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RESEARCH ARTICLE

Eco-friendly drugs from the marine environment: spongeweed-synthesized silver nanoparticles are highly effective on *Plasmodium falciparum* and its vector *Anopheles stephensi*, with little non-target effects on predatory copepods

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Abstract Mosquitoes act as vectors of devastating pathogens and parasites, representing a key threat for millions of humans and animals worldwide. The control of mosquito-borne diseases is facing a number of crucial challenges, including the emergence of artemisinin and chloroquine resistance in *Plasmodium* parasites, as well as the presence of mosquito vectors resistant to synthetic and microbial pesticides. Therefore, eco-friendly tools are urgently required. Here, a synergic approach relying to nanotechnologies and biological control strategies is proposed. The marine environment is an outstanding reservoir of bioactive natural products, which have many applications against pests, parasites, and pathogens. We proposed a novel method of seaweed-mediated synthesis of silver nanoparticles (AgNP) using the spongeweed *Codium tomentosum,* acting as a reducing and capping agent. AgNP were characterized by UV–Vis spectroscopy, Fourier transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDX), and X-ray diffraction (XRD). In mosquitocidal assays, the 50 % lethal concentration (LC_{50}) of *C. tomentosum* extract against *Anopheles stephensi* ranged from 255.1 (larva I) to 487.1 ppm (pupa). LC_{50} of *C. tomentosum*-synthesized AgNP ranged from 18.1 (larva I) to 40.7 ppm (pupa). In laboratory, the predation efficiency of *Mesocyclops aspericornis* copepods against *A. stephensi* larvae was 81, 65, 17, and 9 % (I, II, III, and IV instar, respectively). In AgNP contaminated environment, predation was not affected; 83, 66, 19, and 11 % (I, II, III, and IV). The anti-plasmodial activity of *C. tomentosum* extract and spongeweed-synthesized AgNP was evaluated against CQ-resistant (CQ-r) and CQ-sensitive (CQ-s) strains of *Plasmodium falciparum*. Fifty percent inhibitory concentration (IC_{50}) of *C. tomentosum* were 51.34 μg/ml (CQ-s) and 65.17 μg/ml (CQ-r); *C. tomentosum*-synthesized AgNP achieved IC_{50} of 72.45 μg/ml (CQ-s) and 76.08 μg/ml (CQ-r). Furthermore, low doses of the AgNP inhibited the growth of *Bacillus subtilis, Klebsiella pneumoniae,* and

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Salmonella typhi, using the agar disk diffusion and minimum inhibitory concentration protocol. Overall, *C. tomentosum* metabolites and spongeweed-synthesized AgNP may be potential candidates to develop novel and effective tools in the fight against *Plasmodium* parasites and their mosquito vectors. The employ of ultra-low doses of nanomosquitocides in synergy with cyclopoid crustaceans seems a promising green route for effective mosquito control programs.

Keywords *Anopheles stephensi* · *Codium tomentosum* · Malaria · Mosquito-borne diseases · *Plasmodium falciparum* · *Mesocyclops aspericornis* · Anti-bacterial activity

Introduction

Arthropods are dangerous vectors of deadly pathogens and parasites (Mehlhorn et al. 2012; Benelli 2015a). Among them, mosquitoes (Diptera: Culicidae) represent a key threat for millions of humans and animals worldwide. Their medical and veterinary importance is mainly due to the fact that their act as vectors for a number of pathogens and parasites of public health relevance, including malaria, avian malaria, yellow fever, dengue, Japanese encephalitis, Zika virus, Rift Valley fever, Western equine encephalomyelitis, bancroftian and brugian filariae, canine heartworm disease (*Dirofilaria immitis*), and setariosis (*Setaria* spp.) (Benelli and Mehlhorn 2016; Benelli et al. 2016).

Malaria is caused by *Plasmodium* parasites, mainly *Plasmodium falciparum*, *Plasmodium vivax, Plasmodium ovale,* and *Plasmodium malariae*. In addition, periodic reports highlighted the presence of simian malaria parasites found in humans, most of them implicating *Plasmodium knowlesi. Plasmodium* parasites are vectored to vertebrates through the bites of infected *Anopheles* mosquitoes, which bite mainly between dusk and dawn. According to the latest estimates, there were about 198 million cases of malaria in 2013 and an estimated 584,000 deaths. In Africa, most deaths occur among children living where a child dies every minute from malaria, although mortality rates among children have been reduced by an estimated 58 % since 2000 (Mehlhorn 2008; WHO 2016). In addition, besides the fall of malaria infection rates worldwide, with special reference to sub-Saharan Africa, 2015 was an annus mirabilis for malaria control, due to the Nobel Prize to the Chinese scientist Youyou Tu for the discovery of artemisinin and the development of the first vaccine against *P. falciparum* malaria [i.e., RTS,S/AS01 (RTS,S)] (Benelli and Mehlhorn 2016).

However, malaria prevention and control is facing a number of crucial challenges, including the emergence of artemisinin and chloroquine resistance in *Plasmodium* parasites (Benelli and Mehlhorn 2016), as well as the presence of mosquito strains resistant to synthetic and microbial pesticides (Hemingway and Ranson 2000; Aziz et al. 2016; Naqquash et al. 2016). Therefore, eco-friendly tools are urgently required (Rajkumar and Jebanesan 2008; Azizullah et al. 2014; Benelli et al. 2015a, b, c; Pavela 2015a, b; Govindarajan and Benelli 2016). Notably, botanicals have been used by human communities in different parts of the world as mosquitocidals, adult repellents, and oviposition deterrents, against a wide number of mosquito species (e.g., Benelli 2015a, b). People entering into regions where dengue, malaria, or yellow fever risks exist may protect themselves by use of plant-derived repellents (Mehlhorn et al. 2012; Amer and Mehlhorn 2006a, b). On the other hand, people living in endemic regions have to protect themselves by several strategies at the same time, since infection rates of mosquitoes may be extremely high (Amer and Mehlhorn 2006c, d; Semmler et al. 2009; Benelli 2015a).

Biological control of mosquito vectors using predatory copepods, tadpoles, and fishes also received attention (Marten 2000; Murugan et al. 2015a, b, c; Subramaniam et al. 2016a, b). Predation is an important factor for the maintenance of trophic equilibrium of ecological communities, including aquatic ones. Good examples are odonate young instars, water bugs, tadpoles, fishes, crabs, and copepods acting as natural enemies of mosquito young instars (Bowatte et al. 2013; Murugan et al. 2015d, e, f, g 2016). Cyclopoid copepods are crucial invertebrate predators in zooplankton communities, and are frequently present in lakes, drains, ponds, and reservoirs (Chang and Hanazato 2003; Kumar and Hwang 2006). The cyclopoid *Mesocyclops aspericornis* is a generalist predator that preys on a wide spectrum of food types, ranging from rotifers, cladocerans, mosquito larvae, and fish young instars (Kumar et al. 2012). More generally, several species of copepods, including *M. aspericornis*, *Mesocyclops guangxiensis*, *Mesocyclops longisetus*, and *Mesocyclops thermocyclopoides*, have been reported as potential biological control agents against mosquitoes (Rawlins et al. 1997; Manrique-Saide et al. 1998; Schaper 1999; Murugan et al. 2015b).

