

Research Article



Synthesis and Characterization of Fungus Mediated Silver Nanoparticle for Toxicity on Filarial Vector, *Culex quinquefasciatus*

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Accepted on: 19-11-2013; Finalized on: 31-01-2014.

ABSTRACT

Larvicidal activities of mycosynthesized silver nano-particles (AgNPs) against filarial vector, *Cx. quinquefasciatus*. The AgNPs synthesized by filamentous fungus *Penecillium verucosum*, characterized by UV-Vis spectrophotometer, Fourier transform infrared spectroscopy, scanning electron microscopy, and transmission electron microscopy. Further, laboratory evaluation of fungus mediated nano-particle against larvae and pupae of *Cx. quinquefasciatus*. The characterization studies confirmed the spherical shape and size (3–24 nm) of silver nano-particles. The efficacy of mycosynthesized AgNPs tested concentrations of 25 and 50 ppm against first, second, third, and fourth instar larvae and pupae of *Cx. quinquefasciatus*; LC₅₀ (LC₉₀) values are 4.91 (8.13), 5.16 (8.44), 5.95 (7.76) and 7.83 (12.63) at 25 ppm. Similarly, LC₅₀ (LC₉₀) at 50 ppm were 5.24 (8.66), 5.56 (8.85), 6.20 (10.01), 7.04 (10.92) and 7.73 (11.59) in larval instars and pupae, respectively. The mortality rates were positively correlated with the concentration of AgNPs. Significant ($P < 0.05$) changes in the larval mortality was also recorded between the period of exposure against all instar of larvae and pupae of *Cx. quinquefasciatus*. These finding use of fungus synthesize silver nano-particles is a rapid, eco-friendly, and a single-step approach and potential mosquito larvicidal agents.

Keywords: *Culex quinquefasciatus*, Larvicide, *Penecillium verucosum*, Silver nanoparticle.

INTRODUCTION

Medical importance of mosquitoes vector for the transmission of serious diseases that cause morbidity, mortality, economical loss, and social disruption.¹ *Culex quinquefasciatus*, the primary carrier for nematode parasites that cause filariasis and are widespread over large areas of the tropic and subtropics. Reducing the incidence of this disease is by mosquito control, which is frequently dependent on applications of conventional synthetic insecticides.² Chemical measures in public health programs were initially considered likely to decrease mosquito populations, but these have failed because the constant use of chemical insecticides has often led to disruption of natural biological control system and outbreaks of insect species. Moreover, problems created by using synthetic insecticide include the development of mosquito resistance, environmental pollutions and undesirable effects of humans, mammals and other non target organisms.^{3,4}

Phytochemicals are more or less photosensitive while the use of bacterial protein is high in cost.⁵⁻⁷ More importantly, the incidences of resistance to larvicides of mosquito larvae have been reported.^{8,9} This attempts to develop novel materials as mosquito larvicides are still necessary. With the progress of nano-technology, many laboratories around the world have investigated silver nanoparticles (AgNPs) production as the nano-particle possesses more surface atoms than a microparticle, which greatly improves the particle's physical and chemical characteristics.

Currently, fungi are being utilized in nanotechnology for the production of nano-particles; synthesis using fungi has shown that this environmentally begins and renewable source can be used as an effective reducing agent for synthesis of silver nano-particles (AgNPs). This biological reduction of metal could be utilized for a clean, nontoxic, and environmentally acceptable "green" approach to producing metal nano-particles. It is well known that some microbes such as bacteria, yeast and fungi are potentially useful in the preparation of metal nano-particles under normal air pressure and at room temperature. Many species of fungi have been used in nanotechnology for nanoparticle production, including *Fusarium oxysporum*, *Aspergillus fumigatus*, *Verticillium* spp., and *Chrysosporium tropicum*. The AgNPs formed are highly stable and have significant mosquito larvicidal activity. Hence, in the present study described the synthesis of fungal (*Penicillium verucosum*) nanoparticles for larvicidal and mode of action were studied. In this present work made to attempt on the development of novel fungal toxic metabolite nanoparticles was newer and alternative to plant products against filarial vector, *Cx. quinquefasciatus*.

Nano-particles, generally considered as particles with a size of up to 100 nm, exhibit completely new or improved properties as compared to the larger particles of the bulk material that they are composed of based on specific characteristics such as size, distribution, and morphology.¹⁰ Nanoparticles have attracted considerable attention owing to their various applications. The silver



nanoparticles are reported to possess anti-bacterial¹¹, anti-viral¹², anti-fungal activity.¹³ Synthesis of nanoparticles using plants or microorganisms can potentially eliminate this problem by making the nanoparticles more bio-compatible. Indeed, over the past several years, plants, algae, fungi, bacteria, and viruses have been used for low-cost, energy-efficient, and nontoxic production of metallic nanoparticles.¹⁴

Hence, in this present study made to attempt on laboratory evaluation of mycosynthesized AgNPs were tested against larvae of filarial vector, *Cx. quinquefasciatus*.

