Boron nitride nanoparticles: Preparation, characterization, stability and evaluation of antibacterial activities

Emrah ÖZAKAR [1] * (ID) **, Rukiye SEVİNÇ ÖZAKAR [1]** (ID) **, Mehmet Cemal ADIGÜZEL [2]** (ID)

1   Department of Pharmaceutical Technology, Faculty of Pharmacy, Atatürk University, 25240, Erzurum, Turkey.
2   Department of Microbiology, Faculty of Veterinary Medicine, Atatürk University, 25240, Erzurum, Turkey.
*   Corresponding Author. E-mail: emrahozakar@atauni.edu.tr (E.Ö); Tel. +90-442-231 52 17.

ABSTRACT: In recent years, antibiotics have been the ideal drugs for treating infections caused by microorganisms due to their broad-spectrum effects. However, as a result of the unconscious and widespread use of antibiotics, an important disadvantage of current treatments is the emergence of resistant bacteria. With the development of nanotechnology, nano-sized materials, which have entered our lives, have started to be used frequently in health. Nano-sized materials may form lower resistance compared to conventional antibiotics. Boron nitride has outstanding optical and mechanical properties; It is one of the boron derivatives widely used in biomedical applications today. In this study, boron nitride nanoparticles were obtained by emulsification-solvent evaporation method, characterization (size, zeta potential, PDI, SEM, FTIR and XRD) studies were performed, stability was investigated, and potential antibacterial effect on nine different microorganisms was evaluated by minimum inhibitory concentration (MIC) and agar-well disc diffusion method. In our study, boron nitride nanoparticles were successfully prepared by an emulsification-solvent evaporation method, which is a top-down technique, easily, rapidly and in high yield. Surprisingly, our 466 nm boron nitride nanoparticles with high negative zeta potential (-38.4±0.90 mV) and highly homogeneous particle size distribution (0.144±0.01 PDI) influenced all nine different bacteria. Our negatively charged boron nitride nanoparticles showed a very high inhibitory effect, especially on *Bacillus cereus*, *Escherichia coli* and *Staphylococcus aureus*, even at very low doses (0.02±0.01, 0.80±0.23 and 0.05±0.03 µg/mL, respectively). This study was conducted to prepare nanoparticle formulations of water-insoluble Boron nitride, characterize them, stability and determine their therapeutic effectiveness on nine different bacteria. These findings may make boron nitride nanoparticles not only a reliable and effective antimicrobial agent in health and cosmetics but also an optimal alternative for food preservation.

KEYWORDS: Boron nitride; nanoparticles; stability; antimicrobial activity.

1. INTRODUCTION

In recent years, with the development of technology, many diseases have been discovered and defined in the health field. Among the most important of these, microbial infections are the primary source of chronic diseases and deaths. Some important bacteria, such as *Escherichia coli* and *Staphylococcus aureus*, responsible for soft tissue injuries, skin infections and many other diseases, have become resistant to many drugs. Antibiotics are currently the ideal drugs for treating bacterial infections due to their broad-spectrum effects. However, as a result of the unconscious and widespread use of antibiotics, an important disadvantage of current treatments is the emergence of resistant bacteria. Furthermore, cellular compatibility remains a critical issue to consider when recommending any antibiotic therapy. For these reasons, in the current scenario, scientists are looking for new drug candidates that are economically advantageous, biocompatible, and exhibit antibacterial properties [1].

Nanotechnology today offers the opportunity to develop nanoparticles whose composition, size, shape and surface properties can be changed. Previous studies have also reported that nanoparticles can be taken into cells using various cellular uptake mechanisms [2].

Nano-sized components are promising drug candidates with multiple mechanisms of action leading to the death of microorganisms. They may form lower resistance compared to conventional antibiotics. Studies have shown that many nano-sized materials, such as graphene, silver and gold, can be excellent antimicrobial candidates in various medical applications [3, 4].

Boron is a trace element found in nature. In addition to its widespread use in industrial areas, since the 2000s, studies that it may be useful for the biological functions of human and animal health have increased gradually. Boron is thought to have antibacterial effects against various bacteria and fungi, and these effects are thought to disrupt protein synthesis and the activity of various enzymes in microorganisms [5]. Boron nitride is an effective compound formed by the combination of boron and nitrogen. It is extensively researched in biomedical applications and is reported to be biocompatible in various biological applications [1]. Boron nitride has exceptional optical and mechanical properties as well as thermal conductivity, strong antimicrobial activity and antioxidant capacity [6]. Boron nitride is a hydrophobic compound with insolubility in aqueous media. Therefore, it can be beneficial for biomedical applications with specific functionalization processes. Functionalized boron nitride has also been reported to maintain cell viability [7].

In modern nanomedicine, controlled release and targeting of drugs are among the important goals. By utilizing the extraordinary properties of boron nitride, targeting with physical and/or chemical modifications and/or drug delivery systems can be developed. Comprehensive preclinical studies and *in vivo* tests are needed to investigate the health uses of boron nitride. However, it is also reported that these boron nitride-based nanostructures have a very high potential in diagnosing and treating various diseases in medicine [7].

