Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2022; 10(5): 284-292

Received: 18-07-2022
Accepted: 20-08-2022

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Green silver nanoparticles against *Helicoverpa armigera* and its effects on biochemical, morphological and histological aspects

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DOI: https://doi.org/10.22271/j.ento.2022.v10.i5d.9072

Abstract
The aim of this study was to evaluate the effect of green synthesized silver nanoparticles using aqueous leaf extract of *Ricinus communis* (castor plant) on the life cycle *Helicoverpa armigera.* The synthesized AgNPs were identified using different microscopic techniques: UV-Visible spectroscopy, Scanning electron microscopy (SEM), Energy-disruptive X-ray spectroscopy (EDX) and Transmission electron microscopy (TEM). Insecticidal activity of AgNPs was studied using leaf dipping method. The biochemical studies were analyzed at LC_{50} of AgNPs using different methods (total carbohydrate using phenol-sulphuric acid reaction of Dubois method, total protein using Bradford method, total lipid using sulfo-phospho vanillin of Knight method and non-specific esterases using Van-Asperan method, respectively). UV-vis spectrophotometer confirmed the spectra at 445 nm of AgNPs. Spherical shaped AgNPs of 8.96 nm size were confirmed by TEM and SEM. EDX spectra confirm the chemical composition AgNPs. Higher mortality was observed in 6th instar larvae (70-80% at LC_{50} 175 ppm) than the other instars. The pupa and adult of *H. armigera* also exhibited toxicity against the AgNPs. Effect of AgNPs treatment was investigated on the midgut of 5th instar larva of *H. armigera* using the transmission electron microscope. Accumulation of AgNPs in cell organelles was confirmed by ultra structural studies of insect midgut cell using TEM. The biochemical components of 5th instar larvae showed that, the amount of total carbohydrate, total protein, total lipid and non-specific esterases were significantly decreased After 24 h treatment at LC_{50} of AgNPs. Now, we can summarise that, synthesis of AgNPs using *R. communis* is a green route to control pest population. The synthesized nanoparticles could be used for the improvement of new botanical nano-insecticide or nano-formulation after successful field trials.

Keywords: *Ricinus communis*, silver nanoparticles, *Helicoverpa armigera*, insecticidal activity, biochemical, morphological, histological analysis

Introduction
Helicoverpa armigera is a most polyphagous and cosmopolitan pest species. It is also known as cotton bollworm, corn earworm or old world bollworm. [1]. This insect damage the agriculture crops. In India 80% of population depends on agriculture and Indian economy is largely determined by agricultural productivity. In India, this insect occurs as a major pest in many economically important crops [2].

In past, chemical pesticides used for pest control. Babariya *et al.* [3] control the gram pod borer, *H. armigera* by chemically. The biological and chemical insecticides have been evaluated for their efficacy to control the *T. absoluta* and *H. armigera* on tomato plant [4]. The percentage mortality of *H. armigera* of chemically synthesized Insecticides have been tested [5]. However, haphazard use of chemical pesticide showed resistance by pest (insect, weeds etc.). There is vital require to develop new insecticides which are more environmental safe and target specific due to resistance and high cost of organic insecticides.

Plant extract can be used to develop eco-friendly pesticides to manage pest population. The plant extracts has been evaluated as insecticides against the *H. armigera* [6-12].

Presently, plant synthesized nanoparticles plays a major role in pest management [13-15]. The leaf extract of *E. hitra* and *A. indica* synthesized silver nanoparticles was tested against *H. Armigera, S. litura* and *A. janata* [16-18].

In this study, the effect of green synthesized silver nanoparticles using aqueous leaf extract of *Ricinus communis* (castor plant) was evaluated on the life cycle *Helicoverpa armigera.*

Further, the effect on morphology, histopathology and biochemistry was also evaluated. It is green route of synthesis of AgNPs using *R. communis* to control the *H. armigera*. The synthesized nanoparticles could be development as new botanical nano-insecticide or nano-formulation after successful field trials for reduces crop damage as well as pest population.

Materials and Methods

Leaf Collection and extract preparation

The fresh, healthy and green leaves of the castor plant (*Ricinus communis*) were collected around the area of Yamuna bank, Delhi, India. The leaf extract was prepared by using the described method [19]. Leaves were thoroughly rinsed with tap water followed by distilled water to remove dust and other particles. The rinsed leaves were then air dried for 1-2 h. Then approximately 25 g leaves were cut into fine pieces and put into a 250 mL conical flask containing 100 mL distilled and shake for 1 h in an orbital flask shaker. After 1 h, the extract was filtered through the whatman-1 filter paper and store for the experiment.

