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Research Letter

Boron-reinforced zinc oxide nanoparticles produced by the hydrothermal method: A novel antimicrobial agent

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Abstract

In this study, boron-doped zinc oxide (B/ZnO) nanoparticles (NPs) were synthesized using the hydrothermal method. Different boron (B) concentrations (5%, 10%, 15%, and 20% by weight) were chosen to produce B/ZnO nanocomposites. The antibacterial and anti-biofilm properties of the characterized B/ZnO NPs were also investigated against some pathogenic microorganisms. The NPs had a significant inhibitory effect on the microorganisms. The anti-biofilm analysis revealed that these NPs inhibited the biofilm formed by both *Escherichia coli* and *Pseudomonas aeruginosa* bacteria. Significantly, 20% B-doped ZnO nanocomposites are the most effective nanocomposite and can be used as an alternative to antibiotics for antimicrobial therapy.

Introduction

Antimicrobial resistance has become an increasingly widespread public health problem worldwide. Resistance to antibiotics in infections caused by microorganisms is one of the most important factors that increase morbidity and mortality in infected individuals, negatively affecting healing.[1] Antibiotics are a widely used as a treatment method in the fight against infections; however, their widespread use has led to the emergence of resistant bacteria. Antibiotics are also one of the main strategies used to treat biofilm infections.[2] Biofilms, produced by most pathogenic bacteria, constituted a polymeric layer of sugars, nucleic acids, and proteins that function on biotic and abiotic surfaces to aggregate, adhere, and multiply. The diversity of components in the biofilm structure contributes to the development of resistance, and microorganisms in biofilms show very strong resistance to antimicrobial agents.[3] Hence, novel treatment strategies are needed to overcome bacterial resistance and eliminate biofilm-forming bacteria.

Today, metal oxides are widely used in various fields of science, such as chemistry, biology, physics, and medicine.[4] Metal oxide nanostructures are remarkable materials for scientific applications due to their unique physical and chemical properties. Many numerous methods are used to produce metal oxide nanoparticles (NPs). Liquid phase production techniques such as microwave-assisted synthesis,[5] microemulsion,[6] green synthesis,[7] and hydrothermal/solvothermal[8] are among the techniques detailed in the literature. Among these, hydrothermal synthesis is the most widely utilized environmentally friendly approach due to its cost-effectiveness, straightforward experimental procedures, and production of homogeneous and pure materials.[9]

Recently, NPs have been successfully used in pharmaceutical applications. Their demonstrated antimicrobial properties against numerous pathogenic microorganisms, coupled with their biocompatibility, small size, and expansive surface area, render these materials highly attractive antimicrobial properties.[10] Metal-based NPs such as silver (Ag), copper (Cu), and gold (Au), and metal oxides such as zinc oxide (ZnO), magnesium oxide (MgO), titanium dioxide (TiO_2), and copper oxide (CuO) are known to inhibit the growth of biofilms.[1,11–13] Among these, ZnO has attracted more attention due to its superior properties, including a high surface-to-volume ratio, low cost, and long-term environmental stability. Many researchers have reported potent antimicrobial and anti-biofilm activity of ZnO NPs and suggested as a therapeutic agent for treating biofilms and drug-resistant bacteria.[5,7,14]

Boron, acting as an antibiotic and antiseptic, has therapeutic properties including antifungal, anticoagulant, antidiabetic, antihypertensive, nociceptive, antiparasitic, and antiviral. However, it has also been recognized for its functionality in host defense mechanisms.[15] The high adsorption rate of boron increases its use in drug development diagnosis and treatment.[16] The role of this compound with these superior properties in NP synthesis and biological applications has still not been clarified, and research on this subject has remained quite limited. In the literature, NPs have been synthesized using components such as boron nitrate and boron oxide, and have been reported to have promising antimicrobial effects.[17,18] In a

study, the photoluminescence, photocatalytic and antimicrobial properties of boron-doped TiO_2 nanoparticles obtained by a microwave-assisted solvothermal method have been investigated. The TiO_2 NP obtained by adding 8% B showed more inhibitory activity against *S. aureus* and *E. coli* bacteria than the boron-free TiO_2 NP.[19] Additionally, synthesized bimetallic boron oxide-zinc oxide nanoparticles exhibited strong anticancer, antimicrobial and antioxidant activities.[20] Nevertheless, there is lack of information regarding the synergistic effect of Boron and ZnO at varying concentrations.

