



Research Paper

Assessment of Diethyl Phthalate-induced Neurobehavioural Toxicity and Oxidative Stress in Adult Zebrafish (*Danio rerio*)

Sneha Bibyan and Deepali Jat*

Department of Zoology, Dr Harisingh Gour Central University, Sagar, Madhya Pradesh, India

*Corresponding author email: deepalijat18@gmail.com

Orcid ID- 0000-0001-7908-3889

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Abstract: Diethyl Phthalate is a chemical plasticiser commonly found in a variety of everyday household items, ranging from plastic toys and flooring materials to cosmetics and more. Usually classified as a low-molecular-weight phthalate, it may also be considered as an endocrine disruptor causing severe health-related disorders, including reproductive damage, neurotoxicity, oxidative stress, hormonal imbalance and many more. In relation to the current study, the main focus is to analyse the neurobehavioural effects of DEP on the adult zebrafish brain. Our Preliminary findings indicate that DEP acts as a potent neurotoxicant agent, generating behavioural changes and oxidative stress in the animal model. Further, behavioural analysis was performed to assess anxiety-related behaviour, and it was observed that the compound did not generate any anxiety-related behaviour at the given dose. On further analysis, it was found that the compound has a tendency to generate oxidative stress in the fish. Therefore, the

overall conclusion of the study is that the DEP works in a dose-dependent manner, where the lower doses may generate oxidative stress and the higher dose of the DEP causes hypoactivity in the fish, causing instant mortality. Future studies aimed at clarifying the neuroprotective effectiveness of natural substances against DEP-induced neurotoxicity might offer a fresh approach to treatment.

Keywords: Diethyl Phthalate, Oxidative stress, Neurotoxicant, Dose-dependent study.

Abbreviations: ANOVA: One-way analysis of variance, AOPP: Advanced oxidation of Protein Products, DMSO: Dimethyl sulfoxide, LPO: Lipid Peroxidation, NO: Nitric Oxide, OFT: Open field test, DEP: Diethyl Phthalate

Introduction:

Phthalate esters are synthetic chemical plasticisers extensively used in the

production of polyvinyl chloride (PVC) and other polymer-based products in daily life, including the use of cosmetics, personal care products such as nail paints, fragrances, plastic toys, adhesives, flooring materials and many more (Aminova et al., 2021; Godwin, 2024). The phthalates are classified into two groups based on their molecular weight: a) Low Molecular Weight Phthalate (LMWP), comprising six or fewer carbons, such as dimethyl phthalate (DMP), diethyl phthalate (DEP), and dibutyl phthalate (DBP) and b) High Molecular Weight Phthalate (HMWP), consisting of more than six carbons, such as di(2-ethylhexyl) phthalate (DEHP), diisononyl phthalate (DINP), and diisodecyl phthalate (DIDP) (Wittassek & Angerer, 2008). Among phthalates, Diethyl Phthalate (DEP; CAS No. 84-66-2) is one of the most widely used, especially as a carrier solvent and fixative in deodorants, fragrances, and medicinal compositions (Aldegunde-Louzao et al., 2024). Because of its low molecular weight and moderate lipophilicity, it is rapidly absorbed across the biological membranes, including the blood-brain barrier (Ahmadpour et al., 2021). Unlike other high molecular weight phthalates, DEP faces less scrutiny. Still, since it is ubiquitous in the environment despite having brief biological half-lives, they are consistently found in pseudo-steady states in human bodily fluids such as breast milk, blood, and urine (Hu et al., 2022; Pradhan et al., 2018; Wittassek & Angerer, 2008).

Environmental contamination with phthalates occurs through multiple pathways: industrial discharge into aquatic systems, leaching from consumer products, and runoff from agricultural sites (Baloyi et al., 2021). DEP concentrations have been detected in surface water, sediments, and aquatic animals across a range of geographic

locations, indicating that aquatic ecosystems are important sinks for phthalate esters. Aquatic organisms, especially fish, are exposed to DEP through ingestion, gills, and skin, making them important sentinel species for assessing the risk of environmental neurotoxicity (Deidda et al., 2021; Sefali et al., 2026).

