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Pomegranate Rind-assisted Green Synthesis of ZnO Nanoparticles: Characterization, Antioxidant Activity and Antibacterial Potential against Bacterial Fish Disease

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ABSTRACT

Background: Nanoparticles have gained attention in animal health for their applications as feed additives, antimicrobials, vaccine adjuvants and immunostimulants. This study aims to explore their potential as an eco-friendly alternative to conventional antibiotics in aquaculture.

Methods: In this study, zinc oxide nanoparticles were synthesized from pomegranate rind (P-ZnO NPs), characterized using UV-Visible spectroscopy, X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR) and Field Emission Scanning Electron Microscopy with Energy Dispersive X-ray Spectroscopy (FESEM-EDS) and their antibacterial and antioxidant activities were evaluated by well diffusion and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assays.

Result: P-ZnO NPs exhibited a spherical morphology with an average size of 84.15 nm, strong antioxidant activity (IC_{50} = 98.48±0.89 µg/mL) and significant antibacterial effects against *Photobacterium damselae* subsp. *damselae* (MIC = 6.25 µg/mL), *P. damselae* subsp. *piscicida* and *Aeromonas hydrophila* (MIC = 3.12 µg/mL). These results highlight pomegranate rind mediated ZnO nanoparticles as a green and effective approach for controlling major aquaculture pathogens.

Key words: Antibacterial, Antioxidant, Green synthesis, Nanoparticles, Zinc oxide.

INTRODUCTION

Nanotechnology emerged as a significant sector in science during the early 21st century, with numerous applications in the agriculture, environment, biomedicine and food safety particularly as food additives and antimicrobials (Kavanashree *et al*., 2024; Altammar, 2023). In aquaculture, the increased use of antimicrobials has led to the development of antimicrobial resistant genes in fish bacterial pathogens. This indiscriminate use not only contributes to antimicrobial resistance (AMR) but also results in the accumulation of antibiotic residues in fish (Chen *et al*., 2020), leaching of antibiotic residues into the environment which increase their presence in agricultural runoff, ultimately contributing to the development of antimicrobial resistance in human pathogens highlighting the broader impact of AMR in aquaculture (Miller and Harbottle, 2018).

Green synthesized nanoparticles are a promising alternative to antibiotics, especially plant-based methods are favored for their eco-friendliness and added antioxidant and antibacterial properties (Anjali *et al*., 2025; Sudeepta and Siddhartha, 2024; Keshari *et al*., 2020). Zinc, which is generally regarded as safe, is extensively used as an antimicrobial agent at both micro and nanoscale. When reduced to the nanoscale, its antimicrobial activity is enhanced due to its high surface area to volume ratio and unique bactericidal mechanisms. These include disruption of the bacterial cell membrane, release of Zn^{2+} ions and the formation of reactive oxygen species (ROS) (Jalal *et al*., 2010; Seil and Webster, 2012). Aquaculture, being a vital sector in the food industry, is expected to surpass wild catch production by 2028. However, this sector continues to face challenges due to the prevalence of bacterial

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pathogens (Miranda *et al*., 2016). In this context, green synthesized zinc oxide (ZnO) nanoparticles offer a promising alternative to antibiotics, with demonstrated antibacterial efficacy and low toxicity to human cells (Sirelkhatim *et al*., 2015).

Several *in vitro* studies have demonstrated the antibacterial activity of ZnO nanoparticles against both Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria such as *Salmonella paratyphi*, *Vibrio cholerae* and *Escherichia coli with the* synthesized nanoparticles using *Solanum nigrum* (Ramesh *et al*., 2015). Many studies have documented the antibacterial effecet of ZnO-NPs against fish pathogens such as *Aermonas hydrophila, Edwardseilla tarda, Flavobacterium branchiophilum, Citrobacter spp., Staphylococcus aureus, Vibrio species, Bacillus cereus and Pseudomonas* aeruginosa (Shaalan *et al*., 2017). Pomegranate rind, a bio-waste product of the fruit, contains various bioactive compounds with proven antibacterial properties (Siddiqui *et al*., 2024). In this study, zinc oxide nanoparticles were synthesized using pomegranate rind extract (P-ZnO NPs) and their potential antibacterial effects against major fish pathogenic bacterial species (marine and freshwater origin) were investigated.