In this study, functionalized nanoparticles of boron nitride, one of the unique and feasible drug candidates that can address the deficiencies and limitations in current antibiotic treatments for potential therapeutic applications in the future, were evaluated. This way, the study was conducted to prepare nanosuspension formulations of water-insoluble boron nitride nanoparticles for the first time by an emulsification-solvent evaporation method, to perform characterization studies, to investigate its stability, and to evaluate its potential antibacterial effect on nine different microorganisms by minimum inhibitory concentration (MIC) and agar-well disc diffusion method.

2. RESULTS AND DISCUSSION

2.1. Preparing of boron nitride nanoparticle formulations

The "top-down" technique for nanoparticle preparation is convenient for preparing suitable nano- or micro-sized particles. Compared to the "bottom-up" approach, size consistency and controllability are its most important advantages [8]. The "emulsification-solvent evaporation" method within this approach is widely used to prepare organic or polymeric nanoparticles [9].

In the present study, boron-containing nanoparticles were prepared directly from bulk material (boron nitride) by emulsification-solvent evaporation method without synthesis for the first. With this method, nanoparticles contain fewer method steps compared to other nanoparticle preparation methods in the literature. In addition to obtaining high yields, this method is very simple, easy and fast (Table 1) [10-12].

The highest zeta potential, yield %, and colloidal stability (24 hours later) were obtained with nanoparticles prepared with 12.5 mg boron nitride, and analyses/measurements were carried out with this formulation.

2.2. Yield, particle size, zeta potential, PDI and stability of boron nitride nanoparticles

Nanocrystals or nanosuspensions can be prepared as nanoparticles of pure active substances. It is based on the principle of reducing the size of active substances in bulk form, without using any polymer, by using suitable surfactants and a high-energy device (such as sonication, homogenization) that helps in size reduction. There is a severe need to examine innovative methodologies to minimize excipients that increase the solubility and bioavailability of active substances. Direct size reduction of the active substance is essential to improve its pharmacokinetics. Limited drug loading capacity requires a large amount of carrier material (such as polymer) to ensure proper encapsulation of the drug. This problem appears to be a situation that can be solved by the development of nanocrystals [13].

Since boron nitride was brought directly to the nanosize using various surfactants and high-energy sonication (since it was not encapsulated into any polymeric nanoparticle system), the yield calculation was given instead of the encapsulation efficiency. Our study found that boron nitride nanoparticles (F6 formulation according to Table 3) were obtained with a high yield (93.65%, Table 1). It has been seen that the preparation method is quite efficient and advantageous for preparing boron nitride nanoparticles.

The zeta potential analysis results of the formulations are shown in Table 1. Boron nitride nanoparticles were found to have a high negative zeta potential of -38.4 ± 0.90 mV.

Zeta potential value is essential data in evaluating the stability of nanoparticles. It is accepted that a zeta potential value more significant than ±25 mV keeps nanoparticles stable through electrostatic interaction [6, 14]. High zeta potential values are required to provide a high energy barrier to prevent the recombination of nanoparticles and to maintain stability. It is known that nanoparticles with a more significant zeta potential charge are subject to a much higher repulsion and thus maintain their stability [15].

In our study, boron nitride nanoparticles with negatively charged zeta potential likely provide a high electrostatic barrier considering that the carboxylic acid groups of the surfactants used are predominant. A similar situation was observed after silver nanoparticles formed a complex with green tea extract, and it was reported that the negative zeta potential may have increased due to the presence of carboxylic acids in the structure [16].

The sizes and PDI data of the boron nitride nanoparticles are shown in Table 1. Boron nitride nanoparticles were found to have a size of 466.0 ± 11.33 nm and exhibit a very narrow particle size distribution (PDI) of 0.144 ± 0.01.

PDI is expressed numerically between 0.0 and 1.0. In polymer-based nanoparticulate systems, PDI values below 0.2 are defined as ideal. PDI is a value related to the stability of formulations and the size of the particles formed. High PDI values are accepted as an indicator of big particle size [17]. Looking at the PDI value of the boron nitride nanoparticles in our study, it was observed that the PDI value was less than 0.2 (0.144 ± 0.01) and accordingly showed a more homogeneous particle size distribution.

The short-term colloidal stability, 30th-day and 90th-day results of the prepared boron nitride nanoparticles are given in Table 1. Stability results were compared with freshly prepared formulations as in previously reported studies [18].

It was observed that there was a statistically insignificant ($p > 0.05$) change in the colloidal stability of the formulations after 24 hours and a relatively significant ($p < 0.05$) change in the 30th-day and 90th-day measurements. This may be because boron nitride nanoparticles collapse over time under the influence of gravity, and there is no viscosity enhancer in the environment to slow and/or stop this. As a matter of fact, it has been reported in the literature that silver nanoparticles show significant differences after 24 hours compared to the data when they were first prepared. It is reported that this situation is again caused by the collapse of the particles due to their weight and the aggregation of the collapsed nanoparticles [16].

Table 1. Yield, particle size, zeta potential, PDI and stability results of boron nitride nanoparticles (n=3, 25 °C, X̄±SD).

