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Dietary supplementation of quercetin nanoparticles enhances the growth performance hematological and immunological responses and resistance to *Aeromonas hydrophila* infection in Nile tilapia (*Oreochromis niloticus*) exposed to silver nanoparticles toxicity

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ABSTRACT

The wide incorporation of silver nanoparticles (AgNPs) in aquaculture could result in releasing of these particles into the environment with environmental impacts and health hazards. Thus, this study aimed to investigate the impacts of the dietary supplementation of quercetin nanoparticles (QNPs) on Nile tilapia (*Oreochromis niloticus*) growth, antioxidants, and immunity besides its susceptibility to the infection with *Aeromonas hydrophila* with or without the aqueous exposure to AgNPs. First, the 96-h LC50 value for AgNPs in *O. niloticus* wa estimated to be 19.81 mg/L (the 1/10th of the 96 h LC50 of AgNPs = 1.98 mg/L). Then a total of 240 Nile tilapias (40.00 ± 0.45 g) were randomly distributed into 4 groups (each group contains 60 fish in 3 replicates of 20 fish). The 1st group (control) was fed on a basal diet without QNPs and AgNPs. The 2nd group (QNPs) received the basal diet supplemented with QNPs (400 mg/kg), the 3rd group (AgNPs) was exposed to 1/10th 96-h LC_{50} of AgNPs (1.98 mg/L) and fed on QNPs free diet, while the 4th group (AgNPs+ QNPs) was exposed to AgNPs (1.98 mg/L) and co-supplemented with QNPs (400 mg/kg). Besides, AgNPs resulted in marked elevation in the serum myeloperoxidase (MPO) 8-hydroxy-2-deoxyguanosine (8-OHdG) contents, and declines in lysozyme activity and the levels of nitric oxide (NO) and immunoglobulins (IgM and IgG) in the exposed fish (AgNPs group). These effects were accompanied with significant downregulation in the relative mRNA expressions of *SOD, CAT,* and *GSH* and upregulation in the expressions of *INF-γ, TNF-α,* and *Il-1β*. Feeding QNPs-enriched diet, alone or combined with AgNPs exposure, conversed most of these effects and restored some to the control levels. AgNPs exposure increased mortalities, lowered survival rates and altered Nile tilapia's resistance to *A. hydrophila* infection in the AgNPs exposed fish, whereas co-supplementation with QNPs enhanced their resistance with less mortalities. In summary, the reported immunomodulation, and protective properties of QNPs dietary supplementation, strengthen its applicability as an effective and promising feed supplement to alleviate the AgNPs associated toxicity in fish.

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1. Introduction

Aquaculture is a promising animal-source food-producing sector that covers the gap of food shortage due to overpopulation, especially in countries with nutrition insecurity (Barange, 2018). However, the intensification of fish farming is correlated with the emergence of environmental impacts and health hazards such as the overuse of antimicrobials and the subsequent antimicrobial resistance strains (Harikrishnan and Balasundaram, 2005; Harikrishnan et al., 2009; Herrero et al., 2015; Van Boeckel et al., 2015; Schar et al., 2018). The emergence of nanotechnology overcomes the latter problem through the usage of nanoparticles with antimicrobial and anticancer activities such as nanosilver (AgNPs) (Marin et al., 2015; Simbine et al., 2019). The AgNPs are widely used and considered one of the fast growing nanoproducts because of their low manufacturing cost, high thermal and electrical conductivities, and excellent catalytic properties (Karimi-Maleh et al., 2020; Harikrishnan et al., 2022).

In aquaculture, AgNPs are effective in controlling many microorganisms such as *Vibrio harveyi, Aeromonas hydrophila, Pseudomonas aeruginosa, Staphylococcus aureus, Pseudomonas aeruginosa,* etc. (Vaseeharan et al., 2010; Shaalan et al., 2017; Shaalan et al., 2018; Harikrishnan et al., 2022), particularly with increasing the problem of antibiotic-resistance (Shaalan et al., 2016). Nonetheless, because the AgNPs are metallic nanoparticles, they may leak into bodies of aquatic animals during their production or disposal causing pollution and ecological hazards impacts on survivability, growth, physiological response and stress-associated responses in aquatic animals (Afifi et al., 2016; Kumar et al., 2018; Kumar et al., 2019). In this context, the low exposure of AgNPs (< 10 μg /L) to Nile tilapia enhanced growth, feed utilization, and immunity of fish, however, increasing the concentration (>10 μg / L) decline growth performance, and antioxidant status and induced histopathological changes (Mabrouk et al., 2021). Likewise, the short-term exposure of AgNPs (100 μg/L for 1 h) to Rainbow Trout (*Oncorhynchus mykiss*) lowered the mortalities and clinical manifestations against *A. salmonicida* (Shaalan et al., 2018).

The toxic effects of AgNPs is closely associated to their leakage into aquatic animals' bodies via endocytosis or diffusion, causing mitochondrial dysfunctions and the production of reactive oxygen species (ROS) (Li et al., 2013). The excessive release of ROS disrupts the intracellular metabolism and causes an imbalance of the antioxidant defense system leading to distribution of nucleic acids and protein nature and inhibition of cell proliferation (Afifi et al., 2016). Moreover, the toxic effect of AgNPs is linked with the high affinity of sliver (ionic or nano-form), released following the surface oxidation, to interact with macromolecules containing sulphur such as proteins (Mabrouk et al., 2021). Furthermore, AgNPs can alter fish performance and cause immune suppression, inflammation, metabolic disorders, biochemical disturbances, and depression of growth based on the exposure durations, size and concentrations of the tested AgNPs (Elabd et al., 2019; Hedayati et al., 2019). Therefore, it is crucial to overcome AgNPs associated toxicity.

Overcoming the AgNPs-linked toxicity can be achieved through the application of different antioxidant products such as using natural alternatives to boost fish health and resistance. Quercetin is a potent polyphenolic flavonoid with excellent antioxidant properties. It presents in a variety of vegetables and fruits, and protects tissue from oxidative stress damage (Ibrahim et al., 2021). Additionally, it is effective in treating various allergic, metabolic, cardiovascular and inflammatory diseases because of its antiviral, antibacterial, antidiabetic, anticancer, and antiatherosclerosis properties (Dabeek and Marra, 2019; Ibrahim et al., 2021; Ghafarifarsani et al., 2022). Quercetin can also possess immunostimulant properties, prevent lipid peroxidation, and augment mitochondrial biogenesis (Aguirre et al., 2011). In Nile tilapia, dietary supplementation of quercetin improves growth performance, antioxidant, health, and immunity statuses, and lowers lipids in serum and the whole body (Ghafarifarsani et al., 2022). Besides, dietary supplementation of quercetin can ameliorate the deleterious effect of heavy metal toxicity in tilapia (Ibrahim et al., 2021) and enhance humoral and mucosal immune responses (Ghafarifarsani et al., 2022). Moreover, it induces effective antibacterial interaction against many microorganisms such as *Pseudomonas aeruginosa* (Ouyang et al., 2016) and *A. hydrophila* in Nile tilapia (Ibrahim et al., 2021), and common carp (*Cyprinus carpio*) (Jasim et al., 2022). Despite these effective properties of quercetin, its use is restricted because of poor bioavailability, instability, aqueous solubility, and permeabilities. Thus the nano form of quercetin (QNPs) has been developed with effective characteristics and higher bioavailability (Ibrahim et al., 2021). Consequently, this study aimed to evaluate the impact of QNPs dietary supplementation on growth perormance, hematological,and biochmeical indices and the relative mRNA level of some immune and antioxidant-related genes along with its effect on the immune responses and susceptibility of Nile tilapia to *A. hydrophila* infection following exposure to AgNPs.

