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Phytofabrication of silver nanoparticles using *Annona reticulata* and assessment of insecticidal and bactericidal activities

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Abstract

Aim : Silver nanoparticle synthesis using plant products is now popular due to its environment friendly, easy availability, simple procedure and fascinating properties. In this study, an attempt was made on leaf extract of *Annona reticulata* L silver nanoparticles to synthesize and to assess its bactericidal and insecticidal properties.

Methodology : Using plant material for silver nanoparticle synthesis is one of the bottom up approaches of silver nanoparticle synthesis. In the present study leaf extract of *Annona reticulata* was used for nanoparticle synthesis. The synthesized nanoparticles were characterized by X-Ray Diffraction (XRD), Scanning Electron Microscopy (SEM), Fourier-transform infrared spectroscopy (FTIR) and Energy Dispersive X-Ray Analysis (EDAX). The bactericidal property was evaluated against *E. coli*, *P. aeruginosa*, *S. aureus*, *B. subtilis* and *P. vulgaris* where as insecticidal property was tested against mosquito larvae and *Sitophilus oryzae*.

Results : The XRD spectrum confirmed the formation of silver nanoparticles. SEM observation showed that nanoparticles were 17.33 nm in size and spherical in shape. The EDAX result revealed the presence of silver in the suspension. Silver nanoparticles exhibited higher repellency against *Sitophilus oryzae*. and feeding deterrence. The nanoparticles possessed a strong larvicidal activity when they were used against fourth instar larvae of mosquitoes. The silver nanoparticles showed bactericidal activity against *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*.

Interpretation : The present study strongly suggests that *Annona reticulata* leaf extract can be used to fabricate silver nanoparticles with potent insecticidal and bactericidal properties.

Key words: *Annona reticulata*, Antibacterial activity, Insecticidal activity, Phytofabrication, Silver nanoparticles

Silver nanoparticles synthesis using leaf
extract of *Annona reticulata*

Characterization of
nanoparticles-UV
spectroscopy, FT-IR,
SEM, EDAX & XRD

Insecticidal activity, Insect
repellency test, Feeding
deterrence activity &
Bactericidal activity

Silver nanoparticles exhibit multiactivities such as insecticide,
repellent, deterrent, larvicidal and bactericidal activities

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Introduction

The unique properties of metallic nanoparticles have drawn the attention of many researchers to work on them in comparison to bulk materials. Fabrication of nanoparticles can be done using two approaches top-down and bottom-up. The top-down approach involves synthesis of nanoparticles from bulk material and has a limitation in the form of improper surface nature of nanoparticles and high cost (Thakkar *et al.*, 2010; Mittal *et al.*, 2013). The chemical and biological methods of synthesis belong to the bottom-up approach. In these methods, the possibility of getting uniform particle size is high but the chemical mediated synthesis can cause toxicity to health and environment (Aziz and Jassim, 2018). Hence, the biological mode of synthesis is the most preferred method in these days.

Biological method can be microorganism mediated synthesis or plant mediated; out of which, the former is not preferred due to the possibilities of biohazards and the extra step of culture of microorganisms, besides being expensive (Rajagopal *et al.*, 2015). The advantages of using plants for the synthesis of silver nanoparticles are that they are readily available, show vast diversity, are safe to handle and have various metabolites that may be involved in reduction. Moreover, the plant mediated synthesis is an advance of bioscience in a sense that it is high yielding, involves low cost technology and is non-toxic to vertebrate animals (Chaudhari *et al.*, 2016). Plant materials are considered free of toxins, hence, they can be used in medical fields. It is presumed that synthesized silver nanoparticles have properties of both silver nanoparticles and plant. Hence, plants are considered as "Nature's nanofactory" for nanoparticle synthesis (Jha *et al.*, 2009).

Silver nanoparticles gained much importance over the years due to their remarkable optical, electrical and antimicrobial properties. Silver nanoparticles are considered as good alternative to antibiotics against microorganisms. One gram of silver nanoparticles is known to impart bactericidal properties to hundreds of square meter of substrate material (Thakkar *et al.*, 2010). In addition to this, they have proved effective against wide range of microorganisms, as they prevent the creation of microorganism resistance (Aziz and Jassim, 2018).

