ARTICLE / INVESTIGACIÓN

Antibacterial and anti-biofilm action of cobalt oxide nanoparticles beside persister *Pseudomonas Aeruginosa* isolates

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Abstract: Persister cells of *Pseudomonas aeruginosa* have developed a wide-reaching public health problem. Although this is a medical concern, there is currently no effective means to remove *P. aeruginosa* persister cells. Nanoparticles containing cobalt oxide (Co_3O_4-NPs) were examined for their ability to impact the formation of biofilms and inhibit the growth of bacteria. Researchers found that Co3o4-NPs were effective against *P. aeruginosa*, with inhibition zones ranging from 11 to 19 mm and MIC (Minimum Inhibition Concentration) results of 156 to 312 g/ml. The Co_3O_4-NPs with a titration of 10 mg/mL (76.54 percent) had the maximum biofilm suppression activity, while a titration of 0.156 mg/ml had the lowest (11.50 percent). According to the findings, *P. aeruginosa* biofilms and persister cells can benefit from applying co3o4-NPs.

Key words: Persister cell, SEM, co3o4-NPs, Anti-biofilm action.

Introduction

Antibiotic resistance and biofilms have become a severe hazard to incurable infectious infections. Combating them should be a high priority, with the primary technique being to explore strategies to avoid their establishment[1]. For further information on biofilms, see Biofilms: A community of adherent Bacteria with different metabolites from free-living bacteria[1]. Bacterial infections are thought to be caused by biofilms in 80% of cases[2]. These super constructs are inappropriately resistant to medications and the immunological defense system, creating a significant health concern. A good stress response and survival strategy for bacteria is the formation of biofilms[3,4]. *P. aeruginosa* is a life-threatening human opportunistic pathogen that flourishes in hosts that lack typical defenses, such as those suffering from severe illness or immunologically impaired. In addition to highly ill patients such as those in intensive care units (ICUs)[5], healthful persons can also take it. The most prevalent nosocomial infections that cause death and severe illness in hospitalized patients include[6]. Bacterial persistence, known to create chronic intractable infections, is one of the primary reasons for antibiotic therapy failure. Sub-inhibitory levels of antibacterial medications cause persisters, which are antimicrobial-tolerant cells that do not grow and lack a conventional genetic resistance mechanism, to emerge in populations of bacteria. Persister cells are hereditarily equal to antibacterial sensitive cells in a resident that is not sure of MIC rate or persister cell. However, they take a diverse behavior in that they can survive antibacterial at titer that may be harmful to other cells[7,8]. Persister cells have been identified in bacterial populations before the antibiotic was applied inactive slow growing due to phenotypic variation[9]. In general, nanomaterials have been demonstrated to have stronger microbicide activity than standard antibiotics and have even shown reactivity against numerous bacteria species[10]. The existing work aims to examine the anti-biofilm and anti-persister cell development ability of Cobalt oxide nanoparticles against P. aeruginosa.

Materials and methods

Isolation, Collection, Identification and Growth Circumstances of Bacteria

From a variety of Baghdad-Iraqi hospitals. Fifty different clinical isolates of P. aeruginosa were studied. Bacterial isolates were detected by their characteristic on selective and differential culture media, biochemical assays, and the Vitek-2 system. All bacterial isolates were cultivated on Luria Bertani (LB, Difco Laboratories) agar or in Luria Bertani broth for 18-24 hr. at 37 °C.

Synthesis and Characterization of Cobalt Oxide Nanoparticles

The Sigma-Aldrich Company provided all of the components and the highest quality. UV irradiation was used to create cobalt oxide nanoparticles[11-15]. A 125-watt UV source (with a maximum wavelength of 365 nm) and an ice-bath-cooled Pyrex tube make up the photocell, eliminating an increase in temperature due to UV radiation. Our results were as follows: we added 0.06-mole Ethylene glycol (CH2OH)2 to 75 ml of 0.03-mole cobalt (II) acetate for 20 minutes with a magnetic stirring rod. For 30 minutes, the mixture was exposed to light from a photocell. This morning's precipitation was a dark brown. The material was repeatedly separated and washed using ion-free water in a centrifuge. The material has been dehydrated for 24 hours. After calcining it for three hours at 450 °C, a black powder of co3o4-NPs was produced.

