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Research Article

Formulation of *Clitoria ternatea* Leaves-mediated Silver Nanoparticles to Control *Aedes aegypti* Larvae

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A B S T R A C T

Introduction: Global rise in the *Aedes*-borne diseases and harmful effects of synthetic insecticides has diverted research to explore secondary metabolites in plants as mosquito control agent in the form of nanoparticles. Current study investigated *Clitoria ternatea*-mediated nanoparticles against *Aedes aegypti*.

Methods: The aqueous and hexane leaf extracts of *C. ternatea* were assayed against *Ae. aegypti* early fourth instars. The extract-mediated silver nanocomposites (AgNCs) were synthesized after optimizing the volume and concentration of silver nitrate solution. The synthesis was tracked by the colour change of reaction mixture from pale yellow to dark brown followed by monitoring with UV-Visible spectroscopy and Dynamic Light Scattering.

Results: The biosynthesis of 3 mM, 4 mM and 5 mM AgNCs was traced at 438, 401 and 407 nm, respectively. The average particle size distribution ranged from 34.62 to 60.64 nm and polydispersity index was 0.6-0.7. The 24 h larval exposure with aqueous and hexane leaf extracts demonstrated respective LC_{50} values of 53.057 and 42.179 mg/L, which decreased significantly on larvicidal assay with NCs. The 5mM AgNCs showed the maximum efficiency with LC_{50} of 10.317 mg/L after 24 h. Scanning and transmission electron microscopy images demonstrated a spherical, poly-dispersed structure with diameter in the 1-27 nm range. The assays against non-targets; *Moina* and *Cyclops* ascertained the eco-safety of NCs.

Conclusion: The study demonstrated the *C. ternatea* leaf extract as possible effective mosquito nano-larvicide, alternate to traditional insecticides. Field studies, which could not be held due to the current pandemic, would further ascertain the possible use of these NCs against *Aedes* larvae.

Keywords: Larvicide, Nanocomposites, *Clitoria ternatea*, *Aedes aegypti*, DLS, SEM, TEM

Introduction

Aedes aegypti is one of the most important vectors of arboviruses transmitting several diseases in human beings. The climatic conditions, unsystematic urbanization, continuous movement of goods and people across international borders, and ecological disturbance have enhanced the emergence and resurgence of *Aedes*-borne diseases, especially in the tropical and subtropical regions of the Earth. In India, dengue and Chikungunya are the prime and wide-spreading diseases transmitted by *Ae. aegypti*.[1]

Mosquito control is still the key component of mosquito interventive measures due to the lack of effective drugs and commercial vaccines. All control campaigns usually bank upon sanitation, environmental management, spray of pesticides and use of biological control agents. Increasing disease incidences have augmented the dosages as well as application frequency of chemical pesticides against different developmental stages of mosquito.[2,3] The negative effects of these toxicants on the non-target organisms and local settings, along with emergence of resistant strains have raised alarm in the scientific community. Thus, the development of eco-safe and effective tools as mosquito control interventions is of tremendous importance.

The plants are regarded as an important source of potential novel insecticides as the rich and complex mixture of compounds many of which are reported to be highly advantageous against resistance mosquito strains as individual components or in synergistic combinations.[4] The enormous biodiversity of plants around us yet to be explored though various floral species have been investigated for the purpose. The plant-derived products have been recommended in combination with nanotechnology in order to largely enhance their effects owing to their unique properties and also due to prospective uses in a variety of disciplines.[5,6]

The green synthesis of nanocomposites employing diverse biological agents is being favoured over chemical and physical synthesis due to its consistence, utility ease and environment-safety which can be ascribed to the biodegradable nature of the agent and the lack of hazardous ingredients. Specifically, plant-derived silver nanoparticles (AgNPs) have displayed efficient insecticidal activity against mosquitoes at very low concentrations. Several plants, such as *Aloe vera, Achyranthes aspera, Azadirachta indica, Camellia sinensis, Sesbania drummondii, Hevea brasiliensis, Cymbopogon citratus*, etc. have been studied for the production of silver nanocomposites.[7,8] These formulations are cost and time-effective as well as environment-friendly due to non-requirement of high pressure, energy, temperature and even toxic chemicals.[9] Hence nowadays, botanicals and their secondary metabolites, such as flavonoids, tannins, and ascorbic acid, etc. are preferred agents during the formulation of NPs.

Leguminous plant, *Clitoria ternatea* (butterfly pea or Darwin pea) is a perennial herb which has aroused tremendous interest due to its farming and clinical applications, which range from its utilization as a feed and nitrogen-fixing crop, in processed food additives, cosmetics and pharmaceuticals to a rich source of an eco-friendly insecticide. Several chemical constituents, such as various proteins, triterpenoids, flavonol glycosides, anthocyanins and steroids, have been isolated from *C. ternatea*.[10]

Insecticidal properties of *C. ternatea*, however have been attributed to primarily its protein content.[11,12] The application of 1% w/w and 5% w/w of a 20 kDa protein, finotin, isolated from *C. ternatea*, to the bruchids *Acanthoscelides obtectus* and *Zabrotes subfasciatus*, individually, has demonstrated 100% larval mortality.[11] In addition, the cyclotides, the super stable macrocyclic peptides available in all its tissues have also reported to be effective against insects.[13] The growth inhibitory effects of dietary *C. ternatea* cyclotide, Cter M, has been demonstrated in *Helicoverpa armigera*, while 1.0 µmol/g Cter M peptide eating regimen could induce larval mortality.[12] The toxic properties of cyclotides separated from *C. ternatea* have also been demonstrated by other researchers.[14,15] Application of oil-based cyclotide-rich *C. ternatea* fractionalized extract mixture (1-2% v/v) to transgenic and regular cotton crops, led to larval mortality, feeding deterrence and diminished oviposition in *Helicoverpa* spp.[15] Hence, the use of *C. ternatea* concentrates as the useful alternate to synthetic chemicals with no adverse impacts on the beneficial insects has been recommended.