Nanotechnology has the potential to revolutionize a wide array of applications in the fields of catalysis, sensors, opto-electronics, magnetic devices, drug delivery, anti-microbials, pest management, and parasitology (Scrinis and Lyons 2007; Haverkamp 2010, Govindarajan et al. 2016; Kumar et al. 2016). The utilization of plants for nanoparticle synthesis can be advantageous over other biological processes, because it eliminates the elaborate process of maintaining cell cultures and can also be suitably scaled up for large-scale nanoparticle synthesis (Shankar et al. 2004; Chandramohan et al. 2016; Jaganathan et al. 2016). Recently, several plants have been screened for efficient and rapid extracellular synthesis of metal nanoparticles with different mosquitocidal and anti-splamodial properties (Benelli 2016a, b). The marine

environment is an outstanding reservoir of bioactive natural products, which have many applications against pests, parasites, and pathogens (Kalimuthu et al. 2014; Murugan et al. 2016). Concerning the seaweed-mediated synthesis of nanomosquitocides and anti-plasmodial drugs, good examples included seaweed extracts of *Sargassum muticum* (Madhiyazhagan et al. 2015), *Hypnea musciformis* (Roni et al. 2015), *Ulva lactuca* (Murugan et al. 2015h), and *Centroceras clavulatum* (Murugan et al. 2016).

Codium tomentosum is a species of green seaweeds in the family Codiaceae. Its common names include velvet horn and spongeweed. The spongeweed is native to the north east Atlantic Ocean from the British Isles southwards to the Azores and Cape Verde. It has also been recorded around the coasts of Africa and in various other parts of the world. A lectin named tomentine has been isolated by affinity chromatography from *C. tomentosum*. It shows N-acetylglucosamine-specific activity and has been found to be rich in glycine, threonine, and valin. To the best of our knowledge, there is no information about the insecticidal and anti-plasmodial potential of the spongeweed (WRMS 1967; Valentao et al. 2010).

In this research, we proposed spongeweed-mediated synthesis of silver nanoparticles (AgNP) using *C. tomentosum* as a reducing and capping agent. AgNP were characterized by UV–Vis spectroscopy, Fourier transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM), energy-dispersive x-ray spectroscopy (EDX), and x-ray diffraction (XRD). The potential of the *C. tomentosum* extract and spongeweed-synthesized AgNP were evaluated against chloroquine-sensitive (CQ-s) and chloroquine resistant (CQ-r) strains of *P. falciparum*, as well as against the larvae and pupae of the malaria vector *Anopheles stephensi*. The anti-oxidant and anti-bacteric potential of *C. tomentosum* and AgNP were also assessed. Concerning non-target effect of AgNP in the aquatic environment, the predatory efficiency of *M. aspericornis* copepods was evaluated in standard laboratory conditions and post-treatment with ultra-low doses of spongeweed-synthesized AgNP (i.e., 1 ppm).

Materials and methods

Plant material

C. tomentosum spongeweeds was collected in the Gulf of Mannar (Tamil Nadu, India, latitude from 8° 47′ to 9° 15′ N; longitude from 78° 12′ to 79° 14′ E). *C. tomentosum* spongeweeds were washed with tap water and shade-dried at room temperature for 5 days. Voucher specimens were stored in our laboratories and are available upon request.

DPPH radical scavenging assay

The anti-oxidant activity of the ethanol extract was determined in terms of hydrogen donating or radical scavenging ability using the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), according to the methods by Blois (1958) and Panneerselvam et al. (2016). Sample extract at various concentrations were taken and the volume was adjusted to 100 μl with ethanol. About 5 ml of a 0.1 mM ethanolic solution of DPPH was added to the aliquots of samples and butylated hydroxytoluene (BHT) and rutin were used as standards. Negative control was prepared by adding 100 μl of ethanol in 5 ml of 0.1 mM ethanolic solution of DPPH. The tubes were allowed to stand for 20 min at 27 °C. The absorbance of the sample was measured at 517 nm against the blank (ethanol). Radical scavenging activity of the samples was expressed as 50 % inhibitory concentration (IC_{50}) values, which are the concentrations required to inhibit 50 % of DPPH.

Synthesis and characterization of silver nanoparticles

C. tomentosum aqueous extract was prepared adding 10 g of washed and finely cut spongeweeds in a 300-ml Erlenmeyer flask filled with 100 ml of sterilized double-distilled water and then boiling the mixture for 5 min, before finally decanting it. The extract was filtered using Whatman filter paper no. 1, stored at −4 °C and tested within 5 days. The filtrate was treated with aqueous 1 mM $AgNO_3$ solution in an Erlenmeyer flask and incubated at room temperature. A brown-yellow solution indicated the formation of AgNP, since aqueous silver ions were reduced by the *C. tomentosum* extract generating stable AgNP in water. Silver nitrate was purchased from the Precision Scientific Co. (Coimbatore, India). Green synthesis of AgNP was confirmed by sampling the reaction mixture at regular intervals and the absorption maxima was scanned by UV–Vis, at the wavelength of 200–800 nm in UV-3600 Shimadzu spectrophotometer at 1-nm resolution. Furthermore, the reaction mixture was subjected to centrifugation at 15,000 rpm for 20 min, resulting pellet was dissolved in deionized water and filtered through Millipore filter (0.45 μm). An aliquot of this filtrate containing AgNP was used for scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy, x-ray diffraction (XRD) analysis, and energy-dispersive x-ray (EDX) spectroscopy. The structure and composition of freeze-dried purified AgNP was analyzed by using a 10 kV ultra high-resolution scanning electron microscope with 25 μl of sample was sputter coated on copper stub and the images of AgNP were studied using a FEI QUANTA-200 SEM. The surface groups of the AgNP were qualitatively confirmed by FTIR spectroscopy, with spectra recorded by a PerkinElmer Spectrum 2000

FTIR spectrophotometer. EDX assays confirmed the presence of metals in analyzed samples (Murugan et al. 2015a, b).

Anopheles stephensi rearing

The eggs of *A. stephensi* were collected from water reservoirs in Coimbatore (Tamil Nadu, India) using an "O" type brush. Batches of 100–110 eggs were transferred to 18 cm × 13 cm × 4 cm enamel trays containing 500 ml of water, where eggs were allowed to hatch in laboratory conditions (27 ± 2 °C and 75–85 % R.H.; 14:10 (L/D) photoperiod). *A. stephensi* larvae were fed daily with 5 g of ground dog biscuits (Pedigree, USA) and hydrolyzed yeast (Sigma-Aldrich, Germany) in a 3:1 ratio (Dinesh et al. 2015). Newly emerged larvae, pupae, and adults were collected and used in the experiments.

