Insecticidal activity of *Punica granatum* L. extract for the control of *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) and some of its histological and immunological aspects

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ABSTRACT

The spread of the red palm weevil, *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae), is a serious challenge at present due to the serious damage it causes to the date palm tree. This study investigated the ethanolic extract of *Punica granatum* peel and found that it leads to significant mortalities in both the larvae and adult stages of *R. ferrugineus* after exposure to four concentrations (4.7, 9.3, 18.7, and 37.4 mg/mL). The larvicidal effect reached 48% at day 6 for the 37.4 mg/mL concentration, and adults exhibited full mortality at the same concentration. In addition, the cuticle of treated larvae was investigated histopathologically, and corrugation and a thinning surface were found. Scanning electron microscopy revealed a strong uptake and aggregation of the extract on the glandular pores of the back wings of adults. The impact of *P. granatum* peel extract on the total protein content of the hemolymph of *R. ferrugineus* larvae was also studied. Compared to controls, larvae had 12 different bands before treatment, 4, 3 and 2 bands in common after treatment at concentrations 4.7, 9.3, and 37.4 mg/mL, respectively. These results suggest that *P. granatum* peel extract is a promising alternative to chemical pesticides and can be used as a biopesticide against larvae and adult red palm weevil.

INTRODUCTION

The date palm, *Phoenix dactylifera* (Linn.), is the most important fruit crop in the Middle East and the Arabian Gulf region (Abdullah, 2009). Date palm trees are vulnerable to many insect pests that can cause serious damage to various parts of the tree. The most serious of these insect pests, the red palm weevil *R. ferrugineus* (Oliver), was first recorded in date palm plantations in the Sharkia and Ismailia Governorates of Egypt by Saleh (1992). Currently, management of this insect pest usually involves synthetic insecticides (Mondal and Khalequzzaman, 2006). Accumulated pesticide residues in soil and comestibles are a major public health concern for consumers and representatives of the food industry. However, global trends have shifted toward reductions to the use of synthetic pesticides in agriculture in general and after harvest in particular. The pomegranate, *Punica granatum* Linn. (Lythraceae), is cultivated in areas with a Mediterranean, tropical, and subtropical climate (Mars *et al*., 2000) and in Central Asia (Holland *et al*., 2009). The seeds, fruit, flowers, leaves, and bark of this plant are used to prevent and treat many infectious diseases (Seeram *et al*., 2006). Aqueous extracts of *P. granatum* fruit rind and bark are toxic against *Schistosoma mansoni* tape worms and exhibit molluscicidal activity (Hukkeri, 1993, Tripathi *et al*., 2004; Osman *et al*., 2013). Previous studies have reported the

insecticidal effects of *P. granatum* extract and its inhibiting effects on growth and development (Sharma and Rajguru, 2009; Ghoneim, 2014). The use of insecticides extracted from plants has many advantages for pest control because they are less persistent and can be used without modification of the natural balance of the ecosystem, respecting sustainability principles (Smith, 1989). In the present study, extracts of *P. granatum* were tested, as a natural insecticide, against last instar larvae and adult red palm weevil *R. ferrugineus* (Oliver). Also, its effect on histological and immunological aspects was studied.

MATERIALS AND METHODS
Insect collection and maintenance
Adults and larvae of *R. ferrugineus* were obtained during the summer season from naturally infected date palm trees in the Giza Governorate, Egypt.

Punica granatum peel extract preparation
Punica granatum were purchased from a local market in Egypt. Alcohol extracts of *P. granatum* peel were prepared at the laboratory of Medicinal and Aromatic Plant Research Department, National Research Center, following a previously published method (Tariq *et al.*, 2009). The plant materials were pounded and then extracted using 70% ethanol under reflux. Plant material was macerated at room temperature in a dark place, and the percolate was collected by filtering through cotton wool. The process of maceration percolation was repeated three times. The combined filtrate was completely evaporated in a vacuum rotatory evaporator under pressure at 50°C to obtain a semisolid crude ethanolic extract. The extract was scraped off, transferred to a container, and kept airtight. After evaporation, 106mg crude was obtained, and then it was dissolved in 100ml distilled water for use in bioassays.

