SUPPLEMENTARY MATERIAL

Green-synthesised nanoparticles from *Melia azedarach* seeds and the cyclopoid crustacean *Cyclops vernalis*: an eco-friendly route to control the malaria vector *Anopheles stephensi*?

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Abstract

The impact of green-synthesized mosquitocidal nanoparticles on non-target aquatic predators is poorly studied. In this research, we proposed a single-step method to synthesise silver nanoparticles (Ag NP) using the seed extract of *Melia azedarach*. Ag NP were characterised using a variety of biophysical methods, including UV-vis spectrophotometry, scanning electron microscopy, energy-dispersive X-ray spectroscopy and Fourier transform infrared spectroscopy. In laboratory assays on *Anopheles stephensi*, Ag NP showed LC₅₀ ranging from 2.897 (I instar larvae) to 14.548 ppm (pupae). In the field, the application of Ag NP (10×LC₅₀) lead to complete elimination of larval populations after 72 h. The application of Ag NP in the aquatic environment did not show negative adverse effects on predatory efficiency of the mosquito natural enemy *Cyclops vernalis*. Overall, this study highlights the concrete possibility to employ *M. azedarach*-synthesised Ag NP on young instars of malaria vectors.

Keywords: biological control; biosafety; cyclopoid crustacean; nanobiotechnology; neem

Experimental

Anopheles stephensi rearing

Eggs of *Anopheles stephensi* were collected from local breeding habitats at the National Institute of Communicable Disease Centre (Coimbatore, India) using an “O” type brush. Eggs were transferred to laboratory conditions [$27 \pm 2^\circ\text{C}$, 75-85% R.H., 14:10 (L:D) photoperiod] and placed in 18 x 13 x 4 cm plastic containers containing 500 mL of tap water, waiting for larval hatching. *A. stephensi* larvae were reared in the plastic containers described above, and fed daily with a mixture of crushed dog biscuits (Pedigree, USA) and hydrolyzed yeast (Sigma-Aldrich, Germany) at 3:1 ratio (w:w). Water was renewed every two days. The breeding medium was checked daily and dead individuals were removed. Breeding containers were kept closed with muslin cloth to prevent contamination by foreign mosquitoes. Pupae were collected daily from culture containers, transferred to glass beakers containing 500 ml of water and used in the experiments (Dinesh et al. 2015).

Cyclops vernalis rearing

Copepods were collected from a rural pond (Muthanamkulam, Coimbatore, India) using a mesh net. Collected samples were identified as *C. vernalis* at Department of Zoology of Acharya Nagarjuna University (India). *C. vernalis* was reared following the method reported by Murugan et al. (2015a). Isofemale lines were established from gravid females and maintained at Department of Zoology, Bharathiar University (Coimbatore, India). Gravid females from different isofemale lines were pooled and mass reared in dechlorinated water (pH 7) in fish tanks (15 L) at $27 \pm 2^\circ\text{C}$ and natural photoperiod. Food was *Paramecium* spp. prepared from boiled rice straw water extract and commercial powdered fish food.

Melia azedarach collection and preparation of seed extract

M. azedarach kernels were procured in local neem oil mills close to the Bharathiar University (Coimbatore, India). The material used for preparation of the extract was collected 4 weeks before experiments. The seed extract was prepared adding 10 g of *M. azedarach* crushed kernels in a 300-mL Erlenmeyer flask filled with 100 mL of sterilized double distilled water. The mixture was stored overnight, boiled for 5 min, and finally decanted for 2 days. The extract was filtered using Whatman filter paper No. 1, stored at -15°C and tested within five days.

