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Eco-friendly and cost-effective Ag nanocrystals fabricated using the leaf extract of *Habenaria plantaginea*: toxicity on six mosquito vectors and four non-target species

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Abstract Recently, the biofabrication of metal nanoparticles has gained wide interest owing to its inherent features such as swift, simplicity, eco-friendliness, and cheaper costs. Different green-reducing agents led to the production of nanoparticles with varying toxicity on insects. In the current study, silver nanoparticles (AgNPs) were successfully synthesized using *Habenaria plantaginea* leaf extract. Ag nanoparticles were studied by UV–Vis spectroscopy (UV-Vis), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), atomic force microscopy (AFM), scanning electron microscopy (SEM) coupled with energy-dispersive spectroscopy (EDS), and transmission electron microscopy (TEM). *H. plantaginea* extract and AgNPs were tested for mosquito larvicidal activity on *Anopheles stephensi*, *Aedes aegypti*, *Culex quinquefasciatus*, *An. subpictus*, *Ae. albopictus*, and *Cx. tritaeniorhynchus*. LC_{50} values were 102.51, 111.99, 123.47, 123.96, 136.56, 149.42 μg/ml and 12.23, 13.38, 14.78, 14.37, 15.39, 16.89 μg/ml, respectively. Moreover, *H. plantaginea* aqueous extract and AgNPs were tested against the non-target species *Anisops bouvieri*, *Diplonychus indicus*, *Poecilia reticulata*, and *Gambusia affinis* obtaining LC_{50} values ranging from 831.82 to 36,212.67 μg/ml. Overall, this study showed the effectiveness of *H. plantaginea*-fabricated nanoparticles on a wide range of important mosquito vectors, highlighting their scarce toxicity on four natural enemies predating mosquito larvae and pupae.

Keywords Biofabrication · Biosafety · Biopesticide · AFM, SEM, TEM · Zika virus

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Introduction

Mosquitoes (Diptera: Culicidae) are more than 3600 species distributed worldwide, belonging to 37 well-recognized genera. Mosquitoes have a long slender body, long legs, and long needle-shaped mouthparts. Some species mainly bite at dusk (e.g., *Anopheles*), during the night (e.g., *Culex*) or even during the whole daylight period (e.g., *Aedes albopictus*). Mosquitoes act as vectors for key life-threatening diseases, such as malaria, yellow fever, dengue fever, chikungunya fever, filariasis, encephalitis, and West Nile and Zika viruses, in almost all tropical and subtropical countries, as well as many other parts of the world (Govindarajan et al. 2012; Benelli, 2015a, b; WHO 2014; Dhiman et al. 2010; Benelli et al. 2016; Benelli et al. 2017a,b,c). Humans

have used plant parts, related products, and metabolites in vector control since early historical times. Indeed, plants produce many chemicals, some of which have medicinal and insecticidal properties (Pavela and Benelli 2016a,b; Naqqash et al. 2016; Benelli and Mehlhorn 2016; Pavela 2015; WHO 1980).

The field of nanotechnology is one of the most active areas of research in current material science. The synthesis and characterization of noble metal nanoparticles such as silver, gold, and platinum are an emerging field of research due to their important applications in biotechnology, bioengineering, water treatment, electronics, magnetics, optoelectronics, and pest control (Rafiuddin 2013; Murugan et al. 2015; Benelli and Lukehart 2017). To deal with the abovementioned issues, in latest years, a wide array of plant-borne preparations has been proposed for the green synthesis of nanoparticles, without using high pressure, energy, temperature, or extremely toxic chemicals (Rajan et al. 2015).

Recently, emerging a number of botanicals are screened successfully for nanosynthesis of silver nanoparticles (Ag NPs), including *Acalypha indica* (Krishnaraj et al. 2010), *Citrus limon* (Prathna et al. 2011), *Allium sativum* (Rastogi and Arunachalam 2011), *Trianthema decandra* (Geethalakshmi and Sarada 2012), *Terminalia chebula* (Edison and Sethuraman 2012), *Mimusops elengi* (Prakash et al. 2013), *Ficus religiosa* (Antony et al. 2013), *Morinda citrifolia* (Suman et al. 2013), *Piper pedicellatum* (Tamuly et al. 2013), *Artemisia nilagirica* (Vijayakumar et al. 2013), *Tephrosia purpurea* (Ajitha et al. 2014), *Tribulus terrestris* (Ashokkumar et al. 2014), *Anacardium occidentale* (Balavigneswaran et al. 2014), *Melia dubia* (Kathiravan et al. 2014), *Pulicaria glutinosa* (Khan et al. 2014), *Boerhaavia diffusa* (Kumar et al. 2014), *Azadirachta indica* (Nazeruddin et al. 2014; Murugan et al. 2016), and *Delonix elata* (Sathiya and Akilandeswari 2014). Notably, it has been underlined that different green-reducing herbal preparations led to the production of metal and metal oxide nanoparticles with varying toxicity on insect pests and vectors (see Benelli 2016a,b for reviews).

