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Entomopathogenic fungus generated Nanoparticles for enhancement of efficacy in Culex quinquefasciatus and Anopheles stephensi

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ARTICLE INFO

ABSTRACT

Objective: To evaluate to efficacy of silver and gold generated larvicide with the help of entomopathogenic fungus Chrysosporium tropicum against the *Culex quinquefasciatus* and Anopheles stephensi larvae. **Methods:** The silver and gold nanoparticles were quantified and observed by the Micro−scan reader and X−ray diffraction technique. The micrographs of silver and gold nanoparticles were obtained by the Transmission electron microscope and Scanning electron microscope. The larvicidal efficacy was then performed at six different log concentrations by the probit analysis. **Results:** The characterization study confirmed the spherical shaped and sized (20−50 and 2−15 nm) of silver and gold nanoparticles. The all larval stages of *Cx. quinquefasciatus* were found more susceptible to the synthesized silver nanoparticles. Whereas, the larvae of An. stephensi were found more susceptible to larvicide synthesized with gold nanoparticles. **Conclusions:** The results suggested that the silver and gold nanoparticles generated by the entomopathogenic fungus *C. tropicum* is an environmentally safer and greener approach for mosquito control and new possibility in vector control strategy.

1. Introduction

Mosquitoes are responsible for spreading serious diseases like malaria, filariasis, and dengue etc. Anopheles species are the most important species as they are capable vector for malaria parasites. Anopheles species are the most important species as they are capable vector for malaria parasites. Approximately half of the world′s population is at risk of malaria, particularly those living in lower−income countries. It infects more than five hundred million people per year and kills more than one million [1].

Culex mosquitoes are painful and persistent biters and are responsible for filariasis. Lymphatic Filariasis, commonly known as elephantiasis, is a painful and profoundly disfiguring disease. An estimated one hundred twenty million people in tropical and subtropical areas of the world are infected with lymphatic filariasis; of these, almost twenty five million men have genital disease (most commonly hydrocele) and almost fifteen million, mostly women, have lymphoedema or elephantiasis of the leg. Approximately 66% of those at risk of infection live in the WHO South−East Asia Region and 33% in the African Region [2].

It is known that larvicide play a vital role in controlling mosquitoes in their breeding sites. Two insecticidal bacteria have been used as larvicides to control larvae of nuisance and vector mosquitoes in many countries, Bacillus thuringiensis sp. Israelensis and B. sphaericus [3]. Field studies have shown that both are effective, but serious resistance, as high as 50 000− fold, has evolved where B. sphaericus is used against *Culex mosquitoes*. Unfortunately, the development of resistance against the larvicide in various mosquito populations has also been reported.

It is essential to control mosquito population so that people can be protected from mosquito borne diseases. Fungi and fungus − derived products are highly toxic to mosquitoes, yet

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have low toxicity to non−target organisms [4]. The secondary metabolites of entomopathogenic fungi Chrysosporium [5−6], Fusarium [7] have been screened as a potential larvicides successfully.

Fungi are also been used in nanotechnology for producing nanoparticles. Therefore, present green synthesis has shown that the environmentally benign and renewable source of fungi used as an effective reducing agent for the synthesis of silver and gold nanoparticles. This biological reduction of metal would be boon for the development of clean, nontoxic and environmentally acceptable "green approach" to produce metal nanoparticles. Many of the species of fungi like Fusarium oxysporum [8], Aspergillus [9−10] and Verticillium species [11] used in nanotechnology for nanoparticles production. The formed silver and gold nanoparticles are highly stable and have significant mosquito larvicidal activity. Antiparasitic activities to determine the efficacies of synthesized silver nanoparticles using aqueous leaf extract of Mimosa pudica against the larvae of malaria vector, *An. subpictus*, filariasis vector *Cx. quinquefasciatus* and Rhipicephalus microplus have been evaluated [12]. Nelumbo nucifera synthesized silver nanoparticles using aqueous leaf extract against larvae of An. subpictus and Cx. quinquefasciatus have been observed [13]. The larvicidal potential of silver nanoparticles synthesized using fungus Cochliobolus lunatus against Ae. aegypti and An. stephensi have been observed [14]. Recently, the silver and gold nanoparticles synthesized with C. tropicum have been tested as a larvicide against the Ae. aegypti larvae [15].

The present communication describes the larvicidal effect of silver and gold nanoparticles generated by entomopathogenic fungus C. tropicum. This can be another way to avoid resistance problem effectively minimized while using new fungal based nanolarvicide.

