GREEN SYNTHESIS OF SILVER NANOPARTICLES USING CALOTROPIS GIGANTEA AND THEIR POTENTIAL MOSQUITO LARVICIDAL PROPERTY

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ABSTRACT

In recent years the utilization of secondary metabolites from plant extract has emerged as a novel technology for the synthesis of various nanoparticles. The aim of the present study was to evaluate the effect of plant synthesized silver nanoparticles (AgNPs) using aqueous leaf extract of Calotropis gigantea to control dengue vector Aedes aegypti, malarial vector Anopheles stephensi. The synthesized AgNPs were characterized by UV–vis spectrum, scanning electron microscopy (SEM), Energy-dispersive X-ray spectroscopy (EDX), Fourier transform infrared spectroscopy (FTIR). Synthesized silver nanoparticles (AgNPs) particles were confirmed by analysing the excitation of surface plasmon resonance (SPR) using UV–vis spectrophotometer at 410 nm. SEM analysis of the synthesized Ag NPs clearly showed the clustered and irregular shapes, mostly aggregated and having the size of 20–35 nm. The chemical composition of elements present in the solution was determined by energy dispersive spectrum. The FTIR analysis of the nanoparticles indicated the presence of proteins, which may be acting as capping agents around the nanoparticles. Biosynthesis of nanoparticles may be triggered by several compounds such as carbonyl groups, terpenoids, phenolics, flavonones, amines, amides, proteins, pigments, alkaloids and other reducing agents present in the biological extracts. These results suggest that the synthesized Ag NPs have the potential to be used as an ideal eco-friendly approach for the control of the A. aegypti and A. stephensi. This method is considered as
a new approach to control vectors. Therefore, this study provides first report on the mosquito larvicidal activity of synthesized Ag NPs against vectors.

**Keywords:** Calotropis gigantea, Silver nanoparticles, Aedes aegypti, Anopheles stephensi, Larvicidal activity, Green synthesis.

## INTRODUCTION

Malaria is by far the most important insect transmitted disease (Gilles and Warrell, 1993), remaining a major health problem in many parts of the world and is responsible for high childhood mortality and morbidity in Africa and Asia (Kleinschmidt et al., 2000; Pates and Curtis, 2005). In India, malaria is one of the most important causes of direct or indirect infant, child and adult mortality. About 2 million confirmed malaria cases and 1000 deaths are reported annually, although 15 million cases and 20,000 deaths are estimated by WHO South East Asia Regional Office. *Aedes aegypti*, a vector of dengue that carries the arbovirus responsible for these viral diseases, is widely distributed in the tropical and subtropical zones. Chikungunya, a viral disease caused by both *A. aegypti* and *Aedes albopictus*, was recently considered as an important public health problem in India and other countries like Senegal, West Africa (Yamar et al., 2005). The mosquitoes are potential vectors of many diseases, including malaria, filariasis, dengue, brain fever, etc. There is an urgent need to check the proliferation of the population of vector mosquitoes in order to reduce vector borne diseases by appropriate control methods (Kuppusamy and Murugan, 2009). As mosquitoes are water breeders, their larval stages are attractive targets of pesticides, and it is easy to deal with them in this habitat (Rawani et al., 2010). Use of plant extract for the synthesis of nanoparticles could be advantageous over other environmentally benign biological processes by eliminating the elaborate process of maintaining cell cultures. Recently green silver nanoparticles have been synthesized using various natural products like *Ocimum sanctum* (Ahmad et al., 2010), *Pongamia pinnata* (Raut et al., 2010), *Eclipta prostrata* (Rajkumar and Rahuman, 2011), *Annona squamosa* (Naresh Kumar et al., 2011), *Nerium oleander* (Roni et al., 2013). This tends us to search for a new and easily available green reductant. *Calotropis gigantea* (*C. gigantea*) a common medicinal plant in Indian subcontinent, has purgative, alexipharmic anthelmintic, analgesic, anticonvulsant, anxiolytic, sedative, and antipyretic effect (Argal and Pathak, 2006; Chitme et al., 2005) and is used as a treatment for leprosy, leucoderma, ulcers, tumors, piles, and diseases of the spleen, the liver, and the abdomen (Kartikar and Basu, 1994). Variety of components has been isolated from *C. gigantea*, such as cardenolides, cardiac glycosides (Lhinhatrakool and Suthivaiyakit, 2006), flavonoids (Sen et al., 1992), giganticine (a novel nonprotein amino acid) (Pari et al., 1998). The larvicidal properties of leaf extract of *Calotropis procera* against mosquito larvae of *Anopheles stephensi*, *Culex quinquefasciatus*, and *Aedes aegypti* (Singh et al., 2005). However, there is no scientific report on the use of this plant (*C. gigantea*) in synthesis of silver nanoparticles and its insecticidal activity. In the present study will be the primary report using aqueous leaf extract and synthesized silver nanoparticles of *C. gigantea*. Since, this plant-based, water miscible silver nanoparticle that is rapidly synthesized, cost-effective, and is non-hazardous to human and other organism was examined for antimosquito activity for the first time against dengue vector *A. aegypti*, malarial vector *A. stephensi*.