Habenaria plantaginea Lindl. is a terrestrial species belonging to the family Orchidaceae. It is an endemic plant of south India (Mabberley 2008; Medhi and Chakrabarti 2009). It is very common in the Indian forests of eastern peninsular flora from Periyakombai Hill, at 450–650 m a.s.l. An ovoid-globose tuber giving rise to an erect, glabrous, bracteates stem carrying 3 to 7, sub-basal, clustered, elliptical-oblong to oblong-lanceolate, subacute to acute, sub-sessile, basally clasping leaves that blooms in the late summer and early fill on an erect, laxly 5 to 9 flowered, glabrous, 2 to 7 cm long inflorescence with lanceolate, acuminate, largest towards the base, setaceous margined floral bracts carrying faintly fragrant flowers (Chowdhery 2009). *H. plantaginea* tubers are used in folk medicine to treat cough, asthma, helminthiasis, and snake bites (Maridass et al. 2008; Singh and Sanjiv 2009), as well as for the treatment of tuberculosis and paralysis (Mohammad 2011).

The use of environmentally benign materials such as green-fabricated metal and metal oxide nanoparticles offers numerous benefits of eco-friendliness and compatibility for larvicidal and oviposition-deterrent applications (Benelli and Govindarajan 2017). Here, we proposed a swift method of green synthesis of Ag nanocrystals using the aqueous leaf extract of *H. plantaginea*. Bio-fabricated AgNPs were characterized by UV–vis. spectroscopy, FTIR, XRD, AFM, SEM with EDX, and TEM analyses. The mosquito larvicidal potential of *H. plantaginea* leaf extract and *H. plantaginea*-fabricated AgNPs was tested on six species, *An. stephensi*, *Ae. aegypti*, *Cx. quinquefasciatus*, *An. subpictus*, *Ae. albopictus*, and *Cx. tritaeniorhynchus*. Moreover, we investigated the impact of *H. plantaginea* aqueous extract and *H. plantaginea*-fabricated AgNPs on four non-target species predating Culicidae young instars.

Materials and methods

Chemicals

Silver nitrate ($AgNO_3$) was purchased from Merck (Germany). Normal saline, double distilled water, and demineralized water were used throughout the experiments.

Preparation of *H. plantaginea* leaf extract and nanosynthesis

H. plantaginea leaves were collected during February 2016 in Kodiyakkarai forest, Nagapattinam district, Tamil Nadu, India. The species identity was confirmed at the Department of Botany, Annamalai University, India. The extract was prepared using 50 g of dried leaf powder in 500 mL of boiled and cooled distilled water, following the method reported by Benelli and Govindarajan (2017).

The *H. plantaginea* aqueous extract was challenged with silver nitrate solution (1 mM) under controlled parameters and ambient conditions. The bioreduction was monitored by UV–Vis spectroscopy. AgNPs were characterized by UV–Vis spectroscopy, FTIR, XRD, EDX, SEM, AFM, and TEM.

Larvicidal efficacy

All mosquitoes tested here are laboratory-reared strains of Indian origin. They were originally collected 10 years ago from Vector Control Research Centre, Pondicherry

Following the method by Govindarajan and Benelli (2016), the six mosquito vectors were reared under laboratory condition (25–28 °C). The larvicidal activity was studied as indicated by WHO (2005). The aqueous extract and green-synthesized AgNPs were prepared at different concentrations of 50–300 and 6–35 μg/ml, respectively. Twenty-five reared early third instar-stage larvae were tested in each replicate. Five replicates were done for each concentration. Mortality of the larvae was calculated after 24 h (Benelli et al. 2017d).

Toxicity on non-target predators

We followed the method by Sivagnaname and Kalyanasundaram (2004). The effect of aqueous extract and AgNPs was evaluated on *A. bouvieri*, *D. indicus*, *P. reticulata*, and *G. affinis*. We tested the four non-target organisms as indicated by Benelli et al. (2017d). The aqueous extract of *H. plantaginea* and AgNPs were evaluated at doses >50 times higher if compared to the LC_{50} values estimated for the six mosquito larvae. Ten replicates were done for each dose, plus four replicates of untreated controls.

Data analysis

Mosquito and natural enemy mortality rates were presented as means ± SD. All the statistical analyses were performed by SPSS version 11.5. LC_{50} and LC_{90} values were estimated using probit analysis (Finney 1971), and non-significant chi-squares were calculated (Benelli 2017). In assays focusing on toxicity against non-target organisms, the Suitability Index (SI) was calculated as described by Deo et al. (1988). A probability level of $P < 0.05$ was used for the significance of differences between values.

