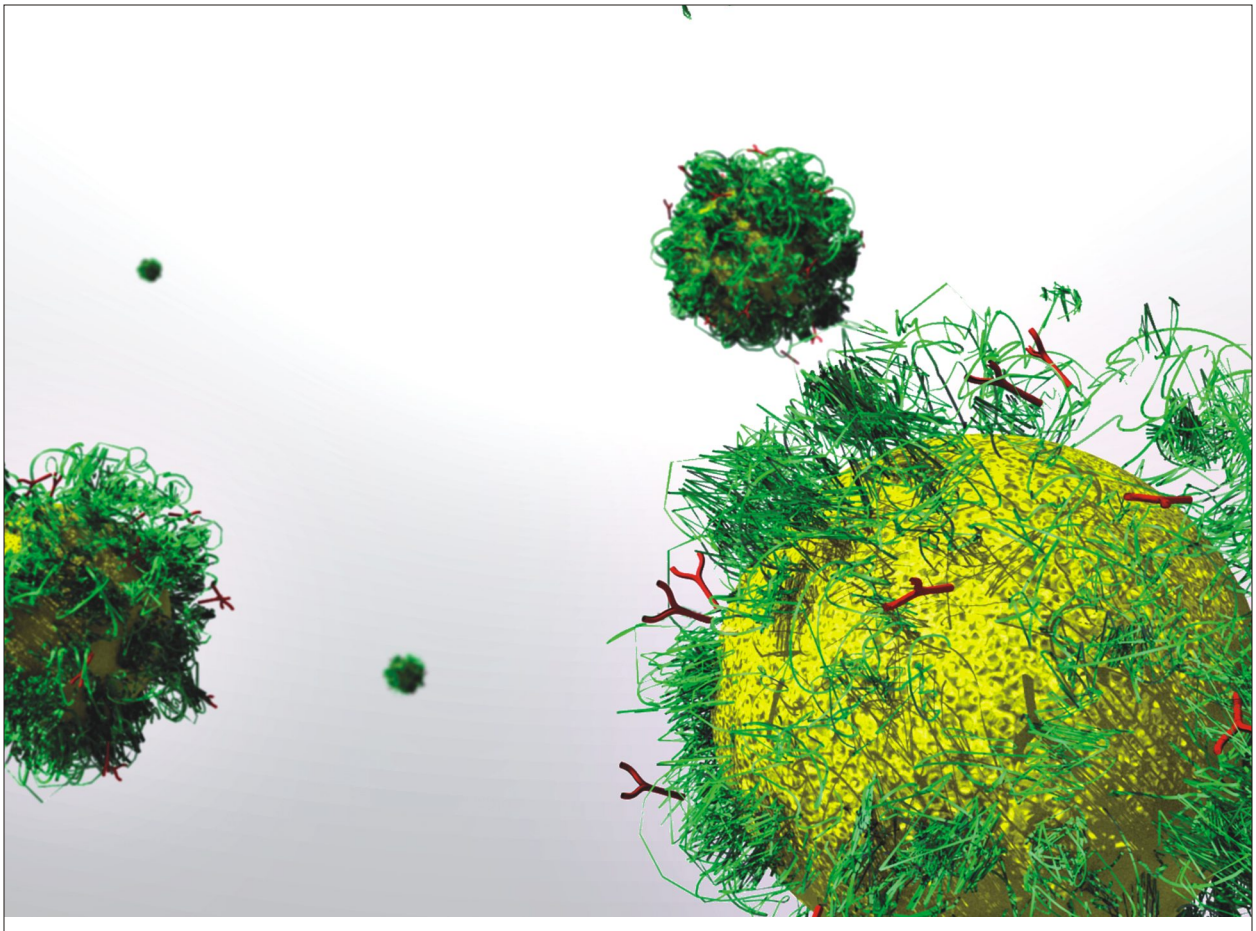

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Green Synthesis, Characterization and Larvicidal Activity of AgNps against *Culex quinquefasciatus* and *Aedes aegypti* Larvae

