique

The effects of pomegranate peel extracts were investigated for their insecticidal activity against the 2nd and 4th instar larvae of cotton leafworm using the dipping technique (Abo-Elghar et al., 2005). Fresh castor oil leaves were cut into discs (4 cm in diameter). The discs were dipped in the prepared concentrations (0.001, 0.00 2, 0.003 and 0.004 %) for the 2nd instar and (0.002, 0.004, 0.006, 0.008 %) for the 4th instar of PPE for 30 seconds and allowed to dry before application under laboratory conditions. Ten larvae were transferred to plastic containers and discs were added after drying for each concentration and each concentration had four replicates. Discs dipped in distilled water served as the negative control group.

Toxicity assessment

Ethanol **(EPPE)**, **M**ethanol **(MPPE)**, **A**cetone **(APPE)** and **H**exane **(HPPE)** **P**omegranate **P**eels **E**xtracts were assessed at 0.1% which is equaled to 1000 ppm. Promising extracts (resulted in 50% mortality) were subjected to detailed toxicity studies to determine their LC_{25}, LC_{50} and LC_{90} values, as well as, the slope of their regression lines, according to **(Finney, 1971)**. Four different concentrations were prepared for each extract (0.001, 0.002, 0.003 and 0.004 %) for the 2nd instar and (0.002, 0.004, 0.006, 0.008 %) for the 4th instar. Mortality percentages were recorded after 24, 48, 72 and 96 hrs. post-treatment and corrected according to the Abbott's formula **(Abbott, 1925)**.

$$\text{Corrected Mortality}(\%) = \frac{\%MT - \%MC}{100 - \%MC} \times 100$$

Where %MT = % larval mortality in treatment.

%MC = % larval mortality in control.

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Biological studies

For studying the impact of LC_{50} on the various biological aspects of *S. littoralis* larvae in the 2nd and 4th instar. 320 larvae were divided into eight replicas, each containing 40 larvae and treated with LC_{50} concentration value using dipping technique for 48 hrs. Larvae were kept in plastic cups and the daily mortality rate of the larvae was recorded until they pupated. The study recorded several parameters, including mortality ratio, larval and pupal duration, pupa weight, pupation rate, adult emergence rate, adult longevity, deformation of different stages and sex ratio. Experimental conditions were adjusted at the temperature of 27 ± 2 °C and a relative humidity of 55%- 65%.

The control group was conducted along with treatment using castor oil discs dipped in distilled water only.

The following biological aspects were determined:

$$1-\ \%\ \text{of larval mortality} = \frac{\text{Number of accumulative dead larvae}}{\text{Total number of larvae}} \text{ X } 100$$

$$2-\ \%\ \text{of deformed larvae} = \frac{\text{Number of deformed larvae}}{\text{Total number of larvae}} \text{X } 100$$

$$3-\ \%\ \text{of pupation} = \frac{\text{Number of pupae}}{\text{Total number of larvae}} \text{ X } 100$$

$$4-\ \%\ \text{of Pupal mortality} = \frac{\text{Number of dead Pupae}}{\text{Total number of pupae}} \text{ X } 100$$

$$5-\ \%\ \text{of adult emergence} = \frac{\text{Number of emerged adults}}{\text{Total number of pupae}} \text{ X } 100$$

$$6-\ \%\ \text{of deformed pupae} = \frac{\text{Number of deformed pupae}}{\text{Total number of pupae}} \text{ X } 100$$

$$7-\ \%\ \text{of maleformed adults} = \frac{\text{Number of malformed adults}}{\text{Total number of adults}} \text{ X } 100$$

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Biochemical studies

Preparation of homogenate sample

The castor oil leaves were treated with LC_{50} of pomegranate extract, while the castor oil leaves used for the control were treated with distilled water. Second instar larvae of *Spodoptera littoralis* were placed to feed on leaves treated with the extract and also the control. After 48 hours, lived larvae were taken, weighed (the weight is not less than half a gram), placed in Eppendorf tubes and then placed in the freezer until frozen. Same technique was repeated with the fourth instar larvae of the insect. Homogenate samples were collected from larvae by homogenizing in insect physiological saline (0.5 g in 5 ml insect physiological saline) and collected in cold tubes (on ice) the samples were centrifugated at 4500 rpm for 5 minutes under cooling (4°C) to remove the tissues. After centrifugation, the supernatant fluid was divided into small aliquots (0.5 ml) and stored at –20 °C until analysis (Dahi et al., 2021)

Determination of total protein content

The protein content of the homogenate samples was determined using folin phenol reagent (Lowry et al., 1951). Two gm of sodium carbonate were dissolved in 0.1 sodium hydroxide to prepare the reagent (1). Reagent (2) was prepared by dissolving 0.5 gm of cupric sulphate in 1% potassium–sodium tartrate. Both reagents were kept at 4 °C till used in the bioassay. Buffer copper sulphate solution was freshly prepared by mixing one part (ml) from reagent (1) with ten parts (ml) from reagent (2). Folin reagent 2N; was prepared by mixing 1 ml of Folin ciocalteus with 2 ml of distilled water. This reagent was freshly prepared. A stock standard of six series of bovine albumin solutions was prepared. For the bioassay, homogenate samples were added to 1 ml of 5% trichloroacetic acid and the precipitated protein was dissolved by boiling for 5 min in 2 ml of 1 N NaOH solution. Then 0.2 ml from this alkaline protein solution was placed in clean test tubes, each containing one ml of reagent (3). After 10 minutes 0.2 ml of folin reagent was added to the mixture and the contents were heated for 2.5 minutes at 50°C (to dissolve the precipitate). The tubes were allowed to stand for 10 minutes to cool at room temperature. The blanks were similarly run

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using 0.2 ml NaOH instead of the hemolymph sample. Reading was measured spectrophotometrically at 750 nm.

