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Mycosynthesis of Silver Nanoparticles (*Trichoderma viride*, *Trichoderma longibrachiatum)* and their Mosquito Larvicidal Efficacy on Dengue Vectors and Acute Toxicity on *Moina macrocopa*

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Current study is based on the larvicidal effect of mycosynthesized siver nanopartices (AgNPs) from *Trichoderma longibrachiatum* (Tl-AgNPs) and *Trichoderma viride* (Tv-AgNPs) on *Aedes aegypti* and *Aedes albopictus* dengue vectors and non-targeted aquatic crustacean, *Moina macrocopa.* Mosquito larvae were exposed to each test concentration (10 to 50 mg L^{-1}) of Tl-AgNPs and Tv-AgNPs separately with three replicates containing 25 larvae each. The AgNPs characterization confirmed the formation of Tl-AgNPs and Tv-AgNPs in the UV-Vis spectrum with the surface plasmon resonance (SPR) band at 430 nm, existence of biomolecules and stabilizing agents on the AgNPs in FTIR spectroscopy and the presence of spherical shape AgNPs in a size range of 15-20 nm in TEM. The total larval exposure period was 48 h in order to evaluate 24 and 48 h larval mortalities and the toxicity effects on *Moina macrocopa* were also evaluated. LC_{50} were recorded as 14.68 mg L^{-1},14.05 mg L^{-1} after 24 h and 13.4 mg L^{-1}, 12.94 mg L^{-1} after 48 h of exposure to *A. aegypti* and *A. albopictus*, respectively for Tl-AgNPs. The LC_{50} for Tv-AgNPs were recorded as 12.08 mg L^{-1},14.05 mg L^{-1} after 24 h and 13.4 mg L^{-1}, 12.94 mg L^{-1} after 48 h exposure for *A. aegypti* and *A. albopictus*, respectively. The Tv-AgNPs are reported to be more toxic for *M. macrocopa* after 48 h exposure with LC_{50} 0.167 ppm, LC_{90} 0.291 ppm for Tl-AgNPs and LC_{50} 0.155, LC_{90} 0.248 ppm for Tv-AgNPs. This study concludes Tl-AgNPs and Tv-AgNPs could be used as potential larvicide for dengue vector control. Results confirmed that Tv-AgNPs are more effective than Tl-AgNPs in controlling mosquito larvae. However, there is a potential threat to other non-targeted organisms in the practical aspects of this treatment.

Keywords: Silver nanoparticles, *Moina macrocopa*, *Trichoderma viride*, *Trichoderma longibrachiatum*, *Aedes albopictus*, *Aedes aegypti*.

INTRODUCTION

Being the major vector-borne disease in the country, Sri Lanka has produced favourable conditions for the transmission of the dengue virus [1] with urbanization and human population growth. With the circulation of all four serotypes (DEN 1,2,3,4) of the dengue virus, the World Health Organization has categorized Sri Lanka as a hyperendemic country. The established vectors are *Aedes aegypti* and *Aedes albopictus*, which belong to the subgenus *Stegomyia*. With these favourable determinants, amiability to sudden dengue outbreaks has rendered the country highly vulnerable over the decades. The first serologically confirmed dengue fever was reported in 1962 in Sri Lanka [2]. However, a rapid rise of over 30,000 cases was reported in 2009 [3]. Since then, the baseline remained as this with higher numbers of around 50,000 reporting during 2012, 2014, 2016, 2017 and 2018. The year 2017 reported the largest outbreak in history, reporting over 180,000 patients (833.9 per 100,000 population) and 414 deaths (case fertility eate: 0.24) throughout the country.

