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ORIGINAL PAPER

Single-step biological fabrication of colloidal silver nanoparticles using *Hugonia mystax:* larvicidal potential against Zika virus, dengue, and malaria vector mosquitoes

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ABSTRACT

Mosquito control is facing key challenges, including outbreaks of new arbovirus threats. We proposed an eco-friendly synthesis of silver nanoparticles (AgNPs) employing a low-cost extract of *Hugonia mystax*. AgNPs were specified by UV, XRD, FTIR and EDX spectroscopy, SEM and TEM. AgNPs were more toxic to *Anopheles stephensi, Aedes aegypti,* and *Culex quinquefasciatus* larvae (LC_{50}: 14.45, 15.86, and 17.46 μg/mL) if compared to aquatic biocontrol organisms *Gambusia affinis, Diplonychus indicus,* and *Anisops bouvieri* (LC_{50}: 2567.15, 1075.16, and 829.63 μg/ml). Overall, we shed light on the mosquito larvicidal efficacy of *H. mystax,* a possible biological resource for low-cost fabrication of AgNPs.

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Introduction

Mosquitoes (Diptera: Culicidae) are important vectors of a wide number of parasites and pathogens, which often represents severe public health problems in tropical and subtropical areas worldwide (Benelli 2015a, 2015b, Benelli et al. 2016a, 2016b, 2016c, Mehlhorn et al. 2012). Mosquito control programs are facing a number of crucial challenges, including the blooming to mosquito resistance to synthetic pesticides and the current outbreaks of new arbovirus threats, such as chikungunya and Zika virus (Benelli and Mehlhorn 2016, Pavela and Benelli 2016). Indeed, current control strategies mainly rely on synthetic pesticides, insect growth regulators, and microbial control agents. However, synthetic chemicals lead to a number of negative implications, including high operational costs, development of resistance and toxic effects on non-target organisms and human health (Benelli 2015a, Naqqash et al. 2016). Limited tools are currently available against the main pathogens and parasites vectored by mosquitoes (Benelli 2015a, Benelli and Mehlhorn 2016).

To deal with the above-mentioned issues, in latest years an increasing number of flora-borne secondary metabolites have been proposed for cost-effective and rapid synthesis of nanoparticles (Benelli 2016a, 2016b, Chao et al. 2016, Hassan et al. 2016, Priyanka et al. 2016a, 2016b, 2016c, Rajan et al. 2015, Ramanibai and Velayutham 2015, Thirunavokkarasu et al. 2013, Velayutham et al. 2013). Recently, in more than one hundred researches, plant-fabricated nanoparticles have been studied for their highly effective mosquitocidal properties (Benelli 2016a, 2016b, Benelli et al. 2015a, 2015b, Govindarajan 2016). For instance, biosynthesized silver nanoparticles (AgNPs) from *Carissa spinarum* (Govindarajan et al. 2016b), *Barleria cristata* (Govindarajan and Benelli 2016a), *Bauhinia variegata* (Govindarajan et al. 2016e), and *Clerodendrum chinense* (Govindarajan et al. 2016d) were most virulent against the larval mosquitoes. Notably, it has been highlighted that differences in the green route employed for fabrication of mosquitocidal nanoparticles lead to different biophysical properties (e.g. size, crystalline structure, and shape) of the synthesized products (see Benelli 2015c, 2016a, 2016b, 2016c for reviews).

The genus *Hugonia* (Linn.) belongs to the family Linaceae. It comprises about 40 species in the world. Two of them, *Hugonia mystax* (Linn.) and *Hugonia ferruginea* (Wight & Arn.) grow in India (Pullaiah and Chennaiah 1997, Santapau and Henry 1983). *H. mystax* locally known as Modirakanni is used for skin diseases by the traditional healers of Tiruvannamalai hills, Tamil Nadu, India. Indeed, the leaves are used for anthelmintic (Padel et al. 2010) and to treat rheumatism (Sutha et al. 2009). Biological activity such as antidiabetic activity of the leaves in rabbits has been also reported (Nammi et al. 2003). To the best of our knowledge, the mosquito larvicidal potential of *H. mystax* is unrevealed.

In this investigation, we proposed a low-cost and swift route to synthesize AgNPs using the aqueous leaf extract

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of *H. mystax*. Green fabricated AgNPs were specified by UV, XRD, FTIR, SEM, TEM, and EDX. The severe toxicity of *H. mystax* leaf aqueous extract and biofabricated AgNPs were assessed against the *Culex quinquefasciatus, Aedes aegypti,* and *Anopheles stephensi* larvae.

Materials and methods

Materials

Fresh leaves of *H. mystax* were collected from the Marakanam reserve forest, located in Tamil Nadu, India. The taxonomist of Annamalai University in India provided identifications of the *H. mystax* leaves. Silver nitrate was acquired from Sigma-Aldrich, Mumbai, India.

