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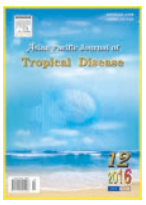

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Phytofabrication of silver nanoparticles using *Elephantopus scaber* and *Azadirachta indica* leaf extract and its effect on larval and pupal mortality of *Culex quinquefasciatus*

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ABSTRACT

Objective: To synthesize silver nanoparticles by using *Azadirachta indica* (*A. indica*) and *Elephantopus scaber* (*E. scaber*) leaf extract as reducing and stabilizing agent and to check the efficacy of silver nanoparticles towards mortality of mosquito larval and pupal stages of *Culex quinquefasciatus* (*Cx. quinquefasciatus*).

Methods: The silver nanoparticles were synthesized using *A. indica* and *E. scaber* leaf extract. The synthesized nanomaterials were characterized using UV-vis spectrophotometer, Fourier transform infrared spectroscopy, transmission electron microscopy and fluorescent microscopy. Finally, the synthesized nanoparticles were applied on fourth instar larval and pupal stages of *Cx. quinquefasciatus* larvae and pupae and mortality was counted for 48 h. Dead larval bodies were electron dispersive X-ray studied to see whether silver has entered into the body or not. Pupal hatching under the influence of these nanoparticles were also studied.

Results: Silver nanoparticles were synthesized from *A. indica* and *E. scaber* leaf which were stable. Mortality results highlighted that LC_{50} and LC_{90} of *A. indica* and *E. scaber* induced silver nanoparticle are 1.64, 4.32 mg/L and 1.43, 4.32 mg/L respectively during 48 h of incubation. EDX study of dead larval body showed a clear signature of silver in their body which suppose to be the main cause of their mortality. Pupal hatching is also hampered by the sublethal doses.

Conclusions: *A. indica* and *E. scaber* may be good source as reducing agent of stable silver nanoparticles. Green synthesized silver nanoparticles may be a good eco-friendly alternative to control mosquito larvae and pupae of *Cx. quinquefasciatus*.

1. Introduction

Mosquitoes are the disease causing vector that are responsible for transmitting malaria, filariasis and many other viral disease like dengue, Japanese encephalitis, yellow fever *etc*.[1]. Mainly three genuses of mosquitoes, namely, *Culex* sp., *Anopheles* sp. and *Aedes* sp. are widely distributed all over the world and cause millons of victims. *Culex quinquefasciatus* (*Cx. quinquefasciatus*) is the vector of filarial parasite *Wuchereria bancrofti*, responsible for human lymphatic filariasis. Moreover, *Culex* sp. transmits causative agents of avian malaria, St. Louis encephalitis, western equine encephalitis, West Nile fever *etc*.[2]. Although, death does not always occur due to filariasis, but it is the second leading cause of disability which imparts severe economic and social burden to the suffered person and his family. According to the report of National Vector Bourne Disease Control Programme[3], in 83 countries all over the world almost 120 million people are infected with this disease and it is predicted that 1.1 billion are at risk. About 40 million people are disabled by the infection of filarial parasite and 76 million people have hidden internal lymphatic infection with apparently normal appearance. According to World Health Organization report, among the infected people worldwide, 70% are from India, Indonesia, Bangladesh and Nigeria. *Cx. quinquefasciatus* is widely distributed all over the world, mainly in tropical and subtropical regions[4]. This mosquito prefers human dwellings for their breeding and they lay eggs in large partially polluted water bodies, rice fields, drains, tanks, ditches where high concentration of decomposed organic matter[5].

To successfully reduce the disease occurrence caused by the dipteral vectors, controlling the disease spreading vector mosquitoes, principally by applying insecticides at their breeding sites is the way of choice[6]. Application of synthetic mosquitocides, namely, dichloro diphenyl trichloroethane, dieldrin, organophosphorus, fenitrothion and propoxur is a practice for several decades, but this chemical insecticides has several demerits including resistance development[7], environmental degradation, bioaccumulation *etc*. Constant application of chemical controlling agents can cause harm to several organisms

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including human race which will ultimately lead to disruption of ecological balance. Prolonged exposure to chemicals may develop resistance mosquito strains. Therefore, a demand stems out for the synthesis of less harmful and more effective mosquito controlling agent. To control this deadly arthropod vector in a regulated way with minimum environmental harm nanomaterials may be an effective way.

Nanotechnology is now an emerging topic of scientific research. Nanomaterials are recently being utilized in various fields such as water treatment, medicine, catalysis, solar energy conversion *etc*. Nanosilver is good conductor, catalytic agent, chemically stable and have good antibacterial property and so it is now opted for several applications[8,9]. In china, nanosilver is used as anti-microbial agent in elevators of public place. Silver nanoparticles (AgNPs) are used for anti-microbial treatment in ointments, fabrics and even in surgery because of its anti-inflammatory, anti-angiogenic and anti-permeable properties[10]. However, for the last few years scientists have highlighted on the effective use of nanomaterials in the field of insect control[1,11,12]. AgNPs can be used as good mosquitocidal agent which is reported by many authors[5,13,14].