This study synthesized boron ZnO (B/ZnO) nanocomposites (NCs) with different B ratios using hydrothermal methods. X-ray diffraction (XRD), scanning electron microscopy (SEM), and Raman spectroscopy were used to confirm the structural and morphological characterization of the synthesized B/ZnO NPs. The antimicrobial and anti-biofilm potentials of the synthesized NCs were then investigated. The synergistic effect that occurs when B and ZnO NPs are conjugated is the subject of interest in this study. No study was found in the literature regarding the antimicrobial and anti-biofilm properties of B-doped ZnO NCs. In this context, this research has the potential to be the first of its kind, making an important contribution to the literature.

Materials and methods

Boron-doped zinc oxide nanocomposite synthesis

Zinc chloride ($ZnCl_2$, reactive grade 98%, Sigma Aldrich), hexamethylene tetramine ($C_6H_{12}N_4$, HMT, Merck), and boric acid (H_3BO_3, 98.5%, Sigma Aldrich) were used in the synthesis of NPs by the hydrothermal method with $ZnCl_2$ and B_2O_3 as the precursor solution.

In total, 0.1M $ZnCl_2$, 0.1M H_3BO_3, and 0.1M $C_6H_{12}N_4$ were dissolved in 100 mL of deionized water. The solution was placed in a Teflon-coated autoclave and kept in a muffle furnace at 140 ℃ for 3 h. B/ZnO particles filtered from the solutions and allowed to cool to room temperature were washed with deionized water to remove the organic components, then annealed in air at 450 ℃ for 1 h for characterization. B/ZnO NPs were obtained by doping different proportions of B (5%, 10%, 15%, 20% by wt) to ZnO.

Boron-doped zinc oxide nanocomposite characterization

XRD was used to investigate the crystal structures of B-doped ZnO nanocomposites by weight using a Philips X'Pert Pro X-ray diffractometer with Cu-Kα radiation. The morphologies of the particles were observed by SEM with a Zeiss EVO 10LS scanning electron microscope. A 302 mW diode laser portable Raman spectrometer (BWS465 B&W Tek Inc.) with 785 nm and 3 cm^{-1} resolution was used for structural measurements.

Antibacterial activity

The antimicrobial activity of B-doped ZnO NPs was determined by the agar well diffusion method against bacteria strains including *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 14579, *Enterococcus faecalis* ATCC 29212, and *Staphylococcus epidermidis* ATCC 12228. In this method, 100 µL of ZnO samples were added into wells of inoculated agar plates from 1-night-activated cultures and incubated at 37℃ for 24 h. Antimicrobial activity was determined by measuring the inhibition zone diameter (mm). The antimicrobial activity was calculated by averaging of three independent experiments.[21]

Determination of the minimum inhibitory concentration

The minimal inhibitory concentration (MIC) was determined using a modified standard of the Clinical and Laboratory Standards Institute (CLSI) liquid microdilution method. *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, *B. cereus* ATCC 14579, *E. faecalis* ATCC 29212, and *S. epidermidis* ATCC 12228 strains were used. MIC assay was performed in 96-well plates using tryptic soy broth (TSB) medium. Cultures were diluted to 1×10^6 CFU/mL after a 1-night incubation at 37℃, and 50 µL of bacteria were inoculated into each well. B-doped ZnO NPs were added to the 96-well plates at different 50 µL concentrations (256–0.5 µM) and incubated at 37℃ for 18 h. The minimum concentration with no visible bacterial growth was defined as the MIC. Gentamicin (Sigma Aldrich, USA) was used as a positive control.[22]

