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Research Article

Fungal Generated Titanium Dioxide Nanoparticles: A Potent Mosquito (*Aedes aegypti*) Larvicidal Agent

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Abstract: Mosquitoes are the most important vectors which transmit wide range of life threatening diseases like malaria and dengue accounting for global mortality and morbidity with increased resistance to common pesticides. Since chemical insecticides pose serious threats to environment, human, and also other non-target organisms, the present study was undertaken to synthesize titanium dioxide nanoparticles using *Aspergillus niger*. The biocidal efficacy of Titanium dioxide nanoparticles against *Aedes aegypti* larvae was investigated and biochemical profile of the treated larvae was also evaluated. The TiO_2 nanoparticles were characterized by UV visible spectroscopy, Scanning Electron Microscopy, and XRD. The larvicidal activity of these nanoparticles against II and III instar larvae of *Aedes aegypti* was found to be concentration dependant. IC_{50} values of TiO_2 nanoparticles against II and III stage larvae were found to be 6.7ppm and 8.4ppm respectively. The results revealed that III instar larvae were more resistant to low concentration of TiO_2 when compared with that of II instar larvae, which might be due to structural development. Biochemical profile in the treated larvae indicates that TiO_2 toxicity reduced the levels of protein, cholesterol, and activities of acid/alkaline phosphatases, and lactate dehydrogenase whereas carbohydrate content was found to be increased in the treated larvae. Fungal generated TiO_2 induces the larval mortality and alter the metabolism of essential biomolecules leading to development of effective strategy to control *Aedes aegypti* at its larval stage.
Keywords: Larvicidal activity, titanium dioxide nanoparticles, *Aedes agypti*, proteins, lipids

INTRODUCTION

Mosquitoes continue to be important vectors that spread a number of human and zoonotic disease pathogens affecting human and animal hosts [1]. They transmit diseases such as malaria (*Anopheles*), filariasis (*Culex*), and dengue (*Aedes aegypti*) resulting in millions of deaths every year, so that they are proven to be the most important group of insects in term of public health [2]. Dengue viruses are transmitted by *Aedes aegypti* mosquitoes predominantly in tropical and subtropical human populations. The main vector *Aedes aegypti* is a cosmo-tropical species that proliferates in water containers in and around residents. Secondary vectors include *Aedes albopictus*, an important vector in Southeast Asia and that has spread to the Americas, western Africa, and the Mediterranean rim; *Aedes mediovittatus* in the Caribbean; and *Aedes polynesiensis* and *Aedes scutellaris* in the western Pacific region. *Aedes aegypti* breeds in many types of household containers, such as water storage jars, drums, tanks, and plant or flower containers [3-4]. No vaccines have been developed to prevent dengue infection and nor the drugs to combat the disease in infected persons. Most of the widely used vector interruption methods are synthetic insecticides based. These synthetic and chemical insecticides not only affect the environment and non-target population but also increase resistance to the vector [5]. Biological control is slow but can be long lasting, inexpensive, and harmless to living organisms and ecosystem; it neither eliminates pathogen nor disease, but brings them into natural balance [6]**.** Fungi and fungus derived products are highly toxic to mosquitoes, yet they have low toxicity or no toxicity to non target organisms. The secondary metabolites of entomo-pathogenic fungi *Chrysosporium*, *Fusarium*, *Asper-gillus*, and *Verticillium* have been screened successfully as potential larvicides. Fungi are also been used for producing nanoparticles since they produce extracellular enzyme to synthesize nanoparticles from materials. Larvicidal activity of silver and gold nanoparticles synthesised by *Chyrosporium tropicum* has been investigated against the *An. stephensi*, *Cx. quinquefaciatus*, and *Ae. aegypti* [7]. Therefore the present investigation was undertaken to the synthesize Titanium dioxide nanoparticles by using *Aspergillus niger* in order to come out with environmentally safe and effective mosquito larvicide againtst *Aedes aegypti*. This necessitates for developing vector control strategy which is the need of the hour for reducing the morbidity.

MATERIALS AND METHODS

Biomass production

Aspergillus niger was cultivated in 250ml Erlenmeyer Flask containing 100ml modified malt extract peptone (MGYP) medium. After adjusting the pH of medium to 6.8, the culture was grown with continuous shaking on a rotary shaker (150 rpm) at 28^{o}C for 72hrs. After 72hrs, fungal balls of mycelia were separated from the culture broth by centrifugation (4000 rpm) at 4^{o}C for 10min and then fungal mycelia were washed with sterile distilled water. The harvested fungal biomass (15 g wet weight) was re-suspended in100ml sterile Milli-Q-Water in 250ml Erlenmeyer flask and again kept on shaker (150 rpm) at 28^{o}C for 62hrs [8].

