International Journal of Entomology Research
www.entomologyjournals.com
ISSN: 2455-4758
Received: 27-02-2024, Accepted: 21-03-2024, Published: 13-04-2024
Volume 9, Issue 4, 2024, Page No. 64-70

Assessment of *Anopheles stephensi* larvicidal activity using silver nanoparticles synthesised from *Andrographis paniculata* leaf extract

Kohila M[1*], Jeyalalitha T[2], Amutha A[3], Muthukumar P[4]
[1] Research Scholar, Department of Zoology, Arulmigu Palaniandavar College of Arts and Culture, Palani, Dindigul, Tamilnadu, India
[2] Associate Professor, Department of Zoology, Arulmigu Palaniandavar College of Arts and Culture, Palani, Dindigul, Tamilnadu, India
[3] Assistant Professor, Department of Zoology, Arulmigu Palaniandavar College of Arts and Culture, Palani, Dindigul, Tamilnadu, India
[4] Department of Zoology, Arulmigu Palaniandavar College of Arts and Culture, Palani, Dindigul, Tamilnadu, India

Abstract
Nowadays, very small sizes of nanoparticles (metals) are being utilized in all scientific research and are still inspiring researchers to discover new extents for their respective worth. In this investigation, it was discovered that synthesised silver nanoparticles (AgNPs) obtained from plant leaf extract were found to reduce the metabolic action of mosquito larvae. The bio-inhibition method using silver nanoparticles proved to be simple, very cost-effective, and convenient. The synthesis of nanoparticles was confirmed by visual detection, in which the leaf extract of *A. paniculata*, a greenish colour, turned into a brown-coloured solution. Further characterization was done by FTIR analysis, SEM, and EDAX. In this study, we assess the larval mortality of *Anopheles Stephensi* against *A. paniculata*-AgNPs.

Keywords: AgNPs, *A. paniculata*, FTIR analysis, SEM, EDAX, Larvicidal activity

Introduction

Mosquitoes transmitted by vector-borne diseases like fleas, ticks, filariasis, dengue fever, malaria, and such types of vectors were reported to be endemic all over the world [1, 2, 3]. According to Khader *et al.* [16], there were 2.5 million malarial cases reported worldwide in 2018, of which 76% accounted for India alone. There are millions of cases of deaths from vector-borne diseases, leading to a severe effect on social and economic advancement [9, 34]. In this aspect, a variety of artificial mosquitocidal agents were developed for controlling mosquitoes. Phyto-compounds were used in earlier days as repellents and insecticides against vectors. It is promising and encourages the researchers to further develop plant-based insecticides [25]. Nowadays, the utilisation of plant-based nanoparticles and nanoemulsions has become widely recognised because of the compounds that may be produced with them that have antibacterial, antioxidant, and anticancer properties [12, 14, 17, 18].

Eco-friendly synthesized nanoparticles are inexpensive, non-toxic, and safer. Hence, we considered it highly beneficial for environmental safety applications and made it easier for maximum synthesize. Moreover, it has been observed that low concentrations of AgNPs are not harmful to higher-category animal cells, especially human cells, but highly toxic to lower-category microbes such as fungi, viruses, and bacteria [33]. Many studies have currently generated environmentally friendly repellents with larval efficacy derived from plant materials. [20, 22]. Various kinds of therapeutic plants are employed in the synthesis of titanium, platinum, silver, copper, and gold nanoparticles, which possess powerful antioxidant, anticancer, and antimicrobial properties [8, 27].

Generally, medicinal herbs have bio-surfactant molecules, which are alkaloids, flavonoids, phenols, saponins, tannins, and glycosides. These compounds have been widely explored for their effectiveness in synthesising AgNPs. The use of eco-friendly, safe substances similar to plant extracts, fungi, and bacteria. This has a number of advantages, including compatibility with medications, environmental friendliness, and other medical therapies, as it doesn't use fatal chemicals [5, 32]. Preparations of larvicides are the most important tools for the control of mosquitoes. The most frequently used insecticides are organophosphates, which inhibit growth, methoprene, and temephos [6, 7, 10, 28]. As these studies concluded, larvicides are used on both natural and man-made bodies of water as a result of the production of AgNPs, which are reducing, stabilising, and capping agents. Therefore, in this investigation, we synthesised the AgNPs by using the *A. paniculata* extract, which has undergone testing on *An. stephensi* larvae. The synthesised AgNPs could be used to suppress mosquito larvae activity. A pictorial representation of the larvicidal activity of the *A. paniculata* AgNPs and the characterization of the AgNPs solution are given in Fig. 1.

