TUJNAS

Original Article

Thamar University Journal of Natural & Applied Sciences 9(1) (2024) 14 – 19

DOI: https://doi.org/10.59167/tujnas.v9i1.2049

Green Synthesis of Zinc Oxide Nanoparticles Using *Allium Sativum* Extract: Evaluation of Antibacterial Activity Against Nosocomial Bacteria

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Received: 20 March 2024. Received (in revised form): 12 April 2024. Accepted: 19 April 2024. Published: 26 June 2024.

Abstract

Zinc oxide nanoparticles (ZnO NPs) are receiving considerable interest in different fields because of their outstanding features. This study investigated the green synthesis of zinc oxide nanoparticles (ZnO NPs) using *Allium sativum* (garlic) extract and rigorously assessed their antibacterial efficacy against a panel of clinically relevant nosocomial pathogens. The obtained ZnO was characterized by X-ray diffraction (XRD) analysis. ZnO NPs synthesized using *A. sativum* extract demonstrated dose-dependent antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Enterococcus faecalis*. *Pseudomonas aeruginosa* exhibited the highest susceptibility to the green-synthesized ZnO NPs. Significantly, ZnO NPs synthesized using 3 mL of *A. sativum* extract displayed superior antibacterial activity compared to those with higher extract volumes. Furthermore, green-synthesized ZnO NPs exhibited significantly enhanced activity compared to conventionally produced (pure) ZnO NPs, particularly against *E. coli*, *P. aeruginosa*, and *S. aureus*. These findings underscore the potential of *A. sativum*-mediated ZnO NP synthesis as a sustainable and highly effective strategy to combat multi-drug resistant bacteria, offering a promising direction for developing novel antibacterial therapeutics.

Keywords: ZnO Nanoparticles; Green Synthesis; *Allium sativum*; Antibacterial Activity; Nosocomial Bacteria

1. Introduction

Nanotechnology has transformed scientific fields by allowing accurate material control at atomic and molecular scales [1, 2]. It also has distinctive physicochemical features due to its size, typically 1 to 100 nm [3]. Zinc oxide nanoparticles (ZnO NPs) are widely studied for their exceptional catalytic, optical, electrical, and antibacterial characteristics[4]. Traditional ZnO NP production is often done through physical and chemical methods, and ecologically friendly procedures have improved "green synthesis" methods for producing ZnO NPs [5, 6]. The strategies minimize environmental effects while preserving nanotechnological progress[5]. Green synthesis utilizes plant extracts and microorganisms to produce ZnO NPs, providing a viable method for sustainable nanoparticle manufacturing that avoids harmful ingredients and reduces energy use [6]. Adopting eco-friendly methods aligns with the increasing need for environmentally sensitive technology. Advancements in green synthesis processes benefit both nanotechnology and environmental sustainability [7].

Green synthesis approaches utilize the natural reducing and stabilizing properties of biological resources such as plant extracts, microbes, and biopolymers to create nanoparticles [8]. Utilizing plants to produce ZnO NPs offers several benefits, such as scalability, cost efficiency, biocompatibility, and eliminating the need for toxic solvents [9, 10]. *A. sativum* (garlic) is a standout choice among these botanical possibilities [11, 12]. *A. sativum* is valued for its wide range of phytochemicals, including organosulfur compounds, phenolic acids, and flavonoids, contributing to its culinary and therapeutic properties [13]. The bioactive components are crucial in decreasing zinc precursor salts and stabilizing newly formed ZnO NPs, thereby preventing them from clumping together [14]. This adaptable method shows great potential for creating nanoparticles sustainably by utilizing natural processes to facilitate environmentally friendly and effective manufacturing procedures [1, 15].

