Phytofabrication of silver nanoparticles using the mangrove associate, *Hibiscus tiliaceus* plant and its biological activity against certain insect and microbial pests

Usha Rani, P., Prasanna Laxmi, K., Vadlapudi, V. and Sreedhar, B.

ABSTRACT

Plants have a rich source of phytochemicals which can produce very stable and active nanoparticles. We report an economic and ecofriendly phytofabrication of nanoparticles using mangrove associate *Hibiscus tiliaceus* leaf extract for the first time. The synthesized NPs were characterized using different analytical methods. Further these bioinspired sliver nanoparticles (AgNP) were evaluated for insecticidal activities against two major agricultural pests, Tobacco cutworm, *Spodoptera litura* F., the cotton bollworm, *Helicoverpa armigera* H., three major stored product pests, flour beetle, *Tribolium castaneum* H., lesser grain borer, *Rhyzopertha dominica* F. and rice weevil, *Sitophilus oryzae* L. and also antibacterial activity against phytopathogens *Xanthomonas campestris* var campestris and *Ralstonia solanacearum* (Smith). For understanding the differences between the biological activity of biosynthesized and chemically synthesized nanoparticles comparisons between the toxicities and antifeedant activities were made. Transmission Electron Microscopic (TEM) studies showed spherical shaped nanoparticles in a size range of 20-65 nm (average mean size 40), while X-ray diffraction pattern revealed face centered cubic (fcc) structure when *H. tiliaceus* leaf was used for bioreduction. Fourier Transform Infrared Spectroscopy (FT-IR) was carried out to identify the proteins that bound specifically on the Ag surface, which increased the stability of the particles. *H. tiliaceus* mediated AgNPs showed excellent antifeedant activity against *S. litura*, *H. armigera*, but were less toxic to all the stored product pests tested, but comparatively higher than the chemically synthesized AgNPs. The green AgNPs exhibited potent antibacterial activity with varying degrees against *X. campestris* and *R. solanacearum* as evidenced by their zone of inhibition at all concentrations.

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INTRODUCTION

Nanotechnology has become prominent discipline in material science. It is now well known that nano particles can be synthesized using several biological materials such as plants, bacteria, fungi, etc. Gardea-Torresdey *et al.* (2003) prepared silver nanoparticles by using living plants of *Medicago sativa* (Alfalfa) for the first time. Since then hundreds of plants have been used for the biological synthesis of nano materials. It is now established that plants and plant materials act as excellent reducing agents, which catalyze the bulk materials into nano form. Among the nanomaterials, silver, due to its antimicrobial property has become more significant for utilizing for various purposes. A number of approaches are available for the synthesis of silver nanoparticles with different sizes and shapes, such as microware irradiation (Yin *et al.*, 2004), chemical reduction (Golubeva *et al.*, 2010), photochemical method (Harada *et al.*, 2009), and electron irradiation (Li *et al.*, 2010), but all these methods use highly toxic and hazardous chemicals, which may pose

potential and environmental risks. Phytosynthesis of nanoparticles not only provides an environmentally benign method but also serves as natural capping agents. Recently scientists are looking with greater interest towards eco-friendly green synthesis methods. Plants have rich source of phytochemicals which can produce very stable and very active nanoparticles. Green methods mainly using a wide variety of phytochemicals like polyphenols, amino acids, proteins, carbohydrates and organic acids act as both reducing and stabilising agents (Adil *et al*., 2015). Using plants for synthesis of nanoparticles is cheap, efficient and requires less maintenance and is more advantageous over other biological sources, as they (plants) are available at required amount. Previously several reports were available on synthesis of silver nanoparticles employing algae, microorganisms, and terrestrial plants, but work on mangrove plants or its associates is almost nil (Satyavani *et al.*, 2014).

Silver, aluminum, gold, zinc, carbon, titanium, palladium, iron and copper have been routinely used for the synthesis of NPs more than decade. The medicinal and preservative properties of silver have been known for over 2,000 years. Because of unique optical, electrical properties of silver, it has been recognised as an important noble metal for the NPs synthesis. Silver nanoparticles are particularly attractive because of their remarkable physico-chemical properties. Several researchers across the world have tested AgNPs for various activities such as antibacterial (Florence *et al.*, 2013), antifungal (Shreya *et al*., 2014), antiviral (Lara *et al.*, 2010) and anticancer (Fathima *et al.*, 2010) properties. One of the important applications of silver is in management of plant diseases (Sang *et al.*, 2012). Agricultural production is reduced worldwide every year due to plant diseases, leading to more investment of money and more efforts for the control. The genus *Xanthomonas* (Proteobacteria) is a diverse and economically important group of Gram-negative bacteria (Sahayaraj *et al*., 2014). Due to the limitations of existing control measures, bacterial wilt caused by *Ralstonia solanacearum* affects a large number of important agricultural crops during the growing season and throughout the postharvest storage (Xiuping *et al*., 2013). Development of resistance to silver in microbes is improbable due to its action on a broad spectrum of targets in the cell (Inoue *et al*., 2002). Compared with other metals, silver exhibits higher toxicity to microorganisms while it exhibits lower toxicity to mammalian cells (Zhao and Stevens, 1998).