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ABSTRACT

The silver nanoparticles (AgNPs) were synthesized using extracellular metabolites of *Streptomyces* sp. GRD (JX512257). The characterization of the synthesized AgNPs was performed by UV-VIS spectrophotometer, FT-IR, Scanning Electron Microscopy (SEM) and X-ray diffraction spectroscopy. The synthesised silver nanoparticles have maximum absorption at 450 nm in UV-VIS spectrophotometer. The FTIR data showed prominent peak in 3401.33, 2922.30, 2364.79, 2116.07, 1643.17, 1375.53, 1424.69 and 1324.03 cm^{-1} ranges. The SEM analysis showed that the AgNPs were spherical and slightly elongated in nature. The XRD peaks revealed the crystalline nature of the silver nanoparticles and the size of the synthesized nanoparticles was found to be in the range of 10-15 nm. The biologically synthesized silver nanoparticles were treated against the mosquito larvae of *Culex quinquefasciatus* and *Aedes aegypti*. The LC_{50} value of synthesized nanoparticle was identified as 1.23 and 1.19 mg L^{-1} for *Culex quinquefasciatus* and *Aedes aegypti* larvae. Further, LC_{90} values are also identified as 2.97 and 4.93 mg L^{-1}, for *Culex quinquefasciatus* and *Aedes aegypti* respectively. Hence the present work suggests that the nanoparticles from *Streptomyces* sp. GRD would be appropriate for developing a biological control method of mosquito larval population.

Key words: Biosynthesis, mosquito, silver nanoparticles, *Streptomyces* sp., GRD, *Aedes aegypti*, *Culex quinquefasciatus*

INTRODUCTION

Mosquito vectors are solely responsible for transmitting diseases such as malaria, dengue, chikungunya, Japanese encephalitis and lymphatic filariasis. *Anopheles* species are the most important species as they are capable vector for malaria parasites. About 3.3 billion people of the world's population are at risk of malaria (WHO, 2012a). *Culex* mosquitoes are painful and persistent biters responsible for filariasis. Lymphatic filariasis is a neglected tropical disease. More than 1.3 billion people in 72 countries worldwide are threatened by lymphatic filariasis, commonly known as elephantiasis. Over 120 million people are currently infected, with about 40 million disfigured and incapacitated by the disease (WHO, 2012b). *Aedes* mosquitoes on the other hand are also painful and persistent biters. *Aedes aegypti* is responsible for dengue. The incidence of

dengue has grown dramatically around the world in recent decades. Over 2.5 billion people of the world's population are now at risk from dengue. WHO currently estimates there may be 50-100 million dengue infections worldwide every year (WHO, 2012c).

Application of chemical insecticides provokes undesirable effects of chemical resistance, toxicity to non-target organism and environmental and human health concerns (Casida and Quistad, 2000). In this regards, nanoparticles exhibits important role in several aspects such as drug delivery, diagnostics, antimicrobial activities etc. (Suber *et al.*, 2005; Rai *et al.*, 2009). Chemical synthesis of nanoparticles requires high energy and are toxic in nature. Biological synthesis of silver nanoparticles provides economic and environmental friendly approach. In this regard the present study mainly focuses on the biological synthesis of silver nanoparticles using *Streptomyces* sp. GRD and the larvicidal activity of the silver nanoparticles against *Aedes* and *Culex* vectors which is responsible for the transmission of dengue and filariasis.

MATERIALS AND METHODS

Collection of microbial isolate: *Streptomyces* sp. GRD (JX512257) was collected from Germplasm, Bioprocess Technology Lab., Department of Microbiology, Bharathidasan University, Tiruchirappalli, 620024, Tamil Nadu, India.

