

Study of the Physicochemical Properties and Anti-biofilm Effects of Synthesized Zinc Oxide Nanoparticles Using *Artemisia* Plant

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Abstract

Introduction: Recently, the biosynthesis of nanoparticles (NPs) using medicinal plants has attracted the attention of researchers due to their low cost and environmental compatibility. The aim of this study was to determine the anti-biofilm effects of zinc oxide (ZnO)-NPs synthesized using the *Artemisia* plant extract on the clinical samples of *Pseudomonas aeruginosa*.

Methods: In this experimental study, the alcoholic extract of *Artemisia* was prepared using the Soxhlet extraction method to synthesize ZnO-NPs. Then, the physical and chemical structures of the NPs were investigated using transmission electron microscopy (TEM), scanning electron microscopy (SEM), and ultraviolet/visible (UV-Vis) techniques. In addition, the gene expression of *ndvB* was analyzed by the polymerase chain reaction method. Finally, anti-biofilm and antimicrobial effects were evaluated using the minimum inhibitory concentration test and microtiter plate assay.

Results: The antimicrobial results showed that ZnO-NPs had a spherical structure approved by the UV-Vis test. Further, ZnO-NPs had inhibitory effects on biofilm formation by *P. aeruginosa* strains. The results demonstrated that ZnO-NPs were effective on the isolations at the lowest and highest viscosities of 3.125 and 100 mg/mL, respectively.

Conclusion: The biosynthesis of ZnO-NPs using the *Artemisia* plant extract is low cost and easy. Moreover, these NPs can be used as a drug with antimicrobial and anti-biofilm effects.

Keywords: ZnO-NPs, *Artemisia*, *Pseudomonas Aeruginosa*, Anti-biofilm

Introduction

Nano-biotechnology deals with a variety of material structures with dimensions up to the size range of 1-100 nm. In recent decades, microbial nanobiotechnology, especially zinc oxide nanoparticles (ZnO-NPs), has provided promising prospects for combating various infectious diseases.^{1,2}

ZnO-NPs offer a unique solution for medical applications and their function is controlled by geometric and optical properties. In addition, they are subjected to biomedical studies and applications including genetics, biosensor development, clinical chemistry, laser phototherapy of cancer cells and tumors, photothermal therapy, photodynamic therapy, and targeted drug delivery. Further, NPs are among the materials that have received special attention due to their special physicochemical properties because they

are chemically stable, non-toxic, and easily functional. Nowadays, numerous methods have been developed for NP synthesis.^{3,4}

For instance, green chemistry is an emerging method for designing and synthesizing pharmaceutical organic compounds and has an easy protocol while being effective in treating various diseases. One of the advantages of compounds synthesized by green chemistry is their biocompatibility and biodegradability over chemicals synthesized by classical methods. Furthermore, these compounds typically have lower production costs.⁵

Secondary metabolites, enzymes, proteins, or other reducing agents play an essential role in the preparation of metal NPs by plants. Moreover, the bio-accumulation of NPs depends on the presence of enzymes and proteins that are involved in their preparation. The



recovery of NPs from plant tissues requires enzymes to destroy cellulose, which is exhausting and expensive. Therefore, using plant extractions for the low and large scale processing of different metal NPs is considered as an easy and convenient alternative to chemical and physical methods. The geranium (i.e., leaf, stem, and root extracts) was first used to produce extracellular NPs.⁶⁻⁸ Arunachalam et al reported the biosynthesis of Zn-NPs using geranium leaf extraction. They also produced triangular and spherical Zn particles using lemon extraction.⁹

Artemisia is one of the largest genera of the family *Asteraceae* or *Compositae* and is rich in substances that have a variety of anti-inflammatory, anti-tumor, antioxidant, and anti-proliferative effects. Additionally, this genus contains high amounts of terpenoids and flavonoids, and more than 160 flavonoid compounds have been extracted from this genus.^{10,11}