Synthesis of titanium dioxide nanoparticles

After incubation, the cell free filtrate was obtained and was added to TiO_2 salt in concentration of 0.1 M (optimum salt concentration determined by preliminary experiment). The entire mixture was transferred into shaker (150 rpm) at 28^{o}C and the reaction was allowed for 48 hrs. The biologically transformed particles were collected periodically and monitored for characterization [9].

Characterisation of Nanoparticles

UV-Vis spectrophotometric analysis

Optical properties of TiO_2 NPs were measured by subjecting the sample to UV-Visible spectrophotometer within the range 200 to 800nm and absorbance was plotted on a graph [10].

Scanning electron microscopic analysis

The morphology and size of the synthesisedn anaoparticles were determined using scaning electron microscope. Scanning Electron Microscopic (SEM) analysis was performed using Joel Model No. 6390 SEM machine. Thin films of the sample were prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the grid, extra powder was removed and was subjected for SEM analysis.

X-Ray Diffraction analysis

Crystal structure, phase composition, phase purity and mean size of the nanoparticles are analysed by X-Ray diffraction spectroscopy. The X-Ray diffraction pattern of the synthesized nanoparticles was recorded between the ranges 10^{o} to 90^{o}.

Collection and maintenance of larvae

II and III instars larva of *Aedes eagypti* strains were procured from National Centre for Disease Control, Mettupalam, Coimbatore, Tamilnadu and were brought to the laboratory safely without disturbance. These larvae were maintained in trays containing distilled water and fed with yeast until larvicidal bioassay.

Screening of larvicidal activity

2nd and 3rd instar larvae of *Aedes aegypti* were procured from National Centre for Disease Control, Mettupalam, Coimbatore, Tamilnadu. The larvae were maintained in trays containing distilled water and supplied with yeast. The larvicidal activity of fungi synthesized titanium dioxide nanoparticles was evaluated as described by World Health Organization method with slight modifications [11]. Different test concentrations of TiO_2 against both 2nd and 3rd instar larvae (2pm, 4ppm, 6ppm, 8ppm and 10ppm) in 200ml of distilled water were prepared. Six replicates each containing 20 larvae was subjected to larvicidal bioassay for all the test concentration and control group (distilled water) as well. Mortality rate was recorded after 24 hour exposure period in case of both the stages of larvae.

Estimation of biochemical parameters:

After the treatment of 24 hours, larvae were removed from the test solution and washed with chilled normal saline. Larval tissue homogenate (10%) was prepared in 0.25M chilled sucrose solution by homogenizer. The homogenate was centrifuged at 700x g for 10 minutes to remove all the cell debris. Supernatant was adopted for estimation of total carbohydrates, lipids, proteins, alkaline phosphatase and acid phosphatase. All the parameters were carried out in triplicate.

Estimation of total proteins: Lowry's method was adopted to estimate protein content in the larvae. Reaction of protein with Folin-Coicalteu become purple blue proportional to the amount of proteins and read at 620 nm. Further protein concentration was calculated with optical density [12].

Estimation of total carbohydrates: Carbohydrate was estimated as described in the method of Nelson [13]. Proteins were removed from the tissue homogenate and the filtrate containing glucose only as reducing substrate was heated with alkaline copper reagent and subsequently treated with Arsenomolybdate reagent. The blue color thus developed was read at 540 nm and protein content was calculated.

Estimation of lipids: Total lipids present in the larval tissue were estimated following the method of Bragdon [14]. Lipid content was separated from the non-lipid components by chloroform- methanol solution and lipid in the aqueous phase was reduced by sulphuric acid-dichromate mixture. The resultant green colour was measured at 600 nm and the concentration of lipid was calculated.

Estimation of acid/alkaline phosphatases

Activity of Acid/Alkaline phosphatases in larval homogenates was evaluated according to Fiske-Subbarow method. 0.1 ml of filtrate was added to 2 ml of buffer substrate and incubated for 1 hour. 0.8mlof acid molybdate and 0.2 ml of ANSA reagent were

added. The final volume was made up to 10 ml with distilled water. The intensity of color developed was measured at 660 nm against reagent blank.

