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# Effect of biosynthesized Silver nanoparticles on growth and development of *Helicoverpa armigera* (Lepidoptera: Noctuidae): Interaction with midgut protease

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**Abstract**

We investigated the effect of Silver nanoparticles (AgNPs) on the gut protease activity of insecticide resistant gram caterpillar, *Helicoverpa armiger*a. The leaf extracts of Peepal tree, *Ficus religiosa* (FR) and banyan tree, *Ficus benghalensis* (FB) mediated biogenic AgNPs were synthesized to modulate the function of gut protease activity in *H. armigera* (Ha). Bioassay with FR and FB AgNPs significantly reduced both larval weight and survival rate of *H.armigera*. The FR and FB AgNPs inhibited the Ha-Gut protease activity by 50 and 70% at 100 µg concentration respectively. The FR and FB silver nanoparticles were interacted and binds with high affinity with protease. The inhibition studies on Ha-gut protease activity may contribute towards developing new IRM (Insecticide Resistant Management) strategies against *H. armigera* to overcome insecticidal resistance issues.

**Key words;** *Helicoverpa armigera*, Biosynthesis, Silver nanoparticles, Gut protease, Tryptophan Fluorescence, Absorbance

## Introduction

Nanoparticles are showing various applications in different fields of agriculture including pest management, herbicide delivery, vector-pest management and nanosensors for pest detection (Rahman et al., 2009; Scrinis and Lyons, 2007). The Silver nanoparticles accumulate in the gut and interact with gut membrane and disrupt the membrane structures in insects (Sharma et al., 2014; Javier et al., 2011). Nanoparticles induced oxidative stress, interference with DNA replication, and protein synthesis (Sharma et al., 2014; Javier et al., 2011).

Plants are used for synthesizing nanoparticles which is a rapid, cost-effective, eco- and, environmentally friendly technique (Huang et al., 2007b; Kumar and Yadav, 2009). It has been reported that medicinally valuable angiosperms have the greatest potential for synthesizing metallic nanoparticles with respect to quality and quantity (Song and Kim, 2009). AgNPs synthesized from leaf extract of *Eclipta prostrata*, is useful to control 4$^{th}$ instar larvae of *Culex quinquefasciatus* and *Anopheles subpictus* have been reported earlier by researchers (Rajakumar and Rahuman, 2011). The larvicidal efficacy of AgNPs synthesized from aqueous leaf extract of *Hibiscus rosa sinensis* against the larvae of *Aedes albopictus* (Sareen et al., 2012).

The gram caterpillar, *Helicoverpa armigera* Hubner (Lepidoptera: Noctuidae), is an extremely destructive pest of many crops in Europe, Asia, and Australia. *H. armigera* is able to adapt to various cropping systems and to develop resistance to insecticides (Kranthi et al, 2001). This pest has been recorded feeding on 182 plant species across 47 families in the Indian subcontinent, of which 56 are heavily damaged (Gunning et al., 1999). The annual losses due to *H. armigera* in cotton and pulses alone have been estimated at US $ 300–500 million in India (Sharma, 2005). This pest has developed resistance to virtually all the

insecticide classes that have been applied to control (Kranthi et al., 2001; Gunning et al., 1999; Sharma, 2005; Srinivas et al., 2004; Ravindra et al., 2010).

The emergence of insecticide resistance due to excessive application of chemical insecticides and also their harmful effects on non-target organisms necessitated an urgent search for new and improvised pest control methods that are economical and effective as well. Insect gut proteases plays a major role in digestion of proteinacious food for their energy and development. Inhibiting gut proteases is one of the major methods for controlling the insect pest. Several researchers have reported the characterization of gut protease inhibitors against *H. armigera* from plants (Ambekar et al., 1996; Raju et al., 2009). *Ficus religiosa* Linn (Peepal tree) and *Ficus benghalensis* Linn (Banyan tree) are more revered trees in Asia having remarkable medicinal properties in curing various life threating diseases (Singh et al. 2015; Gopukumar and Praseetha, 2015). Therefore, in the present study AgNPs were synthesized employing leaf extracts of *F. benghalensis* and *F. religiosa* and its role was investigated in inhibiting the gut protease of *H. armigera*. This is the first time we reported the insecticidal effect of biosynthesized AgNPs on *H.armigera* by inhibiting Ha-gut protease activity.

## Materials and methods

### Chemicals

$AgNO_3$, permethrin, tris-HCl, DDT, sephadex G-200, glycine, NaOH, SDS, Acrylamide, trichloroacetic acid from Himedia, Bengaluru,India, azocasein, DEAE-cellulose were purchaged from sigma Aldrich Bengaluru, India. All other chemicals were of analytical grade.

### Synthesis of Silver nanoparticles

AgNPs were synthesized as per our earlier report (Saware and Venkataraman, 2014a; Saware et al., 2014b). In brief, leaf extracts from *F. benghalensis* (FB) and *F. religiosa* (FR) were

used for synthesis of AgNPs. Fresh and healthy leaves of FR and FB plants were collected and homogenized in pestle and mortar in distilled water. 5 mL of leaf extract was added to 95 mL of a $10^{-3}$ M aqueous $AgNO_3$ solution in a conical flask and exposed for 3 min in microwave oven. Periodically aliquots of the reaction solution were removed and subjected to UV-Vis spectroscopy measurements for surface plasmon resonance study of AgNPs synthesis. Positive (leaf extract) and negative controls (pure $AgNO_3$ solution without leaf extract) were also run simultaneously.

**Insects**

Permethrin-resistant (60-fold) populations of *H. armigera* were used for the study. The *H. armigera* culture was obtained from insect rearing laboratory, ICRISAT, Patancheru, Telangana, India. Insects were reared on chickpea based artificial diet under laboratory conditions at 26 $^{o}$C, 65±5% RH, and 12 h photoperiod (Armes et al., 1992).

