Green synthesis of zinc oxide nanoparticles using Caulerpa chemnitzia and evaluation of their effects against methylparaben-induced organ changes in zebrafish

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Abstract

The increasing use of pesticides like methylparaben (MeP) poses significant environmental and health risks, especially in aquatic ecosystems. This study aimed to synthesize ZnO-NPs using Caulerpa chemnitzia extract (C@ZnO-NPs) and evaluate their physicochemical characteristics and therapeutic potential in mitigating MeP-induced oxidative stress, inflammation and behavioral toxicity in zebrafish. ZnO-NPs were synthesized via a green route using C. chemnitzia extract and characterized using UV-Vis spectroscopy, XRD, FTIR, DLS, zeta potential analysis, SEM-EDAX and HR-TEM. Behavioral assays (Light-Dark Test, Novel Tank Test and T-Maze), biochemical analyses (antioxidants, ROS, LPO and ALT), histopathology, western blotting, and qRT-PCR were performed on zebrafish exposed to MeP. UV-Vis (360 nm peak) and XRD confirmed the formation of crystalline C@ZnO NPs. FTIR and EDAX validated functional group involvement and elemental purity. DLS and zeta potential analyses indicated moderate stability. Behavioral impairments due to MeP were dose-dependently reversed by ZnO-NPs, particularly at 40 mg/L. Antioxidant levels were restored, and oxidative stress and ALT levels were reduced. Histological damage in gill and liver tissues improved with treatment. Inflammatory markers (NF-κB and TNF-α) were significantly downregulated. C@ZnO-NPs effectively ameliorated MeP-induced neurotoxicity, oxidative stress and inflammation in zebrafish, highlighting their potential as a green nanotherapeutic agent in environmental toxicology.

Highlights

- ZnO-NPs successfully synthesized using *Caulerpa chemnitzia* extract; confirmed by UV–Vis, XRD, FTIR, DLS, SEM/TEM.
- ZnO-NPs reversed MeP-induced anxiety-like behavior in zebrafish in light-dark, novel tank, and T-maze tests, showing dose- and time-dependent neuroprotection.
- ZnO-NPs restored antioxidant enzymes, reduced LPO and ROS, and normalized elevated ALT levels, indicating liver and gill protection.
- ZnO-NPs alleviated MeP-induced histopathology in gill/liver and downregulated NF-κB and TNF-α expression.

Introduction

Methylparaben (MP) is a synthetic preservative often used in cosmetics, medicines, and food products (Soni et al. 2002). Despite its ability to prolong shelf life by inhibiting microbial growth, there have been is concerned regarding its possible toxicity and endocrine-disrupting effects (Nowak et al. 2018). Methylparaben is known to mimic estrogen, which can cause hormonal abnormalities, developmental toxicity, and reproductive issues, especially in aquatic organisms (Boberg et al. 2010). Its broad usage has prompted concerns about its environmental reliability and bioaccumulation, particularly in aquatic environments where it has been found in water bodies, resulting in exposure in aquatic species such as zebrafish (Dasmahapatra et al. 2024). Recent research has shown that methylparaben exposure can

induce oxidative stress, inflammation, and damage to numerous organs such as the liver, kidneys, and heart in both mammals and aquatic animals (Calisir et al. 2025; Hu et al. 2022).

Exposure to methylparaben in zebrafish, a frequently used model organism for toxicological studies (Cassar et al. 2019), has been found to cause changes in the structure and function of internal organs such as the gills and liver, as well as developmental problems in embryos (Hu et al. 2023). These toxicological consequences affect not just the organisms, but also the environments in which they live. As a result, there is a critical need for ways to alleviate these negative impacts while also proposing alternate solutions to methylparaben's environmental and health risks (Pereira et al. 2023).

Traditionally, many synthetic chemicals and pharmaceutical medicines have been used to combat the negative effects of environmental pollutants. However, conventional procedures also provide their own set of challenges (Eapen et al. 2024; Miettinen et al. 2021). Pharmaceutical therapies, for example, may increase toxicity, complicating the process of reducing methylparaben side effects. Furthermore, the utilization of chemical agents might contaminate the environment, endangering marine and terrestrial species (Dodge et al. 2015; Chakraborty et al. 2023). Given these constraints, there is an increasing interest in investigating green nanotechnology as an alternate approach. Green nanotechnology refers to the environmentally friendly and sustainable synthesis of nanoparticles (NPs) from natural sources such as plant extracts, algae, or microbes (Bhardwaj et al. 2020). This method not only solves environmental problems, but it also provides a more biocompatible and cost-effective strategy for reducing harmful effects (Ying et al. 2022).

Green synthesis has evolved as a sustainable and environmentally friendly alternative to conventional nanoparticle manufacturing processes. Metal oxide nanoparticles from natural extracts, such as ZnO NPS, are gaining appeal due to their wide range of applications in medicine, the environment, and industry. ZnO NPs manufactured utilizing green methods have amazing qualities such as biocompatibility, low toxicity, and antioxidant potential (Alhujaily et al. 2022). These properties make ZnO NPs a promising choice for therapeutic applications, including the treatment of organ damage induced by environmental contaminants such as methylparaben. These green-synthesized NPs are not only more environmentally friendly, but additionally have increased biological activity, making them useful in combating oxidative stress and inflammation (Saxena et al. 2025; Gupta et al. 2023). Some studies suggested that green synthesis of metal oxide nanoparticles has protective effects in fish liver, gills and embryo through its antioxidants and anti-inflammatory effects (Govahi et al. 2025; Hossam S. El-Beltagi et al. 2024; Kunjiappan et al. 2015; Al-Wakeel, A.H).

Caulerpa chemnitzia, also known as Flattop Seagrape, is a species of seaweed in the *Caulerpaceae* family. It is found along the coast of a large area in Western Australia, from north of Perth to the Kimberley region. The seaweed is known for its edible green algae, CCP has immunomodulatory effects and potential anti-inflammatory and antibacterial activities. *Caulerpa chemnitzia*, a green sea alga, contains various bioactive compounds that act as natural reducing and stabilizing agents in the green synthesis of ZnO NPs (Solomon et al. 2024).

The current study is to examine the manufacture of ZnO NPs using *C.chemnitzia* extract and their ability to reduce the negative effects of methylparaben exposure in zebrafish. The aim of this work is to characterize and assess the effects of green ZnO NPs as therapeutic agents for reducing environmental pollutant-induced organ damage in vivo.

Materials and methods

Chemicals

Zinc acetate dihydrate, sodium hydroxide, and methylparaben were procured from Sigma-Aldrich Pvt. Ltd., Mumbai, India. Primary antibodies TNF-α, Nf-κB and secondary antibodies were purchased from ProteinTech. All other chemicals used are molecular and analytical grade.

Green Synthesis of ZnO NPs

ZnO NPs were synthesized using a green, eco-friendly approach with *C. chemnitzia* extract serving as a biogenic reducing and stabilizing agent. Fresh *C. chemnitzia* biomass was thoroughly washed with distilled water, shade-dried, and ground into a fine powder. For extract preparation, 10 g of the powdered material was boiled in 100 mL of distilled water for 30 minutes and then filtered through Whatman No. 1 filter paper to obtain the clear aqueous extract. $Zn(CH_3COO)_2 \cdot 2H_2O$ served as the precursor for zinc. A 0.1 M aqueous solution of zinc acetate was prepared and mixed with the *C. chemnitzia* extract in a 1:1 volume ratio. The reaction mixture was continuously stirred at 60°C for 3 hours. The pH was adjusted to 9 using 1 M NaOH to promote nanoparticle precipitation. The resulting mixture was incubated at room temperature overnight to ensure complete formation of ZnO NPs. The precipitate was recovered by centrifugation at 10,000 rpm for 15 minutes and washed sequentially with distilled water and ethanol to remove unreacted materials and residual phytochemicals. The obtained product was dried at 80°C for 12 hours and subsequently calcined at 400°C for 2 hours to achieve well-crystallized ZnO NPs.

Characterization

UV-Vis spectroscopy (200–800 nm) was used to determine the optical properties of ZnO NPs and find surface plasmon resonance. X-ray diffraction (XRD) using Cu-Kα radiation (20°-80°) validated crystal structure and phase purity. FTIR analysis (4000 – 400 cm^{-1}) of KBr pellets identified functional groups and bio-capping agents. The particle size, surface charge, and shape were determined by dynamic light scattering (DLS), zeta potential, and transmission electron microscopy (TEM). SEM and EDX spectroscopy were used to investigate surface morphology and elemental composition.

Zebrafish husbandry and experimental design

Adult zebrafish were obtained from a commercial breeding facility in Chennai and are now housed at Mass Biotech (CPCSEA Reg No: 2084/PO/RcBt/19/CCSEA), Padi, Chennai, India. All experimental techniques were authorized by the Institutional Animal Ethics Committee (MB/IAEC/2024/03/14). The fish were acclimatized for 7 days in aerated, dechlorinated tap water maintained at 26.0 ± 2.0°C under a

12:12-hour light-dark photoperiod. Water quality parameters, including pH and dissolved oxygen, were monitored daily using appropriate meters. During acclimatization, the fish were fed commercial flake feed twice daily.

A total of 96 zebrafish were split into six experimental groups of 16 each. The fish were housed in 8 L of dechlorinated tap water, with a fish density of 2 per L. Group 1 served as the control, whereas Group 2 got MeP (50 mg/L) daily for the first seven days. Groups 3, 4, and 5 were exposed to MeP (50 mg/L) for 7 days, followed by daily exposure to ZnO-NPs (10 mg/L, 20 mg/L, and 40 mg/L, respectively) for the next 7 days. Group 6 was exposed to ZnO-NPs (40 mg/L) on a daily basis during the previous week.

After the 14-day trial, the fish were killed with ice and tissue samples were obtained for study of various parameters. Fresh tissues for biochemical and molecular research were maintained at -80°C, whilst histopathological specimens were preserved in 10% formalin.

Behavioral tests

Every 4 days, zebrafish behavioral activity was evaluated using three standard assays. In the Light-Dark Test, anxiety-like behavior was assessed based on the time spent in the light versus dark compartments; decreased time in the light zone indicated heightened anxiety. The Novel Tank Diving Test measured stress-related behavior by recording latency to explore the tank's upper region; increased bottom dwelling reflected anxiety. The T-Maze Test assessed cognitive function by measuring the time spent in the rewarded (green) versus aversive (red) arms, providing insights into spatial memory and decision-making impairments induced by MeP and their potential improvement following ZnO NP treatment.

Biochemical assays

After the experiment, the fish were killed, and the liver and gill tissues were extracted. Tissue homogenates were produced using phosphate buffer at pH 7.4. Standard spectrophotometric methods were used to quantify antioxidant enzyme activities such as superoxide dismutase (SOD) and catalase (CAT) (Elekofehinti et al. 2013), glutathione peroxidase (GPx) (Flohé and Gunzler 1984), and glutathione S-transferase (GST) (Habig et al. 1974), as well as levels of reduced glutathione (GSH) (Jollow et al. 1974) and vitamin C. Lipid peroxidation (LPO) was estimated through malondialdehyde (MDA) levels (Oluyede et al. 2021; Zeb and Ullah 2016), and reactive oxygen species (ROS) were also quantified. Serum alanine aminotransferase (ALT) activity, indicating hepatotoxicity, was measured using a Pars Azmoun commercial kit following the manufacturer's instructions.