Thus, current studies investigated the larvicidal efficacy of the *C. ternatea* leaf extracts and extract-derived silver nanocomposites against *Ae. aegypti*. The biosynthetic process was optimized by altering different parameters during synthesis, such as $AgNO_3$ (silver nitrate) concentration and volume, temperature, catalyst, and others in order to yield an effective formulation. The green nano-larvicides were characterized and assayed against the early fourth instar of *Ae. aegypti* as well as non-target organisms in order to develop an economical, eco-friendly and effective control formulation.

Materials and Methods

Culture of *Aedes aegypti*

Pure line of dengue fever mosquitoes, *Ae. aegypti*, has been maintained in the Insect Pest and Vector Control Laboratory of Acharya Narendra Dev College, University of Delhi, India, at 28±1° C, 80±5% RH and 14 h of Light and 10 h of dark conditions.[16] Daily meal of adults consists of sugary juice of deseeded raisins, while females are intermittently fed upon rat blood required for egg development. The eggs are gathered in a dechlorinated water-filled ovitrap. The young larvae hatched are developed in trays and fed on

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dog biscuits and yeast (3:1). The pupae captured routinely are kept in cages for adult emergence.[16,17]

Preparation of Crude Plant Extract

The newly emerged and delicate leaves of chosen plant *C. ternatea* (Figure 1) were gathered from the college campus [Latitude, Longitude (28°32'21.0" N 77°15'49.8" E)]. After thorough scrutinization for any disease symptoms, the healthy leaves were washed under faucet water followed by autoclaved water to eliminate all the residues and undesirable apparent particulates. The leaves were extracted in hexane and water for investigations.

The hexane extract was prepared with leaves dried in a safe and concealed area at room temperature (28±1 °C) for 20-25 days and subsequently powdered (Figure 2 a, b). An amount of 20 g powder was absorbed in 100 mL of hexane for 10 days and concentrated under low pressure in a vacuum evaporator at 45 °C.

Simultaneously, the aqueous extract of the *C. ternatea* leaves was prepared with finely chopped and grounded fresh leaves. The 20 g leaf powder was heated at 60 °C in 100 mL of autoclaved water for 30 minutes and left undisturbed for around 3 h. The prepared extract was sieved through muslin fabric followed by filtration through Whatman No. 1 paper (125 mm) to clear the particulate matter. The extract thus formed was kept at 4 °C in amber bottles till next use (Figure 2 c).

Figure 1.*Clitoria ternatea* Plant

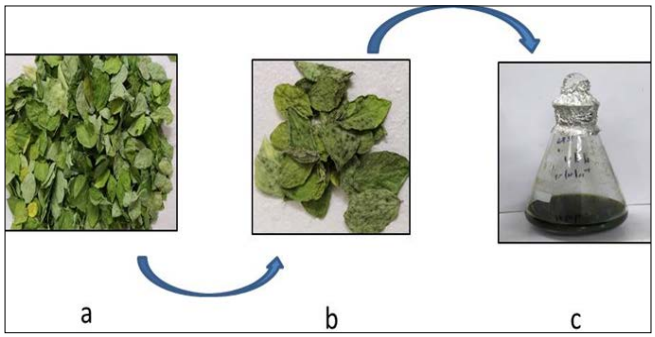

Figure 2.Drying and Extraction of *Clitoria ternatea* Leaves

Formulation of *Clitoria ternatea* Leaf Silver Nanocomposites

The silver nanocomposites (AgNCs) of *C. ternatea* leaf extract were formulated by mixing leaf concentrates and silver nitrate in variable proportions (Figure 3). The 10 mL of the five concentrations of silver nitrate (1, 2, 3, 4 and 5 mM) was mixed individually with different volumes of the leaf extract (0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 mL). The colour of these thirty-five mixtures was monitored as an indication of formulation of nanocomposites.

UV-visible Spectral Analysis of the Silver Nanocomposites Synthesis

The intensity of the 35 mixtures was monitored and analysed after 24 h by UV-Vis spectrophotometry (UV-1800 Shimadzu, Japan) as a confirmation of nanocomposite synthesis. The scanning was conducted in the 200-700 nm frequency range with 1 nm resolution. The silver nitrate solution was taken as the control. Based on the results, three nanocomposites formulated with 10 mL of 3 mM, 4 mM and 5 mM silver nitrate and 2.0 mL volume of plant extract were selected for bulk synthesis.

