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ORIGINAL ARTICLE

Mosquito larvicidal activity of silver nanoparticles synthesised using actinobacterium, *Streptomyces* sp. M25 against *Anopheles subpictus*, *Culex quinquefasciatus* and *Aedes aegypti*

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Abstract The present work reports the larvicidal potential of microbially synthesised silver nanoparticles (AgNPs) by using an actinobacterium, *Streptomyces* sp. M25 isolated from Western Ghats, Tamil Nadu, India. The biomass of *Streptomyces* sp. was exposed to 1 mM silver nitrate ($AgNO_3$) solution. The synthesis of AgNPs was confirmed by visual inspection followed by instrumental analysis such as UV–vis spectroscopy, fourier transform infrared spectroscopy, X-ray diffraction (XRD), energy dispersive X-ray spectroscopy, scanning electron microscopy, atomic force microscopy and transmission electron microscopy (TEM) with selected area electron diffraction. Based on the TEM and XRD analysis, the average size of the AgNPs was determined to be 10–35 nm. The biosynthesised AgNPs exhibited significant larvicidal activity against malarial vector, *Anopheles subpictus* (LC_{50} 51.34 mg/L and χ^2 value of 8.228), filarial vector, *Culex quinquefasciatus* (LC_{50} 48.98 mg/L and χ^2 value of 14.307) and dengue vector, *Aedes aegypti* (LC_{50} 60.23 mg/L and χ^2 value of 4.042), respectively. Similarly, $AgNO_3$ had larvicidal activity against malarial vector, *A. subpictus* (LC_{50} 42.544 mg/L and χ^2 value of 2.561), filarial vector, *C. quinquefasciatus* (LC_{50} 44.922 mg/L and χ^2 value of 1.693) and dengue vector, *A. aegypti* (LC_{50} 39.664 mg/L and χ^2 value of 5.724), respectively. The current study is a rapid, cost effective, eco-friendly and single step approach. The *Streptomyces* sp. M25 is a newly added source for the synthesis of AgNPs with improved larvicidal activity.

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Keywords Biosynthesis · Silver nanoparticles · Mosquito vector · Larvicidal · *Streptomyces* sp.

Introduction

Mosquitoes are the most prevalent vector species, which can able to transmit diseases like, malaria, dengue fever, yellow fever, filariasis, chickungunya, etc., in worldwide (Matasyoh et al. 2011). It also causes some skin and systemic allergic reactions such as angioedema on human beings (Peng et al. 1999). In worldwide, the diseases like malaria, dengue and filariasis are the most significant cause of morbidity and mortality every year (WHO 1996, 2009). In India, these diseases are still the most crucial cause of morbidity and mortality with arising of totally two to three million new cases every year (Sharma et al. 2009). Among *Aedes* species present in India, the dengue vector *A. aegypti* was found as the most prevalent species (86.89 %) occurring throughout the year, while *A. albopictus* and *A. vitattus* were found mostly during monsoon periods in the tropical and subtropical zones. Of the 53 *Anopheles* species present in India, only nine species are most important vectors of malarial disease. The lymphatic filarial vector *Culex quinquefasciatus* is one of the most important vectors and annoying man—biting mosquitoes (Mourya et al. 1989; Das et al. 2002). Lymphatic filariasis is second to malaria as the most significant mosquito borne diseases in India.

Vector control is one of the most serious concerns in developing countries like India, due to the lack of general awareness and socio-economic reasons (Kovendan et al. 2012). The control of these vectors is being strengthened in many areas, but there are significant drawbacks and challenges including the development of resistance to routinely

used synthetic insecticides and phytochemicals day by day and a lack of alternative, cost-effective and safer insecticides led to resurgences in mosquito populations. Increasing insecticide resistances requires the development of new strategies for elongating the use of highly effective mosquito control compounds. Thus, the novel attempts to develop for the synthesis of nanomaterials for control of mosquito larvae in worldwide with the progress of nanotechnology (Patil et al. 2012).

