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Insecticidal Activity of Phytosynthesized Silver Nanoparticles (AgNPs) against *Spodoptera frugiperda* and *Plutella xylostella*

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Abstract

The application of nanotechnology in crop protection holds a significant promise and metal based nanoparticle reported to exhibit insecticidal property. Present study, aims to photosynthesize Silver Nanoparticles (AgNPs) using *Catharanthus roseus* aqueous leaf extract, characterize them and evaluate for their insecticidal efficacy against 2nd and 3rd instar larvae of *Spodoptera frugiperda* and *Plutella xylostella*. Phytosynthesized silver nanoparticles (AgNPs) were initially identified by the change in color from colorless to dark brown. These silver nanoparticles were characterized using UV-Visible spectroscopy that revealed an absorbance peak at 448 nm. Spherical shape with an average nanoparticle size of 48 nm and the presence of elemental silver was confirmed using Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX) spectrum respectively, Atomic Force Microscopy (AFM) analysis showed the surface of the nanoparticles was rough and undulated and the Fourier Transform Infrared Spectroscopy (FTIR) spectrum revealed functional groups of biomolecules in the plant extract responsible for the reduction of silver nitrate to silver nanoparticles. Further, insecticidal bio efficacy data revealed that the highest concentration of AgNPs (8000 ppm) induced 100% and 53.33% mortality at 120 h in 2nd and 3rd instar larvae of *Spodoptera frugiperda* respectively. Whereas, 4000 ppm (Highest concentration) phytosynthesized AgNPs induced 82.75% and 66.66% mortality at 120 h in 2nd and 3rd instar larvae of *Plutella xylostella* respectively. Collectively, this study suggests the potential use of phytosynthesized AgNPs for management of agricultural insect pests.

Keywords: *Catharanthus roseus*; Phytosynthesis; *Plutella xylostella*; Silver nanoparticle; *Spodoptera frugiperda*

Introduction

Insect pests are one of the important limiting biotic factors for sustainable crop production. Conventional pesticides have been routinely used for the management of insect pests. However, injudicious use of pesticides resulted in health and environmental hazards [1,2]. The rising worldwide demand for pesticides will further deteriorate health and environmental biodiversity. Hence, there is a need to explore alternative technologies for sustainable pest management. Nanotechnology is one of the rapidly developing research areas in the last decade and has reported to have wide applications in different fields like pharmaceuticals, medical, diagnostics, electronics, defense, cosmetics, textile, biotechnology and agricultural pest management [3]. The application of nanotechnology in crop protection holds a significant promise, especially, in the management of insect pests and pathogens. Slow and targeted delivery of nanopesticides for efficient vector/pest management and nanosensors for pest surveillance and detection are key components of agricultural nanotechnology. Metal based nanoparticles, nanoemulsions, nano-suspensions and nanocapsules are increasingly reported to be used as insecticides, insect repellents and herbicides [4]. Nanosilver, widely reported to possess biocidal property can be synthesized using physical, chemical and biological/green synthesis methods. Both physical and chemical methods results in the release of toxic byproducts that are not only health and environmental hazardous, but also expensive. On the other hand, green synthesis of metal nanoparticles is inexpensive, eco-friendly, with controlled size and shape [5]. Green synthesis of nanoparticles using microbes and plant extracts has been well documented in the literature [6,7]. However, using plants for nanoparticle synthesis can be beneficial over other biological methods as it eliminates the elaborate process of maintaining cell/microbial cultures and can also be easily scaled up [8]. Recently, green synthesized Silver Nanoparticles (AgNPs) using plant extracts has attracted significant interest among scientists due to their antibacterial, antifungal and larvicidal properties [8-11]. However, reports on investigating the bioefficacy of phytosynthesized silver nanoparticles against agricultural insect pests are very limited. In this context, the present study aims to phytosynthesize silver nanoparticles using *Catharanthus roseus* leaf extract. A *Catharanthus roseus* (also known as Madagascar periwinkle) is one of the important medicinal evergreen herbaceous plants that belong to the family apocynaceae [12]. A *Catharanthus roseus* has been reported to exhibit antibacterial, antifungal, antibiotic, antioxidant, wound healing and antiviral properties [13]. More importantly, phytochemicals present in *Catharanthus roseus* leaves such as carbohydrates, alkaloids, glycosides, flavonoids, tannins, saponins, proteins, amino acids, fats and oils reduces Silver salts (Ag^+) to metallic silver, Ag^0 very efficiently [14]. These bioactive compounds also, reported to possess insecticidal property. Thus, the smart use of such plant extracts with reported insecticidal activity acts as not only an *in situ* reducing agent, but also, enhances surface fictionalization through efficient capping. Further, these green synthesized AgNPs were evaluated for their bioefficacy against two major polyphagous lepidopteran insect pests such as *Spodoptera frugiperda* (fall armyworm) and *Plutella xylostella*

(diamondback moth) which have a reputation of causing significant devastating crop losses globally [15]. *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) commonly known as fall armyworm, is a serious polyphagous insect pest that causes significant damage to cereals and vegetable crops with reported to cause yield loss ranging from 20%-100% [16]. Similarly, *Plutella xylostella* (Linnaeus) (Plutellidae: Lepidoptera) a diamondback moth is another major destructive pest on cruciferous crops reported to cause up to 50% loss with an estimated US$ 168 million annually [17,18].