Antimicrobial resistance is a significant global threat to public health worldwide [16, 17]. Multidrug-resistant bacteria, prevalent in healthcare settings, are diminishing the efficacy of conventional antibiotics [18, 19]. Green synthesis techniques are promising, particularly in producing zinc oxide ZnO NPs [20]. Compared to conventional antibiotics, these nanoparticles exhibit diverse bactericidal mechanisms, offering a promising alternative with less potential for bacterial resistance development [21]. Utilizing environmentally friendly technologies to produce ZnO nanoparticles shows significant promise in combating antibiotic resistance. This action aims to safeguard public health and avert a worldwide health disaster [22, 23].

ZnO NPs have antibacterial solid capabilities due to various processes. Due to their small size and high surface-area-to-volume ratio,

ZnO NPs may make close contact with bacterial cells, which may cause the cell membrane to break and release critical cellular components [24, 25]. Moreover, these nanoparticles can induce oxidative stress in bacteria by increasing reactive oxygen species (ROS) levels, causing damage to DNA, proteins, and lipids, significantly altering cellular structure and vital metabolic processes [26, 27]. Moreover, the breakdown of zinc oxide nanoparticles results in the release of Zinc (II) ions, which disrupt essential enzyme functions and enhance their ability to kill bacteria [28]. Together, these complex tactics cause permanent harm to bacterial cells, leading to their death [29]. Utilizing the antibacterial properties of zinc oxide nanoparticles has excellent potential in fighting microbial infections, providing a diverse and influential approach to tackle antibiotic resistance and improve public health worldwide [11, 30].

This study focuses on the environmentally sustainable synthesis of ZnO NPs using the extract derived from *A. sativum*. Additionally, it comprehensively examines the nanoparticles' efficacy against various medically significant nosocomial bacteria. The overarching goal is to enhance the production process of ZnO NPs, assess their antibacterial potency, and elucidate their mechanisms of action. By contributing to the expanding body of knowledge on environmentally friendly ZnO NP synthesis and their effectiveness against the growing threat of multidrug-resistant bacteria, this research aims to advance nanomedicine strategies to combat global antibiotic resistance. Through these efforts, the study endeavours to foster the development of innovative solutions to address one of the most pressing challenges in modern healthcare.

2. Materials and Methods

2.1 Plant Extract Preparation

The plant extract from raw bulbs of *A.sativum*, procured from the primary market in Dhamar City, Yemen, was obtained via aqueous extraction. The aqueous extract was produced by washing 10 g of *A. sativum*, which was crushed and soaked in 100 ml of sterile distilled water at 60°C for 80 minutes, stirring at 800 rpm using a magnetic stirrer. The mixture was then cooled to room temperature and filtered using Whatman filter paper No. 1. The extract was finally kept at 4°C for future studies [11, 31].

2.2 Synthesis of Pure ZnO NPs

ZnO NPs were synthesized via a precipitation method involving multiple steps. Initially, 14.9 g of zinc nitrate hexahydrate ($Zn(NO_3)_2.6H_2O$) were dissolved in 50 ml of sterile distilled water under magnetic stirring for 30 minutes. Then, 4 g of sodium hydroxide (NaOH) was dissolved in 50 ml of sterile distilled water under magnetic stirring for 15 minutes. The NaOH solution was then slowly added dropwise to the zinc nitrate solution, and the resulting mixture was stirred for 3 hours using magnetic agitation. The mixture was subsequently filtered using Whatman filter paper No. 1 to isolate the precipitates, which were then subjected to three repeated washing cycles with distilled water and ethanol to remove impurities. The mixture was dried in the oven at 60°C overnight and then annealed at 200°C for 2 hours. The dried powders were ground using a mortar and pestle into a fine consistency before being transferred into a sterilized container [11, 32].

2.3 Green Synthesis of ZnONPs

The synthesis of three novel ZnO NPs using *A. sativum* extract followed the same procedures as those used for pure ZnONPs, except for the modification in the first step as varying volumes (3ml, 6ml, 9ml) of *A. sativum* extract were added to the zinc nitrate solution separately after 15 minutes of mixing [33, 34]. This green synthesis method yielded three distinct ZnONP formulations.

