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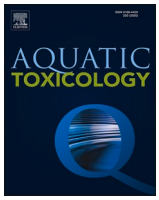

Modulatory effect of thymol on the immune response and susceptibility to *Aeromonas hydrophila* infection in Nile tilapia fish exposed to zinc oxide nanoparticles

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ABSTRACT

Zinc oxide nanoparticles (ZnO-NPs) have many exciting properties that make their use in a continuous increase in various biomedical, industrial, and agricultural applications. This is associated with accumulation in the aquatic ecosystems and fish exposure with consequent deleterious effects. To determine the potential of thymol to counteract the immunotoxic effects of ZnO-NPs, *Oreochromis niloticus* was exposed to ZnO-NPs (⅕ LC_{50} =1.14 mg/L, for 28 days) with or without feeding a thymol-incorporated diet (1 or 2 g/kg diet). Our data demonstrated a reduction of aquaria water quality, leukopenia, and lymphopenia with a decrease in serum total protein, albumin, and globulin levels in exposed fish. At the same time, the stress indices (cortisol and glucose) were elevated in response to ZnO-NPs exposure. The exposed fish also revealed a decline in serum immunoglobulins, nitric oxide, and the activities of lysozyme and myeloperoxidase, in addition to reduced resistance to the *Aeromonas hydrophila* challenge. The RT-PCR analysis showed downregulation of antioxidant (SOD) superoxide dismutase and (CAT) catalase gene expression in the liver tissue with overexpression of the immune-related genes (TNF-α and IL-1β). Importantly, we found that thymol markedly protected against ZnO-NPs-induced immunotoxicity in fish co-supplemented with thymol (1 or 2 g/kg diet) in a dose-dependent manner. Our data confirm the immunoprotective and antibacterial effects of thymol in ZnO-NPs exposed fish, supporting the potential utility of thymol as a possible immunostimulant agent.

1. Introduction

In recent years, nanotechnology rapidly developed and spread worldwide with several applications in various fields, including medicine, biotechnology, agriculture, food production, and aquaculture (Rahman et al., 2022). The excessively released nanomaterials from anthropogenic activities end in their discharge into the aquatic systems causing hazardous effects on humans and aquatic organisms, including fish (Aziz et al., 2022; Shahzad et al., 2017).

Zinc oxide nanoparticles (ZnO-NPs) are among metal nanoparticles that have long been utilized in animal production, animal medicine, aquaculture, environmental remediation, ceramics, paints, and personal care products. Their global production is estimated at 100 and 1000 tons/year (Ma et al., 2013; Rahman et al., 2022; Saddick et al., 2017). The extensive use and high environmental persistence of ZnO-NPs result in inevitable release and bioconcentration in aquatic environments (Abdel-Khalek et al., 2015; Kaya et al., 2015; Ma et al., 2013). It has been estimated that about 3700 tons/year of ZnO-NPs are discharged into aquatic systems worldwide, adversely affecting fish and other aquatic organisms (Keller et al., 2013). The annual output of ZnO-NPs to

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the environment is 550 to 5550 tons, and their production is 10 to 100 times higher than the manufacture of any further nanomaterial (Hazeem, 2022). The behavior of ZnO-NPs in aquatic systems is controlled by many factors, including the water's oxygen level, organic matter content, pH, and ionic strength. In seawater, ZnO-NPs tend to agglomerate and become less mobile due to the high ionic strength. In freshwater, they tend to dissolve quickly, increasing the risk of acute toxicity for freshwater fish (Franklin et al., 2007; Yung et al., 2014).

Prior studies have demonstrated the acute toxicity of ZnO-NPs in fish. The 96-h LC_{50} of ZnO-NPs was shown to be 4.9 mg/L for *Danio rerio* (Xiong et al., 2011), 4.897 mg/L for *Cyprinus carpio* (Subashkumar and Selvanayagam, 2014), 4.1 mg/L for *Oreochromis niloticus* (Alkaladi et al., 2020) and 31.15 mg/L for *Labeo rohita* (Aziz, 2020).

As previously demonstrated, prolonged exposure of fish to ZnO-NPs results in zinc bioaccumulation into different tissues, including the liver, kidney, brain, gills, and muscles (Sibiya et al., 2022). ZnO-NPs affect fish health by inducing various toxic traits, including cytotoxicity, genotoxicity, embryotoxicity, and others (Cong et al., 2017; Sibiya et al., 2022). The primary mechanism of action by which ZnO-NPs induce toxicity is oxidative stress, associated with the enhanced generation of extracellular and intracellular reactive oxygen species (ROS). These ROS can then oxidize proteins and lipids, and DNA causing loss of function and subsequent toxicity (Giordo et al., 2020; Song et al., 2010; Yung et al., 2014). Saddick et al. (2017) reported that ZnO-NPs altered the antioxidant enzyme activities and gene expression in the brain of *O. niloticus* and *Tilapia zillii (Saddick et al., 2017)*. ROS overproduction also reduced mitochondrial membrane potential, leading to mitochondrial dysfunction and activation of the apoptosis pathway mediated by mitochondria and caspases (Guo et al., 2013; Zhao et al., 2016). Other studies have demonstrated that ZnO-NPs may induce toxicity through the Zn ions disintegration from the ZnO-NPs, increasing local concentrations of Zn ions in exposed tissues and internal organs and subsequently inducing oxidative damage (Ng et al., 2017; Vimercati et al., 2020).

The immunotoxicity of ZnO-NPs was previously documented in mice exposed to high doses of ZnO-NPs. These animals presented suppressed immune systems as evidenced by reduced serum concentrations of immunoglobulins (IgG and IgM), tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL1-β), interleukin-10, and T helper-1 cytokines (interferon-γ and interleukin-12p70) (Kargin, 2021; Kim et al., 2014). In crucian carp (*Carassius carassius*), ZnO-NPs induced immune toxicity by elevating the level of neutrophil extracellular traps (NETs); citrullinated histone 3, myeloperoxidase, and neutrophil elastase, and caused oxidative stress (Hong et al., 2022). Rajkumar et al. (2022) have showed that ZnO-NPs generated toxicity in freshwater fish *Cyprinus carpio* by modifying the hematological parameters, the antioxidant defense mechanisms, the oxidative stress encoding genes, and the histomorphology (Rajkumar et al., 2022).

Researchers continuously seek natural antioxidants that can ameliorate the toxic effects of different pollutants on fish (Farag et al., 2021a; Mawed et al., 2022). Thymol (2-isopropyl-5-me) is a naturally occurring monoterpene phenol found in essential oils extracted from thyme (*Thymus vulgaris* L) (Hoseini and Yousefi, 2019) and other plants such as *Origanum* L., *Ocimum gratissimum* L., *Carum copticum* L., *Oliveria decumbens* Vent, *Satureja* L. (Escobar et al., 2020). Thymol molecule has been successfully used in fish nutrition as a feed additive to enhance nutrient bioavailability, growth, productivity, and reproductive performance (Alagawany et al., 2021). Thymol has several beneficial effects, including antioxidant, anti-inflammatory, antimicrobial, antiviral, anticancer, and immunomodulatory effects (Escobar et al., 2020).

The majority of the beneficial effects of thymol are likely dependent on its ability to scavenge highly reactive radicals and enhance the enzymatic and non-enzymatic cellular antioxidants with consequent suppression of oxidative damage to macromolecules, including lipids and protein (Alagawany et al., 2021; Hoseini and Yousefi, 2019).

Oreochromis niloticus (Nile tilapia), a fish native to Egypt and Africa, has spread worldwide due to its commercial value, rapid growth rate, quick adaptability to different diets and harsh environments, and high tolerance to handling procedures and diseases. Moreover, it is an excellent model for ecotoxicological studies (Fontainhas-Fernandes, 1998).

The influence of toxic pollutants on the fish's health status can be explored via assessment of the hematological and biochemical alterations, different markers of the immune responses, and expression of the genes related to the immune response (Kollner et al., 2002). This investigation explored the immunoprotective effect of thymol in ZnO-NPs-exposed Nile tilapia via assessment of the innate immunity variables, the host resistance to *Aeromonas hydrophila,* and the expression of antioxidant and immune-related genes in the liver.

