

# TOXICOLOGICAL EVALUATION OF EDIBLE ZINGIBER ZERUMBET-DERIVED NANOPARTICLES IN ZEBRAFISH (*DANIO RERIO*): AN IN VIVO STUDY

**JEBA MERCY JEYASEELAN<sup>1</sup>, SELVENDRAN THAMARAISELVAN<sup>2</sup>, CHITRA BALASUBRAMANIAN<sup>1</sup>, CHITRA JEYARAJ PANDIAN<sup>3</sup>, AND P THIRUMALAIVASAN<sup>4\*</sup>**

<sup>1</sup>Department of Biotechnology, Srimad Andavan Arts & Science College (Autonomous), (affiliated to Bharathidasan University), Tiruchirappalli - 620 005, India.

<sup>2</sup>Department of Biotechnology Dhanalakshmi Srinivasan University, Chengalpattu - 603 111. Tamil Nadu, India.

<sup>3</sup>Department of Biotechnology, Dr. Umayal Ramanathan College for Women, Karaikudi-630 003, Tamil Nadu, India

<sup>4\*</sup>Department of Biotechnology, The American College, Madurai-625020, Tamil Nadu, India

## \* Corresponding author:

**Dr. P. Thirumalai Vasan**, M. Sc., Ph. D., D. Sc (*Honoris Causa*)

Assistant Professor

Department of Biotechnology, The American College

Madurai- 625020 Tamil Nadu, India

**E-mail:** thirumalaivasan@americancollege.edu.in

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## Abstract

Plant-derived molecular systems have attracted considerable interest as bioactive agents because of their natural origin and therapeutic potential. In the present study, the acetylcholinesterase inhibitory activity and toxicity profile of edible Zingiber zerumbet-derived nanoparticles (EZDNPs) were evaluated in vitro and in vivo using zebrafish. Anticholinesterase activity was assessed using an established enzymatic assay and compared with the reference drug donepezil. EZDNPs did not exhibit detectable toxicity under the tested conditions. Potential genotoxic effects were evaluated using the zebrafish erythrocyte micronucleus assay, while biochemical markers associated with liver function were measured in liver-tissue homogenates to identify treatment-related alterations. The brain, liver, kidney and gills were examined histologically following acute and subacute exposure. EZDNPs showed concentration-dependent AChE inhibitory activity, with no significant increase in the measured genotoxicity-related endpoints or significant alterations in the biochemical and histopathological parameters assessed. These findings support further investigation of EZDNPs as a plant-derived nanomaterial with AChE inhibitory activity and no detectable significant toxicity at the tested doses.

## INTRODUCTION

Plants with medicinal value have been an important source of biologically active compounds that are used in traditional and modern medicine. The wide range of biological activities of plant molecules such as antimicrobial, antioxidant, anti-inflammatory and

neuroprotective properties has led to the derivation of many drugs from them. Recently, there has been a growing interest in studying medicinal plants as potential alternatives to synthetic drugs. This is mostly due to the issues of drug resistance and adverse side effects.

*Zingiber zerumbet* (L.) Smith from the Zingiberaceae family is one of the most extensively studied medicinal plants. It is well-known for its numerous pharmacological properties and its use in traditional medicine [1].

*Zingiber zerumbet*, commonly referred to as bitter ginger or shampoo ginger, is widely distributed in tropical regions of Asia. People have always used the rhizome of this plant to treat a number of illnesses, such as fever, inflammation, digestive problems and infections caused by microbes. Phytochemical investigations have demonstrated the presence of various bioactive compounds in the rhizomes such as flavonoids, phenolic compounds, terpenoids and essential oils. Zerumbone has been identified as the main bioactive compound responsible for the various biological activities of the plant [2]. It is a monocyclic sesquiterpene that has attracted much attention because of its broad spectrum of pharmacological potential. Previous studies have shown that it possesses useful properties by modulating oxidative stress and cellular signaling pathways. It has been reported to induce apoptosis and inhibit growth in different types of tumor cells [3]. Recent studies have suggested its potential neuroprotective effects by protecting neuronal cells from oxidative stress-