AgNPs synthesis

For the synthesis of AgNPs, 30 mL leaves extract was added to 70 mL 1 mM silver nitrate ($AgNO_3$) aqueous solution and kept at room temperature in the incubator. After 24 h, a change in color was observed from light yellow to dark brown color, indicating the formation of AgNPs (Fig. 1A). The AgNPs were further used for characterization using the different techniques.

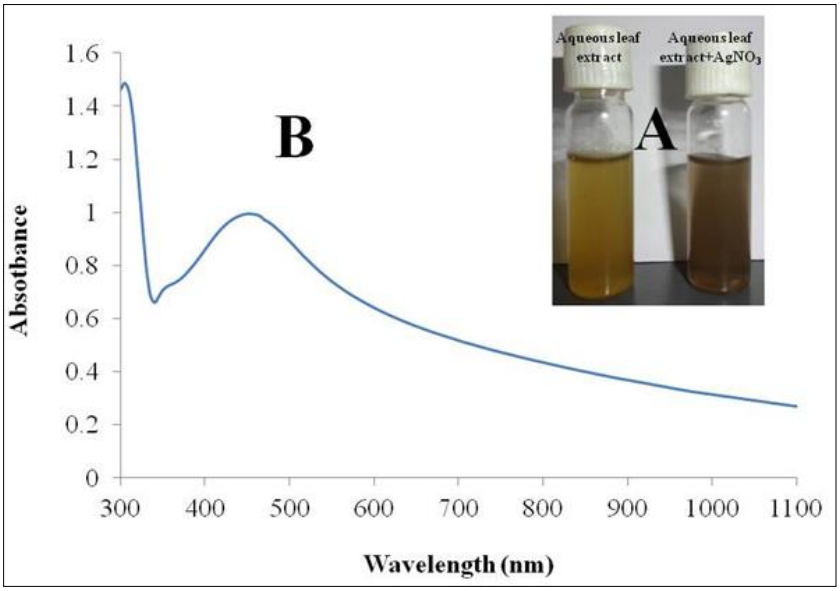

Fig 1: (A) Aqueous leaf extract of *R. communis* without $AgNO_3$ and with 1 mM $AgNO_3$ solution. (B) UV-Visible spectra of synthesized AgNPs.

AgNPs characterization

The presence of AgNPs in the resultant colloidal solution was established through UV-Vis spectroscopy. The production of AgNPs was initially confirmed by the UV-Vis spectrum of the resultant colored colloidal solution. The spectrum was recorded using HALO DB- 20 at the wavelength of 300-1100 nm, 10 nm resolution. After reduction, AgNPs were precipitated at the bottom of the conical flask. This precipitate was centrifuged at 5,000 rpm for 10 min and washed out twice with distilled water and then converted in powder. For electron microscopic studies, the samples of AgNPs were prepared by placing a drop of reaction mixture on a carbon coated copper TEM grid and allowing the water to evaporate. The micrographs of the AgNPs were obtained using Tecnai G^2 transmission electron microscope and scanning electron microscope. Elemental analysis on single particle was carried out by EDX analysis.

Insect rearing

The larvae of *H. armigera* were collected from the Biotechnology Laboratory, Indian Agriculture Research Institute, New Delhi, India. The larvae of *H. armigera* were reared singly on artificial diet in 12 cell trays.

Larvae were provided fresh food until pupation. Freshly formed pupae were transfer to glass jar having moist sponge and blotting paper at the bottom, covered with muslin cloth, which was fastened with rubber bands. Two pairs were released in each earthen pot. The earthen pots were placed in plastic trays containing water to maintain desired level of humidity (>70%) inside the pot. Temperature of laboratory was maintained up to 27±2 °C. Eggs laid on the muslin cloth were removed daily and kept in glass jar for hatching. The neonate larvae were reared and used for the experimental purpose. The equipment and glassware used in the experiments were to be regularly decontaminated in 5% sodium hypochlorite.

Insecticidal activity of AgNPs and statistical analysis

The insecticidal efficacy of synthesized AgNPs was evaluated against the different developmental stages of *H. armigera* including: eggs, larva, pupa and adult (Fig. 2).

