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RESEARCH ARTICLE

Growth Inhibition Effect of Zeolite/Zinc Oxide Nanocomposite against Foodborne Gram-Positive Bacteria

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ABSTRACT

The overuse of antibiotics has led to the increase of multidrug-resistant microorganisms, so there is a need to develop alternatives. Producing nanoparticles with physical and chemical effects and limited resistance is one of the promising methods to combat pathogens, especially foodborne ones. The study aimed to evaluate the antibacterial effects of Nanocomposites of zinc oxide loaded on a zeolite (ZnONCs/Zeolite) and to determine its minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against some Gram-positive foodborne pathogens. Two techniques were used for the ZnONCs/Zeo characterization, including X-ray fluorescence (XRF) and field emission scanning electron microscopy (FE-SEM). The antibacterial potential of ZnONCs/Zeo against common foodborne pathogens (*Staphylococcus aureus, Listeria monocytogenes, Bacillus cereus, Enterococcus faecalis*) was investigated with disk diffusion and broth microdilution assay. SEM images showed good distribution of ZnO-NPs on the zeolite framework, and the cubic structure of the zeolite was well preserved. ZnONCs/Zeo had significant antibacterial activity against the tested bacteria. Thus, significant antibacterial activity was recorded for ZnONCs/Zeo with an inhibition zone (8-25 mm). The results showed that the MIC value of biogenic ZnONCs/Zeo against *S. aureus* was 2 mg/mL, while this value was 4 mg/mL for other bacterial strains. The MBC values of ZnONPs/Zeo against the studied bacteria were varied. Given the promising results of the antibacterial activity of ZnONPs/Zeo, it is suggested that this compound can be used in the food industry as a preservative agent, although further investigations and risk assessment are needed.

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INTRODUCTION

Excessive and indiscriminate use of antibiotics has increased multidrug resistance in microorganisms, thus endangering human and animal health [1, 2]. Hence, there is an urgent need to find alternatives to antibiotics with more effective mechanisms of action. Nanoparticles (NPs) with antimicrobial properties are among the other options to combat multidrug-resistant (MDR) bacteria [3]. The unique properties of nanoparticles, which offer many explicit advantages for biomedical applications, have attracted much attention [4]. Nanomaterials have a broader biocidal spectrum than antibiotics and even react against different types of cells [5]. In addition, inorganic nanoparticles have shown improved biological functions because of characteristics such

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as their structure and size [6].

Inorganic nanoparticles, for instance, silver (AgNPs), gold (AuNPs), and zinc oxide (ZnO NPs), have wide applications in the medical and biological fields as biosensors and for the production of personal care products [7]. Also, the antibacterial properties of metal oxides have been proven due to oxygen production [8]. Among nanoparticles, ZnO NPs in particular are considered vitally effective metal oxide nanoparticles, exhibiting antibacterial properties against a wide range of Gram-positive and Gram-negative microorganisms, as well as main foodborne pathogens [9, 10]. Zinc oxide nanoparticles act by various mechanisms such as generating ROS or producing Zn^{2+} ions that can bind to the bacterial surface, creating unfavorable conditions for the bacteria and subsequently disrupting cell membrane function and cell wall integrity [11]. These multifunctional behaviors of ZnO NPs have made them a possible candidate for use as an antimicrobial agent against foodborne pathogens [12].

Antibacterial agents used in the food industry are classified into two groups: organic and inorganic agents. Inorganic agents are more stable at high pressures and temperatures than organic ones [13]. Metal oxide powders have been proposed as potent antibacterial agents in this field. Recently, metal oxides, which have high stability under severe processing conditions and are commonly recognized as safe ingredients for human and animal health, have gained much attention as antibacterial agents [14]. It has been previously demonstrated that metal oxide nanoparticles, including ZnO NPs, have selective toxicity against bacteria with minimal toxic effects on human cells, suggesting their future applications in the agricultural and food industries [15].