Due to several constraints in dengue vectors such as the development of resistance to synthetic insecticides, transmission of novel and new strains of known arboviruses and the fast spread of vectors, dengue vector controlling strategies have become challenging. Therefore, the employment of synthetic, herbal and microbial larvicides, adult repellents, sterile insect

technique (SIT), bacterial symbiont-based treatments, and transgenic mosquitoes cannot be deemed totally efficient in dengue vector control at this time [4]. In these circumstances, the application of nanotechnology in mosquito control methods may have better potential. The use of nanomaterial would become effective due to the biogenic nature and host-specific activities [5]. Therefore as an alternative to carbamates and pyrethroids, green synthesized nanoparticles have been used [6,7]. In such circumstances, fungal-mediated nanoparticles considered in the present study would be a promising tool. The presence of bioactive molecules in fungal secondary metabolites, fungi and derivatives is toxic to mosquitoes and less toxic to non-target organisms [6]. It has been screened extracellular secondary metabolites as larvicides for many mosquito species [8-10].

Trichoderma fungi are soil-borne, green-spored and are common. *Trichoderma* spp. is a ubiquitous colonizer of cellulose and may also produce chlamydospores and form highly successful colonies due to efficient substrate utilization and secretion of metabolites and enzymes. They are efficient mycoparasites, antagonistic and biocontrol agents [11]. Recently, the larvicidal activity of extracellular extracts of *T. longibrachiatum* and *T. viride* against *Aedes aegypti and Aedes albopictus* larvae was reported [12]. During this study, the extracellular extracts were used to synthesize AgNPs with the expectation of studying them as a potential larvicide for the dengue vectors. Several studies have been carried out previously to assess the efficacy of fungi-mediated nanoparticles against mosquito vectors. AgNPs synthesized using the fungus *Cochliobolus lunatus* [10] and the fungus *Beauveria bassiana* [13] have shown the larval toxicity against *Anopheles stephensi* and *Aedes aegypti*. Herein, the potential of using *Trichoderma longibrachiatum* (Tl) and *Trichoderma viride* (Tv) mediated silver nanoparticles (Tl-AgNP/Tv-AgNP) to control dengue vector larvae, *Aedes aegypti* and *Aedes albopictus* is reported. Simultaneously, the toxicity of these AgNPs on non-targeted aquatic organisms was studied using a model organism. As an model organism available for nanoparticle ecotoxicity tests, cladoceran, *Moina macrocopa* [14] has been selected to assess the effect of Tl-AgNPs and Tv-AgNPs on non-targeted aquatic fauna.

EXPERIMENTAL

Fungal species: *Trichoderma longibrachiatum* (GenBank Accession Number MT507837) and *Trichoderma viride* were sub-cultured and pure cultures were maintained on potato dextrose agar (PDA) plates and stored at 4 ºC.

Maintaining egg sheets and larvae of *Aedes aegypti*, *Aedes albopictus* and *Moina macrocopa*: *Aedes aegypti* egg sheets were obtained from the National Dengue Control Unit and *Aedes albopictus* egg sheets were obtained from Medical Research Institute. Egg sheets were hatched in distilled water in aluminum trays. The larvae were fed with 0.5 mg fish food daily and third instar larvae were taken for the experiment. *Moina macrocopa* samples were obtained from ornamental fish breeding and training center (NAQDA) Rambadagalla, Sri Lanka and fed with green algae *Chlorella* (2×10^4 cells/mL). *Moina macrocopa* neonates of less than 24 h of age were used for the acute toxicity testing.

Fungal biomass: Fungi were cultured in autoclaved Richards' broth liquid media (glucose, agar, potassium nitrate, potassium dihydrogen phosphate, magnesium sulfate and ferric chloride) and maintained in the boiling tubes and incubated at 25-28 ºC. The biomass was harvested by sieving through a Whatman filter paper No. 1 after 7 days. It was washed with deionized water and kept in deionized water for 48 h in dark conditions. The fungal filtrate was obtained by filtering through a Whatman filter paper No. 1 to prepare the AgNPs.

Preparation of AgNPs: The culture filtrate of two fungal spp. (100 mL) was mixed with 0.01 M $AgNO_3$ (100 mL) solution separately and incubated for 96 h in dark conditions at 28 ± 2 ºC. Initial colour and then the colour change after 96 h were recorded. The solution was subjected to centrifugation at 3000 rpm for 20 min (HERMLEZ 206 A). The resulting nanoparticle pellet was washed with deionized water (5 mL) and centrifuged under the same conditions and this process was repeated once. The obtained AgNPs were separated, freeze-dried and stored at 4 ºC.