## MATERIALS AND METHODS

### Materials

Fresh leaves of *C. gigantea* were collected from campus of Bharathiar University, India. The plants were identified by the Taxonomist, Department of Botany, Bharathiar University, Coimbatore, Tamil Nadu, India. The voucher specimen was numbered and kept in our research laboratory for further reference. AgNO₃, were purchased from the Precision Scientific Co, (Coimbatore) and used without further purification.

### Synthesis of silver nanoparticles

Leaves were washed with distilled water and dried for 2 days at room temperature. A plant leaf broth was prepared by placing 10 g of the leaves (finely cut) in a 300mL flask with 100mL of sterile distilled water. This mixture was boiled for 5 min, decanted, stored at - 4°C, and used in our tests within 1 week. The filtrate was treated...
with aqueous 1 mM AgNO₃ solution in an Erlenmeyer flask and incubated at room temperature. Formation of AgNPs was indicated by the brown-yellow coloration of the solution suggesting that aqueous silver ions can be reduced by aqueous extract of plant parts to generate extremely stable silver nanoparticles in water.

**Characterization of silver nanoparticles**

Synthesized silver nanoparticles were confirmed by sampling the reaction mixture at regular intervals and the absorption maxima was scanned by UV–vis spectra, at the wavelength of 200–600 nm in UV-3600 Shimadzu spectrophotometer at 1 nm resolution. Further, the reaction mixture was subjected to centrifugation at 15,000 rpm for 20 min, resulting pellet was dissolved in deionized water and filtered through Millipore filter (0.45µm). An aliquot of this filtrate containing silver nanoparticles was used for SEM, EDS and FTIR studies. The structure and composition of freeze-dried purified silver particles was analyzed by using a 10 kV ultra high resolution scanning electron microscope with 25µl of sample was sputter coated on copper stub and the images of nanoparticles were studied using (FEI QUANTA-200SEM). The surface groups of the nanoparticles were qualitatively confirmed by using Fourier transform infrared (FTIR) spectroscopy. Spectral characterization of silver nanoparticles was done by using a 10 kV ultra high resolution scanning electron microscope (EDS).

**Mosquito rearing**

The eggs of *Aedes aegypti* and *A. stephensi* were collected from National Centre for Disease Control (NCDC) field station of Mettupalayam, Tamil Nadu, India. These were returned to the laboratory and transferred (in approximately the same aliquot numbers of eggs) to 18cm L X 13cmW X 4 cm D enamel trays containing 500mL of water where they were allowed to hatch. Mosquito larvae were reared (and adult mosquitoes held) at 27°C±2°C and 75%–85% RH in a 14:10 (L: D) photoperiod. Larvae were fed 5 g ground dog biscuit and brewers yeast daily in a 3:1 ratio. Pupae were collected and transferred to plastic containers with 500mL of water. The container was placed inside a screened cage (90 cm L X 90 cm H X 90 W) to retain emerging adults, for which 10% sucrose in water solution (v/v) was available ad libitum. On day 5 post emergence, the mosquitoes were provided access to a rabbit host for blood feeding. The shaved dorsal side of the rabbit was positioned on the top of the mosquito cage in contact with the cage screen (using a cloth sling to hold the rabbit) and held in this position overnight. Glass Petri dishes lined with filter paper and containing 50mL of water were subsequently placed inside the cage for oviposition by female mosquitoes.