MATERIALS AND METHODS

Production of mass culture

The fungus, *Penicillium verrucosum* was obtained from Department of Plant Science, Bharathidasan University, Trichy, India and maintained on Nutrient agar slants and used in present study. Inoculum was prepared by using the one loop of culture suspension in nutrient medium incubated at 28°C for 24 h in the environmental shaker at 120 rpm in dark condition. To prepare mass culture for AgNP biosynthesis studies, the fungi, *Penicillium verrucosum* was grown aerobically in a nutrient media containing 0.5 % Peptone, 0.3 % beef extract, 1.5 % agar, 0.5% NaCl. The final pH was adjusted to 6.5±0.2. The flasks were inoculated, incubated on orbital shaker at 29°C and agitated at 120 rpm. After 48 h of incubation, the cell mass was separated by whatman No. 1 paper for filtration and to remove any medium component from the cell mass.

Synthesis of silver nano-particles

Culture filtrate (wet weight) (10 g) was brought into contact with 1Mm of 100ml silver nitrate solution. The flasks were incubated in the incubator shaker at 28°C with shaking speed of 120 rpm in dark condition. Silver nanoparticles were gradually obtained during the incubation period. Simultaneously, controls with cell biomass of fungal filtrate with Milli-Q deionized water and with silver nitrate solution were maintained under same conditions, separately.

Characterization of silver nanoparticles

UV-visible spectrophotometer analysis

After observing colour change, the sample was subjected to mild sonication for 10 minutes. The sample was subjected to scan (300 nm-600 nm) using Thermo-Biomate 3 UV-visible spectrophotometer. Distilled water was taken to adjust the baseline.

Transmission Electron Microscopy (HR-TEM) and Energy Dispersive X-ray Spectra (EDX) analysis

A drop of synthesized nanoparticles solution was placed on carbon coated copper grid and later exposed to infrared light (15 minutes) for evaporation. HR-TEM analysis was performed by using JEOL JEM 2100 instrument operated at an accelerating voltage of 100

keV with resolution of transmission electron microscope. The EDX analysis was carried out using JEOL JEM 2100.

FTIR analysis

FTIR spectroscopic studies were carried out to find possible bio-reducing agent present in the fungal culture. The wavelength spectrum of the cell free extracts before and after the addition of AgNo3, the spectra were recorded using Perkin Elmer make model spectrum RX1 (wavelength range between 4000cm⁻¹ and 400cm⁻¹).

Larval mortality assessment

The test for the larvicidal effect of fungal nanoparticles against *Cx. quinquefasciatus* was conducted in accordance with the WHO standard method.¹⁹ Batches of 50 early 1 to 4th instar larvae of *Cx. quinquefasciatus* were transferred in 100ml of sterilized tape water to a 250 ml enamel bowl containing 1.0 ml of a serial dilution of each fungal nanoparticles extract. Three replicate tests were carried out simultaneously, with a final total of 50 larvae for each concentration. The toxicity of each fungal nanoparticles extract was evaluated with 25µl/100ml (25ppm) and 50µl/100ml (50ppm) tape water after autoclaved, concentrations yielding a range of 0-100% mortality. Controls received tape water, while the untreated larvae were maintained in tape water only. After treatment, symptoms in treated larvae were observed and recorded immediately and at time intervals, and food (Yeast and dog biscuit 3:1 ratio) was offered to the larvae. The larvae were considered dead if, at the end of 24 h, they showed no sign of swimming movements even after gentle touching with a glass rod, as described in the World Health Organization's technical report series. The dead larvae in three replicates were combined and expressed as a percentage mortality of each concentration. Bioassays were performed at 28-34°C.

Morphogenetic variation, behavioral changes and Mode of action

In continuation of bioassays done in respect of above experiments, larval, pupal deformities and inhibition of adult emergence, changes in morphological features and other behavioral aspects were also recorded. The mode of action of fungal toxin to has only been studied in mosquito larvae. After introduced larvae in the required concentration of fungal nano-particle and its toxicity of the nano particle complex by mosquito larvae, the quickly entry in the cuticle and anterior stomach. We noted 12 hrs observation of action of nanoparticles in the mosquito larvae of 4th instar of *Cx. quinquefasciatus*.

Statistical analysis

Probit analysis¹⁶ was used for determination of LC₅₀ and LC₉₀ data from mortality and effect of concentrations were subjected to analysis of variance. Difference between the treatments was determined by Tukey's multiple range test ($P < 0.05$). The DMRT test was carried out two different variance of the sample.