The synthesis of nanoparticles of specific composition and size is a rapidly growing area of materials science research. New methods to the preparation of these materials extend the choice of different properties that can be obtained. Various physical and chemical (Mandal et al. 2005) synthesis methods are aimed for controlling the properties of the particles and for the production of metal nanoparticles. Most of the techniques are still in the developmental stage and various problems are often experienced with the stability of the nanoparticles preparations (Vahabi et al. 2011). One of the major developments in nanotechnology is the synthesis of nanoparticles using living organisms such as plants, microbes, marine organisms and their components (Sadhasivam et al. 2012).

At present, different types of metal nanoparticles are being produced including copper, zinc, titanium, magnesium, gold, alginate and silver (Bhaskara Roa et al. 2010) using microbial sources (Sastry et al. 2003). Among these, AgNPs have received considerable attention due to their attractive physico-chemical properties, surface plasmon resonance, surface-enhanced raman scattering (SERS) or biological applications. Biologically synthesised AgNPs could have many applications, including for both in vivo and in vitro biomedical and industrial research. Addition to this, the AgNPs act as the very good insecticide for the control of mosquito vectors nowadays (Patil et al. 2012).

The present study reports, the mosquito larvicidal activity of silver nanoparticles against *A. subpictus*, *C. quinquefasciatus* and *A. aegypti* has been synthesised using actinobacterium, *Streptomyces* sp. M25 from Western Ghats ecosystem, Tamil Nadu, India.

Materials and methods

Description of producing strain and biomass preparation

Streptomyces sp. M25 was isolated from soil samples collected from Western Ghats (*Lat:* 8°25′N; *Long:* 77°10′E), Tamil Nadu. Viability of culture was maintained on yeast extract malt extract agar (YEME) (Shirling and Gottileb 1966) slants at 4 °C. For the preparation of biomass, mycelial growth was inoculated into 100 mL of YEME broth and incubated at 28 °C for 5 days in rotary shaker with 120 rpm speed. After incubation, the mycelium was separated by centrifugation (5,000 rpm for 10 min at 4 °C). The biomass was collected in sterile screw cap vials after washing twice with sterile distilled water and stored at 4 °C until further use (Balagurunathan et al. 2011).

Biosynthesis of silver nanoparticles

About 10 g of *Streptomyces* biomass was exposed to a 100 mL aqueous solution of 1 mM silver nitrate ($AgNO_3$) in a 500 mL Erlenmeyer flask. The flask was incubated in rotary shaker with 120 rpm at 28 °C. During biosynthesis, the reaction flask was observed for every 24 h for visual colour change from white to brown, which indicated the biosynthesis of AgNPs (Senapati et al. 2005). After colour change, the whole mixture was centrifuged at 5,000 rpm for 15 min and the cells and supernatant was observed to determine the intra (or) extra cellular synthesis of AgNPs. After centrifugation, the quantity of AgNPs was weighed per 100 ml.

Characterisation of silver nanoparticles

UV–Visible and FT-IR analysis

The bioreduction of Ag^+ ions in solution was monitored using UV–vis spectroscopy (Cyberlab double beam model) at a resolution of 1 nm between 190 and 1,000 nm range (Balagurunathan et al. 2011). For the FT-IR analysis, a small amount of the sample was mixed with 100 mg KBr FT-IR grade and pressed into a pellet. The pellet was placed into the sample holder and the FT-IR spectra were recorded in the range of 4,000–400 cm^{-1} in FT-IR spectroscopy at a resolution of 4 cm^{-1} (Bhaskara Rao et al. 2010).

XRD and EDX analysis

Structural characterisation was carried out by X-ray diffraction spectroscopy (Shimadzu XRD 6000), in the operating voltage of 40 kV and a current of 30 mA with Cu Kα radiation ($\lambda = 1.540$ A°). The AgNPs were mixed into 10 mL of deionized water. Then the biosynthesised AgNPs were freeze–dried, powdered and used for XRD analysis (Sadhasivam et al. 2012). EDX measurements of the AgNPs were performed by SEM–EDX (Model Jeol JFSM 6390). The sample was prepared by casting the nanoparticle solution on a glass substrate (Du et al. 2010).