Outbreaks of foodborne diseases occur continuously worldwide due to the consumption of food contaminated with pathogens, and high mortality rates from diarrheal diseases have been reported [16]. Some major foodborne pathogens associated with serious health threats and human diseases include *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Clostridium*, and *Salmonella* [17]. *S. aureus* is one of the major groups of foodborne pathogens that has a varied range of pathogenicity and a high capacity to develop resistance to various antibiotics [18]. *S. aureus* enterotoxins cause food poisoning, which is associated with intestinal dysfunction and multiple symptoms such as fever, nausea, vomiting, diarrhea, etc [16]. *Enterococcus faecalis* is an opportunistic pathogen found in both humans and animals as a commensal and commonly contaminates raw food materials, including milk, meat, and vegetables, causing food poisoning [19].

Listeria monocytogenes is a ubiquitous food pathogen that can survive and proliferate in various harsh environments and foods [20]. These characteristics have made this pathogen a major concern in the food industry. Despite the low incidence of listeriosis compared to other foodborne diseases (such as salmonellosis), its high hospitalization and mortality rates, especially in high-risk groups, make this pathogen a serious threat to public health [21]. *Bacillus cereus* is an opportunistic foodborne pathogen that causes vomiting or diarrhea-like food poisoning, usually mild and self-limiting. Moreover, this pathogen can also cause severe systemic infections [22]. Therefore, the present study was conducted to evaluate the antimicrobial activity of ZnO NPs against several Gram-positive bacteria, mainly foodborne pathogens.

MATERIALS AND METHODS

Nano ZnO/Zeo Composite preparation

First, compounds of ZnO/Zeo nanocomposite, ZnO/Zeo, and zeolite were prepared by a method previously defined by Partoazar et al with minor changes [23]. Zeolite powder was mixed in deionized water for about 1 hour and then filtered through cellulose filter paper. After three washes, subsequent drying was carried out at 80°C and kept away from moisture. Then, zeolite powder (10 g) and Zn (CH3CO2)2·2H2O (7 g) were added to 100 ml of distilled water, and by continuous stirring at 60°C for 1 h, Zn2+-exchanged zeolites were obtained. Next, to produce nanoparticles on the zeolite bed, NaOH 1 M solution was slightly added to the suspension till pH = 12 was obtained. After 2 h, the composite materials were washed and filtered with DW to eliminate the residual zinc acetate from the media. After drying the nanoparticles overnight at 80°C, they were calcined for 2 hours at 400°C. It should be noted that, to form the ZnO/Zeo composite, the above steps were carried out completely without adding NaOH solution after the reflux reaction. X-ray fluorescence (XRF, PW2404; Philips) system was used to characterize zeolite elementals to determine their ZnO percentage. Finally, the ZnO nanomaterials were evaluated

in terms of morphology by the FESEM system (MIRA3 TESCAN).

Bacterial Strain

The strains used in the current study, i.e., *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, *L. monocytogenes* ATCC 13932, and *B. cereus* ATCC 11778, were obtained from The Iranian Biological Resource Center. The strains were aerobically grown in the tryptic soy broth (TSB, Merck, Germany) for 24 h at 37◦C.

Antibacterial Activity Assessment of Zeo/ZnONPs
Disk diffusion assay

The disk diffusion was done to qualitatively screen bacterial susceptibility and select suitable ZnONPs/Zeo concentration for the broth microdilution method. The disk diffusion method was accomplished by swabbing fresh cultures onto Mueller Hinton agar plates and placing sterile filter paper discs (Whatman No.1, 6 mm diameter). Then, 10 mL from sterile ZnONCs/Zeo, ZnO/Zeo, and zeolite alone was dispensed onto the surface of each one. The concentration of bacterial cultures was equivalent to the standard of 0.5 MacFarland and about 1.5 $\times 10^8$ CFU/mL; the ZNO-NPS suspension at a concentration of 0.5 to 16 mg/ml was tested. After the incubation of the plates overnight at the optimal temperature for each strain, the diameter of growth inhibition zones was measured. Positive and negative controls included zinc acetate and the medium alone, respectively. Each experiment was repeated three times, and the inhibition zones were recorded as the mean standard deviation.

