



Adulticidal, Ovicidal and Repellent Activity of ZnO and TiO₂ Nps against Anopheles Stephensi, Aedes Aegypti and Culex Quinquefasciatus

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ABSTRACT:

Synthetic insecticides are used to control mosquitoes, but in the process, they contaminate soil, water, and air. They also have side effects on populations that are not intended targets, particularly humans, and in recent years, resistance to these insecticides has grown significantly. Inorganic nanoparticles such as zinc oxide (ZnO) and titanium dioxide (TiO₂) are far safer for humans and animals. Therefore, the current study was aimed to evaluate the adulticidal, ovicidal and repellent activity of ZnO and TiO₂ nanoparticles, in combination, against *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus*. The adulticidal activity was performed at 3, 6, 9, 12 and 15 ppm of a combination of ZnO and TiO₂ NPs. The adult mortality values of LC₅₀ were 4.415, 4.585, and 4.757, respectively, while the LC₉₀ mortality values were 11.159, 12.778, and 14.168 for *An. Stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*, respectively. In combination, the selected mosquito eggs were treated with ZnO and TiO₂ NPs at concentrations of 2, 4, 6, 8 and 10 ppm. The egg hatchability rate was found to be good at 8 and 10 ppm. The repellent activity was performed at different concentrations, i.e., 0.5, 1 and 1.5 mg/cm², at various intervals viz., 2, 4, 6, 8, and 10 hours. All the selected concentrations showed good repellent activity for up to 6 hours, but 1.5 mg/cm² showed a prominent repellent effect against the selected mosquitoes. Thus, the present results confirmed that ZnO and TiO₂ NPs, in combination, possess the potential to manage mosquito populations.

INTRODUCTION

Compared to other arthropods, mosquitoes are among the most dangerous insects on the planet. Through bites, infected mosquitoes can transmit human diseases, including malaria, dengue, chikungunya, yellow fever, zika, West Nile, lymphatic filariasis, etc. [1-3]. In 2021, the World Health Organisation recorded 247 million cases and 619,000 deaths from malaria [4,5]; annually, 100–400 million cases of dengue fever occurred [6]; and in 2018, 51 million cases of lymphatic filariasis were reported worldwide [7]. *Anopheles stephensi*, *Aedes aegypti*, and *Culex quinquefasciatus* are the main vectors of malaria, dengue, and lymphatic filariasis transmission, respectively. The best defence against diseases spread by mosquitoes is to use insecticides to eradicate adult mosquitoes or repellents to increase one's protection against female adult mosquito bites [8-11].

The primary weapon in the fight against mosquitoes is synthetic insecticides, like compounds containing organochlorine and organophosphate. However, the extensive use of synthetic pesticides to manage mosquito vectors has led to the persistence and buildup

of non-biodegradable chemicals in the ecosystem, the emergence of pesticide resistance in vectors, and harmful effects on organisms that are not intended targets [12-14]. These days, the development of nanoparticles and nanobiotechnology, which is gradually acknowledged as a future emerging technology because of its extraordinary new benefits, could be utilised to control disease outbreaks and vectors [15-17]. Because of their many uses in the fields of antimicrobial agents, biomarkers, diagnostics, cell labelling, drug delivery, cancer therapy, mosquito control, etc., NPs have drawn much attention [18]. ZnO and TiO₂ NPs are highly sought-after among inorganic NPs due to their ease of preparation, low cost, and relative safety for humans and animals [19]. Our previous investigation also confirmed the larvicidal activity of ZnO and TiO₂ NPs against *Aedes aegypti*, *Culex quinquefasciatus*, and *Anopheles stephensi* larvae [20]. Therefore, the current study aimed to evaluate the adulticidal, ovicidal and repellent activity of ZnO and TiO₂ NPs, 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 and the reagents used were of analytical grade. All chemicals were purchased from Merck, Himedia, Mumbai, India.