Materials and Methods

Experimental section

Source of leaf: Fresh leaves of *Catharanthus roseus* were collected from UAS, Dharwad campus. Leaves were thoroughly washed several times with tap water then distilled water to remove the dust particles and air dried at room temperature.

Preparation of the *Catharanthus roseus* aqueous leaf extract

The aqueous plant extract was prepared following protocol described with minor modifications. Briefly, 20 g fresh leaves of *Catharanthus roseus* was ground using pestle and mortar in 100 ml of distilled water (20% aqueous plant extract) and filtered through what man No. 41. The resultant filtrate was centrifuged at 12,000 rpm for 10 min and the supernatant was collected and stored at refrigerator at 4°C until further use [19].

Preparation of Silver Nitrate ($AgNO_3$) solution

The Silver Nitrate ($AgNO_3$) was procured commercially from the Sisco Research Laboratories Pvt. Ltd. (SRL India). This silver nitrate solution was used as a precursor for the synthesis of Silver Nanoparticles (AgNPs). The different concentration of silver nitrate solution was prepared by dissolving 0.1 g of Silver Nitrate ($AgNO_3$) in 100 ml of distilled water to obtain 1000 ppm A_gNO_3 solution. Similarly, 500, 1000, 2000, 4000, 8000 and 16000 ppm $AgNO_3$ was prepared by dissolving 0.05 g, 0.1 g, 0.2 g, 0.4 g, 0.8 g and 1.6 g respectively in 100 ml of distilled water.

Phytosynthesis of Silver Nanoparticles (AgNPs) using catharanthus roseus aqueous leaf extract

For the phytosynthesis of Silver Nanoparticles (AgNPs), Silver Nitrate ($AgNO_3$) was used as a precursor and *Catharanthus roseus* aqueous leaf extract was used as both reducing and capping agent. 100 ml of different concentrations such as 500, 1000, 2000, 4000, 8000 and 16000 ppm of aqueous silver nitrate was prepared in a 250 ml conical flask. Then 2.5 ml of plant extract was added to 20 ml aqueous silver nitrate solution. The mixture was boiled and stirred for 60°C for 30 min on a magnetic hot plate with optimum catalytic reaction conditions [20]. As a result, the catalytic reaction reduced pure Ag (I) ions to Ag (0). The formation of silver nanoparticles can be ascertained by monitoring the change in color [19].

Characterization of phytosynthesized Silver Nanoparticles (AgNPs)

UV-Visible spectroscopy and Particle size analysis: UV-Visible spectroscopy analysis was used to ascertain the formation of AgNPs. Briefly, AgNPs were suspended in deionized water and sonicated for uniform distribution of nanoparticles. Then, 4 ml of sonicated AgNPs was analyzed in UV-Vis Spectroscopy (UV-1800 Shimadzu) with the wavelength range of 200 nm-700 nm at room temperature. AgNPs typically absorb between 420 nm-450 nm, confirming the presence of AgNPs [21]. Particle Size Analyzer (Nicomp NANOZ Z3000 PSS) was used to assess the particle size and their mean distribution. Briefly, AgNPs were sonicated for the homogenous mix of nanoparticles and then 1 ml of sonicated AgNPs were analyzed using PSA. Mean particle size with their percent distribution was recorded as per standard protocol [22].

Scanning Electron Microscope (SEM) and Energy Dispersive X-ray spectroscopy (EDX) study

For SEM (Carl Zeiss-EVO-18-UK) micrographs, powdered and dried form of nanoparticles were sprinkled uniformly on a double sided carbon tape with a spatula and pressed lightly to seat on the carbon tape and turn the sample holder upside down and tap it to remove loose material. After sample preparation on the aluminium stubs, the nanoparticles were coated with gold by sputtering instrument to eliminate the charge effect and subjected to SEM under vacuum. SEM images the sample by scanning the surface with a focused beam of electrons. The electrons interact with atoms in the sample, producing signals that digitize the surface morphology and size of the particles. SEM with EDX (X-Maxn 80, Oxford instruments Pvt Ltd) generates information about the chemical composition of a sample, including elements are present, their percent distribution/ concentration [23].

Atomic Force Microscopy (AFM) study

Atomic Force Microscopy (Tri-A SPM, APE. Research Italy) was used to characterize the width, thickness and topography of Silver Nanoparticles (AgNPs). Briefly, sonicated nanoparticle sample was coated on mica or glass substrate sheet and allowed to dry under room temperature. The entire setup was then incubated overnight in a hot air oven at 30°C. Samples were brought to room temperature before AFM analysis. Silver nanoparticles were characterized in dynamic mode using HQ-CSC-7 cantilever. The 3D image of nanoparticles with respect to width, thickness and topography was recorded and filtered for the profile and surface roughness was measured using SPM control software [24].

Fourier Transform Infrared spectroscopy (FTIR) study

The presence of functional groups in biomolecules of the plant extract used for the phyto synthesis of silver nanoparticles was studied by FTIR spectrometer (IR-Tracer 100, Shimadzu) with Attenuated Total Reflectance (ATR) technique. The unique advantage of ATR-FTIR imaging is, it requires minimal or no sample preparation prior to spectral measurements because the penetration depth of IR light in the sample for ATR measurements is independent of sample thickness. Briefly, 1 to 2 drops of silver nanoparticle solution were loaded onto the ATR sample loader and spectral measurements and done in the range of 4000-400 cm^{-1} using standard protocols [25].