Microdilution assay

The microdilution method was done to determine the minimal inhibitory concentrations (MIC) of ZnONCs/Zeo, ZnO/Zeo, and zeolite alone against studied bacteria based on clinical and laboratory standards (CLSI). Briefly, 100 ml of freshly bacterial cultures (10^6 CFU/ml) was poured in microplate 96-wells, then 100 ml of serial dilutions of ZnONCs/Zeo (final concentrations of 1 to 16 mg/ml). Bacterial suspension and medium were used as positive and negative controls, respectively. After overnight incubation, the MIC was considered as the lowest ZnONCs/Zeo concentration that entirely repressed bacterial growth, i.e., a colorless well. To evaluate MBC values, an amount of 10 µl of inoculum was removed from the wells that showed no visible turbidity and transferred to Trypticase Soy Agar (TSA). The lowest concentration of ZnONCs/Zeo caused the removal of studied bacteria on MHA was described as MBC.

RESULTS

Composite Analysis

The XRF technique was used to assess the percentage of ZnO in the experimental compositions. Based on the results, the NC sample contained 25.149% ZnO among other elements, while the value for ZnO/Zeo was 8.35% (Table 1). The results obtained from FE-SEM imaging at low and high magnifications showed the crystal structure of zeolite materials and ZnO nanoparticles formed on zeolite surfaces, respectively (Fig 1).

Antibacterial Activity Assessment of Zeo/ZnONPs
Disk diffusion method

Different foodborne bacterial strains were surveyed for their susceptibility to ZnONCs/Zeo. In the study of the susceptibility of different bacterial strains to ZnONCs using the disk diffusion method, the dimensions of the inhibition zone around the disk exceeding 8 mm were considered significant. The size of the inhibition growth zone of the tested bacteria around each disk ranged from 8 to 25 mm. The zone of inhibition for *L. monocytogenes* and *S. aureus* strains was recorded at concentrations equal to and higher than 1 mg/mL and 2 mg/mL, respectively. Furthermore, the zone of growth inhibition for *B. cereus* was recorded at concentrations equal to and higher than 8mg/mL, while the growth of *E. faecalis* was inhibited only at a concentration of 16 mg/mL. As shown in Figure 2, the growth inhibitory effect of ZnONCs/Zeo against the tested bacteria was obtained in a concentration-dependent manner.

Table 1. Percentages obtained from various elements in the studied compounds using the XRF system.

	XRF analysis/ (wt. percentage)										
Composition	**ZnO**	**SiO_2**	**Al_2O_3**	**CaO**	**MgO**	**Fe_2O_3**	**P_2O_5**	**K_2O**	**TiO_2**	**SO_3**	**MnO**
ZnO/Zeo	8.358	65.819	8.905	3.661	0.568	1.291	0.031	1.324	0.144	0.153	0.053
ZnONCs/Zeo	25.149	50.232	7.643	4.333	0.478	1.154	0.03	1.029	0.117	0.102	0.039

