ORIGINAL ARTICLE

EXPLORATION OF DISTINCTIVE MENYANTHES TRIFOLIATA AS GENERATED GREEN NANOPARTICLES: REPORT THEIR LETHAL TOXICITY AGAINST *Aedes Aegypti*

| Siva Kamalakannan [1*] | Marimuthu Ramar [1] | Ponnan Arumugam [1] | Kalimuthu Kovendan [2] | Balamurugan Chandramohan [1] | Douglas Vieira Thomaz [3] | Panagal Mani [4] | Selvaraju Raja [5] | and | Kadarkarai Murugan [1] |

[1.] 1Division of Medical Entomology | Department of Zoology | Bharathiar University | Coimbatore-641 046, Tamil Nadu | India |
[2.] Division of Vector Biology and Control | Department of Zoology | Annamalai University | Annamalai Nagar, 608 002 | India |
[3.] Department of Pharmacy | Federal University of Goiás, Goiânia - 74605-170, Goiás, Brazil |
[4.] Department of Biotechnology, Annai College of Arts and Science | Kumbakonam |Tamil Nadu | India |
[5.]PG & Research Dept. of Zoology | Kongunadu Arts and Science College | GN Mills, Coimbatore- 641029, Tamil Nadu | India |

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ABSTRACT

Background: Biofabrication of silver nanoparticles from plant origin provides more resistance against mosquitoes. In the present study, silver nanoparticles (AgNPs) synthesized from the novel plant, *Menyanthes trifoliata* for the control of chikungunya vector, *Aedes aegypti*. **Objective:** The nanoparticles were evaluated for their toxicity of larvae, pupae and insect growth regulatory activity, persistent study against *Aedes aegypti*. **Methods**: Nanoparticles were analyzed with UV-vis spectrophotometry, SEM, XRD, FTIR, and EDAX. The characterization studies confirmed that AgNPs are of size 10-50nm and spherical in shape. The efficacy of plant-synthesized AgNPs was tested against different concentrations ranging from 5-25 µg/mL against L_1 to L_4 larval instars and pupae of *A. aegypti*. **Results**: Observed value of LC_{50} = 8.17, 9.78, 12.96, 16.73 and 20.11 µg/mL; the LC_{90} = 19.57, 24.25, 29.64, 36.95 and 41.81 µg/mL for treated all larval instars and pupae, respectively. The mortality rates were positively correlated with the concentration of AgNPs. Significant ($P<0.05$) changes in larval mortality were also recorded between the period of exposure to all larval instars of A. aegypti. The larval and pupal growth was significantly diminished (IGR activity) by the action of AgNPs and its emergence inhibition EI_{50} and EI_{90} values are 1.87 (10.58) significantly noted against *A. aegypti*. **Conclusion**: The study reported that the toxic nature of AgNPs had a significantly higher impact on the third instars, followed by other stages, and their effect lasted up to a 12-week period. Larval midgut layer strongly disrupted and it's showed in the histology section. These findings reported *M. trifoliata* synthesized AgNPs are rapid, eco-friendly, and novel mosquito control agents.
Keywords: *Menyanthes trifoliate, Silver Nanoparticles, Mortality, Insect growth regulatory, Histology*

1. INTRODUCTION

Diseases transmitted by mosquitoes result in millions of deaths per year worldwide [1]. Vector-borne diseases have resulted in the loss of human economy both in terms of medical care costs and diminished productivity. They are a significant threat to human health, and considerable national and international efforts are required to counter it [2, 3, 4]. *Aedes aegypti* and *Aedes albopictus* are considered as vectors for dengue fever (DF) in Southeast Asia (CDC 001). Over the past 30 years, the incidence and geographical distribution of DF have increased dramatically. About 2.5 billion people worldwide have been estimated to be at risk of DF. Moreover, a hundred million cases arise annually, including 500,000 cases of Dengue Hemorrhagic Fever (DHF) [5]. It should also be noted that synthetic insecticides have reportedly resulted in several ecological issues due to its persistent residual accumulation in the environment, development of resistance in target vectors, and chronic effects on non-target organisms [6].

To overcome these problems, silver nanoparticles (AgNPs) have been studied extensively for their interesting biophysical properties in recent years [7, 8]. Biosynthesis of nanoparticles using plant extracts is currently under research. Compared to other environmentally benign biological processes, the use of plants for nanoparticles synthesis is advantageous as it eliminates the elaborate process of maintaining cell and microbial cultures. In addition, the biosynthesis of nanoparticles would be more useful if the plant extracts are synthesized in a controlled manner for monitoring their size and shape [9, 10, 11, 12].

In the present study, nanoparticles synthesized from *Menyanthes trifoliata* leaf extracts were used to study mosquitocidal and insect growth regulatory activity against *A. aegypti.* This herb contains saponin, menyanthoside, iridoid glycosides, foliamenthin, dihydrofoliamenthin, menthiafolin, and loganin. It also contains pyridine alkaloids including gentianine; coumarins (scopoletin); phenolic acids such as caffeic, protocatechuic, ferulic, sinapic, and vanillic; and flavonoids including rutin and hyperoside. Choleretic action of the herb is attributed to the synergistic action of caffeic and ferulic acids and iridoid glycosides. Scoparone and scopoletin (coumarins isolated from the aerial

parts) exhibited antihepatotoxic, choleretic, and cholagogue properties. Rhizomes contain dihydrofoliamenthin, loganin, menthiafolin, and a triterpenoid saponin menyanthoside [13]. For mosquito control, important phytochemicals from *M. trifoliata* can be synthesized and characterized with the help of AgNPs. Generally, nanoparticles of size up to 100 nm are considered because they exhibit improved new properties, including specific size, distribution, and morphology, compared to the larger particles [14]. Due to their catalytic activity, electronic properties, optical properties, and magnetic activity, nanoparticles are considered to be of much importance [15, 16]. The synthesis of various nanoparticles from palladium, selenium, platinum, gold, and silver using algae, fungi, bacteria and plant extracts has been reported in the literature [17, 18, 19, 20, 21, 22].

In recent years, biosynthesis of nanoparticles using plant extracts has received more attention than other chemical and physical methods, including the use of microbes, since it is not required to maintain an aseptic environment [23, 24, 25]. Green AgNPs have been synthesized using various natural products [26, 27, 28, 29] that had multiple effects on insects such as mortality, antifeedant activity, growth regulation, fecundity, suppression and sterilization, oviposition, and changes in biological fitness [30].
Given this background, there is a need for a critical study to develop novel insecticides to replace existing products in the areas where resistance has rendered these products obsolete or extend the operational lifespan of existing insecticides. The present study deals with the characterization of AgNPs synthesized from the plant *M. trifoliata* and their mosquitocidal, IGR activity on chikungunya vector, *Aedes aegypti*.

2. MATERIALS AND METHODS

2.1. Collection of plant materials:

The plant *M. trifoliata* (Buckbean) (34°5′24″N and 74°47′24″E) was collected during the flowering season (January to April) from Srinagar, Jammu & Kashmir, India. The plant was taxonomically identified at the Botanical Survey of India, South Circle, TNAU Campus, Coimbatore and voucher.

