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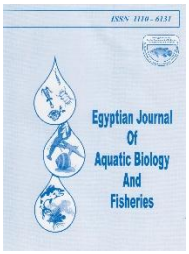


# Green Synthesis of ZnO and Se Nanoparticles Based on *Achillea fragrantissima* (Forssk.) Extract and Their Larvicidal, Biological and Ultrastructural Impacts on *Culex pipiens* Linnaeus

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**ABSTRACT**

The management of pests is facing economic and ecological challenges worldwide due to environmental and health hazards caused by most chemical synthetic pesticides. Effective safe alternatives are needed as botanical extracts with significant insecticidal activity. In the present work, zinc oxide (ZnO) and selenium nanoparticles (SeNPs) were synthesized *via* green approaches using *Achillea fragrantissima* (Forssk.) Sch.Bip extract. The particle size and the zeta potential of prepared NPs formulations were characterized by the transmission electron microscope (TEM) and dynamic light scattering (DLS). Then, the efficacy of green synthesized ZnONPs and SeNPs was evaluated against the 3rd instar larvae of *Culex pipiens* Linnaeus, 1758, which is considered a disease vector. The larvicidal activity of SeNPs against the 3rd instar larvae of *Cx. pipiens* appear to be more potent than ZnONPs and *A. fragrantissima* extract. The $LC_{50}$ values after 48 hrs of exposure recorded 0.07, 23.74 and 89.99 ppm for SeNPs, ZnONPs and *A. fragrantissima* extract, respectively. In addition, fecundity of females emerged from treated larvae with SeNPs, ZnONPs was significantly affected compared to the control. Furthermore, the total metabolites and enzymatic activities were greatly affected in all tested stages of *Cx. pipiens*. Severe ultrastructural deformities were observed in the midgut of treated *Cx. pipiens* larvae. Overall, the nano-formulations of *A. fragrantissima* improve their potencies more than the *A. fragrantissima* extract only.

## INTRODUCTION

Mosquitoes are well known for their public health importance since they cause major health problems being the biggest killers in the world; it is responsible for killing about 750,000 human/ year **(Edwards, 2019)**. In Egypt, *Culex pipiens* Linnaeus, 1758 (Diptera: Culicidae) has been declared as a vector of several diseases **(Abd El-Samie & Abd El-**

**Baset, 2012; El-Zayyat *et al.*, 2017)**. It transmits Rift Valley fever virus **(Dodson *et al.*, 2012)**, Japanese encephalitis, *Wuchereria bancrofti* **(Chancey *et al.*, 2015)** and the West Nile virus **(Ahmed, 2016)**. In addition, this species is accredited for the transmission of human lymphatic filariasis in Egypt **(Joseph *et al.*, 2011; Dyab *et al.*, 2015; El-Naggar *et al.*, 2017)** and it has been recorded in all governorates **(Ammar *et al.*, 2012; Abdel-Shafie *et al.*, 2016)**.

The overuse and misuse of chemical insecticides for preventing diseases vectored by the mosquito have created many problems related to human and environmental hazards due to accumulations of toxic residues in the food chain, as well as the emergence of insecticide resistance among the vectors **(Acevedo *et al.*, 2009; Bonner & Alvanja, 2017)**. Alternatively, bio-insecticides of plant origin including plant extracts and essential oils are an appealing alternative strategy to combat vectors due to their larvicidal, adulticidal and repellent properties **(Bekele, 2018)**. *Achillea fragrantissima* (Forssk.) Sch. Bip (Family: Asteraceae) is a desert herb, used in the Arab region for the treatment of respiratory diseases, gastrointestinal diseases and diabetes. *A. fragrantissima* is rich in flavonoid, sesquiterpene and phenolic active constituents **(Hijazi *et al.*, 2019).** *A. fragrantissima* oil exhibited insecticidal activity against different stored product beetles and weevils **(Nenaah, 2014)** and potential repellent and mosquitocidal activities against *Cx. pipiens* L. female **(Al-Sarar *et al.*, 2020)**.

Novel applications of nanoparticles are growing rapidly in the field for introducing particles at dimensions ranging from approximately 1 to 100 nm due to their completely new or enhanced properties based on size, distribution and morphology **(Shafiq *et al.*, 2007)**. Nanoparticles at the nanoscale had distinct physical, chemical and biological properties. They showed novel characteristics such as more chemical reactivity and extra strength, compared to their counterparts at larger sizes **(Elghanian *et al.*, 1997; Khan *et al.*, 2022)**. They offer a great increase in surface area, therefore, enhancing the affinity to reach the target. Furthermore, they rapidly penetrate and selectively accumulate in different insect cells (**Nenaah *et al.*, 2015)**. Thus, the main objective of the present study was the assessment of toxicity and effects of green synthesized zinc oxide and selenium nanoparticles (ZnONPs and SeNPs) based on *A. fragrantissima* ethanolic extract on the biological, biochemical and ulterastructural parameters of *Cx. pipiens*.

## MATERIALS AND METHODS

### Preparation of ethanolic plant extract

Dry *A. fragrantissima* leaves (100 gm.) have been ground using a mortar. The resulting powder was extracted using the Soxhlet system, with 300 ml of absolute ethanol. The obtained solution was evaporated by a rotary evaporator (Labo-Rota C311) at 40ºC for 2–3 hrs, and the crude extract was kept at 4ºC **(Aina *et al.*, 2007)**.

## Synthesis of nanoparticles

### *Synthesis of ZnO nanoparticles*

The *A. fragrantissima* extract was diluted using ethanol and dropwise added to distilled water under vigorous stirring at about 80$^{o}$C. Zinc acetate (0.2 g) was dissolved in 20ml of distilled water and added dropwise to the extract solution under magnetic stirring. The solution mixture was left under heating for about 4 hours. The pH of the solution was adjusted to alkaline using a few drops of NaOH (0.5 M). The solution turned turbid with the appearance of white color participation, indicating the formation of ZnO nanoparticles (ZnONPs) **(Malik *et al.*, 2022)**.