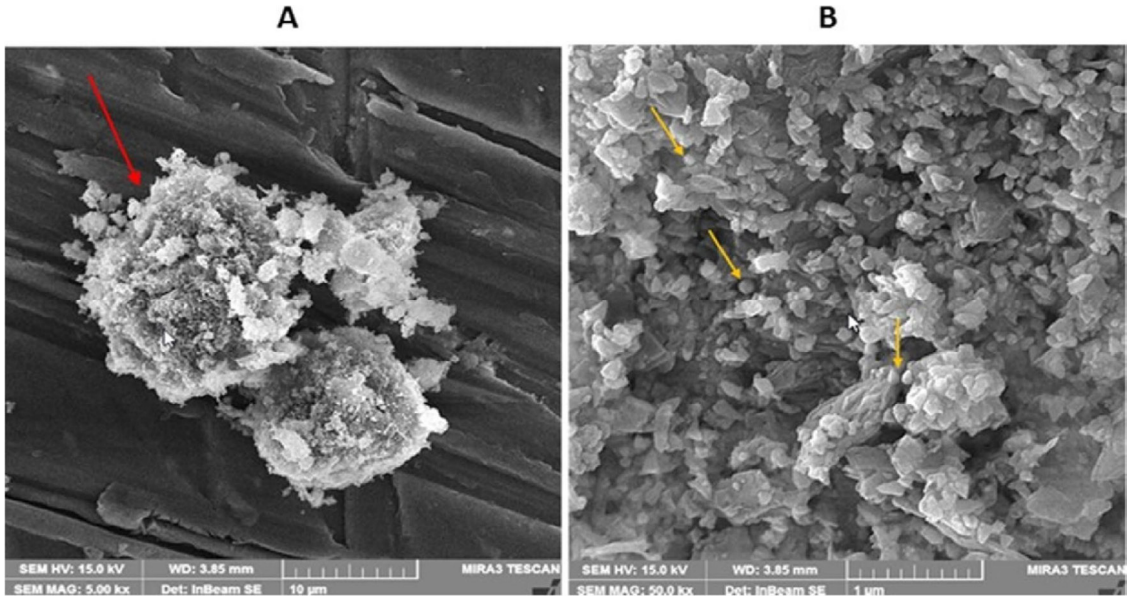

Fig. 1. FE-SEM imaging of zinc oxide nanocomposite materials. A. The crystalline form of the ZnONCs/Zeo is shown by red arrows. B. Doped ZnO nanoparticles on the zeolite composite surface are shown by yellow arrows.

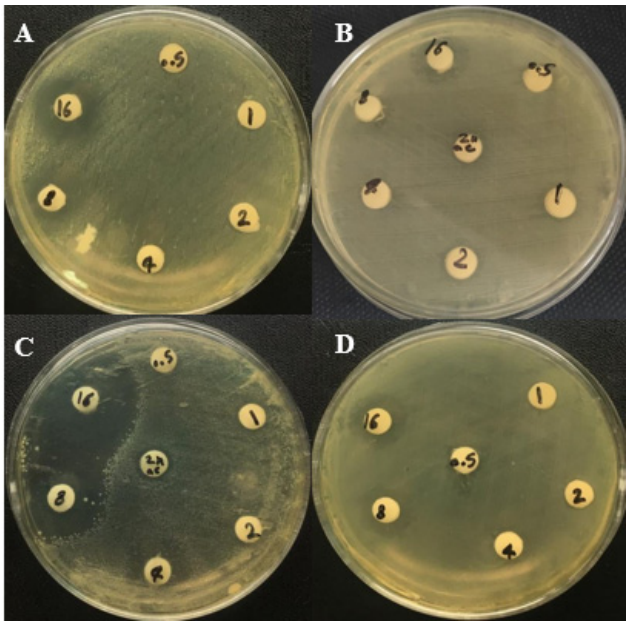

Fig. 2. The susceptibility of different bacterial strains to ZnONCs using the disk diffusion method. Formation of zone of inhibition (A) *S. aureus*, (B) *E. faecalis,* (C) *L. monocytogenes*, and (D) *B. cereus.*

Microdilution assay

The MIC value, as the lowest concentration of zeolite, ZnO/Zeo, and ZnONPs/Zeo to inhibit bacterial growth, was individually determined for each bacterial strain. The results showed that the MIC value of biogenic ZnONPs/Zeo against *S. aureus* (ATCC 25923) was 2 mg/mL, while this value was 4 mg/mL for other bacterial strains. It is worth noting that none of the ZnO/Zeo and zeolite compounds inhibited the growth of the studied bacteria, while the MIC of the positive control (zinc acetate) against all studied bacteria was 8 mg/

Table 2. Results of the susceptibility of the studied strains to different concentrations of ZnONCs/Zeo by the disk diffusion method.