Histopathological analysis

Gill and liver tissues were fixed in 10% buffered formalin and then dehydrated using a graded ethanol series. Following dehydration, the tissues were cleaned with xylene and embedded in paraffin wax. Thin slices (5 µm) were cut using a microtome and stained with hematoxylin and eosin (H&E) to reveal cellular characteristics. The stained slices were then examined under a light microscope for histological analysis.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted using TRIzol Reagent (Qiagen, Germany) according to the manufacturer's instructions. RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Scientific, USA). One microgram of RNA was reverse-transcribed into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, USA). Quantitative real-time PCR (qRT-PCR) was carried out on a Bio-Rad CFX96 Touch™ system using 2× SYBR Green PCR Master Mix (Qiagen, Germany). Each 20 µL reaction contained 10 µL of master mix, 1 µL each of forward and reverse primers (10 µM) (Table 1), 2 µL of cDNA, and 6 µL of nuclease-free water. The cycling conditions were 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. GAPDH was used as the reference gene, and relative expression levels were calculated using the 2^−ΔΔCt method. Results were expressed as fold changes relative to the control group.

Table 1
Primers used in this study

	Forward primers (5'→3')	Reverse primers (3'→5')	Size
TNF-α (BU744228)	CCCTGGTCAACAAGCCTAAA	ATCTGGCGTCAGCTTTCACT	180 bp
NF-κB (BC162402)	GCGGAGTTTGAGAAGTCTGG	CTTCTCGTCTTTGCCTTTGG	213 bp
GAPDH (AY818346)	ACTCCACTCATGGCCGTTAC	TGAGCTGAGGCCTTCTCAAT	184bp

Western blot analysis

To maintain protein integrity, RIPA buffer was treated with protease inhibitors and used to extract proteins from gill and liver tissues. The protein content was measured using the Lowry technique. Proteins were isolated using SDS-PAGE and then transferred to PVDF membranes. Membranes were blocked with 5% BSA and treated overnight at 4°C with primary antibodies against TNF-α and NF-κB. Secondary antibodies were then HRP-conjugated. Protein bands were identified using ECL and expression levels were measured using densitometry, normalized to β-actin.

Statistical analysis

The statistical analysis was done using GraphPad Prism 9. One-way ANOVA was used to compare groups, followed by the Newman-Keuls post-hoc test. A p-value of < 0.05 was judged statistically significant.

Results

UV-Visible spectroscopy

Figure 1 presents the UV–Visible spectrum of green synthesized ZnO NPs using Caulerpa chemnitzia extract. A distinct absorption peak observed at 360 nm indicates the surface plasmon resonance (SPR) characteristic of ZnO NPs, confirming their successful green synthesis.The broad absorption range further suggests the presence of nanoscale particles and the possible influence of phytochemicals from the extract in stabilizing and reducing the nanoparticles.

XRD

Figure 2 depicts the XRD pattern of green produced ZnO NPs with Caulerpa chemnitzia extract. ZnO's hexagonal wurtzite structure is confirmed by significant diffraction peaks at certain 2θ values corresponding to the (100), (002), (101), (102), (110), (103), (200), (112), and (201) planes. The sharp and well-defined peaks indicate good crystallinity, and the absence of additional peaks confirms the phase purity of the synthesized ZnO NPs.

FTIR

The FTIR spectrum of *Caulerpa chemnitzia* revealed prominent peaks at 3282 cm^{-1} (O–H stretching), 1635 cm^{-1} (C = O stretching), 1417 cm^{-1} (C–H bending), and 1097 cm^{-1} (C–O stretching), indicating the presence of polyphenols and proteins. The green-synthesized ZnO NPs (C@ZnO NPs) exhibited shifts in these peaks to 3381, 1643, 1453, and 1097 cm^{-1}, confirming the interaction between plant metabolites and ZnO. Additionally, peaks at 635 and 610 cm^{-1} in the ZnO NPs spectrum correspond to Zn–O bond vibrations, validating successful nanoparticle formation. These results confirm the role of *C. chemnitzia* extract in the biogenic synthesis and stabilization of ZnO NPs.

Dynamic light scattering and zeta potential

Figure 4 shows the DLS analysis of green synthesized ZnO NPs using *Caulerpa chemnitzia* extract, indicating an average hydrodynamic diameter of 119.4 nm. The narrow distribution reflects a uniform size range of nanoparticles. Figure 5 presents the zeta potential analysis of ZnO NPs using *Caulerpa chemnitzia* extract, with a mean zeta potential of -23.0 mV and electrophoretic mobility of -0.000178 cm^2/Vs, suggesting moderate stability of the colloidal system. The negative surface charge indicates electrostatic repulsion between particles, which helps prevent agglomeration and enhances dispersion stability in suspension.

Morphological and elemental analysis

Figure 6 shows SEM images, EDAX analysis and HR-TEM images of green synthesized ZnO NPs using *Caulerpa chemnitzia* extract. The SEM micrographs reveal a transition from irregularly shaped agglomerates to well-defined hexagonal and rod-like ZnO NPs with increasing magnification, indicating successful crystallization (Fig. 6A). The EDAX spectrum confirms the presence of zinc (Zn), oxygen (O), and carbon (C), with elemental mapping demonstrating a uniform distribution of Zn and O across the

sample. The elemental composition includes 51.35 wt.% O, 22.68 wt.% C, and 25.98 wt.% Zn, validating the formation of ZnO NPs (Fig. 6B). These results confirm effective green synthesis and elemental purity. The morphological features and size of the biosynthesized ZnO NPs were investigated using HR-TEM, as shown in Fig. 6C. The micrograph revealed that the ZnO NPs are predominantly quasi-spherical to slightly polyhedral in shape with moderate agglomeration, which is typical for NPs stabilized by phytochemicals in plant extracts. The average particle size was calculated to be approximately 44 nm, with most particles distributed within the 30–60 nm range.

Light-dark test

The light-dark test results demonstrate that MeP (50 mg/L) exposure significantly reduced the time spent in the light zone while increasing the time in the dark zone over 16 days, indicating heightened anxiety-like behavior (Fig. 7). However, co-treatment with ZnO-NPs, particularly at 20 and 40 mg/L, progressively ameliorated these effects, with a notable increase in light zone exploration and decreased dark zone preference. ZnO-NPs alone showed behavior comparable to the control, suggesting a protective effect. These findings indicate that ZnO-NPs mitigate MeP-induced anxiety in a dose-dependent manner, restoring near-normal behavioral patterns over the experimental period.

Novel tank test

The novel tank test results show that MeP (50 mg/L) exposure significantly increased bottom latency and reduced top latency across all days, indicating heightened anxiety-like behavior (Fig. 8). Co-treatment with ZnO-NPs dose-dependently reversed these effects. The 40 mg/L ZnO-NPs co-treatment notably normalized both top and bottom latency percentages, approaching control levels by day 16. ZnO-NPs alone showed no adverse behavioral changes, confirming their safety. These findings suggest that ZnO-NPs effectively mitigate MeP-induced anxiety, with 40 mg/L showing the most therapeutic potential. The consistent improvement over time further validates the anxiolytic role of ZnO-NPs in MeP-induced behavioral toxicity.

T-maze

The T-maze behavioral study revealed that treatment to methylparaben (MeP, 50 mg/L) significantly reduced cognitive performance, as seen by increased time spent in the red arm and decreased time in the green arm on days 4, 8, 12, and 16 when compared to the control group (Fig. 9). This shows a lack of spatial memory and decision-making skills. Co-treatment with ZnO-NPs at concentrations of 10, 20, and 40 mg/L resulted in dose- and time-dependent improvements in performance. Notably, the group receiving MeP + ZnO-NPs (40 mg/L) exhibited near-normal behavior by day 16, spending significantly less time in the red arm and more in the green arm, indicating restored memory and learning capacity. ZnO-NPs alone (40 mg/L) did not cause any behavioral abnormalities, supporting their biocompatibility. These results suggest that ZnO-NPs confer neuroprotective effects against MeP-induced neurotoxicity and cognitive dysfunction, likely by mitigating oxidative stress and enhancing neural function over time.

Biochemical changes

Antioxidants and oxidative stress markers

Exposure to MeP significantly reduced antioxidant enzyme activities in the gill and liver tissues, as reflected by lowered levels of SOD, CAT, GPx, GSH, GST and vitamin C, alongside increased oxidative stress markers such as LPO and ROS (Figs. 10 and 11). However, co-treatment with ZnO-NPs showed a dose-dependent protective effect. Increasing concentrations of ZnO-NPs gradually restored the antioxidant enzymes toward normal levels, with the highest dose (40 mg/L) approaching values comparable to the control group. Similarly, the elevated LPO and ROS levels induced by MeP were progressively reduced with ZnO-NP supplementation. The group treated with ZnO-NPs alone displayed antioxidant and oxidative stress profiles nearly identical to the control, indicating the inherent safety and beneficial properties of ZnO-NPs.

Liver marker enzyme

The serum ALT levels increased notably in the MeP-treated group compared to the control, indicating hepatic stress (Fig. 12). Co-treatment with ZnO-NPs led to a dose-dependent reduction in ALT levels, suggesting a protective effect. At higher concentrations of ZnO-NPs, ALT levels approached those of the control group. Treatment with ZnO-NPs alone showed no significant deviation from control values, reinforcing their safety. Overall, ZnO-NPs appeared to mitigate MeP-induced hepatotoxicity in a concentration-dependent manner.

Histopathological changes

Figure 13A (a-f) shows the histological features of adult zebrafish gill tissue from both the control and treatment groups. The control group had normal gill architecture, with no pathological alterations. In contrast, MeP-treated fish gills exhibited significant toxicity, including hyperplasia, lamellar fusion, filament disintegration, dilated marginal channels, and epithelial lifting. Co-treatment with ZnO-NPs significantly reduced these histopathological changes, resulting in decreased epithelial lifting, lamellar fusion, marginal channel dilation, and secondary lamellar shortening.

Figure 13B (a-f) shows the histological analysis of adult zebrafish liver tissue from both the control and treatment groups. The control group showed normal liver morphology, including well defined hepatocytes. In contrast, MeP-treated liver tissues showed significant histopathological changes, including sinusoidal and venous congestion, vacuole formation, cytoplasmic vacuolation of hepatocytes, and necrosis. Supplementation with ZnO-NPs at 10, 20, and 40 mg/L markedly reduced these alterations, showing improved liver architecture with decreased hepatocyte irregularity, vacuole formation, and cytoplasmic vacuolation

Effect of C@ZnO NPs on mRNA and protein expression

The mRNA and protein expression levels of C@ZnO-NPs on MeP-induced gill and liver damage were determined using qRT-PCR (Fig. 14) and western blot analysis (Fig. 15). MeP exposure significantly increased NF-κB and TNF-α mRNA and protein levels in both gill and liver tissues. Treatment with C@ZnO-NPs at 10, 20, and 40 mg/L considerably reduced the increased expression of NF-κB and TNF-α in the tissues. These findings suggest that C@ZnO-NPs effectively modulate key inflammatory proteins during MeP-induced gill and liver injury.

Discussion

The current work effectively demonstrated the green production of ZnO NPs utilizing *Caulerpa chemnitzia* extract, as proven by UV-Visible spectroscopy, XRD, FTIR, DLS, zeta potential, SEM, and EDX studies. The strong UV-Vis absorption peak at 360 nm corresponds to the SPR characteristic of ZnO NPs, demonstrating nanoscale production aided by phytochemical-mediated reduction and stability. Hameed et al., (2023) reported that green synthesized ZnO-NPs using *Spirogyra hyalina* shows UV-vis absorption peak at 363 nm. XRD patterns reveal sharp diffraction peaks confirming a well-crystallized hexagonal wurtzite structure with an average crystallite size of ~ 25 nm, consistent with previous reports on biogenic ZnO synthesis using *Arthrospira platensis* (El-Belely et al., 2021). FTIR spectra reveal shifts in characteristic peaks (O–H, C = O, C–H, and C–O) that confirm binding interactions between ZnO and the bioactive polyphenols and proteins of *C. chemnitzia*, implicating these metabolites as reducing and capping agents during nanoparticle formation (Elrefaey et al. 2022). The formation of ZnO was confirmed by 600 cm^{-1} peak (Fakhari et al., 2019). The DLS and zeta potential results indicate stable colloidal dispersion of ZnO-NPs with moderate negative charge (-23.0 mV), preventing agglomeration via electrostatic repulsion and enhancing bioavailability, consistent with prior reports of plant-mediated ZnO NPs (Asif et al. 2021). SEM and EDX analyses further validate the morphology and elemental purity, showing well-defined hexagonal and rod-like ZnO structures with uniform Zn and O distribution. Similarly, (Samei et al. 2019) synthesized ZnO-NPs using *Raphidocelis subcapitata* microalgae which shows rod shape in SEM analysis.