Preparation of plant leaf extract

First, the *H. mystax* leaves were dried in the shade and then an electric grinder was used to turn them into finely ground powder. Subsequently, 50 g of the sample were mixed with 0.5 L of boiled and cooled distilled water, continually stirring the mixture on a magnetic stirrer, in order to acquire an aqueous extract. The dried leaf powder suspension was left to settle for 3 h and then was filtered by using Whatman n. 1 filter paper, finally, the filtrate was stored at 10 °C, until the time of the experiment.

Green fabrication of AgNPs

The leaves were thoroughly washed and finely ground. Then we took 10 g of the resulting powder and mixed it with 100 mL of sterilized, double-distilled water, in a 300-ml Erlenmeyer flask. The mixture was left to boil for 5 min before transfusion. Filtered the extract through Whatman filter paper n. 1 and then stored it at −15 °C. Treated the filtrate with aqueous 1 mM $AgNO_3$ solution, which was obtained by dissolving 21.2 mg of $AgNO_3$ powder in 125 mL water purified with Milli-Q technology (Merck KGaA, Darmstadt, Germany). The treatment took place in an Erlenmeyer flask, and the mixture was left at room temperature. 12 mL of the leaf extract were used to reduce 88 mL of a 1 mM silver nitrate aqueous solution. The reaction lasted 10 min at room temperature and resulted in the formation of Ag nanoparticles, as evident by the brown–yellow coloring of the solution.

Characterization of synthesized ag nanoparticles

The UV (UV-160v, Shimadzu, Japan) were used to monitor the bioreduction of the Ag^+ ions. Subsequently, we employed energy dispersive X-ray spectrum (EDX), transmission electron microscopy (TEM Technite 10 Philips, Koninklijke Philips Electronics N.V.) and SEM (Hitachi S3000 H SEM; Thermo Fisher Scientific, Waltham, MA) to analyze the morphology, size, and composition of the Ag nanoparticles. Moreover, examined the purified Ag nanoparticles for biomolecule pressure by employing FT-IR (Nicolet 380, Thermo Scientific) and finally, XRD analysis was used to determine Ag nanoparticles and KBr pellets.

Target organisms

Laboratory reared mosquitoes were collected at Annamalai University's Department of Zoology. The mosquitoes were 3–4 days old after emergence at the time of adult feeding, sustained on water and raisins; a 12-h starvation period preceded feeding. Fed 500 mosquitoes per cage each time, using blood through a Parafilm membrane-fitted unit for 4 h. *Ae. Aegypti* mosquitoes were fed from 12 to 16.00, while *An. stephensi* and *Cx. quinquefasciatus* feeding took place between 18.00 and 22.00. The conditions under which the mosquitoes were kept were 70–85% relative humidity (RH), 28 ± 2 °C temperature and a 12-h photoperiod (Govindarajan et al. 2016b).

Mosquito larvicidal potential

Larvicidal activity of the aqueous leaf extract and AgNPs was evaluated according to the protocol by WHO (2005). Based on the wide range and narrow range tests, aqueous crude extract was tested at 80, 160, 240, 320, and 400 μg mL^{-1} concentrations and AgNPs was tested at 7, 14, 21, 28, and 35 μg mL^{-1} concentrations. About 20 numbers of late third larvae were introduced into a 500-ml beaker containing 249 mL of tap water, and 1 mL of desired concentrations of AgNPs or leaf extract was added. Five replicates were performed.

Bio-safety on non-target organisms

Followed the method described by Sivagnaname and Kalyanasundaram (2004) to assess the mixture's effect on non-target organisms. The biocontrol organisms, against which the impact of the studied plant's extract was examined, were *Gambusia affinis, Diplonychus indicus*, and *Anisops bouvieri*. The biocontrol organisms were gathered in the wild and kept individually in tanks made from cement, with a depth of 30 cm and a diameter of 85 cm. The tanks contained water with a temperature of 27 ± 3 °C, while the external RH was 85%. The evaluation of the aqueous leaf extract and Ag nanoparticles mixture took place with concentrations that reached levels even 50 times higher than the calculated LC_{50} dose for mosquito larvae and monitored the mortality after we exposed them to the mixture for 48 h. Moreover, the organisms were exposed for an additional ten days, in order to assess any possible post-treatment effects on swimming activity and survival (Govindarajan and Benelli 2016b).