Mesocyclops aspericornis rearing

M. aspericornis adults were collected from a pond (Coimbatore, India) using a mesh net. All collected samples were identified as *M. aspericornis* by Dr. Y. Ranga Reddy (Department of Zoology, Acharya Nagarjuna University, India) (Murugan et al. 2015b). *M. aspericornis* was reared following the method reported by Kosiyachinda et al. (2003). 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 ± 1 °C and natural photoperiod. Food was *Paramecium* spp. prepared from boiled rice straw water extract, and commercial powdered fish food.

Larvicidal and pupicidal toxicity in laboratory conditions

Twenty-five *A. stephensi* larvae (I, II, III, or IV instar) or pupae were placed for 24 h in a glass beaker filled with 250 ml of dechlorinated water plus the desired concentration of *C. tomentosum* extract (100, 200, 300, 400, and 500 ppm) or green-synthesized AgNP (10, 20, 30, 40, and 50 ppm). Larval food (0.5 mg) was provided for each tested concentration (Kovendan et al. 2012). Each concentration was replicated five times against all instars. Control mosquitoes were exposed for 24 h to the corresponding concentration of the solvent. Percentage mortality was calculated as follows:

$$\text{Percentage mortality} = (\text{number of dead individuals} / \text{number of treated individuals}) \times 100$$

In vitro cultivation of *Plasmodium falciparum*

Following Murugan et al. (2015h, i), CQ-sensitive (CQ-s) strain 3D7 and CQ-resistant (CQ-r) strain INDO of *P. falciparum* were used in in vitro blood stage culture to test the anti-malarial efficacy of *C. tomentosum* ethanol extracts. The culture was maintained at G. Kuppusamy Naidu Memorial Hospital (Coimbatore, India). *P. falciparum* culture was maintained according to the method described by Trager and Jensen (1976), with minor modifications. *P. falciparum* (3D7) cultures were maintained in fresh O^{+ve} human erythrocytes suspended at 4 % hematocrit in RPMI 1640 (Sigma) containing 0.2 % sodium bicarbonate, 0.5 % albumax, 45 μg/l hypoxanthine, and 50 μg/l gentamycin and incubated at 37 °C under a gas mixture of 5 % O_2, 5 % CO_2, and 90 % N_2. Every day, infected erythrocytes were transferred into a fresh complete medium to propagate the culture. For *P. falciparum* (INDO strain) in culture medium, Albumax was replaced by 10 % pooled human serum.

Anti-plasmodial assays

Control stock solutions of CQ were prepared in water (Milli-Q grade). The tested extract and the AgNP suspension were prepared in dimethyl sulfoxide (DMSO). All stocks were diluted with culture medium to achieve the required concentrations. In all cases except CQ, the final solution contained 0.4 % DMSO (which was found to be non-toxic to the parasite). Then, the seaweed extract and AgNP were placed in 96-well flat-bottom tissue culture-grade plates.

The extract of *C. tomentosum* and AgNP were evaluated for anti-malarial activity against *P. falciparum* strains 3D7 and INDO. For drug screening, SYBR green I-based fluorescence assay was used following the method by Smilkstein et al. (2004) and Murugan et al. (2015h, i). Sorbitol-synchronized parasites were incubated under normal culture conditions at 2 % hematocrit and 1 % parasitemia in the absence or presence of increasing concentrations of the tested drugs. CQ was used as positive control. After 48 h of incubation, 100 μl of SYBR Green I solution {0.2 μl of 10,000 X SYBR Green I (Invitrogen)/ml} in lysis buffer [Tris (20 mM; pH 7.5), EDTA (5 mM), saponin (0.008 %; *w/v*), and Triton X-100 (0.08 %; *v/v*)] was added to each well and mixed gently twice with a multi-channel pipette and incubated in the dark at 37 °C for 1 h. Fluorescence was measured with a Victor fluorescence multi-well plate reader (PerkinElmer) with excitation and emission wavelength bands centered at 485 and 530 nm, respectively. The fluorescence counts were plotted against the drug concentration and the 50 % inhibitory concentration (IC_{50}) was determined by an analysis of dose–response curves. Results were validated microscopically by the examination of Giemsa-stained smears of extract-treated parasite cultures (Bagavan et al. 2011; Murugan et al. 2015h).

Predation of *Mesocyclops aspericornis* against *Anopheles stephensi* larvae

The predation efficiency of *M. aspericornis* was assessed against *A. stephensi* larvae. For each instar, 100 mosquitoes were introduced, with 10 copepods, in a glass beaker containing 250 ml of dechlorinated water. Mosquito larvae were replaced daily with new ones. For each mosquito instar, four replicates were conducted. Control was 250 ml of dechlorinated water without copepods. All beakers were checked after 1, 2, 3, 4, and 5 days and the number of prey consumed by copepods was recorded. Predatory efficiency was calculated using the following formula:

$$\text{Predatory efficiency} = [(\text{number of consumed mosquitoes}/\text{number of predators})/\text{total number of mosquitoes}] \times 100$$

Predation of *Mesocyclops aspericornis* against *Anopheles stephensi* larvae post-treatment with silver nanoparticles

Here, the predation efficiency of *M. aspericornis* adults was assessed against *A. stephensi* larvae, after a mosquitocidal treatment with AgNP. For each instar, 100 mosquitoes were introduced with 10 copepods in a glass beaker filled with 250 ml of dechlorinated water plus the desired concentration of *C. tomentosum*-synthesized AgNP (i.e., one third of the 50 % lethal concentration (LC_{50}) calculated against first instar larvae of *A. stephensi,* 1 ppm). Mosquito larvae were replaced daily with new ones. For each mosquito instar four replicates were conducted. Control was dechlorinated water plus AgNP, without copepods. All beakers were checked after 1, 2, 3, 4, and 5 days and the number of prey consumed by copepods was recorded. Predatory efficiency was calculated using the above-mentioned formula.

