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Synthesis of silver nanoparticles using *Catharanthus roseus* root extract and its larvicidal effects

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Abstract

Phytosynthesis of silver nanoparticles has attracted considerable attention due to their biocompatibility, low toxicity, cost-effectiveness and being a novel method has an eco-friendly approach. Biological activity of root extracts as well as synthesized silver nanoparticles of *Catharanthus roseus* were evaluated against larvae of *Aedes aegypti* and *Culex quinquefasciatus*. The structure and proportion of the synthesized nanoparticles was defined by exploitation ultraviolet spectrophotometry, X-ray diffraction, fourier transform infrared spectroscopy, energy dispersive X-ray spectroscopy and scanning electron microscopy methods. Reduction of silver ions occurred when silver nitrate solution was treated with aqueous root extract at 60°C. Synthesized silver nanoparticles (AgNPs) were confirmed by analyzing the excitation of surface plasmon resonance (SPR) using UV-vis spectrophotometer at 423 nm. FTIR showed aliphatic amines and alkanes corresponding peaks to be presence of responsible compounds to produced nanoparticles in the reaction mixture. Spherical shaped and crystalline nature of particles was recorded under XRD analysis. Presence of silver metal and 35-55nm sized particles were recorded using EDAX and SEM respectively. Larvicidal activity was observed after 24 hrs of exposure to root extracts and synthesized silver nanoparticles. The highest larval mortality was observed in synthesized silver nanopartiucles against *Aedes aegypti* ($LC_{50=}$ 2.01±0.34; $LC_{90=}$ 5.29±0.07 at 5.0 mgl^{-1} concentration) and *Culex quinquefasciatus* ($LC_{50=}$ 1.18±0.15; $LC_{90=}$ 2.55±0.76 at 3.5 to 5.0 mgl^{-1} concentration) respectively. The present study provides evidence that synthesized silver nanoparticles of *Catharanthus roseus* offer potential source for larvicidal activity against the larvae of both dengue and filariasis vectors.

Key words

Aedes aegypti, Biolarvicidal, *Catharanthus roseus, Culex quinquefasciatus, Root extract,* Silver nanoparticles

Introduction

Mosquitoes are principal vector of many vector borne diseases affecting human beings and animals. These vectors are responsible for transmitting several diseases such as malaria, dengue fever, dengue hemorrhagic fever, dengue shock syndrome, chikungunya, lymphatic filariasis, *Japanese encephalitis* and leishmaniasis etc. and cause thousands of deaths every year (Kumar *et al*., 2007; Gopalan and Das, 2009; Dhiman *et al*., 2010). The mosquito-borne diseases result in avoidable ill-health and death (Kishor, 2006).

The container breeding mosquito, *Aedes aegypti* L. thrives in urban and peridomestic environments where it transmits dengue virus to humans (Gubler and Dengue, 1998; Guha-Sapir and Schimmer, 2005). This disease occurs primarily in the equatorial region of Africa, America, South East Asia and Western Pacific (Amarasinghe *et al*., 2011). The incidence of dengue fever has increased dramatically since 1960s (Whitehorn and Farrar, 2010), with current estimates of the incidence ranging from 50 million to 528 million people infected yearly – over 40 percentages are now at high risk worldwide (Gupta *et al*., 2012). Similarly, *Culex quinquefasciatus* is a vector of lymphatic filariasis

affecting 120 million people throughout the world, and approximately, 400 million people are at risk of contracting filariasis, resulting in annual economic loss of 1.5 billion dollars (WHO, 2002; Santhoskumar *et al*., 2011).

Traditionally, plants and their derivatives were used to kill household and agricultural pests. In all probability, these plants are used to control insects, contained insecticidal phytochemicals that were predominantly secondary compounds produced by plants to protect themselves against herbivorous insects (Shaalan *et al*., 2005). In view of the growing concern regarding pollution by chemical insecticides and acquired tolerance among target species, the merits of phytochemicals present in plants as secondary metabolites, are increasingly recognized. Recent studies have insighted the insecticidal properties of chemicals derived from plant material and concluded that they are environmentally safe, degradable and target specific (Nathan, 2007; Banerjee and Nareendhirakannan, 2011; Ponarulselvam *et al*., 2012; Arjunan *et al*., 2012).

