Biological activity of Pomegranate peels Punica granatum
Silver nanoparticles AgNPs extract against Fourth larvae of
Culex quinquefasciatus mosquito (Diptera: Culicidae)

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Abstract. Present study was included the assessment of biological activity of Pomegranate Peels Punica granatum plant extract toward fourth Culex quinquefasciatus larvae mosquito (Order: Diptera, Family: Culicidae) by testing ethanolic and Silver nanoparticles extracts. Results of present study revealed that Silver nanoparticles extract was more toxic than ethanolic extract and the mortality rates were reached to 90 % after 72 h exposure time when it treated with 5 μg/ml compared to 0% at control treatments. Ethanolic extract also was toxic at concentration 5 μg/ml and mortality rates were reached to 80% after 72 h exposure time. Study of larvae after death showed that penetration of Silver nanoparticles extract was entered inside mid gut of larvae after damage of body wall. SEM analysis indicated to that more deformation found in body wall thus AgNPs were easily penetration through skin of fourth larvae. SEM analysis also was showed that Silver nanoparticles were clustered and irregular or oval shapes.

1. Introduction
Peels of pomegranate P. granatum contain a wide variety of phytochemical compounds such as gallotannins, gallic acid, ellagic acid, punicalins and punicalagins [1, 2]. There are several studies referred to the insecticidal effects of pomegranate juices for instance [3], who tested leaves of pomegranate as insecticidal activity toward the stored grains Rhyzopertha dominiea (Fabr) under optimum conditions, the pomegranate powder leaf gave a high incidence of death and development rate reduction in population.

The pomegranate peels have a several health benefits by their different tannins, alkaloid, flavonoids, and organic acids. The gallic acid and granatin B exhibit an anti-inflammatory activities [4, 5, 6].

The mosquitoes are one of medical important can spread many of diseases in the world like malaria diseases and arboviral such as Dengue fever, West Nile Virus and currently Zika Virus [7]. However, the chemical insecticides dense application to control mosquito vectors after spreading of RVF in the Arabian Peninsula in 2000 [8] and Dengue Fever in 1997 and 2004 [9] resulted in developed resistance in mosquitoes.

In Iraq Cx. quinquefasciatus is abundant in middle and southern regions can cause multiple problems such as biting nuisance and may be serve as carriers for diseases [10]. Nanoparticles are efficient and
ecofriendly in comparison to chemical insecticides especially when they mixed with plant extracts that provided safe and beneficial methods to synthesis of AgNPs that are simple and large amounts of nanoparticles can be prepared in a standard time [11]. Present study was attempted to investigate biological activity of biosynthesis of pomegranate Peels extract nanoparticles (AgNPs) because of their safety, inexpensive and readily available as alternative method to the traditional chemical insecticides against Cx. quinquefasciatus.

2. Materials and Methods

2.1. Preparation of Plant Extracts
Peels of pomegranate P. granatum were prepared according [12]. Dried peels of P. granatum were ground into fine powder via electric grander after drying it at room temperature, then resultant powders were extracted using 70% ethanol. First primary tested of plant extract was conducted via cool aqueous extracts at different concentration. 100g of peels powder mixed with 300ml of the ethanol solvent for 24 h, agitation at 24°C. The mixture was filtered through a Whatman filter paper No. 5 and the flow – throw was dried in a rotary evaporator at 40 C for 2-3h. Then 1g of the extract dissolved in 100ml of 70% ethanol to get 1% as a mother solution.

2.2. Silver Nanoparticles (AgNPs) Extracts Preparation
1 mL of 1mM AgNO3 solution added to 99 mL standard solution of pomegranate Punica granatum extracts Peels, at room temperature with shaking until changing the color to brown that indicating the formation of nanoparticles. The shape of the formed nanoparticles was distinguished using scanning electron microscope (SEM from Central Lab. Faculty of Science/ University of Kufa /Iraq) at an accelerating voltage of 12.50 KV, [13].