MATERIALS AND METHODS

Green synthesis and characterization of nanoparticles

The nanoparticles were synthesized following Fouladi-Fard *et al*. (2022); Naiel *et al*. (2022) and Tilahun *et al*. (2023) with slight modifications. P-ZnO NPs were characterized by UV–Vis spectrophotometer (280-800 nm), FTIR (JASCO FT/IR-6600 Type A spectrometer ,Tokyo, Japan; Serial No: B049361790) (Velmurugan *et al.*, 2024), XRD (XPERT-PRO diffractometer, PANalytical, Netherlands) and crystallite size was calculated using the Debye–Scherrer equation (Esther and Kumar, 2024). Morphology and particle size and elemental composition were examined by FESEM-EDS (TESCAN MIRA3 XMU FE-SEM, Czech Republic) (Abazari *et al*., 2023).

Antioxidant assay

The antioxidant activity of P-ZnO NPs was evaluated using the DPPH assay (Sisco Research Laboratories Pvt. Ltd.) following Hameed *et al*. (2023). Different concentrations of nanoparticles (10-750 µg/mL) and Vitamin C (1 mg/mL, standard) were mixed with DPPH solution (800 µg/mL in methanol) to a final volume of 1.5 mL and incubated in the dark. Absorbance was measured at 517 nm and the percentage scavenging activity was calculated as follows:

$$\text{Scavenging activity (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

Where,

A_0= control absorbance.

A_1= Sample absorbance.

Results were compared with Vitamin C (Baliyan *et al*., 2022; Boopathi *et al*., 2024).

Well diffusion assay

Three pathogenic bacterial strains; *Photobacterium damselae* subsp. *damselae* (*Pdd*), *Photobacterium damselae* subsp. *piscicida* (*Pdp*) and *Aeromonas hydrophila* (*Ah*) maintained at the Department of Fish Pathology and Health Management, Fisheries College and Research Institute (FCandRI), Thoothukudi were used in this study. These isolates were subsequently inoculated in fresh TSB and incubated overnight. The bacterial density was standardized to 0.5 McFarland standard (approximately 1.2×10^7 cfu/ml) and the suspensions were evenly swabbed onto Mueller-Hinton Agar (MHA-HIMEDA) plates. Wells of approximately 6 mm diameter were made using a sterile cork borer. P-ZnO NPs were prepared in dimethyl sulfoxide (DMSO) at concentrations of 20, 40, 60 and 80 µg/mL and added to the wells. The plates were incubated at 37°C for 24 hours, after which the zones of inhibition were measured in millimeters (Balouiri *et al*., 2016).

MIC and MBC determination

The MIC and MBC of P-ZnO NPs were determined by broth microdilution following CLSI (2018) guidelines (Kakian *et al*., 2024). A stock solution (25 µg/mL) in Mueller Hinton Broth (MHB) was serially diluted (25-0.195 µg/mL) in 96-well plates containing bacterial suspensions (~10w cfu/ml). Controls included sterility (MHB only) and growth (MHB with inoculum). Plates were incubated at 37°C for 24 h and MIC was recorded as the lowest concentration without visible turbidity. For MBC, 50 µL from clear wells were spread on MHA and incubated at 37°C for 24 h; the lowest concentration showing no colonies was taken as the MBC.