Numerous phthalate compounds also act as endocrine disruptors, and there is mounting evidence that they are developmental neurotoxicants that can disrupt neuronal differentiation and synaptic plasticity (Lucaccioni et al., 2021; Masuo & Ishido, 2011). DEP has been shown to alter thyroid hormone homeostasis, is a critical regulator of neural development, and modulates steroid receptor signalling pathways in neural tissue (Kömürcü & Yavaş, 2026). Research on rodent models has shown that phthalate exposure during the critical development window leads to alterations in anxiety-like behaviour, reduction in spatial memory, and disturbance in social cognition. These consequences are partially linked to neuroinflammation and monoaminergic neurotransmitter dysregulation (Yu et al., 2024).

The animal model that has emerged as an indispensable vertebrate model for toxicological and neuroscientific research is the Zebrafish, also known as *Danio rerio*. Due to its distinctive combination of biological, genetic, and logistical advantages, sharing approximately 70% homology with the human genome and approximately 84% of human disease-associated genes have identifiable zebrafish orthologues, it is considered one of the best vertebrate models (Adamson et al., 2018). Anatomically, the brain of zebrafish consists of conserved forebrain, midbrain, and hindbrain, and includes neurotransmitter systems, including dopaminergic,

serotonergic, GABAergic, and cholinergic pathways, that are analogous to those in the human brain (Horzmann & Freeman, 2016). Additionally, zebrafish larvae and adults exhibit a wide range of quantitative behaviours, such as social interaction, anxiety-related reactions, locomotor activity, learning, and memory, all of which may be accurately assessed utilising standardised behavioural assay platforms (Martins & Schroeder, 2024).

Despite these findings, the specific neurotoxic mechanisms by which DEP affects the teleost brain and the corresponding behavioural phenotypes remain insufficiently characterised. Although a limited number of studies have investigated the reproductive and developmental toxicity of DEP in zebrafish, the neurotoxic effects of DEP, concerning oxidative stress and perturbation of antioxidant defence, remain to be thoroughly investigated. Given the widespread distribution of DEP in the environment, its presence in surface waters, and the neurological sensitivity of teleosts during development, it is crucial to understand the consequences of DEP. Understanding the neurotoxic mechanisms of DEP in zebrafish not only advances the understanding of aquatic toxicology but also provides preliminary evidence relevant to the phthalate risk in higher vertebrates.

The present study was therefore designed to investigate the neurotoxic potential of DEP in adult zebrafish through an integrative approach combining behavioural and biochemical analyses of brain tissue. Together, this study aims to provide a mechanistic explanation of DEP-induced behavioural alterations in zebrafish and add to the increasing amount of information about the neurobehavioral concerns

associated with phthalate plasticisers in aquatic vertebrates.

Methodology:

Animal Husbandry and Rearing: The wild-type strain of adult zebrafish was collected from the local fish distributors in the Sagar District of Madhya Pradesh. Initially, the fish were selected randomly from the stock population without sex-based segregation and acclimatised in the laboratory conditions for 15 days. The fish were kept in the 10-litre rectangular glass water tank, with a sponge filter for proper aeration and cleanliness of water. The water was changed every other day. The pH of water was maintained at approximately 7-7.5. The temperature of the lab was maintained at 26-28 °C. The photoperiod of the fish was maintained for 12:14 hours (dark: light). For proper nutrition, the fish were fed with TetraBits commercial food *ad libitum*.

Drug and Experimental Design: DEP (84-66-02) was obtained from Sigma-Aldrich. The adult zebrafish were divided into the following groups:

- (a) Control (n=10): fish were provided with the home tank water.
- (b) Vehicle control (n=10): fish were provided with home tank water along with 0.01% DMSO
- (c) DEP (Dose I) (n=10): fish were provided with home tank water along with 10µl/L of DEP for 2 hours per day continuously for 12 days.
- (d) DEP (Dose II) (n=10): fish were provided with home tank water along with the combination of 50µl/L of DEP for 2 hours per day continuously for 12 days.
- (e) DEP (Dose III) (n=10): fish were provided with home tank water along with the combination of 100µl/L of

DEP for 2 hours per day continuously for 12 days.

- (f) DEP (Dose IV) (n=10): fish were provided with home tank water along with 150µl/L of DEP for 2 hours per day continuously for 12 days.

After exposure to the toxicant, the fish were returned to their home tank in the housing system.