Biological synthesis of nanoparticles has been found to be an easy, rapid, efficient and a completely unique methodology to eco-friendly approach. The biologically produced silver nanoparticles have attracted considerable attention owing to their diverse properties like anti-microbial (Fayaz *et al*., 2010; Hashemabadi *et al*., 2014), anti-fungal (Ales Pana *et al*., 2009), anti-bacterial (Krishnaraj *et al*., 2010), anti-viral (James *et al.,* 2008), anti-HIV (Lara *et al*., 2010), anti-inflammatory (Nadworny *et al*., 2010), anti-plasmodial (Ponarulselvam *et al*., 2012) and anti-larvicidal activity towards medically challenged pathogens and dreadful vectors (Santhoshkumar *et al*., 2011; Arjunan *et al*., 2012) .

Catharanthus roseus, belonging to Apocynaceae family, is an important medicinal plant producing more than 130 mono-terpeniods and indole alkaloids (Malabadi *et al*., 2009, 2012). *Catharanthus roseus* plant was previously proved to have anticancer, anti-hypertension and antibacterial activities (Malabadi *et al*., 2012). From the literature consulted, there is no knowledge about the mosquito larvicidal activity of synthesized silver nanoparticles (AgNPs) from *Catharanthus roseus,* especially root extract. In pursuit of all these considerations, the present investigation was carried out to synthesize plant-based silver nanoparticles from root extract of *Catharanthus roseus* and evaluation of their mosquito larvicidal activity against *Aedes aegypti* and *Culex quinquefasciatus* vectors.

Materials and Methods

Preparation of silver nanoparticles (AgNPs): *Catharanthus roseus* roots were collected from Thaiyilpatti village near Sattur town. Twenty five gram of freshly harvested plant roots were washed thoroughly in tap water for 10 min to remove dust particles and rinsed briefly in deionized water in shade. The droplets were removed with paper towel and dried for 3 days at room temperature. After that, roots were stirred with 150 ml de-ionized water boiled at 60°C for 30 min and the boiled crude extract was filtered through Whatman filter paper (No. 1) to obtain the filtrate and it was stored at 4°C for further studies. 90 ml of 1 mm concentrated silver nitrate solution was prepared using sterile double distilled water. 10 ml of plant root filtrate was introduced into prepared silver nitrate solution and this reaction mixture was kept without disturbance at room temperature. Rapid synthesis of AgNPs was observed after 5 hrs, incubation through visual observation of colour change from pale yellow to dark brown in the reaction mixture. The fully reduced solution was centrifuged at 8000 rpm for 30 min. The supernatant liquid was discarded and the pellet obtained was redispersed in deionized water. Centrifugation process was repeated three to five times to wash off any absorbed substance on the surface of the silver nanoparticles.

Characterisation of AgNPs: Preliminary characterization of silver nanoparticles was carried out using UV-visible spectroscopy. Reduction of silver ions to nanoparticles form was monitored by measuring the UV-visible spectra at 300nm to 700 nm. Distilled water was used as blank. The purified liquid of silver nanoparticles was subjected to Fourier Transform Infrared spectroscopy analysis (FTIR) for analysis of functional groups present in synthesized nanoparticles. These measurements were carried out on a spectrum RX1-one instrument in diffused reflectance mode at a resolution of 4 cm^{-1} in KBr pellets for comparison, a drop of 20% *Catharanthus roseus* broth was mixed with KBr powder and pelletized after drying properly. The pellets were later subjected to FTIR spectroscopy measurement. For XRD studies, dried nanoparticles were coated on XRD grid and the spectra were recorded by using Philips PW 1830 X-ray generator operated at a voltage of 40 kV and a current of 30 mA with Cu K1 radiation in the range of 2 angles from 10^0 and 90^0. The size of nanoparticles was confirmed by using Philips scanning electron microscope (SEM: Hitachi H-7100) equipped with an EDX attachment. Micrograph and the corresponding EDX spectrum were recorded by focusing on clusters of particles. Electron microscope instrument coupled with an energy dispersive X-ray spectrophotometer (EDX) operated at an accelerating voltage at 20 keV.

