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ANTIBACTERIAL AND ANTIBIOFILM EFFECTS OF BISMUTH NANOPARTICLES PRODUCED BY *BACILLUS SUBTILIS* AGAINST MULTIDRUG-RESISTANT *Pseudomonas aeruginosa*

Ghaidaa Raheem Lateef Al-Awsi[1], Gulboy Nasir[2], Majida M. Meteab Alshammari[3] and Abdolmajid Ghasemian[4]*

[1]*Department of Radiological Techniques, Al-Mustaqbal University College, Iraq*
[2]*College of Agricultural Engineering Sciences, University of Baghdad, Iraq*
[3]*College of Medicine, Jabir Ibn Hayyan Medical University, Al-Najaf, Iraq*
[4]*Department of Biology, Central Tehran Branch, Islamic Azad University, Tehran, Iran*

*Corresponding Author: majidghasemian86@gmail.com

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Abstract

This study aimed to determine the effect of green bismuth oxide (BiO) NPs against multidrug-resistant (MDR) *Pseudomonas aeruginosa* (*P. aeruginosa*) from wound infections. Among 450 wound samples collected from patients admitted to the hospital, 200 *P. aeruginosa* isolates were identified. MDR strains of *P. aeruginosa* were detected by disc diffusion method. BiO NPs were synthesized using wild *Bacillus subtilis* (*B. subtilis*) strain and infrared spectroscopy, X-ray diffraction and scanning electron microscopy techniques. The antibacterial effect of the NPs compared to antibiotics against MDR strains was evaluated using a standard disk diffusion method. BiO NPs were synthesized at 0.005 M concentration of solution. According to the SEM images, the BiO NPs were irregular in size and ranged from 23-41 nm. According to the disk diffusion method, the highest inhibitory effect of BiO NPs was observed against MDR *P. aeruginosa* at 2000 ppm. The MIC and MBC of Bi-O NPs at 2000 ppm and >2000 ppm was observed against 97/200 (47.5%) of MDR *P. aeruginosa*. Eighty percent and 20% of *P. aeruginosa* produced strong and moderate-level biofilms, respectively. In the presence of sub-inhibitory concentration (1200 ppm) of Bi-ONPs, 52 (26%) isolates produced strong-level biofilms (p=0.001) and 22 (11%) produced moderate-level biofilm (p=0.029), while 53% of remaining isolates produced weak biofilms. The results exhibited a significant decrease in biofilm formation in the presence of Bi-O NPs. BiO NPs exerted an antibacterial effect that 2000 ppm and had a significant inhibitory effect against *P. aeruginosa* biofilms.

INTRODUCTION

In recent years, due to the developed infections caused by extensive antibiotic-resistant bacterial strains, interest has been considered for novel and more efficient application of antimicrobial compounds [1-3]. For this reason, nanotechnology and nano-biotechnology are emerging areas of recent research with great interest [3, 4]. The synthesis of nanometer-sized particles with antimicrobial properties known as nano-antibiotics is a promising insight towards the achievement in the synthesis of novel compounds [5]. Various physical, chemical and biological methods are currently known to produce NPs. Physical and chemical methods are very expensive and toxic as hazardous chemicals are applied [5]. Whereas in the biological approach, bacteria, fungi, or plant extracts are used to synthesize the NPs [5]. The biosynthesis of NPs has recently received much attention in the field of medical research [6].

In the biological process, redox reactions are carried out by enzymes produced by microorganisms or plant chemicals [7]. The bio-production of NPs is cost-effective, easy and environmentally compatible [8].

Microorganisms, when exposed to metal ions, are capable of reducing or accumulating metals within or over the cell wall. This accumulation often results in the production of particles that are packaged in nanoscale sizes. In particular, due to the decrease in the size of the NPs, the contact surface of these particles increases and at the lowest concentration can react with the bacterial cell surface [9, 10]. The bismuth metal is the most fragile, crystalline and most natural diamagnetic material [11]. It is usually found in forms of Bismuth sulfide, Bismuth and Bismuth carbonate. Additionally, this metal contains high electrical resistance and its thermal conductivity is lower than any other metal after mercury [3, 11]. Bismuth is commonly used in medicine to treat gastrointestinal disorders, as well as syphilis and tumors, and as radioisotope treatment. Bismuth NPs have recently been used as broad-spectrum antimicrobial molecules [3, 12-14].