Morphometrical Analysis of Zebrafish:

The initial and final body weight after the completion of the experiment was measured using a weighing machine, and the data were analysed using ANOVA. To weigh the fish before and after the experiment, zebrafish from each group were kept in an ice-filled water box.

Behaviour Analysis: Behavioural analysis was done to assess the cognitive and social behaviour of the fish and was performed on the last day of the experiment. To record the behaviour of zebrafish, the fish were acclimatised to the conditions in which they were placed in a round glass tank with a capacity of 4 litres for 10 minutes, and their behaviour was recorded using a digital imaging system, a Logitech B525 HD webcam. Further, the analysis was performed using Toxtrac software (<https://sourceforge.net/projects/toxtrac/>).

The parameters measured during the analysis are average speed, total distance travelled, frozen events, mobile average speed, and number of explored areas. Average speed helps to measure the mean speed of the fish during the test duration. Frozen events help us to analyse the period during which the fish remain completely immobile during the period of analysis. Mobile average speed helps us calculate the average speed of the fish only during the period when the fish were moving actively.

Preparation of brain Homogenate: After the exposure of the DEP to the fish on the

12th day, the fish were euthanised by giving the cold shock treatment and decapitated. The brain was isolated and cleaned using the ice-cold saline buffer. The brains were homogenised in phosphate-buffered saline (pH 7). The mixture was further centrifuged at 4°C in a cooling centrifuge. The supernatant was collected and stored in -20°C.

Biochemical Analysis: As mentioned, the method to measure the malondialdehyde (MDA) concentration, lipid peroxidation (LPO), was performed with the help of a spectrophotometer at the absorbance at 532 nm (Nahar & Jat, 2021). The analysis of advanced oxidation of protein products (AOPP) was done using previously reported methods. The mixture was analysed using a spectrophotometer at an absorbance of 340 nm, and values are expressed in µmol/mg protein (Mahajan et al., 2018). To quantify the nitric oxide (NO) in the reaction mixture, a protocol optimised in the laboratory was utilised, and the reaction mixture was analysed with the help of a spectrophotometer at an absorbance of 548 nm. The final result was expressed in µmol/mg protein (Jensen, 2007). The activity of Acetylcholinesterase was analysed using the optimised protocol from the lab by the use of spectrophotometer at an absorbance of 412 nm (Worek et al., 2012).

Statistical Analysis: All the experimental data mentioned here are represented as Mean ± SEM. Data interpretation and graph formation were done using GraphPad Prism software version 9.5.1 (GraphPad Software, San Diego, CA, USA). Data was analysed using One-way ANOVA followed by Tukey's post-hoc test to measure the alterations between the groups (*p < 0.05, **p < 0.01, ***p < 0.001, ns; non-significant).

Results:

Mortality Rate of Zebrafish after exposure to DEP: After continuous exposure to DEP for 12 days, it was observed that the fish showed a dose-dependent increase in mortality. It was found that there was no mortality in the control, vehicle control, Dose I and Dose II during the 12-day exposure period. In the Dose III group, the first death was seen on the 6th day of the exposure period, and the fish were seen in a hypoactive state during the exposure period; all the fish were lost within 6 days. The fish of the Dose IV group

were highly hypoactive during the exposure and even after the exposure period, and the highest mortality can be seen in this group. After the 3rd day, the death of the fish can be seen, and within a week, all the fish were dead (Fig.1). Therefore, only doses I and II were considered for further behavioural and biochemical study.

Body weight: The body weight of the zebrafish was measured before the initiation of the experiment and after the completion of the experiment. It was observed that the body weight had reduced in all the groups after the completion of the experiment, showing no significant difference (Fig.2).

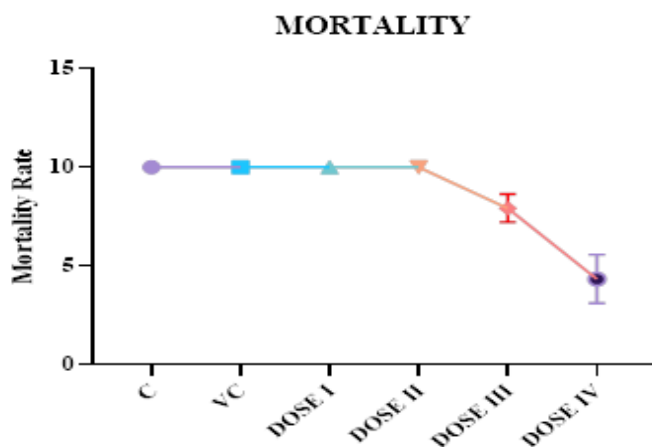


Figure: 1. Graph showing the mortality rate of the fish after the administration of DEP for 12 days. Data is represented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; compared to control group