SEM and TEM observation

Scanning electron microscopy (Model Jeol JFSM 6390) and TEM (Model Hitachi H-500) analysis as well as SAED

pattern was performed for identify the size and shape of the nanoparticles. After synthesis of AgNPs, thin film of the sample that was prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the grid. Extra solution was removed using a blotting paper and then the film on the grid was allowed to dry by putting it under a mercury lamp for 5 min. Then, the film was magnified at different magnifications for the observation of AgNPs (Mukherjee et al. 2001).

Atomic force microscope observations

To perform AFM analysis, the nanoparticles were mixed in methanol and a drop of the mixture was deposited on a silicon slide and the solvent was allowed to evaporate. Then, the thin film (1 × 1 cm^2) containing the sample was analysed using AFM (Model Nanosurf A) (Sadhasivam et al. 2010).

Larvicidal activity

Test larvae

For preliminary screening, the malarial vector larvae of *A. subpictus*, filariasis vector larvae of *C. quinquefasciatus* and dengue vector *A. aegypti* were obtained from the National Center for Disease Control Research Institute, Coonoor, Tamil Nadu, India. The larvae were kept in plastic enamel trays containing dechlorinated tap water and maintained (Patil et al. 2010).

Mosquito larvicidal bioassay

The toxicity test was performed by placing 25 mosquito larvae into 200 mL of sterilized double–distilled water with microbially synthesised AgNPs and $AgNO_3$ to the designed concentrations (25, 20, 15, 10 and 5 mg/L) into a 250 mL beaker. Distilled water used as control for each individual concentrations. Mortality was estimated after 24 h to calculate the acute toxicities on larvae of *A. subpictus*, *C. quinquefasciatus* and *A. aegypti* (Jayaseelan et al. 2011). The 16:8 h light/dark cycle was tested using the AgNPs concentrations of 100, 80, 60, 40 and 20 mg/L and the $AgNO_3$ concentrations of 50, 40, 30, 20 and 10 mg/L with a final volume of 200 mL in a 250 mL glass beaker. A negative control (Mosquito larvae without nanoparticles and silver nitrate) was used in all experiments, and all conditions were tested in five replicates.