Laboratory bioassay studies

Insect rearing and culture: The lepidopteran insect pests such as *Spodoptera frugiperda* and *Plutella xylostella* was maintained in the insectary, department of biotechnology, university of agricultural sciences, Dharwad. To initiate the culture, egg mass and larvae were

collected from the field in the season. Standard insect rearing protocols were followed for culturing both the insects [26,27]. Different instars of *Spodoptera frugiperda* and *Plutella xylostella* larvae were reared on freshly collected, washed, air dried castor and cabbage leaves respectively in the laboratory conditions.

Bio efficacy studies using leaf dip method

For standard leaf dip bioassay for *Plutella xylostella* and *Spodoptera frugiperda*, fresh cabbage and caster leaves respectively were collected, thoroughly washed and air dried. Then the appropriate size of leaf discs were dipped in the pre defined concentrations of plant extract, silver nitrate, green synthesized AgNPs and recommended insecticide solutions for 1 min and shade dried on the tissue paper for 30 min. These treated leaf discs were placed in the petri plates (90 mm x 15 mm size) and thirty synchronized starved larvae in each petri plate were released and allowed to feed on them. Larval mortality was recorded at every 24 h in all the treatments until the end of the experiments. Three biological replicates were maintained for statistical reliability.

Statistical analysis

For each test group, a minimum of 30 second and third instar larvae per replicate and three such independent biological replicates were used in each experiment. The entire bioassay experiment was repeated to achieve statistical reliability. Resultant data were subjected to Kaplan and Meier survival analysis using the graph pad prism program version 8.4.3.

Results and Discussion

Phytosynthesis of Silver Nanoparticle (AgNPs) from *Catharanthus roseus* aqueous leaf extract

For the phytosynthesis of AgNPs, different concentrations (in ppm) of silver nitrate solution was prepared and 20% aqueous leaf extract of *Catharanthus roseus* was used as a reducing and capping agent. Later, mixture containing both the silver nitrate solution and *Catharanthus roseus* aqueous leaf extract (20%) was boiled on hot plate at 60°C for 30 min. The change in solution color from colorless to dark brown was the primary indication of formation of Silver Nanoparticles (AgNPs) (Figure 1). Consistently, a similar change in color was reported from colorless to brown-gray upon addition of *Catharanthus roseus* leaf extract to $AgNO_3$ solution asAgNPs formed in previous studies [28,29].

Characterization of green synthesized Silver Nanoparticles (AgNPs) UV-Visible absorption spectrum and Particle size distribution of phytosynthesized Silver Nanoparticles (AgNPs)

Further confirmation of AgNPs formation was done using UV-Visible spectrophotometer (UV 1800 SHIMADZU). Absorbance range between 420 nm-450 nm was, in particular, used as an indicator to confirm that the Ag^+ has been reduced to metallic Ag^o [30,31]. In the UV spectrum, a single, strong and broad Surface Plasmon Resonance (SPR) peak was centered at 448 nm that confirming the formation of AgNPs. Further mean diameter and particle size distribution of phytosynthesized silver nanoparticles using *Catharanthus roseus* aqueous leaf extract was characterized using particle size analyzer (Nicomp NANOZ Z3000 PSS). Different concentration of silver nanoparticles synthesized revealed varied mean diameter and particle size distribution. In specific, 500, 1000, 2000, 4000, 8000 and 16000 ppm AgNPs revealed particle size with mean diameter of 284.3 nm, 152.1 nm, 84.4 nm, 50.4 nm, 48.9 nm and 69.3 nm respectively. Since 8000 ppm AgNPs resulted in lowest particle size (48.9 nm) (Figure 1), it was decided to use 8000 ppm as a highest concentration for all bioassay studies. The comparable silver nanoparticles size was observed in various other studies also, for instance, *Catharanthus roseus* leaf extract mediated silver nanoparticle size was reported to be 35 nm-55 nm with plasmon resonance peak at 400 nm, 20 nm to 50 nm with a peak absorbance at 500 nm, 49 nm with surface plasmon resonance absorption peak at 425 nm and 40 nm-60 nm [32-34].

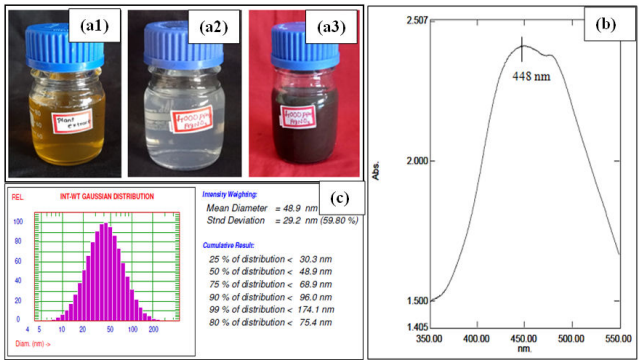

Figure 1: Phytosynthesized Silver Nanoparticles (AgNPs) using *Catharanthus roseus* aqueous leaf extract; (a1): Fresh aqueous leaf extract; (a2): Silver Nitrate ($AgNO_3$) before addition of leaf extract; (a3): After addition of leaf extract; (b): UV-Visible absorption spectrum of phytosynthesized Silver Nanoparticles ($AgNP_s$); (c): Mean particle size distribution of 8000 ppm Silver Nanoparticle (AgNPs) synthesized using *Catharanthus roseus* aqueous leaf extract.