Treating bacterial infections have been problematic in recent decades due to their resistance to antibiotics, and the 21st century is called the century of antibiotic failure. Studies on different bacteria have shown that the presence of ZnO-NPs can have a significant role in controlling bacterial biofilms and thus preventing bacterial viability. *Pseudomonas aeruginosa* is a gram-negative bacillus and an opportunistic bacterium with pathogenic potency in humans, animals, and plants. This bacterium is one of the most important causes of nosocomial infections in a wide range of immunocompromised patients with malignancies, cystic fibrosis, burns, and the like. In addition, different strains of this bacterium can cause similar symptoms and death in animals with the weakened immune system. Bacterial biofilm formation confers the organism resistant to many antibiotics, thus more research is needed to find suitable alternatives to antibiotics.¹²⁻¹⁴

In this regard, this study aimed to produce ZnO-NPs with plausible physicochemical properties using the *Artemisia* extract. Then, the study investigated the physical and chemical properties and antimicrobial effects of the synthesized ZnO-NPs on the clinical isolates of *P. aeruginosa*.

Materials and Methods

Plant Collection and Extraction

The *Artemisia* plant was obtained from the Bank of Iran Biological Reserve Center and then approved by the botanical section with the herbarium number of 1342. The aerial parts of the plant were air-dried and then completely dried in the shade for extraction. The leaves were thoroughly powdered by an electric milking machine and kept in glass containers. In addition, the powder was extracted using the Soxhlet extraction method. Next, 50 g of the plant leaf powder was added to 500 mL of the methanol solvent. The extraction was conducted for 12 hours, and finally, the solvent was removed by a rotary

evaporator (Rv10 digital, German).

The obtained solid powder by distillation was doubled in volume and the extract was stored at 4°C until use for ZnO-NPs synthesis.

Green Synthetic Route of Zinc Oxide Nanoparticles

The metal ions were synthesized by the *Artemisia* extract through high purity synthesis in different sizes. Further, ZnO-NPs were synthesized by adding different volumes of the *Artemisia* extract to various viscosities of HACUI4 (purchased from Merck) salt under different stirring conditions. Two hours after the reaction, the precipitate was washed three times with distilled water (13 000 rpm for 20 minutes). The final washings were performed with ethanol and the resulting product was incubated at 75°C for 2 hours.

Characterization of Zinc Oxide Nanoparticles

Dynamic Light Scattering

The particle size distribution was determined by Malvern Zetasizer (Nano ZS) at room temperature and the angle of 90°. To evaluate the above-mentioned parameters, the device was calibrated based on the aqueous phase (RI: 1.3), and then the synthesized NPs were passed through a syringe filter with a size of 0.22 mm.

Transmission Electron Microscopy

To investigate the morphology and confirm the ZnO-NPs size, a drop of the suspension sample was placed on the carbon film grid and, after being dried at laboratory temperature, it was imaged using a transient electron microscope (LEO 906, Zeiss100 kV) with the accelerator voltage of 120 kV.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) images and point-to-point studies were carried out to investigate the size and morphology of NPs after covering with Zn at a voltage below 30 kV under vacuum pressure (10^{-5} Torr) using SEM. Then, ZnO-NPs were dissolved in water and the resulted suspension was subjected to SEM imaging.

Collecting Clinical and Microbiological Samples

In this study, 50 suspected *P. aeruginosa* specimens were collected from 50 patients who referred to Imam Khomeini hospital in Tehran. For this purpose, informed consent forms were from all patients, and the purpose of the study was explained to them. The collected samples included urine, wound, cerebrospinal fluid, blood, and sputum which were cultured on eosin-methylene blue and MacConkey agar medium after their transfer to the microbiology research laboratory of Roudehen Azad University. After the preparation of slides and the observation of Gram-negative bacilli, routine biochemical tests such as the culture in sulfur indole motility, triple sugar iron, and methyl red/Voges-Proskauer media, as

well as citrate media and oxidase test were performed to confirm *P. aeruginosa*. Then, the bacteria were studied and stored in trypticase soy broth and glycerol at 70°C for subsequent tests.