RESULTS

Optimization of synthesis of AgNPs

Effect of Temperature

Increasing the temperature (37°C to 100°C) shows variation in the generation of AgNPs. Synthesis was found higher at 90°C compare to other temperature, AgNPs synthesized at 90°C shows SPR peak at 420 nm of absorbance in UV–Vis spectrum. The result depicts those fungal compounds having thermo stable properties mainly involved in the fabrication of AgNPs.

Effect of pH

Efficient synthesis of AgNPs was observed in lower pH which shows clear and sharp SPR peak at 420 nm. In acidic pH uniformly distributed small-sized (10nm to 55nm) spherical nanoparticles were observed whereas at higher or alkaline pH the size of AgNPs was comparatively large, irregular shape and highly dispersed due to increase in the absolute value of the negative zeta potential.

Effect of reaction time

Synthesis of AgNPs was improved with increasing the reaction time duration from 0 minutes 1 hour intense SPR peak was observed at 420 nm. The complete synthesis of AgNPs occurs within 1 hour time. This result indicates the rapid synthesis of AgNPs with high stability.

Effect of metal ions concentrations

Effect of metal ion concentration on AgNPs synthesis and their shape and size was evaluated. Higher metal ion concentration (1.0mM AgNO_3) displays enormous yield of AgNPs which shows SPR peak at 420 in UV–Vis spectrum, whereas lower concentration shows decreased SPR peak.

Stoichiometric proportion of silver nitrate and fungus metabolites

Formation of yellowish brown colour indicates the synthesis of AgNPs, as the fungus metabolites quantity (1to 10mL) increases the synthesis of AgNPs also gets increased. AgNPs synthesized in the Stoichiometric proportion of 10ml of fungal metabolites with 90ml of AgNO_3 shows intense SPR peak at 420nm in UV-Visible spectroscopy.

Characterization of AgNPs

UV-Visible spectrophotometer

The Surface plasmon resonance (SPR) of the AgNPs produces a peak at 420nm which indicates the dispersion of silver nano-particles (figure 2)

FTIR interpretation

FTIR absorbance spectrum were observed at 3387cm^{-1} , 2077cm^{-1} , 1637cm^{-1} and 692 cm^{-1} which indicates the functional group of the fungus component involves in the reduction and act as capping agent (Figure 3)

Frequency range(cm^{-1})	Functional group
3418	O-H stretch
2076	Si-H Structure
1639	C=C Structure
694	C-H



Figure 1: Extraction fungal nanoparticle

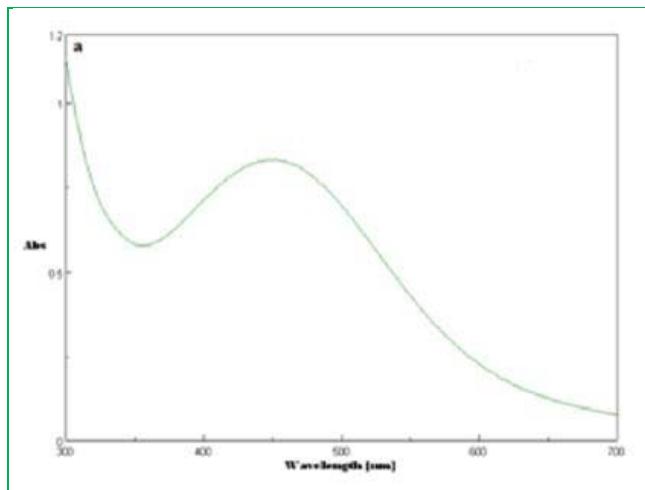


Figure 2: UV-Visible spectra

FTIR results clearly indicate that water soluble compounds present in the fungal metabolites principally involved in the reduction and stabilization of AgNPs.

Bio synthesis of silver nanoparticles using fungi currently under exploitation. The development of biologically inspired experimental process for the synthesis of silver nanoparticles is evolving into an important branch of nano technology. The present study deals with the synthesis of silver nanoparticles using fungal cell free extract. Results noted at 37°C for 48 hours.

Formation of the silver nanoparticles exhibit reddish brown in water.¹⁷ Figure 1 shows the color changes before (a) and after (b) the process of reduction of Ag^{+} to Ag nanoparticles. After in of the conversion process silver nano particles in solution. These colors arise due to excitation of surface Plasmon vibrations in the silver metal nano-particles.¹⁸

Table 1: Probit equations and susceptibility of *Cx. quinquefasciatus* against silver nanoparticles generated by *Penicillium verrucosum* with 25 ppm concentration.

