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Research Article

Effect of ZnO and TiO₂ (Anatase) Nanoparticles And Their Combination In Controlling Mosquito Population Of *Anopheles Stephensi*, *Aedes Aegypti* And *Culex Quinquefasciatus*: An In Vitro Study

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ABSTRACT

Both zinc oxide nanoparticles (ZnO NPs), as well as titanium dioxide nanoparticles (TiO₂ NPs, anatase), are being used in many fields, and have also been previously confirmed to have potential mosquito population control properties. Therefore, this study intended to evaluate the effect of ZnO, TiO₂ and combination of ZnO and TiO₂ NPs on larvae of *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus*. At various concentrations for 24 hours (2, 4, 6, 8 & 10 ppm), ZnO, TiO₂ and combination of ZnO and TiO₂ NPs were treated on larvae of *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*. Results revealed that treatment of NPs significantly increased the mortality of larvae. *Anopheles stephensi* larval mortality LC₅₀ values were 3.609, 3.478 and 2.612 ppm while LC₉₀ values were 13.08, 11.942 and 8.305 ppm, respectively for ZnO, TiO₂ and combination of ZnO and TiO₂ NPs. In *Ae. Aegypti* larva, LC₅₀ ranges of ZnO, TiO₂ and combination of ZnO and TiO₂ NPs were 3.756, 3.455 and 2.594 ppm, while LC₉₀ values were 12.204, 10.543 and 9.555 ppm, respectively at 24 hours exposure. LC₅₀ value of ZnO, TiO₂ and combination of ZnO and TiO₂ NPs against *Cx. quinquefasciatus* larvae were 3.896, 3.763 and 2.589 ppm, while LC₉₀ values were 13.567, 13.178 and 8.680 ppm, respectively at 24 hours exposure. Furthermore, the histopathological changes were observed in the ZnO and TiO₂ NPs in combination treated midgut region of 3rd instar larvae of selected mosquitoes. The combination of ZnO and TiO₂ NPs provided prominent larval mortality at low dose than other individual NPs treatments. The present investigation clearly concludes that the combination of ZnO and TiO₂ NPs treatment would be a good candidate for controlling

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the mosquito Population

INTRODUCTION

The disease-causing vectors, Mosquitoes, are responsible for transmitting numerous human diseases like filariasis, malaria, and many other viral diseases like dengue, Japanese encephalitis, Zika and West Nile virus [1]. Mainly three genera of mosquitoes, namely, *Culex* sp. *Anopheles* sp. and *Aedes* sp., are widely distributed all over the world and are responsible for millions of fatalities. Among all, the most danger vector diseases such as dengue, malaria and filariasis are transmitting to humans by *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus*, respectively [2]. According to the report of WHO (2020), 219 million cases of malaria has been estimated globally, that results in approximately 400,000 deaths every year. Moreover, approximately 3.9 billion people in over 129 countries are also at risk of contracting dengue. Every year an estimated 96 million symptomatic cases and 40,000 approximate deaths have been reported for dengue [3]. According to the report of National Vector Borne Disease Control Programme, in 83 countries, almost 120 million people are infected with human filariasis, and it is predicted that around 1.1 billion are at risk [4]. The spreading of these diseases by mosquitoes is mainly due to the ever-increasing urbanization and is associated with anthropogenic activities. Currently, no effective mosquito population control treatments, without resistance, are available, thus leading to the search for alternative sources to prevent these mosquito-borne diseases [5]. At present, many chemically synthesized insecticides such as dichlorodiphenyltrichloroethane, dieldrin, organophosphorus, fenitrothion and synthetic pyrethroids are used for controlling the mosquito population. But the residues of these insecticides have an extremely harmful impact on the whole biosphere and are also prone to resistance [6].

Recently, nanoparticles (NPs) have received considerable attention in due to their wide range of applications in the fields of antimicrobial agents, biomarkers, diagnostics, cell labelling, drug delivery, cancer therapy, mosquito control, etc., [7]. Among the inorganic NPs, Zinc oxide and titanium dioxide nanoparticles are of high interest as they can be prepared easily, inexpensive, and are considerably safer materials for human beings and animals [8]. Zinc oxide NPs is one of the important metal oxide nanoparticles that is employed in various fields due to their peculiar physical as well as chemical properties [9]. It is a new type of the low-cost and low-toxicity nanomaterial, have attracted tremendous interest in various biomedical fields, including anticancer, antibacterial, antioxidant, antidiabetic, and anti-inflammatory activities, as well as for drug delivery and bioimaging applications [10]. TiO₂ is one of the most popular commercially available nano-size materials that has found application in a variety of fields due to its wide availability, biocompatibility, low cost and non-toxicity and high chemical stability [11]. Previously, several studies proved that the green synthesised Zinc oxide nanoparticles and titanium dioxide nanoparticles are good candidature to control the mosquitoes [12, 13]. Thus, this current study aimed to investigate the larvicidal activity of the zinc oxide and Titanium (IV) oxide (anatase) individual and in combination against *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus*.

