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Dietary α -sitosterol mitigates titanium dioxide nanoparticle-induced hemato-immunotoxicity, oxidative stress, and gene expression dysregulation in *Oreochromis niloticus*

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Introduction: Titanium dioxide nanoparticles (TDNPs) are widely used in food industries, agricultural and consumer products, and diagnostic purposes, leading to their potential release into aquatic environments and associated physiological risks to non-target aquatic organisms, particularly fish. α -Sitosterol (STL), a phytosterol, acts by enhancing antioxidant defenses and modulating inflammatory signaling pathways. Hence, this study investigated whether dietary STL can protect Nile tilapia (*Oreochromis niloticus*) from TDNPs-induced toxicity during a 60-day exposure.

Methods: In this study, 300 Nile tilapia were allocated into four groups. The control group received a basal diet, the STL group was fed a diet supplemented with 80 mg STL/kg, the TDNPs group was exposed to 10 mg/L of TDNPs in water, and the TDNPs + STL group was exposed to TDNPs and fed the STL-supplemented diet.

Results: Dietary STL supplementation markedly improved growth performance, with increases of 33%–60% in final body weight, weight gain, and daily growth rate, and a 29% reduction in feed conversion ratio compared to TDNPs-exposed fish. STL supplementation also restored hematological parameters altered by TDNPs exposure, including significant recovery of red blood cells, hemoglobin, packed cell volume, and white blood cells, thereby reversing macrocytic normochromic anemia and leukopenia. Furthermore, STL significantly decreased elevated serum alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, urea, and creatinine levels induced by TDNPs, and normalized lipid profiles by reducing total cholesterol, triglycerides, and low-density lipoprotein cholesterol, while elevating high-density lipoprotein cholesterol. STL-fed fish also exhibited significant reductions in stress biomarkers (glucose and cortisol) and enhanced

innate immune responses, as evidenced by higher lysozyme, complement 3, nitric oxide, nitro blue tetrazolium, and phagocytic activity. Antioxidant status was strengthened through increased superoxide dismutase, catalase, and glutathione peroxidase activities and reduced malondialdehyde levels. At the molecular level, STL supplementation downregulated endoplasmic reticulum stress-related genes (*chop*, *jnk*, *xbp-1*, and *perk*), while upregulating autophagy-related genes (*beclin-1* and *lc3-ii*) and downregulating *mtor* and *p62*. Histological analysis confirmed STL's protective effects, showing marked recovery of intestinal, hepatic, renal, and splenic structures.

Conclusion: These findings demonstrated that STL confers multi-level protection against TDNPs-induced oxidative, metabolic, and cellular stress, highlighting its potential as a functional dietary supplement for mitigating nanotoxicity in aquaculture.

KEYWORDS

titanium dioxide nanoparticles, alpha sitosterol, *oreochromis niloticus*, oxidative stress, endoplasmic reticulum stress, autophagy, gene expression, immunity

1 Introduction

Nanoparticles (NPs) are increasingly used in agriculture and aquaculture due to their potential to improve food security and resource efficiency (Alam et al., 2024; Khan et al., 2024). However, their release into aquatic environments raises environmental and ecotoxicological concerns, particularly for non-target organisms such as fish (Ameen et al., 2021; Min et al., 2023; Atanda et al., 2025). Titanium dioxide nanoparticles (TDNPs) rank among the most produced and extensively utilized nanomaterials across agriculture, consumer products, and environmental sectors (Sungur, 2021). In agriculture, they are experimentally applied as nanofertilizers, photocatalytic agents for pesticide degradation, and antimicrobial coatings to enhance plant growth and protect crops from pathogens (Lyu et al., 2017; Ko and Hwang, 2019; Rodríguez-González et al., 2019). Moreover, TDNPs have been experimentally explored in aquaculture for various purposes, including photocatalytic water disinfection and anti-infective applications (Alexpandi et al., 2020) and degradation of antibiotic residues in aquaculture wastewater in the Mekong Delta, Vietnam (Do et al., 2019). Yet, accidental environmental release of TDNPs can result in bioaccumulation in plankton and transfer through the food web, posing risks to multiple trophic levels (Abdel-Latif et al., 2020; Rashid et al., 2021). Field measurements indicate TDNPs levels in contaminated waters ranging from 1 to 200 µg/L near industrial sites, highlighting the potential for environmental exposure (Kiser et al., 2009; Kozliak and Paca, 2012; Maiga et al., 2019; Liu et al., 2022).

Exposure to TDNPs has been shown to induce physiological, immunological, and histological disturbances in fish. For instance, increased activities of liver enzymes such as alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate

aminotransferase (AST) have been reported in *Clarias gariepinus* exposed to 1–10 mg/L TDNPs (Tunçsoy, 2021). Immunotoxic effects, including lysosomal destabilization and impaired phagocytosis, were observed in fathead minnows (*Pimephales promelas*) following intraperitoneal TDNPs injection (10 µg/g b.wt) (Jovanović et al., 2015). In neotropical detritivorous fish (*Prochilodus lineatus*), 1–50 mg/L TDNPs exposure for 14 days reduced red blood cell (RBC), white blood cell (WBC), and lymphocyte counts (Carmona et al., 2019). Histopathological changes in the intestine and liver were reported in Nile tilapia (*Oreochromis niloticus*) exposed to 1 mg/L TDNPs for 21 days (De Silva and Pathiratne, 2023), while biochemical and tissue alterations were also observed in *Danio rerio* fed 200 µg/g TDNPs for 14 days (Cunha and De Brito-Gitirana, 2020). Oxidative stress from reactive oxygen species (ROS) is a key mechanism underlying these effects (Vineetha et al., 2021). TDNPs can further disrupt endoplasmic reticulum (ER) function, activating ER stress pathways and autophagy, contributing to cellular damage (Zhou et al., 2024, 2025).

Despite various reports on TDNPs-induced toxicity in fish, little is known about dietary strategies that could mitigate these harmful effects, especially in commercially important species such as Nile tilapia (*O. niloticus*) (Vineetha et al., 2021). Nile tilapia is one of the most widely cultivated aquaculture species globally, ranking as the third-most significant in aquaculture, due to its fast growth, broad environmental tolerance, high feed conversion, and ease of reproduction in captivity (FAO, 2022; Geletu and Zhao, 2023). In 2013, the global production of tilapia reached 4.8 million tons, with Nile tilapia accounting for over 70% of this total (McAndrew et al., 2016). Egypt is a leading producer of Nile tilapia, with production estimates varying by source and year; recent reports indicate an output exceeding 1.1 million tons in 2023, while earlier figures reached approximately 1.6 million tons in 2021, positioning Egypt

as the largest producer in Africa and the third largest globally, after China and Indonesia (El-Sayed, 2021; Alliance, 2023). Additionally, because of its biological suitability as a model species for toxicological and nutritional research, Nile tilapia serves as an ideal tropical fish model for evaluating various forms of nanotoxicity including TDNPs (Perera and Pathiratne, 2014; De Silva and Pathiratne, 2023; Ribeiro et al., 2025).

Phytosterols possess a broad spectrum of physiological activities, comprising anti-inflammatory, antioxidant, immunomodulatory, and antimicrobial effects, along with protective roles in gut and liver health (Feng et al., 2020). They are known to reduce cholesterol absorption and lower low-density lipoprotein cholesterol (LDL-C) levels (Li et al., 2022), thereby contributing to improved growth performance in animals (Ding et al., 2021). Moreover, phytosterols can decrease malondialdehyde (MDA) concentrations while enhancing the antioxidant enzyme activities comprising superoxide dismutase (SOD) and glutathione peroxidase (GPx) (Abdel-Moneim et al., 2020). Among these, α -sitosterol (α -STL), a bioactive phytosterol produced by *Streptomyces misakiensis*, has demonstrated promising biological properties (Abdelaziz et al., 2024). Dietary supplementation with phytosterols has been shown to elevate immunoglobulin levels and upregulate the mRNA expression of immune-related markers, including interleukin-10 and β -defensin in gilthead seabream (*Sparus aurata*) (Costas et al., 2014). β -Sitosterol (β -STL) specifically exhibits notable *in vitro* anti-apoptotic and anti-inflammatory effects by downregulating caspase-3 and interleukin-1 β , while upregulating B-cell lymphoma-2 (Bcl-2) expression (Fan et al., 2023). Furthermore, β -STL mitigated cerebral injury by modulating pathways associated with ER stress, cholesterol overload, and neuronal apoptosis (Tang et al., 2024). Dietary supplementation with α -STL at 80 mg/kg for 60 days enhanced antioxidant status and growth performance, strengthened the immune response, and regulated gene expression in Nile tilapia following *Candida albicans* infection (El-Houseiny et al., 2025), highlighting its potential as a protective dietary intervention against cellular stress and toxicity.

Based on the aforementioned STL biological activities, we hypothesized that it could mitigate TDNPs impacts in tilapia. Therefore, the present study aimed to (i) investigate the toxic effects of TDNPs on growth, hematobiochemical parameters, stress responses, immune status, antioxidant defenses, ER stress, autophagy, and tissue histopathology in Nile tilapia, and (ii) evaluate the potential protective role of STL supplementation in mitigating TDNPs-induced adverse effects. To our knowledge, this work represents the first *in vivo* investigation of the protective efficacy of STL against TDNPs toxicity in Nile tilapia.

2 Materials and methods

2.1 Tested chemicals

TDNPs (99.98% purity and molecular weight 79.87 g/mol) were obtained from Alpha Chemica, Mumbai, India. The same batch of TDNPs was previously characterized by one of the current authors

(Abd-Elhakim et al., 2023). Characterization revealed that the particles had a uniform spherical morphology with a primary size distribution ranging from 6 to 19 nm, as determined by transmission electron microscopy. X-ray diffraction analysis confirmed the rutile crystalline structure, while dynamic light scattering showed a hydrodynamic diameter of approximately 12 nm. The zeta potential was measured at -25 mV, indicating moderate stability in suspension. To maintain dispersion and minimize aggregation during exposure, the TDNPs suspensions were sonicated for 15 min before use and kept under continuous mild aeration and stirring in the stock solution.

α -STL used in this study was derived from *S. misakiensis*, as previously isolated, identified, and characterized in El-Houseiny et al. (2025), following the protocol described by Abdelaziz et al. (2024). Sigma-Aldrich Co. of St. Louis, Missouri, USA, supplied all of the analytical grade chemicals and reagents used in this study.

2.2 Experimental fish

The male Nile tilapia fingerlings (an average body weight of 11.79 ± 0.33 g) were obtained from the Fish Research Centre at Zagazig University, Egypt. Prior to the trial, the fish were acclimated in a laboratory environment, housed in glass aquariums containing 70 L of dechlorinated tap water, and fed a basic diet to minimize external stress factors.

Throughout the trial, key water quality parameters in the aquariums, including pH, dissolved oxygen, water temperature, and ammonia, were regularly monitored using an automated Hanna HI-9147 probe and ammonia test kits (HACH, HACH Co., Loveland, CO, USA). Water parameters were maintained within optimal ranges through continuous aeration, thermostatically controlled heaters, and daily monitoring to ensure uniform environmental conditions across all tanks. Tanks were positioned to avoid direct light and drafts, and water circulation was standardized using identical aeration setups. All parameters remained stable and within the optimal ranges for fish husbandry, as recommended by Boyd and Tucker (2012), with mean $\pm$ SD values as follows: pH at 6.5 ± 0.5 , temperature at 27.3 ± 0.05 °C, ammonia concentration at 0.03 ± 0.01 mg/L, and dissolved oxygen at 6.80 ± 0.5 mg/L. A controlled photoperiod of 12 h of light and 12 h of dark was maintained throughout the study. The experiment was conducted under the regulation of Zagazig University's Animal Use in Research Committee (approval number ZU-IACUC/2/F/85/2025), adhering to the ethical guidelines of the National Institutes of Health (NIH) for the Care and Use of Laboratory Animals in Scientific Research.