Pests are very important antagonists against agricultural production systems and urgently we need to develop green and safer alternative methods of controlling them. NPs are showing promise in different fields of agricultural biotechnology and helping in production of newer pesticides, insecticides and insect repellents. Therefore, extensive studies are being carried out to screen biogenic nanoparticles for pesticidal property. *Hibiscus tiliaceus* Linn (Malvaceae) is a mangrove associate commonly known as sea hibiscus or beach hibiscus. It mainly grows at coastal ecosystems of the tropics and sub-tropics throughout regions of the world. Many studies are restricted to usage of mangroves and their associate mangroves because they grow in very harsh environment. Marine environmental conditions are extremely different from terrestrial ones; it is surmised that mangroves and mangrove associates have diverse group of compounds which aid in tolerating salinity and other types of stress conditions. These chemicals which may vary from chemicals from the terrestrial plants are expected to produce different sized and shaped nanoparticles. In spite of this, suspected variation between marine mangrove plants and normal terrestrial or other aquatic plants. Till now very few mangroves and their associate plants have been exploited for the purpose of nanomaterial bio synthesis. There is a huge gap of knowledge between terrestrial and

marine plant mediated biofabrication of silver nanoparticles. Our main objective is the biological synthesis of silver nanoparticles using marine plant *H. tiliaceus*, characterization followed by assessing their toxicity effects on a few major agricultural pests, the tobacco hornworm, *Spodoptera litura* F., the cotton bollworm, *Helicoverpa armigera* H., stored product pests, *Tribolium castaneum* H., *Rhyzopertha dominica* F. and *Sitophilus oryzae* L. and also antibacterial activity against phytopathogens *Xanthomonas campestris* var *campestris* and *Ralstonia solanacearum* biovar.

MATERIALS AND METHODS

Plant Materials

Marine plant *Hibiscus tiliaceus* Linn (Malvaceae) was collected from Pedavalasa village (Thallarevu Mandal), 10 km from coringa mangrove forest which is near to Yanam, Kakinada, Andhra Pradesh, India during January, 2015. The fresh leaves of Castor (Kiran var.) plants grown in the laboratory fields were used for rearing of *S. litura* as well as for the antifeedant assays.

Insect cultures

The test insects include agricultural pests, *Spodoptera litura*, the *H. armigera*, stored product pests, *T. castaneum*, *R. dominica* and *S. oryzae*. The larvae of *S. litura* used in this study were obtained from a laboratory colony maintained in the Biology and Biotechnology Division, Indian Institute of Chemical Technology, Hyderabad, India. Neonate larvae that emerged from single egg mass on the same day were reared continuously on fresh castor leaves (*Ricinus communis* L. var Kiran) at room temperature (28 ± 2º C), 65 ± 5% RH and 16: 8 L: D photo period in the laboratory. Big sized plastic bins were used for rearing and fresh castor leaves collected in the morning time were provided as food. The leaves were inserted into conical flasks (50 mL cap) containing tap water and placed in the centre of the plastic tub. The tubs were cleaned every day and fresh diet was provided every day. Third instar larvae (8-day old) were used in the study.

H. armigera larvae were collected from pigeon pea fields at ICRISAT, Patancheru, and Hyderabad, India. They were reared individually in 7.5 mL cells of 50-well tissue culture plates on chickpea based artificial diet (Armes *et al.*, 1992) in the laboratory. After pupation, they were transferred to small round plastic tubs (45 cm dia). Adults were provided with 10% honey-water soaked cotton swabs for feeding and the plastic tubs were covered with fine muslin cloth, where usually the eggs are laid by the female moths. The eggs were collected daily on the surface of the cloth. These laboratory-reared 3rd instar larvae were used for bioassays. The stored product pests, *T. castaneum* and *R. dominica* were reared in 1kg jars containing dry seeds of Jowar (*Sorghum vulgare* L.) whereas *S. oryzae* were reared on whole wheat (*Triticum aestivum* L.). All insect cultures were maintained at 28 ± 2° C and 65 ± 5% relative humidity.

Microbial strains

Two grams negative bacteria *Xanthomonas campestris* (B-001239) and *Ralstonia solanacearum* (B-00418) were procured from the National Bureau of Agriculturally Important Microorganisms Culture Collection (NAIMCC), Mau Nathbhanjan, U.P (India) and maintained in the laboratory.