Preparation of crude metabolites: Mycelium (10 g) from a fungal culture was extracted in 100 mL of ethyl acetate at room temperature for five days. After straining the mixture with muslin cloth, the mycelium was separated away. This process was done three times and the resulting filtrates were centrifuged at 3000 rpm for 20 min to remove any remaining debris. The liquid was then poured into a round-bottom flask and concentrated using a rotary evaporator set to 40 ºC. A concentration series of aqueous crude fungal extract was prepared using the obtained solid crude.

Characterization of AgNPs

Visual observation: Initial colour and the colour change of the solution was recorded after 96 h of incubation.

UV-visible analysis: UV-Vis spectrophotometer (Orion Aquamate, 8000) was used to observe the surface plasmon resonance (SPR) band from 200 to 800 nm using a resolution of 5 nm.

TEM analysis: The size and shape of the synthesized AgNPs were determined using a Hitachi TEM 9500 microscope that operated at 300 kV. Samples were prepared by making a dispersion of AgNPs (1 mg nanoparticles/1 mL solvent) and drop casting 200 µL of sample on a carbon-coated copper grid (300 mesh). At room temperature, the samples were dried out through the solvent evaporation.

FTIR analysis: Fourier transformed infrared spectrophotometer (Perkin-Elmer) at the Department of Chemistry, University of Kelaniya, Sri Lanka was used to obtain the FTIR spectrum recorded from 4000 to 750 cm^{-1} by mixing the dried AgNP powder and fungal crude separately with KBr.

Dengue vector larval toxicity test: Test concentrations of *Trichoderma longibrachiatum* and *Trichoderma viride* induced AgNPs and their crude extracellular fungal metabolites were produced separately into a final volume of 200 mL into glass beakers. 25 Larvae of *Aedes aegypti* and *Aedes albopictus* in their third instar were subjected to each test concentration and

control (distilled water) sample, with three replicates of each. The control samples served as a measure of comparison. Every day, food for the larvae was delivered. After 24 and 48 h of exposure, data on mortality rates were collected.

In order to determine the overall mortality rate, moribund larvae were counted and added to the total number of dead larvae. Those larvae are considered to be dead if they do not move in response to a needle being inserted into either the siphon or the cervical region of their bodies. Those larvae that did not demonstrate the typical diving response when the water was disturbed were considered moribund [15]. Percentage mortality was determined after applying Abbott's formula [16] to account for control mortality:

$$\text{Mortality (\%)} = \frac{\text{No. of dead larvae}}{\text{No. of larvae introduced}} \times 100$$

Acute toxicity analysis: Ten randomly selected neonates were placed in 100 mL exposure media ranging from 0.1 mg^{-1} to 0.325 mg L^{-1} (extracellular crude metabolites and nanoparticles) in glass beakers separately. Neonates were not fed during the experiments. The experiments was carried out in three replicates. Dead neonates were recognized by lightly touching the individual for 18 s with a dissecting needle and immobile people were deemed dead. Mortality data were obtained after 48 h of exposure.

Statistical analysis: Probit analysis was done for larval mortality and neonate mortality data for calculating LC_{50}, LC_{90} at 95% fiducial limits of upper confidence limit and lower confidence limit. Chi-square values were also calculated using the software developed by IBM SPSS version 20. The statistically significant results were considered with $p < 0.01$.

RESULTS AND DISCUSSION

Characterization of AgNPs

Visual observation of the colour development of the test solution mixture: The fungal filtrate and 0.1 mM $AgNO_3$ mixture turned from pale yellow to a brick colour after 96 h of incubation confirming the formation of the AgNPs (Fig. 1).

UV-visible studies: A peak at 430 nm corresponding to the plasmon absorbance of silver nanoparticles was observed in UV-visible spectrum (Fig. 2).