2.3. Identification and Rearing of Mosquito
Rearing of mosquitoes was done according to [14]. The samples of mosquito larvae were collected from natural pools, then the larvae were identified morphologically and were sent to certify by Natural Museum/Baghdad as Cx. quinquefasciatus. About (100-150) larvae were placed in (25 x15 x8) cm plastic container containing water from pool. The larvae were supplied a mixture of yeast and fine bread at equal amounts as food. Eggs were obtained from a live pigeon that attached to its legs and wings and placed inside breeding cage during night. The females can take the blood as meal. The received eggs placed in a small plastic container with a amount of water and a larval culture to obtain the suitable number of tested larvae.

2.4. Bioassay Experiments
Larvicidal activity of the Peels of pomegranate loaded as Silver nanoparticles (AgNPs) tested on the fourth larval instars of Cx. quinquefasciatus was assessed by using the standard methods [15]. Different concentrations (0, 1, 2, 3, 4 and 5) g/ml of (AgNPs) extracts were prepared. For each treatment three replicates as well as control treatment was prepared. The experiments were carried out under (27 ± 2) °C, RH (70± 10) % light time (12-12h). The control treatments were occurred by adding 0.1ml ethanol 70% only with the same amount of water. Mortality rate noted daily, the dead pupae and larvae were continuously removed until adult appearance.

2.5. Statistical Analysis
The data was analysis as a Factorial Experiments (Completely Randomized Design C.R.D). The results were compared by using Least Significant Difference Test (L.S.D), *P< 0.05* [16].When necessary, the mortality percentage in the treatments were corrected by using Abbott method [17].

3. Results and Discussions
3.1. SEM Morphological Characterization

The scanning electron micrographs clearly showed that clustered of irregular or oval shapes of silver nanoparticles, an average size 40-100 nm, with inter particle distance the magnified done at (1629-1753 X), figure (1).

![SEM image showing crystalline silver nanoparticles as uniform and aggregates.](image-url)

3.2. Larvicidal Effects of Peels of Pomegranate Extract and its Silver Nanoparticles on Cx. quinquefasciatus larvae

The results showed in table (1), the effects of the ethanol pomegranate Peels extract with and without silver nitrate toward the fourth larvae of Cx. quinquefasciatus. No results findings after 24 h then after 48 the AgNPs extract was highly toxic when treated with high concentration (5) μg/ml, mortality rates were recorded (85) % and were increased after exposure time 72 h it reached to (90) % compared to (0) % at control treatments. After 48h ethanolic extract also was recorded mortality rates were (70) % and were increased to 80% after 72 h. Lower concentration revealed less toxic effects against fourth larvae at concentrations 1 μg/ml the mortality rates were (26.6) % compared to (0) % at control treatments. Statistical analysis showed that there were significantly differences at (P 0.05) % between values.

| Conc. μg/ml | Ethanol extract | Silver nanoparticle extract AgNPs |
|------------|-----------------|----------------------------------|
|            | Larval Mortality (%) | Larval Mortality (%) | L.S.D |
|            | (24) h | (48) h | (72) h | (24) h | (48) h | (72) h | P >0.05 |
| Control    | 0.0±  | 0.0±  | 0.0±  | 0.0±  | 0.0±  | 0.0±  | 00      |
| 1.0        | 0.0±  | 0.0±  | 10±   | 0.0±  | 26.6± | 30±   | 2       |
| 2.0        | 0.0±  | 10±   | 30±   | 0.0±  | 30±   | 50±   | 2       |
| 3.0        | 0.0±  | 20±   | 40±   | 0.0±  | 60±   | 70±   | 2       |
| 4.0        | 0.0±  | 50±   | 70±   | 0.0±  | 70±   | 90±   | 2       |
| 5.0        | 0.0±  | 70±   | 80±   | 0.0±  | 85±   | 90±   | 2       |
| L.S.D      | 00    | 3.5   | 2.5   | 00    | 3     | 2     | 2       |
P >0.05     | 2.5   | 2     | 2     |
From data reviewed above apparently showed that mixing of the extract and the prepared AgNPs were found more active as insecticide against the 4th larvae of Cx. quinquefasciatus. Several previous studies indicated that silver nanoparticles could provide powerful larvicidal effects. The biological activity of the AgNPs may be contributed to penetrate of AgNPs through the larval membrane which leads to the death of the delicate larvae [18] as well as, the increased activity might be attributed to small size of the particles that facilitate its passage through the body wall to inside the cells which might reverse negatively the ecdysis addition to other physiological processes [19].

