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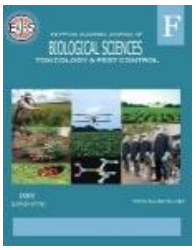

Efficacy of Methanol Leaf Extract, Biosynthesized Silver and Chitosan Nanoparticles Using *Nerium oleander* against *Musca domestica*

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ABSTRACT

Plant-mediated synthesis of nanomaterials has attracted considerable attention in recent years due to its cost-effectiveness and eco-friendly nature. In the present study silver and chitosan nanoparticles were successfully synthesized using *Nerium oleander* leaf extract as a bio-reducing agent. Results recorded from UV–vis spectrum, HRTEM and FTIR supported the biosynthesis and characterization of silver and chitosan nanoparticles. From HRTEM analysis, the AgNPs have spherical shape morphology with an average size of about 4-32 nm. CsNPs were ranged between 34-65 nm and the shape seem spherical with dark parts confirmed the capsulated plant extract. Larvicidal activity of leaf extract of *N. oleander* and synthesized silver and chitosan nanoparticles was carried out against *M. domestica*. CsNPs showed the utmost toxicity against *M. domestica* after 48h exposure with LC_{50} value of 0.64 ppm and LC_{50} values of leaf extract and AgNPs were 73.024 and 2.18 ppm respectively. A significant prolongation in the larval duration was observed in treated larvae. All the tested compounds induced reduction in the pupation rate and adult emergence and caused noticeable larval, pupal, and adult abnormalities. The potency in killing *M. domestica* larvae and stability of *N. oleander*-CsNPs have made this product a good candidate for the development of a novel natural larvicide.

INTRODUCTION

The house fly *Musca domestica*, is not only a nuisance insect but also can transmit many diseases. Chemical insecticides are commonly used for the management of house fly populations. The environmental harmful impacts and the huge cost of chemical insecticides encouraged entomologists to search for an eco-friendly and cheaper alternative way (Abd El-Hamid *et al.,* 2018). Procedures of environmentally benign nanoparticles do not use any toxic chemicals in the protocol of synthesis but use synthetic methods based on naturally occurring bio-materials which provide an alternative means for obtaining these nanoparticles. Over the environmentally benign biological processes, the use of plant extracts for the synthesis of nanoparticles could be advantageous (Subbaiya *et al.*, 2014; Gul *et al.*, 2016; Ga'al *et al.*, 2018; Nalini *et al.*, 2019 and Pilaquinga *et al.*, 2019). Recently silver nanoparticles are attracting considerable attention because of their widespread applications (Morej'on *et al.*, 2018). Chitosan is a biodegradable, biocompatible, and non-toxic polycationic polymer with low

immunogenicity. The unique Physico-chemical properties of chitosan nanoparticles are their small particle size, high surface area, and compactness (Anand *et al.,* 2018). Roni *et al.,* (2013) suggest that the use of *Nerium oleander* to synthesize AgNPs is an environmentally safer and greener approach for mosquito control. Therefore, the present study has been devoted to evaluating the synthesized silver and chitosan nanoparticles using *Nerium oleander* as larvicidal agents against the housefly *Musca domestica*.

MATERIALS AND METHODS

***Musca domestica* Rearing:**

The initial culture of *Musca domestic* used in the present investigation was obtained from a susceptible strain colony in the laboratory of the medical insect research center, Dokki, Giza. The methodology of Elkattan et al. (2011) was used for rearing the larvae of house fly in Entomology Laboratory, Faculty of Science, Benha University.

Plant Materials and Chemicals:

Fresh matured leaves of *Nerium oleander* collected from a plant nursery in Benha city, Qalyobia Governorate. The leaves were thoroughly washed with distilled water, shade dried for a period of 4 days, blended and made into a fine coarse powder. Silver nitrate (AgNO3), glacial acetic acid, and Tri-polyphosphate (TPP) were purchased from Sigma-Aldrich Company. Chitosan (low molecular weight) purchased from Merck Company. Deionized water was obtained from the National Research Centre, and the technique was made in Organic Microanalysis Unit, National Research Centre, Dokki, Giza, Egypt.

