

One-step synthesis of polydispersed silver nanocrystals using *Malva sylvestris*: an eco-friendly mosquito larvicide with negligible impact on non-target aquatic organisms

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Abstract The synthesis of eco-friendly nanoparticles is ever-green branch of nanoscience with a growing number of bio-medical implications. In this study, we investigated the synthesis of polydisperse and stable silver nanoparticles (AgNP) using a cheap leaf extract of *Malva sylvestris* (Malvaceae). Bio-reduced AgNP were characterized by UV-visible spectrophotometry, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction analysis (XRD), atomic force microscopy (AFM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The acute toxicity of *M. sylvestris* leaf extract and green-synthesized AgNP was evaluated against larvae of the malaria vector *Anopheles stephensi*, the dengue vector *Aedes aegypti* and the filariasis vector *Culex quinquefasciatus*. Compared to the leaf aqueous extract, AgNP showed higher toxicity against *A. stephensi*, *A. aegypti*, and *C. quinquefasciatus* with LC₅₀ values of 10.33, 11.23, and 12.19 µg/mL, respectively. Green-synthesized AgNP were found safer to non-target organisms *Diplonychus indicus* and *Gambusia affinis*, with respective LC₅₀ values ranging from 813.16 to 1044.52 µg/mL. Overall, this research firstly shed light on the mosquitocidal potential of *M. sylvestris*, a potential bio-resource for rapid,

cheap and effective synthesis of polydisperse and highly stable silver nanocrystals.

Keywords Biosafety · Biosynthesis · Mosquito-borne diseases · Nanotechnology · Zika virus

Introduction

Nanotechnology is a developing interdisciplinary field of research interspersing material science, bionanoscience, and technology. Remarkable advances are made in the field of biotechnology and nanotechnology to harness the benefit of life sciences, health care, and industrial biotechnology (Gardea-Torresdey et al. 2002; Lee et al. 2003; Benelli 2016; Murugan et al. 2016a, b, c). A reliable and eco-friendly process for synthesis of metallic nanoparticles is an important step in the field of nanotechnology. Many materials are synthesized in nano-size for various applications including medicine, engineering, biomedical electronics, and pest management (Kalimuthu et al. 2008; Smitha et al. 2008). Metals are commonly used for the synthesis of nanoparticles relying to chemical and biological methods. However, chemical methods usually involve use of highly toxic chemicals for synthesis of nanoparticles, which makes them certainly unsuitable for a number of purposes of public health importance. An alternative, eco-friendly, and advantageous approach to chemical methods is the biological nanosynthesis (Benelli 2016).

Recently, biological synthesis of silver nanoparticles has received a special attention due to its cheap and eco-friendly features. Current research shows that rapid green synthesis of metal nanoparticles can be easily performed using metabolites from bacteria, fungi, and plants. In particular, plant-mediated synthesis has been successfully applied for the production of silver nanoparticles, due to its faster rate of synthesis and also

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the possibility to reduce the steps in downstream processing (Huang et al. 2007; Salam et al. 2012).

Mosquitoes (Diptera: Culicidae) are vectors of high public health importance. *Anopheles stephensi*, *Aedes aegypti*, and *Culex quinquefasciatus* are vectors of many pathogens and parasites accounting for huge mortality and morbidity worldwide, including malaria, dengue, Zika virus, Japanese encephalitis and filariasis (Benelli and Mehlhorn 2016; Benelli et al. 2016). The better strategy to lower the incidence of mosquito-transmitted diseases is to avoid mosquito biting using skin repellents, insecticide-treated bed nets, and targeting mosquito young instars with pesticides (Benelli 2015a). In larval stage, mosquitoes have less mobility in breeding habitat, so devising control measures at this stage is comparatively easy (Howard et al. 2007). Current practice aimed to the control of mosquito larvae mainly relies to the use of synthetic pesticides carbamates, organophosphates, and pyrethroids. In the early days of their use, insecticides showed success in reducing vector population, but the frequent and blind use of insecticides increases selection pressure on mosquitoes creating resistance and also had negative impact on human health and non-target species (Raghvendra 2002; Sarwar et al. 2009). In view of these facts, plant-borne control tools can be considered a safer and effective alternative (Benelli 2015b; Pavela 2015a, b).

Nowadays, a growing number of plants are screened successfully for nanosynthesis of silver nanoparticles (AgNP), including *Sida acuta* (Veerekumar et al. 2013), *Barleria cristata* (Govindarajan and Benelli 2015), *Chomelia asiatica* (Muthukumaran et al. 2015a), *Gmelina asiatica* (Muthukumaran et al. 2015b), *Feronia elephantum* (Veerakumar et al. 2014a), and *Heliotropium indicum* (Veerakumar et al. 2014b). Phyto-synthesized silver nanoparticles (AgNP) have been recently proposed as effective mosquito larvicides and are gaining importance over synthetic chemical pesticides because of their reduced harmful effects to non-target species and novelty in the mechanism(s) of action (Patil et al. 2012a; Benelli 2016).

Malva sylvestris (Malvaceae), also known as common malva, is an herb widely present in India, growing to a height of 4 ft. In traditional medicine, the most commonly used parts of the plant are flowers and leaves without petiole. The active ingredients include mucilage, tannins, malvyn, and malvidin (Tomoda et al. 1989). The plant leaves are also rich in nutraceuticals such as antioxidants (phenols, flavonoids, carotenoids, and tocopherols), unsaturated fatty acids (e.g., α -linolenic acid), and minerals (Barros et al. 2010). The anti-inflammatory, antimicrobial, and antioxidant activities of this plant have been documented by many studies (Billeter et al. 1991; Pirbalouti et al. 2010). However, its mosquitocidal activity is currently unknown.