SEM micrographs with Energy Dispersive X-ray (EDX) spectrum obtained for phytosynthesized Silver Nanoparticles (AgNPs)

SEM micrograph obtained for silver nanoparticles synthesized using *C. roseus* leaf extract showed high density nanoscale particles (48 nm) with spherical shape. In the previous studies, SEM micrographs showed spherical shape AgNPs and the presence of elemental silver through EDX spectrum [32-35]. Metallic silver nanocrystals generally show a typical optical absorption peak approximately at 3 KeV to 4 KeV due to surface plasmon resonance [36]. In the present study also, silver nanoparticles synthesized using *Catharanthus roseus* leaves extract showed a sharp peak at 3 KeV indicating the presence of metallic silver. In addition, the EDX profile also showed a sharp peak for other elements such as carbon, oxygen and gold which may have originated from the biomolecules that are bound to the AgNPs surface. The EDX spectrum analysis showed 23.27% silver content, 56.12% carbon, 13. 06% oxygen and 7.55% Gold (Au) (Figure 2).

Atomic Force Microscopy (AFM) image of phytosynthesized Silver Nanoparticles (AgNPs)

The surface topology of phytosynthesized Silver Nanoparticles (AgNPs) was studied by Atomic Force Microscopy (AFM) in dynamic

mode by using HQ-CSC-17 cantilever. The 3D image of nanoparticles in terms of width, thickness and topography was observed. The profile was filtered and surface roughness measured using SPM control software revealed surface of the nanoparticles were rough and undulated.

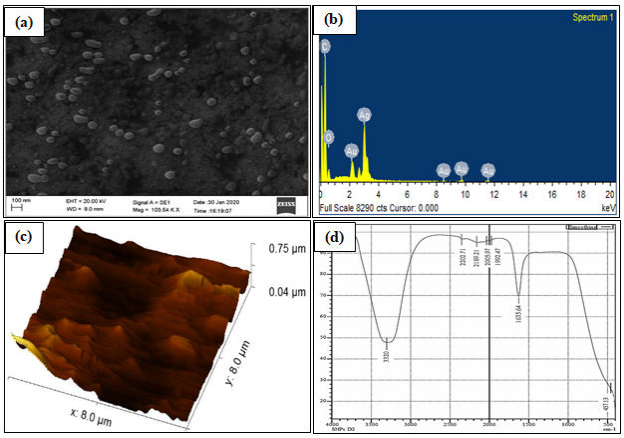

Figure 2: (a): SEM micrograph of green synthesized silver nanoparticles (AgNPs); (b): EDX spectrum; (c): Atomic Force Microscopy (AFM) image; (d): Fourier Transform Infrared (FTIR) spectrum of green synthesized Silver Nanoparticles (AgNPs) using *Catharanthus roseus* aqueous leaf extract.

Fourier Transform Infrared (FTIR) spectrum of phytosynthesized Silver Nanoparticles (AgNPs)

FTIR spectrum (SHIMADZU FTIR model TR-Tracer 100 spectrophotometer) of phyto synthesized silver nanoparticles using aqueous *Catharanthus roseus* leaf extract showed distinct peaks at around 457.13, 1635.64, 1992. 47, 2005.97, 2189.21, 2202.71 and 3320 cm^{-1}. The absorption peak between 1632-1638 cm^{-1} represents characteristic amino acids containing NH_2 groups, amide I band. In particular, the peak at 1635 cm^{-1} was due to -C=O-stretching. The peaks at 1992. 47 cm^{-1} indicating medium C=C=C stretching showed the presence of an allene class of compounds. The peak at 2005.97 cm^{-1} could be assigned to cyanide ion, thiocyanate ion and other related ions. The absorption band at 2189.21 cm^{-1} and 2202.71 cm^{-1} attributed to C≡C stretching showed the presence of an alkyne class of compounds. The existence of a peak at 3320 cm^{-1} could be due to strong and broad O-H stretching of alcohol. Finally, the absorption peak at 457.13 cm^{-1} might be due to the S-S stretching of aryl disulfides. In consistent with the present data, FTIR analysis identified the presence of similar biomolecules (C-O and N-H stretching, presence of carboxylic acid and amide groups) in the *Catharanthus roseus* leaf extract responsible for the bio reduction of Ag^+ ions from $AgNO_3$ to AgNPs [33,34].

Bioefficacy of phytosynthesized silver nanoparticles on lepidopteran pests

Bioassay studies were conducted to evaluate the efficacy of phytosynthesized Silver Nanoparticles (AgNPs) using *Catharanthus roseus* aqueous leaf extract against 2nd and 3rd instar larvae of *Spodoptera frugiperda* and *Plutella xylostella* using the standard leaf dip method. Different concentrations of phytosynthesized silver nanoparticles, plant extract per se, Silver Nitrate ($AgNO_3$) solution per se, along with control (water) and positive control as emamectin benzoate at 0.25 g/l and Coragen at 0.3 ml/l for *Spodoptera frugiperda* and *Plutella xylostella* respectively, were used for bioassay study. The mortality rate was recorded every 24 h until the end of the study (120 h). From the resultant data, mean percent mortality ± SE and Hazard Ratio (HR) with respective 95% of Confidence Interval (CI) ratio was computed using graph pad prism program version 8.4.3.