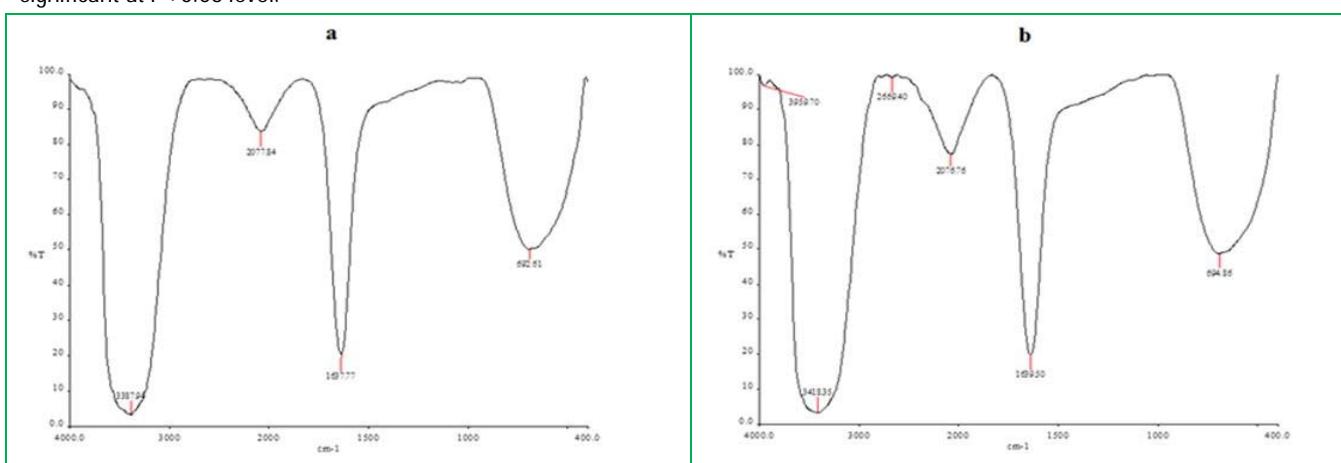
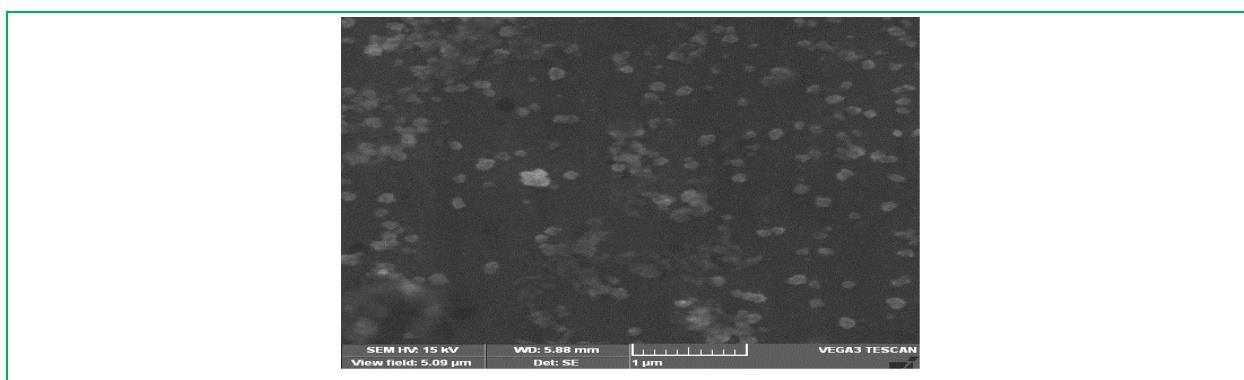
Stages	24 hrs % of Mortality/ (Mean \pm SE)	% Value of LC ₅₀ LC ₉₀	95% Confidential Limit		Regr. Coeff. X(Y)	Chi Square Value (x2)
			LCL LC ₅₀ (LC ₉₀)	U CL LC ₅₀ (LC ₉₀)		
I	100(30 \pm 0.24)	4.91 (8.13)	4.40 (7.45)	5.40 (9.08)	0.3991(-1.196)	2.278
II	100(28.8 \pm 0.24)	5.16 (8.44)	4.66 (7.74)	5.65 (9.41)	0.3917(-2.025)	2.653
III	96 (25.2 \pm 0.23)	5.95 (7.76)	5.40 (8.93)	6.50 (10.97)	0.3358(-1.999)	0.226
IV	92 (21.2 \pm 0.24)	6.87 (11.23)	6.27 (10.15)	7.53 (12.87)	0.2942(-2.023)	0.325
Pupa	86 (17.6 \pm 0.25)	7.83 (12.63)	7.16 (11.27)	8.67 (14.85)	0.267(-2.092)	0.309

*Required concentration 25 μ l/100ml (25ppm); LCL – Lower Confidential Limit, UCL- Upper Confidential Limit; Chi-Square value significant at P< 0.05 level.

Table 2: Probit equations and susceptibility of *Cx. quinquefasciatus* against silver nanoparticles generated by *Penicillium verrucosum* With 50 ppm concentration.