Estimation of lactate dehydrogenase

The King (1965) method was followed for evaluating lactate dehydrogenase activity (LDH). 0.2 ml NAD^+ solution was added to the test tubes containing 1 ml of the buffered substrate. The sample (0.01 ml) was also added to the test tubes. Test tube samples were incubated for exactly 15 min at 37°C and then arrested by adding 1 ml of color reagent (2, 4-dinitrophenyl hydrazine) to each tube. The incubation then continued for an additional 15 min. After the contents were cooled to room temperature, 10 ml of 0.4 N NaOH was added to each tube to make the solutions strongly alkaline. At exactly 60 s after the addition of alkali to each tube, the intensity of color was measured at 440 nm.

RESULT AND DISCUSSION

UV-Visible analysis

The production of TiO_2 nanoparticles by *Aspergillus niger* was characterized by UV-Visible spectroscopy. Figure 1 shows the absorption spectra at 202 nm and 752 nm which are due to the surface Plasmon resonance /vibration in the reaction mixture. The absorption peak is an evidence for the formation of nanoprticle in the fungal culture. The UV visible spectra attribute to the surface Plasmon response (SPR) properties of titanium dioxide nanoparticles. These unique and tunable optical properties along with SPR depend on size, shape and distribution of nanorange particles of Titanium dioxide [15].

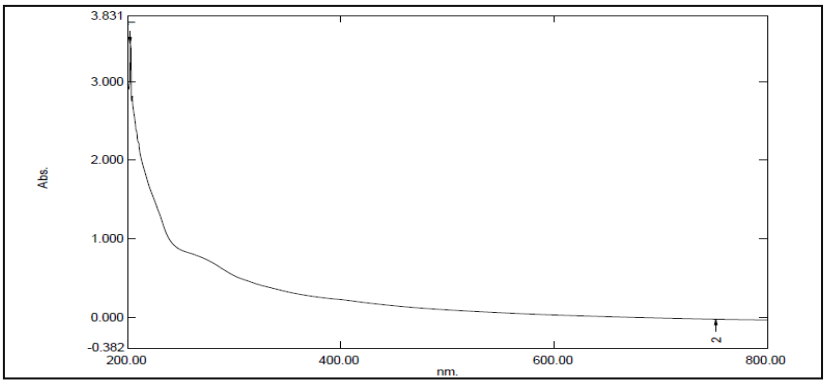

Fig-1: UV-Visible spectrum of titanium dioxide nanoparticles

SEM Analysis

The scanning electron microscope images confirm the presence of nanoparticles. It clearly reveals the morphology and size of the synthesized nanoparticles. Observation in figure 2 reveals that the size of nanoparticles is found to be in the range of 73.58 to 106.9 nm. The synthesized nanoprticles are almost spherical, with high homogeneity and have a narrow size distribution.