Data analysis

We employed probit analysis to evaluate mortality data. The Finney (1971) method was used to calculate LC_{50} and LC_{90} concentrations. Furthermore, we adopted the formula be Deo et al. (1988) to calculate the Suitability Index (SI) for every single biocontrol organisms, as follows:

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

For data analysis, used the SPSS Statistical Software Package, version 16.0 (IBM, Armonk, NY). We adopted a

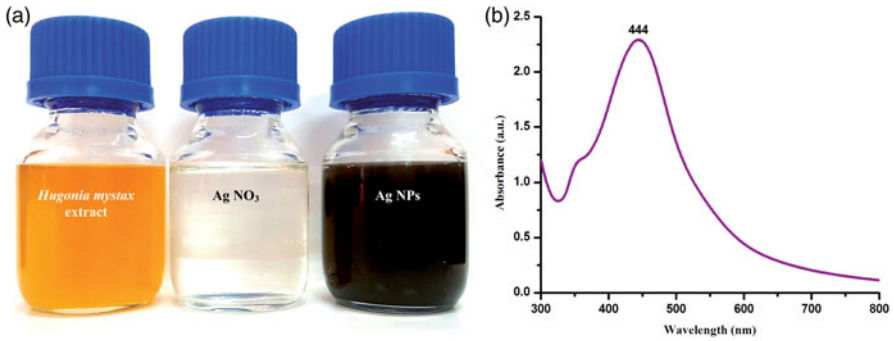

Figure 1. (a) Color intensity of *Hugonia mystax* aqueous extract before and after the reduction of silver nitrate (1 mM). The color change indicates Ag^{+} reduction to elemental nanosilver. (b) UV-visible spectrum of silver nanoparticles after 180 min from the reaction.

$P < .05$ as a level of probability, to assess the statistical significance of the differences between values.

Results and discussion

Fabrication of AgNPs

After incubating it for 180 min in a black chamber, the initially colorless mixture took a dark brown color, providing evidence of Ag nanoparticle formation (Figure 1(a)). The brown color exhibited by the Ag nanoparticles created by *H. mystax* may be attributed to the stimulation of surface Plasmon (SPR) vibrations (Veerakumar et al. 2014, Vijayakumar et al. 2013, Vivek et al. 2012, Zhang et al. 2011). SPR bands are affected by the Ag nanoparticles' shape, size, composition, morphology, and dielectric environment (Benelli 2016a, 2016b, Govindarajan 2016, Muthukumaran et al. 2015a, Shahverdi et al. 2007, Singh et al. 2010). Further examination revealed that spherical Ag nanoparticles are responsible for absorption around the 400–480 nm range in the UV-visual spectrum (Sanpui et al., 2008, Shameli et al. 2012). These bands correspond to the absorption of Ag nanoparticles, while broad SPR was exhibited at 444 nm, in the UV-vis absorption spectrum (Figure 1(b)).

Spectroscopic and microscopic characterization of Ag nanoparticles

The green fabricated Ag nanoparticles' crystalline nature was confirmed via XRD intensities of diffraction peaks at (111), (200), (220), and (311), which coincided with 38.74°, 44.27°, 63.88°, and 77.65°. The resulting XRD pattern underlined that Ag nanoparticles exhibited both hexagonal and cubic structures (Figure 2). Similar findings were stated previously (Veerakumar et al. 2013) for Ag nanoparticles biosynthesized using *Sida acuta,* which exhibited both hexagonal and cubic structures.

Figure 3 shows the FTIR spectrum of *H. mystax*-synthesized AgNPs. Over three repetitions, we noted distinguished absorbance bands at around 3356, 2933, 2392, 1622, 1488, 1384, 1192, 1146, 1095, 931, 825, 719, 661, and 593 cm^{-1}. The aforementioned peaks imply N–H bending (aromatic amines), C–H stretching (alkanes), C=C stretching (alkanes), C–H bonds (methyl and methylene), H-bonds (alcohols, phenol), O–H stretching, and C–O stretching (carboxylic group),

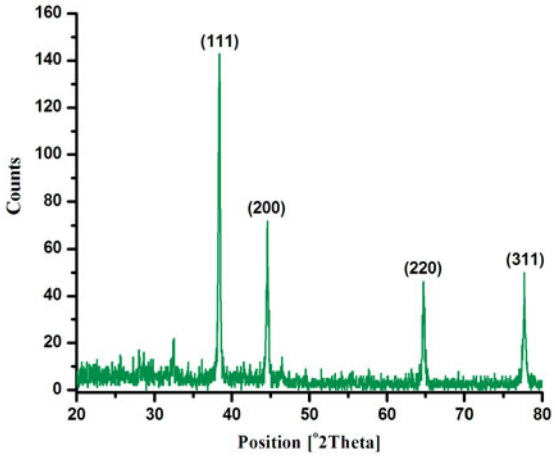

Figure 2. XRD pattern of silver nanoparticles biofabricated using the *Hugonia mystax* aqueous extract.

respectively. According to the observed bands, we infer that there are flavonoids and terpenoids present in the mixture, as potential reducing compounds (Huang et al. 2007, Ruparelia et al. 2008, Wazed Ali et al. 2001), which may also be able to explain the Ag nanoparticles' efficient capping and stabilization.