Previous studies have reported the synthesis of silver nanoparticles using *Annona squamosa* and their *in-vivo* cytotoxic effect (Vivek *et al.*, 2012) and antibacterial activity (Jagtap *et al.*, 2013). Present study reports the phytofabrication of silver nanoparticles using *Annona reticulata* which is an important medicinal plant that possesses insecticidal and bactericidal properties. It is presumed that the silver nanoparticles fabricated using leaves of this plant may have potent insecticidal and bactericidal properties. Hence, the present study aimed to fabricate silver nanoparticles using leaves of *A. reticulata*, and the efficacy of the same for controlling the stored insect pest, mosquito larva and pathogenic bacteria was evaluated.

Materials and Methods

Preparation of *Annona reticulata* L. leaf extract : Custard apple (*Annona reticulata* L.) leaves were collected from Oothur, Kodaikanal, Tamil Nadu, India. Ten gram of matured leaves (8-9 cm) were washed with double distilled water and chopped into 10 mm in size and air dried at room temperature. The dried chopped leaves were boiled for 15 min at 60°C in 100 ml double distilled water. After boiling, the extract was purified by filtering through Whatman No. 1 filter paper and kept at 4°C until use.

Phytofabrication of silver nanoparticles and its characterization: Reaction mixture was prepared by mixing 1 mM silver nitrate (90 ml) with leaf extract (10 ml) and kept at room temperature in dark condition for reduction. The colour change in the reaction mixture indicated the completion of reduction reaction. The colour change in the reaction mixture was checked periodically. After colour change, the solution was filtered through Whatman No. 1 filter paper and the absorbance was read at 300 to 700 nm using UV-Visible spectrophotometer. The silver nanoparticle solution was purified by continuous centrifugation (1000 rpm for 10 min) and redispersed in double distilled water. The synthesized silver nanoparticles were subjected to Fourier-transform infrared spectroscopy (FTIR), Scanning Electron Microscopy (SEM), Energy-dispersive X-ray spectroscopy (EDAX) and X-ray Diffraction (XRD) analysis for studying functional groups, shape, size and presence of elemental silver, respectively. These analysis were carried out at University Science Instrumentation Centre, Alagappa University, Karaikudi.

Insecticidal activity: Insecticidal activity of silver nanoparticles was assessed in stored insect pest, *Sitophilus oryzae*. Thirty adult insects were obtained from our laboratory where it was reared from infested rice collected from local market. The insect specimens were identified in the Department of Entomology, Tamil Nadu Agricultural University, Madurai. The toxicity of synthesized nanoparticles against *S. oryzae* was assessed (Subramanyan and Roesli, 2000).

Repellency test: The repellency of synthesized nanoparticles, leaf extract and silver nitrate against *S. oryzae* was assessed by the procedure adopted from Auamcharoen *et al.* (2012). Briefly, Whatman no 1 filter paper (9 cm) was divided into two equal halves such as control side and experimental side (treated with synthesized nanoparticles (100 µl) / leaf extract (100 µl)/ silver nitrate (100 µl of 1 mM AgNO₃). The control half was not treated. The insects (10 nos) were released on the middle of the paper and the number of insects present in each side of the filter paper at different time intervals was counted (1 hr each for five times).

Feeding deterrence activity : Antifeedant activities were evaluated by the method of Mahdi and Rahman (2008) with slight modifications. Five gram of rice impregnated with synthesized nanoparticles (1ml), leaf extract (1%) and silver nitrate (1mM AgNO₃) were placed in separate boxes. Then, 10 adult *S. oryzae* were allowed to feed for 7 days. After feeding period, grains were weighed and weight loss was measured.

Histopathological findings of *Sitophilus oryzae* after exposure to silver nanoparticles: The silver nanoparticle treated adult *Sitophilus oryzae* after death were subjected to routine histological analysis. The dead insects were fixed in alcoholic Bouin's solution for 24 hrs. Each insect was subjected to microtechnique through serial dilution, infiltration, embedding and staining following standard procedure (Humason, 1979).

Larvicidal activity: The mosquito larvae (4th instar) of *Aedes aegypti* (10 for each concentration) and *Culex quinquefasciatus* (10 for each concentration) were placed in 100 ml of water containing various concentrations (10 ppm, 20 ppm, 30 ppm, 40 ppm and 50 ppm) of silver nanoparticles. Sterile water without nanoparticles and leaf extract served as control (Gnanadesigan et al., 2011).