In this research, we proposed a cheap and rapid method of synthesis of polydispersed AgNP using the aqueous leaf extract of *M. sylvestris*. Bio-reduced AgNP were characterized by UV–Vis spectrophotometry, Fourier transform infrared

spectroscopy (FTIR), X-ray diffraction analysis (XRD), atomic force microscopy (AFM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). The acute toxicity of *M. sylvestris* leaf extract and green-synthesized AgNP was evaluated against the larvae of malaria vector *A. stephensi*, the dengue vector *A. aegypti*, and the filariasis vector *C. quinquefasciatus*. Furthermore, we evaluated the biotoxicity of *M. sylvestris* aqueous extract and green-synthesized AgNP on three non-target aquatic organisms sharing the same ecological niche of *Anopheles* and *Aedes* mosquitoes, *Diplonynchus indicus*, and *Gambusia affinis*.

Materials and methods

Materials

Silver nitrate was procured from Merck, India. The glassware was acid-washed thoroughly and then rinsed with Millipore Milli-Q water. Healthy and fresh leaves of *M. sylvestris* were collected from Nilgiris, Western Ghats (11° 10' N to 11° 45' N latitude and 76° 14' E to 77° 2' E longitude), Tamil Nadu State, India. The identity was confirmed at the Department of Botany, Annamalai University, Annamalai Nagar, Tamil Nadu. Voucher specimens were numbered and kept in our laboratory and are available upon request.

Preparation of plant extracts

Leaves of *M. sylvestris* were dried in the shade and ground to fine powder in an electric grinder. Aqueous extract was prepared by mixing 50 g of dried leaf powder with 500 mL of water (boiled and cooled distilled water) with constant stirring on a magnetic stirrer. The suspension of dried leaf powder in water was left for 3 h and filtered through Whatman n. 1 filter paper, and the aqueous filtrate were stored in an amber-colored airtight bottle at 10 °C temperature until testing.

Synthesis of silver nanoparticles

Ten grams of thoroughly washed and finely cut leaves were added in a 300-mL Erlenmeyer flask along with 100 mL of sterilized double-distilled water, and the mixture was boiled for 5 min before finally decanting it. The colloidal extract was filtered with Whatman filter paper no. 1, stored at –15 °C, and tested within a week. The filtrate was treated with aqueous 1 mM AgNO₃ (21.2 mg of AgNO₃ powder in 125 mL of Milli-Q water) solution in an Erlenmeyer flask and incubated at room temperature. Eighty-eight milliliters of an aqueous solution of 1 mM silver nitrate was reduced using 12 mL of leaf extract at room temperature for 10 min, resulting in a brown–yellow solution indicating the formation of AgNP.

Characterization of synthesized silver nanoparticles

The bioreduction of Ag^+ ions was monitored using a UV–Vis spectrophotometer (UV-160v, Shimadzu, Japan). Analysis on size, morphology, agglomeration pattern, and dispersed nature of AgNP was performed by atomic force microscopy (Agilent Technologies AFM- 5500), scanning electron microscopy (Hitachi S3000 H SEM), and transmission electron microscopy (TEM Technite 10 Philips). The purified AgNP were examined for the presence of biomolecules using FTIR spectroscopy (Thermo Scientific Nicolet 380 FT-IR Spectrometer) KBr pellets, and crystalline AgNP were determined by XRD analysis.

Mosquito rearing

Following the method by Govindarajan and Benelli (2015), laboratory-bred pathogen-free strains of mosquitoes were reared in the vector control laboratory, Department of Zoology, Annamalai University. At the time of adult feeding, these mosquitoes were 3–4 days old after emergences (maintained on raisins and water) and were starved for 12 h before feeding. Each time, 500 mosquitoes per cage were fed on blood using a feeding unit fitted with Parafilm as membrane for 4 h. *A. aegypti* feeding was done from 12 noon to 4:00 p.m. and *A. stephensi* and *C. quinquefasciatus* were fed during 6:00 to 10:00 p.m. A membrane feeder with the bottom end fitted with Parafilm was placed with 2.0 mL of the blood sample (obtained from a slaughter house by collecting in a heparinized vial and stored at 4 °C) and kept over a netted cage of mosquitoes. The blood was stirred continuously using an automated stirring device, and a constant temperature of 37 °C was maintained using a water jacket circulating system. After feeding, the fully engorged females were separated and maintained on raisins. Mosquitoes were held at 28 ± 2 °C, 70–85 % relative humidity, with a photoperiod of 12-h light and 12-h dark.

Acute toxicity against mosquito larvae

Larvicidal activity of the aqueous crude extract and AgNP from *M. sylvestris* was evaluated according to World Health Organization (2005). The aqueous extract was tested at 70, 140, 210, 280, and 350 $\mu\text{g mL}^{-1}$ concentrations, and AgNP was tested at 5, 10, 15, 20, and 25 $\mu\text{g mL}^{-1}$ concentrations. Twenty numbers of late III instar larvae were introduced into a 500-mL glass beaker containing 250 mL of dechlorinated water, plus the desired concentrations of leaf extract or AgNP. For each concentration, five replicates were performed. Larval mortality was recorded at 24 h after exposure, during which no food was given to the larvae. Each test included a set control groups (silver

nitrate and distilled water) with five replicates for each individual concentration.

Biotoxicity on non-target organisms

The effect of non-target organisms was assessed following the method by Sivagnaname and Kalyanasundaram (2004). The effect of aqueous extract and AgNP was tested against non-target organisms *D. indicus* and *G. affinis*. The species were field collected and separately maintained in cement tanks (85-cm diameter and 30-cm depth) containing water at 27 ± 3 °C and relative humidity 85 %.