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Persister Cells Detection

The test for detecting persister cells was produced by applying rabidly killing techniques and freshly cultured cells with lytic solutions[16]. For the McFarland standard of 0.5 McFarland, *P. aeruginosa* isolates were cultured for 18-24 hours at 37 °C in Luria Bertani broth. Then, 200µL of buffer lysis solution was poured into a sterile 10 mL sterile test tube, vortex spun for 10 seconds, and then for 10 minutes were incubated at room temperature. After that, 2 µL 00 of the enzymatic lysis solution was gently poured over the mixture. Finally, incubate for 15 minutes at 200 rpm at 37 °C in a shaking incubator. A 10 µL smear on Luria Bertani agar was used to determine the frequency of persister cells, and the plates were incubated for 18-24 hours at 37 °C.

Biofilm Assay

According to (17), the following was done to evaluate the biofilm of *P. aeruginosa*: First, in brain heart infusion (BHI) broth, they were cultivated for 18-24 hours. The growth was then diluted to 1:100 and pipetted onto the wells of a 96-well polystyrene flat bottom plate containing BHI broth medium with 2 percent (w/w) sucrose, where it was cultivated for 24 hours at 37 °C, after being rinsed 3 times in phosphate-buffered saline and leaving it to dry at room temperature earlier, being colored for fifteen minutes with 200µL of 1% crystal violet. The stain was eluted using 200µL of absolute ethanol after aeration at room temperature, and OD was measured using an ELISA reader at 630 nm. The experiment was done three times for each isolate. Separate groups of test isolates were tested for adherence, each with a different mean optical density value.

No biofilms if the OD ≤ ODc, weak biofilms, If ODc < OD ≤ 2 × ODc

Moderate biofilms, if 2 × ODc < OD ≤ 4 × ODc and if 4 × ODc < OD, strong biofilms

Antimicrobial Effect and MIC of Co3O4-Nps

To study the inhibitory impact of Co_3O_4-Nanoparticales against persistent *P. aeruginosa* Strains, according to (30), agar well diffusion methods were utilized. DMSO in the other served as a negative control, and an antibiotic disc in the other well was a positive control, the co3o4-Nanoparticales in the latter well. In addition, the MIC was determined using a 96-well polystyrene microtiter plate. 100µL of (MHB) Muller Hinton broth was put on a microtiter plate for each well, and then 100µL Co_3O_4-NPs (10 mg/mL) was added to the first vertical row from A1 to A10, followed by 1:2 serial dilutions. The remaining 100 mL of the last well had been abandoned. After 24 hours of incubation, 5 µL of bacterial suspension (108 CFU/mL or 105 CFU/well) was added to all wells except the negative control row from A12 to H12. After dyeing with 10µL of resazurin dye were put to each well and then incubated for around four hours at 37 °C to get prepared for reading, the effects of diverse titer of Co_3O_4-NPs for the microbiological growth were determined by utilizing UV visible spectrophotometer[18].