Chemicals

The solvents and other chemicals used were purchased from Himedia Laboratories Limited, Mumbai. The chemically synthesized nano silver was obtained from Nanolabs. Silver nitrate ($AgNO_3$) used for the synthesis of Ag nanoparticles was purchased from Sigma-Aldrich, USA.

Synthesis and purification of AgNPs

Collected mangrove associate *H. tiliaceus* plants were washed thoroughly with double distilled water and incised into small pieces then finely cut plant materials were weighted (5g) and transferred to 250 mL Erlenmeyer flasks containing 100 ml of double distilled water. After mixing it thoroughly this solution was boiled for 5 minutes at 100^0 C and later filtered through Whatman No.1 filter paper. For the synthesis of AgNPs, 10 mL of leaf broth was mixed with 190 mL of 1 mM $AgNO_3$ aqueous solution and further exposing the reaction mixture directly to sunlight

irradiation at CSIR- Indian Institute of Chemical Technology, Hyderabad, India from the time period between 11 a.m. to 2 p.m. under clear sky condition as there is ample of light and ambient temperature for the photoreduction process. Reduction occurs slowly and colour will change to brown. The Ag NPs obtained by centrifuging at 10,000 rpm for 10 min and were washed three times with deionized water to remove any water soluble material.

Characterization

To determine the time point of maximum production of silver nanoparticles, the absorption spectra of the samples were taken 300 to 800 nm using a UV–vis spectrophotometer (Spectramax M3 molecular devices) operating at the resolution of 1 nm. Size and zeta potential of the synthesized silver nanoparticles were determined by using nanoparticle analyzer (Nano ZS90 instrument, Malvern, UK), Transmission electron microscope (TEM) was performed on a FEI Tecnai F12 (Philips Electron Optics, Holland) instrument operated at 100 kV, X-ray diffraction (XRD) patterns were recorded on Bruker D-8 Advance power XRD, FT-IR measurements was carried out using Thermo Nicolet Nexus 670 spectrometer in the diffuse reflectance mode at a resolution of 4 cm^{-1} in KBr pellets.

Zeta potential measurement and dynamic light scattering (DLS) analysis

The nano material is sonicated for nearly 30 minutes in deionized water and 20 µl of dispersed solution is filled in cuvette for measuring the zeta potential and 2ml was used for dynamic light scattering (DLS) analysis and the method is run for 3 cycles to get average value for both Zeta potential and size using Nano ZS90 instrument, Malvern, UK.

Antifeedant assay

Antifeedant activity of biosynthesized AgNPs from *H. tiliceus* leaf extract was assessed against two lepidopteran agricultural pests, *S. litura* and *H. armigera*. The experiments were conducted with *S. litura* using leaf-disc bioassay method (Devanand and Usha Rani, 2008). The method consists of exposing a known area of surface treated castor leaf disc to starved larvae which are in active feeding stage and later measuring the quantity of leaf disc consumed. A small circular disc (21 cm^2) was cut from the fresh castor leaves. Biogenic silver NPs synthesized from *H. tiliceus* leaf and the synthetic AgNPs at different concentrations (50, 100, 150 and 200 µg/ 21cm^2) were applied separately on the upper surface of the leaf disc with the aid of a micro pipette. After evaporating the solvent for about 5 min at room temperature, leaf discs were kept in individual Petri plates (9 cm dia) lined with a wet filter paper to prevent desiccation. In each Petri plate a single pre starved (for 3hrs) 3rd instar larvae of *S. litura* was introduced and was allowed to feed on treated discs for a period of 24 hours. The leaf discs sprayed with distilled water alone were the controls. All the bioassays were repeated three times and there were ten replicates per each trial. The leaf area consumed was measured after 24 hrs in both treated and control leaf discs using leaf area meter (Area meter AM 300, ADC Bioscientific Ltd).

A different method has been employed to test the antifeedant activity of *H. armigera* larvae. Since the larvae were grown on artificial diet, the same diet was used for evaluation of the compounds. For testing antifeedant activity of biosynthesized and synthetic AgNPs against *H. armigera*, the compound was mixed in the dry portion of the artificial diet. The distilled water (carrier solvent) was then evaporated. The diet treated with distilled water alone was termed as control. Single 3rd instar larva of *H. armigera* was placed on 1 g fresh weight of diet in an individual cup (30 ml) (Koul *et al.*, 1990). The cups were transferred to a plastic container lined with moist filter paper to maintain the humidity. The amount of diet consumed by larva was measured at 24 hrs in both treated and control cups by weighing the left over diet. The antifeedant index (AI) for both the Lepidopteran insects was calculated using the following formula

$AI = (C-T)/(C+T) \times 100$,

where

C is the consumption of control and

T is the consumption in treated as described by Devanand and Usha Rani, (2008).