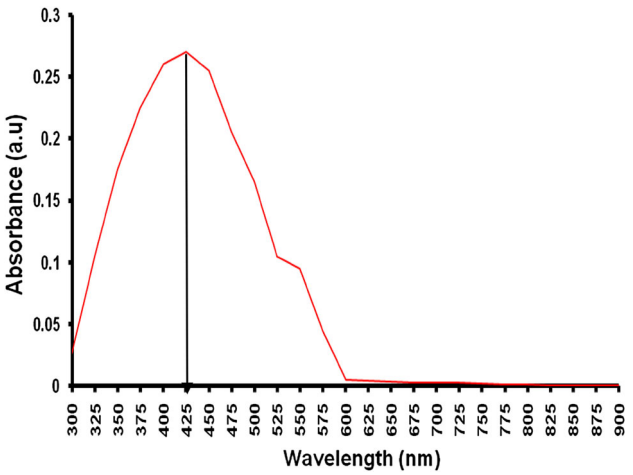

Fig. 1 UV–vis spectrum shows a characteristics peak at 425 nm

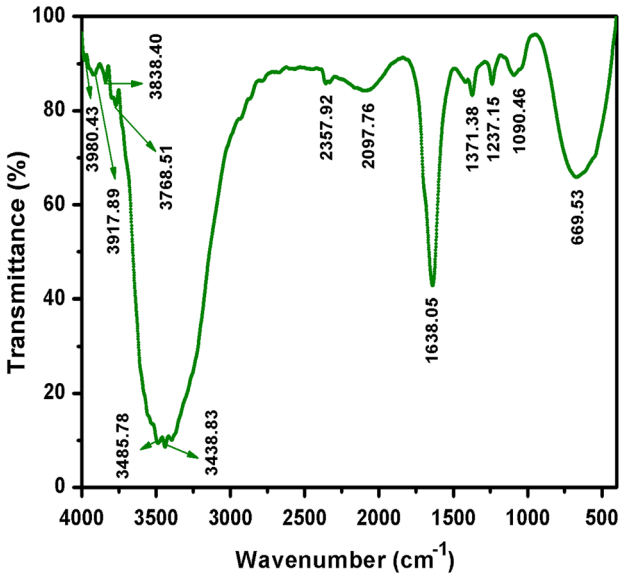

Fig. 2 FT-IR spectrum shows different functional peaks

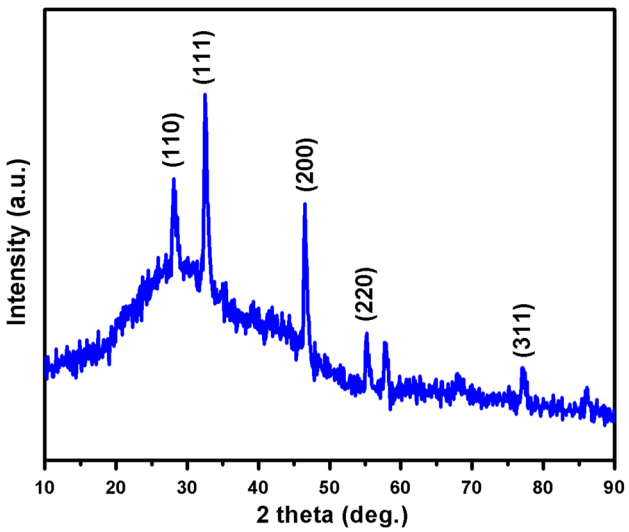

Fig. 3 XRD pattern of actinobacterially synthesised AgNPs

Dose–response bioassay

Based on the preliminary screening results, microbially synthesised AgNPs and $AgNO_3$ were subjected to dose–response bioassay for larvicidal activity against the larvae of *A. subpictus, C. quinquefasciatus* and *A. aegpyti*. Different concentrations ranging from 20 to 100 mg/L (for microbially synthesised AgNPs) and 10–50 mg/L (for silver nitrate) were prepared for larvicidal activity. The numbers of dead larvae were counted after 24 h of exposure, and the mortality percentage was reported from the average of five replicates. However, at the end of 24 h, the selected test samples turned out to be equal in their toxic potential (Rahuman et al. 2008).

Statistical analysis

All results were revealed as the mean ± standard deviation (SD) of the mean (SEM) values. Statistical significant difference was calculated using Chi square followed by regression co-efficient to compare with control group. A significance level of $P < 0.05$ was considered to be statistically significant.

Results and discussion

Biosynthesis of silver nanoparticles

Observed that upon the addition of biomass into the flask containing the $AgNO_3$ solution the colour of the solution was changed into brown colour within 72 h. This indicates the extracellular synthesis of AgNPs. The visual observation of colour change in $AgNO_3$ solution was reported by various research groups (Vahabi et al. 2011). In UV–spectral analysis, the maximum absorbance peak observed at 425 nm in the visible region (Fig. 1). These colour changes arise because of the excitation of surface plasmon vibrations with the AgNPs (Ahmad et al. 2003). Dry weight of the biosynthesised AgNPs is 65 mg/100 ml.

Characterisation of silver nanoparticles

The FT-IR spectrum shows the prominent absorption bands at 3485, 3438, 1638, 1371, 1237, 1090 and 669 cm^{-1} (Fig. 2). The bands observed at 3,485 cm^{-1} is due to OH stretching of water molecules. Another band observed at 3,438 cm^{-1} corresponds to the stretching vibration of primary amines respectively and their corresponding bending vibrations were observed at 1,638 cm^{-1}. The two bands observed at 1,371 and 1,237 cm^{-1} can be assigned to the C–N stretching vibration of aromatic and aliphatic amines