Results and discussion

Toxicity on mosquitoes and non-target predators

The larvicidal activity of *H. plantaginea* aqueous leaf extract and *H. plantaginea*-synthesized AgNPs was studied here. Our results showed promising larvicidal activities (Tables 1 and 2) on the early third instar larvae of the six mosquito species after 24 h of exposure; the *H. plantaginea* extract had LC_{50} values of 102.51, 111.99, 123.47, 123.96, 136.56, and 149.42 μg/ml on *An. stephensi*, *Ae. aegypti*, *Cx. quinquefasciatus*, *An. subpictus*, *Ae. albopictus*, and *Cx. tritaeniorhynchus*, respectively (Table 1). Knowledge about mosquito botanical larvicides has been recently critically discussed by Pavela (2015). While essential oils are more difficult to formulate in a polar solvent like water, aqueous plant extracts represent an easier choice (Pavela and Benelli 2016b). However, in most of the cases, the precise analysis of their chemical constituents is difficult to conduct, requiring NMR and HPTLC. Concerning the activity of plant extracts on mosquitoes, Govindarajan et al. (2008) reported that *Ac. indica* leaves extracted with different solvents, i.e., benzene, chloroform, ethyl acetate, and methanol, acted as larvicides on *An. stephensi* with LC_{50} of 19.25, 27.76, 23.26, and 15.03 ppm, respectively. Later, Govindarajan (2009) investigated the bio-efficacy of *Cassia fistula* leaf extract with different solvents, i.e., methanol, benzene, and acetone, on larvae of the chikungunya vector *Ae. aegypti*, showing LC_{50} of 10.69, 18.27, and 23.95 mg/l, respectively. Larvicidal activity of *Ficus benghalensis* leaves extracted with methanol, benzene, and acetone and tested on *Cx. quinquefasciatus*, *Ae. aegypti*, and *An. stephensi* achieved LC_{50} of 41.43, 58.21, and 74.32 ppm; 56.54, 70.29, and 80.85 ppm; and 60.44, 76.41, and 89.55 ppm, respectively (Govindarajan 2010). The mosquito larvicidal potential of benzene and ethyl acetate extracts of *Ervatamia coronaria* and *Caesalpinia pulcherrima* leaves on *An. stephensi*, *Ae. aegypti*, and *Cx. quinquefasciatus* was showed by LC_{50} and LC_{90} values of 79.08, 89.59, and 96.15 ppm and 150.47, 166.04, and 174.10 ppm, respectively (Govindarajan et al. 2011). Rajeswary and Govindarajan (2013) studied that the mosquito larvicidal properties of *Ageratina adenophora* against *Cx. quinquefasciatus*, *Ae. aegypti*, and *An. stephensi* with the leaf methanol extract LC_{50} of 144.86, 132.82, and 113.08, respectively. Govindarajan and Sivakumar (2014) investigated the larvicidal effect of crude hexane, benzene, chloroform, ethyl acetate, and methanol solvent extracts of *Erythrina indica* on *An. stephensi*, *Ae. aegypti*, and *Cx. quinquefasciatus*. The highest larval mortality was obtained by a treatment with leaf methanol extract, with LC_{50} and LC_{90} values of 69.43, 75.13, and 91.41 ppm and 125.49, 134.31, and 167.14 ppm, respectively.

The use of botanical extracts to fabricate nanomaterials improves their toxicity against mosquito vectors (Benelli 2016a,b). In our assays, the larvicidal activity of *H. plantaginea*-synthesized AgNPs showed LC_{50} values of 12.23, 13.38, 14.78, 14.37, 15.39, and 16.89 μg/ml, respectively (Table 2). These values are extremely low if compared to the activity of the most tested raw plant extracts. However, in some cases, lower values have been estimated for other green-fabricated nanoparticles, showing that different botanicals used as reducing and capping agents plant also a role in determining the bioactivity of the nanoparticles. For example, *Pongamia pinnata*-mediated synthesized AgNPs acted as an effective mosquito larvicidal on *Ae. albopictus* with the LC_{50} of 0.25 ppm (Naik et al. 2014). The larvicidal potential of *Leucas aspera*-synthesized

Table 1 Larvicidal activity of *Habenaria plantaginea* aqueous leaf extract on the mosquito species *Anopheles stephensi*, *Aedes aegypti*, *Culex quinquefasciatus*, *An. subpictus*, *Ae. albopictus*, and *Cx. tritaeniorhynchus*

Mosquito species	Dose (μg/ml)	Mortality (%) ± SD[a]	LC_{50} (μg/ml) (LCL-UCL)	LC_{90} (μg/ml) (LCL-UCL)	Slope	Regression equation	χ^2 (*d.f.*)
An. stephensi	50	27.5 ± 1.2	102.51 (90.72–112.90)	202.16 (187.49–221.45)	3.22	$y = 11.55 + 0.366x$	4.885 (4) n.s.
	100	49.2 ± 0.8					
	150	68.4 ± 0.6					
	200	87.3 ± 1.2					
	250	100.0 ± 0.0					
Ae. aegypti	50	23.8 ± 0.4	111.99 (100.35–122.48)	216.24 (200.63–236.81)	2.89	$y = 7.02 + 0.374x$	3.455 (4) n.s.
	100	45.6 ± 1.2					
	150	64.3 ± 0.8					
	200	83.2 ± 0.6					
	250	98.4 ± 1.2					
Cx. quinquefasciatus	50	19.4 ± 0.8	123.47 (112.15–133.98)	230.82 (214.27–252.73)	2.53	$y = 1.88 + 0.381x$	2.875 (4) n.s.
	100	41.8 ± 1.2					
	150	59.2 ± 0.6					
	200	78.6 ± 0.4					
	250	96.3 ± 1.2					
An. subpictus	60	28.5 ± 0.8	123.96 (109.98–136.35)	243.11 (225.49–266.25)	3.12	$y = 10.95 + 0.307x$	5.772 (4) n.s.
	120	47.3 ± 0.6					
	180	66.7 ± 1.2					
	240	88.4 ± 0.4					
	300	100.0 ± 0.0					
Ae. albopictus	60	24.7 ± 1.2	136.56 (122.34–149.40)	265.53 (246.09–291.28)	2.99	$y = 6.76 + 0.308x$	2.757 (4) n.s.
	120	43.8 ± 0.6					
	180	61.9 ± 0.4					
	240	83.6 ± 1.2					
	300	97.3 ± 0.8					
Cx. tritaeniorhynchus	60	20.3 ± 0.6	149.42 (135.77–162.13)	279.70 (259.53–306.44)	2.54	$y = 1.61 + 0.317x$	1.766 (4) n.s.
	120	39.7 ± 1.2					
	180	58.2 ± 0.8					
	240	79.6 ± 0.4					
	300	95.4 ± 1.2					