Contact toxicity against stored grain pests

The insecticidal activity of biosynthesized and synthetic nano silver against adults of three stored product insects was evaluated by direct contact application assay (Kim *et al.,* 2003; Usha Rani and Rajasekharareddy, 2010). The nano silver were prepared in distilled water at different concentrations (50, 100 and 150 µg /100µL) applied on filter papers (Whatman No. 1, cut into $5cm^2$ pieces) separately. Distilled water was allowed to evaporate for 10-15 min prior to introduction of insects. Then each paper (dried) was placed at the bottom of a Petri plate (5.5cm diameter x 1.2cm) and 10 adults each of *T. castaneum, R. dominica*, and *S. oryzae* were placed in each petri plate and covered with a lid. The inner side of the lid was coated with Vaseline to prevent insect staying on lid. Controls received 100 µL distilled water alone. There were a total of 15 replicates per treatment and the treatments were done on three different days (N=45). Mortality percentages were measured after exposure for 24, 48, and 72 hrs of treatment.

Antimicrobial assay by agar well diffusion method

AgNPs synthesized using *H. tiliaceus* was tested for antibacterial activity by agar well diffusion method according to Sahayaraj *et al.* (2014) with minute modifications against *X. campestris* and *R. solanacearum.* A single colony of test strain was growth over night in nutrient broth on a rotary shaker (200 rpm) at 35^o C after 24 hours a loop full of bacterial culture was swapped homogeneously onto the individual plates using sterile cotton swabs on Muller- Hinton agar (MHA) medium (SRL Laboratories Limited, Mumbai). Wells of 6 mm diameter were prepared using gel puncture. Different concentration of AgNPs (25, 50, 75, 100,150 and 200 µg/ml) was impregnated in each well and antibiotic chloramphenicol (0.1 %) (Himedia Laboratories Limited, Mumbai) and deionised water were used as positive and negative control respectively. After 24 hours incubation at 37ºC zone of inhibition was measured in millimetres and was recorded as mean ± SD of the triplicates experiments.

Statistical analysis

The results of presented data were analysed by using analysis of variance (ANOVA), and the means were statistically compared by Tukey's test, where p values less than 0.001 were considered to be significantly different. Each treatment was in triplicate for statistical validity, and the data were analyzed with standard statistical software Sigma stat 3.0.

RESULTS AND DISCUSSIONS

UV–visible absorption spectrometry

In order to prove the value of marine plants in green nanotechnology, the synthesis of silver nanoparticles using a mangrove plant, *H. tiliaceus* leaf extract has been reported for the first time. Reduction of $AgNO_3$ was visually evident from the colour change (brownish-yellow) of reaction mixture after 25 min of reaction and this is observed due to excitation of Surface Plasmon Resonance (SPR) phenomena (Mulvaney, 1996). Nanoparticles size also determines the colour of the solution, the smaller the size of AgNPs, the greater the colour shift towards red (Mock *et al.,* 2002). In the biofabrication plant metabolites helped in the reduction of $AgNO_3$ to AgNPs monitored by the UV–Vis spectroscopy. This instrument was also used to examine the size and shape of controlled nanoparticles in aqueous suspensions (Wiley *et al.,* 2006). Noble metals are known to exhibit unique optical properties due to the phenomenon of surface plasmon resonance (SPR). The UV–Vis absorption spectra was recorded (Fig. 1) from the resulting solutions that showed the characteristic surface plasmon resonance (SPR) band at about 420 nm. This is similar to the SPR with characteristic peaks of silver nanoparticles prepared by Awwad *et al*. (2013). The exact mechanism of silver nanoparticles synthesis by plant extracts is not yet fully understood.

Zeta potential measurement and dynamic light scattering (DLS) analysis

Zeta potential determined the particle size distribution and the stability of colloidal nanoparticles and DLS measures

hydrodynamic diameter of the hydrosol (particle suspension). The zeta potential and DLS graph of AgNPs synthesized using *H. tiliaceus* showed that the particles carry a charge of -29.3 mV and average size of 75 nm (Fig. 2).

