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

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**Objective:** To assess the larvicidal activity of titanium dioxide nanoparticles (TiO₂NPS) 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 Ti(OH)₂ for the reduction under continuous stirring for 4 h at 50 °C. Synthesized TiO₂NPS 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 TiO₂NPS synthesized by *M. citrifolia* root extract showed band at 3 426 cm⁻¹ showed O–H bond. 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*.

**Conclusions:** TiO₂NPS 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 year¹. Still mosquitoes are the important vector insect in public health viewpoint owing to the problems associated...
with chemical insecticides, including toxicity to non-target organisms, environmental and human health concerns and unavailability of vaccines for many mosquito 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 countries 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 mosquitoide 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$_2$NPs) were synthesized from Bacillus subtilis[17], Eclipta prostrata[18], Lactobacillus and Nyctanthes arboristria leaves[19,20]. TiO$_2$NPs 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, 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$_2$NPs 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$_2$NPs

The aqueous solution of TiO(OH)$_2$ (5 mmol/L) was prepared and used for the synthesis of TiO$_2$NPs. 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$_2$NPs 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$_2$NPs 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$_2$NP$_5$ 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 kV 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$_2$NP$_5$ solution was centrifuged at 10000 r/min for 20 min. The solid residue containing TiO$_2$NP$_5$ 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$_2$NP$_5$ were analyzed by FE–SEM performed on a Philips instrument equipped with an EDX attachment, and for transmission electron microscopy (TEM) analysis TiO$_2$NP$_5$ 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.

2.5. Dose response bioassay

Based on the preliminary screening results, crude aqueous extract of *M. citrifolia* and synthesized TiO$_2$NP$_5$ 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$_2$NP$_5$) 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$_2$NP$_5$ to non–target fish Poecilia reticulata (P. reticulata)

To determine the toxicity of biosynthesized TiO$_2$NP$_5$, a non–target organism *P. reticulata* was taken to the laboratory from the aquarium pet shop Chennai and acclimated to the laboratory environment for about 5 days. 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$_2$NP$_5$. 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$_2$NP$_5$ 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$_2$NP$_5$ 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, whereas 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.
Table 1
Mortality of larvae An. stephensi, Ae. aegypti and Cx. quinquefasciatus at various concentrations of M. citrifolia root aqueous extract.

| Mosquito       | Hours | LC50 (mg/L) | LC90 (mg/L) | Regression equation | UCL (mg/L) | LCL (mg/L) |
|----------------|-------|-------------|-------------|---------------------|------------|------------|
| An. stephensi  | 24    | 59.097      | 83.520      | Y=-0.668+0.049     | 12.551     | 31.832     |
|                | 48    | 77.030      | 91.190      | Y=-0.338+0.046     | 35.064     | 59.097     |
| Ae. aegypti    | 24    | 23.711      | 59.097      | Y=-1.515+0.048     | 22.118     | 51.190     |
|                | 48    | 33.691      | 48.169      | Y=-0.760+0.046     | 11.051     | 28.291     |
| Cx. quinquefasciatus | 24    | 29.794      | 43.257      | Y=-1.377+0.083     | 28.328     | 39.911     |
|                | 48    | 21.636      | 31.736      | Y=-0.733+0.033     | 20.107     | 30.221     |

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

Table 2
Mortality of larvae An. stephensi, Ae. aegypti and Cx. quinquefasciatus at various concentrations of TiO2NPx.

| Mosquito species | Hours | LC50 (mg/L) | LC90 (mg/L) | Regression equation | UCL (mg/L) | LCL (mg/L) |
|------------------|-------|-------------|-------------|---------------------|------------|------------|
| An. stephensi    | 24    | 13.620      | 21.636      | Y=-0.668+0.049     | 12.551     | 31.832     |
|                  | 48    | 5.032       | 21.875      | Y=-0.454+0.090     | 4.273      | 19.893     |
| Ae. aegypti      | 24    | 23.711      | 43.257      | Y=-1.515+0.048     | 22.118     | 51.190     |
|                  | 48    | 16.292      | 31.685      | Y=-0.760+0.046     | 11.051     | 28.291     |
| Cx. quinquefasciatus | 24    | 29.794      | 43.257      | Y=-1.377+0.083     | 28.328     | 39.911     |
|                  | 48    | 21.636      | 31.736      | Y=-0.733+0.033     | 20.107     | 30.221     |

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

quintiquefasciatus were 90.960 and 83.520 mg/L while LC50 were 143.257 and 185.640 mg/L. Regarding the synthesized TiO2NPx, LC50 values at 24 h and 48 h against An. stephensi were 13.620 and 5.032 mg/L while LC90 were 35.064 and 21.875 mg/L. LC50 values against Cx. quinquefasciatus were 29.794 and 21.636 mg/L while LC90 were 43.257 and 31.736 mg/L and LC50 values against Ae. aegypti were 23.711 and 16.292 mg/L while LC90 were 31.685 and 43.257 mg/L. The XRD of TiO2NPx 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 TiO2NPx corresponded to amatase crystalline form when compared with the JCPDS data (File No. 89-4203). The FTIR for TiO2NPx synthesized by M. citrifolia root extract showed band at 3 426 cm \(^{-1}\), 1637 cm \(^{-1}\) and 714 cm \(^{-1}\) (Figure 2). The 3 426 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[28]. 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 TiO2 (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 TiO2NPx. 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 TiO2NPx were 20.46–39.20 nm (Figure 5). The TiO2 nanoparticles did not exhibit any noticeable effects on P. reticulata after either 24 or 48 h of exposure at their LC50 and LC90 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](image1.png)

![Figure 2](image2.png)
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$_2$NPS was noted. However, the activity was observed in both aqueous extracts of *M. citrifolia* and the synthesized TiO$_2$NPS. 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$_2$NPS 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 prostrata* showed larvicidal activity against *Cx. quinquefasciatus* (LC$_{50}$=27.49 and 4.56 mg/L; LC$_{90}$=70.38 and 13.14 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–Lam *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 20=25.25° (101) and 48.0° confirm the characteristic faces for anatase form of TiO$_2$[37]. In a related previous 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$_2$NP$_e$ 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.

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**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$_2$NP$_e$. 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$_2$NP$_e$ are highly stable and have significant anti–parasitic activity.

**Related reports**

The FTIR for TiO$_2$ nanoparticles synthesized by _M. citrifolia_ root extract showed bands 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; 1 637 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 $^\ddagger$ 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$_2$NP$_e$, possessing significant larvicidal activity. In conclusion, an attempt has been made to evaluate the role of _M. citrifolia_ extracts and synthesized TiO$_2$NP$_e$ against _An. stephensi_, _Cx. quinquefasciatus_ and _Ae. aegypti_.

**Applications**

This research work reveals high efficacy of TiO$_2$NP$_e$ 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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