### *Synthesis of selenium nanoparticles*

The *A. fragrantissima* extract was dissolved in ethanol and added dropwise to 100ml of deionized water under magnetic stirring at a temperature of 80$^{o}$C. Selenium oxides (0.2 g) were dissolved in 20ml of deionized water and dropwise added to the above solution under mild stirring. The solution mixture was stirred and heated to 60$^{o}$C for about 2 hours. The solution turned red in color, with some participated red particles, indicating selenium nanoparticles (SeNPs) formation **(Abdel Fattah *et al.*, 2021; Abo El-Fadl *et al.*, 2022)**.

## Characterization of ZnO and Se nanoparticles

### *Transmission electron microscope*

The particle size and shape of the prepared nanoparticles were evaluated using a transmission electron microscope (HR-TEM, JEOL- JEM-2100). The dilute ZnONPs and SeNPs suspension was subjected to a sonicator (Ultrasonics, USA/digital ultrasonic cleaner cd 4830) at a frequency of 30 kHz and power output 750 W for 60 minutes in a sonication water bath before examination according to **Anjali *et al.* (2012)**. The suspension was dropped onto the testing grid (one or two drops) and left for drying before investigation.

### *Dynamic light scattering (DlS)*

The suspension of ZnONPs and SeNPs was firstly sonicated to guarantee the good dispersion of these particles in aqueous media and measured directly by dynamic light scattering (DLS) technique according to **Sugumar *et al.* (2014)**, using zeta sizer instrument (Nano-ZS, Malvern Instruments Ltd., Zeta sizer Ver, 704, UK) in National Research Center.

## Tested mosquitoes

### *Culex pipiens culture*

Larvae of *Cx. pipiens* provided by the Research Institute of Medical Entomology, Giza, Egypt, and self-perpetuating colonies were established and maintained in the insectary of

the Entomology Department, Faculty of Science, Ain Shams University. Mosquitoes were reared according to **Abdel-Haleem *et al.* (2023)** under controlled conditions of temperature (27±2 ºC), relative humidity, R.H. (70 ± 5%) and light: dark period (14:10 hrs). Early third larval instars were used for toxicological studies.

***Bioassay tests***

The larvicidal efficacy of *A. fragrantissima* ethanolic extract, ZnONPs and SeNPs were assayed against 3rd instar larvae of *Cx. pipiens*. The larvae were treated with different concentrations of nanoparticles according to the standard **(WHO, 2005)** protocol. Four concentrations of SeNPs (0.01, 0.05, 0.1, and 0.2 ppm) and ZnONPs (5, 20, 50, and 75 ppm) were used. While for *A. fragrantissima*, ethanolic extract (40, 80, 120, and 200 ppm) concentrations were prepared in ethanol. For each treatment, each concentration was added to 100 ml glass beakers. Three replicates of twenty-five early 3rd larval instar were introduced to each concentration. For control, distilled water with a drop of ethanol only was used. After 24 and 48 hours of exposure, percentage mortality was recorded **(El Hadidy *et al.*, 2022)**.

***The reproductive potential of emerged females***

Sub-lethal concentration ($LC_{25}$) of tested formulations was applied to 3rd larval instar of *Cx. pipiens*. The emerged females from these treatments and untreated were introduced to evaluate the following parameters.

**Fecundity.** The emerging adult females from the treated 3rd instar larvae were collected and transferred to the wooden cages (20×20×20 cm) by using an electric aspirator recommended by (WHO). They were fed on a 10% sugar solution for three days. The adult males and females were left for one day without a sugar solution. On the fifth day, the starved females took a blood meal from a pigeon and were allowed to lay egg rafts on clean water (oviposition traps) in the cages. The number of eggs in each egg raft was counted by using binocular (BEL®) and then the mean values were taken **(Tantawy *et al.*, 2022)**.

**Egg – hatchability.** The eggs were sorted into two categories (hatched and non-hatched eggs) according to the method of **El-Sheikh (2002)**. The egg-hatchability was calculated by using the following equation:

Egg-hatchability % = A / B × 100

Where, A: total number of hatched eggs & B: total number of laid eggs.

**Sterility index (SI).** The sterility percentage was estimated according to the formula of **Toppozada *et al.* (1966)**:

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

Where, a: number of eggs laid / female in treatment;

b: percentage of hatched eggs in treatment;

A: number of eggs laid / female in control, &

B: percentage of hatched eggs in control.

**Biochemical activity**

Tissue samples of untreated and treated 3$^{rd}$ instar larvae and emerged pupae and adults (males and females) with $LC_{50}$ of the ZnONPs, SeNPs, and *A. fragrantissima* extract after 48 hrs of treatment were homogenized using treatment buffer (1gm insect body/ 1ml) in a chilled glass Teflon tissue grinder for 3 minutes. Homogenates were centrifuged at 14000 r.p.m for 15 minutes at -5˚C in a refrigerated centrifuge. The supernatant was stored at -3˚C till use. Three replicates were carried out for each test.

***Determination of total protein***

The total protein was determined by using Coomassie brilliant blue G-250 reagent (CBB) according to **(Bradford, 1976)** technique. An amount of 50mg of Coomassie brilliant blue (G-250) was dissolved in 25ml of ethanol (95 %). Then, a volume of 50ml of phosphoric acid (85 % (w/v) was mixed into the solution. Then, the solution was diluted to a final volume of 500ml, (25µl) of sample solutions that were titrated into a test tube, and then the phosphate buffer (pH 6.6) was added to reach 0.05ml. After that, 2.5ml of protein reagents was added to the sample and mixed well. Absorbance (595 nm) was adjusted between 2 minutes and 1 hour against a blank sample.