Anti-Biofilm Activity of Co3o4-Nps

Co3O4-NPs' anti-biofilm impact on persistent *P. aeruginosa* strains was studied following[19]. The isolates of P. aeruginosa were injected in BHI broth at 37 degrees Celsius. After that, 20 µL of bacterial suspension was put into each microtiter plate well, containing 80 µL of BHI broth with 2 % sucrose. 100 µL of co3o4-NPs was added and mixed well before incubating at 37°C for 24 hours. Then, the plate was allowed to dry at room temperature for two further washes in PBS. Cells of Bacteria that cannot be linked to one another were removed. Biofilms were stained with 200 µL of 1% crystal violet solution. The stain was eluted with 200µL of absolute ethanol, and the OD was calculated using an ELISA reader at 630nm. Only B.H.I broth was used as a negative control. The experiment was done three times for each bacterial isolate. The following equation was used to calculate the rate of inhibition rate of Co_3O_4-NPs:

Rate of inhibition (%) =(OD of control – Od of treated) / (OD of control) × 100

Results

Persister Cells Detection

Rapidly killing approach, used to examine the formation of persister cells in P. aeruginosa isolates. According to the findings, only 5 out of 50 P. aeruginosa isolates (PA4, PA5, PA21, PA27, and PA47) had persister cells[20] seen in Figure 1.

As a result, it is an optimal procedure that operates without influencing the culture's bacterial population size, strain, or physiological state.

Cobalt Oxide Nanoparticles, Synthesis and Characterization

A distinctive XRD design of the precursor synthesized at 450 °C for three hours is shown in Figure 2. All of the reflection peaks in this pattern (JCPDS Card File No. 76–1802) might be easily attributed to crystalline cobalt with oxygen. In this pattern, there were no noticeable impurity peaks. The diffraction peaks of 2θ = 19.50°, 31.37°, 37.02°, 39.10°, 44.97°, 55.84°, 59.58°, 65.46°, and 77.62° appeared. The mean crystallite sizes were 25 nm.

The ingredient of the created elements at 450°C was fixed by EDS analysis. Figure 3 shows that the result contained just cobalt (Co) and oxygen (O) elements, with a cobalt/oxygen atomic ratio of roughly 3:4, which is compatible with the predicted value of Co3O4. No additional elements are found, indicating that the Co3O4 NPs are highly pure.

SEM was used to evaluate the morphology of the created units. The SEM image of produced Co_3O_4 nanoparticles is shown in Figure 4. The nanoparticles have a sphere-like morphology in the SEM picture, and they are created of agglomerated collected particles representing connectivity well among particles. The size of nanoparticles, as well as their homogeneity and size distribution, can be detected by using SEM. The SEM image shows that the particles are evenly scattered. The mean particle size was randomly estimated from the figures to be 37nm.

Co3O4-Nps, Antibacterial Activity And MIC

On persistence, Co_3O_4-Nanoparticales were proven to have antibacterial efficacies against *P. aeruginosa*. Co3O4-NPs displayed antibacterial solid activity in the agar-well diffusion technique, with inhibition zones (11-19 mm) at 10.000g /ml. According to the MIC results, Co_3O_4-NPs had high antibacterial activity (156.25 -312.5 g/ml), as demonstrated in Table 1 and Figure 5 A, B. The ability of persistent *P. aeruginosa* to proliferate was often reduced during 16 hours of Co_3O_4-NPs exposure, as was also observed.