2. Materials and methods

2.1 Fungus strain, preparation of broth and culture of C. tropicum

The fungal strain of *C. tropicum* (MTCC 2828) was obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology Chandigarh India. This strain was routinely maintained in our laboratory on Sauboraud's Dextrose Agar (SDA) medium at 25℃.

The broth was prepared for culture of *C. tropicum* by the method of Gardner and Pillai [16]. *C. tropicum* was grown in Sauboraud's Dextrose Broth (SDB). Five 250 ml conical flask, each containing 100 ml Sauboraud's dextrose broth (Dextrose 40 g, peptone 10 g, deionized water 1000ml) were autoclaved at 20 psi for 20 min. The broth was supplemented 50 μg/ml chloramphenicol as a bacteriostatic agent. C. tropicum colonies grown on the SDA plates were transferred to each flask using the inoculation needle. The conical flasks inoculated with *C. tropicum* were incubated 250C for 15 days.

2.2 Collection and maintenance of mosquito larvae in laboratory

Mosquito larvae were collected from various localities, including urban, rural and semi−urban regions of Agra (27 °, 10′N, 78o05′E), India and reared in deionized water containing glucose and yeast power. The colonies of *Cx. quinquefasciatus* and An. stephensi were maintained in the laboratory at a temperature of 25 ℃, with a relative humidity of 75±5% and 14h photoperiod. The larvae of *Cx. quinquefasciatus* and *An. stephensi* were maintained in separate enamel containers as per the standard method [17].

2.3 Synthesis and characterization of silver and gold nanoparticles

After incubation the fungal biomass was separated from the medium by filtration through whatman−1 filter paper and washed thrice in sterile distilled water to remove any nutrient media that might interact with the gold ions. Approximately 10g of *C. tropicum* biomass was transferred to a 250 ml conical flask containing 100 ml of distilled water and incubated for 72h at 25 ℃ and then the aqueous solution components were separated by filtration using Whatman−1 filter paper. To this solution (liquid fungal), AgNO3 and HAuCl4 (10−3M) was added and kept for 72h at 25℃. Simultaneously, control with fungal liquid of C. tropicum without AgNO$_3$ and HAuCl4 was maintained under same conditions, separately.

Periodically, aliquots of the reaction solutions were removed and their absorption was measured in a Micro−Scan reader model no. MICROSCAN MS5608A. Then the solution was converted in the powder for XRD measurement. For XRD study, dried nanoparticles were coated on XRD grid and the spectra were recorded by using Bruker AXS D_8 Advance. The micrographs of silver and gold nanoparticles were obtained by Philips CM−10 Transmission electron microscope and confirmed by the Scanning electron microscope. Elemental analysis on single particle was carried out by EDX analysis.

2.4 Bioassays, data management and statistically analysis

Larvicidal activity of silver and gold nanoparticles generated by *C. tropicum* against *Cx. quinquefasciatus* and *An. stephensi* was assessed by using the standard method [18]. All mosquito larvae of *Cx. quinquefasciatus* and *An. stephensi* were separated and placed in a container in microbe free deionized water. After that different test concentrations of silver and gold nanoparticles in 100ml deionized were prepared in 250−ml beakers. Bioassays were conducted separately for each instar at six different log test concentrations (0.77, 1.07, 1.25, 1.38, 1.47, 1.55 ppm and 0.30, 0.60, 0.77, 0.90, 1.00, 1.08 ppm, respectively) of aqueous gold and silver nanoparticles. To test the larvicidal activity of silver and gold nanoparticles, 20 larvae of each stage were separately exposed to 100ml of test concentration. Similarly, the control (without silver and gold nanoparticles) was run to test the natural mortality. Thereafter, we could further examine the mortality which was determined after different time of the treatment, the experiment time. No food was offered to the larvae during the experiments. Experiments were replicated thrice to validate the results.

The data on the efficacy was subjected to probit analysis [19]. The control mortality was corrected by Abbott's formula [20]. The relationship between probit and log concentrations were established as probit equations and probit regression lines were drown for each of larval stage.