The larvicidal activity of AgNPs was tested against the I-VI instar larvae, pupa, adult and eggs of *H. armigera* at different concentration (250, 500, and 1000 ppm, respectively) after 24 h of exposure. In the experiment, the *R. communis* leaves were cut into 2 cm × 2 cm discs and dipped in 250, 500 and 1000 ppm concentrations of AgNPs. The treated leaves were provided to the larvae. The leaves were provided to larvae at 24 h interval up to 6 days. Larvicidal mortality was recorded after 24 h up to 6 days of treatment. Three replicates were maintained for each treatment with 10 larvae per replicate. For evaluating the pupicidal, adulticidal and ovicidal activities, the 5^{th} instar larvae were fed on the castor leaves treated at median lethal concentration (LC_{50}) of AgNPs. Pupa,

adult and eggs duration, % of pupal, % of adult emergence and % hatchability were recorded [20]. In control test, leaves were treated with distilled water only. Mortality rates (MR) were measured according to Eslin & Pre'vost, [21] using the following formula:

$$MR\ (\%) = \frac{\text{Number of dead larvae}}{\text{Intial number of larvae}} \times 100$$

The data on the efficacy was subjected to probit analysis [22]. The control mortality was corrected by Abbott's formula [23].

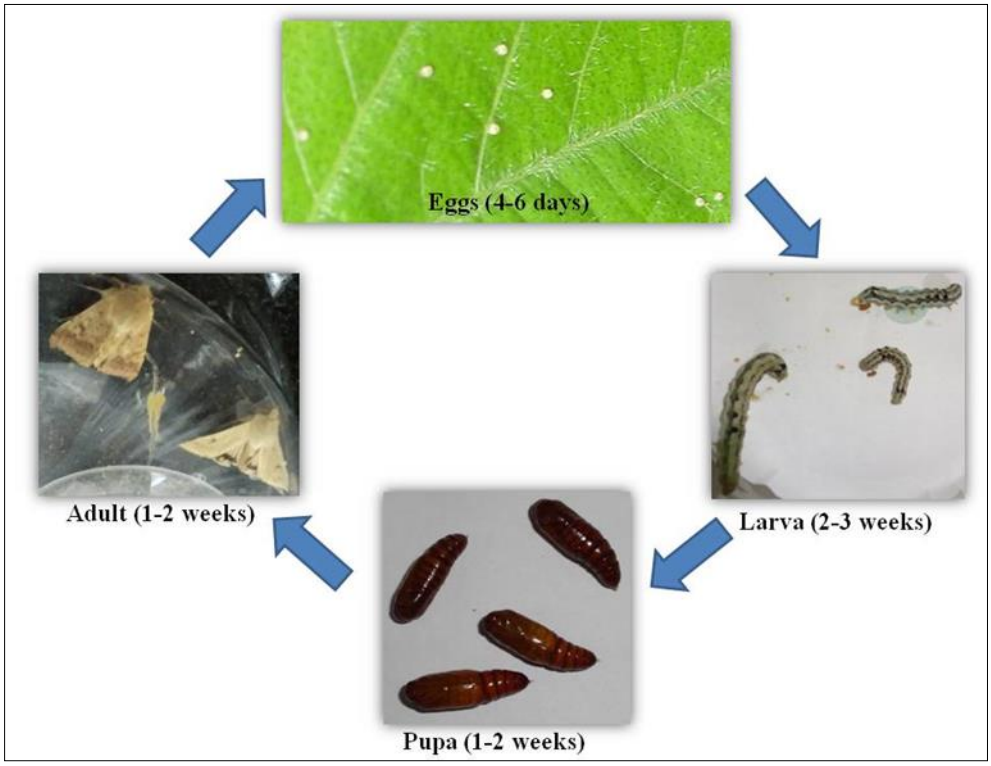

Fig 2: Developmental stages of *H. armigera*.

Histopathological analysis

Fifth instar larvae of *H. armigera* were isolated from the laboratory colonies and maintained on the artificial diet. The larvae were starved for several hours and then fed on *R. communis* leaves treated with LC_{50} of AgNPs for 24 h. In control, the larvae were fed on *R. communis* leaves treated with distilled water. The histopathological effect of AgNPs on the midgut of 5th instar larva of *H. armigera* was investigated using the transmission electron microscope.