**Larval/pupal toxicity test**

Twenty-five larvae (instars 1-4) or pupae were placed in 249mL of de-chlorinated water in a 500mL glass beaker, and 1mL of the desired concentration of silver nanoparticles was added. In fact, 0.5mg larval food was provided for each test concentration. Tests of each concentration against each instar and the pupae were replicated thrice. In each case, the control comprised 25 larvae or pupae in 250mL of distilled water. Control mortality was corrected by using Abbott’s formula (Abbott, 1925) and percentage mortality was calculated as follows:

\[
\text{Percentage mortality} = \frac{\text{Number of dead larvae/pupae}}{\text{Number of larvae/pupae introduced}} \times 100
\]

The LC₅₀ and LC₉₀ were calculated from toxicity data by using probit analysis (Finney, 1971).

**Statistical analysis**

The average larval, pupal and adult mortality data were subjected to probit analysis for calculating LC₅₀, LC₉₀ and other statistics at 95 % fiducial limits of upper fiducial limit and lower fiducial limit, and Chi-square values were calculated using the SPSS Statistical software package 16.0 version was used. Results with \( P<0.05 \) were considered to be statistically significant.

**RESULTS AND DISCUSSION**

**Spectral characterization**

**UV–vis spectrum of silver nanoparticles**

Reduction of silver ions in the aqueous solution of silver during the reaction with the ingredients present in the plant leaf extract was observed by the UV-vis spectroscopy. The change in color was noted by visual observation in the *C. gigantea* leaf extract when it was incubated with AgNO₃ solution. *C. gigantea* leaf extract without AgNO₃ did not show any change in color (Figure 2a). The color of the extract changed to
light brown within an hour and then later changed to dark brown during the 2hrs incubation period. No significant change occurred after 2hrs. The brown color could be due to the excitation of surface plasmon vibrations, typical of the silver nanoparticles (Ahmad et al., 2003; Krishnaraj et al., 2010). Figure 2b displays the UV–vis spectra of leaf solution as a function of reaction time. The strong resonance centered at 410 nm was clearly observed and increased in intensity with time. It might arise from the excitation of longitudinal plasmon vibrations in silver NPs in the solution. Reduction of silver ions present in the aqueous solution of silver complex during the reaction with the ingredients present in the leaves of C. gigantea extract observed by the UV–vis spectroscopy revealed the presence of silver nanoparticles may be correlated with the UV–vis spectra. These changes were attributed to the excitation of surface plasmon resonance (SPR) in the metal nanoparticles (Natarajan et al., 2010). Ag nanoparticles were observed to be stable in solution and show very little aggregation. Besides, the plasmon bands were broadened with an absorption tail in the longer wavelengths, which may be due to the size distribution of the particles (Ahmad et al., 2003).

FT-IR spectra

FTIR spectroscopy analyses were carried out to identify the biomolecules responsible for capping of the bioreduced AgNPs synthesized using plant extract. The FTIR spectra of aqueous silver nanoparticles prepared from the C. gigantea leaf extract (Figure 3) show transmittance peaks at 612.28 and 866.84 (C–H bend alkenes), 1114.65 (C–O stretch alcohols), 1384.64 (CH\textsubscript{2} bend alkenes), 2923.56 (C–H stretch alkenes) and 3443.28 (O–H stretching due to alcohols group). These peaks indicate that the carbonyl group formed amino acid residues and that these residues “capped” the silver nanoparticles to prevent agglomeration, thereby stabilizing the medium (Sathyavathi et al., 2010). When the metal nanoparticles form in solution, they must be stabilized against the van der Waals forces of attraction which may otherwise cause coagulation. Physisorbed surfactant and polymers may cause steric or electrostatic barriers or purely electrostatic barriers around the particle surface and may thereby provide stabilization (Muluveney, 1996). FTIR peaks that were corresponding to aromatic rings, geminal methyls, and ether linkages indicate the presence of flavones and terpenoids responsible for the stabilization of the AgNPs synthesized by the Sesuvium portulacastrum leaf extract (Nabikhan et al., 2010).