[a] Values are mean ± SD of five replicates

Table 2 Larvicidal activity of Ag nanoparticles synthesized using the *Habenaria plantaginea* leaf extract on six mosquito vectors

Mosquito species	Dose (μg/ml)	Mortality (%) ± SD[a]	LC_{50} (μg/ml) (LCL-UCL)	LC_{90} (μg/ml) (LCL-UCL)	Slope	Regression equation	χ^2 (*d.f.*)
An. stephensi	6	28.4 ± 0.6	12.23 (10.83–13.46)	24.02 (22.28–26.29)	3.14	$y = 11.54 + 3.067x$	4.637 (4) n.s.
	12	47.6 ± 1.2					
	18	69.3 ± 0.8					
	24	88.4 ± 0.4					
	30	100.0 ± 0.0					
Ae. aegypti	6	23.9 ± 0.8	13.38 (12.00–14.63)	25.75 (23.90–28.17)	2.84	$y = 6.91 + 3.132x$	2.896 (4) n.s.
	12	44.7 ± 1.2					
	18	65.8 ± 0.6					
	24	83.6 ± 0.4					
	30	98.4 ± 1.2					
Cx. quinquefasciatus	6	19.5 ± 0.4	14.78 (13.43–16.04)	27.55 (25.58–30.13)	2.50	$y = 1.66 + 3.197x$	2.320 (4) n.s.
	12	40.2 ± 1.2					
	18	61.6 ± 0.8					
	24	78.4 ± 0.6					
	30	96.3 ± 1.2					
An. subpictus	7	27.4 ± 1.2	14.37 (12.74–15.82)	28.19 (26.16–30.86)	3.13	$y = 11.19 + 2.633x$	4.520 (4) n.s.
	14	48.2 ± 0.8					
	21	69.5 ± 0.6					
	28	87.3 ± 1.2					
	35	100.0 ± 0.0					
Ae. albopictus	7	24.9 ± 0.8	15.39 (13.73–16.88)	30.12 (27.92–33.01)	3.09	$y = 8.35 + 2.636x$	3.150 (4) n.s.
	14	45.7 ± 0.6					
	21	66.3 ± 1.2					
	28	83.2 ± 0.8					
	35	98.4 ± 1.2					
Cx. tritaeniorhynchus	7	20.5 ± 0.4	16.89 (15.28–18.36)	31.87 (29.58–34.89)	2.62	$y = 3.12 + 2.711x$	1.942 (4) n.s.
	14	41.2 ± 1.2					
	21	62.9 ± 0.6					
	28	79.4 ± 0.8					
	35	96.3 ± 1.2					

[a] Values are mean ± SD of five replicates

Table 3 Toxicity of *Habenaria plantaginea* aqueous leaf extract against four predators of mosquito young instars

Non-target organism	Dose (μg/ml)	Mortality (%) ± SD[a]	LC_{50} (μg/ml) (LCL-UCL)	LC_{90} (μg/ml) (LCL-UCL)	Slope	Regression equation	χ^2 (*d.f.*)
Anisops bouvieri	4000	26.9 ± 0.4	8338.26 (7434.28–9144.90)	16,072.88 (14,925.77–17,574.06)	2.87	$y = 9.82 + 0.005x$	5.907 (9) n.s.
	8000	48.2 ± 1.2					
	12,000	65.7 ± 0.6					
	16,000	89.4 ± 0.8					
	20,000	100.0 ± 0.0					
Diplonychus indicus	6000	27.9 ± 1.2	12,649.20 (11,225.65–13,912.40)	24,931.49 (23,118.37–27,316.23)	3.21	$y = 10.17 + 0.003x$	2.749 (9) n.s.
	12,000	44.7 ± 0.8					
	18,000	68.3 ± 0.6					
	24,000	87.2 ± 1.2					
	30,000	98.6 ± 0.4					
Poecilia reticulata	15,000	25.6 ± 0.8	32,523.97 (29,242.09–35,495.42)	61,282.89 (56,995.89–66,866.94)	2.61	$y = 6.85 + 0.001x$	3.929 (9) n.s.
	30,000	43.2 ± 1.2					
	45,000	66.8 ± 0.6					
	60,000	88.3 ± 0.4					
	75,000	99.4 ± 1.2					
Gambusia affinis	15,000	21.7 ± 0.6	36,212.67 (32,940.26–39,253.84)	66,813.86 (62,142.72–72,934.33)	2.41	$y = 1.5 + 0.001x$	3.367 (9) n.s.
	30,000	39.5 ± 1.2					
	45,000	58.3 ± 0.8					
	60,000	83.9 ± 0.4					
	75,000	97.2 ± 1.2					