The aqueous extract and AgNP of *M. sylvestris* was evaluated at concentrations of 50 times higher the LC_{50} dose for mosquito larvae. Ten replicates will be performed for each concentration along with four replicates of untreated controls. The non-target organisms were observed for mortality and other abnormalities such as sluggishness and reduced swimming activity after 48-h exposure. The exposed non-target organisms were also

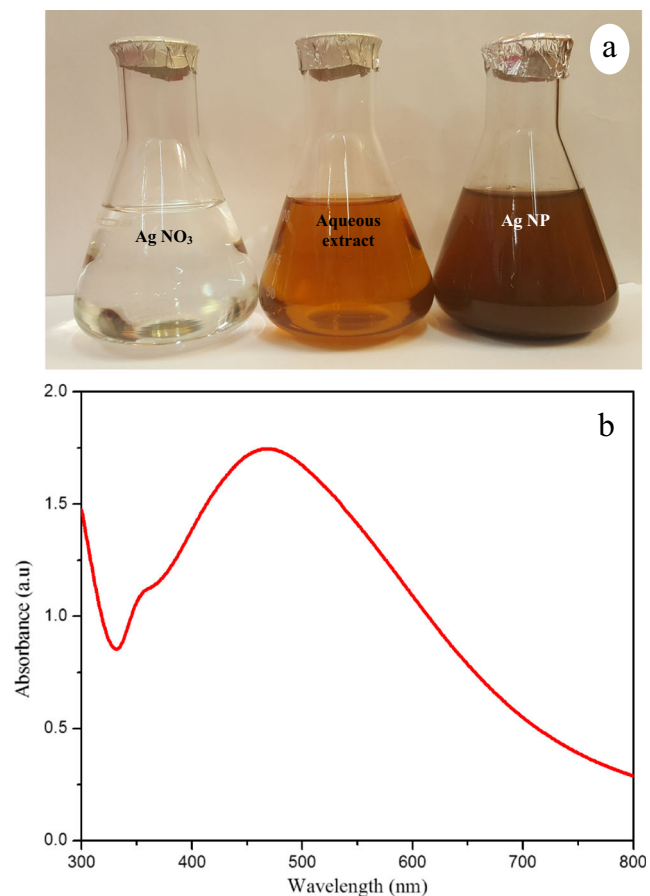


Fig. 1 a Color intensity of *Malva sylvestris* aqueous extract before and after the reduction of silver nitrate (1 mM). The color change indicates Ag^+ reduction to elemental nanosilver. b UV-visible spectrum of silver nanoparticles after 180 min from the reaction

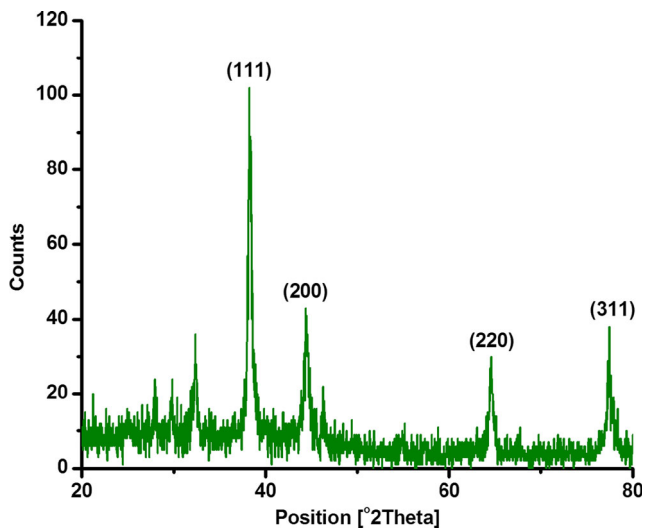


Fig. 2 XRD pattern of silver nanoparticles synthesized using the *Malva sylvestris* aqueous extract

observed continuously for 10 days to understand the post-treatment effect of this extract on survival and swimming activity.

Data analysis

Mortality data were subjected to probit analysis. LC_{50} and LC_{90} were calculated using the method by Finney (1971). In experiments evaluating biotoxicity on non-target organisms, the Suitability Index (SI) was calculated for each non-target species using the following formula (Deo et al. 1988).

$$SI = \frac{LC_{50} \text{ of non-target organisms}}{LC_{50} \text{ of target vector species}}$$

All data were analyzed using the SPSS Statistical Software Package version 16.0. A probability level of $P < 0.05$ was used for the significance of differences between values.

Results and discussion

Biosynthesis and characterization of silver nanoparticles

After incubation with silver nitrate in dark room, the pale yellow colloidal solution turned into a dark brown suspension, which indicated AgNP synthesis (Fig. 1a). *M. sylvestris*-