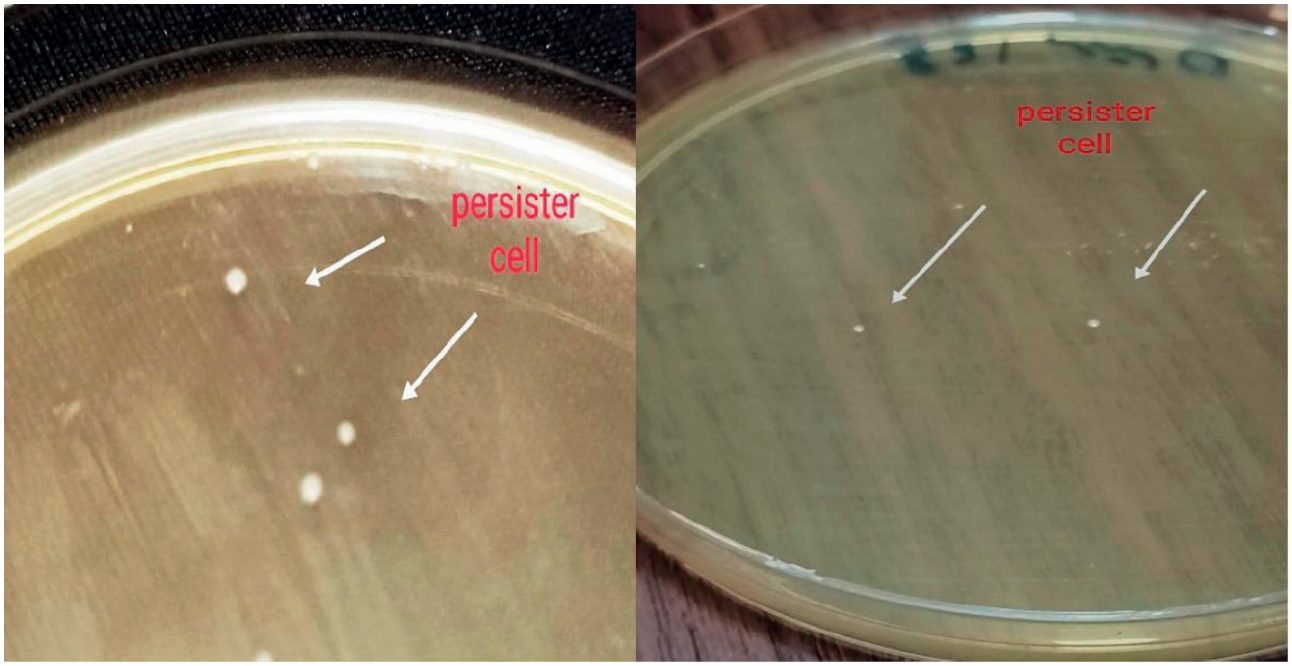

Figure 1. Formation of Persister cell in *P. aeruginosa* By Rapidly Killing Method.

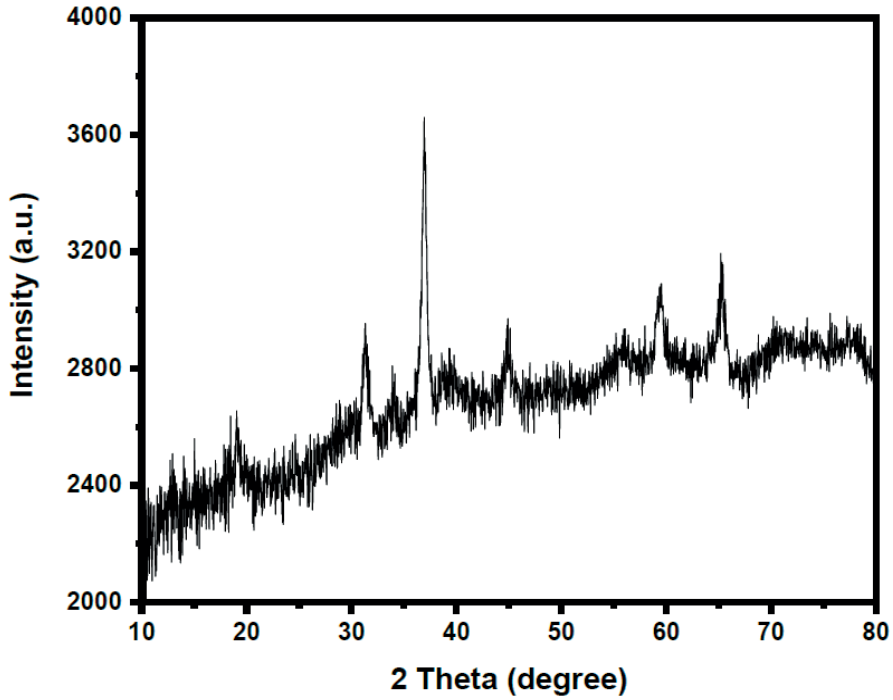

Figure 2. XRD Pattern Of Co_3O_4 -Nanoparticles.

