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Assessment of titanium dioxide nanoparticle as treatment of *Aeromonas hydrophila* infection in *Oreochromis niloticus*

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ABSTRACT. Nanoproducts became widely used materials all over the world. Antimicrobial properties of titanium dioxide (TiO₂) nanoparticle (NP) were examined against *Aeromonas hydrophila* (*A. hydrophila*) bacteria and the minimum inhibitory concentration (MIC) was found to be 20 µg/ml of TiO₂NP. In addition, the treatment efficacy of TiO₂NP was examined in *Oreochromis niloticus* (*O. niloticus*) infected with *A. hydrophila*. One hundred and eighty fish (54±2.4 g b.w.) were divided into six groups (G). *O. niloticus* in G1, G2 and G3 were fed for 30 days with 0, 20 and 100 µg/g b.w. TiO₂NP, respectively, while G4, G5 and G6 were i.p. injected with 0.2 ml distal water, 20 and 100 µg/g b.w. TiO₂NP, respectively, for three times with ten days of interval. The blood parameters as well as some of the biochemical parameters of *O. niloticus* that received high dosage of TiO₂NP were significantly affected regardless to the administration route. Elevation of the activities of glutathione peroxidase (GPx) and metallothioneine (MT) were recorded with the high dosage. Furthermore, *O. niloticus* subjected to high dosage of TiO₂NP had the lower survival rate (SR%) especially with the injection route (50%). On the other hand, no significant changes were demonstrated with the perceived TiO₂NP MIC. The mortality rate (MR%) of challenged *O. niloticus* against *A. hydrophila* was decreased in case of TiO₂NP MIC exposure, as G2 and G5 revealed 20 and 30%, respectively. Therefore, the 20 µg/g b.w. of TiO₂NP could safely protect *O. niloticus* against *A. hydrophila* infection since no health hazards was observed. Meanwhile, health status of *O. niloticus* was adversely affected with high dosage of TiO₂NP irrespective to the route of administration.

Keywords: TiO₂, nanoparticles, *Oreochromis niloticus*, *Aeromonas hydrophila*, antioxidant.

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INTRODUCTION

Nanotechnology has provided the global market with novel nano-products with unique properties and functions that has emerged rapidly affecting economic sectors (Bour *et al.*, 2015). Nano-products have different physicochemical properties than their bulk forms; they are define as materials with a size between 1 and 100 nm on at least one dimension, which provides surface area to volume ratio. Titanium nanoparticles (TiO₂NP) is one of the most manufactured NP worldwide; its production was expected to reach 201,000 tons during 2015 (Markets, 2015). TiO₂NP is widely used in the production of paints, coatings, plastics, papers, inks, foods, pharmaceuticals, cosmetics and toothpaste (Menard *et al.*, 2011 and Shi *et al.*, 2013).

The most frequently isolated bacterial pathogen in warm freshwater fishes is *A. hydrophila*, a Gram-negative motile rod bacterium that always associated with diseases outbreaks in the aquatic environment (Angka, 1990; Esteve *et al.*, 1993). *A. hydrophila* infection causes a systemic disease resulting in ulcerative dermatitis, tail or fin rot, ocular ulceration, which leads to hemorrhagic septicemia, the most common cause of mortality in the acute form is rapid septicemia (Cipriano, 2001).

Fish producers have used antibiotics and chemicals (malachite green, formalin, methyl blue, potassium permanganate and copper sulphate) as treatments for fish diseases, which unfortunately had severe impacts on fish consumers and environment. Therefore, a demand for new antibacterial agent that can avoid such hazards become essential (Sakr *et al.*, 2014). Ravikumar *et al.* (2011) claimed that metal oxide nanoparticles antimicrobial property could be due to the reactive oxygen species mechanism. Nano-TiO₂ is a safe product (Rowe *et al.*, 2003; Jacobs *et al.*, 2010) and could be used as an additive in protocols for removal of arsenic from drinking water (EPA 2010). However, nano-TiO₂ had immune suppressive effect on fish health that enhance mortalities in fish exposed to infectious bacteria (Jovanovic *et al.*, 2015). Despite the high investments in nanotechnology the studies related with the antimicrobial property of metal oxide nanoparticle against bacterial fish diseases are too limited (Vale *et al.*, 2016). Therefore, this study was designed to investigate the potential antimicrobial

role of TiO₂NP. Furthermore, the impacts of TiO₂NP on *O. niloticus* health were evaluated.