	Freshly prepared	After 24h later (Colloidal Stability)	30th-day Stability	90th-day Stability
Yield (%)	93.65 ± 2.17	—	—	—
Particle Size (nm)	466.0 ± 11.33	473.9 ± 14.21	491.1 ± 14.77	548.6 ± 23.61
PDI	0.144 ± 0.01	0.208 ± 0.04	0.223 ± 0.12	0.254 ± 0.19
Zeta Potential (mV)	-38.4 ± 0.90	-36.9 ± 2.31	-34.0 ± 1.95	-29.7 ± 2.52

2.3. Morphological analysis

Polymeric nanoparticles obtained by nanoprecipitation or "emulsification-solvent evaporation" are generally characterised by a spherical shape caused by surface tension since they exhibit minimum surface area and polydisperse size distribution. However, nanoparticles prepared with inorganic materials are also likely to show shapes such as cubes, rods, discs or stars with the contribution of the auxiliary materials used [8]. SEM images of boron nitride and boron nitride nanoparticles in our study are given in Figure 1. In the image, boron nitride bulk material was found to be irregular and amorphous solid particles, while boron nitride nanoparticles were not spherical but uniformly three-dimensional and circular. When they were evaluated together with the zetasizer results obtained, it was seen that they were compatible in terms of size.
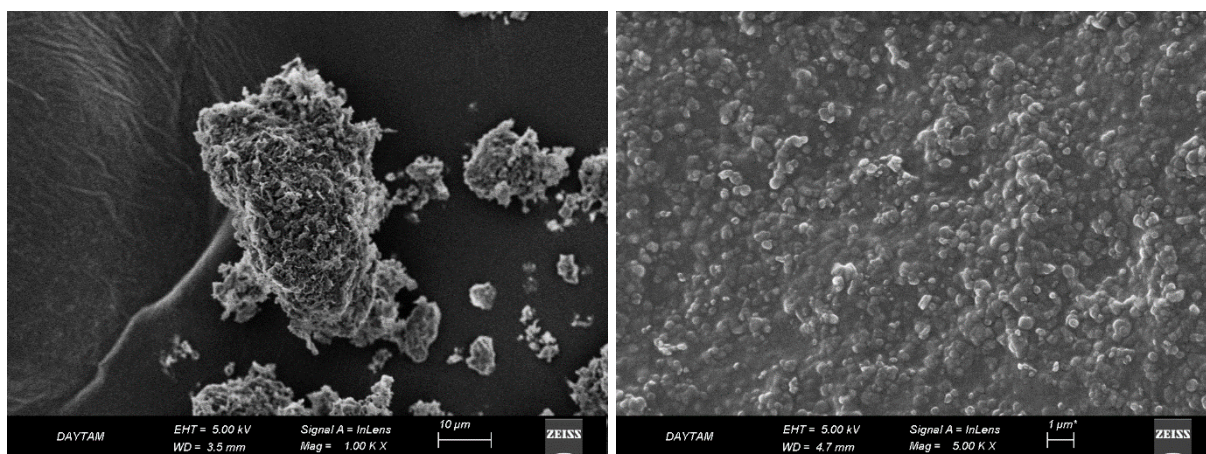
Figure 1. SEM images of boron nitride (left) and boron nitride nanoparticles (right)

2.4. XRD analysis

XRD was used to analyze the phase and crystal structure. As shown in Figure 2, four peaks corresponding to planes (002), (100), (004) and (110) of pure boron nitride were observed at 26°, 41°, 55° and 76°, respectively. These peaks agree with the literature [19, 20]. The diffraction peaks observed in boron nitride nanoparticles are in good agreement with the presence of boron nitride. All peaks were observed at the same angle [6].

The peak intensity seen in boron nitride nanoparticle is much higher than in boron nitride nanoparticles may be due to the surfactants used in the formulation coating the surface of boron nitride nanoparticles [21]. In summary, it can be said that the boron nitride in the formulation did not change into any undesired form, and boron nitride in the formulation shows all the characteristic peaks.
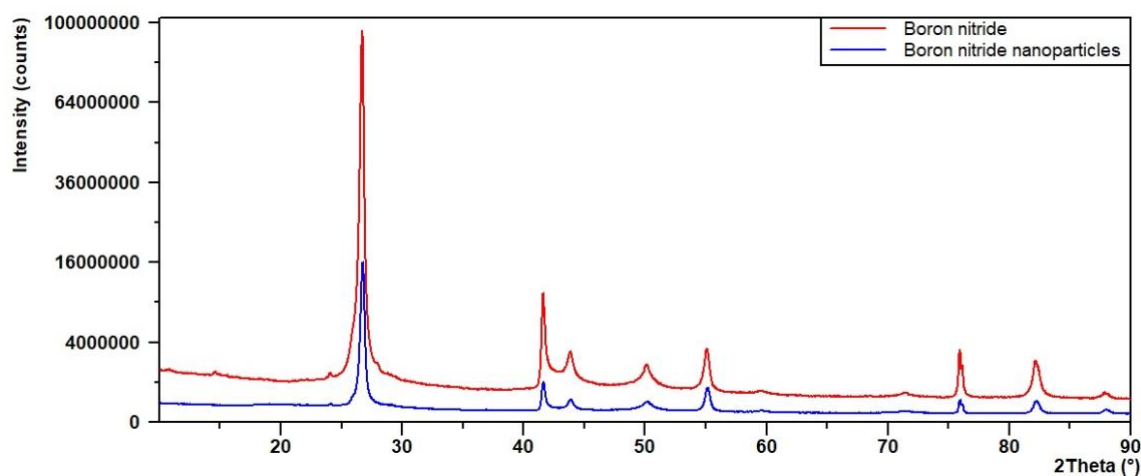