AgNPs can be synthesized by chemical process such as sodium borohydride reduction of monovalent silver ion to zero valent AgNPs[15]. UV irradiation induced polymetaacrylic acid cap for mosquito control by AgNPs were used by Sap-Iam *et al*.[16]. Biological synthesis of AgNP is more advanced than chemical and physical methods because green synthesis of AgNPs is cost effective and environment friendly. AgNPs can be synthesized biologically where fungi or bacteria are used for the reduction of silver ions to colloidal nano silver[17-19]. With the advent of utilization of micro organisms for the production of nanometals, use of plant biomolecules has also become a source of reducing agents. Many plants are utilized in bioremediation to purify heavy metals or to accumulate them. Hyper accumulator plants have some distinct mechanism to reduce it to lower oxidation state or chelation with bio ligands[20]. Those biomolecules of plants can be a possible agent to reduce low reduction potential metals to synthesize metal nanoparticles which is less time consuming, more cost effective, environment friendly and a single step process than physical and chemical methods[21]. Literature study reveals plant extracts are used to reduce silver ions as they contains several biomolecules like alkaloid, flavonoid, proteins, lectins, triterpenes, phenolics *etc*. These biomolecules are responsible for reduction of silver ions to nanoparticles and subsequent stabilization of nanoparticles by their capping effect to prevent the formed nanoparticles from being agglomerized. Some workers reported that proteins, carbohydrates and polyphenols are involved in AgNP synthesis[22] although the exact mechanism is still not clear. In this article, we report eco friendly and green synthesis of AgNPs using *Azadirachta indica* (*A. indica*) and *Elephantopus scaber* (*E. scaber*) leaf extracts as reducing and stabilizing agent, both of which has medicinal and aromatic property. Various bioactive molecules are present in these two leave extracts that can be utilized for reduction of metal solution to nanoparticles.

A. indica (common name: neem), typical for tropical and subtropical regions can be found in Indian subcontinent, Nepal, Bangladesh, Pakistan and Sri Lanka. Ayurvedic products made from neem is very popular in India for several years because it has medicinal properties such as antidiabetic, antimicrobial, antihelminthic, contraceptive, improve liver function. Various parts of neem contains diverse range of biochemicals. Govindachari *et al*.[23] reported antibacterial and antifungal property of quercetin and and ß-sitosterol, first phenolic compound isolated from fresh neem leaves. They also reported other major active compounds such as nimbin, nimbidin, salanin, 6-deacetylnimbin, azadiradione, epoxy-azadiradione *etc*. So, neem leaves are a good choice to reduce silver ions to its zero state[24].

E. scaber is a flowering plant of family compositae and found in moist forests of tropical and subtropical regions *i.e.,* Indian subcontinent, Eastern and Southeast Asia, Northern Australia and tropical Africa. This plant is used as a traditional medicine as astringent agent, cardiac tonic, diuretic, anti microbial *etc*. *E. scaber* contains a germacranolide sesquiterpene lactone named elephantopin, iso-17,19- dihydro-deoxy elephantopin, 17,19-dihydrodeoxyelephantopin, and 8-hydroxyl Naringenin which mainly acts as anti bacterial agent[25,26].

Our objective was to study the efficiency of the plant extracts *A. indica* and *E. scaber* as a reducing agent for the rapid, single step and eco friendly synthesis of AgNPs as well as the effectivity of the synthesized nanoparticles as an environment friendly mosquitocidal agent. These two plant extract were proved to be excellent reducing agent for the synthesis process. These two plant mediated nanoparticles showed notable larvicidal and pupicidal activity. Moreover, they are supposed to be toxic for pupal hatching when used in sub lethal doses.

2. Materials and methods

2.1. Preparation of nanoparticle synthesis

$AgNO_3$ crystal extra pure was purchased from Merck. Double distilled water was used for synthesis process.

2.2. Collection of plant material

Fresh neem (*A. indica*) leaves (Figure 1a) were collected from the garden of The Deptt. of Environmental science, The University of Burdwan (23°16' N, 87°54' E) and *E. scaber* leaves (Figure 1b) were collected from the adjoining areas of Sonamukhi forest, Bankura (23°30' N, 87°42' E) where it is abundantly available.

Figure 1. a: Neem (*A. indica*); b: Hasti pada (*E. scaber).*

2.3. Preparation of aqueous extract of plants

The collected leaves were surface cleaned with running tap water to remove the debris and other contaminated organic contents, followed by double distilled water. Washed leaves were then air dried for 48 h at room temperature. Aqueous extract of the leaves were prepared by mixing 5 g of dried leaf with 100 mL double distilled water and boiling at 95 ℃ for 10 min with continuous stirring. The filtrates were then filtered by Whatman filter paper. The extracts can be kept in refrigerator for 1 week for further use.

2.4. Synthesis of AgNPs

$AgNO_3$ solution (1 mmol/L) was prepared by dissolving calculated amount of $AgNO_3$ in double distilled water. The plant extracts prepared were used for reduction of Ag^+ to Ag^0. Plant

extract and $AgNO_3$ solution was mixed at the ratio of 1:9 and kept in dark condition to prevent photoreactivation of $AgNO_3$. The color of $AgNO_3$ turned from colorless to reddish brown after few minutes of incubation which turned deep with time. The final nano colloid was centrifuged twice to discard any un-interacted biological molecules at 10 000 r/min for 15 min in a Remi research centrifuge. The final pellet of AgNP was collected and dried in lyophilizer. The synthesized AgNP powder was used for further characterization.