Biofilm Inhibition Activity

The ability of Co_3O_4-NPs to suppress biofilm formation by persister cells of P.aeruginosa isolates was examined in this work. Co_3O_4-NPs were examined for their average inhibition rate against *P. aeruginosa*. Co_3O_4 nanoparticles greatly assisted biofilm reduction. Biofilm was inhibited to a percentage of 76.54 percent at 10 mg/ml and 0.156 mg/ml. Because of this, co3o4-NPs had a considerable effect on P. aeruginosa persister cell biofilms.

If we can diminish persister, we can reduce bacterial resistance to medicines and therapies, which will help treat chronic infections. We assessed the existence of the creature utilizing a quick-killing strategy. Following testing with lysis buffer solution and enzymatic lysis solutions, persister cells in *P. aeruginosa* isolate. Current work indicates the inhibitory zones of Co_3O_4-NPs ranged from 11- 19 mm in a concentration of 10.000µg/ml to remove *P. aeruginosa* persister cells. For *P. aeruginosa* isolates, the MIC of Co_3O_4-Nanoparticales ranged from 156.25 to 312.5 µg/ml. Results showed that Co_3O_4-NPs demonstrated a significant anti-biofilm activity at 76.54 percent present in co3o4-Nanoparticales at a concentration of 10000 µg/mL correspondingly.

Discussion

Methods that depend on antibacterial titer are substantially slower than the rapid killing method of persister cell isolation. Further studies can be conducted on the isolated