TEM studies: Monodispersed roughly spherical-shaped TEM micrograph images (Fig. 3) obtained with an average size ranging from 15 nm to 20 nm of AgNPs.

FTIR studies: The FTIR spectrum of extracellularly biosynthesized Tl-AgNPs, Tv-AgNPs and the fungal crudes are shown in Fig. 4. Major peaks were identified at 1700, 1452, 1101 and 3400 cm^{-1} in all four spectra. The peak at 1700 cm^{-1} corresponds to C=O stretching, whereas peak at 1452 cm^{-1} is assigned to CH_3 bending. The peak between 1400-1000 cm^{-1} corresponds to the C-O stretching. This substantiates the existence of biochemicals derived from fungal extracts as well as agents for capping and stabilizing.

Larval toxicity test: Mortality rates of *Aedes aegypti* and *Aedes albopictus* mosquito larvae exposed to Tv-AgNPs, Tl-AgNPs and extracellular crude metabolites are depicted in Fig.

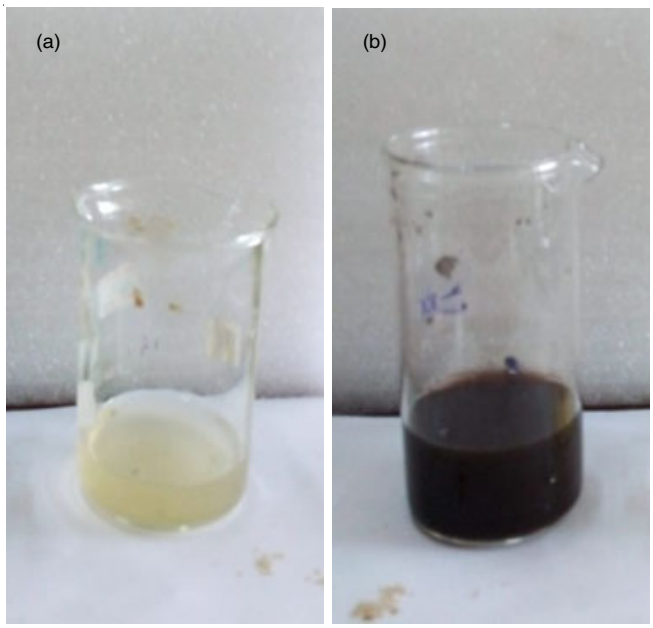

Fig. 1. (a) Initial colour (b) Colour after 96 h of incubation time of samples containing fungal filtrate and 0.01 M:$AgNO_3$

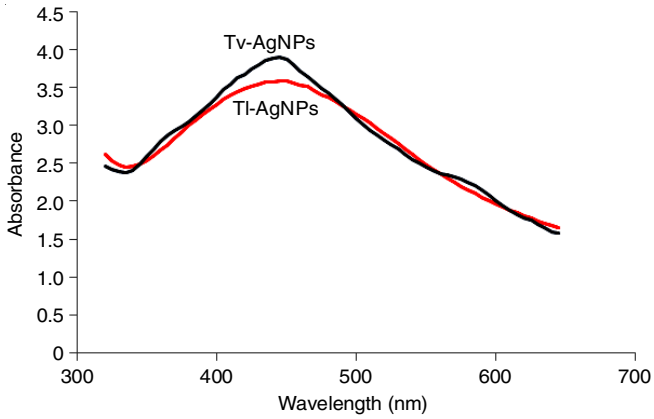

Fig. 2. UV-vis spectrum with a characteristic peak between 400 nm and 500 nm

5. A rise in treatment concentration and an extension of exposure period from 24 h to 48 h has led to an increase in the mortality rate. The efficacy of both mycosynthesized Tl-AgNPs and Tv-AgNPs at all the tested concentrations (10-50 mg L^{-1}) against the third instar larvae of *A. albopictus* was higher than that of *A. aegypti*. The LC_{50} and LC_{90} values of Tl-AgNPs, Tv-AgNPs and extracellular crude metabolites at 24 and 48 h for *A. aegypti* and *A. albopictus* are given in Tables 1 and 2. The efficacy of Tv-AgNPs was higher than that of Tl-AgNPs for both vector species. The mortality of both larvae positively correlated with the concentration of AgNPs. Both *A. aegypti* and *A. albopictus* larvae showed 100% mortality at 50 mg L^{-1} concentration of both Tl-AgNPs and Tv-AgNPs after 24 h exposure.

