#### Research Article

# An Evaluation of Antibacterial, Antioxidant, and Biocompatibility (Hemocompatibility) Nature of Green Synthesized ZnONPs: An In-vitro Approach

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#### **Abstract**

**Introduction:** Zinc oxide nanoparticles (ZnONPs) derived from plant extracts are garnering attention in medical applications due to their unique properties and eco-friendly synthesis, which enhance antimicrobial, antioxidant, anti-inflammatory, and anticancer effects. This research investigated the antibacterial and biocompatibility properties of previously characterized ZnONPs against bacterial pathogens, with biocompatibility evaluated through a hemocompatibility study using human blood samples.

**Methods:** The bactericidal efficacy of phyto-synthesized ZnONPs was evaluated using the standard agar well-diffusion method against bacterial pathogens including *E. coli, P. aeruginosa, S. aureus, K. pneumoniae,* and *S. flexneri.* Moreover, their antioxidant capacity was evaluated using various nonenzymatic in-vitro assays, including ABTS, DPPH, TRP, and TAC assays.

**Results:** The antibacterial efficacy of presynthesized and characterized ZnONPs showed dosage-dependent bactericidal activity against bacterial pathogens including *Pseudomonas aeruginosa, Streptococcus pneumoniae, Staphylococcus aureus, Escherichia coli,* and *Klebsiella pneumoniae.* At a concentration of 800 µg mL<sup>-1</sup>, the ZnONPs exhibited significant free radicals scavenging and reducing capabilities as demonstrated by DPPH, TAC, TRP, and ABTS assays. Moreover, the ZnONPs demonstrated excellent biocompatibility, as evidenced by hemocompatibility assay.

**Discussion:** Phytochemicals derived from plants, including alkaloids, flavonoids, and tannins, stabilize nanoparticles, thereby enhancing their antibacterial effectiveness against Grampositive and Gram-negative bacteria. Green synthesized ZnONPs demonstrate reduced toxicity to human cells, notable antimicrobial effectiveness, strong antioxidant characteristics, and low cytotoxicity, making them beneficial for anti-aging therapies, wound healing, drug delivery, and tissue engineering.

**Conclusion:** Green-synthesized ZnONPs demonstrate significant dose-dependent antibacterial efficacy against pathogenic bacteria such as *P. aeruginosa*, *S. pneumoniae*, *S. aureus*, *E. coli*, and *K. pneumonia*. The antioxidant test results demonstrated a significant dose-dependent capacity of DPPH, TAC, TRP, and ABTS to eliminate free radicals. The antibacterial and antioxidant properties of ZnONPs exhibited notable biological compatibility.

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**Keywords:** ZnONPs, pathogenic bacteria, antibacterial activity, antioxidant activity, DPPH, hemocompatibility

#### 1. Introduction

Nanotechnology has emerged as a proven cutting-edge technology through various applications in chemical, medicinal, mechanical, and food manufacturing sectors [1, 2]. Nanotechnologies are applicable in computation, energy production, optical science, drug delivery, and biological sciences [3]. Numerous nanoscale devices are being developed following the advent of nanotechnologies, utilizing various methods including chemical, physical, and eco-friendly approaches [4]. The synthesis of green nanoparticles (NPs) is a versatile technique that can be efficiently prepared and engineered [5]. Metallic oxide NPs have garnered significant attention over the past decade due to their diverse applications in various technical fields [6]. ZnONPs constitute an intriguing inorganic material with numerous advantages [7]. ZnONPs are utilized in various domains, including energy conservation, clothing, electronic devices, healthcare, catalytic processes, beauty products, semiconductors, as well and chemical-based sensing [8]. The nanoparticles are nontoxic and biocompatible, with excellent applications in targeted drug delivery, wound management, and biological imaging. As we age, free radical reactions are expected to induce gradual detrimental changes throughout our tissues. [9].