[a] Values are mean ± SD of ten replicates

AgNPs on *Ae. aegypti* has been also studied, reporting an LC_{50} of 8.5632 mg/l (Suganya et al. 2014). Green synthesis of AgNPs using the leaf extract of *Pithecellobium dulce* on the filariasis vector *Cx. quinquefasciatus* showed an LC_{50} of 21.56 mg/L (Raman et al. 2012). About fungi, *Agaricus bisporus*-mediated fabrication of AgNPs on *Culex* vectors showed dose-dependent mortality rates when tested at 5 mg/L (100% mortality), 2.5 mg/L (81%), 1.25 mg/L (62%), 0.625 mg/L (28%), and 0.312 mg/L (11%) (Dhanasekaran and Thangaraj 2013). Biofabrication of AgNPs using

Table 4 Toxicity of Ag nanoparticles prepared using the *Habenaria plantaginea* leaf extract on four predators of mosquito young instars

Non-target species	Dose (μg/ml)	Mortality (%) ± SD[a]	LC_{50} (μg/ml) (LCL-UCL)	LC_{90} (μg/ml) (LCL-UCL)	Slope	Regression equation	χ^2 (*d.f.*)
Anisops bouvieri	400	27.4 ± 0.6	831.82 (741.73–912.24)	1601.37 (1487.37–1750.37)	2.85	$y = 9.79 + 0.047x$	4.819 (9) n.s.
	800	46.2 ± 1.2					
	1200	68.3 ± 0.8					
	1600	88.9 ± 0.4					
	2000	100.0 ± 0.0					
Diplonychus indicus	600	24.8 ± 1.2	1286.90 (1148.64–1410.52)	2489.26 (2311.35–2722.37)	2.92	$y = 8.68 + 0.031x$	2.389 (9) n.s.
	1200	47.4 ± 0.8					
	1800	66.5 ± 0.6					
	2400	87.2 ± 1.2					
	3000	98.6 ± 0.4					
Poecilia reticulata	1500	28.9 ± 0.8	3102.22 (2745.75–3417.22)	6141.95 (5693.83–6731.49)	3.30	$y = 11.2 + 0.012x$	3.859 (9) n.s.
	3000	46.2 ± 0.6					
	4500	67.4 ± 1.2					
	6000	88.6 ± 0.4					
	7500	99.1 ± 1.2					
Gambusia affinis	1500	23.6 ± 0.8	3486.10 (3133.90–3806.23)	6717.78 (6228.99–7364.49)	2.86	$y = 5.47 + 0.012x$	1.375 (9) n.s.
	3000	41.9 ± 0.6					
	4500	62.3 ± 1.2					
	6000	83.4 ± 0.4					
	7500	96.2 ± 1.2					

[a] Values are mean ± SD of 10 replicates

Table 5 Suitability index of four non-target predators over mosquito young instars exposed to *Habenaria plantaginea* aqueous leaf extract and green-synthesized Ag nanoparticles

Treatment	Non-target species	*An. stephensi*	*Ae. aegypti*	*Cx. quinquefasciatus*	*An. subpictus*	*Ae. albopictus*	*Cx. tritaeniorhynchus*
Habenaria plantaginea leaf extract	*Anisops bouvieri*	81.34	74.45	67.53	67.26	61.05	55.80
	Diplonychus indicus	123.39	112.94	102.44	102.04	92.62	84.65
	Poecilia reticulata	317.27	290.41	263.41	262.37	238.16	217.66
	Gambusia affinis	353.25	323.35	293.29	292.13	265.17	242.35
Ag nanoparticles	*Anisops bouvieri*	68.01	62.16	56.28	57.88	54.04	49.24
	Diplonychus indicus	105.22	96.18	87.07	89.55	83.61	76.19
	Poecilia reticulata	253.65	231.85	209.89	215.88	201.57	183.67
	Gambusia affinis	285.04	260.54	235.86	242.59	226.51	206.40

Nerium oleander leaf extract against first to fourth instar larvae of *An. stephensi* led to LC_{50}values of 20.60, 24.90, 28.22, and 33.99 ppm, respectively (Roni et al. 2013). Also, green-synthesized Ag nanocrystals fabricated with *Murraya koenigii* leaf extract were toxic to *An. stephensi* larvae (I-IV) and pupae, with LC_{50} of 10.82, 14.67, 19.13, 24.35, and 32.09 ppm (Suganya et al. 2013). Concerning the potential mechanisms of action of nanoparticles, very little is known and further research is still needed (Benelli 2016b).

In our non-target assays, we tested *H. plantaginea*-synthesized AgNPs and the related aqueous plant extract on insects (*A. bouvieri* and *D. indicus*) and fishes (*P. reticulata* and *G. affinis*). We observed moderate toxicity on *A. bouvieri*, *D. indicus*, *P. reticulata*, and *G. affinis* with LC_{50} values ranging from 831 to 36,212 μg/ml (Tables 3 and 4). The estimated SI indicated that *H. plantaginea*-fabricated AgNPs were less toxic to the four non-target organisms if compared to the targeted mosquito larval populations (Table 5). Recently, a growing number of research evidences highlighted the eco-friendly nature of plant extracts, essential oils as well as AgNPs fabricated using botanicals on non-target species predating mosquitoes (Benelli 2016a,b). For example, the *Atlantia monophylla* extract was safe to aquatic mosquito predators *G. affinis*, *P. reticulata*, and *D. indicus*, with LC_{50} values of 23.4, 21.3, and 5.7 mg/l (Sivagnaname and Kalyanasundaram 2004). *Acacia caesia*-synthesized AgNPs were safer to three non-target *A. bouvieri*, *D. indicus*, and *G. affinis*, with LC_{50} ranging from 684 to 2245 μg/ml (Benelli et al. 2017d). Essential oil from *Amomum subulatum* was safer to *D. indicus*, *G. affinis*, and *P. reticulata* with LC_{50} range of 3123–9104 μg/ml (Govindarajan et al. 2017). Also, green-synthesized AgNPs obtained using the leaf extract of *Rubus ellipticus* as reducing and capping agent were scarcely toxic to *A. bouvieri*, *D. indicus*, and *G. affinis*, with LC_{50} from 896 to 2261 μg/ml (AlQahtani et al. 2017). Further researches aimed to understand the fate and long-term toxicity of residual nano-Ag concentrations in water bodies are ongoing (Banumathi et al. 2017).