Efficacy of extracellular crude metabolites of all the tested concentrations for the efficacy of *T. viride* extracellular crude metabolites was higher than that of *T. longibrachiatum* for *A. aegypti* and *A. albopictus*. However, *A. albopictus* was found to be the most susceptible vector for both crude extracellular

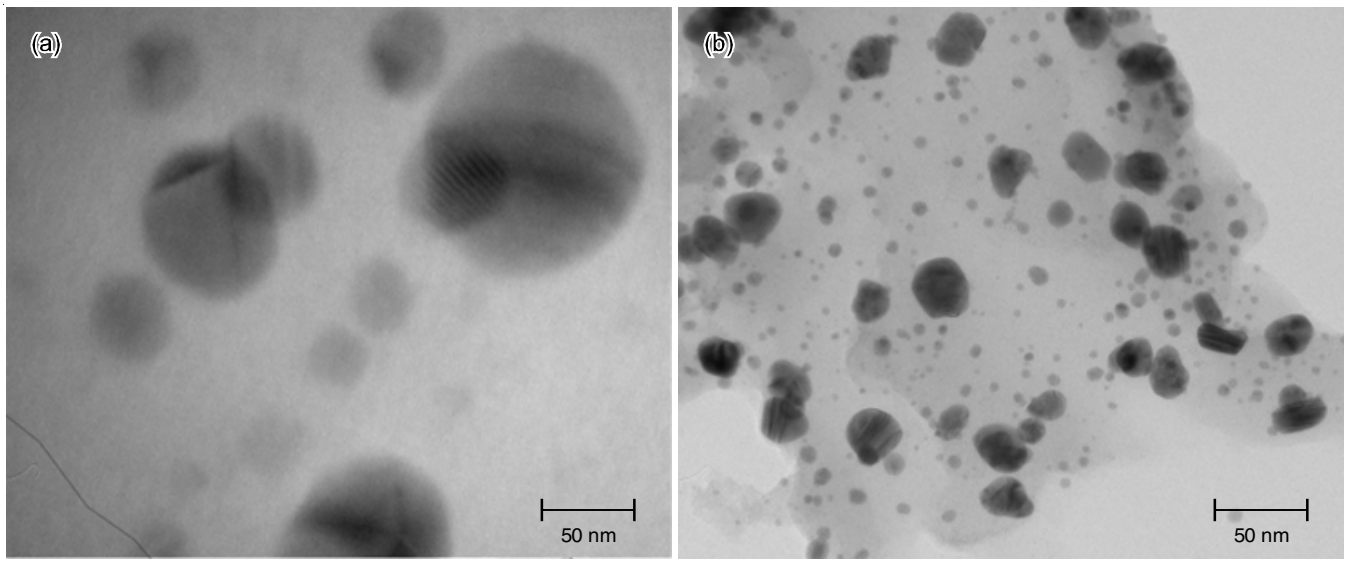

Fig. 3. Transmission electron microscopy images of (A) Tl-AgNPs (scale bar corresponds to 50 nm) and (B) Tv-AgNPs (scale bar corresponds to 50 nm)

TABLE-1
LC_{50}, LC_{90} OF *T. longibrachiatum* AND *T. viride* MEDIATED SILVER NANOPARTICLES AND EXTRACELLULAR CRUDE METABOLITES AGAINST THIRD INSTAR LARVAE OF *A. aegypti*