RESULTS AND DISCUSSION

Green synthesis and characterization of the nanoparticles

The formation of nanoparticles was initially indicated by a visible color change in the reaction mixture (Fig 1). A transition from brown to a pale yellow precipitate suggested the successful synthesis of nanoparticles. The colloidal solution containing P-ZnO NPs exhibited a prominent peak at 375 nm (Fig 2). FTIR showed prominent many absorption bands (Fig 3). The broad band at 3506.06 cm{ ^{1} corresponds to O-H stretching vibrations, indicating the presence of phenolic compounds. The peaks at 2923.59 cm^{-1} and 2853.24 cm^{-1} are attributed to the asymmetric and symmetric stretching vibrations of $-CH_2$ groups indicating the presence of aliphatic compounds. The absorption at 2358.57 cm^{-1} is associated with O=C=O stretching vibrations. The band at 1745 cm^{-1} corresponds to the C=O stretching of carboxyl functional groups. Peaks at 1630.55 cm^{-1} and 1457.30 cm^{-1} are indicative of N–H bending vibrations. The strong band at 1114.80 cm^{-1} is assigned with the possible metal oxygen (Zn-O) bonding, resulting in the formation of ZnO. The bands at 988.42 cm^{-1}, 886.27 cm^{-1} and 792.28 cm^{-1} correspond to C-H bending vibrations of aldehydes or aromatic compounds. The sharp band at 618.52 cm^{-1} is due C-Cl stretching vibrations. The bands at

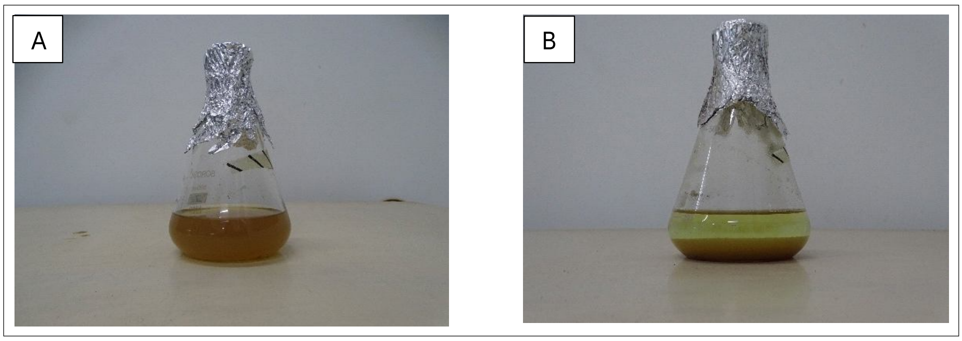

Fig 1: (A) Represents the crude pomegranate rind extract after the addition of zinc nitrate hexaydrate (B) Shows the color change following P-ZnO NPs synthesis, with the precipitated nanoparticles settled at the bottom.

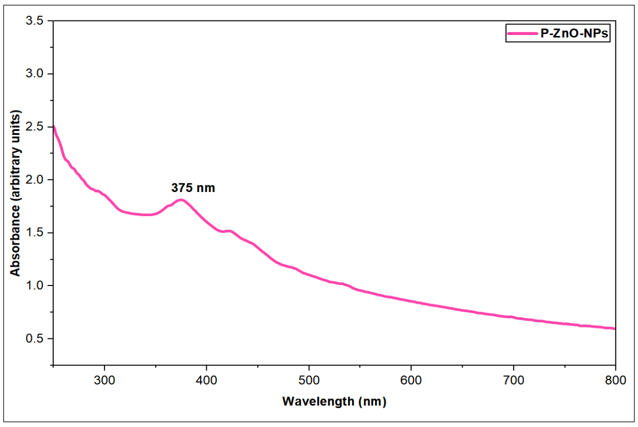

Fig 2: UV absorption spectra of the P-ZnO NPs.