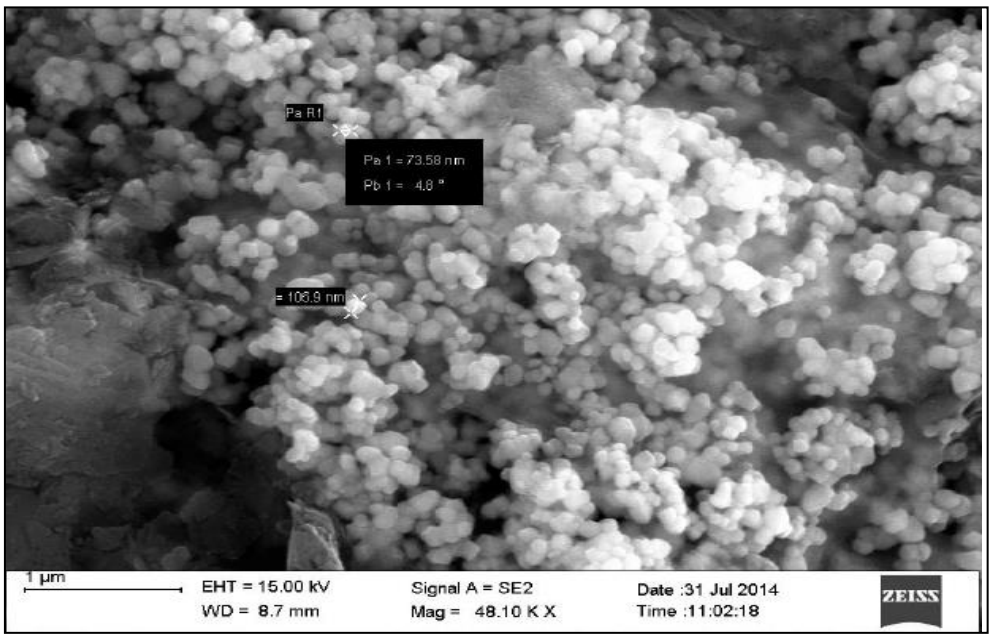

Fig-2: SEM image of TiO_2 nanoparticles

X-Ray Diffraction pattern

In XRD method, Crystal structure and crystallite size were determined by calculating through Debye Scherrer equation where scattering angle was considered. Broadening of a particular peak in a diffraction pattern along with particular planar reflection was observed in the crystal unit cell. The X Ray diffraction pattern reveals three characteristic peaks cantered at 25^o , 47^o, 53^o. Broader peaks indicate the smaller size of crystallite and vice versa due to random arrangement of crystallites. Tall cum narrow peaks revealed the individual crystallite structure. This indicates the presence of nanomaterials . Figure 3 represents the XRD patterns of TiO2 prepared by *Aspergillus niger*. XRD pattern clearly indicates that anatase form of TiO2 was obtained by this method. Indication of reduction in crystallite size was attributed to broadening of peak in the case of TiO2 prepared by biosynthesis. Peaks obtained corresponds to the indication of anatase form [16].

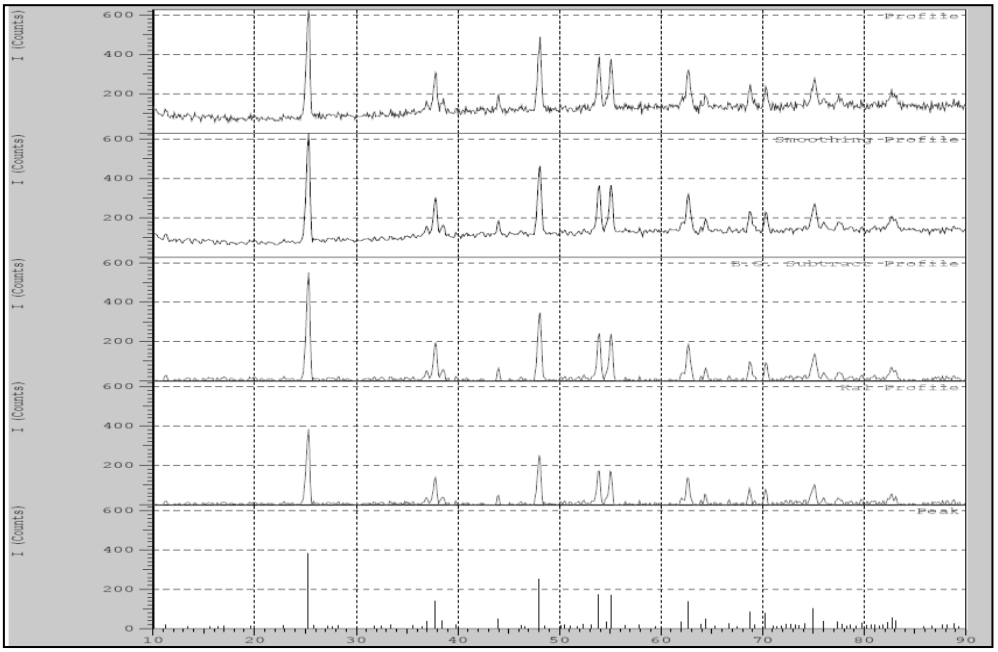

Fig-3: XRD pattern of TiO_2 nanoparticles prepared by fungal culture

Larvicidal activity of TiO_2 nanoparticles

Mortality rate

Mortality rate was calculated using WHO guidelines against both second and third instar larvae of *Aedes aegypti* mosquitoes. Percentage mortality of II and III instar larvae exposed to different concentrations (2, 4, 6, 8 and 10 ppm) of titanium dioxide nanoparticles has been shown in Table 1. The LC_{50} and LC_{90} values for II instar larvae were 6.7 and 11.9ppm respectively. Similarly, the LC_{50} and LC_{90} values for III instar larvae were 8.4 and 14.9ppm. Mortality or toxicity induced against mosquito larvae was found to be concentration dependant i.e. mortality rate was found to be increased in treated larvae as the concentration of nanoparticles increased (Figure 4).