Bulk Synthesis of *Clitoria ternatea* Leaf Silver Nanocomposites

A volume of 100 mL of silver nitrate (3 mM, 4mM, 5 mM) mixed separately with 20 mL of *C. ternatea* leaf extract was added with 0.5 mL of 1M NaOH (reducing agent). The mixtures were kept at room temperature 37 ± 2 °C in dark for 24 h. The nanocomposites were isolated from the mixture by centrifugation (REMI-C-24) at 12,000 rpm for 15 min at 4°C. The supernatant was collected and centrifuged again at 12,000 rpm. The pellets were dried and were stored for conducting larvicidal bioassays and characterization.

Dynamic Light Scattering (DLS) Analysis

Dynamic Light Scattering (DLS) was used to examine the particle size of the formulated silver nanocomposites. After centrifugation twice at 12,000 rpm, the mixtures were filtered through a 0.22 m Millipore membrane (Millex GV, Merck Millipore). The 1 mL of filtered solution of AgNCs was used for the size measurement and distribution of particles. The average particle size distribution of nanocomposites was analyzed based on intensity, volume and number.

Larvicidal Bioassay with *Clitoria ternatea* Crude Leaf Extracts and Nanocomposites

The larvicidal efficacy of hexane and aqueous leaf extract of *C. ternatea* was estimated against early fourth instars of the susceptible strain of *Ae. aegypti*. The assay was conducted under controlled temperature of 28±1° C, using standard WHO protocol[18] with minor modifications.[19,20] The *Ae. aegypti* larvae were exposed to a range of extract and AgNCs concentrations for 24h and 48h. A total of 20

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early fourth instar larvae of *Ae. aegypti* were exposure to the 200 mL of dechlorinated water-extract/ NC mixture (199:1). The fatality count of the larvae recorded after 24 h was subjected to probit analysis to determine LC_{50} and LC_{90} values.[19] Concurrent control assays were performed with the ethanol. Three replicates were set up for each concentration. Equal number of controls were set up simultaneously with absolute ethanol.

Statistical Analysis

The % larval mortality was calculated in each bioassay. In case of more than 10% pupation in any assay, the test was discarded and repeated. Likewise, in case of control mortality ranging from 5%-20%, the mortality of treated groups was corrected according to Abbott's formula,[21] while the test was rejected if larval mortality was > 20%.

Abbott's Formula:

$$\% \text{ Corrected mortality} = \frac{\% \text{ Test mortality} - \% \text{ Control mortality}}{100 - \% \text{ Control mortality}} \text{ X } 100$$

Lethal concentrations (LC) causing 50% and 90% larval mortality (LC_{50} and LC_{90}, respectively) were calculated from a log dosage-probit mortality regression line using computer software programs SPSS 22.0.[3] In addition, the 95% confidence intervals (CI) of each lethal concentration, and other statistical parameters including slope, standard error (SE), regression coefficient (RC) and chi square values were computed. The LC_{50} values were considered significantly different when their 95 % CI did not overlap.[22]

Scanning Electron Microscopy (SEM)

The aqueous solution of the prepared AgNCs was repeatedly centrifuged. Thin films of the solution were prepared and dried under a mercury lamp for 5 min. The composites were scrutinized under high-resolution scanning electron microscope (Zeiss Model: V5.05) (Sigma) at an accelerating voltage of 20 KeV.

Transmission Electron Microscopy (TEM)

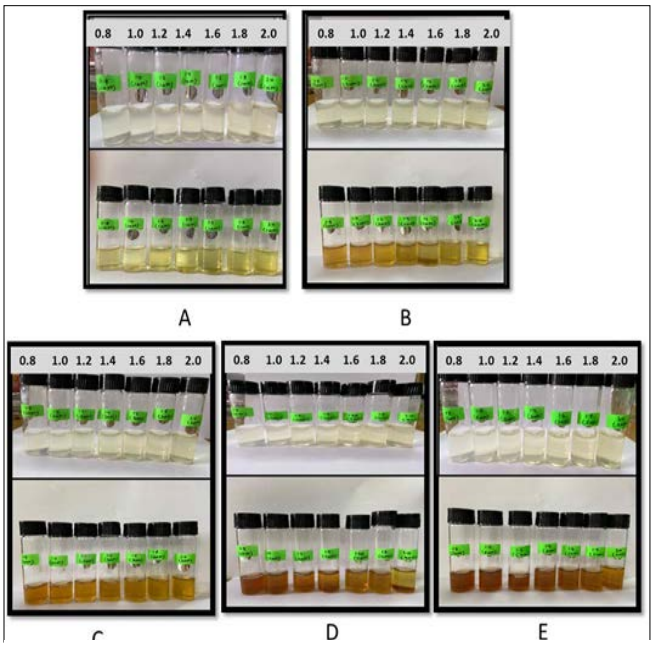
A droplet of *C. ternatea*-nanocomposites was placed on carbon-coated copper grid parafilm for 10-20 min. The structure of AgNCs was also scrutinized under a Transmission Electron Microscope (FEI Tecnai G2 30 S-TWIN) at an accelerating voltage of 300 kV.

Toxicity Assessment of Nanocomposites on the Non-target Organisms

The NCs prepared were assessed for toxic effects on *Gambusia affinis* and *Moina macrocopa*. The 1 mL of AgNCs solution (at LC_{50} value obtained against *Ae. aegypti*) was added to the 499 mL of water to which ten healthy organisms were added. The control set-ups were exposed to dechlorinated tap water. Five replicates were run for each assay. Observations were made after 24 h and 48 h of exposure for lethality and behavioural abnormalities, such as sluggishness and reduced swimming activity. The delayed post-treatment effects were also evaluated till next 5 days.