The biofilm-forming bacteria are resistant to common antibiotics and environmental conditions (such as immune responses) via producing biofilm materials that withstand in the patient's body for a long time [15-17]. Here, for the first time, we assessed the bio-production of BiO NPs using *B. subtilis*. Therefore, this study aimed to investigate the antimicrobial and anti-biofilm effects of BiO NPs synthesized using *B. subtilis* isolated from forest park soil against MDR clinical *P. aeruginosa* strains.

MATERIALS AND METHODS

Isolation of *Bacillus subtilis*

One-hundred soil samples were collected from different areas of the forest park of west Baghdad, Iraq at a depth of 7-11 cm in sterile conditions and transferred to the laboratory in 50 mL vials. Samples were screened by sterile 2mm diameter filters. One gram of filtered soil was added to the test tube containing 7 mL of sterile distilled water. The suspensions were placed in Ben Marie (80 °C) for 5 minutes to leave only spore-forming bacteria (such as *Bacillus* and *Clostridium spp*). The samples were purified using the Pure Plate method. As a result, serial dilutions were prepared in nine tubes and 1 mL of each suspension was cultured onto the nutrient agar medium (Merck, Germany). Plates were then incubated at 30° C for 24 hours in aerobic conditions [18]. Suspected colonies with a rough surface were taken and purified. After gram staining, biochemical tests were used to identify the bacterial species. Moreover, the Bsub5F (5'-AAG TCG AGC GGA CAG ATG G-3') and Bsub3R (5'-CCA GTT CCA ATG ACC CTC CCC-3') were used to amplify species-specific gene [19, 20].

Isolation of Bismuth Nitrate-Resistant Bacteria

BiO-sulfide agar (Merck, Germany) was used to evaluate the ability of the isolates for bismuth nitrate resistance. Isolates from the soil were cultured linearly onto this medium and incubated at 30 °C for 24 hours for growth onto the medium.

Synthesis of BiO NPs

Various concentrations of bismuth-oxide nitrate (BiON) (0.01-1 mol/L) were prepared and added to *B. subtilis* broth culture media. *B. subtilis* was cultured in a turbidity equal to the half McFarland concentration in 50 mL nutrient broth medium (Merck, Germany) at 35 ° C for 24 h. Then it was centrifuged at 4000rpm (4°C) for 20 minutes and the supernatant was used to produce extracellular BiO NPs. Then, the BiON was added to each of the supernatants in a new tube (10% volumetric/volumetric ratio) and incubated at 35 ° C for 24 h. In case of color change in medium (brown with a metallic sheen), samples were centrifuged at 8000rpm for 20 minutes. The precipitate was then washed three times with normal saline and centrifuged again. This was done to ensure complete removal of other materials, and the precipitate was incubated at 40 ° C for 48h for complete drying [8, 21].

Confirmation Tests for BiO NPs

After observing ambient discoloration and initial confirmation, the NPs powder was analyzed by Nicolet 8700 infrared spectrometry (Thermo Scientific) in 400-4000 cm^{-1} wavelength for detection of Bi-O bonds and also scanning electron microscopy (EM-3200 KYKY) for examination of their structure, shape and size [22, 23].

Crystal Characterization of BiO NPs by X-ray Diffraction

The powder produced by bacterial supernatant was incubated for 2 hours at 550 ° C in the furnace for calcination. They were then sent to the research laboratory to draw the diagrams and examine the samples in terms of crystalline phase determination by the X-ray diffraction (STADI P STOE) apparatus, and their size was determined by the Scherrer-Debye formula [8, 23].

Sample Collection from Hospitalized Patients

The ethical approval of this study was given by the University of Baghdad. Wound sampling including 450 samples from patients admitted to the city hospital was conducted from August 2017 to August 2019. Then, the sytrimide agar

medium (Merck, Germany) was used to isolate suspected colonies of *P. aeruginosa*. Conventional biochemical tests and polymerase chain reaction (PCR) technique (for *oprL* amplification) were employed to identify the isolates.

Molecular Identification

Genomic DNA was extracted from bacterial isolates using DNA Extraction Kit for Gram-negative Bacteria (Pishgam, Iran). The L lipoprotein (*oprL*) gene was amplified using forward: 5'-ATGGAAATGCTGAAATTCGGC-3' and reverse: 5'- CTTCTTCAGCTCGACGCGACG-3' primers giving a 504bp product.

