Nanomed. J. 6(3):223-231, Summer 2019

RESEARCH PAPER

Antibacterial and anti-biofilm effects of microwave-assisted biologically synthesized zinc nanoparticles

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ABSTRACT

Objective(s): The present study aimed to investigate the antibacterial and anti-biofilm potential of the non-oxidized form of zinc nanoparticles (Zn NPs) prepared by a 'green approach' using the Lavandula vera extract with microwave irradiation.

Materials and Methods: After synthesis of Zn NPs, the microdilution and disk diffusion methods was applied for antimicrobial evaluation followed by anti-biofilm activity measurement using crystal violet colorimetric assay procedure.

Results: The obtained results demonstrated the production of spherical Zn NPs within the size range of 30-80 nanometers. The measured minimum inhibitory concentration of the Zn NPs and ZnSO4 against the biofilm-producing and clinically isolated pathogens of Staphylococcus aureus, Pseudomonas aeruginosa, and Proteus mirabilis was estimated to be more than 2560 µg/ml. In addition, a non-significant increase ($P>0.05$) was observed in the antibacterial activity against methicillin-resistant S. aureus after the addition of the Zn NPs (500 µg/disk) to the antibiotic discs containing tobramycin, erythromycin, tetracycline, azithromycin, and kanamycin compared to ZnSO4. On the other hand, the Zn NPs significantly decreased the biofilm formation of P. mirabilis compared to P. aeruginosa ($P<0.05$). Biofilm formation by S. aureus also reduced to 68.3±2.1% in the presence of the Zn NPs (640 µg/ml), which was considered significant compared to P. mirabilis and P. aeruginosa at the same concentration ($P<0.05$).

Conclusion: To sum up, the biofilm inhibitory activity of Zn NPs at higher concentrations than 160 µg/ml against S. aureus and P. mirabilis was more significant compared to the inhibitory effects of ZnSO4. However, further investigations are required in order to determine the antibacterial and anti-biofilm mechanism of Zn NPs.

Keywords: *Antibacterial Activity, Zinc, Nanoparticle, Nanobiotechnology, Green Synthesis*

How to cite this article
Shakibaie M, Alipour-Esmaeili-Anari F, Adeli-Sardou M, Ameri A, Doostmohammadi M, Forootanfar H, Ameri A. Antibacterial and Anti-biofilm Effects of Microwave-assisted Biologically Synthesized Zinc Nanoparticles. Nanomed J. 2019; 6(3):223-231. DOI: 10.22038/nmj.2019.06.00009

INTRODUCTION

Biofilm formation is a critical route used by gram-negative and gram-positive bacteria for irreversible attachment to surfaces through a self-produced extracellular matrix composed of polysaccharides, lipids, proteins, and nucleic acids [1, 2]. It is a defense mechanism through which bacterial cells protect themselves against harsh conditions, such as exposure to common antibiotics and host immune system [3]. Furthermore, biofilm production results in the enhancement of water and nutrition absorbance, playing a reinforcing role in horizontal gene transfer, which in turn optimizes

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Note. This manuscript was submitted on December 28, 2018; approved on March15, 2019

the colonization and proliferation of bacterial cells and promotes antibiotic resistance [4]. Many infectious diseases are caused by *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Streptococcus mutans*, which are biofilm-dependent and difficult to be treated [5, 6]. Furthermore, biofilm formation is associated with severe problems in various food industries, including brewing [7], meat [8], and fish processing [9], and ready-to-eat food industries [10].

Considering the protective effects of biofilm on the entrapped cells, along with the development of antibiotic-resistant species, the application of common antibiotics against biofilm formation seems ineffective [11]. Several studies have been focused on new alternatives with valuable properties, such as the development of non-resistant species, fewer side-effects, and higher antimicrobial potency [12, 13]. The application of metal-based antimicrobial agents with their multi-target activities has been reported to hinder the development of resistant species, attracting the attention of researchers [14, 15]. Despite the key role of metal ions (e.g., iron and zinc) in bacterial cell metabolism, they might be toxic to bacteria at some critical pH and concentrations [16].