2.5. Characterization of synthesized nanoparticles

The formation of AgNP was determined visually by observing the color change from colorless to reddish brown. Confirmation of AgNP was done by using UV-vis spectrophotometer (Perkin-Elmer, Lamda) operated at 2 nm resolution at 300–700 nm wavelength range. The transmission electron microscopy (TEM) study was done using JEOL, Jem-2100 instrument operated at 200 kV. Samples for this analysis were prepared by coating the aqueous AgNP on copper grid of 300 mesh size coated with carbon and then allowed to dry in vacuum at 25 ℃ overnight. Fluorescent microscopic study was done with a thin film of AgNP solution spread over a clean glass slide (blue star) with a microscope (SD 1000). Fourier transform infrared spectroscopy (FTIR) study was performed to know about the functional groups present in the nanoparticles. The powdered forms of nanoparticles were used for the FTIR study using an instrument of BRUKER (Tensor 27).

2.6. Collection and rearing of larvae and pupae

Egg rafts of *Cx. quinquefasciatus* were collected from drains with a dipper and taken to the laboratory. Larvae were fed with a small amount of ground dog biscuit which moulted to subsequent stages. The pupae were collected with a dropper and transferred to a glass beaker. The beaker was placed inside a screened cage (90 cm × 90 cm × 90 cm) to retain emerging adults, for which 10% sucrose in water solution were given. After emergence, the mosquitoes were fed with cotton ball soaked with glucose solution. Identification of adult mosquitoes were done using the identification key of Service[27] and Laird[28]. The mosquitoes were provided with blood feeding. For oviposition, Petri plates with water and a piece of filter paper was placed inside the cage[29]. After hatching of the eggs the fourth instar larvae and pupae were used in the experiment.

2.7. Determination of mosquito larvicidal activity of synthesized AgNP

Mosquitocidal ability of green synthesized AgNPs was performed by the method of World Health Organization[30] with some modifications. The bioassay was performed at room temperature of (27 ± 1) ℃. AgNP solution was applied at the doses of 0.1, 0.5, 1.0, 2.0 and 5.0 mg/L. Thermocol pans were taken to perform the test. Each pan was filled up with 200 mL of dechlorinated tap water with the dose of NP where 20 fourth instar larvae were released. Each dose had three replicates along with a control, *i.e.* no AgNP. The whole experiment was performed thrice. The larvae were incubated with the nanoparticles for 48 h and the numbers of dead larvae were counted after 24 h and 48 h.

2.8. Determination of pupicidal activity of synthesized AgNP and effect on pupal hatching

Twenty pupae were released at every thermocol pans containing 200 mL of dechlorinated water with the following doses: 0.1, 0.5, 1.0, 2.0 and 5.0 mg/L with three replicates. Numbers of dead pupae were counted after 24 and 48 h.

Same set of experiment was conducted to check the percentage of pupal hatching at 24 and 48 h intervals when grown up under the same concentrations of AgNP.

2.9. Statistical analysis

The percent mortality was calculated by Abbott's formula[31].

$$\text{Percentage mortality} = \frac{\text{Number of dead larvae or pupae} \times 100}{\text{Number of larvae or pupae introduced}}$$

The average mortality data were calculated with respect to the control and other statistics as 95% confidencial limits of upper and lower confidence limit by following arithmetic statistical manual[32]. The basic statistics including regression analysis, ANOVA and other statistical calculations has been done by SPSS 20.0.

2.10. Estimation of total phenolics in leaves

Leaf extracts are used to reduce silver ion to zero valent state by phenolics, flavanoids, terpenoids, alkaloids, triterpenoids, lectins and many compounds[1]. The plants which have been used in the present work are chosen mainly because of their high content of active biomolecules. Phenolic content of leaves of *A. indica* and *E. scaber* was determined using spectrophotometric method of Mallick and Singh[33]. Standard curve was prepared by phenolic acid (gallic acid). Methanolic extracts of the leaves were prepared in the concentration of 1 mg/mL, centrifuged and the supernatant was evapored in a hot plate until it dries. Then, the dried extract was mixed with 5 mL distilled water. The reaction mixture was prepared by mixing 0.5 mL of plant extract which was made upto 3 mL with distilled water, 0.5 mL of 10% Folin-Ciocalteu's reagent and 2 mL 20% Na_2CO_3. Blank was concomitantly prepared, containing 3 mL distilled water, 0.5 mL of 10% Folin-Ciocalteu's reagent and 2 mL 20% Na_2CO_3. The solutions were placed in a boiling water bath for just one minute, then after cooling, the absorbance was measured at 650 nm. Concentration of total phenolics was determined from the standard graph and subsequent calculations.

2.11. Electron dispersive X-ray (EDX) of mosquito larval body

A few dead mosquito larval bodies were washed thoroughly with distilled water to remove any kind of organic or inorganic material from its body surface. Then the bodies were dried at 40 ℃ for 1 h in hot air oven. The dried larvae were then ground with a clean mortar and pestle. The dust of mosquito larvae were studied under scanning electron microscope and energy dispersive X-ray spectroscopy (Zeiss, Evo-18, Special edition).