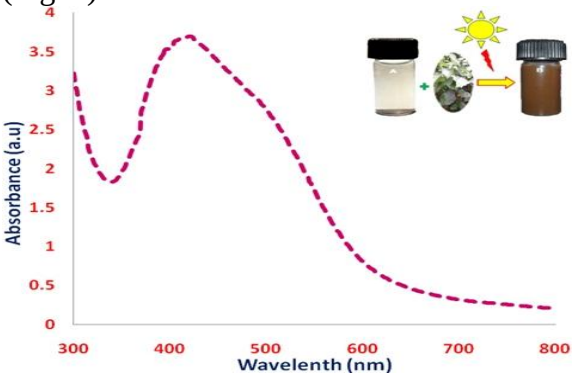

Fig.1. Shows the UV-vis absorption spectra of colloidal AgNPs synthesized using plant *H. tiliaceus.*

In our results zeta potential value is strongly negative as it clearly indicates that particles have long-term stability, good colloidal nature and high disparity of synthesized nanoparticles due to negative–negative repulsion between the particles. We found single peak in the DLS measurement which indicates there are no particle aggregation/ agglomeration (Rajasekharreddy and Usha Rani, 2014).

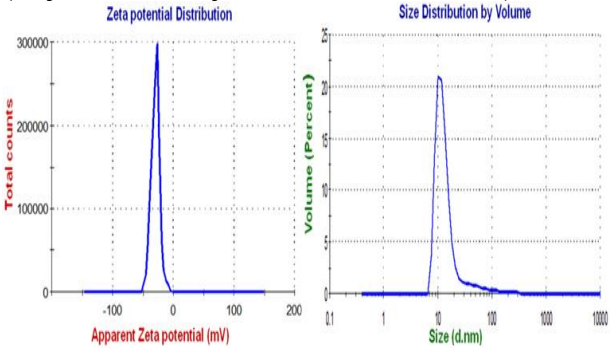

Fig. 2. Zeta potential analysis and Dynamic light scattering technique (DLS) graphs.

Morphology and size

TEM has been used to identify the shape, size, and morphology of nanoparticles. The study of the synthesized nano particles in TEM and the observations of TEM images at 100 and 200 nm resolutions (Fig. 3) elucidated that all the AgNPs obtained from *H. tiliaceus* leaf extract are polydispersed with either irregular or spherical in shape and the size varied from 20-65 nm. In TEM analysis one should use single drop of sample on carbon film that cannot fully represent the entire solution and while acquiring image electron beam somewhat alter the arrangement of the nanoparticles (Popov *et al.*, 2006). However, the repeated verification of the images provided the evidence that the particle sizes are varied. We have analyzed particle size with TEM and DLS. Both suggested different sizes. This variation occurred because DLS analysis includes the ligand shell and determines the hydrodynamic size whereas TEM look at only metallic core (Kasture *et al.*, 2008). TEM is the technique which also gives the selected area electron diffraction (SAED) patterns which reveal the distribution and crystalline nature of particles in the focusing zone clearly (Fig.3).

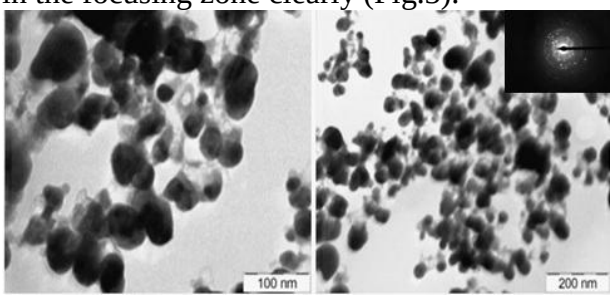

Fig. 3. Transmission electron micrographs of AgNPs the scale bar corresponds to 100 nm and 200 nm (SAED pattern).

Fourier transform infrared spectroscopy (FT-IR) analysis

FTIR analysis was carried out to identify the possible functional groups, which may be responsible for synthesis and stabilization (capping) of AgNPs. The FTIR spectrum of AgNPs synthesized by using *H. tiliaceus* leaf extract was shown in (Fig. 4). The peaks around 3,655 cm^{-1} are indicative of –OH stretching vibrations from different phenolic group (Koyel *et al.*, 2013) and alcohols, 3404.79 cm^{-1} is due to the presence of hydrogen bond N-H stretching of 1°, 2° amines, amides. The band observed at 2922.35 cm^{-1}, 2852.61 cm^{-1} and 2341.45 cm^{-1} could be assigned to C–H stretch of alkanes. 1587 cm^{-1} could be assigned to C–C stretch (in–ring) N–H bend of 1° amines. This is due to the carbonyl (C=O) amide linkage indicative of amino acids suggesting the presence of

proteins on the surface of Ag particles (Jayanta *et al.*, 2014), whereas 581.45 cm^{-1} is a weak band. FTIR spectrum revealed that sharp and strong absorption bands at proteins which helped as efficient capping and stabilization agents were present in the plant extract for AgNPs synthesis. These capping agents stabilize the NPs and prevent them from aggregation in the solution in the green synthesis. Our FT-IR spectroscopic study confirmed that the plant extract has the ability to perform dual functions of reduction and stabilization of AgNPs (Johnson and Joy, 2015).