Figure 4(a, b) shows the SEM investigation of the Ag nanoparticles' morphology and size distribution. The Ag nanoparticles show an almost spherical morphology, corresponding to the SPR band's shape in the UV-vis spectrum. The mean size of the particles as studied from all the SEM images was between 40 and 90 nm. Figure 5 shows the EDX profile of Ag nanoparticles. The typical absorption peak of metallic Ag nanoparticles appears at around 3 keV due to SPR (Panneerselvam et al. 2016). TEM images revealed finely configured spherical Ag nanoparticles of crystalline nature, with sizes varying from 10 to 75 nm (Figure 6), and a mean size of 35 nm. Another noteworthy observation was the capping of Ag nanoparticles by a thin coating of biomolecules, with stabilizing properties, resulting in poly-dispersions of the nanoparticles, with no direct contact, and prolonged stability (Govindarajan and Benelli 2016b, Thirunavukkarasu et al. 2010).

Mosquito larvicidal properties

Both the *H. mystax* aqueous leaf extract and the Ag nanoparticles have affected the survival of all the tested mosquito

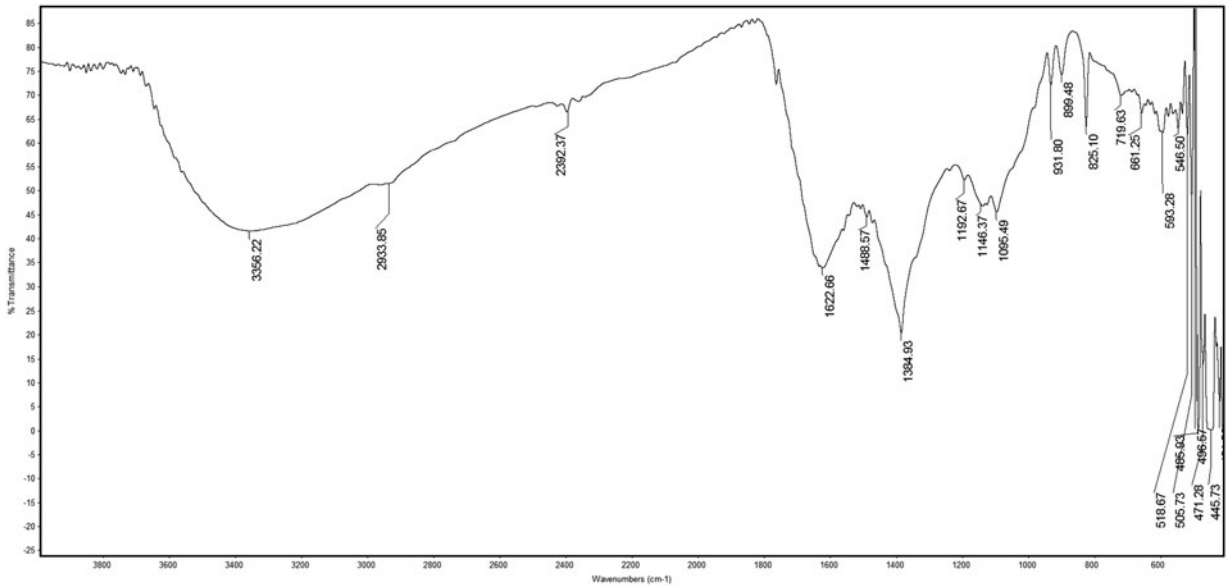

Figure 3. FTIR spectrum of silver nanoparticles biofabricated using the *Hugonia mystax* aqueous extract.

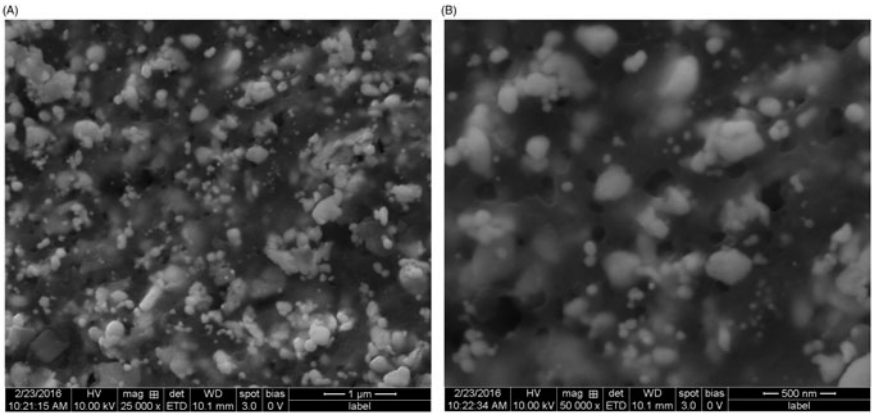

Figure 4. Scanning electron microscopy (SEM) of *Hugonia mystax* silver nanoparticles (A. 25 000X; B. 50 000X).