***Determination of total carbohydrate***

Total carbohydrate contents were determined in untreated and treated tissue samples according to the method of **Singh and Sinha (1977)**, using anthrone reagent. A volume of 72ml of concentrated $H_2SO_4$ was added to 28ml of distilled $H_2O$ and 50mg of anthrone was poured on warm water, with vigorous shaking to prepare anthrone reagent. The sample solution (100 µL) was diluted with $H_2O$ to 1ml, and 5ml of anthrone reagent was mixed well with the sample. 5ml of anthrone reagent and 1.1ml of $H_2O$ were mixed to prepare a blank. Both sample and blank were placed in the water bath for 10 minutes and then cooled for 15 minutes to 27°C. The absorbance of the sample was recorded at 620 nm.

***Determination of total lipid***

Total lipid was determined in untreated and treated tissue samples according to the method described by in the study of **Knight *et al.* (1972)**, using a phospho-vanillin reagent. Pure vanillin (0.6 gm) was added to 10ml of ethanol and then mixed with 90ml of $H_2O$ and 400 ml of concentrated phosphoric acid. The mixture was kept in a dark container bottle (27°C). In a test tube, 250µl of sample solution was mixed with 5ml of concentrated $H_2SO_4$ and placed in a water bath for 10 minutes then cooled to room temperature. 500µl of the digest was mixed with a phospho-vanillin reagent to start the reaction. 45 minutes later, the appeared color was recorded at 525nm against a reagent blank (500 µL of $H_2O$ and 6.0 mL of phospho-vanillin reagent).

***Determination of GST activity***

The glutathione-S-transferase activity was determined in untreated and treated tissue samples according to the method of **Habig *et al.* (I974)**. 200µl of larval homogenate, 1 ml of the potassium salt of phosphate buffer (pH 6.5) and 100µl of GSH were mixed to prepare the reaction solution. Then, 25µl of the substrate CDNB solution was added to start the reaction. The concentration of CDNB and GSH was adjusted to 1mM and 5mM, respectively. The enzyme and substrate solution were incubated for 5 minutes at 30˚C. The absorbance of the mixture solution was recorded at 340nm against blank.

***Determination of ATPase activity***

The activity of ATP-ase was determined according to **Amaral *et al.* (2001)**. The amount of inorganic phosphate resulting from ATP hydrolysis by ATP-ase activity was measured. 150 mM NaCl, five mM ATP $Na_2$-TRIS and 15 mM KCl in 30 mM Histidine HCl-TRIS were mixed to prepare the test solution then pH was adjusted to be 7.5. The ATP-ase was mixed with this solution to complete the final volume (0.5ml). The enzyme solution mixture was incubated at pH 7.5 (37 ˚C), and then ATP-solution was dropped to start the reaction, and afterwards, the mixture was incubated at 37˚C for 30 minutes. 100 ul SDS (5%) was added to terminate the reaction. The resulting inorganic phosphate was determined by using the phosphorus kit (a Quimica Clinica Applicada S.A. (Spain) commercial kit). The molybdate reacts with phosphorus resulting in phosphor-molybdate, which is reduced to a molybdenum blue that is measured photo-metrically at 650nm, using a spectrophotometer against blank.

**Ultrastructure studies**

The malformations in the midgut of treated third larval instar of *Cx. pipiens* with $LC_{50}$ of nanoparticle formulations for 24 hrs were distinguished by transmission electron microscopy (TEM) compared to untreated. For transmission electron microscopy, the ultrathin cross sections of the midgut were made by a Reichert Supernova

ultramicrotome. The treated midguts were fixed in a mixture of glutaraldehyde (2.5%), and paraformaldehyde (4%) in (0.1 M) phosphate buffer (pH 7.3) and then fixed with (1%) osmium tetroxide solution. The midgut samples were fixed and dehydrated in the same buffer. Serial dilutions of acetone solutions were prepared, and then they were embedded in an epoane. Lead citrate and uranyl acetate were used to stain ultrathin sections according to **Reinbold *et al.* (2001)**. Untreated and treated samples were examined by SEO PEM-100TEM at the Electron Microscopy Unit, Faculty of Science, Ain Shams University, Cairo, Egypt.

### Statistical analysis

Biological data were expressed as mean ±SE. The mortality data were analyzed to estimate $LC_{50}$, $LC_{95,}$ and slope with standard error values using multiple linear regressions **(Finney, 1971)**. According to the Tukey test, significance was assessed at $P< 0.05$, and statistical values that are separated by the same letter are not significantly different (one-way ANOVA). The statistical analyses were carried out using Minitab version 17.

## RESULTS

### *1.* Characterization of ZnONPs and SeNPs

#### *1.1. Transmission electron microscope (TEM)*

The shape and size of the synthesized nanoparticles ZnONPs and SeNPs were assessed using TEM measurements. ZnONPs size ranged from 5- 15nm in diameter, as shown in Fig. (1a, b). While SeNPs size ranged from 20- 40nm in diameter with the appearance of some aggregated particles, forming larger sizes of about 80 to 100nm, as shown in Fig. (1c, d).