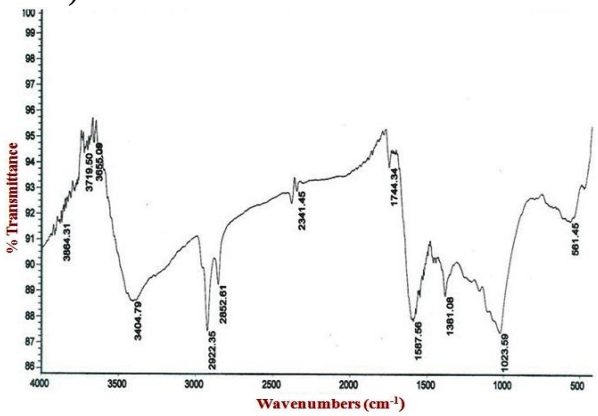

Fig. 4. Shows FTIR spectrum AgNPs synthesized by using *H. tiliaceus* leaf extract.

XRD analysis

XRD is mainly used to study the size, shape, lattice parameter determination and phase fraction analysis of the unit cell of synthesized AgNPs. X-ray diffraction is used to assess the crystalline structure and preferred orientation in powder solid samples of the AgNPs. XRD analysis clearly showed (Fig. 5.) three distinct diffraction high peaks at *2θ*, values of (38.15º, 46.24º, 64.44º and 76.86 º) for *H. tiliaceus* plant mediated AgNPs which can be indexed (111), (200), (222) and (311) reflection plates of face centered cubic (fcc) structure of silver. The unidentified additional crystalline peaks are also apparent in many works in the XRD pattern that includes the relevant *2θ* range (Haytham *et al.*, 2015). In our present observation, XRD pattern clearly illustrates these four intense broad peaks reflecting a high degree of crystallinity and smaller size of the AgNPs. Similar results were obtained with AgNPs synthesised using terrestrial plants *Carica papaya, Datura metel* and *Solanum melongena* (Rajasekharreddy *et al.*, 2010).

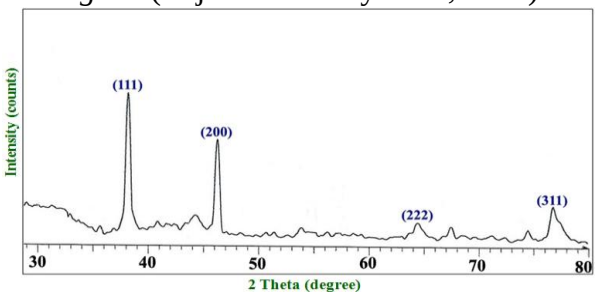

Fig. 5. Shows XRD pattern analysis of silver nanoparticles synthesized by *H. tiliaceus* extract with silver nitrate aqueous solution.

Antifeedant assays

Antifeedant property of each of the biogenic and synthetic silver was assessed against the third instar larvae of *S. litura* by comparing the leaf area consumed in the treated leaves and the normal untreated or solvent treated leaf discs (controls). The results are presented in Table 1. When compared with control, reduced food intake (15±3.2, 15.7±1.2±, 21.3±3.2 and 25.1±3.3% of leaf area) was observed with the treatments of synthetic silver at 50, 100, 150, 200 µg /21 cm^2 concentrations, respectively. The highest percent antifeedant activity was observed in the biogenic silver treated leaf disc (55.5 ± 8.2 %) at 50 µg conc. 200 µg/21cm^2 and 30.1 ±0.7% for *H. armigera* Hence when compared with synthetic silver and solvent control, biogenic silver showed significant (df = 15,171; F=440.95; P<0.001) activity against *S. litura* at all concentrations 50 µg/21cm^2, 100 µg/21cm^2, 150 µg/21cm^2 and 200 µg/21cm^2 respectively.

Table 1. Percent antifeedant activity of biogenic and synthetic AgNPs against *S.litura and H. armigera.*

	S. litura				*H. armigera*			
AgNPs type	50µg/21cm^2	100µg/21cm^2	150µg/21 cm^2	200µg/21cm^2	50µg/1gm	100µg/1gm	150µg/1gm	200µg/1gm
Biogenic	55.5±8.2[a]	64.2±5.5[b]	78.1±4.4[c]	94.1±2.0[d]	12 ± 0.1[e]	16 ± 0.6[f]	20 ± 0.6[g]	30.1±3.3[h]
Synthetic	15.7±1.2[a]	15±3.2[b]	21.3±3.2[c]	25.1±3.3[d]	11 ± 0.1[e]	15 ± 0. 6[f]	17 ± 0.8[g]	19±3.2[h]

Values are mean ±Standard error (ANOVA followed by TUKEY test performed); Means within a column followed by the same letter are significantly different at P< 0.001

The activity was further enhanced with increase in concentration from 100 to 200 µg for both *S. litura* and *H. armigera* in dose dependent manner with the highest antifeedant activity of 94.1±2.0% obtained for *S. litura* at *H. armigera* appears to have a better detoxification system by which lower antifeedant activity is observed and hence provides an interesting topic for further research as why higher antifeedant activity has occurred in one Lepidopteran pest compared to others.