Anti-bacterial activity

C. tomentosum-synthesized AgNP were tested against *Bacillus subtilis, Klebsiella pneumoniae* and *Salmonella typhi*. All bacteria strains were provided by Microbial Type Culture, Collection and Gene Bank Institute of Microbial Technology, Sector 39-A, Chandigarh-160036 (India). For all species, bacterial cultures 18–24-h old were used for the preparation of testing cultures. All bacteria were grown in nutrient broth: peptone (5 g/l), hydrolyzed yeast extract (1.50 g/l), beef extract (1.50 g/l), and sodium chloride (5 g/l), pH 7.4. Thirteen grams of nutrient broth was suspended into 100 ml of distilled water. Twenty-five milliliters of nutrient broth was transferred in each of four conical glass flasks and autoclaved at 121 °C for 15 min (15 psi). Then, each bacterial strain was inoculated and incubated at 37 °C for 24 h. After this phase, the culture attained 2×10^{-6} cfu/ml and was used for anti-bacterial assays. The anti-bacterial activity of *C. tomentosum*-synthesized AgNP was assessed using the agar disk diffusion method (Dinesh et al. 2015). The tested bacteria strains were swabbed on Muller-Hinton agar medium plates. Three sterilized filter paper disks treated with three different concentrations of the tested compounds were inserted in each plate. The plates were incubated at 37 °C for 24 h. After the incubation, the zones of inhibition radius were measured using a photomicroscope (Leica ES2, Germany).

Data analysis

SPSS software package 16.0 version was used for all analyses. Data from DPPH radical scavenging assay and mosquitocidal experiments were analyzed by probit analysis, calculating IC_{50} and LC_{50} values, respectively (Finney 1971). In anti-plasmodial assays, values were expressed as percentage growth inhibition. The concentration causing 50 % inhibition of parasite growth (IC_{50}) was calculated from the drug concentration-response curves. Bacteria inhibition growth data were transformed into arcsine$\sqrt{}$proportion values and analyzed using a two-way ANOVA with three factors (i.e., tested dose and targeted species). Means were separated using Tukey's HSD test ($P < 0.05$).

Copepod predation data were analyzed by JMP 7 using a weighted generalized linear model with one fixed factor: $y = X\beta + \varepsilon$ where y is the vector of the observations (i.e., the number of consumed preys), X is the incidence matrix, β is the vector of fixed effects (i.e., the targeted 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 and discussion

Synthesis and bio-physical characterization of silver nanoparticles

In our experiments, UV–Vis spectrum showed a maximum absorbance peak at 420 nm, which increased over time during the incubation of silver nitrate with the *C. tomentosum* extract (Fig. 1). The broadness of the peak is a good indicator of the size of the nanoparticles. As the particle size increases, the peak becomes narrower, with a decreased bandwidth (Petit et al. 1993; Kong and Jang 2006). It is generally recognized that UV–Vis spectroscopy could be used to examine the size and shape-controlled nanoparticles in aqueous suspensions (Shrivastava and Dash 2010). As a confirmation, the *C. tomentosum* frond extract without $AgNO_3$ did not show any change in color over time. *C. tomentosum*-mediated

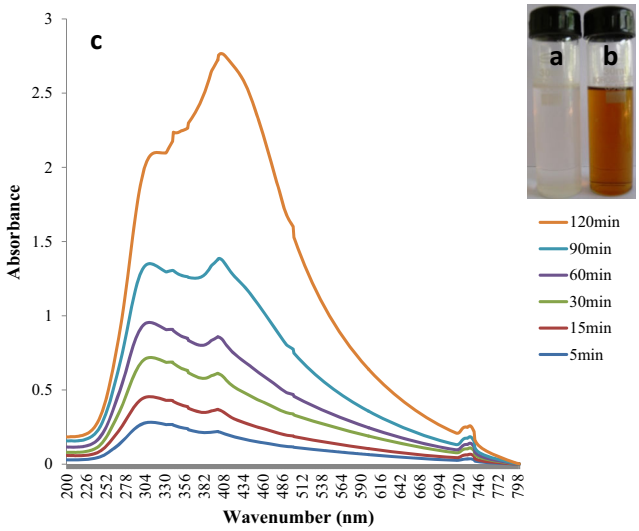

Fig. 1 Chromatic variation of the *Codium tomentosum* leaf extract **a** before and **b** after the process of reduction of Ag^{+} to Ag nanoparticles. **c** UV-visualization of the absorption spectra of *Codium tomentosum*-synthesized Ag nanoparticles at different time intervals

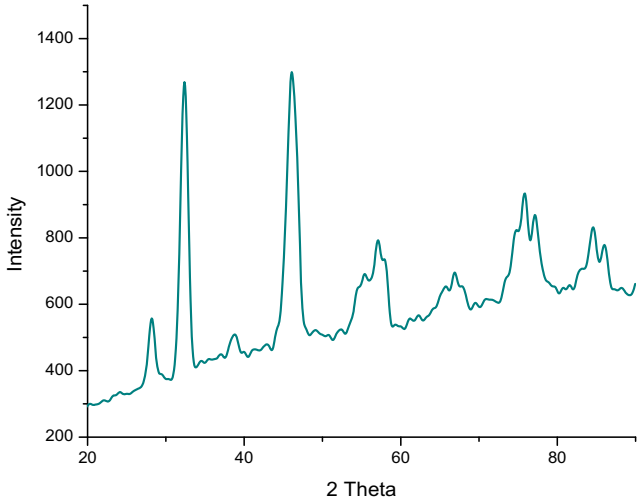

Fig. 2 X-ray diffraction pattern of *Codium tomentosum*-synthesized silver nanoparticles

reduction of silver ions to silver nanoparticles was linked with changes in the UV–Vis spectra. The appearance of the yellowish-brown color was an indication of the synthesis of colloidal AgNP in the medium. The dark color may be due to the excitation of surface plasmon vibrations, typical of the AgNP (Ahmad et al. 2003; Dinesh et al. 2015). In agreement with our results, a peak with maximum absorption at 410 nm characterized the synthesis of *Aloe vera*-fabricated AgNP (Dinesh et al. 2015), while peaks with maximum absorption at 450 and 420 nm were observed for *Moringa oleifera*-fabricated AgNP (Sujitha et al. 2015) and *Phyllanthus niruri*-synthesized AgNP (Suresh et al. 2015), respectively.

In XRD analyses, Bragg's reflections corresponding to the (111), (200), (220), (311), and (222) sets of lattice planes were observed, showing that the AgNP were crystalline in nature (Fig. 2). XRD results suggest that crystallization of the bio-organic phase occurred on the surface of the AgNP. The XRD pattern observed in this study was consistent with previous reports (Bar et al. 2009), including seaweed-mediated processes (Madhiyazhagan et al. 2015). Sathyavathi et al. (2010) reported diffraction peaks at 44.50°, 52.20°, and 76.7° 2θ, which correspond to the (111), (200), and (220) facets of the face-centered cubic crystal structure. Dubey et al. (2009) studied the size of silver nanocrystals as estimated from the full width at half-maximum of (111) peak of silver using the Scherrer's formula was 20–60 nm.