2.3 Formulation of diet and experimental plan

In accordance with the Nutrient Requirements of Fish and Shrimp (Jobling, 2012), the ingredients for the experimental diet presented in Table 1 were formulated to adequately fulfill the dietary needs of the fish. The diet's ingredients were thoroughly

TABLE 1 Ingredients and proximate chemical analysis of the experimental diets (g/kg diet, on a dry matter basis).

Ingredients	Control	STL
Fish meal	110	110
Soybean meal	420	420
Ground corn	240	240
Wheat bran	100	100
Corn oil	40	40
Starch	40	39.92
α -Sitosterol (STL)	–	0.08
Cod liver oil	20	20
Mineral and Vitamin premix ¹	30	30
Chemical analysis		
Crude protein (N $\times$ 6.25)	308.8	308.8
Crude lipids	75.0	75.0
Crude fiber	52.8	52.8
Ash	72.2	72.23
Nitrogen-free extract ²	491.2	491.1
Gross energy (kcal kg ⁻¹) ³	4,472	4,472

¹Vitamin and mineral premix (Agri-Vet, 10th Ramadan city, Egypt; per kg of diet) provides: vitamin D3–600 IU, vitamin A 4,000 IU, vitamin E 20 mg, vitamin B1 3.6 mg, vitamin K3–5 mg, vitamin B2–6 mg, vitamin B3 14.4 mg, vitamin B5–12 mg, vitamin B12 0.02 mg, vitamin B6 3.5 mg, folic acid 0.9 mg, biotin 0.07 mg, vitamin C 50 mg, inositol 300 mg, Fe 30 mg, Mg 15 mg, Cu 4 mg, Zn 42 mg, Co 0.11 mg, K 75 mg, Se 0.04 mg, Mn 1.6 mg, I 0.4 mg, and Mo 0.005 mg.

²Calculated by difference (100 – protein% + lipids% + ash% + crude fiber%).

³Gross energy (GE) was calculated as 5.65, 9.45, and 4.11 kcal/g for protein, lipid, and NFE, respectively.

mixed before being processed into pellets, air-dried for 24 h at ambient temperature, and subsequently kept at 4 °C until needed. In line with the [AOAC \(2006\)](#) guidelines, the crude protein, moisture, crude fat, crude fiber, and total ash content of each diet investigated were analyzed using the forced-air oven, macro-Kjeldahl method, ether extraction technique, and muffle furnace, respectively.

During the acclimation and experimental phases, fish were provided with a basal diet equivalent to 3% of their body weight per day. Feeding was conducted twice daily at 09:00 and 16:00; such time points were selected based on the diurnal feeding behavior of Nile tilapia. These time points were chosen because tilapia exhibit peak feeding activity during the mid-morning and mid-afternoon under standard photoperiods, which optimizes feed intake efficiency and growth performance in fingerlings ([Souza et al., 2014](#); [Hamed et al., 2024](#)). Fish were fed to apparent satiation to ensure adequate nutrient intake without overfeeding. The feeding amount was adjusted biweekly based on changes in biomass, which was determined by sampling and weighing a representative subset of fish from each group. To maintain optimal water quality and minimize the accumulation of waste, uneaten feed and feces were removed daily using a siphoning method. Additionally, 50% of the water in each tank was replenished every other day using pre-

aerated and temperature-matched dechlorinated tap water to avoid inducing stress due to sudden environmental changes.

After acclimation, 300 fish were randomly assigned to four treatment groups, with each group containing 75 fish. Each treatment was further divided into five replicates of 15 fish per replicate. The first group (Control) was sustained in clean glass aquariums and was given a basic diet without any additives. The second group (STL) was also housed in clean glass aquariums and was provided with diets that included 80 mg/kg STL. The selected STL dose was based on the findings of [El-Houseiny et al. \(2025\)](#), who performed a full gradient (0, 20, 40, 60, and 80 mg/kg) and found maximal growth, antioxidant, and immune benefits at 60–80 mg/kg; thus, the highest effective dose was chosen. The third group (TDNPs) was exposed to TDNPs at a concentration of 10 mg/L. This exposure level reflects an environmentally relevant sublethal concentration validated by [Vineetha et al. \(2021\)](#) over 14 days to mimic realistic exposure scenarios in aquaculture effluents and demonstrated clear biochemical and histopathological endpoints. The fourth group (STL + TDNPs) received STL and was also exposed to TDNPs at the previously specified levels. To ensure stable concentration and homogeneity, and to minimize fluctuations in TDNPs exposure levels, 50% of the tank water was replaced daily with well-aerated water containing freshly prepared TDNPs suspensions. Exposures were performed for 60 days. All experimental procedures, including feeding, sampling, and measurements, were conducted in a blinded manner. Investigators responsible for outcome measurements were blinded to group allocations, with fish tanks, diets, and sample containers coded to prevent observer bias during assessments of growth, hematological, biochemical, molecular, and histopathological endpoints.

2.4 Blood and tissue sampling collection

After the experimental period, fish were anesthetized with 100 mg/L benzocaine solution (Al-Nasr Pharmaceutical Chemicals Co., Oubour, Qalyubia, Egypt). Previous studies in Nile tilapia have demonstrated that immersion in benzocaine at 100 mg/L induces stage III anesthesia within 2–3 min and full recovery within 5–7 min, thereby minimizing handling stress and avoiding significant alterations in hematological and physiological parameters ([Okamura et al., 2010](#); [Weinert et al., 2015](#)). Approximately 0.5 mL of blood was collected from the caudal vein of each fish using a sterile 3-mL syringe fitted with a 24-gauge needle. Three separate batches of blood samples were collected, totaling 15 samples per group (3 samples per replicate). The first batch was obtained in EDTA tubes for blood cell count analysis. The second batch was collected in heparinized tubes for evaluating phagocytic and respiratory burst activities. The third batch was collected in plain tubes (without anticoagulants), allowed to clot at room temperature, and then centrifuged at 664 $\times g$ for 15 min to obtain serum, which was stored at –20°C for subsequent biochemical analyses. Following blood collection, fish were euthanized by an overdose of benzocaine solution (250 mg/L) ([Neiffer and Stamper, 2009](#)). Liver tissues were collected from 15 fish per group (three samples per replicate), thoroughly trimmed of connective tissue and fat, rinsed

with chilled 0.9% saline, and blotted dry. The liver samples were homogenized in 10 volumes of phosphate-buffered saline (pH 7.4). The hepatic homogenate underwent a gentle centrifugation at $664 \times g$ for 15 min in a chilled centrifuge set to 4°C. After separation, the clear supernatant was carefully collected and preserved at -80°C until assessing oxidative and antioxidant indicators.

For gene expression analysis, an additional 15 liver samples per group (3 samples per replicate) were preserved in TRIzol reagent (Thermo Scientific, USA) and stored at -80°C for RNA extraction to assess ER stress- and autophagy-related genes. Furthermore, liver, spleen, anterior intestine, and anterior kidney samples were randomly collected from 15 fish per group (3 samples per replicate) and fixed in 10% neutral buffered formalin for histopathological investigation. All tissue sample analyses were performed in a blinded manner, with sample identity coded to prevent bias during evaluation. The sample allocation and number of fish tested for each parameter within the experimental groups are demonstrated in [Supplementary Figure S1](#).

2.5 Growth performance analysis

At the beginning and end of the feeding trial, the initial body weight (IBW) and final body weight (FBW) of the fish were recorded. Growth performance indices were then calculated following the equations of [Jobling \(1995\)](#), including:

Survival Rate(%)

$$= (\text{Number of fish at the beginning of the experiment} / \text{Number of fish at the end of the experiment}) \times 100$$

$$\text{Weight Gain (WG, g)} = \text{FBW} - \text{IBW}$$

$$\text{Daily Weight Gain (DWG, g/day)} = \text{WG}/60 \text{ days}$$

$$\text{Specific Growth Rate (SGR, \% /day)}$$

$$= [(\ln \text{FBW} - \ln \text{IBW})/60 \text{ days}] \times 100$$

$$\text{Feed Intake (FI, g feed/fish)}$$

$$= \text{Total feed consumed}/\text{Number of fish per aquarium}$$

$$\text{Feed Conversion Ratio (FCR)} = \text{Total FI}/\text{Total WG}$$

$$\text{Condition Factor} = (W/L^3) \times 100 \text{ where } W$$

$$= \text{body weight(g) and } L = \text{total length(cm)}$$

2.6 Assessment of hematological indices

Hematological indices, including WBC count, RBC count, hematocrit, and hemoglobin (Hb) concentration, were assessed

following the method defined by [Bain et al. \(2016\)](#). Additionally, mean corpuscular volume (MCV) percentage, mean corpuscular hemoglobin (MCH), and packed cell volume (PCV) were measured by the microhematocrit technique ([Hrubec et al., 2000](#)). Methanol-fixed blood smears were Wright–Giemsa-stained to analyze the differential leukocyte count. Leukocytes were identified and classified based on morphological characteristics such as nuclear shape, cytoplasmic granules, and cell size, following the criteria described by [Hrubec et al. \(2001\)](#). All hematological assays were previously validated for use in *O. niloticus* and other tilapia species, ensuring methodological reliability ([Hrubec et al., 2000](#); [Sayed and Moneeb, 2015](#); [Bain et al., 2016](#)). To confirm intra-assay precision, 10% of randomly selected samples were analyzed in duplicate, yielding a mean coefficient of variation (CV) of <5% across all hematological parameters.

2.7 Hepatorenal function indicators, lipid profile, and stress markers

To evaluate various biochemical markers in serum, specific enzymatic and colorimetric assays were conducted using commercially available Spinreact kits (Esteve De Bas, Girona, Spain) consistent with the manufacturer's instructions. ALT activity was measured using the Spinreact kit, catalog no. BEIS45-E. The assay is based on ALT catalyzing the transamination of alanine and α -ketoglutarate to form glutamate and pyruvate. Pyruvate is then converted to lactate by lactate dehydrogenase (LDH) and NADH. The decrease in NADH absorbance at 340 nm, monitored photometrically, reflects ALT activity, according to [Murray and Kaplan \(1984a\)](#). AST activity was determined using the Spinreact kit, catalog no. BEIS46-E. In this assay, an amino group is reversibly transferred from aspartate to α -ketoglutarate, forming oxaloacetate and glutamate. The resulting oxaloacetate is then converted into malate through the action of malate dehydrogenase (MDH), a reaction that concurrently involves the oxidation of NADH. The decline in NADH absorbance at 340 nm corresponds to AST activity, following the method described by [Murray and Kaplan \(1984b\)](#). ALP activity was assessed with the Spinreact kit, catalog no. MDBEIS44-I. The assay depends on ALP's hydrolysis of p-nitrophenyl phosphate to 4-nitrophenoxide, which generates a yellow color measurable photometrically at 405 nm. The yellow color intensity is proportionate to ALP activity, as detailed by [Wenger et al. \(1984\)](#). Serum creatinine levels were determined by the Spinreact kit, catalog no. MDBSIS13-E. The assay is based on creatinine reacting with sodium picrate to form a red complex, which is quantifiable at 505 nm, per the method of [Fossati et al. \(1983\)](#). Urea concentration was estimated by the Spinreact kit, catalog no. BSIS35-I, which is based on the reaction with o-phthalaldehyde in an acidic medium, forming a chromogenic complex measurable at 510 nm, as defined by [Kaplan \(1984\)](#).