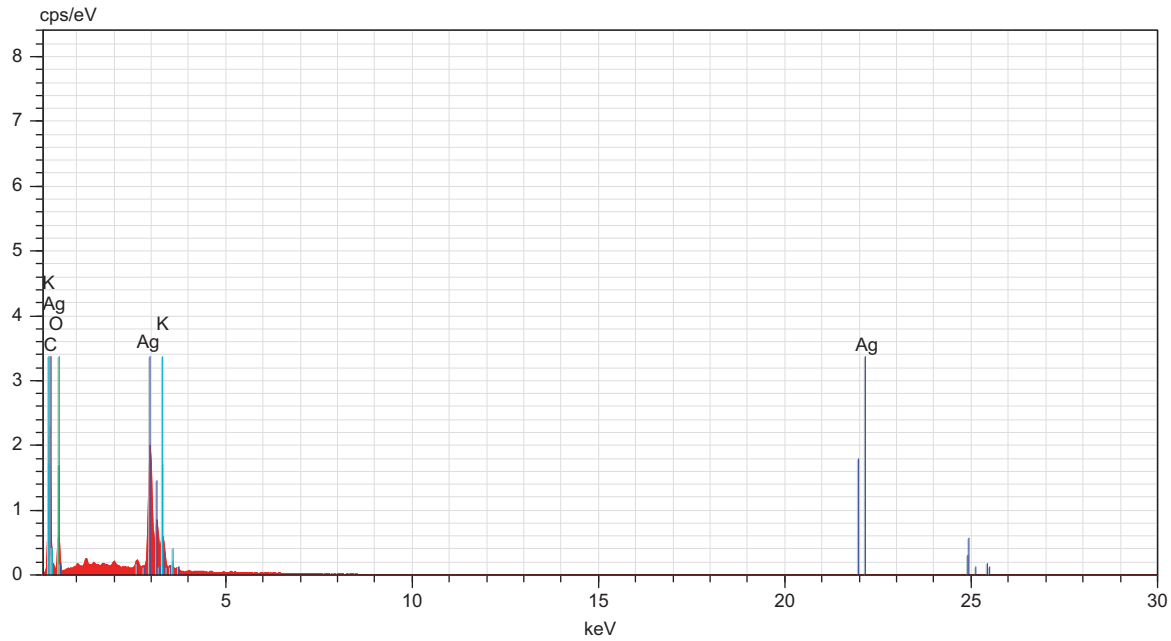

Figure 5. Energy dispersive X-ray (EDX) spectrum of silver nanoparticles biofabricated using the *Hugonia mystax* aqueous extract, showing presence of different phyto-elements as capping agents.

larvae (Tables 1 and 2). The *H. mystax*-synthesized AgNPs exhibited increased toxicity against *Cx.* quinquefasciatus (LC_{50} 17.46 µg/mL), *Ae. aegypti* (LC_{50} 15.86 µg/mL), and *An. stephensi* (LC_{50} 14.45 µg/mL) (Table 2), compared to the aqueous leaf extract alone. Recently, significant efforts have been carried out to investigate the mosquito larvicidal activity of flora-derived substances (Pavela 2015a, 2015b). For instance, Govindarajan et al. (2016c) assessed the acute toxicity of *P. barbatus* essential oil against *Cx. tritaeniorhynchus, Ae. albopictus* and *An. subpictus* larvae, and found LC_{50} values of 94.34, 87.25, and 84.20 µg/ml, respectively. Also, the larvicidal toxicity of the *Cardiospermum halicacabum* leaf extract (testing solvents with different polarity, i.e. chloroform, methanol, ethyl acetate, hexane, and benzene) was assessed against *Ae. aegypti* and *Cx. quinquefasciatus*. LC_{50} were 154.95, 150.44, 183.36, 193.31, and 174.24 ppm, and 164.54, 156.80, 192.31, 200.02, and 182.51 ppm, respectively (Govindarajan 2011).

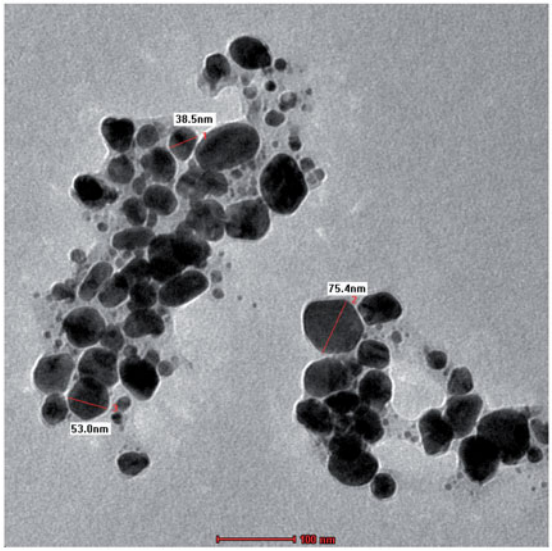

Figure 6. Transmission electron microscopy (TEM) of silver nanoparticles biofabricated using the *Hugonia mystax* aqueous extract.