Green-synthesis and characterization of silver nanoparticles

Following the method reported by Dinesh et al. (2015), the *M. azedarach* seed extract was transferred into an Erlenmeyer flask, treated with an aqueous solution of 1 AgNO_3 (1 mM), and incubated at room temperature. A brown-yellow solution indicated the formation of silver nanoparticles (Ag NP), since aqueous silver ions were reduced by the seed extract generating stable Ag NP in water. Silver nitrate was purchased from the Precision Scientific Co. (Coimbatore, India). The presence of Ag NP was confirmed by sampling the reaction mixture at regular intervals, and the absorption maxima was scanned by UV-vis spectroscopy at the wavelength of 200–600 nm, using a UV-3600 Shimadzu spectrophotometer at 1-nm resolution. The reaction mixture was subjected to centrifugation at 15,000 rpm for 20 min. The resulting pellet was dissolved in deionized water and filtered through a Millipore filter (0.45 μm). An aliquot of the filtrate containing Ag NP was used for SEM, FTIR and EDX (Murugan et al. 2015a). FTIR spectroscopy was carried out using a Perkin-Elmer Spectrum 2000 FTIR spectrophotometer in the diffuse reflectance mode operating at a resolution of 4 cm^{-1} . TEM was performed using a JEOL model 1200 EX instrument operating at an accelerating voltage of 120 kV. Samples were prepared by placing drops of Ag NP formulation on carbon-coated TEM grids. The film on TEM grid was allowed to dry for 5 min in laboratory condition. EDX analyzed the presence of metals in the sample. EDX was conducted using a JEOL-MODEL 6390.

Larvicidal and pupicidal assays in laboratory conditions

Following the methods reported by Murugan et al. (2015), 25 mosquito larvae (I, II, III or IV instar) or pupae were exposed for 24 h in a 500-mL glass beaker filled with dechlorinated water plus *M. azedarach* seed extract (20, 40, 80, 160 and 320 ppm) or green-synthesised Ag NP (2, 4, 8, 16 and 32 ppm). Larval food (0.5

mg) was provided for each tested concentration. Each concentration was replicated five times. In the control, 25 larvae or pupae were transferred in 250 mL of dechlorinated water. No mortality was observed in the control. Percentage mortality was calculated as follows: Percentage mortality = (Number of dead individuals/Number of treated individuals)*100

Larvicidal assays on Anopheles stephensi in the field

The *M. azedarach* seed extract and green synthesised silver Ag NP were applied in six external water reservoirs at the National Institute of Communicable Disease Centre (Coimbatore, India), using a knapsack sprayer (Private Limited 2008, Ignition Products, India). Pre-treatment and post-treatment at 24, 48 and 72 h was conducted using a larval dipper. The larvicidal efficacy was assessed on third- and fourth-instar larvae. Larvae were counted and identified to specific level. Specific identification highlighted that more than 96% of all surveyed larvae belong to *A. stephensi*. Six trials were conducted for each test site with similar weather conditions (27±2°C; 79% R.H.). The required quantity of mosquitocidal was calculated on the basis of the total surface area and volume (0.25 m³ and 250 L); the required concentration was prepared using 10×LC₅₀ values on I instar larvae (Suresh et al. 2015). The percentage reduction of the larval density was calculated using the formula: Percentage reduction = (C – T)/ C × 100, where C is the total number of mosquitoes in control and T is the total number of mosquitoes in treatment.

Predation efficiency of Cyclops vernalis on Anopheles stephensi

C. vernalis adults were collected from small water ponds in Coimbatore (Tamil Nadu, India) and transferred to laboratory conditions [27 ± 2°C and 75-85% R.H.;14:10 (L:D)] photoperiod. In the first experiment, the predation efficiency of *C. vernalis* was assessed on II and III instars larvae of *A. stephensi*. For each replicate, 200 mosquito larvae were introduced, with 20 *Cyclops vernalis*, in plastic cups (5L) containing dechlorinated water. For each instar, five replicates were conducted. Control was 5 L of dechlorinated water without copepods. All experimental arenas were checked after 12 h (daylight time) and 24 h (night time) and the number of preys consumed by the copepods was recorded. After each checking over time, the predated mosquito larvae were replaced with new ones. Predatory efficiency was calculated using the following formula: Predation efficiency = [(Number of consumed mosquitoes/Number of predators)/Total number of mosquitoes]*100

In the second experiment, the predation efficiency of *C. vernalis* was assessed on *A. stephensi* larvae, after a mosquitocidal treatment with Ag NP. Both for II and III instar larvae, 200 mosquitoes were introduced with 20 copepods in a 5 L plastic cups filled with dechlorinated water plus 1 mL of the desired concentration of nanoparticles (i.e. 1 ppm, 1/3 of the LC₅₀ calculated on I instar larvae of *A. stephensi*). For II and III instar larvae, five replicates were conducted. Control was dechlorinated water without copepods. All experimental arenas were checked after 12 h (daylight time) and 24 (night time) h and the number of preys consumed by copepods was recorded. Predation efficiency was calculated using the above-mentioned formula.

