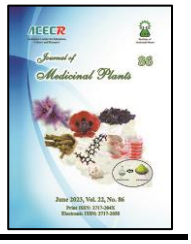
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Research Article

Green synthesis of ZnO nanoparticles from *Foeniculum vulgare* Mill. seed extract and its antibacterial effects on foodborne bacteria

Parisa Salari[1], Mansour Ghaffari Moghaddam[2], Masoumeh Bahreini[1], Mohammad Reza Sharifmoghadam[1,*]

[1] *Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran*
[2] *Department of Chemistry, Faculty of Science, University of Zabol, Zabol, Iran*

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ABSTRACT

Background: *Foeniculum vulgare* Mill. seeds contain polyphenolic compounds which can be considered a suitable option for the green synthesis of nanoparticles. **Objective:** In this study, the antibacterial activity against foodborne bacteria of ZnO nanoparticles synthesized from the aqueous extract of *Foeniculum vulgare* Mill. seeds was evaluated. **Methods:** The synthesized ZnO nanoparticles were characterized using different analyses. The minimum inhibitory and bactericidal concentration of the nanoparticles were investigated against standard foodborne bacteria, *S. aureus*, *Y. enterocolitica*, *E. coli O157:H7*, and *B. cereus*, using the broth microdilution method. **Results:** UV-Vis spectroscopy analysis indicated an absorption peak at 231 nm which confirms the formation of ZnO nanoparticles. In addition, the X-ray diffraction pattern is consistent with the JCPDS cards, which also means the formation of ZnO nanoparticles. The results of the electron microscope revealed that the nanoparticles had a hexagonal shape with an average size of 50 nm, which is in agreement with the results obtained from the dynamic light scattering analysis. In addition, the minimum inhibitory concentration of ZnO nanoparticles against gram-negative and gram-positive bacteria, *Y. enterocolitica*, *E. coli O157:H7*, *S. aureus*, and *B. cereus* were 62.5, 62.5, 31.25 and 500 µg/ml, respectively. **Conclusion:** ZnO nanoparticles synthesized from *Foeniculum vulgare* Mill. seed extract had an appropriate antibacterial effect against foodborne bacteria.

1. Introduction

Nanoparticles usually refer to particles with one dimension less than 100 nm [1]. Nanoparticles include various types of metal, polymers, fullerene, and ceramic [2]. Although some physical and chemical processes are

Abbreviations: FDA, The United States Food and Drug Administration; UV-Vis, Ultraviolet-Visible Spectroscopy; XRD, X-Ray Diffraction Spectroscopy; JCPDS, Joint Committee on Powder Diffraction Standards; FT-IR, Fourier Transform Infrared Spectrometer; FE-SEM, Field Emission Scanning Electron Microscopes; TEM, Transmission Electron Microscopes; DLS, Dynamic Light Scattering; EDAX, Energy Dispersive X-Ray Analysis; ZnO, Zinc Oxide; $Zn(NO_3)_2.6\ H_2O$, Zinc Nitrate Hexahydrate; $Na_4P_2O_7$, Sodium Pyrophosphate; MIC; Minimum Inhibitory Concentration; MBC, Minimum Bactericidal Concentration; TTC, 2,3,5-Triphenyl Tetrazolium Chloride; PDI, Polydispersity Index

*Corresponding author: Sharif@um.ac.ir

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involved in the synthesis of different nanoparticles, biological methods are more important due to their lower toxicity, cheapness, and compatibility with the environment [3]. Both plants and microbes could be the sources of nanoparticle synthesis in a green or natural way [4]. However, an essential advantage of using plant sources rather than microorganisms is that ions reduce more quickly and become more stable in metal nanoparticles [5]. In addition, by plant sources, we can synthesize different sizes and shapes of nanoparticles [6].

The United States Food and Drug Administration (FDA) has approved the use of ZnO nanoparticles due to their activity against bacteria and being environmentally friendly [7]. There are many applications of ZnO nanoparticles; For instance, in the food industry as a food preservative, in the cosmetic-health industry as an ultraviolet ray absorber, in medicine as an anti-cancer and antimicrobial agent, and also in many other sciences, it has a function [8].

Consuming food contaminated with microbes or microbial toxins can lead to food poisoning or foodborne diseases [9]. Animal food products such as meat, eggs, and dairy products are the main sources of transmission of diseases related to foodborne microbes. It has been determined that gram-negative bacteria are more involved in foodborne diseases than gram-positive ones when it comes to food poisoning [10].

