ORIGINAL ARTICLE

Antimicrobial Performance of Zinc Oxide Nanoparticles with Green Synthesis Against Gram-Positive and Gram-Negative Bacteria

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ABSTRACT

Background: Increasing use of antibiotics in the treatment of bacterial infections has increased resistance against them. The development of new antimicrobial drugs cannot overcome the continuous and rapid spread of this resistance.
Aim: To determine the antimicrobial efficacy of green zinc oxide nanoparticles against gram-positive and gram-negative bacteria.
Methods: In this study, *Escherichia coli* (ATCC25922), *Staphylococcus aureus* (ATCC25923), *Pseudomonas aeruginosa* (ATCC27853) were tested as microorganisms. High purity zinc oxide nanoparticles were prepared by green and environmentally friendly method. In order to identify the target bacteria, biochemical tests were performed for gram-negative bacteria and specific tests were performed for gram-positive bacteria. Then, the antimicrobial properties of zinc oxide nanoparticles were investigated by MIC method, disk diffusion (measuring the diameter of growth inhibition zone) and exposure method.
Results: The results of MIC showed that zinc oxide nanoparticles with green synthesis method have antimicrobial effect against *Escherichia coli*, *Staphylococcus aureus* and also the antibacterial activity of zinc oxide nanoparticles increases with increasing concentration and decreasing nanoparticle size. The results of nanoparticle impregnated disks showed that Escherichia coli was more susceptible than Staphylococcus aureus. The results of exposure showed that after counting the bacterial colonies, the number of *Escherichia coli* and *Staphylococcus aureus* colonies decreased and this was a confirmation of the antimicrobial properties of zinc oxide nanoparticles.
Conclusion: Today, researchers believe that zinc oxide nanoparticles can be used as an alternative to antibiotics and even in synergism with antibiotics to treat bacterial infections.
Keywords: Zinc oxide nanoparticles with green synthesis, *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa*

INTRODUCTION

Along with the rapid development of human life, the control of harmful microorganisms is inevitable. A wide range of microorganisms are in balance with the human living environment, but their rapid and uncontrolled growth can lead to serious problems. Nosocomial infections are one of the many problems around the world and controlling the spread of these infections, especially in hospitals, is a serious challenge[(1)].Numerous antibiotics have been used to inhibit the growth and destruction of microbes. However, the development of resistance and the emergence of side effects have severely limited the use of these agents. However, nanoscale biological compounds have unique physical and chemical properties that the performance of several categories of nanocarriers in recent years and antimicrobial nanoparticles have been proven in the treatment of infectious diseases. Also, the use of nanoparticles as markers in molecular detection instead of current markers has increased the sensitivity of selectivity and multi-dimensional detection capacity[(2, 3)].

Some metal nanoparticles have inherent antimicrobial activity. These particles are used to control infectious diseases. Microbial pathogens are not able to develop resistance to these particles. Antimicrobial nanoparticles (NPs) offer many advantages over conventional antibiotics in reducing drug side effects, resistance, and treatment costs[(4)]. Drug nanocarriers also have a significant effect on improving the pharmacokinetics of drugs and reducing side effects. Theoretically, nanocarriers are kept longer in the body than antibiotic molecules, which is useful for long-term therapeutic effects. Today, nanotechnology is growing in all areas of immunization, drug design, drug delivery, diagnosis and control of infectious diseases [(5)].

Some ceramic oxides, calcium oxide, magnesium oxide, as well as many nanoparticle oxides such as zinc oxide and copper nanoparticles have shown significant antimicrobial activity. The antimicrobial properties of nanoparticles have attracted the attention of researchers and industry owners. Metal nanoxide is very active and has antibacterial activity against gram-positive and negative bacteria[(6-10)] .According to a study by Atmaca et al. (1998) Zinc oxide and zinc acetate have antimicrobial activity against *Staphylococcus aureus*,*Staphylococcus epidermis* and *Pseudomonas aeruginosa* in Mollerhinton broth.The effect of zinc oxide on *Staphylococcus aureus* and *epidermis* was greater than *Pseudomonas aeruginosa*[(11)]. Also,Xie et al. (2011) concluded that the MIC for *Campylobacter jejuni* varies from 0.025 to 0.05 mg/ml and zinc oxide nanoparticles at this concentration have bactericidal activity for *Campylobacter jejuni* and are not purely bactrostatic[(12)]. In another study, Sinha et al. (2012) investigated the toxicity of two common nanoparticles, silver and zinc oxide on mesophilic and halophilic bacteria.The results showed that the toxicity of nanoparticles was more effective on gram-negative bacteria. Zinc nanooxide reduced the growth rate of *Enterobacter strains* by 50% and the growth rate of