MATERIALS AND METHODS

Chemicals

The zinc oxide (nanopowder - 21.32 nm) and TiO₂ (anatase nanopowder, size ranging between 20.46-39.20 nm) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals as well as the reagents used were of analytical grade and were purchased from Merck, Himedia, Mumbai, India.



Collection of eggs and maintenance of larvae

The eggs of *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus* were collected from VCRC (Vector Control Research Centre), Puducherry, a unit of ICMR, using an “O”-type brush. These eggs were then brought to the laboratory and transferred to 18×13×4-cm enamel trays that contained 500 mL of water for the hatching process. All the mosquito larvae were fed with pedigree dog biscuits and yeast at a ratio of 3:1. The feeding was continued till all the larvae transformed into pupal stage. The experimental mosquito was reared in the Vector Control Laboratory at the Department of Zoology, Annamalai University.

Larvicidal activity

Larvicidal activities of ZnO and TiO₂ NPs were determined in terms of the LC₅₀ and LC₉₀ by using a standard procedure by WHO [14] with slight modifications. The early fourth instar (twenty) of *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* were transferred to 500 mL bowls that contained 249 mL of de-chlorinated tap water. The ZnO and TiO₂ NPs were dissolved in distilled water to prepare a serial dilution of test dosage and then mixed in 249 mL tap water containing larvae. Six replicates were run simultaneously with different dosages 2, 4, 6, 8 and 10 ppm of ZnO, TiO₂ and combination ZnO and TiO₂ NPs (equal ratio) along with the control (1 mL of ethanol alone to 249 mL of tap water). The bioassay was performed at room temperature at 26 ± 2°C, with 60–80% relative humidity, during that time, no food was provided to the larvae. The mortality of larvae was recorded 24 h post-treatment, and LC₅₀ and LC₉₀ were evaluated by probit analysis [15].

Histopathology

The 3rd instar larvae treated with LC₅₀ concentrations, i.e., at the concentration of 2.589, 2.594 and 2.612 ppm of a combination of ZnO and TiO₂ NPs on *An. Stephensi*, *Ae. aegypti* and *Cx.*

quinquefasciatus, respectively, after 24 hours treatment, the alive larvae were collected for histopathological examination. The larvae were rinsed with distilled water before fixation with the Bouin's solution, followed by dehydration in graded ethanol and toluene series. Then, the larvae were embedded in paraffin, sectioned and stained with Haematoxylin and Eosin before the examination using compound microscope [16].

Statistical analysis

Data were arranged in an Excel sheet; statistical analysis of the experimental data was performed using the computer software Stat Plus 2009 (Analyst Soft, Canada) to find the lethal concentration against larvae (LC₅₀ and LC₉₀) out in 24 hours by probit analysis with a reliability interval of 95%. Additionally, to determine if there was a significant statistical difference among different doses of NPs against mosquito larvae, student's t-test was used to analyse the difference of the percentage of mortality.

RESULTS

Effect of ZnO, TiO₂ and combination of ZnO and TiO₂ NPs on selected mosquito larvae

The results for larvicidal toxicity effect of zinc oxide, anatase and combination of zinc oxide and anatase against *An. Stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* was presented in Table 1, 2 and 3, respectively. After 24 hours of exposure of ZnO, TiO₂ and combination of ZnO and TiO₂ NPs against *An. Stephensi*, the larval mortality of LC₅₀ values were 3.609, 3.478 and 2.612 ppm while LC₉₀ values were 13.08, 11.942 and 8.305 ppm, respectively. In larva of *Ae. Aegypti*, the LC₅₀ ranges of ZnO, TiO₂ and combination of ZnO and TiO₂ NPs were 3.756, 3.455 and 2.594 ppm while LC₉₀ values were 12.204, 10.543 and 9.555 ppm, respectively at 24 hours exposure. The LC₅₀ value of ZnO, TiO₂ and combination of ZnO and TiO₂ NPs against *Cx. quinquefasciatus* larvae were 3.896, 3.763 and 2.589 ppm while LC₉₀ values were 13.567, 13.178 and 8.680 ppm, respectively



at 24 hours exposure. Among the three species, the larvae of *An. Stephensi* was showing high mortality followed by *Ae. aegypti* and *Cx. quinquefasciatus* at all the concentrations of ZnO, TiO₂ and combination of ZnO and TiO₂ NPs, while at the concentration of 10 ppm, all the larvae of mosquitoes showed 100% mortality. The present results presented that the combination of nanoparticles (ZnO and TiO₂) was showing prominent larvicidal activity than individual treatment of ZnO and TiO₂.