FTIR spectroscopy shed light on the identity of biomolecules from *C. tomentosum*, which may be responsible for synthesis and stabilization of AgNP. The FTIR spectrum of AgNP prepared from the *C. tomentosum* extract showed peaks at 3496, 2970, 1498, 1479, and 593/cm (Fig. 3). The broad intense band close to 3402/cm may be assigned to the N–H stretching frequency arising from peptide linkages (Mukherjee et al. 2008). The presence of peak at 3416/cm could be ascribed to O–H group in polyphenols or proteins/enzymes or polysaccharides (Susanto et al. 2009). The peak located at 1640 cm^{-1} could be assigned to the C=O stretching in carboxyl groups or C=N bending in amide groups (Bankara et al. 2010). The strong band at 1635/cm may be due to amide I vibrations, corresponding to stretching of carbonyl groups in amide linkages (Nagajyothi et al. 2014). FTIR peaks corresponding to aromatic rings, geminal methyls, and ether linkages may indicate the presence of flavones and terpenoids responsible for the stabilization of the AgNP (Nabikhan et al. 2010). FTIR spectroscopy revealed that the carbonyl groups from amino acid residues have the stronger ability to bind metal, indicating that the proteins could form a capping layer on AgNP, preventing agglomeration and thereby stabilizing the medium (Benelli 2016a).

SEM analyses showed that *C. tomentosum*-synthesized AgNPs were predominantly irregular in shape, with a large distribution of sizes, mainly ranging from 20 to 40 nm (Fig. 4).

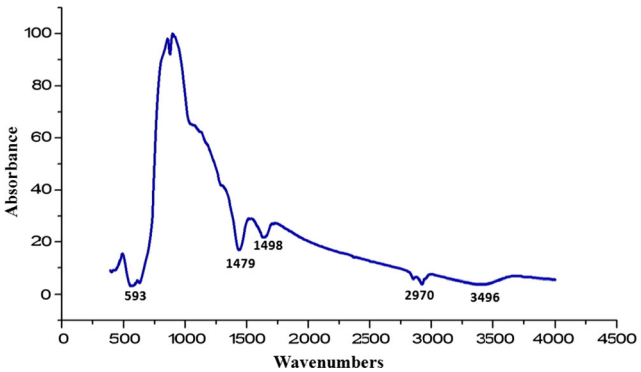

Fig. 3 Fourier transform infrared (FTIR) spectra of vacuum-dried *Codium tomentosum*-synthesized silver nanoparticles

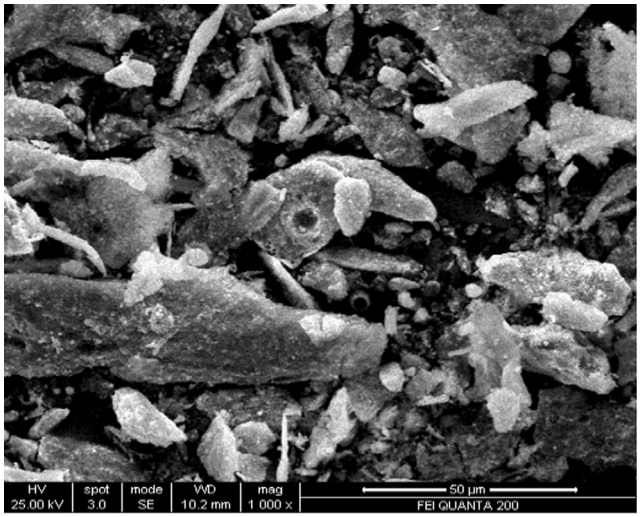

Fig. 4 Scanning electron microscopy (SEM) micrograph showing the morphological characteristics of silver nanoparticles synthesized using the *Codium tomentosum* leaf extract

Notably, the shape of metal nanoparticles considerably changes their optical and electronic properties (Xu and Käll 2002). As a general trend, the shape of plant-synthesized AgNPs was mostly spherical (Benelli 2016a) with exception of neem (*Azadirachta indica*), which yielded polydisperse particles both with spherical and flat nanoparticles of 5–35 nm in size (Shankar et al. 2004). Concerning seaweeds, Madhiyazhagan et al. (2015) studied *S. muticum*-synthesized AgNP indicating that they were mostly spherical, well-dispersed and ranged in sizes 43–79 nm. The EDX pattern showed the chemical composition of *C. tomentosum*-synthesized AgNPs (Fig. 5). The sharp optical absorption band peak at 3 keV is a typical absorption range of metallic silver nanocrystallites (Magudapathy et al. 2001; Fayaz et al. 2010; Ali et al. 2011; Kaviya et al. 2011). Furthermore, the other weak elemental signals are probably due to x-ray emission of constituents from proteins enzymes and other metabolites from the *C. tomentosum* extract. These constituents often surround AgNP with a thin layer of capping organic material from the plant broth and this enhance the AgNP stability in the solution for several weeks after synthesis (Dinesh et al. 2015; Suresh et al. 2015).

Anti-oxidant activity

Anti-oxidants are vital substances that play an important role in disease prevention, protecting the body from the damage caused from free radical-induced oxidative stress. Various phytochemicals, like polyphenols, are widely present in plants and have been reported to act as free radical scavengers. Nowadays, plants are extensively screened for anti-oxidant potential (Lubbad et al. 2015). The anti-oxidant activity is important in malaria treatment since oxidative stress normally follows malaria infection. This is due to elevated production of reactive oxygen species (Griffiths et al. 2001; Muller et al. 2004). Screening for anti-oxidant-rich materials, we focused on *Codium* genus, which possess anti-genotoxic (Celikler et al. 2009), anti-bacterial (Alghazeer et al. 2013), cytotoxic (Manilal et al. 2009), and anti-cancer (Jongsuvat 1981) properties. Here, the DPPH radical scavenging activity was detected and compared with BHT and rutin. Rutin showed higher free radical scavenging activity (IC_{50} = 7.13 µg/ml) against DPPH radicals if compared to the synthetic anti-oxidant BHT (IC_{50} = 9.04 µg/ml). The *C. tomentosum* extract also showed good anti-DPPH activity (IC_{50} = 13.42 µg/ml). *C. tomentosum* extract seems to contain a good amount of hydrogen donor molecules, which may help to reduce free radicals in DPPH$^{\bullet}$ scavenging assays. The high scavenging property of plant extracts may be due to hydroxyl groups existing in the phenolic chemical structures (Sanaa and Shanab 2007; Nahak and Sahu 2010).