3. Results

3.1 Micro−scan reader analysis of silver and gold nanoparticles

The presence of AgNPs and AuNPs in the cell free extract of C. tropicum was determined by visible analysis. The cell free extract of *C. tropicum* before immersion in AgNO$_3$ and HAuCl$_4$ was white in colour. Whereas, after exposure to 10−3 M aqueous solution of AgNO$_3$ and HAuCl$_4$ for 72h it turned into dark brown and ruby red in colour. The conversion of colourless solution to coloured solution in the presence of AgNO$_3$ and HAuCl4 was the indication of synthesis of Ag and Au nanoparticles. Further the AgNPs and AuNPs generated by C. tropicum have been characterized by micro−scan reader after incubation. The Micro−scan reader spectra recorded form a fungal liquid before immersion of AgNO3 and HAuCl$_4$ (curve 1) and after immersion in 10−3 M aqueous solution of AgNO$_3$ and HAuCl$_4$ for 72h (curve 2) (Figure 1a, b). The fungal liquid exposed to Ag$_+$ and Au3$_+$ ions show a distinct and fairly broad absorption band centered at 480 and 530 nm. The presence of these broad resonances indicates and aggregated structure of the silver and gold particles in the fungal liquid (cell free extract).
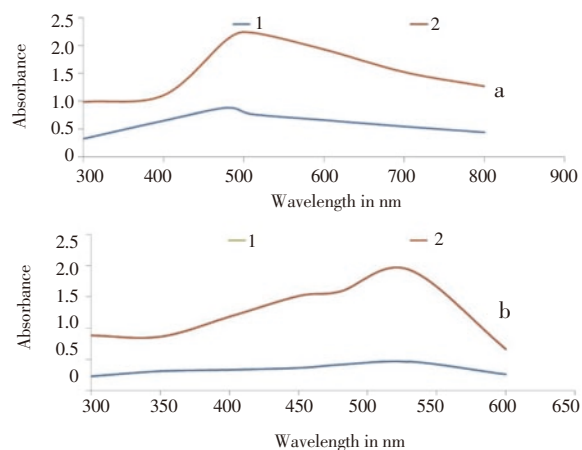

Figure 1a. Micro−Scan spectra of silver and b. gold nanoparticles generated by C. tropicum before (without AgNO3 and HAuCl4) (1) and after immersion in 10−3 M aqueous AgNO3 and HAuCl4 solution for 72h (2).

3.2 XRD analysis of silver and gold nanoparticles

The XRD patterns were obtained for the silver and gold nanoparticles synthesized by the *C. tropicum*. The presence of the intense peaks (38.4o, 44.4o and 64.2o) of the silver and gold nanoparticles (111), (200) and (211) appeared which are indexed as crystalline silver and gold face centered cubic phase. The XRD pattern clearly shows that the silver and gold

nanoparticles formed by the reduction of Ag$^+$ and AuCl$^-$4 ions by *C. tropicum* are crystalline in nature.

3.3 TEM, SEM and EDX analysis of silver and gold nanoparticles

After bioreduction, silver and gold nanoparticles were precipitated at the bottom of conical flask. This precipitate was washed out twice with double distilled water and then analyzed by employing Philips CM^{-10} Transmission Electron Microscope. The sample of silver and gold nanoparticles synthesized using fungal liquid was prepared by placing a drop of reaction mixture over copper grid and allowing water to evaporate. TEM micrograph of silver and gold nanoparticles has shown the spherical shaped and 20−50, 2−15 nm sized.

Scanning electron microscope pictures of silver and gold nanoparticles synthesized with C. tropicum have been obtained after 72h. The bright area clearly is seen in the image. The SEM images are showing distinctly the high density silver and gold nanoparticles synthesized by C. tropicum confirmed the development of silver and gold nanostructures.

Figure 2a, b shows the EDX (energy dispersive analysis of X−rays) spectrum recorded in the spot− profile mode from one of the densely populated silver and gold nanoparticles region on the surface of the film. Strong signals from the silver and gold atoms in the nanoparticles are observed, while weaker signals from Na, Ag, S, Pb and O signals are likely to be due to X−ray emission from proteins/enzymes present in the cell wall of the biomass.
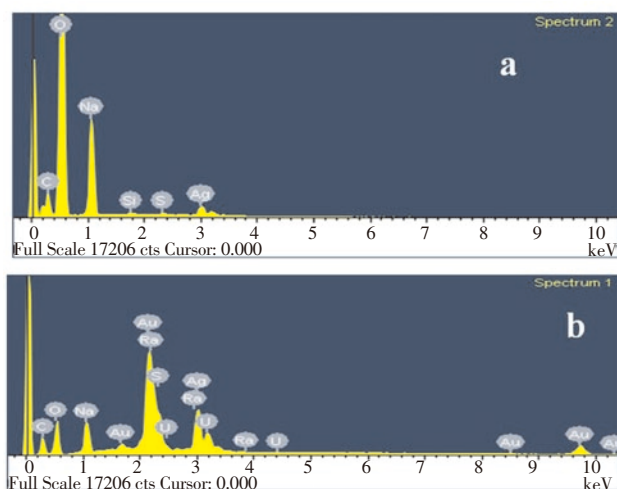

Figure 2a. EDX spectrum of silver and b. gold nanoparticles generated by C. tropicum.

