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Brine Shrimp Lethality Assay for *in vitro* Toxicity Assessment of Eco-friendly Synthesized ZnO Nanoparticles

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ABSTRACT

Background: Zinc is an essential micronutrient as well as an effective antimicrobial agent, widely used in fish health management. Due to its relatively non-toxic nature, zinc is also employed in the green synthesis of nanoparticles. However, concerns remain regarding its potential toxicity, particularly in aquaculture and fish health applications.

Methods: Pomegranate peel-mediated ZnO nanoparticles (PP-ZnO NPs) were synthesized *via* a green route. The nanoparticles were characterized using transmission electron microscopy (TEM) to determine particle morphology and size, dynamic light scattering (DLS) to assess hydrodynamic size distribution and polydispersity index (PDI) and zeta potential analysis to evaluate surface charge and colloidal stability. *In vitro* toxicity was assessed using the brine shrimp lethality assay (BSLA) to determine the effect of PP-ZnO NPs on shrimp survival.

Result: TEM analysis revealed that the PP-ZnO NPs possessed a hexagonal wurtzite structure with an average size of 54±8 nm. DLS analysis showed that the nanoparticles were moderately monodispersed, with a PDI of 0.345, indicating slight agglomeration. Zeta potential measurements confirmed a negative surface charge (-41.5 mV), suggesting good colloidal stability. BSLA demonstrated that the PP-ZnO NPs were non-toxic to brine shrimp, indicating their biocompatibility and safety for potential applications.

Key words: Brine shrimp, Lethality assay, Nanoparticles, Pomegranate peel, Zinc oxide.

INTRODUCTION

Nanoparticles used in human and veterinary medicine are known for their applications in therapeutics, vaccination, drug delivery and as antimicrobials (Kumar *et al*., 2023; Youssef *et al*., 2019). These nanoparticles also find their application in animal health as immunoostimulants, as prophylactics, as antimicrobials and in feed formulation (Khan *et al*., 2024; Afifi *et al*., 2016). Among the different types of nanoparticles the plant based nanoparticles are gaining importance because of easier and ecofriendly synthesis (Ying *et al*., 2022). These green synthesis nanoparticles are used as alternatives to commercial drugs ensuring the sustainable and reliable aquaculture needs (Fajardo *et al*., 2022).

There are many studies highlighting the application of nanoparticles in both fish health and nutrition. Nanotechnology is used in the application of nanovaccine delivery against fish diseases and also in the shrimp farming, for the development of pathogen free seedlings and enhancement of growth in shrimps (Dasari *et al*., 2024). In case of disease management in aquaculture the metal nanoparticles such as gold, silver and ZnO nanoparticles are widely used. For example antibacterial activity against fish bacterial pathogens *Vibrio harveyii*, *Vibrio parahaemolyticus*, *Vibrio anguillarum* and *Vibrio vulnificus* has been demonstrated by Fatima *et al*. (2020), using silver nanoparticles (Fatima *et al*., 2020). Zinc oxide nanoparticles acted as good bacterial agents against *Aeromonas hydrophila* and *Vibrio parahaemolyticus* in *Oreochromis mossambicus* (Abinaya *et al*., 2023).

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Despite the enormous applications of nanoparticles in aquaculture, their potential toxicity remains a major concern. Several studies have highlighted the adverse effects of nanoparticles in human and animal cells (Riyaz *et al*., 2025). To address this issue, green-synthesized nanoparticles have emerged as an alternative; however, even these environmentally friendly nanoparticles may exhibit low levels of cytotoxicity (Kazemi *et al*., 2023). Among various metal-based nanoparticles, zinc oxide (ZnO) nanoparticles are safe and are reported to exert comparatively lower toxic effects compared to other metallic nanoparticles (Anjum *et al*., 2021). In this context, the present study evaluates the *in vitro* toxicity of pomegranate peel-mediated ZnO nanoparticles to ensure their safety prior to applications in aquaculture.

MATERIALS AND METHODS

DLS and zeta potential measurement

The experimental research work was carried out at the Department of Fish Pathology and Health Management, Fisheries College and Research Institute, Thoothukudi – 628008, during the period from April 2022 to April 2025. The pomegranate peel-capped ZnO nanoparticles (PP-ZnO NPs) were synthesized according to Abdelmigid *et al*. (2022) with slight modifications (Abdelmigid *et al*., 2022). The size distribution, surface charge and colloidal stability of the PP-ZnO NPs were evaluated using dynamic light scattering (DLS) and zeta potential measurements. PP-ZnO NPs were prepared at a concentration of 100 µg/mL in deionized water and sonicated for 2 minutes to ensure homogeneous dispersion and to disrupt possible agglomerates. The analysis was carried out using a HORIBA SZ-100 Particle Size and Zeta Potential Analyzer (Version 2.80, HORIBA Scientific, Japan) at a controlled temperature of 25.0±0.1°C (Chakra *et al*., 2025).