Stages	24 hrs % of Mortality/ (Mean \pm SE)	% Value of LC ₅₀ LC ₉₀	95% Confidential Limit		Regr. Coeff. X(Y)	Chi Square Value (x2)
			LCL LC ₅₀ (LC ₉₀)	U CL LC ₅₀ (LC ₉₀)		
I	100(28.4 \pm 0.24)	5.24 (8.66)	4.72 (7.93)	5.75 (9.68)	0.3756(-1.971)	3.411
II	99 (27 \pm 0.25)	5.56 (8.85)	5.05 (8.15)	6.05 (9.84)	0.3887(-2.162)	1.131
III	94 (24 \pm 0.24)	6.20 (10.01)	5.65 (9.16)	6.76 (11.25)	0.3362(-2.086)	1.491
IV	94 (20.2 \pm 0.26)	7.04 (10.92)	6.49 (9.97)	7.65 (12.34)	0.3301(-2.325)	1.758
Pupa	91 (17.2 \pm 0.28)	7.73 (11.59)	7.16 (10.57)	8.39 (13.16)	0.3316(-2.564)	2.084

*Required concentration 50 μ l/100ml (50ppm); LCL – Lower Confidential Limit, UCL- Upper Confidential Limit; Chi-Square value significant at P< 0.05 level.

**Figure 3:** FTIR interferogram of **a.** culture filtrate, **b.** synthesized AgNPs**Figure 4:** SEM image of nanoparticle.

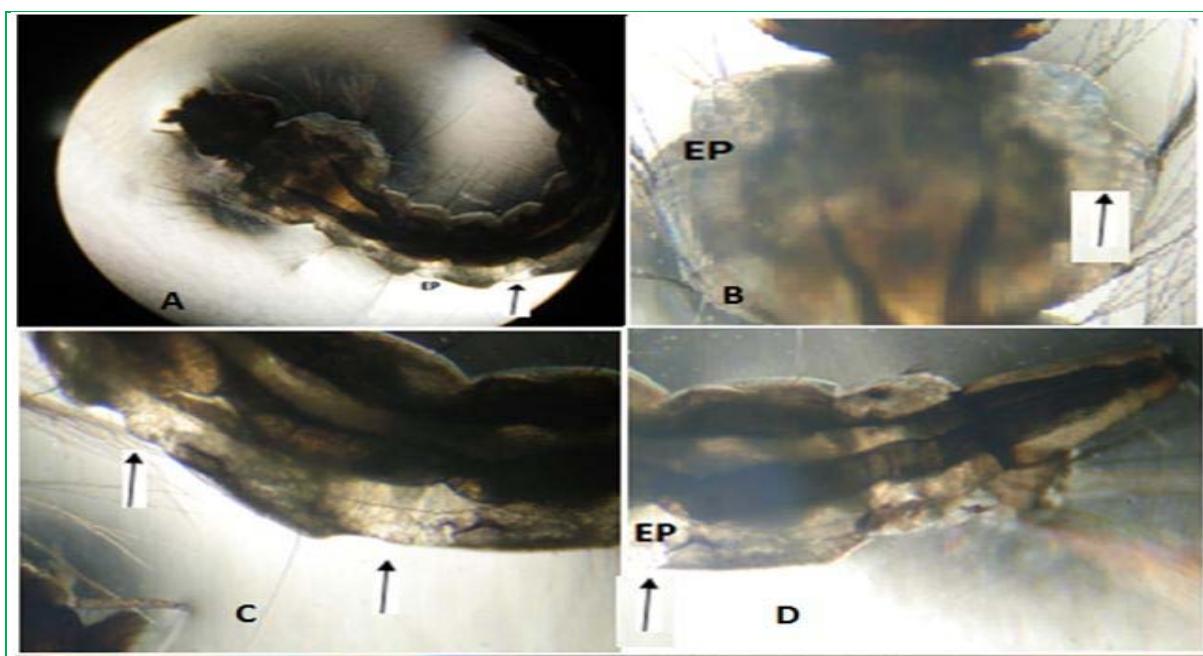


Figure 5: Optical microscopy view (10X) A) Infected larvae B) Head region C) Anterior region D) tail region for epithelial layer damage for the treatment of *Pencillium verrucosum* mediated silver nanoparticles; EP-Epithelial layer.