Effect of ZnO and TiO₂ NPs (in combination) on the midgut of third-instar larvae

The figure 1 shows the longitudinal cross-section through the anterior midgut of the 3rd instar larvae of control and combination of ZnO and TiO₂ NPs treated *An. Stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*, respectively. The control larval midgut displayed well-developed brush border, distinct basal membrane, digestive cells, presence of food bolus, normal intestinal epithelial, fat body and peritrophic membrane, whereas the 3rd instar larvae treated with LC₅₀ concentrations, i.e., at the concentration of 2.589, 2.594 and 2.612 ppm of a combination of ZnO and TiO₂ NPs on *An. Stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*, respectively, after 24 hours treatment, displayed degenerated brush border, digestive cells, basal membrane and digestive cells. Additionally, cellular vacuolization, degenerated peritrophic membrane, distributed food bolus, vacuolated intestinal epithelial, along with smaller fat bodies were also observed in the nanoparticle-treated groups.

DISCUSSION

Mosquito control focuses on reducing the longevity as well as the population of mosquitoes to lessen their danger on human and animal health. Larvicides play a very vital role in controlling the mosquito population in their breeding sites, but these also have a negative impact on the beneficial and non-target organisms [17]. 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 [18]. Hence, researchers are trying to find out new insecticides as an alternative to chemical insecticides such as organochlorine and organophosphate compounds. Therefore, the goal of this present study is to explore the effects of ZnO, TiO₂ and a combination of ZnO and TiO₂ NPs against larvae of *An. Stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*. ZnO and TiO₂ NPs are applied in many fields, including pharmacology due to they being inexpensive as well as being relatively safe material for human beings and animals [8]. Previously, many researchers confirmed that green synthesised ZnO and TiO₂ NPs are a good candidate to control the mosquitoes [19, 20]. In this investigation, 24 hours exposure of ZnO and TiO₂ NPs brought significant larval mortality against larva of *An. Stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*. The mechanism of the larvicidal effect of ZnO and TiO₂ NPs is unknown. But, may be due to the penetration through treated larval membrane and interaction with cellular molecules resulting in the death of larvae or after they reach their midgut epithelial membrane, the enzymes were inactivated and generate peroxides, leading to cell death. This is the first work reported on ZnO and TiO₂ NPs on the control of the mosquito population. This result was supported by Suman et al. [19], who stated that the titanium dioxide nanoparticles synthesized using *Morinda citrifolia* root extract was showing larvicidal activity against *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus*. Meanwhile, Ashokan et al. [20] reported that the green synthesised zinc oxide using *Myristica fragrans* having larvicidal activity against dengue vector. The combination of zinc oxide and anatase treatment showed prominent larval mortality against *An. Stephensi*, *Ae. aegypti* and *Cx. Quinquefasciatus* than individual treatment of



ZnO and TiO₂ NPs which may be due to their synergistic effects. Further, the histopathological changes observed in the ZnO and TiO₂ NPs in combination treated midgut region of 3rd instar larvae of *An. Stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* displayed numerous histological damages. After 24 hours of treatment, a reduction of fat body cells was observed in all three treated larvae groups and such changes were also previously reported by Suman et al. [19]. Additionally, the vacuolation observed in the histology of treated larvae midgut region may be an indication that the cells are undergoing a cell death process, which may be caused due to the presence of toxic substances, i.e., the treatment compounds. The treatment compounds may also be responsible for the alteration in the microvilli size in the midgut of the treatment-exposed larvae, and such changes have also been previously reported by Soni and Dhiman [21].

CONCLUSION

The present investigation proved that the combination of ZnO and TiO₂ NPs could be a potential candidate for controlling mosquito species population at their breeding sites as well as in their habitats. However, further examination is required to prove their long-term effects as potent insecticides in the field conditions and on the ecosystem.

