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RESEARCH ARTICLE

Multipurpose effectiveness of *Couroupita guianensis*-synthesized gold nanoparticles: high antiplasmodial potential, field efficacy against malaria vectors and synergy with *Aplocheilus lineatus* predators

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Abstract Mosquito-borne diseases represent a deadly threat for millions of people worldwide. According to recent estimates, about 3.2 billion people, almost half of the world's population, are at risk of malaria. Malaria control is particularly challenging due to a growing number of chloroquine-resistant *Plasmodium* and pesticide-resistant *Anopheles* vectors. Newer and safer control tools are required. In this research, gold nanoparticles (AuNPs) were biosynthesized using a cheap flower extract of *Couroupita guianensis* as reducing and stabilizing agent. The biofabrication of AuNP was confirmed by UV–vis spectrophotometry, Fourier transform infrared (FTIR) spectroscopy, transmission electron microscopy (TEM), energy-dispersive X-ray (EDX) spectroscopy, X-ray diffraction (XRD), zeta potential, and particle size analysis. AuNP showed different shapes including spheres, ovals, and triangles. AuNPs were crystalline in nature with face-centered cubic geometry; mean size was 29.2–43.8 nm. In laboratory conditions, AuNPs were toxic against *Anopheles stephensi* larvae, pupae, and adults. LC_{50} was 17.36 ppm (larva I), 19.79 ppm (larva II), 21.69 ppm (larva III), 24.57 ppm (larva IV), 28.78 ppm (pupa), and 11.23 ppm (adult). In the field, a single treatment with *C. guianensis* flower extract and AuNP ($10 \times LC_{50}$) led to complete larval mortality after 72 h. In standard laboratory conditions, the predation efficiency of golden wonder killifish, *Aplocheilus lineatus,* against *A. stephensi* IV instar larvae was 56.38 %, while in an aquatic environment treated with sub-lethal doses of the flower extract or AuNP, predation efficiency was boosted to 83.98 and 98.04 %, respectively. Lastly, the antiplasmodial activity of *C. guianensis* flower extract and AuNP was evaluated against CQ-resistant (CQ-r) and CQ-sensitive (CQ-s) strains of *Plasmodium falciparum*. IC_{50} of *C. guianensis* flower extract was 43.21 μg/ml (CQ-s) and 51.16 μg/ml (CQ-r). AuNP IC_{50} was 69.47 μg/ml (CQ-s) and 76.33 μg/ml (CQ-r). Overall, our results showed the multipurpose effectiveness of *C. guianensis*-synthesized AuNPs, since they may be proposed as newer and safer tools in the fight against CQ-r strains of *P. falciparum* and for field control of malaria vectors, in synergy with wonder killifish predators.

Keywords Chloroquine · Flower-mediated nanosynthesis · Malaria · Nanobiotechnology · *Plasmodium falciparum* · Golden wonder killifish

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Introduction

Malaria is a life-threatening disease caused by parasites that are transmitted to people through the bites of infected female mosquitoes. According to the latest estimates, there were about 198 million cases of malaria in 2013 and an estimated 584,000 deaths (WHO 2014). About 3.2 billion people, almost half of the world's population, are at risk of malaria. Sub-Saharan Africa carries a disproportionately high share of the global malaria burden. In 2015, the region was home to 89 % of malaria cases and 91 % of malaria deaths. Furthermore, a large number of cases are currently reported also from India, where a great increase in the incidence of *Plasmodium* parasites resistant to commonly used drugs (e.g., chloroquine, CQ, hereafter), has been registered in a number of endemic areas, and has been identified as one of the main causes for high malaria-related mortality (Jensen and Mehlhorn 2009; WHO 2014). Thus, there is an urgent need to identify alternative drugs for malaria treatment. Since a number of currently employed drugs originated from medicinal plants (e.g., artemisinin and quinine), further species reported by Indian and Chinese traditional medicine can be surveyed to identify novel compounds active against *Plasmodium* parasites (e.g., Siems et al. 1999; Bhat and Surolia 2001; Bagavan et al. 2011a, b; Murugan et al. 2015a, b).

People entering into regions, where malaria, dengue, or yellow fever risks exist may protect themselves by the use of chemical or plant-derived repellents (Mehlhorn et al. 2012; Amer and Mehlhorn 2006a, b). However, 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 (Benelli 2015a). *Anopheles* populations are usually targeted using synthetic insecticides (Amer and Mehlhorn 2006c, d; Semmler et al. 2009). However, chemical insecticides used against mosquito vectors have showed negative effects on non-target organisms (Service 1977; Chandra et al. 2008; Ohba et al. 2010; Rao and Kavitha 2010) and human health and also induce resistance in a number of vector species (Hemingway and Ranson 2000). In this scenario, eco-friendly tools have been implemented to enhance control of mosquito vectors, with special reference of botanical mosquitocidals (see Azizullah et al. 2014; Benelli 2015b; Benelli et al. 2015 and Pavela 2015 for recent reviews).

Biological control agents have been used to reduce mosquito vector populations with moderate environmental impact (e.g., Yap 1985; Voyadjoglou et al. 2007; Kamareddine 2012). In particular, several fish species have been successfully employed to control aquatic stages of both Anopheline and Culicine mosquitoes (e.g., Louca et al. 2009; Rao and Kavitha 2010; Patil et al. 2012; Chobu et al. 2015; Murugan et al. 2015c; Subramaniam et al. 2015).

In modern material science, nanotechnology plays a remarkable role, due to salient features, such as manipulating nanoscale structures, engineering of atoms, and designing of materials with enhanced properties (Jain et al. 2009). Currently, the development of green routes for nanosynthesis is essential for a wide array of research fields, particularly medicine, parasitology, and pest management (Mubarak Ali et al. 2011). Green nanosynthesis helps to avoid high energy inputs and replace the hazardous chemical, minimizing harmful pollution to the environment (Bharathi et al. 2014; Huang et al. 2007). In the latest years, biological routes for fabrication of metal nanoparticles have been suggested as possible eco-friendly alternatives to classic chemical and physical methods (Mohanpuria et al. 2008), with a special focus on nanosynthesis of mosquitocides and antiplasmodial drugs (Benelli 2016).

Couroupita guianensis (Lecythidaceae), commonly known as a cannonball tree, is widely used in Indian traditional medicine for the treatment of a broad spectrum of diseases, since it possesses antibiotic, antifungal, (Al-Dhabi et al. 2012), antidepressant (Kulkarni et al. 2011), antiseptic, and analgesic (Geetha et al. 2004) activities. Fruits can be used to cure stomachache, while the leaf juice cures skin diseases and is used as a treatment for malaria by shamans of South America (Kumar et al. 2011). Recently, *C. guianensis* leaf and fruit extracts were selected for rapid and cost-effective synthesis of silver nanoparticles toxic against the dengue vector *Aedes aegypti*. It has been showed that various physiological conditions such as temperature, pH, concentration of metal ions, stoichiometric proportion of reaction mixture, and reaction time showed influence on the size, dispersity, and synthesis rate of silver nanoparticles (Vimala et al. 2015). The majority of green-synthesized metal nanoparticles are silver ones (Benelli 2016). However, also gold nanoparticles (AuNP) have been fabricated using cheap extracts of several plant species, such as *Cymbopogon citratus* (Murugan et al. 2015d), *Terminalia arjuna*, (Gopinath et al. 2013) and *Stoechospermum marginatum* (Rajathi et al. 2012). Green-synthesized AuNPs have been recently proposed as newer and safer control tools against mosquito vectors of medical and veterinary importance (Murugan et al. 2015d; see Benelli 2016 for a recent review).