Serum cholesterol and triglyceride concentrations were assessed using Spinreact kits (catalog nos. TKBSIS48-I and MXBSIS49-I), following the methods of [Allain et al. \(1974\)](#) and [Fossati and Prencipe \(1982\)](#), respectively. The serum levels of high-density

lipoprotein cholesterol (HDL-C) and LDL-C were measured by colorimetric Biodiagnostic kits (catalog nos. CH 12–30 and CH 12 31, respectively) per the protocols of [Lopes-Virella et al. \(1977\)](#) and [Assmann et al. \(1984\)](#), respectively. Serum cortisol concentrations were assessed by a commercial ELISA (enzyme-linked immunosorbent assay) kit (MyBioSource Inc., San Diego, CA, USA; catalog no. MBS704055), while serum glucose amounts were determined colorimetrically via a kit from the same provider (catalog no. MBS169259).

All biochemical assays were validated for fish serum applications and cross-referenced with studies on *O. niloticus* and related species ([Dawood et al., 2020](#); [Zahran et al., 2021](#); [Reda et al., 2025](#)). Analytical reproducibility was confirmed by duplicate analysis of 10% of samples, with intra-assay and inter-assay CVs of <6% and <8%, respectively.

2.8 Serum immune parameters

The assessment of complement 3 (C3) levels in fish serum was performed by MyBioSource Co. kits (California, USA), based on the company's directives (catalog no. MBS281020). C3 concentrations were measured by a two-site sandwich ELISA. Wells pre-coated with a C3-specific antibody captured C3 from samples and standards. After washing, a biotin-conjugated C3 antibody was added, followed by streptavidin-HRP. Substrate was then applied, producing a color proportional to C3 concentration, which was measured at 450 nm after stopping the reaction.

Total nitric oxide (NO) production was assessed by a colorimetric kit from MyBioSource (catalog no. MBS480450) via reducing nitrate to nitrite via the NADH-dependent enzyme nitrate reductase. The generated nitrite was then quantified by the Griess reagent ([Bryan and Grisham, 2007](#)). The final reaction product was detected spectrophotometrically at 540 nm. Serum lysozyme activity was determined using the turbidimetric method described by [Ellis \(1999\)](#) with a suspension of *Micrococcus lysodeikticus* (0.2 mg/mL) prepared in 0.05 M phosphate-buffered saline (PBS) (pH 6.2). A mixture containing 0.75 mL of the *M. lysodeikticus* suspension and 0.25 mL of serum was incubated for 5 min at 25 °C. The decrease in optical density at 540 nm was recorded every minute using a 5010 Photometer (BM Co., Germany). Lysozyme activities were calculated based on a standard curve of serially diluted chicken egg white lysozyme (Sigma, USA).

Leukocyte phagocytic activity in fish blood was measured following [Siwicki et al. \(1994\)](#). Whole blood was diluted with PBS, and leukocytes were enumerated and adjusted to a final concentration of 1×10^6 cells/mL. Yeast cells, previously coated to enhance recognition, were mixed with the leukocyte suspension at a 1:100 ratio. The mixture was then left to incubate for 30 min at 25 °C. After incubation, the cells were stained and examined under a microscope, enabling the identification and quantification of those actively engaged in phagocytosis. In parallel, the cells' respiratory burst activity was measured using the nitro blue tetrazolium (NBT) reduction method, following the approach outlined by [Rook et al. \(1985\)](#). Leukocytes (1×10^6 cells/mL) were incubated with NBT and

phorbol myristate acetate at 25 °C for 30 min. Formazan-positive cells were counted microscopically, and formazan was also quantified spectrophotometrically at 620 nm after dissolving in dimethyl sulfoxide.

All immune assays were validated for the tilapia species as previously reported ([Dawood et al., 2020](#); [Zahran et al., 2021](#)). Intra-assay and inter-assay CVs were <6% and <8%, respectively, confirming high reproducibility of the immune biomarker assessments.

2.9 Assessment of the hepatic homogenate oxidative stress markers

Hepatic MDA levels were quantified by commercial kits from Biodiagnostic Co. (Cairo, Egypt, catalog no. MD 25 29), following the scheme established by [Mihara and Uchiyama \(1978\)](#). This assay measures thiobarbituric acid reactive substances (TBARS), with MDA concentration serving as the indicator of lipid peroxidation. Catalase (CAT) activity in liver supernatants was determined by Biodiagnostic kits (catalog no. CA 25 17) based on [Aebi \(1984\)](#). The assay monitors the decomposition of hydrogen peroxide by CAT, tracking the decrease in absorbance at 240 nm as a measure of enzymatic activity. SOD activity in liver tissues was assessed using Biodiagnostic kits (catalog no. SD 25 21), following the approach of [Nishikimi et al. \(1972\)](#). This process relies on SOD's capacity to hinder the phenazine methosulfate-driven reduction of nitroblue tetrazolium, thereby quantifying enzymatic activity. For GPx, the EnzyChrom™ Glutathione Peroxidase Assay Kit (EGPX-100) from Bio-Assay Systems (California, USA) was employed, based on the protocol by [Paglia and Valentine \(1967\)](#). This colorimetric assay measures the consumed nicotinamide adenine dinucleotide phosphate in enzyme-coupled reactions, with the reduction in absorbance at 340 nm reflecting the sample GPx activity. Assay validation and performance were confirmed according to manufacturer-provided quality controls, with recovery rates between 95% and 104% and CVs below 6%. The applicability of these oxidative stress assays to *O. niloticus* has been documented in previous studies ([Abdel-Khalek et al., 2015](#); [Soror et al., 2021](#); [El-Naby et al., 2025](#)).

2.10 Hepatic endoplasmic stress and autophagy-related gene analysis

Frozen liver tissues (100 mg) were homogenized in 1 mL of TRIzol reagent, and total RNA was subsequently isolated using the easyRED™ extraction kit (iNtRON Biotechnology, Korea), following the producer's procedure. The extracted RNA was then transformed into first-strand cDNA by the QuantiTect® Reverse Transcription Kit (Qiagen, Germany). Based on the accession numbers listed in [Supplementary Table S1](#), primer sequences of ER stress-related genes [C/EBP homologous protein (*chop*), c-Jun N-terminal kinase (*jnk*), X-box binding protein-1 (*xbp-1*), and protein kinase R-like ER kinase (*perk*)] and autophagy-related genes [*beclin-1* and light chain 3 phosphatidylethanolamine

conjugate (*lc3-ii*), mammalian target of rapamycin (*mtor*), and *p62*] were designed by the NCBI Primer-BLAST tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The method of Sreedharan et al. (2018) was used to assess the efficiency of the primer. Using the QuantiTect[®] SYBR[®] Green PCR kit (Qiagen, Germany), amplification was carried out on a Rotor-Gene Q thermocycler under the following thermal profile: the reaction began with an initial denaturation at 95°C for 10 min, then proceeded through 40 amplification cycles involving denaturation at 95°C (15 s), annealing at 60°C (30 s), and extension at 72°C (30 s). The reference gene was selected based on the approach described by Rojas-Hernandez et al. (2019). To assess expression stability across the experimental groups, three housekeeping genes were evaluated: glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), β -actin, and elongation factor 1- α (*ef-1- α*). Among them, *act- β* showed the most stable expression and was therefore chosen as the reference gene. The $2^{-\Delta\Delta CT}$ approach, outlined by Livak and Schmittgen (2001), was adopted to evaluate the relative expression levels of the mRNA profiles for every gene.

2.11 Histopathological study

Intestinal, hepatic, renal, and splenic tissues were placed in 10% formalin for 48 h, subjected to a dehydration process using varying gradations of ethyl alcohol, cleansed in xylene, and subsequently embedded in paraffin blocks, which were then sliced with a microtome to 5- μ m sections. The sections were stained with hematoxylin–eosin to facilitate histopathological evaluation (Suvarna et al., 2018). Five non-overlapping fields per organ per fish were randomly selected for analysis blindly by a pathologist using a 10 $\times$ objective lens. Following the methodology outlined by Bernet et al. (1999), histopathological alterations observed in the liver (including degeneration, necrosis, inflammatory infiltration, and edema), kidney (such as interstitial edema, inflammatory cell infiltration, and glomerular shrinkage), and spleen (characterized by hypocellularity of the white pulp, sinusoidal dilation, and hemosiderin deposition) were evaluated across all experimental fish groups, as summarized in Supplementary Table S2. Histopathological changes were evaluated using a scoring system, and the average score was determined for each fish. Scores ranged from 0 (no observable alteration) to 4 (widespread lesion), indicating the severity of the changes.

2.12 Statistical analysis

To confirm that the data satisfied the homogeneity and normality assumptions, the Kolmogorov–Smirnov and Levene's tests were applied prior to analysis of variance (ANOVA). A one-way ANOVA followed by Tukey's multiple comparison test was performed to detect significant differences among treatments at the end of the experiment. Tukey's test incorporates an error correction procedure to reduce false positives across multiple pairwise

comparisons. Since the histopathological scoring data did not meet the assumptions required for parametric analysis, they were evaluated using the Kruskal–Wallis test followed by Dunn's *post hoc* test with Bonferroni-adjusted *p*-values to minimize Type I error. Results are presented as mean $\pm$ standard error (SE), with statistical significance set at $p < 0.05$. All statistical analyses were performed using IBM SPSS Statistics software, version 11.0 (IBM Corp., Armonk, NY, USA), and all figures were constructed using GraphPad Prism software, version 9.0 (GraphPad Software, San Diego, CA, USA).

3 Results

3.1 Impact on the growth performance of fish

The effects of incorporating STL into the diet of *O. niloticus* and exposure to TDNPs over 60 days are summarized in Table 2. While both the control and TDNPs-exposed groups showed increases in body weight at the end of the experiment, the TDNPs group exhibited significantly ($p < 0.05$) lower growth performance compared to the control. Specifically, fish exposed to TDNPs had a 24.74% lower FBW and condition factor (13.68%), along with significant reductions in WG (40.80%), DWG (40%), SGR (28.99%), and FI (16.55%). Additionally, the FCR was highest in the TDNPs group (increased by 41.10%) among all treatments. Moreover, the TDNPs-exposed fish showed significantly ($p < 0.05$) reduced survival by 20% compared to the control group. In contrast, STL supplementation significantly ($p < 0.05$) improved FBW (33.33%), WG (58.01%), DWG (60%), SGR (32.61%), FI (12.76%), and condition factor (10.26%), while reducing the FCR by 28.77% compared to the control group. Notably, the STL + TDNPs group showed significantly higher survival, condition factor, WG, and DWG, and lower FCR compared to the TDNPs group ($p < 0.05$).