Preparation of Plant Extract:

A plant leaf extract was prepared by mixing 100 g of powder with 300 ml of methanol 70% in one liter dark-colored flask using a magnetic stirrer for 48 hours, then the extract is filtered with (Whatman No. 1) filter paper. The residue is removed and pure leaf extract is obtained. the solvent was evaporated in a Rotary Evaporator (Labo-Rota C11) in a water bath adjusted at 40 ºC for 2-3 hours, then the obtained crude extract was weighed and stored at -4 ºC till used (Fartyal and Kumar, 2014).

Synthesis of Silver Nanoparticles (AgNPs) of *N. oleander*:

AgNPs prepared by mixing 10 ml filtrate (leaf extract) with 90 ml of 1mM aqueous solution of $AgNO_3$ and incubated at room temperature overnight. Dark brown coloration of the solution indicates the formation of silver nanoparticles (Fatima Jafer et al., 2018).

Characterization of AgNPs:

The synthesized AgNPs were monitored by UV-Vis spectroscopy, using UV-Shimadzu spectrophotometer at a wavelength of 200–700 nm. High-Resolution Transmission Electron Microscopy (HRTEM) JEOL (JEM-2100 TEM) was used to visualize the morphology and the size of the synthesized Ag NPs.

Synthesis of Chitosan Nanoparticles (CsNPs) of *N. oleander*:

10 ml filtrate (leaf extract) was diluted in 80 ml deionized water, the solution was sonicated for about one hour. Chitosan solution was prepared by dissolving 2.5 g chitosan in 10 ml (1%) glacial acetic acid using a magnetic stirrer until the solution was transparent. Once dissolved, the chitosan solution was added to the mixture. The mixture was stirred until the solution becomes clear. TPP was added drop by drop to the mixture with continuous stirring at 60°C till the chitosan nanoparticles precipitated (Othman *et al.,* 2018). The initiation of the ionic gelatin mechanism induced by TPP indicated the formation of CsNPs. The capsulated plant extract CsNPs were centrifuged and dry froze to have capsulated CsNPs powder.

Characterization of CsNPs:

UV- Shimadzu spectrophotometer has been used to follow the formation of Nerium capsulated CsNPs and the UV-Vis spectra were recorded between 100-800 nm. Shape and size of CsNPs were practically obtained using (HRTEM), Specimens for TEM measurements were prepared by placing a drop of colloidal solution on 400 mesh Carbon coated Copper grid and evaporating the solvent in the air at room temperature. Fourier transforms infrared (FTIR) measurements were investigated using (8300 FT-IR Shimadzu Spectrophotometer) in the range from 4000 cm-1 to 400 cm-1.

Larval Bioassays:

Larvicidal activities of methanol leaf extract biosynthesized silver, and chitosan nanoparticles of *N. oleander* were evaluated according to Wright (1971) as standard methods. Tests were done by exposing late second instar larvae of *M. domestica* to food contaminated with the tested materials. The methanol leaf extract was tested at 10, 20, 40, 80, and 160 ppm, while AgNPs and CsNPs were tested at 1, 2, 4, 8,16 ppm concentrations. Twenty-five larvae were put in a 250 ml glass beaker containing 10 g of the bait prepared by mixing 10 g artificial diet with 9 ml water, and 1 ml of the prepared concentration of each compound was added. The beakers were wrapped with muslin cloth with the help of a rubber band. The control group reared on media only supplied with water. Three replicates were used and all experiments were carried out at laboratory conditions (27±2°C and 70±5% relative humidity). The larval mortality was recorded at 48h post-treatment and the lethal concentrations (LC_{25} and LC_{50}) were calculated.

Biological Measurements:

To test the effects of the used compounds on some biological aspects. 100 eggs were removed from the stock colony and placed in larval media treated with two different concentrations corresponding to LC_{25} and LC_{50} of each tested compound. Control experiments were done without any treatment. All experiments were repeated three times. The time between egg hatching and pupation was averaged to get larval duration. Survived larvae were daily examined to estimate the pupation rate and successfully emerged adults. Morphological abnormalities of the developmental stages were recorded and photographed.