Exposure time (h)	*T. longibrachiatum* (mg L^{-1}) at 95% confidence limit				*T. viride* (mg L^{-1}) at 95% confidence limit			
	Tl-AgNPs		Crude extract		Tv-AgNPs		Crude extract	
	LC_{50}	LC_{90}	LC_{50}	LC_{90}	LC_{50}	LC_{90}	LC_{50}	LC_{90}
24	14.68 (9.93-18.52)	44.09 (33.72-73.4)	85.27 (50.03-1592.68)	575.41 9159.2-1722099.6)	12.08 (7.24-15.7)	37.71 (28.9-62.5)	40.51 (31.27-565.44)	162.53 (88.3-918.12)
48	13.40 (8.91-16.99)	38.36 (29.89-60.24)	36.56 (28.84-52.8)	133.06 (78.86-513.27)	10.72 (6.23-14.13)	30.98 (24.19-47.84)	33.19 (25.87-46.92)	129.26 (7.12-515.53)

TABLE-2
LC_{50}, LC_{90} OF *T. longibrachiatum* AND *T. viride* MEDIATED SILVER NANOPARTICLES AND EXTRACELLULAR CRUDE METABOLITES AGAINST THIRD INSTAR LARVAE OF *A. albopictus*

Exposure time (h)	*T. longibrachiatum* (mg L^{-1}) at 95% confidence limit				*T. viride* (mg L^{-1}) at 95% confidence limit			
	Tl-AgNPs		Crude extract		Tv-AgNPs		Crude extract	
	LC_{50}	LC_{90}	LC_{50}	LC_{90}	LC_{50}	LC_{90}	LC_{50}	LC_{90}
24	14.05 (9.47-17.73)	40.74 (31.58-65.08)	71.72 (41.52-6657.84)	799.79 (169.5-57000395287)	12.59 (8.13-1.10)	35.99 (28.12-55.97)	32.08 (24.72-45.47)	131.94 (76.25-579.93)
48	12.94 (8.89-1.2)	33.53 (26.73-49.09)	55.23 (34.85-767.66)	586.33 (147.92-3400528.9	11.0 (6.86-14.18)	29.17 (23.13-43.02)	27.8 (21.04-37.35)	113.57 (68.57-421.28)

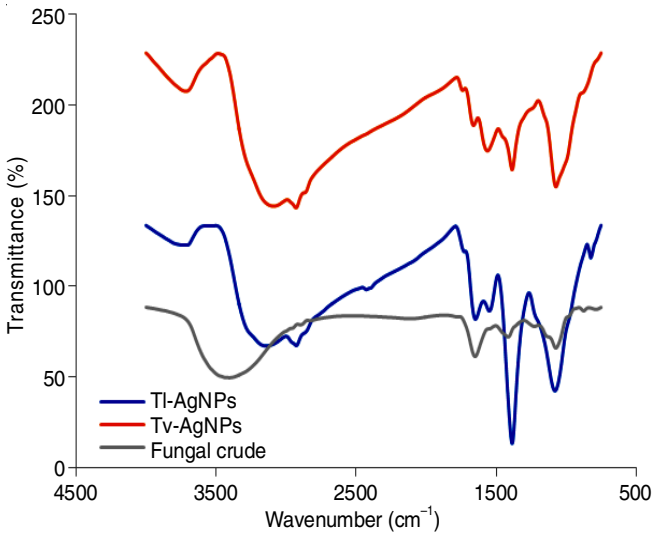

Fig. 4. FT-IR spectra of AgNPs and crude with different functional peaks

metabolites of *T. longibrachiatum* and *T. viride*. Considering the maximum mortalities of crude extracellular extracts, 66.6% and 69.3% maximum mortalities were achieved by *A. aegypti* at 50 mg L^{-1} after 48 h. In *A. albopictus*, maximum mortality was achieved at 50.67 mg L^{-1}, 73.3 mg L^{-1} after 48 h.