These typical variations of aging are quite prevalent among all individuals. Nonetheless, trends shaped by genetic determinants and environmental fluctuations that regulate detrimental free radicals have impacted this prevalent pattern [10]. All these manifest as ailments at particular ages, influenced by both environmental and genetic factors. Cancer and coronary artery disease, two of the most common causes of mortality, are both classified as "free radical" illnesses [11]. Oxidative damage occurs when free radicals exceed the body's ability to regulate them. Consequently, free radicals impair proteins, lipids, and DNA, leading to various human diseases. Consequently, utilizing external sources of antioxidant compounds can assist in mitigating the effects of oxidative stress [12, 13]. Comprehensive global projections of bacterial infection incidence have recently been released for the first time. Bacterial infections were reported to account for over 7 million deaths globally in 2019 [11]. This represents one-eighth of global mortality rates. It also positions bacterial infections as the second leading cause of death globally, surpassed only by ischemic heart disease [14]. Bacterial infections, at present, are underestimated as contributor to global health challenges, necessitating an urgent enhancement of mitigation strategies. The study underscores the critical importance of access to effective antibacterial agents within all healthcare systems. This represents 14% of total global fatalities or one in every eight deaths. Bacterial infections constitute the second leading cause of mortality globally. Five bacterial species (Pseudomonas aeruginosa, Streptococcus pneumoniae, Staphylococcus aureus, Escherichia coli, and Klebsiella pneumoniae) have been responsible for more than 50% of these fatalities. This study aimed to evaluate the antibacterial, antioxidant, and biocompatibility properties of previously green synthesized and characterized ZnONPs against bacterial pathogens (P. aeruginosa, S. pneumoniae, S. aureus, E. coli, and K. pneumoniae), with biocompatibility evaluated through hemocompatibility studies.

### 2. Materials and Methods

#### 2.1. Brief Profile of Test ZnONPs

The ZnONPs used in this study were provided by Chinnathambi et al., [15]. They synthesized these ZnONPs using methanol leaf extract from *Tarenna asiatica* leaves. This nanoparticle has been reported to exhibit larvicidal and pupicidal activity against *Aedes aegypti*, which can cause dengue fever. These nanoparticles were 22.35 nm in size and spherical in morphology.

#### 2.2. Antibacterial Assay

The antibacterial efficacy of ZnONPs against bacterial species including *P. aeruginosa*, *S. pneumoniae*, *S. aureus*, *E. coli*, and *K. pneumoniae* was evaluated using agar well-diffusion methods [16]. About 0.01 mL of ZnONPs in various dosages as 100, 200, 400, and 800  $\mu$ g mL<sup>-1</sup> was individually added on wells of MHA plates inoculated with *P. aeruginosa*, *S. pneumoniae*, *S. aureus*, *E. coli*, and *K. pneumoniae* followed by an incubation for 2 days at 37  $\pm$  1°C. Commercial antibiotics including ciprofloxacin, vancomycin, gentamicin, and amoxicillin served as the positive control. The inhibition diameter size was measured and compared to the positive control.

#### 2.3. Antioxidant Determination

#### 2.3.1. DPPH Assay

Initially, the free radical scavenging potential of ZnONPs was assessed using the standard DPPH assay, adhering to the established protocol with test NPs concentrations ranging from 100 to 800  $\mu g$  mL<sup>-1</sup>, while ascorbic acid served as the positive control. The absorbance of each concentration reaction mixture was measured at 515 nm using a microplate photometer. The percentage of antioxidant activity was calculated using the equation provided below [17].

$$\% FRSA = \left(1 - \frac{Abs}{Abc}\right) \times 100$$

Abc and Abs indicates the absorbances of the negative control and sample, respectively.

#### 2.3.2. ABTS Assay

The antioxidant activity of the test NPs was evaluated according to the modified protocol of Faisal et al., [17] utilizing various dosages (100-800  $\mu$ g mL<sup>-1</sup>) of test ZnONPs. Trolox served as the positive control. The

absorbance of each sample was measured at 734 nm. The standard equation was utilized to ascertain the scavenging percentage.

#### 2.3.3. TRP Assay

Furthermore, the total reducing power assay was conducted to assess the reducing capacity of ZnONPs at varying concentrations (100-800  $\mu g$  mL $^{-1}$ ) adhering to the methodology of Faisal et al., with necessary modifications [17]. Ascorbic acid served as the positive control and absorbance of each reaction completed, the sample was read at 630 nm using a microplate reader. Moreover, the standard equation is employed to compute the percentage of reducing potential.