Results

Figure 3.Preparation of Extract-silver Nitrate Mixtures



A: $AgNO_3$ =1mM; B: $AgNO_3$ = 2mM; C: $AgNO_3$ = 3mM; D: $AgNO_3$ = 3mM: E: $AgNO_3$ = 3mM

Figure 4.Colour Change Observed during Synthesis of Silver Nanocomposites at Constant Volume of *Clitoria ternatea* Leaf Extracts (0.8 to 2.0 mL) with Different Concentrations of Silver Nitrate

Formulation of Silver Nanocomposites with Different Ratios of *C. ternatea* Leaf Extract and Silver Nitrates

The change in the colour of *C. ternatea* leaf extract-silver nitrate mixture indicated the synthesis of silver nanocomposites. The mixtures containing 3 mM, 4 mM and 5 mM of silver nitrate exhibited a noticeable colour change from pale yellow to reddish-brown and finally dark reddish-brown (Figure 4 C-E), while addition of 1 mM and 2 mM silver nitrate did not cause any significant changes (Figure 4 A, 4B). Thus, the mixtures with 1 mM and 2 mM silver nitrate were not considered for further investigations.

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Confirmatory Analysis of the Synthesis of AgNCs

UV-visible Spectral Analysis

The spectra, of the AgNCs formulated with varying volumes of *C. ternatea* leaf extract added to selected concentration of silver nitrate (3nmM, 4mM, 5mM) are presented in Figure 5A which displayed the narrowest and the highest absorbance peak with 2.0 mL *C. ternatea* leaf extract.

The biosynthesis of AgNCs at 3 mM silver nitrate could be traced at 438 nm, while at 4 mM and 5mM, it was held at 401 and 407 nm (Figure 5, Table 1).Relatively wider and less prominent peaks were observed with the lower volumes of the leaf extract and thus were rejected for further assays.

Dynamic Light Scattering (DLS) Analysis of AgNCs

The average particle size distribution of nanocomposites synthesised with 3 mM, 4 mM, and 5 mM silver nitrate was found to be in the range of 34.62-60.64 nm (Table 2, Figure 6); while the polydispersity index was found to be below the desired range of index, i.e. 0.6 to 0.7, indicating the optimal size distribution of nanocomposites. Furthermore, the high photon count rates in each nanocomposite solution suggested that the sample was of good quality and had the best signal for analysis. The optimal signal to noise ratio of the intercept output of dynamic light scattering analysis (0.15 to 0.24) and high percent intensity of 73.5, 82.9, and 80.7 percent further supported the synthesis of nanocomposites under ideal conditions.

Table 1.Spectrophotometric Optimization of Synthesis of Silver Nanocomposites by Varying Volume of *Clitoria ternatea* Leaf Extract (CTLE) Volume and Concentration of Silver Nitrate ($AgNO_3$)

Volume of plant extract	Absorbance (Wavelength in nm) with CTLE in 3 mM of $AgNO_3$ solution	Absorbance (Wavelength in nm) with CTLE in 4 mM of $AgNO_3$ solution	Absorbance (Wavelength in nm) with CTLE in 5 mM of $AgNO_3$ solution
0.8	1.191 (410)	1.323 (401)	1.537 (390)
1.0	1.408 (412)	1.329 (400)	1.541 (394)
1.2	1.328 (395)	1.449 (411)	1.698 (400)
1.4	1.515 (425)	1.578 (427)	1.728 (400)
1.6	1.605 (427)	1.640 (430)	1.856 (405)
1.8	1.752 (435)	1.846 (439)	1.962 (407)
2.0	1.759 (438)	1.917 (440)	2.317 (421)

Table 2.Dynamic Light Scattering Analysis of Average Particle Size Distribution of *Clitoria ternatea* Leaf Extract (CTLE)-mediated Silver Nanocomposites at Different Concentrations of Silver Nitrate

Parameters	Concentrations of silver nitrate (mM)		
	3	4	5
Average size	60.64	39.17	34.62
PdI	0.636	0.451	0.505
Count rate (kcps)	470.3	228.5	414.2
Intercept	0.239	0.155	0.210
Peak 1	44.53	37.42	35.79
Peak 2	32.57	44.58	41.45
Peak 3	81.06	44.36	84.24
% intensity			
Peak 1	73.5	82.9	80.7
Peak 2	18.6	12.3	11.5
Peak 3	7.9	4.8	7.8
St. dev. (d.nm)			
Peak 1	9.12	1.61	12.30
Peak 2	1.70	9.08	10.87
Peak 3	2.67	1.62	2.70

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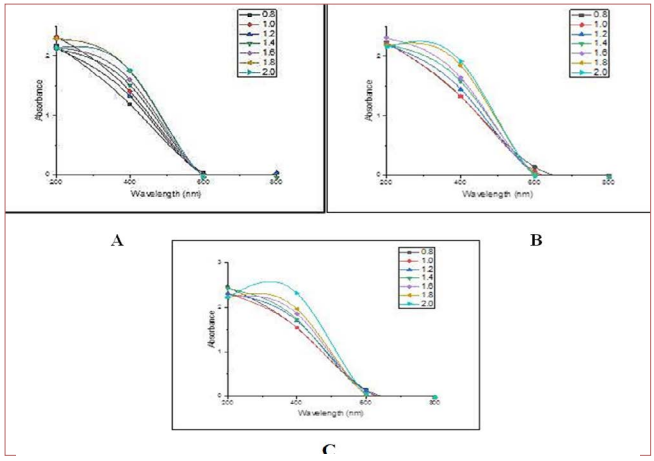