2. Materials and methods

2.1. The preparation and characterization of AgNPs and QNPs

The synthesis and characterization of AgNPs and QNPs is previously described in Farag et al. (2023). The UV–VIS spectroscopy results of characterization of Ag-NPs show the maximum peak at 340 nm. TEM analysis reveals a spherical shape with an average size of 108 nm. The net surface charge is − 33 mV based on the data from the zeta potential analysis. The hydrodynamic size is 89 nm based on the DLS analysis (Fig. 1 A). For QNPs, they showed spherical shape which absorbed UV at 310 nm and had an average size in the range of 45–65 nm based on TEM analysis with a −23 mV charge based on the zeta potential analysis and exact size of 77 nm based on the DLS analysis (Fig. 1B).

2.2. The acute toxicity study [Determination of the median lethal concentration of AgNPs (96-h LC50)]

For estimating the AgNPs 96 h LC50, a total of 80 acclimatized Nile tilapias (*O. niloticus)* (average weight = 40 ± 0.13 g) were divided randomly to 8 equal groups (n = 10 fishes / group). The Nile tilapias were kept in a 30 L capacity aquarium containing 20 L of AgNPs-treated water while the control group was reared in water free of AgNPs. Suspensions of AgNPs with different concentrations (0, 5, 10, 20, 30, 40, 50, and 60 mg/ L) were prepared and dispersed using a bath sonicator for 1 h immediately before it be used without adding any stabilizers according to the protocols reported by Afifi et al. (2016) and Hamed and Abdel-Tawwab (2021). Briefly, every day, the Ag nano-powder was directly dispersed in deionized water for 30 min by the use of ultrasonic vibration (40 kHz) to prevent the aggregation of AgNPs. Besides, a daily determination of Ag concentrations using inductively coupled plasma mass spectrometry (ICP-MS) was done at 0, 12, and 24 h of exposure to authenticate fixed exposure concentrations. Every 24 h, the water treated with AgNPs was changed to ensure a AgNPs constant concentration.Additionally, during the experiment, fish received no food to minimize the feces production and AgNPs absorption in food. The Nile tilapias were exposed to each concentration for 96 h. The mortality, external morphology and swimming behaviors of all groups and the control were daily recorded. Finney's Probit method was used to calculate the 96-h LC_{50} value of AgNPs with 95% confidence limits (Finney, 1971).

2.3. The antidotal study

2.3.1. Fish and formulation of tested diets

A total of 240 healthy *O. niloticus* fish (average weight = 40.00 ± 0.45 g) were used in this study. Fish were purchased from the hatchery (El-Abbassa Fish Hatchery, Al-Sharkia, Egypt). Fish were acclimatized for 2 weeks in dechlorinated tap water -filled glass aquaria.

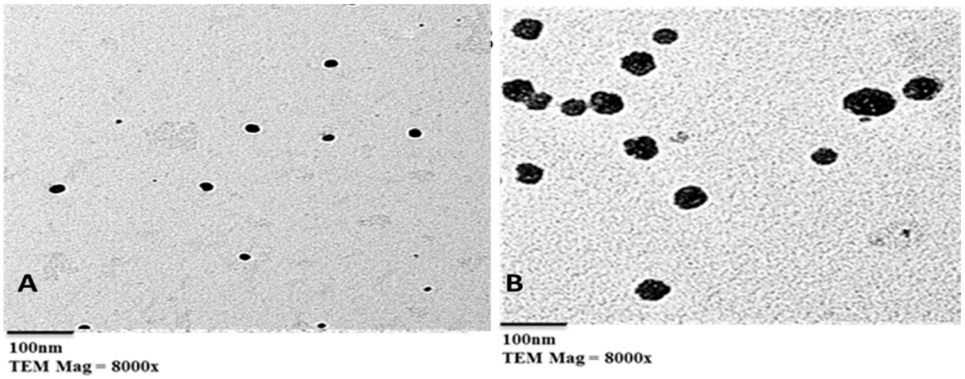

Fig. 1. shows the spherical shape of AgNPs (A) and QNPs (B) under TEM (Farag et al., 2023).

They were kept on basal diet three times daily (at 7:00 a.m., 11:00 a.m., and 4:00 p.m.) at a feeding rate of 5% of the fish biomass, without the addition of the QNPs or AgNPs. Parameters of water quality were adjusted to the recommendations of the American Public Health Association (APHA, 1998). Moreover, all aquaria were subjected to the same rearing conditions (temperature: 25 ± 1.02 °C, pH: 6.9 ± 0.1, ammonia: 0.046 ± 0.005 mg/L and dissolved oxygen: 6.9 ± 0.5 mg/L) with a controlled photoperiod of 10 h light: 14 h dark.

The QNPs was added to the basal diet ingredients (Table 1) at the rate of 400 mg/ kg diet (then be mixed mechanically, pelletized, and thoroughly dry in air at 25 °C for 24 h, then kept at 4 °C until used for fish feeding.

After the acclimatization period, the *O. niloticus* fishes were randomly divided into 4 equal groups (n = 60 fish/group, these 60 fish were subdivided in to 3 replicates and each replicate contains 20 fish). Fish of each replicate were kept in 100 × 50 × 40 cm-sized glass aquaria contaning 160 L of dechlorinated tap water. The 1st group (control) was fed on a basal diet only. The 2nd group was fed on a basal diet supplemented with QNPs (400 mg/kg diet) (QNPs supplemented group), the 3rd group was fed on a basal diet free of QNPs and exposed to 1/10th LC_{50} of AgNPs (1.98 mg/L), while the 4th group (AgNPs / QNPs co-administered group) were fed on a basal diet plus QNPs and exposed to AgNPs (at the previously mentioned concentrations). Feed was introduced to fish 3 times a day (at 7:00 a.m., 11:00 a.m., and 4:00 pm) for 60 days (the period of the experiment) and feed intake was adjusted every two weeks depending on the fish weight gain and total fish biomass.

Table 1
Formulation and calculated composition analysis of the basal diet fed to experimental *O. niloticus* fish.

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*	10
Premix-Vit**	10
Corn oil	30
Total	1000
Calculated composition	
Crude protein%	320.5
Lipid %	45.50
Crude fiber %	42.45
Ash %	73.01
Nitrogen-free extract***	518.54

* 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.

** 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.

*** Nitrogen free extract = 100 − (crude protein + Crude lipids + ash + crude fiber).

2.3.2. Assessment of growth performance and survival of Nile tilapia

At the experimental end (60 days), fish were individually weighed, and the growth parameters were calculated based on formulas formerly published (El-Kassas et al., 2020; Farag et al., 2021).