Table 2. Contact toxicity of the biosynthesized and synthetic nano silver against stored grain pests.

	Dose (µg/cm^2)	*T. casutaneum*	*R. dominica*	*S. oryzae*
Biogenic	50	21.3±0.12^a	22.8±0.32^b	19.4±0.45^a
	100	39.2±0.24^c	41.2±0.24^c	33.2±0.38^c
	150	45.2±0.24^e	42.8±0.24^e	37.4±0.16^e
Synthetic silver	50	17.6±0.12^a	26.8±0.24^a	19.2±0.24^a
	100	31.3±0.13^c	29.8±0.24^c	30.0 ±0.21^d
	150	32.8±0.17^e	31.4±0.16^e	33.8±0.24^e
Control	--	0±0^f	5.1± 0.26^f	9.4± 0.17^f

Values are mean ±Standard error (ANOVA followed by TUKEY test performed); Means within a column followed by the same letter are significantly different at P< 0.001

The results clearly indicated that biogenic silver was the effective treatment at all the concentrations tested compared to synthetic silver and control. Biogenic NPs recorded higher antifeedant activity (12%) at 50 µg concentration compared to solvent control. The antifeedant activity of biogenic silver had increased to 16 and 30.1 % with increase in concentration from 100 to 200 µg respectively. A dose-dependent (Table 1) antifeedant activity was observed against third instar larvae of *H. armigera* for both the biosynthesized and the synthetic AgNPs. Furthermore, the antifeedant effect was lesser in synthetic silver than in the biosynthesized nano silver due to binding with secondary metabolites as already discussed. A dose-dependent feeding inhibition action was observed against third instar larvae of *H. armigera* for both the biogenic and the synthetic silver. However, difference in antifeedant activity between biogenic and synthetic nano silver was negligible may be due to the same quantity of accumulation of AgNPs in the gut by the larvae. Pest management is a very important and tough task now a day because insects are developing resistance to synthetic chemicals.

Table 3. *In vitro* antibacterial potential of biosynthesised AgNPs using *H. tiliaceus* leaf extract

Ag NPs conc. (µg/ml)	Zone of inhibition in (mm)	
	X. campestris	*R. solanacearum*
25	13.8 ± 0.34^a	11.8 ± 0.26^f
50	15.9 ± 0.11^b	13.6 ± 0.34^a
75	17.9 ± 0.11^c	15.6 ± 0.45^b
100	20 ± 0.15^d	19 ± 0.15^c
150	24 ± 0.17^e	22 ± 0.33^h
200	26.6 ± 0.25^f	24.2 ± 0.69^f
Positive control	27.7 ± 0.7^g	25.5 ± 0.37^g

All the means within a column letter are significantly different at P< 0.001

There are reports on effects of other plants assisted biogenic AgNPs on feeding, survival, growth and development of *S. litura* (Anbu Radhika and Sahayaraj, 2014).We found AgNPs synthesized from marine plant *H. tiliaceus* leaf extract showed significantly excellent feeding inhibition activity against *S. litura*. The results suggest that biogenic AgNPs from *H. tiliaceus* were promising with

regard to their use for the protection of agriculture fields from *S. litura.*

Contact toxicity against stored grain pests

The results (Table. 2) indicated that at 50 µg concentration cased mortality of *T. castaneum, R. dominica* and *S. oryzae* were 21.3 ± 0.12 %, 22.8 ± 0.32 % and 19.4 ± 0.45 % respectively with biogenic silver treatment, whereas, in synthetic silver treatment at the same concentration, lower mortality was observed in comparison to control. Biogenic silver treatment at 100 µg concentration, maximum value of mortality against *R. dominica* was noted followed by *T. castaneum* and *S. oryzae* at $p<0.001$. But with synthetic silver treatment, lower mortality i.e 31.3±0.13 %, 29.8±0.24 % and 30.0±0.21 % against *T. castaneum, R. dominica* and *S. oryzae* respectively was observed compared to the solvent control. At the highest concentration of 150 µg biogenic silver treatment was found to be comparatively toxic and killed 45.2 % of *T. castaneum,* 42.8 % of *R. dominica* and 37.4 % of *S. oryzae* insects after 24hrs. It was clear from the results that synthetic silver treatment was not significantly effective in causing mortality against all the treated insects. This indicated that the biogenic silver was remarkably more potent than the synthetic silver nano particles. Hence the most toxic sample was biogenic silver nano particle and the least toxic was synthetic silver nanoparticles compared to control which is due to the action of capping agents (secondary metabolite moieties such as phenols and amide groups elucidated in FTIR) attached to the AgNPs. The use of nanoparticles in pest control is still at early stage and in recent years, nanoparticles have received much attention for controlling an stored insect pests (Vani and Brindhaa, 2013). But in our study moderate toxic effects were observed with biogenic AgNPs on stored insect pests *T. castaneum, R. dominica* and *S. oryzae* adults; this is due to the thickness of the cuticle, lower dose used (50 µg/ 21 cm^2) and low penetrability of nanoparticles.