Statistical Analysis:

Probit analysis was performed to calculate the LC_{50} values using SPSS software package version 20 Program. The values for probit equations (y), chi-square, LC_{50}, and 95% confidence limits (lower and upper) were obtained for tested concentrations after 48h. Also, the data were subjected to analysis of variance and compared with One Way analysis of variance (ANOVA), Finney (1971).

RESULTS AND DISCUSSION

Characterization of Biosynthesized Silver Nanoparticles:

The fresh extract of *N. oleander* was characterized by its green color. Transformation of the color to dark brown (fig.1a) occurred after the addition of $AgNO_3$ solution to the plant extract indicates the formation of AgNPs due to the reduction of Ag ions by the ingredients present in the leaves of *N. oleander* extract.

The UV–Vis spectrum of the synthesized silver nanoparticles is shown in figure (1b). The maximum absorbance was found at a wavelength of 430 nm, due to the excitation of surface plasmon resonance (SPR) of the synthesized AgNPs (Ga'al *et al.*, 2018); suggesting that, the nanoparticles have been formed and dispersed in the aqueous solution with no aggregation formation. Similar findings were reported by (Roni *et al.*, 2013; Subbaiya *et al.*, 2014; Pal *et al.*, 2018 and Sutthanont *et al.*, 2019).

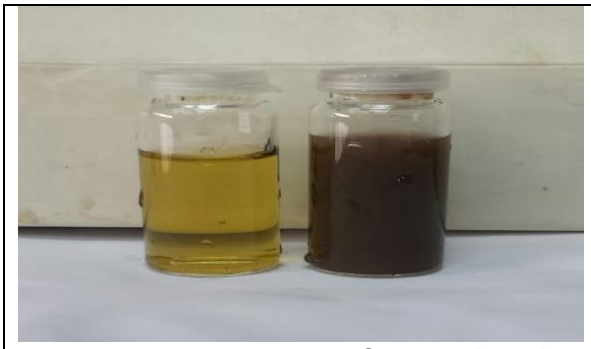


Fig.(1a) Preparation of *N. oleander*-AgNPs

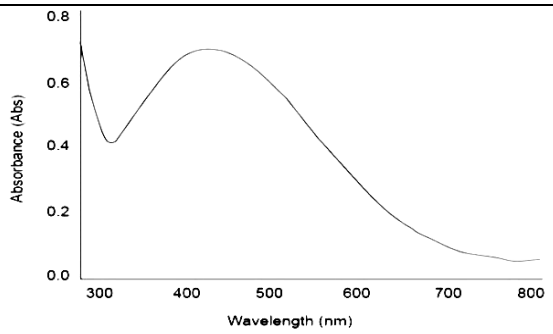


Fig. (1b) UV-Vis spectroscopy of *N. oleander*-AgNPs

TEM analysis was carried out in order to investigate the morphology and size of the AgNPs figure (2). The synthesized silver nanoparticles obtained using this green method have spherical shape morphology with an average size of about 4-32 nm, with the appearance of some clusters. The average particle size was measured using Image J program and it showed that the majority of the particle size around 6 nm and this is a good resemblance with the shape of SPR band observed in the UV–Vis spectra. (Ga'al *et al.,* 2018 and Shehata and Mahmoud, 2019).

