Insecticidal activities of pomegranate peels and leave crude juices

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Abstract

The present study was conducted to envisage the insecticidal activities of pomegranate crude juices on cotton leaf worm, *Spodoptera littorallis*. Pomegranate peels and leave crude juices were obtained by hydraulic press and applied as a natural insecticide against cotton leaf worm during its whole life cycle. The data indicated that there was a direct relationship between the phenolic and flavonoid contents of pomegranate crude juices and the studied parameters relevant to cotton leaf worm life cycle. The studied parameters were larvae and pupae mortalities, adult emergence, hatchability and sterility. Furthermore, histopathological examinations were performed on the cuticle and mid gut of untreated and treated larvae with pomegranate crude juices. Generally speaking, pomegranate crude juices can be applying as a natural insecticide against cotton leaf worm since it is nearly priceless, safe agent, biodegradable, alternative to hazardous synthetic insecticides and harmless to the environment.

Keywords: Pomegranate peels and leave crude juices, Polyphenols, Flavonoids, Cotton leaf worm, Insecticidal activity, Microscopical examination

Running title: Insecticidal activities of pomegranate crude juices

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Introduction

Pomegranate is one of the important and oldest fruits of tropical and subtropical regions, which originated in the Middle East. The world pomegranate production amounts to approximately 1.5 tons (FAO STAT-FAO, 2014) and the peels amounts to approximately 60% of the pomegranate fruit weight (Lansky and Newman, 2007). It is widely reported that pomegranate exhibits antivirus, antioxidant, anticancer, antiproliferative activities (Adhami and Muhtar, 2006 and Farag *et al*. 2015a). For centuries, the barks, leave, flowers and seeds of this plant have been used to ameliorate some diseases (Dandekar *et al*., 2008).

Peels of pomegranate contain a wide variety of phytochemical compounds such as gallotannins, gallic acid, ellagic acid, punicalins and punicalagins (Reddy *at el*, 2007 and Farag *et al*., 2015a). The chemical interactions that occur among living organisms including plants, insects and microorganisms are called allelopathy, which induces direct and indirect harmful or beneficial effects through the production of allelochemicals

Several reports indicated the insecticidal effects of pomegranate juices of various botanical parts. For instance, Ghandi and Pillar (2011) tested leave of *Punica granatum* (pomegranate) for their insecticidal activity against the stored grains pest, *Rhyzopertha dominiea* (Fabr) under laboratory conditions. Pulverized leaf of pomegranate produced high incidence of mortality and reduced the rate of development in medium resulting in significant reduction in population. Nirjara and Sujatha (2011) mentioned that the mortality over the controls ranged from 18-71% with pomegranate leave in wheat medium. The seed production over controls in wheat extended from 27-75% with pomegranate. Mohammed (2012) investigated the methanolic extracts of five plant materials for insecticidal activity against flower beetles *Tribolium confusum* using different concentrations on filter paper. The results revealed that the pomegranate leave extract with some variations had lethal effects against the pest as compared with the untreated check.

Highest mortalities were recorded by Ben Hamouda, *et al.* (2014) on *Tribolium castaneum* larvae treated with peel extracts of pomegranate. The mortalities were 72% and 56% for topical application and ingestion treatment, respectively. The treatment with this botanical insecticide may be promising in protecting stored grains from *ocleopteran* pest infection. Eldiasty *et al*. (2014) evaluated pomegranate and apricot extracts against mosquito *Culex pipiens* larvae. Different degree of potency apricot extract appeared more effective than pomegranate extract. The potency was increased when make binary mixture of both extracts. Jin-Soun (2015) investigated the insecticidal effect from ethanol extracts of root peel, stem peel and fruit peel of pomegranate on house dust mite which applied by direct contact method at different concentrations and exposure time of 24 h. At concentration extract 1 mg/40 µl, a complete mortality of 100% was observed from the root peel, stem peel and fruit peel of pomegranate.

The aforementioned data clearly demonstrate the effects of pomegranate peels and leave extracts obtained by various solvents of different polarities in different area of scientific research. However, very limited studies were conducted on the use of pomegranate peels and leave crude juices. Hence, the present work was performed to study the insecticidal activities of pomegranate peels and leave crude juices of Wonderful variety. In other words, the pomegranate crude juices were applied to study their insecticidal activities on the whole life circle of the 4th instar larvae of cotton leaf worm.