induced damage and inflammatory response indicating its potential role in treatment of neurodegenerative disorders [4]. *Zingiber zerumbet* has been shown to have antimicrobial activity and has potential use in cancer and neurological studies. Rhizome extracts of this plant have been shown to inhibit the growth of a variety of pathogenic microorganisms including Gram-positive and Gram-negative bacteria [5]. The results highlight the importance of this plant as a source of natural antimicrobial compounds. The antioxidant activity of *Zingiber zerumbet* extracts can also be an important feature for their ability to protect cells from damage by reactive oxygen species [6]. Recent progress in nanotechnology provides new avenues for improving the biological activity of plant-derived compounds. One such development is the plant-mediated synthesis of nanoparticles which has received much attention due to its eco-friendly nature, cost effectiveness and production of biologically active nanomaterials. In green synthesis systems,

phytochemicals present in plant extracts act as natural reducing and stabilizing agents during the formation of nanoparticles, hence eliminating the use of hazardous chemical reagents [7]. The nanoparticles derived from plants due to

their nanoscale size and large surface area exhibit enhanced interactions with biological systems which may improve their antimicrobial and pharmacological properties [8]. *Zingiber zerumbet* has long been known as a valuable medicinal plant with therapeutic benefits. Its rhizome contains multiple bioactive substances, including zerumbone, which exerts useful biological effects [9]. Converting these phytoconstituents into nanoparticles may improve their stability, bioavailability and biological activity. Edible *Zingiber zerumbet*-derived nanoparticles (EZDNPs) represent an innovative approach to develop plant-based therapeutic agents through nanobiotechnology; however, with increasing interest in plant-derived nanomaterials, the potential for biotoxicity must be thoroughly evaluated prior to clinical use. Physicochemical characteristics, particle size and the exposure level of nanoparticles affect how nanoparticles interact with biological systems and should be evaluated as part of the toxicity assessment process [10].

The zebrafish (*Danio rerio*) model has become a favoured vertebrate model organism for nanotoxicology, due to its genetic similarity to humans, short developmental cycle and sensitivity to environmental alterations. Previous

studies have demonstrated that nanoparticles induce oxidative stress, DNA damage and tissue alteration in the zebrafish model [11, 12]. The aim of the present study was to evaluate anticholinesterase activity and safety profile of EZDNPs in zebrafish by assessing genotoxicity, biochemistry and histopathology.

## MATERIALS AND METHODS

### Maintenance and Husbandry of Experimental Animals

Adult zebrafish were maintained under standard laboratory husbandry conditions in a recirculating aquatic housing system. Fish were housed under a regulated photoperiod of 14 h light and 10 h darkness at  $28 \pm 0.5$  °C. To maintain conductivity and dissolved oxygen levels suitable for laboratory culture, the system water was filtered and aerated and maintained at pH 7.0-7.5. Fish were kept at regulated stocking densities and fed a combination of commercial dry feed and live feed twice daily. Feeding was adjusted according to tank density and biomass. Animal health and water quality were monitored throughout the study. All experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC) of Mass Biotech under approval number MB/IAEC/2024/01/11 [13, 14].

## Preparation of Edible Nanoparticle Suspensions

*Zingiber zerumbet* rhizomes (100 g) were homogenized in 100 mL distilled water, filtered and sequentially centrifuged at 4,000 ×g (20 min) and 10,000 ×g (40 min) at 4 °C. The supernatant was subjected to 60% sucrose gradient ultracentrifugation at 150,000 ×g for 120 min (Beckman Coulter Optima L-100XP). The nanoparticle-containing sucrose fraction was collected and stored at -80 °C. Protein content was quantified using the Bradford assay with BSA [15].