2.4 Characterization of ZnONPs

X-ray diffraction (XRD) analysis was executed on the samples using an XRD-6000 instrument to investigate their crystal structure.

2.5 Cultivation and Identification of Nosocomial Bacteria

Nosocomial bacteria for antimicrobial activity assessment were procured from the laboratories of Al-Hikma University, Dhamar City, Yemen. These bacteria included *S. aureus*, *E. faecalis*, *K. pneumoniae*, *P. aeruginosa*, and *E.coli*. Confirmation of bacterial identity was performed using standard microbiological techniques [35, 36].

2.6 Antimicrobial Activity of ZnONPs

The antimicrobial effects of ZnO NPs synthesized using a green method using *A. sativum* extract and a non-green method were evaluated against nosocomial bacteria using the disc diffusion assay [37, 38]. Various concentrations (5, 25, 50, and 100 mg/mL) of ZnO NPs using sterile distilled water as the solvent, including unprocessed controls and the positive control using the gentamycin as a reference, were prepared and tested against the identified nosocomial bacteria. Bacterial suspensions were adjusted to a standardized inoculum density of 1.5 x 10^8 CFU/mL using the 0.5 McFarland standard [39, 40]. The suspensions were streaked onto Mueller-Hinton agar plates and then incubated with discs containing the ZnO NPs (each disc was saturated with 40µ)at 37°C for 24 hours. The diameter of the inhibition zone around the discs was measured to determine the antibacterial activity of ZnO NPs [31, 41].

3. Results and Discussions

3.1 ZnO NPs Characterization

X-ray diffraction (XRD) analysis was employed to characterize the crystal structure and particle size of ZnO NPs synthesized via the green method [25]. The XRD pattern of pure ZnO NPs and those prepared with varying volumes (3, 6, and 9 ml) of *A. sativum* extract (Figure 1) revealed characteristic diffraction peaks at 2-theta values of 32.14°, 34.80°, and 36.64°. These peaks align with the (100), (002), and (101) lattice planes, respectively, confirming the hexagonal wurtzite structure of ZnO (JCPDS 36-1451). The absence of impurity peaks within the diffraction patterns suggests the complete reduction of the precursor and demonstrates the synthesis of highly crystalline ZnO NPs [25]. The crystallite size (D) was measured by $D=0.9\lambda/ \beta COS(\theta)$, where D is crystallite size, K is the geometric factor (0.9), λ is X-ray wavelength (0.154 nm), β is FWHM of the diffraction peak (in radian), and θ is the diffraction angle [42, 43].

Table 1: The Measured and Calculated XRD Parameters for Crystallite Size.

The prepared samples	2-theta	FWHM (β)	Crystallite size(nm)(D)	Average Crystallite size(nm)(D_{ave})
ZnO-pure	32.140	0.330	25.05447	27.82649
	34.801	0.238	34.98256	
	36.639	0.357	23.44243	
ZnO-3mL	32.260	0.376	21.99594	23.9813
	34.881	0.288	28.91553	
	36.720	0.398	21.03243	
ZnO-6mL	32.220	0.358	23.09955	25.1718
	34.860	0.273	30.50254	
	36.719	0.382	21.9133	
ZnO-9mL	32.200	0.351	23.55904	25.42197
	34.840	0.274	30.38955	
	36.641	0.375	22.31732	