3. Results

3.1. UV-vis spectroscopic study

The beautiful brownish color of AgNP was scanned through UV-vis spectrophotometer. Surface plasmon resonance of AgNPs is responsible for the brown colour of this solution[34]. The gradual formation of nanoparticles due to bioreduction of Ag^+ ions by the

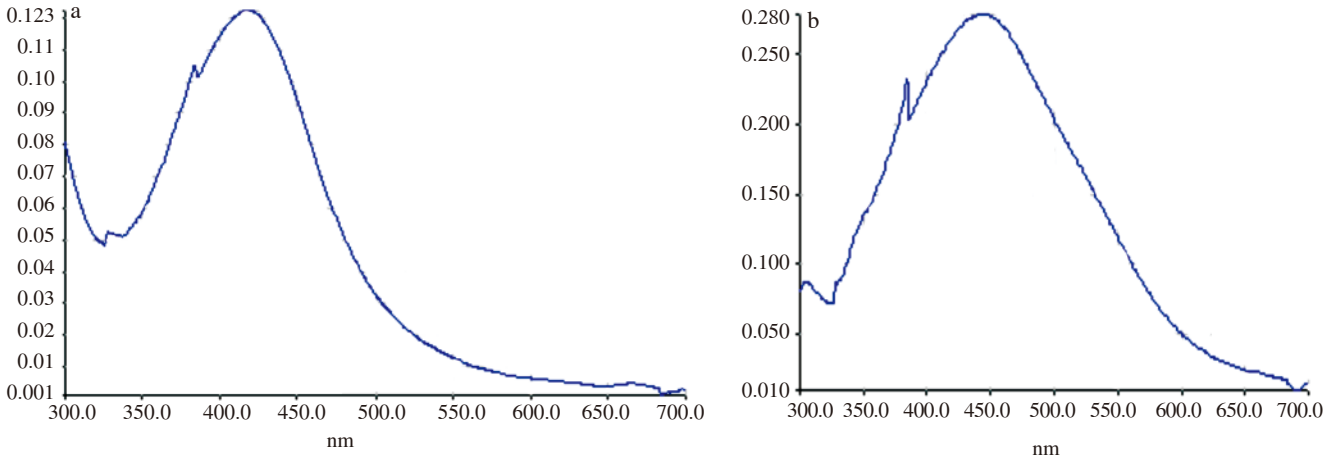

Figure 2. UV-vis spectra of silver nanoparticles synthesized from *A. indica* (a) and *E. scaber* (b) showing peak at near 420 nm because of surface plasmon resonance of silver nanoparticles.

extract of *A. indica* and *E. scaber* can be observed by changes in absorption spectra. The nanoparticle solution was scanned for absorbance by taking aliquots from the reaction mixture. The characteristic surface plasmon absorption spectral band of neem mediated AgNP showed at 450 nm (Figure 2a) and *Elephantopus* mediated AgNP showed at 445 nm (Figure 2b).

3.2. TEM analysis

TEM analysis was done to visualize the shape of the synthesized nanoparticles as well as measure the diameter of the biogenically synthesized AgNPs. The images showed that the nanoparticles are crystalline and and most of them are poly-hedral and semi-spherical. Figures 3a and 3b show a representative TEM photograph of the synthesized AgNP from fresh leaves of *A. indica* and *E. scaber,* respectively.

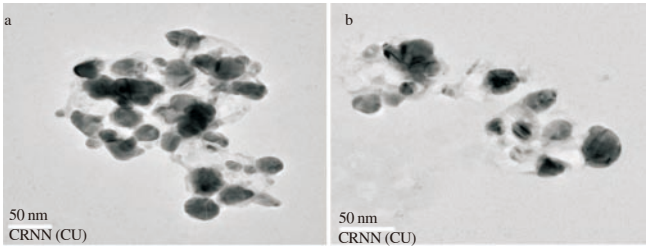

Figure 3. TEM images of quasi spherical silver nanoparticles synthesized from *A. indica* (a) and *E. scaber* (b).

3.3. Fluorescent microscopic study

Fluorescence nature of AgNP was used to characterize AgNP which showed beautiful fluorescent property and spherical fluorescent AgNPs can clearly be seen (Figure 4a and 4b).

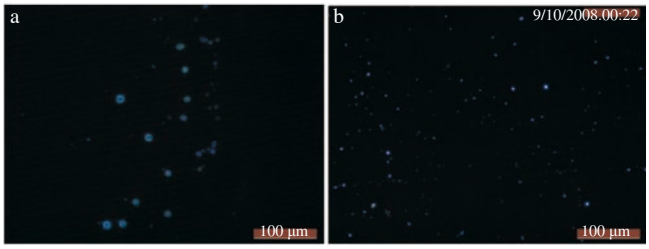

Figure 4. Fluorescent microscopic image of silver nanoparticles synthesized from *A. indica* (a) and *E. scaber* (b).