3.2 Effects on hematological indices

Exposure of the fish to water contamination by TDNPs for 60 days led to a significant reduction ($p < 0.05$) in levels of RBCs (45.16%), HB (33.54%), PCV (28.94%), WBCs (36.79%), heterophils (32.74%), lymphocytes (30.99%), eosinophils (35.48%), and monocytes (56.98%), while MCV (29.60%) and MCH (21.44%) increased when compared to the control fish (Table 3). There was no significant change in MCHC between the treated groups ($p > 0.05$). The observed increase in MCV and MCH, along with a normal MCHC in the TDNPs group, indicated macrocytic normochromic anemia. In contrast, STL supplementation led to a significant ($p < 0.05$) increase in RBC (11.06%), PCV (5.34%), and lymphocyte (3.76%) levels compared to the group without supplementation. All altered hematological parameters were significantly ($p < 0.05$) corrected by the STL supplementation in the TDNPs-exposed fish.

TABLE 2 Effects of titanium dioxide nanoparticle (TDNPs) exposure and/or dietary α -sitosterol (STL) supplementation for 60 days on the growth performance of *Oreochromis niloticus*.

	Control	STL	TDNPs	STL + TDNPs
Initial body weight (g)	11.83 $\pm$ 0.73	12.03 $\pm$ 0.54	11.33 $\pm$ 0.88	11.97 $\pm$ 0.32
Final body weight (g)	27.00 $\pm$ 1.15 ^b	36.00 $\pm$ 1.15 ^a	20.32 $\pm$ 1.03 ^c	23.47 $\pm$ 0.35 ^{bc}
Weight gain (g)	15.17 $\pm$ 0.44 ^b	23.97 $\pm$ 0.65 ^a	8.98 $\pm$ 0.30 ^d	11.50 $\pm$ 0.40 ^c
Daily weight gain (g/day)	0.25 $\pm$ 0.01 ^b	0.40 $\pm$ 0.01 ^a	0.15 $\pm$ 0.01 ^d	0.19 $\pm$ 0.01 ^c
Specific growth rate (%)	1.38 $\pm$ 0.03 ^b	1.83 $\pm$ 0.03 ^a	0.98 $\pm$ 0.05 ^c	1.12 $\pm$ 0.04 ^c
Feed intake (g)	22.17 $\pm$ 0.44 ^b	25.00 $\pm$ 0.58 ^a	18.50 $\pm$ 0.29 ^c	20.00 $\pm$ 0.58 ^{bc}
Feed conversion ratio	1.46 $\pm$ 0.01 ^c	1.04 $\pm$ 0.01 ^d	2.06 $\pm$ 0.06 ^a	1.74 $\pm$ 0.05 ^b
Survival rate	97.78 $\pm$ 2.22 ^a	100.00 $\pm$ 0.00 ^a	80.00 $\pm$ 3.85 ^b	93.33 $\pm$ 3.85 ^a
Condition factor	2.34 $\pm$ 0.01 ^b	2.58 $\pm$ 0.03 ^a	2.02 $\pm$ 0.03 ^c	2.23 $\pm$ 0.04 ^b

Data are presented as mean $\pm$ standard error (n = 5 aquariums per treatment and 15 fish per aquarium). Values with different superscript letters (a, b, c, and d) within the same row indicate statistically significant differences among groups (p < 0.05). Statistical comparisons were performed using one-way ANOVA followed by Tukey's multiple comparison test.

3.3 Impact on hepatic function parameters, lipid profile, and renal injury markers

The modifications in serum liver enzymes, lipid profiles, and kidney damage indicators in fish subjected to TDNPs and/or diets comprising STL for 60 days are shown in Table 4. Exposure to TDNPs significantly (p < 0.05) increased the levels of serum enzymes [ALT (151.36%), AST (147.62%), and ALP (48.93%)], as well as urea (62.63%) and creatinine (86.79%). The combination of STL supplementation and TDNPs exposure significantly (p < 0.05) influenced the serum levels of ALT, ALP, AST, urea, and creatinine. Incorporating STL into the diet displayed no significant alterations in the mentioned variables (p > 0.05) when compared with the group receiving a non-supplemented diet.

In terms of the lipid profile, a significant decrease (p < 0.05) was detected in total cholesterol (18.43%), triglycerides (24.87%), and LDL-C (13.65%), while HDL-C increased by 24.51% after the fish were administered STL for 60 days (Table 4). On the other hand, exposure to TDNPs significantly elevated the lipid profile compared to the other groups. Notably, the STL + TDNPs group exhibited a significant (p < 0.05) reduction in cholesterol, triglyceride, and LDL-C levels, along with an increase in HDL-C, compared to the TDNPs group. These values approached those of the control group, with differences of only 4.53%, 4.92%, 10.86%, and 10.98%, respectively.

3.4 Stress indicators

Fish in the TDNPs group exhibited notably higher levels of blood glucose (27.81%) and cortisol (57.70%) compared to fish in other groups (p < 0.05). In contrast, compared to fish-fed basal diets without supplements, the inclusion of STL supplements led to significant reductions (p < 0.05) in glucose (19.10%) and cortisol (17.42%) levels. The dietary supplementation of STL to TDNPs-exposed groups normalized the glucose and cortisol levels (Table 4).

3.5 Effects on innate immunity parameters

The effects of TDNPs and/or STL on the non-specific immunological parameters of *O. niloticus* after a 60-day exposure are presented in Figure 1. The TDNPs group displayed a significant (p < 0.05) decrease in lysozyme activity (37.58%), C3 (21.72%), NO (52.44%), phagocytic activity (48.63%), and NBT (46.67%) compared to the control group. Conversely, the STL group demonstrated significantly (p < 0.05) higher levels of lysozyme activity (56.22%), C3 (13.74%), NO (37.5%), phagocytic activity (67.95%), and NBT (73.33%) when compared to the control group. In contrast, the immunological parameters in the serum of the STL + TDNPs group were improved compared to those of the TDNPs group, with levels nearly aligning with control values (p < 0.05) at 9.77%, 6.38%, 12.5%, 9.32%, and 20%, respectively.

3.6 Effects on hepatic antioxidant variables

As represented in Figure 2, fish that were exposed to TDNPs exhibited significantly (p < 0.05) lower levels of enzymatic antioxidants [CAT (46.23%), SOD (47.60%), and GPx (35.61%)] compared to the control fish, while displaying considerably (p < 0.05) higher levels of MDA (105.43%). Notably, liver tissues from fish fed a diet enriched with STL showed significant (p < 0.05) enhancements in CAT (27.64%) and SOD (14.03%) levels when compared to fish on a non-supplemented diet. In the TDNPs-exposed group, co-supplementation with STL significantly (p < 0.05) improved the aforementioned parameters, with values closely resembling those of the control group.

3.7 Impact on mRNA expression of genes involved in ER stress

As shown in Figure 3, the mRNA expression levels of *chop* (9.15-fold), *jnk* (12.66-fold), *xbp-1* (9.31-fold), and *perk* (12.50-fold)

TABLE 3 Effects of titanium dioxide nanoparticle (TDNPs) exposure and/or dietary α -sitosterol (STL) supplementation for 60 days on hematological indices of *Oreochromis niloticus*.

	Control	STL	TDNPs	STL + TDNPs
Erythrogram				
RBCs ($10^6/\mu\text{L}$)	2.17 ± 0.04^b	2.41 ± 0.04^a	1.19 ± 0.02^c	2.07 ± 0.04^b
HB (g/dL)	9.57 ± 0.60^a	10.93 ± 0.12^a	6.36 ± 0.06^c	7.95 ± 0.09^b
PCV (%)	22.67 ± 0.35^b	23.88 ± 0.07^a	16.11 ± 0.11^d	20.81 ± 0.17^c
MCV (fL)	104.50 ± 1.81^b	99.17 ± 1.26^b	135.43 ± 2.64^a	100.57 ± 0.97^b
MCH (pg)	44.03 ± 1.96^{bc}	45.40 ± 1.16^b	53.47 ± 1.16^a	38.40 ± 0.31^c
MCHC (%)	42.13 ± 2.09^{ab}	45.77 ± 0.58^a	39.47 ± 0.09^b	38.20 ± 0.12^b
Leucogram				
WBCs ($10^3/\mu\text{L}$)	4.43 ± 0.05^a	4.62 ± 0.04^a	2.80 ± 0.04^c	4.09 ± 0.07^b
Heterophils ($10^3/\mu\text{L}$)	1.13 ± 0.02^a	1.18 ± 0.01^a	0.76 ± 0.01^c	1.03 ± 0.02^b
Lymphocytes ($10^3/\mu\text{L}$)	2.13 ± 0.02^b	2.21 ± 0.01^a	1.47 ± 0.01^c	2.07 ± 0.01^b
Eosinophils ($10^3/\mu\text{L}$)	0.31 ± 0.01^{ab}	0.33 ± 0.01^a	0.20 ± 0.01^c	0.27 ± 0.01^b
Monocytes ($10^3/\mu\text{L}$)	0.86 ± 0.01^a	0.90 ± 0.01^a	0.37 ± 0.01^c	0.72 ± 0.02^b

Data are presented as mean $\pm$ standard error ($n = 15$). Values with different superscript letters (a, b, and c) within the same row indicate statistically significant differences among groups ($p < 0.05$). Statistical comparisons were performed using one-way ANOVA followed by Tukey's multiple comparison test. RBCs, red blood cell count; Hb, hemoglobin; PCV, packed cell volume; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBCs, white blood cell count.

in the TDNPs group were significantly elevated compared to the control group ($p < 0.05$). Yet, in the STL + TDNPs group, the mRNA expression levels of *chop* (3.28-fold), *jnk* (5.66-fold), *xbp-1* (3.22-fold), and *perk* (5.03-fold) were substantially lower than those in the TDNPs group ($p < 0.05$). When comparing the control and STL groups, no statistically significant change was found ($p > 0.05$).

3.8 Impact on mRNA expression of genes involved in autophagy

As displayed in Figure 4, there were no significant variations ($p > 0.05$) in the expression levels of *beclin-1*, *mtor*, *lc3-ii*, and *p62* when comparing the STL group to the control group ($p > 0.05$). In the group

TABLE 4 Effects of titanium dioxide nanoparticle (TDNPs) exposure and/or dietary α -sitosterol (STL) supplementation for 60 days on some serum biochemical indices of *Oreochromis niloticus*.