2.2. *A. aegypti* rearing:

The eggs of *A. aegypti* were collected from Kalveerampalayam at Coimbatore, India, using "O"-type brush. These eggs were brought into the laboratory and transferred to 18 × 13 × 4-cm enamel trays containing 500-mL of tap water before larval hatching. The larvae were daily fed a mixture of dog biscuits and hydrolyzed yeast (3:1 ratio). The pupae were collected from the rearing trays and transferred to plastic containers (12 × 12 cm) containing 500 mL of water with the help of a dipper. The plastic jars were kept in a 90 × 90 × 90-cm mosquito cage for adult emergence. The mosquito larvae were maintained at 27+2°C, 75-85% relative humidity, and photoperiod of 14:10 (light/dark). A 10% sugar solution was provided for a period up to 3 days before blood feeding.

2.3. Preparation of plant extract:

Excised leaves were washed with tap water and shade dried at room temperature (22±25^{o}C), with the help of an electrical blender. The plant leaves were grounded into fine powder. From each sample, ½ kg of powder material was extracted with the help of methanol using soxhlet apparatus [31]. The solvent was evaporated by rotary vacuum evaporator. To make concentration, 1 g of plant residue was dissolved in 100 mL of acetone. From the stock, the desirable concentration was prepared. To make 1 ppm, one milligram sample was dissolved in 1000 mL of solvent and to make 1% concentration, 1g was mixed with 100 mL of distilled water.

2.4. Synthesis and Characterization of AgNPs:

Silver nitrate ($AgNO_3$) 1mM purchased from Sigma-Aldrich Chemical Pvt. Ltd. was added to plant extract to make a solution of 200 mL. A change in the color of the solution was observed during the heating process. The reactions were carried out in darkness (to avoid photo activation of $AgNO_3$) at room temperature. Suitable control was maintained during the entire course of the experiments. Complete reduction of $AgNO_3$ to Ag^+ ions was confirmed by the changes in color from colorless to colloidal brown. After irradiation, the dilute colloidal solution was cooled to room temperature and kept aside for 24 h to complete bio-reduction and saturation denoted by UV-visible spectro-photometric scanning. Then, the colloidal mixture was sealed and stored properly for future use. The formation of AgNPs was furthermore confirmed by spectrophotometric analysis.

The reduction of pure Ag^+ ions was monitored by measuring the UV-vis spectrum of the reaction medium at four hours after diluting a small aliquot (Approx. 0.5 g) of the sample in 5 mL distilled water. UV-vis spectral analysis was conducted by using UV-vis spectrophotometer (Cary 4000 UV-vis spectrophotometer). The biomass after reaction spontaneously precipitates at the bottom of the conical flasks at the one hour mark. The suspension above the precipitate was sampled for SEM-EDX observations. SEM samples of the aqueous suspension of nanoparticles were fabricated by dropping the suspension onto a clean electric glass and allowing water to completely evaporate. The samples were coated by carbon and SEM analyses were performed on a Hitachi s-3500N. The AgNPs' solutions were

purified by repeated centrifugation at 5000 rpm (30 x 1.5/ 2.0 mL) for 20 minutes followed by redispersion of the pellet of AgNPs into 10 mL of deionized water. After freeze drying of the purified nanoparticles, the structure and composition were analyzed by XRD (Rigaku RINT 2100 series). The dried mixture of AgNPs was collected for the determination of the formation of AgNPs by an X'Pert Pro X-ray diffract meter operated at a voltage of 40 kV and a current of 30 mA with Cu Kα radiation in a θ-2θ configuration. To remove any free biomass residue or compound that is not the capping ligand of the nanoparticles, the residual solution of 100 mL, after reaction was centrifuged at 5000 rpm (30 x 1.5/ 2.0 mL) for 10 minutes and the resulting suspension was redispersed in 10 mL sterile distilled water. The centrifuging and redispersing process was repeated three times. Thereafter, the purified suspension was freeze dried to obtain dried powder. Finally, the dried nanoparticles were analyzed by FTIR (Bruker model, TENSOR 37).

2.5. Larvicidal activity:

Larvicidal activities of the plant mediated AgNPs were determined by following the standard procedure [32]. Fifty early fourth instar larvae and pupae of *A. aegypti* (obtained from the cyclic colony that was maintained for the past 6 months at the laboratory) were transferred to 249 mL of tap water taken in to 500 mL bowls. Five replicates were set up for each test concentration and the AgNPs were tested at concentrations ranging from 5 to 25 µg/mL. Bioassay was conducted at room temperature 27 ± 3°C and 85% relative humidity. In case of the experiment for determining pupicidal activity, the mouth of each bowl containing pupae was covered with muslin cloth to prevent the escape of any emerged adult mosquitoes. Mortality in larvae / pupae was recorded 24 h post treatment.

Percentage mortality = (number of dead individuals/number of treated individuals) ×100 (eq.1)

2.6. Test for Insect Growth Regulatory Activity (IGR):

The methanolic leaf extract of *M. trifoliata* and AgNPs were tested individually for the developmental activity of freshly emerged first instar larvae of *A. aegypti* [33]. Tests can be used plant extract and AgNPs for developmental activity conducted at different concentrations ranging from 2-10 µg/mL. The desired concentration of the test solution was achieved by adding 1.0 ml of appropriate stock solution to 249 ml of dechlorinated water. Five replicates for each concentration were set up. All larvae were monitored till adult emergence and were provided with larval food. Observations were conducted at 24 h intervals and the dead larvae and pupae were daily removed and counted. The developmental stages of larvae, pupae and adults were recorded. The emergence inhibition concentrations (EI_{50}) and (EI_{90}) were derived from the experimental data through probit analysis [34].

2.7. Adulticidal assays:

Adulticidal bioassay was performed following the method by WHO, 1981 [35]. The plant extract and AgNPs were tested at 5, 10, 15, 20 and 25 µg/mL. The *M. trifoliatea* leaf extract and AgNPs were applied on Whatman no. 1 filter papers (size 12 cm × 15 cm) lining a glass tube (diameter 30 mm, length 60 mm). Control papers were treated with silver nitrate and distilled water. Twenty female mosquitoes were collected and gently transferred into a plastic holding tube. The mosquitoes were allowed to acclimatize in the holding tube for one hour and then exposed to the test paper for one hour. At the end of the exposure period, the mosquitoes were transferred back to the holding tube and kept for 24 h for the recovery period. A pad of cotton soaked with 10% glucose solution was placed on the mesh screen. Each test included a set control groups (silver nitrate and distilled water) with five replicates for each individual concentration.

2.8. Persistence study of AgNPs:

Third instar larvae were tested at highest 25 µg/mL AgNPs and further evaluated for persistent toxicity assay against *A. aegypti*. A total of 10 larvae were exposed with single concentration. The concentration was persist 2 week duration then after larvae was introduced and examine for 24 h mortality, and further its study extend up to 12 week persistent period (i.e. 2, 4, 6, 8, 10, 12 week interval). Persistence study was run under a temperature of 27°C followed by untreated control.

2.9. Histology of larval midgut of *A. aegypti:*

A small proportion (Mosquito larvae and adult in the experiment and control) were selected at random for histological analysis. The mosquito tissue or body was taken in the experiment. Then the tissue were fixed in 10% neutral buffered formalin and transferred to the sectioning, where they were embedded in paraffin blocks, sectioned at 5 micro meters, and stained with hematoxylin and eosin or Gomori's methenamine silver nitrate, for histological examination. Tissue was examined for changes that could have resulted from mycotoxin damage or mycotic infection.