Application of nanoparticles alone or in combination with antibiotics could be an alternative strategy in this regard owing to the extraordinary properties of nanoparticles, including surface plasmon absorption and enhanced catalytic activity [17-19]. Zinc (Zn) is an essential micronutrient for nearly all organisms, which has regulatory and metabolic functions in more than 300 human enzymes [20]. Furthermore, zinc is a critical ion for bacterial growth, virulence factor expression, and pathogenicity development [21]. Several studies have investigated the development of zinc-containing nanoparticles with antimicrobial and antifungal properties. For instance, the antibacterial properties of biomimetically synthesized zinc oxide nanoparticles (ZnO NPs) have been demonstrated against various bacterial species, such as *S. epidermidis*, *Listeria monocytogenes*, *Klebsiella pneumonia*, *P. aeruginosa*, and *E. coli* [22]. Another study in this regard also reported the anti-biofilm activity of chitosan/ZnO composite against *Vibrio parahaemolyticus* and *Bacillus licheniformis* [23].

According to reports, the antibacterial properties of ZnO NPs are associated with their oxidative stress induction, especially through interaction with the cell membrane and interrupting its function by reactive oxygen species (ROS) generation [24]. In addition, it has been demonstrated that the combination of zinc-based nanoparticles (Zn NPs) with conventional antibiotics exerts significant positive effects on the growth inhibitory properties of antibiotics [24]. Current findings have also indicated that the combination of ZnO NPs with ciprofloxacin and ceftazidime could increase the antibacterial activity of these antibiotics, thereby leading to antibiotic uptake augmentation and changes in the bacterial cell morphology [25].

Most of the studies in this regard have used the oxidized form of the Zn NPs or nanocomposites containing ZnO NPs. The present study aimed to use a 'green approach' with the application of the *Lavandula vera* extract combined with microwave irradiation in order to prepare the non-oxidized form of Zn NPs and evaluate the antibacterial and anti-biofilm effects of the synthesized Zn NPs against biofilm-producing and clinically isolated bacterial pathogens, including *S. aureus*, *P. aeruginosa*, and *P. mirabilis*. Moreover, the combined effects of the produced Zn NPs with various antibiotics were assessed against methicillin-resistant *S. aureus* (MRSA).

MATERIALS AND METHODS

Chemicals and bacterial pathogens

In this study, Muller-Hinton broth (MHB), 2, 3, 5-triphenyl-2H-tetrazolium chloride (TTC), nutrient broth (NB), NaH_2PO_4, Na_2HPO_4, and zinc sulfate heptahydrate were provided by Merck Chemicals (Darmstadt, Germany). The antibiotic disks were obtained from Mast Company (Merseyside, UK). The clinical isolates of MRSA, *S. aureus*, *P. aeruginosa*, and *P. mirabilis* were applied in the antibacterial examinations [26].

Synthesis and determination of the properties of Zn NPs

The Zn NPs were prepared using a method previously described [27]. Initially, the *Lavandula vera* extract was prepared by the addition of the herbal powder (5 g) to deionized water (100 ml) and heating at the temperature of 80°C for 30 minutes, followed by centrifugation at 8000 rpm for five minutes, in order to remove the plant remnants. Afterwards, the prepared extract (10 ml) was added to a reaction vessel containing the

$ZnSO_4$ solution (1 mM, 40 ml) and bubbled by pure argon. For the reduction of the Zn^{2+} ions to dark grey Zn NPs, the reaction vessel was irradiated using a microwave (850 W, 60 seconds), and the synthesized Zn NPs were washed with chloroform, ethanol, and deionized water.

The shape and elemental analysis of the prepared nanostructures were inspected using the energy dispersive X-ray (EDX)-equipped SEM apparatus (model: KYKY-EM3200). In addition, a transmission electron microscopy (TEM) apparatus (model: Zeiss 902A) at the accelerating voltage of 80 Kv in order to obtain the TEM micrographs. The size distribution profile of the nanostructures was achieved using Zetasizer MS2000 (Malvern Instruments, UK).