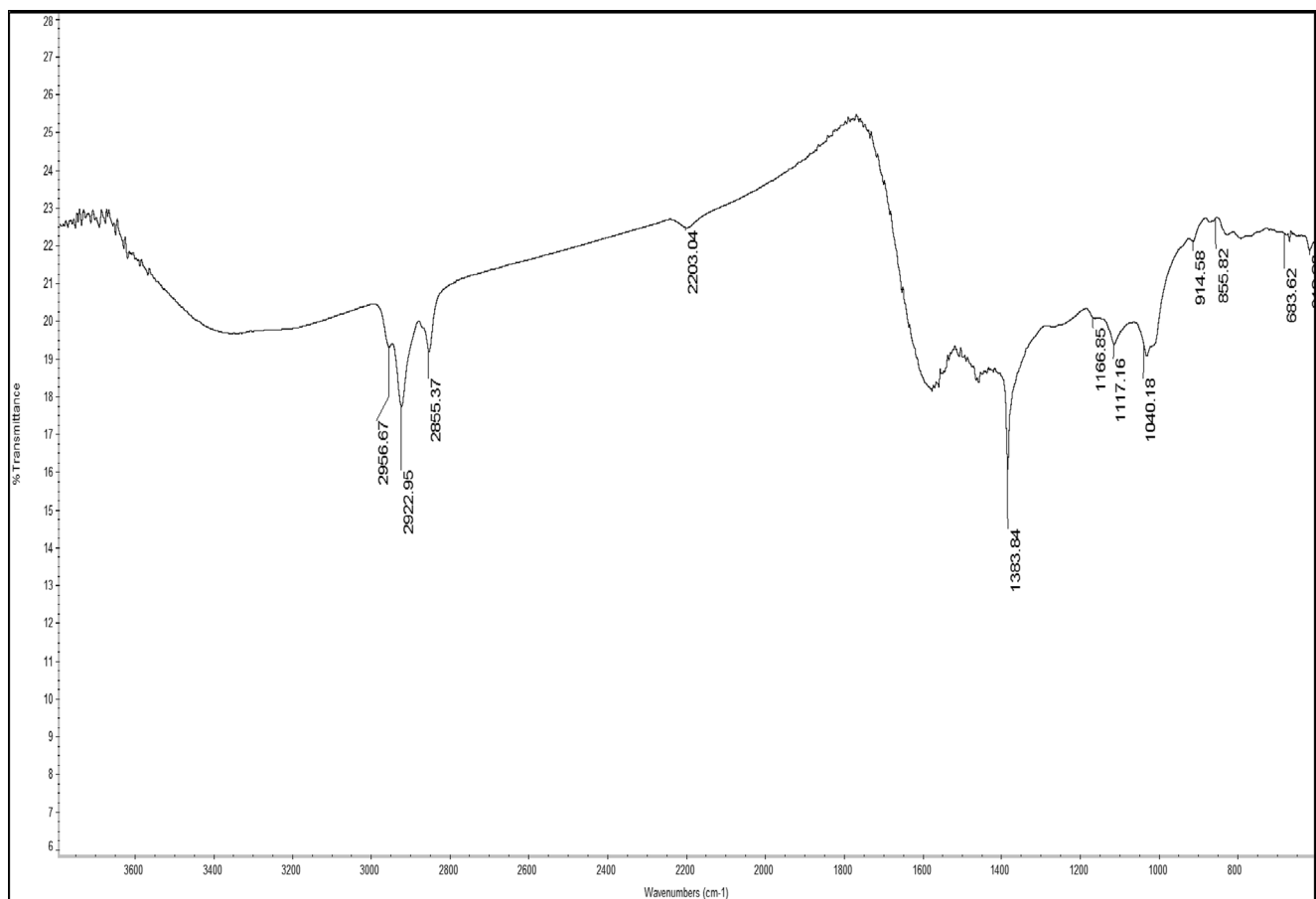


Fig. 3 FTIR spectrum of silver nanoparticles synthesized using the *Malva sylvestris* aqueous leaf extract