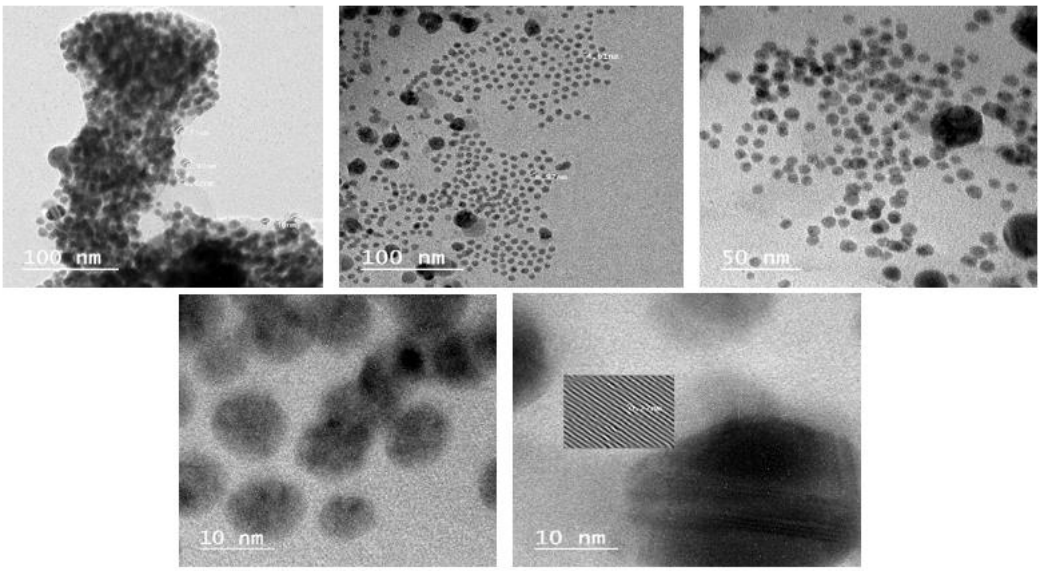


Fig. 2: TEM image of biosynthesized AgNPs of *N. oleander*

Characterization of Biosynthesized Chitosan Nanoparticles:

After the addition of chitosan to a solution containing plant extract with stirring at 60 ºC and TPP was added dropwise to capsulate the plant extract, the precipitation is the indication of nanoparticles formation (Hosseini *et al.,* 2013, Asiri *et al.,* 2018 and Othman *et al.,* 2018). The synthesis of CsNPs was confirmed by UV-visible spectral analysis. UV-Vis absorption spectrum of CsNPs is shown in figure (3). According to the surface plasmon resonance peaks in absorption spectra, CsNPs colloidal solution shows an absorption peak at wavelength 330 nm; suggesting that the nanoparticles have been formed and have been dispersed in the aqueous solution with no aggregation formation (Divya and Jisha, 2018).

FTIR analysis was used to verify that, the nanoparticles were indeed coated with

the plant extract used for their synthesis. This is important because plant extract does not only act as a reducing agent but also its organic fractions overlay the nanoparticles (Morej´on *et al.*, 2018). FTIR spectra of *N. oleander*-CsNPs are shown in figure (4). In general, chitosan powder shows characteristic peaks at 3433 (-OH and $-NH_2$ stretching), 2920 (-CH stretching), 1647 (amide I), 1088 (C-O-C stretching) and 591 cm-1 (pyranoside ring stretching vibration) (Murugan *et al.*, 2017). For CsNPs (fig.4-S) the peak of amide I ($-NH_2$ bending) shifted from 1647 to 1685 cm^{-1}, and new peaks appeared at 1338 (C-O-C stretch) and 1560 cm^{-1} (amide II), implying the complex formation via electrostatic interaction between NH_3+ groups of chitosan and phosphoric groups of TPP within the nanoparticles (Yoksan *et al.*, 2010). The addition of *N. oleander* extract (fig.4-N) resulted in a remarkable decrease in the intensity of ($-NH_2$) and (-CH) stretching peaks 3449 and 2922 cm^{-1}, indicating that, an increase in the hydrogen bonds may be formed between the amino group of the CsNPs and the hydroxylic groups come from the plant extract. These results indicated that the plant extract was encapsulated into the chitosan nanoparticles.