Weight gain (BWG) (g) = final body weight (FW) – initial body weight (IW); feed intake (FI) (g) = Splitting the total amount of the feed offered to fish in the experiment by the total number of fish (feed/fish); feed conversion ratio (FCR) = FI / WG; specific growth rate (SGR) (%/day) = 100 [(LnFBW – LnIBW) / T] (T is feeding period); survival rates (SR) (%) = (the final number of fish / initial number of fish) × 100.

2.3.3. Collection of blood and tissue samples

At the experimental end (60 days), two sets of blood samples (2 mL for each) were obtained by vein puncture from the caudal vein using sterile syringes. The first set was collected without anticoagulant and centrifuged for 20 min at 1075 g for serum separation. Sera were kept at − 20 °C till the assessing of physiological parameters. The other blood samples were immediately collected on EDTA for hematological assay. Liver tissue sections were sampled from sacrificed fish, following dissection, and quickly frozen in liquid nitrogen and stored at − 80 °C til the total RNA extraction.

2.3.4. Biochemical and hematological analysis

The complete blood picture as well as total and differential leukocytes count (WBCs) were estimated by the automatic cell counter (Hospitex Hema screen 18, Italy) according to Dacie and Lewis (1995).

The total protein and albumin were determined according to Doumas et al. (1981) using colorimetric kits (BIOMED Diagnostic, Egy-Chem kits, Egypt). Besides, glucose was estimated as described in Teixeira (et al. (2018).

2.3.5. DNA damage and immune response assays

8-hydroxy-2-deoxyguanosine (8-OHdG) was estimated in serum by the Fish ELISA kit (Cat No. MBS032638; MyBioSource Co. San Diego, USA) according to the manufacturer protocol.

The lysozyme activity was spectrophotometry analyzed in serum according to the method of (El-Kassas et al., 2022). The myeloperoxidase (MPO) and nitric oxide (NO) contents were determined calorimetrically in the serum using commercial kits from Biodiagnostic Co.

Egypt following the manufacturer's protocols. Commercial fish ELISA kits for measuring the levels of Immunoglobulin M and G (IgM: Cat No. MBS035038 and IgG: MBS043814, respectively) were used (MyBioSource, San Diego, USA).

2.3.6. qRT-PCR analysis of genes of stress and immunity

Extraction of total RNA from the liver tissues, the reverse transcription of cDNA and the qPCR analysis were performed as described in our previous study Farag et al. (2023). RNA was extracted from the hepatic tissue, and its integrity and concentration were checked by 1% agarose and spectrophotometry. First-strand cDNA was synthesized using a QuantiTect RT kit (Qiagen, Germany). Real-time PCR was performed using a QuantiTect SYBR Green PCR kit (Qiagen, Germany) and a Rotor-Gene Q apparatus. The thermocycler condition was 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. The forward and reverse sequences of primers are tabulated in Table 2. The relative expression patterns of mRNA expression of the studied genes were calculated by the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.3.7. Challenge with Aeromonas hydrophilia

By feeding trial end, the impact of dietary supplementation of QNPs on the resistance ability of Nile tilapia, following AgNPs exposure, to *A. hydrophila* was investigated. Twenty fish /group were challenged with to *A hydrophila*. They were intraperitoneally injected with 0.1 mL cell suspension (with 1.5×10^7 cells/mL) and observed daily for 2 weeks with recording of clinical signs and mortality. *A. hydrophila* was obtained from Agricultural microbiology department, faculty of Agriculture, Zagazig University, Egypt.

2.3.8. Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze the data using SPSS (version 22.0, SPSS Inc., USA). The means of studied groups were compared by Tukey's multiple comparisons post hoc test and the statistical significances were approved at $p < 0.05$. The analyzed data were presented as means ± SE (standard error).

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

Genes	Sequence (5′−3′)	Annealing temperature (oC)	References
SOD	F-GACGTGACAACACAGGTTGC R-TACAGCCACCGTAACAGCAG	60	(Ibrahim et al., 2021)
CAT	F-TCAGCACAGAAGACACAGACA R-GACCATTCCTCCACTCCAGAT	60	(Ibrahim et al., 2021)
GSH-Px	F-CCAAGAGAACTGCAAGAACGA R-CAGGACACGTCATTCCTACAC	60	(Ibrahim et al., 2021)
IL-1β	F-CAAGGATGACGACAAGCCAACC R-AGCGGACAGACATGAGAGTGC	60	(Abdo et al., 2022)
TNF-α	F-GGAAGCAGCTCCACTCTGATGA R-CACAGCGTGTCTCCTTCGTTCA	60	(Abdo et al., 2022)
IFN-γ	F: AAGAATCGCAGCTCTGCACCAT R: GTGTCGTATTGCTGTGGCTTCC	60	(El-Kassas et al., 2022)
β-actin	F-CAGCAAGCAGGAGTACGATGAG R-TGTGTGGTGTGTGGTTGTTTTG	60	(Abdo et al., 2022)

SOD= superoxide dismutase, CAT= catalase, GHS-PX= glutathione peroxidase, IL-1β=interleukin1-beta, TNF-α = tumor necrosis factor α, β-actin= Beta-actin, IFN-γ = interferons

3. Results

3.1. LC50 value of AgNPs and behavioral responses in Nile tilapia

The probit analysis results showed that the 96-h LC50 value for AgNPs in *O. niloticus* was 19.81 mg/L; with lower and upper confidence limits of 9.16 and 28.46 mg/L, respectively, as presented in Fig. 2.

Besides, marked behavioural changes of AgNPs-exposed fish were listed in Table 3. The exposure of Nile tilapia to AgNPs induced changes that varied from hyperventilation, respiratory distress, air gulping, uncoordinated swimming, and sluggish movement. The scores and severity of these changes increased with increasing AgNPs concentration. There were no obvious behavioural changes in the control group (0 exposures).

3.2. Growth performance parameters following the separate and concurrent exposure to AgNPs and QNPs

Table 4 illustrates the impact of separate or combined exposures to AgNPs (aqueous exposure) and QNPs (dietary exposure) on Nile tilapia growth performance. The dietary supplementation of QNPs increased the growth of Nile tilapia as confirmed by the higher final body weight (FW) relative to control supplementation. The exposure of Nile tilapia to 1/10th of the 96-h LC50 of the AgNPs was associated with a significant reduction of fish's FW. QNPs supplementation to AgNPs-exposed tilapias succeeded to improve the AgNPs-linked reduction of body weight. The increases in FW were related to the increases in body weight gain (BWG). A Marked increase of BWG was reported in QNPs supplemented group. Additionally, QNPs supplementation to AgNPs-exposed fishes improved the reduction of BWG caused by AgNPs exposure. SGR (%) was also, markedly improved with the separate QNPs supplementation and was retained at a normal level when QNPs were supplemented with exposure to AgNPs.

Feed intake (FI) was also, influenced by QNPs supplementation either alone or with the aqueous exposure to AgNPs. Significant reductions of FI were reported in the case of the QNPs, QNPs + AgNPs, and control groups compared to AgNPs alone. The reduction of FI in these groups caused an improvement in FCR (Table 4). Besides, the survivability of Nile tilapia was also, altered by either separate QNPs dietary supplementation or with AgNPs exposure. The survival rate was 97.59 in control and 99.17% in QNPs-supplemented fish, respectively. Whereas, a significant drop in the tilapia's survivability to 80.46% was recorded in the case of AgNPs exposure. This decline improved and retained to normal in the case of concurrent supplementation of QNPs with AgNPs.