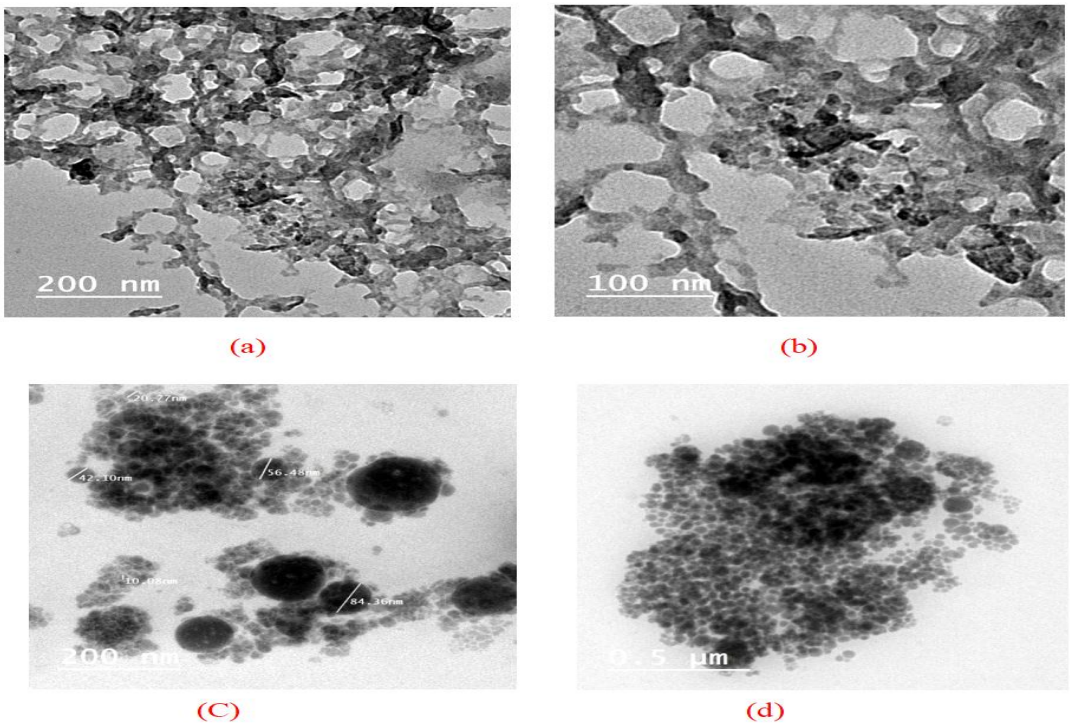


**Fig. 1.** TEM image **(a,b)** showing ZnO nanoparticles (ZnONPs) with size ranging from 5-15nm in diameter, and TEM image **(c,d)** showing selenium nanoparticles (SeNPs) of size ranging from 20- 40nm in diameter.

### *1.2. Particle size and dynamic light scattering (DLS)*

The particle size distribution of the synthesized ZnONPs was determined by dynamic light scattering (DLS) measurements, as displayed in Fig. (2a). The particle size has an average diameter of 219nm, as indicated in Fig. (2a). While, the droplet size distribution of the prepared SeNPs was assessed using dynamic light scattering (DLS) measurements as presented in Fig. (2b). The particle size estimated from Fig. (2b) has an average diameter of 190nm.

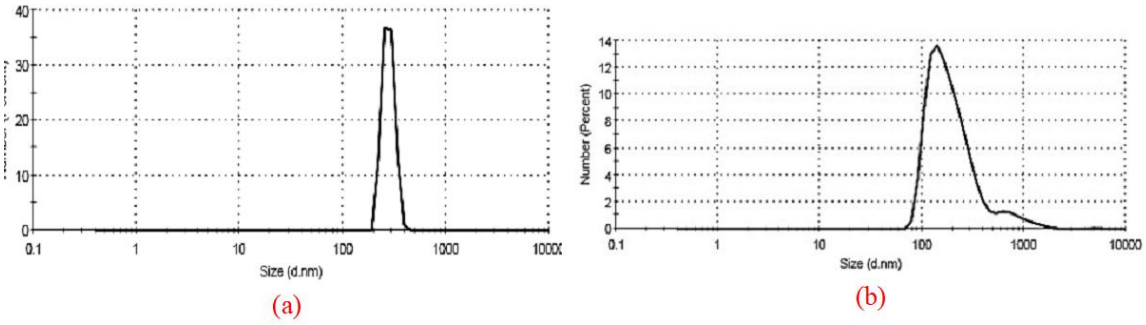


**Fig. 2.** Dynamic light scattering **(a)** Droplet size distribution of the ZnONPs, and **(b)** Droplet size distribution of the SeNPs.

## 2. Larvicidal activity

The larvicidal efficacies of *A. fragrantissima* ethanolic extract, ZnONPs and SeNPs were evaluated against the 3rd instar larvae of *Cx. pipiens*, as shown in Table (1). The toxicity values varied according to the concentrations of the used formulations. Larval mortality gradually increased with the increase in concentration. After 24 hrs of exposure, SeNPs was more potent than ZnONPs and *A. fragrantissima* extract with $LC_{50}$ values 0.17, 133.77, and 134.29 ppm, respectively. It was noted that *A. fragrantissima* extract and ZnONPs were convergent in efficiency after 24 hrs of exposure, while at 48 hrs of exposure, the toxicity of ZnONPs was greatly enhanced, compared to *A. fragrantissima* with $LC_{50}$ 23.74 and 89.99 ppm, respectively. In addition, the toxicity of SeNPs increased at 48 hrs of exposure, therefore the time of exposure significantly increases the tested formulations' toxicity. The slope values of probit regression lines showed moderate homogeneity in response to tested nanoparticles and *A. fragrantissima* extract.

**Table 1.** Lethal concentration values of ZnONPs, SeNPs, and *Achillae fragrantissima* ethanolic extract against 3rd instar of *Culex pipiens* larvae 24 and 48 hrs post-treatment

| Tested compound (ppm) | **24hrs of exposure** | | | **48hrs of exposure** | | |
|---|---|---|---|---|---|---|
| | **$LC_{50}$ (Co. limits) *** | **$LC_{95}$ (Co. limits) *** | **Slop± SE**** | **$LC_{50}$ (Co. limits)*** | **$LC_{95}$ (Co. limits)*** | **Slop± SE**** |
| **ZnONPs** | 133.77 (77.34–234.25) | 1290.72 (301.56-5734.37) | 1.67± 0.13 | 23.74 (19.77-28.47) | 104.60 (75.95-145.03) | 2.55±7.61 |
| **SeNPs** | 0.17 (0.13-0.24) | 1.43 (0.58-3.70) | 1.80± $9.04X10^{-2}$ | 0.07 (0.06- 0.09) | 0.51 (0.31-0.87) | 2.00± $6.28X10^{-2}$ |
| ***A. fragrantissima*** | 134.29 (119.83–150.53) | 342.07 (257.46– 454.98) | 4.05±0.26 | 89.99 (79.29– 102.14) | 287.72 (214.51-386.58) | 3.26±0.16 |

*Co. limits coefficient limits. **SE standard error.