3.4. FTIR study

The nature of plant extract as reducing agent and stabilizing agent is probably due to the presence of various functional groups which was confirmed from FTIR study of AgNPs (Figure 5a and 5b). The AgNPs synthesized from *A. indica* leaf extracts. The bands at 2350 cm^{-1}, 1631 cm^{-1} and 1402 cm^{-1} which corresponds to the functional groups –C-O, -'C=O and –C=C aromatic group. Stretching and peaks at 1294 cm^{-1} and 808 cm^{-1} corresponds the –C-N and –S=O stretching vibration respectively (Figure 5a). On the other hand, nanoparticles produced from *E. scaber* leaf extract also provide distinct sharp peaks at 2914 cm^{-1}, 2345 cm^{-1} and 2129 cm^{-1} which attributes the stretching vibration of alkyl -C-H and alkynes –C=-C-H, respectively. The observed peaks at 1278 cm^{-1}, 1033 cm^{-1} and 800 cm^{-1} are mainly –C-N, –S=O and aromatic –C-H stretching, respectively (Figure 5b). Therefore, the synthesized nanoparticles were surrounded by proteins and metabolites such as terpenoids. From the IR study it is clear that the carbonyl groups from the amino acid residues and proteins played a vital role towards stabilization of AgNP, which subsequently prevent the nanoparticles from agglomeration. This observation clearly suggested that plant biomoleculs can reduce as well as stabilize the metals in aqueous medium. The presence of flavanones or terpenoids on the nanoparticle surface was confirmed by the -C=O stretching vibration[35,36].

3.5. Mosquito larvicidal bioassay study

The biogenically synthesized AgNPs from leaves of *A. indica* and *E. scaber* was applied separately on 4th instar larvae of *Cx. quinquefasciatus*. Table 1 represents the results of the rate of mortality of larvae of *Cx. quinquefasciatus* when they are exposed to different concentrations of (0.1, 0.5, 1.0, 2.0 and 5.0 mg/L) of aqueous solution of AgNP for 24 h and 48 h. The lowest concentration (0.1 mg/L) of AgNP synthesized from both the plant extracts showed no mortality after 24 h. The 5 mg/L concentration of synthesized AgNPs from *A. indica* leaves showed 85% mortality after 24 h and 100% after 48 h whereas AgNP synthesized from *E. scaber* leaves showed 93.33% and 100% mortality after the same time interval respectively as depicted in Table 1. The intermediate doses showed moderate mortality. Almost similar pupicidal activity was seen where 0.1 mg/L concentration shows no mortality in pupa but 5 mg/L concentration of AgNP synthesized from *A. indica* leaf extract showed 100% mortality of pupae after 48 h and AgNP

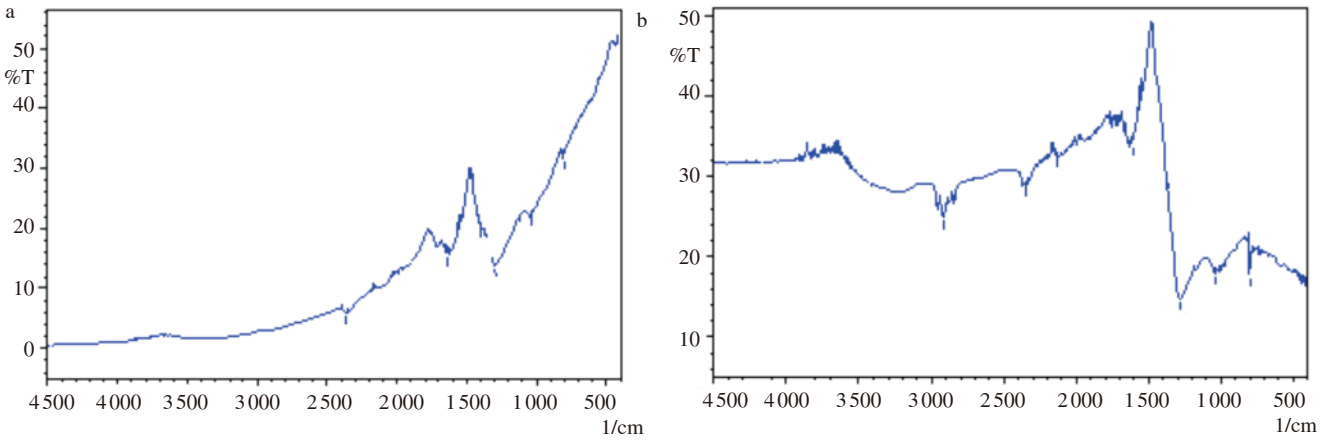

Figure 5. FTIR spectra of silver nanoparticles synthesized from *A. indica* (a) and *E. scaber* (b).

synthesized from *E. scaber* showed 98.33% mortality after 48 h (Table 2). The extracts of both the plants have minimum effect on the larvae and pupae when applied in the same concentration as in the nanoparticle solution. The same concentrations of $AgNO_3$ solution alone have a negligible effect on mosquito larvae and pupae.

Table 3 represents the LC_{50} and LC_{90} values[37], of 4th instar larvae and pupae of *Cx. quinquefasciatus* by synthesized nanoparticles in a greener way which indicates LC_{50} and LC_{90} value of nanoparticles synthesized by *A. indica* are 0.898 mg/L and 2.184 mg/L respectively whereas, the same for AgNP synthesized fron *E. scaber* were 0.991 amd 2.108 mg/L.

Soon after addition of nanoparticles on the larvae caused rapid movement. However, after few minutes larvae become sluggish compared to that of the control larvae (without nano)". The mosquito larvae surely injested the nanoparticles because under microscopic examination alimentary canal of the dead larvae were seen packed with nanoparticles (Figure 6).