For electron microscopic investigation, the midgut of control and treated larvae was dissected and fixed immediately in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer for 8-12 h at 40 °C. Post fixation was done in 2% osmium tetraoxide buffer at pH 7.3 for 2 h at 4 °C. The tissues were subjected to dehydration in graded ethanol series (30-100%). Embedding using 1:1 araldite-propylene oxide for 3 h. Finally, the samples were transferred to a bath of fresh araldite and left overnight and then the tissues were embedded in araldite-filled capsules. Ultra sections were prepared using microtome were mounted on copper grids and double stained with uranyle acetate and lead citrate before examination. The sections were examined and photographed using Tecnai G^2 transmission electron microscope.

Biochemical analysis

Tissue preparation

The tissue preparation was done by the described method with some modifications [24]. Total body tissue samples were collected from late 6th instar larvae treated as 5th instars larvae fed on treated leaves with LC_{50} values of AgNPs. The insect bodies were homogenized in distilled water (1 gm insect bodies/ 5 ml) using chilled glass teflon tissue grinder for 3 min. Homogenates were centrifuged at 8000 rpm for 10 min at 4 °C in a refrigerated centrifuge. The supernatant can be used directly of stored at 4 °C until use for biochemical analysis. Samples of non-treated also were prepared in the same manner.

Total carbohydrate

The total carbohydrates were determined by the phenol-sulphuric acid reaction of Dubois *et al.* [25] with some modifications. Briefly, 200 µL sample was pipette into a 10 mL test tube containing 1800 µl distilled water, 1 mL of 5% phenol in water, 5 mL concentrated H_2SO_4, after then leave for 10-20 min at room temperature for cooling. After then the measurement was taken at λ = 490 nm against a blank sample. All measurements were performed in triplicate.

Total protein

The total proteins were determined by the method of Bradford, [26] with some modifications. Briefly, 100 µL samples was pipette into a 10 ml test tube. 3 mL of Bradford reagent were added to the test tubes and incubate at room temperate for 10 min. After then the measurement was taken at λ = 595 nm against a blank sample. All measurements were performed in triplicate.

Total lipid

The total lipids were determined by the Sulfo-phospho vanillin method of Knight *et al.* [27] with some modifications. Concentrated H_2SO_4, 5 mL, was added to a test tube containing 0.1 mL of treated and non-treated samples. The

test tube was heated for 10 min in a boiling water bath, cooled, and a 0.4 mL aliquot was placed in a clean, dry tube labelled "unknown". A blank contained 0.4 mL of concentrated H_2SO_4. To each was added 6 mL of phospho-vanillin reagent. The tubes were then kept in dark for 45 min. After then the measurement was taken at λ = 525 nm against a blank sample. All measurements were performed in triplicate.

Non-specific esterases

Nonspecific esterases, α-esterase and β-esterase, were determined calorimetrically according to the method described by Van Asperen, [28] using α-naphthyl acetate and β-naphthyl acetate as substrate, respectively. In experiment, 1 mL of sample is mixed with 5 mL of substrate-solution. After 30 min incubation at 27 °C, 1 mL of diazoblue laurylsulphate solution (DBLS) is added. A strong blue color in case of α-naphthol and a strong red color in the case of β-naphthol were produced. After then the measurement was taken at λ = 600 nm for α-naphthol and 555 nm for β-naphthol, respectively.

Results and discussion

UV-visible, TEM, SEM and EDX analysis

As mentioned above after visible analysis, AgNPs formation was confirmed by the UV-Vis spectroscopy. Fig. 1B shows the distinct peak of UV-Vis spectra of AgNPs at ~445 nm. The similar result was observed [29].

TEM analysis further established the presence of AgNPs. The AgNPs were spherical in shape and 8.96 nm in size (Fig. 3A). Scanning electron microscope pictures show the AgNPs synthesized using *R. communis* (Fig. 3B). The SEM images distinctly show the high density AgNPs synthesized using *R. communis* confirming the development of Ag nanostructures.

The EDX attachment with the SEM is known to provide information on the chemical analysis of the field that are being investigated or the composition at specific location (spot EDX). The representative profile of the spot EDX analysis was obtained by focusing on the AgNPs (Fig. 3C).