In this research, AuNP were biosynthesized using a cheap flower extract of *C. guianensis* as reducing and stabilizing agent. The biofabrication of AuNP was confirmed by UV–vis spectrophotometry, Fourier transform infrared (FTIR) spectroscopy, transmission electron microscopy (TEM), energy-dispersive X-ray (EDX) spectroscopy, X-ray diffraction (XRD), zeta potential, and particle size analysis. In laboratory conditions, AuNP acute toxicity was tested against larvae, pupae, and adults of the malaria vector *Anopheles stephensi*. Field experiments were carried out in water storage reservoirs on larval population of *A. stephensi*. Furthermore, the predation efficiency of the golden wonder killifish against *A. stephensi* larvae was evaluated in standard laboratory

conditions and in aquatic environments treated with sub-lethal doses of the *C. guianensis* flower extract or AuNP. Lastly, the antiplasmodial potential of *C. guianensis* flower extract and AuNP was evaluated against CQ-resistant (CQ-r) and CQ-sensitive (CQ-s) strains of *Plasmodium falciparum*.

Materials and methods

Plant material

Fresh flowers of *C. guianensis* were collected from the Vinayagar Temple (Kovai Medical Centre Hospital Campus, India). The plants were authenticated at Botanical Survey of India. Voucher specimens were deposited at Zoology Department, Bharathiar University (voucher ID: CORGUA1-3).

Anopheles stephensi rearing

Mosquitoes tested in this study were from a laboratory-reared pathogen-free strain of *A. stephensi* originally established as described by Dinesh et al. (2015). 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 × 13 × 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)]. *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. Newly emerged larvae and pupae and 2-day-old adults were collected and used in the experiments (Dinesh et al. 2015).

Flower extract, green synthesis, and characterization of gold nanoparticles

C. guianensis flowers were washed with distilled water and dried in shade for 2 days at room temperature. Flower extract was prepared by placing 5 g of finely cut flowers in a 300-ml Erlenmeyer flask filled with 100 ml of sterile distilled water. The mixture was boiled for 5 min, decanted, and stored at −4 °C. Within 5 days, the *C. guianensis* flower extract was treated with aqueous $HAuCl_4$ 10^{-3} M and kept in an Erlenmeyer flask for 72 h at 25 °C. Color change indicated the formation of AuNP, since aqueous gold ions were reduced by the flower extract generating stable AuNP in water. $HAuCl_4$ was purchased from the Precision Scientific Co. (Coimbatore, India). Following Roni et al. (2015), the tested concentrations were given in amounts of the stock solution (i.e., the obtained suspension of green-synthesized AuNP or the original flower extract from *C. guianensis*) used to treat a given aquatic environment.

Green-synthesized AuNP were characterized by UV–vis spectrophotometry, FTIR spectroscopy, TEM, EDX spectroscopy, and XRD and analyzed for size. In UV–vis assays, the bio-reduction of $HAuCl_4$ in the aqueous medium was monitored by periodic sampling of aliquots (2 ml), measuring UV–vis spectrum in 10-mm quartz cuvette with a systronics. We used a UV–vis spectrophotometer (resolution: 1 nm) at 500 and 680 nm with a scanning speed of 1856 nm/min. OD values were taken up to 3 days at regular intervals. Then, samples were centrifuged at 42,000 rpm for 10 min, pellets were dried, and the nanopowder obtained was used for further analyses. The optical properties of green-synthesized AuNP were monitored on a Hewlett-Packard diode array spectrophotometer (model HP-8452) operating at a resolution of 2 nm.

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 AuNP solutions on carbon-coated TEM grids. The film on the TEM grid was allowed to dry for 5 min in laboratory condition. XRD analysis of drop-coated films on glass substrates from the AOT-capped AuNP was carried out on a Phillips PW1830 instrument operating at 40 kV and current of 30 mA with Cu $K\alpha$ radiation. EDX analyzed the presence of metals in the sample (Dinesh et al. 2015; Suresh et al. 2015). The mean size of AuNP was calculated using the Debye-Scherrer equation by determining the width of the (111) and the similar Bragg reflection (Kasthuri et al. 2009). The particle size of AuNP was determined by using the particle analyzer Malvern Zetasizer; AuNP size was analyzed measuring the size-dependent fluctuation of scattering of laser light on AuNP.

Concerning FTIR measurements, samples were prepared as described for XRD analysis and measured using Shimadzu 8400 s with a spectral range of 4000–400 cm^{-1} with resolution of 4 cm^{-1}. FTIR samples were prepared similarly as for powder diffraction measurements. FTIR spectra of leaf extracts sampled before and after the synthesis of AuNP were compared to discuss possible function groups involved in AuNP formation (Dinesh et al. 2015; Suresh et al. 2015).

Larvicidal and pupicidal toxicity

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 in a 500-ml glass beaker, and 1 ml of the desired concentration of *C. guianensis* flowers extract or green-synthesized gold nanoparticles was added. Larval food (0.5 mg) was provided for each tested concentration (WHO 2005; Kovendan et al. 2012; Dinesh et al. 2015). 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:

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

Adulticidal toxicity

Adulticidal experiments were performed following the methods reported by Subramaniam et al. (2015). The flower extract was tested at 50, 100, 150, 200, and 250 ppm. Green-synthesized AuNP were tested at 5, 10, 15, 20, and 25 ppm formulated in 5 ml of aqueous solution. For each tested dosage, five replicates were carried out. The flower extract or AuNPs were applied on Whatman no. 1 filter paper (size 12 × 15 cm) lining a glass holding tube (diameter 30 mm; length 60 mm). In control treatments, filter paper was treated with either the same volume of distilled water or $AgNO_3$ (1 mM) in aqueous solution. In each test, 20 mosquito females were gently transferred into another glass holding tube. The mosquitoes were allowed to acclimatize in the tube for 1 h and then exposed to a test tube lined with treated or control paper for 1 h. At the end of exposure period, the mosquitoes were transferred back to the original holding tube, kept for a 24-h recovery period, then mortality was recorded. A pad of cotton soaked with 10 % (*w*:*v*) glucose solution was placed on the mesh screen at the top of the holding tube (Suresh et al. 2015).

Larvicidal assays in the field

C. guianensis flowers extract and *C. guianensis*-synthesized AuNPs were applied in six external water storage reservoirs at the National Institute of Communicable Disease Centre (Coimbatore, India), using a knapsack sprayer (Private Limited 2008, Ignition Products, India). Following the method described by Suresh et al. (2015), pre-treatment and post-treatment observations were conducted at 24, 48, and 72 h using a larval dipper. Toxicity was assessed against III and IV instar larvae. Larvae were counted and identified to specific level. Following Suresh et al. (2015), we identified a sample of 100 larvae per reservoir. More than 93 % of all surveyed larvae belong to *A. stephensi*. Six trials were conducted for each test site with similar weather conditions (28 ± 2 °C; 80 % R.H.). The required quantity of mosquitocidal was calculated on the basis of the total surface area and volume (0.25 m^3 and 250 l); the required concentration was prepared using $10 \times LC_{50}$ values (Murugan et al. 2003; Subramaniam et al. 2015). Percentage reduction of the larval density was calculated using the formula:

$$\text{Percentage reduction} = (C–T)/C \times 100$$

where C is the total number of mosquitoes in the control and T is the total number of mosquitoes in the treatment.