Fennel or green anise with the scientific name (*Foeniculum vulgare* Mill.) is a two-year, herbaceous plant from the Umbelliferae family whose seeds have many antioxidant and antimicrobial properties [11]. Among the valuable properties of fennel seed, its hepatoprotective, anti-inflammatory, and anticarcinogenic properties stand out. The presence of polyphenols can contribute to the antioxidant activity of this plant [12]. Since the antioxidant compounds cause the reduction of metal ions and increase the stability of nanoparticles, the task of green synthesis of nanoparticles is the responsibility of these compounds [13].

Reducing the required temperature and time, as well as saving energy, has made ultrasonic waves one of the best ways of extracting plants. Moreover, this technique could be employed on an industrial scale due to its low cost [14].

In this study, ZnO nanoparticles were synthesized via ultrasonic processing from *Foeniculum vulgare* Mill. seeds aqueous extract. Furthermore, their antibacterial properties were assessed against four of the most common gram-positive and gram-negative foodborne bacteria. In order to verify the nanoparticle synthesis, X-ray diffraction, UV-Vis spectrophotometry, Fourier transform infrared spectroscopy, field emission scanning electron microscopy, transmission electron microscopy, dynamic light scattering, and zeta potential were used.

2. Materials and Methods

2.1. Preparation of required materials and bacterial strains

Zinc nitrate hexahydrate ($Zn(NO_3)_2.6H_2O$) was purchased from BDH, England. The Fennel seeds were purchased from a local store in Mashhad (voucher sp.no E-1372 FUMH). Additionally, foodborne bacterial strains, including *Bacillus cereus* (PTCC 1015), *Yersinia enterocolitica* (PTCC 1785), and *E. coli O157:H7* (PTCC 1860) were obtained from the Iran Scientific and Industrial Research Organization. *Staphylococcus aureus* strain (PTCC 1784) was also provided from the microbiology laboratory of the Faculty of Science at Ferdowsi University of Mashhad.

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2.2. Preparation of herbal extract

First, fennel seeds were identified and approved by Research Center for Plant Sciences at Ferdowsi University of Mashhad (voucher sp.no E-1372 FUMH). After washing and drying the seeds away from direct light, the best ones were separated and ground. Then, the obtained aromatic powder was stored in a clean glass container covered with aluminum foil at 4 °C. In order to prepare the extract, 10 grams of herbal powder were mixed with 100 ml of distilled water, and the extraction process was performed in an ultrasonic device (Panasonic Japan model 2600s, frequency 28±5%) for 32 minutes at a temperature of 30°C. After that, the final extract was filtered with filter paper and stored in a dark-colored glass at refrigerator temperature.

2.3. Synthesis of ZnO nanoparticles

90 ml of 0.1 M zinc nitrate solution and 10 ml of aqueous plant extract were mixed. This solution was kept overnight in a constant incubator at 28 °C in the dark. The mixture was then washed several times with distilled water and centrifuged at 10,000 rpm for 15 minutes. The supernatant was discarded, and the remaining sediment was dried in an oven at 80°C for 3 hours. After grinding with a mortar, the powder was calcined for 2 hours in a furnace at 600°C. Finally, the white powder of ZnO nanoparticles was obtained (Fig. 1).