## 3. The reproductive potential of emerged females

The emerged females from treated larvae with the sub-lethal concentration of *A. fragrantissima* ethanolic extract, ZnONPs and SeNPs revealed that they adversely affect the female fecundity and fertility, as shown in Table (2). The fecundity was significantly decreased after treatment with SeNPs, ZnONPs and *A. fragrantissima* ethanolic extract relative to the untreated. The most considerable effect was achieved by SeNPs (105.13), followed by ZnONPs (177.25) and *A. fragrantissima* ethanolic extract (201.25). Likewise, the fertility of produced eggs was greatly affected, and the egg hatchability decreased from 97.88% for the untreated to 19.73%, 31.24%, and 49.02% for SeNPs, ZnONPs and *A. fragrantissima* ethanolic extract, respectively. Consequently, SeNPs showed high sterility index of 91.66, while 77.73 and 60.32 were recorded for ZnONPs and *A. fragrantissima* ethanolic extract, respectively.

**Table 2.** Effect of sub-lethal concentration of ZnONPs, SeNPs and *Achillea fragrantissima* ethanolic extract on fecundity, fertility and sterility index of *Culex pipiens* females.

| **Tested compounds** | **No. of eggs laid** **Mean ±SE** | **No. of hatched eggs** | | **Sterility index** |
|---|---|---|---|---|
| | | **Mean ±SE** | **%** | |
| **Untreated** | 254±0.97[a] | 248.63±0.66[a] | 97.88 | 0.0 |
| **ZnONPs** | 177.25±0.66[c] | 55.38±0.66[c] | 31.24 | 77.73 |
| **SeNPs** | 105.13± 33[d] | 20.75±0.62[d] | 19.73 | 91.66 |
| ***A. fragrantissima* ethanolic extract** | 201.25± 0.86[b] | 98.66± 36[b] | 49.02 | 60.32 |

Means with the same letters are not significantly different. Each value represents the mean of three replicates.

*SE Standard error

## 4. Biochemical studies

It was important to investigate the biochemical changes in the main body metabolites (lipids, proteins and carbohydrates) and the activity of two enzymes (ATPase and GST) of *Cx. pipiens* larvae after 48 hrs of treatment with $LC_{50}$ of ZnONPs and SeNPs. Moreover, the effects of treatments were addressed on the emerged pupae, males and females. The data are summarized in Table (3). ZnONPs significantly increased the total proteins of treated larvae relative to the untreated. In contrast, the SeNPs significantly inhibited the total proteins. These effects have extended to pupae and adult males and females. The pupal proteins were non-significantly affected by treatment with ZnONPs, while SeNPs significantly decreased the pupal protein contents. SeNPs greatly decreased the total proteins in treated males and females, with percent changes of -7.13% and -8.35%, compared to the untreated. Both ZnONPs and SeNPs considerably decreased the total lipid contents in larvae, pupae, males and females compared to the untreated ones. The total lipids contents significantly varied between ZnONPs and SeNPs treatments in treated larvae and emerged pupae and females, but non-significantly differed after treatment with ZnONPs and SeNPs in males with inhibition means of 2.55 and 2.48 (mg/ gm. b. wt). The total carbohydrate contents remarkably decreased in larvae, pupae, males and females after treatment with ZnONPs and SeNPs. SeNPs exhibited more inhibition effect than ZnONPs in larvae (-17.56%), pupae (-15.09%), males (-11.89%) and females (-13.52%). Furthermore, the tested enzymes of larvae were greatly affected by treatment with tested nanoparticle formulations, and these effects extended to the emerged pupae and adults. The ATPase activity was considerably inhibited, while the GST activity was greatly enhanced after ZnONPs and SeNPs treatments. SeNPs significantly inhibited ATPase activity in larvae (-64.78%), pupae (-52.87%), males (-30.99%) and females (-31.46%). Additionally, ZnONPs showed a significant decrease in ATPase activity relative to the untreated larvae (-19.71%), pupae (-36.30 %), males (-26.90%) and

females (-26.40%). The GST activity was greatly increased due to treatment with the ZnONPs and SeNPs. The activity of GST greatly increased in larvae (185.97%), pupae (205.94%), males (189.57%) and females (186.37%) treated with ZnONPs, compared to SeNPs and the untreated.

**Table 3.** Effect of ZnONPs and SeNPs treatments on total protein, lipid, carbohydrate, ATPase and GST of larvae, pupae, males and females of *Culex pipiens*