MATERIALS AND METHODS

Chemicals

Titanium dioxide nanoparticles (TiO₂NP) (Sigma Aldrich Corp, St. Louis, MO, USA) anatase, nano powder, < 25 nm, purity 99.7%, average zeta potential of 16.4 mV, conductivity of 16 mS/cm. The aggregate size had an average diameter of 86 nm, zeta potential of 8.87 mV and conductivity of 15.4 mS/cm.

Bacteria isolation and identification

A. hydrophila was isolated from diseased fish that were collected from a private freshwater fish farm. Bacterial swabs were obtained from fish hepatopancreas, spleen and kidneys according to Woo and Bruno (2014). Swabs were inoculated onto tryptic soya broth then the inoculum was streaked onto Rimler Schotts agar and incubated at 37°C for 24 h. *A. hydrophila* was inoculated onto tryptic soy agar (Oxoid, Canada) then incubated in 28°C for 24 h according to Austin and Austin (2012). Bacterial strain was confirmed by the polymerase chain reaction (PCR). DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged. Nucleic acid was eluted with 100 µl of elution buffer. Primers used were supplied from Metabion (Germany) and they are listed in Table 1. PCR amplification (35 cycles) was performed in a 25-µl reaction containing 12.5 µl of Emerald Amp Max PCR master mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 5.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler. Analysis of the PCR products was performed by a gel documentation system (Alpha Innotech, Biometra) and the data were analyzed through computer software Chip PCR (Rodiger and Burdukiewicz 2013).

Table 1. Primers sequences, target gene, amplicon size and cycling conditions

| Target gene | Primers sequences 5'-3' | Amplified segment (bp) | Annealing temperature | Reference |
|----------------------------------|--|---------------------------|--------------------------|------------------------------|
| <i>A. hydrophila</i> 16S rRNA | GAAAGGTTGATGCCTAATACGTA CGTGCTGGCAACAAAGGACAG | 625 | 50°C 40 sec. | Gordon <i>et al.</i> 2007 |

Determination of TiO₂NP minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was performed according to **Ravikumar *et al.* (2011)**. Briefly, 50 µl of 24h old *A. hydrophila* inoculum (corresponding to a concentration of 5 x 10⁵ CFU) were exposed to a dilution series of TiO₂NP ranging from 500 to 10 µg/ml (500, 400, 300, 200, 100, 60, 50, 40, 30, 20 and 10 µg/ml). The culture was allowed to grow at 37°C for 48h and the whole setup was triplicated, while tryptic soya broth alone was considered as the negative control. The MIC of the nanoparticles was defined as the lowest concentration of the agent that restricted the growth of bacteria in the culture media.

Experimental design

Two hundred *O. niloticus* with an average 54±2.4 g b.w. was acclimated for two weeks at laboratory condition; water temperature 25.5±1.5°C, dissolved oxygen 5.2±0.5 mg/l, pH 7.5±0.4. Solid wastes of fish were removed daily with the exchange of one third of aquarium water. Following acclimation, one hundred and eighty *O. niloticus* were divided into six groups (G1-G6). Each group had three subdivisions (replicates) and fish were randomly distributed into 18 glass aquariums (50x50x40 cm), ten fish per aquarium. The Institutional Aquatic Animal Care and Use Committee, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University have approved the procedures.