Determination of total carbohydrate content

The total carbohydrate content of the prepared homogenate was determined according to Singh & Sinha, (1977). Anthrone reagent was prepared by adding 28 ml of distilled water to 72 ml of concentrated H_2SO4; while this mixture was still warm, 50 mg of anthrone was added with vigorous shaking. Standard mixtures of glucose were prepared by dissolving 250 mg of glucose in 100 ml of distilled water, from which standard solutions were prepared to cover the range of carbohydrate content. An aliquot of 0.2 ml of the homogenate was diluted with 1 ml of distilled water; then treated with 5 ml anthrone reagent. A blank containing 1.2 ml distilled water and 5 ml anthrone reagent was prepared. All test tubes of the unknown and the blank were placed in a boiling water bath for 10 minutes and then left to cool for 15 minutes at room temperature. Readings were measured pectrophotometrically at 620 nm.

Determination of total lipid content

The total lipid content of the homogenate was determined by the phosphovanillin method (Barnes & Blackstock, 1973).Vanillin solution was prepared by dissolving 0.6 gm vanillin in 8-10 ml of absolute ethanol. The solution was completed to a volume of 100 ml with distilled water with continuous stirring for complete mixing. The formed phosphovanillin reagent was stored in a dark bottle at room temperature. One ml of chloroform-methanol mixture (v/v) was placed into a clean dry test tube containing 0.2 ml of the homogenate sample, with vigorous shaking for complete extraction of the lipid. The contents were centrifuged at 3000 rpm for 10 minutes. By using a water pump, the upper phase as much as possible was removed by siphoning and then the extracted lipid (lower phase) was mixed thoroughly with 0.2 ml of saline solution (0.9 % NaCl). The mixture was allowed to separate into two phases (upper aqueous and lower organic phase) by centrifuging at 3000 rpm for 15 minutes. The upper phase was removed by siphoning. 0.5 ml concentrated H_2SO4 was added to the extracted lipids, the contents were heated for 5 minutes in a boiling water bath and then left to cool at room temperature. A blank was

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simultaneously run which contained 0.5 ml concentrated H_2SO4. The phosphoric acid-vanillin reagent (2.5 ml) was added to the unknown and the blank with vigorous shaking. The mixture was left at room temperature for 10 minutes. The developed color was measured spectrophotometrically at 540 nm against the blank.

Statistical analysis

The LC_{25}, LC_{50} and LC_{90} values were estimated using the "LdP Line®" software, Bakr (2007) according to the method described by Finney et al., (1971). Mortality percentages were corrected using Abbott's formula (Abbott, 1925).

The significance of the means was tested using one-way analysis of variance **(ANOVA)**, and the means were compared using the Duncan's multiple range tests at 5 % level of probability using the software Statistical Package for Social Sciences **(SPSS)** version 27.0 for windows.

RESULTS

Yield calculation of prepared pomegranate peel extracts

Data in Figure 1 demonstrated the percentage yield of the four pomegranate peel extracts tested. EPPE resulted in the highest % yield (48%) and APPE recorded the lowest %yield with a value equal to 35%.

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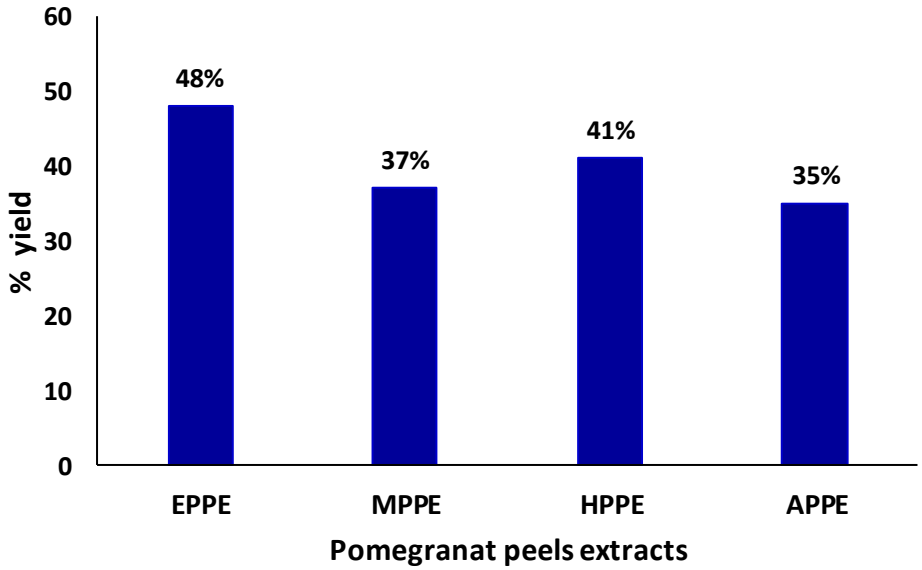

Figure 1. Percentage yield for the tested pomegranate peel extracts with different solvents (Ethanol (EPPE), Methanol (MPPE), Acetone (APPE) and Hexane (HPPE) Pomegranate Peels Extracts)

Toxicity of pomegranate peel extracts against *Spodoptera littoralis* larvae

The toxicity of four pomegranate peel extracts against 2nd and 4th instar larvae of cotton leafworm was evaluated and mortality % was recorded after 24, 48, 72, and 96 hrs. Table 1 showed the LC_{25}, LC_{50}, and LC_{90} values of the four extracts against 2nd instar larvae. EPPE was the most effective extract after 96 hrs. with LC_{50} value equaled 0.0014% followed by HPPE, MPPE and APPE,. The LC_{50} values obtained by EPPE, MPPE, and APPE were increased by increasing the time of exposure while HPPE recorded the same LC_{50} value after 24 and 48 hrs. Similar results were obtained in the toxicity assessment against 4th instar cotton leafworm larvae as presented in Table 2. As the ethanolic extract of pomegranate peel revealed the highest toxic effect, it was subjected to further biological and biochemical studies.