3.3. Hematological and protein profile changes in response to QNPs dietary supplementation and AgNPs exposure

Neither the exposure of Nile tilapia to AgNPs nor the separate and co-supplementation with QNPs altered the assessed hematological variables such as Hb, RBCs, PCV%, MCV, MHC, and MCHC (Table 5). Only WBCs count and the count of lymphocytes and monocytes exhibited significant changes following AgNPs exposure; however they were improved to normal levels when QNPs supplemented either alone or with AgNPs. On the other hand, the other leukogram parameters displayed no significant differences between all experimental groups.

The exposure to 1/10th of the 96 h LC50 of AgNPs for 60 days significantly lowered total protein and albumen levels. QNPs dietary supplementation (alone or with AgNPs) returned the levels of total protein and albumen to the normal levels (control). Moreover, QNPs as dietary supplement maintained the glucose without change relative to the control. However, glucose levels demonstrated a significant increase in the case of the AgNPs-exposed group relative to the control; this effect was completely abolished upon the concomitant supplementation of QNPs with AgNPs (Table 6).

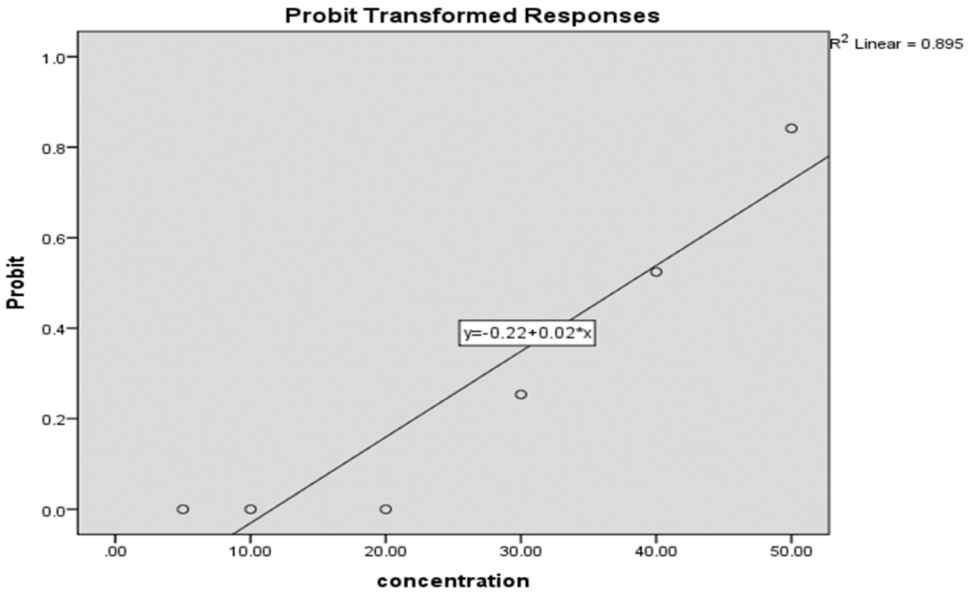

Fig. 2. Probit analysis results to calculate the 96-h LC50 of AgNPs in *O. niloticus*.

Table 3
Behavioral changes of *O. niloticus* after 96 h exposure to different AgNPs concentration.

Behavior	AgNPs concentration (mg / L)							
	Control (0)	5	10	20	30	40	50	60
Air gulping	-	-	+	+ +	+ +	+ +	+ ++	+ ++ +
Respiratory distress	-	-	+	+ +	+ +	+ ++	+ ++	+ ++ +
Sluggish movement	-	-	+	+ +	+ +	+ ++	+ ++	+ ++ +
Uncoordinated swimming	-	-	+	+ +	+ ++	+ +	+ ++	+ ++ +
Hyperventilation	-	-	-	+ +	+ +	+ ++	+ ++	+ ++ +

-donates no clinical signs; x indicates mild signs; xx refers to moderate response; xxx and XXXX represent strong and very strong responses, respectively.

Table 4
Effects of separate and concurrent exposure to AgNPs and QNPs on growth performance parameters.

	Control	QNPs	AgNPs	AgNPs+ QNPs	p-value
IW (g)	40.47 ± 0.18	40.52 ± 0.19	40.45 ± 0.09	40.48 ± 0.20	0.995
FW (g)	76.13 ± 2.50^{b}	84.22 ± 1.68^{a}	66.05 ± 0.60^{d}	71.57 ± 3.00^{c}	0.002
BWG (g)	35.65 ± 2.66^{b}	43.71 ± 1.66^{a}	25.60 ± 0.57^{c}	31.09 ± 3.18^{b}	< 0.001
SGR (%)	59.42 ± 4.42^{b}	72.87 ± 2.76^{a}	42.66 ± 0.95^{c}	51.82 ± 5.30^{b}	0.003
FI (g)	50.60 ± 0.15^{b}	49.39 ± 0.51^{b}	55.73 ± 1.19^{a}	52.16 ± 0.48^{b}	0.001
FCR	1.43 ± 0.11^{bc}	1.13 ± 0.05^{c}	2.17 ± 0.07^{a}	1.70 ± 0.17^{b}	0.001
Survival (%)	97.59 ± 0.18^{ab}	99.17 ± 0.18^{a}	80.46 ± 2.06^{c}	92.44 ± 1.82^{b}	< 0.001

IW (g)= initial weight, FW (g)= final weight, BWG (g)= body weight gain, SGR (%)= specific growth rate, FI (g)= feed intake, FCR= feed conversion ratio, Survival rate (%). Results are represented as Means ± SE. Different lowercase letters (a, b, c, d) donate statistical significances in the same raw at $P < 0.05$.

3.4. The immune and oxidative stress response of Nile tilapia to AgNPs and/or QNPs treatments

Table 6 presents the immunological indices of Nile tilapia exposed to AgNPs and the dietary enrichment of QNPs. Nile tilapia-fed basal-QNPs enriched diet exhibited normal immune response as the control fish as confirmed by non-altered immunoglobulins levels (IgM and IgG), lysozyme activities, and NO levels. Besides, a significant decrease in MPO

Table 5
The consequence of QNPs dietary supplementation (400 mg/ kg diet) on hematological variables of Nile tilapia exposed to AgNPs (1/10th LC50; 1.98 mg/ L) for 60 days.