| Nano-particles | Total protein (µg/gm.b.wt) ± SE* | % Change | Total lipid (µg/gm.b.wt)± SE* | % Change | Total carbohydrate (µg/gm.b.wt) ± SE* | % Change | ATPase (umoles Pi/min/mg protein) ± SE* | % Change | GST (µ mole sub conjugated / min/mg protein) ± SE* | % Change |
|---|---|---|---|---|---|---|---|---|---|---|
| **Larvae** | | | | | | | | | | |
| **Control** | 5.60±0.04 [b] | - | 2.57±0.01 [a] | - | 3.53±0.01 [a] | - | 1.42±0.00 [a] | - | 2.71±0.00 [a] | - |
| **ZnONPs** | 7.22±0.02[a] | +28.9 | 2.39 ±0.02 [b] | -7.00 | 3.32±0.01 [b] | -5.94 | 1.14±0.02 [b] | -19.71 | 7.75±0.10 [b] | +185.97 |
| **SeNPs** | 4.37 ± 0.01 [c] | -21.96 | 1.96 ± 0.01 [c] | -23.74 | 2.91 ±0.00 [c] | -17.56 | 0.50±0.00 [c] | -64.78 | 5.78 ±0.10 [c] | +113.28 |
| **Pupae** | | | | | | | | | | |
| **Control** | 5.82±0.03 [a] | - | 2.67±0.01 [a] | - | 3.71±0.01 [a] | - | 1.57±0.01 [a] | - | 2.86±0.05 [a] | - |
| **ZnONPs** | 5.67±0.02 [a] | -2.57 | 2.46±0.01 [b] | -7.87 | 3.41±0.01 [b] | -8.08 | 1.00±0.05 [b] | -36.30 | 8.75±0.13 [b] | +205.94 |
| **SeNPs** | 4.80±0.15[b] | -17.52 | 2.31±0.00 [c] | -13.48 | 3.15 ±0.03 [c] | -15.09 | 0.74±0.00[c] | -52.87 | 6.15±0.03 [b] | +115.03 |
| **Males** | | | | | | | | | | |
| **Control** | 6.03±0.04 [a] | - | 2.71±0.13 [a] | - | 3.87±0.01[a] | - | 1.71±0.01[a] | - | 3.07±0.03[a] | - |
| **ZnONPs** | 5.82±0.02 [b] | -3.48 | 2.55±0.01[b] | -5.90 | 3.50±0.04[b] | -9.56 | 1.25±0.01 [b] | -26.90 | 8.89±0.01[b] | +189.57 |
| **SeNPs** | 5.60 ±0.00[c] | -7.13 | 2.48 ±0.01 [b] | -8.49 | 3.41± 0.00 [c] | -11.89 | 1.18± 0.01[c] | -30.99 | 8.8 ± 0.00 [c] | +186.64 |
| **Females** | | | | | | | | | | |
| **Control** | 6.11±0.01 [a] | - | 3.06±0.03 [a] | - | 3.92±0.00 [a] | - | 1.78±0.01 [a] | - | 3.23±0.01[a] | - |
| **ZnONPs** | 5.85±0.02 [b] | -4.25 | 2.64±0.01 [b] | -13.72 | 3.58±0.04 [b] | -8.67 | 1.31±0.01[b] | -26.40 | 9.25±0.0[b] | +186.37 |
| **SeNPs** | 5.60±0.00[c] | -8.35 | 2.24±0.01[c] | -26.80 | 3.39±0.00[c] | -13.52 | 1.22±0.01[c] | -31.46 | 8.89±0.00[c] | +175.23 |

Means with the same letters are not significantly different. Each value represents the mean of three replicates. *SE Standard error.

## 5. Ultrastructural studies using transmission electron microscopy (TEM)

Ultrastructural studies for the untreated and treated 3rd instar larvae of *Cx. pipiens* after 24 hrs of exposure to ZnONPs and SeNPs were examined.

### *5.1. Ultrastructure of untreated Culex pipiens larval midgut*

The TEM microphotograph of the cross-sectioned midgut of the untreated larvae Fig. (3a, b), showed a normal structure of the midgut, which consists of a single layer of ciliated columnar cells resting on a basement membrane and surrounded by circular and longitudinal muscle fibers. Epithelial cells are surrounded by typical cell organelles including a relatively large rounded nucleus; the nucleus is surrounded by an intact nuclear membrane, and chromatin is condensed in the centre of the nucleus. Many microvilli (mv) are observed on the surface of epithelial cells with a typical brush border; the peritrophic membrane (pm) protects the epidermal layer from the food callus (fc) Fig. (3a, b).

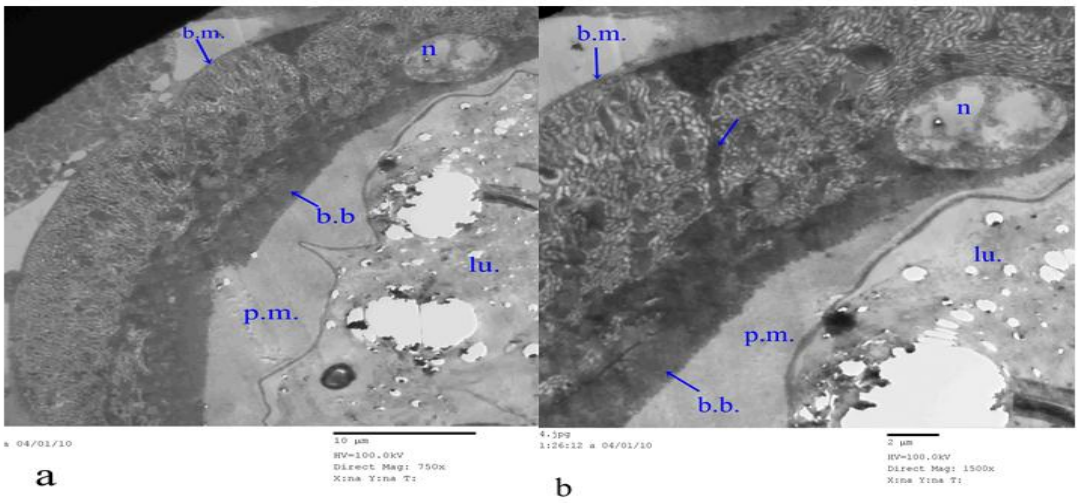


**Fig. 3.** TEM microphotograph of the cross-sectioned midgut of untreated *Cx. pipiens* larvae, showing: **(a & b)** the untreated larvae, **(a)** 750x, **(b)** 1500x. (lu.) lumen, (b.m.) basement membrane, the peritrophic membrane (p.m.), (b.b.) brush border, (n) nucleus.

### *5.2. Ultrastructure of treated Culex pipiens larval midgut with ZnONPs and SeNPs*

The TEM microphotograph of the cross-sectioned midgut of the treated 3$^{rd}$ instar larvae of *Cx. pipiens* was demonstrated in Fig. (4a, b & c). The midgut epithelial cells showed many changes including destructed epithelial cells with vacuolated cytoplasm due to the degeneration of cell organelles in the cytoplasm and scattered brush border (Fig. 4a). The midgut epithelial cells showed elongated apically located nuclei with scattered chromatin in comparison with the untreated. Swollen destructed cells, masses of cellular material appeared in the lumen. The epithelial cells were not firmly attached to the basement membrane finally, the epithelium lost its normal appearance as shown in Fig. (4b). Microvilli were disrupted and highly damaged leading to a midgut vacuolization appearance (Fig. 4c).