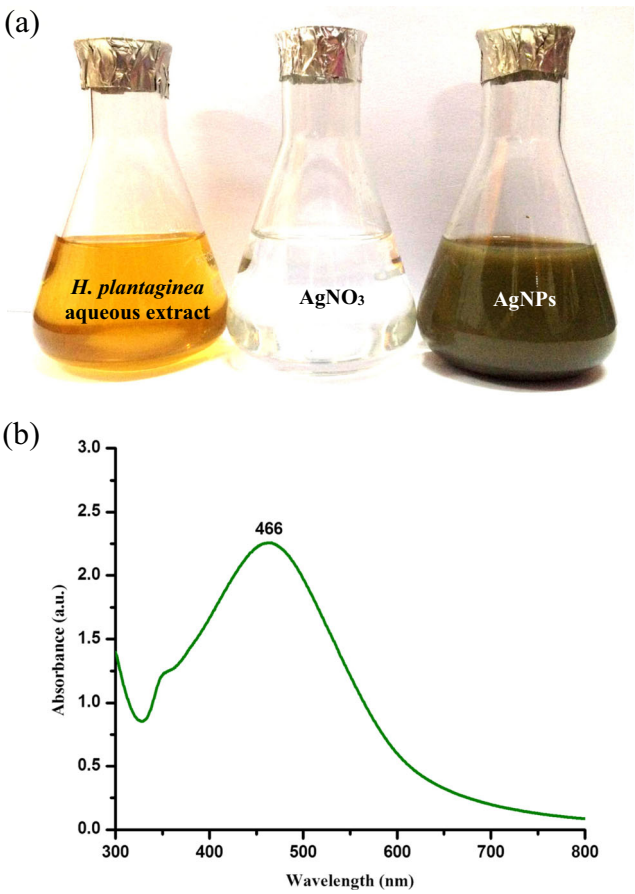

Fig. 1 **a** Color of the *Habenaria plantaginea* aqueous extract before (*left*) and after (*right*) the reduction of $AgNO_3$ (1 mM). **b** UV-visible spectrum of green Ag nanoparticles synthesized using the leaf extract of *H. plantaginea*

Bionanosynthesis and nanocharacterization

UV–Vis spectroscopy

The reduction of silver nitrate to AgNP by the leaf extract of *H. plantaginea* was confirmed by measuring the UV–Vis spectrum of the nano- suspension. The silver nitrate solution was added to the yellow aqueous leaf extract. The color change to brown confirmed the formation of AgNP (Fig. 1a). This color change was due to the reduction of Ag^{+} to Ag^{0} by various biomolecules present in the leaf extract. The absorption spectrum of AgNP was recorded and is depicted in Fig. 1b. The AgNP showed a characteristic absorption peak at a wavelength of 466 nm due to surface plasmon resonance (SPR). The spherical shape of the AgNP was confirmed by the kmax of 466 nm. According to the literature (Prathna et al.