Collection of eggs and larvae maintenance

Using an "O"-style brush, the eggs of *Anopheles stephensi*, *Aedes aegypti*, and *Culex quinquefasciatus* were removed from the VCRC (Vector Control Research Centre), Puducherry, an ICMR unit. After that, the eggs were taken to the lab and placed in enamel trays measuring 18 by 13 by 4 cm, each holding 500 millilitres of water to facilitate hatching. In a 3:1 ratio, pedigree dog biscuits and yeast were fed to the mosquito larvae. Feeding was maintained until every larva reached the pupal stage. The experimental mosquito was raised in the Department of Zoology at Annamalai University's Vector Control Laboratory.

Adulticidal bioassay

The WHO method (1996) was used as the basis for the adulticidal bioassay. ZnO and TiO₂ NPs were combined and diluted in water to create different concentrations (3, 6, 9, 12, and 15 ppm), and the adulticidal activity was evaluated for the same. Twenty female mosquitoes were carefully gathered and put inside a holding tube made of plastic. Before spending an hour on test paper, the mosquitoes were allowed to acclimatise for one hour in holding tubes. After exposure, the mosquitoes were returned to the holding tube and left for a full day to recuperate. A cotton pad dipped in a 10% glucose solution was positioned on the mesh screen. Every experiment was repeated six times with the appropriate control. After the 24-hour recovery period, the mortality rate of mosquitoes was ascertained. LD₅₀ and LD₉₀ were computed with 95% confidence limits using log probit analysis [21].

Ovicidal activity

We measured ovicidal activity on a subset of mosquito eggs using Su and Mull's [22] method. ZnO and TiO₂ NPs were prepared in various concentrations by diluting them in water, with the final concentrations ranging from 2, 4, 6, 8, and 10 ppm. One hundred these mosquito species' eggs were exposed to every NP concentration. Following treatment, each

concentration's eggs were counted under a microscope and then individually moved to distilled water cups for assessment of hatching. Every experiment was carried out six times with the proper control. The following formula was used 48 hours after treatment to determine the hatch rates.

Percentage of egg mortality = $\frac{\text{No. of hatched larvae}}{\text{Total No. of eggs}} \times 100$

Repellent activity

The repellent study used the WHO's [23] methodology. One hundred blood-starved female mosquitoes, aged three days, were housed in a 45 cm × 30 cm × 45 cm net cage. On the day of the assay, the volunteer did not come into contact with lotions, perfumes, or scented soaps. Only the dorsal 25 cm of each volunteer's arm was visible; rubber gloves protected the remaining skin. ZnO and TiO₂ NPs were added to the control group in combinations at 0.5, 1, and 1.5 mg/cm². The treated arms were added to the cages simultaneously, with ethanol as the control group. Whether the target mosquitoes are daytime or nighttime biters determines when to conduct the test. *Ae. aegypti* was tested from 7:00 to 17:00 during the day, and *Cx. quinquefasciatus* and *An. stephensi* were tested from 19:00 to 5:00 at night. The mosquitoes were activated by gently tapping the sides of the experimental cages after the control, and treated arms were simultaneously added to the mosquito cage. There were six repetitions of each test concentration. The volunteer tested each concentration every five minutes by placing the treated and control arms in the same cage for a full minute. The mosquitoes that touched the hand were noted and subsequently shook off to prevent any blood from being consumed, providing a five-minute window of protection. The following formula was used to determine the percentage of repellency.

Repellency (%) = $\frac{(Ta - Tb)}{Ta} \times 100$

Ta and Tb represent the number of mosquitoes in the treated and control groups, respectively.

Statistical analysis

The lethal concentration against adult mosquitoes (LC₅₀ and LC₉₀) for 24 hours was determined using probit analysis. Additionally, the difference in the percentage of mortality was analysed using a student's t-test to see if there was a significant statistical difference between different doses of ZnO and TiO₂ NPs combined against mosquito larvae. The results with distinct superscripts



(a, b, and c) differed significantly at $p < 0.05$ for each experimental group.