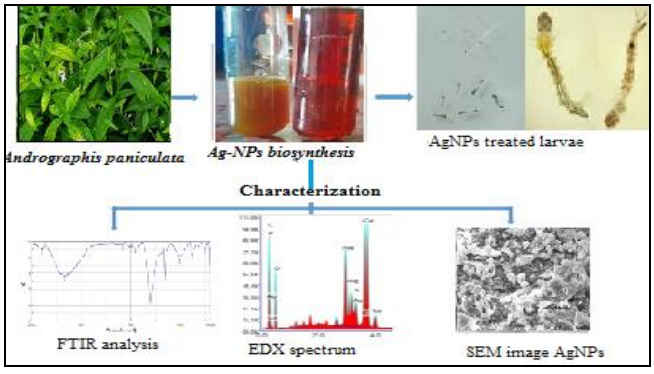

Fig 1: Graphical representation depicting the larvicidal activity of the ethonolic leaf extracts *A. paniculata*, characterization along with its effect on larval forms.

Materials and Methods

Preparation and Purification of AgNPs

According to Prasetyo *et al*. [29], the synthesis of AgNPs was carried out with slight modifications. A 1 mM $AgNO_3$ solution was prepared in DDH_2O (Double Distilled water). 200 mL of a solution of 1 mM $AgNO_3$ was treated with 100 mL of culture supernatant in a 500 mL conical flask. Equal amounts of ethanolic leaf extract and $AgNO_3$ liquid were kept in the shaker at 150 rpm and maintained in a dark condition for 24 to 72 hours at normal temperature. The $AgNO_3$ reduction was monitored by a noticeable color change in the leaf extract. The resulting AgNPs liquid was filtered using a series of centrifugations at 10,000 rpm for ten minutes. After discarding the supernatant, the pellet was dissolved in double-distilled water and either dried on watch glasses or in a hot air oven set to 25 to 30 ^oC.

Collection of eggs and maintenance of larvae, pupae, and adults

An stephensi eggs were procured from the Indian Council of Medical Research-Vector Control Research Centre (ICMR-VCRC) located in Madurai. After being transported to the lab, the eggs were placed in 20 x 15 x 6 cm enamel trays with 500 ml of water and left to hatch into larvae. The culture of mosquito larvae was kept alive in the lab. Dog biscuits and yeast were fed to the mosquito larvae in a 3:1 ratio. The larva was fed continuously until it changed into a pupa. Using a dipper, the pupae were taken out of the rearing trays and placed into 12x12 cm plastic containers with 500 ml of water inside.

Characterization of silver nanoparticles (AgNPs)

SEM and EDAX analysis of AgNPs

The SEM and EDAX studies were done using an electron microscope (VEGA 3 TESCAN). The synthesized AgNPs were centrifuged at 10,000 rpm for 15 minutes, and the supernatant was discarded. The pellets were mixed with distilled water for centrifugation at 10,000 rpm for 10 minutes. After the pellets were dried in a hot air oven at 50 ^oC, a tiny quantity of the dried samples could be dropped onto a copper grid to be carbon-coated. The films on the SEM grid, a carbon-coated copper grid, were exposed to a mercury lamp for five minutes in order to dry. The size, structure, morphological characteristics, and micrograph pictures of the synthesized AgNPs from the leaf extracts were noted and examined. The images were implanted with information regarding the size of the contents, the applied voltage, and their magnification.

FTIR analysis (Fourier Transform Infrared Spectrophotometer)

The FTIR spectra were recorded using a FTIR–8400s SHIMADZU. The pellet for analysis was made from synthesised AgNps from *A. paniculata* and KBr (1:1 ratio), and the background calibrations have been carried out using a pure KBr pellet.

