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Antibacterial Effect of Biosynthesized Nanoparticles of Ferric Chloride Against MDR-*Pseudomonas aeruginosa*

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Abstract

Background. Multidrug-resistant (MDR) *Pseudomonas aeruginosa* poses a serious challenge in healthcare due to limited effective treatments. Biosynthesized iron oxide nanoparticles (FeO-NPs) have emerged as a promising alternative, showing potential antibacterial activity against MDR bacteria. **Aim.** This study synthesized and characterized biosynthesized iron oxide nanoparticles (FeO-NPs). It also evaluated their antibacterial activity against multidrug-resistant *Pseudomonas aeruginosa* from clinical samples. **Methods.** A total of 150 burn wound samples were collected between September and October 2023 from Medical City Hospital, Imam Ali Hospital, and Yarmouk Hospital. The samples were obtained for the isolation of pathogenic Pseudomonas aeruginosa. After isolation and laboratory identification, 70 samples were confirmed to be pathogenic P. aeruginosa only. **Results**. After performing conventional diagnostic procedures, including macroscopic and microscopic examinations, followed by standard biochemical tests, 70 isolates were identified as Pseudomonas aeruginosa out of 120 clinical burn swab samples. **Conclusion.** Biosynthesized FeO-NPs showed strong activity against multidrug-resistant *Pseudomonas aeruginosa* (18 mm at 1000 mg/mL). They may serve as eco-friendly alternatives to combat antibiotic resistance.

Keywords: Multidrug-Resistant; *Pseudomonas aeruginosa*; Fe_2O_3; Nanoparticles.
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Introduction

Pseudomonas aeruginosa commonly exhibits both multidrug resistance (MDR), in which there is resistance to at least one agent in three or more antimicrobial categories, and extensive drug resistance (XDR), which indicates that the bacteria remain sensitive to only one or two categories of antibiotics. (1, 2). The MDR-*P. aeruginosa* is becoming more prevalent as a cause of death in burn patients. In pediatric burn Intensive Care Units, 86% of deaths after sepsis are attributed to MDR organisms, with *P. aeruginosa* being the primary culprit in burns patients, which leading to (4 - 60%) nosocomial infections in various parts of the world (3, 4). Hence, it is imperative to expedite the development of novel antibacterial therapies to tackle these escalating issues. Over the past few decades,

there has been a growing development and use of nanotechnologies to tackle the problem of bacterial resistance, yielding encouraging results. (5).Nanotechnology has experienced a surge in popularity in recent years. Nanotechnology is a multidisciplinary field that includes encompasses technology, engineering, and science at the nanoscale. It has applications in various scientific disciplines, including engineering, materials science, physics, biology, and chemistry (6).

Nanoparticles serve as the fundamental building blocks of nanotechnology. Nanoparticles are minuscule particles that have a size smaller than 100 nm as objects ranging in size from 1- 100 nm and can be composed of carbon, metal, metal oxides, or organic compounds (7). Iron oxide nanoparticles are a type of metal nanoparticle that has recently been produced. They are known for their distinctive micro configuration and features, such as being superparamagnetic and having a strong coercive force. (8). Various methodologies have been devised for the production of hematite particles. One potential approach is the utilization of bacteria, specifically *Escherichia coli*, as an alternative to the traditional chemicals and physical processes. This eco-friendly method allows the creation of metal or metal oxide nanoparticles. (9). The nanoparticles that are produced through the biological process exhibit enhanced catalytic reactivity, increased specific surface area, and better enzyme and metal salt properties. (10).

The biological process is a more sustainable and energy-efficient method that is also environmentally favorable. (11, 12). In this study, we investigated the antibacterial effect of biosynthesized nanoparticles of ferric chloride (Fe_2O_3- NPs) on MDR *P. aeruginosa*. And focused on examining the microbial synthesis of Fe_2O_3 nanoparticles. This project involves utilizing the bacterial strain *E. coli* as a reducing agent to produce Fe_2O_3 nanoparticles. The particles will be characterized using a UV-visible spectrometer, Atomic Force Microscopy (AFM), and Fourier Transform Infrared Spectroscopy (FTIR) analysis.

Materials and methods

Samples collection

A total of 150 burn wound samples were collected between September and October 2023 from Medical City Hospital, Imam Ali Hospital, and Yarmouk Hospital. The samples were obtained for the isolation of pathogenic *Pseudomonas aeruginosa*. After isolation and laboratory identification, 70 samples were confirmed to be pathogenic *P. aeruginosa* only.

Isolating and Identifying *P. aeruginosa* in the Laboratory Isolation of *Pseudomonas. Aeruginosa*

The clinical burn swabs were subjected to culture on various types of agar, including Blood, MacConkey, Brain heart infusion agar, and *P. aeruginosa* Cetrimide agar, which is a selective medium. (13, 14). Then, the cultures were incubated at a temperature of 37ºC for 18 - 24 hours.The suspected bacterial isolates, perhaps belonging to *Pseudomonas aeruginosa*, were inoculated on Cetrimide agar to exhibit the distinctive characteristics of this bacterium, such as a blue-greenish hue and the presence of a fruity odor.