UV-Visible spectral analysis of AgNPs

UV-Vis absorption spectra of Silver nanoparticles synthesized by fungal culture filtrates with 1mM Silver nitrate

UV-VIS absorption spectrophotometer is used to investigate the SPR phenomenon. Silver nanoparticles exhibits interesting optical properties directly related to Surface Resonance (SPR) which is highly dependent on the morphology of the nanoparticles. Reduction of Ag⁺ ions during exposure to the extract of fungal culture filtrate was followed by UV-spectroscopy. It was observed that silver nanoparticles exhibit reddish-brown color, absorbance value at 430nm. The color appeared due to the excitation of the surface Plasmon vibrations of the metal nanoparticles. Many research groups have observed absorption maxima of colloidal silver Solution between 410 to 440 nm which is assigned to surface plasmon of various metal nanoparticles.¹⁹

FTIR analysis

FTIR spectroscopy analysis was carried out to identify the possible biomolecules responsible for the reduction of Ag⁺ ions. FTIR interferogram of both culture filtrate and AgNPs shows variation in the stretches at 3418, 2076, 1639 and 694cm⁻¹ which corresponds to primary amines (N-H) and carbonyl (C=O) functional groups may be present in the amino acid residues and proteins in the cell filtrate. This result highly corroborates with the results of Saravanan *et al.*,²⁴ reported that the proteins can bind to nanoparticles through the functional group and it increases the stabilization of nanoparticles.

Larvicidal activity

The table 1 and 2 provide the fungal synthesized nanoparticle (*P.vercosum*) on various stages (I, II, III, IV and

pupae) of *Cx. quinquefasciatus*. Considerable mortality was evident after the treatment of silver nano-particle for all larvae and pupae. Mortality was increased as concentration increased. The percentage mortality of I instar was 100% at 25µl/100ml (25ppm) concentration and it was further increased mortality in II instar. Similar trend has been noted for all the instars (III and IV). However the percentage of pupal mortality was 86% at 10ppm concentration. The effect on larval and pupal mortality was concentration dependent (Table1). The LC₅₀ (LC₉₀) values, regression equation and chi-square values are 4.91 (8.13), Y= -1.1963 + 0.3991, χ^2 values are 2.278 in I instar larvae and in pupa the LC₅₀ (LC₉₀), regression equation, chi-square values are 7.83 (12.63), Y= -2.092 + 0.267, χ^2 values 0.309 in pupa treated with 25ppm of fulgal mediated nano-particle. The DMRT values are significant at 5% level. The chi-square values are significant at P< 0.05 level. Similarly, 50ppm, the % of larval mortality was 100% in I instar and 91% in pupa. The LC₅₀ (LC₉₀), regression equation and chi-square values of I instar was about 5.24 (8.66), Y= -1.9716 + 0.3756 and χ^2 = 3.411. In pupal treatment the LC₅₀ (LC₉₀), regression equation and chi-square values are 7.73 (11.59), Y= -2.564 + 0.3316 and χ^2 = 2.084.

Morphological variation

After treatment with a lethal dosage (LC₅₀) of each fungal nanoparticles synthesis, the dead larvae were studied for morphological alterations under optical microscopy. Larvae mounted with Hoyer's medium on a microscope slide were scrutinized under light microscopy. Morphological changes in body segments including the head, thorax, and abdomen, and other organs such as the eyes, antennae, mouth brushes, setae, saddle, and anal gills were observed, Photographed. For Symptomatological observations on the larvae treated

with the fungal nano-particles. 4th instar larvae were still active immediately after exposure to LC₅₀ of each nanoparticles concentration, and the feeding process and normal zigzag motion of these treated larvae were clearly seen. However, after 5 min of exposure, abnormal evidence of excitation, restlessness, and sluggishness was initially observed. Excitation and restlessness persisted for between 30-60 min, and other anomalous motions were seen such as a coiling movement. The treated larvae frequently sank down and floated up again quickly. During the period of 60 - 100 min, some larvae showed more toxic symptoms.

Observations on the morphological alterations of treated 4th instar larvae revealed that most organs, except anal papillae (gills), had a normal structural appearance. Under optical microscopes, both treated and control larvae showed similarities in morphological architecture and cuticular sculpturing of the head, thorax, and abdomen segments, and other organs such as the eyes, antennae, mouth brushes, setae, saddle, siphon, and ventral brushes. A distinct difference, however, was the cuticle layer damage observed in the treated larvae. These results therefore indicated that the toxic effect of nanoparticles is predominantly on the cuticle layer, leading to their morphological deformation. In histological study, the nanoparticles treated 4th instar larval midgut was sectioned under microscopy. These histology observations clearly indicate that the fungal toxic nanoparticles affect specifically on the cuticles or epithelial layer of *Cx. quinquefasciatus*.

DISCUSSION

Over the last 5 decades the indiscriminate use of synthetic insecticides in agriculture and public health programs for the control of pest species has created multifarious problems viz. insecticide resistance, environmental pollution, toxic hazards to humans and other non-target organisms. In attempt to overcome these problems, great emphasis has been recently placed on the research and development of forms of pest control using plant products and microbial origin. Studies on natural products as larvicides have indicated that they could provide possible alternative to synthetic insecticides.