halophilic bacteria by 80%[1]. The reason for the greater effect on halophilic bacteria is due to the greater amount of negative charge on the cell surface.The small effect of these nanoparticles on gram-positive bacteria has been reported due to the presence of a thicker layer of peptidoglycan in these bacteria.

Today, nanoparticles are produced by various chemical methods that have disadvantages such as instability of the solution, non-uniform particle size, impurity of nanoparticles, low efficiency and require advanced equipment for production[13]. Therefore, researchers turned to nanoparticle production biological systems that have minimal environmental hazards and simple and biocompatible production methods.In recent years, a large number of living organisms such as bacteria, fungi, algae, plants, plant extracts and their metabolites have been mediated for the synthesis of nanoparticles, but the identification of plant systems as potential natural nanoparticles has made it very attractive for nanoparticle biosynthesis.

In the synthesis of green nanoparticles, harmful chemical compounds and solvents that used in the chemical method replaced with natural compounds and biological agents in plant extracts such as enzymes, carbohydrates and trinoids. Therefore, the synthesis of nanoparticles using natural resources leads to a reduction in the synthesis process and a reduction in the use of energy and chemical solvents that are destructive to the environment. Therefore, in line with the objectives of green chemistry, the synthesis of zinc oxide nanoparticles was performed using coffee powder extract as a biological resource without the use of reducing chemical agents.

MATERIAL AND METHODS

This experimental study was performed during two stages including preparation of zinc oxide nanoparticle suspension and performing tests to determine the antimicrobial potential of nanoparticles.

Preparation of zinc oxide nanoparticles: Zinc oxide nanoparticles were in the size of 20 nm and in powder form and purity was over 99%. The physical and structural properties of the nanoparticles have been examined by SEM and TEM. Zinc oxide nanoparticles are used to produce zinc nanoparticles using coffee powder extract. At first, 10 g of coffee powder in 100 ml of distilled water is prepared using a magnetic stirrer and a 4500 rpm centrifuge.Next, Whatman Supernatant filter paper and aqueous zinc acetate solution is used as a precursor. After that, white powder is obtained by using different degrees of heat.Finally, the size and shape of particles is determined by using X-ray diffraction electron microscopy.

At first, to prepare different concentrations of zinc oxide nanoparticles, four sterile test tubes were considered and 0.9 cc of distilled water with sterile pipette was added to each of them.Then, 0.1 g of zinc oxide nanoparticles were weighed with a digital scale and added to the first test tube and completely homogenized with a vortex for 5 minutes. Next, with a sterile sampler, 0.1 ml was picked up from the first tube and added to the second tube, and again it goes from the second test tube to the third test tube and to the end. Finally, all test tubes were homogenized with Vertex device.

Bacterial samples: Microorganisms used for antimicrobial testing included*S. aureus* standard strain (ATcc25923) of gram-positive type. Also, *E. coli* standard strain ATcc25922) and *P.aeruginosa* standard strain ATcc27853) were selected from gram-negative type and these bacteria were obtained from Mahmoudieh laboratory. Because the disc had to be cultured for 24 or 48 hours, the bacteria were first prepared on BHI agar medium and cultured on an 8 cm plate and incubated. Then, to prepare the bacterial suspension, three long tubes were selected and 3 cc of distilled water was poured into each tube and sterilized by autoclave. Then, a colony was separated by using a sterile loop from each bacteriumthat had been cultured for 24 hours and dissolved in each tube next to the flame and placed on the vortex. Finally, the turbidity was compared with a half-McFarland tube.

Disk diffusion test: At first, the suspension prepared from each bacterium was densely cultured with a sterile swab on a Müller-Hinton agar culture. Then, each disk was fixed with a sterile forceps at certain intervals with a low flame and pressure on the culture medium.Finally, the plate was closed and incubated at 37°C for 24 hours.