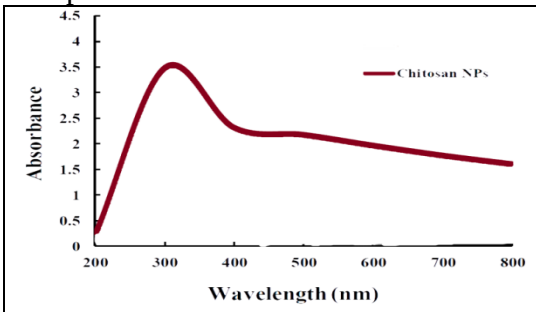


Fig. 3: UV-Vis spectroscopy of CsNPs

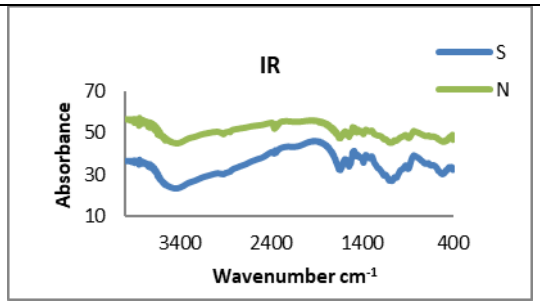
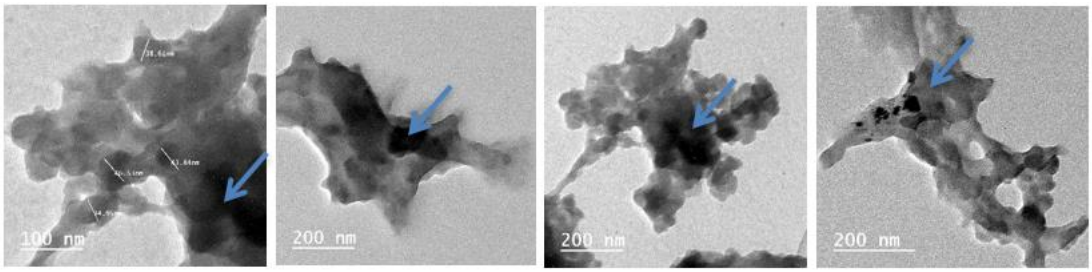


Fig. 4: FTIR spectra S; CsNPs, N; *N. oleander*-CsNPs

The morphology of biosynthesized CsNPs characterized by TEM, and the images are shown in figure (5). The capsulated plant extract can be confirmed as dark parts. CsNPs exhibited spherical shape morphology with an average size of about 34-65 nm and no aggregations were formed (Yang *et al.*, 2018).



Arrow refers to *N. oleander* extract

Fig.5: TEM image of biosynthesized CsNPs of *N. oleander*

Larvicidal Activity of Tested Materials Against 2nd instar Larvae of *M. domestica*:

The insecticidal activity of methanol leaf extract biosynthesized silver and chitosan nanoparticles of *N. oleander* were evaluated against 2nd instar larvae of *M. domestica* and the results are given in table (1). The mortality percentage was directly proportional to the concentration. The larval mortality was 16.66% at 10 ppm concentration, while at high concentration (160 ppm), it was increased to 78.33%. The 95% confidence limits were 59.73-89.33. The chi-square value was significant at ($p<0.05$) level. The control showed no mortality in all assays. The larvicidal activity of *N. oleander* leaf extract could be explained by the action of phytochemical components

that are involved in plant self-protection including sterols with an average rate of 27.57%, phenols with an average rate of 23.67%, hydrocarbons (20.531%) and phenyl propanoids (11.49%) as indicated from gas chromatography-mass spectrometry (GC-MS). Kazim (2013) reported that the efficacy of aqueous extracts of *N. oleander* was significantly higher than those of the *Sidr Ziziphus* and *Elias Myrthus* against the larval stage of the housefly. Similarly, various concentrations of aqueous extract of *N. oleander* are effective against adult houseflies, *M. domestica*. The maximum mortality (83%) was observed at the highest concentration (100ppm) (Mohammed, 2018). Some researchers have determined the larvicidal activity of *N. oleander* extracts of various plant parts against other insects (Behravan *et al.,* 2017; Raveen *et al.,* 2017 and Semiz, 2017).