Larvicidal activity of AgNPs

Insect rearing: The eggs of *Aedes aegypti* and *Culex quinquefasciatus* were collected from the Centre for Research in Medical Entomology, Madurai, Tamil Nadu. These were transferred to 18 cm length x 13 cm width x 4 cm high Denamel trays containing 500ml of water where they were allowed to hatch. Mosquito larvae were reared at 27°C±2°C and 75%–85% relative humidity (RH) in a 14:10 (L:D) photoperiod. Larvae were fed 5g ground dog biscuit and brewers yeast daily in 3:1 ratio.

Larvicidal activity was assessed by the procedure of WHO with some modification (Abdul Rahuman *et al*., 2009). Synthesized AgNPs toxicity test was performed by placing 20 mosquito larvae in 200 ml of sterilized double distilled water with AgNPs in 250 ml beaker (Borosil). 1000 mg of synthesized AgNPs was first dissolved in 1 l of Milli Q water (stock solution). From stock solution, nanoparticle solutions were diluted using Milli Q water as solvent according to the desired concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5 mgl^{-1}). Each test included a set control group (root extract) with six replicates for each individual concentration. Mortality was assessed after 24 hrs to determine acute toxicities on fourth instar larvae of *Aedes aegypti* and *Culex quinquefasciatus*.

Dose-response bioassay: During laboratory trial, synthesized AgNPs were subjected to a dose–response bioassay for larvicidal activity against *Aedes aegypti* and *Culex quinquefasciatus*. Different concentrations ranging from 0.5$mg1^{-1}$, 1.0$mg1^{-1}$, 1.5$mg1^{-1}$, 2.0$mg1^{-1}$, 2.5$mg1^{-1}$, 3.0$mg1^{-1}$, 3.5$mg1^{-1}$, 4.0$mg1^{-1}$, 4.5$mg1^{-1}$ and 5 $mg1^{-1}$ for synthesized AgNPs were prepared for larvicidal activity. The numbers of dead larvae were counted after 24 hrs of exposure, and percent mortality was reported from the average of six replicates. However, at the end of 24 hrs, selected test samples turned out to be equal in their toxic potential.

Statistical analysis: Data corresponding to the performance of larvae with deleterious effect of AgNPs were subjected to analysis of variance and average mortality data was calculated $LC_{50,}$ $LC_{90,}$ lower confidence limit and upper confidence limit values at 95% using SPSS software package 9.0 version. Results with p value <0.05 were considered to be statistically significant.

Results and Discussion

The biological synthesis of metal nanoparticles is attracting considerable attention among the researchers due to their potential applications for the development of novel technologies (Singh and Balaji Raja, 2011). Synthesized AgNPs were detected following colour change by visual observations due to the progress of reaction between 1mM silver nitrate solution (90 ml) and active compounds of *C. roseus* root filtrates (10 ml). The colour of silver nitrate solution changed initially from yellow to light brown; after 10 min the colour changed to dark brown. Dark brown colour indicated the production of silver nanoparticles in the reaction mixture (Fig. 1 A1). *C. roseus* root filtrate was able to reduce silver ions and produce silver nanoparticles. It is reported that colour change in solution might be due to excitation of surface plasmon vibrations in the nanoparticles and might also be attributed to the presence of large amount of phytochemicals and the wide range of metabolites present in plant extract (Jha and Prasad, 2010; Arun *et al.,* 2011).

The synthesized AgNPs were continuously readout using UV-Visible absorption spectrum in the ranges of wavelength from 300 nm to 700 nm. The particles have increasingly sharp absorbance maximum peak at 423 nm and gradually decreased while nanometer increased respectively (Fig. 1 A2). A strong responsible peak was recorded at 423 nm in UV-Vis spectrum, indicating complete reduction of $AgNO_3$ and AgNPs were dispersed in the reaction mixture without aggregation. Silver nanoparticle formed at 400 nm confirmed the occurrence of silver nanoparticles which were reported by Harajyoti and Ahmed (2011). Mano Priya *et al.* (2011) reported that silver nanoparticles formed at 380 nm confirmed the occurrence of silver nanoparticles. The biologically synthesized nanoparticles were stable for more than six months and showed very little aggregation (Agalya Priyadarshini *et al*., 2012). Besides, the plasmon bands were broadened throughout absorption in accountable wave-lengths, which could exhibit the size, shape and distribution of particles (Ahmad *et al*., 2003) within the binary compound suspensions.