Determination of antimicrobial activity
Microdilution method

In order to determine the minimal inhibitory concentration (MIC) of the Zn NPs and $ZnSO_4$, a previously described microdilution protocol was applied [28]. In brief, 180 microliters of MHB supplemented with various concentrations of Zn NPs (0.625-2560 µg/ml) and $ZnSO_4$ (0.625-2560 µg/ml) was separately inserted to 96-well sterile microplates. To obtain 10^5 CFU/ml in each well, 20 microliters of freshly cultured *S. aureus*, *P. aeruginosa*, and *P. mirabilis* was added, and the microplates were placed in an incubator at the temperature of 37°C for 24 hours. At the next stage, 20 microliters of the TTC solution (0.5 mg/ml) was placed in each well, followed by incubation for two hours and visualization of the related pink color change. MIC was considered as the lowest concentration where no visible color change occurred. In parallel to each experiment, ciprofloxacin was also used as the positive control, while sterile MHB was considered as the negative control. Three replicates of each experiment were performed on various days, and the mean values of the obtained results were reported.

Disk-diffusion assay

The susceptibility of the clinically isolated pathogens and MRSA to the Zn NPs and $ZnSO_4$ was determined using the disk-diffusion method. Initially, an overnight culture of MRSA was prepared in MHB (37°C, 150 rpm) and diluted with sterile normal saline in order to reach the 0.5 McFarland standard. Afterwards, the diluted culture was spread on the surface of the MHA plates, and the plates were mounted using sterile antibiotic disks. The blank disks were loaded with the Zn NPs (500 µg/disk) or $ZnSO_4$ (500 µg/disk), and the antibiotic discs mounted by the Zn NPs (500 µg/disk) or $ZnSO_4$ (500 µg/disk) were placed on the surface of the inoculated media. Following that, incubation was performed at the temperature of 37°C overnight, and the growth inhibition zones were measured. Three replicates of the described experiment were carried out, and the mean values of the observed inhibition zones were reported.

Biofilm Inhibition assay

A previously described microtiter method was used to measure biofilm formation by the mentioned isolates with some modifications [26]. In brief, the fresh inoculum of *S. aureus*, *P. mirabilis*, and *P. aeruginosa* was prepared in tryptic soy broth (TSB) medium supplemented by glucose (10 g/l to reach the optical density [OD_{650}] of 0.13, equal to 0.5 McFarland standard [1.5×10^8 CFU/ml]). Afterwards, 100 microliters of the bacterial suspensions (~10^6 CFU/ml) was separately added to the 96-well microplate, and 100 microliters of the TSB medium containing the Zn NPs and $ZnSO_4$ was separately added to reach the concentration of 5-640 µg/ml. The prepared microplates were incubated overnight at the temperature of 37ºC.

At the next stage, the unattached bacterial cells were isolated from the formed biofilm, and each well was washed three times using sterile phosphate buffered solution (10 µl, pH: 7.2) in order to remove the free cells, followed by the fixation of the biofilm by methanol exposure (150 µl) and preservation at the temperature of 25ºC for 20 minutes. Following that, methanol was replaced by 1% w/v crystal violet solution (200 µL), and each well was gently washed with sterile distilled water and preserved at the temperature of 25ºC to be dried. Finally, 200 microliters of glacial acetic acid (33% v/v) was inserted into each well, and the absorbance was recorded at the wavelength of 570 nanometers using the Synergy2 multi-mode microplate reader (BioTek, USA). Three replicates of the mentioned procedure were performed, and the mean values of the obtained data were reported.