Predation efficiency of *Aplocheilus lineatus*

A. lineatus fishes were provided by the Tamil Nadu Fisheries Department (Mettur Dam, Salem, Tamil Nadu, India) and maintained in laboratory at 27 ± 3 °C and R.H. 85 % in cement tanks (120 cm diameter, 60 cm depth) filled with field-collected water. All experiments were carried out from 15 June to 25 July 2015. In standard laboratory conditions, the predation efficiency of *A. lineatus* was assessed against IV instar larvae of *A. stephensi*; 200 IV instar larvae of *A. stephensi* were introduced with 1 *A. lineatus* adult in a 2-l glass arena filled with dechlorinated water. Five replicates were conducted. Control arenas contained dechlorinated water only. All arenas were checked every 24 h for 5 days, and the number of missing prey, assumed to be eaten by the fish, was recorded. Missing mosquito larvae were replaced after each daily check with new ones. Following Subramaniam et al. (2015), predation efficiency was calculated using the following formula:

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

Predation assays post-treatment with gold nanoparticles: here, 200 mosquito larvae were introduced, with 1 adult *A. lineatus*, in glass cups (2 l) containing dechlorinated water plus 1/3 of the LC_{50} calculated against IV instar larvae of *A. stephensi* (Murugan et al. 2015c; Subramaniam et al. 2015). Five replicates were conducted. Control was 2 l of AuNP-contaminated water plus 200 larvae, without *A. lineatus*. All experimental cups were checked after 24 h, and the number of preys consumed by mosquito fish was recorded. After each checking, the predated mosquito larvae were replaced with new ones; 5 replicates were made with and without predator (control), before and after the treatment of

AuNP of *C. guianensis*, separately. Using the same predator individual, the rate of predation was observed for five consecutive days. The prey density is being set to the same value after every 24 h. The fish predation efficiency was calculated using the abovementioned formula.

In vitro cultivation of *Plasmodium falciparum*

Following the method reported by Murugan et al. (2015a), CQ-sensitive strain 3D7 and CQ-resistant strain INDO of *P. falciparum* were used in in vitro blood stage culture to test the anti-malarial efficacy of *C. guianensis* flower 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.

Antiplasmodial assays

Control stock solutions of CQ were prepared in water (Milli-Q grade); the tested extracts 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, drugs and tested extracts were placed in 96-well flat-bottom tissue culture-grade plates.

The *C. guianensis* flower extracts 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). Sorbitol-synchronized parasites were incubated under normal culture conditions at 2 % hematocrit and 1 % parasitemia in the absence or presence of increasing concentrations of plant extracts. CQ was used as positive control. After 48 h of incubation, 100 μl of SYBR Green I solution {0.2 μl of 10,000 × 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. 2011a, b).

Data analysis

All data were analyzed using the SPSS Statistical Software Package version 17.0. Mosquito mortality data from laboratory assays were analyzed by probit analysis, calculating LC_{50} and LC_{90} following the method described by Finney (1971). Mosquito adulticidal data were analyzed using a two-way ANOVA with two factors (i.e., the mosquitocidal treatment and the dose). Mosquito larval density data from field assays were analyzed using a two-way ANOVA with two factors (i.e., the mosquitocidal and the elapsed time from treatment). Means were separated using Tukey's HSD test. In all analyses, a probability level of $P < 0.05$ was used for the significance of differences between values.

Fish predation data were analyzed by JMP 7 using a weighted general linear model with two fixed factors: $y = X\beta + \varepsilon$ where y is the vector of the observations (the number of consumed preys), X is the incidence matrix, β is the vector of fixed effects (i.e., the mosquitocidal and targeted mosquito instar), and ε is the vector of the random residual effect. In all analyses, a probability level of $P < 0.05$ was used for the significance of differences between values.

In antiplasmodial assays, all values are expressed as percentage growth inhibition. The concentrations causing 50 % inhibition of parasite growth (IC_{50}) were calculated from the drug concentration–response curves.

Results and discussion

Characterization of *Couroupita guianensis*-synthesized gold nanoparticles

In our experiments, the production of AuNP was observed within 120 min after that the aqueous *C. guianensis* flower extract was added to $HAuCl_4$ solution. Color changed from colorless to pale yellow to dark brown coloration (Fig. 1a). The UV–vis spectrum of AuNP synthesized using the flower extract of *C. guianensis* is reported in Fig. 1b. The formation of AuNP was confirmed by an absorption peak at 560 nm, probably due to excitation of surface plasmon resonance bands (Mulvaney 1996; Shankar et al. 2004; Kasthuri et al. 2009). Similarly to our findings, the formation of AuNP using *Stoechospermum marginatum* was confirmed by an absorption peak at 550 nm (Rajathi et al. 2012). Recently, Lallawmawma et al. (2015) showed that the *Jasminum nervosum*-mediated biosynthesis of AuNP evoked a

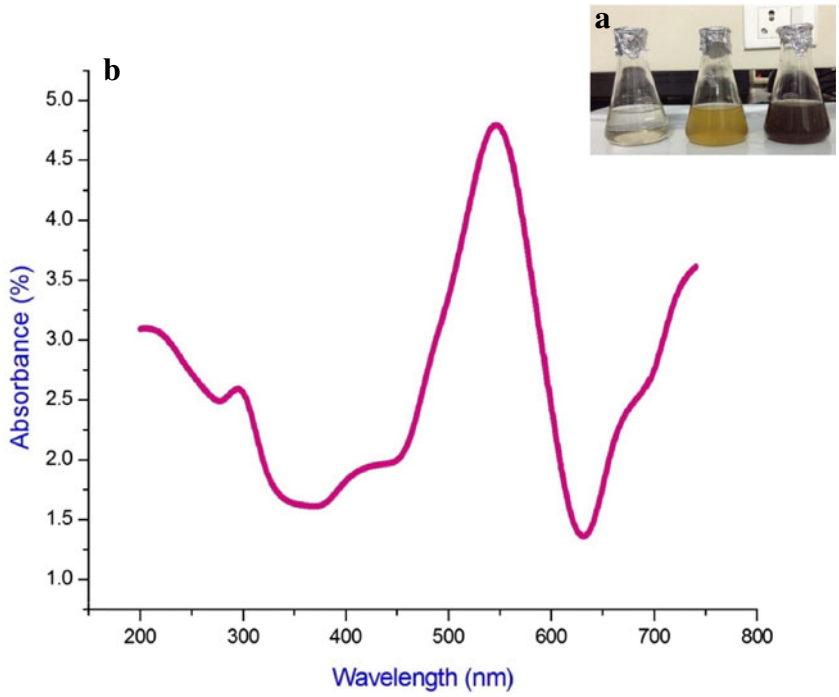


Fig. 1 **a** Color of the aqueous $HAuCl_4$ 10^{-3} M (*left*); the flower extract of *Couroupita guianensis* before (*center*) and after (*right*) the process of reduction of Au^{+} to Au nanoparticles. **b** UV visualization of the absorption spectrum of gold nanoparticles synthesized using *C. guianensis* after 120 min from the reaction

maximum absorbance peak at 550 nm. TEM highlighted the presence of spherical and oval shapes of AuNP, with size ranging from 29.2 nm to 43.8 nm (Fig. 2). In agreement with our results, Singaravelu et al. (2007) reported monodisperse AuNP (8–12 nm) fabricated using *Sargassaum wightii* as reducing agent in a 10^{-3} M