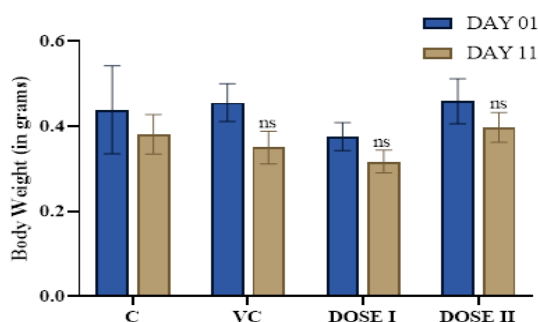


Figure: 2. Graph showing the Body weight of the fish before and after the administration of DEP for 12 days. Data is represented as mean ± SEM. ns=non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; compared to control group.

Behavioural Analysis: DEP appears to act in a dose-dependent manner, with lower doses affecting zebrafish behaviour and higher doses potentially leading to fish death. A behaviour change might be due to anxiety or stress initiated by the exposure to DEP. The exposure of DEP to the fish showed a significant increase in average speed [$F(3,16) = 381, p < 0.0001$] (Fig 3A), mobile average speed [$F(3,16) = 71.3, p < 0.0001$] (Fig 3B), total distance travelled

[$F(3,16) = 98.7, p < 0.0001$] (Fig 3C), area explored [$F(3,16) = 24.2, p < 0.0001$] (Fig 3D). Dose I exposed fish showed no significant differences in any of the above-mentioned factors compared to the control and vehicle control fish. Tukey's post hoc analysis showed that various parameters of behavioural analysis have increased significantly. Fig 3E displays the trajectory graph of the fish prepared using Toxtrac software.

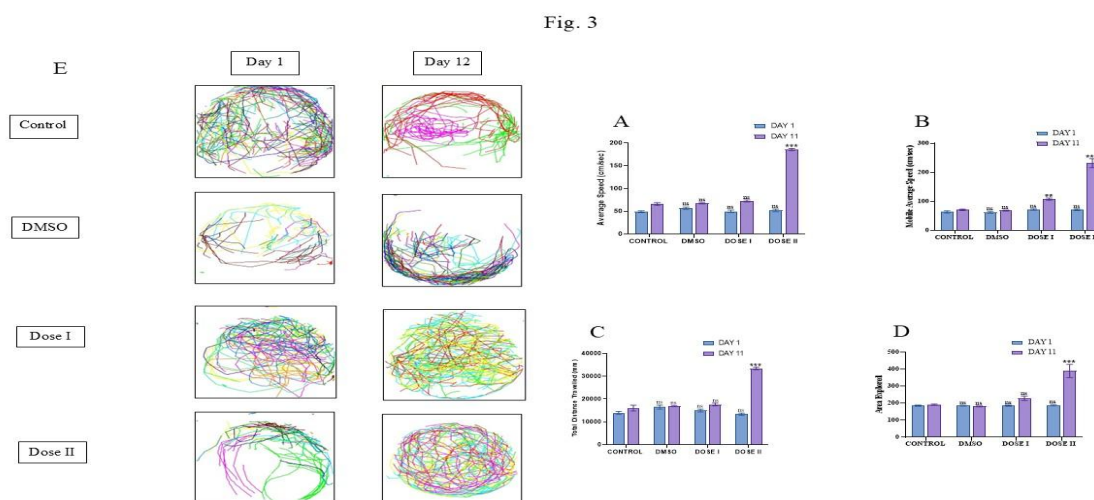


Figure: 3. Graphs showing the effect of DEP on behavioural analysis of the fish before and after the experiment. a) Average Speed, b) Mobile average speed, c) total distance travelled, d) Area explored by the fish, and e) Frozen events. Data is represented as mean ± SEM. ns=non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; compared to control group. A trajectory plot was prepared using Toxtrac software.