Figure 2. XRD diffractogram of boron nitride and boron nitride nanoparticles (25 °C)

2.5. FT-IR analysis

FTIR spectra of boron nitride and boron nitride nanoparticles are shown in Figure 3 and given in Figure 4, respectively. Two prominent characteristic peaks (754 cm^{-1} and 1353 cm^{-1} in Figure 6) observed in the pure boron nitride spectra were also observed in the IR spectrum of boron nitride nanoparticles (756 cm^{-1} and 1351 cm^{-1}). These regarded bands are associated with the characteristic peaks of boron nitride [22, 23]. The IR peak at 1353 cm^{-1} is due to the B-N stretching vibration, and the IR peak at 756 cm^{-1} is due to the stretching vibration at B-N-B [24].

In our study, it was observed that boron nitride and boron nitride nanoparticles have similar band strains and give similar fingerprint peaks. With these results, it was seen that there was no undesirable interaction. When nanoparticular studies with boron nitride are examined, similar peaks are observed [11, 25].

3500-3000 cm⁻¹ around peak may have shifted to the 3000-2500 cm⁻¹ range due to the surfactants' effect or the preparation method. The decrease in amplitude may have occurred due to the effect of surfactants adsorbed on nanoparticles.
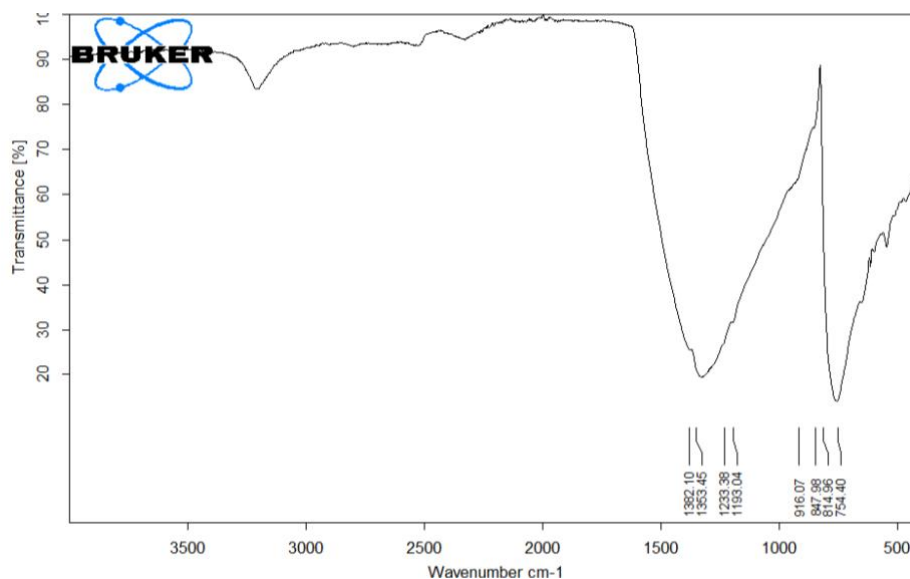

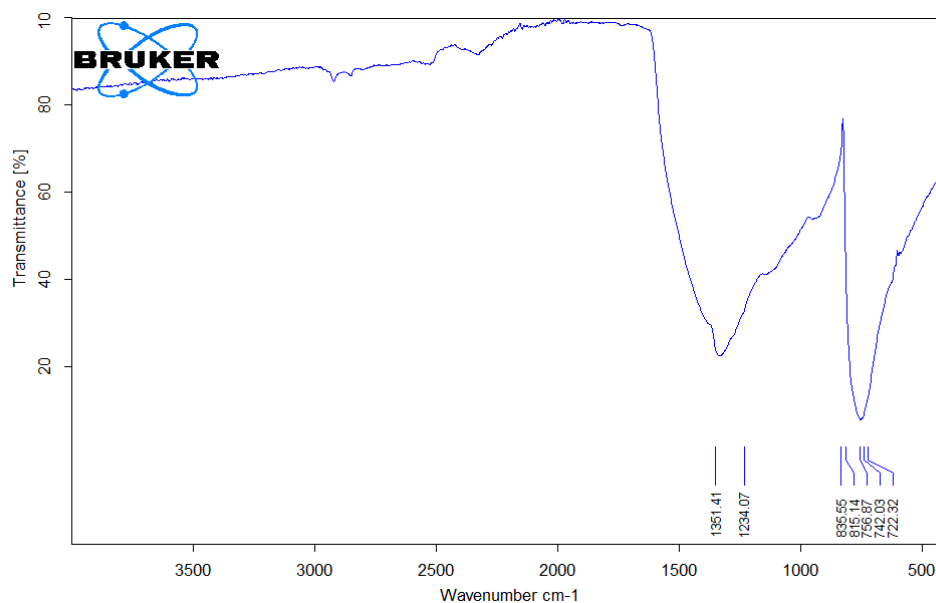**Figure 3.** FT-IR spectra of boron nitride (25 °C).

