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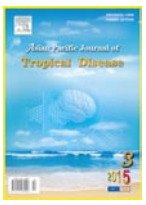

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Larvicidal activity of titanium dioxide nanoparticles synthesized using *Morinda citrifolia* root extract against *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus* and its other effect on non-target fish

Thodhal Yoganandham Suman[1], Radhika Rajasree Santha Ravindranath[1*], Devan Elumalai[2], Patheri Kuny Kaleena[2], Rajendiran Ramkumar[3], Pachiappan Perumal[3], Lakshminarayanan Aranganathan[1], Paneer Selvam Chitrarasu[4]

[1]*Centre for Ocean Research (NIOT-SU Collaborative Research Centre), Sathyabama University, Chennai 600119, Tamil Nadu, India*

[2]*Department of Zoology, Presidency College, Chennai 600005, Tamil Nadu, India*

[3]*Department of Biotechnology, Periyar Pakalinagar, Periyar University, Salem-636011, Tamil Nadu, India*

[4]*Unit of Aquaculture and Aquatic Toxicology, Post Graduate and Research Department of Zoology, The New College, Chennai 600014, Tamil Nadu, India*

PEER REVIEW

Peer reviewer

C. Jayaseelan, Ph.D., Senior Research Fellow, Unit of Nanotechnology and Bioactive Natural Products, Department of Zoology, C. Abdul Hakeem College, Melvisharam-632 509, Vellore District, Tamil Nadu, India.
Tel: +91 98434 29490/04172 269009
Fax: +91 04172 269487
E-mail: jayaseelancahc@gmail.com

Comments

The present paper demonstrated the use of a natural, low-cost biological reducing agent. The manuscript is well organized and potentially interesting, and addresses an important topic.
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ABSTRACT

Objective: To assess the larvicidal activity of titanium dioxide nanoparticles (TiO_2NP_S) synthesized from the root aqueous extract of *Morinda citrifolia* (*M. citrifolia*) against the larvae of *Anopheles stephensi (An. stephensi)*, *Aedes aegypti (Ae. aegypti)* and *Culex quinquefasciatus (Cx. quinquefasciatus)*.

Methods: The *M. citrifolia* broth solution was prepared by taking 8 g of the powdered root of *M. citrifolia* in 250 mL Erlenmeyer flask along with 100 mL of distilled water and boiled for 5 min. About 20 mL of *M. citrifolia* root extract was added into the 80 mL of an aqueous solution of 5 mmol/L $TiO(OH)_2$ for the reduction under continuous stirring for 4 h at 50 °C. Synthesized TiO_2NP_S were characterized by X-ray diffraction, Fourier transform infrared spectroscopy, field emission scanning electron microscopy and energy dispersive X-ray spectroscopy. X-ray diffraction confirmed the crystalline nature of the nanoparticles. Toxicity studies were carried out against non-target fish species *Poecilia reticulata*, the most common organism in the habitats of *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*.

Results: The Fourier transform infrared spectroscopy for TiO_2NP_S synthesized by *M. citrifolia* root extract showed band at 3 426 cm^{-1}, 1 637 cm^{-1} and 714 cm^{-1}. The 3 426 cm^{-1} showed O-H stretching due to alcoholic group; 1 637 cm^{-1} showed N-H bend due to alcoholic group. In particular, the 1 637 cm^{-1} indicated the presence of H bend bond for 1° for proteins. A peak was observed around 714 cm^{-1} due to Ti-O-O bond. Field emission scanning electron microscopy and transmission electron microscopy showed the spherical nature of the nanoparticles with a size of 20.46-39.20 nm. The biosynthesized TiO_2NP_S showed maximum activity against the larvae of *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* when compared to the aqueous extract of *M. citrifolia*. Toxicity studies revealed no toxicity towards *Poecilia reticulata* at LC_{50} and LC_{90} doses of TiO_2NP_S.

Conclusions: TiO_2NP_S could be used along with *Poecilia reticulata* in integrated vector control.