CONFLICT OF INTEREST:

The authors declare no conflict of interest

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Table 1: Larvicidal activity of ZnO, TiO₂ and combination of ZnO and TiO₂ NPs against against An. stephensi

Nanoparticles	Concentration (ppm)	Mortality of larvae (%)	LC ₅₀ (ppm) (LCL-UCL)	Regression Equation	LC ₉₀ (ppm) (LCL-UCL)	x ² df=3
ZnO NPs	2	34.64 ± 2.64 ^a	3.609 (2.499-5.214)	Y-2.3686x + 3.6807	13.08 (9.019-18.184)	0.468
	4	46.48 ± 3.54 ^b				
	6	59.12 ± 4.50 ^c				
	8	78.15 ± 5.98 ^d				
	10	91.47 ± 6.97 ^e				
TiO₂ NPs	2	35.2 ± 2.72 ^a	3.478 (2.431-4.976)	Y-2.4613x + 3.6690	11.942 (8.348-17.085)	0.483
	4	48.6 ± 3.67 ^b				
	6	60.25 ± 4.59 ^c				
	8	81.14 ± 6.18 ^d				
	10	92.5 ± 6.4 ^e				
Combination of ZnO and TiO₂ NPs (Equal volume)	2	44.98 ± 3.13 ^a	2.612 (1.830-3.727)	Y-2.6952x + 3.879	8.305 (5.820-11.849)	0.502
	4	60.67 ± 4.22 ^b				
	6	74.18 ± 5.15 ^c				
	8	95.43 ± 6.62 ^d				
	10	100.00 ± 6.95 ^e				

Significant at p<0.05; Control nil mortality; LC50: lethal concentration that kills 50% of the exposed larvae or pupa; LC90: that kills 90% of the exposed larvae or pupa; LFL lower fiducial limit; UFL upper fiducial limit; x² : Chi-square value; df: degrees of freedom.

Table 2: Larvicidal activity of ZnO, TiO₂ and combination of ZnO and TiO₂ NPs against Ae. Aegypti

Nanoparticles	Concentration (ppm)	Mortality of larvae (%)	LC ₅₀ (ppm) (LCL-UCL)	Regression Equation	LC ₉₀ (ppm) (LCL-UCL)	x ² df=3
ZnO NPs	2	31.2 ± 2.17 ^a	3.756 (2.672-5.278)	Y=2.5855x + 3.5162	12.204 (8.684-17.150)	0.423
	4	45.4 ± 3.15 ^b				
	6	58.12 ± 4.04 ^c				
	8	79.15 ± 5.50 ^d				
	10	92.47 ± 6.43 ^e				
TiO₂ NPs	2	33.2 ± 2.31 ^a	3.455 (2.484-4.806)	Y=2.7315x + 3.5296	10.543 (7.580-14.664)	0.494
	4	48.6 ± 3.38 ^b				
	6	65.2 ± 4.53 ^c				
	8	82.1 ± 5.71 ^d				
	10	94.5 ± 6.57 ^e				

Combination of ZnO and TiO₂ NPs (Equal volume)	2	45.98 ± 3.20 ^a	2.594 (1.757-3.830)	Y=2.3744x +4.0187	9.555 (6.474-14.108)	0.536
	4	59.67 ± 4.15 ^b				
	6	71.18 ± 4.95 ^c				
	8	93.43 ± 6.49 ^d				
	10	100.00 ± 6.95 ^e				

Significant at p<0.05; Control nil mortality; LC50: lethal concentration that kills 50% of the exposed larvae or pupa; LC90: that kills 90% of the exposed larvae or pupa; LFL lower fiducial