Data analysis

Mosquito toxicity data were subjected to ANOVA with two factors (i.e. laboratory experiments: the targeted instar and the dosage; field experiments: the tested compound and the time elapsed from treatment). Means were separated using Tukey's HSD test. The average mosquito mortality data were subjected to probit analysis. LC₅₀ and LC₉₀ were calculated using the method by Finney (1971). Data were analyzed using the SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). A probability level of P < 0.05 was used for the significance of differences between values.

Predation efficiency data were analyzed by JMP 7 (SAS, 1999) using a weighted generalized linear model with one fixed factors: $y = XB + \epsilon$ where y is the vector of the observations (i.e. number of consumed preys), X is the incidence matrix, B is the vector of fixed effects (i.e. the presence of Ag NP and the targeted mosquito instar) and ϵ is the vector of the random residual effect. A probability level of P < 0.05 was used for the significance of differences between values.

Results

Table S1. Acute toxicity of *Melia azedarach* seed extract on larvae and pupae of the malaria vector *Anopheles stephensi*.

Target	Mortality (%) $\pm$ SD					LC ₅₀ (LC ₉₀)	LC ₅₀ (LC ₉₀) 95% Confidence Limit		Regression equation	χ^2
	20 ppm	40 ppm	80 ppm	160 ppm	320 ppm		LCL	UCL		
I	42.61 $\pm$ 3.01 ^a	49.57 $\pm$ 2.09 ^a	57.24 $\pm$ 2.12 ^b	69.11 $\pm$ 2.37 ^c	83.70 $\pm$ 1.84 ^d	46.073 (383.546)	1.613 (314.785)	77.773 (507.030)	y=0.175+0.04 x	1.074 n.s.
II	38.90 $\pm$ 3.10 ^a	46.71 $\pm$ 2.14 ^b	56.59 $\pm$ 1.48 ^c	61.15 $\pm$ 1.40 ^c	76.19 $\pm$ 1.15 ^d	70.686 (494.550)	20.011 (390.256)	108.005 (708.626)	y=214+0.003x	2.324 n.s.
III	32.24 $\pm$ 1.08 ^a	40.95 $\pm$ 1.41 ^b	49.65 $\pm$ 1.17 ^c	59.174 $\pm$ 2.33 ^d	73.42 $\pm$ 0.98 ^e	118.006 (535.088)	78.843 (424.450)	156.544 (756.668)	y=363+0.003x	3.255 n.s.
IV	29.73 $\pm$ 2.00 ^a	35.73 $\pm$ 1.98 ^{ab}	39.11 $\pm$ 2.03 ^b	46.59 $\pm$ 1.48 ^c	61.10 $\pm$ 2.06 ^d	201.265 (709.750)	155.175 536.315	277.573 (1116.823)	y=507+0.003x	0.517 n.s.
Pupae	25.62 $\pm$ 1.03 ^a	31.10 $\pm$ 3.49 ^{ab}	37.75 $\pm$ 4.53 ^b	43.67 $\pm$ 3.15 ^b	73.42.33 $\pm$ 2.36 ^c	253.871 (808.671)	196.337 (595.304)	373.352 (1357.290)	y=586+0.002x	1.537 n.s.

Mortality rates are means $\pm$ SD of five replicates

No mortality was observed in the control

Within each row, means followed by the same letter(s) are not significantly different (P<0.05)

LC₅₀ = lethal concentration that kills 50 % of the exposed organisms

LC₉₀ = lethal concentration that kills 90 % of the exposed organisms

LCL = lower confidence limit

UCL = upper confidence limit

χ^2 = chi-square value

n.s. = not significant (P <0.05)

Table S2. Acute toxicity of *Melia azedarach*-synthesised silver nanoparticles on larvae and pupae of the malaria vector *Anopheles stephensi*.