Acute toxicity test: The mean percentage mortality of *Moina macrocopa* for Tl-AgNP and Tv-AgNp concentrations are shown in Fig. 6. The percentage of mortality has increased with the increasing concentration of AgNPs and exposure time from 24 h to 48 h LC_{50} and $LC_{90}$of both mycosynthesized Tl-AgNPs and Tv-AgNPs at all the tested concentrations (10-50 mg L^{-1}) against the third instar larvae of *A. albopictus* and *A. aegypti* were significantly higher than the tested concentrations (0.1-0.325 mg L^{-1}) for *Moina macrocopa* neonates. These predetermined tested concentration series were selected as there were 100% mortalities for *M. macrocopa* at 10-50 mg L^{-1} concentration range for AgNPs. The LC_{50} values were recorded as 0.167 mg L^{-1}, 0.155 mg L^{-1} and LC_{90} values were 2.91 mg L^{-1}, 2.48

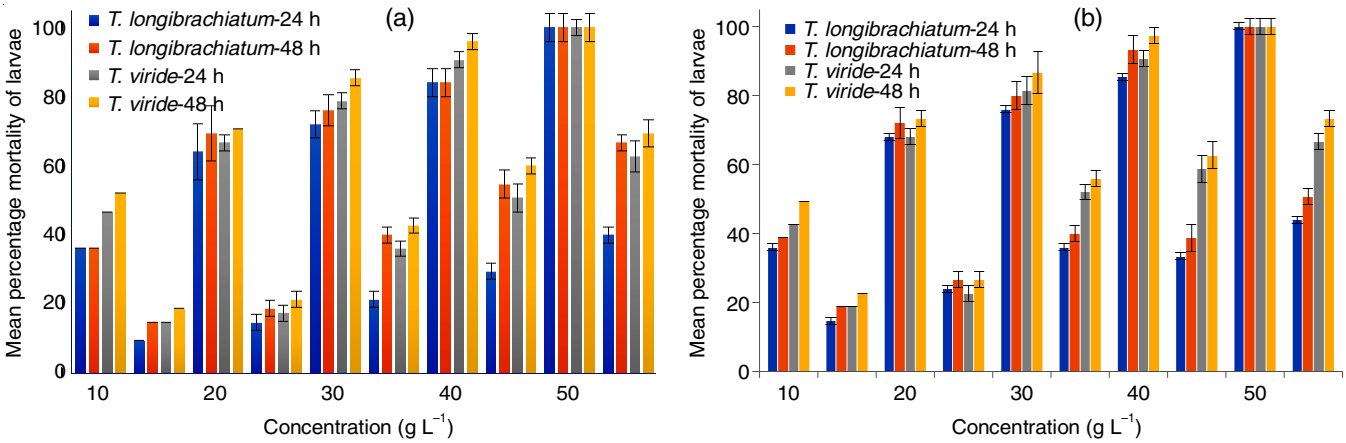

Fig. 5. Mean percentage mortality of (a) *A. aegypti* and (b) *A. albopictus* mosquito larvae exposed to Tl-AgNPs, Tv-AgNPs and extracellular crude metabolites for a period of 24 and 48 h (n = 25)

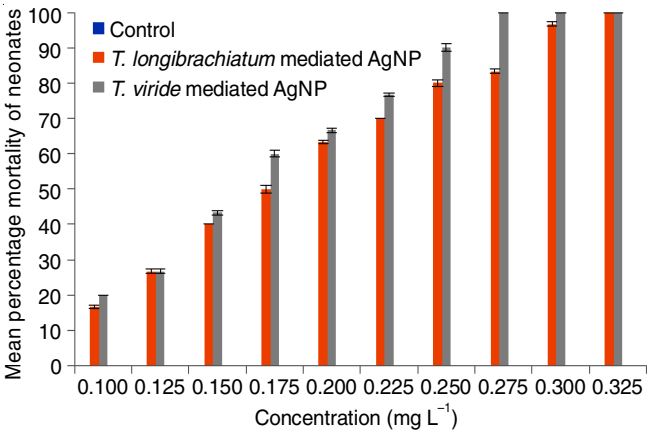

Fig. 6. Mean percentage mortality of *Moina macrocopa* neonates exposed to Tl-AgNPs, Tv-AgNPs and extracellular crude metabolites for a period of 48 h and 48 h (n = 10)

mg L^{-1} after 48 h for TlAgNPs and TvAgNPs. There were no recorded mortalities of *M. macrocopa* for extracellular crude metabolites of *T. longibrachiatum* and *T. viride* (Table-3). Both types of Tl-AgNPs and Tv-AgNPs showed aggregations and adhesion on the carapace, appendages and digestive tract (Fig. 7).