	Control	QNPs	AgNPs	AgNPs+ QNPs	p-value
Hb (g/dl)	7.75 ± 0.12	7.44 ± 0.23	7.28 ± 0.12	7.33 ± 0.16	0.251
RBCs (10^{6}/μl)	2.22 ± 0.17	2.74 ± 0.12	2.37 ± 0.07	2.42 ± 0.09	0.076
PCV (%)	21.18 ± 0.03	21.01 ± 0.00	21.05 ± 0.56	21.63 ± 0.26	0.504
MCV (fl)	93.80 ± 3.90	93.97 ± 2.62	96.78 ± 3.59	92.33 ± 1.28	0.771
MCH (Pg)	32.41 ± 0.14	32.26 ± 1.37	32.24 ± 1.56	32.66 ± 0.77	0.992
MCHC (%)	35.13 ± 0.61	34.40 ± 1.31	34.12 ± 0.61	33.80 ± 0.40	0.695
WBCs (10/ mm3)	5.37 ± 0.11^{ab}	6.56 ± 0.40^{a}	4.36 ± 0.56^{b}	5.45 ± 0.28^{ab}	0.021
Lymphocytes	2.70 ± 0.14^{a}	2.99 ± 0.46^{a}	1.42 ± 0.03^{b}	2.45 ± 0.27^{ab}	0.018
Monocytes	0.60 ± 0.05^{ab}	0.65 ± 0.03^{a}	0.45 ± 0.01^{b}	0.59 ± 0.03^{ab}	0.020
Eosinophils	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.848
Basophils	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.000

Hb- Hemoglobin, RBCs- Red blood corpuscles, MCV- Mean corpuscular volume, MCH- Mean corpuscular hemoglobin, MCHC- Mean corpuscular hemoglobin concentration, TLCs- Total leukocyte counts, H- Heterophil, L- Lymphocyte. Results are represented as Means ± SE. Different lowercase letters (a, b, c, d) donate statistical significances in the same raw at $P < 0.05$.

Table 6
Immune response of Nile tilapia exposed to AgNPs (1/10th LC50; 1.98 mg/L) for 60 days with and without QNPs dietary supplementation.

	Control	QNPs	AgNPs	AgNPs+ QNPs	p-value
Total protein (g/dl)	4.14 ± 0.04^{a}	4.35 ± 0.09^{a}	2.49 ± 0.33^{b}	4.17 ± 0.09^{a}	< 0.001
Albumin (g/dl)	1.47 ± 0.07^{a}	1.67 ± 0.01^{a}	1.09 ± 0.07^{b}	1.46 ± 0.07^{a}	< 0.001
Glucose (mg/dl)	75.24 ± 0.05^{b}	65.53 ± 3.27^{b}	124.44 ± 7.74^{a}	75.17 ± 0.12^{b}	< 0.001
IgG (mg/dL)	80.53 ± 0.23^{b}	87.75 ± 0.68^{a}	43.66 ± 1.97^{c}	43.87 ± 1.95^{c}	< 0.001
IgM(mg/dL)	8.34 ± 0.11^{a}	8.20 ± 0.04^{a}	3.88 ± 0.05^{c}	7.28 ± 0.09^{b}	< 0.001
Lysozyme (U/L)	16.08 ± 0.08^{a}	17.16 ± 0.14^{a}	6.07 ± 0.59^{b}	16.31 ± 0.22^{a}	< 0.001
NO (μmol/L)	54.41 ± 0.35^{a}	54.15 ± 1.00^{a}	20.94 ± 0.42^{b}	22.04 ± 0.58^{b}	< 0.001
MPO (U/L)	2.93 ± 0.01^{c}	1.94 ± 0.00^{d}	6.45 ± 0.35^{a}	4.21 ± 0.10^{b}	< 0.001

Results are represented as Means ± SE. Different lowercase letters (a, b, c, d) donate statistical significances in the same raw at $P < 0.05$.

level relative to the control was reported in QNPs group. AgNPs significantly lowered these indices with a distinctive elevation of MPO level. Remarkably, concurrent dietary supplementation of QNPs with AgNPs exposure alleviated these effects.

The aqueous exposure of Nile tilapia to AgNPs significantly increased the serum 8- OHdG levels, relative to the control (Fig. 3). However, QNPs supplementation alone maintained its level as the control. Besides, the co-treatment of AgNPs exposure with QNPs counteracted the elevation of serum 8- OHdG level compared to AgNPs only.

3.5. Variations of the transcriptomic profile of antioxidant and immune-related genes

Dietary supplementation of QNPs maintained the relative mRNA expression of *CAT, SOD,* and *GSH-Px* within the normal levels. On the hand, significant downregulations of these genes occurred in response to AgNPs exposure. The dietary supplementation of QNPs to AgNPs-exposed fish alleviated these downregulations except for the *GSH-Px* gene which was still significantly less than the control (Fig. 4). Likewise, the proinflammatory cytokines genes such as *IFN-γ, TNF-α,* and *IL-1β,* QNPs dietary addition did not modify their mRNA expressions. However, when fishes were exposed to AgNPs, these genes exhibited significant upregulation. This upregulation was completely abolished in the case of *TNF-α,* and *IL-1β* genes when the AgNPs were co-treated with QNPs dietary supplementation ($P < 0.05$). Though, for the *IFN-γ* gene, the co-supplementation of QNPs with AgNPs lowered its mRNA expression but still higher than the control (Fig. 5).

3.6. Nile tilapia's resistance against A. hydrophila infection

The active immune response of *O. niloticus* following the dietary supplementation of QNPs and/or AgNPs exposure was further assessed by measuring the fish's resistance to the *A. hydrophila* challenge (Table 7). The mortality rate (%) in normally farmed fish reached 5%, with a high survival rate reaching 95%. The QNPs-supplemented group had no mortalities (0%). The most significant highest post-challenge mortalities were observed in the fish exposed to AgNPs alone (25%) with the lowest survival rate (75%). The mortalities were decreased to 10%, and 90% survival rate, with QNPs supplementation with AgNPs.

4. Discussion

Despite the beneficial effects of AgNPs as effective antimicrobial agents, their extensive uses in industrial and research fields possibly cause pollution of aquatic ecosystems with negative impacts on aquatic animals' life ((Harikrishnan et al., 2009; Jin et al., 2010; Harikrishnan et al., 2010; Srinonate et al., 2015; Hamed and Abdel-Tawwab, 2021). The toxicity of AgNPs is regulated by many factors including the size of their particles, dose, time of exposure, shape, and coating materials (Akter et al., 2018; Mansour et al., 2021). In this regard, the results of the current study estimated the 96-h LC50 of AgNPs in Nile tilapia to be 19.81 mg/L which differed from other studies. For example; the 96-h LC50 values of AgNPs were reported to be 19.5 and 20 mg/L in the case of Nile tilapia (*Oreochromis niloticus*) and redbelly tilapia (*Tilapia zillii*), respectively (Afifi et al., 2016) while 12.6 mg/L in Mozambique tilapia (*O. mossambicus*) (Govindasamy and Rahuman, 2012). Moreover, it was determined to be 177.5 mg/L in channel catfish (*Clarias batrachus*) (Pandit and Sinha, 2019), 0.34 mg/L and 0.53 mg/L in silver carp (*Hypophthalmichthys molitrix*), and goldfish (*Carassius auratus*), respectively (Hedayati et al., 2012). However, it was 100 mg/L in another study on Nile tilapia (Thummabancha et al., 2016), and 164.02 mg/L in *C. batrachus* (Ali and Tripathi, 2014). The different values of the 96-h LC50 between different fish species and within the same species might be related to age, body weight, sex, feeding condition of the tested fish, water temperature and ambient conditions (Shaluei et al., 2013).