2. Materials and methods

2.1. Tested chemicals

2.1.1. Preparation and characterization of zinc oxide nanoparticles

The preparation of zinc oxide nanoparticles (ZnO-NPs) started with dissolving 12 g of Zn $(NO_3)_2{\cdot}6H_2O$ (Sigma-Aldrich Chemical Co.; St. Louis, MO, USA) in distilled water (1 L) to have a 5 mM final nominal concentration. This solution was then stirred for 25 min with 10% NaOH solution over a hot plate magnetic stirrer and then stirred at 70 °C for 2 h. This was followed by cooling and filtering the obtained solution (El-Saadony et al., 2021).

Several protocols were applied for characterizing the ZnO-NPs. The ZnO-NPs' optical absorption spectra were measured using a UV-vis LaxcoTM double-beam spectrophotometer. Transmission electron microscopy (TEM, JEOL 1010, Japan) was used to determine the diameter and size of the ZnO-NPs. Moreover, the size of particles in the colloidal solution was assessed using the dynamic light scattering analysis (DLS, Malvern Hills, UK). Finally, the relative stability and surface charges of ZnO-NPs were studied by applying the zeta potential analysis.

2.1.2. Thymol

Thymol (2-Isopropyl-5-methylphenol); 98.5% purity; Linear Formula: 2-$[(CH_3)_2CH]C_6H_3$-5-$(CH_3)OH$; MW: 150.22, CAS Number: 89–83–8 was purchased from Sigma- Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Fish maintenance and tested diets formulation

Four hundred forty *O. niloticus* fish weighing 10–12 g were used to assess the acute toxicity in the main experimental study. The fish were purchased from a local Fish Hatchery (El-Abbassa, Al-Sharkia, Egypt). The fish spent a two-week-acclimatization period in dechlorinated tap water aquaria. They received a formulated basal diet three times daily (at a rate of 5% of the fish weight) without chemical exposure. The critical water quality parameters employed by the American Public Health Association were applied (Rice et al., 2012). The same environmental conditions comprising temperature, dissolved oxygen (DO), pH, and ammonia (NH_3); controlled photoperiod (10 h light/14 h dark cycle) were applied on all aquaria.

The basal diet (Table 1) was supplemented with thymol at a dietary rate of 1 or 2 g/kg diet, followed by mechanical mixing of all the feed ingredients, pelletization, and exposure to air at 25 °C for 24 h for complete dryness, and then stored until be used at 4 °C.

2.3. Lethal toxicity study to estimate the median lethal concentration (96-h LC_{50})

The LC_{50} of ZnO-NPs was estimated in the previously acclimatized fish (n = 80; weighing 40 ± 0.11 g) randomly allocated into eight groups, ten fish each. During the investigation period, no food was provided to the fish to minimize the absorption of NPs' food absorption

Table 1
Calculated composition analysis and formulation of the basal diet fed to *O. niloticus*.

Items	Control
Ingredient (%)	
Yellow corn	210
Soybean meal 48% CP	200
Fish meal	150
Corn gluten 60% CP	130
Rice bran	110
Wheat middlings	150
Premix-Min [a]	10
Premix-Vit [b]	10
Corn oil	30
Total	1000
Calculated composition (%)	
Crude protein	32.05
Lipid	4.55
Crude fiber	4.24
Ash	7.30
Nitrogen free extract [c]	51.85

[a] Composition of mineral premix kg−1: manganese, 53 g; zinc, 40 g; iron, 20 g; copper, 2.7 g; iodine, 0.34 g; selenium, 70 mg; cobalt, 70 mg and calcium carbonate as carrier up to 1 kg.
[b] Composition of vitamin premix kg−1: vitamin A, 8000,000 IU; vitamin D3, 2000,000 IU; vitamin E, 7000 mg; vitamin K3, 1500 mg; vitamin B1, 700 mg; vitamin B2, 3500 mg; vitamin B6, 1000 mg; vitamin B12, 7 mg; biotin, 50 mg; folic acid, 700 mg; nicotinic, 20,000 mg; pantothenic acid, 7000 mg.
[c] Nitrogen free extract = 100 − (crude protein + crude lipids + ash + crude fiber).

and feces production without changing the aquaria water. Various suspension concentrations of the ZnO-NPs were prepared, and a bath sonicator was used for the dispersion of nanoparticles for 1 h immediately before being used without the addition of any stabilizing agents.

Range finding tests were conducted as previously reported (Mahmoud et al., 2020; Saddick et al., 2017), followed by the lethal toxicity study using gradual concentrations of ZnO-NPs suspension (0, 1, 3, 5, 7, 9, 11, and 14 mg/ L) in triplicate. Ten *O. niloticus* fish were exposed, for 96 h, to each concentration of ZnO-NPs (20 L-treated water in a 30 L aquarium). In contrast, unexposed control fish were reared in clean distilled water without chemical treatment. The swimming behavior, outer morphology, and mortalities of fish were monitored regularly each day and then compared with the control fish to estimate the 96-h LC_{50} value of ZnO-NPs, by Finney's statistical method with a 95% confidence interval (Finney, 1971).

2.4. Antidotal investigation: modulatory effect of thymol against ZnO-NPs immunotoxicity

Acclimatized fish were randomly distributed equally to six equal groups (60 fish each, in three replicates, 20 fish/replicate). The unexposed control fish received a basal diet and were kept, without any chemical treatment, in clean water glass aquaria. The THY1 and THY2-supplemented fish groups received diets enriched with thymol at dietary levels of 1 and 2 g/kg, respectively, and were kept in clean water glass aquaria. The ZnO group was exposed to a nominal concentration of 1.14 mg/L (equivalent to ⅕ LC_{50}) of ZnO-NPs by adding the ZnO-NPs to water and fed on a basal diet for 28 days. The fifth (ZnO-THY1) and the sixth (ZnO-THY2) groups received ZnO-NPs (1.14 mg/L) and were fed on low (1 g/kg) and high (2 g/kg) thymol-supplemented diets, respectively, for 28 days.

The tanks' water was changed daily before adding ZnO-NPs at the concentration indicated before. A suction pump was used to remove about 80% of the water, feed, and fecal matter, followed by adding clean water and freshly sonicated ZnO-NPs to each tank.

Throughout the experimental period, fish were fed thrice daily (7:00 a.m., 11:00 a.m., and 4:00 p.m.) at a dietary rate equivalent to 5% of the fish biomass. The feed requirements were calculated every week according to the fish's weight gain, and the signs of toxicity were monitored.

2.4.1. Water quality parameters

The quality of water was regularly investigated throughout the experiment, two times each week by the use of portable digital instruments to measure water temperature, pH, and dissolved oxygen (Martini Instruments Model 201/digital), whereas a colorimetric method was used to assess the level of total ammonia.

2.4.2. Tissue harvest

After the exposures, two sets of blood samples were withdrawn from the caudal blood vessels by sterile syringes. The first sample set was collected without using anticoagulant and then subjected to centrifugation for 20 min at 1075 g to obtain serum, which was stored at - 20 °C until physiological and immunological variables estimation. The other blood set was collected for immediate hematological profiling using EDTA as an anticoagulant. Spinal cord sectioning was performed to euthanize the fish, followed by dissection of the liver, which was frozen instantly in liquid nitrogen and stored at −80 °C for future use in total RNA extraction.

2.5. Hematological variables

Different indices of blood cells, including hemoglobin concentration, RBC count, packed cell volume (PCV), and leukocyte counts (total and differential), were investigated with the aid of an automatic cell counter (Hospitex Hema screen 18, Italy) as previously described (Bain et al., 2016).

2.6. Serum protein profile

The method employed by Doumas et al. (1981) was followed for colorimetric quantitation of the levels of total protein and albumin in serum using the test kits of BIOMED Diagnostic Egy Chem (Egypt) (Doumas et al., 1981).