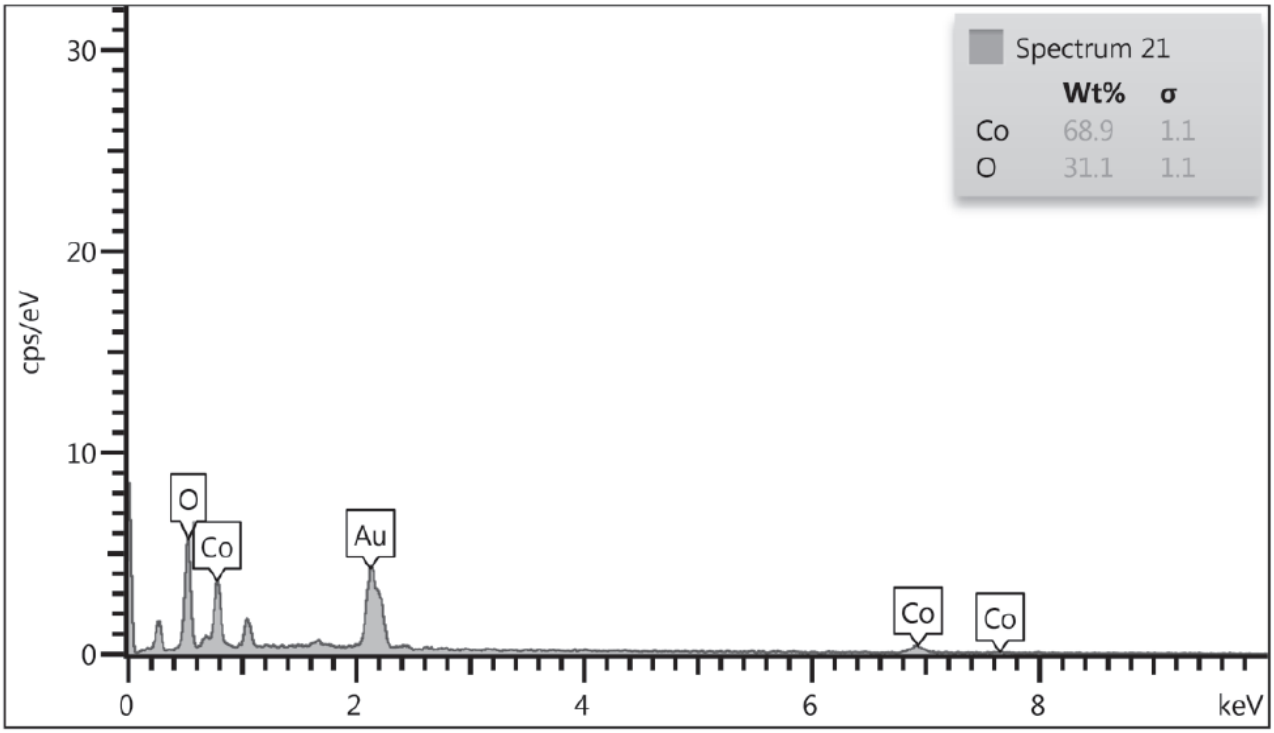

Figure 3. EDS Measurement of S the Co_3O_4 Nps.

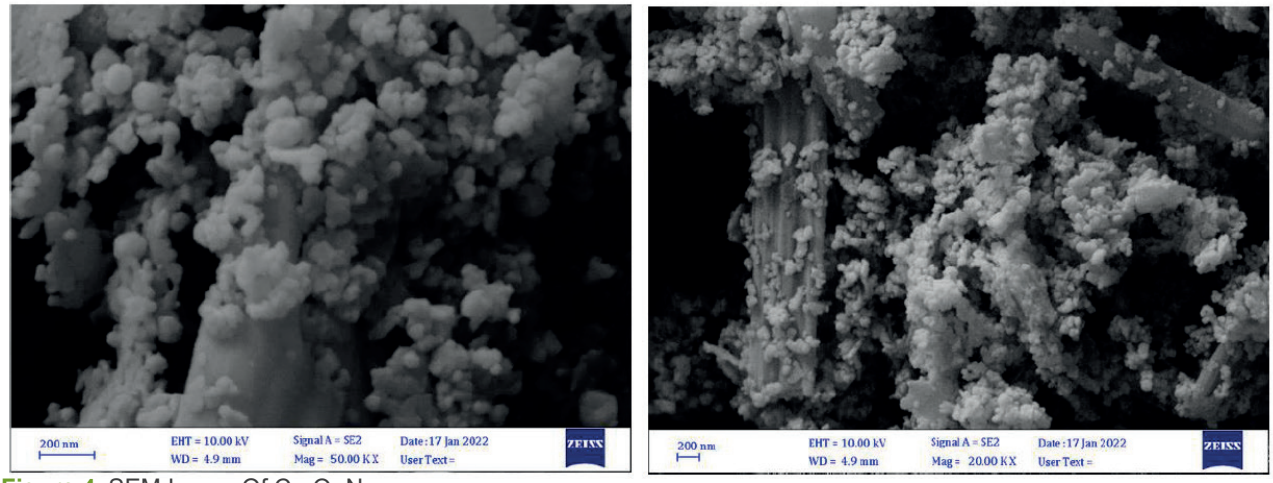

Figure 4. SEM Image Of Co_3O_4 Nps.

NO	*P. aeruginosa* Isolate	MIC of co3O4-NPs (µg /ml)	Inhibition zone (mm)
1	PA 4	156,25	11
2	PA 5	156,25	13
3	PA 21	156.25	11
4	PA 27	312,50	19
5	PA 47	156,25	13

Table 1. Antibacterial Effect of Co_3O_4-NPs Against Persister Cell of *P. aeruginosa* Isolates.