The housefly larvicidal potency of *N. oleander* is much improved in combination with AgNPs. The LC_{50} value was 2.18 ppm in larvae treated with *N. oleander*-AgNPs which is an over 33-fold increase in the toxicity than the crude leaf extract (LC_{50}= 73.024 ppm) against *M. domestica* larvae. The high larvicidal efficacy of synthesized AgNPs using plant extracts may be due to, its ability in exoskeleton permeation, then penetration into insect cells, where they restrict macromolecules like DNA and proteins, changing their structure, therefore their function and finally causes the loss of cellular function & cell death (Benelli, 2016). Results confirm the previous report by Roni *et al.* (2013) who demonstrated that the synthesized *N. oleander*-AgNPs are effective against *A. stephensi* than the aqueous extract, Gul *et al.* (2016) who observed that, the synthesized AgNPs of melon exhibited improved activities as compared to melon leaves extract against *M. domestica* adults and Abd El-Hamid *et al.* (2018) found that, the chemically synthesized AgNPs have toxicity on third instar larvae of *M. domestica* in the two methods of treatment (dipping and feeding). Additionally, several authors recorded the highest mortality of biosynthesized silver nanoparticles compared to crude extracts of different plants against many mosquito species (Morej´on *et al.,* 2018; Shehata and Mahmoud, 2019 and Sutthanont *et al.,* 2019).

Table 1: Larvicidal activity of leaf extract, synthesized silver and chitosan nanoparticles of *N. oleander* against *M. domestica* after 48h of treatment

Compound	Conc.	(ppm)					Slope	LC_{25}	LC_{50}	95% Confidence Limit		X^2 (*df*=4)
		10	20	40	80	160				LFL	UFL	
Leaf extract	M% ±SD	16.66 ±0.09	23.33 ±0.10	33.33 ±1.10	51.66 ±0.64	78.33 ±0.75	1.17 ±0.09	19.31	73.02	59.73	89.33	5.21
	Conc.	**1**	**2**	**4**	**8**	**16**						
AgNPs	M% ±SD	25.00 ±1.0	43.33 ±1.5	61.66 ±1.4	76.16 ±0.8	86.36 ±1.2	1.16 ±0.10	1.40	2.18	1.92	3.43	3.31
CsNPs	M% ±SD	65.00 ±0.8	76.66 ±1.2	80.00 ±1.9	83.33 ±2.0	100.0 ±1.9	1.09 ±0.09	0.155	0.64	0.513	0.799	6.90

LCL: lower confidence limit; UCL: upper confidence limit; X^2: Chi-square value; *df* : degrees of freedom; Significant at $p < 0.05$ level; M%: mortality %; SD: standard deviation

The most interesting aspect in the present study is the large potentiation in the lethal effect when *N. oleander* leaf extract was used to synthesize CsNPs, reaching an LC_{50} of 0.64 ppm. The toxicity of *N. oleander*-CsNPs is at least 114- fold higher than that of the methanol leaf extract alone, and three-fold higher than that of *N. oleander*-AgNPs as evidenced by the difference in LC_{50} values. The higher mortality rates at lower concentrations of chitosan nanoparticles were similar to the previous reports of silver nanoparticles, from this observation, the biosynthesized CsNPs may have a similar effect on the housefly larvae. Results are consistent with earlier reports of Murugan *et al.* (2017) who reported that chitosan was toxic against *Anopheles sundaicus* larvae even at

low concentrations, while Chitosan nanoparticles were highly toxic, Rajesh and Mahadik (2017) observed that chitosan NPs started mortality at lower concentrations than silver nanoparticles against *Culex pipens*, Anand *et al.* (2018) reported that the raw chitosan have a negligible larvicidal effect against *A. aegypti* when compared to their corresponding nanoparticles, Dhandapani *et al.* (2019) found that, Cs-TPP nanoparticles are better than Cs nanoparticles to mosquito control and Ninan *et al.* (2019) concluded that neem oil-loaded chitosan/alginate/gelatin capsules have potential larvicidal activity against *C. quinquefasciatus*.

Effect of The Tested Materials on Some Biological Aspects of *M. domestica*:

The effects of the tested compounds on the development of *M. domestica* are shown in table (2). The larval duration of the control was 8.6±0.23 days. A significant prolongation in the larval duration was observed in treated larvae. The longest larval duration (11.8 ± 0.20 days) was observed in larvae treated with LC_{50} of AgNPs followed by (11.6 ± 0.12 days) in larvae treated with LC_{50} of CsNPs. On the other hand, there was an insignificant effect on the larval duration after treatment with LC_{25} of methanol leaf extract as compared with the control. Such result was in agreement with those obtained by Ahmad *et al.* (2015) who reported that, the extracts of ginger, tobacco, neem, and basil significantly influenced the life history of the house fly, and Attaullah *et al.* (2019) tested some weed extracts against *M. domestica* and found good effects.