2.7. Stress-related assays

The Cayman Chemical Company kit was used to assess the serum cortisol level. The serum glucose level was measured as previously described (Teixeira et al., 2018), whereas the glucagon serum level was determined using ELISA kits from MyBioSource.com with a catalog number MBS034316.

2.8. Aeromonas hydrophilia challenge test

Fish resistance to the *A. hydrophila* challenge was investigated at the end of the experiment. Twenty fish from each group were inoculated I/P with 0.1 mL bacterial cell suspension containing 1.5×10^7 cells/mL. Challenged fish were observed for two weeks to record the clinical signs and fish mortalities.

2.9. Immune response assays

Serum immunoglobulins M and G (IgM and IgG) were measured using the fish ELISA kits supplied commercially by MyBioSource, San Diego, USA (Catalog No. MBS035038 and MBS043814, respectively). The activity of serum lysozyme was spectrophotometrically determined by the lysis of Micrococcus lysodeikticus freeze-dried particles (Stolen et al., 1990). Serum nitric oxide (NO) level was measured calorimetrically (Montgomery and Dymock, 1961). Serum myeloperoxidase (MPO) was estimated using ELISA kits from MyBioSource.com with a catalog

number MBS016324, following the manufacturer's instructions.

2.10. Transcriptional analysis of antioxidant enzymes and immune-related genes in the liver

Total RNA was extracted from frozen liver specimens using Trizol reagent (easyREDTM, iNtRON Biotechnology, Korea). The extracted RNA was used to synthesize the first strand of cDNA by a Quantitect® Reverse Transcription kit (Qiagen, Germany), following the kit's protocol. The forward and reverse sequences of primers of the SOD, CAT, TNF-α, and IL-1β genes are shown in Table 2, using β-actin as a housekeeping gene. The qPCR analysis was conducted in a Rotor-Gene Q instrument with a QuantiTect® SYBR® Green PCR kit (Qiagen, Germany) under the following thermocycler condition: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s and 72 °C for 30 s. Each gene's relative mRNA expression pattern was calculated using the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.11. Statistical analysis

All experimental data were analyzed statistically via applying a One-way Analysis of Variance (ANOVA) using SPSS version 17 (IBM, USA). Tukey's multiple comparisons post hoc test was performed to compare the means of different groups, where the statistical significance was approved at $p < 0.05$. The analyzed data were presented as means ± SEM (standard error of the mean).

3. Results

3.1. ZnO-NPs characterization

Fig. 1 shows the characterization of zinc oxide nanoparticles (ZnO-NPs) using four devices. The UV–VIS spectroscopy analysis was performed to estimate the optical property of ZnO-NPs by LaxcoTM dual-beam spectrophotometer in the 200–700 nm range. The results showed a maximum peak at 340 nm (Fig. 1A). Moreover, the ZnO-NPs seemed spherical, with a 108 ± 2 nm average size (Fig. 1B). In contrast, the particle size was 89 nm, depending on the Brownian motion in suspension (Fig. 1C). Finally, the zeta potential analysis was performed to determine the ZnO-NPs surface charge, ensuring synthesized nanoparticles' stability where the net surface charge was −33 mV (Fig. 1D). To confirm the stability of the nanoparticles, the zeta potential analysis was applied, and the surface charge was found to be −33 mV (Fig. 1D).

3.2. LC_{50} value of Zno-NPs and behavioral responses in exposed fish

Concerning the 96-h LC_{50}, we have estimated it to be 5.71 mg/L, with upper and lower confidence limits of 7.33 and 4.05 mg/L, respectively (Fig. 2). The associated fish mortalities increased with increasing the ZnO-NPs levels during the 96-h period. No clinical signs of intoxication were observed in the control fish and those treated with the lowest level (1 mg/L) of ZnO-NPs. Conversely, neurobehavioral signs of intoxication were noticed in the alive and dying fish subjected to higher levels (>1 mg/L) of the ZnO-NPs. These included uncoordinated swimming, respiratory distress, gulping, lethargy movement, and hyperventilation (Table 3).

3.3. Water quality parameters

All aquaria showed no significant alterations in the pH value. The water temperature displayed a similar result, except for a significant elevation in the ZnO-THY1 group, compared to other groups. The ZnO-NPs significantly reduced the level of DO, whereas they elevated the ammonia concentration compared to the control. Co-supplementation with thymol restored the control values for DO and ammonia in both ZnO-THY1 and ZnO-THY2 groups (Table 4).

3.4. Effects on hematological variables

No significant differences were observed among all experimental groups concerning all the erythrogram variables (hemoglobin level, RBC count, and PCV). Regarding the leukogram indices, both thymol-supplemented groups showed no significant differences from the control values. Exposure to ZnO-NPs reduced total WBC and lymphocytic counts relative to their corresponding controls. Co-administration of thymol at the two dose levels did not ameliorate the toxic impact of ZnO-NPs on WBC count while improving the lymphocytic count in a dose-dependent manner, where the control value was restored at the highest dose level (Table 5).

3.5. Effects on protein profile and stress markers

As shown in Table 6, the food incorporated with thymol presented no effects on protein profile variables, except for a significant elevation of serum albumin level in THY1 and THY2 groups, compared to the control.

The ZnO group displayed significant decreases in total protein, albumin, and globulin serum levels compared to the corresponding control values. The supplementation of thymol combined with ZnO-NPs exposure improved total protein and globulin reductions in the ZnO-THY1 group, but the control values were not restored. On the other hand, the ZnO-THY2 group displayed improvement in the three variables, and the control value was attained for globulin.

Regarding the stress indicators, fish exposed to ZnO-NPs displayed higher serum cortisol levels than the control. Compared to the ZnO group, the concurrent supplementation of thymol with ZnO-NPs exposure mitigated the toxic effect on cortisol. However, the control range was restored only in the ZnO-THY2 group. A significant increment was noticed for serum glucose in the group treated with ZnO-NPs when compared with the control. Thymol-based food abolished the toxic effects of ZnO-NPs in both ZnO-THY1 and ZnO-THY2 groups, where the control levels were attained. Finally, no significant variations were recorded among all experimental groups concerning the serum glucagon level.

3.6. Immunological response variables

Fig. 3 shows the effect of ZnO-NPs exposure with or without thymol coadministration on various innate immunity indices. Both groups of

Table 2
Primer sequences (forward and reverse) used for real-time qPCR analysis.

Genes	Forward (5′−3′)	Reverse (5′−3′)	Accession No. / References
SOD	CATTCCATCATTGGCCGTACT	CCACCTTTGCCCAAGTCATC	BC_082800.1
CAT	GTACAGGCCGGCTCTCACA	ACCCGTGCTTTACAGGTTAGCT	NM_012520.2
TNF-α	GGAAGCAGCTCCACTCTGATGA	CACAGCGTGTCTCCTTCGTTCA	Dawood et al. (2020)
IL-1β	CAAGGATGACGACAAGCCAACC	AGCGGACAGACATGAGAGTGC	Dawood et al. (2020)
β-actin	CAGCAAGCAGGAGTACGATGAG	TGTGTGGTGTGTGGTTGTTTTG	Dawood et al. (2020)

Superoxide dismutase (SOD), Catalase (CAT), tumor necrosis factor-α (TNF-α), Interleukin 1β (IL-1β), and Beta-actin (β-actin).