The toxic effect of AgNPs is closely correlated to the oxidation of its surface and the release of silver (Ag) ions, which interact with the biological macromolecules causing mitochondrial dysfunction and the generation of ROS (Afifi et al., 2016). The current results revealed that Nile tilapia exposed to 1.98 mg/L of AgNPs for 60 days exhibited a significant reduction in its growth parameters, and feed utilization indices such as low final weight and gain, increased FCR, and low SGR. The AgNPs-linked negative growth performance could be attributed to

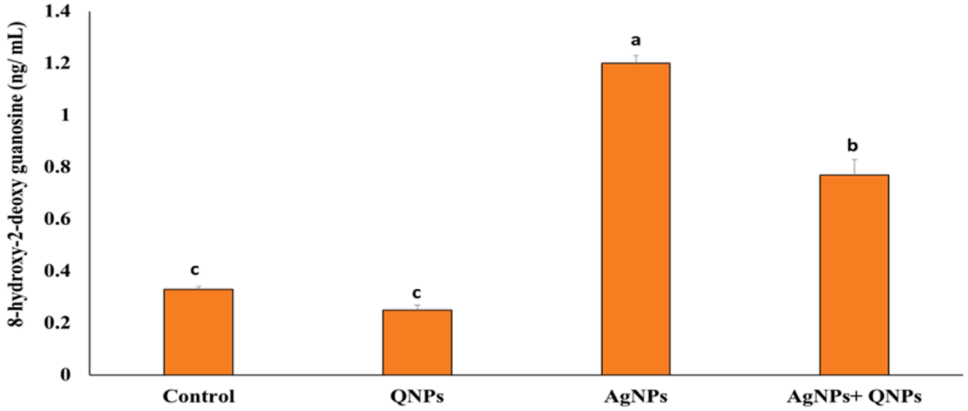

Fig. 3. represents means ± SE of the serum 8- OHdG levels in response to AgNPs aqueous exposure and /or QNPs dietary supplementation. Different lowercase letters (a, b, c) donate statistical significances at $P < 0.05$.

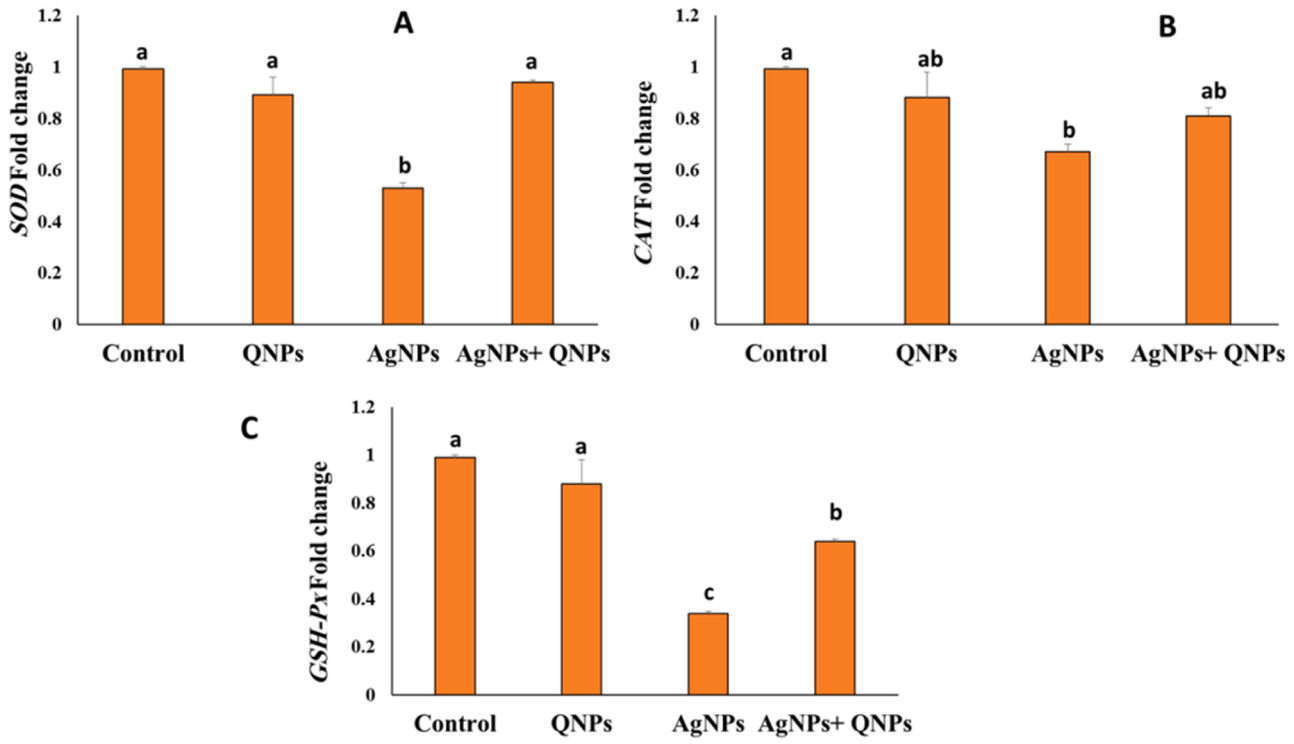

Fig. 4. shows the relative mRNA expression level of *SOD, CAT* and *GSH-Px* in the tilapia liver exposed to AgNPs for 60 days with /without QNPs dietary supplementation (n = 9/group). A represents *SOD* fold changes, B shows *CAT* relative mRNA level, C represents *GSH-Px* mRNA copies. Values are shown as means ± SE. Different lowercase letters (a, b, c) donate statistical significances at $P < 0.05$.