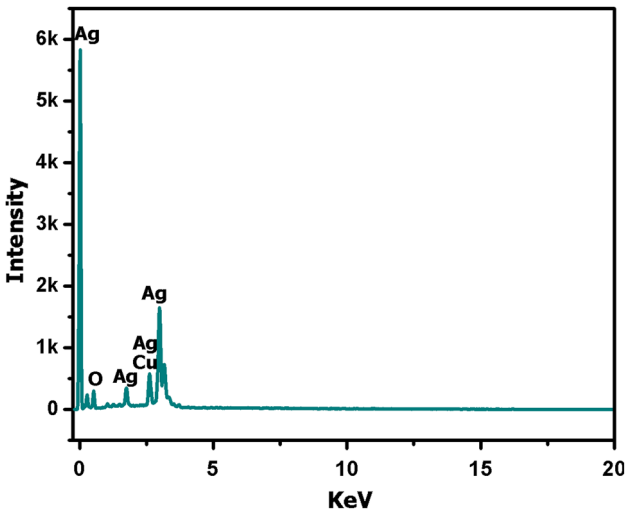

Fig. 4 EDX pattern shows strong Ag signals

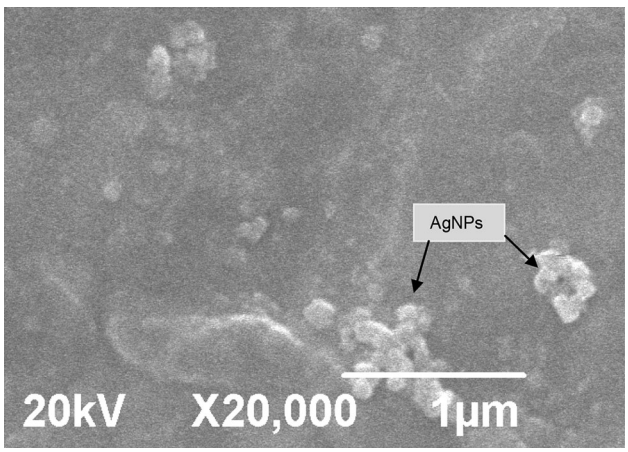

Fig. 5 Typical SEM image showing the spherical morphology and agglomeration of AgNPs at 45,000×

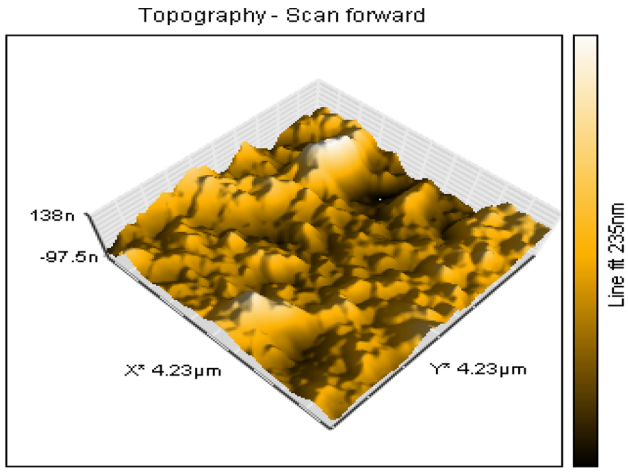

Fig. 6 Typical AFM image showing the 3D image topography of AgNPs at 235 nm

respectively. NO_3^- ions were free in the actinobacterial surface; it was proved by two bands observed at 1,090 and 669 cm^{-1}. Based on the overall observations, protein can bind to nanoparticles either through free amine groups or cysteine residues. Additionally, it binds with electrostatic attraction of negatively charged carboxylate groups of enzymes present in the cell wall of actinobacterial mycelium (Vahabi et al. 2011).

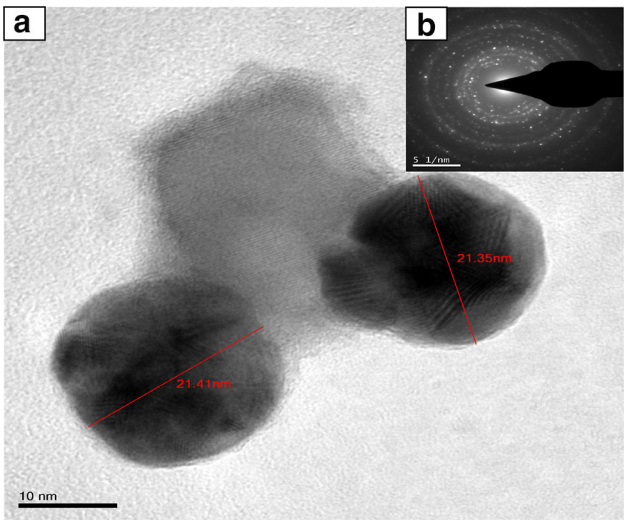

Fig. 7 **a** TEM image shows the clear shape and size of the AgNPs at 10 nm scale. **b** SAED pattern of microbially synthesised AgNPs