A: $AgNO_3$ = 3mM; B: $AgNO_3$ = 4mM; C: $AgNO_3$ = 5mM

Figure 5.UV-Vis Spectra of Silver Nanocomposites formed with Different Volumes of *Clitoria ternetea* Leaf Extract (0.8 to 2.0 mL) Added to 10 mL of Different Concentrations of Silver Nitrate

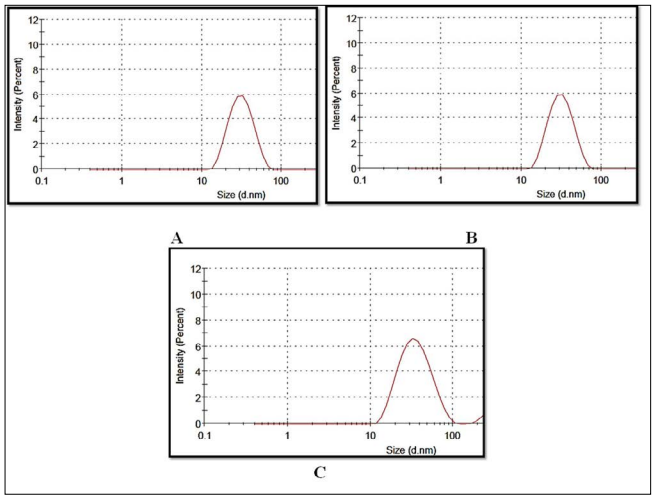

Figure 6.Dynamic Light Scattering Analysis of the Size Distribution of *Clitoria ternatea* Leaf Extract (CTLE) - mediated Silver Nanocomposites at (A) 3 mM, (B) 4 mM and (C) 5 mM Silver Nitrate

Table 3.Larvicidal Bioassay of *Clitoria ternatea* Leaf Extract against early Fourth Instar of *Aedes aegypti* after 24 h of Exposure

Parameters	*Clitoria ternatea* Leaf Extracts	
	Hexane Extract (HE)	Aqueous Extract (AE)
LC_{30} (mg/L)	25.151	34.291
95% Fiducial limits	17.144-36.897	24.157-48.677
LC_{50} (mg/L)	42.179	53.057
95% Fiducial limits	28.751-61.879	37.377-75.316
LC_{90} (mg/L)	149.230	154.170
95% Fiducial limits	101.721-218.929	108.608-218.847
χ^2 (df)	0.241 (5)	0.940 (5)
Slope ± SE	2.361 ± 0.085	2.781 ± 0.078

LC30 - Lethal Concentration at which 30% larvae are killed, LC50 - Lethal Concentration at which 50% larvae are killed, LC90 – Lethal Concentration at which 90% larvae are killed, SE = Standard error. χ2 = chi-square and df = degree of freedom.

Table 4.Larvicidal Bioassay of *Clitoria ternatea* Leaf Extract against Early Fourth Instar of *Aedes aegypti* after 48 h of Exposure

Parameters	*Clitoria ternatea* Leaf Extracts	
	Hexane extract	Aqueous extract
LC_{30} (mg/L)	24.393	26.373
95% Fiducial limits	17.855-33.325	18.306-37.995
LC_{50} (mg/L)	34.527	42.267
95% Fiducial limits	25.273-47.17	29.295-60.804
LC_{90} (mg/L)	80.708	133.166
95% Fiducial limits	59.076-110.262	87.982-182.617
χ^2 (df)	0.879 (5)	0.902 (5)
Slope ± SE	3.491 ± 0.069	2.576 ± 0.081

LC30 - Lethal Concentration at which 30% larvae are killed, LC50 - Lethal Concentration at which 50% larvae are killed, LC90 – Lethal Concentration at which 90% larvae are killed, SE = Standard error. χ2 = chi-square and df = degree of freedom.

Table 5.Larvicidal Bioassay of Silver Nanocomposites of *Clitoria ternatea* Leaf Extract against Early Fourth Instar of *Aedes aegypti* after 24 h of Exposure

Parameters	*Clitoria ternatea* leaf AgNCs		
	3 mM	4 mM	5 mM
LC_{30} (mg/L)	6.101	3.785	2.015
95% Fiducial limits	4.084-9.115	1.867-7.673	0.743-5.469
LC_{50} (mg/L)	10.325	11.396	10.317
95% Fiducial limits	6.911-15.425	5.621-23.105	3.802-27.996
LC_{90} (mg/L)	37.353	168.527	93.056
95% Fiducial limits	25.003-55.803	83.121-341.387	34.293-252.699
χ^2 (df)	0.941 (4)	1.098 (4)	0.703 (4)
Slope ± SEM	2.354 ± 0.089	1.098 ± 0.157	0.756 ± 0.221

LC30 - Lethal Concentration at which 30% larvae are killed, LC50 - Lethal Concentration at which 50% larvae are killed, LC90 – Lethal Concentration at which 90% larvae are killed, SE = Standard error, χ2 = chi-square and df = degree of freedom.