Exposure to MeP induces significant behavioral changes in zebrafish, including increased anxiety behavior such as reduced exploration and erratic swimming (Xiang et al. 2024). Cognitive impairments are also observed, with deficits in learning and memory tasks (Kim et al. 2022). These effects are likely linked to MeP-induced neurotoxicity and disruption of cholinergic signaling (Thakkar et al. 2022). In the present study behavioral assays highlight the neuroprotective and anxiolytic potential of C@ZnO NPs against MeP-induced toxicity. The light-dark and novel tank tests revealed that MeP exposure significantly induced anxiety-like behaviors, reflected by decreased exploration and altered latency metrics. C@ZnO NPs co-treatment ameliorated these effects in a dose-dependent manner, with 40 mg/L showing near-normal behavior restoration. T-maze results corroborate cognitive impairments induced by MeP, manifesting as spatial memory and decision-making deficits. C@ZnO NPs reversed these impairments dose and time-dependently, indicating restoration of neural function and learning ability. These findings are consistent with previous reports indicating that green-synthesized nanoparticles can

alleviate anxiety, enhance spatial memory and decision-making behavior, and reduce neurotoxicity through antioxidant and anti-inflammatory pathways (Hammad et al. 2024).

Zebrafish are frequently used as model organisms for testing water toxicity due to their genetic closeness to humans and transparent physiology (Bambino et al. 2017). Gills are the principal sites for gas exchange, osmoregulation, and nitrogenous waste excretion in zebrafish (Evans et al. 2005). Because of their close interaction with the aquatic environment, they are extremely sensitive to waterborne contaminants and serve as early indicators of toxicity (Macirella et al. 2017). The liver functions as the central organ for metabolism, detoxification, and nutrient storage, playing a crucial role in processing and eliminating toxins through enzymatic pathways (Goessling et al. 2015). Both organs respond to xenobiotic stress by triggering oxidative stress and activating inflammatory responses (Singh et al. 2024; Chen et al. 2012). In this work, we looked at how C@ZnO NPs protected adult zebrafish gills and livers against MeP-induced oxidative stress and inflammation.

Oxidative stress arises when the production of ROS exceeds the capacity of the body's antioxidant defense system, leading to cellular and molecular damage. Methylparaben (MeP) has been shown to cause oxidative stress by producing ROS and affecting mitochondrial activity. In adult fish, exposure to 50 mg/L of MeP has been shown to significantly reduce phase I biotransformation enzyme activity (ethoxyresorufin O-deethylase), increase lipid peroxidation (LPO) in gill tissues, and elevate the frequency of micronuclei in erythrocytes (de Carvalho Penha et al. 2021). Prolonged exposure to MeP may contribute to inflammation, DNA damage, and altered cellular signaling pathways (Bereketoglu et al. 2019). Furthermore, research indicates that MeP might interfere with antioxidant enzyme activity, raising concerns about its long-term health implications (Bereketoglu and Pradhan, 2019; Xiang et al. 2024). In the current study, MeP exposure dramatically lowered antioxidant enzyme activity, including SOD, CAT, GPx, GST, GSH, and vitamin C, while raising oxidative stress indicators including LPO and ROS. Treatment with Ca@ZnO NPs effectively restored antioxidant defense mechanisms in a dose-dependent manner and mitigated oxidative damage. Supporting this, Tanna et al. (2018) and Solomon et al. (2024) reported potent free radical scavenging activity of Caulerpa species and their nanoformulations. Similarly, (Janarathanam et al. 2024) demonstrated that hamamelitannin-conjugated ZnO NPs restored antioxidant enzyme levels in zebrafish larvae exposed to hydrogen peroxide-induced oxidative stress.

Hepatotoxicity refers to liver damage caused by toxic substances. Hu et al. (2022) reported that MeP impairs liver enzyme function by elevating ALT levels in adult zebrafish. In the present study, MeP exposure significantly increased ALT activity, indicating hepatocellular damage. Treatment with C@ZnO NPs effectively reduced ALT levels in a dose-dependent manner. This reduction suggests a protective role of the nanoparticles against MeP-induced hepatic injury. The findings support the hepatoprotective potential of green-synthesized C@ZnO NPs. This observation is in agreement with the results of (Govahi et al. 2025), who demonstrated that ZnO NPs synthesized using *Scrophularia striata* extract significantly reduced ALT levels in common carp, indicating reduced hepatic damage. Furthermore, the current results are further corroborated by the study of (Hossam S. El-Beltagi et al. 2024), in which oral administration of green-synthesized ZnO NPs derived from *Moringa oleifera* effectively suppressed the elevation of key

hepatic biomarkers such as ALT, AST, ALP, LDH, and GGT in carbon tetrachloride (CCl_4)-treated albino rats.

Histopathological analyses further validated biochemical and behavioral data. MeP-induced damage in gill and liver tissues was characterized by structural disruptions including hyperplasia, epithelial lifting, necrosis, and vacuolation. Similarly, exposure to MeP and methylparaben has been reported to alter the structure of gill and liver tissues in *Catla catla* and zebrafish (*Danio rerio*) (Selvi et al. 2015; Hu et al. 2022). C@ZnO NPs supplementation reduced these pathological changes significantly, preserving tissue integrity and cellular morphology in a concentration-dependent fashion. Kunjiappan et al. (2015) reported that acetaminophen (APAP) treated common carp fish (Cyprinus carpio L.) showed severe structural alterations, including complete loss of hepatic architecture, central vein necrosis, fatty changes, sinusoidal congestion, hepatocellular hyperplasia, and apoptosis. However, fish treated with APAP in combination with green-synthesized gold nanoparticles (APAP + GNaP) displayed marked improvement in liver morphology, characterized by reduced hepatocellular damage, mild vacuolar degeneration, and absence of congestion. These histological features were nearly restored to normal in fish treated with GNaP alone, indicating the potent hepatoprotective effect of green-synthesized nanoparticles. These results support the protective role of biogenic nanoparticles in mitigating xenobiotic-induced hepatic damage, in agree with the current study's findings where C@ZnO NPs ameliorated MeP-induced histopathological alterations in zebrafish liver tissues.

Xenobiotic-induced tissue damage triggers inflammation, which is often mediated by signaling molecules including NF-κB and TNF-α (Wang et al. 2023). These indicators serve critical roles in starting and maintaining inflammatory cascades, especially in organs such as the gill and liver, which are directly involved in detoxification and environmental interaction. The study found that MeP exposure dramatically increased NF-κB and TNF-α expression levels in adult zebrafish gill and liver tissues, indicating a strong inflammatory response. Co-treatment with C@ZnO NPs at concentrations of 10, 20, and 40 mg/L resulted in a dose-dependent reduction of these inflammatory markers. The observed suppression of NF-κB and TNF-α expression at both transcriptional and translational levels suggests that C@ZnO NPs exhibit significant anti-inflammatory activity. Al-Wakeel, A.H et al. (2024) concurred with these findings, demonstrating that dietary supplementation with green-synthesized selenium nanoparticles (SeNPs) in Nile tilapia did not induce TNF-α or NF-κB activation. This absence of an inflammatory response was supported by reduced oxidative stress. Given that ROS are upstream activators of NF-κB, the observed inhibition suggests that SeNPs help prevent the activation of pro-inflammatory cascades. These results reinforce the role of green-synthesized nanoparticles in modulating inflammation by targeting key inflammatory mediators such as TNF-α and NF-κB. In addition, these findings are further supported by the study "Synergistic effect of zinc oxide–cinnamic acid nanoparticles for wound healing management" by (Jehad Zuhair Tayeb et al. 2024). Zinc oxide-cinnamic acid nanoparticles at 80 µg/mL significantly reduced TNF-α and IL-1β expression in a wound-induced zebrafish model, relative to the control group.

Conclusion

The green production of ZnO NPs (C@ZnO NPs) with Caulerpa chemnitzia extract was successfully validated by UV-Visible spectroscopy, which revealed a distinct absorption peak at 360 nm, indicating particle generation. XRD analysis revealed the hexagonal wurtzite structure and nanoscale crystallinity, while FTIR spectra confirmed the role of phytochemicals in nanoparticle stabilization. DLS and zeta potential analyses indicated uniform particle size and moderate colloidal stability. SEM-EDAX results demonstrated defined morphology and elemental purity. Biologically, C@ZnO NPs effectively mitigated MeP-induced anxiety-like behaviors and cognitive impairments in zebrafish, as shown by behavioral assays (LDT, NTT and T-maze). Biochemical evaluations indicated restoration of antioxidant defenses and reduced oxidative stress. Histopathological analysis revealed tissue recovery in gills and liver, further supported by downregulation of inflammatory markers (NF-κB, TNF-α) at both mRNA and protein levels. These findings collectively demonstrate that green-synthesized C@ZnO NPs possess significant neuroprotective and anti-inflammatory potential, suggesting their applicability in mitigating pesticide-induced toxicity through eco-friendly nanotechnology. In the future, detailed molecular studies are warranted to understand the mechanistic pathways through which ZnO NPs confer neuroprotection. Long-term toxicity and biodistribution studies in higher models will also be essential to validate their biomedical applicability. The promising results suggest that green-synthesized ZnO NPs may serve as potential therapeutic agents against pesticide-induced neurotoxicity and oxidative stress-related disorders.

Declarations

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Authors' Contributions

Conceptualization was carried out by CP and GA. Data curation was handled by CP and MKS. Methodology was developed by MKS, CP, and GA. The original draft was written by CP, MKS, and GA, while the review and editing were performed by CP and MKS. All authors have read and agreed to the published version of the manuscript.

Ethical Approval

The study was approved by the Institutional Animal Ethics Committee (IAEC) under Proposal No. MB/IAEC/2024/03/14. All experimental procedures were conducted in strict accordance with the guidelines and regulations of the Committee for the Purpose of Control and Supervision of Experimental Animals (CCSEA), ensuring the ethical treatment and welfare of animals throughout the study.

Consent to Participate

Not Applicable

Consent to Publish

Not applicable.

Competing Interests

The authors declare no conflicts of interest.