KEYWORDS

Morinda citrifolia, Titanium dioxide nanoparticles, Larvicidal activity

1. Introduction

Mosquitoes are important vectors of human diseases, especially in the tropics as it kills millions of people every year[1]. Still mosquitoes are the important vector insect in public health viewpoint owing to the problems associated

*Corresponding author: Dr. Radhika Rajasree S.R., Scientist-D and Group Head, Centre for Ocean Research, Sathyabama University, Jeppiaar Nagar, Rajiv Gandhi Road Chennai-600119, Tamil Nadu, India.
Tel: 9840927503
E-mail: radhiin@gmail.com
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with chemical insecticides, including toxicity to non-target organisms, environmental and human health concerns and unavailability of vaccines for many mosquitoes borne diseases[2,3]. Control of mosquito populations is the only available option to reduce the incidence of vector-borne diseases like malaria, filariasis, dengue and chikungunya in many tropical countries especially India[4]. *Aedes aegypti* (*Ae. aegypti*) is a carrier of dengue fever virus causing dengue, chikungunya, and dengue hemorrhagic fever[5]. According to the WHO report of the year 2009, two fifths of the world population is under risk of dengue infection (WHO index) and in the year 2010, 28 292 cases of infection and 108 deaths were reported in India[6]. The incidence of dengue has grown dramatically around the globe in recent decades; over 2.5 billion people (40% of the world's population) are at risk from dengue. WHO currently estimates that there may be 50-100 million dengue infections worldwide every year[6]. *Culex quinquefasciatus* (*Cx. quinquefasciatus*) is the main vector for lymphatic filariasis. Around 120 million people are infected worldwide and 44 million people have common chronic clinical manifestation. According to WHO[7], about 90 million people worldwide are infected with *Wuchereria bancrofti*, the lymphatic dwelling parasite, and ten times more people are at the risk of being infected. In India alone, 25 million people harbor microfilaria and 19 million people suffer from filarial disease manifestations[8]. *Anopheles stephensi* Liston (*An. stephensi*), is the major human malaria mosquito vector prevalent in various states including the Middle East and South Asia[9], which harbors and transmits the malarial protozoan parasite *Plasmodium falciparum*[10]. In addition to malaria vector, *Ae. aegypti* L. is another leading mosquito vector for RNA viruses which causes dengue and yellow fever[11].

Mosquito control is being strengthened in many areas, but there are significant challenges, including an increasing mosquito resistance to insecticides and a lack of alternative, cost-effective, and safe insecticides. The role of phytochemicals is one such strategy that may be suitable for mosquito control. Therefore, attempts to produce novel materials as mosquitocide are still necessary. Biologically active plant materials have attracted considerable interest in mosquito control programs in the recent times. Many works on plant extracts and their active constituent compound against mosquito have been carried around the world[12]. In a recent study[13], Suman reported the toxic effect of methanol extract of *Ammania baccifera*, against *Cx. quinquefasciatus* and *Ae. aegypti*. A substance derived from plants has drawn a greater attention for researchers and round about 2 000 plant species are formerly identified for their insecticidal activities[14].

In late years, nanoparticle/polymer composites have become important owing to their diminished size and large surface area and because they display unique properties not considered in bulk materials. As a result, nanoparticles have useful applications in photovoltaic cells, optical and biological sensors, conductive materials, and coating formulations[15]. The plant-mediated biosynthesis of nanoparticles is advantageous over chemical and physical methods because it is a cost-effective and environmentally-friendly method, where it is not necessary to use high pressure, energy, temperature, and toxic chemicals[16]. The titanium dioxide nanoparticles (TiO_2NP_S) were synthesized from *Bacillus subtilis*[17], *Eclipta prostrate*[18], *Lactobacillus* and *Nyctanthes arbortristis* leaves[19,20]. TiO_2NP_S synthesized from *Aeromonas hydrophila* and *Catharanthus roseus* possess antibacterial activity and antiparasitic activity[21,22].