Morphological characterization

Scanning electron micrographs enabled visualization of the size and shape of the silver nanoparticles (Figure 4). SEM analyses of the synthesized AgNPs clearly showed clustered and irregular shapes, mostly aggregated and having an average size of 20–35 nm with interparticle distance, which magnified at ×20,000 and ×30,000 times. The rough morphology of the NPs provides excellent larvicidal activity for the synthesized nanoparticles. In addition, the SEM image shows that the “capped” silver particles were stable in solution for at least 8 weeks. The energy-dispersive X-ray spectroscopy (EDX) attachment present with the SEM was known to provide information on the chemical analysis of the fields being investigated or the composition at specific locations (spot EDX). Figure 4c shows a representative profile of the spot EDX analysis, obtained by focusing on AgNPs. Silver nanoparticles were synthesized using leaf extract of Acalypha indica from the control silver nitrate obtained was greater than 1,000 nm size, whereas synthesized silver nanoparticles measured 20–30 nm in size (Sathyavathi et al., 2010). SEM analysis showed the particle size between 25 and 110 nm as well as the cubic structure of the nanoparticles (Khandelwal et al., 2010).

Larvicidal activity of synthesized AgNPs

In the present study was carried out to establish the larvicidal activity of synthesized silver nanoparticles (AgNPs) using leaf extract of C. gigantea (Apocynaceae) against the first-fourth instar larvae and pupae of A. aegypti and A. stephensi was determined (Table 1 and Figure 1). Range of concentrations of synthesized AgNPs (50, 25, 12.5, 6.25, 3.125 ppm) and aqueous crude latex (250, 200, 150, 100, 50 ppm) were tested against larvae of A. aegypti and A. stephensi. The synthesized AgNPs from C. gigantea were highly toxic than aqueous leaf extract in both mosquito species. The comparative LC\textsubscript{50} and LC\textsubscript{90} values of synthesized AgNPs against the first to fourth instar larvae and pupae of A. aegypti (LC\textsubscript{50}= 7.18 ppm, 5.93 ppm, 11.60 ppm, 17.56 ppm and 28.87; LC\textsubscript{90} = 24.33 ppm, 34.01 ppm, 51.92 ppm, 63.38 ppm and 83.88 ppm against the larvae and pupae
of A. stephensi (LC₅₀ = 4.24 ppm, 4.98 ppm, 8.84 ppm, 15.31 ppm and 26.35 ppm; LC₉₀ = 17.90 ppm, 27.06 ppm, 38.79 ppm, 57.16 ppm and 80.96 respectively. The possible larvicidal activity may be due to penetration of nanoparticles through membrane. Similarly, the bioactivity of latex-producing plant Pergulariadaemia as well as AgNPs against the larval instars of Aedes aegypti and A. stephensi mosquito larvae was determined, the results AgNPs shows excellent larvicidal activity of first, second and third instar larvae and fourth-instar larvae did not exhibit any noticeable effects after either 24 or 48 h of exposure at their LC₅₀ and LC₉₀ values (Chandrashekar et al., 2012). AgNPs synthesized using Euphorbia hirta plant leaf extract against malarial vector A. stephensi was determined, the highest larval mortality was found in the synthesized AgNPs against the first to fourth instar larvae and pupae of values LC₅₀ (10.14, 16.82, 21.51, and 27.89 ppm, respectively), LC₃₀ (31.98, 50.38, 60.09, and 69.94 ppm, respectively), and the LC₅₀ and LC₉₀values of pupae of 34.52 and 79.76 ppm, respectively (Agalya Priyadarshini et al., 2012).