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Table 1. Toxicity of four pomegranate peel extracts against 2nd larval instar of *Spodoptera littoralis* at different exposure time intervals.

TIME (HRS)	LC_{25} (%)	LC_{50} (%)	95% FIDUCIAL LIMIT		LC_{90} (%)	95% FIDUCIAL LIMIT		SLOPE ± S.E.
			Lower	Upper		Lower	Upper	
EPPE								
24	0.0016	0.0028	0.0025	0.0032	0.0085	0.0065	0.0128	2.7107 ± 0.3311
48	0.0012	0.0022	0.0019	0.0024	0.0066	0.0053	0.0093	2.6448 ± 0.3114
72	0.0009	0.0015	0.0013	0.0017	0.004	0.0034	0.0048	3.1241 ± 0.3230
96	0.0008	0.0014	0.0012	0.0015	0.0035	0.003	0.0042	3.1524 ± 0.3308
MPPE								
24	0.0019	0.0034	0.0031	0.0037	0.0103	0.0085	0.0135	2.6517 ± 0.2437
48	0.0015	0.0025	0.0023	0.0028	0.0069	0.006	0.0083	2.9286 ± 0.2396
72	0.0012	0.002	0.0018	0.0022	0.0055	0.0048	0.0065	2.9012 ± 0.2360
96	0.0012	0.002	0.0018	0.0022	0.0055	0.0048	0.0065	2.9012 ± 0.2360
APPE								
24	0.002	0.0033	0.003	0.0036	0.008	0.0067	0.0102	3.2930 ± 0.3140
48	0.0016	0.0027	0.0025	0.003	0.0071	0.006	0.0089	3.0881 ± 0.2851
72	0.0014	0.0022	0.002	0.0024	0.0058	0.005	0.007	3.1128 ± 0.2756
96	0.0013	0.0022	0.002	0.0024	0.0058	0.005	0.007	3.0797 ± 0.2741
HPPE								
24	0.0011	0.0022	0.0019	0.0025	0.0075	0.0058	0.0116	2.3586 ± 0.3039
48	0.0011	0.0022	0.0019	0.0025	0.0075	0.0058	0.0116	2.3586 ± 0.3039
72	0.0011	0.0022	0.0019	0.0025	0.0075	0.0058	0.0116	2.3586 ± .3039
96	0.001	0.0019	0.0016	0.0021	0.0063	0.005	0.0091	2.4333 ± 0.3029

*** EPPE=** Ethanol Pomegranate Peels Extracts, **MPPE=** Methanol Pomegranate Peels Extracts, **APPE=** Acetone Pomegranate Peels Extracts and **HPPE=** Hexane Pomegranate Peels Extracts.

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Table 2. Toxicity of four pomegranate peel extracts against 4th larval instar of *Spodoptera littoralis* at different exposure time intervals.

TIME (HRS.)	LC_{25} (%)	LC_{50} (%)	95% FIDUCIAL LIMIT		LC_{90} (%)	95% FIDUCIAL LIMIT		SLOPE ± S.E.
			Lower	Upper		Lower	Upper	
EPPE *								
24	0.0038	0.0063	0.0058	0.0069	0.0162	0.0135	0.0209	3.1299 ± 0.3009
48	0.0027	0.0048	0.0043	0.0053	0.0142	0.0118	0.0183	2.7130 ± 0.2649
72	0.0019	0.0032	0.0028	0.0036	0.009	0.0078	0.0108	2.8893 ± 0.2690
96	0.0012	0.0022	0.0008	0.0026	0.007	0.0056	0.0171	2.5111 ± 0.2976
MPPE *								
24	0.0047	0.007	0.0065	0.0076	0.0149	0.0128	0.0184	3.8848 ± 0.3673
48	0.0036	0.0057	0.0052	0.0062	0.0136	0.0117	0.0166	3.3752 ± 0.3017
72	0.0028	0.0045	0.0041	0.0049	0.0111	0.0097	0.0133	3.2818 ± 0.2821
96	0.0028	0.0045	0.0041	0.0049	0.0111	0.0097	0.0133	3.2818 ± 0.2821
APPE *								
24	0.0044	0.0071	0.0065	0.0078	0.0174	0.0144	0.0227	3.2897 ± 0.3261
48	0.0032	0.0056	0.005	0.0062	0.0162	0.0133	0.0214	2.7725 ± 0.2743
72	0.0024	0.0043	0.0039	0.0048	0.013	0.0109	0.0166	2.6825 ± 0.2615
96	0.0024	0.0043	0.0039	0.0048	0.013	0.0109	0.0166	2.6825 ± 0.2615
HPPE *								
24	0.0032	0.0054	0.0048	0.006	0.0144	0.0116	0.02	2.9886 ± 0.3379
48	0.0032	0.0054	0.0048	0.006	0.0144	0.0116	0.02	2.9886 ± 0.3379
72	0.0032	0.0054	0.0048	0.006	0.0144	0.0116	0.02	2.9886 ± 0.3379
96	0.0023	0.0042	0.0037	0.0047	0.0134	0.0106	0.0193	2.5340 ± 0.3073