### In vitro anticholinesterase activity

The anticholinesterase activity of EZDNPs was determined spectrophotometrically. A stock suspension of EZDNPs was prepared at. Aliquots of this stock, corresponding to 200-1000 µL of EZDNPs per reaction and final concentrations of 2-10 mg/mL in a 1-mL assay volume, were tested. A fixed 10-µL aliquot of AChE solution with constant enzymatic activity was used in every reaction and incubated with the EZDNP sample for 45 min at room temperature. Subsequently, 125 µL of 3 mM DTNB was added and the final volume was adjusted to 1 mL with Tris-HCl buffer (pH 8.0). The reaction was initiated by adding 25 µL of 15 mM ATCI and absorbance was monitored at

412 nm for 4 min using a UV-visible spectrophotometer. Donepezil was used as the positive control and an enzyme control without the test sample was included. Percentage inhibition was calculated as  $[(E - S)/E] \times 100$ , where E represents enzyme activity without the sample and S represents enzyme activity in the presence of the sample. Each concentration was analyzed in three technical replicates [16].

### Acute Toxicity Study

Adult zebrafish were used for the acute toxicity study, which was conducted with reference to OECD Test Guideline 203. EZDNPs were incorporated into a composite pellet diet (0.3 mm diameter) and administered orally at doses of 1, 5 and 10 mg/kg body weight. Forty fish were randomly assigned to four groups (n = 10 per group): one untreated control group and three EZDNP-treated groups. Each fish was weighed before being placed in a 1-L beaker containing 500 mL of dechlorinated water. Following oral administration and feed consumption, the fish were transferred to fresh water. Mortality and survival were monitored for 96 h [17].

### Subacute Toxicity Study

A 14-day semi-static toxicity study was conducted using 40 healthy adult zebrafish (>6 months old) to evaluate the

effects of EZDNPs. Fish were randomly divided into four groups ( $n = 10$  per group): a vehicle-control group and three EZDNP-treated groups administered

doses of 1, 5 and 10 mg/kg body weight. The vehicle-control group received 0.1% DMSO without EZDNPs. Fish were maintained in 5 L of dechlorinated tap water, fed once daily and uneaten feed was removed after 1 h to maintain water quality [18].

### **Evaluation of Erythrocyte Abnormalities**

On sampling days, fish were carefully removed from the tanks and blotted dry to remove excess water and mucus. Peripheral blood samples ( $n = 5$  per group) were collected from the caudal vein using a sterile syringe. Blood smears were immediately prepared on clean glass slides, air-dried for 15 min and fixed in 70% methanol for 10 min. Slides were stained with 4% Giemsa for 7 min, rinsed with water, air-dried and mounted with DPX. Micronuclei and other erythrocyte nuclear abnormalities were examined as separate endpoints under a light microscope using a 40 $\times$  objective [19].

### **Liver Function Biomarkers**

After the experimental period, five fish from each group were euthanized in accordance with the approved protocol

and liver tissues were collected. Each liver sample was homogenized separately in an appropriate volume of ice-cold homogenization buffer. The homogenates were centrifuged at  $1,800 \times g$  for 10 min at 4 °C and the resulting supernatants were used to determine alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activities [20].

### **Histological Assessment**

Major organs, including the liver, kidney, brain and gills, were collected and preserved in 10% neutral-buffered formalin after the experimental period. Tissues were processed, embedded in paraffin, sectioned at 4  $\mu$ m and stained with hematoxylin and eosin (H&E). Sections were examined under a light microscope for histopathological alterations [21].

### **Statistical Analysis**

All data were analyzed using IBM SPSS Statistics, version 25. Individual fish were treated as biological replicates; five independent liver-tissue samples were analyzed per group for biochemical endpoints. Laboratory measurements were performed in three technical replicates and the mean of the technical replicates was used as the value for each biological replicate. Data are

presented as mean  $\pm$  standard deviation. Differences among groups were analyzed using one-way analysis of variance (ANOVA), followed by Fisher's least significant difference (LSD) post hoc test. Differences were considered statistically significant at  $p < 0.05$  [22].

## RESULTS

### Assay for in vitro inhibition of acetylcholinesterase (AChE)

EZDNPs inhibited acetylcholinesterase activity in a concentration-dependent manner. Dose-response analysis yielded a reported  $IC_{50}$  value of  $1.838 \pm 0.010$  mg/mL based on linear interpolation of three replicate measurements (mean  $\pm$  SD,  $n = 3$ ). Under the same experimental conditions, donepezil, the reference standard, showed a reported  $IC_{50}$  value of  $3.549 \pm 0.089$  mg/mL based on linear interpolation of triplicate measurements (mean  $\pm$  SD,  $n = 3$ ) (Fig. 1).