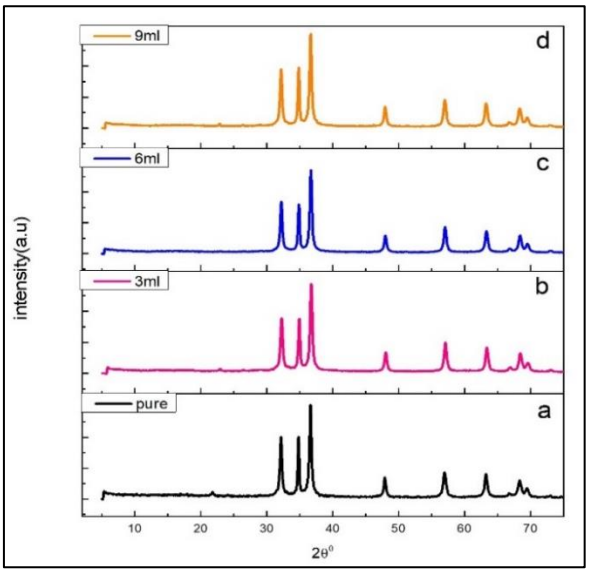

Figure 1: XRD pattern of synthesized ZnO nanoparticles, (a) pure, (b) 3mL, (c) 6mL and (d) 9mL of garlic extract.

3.2 Antibacterial Activity of ZnO Nanoparticles

ZnO NPs demonstrated varied antibacterial activity (Figure 2) against nosocomial bacteria, including *S. aureus*, *E. faecalis*, *K. pneumoniae*, *P. aeruginosa*, and *E. coli*. The antibacterial effectiveness of ZnO NPs was often dose-dependent, and susceptibility differences were observed among bacterial species.

Table 2 displays the results of the antibacterial effectiveness of ZnO NPs prepared by a green synthesis process using 3 mL of *A. sativum* extract. The size of inhibition zones (ZOIs) was used as the criterion to assess the antibacterial efficacy of the nanoparticles against nosocomial bacteria [44]. ZnO NPs showed different growth inhibition degrees on all examined nosocomial bacteria, including *S. aureus*, *E. faecalis*, *K. pneumoniae*, *P. aeruginosa*, and *E. coli*. ZnO NPs displayed antibacterial activities that varied based on concentration. ZnO NPs at concentrations ranging from 5 to 100 mg/mL increased the zone of inhibition for most bacterial species. The highest growth inhibition was usually seen at concentrations of 50 mg/mL or 100 mg/mL. At times, a smaller zone of inhibition was noticed at 100 mg/mL compared to 50 mg/mL for certain species, suggesting potential saturation effects at very high doses of ZnO nanoparticles [45]. ZnO NPs showed different levels of effectiveness against various hospital-acquired bacteria, with *P. aeruginosa* consistently being the most susceptible and showing the most significant zone of inhibition at all concentrations. *E. faecalis* showed the lowest susceptibility, with much lower ZOIs than other bacteria. The variations in susceptibility could be due to the differences in their cell wall composition as gram-positive bacteria possess a thick peptidoglycan layer and negatively charged teichoic acids, potentially leading to stronger initial attraction to ZnO NPs but making it more difficult for the nanoparticles to penetrate and cause internal damage. Conversely, gram-negative bacteria have a thinner peptidoglycan layer and an outer membrane rich in lipopolysaccharides (LPS). The thinner cell wall makes gram-negative bacteria more vulnerable to ZnO NP penetration, and the LPS layer of the outer membrane is susceptible to disruption by the nanoparticles [43]. Gram-negative bacteria similar to *P. aeruginosa* and *E. coli*, which have thinner peptidoglycan coatings, are more susceptible to the disruptive effects of ZnO NPs [46]. ZnO NPs exhibit antibacterial properties through multiple pathways, including reactive oxygen species production, such as hydrogen peroxide, superoxide anion, and hydroxyl radicals [15]. These ROS cause oxidative harm to bacterial cell components such as membranes, proteins, and DNA, leading to the eventual death of the cell [4, 22].

Table 2: The Antibacterial Activity of ZnO Nanoparticles Synthesized by the Green Method using 3ml of *A. Sativum* Extract.

