IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

Preliminary Toxicological Assessment of Hesperidin-Loaded Iron Oxide Nanoparticles in Brine Shrimp and Zebrafish Embryo Models

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Abstract

The increasing biomedical application of nanoparticle-based delivery systems necessitates comprehensive safety evaluation prior to translational development. The present study aimed to investigate the preliminary toxicological profile of hesperidin-loaded iron oxide nanoparticles (Hesp-IONPs) using alternative aquatic model systems, namely the brine shrimp (*Artemia salina*) lethality assay and the zebrafish (*Danio rerio*) embryo acute toxicity model. In the brine shrimp assay, nauplii were exposed to graded concentrations of Hesp-IONPs (1–160 µg/mL) for 24 hours, and percentage live nauplii was determined to estimate the effective concentration (EC_{50}). A concentration-dependent increase in mortality was observed, with an EC_{50} value of approximately 33.60µg/mL, indicating the safety of the prepared nanoparticle. Further developmental toxicity assessment was performed in zebrafish embryos exposed to varying concentrations of Hesp-IONPs up to 72 hours post-fertilization (hpf). Survival rate and hatching rate were evaluated. The results demonstrated a dose- and time-dependent reduction in survival and hatching rates at elevated concentrations. However, the lower concentrations exhibited only negligible morphological abnormalities and maintained acceptable survival rates, suggesting a favourable safety margin within the therapeutic range. Overall, the findings provide preliminary evidence that Hesp-IONPs exhibit moderate concentration-dependent toxicity at higher doses, while demonstrating relative biocompatibility at lower exposure levels. These results support the continued investigation of Hesp-IONPs as a potential nanocarrier system, while emphasizing the importance of dose optimization for safe biomedical applications.

Keywords: *Hesperidin; Iron oxide nanoparticles; Nanotoxicology; Brine shrimp lethality assay; Zebrafish embryo toxicity.*

Introduction

Cancer remains a major global health problem, characterized by deregulated cell proliferation, evasion of apoptosis, metabolic reprogramming and the capacity to invade and colonize distant organs (Johariya *et al*., 2023). Tumour biology in bone is particularly challenging because osseous tissue presents a dense extracellular matrix and a distinct microenvironment that impedes drug penetration and fosters chemoresistance and metastatic spread (Liu *et al*., 2025). Osteosarcoma, the most common primary malignant bone tumour, predominantly affects children and young adults and frequently demonstrates aggressive behaviour and early dissemination, underlining the need for more selective and effective therapeutic platforms (Beird *et al*., 2022).

Phytochemicals such as the citrus flavanone glycoside hesperidin exhibit a broad spectrum of bioactivities relevant to cancer prevention and therapy, including antioxidant, anti-inflammatory and antiproliferative effects (Yi *et al*., 2017). However, hesperidin's clinical translation is limited by poor aqueous solubility, low intestinal absorption, extensive first-pass metabolism and rapid elimination, which together produce low and variable systemic bioavailability following conventional dosing. Strategies that enhance solubility, protect the molecule from premature metabolism, and improve tumour delivery are therefore needed to realize hesperidin's therapeutic potential (Hoang *et al*., 2025).

Nanoparticle-based delivery offers a route to overcome these limitations by increasing apparent solubility, prolonging circulation time, and enabling targeted accumulation at tumour sites via passive (EPR) and active targeting mechanisms (Attia *et al*., 2019). Among nanocarriers**,** iron oxide nanoparticles (IONPs) have particular advantages for oncological applications: they are biocompatible, can be synthesized with superparamagnetic properties, permit facile surface functionalization for drug loading and targeting, and enable magnetic guidance and multimodal theranostics (imaging + therapy). These attributes make IONPs attractive candidates for delivering poorly bioavailable phytochemicals such as hesperidin to bone tumours while simultaneously offering diagnostic imaging capability (Ajinkya *et al*., 2020; Salehirozveh *et al*., 2024).

Nevertheless, the biological behaviour and safety of metal-based nanocarriers must be rigorously evaluated. Metal oxide nanoparticles can produce concentration-dependent oxidative stress, perturb developmental processes and provoke organ-specific toxicity depending on size, coating, agglomeration state, dose and route of exposure (Chrishtop *et al*., 2021; Wadhawan *et al*., 2025). The zebrafish embryo model and brine shrimp (Artemia) assays are established, cost-effective *in vivo* screens for nanoparticle developmental and acute toxicity: zebrafish embryos permit sensitive readouts of mortality, hatching, morphological malformations, cardiac function and behavioural endpoints, while the brine shrimp lethality assay provides a rapid measure of acute cytotoxicity useful as a preliminary screen. These model systems are widely used to screen metal and metal-oxide nanoparticles, including iron oxide, and to inform subsequent mammalian testing (Haque & Ward, 2018; Jagdale *et al*., 2020).