*** EPPE=** Ethanol Pomegranate Peels Extracts, **MPPE=** Methanol Pomegranate Peels Extracts, **APPE=** Acetone Pomegranate Peels Extracts and **HPPE=** Hexane Pomegranate Peels Extracts

Biological effects of pomegranate peel extract on the 2nd and 4th larval instar of *S. littoralis*

Data in Table (3) represented the biological effect of the sub lethal concentration (LC_{50}) of EPPE on larval duration, pupal duration, and adult longevity. Treatment with EPPE resulted in a significant decrease larval durtion of pretreated 2nd instar to (10.88 days) and (12.5 days) for the control. Similarly, there was a significant decrease in larval duration for the pretreated 4th instar to (9.00 days) and (10.5 days) for control. There was also, a significant reduction in pupal duration in both the 2nd and 4th instar. The mean pupal duration for the 2nd instar was (7.47 days) and for control was (8 days). The 4th instar pupal duration was (8.54 days) compared to (9 days) for the control. Both male and female moth's

longevity was reduced significantly in case of pretreated 2nd instar with EPPE, where it recorded (4 and 6 days) compared with the control (8.4 and 9.2 days), respectively. On the contrary pretreated 4th instar showed insignificant prolongation in males and females longevity where it recorded (7.86 & 9.71 days) compared to the control (4.71 & 5.14 days), respectively.

Table 3. Effect of LC_{50} value of EPPE on larval duration, pupal duration and adult longevity when treated at 2nd and 4th instar larvae of cotton leafworm *S. littoralis*.

COMPOUNDS	LARVAL DURATION (MEAN DAYS ±S.D.)		PUPAL DURATION (MEAN DAYS ±S.D.)		ADULT LONGEVITY (MEAN DAYS ± S.D.) %			
	2nd	4th	2nd	4th	2nd ♀	2nd ♂	4th ♂	4th ♀
Pomegranate	10.88 [b] ± 0.791	9.00 [b] ± 0.310	7.47 [b] ± 0.511	8.54 [b] ± 0.262	4.00 [b] ± 0	6.00 [b] ± 2.83	7.86 [a] ± 2.85	9.71 [a] ± 2.87
Control	12.5 [a] ± 0	10.5 [a] ± 0	8.00 [a] ± 0	9.00 [a] ± 0	8.40 [a] ±2.19	9.2 [a] ±1.79	4.71 [b] ± 1.60	5.14 [b] ± 1.46
F value	33.8	154.5	7.59	21.40	20.20	7.10	6.45	14.01
L.S.D $_{0.05}$	0.599	0.255	0.420	0.215	2.26	2.77	2.70	2.65

* Means in same column followed by the same letter are not significantly different.

Treatment with EPPE resulted in the appearance of pupal deformities and adult malformations were observed**.** Table 4 showed the total deformation at the pupal stage. EPPE resulted in the highest total deformation (50.5 %) in pretreated 2nd instar whereas caused the lower deformation for 4th instar (18.5 %), compared with control (15.79 and 7.9 %) respectively. Moreover, EPPE caused varying degrees of deformities in adult *S. littoralis* when treated at the 2nd and 4th instar. Adult females displayed a higher effect than males when treated at the 2nd instar, with pretreatment of 81.8%, and. 53.5% respectively Conversely, males displayed a higher effect than females in the control group, with a value of 28.6% and 11.1% respectively. At the 4th instar, the deformity value in males was higher than in females, 22.2% and 15.4%, respectively. On the contrary, the control treatment males showed no deformation unlike females where deformation percentages were 6.7%. The total malformation recorded its maximum value (60.98%) in the 2nd instar. Less

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deformities were observed in the 4th instar (18.2%) where the control for 2nd and 4 recorded (18.75% and 2.86%) respectively.

Table (4): Effect of LC_{50} values of Pomegranate peel extract on deformations of pupae and adult malformations treated at the 2nd and 4th instar larvae of cotton leafworm *S. littoralis*.

COMPOUNDS	PUPAE DEFORMATION %				TOTAL DEFORMATION %		ADULT MALFORMATION %				TOTAL MALFORMATION %	
	2nd		4th		2nd	4th	2nd		4th		2nd	4th
	♂	♀	♂	♀			♂	♀	♂	♀		
Pomegranate	43.08	4.71	10	23.53	50.5	18.5	53.3	81.8	22.2	15.4	60.98	18.2
Control	22.22	10.00	0	16.67	15.79	7.9	28.6	11.1	0	6.7	18.75	2.86

Morphological effects of pomegranate peel extract on the 2nd and 4th larval instar of *S. littoralis*

Figures 2, 3 and 4 illustrate the deformities in larval and pupal stages as well as malformed adults resulting from pretreatment with EPPE. The following abnormal changes for treated larvae compared to the control were observed in Figs 2: (1) Various symptoms of incomplete molting with old cuticle and abdominal constriction (Figs 2 B-E), (2) intermediate larval-pupal malformations (Figs 2 F-K). As shown in Fig 3, the malformed pupae resulting from the application of EPPE were compared to control. (1) deformed body (Figs 3 B-D), (2) pupa failing to shed exuvia (Figs 3 E), (3) pupal adult with intermediate malformation, adult failing to emerge (Figs 3 F-I).