Statistical analysis

Data analysis was performed in SPSS version 15 (SPSS Inc., Chicago) using one-way analysis of

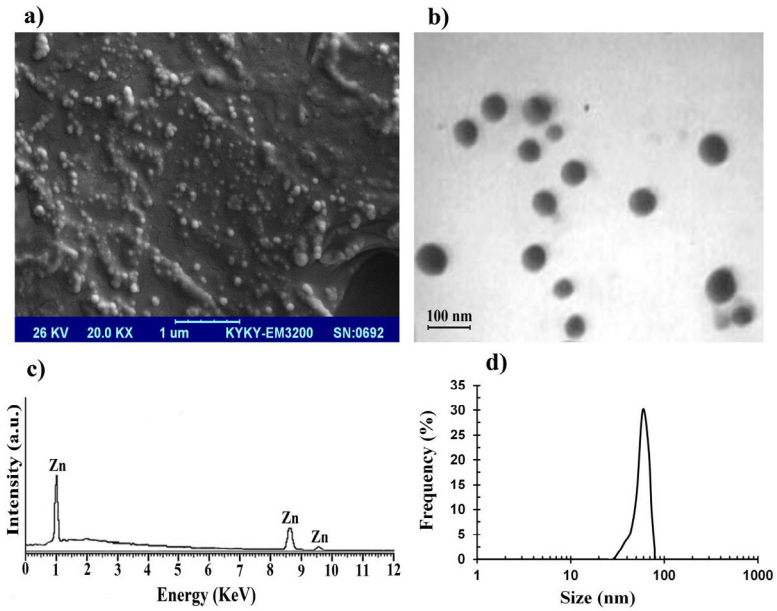

Fig 1. a) Scanning Electron Micrograph; b) Transmission Electron Image; c) Energy Dispersive X-ray Profile; d) Particle Size Distribution Pattern of Microwave-assisted Biosynthesized Zn NPs using L. vera Leaf Extract

variance (ANOVA).

RESULTS

Synthesis and characterization of the Zn NPs

The scanning electron microscope (SEM) image of the synthesized Zn NPs showed the dispersion of the nanostructures with some aggregation (Fig 1-a), and the TEM image indicated spherical nanostructures (Fig 1-b). The elemental microanalysis of the Zn NPs using the EDX method revealed zinc absorption bands (ZnLα1, ZnKα1, and ZnKβ1 at 1.01, 8.64, and 9.57 keV, respectively) (Fig 1-c). Such signals (weight percentage: 100%) without another atom signal confirmed the formation of zinc nanostructures (Fig 1-c).

Fig 1-d depicts the corresponding size distribution pattern of the Zn NPs as measured by laser light scattering, and one modal peak was evident within the range of 30-80 nanometers, and the NPs with the size of 58 nanometers had the highest frequency.

Antimicrobial activity of the Zn NPs

To measure the MIC of the Zn NPs and $ZnSO_4$ against the clinical pathogenic isolates, the serial dilution approach was applied. The measured MIC of the Zn NPs and $ZnSO_4$ on the *S. aureus*, *P. mirabilis*, and *P. aeruginosa* isolates were higher than 2560 µg/ml. Moreover, in case of ciprofloxacin, the obtained MIC was estimated at 1, 0.125, and 0.5 µg/ml for *S. aureus*, *P. mirabilis*, and *P. aeruginosa*, respectively.

The antibacterial effects of various antibiotics alone or along with a sub-inhibitory concentration of the Zn NPs (500 µg/disk) or $ZnSO_4$ (500 µg/disk) on the MRSA were evaluated using the disk-diffusion method (Fig 2).