Larvicidal Bioassay

The larvicidal activity was evaluated by the method of WHO [35], with some modifications, and as per the procedure of Rahuman *et al.* [30]. The laboratory bioassay was carried out with different concentrations of silver nanoparticles of *A. paniculata*, such as 20, 40, 60, 80, and 100 ppm, against 15 larvae of the 1st, 2nd, 3rd, and 4th instars. Each group was placed in a bioassay-testing container with 500 ml of water. In each treatment, four replicates were maintained for bioassay, and distilled water was used as the control. The mortality of larvae was recorded as the average of four replicates after 48 hours of post-treatment. The LC_{50} and LC_{90} values were found after 48 hours. The percentage of larval mortality was calculated using formula (1), and corrected percentages of mortality were done when necessary using Abbot's formula (2).

$$\text{Percentage of larvicidal activity} = \frac{\text{Number of dead larva}}{\text{Total number of larva introduced}} \text{ X } 100 \quad (1)$$

$$\text{Corrected percentage of mortality} = \left(1 - \frac{n \text{ in T after treatment}}{n \text{ in C after treatment}}\right) \text{X } 100 \quad (2)$$

Where, n is number of pupae, T – is the treated, and C is the control.

LC_{50} $LC_{90,}$ 95 % confidence limit of lower confidence limit (LCL) and upper confidence limit (UCL).

Statistical Analysis

The larval mortality rates were analysed by probit analysis, and for calculating $LC_{50,}$ $LC_{90,}$ 95 % confidence limits, values were found out by using the Reddy *et al.* [31] method. The SPSS software package version 22.0 was used.

Results and Discussion

Biosynthesis of AgNPs by *A. paniculata* was employed as larvicidal analysis, as reported in this research work. The saturated Ag ions were reduced to AgNPs when added to the *A. paniculata* leaf extract. The colour of the aqueous solution turned from greenish to brown colour after 24 to 48 h of the incubation. The changing colour could be the formation of AgNPs (Fig. 2). The development and constancy of the reduced AgNPs in the aqueous substance were visualized by a UV-Vis spectrophotometer. The spectrophotometer curve shows greater than before absorbance in various time intervals (1 h, 24 h, and 48 h), and the curve peaks were observed at 425 nm, corresponding to the surface plasmon resonance (SPR) of AgNPs (Fig. 3). This 425 nm value connected to a usual nanoparticle-core shell size of 10 to 12 nm, this size concluded by the shape of the spectrum. It shows a maximum scattering of nanoparticle size in view of the fact that the hit the highest point (peak) is wide but symmetrical. The highest absorbance at 425 nm confirms the synthesis of AgNPs due to the reduction of silver ions by the phytocompounds of *A. paniculata*. The peak value dispersion can be indicating that, it has wide spaces present among the minute particles. This study was in agreement with an earlier report that the absorbance at around 430 nm for Ag^+ is a Nobel metal particle [24].

Fig 2: Biosynthesized silver nanoparticles of *A.paniculata,* 24 h colour changes