MATERIALS AND METHODS

1. Plant samples

Ripe pomegranate fruits were collected in October, 2014 from pomegranate trees in El- Menia governorate, Egypt. Samples of ripe pomegranate fruits were handpicked from different trees of Wonderful cultivar. The plant was authenticated by Dr. Abdalatif, A. M., Associate Prof. of Horticulture

Department, Faculty of Agriculture, Cairo University. The English, scientific and family names of the plant under study are: Pomegranate, *Punica granatum* L. and *Lythraceae*, respectively.

2. Preparations of pomegranate leave and peels crude juices

Leave and peels of ripe pomegranate fruits were manually separated, cleaned from dust followed by seed removal then mechanically pressed by a Carver hydraulic laboratory press (Carver model C S/N 37000- 156; Fred S. Carver nc, Menomonee Falls, WI, USA). The resultant crude juices were concentrated using freeze- dryer (Labconco Corporation, Kansas City, M.O. USA) and kept in brown bottles at -5ºC until use.

3. Total polyphenolic content (TPP)

The total phenolic compounds in the pomegranate peels and leave crude juices were determined by the Folin- Ciocalteau method (El-falleh *et al*., 2012). TPP contents in the crude juices were calculated and expressed as gallic acid equivalent per g dry weight (mg GAE/g DW) by reference to regression equation of standard curve (Y=0.018x – 0.039, R^2 =0.986).

4. Flavonoid content

The colorimetric aluminum chloride method (El-falleh *et al*., 2012) was used for the determination of the total flavonoid content of the pomegranate crude juices. The concentrations of flavonoids in the pomegranate crude juice samples were calculated from the regression equation of calibration plot (Y=0.010x-0.143, R^2=0.989) and expressed as mg quercetin equivalent /g of dry weight sample.

5. Insecticidal activity

a. Insect type

The insecticidal studies were performed at the standard laboratory culture of Plant Protection Institute, Agriculture Research Centre, Dokki, Giza, Egypt. The Egyptian cotton leaf worms, *Spodoptera littoralis* (Boisd.) were reared on castor-bean plant leave (*Ricinus communis*) under 25±2ºC and 65±5% (R.H) for six successive generations with the same food. The pre-pupae were allowed to pupate in clean jars containing 2 cm high dry saw dust. The resulting pupae were transferred to glass jars containing filter papers and kept in suitable cages (35 × 35 × 35 cm) for mating and emerge moths. Emerged moths were fed on a piece of cotton dipped in 10% sugar solution and then eggs were daily collected.

b. Bioassay (toxicity test)

Thin- layer film technique (contact toxicity), reported by Ascher and Mirian (1981) was used as a method to determine the toxicity of pomegranate peels and leave crude juices. Serial quantities of the pomegranate peels and leave crude juices were used to determine LC_{25} and LC_{50} for each juice. The LC_{25} and LC_{50} values of the pomegranate peels and leave crude juices were calculated by using Ldp line program. In the bioassays for 4th larvae instar of *S. littoralis* were reared in petri–dish (9 cm) containing an aliquot from each juice (little amount of methanol was added for diffusion). Control larvae were treated with methanol only. Treated insects were held at 25±2ºC and 65±5% R.H. Mortalities were determined after treatment with pomegranate crude juices and corrected using Abbott's formula (1925). The corrected percentages of mortalities were statistically computed according to the method of Finney (1971).

(1) Biotic potential and Biological aspects

The LC_{50} values of each pomegranate crude juices were applied on the 4th instar larvae of *S. littoralis* and under constant temperature (25±2°C) and relative humidity (65±5% R.H) to determine the following criteria: mortality (%) of larval, pupal stages and adult emergence. The normal adults of the insect were grouped in pairs and each pair was placed in a small cage, provided with stripes of porous filter papers and hanged at oviposition sites and provided with 10% sugar solution for feeding. The eggs were collected and scored for hatch as well as percent of hatchability. The percentage of sterility was calculated according to the equation of Toppozada *et al*. (1966).