3.4 Efficacy of AgNPs and AuNPs against An. stephensi larvae

The larvae of An. stephensi were found susceptible to the C. tropicum AgNPs. The mortality was recorded after 24h. The efficacy for first instars (LC$_{50}$ 1.77, LC$_{90}$ 12.30, LC$_{99}$ 13.18 ppm), for second instars (LC$_{50}$ 10, LC$_{90}$ 15.13, LC99 17.78 ppm), for third instars (LC$_{50}$ 4, LC$_{90}$ 12.30, LC$_{99}$ 12.88 ppm) and for fourth instars (LC$_{50}$ 12, LC$_{90}$ 12.30, LC$_{99}$ 19.49 ppm) were recorded with their probit equations and confidential limits (Table 1). The

Table 1

Probit equations and susceptibility of An. stephensi against silver and gold nanoparticles generated by C. tropicum with 95% confidential limits (C.L.).

	Instars	Probit equations	LC_{50}	LC_{90}	LC_{99}
Silver nanoparticles	First	Y=0.59+6.52x	1.77 (0.60−2.94)	12.30 (11.18−13.42)	13.18 (12.04−14.3)
	Second	Y=0.41+5.52x	10 (8.88−11.12)	15.13 (13.96−16.3)	17.78 (16.58−18.98)
	Third	Y=0.48+6.12x	4 (2.93−5.07)	12.30 (11.18−13.42)	12.88 (11.74−14.02)
	Fourth	Y=0.41+5.38x	12 (10.86−13.14)	12.30 (11.18−13.42)	19.49 (18.21−20.77)
Gold nanoparticles	First	**	**	**	**
	Second	**	**	**	**
	Third	Y=0.164+4.25x	12 (10.88−12.12)	36 (34.86−37.14)	47.86 (46.69−49.03)
	Fourth	Y=0.17+3.86x	24 (22.88−24.12)	38.01 (36.84−39.18)	70.79 (69.54−72.04)

(** 100% mortality was observed)

Table 2

Probit equations and susceptibility of Cx. quinquefasciatus against silver and gold nanoparticles generated by C. tropicum with 95% confidential limits (C.L.).

	Instars	Probit equations	LC50	LC90	LC99
Silver nanoparticles	First	**	**	**	**
	Second	**	**	**	**
	Third	Y=0.34+5.97x	6(4.96−7.04)	12.30 (11.18−13.42)	14.79 (13.65−15.93)
	Fourth	Y=0.34+5.73x	8 (6.96−9.04)	13.18 (12.14−14.22)	16.59 (15.55−17.63)
Gold nanoparticles	First	Y=0.17+4.50x	6 (4.83−7.17)	30 (28.91−31.09)	38.90 (37.7−40.02)
	Second	Y=0.16+4.01x	18 (16.91−19.09)	36.30 (35.16−37.44)	60.25 (59.02−61.48)
	Third	Y=0.14+3.8x	24 (22.88−25.12)	40.73 (39.56−41.9)	77.62 (76.34−78.9)
	Fourth	Y=0.13+3.57x	30 (28.86−31.14)	52.48 (51.25−53.71)	102.31 (100.98−103.66)

(** 100% mortality was observed)

mortality rate was r = 0.90, 0.92, 0.90 and 0.97 for first, second, third and fourth instars. The chi−square values calculated at 4 df were 47.15, 32.53, 40.37 and 30.91 for first, second, third and fourth instars. These chi−square values for first, second, third and fourth instars of An. stephensi were found higher than the critical value of chi−square at 0.05 significance level. The probit regression lines drown for each of larval stage of An. stephensi (Figure 3a). The observed LC values have shown the degree of susceptibility of fungal AuNPs amongst the four larval stages of *An. stephensi* in order of first instar > second instar < third instar > fourth instar.
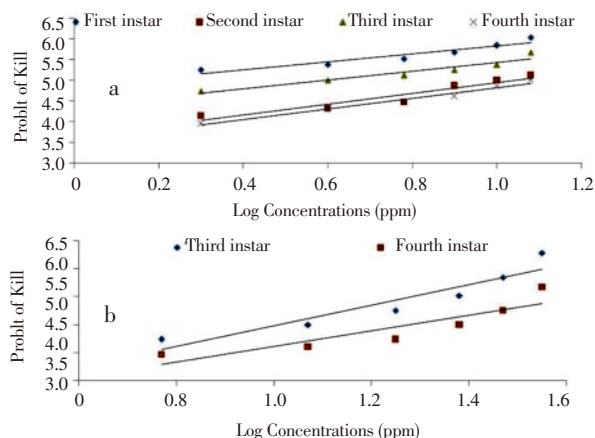