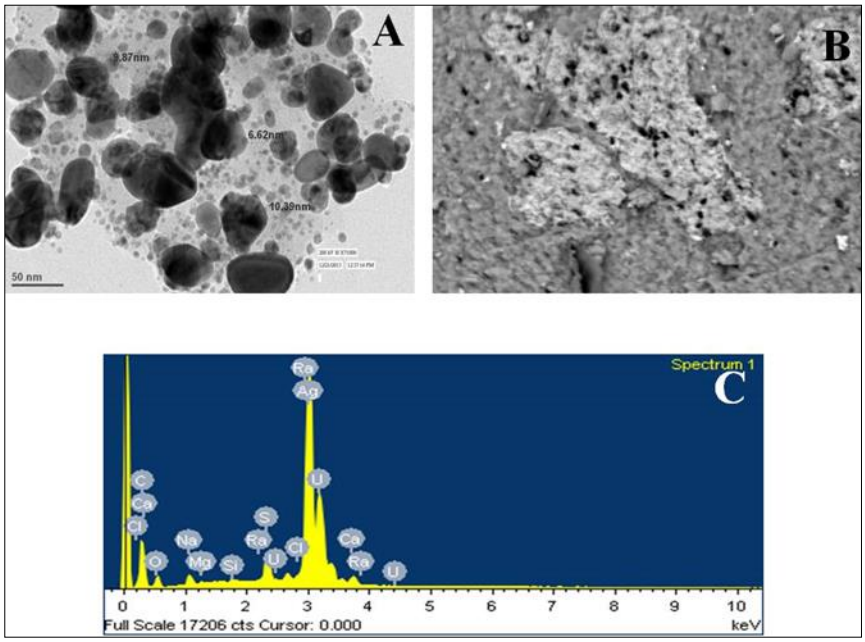

Fig 3: (A) TEM (B) SEM and (C) EDX images of synthesized AgNPs.

Insecticidal activity of AgNP

The larvicidal activity of AgNPs was tested against the I-VI instar larvae of *H. armigera* at the 250, 500 and 1000 ppm concentrations, respectively. The significant mortality was observed in all larval stages (Fig. 4A). The 6th instar larvae showed the highest mortality 70, 80 and 90% which was statistically significant compared with control (Fig. 4B). The LC_{50} values were 250 ppm for 1st, 3rd and 4th instar, 500 ppm for 5th instar and 175 ppm for 6th instar larvae with respect to their R^2, χ^2 and p value (Table 1). The larvicidal effect of silver nanoparticles synthesized using leaf extract of *E. hitra* has been evaluated against the first to fourth instar larvae and pupae of *H. armigera* [16]. The results showed that, the considerable larval mortality was found in the synthesized AgNPs against the first to fourth instar larvae and pupae of *H. armigera*. The antifeedant and larvicidal activities of silver nanoparticles synthesized using aqueous leaf extract of *A. indica* have been evaluated against third instar larvae of *H. armigera* [17]. They observed that the maximum antifeedant and larvicidal efficacy in crude aqueous and synthesized AgNPs against *H. armigera* larvae was (LC_{50} 127.49, 84.56 mg/L; 766.54 and 309 mg/L), respectively. However, the impact of silver nanoparticles on growth and feeding responses of *S. litura* and *A. janata* has been studied [18].

For evaluating the Pupicidal, adulticidal and ovicidal activities the 5th instar larvae were fed on the castor leaves treated with median lethal concentration (LC_{50}) of AgNPs for 72 h. After 72 h treatment the larvae were fed with normal diet and observed till conversion of pupae, pupae to adult and eggs hatchability. The % of pupation was highly reduced from 98% (control) to 40% after larval feeding with AgNPs treated leaves. Percentage of adult emergence also decreased from 95% for control treatment to 45% when AgNPs were used. 90-100% hatchability was observed in control group than the treatment (0% hatchability). Similar, results were observed by [30, 16].