Table 2: Effect of the tested compounds on some biological aspects of *M. domestica*

Compounds		Larval duration (days) (Mean+SD)	Change %	Pupation rate	% inhibition in pupation	% Adult emergence	% inhibition in Adult emergence
Control		8.6±0.23	-	100	-	100	-
Methano l extract	LC_{25}	9.3±0.06^{NS}	8.14	93.33±9.1 ^{NS}	6.67	84.0±7.1*	16.00
	LC_{50}	9.5±0.12*	10.47	83.33±7.5*	16.67	75.0±4.2**	25.00
AgNPs	LC_{25}	10.6±0.21**	23.26	83.33±5.8*	16.67	60±6.1**	40.00
	LC_{50}	11.8±0.20**	37.21	63.33±5.5**	36.67	31.58±3.4**	68.42
CsNPs	LC_{25}	10.3±0.40**	19.77	84.67±9.4*	15.33	82.31±8.4*	17.69
	LC_{50}	11.6±0.12**	34.88	73.33±6.7**	26.67	40.91±5.3**	59.09

** = Significant at 1% level, * = Significant at 5% level, NS = Non-Significant

All the tested compounds induced reduction in the pupation rate and percent of adults emerged from treated larvae. The inhibition of adult emergence reached 25.00, 68.42, and 59.09 % in insects treated with LC_{50} of crude extract, AgNPs and CsNPs, respectively. The pupation rate and the percentage of adult emergence were decreased as the concentrations increased. The disturbing effects on insect development including prolongation of larval duration and inhibition of molting may be due to, the interference of the active metabolites of the plant with bio-formation of the ecdysone hormone which affects cytochrome- P450 involved in the control of molting process in insects. Results in agreement with those obtained by Bobi *et al.* (2017) who observed that development of *M. domestica* larvae to pupae decreased with increasing the concentration of the selected plant extracts. A similar observation was also reported, reduction of the percentage of pupation and adult emergence by 33% and 75% respectively, after treatment of 3rd larval instar of *M domestica* with chemically synthesized AgNPs (Abd El-Hamid *et al.*, 2018),

and Kamel *et al.* (2019) revealed a significant prolongation in larval and pupal duration, in *M. domestica* treated with *Mentha piperita* and *Moringa oleifera* extracts. A significant reduction in pupation percent and adult emergence.

Morphological abnormalities:

Distinct morphological abnormalities were recorded in treated insects. Some deformed larvae were compressed and shrink, pigmented larvae, and larval-pupal intermediates which had parts of pupal cuticle with persisting last larval skin in their anterior end (figure,6). Some of the treated larvae were able to pupate, however, the resulting puparia of some individuals was deformed such as small-sized pupae and elongated pupae and pupal-adult intermediates (figure,7). Most pupae failed to metamorphosed completely to adults and remained concealed in the puparia however, some emerged adults had various deformations such as severely crumpled wings and legs (figure,8). Abnormalities may be attributed to the metamorphosis inhibiting effect of used compounds as a result of the disturbance of hormonal control.El Kattan *et al.* (2011) recorded various morphological abnormalities in all stages of *M. domestica* treated with *L. camara*, *P. zonale*, *C. macrocarpa,* and *A. nilotica*. Similarly, El Sherbini and HanyKamel (2015) reported developmental abnormalities in *M. domestica* larvae and pupae after treatment with *Fortunella crassifolia* extract, and Abdel Razik (2017) who found that distinct malformations of larvae, pupae, and adult of *M. domestica* were induced after treatment of 2nd instar larvae with some plant extracts. Moreover, Abd El-Hamid *et al.* (2018) reported the same findings on treatment with chemically synthesized AgNPs.