Immersion test
Dipping (immersion) tests were carried out according to the procedure published by Khater *et al.* (2013) to determine the efficacy of *P. granatum* L extract against adult and last instar larvae of *R. ferrugineus*. Four concentrations (4.7, 9.3, 18.7, and 37.4

mg/mL) were prepared. The procedures were repeated five times for each concentration, and 5 insects were used per replicate in each test, such that 25 insects were used for each concentration. Each group of insects was immersed for 1 min in each concentration, and the solution was continuously stirred. The negative control was treated with distilled water. The immersed insects were placed in a plastic pot with filter papers (Whatman Grade 1) and were kept at 27±2°C and 80±5% RH. Insect mortality was observed daily for 1 week. Live and dead insects were counted. Both larvae and adults were considered alive if they exhibited normal behavior when physically stimulated with wooden dowels; insects that did not exhibit movement or any other signs of life were considered dead.

Light microscopic observations of larvae
Five last instar larvae were dipped into 37.4 mg/mL extract of *P. granatum* for 1 min. After 24 hrs, the exposed larvae were fixed in 10% formalin buffer solution for 24 hrs. The specimens were dehydrated, cleared, and embedded into paraffin blocks. Paraffin sections 5 μm thick were prepared, stained with hematoxylin and eosin, and examined microscopically.

Scanning electron microscope (SEM) examination of adults
Five *R. ferrugineus* adults were dipped in 37.4 mg/mL extracts for 1 min. immediately after each insect was dead, it was placed into 2.5% glutaraldehyde for 24 hrs. The samples were dehydrated in a graded acetone series of 70, 80, 90, and 100% for 1 h and were dried with a dryer (Blazer Union, F1–9496 Blazer/Fürstentun Liechtenstein) using liquid carbon dioxide. Specimens mounted on SEM stubs were coated with gold using a S15OA Sputter Coater. Coated larvae and nymphs were examined via SEM.

Sodium dodecyl sulphate polyacrylamide gel-electrophoresis (SDS-PAGE)
Hemolymph, 50–100 µg, of the control and exposed larvae with *P. granatum* L. extract at three concentrations were electrophoresed by using 10% gel under reducing conditions (Laemmli, 1970). Finally, the gel was

photographed after stained with Coomassie Brilliant Blue R250 stain.

Statistical Analysis

The mortality results of the third instar larvae and adult of RPW were statistically analyzed by ANOVA followed by Duncan test using the SPSS computing program. LC50 values were also calculated log-concentrations probit model using Ldp line R software.

RESULTS

P. granatum extract against _R. ferrugineus_

A comparison with the control indicated that the extract had a significant effect on the larval stage. The daily percentages for mean mortality are given in Table 1. The mean mortality of larvae increased with increased concentrations. The larvicidal effects reached 48% at day 6 at the 37.4 mg/mL concentration, and the other concentrations caused mortalities ranging from 4 to 44% (Table 1). The LC_{50} was calculated for each day, and the results are presented in Table 1. Dipping larvae in a 37.4 mg/mL concentration of extract induced thinning and a corrugated cuticular surface together with the separation of the inner cellular layer of the epidermal cells in some regions of the procuticle (Fig. 1 a,b), while the midgut region exhibited a normal appearance (Fig. 1c,d).

Table 1. Daily mortality of last instar larvae of _Rhynchophorus ferrugineus_ treated with different concentrations of _Punica granatum_ L. extract (means ± SEs).

	Mean mortality percentages ± SEs				
	Concentrations (mg/mL)				
Interval /(days)	**4.7**	**9.3**	**18.7**	**37.4**	**LC_{50}**
1	ᵃ4±4	ᵃ8±4.8	16±7.4	16±7.4	-
2	ᵃ4±4	ᵃ8±4.8	24±11.6	28±8	-
3	ᵃᵇ12±4.8	ᵃ12±4.8	28±12	36±4	132.44
4	ᵃᵇ16±7.4	ᵃ20±8.9	32±13.5	36±4	192.51
5	ᵇᶜ28±8	ᵃᵇ28±8	32±13.5	44±7.4	181.8
6	ᶜ44±11.6	ᵇ44±11.6	44±11.6	48±17.8	377.6
F	4.6	3.3	0.57	2.9	-
P value	0.004**	0.019*	Ns	0.03*	-

For each concentration, 25 larvae were treated. SE: standard error of the mean (n = 5). Values marked with letters (a, b, c) are significantly different (P < 0.05). *significant values, **highly significant, Ns: non-significant. LC_{50}: Lethal concentrations that killed 50% of larvae.