	Control	STL	TDNPs	STL + TDNPs
ALT (U/L)	25.33 ± 1.20^b	23.00 ± 1.15^b	63.67 ± 4.67^a	34.00 ± 2.08^b
AST (U/L)	29.67 ± 0.88^b	25.33 ± 2.03^b	73.47 ± 4.28^a	31.17 ± 0.73^b
ALP (U/L)	36.93 ± 1.10^b	35.37 ± 0.84^b	55.00 ± 2.89^a	40.00 ± 1.15^b
Urea (mg/dL)	2.81 ± 0.06^b	2.81 ± 0.09^b	4.57 ± 0.23^a	3.05 ± 0.08^b
Creatinine (mg/dL)	0.53 ± 0.02^{bc}	0.46 ± 0.02^c	0.99 ± 0.01^a	0.61 ± 0.02^b
TC (mg/dL)	110.33 ± 1.45^b	90.00 ± 1.15^c	127.33 ± 1.45^a	115.33 ± 1.45^b
TG (mg/dL)	92.77 ± 1.25^b	69.70 ± 1.42^c	107.33 ± 1.45^a	97.33 ± 0.88^b
HDL-C (mg/dL)	26.77 ± 0.72^b	33.33 ± 0.88^a	20.17 ± 0.60^c	23.83 ± 1.01^{bc}
LDL-C (mg/dL)	75.77 ± 1.97^c	65.43 ± 1.44^d	93.83 ± 1.64^a	84.00 ± 2.08^b
Glucose (mg/dL)	74.33 ± 2.33^b	60.13 ± 0.59^c	95.00 ± 2.65^a	81.00 ± 2.08^b
Cortisol (ng/mL)	56.10 ± 1.24^b	46.33 ± 1.45^c	88.47 ± 1.35^a	60.67 ± 0.88^b

Data are presented as mean $\pm$ standard error ($n = 15$). Values with different superscript letters (a, b, and c) within the same row indicate statistically significant differences among groups ($p < 0.05$). Statistical comparisons were performed using one-way ANOVA followed by Tukey's multiple comparison test. ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

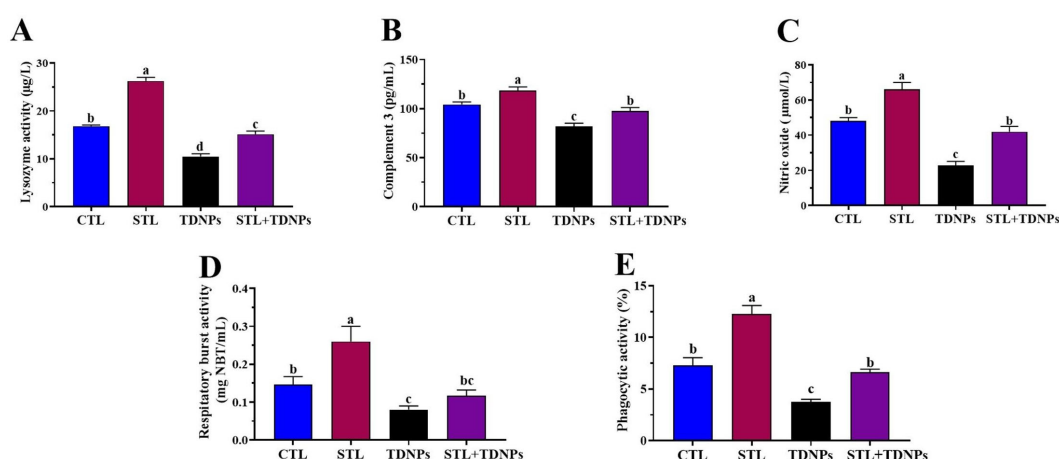


FIGURE 1

Effects of titanium dioxide nanoparticle (TDNPs) exposure and/or dietary α -sitosterol (STL) supplementation for 60 days on serum innate immune parameters in *Oreochromis niloticus*: (A) lysozyme activity, (B) complement 3 (C3) content, (C) nitric oxide (NO) concentration, (D) respiratory burst activity, and (E) phagocytic activity (%). Data are presented as mean $\pm$ standard error ($n = 15$). Statistical comparisons were performed using one-way ANOVA followed by Tukey's multiple comparison test. Bars with different superscript letters (a, b, and c) indicate statistically significant differences among groups ($p < 0.05$).

exposed to TDNPs, a significant increase ($p < 0.05$) in *mtor* (7.15-fold) and *p62* (10.57-fold) gene expression and a significant decrease ($p < 0.05$) in *beclin-1* (0.21-fold) and *lc3-ii* (0.28-fold) gene expression were observed when compared to the control group. Conversely, the group

treated with both STL and TDNPs exhibited notable downregulation of *mtor* (3.01-fold) and *p62* (3.15-fold) genes, along with significant upregulation ($p < 0.05$) in *beclin-1* (0.67-fold) and *lc3-ii* (0.90-fold) gene expression in comparison to the group exposed to TDNPs.

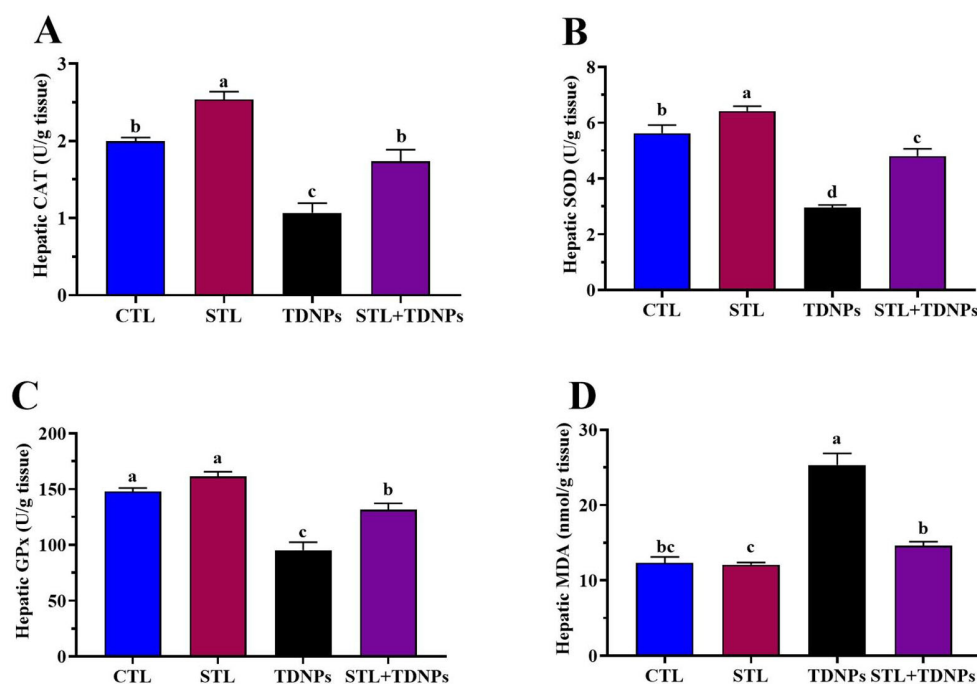


FIGURE 2

Effects of titanium dioxide nanoparticle (TDNPs) exposure and/or dietary α -sitosterol (STL) supplementation for 60 days on hepatic oxidative stress biomarkers in *Oreochromis niloticus*: (A) catalase (CAT) activity, (B) superoxide dismutase (SOD) activity, (C) glutathione peroxidase (GPx) content, and (D) malondialdehyde (MDA) concentration. Data are presented as mean $\pm$ standard error ($n = 15$). Statistical comparisons were performed using one-way ANOVA followed by Tukey's multiple comparison test. Bars with different superscript letters (a, b, c, and d) indicate statistically significant differences among groups ($p < 0.05$).

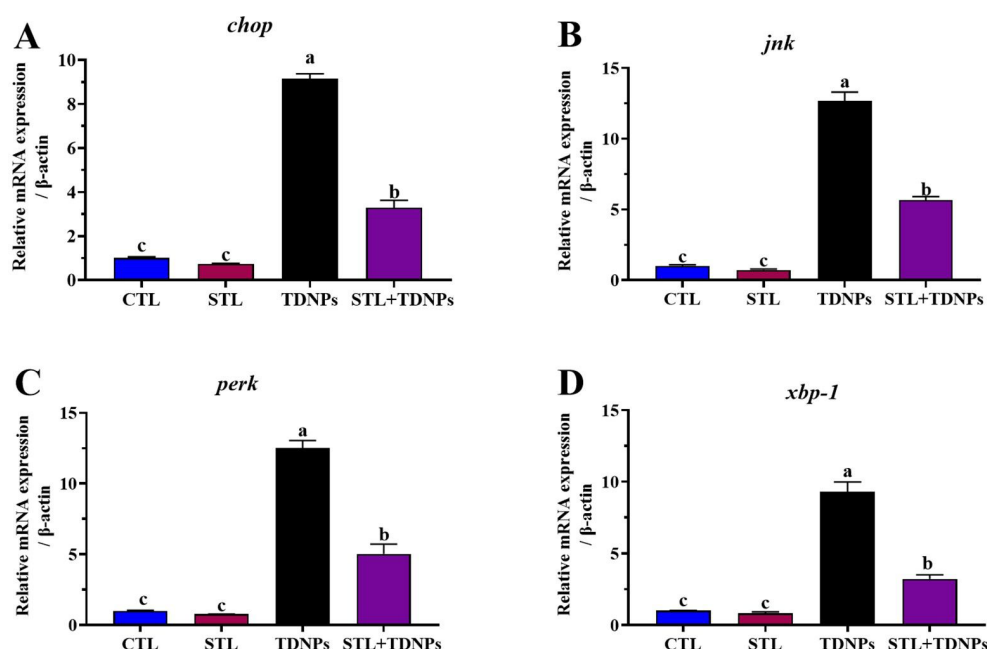


FIGURE 3

Effects of titanium dioxide nanoparticle (TDNPs) exposure and/or dietary α -sitosterol (STL) supplementation for 60 days on hepatic mRNA expression of ER stress-related genes in *Oreochromis niloticus*: (A) C/EBP homologous protein (*chop*), (B) c-Jun N-terminal kinase (*jnk*), (C) protein kinase RNA-like endoplasmic reticulum kinase (*perk*), and (D) X-box binding protein 1 (*xbp-1*). Data are presented as mean $\pm$ standard error ($n = 15$). Statistical comparisons were performed using one-way ANOVA followed by Tukey's multiple comparison test. Bars with different superscript letters (a, b, and c) indicate statistically significant differences among groups ($p < 0.05$).

3.9 Histopathological investigations

Histological examinations revealed organ-specific pathological alterations following TDNPs exposure, with varying degrees of severity across the intestine, liver, kidney, and spleen. Representative photomicrographs for all organs are presented in Figures 5–8, while corresponding lesion scores are detailed in Table 5. Notably, STL dietary supplementation demonstrated a consistent protective effect across all examined tissues, reducing the extent and severity of TDNPs-induced damage.

3.9.1 Intestine

Histological sections of intestines from the control (Figure 5A) and STL-supplemented groups (Figure 5B) showed normal architecture, including intact mucosal villi, submucosa, musculosa, and serosa. The STL group exhibited more elongated and branched villi. In contrast, the intestines of the TDNPs-exposed group (Figure 5C) demonstrated severe pathological alterations, including significant desquamation of mucosal epithelium (3.20 ± 0.20), submucosal edema (1.80 ± 0.25), and leukocytic infiltration (2.70 ± 0.30), all of which were significantly higher ($p < 0.05$) compared to control and STL groups. Co-treatment with STL (Figure 5D) ameliorated these effects, as indicated by significantly reduced lesion scores for mucosal desquamation (0.90 ± 0.28) and submucosal edema (0.50 ± 0.17).

3.9.2 Liver

Liver tissues from control (Figure 6A) and STL groups (Figure 6B) maintained normal histo-morphological configurations,

including pancreatic acini and hepatocytes. However, TDNPs exposure (Figure 6C) caused significant ($p < 0.05$) hepatic degeneration, characterized by necrotic hepatocytes (2.20 ± 0.25), degenerated hepatocytes (2.90 ± 0.23), perivascular inflammatory infiltrates (2.60 ± 0.31), and congestion of blood vessels (2.70 ± 0.30), all significantly elevated versus control. STL co-treatment (Figure 6D) led to marked improvement, with significantly ($p < 0.05$) lower scores for hepatocellular degeneration (0.70 ± 0.26), necrosis (0.30 ± 0.15), and inflammatory infiltrates (0.70 ± 0.21).