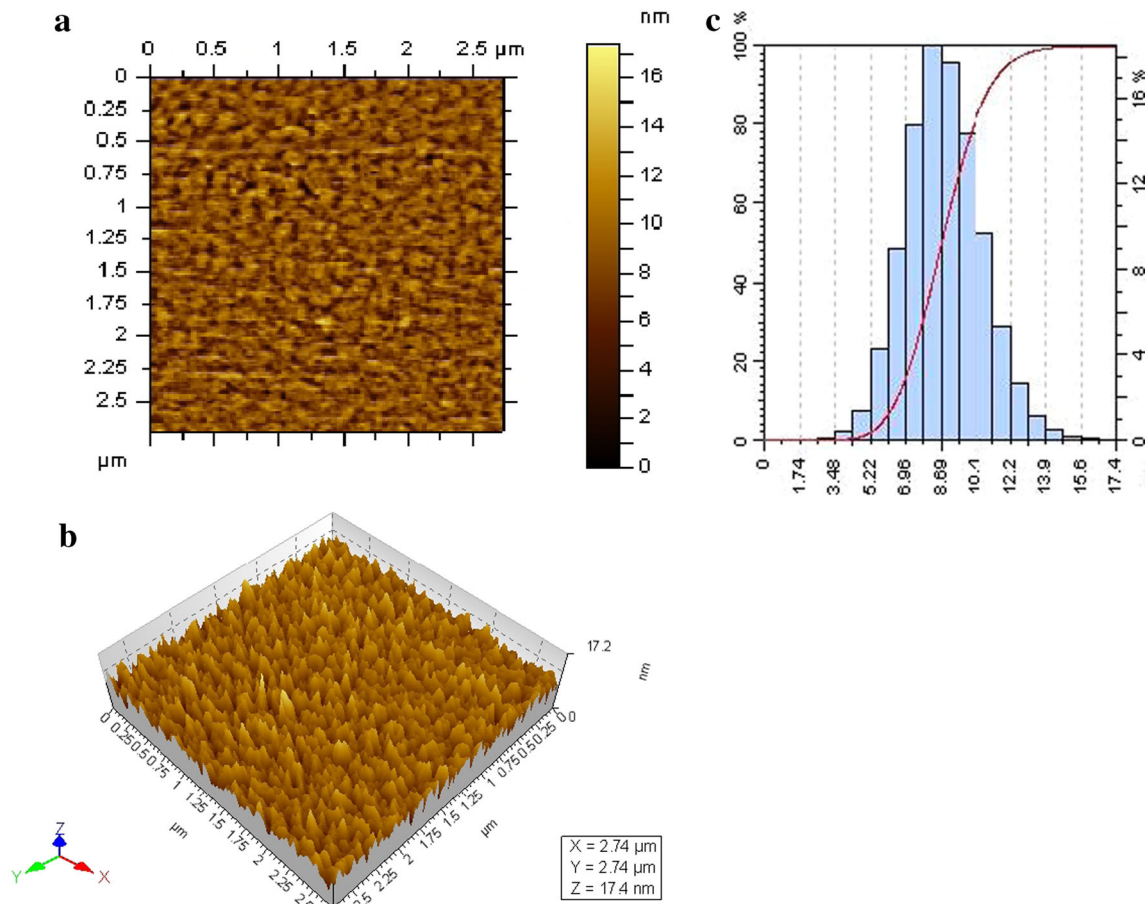


Fig. 4 AFM micrograph of synthesized silver nanoparticles from *Malva sylvestris*, **a** 2.5 μm resolution studies 3- to 17-nm size, spherical shaped, polydispersed particles, **b** 3D image of AgNP analyzed by NOVA-TX software, and **c** histogram showing the particle size distribution

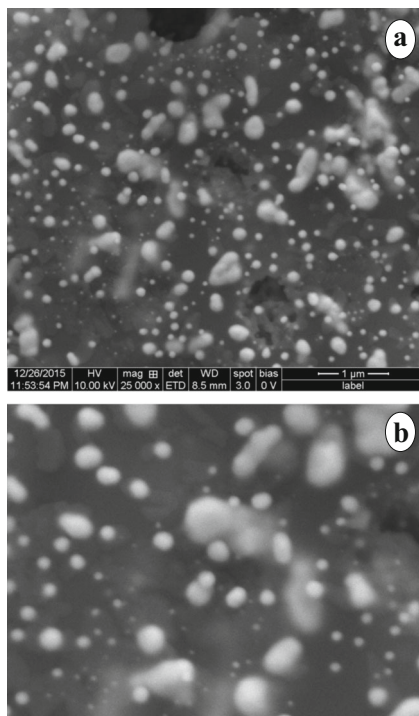


Fig. 5 Scanning electron microscopy (SEM) images of *Malva sylvestris* silver nanoparticles at different resolutions (**a** $\times 25,000$ and **b** $\times 50,000$)

synthesized AgNP exhibited a brown color, due to excitation of surface plasmon vibrations (Govindarajan and Benelli 2015). The surface plasmon resonance bands are influenced by size, shape, morphology, composition, and dielectric environment of AgNP. Previous research has shown that spherical AgNP contribute to the absorption band at around 400–480 nm in the UV–Vis spectrum (Shameli et al. 2012). These bands corresponded to that of AgNP, and the UV–Vis absorption spectrum showed broad surface plasmon resonance at 449 nm (Fig. 1b). The synthesized AgNP were stable in solution for at least 4 weeks after nanosynthesis.

The crystalline nature of AgNP was studied by XRD analysis (Fig. 2). XRD patterns confirmed the crystalline nature of synthesized AgNP. Four diffraction peaks were observed at 38.22, 44.37, 64.53, and 77.46 and represent the (111), (200), (220), and (311) reflections and the face-centered cubic structure of metallic silver, respectively. Similar phenomena were reported earlier (Govindarajan et al. 2015). For instance, AgNP fabricated using *Trianthema decandra* showed interplanar distance that corresponds to mixed phase of cubic and hexagonal structures (Geethalakshmi and Sarada 2010).

The FTIR spectrum of AgNP synthesized with *M. sylvestris* leaf extract is shown in Fig. 3. The peak at

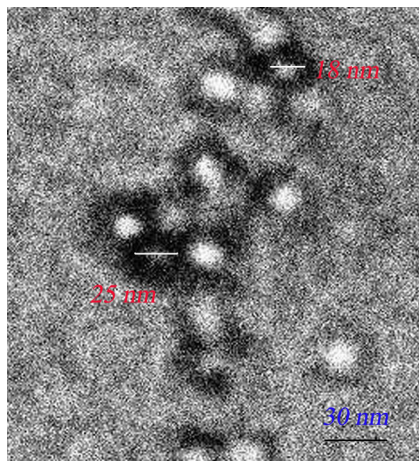


Fig. 6 TEM micrograph of silver nanoparticles synthesized using the *Malva sylvestris* aqueous extract

2922 cm^{-1} could indicate carboxylic acid (Li et al. 2007). The 2855 cm^{-1} indicates the presence of methylene group (CH_2) next to OH or NH_2 functional group. The band at 1383 cm^{-1} corresponds to C–C stretching of aromatic amine. The peak at 1167 and 1117 cm^{-1} probably indicates the presence of C–O stretching alcohols, carboxylic acids, esters, and ethers. The peak obtained at 855 cm^{-1} witnessed the bending vibrations of C–H groups of phenyl rings. The peak near 683 cm^{-1} is assigned to CH out of plane bending vibrations of substituted ethylene systems $-\text{CH}=\text{CH}$ (Veerekumar et al. 2013). Overall, the rapid reduction of silver ions might be linked with the presence of water-soluble phytochemicals such as