Mathivanan et al. (2010) showed that the *Ervatamia coronaria* methanol leaves extract on *Cx. quinquefasciatus*, *Ae. aegypti*, and *An. stephensi* larvae resulted in LC_{50} values of 72.41, 65.67, and 62.08 mg/L, respectively. Finally, high larvicidal activity was discovered for the essential oil extracted from *Clausena anisata*, with 24 h LC_{50} values of 140.96, 130.19, and 119.59 ppm, respectively (Govindarajan 2010).

Bio-synthesized Ag nanoparticles appeared to have increased larvicidal toxicity at lower concentrations, compared to botanicals alone (Benelli 2016c). In recent years, there has been a heightened interest in the exploration of several plants as sources of reducing agents for the green fabrication of Ag nanoparticles with mosquitocidal properties (Benelli 2015c, 2016a, 2016b). Controls, including experiments trial Ag^{+} particles at the similar applications as Ag nanoparticles, revealed no increased mortality, in accordance with the findings by Marimuthu et al. (2011) and Govindarajan and Benelli (2016b).

Every plant is unique with respect to the nature and combination of compounds it contains that exhibit mosquitocidal and/or repellent properties (Benelli 2015b, 2016a, 2016b, 2016c, Haldar et al. 2013). Combining nanoparticles with bioactive substances, greater antivectorial efficacy has been achieved. In the current experiment, the addition of bio-stabilized AgNPs increased the mixture's larvicidal activity by many folds. Our findings are in concordance with the research of Muthukumaran et al. (2015b), who investigated the mosquito larvicidal action of AgNPs that were fabricated using *Gmelina asiatica* against *Cx. quinquefasciatus* (LC_{50} 27.83 µg/mL), *Ae. aegypti* (LC_{50} 25.77 µg/mL) and *An. stephensi* (LC_{50} 22.44 µg/mL. Govindarajan et al. (2016a) investigated the toicity of *Malva sylvestris*-fabricated AgNPs on *Cx. quinquefasciatus*, *Ae. Aegypti,* and *An. stephensi* larvae. Nanoparticles exhibited increased toxicity against *Cx. quinquefasciatus* (LC_{50} 12.19 µg/mL), *Ae. aegypti* (LC_{50} 11.23 µg/mL), and *An. stephensi* (LC_{50} 10.33 µg/mL) compared to the leaf extract alone. The same research team examined the acute

Table 1. Larvicidal potential of *Hugonia mystax* 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 *(d.f.)*
A. stephensi	80	28.4 ± 1.2	162.66 (143.23–179.69)	326.16 (302.17–357.79)	3.57	$y = 12.51 + 0.225x$	3.626 (4) n.s.
	160	49.6 ± 0.4					
	240	67.5 ± 0.8					
	320	88.3 ± 1.2					
	400	99.2 ± 0.6					
A. aegypti	80	23.5 ± 0.8	181.75 (162.53-199.05)	355.53 (329.42-390.14)	3.17	$y = 7.11 + 0.23x$	1.323 (4) n.s.
	160	45.3 ± 0.6					
	240	62.6 ± 1.2					
	320	84.2 ± 0.4					
	400	96.1 ± 0.8					
C. quinquefasciatus	80	19.3 ± 0.6	199.47 (181.13–216.52)	374.51 (347.41–410.49)	2.57	$y = 1.81 + 0.237x$	1.616 (4) n.s.
	160	41.5 ± 1.2					
	240	57.4 ± 0.4					
	320	80.2 ± 0.8					
	400	94.6 ± 0.6					

[a]Values are mean ± SD of five replicates.
No mortality was noticed in the control.
SD = standard deviation.
LCL–UCL = 95% lower and upper confidence limit.
χ^2 = chi square.
d.f. = degrees of freedom.
n.s. = not significant at $\alpha = 0.05$ level.