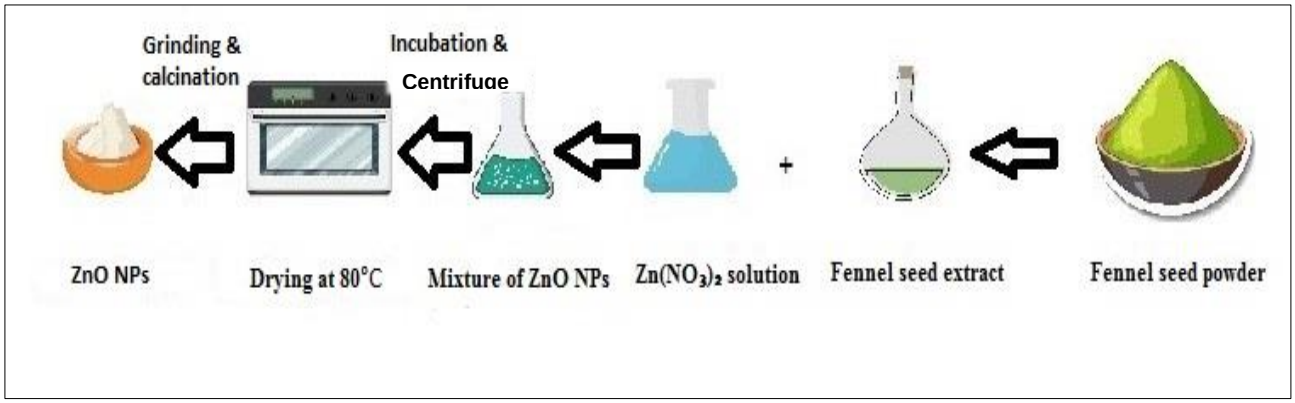


Fig. 1. Schematic figure of the stages of ZnO nanoparticle synthesis

2.4. Characterization of ZnO nanoparticles

XRD analysis was performed to determine the crystal size of synthetic nanoparticles. The phases were created at the wavelength of 1.541874 A˚ in the 2θ range of 20 to 80 degrees through Cu K (α). For the optical study of nanoparticles, UV-Vis analysis is carried out in the wavelength range of 190-899 nm. In order to check the type of connections and chemical morphology of nanoparticles, FT-IR is used in the wavelength range of 400-4000 cm. In addition, elemental analysis with EDAX and frequency distribution of existing elements with mapping were studied. DLS was used to measure the size of the particles. Also, for evaluating the stability of the particles, zeta potential was used. The method described by Espitia et al. was used to prepare a ZnO nanofluid. ZnO nanoparticles were dispersed using sodium pyrophosphate ($Na_4P_2O_7$) [15]. In this regard, 0.14 g of sodium pyrophosphate and 0.0026 g of ZnO nanoparticles were added to 15 ml of deionized water. Then the solution was dispersed for 2 minutes through a 200 W ultrasonic probe with a diameter of 1 cm. Finally, the structure and size of nanoparticles were analyzed by using TEM and FE-SEM.

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2.5. Investigating the antibacterial effects of ZnO nanoparticles on foodborne bacteria

The MIC of ZnO nanoparticles against foodborne bacteria including *S. aureus, Y. enterocolitica, E. coli O157:H7,* and *B. cereus* was evaluated using the microdilution method according to the CLSI protocol [16]. Accordingly, nanoparticles with a concentration of 2000 µg/ml were first added to the nutrient broth culture medium, which was then autoclaved for sterilization and stirred for 24 hours at room temperature [17]. Afterward, 100 microliters of a serial dilution of nanoparticles from 1000 to 1.953 µg/ml were added into each well in each row of a sterile microplate from left to right. In addition, 100 microliters of sterile nutrient broth medium were transferred to each well. A suspension of 0.5 McFarland turbidity (1.5×10^8 CFU/ml) was prepared from each bacterial strain using a spectrophotometer at a wavelength of 600 nm and diluted in a ratio of $\frac{1}{20}$ to reach a concentration of approximately 5×10^6 CFU/ml. Then, 20 µl of each bacterial suspension was added into each well of the microplate which was incubated for 24 hours at 37°C. Moreover, some wells in the microplate were considered positive controls (culture medium with only bacteria) and negative controls (culture medium alone) for each strain. To determine the MIC, 50 µl of 2,3,5-triphenyl tetrazolium chloride (TTC) was added to each well of the microplate which was incubated again for 1 hour at 37 °C. The minimum inhibitory concentration (MIC) of ZnO nanoparticles, which prevent the strains from growing caused the wells to remain colorless. In order to determine the minimal bactericidal concentration (MBC), 10 µL of bacterial suspensions were taken from wells corresponding to MIC, 2 × MIC, and 4 × MIC, plated on MHA. Then the plates were incubated at 37 °C for 24 hours. The minimum concentration of ZnO nanoparticles thatkilled all bacterial strains was determined as MBC. All experiments were repeated three times.

3. Results

Based on the results of the XRD analysis in Fig. 2, the peaks of the synthesized nanoparticles correspond to the standard peaks of JCPDS cards. The values of the lattice constants of the obtained morphology are also a = 3.24 and c = 5.20 A˚. As shown in the Debye-Scherrer equation (1):

$$D=0.9\lambda/\beta\cos\theta \qquad (1)$$

the average crystal size of synthesized ZnO nanoparticles is 34.54 nm. In this equation, D, λ, β, and θ refer to the crystal size of nanoparticles, the X-ray wavelength, the width at half the height of the phase peak, and the angle of the maximum diffraction peak, respectively [18]. Furthermore, the sharpness of the existing peaks confirms that the synthesized nanoparticles are crystalline in structure.

The optical properties of synthesized nanoparticles are shown in Fig. 2 by UV-Vis analysis. The absorption peak at 231 nm in Fig. 2 indicates the formation of ZnO nanoparticles. The acceptable range of ZnO nanoparticle absorption in UV-Vis spectroscopy is 200-400 nm [19].

FT-IR analysis determines the molecules' vibrations and functional groups in the sample structure, which is presented in Fig. 2.