Following the detection of the TiO₂NP MIC, the nanomaterial was dispersed in distal water by sonication to form a stock solution. Fish groups were either fed (for 30 days) or injected with TiO₂NP MIC. G1 (control) was fed a commercial fish diet with no additives, G2 was fed diet contained the revealed MIC of TiO₂NP per gram of fish body weight, while G3 was fed with a high dosage (100 µg/ g b.w.) of TiO₂NP per gram of fish body weight. On the other hand, fish under anesthesia with tricaine methanesulfonate (MS222; Sigma, St. Louis, MO, USA) were injected i.p. with 0.2 ml distal water (G4, control), with the obtained MIC of TiO₂NP suspended in 0.2 ml distal water (G5) or with high dosage (100 µg/g b.w.) of TiO₂NP suspended in 0.2 ml distal water (G6). Injections were repeated three successive times with ten days interval. By the due time, fish were euthanized by immersion in MS222 solution (250 mg/L; 25 to 30°C) that assumed to cause rapid unconsciousness, followed by decapitation and exsanguination (gill cut) according to Anonymous (2007).

By the end of the experimental period, the fish were counted to determine the survival rate percentage (SR %) according to the following formula:

$$\text{SR \%} = \frac{\text{Number of fish at the end}}{\text{Number of Fish at the beginning of the Experiment}} \times 100$$

Hematological and biochemical analyses

For hematological analyses, blood samples were collected in vacutainers containing heparin (30 IU/l of blood) as an anticoagulant. For the serum biochemical analysis, blood samples were collected into vacutainers without anticoagulant, and serum was separated by centrifugation at 1000 g for 10 min and stored in a freezer at -20°C until use.

Red blood cells (RBCs) and white blood cells (WBCs) were counted by a haemocytometer according to Stoskopf (1993). Blood haemoglobin (Hb) was assessed by cyanomethemoglobin method (Drubkin, 1964). Packed cell volume (PCV) was determined by centrifuging heparinized blood in a capillary tube at 10,000 RPM for five minutes. In addition, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated according to the formulas mentioned by Dacie and Lewis (1975) as follows:

$$\text{MCHC (g/dl)} = (\text{HB} / \text{PCV}) \times 100,$$

$$\text{MCH (pg)} = (\text{HB} / \text{RBCs}) \times 10,$$

$$\text{MCV (}\mu\text{m}^3\text{)} = (\text{PCV} / \text{RBCs}) \times 10.$$

Total protein (TP) was measured according to Weichselbaum (1946), albumin (Alb) was determined by colorimetric methods (Doumas *et al.*, 1971), while globulin concentrations (Glo) were determined by subtracting the Alb concentration from the concentration of TP according to Coles (1974). Serum creatinine was assessed according to Henry (1974). Liver enzymes aspartate amino transaminase (AST) and alanine amino transaminase (ALT) were determined according to Reitman and Frankel (1957).

At the end of the experimental period, the liver and spleen were carefully removed and weighed. Somatic indices including hepatosomatic index (HSI) and splenosomatic index (SSI) were calculated (Htunhan, 1978) as follows:

$$\text{HSI} = \frac{\text{weight of the liver}}{\text{fish body weight}},$$

$$\text{SSI} = \frac{\text{weight of the spleen}}{\text{fish body weight}}.$$

Liver tissues were examined for antioxidants activities. Glutathione peroxidase (GPx) activity was measured according to the method described by Mohandas *et al.* (1984). Briefly, the assay mixture was added to 0.2-0.3 mg protein of liver and 0.25 mmol/l hydrogen peroxide in a final volume of 1.0 ml NADPH. The activity of the enzyme was recorded at 340 nm at 25°C and was expressed as nmol of NADPH oxidized/min/mg protein by Enzyme-Linked Immunosorbent Assays (ELISA).

Standard ELISA protocol (Derango and Page 1996) was applied to measure the metallothionein content. Briefly, the primary antibody against metallothionein (Ab 36882) and the secondary antibody (Ab 6721) were purchased from Abcam, UK. An automatic micro titer plate ELISA reader (Wipro, India) was used to measure the absorbance at 650 nm. A second reading was recorded at 450 nm after addition of 2.0 M H₂SO₄ that stops the reaction. The high precision metallothionein value was achieved by plotting the ELISA values in the regression curve that obtained from a standard curve (Hornitzky and Searson 1986).