RESULTS

The adulticidal activity of the combination of ZnO and TiO₂ NPs against mosquitoes

The effect of different concentrations of a combination of ZnO and TiO₂ NPs like 3, 6, 9, 12 and 15 ppm against adult selected mosquitoes is presented in table 1. Prominent mortality was observed at the concentration of 15 ppm treatment and was 98.24, 97.65 and 95.47 for *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*, respectively.

The adult mortality values of LC₅₀ were 4.415, 4.585, and 4.757, respectively, while the LC₉₀ mortality values were 11.159, 12.778, and 14.168 for *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*, respectively. The chi-square values were 0.770, 0.539 and 0.657, which displayed the adulticidal activity at a significant level of $p < 0.05$. Additionally, when the concentration of NPs increased, the mortality of adult-selected mosquitoes also augmented.

Ovicidal activity of the combination of ZnO and TiO₂ NPs against mosquitoes

The selected mosquito eggs were exposed to different concentrations of ZnO and TiO₂ NPs in combination (2, 4, 6, 8 & 10 ppm), as represented in table 2. The egg hatchability rate significantly declined in all the selected concentrations exposed to mosquito eggs compared to control. The hatchability rate of *An. stephensi* was 45.67, 13.64, 4.6, 0 and 0 per cent at concentrations of 2, 4, 6, 8 and 10 ppm NPs, respectively.

The *Ae. aegypti* of egg hatchability were 47.34, 17.22, 6.45, 0 and 0 at a concentration of 2, 4, 6, 8 & 10 ppm,

respectively. Meanwhile, the egg hatchability of *Cx. quinquefasciatus* was 49.68, 19.32, 10.07, 1.89 and 0 at 2, 4, 6, 8 & 10 ppm of NPs. Thus, the egg hatchability was significantly augmented when the concentration of ZnO and TiO₂ NPs, in combination, was increased.

Repellent activity of ZnO and TiO₂ NPs (in combination) against mosquitoes

In combination, the repellent activity (laboratory condition) of ZnO and TiO₂ NPs were examined against *An. Stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* at different concentrations like 0.5, 1 and 1.5 mg/cm² at various intervals, i.e., 2, 4, 6, 8, and 10 hours (Table 3). The biting protection against *An. Stephens* was 90.1, 97.2, and 98.82 per cent at 0.5, 1.0, and 1.5 mg/cm² concentrations of combined NPs for 2-hour intervals, respectively. While the repellent activity against *Ae. Aegypti* was found to be 88.83, 97.80, and 98.63 at 0.5, 1, and 1.5 mg/cm² concentrations of a combination of ZnO and TiO₂ for 2 hours. The biting protection of 85.86, 96.64 and 98.02 per cent during 2 hours at 0.5, 1 and 1.5 mg/cm² of combined NP concentration against *Cx. quinquefasciatus*, respectively.

The prominent repellent activity of NPs was against *An. stephensi* followed by *Ae. aegypti* and *Cx. quinquefasciatus*, respectively. All the selected concentrations of NPs showed repellent activity against mosquitoes chosen for up to 6 hours. However, the effectiveness of the repellent significantly varied between every concentration and interval. However, an increase in the exposure duration displayed a reduction in repellent activity, and it was directly comparable to the concentration of the combined NPs.