According to the DLS results, the average diameter of the synthesized ZnO nanoparticles is about 66 nm (Fig. 3). ZnO nanoparticles have a zeta potential of approximately -35 mV at pH = 7.7 (Fig. 3), which indicates proper stability [20]. In addition, the nanoparticle dispersion

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index (PDI) is about 0.33, which reveals the appropriate homogeneity of the ZnO nanofluid.

Fig. 4 shows the FE-SEM images of ZnO nanoparticles produced at magnifications of 500 and 200 nm. The hexagonal state of the nanoparticles reveals that ZnO nanoparticles are formed (Fig. 4). In addition, the maps of zinc and oxygen elements of synthesized nanoparticles with a magnification of 500 nm are shown in Fig. 4.

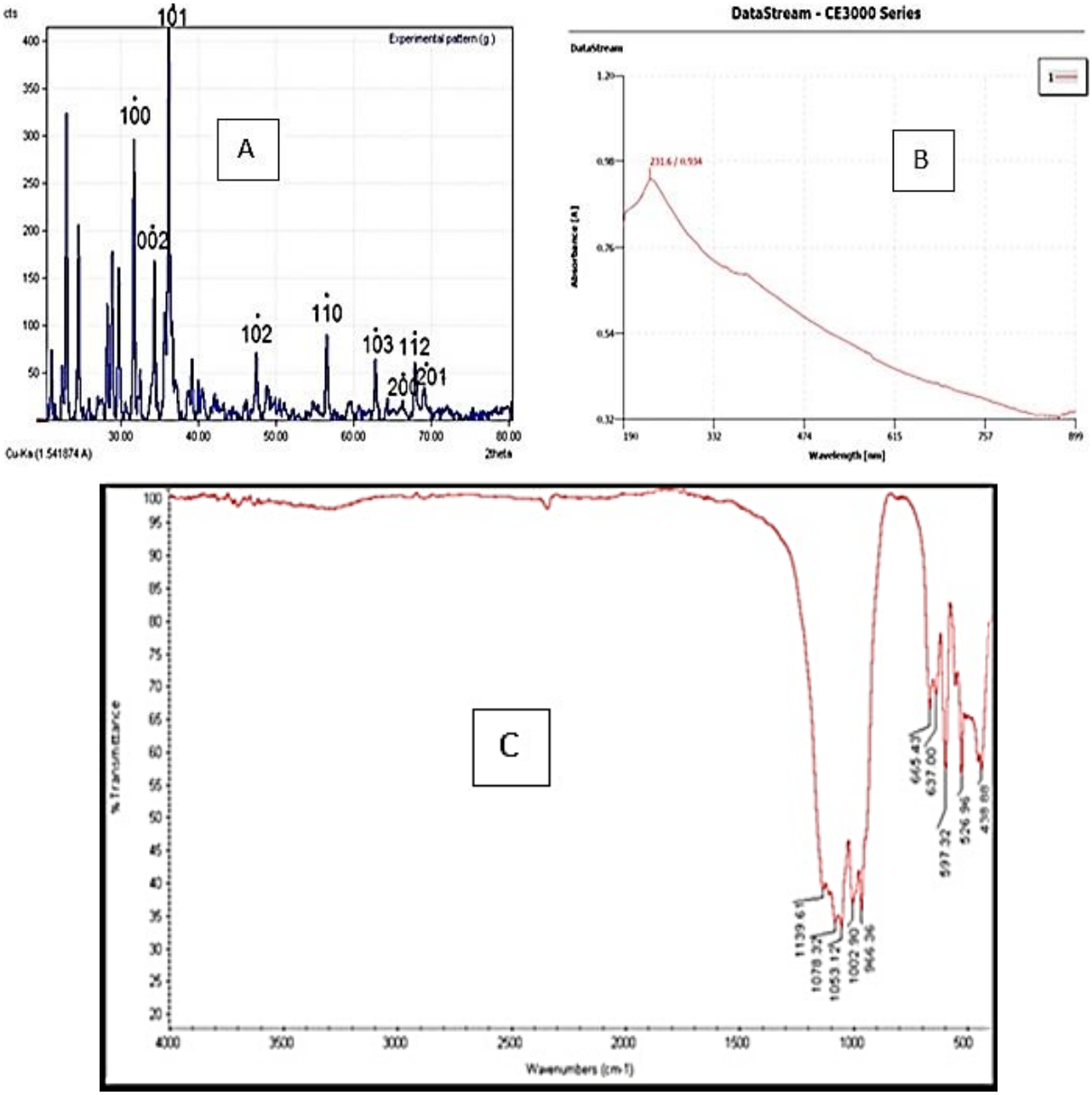


Fig. 2. (A) XRD pattern, (B) UV-Vis spectrum, and (C) FT-IR spectrum of synthesized ZnO nanoparticles.