Nosocomial Bacteria	Inhibition zone (mm) of different Concentrations (mg/ml)			
	5	25	50	100
S. aureus	11	12	16	13
E. faecalis	9	12	15	13
K. pneumoniae	10	16	17	15
P. aeruginosa	13	13	15	13
E. coli	12	15	16	14

From Table 3, the antibacterial effectiveness of ZnO NPs synthesized utilizing 6 mL of *A. sativum* showed that *S. aureus* recorded the most significant susceptibility, with inhibitory zones rising from 9 mm to 16 mm as the ZnO NPs increased from 5 mg/mL to 50 mg/mL. A modest decrease in activity (8 mm inhibitory zone) was observed at the highest dose of 100 mg/mL, possibly due to nanoparticle aggregation reducing their effective surface area [23]. The antibacterial effectiveness of ZnO NPs differed amongst bacterial species [27]. *E. faecalis* was not inhibited at 5 mg/mL but exhibited moderate sensitivity at higher dosages. *K. pneumoniae* and *P. aeruginosa* showed reduced susceptibility at lower ZnO NP doses but became more sensitive as the concentrations of ZnO NP increased. *E. coli* showed sensitivity at all doses tested. The relationship between the concentration of ZnO NPs and their ability to suppress *E. coli* growth is directly proportional, believed to be due to electrostatic interactions between the negatively charged surface of the bacterial cell and the positively charged ZnO NPs, leading to rupture of the cell membrane [47]. ZnO NPs cause damage to the bacterial cell membrane by physically interacting with it, potentially through electrostatic interactions. This interaction results in membrane instability, increased permeability, and leaking of cellular components [48, 49].

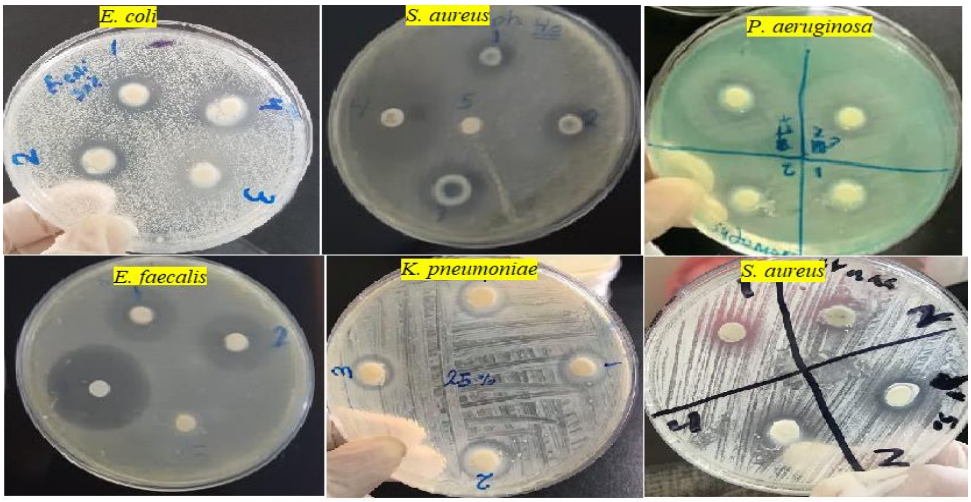

Figure 2: Images depicting ZnO-NP induced zones of inhibition in nosocomial bacteria.

Table 3: The Antibacterial Activity of ZnO Nanoparticles Synthesized by the Green Method using 6 ml of *Allium Sativum* Extract.

Nosocomial Bacteria	Inhibition zone (mm) of different Concentrations (mg/ml)			
	5	25	50	100
S. aureus	9	10	16	8
E. faecalis	-	14	15	13
K. pneumoniae	-	12	14	14
P. aeruginosa	14	12	14	12
E. coli	10	12	15	13