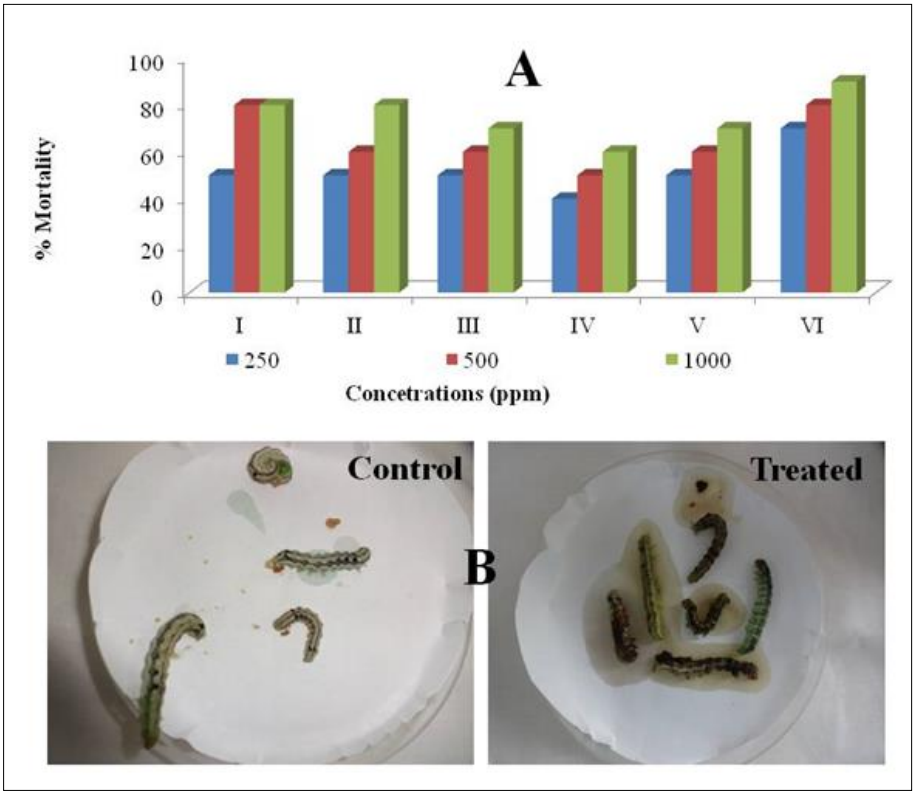

Fig 4: (A) % mortality at different concentrations of synthesized AgNPs against larvae (I-VI) of *H. armigera*. (B) Larvae of *H. armigera* before treatment (control) and after treatment with synthesized AgNPs (treated).

Morphological changes

The larvae of *H. armigera* were found affected after acute exposure of AgNPs (Fig. 5). The figure 5 showed the deformities appeared in the larvae, pupae and adults during the treatment. The larvae colors were turned to dark greenish. The pupae were not moulted in to the adults after treatment. The wings of treated adults were not completely developed. The eggs laid by the treated adults were not hatched. Abd El-Wahab & Anwar [31] have shown malformation and morphological changes in the *Spodoptera littoralis* by nanoparticles adsorption through the integument.

Fig 5: Morphological changes (deformities) in larva, pupa and adult of *H. armigera* before and after treatment with synthesized AgNPs.

Histological changes

Figure 6A-C showed midgut of the larvae fed on the without AgNPs treated castor leaves appearance of the columnar cells, mictovilli (MV), Tracheolrs (Tr), mictochondria (M), rough endoplasmic reticulum (rER), nucleus (N), golgi bodies (GB) and goblet cavity (Gc).

The midgut of the larvae fed on the castor leaves treated with AgNPs showed the goblet cells suffered from degeneration in

their cytoplasm (Fig. 7A). The microvilli (MV) were disrupted showing degenerative appearance at the apical region (Fig. 7B). The goblet cells occurred with enlarged and distorted cavities (GC), vacuolation in nucleus (N) and vacuolar degeneration (Vd) (Fig. 7C). In Fig. 7d-f, the cytoplasm showed increased vacuolation (V), destroyed microvilli (MV), vacuolated organelles (Vo), degenerated lumen cells (Lm), destroyed rough endoplasmic reticulum (rER) and peritrophic membrane (Pm). Similarly, localization of gold nanoparticles in the rough endoplasmic reticulum and vesicles of *Drosophila* were observed previously [32]. Also, the ultrastructural cell damage has been demonstrated in the *H. armigera* larvae fed on *B. thuringiensis* crystals and Bt-tomato plants [33].