3.9.3 Kidney

Renal tissues from control (Figure 7A) and STL groups (Figure 7B) showed preserved glomeruli and tubules. Conversely, the TDNPs group (Figure 7C) exhibited severe renal damage, including necrotic tubular epithelium (2.90 ± 0.23), hypocellular glomeruli (1.80 ± 0.25), and vascular congestion (2.90 ± 0.23), all significantly ($p < 0.05$) increased compared to controls. STL supplementation (Figure 7D) attenuated renal injury, as evidenced by reduced lesion scores for necrotic tubules (0.40 ± 0.16), glomerular damage (0.70 ± 0.21), and congestion (0.70 ± 0.21), with statistically significant ($p < 0.05$) improvement over the TDNPs group.

3.9.4 Spleen

Spleen sections from control (Figure 8A) and STL groups (Figure 8B) had normal cytoarchitecture with intact white pulp and melanomacrophage centers. The TDNPs group (Figure 8C) displayed prominent splenic alterations, including depleted white

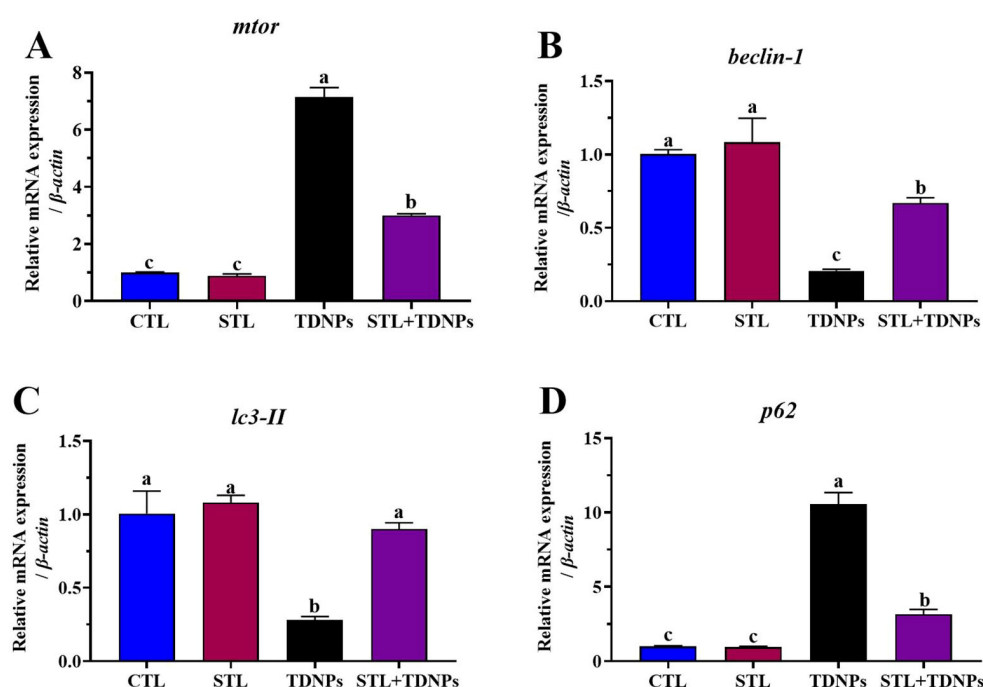


FIGURE 4

Effects of titanium dioxide nanoparticle (TDNPs) exposure and/or dietary α -sitosterol (STL) supplementation for 60 days on hepatic mRNA expression of autophagy-related genes in *Oreochromis niloticus*: (A) mammalian target of rapamycin (*mtor*), (B) *beclin-1*, (C) light chain 3 phosphatidylethanolamine conjugate (*lc3-II*), and (D) *p62*. Data are presented as mean $\pm$ standard error ($n = 15$). Statistical comparisons were performed using one-way ANOVA followed by Tukey's multiple comparison test. Bars with different superscript letters (a, b, and c) indicate statistically significant differences among groups ($p < 0.05$).

pulp (2.20 ± 0.20), increased melanomacrophage centers (2.80 ± 0.25), and pelosis (2.90 ± 0.28), which were significantly ($p < 0.05$) higher than in controls. The STL + TDNPs group (Figure 8D) displayed significant ($p < 0.05$) restoration in splenic architecture, with white pulp depletion reduced to 0.50 ± 0.17 and melanomacrophage score lowered to 1.90 ± 0.18 .

Lesion scores summarized in Table 5 underscore the differential susceptibility of each organ to TDNPs-induced toxicity. Among the tissues examined, the liver and kidney exhibited the highest lesion severity, followed by the spleen and intestine. STL supplementation significantly attenuated pathological changes in all organs, with the most notable protective effects observed in renal and hepatic tissues.

4 Discussion

In the current study, dietary STL markedly ameliorated the multiple adverse effects induced by 60-day waterborne exposure to 10 mg/L TDNPs in Nile tilapia. TDNPs alone caused growth suppression, hematological disruption, immunosuppression, oxidative stress, ER stress, and autophagy dysregulation. In contrast, STL co-treatment restored these parameters toward control levels, likely via improved intestinal integrity, enhanced antioxidant defenses, and modulation of stress- and autophagy-related gene expression.

4.1 Growth performance and intestinal morphology

Herein, fish exposed to 10 mg/L TDNPs for 60 days exhibited a significant reduction in growth performance indicators compared with controls, consistent with growth retardation reported in carp under similar TDNPs exposures (e.g., 1 mg/L TDNPs for 10 days; Haghighat et al., 2021). The observed villus atrophy and epithelial desquamation in the anterior intestine of TDNPs-exposed fish point to impairment of nutrient absorption due to oxidative and inflammatory injury to the intestinal mucosa, reducing digestive enzyme activity and nutrient uptake, thereby reducing growth (Cunha and De Brito-Gitirana, 2020; MaChado Kayser et al., 2022). In contrast, STL supplementation (80 mg/kg diet) preserved villus height and mucosal integrity, and improved FI, feed efficiency, and growth performance. Mechanistically, STL's antioxidant properties limit ROS-induced lipid peroxidation in enterocytes, while its membrane-stabilizing and anti-inflammatory effects maintain barrier function and nutrient transport capacity (El-Houseiny et al., 2025). Additionally, improved activities of intestinal digestive enzymes, particularly protease activity under STL, have been reported in largemouth bass (*Micropterus salmoides*) receiving phytosterol-enriched diets (0.1% and 0.5%) for 56 days, which could consequently enhance feed utilization efficiency (Liang et al., 2024).

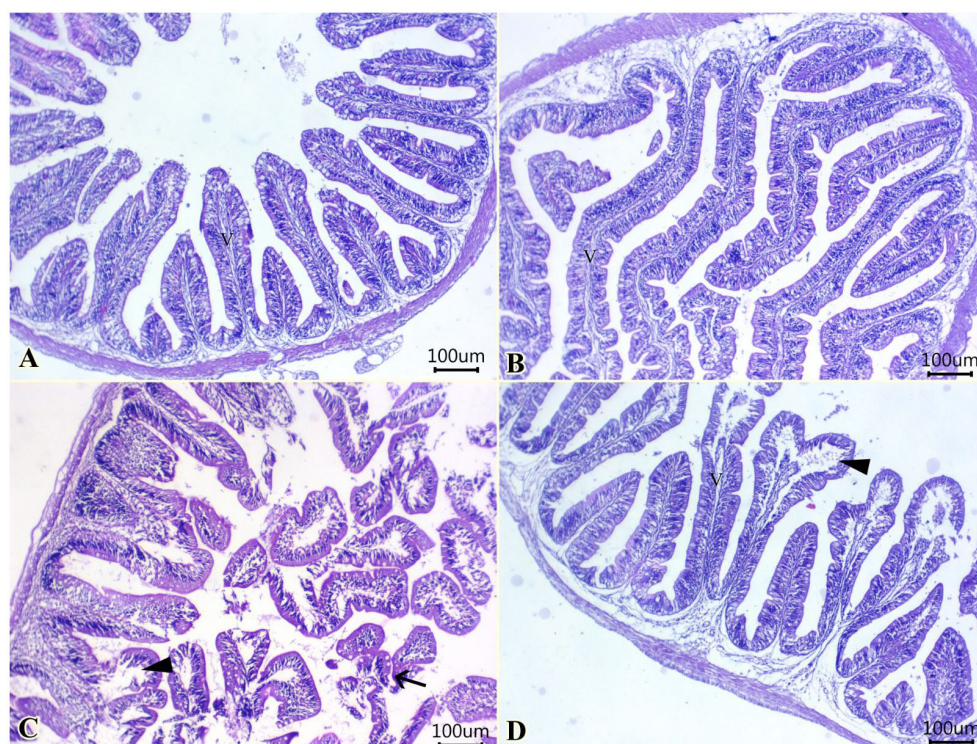


FIGURE 5

(A–D) Representative photomicrographs of H&E-stained sections from the intestine showing normal mucosal villi (V), submucosa, muscosa, and serosa in both control (A) and α -sitosterol (STL)-treated fish (B). Desquamated sheets of mucosal epithelium (arrow), and edema within lamina propria, submucosa (arrowhead) in titanium dioxide nanoparticle (TDNPs)-exposed fish (C). Mild edema within the submucosal layer (arrowhead) at the STL + TDNPs group (D) with apparently normal villi, and the muscular layer. H&E bar, 100 μ m.

4.2 Hematological parameters

TDNPs exposure macrocytic normochromic anemia, evidenced by decreased RBC, Hb, and PCV and increased MCV and MCH, which is probably mediated by erythrocyte oxidative injury, hemolysis, and membrane lipid oxidation (Vasantharaja et al., 2015). Specifically, TDNPs exposure in *T. carolinus* (3.0 μ g/g by I/P injection) provoked the formation of various erythrocyte nuclear aberrations and micronuclei and subsequently diminished erythrocyte viability (Vignardi et al., 2015). Additionally, the electrostatic interactions between TDNPs surfaces and negatively charged erythrocyte membrane lipids could cause structural perturbations, leading to echinocyte formation and leakage of Hb (Li et al., 2008). Leukopenia and reduced differential counts further confirm TDNPs interference with hematopoietic tissues, possibly through direct ROS damage to spleen and kidney progenitor cells (Carmo et al., 2019).

Instead, STL co-treatment restored RBC, Hb, PCV, and WBC counts, likely through reinforcement of antioxidant defense systems (CAT, SOD, and GPx) that protect hematopoietic niches from ROS-induced damage, reflected in the enhanced splenic architecture (El-Houseiny et al., 2025). STL may also enhance hepatic erythropoietic support via upregulation of antioxidant gene expression and improved protein synthesis capacity (Ji et al., 2021). These mechanistic effects collectively indicate that STL acts as both an antioxidant and a cytoprotective modulator of erythropoiesis under NPs stress.

4.3 Hepatic and renal function biomarkers

Exposure to TDNPs significantly increased serum ALT, AST, and ALP levels and elevated urea and creatinine, indicating hepatic enzyme leakage and renal dysfunction due to oxidative and inflammatory tissue damage (Banaee et al., 2019). Histopathological findings of hepatic necrosis and renal tubular degeneration confirm this hepatonephrotoxicity. In contrast, STL co-administration lowered these enzymes and renal indices, indicating hepatoprotective and nephroprotective actions. Comparable hepatoprotective effects of dietary STL (60 and 80 mg/kg diet for 60 days) were recently reported in Nile tilapia by El-Houseiny et al. (2025). STL's hepatoprotective and nephroprotective mechanisms likely involve attenuation of oxidative stress via radical scavenging, stabilization of cellular membranes, and upregulation of endogenous antioxidant systems (Gumede et al., 2024). STL has been shown to modulate lipid metabolism, suppress inflammatory cytokine expression, and promote hepatocellular regeneration (Devaraj et al., 2020). These combined effects support restoration of hepatic enzyme activities and renal function, reducing serum urea and creatinine accumulation.