The efficacy of various concentrations of Pedilanthus tithymaloides aqueous leaf extract (0.2, 0.4, 0.6, 0.8, and 1.0 %) against the developmental stages of dengue vector A. aegypti. The maximum mortality, 40, 36, 32, and 26 %, was observed at 1 % concentration level in all instars and pupa, respectively, and their LC₅₀ values are 1.19, 1.39, 1.77, 1.46, and 1.97 % on treatment with aqueous leaf extract alone (Sundaravadiavel et al., 2012). With regards of previous study the aqueous leaf extract of C. gigantea at various concentrations against dengue vector A. aegypti, malarial vector A. stephensi is given in the Table 2; Figure 1. From the aqueous leaf extract of A. aegypti (LC₅₀=89.04, 109.63, 138.61, 176.82 and 299.49ppm; LC₉₀= 179.37, 227.24, 270.77, 320.49 and 542.35ppm), and against the larvae and pupae of A. stephensi (LC₅₀=86.02, 113.45, 143.98, 175.04 and 280.23ppm; LC₉₀= 182.61, 232.79, 287.30, 326.09 and 519.86ppm) respectively. The chi-square value was significant at p ≤ 0.05 level. The results of larvicidal activity clearly indicates that the percentage of mortality being directly proportional to concentration of the extract. After exposure to the test concentrations, the treated larvae exhibited restlessness, sluggishness, tremors, and convulsions followed by paralysis at the bottom of the bowl. The larvicidal effect of aqueous crude leaf extracts, silver nitrate, and synthesized silver nanoparticles of Mimosa pudica showed that the highest mortality was found in synthesized AgNPs against the larvae of Anopheles subpictus (LC₅₀=8.89, 11.82, and 0.69 ppm) and against the larvae of Culex quinquefasciatus (LC₅₀=9.51, 13.65, and 1.10 ppm) (Marimuthu et al., 2011). To our best of knowledge there is no report in the literature for the control of mosquito opulation by using C. gigantea. This is an ideal eco-friendly approach for the control of against dengue vector A. aegypti, malarial vector A. stephensi.

Table 1. Effect of exposure to C. gigantea aqueous leaf extract on mortality in larvae and pupae of A. aegypti and A. stephensi.

<table>
<thead>
<tr>
<th>Life stages (Instars)</th>
<th>Percentage of larval and pupal mortality</th>
<th>Slope</th>
<th>LC₅₀/LC₉₀</th>
<th>95% confidence limit</th>
<th>( t^2 ) (df=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration (ppm)</td>
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<td></td>
<td></td>
<td></td>
<td>LC₅₀ (LCL)</td>
<td>LC₉₀ (UCL)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>31.8±2.8; 52.4±3.0</td>
<td>81.2±2.6</td>
<td>93.0±1.3</td>
<td>100±1.0</td>
<td>0.354</td>
</tr>
<tr>
<td>II</td>
<td>26.8±2.6; 41.4±2.2</td>
<td>70.6±2.4</td>
<td>85.2±2.0</td>
<td>92.2±1.2</td>
<td>0.349</td>
</tr>
<tr>
<td>A. aegypti</td>
<td>20.4±2.8; 29.8±3.2</td>
<td>72.8±3.2</td>
<td>84.2±3.0</td>
<td>138.61 (270.77)</td>
<td>0.341</td>
</tr>
<tr>
<td>III</td>
<td>12.8±2.2; 20.6±2.6</td>
<td>60.3±3.2</td>
<td>71.2±2.2</td>
<td>176.82 (320.49)</td>
<td>0.314</td>
</tr>
<tr>
<td>IV</td>
<td>9.0±2.0; 16.2±2.4</td>
<td>20.0±2.0</td>
<td>29.8±3.2</td>
<td>40.2±2.8</td>
<td>0.152</td>
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<td>Pupa</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>33.8±2.4; 55.2±2.2</td>
<td>80.2±2.4</td>
<td>91.6±2.6</td>
<td>100±1.0</td>
<td>0.337</td>
</tr>
<tr>
<td>II</td>
<td>25.2±2.0; 41.8±2.6</td>
<td>69.2±3.2</td>
<td>80.2±2.8</td>
<td>93.2±1.2</td>
<td>0.348</td>
</tr>
<tr>
<td>A. stephensi</td>
<td>20.2±2.8; 31.2±2.8</td>
<td>55.8±3.4</td>
<td>71.8±3.0</td>
<td>80.2±2.4</td>
<td>0.321</td>
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<tr>
<td>IV</td>
<td>15.2±2.0; 23.0±3.0</td>
<td>42.2±2.2</td>
<td>63.8±2.4</td>
<td>70.4±2.0</td>
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<td>Pupa</td>
<td>10.2±2.4; 16.2±2.6</td>
<td>26.4±2.2</td>
<td>34.2±2.0</td>
<td>42.0±2.6</td>
<td>0.163</td>
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</tbody>
</table>