### Micronucleus assay

The micronucleus assay was used to assess micronuclei and other nuclear abnormalities, including blebbed, lobed and notched nuclei, in peripheral erythrocytes from control and nanoparticle-treated zebrafish (Fig. 2). Compared with the control group, EZDNP exposure did not cause a

significant increase in the measured nuclear abnormalities. The few abnormalities observed were consistent with baseline variation. No significant increase in the measured genotoxicity-related endpoints was observed after 96 h of acute exposure or 14 days of subacute exposure.

### Biochemical assays

Hepatic biochemical markers, including AST, ALT and ALP (Fig. 3), were measured in liver-tissue homogenates following acute and subacute exposure to EZDNPs. Enzyme activities in the EZDNP-treated groups were comparable to those in the control group. No significant alterations were observed in the measured biochemical parameters.

### Histological Analysis

Histological examination following acute and subacute exposure showed no significant structural alterations in the major organs of zebrafish. Brain sections showed preserved neuronal and progenitor-cell morphology comparable to the control group. Liver sections showed preserved hepatocyte architecture, kidney tissues showed well-preserved proximal and distal tubules and gill tissues retained intact lamellar structures. Overall, exposure to EZDNPs at the tested

treatment levels did not result in significant histopathological abnormalities (Fig 4 and 5).

## DISCUSSION

EZDNPs were evaluated using an in vitro enzyme-inhibition assay and in vivo zebrafish toxicity studies. The nanoparticles showed significant acetylcholinesterase (AChE) inhibitory activity and did not produce a significant increase in the measured genotoxicity-related endpoints or significant alterations in the biochemical and histopathological parameters assessed at the tested treatment levels. Therefore, *Z. zerumbet*-derived nanoparticles may represent a source of natural product-based compounds for further pharmacological investigation. AChE is essential for cholinergic neurotransmission because it rapidly hydrolyses acetylcholine at synapses. Inhibition of this enzyme is an established symptomatic treatment approach for Alzheimer's disease [23, 24]. In the present study, EZDNPs inhibited AChE in a concentration-dependent manner, with a reported IC<sub>50</sub> value of 0.69 mg/mL. This observation indicates potential relevance to anticholinesterase research; however, direct neuroprotective effects were not evaluated and require further mechanistic investigation.

Natural products remain valuable sources of cholinesterase inhibitors. Plant secondary metabolites, including alkaloids, flavonoids, terpenoids and phenolic compounds, may interact with the catalytic site of AChE and inhibit its activity [25, 9]. *Z. zerumbet* rhizomes contain several bioactive compounds [26, 5]. Previous studies have reported that *Z. zerumbet* can modulate oxidative-stress pathways and protect neuronal cells from injury. Owing to their small size and large surface area, plant-derived nanoparticles may show enhanced interactions with biological targets compared with crude extracts [27]. The AChE inhibitory activity observed in this study may be associated with the combined effects of nanoscale properties and the bioactive phytochemicals present in EZDNPs [28]. The micronucleus assay is widely used to assess chromosomal damage and genotoxicity in aquatic organisms [29]. Zebrafish erythrocytes are suitable for this purpose because their intact nuclei are readily examined microscopically [30]. Micronuclei and other nuclear abnormalities were assessed separately. Zebrafish exposed to EZDNPs for 96 h showed no significant increase in micronuclei or other measured nuclear abnormalities compared with the control group. The observed abnormalities remained within the range measured in

the control group, indicating no significant increase in the assessed genotoxicity-related endpoints under the tested conditions. Previous studies have reported increased micronucleus formation following zebrafish exposure to toxic substances such as fenitrothion, erythromycin and lincomycin [31-33].

AST, ALT and ALP are commonly measured as biochemical indicators associated with liver function and hepatocellular injury [34]. Altered activities of these enzymes in liver-tissue homogenates may indicate treatment-related hepatic changes. In the present study, no significant differences in AST, ALT, or ALP activities were observed between the control and EZDNP-treated groups. All measured enzyme activities remained within the ranges observed in the control group, indicating no significant alteration in the assessed hepatic biochemical parameters.