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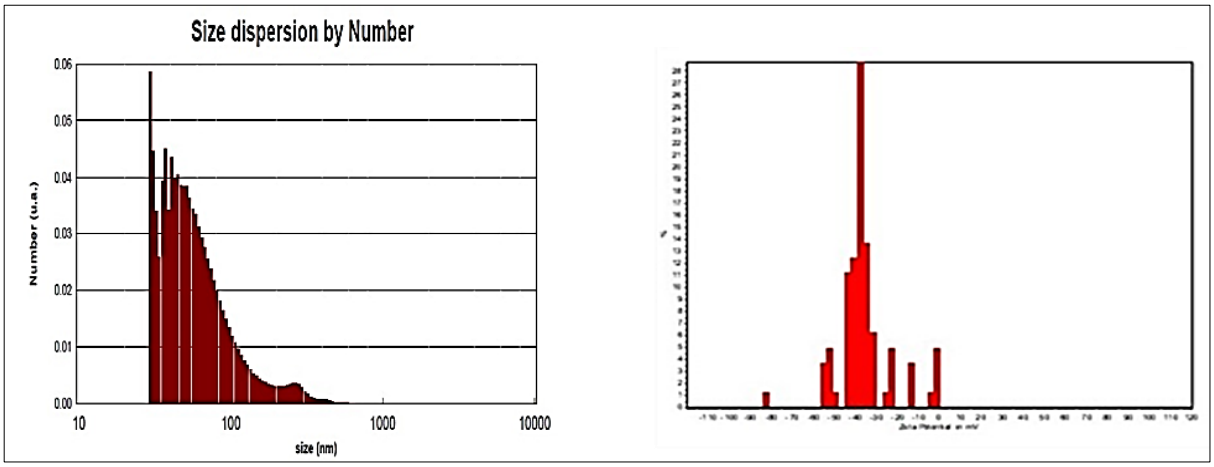


Fig. 3. (A) The average diameter image and (B) the zeta potential diagram of synthesized ZnO nanoparticles.