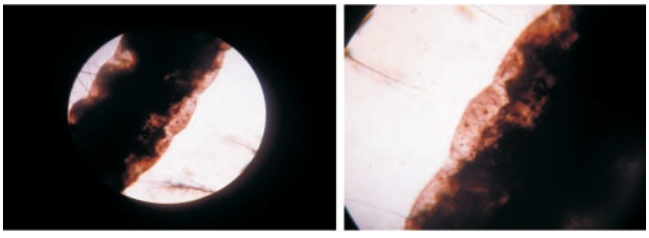

Figure 6. Microscopic image of dead larval body shows blackness of its body due to penetration of AgNP in its body.

3.6. Determination of percentage pupal mortality and percent pupal hatching

Pupal mortality of larval stage of *Cx. quinquefasciatus* by AgNP follows a dose dependent relationship. 0.1 mg/L treatment from both *A. indica* and *E. scaber* showed no mortality after 24 h but after 48

Table 1
Percent larval (fourth instar) mortality caused by AgNP synthesized by *A. indica* (Neem) and *E. scaber* (Hasti pada).

Plant species used to synthesize Ag NP	Concentration (mg/L)	% Larval mortality ± SD[a] after 24 h	Regression equation	R^2 value	% Larval mortality ± SD[a] after 48 h	Regression equation	R^2 value
A. indica (Neem)	0.1	0.00 ± 0.00			8.33 ± 2.88		
	0.5	26.66 ± 2.88			45.00 ± 5.00		
	1.0	30.00 ± 0.00	$Y = 15.18x + 9.539$	0.93	65.00 ± 5.00		
	2.0	36.66 ± 2.88			80.00 ± 0.00	$Y = 15.20x + 33.51$	0.75
	5.0	85.00 ± 0.00			100.00 ± 0.00		
	0.1	0.00 ± 0.00			3.33 ± 2.88		
E. scaber (Hasti pada)	0.5	20.00 ± 5.00			35.00 ± 5.00		
	1.0	38.33 ± 2.88	$Y = 17.24x + 9.672$	0.94	66.60 ± 2.88	$Y = 16.44x + 28.71$	0.71
	0.1	45.00 ± 5.00			80.00 ± 5.00		
	0.5	93.33 ± 2.88			100.00 ± 0.00		

No mortality was observed in the control. [a]: Values are mean ± SD of three replicates.

Table 2
Percent pupal mortality caused by AgNP synthesized by *A. indica* (Neem) and *E. scaber* (Hasti pada).

Plant Species used to synthesize Ag NP	Concentration (mg/L)	% Larval mortality ± SD[a] after 24 h	Regression equation	R^2 value	% Larval mortality ± SD[a] after 48 h	Regression equation	R^2 value
Azadiracta indica (Neem)	0.1	0.00 ± 0.00			8.33 ± 2.88		
	0.5	0.00 ± 0.00			11.66 ± 2.88		
	1.0	18.33 ± 2.88			31.66 ± 5.77	$Y = 19.05x + 9.886$	0.95
	2.0	51.66 ± 2.88	$Y = 20.93x - 2.340$	0.96	61.66 ± 2.88		
	5.0	98.33 ± 2.88			100.00 ± 0.00		
	0.1	0.00 ± 0.00			5.00 ± 5.00		
E. scaber (Hasti pada)	0.5	6.66 ± 2.88			13.33 ± 2.88		
	1.0	8.33 ± 2.88	$Y = 18.24x - 6.042$	0.97	15.00 ± 0.00	$Y = 19.30x + 1.133$	0.99
	0.1	23.33 ± 2.88			40.00 ± 0.00		
	0.5	88.33 ± 7.63			98.33 ± 0.00		

No mortality was observed in the control; [a]: Values are mean ± SD of three replicates.

Table 3
Probit analysis of mosquito larval and pupal mortality after 24 hours.

Plant species used to synthesize Ag NP	Life stage of *Cx. quinquefasciatus*	LC_{50} (mg/L)	95% Confidence limit LCL-UCL	LC_{90} (mg/L)	95% Confidence limit LCL-UCL	χ^2 ($df = 3$)
A. indica (Neem)	Larva (fourth instar)	0.898	0.492–2.631	2.184	1.403–17.356	23.161
	Pupa	2.112	1.321–10.324	3.363	2.259–25.190	26.497
E. scaber (Hasti pada)	Larva (fourth instar)	0.991	0.190–3.425	2.108	1.362–18.981	28.948
	Pupa	3.140	2.876–3.443	5.104	4.673–5.656	3.884

LC_{50} lethal concentration that kills 50% of the exposed organisms; LC_{90} lethal concentration that kills 90% of the exposed organisms; UCL: 95% Upper confidence limit; LCL: 95% Lower confidence limit,

h showed 8.33% and 13.33% mortality repectively. Whereas, the highest does of AgNP *i.e.*, 5 mg/L, reduced by *A. indica* showed 100% mortality after 48 h and AgNP reduced from *E. scaber* showed 98.33% mortality.

Pupal hatching is also related with the dose of AgNP where increment in AgNP dose showed decreased pupal hatching to adult. *A. indica* reduced AgNP treated pupae shows 88.33% hatching at 0.1 mg/L dose after 48 h and no hatching occurred at 5 mg/L concentration. Similarly, 0.1 mg/L dose of *E. scaber* induced AgNP 85% hatching and none of the pupae hatched to adult those were treated with 5 mg/L AgNP (Table 4).