Figure 4. FT-IR spectra of boron nitride nanoparticles (25 °C).

2.6. Assays for antibacterial activity of boron nitride nanoparticles MIC and disc diffusion assay

The antimicrobial activity of boron nitride nanoparticles was tested against reference bacterial strains including *Bacillus cereus* ATCC 10987, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Klebsiella pneumoniae* ATCC 27736, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC 12453, *Streptococcus agalactiae* ATCC 12986, *Staphylococcus aureus* ATCC 29213, and *Salmonella enterica subsp. enterica serovar* Typhimurium ATCC 14028. The bacteria were grown on MH agar for 24 hours at 37 °C to obtain a pure culture. In addition to being effective on all bacterial strains, especially on *Proteus mirabilis* ATCC 12453 (MIC value 0.1µg/µl), *Bacillus cereus* ATCC 10987 (MIC value 0.02µg/µl) and *Staphylococcus aureus* ATCC 29213 (MIC value 0.05µg/µl) It was effective even at much lower doses (Figure 5). At the same time, disc diffusion results showed these bacteria strains were effective (Figure 6). It has been observed that the antibacterial activity of boron nitride nanoparticles when they had nano-size with a high zeta potential, shows a significantly strong interaction with bacteria.
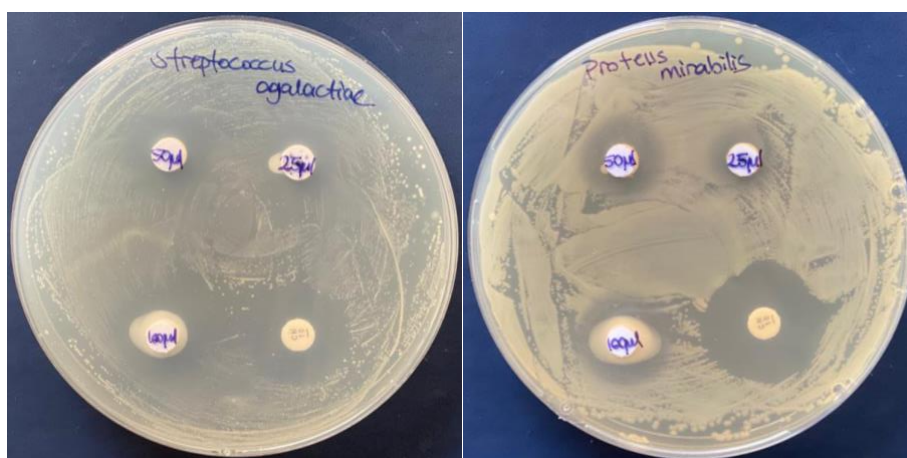
Table 2. MIC and agar-well diffusion test results of boron nitride nanoparticles in the current study (n=3, X̄±SD).

Reference Bacteria Strains	MIC (µg/mL) *	Disc Diffusion (mm)		
		100µl	50µl	25µl
Bacillus cereus ATCC 10987	0.02±0.01	15±0.25	13±0.60	10±0.15
Proteus mirabilis ATCC 12453	0.10±0.06	15±0.10	13±0.21	10±0.10
Streptococcus agalactiae ATCC 12986	0.40±0.12	20±0.26	16±0.38	12±0.06
Staphylococcus aureus ATCC 29213	0.05±0.03	13±0.29	10±0.25	-
Escherichia coli ATCC 25922	0.80±0.23	-	-	-
Enterococcus faecalis ATCC 29212	0.10±0.06	-	-	-
Klebsiella pneumonia ATCC 27736	0.40±0.23	-	-	-
Pseudomonas aeruginosa ATCC 27853	3.10±1.79	-	-	-
Salmonella enterica subsp. *enterica* serovar Typhimurium ATCC 14028	0.40±0.12	-	-	-

*Boron nitride nanoparticle concentration: 12.5 mg/mL

Figure 5. MIC results (BC: *Bacillus cereus*, EC: *Escherichia coli*, EF: *Enterococcus faecalis*, KP: *Klebsiella pneumonia*, PM: *Proteus mirabilis*, SA: *Streptococcus agalactiae*, SAL: *Salmonella enterica subsp. enterica* serovar Typhimurium, SAU: *Staphylococcus aureus*)

Figure 6. The agar-well diffusion test results against *Proteus mirabilis* and *Streptococcus agalactiae*. A gentamicin disc (10 µg) was used as a control. The 4 mm diameter well included 25, 50, and 100 µl volumes of prepared nanoparticles in the current study.

Bacterial cell membrane is generally negatively charged. These charges are reflected on the cell membrane as a whole of intracellular organelles and vital activities. Gram-negative bacteria are equipped with lipopolysaccharides with negative electrical potential on the surface of their cell membranes and peptidoglycan structures located in the space between these membranes. Gram-positive bacteria, conversely, have teichoic acid, lipoteichoic acid and some other surface proteins that carry negative electrical potential in addition to peptidoglycan structures. As a result, these molecules collectively create a negative electric potential across the bacterial cell membrane [26].

Nanoparticles can carry a positive, negative or neutral charge on their surface. This leads to interaction with the electrical charge on the cell membrane of bacteria and is the most important factor determining the antimicrobial activity of nanoparticles [27].