#### 2.3.4. TAC Assay

The total antioxidant capacity of ZnONPs was assessed according to the modified protocol of Faisal et al., [17], utilizing various dosages (100-800  $\mu g$  mL $^{-1}$ ) of NPs. According to the aforementioned assays, ascorbic acid served as a positive control. The absorbance of each concentration of reaction blends was measured at 630 nm using a standard microplate reader. By following the standard equation, the TAC percentage was calculated.

## 2.4. Hemocompatibility Assay

The biocompatibility of green-fabricated ZnONPs, exhibiting notable antibacterial and antioxidant properties, was assessed using a standard hemocompatibility assay protocol on human blood samples in a 96-well plate [18]. Various dosages (100-800  $\mu g$  mL<sup>-1</sup>) of ZnONPs were utilized with a healthy human blend sample. About 0.5% of Triton X-100 was used as positive control and the absorbance of both the sample and control was assessed at 540 nm.

#### 2.5. Statistical Analysis

Each analysis was conducted in triplicates to ensure the reproducibility and precision of the results. The mean and standard error values were used for tables and figures. One-way ANOVAs were conducted to ascertain the statistical significance of the test and control samples using SPSS. The linear regression analysis was conducted to determine the  $IC_{50}$  value.

#### 3. Results

P. aeruginosa (26  $\pm$  0.1 mm), S. pneumonia (21  $\pm$  0.1 mm), S. aureus (24  $\pm$  0.1 mm), E. coli (25  $\pm$  0.1 mm), and K. pneumonia (16  $\pm$  0.1 mm) at 800  $\mu$ g mL<sup>-1</sup> of ZnONPS (Table 1). The material under investigation possesses redox capability, allowing the conversion of Fe<sup>3+</sup> ions to Fe<sup>2+</sup> ions. ABTS (2,2'-azino-bis(3ethylbenzothiazoline-6-sulfonic acid), as well as DPPH (2,2-diphenyl-1-picrylhydrazyl) antioxidant assays were conducted to validate the TAC as well as TRP the results. DPPH is a stable free radical that can be reduced by accepting an H atom or an electron from a donor, resulting in the formation of a colorless diphenyl picrylhydrazine molecule. The spectrophotometric methods rely on the cooling of solid-colored free radicals of DPPH as well as ABTS, which signifies the scavenging ability of the antioxidant sample. The study revealed that all tested quantities exhibited exceptional free radical scavenging capabilities, as detailed in Table 2. The antioxidant activity assay results indicated significant activity at an elevated dosage (800  $\mu g \ mL^{-1}$ ) as follows: for DPPH 78  $\pm$  1.2%, TAC 72  $\pm$  1.1%, TRP 52  $\pm$ 1.5%, and ABTS 61  $\pm$  1.6% (Table 2). Figures 1-4 depict the antioxidant capabilities of ZnONPs in DPPH, TAC, TRP, and ABTS scavenging and reduction assays. The IC<sub>50</sub> values for DPPH, TAC, TRP, and ABTS were determined to be 365  $\mu$ g mL<sup>-1</sup> (Figure **1**b), 435  $\mu$ g mL<sup>-1</sup> (Figure **2**b), 780  $\mu$ g mL<sup>-1</sup> (Figure **3**b), and 758 µg mL<sup>-1</sup> (Figure 4b), respectively. These antioxidant properties of ZnONPs may be attributed to the presence of bioactive compounds that are likely involved in their synthesis and stabilization. The biocompatibility of ZnONPs was evaluated through a hemolysis study using different dosages. Table 3 illustrates that the hemolytic activity of ZnONPs at a concentration of 800  $\mu g$  mL<sup>-1</sup> was 3.69  $\pm$  0.17%. The findings indicate that the green synthesized ZnONPs can be utilized effectively at concentrations up to 700 µg mL<sup>-1</sup>, at which level they exhibit biocompatibility. Exceeding that threshold may induce mild hemolysis; nevertheless, it can be utilized for medical applications such as antibacterial and antioxidant pharmaceuticals.

Table 1: Antibacterial ability (zone of inhibition in mm) of green synthesized ZnONPs against test bacterial pathogens.

