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RESEARCH ARTICLE

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Antibacterial Effects of Synthesized ZnO: Ag Nanoparticles against *Listeria monocytogenes*

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ABSTRACT

Background and objective: Listeriosis is considered as one of the most significant food-borne diseases. Nanotechnology is perceived as a promising solution to produce and develop such new antibacterial substances.

Method: The present study synthesized ZnO and Ag-doped ZnO nanoparticles (ZnO:Ag NPs). The products of this study were under the effect of characterization by X-ray diffraction (XRD), scanning electron microscope (SEM) and Fourier-transform infrared spectroscopy (FTIR). This study tried to examine the antibacterial activities attributed to ZnO and ZnO:Ag NPs against *Listeria monocytogenes* by agar well diffusion method at six concentrations of 100, 50, 25, 12.5, 6.25, 3.12 µg/ml.

Results: A polyhedral form of the abovementioned unified nanoparticles was shown by SEM micrographs. Among the mentioned concentrations, treatment of 100, 50 and 25 µg/ml of ZnO:Ag NPs was completely inhibited the growth of *L. monocytogenes* in 24 h.

Conclusion: The results indicated that the appearance of Ag NPs in their hybrids (ZnO:Ag NPs) although in low content (<25µg/ml) had effective antibacterial activities and it can be used as a drug candidate.

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KEYWORDS

ZnO, ZnO:Ag NPs, Antibacterial activity, *Listeria monocytogenes*, agar well diffusion.

INTRODUCTION

Antimicrobial resistance is a significant health problem in all over the world (1). The combination of Nano-sized drug can be regarded as an important option in improving pharmaceutical products (1, 2). A bulk of research studies revealed that nanoparticles (NPs) including metal ions and metal oxide contain antibacterial features. These kinds of particles are among the most applicable particles and materials (3, 4). A multitude of researches have been performed on some NPs including zinc, silver, and copper with respect to the power of their antibacterial activity in decreasing development of various microorganisms (5). Silver nanoparticles have been implemented widespread amongst other inorganic antibacterial agents (6). Silver NPs release silver ions in liquids indicating an extensive gamut of the antimicrobial manoeuvres (7). Silver nanoparticles have the ability in controlling the growth of bacteria and are applicable in medical equipment, dental composite materials, and textile materials. Silver was applied in topical preparations and bandages saturating for limiting bacterial development in injured skin (8, 9). ZnO NPs is applied remarkable and differently with respect to its exceptional electrical, optical, catalytic, physical, and chemical features. The nature of ZnO NPs is biodegradable and nontoxic (10, 11). Meanwhilst, eukaryotic cells are insusceptible to low concentrations of ZnO. Thus, ZnO particles which have one dimension in the range of 1–100 nm, lead to expanded reactions, while using the bacterial surface in the role of an efficacious bactericidal agent facing either Gram-positive and Gram-negative bacteria such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus* and *Bacillus subtilis* (5, 12).