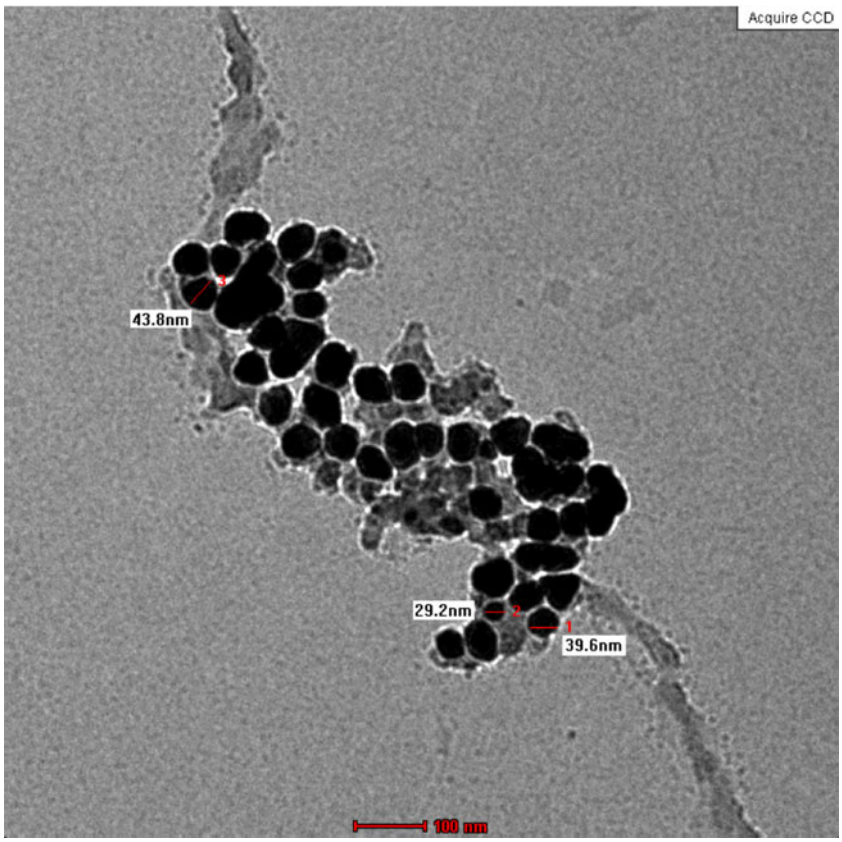


Fig. 2 Transmission electron micrograph (TEM) of green-synthesized gold nanoparticles obtained by reduction of $HAuCl_4$ with the flower extract of *Couroupita guianensis*

$HAuCl_4$ solution. AuNPs produced using *Zizyphus mauritiana* were nanospherical and ranged 20–40 nm in size with 32 nm in average size (Sadeghi 2015a). Moreover, Sadeghi et al. (2015b) reported the TEM image of *Stevia rebaudiana*-synthesized AuNPs showing that these nanoparticles are spherical and uniformly distributed with sizes from 5 to 20 nm.

EDX analysis reveals that strong signal in the Au region, a sharp optical absorption peak between 2 and 3 keV, indicates the presence of gold nanocrystallites in the nanoparticle suspension (Fig. 3). The weaker carbon peaks were possibly due to the biomolecules coating AuNP (Chandran et al. 2006). Other elemental signals were probably due to elements composing the enzymes and/or proteins present in the flower extract of *C. guianensis* (see also Zayed and Eisa 2014; Raju et al. 2011). The XRD pattern showed intense peaks corresponding to (111), (200), and (220) Bragg reflection based on the face-centered cubic structure of AuNP (Bankar et al. 2010; Inbakandan et al. 2010). Thus, XRD highlighted that AuNPs formed by the reduction of $HAuCl_4$ with *C. guianensis* flower extract were crystalline in nature (Fig. 4), as previously reported for earlier studies where gold nanosynthesis was mediated by *Cacumen platycladi*, *Zingiber officinale*, and *Acacia nilotica* metabolites (Zhan et al. 2011; Velmurugan et al. 2014; Majumdar et al. 2013, 2015).

The FTIR spectrum of AuNP fabricated with the *C. guianensis* flower extract is shown in Fig. 5. Strong intense bands appeared at 422.41 (C=C stretch nitro groups of aromatics), 1456.26 (C–H bending in alkanes), 1514.12 (N–O asymmetric stretching in nitro compounds), 1641.42 (N–H bending in amines I), 2362.80 (nitrile C≡N stretching), and 3421.72 (amine N–H stretching) (Caruso et al. 1998). These compounds may play a major role in the reduction of gold ions to AuNP (see also Susanto et al. 2009). In addition, it can be inferred that some molecules from *C. guianensis* extract were responsible of coating/capping action on biofabricated AuNP (Benelli 2016).

Zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle which indicates information about the surface charge of particles (Delgado et al. 2005). In our analysis, zeta potential of AuNP was −18.9 mV, and the particle size distribution of the AuNP determined by dynamic light scattering is shown in Fig. 6. In agreement with TEM results, the distribution of particle diameters showed a main peak located between 1 and 40 nm and the sizes ranging from 10 to 110 nm with an average particle size of 20 nm (Fig. 6). Similarly, Dwivedi and Gopal (2010) noted that *Chenopodium album*-synthesized silver and gold nanoparticles were stable under a wide pH range due to their high zeta potential.