These findings are broadly consistent with previous reports describing the hepatoprotective effects of *Zingiber zerumbet* and *zerumbone* [28, 5]. *Zerumbone* has been reported to reduce oxidative stress and support liver architecture in experimental models [27]. Nevertheless, the present findings are limited to the measured biomarkers, treatment levels and exposure periods and should not be interpreted as evidence

of universal safety.

Histopathological examination did not reveal significant tissue alterations at the tested treatment levels. The gills, liver, kidneys and brain were evaluated for structural integrity and no significant differences were observed between the control and EZDNP-treated groups. Brain tissue showed preserved neuronal morphology, kidney tissue showed preserved proximal and distal tubules and liver tissue retained normal hepatocyte architecture without evident necrosis or inflammatory-cell infiltration.

Gill tissues retained an intact lamellar structure comparable to that of the control group. Because gills are particularly sensitive to toxic exposure, structural changes may occur following exposure to harmful chemicals [34]. No significant gill lamellar damage was observed following EZDNP exposure under the experimental conditions.

The present observations are consistent with a previous report [5] describing limited histological alterations following treatment with *Zingiber zerumbet* extract. In contrast, exposure to certain synthetic chemicals, pesticides, or heavy metals can produce tissue injury and inflammatory responses in zebrafish. The present findings support the absence of detectable significant



histopathological alterations at the tested treatment levels and exposure periods.

## CONCLUSION

The present study demonstrated concentration-dependent in vitro inhibition of acetylcholinesterase by EZDNPs. This activity indicates potential relevance to anticholinesterase applications, although direct neuroprotective effects were not evaluated and require further mechanistic investigation. In zebrafish, no significant increase was observed in the measured genotoxicity-related endpoints and no significant alterations were detected in the assessed liver-homogenate biochemical markers or histopathological features at the tested treatment levels. These findings support further investigation of EZDNPs as a plant-derived nanomaterial with AChE inhibitory activity and no detectable significant toxicity under the experimental conditions used in this study.

## Conflict of Interest

The authors declare that they have no financial or non-financial conflicts of interest related to this study.

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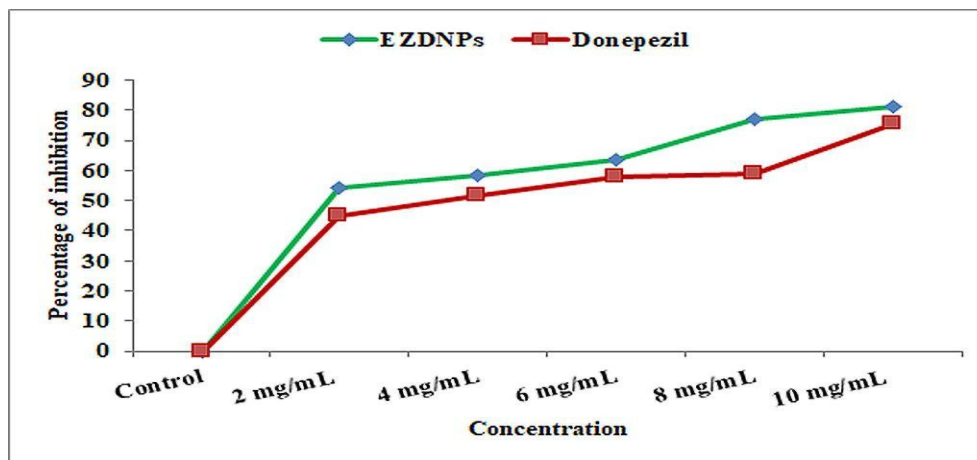
**Figure**