Table 1: Percentage mortality of II and III instar larvae exposed to TiO2

Aedes aegypti Instar	Exposure Time (hrs)	Concentration of TiO_2 (ppm)	% Mortality
II	24	2	13.15±5.24
		4	28.94±5.24
		6	56.1±5.16
		8	65.78±5.58
		10	74.52±5.84
III	24	2	7.2±5.84
		4	19.78±5.84
		6	29.72±3.76
		8	54.05±5.24
		10	60.32±4.16

LC_{50} (50% larval mortality) was observed to be 6.7 ppm against II instar larvae and 8.4 ppm against III instar. The same result indicates that higher concentration is required to induce toxicity against III

stage larvae (Table 2). The resistance might be due to the structural and functional development occurred in III instar larvae when they grow. Our findings are in good accordance with the work done by Namita *et al.*, (2013) who demonstrated the larvicidal efficacy of silver nanoparticles synthesized by *Aspergillus niger*. Further, this study also proved that the mortality rate is higher in second instar larvae when compared to third instar larvae when the larvae was exposed to constant (10ppm) nanopaticles [17]. This necessitates the need for increasing the nanoparticle concentration to increase the mortality rate against well developed and higher stage larvae [18].

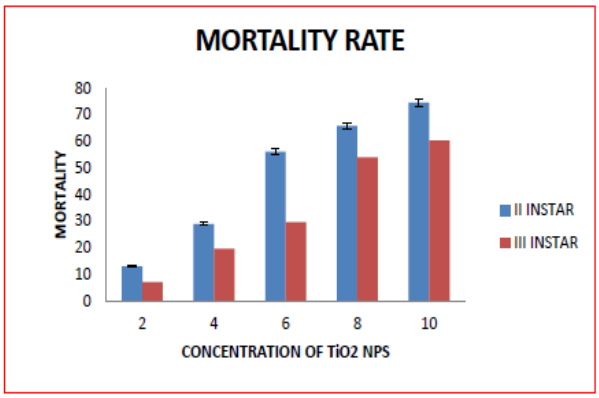

Fig-4: Percentage mortality induced by TiO_2 Treatment

Table 2: LC_{50} values of TiO_2 against *Aedes aegypti* instars

Instar (Larvae)	LC_{50} (ppm)	LC_{90} (ppm)	LC_{99} (ppm)
II	6.7	11.9	13.2
III	8.4	14.9	16.2

Status of protein, carbohydrate and lipid

The level of protein in the tissues of II and III instar larvae exposed to TiO_2 nanoparticles at various concentrations is depicted in Table 3 and 4. Protein content estimated in the treated larvae was found to be decreased which might be directly relevant to the mortality rate. Higher the toxicity lower the protein level in the mosquito larvae hence protein content is significantly down regulated when the toxicity is increased (Figure 5). Shift in protein metabolism might be due to the stress imposed on the larvae which suppresses the synthesis of proteins or increases the utilization of protein for energy. This shows that during stress conditions proteolysis dominates the synthesis of protein. According to Nath *et al* (1997), this could result from the breakdown of protein into amino acids, so with the entrance of these amino acids to TCA cycle as a keto acid, they will help to supply energy for the insect [19]. Hence, protein depletion in tissues is due to physiological mechanism and might play a role in compensatory mechanisms under insecticidal stress, to provide intermediates to the Krebs cycle, by retaining free amino acid content in haemolymph [20]. Our results are in par with the findings of Sugumar *et al.*, (2010) who revealed the diminished protein content in *Culex* larvae upon the insecticidal treatment with eucalyptus oil nanoemulsion [21].

Upon exposure to synthesized TiO_2 nanopatrticle induced toxicity has drastically reduced the protein content in the larval tissues in concentration dependant manner which suggests that interference in carbohydrate metabolism has a good impact on nanoparticle treatment [22]. A study conducted by Gade *et al.*, (2004) states that carbohydrates are mainly mobilized from glycogen reserves in fat body under stress condition resulting in increased level of soluble carbohydrate in haemolymph. Insects generally hydrolyze polysaccharides into simple sugars and absorb them mostly in the form of glucose in the midgut, causing an immediate increase in the concentration of glucose in the hemolymph. Some of the glucose is used directly in the process of glycolysis, part is converted into trehalose, and the rest is stored as glycogen in the fat body [23]. It also complies with the study of Djeghader *et al.*, (2013) who investigated the biological effect of benzoyl phenyl urea derivative on larvae *Culex pipens* [24].