518.50 cm^{-1} and 464.93 cm^{-1} fall within the characteristic range of Zn-O stretching vibrations, confirming the successful synthesis of ZnO nanoparticles. The XRD diffraction profile of the synthesized nanoparticles exhibited distinct peak positions at 2θ values of 32.18°, 34.84°, 36.64°, 47.96°, 57.00°, 63.26°, 66.76°, 68.37°, 69.66°, 72.93° and 77.33°. These peaks were indexed to the (100), (002), (101), (102), (110), (103), (200), (112), (201), (004) and (202) in good agreement with the standard diffraction data reported in JCPDS file No. 36-1451 (Fig 4). The average crystallite size of the ZnO nanoparticles was found to be approximately 24.4 nm**.** The SEM images revealed spherical-shaped nanoparticles with an average particle size of 82.15 nm (ImageJ.exe software) (Fig 5). The EDS spectrum showed zinc, oxygen and carbon weight percentage as 73.53% 18.65% and 7.82% respectively. The EDS peaks were observed between 1 and 10 keV, with a prominent zinc peak at approximately 1 keV and another at 8.6 keV, along with an oxygen peak around 0.5 keV (Fig 5).

Antioxidant activity

DPPH assay revealed dose-dependent increase in scavenging activity with increasing concentrations of nanoparticles (10, 50, 150, 250, 350, 500 and 750 µg/mL). The IC_{50} value of the synthesized nanoparticles was 98.48±0.89 µg/mL, whereas the IC_{50} value for Vitamin C was 2.33±1.06 µg/ mL (Fig 6).

Antibacterial activity

The antibacterial efficacy of P-ZnO NPs was assessed against three pathogenic bacteria *Pdd*, *Pdp* and *Ah* using the agar well diffusion assay and microbroth dilution assay. Wells of 6 mm diameter were created on Mueller-Hinton Agar (MHA) plates, into which P-ZnO NPs at concentrations of 20, 40, 60 and 80 µg/ml were applied. Cefixime was used as a positive control for *Pdd* and *Pdp* and ofloxacin for *Ah*. The synthesized P-ZnO NPs exhibited clear dose-dependent antibacterial activity against all three pathogens in well diffusion assay (Table 1, Fig 7) and MIC and MBC values were also calculated (Table 2).

Nanoparticles are recognized as potent therapeutic and prophylactic agents due to their nanoscale size (Wang *et al*., 2016). Their antibacterial action involves ROS generation, membrane disruption and inhibition of electron transport or efflux pumps (Aflakian *et al*., 2023). In this study, ZnO nanoparticles were green-synthesized from pomegranate rind waste rich in bioactive compounds with antibacterial properties (Alexandre *et al*., 2019; Chen *et al*., 2020; Siddiqui *et al*., 2024) providing a sustainable route for nanoparticle production (Suresh *et al*., 2015).

Characterization confirmed the successful synthesis of P-ZnO NPs with UV-Vis absorption peak at 375 nm (Sharma *et al*., 2025). The FTIR spectrum of P-ZnO NPs revealed the presence of various functional groups in the reduction and stabilization of nanoparticles (Jayachandran *et al*. 2021; Lakshmi *et al*., 2024; Monika *et al*., 2024; Golzarnezhad *et al*., 2025; Krithika *et al*., 2021). SEM images showed spherical nanoparticles with an average size of 82.15 nm, while EDS confirmed elemental composition (Zn: 73.53%, O: 18.65%, C: 7.82%) (Saravanan *et al*., 2025), where carbon likely originated from phytochemical capping agents (Jayappa *et al*., 2020). The P-ZnO NPs exhibited strong antioxidant activity with an IC_{50} value of 98.48±0.89 µg/mL. Comparable studies with pomegranate rind-mediated ZnO NPs reported an IC_{50} of 124 µg/mL in barley seed germination assays (Shaban *et al*., 2024), suggesting