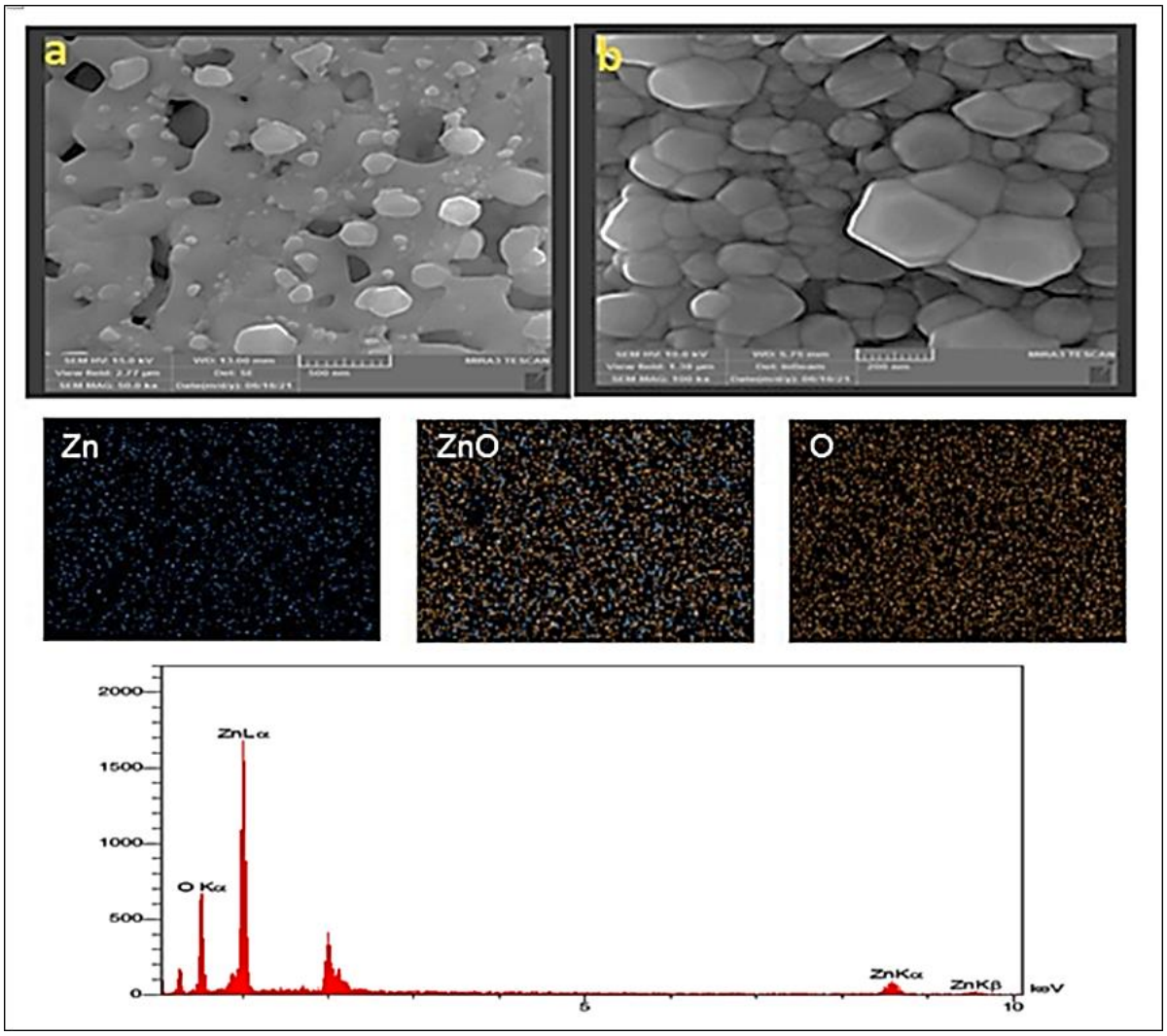


Fig. 4. (A) FE-SEM image with (a) 500 and (b) 200 nm magnification (B) maps of ZnO NPs, Zn, and O at 500 nm magnification, and (C) EDAX spectrum.

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The EDAX spectrum of ZnO nanoparticles in Fig. 4 confirms that zinc and oxygen are the major available elements in the sample.

The TEM image of synthesized nanoparticles in Fig. 5 depicts the hexagonality of the synthesized ZnO nanoparticles. The approximate size of ZnO nanoparticles is 50 nm, which is close to the result of the DLS analysis.

The size of ZnO nanoparticles is also an essential criterion for their performance. For example, it has been determined that ZnO particles with a size of 50-500 nm prevent the growth of bacteria, and the smaller ones are more effective [21].

In the case of measuring the antibacterial effects of ZnO nanoparticles against foodborne bacteria, the results are shown in Table 1.

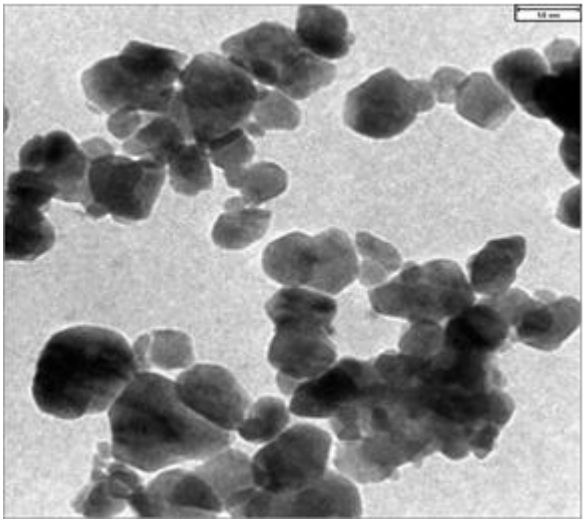


Fig. 5. Transmission electron microscope image of synthesized ZnO nanoparticles

Table 1. MIC* and MBC** results of ZnO nanoparticles against foodborne bacteria

	MIC (µg/ml)	**MBC (µg/ml)**
B.cereus	500	500
E.coli O157:H7	62.5	-
S.aureus	31.25	-
Y.enterocolitica	62.5	-

*: Minimum Inhibitory Concentration
**: Minimum Bactericidal Concentration

4. Discussion

Although there are different ways to produce nanoparticles, biological methods are preferred for many reasons, such as cost-effectiveness in terms of time and cost, ease of working with biological resources, and higher environmental security [22]. Moreover, among the green synthesis resources, plants pose a much lower risk than microorganisms, which is crucial in the transfer of medicinal substances [23]. The seeds of medicinal plants such as fennel, fenugreek, *Mentha pulegium*, *Ficus carica*, and *Silybum marianum*, which contain phenols and antioxidants can contribute to nanoparticle synthesis [13, 24-27]. Among the extraction methods, ultrasound waves, especially the ultrasonic bath, are recommended due to their indirect interaction with the extract, which could be harmless to the substances. In addition, frequencies between 20 and 40 kHz can generally