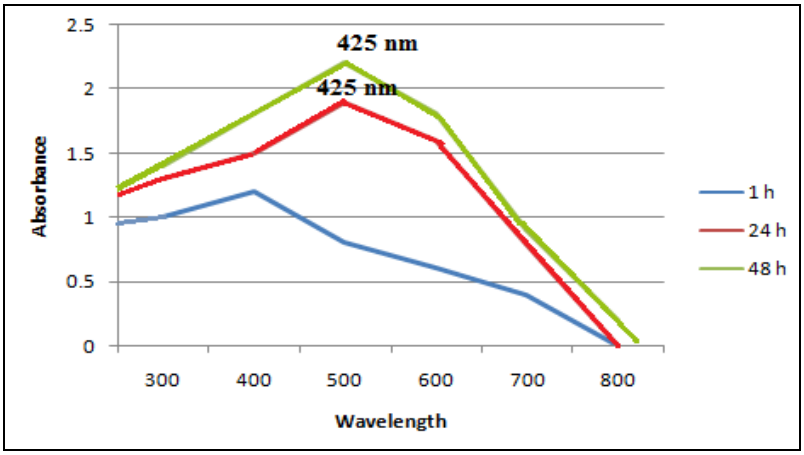

Fig 3: UV-vis spectra AgNPs synthesized using *A. paniculata* extract

Fourier Transform Infrared Spectroscopy analysis of AgNPs

The FTIR analysis was made to verify the probable types of bio-molecules present in the in the samples, especially to find out the functional groups of the samples. In this study, FTIR analysis is responsible for the capping and well-organization of the AgNPs synthesized by *A. paniculata* L. extract. The FTIR spectra of the synthesised *A. paniculata*-mediated AgNPs showed a total of 10 characteristic absorption peaks, as shown in Fig. 4. The bands at 3787.51 cm^{-1} and 3332.39 cm^{-1} can be assigned to O-H stretching. The band at 2168.56 cm^{-1} can be assigned S-CΞN stretching. The bands at 1994.03 cm^{-1} and 1911.11 cm^{-1} can be assigned to C=C=C stretching. The band at 1608.34 cm^{-1} can be assigned to Nitro compounds; 1313.29 cm^{-1} can be assigned to Nitro compounds, 776.208 cm^{-1} can be assigned to C-H bending. The bands at 509.115 cm^{-1} can be assigned to C-I stretching. The peak values of the compound and its functional groups are given in Table 1. This result always gives information about the role of *A. paniculata* L. extract as a reducing and capping agent. Similar results were confirmed in the study of Nasir *et al.* [24] on *Allium sativum* L extract. This result also concluded the results of Jazem A Mahyoub [15], who confirmed that 776.208 cm^{-1} can be assigned to C-H bending and 509.115 cm^{-1} can be assigned to C-I stretching.

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Table 1: FTIR analysis for the identification of functional groups from *A. paniculata*-AgNps

Sl. No	Peak value (cm^{-1})	Functional group	Compound class
1.	3787.51	O-H stretching	alcohol
2.	3332.39	O-H stretching	alcohol
3.	2168.56	S-CΞN stretching	thiocyanate
4.	1994.03	C=C=C stretching	allene
5.	1911.11	C=C=C stretching	allene
6.	1608.34	N-O stretching	nitro compound
7.	1313.29	N-O stretching	nitro compound
8.	1016.3	Unknown	Unknown
9.	776.208	C-H bending	1,3-disubstituted
10.	509.115	C-I stretching	halo compound

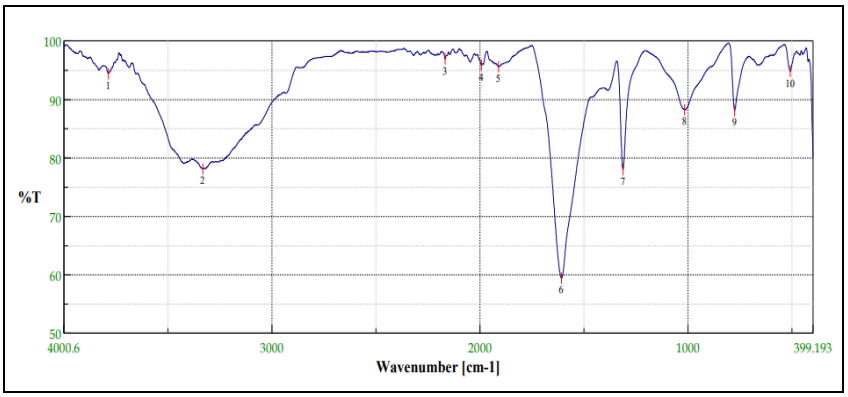

Fig 4: FTIR analysis of AgNPs peaks values synthesized from *A. paniculata* ethanolic leaf extract

SEM and EDAX spectrum analysis of *A paniculata*-AgNPs

SEM images of AgNPs synthesised from *A. paniculata* leaf extracts are different sizes and shapes (Figure 5). The particle size of the biosynthesized nanoparticles was found to be between 50 nm and 192 nm. The SEM images show that each nanoparticle is spherical or pseudospherical in shape, and some have an undefined morphology of agglomeration. This result was confirmed by the results of AgNPs present in *Annona glabra* (Annonaceae) extract [4], and another study was also reported using *A. senegalensis* and *C. obstufolia* leaves [26]. Despite that, we found some little difference in the sizes of the AgNPs.

In this study, separate molecules were analysed with the energy dispersive absorbance X-ray spectrum (EDAX spectrum), which showed the presence of carbon, oxygen, silver, potassium, and calcium atoms (Table 2). The presence of Ag atoms (Fig. 6) has strong peaks that were observed approximately at 3 keV. These results were in conformity with those of Loganathan *et al.* [19], and he observed that synthesised AgNPs of *Knoxia sumatrensis* extract in EDAX confirmed the presence of silver.