Table 4 shows the antibacterial characteristics of ZnO NPs synthesized using 9 mL of *A. sativum* extract. The ZnO NPs showed varying antibacterial effectiveness against the range of nosocomial bacteria examined. ZnO NPs' impact on *S. aureus* depended on the concentration used. An inhibitory zone of 10 mm was observed at a dosage of 5 mg/mL. At greater dosages of 25, 50, and 100 mg/mL, inhibitory zones measuring 12 mm and 6 mm were found. The reduced inhibition at a concentration of 100 mg/mL indicates a potential for nanoparticle aggregation, leading to a decrease in the surface area accessible for antibacterial action [50]. *E. faecalis* showed inhibition zones of 14 mm, 16 mm, and 15 mm at 25, 50, and 100 mg/mL of ZnO NPs, respectively, demonstrating a clear relationship between ZnO NP concentration and its effectiveness in inhibiting this bacteria. *K. pneumoniae* showed larger inhibitory zones at greater concentrations of ZnO NPs. Specifically, at 25, 50, and 100 mg/mL dosages, the zones measured 15 mm, 14 mm, and 13 mm, respectively. The minor decrease in zone size as the concentration increases may be due to possible aggregation effects [51]. *P. aeruginosa* was susceptible to all concentrations of ZnO NPs, showing zone diameters of 13 mm (5 mg/mL), 15 mm (25 and 50 mg/mL), and 14 mm (100 mg/mL). This sensitivity had a less concentration-dependent trend compared to other bacteria. ZnO NPs had different impacts on *E. coli*, resulting in zone sizes of 10 mm (5 mg/mL), 12 mm (25 mg/mL), a notable increase to 18 mm (50 mg/mL), and then a reduction to 13 mm (100 mg/mL). Based on these findings, an ideal antibacterial dosage of around 50 mg/mL is recommended for combating Escherichia coli. The differences in antibacterial effectiveness shown in various bacteria may be due to variations in cell wall composition [52]. Gram-positive bacteria such as *E. faecalis* and *S. aureus* have a dense peptidoglycan coating that may shield ZnO NPs [53]. On the other hand, Gram-negative bacteria, including *K. pneumoniae*, *P. aeruginosa*, and *E. coli,* have a weaker peptidoglycan layer and an outer membrane, making them more vulnerable to the antibacterial properties of ZnO NPs[54-56]. Generally, ZnO NPs can produce zinc ions that penetrate bacterial cells and interfere with essential metabolic processes such as enzyme activity and DNA replication, leading to differences in antibacterial effectiveness against various bacteria [25, 57].

Table 4: The Antibacterial Activity of ZnO Nanoparticles Synthesized by the Green Method using 9 ml of *Allium Sativum* Extract.

Nosocomial Bacteria	Inhibition zone (mm) of different Concentrations (mg/ml)			
	5	25	50	100
S. aureus	10	-	12	6
E. faecalis	-	14	16	15
K. pneumoniae	-	15	14	13
P. aeruginosa	13	15	15	14
E. coli	10	12	18	13

Table 5 shows the antibacterial effectiveness of ZnO NPs synthesized using the non-environmentally friendly approach. The ZnO NPs showed antibacterial activity that increased with the dosage against all microorganisms tested. Significantly, there were modest antibacterial effects against *S. aureus*, with the ZOI increasing from 10 mm at 5 mg/mL to 14 mm at 50 mg/mL and then slightly decreasing at 100 mg/mL. ZnO NPs were efficient against *E. faecalis*; however, no antibacterial activity was observed at the lowest dose of 5 mg/mL. The most significant antibacterial effect was seen against *K. pneumoniae*, especially at concentrations of 25 mg/mL and 50 mg/mL, resulting in a zone of inhibition of 15 mm. Significant antibacterial activity was shown against *P. aeruginosa*, with larger inhibition zones as the concentration of ZnO NPs increased. *E. coli* showed similar antibacterial effects as other bacteria, and the size of the zone of inhibition was directly related to the concentration of nanoparticles [58]. The results highlight the varying antibacterial properties of ZnO NPs synthesized using the non-green method on important hospital-acquired pathogens, depending on the dosage. These effects can be explained by various mechanisms, such as oxidative stress caused by the production of reactive oxygen species, damage to the integrity of cell membranes of bacteria, and the release of zinc ions that disrupt crucial cellular functions [8, 59]. The effectiveness of nanoparticles as antibacterial agents can vary between bacteria due to the existence of efflux pumps. These pumps function as a defence mechanism, actively expelling nanoparticles from bacterial cells. By decreasing the accumulation of nanoparticles within the cell, efflux pumps can significantly reduce their antibacterial impact, contributing to differences in susceptibility across bacteria [21, 60].