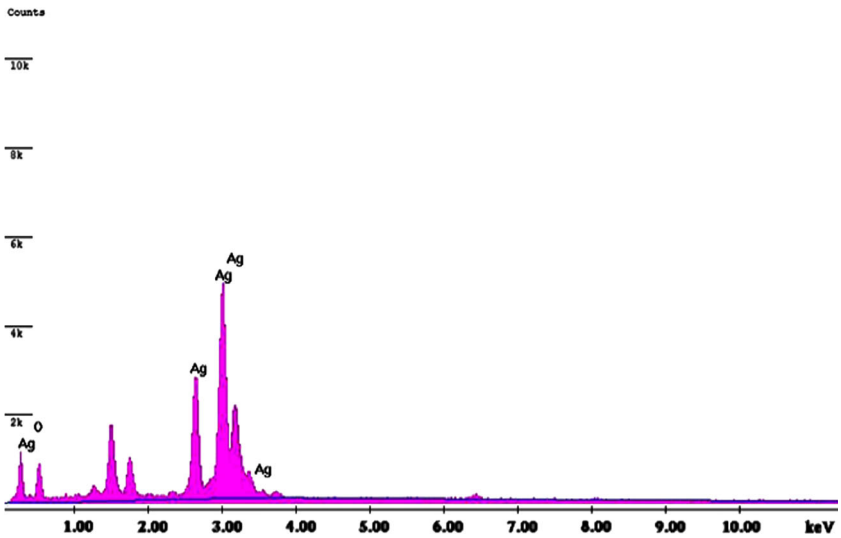

Fig. 5 Energy-dispersive x-ray (EDX) spectrum of silver nanoparticles synthesized using the leaf extract of *Codium tomentosum*

Table 1 Acute toxicity of *Codium tomentosum* extract against larvae and pupae of the malaria vector *Anopheles stephensi*

Target	LC_{50} (LC_{90}) (ppm)	95 % Confidence limit		Regression equation	χ^2
		LCL LC_{50} (LC_{90}) (ppm)	UCL LC_{50} (LC_{90}) (ppm)		
Larva I	255.1 (576.9)	222.4 (519.1)	284.4 (663.2)	$y = -1.016 + 0.004\,x$	1.532 N.S.
Larva II	291.7 (631.4)	259.9 (564.1)	322.9 (734.2)	$y = -1.101 + 0.004\,x$	0.825 N.S.
Larva III	340.6 (683.5)	309.6 (608.3)	375.2 (799.7)	$y = -1.273 + 0.004\,x$	0.383 N.S.
Larva IV	410.7 (775.2)	374.6 (680.4)	458.6 (928.3)	$y = -1.444 + 0.004\,x$	1.179 N.S.
Pupa	487.1 (896.1)	437.5 (766.2)	564.5 (1124.1)	$y = -1.527 + 0.003\,x$	0.431 N.S.

No mortality was observed in the control

LC_{50} lethal concentration that kills 50 % of the exposed organisms, *LC_{90}* lethal concentration that kills 90 % of the exposed organisms, *LCL* lower confidence limit, *UCL* upper confidence limit, χ^2 chi-square; *N.S.* not significant ($\alpha = 0.05$)

Acute toxicity on *Anopheles stephensi* young instars

Here, the extract of *C. tomentosum* was effective on all larval instars (I–IV) and pupae of *A. stephensi*. LC_{50} values were 255.1 ppm (larva I), 291.1 ppm (II), 340.6 ppm (III), 410.7 ppm (IV), and 487.1 ppm (pupa) (Table 1). Toxicity results indicated that the percentage of mortality was proportional to the concentration of the tested *C. tomentosum* extract. Currently, different seaweed species have been studied as possible sources of bioinsecticides (Abbassy et al. 2014; Kalimuthu et al. 2014). For instance, Khanavi et al. (2011) reported the larvicidal potential of *Chondria dasyphylla* and *Sargassum swartzii* against III and IV instar larvae of the malaria vector *A. stephensi*. Kumar et al. (2012) studied the larvicidal and pupicidal activity of the brown seaweed *Sargassum wightii* towards *Anopheles sundaicus*, with LC_{50} ranging from 0.88 mg/l (I instar) to 1.171 mg/l (pupae). Asha et al. (2012) showed that the thallium hexane, chloroform, methanol, and water extract of *Ulva fasciata* and *U. lactuca* exhibited dose-dependent toxicity against third instar nymph of *Dysdercus cingulatus*.

Interestingly, *C. tomentosum*-synthesized AgNP achieved higher toxicity on *A. stephensi* young instars, with LC_{50} of 18.1 ppm (I), 20.3 ppm (II), 23.9 ppm (III), 29.6 ppm (IV), and 40.7 ppm (pupae) (Table 2). Concerning the green synthesis of nano-formulated mosquitocides, a growing number of AgNP has been reported for their larvicidal and pupicidal toxicity against different mosquito vectors of public health importance (Benelli 2016a). As regards to seaweeds, our results are comparable with the toxicity of *Caulerpa scalpelliformis*-synthesized AgNP against *Culex quinquefasciatus*, with LC_{50} values of 3.08 ppm (I), 3.49 (II), 4.64 (III), 5.86 ppm (IV), and 7.33 (pupa) (Murugan et al. 2015a). Furthermore, Madhiyazhagan et al. (2015) reported that the *S. muticum*-synthesized AgNP were highly toxic against larvae and pupae of *Aedes aegypti*, *A. stephensi*, and *C. quinquefasciatus*. Recently, Murugan et al. (2016) showed that AgNP synthesized using an aqueous extract of *C. clavulatum* were highly effective against larvae and pupae of *A. aegypti*, with LC_{50} ranging from 21.460 ppm (I) to 33.877 ppm (pupa). To our mind, the mosquitocidal action of AgNP against malaria mosquitoes may be attributed to the small size of the green-synthesized

Table 2 Acute toxicity of *Codium tomentosum*-synthesized silver nanoparticles against larvae and pupae of the malaria vector *Anopheles stephensi*

Target	LC_{50} (LC_{90}) (ppm)	95 % Confidence limit		Regression equation	χ^2
		LCL LC_{50} (LC_{90}) (ppm)	UCL LC_{50} (LC_{90}) (ppm)		
Larva I	18.1 (43.0)	3.0 (34.2)	25.3 (69.7)	$y = 0.930 + 0.051\,x$	10.05 N.S.
Larva II	20.3 (48.6)	17.0 (44.2)	23.1 (54.9)	$y = -0.925 + 0.045\,x$	3.474 N.S.
Larva III	23.9 (54.9)	20.6 (49.6)	26.8 (62.7)	$y = -0.992 + 0.041\,x$	2.663 N.S.
Larva IV	29.6 (67.4)	26.1 (59.4)	33.0 (80.2)	$y = 1.005 + 0.034\,x$	1.444 N.S.
Pupa	40.7 (89.7)	36.0 (75.1)	47.4 (117.0)	$y = -1.066 + 0.026\,x$	0.337 N.S.