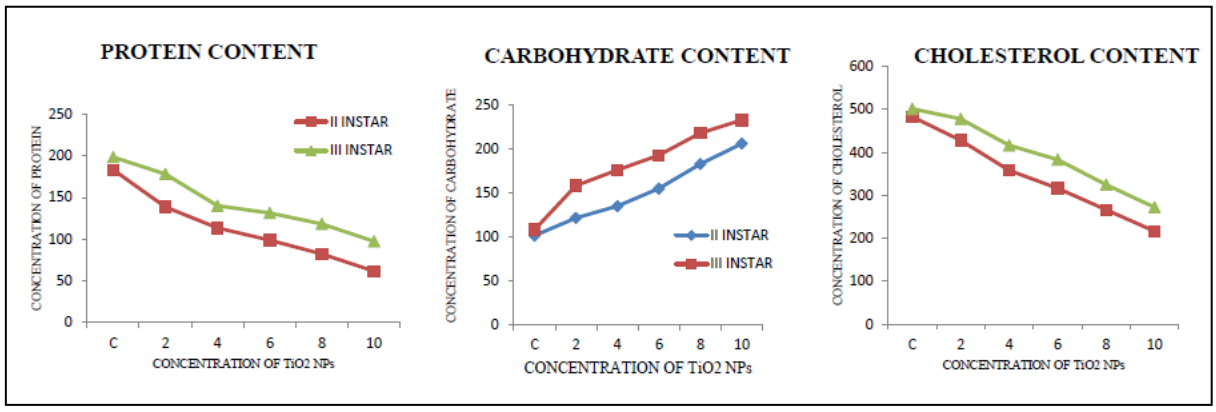

Fig- 5: Level of protein, carbohydrate and lipid contents in the nanopartciles treated larvae

Table 3: Level of protein, carbohydrate, and lipid in II Instar Larvae

TiO_2 NPs	Protein(µg/g)	Carbohydrate(µg/g)	Lipid(µg/g)
Control	183.3 ± 5.77	121.35.77	483.3±14
2ppm	138.7± 2.31a*	134.7±5.77a^{ns}	428.3±5.77 a*
4ppm	113.3± 5.77a*	154.7±5.77a*	358.3±28.87a*
6ppm	98.7± 2.31a*	182.7±5.77a*	316.7±14.43 a*
8ppm	82.0±3.46a*	206.0±0a*	266.7±14.43 a *
10ppm	61.3±2.31a*b*	101.3±5.77a*b*	216.7±14.43a*b*

TiO_2 NP treatment significantly decreases the level of lipids in both second and third instar larvae. This reduction was indirectly proportional to the concentration of TiO_2 NP and mortality. Lipids constitute the major portion of cell membrane and they are also important for cell maintenance, reproduction, and embryonic growth. The decrease in cholesterol levels might be due to the inhibited lipid synthesis and mobilization of the stored lipid through gradual unsaturation of lipid molecules [25]. Similar findings was observed when Sak *et al* who treated the mosquito larvae with the extract of *Pimpala turionella*. Our results are in accordance with the observations of Senthil *et al*., (2009) who demonstrated the reduction in total lipid level of *Anoepheles stephensi* larvae treated with some plant based insecticidal preparations which imposed stress in the larval species [26].

Table 4: Level of protein, carbohydrate, and lipid in III Instar Larvae

TiO_2 NPs	Protein(µg/g)	Carbohydrate(µg/g)	Lipid(µg/g)
Control	198.67±2.31	158.00±10.0	501.67±2.89
2ppm	178.33±2.89a*	176.00 ±0.00a^{ns}	478.33±5.77a^{ns}
4ppm	140.00±4.00a*	192.67±5.77a*	416.67±14.43a*
6ppm	131.33±2.31a*	218.00±0.00a*	383.33±14.43a*
8ppm	118.33±7.64a*	232.67±0.00a*	325.00±0.00a*
10ppm	97.33±2.31a*b*	108.00±0.00a*b*	272.50±3.54a*b*