Control-nil mortality, LFL - lower fiducial limit; UFL - upper fiducial limit, \( x^2 \) - Chi-square value, df - degrees of freedom, Each value is the mean ± SD of five replicates, *Significant at p < 0.05 level.
Table 2. Effect of exposure to silver nanoparticles on mortality in larvae and pupae of A. aegypti and A. stephensi.

<table>
<thead>
<tr>
<th>Species</th>
<th>Life stages (Instars)</th>
<th>Percentage of larval and pupal mortality</th>
<th>95% confidence limit</th>
<th>( \chi^2 )</th>
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<tr>
<td></td>
<td>Concentration of AgNPs (ppm)</td>
<td></td>
<td>LC(<em>{50}) (LC(</em>{90}))</td>
<td>(LCL  UCL) ( \chi^2 ) (df=3)</td>
</tr>
<tr>
<td></td>
<td>3.125</td>
<td>6.25</td>
<td>12.5</td>
<td>25</td>
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<td>A. aegypti</td>
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<td>40.2±2.1</td>
<td>55.6±3.6</td>
<td>71.2±2.0</td>
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<td>II</td>
<td>38.6±2.0</td>
<td>50.4±2.8</td>
<td>68.2±2.8</td>
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<td>III</td>
<td>33.8±2.8</td>
<td>42.4±5.5</td>
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<td>IV</td>
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<td>39.4±2.0</td>
<td>48.6±2.2</td>
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<td>Pupa</td>
<td>20.8±2.6</td>
<td>30.2±2.4</td>
<td>39.4±2.1</td>
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<tr>
<td>A. stephensi</td>
<td>I</td>
<td>43.6±2.0</td>
<td>55.4±2.6</td>
<td>84.6±1.2</td>
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<td>II</td>
<td>40.8±2.2</td>
<td>53.4±3.1</td>
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<td>III</td>
<td>35.6±2.6</td>
<td>44.2±2.8</td>
<td>59.6±3.2</td>
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<td>IV</td>
<td>30.6±3.0</td>
<td>38.2±2.1</td>
<td>49.6±2.5</td>
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<td></td>
<td>Pupa</td>
<td>25.4±2.0</td>
<td>30.8±2.8</td>
<td>41.2±3.0</td>
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</table>

Control-nil mortality, ‘LFL’ - lower fiducial limit; UFL - upper fiducial limit, \( \chi^2 \) – Chi-square value, df- degrees of freedom, Each value is the mean ± SD of five replicates, *Significant at \( P < 0.05 \) level

Figure 1. Larval and pupal toxicity effect of C. gigantea aqueous leaf extract and synthesized AgNPs against Aedes aegypti, A. stephensi.
Figure 2. *a* Aqueous solution of AgNO₃ with *C.gigantea* leaf extracts (left) before adding the leaf extract and (right) after addition of leaf extract at 120 min. *b* UV–Vis spectra of aqueous silver nitrate with *C.gigantea* leaf extract at different time intervals.

Figure 3. FTIR spectra of vacuum-dried powder of synthesized AgNPs from *C.gigantea* leaf extract.
Figure 4. SEM micrograph showing the morphological characteristics of silver nanoparticles synthesized using leaves of *C. gigantea* leaf extract. **a.** Lower magnification. **b.** Higher magnification. **c.** EDX showing the chemical constituents of the synthesized nanoparticles.
CONCLUSIONS
In conclusion, the presented green synthesis shows that the environmentally benign and renewable source of *C. gigantea* can be used as an effective reducing agent for the synthesis of AgNPs. This biological reduction of metal would be boon for the development of clean, nontoxic, and environmentally acceptable green approach to produce metal nanoparticles, involving organisms even ranging to higher plants. The formed Ag NPs are highly stable and have significant larvicidal activity.

CONFLICT OF INTEREST STATEMENT
Authors declare that they have no conflict of interest.

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