Given the complementary goals of improving hesperidin's bioavailability and achieving targeted bone-tumour delivery while ensuring acceptable safety, the present study therefore developed and characterized hesperidin-encapsulated iron oxide nanoparticles (Hesp-IONPs) and evaluated their antioxidant capacity, in vitro anticancer efficacy against an osteosarcoma model, and preliminary *in vivo* toxicity using established

small-animal screens (brine shrimp and zebrafish embryos). Evaluating Hesp-IONPs across physicochemical, cellular and organismal endpoints offers an integrated assessment of whether magnetic nanocarriers can simultaneously enhance therapeutic effect and maintain an acceptable safety margin, information essential for rational progression toward preclinical animal studies and potential clinical translation.

Materials and Methods

Brine Shrimp Lethality Assay

The preliminary cytotoxicity of the synthesized hesperidin-loaded iron oxide nanoparticles (Hesp-IONPs) was evaluated using the brine shrimp (*Artemia salina*) lethality assay (Kumar *et al*., 2012). Artificial seawater was prepared by dissolving 2g of iodine-free salt in 200 mL of distilled water and mixed thoroughly until complete dissolution. The prepared saline solution was used as the hatching and experimental medium. Freshly hatched brine shrimp nauplii were collected under illumination after 24–48 hours of incubation. Six-well ELISA plates were used for the assay, and each well was filled with 10–12 mL of the prepared saline water. Ten active nauplii were carefully transferred into each well using a micropipette. Hesp-IONPs were added to the respective wells to obtain final concentrations of 1, 5, 10, 20, 40, 80, and 160µg/mL. A control group containing only saline water without nanoparticles was maintained under identical experimental conditions. All treatments were performed in triplicate. The plates were incubated at room temperature for 24 hours under normal laboratory lighting conditions. After the incubation period, the number of surviving nauplii in each well was counted manually using a magnifying lens. Nauplii that showed no movement even after gentle agitation were considered dead. The percentage live nauplii was calculated using the following formula:

(Number of dead nauplii / **(**number of dead nauplii + number of live nauplii)) × 100

The EC_{50} value was determined by plotting percentage live nauplii against concentration.

Zebrafish embryonic toxicology evaluation of Hesp-IONPs (Wang *et al*., 2016)

Fish Maintenance and Hesp-IONPs treatment

Wild-type zebrafish (*Danio rerio*) were acquired from local Indian vendors and were housed in individual tanks under controlled conditions of temperature ($28^{o}\pm2^{o}C$), light/dark cycle (14:10 h), and pH (6.8–8.5). The fishes were fed with commercially available dry blood worms or optimum food twice daily. Zebrafish embryos were obtained by crossing one female and three males per breeding tank, and viable eggs were collected and rinsed at least three times with freshly prepared E3 medium without methylene blue. The study involved the placement of fertilized eggs in culture plates of varying well sizes (6, 12, and 24 wells) with 20 embryos per 2mL solution per well. The experimental treatment and control groups were replicated three times. To prepare the experimental treatment, a stock suspension of Hesp-IONPs with five different concentrations was freshly made and added directly to the E3 medium. The solution was sonicated for 15 minutes to disperse the nanoparticles while maintaining a pH range of 7.2-7.3. Healthy fertilized embryos were exposed to different concentrations of Hesp-IONPs ranging from (1, 5, 10, 20, 40, 80 & 160µg/mL) for 24 to 72 hours post fertilization. The Hesp-IONPs were added to the E3 medium where the embryos were incubated. Control groups were also included in the experiment. Dead embryos were removed from the

nanoparticles exposed groups every 12 hours. All experimental plates were wrapped in foil to exclude light and maintained at 28°C.

Zebrafish embryonic toxicity evaluation (Wang et al., 2016)

Throughout the exposure period following fertilization, the developmental stages of Zebrafish embryos were monitored using a stereo microscope. The embryos were subjected to various concentrations of Hesp-IONPs (1, 5, 10, 20, 40, 80 & 160µg/mL) for 24-72hpf. Embryonic mortality and hatching rates were assessed at 24-hour intervals. The study endpoints included embryo/hatchling mortality, hatching rate, and the identification and documentation of any malformations among the embryos and larvae in both control and treatment groups. Photographs of malformed embryos were captured using a COSLAB - Model: HL-10A light microscope and the percentage of abnormal embryos was recorded every 24 hours.