Fig. 1. Transverse H and E-stained sections of **(a)** the cuticle, muscles of normal last instar larvae of _Rhynchophorus ferrugineus_, **(b)** cuticle 24 h after being dipped in 37.4 mg/mL _Punica granatum_ L. extract **(c)** midgut of normal control of the last instar larvae of _Rhynchophorus ferrugineus_ **(d)** midgut 24 hrs after being dipped in 37.4 mg/mL _Punica granatum_ L. extract. (10×, 40×). Cuticle (cu), muscle layer (ml), basement membrane (bm), goblet cells (gc), villi (v), epithelium cells (ep), interspace (is), and lumen (lu) (10×).
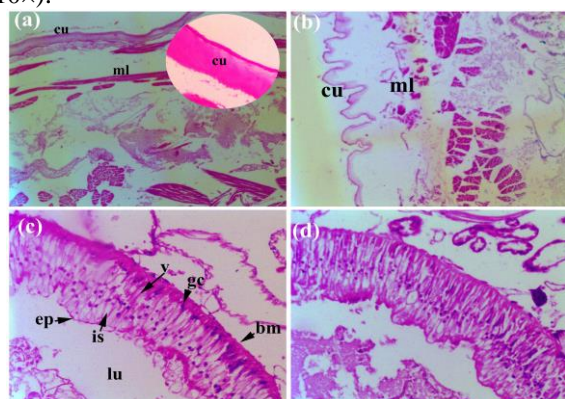
Adult _R. ferrugineus_

Comparisons with the control revealed significant mortality in adult red palm weevils after exposure to the extract. The daily mean mortality percentages are presented in Table 2. The strongest effects of the extract were observed at day 6 at the 37.4 mg/mL concentration, which had a mortality of 100%. However, other concentrations caused mortality as well, ranging from 4% to 88%. The LC_{50} was calculated each day, and was 28.8, 6.875, 7.584, and 7.67 mg/mL on days 3, 4, 5, and 6, respectively (Fig. 1).

Scanning electron microscopic observations

The normal red palm weevil, of the order Coleoptera, has two pairs of wings. The front wings are strong (Fig. 2A) and brown in color, and they feature groups of lines. These wings are not used in flying. Instead, they protect the hind wings. SEM observation of the hind

wings revealed many glandular pores (Fig. 2C). The body wall is curled and resembles superimposed sheets (Fig. 2B). The cuticle of the abdominal thorax segments is densely coated with sensillae, which are circular and surrounded by many small pegs of trichoid sensillae at the circular edges (Fig. 2D). The main function of the sensillae is as a chemoreceptor.

Table 2. Daily percentage mortality of adult *Rhynchophorus ferrugineus* treated with different concentrations of *Punica granatum* L. extracts (means ± SE).

	Mean mortality percentages ± SE				
	Concentrations (mg/mL)				
Interval time/Days	4.7	9.3	18.7	37.4	LC_{50}
1	[a]4 ±4	4±4	[a]8±4.8	[a]8±4.8	-
2	[a]12±4.8	36±19.3	[b]48±13.5	[b]52±10.1	-
3	[ab]28±12	36±19.3	[b]56±16	[b]60 ±8.9	28.8
4	[b]52±13.5	60±20.9	[b]76±16	[c]80±6.3	6.875
5	[b]52±13.5	64±22.2	[b]80±15.4	[c]92±4.8	7.584
6	[b]56±13.2	64±22.2	[b]88±12	[c]100±0	7.67
F	4.2	1.5	4.6	24.8	-
P value	0.007*	Ns	0.004*	<0.001**	-

For each concentration, 25 larvae were treated for each concentration. SE: standard error of the mean (n = 5). Values marked with letters (a, b, c) are significantly different (P < 0.05). *significant values, **highly significant, Ns: non-significant. LC_{50}: lethal concentrations that killed 50% of adults.

When adult *R. ferrugineus* were dipped in 37.4 mg/mL of *P. granatum* extract for 1 min, strong uptake by the cuticle was observed (Fig. 3F). The extract was aggregated on the glandular pores of the hind wing, and the front wings appeared normal (Fig. 2E, G). The sensory sensillae appeared to shrink, and the sensory hair showed irregularly and comprised in its socket (Fig 2H).

Protein profile of hemolymph of *R. ferrugineus*

A total of 12 protein bands were observed in the normal animals at molecular weights of 26 -263 KDa (Fig. 3). However, after exposure to 4.6, 9.3, and 37.3 mg/mL concentrations, the hemolymph SDS dissociated proteins were separated into 8, 6, and 5 bands at mol wt in the ranges 27–254, 29-255, and 30-265 KDa, respectively. Four, three, and two protein bands were shared with the normal control at the extract concentrations of 4.6, 9.3, and 37.3 mg/mL. New protein bands were stimulated at all concentrations at mol wt in the ranges 27–254, 29–255, and 30-265 KDa, respectively. A common band appeared between concentrations at a mol wt of 83 KDa. In addition, seven protein bands disappeared at all concentrations in the mol wt range 26-243 KDa.