The components of the master mix used in the reaction included: double-distilled H_2O (18 µL), 10 pmol of each primer (1 µL of each), PCR buffer (2.5 µL), DNA template (1µL), dNTP (1µL), Taq polymerase (0.5 µL), in a final volume of 25µL. The 100 bp DNA marker was used to confirm the molecular weight of the amplified products. The PCR program was as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30s, annealing at 55 ° C for 45s, extension at 74 °C for 1 min and final extension at 74 °C for 5min. Genomic samples were electrophoresed on a 1% agarose gel using a horizontal electrophoresis system. Then, the gel was observed in a 504 nm wavelength UV trans-illuminator [18]

Antibiotic Resistance Pattern

The disk diffusion method was used for the susceptibility test (Bauer Kirby method and CLSI version 2018). Following antibiotics were assessed including gentamicin (10 µg), ciprofloxacin (5 µg) tetracycline (30 µg), colistin (30 µg), fosfomycin (30 µg), amikacin (30µg), imipenem (10µg), ampicillin (10 µg) were determined for *P. aeruginosa* isolates. Plates were incubated at 37 ° C for 24 hours [24]. *Staphylococcus aureus* ATCC25923 and *Escherichia coli* ATCC25922 were used as the quality control of the disks.

Antimicrobial traits of BiO NPs

To investigate the antimicrobial effect of BiO NPs produced by *B. subtilis*, 100µL of bacterial suspension was lawn onto the Mueller Hinton agar (MHA) medium surface. The blank disks were applied with several dilutions of BiO NPs solution including 100ppm, 300ppm, 600ppm, 900ppm, 1200ppm and 2000ppm. The discs were then placed at 40 °C until completely dried and were then prepared using sterile forceps to place onto the MHA medium. Finally, the plates were incubated at 37 ° C for 24 hours. After this time, the growth zone diameter was measured.

Determination of Minimum Inhibitory Concentration of BiO NPs

The broth dilution method was used to evaluate the minimum inhibitory (MIC) and bactericidal concentration (MBC) of BiO NPs in MH broth medium. One milliliter of bacterial suspension equal to half Mac Farland turbidity was inoculated to each dilution and serial dilution of BiO NPs was prepared and subjected to microbial suspensions. The cultures were incubated at 37°C at aerobic conditions for 18-22h [24].

Biofilm Formation

Single colonies of pure cultures of *P. aeruginosa* isolates were cultured in Luria Bertani (LB) medium (having 0.5 McFarland standard turbidity). In the next step, each isolate and control strain were added to 96 well polystyrene plate containing LB medium and 1% glucose. The negative control included the LB medium with 1% glucose. The plates were incubated at 37 °C for 24 h. After that, the wells were slowly evacuated and washed with sterile H_2O (pH 7.4). Then, 200 µL of pure methanol was added to each well for fixation and kept at ambient temperature for 10 minutes. After evacuation, the crystal violet dye (1% w / v) was added and kept at room temperature for 20 minutes and washed using H2O. Next, 33% (v/v) glacial acetic acid was added and the wells absorbance rate was read using enzyme-linked immunosorbent assay (ELISA) reader (BioTek ELx808) at 630nm. Each test was repeated three times. For analysis of biofilm formation level (strong, moderate and weak levels), the test mean absorbance was compared to that of the negative control as described previously [15, 25]. Furthermore, a strong biofilm-producing *P. aeruginosa* was used as positive control of the test.

Antimicrobial Effect of BiO NPs and Ciprofloxacin

BiO NPs (1200ppm, 50µg) were subjected to 96-well plates of biofilm formation assay singly and together with ciprofloxacin (500ppm, 50µL). Ciprofloxacin solution was sterilized by filtration and added to the soluble BiO NPs and their effect was evaluated against the biofilms of *P. aeruginosa*. The workflow was as biofilm formation into 96-well plates with similar conditions. The optical absorption of biofilm was evaluated by an ELISA reader using the same conditions as aforementioned (BioTek ELx808).

Statistical Analysis

Data was analyzed using SPSS software version 20. The difference between optical absorption and mean values were compared using Chi Square and one-way analysis of variance (ANOVA) tests and the significance level was set at <0.05.