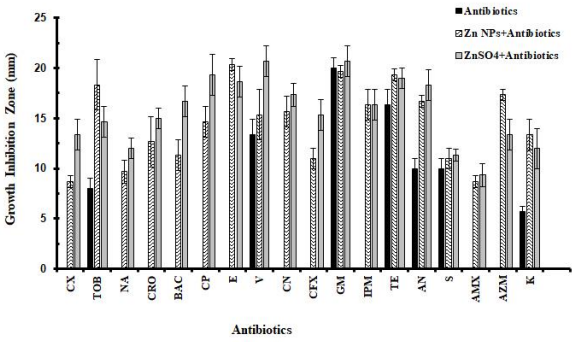

Fig 2. Inhibitory Effects of Various Antibiotics Alone and Combined with Zn NPs and ZnSO4 against MRSA (TOB [Tobramycin; 10 µg/disk], NA [nalidixic acid; 30 µg/disk], CRO [ceftriaxone; 30 µg/disk], CP [ciprofloxacin; 5 µg/disk], BAC [bacitracin; 0.04 unit/disk], V [vancomycin; 30 µg/disk], CN [cephalexin; 30 µg/disk], CFM [cefixime; 5 µg/disk], GM [gentamicin; 10 µg/disk], TE [tetracycline; 30 µg/disk], AN [amikacin; 30 µg/disk], S [streptomycin; 10 µg/disk], AMX [amoxicillin; 25 µg/disk], CX [cloxacillin; 5 µg/disk], E [erythromycin; 15 µg/disk], ME [methicillin; 5 µg/disk], IPM [imipenem; 10 µg/disk], AZM [azithromycin; 15 µg/disk], K [kanamycin; 30 µg/disk])

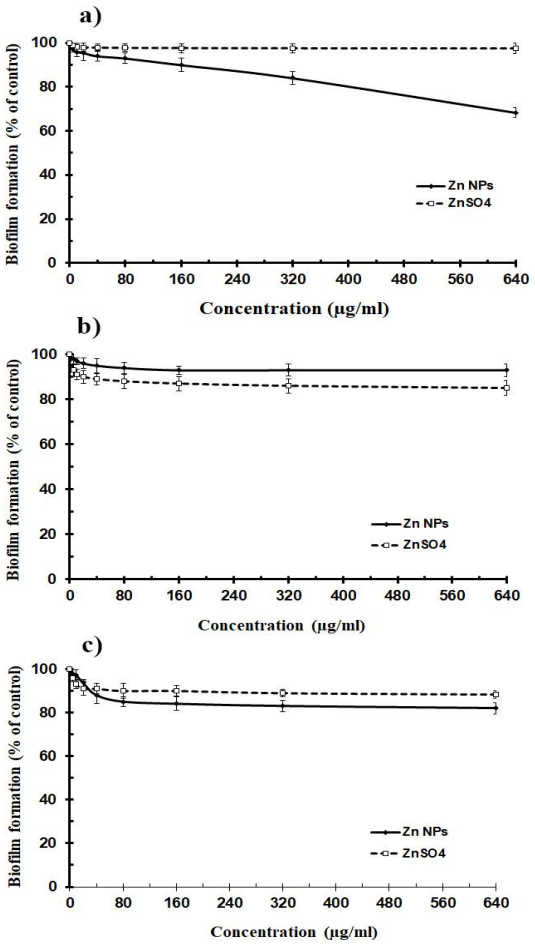

Fig 3. Inhibitory Effects of Zn NPs and ZnSO4 on Biofilm Formation by a) S. aureus, b) P. aeruginosa and c) P. mirabilis (Wells containing the bacteria without Zn NPs and ZnSO4 were considered as control; results expressed as mean±SD; n=3)

The disks containing 500 µg/disk of the Zn NPs or $ZnSO_4$ showed no inhibition zones on the MRSA. In case of the antibiotic disks, only streptomycin, tobramycin, tetracycline, gentamicin, vancomycin, amikacin, and kanamycin could inhibit the growth of the tested MRSA (Fig 2).