Sterility, % = 100 – [a × b /A × B] × 100

Where:

a = No. of eggs laid / female in treatment.

b = % of hatch in treatment.

A = No. of eggs laid / female in control.

B = % of hatch in control.

(2) Toxicity index

The equation of Sun (1950) was applied to evaluate the efficiencies of pomegranate crude juices as follows:

Toxicity index = LC_{50} of the most effective compound / LC_{50} of the compound used × 100

In this equation the most toxic compound has been given 100 units on the toxicity index scale.

(3) Histopathological examination

At the end of the experimental period whereas the values LC_{50} of the pomegranate peels and leave crude juices were applied by contact toxicity on *S. littoralis* larvae and dissected after 48 h from exposure . Cuticle and mid gut of untreated and treated larvae were removed then stored in a neutral formalin solution (10%) and embedded in a paraffin wax. The organs were sectioned at the thickness of 5 µm then stained with Ehrlisch´s haematoxylin and eosin (H&E) according to Bancroft and Stevens (1996). The samples were examined under ordinary electron microscope (400 magnifications).

6. Statistical analysis

The statistical analysis was performed using SPSS statistical software (Landau and Everitt, 2004). Differences in mortality percentages of 4th instar larvae of cotton leaf worm were compared using one-way analysis of variance (ANOVA). Data were expressed as mean ± standard error. The statistical significance of differences between individual means was determined by student "t" test for paired observations.

RESULTS AND DISCUSSION

Several researchers have been studied the constituents and characteristics of internal sap of plant parts through extraction with different solvents of varied polarities (Miguel *et al.*, 2004 and Tiwari *et al.*, 2011). In the present work, the internal plant sap was obtained by mechanical press without recourse to solvents. One has to point out that the pomegranate botanical parts are safe natural plant organs and obtained from annual pruning of pomegranate trees and are regarded as waste materials. It is well known that some solvents might possess side deleterious effects on human being organs. Therefore, the main target of the present work was to obtain the internal plant sap in its native form to study the insecticidal activities of pomegranate peels and leave crude juices and to mimic the nature conditions for insecticides administration.

1. Total phenolics and flavonoids of pomegranate peels and leave crude juices

Table 1 presents the quantities of total polyphenols and flavonoids of pomegranate leave and peel crude juices. The levels of polyphenols and flavonoids varied according to the pomegranate botanical part. Peel crude juice contained higher amounts of total polyphenols and flavonoids, being about 1.22 and 1.43 times as great as that in leave crude juice, respectively. Similar results were obtained by El-falleh *et al*. (2012) and Farag *et al*. (2015b). One has to point out that phenolic compounds are important components since its used as a general disinfectant at homes and retards the progression of arteriosclerosis and reduces the incidence of heart disease (Gil *et al*., 2000, Miguel *et al*, 2004 and Houston, 2005).

2. Insecticidal activity

The secondary metabolites of plants are vast compounds with a wide range of biological activity. Steroids, phenolic compounds and tannins possess great impact on insecticidal activities. It has been established that different plant extracts induced great toxicity against store insects due to the presence of different classes of bioactive compounds (Mostafa, *et al*., 2012). There is an urgent need to develop low cost, biodegradable to non-toxic products, alterative to synthetic insecticides and ecofriendly. Therefore, the present work was focused on use of pomegranate peels and leave crude juices as natural insecticides. The results show that LC_{25} and LC_{50} values for peels crude juice (320 ppm and 630 ppm) were higher than leave crude juices (280 ppm and 610 ppm).

Toxicological studies

(1) Morphology changes in cotton leaf worm

The comparison between untreated (control) larvae and treated ones with pomegranate juices (Figure 1) led to deduce that larvae size was obviously squeezed as the result of treating with pomegranate leave crude juice. On the other hand, larvae failed to free themselves from their old larval skin when treated with pomegranate peels crude juice.