Infection trial

By the end of the experiment (after 30 days), 10 fish were randomly collected from each group and injected i.p. with 0.3 x10⁸ cfu/ml of *A. hydrophila* according to Schaperclaus *et al.* (1992). Pure saline solution (0.65%) was parallel injected in a similar fashion, in three fish, for negative control injection (Boijink *et al.*, 2001). *O. niloticus* were kept in the

same experimental condition and under observation. Mortality rate (MR) % were estimated following 14 days as follows:

$$\text{MR \%} = \text{No. of death in specific period} / \text{Total population during that period} \times 100.$$

Statistical analysis

Statistical analysis was performed by using the analysis of variance (ANOVA). All statistics were run on the computer using the SPSS program (SPSS, 2004).

RESULTS

Determination of TiO₂NP MIC

To determine MIC, a series of TiO₂NP dilutions ranging from 500 µg/ml to 10 µg/ml were mixed with adjusted *A. hydrophila* concentration while negative control contained only inoculated broth and the time and temperature of incubation being 48 h and 37°C, respectively. MIC was determined to be 20 µg/ml, which was the minimum concentration that visually inhibited the growth of the microorganism.

The SR % in different tested groups (Table 2) revealed that it was significantly decreased in fish subjected to high dosage of TiO₂NP (100 µg/g b.w.), especially through the injection route (50% in G6). Meanwhile, the SR % was boosted in groups exposed to TiO₂NP MIC regardless to the administration route (G2 and, G5).

Table 2. Survival rate percentage of *O. niloticus* in different experimental groups

| Items | Feed | | | Injection | | |
|-------|-------------------------|------------------------|------------------------|-------------------------|-------------------------|----------------------|
| | G1 Control | G2 20 µg/g | G3 100 µg/g | G4 Control | G5 20 µg/g | G6 100 µg/g |
| No | 30 | 30 | 30 | 30 | 30 | 30 |
| SR% | 83.3 ^{ab} ±3.3 | 86.7 ^a ±3.3 | 66.7 ^b ±8.8 | 73.3 ^{ab} ±3.3 | 76.7 ^{ab} ±3.3 | 50 ^c ±5.8 |

No=Number of fish, SR= Survival rate. Different letters in the same row are significantly different at P≤0.05.

Hematological and biochemical analyses

As shown in Table 3, *O. niloticus* received high dosage of TiO₂NP (100 µg/g b.w.) displayed sever decrease in blood indices irrespective to the administration route. RBCs and Hb were severely decreased in G3 and G6, which received high TiO₂NP dosage,

presenting 1.67 and 1.57 X10⁶; 5.01 and 4.71 g/dl, respectively. In the same line WBCs were decreased to 65 and 61.2 X10⁶ in G3 and G6, respectively. While no significant differences were observed in MCV, MCH, and MCHC.