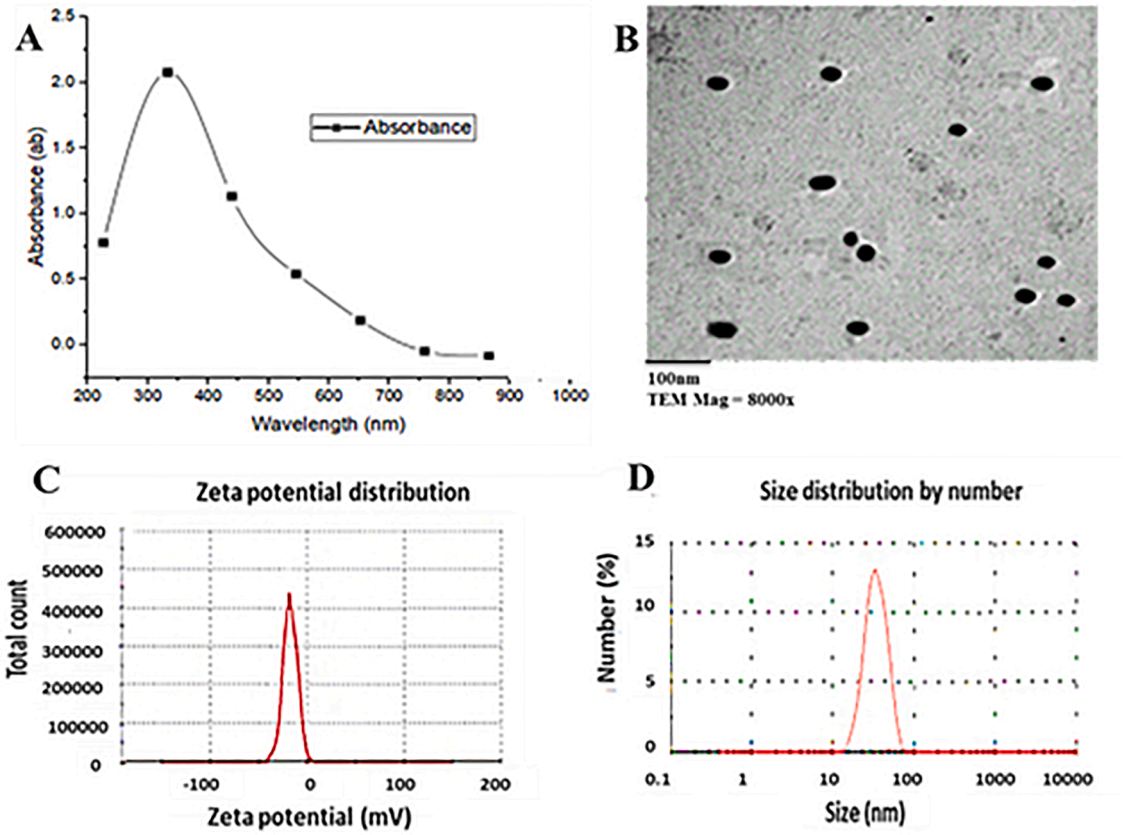

Fig. 1. Characterization of ZnONPs by four devices: UV–VIS spectroscopy analysis showed the maximum peak at 340 nm (**A**). The ZnO-NPs were spherical with an average size of 108 nm (**B**). The size was estimated based on the Brownian motion of the ZnO-NPs in suspension, where the exact size was 89 nm (**C**). The zeta potential analysis was carried out to determine the surface charge of ZnO-NPs, which ensures the stability of synthesized nanoparticles, where the net surface charge was −33 mV (**D**).

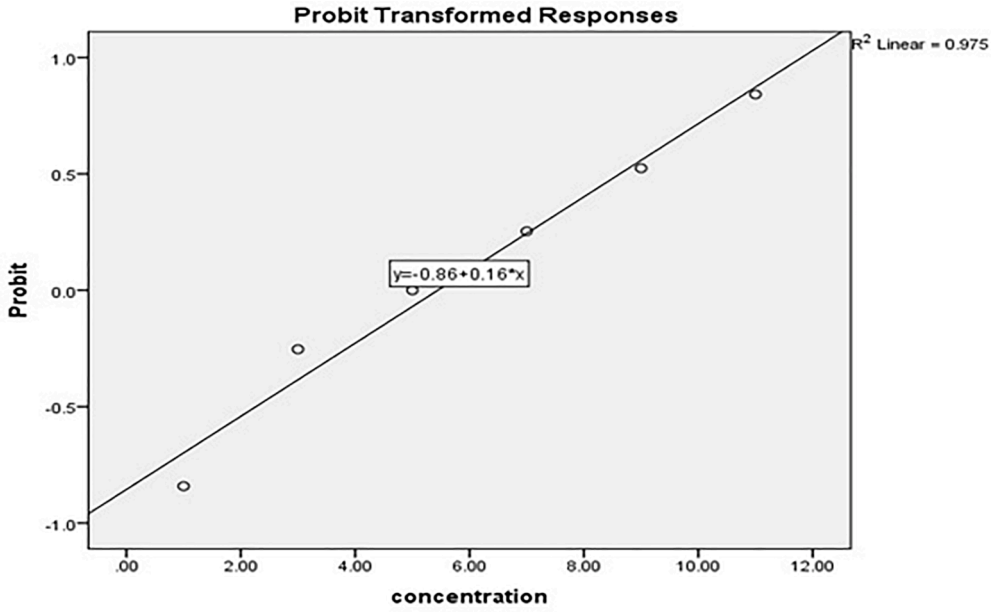

Fig. 2. Probit analysis of 96-h LC_{50} calculation of ZnO-NPs in *O. niloticus*.

fish fed a thymol-enriched diet (THY1 and THY2) showed no significant variation from control values with respect to all markers. Compared to the unexposed control fish, the ZnO group presented significant reductions in serum IgM, IgG, NO, and lysozyme activity, while the MPO activity was elevated. The thymol-supplemented groups with concurrent ZnO-NPs exposure revealed significant improvement in immunity indices in a dose-dependent manner. In the ZnO-THY1 group, the fish showed improvement in serum IgM and MPO activity relative to the ZnO

Table 3
Behavioral changes in *O. niloticus* exposed to different ZnO-NPs concentrations for 96 h.

behavior	experimental groups (znonps; mg / l)							
	Control (0)	1	3	5	7	9	11	14
Air gulping	–	–	–	+	+	++	++++	++++
Respiratory distress	–	–	–	+	+	+++	+++	++++
Sluggish movement	–	–	–	+	+	++	+++	++++
Uncoordinated swimming	–	–	–	–	++	++	+++	++++
Hyperventilation	–	–	–	–	–	++	+++	++++

None -, mild +, moderate ++, strong +++, very strong +++.

Table 4
Water quality measurements in the aquaria in response to ZnO-NPs exposure (⅕ LC_{50}; 1.14 mg/L) and supplementation of thymol (1 and 2 g/kg diet) for 28 days.

	Control	THY1	THY2	ZnO	ZnO - THY1	ZnO - THY2
Temp. (°C)	27.12 ±0.100[b]	27.13 ±0.024[b]	27.03 ± 0.012[b]	27.08 ± .038[b]	29.46 ±0.228[a]	27.13 ± 0.052[b]
pH	7.013 ± 0.009	7.010 ± 0.006	7.013 ± 0.009	7.003 ± 0.003	7.013 ± 0.009	7.010 ±0.006
DO (mg/L)	6.45 ± 0.164[a]	6.47 ± 0.165[a]	6.63 ± 0.078[a]	4.84 ± 0.270[b]	6.05 ± 0.024[a]	6.06 ± 0.021[a]
NH_3 (mg/L)	0.083 ±0.013[b]	0.080 ± 0.015[b]	0.070 ± 0.015[b]	0.233 ± 0.055[a]	0.120 ± 0.015[b]	0.086 ± 0.012[b]

Values are mean ± SEM for three replicate/group, values are not sharing a common superscript letter (a,b) differ significantly at $p < 0.05$. THY1: thymol 1g/kg diet, THY2: thymol 2g/kg diet, ZnO-NPs: Zinc oxide nanoparticles (1.14 mg/L), ZnO-THY1: ZnO-NPs (1.14 mg/L) + thymol 1g/kg diet, ZnO-THY2: ZnO-NPs (1.14 mg/L) + thymol 2g/kg diet, Temp: temperature, DO: dissolved oxygen, NH_3: ammonia.

group, and only the MPO activity returned to the control range. In contrast, the ZnO-THY2 group presented significant amelioration of the toxic effect of ZnO-NPs on all variables, compared to ZnO-group, and control values were attained for all parameters except for NO, supporting the immune-boosting effect of thymol, especially at the highest dose level.