(2) Effect of pomegranate peels and leave crude juices on the biotic potential and some biological aspects

Data in Table (2) illustrate the mortality percentages of the 4th instar larvae of cotton leaf worm up to the papal stage due to treatments with pomegranate peels and leave crude juices at LC_{50} value. Both pomegranate peels and leave crude juices caused incredible increase in larvae mortality, being 13.61 and 11.25 times as great as that for untreated larvae. This means that peels crude juice induced greater mortality effect than caused by leave crude juice. The conversion of larvae to pupa was also affected by pomegranate crude juices. Peel crude juice induced more pronounced effect than crude leave juice, being about 3.5 and 1.4 times greater than the untreated larvae, respectively. In other words, the percentage pupae mortality was in the following order due to application of pomegranate crude juices, i.e., peel juice > leave juice > untreated ones. Similarly, the percentage of adult emergence followed the same aforementioned arrangement. This set of experiments demonstrates that pomegranate juices induced a detrimental effect on the entire developmental stages of the cotton leaf worm. These results led to suggest that pomegranate peels and leave crude juices can be administered as natural insecticides and safe agent to halt the life of cotton leaf worm.

The results for hatchability % demonstrated that in the untreated female moth laid down eggs to a value of 96.35%. Whilst, the values of hatchability % for treated moth with pomegranate peels and leave crude juices were affected, being 0.8 and 0.7 times lower than the control moth. The sterility data of the moth treated with pomegranate peels crude juice induced greater sterility than moth treated with pomegranate leave crude juice. In this respect, the egg sterility due to applications of peel crude juice was about 1.11 times as great as that induced by using leave crude juice. Generally speaking, peels crude juice remarkably influenced the entire circle life of the cotton leaf worm and can be used to lessen the deleterious effect on cotton plant.

It is well known that essential oils possessed an obvious effect on the entire developmental stages of insects. Egypt is famous by cultivation cotton plants long time ago and considered as a major source of hard currency. It is well established that cotton leaf worm exhibited a big hazard effect on cotton plants. Consequently, it was considered of interest to study of the effect of some plant extracts as insecticides towards the cotton leaf worm. In this context, Farag *et al*. (1994) used thyme and clove essential oils to cause detrimental effect on the various developmental stages of *S. littoralis*. Their results demonstrated the remarkable detrimental effect on the larvae and pupal stages and reduced the egg production. The idea behind this work is that pomegranate peels and leave crude juices contained some phenolic compounds and might suppress the activity of the cotton leaf worm. In fact, the data of this work encourage the use of pomegranate juices as natural safe insecticides.

Several authors have demonstrated the influence of certain essential oils and their insecticidal effects. For instance, Meisner, *et al*. (1982) found that carvone exhibited highest activity as phagodeterrent against *S. littoralis*. In addition, Krishnarajah *et al*. (1985) reported that p-cymene was most effective in knock-down potency which was found in higher percentages in thyme oil.

It is well known that juvenile hormone (JH) is quite important in both insect development and reproduction. In addition, cytochrome p-450 oxidase system is essential for the maintenance of balanced JH titers and slight changes in the p-450 system which affects the dynamic characteristics of insect population. Consequently, the insecticidal activity of thyme and clove essential oils and their components on the various developmental stages of cotton leaf worm was due to the inactivation of JH by altering the microsomal cytochrome p-450 oxidase system (Bowers and Nishid, 1980). .

It appears that there is a relationship between the chemical structure of the phenolic moieties in the coumarin of celery and murraya leave extracts and its insecticidal activity. These compounds contain aromatic nucleus attached with a polar functional group (OH). The wide spread use of phenol and chlorophenol and related compounds as disinfectants is well established. It is well known that phenolic hydroxyl group is quite reactive and easily forms hydrogen bonds with active sites of enzymes (Farag, *et al*., 2003). The data indicate that the celery murraya leave extracts which contain coumarin caused high mortality of cowpea beetles and induced quite harmful effect on brain tissue of the insect.