Figure 3a. Relationship between probit of kill and log concentration of C. tropicum generated silver and b. gold nanoparticles showing probit regression line in instar of An. stephensi in the laboratory after 24 and 72h.

The larvae of *An. stephensi* were found susceptible to the *C. tropicum* AuNPs. The mortality was recorded after 72h. The first and second instars have shown 100% mortality against the C. tropicum AuNPs after 72h. Whereas, for third instars (LC50, 12 ppm, LC_{90} 36 ppm, and LC_{99} 47.86 ppm) and fourth instars (LC50 24 ppm, LC_{90} 38.01 ppm and LC99 70.79 ppm) were calculated with their probit equations and confidential limits (Table 1). The mortality rate were r = 0.88 and 0.80 for third and fourth instars. The chi−square values calculated at 4 df were 18.36 and 15.21 for third and fourth instars. These chi−square values for third and fourth instars of *An. stephensi* were found higher than the critical value of chi−square at 0.05 significance level. The probit regression lines drown for each of larval stage of *An. stephensi* (Figure 3b). In control group no mortality could be observed. The observed LC values have shown the degree of susceptibility of fungal AuNPs amongst the four larval stages of An. stephensi in order of first instar > second instar > third instar > fourth instar.

3.5 Efficacy of AgNPs and AuNPs against Cx. quinquefasciatus larvae

The *C. tropicum* AgNPs were found effective against all larval instars of *Cx. quinquefasciatus*. The mortality was scored after 1h of exposure. The first and second instar larvae have shown the 100% mortality to the silver nanoparticles generated by *C. tropicum*. The efficacy for third instars (LC_{50} 6, LC_{90} 12.30, LC_{99} 14.79 ppm) and for fourth instars (LC_{50} 8, LC_{90} 13.18,

LC$_{99}$ 16.59 ppm) were observed with their probit equations and confidential limits (Table 2). The mortality rate was r = 0.98 and 0.99 for third and fourth instars. The chi–square values at 4df were 37.02 and 34.25 for third and fourth instars. These chi–square values for third and fourth instars of *Cx. quinquefasciatus* were found higher than the critical value of chi–square at 0.05 significance level. The probit regression lines drown for each of larval stage of *Cx. quinquefasciatus* (Figure 4a). The observed LC values have shown the degree of susceptibility of fungal AgNPs amongst the four larval stages of *Cx. quinquefasciatus* in order of first instar > second instar > third instar > fourth instar.
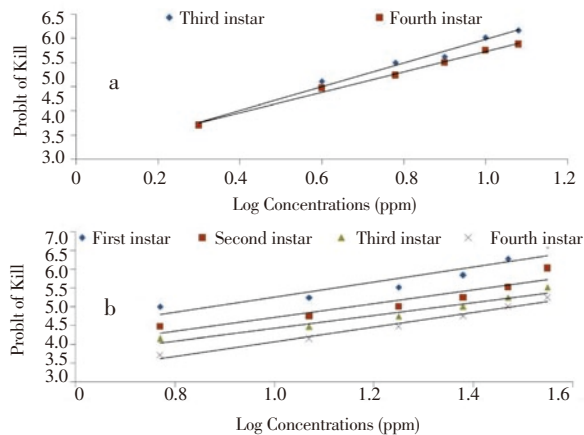

Figure 4a. Relationship between probit of kill and log concentration of *C. tropicum* generated silver and b. gold nanoparticles showing probit regression line in instar of Cx. quinquefasciatus in the laboratory after 1 and 72h.