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Tayel et al. showed that the Gram-positive strains are more vulnerable to NPs associated to the structure and thickness of the membrane and cell wall (13, 14). Zinc oxide (ZnO) is a perfect antibacterial element due to its more biocompatibility and stability in compare to organic antibacterial agents. It is suggested that morphology, shape and size of ZnO NPs, and added dopants into ZnO host matrix have the ability of tuning different characteristic and increasing attributed antibacterial activity (15). Raghupathi et al. (3, 14) stated that smaller size ZnO NPs could show greater antibacterial activity in comparison to greater size NPs on either Gram-positive and Gram-negative bacteria. ZnO NPs did not indicate any antibacterial activity versus the spores that are resistant to high-temperature and high-pressure, and factors of crystal structure of nanoparticles did not show any significant effect on their antibacterial activities. Mahapatra et al. (16) showed that decreased size of particle and the elevated presence of doping element are the result of increased solubility of doping element in the base material. Therefore, adding up silver in zinc oxide could lead to different size in particles and impose an effect on its antibacterial activity. Doping of Ag in ZnO NPs induces remarkably imperfections in the band structure of ZnO NPs and tailors its band gap. Finally, a considerable effect on the antibacterial activity of ZnO NPs is imposed by Ag doping; Available literature revealed that the influential bactericidal activity of ZnO:Ag NPs is due to their size and concentration (3). A study on antimicrobial activity of ZnO and TiO2 nanoparticles compared with ZnO:Ag and TiO2:Ag NPs against Gram-positive bacteria *Staphylococcus aureus* and Gram negative *Escherichia coli*, observed that oxides doped not with silver compared with doped, no antimicrobial activity or significantly reduced. Hybridization of metals such as Au, Ag and Pd with semiconductor oxides is an efficient strategy for breaking semiconductor oxides' gaps (10). In this study, according to our expectations, the addition of silver to the zinc oxide compound has led to a significant increase in its antimicrobial effects. The genus *Listeria* is located in the *Clostridium* sub-branch of Gram-positive bacteria with respect to its low G+C content of genome. Six species were identified as *Listeria monocytogenes, L. innocua, L. ivanovii, L. seeligeri, L. welshimeri* and *L. grayi*. Two species are pathogeni including *L. monocytogenes* in humans and *L. ivanovii* in other mammals (17, 18). Pathogenic infection resulted of *L. monocytogenes* can lead to listeriosis and influences people who are pre-disposed by background disease influencing their immune system including cancer or AIDS and other cases including elderly, pregnancy, newborn babies or fetuses. Indications of the disease are like influenza and can lead to serious consequences including meningitis, septicemia, spontaneous abortion or listeriosis of the newborn. Although, the incidence of Listeriosis as a severe food-borne infection is low, mortality rates are on average approach of 30% and extended infections and intoxications of *Salmonella* and *Clostridium botulinum* (19, 20). The present study did a research on synthesizing ZnO and ZnO:Ag NPs and assessing the antibacterial activity against *L. monocytogenes*. Recently, a bulk of research studies has been done on the antimicrobial effects of ZnO NPs on gram-positive and gram-negative bacteria. However, the effects of zinc oxide doped with silver on *L. monocytogenes* very few studies have been done. Although, most researches were done on standard bacteria, this study examined antibacterial effects of ZnO:Ag NPs on strains isolated from local cheeses. In the present research, *L. monocytogenes* was not isolated from other traditional dairy products. The present study examined the antibacterial activities of ZnO and ZnO: Ag NPs opposed to *L. monocytogenes* by agar well diffusion method at six concentrations of 100, 50, 25, 12.5, 6.25 and 3.12 µg/ml and the deionized water was considered as control.

MATERIALS AND METHODS

Synthesis of ZnO and ZnO: Ag NPs

This study employed high purity zinc nitrate ($Zn(NO3)_2$), silver nitrate ($AgNO_3$) and sodium hydroxide (NaOH) solution as the precursor and isopropanol ($(CH3)_2CHOH$) as the solvent for synthesizing the undoped and Ag-doped ZnO nanostructures. In a typical synthesizing of pure ZnO NPs, 0.05 M zinc nitrate was dissolved in 100 ml of propanol and 0.1 M NaOH solution was dissolved in 100 ml propanol. NaOH solution was added drop wise to zinc nitrate solution. After that, a white-colored gel was fabricated and kept for ageing overnight. Comparably, synthesizing Ag doped ZnO NPs, 0.1 M zinc nitrate solution with milimolar silver nitrate and NaOH solution was added and the stirring for 30 minutes. The produced glassy like white-colored gel was put aside to age through the night. The solution was centrifuged at high speed (10,000 rpm) for 5 min to collect the Nanohybrids. The residuary precursors and agents were put aside following some rounds of centrifugation by adding fresh distilled water. It was dried in oven at 100°C for 2 hrs.

Characterization

Researcher examined sample's structure by X-ray diffraction (XRD) method which took advantage of a XRD6000, Shimadzu system with CuKα radiation (0.15406 nm). In this study, scanning electron microscope (SEM) (CM120, Philips, Netherlands) assessed distribution of different NPs regarding their diameter and size. Fourier transform infrared

spectroscopy (FTIR) absorption was assessed for KBr-supported samples over a frequency range of 400–4000 cm^{-1} and at a resolution of 4 cm^{-1}, using a model SHIMADZU, FTIR-8400S.