Figure 4, showed that EPPE treatment caused shrinkage to the moth appendages especially the wings, as compared to non-treated moths. (1) wrinkled unexpanded wings. (Fig 4 B-E)

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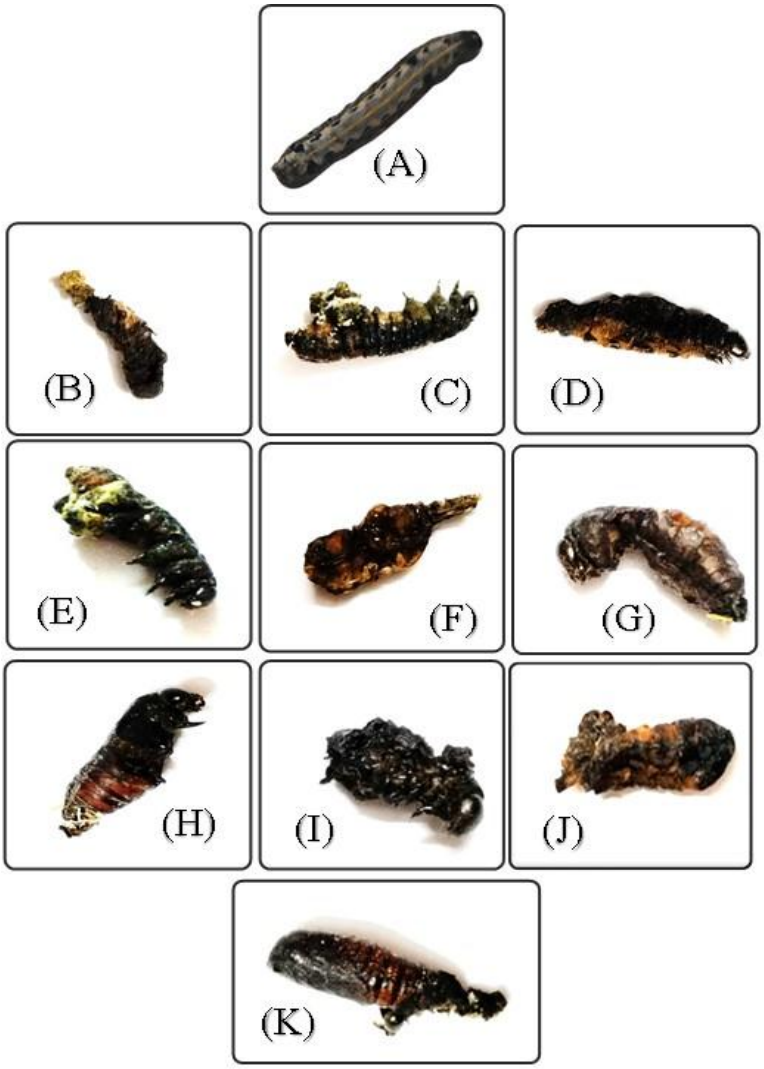

Figure 2. Morphological deformations of *S. littoralis* larvae resulting from treatment of 2nd and 4th instar larvae with Ethanol Pomegranate Peel Extract (EPPE). **(A)** Normal larva. **(B, C, D, E)** Various symptoms of incomplete molting with old cuticle and abdominal constriction. **(F, G, H, I, J, K)** intermediate larval-pupal malformations.

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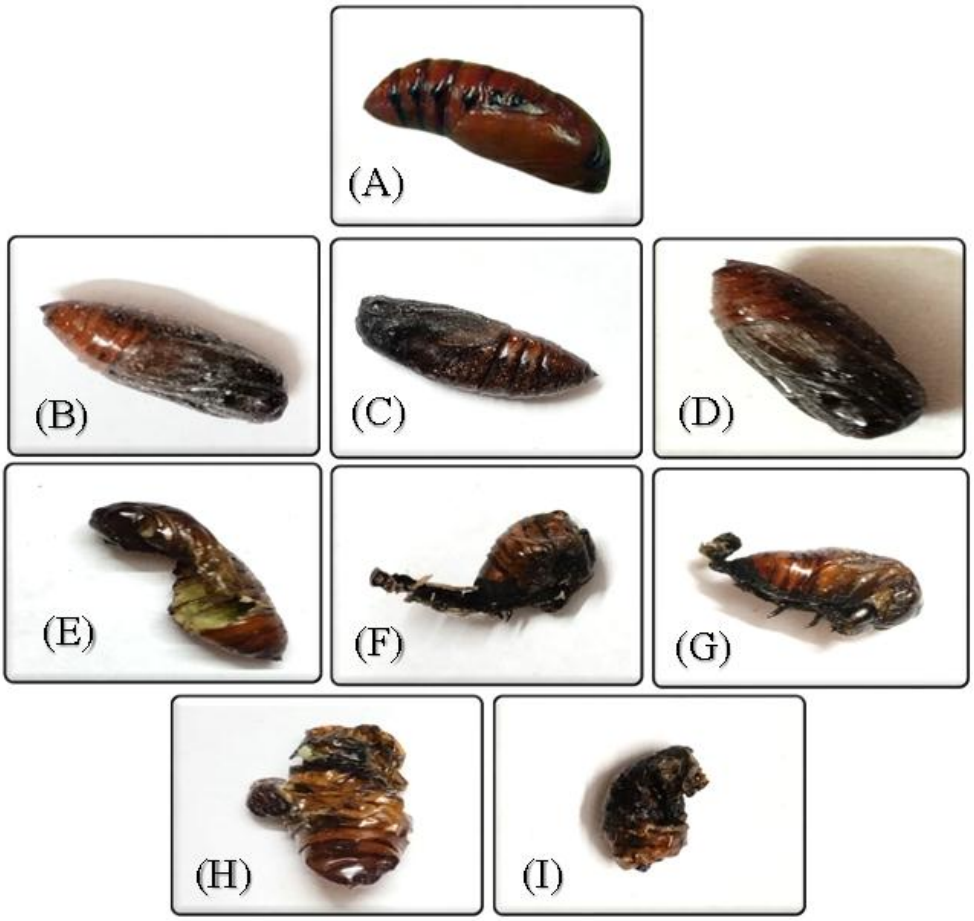