In addition, antibiotic (Bio-Rad disks) resistance was assessed by the disk diffusion method (Kirby-Bauer method) based on the Clinical and Laboratory Standards Institute (CLSI) (100) in which 0.5 McFarland equivalent microbial suspension was cultured in the Mueller-Hinton agar medium. The applied standard antibiotic discs included ciprofloxacin (5 µg), chloramphenicol (30 µg), polymyxin (300 µg), erythromycin (15 µg), meropenem (10 µg), ceftazidime (30 µg), cefotaxime (30 µg), tetracycline (30 µg), and tobramycin (10 µg) on the Mueller-Hinton agar medium with the sterile single-sided swab test. The space between the disks was 20 and 16 mm from the wall, and incubation lasted for 18-24 hours at 37°C. Eventually, the results were evaluated by measuring the growth zone diameter and comparing them with a standard table.

Phenotypic Evaluation of Biofilm Formation

The applied method in phenotypic testing was the one that was used by Freeman et al. In this method, Congo red agar culture medium was utilized for 24 hours at 37°C.¹⁵

PCR and Electrophoresis

For DNA extraction, bacteria were cultured in the Luria-Bertani broth medium for 12 hours, and the Bio Flux kit was used for extraction.¹⁵ To isolate PCR bands, estimate the molecular weight of the bands, and to observe the quality of the purified DNA, they were subjected to electrophoresis (agarose gel 2%) after ethidium bromide staining, and finally, visualized by a transilluminator in a dark room with a UV lamp at 254 nm. In this wavelength, DNA has the highest optical absorption. Eventually, the gels were photographed for closer examination.

Detection of the *ndvB* Gene

The *ndvB* affects the expression of multiple genes that are involved in biofilm formation. This gene was amplified using the primers listed in Table 1.

Evaluation of the Antimicrobial Activity of ZnO-NPs by the Minimum Inhibitory Concentration Method

The minimum inhibitory concentration (MIC) test was performed using the microplate dilution method according to the CLSI standards (CLSI 2017) to check sensitivity to antibiotics. The inoculation of microorganisms was

obtained after 12 hours of culturing in the liquid medium to obtain standard McFarland turbidity. The NPs were prepared at the concentrations of 3.125, 6.25, 12.5, 25, 50, and 100 µg/mL. Subsequently, 96 well plates were provided by a 95 µL dispenser from the Mueller-Hinton broth medium and 5 µL microbial inoculum inside each well. Next, 100 µL of NPs with different concentrations were poured into the wells. The bottom well containing 95 µL of the Mueller-Hinton broth medium and 5 µL of microbial inoculation per row were used as controls. The obtained volume in all wells was 200 µL. Ultimately, the plate was stirred on the shaker for 60 seconds and then incubated at 37°C for 24 hours and examined again. Each test was repeated three times.

Phenotypic Evaluation of Anti-biofilm Effects

The microliter definition method was used to study the quantitative effects of ZnO-NPs on the biofilm.¹⁵ To this end, 50 µL of non-lethal concentrations of ZnO-NPs were incubated at 37°C for 24 hours after 24 hours of culturing of each isolate and bringing the opacity to about 0.5 McFarland (the optical absorption of about 0.08-0.1 at 625 nm wavelengths). Bacteria attached to the wall were immobilized with 250 µL of 96% ethanol after 15 minutes. After the plates were dried, 200 µL of the crystal violet was added and incubated for 15 minutes. Then, the biofilm was evaluated by adding 200 µL of acetic acid 33% to each well, and finally, its absorption at 492 nm wavelength was read by an enzyme-linked immunosorbent assay reader.

Results

The reaction was carried out at room temperature after adding the dried plant extract to the Zn salt solution, and color change showed ZnO-NP formation (Figure 1). The resulted brown solution was analyzed by dynamic light scattering (DLS), SEM, transmission electron microscopy (TEM), and ultraviolet/visible (Uv-Vis) techniques. Figure 2 displays the maximum absorption rate of solutions containing ZnO-NPs at 322 nm. The DLS study showed that the average size of lipid NPs containing the essence



Figure 1. Color Change During the Synthesis of ZnO-NPs by the *Artemisia* Plant Extract.