Selection and preparation of *Listeria monocytogenes*

In this study, 200 fresh and traditional cheese samples were collected from various locations in Iran. 7 samples (3.5%) of *L. monocytogenes* were isolated using the cold enrichment method. Briefly, 25g samples of cheese were aseptically added to 225ml of *Listeria* Enrichment broth and homogenized in a Stomacher. The homogenized samples were incubated at 4°C. After one week of incubation, colonies were streaked onto *Listeria* Enrichment Agar. Typical *Listeria*-like colonies were purified on BHI agar medium and recognized by applying morphological, cultural, and biochemical tests. Specifically, Gram stain, catalase test, motility, ß-hemolysis, and production of acids from rhamnose and xylose were employed for clarifying Bergey's manual, identifying the species belonged to the genus. One standard bacterial strain (*L. monocytogenes* ATCC7644) was tested.

Determination of Antibacterial Activity

The antimicrobial activity of ZnO and ZnO:Ag NPs on isolated *L. monocytogenes* from traditional cheese and *L. monocytogenes* ATCC7644 was specified by proper diffusion approaches and specifying the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) by microdilution tests. The incubation of bacterial cultures was regularly performed at 37°C in Mueller Hinton broth (MHB, Merck Germany). The cells were suspended into sterile PBS to attain turbidity parallel to 0.5 MacFarland (1.5×10^8 CFU/mL) standard. Petri dishes with 20 ml of Muller Hinton Agar (MHA, Merck Germany) were provided and previously inoculated with 0.1 ml of a 24 h broth culture of *Listeria monocytogenes*. After solidification, the dishes were stored in a refrigerator for 1 hour. For providing colloidal suspension of the synthesized nanostructures, 10 µL of ZnO:Ag nanoparticles suspensions (0.06 mg of ZnO or ZnO:Ag NPs were added to 6 ml of MHB) were poured into of each well (6mm). After overnight incubation at 37°C, various sizes of inhibition zone (the clear zone around of the colonies) were assessed on every plate. The experiment was done three times to validate repeatability.

RESULTS AND DISCUSSIONS

Characterization of prepared ZnO and ZnO:Ag nanoparticles

The structure of ZnO and ZnO:Ag nanostructures were synthesized and examined by analyzing the X-Ray diffraction data (method using a XRD6000, Shimadzu system with CuKα radiation). Figure 1 a and b indicate the XRD results of ZnO and ZnO:Ag NPs. The diffraction peaks of the synthesized ZnO samples and the crystalline wurtzite ZnO structure were the same regarding JCPDS standard card 36-1451. The ionic radius Ag^{1+} (0.57 A) and Zn^{2+} (0.60 A) is the same. Therefore, substitution by silver should not lead to fundamental changes in the lattice parameters. This study used Scherrer formula for assessing average sizes of all samples. The lower crystallite size by doping could be due to the lower ionic radius of Ag^{1+} in compare to Zn^{2+}. The SEM micrograph along with size distribution histogram of ZnO:Ag nanoparticles are presented in figure 2 a. The results showed the average grain size of 27.2 nm for ZnO and 22.4 nm for ZnO:Ag Nanoparticles. For assessing the particle size from XRD, all peaks were utilized and a mean value was obtained. On the other hand, the quantity of the particles was straightly scanned by SEM. Thus, there would be some difference between the particle sizes acquired from XRD and SEM. FTIR analysis was conducted to examine the surface chemistry and modes of vibrations of chemical bonds, which exist in every prepped sample. The FTIR spectrum of pure ZnO showed three peaks. With the addition of silver, the intensity of the peaks increased (Figure 3).

Antibacterial activity of ZnO and ZnO:Ag NPs

The prepared antibacterial nanoparticles towards *L. monocytogenes* were performed by using culture turbidity (0.5 MacFarland Standard) as qualitative assessment of cell development. Observing increase in antimicrobial activity of ZnO NPs may be resulted from size, morphology and soluble Zn^{2+} ions. Due to smaller size of nanoparticles than size of bacterium, nanoparticles could simply attach to the cell wall of the bacterium because they are smaller than bacterium and this difference result in bacterium death (21). According to the results of Table 1, the antimicrobial effect of ZnO NPs on the standard strains of *L. monocytogenes* ATCC7644 was much less than the antimicrobial activity of nanoparticle ZnO:Ag NPs, respectively. Amongst the six concentrations studied, the highest concentration of ZnO and ZnO: Ag NPs was 100 µg/ml. While at 12.5 µg/ml concentration only ZnO:Ag NPs affected on *L. monocytogenes* ATCC7644 but ZnO NPs was ineffective. The antimicrobial effect of ZnO NPs on *L. monocytogenes* strain isolated from local cheese was also significantly less than the antimicrobial effect of ZnO:Ag NPs. The isolated strain compared with standard strain had less inhibition zone and was more resistant to the antimicrobial effects of both mentioned nanoparticles. In the concentration of 3.125 µg/ml, none of the nanoparticles were able to antimicrobial effects. The most inhibition zone