Target	Mortality (%) $\pm$ SD					LC ₅₀ (LC ₉₀)	LC ₅₀ (LC ₉₀) 95% Confidence Limit		Regression equation	χ^2
	2 ppm	4 ppm	8 ppm	16 ppm	32 ppm		LCL	UCL		
I	48.14 $\pm$ 2.50 ^a	52.47 $\pm$ 1.67 ^a	69.55 $\pm$ 1.78 ^b	88.23 $\pm$ 1.48 ^c	100 $\pm$ 0.00 ^d	2.897 (16.385)	0.875 (14.068)	4.395 (20.029)	y = 0.275 + 0.095x	0.660 n.s
II	43.16 $\pm$ 2.35 ^a	47.36 $\pm$ 1.31 ^a	59.61 $\pm$ 1.77 ^b	78.55 $\pm$ 1.23 ^c	99.26 $\pm$ 0.19 ^d	4.800 (21.082)	2.830 (18.246)	6.423 (25.365)	y = 0.379 + 0.079x	1.056 n.s
III	37.57 $\pm$ 1.79 ^a	43.55 $\pm$ 1.84 ^b	56.53 $\pm$ 1.79 ^c	70.34 $\pm$ 1.52 ^d	95.23 $\pm$ 1.42 ^e	6.554 (26.611)	4.375 (23.062)	8.443 (31.917)	y = 0.419 + 0.066x	0.684 n.s
IV	32.48 $\pm$ 1.30 ^a	39.35 $\pm$ 1.36 ^b	49.29 $\pm$ 1.69 ^c	67.53 $\pm$ 1.53 ^d	83.67 $\pm$ 1.93 ^e	9.372 (36.162)	6.743 (30.808)	11.790 (44.643)	y = 0.448 + 0.048x	2.337 n.s
Pupa	29.59 $\pm$ 1.49 ^a	32.32 $\pm$ 1.69 ^a	46.54 $\pm$ 1.55 ^b	54.45 $\pm$ 1.39 ^c	71.44 $\pm$ 1.69 ^d	14.548 (50.311)	11.385 (41.152)	18.169 (66.836)	y = 0.521 + 0.036x	2.634 n.s

Mortality rates are means $\pm$ SD of five replicates

No mortality was observed in the control

Within each row, means followed by the same letter(s) are not significantly different (P<0.05)

LC₅₀ = lethal concentration that kills 50 % of the exposed organisms

LC₉₀ = lethal concentration that kills 90 % of the exposed organisms

LCL = lower confidence limit

UCL = upper confidence limit

χ^2 = chi-square value

n.s. = not significant (P < 0.05)

Table S3. Field toxicity of *Melia azedarach* seed extract and green-synthesized silver nanoparticles on larval population of *Anopheles stephensi* in water storage reservoirs.

Treatment	<i>Anopheles stephensi</i> larval density (%)			
	Before treatment	24 h	48 h	72 h
<i>Melia azedarach</i> seed kernel extract (10×LC ₅₀)	418.15±20.49 ^a	62.89±8.84 ^b	40.27±7.15 ^c	25.10±6.70 ^d
Green-synthesized Ag nanoparticles (10×LC ₅₀)	419.88±41.19 ^a	52.6±12.38 ^b	31.39±11.79 ^b	0.00±0.00 ^c

Mortality rates are means±SD of six replicates

Within each row, means followed by the same letter(s) are not significantly different (P<0.05)

Figure S1. Chromatic variation of the *Melia azedarach* seed extract before (a) and after (b) the process of reduction of Ag^+ ions to Ag^0 nanoparticles. (c) UV-visualization of the absorption spectra of silver nanoparticles synthesised using *M. azedarach* seed extract plus an aqueous solution AgNO_3 (1 mM) over time.

Figure S2. Transmission electron micrograph of silver nanoparticles green-synthesized using *Melia azedarach* seed extract.

Figure S3. Energy-dispersive X-ray spectrum (EDX) of the silver nanoparticles synthesised using the *Melia azedarach* seed extract.

Figure S4. Fourier transform infrared (FTIR) spectrum of vacuum-dried powder of silver nanoparticles green-synthesised using the *Melia azedarach* seed extract.

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