Transmission electron microscopy

The synthesized PP-ZnO NPs were analyzed using transmission electron microscopy (TEM) to determine their surface morphology, particle size and crystalline nature. A nanoparticle suspension was prepared by dispersing PP-ZnO NPs in deionized water at a concentration of 50 µg/mL, followed by sonication for 10 minutes to achieve a homogeneous dispersion and to break up any agglomerates. A drop of the suspension was then placed on a carbon-coated copper grid (300 mesh) and the grid was allowed to air-dry at room temperature prior to imaging (Abdelmigid *et al*., 2022). Particle size was measured from the TEM micrographs using ImageJ software.

Brine shrimp lethality assay

Brine shrimp lethality assay **(**BSLA) was conducted to determine whether the PP-ZnO NPs had any adverse effects on the brine shrimp's (*Artemia salina*) survival *in vitro* and its behaviour was compared to the blank solution (no toxicant) and the positive control (known toxicant- $K_2Cr_2O_7$). The findings from this study will contribute to the understanding of the potential environmental impact of the PP-ZnO NPs solution and its safety implications (Sahgal *et al*., 2010). Each test group consisted of 30 brine shrimp placed in a 25 ml saline solution mixed with different concentrations of the sample (10,30,80,100 and 200 µg/ml). To establish a point of comparison, a control group (blank) was also set up, consisting of 30 brine shrimp in brine solution without the test substance (PP-ZnO NPs.) A positive control group was included using potassium dichromate ($K_2Cr_2O_7$) at a concentration of 1 mg/ml, a known toxicant. Shrimp behaviour and mortality were recorded at multiple time points at 1, 2, 4, 6 and 24 hours post exposure (hpe) to PP-ZnO NPs. The final mortality rate was assessed after 24 hours for all groups: sample, blank and positive control. The goal was to determine whether the nanoparticles negatively affected the survival or behaviour of brine shrimp in comparison to the controls.

RESULTS AND DISCUSSION

Dynamic light scattering and Zeta potential measurement

Dynamic light scattering (DLS) is a technique used to determine the hydrodynamic diameter of dispersed particles. The PP-ZnO NPs exhibited an average particle size of 259 nm, with the majority of particles distributed around 230 nm. These results indicate that most PP-ZnO NPs fall within the 200-300 nm range. The polydispersity index (PDI) was 0.345, suggesting good to moderate dispersion of the nanoparticles. The Z-average value was higher (564.5 nm), which reflects the presence of some larger aggregates within the dispersion. The PP-ZnO NPs nanoparticles exhibited a zeta potential of -41.5 suggesting a strong negative surface charge, indicative of good colloidal stability (Fig 1).

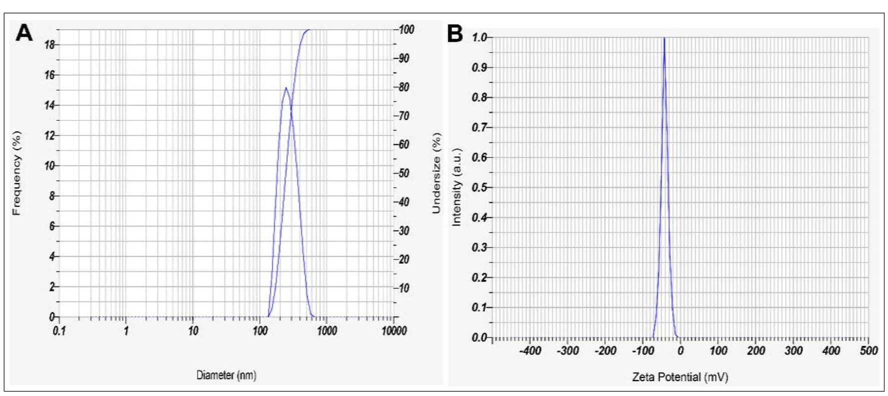
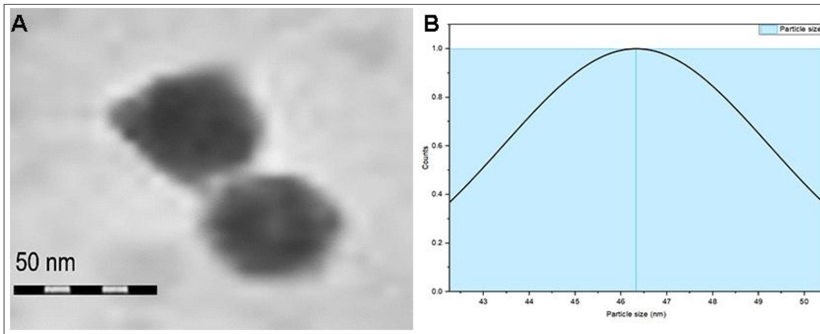


Fig 1: DLS (A) and Zeta potential (B) of the PP-ZnO NPs.