3.7. Host resistance against A. hydrophila

The challenge with bacterial agents like *A. hydrophila* is an excellent assay that can weigh the reaction of the active immune responses of fish. The control fish showed 5% mortalities, while the two thymol-supplemented groups presented no deaths (Table 6). The post-challenge mortality in the ZnO group reached 15%, whereas this value was reduced to 10% upon thymol co-supplementation (at either 1 or 2 g/kg diet) with simultaneous ZnO-NPs exposure.

The fish exhibited difficulty in breathing, having the manner of speedy respiration, rapid operculum movement, and lack of response to the escape reflex. Furthermore, fish from the ZnO-exposed group presented slight skin darkness with a thick mucus layer. At necropsy examination, the internal organs appeared severely congested, and the

Table 5
Effect of supplementation of thymol (1 and 2 g/kg diet) on hematological variables of *O. niloticus* exposed to ZnO-NPs (⅕ LC_{50}; 1.14 mg/L) for 28 days.

	Control	THY1	THY2	ZnO-NPs	ZnO - THY1	ZnO - THY2
HB (g/dl)	7.66±0.052	7.67±0.088	7.68±0.065	7.69±0.072	7.69±0.072	7.69±0.072
RBCs (10^6/µl)	2.91±0.030	2.91±0.033	2.92±0.017	2.9 ± 0.020	2.91±0.025	2.90±0.028
PCV (%)	24.14±0.072	24.20±0.526	24.09±0.653	24.09±0.653	23.73±0.372	23.74±0.376
WBCs (10/mm3)	6.63±0.057[a]	6.63±0.118[a]	7.11±0.327[a]	4.64±0.074[b]	4.68±0.073[b]	5.16±0.103[b]
Lymphocyte	2.70±0.138[a]	2.82±0.137[a]	2.89±0.357[a]	1.41±0.031[b]	2.06±0.035[ab]	2.61±0.180[a]
Monocyte	0.626±0.014	0.633±0.014	0.636±0.017	0.626±0.008	0.633±0.024	0.626±0.008
Eosinophil	0.010±0.000	0.003±0.003	0.006±0.003	0.006±0.006	0.006±0.003	0.003±0.003
Basophil	0.0010±0.001	0.0007±0.001	0.0007±0.000	0.0013±0.000	0.0003±0.000	0.0010±0.001

Values are mean ± SEM, values are not sharing a common superscript letter (a,b) differ significantly at $p < 0.05$. THY1: thymol 1g/kg diet, THY2: thymol 2g/kg diet, ZnO-NPs: ZnO nanoparticles (1.14 mg/L), ZnO-THY1: ZnO-NPs (1.14 mg/L) + thymol 1g/kg diet, ZnO-THY2: ZnO-NPs (1.14 mg/L) + thymol 2g/kg diet, HB: hemoglobin, RBCs: red blood cells, PCV: packed cell volume, WBCs: white blood cells.

Table 6
Effect of supplementation of thymol (1 and 2 g/kg diet) on protein profile, stress variables and host resistance against *A. hydrophila* in *O. niloticus* exposed to ZnO-NPs (⅕ LC50; 1.14 mg/L) for 28 days.

	Control	THY1	THY2	ZnO-NPs	ZnO -THY1	ZnO -THY2
TP (g/dl)	3.83±0.049[a]	3.91±0.037[a]	3.92±0.040[a]	2.25±0.021[d]	3.12±0.012[c]	3.52±0.017[b]
Albumin (g/dl)	1.47±0.063[b]	1.74±0.020[a]	1.77±0.008[a]	0.90±0.040[d]	1.07±0.080[cd]	1.16±0.043[c]
Globulin (g/dl)	2.36±0.089[a]	2.17±0.057[a]	2.14±0.035[a]	1.35±0.020[c]	2.05±0.068[b]	2.36±0.060[a]
Stress variables						
Cortisol (ng/mL)	5.62±0.078[c]	5.57±0.084[c]	5.57±0.108[c]	9.16±0.146[a]	7.43±0.158[b]	5.75±0.04[c]
Glucose (mg/dl)	82.75±2.685[b]	80.20±0.030[b]	80.33±0.885[b]	101.28±0.609[a]	81.68±0.658[b]	80.76±0.392[b]
Glucagon (ng/mL)	4.33±0.055	4.33±0.046	4.28±0.117	4.30±0.127	4.34±0.052	4.35±0.127
Host resistance						
Dead fish/group	1/20	0/20	0/20	3/20	2/20	2/20
Survival%	95	100	100	85	90	90
Mortality rate (%)	5	0	0	15	10	10

Values are mean ± SEM, values are not sharing a common superscript letter (a,b,c, d) differ significantly at $p < 0.05$. THY1: thymol 1g/kg diet, THY2: thymol 2g/kg diet, ZnO-NPs: Zinc oxide nanoparticles (1.14 mg/L), ZnO-THY1: ZnO-NPs (1.14 mg/L) + thymol 1g/kg diet, ZnO-THY2: ZnO-NPs (1.14 mg/L) + thymol 2g/kg diet, TP: total protein.