No mortality was observed in the control

LC_{50} lethal concentration that kills 50 % of the exposed organisms, *LC_{90}* lethal concentration that kills 90 % of the exposed organisms, *LCL* lower confidence limit, *UCL* upper confidence limit, χ^2 chi-square, N.S. not significant ($\alpha = 0.05$)

Table 3 Predation of the copepod *Mesocyclops aspericornis* against larvae of *Anopheles stephensi*

Targeted prey	Consumed preys (*n*)						Total predation (*n*)	Consumed preys per copepod per day (*n*)
	Control	Day 1	Day 2	Day 3	Day 4	Day 5		
Larva I	0	89 ± 1.1	85 ± 4.7	80 ± 6.6	78 ± 5.0	75 ± 3.8	407	8.1a
Larva II	0	75 ± 5.9	72 ± 4.1	65 ± 3.9	60 ± 3.6	55 ± 5.0	327	6.5b
Larva III	0	25 ± 3.6	21 ± 4.6	17 ± 3.9	13 ± 2.0	9 ± 1.9	85	1.7c
Larva IV	0	16 ± 3.6	13 ± 3.6	10 ± 3.8	6 ± 0.9	3 ± 0.4	48	0.9d

Predation rates are means ± SD of four replicates (10 copepods vs. 100 mosquitoes per replicate)

Control was water without copepods

Within the column, means followed by the same letter(s) are not significantly different (generalized linear model, $P < 0.05$)

nanoparticles, which allows passage through the insect cuticle and into individual cells where they interfere with molting and other physiological processes (Arjunan et al. 2012; Murugan et al. 2015b; Benelli 2016b).

Copepod predation post-treatment with spongeweed-synthesized silver nanoparticles

M. aspericornis adults actively predate *A. stephensi* young larval instars. The predatory efficiency per copepod per day was 8.1, 6.5, 1.7, and 0.9 larvae (larva I, II, III, and IV, respectively) (Table 3). Our results are in agreement with previous evidences on other species. Indeed, adult copepods belonging to other species have been found effective to control young larval instars of different mosquitoes, including the arbovirus vectors *Aedes albopictus* and *A. aegypti*, while little predation rates have been observed against late instar larvae (Kay et al. 1992; Schreiber et al. 1993; Marten et al. 1994; Murugan et al. 2015a, b).

Post-treatment with spongeweed-synthesized AgNP, the predatory efficiency of a single *M. aspericornis* per day was comparable to standard laboratory conditions, i.e., 8.3, 6.6, 1.9, and 1.1 larvae (larva I, II, III, and IV, respectively) (Table 4). Also, in this experiment, copepods were effective predators of first and second instars of mosquitoes, while they are not active control agents against late larval instars. The higher predation rates of *M. aspericornis* against *A. stephensi* young larvae may be due to the impact of nanoparticles treatment on the prey organism, since they can affect the physiological and metabolic activities, thus motility. This has been hypothesized also by Murugan et al. (2011), reporting higher predation rates of the copepod *M. aspericornis* against *A. aegypti*, post-treatment with neem seed kernel extract. In addition, Murugan et al. (2015a) showed that seaweed-synthesized AgNPs did not reduce the predation of copepod *M. longisetus* against the filariasis vector *C. quinquefasciatus*. Furthermore, Murugan et al. (2015b) showed that very low doses (i.e., 1 ppm) of lemongrass-synthesized gold nanoparticles may help to control malaria and dengue vectors boosting early instar mosquito larvae predation by *M. aspericornis*. Overall, we hypothesize that low doses of green-synthesized AgNPs reduce motility of mosquito larvae, thus enhancing the predation success of mosquito natural enemies (Murugan et al. 2015c,d,e; Subramaniam et al. 2015).

Anti-plasmodial assays

In Asian traditional medicine, marine plants are used to cure many of the infectious and non infectious diseases

Table 4 Predation of the copepod *Mesocyclops aspericornis* against larvae of *Anopheles stephensi* after a treatment with seaweed-synthesized silver nanoparticles (1 ppm)

Targeted prey	Consumed preys (*n*)						Total predation (*n*)	Consumed preys per copepod per day (*n*)
	Control	Day 1	Day 2	Day 3	Day 4	Day 5		
Larva I	0	93 ± 6.6	90 ± 4.1	85 ± 4.2	78 ± 6.09	73 ± 5.1	419	8.3a
Larva II	0	83 ± 6.2	68 ± 8.3	65 ± 4.0	60 ± 5.2	58 ± 3.0	334	6.6b
Larva III	0	24 ± 3.5	22 ± 3.5	19 ± 4.9	17 ± 3.06	13 ± 2.9	95	1.9c
Larva IV	0	15 ± 0.9	14 ± 0.6	13 ± 0.7	8 ± 0.8	5 ± 1.9	55	1.1d

Predation rates are means ± SD of four replicates (10 copepods vs. 100 mosquitoes per replicate)

Control was water without copepods

Within the column, means followed by the same letter(s) are not significantly different (generalized linear model, $P < 0.05$)

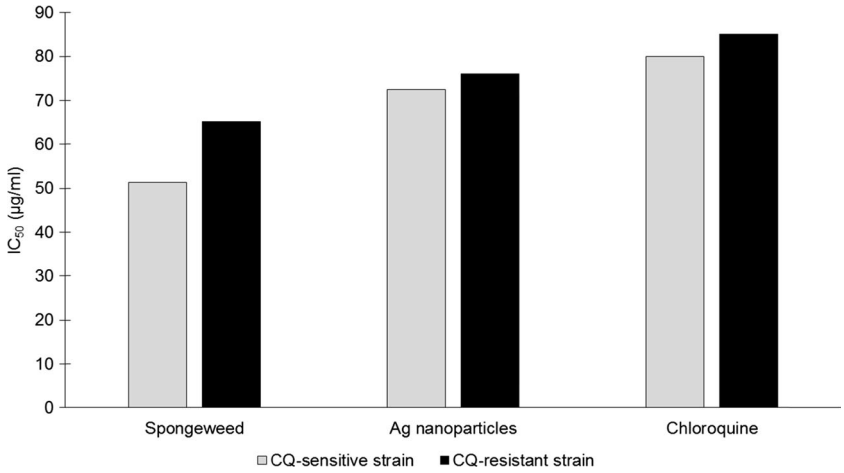

Fig. 6 Growth inhibition of chloroquine-resistant and chloroquine-sensitive strains of *Plasmodium falciparum* post-treatment with chloroquine, *C. tomentosum* extract and seaweed-synthesized silver nanoparticles

