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# *In vivo* and *in vitro* effectiveness of *Azadirachta indica*-synthesized silver nanocrystals against *Plasmodium berghei* and *Plasmodium falciparum*, and their potential against malaria mosquitoes

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ABSTRACT

Malaria transmission is a serious emergence in urban and semiurban areas worldwide, becoming a major international public health concern. Malaria is transmitted through the bites of *Anopheles* mosquitoes. The extensive employ of synthetic pesticides leads to negative effects on human health and the environment. Recently, plant-synthesized nanoparticles have been proposed as highly effective mosquitocides. In this research, we synthesized silver nanoparticles (AgNP) using the *Azadirachta indica* seed kernel extract as reducing and stabilizing agent. AgNP were characterized by UV–vis spectrophotometry, SEM, EDX, XRD and FTIR spectroscopy. The *A. indica* seed kernel extract was toxic against *Anopheles stephensi* larvae and pupae, $LC_{50}$ were 232.8 ppm (larva I), 260.6 ppm (II), 290.3 ppm (III), 323.4 ppm (IV), and 348.4 ppm (pupa). AgNP $LC_{50}$ were 3.9 ppm (I), 4.9 ppm (II), 5.6 ppm (III), 6.5 ppm (IV), and 8.2 ppm (pupa). The antiplasmodial activity of *A. indica* seed kernel extract and AgNP was evaluated against CQ-resistant (CQ-r) and CQ-sensitive (CQ-s) strains of *Plasmodium falciparum*. $IC_{50}$ of *A. indica* seed kernel extract were 63.18 μg/ml (CQ-s) and 69.24 μg/ml (CQ-r). *A. indica* seed kernel-synthesized AgNP achieved $IC_{50}$, of 82.41 μg/ml (CQ-s) and 86.12 μg/ml (CQ-r). However, *in vivo* antiplasmodial experiments conducted on *Plasmodium berghei* infecting albino mice showed moderate activity of the *A. indica* extract and AgNP. Overall, this study showed that the *A. indica*-mediated fabrication of AgNP is of interest for a wide array of purposes, ranging from IPM of mosquito vectors to the development of novel and cheap antimalarial drugs.



## 1. Introduction

The veterinary and medical importance of mosquitoes (Diptera: Culicidae) is mainly due by their capability to act as vectors for a number of important diseases and parasites, including malaria, avian malaria, yellow fever, dengue, Zika virus, Rift valley fever, Western equine encephalomyelitis, bancroftian and brugian filariae, canine heartworm disease (*Dirofilaria immitis*) and setariosis (*Setaria* spp.) (Mehlhorn et al., 2012; Benelli, 2015a). Mosquito-borne diseases are endemic in more than over 100 countries, causing mortality of nearly two million people every year (Jang et al., 2002; Taubes, 2000). The World Health Organization estimates that each year at least one million children were affected by mosquito-borne diseases and die. Worldwide, more than 2100 million people are at risk of malaria, filariasis, Japanese encephalitis, dengue fever, chikungunya and yellow fever (Becker et al., 2003; Benelli and Mehlhorn, 2016).

The neem tree (*Azadirachta indica* A. Juss.; Meliaceae) is traditionally labeled as “the village pharmacy” on account of its multifaceted healthful properties. Leaves and seeds of *A. indica* yield limonoid compound with excellent insecticidal activity against many phytophagous pests (Pavela et al., 2004; Pavela and Bárnet, 2005; Mordue and Nisbet, 2000). Dried *A. indica* leaves are commonly used by rural populations for protection against infestation of foodstuff insect pests. Smoke

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produced by burning of *A. indica* leaves is used for the protection against mosquitoes and have been traditionally used in the management of skin conditions such as eczema, psoriasis and certain fungal infections (Schmutterer, 1990; Tewari, 1992). *A. indica* seed oil contains at least 100 biologically active compounds. Among them, major constituents are a highly oxidized tetranortriterpenoids as e.g. azadirachtin, nimbin, nimbidin and nimbolides (Dua et al., 2009; Egho, 2012). In particular, azadirachtin is contained, which is considered as one of the most efficient insect growth regulators of plant origin, moreover showing repellent and antifeedant effects against more than 200 species of phytophagous insects (Mordue and Nisbet, 2000). For this reason, botanical insecticides based on extracts or seed oil from this plant rank among the most widely used both in plant and agricultural product protection (Mordue and Nisbet, 2000; Pavela et al., 2009). In addition, many formulations deriving from *A. indica* seed oil show antifeedancy, fecundity suppression, ovicidal and larvicidal activity, insect growth regulation and/or repellence against many arthropod pests of medical and veterinary importance (Semmler et al., 2009; Mehlhorn, 2011; Benelli et al., 2015a, 2015b). For instance, the concentrate extract of *A. indica* seeds [e.g. MiteStop, developed by the University spin-off company Alpha-Biocare (Dusseldorf, Germany)] is effective against ticks, house dust mites, cockroaches, raptor bugs, cat fleas, bed bugs (Schmahl et al., 2010), biting and bloodsucking lice (Al-Quraishy et al., 2011, 2012; Abdel-Ghaffar et al., 2012), *Sarcoptes scabiei* mites infesting dogs (Abdel-Ghaffar et al., 2008), poultry mites (Abdel-Ghaffar et al., 2009; Locher et al., 2010) and beetle larvae parasitizing the plumage of poultry (Walldorf et al., 2012). Other advantages arising from the use of *A. indica* based products are no induction of resistance, due to their multiple modes of action against pests and low toxicity rates against vertebrates (Benelli et al., 2015a, 2015b).