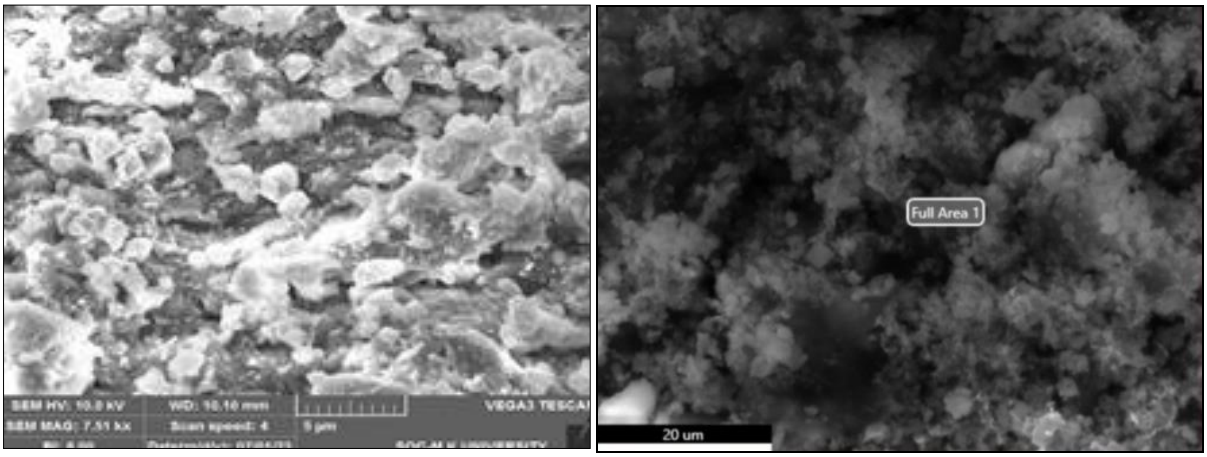

Fig 5: SEM image of AgNPs synthesized from *A paniculata* ethanolic leaf extract

Table 2: EDAX spectrum of *A paniculata*-AgNPs

Element	Weight %	Atomic %	Net Int.	Error %	Kratio	Z	A	F
C K	36.22	50.46	966.29	5.79	0.2162	1.0591	0.5637	1.0000
O K	40.96	42.84	643.65	10.23	0.0473	1.0226	0.1131	1.0000
AgL	10.84	1.68	1553.03	4.19	0.1051	0.7380	1.3053	1.0061
K K	1.34	0.58	356.73	3.28	0.0120	0.8856	0.9877	1.0185
CaK	10.64	4.44	2225.53	2.23	0.0866	0.9034	0.8969	1.0059

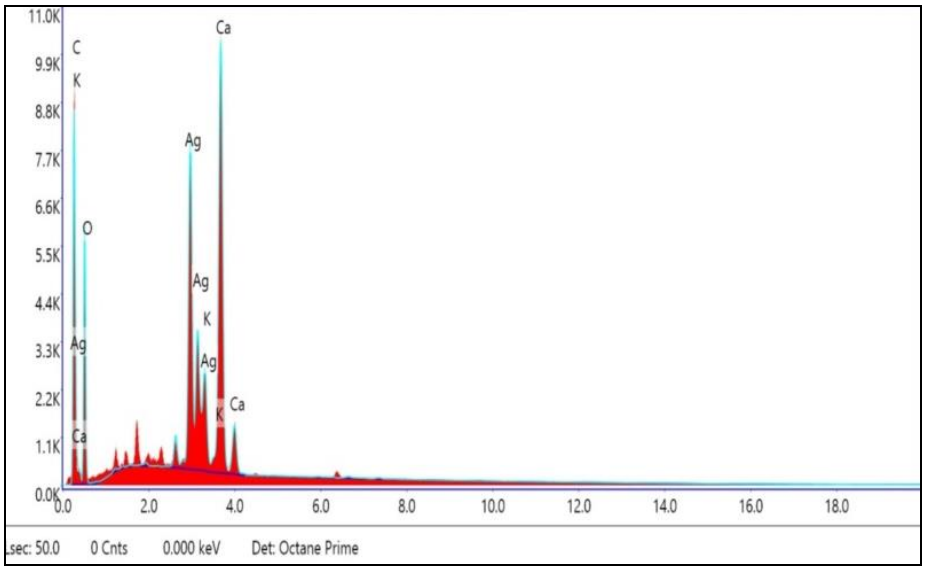

Fig 6: EDAX analysis of AgNPs synthesized from *A paniculata* ethanolic leaf extract