Table 5: The Antibacterial Activity of Non-Green Synthesized ZnO Nanoparticles

Nosocomial Bacteria	Inhibition zone (mm) of different Concentrations (mg/ml)			
	5	25	50	100
S. aureus	10	11	14	9
E. faecalis	-	10	14	12
K. pneumoniae	-	15	15	11
P. aeruginosa	11	13	14	12
E. coli	11	12	13	12

The ZnO NPs synthesized by the green method showed a significantly greater inhibitory zone against *S. aureus* than the findings of Ali *et al*. (2018)[60]. The findings are consistent with Nezamabadi et al. (2020) [61]. The effectiveness against *K. pneumoniae* remained consistent, as Ifeanyichukwu et al. (2020) [2]. ZnO NPs showed a gradual rise in inhibition against *K. pneumoniae* as the concentration increased to 50 mg/mL, in line with Al-Badaii et al.'s findings in 2023. Siddiqi et al. (2018) suggested that the antibacterial properties of ZnO NPs on gram-negative bacteria could be due to the rupture of the cell membrane and potential influence on genetic material [62]. The antibacterial activity against *E. coli* was consistent with the results of Ifeanyichukwu et al. (2020) [2]. The results of *P. aeruginosa* were higher than those reported by Al-Badaii et al. (2023) [31]. This study shows the considerable antibacterial effectiveness of ZnO NPs against several nosocomial bacteria. The results emphasize the varying bacterial susceptibility based on concentration and demonstrate the efficacy of green production methods. The results endorse the possible application of ZnO NPs in creating innovative antibacterial methods to address healthcare-related illnesses, especially as multi-drug resistance becomes a pressing global issue.

4. Conclusions

This study investigated the antibacterial potential of ZnONPs synthesized using *A. sativum* extract against various nosocomial pathogens. The findings revealed a dose-dependent increase in the inhibitory effect of ZnONPs on *S. aureus*, *E. coli, P. aeruginosa*, and *K.pneumoniae*. *E. coli* exhibited the highest susceptibility, followed by *P. aeruginosa*, *S. aureus*, and *K. pneumoniae*. Conversely, *E. faecalis* displayed the most vigorous resistance. Interestingly, ZnONPs synthesized using 3 ml of *A. sativum* extract demonstrated superior antimicrobial activity against all tested bacteria compared to those with higher extract volumes (6 ml and 9 ml), suggesting an optimal concentration for biomolecule-mediated nanoparticle synthesis and stabilization. Pure ZnONPs exhibited significant antimicrobial activity at higher concentrations (second and third), moderate activity at the fourth concentration, and minimal to no activity at the lowest concentration. However, for *S. aureus* and *K. pneumoniae*, pure ZnONPs displayed greater efficacy than ZnONPs synthesized with 6 ml and 9 ml of *A. sativum* extract. Conversely, the pure ZnONPs showed weaker activity against *E. coli*, *E. faecalis*, and *P. aeruginosa* than those synthesized using the green method with *A. sativum* extract. These findings highlight the potential of *A. sativum* extract-mediated ZnO NP synthesis as a promising strategy to enhance antibacterial activity against a broad spectrum of nosocomial pathogens, particularly *E. coli*, *P. aeruginosa*, and *S. aureus*. Further research is warranted to explore the underlying mechanisms of action and optimize synthesis parameters for broader applications.

Data Availability

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

The authors thank the Faculty of Medical Sciences at Al-Hikma University in Dhamar, Yemen, for granting access to their research facilities. Additionally, appreciation is extended to the Faculty of Applied Sciences in Dhamar, Yemen, for making their laboratory facilities available for this study.

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