Effect of TiO_2 toxicity on Acid phosphatase, Alklaine phosphatase and Lactate Dehydrogenase

Acid phosphatase hydrolyze a variety of orthophosphate esters and are capable of catalyzing transphosphorylation reactions to the phosphate pool in order to synthesize biomolecules such as adenosine triphosphate (ATP), and genetic materials [27]. The reduced ACP activity might be due to the strong inhibition of ecdysone, which is further accompanied by subsequent decrease in number of lysosomes and followed by decreasing the lysosomal ACP activity. Moreover, the decreased levels of ACP activity suggest a reduced phosphorous liberation for suppressed metabolism and down regulated enzyme regulation (Table 5A and 5B). Similar observation in terms of down regulated ACP activities was also reported by EL-Sheik et al 2002who demonstrated the insecticidal activity of pyriproxyfen against *A. ipsilon* [27].

The levels of ALP in the larvae treated with titanium dioxide nanoparrticles has been shown in table 4.The enzyme is active inside the cells where this acts in synthesis of fibrous protein, growth and development of tissue [28]. The alkaline phosphatase is a set of

hydrolytic enzymes that hydrolyze phosphomonoesters under the alkaline condition. Different stress and disease causes considerable decrease in the activity of ALP [29]. The reduced ALP activity is attributed to interference in developmental disturbance posed by insecticide treatment against mosquito larvae of *C.pipiens*. Wu-Tsiu and his coworkers have assessed the impact cyromazine on the ALP activity upon treating the mosquito *Culex pipens* larvae in his laboratory [30].

Lactate dehydrogenase (LDH) is a parameter widely used in toxicology and in clinical chemistry to diagnose cell tissue and organ damage. LDH is involved in the production of energy, during the requirement of considerable amount of additional energy is rapidly required [31]. Senthilnathan *et al.*, showed the LDH activity in *Spodoptera litura* (Fabricius) decreases in midgut after feeding on *Ricinus communis* L. treated with azadirachtin and nucleopolyhedrovirus, which demonstrates the low nutritional efficiency of the larvae [32]. LDH level in the larvae treated with TiO_2 nanoparticles was found to be decreases dependent upon the concentration of TiO_2 NP and mortality percentage. This reduction in the enzyme activity might be due to stress produced by larvicidal treatment [31].

Table 5 A: Activity of ACP, ALP and LDH enzymes in II instar larvae

TiO_2 NPs	**ACP (IU/L)**	**ALP(IU/L)**	**LDH(IU/L)**
Control	60.7±1.15	18.33±0.58	0.63±0.03
2ppm	49.0±1.41 a*	15.67±0.58 a*	0.53±0.03a*
4ppm	41.0±1.41 a*	13.00±74.48 a*	0.48±0.03 a*
6ppm	34.0±1.73 a*	11.33±0.58 a*	0.43±0.06 a *
8ppm	30.0±0 a*	8.67±0.58 a*	0.28±0.03 a *
10ppm	21.3±1.15a*b*	7.00±0.00 a*b*	0.13±0.03a*b*

Table 5B: Activity of ACP, ALP and LDH enzymes in III instar larvae

TiO_2 NPs	**ACP(IU/L)**	**ALP(IU/L)**	**LDH(IU/L)**
Control	57.00±1.73	19.33±0.58	0.58±0.03
2ppm	51.33±1.15a*	18.00±0.00a	0.50±0.00a*
4ppm	42.00±0.00a*	16.67±0.58a*	0.38±0.03a*
6ppm	38.67±1.15a*	15.33±0.58a*	0.28±0.03a*
8ppm	30.67±2.31a*	12.33±0.58a*	0.22±0.03a*
10ppm	25.33±1.15a*b*	9.67±0.58a*b*	0.13±0.03a*b^{ns}

Values are mean ± SD of three samples in each group
Group comparison
a – control Vs all
b – 2ppm Vs 10 ppm
Statistical significance
* - Significant (P< 0.05)
ns - not significant

CONCLUSION

The TiO_2 nanoparticles synthesized from *Aspergillus niger* has been used to detect the larvicidal activity against *Aedes aegypti*. The mortality rate and change in biochemical parameters indicate that these nanoparticals can be used effectively to control mosquito population. In conclusion, our results claim that *Aspergillus niger* releases enzymes capable of synthesizing TiO_2 nanoparticles that can be suggested to develop safer and effective larvicide alternative to chemical methods of mosquito control.

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