Figure 3. Pupal deformities of *S. littoralis* as a result of treatment with LC_{50} of Ethanol Pomegranate Peel Extract (EPPE). **(A)** Normal pupa, **(B, C and D)** deformed body, **(E)** pupa failing to shed exuvia, **(F, G, H. I)** pupal adult with intermediate malformation, adult failing to emerge.

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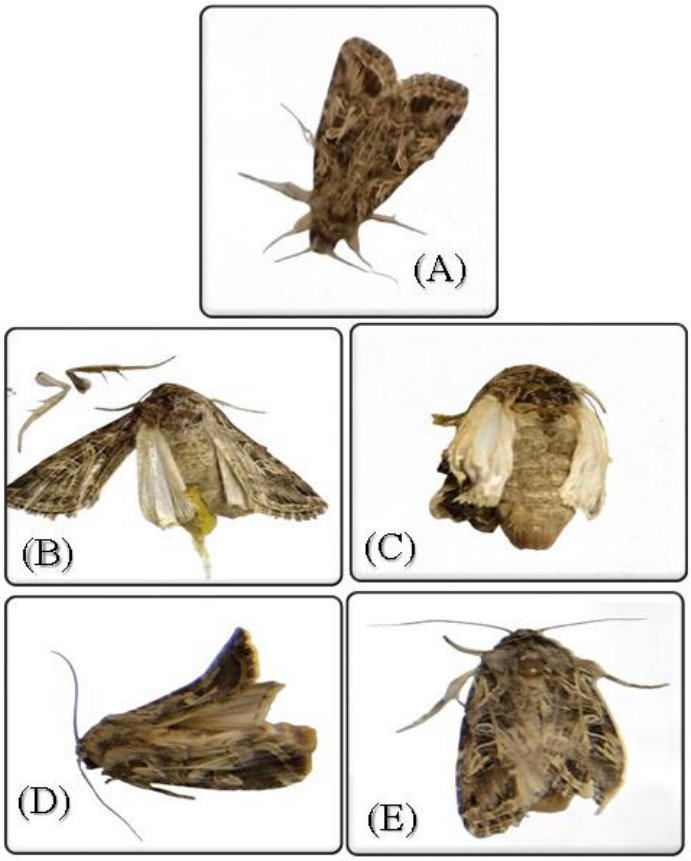

Figure 4. Abnormalities in *S. littoralis* moths after treatment with Ethanol Pomegranate Peel Extract (EPPE) at 2nd and 4th instar larvae **(A)** Normal *S. littoralis* moth, **(B, C, D, E)** wrinkled unexpanded wings.

Table 5 represented the effect of pretreatment with (EPPE) on pupation percentage, pupal weight, adult emergence, and sex ratio. Pretreatment with EPPE resulted in a highly significant reduction in pupal weight for both 2nd and 4th instar treated with LC_{50} values (0.157 and 0.210 g), respectively. compared to the control (0.255 and 0.261 g, respectively). The pupation percentage was reduced to 30.94% for the 2nd instar and 9.64% for the 4th instar when compared with the control (95 %).

Adult emergence percentages were reduced to 81% on pretreated 4th instar compared to the control (92.1%). Highest reduction in the adult emergence percentages (41.4%) in pretreated 2nd instar than the control group (84.2%) was observed. EPPE pretreatment affected the sex ratio by producing males more than females in the 2nd instar (2.73:1)

compared to the control (0.78:1). However, in the 4th instar larvae, the sex ratio of males less than females (0.69: 1) compared with the control (1.33: 1).

Table 5. Effect of LC_{50} values of Pomegranate peel Extract on pupation percentage, pupal weight, adult emergence and sex ratio of 2nd and 4th instar of *S. littoralis*.

COMPOUNDS	PUPATION %		PUPAL WEIGHT (GM) ±S.D.		ADULT EMERGENCE%		SEX RATIO 2ND	SEX RATIO 4TH
	2nd	4th	2nd	4th	2nd	4th	♂ ♀	♂ ♀
Pomegranate	30.94	9.64	0.157 b ±0.019	0.210 b ± 0.025	41.4	81.5	2.73:1	0.69:1
Control	95	95	0.255 a ±0.006	0.261 a ±0.008	84.2	92.1	0.78:1	1.33:1
F value	---	---	196.9	24.2	---	---	---	---
L.S.D$_{0.05}$	---	---	0.015	0.022	---	---	---	---

* Means in same column followed by the same letter are not significantly different.

Influence of EPPE on total proteins, lipids, and carbohydrates in *S. littoralis*

Figure 5 illustrated the influence of EPPE treatment on biochemical parameters of 2nd and 4th instar larvae of *S. littoralis*. Treatment with EPPE resulted in a highly significant decrease in protein level for 2nd instar as compared to the control (200.6 and 241.3 mg/g body wt. respectively). This level was significantly decreased for 4th instar larvae compared to the control (332 and 379.2 mg/g body wt. respectively). The total carbohydrate level showed a highly significant decrease for both 2nd and 4th instar larvae compared to control.