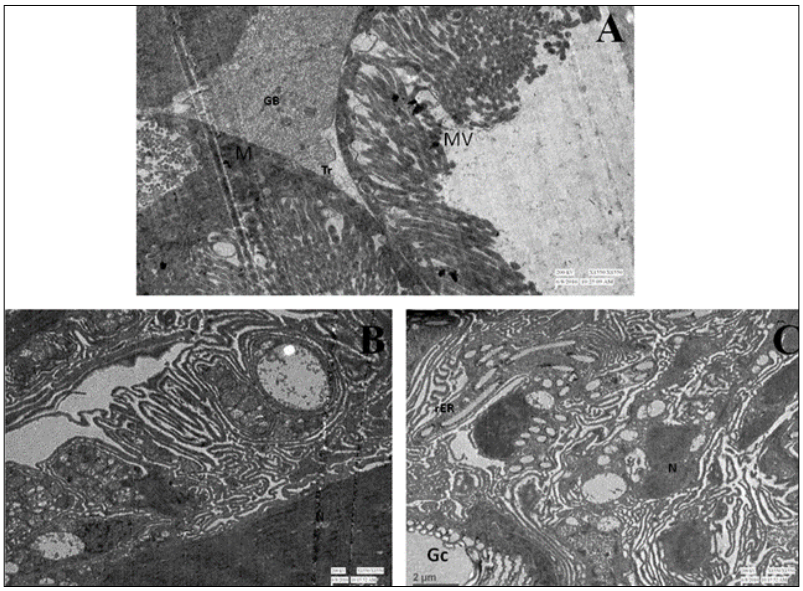

Fig 6: A-C Midgut of the larvae of *H. armigera* fed on the castor leaves without AgNPs treatment (control) appearance of the columnar cells, mictovilli (MV), tracheolrs (Tr), mictochondria (M), rough endoplasmic reticulum (rER), nucleus (N), golgi bodies (GB) and goblet cavity (Gc).

Biochemical changes

Changes in carbohydrates, proteins and lipids

As seen in Table 2, treatment of 6th instar of *H. armigera* at LC_{50} of AgNPs caused a reduction in the total carbohydrates from 4.614 to 2.72 µlmol/ml, giving a -40.85% decrease than their value in the control. Rashwan, [30] observed the reduction in total carbohydrate after treatment with rynaxypyr and spinetoram by -73.93 and -54.97% relative to control. Similar, results were observed by [34-35]. A slight reduction of -1.59% in protein content, as its value was reduced from 0.439 in the control to 0.432 mg/ml in treated larvae. El-barky *et al.* [35] recorded significant decrease in protein content by -69.87% after treating *S. littoralis* larvae by LC_{50} of spinosad compared to the control group, they indicated that the reduction in protein content may be due to inhibition of DNA and RNA synthesis. Similar, results were recorded in the previous studies [36, 24].

While, total lipid content was decreased from 1.048 to 0.965 mg/mL, given a -7.91% decrease than their value in the control. Further, Rashwan, [30] recorded the significant reduction in total lipids by -46.98% and -29.44% for rynaxypyr and spinetorm, respectively. Similar, results were observed by [37].

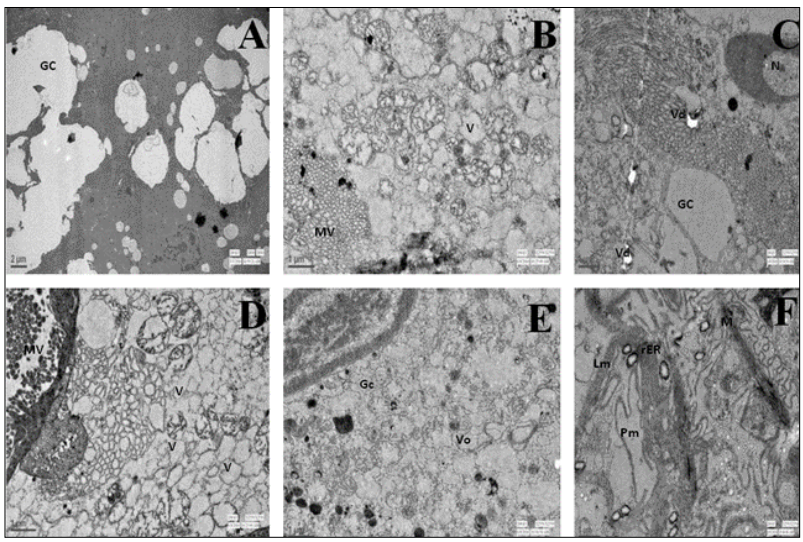

Fig 7: A-F degeneration in their cytoplasm, destroyed microvilli (MV), goblet cells with enlarged and distorted cavities (GC), vacuolation in nucleus (N), vacuolar degeneration (Vd), increased vacuolation (V), vacuolated organelles (Vo), degenerated lumen cells (Lm), destroyed rough endoplasmic reticulum (rER) and peritrophic membrane (Pm).