Collection and identification of mosquito larvae: The mosquito eggs and egg raft of *Culex quinquefasciatus* and *Aedes aegypti* were collected from Centre for Research in Medical Entomology (CRME), Madurai, Tamilnadu. The collected egg was kept in a plastic tray containing dechlorinated water, in order to hatch out larvae. The reared larvae were maintained for five days in standard environment (28±2)°C temperature and 14:10 light and dark period cycle; the larvae were fed with powdered mixture of dog biscuits and yeast powder in 3:1 ratio. Further the larvicidal activity was assessed by the WHO guideline with some modification of Rahuman *et al.* (2000) methods.

Biosynthesis of silver nanoparticles: *Streptomyces* sp. GRD was grown in Starch casein broth medium until the growth reached stationary phase. After incubation, the medium was harvested and centrifuged at 10,000 rpm for 10 min to remove cell debris. The collected supernatant was challenged with 1 mM silver nitrate ($AgNO_3$) as precursor and the flasks were incubated at room temperature in a shaker and was observed for colour change.

Separation and purification of AgNPs: After the completion of the reaction, the content was centrifuged at 15000 rpm for 5 min. The pellet was washed thrice with sterile deionized distilled water to remove excess of silver ions from synthesized silver nanoparticles. The AgNP's pellet was freeze dried in a lyophilizer (Lark penguin classic) and used for quantification and further characterization studies.

Characterization of silver nanoparticles

UV visible of silver nanoparticles: The formation of AgNPs was characterized by UV visible spectroscopy using UV/VIS Spectrometer (PG-Instruments). Steady-state emission spectra were measured using a RF 200-700 spectrophotometer.

Fourier transform infrared (FT-IR) analysis of silver nanoparticles: The samples were analysed for their variations in chemical groups using FT-IR spectroscopy (Perkin-Elmer Spectrum-One instrument in the diffuse reflectance mode at a resolution of 4 cm^{-1} in KBr pellets). The wavelength of light absorbed is characteristic of the chemical bond can be seen in this annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined.

SEM analysis of the synthesized silver nanoparticles: The surface morphology of silver nanoparticles was observed with a Scanning Electron Microscope (SEM) JEOL Model JSM-6390LV with secondary electron detectors at an operating voltage of 15 kV with a magnification of 9.65 kx.

X-ray diffraction analysis of AgNPs: The freeze-dried silver nanoparticles were used for X-ray Diffraction (XRD) analysis. XRD patterns were recorded operating at 40 kV and a current of 30 mA with Cu Ka radiation (l = 1.54 A°). The diffracted intensities were recorded from 0-80 2θ angles. The crystalline domain size was calculated from the width of the XRD peaks, using the Scherrer equation:

$$D = \frac{0.94\,\lambda}{\beta \mathrm{Cos}\theta}$$

Where:

D = Average crystallite domain size perpendicular to the reflecting planes
λ = X-ray wavelength
β = Full width at half maximum (FWHM)
θ = Diffraction angle

Mosquito larvicidal activity of actinobacterial derived AgNPs: The toxicity test of actinobacterial derived AgNPs was performed by WHO method with some modification by placing around 25, III and IV instar larvae into 200 mL of sterilized double-distilled water with AgNPs into a 250 mL beaker. The nanoparticle solutions were diluted using double-distilled water as a solvent according to the desired concentrations (5, 4, 2, 1, 0.5 mg L^{-1}). Each test included a set control group (silver nitrate and distilled water) with four replicates for each individual concentration. Mortality was evaluated after 24 h to determine the acute larvae toxicities in *Culex* sp. and *Aedes* sp. (Finney, 1971).

Dose response bioassay: Based on the preliminary screening results, synthesized AgNPs were subjected to dose-response bioassay for larvicidal activity against the larvae of *Culex quinquefasciatus* and *Aedes aegypti*. Different concentrations of synthesized AgNPs ranging from 0.1-5 ppm were prepared for larvicidal activity of mosquitoes. The numbers of dead larvae were counted after 24 h of exposure and the percentage of mortality was reported from the average of four replicates.