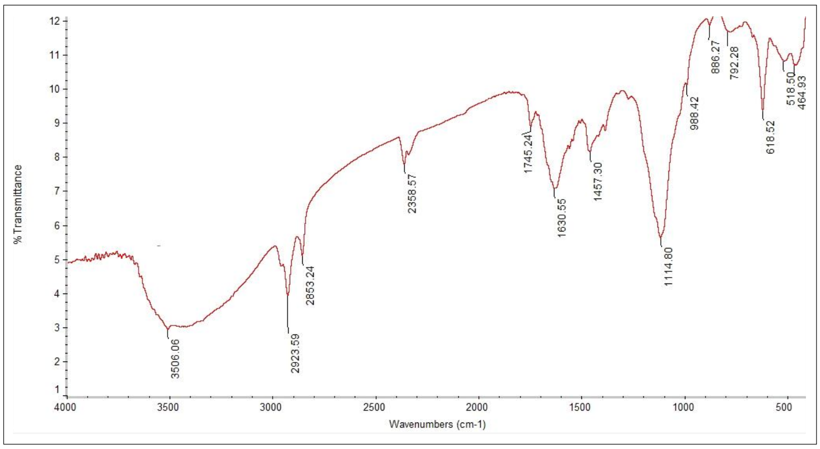

Fig 3: FTIR of the P-ZnO NPs.

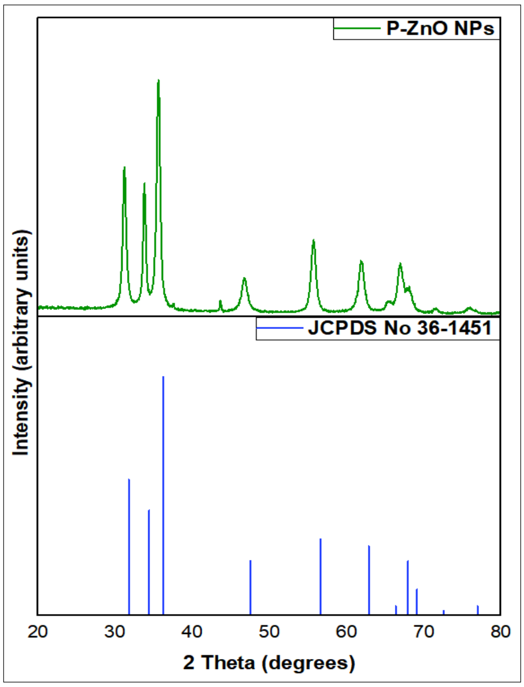

Fig 4: X-ray diffraction (XRD) pattern of P-ZnO NPs and standard JCPDS card No. 36-1451.

Table 1: Mean Zone of Inhibition (mm±SD) for different bacteria at varying P-ZnO NPs concentrations.

Concentrations (µg/ml)	Mean ZOI (mm±SD) for different bacterial isolates		
	Pdd	*Pdp*	*Ah*
20	9.93±0.12	8.97±0.15	8.97±0.20
40	10.97±0.15	10.97±0.15	10.97±0.15
60	13.97±0.06	13.90±0.17	13.90±0.17
80	17.93±0.12	19.70±0.61	19.70±0.12
Positive	30.90±0.10	20.97±0.06	20.97±0.15
Negative	0±0	0±0	0±0

that the lower IC_{50} in the present study reflects a comparatively higher antioxidant capacity.