Morinda citrifolia L. (Rubiaceae) (*M. citrifolia*), known as "Noni", is widely distributed in tropical Asia, India, and the Pacific Islands. Almost all parts of this plant, including fruits, flowers, leaves, bark, stem, and roots have been used as food, medicine, and fabric dyes for more than 2 000 years by the Polynesian people[23]. The roots of *M. citrifolia* produce various anthraquinones that exhibit larvicidal activities against *Ae. aegypti*[24]. *M. citrifolia* leaf extract was previously shown to possess larvicidal activity against *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*[25]. Hence the present study aims to investigate the larvicidal activity of TiO_2NP_S synthesized using *M. citrifolia* which is not yet been explored.

2. Materials and methods

2.1. Preparation of the plant extract

Fresh plant material was collected from Periyar University, Salem, Tamil Nadu, India. The taxonomic identification was made by Dr. Mujeerafathima, Department of Plant Biology and Biotechnology, Nandanam Government Arts College, Chennai, India. The voucher specimen was numbered and deposited in our botany herbarium. About 8 g of the *M. citrifolia* root powder was boiled at 60 °C for 15 min in 100 mL of distilled water and filtered through Whatman No. 1 filter paper. The filtered *M. citrifolia* root extract was stored in the refrigerator at 4 °C for further studies.

2.2. Synthesis of TiO_2NP_S

The aqueous solution of $TiO(OH)_2$ (5 mmol/L) was prepared and used for the synthesis of TiO_2NP_S. About 20 mL of boiled *M. citrifolia* root extract was added into 80 mL of aqueous solution of 5 mmol/L $TiO(OH)_2$ for the reduction at 50 °C for 4 h with continuous stirring.

2.3. Characterization of nanoparticles

TiO_2NP_S reaction mixture was centrifuged at 60000 r/min for 40 min and the resulting pellet was dissolved in deionized water and filtered through Whatmann filters (0.45 μm). An aliquot of this filtrate containing TiO_2NP_S was used for

X-ray diffraction method (XRD), Fourier transmission electron microscopy (FTIR), field emission scanning electron microscopy (FE-SEM) and energy dispersive X-ray spectroscopy (EDX). XRD measurement of *M. citrifolia* root extract reduced TiO_2NP_S was carried out on films of the respective solution drop coated onto a glass substrate on a Rigaku smart lab instrument operated at a voltage of 9 kW and a current of 30 mA with CuKa radiations. For FTIR measurement, dry powder of the nanoparticles was obtained in the following manner: the synthesized TiO_2NP_S solution was centrifuged at 10000 r/min for 20 min. The solid residue containing TiO_2NP_S solutions was dispersed in sterile deionized water and washed for three times to remove the unattached biological impurities. The pure residue was then dried completely in an oven at 70 °C overnight. The powder obtained was subjected to FTIR measurement carried out on a Perkin-Elmer Spectrum One with an instrument resolution of 4 cm^{-1} in potassium bromide pellets. The surface morphology and composition of TiO_2NP_S were analyzed by FE-SEM performed on a Philips instrument equipped with an EDX attachment, and for transmission electron microscopy (TEM) analysis TiO_2NP_S were prepared on carbon-coated copper TEM grids. TEM measurements were performed on a JEOL model 1200EX instrument operated at an accelerating voltage of 120 kV and later with an XDL 3000 powder.

2.4. Larvicidal bioassay

One gram of aqueous *M. citrifolia* root extract was first dissolved in 100 mL of distilled water (stock solution). From the stock solution, 200, 150, 100, 75 and 50 mg/L was prepared with dechlorinated tap water for a bioassay using the root extract of *M. citrifolia*. The larvicidal activity was assessed following WHO (1996) and as per the method of Rahuman *et al*[26,27]. For the bioassay test, larvae of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* were taken in five batches of 20 in 249 mL of water and 1 mL of aqueous plant extract (200, 150, 100, 75 and 50 mg/L). Control was set up with dechlorinated tap water. The number of dead larvae was counted after 24 h of exposure, and the percentage of mortality was reported from the average of five replicates. The experimental media in which 100% mortality of larvae occurs alone was selected for dose-response bioassay. Synthesized TiO_2NP_S toxicity tests were conducted using a multi-concentrations test, consisting of a control and different concentrations of nanoparticles. Each test was performed by placing 20 mosquito larvae into 200 mL of sterilized double distilled water with nanoparticles into a 250-mL beaker (Borosil). The nanoparticle solutions were diluted using double distilled water as a solvent for the desired concentrations (100, 50, 25, 12 and 5 mg/L).