Histological studies include the study of minute morphological structure of a tissue under the microscope and the changes that occur in it under various conditions such as the effects of any experiment done over that tissue [36].

The time honored paraffin embedding and haematoxylin-eosin staining technique was used for the preparation of the sections. The various steps involved in the preparation of sections for the histological studies are as follows [37].

2.10. Data analysis:

The mosquito toxicity data were subjected to ANOVA followed by Tukey's HSD test. Mosquito mortality data were also subjected to probit analysis. LC_{50} and LC_{90} were calculated using the method used by Finney [34]. The analytical data together with tables are presented in appropriate places in the manuscript. SPSS software package 16 version was used for computing all the data including probit analysis, correlation equation, SE and mean of the sample.

3. RESULTS

3.1. Synthesis and characterization of AgNPs:

The generation of AgNPs varied with increasing temperature (37°C to 100°C). AgNP synthesis was found to be higher at 90°C; AgNPs synthesized at 90°C showed surface plasmon resonance (SPR) peak at 420 nm of absorbance in UV-Vis spectrum at 240 minutes. The result demonstrates that plant compounds having thermo stable properties are mainly involved in the fabrication of AgNPs. Efficient syntheses of AgNPs were observed at lower pH, and a clear and sharp SPR peak was observed at 420 nm. In acidic pH, uniformly distributed, small-sized (10 to 50 nm), spherical nanoparticles were observed, whereas in alkaline pH, the size of AgNPs was comparatively larger, irregularly shaped, and highly dispersed due to an increase in the absolute value of the negative zeta potential.

The synthesis of AgNPs improved with increasing reaction time; intense SPR peak was observed at 420 nm when the reaction time ranged between 0 to 1 h. The complete synthesis of AgNPs occurs within four hours' time. This result indicates the rapid synthesis of AgNPs with high stability. The effect of metal ion concentration on AgNPs synthesis and their shape and size were also evaluated. Higher metal ion concentration (1.0 mM $AgNO_3$) results in an enormous yield of AgNPs, which shows SPR peak at 420 nm in UV-vis spectrum, whereas a lower concentration shows decreased SPR peak. The formation of yellowish brown color indicates the synthesis of AgNPs, and as the plant metabolites' quantity increases (1 to 10 mL), the synthesis of AgNPs also gets increased. AgNPs synthesized in the stoichiometric proportion of 10 mL of plant metabolites with 90 mL of $AgNO_3$ showed intense SPR peak at 420 nm in UV-visible spectroscopy, indicating the dispersion of AgNPs (Figure 1).

a)

b)

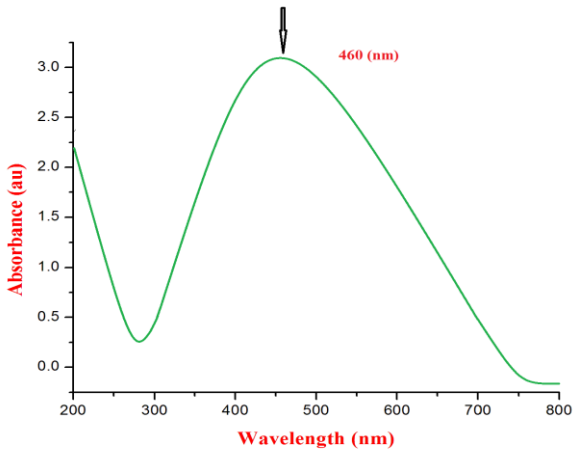

Figure 1: (a) Color intensity of the *Menyanthes trifoliata* aqueous extract before and after the reduction of silver nitrate (1mM). The color change indicates Ag^+ reduction to elemental nanosilver. **(b)** UV-visible spectrum of silver nanoparticles after 180 min from the reaction.

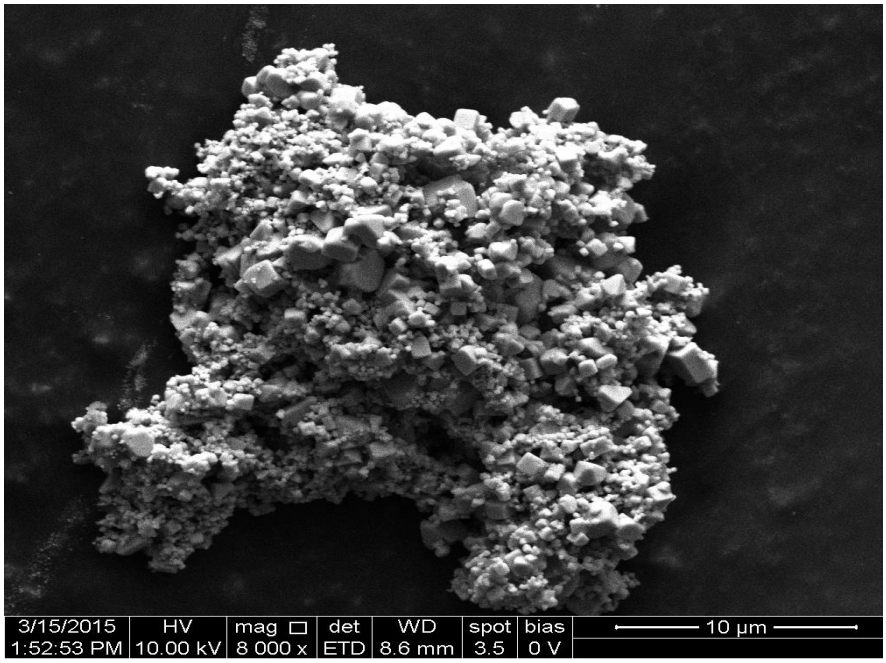

Figure 2: Scan electron microscopy (SEM) of *Menyanthes trifoliata*-fabricated silver nanoparticles.