Anti-biofilm activity

Biofilm-producing *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 strains were used to determine the anti-biofilm activity of B-doped ZnO NPs. Following serial dilutions, 100 mL of test compounds (1.2–20 mg/mL) and 100 mL of bacterial culture (OD 600 = 0.132) activated in TSB medium at 37℃ were transferred to 96-well polystyrene microtiter plates. After 24 h of incubation at 37℃, cells adhered to the wells were rinsed carefully with distilled water and allowed to air dry. The dyeing process was carried out with 0.4% (w/v) crystal violet in an aseptic environment and then rinsed with distilled water. The crystal violet was dissolved with 200 mL of ethanol. TSB medium (100 mL) was used as a negative control, and bacterial cultures without a compound were used as a positive control. The wells were measured spectrophotometrically at 595 nm (BioTek Epoch 2 Microplate). The study was repeated three times.[23] Anti-biofilm activity was determined by the formula:

$$\text{Anti} - \text{biofilm activity (\%)} = (1 - \text{OD sample/OD control}) \times 100.$$

Statistical analysis

Chi-square statistics were used for statistical analysis in all study descriptive statistics groups. The significance of (P-0.01) was determined. Social Science Statistical Package

for Windows (SPSS) was used to perform all analyzes (version23.0, SPSSInc, Chicago, III).

Results

Boron-doped zinc oxide nanocomposite characterization

B-doped ZnO nanocomposite materials were successfully synthesized using the hydrothermal method. B/ZnO NPs were produced using different B concentrations (5%, 10%, 15%, and 20% by weight). Structural characterization of B/ZnO NCs showed that they were formed in the ZnO phase with a hexagonal wurtzite structure. The XRD peak intensity decreased systematically with the addition of B. Morphological analyses exhibited that pure ZnO and B/ZnO NPs grew as spherical hexagonal rods [Fig. 1(a)]. The SEM images showed that the hexagonal bars got smaller as B supplementation increased. As a result, it was determined that B supplementation disrupted the crystal structure of ZnO. SEM images of ZnO and B-reinforced ZnO (5% B/ZnO, 10% B/ZnO, 15% B/ZnO, and 20% B/ZnO) particles are shown in Fig. 2. The pure ZnO particles grew as micro-size spherical hexagonal rods. As the B supplementation increased, the hexagonal rods became smaller and nano-size. It was observed that 5% B/ZnO and 10% B/ZnO nanocomposites were again in the form of spherical hexagonal rods, but they were not homogeneous. Their dimensions were determined to be around 200 nm. Particle sizes of 15% B/ZnO and 20% B/ZnO NPs were between 80 and 100 nm.

The Raman spectra of ZnO and B-doped ZnO (5% B/ZnO, 10% B/ZnO, 15% B/ZnO, and 20% B/ZnO) NPs are shown in Fig. 1(b). From the Raman spectra of all NPs, peaks belonging to the E2 high vibration mode were observed around 437 cm^{-1} for hexagonal wurtzite ZnO particles.[24] The peak (E2H–E2L) around 327 cm^{-1} corresponds to the multiphonon mode. A shift E1(LO) peak of 584 cm^{-1} was not found.

Antimicrobial activity

The antibacterial activity of newly synthesized B-doped ZnO NCs was evaluated against different bacterial strains. The results demonstrated that these NCs exhibited strong antimicrobial activity (Table I) against clinically significant pathogens such as *E. coli, P. aeruginosa* and *B. cereus*. Considering the antimicrobial activity of B-doped ZnO NCs at different rates, it was observed that the effectiveness of ZnO NPs was lower, and the antimicrobial activity increased compared to the added B ratios. As seen in Fig. 3(a), the antimicrobial activity was higher at the 20% ZnO:B ratio depending on the concentration. Particularly, 20% B/ZnO NCs exhibited a low MIC value against the microorganisms studied. The MIC values of the components exhibited similar results when compared to the gentamicin.

Anti-biofilm activity

E. coli ATCC 25922 and *P. aeruginosa* ATCC 27853 reference strains were used to determine the anti-biofilm activity of ZnO NCs containing different concentrations of B compounds. An increase in biofilm inhibition was observed depending on both the concentration and the percentage of boron. At a 20 mg/ml concentration, the 20% B/ZnO compound showed a biofilm inhibition of 46.14% for *E. coli* ATCC 25922 [Fig. 3(b)] and 42.21% for *P. aeruginosa* ATCC 278533 [Fig. 3(c)]. The anti-biofilm effect of the tested compounds on *E. coli* was higher than on the *P. aeruginosa* strain.