2.5. Dose response bioassay

Based on the preliminary screening results, crude aqueous extract of *M. citrifolia* and synthesized TiO_2NP_S were subjected to dose-response bioassay for larvicidal activity against the larvae of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*. Different concentrations ranging from 50 to 200 mg/L (aqueous root extracts) and 5 to 100 mg/L (for synthesized TiO_2NP_S) were prepared for larvicidal activity. The number of dead larvae was counted after 24 h of exposure, and the percentage of mortality was reported from the average of five replicates.

2.6. Toxicity of TiO_2NP_S to non-target fish Poecilia reticulata (P. reticulata)

To determine the toxicity of biosynthesized TiO_2NP_S, a non-target organism *P. reticulata* was taken to the laboratory from the aquarium pet shop Chennai and acclimatized to the laboratory environment for about 5 d. They were fed with commercial feed pellets, and healthy *P. reticulata* was used for the experiments. Assessment of toxicity was carried out by the following procedure at LC_{50} value and then at LC_{90} value[28]. A total of 30 *P. reticulata* were placed in a rectangular glass tank containing 400 mL water solution in three replicates. Each group of 30 fish was exposed to a test solution of TiO_2NP_S. A control consisting of 30 fish in dechlorinated tap water, was studied at the same time. The number of dead fish was recorded first at 24 h and 48 h and the percentage mortalities were recorded. All of these bioassay tests were conducted at room temperature of approximately 27-28 °C, without aeration or renewal of water.

2.7. Statistical analysis

The average larval mortality data were subjected to probit analysis (FORTRAN) for calculating LC_{50} and LC_{90}. Other analysis at 95% fiducial limit of upper confidence limit and lower confidence limit values were calculated by using software SPSS 12.0. Results with $P<0.05$ were believed to be statistically important.

3. Results

The regression equation (based on the probit analysis) between the concentration of aqueous root extract and synthesized TiO_2NP_S against 4th instar larvae of *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* after 24 h and 48 h of exposure are represented in Tables 1 and 2. The result clearly indicated that the synthesized TiO_2NP_S at very low concentration was toxic against all three mosquito species when compared with the aqueous root extract of *M. citrifolia*. For aqueous root extract of *M. citrifolia*, the 24 h and 48 h LC_{50} values against *An. stephensi* were 77.030 and 88.890 mg/L where LC_{90} values were 192.399 and 171.294 mg/L. LC_{50} values against *Ae. aegypti* at 24 h and 48 h were 87.046 and 76.833 mg/L and LC_{90} were 171.352 and 152.996 mg/L. LC_{50} values against *Cx.*

Table 1

Mortality of larvae *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* at various concentrations of *M. citrifolia* root aqueous extract.

Mosquito	Hours	LC_{50} (mg/L)	LC_{90} (mg/L)	Regression equation		UCL (mg/L)		LCL (mg/L)	
				LC_{50} (mg/L)	LC_{90} (mg/L)	LC_{50}	LC_{90}	LC_{50}	LC_{90}
An. stephensi	24	77.030	192.399	$Y=-5.709+0.080X$	$Y=-2.206+0.168X$	78.060	200.857	74.128	185.715
	48	88.890	171.294	$Y=-4.243+0.053X$	$Y=-2.645+0.016X$	91.778	192.911	86.641	178.590
Ae. aegypti	24	87.046	171.352	$Y=-4.475+0.054X$	$Y=-1.476+0.046X$	88.496	179.768	81.437	164.057
	48	76.833	152.996	$Y=-6.213+0.084X$	$Y=-1.600+0.017X$	77.486	160.640	76.147	145.096
Cx. quinquefasciatus	24	90.960	143.257	$Y=-5.804+0.063X$	$Y=-2.378+0.170X$	92.796	205.670	91.190	227.690
	48	83.520	185.640	$Y=-8.295+0.099X$	$Y=-2.565+0.019X$	84.478	191.510	82.427	206.801

UCL: Upper confidence limit; LCL: Lower confidence limit.