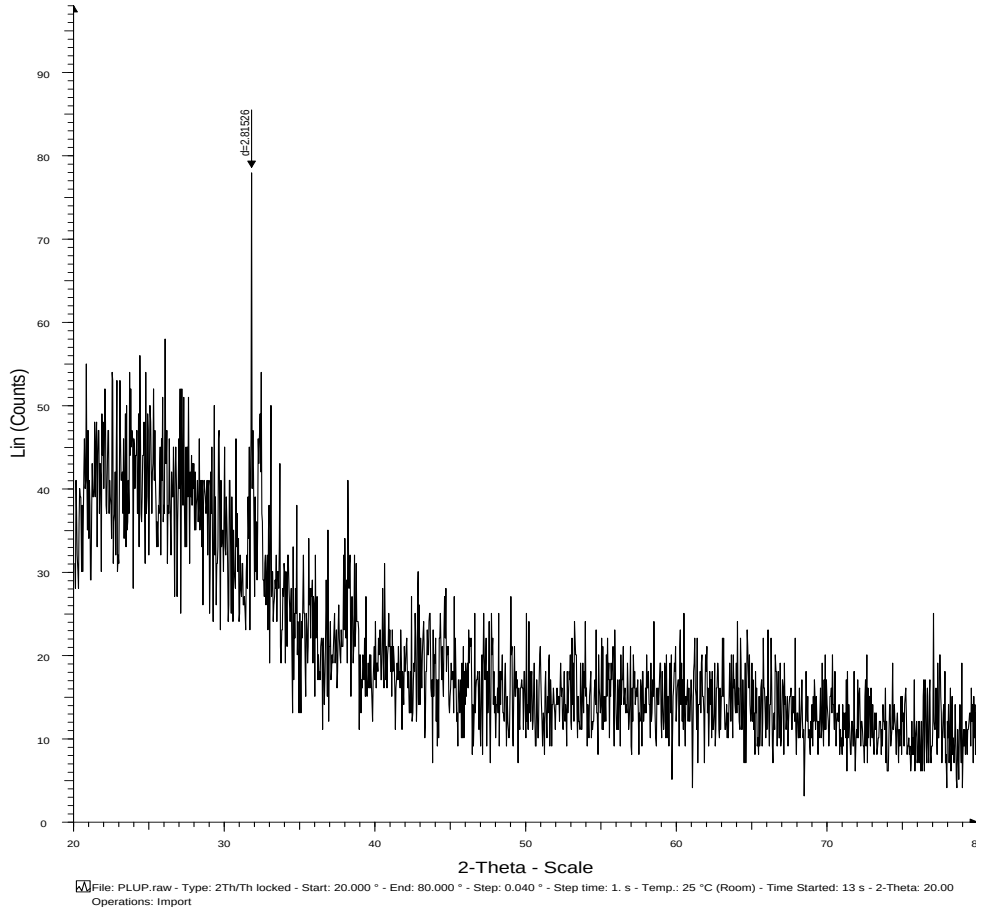

Figure 3: XRD pattern of silver nanoparticles bio-fabricated using the *Menyanthes trifoliata* aqueous leaf extract.

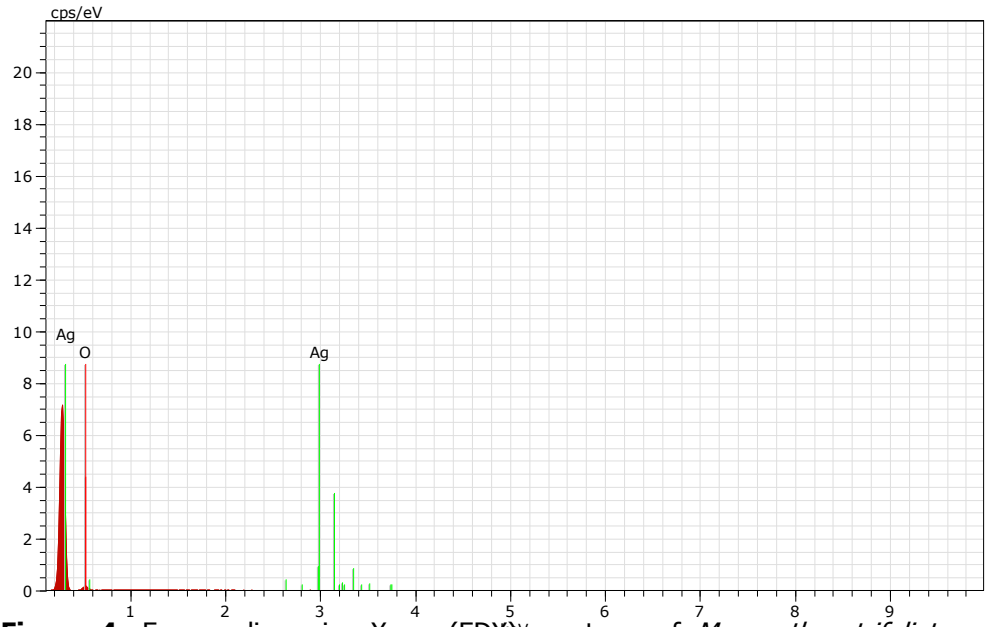

Figure 4: Energy dispersive X-ray (EDX) spectrum of *Menyanthes trifoliata*-synthesized silver nanoparticles showing the presence of different phyto-elements as capping agents.

Spectrum: 3 2961

El	AN	Series	unn. C [wt.%]	norm. C [wt.%]	Atom. C [at.%]	Error (1 Sigma) [wt.%]
O	8	K-series	92.79	92.79	98.86	36.93
Ag	47	L-series	7.21	7.21	1.14	3.27
Total:			100.00	100.00	100.00	

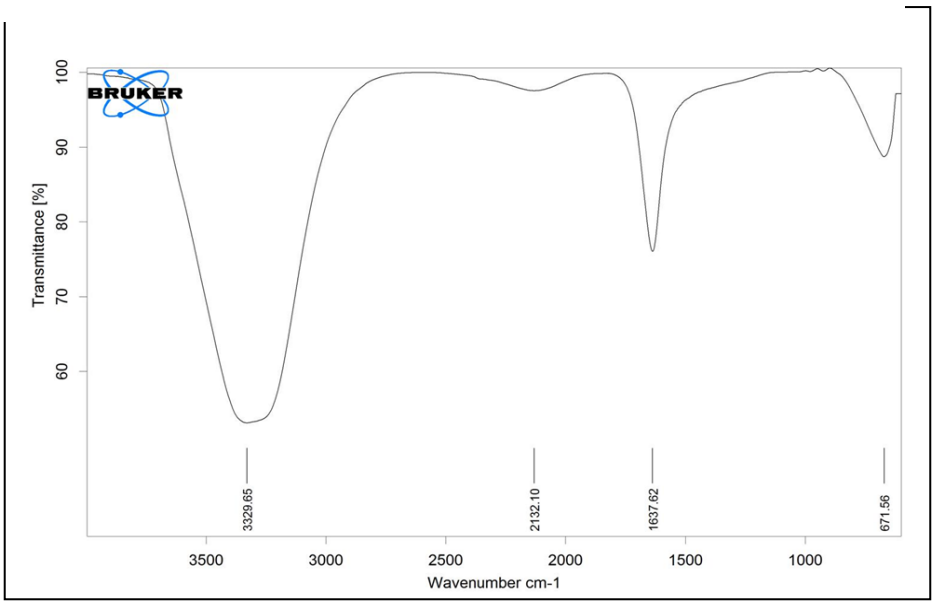

Figure 5: Fourier Transfer Infra-Red (FTIR) spectrum of silver nanoparticles bio-fabricated using the *Menyanthes trifoliata* aqueous leaf extract.

XRD analysis (Figure 3) showed an intense peak at 3θ values of 2.815°, Bragg's reflection based on the face-centered cubic structure of AgNPs. FTIR absorbance spectrum was observed at 3329.65, 2132.10, 1637.62 and 67.56 cm^{-1}, indicating the functional group of the plant component is involved in the reduction and acts as a capping agent.