Statistical analysis: The average larval mortality data were subjected to probit analysis for calculating LC_{50}, LC_{90} and other statistics at 95% of upper confidence limit and lower confidence limit and chi-square values were calculated using the software developed by Reddy *et al.* (1992). Results with $p<0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

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 nanoparticles are considered to be potential agents for various biological applications including antimicrobial, its application as a mosquito larvicidal agent was investigated.

The present study was focused on the extracellular synthesis of AgNPs from *Streptomyces* sp. GRD. When $AgNO_3$ was added to the supernatant of *Streptomyces* sp. GRD the colour of the supernatant changed from yellow to dark brown and in control there was no colour change (Fig. 1). The similar reports of colour change during extracellular biosynthesis of AgNPs were also reported in other studies (Duran *et al.*, 2007; Vigneshwaran *et al.*, 2007; Dhanasekaran and Thangaraj, 2012). The biological synthesized AgNPs recognized that surface Plasmon resonance peak in the UV-Visible spectrophotometer absorption spectra of the silver nanoparticles synthesized by biological reduction showed an absorption peak at 450 nm (Fig. 2).

Generally, UV-VIS spectroscopy can be used to examine size and shape of the controlled nanoparticles in aqueous suspension. The results of the UV-VIS absorption showed increasing

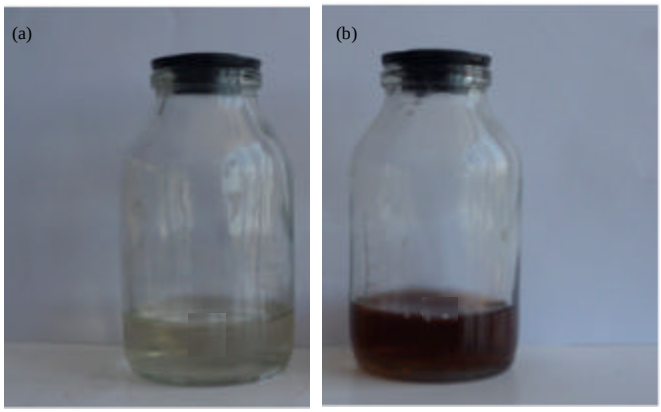

Fig. 1(a-b): Synthesis of silver nanoparticles by the *Streptomyces* sp. GRD. (a) Broth before the addition of $AgNo_3$ and (b) Synthesis mixture showing the formation of silver nanoparticles