4.4 Serum lipid profile and stress indices

Herein, the dyslipidemia observed in TDNPs-exposed fish (elevated TC, TG, and LDL-C and reduced HDL-C) reflects

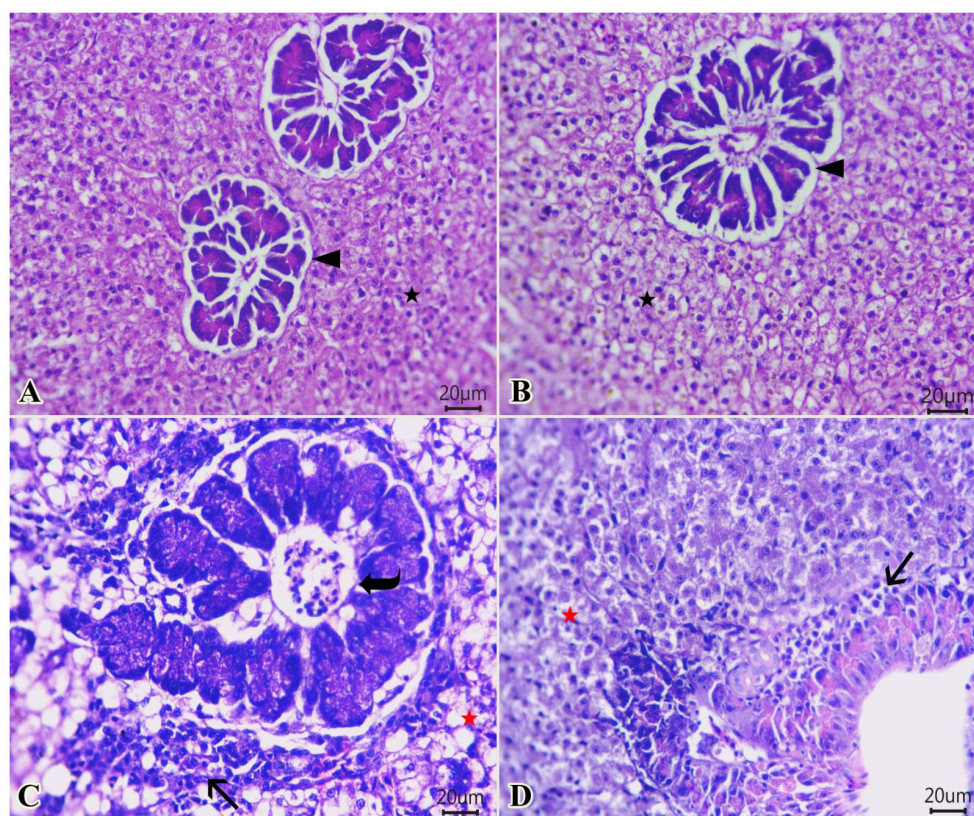


FIGURE 6

(A–D) Representative photomicrographs of H&E-stained sections from liver showing normal histo-morphological structures of hepatocytes, pancreatic acini, and vascular tissues in both control (A) and STL-treated fish (B). Diffuse areas of necrosis (red star) and hydropic degeneration within a large number of hepatocytes, peripancreatic round cell infiltrations (arrow), and congested portal veins (curved arrow) in TDNPs-exposed fish (C). Unicellular necrotic hepatocytes (red star) and a minute number of peripancreatic lymphocytes (arrow) at the STL + TDNPs group (D). H&E bar, 20 μm.

disrupted hepatic lipid metabolism and oxidative damage to lipoproteins. TDNPs can interfere with lipid homeostasis by inducing ER stress and oxidative modification of apolipoproteins (Anagha et al., 2019). Furthermore, TDNPs-exposed fish showed elevated serum glucose and cortisol levels, indicating stress-induced hyperglycemia, possibly due to activation of gluconeogenic pathways and glycogenolysis (Banaee et al., 2019; Hajirezaee et al., 2020). TDNPs-induced oxidative stress may activate the hypothalamic–pituitary–interrenal axis, increasing cortisol production and subsequently promoting gluconeogenesis, which leads to elevated glucose levels (Canli et al., 2018; Carmo et al., 2019).

In contrast, STL supplementation markedly reduced LDL-C, TC, and TG, and elevated HDL-C probably by inhibiting intestinal cholesterol absorption (competitive uptake with phytosterols) and downregulating hepatic 3-hydroxy-3-methylglutaryl-CoA reductase, while upregulating LDL receptor expression (Chen et al., 2020). In addition, phytosterols have been reported to alter cholesterol metabolism by reducing endogenous cholesterol synthesis and increasing cholesterol catabolism (Jiang et al., 2024). Moreover, STL's hypolipidemic and antioxidant properties may protect hepatocytes from steatotic injury. Furthermore, herein, STL co-treatment significantly lowered both glucose and cortisol

serum concentrations. STL mitigated these stress responses, possibly through stabilization of glucose metabolism, enhanced insulin sensitivity, and inhibition of cortisol overproduction (Ramalingam et al., 2020).

4.5 Innate immunity and phagocytic function

In this study, TDNPs exposure caused immunosuppression as evidenced by reduced lysozyme activity, C3, NO, and phagocytic activities, likely due to NPs accumulation in macrophages and interference with ROS-dependent microbial killing (Couleau et al., 2012; Hajirezaee et al., 2020). Furthermore, exposure to TDNPs has been shown to significantly alter the expression of various pro-inflammatory cytokines, leading to an imbalance that contributes to an inflammatory response capable of suppressing normal immune function (Vineetha et al., 2021; Sherif et al., 2022). In contrast, STL co-treatment restored innate immune responses through enhancement of macrophage phagocytic activity, increased production of lysozyme and complement factors, and stabilization of splenic and renal immune tissue architecture. STL's immunostimulatory action may derive from membrane incorporation in leukocytes, facilitating receptor-mediated

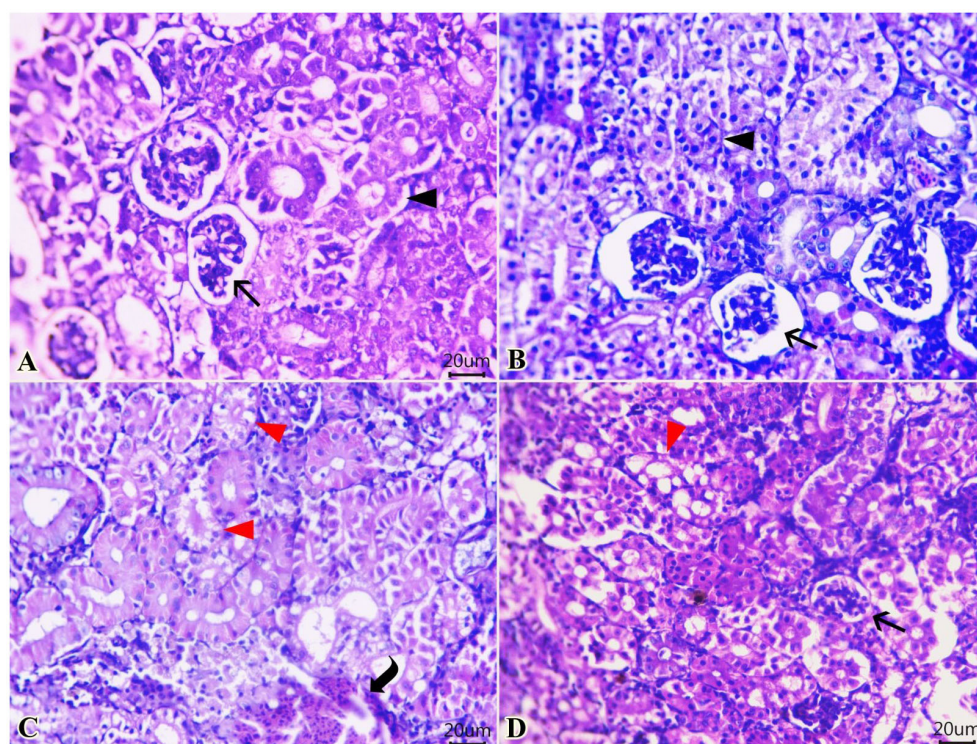


FIGURE 7

(A–D) Representative photomicrographs of H&E-stained sections from the kidney showing normal morphology of renal tubules (arrowheads), glomerular tufts (arrows), and stromal tissues in both control (A) and STL (B). Oncotic necrosis in a large number of renal tubular epithelium (red arrowheads) beside congested renal vasculature (curved arrow) in TDNPs-exposed fish (C). Improved renal parenchyma with less tubular necrosis (red arrowhead) and apparent normal glomerular tufts (arrow) at the STL + TDNPs group (D).

pathogen recognition and complement activation (Shi et al., 2013; Luhata and Usuki, 2021). Moreover, phytosterols are known to modulate immune cell signaling via the NF- κ B pathway, leading to enhanced cytokine balance and immune surveillance (Song et al., 2022). In this respect, Reyes-Becerril et al. (2022) reported that a phytosterol-rich plant extract, such as the aqueous root extract of *Cylindropuntia cholla*, exhibited a significant immunomodulatory activity in primary cultures of tilapia peripheral blood leukocytes, as evidenced by increased phagocytic activity, respiratory burst, and NO production. Similarly, STL supplementation (60 and 80 mg/kg) for 60 days in Nile tilapia led to increased immune resilience and reduced inflammation in vital organs (El-Houseiny et al., 2025). Moreover, dietary β -STL (1%–2%) improved intestinal immune function by increasing lysozyme and complement levels, while augmenting anti-inflammatory markers and dwindling pro-inflammatory cytokines in large yellow croaker (*Larimichthys crocea*) (Song et al., 2022).