Table 2. Larvicidal potential of AgNPs synthesized using the *Hugonia mystax* leaf extract against the *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 *(d.f.)*
A. stephensi	7	27.6 ± 0.8	14.45 (12.84–15.88)	28.11 (26.09–30.76)	3.00	$y = 10.64 + 2.651x$	5.800 (4) n.s.
	14	48.5 ± 0.4					
	21	66.2 ± 1.2					
	28	89.3 ± 0.6					
	35	100.0 ± 0.0					
A. aegypti	7	23.2 ± 1.2	15.86 (14.28–17.30)	30.11 (27.97–32.91)	2.68	$y = 5.57 + 2.721x$	3.063 (4) n.s.
	14	44.6 ± 0.6					
	21	62.4 ± 0.8					
	28	85.3 ± 0.4					
	35	98.1 ± 0.8					
C. quinquefasciatus	7	19.5 ± 0.4	17.46 (15.88–18.94)	32.57 (30.23–35.66)	2.50	$y = 1.3 + 2.729x$	1.597 (4) n.s.
	14	40.2 ± 1.2					
	21	58.4 ± 0.8					
	28	79.6 ± 0.4					
	35	95.3 ± 0.6					

[a]Values are mean ± SD of five replicates.
No mortality was noticed in the control.
SD = standard deviation.
LCL–UCL= 95% lower and upper confidence limit.
χ^2 = chi square.
d.f. = degrees of freedom.
n.s. = not significant at $\alpha = 0.05$ level.

toxicity of Ag nanoparticles biosynthesized with the mediation of *C. spinarum* leaf extract against *Cx. tritaeniorhynchus*, *Ae. albopictus* and *An. subpictus* larvae, obtaining LC_{50} values of 10.04, 9.01, and 8.37 µg/mL, respectively (Govindarajan et al. 2016b); Furthermore, bio-fabricated AgNPs that were biosynthesized through *B. cristata* mediation exhibited larvicidal efficacy against third instar larvae of *Cx. tritaeniorhynchus* (LC_{50} 15.01 µg/mL), *Ae. albopictus* (LC_{50} 13.49 µg/mL), and *An. subpictus* (LC_{50} 12.46 µg/mL); whereas aqueous leaf extract achieved the following LC_{50} values: 146.31, 135.32, and 124.27 µg/mL, respectively (Govindarajan and Benelli 2016a). *B. variegata*-derived aqueous leaf extract showed toxicity against *Cx. tritaeniorhynchus* (LC_{50} 157.24 µg/mL), *Ae. albopictus* (LC_{50} 145.08 µg/mL), and *An. subpictus* (LC_{50} 132.78 µg/mL). The toxicity of Ag nanoparticles was substantiated against Cx. *tritaeniorhynchus*, *Ae. albopictus* and *An. subpictus,* with respective LC_{50} values of 51.92, 46.16, and 41.96 µg/mL (Govindarajan et al. 2016e). Moreover, in addition to their acutely toxic properties, it has been found that very low concentrations of Ag nanoparticles result in the mosquito larvae displaying reduced motility, thereby rendering them more vulnerable to odonate nymphs and other natural enemies of mosquitoes (Benelli 2015c, 2016a, 2016b).

Toxicity on bio-control aquatic organisms

Interestingly, non-target toxicity testing revealed negligible effects against *G. affinis, D. indicus,* and *A. bouvieri* adults, as shown by the achieved LC_{50} values that ranged from 829 to 32192 µg/ml (Tables 3 and 4). Furthermore, our scrutiny pointed that swimming activity and longevity of the studied species were not affected up to a week following testing. SI indicated that *H. mystax*-mediated AgNPs exhibited decreased toxicity against the tested non-target organisms, compared to the targeted mosquito larvae (Table 5). There is currently limited knowledge regarding mosquitocidal essential oil, plant extracts, and green-fabricated NPs toxicity against bio-control organisms (Benelli 2016a, 2016b). Recently, the *Zanthoxylum monophyllum* essential oil, along with its main chemical compounds, were found non-toxic to the predatory fish *G. affinis*. As the LC_{50} for *G. affinis* was calculated at 4234 µg/mL, it is inferred that the essential oil is safe for the fish species, resulting in a high SI value, ranging from 86.39 for the robust *Cx. tritaeniorhynchus* larvae to 102.02 for the more fragile *An. subpictus* larvae (Pavela and Govindarajan 2016). Govindarajan et al. (2016a) investigated that the *M. sylvestris* – fabricated AgNPs also exhibited minimal biotoxicity against aquatic mosquito predators *D. indicus* (LC_{50} 813 µg/mL) and *G. affinis* (LC_{50} 10 459 µg/mL). Furthermore, Govindarajan et al. (2016b) assessed the toxicity of *C. spinarum* aqueous extract and biosynthesized AgNPs on the bio-control organisms *A. bouvieri*, *D. indicus,* and *G. affinis*. Toxicity testing revealed minimal toxicity, obtaining LC_{50} values in the range of 424–6402 µg/mL.