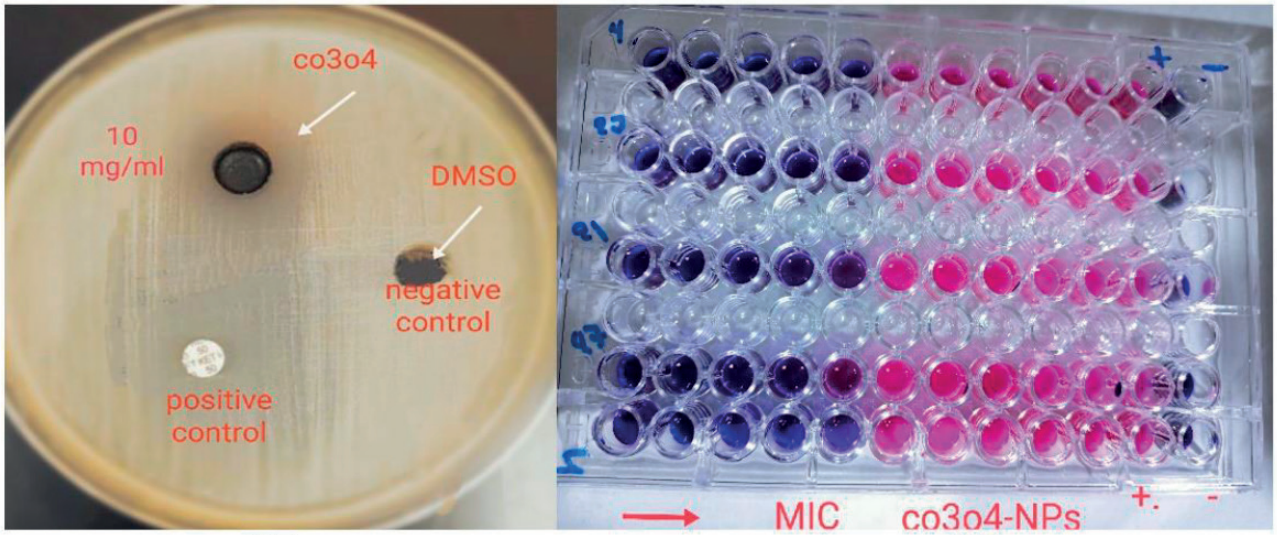

Figure 5. Antibacterial Activity Of Co_3O_4-Nps Against Persister Cell Of *P. aeruginosa* Isolates By A-Agar-Well Diffusion Method B-Micro Titer Plate Method.

Biofilms inhibition rate (%)	Co3o4-NPs concentration (µg/ml)
76.54	10000
63.34	5000
49.20	2500
37.64	1250
25.02	625
19.10	312.5
11.50	156.25

Table 2. AntiBiofilm Ability Of Co_3O_4-Nps Against Persister *P. aeruginosa* Isolates.

persister cells because the fast killing approach is stable after isolation[16].

The reflection peaks could be easily attributed to crystalline cubic phase Co_3O_4. The crystallite sizes of the as-produced were calculated by using the Scherer formula[21-23].

P. aeruginosa is a hazardous microorganism that is a source of various infections in the community and hospitals. In recent years, the incidence of multidrug-resistant *P. aeruginosa* isolates has become a global public clinical issue[24]. Bacterial persistence refers to the survival of a minor proportion of germs after being eliminated by bactericidal drugs at lethal levels. Co_3O_4-NPs were also tested against *P. aeruginosa* persister cells. It was evaluated as an antibacterial and suppression of biofilm formation agent concern to diverse reports[27]. The antibacterial activity of Co_3O_4-NPs was improved against a wide range of microbial diseases[28]. In general, the effects of Nanoparticles Co_3O_4 against bacteria were summarized as follows: electrostatic interaction causes mechanical harm to the cell wall, oxidative stress from the formation of reactive oxygen species (ROS), and damage to protein activities and cell structures from metal cation release[29].

Conclusions

To boost their bactericidal potency, Co_3O_4-nps were synthesized utilizing a photolysis method. The current work was the first to indicate that Co_3O_4-NPS may effectively suppress biofilm and persister cell production of *P. aeruginosa*.

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Conflicts of Interest

The authors declare no conflict of interest.

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