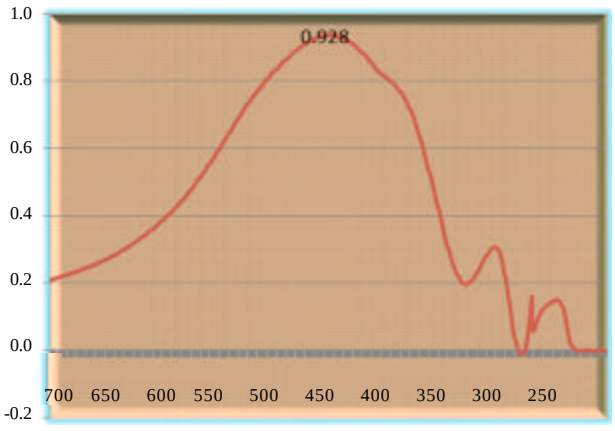

Fig. 2: UV spectra of silver nanoparticles synthesized by *Streptomyces* sp. GRD

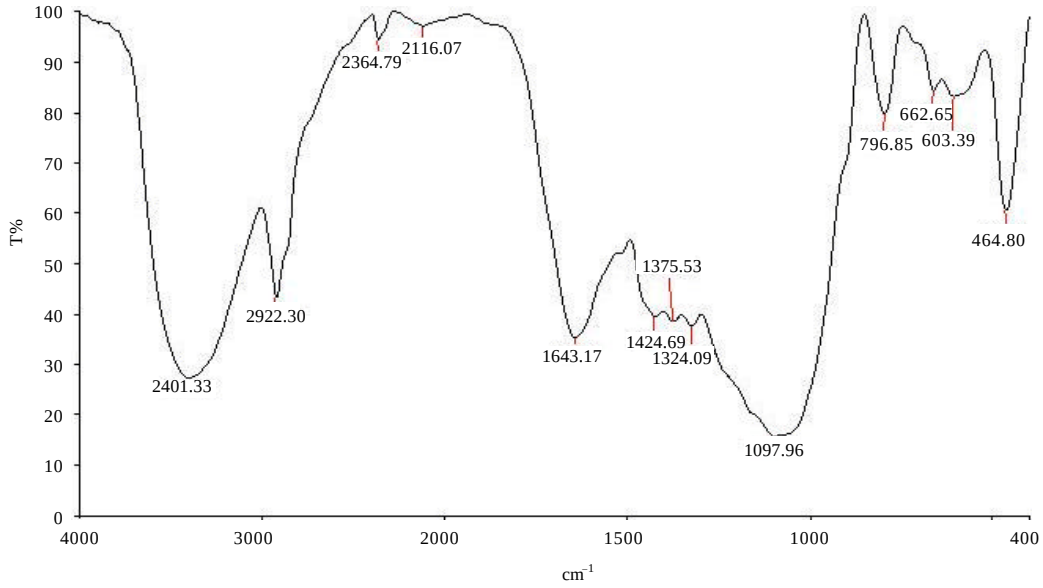

Fig. 3: FTIR spectra of silver nanoparticles synthesized by *Streptomyces* sp. GRD

colour intensity with increased time intervals and this might be due to the production of the silver nanoparticles (Romero and Srivastava, 2010) and the formation of the brownish yellow colour might be due to the surface plasmon vibration of the synthesised silver nanoparticles (Krishnaraj *et al.*, 2010).

Further the result of FT-IR spectra of the actinobacterial synthesized AgNPs revealed prominent peaks with different values such as 3401.33, 2922.30, 2364.79, 2116.07, 1643.17, 1375.53, 1424.69 and 1324.03 cm^{-1} (Fig. 3). The spectrum shows the band at 3500-3300 cm which corresponds to a primary amine NH band; similarly, bands at 2364.79 and 2116.07 cm^{-1} correspond to a secondary amine C-H band and a primary amine CN stretch vibration of the protein, respectively. There is no corresponding vibrations for C = O stretching. The intense band at 46480 cm^{-1} is assigned to O-H. The two bands at 1424.69 and 1324.03 cm^{-1} correspond to the C = O stretching vibrations of aliphatic and aromatic amines, respectively.

The results of the FTIR used to the stabilization of the synthesised silver nanoparticles. The prominent peaks of the FTIR results are showing the correspond values to the amide group (N-H stretching-3426.89), aliphatic group (cyclic CH_2-2925.49), methyl group (CH -2869.56), alkane group (CH-2346.95) alkene (CC-1631.49 and 669.178) and ether groups (COC-1031.73). The observed peaks are consider as major functional groups in different chemical classes such as flavinoides, triterpenoids and polyphenols (Nabikhan *et al.*, 2010). Hence, the terpenoids are proven to have good potential activity to convert the aldehyde groups to carboxylic acids in the metal ions, further, polyphenols are also proven to have potential reducing agent in the synthesis of the silver nanoparticles. Further, amide groups are responsible for the presence of the enzymes and these enzymes are responsible for the reduction synthesis and stabilization of the metal ions (Prasad and Elumalai, 2011; Mukunthan *et al.*, 2011).