**Bioassays of *H. armigera* using AgNPs**

The third instar larvae were divided into eight groups of ten insects each. Bioassays on AgNPs were conducted by rearing the third instar larvae on artificial diet containing FR and FB AgNPs (6.6, 13.3, 26.6, 40, 53.3, and 66.6 µl/gm diet) respectively, FR (6.66 µl/gm diet) + permethrin (100µM/gm diet) and FB (6.66 µl/gm diet) + permethrin (100µM/gm diet), separately. One set of larvae were reared on diet containing permethrin (100 µM/gm diet) alone and another set was left untreated as control. The initial weights of the larvae were recorded before releasing in the artificial diets. There were three replications for each treatment and 10 larvae in each replication in completely randomized design. The larval weights were recorded 5 days after initiating the experiment.

**Isolation and purification of Ha-Gut protease**

Fourth instar *H. armigera* larvae (n = 50) were washed in cold 50 mM Tris–HCl buffer, pH 7.4 and dissected to remove the gut. The gut was then homogenized in pestle and

mortar in 50 mM Tris–HCl buffer, pH 7.4 at 4°C. The homogenate was centrifuged at 10,000g for 10 min at 4°C. The supernatant was then subjected to ammonium sulfate precipitation to attain 70% saturation and allowed to stand for 4 h at 4°C. The precipitate was collected by centrifugation at 8000 g for 10 min and dissolved in 50 mM Tris–HCl buffer, pH 7.4. The above fraction was loaded on to a DEAE-cellulose column (2.5×5 cm) equilibrated with 50 mM Tris–HCl buffer, at pH 7.4, 1 mM dithiothreitol (DTT) and the column was washed with the same buffer. The column was further washed with 20 mL of buffer until no absorbance at 280 nm was detected in the eluate. The adsorbed proteins were then eluted with 50 ml of Tris-HCl buffer containing gradient NaCl (0.1M to 2M) and 1.5 ml fractions were collected. The fractions containing maximum protease activity were pooled, concentrated, dialyzed against 50 mM Tris–HCl buffer, pH 7.4. The dialyzed sample were loaded on to the gel filtration using a sephadex G-200 (1×60 cm) column equilibrated with 50 mM Tris–HCl buffer, 7.4. The active fractions were pooled concentrated and stored at -20 $^{o}$C for further use.

**Protein estimation**

Protein concentration was determined by the method of (Lowry et al., 1951) using bovine serum albumin as a standard.

**Protease assay**

The protease assays were carried as described earlier (Giri et al 1998). Briefly, the assay medium containing 60 µl of midgut extract and 200 µl of 1% azocasein (in 50 mM Glycine–NaOH buffer, pH 8.0) and incubated at 37 °C for 30 min. The enzyme reaction was terminated by the addition of 300 µl of 5 % trichloroacetic acid. The assay mixture was centrifuged at 10,000 rpm for 10 min, and 560 µl of 1 M NaOH was added to the supernatant and absorbance was measured at 450 nm. One unit enzyme activity was defined as the amount of enzyme that increases absorbance by 1 OD under the given assay conditions.

Protease activity in the gel was detected as described earlier (Michaud et al, 1993). 10 % SDS-polyacrylamide gels was prepared according to (Laemmli, 1970). 0.5 % w/v azocasein was dissolved in the gelmix prior to polymerization. 200 µg of gut extract was loaded on to gel, after electrophoresis, gel was washed in 1% triton X-100 to remove SDS and incubated in 50 mM Tris–HCl buffer, pH 8.0, for 2 h at 37 °C. The gel was then stained with coomassie blue.

**Measurement of binding affinity of AgNPs with midgut protease**

The binding of FR and FB AgNPs with purified Ha-gut protease was determined by Trp fluorescence quenching titrations using spectrofluorometer (Liu et al, 2000). Purified midgut protease (50 µg/ 2 mL) was titrated in 50 mM Tris-HCl buffer, pH 8 with increasing concentrations of FR and FB AgNPs (5, 10, 20, 30, 40, 50 µl) respectively, while quenching of Trp fluorescence was monitored at 340 nm following excitation at 280 nm (slit width for both, 5 nm).

**Absorbance spectra of midgut protease**

Absorbance spectra was measured between 200 and 600 nm in 1cm quartz cuvette containing midgut protease (50µg /ml) in 50 mM Tris-HCl buffer, pH 8 at room temperature using U-3010 spectrophotometer (Tokyo. Japan) with addition of increasing concentration of AgNPs.

**Statistical analysis**

Data were subjected to one-way analysis of variance (ANOVA) to judge the significance of differences between the treatments by using F-test, while the significance of differences between the treatment means was judged by least significant difference (LSD) at $p < 0.05$. Statistical analysis was performed in MS Excel 2010v.

**Results**

**Effect of AgNPs on growth and survival of *H. armigera***

Diet supplemented with lower concentration of AgNPs (6.6 µl/gm of diet) had no effect on larval weight. But diet containing AgNPs (6.6 µl/gm diet) and permethrin (100 µM/g diet) inhibited larval growth and survival rate (Fig. 1 and 2) compared with control larvae. Comparatively, the rate of weight gain and survival rate were significantly reduced in a dose-dependent manner with increasing concentrations of AgNPs (Fig 1 and 2)

**Protease activity in gel**

*H. armigera* gut extract shows two isozymes of proteases and we purified protease-I and the purity of proteins were checked with SDS and native page. The presence of proteolytic activity in gut extracts of *H. armigera* was shown by an in-gel assay based on digestion of casein (Fig. 3).

**Midgut protease activity**

Ha-Gut protease activity increases slightly (10-20%) at lower concentration and decreased with increasing concentrations of FB and FR AgNPs (Fig. 4). FB and FR at 100 µl inhibited Ha-gut protease activity by 60% and 70% respectively, Silver nitrate and leaf extract alone does not have any inhibitory effect on gut protease activity. While, with combination of FR and FB AgNPs inhibited 80 % of protease activity (Fig. 5).