Table 2

Mortality of larvae *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* at various concentrations of TiO_2NP_S.

Mosquito species	Hours	LC_{50} (mg/L)	LC_{90} (mg/L)	Regression equation		UCL (mg/L)		LCL (mg/L)	
				LC_{50} (mg/L)	LC_{90} (mg/L)	LC_{50}	LC_{90}	LC_{50}	LC_{90}
An. stephensi	24	13.620	35.064	$Y=-0.668+0.049X$	$Y=-0.338+0.046X$	14.845	39.526	12.551	31.832
	48	5.032	21.875	$Y=-0.454+0.090X$	$Y=-0.1000+0.0631X$	5.711	24.587	4.273	19.893
Ae. aegypti	24	23.711	59.097	$Y=-1.154+0.048X$	$Y=-0.562+0.321X$	25.662	73.266	22.118	51.190
	48	16.292	31.685	$Y=-0.760+0.046X$	$Y=-1.317+0.082X$	18.626	34.255	14.015	29.821
Cx. quinquefasciatus	24	29.794	43.257	$Y=-1.173+0.038X$	$Y=-1.300+0.059X$	31.356	48.169	28.328	39.911
	48	21.636	31.736	$Y=-0.733+0.033X$	$Y=-1.377+0.083X$	33.691	45.852	20.107	30.221

UCL: Upper confidence limit; LCL: Lower confidence limit.

quinquefasciatus were 90.960 and 83.520 mg/L while LC_{90} were 143.257 and 185.640 mg/L. Regarding the synthesized TiO_2NP_S, LC_{50} values at 24 h and 48 h against *An. stephensi* were 13.620 and 5.032 mg/L while LC_{90} were 35.064 and 21.875 mg/L. LC_{50} values against *Cx. quinquefasciatus* were 29.794 and 21.636 mg/L while LC_{90} were 43.257 and 31.736 mg/L, and LC_{50} values against *Ae. aegypti* were 23.711 and 16.292 mg/L while LC_{90} were 31.685 and 43.257 mg/L. The XRD of TiO_2NP_S synthesized using *M. citrifolia* root extract showed the presence of broad peaks at 25.25, 37.79, 48.03, 55.06, 62.10, 68.75 and 70.28 degrees (Figure 1). The lattice parameter obtained for TiO_2NP_S corresponded to anatase crystalline form when compared with the JCPDS data (File No. 89-4203). The FTIR for TiO_2NP_S synthesized by *M. citrifolia* root extract showed band at 3426 cm^{-1}, 1637 cm^{-1} and 714 cm^{-1} (Figure 2). The 3426 cm^{-1} shown O-H stretching due to alcoholic group; 1637 cm^{-1} shown N-H bend due to alcoholic group. In particular, the 1637 cm^{-1} indicated the presence of H bend bond for 1° for proteins. The peak at 714 cm^{-1} was due to Ti-O-O bond[29]. The surface of nanoparticles was investigated using FE-SEM (Figure 3). The observed micrograph showed synthesized nanoparticles aggregates and spherical form. Energy dispersive analysis of X-rays of the synthesized product gave distinct elemental signals of TiO_2 (Figure 4). Other elemental signals include C and O, which may be recorded from the biomolecules that are bound to the surface of nano titanium dioxide. EDX proved the chemical purity of the synthesized TiO_2NP_S. Mostly nanoparticles were spherical, oval and triangle in shape and mostly aggregated, and few individual particles are present. TEM revealed that the sizes of the TiO_2NP_S were 20.46-39.20 nm (Figure 5). The TiO_2 nanoparticles did not exhibit any noticeable effects on *P. reticulata* after either 24 or 48 h of exposure at their LC_{50} and LC_{90} values against fourth instar larvae of *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*. This suggests that these nanoparticles could be used along with this predatory fish in integrated vector control.