In our study, it was surprising that boron nitride nanoparticles with a zeta potential of -38.4±0.90 mV were especially influential on all nine different bacteria. It was remarkable that nanoparticles with the same potential charge as the potential of the bacterial cell membrane caused bacterial inhibition. Negatively charged boron nitride nanoparticles were found to be highly effective against *Proteus mirabilis* in both MIC and agar-well disc diffusion studies.

A study reported that positively charged silver nitrate nanoparticles were much more effective on bacteria than negative and neutral nanoparticles. The authors also showed that nanoparticles with positive, negative and neutral charges were ineffective against Proteus bacteria. They reported that this situation may have properties or mechanisms that enable some bacterial species to form resistance to the charge on the surface of nanoparticles [28].

It has also been reported in the literature that bacteria can modify the electrical charge of their surfaces by changing the structure of phospholipids in the cell membrane and develop resistance to cationic antimicrobial peptides [29].

Studies on metallic nanoparticles in the literature are increasing day by day. Especially metallic nanoparticles reduced to nano-size have been reported to be highly effective. For example, in a study in which silver (Ag) nanoparticles with positive zeta potential were synthesized, antimicrobial activity studies were carried out on *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*. Silver nanoparticles were reported to have no effect against *Bacillus cereus*, while they were effective against *Escherichia coli* and *Staphylococcus aureus* (60 and 40 µg/mL, respectively) [30]. In another study, MIC values of 6.7 and 3.1 µg/mL were reported for synthesized silver nanoparticles on *Escherichia coli* and *Staphylococcus aureus*, respectively [31]. In another study conducted on Gram-negative and Gram-positive bacteria samples with silver nanoparticles synthesized as positively charged, it was mentioned that nanoparticles had an antimicrobial effect when MIC values were considered. It was also mentioned that a predominantly negative charge is an advantage [32]. In another study with negatively charged zinc oxide (ZnO) nanoparticles, antimicrobial activity was tested on *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus* and *Pseudomonas aeruginosa* bacteria and the most effective MIC value was reported to be 250 µg/mL [33]. When these results were compared with the findings of our study, it was observed that negatively charged boron nanoparticles were highly influential on *Bacillus cereus* (0.02±0.01 µg/mL). At the same time, it was found that it exhibited an inhibitory effect on *Escherichia coli* and *Staphylococcus aureus* even at very low doses (0.80±0.23 and 0.05±0.03 µg/mL, respectively).

Most examples in the literature emphasize that the necessary condition to increase antibacterial activity is to have a positive particle surface charge. Naturally, the positive charge allows a more effective electrostatic interaction with the negative charge of the bacterial cell wall. However, this expected effect on antimicrobial activity clearly contradicts our experimental data. Especially considering that cationic nanoparticles are more cytotoxic than nanoparticles with neutral or negative surface charge, it is seen that our nanoparticles with high antibacterial activity constitute another important advantage in terms of safe use in mammalian cells and tissues [32].

This study aimed to investigate the antimicrobial effectiveness of boron nitride by bringing it to nanosize since it is not water-soluble. The size was reduced by using surfactants and high-energy sonication. Thus, boron nitride was dispersed in colloidal form in the aqueous environment as nanosized particles, allowing it to interact with bacteria. Thus, the antimicrobial activity of boron nitride, which is immiscible with water in bulk material, has been demonstrated by bringing it to nanosize. This antimicrobial effect is also crucial because it is achieved with nanoparticles with the same charge rather than nanoparticles with opposite charges, which have many examples in the literature. Here, it is thought that boron nitride nanoparticles with the same charge as the negatively charged bacterial cell membrane show antimicrobial activity through a different mechanism.

3. CONCLUSION

In our study, boron nitride nanoparticles were prepared by emulsification-solvent evaporation, a top-down technique, for the first time. Boron nitride nanoparticles, which are insoluble except in strong alkalis, were successfully obtained easily, rapidly and in high yield for the first time in this study. Our boron nitride nanoparticles with a size of 466 nm with a high negative zeta potential (-38.4±0.90 mV) and a very homogeneous particle size distribution (0.144±0.01 PDI) were found to be compatible with pure boron nitride by FTIR and XRD analyses. It was found to be highly effective on nine different Gram-positive and Gram-negative bacteria in MIC and disc diffusion experiments. In contrast to the antibacterial activity of positively charged nanoparticles in the literature, it exhibited a high antibacterial activity with a negative charge. These properties may make boron nitride nanoparticles not only a reliable and effective antimicrobial agent in health and cosmetics but also an optimal alternative for food preservation.

4. MATERIALS AND METHODS

4.1. Materials

Boron nitride, Pluronic F68, and dichlorometan were purchased from Sigma-Aldrich (USA). Tween 40 was purchased from Merck (Germany). Lactic acid was obtained from Doğa İlaç (Turkey). Mueller Hinton (MH) broth was purchased from Oxoid (United Kingdom). Ultrapure water was used in all analysis and studies (18.2 MΩ·cm, TOC ≤ 4 ppb, Merck Millipore Direct-QTM 3, Germany).