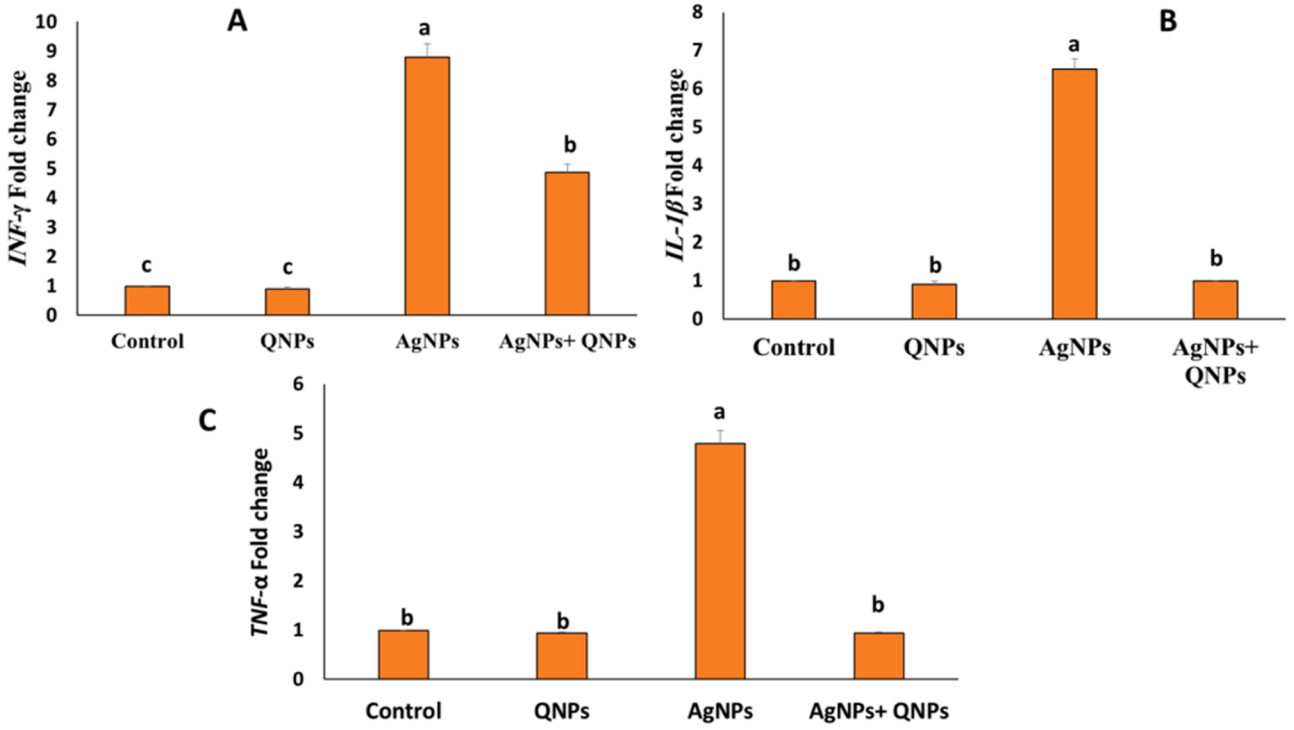

Fig. 5. shows the relative mRNA expression level of *IFN-γ, TNF-α,* and *IL-1β* in the tilapia liver exposed to AgNPs for 60 days with /without QNPs dietary supplementation (n = 9/group). A represents *IFN-γ* fold changes, B shows *IL-1β* relative mRNA level, C represents *TNF-α* mRNA copies. Values are shown as means ± SE. Different lowercase letters (a, b, c) donate statistical significances at $P < 0.05$.

the toxic release of Ag^{+} ions from AgNPs in the aquatic environment and its uptake (Shah and Mraz, 2020). In addition, it might be correlated with increasing the protein loss, oxidative phosphorylation and impairing the synthesis of protein (van Aerle et al., 2013) as well as, downregulating the expression levels of the growth hormone gene and insulin-like growth factor gene (Mansour et al., 2021). The impairment

Table 7
Effect of QNPs supplementation on *O. niloticus* challenged with *A. hydrophila* (1.5 mL of 10^7 bacterial cells mL^{-1}) under AgNPs exposure.

Items	Total number	The number of dead fish	Survival %	mortality %
Control	20	1	95	5
QNPs	20	0	100	0
AgNPs	20	5	75	25
AgNPs+ QNPs	20	2	90	10

of protein synthesis and increasing protein loss might explain the estimated reduction of serum protein and albumen levels in the case of the AgNPs-exposed group. These findings were similar to the previous studies conducted on goldfish (*Carassius auratus gibelio)* (Forouhar Vajargah et al., 2019), Yellow Perch (*Perca flavescent)* and African catfish *(Clarias gariepinus*) (Mahboub et al., 2021) which showed retarded growth performance with increasing AgNPs dose.

Conversely, the Nile tilapias fed on the QNPs-supplemented diet, either alone or with AgNPs exposure, displayed improved growth performance and amelioration to the negative effect of AgNPs toxicity, respectively. The remarkable enhancement of Nile tilapia growth in the case of the QNPs-supplemented group and its improving effect on the AgNPs-impaired growth perhaps are resulted from stimulating the growth of the beneficial bacteria of the gut (acting as a probiotic), which can inhibit the growth of pathogenic ones (Ibrahim et al., 2021). Moreover, it may be attributed to its polyphenolic flavonoids that upregulate the expressions of growth-related genes which increased with using QNPs and this also explain the enhanced growth in QNPs alone group compared to control (Ibrahim et al., 2021; Farag et al., 2023).

The assessment of the hematological profile is an indispensable indicator for screening the alteration caused by toxicant exposure (Abdel-Mageid et al., 2020). In this study, the AgNPs exposure did not change most of the assessed blood parameters such as PCV,Hb, MCH, MCV, MCHC, and RBCs counts. However, AgNPs significantly decreased the counts of WBCs, lymphocytes, and monocytes. These effects might be linked to the small dose of AgNPs as we used only one-tenth (1/10th) of the 96 h LC50. On the other hand, the previous studies conducted on African catfish (*Clarias gariepinus*) (Mahboub et al., 2021), rainbow trout (Imani et al., 2015), *L. rohita* fish (Khan et al., 2017), and Nile tilapia fingerlings (Mansour et al., 2021) reported marked changes in hematological parameters of these fish species. These hematotoxicological effects of AgNPs exposure are probably linked with the excessive production of ROS which destroys the cell membrane integrity of hematopoietic cells and disrupts their functions (Shaluei et al., 2013). The high content of polyunsaturated fatty acids in the cell membrane of blood cells makes them targets for ROS-induced peroxidation causing cell lysis (Massarsky et al., 2014). The differences in the findings between studies conducted on AgNPs exposure may be correlated with changes in nutrition, age, fish species, environmental pollution, fish's health, the dose of AgNPs, and the route of exposure (Afifi et al., 2016).

Dietary supplementation of QNPs also did not alter most of the assessed hematological indices and restored the AgNPs-induced reduction of the leukocyte count. This effect might be associated with the protective role of QNPs on liver and kidney tissues from damage from AgNPs exposure and preventing the metabolic stress generated by AgNPs exposure. The protecting role of QNPs might be related to maintaining the leukocyte redox by lowering the ROS synthesis, preventing peroxidation, protecting cell membrane integrity as well as enhancing leukocyte proliferation (Farag et al., 2021; Ibrahim et al., 2021).

Furthermore, the diminishing contents of total protein and albumen after AgNPs exposure were restored to normal by QNPs dietary supplementation may be due to its protective effects on liver and kidney functions because of its high contents of flavonoids. This influence was augmented by its incorporation into the nanoform that increases its stability and bioavailbility (Ibrahim et al., 2021). Moreover, the QNPs improved the AgNPs-associted reduction in the IgG, IgM, lysozyme, and NO levels. This effect may be connected with increasing WBCs, monocytes, and lymphocytes counts in the case of combining treatment of QNPs with AgNPs exposure compared to AgNPs only. The later effect is probably linked with the immune-stimulatory impact of QNPs on immune cells inducing marked higher levels of immunoglobulins, respiratory burst, lysozymes and total protein levels because of its higher contents of flavonoids (Wang et al., 2020). The higher lysozyme activity is necessary to hydrolyze pathogenic bacteria by destroying the β-(1,4) glycosidic bonds between the N-acetylglucosamine and N-acetylmuramic acid (Bao et al., 2017). Moreover, the higher activity of MPO in the case of the AgNPs-exposed group may be explained by the self-defense of fish to AgNPs-induced stress as well as the antibacterial properties of AgNPs which increase with increasing its dose (Mabrouk et al., 2021). Whereas, dietary supplementation of QNPs to AgNPs-exposed fish lowered MPO levels compared to AgNPs only but still higher than control. This indicates the immunostimulatory effect of QNPs which increases the phagocytic activity in killing microorganisms (Wang et al., 2020). Besides, restoring the IgG and IgM levels in the case of the QNP-AgNPs group might be correlated with the higher polysaccharides and flavonoids in QNPs (Ibrahim et al., 2021).