RESULTS AND DISCUSSION

Synthesis of BiO NPs

Falcons containing bismuth nitrate solution were discolored after 9 days and the yellow color of culture media was changed to darkened metallic brown indicating NP synthesis. This indicated that isolates of *B. subtilis* were capable of producing extracellular BiO NPs at this concentration.

Infrared spectroscopy

The vibrational wavelengths of BiO NPs produced by *B. subtilis* were observed at 583cm^{-1}, and 680, 737 and 3437 wavelengths. Absorbance at 3437 cm^{-1} and 400-700 cm^{-1} reflected H-O and Bi-O vibrations (Fig. 1).

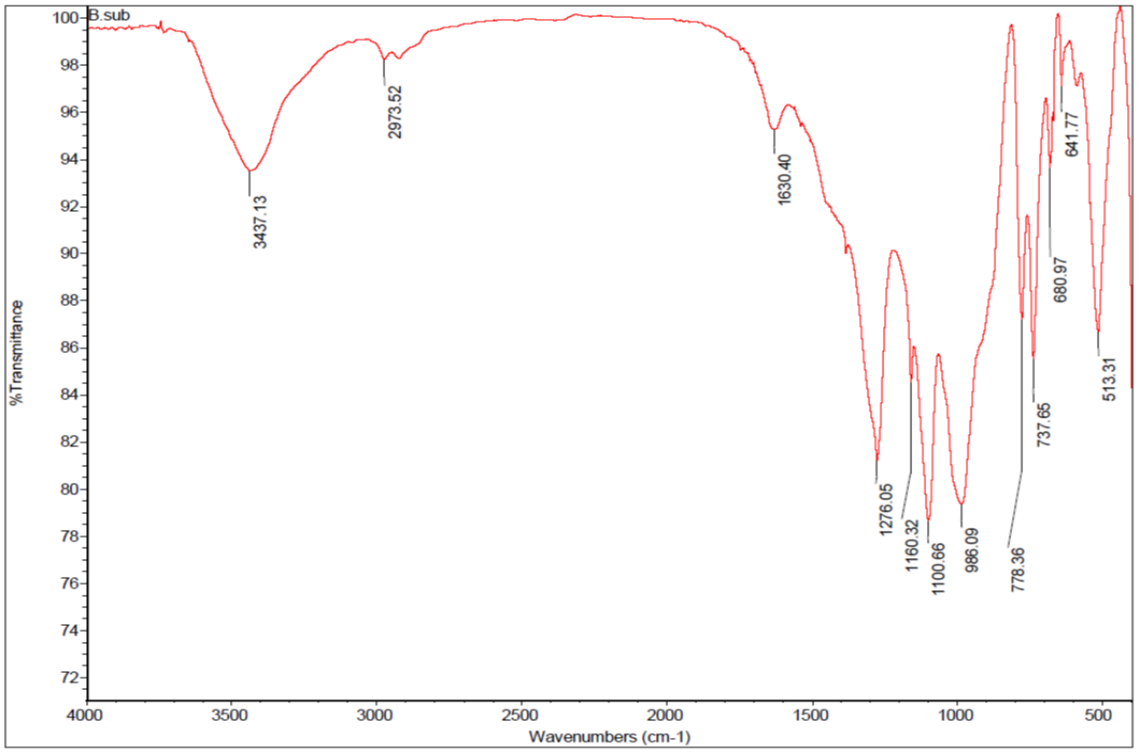

Fig. 1. Infrared spectroscopy of BiO NPs produced by *B. subtilis*

Scanning Electron Microscopy

According to the SEM images, the BiO NPs produced by *B. subtilis* were irregular in size range of 23-41 nm (Fig. 2). All the NPs produced were less than 100 nm in size.

X-ray Diffraction

X-ray diffraction pattern of BiO NPs was identified in the range of (2Th) 10 to 80 angle considering standard of (JCPDS = 00-027-0053); with spectral information including (002), (-112), (-121), (-202), (-223), (-322), and (-143). According to the Debye Scherrer formula (D = 0.9λ/ βCos θ) average size of NPs synthesized by *B. subtilis* was estimated to be 44 nm (Fig. 3).