Another drawback with use of chemical insecticides is the failure of many vector control campaigns resulting in the vector resurgence in epidemic zones. The use of commercially available conventional synthetic insecticides has raised serious ecological, economical and environmental problems. They also contribute towards the development of resistance in the target insect species. All these factors led to search for safer and more compatible alternatives among which natural products are of first importance.²¹

The morphology of the NPs was spherical. It was noticeable that the edges of the particles were lighter than the centers, suggesting that biomolecules, such as proteins in *E. prostrata* capped the silver NPs. It was

utilized to characterize the particles and their sizes and distribution by taking micrograph from drop-coated films of the silver nanoparticles synthesized by the treatment of silver complex solution with fungal filtrate for 6 h. From the spectroscopic and imaging data obtained, it is obvious that 6 h is insufficient for the reduction of all silver ions present in solution, since the maximum absorbance at this time is much lower than the one obtained after 24 h, and therefore it is observed in the TEM images an increase on the number of particles and on the size of the AgNPs with reaction time. Most of the silver nanoparticles in TEM images are not in physical contact but they are separated by a fairly uniform interparticle distance.²² Jha et al.,²³ reported that compounds apigenin and kaempferol isolated from *Eclipta* leaf may be responsible for AgNPs reduction. SEM analysis of the synthesized silver nanoparticles was clearly distinguishable owing to their size difference. SEM image of nanoparticles observed from the micrograph majority are spherical with a small percentage of elongated particles and ranged in size of 35–60 nm with an average size of 45 nm. Uniformly distributed silver nanoparticles on the surface of the pellets are observed. The uniform size of the silver nanoparticles suggests that the particles on the pellets and in the solution may have the same size. SEM analysis of the AgNO₃ and synthesized AgNPs were clearly distinguishable owing to their size difference.

The stability of the silver nanoparticles can be attributed to the formation of silver electride that may form a thin layer on the aqueous surface of the reaction mixture. This silver electride possibly may convert the silver to nano silver. The protein present further is believed to cap the silver nanoparticles formed, restricting the agglomeration of the particles and thus checking the size and shape. The exact mechanism of the formation of these nanoparticles in these biological media is unknown. Presumably biosynthetic products or reduced cofactors play an important role in the reduction of respective salts to nanoparticles. It seems quite probable that the phenols play an important part in the reduction of ions to AgNPs as the concept of antioxidant action of phenol compounds is not new. Therefore, in combination with mosquito nets or other vector control measures, such plants synthesized AgNPs may have significant impact on malaria and filariasis incidence and can be potential candidates to be considered in integrated vector control programs. Fungus synthesized AgNPs, being readily available and their application methods being simple and affordable may be useful in protecting filariasis.

In the present study, younger larvae are more susceptible than older ones. Late fourth instars that have ceased feeding or feed little before pupation are much less susceptible because of lack of ingestion of a lethal dose in a short period of time. Early instars definitely be killed by dosages and concentrations that was induced some mortality in older larvae. Larval instars prevail in the breeding environments. Similarly, administration of maximum dosages geared to kill older larvae was to be



necessary to control these heterogeneous larval populations.²⁴

The broad spectrum antimicrobial properties of silver nanoparticles have attracted researchers to evaluate their potential against the parasites of world's most threatening diseases, malaria, and dengue. Synthesis of AgNPs using microorganisms like bacteria, fungi, actinomycetes, and extracts of various plant parts have advantage over other processes, as they are environment friendly. Fungi are beneficial than other biological agents for AgNP synthesis as bearing high metal uptake and tolerance and better wall binding capability²⁵. Fungi secrete copious amount of proteins, fungal biomass can be produced on large scale, and easy recovery of silver nanoparticles attracted the researchers. In addition, myco-synthesized silver nano-particles have good monodispersity, controlled size, and shape with hydrophilic nature.²⁶

The FTIR spectrum of extracellular biosynthesized silver nanoparticles is shown in Figure 3. The spectrum shows the presence of bands mainly at 3418, 2076, 1639 and 694cm⁻¹. The band at 3418 corresponds to C=O stretching non-conjugate vibrations. The intense band at 2076 cm⁻¹ is assigned to C=N. The two bands at 1639 and 694 cm⁻¹ correspond to the C–N stretching vibrations of aliphatic and aromatic amines, respectively. Similar kinds of bands are reported by researchers.²⁷ SEM micrograph of *P. verucosum* biomass (Figure 4) shows the Ag adsorbed on fungal mycelial filaments reducing Ag ions to nano-silver which is further characterized and evident by TEM studies. The micrograph shows silver aggregates on mycelium of *C. lunatus*; silver nanoparticles were characterized using SEM by various investigators.