EPPE treatment resulted in a weak significant increase in total lipid compared with the control (327.14 and 323.06 mg /g body weight, respectively) for the 2nd instar. In contrast, the treatment showed a non-significant in total lipid compared with control (395.86 and 394.64 mg/g body wt.) for the 4th instar.

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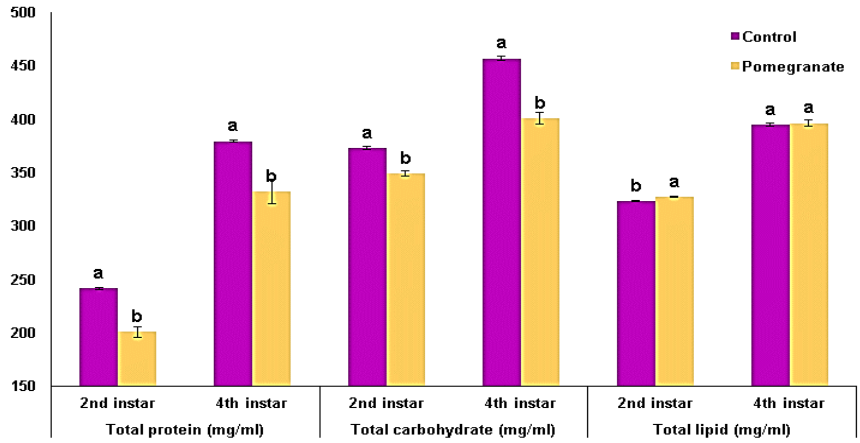

Figure 5. The effect of EPPE treatment on biochemical parameters of 2nd and 4th instar larvae of *S. littoralis*. (Values with the same letter are not significantly different)

DISCUSSION

The present study is concerned with investigating the insecticidal activity of pomegranate peel extracts using four different solvents with different polarities (ethanol, methanol, acetone, and hexane) against 2nd and 4th instar larvae of *S. littoralis*. Extracts were prepared by low heat method using Soxhlet apparatus and toxicity test was applied using dipping technique and mortality percentage was recorded for different time intervals (24, 48, 72, and 96 hrs.). The obtained results revealed that pomegranate peel extract possessed a toxic effect against 2nd and 4th instar larvae and the toxicity was increased by the increase in the exposure time. These findings are in agreement with **Farag & Emam (2016)** who proved the efficacy of the juice of pomegranate peels against *S. littoralis* larvae. **Elfalleh et al., (2012)** have referred to this toxic effect as the high content of polyphenols and flavonoids in pomegranate peels. Moreover, 2nd instar was more susceptible to the tested extracts than 4th instar. **Ismail (2020)** stated that the toxicity of tested pesticides may be decreased with the development of the larval instar. The data of the present study indicated that ethanol pomegranate peel extract (EPPE) possessed the highest toxic effect against 2nd and 4th instar larvae. Such results could be related to the high polarity of ethanol. **Ladhari et**

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al., (2013) stated that the extract of the high polarity solvents contained more bioactive molecules than those of medium polarity solvents.

As EPPE revealed the highest toxic effect, it was subjected to biological and biochemical studies. The pretreatment with EPPE resulted in remarkable biological changes in larval and pupal duration as well as a disturbance in adult emergence. Moreover, EPPE resulted in obvious deformations in larval and pupal stages, and adult malformation was also recorded along with a disturbance in sex ratio when compared with control group. **Bowers & Nishid, (1980),** have referred to these developmental disturbances as the imbalance of juvenile hormone (JH) which is responsible for development and reproduction in insects and the exposure to botanical extracts inactivate JH through the alteration of the microsomal cytochrome P-450 oxidase system.

In the present study, pomegranate treatment showed noticeable and tangible results in their effects on *S. littoralis* larvae, which was reflected in the results for the main metabolite components. Physiological studies of total protein determination are very important because it is considered a major component necessary to an organism for its development and reproduction and to perform its vital activities. The results showed that total protein was decreased in all treatments in the 2^{nd} and 4^{th} instar larvae of *S. littoralis* treated with the tested compound. These results were in agreement with those obtained by **Emam (2022)** who found that the total protein was significantly decreased with pomegranate leaf and peel crude extract when compared with untreated Black cutworm larvae. The significant reduction in total soluble protein content can be attributed to the inhibition of DNA synthesis and reduced enzymatic activity **(Hamouda, 2002)**. The insecticide-induced stress probably reduced the volume of the hemolymph and decreased the total protein content **(Sugumaran, 2010).**

This indicated that the growth inhibition resulting from the treatment caused a severe reduction in digestion. This phenomenon could be due to the breakdown of proteins into their respective amino acids, which could help provide energy for the insect to survive. **Etebari et al., (2007)** and **Sak et al., (2006)** reported that insecticides decreased the protein

amount of an insect body. Reduction in protein levels may be due to their effect on protein metabolism and the utilization of lipid reserves to generate energy as a result of stress conditions**.**