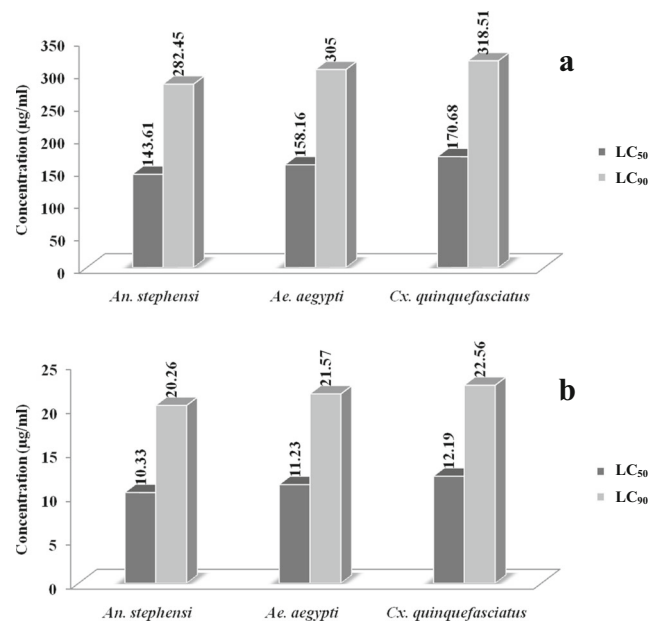


Fig. 7 Larvicidal activity of aqueous leaf extract (a) and silver nanoparticles (b) synthesized using the *Malva sylvestris* against the mosquito vectors *Anopheles stephensi*, *Aedes aegypti*, and *Culex quinquefasciatus*

flavones, quinones, and organic acids present in the leaf extract of *M. sylvestris*.

AFM is a primary tool for analyzing size, shape, and agglomeration pattern and offers visualizations of three-dimensional views of the nanoparticles unlike the electron microscopes. It has an advantage over combination of high

Table 1 Larvicidal activity of *Malva sylvestris* aqueous leaf extract against the mosquito vectors *Anopheles stephensi*, *Aedes aegypti*, and *Culex quinquefasciatus*

Mosquito species	Concentration (µg/mL)	Mortality (%) ± SD ^a	LC ₅₀ (µg/mL) (LCL-UCL)	LC ₉₀ (µg/mL) (LCL-UCL)	Slope	Regression equation	χ^2 (df)
<i>An. stephensi</i>	70	28.7 ± 0.8	143.61 (127.25–158.09)	282.45 (262.00–309.27)	3.18	$y = 11.23 + 0.263x$	3.613 (4) n.s.
	140	46.5 ± 0.6					
	210	68.3 ± 0.6					
	280	89.6 ± 0.4					
	350	99.2 ± 0.6					
<i>Ae. aegypti</i>	70	25.5 ± 1.2	158.16 (141.89–172.88)	305.00 (282.98–334.02)	2.88	$y = 6.41 + 0.268x$	2.301 (4) n.s.
	140	41.3 ± 0.6					
	210	63.9 ± 0.6					
	280	85.4 ± 0.8					
	350	97.2 ± 0.4					
<i>Cx. quinquefasciatus</i>	70	21.8 ± 0.4	170.68 (155.00–185.24)	318.51 (295.85–348.36)	2.51	$y = 1.91 + 0.275x$	2.035 (4) n.s.
	140	38.6 ± 0.6					
	210	59.2 ± 0.4					
	280	82.3 ± 0.4					
	350	96.1 ± 0.8					

No mortality was observed in the control

SD standard deviation, LC₅₀ lethal concentration that kills 50 % of the exposed organisms, LC₉₀ lethal concentration that kills 90 % of the exposed organisms, UCL 95 % upper confidence limit, LCL 95 % lower confidence limit, χ^2 chi square, df degrees of freedom, n.s. not significant ($\alpha = 0.05$)

^a Values are mean ± SD of five replicates

Table 2 Larvicidal activity of silver nanoparticles synthesized using the *Malva sylvestris* leaf extract against the mosquito vectors *Anopheles stephensi*, *Aedes aegypti*, and *Culex quinquefasciatus*

Mosquito species	Concentration (µg/mL)	Mortality (%) ± SD ^a	LC ₅₀ (µg/mL) (LCL-UCL)	LC ₉₀ (µg/mL) (LCL-UCL)	Slope	Regression equation	χ ² (df)
<i>An. stephensi</i>	5	27.3 ± 1.2	10.33 (9.15–11.36)	20.26 (18.79–22.18)	3.14	y = 10.95 + 3.686x	4.06 (4) n.s.
	10	48.9 ± 0.8					
	15	66.5 ± 0.8					
	20	89.2 ± 0.6					
	25	99.3 ± 0.4					
<i>Ae. aegypti</i>	5	23.4 ± 0.6	11.23 (10.08–12.27)	21.57 (20.02–23.59)	2.82	y = 6.45 + 3.77x	2.326 (4) n.s.
	10	45.6 ± 0.4					
	15	62.3 ± 0.4					
	20	86.5 ± 1.2					
	25	97.2 ± 0.8					
<i>Cx. quinquefasciatus</i>	5	19.6 ± 0.6	12.19 (11.09–13.22)	22.56 (20.97–24.63)	2.43	y = 1.43 + 3.886x	1.778 (4) n.s.
	10	41.2 ± 0.6					
	15	59.4 ± 0.4					
	20	82.1 ± 0.8					
	25	96.3 ± 1.2					

No mortality was observed in the control

SD standard deviation, LC₅₀ lethal concentration that kills 50 % of the exposed organisms, LC₉₀ lethal concentration that kills 90 % of the exposed organisms, UCL 95 % upper confidence limit, LCL 95 % lower confidence limit, χ² chi square, df degrees of freedom, n.s. not significant (α = 0.05)

^a Values are mean ± SD of five replicates

resolution samples, does not have to be conductive, and does not require the high-pressure vacuum conditions. The 2.5-µm resolution studies of green-synthesized AgNP with AFM reveal that the particles are polydispersed, spherical in shape, having the size range from 3 to 17 nm, and having no agglomeration observed between the particles (Fig. 4a). Raw data obtained from this AFM microscope is treated with a specially designed image processing software (NOVA-TX) to further exploit the 3D image of nanoparticles (Fig. 4b). The average particle size obtained from the corresponding diameter distribution was about 8 nm (Fig. 4c).