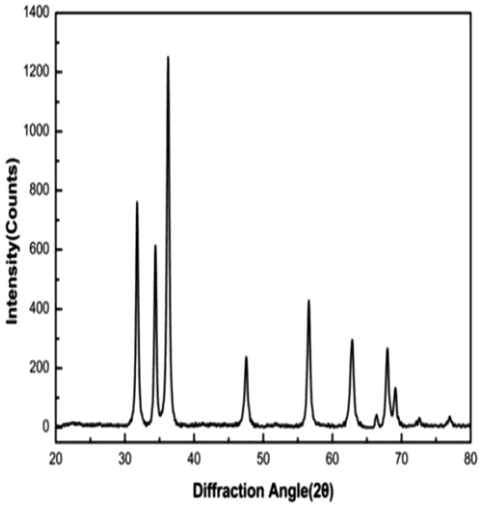
Antibacterial test by MIC method: At first, different concentrations of zinc oxide nanoparticles are prepared and each test tube is numbered from different dilutions. Then, 0.1 cc of the first tube containing zinc nanoxide and 0.9 cc of distilled water is added to the first tube containing the nutrient broth medium and it is mixed by vortex.Next, 0.1 cc of tube number 1 transferedto the tube number 2 by a sterilized pipette in the fore and next to the flame and mix again and thus it is done in this way until the last tube. Then 0.1 cc is picked up from the last tube and discarded. After that, 0.1 cc of the microorganism suspension was added to the tubes containing the liquid culture medium and zinc oxide nanoparticles. Finally, the test tubes were placed on a vortex to mix the culture medium with a solution of oxidized nanoparticles and bacteria, then the tubes were incubated for 24 hours at 37 °C. The lowest concentration at which no turbiditywas observed, it was considered as the minimum inhibitory concentration. Then, 100 µl of each of the test tubes containing the bacteria is picked up and transferred to the Müller Hinton Agar plate, then the plates are heated at 37 °C for 24 hours. After this period, the plates are examined for the growth of microorganisms on them, the lowest concentration at which no growth was observed, it was considered as the minimum lethal concentration (MBC).

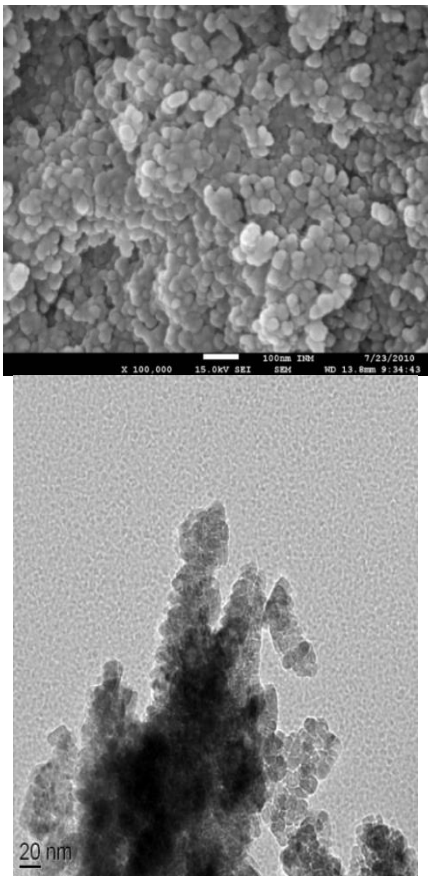
RESULTS

Zinc oxide nanoparticle results: X-ray diffraction has been used to survey the crystal structure of zinc oxide nanoparticles. Figure 1 shows the XRD image of a zinc oxide nanoparticle. Also, figure 2 shows the SEM image of the zinc oxide nanoparticle that used in this study. SEM and TEM electron microscopy on the zinc oxide nanoparticles showed that the zinc oxide particles have a diameter of 20 nm and also the nanoparticles have a purity of over 99%.