Results noted at 37°C for 48 h (Figure 4). In addition, triangular, pentagonal, and hexagonal structures of the nanoparticles were also observed. The EDX proved the chemical purity of the synthesized AgNPs (Figure 5). The reduction of Ag^+ ions was followed by UV-spectroscopy, which exhibited the yellowish brown color of AgNPs with the absorbance value at 420 nm. The occurrence of the color could be due to the excitation of the surface plasmon vibrations of the metal nanoparticles. FTIR spectroscopy analysis was carried out to identify the possible biomolecules responsible for the reduction of Ag^+ ions. FTIR interferogram of both plant extract and AgNPs showed variation in the stretches at 3329, 2132, 1637 and 671cm^{-1} that corresponded to primary amines (N-H) and carbonyl (C=O) functional groups present in the amino acid residues and proteins in the plant extract [38]. The FTIR results clearly indicated that water soluble compounds present in the plant compound are principally involved in the reduction and stabilization of AgNPs (Figure 5).

3.2. Larvicidal, pupicidal and adulticidal potential against *A. aegypti:*

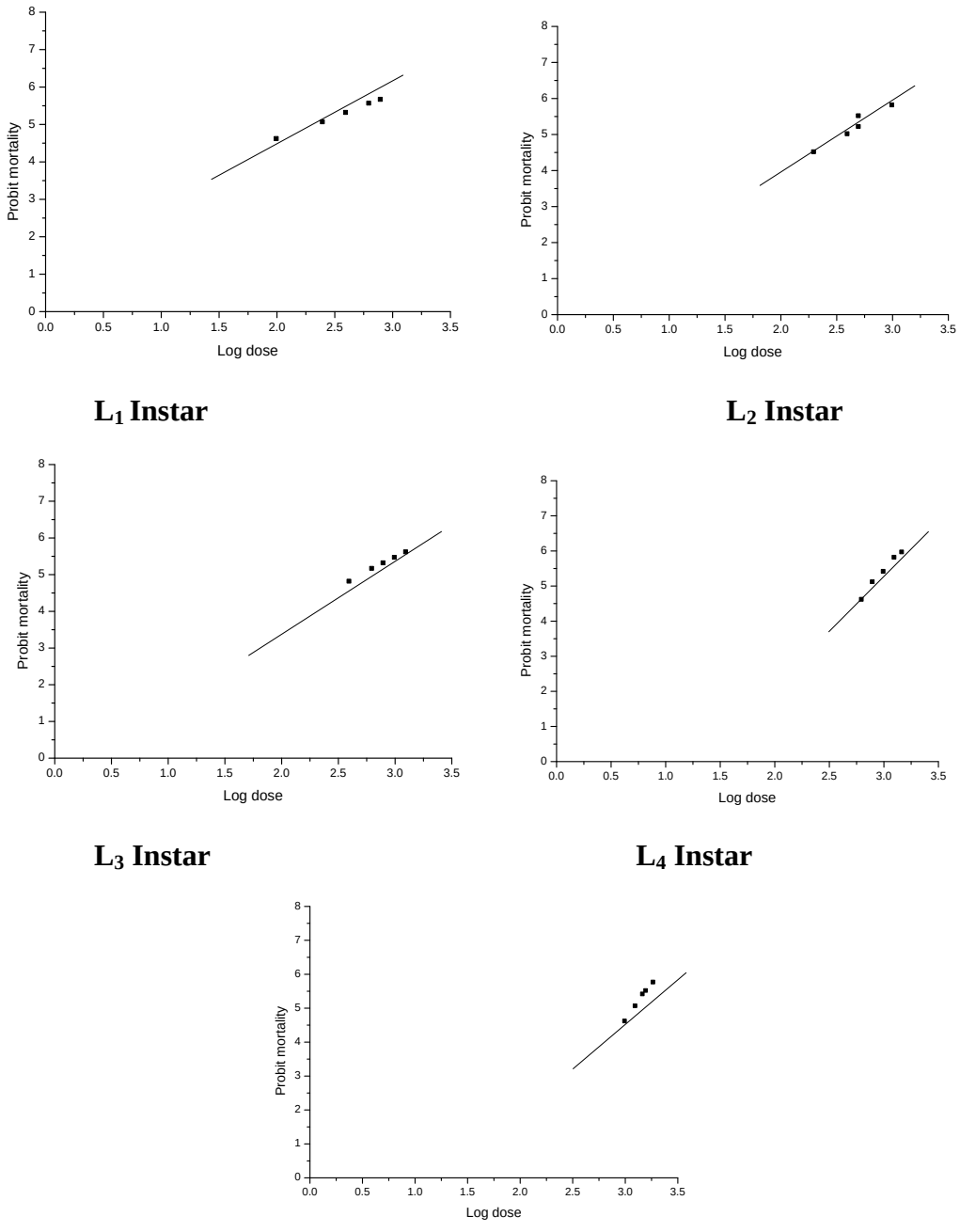

Figure 6: Probit regression line for mortality of *Menyanthes trifoliata* against the different larval instars and pupal stages of *Aedes aegypti.*

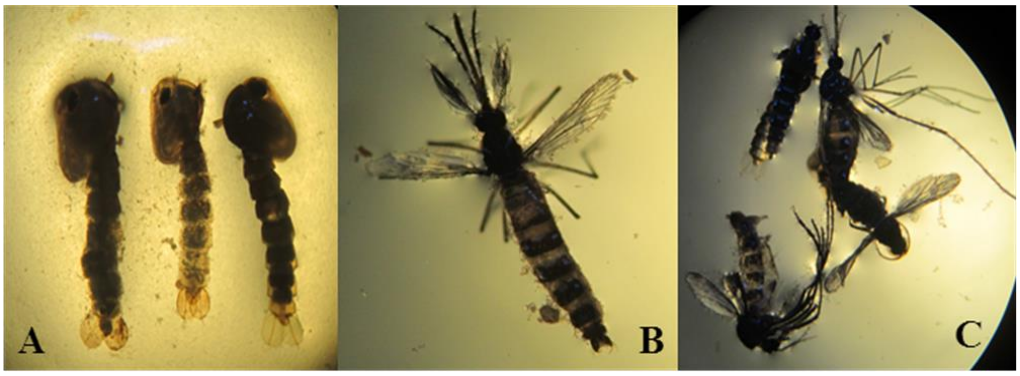

Figure 7: Immature growth stage of *Aedes aegypti* - A) Pupae retain larval character B) Adult retain larval character C) Under development of wings.

Methanolic leaf extract of *M. trifoliate* and AgNPs treated various larval stages (L_1, L_2, L_3 and L_4) and pupae of *A. aegypti* (Figure 6). Considerable mortality at all larval stages were evident post *M. trifoliate* mediated AgNP treatment. The mortality based on concentration dependent. The percentage mortality of L_1, L_2 instars was 100% at 25 µg/mL of higher concentration and it was further observed and remarkable mortality in L_3 and L_4 instars. Similar trends were noted for all the instars including pupae. The effect on larval mortality was concentration dependent. The plant-mediated AgNPs had LC_{50} and LC_{90} values of 8.17 (19.57), 9.78 (24.25), 12.96 (29.64), 16.73 (36.95), and 20.11 (41.81) for L_1, L_2, L_3 and L_4 and pupae, respectively. The result revealed that the mortality rate increased as the exposure concentration was increased, and the larvae also underwent melanization slowly. The regression equation values were -0.919 (0.112), -0.867 (0.089), -0.996 (0.077), -1.061 (0.063) and -1.188 (0.059) for L_1, L_2, L_3, L_4 and pupae, respectively, and the curve fitted in the figure. Chi-square values were 5.004, 2.015, 1.731, 1.441 and 0.760 for L_1, L_2, L_3, L_4 instars and pupae treated with AgNPs, respectively.