Fig. 2. A–D). Scanning electron micrograph of the normal adult red palm weevil *Rhynchophorus ferrugineus*, showing **A)** the surface of the front wing, **B)** the cuticle thorax, **C)** glandular pores on the surface of the hind wing (arrow), and D) the sensillae coeloconica on the abdomen. **E–H)** Scanning electron micrograph of adult 24 h after being dipped in 37.4 mg/mL *Punica granatum* L extract showing **E)** the normal appearance of the front wing surface, **F)** the abnormal surface of the cuticle thorax, **G)** an accumulation of the extract around the glandular pores (arrow), and **H)** the abnormal sensillae coeloconica (inset).
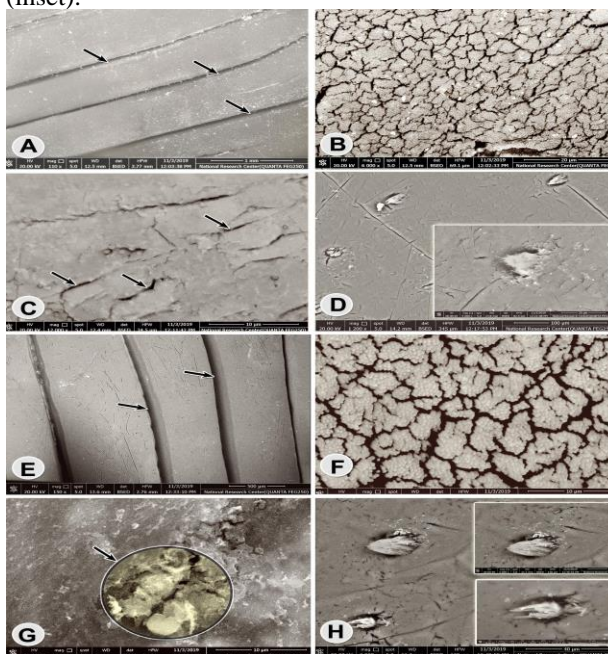
Fig. 3. Total protein of the hemolymph of *Rhynchophorus ferrugineus* last instar larvae treated with different concentrations of *Punica granatum* L. extracts, according to 10% SDS-PAGE electrophoresis. Lane M represents molecular weight. Lane C represents the control, or untreated hemolymph. Lanes 1, 2, and 3 represent treatment with 4.7, 3.9, and 37.4 mg/mL extract, respectively.
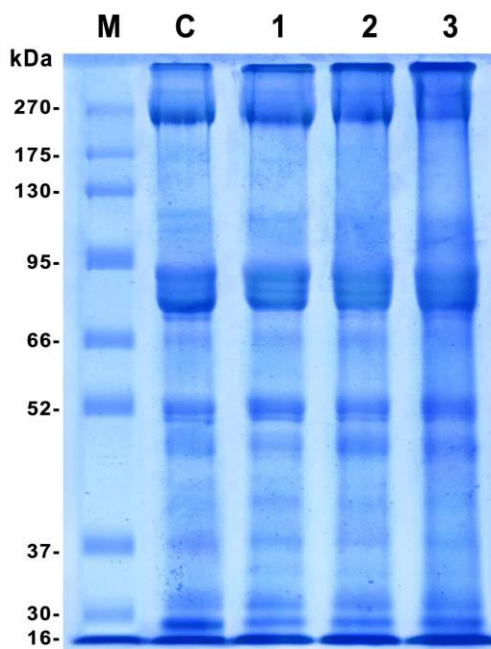
DISCUSSION

Full mortality was found in adults of *R. ferrugineus* at a 37.4 mg/mL concentration of *P. granatum* at day 6. However, larval mortality reached only 48% at the same time and concentration. Hamouda *et al*. (2014) found that an ethanol extract of *P. granatum* was the most toxic to the red flour beetle *Tribolium castaneum*, expressed by 56% larval mortality after ingestion. In addition, Mohammed (2013) reported that *P. granatum* extract showed good repellent activity against adults and larvae of the confused flour beetle, *T. confusum*, at a concentration of 2.5%, ranging from 20–100 % at 2 to 5 h after release for adults. It was found that as the concentration increased, the repellent effects also increased. In addition, a significant difference was shown between treatments at concentrations of only 2.5% and 5% for adults. However, there were significant differences found at all concentrations between the treatments for the larval stage. The authors demonstrated that the repellent response for *T. confusum* is higher for adults than for larvae,