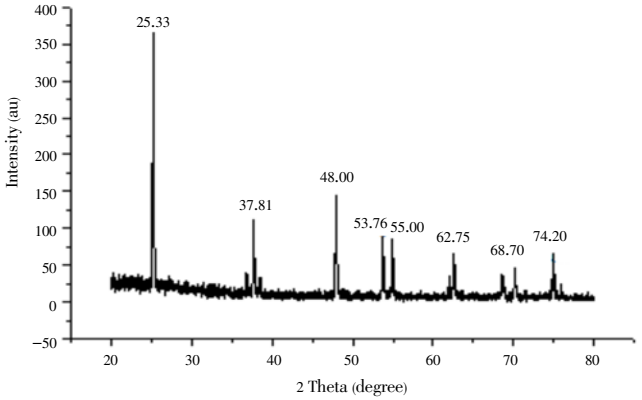

Figure 1. XRD images of biosynthesized TiO_2NP_S.

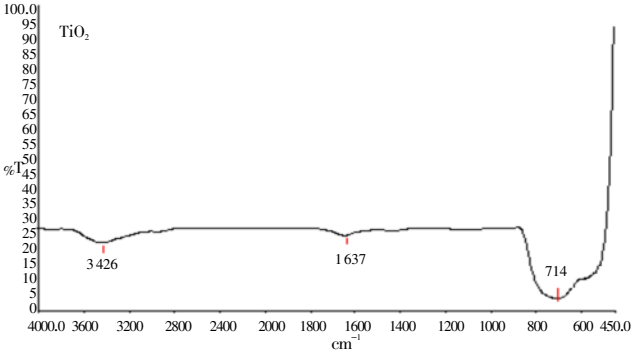

Figure 2. FTIR images of biosynthesized titanium dioxide nanoparticles.

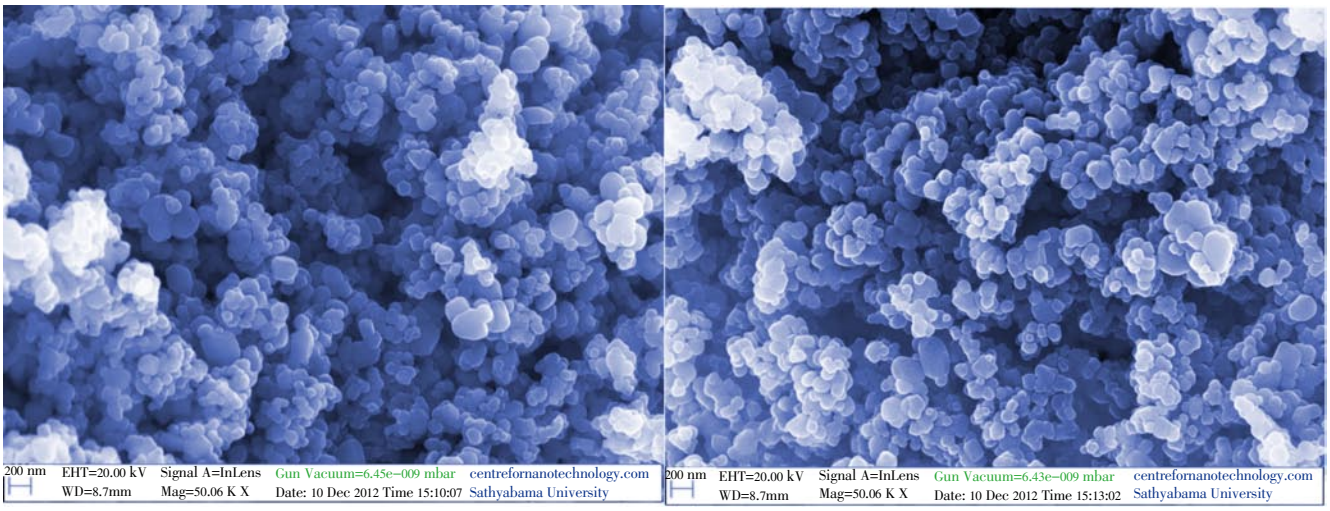

Figure 3. FE-SEM images of biosynthesized TiO_2NP_S.