Mortality (100%) was observed at 25 ppm concentration against all instars treated *Cx. quinquefasciatus*. While at 25 ppm concentration, mortality against fourth instars of *Cx. quinquefasciatus* increased to 100% in 1 and 2nd instar where as 3rd and 4th instar 96, 92%, respectively. The pupal mortality was reported 86%. The LC₅₀ and LC₉₀ along with upper and lower confidence limit values and regression equation of *Cx. quinquefasciatus* against myco-synthesized AgNPs for second, third, and fourth instars are presented in Table 1 using the mortality rates as the dependent variable Y and the dose as the independent component X. In case of all three larval instars (second, third, and fourth), Y is positively related to the X indicating that mortality rate increases with the increasing rate of dose. Mortality rates and difference in the susceptibility of second, third, and fourth larval stages of against myco-synthesized AgNPs was observed. Similarly 50ppm concentration, the percentage of morality and LC₅₀ and LC₉₀ are observed. The higher mortality rates at lower doses are comparable with earlier reports. Marimuthu et al.,²⁹ reported bioactivity of synthesized AgNPs against the larvae of *A. subpictus*, *Cx. quinquefasciatus* and *R. microplus* (LC₅₀=13.90, 11.73, and 8.98 ppm), respectively.

In the present study, we have synthesized the silver nanoparticles using *P. verucosum*. These nanoparticles have been characterized through transmission electron microscopy, and further confirmed by scanning electron microscopy. The characterization study confirmed the spherical shape and size (2-15 and 20-50 nm) of gold and silver nanoparticles. These silver nano-particles have been tested as a larvicide against *Culex* larvae. The larvicidal efficacy was noted when performed against all instars of *Cx. quinquefasciatus* at 5 different log concentrations, and significant results could be observed. The larval mortality was observed after different time of exposures. The mortality values were obtained using the probit analysis. The larvae were found to be highly susceptible for the silver nanoparticles. The first and second instar larvae have shown 100% mortality against the silver nano-particles after 24 h exposure. The results could suggest that the use of fungus *P. verucosum*, silver nanoparticles is a rapid, environmentally safer, and greener approach for mosquito control. This could lead us to a new possibility in vector control strategy.

These findings after treatment with fungus nano-particle leads to damaged anal papillae, with a shrunken cuticle border and destroyed surface with loss of ridge-like reticulum were found under optical and scanning electron microscopes, respectively. Similarly, Green et al.,³⁰ reported distinct features of alteration such as highly swollen anal papillae of *Ae. aegypti* larvae after treatment with whole oil of *Tagetes minuta*. Structural deformation of anal papillae probably led to their dysfunction, which may be intrinsically associated with the death of mosquito larvae.

Ultra structural observations on *A. aegypti* larvae after treating with ethanolic extract of *Derris urucu* roots revealed an imperfect peritrophic matrix and extensive damage of the midgut epithelium.³¹ Correspondingly, the toxic effect of *P. verucosum* on *Cx. quinquefasciatus* larvae was mainly in the midgut, showing partial or total cell destruction, high citoplasmatic vacuolization, increased sub peritrophic space and cell hypertrophy, and the epithelium did not maintain its monolayer appearance. Generally, insecticides enter insects by cuticular penetration and/or ingestion and then pass throughout the interior of the insect to the site of action.³² Although these reports provided some evidence regarding the action site of these bioactive compounds, the mechanism causing mortality of mosquito larvae is still unknown and needs to be studied further. However, this study explained the potential of fungus mediated silver nano-particle could be alternative to plant origin of insecticide against mosquito larval species and its benefit to developing new types of larvicides used for mosquito control.

The use of natural product chemistry coupled with nanotechnology that reduces mosquito populations at the larval stage can provide many associated benefits to vector control. Since silver nano-particles are considered to be potential agents for various biological applications



including antimicrobial, its application as a mosquito larvicultural agent was investigated. The data obtained from the present study clearly indicate that silver nanoparticles could provide excellent larval control of *Cx. quinquefasciatus*. The mechanism which causes the death of the larvae could be the ability of the nano-particles to penetrate through the larval membrane. The silver nanoparticles in the intracellular space can bind to sulphur-containing proteins or to phosphorus containing compounds like DNA, leading to the denaturation of some organelles and enzymes. Subsequently, the decrease in membrane permeability and disturbance in proton motive force causes loss of cellular function and finally cell death.³³

CONCLUSION

The prospect of utilizing natural products for synthesizing silver nano-particles and testing its efficacy in controlling mosquitoes as larvicides was a recent phenomenon facilitating the development of a more potent and environmentally safe pesticide. Identification of the bioactive principles involved and their mode of action and field trials are necessary to recommend an effective formulation as an anti-mosquito product in control programs. Thus the timing of treatments depends on the knowledge of the biology of targets as well as non-target species. We conclude that the application of furigus mediated SNP to kill larva, pupa of filarial vector could significantly reduce parasite transmission and therefore lead to reduced filarial risk.

Acknowledgement: The authors thanks to Professor and Head (i/c) during 2012 in the department of Biotechnology, for providing necessary facility to conduct experiment. I extent my grateful thanks to center for Nano science and Technology for sample analysis.

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Source of Support: Nil, **Conflict of Interest:** None.