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prepare a higher-quality extract [28]. While other extraction methods, such as soxhlet and maceration, are time and energy-consuming, ultrasound could be a top priority. Soxhlet extraction also has other drawbacks with negative impacts on the environment, such as using large quantities of samples and expensive flammable solvents [29]. Also, sensitive compounds to temperature are degraded by applying the soxhlet method [30]. Further, forthcoming purification is crucial to maceration extraction [31]. It is worth mentioning that extraction by ultrasonic waves is also beneficial for plants containing polyphenols [32]. Many researchers have shown that herbal extracts can be used to synthesize various nanoparticles, including ZnO, TiO2, and iron oxide [33-36]. Compared to other nanoparticle synthesis methods like microwaves, ultrasound produces smaller and more monotonous nanoparticles, which is more effective for the biosynthesis of metal nanomaterials [37]. Thus, ultrasound can increase extraction yields while minimizing negative effects on compound quality, for instance, by facilitating solvent penetration [32, 38]. ZnO nanoparticles have numerous properties in the pharmaceutical, food, electronic, and medical industries. In addition, they have been approved for use by the FDA; have received much attention [39, 40].

As shown in Fig. 2, the XRD peaks, which include 100, 002, 101, 102, 110, 103, 200, 112, and 201, represent the hexagonality of ZnO nanoparticles which is the most stable state possible [41, 42].

The absorption FT-IR peaks around 438, 526, and 597 cm^{-1} indicate stretching vibrations of the Zn-O bond, confirming the formation of ZnO nanoparticles. The region between 400 and 600 cm^{-1} indicates the absorption spectra of the Zn-O bond [43]. Additionally, the absorbed spectrum at 1053 cm^{-1} demonstrates the C-OH stretching band [44]. Wave numbers between 675 and 1000 cm^{-1} correspond to absorption spectra of the = CH bond [45], which appears around 966 cm^{-1}.

The shape of the ZnO nanoparticle has unique features. For example, it has been reported that this hexagonal nanoparticle has a better effect against resistant bacteria such as *S. epidermidis* and other critical pathogenic bacteria such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* [46].

According to the study of Thi et al. in 2020, who synthesized ZnO nanoparticles using orange peel extract, increasing the temperature for baking nanoparticles has a direct relationship with increasing their size. In addition, the weight loss of these nanoparticles, which were hexagonal between 500 and 900 °C, was shallow [47]. Likewise, in 2018, Khatami et al. set the calcination conditions of nano ZnO at 600 °C for 2 hours, which resulted in the formation of particles with a size of 10-90 nm [48]. Rafique et al. used plant extract to synthesize ZnO nanoparticles. According to peaks such as 100, 101, 102, 110, 112, 002, and 103, they realized the existence of the hexagonal structure of these nanoparticles [49].

According to the antibacterial effect, *S. aureus* and *B. cereus* were the most sensitive and resistant strains to ZnO nanoparticles, respectively, because they are different in their cell wall composition. Gram-negative bacteria have negative electrical charges and lipopolysaccharide, which make it much easier for ZnO nanoparticles to adhere to their membrane [50]. While gram-positive bacteria have a thick peptidoglycan cell wall probably making them more resistant to ZnO nanoparticles. Barani et al. reported the MIC of ZnO nanoparticles produced by the green method against *B. subtilis, L. innocua, P. aeruginosa,* and

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E. coli bacteria which was 500, 1000, 62.5, and 125 µg/ml, respectively [17]. In a study performed by Chegini et al., the ZnO nanoparticles produced from ethanolic extracts of *Satureja sahandica Bornm* plant leaves showed MICs of 625 and 1250 µg/ml against gram-negative bacteria, *P. aeruginosa* and *E. coli*, as well as 625 µg/ml and 2500 µg/ml against gram-positive bacteria, *B. cereus* and *S. aureus*, respectively [51].

Overall, in this study, ZnO nanoparticles were synthesized from an aqueous extract from *Foeniculum vulgare* Mill. seeds by ultrasonic extraction. Moreover, their antibacterial effect was evaluated against some of the most important foodborne bacteria. Primary identification of biosynthesized nanoparticles was confirmed via UV-Vis spectroscopy and XRD. Nanoparticles averaged 50 nanometers in size, based on TEM analysis. The minimum inhibitory concentration of hexagonal ZnO nanoparticles against gram-negative bacteria including *Y. enterocolitica*, *E. coli O157:H7*, *S. aureus*, and *B. cereus* achieved better results than the ones obtained by the researchers mentioned earlier, probably due to their size. As a consequence, ZnO nanoparticles can be used in the food industry due to their antibacterial properties.

5. Conclusion

This study focuses on fennel seeds to biosynthesize ZnO nanoparticles by using ultrasonication. The nanoparticles were also assessed for antibacterial activity. According to the different characterization analyses like UV-Vis, XRD, EDX, FT-IR, DLS, zeta potential, TEM, and FE-SEM, ZnO nanoparticles were synthesized successfully. Additionally, these nanoparticles have the appropriate function of inhibiting the growth of foodborne bacteria.