Table 1: Effect of methanolic leaf extract of *Menyanthes trifoliata* on the insect growth regulatory activity chikungunya vector, *Aedes aegypti*

Conc. (µg/mL)	Mean duration of each instars (Days) Mean ± SE (100 L/expt.)					Total no. of days	Mortality (%)	Total emergency (%)	EI_{50} EI_{90} (%)
	L_1- L_2	L_2-L_3	L_3-L_4	Pupa	Adult				
2	1.6 ±0.3^{ef}	2.8 ±0.29^{e}	5.6 ±0.3^{e}	2.1 ±0.2^{de}	1.8 ±0.5^{f}	12.5 ±1.5^{f}	55	45	1.17 (9.74)
4	1.5 ±0.4^{de}	3.1 ±0.32^{d}	5.5 ±0.4^{d}	2.6 ±0.3^{d}	2.1 ±0.2^{de}	14.8 ±1.7^{de}	62	38	
6	1.8 ±0.5^{d}	3.6 ±0.31^{c}	6.1 ±0.3^{cd}	3.1 ±0.4^{bc}	2.6 ±0.3^{d}	17.2 ±1.7^{d}	70	30	
8	2.3 ±0.3^{b}	3.8 ±0.33^{b}	6.3 ±0.2^{c}	3.3 ±0.3^{b}	3.1 ±0.1^{bc}	18.8 ±1.5^{c}	80	20	
10	2.8 ±0.3^{b}	4.1 ±0.43^{a}	7.3 ±0.1^{b}	3.3 ±0.1^{b}	3.3 ±0.4^{b}	20.8 ±1.6^{b}	85	15	
Control	1.13 ±0.3^{a}	2.18 ±0.3^{a}	3.14 ±0.3^{a}	4.8 ±0.3^{a}	1.3 ±0.3^{a}	12.58 ±1.5^{a}	5	95	

Within the column means followed by the same letter(s) are not significantly different at 5% level of DMRT**; L_1, L_2, L_3, L_4**: Larval Instars; **EI**: Emergency Inhibition.

Table 2: Effect of *M. trifoliata* synthesized silver nanoparticles on the insect growth regulatory activity of chikungunya vector, *Aedes aegypti*

Conc. (µg/mL)	Mean duration of each instars (Days) Mean ± SE(100 L/ expt.)					Total no. of days	Mortality (%)	Total emergency (%)	EI_{50} EI_{90} (%)
	L_1- L_2	L_2-L_3	L_3-L_4	Pupa	Adult				
2	2.16±0.3^{d}	3.33±0.22^{e}	6.33±0.30^{e}	2.66±0.31^{e}	3.16±0.43^{e}	17.64±1.6^{f}	70	30	1.87 (10.58)
4	2.33±0.3^{d}	4.16±0.17^{d}	7.16±0.27^{d}	3.33±0.31^{c}	4.33±0.31^{d}	21.31±1.5^{de}	80	20	
6	3.16±0.3^{c}	4.83±0.14^{d}	7.66±0.43^{d}	3.50±0.4^{c}	4.5±0.53^{d}	23.65±1.9^{d}	88	18	
8	3.33±0.3^{c}	6.16±0.32^{c}	6.83±0.31^{c}	5.33±0.31^{d}	6.33±0.26^{c}	27.98±1.5^{c}	94	6	
10	4.16±0.3^{a}	7.33±0.21^{b}	8.33±0.33^{b}	4.83±0.5^{b}	5.83±0.29^{b}	30.48±1.7^{b}	98	2	
Control	1.16 ±0.3^{a}	2.16 ±0.3^{a}	3.16 ±0.3^{a}	4.8 ±0.3^{a}	1.3 ±0.3^{a}	12.58 ±1.5^{a}	7	93	

Within the column means followed by the same letter(s) are not significantly different at 5% level of DMRT; **L_1, L_2, L_3, L_4**: Larval Instars; **EI:** Emergency Inhibition.

Table 1 provides the effects of methanolic leaf extract on IGR activity of dengue vector *A. aegypti*. When *M. trifoliata* was at 2 µg/mL, the adult emergence was raised to 12.5 days; at 4 µg/mL, the adult emergence was raised to 14.8 days and when it was further increased at 10 ppm, the adult emergence was raised to 20.8 days. In control settings, the adult emerged at 12.58 days. The EI_{50} (EI_{90}) values were 1.17 (9.74). Similarly, when AgNPs were treated at 2 µg/mL, the adult emerged at 17.64 days, and at 10 µg/mL, the adult emerged at 30.48 days. The EI_{50} (EI_{90}) value

was 1.87 (10.58). The emergence of larval, pupal and adult stages was the maximum extended in the treated group than in the control group.

Table 3: Adulticidal activity of the *M. trifoliata* leaf extract and green-synthesized silver nanoparticles against the dengue vector, *Aedes aegypti*

Treatment	Conc. (µg/mL)	Mortality (%)	LC_{50} (µg/mL) (LCL-UFL)	LC_{90} (µg/mL) (LCL-UCL)	Regression equation	χ^2 (*d.f* = 4)
M. trifoliate extract	Control 5 10 15 20 25	0.0±0.0[a] 24.5±1.23[b] 38.2±2.73[c] 49.1±1.57[d] 64.7± 2.65[f] 78.6 ±1.90[g]	145.92 (129.43-162.05)	322.31 (286.65-377.73)	*y*= -1.060 +0.007 *x*	0.326 n.s.
Ag nanoparticles	Control 5 10 15 20 25	0.0 ± 0.0[a] 37.6± 3.04[c] 49.4 ± 1.92[d] 61.7 ± 2.68[e] 76.3 ± 3.00[g] 89.4± 2.40[h]	14.99 (12.88- 16.64)	31.97 (29.14- 36.28)	*y*= -1.132 +0.075 *x*	1.186 n.s.

No mortality was observed in the control; Mortality is expressed as mean±SD of five replicates; Within a column means followed by the same letter(s) are not significantly different at 5% level by Tukey's HSD test; ***LCL*** = lower confidence limit; ***UCL*** = upper confidence limit; χ^2 = chi-square; ***d.f*** = degrees of freedom; **n.s**. = not significant (α = 0.05).

Table 4: Effect of *M. trifoliata* and synthesized silver nanoparticles on *Aedes aegypti* at different period of exposure.

Exposure interval (Week)	Concentration (ppm)			
	M. trifoliate leaf extract		Ag nanoparticles	
	0	25	0	25
2	2.5±2.5 [b]	70±2.88 [i]	2.5±2.5 [b]	100±2.5 [bd]
4	20±9.12 [d]	68.5±6.29 [h]	25±8.6 [de]	94.5±4.78 [b]
6	15±15 [c]	57.5±4.78 [g]	15±15 [c]	95±5.0 [b]
8	2.5±2.5 [b]	50±17.55 [f]	5±2.88 [b]	100.5±2.5 [bc]
10	0±0 [a]	50.5±2.5 [e]	0±0 [a]	95±2.88 [b]
12	0±0 [a]	32±6.45 [d]	0±0 [a]	100±0.0 [a]

No mortality was observed in the control; Mortality is expressed; as Mean±SD of five replicates.