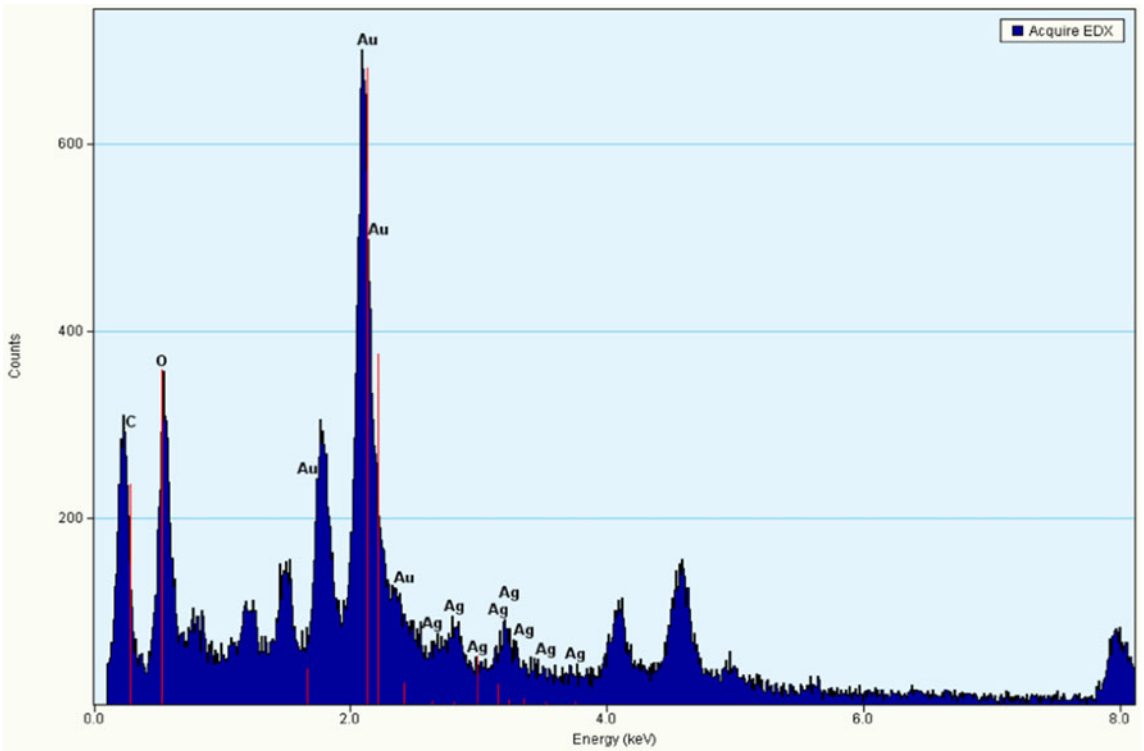


Fig. 3 Energy-dispersive X-ray (EDX) profile of gold nanoparticles biosynthesized using the flower extract of *Couroupita guianensis*

Larvicidal and pupicidal potential

In laboratory conditions, the flower extract of *C. guianensis* was toxic against larval instars (I–IV) and pupae of *A. stephensi.* LC_{50} values were 199.20 ppm (I), 225.78 ppm (II), 257.17 ppm (III), 307.72 ppm (IV), and 363.25 ppm (pupae) (Table 1). A dose-dependent effect was found, in agreement with a number of previously reported plant-borne mosquitocidals (Nicoletti et al. 2012; Panneerselvam et al. 2013a, b, c; Roni et al. 2015; Subramaniam et al. 2012a, b). For instance, Roni et al. (2013) reported that aqueous leaf extract of *Nerium oleander* exhibited dose-dependent larval toxicity against *A. stephensi*. Recently, Murugan et al. (2015e) studied the larvicidal and pupicidal activity of *Aristolochia indica* leaf extract against *A. stephensi*, with LC_{50} ranging from 262.66 ppm (I) to 565.02 ppm (pupae). Murugan et al. (2015f) also reported that *Datura metel* leaf extract have LC_{50} values ranging from 34.693 ppm (I instar) to 81.500 ppm (pupae) against *A. stephensi*. However, it should be also noted that a number of plant extracts, as well as essential oils, have been found more effective against Culicidae young instars, if compared to the *C. guianensis* flower extract (see Pavela 2008, 2009, 2015; Benelli 2015b).

Green-synthesized AuNPs were highly effective against *A. stephensi* larvae and pupae, with LC_{50} of 17.36 ppm (I), 19.79 ppm (II), 21.69 ppm (III), 24.57 ppm (IV), and 28.78 ppm (pupae) (Table 2). In the latest years, a growing number of terrestrial and aquatic plants have been proposed for eco-friendly biosynthesis of gold and silver nanoparticles (see Benelli 2016 for a dedicated review). A number of studies focused on the control of malaria mosquitoes. A good example is Murugan et al. (2015d), which highlighted the larvicidal potential of *Cymbopogon citratus*-synthesized AuNP against *A. stephensi*. Concerning filariasis vectors, *Jasminum nervosum*-mediated synthesis of AgNPs and AuNPs was performed to test their acute toxicity against III instar larvae of

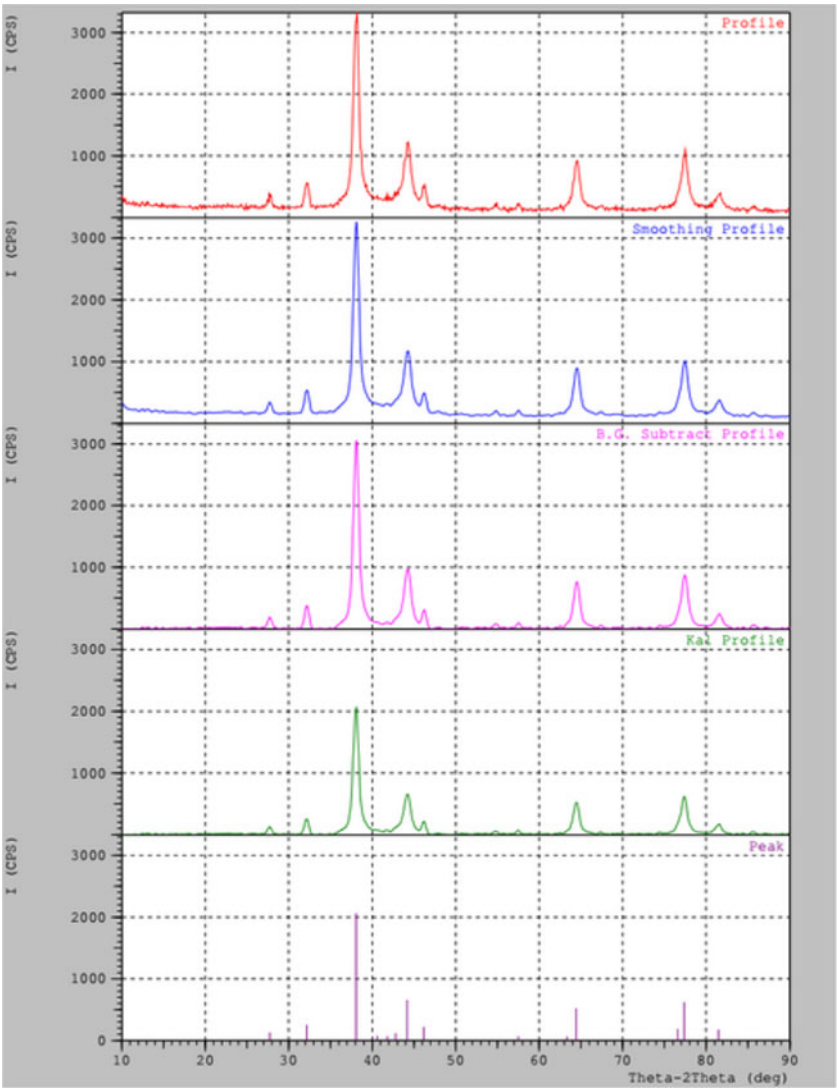
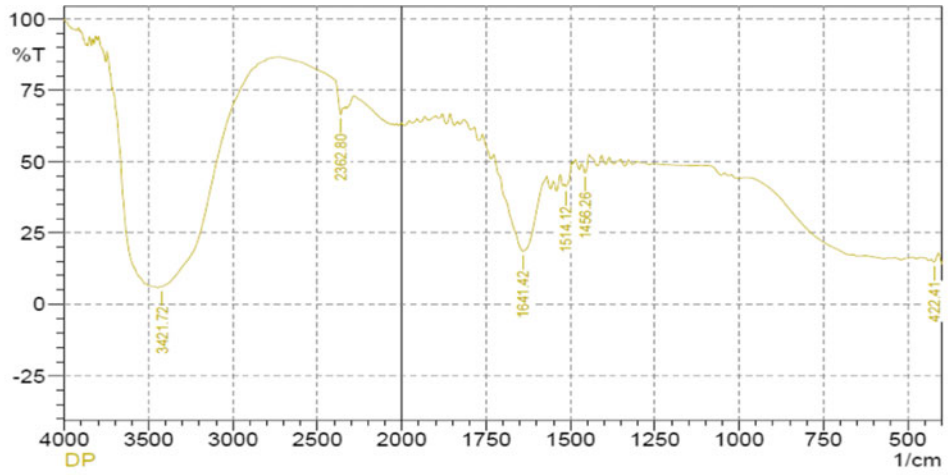