**Figure 1: In vitro acetylcholinesterase (AChE) inhibitory activity of EZDNPs and donepezil.**

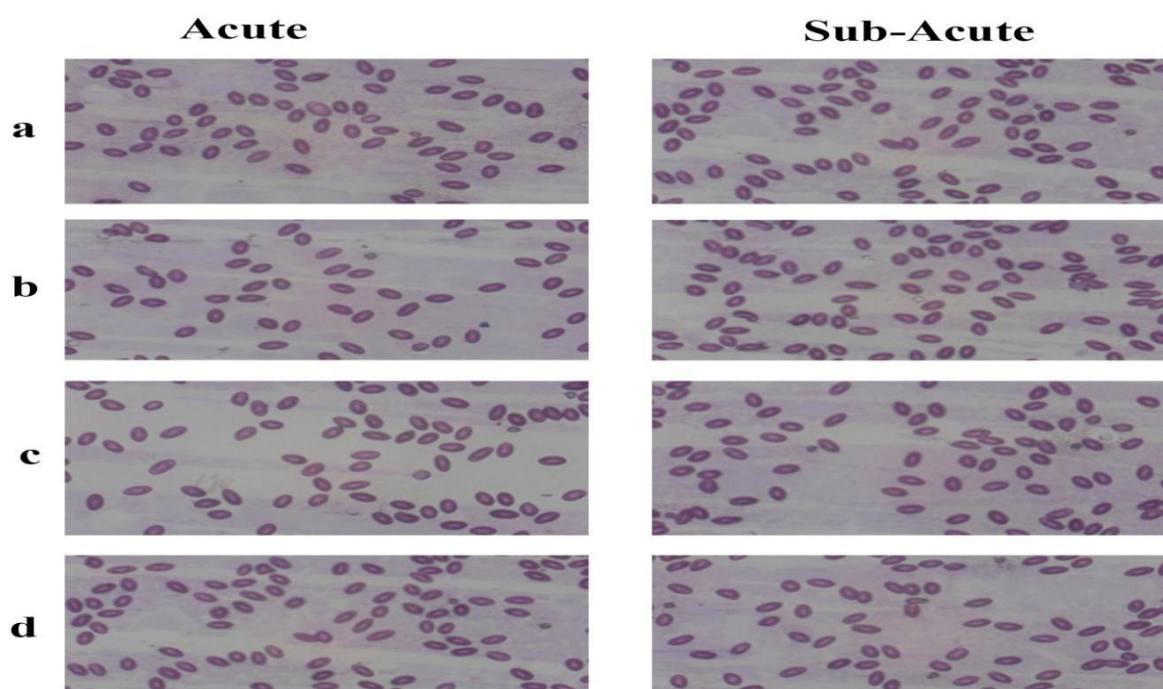


**Figure 2: Micronucleus assay in zebrafish erythrocytes following EZDNP exposure.**

Images from acute and subacute toxicity studies showing zebrafish peripheral erythrocytes: (a) control, (b) 1 mg/kg EZDNPs, (c) 5 mg/kg EZDNPs and (d) 10 mg/kg EZDNPs.