The AgNPs as a larvicidal activity may be due to denaturation of the sulfur-containing proteins, or phosphorus containing compounds like DNA that leads to the denaturation of organelles and enzymes [20], and reduces the cellular membrane permeability and reduction in ATP synthesis which finally causes cell death [21]. Similar findings [22] were revealed that mosquito larvicidal bioassay with the AgNPs synthesized by dried green fruits of Drypetes roxburghii against two species of mosquito Cx. quinquefasciatus and Anopheles stephensi. Study [23] who indicated that the larvicidal activity of silver nanoparticles synthetize by Swietenia mahagoni (L.) Jacq. Leaf extract against An. stephensi larvae and group of Cx. vishnui and Cx. quinquefasciatus by concentration 80 ppm was exhibit a significantly higher than the mortality rates by 20 ppm, 40 ppm and 60 ppm of AgNPs of S. mahagoni leaf extract. From other side the biological activity of ethanolic plant extract might be due to multiple compounds including phenolic, terpenoids, and alkaloids especially tannins were identified in ethanol extract by study of [24]. The tannin formed irreversible complex with the proteins containing proline lead to inhibit cell protein synthesis, this might be attributed for the mortality in Cx. quinquefasciatus larvae [25].

3.3. Histological Examination

Results of present study clearly showed that the penetration by AgNPs plant extract of mid gut along the elementary canal after 72 h when dead fourth larva Cx. quinquefasciatus was treated with 5 μg/ml of AgNPs plant extract which tested by light microscope at (40X) (figure 2).

![Figure 2](image-url)

**Figure 2.** Fourth larva of Cx. quinquefasciatus treated with 5 μg/ml of AgNPs plant extract of pomegranate Peels P. granatum.

Results of SEM also indicated more damaged and clearly changes in body wall of fourth larva Cx. quinquefasciatus revealed more shrinkage when it treated at 5 μg/ml of AgNPs plant...
extract after 72 h compared to control treatments that appeared more distended tissues figure (3).

Figure 3. Fourth larva of Cx. quinquefasciatus tested by SEM (A and B) treated with 5 μg/ml of AgNPs plant extract of pomegranate Peels P. granatum. (C and D) control treatments.

From data of present study mentioned above demonstrated that larvicidal effects, included multiple alterations in body wall and then penetration of AgNPs plant extract inside alimentary canal. If the body wall was damaged or injured penetration by AgNPs plant extract would be easier passage that proved the plant extract mixing with silver nitrate has led to penetration and this was agreed with many previous studies. The study of [26] reported the silver particles processing from Melia dubia leaves extracts were higher effective than the crude extract against mosquito larvae Cx. quinquefasciatus. The alterations were found in the mid gut and body wall may be explained according to [26] because the midgut which responsible for digestion process of larva which direct interaction with the toxic compounds, and so initiating death. Also progressive damage of the intestinal tissue of larvae, finally, resolve the gut cells content with hemolymph, which is responsible for larval mortality. The study [27] confirmed the activity of aqueous extract of Eucalyptus leaves acts on Cx pipiens larvae and hypertrophy was observed in intestinal cells which began to pull away from each other. The results of present study agreed with [28], that reported clear damage of the epithelial cells of the midgut and intestinal tissue muscles and cuticle
of Cx pipiens larvae were the most severely damaged when treatment by the Citrus limon and Allium sativum oils. The study [29] appeared aqueous leaves extract of Ricinus communis induces histopathological changes at different levels of the body leading to disorganization of movements followed by immobilization and subsequent death of the fourth larvae of Cx pipiens.

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