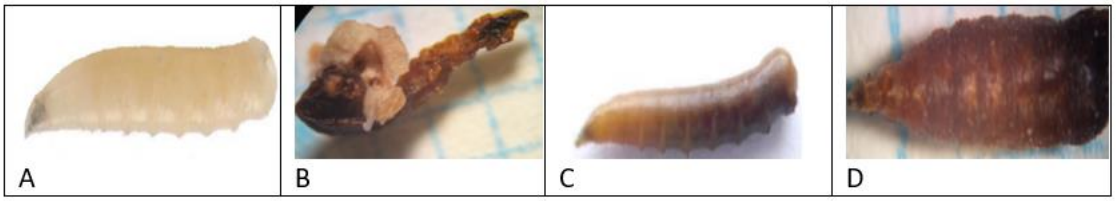


Fig. 6. (a) control larva (b) compressed and shrink larva (c) darkened larva (d) larval-pupal intermediate

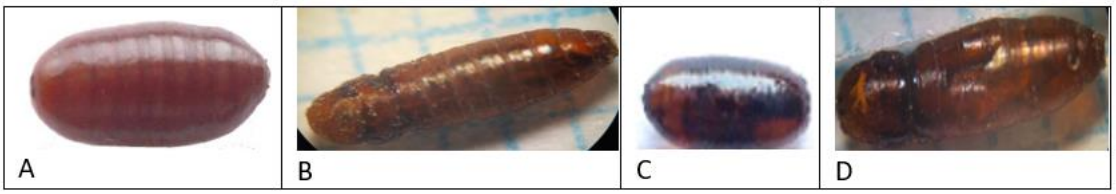


Fig. 7. (a) control pupa (b) elongated pupae, (C) small-sized pupa, (D) pupal-adult intermediate

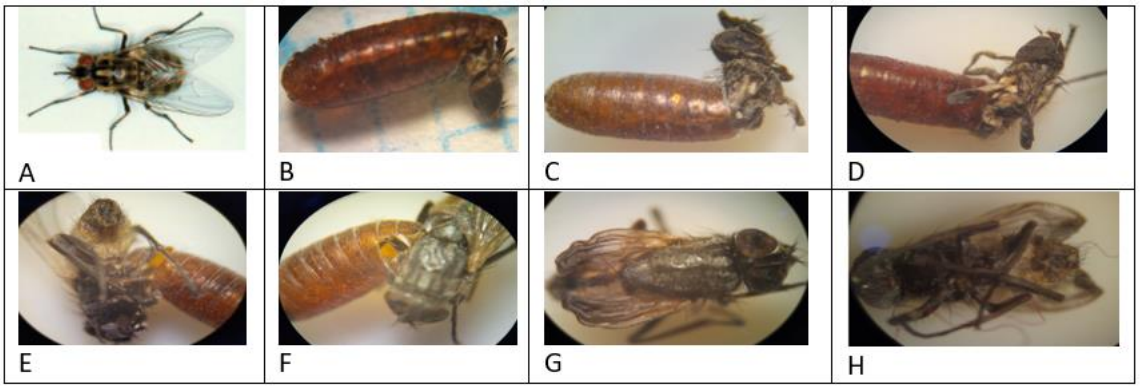


Fig. 8. (a) control adult (b-f) incomplete adult eclosion (g,h) adult with crumpled wings and broken legs

CONCLUSION

This study is the first report on the successful synthesis of chitosan nanoparticles using *N. oleander* as a reducing and capping agent. Our study showed a simple and cost-effective method for the synthesis of AgNPs and CsNPs. The biosynthesized silver and chitosan nanoparticles augmented the larvicidal activity of *N. oleander* against *M. domestica* larvae by 33 and 114 times than crude extract respectively. It could be concluded that the rapid biological synthesis of silver and chitosan nanoparticles using *N. oleander* leaf extract would be an effective potential alternative green larvicide for the control of house fly at the developmental stages with an eco-friendly approach. Further study is needed to identify the mechanisms by which nanoparticles exert their toxic effect on their intended target species and the cytotoxic effects on the non-target species.

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