(Bandaranayake 1998). Several seaweeds have shown a wide range of bioactive properties. Recent research elucidates that plant metabolites are a useful arsenal in the fight against malaria. For instance, Ravikumar et al. (2011b) reported that seaweeds are a good source of compounds which can be used for the development of anti-plasmodial drugs. Here, the anti-plasmodial activity of *C. tomentosum* extract and *C. tomentosum*-synthesized AgNP was evaluated against CQ-resistant (CQ-r) and CQ-sensitive (CQ-s) strains of *P. falciparum* when compared to chloroquine. *C. tomentosum* IC_{50} were 51.34 μg/ml (CQ-s) and 65.17 μg/ml (CQ-r), while *C. tomentosum*-synthesized AgNP IC_{50} were 72.45 μg/ml (CQ-s) and 76.08 μg/ml (CQ-r). IC_{50} of choloroquine were 80.00 μg/ml (CQ-s) and 85.00 μg/ml (CQ-r) (Fig. 6). In agreement with our findings, Ravikumar et al. (2011a) studied that the methanolic extracts of two marine plants *Chaetomorpha antennina* and *Aegiceras corniculatum* showed maximum suppression of parasitemia, 63.50 ± 0.408 % at 1.5 mg/ml against *P. falciparum*. Kamaraj et al. (2012) reported that the leaf extract of *Eclipta prostrata* showed moderate anti-plasmodial activity against *P. falciparum* with the IC_{50} value of 30 μg/ml. Recently, Murugan et al. (2015i) studied that the anti-plasmodial activity of *Senna occidentalis* and *Ocimum basilicum* against CQ-resistant (CQ-r) and CQ-sensitive (CQ-s) strains of *P. falciparum;* IC_{50} of *S. occidentalis* were 48.80 μg/ml (CQ-s) and 54.28 μg/ml (CQ-r), while *O. basilicum* IC_{50} were 68.14 μg/ml (CQ-s) and 67.27 μg/ml (CQ-r). Furthermore, to the best of our knowledge, the anti-plasmodial activity of green-synthesized metal nanoparticles has been scarcely studied (Benelli 2016a, b; Benelli and Mehlhorn 2016). For example, Murugan et al. (2015h) reported that anti-plasmodial activity of seaweed-synthesized AgNP against CQ-resistant (CQ-r) and CQ-sensitive (CQ-s) strains of *P. falciparum,* IC_{50} of *U. lactuca* extract were 57.26 μg/ml (CQ-s) and 66.36 μg/ml (CQ-r), while *U. lactuca*-synthesized AgNP IC_{50} were 76.33 μg/ml (CQ-s) and 79.13 μg/ml (CQ-r). In addition, AgNP synthesized using *Catharanthus roseus*, and *Couroupita guianensis* are active against blood stages of *P. falciparum* (Ponarulselvam et al. 2012, 2015; Subramaniam et al. 2016b). Notably, the anti-plasmodial property of the above-mentioned plant extracts may be attributed to the presence of phytochemicals conferring protective and anti-oxidative activity against oxidative stress induced in the host parasitized red blood cells by the malarial parasites (Becker et al. 2004; Nethengwe et al. 2012).

Table 5 Growth inhibition induced by *Codium tomentosum*-synthesized silver nanoparticles against three pathogen bacteria

Treatment (mg/ml)	Inhibition zone (mm)		
	Bacillus subtilis	*Klebsiella pneumoniae*	*Salmonella typhi*
Ag nanoparticles 50	12.42 ± 0.56b	11.22 ± 0.23b	14.64 ± 0.43b
Ag nanoparticles 100	16.80 ± 0.98c	15.48 ± 0.52c	17.46 ± 0.64c
Ag nanoparticles 150	18.36 ± 1.02d	17.68 ± 0.28d	20.24 ± 0.84d
Tetracycline 100	5.20 ± 0.78a	6.22 ± 0.54a	4.48 ± 0.96a

Values are mean ± standard deviation of four replicates

Tetracycline was tested as positive control

Growth inhibition zones were not recorded in negative control, where no anti-biotic drugs were administered

Within each column, different letters indicate significant differences (ANOVA, Tukey's HSD test, $P < 0.05$)

Table 6 Chromatic variation in bacterial cultures after treatment with 1 mM of silver ions

Elapsed time	*Bacillus subtilis*	*Klebsiella pneumoniae*	*Salmonella typhi*
1 min	−	−	−
10 min	−	−	−
30 min	−	−	−
1 h	+	+	+
2 h	+	+	+
4 h	++	++	++
8 h	++	++	++
16 h	+++	+++	+++
24 h	+++	+++	+++

− no color variation, + variation to pale yellow, ++ variation to light brown, +++ variation to dark brown

Anti-bacterial activity

Here, we have investigated the anti-bacterial activity of AgNP synthesized using the anti-oxidant-rich spongeweed *C. tomentosum*. AgNP showed good anti-bacterial properties against *Bacillus subtilis, Klebsiella pneumoniae*, and *Salmonella typhi*. At the maximum concentration tested (150 ppm), they showed strong inhibitory action against *B. subtilis* (zone of inhibition 18.36 mm), *K. pneumoniae* (zone of inhibition 17.68 mm), and *S. typhi* (zone of inhibition 20.24 mm) (Table 5). Aqueous silver ions exposed to bacterial organisms were reduced in solution, leading to the formation of silver hydrosol. The bacterial biomass was pale yellow before the addition of silver ions, and then changed to light and dark brown, evidencing the formation of silver nanoparticles (Table 6). Similarly, Devina Merin et al. (2010) have reported the anti-bacterial activity of AgNP synthesized from marine microalgae. Dinesh et al. (2015) focused on the anti-microbial activity of *A. vera*-synthesized AgNP tested at 300 ppm against three bacteria species. *A. vera*-fabricated AgNP led to zones of inhibition higher than 19 mm in all targeted bacteria. Recently, Madhiyazhagan et al. (2015) reported that the *S. muticum*-synthesized AgNPs showed good anti-bacterial properties against *B: subtilis*, *K. pneumoniae*, and *S. typhi*. AgNP tested at 50 ppm evoked growth inhibition zones larger than 5 mm in all tested bacteria, while inhibition zones were larger than 10 mm when nanoparticles were tested at 150 ppm. Also, plant-mediated synthesis of silver nanoparticles using *Petroselinum crispum* showed appreciable anti-bacterial efficacy against three bacteria: *K. pneumoniae*, *Escherichia coli,* and *Staphylococcus aureus* (Roy et al. 2015). The exact mechanisms of nanoparticle toxicity against various bacteria are not understood completely. Nanoparticles are able to attach to the membrane of bacteria by electrostatic interaction, and disrupt the integrity of the bacterial membrane (Thill et al. 2006). Nanotoxicity is generally triggered by the induction of oxidative stress by free radical formation (Nel et al. 2009; Soenen et al. 2011).

Conclusions

Mosquito-borne disease control is facing a number of crucial challenges, including the emergence of artemisinin and chloroquine resistance in *Plasmodium* parasites, as well as the presence of mosquito vectors resistant to synthetic and microbial pesticides. Therefore, eco-friendly tools are urgently required. Here, a synergic approach relying to nanotechnologies and biological control strategies is proposed. Our study highlighted the multipurpose effectiveness of *C. tomentosum*-synthesized AgNP, as an eco-friendly tool in the fight against malaria, since they showed both anti-vectorial and anti-plasmodial activity, even on *Plasmodium* strains resistant to chloroquine. Other bioactivities, like bacterial growth inhibition and the employ of nanoparticles in synergy with natural enemies may also represent important solutions for integrated pest management (see also Benelli 2016a).

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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.

Informed consent Informed consent was obtained from all individual participants included in the study.

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