Antibiotic Susceptibility of Isolates

Susceptibility of 200 *P. aeruginosa* strains to antibiotics included ciprofloxacin 100%, imipenem 100%, ampicillin 100%, tetracycline 100%, amikacin 56% gentamicin 56%, fosfomycin (18%) and colistin 10%. Therefore, all of the isolates were resistant to more than three antibiotics each from a different class (ciprofloxacin, imipenem/or ampicillin and tetracycline) and were multidrug-resistant (MDR) *P. aeruginosa*.

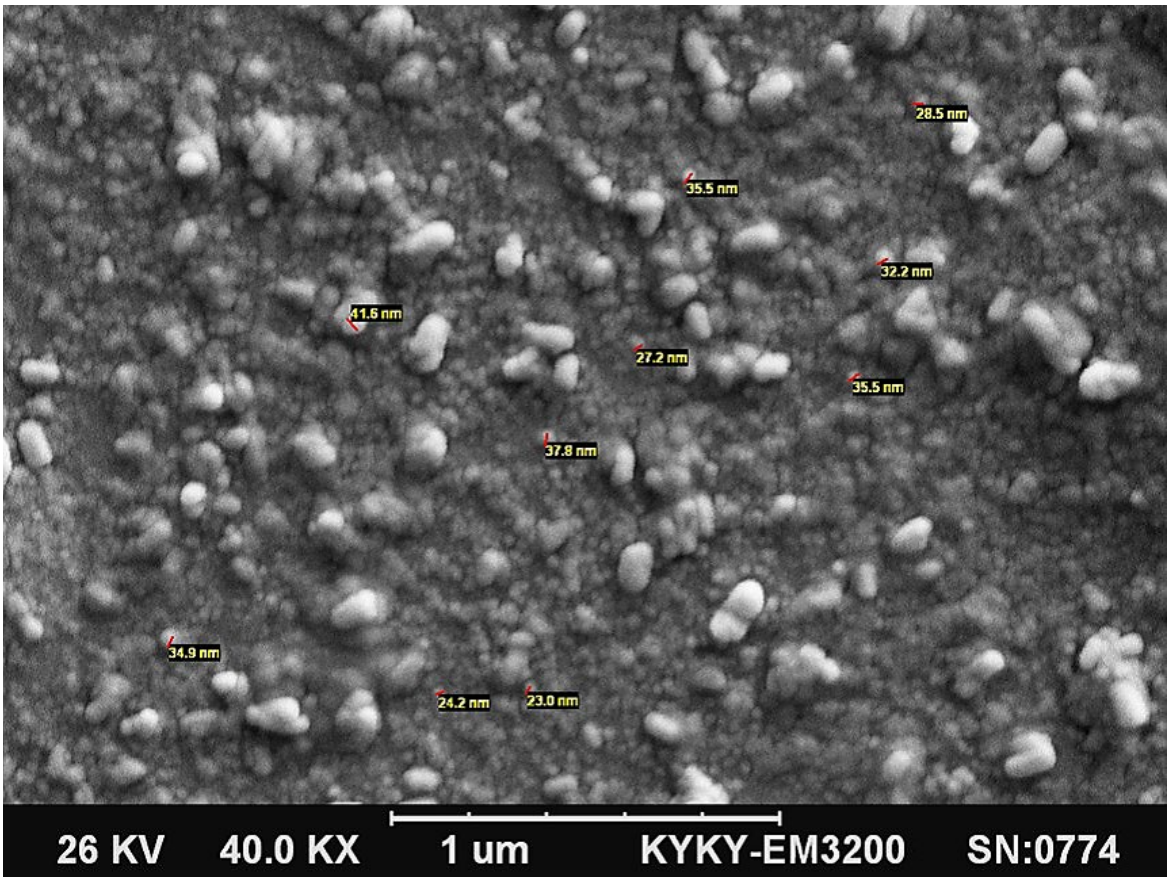

Fig. 2. The SEM image and size range of NPs

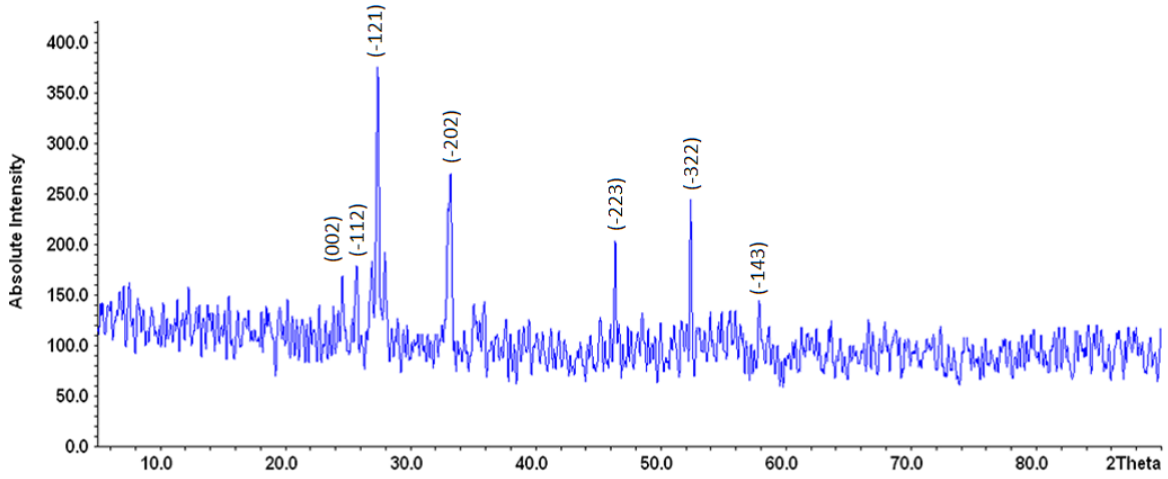

Fig. 3. The X-ray diffraction pattern of BiO NPs

Antimicrobial Effect of BiO NPs

According to the disk diffusion method, the highest inhibitory effect of BiO NPs was observed against MDR *P. aeruginosa* with mean 17.1±0.5 mm zone diameter at 2000 ppm against while lower concentrations exhibited lower zone diameters. The mean zone diameter for each concentration has been exhibited in Table 1.

Table 1. The mean zone diameter and growth inhibition of MDR-*P. aeruginosa* in exposure to various BiO NP concentrations

BiO NP concentration	Mean diameter	%MIC	%MBC
100ppm	1.7±0.3mm	0	0
300ppm	2.5±0.5mm	0	0
600ppm	6.5±0.5mm	0	0
900ppm	9.5±0.5mm	0	0
1200ppm	12.5±0.5mm	0	0
2000ppm	17.1±0.5mm	97	ND
P value	<0.001	<0.001	-

BiO NP: bismuth-oxide nitrate, %MIC: percentage of isolates with growth inhibition, %MBC: % of minimum bacteriocidal effect, ND: not determined

Minimal Inhibition Concentration of BiO NPs

The MIC and MBC of BiO NPs were 2000 and >2000 ppm, respectively which was observed against 97/200 (47.5%) of MDR *P. aeruginosa*. Other isolates exhibited higher MICs and MBCs.