Fig. 4 X-ray diffraction (XRD) pattern of gold nanoparticles biosynthesized using the flower extract of *Couroupita guianensis*



No.	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	422.41	14.78	1.82	430.13	408.91	17.01	0.61
2	1456.26	46.03	4.74	1467.83	1444.68	7.32	0.5
3	1514.12	41.15	2.31	1517.98	1494.83	8.27	0.43
4	1641.42	18.51	2.47	1728.22	1635.64	46.64	0.16
5	2362.8	66.45	2.99	2735.06	2347.37	33.62	-10.03
6	3421.72	6.07	1.27	3431.36	2735.06	307.67	-133.7

Fig. 5 Fourier transform infrared (FTIR) spectrum of vacuum-dried powder of gold nanoparticles biosynthesized using the flower extract of *Couroupita guianensis*

Culex quinquefasciatus, with LC_{50} and LC_{95} values of 57.40 and 144.36 μg/ml for AgNPs and 82.62 and 254.68 μg/ml for AuNPs (Lallawmawma et al. 2015). Low doses of silver nanoparticles biosynthesized using *Euphorbia hirta* leaf extract have been reported as highly toxic against *A. stephensi*, with LC_{50} values ranging from 10.14 ppm (I instar larvae) to 34.52 ppm (pupae) (Priyadarshini et al. 2012). It has been hypothesized that the toxicity of AgNP against dengue vectors may be attributed to the small size of these 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).

Adulticidal potential

In adulticidal experiments conducted in the laboratory, the *C. guianensis* flower extract and nanoparticles showed LC_{50}

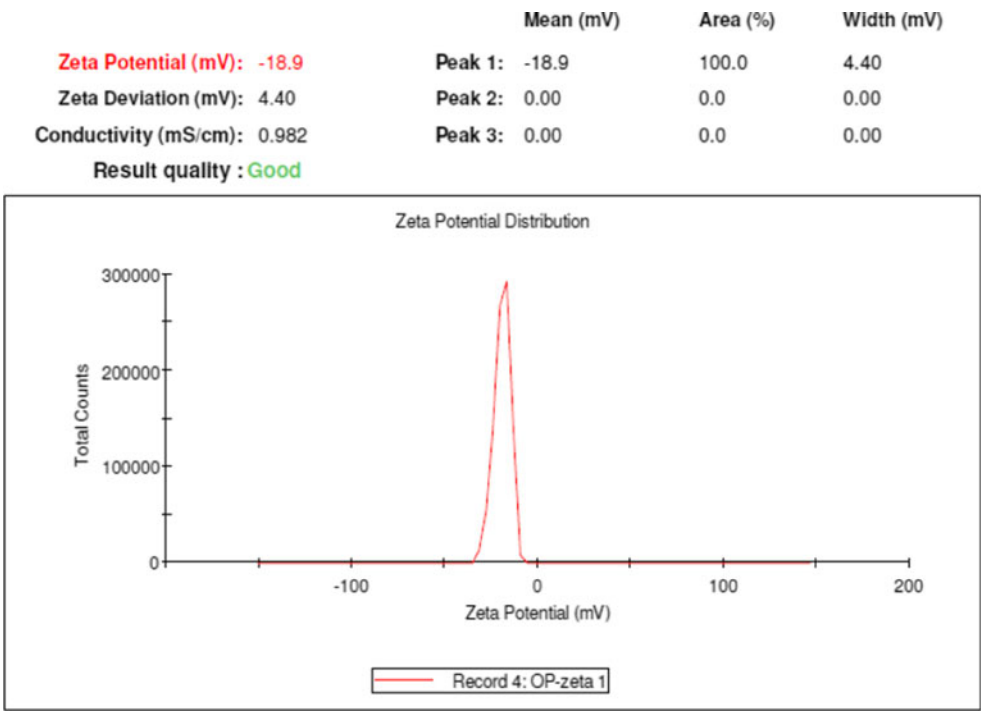


Fig. 6 Size analysis of gold nanoparticles biosynthesized using the flower extract of *Couroupita guianensis*

Table 1 Larvicidal and pupicidal toxicity of *Couroupita guianensis* flower extract against the malaria vector *Anopheles stephensi*

Target	Regression equation	LC_{50} (LC_{90})	95 % confidence limit LC_{50} (LC_{90})		χ^2 (d.f. = 4)
			Lower	Upper	
Larva I	$y = 0.885 + 0.004x$	199.20 (487.62)	101.11 (402.76)	259.31 (686.74)	5.50 n.s.
Larva II	$y = 0.815 + 0.004x$	225.78 (580.69)	185.82 (516.95)	258.62 (679.76)	2.28 n.s.
Larva III	$y = 0.876 + 0.003x$	257.17 (633.38)	218.88 (559.50)	290.89 (751.27)	0.44 n.s.
Larva IV	$y = 0.961 + 0.003x$	307.72 (718.30)	270.39 (625.17)	346.07 (874.12)	0.18 n.s.
Pupa	$y = 1.048 + 0.003x$	363.25 (807.51)	323.54 (691.11)	412.86 (1012.65)	0.09 n.s.

No mortality was observed in control

LC_{50} lethal concentration that kills 50 % of the exposed organisms, *LC_{90}* lethal concentration that kills 90 % of the exposed organisms, χ^2 chi-square value, *d.f.* degrees of freedom, *n.s.* not significant ($\alpha = 0.05$)

and LC_{90} of 133.96 and 11.23 ppm and 287.65 and 24.61 ppm, respectively (Table 3). Plant-borne extracts and essential oils can be tested for their adulticidal properties against a number of mosquitoes of economic importance (e.g., Amerasan et al. 2012, 2015; Govindarajan and Sivakumar 2012; Panneerselvam and Murugan 2013). In particular, 88 % adult mortality was observed from *Pelargonium citrosa* leaf extracts at 2 % concentration against *A. stephensi* (Jeyabalan et al. 2003). The ethanol extract of *Citrus sinensis* showed LC_{50} and LC_{90} values 320.38 and 524.57 ppm against *A. aegypti* adults (Murugan et al. 2012). On the other hand, limited information is available about the adulticidal effect of green-synthesized metal nanoparticles against mosquito vectors (Benelli 2016). Naresh Kumar et al. (2012) reported a reduction in adult longevity (4.2 days for males and 11.7 days for females, dose 10 ppm) in *A. stephensi* adults after treatment with 10 ppm of silver nanoparticles produced using *Annona squamosa* extract. Suresh et al. (2015) recently showed that *Phyllanthus niruri*-synthesized silver nanoparticles are highly toxic against *A. aegypti* adults, with an LC_{50} of 6.68 ppm. Recently, Subramaniam et al. (2015) highlighted the adulticidal properties of *Mimusops elengi*-synthesized silver nanoparticles, which are highly toxic against *A. stephensi* and *A. albopictus*. Notably, the mode of action of green-fabricated metal nanoparticles against Culicidae adults has not been elucidated (Benelli 2016).