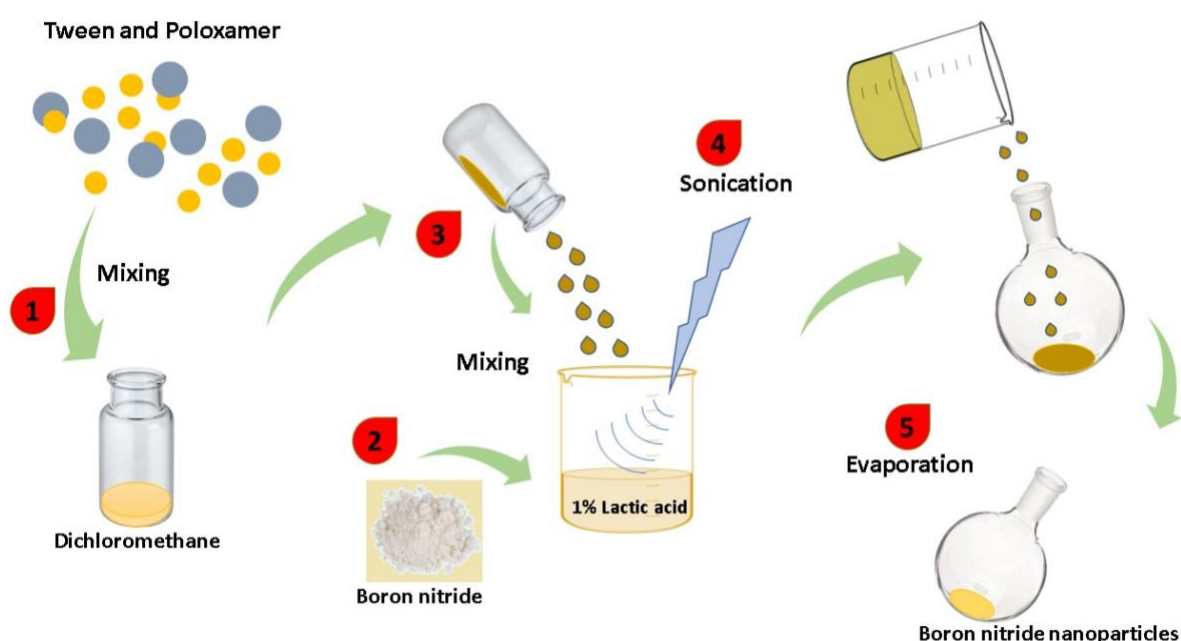#### 4.2. Preparing of boron nitride nanoparticle formulations

"Emulsification-solvent evaporation" method was used for the preparation of boron nitride nanoparticles [34]. In a glass vial, boron nitride (such as 2.5 mg, 7.5 mg or 12.5 mg) was added to 1 mL of 1 % (w/v) lactic acid solution. In another vial, Tween 40 (such as 2.5 mg, 5 mg or 10 mg) and Pluronic F68 (such as 2.5 mg, 5 mg or 10 mg) were added, and dichloromethane (such as 1 mL, 2 mL or 3 mL) was added. Both vials were stirred and dissolved for 10 min on a multi-point magnetic stirrer (2mag, mix 15 eco, Germany) operating at 750 rpm. Then, the organic phase vial was added to the aqueous solution containing boron nitride and sonicated with a 60% power/2 cycles ultrasonic probe (Bandelin, Sonopuls HD 2070, Germany) for 2 min. Dichloromethane was removed from the resulting emulsion with an evaporator (Heidolph, Laborata 4001, Germany) rotating at 60 rpm at 40 °C. Boron nitride nanoparticles were obtained due to the removal of the organic phase. These nanoparticles were centrifuged in a refrigerated centrifuge (Kubota 3780, Japan) at 10,000 rpm for 45 minutes to remove surfactants that did not interact with boron nitride nanoparticles and possible residual solvents. The precipitated boron nitride nanoparticles were suspended with 1 mL of ultrapure water and frozen at (-)20 °C for one day. Subsequently, boron nitride nanoparticles were subjected to lyophilisation for one day (Martin Christ, Alpha 1-4 LD plus, Germany). As a result, boron nitride nanoparticles in dry powder form were obtained. These nanoparticles were stored in a moisture-proof and dark environment. The formulations were studied in three replicates. The preparation of boron nitride nanoparticles by emulsification-solvent evaporation method is schematically shown in Figure 7.

The preparation efficiency of the boron nitride nanoparticles obtained during the pre-formulation studies was calculated based on their dry powder and lyophilized states. The formulations were evaluated with zeta potential measurements performed on freshly prepared formulations and after keeping them in the dark at room temperature for one day (colloidal stability). Since the F6 formulation had more stable and higher measured zeta potential values and obtained the highest yield, the F6 formulation (containing 12.5 mg boron nitride) was used for further experiments and analysis.

Table 3. Preformulation study results of boron nitride nanoparticles (n=3, 25 °C, X̄±SD).