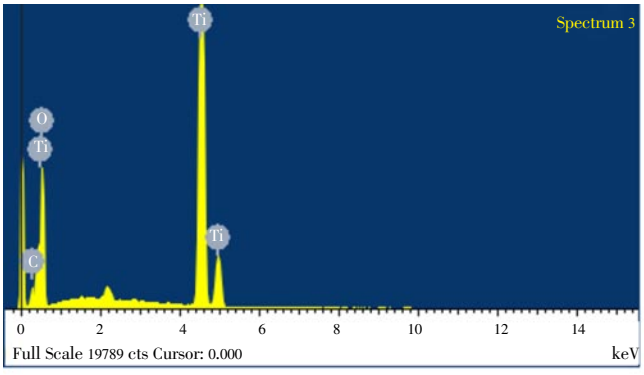

Figure 4. EDAX images of biosynthesized TiO_2NP_S.

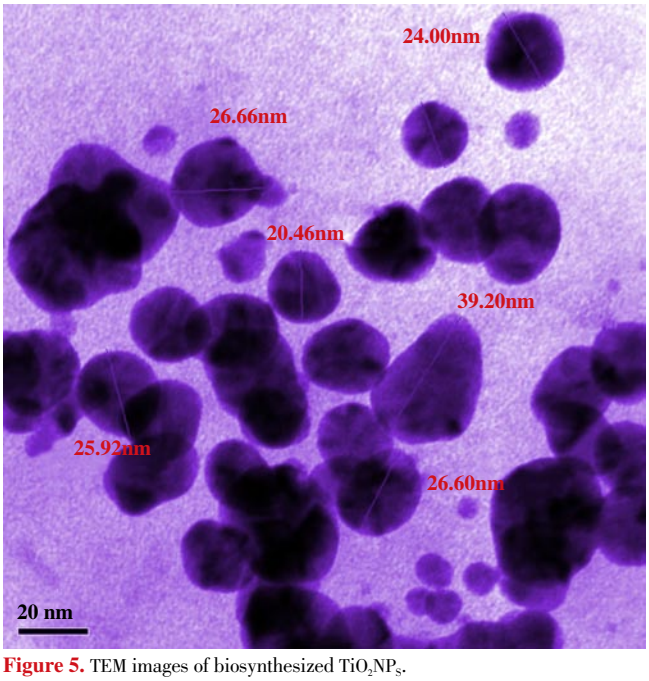

Figure 5. TEM images of biosynthesized TiO_2NP_S.

4. Discussion

Larval mosquito control, particularly in sensitive environments, has come to rely heavily on a small number of materials with a high degree of target specificity[30]. In the present work, the larvicidal activity of aqueous root extracts and synthesized TiO_2NP_S was noted. However, the activity was observed in both aqueous extracts of *M. citrifolia* and the synthesized TiO_2NP_S. Similarly, Suman *et al.* studied the activity of aqueous aerial extract and synthesized silver nanoparticles of *Ammannia baccifera* against the larvae of *Anopheles subpictus* (*An. subpictus*) and *Cx. quinquefasciatus*, and synthesized silver nanoparticles showed the highest mortality (LC_{50}=29.54, 22.32 mg/L)[31]. The maximum adulticidal activity of aqueous leaf extracts and synthesized TiO_2NP_S of *Catharanthus roseus* were observed against *Hippobosca maculata* and *Bovicola ovis*[32]. The synthesized zinc oxide nanoparticles against *Rhipicephalus microplus* and *Pediculus humanus capitis* and the larvae of *An. subpictus* and *Cx. quinquefasciatus* showed LC_{50} values of 29.14, 11.80, 11.14, and 12.39 mg/L, respectively[22]. The larvicidal activity of silver nanoparticles synthesized by filamentous fungus *Cochliobolus lunatus* was tested in various concentrations (10, 5, 2.5, 1.25, 0.625, and 0.3125 mg/L) against second, third, and fourth instar larvae of *Ae. aegypti* (LC_{50}=1.29, 1.48, and 1.58 mg/L; LC_{90}=3.08, 3.33, and 3.41 mg/L) and *An. stephensi* (LC_{50}=1.17, 1.30, and 1.41 mg/L; LC_{90}=2.99, 3.13, and 3.29 mg/L[33]. Synthesized silver nanoparticles using *Cocos nucifera* coir extracts against fourth instar larvae of *An. stephensi* and *Cx. quinquefasciatus* showed LC_{50} value of 4.75, 17.10, 2.42 and 6.50 mg/L[34]. Aqueous extract and synthesized silver nanoparticles from *Eclipta prostrate* showed larvicidal activity against *Cx. quinquefasciatus* (LC_{50}=27.49 and 4.56 mg/L; LC_{90}=70.38 and 13.14 mg/L) and *An. subpictus* (LC_{50}=27.85 and 5.14 mg/L; LC_{90}=71.45 and 25.68 mg/L[30]. The mosquito larvicidal activity of UV irradiation-induced silver nanoparticles were found to decrease the survival of fourth instar larvae of *Ae. aegypti* by 88% after 24 h of exposure at 1 mg/L concentration[35]. Sap-Iam *et al.* reported bioactivity of synthesized silver nanoparticles against the larvae of *An. subpictus*, *Cx. quinquefasciatus* and *Rhipicephalus microplus* (LC_{50}=13.90, 11.73, and 8.98 mg/L), respectively[36]. The XRD peaks at 2θ=25.25° (101) and 48.0° confirm the characteristic faces for anatase form of TiO_2[37]. In a related pervious study, it has been observed that the crystal structure of nano-TiO_2 contributed to cytotoxicity, with anatase TiO_2 showing more toxicity than rutile TiO_2[38]. In previous report, Velayutham