Pdd is a marine opportunistic pathogen with zoonotic significance, infecting diverse fish species, while *Pdp*, derived from *Pdd* through gene loss, is linked to major outbreaks (Baseggio *et al*., 2022; Andreoni and Magnani, 2014). Ah is a virulent facultative anaerobe in carps with zoonotic potential (Semwal *et al*., 2023). Well diffusion assays showed dose-dependent inhibition, with AH most sensitive (20.0±0.0 mm at 80 µg/mL), followed by *Pdd* (18.0±0.0 mm) and *Pdp* (16.0±0.0 mm). MIC/MBC assays confirmed efficacy, with values of 6.25/12.5 µg/mL for *Pdd* and 3.12/6.25 µg/mL for both *Pdp* and *Ah* (Dube, 2024). These results are comparable to previous studies where CuO nanoparticles (100 µg/mL) produced inhibition zones of 17-21 mm against *Pseudomonas fluorescens*, *Vibrio parahaemolyticus* and *Flavobacterium branchiophilum* (Kumar *et al*., 2015). Similarly, Gum Arabic-silver nanoparticles inhibited *A. hydrophila* and *P. aeruginosa* with MIC values of 1.625 and 3.25 µg/mL, respectively. MIC and MBC assays further supported the antibacterial efficacy of P-ZnO NPs. Pdd showed MIC and MBC values of 6.25 and 12.5 µg/mL, respectively, while both *Pdp* and *Ah* exhibited MIC of 3.12 µg/mL and MBC of 6.25 µg/mL. Several studies have reported nanoparticles effectiveness against diverse fish pathogens including *Vibrio harveyi*, *Edwardsiella tarda*, *Flavobacterium branchiophilum*, *Citrobacter spp.*, *Staphylococcus aureus*, *Bacillus cereus* and *Pseudomonas aeruginosa* (Shaalan *et al*., 2017). The rising concern of antibiotic resistance in aquaculture is notable, as tetracycline use in fish farms has been linked to resistant strains. For instance, tetracycline, widely used in fish farms, has been linked to resistant bacterial strains (Shaalan *et al*., 2017; Tuševljak *et al*., 2013). Also resistance in fish pathogens such as *Aeromonas salmonicida*, *Photobacterium*, *Yersinia ruckeri*, *Vibrio* spp., *Listeria*, *Pseudomonas* and *Edwardsiella* poses risks of resistant gene transfer to human pathogens (Sørum, 2008; Swain

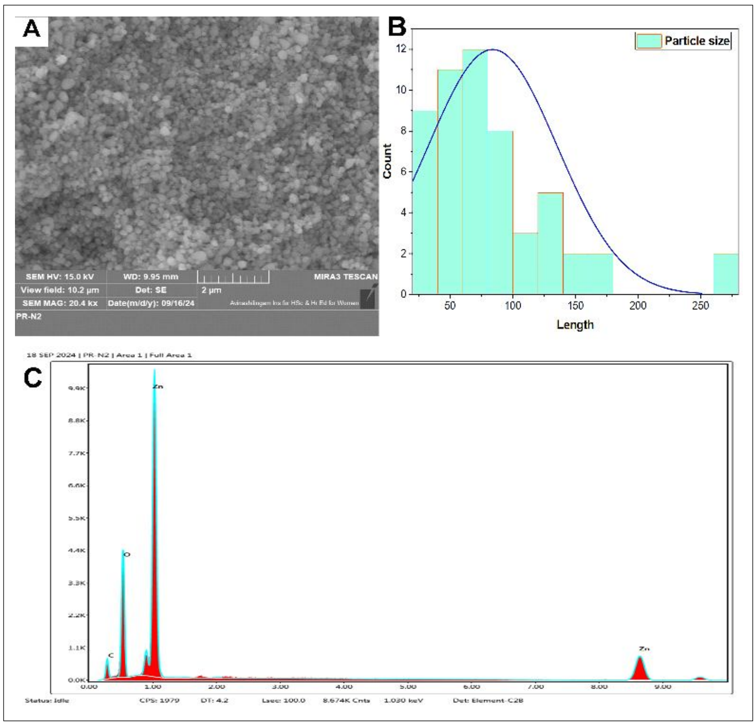

Fig 5: (A) FESEM image of P-ZnO NPs shows well-dispersed spherical nanoparticles (B) Average particle size of 32.4 nm, (C) EDS of P-ZnO NPs confirms the elemental composition: Zn-73.53%, O-18.65% and C-7.82%.