FTIR analysis was performed to find the possible functional groups and biomolecules for capping and efficient stabilization of bio-metal AgNPs synthesized by root filtrates in the reaction mixture. This spectrum showed many absorption bands indicating the presence of active functional groups in the synthesized silver nanoparticles (Fig. 1 B). Intensity peaks slightly increased for the period of silver nanoparticles synthesis like 1033.85 cm^{-1}, 1381.03 cm^{-1}, 2850.71 cm^{-1}, as well as some intensity peaks decreased like 505.35cm^{-1}, 451.34cm^{-1}, 437.84 cm^{-1} and 416 cm^{-1}. The absorption bands seen at 1033.85 cm^{-1} were assigned to the stretching vibrations of aliphatic amines, peaks at 1381.03 cm^{-1} indicated C-H rock medium to alkanes and peaks at 2850.71 cm^{-1} indicated C-H stretch medium alkanes. The presence of active functional groups in root extracts refused in swift reduction of silver ions to silver nanoparticles. Silver nanoparticles obtained in the present study might be surrounded by proteins having the functional groups such as amines and alkenes. Similar findings of proteins with reduced silver was reported by Soni and Prakash (2011) from fungal extract and Sudha Lakshmi (2011) from leaf extract of *Cleome viscosa*. Tripathi *et al.* (2013) suggested that functional residues had stronger capability to bind silver nanoparticles to prevent agglomeration and provides longer stability and also stated that the biomolecules present in the plant extract might play dual role *i.e.,* silver nanoparticle synthesis as well as stabilization of the synthesized particles.

XRD pattern showed three intense peaks (28, 32.4 and 46.5.) in the whole spectrum of 2 θ value ranging from 20° to 80° (Fig. 1 C). The average estimated particle size of the sample was 35-55 nm. It is reported that the biologically synthesized AgNPs lattice were unaffected by other molecules in plant extract and their size was determined to be about 21 nm (Krishnaraj *et al.*, 2010) and 62 nm (Sivakama Valli and Vaseeharan, 2012) respectively. Baishya *et al.* (2012) reported that XRD pattern obtained for silver nanoparticles showed a number of Bragg's reflections that might be indexed on the basis of the face centered