was in concentration 100 µg/ml (Table 2). MIC and MBC of *L. monocytogenes* ATCC7644 and *L. monocytogenes* isolated of cheese treated with ZnO and Ag-doped ZnO NPS in table 3, 4 has been shown.

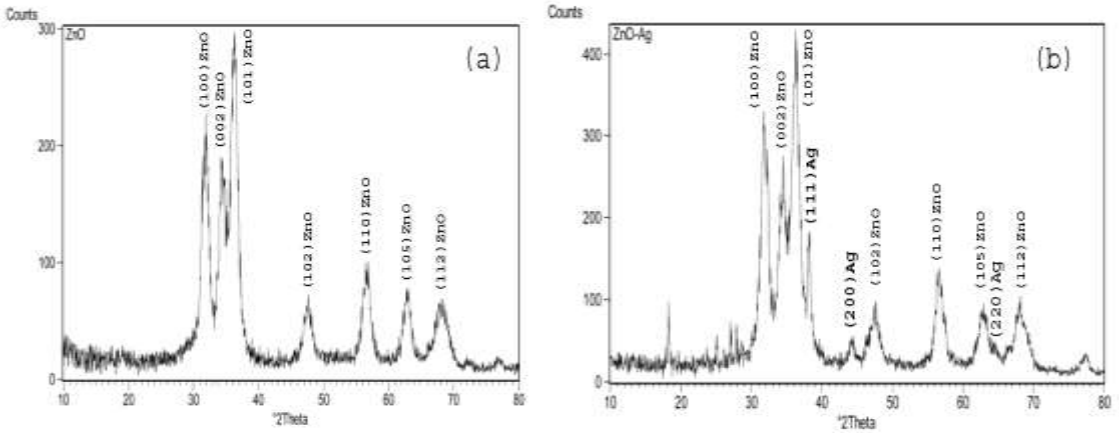

Figure 1: XRD pattern of pure ZnO (a) and ZnO:Ag (b) nanoparticles.

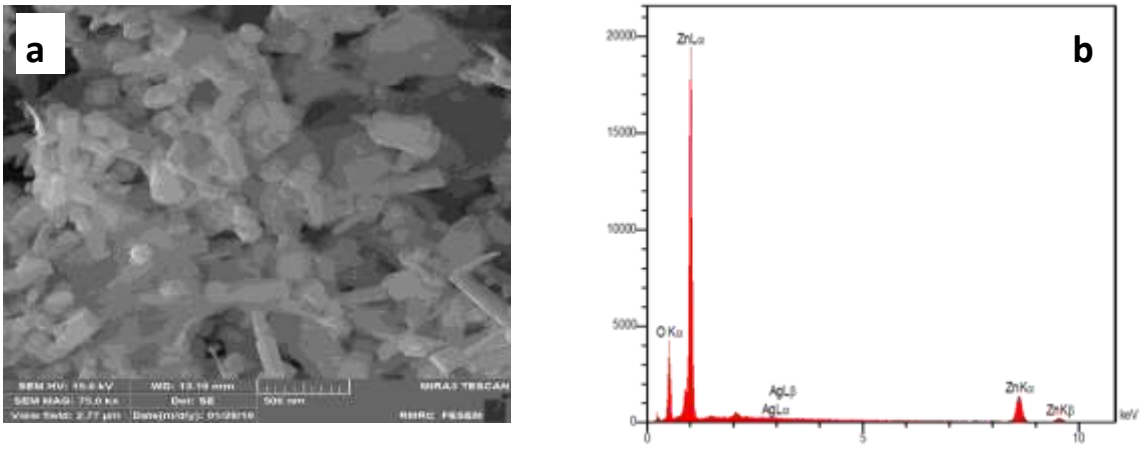

Figure 2: SEM image of ZnO:Ag nanostructure powder (a) and EDX spectrum of ZnO:Ag(b).