Statistical Analysis

All experiments were performed in duplicate or triplicate, and the data were expressed as Mean ± standard error mean (SEM). Statistical analyses were carried out using GraphPad Prism (Version 8.0, GraphPad Software, San Diego, CA, USA). For the brine shrimp lethality assay, percentage live nauplii was calculated for each concentration after 24 hours of exposure. The effective concentration (EC_{50}) was determined by linear regression analysis. For the zebrafish embryo acute toxicity assay, survival rate and hatching rate were recorded at 24, 48, and 72 hours post-fertilization (hpf). Two-way analysis of variance (Two-Way ANOVA) was used to evaluate the effects of concentration and exposure time on survival and hatching rates. When significant differences were detected, Tukey's multiple comparison test was applied for post hoc pairwise comparisons. A p-value of <0.05*, <0.01* and <0.001*** was considered statistically significant.

Results and Discussion

Brine Shrimp Lethality Assay

The acute toxicity of hesperidin-loaded iron oxide nanoparticles (Hesp-IONPs) was initially evaluated using the brine shrimp (*Artemia salina*) lethality assay. Exposure of nauplii to increasing concentrations of Hesp-IONPs (1–160 µg/mL) for 24 hours produced a concentration-dependent decline in viability (Fig. 1). Control groups consistently exhibited near-complete survival, confirming assay reliability. Survival rates remained statistically comparable to control at lower doses (1–10 µg/mL), but a significant reduction was observed beginning at 20 µg/mL ($p < 0.01$) and became more pronounced at 40–160 µg/mL ($p < 0.001$). The estimated median effective concentration (EC_{50}) was 33.60 µg/mL, indicating moderate lethality under these conditions. These observations are consistent with the use of *Artemia salina* as a rapid preliminary toxicity model for nanostructures, where brine shrimp lethality has been shown to correlate with broader cytotoxic potential and provide a cost-effective alternative to more resource-intensive *in vitro* assays (de Paiva Pinheiro *et al*., 2024).

Previous studies using *Artemia* models indicate that the nauplius stage is sensitive to nanoparticle exposure, and metal oxide nanoparticles often exhibit dose-dependent toxicity profiles in these assays. Conversely, some engineered nanoparticles (e.g., silica nanoparticles) show minimal lethal effects at comparable exposures, illustrating how surface chemistry and particle composition influence biocompatibility

(Gambardella *et al*., 2014; Zhu *et al*., 2017). The moderate concentration dependence observed in the present study suggests that hesperidin encapsulation may mitigate some inherent nanoparticle cytotoxicity, likely due to hesperidin's antioxidant properties that can counterbalance oxidative stress induced by metal cores.

Oxidative stress is recognized as a key mechanism underlying nanoparticle toxicity in aquatic organisms, often leading to protein, lipid, and DNA damage at elevated exposures. Although brine shrimp lethality does not directly model vertebrate development, it provides an early indication of potential bioactivity and the necessity for follow-up evaluation in more complex organisms (Manke *et al*., 2013; Nhamussua *et al*., 2026). The observed EC_{50} aligns with previously reported nanocarrier systems where moderate fatality thresholds were indicative of manageable toxicity for biomedical applications.

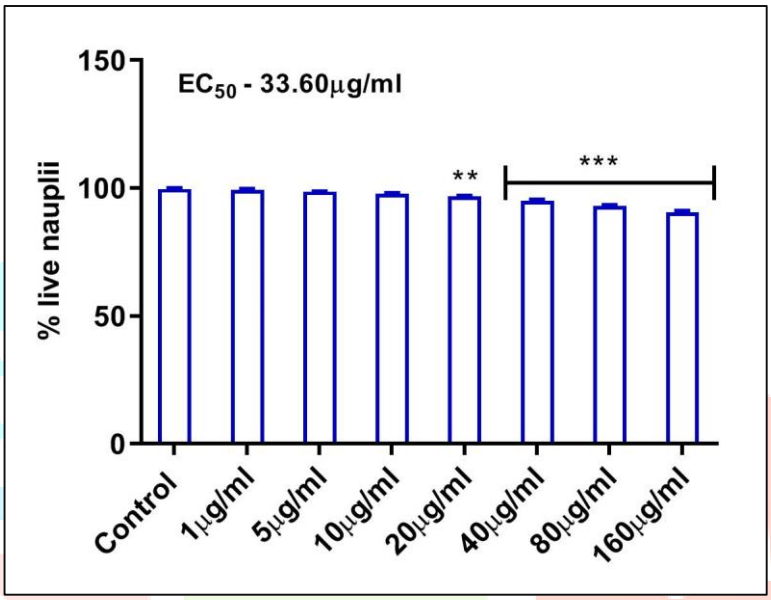

Fig.1. Brine shrimp lethality assay showing the effect of Hesp-IONPs on Artemia salina nauplii after 24 h exposure