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Larvicidal Efficacy with Crude *Clitoria ternatea* Extracts

The hexane (HE) and aqueous (AE) extract of *C. ternatea* leaves assessed for their larvicidal potential against *Ae. aegypti* early fourth instars displayed higher toxicity of hexane extracts after 24 h and 48 h (Tables 3, 4). The assay with HE resulted in LC_{50} value of 42.179 mg/L after 24 h of exposure which showed 18.14% increased efficacy in next 24 h (Figure 7a). In comparison, the bioassays with the AE against *Ae. aegypti* resulted in 25.79% lower larval toxicity than HE with LC_{50} values of 53.057 and 42.267 mg/L after 24 h (Table 3) and 48 h (Table 4) of exposure, respectively (Figure 7b). The overlapping of the d-m-r lines of HE and AE at 48 h denotes no significant differences at LC_{50} level of dose (Figure 4b) ($p > 0.05$).

Larvicidal Efficacy with *Clitoria ternatea* Leaf AgNCs

The results of the larvicidal potential of AgNCs against *Ae. aegypti* are depicted in Table 4 and 5. The results obtained clearly show the considerable efficacy of all the three AgNCs formulated with different concentration of silver nitrate (3mM, 4mM and 5mM) than the crude extract.

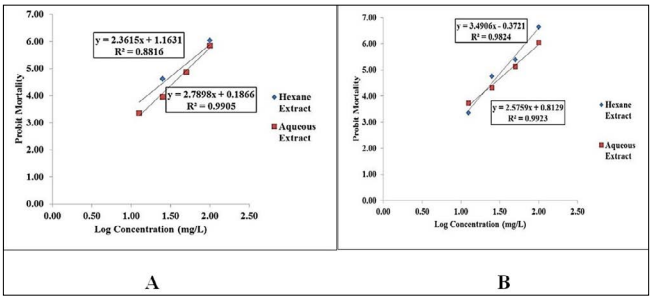

Figure 7.Dosage-mortality Regression Lines on Exposure of *Aedes aegypti* Early Fourth Instars to the Hexane and Aqueous Leaves Extract of *Clitoria ternatea* for A) 24 Hours and B) 48 Hours.

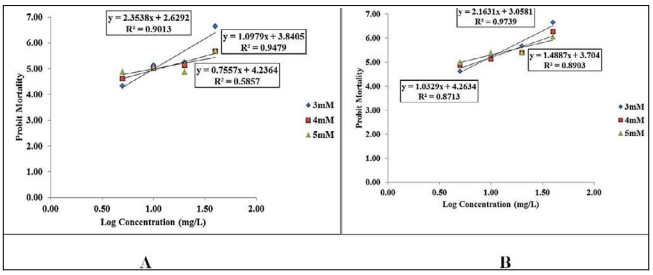

Figure 8.Dosage-mortality Regression Lines on Exposure of *Aedes aegypti* Early Fourth Instars to the Silver Nanocomposites of *Clitoria ternatea* Leaves Extract for a) 24 Hours and b) 48 Hours

The AgNCs (3mM $AgNO_3$) resulted in the respective LC_{50} values of 10.325 and 7.923 mg/L after 24 h and 48 h of larval exposure (Tables 5, 6) displaying 4.1-fold and 4.36-fold higher efficacy than the AE extract. Among the three AgNCs, those with 5mM silver nitrate showed the maximum efficiency with LC_{50} of 10.317 mg/ L after 24 h (Figure 8a) and just 5.174 mg/ L after 48 h of larval exposure (Figure 8b). The overlapping of the d-m-r lines of three AgNCs denotes no significant differences at toxicity level (Figure 8) ($p > 0.05$).

Table 6.Larvicidal Bioassay of Silver Nanocomposites of *Clitoria ternatea* Leaves Extract against Early Fourth Instar of *Aedes aegypti* after 48 h of exposure

Parameters	Clitoria ternatea leaf AgNCs		
	3 mM	4 mM	5 mM
LC_{30} (mg/L)	4.655	2.282	1.588
95% Fiducial limits	2.987-7.254	1.069-4.869	0.719-3.508
LC_{50} (mg/L)	7.923	7.220	5.174
95% Fiducial limits	5.084-12.348	3.383-15.408	2.342-11.429
LC_{90} (mg/L)	31.331	22.847	16.857
95% Fiducial limits	20.105-48.824	10.706-48.755	7.631-37.236
χ^2 (df)	0.993 (4)	0.954 (4)	0.963 (4)
Slope ± SEM	2.163 ± 0.098	1.050 ± 0.168	1.033 ± 0.176

LC30 - Lethal Concentration at which 30% larvae are killed, LC50 - Lethal Concentration at which 50% larvae are killed, LC90 – Lethal Concentration at which 90% larvae are killed, SE = Standard error. $\chi 2$= chi-square and df = degree of freedom.