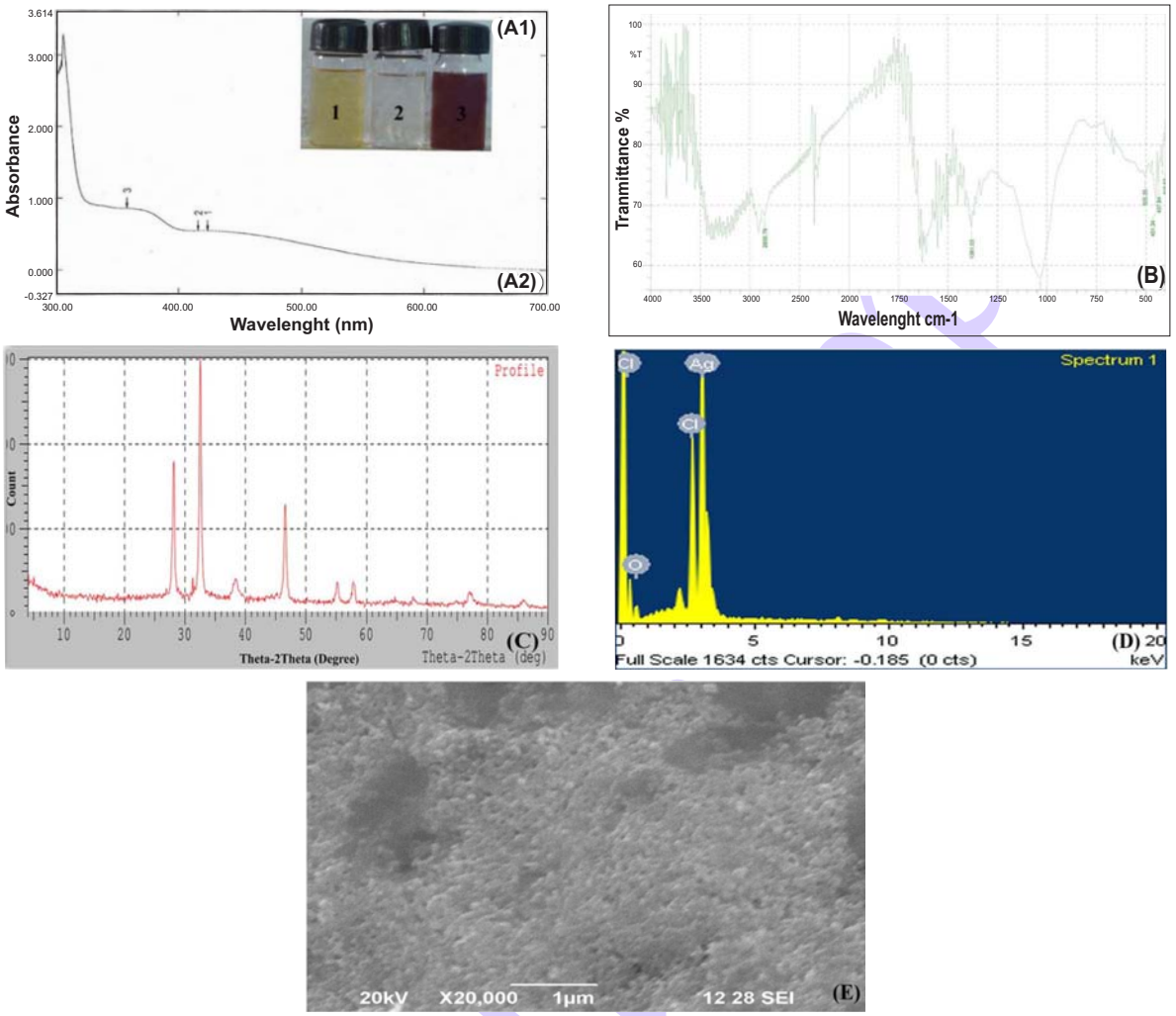

Fig. 1.: A1: Photographs showing changes in color after adding $AgNO_3$ before reaction and after reaction time of 24 hrs; (1) root extract of *Catharanthus roseus;* (2) 1mM $AgNO_3$ and (3) 1mM $AgNO_3$ with root extract after 24 hrs of incubation. A2: UV-vis spectra of synthesized AgNPs from *Catharanthus roseus*. B: FTIR spectrum of AgNPs; C: XRD patterns of AgNPs; D: EDAX spectrum of AgNPs; E: SEM image of AgNPs from *Catharanthus roseus* at magnification of 20kv X20,000

cubic structure of silver. This findings exhibited that the produced nano sized silver particles, were viable, stable, potential and bio-mineralized form of crystalline in nature due to capping of root filtrates containing bio-active ingredients.

EDAX provides supporting confirmation for the formation of silver nanoparticles. In the present study, EDAX spectrum showed signal of silver which confirmed the presence of silver nanoparticles (Fig. 1 D). The signal was observed at 3 KeV, which was typical for silver nanoparticles due to surface plasmon resonance. The other spectral signals such as Cl and O were also noticed in the EDAX spectrum. The signals other than silver signal in EDAX spectrum may arise from the organic content of leaf extract that were already bound with the surface of silver nanoparticles. Forough and Farhadi (2010) suggested that the EDAX spectrum of solution containing silver nanoparticles confirmed the presence of elemental silver signals. Similar finding confirmed the reports of Suman *et al.* (2013) who reported the synthesis of silver nanoparticle using *Ammannia baccifera* and the findings of Umesh *et al.* (2013) on seed extract of *Artocarpus heterophyllus*.