**Effect of Silver nanoparticles on the tryptophan fluorescence spectra**

Fluorescence intensity of Trp residues in midgut protease-I was decreased with increasing concentration of FR and FB nanoparticles (0–50 µl). However, there was no significant emission shift with the addition of these nanoparticles but with a slight shift on the Trp residues fluorescence emission (Fig. 6A-B).

**Interaction of AgNPs with Ha-gut protease**

The $K_d$ values for binding were estimated by fitting fluorescence quenching data to an equation that describes a single binding site (Fig. 7). The $K_d$ values for binding of FR and FB AgNPs with midgut protease-I was 7µg and 10µg, respectively.

**UV-visible absorbance spectra of Ha-gut protease**

Absorbance spectra showed increase of absorbance with increasing concentrations of AgNPs with a blue shift (3-10 nm) (Fig.8).

**Discussion**

We studied the effect of different concentrations of FR and FB AgNPs on larval growth and survival, and Ha-gut protease activity. AgNPs at lower concentrations had no effect on larval weight and survival. Permethrin alone had little effect on larval weight or development of the pest as they had developed resistance to various insecticides (Gunning and Moores, 1999). However combination of AgNPs with permethrin significantly reduced the body weight and survival rate. The higher concentration of FR and FB AgNPs alone had effectively reduced the larval body weight and increased mortality rates compared to control larvae.

Similar reports were reported earlier that larval and pupal body weights decreased when fed on increasing concentrations of AgNPs in *Spodoptera litura* F. and *Achaea janata* L., and the nanoparticles were found to be accumulated in the larval guts (Jyothsna and Usha Rani, 2014).

Larvicidal activity of AgNPs using *Nelumbo nucifera* leaf extract against *Culex quinquefasciatus* and *Anopheles subpictus was reported earliesr* reserchers (Santhoshkumar et al., 2011). Further, larvicidal potentiality of synthesized AgNPs using leaf aqueous extract of *Tinospora cordifolia* Miers against *An. Subpictus* and *Cx. Quinquefasciatus* was revealed (Jayaseelan et al., 2011). The larvicidal potential of AgNPs synthesized using fungus

*Cochliobolus lunatus* against two species of mosquitoes *Aedes aegypti* and *An. stephensi* Liston have been reported by (Salunkhe et al., 2011).

The insect digestive proteases catalyze the dietary protein to release the free amino acids and supply essential nutrients for growth and development. Earlier we have reported that the biosynthesized Silver nanoparticles modulates amylase activity in *H. armigera* (Kantrao et al., 2014). In the present study, it is observed that AgNPs stimulated Ha-gut protease activity at lower concentrations and inhibited at higher concentrations. Gole et al, reported increase of trypsin activity with immobilized protease with nanoparticles (Gole et al., 2001). The increased protease activity was due to the accumulation of protease on the nanoparticle surface bringing more substrate available to the active site of enzyme, whereas the decrease in protease activity was due to more AgNPs binding to the substrate binding site or the protease buried inside the nanoparticles and making no substrate available for binding of protease. Similar results were reported earlier, where, gold nanoparticles, enhanced trypsin activity at lower concentration, while, at higher concentration inhibited trypsin activity (Min et al., 2009; Deka et al., 2002). Studies on interaction of AgNPs with α-amylase were carried out to study the effect of rapid degradation of starch hydrolysis (Ernest et al., 2012).

FB AgNPs had no apparent shift in λem on the fluorescence spectra of Ha-gut protease. However, in presence of FR AgNPs 4-5 nm λem red shift was observed for the midgut protease-I (Fig. 6A). These results suggest there is an association between the protein and the nanoparticles with a change of Trp environment. The tryptophan fluorescence measurement data indicate that FR and FB AgNPs bind to Ha-Gut protease with high affinity quenching by almost 40–60%. The low $K_d$ values for the both the AgNPs suggests high affinity towards midgut protease.

Absorbance of the midgut protease was increased in presence of FR and FB AgNPs indicates that conformational changes of protein occur due to exposure of more Trp residue into the medium on binding of AgNPs.

## CONCLUSIONS

The synthesized FR and FB AgNPs showed effect on growth, development and insecticidalcidal activity towards *H. armigera* larvae by inhibiting midgut proteases. Emulating nature's strategy by empowering AgNPs as insecticidal molecules may be an effective strategy to control insect pests that carries a lower toxicological burden on the environment. Further this information will provide a rational basis for the design of new nanoparticles to control insecticide resistance.

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**Figure Legends**

**Fig. 1**. Effect of AgNPs on growth of *H. armigera* larvae. (A) Effect of Silver nanoparticles on pupae formation and adult emrgence, (Demorphism of pupae). (B) Effect of Silver naoparticles on larval weight, a) control; b) FR treated and c) FB treated larvae. (C) *H. armigera* larvae were fed on diet containing FB and FR Silver nanoparticles (6.6, 13.3, 26.6, 40, 53.3, 66.6 µl/gm diet), permethrin (100 µM/gm diet) and AgNPs (6.6 µl/gm diet) + permethrin (100 µM). The experiment was performed in three replicates (* significantly different from control at $p < 0.05$).

**Fig. 2**. Effect of AgNPs on survival of *H. armigera* larvae. Larvae were fed on diet containing FB and FR AgNPs (6.6, 13.3, 26.6, 40, 53.3, 66.6 µl/per gram diet), permethrin (100 µM/gm diet) and AgNPs (6.6 µl/gm diet) + permethrin (100 µM/gm). The experiment was performed in three replicates (* significantly different from control at $p < 0.05$).

**Fig. 3.** Detection and purity of *H. armigera* gut protease isozymes. A) Native PAGE analysis of Ha-gut protease activity. Lane - 1, crude extract, lane - 2, DEAE-cellulose column Purified protein. B) SDS PAGE analysis of purified protease-I, lane 1-crude extract, lane 2, purified protein.