Bio efficacy study against *Spodoptera frugiperda* larvae

The bio efficacy of plant extract per se, $AgNO_3$ per se (8000 ppm) and different concentrations of phytosynthesized Silver Nanoparticles (AgNPs)-1000, 2000, 4000 and 8000 ppm in terms of their ability to induce mortality were compared with the control (water). When compared to control, plant extract alone (p=0.0206) and $AgNO_3$ per se (0.0205) induced marginally significant mortality in 2nd instar *Spodoptera frugiperda* larvae. However, in case of 3rd instar larvae, there was no significant change in mean mortality (Plant Extract (PE): p=0.153; $AgNO_3$: p=0.153). Comparatively, 2nd instar *Spodoptera frugiperda* larvae were more vulnerable to AgNPs toxicity than 3rd instar larvae. Specifically, 8000 ppm AgNPs induced mean mortality of 48.27%, 51.72%, 79.31%, 86.20% and 100% in 2nd instar, whereas in 3rd instar it was 6.66%, 13.33%, 20 %, 33.33% and 53.33% at 24, 48, 72, 96 and 120 h respectively (p-value <0.0001). Similarly, at 4000 ppm AgNPs, mean mortality was found to be 36.66%, 43.33%, 53.33%, 60.00% and 66.66% in 2nd instar and in 3rd instar it was recorded 3.33%, 6.66%, 13.33%, 26.66% and 40% at 24, 48, 72, 96 and 120 h respectively (p-value <0.0001). In 2000 ppm AgNPs, mean mortality was observed by 20%, 23.33%, 26.66%, 26.66%, 30% and 3.33%, 6.66%, 16.66%, 26.66% and 30.00% at 24, 48, 72, 96 and 120 h in 2nd and 3rd instar respectively (p-value 0.0012). In the final concentration tested (1000 ppm AgNPs), mean mortality observed was 3.33%, 3.33%, 6.66%, 20% and 26.66% in 2nd instar (p=0.0026), whereas in 3rd instar, it was 0.00%, 3.33%, 10.00%, 13.33% and 20.00% at 24, 48, 72, 96 and 120 h respectively (p=0.0104). As expected emamectin benzoate (at 0.25 g/l) induced 100% mortality within 24 h in both 2nd and 3rd instar larvae of Spodoptera frugiperda (Table 1 and Figure 3).

Groups	2nd instar						3rd instar					
	Mean % mortality (± SEM)					Log-rank test (p- value)	Mean %mortality (± SEM)					Log-rank test (p-value)
	24 h	48 h	72 h	96 h	120 h		24 h	48 h	72 h	96 h	120 h	

Control (Water)	0	0	0	0	0		0	0	0	0	0	
Plant extract (20%)	6.66 (4.55)	6.66 (4.55)	6.66 (4.55)	6.66 (4.55)	16.66 (6.80)	0.0206*	0	0	0	0	6.66 (4.55)	0.1538 NS
$AgNO_3$ (8000 ppm)	0	0	3.33 (3.27)	13.33 (6.20)	16.66 (6.80)	0.0205*	0	3.33 (3.27)	6.66 (4.55)	6.66 (4.55)	6.66 (4.55)	0.1538 NS
1000 ppm AgNPs	3.33 (3.27)	3.33 (3.27)	6.66 (4.55)	20.00 (7.30)	26.66 (8.07)	0.0026**	0	3.33 (3.27)	10.00 (5.47)	13.33 (6.20)	20.00 (7.30)	0.0104*
2000 ppm AgNPs	20.00 (7.30)	23.33 (7.72)	26.66 (8.07)	26.66 (8.07)	30.00 (8.36)	0.0012**	3.33 (3.27)	6.66 (4.55)	16.66 (6.80)	26.66 (8.074)	30.00 (8.36)	0.0012**
4000 ppm AgNPs	36.66 (8.79)	43.33 (9.04)	53.33 (9.10)	60.00 (8.94)	66.66 (8.60)	<0.0001 ****	3.33 (3.27)	6.66 (4.55)	13.33 (6.20)	26.66 (8.074)	40.00 (8.94)	0.0001***
8000 ppm AgNPs	48.27 (9.27)	51.72 (9.27)	79.31 (7.52)	86.2 (6.40)	100.00 (0.00)	<0.0001 ****	6.66 (4.55)	13.33 (6.20)	20.00 (7.30)	33.33 (8.60)	53.33 (9.10)	<0.0001 ****
Emamectin benzoate(0.25 g/l)	100.00 (0.00)	-	-	-	-	<0.0001 ****	100.00 (0.00)	-	-	-	-	<0.0001 ****

Note: * Different treatments compared with control (water); ** Mean percent mortliaty; *** Significance level at ≤ 0.05 computed using graph pad prism with log rank test. **** Values in parenthesis are standard error mean (± SEM)

Table 1: Mean percentage mortality (± SEM) of Spodoptera frugiperda 2nd and 3rd instar larvae exposed to control and different concentrations of AgNPs.