Biochemical Analysis: The universality of the DEP is one of the major risk factors and may be the reason for numerous neurodegenerative disorders. Oxidative damage in the tissues is caused by the synthesis and buildup of free radicals. Oxidative stress is the difference between the generation, buildup, and removal of free radicals in the tissue, and it may be detected using a variety of biochemical tests, including AOPP, NO, LPO, and AChE activity.

Biochemical assays concerned with oxidative stress are shown in Fig.4. The results of this study were analysed using one-way ANOVA, and the analysis presents the significant effect of DEP on the Oxidative stress biomarkers: AOPP [$F(3,8) = 9.64, p < 0.05$] (Fig 4A), NO [$F(3,8) = 6.61, p < 0.05$] (Fig 4B), LPO [$F(3,8) = 12.9, p < 0.05$] (Fig 4C) and Acetylcholinesterase activity [$F(3,8) = 30.8, p = 0.0001$] (Fig 4D). Tukey's post hoc analysis showed that levels of

oxidative stress biomarkers increased, and the activity of acetylcholinesterase

decreased significantly ($p < 0.05$).

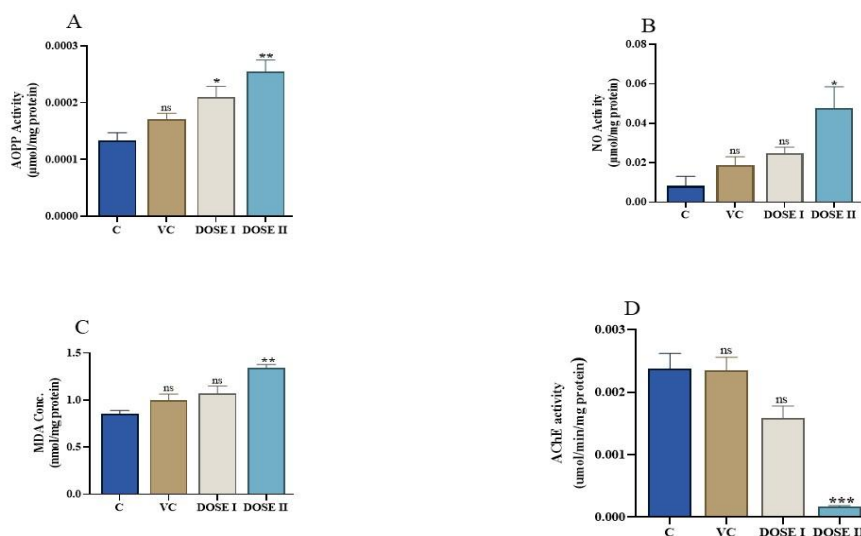


Figure: 4. Graphs showing the effect of DEP on biochemical analysis of the fish after the experiment. a) AOPP activity, b) NO activity, and c) MDA concentration. Data is represented as mean $\pm$ SEM. ns=non-significant, * $p < 0.05$, ** $p < 0.01$, * $p < 0.001$; compared to control group.**

Discussion:

The study shows that exposure of zebrafish to DEP produces dose-dependent effects on mortality, behavioural alterations, and oxidative stress biomarkers in the fish and acetylcholinesterase activity. The mortality curve clearly showed that the fish survived the two lower DEP dose concentrations and survived for the full period of the experiment, i.e., 12 days. The third concentration (Dose III) caused the mortality by day 6, and the highest concentration of the dose (Dose IV) caused the rapid mortality within 2-3 days. The steep increase in toxicity between Dose II and Dose III verifies that DEP at sufficiently high exposures may quickly harm the nervous and circulatory system of the organism.

There is a gradual increase in the lethality across doses that is consistent with classical toxicant dose-response relationships. Rapid death at the Dose IV indicates acute systemic failure, which is more likely to be mediated by oxidative damage, metabolic disruption, or respiratory collapse. If we compare the survival rate at the intermediate dose versus the highest dose suggests a point at which homeostatic and compensatory antioxidant systems become overburdened. Previous studies on phthalates and their related compounds have shown similar dose-dependent mortality on aquatic organisms, which often occurs due to the disruption of mitochondrial function, an increase in reactive oxygen/nitrogen species, and impaired gill or cardiac physiology (Manna & Firdous, 2025).