Histopathology of mosquito larvae after exposure to silver nanoparticles: Histopathological observations of the effects of

synthesized silver nanoparticles were conducted on fourth instar larvae of *Aedes aegypti* and *Culex quinquefasciatus*. Larvae treated with synthesized silver nanoparticles, leaf extract and AgNO₃ and untreated larvae were fixed, dehydrated and mounted in paraffin blocks. Sections of 8 µm thickness of larval tissue were made, mounted on glass slides and stained with haematoxylin and eosin for histopathological examination under light microscope.

Bactericidal activity : Agar diffusion assay was used to determine the antibacterial activity of nanoparticles against pathogenic microorganisms like *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis* and *Proteus vulgaris*.

Statistical analysis: The data presented in tables of repellency test, feeding deterrence activity and antibacterial activity are mean ± SE. Data corresponding to the performance of larvicidal effect of AgNPs were subjected to analysis of variance and

Fig. 1 : Histological sections of *Sitophilus oryzae* after exposure to silver nanoparticles, *A. reticulata* leaf extract and AgNO₃; (A) Control; (B) silver nitrate; (C) leaf extract and (D) synthesized silver nanoparticles; (EL- Epithelial layer; ML- Membrane layer).

Fig. 2 : Histological sections of 4th instar larvae of *Aedes aegypti* and *Culex quinquefasciatus*; after exposure to silver nanoparticles, leaf extract and AgNO₃. (A) Control; (B) Silver nitrate treated; (C) *A. reticulata* leaf extract treated and (D) Synthesized silver nanoparticle treated (EP-Epithelial cells; PM- Peritrophic matrix; L-Lumen; MV- Microvilli).

Table 1: Repellency (%) of synthesized silver nanoparticles (Ag NPs), leaf extract and silver nitrate (Ag NO₃) against *Sitophilus oryzae* adults

Concentration of samples (100 µl cm ⁻²)	Duration of exposure				
	1 hr	2 hr	3 hr	4 hr	5 hr
Control + AgNO ₃	10	30	30	40	50
Control + Silver nanoparticles	30	40	50	80	90
Control + leaf extract	20	20	30	40	60

Temperature = 24±1°C; Relative humidity = 65-70%; 16L: 8D photoperiod. The values are % repellency

average mortality data was calculated LC₅₀, LC₉₀ lower confidence limit and upper confidence limit values at 95% using EPA Probit analysis program (version 1.5). P<0.05 was considered as significantly different for all statistical tests.

Results and Discussion

Silver nanoparticle synthesis in reaction mixture was easily predicted initially by visible observation. In the present study, the colour change (watery to yellowish brown) occurred in the reaction mixture after 24 hrs. It indicates the reduction of silver ions in the reaction mixture and possible silver nanoparticle synthesis. It is well known that excitation of surface plasmon vibrations of silver nanoparticles in solution show yellowish - brown colour (Thirumurgan et al., 2010; Mittal et al., 2013).

After colour change in the filtered reaction mixture was detected by UV-Vis spectrophotometer. In the present study, the absorption spectra of the reaction mixture showed absorbance peak at 455 nm and broadening of peak indicated that the particles were polydispersed. The UV signature at 440-460 nm proved the presence of silver nanoparticles in the reaction mixture (Emeka et al., 2014). Similar phenomenon was reported in *Argemone mexicana* leaf extract that exhibited the broadening peaks in the reaction media with absorbance peak at 440 nm (Geethalakshmi and Sarad, 2010; Singh et al. 2010). Swamy and Prasad (2012) obtained absorbance peak at 423 nm when leaf extract of *Santalum album* was used for silver nanoparticle synthesis.

FTIR spectral analysis revealed the functional groups in the synthesized silver nanoparticles which are responsible for capping and stabilization of silver nanoparticles. The spectrum showed the presence of ten active functional groups in the synthesized silver nanoparticles. The bands appeared at 3438.84 cm⁻¹, 2931.45 cm⁻¹, 2881.45 cm⁻¹, 1741.60 cm⁻¹, 1670.24 cm⁻¹, 1454.23 cm⁻¹, 1384.79 cm⁻¹, 1340.43 cm⁻¹, 1080.06 cm⁻¹ and 1043.42 cm⁻¹ corresponding to N-H stretching of aliphatic amides, O-H stretching of carboxylic acid, C-H stretching of alkanes, C=O stretching of ketones, C-H bond of alkanes, CH₃ C-H bond of alkanes, C-F, C-O stretching of alcohols, respectively. Similar trend was reported by Tripathi et al. (2013). Silver nanoparticles were synthesized using *Ficus panda* leaf which revealed the presence of functional groups like alkene, amide, alkane, ester

and ether on synthesized nanoparticles. These molecules may surround the synthesized silver nanoparticles and play dual role such as synthesis and stabilization of nanoparticles (Tripathi et al., 2013). The morphology and size of the biosynthesized silver nanoparticles were provided by SEM images. The diameter of synthesized nanoparticles was 15-25 nm. SEM images showed that the silver nanoparticles were synthesized in high density by *Annona reticulata* leaf extract. The shape of the silver nanoparticles was found to be spherical in nature.