SEM analysis was performed in order to investigate the morphology and size distribution of AgNP (Fig. 5a, b). SEM

showed that the morphology of AgNP is mostly spherical, in agreement with the shape of SPR band in the UV–vis spectrum. The average particle size measured from all the SEM images is around 25 to 50 nm. Metallic AgNP generally show typical absorption peak approximately at 3 keV due to surface plasmon resonance (Magudapatty et al. 2001). TEM micrograph confirms the presence of polydispersed AgNP, showing fine configuration of crystalline, spherical AgNP, with size ranging between 18 and 25 nm (Fig. 6) and mean size of 20 nm. It was also noted that AgNP bound with thin layer of biomolecule coating on their surface which act as stabilizing agent; therefore, AgNP were polydispersed without direct contact and stable for long period of time (Vignesh et al. 2013).

Table 3 Biototoxicity of *Malva sylvestris* aqueous leaf extract against two non-target organisms sharing the same ecological niche of mosquito vectors

Non-target organism	Concentration (µg/mL)	Mortality (%) ± SD ^a	LC ₅₀ (µg/mL) (LCL-UCL)	LC ₉₀ (µg/mL) (LCL-UCL)	Slope	Regression equation	χ ² (df)
<i>D. indicus</i>	4000	26.8 ± 0.8	8282.25 (7379.61–9086.64)	15964.04 (14826.85–17450.54)	2.87	y = 10.09 + 0.005x	4.926 (4) n.s.
	8000	48.2 ± 1.2					
	12000	67.4 ± 1.2					
	16000	89.3 ± 0.8					
	20000	100.0 ± 0.0					
<i>G. affinis</i>	5000	27.5 ± 0.6	10459.13 (9234.44–11537.88)	20958.91 (19416.23–22993.85)	3.56	y = 11.14 + 0.004x	1.276 (4) n.s.
	10000	46.8 ± 0.6					
	15000	67.4 ± 0.8					
	20000	88.6 ± 0.8					
	25000	97.2 ± 0.6					

No mortality was observed in the control

SD standard deviation, LC₅₀ lethal concentration that kills 50 % of the exposed organisms, LC₉₀ lethal concentration that kills 90 % of the exposed organisms, UCL 95 % upper confidence limit, LCL 95 % lower confidence limit, χ² chi square, df degrees of freedom, n.s. not significant (α = 0.05)

^a Values are mean ± SD of five replicates

Table 4 Biotoxicity of silver nanoparticles synthesized using the *Malva sylvestris* leaf extract against two non-target organisms sharing the same ecological niche of mosquito vectors

Non-target organism	Concentration (µg/mL)	Mortality (%) ± SD ^a	LC ₅₀ (µg/mL) (LCL-UCL)	LC ₉₀ (µg/mL) (LCL-UCL)	Slope	Regression equation	χ ² (df)
<i>D. indicus</i>	400	29.4 ± 1.2	813.16 (718.55–896.50)	1610.57 (1493.10–1765.02)	3.31	y = 12.03 + 0.046x	5.273 (4) n.s.
	800	47.3 ± 0.6					
	1200	68.5 ± 0.4					
	1600	88.4 ± 0.6					
	2000	100.0 ± 0.0					
<i>G. affinis</i>	400	26.8 ± 0.6	1044.52 (923.55–1151.24)	2080.06 (1927.94–2280.32)	3.42	y = 10.95 + 0.036x	1.733 (4) n.s.
	800	48.2 ± 0.6					
	1200	66.5 ± 0.4					
	1600	89.3 ± 0.8					
	2000	97.4 ± 1.2					

No mortality was observed in the control

SD standard deviation, LC₅₀ lethal concentration that kills 50 % of the exposed organisms, LC₉₀ lethal concentration that kills 90 % of the exposed organisms, UCL 95 % upper confidence limit, LCL 95 % lower confidence limit, χ² chi square, df degrees of freedom, n.s. not significant (α = 0.05)

^a Values are mean ± SD of five replicates

Acute toxicity against mosquito larvae

Both the *M. sylvestris* leaf extract and AgNP showed dose-dependent larvicidal effect against all tested mosquito species (Table 1; Fig. 7a). Compared to the leaf aqueous extract, AgNP showed higher toxicity against *A. stephensi*, *A. aegypti*, and *C. quinquefasciatus* with LC₅₀ values of 10.33, 11.23, and 12.19 µg/mL, respectively (Table 2; Fig. 7b). Furthermore, AgNP showed enhanced larvicidal effect even at lower concentrations compared to plant derivatives alone, which denotes that AgNP potentially

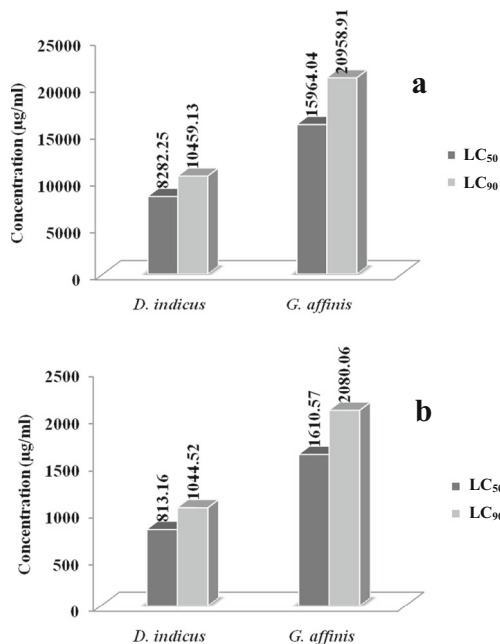


Fig. 8 Biototoxicity of aqueous leaf extract (a) and silver nanoparticles (b) synthesized using the *Malva sylvestris* against two non-target organisms sharing the same ecological niche of mosquito vectors