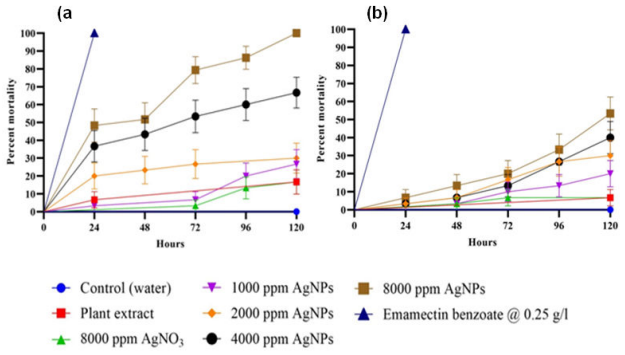

Figure 3: Mean percent mortality (± SEM) of *Spodoptera frugiperda* 2nd (a) and (b) 3rd instar larvae exposed to different treatments.

The toxicity of different concentrations of AgNPs (1000, 2000, 4000 and 8000 ppm) in comparison with the $AgNO_3$ was computed using hazard ratio with 95% CI and log rank test through the graph pad prism program. Resultant statistical analysis revealed the hazard ratio (with 95% CI) of 1000, 2000 ppm AgNPs was 1.67 (95% CI: 0.56-4.95; p=0.3459) and 2.06 (95% CI: 0.72-5.89; p=0.1723) implying comparatively AgNPs toxicity was higher with statistically non-significant when compared to $AgNO_3$ toxicity for 2nd star larvae. However, at higher AgNPs concentration 4000 and 8000 ppm, the hazard ratio was 5.71 (95% CI: 2.57-12.67; p<0.0001) and 11.20 (95% CI: 5.51-22.75; p<0.0001) suggesting significantly higher toxicity against 2nd instar larvae when compared to $AgNO_3$ per se. Similarly, mortality data of 3rd instar larvae revealed 1000 and 2000 ppm AgNPs toxicity level was higher with no significance to marginally significant change was observed when compared to $AgNO_3$ alone. The hazard ratio for 1000 ppm was 3.07 (95% CI: 0.76-12.28; p=0.1434) and for 2000 ppm was 4.83 (95% CI: 1.48-15.77; p=0.0232). As expected the hazard ratio for 4000 and 8000 ppm was 6.64 (95% CI: 2.32-18.97; p=0.0033) and 9.53 (95% CI: 3.77-24.10; p<0.0001). This data clearly indicates the toxicity (hazard ratio) of all the tested AgNPs were higher compared to $AgNO_3$ per se. Hence, AgNPs were able to induce significantly higher mortality specially, at higher concentrations in 2nd and 3rd instar larvae of *Spodoptera frugiperda*. Although there were no single reports on bioefficacy of green synthesized silver nanoparticles specifically against *Spodoptera frugiperda* larvae, comparable mortality was reported in related species such as *Spodoptera litura* using other than *Catharanthus roseus* leaf extract. [37] reported that soya based AgNPs (87 nm) induced 100% mortality in 2nd instar *Spodoptera litura* larvae at 8000 and 10000 ppm within 72 h and in 3rd instar larvae at 96 h at 10000 ppm specifically. Similarly, Bharani reported that pomegranate peel extract mediated silver nanoparticles at the highest concentration of 100 µg induced 100%, 100%, 86.4%, 78.4%, 61.3% and 58.2% in 1st 2nd, 3rd, 4th, 5th and 6th, instar larvae of *Spodoptera litura* respectively. More recently, in another related species *Spodoptera littoralis* (cotton leaf worm), the larvicidal activity of Silver Nanoparticles (AgNPs) synthesized using borago officinalis leaf extract was demonstrated. 100% mortality in 3rd instar larvae of *Spodoptera littoralis* was reported within 24 h of exposure at 4.0 mg/g AgNPs concentration [38,39].

Bio efficacy study against *Plutella xylostella* larvae

The insecticidal efficacy of different concentrations of green synthesized Silver Nanoparticles (AgNPs) (1000, 2000 and 4000 ppm), plant extract per se, Silver Nitrate solution ($AgNO_3$) per se were compared with control/ water in terms of their ability to induce percent mean mortality. When compared to control, plant extract per se (p=0.157) and $AgNO_3$ per se (p=0.2322) did not induce significant mortality in 2nd instar of *Plutella xylostella* larvae. But in the case of 3rd instar larvae, plant extract per se induced marginally significant (Plant Extract (PE): p=0.0495) mortality, $AgNO_3$ per se did not induce any significant mortality ($AgNO_3$: p=0.1570). Comparative mortality data revealed 2nd instar *Plutella xylostella* larvae were more susceptible to AgNPs toxicity than the 3rd instar larvae. In specific, mortality rate induced by 4000 ppm AgNPs were 37.93%, 65.51%, 79.31%, 82.75% and 82.75% in 2nd instar larvae, whereas in 3rd instar larvae it was 33.33%, 36.66%, 46.66 %, 46.66% and 66.66% at 24, 48, 72, 96 and 120 h respectively (p-value <0.0001). Similarly, at 2000 ppm AgNPs, mortality rate was observed to be 23.33%, 30.00%, 36.66%, 50.00% and 63.33% in 2nd instar larvae and in case of 3rd instar larvae it was found 26.66%, 30.00%, 43.33%, 46.66%, 53.33%at 24, 48, 72, 96 and 120 h respectively (p-value <0.0001). Finally, 1000 ppm AgNPs showed mean mortality rate of 6.66%, 13.33%, 13.33%, 50,00%, 50.00% in 2nd instar larvae (p-value=0.0002) and 20.00%, 33.33%, 33.33%, 36.66%, 50.00% in 3rd instar larvae at 24, 48, 72, 96 and 120 h respectively (p-value<0.0001). However, recommended insecticide, Coragen (0.3 ml/l) exhibited 100% mortality at 120 h in 2nd instar larvae, whereas only 86.66% mortality in 3rd instar larvae (Table 2 and Figure 4).