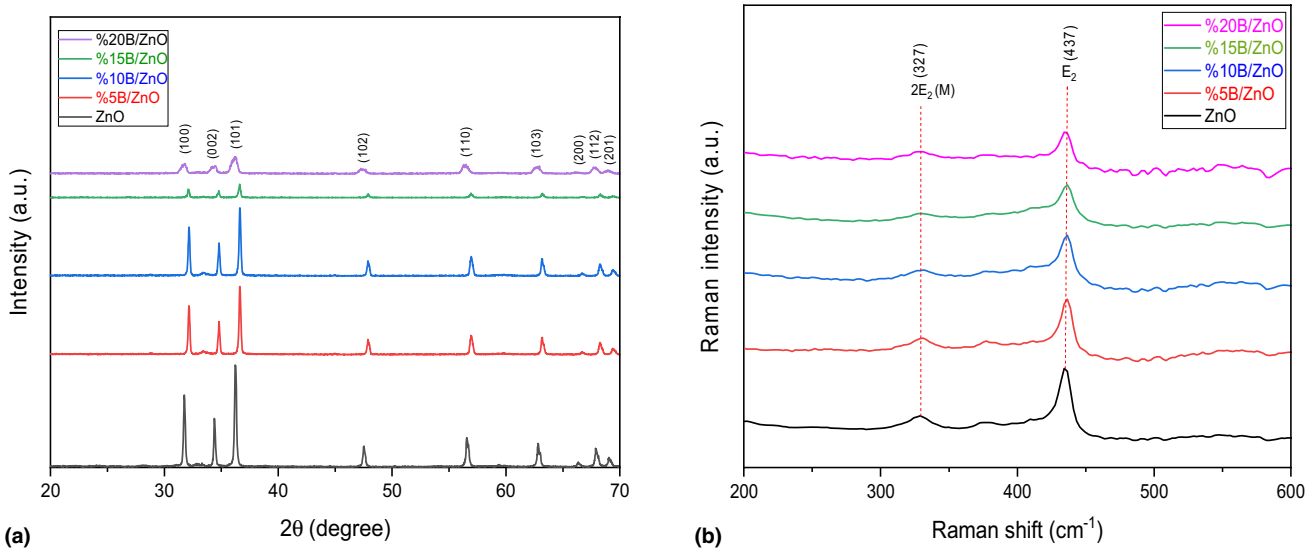


Figure 1. (a) X-ray diffraction patterns of zinc oxide and boron-doped zinc oxide nanoparticles. (b) Raman spectra of the nanoparticles.