(A) showing hexagonal shaped PP-ZnO NPs (B) average size of PP-ZnO NPs.

Fig 2: Transmission electron microscopy (TEM) analysis.

Table 1: Mortality pattern recorded at 24 hours post exposure using the brine shrimp lethality assay.

Sample Code	Concentration (µg/ml)	Mortality of Brine shrimp (no. of shrimps dead) (hours)					
		1hr	2 hr	4 hr	6 hr	24 hr	% Mortality (24 hr)
PP-ZnO NPs	100	0	0	0	0	0	
	20	0	0	0	0	2	7
	50	0	0	0	0	2	7
	100	0	0	0	0	3	10
	200	0	0	0	1	2	10
Control $K_2Cr_2O_7$	1 (mg/ml)	30	-	-	-	-	100
Blank	Saline water	0	0	0	0	0	0

Transmission electron microscopy

Transmission electron microscopy (TEM) analysis revealed well monodispersed hexagonal shaped PP-ZnO NPs with an average size of 54±8 nm (Fig 2).

Brine shrimp lethality assay

The brine shrimp lethality assay (BSLA) was conducted to reveal the invitro toxic potential of the PP-ZnO NPs at invitro at different concentrations (10, 20, 50, 100 and 200 µg/ml). A total of 30 nauplii (N=30) were exposed to above concentration and the mortality was recorded at different time intervals (1, 2, 4, 6 and 24 h). The positive control (potassium dichromate ($K_2Cr_2O_7$), 1 mg/ml) exhibited complete mortality (100%) within 24 h, while the blank (saline water) showed no mortality, confirming the reliability of the assay. The mortality pattern at different time interval has been shown in the Table 1. Thus, compared with the positive control (highly toxic) and the blank (non-toxic), the PP-ZnO NPs can be considered non-toxic under the tested conditions.

Nanotechnology has its application in medicine and veterinary pharmacology. Zinc oxide nanoparticles are considered more reliable and non-toxic when compared to other metal nanoparticles. In this study the pomegranate peel assisted zinc oxide nanoparticles were synthesized and after their initial color change from brown to pale yellow they were subjected to characterization using DLS, Zeta and TEM. DLS and Zeta confirmed the particle's hydrodynamic size in the range of 200-300 nm and with the zeta value of -41.5 mV. The TEM revealed further the particles are in nano range approximately 54±8 nm. Similar size and zeta potential have been reported in studies (Guo *et al*., 2021).

The Brine shrimp lethality assay is an easy technique to find the toxicity associated with the nanoparticles. This is because, they are non-selective filter feeders like daphnia and they can ingest very small particles less than 50 micrometres making them a suitable candidate for conducting *in vitro* toxicity studies (Ates *et al*., 2013). The reason is that these brine shrimps are highly resistant to metal toxicants making them reliable for conducting toxicity studies in nanomaterials. The BSLA conducted revealed the nanoparticles are non-toxic in nature with the mortality not more than ten percent, which highlights they can be used in animal studies as vaccine adjuvants, drug delivery and antibiotic alternatives. Scientists are exploring on the use of nanoparticles to replace synthetic drugs as these nanoparticles are way more efficient in controlling pathogens because of their size in nanometers (Casiano-Muñiz *et al*., 2024). Several studies have reported the LC50 values for the BSLA for nanoparticles in the range of 70-100 µg/ml (Ates *et al*., 2013). From this it is understood that the nanoparticles synthesized in this study is non toxic as compared with earlier reported studies.

CONCLUSION

Thus, these nanoparticles that are prepared with pomegranate peel indicates the sustainable synthesis of nanoparticles. TEM revealed that the nanoparticles were more or less hexagonal shape with the size of 54±8 nm. The BSLA conducted showed that the PP-ZnO NPs are non-toxic highlighting its application in treatment based alternatives, as micronutrient in feed incorporations and also in vaccine based drug delivery.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Animal ethics

The Brine Shrimp Lethality Assay (BSLA) does not involve vertebrate models and the experiment was conducted invitro. Hence, no approval from the Institutional Animal Ethics Committee was required.

Informed consent

The Brine Shrimp Lethality Assay (BSLA) does not involve vertebrate models and the experiment was conducted invitro. Hence, no approval from the Institutional Animal Ethics Committee was required.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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