	2nd instar						3rd instar					
Groups	**Mean% mortality (± SEM)**					**Log-rank test**	**Mean %mortality (± SEM)**					**Log-rank test (p-value)**
	24 h	**48 h**	**72 h**	**96 h**	**120 h**		**24 h**	**48 h**	**72 h**	**96 h**	**120 h**	**(p-value)**
Control (water)	0	0	0	3.33 (3.27)	6.66 (4.55)		0	0	0	3.33 (3.27)	3.33 (3.27)	
Plant extract (20%)	0	0	3.12 (3.07)	9.37 (5.15)	18.75 (6.90)	0.157NS	3.22 (3.17)	9.67 (5.31)	9.67 (5.31)	16.12 (6.60)	19.35 (7.09)	0.0495*
$AgNO_3$ (4000 ppm)	0	3.33 (3.27)	3.33 (3.27)	6.66 (4.55)	16.66 (6.80)	0.2322 NS	6.66 (4.55)	6.66 (4.55)	13.33 (6.20)	13.33 (6.20)	13.33 (6.20)	0.157 NS
1000 ppm AgNPs	6.66 (4.55)	13.33 (6.20)	13.33 (6.20)	50.00 (9.12)	50.00 (9.12)	0.0002***	20.00 (7.30)	33.33 (8.60)	33.33 (8.60)	36.66 (8.79)	50.00 (9.12)	<0.0001****
2000 ppm AgNPs	23.33 (7.72)	30.00 (8.36)	36.66 (8.79)	50.00 (9.12)	63.33 (8.79)	<0.0001****	26.66 (8.07)	30.00 (8.36)	43.33 (9.04)	46.66 (9.10)	53.33 (9.10)	<0.0001****
4000 ppm AgNPs	37.93 (9.01)	65.51 (8.82)	79.31 (7.52)	82.75 (7.014)	82.75 (7.014)	<0.0001****	33.33 (8.60)	36.66 (8.79)	46.66 (9.10)	46.66 (9.10)	66.66 (8.60)	<0.0001****
Coragen (0.3 ml/l)	20.00 (7.303)	46.66 (9.10)	80.00 (7.303)	90.00 (5.477)	100.00 (0.00)	<0.0001****	33.33 (8.60)	40.00 (8.94)	60.00 (8.94)	73.33 (8.07)	86.66 (6.20)	<0.0001****

Note: * Different treatments compared with control (water); *** Mean percent mortliaty and significance level at ≤ 0.05 computed using Graph Pad Prism with Log-rank test. **** Values in parenthesis are standard error mean (**±** SEM)

Table 2: Mean percentage mortality (± SEM) of *Plutella xylostella* 2nd and 3rd instar larvae exposed to control and different concentrations of AgNPs.

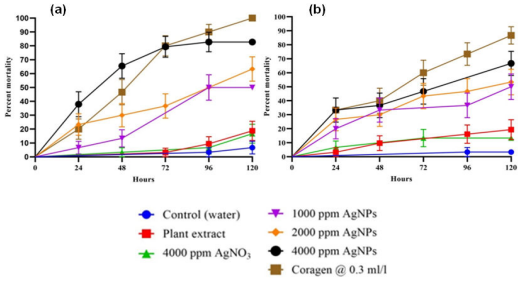

Figure 4: Mean percent mortality (± SEM) of *Plutella xylostella* 2nd (a) and (b) 3rd instar larvae exposed to different treatments.

Hazard ratio with 95% CI and Log-rank test was computed using the graph pad prism program to compare the toxicity of different concentrations of AgNPs (1000, 2000 and 4000 ppm) with $AgNO_3$ per se. Resultant data revealed that the hazard ratio (with 95% CI) of 1000 AgNPs was 3.54 (95% CI: 1.47 to 8.54; p=0.0046) indicating 3.54 fold higher AgNPs toxicity with marginally significant change when compared to the toxicity of $AgNO_3$ per se against 2nd instar larvae. However, for 2000 and 4000 ppm AgNPs were recorded to be 5.26 (95% CI: 2.33 to 11.84; p<0.0001) and 8.32 (95% CI: 3.91 to 17.73; p<0.0001) respectively suggesting significantly higher AgNPs toxicity against 2nd instar larvae when compared to $AgNO_3$ per se (Table 3). Similarly, 3rd instar larvae mortality data revealed that the toxicity level of 1000 and 2000 ppm AgNPs were significantly higher compared to $AgNO_3$ per se. The hazard ratio obtained for 1000 ppm AgNPs was 4.34 (95% CI: 1.76 to 10.70; p=0.0029) and at 2000 ppm AgNPs, it was 4.72 (95% CI: 1.96 to 11.39; p<0.0011). The toxicity level of 4000 ppm AgNPs was much higher with an enhanced significant level (hazard ratio-6.19; 95% CI: 2.76 to 13.84; p<0.0001). The above data clearly indicated, the toxicity of the tested concentration of AgNPs was higher compared to $AgNO_3$ per se. Hence AgNPs were able to induce significantly higher mortality especially at higher concentration in 2nd and 3rd instar larvae of *Plutella xylostella* (Table 3).