Evaluation of Biofilm Formation Ability

Eighty percent and 20% of *P. aeruginosa* produced strong - level and moderate biofilms, respectively in the absence of Bi-O NPs. In the presence of sub-inhibitory concentration (1200 ppm) of Bi-ONPs (treatment an overnight), 52 (26% of) isolates produced strong-level biofilms (p=0.001) and 22 (11%) produced moderate-level biofilm (p=0.029), while 53% of remaining isolates produced weak biofilms. The results exhibited a significant decrease in biofilm formation in the presence of BiO NPs. The synergistic anti-biofilm effect of BiO NPs and ciprofloxacin led to inhibition in the biofilm formation by MDR-*P. aeruginosa* (Table 2).

Table 2. The biofilm formation ability of MDR-*P. aeruginosa* in various conditions

Biofilms	**Non-treatment**	**BiO NP**	**BiO NP plus Cip**	***p value***
Strong-level	80% (n=160)	26% (n=52)	19% (n=38)	<0.0001
Moderate-level	20% (n=40)	11% (n=22)	8% (n=16)	<0.0001

BiO NP: bismuth-oxide nanoparticle (1200ppm), Cip: ciprofloxacin (500ppm), n: number

Nanoparticles contain high volume-to-surface ratio which exert efficient antibacterial effects. Application of microbial cells for synthesis of nanoparticles has attracted particular attention [26]. Some bacteria are capable of producing intracellular and extracellular nano-sized metals applied for metallic NPs synthesis such as gold, silver, cadmium, sulfide, bismuth and zinc [27]. Owing to requirement of high temperature, pressure and time, costs and toxicity behind NPs synthesis by chemical and physical methods, bioassay using microorganisms is preferred [26, 28, 29].