The *C. tropicum* AuNPs were found effective against all larval instars of *Cx. quinquefasciatus*. The mortality was scored after 72h of exposure. The first instars were found more susceptible and other instars were found more tolerant to the *C. tropicum* AuNPs. The efficacy for first instars (LC$_{50}$ 6 ppm, LC$_{90}$ 30 ppm, and LC99 38.90 ppm), second instars (LC$_{50}$ 18 ppm, LC90 30.36 ppm, and LC$_{99}$ 60.25 ppm), third instars (LC$_{50}$ 24 ppm, LC$_{90}$ 40.73 ppm, and LC$_{99}$ 77.62 ppm) and fourth instars (LC$_{50}$ 30 ppm, LC$_{90}$ 52.48 ppm, and LC$_{99}$ 102.31 ppm) were recorded with their probit equations and confidential limits (Table 2). The mortality rates were *r* = 0.82, 0.95, 0.97, and 0.99 for first, second, third, and fourth instars, respectively. The chi–square values at 4 df were 20.57, 16.73, 14.63, and 12.92 for first, second, third, and fourth instars, respectively. The probit regression lines drown for each of larval stage of *Cx. quinquefasciatus* (Figure 4b). In control group no mortality could be observed. The observed LC values have shown the degree of susceptibility of fungal AuNPs amongst the four larval stages of *Cx. quinquefasciatus* in order of first instar > second instar > third instar > fourth instar.

4. Discussion

Unlike other mosquito control agents, the entomopathogenic fungi synthesized AuNPs unique because fungal AuNPs have the ability to directly infect the host insect by penetrating into the cuticle and do not need to ingest by the insect to cause disease. There are preferential advantages when we use fungal AuNPs as biocontrol agent for mosquitoes. The fungal AuNPs have very narrow range, and considerable progress has been made in recent years in development of environmentally benign spores and mycelium–based biocontrol agent for the mosquito population. Fungal biocontrol agents have reduced inputs of harmful synthetic chemical pesticide in agriculture, horticultural, and forest system. Toxicity of silver nanoparticles in zebra fish models have been recorded [21]. Using starch and bovine serum albumin (BSA) as capping agents, silver nanoparticles were synthesized to study their deleterious effects and distribution pattern in zebra fish embryos (Danio rerio). Toxicological endpoints like mortality, hatching, pericardial edema and heart rate were recorded. A concentration–dependent increase in mortality and hatching delay was observed in Ag–NP treated embryos. Additionally, nanoparticles treatments resulted in concentration–dependent toxicity, typified by phenotypes that had abnormal body axes, twisted notochord, slow blood flow, pericardial edema and cardiac arrhythmia. Ag$_+$ ions and stabilizing agents showed no significant defects in developing embryos.

The use of nanoparticles in insects and their potential for use in insect pest management have been focused [22]. The larvicidal potential of the hexane, chloroform, ethyl acetate, acetone, methanol, and aqueous leaf extracts of Nelumbo nucifera and synthesized silver nanoparticles using aqueous leaf extract against fourth instar larvae of An. subpictus and *Cx. quinquefasciatus* have been tested [13]. Larvae were exposed to varying concentrations of plant extracts and synthesized silver nanoparticles for 24 h. Whereas, in our work the gold nanoparticles were synthesized using keratinophilic fungus *C. tropicum* and mortality was observed after 24, 48, and 72h.

The formulation of water dispersible nanopermethrin has been investigated for its larvicidal property [23]. The results extrapolated that nanopermethrin could serve selectively as a potential larvicide. The larvicidal potential of silver nanoparticles synthesized using fungus Cochliobolus lunatus against Aedes aegypti and Anopheles stephensi has been tested [14]. They also tested the potential of *C. lunntus* silver nanoparticles against non–target fish species Poecilia reticulata, the most common organism in the habitats of *A. aegypti* and *A. stephensi* showed no toxicity at LC$_{50}$ and LC$_{90}$ doses of the AgNPs. Recently, the efficacy of fungus mediated silver and god nanoparticles have been tested against the Ae. aegypti larvae [15]. Whereas, in the present study we have tested the *C. tropicum* synthesized gold nanoparticles against the all larval instars of *Cx. quinquefasciatus*, and *An. stephensi*.

5. Conclusions

In the present investigation we have tested the silver and gold nanoparticles generated by entomopathogenic fungus *C. tropicum* against the malaria and filariasis vector larvae *An.* stephensi and *Cx. quinquefasciatus* in the laboratory. The silver nanoparticles were found more effective than the gold nanoparticles on mosquito control. We can now conclude that the fungus generated silver and gold nanoparticles could be a better, environmentally safer and green approach for vector larvae control.

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