XRD pattern shows sharp peaks at the diffraction angles at 32.57°, 46.55°, 55.21° and 77.11° (Fig. 3) Corresponding to (111), (200), (220) and (311) Bragg's reflection, in the whole spectrum of 2θ values ranges from 10° to 90° respectively. The sharp intense peaks of the spectrum clearly indicated that the particles are in the nanoregime and also crystalline in nature. The size of the crystallite AgNPs was calculated using Debye–Scherrer formula (Sadhasivam et al. 2012). The calculated crystallite size of Ag particle is ~11.5 nm. The EDX analysis study shown in Fig. 4. The strong Ag signal confirms the presence of metallic AgNPs and their crystalline nature (Ahmad et al. 2003). The SEM observation showed that the presence of AgNPs clearly (Fig. 5). The topography of biosynthesised AgNPs were obtained from 3D image (Fig. 6) of AFM. The surface morphology was predicted, through image scanning in an area of 4.23 × 4.23 μm. Representative TEM images in Fig. 7a shows the well dispersed AgNPs and the morphology and size of the AgNPs are displayed more clearly in the figure at higher magnification. The AgNPs were mostly spherical in shape and showed a large distribution of sizes in the range of 10–35 nm. The SAED pattern of the AgNPs suggests clear single crystallinity showed in Fig. 7b. The nanoparticle shapes are resolved by the relative growth rate in different crystallographic directions. Based on the experimental results the interaction among biomolecules and surface of AgNPs were very strong. The crystal growth prejudiced by the inhibition of

Table 1 Larvicidal activity of the microbially synthesised AgNPs

Species	Concentrations (mg/L)	Percent mortality ± SD	LC_{50} (mg/L)	95 % confidence interval for LC_{50}		r^2	χ^2 ($df = 4$)	Regression equation
				LCL (mg/L)	UCL (mg/L)			
Anopheles subpictus	100	100 ± 0.00	51.34	47.72	55.22	0.962	8.228	y = −24.5x + 128.9
	80	82 ± 2.36						
	60	66 ± 1.89						
	40	21 ± 3.33						
	20	8 ± 1.00						
Culex quinquefasciatus	100	100 ± 0.00	48.98	44.81	53.53	0.956	14.307	y = −23.8x + 130.4
	80	96 ± 2.25						
	60	54 ± 1.96						
	40	32 ± 2.50						
	20	13 ± 3.78						
Aedes aegypti	100	96 ± 2.24	60.232	55.854	65.032	0.972	4.042	y = −21.7x + 110.9
	80	62 ± 2.74						
	60	42 ± 2.73						
	40	21 ± 3.33						
	20	8 ± 1.00						

Control−nil mortality, a mean value of five replicates, significant at $P < 0.05$ level, *LC50* lethal concentration that kills 50 % of the exposed larvae *UCL* upper confidence limit, *LCL* lower confidence limit, *r2* regression co-efficient, *χ2* Chi square, *df* degrees of freedom