A large peak of silver in the EDAX result confirmed the presence of silver in the suspension. The rest of the peaks may be attributed to the presence of other compounds in the leaf extract. This result is consistent with the earlier reports in which *Elettaria cardamomom* (Gnana Jobitha et al., 2012) exhibited silver nanoparticles. The XRD pattern of silver nanoparticles revealed different intensity peaks in the whole spectrum of 2θ values ranging from 0 to 90°. The silver nanoparticles produced in the present experiment were present in the form of nanocrystals as evidenced by the peaks at 2θ values of 38.40, 32.50 and 28.10 corresponding to the diffraction exhibited from 4 - 90° at range of 2θ. The average estimated particle size of the sample was 17.33 nm. The insecticidal activity of synthesized nanoparticles against *Sitophilus oryzae* was evaluated. In the toxicity assay, the synthesized nanoparticles exhibited higher activity against *S. oryzae* adults tested with rice and without rice than leaf extract and silver nitrate. When the insects with rice grains were treated with silver nanoparticles, 20% mortality was observed after 24 hrs. It increased to 40% after 48 hrs at 100 µl concentration. When the insects were treated with leaf extract and silver nitrate alone at 100 µl, only 10% mortality was noticed after 24 hrs and 20% after 48 hrs. When insects without rice grains were treated with silver nanoparticles, 50% mortality was observed after 24 hrs and 100% mortality was observed at the concentration of 100 µl after 48 hrs. When the insects were treated with leaf extract and silver nitrate, 10% and 30% mortality were noticed after 24 hrs and 30% and 50% mortality were noticed after 48 hrs at the concentration of 100 µl, respectively. The reports on insecticidal activity of silver nanoparticles are meager. The present study provides the evidence that synthesized nanoparticles exhibited higher toxicity against *S. oryzae* adults when reared without rice than reared with rice. The synthesized silver nanoparticles were repellent to *S. oryzae* (Table 1). The nanoparticles exhibited higher repellency effect against *S. oryzae* than silver nitrate and leaf extract alone. After releasing insects in the test chamber, they

Table 2 : Larvicidal activity of silver nanoparticles (Ag NPs) synthesized by using *Annona reticulata* leaf extract against larvae of *A. aegypti* and *C. quinquefasciatus*

Species	Concentration of AgNPs (ppm)	% mortality	LC ₅₀ (ppm)	UCL – LCL (ppm)	LC ₉₀ (ppm)	UCL – LCL (ppm)	X ²
<i>Aedes aegypti</i>	10	30 ± 0.745	2.38	2.66 - 2.11	2.81	3.37 – 2.24	7.81
	20	50 ± 0.687					
	30	60 ± 0.816					
	40	80 ± 0.500					
	50	100 ± 0.000					
<i>Culex quinquefasciatus</i>	10	20 ± 0.471	2.26	2.54 - 1.99	2.81	3.23 – 2.26	7.81
	20	30 ± 0.763					
	30	50 ± 0.105					
	40	70 ± 0.745					
	50	100 ± 0.000					

UCL: Upper Confidence Limit; LCL: Lower Confidence Limit; Values are mean of replicates ± S.D.

showed 30-90% of repellency effect to synthesized silver nanoparticles at 1 to 5 hrs. However, this effect was found to be lesser in leaf extract (20-60%) and silver nitrate (10-50%). At the end of the experiment (5 hrs), the 100 µl cm⁻² concentration of synthesized silver nanoparticles showed strongest repellency effect (90%) against *S. oryzae*.