Note. ZnO-NPs: Zinc oxide nanoparticles.

Table 1. The Sequences of Primers Used to Amplify the *ndvB* Gene

Gene	Primer	Nucleotide Sequence	Product Size (bp)
<i>ndvB</i>	Forward	F 5'- TTCTTCTCTGCGCAATG -3'	153
	Reverse	R 5'- TACCGAGACAGGTAACGT -3'	

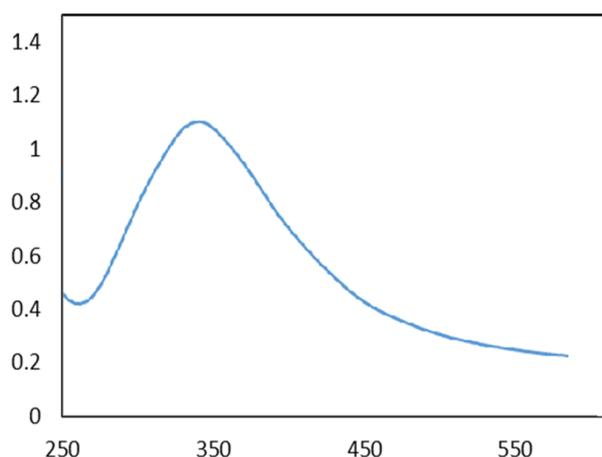


Figure 2. Ultraviolet/visible Spectroscopy of ZnO-NPs Synthesized by the *Artemisia* Plant Extract.

Note. ZnO-NPs: Zinc oxide nanoparticles. As shown, the UV absorption of the synthesized ZnO-NPs was between 250 and 550 nm, and the maximum absorption was at the wavelength of 320 nm.

of ZnO-NPs was 78.86 nm (Figure 3). TEM image results demonstrated that the average size of the NPs was 17 nm (Figure 4). Further, the structure of the NPs was observed to be spherical as demonstrated by SEM microscopy (Figure 5).

Isolation of *Pseudomonas aeruginosa* Strains

In this study, 50 clinical specimens suspected to be *P. aeruginosa* based on green lactose negative colonies were isolated and 20 strains were selected by microscopic and biochemical tests. The concordant agar medium was used for the phenotype evaluation of the biofilm. Biofilm forming bacteria produced black colonies while non-biofilm strains remained the same as the original red. Moreover, all 20 clinical strains were positive for biofilm formation. The molecular analysis of the presence of the biofilm-producing gene (*ndvB*) was performed, which resulted in 153 bp bands according to the designed primer. Figure 6 shows that the *ndvB* gene was present in all strains.

The results revealed that ZnO-NPs were effective on the isolates at the lowest viscosity of 3.125 mg/mL and the highest viscosity of 100 mg/mL. Additionally, the microliter plate method was used to quantitatively study the anti-biofilm effects of ZnO-NPs. Based on the results of this test, not all strains of the sub-MIC of NPs had biofilm formation ability (Figure 7).

Discussion

NPs have shown the potential for reducing the adverse effects of nosocomial infectious on humans health. The bacteria are considered as the most important contributing factors to nosocomial infections. These organisms act in various ways to create pollution and maintain their stability in the environment. One of the most important

of these methods is forming biofilms that play a key role in creating bacterial stability in the environment by converting the bacterial planktonic form to a fixed form. For example, *P. aeruginosa* in the planktonic form is less resistant to ceftriaxone antibiotic compared to the fixed biofilm form.¹⁴ In the biofilm structure, suitable nutrients are provided to the bacteria for growth due to selective transitional openings.¹⁶

Mohsenipour et al reported that the *Euphorbia hebecarpa* ethanolic extract better inhibited the planktonic forms of bacteria in comparison to methanolic extracts.¹⁷ However, these pores appear to be somewhat impermeable to antibiotics, which may be one of the main causes of bacterial resistance and biofilm stability.