The biosynthesized silver nanostructure by using actinobacteria was further demonstrated and confirmed by electron microscopy. The SEM micrographs of nanoparticle obtained in the prepared sample showed that AgNPs are spherical shaped, well distributed without aggregation in solution

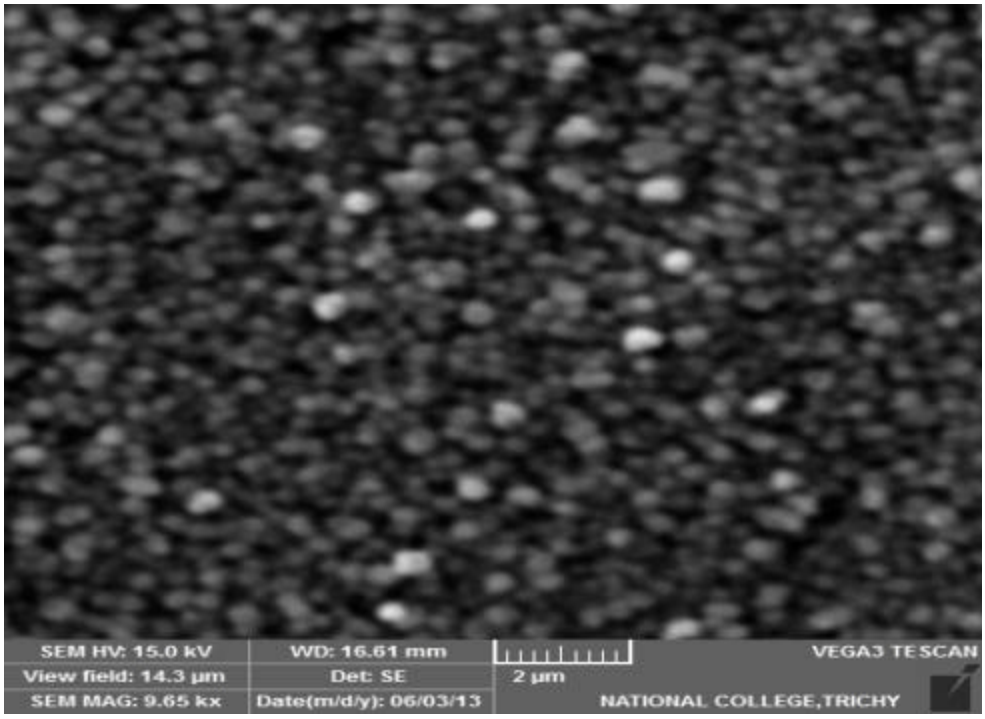

Fig. 4: SEM analysis of silver nanoparticles synthesized by *Streptomyces* sp. GRD

Table 1: Larvicidal activity of silver nanoparticle synthesized by *Streptomyces* sp. GRD extract against *Culex quinquefasciatus* and *Aedes aegypti*

Species	Concentration (mg mL^{-1})	Mortality*±SD	LC_{50}±SE	(UCL–LCL)	LC_{90}±SE	(UCL–LCL)	χ^2 (df = 4)
Culex quinquefasciatus	0.5 (0.5 ppm)	13±2.00	1.23±0.06	1.35-1.10	2.97±0.23	3.42-2.53	15.2
	1.0 (1 ppm)	33±1.82					
	2.0 (2 ppm)	75±2.30					
	4.0 (4 ppm)	96±1.60					
	5.0 (5 ppm)	100±0.00					
Aedes aegypti	0.5 (0.5 ppm)	12±2.60	1.19±0.09	1.37 -1.02	4.93±0.06	6.09-3.75	15.2
	1.0 (1 ppm)	46±0.84					
	2.0 (2 ppm)	58±2.04					
	4.0 (4 ppm)	82±4.24					
	5.0 (5 ppm)	100±0.00					

LC_{50}: Lethal concentration that kills 50% of the exposed larvae, LC_{90}: Lethal concentration that kills 90% of the exposed larvae, UCL: Upper confidence limit, LC: Lower confidence limit, χ^2 chi-square, df: Degree of freedom. Significant at $p<0.05$ level

(Fig. 4). It is known that the shape of metal nanoparticles considerably change their optical and electronic properties (Marimuthu *et al.*, 2011). Similar phenomenon was reported by Chandran *et al.* (2006). X-ray diffraction pattern (XRD) was recorded for the synthesized Ag NPs (Fig. 5). Three distinct diffraction peaks at 38.45°, 44.64° and 64.71° were indexed with the planes (111), (200) and (220) for the face-centered cubic silver. The XRD pattern thus clearly shows that the silver nanoparticles are crystalline in nature. The size of the silver nanoparticles as calculated by the Scherrer equations was found to be in the range of 10-15 nm. Similar results were obtained by Dhanasekaran *et al.* (2013).