Spodoptera frugiperda						***Plutella xylostella***					
Groups		**2nd instar larvae**		**3rd instar larvae**		**Groups**		**2nd instar larvae**		**3rd instar larvae**	
		Hazard Ratio (95% CI)	**Log-rank test p-value**	**Hazard Ratio (95% CI)**	**Log-rank test p-value**			**Hazard Ratio (95% CI)**	**Log-rank Test p-value**	**Hazard Ratio (95% CI)**	**Log-rank Test p-value**
$AgNO_3$ (8000 ppm)	1000 ppm AgNPs	1.67 ↑ (0.56-4.95)	0.3459NS	3.07 ↑ (0.76-12.28)	0.1434NS	$AgNO_3$ (4000 ppm)	1000 ppm AgNPs	3.54 ↑ (1.47-8.54)	0.0046**	4.34 ↑ (1.76-10.70)	0.0029**
	2000 ppm AgNPs	2.06 ↑ (0.72-5.89)	0.1723NS	4.83 ↑ (1.48-5.77)	0.0232*		2000 ppm AgNPs	5.26 ↑ (2.33-11.84)	<0.0001****	4.72 ↑ (1.96-11.39)	0.0011**
	4000 ppm AgNPs	5.71 ↑ (2.57-12.67)	<0.0001****	6.64 ↑ (2.32-18.97)	0.0033**		4000 ppm AgNPs	8.32 ↑ (3.91-17.73)	<0.0001****	6.19 ↑ (2.76-13.84)	<0.0001****
	8000 ppm AgNPs	11.20 ↑ (5.51-22.75)	<0.0001****	9.53 ↑ (3.77-4.10)	<0.0001****		-	--	-	-	-

Note: ** Different treatments of AgNPs compared with $AgNO_3$ 8000 ppm and 4000 ppm; **** Hazard ratio and significance level at ≤ 0.05 computed using graph pad prism with log rank test; CI-Confidence Interval

Table 3: The Hazard Ratio (HR) with 95% CI of *Spodoptera frugiperda* and *Plutella xylostella* 2nd and 3rd instar larvae exposed to different concentration of AgNPs compared to $AgNO_3$ (8000 ppm) and $AgNO_3$ (4000 ppm) respectively.

Previous studies reported that the insecticidal activity of phytosynthesized AgNPs using 8 plant extracts datura, ginger, neem, clove, bitter gourd, eucalyptus, baking and garlic against 3rd instar larvae of *Plutella xylostella* revealed the mortality rate of 84, 88, 92, 88, 84, 80, 88 and 84% at the highest concentration of 2.0 mg/ml, 2.3 mg/ml, 2.4 mg/ml, 2.8 mg/ml, 3.0 mg/ml, 3.0 mg/ml, 4.0 mg/ml and 23 mg/ml respectively [40]. In another study, phytosynthesized AgNPs using seaweed (*Hypnea musciformis*) leaf extract induced significant mortality in both 1st instar larvae and pupae of *Plutella xylostella.* LC_{50} ranged from 24.5 to 38.23 ppm [41].

Conclusion

Collectively, the present study suggests the utility of phytosynthesized silver nanoparticles using *Catharanthus roseus* aqueous leaf extract as potential larvicidal agent against novel invasive insect pest *Spodoptera frugiperda* and notoriously insecticide resistant cruciferous pest *Plutella xylostella*. Silver nanoparticles were successfully synthesized and characterized using *Catharanthus roseus* leaf extract with a size below 100 nm. Further bioefficacy studies against lepidopteran pests revealed that plant extract per se and 8000 ppm $AgNO_3$ per se did not because significant mortality in both 2nd and 3rd instar larvae of *Spodoptera frugiperda* and *Plutella xylostella*. Different concentrations of Silver Nanoparticles (AgNPs) induced progressively varied mortality in both *Spodoptera frugiperda* and *Plutella xylostella* as compared to Silver Nitrate ($AgNO_3$) solution. As anticipated, compared to emamectin benzoate at 0.25 g/l (positive check), the toxicity of Silver Nanoparticles (AgNPs) was significantly less and 2nd instar larvae were more susceptible to AgNPs than 3rd instar larvae. Interestingly, Silver Nanoparticles (AgNPs) at 4000 ppm induced comparable mortality with recommended insecticide coragen (0.3 ml/l) against *Plutella xylostella*. Collectively the present study suggests the promising potentials of phytosynthesized AgNPs for agricultural pest management.

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