Results

	Diam. (nm)	% Number	Width (nm)
Z-Average (d.nm): 78.86	Peak 1: 41.65	100.0	12.13
Pdi: 0.217	Peak 2: 0.000	0.0	0.000
Intercept: 0.952	Peak 3: 0.000	0.0	0.000

Result quality **Good**

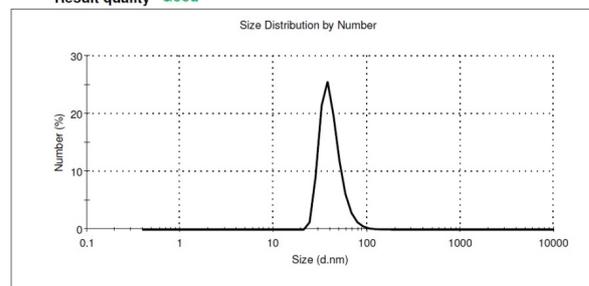


Figure 3. An Example of the Spectrum Obtained From Particle Size Determination and the Dispersion Index in ZnO-NPs Synthesized by the *Artemisia* Plant Extract.

Note. ZnO-NPs: Zinc oxide nanoparticles.

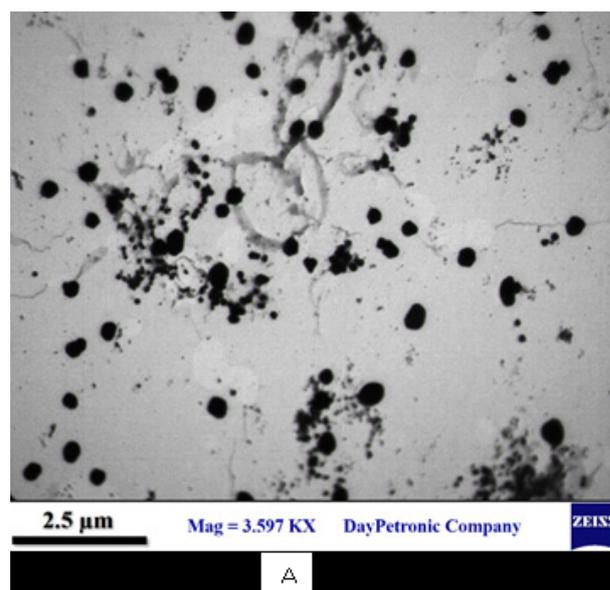


Figure 4. TEM Microscope Image of ZnO-NPs Produced by the *Artemisia* Plant Extract Showing the Spherical Shape of NPs.

Note. TEM: Transmission electron microscopy; ZnO-NPs: ZnO-NPs: Zinc oxide nanoparticles.

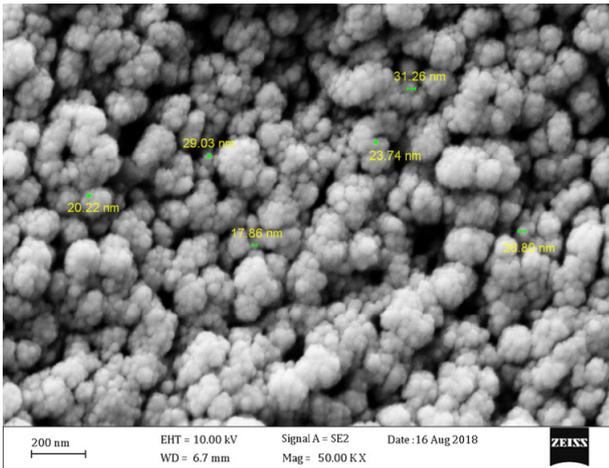


Figure 5. SEM Microscope Image of ZnO-NPs Produced by the *Artemisia* Plant Extract.
 Note. TEM: Transmission electron microscopy; ZnO-NPs: Zinc oxide nanoparticles.