**Fig. 4.** Modulatory effects of AgNPs on Ha-Gut Protease–I activity. (A) Gut protease activity was determined in presence of increasing concentration of (A) FR (▲) and FB (Δ) AgNPs. Both FR and FB AgNPs stimulated protease activity at lower concentrations and inhibited in a dose-dependent manner with increasing concentrations. (B) Predicted schematic diagram of immobilization of protease. (C) Schematic diagram of protease buried in the nanoparticles. Data represent mean ± SE of three independent assays (* significantly different from control at $p < 0.05$).

**Fig. 5**. Determination of Ha-gut protease activity with different treatments. Protease activity was determined as detailed in text, control larvae, Silver nitrate (2 mM), ficus leaf extract (200 µL), FR AgNPs (100 µl), FB AgNPs (100 µl); and combination of both nanoparticles (FR+FB AgNPs). Data represent mean ± SE of three independent assays (* significantly different from control at $p < 0.05$).

**Fig. 6**. Tryptophan fluorescence quenching spectra of Ha-GP. 80µg/ml of purified Ha-GP-I was titrated with (A) FR and (B) FB AgNPs in 50mM Tris–HCl buffer, pH 7.4 (25°C), quenching of Trp fluorescence emission was monitored at 355 nm (slit width, 10 nm)

following excitation at 280 nm (slit width 5 nm). Spectrum 1, corresponds to protein alone and spectra 2–8 and 2-7 corresponds to the protein in presence of increasing concentrations of AgNPs. Each titration was carried with addition of 10µl of corresponding FR and FB AgNPs.

**Fig. 7**. Binding of AgNPs to midgut protease-I as assessed by tryptophan fluorescence quenching. The $K_d$ values for binding were estimated by fitting fluorescence quenching data to an equation that describes a single binding site. Both FR and FB AgNPs interacted with gut protease with relatively high affinity, and the $K_d$ values for binding was 7µg and 10 µg respectively.

**Fig. 8**. Absorbance spectra of Ha-gut protease with AgNPs (A) Absorbance spectra of protease-I with increasing concentration of FB nanoparticles and (B) FR nanoparticles. Line 1, corresponds to protein alone and line 2–4 in presence of AgNPs. Ha-gut protease (50 µg in Tris-HCl buffer, pH 7.4) was titrated with increasing concentration of FB (10, 20, 30 µl) and FR AgNPs (10, 20, 30 µl).

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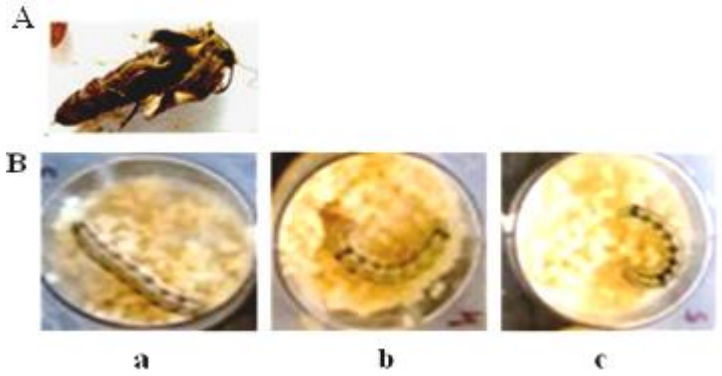


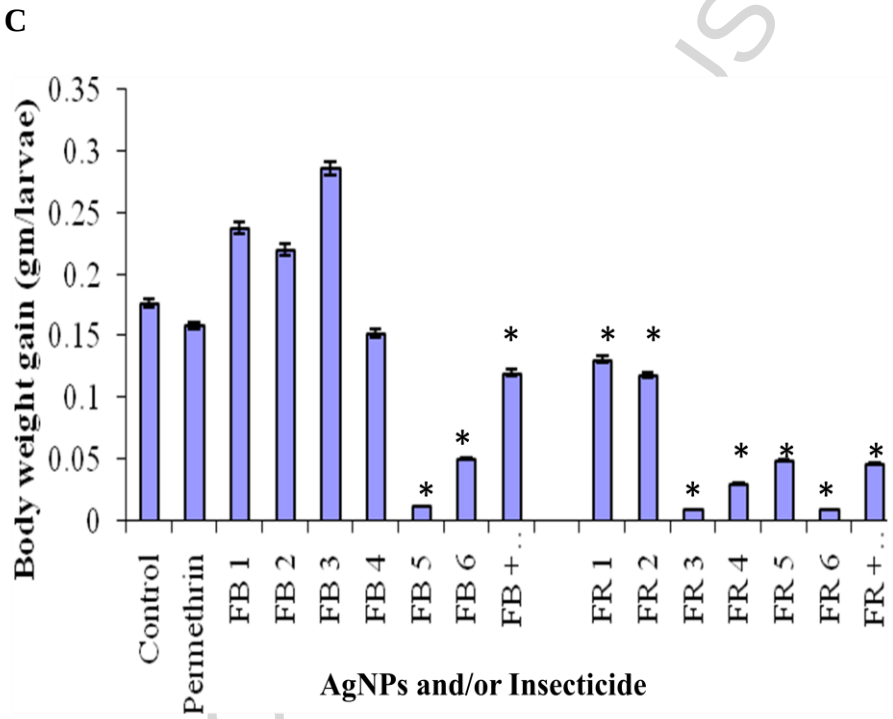


**Fig. 1**. Effect of AgNPs on growth of *H. armigera* larvae. (A) Effect of Silver nanoparticles on pupae formation and adult emrgence, (Demorphism of pupae). (B) Effect of Silver naoparticles on larval weight, a) control; b) FR treated and c) FB treated larvae. (C) *H. armigera* larvae were fed on diet containing FB and FR Silver nanoparticles (6.6, 13.3, 26.6, 40, 53.3, 66.6 µl/gm diet), permethrin (100 µM/gm diet) and AgNPs (6.6 µl/gm diet) + permethrin (100 µM). The experiment was performed in three replicates (* significantly different from control at $p < 0.05$).