increases the bioactivity of plant products. Recently, a growing number of plants have been explored for the green synthesis of mosquitocidal AgNP, and it has been showed that the combination of nanoparticles with bioactive principles bestows improved efficiency (Benelli 2016; Haldar et al. 2013). The present study implicated that the percentage of mosquito larvicidal mortality increased by many folds with the addition of bio-stabilized AgNP. Similarly, Muthukumar et al. (2015b) recently focused on the potential of *Gmelina asiatica*-mediated synthesis of AgNP against *A. stephensi*, *A. aegypti*, and *C. quinquefasciatus*, obtaining higher LC₅₀ values (i.e., 22.44, 25.77, and 27.83 µg/mL, respectively). Murugan et al. (2015) showed that low doses (1 ppm) of *Cymbopogon citratus*-synthesized gold nanoparticles control malaria and dengue vectors and boost early instar mosquito larvae predation by copepods in a nanoparticle-contaminated aquatic environment. Concerning the mechanism(s) of action, the observed larvicidal activity may be attributed to the interaction between silver nanoparticles and the extracellular lipoprotein matrix. This interaction increases the permeability of the plasma membrane of cells. Additionally, an interaction between AgNP and the sulfur-containing proteins or phosphorous containing compound leads to denaturation of organelles and enzymes and reduces ATP synthesis which finally causes the loss of the cellular function and cell death (Sap-Iam et al. 2010; Sondi and Salopek-Sondi 2004).

Biototoxicity on non-target organisms

The biototoxicity of *M. sylvestris* aqueous extract and green-synthesized AgNP was evaluated on non-target organisms *D. indicus* and *G. affinis*. Toxicity treatments achieved negligible toxicity against *D. indicus* and *G. affinis*, with LC₅₀ values

Table 5 Suitability index of different non-target organisms over young instars of *Anopheles stephensi*, *Aedes aegypti*, and *Culex quinquefasciatus* exposed to *Malva sylvestris* aqueous leaf extract and green-synthesized silver nanoparticles

Treatment	Non-target organism	<i>An. stephensi</i>	<i>Ae. aegypti</i>	<i>Cx. quinquefasciatus</i>
Aqueous leaf extract	<i>Diplonychus indicus</i>	57.67	52.36	48.52
	<i>Gambusia affinis</i>	72.83	66.13	61.27
Silver nanoparticles	<i>Diplonychus indicus</i>	78.71	72.40	66.70
	<i>Gambusia affinis</i>	101.11	93.01	85.68

ranging from 813.16 to 10,459.13 $\mu\text{g/mL}$ (Tables 3 and 4; Fig. 8a, b). Focal observations highlighted that longevity and swimming activity of the study species were not altered for a week after testing. SI indicated that *M. sylvestris*-fabricated AgNP were less toxic to the non-target organism tested if compared to the targeted mosquito larval populations (Table 5). Nowadays, moderate knowledge is available about the acute toxicity of mosquitocidal nanoparticles toward non-target aquatic species (Benelli 2016). *Pergularia rubra*- and *Pergularia daemia*-synthesized AgNP did not exhibit any evident toxicity effect against *Poecilia reticulata* fishes, after 48 h of exposure to LC_{50} and LC_{90} values calculated on IV instar larvae of *A. aegypti* and *A. stephensi* (Patil et al. 2012b). Haldar et al. (2013) did not detect toxicity of AgNP produced using dried green fruits of *D. roxburghii* against *P. reticulata*, after 48-h exposure to LC_{50} of IV instar larvae of *A. stephensi* and *C. quinquefasciatus*. Rawani et al. (2013) showed that mosquitocidal AgNP synthesized using *Solanum nigrum* berry extracts were not toxic against two mosquito predators, *Toxorhynchites* larvae and *Diplonychus annulatum*, and *Chironomus circumdatus* larvae, exposed to lethal concentrations of dry nanoparticles calculated on *A. stephensi* and *C. quinquefasciatus* larvae. AgNP biosynthesized using the 2, 7-bis[2-[diethylamino]-ethoxy]fluorence isolate from the *Melia azedarach* leaves did not show acute toxicity against *Mesocyclops pehpeiensis* copepods (Ramanibai and Velayutham 2015). Interestingly, the exposure to extremely low doses (e.g., 1 ppm) of green-synthesized AgNP did not negatively affect the predation efficiency of a number of mosquito predators of relevance for mosquito control (Benelli 2016; Murugan et al. 2015). It has been hypothesized that low doses of plant-synthesized metal nanoparticles may reduce the motility of mosquito larvae, enhancing predation of odonate nymphs and other mosquito natural enemies (Benelli 2016).

Conclusions

The plant-mediated synthesis of the silver nanoparticles is one-pot, eco-friendly, and cost-effective. In the present study, AgNP were rapidly biosynthesized at room temperature using a cheap *M. sylvestris* leaf extract. Bio-reduced silver nanocrystals were polydispersed and stable in solution for at

least 4 weeks. AgNP have excellent anti-mosquito larvicidal activity against third instar larvae of three important mosquito vectors, with LC_{50} values ranging from 10.33 to 12.19 $\mu\text{g/mL}$. Overall, this *M. sylvestris*-based route of nanosynthesis may be considered for the development of newer and safer mosquito larvicides.

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Compliance with ethical standards All applicable international and national guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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