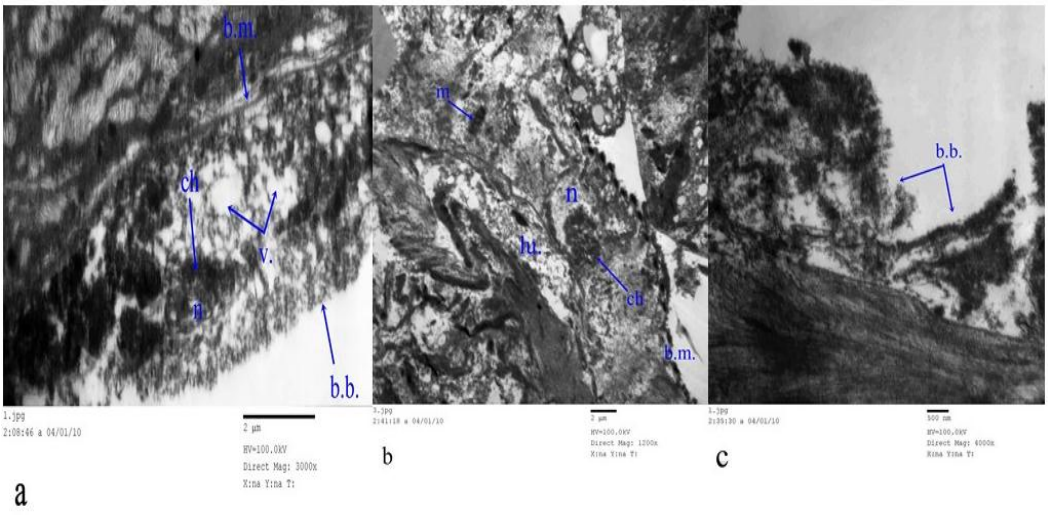


**Fig. 4.** TEM microphotograph of the cross-sectioned midgut of the larvae of *Cx. pipiens* treated with SeNPs and ZnONPs, 24 hrs post-treatment, **(a)** 3000x **(b)** 1200x **(c)** 4000x. (b.m.) basement membrane, (ch) chromatin, (b.b.) brush border, (n) nucleus (p.m.) peritrophic membrane (v), (lu.) lumen, vacuoles (m) mitochondria.

## DISCUSSION

Mosquito control is facing environmental and ecological challenges because of the hazards caused by the application of chemical insecticides. Despite the efficacy of chemical insecticides on mosquito vector populations, vectors rapidly develop resistance towards them **(Meier *et al.*, 2022)**. Other undesirable effects include toxicological effects against non-target organisms and environmental and human health concerns **(Jayaraj *et al.*, 2016)**. In contrast, plant-based insecticides represent potent alternatives to chemical insecticides due to the susceptibility of mosquitoes to different plant constituents such as sterols and terpenoids **(Demirak & Canplot, 2022)**. Therefore, using plant extracts to control mosquitoes is an environmentally safe option compared to chemical insecticides. Literature search shows that the flavonoids, terpenoids, lignans, amino acid derivatives, fatty acids and alkamides such as p-hydroxyphenethylamide IV have been identified in *Achillea* species **(Saeidnia *et al.*, 2011; Patocka & Navratilova, 2019; Alshuail *et al.*, 2022)**. The use of nanoparticles to control mosquitoes becomes increasingly popular owing to their low cost and environmental safety **(Alhag, 2023)**. Lately, botanical extracts synthesized NPs are cost-effective, and their application methods are affordable and simple **(Sowndarya *et al.*, 2017)**. It was suggested that the main toxicity mechanism against mosquito larvae is the penetration of nanoparticles through the larval integument that subsequently affects the enzymes, internal organs and organelles, and cellular function, which eventually leads to cell death **(Selvan *et al.*, 2018; Abinaya *et al.*, 2019)**. It has been found that *A. fragrantissima* has appropriate reducing and stabilizing characteristics during the preparation of ZnONPs and SeNPs. The reduction of ZnO and

Se cation into ZnO and Se has been carried out by the –OH group, which acts as an aldehyde to form ZnONPs and SeNPs and stabilize them; meanwhile, the glucosinolates have been oxidized to gluconic acid. The reduction of ZnONP and SeNP cations can be made by some organic compounds that have reductive groups such as –OH, –SH, –NH, etc.. **(Fouad *et al.*, 2021; Abdelhamid *et al.*, 2022)**. In the present study, the mean droplet size was less than 100nm, which means that it was within the nano range according to **Khan *et al.* (2019)**. DLS is a measure of the stability and uniformity of the droplet size of nanoparticles **(Caputo *et al.*, 2019)**. The low DLS of the formulated nanoparticles results in high uniformity of droplet size and provides long-term stability. The stabilization of the nanoparticles is due to the surfactant since it provides a mechanical barrier to prevent accumulation **(Heinz *et al.*, 2017)**.