SEM image showed that the synthesized silver nanoparticles were clustered (Fig. 1 E). The particles were more or less spherical in shape and their size ranged was 35nm- 55nm. The findings supported the spherical nature of AgNPs produced using leaf extract of *Cissus quadrangularis* and were obtained at 50-60 nm range in their size (Sivakama Valli and Vaseeharan,

Table 1.: Larvicidal activity of root extracts and synthesized silver nanoparticles using C*atharanthus roseus* against fourth instar larvae of *Aedes aegypti and* C*ulex qunifasciatus*

Species	Concentration (mgl^{-1}) means	% Mortality* by (mgl^{-1})	LC_{50} (mgl^{-1})	UCL-LCL (mgl^{-1})	LC_{90}±SE (mgl^{-1})	UCL-LCL (mgl^{-1})	χ^2 (df=5)
Aedes aegypti	0.5	00.00±0.00	2.01±0.34	2.85 - 0.67	5.29±0.07	5.63 - 1.14	4.22
	1.0	28.30±0.68					
	1.5	40.00±0.81					
	2.0	46.60±0.94					
	2.5	50.16±0.67					
	3.0	60.00±1.05					
	3.5	66.60±0.94					
	4.0	83.00±0.68					
	4.5	90.00±0.75					
	5.0	100.00±0.00					
Culex quinquefasciatus	0.5	00.00±0.00	1.18±0.15	1.86 - 0.53	2.55±0.76	3.12 - 1.16	5.58
	1.0	60.00±0.57					
	1.5	73.33±1.94					
	2.0	75.00±0.76					
	2.5	81.66±1.68					
	3.0	91.33±1.91					
	3.5	100.00±0.00					
	4.0	100.00±0.00					
	4.5	100.00±0.00					
	5.0	100.00±0.00					

Control (root extract) - Nil mortality; Significant at $p<0.05$ level. LC_{50} lethal concentration that kills 50% of the exposed larvae; LC_{90} lethal concentration that kills 90% of the exposed larvae; UCL upper confidence limit; LCL lower confidence limit; $\div^2$ chi-square; df degree of freedom; *Mean value of six replicates ± SE

2012). The stable silver nanoparticle of spherical shape, with an average size of 20 nm was synthesized using leaf extract of *Aloe vera* (Chandran *et al.,* 2008) and ~20 nm ranges from black tea leaf extracts (Begum *et al*., 2009). This result is in agreement with the previous report where AgNPs were synthesized using *Syzygium cumini* seed extract and their i*n-vitro* antioxidant (Banerjee and Nareendhirakannan, 2011).

Currently, a novel strategic method is needed to develop potential bio-pesticides to eliminate mosquito vectors responsible causing highly threatening diseases. The extremely potential larvicidal and pupicidal effects of naturally derived bio-products can facilitate to eradicate several infectious diseases (Roopan *et al*., 2013). AgNPs synthesized exploitation *Catharanthus roseus* plant root extract against *Aedes aegypti* and *Culex quinquefasciatus* were determined. The highest larval mortality (100%) was found in the synthesized AgNPs against the fourth instar larvae of *Aedes aegypti* and *Culex quinquefasciatus* and their LC_{50} (2.01 mgl^{-1} and 1.18 mgl^{-1}) and LC_{90} (5.29 mgl^{-1} and 2.55 mgl^{-1}) values were recorded at 5 µl and 3.5 µl concentration level, respectively. The results of upper confidential level (UGL), lower confidential level (LGL) and chi square ($\div^2$) values are mentioned in Table 1. Similarly, larvicidal activity of biologically synthesized AgNPs from various plant extracts was reported by Rajakumar and Abdul Rahuman (2011) from leaf extract of *Eclipta prostrate* against *Culex quinquefasciatus* and *Anopheles subpictus* and observed LC_{50} values of 4.56 mgl^{-1} and 5.14 mgl^{-1}. Agalya Priyadarshini *et al.* (2012) reported maximum larvicidal activity using *Euphorbia hirta* leaf extract against *Anopheles stephensi* at 10.14, 16.82, 21.51, 27.89 and 34.52 ppm LC_{50} concentration. It is reported that the silver nanoparticles synthesized with *Rhizophora mucronata* leaf extract against the larvae of *Aedes aegypti* and *Culex quinquefasciatus* and LC_{50} values of synthesized AgNPs was identified as 0.585 and 2.615 mgl^{-1} and LC_{90} values as 0.891 and 6.291 mgl^{-1}, respectively (Gnanadesigan *et al.,* 2011).

Catharanthus roseus root extract have been used as a reducing and capping agent for synthesis of AgNPs. The formed silver nanoparticles are highly stable and had significant larvicidal activity against larvae of *Aedes aegypti* and *Culex quinquefasciatus*. The present study reveals high efficacy of AgNPs' as a strong anti-larvalcidal agent.

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