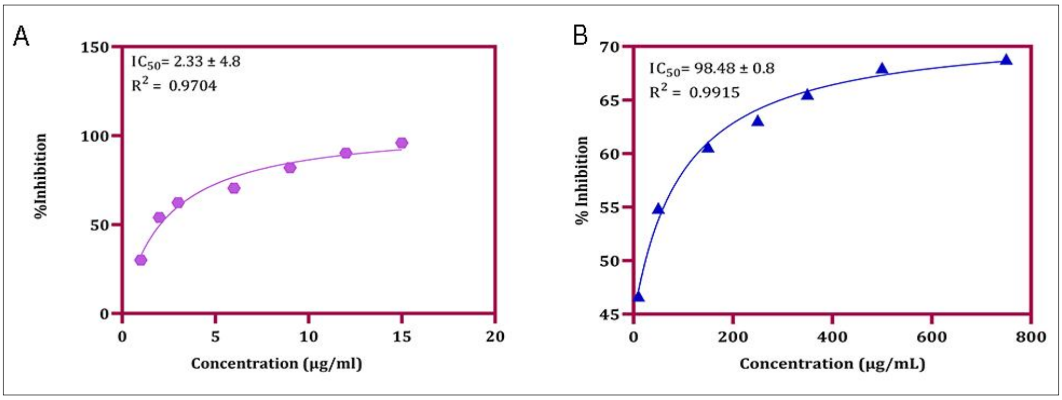

Fig 6: DPPH radical scavenging assay of the P-ZnO NPs (A) and Vitamin C (B).

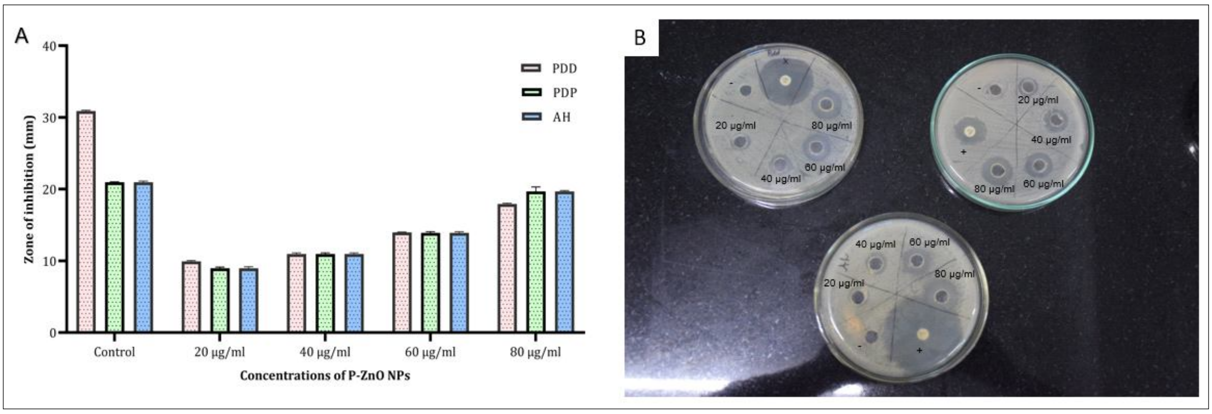

Fig 7: (A) and (B) ZOI for Pdd, Pdp and Ah against P-ZnO NPs.

Table 2: MIC and MBC values of P-ZnO-NPs against aquaculture pathogens.

Bacterial isolate	MIC (µg/mL)	MBC (µg/mL)
Pdd	6.25	12.5
Pdp	3.12	6.25
Ah	3.12	6.25

et al., 2014). Thus Pomegranate rind mediated ZnO nanoparticles (P-ZnO NPs) offer an ecofriendly and sustainable antibacterial alternative in aquaculture.

CONCLUSION

These findings demonstrate the strong antioxidant (IC_{50} = 98.48±0.89 µg/mL) and bactericidal activity of P-ZnO NPs at low concentrations, with MIC values of 3.12-6.25 µg/mL against marine (*Pdd* and *Pdp*) and freshwater (*Ah*) pathogens. Hence, eco-friendly green-synthesized nanoparticles are gaining prominence in aquaculture as sustainable alternatives to conventional antimicrobials.

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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.

Informed consent/ethical approval

This study did not involve any live animals or human participants. All experiments were performed using *in vitro* antibacterial and antioxidant assays and hence no ethical approval was required.

Animal ethics

No live animals were used in this study. All experiments were carried out using bacterial cultures only.

Conflict of interest

The authors declare no conflicts of interest.

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