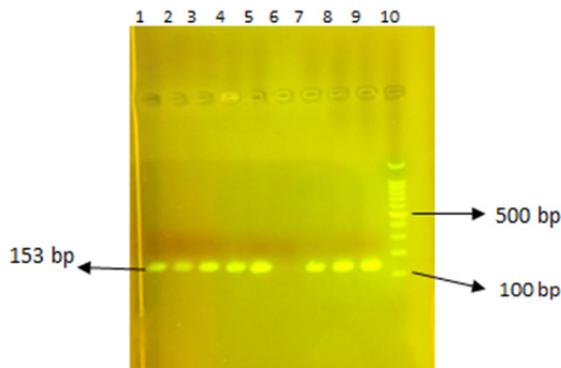


Figure 6. Electrophoresis of the *ndvB* Gene Resulting in 153 bp Bands on 1% Agarose Gel.

Since the biofilm plays the first and most important role against antibiotics and other drugs, using ZnO-NPs may be effective in destructing these structures. Therefore, the identification of biofilm-producing bacteria and the study of the effects of ZnO-NPs on biofilm expression have been the principles that many researchers have been trying to prove and apply to these particles against bacterial biofilms.¹⁸ Various chemical solvents have been used to synthesize ZnO-NPs in recent years. For example, Irvani et al used triethanolamine as a reducing agent to produce the NPs of about 40 nm in size and then investigated the antimicrobial activity of the produced particles.¹⁹ Our results also showed that ZnO-NPs were effective on the isolations at the lowest and highest viscosities of 3.125 and 100 mg/mL, respectively. In another study, Horna et al evaluated the inhibitory effects of bacteria with a high frequency of the *exoU*+/*exoS*+ genotype associated with multidrug-resistant, including *P. aeruginosa*, ampicillin-resistant *Escherichia coli*, and erythromycin-resistant *Streptococcus pyogenes*. They observed that nanotubes had a remarkable bacteriostatic effect on these bacteria.²⁰

Different species of *Artemisia* contain high concentrations of antimicrobial compounds in addition to antioxidants.⁷ It is remarkable that this herb is able to maintain the natural microbial flora of the body. Various reports demonstrated that the antimicrobial activity of *Artemisia* is exerted by various mechanisms including interference with the proton transport chain in mitochondria,²¹ the disruption of proteins, interference with membrane function,²² and the inhibition of the sarcoplasmic reticulum calcium pump.²³ Similarly, Ravindra et al studied the antibacterial activity of curcumin-loaded ZnO-NPs and reported a high antibacterial level.²⁴

The present study examined the MIC of ZnO-NPs against the clinical strains of *P. aeruginosa*, and the results showed that ZnO-NPs had significant lethal effects with the concentrations of 3.125-100 µg/mL. The results further revealed that ZnO-NPs at the lowest concentration of 3.125 µg/mL and the highest concentration of 100 µg/mL were effective on the isolates. Accordingly, our findings confirmed the dose-dependent antibacterial effects of ZnO-NPs against this bacterium. Some studies linking the antibacterial effects of ZnO-NPs against bacteria with high drug resistance (i.e., *P. aeruginosa*, ampicillin-resistant *E. coli*, and ciprofloxacin-resistance *Klebsiella*) represented the bacteriostatic effect by ZnO-NPs.¹⁸ Likewise, Arunachalam et al evaluated the antimicrobial effects of ZnO-NPs against bacteria such as *Staphylococcus aureus* and *E. coli* and reported that ZnO-NP activity was concentration-dependent and had stronger antibacterial effects against Gram-negative bacteria compared to Gram-positive ones. This may highlight the difference between these two groups of bacteria regarding their cell wall structure.⁹ In their study, Agnihotri et al investigated the impact of ZnO-NPs on *E.*