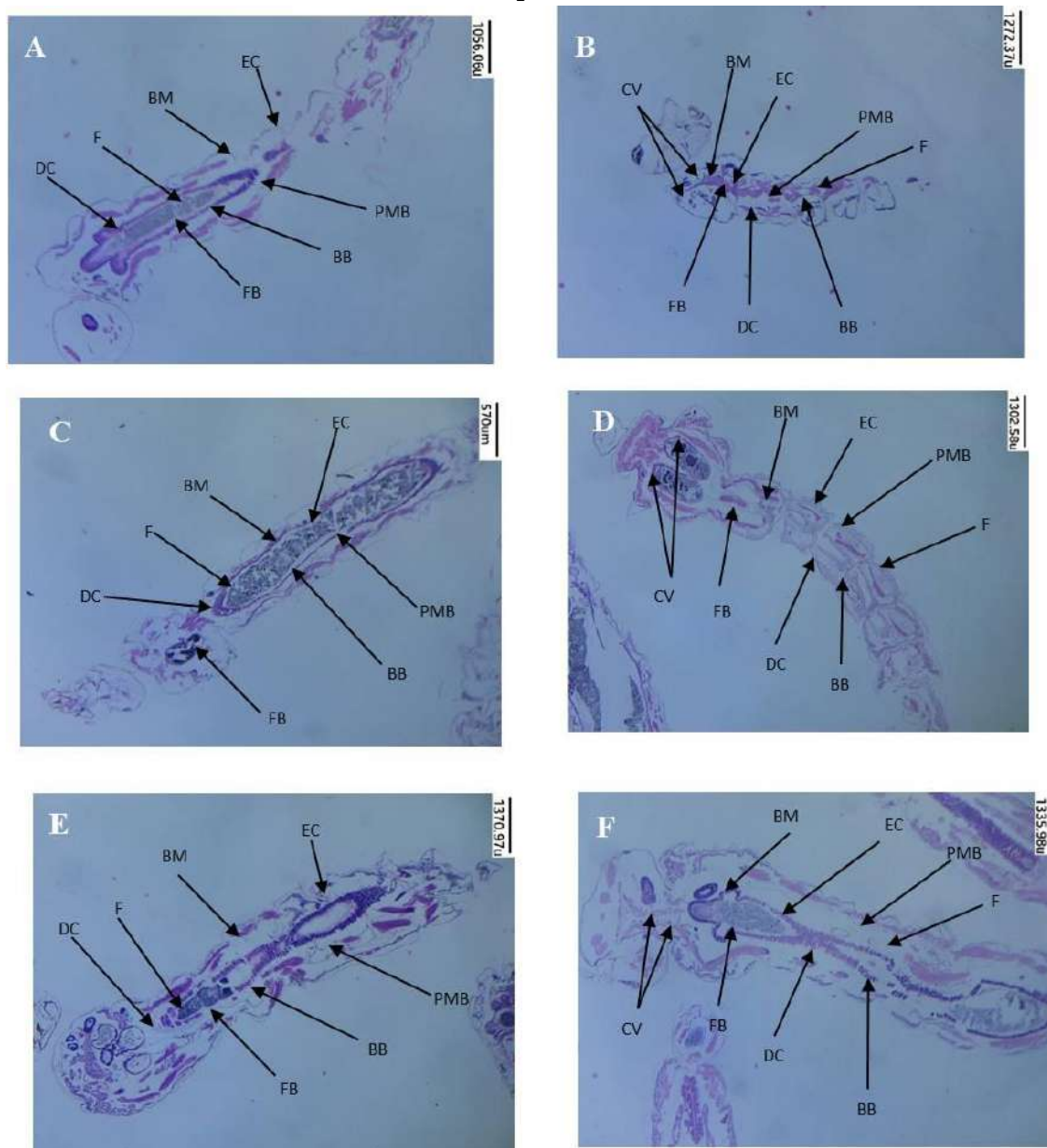
limit; UFL upper fiducial limit; x² : Chi-square value; df: degrees of freedom.

Table 3: Larvicidal activity of ZnO, TiO₂ and combination of ZnO and TiO₂ NPs against Cx. quinquefasciatus

Nanoparticles	Concentration (ppm)	Mortality of larvae (%)	LC ₅₀ (ppm) (LCL-UCL)	Regression Equation	LC ₉₀ (ppm) (LCL-UCL)	x ² df=3
ZnO NPs	2	28.82 ± 2.01 ^a	3.896 (2.716-5.590)	Y=2.3966x + 3.5854	13.567 (9.457-19.463)	0.772
	4	46.4 ± 3.22 ^b				
	6	60.12 ± 4.18 ^c				
	8	76.15 ± 5.29 ^d				
	10	88.47 ± 6.15 ^e				
TiO₂ NPs	2	30.2 ± 2.10 ^a	3.763 (2.618-5.410)	Y= 2.3915 +3.6246	13.178(9.167-18.944)	0.781
	4	47.6 ± 3.31 ^b				
	6	62.2 ± 4.32 ^c				
	8	76.1 ± 5.30 ^d				
	10	89.5 ± 6.22 ^e				
Combination of ZnO and TiO₂ NPs (Equal volume)	2	42.98 ± 3.27 ^a	2.589 (1.776-3.774)	Y=2.4861x + 3.9736	8.680 (5.995-12.652)	0.795
	4	63.67 ± 4.85 ^b				
	6	76.18 ± 5.80 ^c				
	8	92.43 ± 6.57 ^d				
	10	100.00 ± 6.95 ^e				

Significant at $p < 0.05$; Control nil mortality; LC50: lethal concentration that kills 50% of the exposed larvae or pupa; LC90: that kills 90% of the exposed larvae or pupa; LFL lower fiducial limit; UFL upper fiducial limit; χ^2 : Chi-square value; df: degrees of freedom.

Figure 1: The longitudinal cross sections through the anterior midgut of 3rd instar larvae of selected mosquitoes.



A, C & E are controls for *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* 3rd instar larvae, respectively. B, D & F represents the ZnO and TiO₂ NPs (combination) treated *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* 3rd instar larvae, respectively