4.6 Oxidative stress, autophagy, and ER stress

In the current study, TDNPs induced hepatic oxidative stress, as evidenced by decreased activities of CAT, SOD, and GPx, alongside increased MDA levels, indicating lipid peroxidation. This is consistent with previous reports showing that TDNPs generate

ROS, which damage cellular components and trigger lipid peroxidation in fish (Carmo et al., 2019; Tang et al., 2019; Bobori et al., 2020). Elevated ROS levels can further disrupt ER homeostasis, leading to the accumulation of misfolded proteins and activation of the ER stress response (Chen et al., 2021; Uribe-García et al., 2025). This response is mediated by the unfolded protein response, primarily regulated through three ER stress sensors: inositol-requiring enzyme 1 α (IRE1 α), PERK, and activating transcription factor 6 (ATF6) (Credle et al., 2005). Upon ER stress, these sensors are activated following dissociation from binding immunoglobulin protein (BiP), an ER chaperone involved in protein folding (Smith and Wilkinson, 2017). IRE1 α promotes splicing of XBP-1 mRNA and activation of JNK, enhancing the protein-folding capacity, whereas PERK reduces global protein synthesis to alleviate ER load (Jiang et al., 2021; Shi et al., 2023). ER stress is intimately linked to autophagy, a protective mechanism that removes misfolded proteins and damaged organelles (Rashid et al., 2015). Activation of the IRE1 and PERK pathways can induce autophagy via JNK signaling and beclin-1 modulation (Shi et al., 2023). Conversely, the mTOR pathway negatively regulates autophagy by inhibiting AMP-activated protein kinase signaling (Kim et al., 2011). Herein, TDNPs exposure significantly upregulated ER stress-related genes (*chop*, *jnk*, *xbp-1*, and *perk*), while downregulating autophagy-related genes (*beclin-1* and *lc3-ii*) and upregulating *mtor* and *p62*, indicating ER initiation and autophagy inhibition. Similarly, TDNP exposure

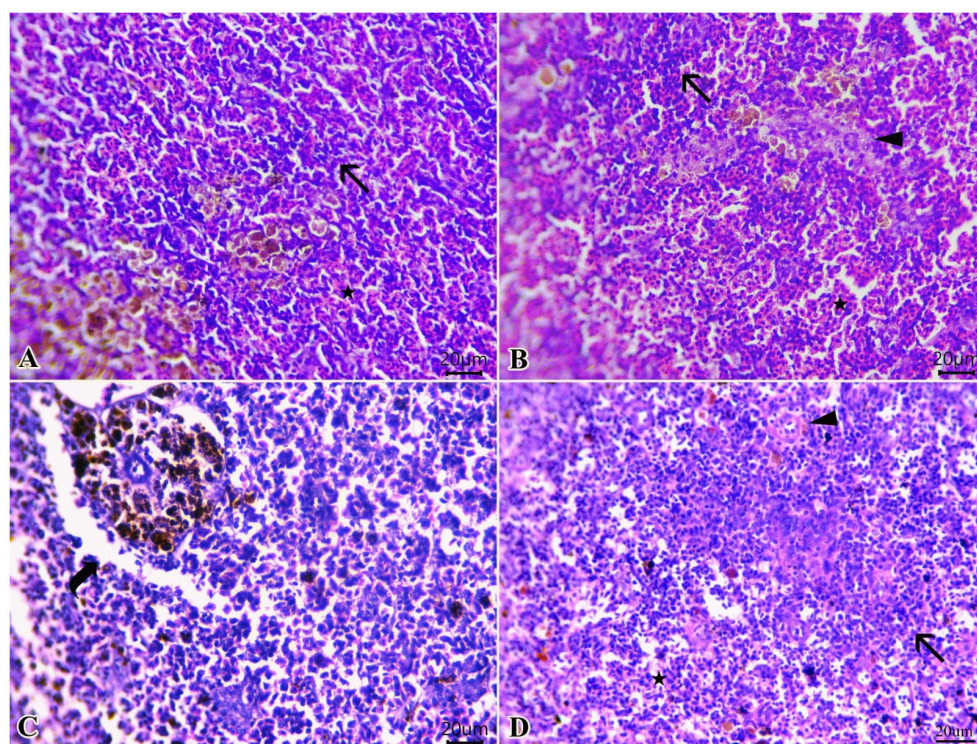


FIGURE 8

(A–D) Representative photomicrographs of H&E-stained sections from spleen showing normal histology of white pulps (arrow) around central arterioles (arrowhead) alternated with red pulps (star) in both control (A) and STL (B). Low density of lymphoid elements, particularly at the white pulp (curved arrow) in TDNPs-exposed fish (C). Re-populated cytoarchitectures of white pulp (arrow) around the ellipsoidal arterioles (arrowhead) beside normal red pulp (star) at the STL + TDNPs group (D). H&E bar, 20 μ m.

has been reported to dysregulate autophagy markers, including *beclin-1*, *lc3*, and *p62*, ultimately leading to autophagy inhibition and cellular necrosis in zebrafish models (Kotil et al., 2017; Siqueira et al., 2021). Collectively, TDNPs initiate a cascade in which oxidative stress triggers ER stress, which, in turn, modulates autophagy pathways, culminating in hepatic cell dysfunction. On the other hand, STL co-treatment restored antioxidant enzyme activities and reduced MDA. Similarly, dietary supplementation with STL at 60 and 80 mg/kg significantly increased hepatic GPx, CAT, and SOD activities in Nile tilapia after 60 days of feeding (El-Houseiny et al., 2025). Dietary STL antioxidant capacity could be strongly related to its role as a hydrogen donor or its capability to directly counteract stable free radicals (Cheng et al., 2019). Of note, STL also normalized UPR gene expression and autophagy markers in the hepatic tissues of TDNPs-exposed fish. Recently, β -STL enhanced autophagic activity by promoting *lc3-i* conversion to *lc3-ii* and *p62* degradation in lipopolysaccharide-stimulated RAW264.7 macrophages (Li et al., 2024). Similarly, in endothelial cells, β -STL suppresses inflammatory cytokine production by regulating MAPK and NF- κ B signaling, which is associated with autophagic activity (Bi et al., 2023). These effects likely stem from STL's free-radical scavenging and membrane-protective properties (Zhang et al., 2023), which alleviate ER stress and permit autophagic flux. Moreover, Tang et al. (2024) reported that a key mechanism through which β -STL could reduce ER stress involves the modulation of cholesterol

metabolism and the inhibition of cholesterol overload, which is a known trigger for ER stress. Moreover, Abo-Zaid et al. (2023) suggested that the antisteatotic effects of β -STL may alleviate lipid accumulation in rats fed a high-fat diet, thereby reducing ER stress. In line with this, our study demonstrated a pronounced cholesterol-lowering effect of STL, with a significant reduction in LDL-C.

However, some limitations of this study should be acknowledged. The experiment was conducted under controlled laboratory conditions using a single exposure concentration (10 mg/L TDNPs) and a single dietary STL dose (80 mg/kg), which may not fully represent environmental exposure scenarios. Natural water systems contain fluctuating NPs concentrations and complex physicochemical interactions that could alter bioavailability and toxicity. Additionally, fish in natural environments are exposed to multiple stressors, including temperature variation, pathogens, and dietary differences, which may influence responses to TDNPs and STL supplementation. The present study focused on a single TDNPs concentration and dietary STL dose; thus, the dose dependency and long-term safety warrant further investigation. Future studies should incorporate dose-response analyses to further elucidate the potential modulatory mechanisms of STL against NPs toxicity. Moreover, these findings were obtained in Nile tilapia under laboratory conditions, and their generalizability to other fish species or ecosystems remains to be validated. Investigations under semi-field or field conditions with

TABLE 5 Effects of titanium dioxide nanoparticle (TDNPs) exposure and/or dietary α -sitosterol (STL) supplementation for 60 days on lesion score of the intestinal, hepatic, renal, and splenic tissues of *Oreochromis niloticus*.

	Control	STL	TDNPs	STL + TDNPs
Intestinal tissue				
Desquamated mucosal sheets	0.00 ± 0.00 ^b	0.10 ± 0.10 ^b	2.70 ± 0.30 ^a	0.50 ± 0.17 ^b
Edema in the submucosa	0.00 ± 0.00 ^c	0.20 ± 0.13 ^c	3.20 ± 0.20 ^a	0.90 ± 0.28 ^b
Leukocytic infiltrates	0.00 ± 0.00 ^b	0.10 ± 0.10 ^b	1.80 ± 0.25 ^a	0.50 ± 0.17 ^b
Hepatic tissue				
Degenerated hepatocytes	0.00 ± 0.00 ^c	0.10 ± 0.10 ^{bc}	2.90 ± 0.23 ^a	0.70 ± 0.26 ^b
Degenerated pancreatic acini	0.00 ± 0.00 ^b	0.10 ± 0.10 ^b	2.50 ± 0.29 ^a	0.60 ± 0.22 ^b
Necrotic hepatic cells	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	2.20 ± 0.25 ^a	0.30 ± 0.15 ^b
Round inflammatory cell infiltration	0.00 ± 0.00 ^b	0.20 ± 0.13 ^b	2.60 ± 0.31 ^a	0.70 ± 0.21 ^b
Congested hepatic blood vessels	0.00 ^c ± 0.00	0.10 ± 0.10 ^c	2.70 ± 0.30 ^a	0.90 ± 0.18 ^b
Renal tissue				
Degenerated renal tubular epithelium	0.00 ± 0.00 ^c	0.10 ± 0.10 ^c	2.90 ± 0.23 ^a	1.00 ± 0.26 ^b
Necrotic tubular epithelium	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	2.90 ± 0.23 ^a	0.40 ± 0.16 ^b
Hypocellular glomerular tufts	0.00 ± 0.00 ^c	0.10 ± 0.10 ^{bc}	1.80 ± 0.25 ^a	0.70 ± 0.21 ^b
Congested renal vasculatures	0.00 ± 0.00 ^c	0.10 ± 0.10 ^{bc}	2.90 ± 0.23 ^a	0.70 ± 0.21 ^b
Splenic tissue				
Depleted white pulp	0.00 ± 0.00 ^c	0.00 ± 0.00 ^c	2.20 ± 0.20 ^a	0.50 ± 0.17 ^b
Melanomacrophage centers	1.20 ± 0.13 ^c	1.20 ± 0.13 ^c	2.80 ± 0.25 ^a	1.90 ± 0.18 ^b
Pelosis	0.00 ± 0.00 ^c	0.20 ± 0.13 ^{bc}	2.90 ± 0.28 ^a	0.80 ± 0.25 ^b
Interstitial edema	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	1.70 ± 0.26 ^a	0.60 ± 0.22 ^b

Data are presented as mean ± standard error ($n = 15$). Values with different superscript letters (a, b, and c) within the same row indicate statistically significant differences among groups ($p < 0.05$). Statistical comparisons were performed using the Kruskal–Wallis test followed by Dunn's *post hoc* test with Bonferroni correction.

varied exposure periods are therefore needed to confirm the ecological relevance and broader applicability of the present results.

5 Conclusion

This study indicated the potential of STL as an effective natural additive for mitigating the harmful effects of TDNPs in *O. niloticus*. The results showed that TDNPs exposure led to reduced body weight, impaired liver and kidney functions, and the onset of macrocytic normochromic anemia, leukopenia, immunosuppression, hyperlipidemia, oxidative stress, ER stress, and increased autophagy in the liver. STL effectively counteracted these adverse effects by preventing weight loss, restoring hepatic and renal functions, improving hematological and immunological parameters, and correcting lipid abnormalities. Moreover, STL reduced oxidative stress, alleviated ER stress, and modulated autophagy. These findings indicate that STL functions as a growth promoter, antioxidant, and immunostimulant, preserving tissue integrity and potentially offering health benefits relevant to human consumption. Therefore, STL may be considered a promising natural supplement for inclusion in aquafeed.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#). Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by Animal Ethics Committee of Zagazig University's, with an approval number ZU-IACUC/2/F/85/2025. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AB: Writing – original draft, Data curation, Methodology, Software, Conceptualization. WE-H: Writing – original draft, Software, Conceptualization, Methodology, Data curation, Investigation. AM: Resources, Software, Writing – review & editing, Conceptualization. YA-E: Visualization, Conceptualization, Software, Writing – review &

editing. AA: Writing – review & editing, Conceptualization, Resources, Software. YA: Conceptualization, Software, Resources, Writing – review & editing. HH: Data curation, Visualization, Writing – review & editing, Software. NA-A: Methodology, Conceptualization, Writing – review & editing, Investigation.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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