Larvicidal and pupicidal toxicity against *A. stephensi* in the field

In field experiments, the application of *C. guianensis* flower extract ($10 \times LC_{50}$) led to *A. stephensi* larval reduction of 39.9, 69.2, and 100 % after 24, 48, and 72 h, respectively. A single

Table 2 Larvicidal and pupicidal toxicity of *Couroupita guianensis*-synthesized gold nanoparticles against the malaria vector *Anopheles stephensi*

Target	Regression equation	LC_{50} (LC_{90})	95 % confidence limit LC_{50} (LC_{90})		χ^2 (d.f. = 4)
			Lower	Upper	
Larva I	$y = 0.922 + 0.053x$	17.36 (41.497)	4.127 (33.426)	24.093 (63.236)	8.83 n.s.
Larva II	$y = 1.090 + 0.055x$	19.79 (43.063)	7.197 (34.486)	26.831 (67.913)	10.61 n.s.
Larva III	$y = 0.967 + 0.045x$	21.69 (50.448)	18.411 (45.869)	24.466 (56.993)	1.51 n.s.
Larva IV	$y = 0.890 + 0.036x$	24.57 (59.972)	20.860 (53.407)	27.788 (70.145)	0.54 n.s.
Pupa	$y = 0.786 + 0.027x$	28.78 (75.704)	24.335 (64.583)	33.021 (95.730)	0.34 n.s.

No mortality was observed in control

LC_{50} lethal concentration that kills 50 % of the exposed organisms, *LC_{90}* lethal concentration that kills 90 % of the exposed organisms, χ^2 chi-square value, *d.f.* degrees of freedom, *n.s.* not significant ($\alpha = 0.05$)

Table 3 Adulticidal toxicity of *Couroupita guianensis* flower extract-synthesized gold nanoparticles against the malaria vector *Anopheles stephensi*

Treatment	Concentration (ppm)	Mortality (%)	LC_{50} (95 % LCL-UCL)	LC_{90} (95 % LCL-UCL)	χ^2 (d.f. = 4)
Couroupita guianensis	50	26.4 ± 1.2^a	133.96	287.65	1.38 n.s.
flower extract	100	38.2 ± 1.8^b	(118.85–148.01)	(259.97–328.27)	
	150	52.8 ± 2.4^c			
	200	68.6 ± 1.8^d			
	250	86.2 ± 1.2^e			
Gold nanoparticles	5	32.2 ± 2.6^a	11.23	24.61	5.74 n.s.
	10	44.0 ± 2.2^b	(7.22–14.07)	(20.51–33.79)	
	15	57.8 ± 2.6^c			
	20	77.4 ± 2.0^d			
	25	95.2 ± 1.8^e			

Mortality rates are means ± SD of five replicates. No mortality was observed in the control. Within each column, means followed by different letters are significantly different ($P < 0.05$)

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 value, *d.f* degrees of freedom, *n.s.* not significant ($\alpha = 0.05$)

treatment with *C. guianensis*-synthesized AuNP ($10 \times LC_{50}$) led to 47.6, 76.7, and 100 % of larval reduction after 24, 48, and 72 h, respectively (Table 4). This is in agreement with earlier research, which pointed out that plant-borne compounds can be cheap and effective larvicidal agents for field purposes (Kovendan and Murugan 2011). For example, the mosquitocidal efficacy of the leaf extract of *Euphorbia hirta* was investigated in a field condition against *A. stephensi*, and larval density was reduced by 13.17, 37.64, and 84.00 % after 24, 48, and 72 h, respectively (Panneerselvam et al. 2013b). Recently, a growing number of plant-synthesized metal nanoparticles have been found effective against mosquito vector larvae in field conditions (e.g., Dinesh et al. 2015; Suresh et al. 2015; Madhiyazhagan et al. 2015; Subramaniam et al. 2015). The enhanced mosquitocidal potential of AuNP, if compared with the flower extract alone, may be linked to the fact that these poly-dispersed AuNPs are stable in the water for several weeks, and this allows them to pass through the insect cuticle and even into individual cells, where they interfere with molting and other physiological processes (Benelli 2016). To the best of our knowledge, the long-lasting efficacy of green-synthesized metal nanoparticles did not have genotoxicity against non-target organisms at doses lower than 12 ppm (Chandramohan et al. 2015).

Predation efficiency of *Aplocheilus lineatus*

In standard laboratory conditions, after 24 h, *A. lineatus* predation of IV instar larvae of *A. stephensi* was 56.38 % (Table 5). The predation efficiency of *A. lineatus* after treatment with ultra-low dosages of *C. guianensis* flower extract and green-synthesized AuNP was higher, reaching 83.98 and 96.04 % (Table 5). No detectable toxicity effects were observed on *A. lineatus* individuals exposed to the AuNP-contaminated aquatic environment (post-treatment observation period 10 days; data not shown). In agreement with our results, Chobu et al. (2015) have reported that the mosquitofish *Gambusia affinis* and *Carassius auratus* were effective predators of *Anopheles gambiae* III instar larvae, and that *G. affinis* is a more efficient predator of *A. gambiae* larvae over *C. auratus*. Tabibzadeh et al. (1971) also showed that *Gambusia* sp. substantially reduced Anopheline larvae in different aquatic habitats and contributed to a reduction in malaria transmission. Concerning the potential toxicity of mosquitocidal nanoparticles against non-target fishes, results are promising (Benelli 2016). Patil et al. (2012) showed that *Pergularia daemia*-synthesized silver nanoparticles were non-toxic against the *Poecilia reticulata*, while they are able to evoke good mortality rates against mosquito vectors

Table 4 *Anopheles stephensi* larval reduction in water storage reservoirs, post-treatment with *Couroupita guianensis* flower extract, and *C. guianensis*-synthesized gold nanoparticles

	Couroupita guianensis flower extract ($10 \times LD_{50}$)				Gold nanoparticles ($10 \times LD_{50}$)			
	Before treatment	24 h	48 h	72 h	Before treatment	24 h	48 h	72 h
Larval density (*n*)	1150 ± 11.41^b	725 ± 10.18^d	262 ± 12.37^e	0.00 ± 0.0^g	1420 ± 18.17^a	780 ± 10.07^c	237 ± 8.82^f	0.00 ± 0.0^g

Larval densities (*n*) are means ± SD of six replicates. Within the row, means followed by different letters are significantly different ($P < 0.05$)