Changes in alpha and beta esterases activity

The activity of alpha and beta esterase in *H. armigera*, 6th instar larvae treated with the LC_{50} of AgNPs is shown in table 2. The activity of alpha and beta esterase in treated larvae was 0.71 mg/ml as compared to 1.06 mg/mL in control, being a decrease by -33.02%. Beta esterase activity was also decreased by -0.55%. The effect of both rynaxypyr and spinetoram on alpha-esterase and beta-esterase activity in total homogenate of *S. littoralis* 5th instar larvae has also been demonstrated [30]. They found the significant reduction by -31.71% in alpha-estrase and -61.81 to 11.18% in beta-esterase. Similar, results were also found in the previous studies [38-40].

Table 1: Larvicidal activity of synthesized AgNPs at various concentrations against *H. armigera.*

Instars	Concentration (PPM)	Percent Morality ± SD	Probit equation	R^2	LC_{50}±CL	χ^2	p
1st	250	50±0.909	y=48.842x-61.531	0.764	250±1.12	2.059	0.357
	500	80±0.889					
	1000	80±0.889					
2nd	250	50±0.909	y=48.217x-66.516	0.956	250±1.12	1.99	0.370
	500	60±0.912					
	1000	80±0.889					
3rd	250	50±0.909	y=32.249x-26.847	0.999	250±1.12	1.94	0.379
	500	60±0.912					
	1000	70±0.645					
4th	250	40±0.874	y=32.249x-36.847	0.999	500±2.122	1.85	0.397
	500	50±0.909					
	1000	60±0.912					
5th	250	50±0.909	y=32.249x-26.847	0.999	250±1.12	1.94	0.379
	500	60±0.912					
	1000	70±0.645					
6th	250	70±0.645	y=32.249x-6.8466	0.999	175±0.146	2.17	0.338
	500	80±0.889					
	1000	90±0.915					

Table 2: Biochemical activities of larva, pupa and adult of *H. armigera* at LC_{50} concentration of Synthesized AgNPs.

	Larva			Pupa			Adult		
	Control	Treated	% Increase or decrease than control	Control	Treated	% Increase or decrease than control	Control	Treated	% increase or decrease than control
Carbohydrate (µlmol/ml)	4.614 ±0.010	2.73 ±0.013	-40.85	4.767 ±0.012	3.208 ±0.010	-32.70	2.172 ±0.010	1.425 ±0.05	-34.39
Total Protein (mg/ml)	0.439 ±0.002	0.432 ±0.002	-1.59	0.857 ±0.011	0.757 ±0.010	-11.66	0.322 0.001	0.17 ±0.001	-16.14
Total lipid (mg/ml)	1.048 ±0.012	0.965 ±0.011	-7.91	1.28 ±0.117	1.048 ±0.012	-18.12	0.868 d=0.013	0.813 ±0.011	-6.33
a-esterase (mg/ml)	1.06 -±0.013	0.71 ±0.002	-33.02	0.96 ±0.005	0.77 -±0.001	-19.79	1.06 0.013	0.80 -±0.010	-24.53
13-esterase (mg/ml)	43.19 ±1.117	42.95 ±1.112	-0.55	44.12 ±1.23	42.02 ±1.114	-4.76	44.12 ±1.23	42.95 ±1.113	-2.65

Conclusion

In the present study, the effect of silver nanoparticles (AgNPs) synthesized using aqueous leaf extract of castor plant (*Ricinus communis*) against *Helicoverpa armigera* was evaluated. It is a novel, cost effective, eco-friendly and green route to synthesis of AgNPs using *R. communis* to control the *H. armigera*. The synthesized nanoparticles could be used for the improvement of new botanical nano-insecticide or nano-formulation after successful field trials which can reduce crop damage as well as pest population.

Acknowledgements

I owe to DST SERB, New Delhi, India for awarding the Young Scientist Project (File no. SB/YS/LS-35/2014). I thank to University of Delhi and ICMR-National Institute of Malaria Research, New Delhi, India for providing the lab facility for conducting the research. I thank to Prof. T. C. Nag, Department of Anatomy, AIIMS, New Delhi, India, providing the TEM facility.

Conflict of interest statement

The authors declare no conflict of interest.

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