Culicidae control is being enhanced in many areas, but there are significant challenges, including an increasing mosquito resistance to insecticides and a lack of alternative, cost-effective and eco-friendly insecticides (Benelli, 2015a). To deal with these crucial challenges, recent emphasis has been placed on plant materials with mosquitocidal properties against mosquitoes of medical and veterinary importance (Mehlhorn et al., 2005; Amer and Mehlhorn, 2006a, 2006b, 2006c, 2006d; Benelli, 2015b; Benelli et al., 2015c; Pavela, 2015). Plant-borne chemicals have been used by human communities in different parts of the world as ovicides, larvicides, adult repellents and oviposition deterrents against a wide number mosquito species of economic importance (e.g. Panneerselvam and Murugan, 2013; Panneerselvam et al., 2013; Benelli et al. 2013a, 2013b, 2015a, 2015b; Govindarajan et al., 2016a, 2016b).

Nanotechnology is a promising field of interdisciplinary research, since it opens up a wide array of opportunities in different fields including pharmacology, electronics, parasitology and pest management (Bhattacharyya et al., 2010). Notably, the use of plant metabolites as reducing and stabilizing agents for the synthesis of mosquitocidal nanoparticles has been attempted, with promising results (Benelli, 2016). Good examples include *Annona squamosa* (Arjunan et al., 2012), *Nerium oleander* (Roni et al., 2013), *Moringa oleifera* (Sujitha et al., 2015), *Caulerpa scalpelliformis* (Murugan et al., 2015a), *Cymbopogon citratus* (Murugan et al., 2015b), *Artemisia vulgaris* (Murugan et al., 2015c) and *Toddalia asiatica* (Murugan et al., 2015d). In several cases, good effectiveness of the synthesized nanoparticles has been confirmed also in field assays, treating water storage reservoirs (see Benelli, 2016 for a review).

*A. indica*-borne metabolites can be used for effective and rapid nanosynthesis of silver nanoparticles (AgNP) (Shankar et al., 2004; Tripathi et al., 2009). However, to the best of our knowledge, the antimalarial and mosquitocidal properties of *A. indica*-synthesized AgNP are unknown. In this research, we proposed a method of green synthesis AgNP using *A. indica* seed kernel extract as reducing and stabilizing agent. In view of an increasing interest in developing bio-insecticides as an alternative to chemical insecticides, this investigation aims to assess the larvicidal and pupicidal of the *A. indica* seed kernel extract and AgNP against malaria, vector, *A. stephensi*, as target species of the vector control. AgNP were characterized by UV–vis spectrum, X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and scanning electron microscopy and energy dispersive X-ray analysis (SEM-EDX). Both the aqueous extract of *A. indica* seed kernel extract and synthesized AgNP were tested in laboratory conditions against young instars of the malaria vector *A. stephensi*. Furthermore, the antiplasmodial activity of *A. indica* seed kernel extract and AgNP was evaluated *in vitro* against CQ-resistant (CQ-r) and CQ-sensitive strains (CQ-s) of the malaria parasite *Plasmodium falciparum*. *In vivo* antiplasmodial experiments conducted on *Plasmodium berghei* infecting albino mice confirmed the activity of the *A. indica* extract and AgNP against *Plasmodium* parasites.

## 2. Materials and methods

### *2.1. Anopheles stephensi rearing*

We tested a laboratory-reared, pathogen-free strain of *A. stephensi*, originally established as reported 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 cm × 13 cm × 4 cm enamel trays containing 500 ml of water, where eggs were allowed to hatch in laboratory conditions (27 °C ± 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. Newly emerged larvae and pupae were collected and used in the experiments (Dinesh et al., 2015).

### *2.2. Synthesis and characterization of silver nanoparticles*

The *A. indica* kernel extract was prepared adding 10 g of neem kernel 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 N. 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 *A. indica* seed kernel extract generating stable AgNP in water. Silver nitrate was purchased from the Precision Scientific Co. (Coimbatore, India).

Synthesis of AgNP was confirmed by sampling the reaction mixture at regular intervals and the absorption maxima was scanned by UV–vis spectrophotometry, at the wavelength of 200–800 nm in UV-3600 Shimadzu spectrophotometer, 1 nm resolution. Furthermore, the reaction mixture was subjected to centrifugation at 15,000 rpm for 20 min; the resulting pellet was dissolved in deionized water and filtered through Millipore filter (0.45 μm). An aliquot (2 ml) of this filtrate containing AgNP was used for scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD) analyses, 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 nanoparticles were studied using a FEI QUANTA-200 SEM. The surface groups of AgNP were qualitatively confirmed by FTIR spectroscopy, with spectra recorded by a Perkin-Elmer Spectrum 2000 FTIR spectrophotometer.

### *2.3. Larvicidal and pupicidal experiments against Anopheles stephensi*

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