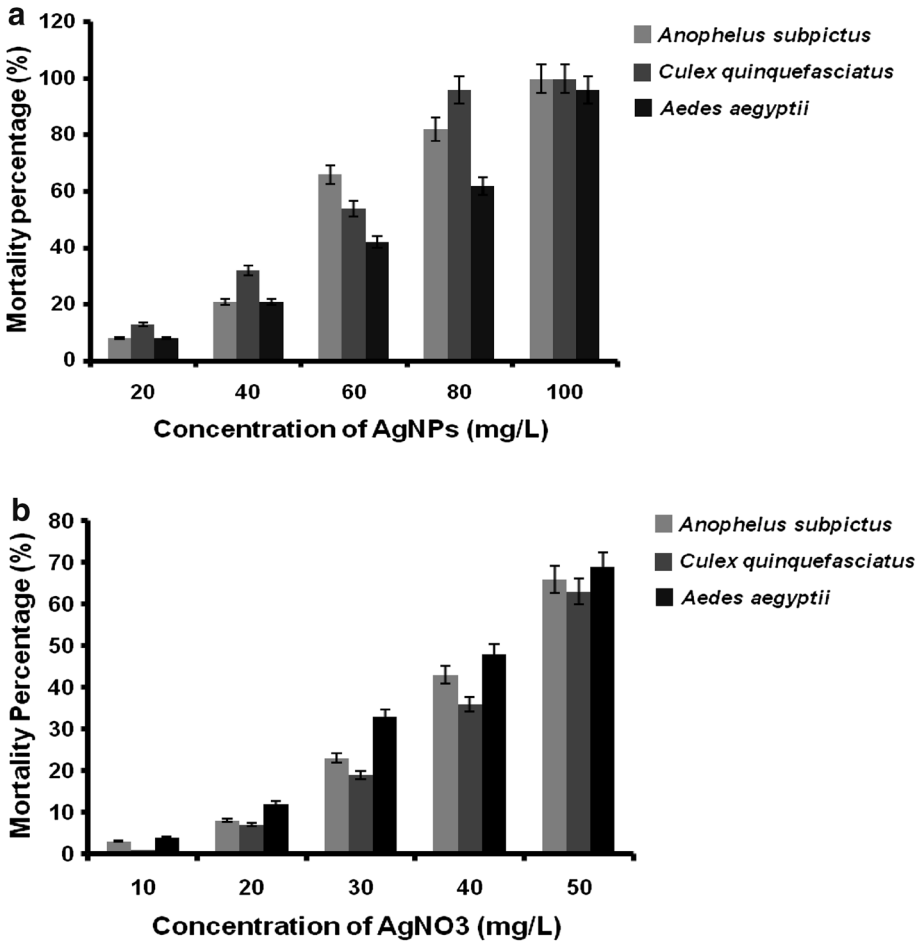

Fig. 8 **a** Mean mortality rate of microbially synthesised AgNPs on three mosquito species at different concentrations. **b** Mean mortality rate of $AgNO_3$ on three mosquito species at different concentrations

silver atom accumulation on the bacterial surface (Sadhasivam et al. 2012; Ahmad et al. 2003).

Larvicidal activity

In the present study, the larvicidal effect of AgNPs synthesised using actinobacterial strain M25 and $AgNO_3$ is noted. The highest mortality was found in synthesised AgNPs against the larvae of *A. subpictus* (LC_{50} 51.34 mg/L, $r^2 = 0.962$ and χ^2 value of 8.228), against the larvae of *C. quinquefasciatus* (LC_{50} 48.98 mg/L, $r^2 = 0.956$ and χ^2 value of 14.307) and against the larvae of *A. aegpyti* (LC_{50} 60.23 mg/L, $r^2 = 0.972$ and χ^2 value of 4.042), respectively (Table 1; Fig. 8a). Similarly, the precursor chemical $AgNO_3$ was found mortality against the larvae of *A. subpictus* (LC_{50} 42.544 mg/L, $r^2 = 0.954$ and χ^2 value of 2.561), against the larvae of *C. quinquefasciatus* (LC_{50} 44.922 mg/L, $r^2 = 0.936$ and χ^2 value of 1.693) and against the larvae of *A. aegpyti* (LC_{50} 39.664 mg/L, $r^2 = 0.983$ and χ^2 value of 5.724), respectively (Table 2; Fig. 8b).The Chi square value was significant at $P < 0.05$. The result indicates, the biosynthesised AgNPs were showed higher toxic effect than their precursor $AgNO_3$ against all the three mosquito species.