Table 1: Adulticidal activity of ZnO and TiO₂ NPs (in combination) against mosquitoes

Species	Combination of ZnO and TiO ₂ NPs Concentration (ppm)	Mortality of larvae (%)	LC ₅₀ (ppm) (LCL-UCL)	Regression Equation	LC ₉₀ (ppm) (LCL-UCL)	χ^2 df=3
<i>An. stephensi</i>	3	37.64 ± 2.87 ^a	4.415 (3.033-6.428)	Y=2.4028x + 3.4507	11.159 (8.768-16.970)	0.770
	6	56.48 ± 4.30 ^b				
	9	75.78 ± 5.77 ^c				
	12	87.65 ± 6.68 ^d				
	15	98.24 ± 7.48 ^e				



<i>Ae. Aegypti</i>	3	36.96 ± 2.81 ^a	4.585(3.349-6.276)	Y=3.0287x + 2.9962	12.778(9.334 - 17.494)	0.539
	6	55.78 ± 4.25 ^b				
	9	73.56 ± 5.60 ^c				
	12	85.87 ± 6.54 ^d				
	15	97.65 ± 7.4 ^e				
<i>Cx. quinquefasciatus</i>	3	35.64 ± 2.71 ^a	4.757(3.415-6.626)	Y=2.7771x + 3.1188	14.168(10.172-19.734)	0.657
	6	53.87 ± 4.10 ^b				
	9	70.58 ± 5.3 ^c				
	12	84.87 ± 6.46 ^d				
	15	95.47 ± 7.27 ^e				

Significant at $p < 0.05$; Control nil mortality; LC₅₀: lethal concentration that kills 50% of the exposed adult mosquito; 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.

Table 2: Ovicidal activity of ZnO and TiO₂ NPs (in combination) against mosquitoes

Concentration ZnO + TiO ₂ NPs (Equal volume)	Control	2 (ppm)	4 (ppm)	6 (ppm)	8 (ppm)	10 (ppm)
	<i>An. stephensi</i>					
	100	45.67 ± 3.48 ^a	13.64 ± 1.04 ^b	4.6 ± 0.81 ^c	NH	NH
	<i>Ae. aegypti</i>					
	100	47.34 ± 3.61 ^a	17.22 ± 1.31 ^b	6.45 ± 0.49 ^c	NH	NH
	<i>Cx. quinquefasciatus</i>					
100	49.68 ± 3.78 ^a	19.32 ± 1.47 ^b	10.07 ± 0.77 ^c	1.89 ± 0.14 ^d	NH	

NH: No hatchability; Significant at $p < 0.05$

Table 3: Repellent activity of ZnO and TiO₂ NPs (in combination) against mosquitoes

Time period (h)	Percentage of protection*		
	Concentration of combination of ZnO and TiO ₂ NPs (mg/cm ²)		
	0.5	1.5	2.5
<i>An. stephensi</i>			
2	90.1 ± 6.86	97.2 ± 7.40	98.82 ± 7.48a
4	76.7 ± 5.84	93.9 ± 7.15	95.81 ± 7.22a
6	59.4 ± 4.52	82.4 ± 6.28	84.2 ± 6.26b
8	49.9 ± 3.80	68.82 ± 5.24	71.83 ± 4.86c
10	0	44.87 ± 3.42	59.93 ± 4.34d
<i>Ae. aegypti</i>			
2	88.83 ± 6.69 ^a	97.80 ± 7.10 ^a	98.63 ± 7.40 ^a
4	74.92 ± 5.76 ^b	92.76 ± 6.50 ^a	92.31 ± 6.86 ^a
6	55.85 ± 4.71 ^c	78.65 ± 5.14 ^b	80.43 ± 6.26 ^b
8	44.98 ± 1.61 ^d	52.76 ± 3.79 ^c	61.24 ± 4.97 ^c
10	0	39.87 ± 3.04 ^d	56.28 ± 2.68 ^d
<i>Cx. quinquefasciatus</i>			
2	85.86 ± 6.54 ^a	96.64 ± 7.01 ^a	98.02 ± 7.37 ^a
4	73.81 ± 5.62 ^b	89.65 ± 6.83 ^{ab}	95.87 ± 7.30 ^a
6	56.62 ± 4.34 ^c	79.87 ± 6.08 ^b	78.54 ± 5.98 ^b
8	40.23 ± 3.06 ^d	56.43 ± 4.30 ^c	60.34 ± 4.60 ^c