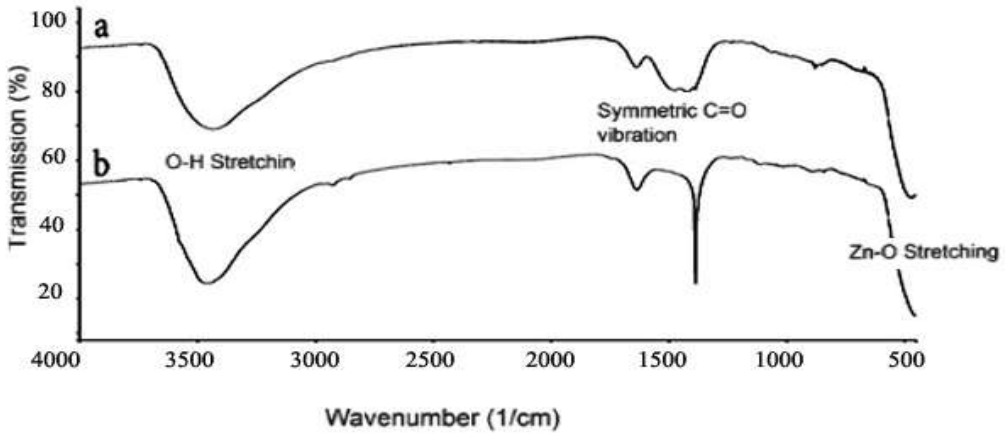

Figure 3: FTIR image of ZnO (a) and ZnO:Ag (b) NPs.

Table 1. Inhibition zone of *L. monocytogenes* ATCC7644 treated with ZnO and Ag-doped ZnO NPS.

Concentrations(µg/ml)	Inhibition zone(mm) ZnO Nanoparticles	Ag-doped ZnO Nanoparticles
100	16±0.1	24±0.0
50	14±0.0	22±0.0
25	12±0.1	18±0.1
12.5	10±0.0	14±0.0
6.25	9±0.1	11±0.1
3.12	6	7

Table 2: Inhibition zone of L. monocytogenes isolated of local cheese treated with ZnO and Ag-doped ZnO NPS.

Concentrations(µg/ml)	Inhibition zone(mm) ZnO Nanoparticles	Ag-doped ZnO Nanoparticles
100	18±0.1	20±0.0
50	16±0.0	18±0.1
25	14±0.1	17±0.0
12.5	6	11±0.1
6.25	6	6
3.12	6	6

Table 3: Determination of MIC and MBC of *L. monocytogenes* ATCC7644 treated with ZnO and Ag-doped ZnO NPS.

Concentrations(µg/ml)	MIC(µg/ml)	MBC(µg/ml)
ZnO	25	50
Ag-doped ZnO	12.5	25

Table 4: Determination of MIC and MBC of *L. monocytogenes* isolated of cheese treated with ZnO and Ag-doped ZnO NPS.