Data Availability Statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Figures

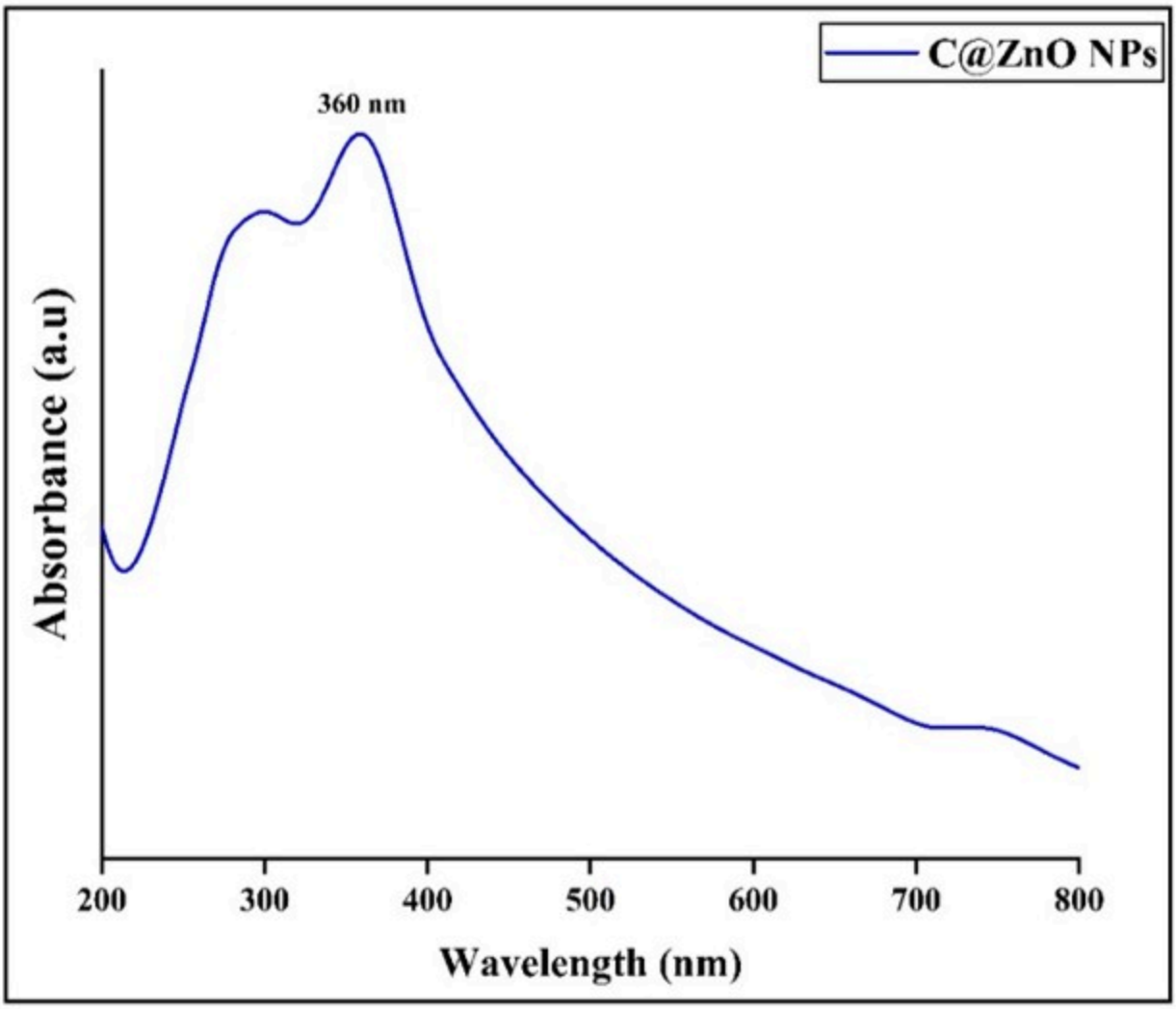

Figure 1

UV-Visible spectrum of C@ZnO NPs

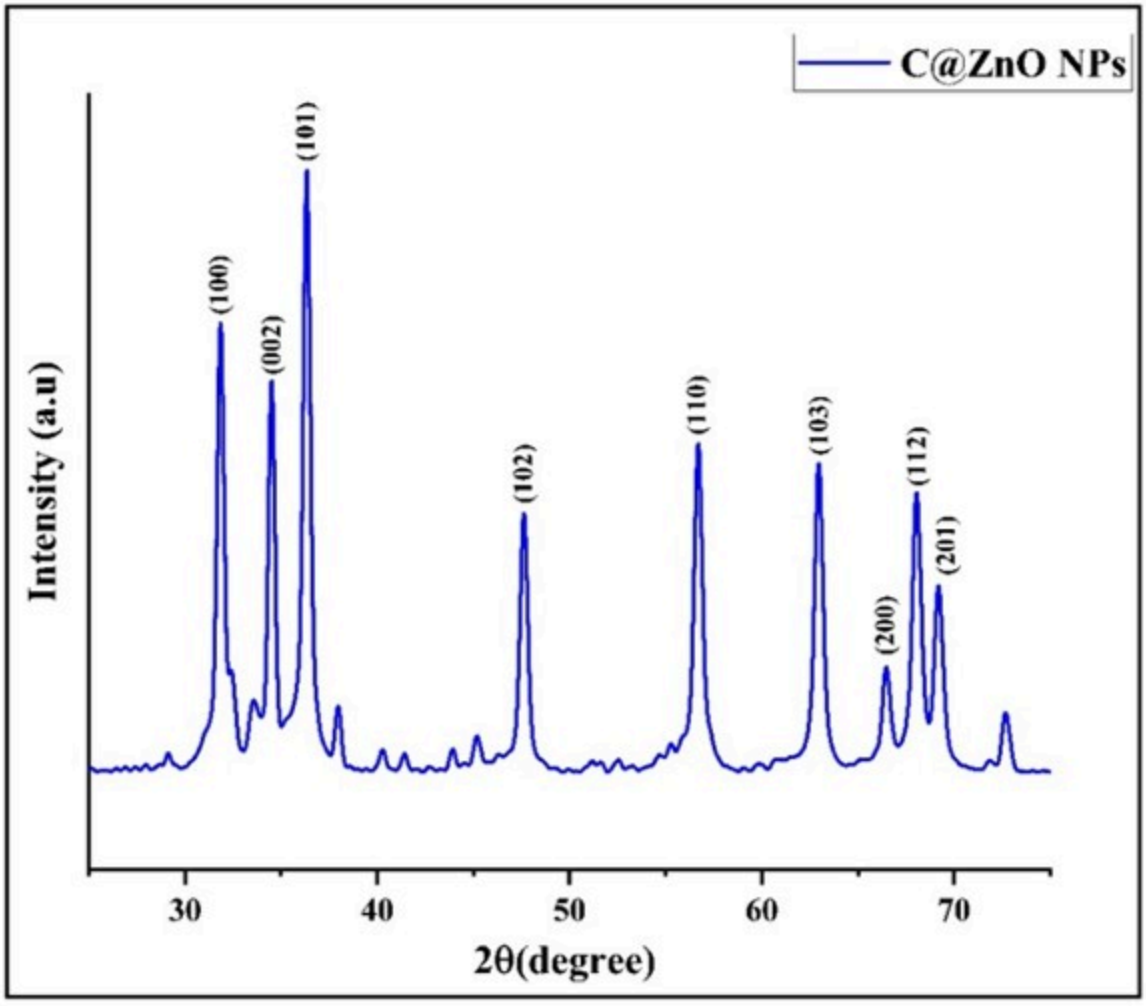

Figure 2

XRD spectrum of C@ZnO NPs

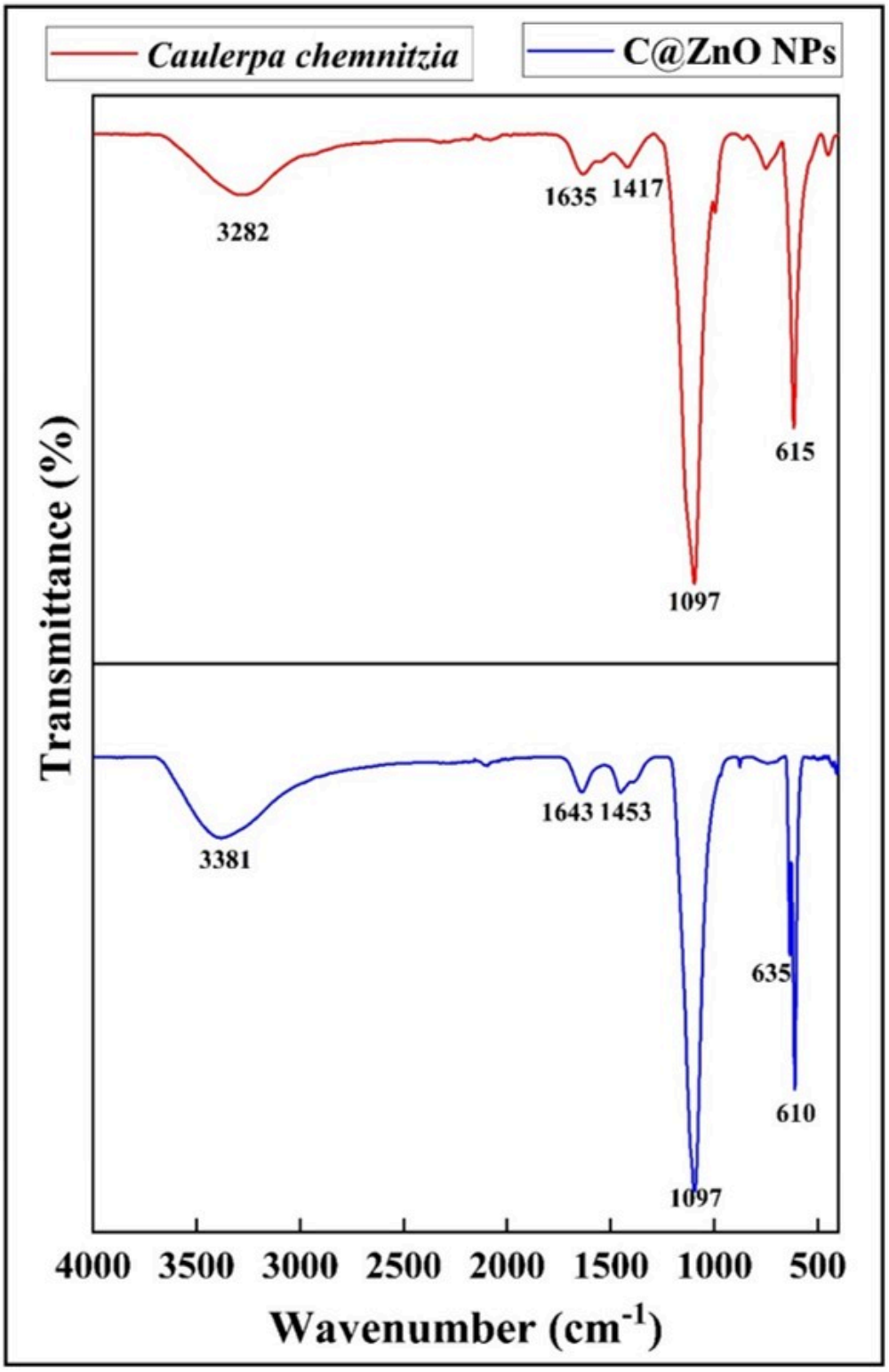

Figure 3

FTIR spectrum of *Caulerpa chemnitzia* extract and C@ZnO NPs

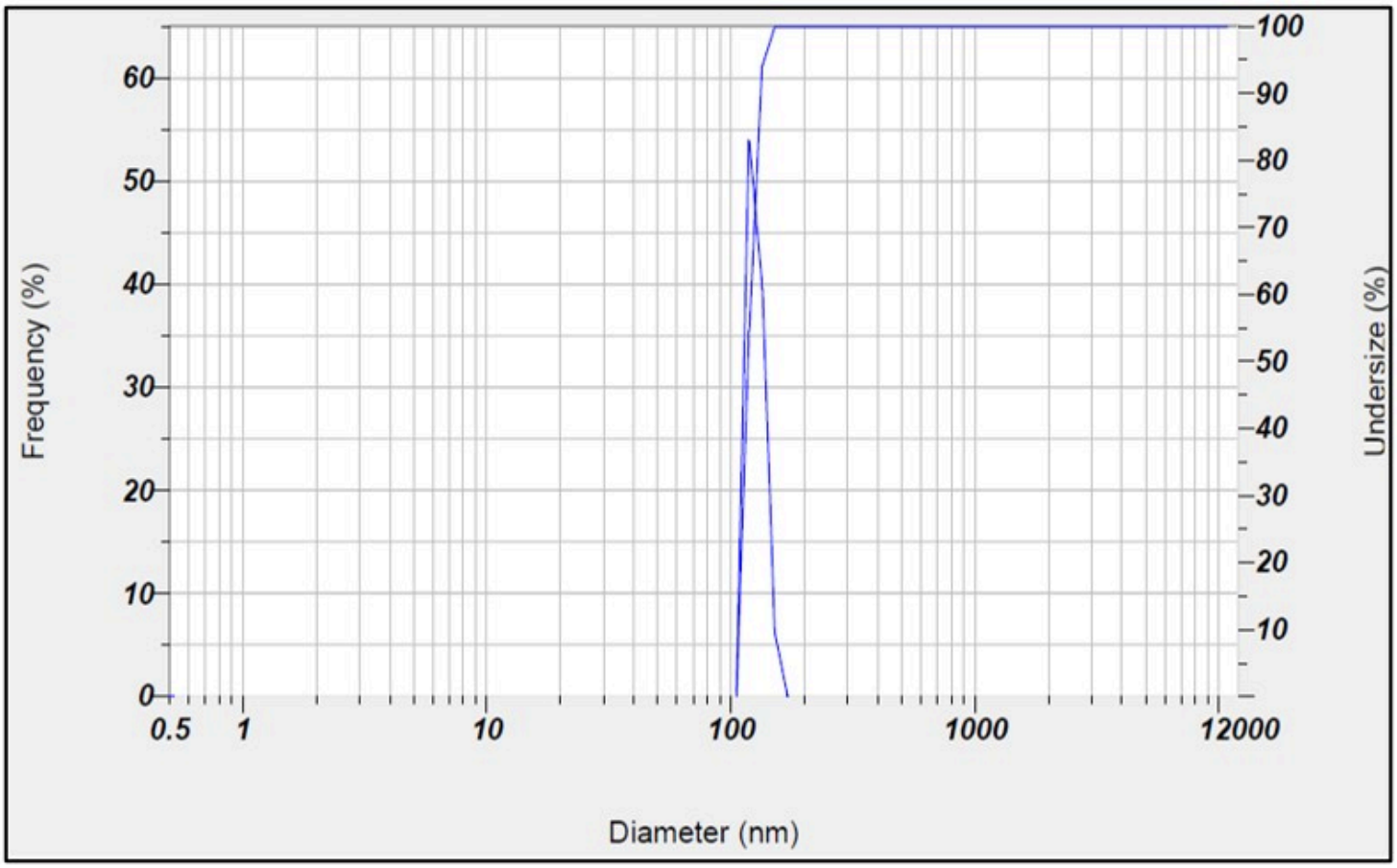

Figure 4

DLS spectrum of C@ZnO NPs

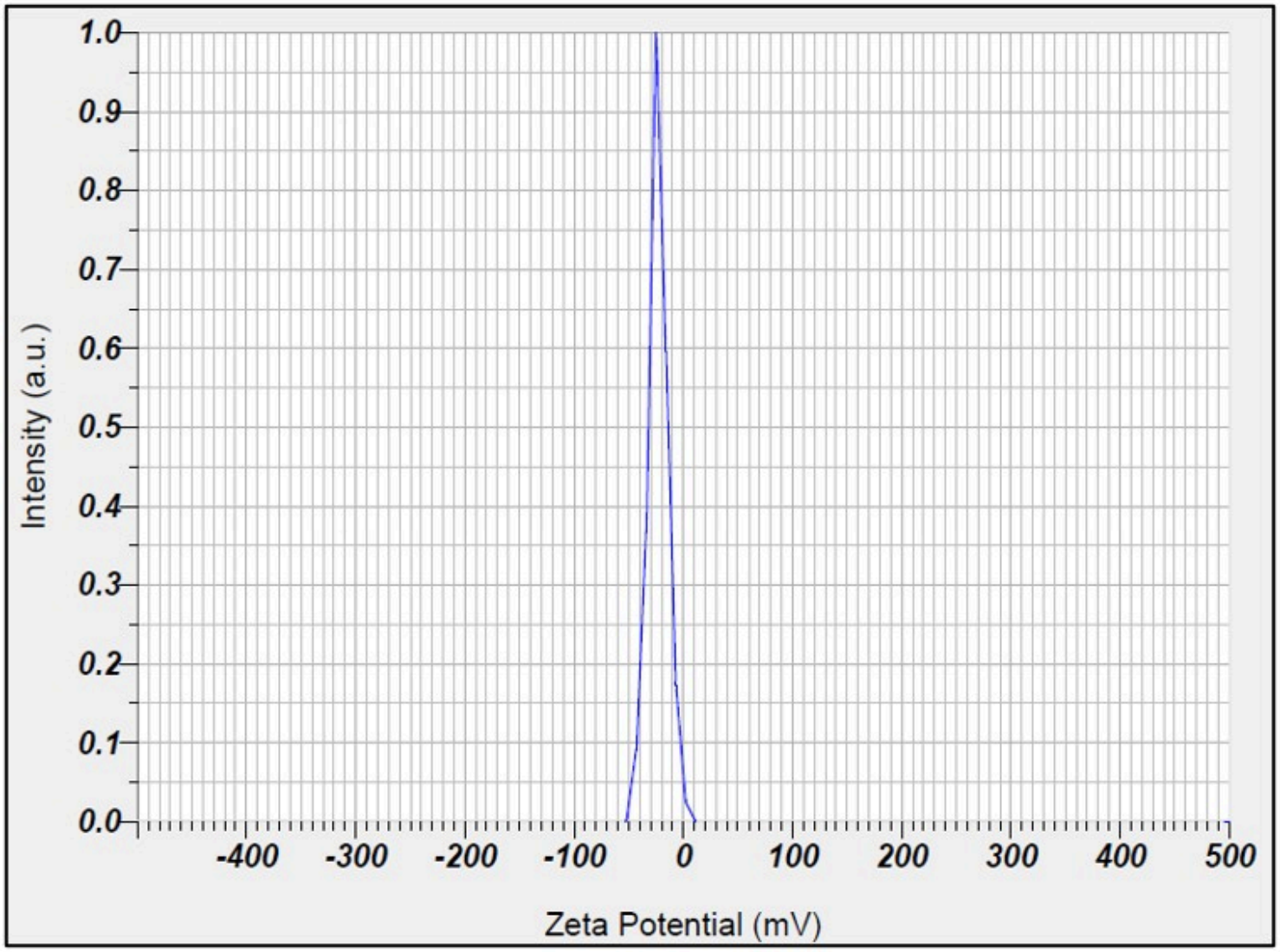

Figure 5

Zeta potential of C@ZnO NPs

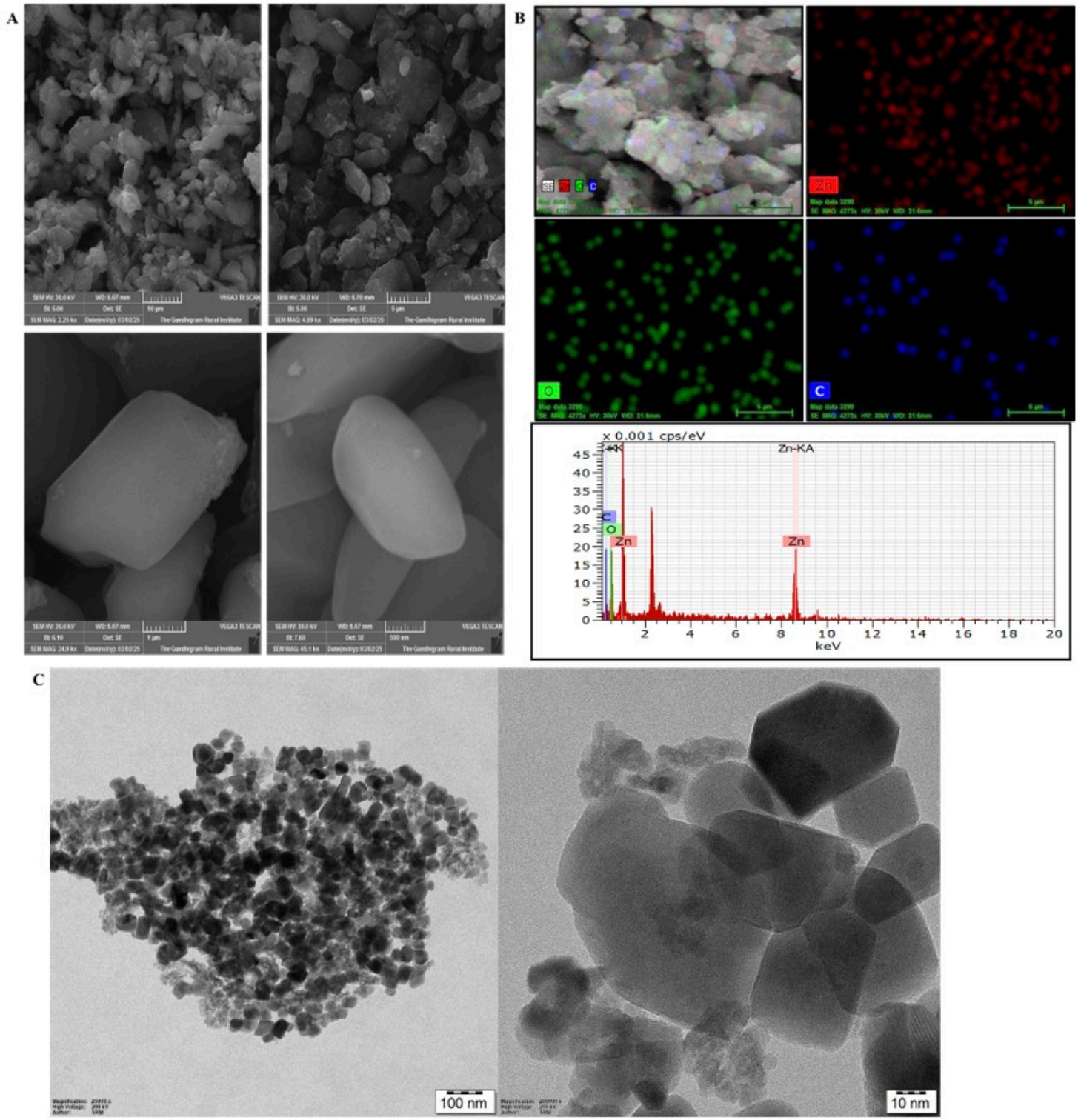

Figure 6

A) SEM, B) EDAX mapping of C@ZnO NPs and C) HR-TEM