Generally, the high glucose level is a stress response, which is correlated with high cortisol levels, to meet the requirements of energy to counteract toxicities (Hamed and Abdel-Tawwab, 2021). Blood glucose levels are strongly influenced by environmental stresses, managemental procedures, nutritional status, seasonal variations, and sexual maturity. Herein, the AgNPs-associated higher glucose levels reflect the Nile tilapia's strong stress response to the excessive release of Ag^+ from AgNPs to the aquatic environment to meet the energy requirements (Shah and Mraz, 2020). Our findings coincided with the results in African catfish, silver carp (*Hypophthalmichthys molitrix)*, Atlantic salmon (*Salmo salar*) and *Clarias gariepinus* (Farmen et al., 2012; Shaluei et al., 2013; Naguib et al., 2020; Mahboub et al., 2021) who reported elevated serum glucose in response to AgNPs. On the other hand, feeding QNPs restored glucose levels as in the control group which may reflect that the bioactive composites in QNPs probably promote glucose uptake, resulting in hypoglycemic effects (Hamed and Abdel-Tawwab, 2021). Besides, it might reflect the possibility of QNPs to alleviate the AgNPs-induced stresses via their anti-inflammatory, antioxidant, and anti-apoptotic effects (Ibrahim et al., 2021).

The AgNPs-induced oxidative stress was also supported by increasing the 8-OHdG level in the case of the AgNPs-exposed group. The high level of the 8-OHdG is considered a stress biomarker as it represents the oxidative damage to DNA because of the eventual tissue injuries (Farag et al., 2021). This toxic effect of the AgNPs aqueous exposure was confirmed by the significant downregulation of *SOD, CAT,* and *GSH* mRNA levels. Where increasing AgNPs levels induced depression in the fish's antioxidant and immune systems, which could be returned to the excessive ROS production and disturbance of the antioxidant system (Mabrouk et al., 2021). In accordance, (Hamed and Abdel-Tawwab, 2021; Mansour et al., 2021) demonstrated significant suppression in the Nile tilapia's expression levels of *SOD* and *CAT* gene expression and their enzyme activities upon AgNPs exposure. Remarkably, the QNPs dietary supplementation restored this effect by increasing the relative mRNA levels of CAT, SOD, and GSH genes reaching the control levels. The QNPs effect is perhaps linked with its antistress effect with antioxidant properties via reducing ROS synthesis (Ibrahim et al., 2021). In this perspective, increasing the SOD, CAT, and GSH expression comprises the key lines of cell defense against excessive free radicals. Increasing SOD catalyzes the O^{2-} into O^2 and H_2O_2, while the GPx and CAT decompose H_2O_2 into O^2 and H_2O to remove excessive free radicals. The QNPs-related impacts might be correlated with enhancing the Nrf2 signaling pathway which is the key regulating factor of oxidative stress by encoding antioxidant enzymes (Wang et al., 2020). Also, may be

correlated with its high contents of polyphenolic and flavonoids constituents (Ibrahim et al., 2021).

In fish, the balance between proinflammatory and anti-inflammatory signaling is crucial to maintain a good immune response (Wang et al., 2020). In this perspective, AgNPs resulted in a distinct proinflammatory response manifested by the upregulations of the proinflammatory cytokines *IFN-γ, TNF-α,* and *IL-1β* relative mRNA levels indicating the proinflammatory role of AgNPs. This proinflammatory response is probably related to altering the p53 gene pathway causing apoptosis, mitochondrial damage, and induction of inflammation (Mansour et al., 2021). Similarly, the exposure of Nile tilapia fingerlings to Ag-NPs induced significant upregulations of *TNF-α* and *TGF-β* genes (Mansour et al., 2021). On the other hand, AgNPs exposure with QNPs dietary supplementation diminished the expressions of *TNF-α, IFN-γ,* and *IL-1β* reaching the control values, except the *IFN-γ* gene, was still higher than the control. The QNPs anti-inflammatory effect may be returned to the regulation of p38 mitogen activated protein kinase (MAPK) signal pathway and NF-κB (Wang et al., 2020). However, the exact mechanisms of quercetin and its nanoform in regulating the inflammatory response in Nile tilapia requires further investigation at the transcriptomic and histological levels.

The foremost findings of AgNPs aquous exposure and the QNPs dietary supplementation were also, correlated with alteration of Nile tilapia response to *Aeromonas hydrophila* infection. Where, high mortalities of Nile tilapia were recorded post-*A hydrophila* infection in the case of AgNPs exposure. This response might be correlated with the synergy-toxic effect of both AgNPs and *Aeromonas hydrophila* infection. In this context, the AgNPs induce oxidative stress manifested by lowering *SOD*, *CAT,* and *GSH* gene expression levels decreased lysozyme and NO activities as well as high 8-OHdG levels besides, upregulating the relative expression levels of *TNF-α, IFN-γ,* and *IL-1β* genes. In addition, the AgNPs-Aeromonas hydrophila high mortilities might be linked with tissue destruction such as skin, gills, and liver that may be because of the disturbances of Na+ /K+ ATPase activity (Mabrouk et al., 2021). Remarkably, feeding QNPs enriched diet, alone or in combination with AgNPs lowered the fish mortalities to 0% and 10% respectively. The QNPs' effects are possibly correlated with their antistress, and anti-inflammatory characteristics due to its active components of flavonoids and polyphenols (Wang et al., 2020). In zebrafish, the bactericidal effect of quercetin was accompanied by increasing the activity of antimicrobial peptides (AMPs) such as LEAP-2 which destroys the cell membrane of bacteria causing the losses of its external and internal barriers as it blocks the bacterial respiratory chain and hydrolyzes the bacterial genomic DNA (Wang et al., 2020).

5. Conclusion

In summary, the aqueous exposure to 1/10th 96-h LCD_{50} of AgNPs retarded Nile tilapias' growth and food utilization indices. Additionally, AgNPs altered Nile tilapia's antioxidant status and immune response to *A. hydrophila* infection. Co-feeding of QNPs with AgNPs improved Nile tilapias' performance by reducing the AgNPs-associated oxidative stress and inflammatory responses via elevating the expressions of antioxidant-related genes and decreased the mRNA levels of inflammatory-linked genes. Therefore, the dietary supplementation of QNPs represents an effective strategy to alleviate AgNPs-associated immunosuppression and oxidative stress in fish.

Ethical statement

The experiment was approved by the Institutional Animal Care and Use Committee, Zagazig University, Egypt (ZU-IACUC/2/F/110/2022).

CRediT authorship contribution statement

All authors (Eman Ismail El behery, Zheng Chuntian, Mayada R. Farag, Mahmoud Alagawany, Seham El-Kassas, Mahmoud M. Azzam, Alessandro Di Cerbo, Eman Wagih) have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

Authors declare no conflict of interests.

Data Availability

The data that has been used is confidential. The data presented in this study are available on request from the corresponding author.

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Data Availability Statement

The data presented in this study are available on request from the corresponding author.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2023.101780.

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