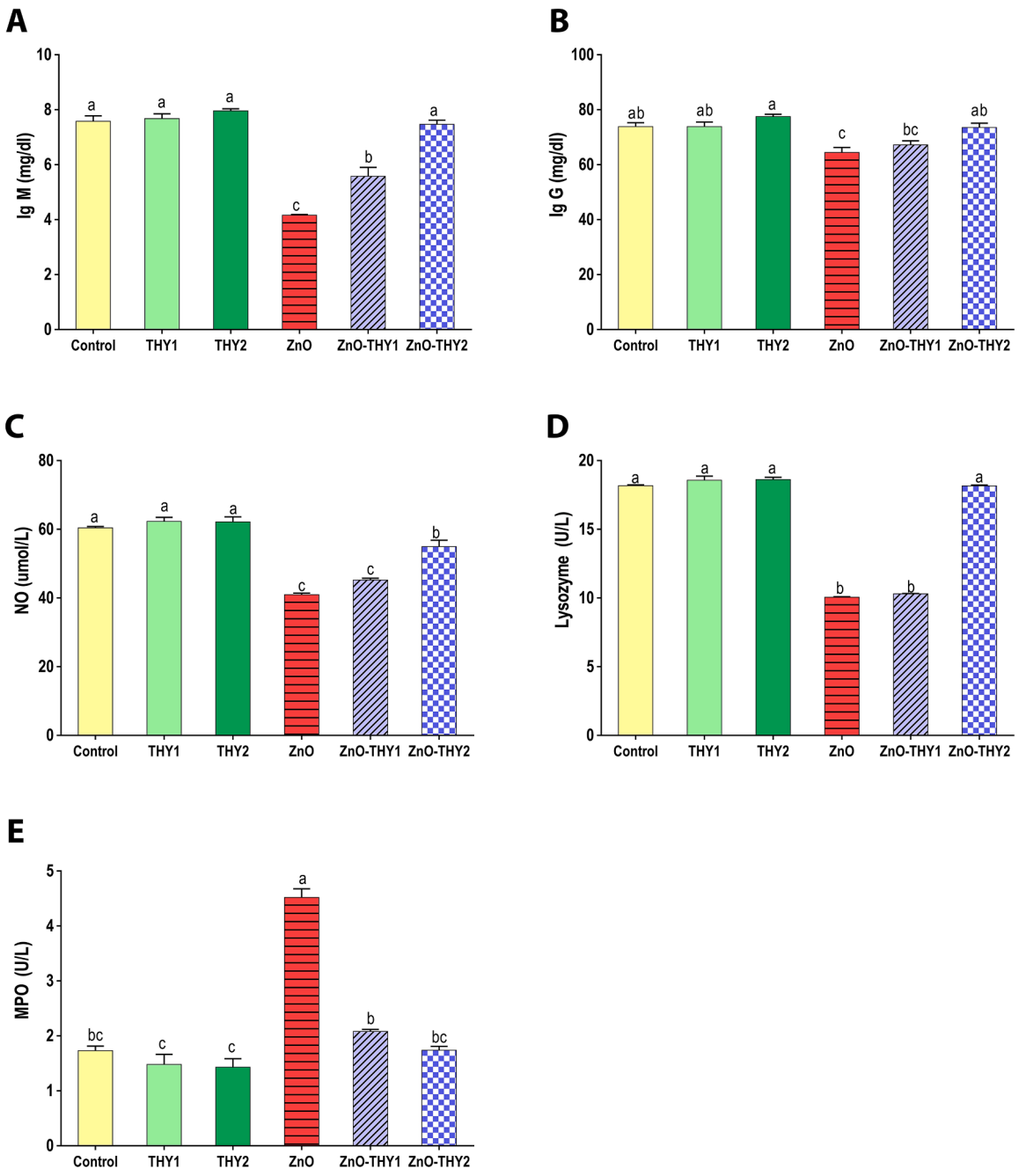

Fig. 3. Effect of supplementation of thymol (1 and 2 g/kg diet) on IgM (A), IgG (B), lysozyme activity (C), NO level (D), and MPO activity (E) in *O. niloticus* exposed to ZnO-NPs (⅕ LC50; 1.14 mg/L) for 28 days. Values are mean ± SEM, bars that are not sharing a common superscript letter (a,b,c) differ significantly at $p < 0.05$.

intoxicated fish's gills revealed excessive slime deposition. These signs were less severe in the combined treatment groups, whereas the control and THY1 and THY2 groups appeared with better health status during the infection.

3.8. Transcriptional profile of enzymatic antioxidants and immune-related genes

As displayed in Fig. 4, the expression pattern of the antioxidant (SOD and CAT) genes and the immune-related genes (TNF-α and IL-1β) in liver tissue showed no significant variation from control values in the two thymol-supplemented groups, except for SOD in the THY2 group, which was higher than control. On the other hand, fish exposed to the ZnO-NPs presented downregulation of the expression of both antioxidant genes and upregulation of the two immune-related genes. Notably, the obtained data indicated a positive impact of a thymol-enriched diet on the gene expression in ZnO-NPs-exposed fish in a manner dependent on the thymol dose. Fish in the ZnO-THY1 group revealed dilution of the toxic effect of ZnO-NPs on SOD and IL-1β gene expression, and the control value was reached only for IL-1β gene expression. Conversely, the ZnO-THY2 group showed improvement in the expression of the four genes relative to the ZnO group, and control levels were restored for all genes except TNF-α.

4. Discussion

Great concerns have been raised from the widespread use of nanoparticles like ZnO-NPs and possible leakage into the various aquatic

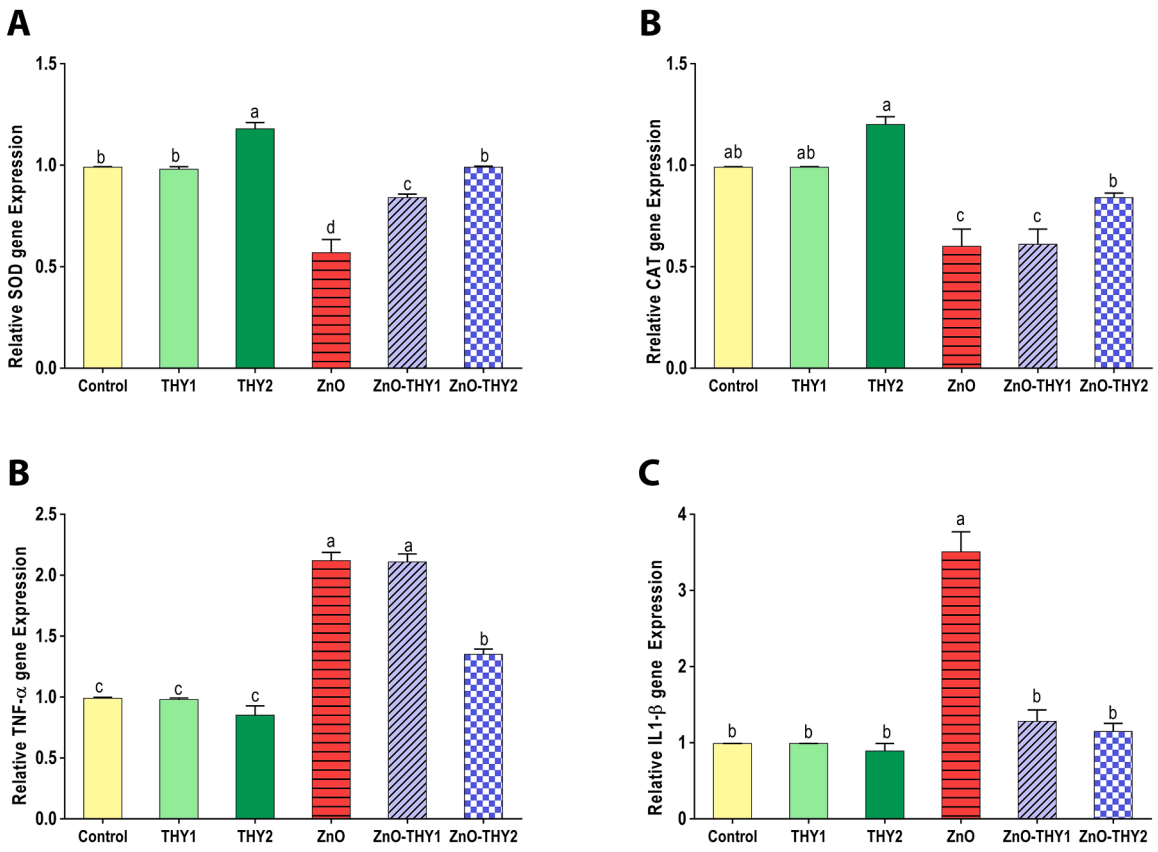

Fig. 4. Effect of exposure to ZnO-NPs (⅕ LC50; 1.14 mg/L) with or without supplementation of thymol (1 and 2 g/kg diet) for 28 days on the expression pattern of SOD (A), CAT (B), and TNF-α (C) and IL1-β (D) in the liver. Values are mean ± SEM, bars that are not sharing a common superscript letter (a,b,c) differ significantly at $p < 0.05$.

systems, with subsequent toxic impacts on aquatic organisms, including fish (Aziz et al., 2022). Critical to preventing the ZnO-NPs-induced deleterious effects in fish is the supplementation of suitable antioxidants of natural origin. Therefore, this study aimed to address the efficacy of thymol-incorporated food (1 or 2 g/kg) to abolish the immunosuppressive impacts resulting from exposure of *O. niloticus* to ZnO-NPs (⅕ LC_{50}, 1.14 mg/L for 28 days).

Our results regarding the characterization of ZnO-NPs demonstrated the exact size of ZnO-NPs to be 89 nm depending upon the Brownian motion of the nanoparticles. The small size of these nanoparticles enables them to cross cell membranes easily, affecting different cellular components (Ma et al., 2013).

The LC_{50} in fish is a beneficial approach to determine the tolerance and safety levels for various aquatic pollutants (Prenter et al., 2004). The data herein demonstrated the estimated 96-h LC_{50} in *O. niloticus* to be 5.71 mg/L with confidence levels of 4.05 and 7.33 mg/L. Our findings agree with Alkaladi et al. (2020), who reported that the 96-h LC_{50} value of ZnO-NPs in Nile tilapia was 4.1 μg/L (Alkaladi et al., 2020). In contrast, the earlier study by Suganthi et al. (2015) reported its value ranging between 100 and 110 ppm (Palani et al., 2015). Moreover, its value was 4.897 mg/L in *C. carpio* (Subashkumar and Selvanayagam, 2014), 25.5 mg/L in *Oncorhynchus mykiss* (Mahmoud et al., 2020), 3 μg/mL in the zebrafish (Giordo et al., 2020), and 31.15 in *Labeo rohita* (Aziz, 2020). The LC_{50} value in fish may vary depending on the species, size, age, sex, genetic makeup, and the differences in environmental circumstances, including water temperature and physicochemical properties (Abdel-Tawwab and Hamed, 2020; Gharaei et al., 2020; Paknejad et al., 2012). Accordingly, Miao et al. (2010) mentioned that the low water pH elevates ZnO-NP toxicity (Miao et al., 2010). Moreover, Ma et al. (2013) demonstrated that the intrinsic physicochemical properties of ZnO-NPs, such as particle shape and size, surface area, and chemical composition, may also alter their toxicity, with the toxicity increasing by decreasing the nanoparticle size (Ma et al., 2013).