Table 5 Predation efficiency of *Aplocheilus lineatus* against IV instar larvae of *Anopheles stephensi* in normal laboratory conditions and post-treatment with *Couroupita guianensis* flower extract or green-synthesized gold nanoparticles

Treatment	Consumed *Anopheles stephensi* larvae per day					Total predation (%)	Predation efficacy per day (*n*)
	Day 1	Day 2	Day 3	Day 4	Day 5		
Standard laboratory conditions	107.4 ± 5.45	118.6 ± 11.01	107.8 ± 5.35	114.2 ± 2.86	115.8 ± 5.80	56.38[a]	112.76[a]
Couroupita guianensis flower extract	168.6 ± 3.28	169.2 ± 2.58	170 ± 2.44	167.4 ± 5.77	164.6 ± 1.51	83.98[b]	167.96[b]
Gold nanoparticles	190.6 ± 2.30	192.4 ± 2.60	193.8 ± 2.04	190.4 ± 2.07	193.2 ± 1.92	96.04[c]	192.08[c]

Predation rates are means ± SD of five replicates (1 fish vs. 200 mosquitoes per replicate). Control was clean water without fishes. Within each column, values followed by different letters are significantly different ($P < 0.05$)

A. stephensi and *A. aegypti*. Recently, Murugan et al. (2015g) showed that seaweed-synthesized silver nanoparticles did not reduce the predation of copepod *Mesocyclops longisetus* against the filariasis vector *C. quinquefasciatus*. In agreement with our data, the predation of *Mesocyclops aspericornis* adults against malaria and dengue mosquitoes was significantly higher post-treatment with very low dosages (i.e., 1 ppm) of AuNP fabricated using lemongrass, *Cymbopogon citratus* (Murugan et al. 2015d). Recently, Subramaniam et al. (2015) also reported that *Mimusops elengi*-synthesized silver nanoparticles did not negatively impact predation rates of the mosquitofish *Gambusia affinis* against *Anopheles stephensi* and *Aedes albopictus*, validating this novel control tool in an environment-friendly perspective. The enhanced predation ability of *A. lineatus* post-treatment with AuNP can be due to the fact that ultra-low doses of metal nanoparticles reduce the motility of mosquito larvae (without impact on natural enemies), allowing higher predation rates (see also Benelli 2016).

Antiplasmodial assays

In antiplasmodial assays, both the *C. guianensis* flower extract and *C. guianensis*-synthesized AuNP showed higher activity against *P. falciparum* if compared to chloroquine. *C. guianensis* IC_{50} was 43.21 μg/ml (CQ-s) and 51.16 μg/ml (CQ-r), while *C. guianensis*-synthesized AuNP IC_{50} was 69.47 μg/ml (CQ-s) and 76.33 μg/ml (CQ-r); IC_{50} of chloroquine was 80.00 μg/ml (CQ-s) and 90.00 μg/ml (CQ-r) (Fig. 7). Screening of plants used in traditional medicine can be helpful to identify newer and safer anti-malarial drugs. El Tahir et al. (1999) reported that the methanol extract of *Annona squamosa* leaves showed high antiplasmodial activity with IC_{50} values of 2 and 30 μg/ml against CQ-s strain 3D7 and CQ-r strain Dd2 of *P. falciparum*. Murugan et al. (2015a) studied the antiplasmodial activity of *Senna occidentalis* and *Ocimum basilicum* on CQ-r and CQ-s strains of *P. falciparum*. IC_{50} of *S. occidentalis* was 48.80 μg/ml (CQ-s) and 54.28 μg/ml (CQ-r), while *O. basilicum* IC_{50} was 68.14 μg/ml (CQ-s) and 67.27 μg/ml (CQ-r). Concerning green-synthesized nanoparticles, moderate knowledge is available.

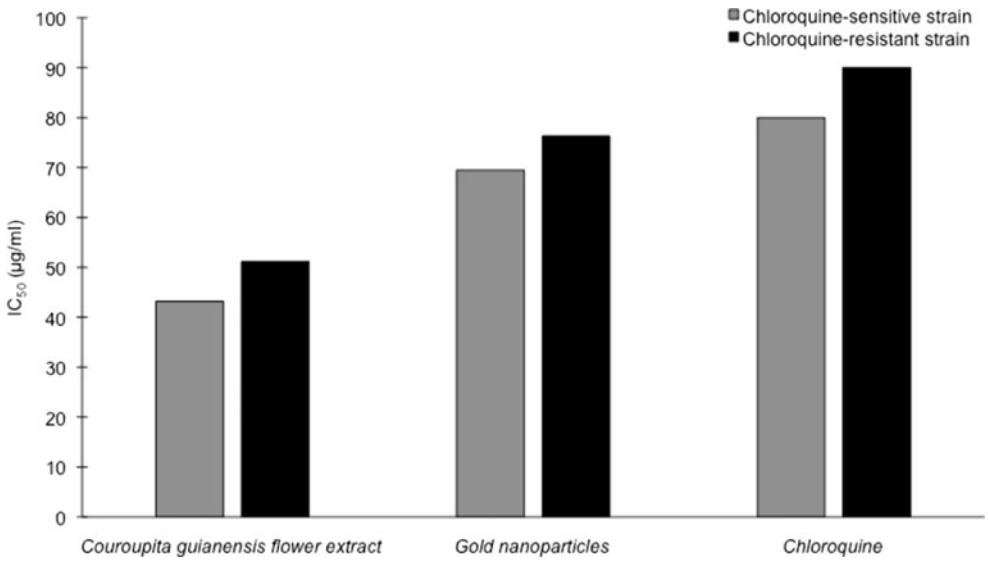


Fig. 7 In vitro growth inhibition of *Plasmodium falciparum* after a treatment with chloroquine, the flower extract of *Couroupita guianensis*, or green-synthesized gold nanoparticles

Concerning nanocomposite antiplasmodial drugs, Murugan et al. (2015b) focused on the antiplasmodial potential of silver nanoparticles produced using the aqueous extract of *Ulva lactuca* against CQ-r and CQ-s strains of *P. falciparum*; IC_{50} of *U. lactuca* was 57.26 μg/ml (CQ-s) and 66.36 μg/ml (CQ-r) while *U. lactuca*-synthesized silver nanoparticles achieved IC_{50} of 76.33 μg/ml (CQ-s) and 79.13 μg/ml (CQ-r). Recently, Rajakumar et al. (2015) validated the antiplasmodial activity of palladium nanoparticles produced using the leaf aqueous extract of *Euphorbia prostrata* in in vivo experiments on *Plasmodium berghei* in Swiss albino mice. Further research on the toxicity mechanism(s) of *C. guianensis*-fabricated AuNP is ongoing.

Conclusions

In this research, we biosynthesized AuNP using a cheap aqueous extract of *C. guianensis* flowers as reducing and stabilizing agent. The biofabricated AuNPs were mostly spherical or subtriangular in shape, crystalline in nature, with face-centered cubic geometry, their mean size was 26.5–43.8 nm. Overall, our results showed the multipurpose effectiveness of *C. guianensis*-synthesized AuNPs, since they may be proposed as newer and safer tools in the fight against CQ-r strains of *P. falciparum* and for field control of malaria vectors in synergy with wonder killifish predators.

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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.

Conflict of interest The authors declare no conflicts of interest.

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

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