Larval and pupal toxicity effect of *A paniculata*- AgNPs against *An. stephensi*

The synthesised AgNPs from *A paniculata* extract exhibited potent larvicidal and pupicidal activity against larval and pupae stages of *An. stephensi*, when exposed at various concentrations (20 ppm–100 ppm) at 48 h of incubation, as presented in Table 3. The synthesised AgNPs from *A paniculata* leaf extract have strong larvicidal activity. The synthesised AgNPs showed a dose- and time-dependent toxic effect against the 1st, 2nd, 3rd, and 4th instar larvae and pupae of *An. stephensi*. In the control groups, there were absence of mortality. The highest mortality rate of 99±0.5 was noticed at 100 ppm concentration in the 1st instar larvae, and the lowest mortality rate was 43±0.4 at 20 ppm concentration in the 4th instar larvae at 48 h of incubation. At the 20 ppm concentration, the highest mortality was 52±0.3 in the 1st instar larvae. In my result, there were potential larvicidal activity was observed at lower concentration of *A paniculata*-AgNPs at 24 h treatment, and this mortality rate was increased with increasing concentration of AgNPs at 48 h treatment. The larvicidal activity increased with increasing concentrations; this is due to some different secondary metabolites present in *A paniculata* [21]. My results are agreement with the earlier reporters, who reported exploring the *Aedes aegypti* and *Culex quinquefasciatus* larvicidal activity of the phytocompound through a dose-dependent response [13].

The LC_{50} values were obtained at 20.748, 23.914, 28.371, and 35.901 for 1st, 2nd, 3rd, and 4th instar larvae, respectively. The LC_{90} values were 79.288, 94.550, 106.384, and 119.256 for 1st, 2nd, 3rd, and 4th instar larvae, respectively. While the concentration of AgNPs was 20 ppm, the pupal mortality rate was 39±0.6. It has been increased to 73±0.2 at 100 ppm. The LC_{50} value of pupal stage after the treatment of *A. paniculata*-AgNPs was 46.608, and the LC90 value was 158.189. The highest LFL LC50 value (24.183) and LC_{90}

value (104.129) were observed for 4th instar larvae. Similarly, the highest UFL-LC_{50} value (44.200) and LC_{90} value (144.707) were observed for 4th instar larvae. The LFL (LC_{50} and LC_{90}) values of 33.311 and 131.063 and the UFL (LC_{50} and LC_{90}) values of 56.436 and 213.178 were noted for pupae. The highest Chi-square value of 4.655 was noted in 1st instar larvae.

A paniculata-AgNPs exhibited good larvicidal activity (Fig. 7). The result also revealed that *A paniculata*-AgNPs is more potent as a mosquito larvicide, which showed 52% mortality even at a concentration of 20 ppm. The findings of this research are consistent with the work of Nganjiwa *et al.* [25]. The maximum larvicidal activity was noticed from synthesised AgNPs of *Chomelia asiatica* (Rubiaceae) medicinal plant, and they could be used to control *An. stephensi* larval forms as an eco-friendly approach [23]. Similar larvicidal activity of AgNPs was reported from *Allium sativum* bulb extracts against Aedes larvae [24] and *Atalantia monophylla* leaf extract against blood sucking malarial vector larvae [11].

Table 3: Larvicidal and Pupicidal efficacy of synthesized *A paniculata*-AgNPs *against An stephensi*

Larval instars and Pupa	Larval mortality ± SD (%) ++Concentrations (ppm)					LC 50 (LC_{90})	95% Confidence limit		Chi-Square Value (χ^2)
	20	40	60	80	100		LFL [LC_{50} (LC_{90})]	UFL [LC_{50} (LC_{90})]	
1st	52±0.3	67±0.4	76±0.8	88±0.2	99±0.5	20.748 (79.288)	9.557 (71.419)	28.521 (90.659)	4.655*
2nd	49±0.8	62±0.5	71±0.6	83±0.2	94±0.5	23.914 (94.550)	11.242 (84.154)	32.508 (110.691)	1.660*
3rd	46±0.2	58±0.4	67±0.5	79±0.4	90±0.5	28.371 (106.384)	15.534 (93.742)	37.081 (126.950)	0.930*
4th	43±0.4	51±0.6	62±0.4	74±0.2	86±0.6	35.901 (119.256)	24.183 (104.129)	44.200 (144.707)	1.075*
Pupae	39±0.6	46±0.5	55±0.8	66±0.4	73±0.2	46.608 (158.189)	33.311 (131.063)	56.436 (213.178)	0.181*

The larval mortality is expressed as mean±SD of five replicates. No mortality was observed in the control; *LFL* – Lower Fiducidal Limit; *UFL* - Upper Fiducidal Limit; χ^2, Chi-Square value. *Significant at P<0.05 level.