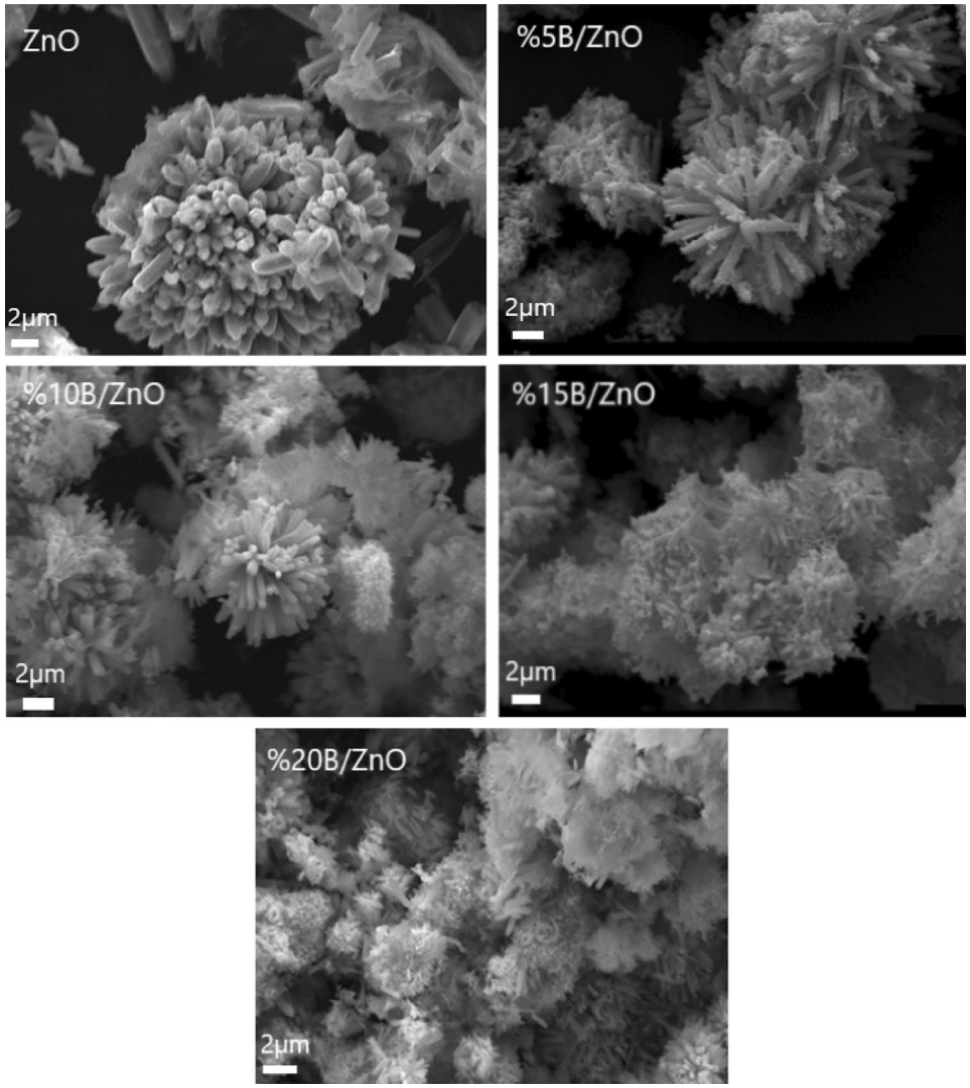


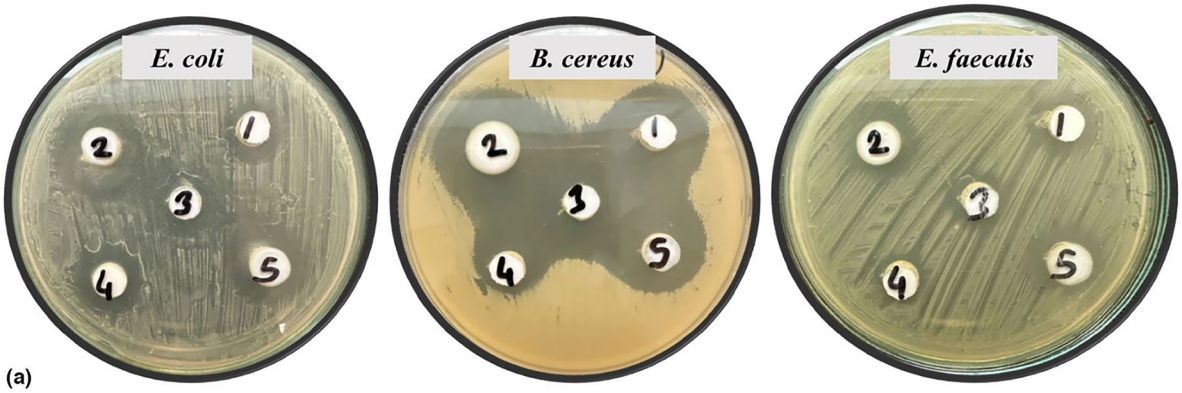
Figure 2. Scanning electron microscope images of zinc oxide and boron-doped zinc oxide nanoparticles.

Table I. Zinc oxide and boron-doped zinc oxide nanoparticles' effects on pathogenic strains.