Larval mortality of *A. aegypti* and *A. albopictus* by the biosynthesized AgNPs was observed after different times of exposures. Probit analysis was performed to obtain mortality values. *A. aegypti* and *A. albopictus* larvae were found to be highly susceptible to AgNPs. These results suggest that the Tl-AgNPs and TV-AgNPs have the potential to be developed as a rapid approach for mosquito control. The mechanism which causes the death of the larvae could be the ability of nanoparticles to penetrate through the larval membrane. The AgNPs in the intracellular space can bind to sulphur-containing proteins

Fig. 7. Light microscopic images of (A) *M. macrocopa* after 48 h of exposure to control (distilled water) (B) *M. macrocopa* after 48 h exposure to 50 mg L^{-1} Tv-AgNPs (b1) Accumulation of Tv-AgNPs inside the digestive tract (b1,d1), heavily adsorbed Tv-AgNPs on the carapace (b2, b3, b4) and on the appendages (b5-b6) (C,D) *M. macrocopa* after 48 h exposure to 50 mg L^{-1} Tl-AgNPs; Tl-Ag NPs adsorbed on the carapace (c2, d2), Accumulation of Tl-AgNPS inside the digestive tract(c1,d1 and on the antennae (d3-d4)

TABLE-3
LC_{50}, LC_{90} OF *T. longibrachiatum* AND *T. viride* MEDIATED SILVER NANOPARTICLES AGAINST *Moina macrocopa* NEONATES AFTER 48 h

Fungal metabolite	LC_{50} (mg L^{-1}) with 95% confidence limit	LC_{90} (mg L^{-1}) with 95% confidence limit
T. longibrachiatum mediated AgNP	0.167 (0.142-0.189)	0.291 (0.248-0.390)
T. viride mediated AgNP	0.155 (0.133-0.714)	0.248 (0.217-0.312)
T. longibrachiatum extracellular crude metabolites	–	–
T. virde extracellular crude metabolites	–	–

or phosphorus-containing compounds like DNA, leading to the denaturation of some organelles and enzymes. It decreases membrane permeability, which lead to the cell death [17].

Acute toxicity experiments on *Moina macrocopa* in this study showed that Tv-Ag NPs and Tl-AgNPs had strong acute toxic effects. Arulvasu *et al.* [18] found that AgNPs had toxic effects on the molting, hatching rate and generation of *Artemia* [19]. Aggregation and adherence of nanoparticles on carapace damage the organisms because the carapace of cladocerans is associated with defense, growth, reproduction and molting of the organism [20,21]. Particularly, it could lead to the ultimate mortality of *Moina macrocopa*.

Conclusion

A successful eco-friendly synthesis of AgNPs using *T. longibrachaitum* (Tl) and *T. viride* (Tv) extracellular extract as reducing and capping agents is acheived. Tv-AgNPs were found to be more efficient as larvicides than Tl-AgNPs, while *A. albopictus* vectors are more susceptible to both Tl-AgNPs and Tv-AgNPs. The Tl-AgNPs and Tv-AgNPs showed toxic effects on the non-targeted aquatic crustacean, *Moina macrocopa* at very low concentrations. This shows a potential threat to other aquatic organisms when applied to natural mosquito breeding water bodies. Hence, a localized application method such as in restricted container types with heavy breeding of dengue vectors such as lift wells, cement tanks in construction sites and water treatment plants is suggested in practical aspects as a larvicide.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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