In the present study, the adulticidal potential of *M. trifoliata* green synthesized AgNPs against *A. aegypti* showed LC_{50} and LC_{90} values of 14.99 µg/mL and 31.97 µg/mL respectively (Table 3). A persistence study was also conducted with 25 µg/mL of AgNPs against the L_3 instar of *A. aegypti*. Mortality was observed at 24 h interval. Larval mortality increased 100% over two-week concentration of persistent period and it remained unchanged up to the 12-week persistent period of being treated AgNPs (Table 4). Similarly, larval mortality 32 % was observed at 2 week persistent period. A notable observation was that plant extract exposure over a longer period of time resulted in the reduction of its toxic nature, but AgNPs' toxicity was higher. It was also evident in the present study that its toxic nature remained stable.

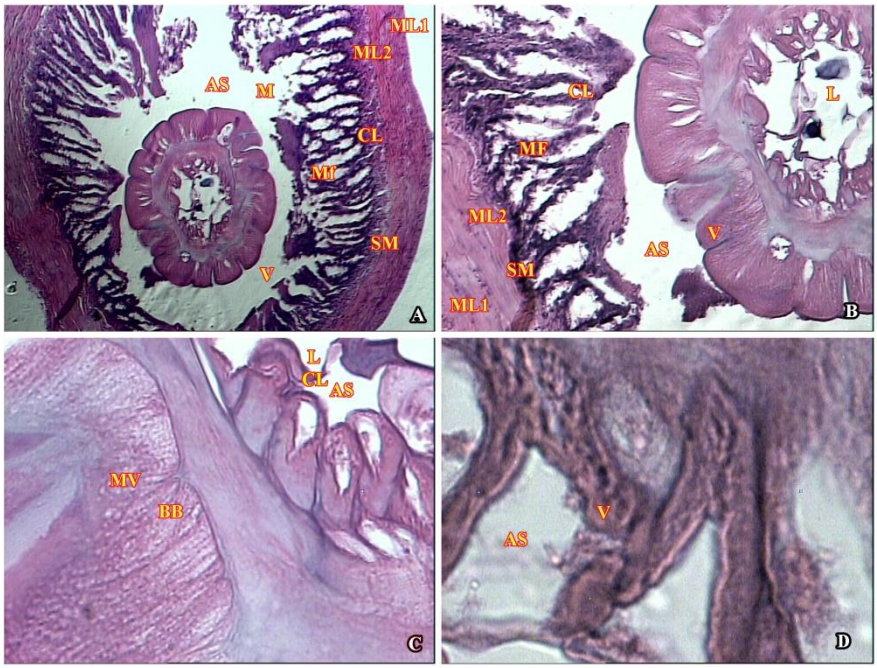

Figure 8. Histological section of midgut of *A. aegypti* in control group showing flattened a), elongated (b) and pyramidal epithelial cells. L, lumen. Hematoxylin-eosin staining. Magnification: 10, 20, 40, 100X view; ML1- Outer Membrane Layer; ML2-Inner Membrane Layer; AS-Abdominal Segment; M-Mitochondria; CL-Cytoplasmic Layer; MF-Muscle Fibres; SM-Sinus membrane; V-Vacuoles; L-Lumen; MV-Microvillus; BB-Brush Border.

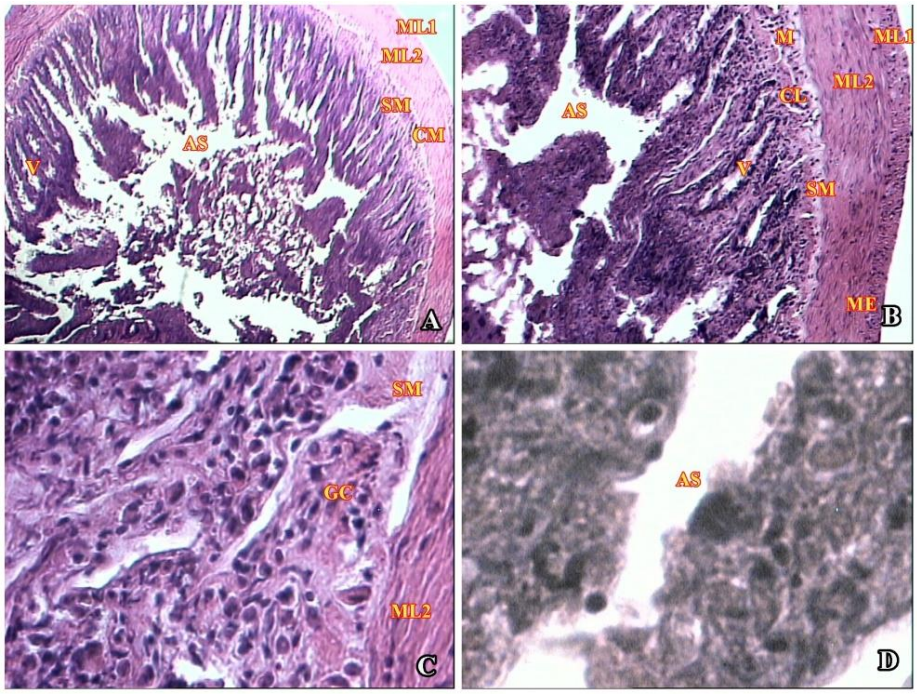

Figure 9. Histological section of midgut of *A. aegypti* in treated group showing flattened a), elongated (b) and pyramidal epithelial cells. L, lumen. Hematoxylin-eosin staining. Magnification: 10, 20, 40, 100X view; ML1- Outer Membrane Layer; ML2-Inner Membrane Layer; AS-Abdominal Segment; M-Mitochondria; CL-Cytoplasmic Layer; MF-Muscle Fibres; SM-Sinus membrane; V-Vacuoles; L-Lumen; MV-Microvillus; BB-Brush Border.

The histology study shows that the mosquito larval midgut was greatly influenced on the plant mediated silver nanoparticles. The larval midgut layer was disturbed due to the action of toxic compound found in the plant. The

concentration was increased and the midgut damages are also increased. In our experiment, the normal larval midgut was clearly seen intestinal cell nucleus, intestinal inner and outer membrane and food vacuole. Whereas, in experimental sets these all the parts are damaged. This was due to toxic chemicals present in the plant compound.

4. DISCUSSION

The indiscriminate use of synthetic insecticides in public health programs for the control of insect species has created various problems: insecticidal resistance, environmental pollution, toxic hazards and effect on non-target organisms. To overcome these problems, different forms of pest control using plant products are currently under development. Studies on natural plant sources such as larvicides have reported it to be a possible alternative to synthetic insecticides.