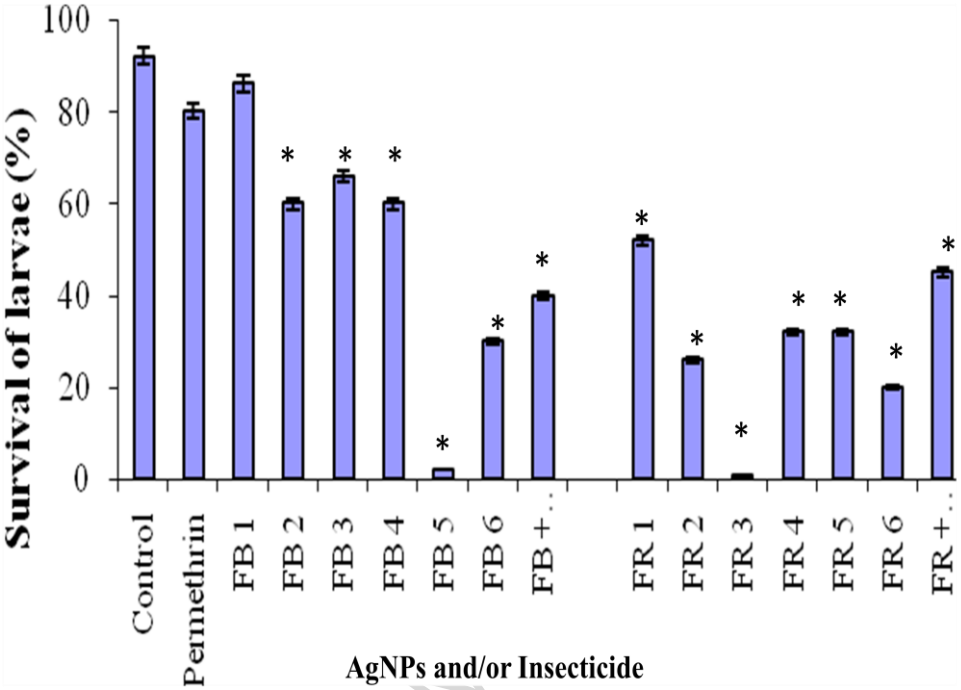


**Fig. 2**. Effect of AgNPs on survival of *H. armigera* larvae. Larvae were fed on diet containing FB and FR AgNPs (6.6, 13.3, 26.6, 40, 53.3, 66.6 µl/per gram diet), permethrin (100 µM/gm diet) and AgNPs (6.6 µl/gm diet) + permethrin (100 µM/gm). The experiment was performed in three replicates (* significantly different from control at $p < 0.05$).

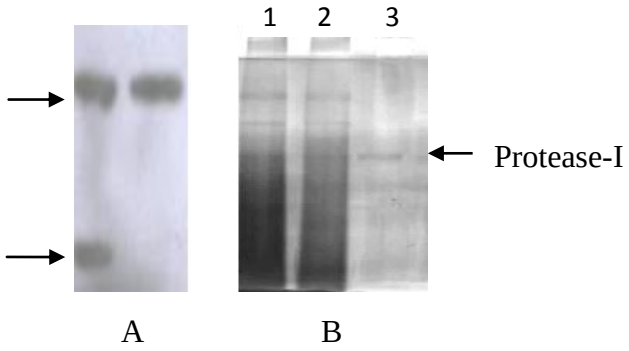


**Fig. 3.** Detection and purity of *H. armigera* gut protease isozymes. A) Native PAGE analysis of Ha-gut protease activity. Lane - 1, crude extract, lane - 2, DEAE-cellulose column Purified protein. B) SDS PAGE analysis of purified protease-I, lane 1-crude extract, lane 2, purified protein.

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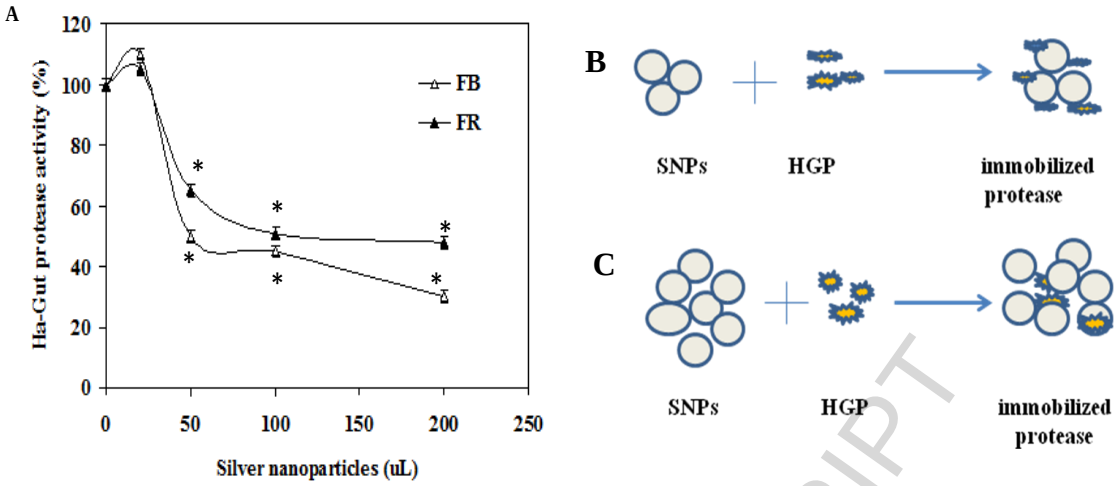
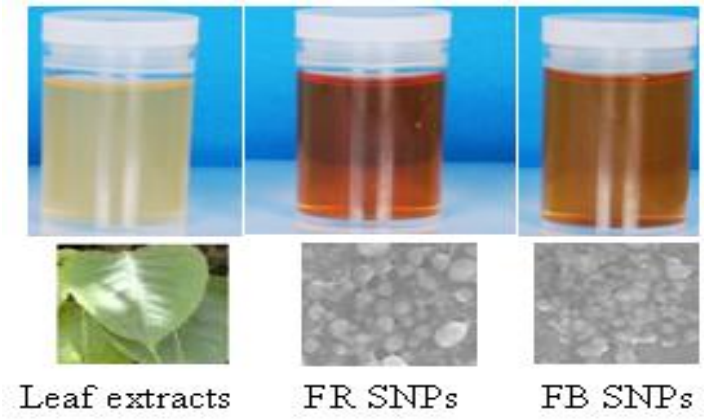