Antibacterial assays

In this study, the antibacterial properly of *H. tiliaceus* mediated AgNPs were investigated against *X. campestris* and *R. solanacearum* with various concentrations and the results obtained are shown in Table 3. The results of antimicrobial activity with a zone of inhibition maximum was found in *X. campestris* (26.6 ± 0.25 mm), followed by *R. solanacearum* (24.2 ± 0.69 mm) at 200 µg/ml concentration of AgNPs in Fig. 6. The lowest zone of inhibition was observed with *X. campestris* (13.8 ± 0.34 mm) and *R. solanacearum* (11.8 ±0.26 mm) and at 25 µg/ml. Chloramphenicol used as reference antibiotic showed variable inhibitory activity on tested bacteria with zone of inhibition of 27.7±0.7% for *X. campestris* and 25.5± 0.37% for *R. solanacearum* but had exhibited higher inhibition compared to the biogenic or synthetic AgNPs.

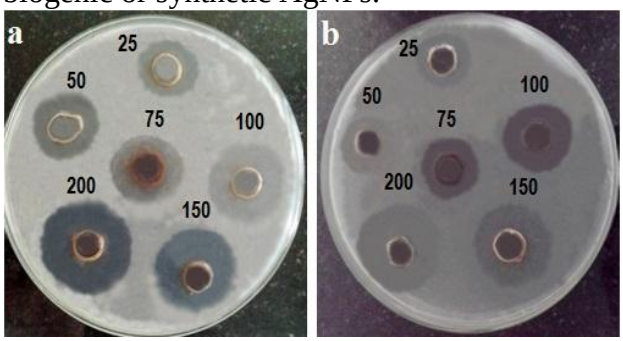

Fig. 6. Zone of inhibition of biosynthesised AgNPs at different concentrations of against **(a)** *X. campestris* **(b)** *R. solanacearum.*

AgNPs are considered as potential source of novel antimicrobial agents, which offer numerous advantages such as broad-spectrum activity and lower tendency to induce resistance. We have used agar well diffusion method for the present antibacterial study because of several advantages. For instatnce it accommodates more sample and clear zone formation for better measurement. Li *et al*. (2010) reported the antibacterial mechanism of SNPs towards *E. coli* as a model organism. Several researchers reported that biologically synthesized AgNPs have significant antibacterial activity on *X. Campestris* (Mahmood *et al.,* 2014).In our results we found that *X. campestries* is more susceptible than *R. solanacearum* towards *H. tiliaceus* mediated AgNPs at all concentrations. This variation was due to in thickness and cell membrane composition between two bacterial

species (Kim *et al.,* 2007). Similar results were reported on dose dependent inhibition of AgNPs synthesized from *L. reticulate* leaf extract (Kumara Swamy *et al.,* 2015). Our finding is consistent with other researchers' reports (Adithan *et al.,* 2015) on *R. solanacearum.* Several studies propose the mechanisms (s) of the bactericidal action of AgNPs. Swarnali *et al.,* (2014) suggested that AgNPs may attach to the surface of the cell membrane, thus disrupting permeability and respiration functions of the cell, and also their enormously high surface area (Geethalakshmi *et al.,* 2013). This explanation was supported by TEM results obtained in this work. Li *et al*. (2011) showed that AgNPs entered bacterial cells and condensed DNA as a result preventing DNA from replication. The present study also proved that *H. tiliaceus* mediated AgNPs have strong antibacterial activity candidates against phytopathogens.

To the best of our knowledge, this is the first time a successful production of AgNPs using aqueous leaf extract of *H. tiliaceus* has been done using direct sunlight exposure method Peter Amaladhas *et al.,* 2013; Goutam Brahmachari *et al.,* 2014). The potential uses and benefits of nanotechnology are enormous including agriculture and this kind of study may also create platform in future for preparing nanopesticides against insect pests and resistant phytopathogens. The present study revealed *H. tiliaceus* mediated AgNPs showed an excellent antibacterial activity against *X. campestries* and *R. solanacearum.* Hence this marine plant was efficient and long searched alternative and could be the answer to antibiotic resistance in pathogens.

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