The results of Disk diffusion: After culturing *E. coli* in Müller-Hinton agar medium and placing antibiotic disks including imipenem, norfloxacin, ciprofloxacin, in addition, a blank disk impregnated with zinc oxide nanoparticles was used. After incubating the diameter of the growth inhibition zone for each disk was measured in millimeters. The results of Table 1 showed that antibiotic ciprofloxacin had the largest diameter of growth inhibition zone in *E. coli* culture. Also, the lowest diameter of growth zone was related to zinc oxide nanoparticles. The results of the antimicrobial effect of zinc oxide nanoparticles on *E. coli* showed that this bacterium was sensitive to the antibiotics and zinc oxide nanoparticles.Also, the results of Table 1 showed that this bacterium is most sensitive to ciprofloxacin antibiotic. Norfloxacin and imipenem antibiotics can both be equally effective against *E. coli*.The highest mean diameter of the growth inhibition zone was 37.5 mm that related to ciprofloxacin antibiotic.

After culturing *S. aureus* and placing antibiotic disks including imipenem, norfloxacin, ciprofloxacin and blank disk impregnated with zinc oxide nanoparticles, the diameter of the growth inhibition zone for each disk was measured in millimeters and its results are given in Table 2. The results show that in *S. aureus* culture, the ciprofloxacin antibiotic has the highest diameter of growth inhibition zone and the lowest diameter of growth aura is related to zinc oxide nanoparticles.The results showed that the diameter of the growth inhibition zone for zinc oxide nanoparticles was 14 mm in *E. coli*, but 12 mm in *S. aureus*. Therefore, it can be concluded that the antimicrobial effect of zinc oxide nanoparticles in *E. coli* is greater than *S. aureus*.





(a)(b)(c)

Figure 1. X-Ray image of zinc oxide nanoparticles (a), SEM electron microscope image of zinc oxide nanoparticles (b), TEM electron microscope image of zinc oxide nanoparticles (c).

The results of blank disks impregnated with various concentrations of nanoparticles: According to Table 3, the results of antimicrobial effect of zinc oxide nanoparticles in different concentrations on *E. coli* show that with decreasing concentration of zinc oxide nanoparticles, the diameter of the growth inhibition zone has decreasedand at a concentration of 10^{-4}, the antimicrobial effect of oxide nanoparticles had a significant reduction. Figure 3 also shows the growth status of *E. coli*.

The results of antimicrobial effect of zinc oxide nanoparticles on *Pseudomonas aeruginosa* showed that *Pseudomonas aeruginosa* was resistant to antibiotics and zinc oxide nanoparticles and no growth was observed at any phase

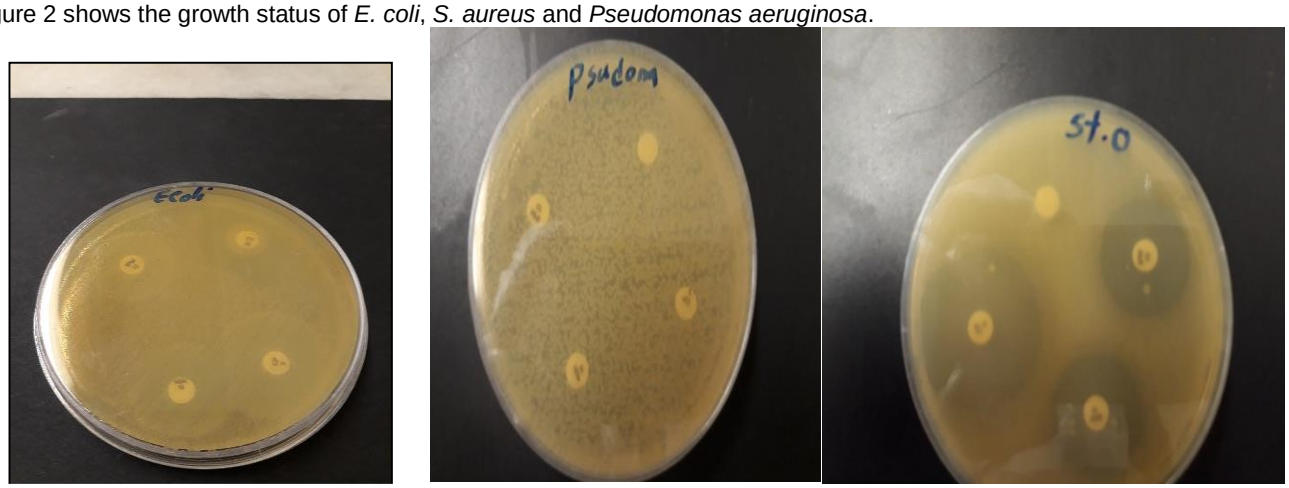
Table 1. The results of diameter of the growth inhibition zone in *E. coli.*