**Fig. 4.** Modulatory effects of AgNPs on Ha-Gut Protease–I activity. (A) Gut protease activity was determined in presence of increasing concentration of (A) FR (▲) and FB (Δ) AgNPs. Both FR and FB AgNPs stimulated protease activity at lower concentrations and inhibited in a dose-dependent manner with increasing concentrations. (B) Predicted schematic diagram of immobilization of protease. (C) Schematic diagram of protease buried in the nanoparticles. Data represent mean ± SE of three independent assays (* significantly different from control at $p < 0.05$).



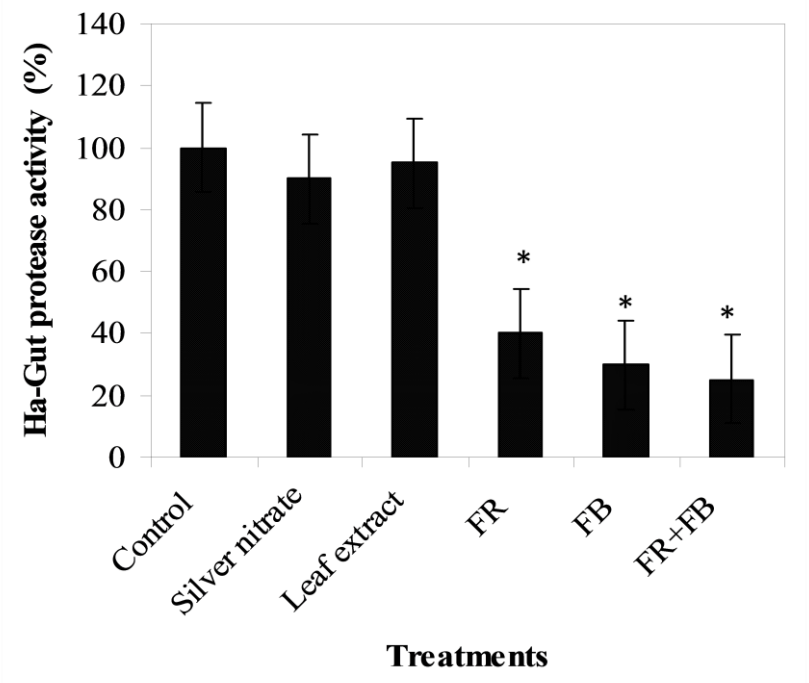




**Fig. 5**. Determination of Ha-gut protease activity with different treatments. Protease activity was determined as detailed in text, control larvae, Silver nitrate (2 mM), ficus leaf extract (200 µL), FR AgNPs (100 µl), FB AgNPs (100 µl); and combination of both nanoparticles (FR+FB AgNPs). Data represent mean ± SE of three independent assays (* significantly different from control at $p < 0.05$).