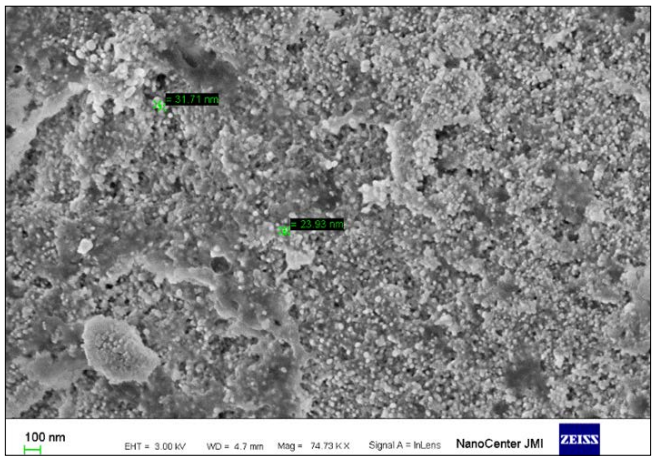

Figure 9.Scanning Electron Microscopy of *Clitoria ternatea* Leaf-nanocomposites

SEM and TEM

The high-resolution SEM of *C. ternatea*-nanocomposites demonstrated spherical for with an average diameter of 27 nm (Figure 9). Most of the nanocomposites were observed in an aggregated form. The TEM characterization of the NCs also displayed the spherical shape of AgNCs with an average size of 16-18 nm with uniform distribution (Figure 10).

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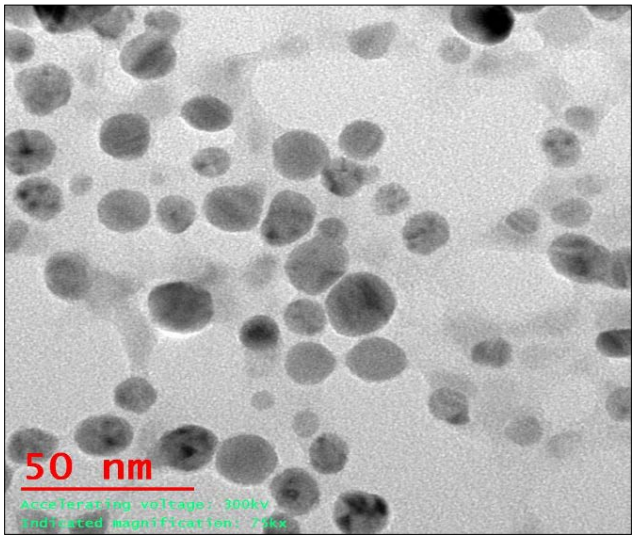

Figure 10.Transmission Electron Microscopy of *Clitoria ternatea* Leaf-nanocomposites

Toxicity Assessment of Nanocomposites on the Non-target Organisms

The evaluation of biotoxicity of *C. ternatea* leaf-nanocomposites AgNCs against *G. affinis* and *M. macrocopa* did not impart any significant toxicity displaying the safety to non-targets of these nanocomposites at concentrations toxic to the mosquito larvae. In addition, prolonged exposure of nontargets for next 5 days did not induce any toxic symptoms or alterations in their longevity and swimming activity.

Discussion

Since past few years, the rise in *Ae. aegypti* - borne diseases has raised widespread alarm. The global occurrence of these diseases has made researchers and vector management organizations alert to explore effective interventions. Despite the numerous control techniques available, dengue vector management has become a tough challenge and primarily depend upon the use of hazardous insecticides. Hence, researchers are now focusing upon plant extracts as an alternative and eco-friendly solution owing to the development of insecticide resistance in the vector and the negative impacts of insecticides on the environment and human health.

The current study investigated the leaves of *C. ternatea* extracted in hexane and water as the larvicidal agent against *Ae. aegypti* early fourth instars. Earlier reports have demonstrated the larvicidal potential of *C. ternatea* extracts possess against different mosquito vectors.[23, 24] The current assays revealed LC_{50} values of 42.179 mg/ L and 53.057 mg/L with *C. ternatea* hexane and aqueous extract, respectively. In contrast, larvicidal assays with methanolic leaf extracts of *C. ternatea* against *An. stephensi* resulted in much higher LC_{50} and LC_{90} values of 555.6 mg/L and 1190.3 mg/L, respectively.[23] On the other hand, the methanolic seed extracts of *C. ternatea* displayed potent and significantly higher larvicidal activity showing LC_{50} and LC_{90} values of 65.2 mg/L and 177.1 mg/L, respectively after 48 h of exposure.[23] A much higher toxicity with the methanolic flower extracts of *C. ternatea* has been reported against early fourth instar larvae of *Ae. aegypti* reporting a toxic level of 1056 mg/L at LC_{50} and 1425 mg/L at LC_{90} dose after 24 hours of exposure which is 24.75 and 10.71 times higher than the dose obtained in the current study.[24]