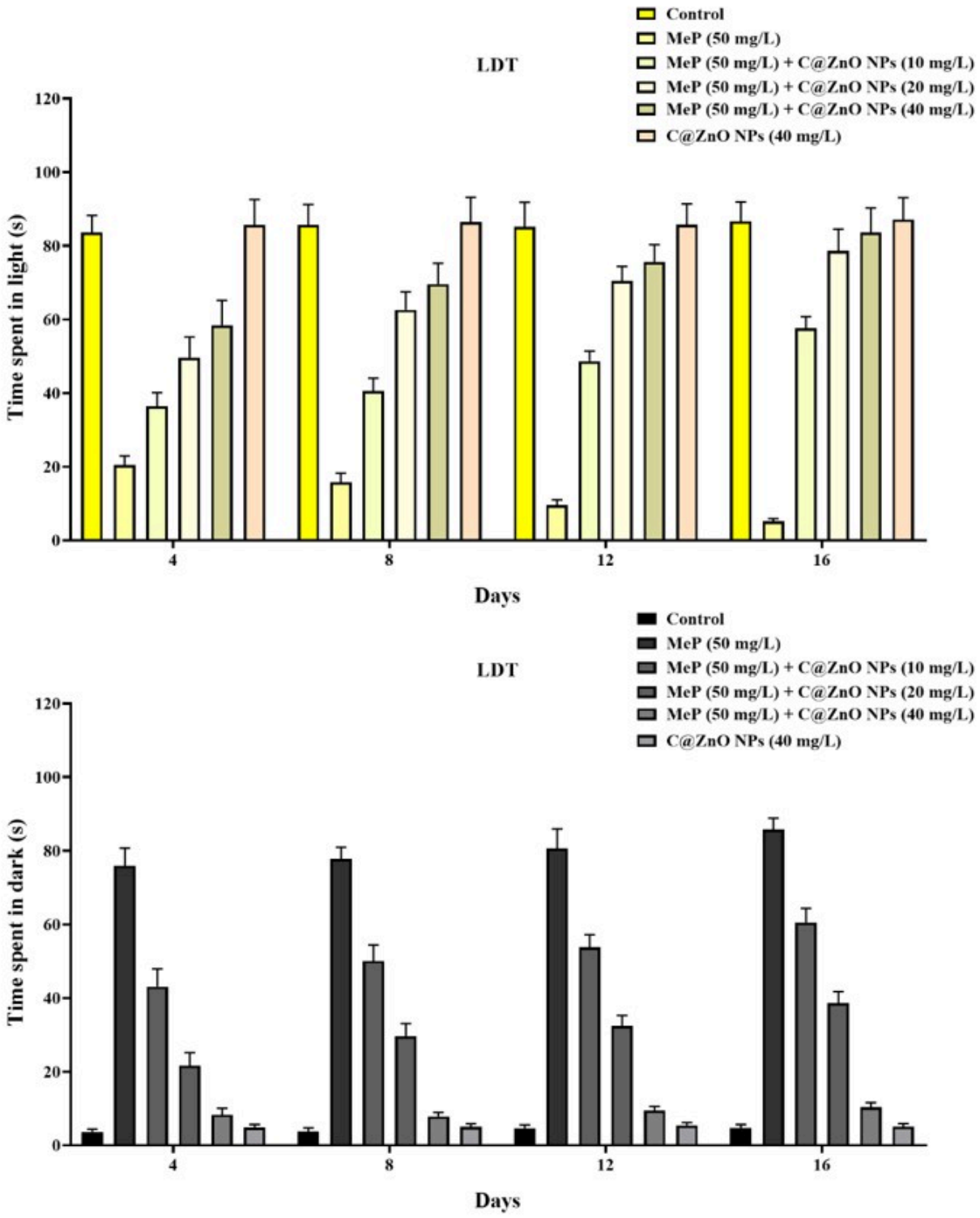

Figure 7

Effects of C@ZnO NPs on anxiety related behavior evaluated by using LDT. The time spent in the light arm (a) and dark arm (b) of the LDT over a period of 4, 8, 12 and 16 days. Time spent in the light area is indicative of reduced anxiety-like behavior, whereas time spent in the dark arm reflects anxiety presence. Data are presented as mean ± SD.

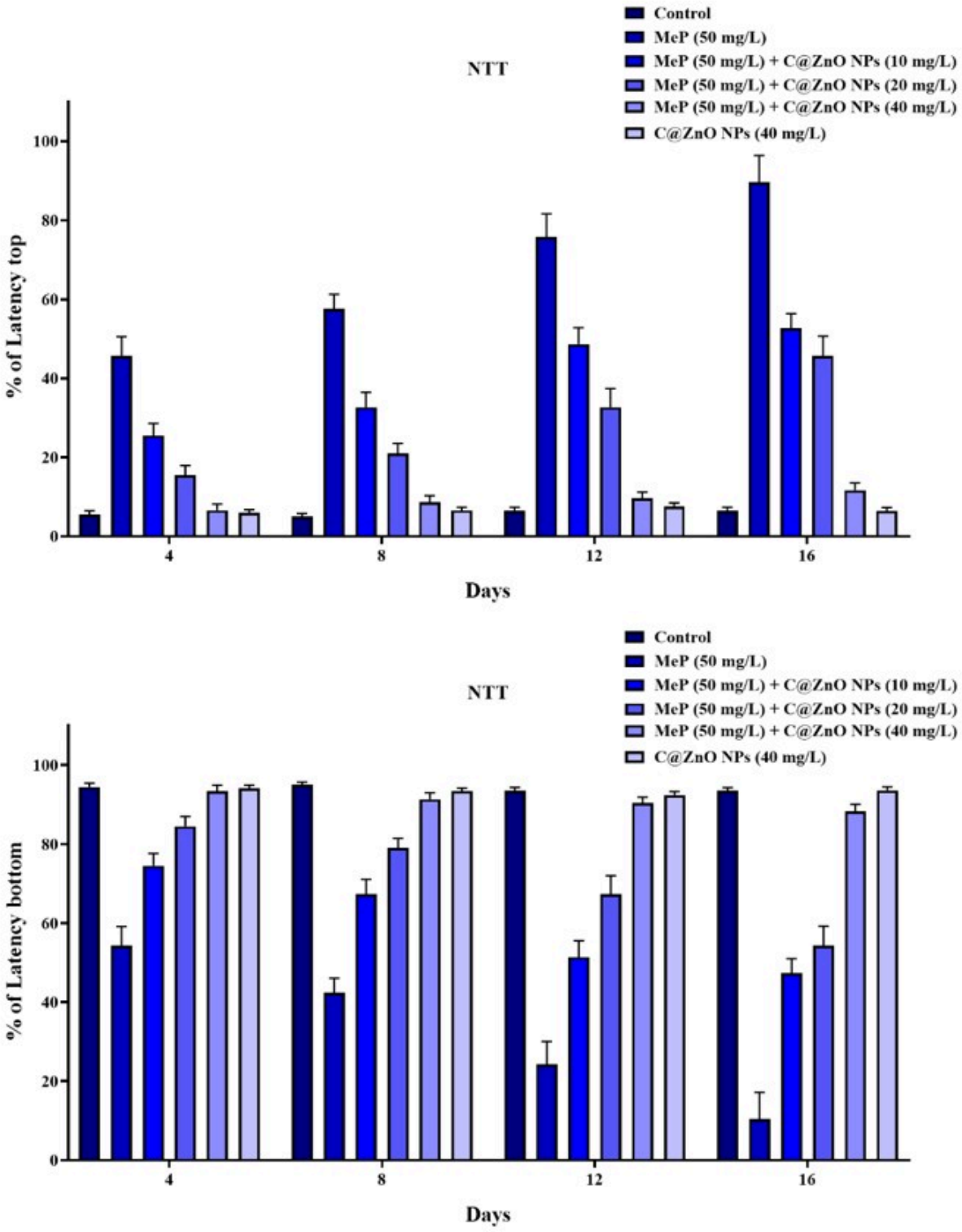

Figure 8

Effects of C@ZnO NPs on anxiety related behavior evaluated by using NTT. The proportion of delay at the top (a) and bottom (b) of the