Table 3. Blood analyses of *O. niloticus* received TiO₂NP. Mean±SE

| Items | Feed | | | Injection | | |
|---------------------------------|----------------------------|----------------------------|----------------------------|----------------------------|----------------------------|----------------------------|
| | G1 | G2 | G3 | G4 | G5 | G6 |
| | Control | 20 µg/g | 100 µg/g | Control | 20 µg/g | 100 µg/g |
| RBCs X10 ⁶ | 2.4 ^a ±0.25 | 2.35 ^a ±0.2 | 1.67 ^b ±0.1 | 2.16 ^a ±0.3 | 2.3 ^a ±0.24 | 1.57 ^b ±0.15 |
| WBCs X10 ³ | 72 ^a ±1.8 | 71 ^a ±3.5 | 65 ^{ab} ±3.3 | 73.4 ^a ±1.5 | 74.5 ^a ±2.7 | 61.2 ^b ±2.1 |
| Hb g/dl | 7.44 ^a ±0.55 | 7.28 ^a ±0.35 | 5.01 ^b ±0.5 | 6.48 ^a ±0.4 | 6.9 ^a ±0.8 | 4.71 ^b ±0.6 |
| PCV % | 23.1 ^a ±1.6 | 22.6 ^a ±0.9 | 15.53 ^b ±1.8 | 20.1 ^a ±0.8 | 21.39 ^a ±0.2 | 15.4 ^b ±0.5 |
| MCV fl | 96.25 ^a ±2.1 | 96.17 ^a ±1.9 | 92.9 ^b ±1.5 | 93 ^b ±0.5 | 93 ^b ±0.4 | 92.7 ^b ±0.8 |
| MCH Pg | 31 ^a ±1.2 | 30.9 ^a ±0.35 | 30 ^a ±1.3 | 30 ^a ±0.2 | 30 ^a ±0.2 | 30 ^a ±0.7 |
| MCHC g/dl | 32.2 ^a ±2.1 | 32.13 ^a ±0.5 | 32.3 ^a ±2.4 | 32.26 ^a ±0.3 | 32.26 ^a ±0.1 | 32.36 ^a ±0.1 |

Different letters in the same row are significantly different at P≤0.05.

For both groups subjected to 100 µg/g b.w. TiO₂NP (G3 and G6), a significant decrease in the values of TP (4.2 and 3.95) as well as Glo (1.8 and 1.52) was observed. Meanwhile, Alb revealed no significant difference among tested groups (Table 4). On the other hand, no significant differences were recorded in TP or Glo in fish exposed to the TiO₂NP MIC through either food or injection routes (G2 and G5).

Liver enzymes, AST and ALT, were significantly increased with the high TiO₂NP dosage in both G3

and G6, regardless to the administration route. Groups received a dosage that corresponds to the MIC were insignificantly different compared with control.

To evaluate the creatinine clearance, which reflects the glomerular filtration rate of fish kidneys, serum creatinine was measured. Values of serum creatinine had the same trend of liver enzymes since high dosage of TiO₂NP (G3 and G6) resulted in a remarkable elevation whatever the administration route was.

Table 4. Serum analyses of *O. niloticus* received TiO₂NP. Mean±SE

| Items | Feed | | | Injection | | |
|-----------------------|----------------------------|----------------------------|---------------------------|----------------------------|---------------------------|----------------------------|
| | G1 | G2 | G3 | G4 | G5 | G6 |
| | Control | 20 µg/g | 100 µg/g | Control | 20 µg/g | 100 µg/g |
| TP g/dl | 5.1 ^a ±0.1 | 5 ^a ±0.25 | 4.2 ^b ±0.12 | 5.2 ^a ±0.3 | 4.9 ^{ab} ±0.2 | 3.95 ^b ±0.38 |
| Alb g/dl | 2.65 ^a ±0.24 | 2.62 ^a ±0.28 | 2.4 ^a ±0.1 | 2.79 ^a ±0.25 | 2.51 ^a ±0.2 | 2.23 ^a ±0.3 |
| Glo g/dl | 2.45 ^a ±0.15 | 2.38 ^a ±0.18 | 1.8 ^b ±0.2 | 2.41 ^a ±0.2 | 2.39 ^a ±0.1 | 1.52 ^b ±0.3 |
| AST U/L | 23 ^d ±2.3 | 29 ^c ±2.4 | 75 ^b ±3.2 | 25 ^d ±1.3 | 36 ^c ±3.9 | 112 ^a ±5.8 |
| ALT U/L | 18 ^c ±1.85 | 20 ^c ±2.7 | 85 ^b ±5.2 | 17 ^c ±2.5 | 28 ^c ±9.3 | 142 ^a ±6.3 |
| Creat mg/dl | 0.2 ^c ±0.02 | 0.71 ^b ±0.05 | 0.8 ^b ±0.06 | 0.18 ^c ±0.01 | 0.81 ^b ±0.1 | 1.3 ^a ±0.02 |

Different letters in the same row are significantly different at P≤0.05. Creat denotes serum creatinine.