Table 2: Larvicidal activity of the *Streptomyces* sp. GRD synthesised silver nanoparticles against *Cx. quinquefasciatus and A. aegypti* larvae

Concentration (mg mL^{-1})	Percentage of mortality*±SD in *Culex quinquefasciatus*	Percentage of mortality*±SD in *Aedes aegypti*
5 (5 ppm)	100±0.00	100±0.00
4 (4 ppm)	96±1.60	82±4.24
2 (2 ppm)	75±2.30	58±2.04
1 (1 ppm)	33±1.82	46±0.84
0.5 (0.5 ppm)	13±2.00	12±2.60

Values are represented in Mean±SD values

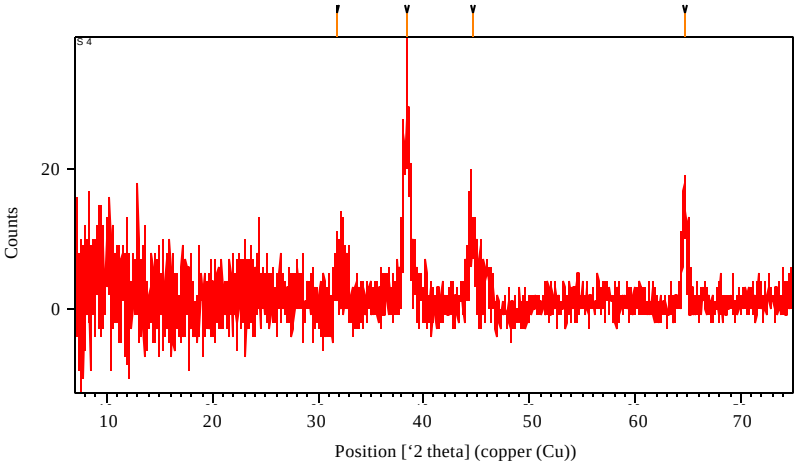

Fig. 5: XRD analysis of the synthesized silver nanoparticles

The result of the present study suggested that, 5 mg L^{-1} concentration of AgNPs showed 100% of early third instar larvae of *Culex quinquefasciatus* and *Aedes aegypti* (Table 2). The results suggested that, the value of LC_{50} was identified as 1.23 and 1.19 mg L^{-1} for *Culex quinquefasciatus* and *Aedes aegypti* larvae. Further, LC_{90} values are also identified as 2.97 and 4.93 mg L^{-1}, for *Culex quinquefasciatus* and *Aedes aegypti* respectively. The results of Upper Confidential Level (UCL), Lower Confidential Level (LCL) and chi squire (χ^2) values are also recorded in Table 1.

The mechanism which causes the death of the larvae could be the ability of the nanoparticles 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 (Rai *et al.*, 2009). The mortality effect of silver nanoparticles on mosquito larvae may be enabled by the small size of the particles, which allows passage through the insect cuticle and into individual cells where they interfere with molting and other physiological processes. Safaepour *et al.* (2009) reported that a 0.5 µg mL^{-1} concentration of silver nanoparticles inhibited cell growth by <30%, whereas at 5 µg mL^{-1}, cell growth was inhibited by >60%.

CONCLUSION

In this conclusion, biological AgNPs synthesized by using *Streptomyces* sp. GRD has been tested against the larvae of *Culex quinquefasciatus* and *Aedes aegypti*. By this approach, it is suggestive that rapid synthesis of nanoparticles would be proper for developing a biological process for mosquito control. As there is no significant detailed report on mosquito larvicidal activity using

AgNPs synthesized from actinobacterial metabolites, this study will attract the researchers to work further in mass production and field application of nanoparticles reduce the mosquito larvae population.

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