NTT for 4, 8, 12, and 16 days. Time spent in the upper zone indicates less anxiety-like behavior, whereas time spent in the bottom zone indicates anxiety present. The data are provided as mean ± SD.

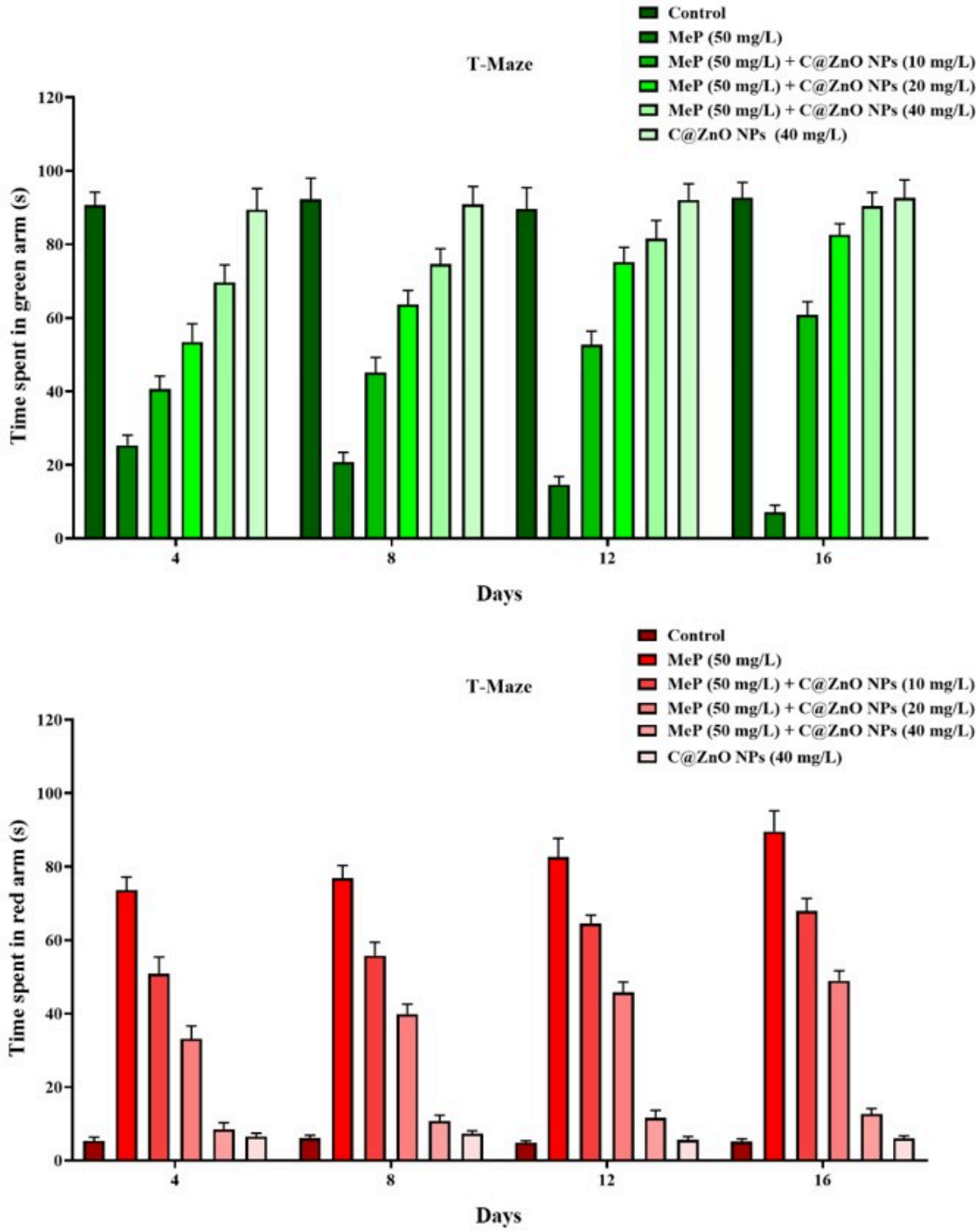

Figure 9

Effects of C@ZnO NPs on anxiety related behavior evaluated by using T-Maze test. The time spent in the green arm (a) and red arm (b) of the T-Maze over a period of 4, 8, 12 and 16 days. Time spent in the green arm is indicative of reduced anxiety-like behavior, whereas time spent in the red arm reflects anxiety presence. Data are presented as mean ± SD.