Our findings showed that the group of fish exposed to ZnO-NPs suffered a reduction of DO level in aquaria water and an elevation of ammonia level. This change in water quality might contribute to the observed immunotoxic effects of ZnO-NPs in fish. Notably, the groups of fish fed on thymol-enriched food concomitantly with ZnO-NPs exposure revealed improvement of the water quality parameters, which might augment the immunoprotective effect of thymol.

The hematological profile can give precious information about aquatic organisms' internal state of health. Our data indicated that exposure to ZnO-NPs significantly declined the total WBC and lymphocytic counts, which could result from disturbed innate immunity and tissue damage (Zhang et al., 2020a). The lymphopenia may occur in response to the stress accompanied by elevated blood cortisol and consequent lymphocyte apoptosis induction or shift of the circulating blood to the inflamed tissues (Chen et al., 2015). In addition, it could result from reduced production of new cells, death of mature cells, and elimination of lymphocyte precursors (Steele, 2002). These toxic effects on leukogram variables might explain the alterations in protein profile, IgM, IgG, and fish resistance to *A. hydrophila*. In agreement with our results, Alkaladi et al. (2015) observed that exposure of *O. niloticus* to 1 and 2 mg/L ZnO-NPs for 7 and 15 days induced lymphopenia and monocytopenia (Alkaladi et al., 2015). Suganthi et al. (2015) reported that exposure of *O. mossambicus* to ZnO-NPs significantly decreased the WBCs count (Palani et al., 2015).

Interestingly, co-administration of thymol concurrently with ZnO-NPs restored the control value for the lymphocytic count at the high dose level of thymol, which validates the immunostimulatory effects and anti-stress properties of thymol. This may be due to the activation of the defensive mechanisms with subsequent lymphocytic proliferation due to

the antioxidant activity of thymol. Similarly, feeding Nile tilapia on diets supplemented with 1% thyme oil significantly increased the lymphocytic count (Valladão et al., 2019). Also, Ahmadifar et al. (2014) have shown that feeding great sturgeon on a diet supplemented with thymol–carvacrol enhanced lymphocyte proliferation (Ahmadifar et al., 2014).

The biochemical blood indices have been approved as valuable assessing tools to appraise fish response to various environmental chemicals. Our data concerning the serum protein pattern revealed reductions in total protein, albumin, and globulin serum concentrations in the ZnO-NPs-exposed group. This could be correlated to the oxidative stress-mediated suppression of protein synthesis by the liver, reduced rate of glomerular filtration, or inhibition of immunoglobulin production by lymphocytes. Additionally, the products of oxidative damage may directly interact with cytoplasmic proteins causing their damage (Halliwell, 2007; Small et al., 2012; Soltanian and Fereidouni, 2017). Consistent with our results, previous studies recorded the modulating effect of ZnO-NPs on serum protein profile in *O. niloticus* (Alkaladi et al., 2015; Hamed et al., 2022) and other fish species such as *Cyprinus carpio* (Mahboub et al., 2022). Intriguingly, supplementation of thymol simultaneously with ZnO-NPs exposure improved the serum protein profile, which might reflect increased protein and immunoglobulin synthesis. Moreover, thymol was reported to have the ability to increase the absorption of proteins and globulin in the lower intestine, causing their serum concentrations to increase (Rahimi et al., 2010). Our results also agree with Hoseini and Yousefi (2019), who recorded a significant increase in serum total protein in *Oncorhynchus mykiss* treated with thyme extract (Hoseini and Yousefi, 2019).

Cortisol, the major stress hormone, has long been a reliable indicator of stress in fish (Sadoul and Geffroy, 2019). Our findings indicated increased serum cortisol levels in ZnO-NP-exposed fish, which is harmonious with the previous investigations in *O. niloticus* (Alkaladi et al., 2020; Goda et al., 2023; Hamed et al., 2022). Under stress conditions, the hypothalamic-pituitary inter-renal axis is triggered, elevating the serum cortisol level and other corticosteroids to cope with the disrupted physiology (Banaee et al., 2019; Gagnon et al., 2006).

Of note, fish in the groups supplied with thymol-enriched food with concurrent ZnO-NPs exposure showed recovery of cortisol levels, indicating the ability of thymol to abolish stress. The decreased cortisol level boosts the fish's local immunity and increases their ability to fight off infections (Parra et al., 2015). In line with our results, Hoseini and Yousefi (2019) recorded a significant decrease in serum cortisol levels in thyme extract-exposed rainbow trout (Hoseini and Yousefi, 2019). Also, Zadmajid and Mohammadi (2017) observed that supplementing gibel carp (*Carassius auratus gibelio*) juveniles with thyme essential oil significantly decreased serum cortisol levels (Zadmajid and Mohammadi, 2017).

Blood glucose is another sensitive indicator of various environmental stressors in fish (Uddin et al., 2018). Our data revealed a significant increment of serum glucose in the fish exposed to ZnO-NPs, suggesting enhanced glycogenolysis with decreased glycolysis as a reaction to stress and elevated levels of glucocorticoids (Odhiambo et al., 2020; Uddin et al., 2018). In addition, the synthesis of glucose from amino acids and proteins may play a part in elevating the blood glucose (Almeida et al., 2001). Similarly, prior reports demonstrated elevated blood glucose in ZnO-NP-exposed *O. niloticus* (Goda et al., 2023; Hamed et al., 2022) and *Cyprinus carpio L.* (Lee et al., 2014; Mahboub et al., 2022). Supplying the ZnO-NPs-exposed fish with thymol alleviated the toxic impact on serum glucose. A similar anti-hyperglycemic effect of thymol was recorded in *Oncorhynchus mykiss* treated with oxytetracycline (Hoseini and Yousefi, 2019) and mice and rats with induced type 2 diabetes (Agarwal et al., 2022; Saravanan and Pari, 2015). The antistress effect exhibited by thymol may be mediated by enhancing the antioxidant activity and the anti-inflammatory and anti-apoptotic properties (Alagawany et al., 2021).

In fish, the innate immune response plays a major role in counteracting the invading pathogens because the acquired immune response is less effective than that of mammals (Kordon et al., 2019). Our data revealed suppression of innate immunity in the fish group exposed to ZnO-NPs, evidenced by reductions of IgG, IgM, and NO serum levels and the lysozyme and MPO activities. IgM, the predominant immunoglobulin molecule in teleost fish, is involved in adaptive and innate immune responses. It can be expressed on the B-cell surface or discharged as an antibody (Flajnik and Kasahara, 2010). It contributes to complement activation, which lyses and opsonizes invading microorganisms (Boshra et al., 2004). The decline of immunoglobulins level inhibits the function and number of B lymphocytes, whereas the reduced NO level indicates the suppression of the phagocytosis (Farag et al., 2021a). The lysozyme is critical for the lysis of bacterial cells and turning on phagocytosis and complement activation. The inhibited activity might result from inhibited function and a reduced number of lysozyme-producing WBCs, such as macrophages and neutrophils (Saurabh and Sahoo, 2008).

The MPO is a reliable marker of neutrophils. When neutrophils are recruited to the sites of inflammation, they recognize and phagocytose the pathogen. MPO plays a crucial role in synthesizing the alkaline milieu for counteracting pathogens. Additionally, MPO is crucial in neutrophil extracellular traps (Arnhold, 2020). Congruent with our observations, *O. niloticus* exposed to ZnO-NPs for six weeks showed a marked decrease in serum IgG (Goda et al., 2023), and mice exposed to ZnO-NPs for 14 days suffered a decline in serum IgG, and IgM (Kargin, 2021). Likewise, exposure of common carp to ZnO-NPs for 30 days decreased the lysozyme activity (Rashidian et al., 2022).