Both of the examined somatic indices (Table 5); HSI and SSI of *O. niloticus* in groups received the TiO₂NP MIC, were insignificantly different from control regardless to the administration route. Meanwhile,

G3 and G6 revealed a significant increase as they recorded 1.76 and 2.4%, respectively. In addition, SSI showed similar trend as that for HSI, with a significant increase in G3 and G6 by 0.42 and 0.86%, respectively.

Table 5. HSI and SSI of *O. niloticus* received TiO₂NP. Mean±SE

| Items | Feed | | | Injection | | |
|-------|--------------------------|--------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| | G1 | G2 | G3 | G4 | G5 | G6 |
| | Control | 20 µg/g | 100 µg/g | Control | 20 µg/g | 100 µg/g |
| HSI % | 1.6 ^c ±0.02 | 1.62 ^c ±0.03 | 1.76 ^b ±0.01 | 1.59 ^c ±0.01 | 1.59 ^c ±0.1 | 2.4 ^a ±0.12 |
| SSI % | 0.33 ^c ±0.001 | 0.34 ^c ±0.002 | 0.42 ^b ±0.01 | 0.31 ^c ±0.01 | 0.32 ^c ±0.01 | 0.86 ^a ±0.01 |

Different letters in the same row are significantly different at P≤0.05.

In hepatic tissues of *O. niloticus*, a significant elevation of antioxidants activities for GPx and MT was remarkable in response to the high dosage of TiO₂NP in spite of the administration route. Groups received TiO₂NP MIC (G2 and G5) were insignificantly different from control groups (G1 and G4) (Table 6).

Infection trial

Ten *O. niloticus* from each group were challenged

against *A. hydrophila*, and MR% was calculated and presented in Table 7. High TiO₂NP dosage, regardless to the administration route, in both G3 and G6 resulted in a higher MR% that recorded 70 and 80%, respectively followed by the control in both G1 and G4 (50%). On the other hand, the lower MR% was observed in G2 and G5 revealing 20 and 30%, respectively for those fish that received the TiO₂NP MIC (20 µg/g b.w.).

Table 6. Antioxidants activities in hepatic tissue of *O. niloticus* received TiO₂NP. Mean±SE

| Items | Feed | | | Injection | | |
|-------|-------------------------|-------------------------|-------------------------|------------------------|------------------------|-------------------------|
| | G1 | G2 | G3 | G4 | G5 | G6 |
| | Control | 20 µg/g | 100 µg/g | Control | 20 µg/g | 100 µg/g |
| GPx | 190.8 ^c ±6.5 | 190 ^c ±4.8 | 253.7 ^b ±3.8 | 195 ^c ±2.8 | 198 ^c ±3.9 | 287.5 ^a ±5.2 |
| MT | 15.7 ^c ±1.2 | 17.3 ^c ±2.75 | 25.9 ^b ±1.8 | 16.8 ^c ±2.4 | 16.2 ^c ±1.8 | 36.7 ^a ±2.2 |

Different letters in the same row are significantly different at P≤0.05.

Table 7. MR% of *O. niloticus* received TiO₂NP and challenged with *A. hydrophila*

| Items | Feed | | | Injection | | |
|-------|---------|---------|----------|-----------|---------|----------|
| | G1 | G2 | G3 | G4 | G5 | G6 |
| | Control | 20 µg/g | 100 µg/g | Control | 20 µg/g | 100 µg/g |
| NO. | 10 | 10 | 10 | 10 | 10 | 10 |
| MN | 5 | 2 | 7 | 5 | 3 | 8 |
| MR% | 50 | 20 | 70 | 50 | 30 | 80 |

NO=Number of fish, MN= Mortality number and MR%= Mortality rate%.

DISCUSSION

Nile tilapia, *O. niloticus*, is a widespread teleost fish in tropical regions where it has a significant economic value in fishery and aquaculture industries. This study highlighted the antimicrobial properties of TiO₂NP and possible impacts on *O. niloticus* health. *A. hydrophila* bacteria is a common pathogen for fish. It is a Gram-negative motile rod and one of the highest isolated bacterial pathogens of freshwater fish in fish farms that occurred in warm climatic countries (Angka, 1990 and Esteve *et al.*, 1993).