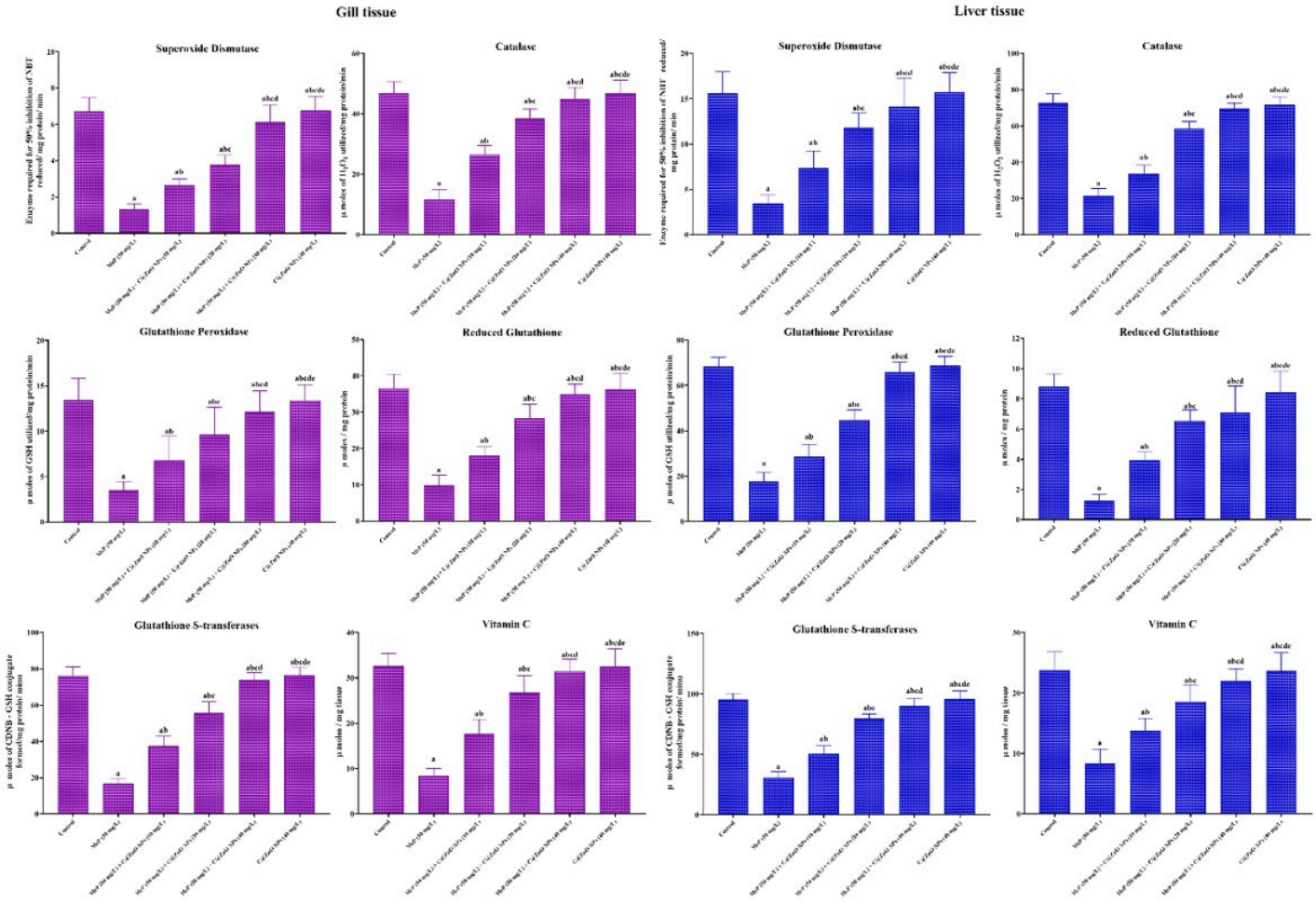

Figure 10

Effect of Ca@ZnO-NPs on enzymatic and non-enzymatic antioxidant levels in gill and liver tissues of control and treated groups. Values are mean ± SD, with different letters indicating statistically significant differences.

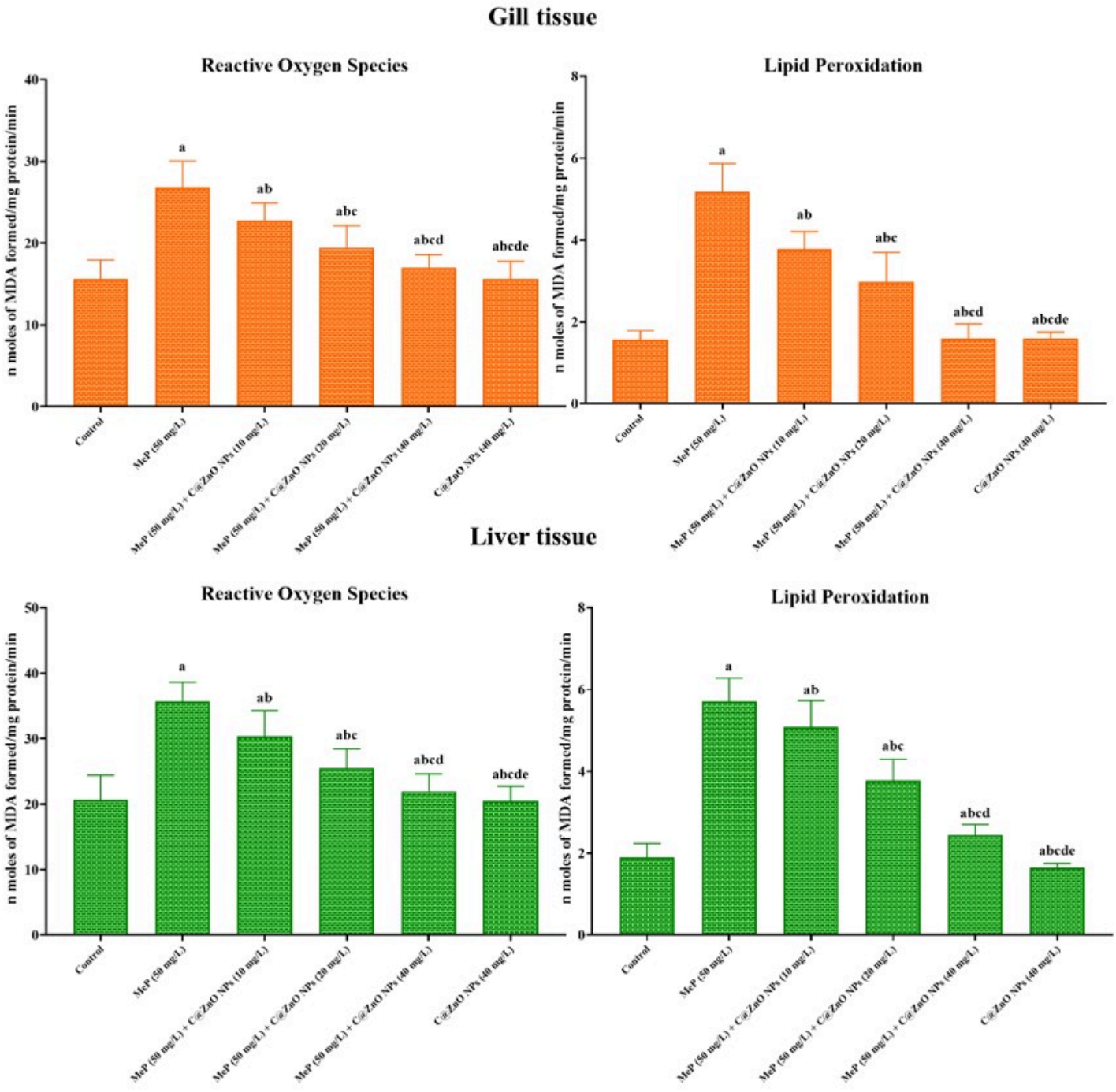

Figure 11

Effect of Ca@ZnO-NPs on ROS and lipid peroxidation in the gill and liver tissue of control and experimental group. Values are mean ± SD, with different letters indicating statistically significant differences.

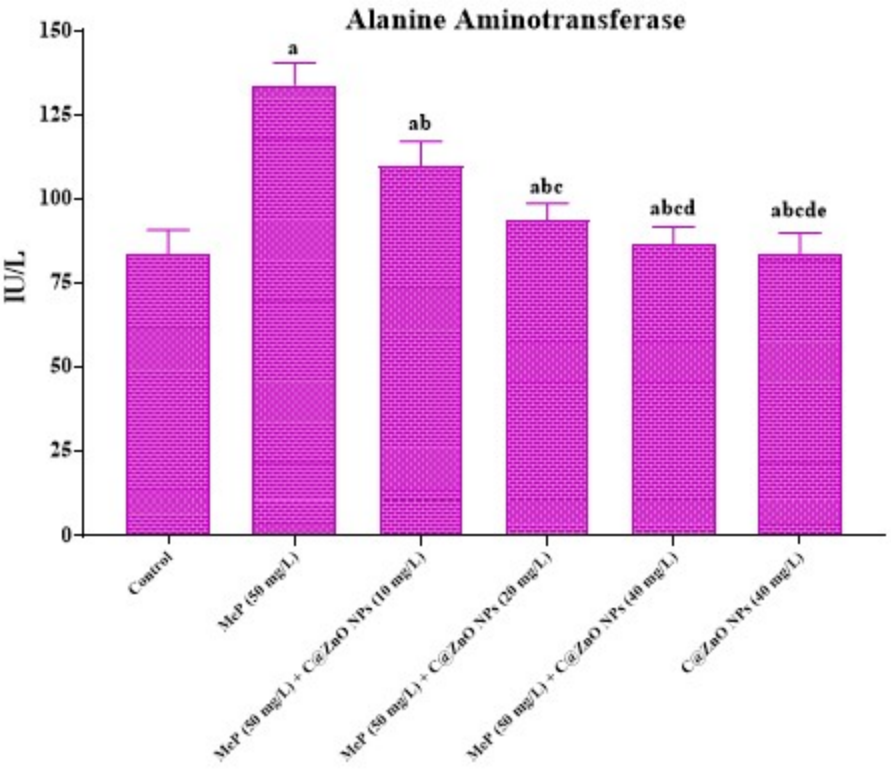

Figure 12

Effect of Ca@ZnO-NPs on ROS and lipid peroxidation in the gill and liver tissue of control and experimental group. Values are mean ± SD, with different letters indicating statistically significant differences.