Antibiotics	The first order of inhibition zone diameter (mm)	The second order of inhibition zone diameter (mm)	The third order of inhibition zone diameter (mm)	Mean	SD
IPm10	30	27.5	25	27.5	2
CP5	38	37.5	37	37.5	0.4
NOR	30	27.5	25	27.5	2
Nano Zno	14	13	12	13	0.8

Table 2. The results of diameter of the growth inhibition zone in *S. aureus.*

Antibiotics	The first order of inhibition zone diameter (mm)	The second order of inhibition zone diameter (mm)	The third order of inhibition zone diameter (mm)	Mean	SD
IPm10	26	25.5	25	25.5	0.4
NOR	25	25	25	25	0
CP5	35	34.5	34	34.5	0.4
Nano Zno	12	12.5	13	12.5	0.4

.Figure 2 shows the growth status of *E. coli*, *S. aureus* and *Pseudomonas aeruginosa*.



(a) (b)(c)

Figure 2. Results of disk diffusion test on bacteria in Cp, Imp, NOR, Nano ZNO disks. *S. aureus*(a),*Pseudomonas aeruginosa* (b), *E. coli* (c).

Table 3. The effect of different concentrations of nanoparticles on *E. coli* by disk method

Blank disks impregnated with zinc oxide	The first order of inhibition zone diameter (mm)	The second order of inhibition zone diameter (mm)	The third order of inhibition zone diameter (mm)	Mean	SD
10^{-1} concentration	20	17.5	15	17.5	2
10^{-2} concentration	10	10	10	10	0
10^{-3} concentration	9	8.5	7	8.2	0.8
10^{-4} concentration	1	1	1	1	0

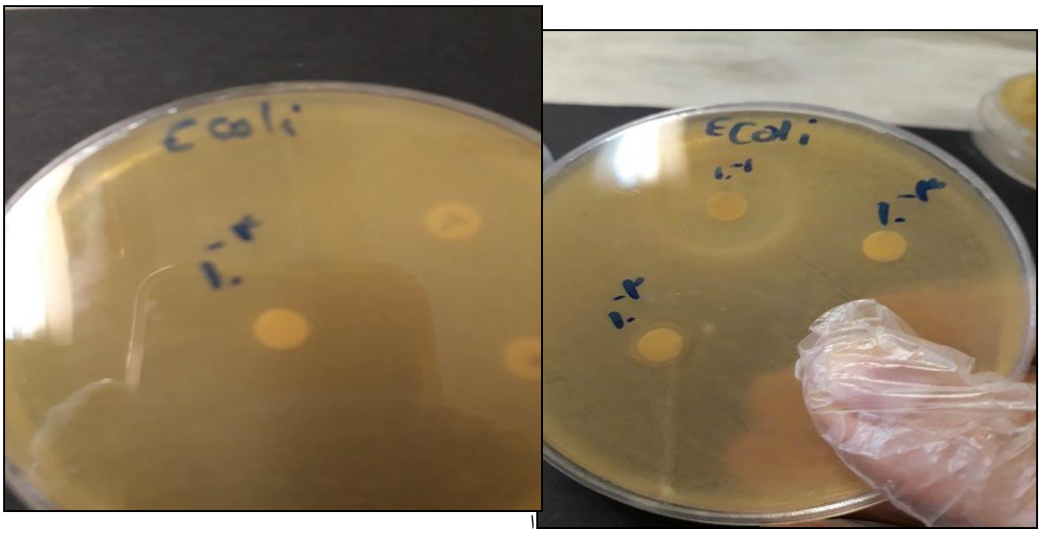


Figure 3. Results of impregnating blank disks with different concentrations of nanoparticles in *E.coli.*

After culturing *S. aureus* and inserting zinc oxide nanoparticle disks, the diameter of the growth inhibition zone was measured and reported in Table 4. According to the results of the table, it can be concluded that the blank disk impregnated with nanoparticles in high concentration has retained its antimicrobial properties, but as the concentration decreases, this

antimicrobial property decreases, so that this antimicrobial property is removed at a concentration of 10^{-4}. Also, the results of*E. coli* bacteria showed that zinc oxide nanoparticles at a concentration of 10^{-4} have not lost their antimicrobial properties, so it can be said that *E. coli* bacteria are more sensitive to zinc oxide nanoparticles.