Author Contribution

P.S carried out the laboratory work and composed the manuscript; M.G.M and M.R.S supervised the project, reviewed and edited the Draft; M.B whose involvement in this project was advisory.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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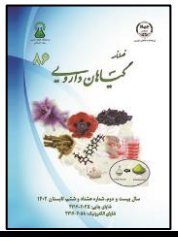
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مقاله تحقیقاتی

سنتز نانوذرات اکسید روی با کمک عصاره بذر گیاه رازیانه و تأثیرات ضدباکتریایی آن بر باکتری‌های منتقله از راه غذا

پریسا سالاری[۱]، منصور غفاری مقدم[۲]، معصومه بحرینی[۱]، محمدرضا شریف مقدم[۱،*]

[۱] گروه زیست شناسی، دانشکده علوم، دانشگاه فردوسی مشهد، مشهد ایران

[۲] گروه شیمی، دانشکده علوم، دانشگاه زابل، زابل، ایران

اطلاعات مقاله

گل‌واژگان:
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عصاره بذر
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منتقله از راه غذا

چکیده

مقدمه: بذرهای رازیانه حاوی ترکیبات پلی فنولی بوده که می‌تواند بعنوان گزینه مناسبی برای سنتز نانوذرات در نظر گرفته شود. **هدف:** در این مطالعه، نانوذرات اکسید روی (ZnO) با استفاده از عصاره آبی بذر رازیانه سنتز شد و فعالیت ضدباکتریایی آنها علیه باکتری‌های منتقله از راه غذا ارزیابی شد. **روش بررسی:** ویژگی‌های نانوذرات ZnO سنتزشده با استفاده از آنالیزهای مختلف مشخص شدند. کمترین غلظت بازدارنده از رشد و کمترین غلظت باکتری‌کشی این نانوذرات بر باکتری‌های استاندارد منتقله از راه غذا شامل استافیلوکوکوس اورئوس، یرسینیا انتروکولیتیکا، اشریشیا کلی (O157:H7) و باسیلوس سرئوس به روش میکرودایلوشن بررسی شد. **نتایج:** با توجه به نتایج حاصل از طیف سنجی مرئی- فرابنفش، نانوذرات ZnO در طول موج ۲۳۱ nm دارای قله جذبی بودند که تأیید کننده تشکیل شدن این نانوذرات می‌باشد. به‌علاوه، الگوی پراش اشعه ایکس مطابق با کارت‌های JCPDS و به معنای تشکیل نانوذرات ZnO می‌باشد. براساس یافته‌های میکروسکوپ الکترونی، نانوذرات تولیدی در این پژوهش شکل هگزاگونال داشتند و به طور تقریبی میانگین اندازه نانوذرات هم ۵۰ nm بود که با اندازه نتایج بدست آمده از آنالیز پراکندگی دینامیکی نور قرابت دارد. همچنین، کمترین غلظت بازدارنده از رشد باکتری‌ها توسط نانوذرات ZnO برای اشریشیا کلی (O157:H7)، یرسینیا انتروکولیتیکا، استافیلوکوکوس اورئوس و باسیلوس سرئوس به ترتیب برابر با ۶۲/۵، ۶۲/۵، ۳۱/۲۵ و ۵۰۰ میکروگرم در میلی‌لیتر بدست آمد. **نتیجه‌گیری:** نانوذرات ZnO به کمک عصاره بذر رازیانه سنتز شد که اثر ضدباکتریایی مناسبی علیه باکتری‌های منتقله از راه غذا داشت.

مخففها: FDA، سازمان غذا و داروی آمریکا؛ UV-Vis، طیف سنجی فرابنفش- مرئی؛ XRD، طیف سنجی پراش اشعه ایکس؛ JCPDS، کمیته مشترک استانداردهای پراش پرتو ایکس؛ FT-IR، طیف سنج تبدیل فوریه فروسرخ؛ FE-SEM، میکروسکوپ الکترونی روبشی نشر میدانی؛ TEM، میکروسکوپ الکترونی عبوری؛ DLS، پراکندگی نور دینامیکی؛ EDAX، طیف سنجی پراش انرژی پرتو ایکس؛ ZnO، اکسید روی؛ $Zn(NO_3)_2.6\ H_2O$، روی اکسید ۶ آبه؛ $Na_4P_2O_7$، سدیم پیروفسفات؛ MIC حداقل غلظت مهار کننده؛ MBC، حداقل غلظت کشندگی؛ TTC، ۲،۳،۵- تری فنیل تترازولیوم کلراید؛ PDI، شاخص پراکندگی

* نویسنده مسؤول: Sharif@um.ac.ir

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