For the analysis of behavioural changes, open field analysis was performed. Open field testing revealed dose-related behavioural deficits that are correlated with mortality and biochemical results. When the fish were administered with sub-lethal doses, i.e., two lower doses, it can be seen that there is an alteration in the locomotor activity and a reduction in anxiety-like behaviour, for instance, a reduction in thigmotaxis behaviour, increased central area exploration, increased total distance travelled, and an increase in the speed with a reduction in frozen events. Whereas at intermediate or high doses, fish have shown delayed responses, disorganised swimming behaviour and hypoactivity before death, consistent with neuromuscular impairment or central nervous system depression.

The behavioural analysis data were difficult to understand when we studied the oxidative stress-mediated neurotoxicity. An increase in ROS/RNS and lipid peroxidation compromises neuronal membrane integrity, synaptic signalling, and mitochondrial ATP production (Alkhalifa et al., 2025). DEP may also interfere in neurotransmitter signalling either directly or indirectly via oxidative damage. Such modulations have also been reported in other phthalates and could explain the changes in anxiety-like and locomotor behaviour that were observed (de Oliveira et al., 2025).

Biochemical assays showed a significant, dose-dependent increase in Advanced Oxidation of protein products (AOPP), Nitric Oxide Assay (NO) and Lipid Peroxidation assay (LPO). The contemporary increase in protein oxidation and LPO encourages the broad oxidative assault on cellular macromolecules. Elevation of AOPP suggests oxidative modification of proteins, which may impair the enzyme function, receptor binding and

disrupt many more functions. Increase LPO suggested that the membrane is damaged and hence disrupts the cellular and organelle membrane, promoting ion leakage, and impairs signalling. Rise in NO levels may suggest an increase in inducible nitric oxide synthase (iNOS) activation secondary to oxidative/inflammatory signalling; NO reacts with superoxide to form peroxynitrite, an aggressive nitrating and oxidising species that further amplifies cellular injury. AChE is an enzyme that is responsible for the hydrolysis of acetylcholine and hence terminating the cholinergic neurotransmission. Reduction in the AChE activity may lead to the accumulation of acetylcholine in the synapses, which may lead to prolonged cholinergic stimulation and impaired neuronal signalling. The observed reduction in AChE activity may be attributed to DEP-induced stress, which may damage neuronal membranes and alter enzyme structure and functions. The reduction in AChE activity, along with impaired locomotor performance observed in DEP-exposed zebrafish, suggests that disruption of cholinergic neurotransmission contributes to the alteration in behaviour of zebrafish.

The relation between higher biomarker levels and decreased survival rate suggests a causative link that oxidative damage may substantially contribute to systemic failure and death. The behavioural deficits and biomarker elevations preceding mortality at sublethal and intermediate doses strengthen the mechanistic interpretation. Altogether, we may suggest that DEP toxicity in fish involves the activation of oxidative and nitrosative stress pathways that compromise neuronal and systemic physiology.

Hence, the study integrating mortality, behaviour, and oxidative stress biomarkers indicates that DEP is capable of causing

neurotoxicity and systemic changes in fish in a dose-dependent manner. The doses that do not cause immediate mortality are capable of inducing measurable behavioural and biochemical disturbances, indicating sublethal ecological consequences such as impaired predator avoidance, feeding, and reproduction. These results support the concern about DEP contamination in the aquatic environment, where high exposure or chronic accumulation may threaten the fish population.

In summary, DEP exposure produced clear dose-dependent toxicity in fish, where sublethal concentrations caused behavioural changes and oxidative damage, whereas higher concentrations caused rapid mortality. There is an elevation in oxidative stress biomarkers, i.e., AOPP, NO and LPO and AChE activity, indicating oxidative and nitrosative stress as a central mechanism linking behavioural impairment to molecular injury and death. Hence, these findings indicate a need for further mechanistic and ecologically oriented studies to determine the environmental risk posed by DEP in aquatic systems.

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and editing, Supervision, Visualisation. All authors read and approved the final manuscript.

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Declarations:

Data availability: The datasets used and analysed during the current study are available from the corresponding author upon reasonable request.

Conflict of interest: The authors declare no conflicts of interest.

Ethics approval: The ethical permission was provided by CPCSEA (05/IAEC/Ex/Vety/2025). The experimental procedures were carried out as per CPCSEA norms and internationally followed standards for the ethical treatment of animals in scientific research. All experimental protocols were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of Vedic Institute of Pharmaceutical Sciences, Sagar (M.P.), India, before the commencement of the study.

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