(3) Histopathological examination of *S. littoralis* treated by pomegranate juices

Pomegranate peels and leave crude juices at LC_{50} dose induced histological damage in the mid gut of 4^{th} instar larvae *Spodoptera littoralis*, as some of the epithelial cells were vacuolated and caused destruction of nuclear content. Microscopical examination of mid gut *S. littoralis* without treating with pomegranate juices (control) was presented in Fig. 2. The mid gut was consisted of normal epithelia cells. Epithelial tissues are known to line the cavities and surfaces of structures throughout the body. Functions of epithelial cells include secretion, selective absorption, protection, transcellular transport and detection of sensation with goblet cells (a goblet cell is a glandular simple columnar epithelial cells whose function is to secrete gel-forming mucins, the major components of mucus. Cuticle of control *Spodoptera littoralis* (Fig. 3) showed normal basement (non-cellular membrane, the layer of epithelium on the basement membrane produces the cuticle), epicuticle, exocuticle and endocuticle with normal hypodermal cells. Microscopical examination of mid gut of *Spodoptera littoralis* treated with pomegranate peel crude juice (Fig. 2) showed necrosis of some epithelial cells lining and loss of great numbers of goblet cells. Cuticle of cotton leaf worm (Fig. 3) showed the appearance of epicuticle, lost most of exo- and endocuticle and hypodermal cells. Microscopical examination of mid gut of *Spodoptera littoralis* treated with pomegranate leave crude juice (Fig. 2) showed most of columnar cells degenerated, only few numbers were necrosis. Cuticle (Fig.3) showed losses of some hypodermal cells. In addition some parts elicited thickening of exo and endocuticle with degenerative changes.

The data of Salam and Ahmed (1997) and Younes *et al.* (1999) illustrate that similar observations were seen for neem extract and other plant extracts against *S. littoralis, S. gregaria* and various insect species belonging to different order. The *Melia azedarach* extract caused destruction of epithelial cells against *S. littoralis*. Also, degeneration of the epithelial cells and decay of its boundaries were found when *S. littoralis* larvae treated with the extracts of both *Clerodendro inerme* and *Conyza dioscoridis* (Emara and Assar, 2001).

The outcomes of the present study encourage the use of pomegranate peels crude juice as a natural insecticide since it is almost priceless, safe agent obtained from natural organs by annual pruning of pomegranate trees and regarded as a waste materials and ecofriendly.

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Table 1. Total polyphenolic and flavonoid contents of pomegranate peels and leave crude juices.

Parameter	**Peel crude juice**	**Leave crude juice**
Total polyphenolics (TPP) (GAE mg/g dry weight)	58.63±0.129a	48.02±0.071b
Total flavonoids (TF) (Q E mg/g dry weight)	47.32±0.032a	33.02±0.009b

Values are means of three replicates of each parameter ± standard error. Means within each row followed by the same letter are not significantly different at $p > 0.01$.
GAE and QE refer to gallic acid and quercetin, respectively.

Table2. The effect of pomegranate peels and leave crude juices on the biotic potential and some biological aspect of 4th instar larvae of *Spodoptera littoralis*.

Biotic potential and biological aspect (%)	Treatments		
	Control	Leave crude juice	Peels crude juice
Larvae mortality	5.00±0.01a	56.25±0.05b	68.05±0.08c
Pupae mortality	5.00±0.05a	7.00±0.03b	17.50±0.05c
Adult emergence	85.00±0.09a	25.75±0.06b	23.00±0.03c
Egg hatchability	96.35±0.01a	77.25±0.03b	68.05±0.05c
Sterility	-------	72.58±0.03a	80.52±0.06b

Values are means ± SE of measurements.

Means within each row followed by different letters were significantly different at P < 0.01.

Fig.1. Morphology changes in malformed of the 4th instar larvae of *Spodoptera littoralis* treated by pomegranate juices:

a. Normal larvae
b. Squeezed size of treated larvae with pomegranate leave crude juice

c. Treated larvae with pomegranate peels crude juice failed to free themselves from their old larval cuticle.