Concentrations(µg/ml)	MIC(µg/ml)	MBC(µg/ml)
ZnO	50	100
Ag-doped ZnO	12.5	25

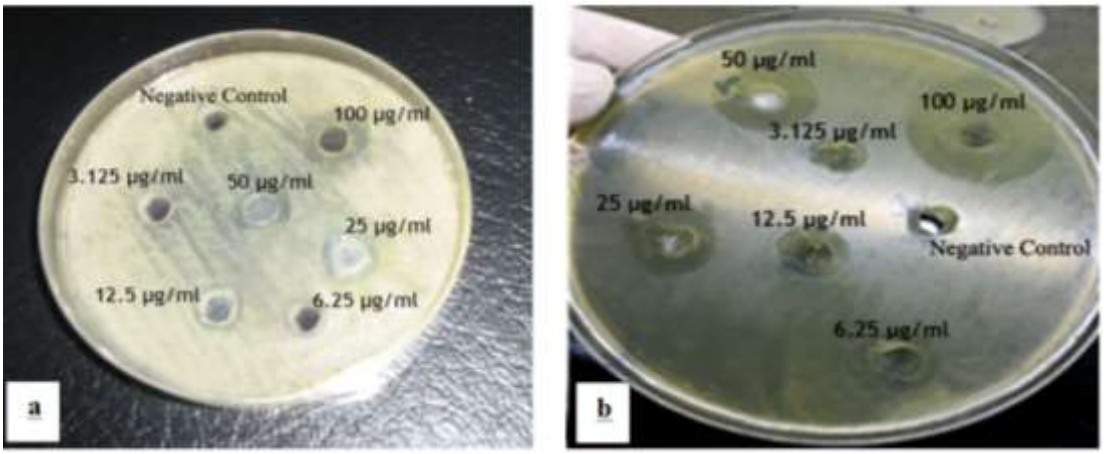

Figure 4: Antibacterial activity of ZnO (a) and ZnO:Ag (b) NPs against *L. monocytogenes* ATCC7644

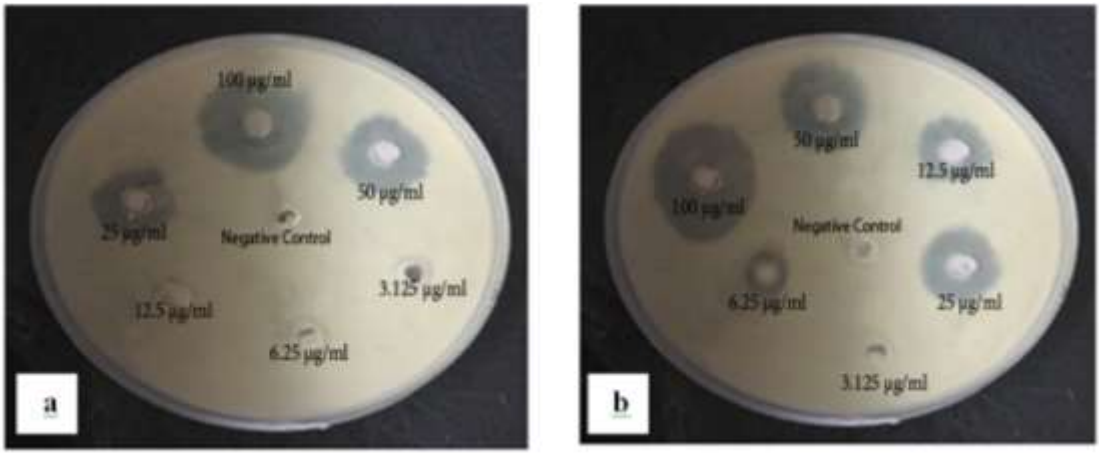

Figure 5: Antibacterial activity of ZnO (a) and ZnO:Ag (b) NPs against *L. monocytogenes* isolated of cheese.

The prevalence of pathogenic microorganisms in cheese depends on the quality of the milk used, the thermal processes of the milk and the storage temperature of the cheese, and the duration of distribution and sale (5, 22). Nanotechnology has many applications in new approaches to biology for the diagnosis and treatment of pathogens (21). A variety of methods are used to synthesize ZnO nanostructures such as hydrothermal synthesis, chemical deposition or physical vapor, precipitation in aqueous solution, and sol-gel process (23). Today, Ag and ZnO nanopowders are used as drugs with antibacterial and antifungal properties in skin creams (15). Due to the advantage of low temperature during synthesis, in the present study, chemical precipitation technique was implemented as a mean for the synthesis of ZnO:Ag NP (2). Degradation of silver to zinc oxide enhanced antibacterial activity and was suitable for either gram-positive and gram-negative pathogens (21). The results of MIC and MBC showed that *L. monocytogenes* were more sensitive to lower concentrations of ZnO:Ag NPs compared to ZnO NPs alone.

Conclusion

In general, ZnO and ZnO:Ag NPs were prepared by a precipitation method. The results of XRD, SEM and FTIR confirmed the presence of silver on zinc oxide. However, both of Ag and ZnO nanoparticles have a lot of activity in preventing the growth of different bacteria. Ag-doped ZnO NPs had a higher inhibition of zone than zinc oxide NPs. As expected, the addition of silver to the zinc oxide increases its antimicrobial effect. The small size of the synthesized nanoparticles has a major role in the antimicrobial effects of these nanoparticles. Thus, it could be expected that ZnO:Ag nanoparticles should be used as valid antibacterial drugs.

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