Effects of the Zn NPs and $ZnSO_4$ on biofilm formation

Fig 3 shows the inhibitory effects of the Zn NPs and $ZnSO_4$ on the biofilm formation of *P. aeruginosa*, *S. aureus*, and *P. mirabilis*. Biofilm formation by *S. aureus* decreased with the increased Zn NPs and $ZnSO_4$ to 640 µg/ml, reaching 68.3±2.1% and 97.4±2.3%, respectively (Fig 3-a). In case of *P. aeruginosa*, the rate of biofilm formation decreased to 93±2.8% and 85±3.3% in the presence of the Zn NPs and $ZnSO_4$ (0-640 µg/ml), respectively (Fig 3-b). In addition, the rate of biofilm formation in *P. mirabilis* reduced to 82±2.6% and 88.3±1.7% in the presence of 640 µg/ml of the Zn NPs and $ZnSO_4$, respectively and remained constant at higher concentrations than 80 µg/ml (Fig 3-c). Fig 4-a and Fig 4-b show the comparison of biofilm formation by *S. aureus*, *P. aeruginosa*, and *P. mirabilis* treated with the Zn NPs and $ZnSO_4$, respectively.

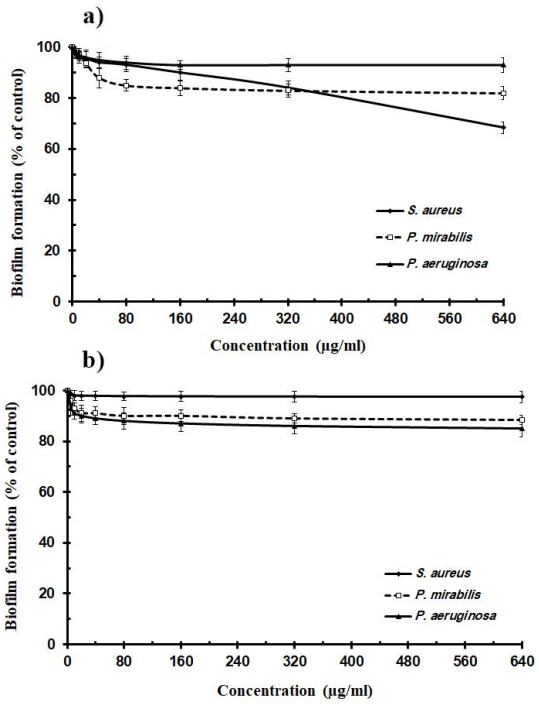

Fig 4. Biofilm Formation by S. aureus, P. aeruginosa, and P. mirabilis Treated with Various Concentrations (5-640 µg/ml) of a) Zn NPs and (b) ZnSO4 (Wells containing the isolates without Zn NPs and ZnSO4 considered as control; results expressed as mean±SD in three replicated experiments)

DISCUSSION

Today, the biomedical application of nanostructures for therapeutic and preventive purposes has attracted the attention of researchers [19, 29, 30]. In the present study, the antimicrobial activity of the produced Zn NPs (30-80 nm) and $ZnSO_4$ in the clinical isolates of *P. aeruginosa*, *S. aureus*, and *P. mirabilis* had no significant difference, and the measured MICs were higher than 2,560 µg/ml. As for the other nanoparticles, the toxic activity of tellurium nanorods on *S. aureus*, *P. mirabilis*, and *P. aeruginosa* has been reported to be lower compared to potassium tellurite [31]. These nanoparticles have shown low cytotoxicity on the HepG2, A549, MCF-7, and

HT1080 cell lines [32]. In contrast, Nazari et al. [33] reported the MICs of bismuth NPs (<5 nm) against various clinical isolates of *H. pylori* to be within the range of 60-100 µg/ml. Furthermore, at lower concentrations than 280 µg/ml of bismuth subnitrate, no antibacterial effects were reported in the mentioned study.

Several pathways have previously been reported to be the possible mechanisms for the antibacterial effects of ZnO NPs, including ROS formation (hydrogen peroxide, hydroxyl radicals, and peroxide), Zn^{2+} ion release, internalization of nanoparticles into bacteria, and electrostatic interactions [34]. Furthermore, He et al. [35] have claimed that ZnO NPs exhibited fungicidal activities against *Botrytis cinerea* and *Penicillium expansum* through the deformation of fungal hyphae and preventing the development of conidiophores and conidia, respectively. The current research aimed to assess the antibacterial and anti-biofilm effects of microwave-assisted synthesized Zn NPs. The disks containing only Zn NPs (500 µg/disk) showed no growth inhibition zones for the tested MRSA (Fig 2). It is notable that the exhibition of antimicrobial effects often requires the diffusion of the tested material through the culture media, while Zn NPs have low solubility for the diffusion and exhibition of antimicrobial outcomes in the disk-diffusion assay. However, the antibacterial mechanism of the Zn NPs remained unclear, and further investigation is required in this regard.