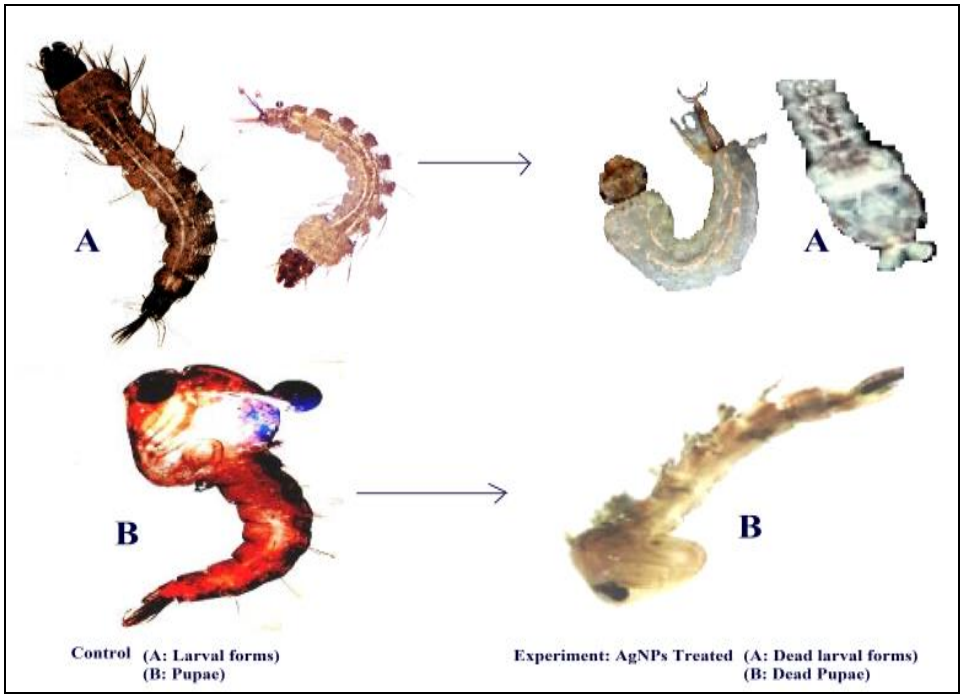

Fig 7: Group A: Control 4th instars larvae to AgNPs treated dead larvae
Group B: Control Pupae to AgNPs treated dead Pupae

Conclusion

Mosquitoes transmit many diseases; therefore, scientists sought to develop alternative insecticides that are easily biodegradable. Plant based insecticides are highly effective, safe, easily degradable and environmentally acceptable. Therefore, plant-based insecticides were considered as a viable alternative for mosquito control. Our country has rich plant biodiversity, thus, we decided plants can be used as insecticides. The biosynthesis of aqueous *A paniculata* leaf extract has suitable alternatives for controlling mosquito larvae. Accordingly, we determined that AgNPs synthesized from *A. paniculata* leaf extract could act as a larvicidal agent against *An. stephensi*. The synthesized AgNPs was an effective capping and reducing agent. In the present study, *A paniculata* leaf extract was used to reduce Ag ions to form AgNPs. Application of *A. paniculata* -AgNPs was found as an eco-friendly larvicidal agent against *An stephensi*. This study we found out *A. paniculata*- AgNPs has significant larvicidal and pupicidal activity. *An stephensii* larvicidal and pupicidal activity is mainly due to the specificity of AgNPs and their tiny size. Hence, it can easily interact incredible with the larval stage and produce protein-degradative effects on its body, or the silver nanoparticle might alter the sodium-potassium channels in the larval nervous system, and that could be the reason for the larvae exhibiting immobility when induced by a stimulus that leads to larval death. It was a major reason for larval and pupal deaths.

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