In the present study, the larvicidal potency of the synthesized nanoparticles was higher than that exhibited by *A. fragrantissima* ethanolic extract. Whereas, the SeNPs showed higher toxicity than both ZnONPs and *A. fragrantissima* ethanolic extract, with $LC_{50}$ values of 0.07, 23.74, and 89.99 ppm, respectively. Similar studies demonstrated that the biosynthesized SeNPs of *Clausena dentata* showed good larvicidal efficacy against larvae of *A. aegypti, Cx. quinquefasciatus* and *A. stephensi,* compared to *Clausena dentata* **(Sowndarya *et al.*, 2017)**. Additionally, the SeNPs of *D. indica* leaf extract showed larvicidal and pupicidal activities against two mosquito species **(Krishnan *et al.*, 2020)**. Similar observations were noticed upon treating *Cx. tritaeniorhynchus* larvae with ZnONPs, based on *Cucurbita* seed extract **(Velsankar *et al.*, 2019)**. In addition, the CuONPs synthesized by using *A. fragrantissima* extract were highly efficient against *Cx. pipiens* compared to the *A. fragrantissima* extract only **(Al-Ghabban & Eldiasty, 2022)**. Overall, all previous studies reported that the efficacy of the NPs based on botanical extracts was higher than with the botanical extracts only. This might be attributed to the tiny size of nanoparticles, which makes them easier and faster to penetrate through larval tissue and reach their targets with high potency in larval mortalities **(Alhag, 2023)**. The present study showed a reduction in the number of eggs laid by females that emerged from treated larvae with SeNPs, ZnONPs and *A. fragrantissima* extract, respectively, relative to the untreated. On comparing the number of hatched eggs of untreated and treated with NPs, it was observed that SeNPs, ZnONPs, and *A. fragrantissima* ethanolic extract treatments reduced the hatched eggs and increased the sterility indices. **Benelli (2018)** reported that AgNPs are known to reduce protein synthesis and gonadotrophin release, leading to developmental and reproductive failure. Moreover, AgNPs caused changes in esterase activity, which vary the juvenile hormones and their analogs levels and cause negative effects on insect reproduction **(Yasur & Rani, 2015)**. The larvicidal efficiency of the SeNPs and ZnONPs synthesized using *A. fragrantissima* might be due to intracellular toxic effects inside cuticular and peripheral cells (**Krishnan *et al.*, 2020)**. In addition, it may cause denaturation of the phosphorous-containing compounds (DNA) or sulfur-containing proteins, resulting in the denaturation of organelles and the cell

membrane permeability, which finally leads to cell death **(Sowndarya *et al.*, 2017)**. It is worth mentioning that, nanoparticles accumulate in the gut cells and digestive tract, where the AgNPs highly accumulated in the nucleolus, rough endoplasmic reticulum and mitochondria in the guts of treated lepidopteran larvae **(Yasur & Rani, 2015)**. Furthermore, the larvicidal potency against *Cx. pipiens* larvae occurring by SeNPs and ZnONPs might be due to the accumulation and severe alterations in the alimentary canal as tissue layers rupturing, cortex region and epithelial cells structure, which was detected by ultrastructure examination. The insect midgut is the main site of absorption which acts an important role in metabolic activity. Remarkably, nanoparticles also may be deposited in the siphon opening and cause larval suffocation. **(Velsankar *et al.*, 2019)**. Interestingly, similar works have reported that the histopathological studies of botanical extracts-based nanoparticles treated larvae detected the presence of deformities in epithelial cells and tissue structures of the mid-gut **(Banumathi *et al.*, 2017; Ibrahim *et al.*, 2022)**.

It was noted that, not only the histological and biological alterations were detected but also the levels of metabolites and activity of detoxification enzymes were altered in treated larvae and all emerged stages. The production of oxygen-free radicals is a common consequence of nanoparticle interactions with intercellular components **(Alhag, 2023)**. In addition, NPs cause toxic effects by interfering with normal biochemical reactions of the cell **(Yasur and Rani, 2015),** leading to enzymes denaturation and inhibition of enzymes activities that reduce ATP synthesis in all tested stages **(Sowndarya *et al.*, 2017)**. In addition, it has been shown that the increased activity of insect detoxification enzymes is associated with the increased detoxification of the used insecticides; these findings agree with the obtained results of GST activity. Increased GST activity was detected in insects from metal-contaminated habitats **(Yasur & Rani, 2015)**. Overall, the activity of enzymes varies depending on treatment concentration and nanoparticle accumulation in the insect through its life cycle. Furthermore, the metabolites of treated larvae with ZnONPs and SeNPS and all emerged stages were significantly inhibited. In contrast, the total protein of treated larvae with ZnONPs was greatly elevated. This was interpreted as nanoparticles affect gene expression and induce immune responses leading to variations in carbohydrate, protein and lipid metabolism associated with cellular toxicity that influences the reproduction and development of the insect. Moreover, nanoparticles cause altered biochemical activity, production of reactive oxygen species, and disruption of nutrient intake resulting in the failure of insects to reproduce and grow normally **(Shahzad & Manzoor, 2021)**. Carbohydrates are responsible for the normal functioning of the female and male reproductive systems and the development of the embryo. In males, sugars are the major constituent of the reproductive glands and testes. In the female's system, carbohydrates are important for vitellogenesis and the formation of glycosaminoglycan in the membrane and chorion. The vitellogenesis process involves the accumulation of protein, carbohydrate and lipid yolk

in the oocyte of the embryo to meet its metabolic and structural needs (**Osman *et al.*, 2015)**. In general, an obvious significant reduction in protein levels was observed in the treated larvae. The drop in protein contents of the treated samples might be due to protein binding with foreign compounds such as tested nanoparticles or might be due to protein converted into simple amino acids to release energy **(Derbalah *et al.*, 2014)**.

## CONCLUSION

From the present study, based on $LC_{50}$ values, the ZnONPs and SeNPs showed higher toxicity against *Cx. pipiens* larvae than *A. fragrantissima* ethanolic extract. In addition, it was noticed that, SeNPs, ZnONPs and *A. fragrantissima* ethanolic extract treatments reduced the number of eggs laid by females that emerged from treated larvae, decreased the hatched eggs and increased the sterility indices. The tested nanoparticles showed different ultrastructural deformities in the larval midgut cells. Besides, biochemical alterations were detected in all tested stages of *Cx. pipiens*. Consequently, it was concluded that, the ZnONPs and SeNPs synthesized via green approaches using *A. fragrantissima* ethanolic extract showed strong efficacy against *Cx. pipiens* larvae thus they can contribute to the integrated management programs.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Ethical statement**

All experiments in this research were approved by the Ethics Committee of the Faculty of Science, Ain Shams University, Cairo, Egypt (Approval code: ASU-SCI/ENTO/ 2023/3/2).

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