Bacterial pathogens	Var	Positive control			
	100	200	400	800	
P. aeruginosa	11 ± 0.8*	15 ± 0.5**	19 ± 1.2*	26 ± 0.3*	28 ± 1.5
S. pneumoniae	8 ± 0.4	12 ± 0.3*	16 ± 1.0*	21 ± 0.2*	22 ± 1.3
S. aureus	13 ± 0.3*	14 ± 0.1*	18 ± 1.3*	24 ± 0.1*	28 ± 1.5
E. coli	10 ± 0.4*	13 ± 0.3	19 ± 1.4*	25 ± 0.3*	29 ± 1.6
K. pneumoniae	8 ± 0.3*	11 ± 0.2	13 ± 0.9*	16 ± 0.5*	22 ± 1.4

The mentioned values are the mean and standard error of triplicates. \* States p < 0.05; \*\* p < 0.001 while compared with control

ZnONPs (μg mL <sup>-1</sup> )		Antioxidant activities (%) in various assays					
	DPPH	TAC	TRP	ABTS	Control		
100	28 ± 1.5	23 ± 1.2	21 ± 1.2	17 ± 1.3	31 ± 1.2		
200	34 ± 1.1	31 ± 1.3	29 ± 1.6	26 ± 1.7	48 ± 1.9		
400	52 ± 1.8	46 ± 1.4	41 ± 1.8	35 ± 1.4	62 ± 1.5		
800	78 ± 1.2**	72 ± 1.1**	52 ± 1.5	61 ± 1.6*	81 ± 1.5		

Table 2: Antioxidant potential of ZnONPs analysis by various assays.

The mentioned values are mean and standard error of triplicates. \* States p < 0.03; \*\* p < 0.001 while compared with control

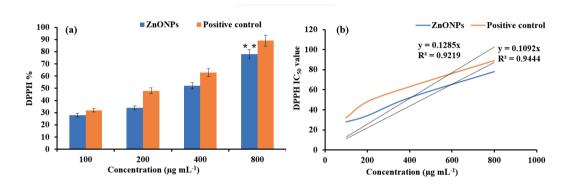
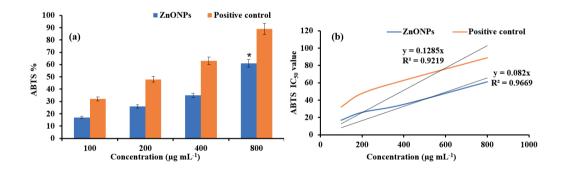


Figure 1: DPPH activity of ZnONPs compared with control. (a): DPPH scavenging percentage (b): DPPH IC<sub>50</sub> value. \*p < 0.001 while compared with control.



**Figure 2**: ABTS scavenging activity of ZnONPs compared with control. **(a)**: ABTS scavenging percentage **(b)**: ABTS IC<sub>50</sub> value. \* States p < 0.03 while compared with control.

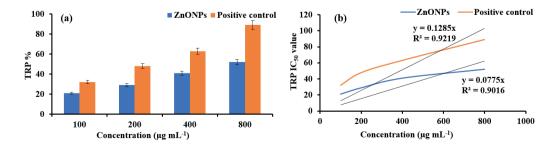


Figure 3: TRP activity of ZnONPs compared with control. (a): TRP scavenging percentage (b): TRP IC<sub>50</sub> value.

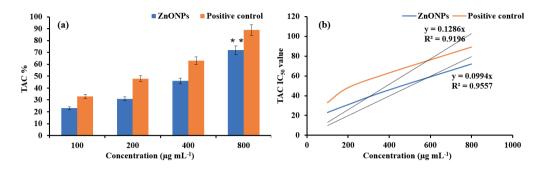


Figure 4: TAC activity of ZnONPs compared with control. (a): TAC scavenging percentage (b): TAC IC<sub>50</sub> value. \*\* p < 0.001 while compared with control.

Table 3: Hemolysis proportions (%) of ZnONPs.