The challenge-induced mortality test is advantageous in measuring the function of the active immune response (Rehberger et al., 2017). Herein, we have recorded elevated mortalities in fish exposed to ZnO-NPs after challenge with *A. hydrophila*, compared to control fish, which reflects compromising of the active components of the immune system by the ZnO-NPs. The observed suppression of the fish immune response by ZnO-NPs could be a sequela of the oxidative toxic insult on immune components with subsequent leukopenia and lymphopenia. Additionally, it might disrupt the production of inflammatory cytokines and enhance apoptotic activity in the immune cells and lymphoid tissues (Farag et al., 2021a). In addition, ZnO-NPs may induce immunotoxic effects by elevating the level of neutrophil extracellular traps (NETs) composed of myeloperoxidase, citrullinated histone 3, and neutrophil elastase. The increase in NETs may depend on ROS and nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase and partial processes of the glycolysis (Hong et al., 2022).

Notably, co-supplementation of thymol to the ZnO-NPs exposed fish improved the immune response variables and augmented the resistance to *A. hydrophila,* as evidenced by decreased mortalities, demonstrating the immunostimulant potential of thymol. This correlated well with the recorded improvement in the transcription pattern of SOD, CAT, and immune-related genes such as TNF-α and IL1-β indicating the antioxidant, free radical scavenging, and anti-inflammatory activities of thymol could be accepted as possible mechanisms in enhancing the immune response of fish.

The immunoprotective effect of thymol could result from the antioxidant activity of thymol (Alagawany et al., 2021) with activation of the innate immune components, including immunoglobulins, lysozyme, and complement molecules (Ibrahim et al., 2022) and increased lymphocyte proliferation and phagocytic rates (Khalil et al., 2020). In accordance with our findings, the immunostimulant influence of thymol was demonstrated in the prior investigations on different species of fish via increasing lysozyme activity and immunoglobulin levels (Amer et al., 2018; Hoseini and Yousefi, 2019; Ibrahim et al., 2022; Kong et al., 2021; Wassif and Mohammed, 2022). Li et al. (2022) documented that supplementation of *Channa argus* with thymol markedly ameliorated the deltamethrin-induced immunotoxicity by elevating the serum levels of humoral immune markers (IgM and lysozyme, C3 and C4) (Li et al., 2022).

In line with our findings, the antibacterial effect of thymol was previously demonstrated in catfish challenged with *A. hydrophila* (Dong et al., 2020; Zheng et al., 2009) and snakehead fish (*Channa argus*) challenged with *Aeromonas veronii* (Kong et al., 2021). Thymol might protect the fish from *A. hydrophila* via inhibition of aerolysin production and synthesis of biofilm by downregulation of expression of aerA, ahyI, and ahyR genes and suppressing the quorum sensing system (Dong et al., 2020). Also, thymol supplementation increased survival and decreased deaths in the *A. hydrophila-A. sobria*-challenged Nile tilapia (Wassif and Mohammed, 2022) and by downregulating the bacterial virulence genes expression such as hemolysin and aerolysin (Ibrahim et al., 2022).

Alterations of the gene expression patterns can be considered the earliest bioindicators of stress (Dondero et al., 2006). Our findings revealed downregulation of the expression of the enzymatic antioxidant genes SOD and CAT in liver tissues of ZnO-NPs-exposed fish, which may contribute to the recorded suppression of innate immunity components and fish resistance to bacterial challenge. The immunocytes are highly vulnerable to disruption in the antioxidant status because they perform their immune function by increasing the ROS (Farag et al., 2021b). In addition, the immunocyte plasma membranes are more susceptible to peroxidation due to their high content of polyunsaturated fatty (Ince, 2020). Both SOD and CAT are critical defensive enzymes against ROS and oxidative damage. The suppression of their activities and the downregulation of their mRNA expression could indicate an oxidant elimination (Moreno et al., 2005). Thus, oxidative stress represents a possible mechanism underlying the deleterious effects of ZnO-NPs in fish. Consistent with our data, prior studies demonstrated that *niloticus* exposed to ZnO-NPs showed reduced SOD and CAT activities and mRNA expression in muscles and the brain (Abdelazim et al., 2018; Saddick et al., 2017). Similar findings were observed by Mahboub et al. (2022) in the liver of *Cyprinus carpio* treated with to ZnO-NPs (Mahboub et al., 2022).

Notably, SOD and CAT gene expressions were improved when ZnO-NPs-exposed fish were co-supplemented with thymol, reflecting the potent antioxidant activity of thymol. Such an antioxidant activity of thymol was supported by literature findings that elucidated that dietary inclusion of thymol to Nile tilapia upregulated the expression patterns of SOD and CAT genes resulting in enhanced activity of both antioxidant enzymes (Abd El-Naby et al., 2020; Amer et al., 2018; Magouz et al., 2022). The antioxidant activity of thymol could be mediated by activating the Nrf2 pathway (Mostafa et al., 2021; Yao et al., 2018). Li et al. (2022) demonstrated the protective antioxidant effect of thymol in deltamethrin-exposed *Channa argus* via upregulation of the expression patterns of Nrf2 and Cu/Zn SOD in the spleen and liver (Li et al., 2022).

Moderate expression of the proinflammatory cytokine genes is crucial for conserving the natural and acquired immune balances and increasing fish's resistance against stressors and infection. Overexpression of these genes after exposure to environmental toxicants is correlated with the immunotoxic effects provoked by these chemicals (Abou-Zeid et al., 2021). Our data revealed upregulation of the TNF-α and IL1-β genes in liver tissue of ZnO-NPs-exposed Nile tilapia, parallel to suppression of the innate immunity elements. Generally, oxidative stress is entangled with the activation of immune-related cytokines. The overexpression of TNF-α and IL-1β may be linked to the activation of the ROS-NF-κB-NLRP3 signaling pathway (Liang et al., 2017). NF-κB is a critical transcription factor that directly regulates the transcription levels of the immune-related genes TNF-α and IL-1β (Wang et al., 2014). Consistent with our data, rats intoxicated with ZnO-NPs presented overexpression of the immune-related genes such as TNF-α and IL-1β in the brain (Amer and Karam, 2018; Attia et al., 2018). A similar effect was observed in the ileal mucosa of weaned piglets exposed to ZnO-NPs (Wang et al., 2018).

Notably, fish co-supplemented with thymol alleviated the ZnO-NPs-induced gene upregulation. This could be due to the inhibition of the NF-κB and MAPK signaling pathways (Liang et al., 2014; Yao et al., 2018). In line with our findings, Zhang et al. (2020b) have shown that dietary supplementation of koi carp with oregano essential oil significantly downregulated TNF-α expression in the intestine (Zhang et al., 2020b). Li et al. (2022) elucidated that thymol attenuated deltamethrin-induced immunotoxicity by decreasing the expression pattern of NF-κB, TNF-α, p65, and IL-1β in the *Channa argus* liver and spleen (Li et al., 2022).

5. Conclusion

Given the entirety of these data, it can be concluded that exposure of Nile tilapia to ZnO-NPs is associated with suppression of the innate immune response and ability of fish to resist the *A. hydrophila*, probably through affection of the antioxidant status and the expression of immune-related genes. Co-supplementation of thymol boosted the immune system in the ZnO-NPs-exposed fish, most likely by enhancing the antioxidant status and the transcription pattern of immune-related genes. Our findings implicate thymol as a valuable immunoprotective and antibacterial against ZnO-NPs in *O. niloticus*, although future studies should focus on the mechanisms underlying this immunostimulant effect to fill the gaps in our knowledge.

Ethical statement

All investigation protocols were performed under the approval of the Institutional Animal Care and Use Committee, Faculty of Veterinary Medicine, University of Sadat City (approval number: VUSC-024-1-21).

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

Acknowledgement

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