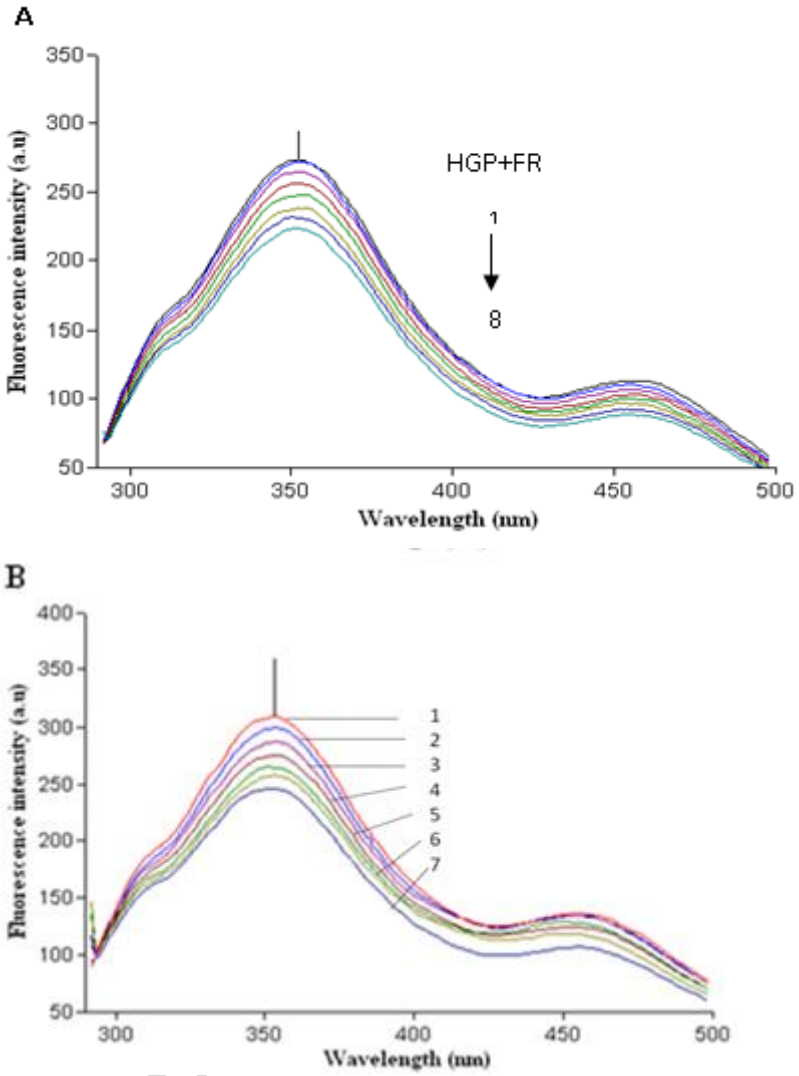



**Fig. 6**. Tryptophan fluorescence quenching spectra of Ha-GP. 80µg/ml of purified Ha-GP-I was titrated with (A) FR and (B) FB AgNPs in 50mM Tris–HCl buffer, pH 7.4 (25°C), quenching of Trp fluorescence emission was monitored at 355 nm (slit width, 10 nm) following excitation at 280 nm (slit width 5 nm). Spectrum 1, corresponds to protein alone and spectra 2–8 and 2-7 corresponds to the protein in presence of increasing concentrations of AgNPs. Each titration was carried with addition of 10µl of corresponding FR and FB AgNPs.

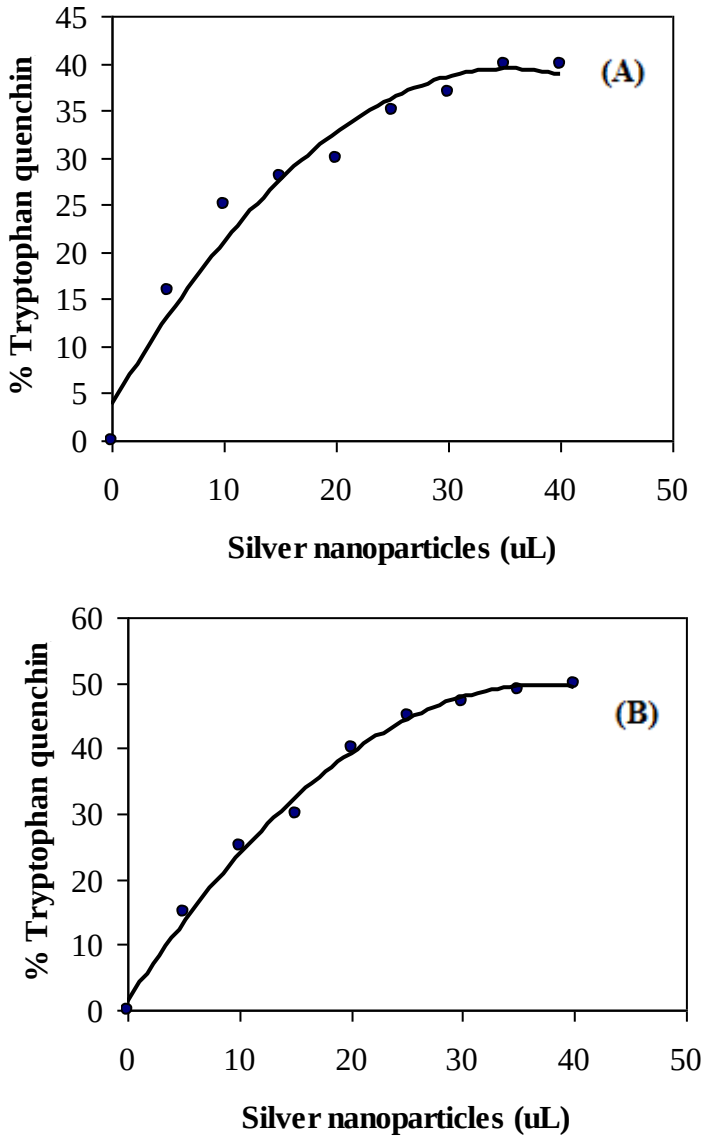



**Fig. 7**. Binding of AgNPs to midgut protease-I as assessed by tryptophan fluorescence quenching. The $K_d$ values for binding were estimated by fitting fluorescence quenching data to an equation that describes a single binding site. Both FR and FB AgNPs interacted with gut protease with relatively high affinity, and the $K_d$ values for binding was 7µg and 10 µg respectively.