Nanoparticles have a better and different quality compared to other forms of the same element. A small amount of them can have a great deal of antibacterial effect (Karimipour and Tanomand, 2016). The inhib-

itory effect of the nanoparticles may occur from their interference in the biological mechanisms of the bacteria as they penetrate the cell wall of the bacteria and change its properties. This increases the penetrability of cell membrane and interrupt the control of material intake and output from the cytoplasm. In the present study, the TiO₂NP MIC for *A. hydrophila* was determined to be 20 µg/ml. High SR % of *O. niloticus* was recorded with the determined MIC regardless to the administration route.

Concerning blood indices, high dosage of TiO₂NP (100 µg/g b.w.) displayed drastic impacts in both fed and injected fish although injected group (G6) revealed higher response. Meanwhile, no significant difference was observed in the tested blood indices

between the control groups and those received the TiO₂NP MIC. A number of studies have suggested that TiO₂NPs could pose toxicity to several aquatic organisms including microbes, algae, invertebrates and fish (Chen et al., 2012). In a species of ark clam known as the blood clam *Tegillarca granosa*, Shi et al. (2017) treated the clam with 10 and 100 mg/l TiO₂NP for 30 days. The authors reported that RBCs were significantly decreased from 79.76% to 70.98% in comparison to the negative control. In the same line, Barmo et al. (2013) and Balbi, et al. (2014) observed a reduction in different blood indices and phagocytic activity of the saltwater mussels *Mytilus galloprovincialis* in vivo acute toxicity (96 h) of TiO₂NP (size 15-60 nm) with a dosages 1-100 mg/l. The findings of the present study could be explained by the fact that high dosages of TiO₂NP perform a physical stress, which damaged blood cells. In agreement, Reeves et al. (2008) stated that the physical stress of TiO₂NP disrupted membranes of blood cell that was induced by the adhesion of TiO₂NP.

The examined liver enzymes of *O. niloticus* AST and ALT of fish exposed to high TiO₂NP were significantly increased by several times relative to control fish, while those subjected to the TiO₂NP MIC slightly increased. Along with the present results, Wang et al. (2007), Chen et al. (2009), Duan et al. (2009) and Liu et al. (2009) stated that the activity of a number of enzymes, including AST and ALT, were increased in mice treated with TiO₂NP. This was attributed to the increase in cellular membranes damages leading to liver enzymes leaking out. On the other hand, serum creatinine in fish exposed to the selected high TiO₂NP dosage was significantly increased by 4 to 7.2 times in G3 and G6, respectively with regard to the control. Meanwhile, fish groups subjected to TiO₂NP MIC also revealed a significant increase by 3.5 and 4.4 times in G2 and G5, respectively. In agreement, Banaee et al. (2016) found a significant increase ($P < 0.05$) in creatinine levels 1.17 to 1.08 of *Cyprinus carpio* subjected to TiO₂NP compared to control group 0.14 to 0.17, respectively.

HSI is a widely known bioindicator of contaminant exposure (Sadekarpawar and Parikh, 2013). Because the liver is so important in detoxification, exposure to contaminants can lead to an increase in liver size from hypertrophy (an increase in size), hyperplasia (an increase in number) of hepatocytes (Sole et al., 2010), or both. On the other hand, fish spleen acts primarily as a blood filter, and plays important roles

in regard to red blood cells and the immune system. Fish with larger spleens, may simply have a greater filtering capacity and thus increased immune function (Hadidi et al., 2008). *O. niloticus* which injected with a high dosage of TiO₂NP (G6) revealed high HSI as well as SSI, which may indicate the suffering of fish from hepatomegaly as well as splenomegaly, respectively. This could be attributed to TiO₂NP generation of free radicals, which in turn initiate an inflammatory response that leads to hepatocytes swelling together with dilatation of the central vein, increased permeability hepatocytes membrane and the endothelial lining of blood vessels (Johar et al., 2004 and Alarifi et al., 2013).