The total soluble carbohydrate content was significantly decreased in pomegranate peel-treated larvae, while there was a very significant increase in the percentage of pomegranate peel-treated larvae compared with the control. This result agreed with **Sobhi et al., (2020)**, who reported that eucalyptus oil caused a significant reduction in the total carbohydrate content in the 4 instar larvae of *S. littoralis* after treatment. The carbohydrate content supplies the body with glucose, which provides an energy source for the synthesis of pupal and adult tissues, especially the cuticle. Carbohydrates are necessary for the normal functioning of male and female reproductive systems and for embryo development. In males, sugars form an important constituent of the testes, and most carbohydrates in the reproductive system are present in the testes. In the female system, carbohydrates are necessary for vitellogenesis and the formation of glycosaminoglycans present in the vitelline membrane and chorion. Vitellogenesis involves the accumulation of carbohydrates, lipid and protein yolks within the oocyte to meet the structural and metabolic needs of the developing embryo **(Chippendale, 1978).**

The decrease in total carbohydrates may be due to the requirement of glucose as an energy source for development and growth. The decrease in total soluble carbohydrate content of untreated pupae could be attributed to the use of glucose to support all life processes of glucose, which plays an important role in energy supply and adult maturation (sperm and egg development) and builds up new chitin **(Tolba, 2006; El-Sheikh et al. 2005; El-Sheikh2006)**. The significant increase in total carbohydrates induced by chlorpyrifos may be related to the cellular damage caused by chlorpyrifos, resulting in glycogen conversion to monosaccharides, glucose, and hydrolysis **(Dahi et al., 2017)**. Furthermore, it may be due to the mode of action of CSF in enhancing acetylcholine esterases, and the latter requires much more glucose, so the rate of anabolism of glycogen increases significantly. In addition, the disparity between the rate of carbohydrate synthesis

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and biodegradation might cause a change in the level of carbohydrates in insect tissues as a response to this treatment under stress **(Bobrovskikh and Gruntenko 2023)**.

The present findings showed a significant increase in the total soluble lipid content in the pomegranate peel treatments. These results are in agreement with those obtained by **Mageed et al., (2018),** who recorded significantly increase in total lipids values for flufenoxuron, chlorfluazuron, triflumuron, in comparing to control. The significant increase in total lipids may be due to the intensive breakdown of the lipoprotein consisting of the exoskeleton of the insect body and degenerated cuticle, which helped to release much more lipids in the haemolymph. In addition, the disparity between the rates of lipid synthesis and biodegradation might cause a change in the levels of lipids in the tissues of the insect as a response to this treatment **(Abuldahab et al., 2011).**

CONCLUSION

In conclusion, the present results indicate that pomegranate peel extract possessed a toxic effect on *S. littoralis* and inhibited growth through different metabolic processes. The tested extract was highly incorporated during the 2nd instar more than the 4th instar larvae. The biological and biochemical studies have proved the efficacy of pomegranate peel extract as Insect growth regulator especially against the early larval stage. The obtained results strongly recommended the use of pomegranate peel extract to develop an economic bio-pesticide for use in integrated pest management programs of *S. littoralis* for the sustainability of crop production.

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التأثيرات السامة والتغيرات البيوكيميائية والبيولوجية لمستخلص قشر الرمان (*Punica granatum*) على الأعمار اليرقية المختلفة لدودة ورق القطن *Spodoptera littoralis* (Boisd.)

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المستخلص

يمثل استكشاف المبيدات الخضراء أحد أهم الطرق الحديثة للحفاظ على الزراعة المستدامة وتجنب المخاطر الصحية والبيئية الناجمة من الاستخدام المفرط للمبيدات الكيميائية في مكافحة دودة ورق القطن *Spodoptera littoralis*. لذلك فقد اهتمت الدراسة الحالية بتقييم التأثيرات السمية لأربعة مستخلصات مختلفة من قشر نبات الرمان ضد كلاً من الطور اليرقي الثاني والرابع لدودة ورق القطن خلال فترات تعرض مختلفة (24، 48، 72 و96 ساعة). وقد نتج عن مستخلص قشر الرمان الإيثانولي (EPPE) أعلى تأثير سمي بتركيز نصفي قاتل مساو (0.0014 و0.0022%) بعد 96 ساعة ضد يرقات العمر الثاني والرابع على التوالي. في حين كانت قيم مستخلصات قشور الرمان من الهكسان(HPPE) والميثانول (MPPE) والأسيتون(APPE) ، (0.0019 و0.0042 %)، (0.002 و0.0043 %)، (0.0022 و0.0045 %) على التوالي. أدت المعالجة المسبقة بالتركيز النصفي القاتل للمستخلص الأكثر فعالية (EPPE) بعد 48 ساعة إلى حدوث تغيرات بيولوجية تمثلت في انخفاض معنوي شديد في نسبة التشرنق (30.94 و41.4%) ليرقات العمر الثاني والرابع على التوالي بالمقارنة بالمجموعة الضابطة (95 و84.2 %). كما أثرت المعالجة المسبقة بالمستخلص بشكل كبير على نسبة الجنس (كان عدد الذكور الذين ظهروا أكبر من عدد الإناث). أحدثت المعالجة بـ (EPPE) انخفاض معنوي شديد في إجمالي محتوي البروتين والكربوهيدرات القابلة للذوبان في كلا العمرين الثاني والرابع. كما زاد المحتوى الكلي للدهون زيادة ضعيفة في الطور الثاني، ولكن لم يتم تسجيل فرق كبير في المعالجة في الطور الرابع عند مقارنتها بالمجموعة الضابطة. توصي الدراسة الحالية باستخدام مستخلص قشر الرمان في برامج المكافحة المتكاملة لحشرة دودة ورق القطن وخاصة ضد الطور اليرقي المبكر.

الكلمات المفتاحية: دودة ورق القطن؛ مستخلص قشر الرمان؛ نشاط المبيدات الحشرية؛ التغيرات البيولوجية؛ دراسات كيميائية حيوية

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