	ZnONCs/Zeo(mg/ml)							
Bacterial strain	**0.5**	**1**	**2**	**4**	**8**	**16**	**PC**	**NC**
S. aureus	0	0	11	12	14	17	10	0
E. faecalis	0	0	0	0	0	8	10	0
B. cereus	0	0	0	0	9	11	10	0
L. monocytogenes	0	9	12	15	20	25	10	0

PC: Positive control, **NC**: negative controls**,** Results are expressing of zone inhibition in mm

Table 3. The antibacterial activity of ZnONPs/Zeo and zinc acetate against bacterial strains was measured as MIC* and MBC*

	ZnONPs/Zeo		zinc acetate	
Bacterial strain	**MIC value (mg/ml)**	**MBC value (mg/ml)**	**MIC value (mg/ml)**	**MBC value (mg/ml)**
S. aureus	2	8	8	8
E. faecalis	4	64	8	8
B. cereus	4	16	8	8
L. monocytogenes	4	4	8	8

* MIC: minimal inhibitory concentration, MBC: minimal bactericidal concentration

ml. The MBC values of ZnONPs/Zeo against the studied bacteria were varied. (Table 3).

DISCUSSION

In this study, four foodborne pathogen strains were used to investigate the antimicrobial potential of ZnONPs/Zeo to increase the reality and applicability of the study. The bacterial growth Inhibition was assessed by treatment with ZnONPs/Zeo in both solid media (qualitative test) and broth (quantitative test) to provide further possibilities for using ZnONPs/Zeo as an antibacterial agent. Previous studies have shown that metal oxide nanomaterials are involved in cell death, disrupting mitochondrial function, lactate dehydrogenase leakage, and altering cell morphology [24, 25].

The antibacterial activity of ZnONPs/Zeo was much higher than that of ZnO/Zeo. This can be explained by the fact that smaller particles usually have a higher surface-to-volume ratio, making them a more efficient agent for antibacterial activity [26]. The production of hydrogen peroxide (H2O2) from the ZnO surface provides another explanation for the antibacterial activity of ZnO [27]. On the other hand, it has been suggested that the concentration of H2O2 produced from the surface rises with the diminishing size of particles [27]. ZnO is typically unstable in solution, and with the production of H2O2, the concentration of Zn2+ ions increases because of ZnO decomposition, which is another reason for ZnO's antibacterial activity [14].

In the present study, two different methods were used, including growth inhibition zone assay, which indicates both bacteriostatic and bactericidal activity of ZnO, and MIC determination, which focuses on the bactericidal activity of nanoparticles. By examining the antibacterial activity using the disk diffusion method, it was found that ZnONPs/Zeo inhibited the growth of all the bacteria tested in a concentration-dependent manner. Overall, the growth inhibition zone was observed in *E. faecalis* and *B. cereus* at high concentrations. The MBC and MIC values of ZnONPs/Zeo showed that the growth of the tested bacteria was inhibited by increasing the concentration of nanoparticles. The MIC value for *S. aureus* was estimated to be 2 mg/mL and for other strains to be 4 mg/mL, and the MBC ranged from 4 to 64 mg/mL for different strains. A previous study has shown that ZnO nanoparticles have antimicrobial activity against *Salmonella enteritidis*, *L. monocytogenes*, and *Escherichia coli* O157:H7 [28]. ZnO NPs damage the bacterial cell membrane, leading to leakage of intracellular contents and, ultimately, bacterial cell death [29].

CONCLUSIONS

According to the results of the present study, it can be concluded that ZnONPs/Zeo can be proposed as an effective and potent antibacterial agent against foodborne pathogens. Therefore, the application of ZnONPs/Zeo as a preservative against foodborne bacteria in the food industry can be suggested after confirming its biosafety or toxicity.

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CONFLICTS OF INTEREST

No competing interests to declare.

DATA AVAILABILITY STATEMENT

Data is available on request from the corresponding authors.

AUTHOR CONTRIBUTIONS

Mohammad Mehdi Soltan Dallal: Conceptualization, Project administration, **Alireza Partoazar,** Conceptualization, **Katayoun Samimi-Rad:** Conceptualization**, Zahra Dargahi,** Investigation**, Samira Karimaei:** Investigation, Formal analysis, Writing-reviewing and editing.

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