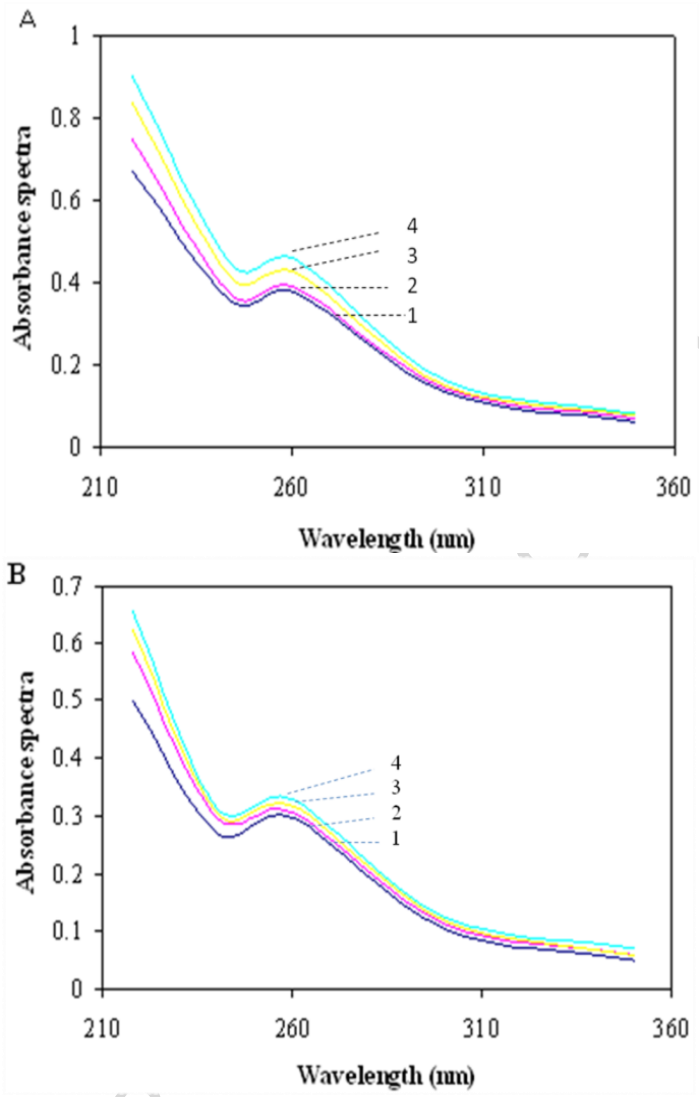
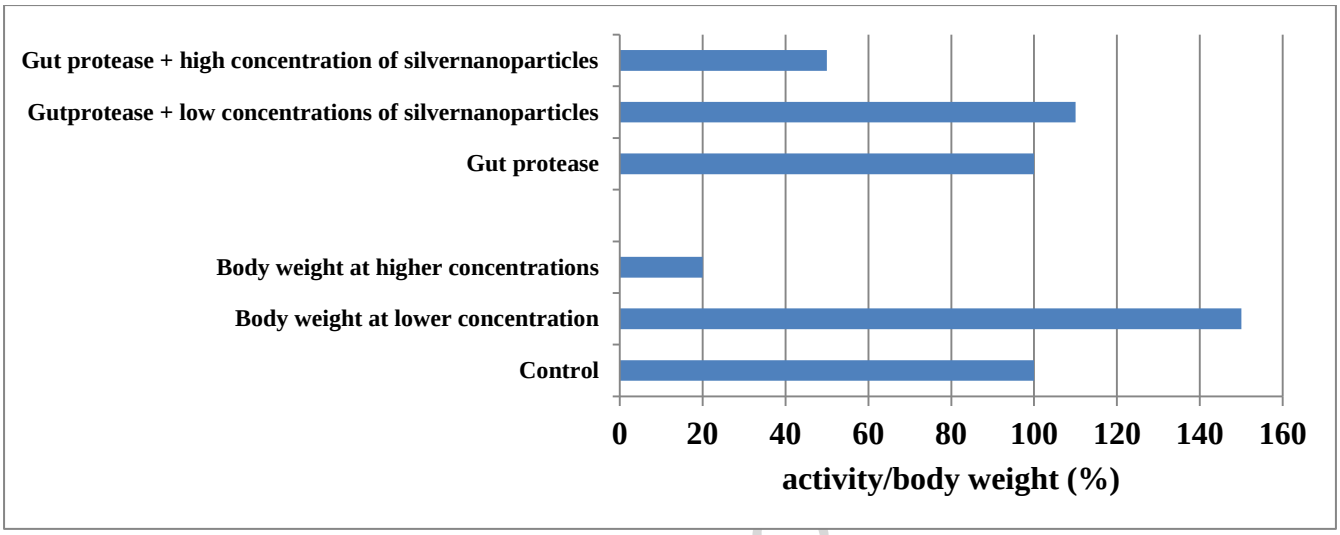



**Fig. 8**. Absorbance spectra of Ha-gut protease with AgNPs (A) Absorbance spectra of protease-I with increasing concentration of FB nanoparticles and (B) FR nanoparticles. Line 1, corresponds to protein alone and line 2–4 in presence of AgNPs. Ha-gut protease (50 µg in Tris-HCl buffer, pH 7.4) was titrated with increasing concentration of FB (10, 20, 30 µl) and FR AgNPs (10, 20, 30 µl).

## Graphical abstract



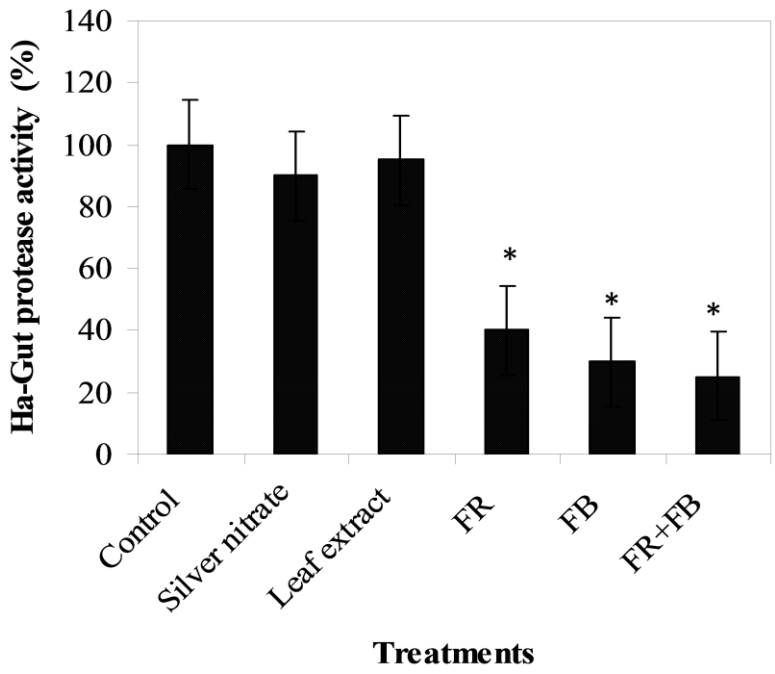




**Figure**. Effect of green synthesized FR and FB silver nanoparticles on body weight and gut protease activity of insecticide resistance pest *Helicoverpa armigera*

**Research Highlights**

- Inhibitory & modulatory effect of green synthesised FR and FB AgNPs on growth of *H.armogera*
- Purification of Gut protease of *H.armigera* and its interactions of FR and FB AgNPs.
- Conformational changes of protein observed after binding of AgNPs