The feeding deterrence effect was noticed higher in the rice treated with synthesized silver nanoparticles than that of leaf extract and silver nitrate. The study showed the mean weight loss on rice treated with synthesized silver nanoparticles was lower (0.13±0.007 g) compared to control (0.74±0.030 g), silver nitrate (0.48± 0.119 g) and leaf extract (0.31± 0.016 g). Silver nanoparticles induced histological changes in the mid gut of *Sitophilus oryzae*. They destroyed the membrane and epithelial layer of mid gut (Fig. 1). The degradation of layers was found to be higher with silver nanoparticles than the leaf extract and silver nitrate solution. The studies on histopathological changes in the *Sitophilus oryzae* treated with silver nanoparticles are meagre. The present study provides the first evidence that

histopathological changes in the mid gut of *S. oryzae* were due to the application of silver nanoparticles.

The maximum (100 %) larvicidal activity was observed at 50 ppm concentration of synthesized silver nanoparticles against fourth instar larvae of *A. aegypti* and *C. quinquefasciatus* (Table 2). The LC₅₀ values were determined as 2.38 and 2.26 ppm for *A. aegypti* and *C. quinquefasciatus* whereas the LC₉₀ values were found to be 2.81 and 2.81 ppm for *A. aegypti* and *C. quinquefasciatus* respectively (Table 2). These results are in confirmation with the previous study of Sarah *et al.* (2012) who reported the larvicidal activity of silver nanoparticles against *Aedes albopictus* larvae. Compared to control (untreated larvae), the mosquito larvae treated with silver nanoparticles, leaf extract and silver nitrate showed histological changes (Fig.2 a,b). Disorganized cellular architecture was noticed in larvae of both mosquitoes when treated with silver nanoparticles. It is presumed that the larval death is caused due to the penetration of silver nanoparticles in to the larval skin. It is also possible that silver nanoparticles damage some cellular organelles and enzymes

Table 3 : Antibacterial activity of synthesized silver nanoparticles (Ag NPs) using aqueous leaf extract of *Annona reticulata* against bacterial pathogens

Bacterial strains	Zone of inhibition (mm)						
	Control		Different conc. of synthesized AgNPs				
	AgNO ₃ (20 µl)	Plant extract (20 µl)	10 µl	20 µl	30 µl	40 µl	50 µl
<i>E. coli</i>	16±0.353	0	12±0.414	12±0.353	13±0.353	13±0.059	13±0.250
<i>P.aeruginosa</i>	25±0.790	0	13±0.250	15±0.239	17±0.414	17±0.750	18±0.790
<i>S.aureus</i>	0	0	10±0.250	11±0.216	11±0.216	13±0.544	13±0.612
<i>B. subtilis</i>	19±0.250	0	11±0.250	15±0.353	15±0.414	16±0.353	16±0.250
<i>P. vulgaris</i>	0	0	0	0	0	0	0

Values are mean of replicates ± S.E.

(Rai et al. 2009; Rajagopal et al., 2015). The membrane permeability change along with disturbance in proton motive force may also cause loss of cellular function and finally cell death (Sarah et al., 2012). The antibacterial activity assay was carried out on human pathogens like *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis* and *Proteus vulgaris* by standard well diffusion method. Antibacterial efficacy was observed higher in silver nanoparticles than silver nitrate solution and leaf extract alone (Table 3). Bactericidal efficacy at 50 µl concentration of synthesized silver nanoparticles was more effective against the tested bacterial strains. The highest antibacterial activity was observed against *P. aeruginosa* (18±0.790 mm zone of inhibition) followed by *B. subtilis* (16±0.250 mm), *E. coli* (13±0.250 mm) and *Staphylococcus aureus* (13±0.612 mm). However, no activity was noticed against *Proteus vulgaris*.

At present, there is an urgent need to construct antibacterial drugs from other sources as microorganisms develop resistance against most antibiotics. The present study has proved that the synthesized AgNPs possess potent bactericidal activity. The lethal activity of silver nanoparticles on bacteria is still obscure. It is suggested that silver nanoparticles may bind on the bacterial cell wall, and subsequently enter the cell membrane and alter the membrane permeability leading to cell death (Thakkar et al., 2010; Ramamurthy et al., 2013; Aziz and Jassim, 2018). Seralathan et al. (2014) reported that differential bactericidal activity of silver nanoparticles may be due to the presence of capping agents and stabilizers which are used in the synthesis of silver nanoparticle. It is concluded that the silver nanoparticles synthesized from *A. reticulata* exhibit multi-activities such as insecticidal, repellent, deterrent, larvicidal and bactericidal activities. It would be very helpful to design nanoformulations in pesticide industry as well as in therapeutics.

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