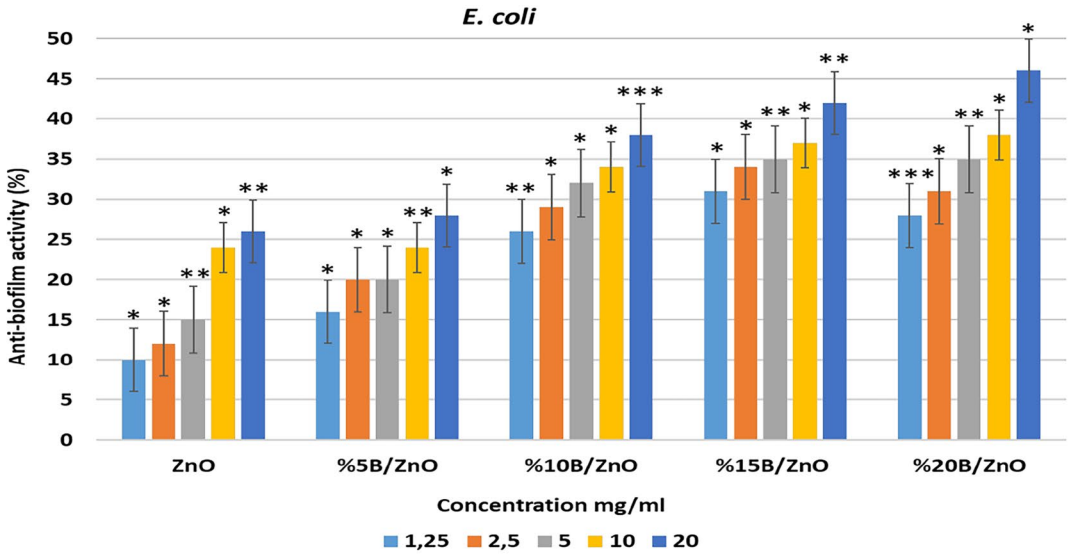
Microorganism Nanoparticle	*E. coli ATCC* 25922	*P.aeruginosa ATCC* 27853	*S. aureus ATCC* 25923	*E. faecalis* ATCC 29212	*S. epidermidis* ATCC 12228	*B. cereus ATCC* 14579
Antimicrobial zone diameter						
ZnO	14±0.3	11±0.7	11±1.3	16±0.4	16±0.4	27±0.8
5% B/ZnO	17±1.2	17±0.4	14±0.6	20±0.7	18±0.8	25±0.4
10% B/ZnO	15±1.5	17±0.9	16±0.4	15±1.6	22±1.2	24±1.1
15% B/ZnO	20±0.7	20±1.6	18±0.3	14±0.7	21±1.3	20±0.6
20% B/ZnO	21±0.4	25±0.9	25±1.0	18±1.3	22±0.9	26±0.7
MIC[a] (uM)						
ZnO	32	128	128	32	16	0.5
5% B/ZnO	16	16	64	16	8	0.5
10% B/ZnO	8	16	16	8	2	0.5
15% B/ZnO	4	8	16	8	4	1
20% B/ZnO	2	1	1	2	2	0.5
CN[b]	0.5	1	0.5	1	1	0.5

[a]Minimum inhibitory concentration (MIC) was determined as the lowest concentration that inhibits bacterial growth determined in three independent experiments performed in triplicate.