Conclusions

In this study, the *H. mystax*-mediated synthesis of AgNPs was reported as a one-step, low-cost and effective fabrication route of mosquitocidal. Indeed, we biosynthesized AgNPs at room temperature, using inexpensive and non-toxic *H. mystax* leaf extract as starting raw material. A variety of bio-physical methods, including XRD analysis, SEM, TEM, EDX spectroscopy, FTIR spectroscopy, and UV-vis spectrophotometry were used to characterize the bio-reduced Ag nanoparticles. We assessed the effect of *H. mystax* aqueous leaf extract and AgNPs against larvae of filariasis and St. Louis encephalitis vector *Cx. quinquefasciatus,* the dengue and Zika virus vector *Ae. aegypti,* and the malaria vector *An. stephensi.* Our results showed that the bio-fabricated Ag nanoparticles exhibited

Table 3. Effect of *Hugonia mystax* aqueous leaf extract against three bio-control organisms sharing the same ecological niche of *Anopheles* and *Aedes* 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 (*d.f.*)
Gambusia affinis	15,000	25.3 ± 1.2	32192.80 (28794.52–35240.89)	61796.55 (57392.75–67567.76)	2.82	$y = 8.44 + 0.001x$	5.839 (4) n.s.
	30,000	47.6 ± 1.4					
	45,000	65.2 ± 1.5					
	60,000	86.4 ± 0.8					
	75,000	100.0 ± 0.0					
Diplonychus indicus	7000	28.5 ± 0.5	14756.43 (13094.32–16230.74)	29116.24 (26987.41–31924.09)	3.24	$y = 10.41 + 0.003x$	6.229 (4) n.s.
	14,000	45.3 ± 1.2					
	21,000	67.4 ± 1.8					
	28,000	85.2 ± 1.5					
	35,000	100.0 ± 0.0					
Anisops bouvieri	6000	29.2 ± 1.4	12251.72 (10819.31–13512.43)	24358.07 (22574.93–26706.32)	3.38	$y = 12.02 + 0.003x$	5.308 (4) n.s.
	12,000	47.4 ± 1.8					
	18,000	68.5 ± 0.5					
	24,000	87.4 ± 1.5					
	30,000	100.0 ± 0.0					

[a]Values are mean ± SD of five replicates.
No mortality was noticed in the control.
SD = standard deviation.
LCL–UCL= 95% lower and upper confidence limit.
χ^2 = chi square.
d.f. = degrees of freedom.
n.s. = not significant at $\alpha = 0.05$ level.

Table 4. Effect of synthesized AgNPs using the *Hugonia mystax* leaf extract against three bio-control organisms sharing the same ecological niche of *Anopheles* and *Aedes* 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 (*d.f.*)
Gambusia affinis	1200	28.5 ± 1.5	2567.15 (2281.50–2821.28)	4584.36 (4263.30–4996.01)	3.22	$y = 9.81 + 0.015x$	7.009 (4) n.s.
	2400	44.4 ± 1.2					
	3600	66.2 ± 0.8					
	4800	84.3 ± 1.4					
	6000	100.0 ± 0.0					
Diplonychus indicus	500	26.2 ± 1.8	1075.16 (956.76–1180.59)	2109.57 (1955.68–2312.77)	3.14	$y = 9.48 + 0.037x$	6.724 (4) n.s.
	1000	47.4 ± 1.3					
	1500	65.8 ± 0.5					
	2000	83.4 ± 1.2					
	2500	100.0 ± 0.0					
Anisops bouvieri	400	29.3 ± 0.8	829.63 (733.95–914.10)	1647.57 (1526.66–1807.10)	3.36	$y = 11.43 + 0.045x$	6.043 (4) n.s.
	800	46.4 ± 1.2					
	1200	67.2 ± 1.8					
	1600	86.5 ± 0.5					
	2000	100.0 ± 0.0					

[a]Values are mean ± SD of five replicates.
No mortality was noticed in the control.
SD = standard deviation.
LCL–UCL= 95% lower and upper confidence limit.
χ^2 = chi square.
d.f. = degrees of freedom.
n.s. = not significant at $\alpha = 0.05$ level.

Table 5. Suitability index of three bio-control organism over young instars of *Anopheles stephensi, Aedes aegypti,* and *Culex quinquefasciatus* exposed to *Hugonia mystax* aqueous leaf extract and AgNPs.

Treatment	Species	*A. stephensi*	*A. aegypti*	*C. quinquefasciatus*
Hugonia mystax aqueous leaf extract	*Gambusia affinis*	197.91	177.12	161.39
	Diplonychus indicus	90.71	81.19	73.97
	Anisops bouvieri	75.32	67.40	61.42
Green-fabricated Ag nanoparticles	*Gambusia affinis*	177.65	161.86	147.03
	Diplonychus indicus	74.40	67.79	61.57
	Anisops bouvieri	57.41	52.30	47.51

high larvicidal action versus the third instar of these tested mosquitoes, achieving LC_{50} values that ranged from 14 to 17 μg/mL. A further noteworthy point is that the low toxicity testing against natural mosquito predators stressed the eco-friendly profile of these bio-fabricated nanoparticles. Therefore, we proposed the *H. mystax*-mediated biosynthetic pathway as a future good sustainable route for nanomosquitocide fabrication.

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