Table 4
Percent pupal hatching incubated in AgNP solution synthesized by *A. indica* (Neem) and *E. scaber* (Hasti pada).

Plant species used to synthesize Ag NP	Concentration (mg/L)	% Pupal emergence after 24 h	% Pupal emergence after 48 h
A. indica (Neem)	0.1	36.66 ± 2.88	88.33 ± 2.88
	0.5	25.00 ± 5.00	90.00 ± 5.00
	1.0	11.66 ± 2.88	53.33 ± 2.88
	2.0	30.00 ± 0.00	35.00 ± 0.00
	5.0	0.00 ± 0.00	0.00 ± 0.00
E. scaber (Hasti pada)	0.1	35.00 ± 0.00	85.00 ± 5.00
	0.5	30.00 ± 0.00	81.66 ± 2.88
	1.0	16.66 ± 2.88	78.33 ± 2.88
	2.0	21.66 ± 2.88	51.66 ± 5.77
	5.0	0.00 ± 0.00	0.00 ± 0.00

3.7. Determination of total phenolics from plants

The phenolic content in the two leaves *A. indica* and *E. scaber* which were selected for the reduction of silver ion to its zero state were recoreded as 3.57 and 4.88 mg/g fresh weight, respectively. In reduction of silver nitrate to its nano form the plant derived reducing agents were proteins, phenolics, flavonoids, tannins *etc.* So, the high phenolics content in the leaves of these two leaf samples were probably responsible for the rapid reduction of silver ions to nanosilver.

3.8. EDX of dead larval bodies

From the EDX study it is clear that the larval body dust showed 1.35% (weight) Ag in that particular area of the mosquito sample (Figure 7a and 7b). So, from this observation it is clear that AgNP has surely penetrated into the larval body which is possibly the cause of death (Figure 8).

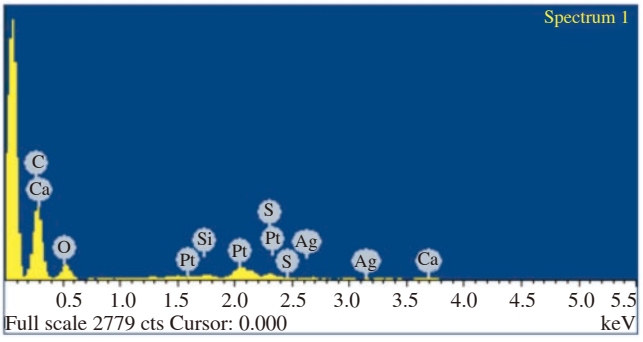

Figure 7. EDX spectrum showing presence of silver in chemical composition of larval body.

4. Discussion

Nanoparticles as mosquito control agent are a new breakthrough in recent research. Because, nanoparticles synthesized in a greener

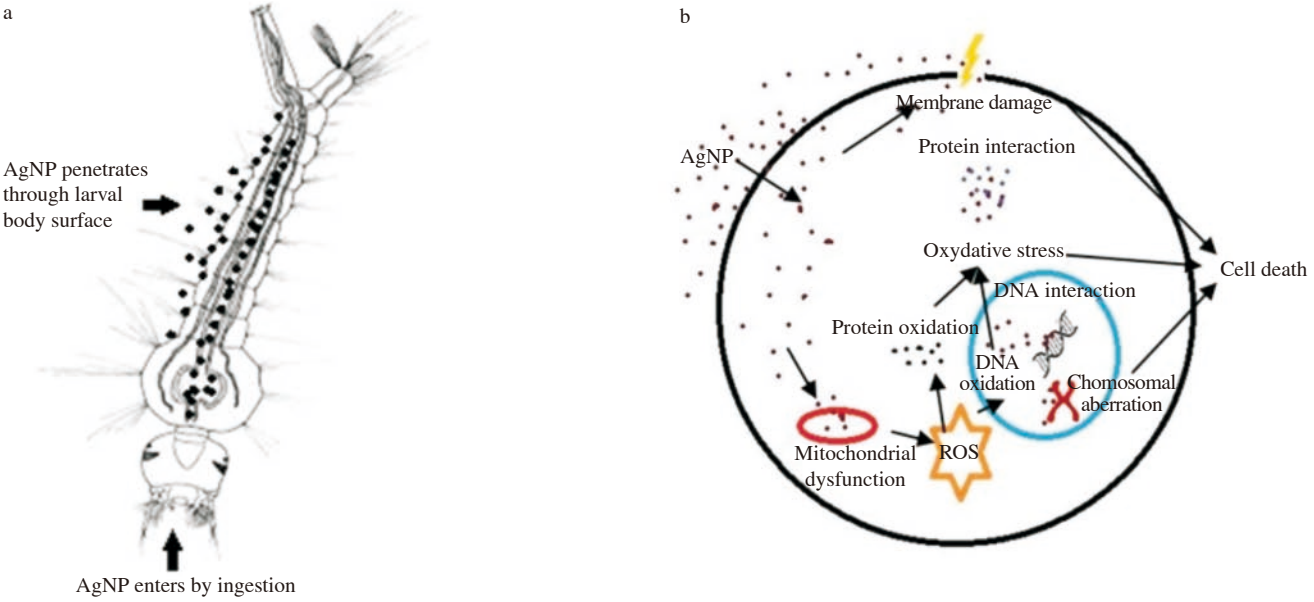

Figure 8. Proposed mechanism of (a): Route of AgNP into mosquito larval body; (b): Mechanism of AgNP interacting with body cell and causing death.