Zebrafish Embryonic Toxicity Evaluation

Zebrafish (*Danio rerio*) embryos are widely recognized as a sensitive vertebrate model for nanoparticle toxicity assessment due to their transparent chorion, rapid development, and genetic similarity to humans (Haque & Ward, 2018). The present findings demonstrate that Hesp-IONPs induced moderate, concentration-dependent embryotoxic effects, primarily manifested as reduced survival and delayed hatching rather than severe teratogenic deformities. Zebrafish embryos exposed to Hesp-IONPs at 40µg/mL (selected based on the brine shrimp EC_{50} of 33.60µg/mL) exhibited time-dependent alterations in survival and hatching parameters. Representative micrographs (Fig.2. A–C) demonstrate normal embryonic morphology at 24 hpf, while mild developmental delay and reduced spontaneous movement were observed at 48hpf. By 72hpf, larvae displayed slight pigmentation irregularities and reduced motility compared to controls, although gross malformations were absent.

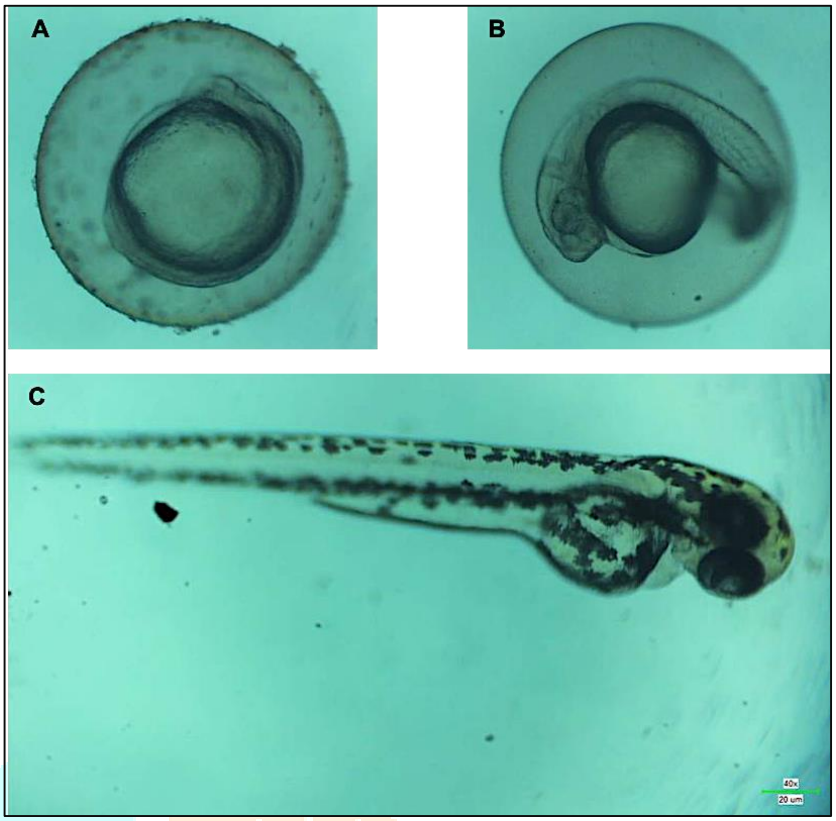

Fig.2. Representative morphological changes in zebrafish embryos exposed to Hesp-IONPs (40 µg/mL). (A) Embryo at 24 hours post-fertilization (hpf) showing intact chorion and normal early developmental morphology. (B) Embryo at 48hpf demonstrating visible organogenesis with mild developmental delay and reduced spontaneous movement. (C) Larva at 72 hpf displaying complete body elongation, pigmentation, and yolk sac absorption, with slight reduction in motility compared to control. Images were captured under light microscopy (40× magnification; scale bar = 20 µm).

Quantitative survival analysis revealed a concentration-dependent and time-dependent reduction in viability (Fig. 3. % Survival Rate). At 24 hpf, survival remained comparable to control (>95%). However, a gradual decline was observed at 48 hpf and became more evident at 72 hpf, particularly at higher concentrations (80–160 µg/mL), with statistical significance ($p < 0.05$ to $p < 0.001$). The calculated LC_{50} value was 46.18 µg/mL, indicating moderate embryotoxicity.