In the present study, *B. subtilis* isolated from soil of the forest park in Baghdad was able to discolor the solution by reduction of BiO N at a concentration of 0.005 M at 30 °C after 24 h of incubation. After observing the color change from dark yellow to dark brown and initial confirmation of the production of BiO NPs, chemical properties and finally NP synthesis was confirmed by infrared spectroscopy analysis, X-ray diffraction and SEM. The results of SEM revealed that *B. subtilis* was able to synthesize irregular BiO NPs with 23-41 nm size.

Our research showed that BiO NPs were effective against planktonic form of MDR-*P. aeruginosa* in a concentration-dependent behavior, particularly at 2000 ppm. In a study, subcitrate bismuth NPs with 170- 200 nm sizes and concentrations of 350 - 3500 ppm were assessed against *Klebsiella pneumoniae* isolates where 3500 ppm exerted antibacterial effect. Extracellular silver and zinc NPs have been synthesized in ranges of 5–60 nm (43–142 nm) and 20–30 nm, respectively using *B. subtilis*. They also exhibited that the bacterial species ability and various sizes of NPs may be influenced by application of different precursors [14, 30, 31]. Noticeably, the antibacterial potential of NPs is related to their size and shape [3, 8].

Our findings revealed a direct relationship between the concentration of NPs and the rate of bacterial elimination. Indeed, concentrations higher than 1200ppm exerted antibacterial effects in the disk diffusion and broth dilution methods.

One study described the antimicrobial effect of 100 nm size chemically synthesized silver NPs at concentrations of 5-10 µg /mL against *Staphylococcus aureus*, *Salmonella typhi* and *Escherichia coli* in a dose-dependent effect. They also synthesized BiO NPs chemically in a range of 31.99-89 nm and demonstrated the antimicrobial effect of these NPs on *P. aeruginusa*, *Escherichia coli* at concentrations of 2.29-11 ppm [32, 33]. The results of their work showed that different concentrations of these NPs had no significant inhibitory effect against the bacteria isolates. The difference of their findings with those from us mostly include due to differences in the strains studied shape/size of the NPs or different synthetic methods.

Biofilm formation plays a central role in the pathogenesis of *P. aeruginosa* . We observed that 160/200 (80%) and 40/200 (20%) of isolates produced strong-and moderate-level biofilms, respectively. It is noteworthy that the application of 1200ppm BiO NP decreased biofilm formation significantly where 52 (26%) and 22 (11%) of them produced strong and moderate-level biofilms, respectively ($p<0.0001$). Additionally, the exposure of 1200ppm BiO NP plus 500ppm ciprofloxacin decreased biofilm formation significantly where 38 (19%) and 16 (8%) of them produced strong and moderate-level biofilms, respectively ($p<0.0001$). In a study, rod shape FeOOH nanoparticles in size of 40 nm inhibited the biofilm formation by *P. aeruginosa* in a concentration-dependent manner [34]. In a study by Salomoni et al., 5µg/mL of commercial 10 nm silver NPs conferred antibacterial effect against *P. aeruginosa* [35]. Besides, MDR-*P. aeruginosa* strains were susceptible to AgNPs with MIC range of 1.406–5.625 µg/mL and the MBC range of 2.813–5.625 µg/mL [36]. There is a concern about the possible cytotoxicity of NPs in the environment and human body. Hence, more in-depth disclosure of their impacts is needed. However, due to the substantial antimicrobial effects of various compounds of NPs, they are promising agents in the era of extensive drug resistance [26]. [2, 3, 6, 8, 37]. In this study, BiO NPs were synthesized by the green method with irregular shapes ranging 23-41 nm using *B. subtilis*. BiO NPs at 2000 ppm had a significant inhibitory effect against MDR-*P. aeruginosa* planktonic form (MIC=2000ppm and MBC>2000ppm) which was concentration-dependent. Furthermore, sub-MIC level of BiO NPs exerted higher anti-biofilm effect significantly as compared to untreated isolates. Moreover, synergistic effect of this compound plus ciprofloxacin provided promising insights for combination therapies with antibiotics.

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This study was performed by the authors equally.

CONFLICT OF INTEREST

None to declare by the authors

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