10	0	32.76 ± 2.49^d	50.87 ± 3.87^d
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Significant at $p < 0.0$

DISCUSSION

Currently, vector control is a very effective means of preventing and controlling infectious diseases. However, environmental changes, pesticide resistance, and population growth have created new challenges for current vector control. Recently, metal NPs have been explored for their potential use in mosquito population control. This is due to their toxicity against mosquitoes at all life stages [24-30]. The present results also confirmed that the combination of ZnO and TiO₂ NPs possess good adulticidal, Ovicidal and repellent activity against selected mosquitoes *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus*.

Adulticide is the killing of adult mosquitoes by the application of pesticides. The ability to control adult mosquitoes is an essential component of mosquito-borne disease control [31]. Synthetic insecticides are used to eradicate the adult mosquito population; meanwhile, increased and continuous applications of these insecticides induce resistance to mosquitoes [32,33]. To overcome this issue, alternative vector control techniques are required. The results demonstrated that the treatment of the combination of ZnO and TiO₂ NPs at 15 ppm nearly killed 95% of selected adult mosquitoes. The LC₅₀ adult mortality values were 4.415, 4.585 and 4.757 for *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*, correspondingly. In combination, the ZnO and TiO₂ NPs may kill adult mosquitoes at lower concentrations due to their synergistic effects by inhibiting digestion and/or altering their growth, development and behaviour. The present result was supported by Amuthavalli et al., [34], who reported that the green synthesised ZnO NPs using *Lawsonia inermis* leaves displayed high toxicity against *Anopheles stephensi* larvae and pupa. Additionally, Suman et al., [35] reported that the TiO₂ NPs synthesised using *Morinda citrifolia* root extract demonstrated larvicidal activity against *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus*.

The mosquito control at the egg stage is a novel approach and simple method to eradicate the mosquito population [36]. Ovicidal substances can impede embryo growth, decrease larvae' ability to survive inside eggs, or stop eggs from hatching [37]. The present investigation discovered that while fresh eggs

treated with ZnO and TiO₂ NPs combined showed impaired embryo development, suggesting ovicidal activity, fresh eggs treated with ZnO and TiO₂ NPs combined showed embryogenesis in progress. *Aedes aegypti* oocytes, eggshells, and eggs all contain chitin, which is crucial to the viability of the eggs [38]. ZnO and TiO₂ NPs' ovicidal activity may be related to lectin's binding to chitin in eggshells, inhibiting hatching by encouraging this structure's disruption.

Applying synthetic repellents to exposed skin to shield against mosquito bites is a popular strategy for lowering the spread of malaria and painful bites [39]. However, questions exist regarding their safety and toxicity [40]. Because of these restrictions, scientists have created new substances to repel mosquitoes and stop mosquito bites. In the present study, results displayed that a combination of ZnO and TiO₂ NPs showed prominent repellent activity against *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus* at a concentration of 0.5, 1 and 1.5 mg/cm² for first 6 hrs. However, the mosquito biting protection significantly varied between every concentration and interval. Repelling activity decreased with increased exposure time, directly correlated with the ZnO + TiO₂ NP concentration. ZnO and TiO₂ NPs worked well together to repel mosquitoes, which may have something to do with their capacity to obstruct mosquito sensory organs. Furthermore, earlier research has demonstrated that ZnO and TiO₂ NPs work well as pesticides and as agents for pest control [41,42]. However, this study is the first to report that ZnO and TiO₂ NPs, in combination, have adulticidal, ovicidal, and repellent properties against selected mosquitoes.

CONCLUSION

The current study provided substantial evidence that ZnO and TiO₂ NPs combined have adulticidal, ovicidal, and repellent properties against specific mosquito species, including *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus*. More research is necessary to determine whether combining ZnO and TiO₂ NPs affects organisms that are not the intended target.



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