Table 4. The results of the effect of different concentrations of nanoparticles in bacteria s.

Blank disks impregnated with zinc oxide	The first order of inhibition zone diameter (mm)	The second order of inhibition zone diameter (mm)	The third order of inhibition zone diameter (mm)	Mean	SD
10^{-1} concentration	11	11.5	12	11.5	0.4
10^{-2} concentration	10	10	10	10	0
10^{-3} concentration	9	8.5	8	8.5	0.4
10^{-4} concentration	0	0	0	0	0

Finally, the results of antimicrobial effect of oxide nanoparticles with different concentrations on *P. aeruginosa* showed that this bacterium was resistant to different concentrations of zinc oxide nanoparticles and no growth was observed.Figure 4 also shows the growth status of *S. aureus* and *P. aeruginosa*.

(a)(b)

Figure 4. Results of impregnating blank disks with different concentrations of nanoparticles. *S. aureus*(a), *P. aeruginosa*(b).

Exposure results: Different dilutions were prepared from *E. coli*, *S. aureus* and *P. aeruginosa* and the antibacterial effect of zinc oxide nanoparticles against these bacteria was investigated in Table 5.

Table 5. Results of antimicrobial effect of zinc oxide nanoparticles in the exposure method.

Bacteria	Number of control colonies	Number of colonies in the presence of zinc oxide nanoparticles
E. coli	300	No colonies were observed
P. aeruginosa	uncountable	uncountable
S. aureus	300	4

DISCUSSION

In the present study, coffee powder extract was used to prepare zinc oxide nanoparticles and the sample was identified by TEM and SEM electron microscopy. Then, the effect of these zinc oxide nanoparticles on gram-positive and gram-negative bacteria was investigated.The use of plant extracts has been one of the cleanest and most biocompatible methods used for large-scale production.The components in coffee powder can act as pharmaceutical agents for the rapid biosynthesis of formed metal nanoparticles.

The results of the antimicrobial effect of zinc oxide nanoparticles on *E. coli* were investigated and the results showed that this bacterium was sensitive to imipenem, neurofluxacin and ciprofloxacin antibiotics and the largest diameter of growth inhibition zone is related to ciprofloxacin which was 38 mm.The diameter of the non-growth halo in the blank disk impregnated with the concentration of zinc oxide nanoparticles in *E .coli* was 14 mm, while in *S. aureus* the blank disk contained nanoparticles equal to 12 mm. In fact, it can be concluded that the antimicrobial effect of zinc oxide nanoparticles against *E. coli* was greater than *S. aureus.*

Sinha et al. (2011) studied the antimicrobial effects of zinc oxide nanoparticles.The results of that study showed that the gram-negative species of *Enterobacteriaceae* were more sensitive than the gram-positive bacterium *Bacillus subtilis*. The cause of resistance of gram-positive bacteria has been related to the presence of a thick layer of peptidoglycan[15,16]. The findings of the present study are consistent with previous research.

The results of our study showed that *P. aeruginosa* resistant to the antibiotics ciprofloxacin, imipenem and neurofluxacin and even showed resistance to blank disks impregnated with zinc oxide nanoparticles that indicate an increasing trend in the antibiotic resistance of P. aeruginosa.Rajaei et al. (2015) investigated that the

isolates of P. aeruginosa resistance to ciprofloxacin, amikacin, ceftriaxone, cefotaxime and imipenemantibiotics[16,17]. Widespread resistance of P. aeruginosa to cephalosporins (ciprofloxacin, ceftriaxone, cefotaxime and zoxime stiffness) and imipenem leads to the further spread of these resistant strains. Therefore, in the present study, most attention was paid to finding a suitable solution to combat this bacterium. Saadat et al. (2012) by evaluating and comparing the antibacterial activity of zinc oxide nanoparticles and ethylene diamine tetraacetic acid on *P. aeruginosa* showed that zinc oxide nanoparticles in comparison with ethylene diamine tetraacetic acid have desirable antibacterial properties against *P.aeruginosa*[18.]