Formulation Code	Boron nitride (mg)	Pluronic F68 (mg)	Tween 40 (mg)	Yield %	Freshly prepared (zeta potential) mV	After 24 h later (zeta potential) mV
F1	2.5	5.0	10.0	77.16 ± 3.29	-16.7 ± 1.03	-15.9 ± 1.24
F2	2.5	10.0	5.0	89.38± 2.09	-19.2 ± 0.64	-18.0 ± 0.97
F3	7.5	5.0	10.0	87.49± 1.83	-25.9 ± 0.99	-22.3± 1.36
F4	7.5	10.0	5.0	84.42 ± 1.68	-28.6± 1.50	-27.6± 2.41
F5	12.5	5.0	10.0	88.39± 2.40	-34.6± 2.82	-31.4± 2.08
F6	12.5	10.0	5.0	93.65 ± 2.17	-38.4 ± 0.90	-36.9 ± 2.31

Figure 7. Boron nitride nanoparticles preparation by emulsification-solvent evaporation method

4.3. Particle size, zeta potential, PDI and yield of boron nitride nanoparticles

The % yield of lyophilised boron nitride nanoparticles after lyophilisation will be determined by proportioning the amount of material used at the beginning with the amount after lyophilisation.

Lyophilised boron nitride nanoparticles will be diluted 1/10 with ultrapure water, and particle size, zeta potential and PDI measurements will be analysed on these samples with a zetasizer (Malvern Zetasizer Nano ZSP, United Kingdom). Each measurement will be performed in at least three replicates [35].

4.4. Morphological analysis

Images were taken with SEM (Zeiss Sigma 300, Germany) to obtain information about the surface structure and morphology of lyophilized boron nitride nanoparticles. For this purpose, pure boron nitride and lyophilised boron nitride nanoparticles will be examined. They will be coated with 100 Å thick gold to make them conductive. This way, boron nitride nanoparticles' surface structure, size and shape compared to bulk material will be determined [7].

4.5. XRD analysis

X-rays can be used to detect unwanted changes in the crystal lattice of a substance. X-ray diffractograms of pure boron nitride and boron nitride nanoparticles will be taken with an XRD (Malvern

PANanalytical Empyrean, United Kingdom) with Cu-K α radiation (λ=1.541874 Å) conditioned at 45 kV and 40 mA to determine whether there are unwanted interactions when developing formulations. The measurements will be performed in the 2θ range from 10-60° with a 2°/min scanning speed [36].

4.6. FT-IR analysis

This analysis will be carried out to determine whether there are any undesirable chemical interactions between boron nitride and the surfactants forming the nanoparticles. For this purpose, the spectra of pure boron nitride and boron nitride nanoparticles will be taken from powder samples with an FTIR spectrophotometer (Bruker VERTEX 70v, Germany) with an ATR module. Measurements will be performed in the spectral range of 400-4000 cm^{-1} with a resolution of 4 cm^{-1} [37].

4.7. Stability of boron nitride nanoparticles

The stability of the formulations was examined for 24 hours, 30th-day and 90th-day to determine the changes in PDI, zeta potential and particle size at the end of the incubation period. In this context, freshly prepared formulations were kept in a dark environment at 25±2°C and 60±5% relative humidity 90 days. They were re-analysed for zeta potential, PDI and particle size. The obtained data were compared with the data of freshly prepared formulations [38].

4.8. Assays for antibacterial activity of boron nitride nanoparticles

The antibacterial activities of the resultant boron nitride nanoparticles were evaluated by determining the Disc diffusion and MIC for nine bacteria. The detailed protocol is described below.

The antimicrobial activity of the prepared boron nitride nanoparticles was tested using an agar-well diffusion and broth microdilution tests [39]. The boron nitride nanoparticles were tested against pure culture of *Bacillus cereus* ATCC 10987, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Klebsiella pneumoniae* ATCC 27736, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC 12453, *Streptococcus agalactiae* ATCC 12986, *Staphylococcus aureus* ATCC 29213, and *Salmonella enterica subsp. enterica serovar* Typhimurium ATCC 14028.

An agar-well diffusion assay was performed after spreading 100 µl of 24 h 0.5 McFarland bacterial culture on the agar surface. Each boron nitride nanoparticles were placed in 4mm diameter wells as 25, 50, and 100 µl volumes. After an incubation period, the inhibition zone was measured with a digital caliper. The gentamicin disc (10 µg) was used as a control. The assay was performed in triplicate.

The broth microdilution method was performed for detecting the MIC [39]. The study was carried out with 12.5 mg lyophilized boron nitride nanoparticles per milliliter. Briefly, twofold serial dilutions of the boron nitride nanoparticles in cation-adjusted MH broth were prepared in a 96-well microplate. A bacterial inoculum at a density of 0.5 McFarland was prepared from 24 h bacterial culture in MH broth. The inoculum was diluted 1:100 in tryptic soy broth, and then distributed to the wells in 50 µL volumes. The lid was placed on the microplate, which was then incubated for 24 hours at 37 °C. After incubation, the plate was assessed using an ELISA reader device (BioTek, Power Wave XS2) to measure optical density at a wavelength of 600 nm (OD600). The results compared with the negative control (including either MH broth or MH broth and bacteria) that included in the last two wells on the plate. The MIC test was performed at least in triplicate for nanoparticle formulations.

4.9. Statistical evaluations

Statistical evaluations were performed to determine between zeta potential, PDI, particle size for stability, and antibacterial activity of formulations with the one-way analysis of variance (ANOVA) by IBM SPSS Statistics 20 program. The data were presented as the mean ± standard deviation ($\overline{X}$±SD). Differences were considered to be statistically significant at $p < 0.05$.

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