One of the approaches to overcoming antibiotic resistance as a major clinical and public health problem is the combined use of antibiotics with other macro or nanostructures [19]. Previous studies have denoted that the antimicrobial effects of penicillin G, amoxicillin, erythromycin, clindamycin, and vancomycin increase with the combined use of these agents with silver nanoparticles (22.5 nm) against *E. coli* and *S. aureus* [36]. Moreover, the addition of zinc-containing compounds (e.g., Zn acetate) to erythromycin has been reported to enhance the antibacterial effects against *Propionibacterium* spp., especially erythromycin-resistant strains [37]. On the other hand, chemically synthesized ZnO NPs (20-45 nm) have been reported to improve the inhibitory effects of ciprofloxacin against *S. aureus* and *E. coli* by 27% and 22%, respectively [38].

In the present study, the growth inhibition zone of the tested MRSA was observed in the presence of gentamicin, vancomycin, kanamycin, tobramycin, amikacin, tetracycline, and streptomycin (Fig 2). Furthermore, the combined antibacterial effects of $ZnSO_4$ with cloxacillin, nalidixic acid, ceftriaxone, bacitracin, ciprofloxacin, cefixime, and amoxicillin against the MRSA were more significant compared to the Zn NPs ($P<0.05$). On the other hand, an insignificant increase was observed in the growth inhibition zone diameter when the antibiotic disks of tobramycin, erythromycin, tetracycline, azithromycin, and kanamycin were combined with the Zn NPs compared to $ZnSO_4$ ($P>0.05$) (Fig 2).

According to the findings of the current research, the tested MRSA was resistant to cloxacillin, nalidixic acid, ciprofloxacin, bacitracin, ceftriaxone, erythromycin, cephalexin, cefixime, imipenem, amoxicillin, and azithromycin. Interestingly, the antibacterial effects significantly increased with the addition of the Zn NPs or $ZnSO_4$ to the mentioned antibiotic disks ($P<0.05$), which showed no inhibitory effects on the MRSA alone. Furthermore, the presence of the Zn NPs or $ZnSO_4$ had no significant effects on the antibacterial activity of gentamicin and tetracycline on the tested MRSA ($P>0.05$).

According to Thati et al. [39], ZnO NPs potentiate the antibacterial activities of beta lactams, cephalosporins, aminoglycosides, glycopeptides, macrolides, and lincosamides against MRSA, among which the highest increase belonged to penicillin G and amikacin. Interestingly, the produced Zn NPs in the present study enhanced the effects of antibiotics that interfered with the cell wall and peptidoglycan synthesis (cloxacillin, bacitracin, ceftriaxone, cephalexin, cefixime, imipenem, and amoxicillin), while showing no inhibitory effects on the MRSA alone. Furthermore, the Zn NPs significantly increased the growth inhibition zone of azithromycin and erythromycin (protein synthesis inhibitors), as well as nalidixic acid and ciprofloxacin (DNA gyrase inhibitors), against MRSA ($P<0.05$).

Bacterial biofilms could increase antibiotic resistance by preventing the antibiotic penetration within the biosynthesized matrix [40]. This effect of biofilms places them among the most important concerns in therapeutic medicine, urging the discovery of novel strategies, such as the use of specific nanostructures [26]. For instance, biofilm formation by *E. coli* and *K. pneumonia* has been reported to reduce in the presence of silver nanoparticles at the concentration of 50 µg/ml [41]. Furthermore, Hernandez-Delgadillo et al.

[42] have reported that chemically synthesized bismuth NPs (3.3 nm) could completely prevent the biofilm formation by *S. mutans*. However, literature review showed no reports on the anti-biofilm effects of biogenic Zn NPs.