S. No	Concentration (µg mL <sup>-1</sup> )	Hemolysis (%)
1	800	3.69 ± 0.17
2	400	0.73 ± 0.22
3	200	0.55 ± 0.11
4	100	0.21 ± 0.05

The mentioned values are the mean and standard error of triplicates

#### 4. Discussion

The ZnONPs previously obtained through phytofabrication, exhibited significant antibacterial and antioxidant properties at elevated dosages. The hemocompatibility results indicate that the ZnONPs exhibited significant biocompatibility potential. The overall results indicated that the antibacterial and antioxidant activities are dose dependent. ZnONPs are believed to exhibit superior antibacterial properties against various bacterial strains due to intrinsic differences in bacteria cell membranes [18]. The external lipopolysaccharide membranes of Gram-negative bacteria, which confer resistance to antimicrobial agents, are the source of variations in morphological changes and structural composition [19]. Grampositive microbes are especially susceptible to permeation barriers due to the absence of an external peptidoglycan layer. Gram-negative micro-organisms are generally more resistant to antibacterial treatments and present a barrier to diffusion due to their more complex cell membranes compared to Grampositive bacteria [20]. The presence of bioactive compounds on the surface of nanoparticles, which function as capping and stabilizing agents, may account for certain antibacterial properties. Numerous studies indicate that the hydroxyl and carboxylic acid groups of phenolic compounds present in plant extracts are responsible for the formation of ZnONPs [21]. The hydroxyl molecules are responsible for the reduction of  $Zn^{2+}$  ions to  $Zn^{0}$ , while carboxyl groups function as stabilizers [22]. This may initiate the formation of the Zn<sup>0</sup>-phenolate complex through a neutralizing effect, leading to the nucleation, growth, and capping of nanoparticles at an optimal temperature. Subsequently, at elevated temperatures, the entire structure undergoes direct decomposition, resulting in the formation of ZnONPs [23-25]. Although most NPs will interact with blood at some stage within the body's tissues [26-28], the relationships between NPs and blood cells remain inadequately understood, and the importance of evaluating NPs' hemocompatibility has been significantly obscured [29, 30].

### 5. Conclusion

The green-mediated ZnONPs (pre-characterized) exhibit significant dose-dependent antibacterial efficacy against bacterial pathogens, including *P. aeruginosa, S. pneumoniae, S. aureus, E. coli*, and *K. pneumonia*. The antioxidant assay results showed considerable dose-dependent free radicals scavenging potential on various free radicals such as DPPH, TAC, TRP, and ABTS. The antibacterial and antioxidant properties of ZnONPs demonstrated significant biocompatibility. The efficacy and practical applications, biocompatibility of test NPs, in-vivo, and animal model studies must be performed.

#### 6. Limitations

Unfortunately, due to financial constraints, I am unable to perform these studies to ensure the ideal dosage, efficacy, safety, and mechanistic insights of test NPs for potential therapeutic applications. These are the limitations and considerations of this study. However, in-vivo, animal model, and mechanistic insights-related studies will be performed in the future to scientifically understand the NPs interaction mechanisms in biomedical applications and biosafety of the ZnONPs.

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#### Statement of Ethics

The studies involving human volunteer (blood donor) participants were planned, conducted, and reported in accordance with the World Medical Association (WMA) Declaration of Helsinki.

# **Ethical Approval**

The volunteer (blood donor) provided written informed consent for the publication of this study. No ethical issues emerged during the study, as it complied with established protocols. A written statement

was obtained from the institute's ethical committee (SU/CLAR/RD/025/2023; dated August 24, 2023). I followed the required precautions for in-vitro studies and all laboratory facilities were utilized solely for this study, and ensured compliance with the institutional regulations and protocols.

#### **Informed Consent Statement**

Informed consent, in written form, was acquired from the volunteer (blood donor) for the publication of this data and associated findings, adhering to ethical principles and institutional regulations.

#### **Conflict of Interest**

The author declares that there is no conflict of interest.

## **Artificial Intelligence (AI) Disclosure Statement**

Al-Unassisted Work.

## **Funding/Support Sources**

No funding.

#### **Author Contribution**

Mathiyazhagan Narayanan: Conceptualization, data analysis, writing, and reviewing the manuscript.

# **Data Sharing Statement**

All data generated during this report have been included in the article.

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