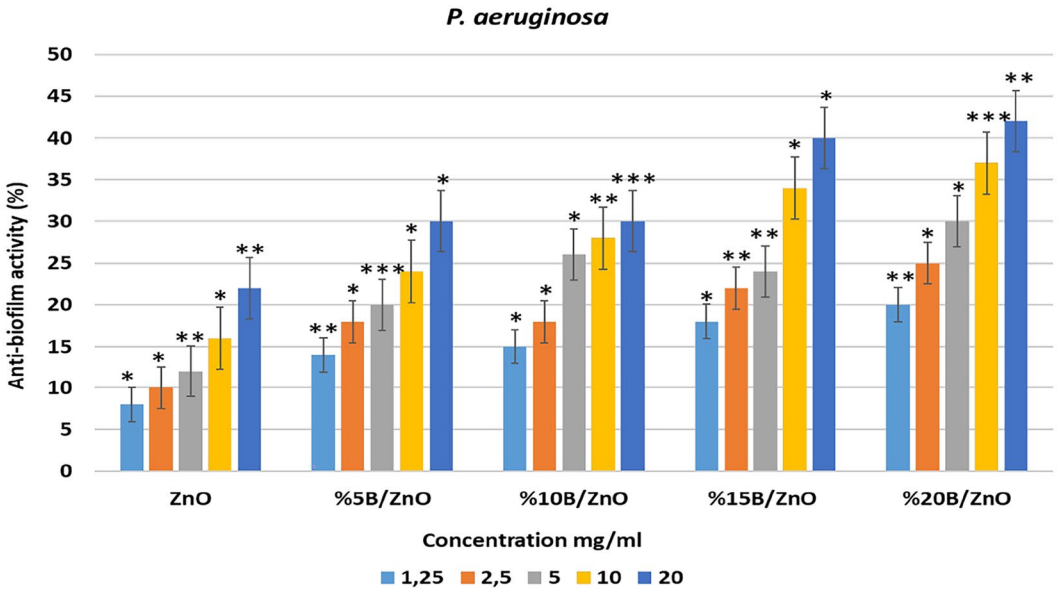
[b]CN: Gentemicin, B/ZnO: boron/zinc oxide.



(a)



(b)



(c)

Figure 3. (a) Inhibition zone activities of the nanoparticles on pathogenic strains, 1: ZnO, 2: 5% B/ZnO, 3: 10% B/ZnO, 4: 15% B/ZnO, and 5: 20% B/ZnO. (b) Anti-biofilm activity of the nanoparticles on *E. coli* (c) Anti-biofilm activity of the nanoparticles on *P. aeruginosa*. The statistical significance values ***, **, and * denote significance at $p < 0.0001$, $p < 0.01$, and $p < 0.05$, respectively, whereas 'ns' denotes non-significance.

Discussion

The distinctive properties of NPs, such as nano-size, high surface-to-volume ratio, ability to move at the cellular level, surface adaptability, multifunctionality, and biocompatibility, have made their use possible as an alternative microbial therapy. ZnO NPs are considered less toxic and biosafe among metal-based NPs. They have been used effectively in the fight against many diseases, including in the diagnosis and treatment of cancer, and even as a drug carrier.[25] It is known that ZnO NPs have a broad-spectrum antimicrobial effect on many microorganisms, including different drug-resistant pathogens.[26] The potent antimicrobial effects of these NPs are associated with their ability to generate reactive oxygen species (ROS) and induce cell apoptosis. NPs interact directly with the bacterial cell wall. NPs penetrating into the cell through nano-sized pores in the bacterial cell membrane trigger ROS production. This damages the DNA and proteins of the bacteria and causes irreparable oxidative damage.[7,27] In a study by Kaushik et al. on the antimicrobial activity and wound healing potential of ZnO NPs, Their findings revealed a strong microbial effect against *E. coli, S. aureus, S. enterica typhimurium*, *Aspergillus flavus, Aspergillus fumigatus*, and *Candida albicans* Additionally, they noted that smaller-sized ZnO NPs exhibited higher activity. They also reported that ZnO NPs played an active role in wound healing using fibroblast cells.[28]

Infections caused by antibiotic-resistant bacteria are recognized as a major global health threat. Most of these microbial infections are associated with biofilm. *E. coli* and *P. aeruginosa* are among the most important biofilm-producing microorganisms associated with infection. Biofilm is the extracellular polysaccharide (EPS) matrix adhering microorganisms. The structure of EPS consists of polysaccharides, proteins, glycolipids and extracellular DNA (eDNA). By forming a biofilm, many microorganisms can protect themselves from effects such as host immune response, antimicrobial therapy, and adverse environmental conditions. EPS in the biofilm limits or slows down the diffusion of antibiotics.[3] The researchers reported that ZnO NPs synthesized in different sizes showed significant antibacterial and antibiofilm activity against Gram-positive *Staphylococcus aureus* and Gram-negative *Proteus vulgaris*. At 250 µg/ml ZnO NP concentration, maximum biofilm inhibition of 67.3% and 58.18% was observed against *S. aureus* and *P. vulgaris*.[14] These findings are consistent with our study results and indicate that ZnO NPs are dangerous for the tested bacterial species. This means that ZnO NPs have great potential as an alternative antibacterial agent to conventional antibiotics.