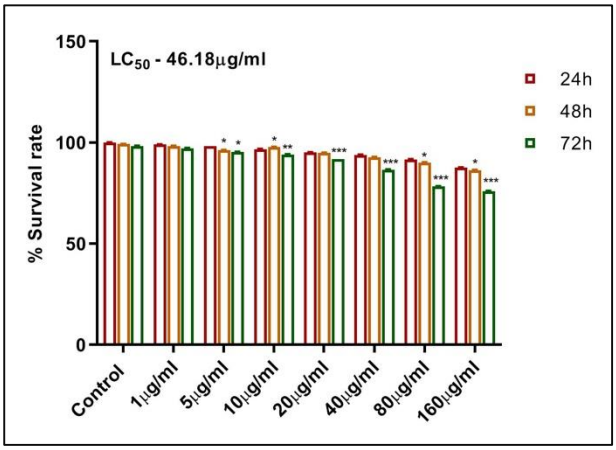

Fig.3. Effect of Hesp-IONPs on zebrafish embryo survival rate

Similarly, hatching rate analysis (Fig.4. % Hatching Rate) demonstrated a significant delay at 48 hpf and 72 hpf in embryos exposed to ≥20 µg/mL. At 160 µg/mL, hatching was markedly reduced ($p < 0.001$), with an estimated LC_{50} of 42.76µg/mL for the hatching endpoint.

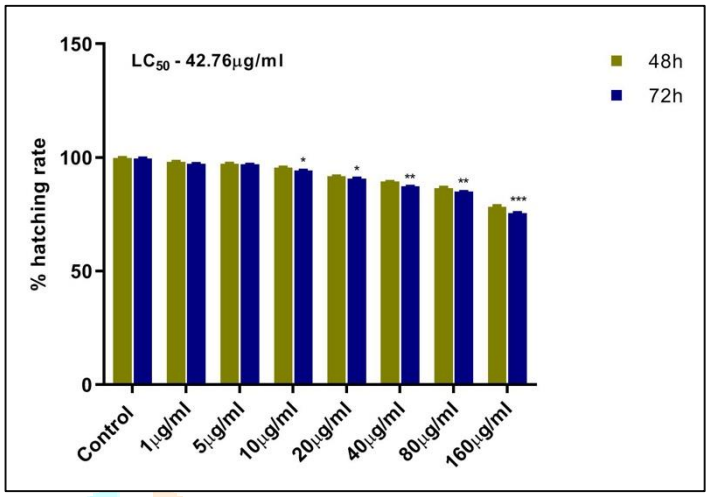

Fig.4. Effect of Hesp-IONPs on zebrafish embryo hatching rate

The time-dependent decline in survival aligns with previous reports indicating that iron oxide nanoparticles can induce oxidative stress and mitochondrial dysfunction in zebrafish embryos at higher concentrations (Jurewicz *et al*., 2020). Iron-based nanomaterials may catalyze reactive oxygen species (ROS) generation via Fenton-type reactions, leading to developmental impairment (Saghir *et al*., 2026). However, the absence of pronounced morphological abnormalities in the present study suggests that hesperidin encapsulation may attenuate severe oxidative injury. Hesperidin is known to possess strong antioxidant and free radical–scavenging properties (Hoang *et al*., 2025), which could partially counteract nanoparticle-mediated ROS generation.

Delayed hatching observed at ≥20 µg/mL is a common sublethal endpoint in zebrafish nanotoxicology. Hatching inhibition has been attributed to nanoparticle accumulation on the chorionic membrane, which may interfere with chorionase enzyme activity or oxygen diffusion (Abou-Saleh *et al*., 2019). Metal oxide nanoparticles, including Fe_3O_4, have previously been shown to reduce hatching rates in a concentration-dependent manner. The LC_{50} values obtained in this study (42–46µg/mL range) fall within the moderate toxicity classification commonly reported for surface-modified iron oxide nanoparticles (Magro *et al*., 2018). Importantly, the observed LC_{50} was higher than the brine shrimp EC_{50} (33.60 µg/mL), suggesting comparatively lower sensitivity in the vertebrate model at early developmental stages. This pattern is consistent with earlier comparative toxicity studies demonstrating species-specific variability between invertebrate and zebrafish embryo assays (Teixido *et al*., 2022).

Conclusion

Overall, the findings indicate that Hesp-IONPs exhibit moderate embryotoxicity at higher concentrations, primarily affecting survival and hatching kinetics rather than inducing severe structural deformities. The relatively preserved morphology supports the hypothesis that bioactive surface functionalization with hesperidin may improve nanoparticle biocompatibility. Nevertheless, concentration-dependent effects underscore the importance of dose optimization for biomedical translation.

Authors' Contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Competing Interests

Authors have declared that no competing interests exist.

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