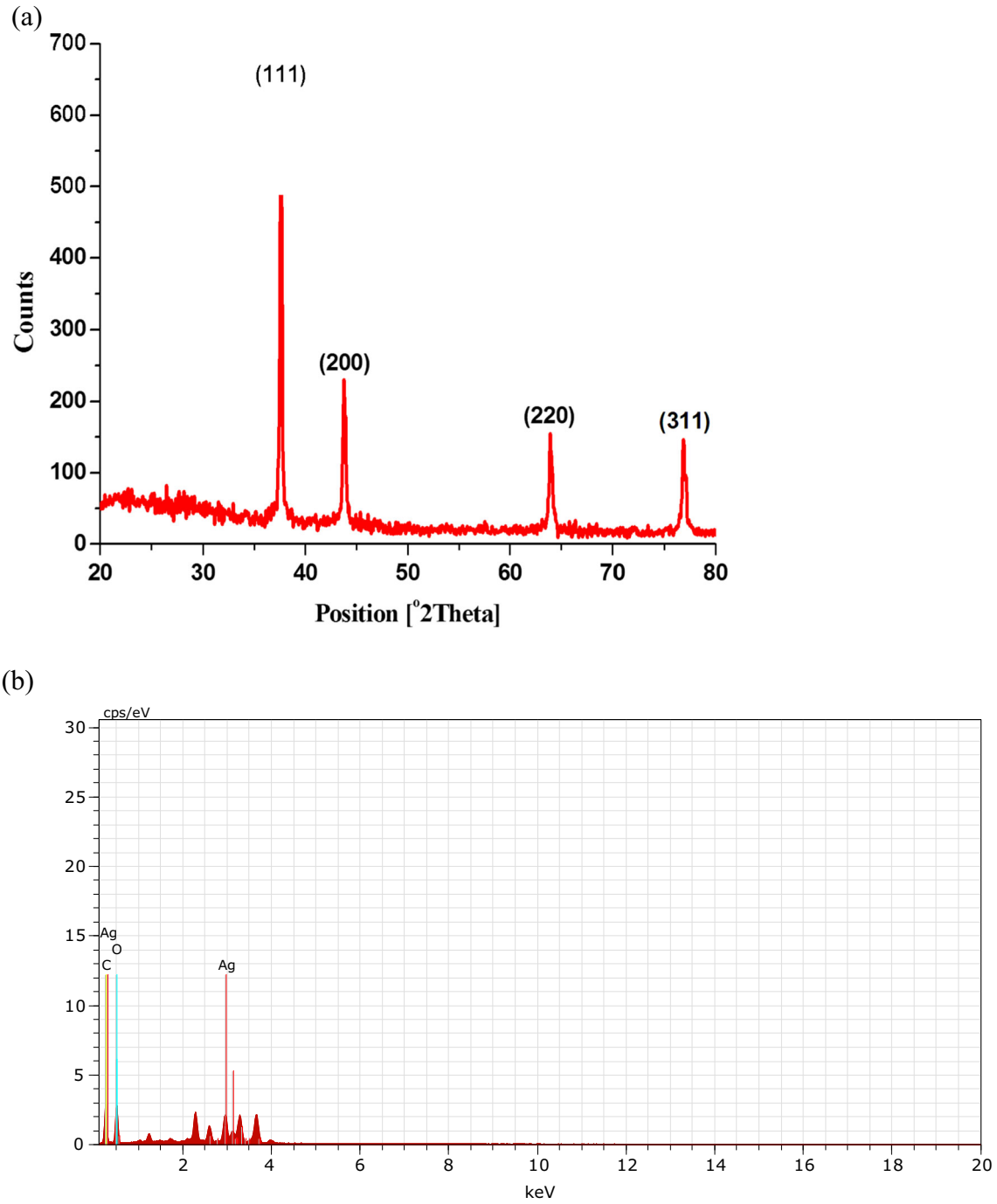

Fig. 2 XRD pattern (**a**) and EDX spectrum (**b**) of Ag nanoparticles fabricated using the leaf extract of *H. plantaginea*

2011), absorption bands in the range 400–480 nm in the UV–Vis spectrum correspond to spherical-shaped metallic nanoparticles. The presence of a single peak in the figure corroborated the spherical shape of *H. plantaginea*-synthesized AgNPs according to Mie theory (Prathna et al. 2011).

XRD and EDX analyses

The X-ray diffraction (XRD) profile of *H. plantaginea*-synthesized AgNPs is depicted in Fig. 2a. The four distinct peaks at $2\theta = 38.52, 44.28, 63.97$, and 78.34 were interpreted as (1 1 1), (2 0 0), (2 2 0), and (3 1 1) lattice planes respectively, showing the face-centered cubic (fcc) structure of metallic Ag (Maity et al. 2011, 2012).

Energy-dispersive spectrum revealed the presence of elemental silver in the sample (Fig. 2b), as underlined by the sharp peak at 3 keV. The occurrence of other peaks (O and C) was presumably related with capping action of metabolites from *H. plantaginea* extract (Madhumitha et al. 2015).

FTIR spectroscopy

As shown by Fig. 3, the FTIR spectrum exhibited a number of major peaks at 3461.11, 2918.18, 2849.30, 2427.91, 1639.84, 1382.55, 1115.14, 1046.26, 841.64, and 788.97 cm^{-1}. The peak at 3461.11 could be due to –OH stretching from alcohols and phenols in the leaf extract. The small band at 2918.18 can be due to the C–H stretching of alkanes. The possible presence of carboxylic acid (O–H stretch) was observed at 2849.30 and 2427.91. The medium band observed at 1639.84 implied the stretching vibrations of C=C functional groups of aromatics. A strong peak at 1382.55 denoted the bending vibrations of sulfate (S=O stretching) and suggested the possible binding of S-NPs with the proteins present in the extract. The small peaks at 1115.14 can be due to the P=O stretching of phosphine oxide present in the extract. The band observed at 1046.26, 841.64, and 788.97 can indicate P=OR and S=OR stretching modes of esters from the *H. plantaginea* extract (Mishra and Sardar 2012; Tamboli and Lee 2013; Perni et al. 2014).

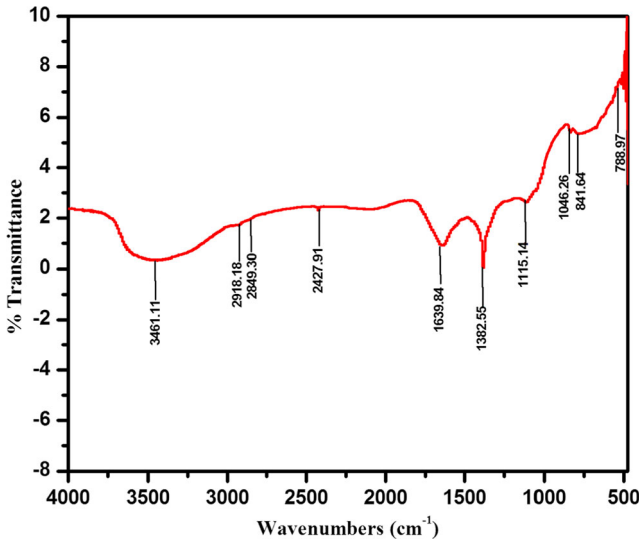

Fig. 3 FTIR spectrum of green synthesized Ag nanoparticles using the leaf extract of *H. plantaginea*