Since most of the plant-based products are not as effective as synthetic insecticides, their use in large-scale mosquito control programs under epidemic conditions may not be acceptable. Therefore, the use of herbal products should be promoted through community-based vector control programs. In the present study, locally available methanolic extract of *M. trifoliata* mediated AgNPs showed considerable higher larvicidal activity against *A. aegypti.*

It was found that the nanoparticles were spherical in shape, with their edges being lighter than the centers, suggesting that biomolecules such as proteins in *M. trifoliata* capped the AgNPs. This finding is in accordance with the results of Kamalakannan et al., 2014 [38] who reported that proteins bind to nanoparticles through the functional group, increasing the stability of nanoparticles. It was utilized to characterize the particles and their sizes and distribution by taking micrograph from drop-coated films of the silver nanoparticles synthesized by the treatment of silver complex solution with *M. trifoliata* leaf extract for 4 h. It was also found that four hours for the reduction of all silver ions present in solution is insufficient since it has a maximum absorbance much lower than the one obtained after 24 h [39]. Most of the AgNPs in SEM and TEM images are separated by a fairly uniform interparticle distance [40]. Jha et al., 2009 reported that apigenin and kaempferol isolated from *Eclipta* leaf may be responsible for the synthesis of nanoparticles [41]. Similarly, the synthesized AgNPs obtained from the plant compounds of triterpenoid, saponin, and menyanthoside, were clearly distinguishable in size. Most of the nanoparticles were spherical in shape with a size range of 40-60 nm (Figure 2). Moreover, it was also observed that the AgNPs were uniformly distributed on the surface of the pellets. SEM micrographs of the synthesized AgNPs of *M. trifoliata* were magnified at ×4000 and measured at 10 µm.

In the present investigation, the immediate reduction of AgNPs was observed, which might be due to the water soluble phytochemicals such as diterpenoids and flavonoids. This has also been reported in *A. paniculata* [42, 43, 44, 45]. A significant reduction in reaction time was also observed for *M. trifoliata* leaf, which is an important result as it enables this biosynthesis method to be at par with the other plant-assisted biosynthesis routes for AgNPs that are currently much more rapid.

In earlier studies, AgNPs synthesized from *Pedilanthus tithymaloides* were investigated for their efficacy against the dengue vector *A. aegypti* by exposing the larvae to varying concentrations of AgNPs for 24 h [46, 47]. The present study showed AgNPs' 100% mortality rate with regard to L_1 and L_2 instars of *A. aegypti*. LC_{50} and LC_{90} values of AgNPs against the larval stages were found to be higher than those treated with plant extract alone. Similarly, the larvicidal property of AgNPs using *Belosynapsis kewensis* leaf extract against *A. stephensi* and *A. aegypti* (LC_{50} and LC_{90}) were also analyzed. The LC_{50} and LC_{90} values of AgNPs against *A. stephensi* were 78.4 ppm and 144.7 ppm, and those against *A.aegypti* were 84.2 ppm and 117.3 ppm, respectively [48]. Similarly, in the present study, *M. trifoliata* mediated AgNPs achieved LC_{50} and LC_{90} values of 1.131 (0.381), 0.196 (0.507), 0.204 (1.230), 0.212 (1.870) and 0.3507 (4.508) for L_1, L_2, L_3, L_4 instars and pupae respectively.

Acetone leaf extract of *M. tinctoria* was tested for its efficiency against third instar larvae of *A. aegypti*. The larvae were subjected to different concentrations (2 to 24 ppm). Third instar larvae of *A. aegypti* was treated with biosynthesized AgNPs, and the percentage of larval mortality was assessed against various concentrations. The LC_{50} value of synthesized AgNPs was 3.631 ppm [49]. In our present study, 5-25 µg/mL concentration of methanolic leaf extract mediated AgNPs was used, and both L_1 and L_2 instars showed 100% larval mortality at 24 h.

Methanolic leaf extract of *A. vasica* along with AgNPs significantly inhibits adult emergence. The percentage of adult emergence inhibition was 08.0±1.3, 14.0±1.3, 30.0±1.5, 45.5±1.5, 58.0±3.2, 76.0±2.4 and 96.0±2.3 % against the larvae of *Cx. Quinquefasciatus*, respectively, for the concentrations 250, 500, 750, 1000, 1,250, 1,500 and 1,750 ppm. The EI_{50} value was 623.12 ppm, and the EI_{90} value was 1254.35 [50]. In the present study deals with *M. trifoliata* mediated silver nanoparticles are proven record for the significant inhibition of adult emergence. The adult emergence inhibition (EI_{50} = 1.87 and EI_{90} = 10.58) was noted in the table 2.

Earlier study report that adulticidal activity of the essential oil isolated from *Mentha longifolia* against the house mosquito *C. pipiens* was screened with the help of the fumigant toxicity assay by Oz et al. [46]. Similarly, adult mortality due to the effect of ethanol extract on *C. sinensis*, *An. Stephensi*, and *Ae. aegypti* showed LC_{50} and LC_{90} values of 272.19 and 457.14 ppm, 289.62 and 494.88 ppm and 320.38 and 524.57 ppm respectively [56]. Adult mortality due to the effect of methanol extract of *Andrographis paniculata* on *C. quinquefasciatus* and *A. aegypti* showed LC_{50} and LC_{90} values of of 149.81, 172.37 ppm and 288.12, 321.01 ppm respectively [51]. The LC_{50} and LC_{90} values of *A. alnifolia* leaf extracts hexane, benzene, ethyl acetate, acetone and methanol on *C. quinquefasciatus* adults were 383.59, 354.13, 327.74, 314.33 and 291.71 ppm, respectively. *P. nilgiriensis* synthesized AgNPs tested against *A. aegypti* adults achieved LC_{50} and LC_{90} values of 14.27 µg/mL and 25.68 µg/mL respectively [52].

AgNPs in intracellular space can bind to sulphur-containing proteins or phosphorus-containing proteins compounds such as DNA, leading to the denaturation of some organelles and enzymes [38], which leads to the death of the insect [53]. Biosynthesized AgNPs of *An. paniculata* significantly reduced the weight of the animal, 50% concentration showed (687.3±18.1) higher growth than control (606.7±17.5) of the *S. litura*. Our study reports that AgNPs containing plant protein affects the growth and development of *A. aegypti* larvae, pupae and adult. The larval and pupal duration was extended and pupa retains the larval character and wings are under development was noted in the experiment. The persistent study proved that plant mediated AgNPs retain their toxic nature for a long period of time.

In the present histology study, the larva exposed to different concentration of nanoparticle and it may due to toxin production of insecticides may influence to the larval midgut and also arrest the ion transport mechanism as well as nutrient exchange. It's clearly indicated that the dead of the larva due to toxin metabolites produced from the plant. The plant protein present is further believed to cap the AgNPs formed, thereby restricting the agglomeration of the particles and checking their size and shape. Plant synthesized AgNPs may have significant impact on dengue incidence and can be considered to be potential candidates in integrated vector control programs. Our findings conclude that plant mediated AgNPs effectively control larvae and pupae and inhibit adult development, which could significantly reduce parasite transmission and therefore lead to reduced dengue risk.

5. CONCLUSION

The plant protein present is further believed to cap the AgNPs formed, thereby restricting the agglomeration of the particles and checking their size and shape. Plant synthesized AgNPs may have significant impact on dengue incidence and can be considered to be potential candidates in integrated vector control programs. Our findings conclude that plant mediated AgNPs effectively control larvae and pupae and inhibit adult development, which could significantly reduce parasite transmission and therefore lead to reduced dengue risk.

Declaration of interest: None

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