The synthesis of nanocomposites has engrossed extensive consideration due to their inimitable properties and budding applications.[25] Several approaches have been tried for their creation; nevertheless, as compared to chemical and physical procedures, the green path of synthesis is deemed more simplistic, trustworthy, and eco-friendly. In pharmaceutical and biological applications, production of nanocomposites using environment-friendly materials offer various advantages.[26] Utilization of such nanoparticles in mosquito control interventions has gained great interest. Keeping that in mind, the current study formulated silver nanocomposites from investigated plant, *C. ternatea* with an assumption that nanocomposites will exhibit increased efficiency at much lower concentrations against *Ae. aegypti.* Further, the nanocomposites were formulated with only aqueous extracts as these are considered cost-effective and eco-friendly in comparison to the extracts in organic solvents.[27] The formation of silver nanocomposites in the aqueous solution of *C. ternatea* leaves was confirmed by visual colour change after 24 h of incubation at room temperature. Comparable colour change from pale yellow to brownish has been documented while synthesizing silver nanoparticles from the leaves of *A. aspera*.[8,28] Such change in colour of the reaction mixture is proposed to be positively correlated with $AgNO_3$ concentration in the solution which led to the surface plasmon vibrations.[29,30] In addition, a specific combination of reductant volume, temperature and amount of NaOH resulted in a quick colour shift. UV-Visible spectroscopy of the formulated nanocomposites was conducted as a simple and quick confirmatory tool of the biosynthesis. A narrow, conspicuous and the highest peak of SPR in the absorption spectra of *C. ternatea* nanocomposites formed with 2.0 mL leaf extract was observed at a wavelength ranging from 420-440 nm denoting the maximal silver nitrate reduction. In accordance with our results, the synthesis of silver nanoparticles using aqueous peel extract of *C. ternatea* has been obtained at 420 nm.[31] On the other hand, the UV-Vis spectra of silver nanoparticles generated from the fresh *C. ternatea* flowers resulted in a large surface plasmon peak at 430 nm, similar to our results.[32]

It has been proposed that optimal synthesis of silver nanocomposites is confirmed by the absorbance peak

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values ranging from 400 to 500 nm wavelengths which supports our results.[33] Similar results with the most intense peak at 417 mm was recorded with 4 mM *A. aspera* leaf nanocomposites.[28] Likewise, absorption peaks of silver nanoparticles of *Moringa oleifera* leaf extract was demonstrated in the range of 425-435 nm.[34] Similarly, the absorption spectra of silver nanoparticles of mangosteen leaf extract generated an absorbance peak at 438 nm in reaction medium.[35]

The formulated NCs were also characterized by DLS which measured the hydrodynamic diameter, the diameter of the particle, plus ions or molecules that are attached to the surface and moves with the AgNPs in solution, and % intensity of the AgNCs.[36] The mean diameter of 3mM to 5mM NCs ranged from 34.62-60.64 nm similar to those obtained when the mean size of *Chamaemelum nobile* AgNPs was measured.[37] An average size of 47.3 nm was recorded with a range from 39 nm to 78.5 nm. Likewise, the size of *A. aspera* leaves-derived NPs ranged from 32.48-61.02 nm; while *Ipomea carnea*-mediated AgNPs varied from 30-130 nm.[28,38]

The nanoformulations exhibited 3-4-fold higher larvicidal potential against early fourth instars of *Ae. aegypti* in comparison to the crude extracts of *C. ternatea*, the efficacy augmenting with the concentration of silver nitrate used and the exposure duration. Thus, larval exposure with NCs formed with 5 mM silver nitrate for 48 h imparted the most efficacious toxicity. In contrast, the silver NCs synthesized with 4 mM silver nitrate and *A. aspera* leaf and stem extracts were found most effective against *Ae. aegypti* larvae.[8, 28]

Several reports have recommended the green synthesis of nanocomposites as a control agent of *Ae. aegypti* using different parts of different plants; *Ricinus communis leaves*[39]; *Solanum mammosum* fruits[40]; *Chomelia asiatica* leaves[41], *Catharanthus roseus* roots[42], *Holarrhena antidysenterica* bark[43], to name a few. The nanocomposites made from *C. ternatea* leaves have yet to be tested as a mosquito control agent. As per our knowledge, this is the first study with *C. ternatea* silver nanocomposites against *Ae. aegypti* larvae, though similar NPs have been investigated for possible use against *Escherichia coli, Klebsiella pneumoniae, Salmonella pyogenes*, and *Staphylococcus aureus*.[32]

The biophysical characterization of *C. ternatea* leaf-AgNCs by SEM and TEM revealed their spherical shape, polydispersity and an average diameter of 1-27 nm. Similar results have been reported with other plant-mediated NCs. The formulations from *A. aspera* leaf extracts were spherical ranging from 7 - 14 nm, while that of stem varied from 1-25 nm.[8,28]

The AgNCs synthesized from *C. ternatea* leaf extract also did not impart any lethal effects on the non-target organisms at the concentration lethal to the *Ae. aegypti* larvae. These results are in agreement with the findings of Sharma A et al.[28] who revealed the insignificant toxicity of plant-derived AgNCs against three non-target organisms; *Daphnia magna, G. affinis* and *M. macrocopa*. These results are highly encouraging recommending the use of these NCs in the vector control program. The field studies would further ascertain their use which could not be held due to COVID restrictions in India.

Conclusion

The development of a robust and environmentally acceptable approach, for synthesis of metallic nanoparticles is a vital necessity in the mosquito management programs. Current study formulated *C. ternatea* leaf extracts-mediated silver nanocomposites as a natural, low-cost and eco-safe agent against larvae of *Ae. aegypti*. The synthesized nanocomposites were found considerably effective against the *Ae. aegypti* larvae. The study demonstrated the *C. ternatea* leaf extract as possible effective mosquito nano-larvicide, alternate to traditional insecticides. Field studies, which could not be held due to the current pandemic, would further ascertain the possible use of these NCs against *Aedes* larvae.

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Conflict of Interest: None

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