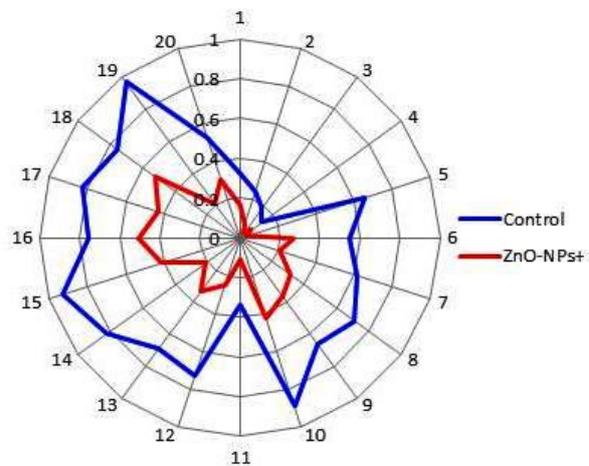


Figure 7. Quantitative Study of Biofilm Production in Strains Affected by ZnO-NPs.
 Note. ZnO-NPs: Zinc oxide nanoparticles.

coli, *P. aureus*, and *Bacillus subtilis*. Based on their finding, the lowest MICs of ZnO-NPs on the three strains were 3, 2, and 19 µg/mL, respectively, with an average diameter of 7 nm. Additionally, the lowest inhibitory concentrations were reported for nano-medium with a diameter of 70 nm for *E. coli* and *P. aureus* as 34 and 25 µg/mL, respectively.²⁵ Gupta et al reported similar results demonstrating the significant antibacterial activity of ZnO-NPs against *S. aureus* MTCC 9760, *S. pyogenes* MTCC 1926, *B. cereus* MTCC 430, *P. aeruginosa* MTCC 424, *P. mirabilis* MTCC 3310, and *E. coli* MTCC 40. In addition, the synthesized ZnO-NPs showed antibacterial efficacy against both Gram-positive and Gram-negative pathogens.²⁶

Further, Ansari et al found the ability of biofilm formation by *Pseudomonas* strains using the Congo red agar culture, and the results of SEM revealed that ZnO-NPs prevented the growth of bacteria and biofilm formation.²⁷ Similarly, Chikkanna et al studied the inhibitory effect of ZnO-NPs and chlorhexidine on biofilm formation by *P. aeruginosa* strains.²⁸ The results of a recent study also showed that this compound had anti-biofilm properties and could destroy the ability to form biofilms.²⁶ Some previous studies reported the anti-biofilm effects of NPs^{29,30} indicating that the highest MIC values of the essential oil were determined 100 ppm against *E. coli*, and the highest MIC value for *K. pneumoniae* was 250 ppm.³¹

The extraordinary ability of Zn as an antimicrobial agent has encouraged many researchers to use Zn-NPs in anti-microbial research.³² For instance, Garza-Cervantes et al compared the antimicrobial activity of ZnO-NPs and several antimicrobial compounds against biofilm-producing *P. aeruginosa* strains. The results of the recent study showed that using ZnO-NPs in combination with antimicrobial agents reduced biofilm formation, increased treatment efficacy, and prevented the formation of resistant strains.³³ Finally, Tiwari et al studied the effects of ZnO-NPs as antibacterial agents and demonstrated that ZnO-NPs reduced the growth of microbial agents.³⁴

Conclusion

Various investigations showed that ZnO-NPs have a remarkable ability to inhibit bacterial infectious diseases. More precisely, ZnO-NPs had inhibitory effects on *P. aeruginosa* strains. Overall, they can be used as antimicrobial and anti-biofilm drugs.

Ethical Approval

The study protocol was approved by the Ethics Committee of Islamic Azad university of Roudehen Branch, Roudehen, Iran (Code No: 113305609710002).

Conflict of Interest Disclosure

There is no conflict of interests in this study.

Informed Consent

Not applicable.

Funding/Support

None.

Authors' Contribution

MT: Designing the study and conducting all testes and data collection, contributing to writing and editing the manuscript and approving the final version of the manuscript.; SG: conducting all testes and data collection Participating in data collection and analysis.

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