et al. reported that the peak of FTIR spectrum of synthesized TiO_2NP_S at 714 cm^{-1} was due to Ti-O-O bond[32]. In recent study, silver nanoparticles synthesized using *Pergularia daemia* showed non toxicity towards the non-target fish *P. reticulata*[39].

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

Authors are grateful to the Management of Sathyabama University for providing necessary facilities and Center for Nanoscience and Nanotechnology, Sathyabama University, Jeppiaar Nagar, Chennai for analyzing the samples by XRD and FE-SEM.

Comments

Background

The present study demonstrated that the use of a natural, low-cost biological reducing agent: *M. citrifolia* root extract (aqueous) could produce metal oxide nanostructures, through efficient green nano chemistry methodology, avoiding the presence of hazardous and toxic solvents and waste; furthermore, the nanostructures showed excellent larvicidal activity.

Research frontiers

The present green synthesis shows that the environmental benign and renewable source of *M. citrifolia* is used as an effective reducing agent for the synthesis of TiO_2NP_S. This biological reduction of metal would be a boon for the development of clean, nontoxic, and environmentally acceptable green approach to produce metal nanoparticles, involving organisms even ranging to higher plants. The formed TiO_2NP_S are highly stable and have significant anti-parasitic activity.

Related reports

The FTIR for TiO_2 nanoparticles synthesized by *M. citrifolia* root extract showed band at 3 426 cm^{-1}, 1 637 cm^{-1} and 714 cm^{-1}. The 3 426 cm^{-1} shows O-H stretching due to alcoholic group; 1637 cm^{-1} shows N-H bend due to alcoholic group. In particular, the 1 637 cm^{-1} indicates the presence of H bend bond for 1° for proteins. Velayutham *et al*. (2012) reported that the peak of FTIR spectrum of synthesized titanium dioxide nanoparticles at 714 cm^{-1} was due to Ti-O-O bond.

Innovations & breakthroughs

This method is considered as an innovative approach for the synthesis of TiO_2NP_S possessing significant larvicidal activity. In conclusion, an attempt has been made to evaluate the role of *M. citrifolia* extracts and synthesized TiO_2NP_S against *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*.

Applications

This research work reveals high efficacy of TiO_2NP_S as a strong larvicidal agent. The surface reactivity facilitated by capping enables these functionalized nanoparticles as promising candidates for various pharmaceutical, biomedical, and environmental applications.

Peer review

The present paper demonstrated the use of a natural, low-cost biological reducing agent. The manuscript is well organized and potentially interesting, and addresses an important topic.

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