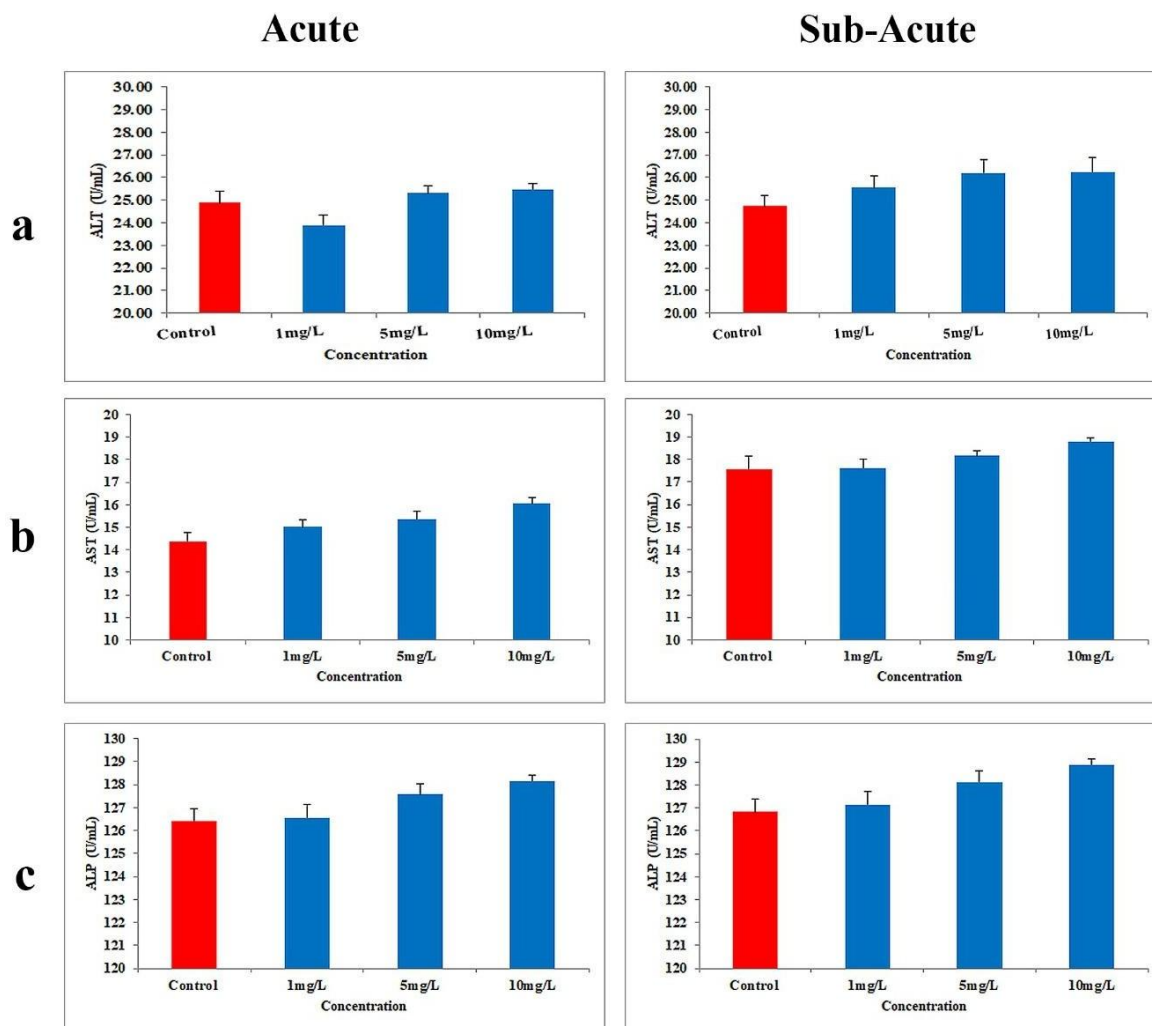


**Figure 3: Biochemical examination of zebrafish following EZDNP exposure. Activities of (a) ALT, (b) AST and (c) ALP measured in liver-tissue homogenates from the control and EZDNP-treated groups during acute and subacute exposure. Four groups were included: (a) control, (b) 1 mg/kg EZDNPs, (c) 5 mg/kg EZDNPs and (d) 10 mg/kg EZDNPs.**