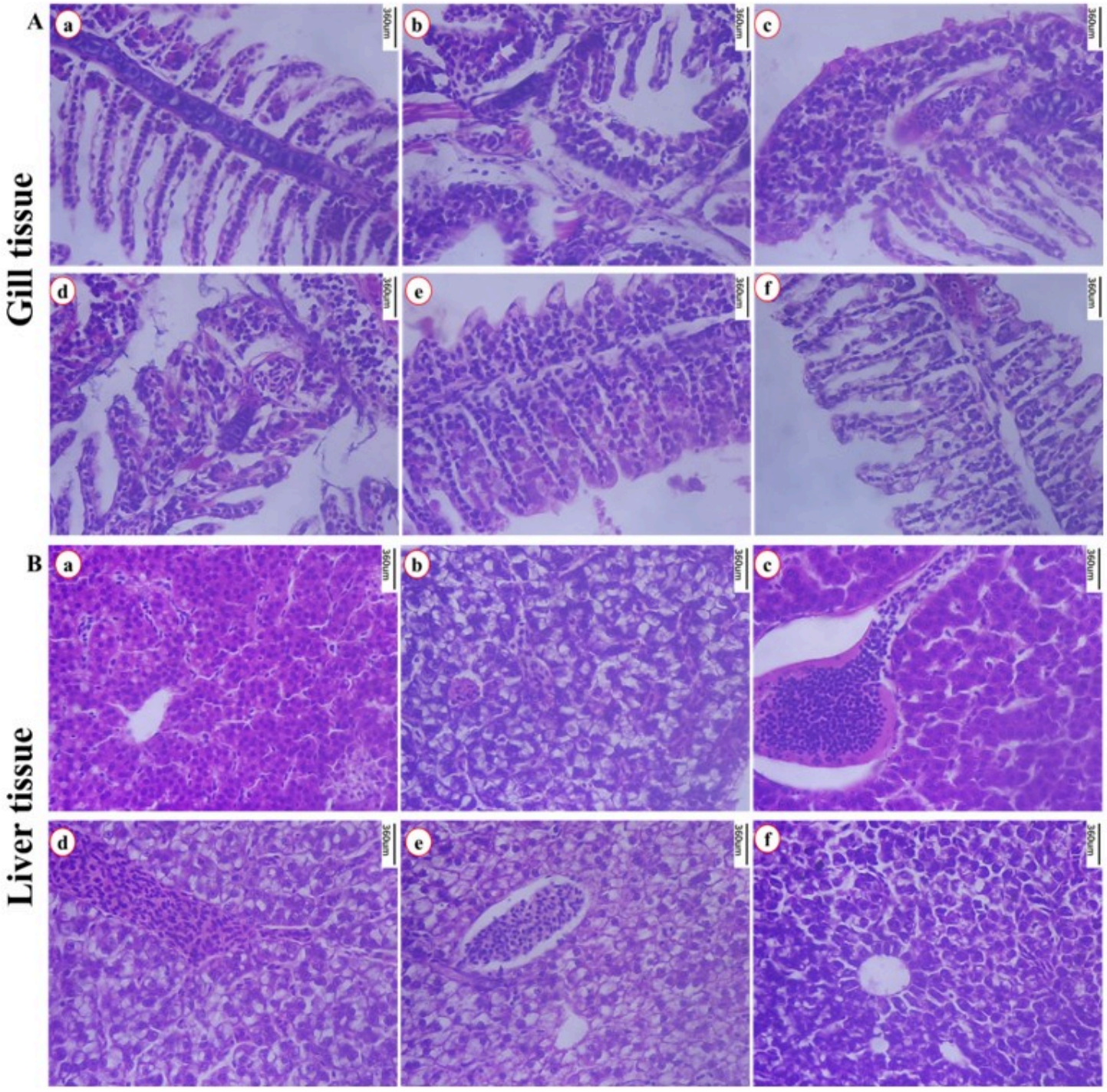

Figure 13

Histological changes in gill and liver tissues of zebrafish following 14 days of exposure to MeP and Ca@ZnO-NPs. A) Gill histology: (a) The control group exhibited normal histological characteristics such as gill filaments (F), primary lamellae (PL), and secondary lamellae (SL). (b) Zebrafish treated to 50 mg/L MeP showed significant changes, including hyperplasia (black arrow), lamellar fusion (red arrow), filament erosion (blue arrow), and epithelial lifting or dilated marginal channels (green arrow). (c, d, f) Co-exposure to MeP (50 mg/L) with Ca@ZnO-NPs at 10, 20, and 40 mg/L resulted in decreased pathological

alterations such as epithelial lifting (green arrow), lamellar fusion (red arrow), and secondary lamellar shortening (pink arrow). B) Liver histology: (a) The control group has normal liver architecture, with hexagonally distributed hepatocytes and centrally placed nuclei. (b) MeP treatment at 50 mg/L resulted in sinusoidal and venous congestion (red arrow), cytoplasmic vacuolation (green arrow), necrosis (yellow arrow), and hepatic plate disruption (arrowhead). (c, d, f) Co-treatment with MeP and Ca@ZnO-NPs (10, 20, and 40 mg/L) attenuated these effects, resulting in moderate hepatocyte shape irregularities (black arrow), cytoplasmic vacuolation (green arrow), and necrosis (yellow arrow). (Hematoxylin and eosin staining; magnification: 40x

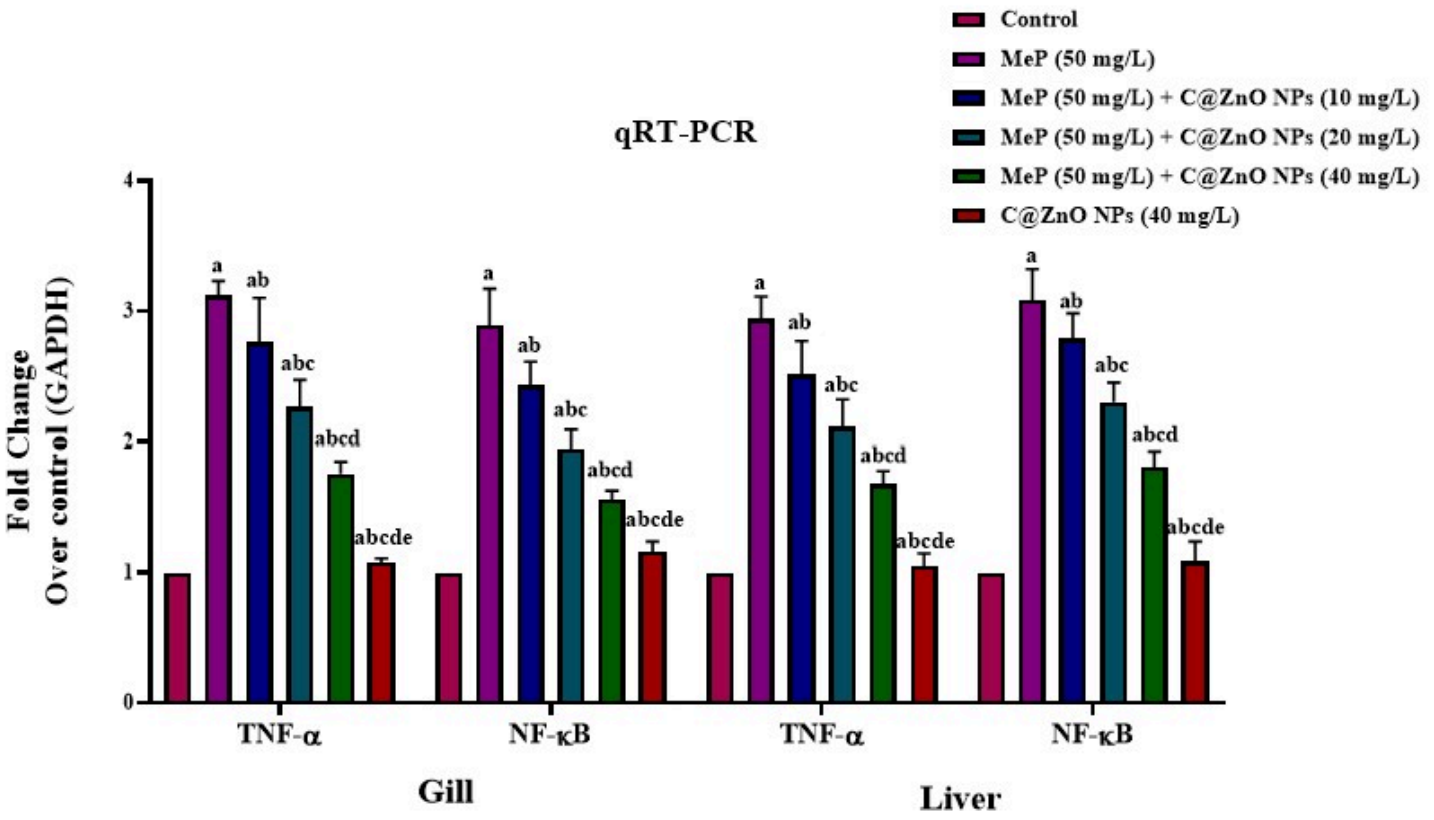

Figure 14

Effect of Ca@ZnO NPs on mRNA expression of inflammatory marker in the gill and liver tissue of control and experimental group analysed by using qRT-PCR. Values are mean ± SD, with different letters indicating statistically significant differences.

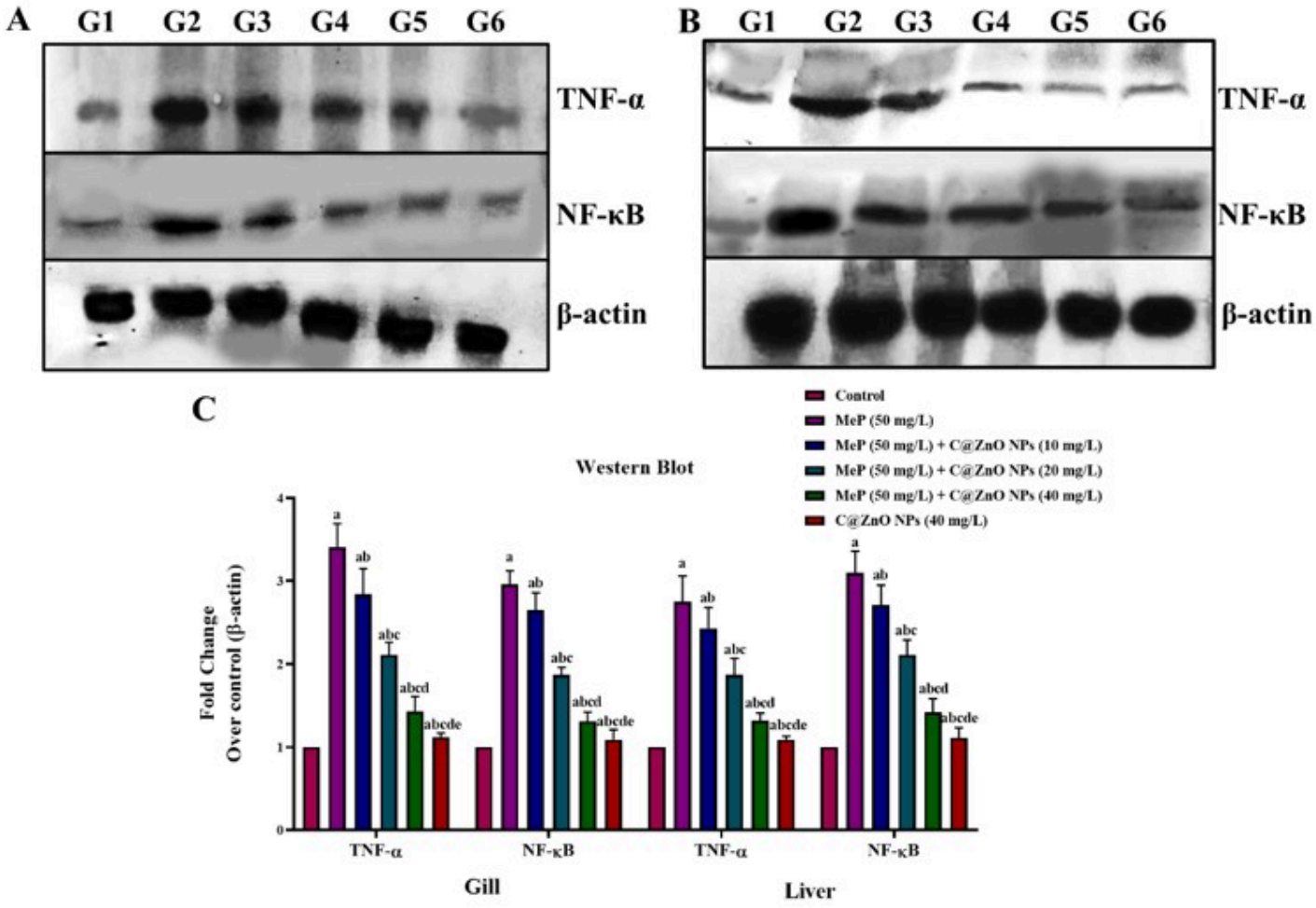

Figure 15

Effect of Ca@ZnO-NPs on protein expression of inflammatory marker in the gill and liver tissue of control and experimental group. A) Western blotting bands of gill tissue, B) Western blotting bands of liver tissues and C) Bar figures of protein expressions of TNF-α and NF-κB in the gill and liver tissue. G1-Control; G2-MeP (50mg/L); G3-MeP (50mg/L) + C@ZnO NPs (10mg/L); G4-MeP (50mg/L) + C@ZnO NPs (20mg/L); G5-MeP (50mg/L) + C@ZnO NPs (40mg/L); C@ZnO NPs (40mg/L). Values are mean ± SD, with different letters indicating statistically significant differences.

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