According to the results of the present study, the effects of the Zn NPs on the biofilm formation by *S. aureus* and *P. mirabilis* were more significant compared to $ZnSO_4$ at higher concentrations than 160 µg/ml ($P<0.05$) (Figs 3-a & 3-c). In this regard, Lechevallier et al. [43] stated that zinc sulfate (3 mg/ml) could inhibit the biofilm of *K. pneumoniae*, resulting in 90% reduction in the viable counts. However, the anti-biofilm effects of $ZnSO_4$ (as the source of Zn^{2+} ions) on *P. aeruginosa* were reported to be more significant compared to the zero-valent Zn NPs at higher concentrations than 160 µg/ml ($P<0.05$) (Fig 3-b). Similar effects have been denoted in selenium dioxide (as the source of Se^{4+} ions) on the biofilm formation by *S. aureus*, *P. aeruginosa*, and *P. mirabilis*, which are considered to be more significant than the zero-valent Se NPs ($P<0.05$) [24].

In the present study, the inhibitory effect of biogenic Zn NPs at sub-MIC concentration (80-640 µg/ml) on the biofilm formation by *P. mirabilis* was more significant compared to *P. aeruginosa* ($P<0.05$) (Fig 4-a). In addition, the biofilm formation by *S. aureus* reduced to 68.3±2.1% in the presence of the Zn NPs (640 µg/ml), which significantly decreased compared to *P. mirabilis* and *P. aeruginosa* at the same concentration ($P<0.05$). The anti-biofilm effects of $ZnSO_4$ on *P. aeruginosa* and *P. mirabilis* were also more significant compared to *S. aureus* ($P<0.05$) (Fig 4-b). On the other hand, the effects of $ZnSO_4$ on the biofilm formation by *P. aeruginosa* were more considerable compared to *P. mirabilis*, while the effects were not considered significant ($P>0.05$) (Fig 4-b).

Dental composites modified by ZnO NPs (10% w/w) have been reported to decrease the biofilm formation by *S. sobrinus* by 80%, which is a statistically significant suppression of biofilm growth compared to unmodified composites [44]. In contrast, Salem et al. [45] observed that ZnO nanostructures (90-100 nm) enhanced the biofilm formation by *Vibrio cholera*, suggesting that ZnO NPs inhibited the adenylyl cyclase enzyme, thereby leading to the reduced production of cAMP, which is a depression factor for the biofilm formation by *V. cholerae* [45]. Seemingly, the chemical composition of zinc-containing compounds and type of the treated pathogens play a key role in the inhibition of biofilm formation.

CONCLUSION

The results of the disk-diffusion method strongly demonstrated that the simultaneous application of Zn NPs and $ZnSO_4$ with various antimicrobial agents could enhance the antimicrobial activity of the tested antibiotics against MRSA. This observation is considered of great importance in case of antibiotics such as nalidixic acid, ceftriaxone, ciprofloxacin, bacitracin, cephalexin, amoxicillin, cloxacillin, erythromycin, imipenem, and azithromycin, which showed no inhibitory effects when used alone. According to the results, the biofilm inhibitory activity of Zn NPs at higher concentrations than 160 µg/ml against *S. aureus* and *P. mirabilis* was more significant compared to the inhibitory effects of $ZnSO_4$. With respect to the observed antibacterial and anti-biofilm activities, further investigations are required to clarify the action mechanism of biologically produced Zn NPs.

ACKNOWLEDGMENTS

This research project was supported by Young Researcher Grant (award number: 972327) from the National Institute for Medical Research Development (NIMAD) in Tehran, Iran. Hereby, we extend our gratitude to the Deputy of Research at Kerman University of Medical Sciences in Kerman, Iran for the financial support of this study. We would also like to thank the Herbal and Traditional Medicine Research Center at Kerman University of Medical Sciences and Iranian Nanotechnology Initiative Council for assisting us in this research project.

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