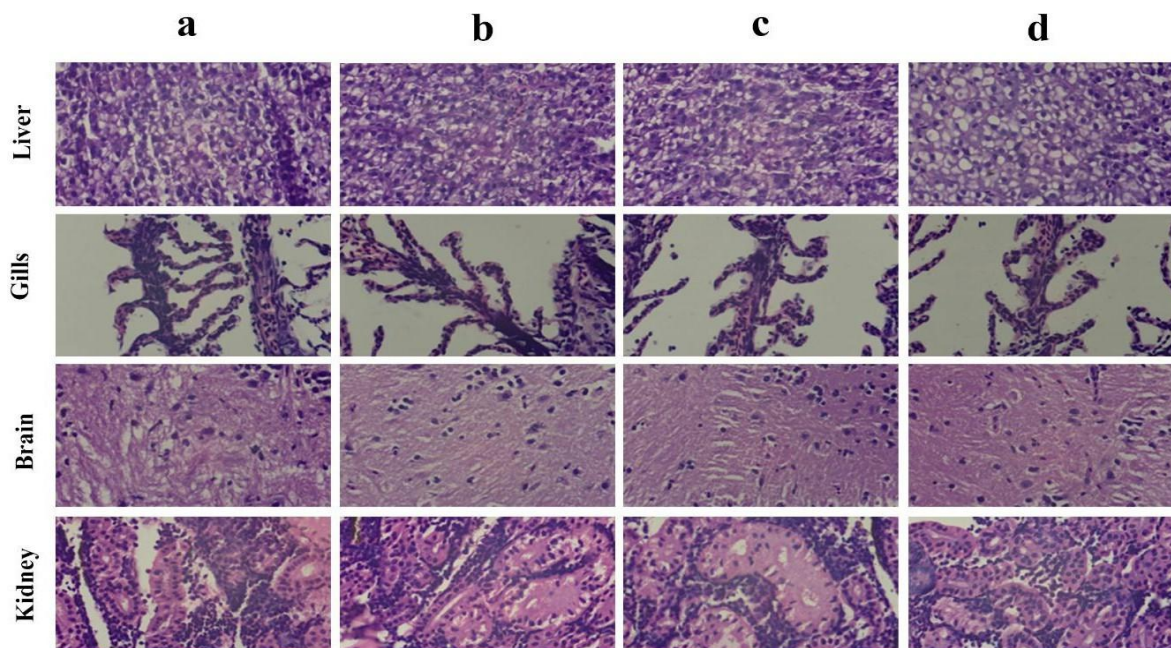


**Figure 4: Histopathological examination of zebrafish organs following acute EZDNP exposure. Micrographs of liver, gill, brain and kidney tissues from (a) control, (b) 1 mg/kg EZDNP-treated, (c) 5 mg/kg EZDNP-treated and (d) 10 mg/kg EZDNP-treated groups, showing preserved tissue architecture (H&E staining; magnification: approximately 40 $\times$ ).**

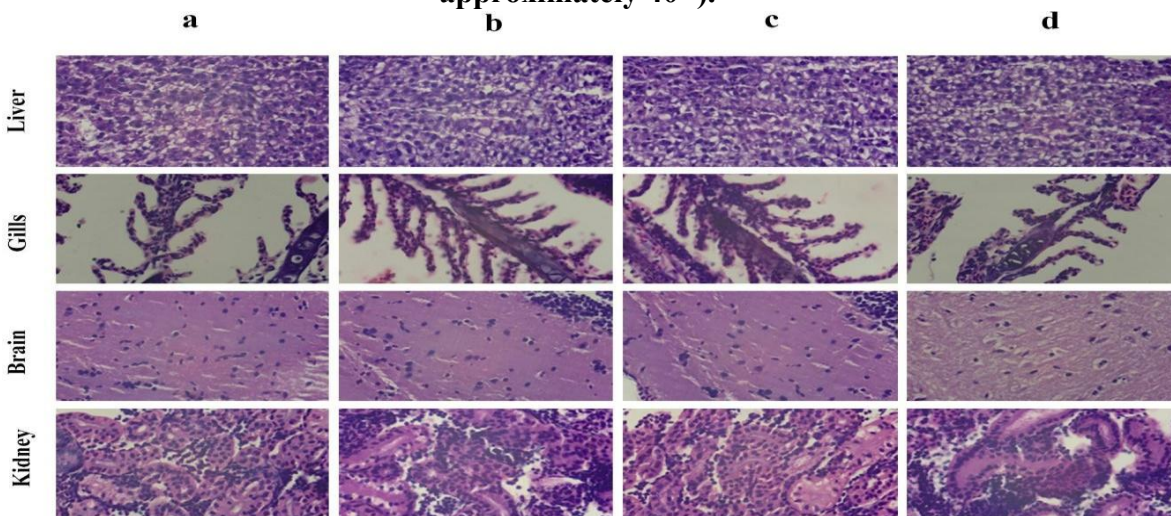


**Figure 5: Histopathological examination of zebrafish organs following subacute EZDNP exposure. Micrographs of liver, gill, brain and kidney tissues from (a) control, (b) 1 mg/kg EZDNP-treated, (c) 5 mg/kg EZDNP-treated and (d) 10 mg/kg EZDNP-treated groups, showing preserved tissue architecture (H&E staining; magnification: approximately 40 $\times$ ).**