According to the results obtained from blank disks impregnated with different concentrations of zinc nanooxide, it was observed that in *E. coli*, with increasing concentration of zinc oxide nanoparticles, the diameter of the growth inhibition zone in *E. coli* has increased.So that at the highest concentration of the diameter of growth inhibition zone was20 mm in *E. coli*.On the other hand, it is the same in *S. aureus*, but the diameter of the non-growth halo in this bacterium was 11 mm, but in *P. aeruginosa*, despite the blank disks impregnated with different concentrations of nanoparticles, the diameter of the growth halo diameter was zero and resistance was observed. Hosseinzadeh et al. (2012) investigated the antimicrobial effect of zinc oxide nanoparticles against different bacterial strains. The results showed that zinc oxide nanoparticles were effective against *E. coli*, *S. aureus* and *S. epidermidis*, respectively.Also, this study showed that with increasing the concentration of zinc oxide nanoparticles on bacteria, the diameter of the growth inhibition zone increased[(1)]. Therefore, it can be said that the previous research is in line with the subject of our study.

The results of antimicrobial effect on *P. aeruginosa* showed that this bacterium showed resistance to different concentrations of zinc oxide nanoparticles by green synthesis method.Jafari et al. (2011) studied the antibacterial properties of chemically synthesized zinc oxide and silver nanoparticles in a combined and alone on *Pseudomonas aeruginosa, Bacillus subtilis, Salmonella gallinarum, Escherichia coli, Staphylococcus aureus*.The results showed that *Escherichia coli, Salmonella gallinarum*and *Pseudomonas aeruginosa* were more sensitive to the combination of two nanoparticles than *Staphylococcus aureus* and Bacillus subtilis strains and the combination of zinc and silver nanoparticles also increased bactericidal activity[(14)].Saadat et al. (2012) investigated the antimicrobial activity of chemically synthesized zinc oxide nanoparticles in comparison with the antibiotic imipenem against *P. aeruginosa*. The results showed that the zinc oxide nanoparticles inhibited the growth of *P.aeruginosa*([15)].However, the results of this study were not consistent with our study and this is due to the synthesis of zinc oxide nanoparticles by the green method in which *P. aeruginosa* has shown resistance to zinc oxide nanoparticles. But, in the synthesis of zinc oxide nanoparticles by chemical method, it has been shown that this zinc oxide nanoparticle was sensitive against *P. aeruginosa*.

The results of the exposure method showed that zinc oxide nanoparticles produced by green synthesis method can have the greatest antimicrobial effect against *E. coli* and these zinc oxide nanoparticles were more sensitive to *E. coli* and less sensitive to *S. aureus*, and *P. aeruginosa* was also resistant to this nanoparticle.*E. coli* as gram-negative bacteria have a lipopolysaccharide layer on the outside of their cell wall and have a thin layer of peptidoglycan under that.Lipopolysaccharides are not as rigid as peptidoglycans due to the weak link between lipids and polysaccharides.Lipopolysaccharides are negatively charged and nanoparticles are positively charged. Therefore, it can be concluded that gram-negative bacteria (e.g.*E. Coli*) are more sensitive to zinc oxide nanoparticles comparison to gram-positive bacteria (e.g. *S. aureus*).The researchers suggested that zinc oxide nanoparticles may be essentially bounded to the outer space of the cell membrane, possibly penetrating into the cell at high concentrations[(1)]. The results of our study are consistent with the results of previous studies.

CONCLUSION

Due to the great problem of drug resistance that threatens the future of public health. Therefore, finding an effective solution to combat pathogenic microorganisms is very important. New technologies can play an important role in this regard. The use of green synthesized zinc nanoparticles due to the use of green bioreactors instead of toxic and polluting chemicals as well as the synthesis at ambient temperature and pressure instead of specific conditions, significantly reduces costs, speeds up and reduces environmental pollution compared to common chemical and contaminating methods of nanoparticle synthesis.In this study, biocompatible nanoparticles based on zinc oxide were tested in accordance with green chemistry standards and its antimicrobial effects on gram-positive and negative bacteria resistant to common antibiotics were investigated.

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