In the present study, significant differences were only demonstrated in TP and Glo levels in fish exposed to the high examined TiO₂NP level. Banaee et al. (2016) observed a decrease in total protein as well as globulin levels in *C. carpio* exposed to 125 µg/l of TiO₂NPs for 21 days that was attributed to reduced protein and globulin synthesis in hepatocytes. The authors claimed that the decrease in protein levels might be related to malnutrition, increased energy cost of homeostasis, tissue repair and the detoxification mechanism under stress conditions. Meanwhile, Griffith et al. (2009) explained such decrease by the effect of TiO₂NPs exposure on the expression of genes involved in protein synthesis.

The increase activity of both AST and ALT besides the observed high HSI indicated that the fish hepatic tissue was adversely affected with the high TiO₂NP dosage. Therefore, antioxidants activity of GPx and MT were assessed to confirm this observation. Antioxidants activity were in the same line of the above mentioned results of liver enzymes and somatic indices since GPx and MT increased with the high TiO₂NP dosages while there was an insignificant difference between groups received MIC and control. A number of studies have painted a picture, which is in line with the currently predominant paradigm that nanomaterial toxicity is associated with the induction of oxidative stress (Lammel & Sturve, 2018). These authors have suggested that TiO₂NPs induce the formation of ROS leading to damage of biological macromolecules including lipids, proteins and DNA, and consequently to loss of vital cellular functions and cell death. Firat and Bozat (2018) reported that acute exposure of TiO₂NPs caused decreases in activities of a number of enzymes including Gpx (37%), while its exposure for 14 days increased the activity of the

enzyme (32%). This was attributed to the fact that TiO₂NPs can potentially cause oxidative stress, which may lead to disturbance in the antioxidant enzymes systems by either stimulating or inhibiting their activities. Xiong *et al.* (2011) also observed that ROS in zebra fish exposed to 50 mg/l TiO₂NP, was elevated by 139.7% to 178.1% relative to the control group due to their protein carbonyl content. Similar changes were observed in zebrafish (Bar-Ilan *et al.*, 2013) and rainbow trout (Boyle *et al.*, 2013). In contrast, Federici *et al.* (2007) observed that rainbow trout subjected to high level of TiO₂NP (0.1-1 mg/l) for 14 days naturally still had the ability to scavenge the ROS. This conflict between the results of the present study and other studies could be explained by differences in animal's species as well as dosage, administration route, and duration of exposure to TiO₂NP.

O. niloticus challenged with *A. hydrophila* and either fed or injected with TiO₂NP MIC represented low MR%; 20 and 30% in fed and injected fish, respectively. Whereas fish treated with high TiO₂NP dosage showed a high MR% both in fed and injected groups revealing 70 and 80%, respectively. These results were anticipated due to the immunosuppression and compromised health status of the fish that occur, possibly because of high ROS activity, which was reflected by increased AST, ALT, creatinine, HSI, and SSI along with low WBCs, TP, and Glo; the decrease in globulin

level may reduce the resistance of fish to pathogens (Griffitt *et al.*, 2009). Jovanovic *et al.* (2015) stated that Nano-TiO₂ is immunotoxic to fish and reduces the bactericidal function of fish neutrophils. The authors demonstrated that *Pimephales promelas* exposed to nano-TiO₂ (2 and 10 mg/g b.w.) and challenged with *A. hydrophila* or *Edwardsiella ictaluri* revealed a decrease in neutrophil phagocytosis rate, which resulted in increased fish mortality.

CONCLUSIONS

TiO₂NP had potential antibacterial properties and its MIC was determined to be 20 µg/g b.w. for *A. hydrophila* infection. This concentration proved no adverse impact on the health status of *O. niloticus*. The more appropriate route of administration was via fish feed. Meanwhile, irrespective to the route of administration, high dosage of TiO₂NP had immunosuppression as well as ROS generation effect therefore, not recommended to be used in fish treatment.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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