The larvicidal efficacy of *Cochliobolus lunatus* synthesised silver nanoparticles (AgNPs) was checked against larva of *A. aegypti* and *A. stephensi* at different concentrations (10, 5, 2.5, 1.25, 0.625 and 0.3125 ppm). The test was determined against second, third, and fourth instar larvae of *A. aegypti* (LC_{50}-1.29, 1.48 and 1.58; LC_{90}-3.08, 3.33 and 3.41 ppm) and *A. stephensi* (LC_{50}-1.17, 1.30 and 1.41; LC_{90}-2.99, 3.13 and 3.29 ppm) were observed (Salunkhe et al. 2011). The larvicidal activity of silver nanoparticles against malarial vector, *A. subpictus* and filariasis vector, *C. quinquefasciatus* was reported by Jayaseelan et al. (2011). The results suggested that the optimal times for measuring mortality effects of synthesised AgNPs were 33 % at 5 min, 67 % at 15 min, and 100 % after 1 h. The maximum activity was observed in the synthesised silver nanoparticles against lice, *A. subpictus* and *C. quinquefasciatus*(LC_{50} = 12.46, 6.43 and 6.96 mg/L; $\gamma^2 = 0.978$, 0.773 and 0.828), respectively. The maximum efficacy of AgNPs was observed against the larvae of *A. subpictus, C. quinquefasciatus,* and *Rhipicephalus microplus* (LC_{50} = 13.90,

Table 2 Larvicidal activity of the silver nitrate ($AgNO_3$)

Species	Concentrations (mg/L)	Percent mortality ± SD	LC_{50} (mg/L)	95 % confidence interval for LC_{50}		r^2	χ^2 ($df = 2$)	Regression equation
				LCL (mg/L)	UCL (mg/L)			
Anopheles subpictus	50	66 ± 2.36	42.544	38.41	47.126	0.954	2.561	y = −16.1x + 76.9
	40	43 ± 2.74						
	30	23 ± 2.73						
	20	8 ± 1.00						
	10	3 ± 2.74						
Culex quinquefasciatus	50	63 ± 2.73	44.922	42.388	47.612	0.936	1.693	y = −15.3x + 71.1
	40	36 ± 2.23						
	30	19 ± 2.23						
	20	7 ± 2.74						
	10	1 ± 1.00						
Aedes aegypti	50	69 ± 4.18	39.664	36.296	43.356	0.983	5.724	y = −16.6x + 83
	40	48 ± 2.74						
	30	33 ± 2.73						
	20	12 ± 3.33						
	10	4 ± 2.23						

Control–nil mortality, a mean value of five replicates, significant at $P < 0.05$ level, *LC50* lethal concentration that kills 50 % of the exposed larvae *UCL* upper confidence limit, *LCL* lower confidence limit, *r2* regression co-efficient, *χ2* Chi square, *df* degrees of freedom

11.73 and 8.98 mg/L, $r^2 = 0.411$, 0.286 and 0.479), respectively (Marimuthu et al. 2011). Priyadarshini et al. (2012) revealed that the biolarvicidal and pupicidal activity of silver nanoparticles against *A. stephensi*. Based on their report, the highest larval mortality was found in the synthesised silver nanoparticles against the first to fourth instar larvae and pupae of values LC_{50} (10.14, 16.82, 21.51 and 27.89 ppm, respectively), LC_{90} (31.98, 50.38, 60.09 and 69.94 ppm, respectively), and the LC_{50} and LC_{90} values of pupae of 34.52 and 79.76 ppm, respectively. The larvicidal activity of silver nanoparticles from plant source against *A. aegypti* and *A. stephensi* was also a very successful one (Patil et al. 2012). Based on these reports, the AgNPs synthesised in this present study showed improved toxicity against all the three mosquito vectors. Additionally, based on the available literatures this study is a novel report on the synthesis of AgNPs with larvicidal activity by using bacterial strains, especially in the genus of *Streptomyces*.

Conclusion

The present study demonstrated that the excellent larvicidal activity of actinobacterially synthesised AgNPs against mosquito vectors. Thus, the AgNPs have potential to be developed a novel larvicidal agents. However, the larvicidal action mode of AgNPs needs further investigations. This study is a newly added source for the synthesis of AgNPs by using actinobacteria in particularly *Streptomyces* sp. Moreover, these results could be very useful in the research for selecting novel and more selective larvicidal compounds. This report will also lead to the development of a coherent biosynthetic procedure for other metal nanomaterials such as gold and platinum with the actinobacterial strains from various ecosystems against almost all the vectors, which are responsible for transmit many diseases among human civilization.

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