way are less toxic than conventional chemical pesticides. Moreover, nanoparticles are easily degraded to its non toxic form after sometimes in the environment imposing least harm to that. The field of green synthesis of AgNPs using plant extract has been explored by many researchers as various plant contains different kind of biomolecules which are responsible for reduction and stabilization of metals to its nano forms. In our present work *A. indica* and *E. scaber* are chosen for that purpose. Both the plant extracts showed tremendous efficiency towards the reduction of silver ions to its zero state.

Initial analysis of synthesized AgNPs in aqueous solution was done by UV-vis spectroscopy. The sharp absorption band was detected due to free electron of AgNPs which gives rise to a surface plasmon resonance and it is the result of combined vibration of the electrons of AgNPs in resonance with the light wave[38]. In present research surface plasmon resosnance spectrum of AgNP was recorded at 450 nm and 445 nm for *A. indica* and *E. scaber* induced nanoparticles, respectively. The results of UV-vis absorption showed increasing color intensity with increased observation time. This is probably due to more production of AgNPs[39]. TEM image provided further insight into the morphology along with the particle size distribution of AgNPs. The data obtained from transmission electron micrograph showed distinct shape and size of the AgNP (Figure 3). The particles from both the origin were spherical in shape in the range of below 50 nm and more or less uniformly distributed without significant agglomeration (Figure 3). Almost similar TEM image of AgNPs was observed by Selvi and Sivakumar[40] for the synthesis of AgNP by *Fusarium oxysporum*.

FTIR was done to identify the possible biomolecules responsible for the reduction of Ag^+ ions and capping the bioreduced AgNP by proteins[41]. FTIR of the synthesized AgNP is depicted in Figure 5. The distinct sharp peaks at 3 327 cm^{-1} correspond to –OH stretching of alcoholic compounds. However, peaks at 1 506/1 600 cm^{-1} corresponds to nitrite (-C=N) groups. Almost similar IR spectra of AgNPs were reported by Medda *et al.*[42] synthesized from *Aloe vera* leaf extract.

The mechanism that how nanoparticles lead to death of mosquito is still not clear, but it can be assumed that nanoparticles easily cross larval membrane due to its smaller size. After entering into the body, the nanoparticles interact with the biological molecule to generate reactive oxygen species which ultimately leads to cell death[43]. Another worker reported that AgNPs penetrated cell membrane by the attraction of cell membrane and silver ion[44]. In addition, nanoparticles may have caused perforation in cellular membrane of mosquito or lead to a dissipation of proton motive force. Nanoparticles interacted with the larval body in two ways- AgNP crossed the larval body surface as they are small enough to be penetrated through the cell membranes and by ingestion (Figure 8a). Death of the larvae may be caused by oxidative stress from reactive oxygen species, membrane damage, DNA and chromosomal denaturation[45,46] which are hypothetically explained in Figure 8b.

When synthesized AgNPs were applied for pupae, pupal mortality was not as much as larvae for the lower doses, although, limited number of pupae hatched after the stipulated time. Moreover, higher dose of nanoparticles showed 100% mortality. In the present investigation, AgNP showed the larvicidal activity against *Cx. quinquefasciatus* and also act as growth inhibitor of pupae. Vector management is one of the major issues due to the capacity of resistance against insecticides. Therefore, an urgent need has emerged to develop the new insecticide[47]. Hence, the invention of nanometals using *A. indica* and *E. scaber* as represented here, can provide a new product to control mosquitoes by replacing the synthetic larvicidal products as this route would make available larvicides to prevent several dreadful diseases.

Mosquito larvae were exposed with AgNPs and the dead larvae were subjected to SEM with EDX analysis to ascertain the incorporation of AgNP in the internal body parts of mosquito larvae. In the SEM picture a crushed body part of the mosquito can be seen which were analyzed (Figure not supplied). However, EDX spectrum showed distinct peaks of silver which clearly proved that silver have entered to the internal body parts of mosquito larva (Figure 7).

Leaf extract of *A. indica* and *E. scaber* could be effectively utilized as reducing as well as stabilizing agent for the synthesis of AgNPs. TEM analysis clearly revealed that size of the AgNPs from both the plant species are less than 60 nm. This eco-friendly, non-toxic and rapidly synthesized AgNPs are highly stable and have significant mosquito larvicidal activity against *Cx. quinquefasciatus*. Present study also highlighted that synthesized AgNPs from both the leaf extracts have immense potentiality towards hampering of pupal hatching at sublethal dose. EDX analysis of dead larvae body suggests that larval mortality is due to diffusion of AgNPs inside the larval body. However, more research is needed to support the present findings. Finally, it can be suggested that the synthesized AgNPs can be applied for other pest/insect control and there is a wide scope for detailed investigation for controlling the agricultural pathogens.

Conflict of interest statement

We declare that we have no conflict of interest.

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