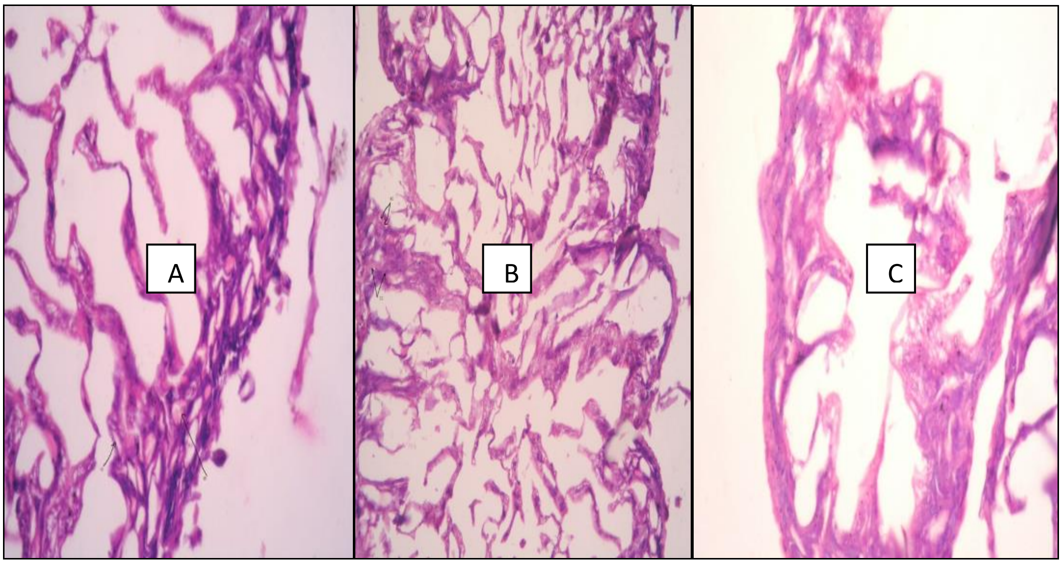

Fig. 2. Microscopical examination of the 4th instar larvae mid gut of *S. littorallis*

A: Untreated larvae showing normal architecture
B: Treated larvae with pomegranate peels crude juice
C: Treated larvae with pomegranate leave crude juice

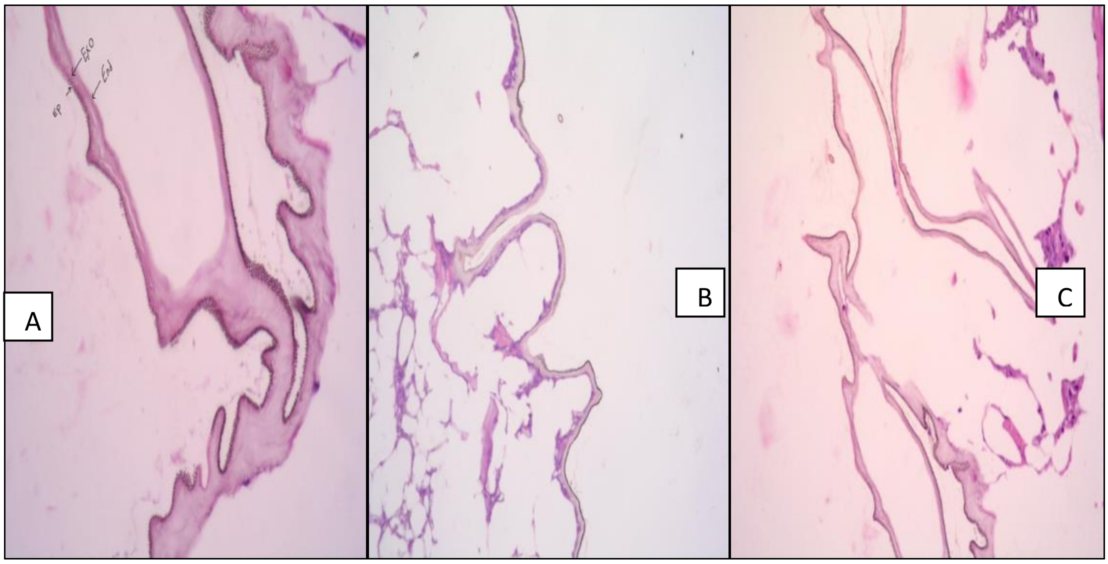

Fig.3. Microscopical examination of the 4th instar larvae cuticle of *S. littorallis*.

A: Untreated larvae
B: Treated larvae with pomegranate peels crude juice
C: Treated larvae with pomegranate leave crude juice