However, the conjugation of NPs with certain materials can increase their antimicrobial effect. For instance, Ahmad et al. synthesized ZnO, ZnO–Cu, and rGO/ZnO-Cu nanomaterials and evaluated their antimicrobial properties. Their findings showed that all nanomaterials inhibited microorganisms, but the rGO/ZnO-Cu NCs had a greater microbicidal effect than the other NPs.[29] The use of chitosan, a polymer substance, together with ZnO to form nanocomposites, caused a stronger inhibition of pathogenic microorganisms compared to ZnO NPs.[30] When the enzyme pancreatin (PK) was added to ZnO NPs and its antibacterial, anti-biofilm activity and mechanism of action were investigated against methicillin-resistant *Staphylococcus aureus* (MRSA), ZnO NPs-PK was shown to have antibacterial, anti-biofilm, anti-motility, and anti-virulence properties against MRSA. They also found that ZnO NPs-PKs were more potent in eradicating MRSA than compared to ZnO NPs or PK alone. In another study, bimetallic boron oxide–zinc oxide nanoparticles (B_2O_3–ZnO NPs) were synthesized and examined for their anticancer, antimicrobial, and antioxidant activities. B_2O_3–ZnO NPs exhibited promising antibacterial activity against *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus*. Moreover, B_2O_3–ZnO NPs displayed antioxidant and anticancer activities against Caco 2 (Caucasian colon adenocarcinoma) cell line.[20] Furthermore, boron-doped TiO_2 NPs synthesized at different rates using microwave-assisted solvothermic method were analyzed their antibacterial properties against *S. aureus* and *E. coli*. As boron addition increased, TiO_2 NPs caused an increase in the inhibition zones of *S. aureus* and *E. coli* bacteria from 8.66 mm to 15.61 mm and from 9.04 mm to 13.65 mm, respectively. These results are compatible with our study.[19] In the present study, we examined the microbial effect of the B mineral, known for its beneficial impact on human health, when conjugated with ZnO NPs. We observed an enhancement in the antimicrobial and anti-biofilm effects with increased B supplementation. This synergistic effect may present a new treatment strategy for drug-resistant microorganisms.

Conclusion

In this study, B-doped ZnO nanomaterials were successfully synthesized via hydrothermal method with different B additions (5–10–15–20% at weight Examination of the morphological structures of the synthesized NPs revealed spherical hexagonal rods for both pure ZnO and B/ZnO NPs. The hexagonal rod shapes were found to decrease with increasing boron reinforcement, and particle sizes of 15% B/ZnO and 20% B/ZnO NPs were between 80 and 100 nm. Structurally, B/ZnO nanocomposites exhibited a hexagonal wurtzite configuration. All synthesized NPs showed the strongest antimicrobial effect on *E. coli*, *P. aeruginosa* and *B. cereus* pathogens. Moreover, this inhibitory property was observed to escalate with higher concentrations of B supplementation. The 20% B/ZnO compound showed a biofilm inhibition of 46.14% for *E. coli* and 42.21% for *P. aeruginosa* at a concentration of 20 mg/ml. In conclusion, the synergistic combination of B and ZnO NPs displayed remarkable efficacy against the tested pathogenic microorganisms. Hence, B-supplemented ZnO NPs can be considered as an alternative to conventional antibiotics serving as promising microbial agents.

Author contributions

Concept: AT, EÇ; Design: AT, EÇ; Data Collection or Processing: AT, EÇ, ST; Analysis or Interpretation: AT, EÇ, ST, EK; Literature Search: AT, EÇ, ST; Writing: AT, EÇ, ST, EK.

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Data availability

The data that support the findings of this study are available online upon reasonable request from the authors.

Declarations

Conflict of interest

No conflict of interest was declared by the authors.

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