which may be attributed to the fact that the chemoreceptors are better developed in adults than in larvae. Similarly, Koide *et al*. (1998) reported that toxicity caused by *P. granatum* is due to the astringent properties of the tannins contained in the peel of the fruit, which halt infestation. In addition, Kumar *et al*. (2018) demonstrated that secondary compounds from the plants include alkaloids, terpenoids, phenolics, flavonoids, and other minor chemicals that can affect insects in several ways. Yadav *et al*. (2019) tested ethanol extracts from the leaves of *Eucalyptus gluaca*, *Melia azadrach*, *Mentha arvensis*, and *Olea europaea* and the pericarp of *P. granatum* against *T. castaneum*. They found strong toxicity effects in adults at a 5% concentration of *M. arvensis* followed by *E. gluaca*, *O. europaea*, *M. azedrach*, and *P. granatum*. For the larval stage, an extract of *O. europaea* and *E. gluaca* at concentrations of 7.5% and 1%, respectively, caused high mortality, with lower mortality caused by *M. arvensis*, *M. azedrach*, and *P. granatum*. These efficient natural products could prove to be a rich source of a variety of organic chemicals for the development of successful pest control agents (Kamruzzaman *et al*., 2005). A similar result was found by Auamcharoen (2012), who showed that adult *T. castaneum* were significantly more susceptible to the fumigant *Evodia rutaecarpa* than were the larvae. In our work, the *P. granatum* extract at a concentration of 37.4 mg induced histological damage to the cuticle of the last instar larvae, while the midgut exhibited a normal appearance. The same result was found in treatment of the larvae of *R. ferrugineus* with two crude plant extracts of *Carapichea ipecacuanha* and *Eucalyptus camaldulensis* (Hussein *et al*., 2018). In addition, El-Bokl *et al*. (2010) investigated the histology of the ovary and testis of the pre-pupae stage of *R. ferrugineus* after exposure to a range of concentrations of a natural plant extract (neem). They found that the extract disrupted gamete production and led to the degeneration and necrosis of germ cells, among other effects. Shalaby *et al*. (2016) suggested that

light microscope observations could be used to determine the ability of oil extract to penetrate the larval cuticle. In the dipping assay, the transcuticular uptake of the oil extract could be its main route for entry into the *Lucilia sericata* larvae. In the present work, the cuticle of the last instar larvae appeared corrugated and thinner than normal, and the epidermal cell layer was disrupted. At the same time, the gut remained unaffected by the extract treatment.

SEM observations have not previously been performed to determine changes in adult *R. ferrugineus* after being dipped in *P. granatum* extract. Thus, our results were compared with those obtained for other insects. Similar cuticular changes were observed by Aly *et al.* (2010), who found that adult *Schistocerca gregaria* showed morphological deformities after exposure to wild plant extracts of *Fagonia bruguieri*. Prates *et al.* (1998) and Lee *et al.* (2002) concluded that the insecticidal constituents of many plant extracts and essential oils are mainly monoterpenoids, which are typically volatile and can be toxic by penetrating the insect cuticle or its respiratory system. Their results match those of El Nagar *et al.* (2012), who found that treatment of early pupae of the cowpea weevil (*Callosobruchus maculatus* F) with peppermint oil (*Mentha piperita*) caused malformation and disorientation in the direction of antennal sensilla, their fusion, and abnormalities in their shape, particularly in the trichoid sensilla, which are specific to the female sex pheromones. Similarly, Soryia *et al.* (2009) and Baker *et al.* (2010) demonstrated abnormalities in the antennae of adult *C. maculate* and the larvae of the black carpet beetle (*A. fasciatus*) after treatment with lufenuron, *Mentha piperita*, *Ocimum basilicum*, *Citrus limon*, and *Citrus sinensis* volatile oils. They concluded that the deformities caused by these oils could lead to the failure of treated males to mate with females, which would ultimately lower the population of the targeted insect.

The SDS dissociated proteins of hemolymph of the last instar larvae of *R. ferrugineus* showed the appearance and absence of certain bands after exposure to *P. granatum* extract. These results supported the work of Gnanamani and Dhanasekaran (2014), who showed that the total protein concentration in the hemolymph of *Pericallia ricini* larvae was significantly altered as a result of treatment with plant extracts. However, Medhini *et al.* (2012) found that, compared to control, protein and carbohydrate contents were reduced in all of the tissues of the treated *Spodoptera litura* larvae, irrespective of the *Calendula officinalis* extracts tested.

In conclusion, our results indicate that *P. granatum* peel extract is a suitably effective candidate for the biocontrol of the larvae and adults of the red palm weevil and can be used as a bio-pesticide. In a further study, the field application will be carried out .

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