AFM, SEM, and TEM

H. plantaginea-synthesized AgNPs studied using AFM showed polydispersed and spherical structures, with size from 0.1 to 29 nm (Fig. 4). SEM (Fig. 5a) highlighted well-defined spherical *H. plantaginea*-synthesized AgNPs. The average particle size within the selected area of SEM was 50 nm. The result is comparable with the *Annona squamosa* leaf extract-mediated silver nanoparticles reported by Kumar et al. (2012). Lastly, TEM (Fig. 5b) revealed that

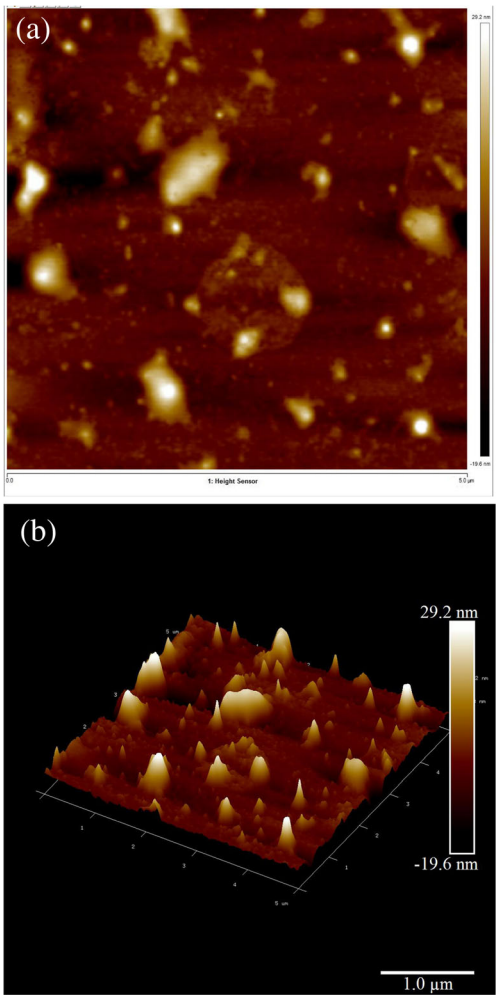

Fig. 4 AFM micrograph of Ag nanoparticles fabricated using the *H. plantaginea* extract (**a**) 1.0 µm resolution studies 29.2 nm size, spherical shaped, poly-dispersed particles. **b** 3D image of Ag nanoparticles analyzed by NOVA-TX software

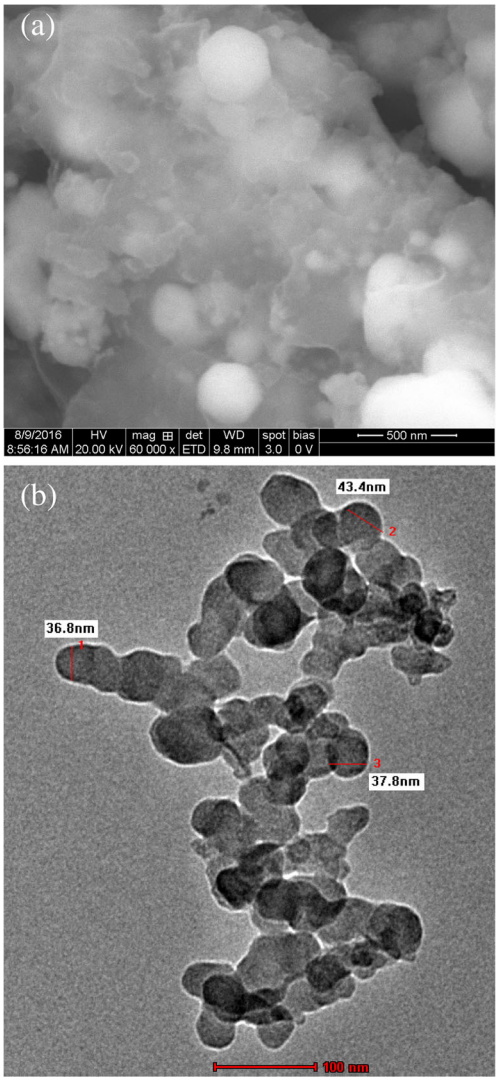

Fig. 5 SEM (**a**) and TEM (**b**) of *H. plantaginea*-fabricated Ag nanoparticles

H. plantaginea-synthesized AgNPs are spherical in shape and are uniformly distributed without significant agglomeration. The crystalline nature of AgNPs is in good agreement with XRD results. *H. plantaginea*-synthesized AgNP size ranges from 20 to 50 nm, with average size of 37.8 nm (see also Roopan et al. 2013).

Conclusions

Overall, the *H. plantaginea*-mediated nanosynthesis led to the production of nanoparticle homogeneous in size, with crystalline structure, as showed by TEM, AFM, and XRD data, respectively. Furthermore, our experiments showed the high efficacy of *H. plantaginea*-fabricated nanocrystals against a wide range of important mosquito vectors, highlighting their scarce toxicity on four natural enemies predating mosquito larvae and pupae.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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