Identification of *Pseudomonas. Aeruginosa*

Common techniques are used to detect *P. aeruginosa* in the laboratory, which involves observing the physical characteristics and appearance of microbial colonies that have been developed in the culture media. (15). A single colony was chosen from each primary positive culture, and it was characterized by its morphological traits, such as texture, elevation, edge, translucency, pigmentation, shape, and size. A Gram stain was conducted on a bacterial smear to analyze the cellular morphology of bacterial cells, including their

Gram reaction, shape, spores, presence of capsules, organization, and other properties. (16). Cetrimide agar is a selective media for pseudomonas (17). The identification of the bacteria *P. aeruginosa* was conducted using the VITEK 2 system. All probable bacterial isolates of *P. aeruginosa* were exposed to various biochemical assays that include oxidase, catalase, Simmons citrate agar test, and other tests.

Culture media

Blood agar, MacConkey agar**,** Muller Hinton agar ,The user mentions the use of Brain Heart infusion agar medium .and the selective media of *P. aeruginosa* cetrimide agar (18).

Biochemical tests

The subsequent biochemical assays were employed to ascertain the identity of the bacterial isolates, oxidase**,** catalase, and Simmons citrate agar test. Furthermore, the VITEV2 system was used.

Antibiotic sensitivity test

The disc diffusion method is considered the most reliable technique for confirming and assessing the susceptibility of bacteria to antibacterial drugs. (19). In this test, isolated bacterial colonies were chosen and suspended in nutrient media. The colonies were then standardized by using the turbidity test, specifically by employing MacFarland no. 0.5. The standardized suspension is cultivated and spread onto the Muller Hinton agar plate, and the various antibiotic discs are placed on the inoculation plates. Thirteen various antimicrobial discs were allowed to be spread within the hardened agar according to CLSI 2023, which include: Imipenem (10µg)**,** Meropenem (10µg)**,** Ceftazidime (30µg)**,** Ciprofloxacin (5µg)**,** Piperacillin-Tazobactam (100/10µg)**,** Gentamicin (10µg)**,** Aztreonam (30µg)**,** Amikacin (30µg)**,** Levofloxacin (5µg)**,** Tobramycin (10µg)**,** iron oxide nanoparticles was stored in a light-free container. The procedure of producing Cefepime (30µg)**,** and we chose 2 that are not found in the guidelines of the clinical and laboratory Standards Institute, then the standards were compared **(**CLSI,2023**),** which **i**ncludes**:** Nalidixic acid (30 µg/disk) and Tigycyclin (15µg/disk**)**.

Production of biomass from *E. coli*

The bacterial strain *E. coli* was cultivated in (brain heart infusion) broth medium to generate biomass for biosynthesis. The culture flask was placed on an orbital shaker and incubated at room temperature after stirring at a speed of 220 rpm. Subsequently, the biomass was collected during 24 hours of growth. Nanoparticles were synthesized using the collected cells.

Biological synthesis of Iron Oxide (Fe_2O_3) nanoparticles

The biological technique approach was employed to synthesize iron oxide nanoparticles by utilizing Ferric chloride ($FeCl_3$) with modification. The intracellular extract produced by *E. coli* was used in the manufacture of Fe_2O_3 nanoparticles. The standard procedure was done as follows: 20 grams of Ferric chloride ($FeCl_3$) were mixed in 250 ml of the solution of intracellular extract that was obtained from *E. coli*. The mixture was dispersed by using an ultrasonication bath for 10 minutes to ensure thorough mixing of the components. The flask was then placed in a shaker in the dark room overnight. Next, the mixture was subjected to centrifugation for 20 minutes at a speed of 8000 rounds per minute (rpm). The residual solution containing iron oxide nanoparticles was rinsed twice with deionized distilled water to remove any remaining intracellular remnants. The nanoparticles which were obtained from the precipitation process were dried in the oven at the temperature of 40°C for one night. Ultimately, a brown powder consisting of nanoparticles was according to the mentioned reference with some modifications. (20, 21).

Characterization of Fe_2O_3 NPs

The presence of Fe_2O_3 nanoparticles was verified by measuring the wavelength of the reaction mixture by using UV-visible spectroscopy Optima (Japan). An atomic force microscope (AFM) UNICCO / USA was used to study the surface morphology and diameter of NPs. The functional groups were identified through analysis by using Fourier Transform Infrared Spectroscopy (FTIR) Shimadzu (Japan).

Fe_2O_3 Nanoparticles' Antibacterial Activity

The *P. aeruginosa* was cultivated on M H-agar by utilizing the method of microbial suppression known as minimum inhibitory concentration (MIC), which involved synthesizing Fe_2O_3 nanoparticles from external sources. (22, 23).

Results

After performing conventional diagnostic procedures, including macroscopic and microscopic examinations, followed by standard biochemical tests, 70 isolates were identified as Pseudomonas aeruginosa out of 120 clinical burn swab samples.

Antibiotics resistance in *P. aeruginosa*

Out of the total of 120 isolates, only 70 isolates were subjected to susceptibility test (disc diffusion method) for 13 different antibiotics that were suggested by the CLSI (2023) (24). The guidelines and (Figure 1) demonstrate different levels of resistance. In this investigation, *P. aeruginosa* exhibited the highest percentage of resistance to Nalidixic acid (NA), followed by Tobramycin (TOB), Gentamicin (CN), and Ceftazidime (CAZ). Among the selected anti-biotics are: Imipenem (IPM), Meropenem (MEM), Ciprofloxacin (CIP), Piperacillin-tazobactam (PIT), Aztreonam (AT), Amikacin (AK), Levofloxacin (LE),Cefepime(CPM), Tigycyclin(TGC).

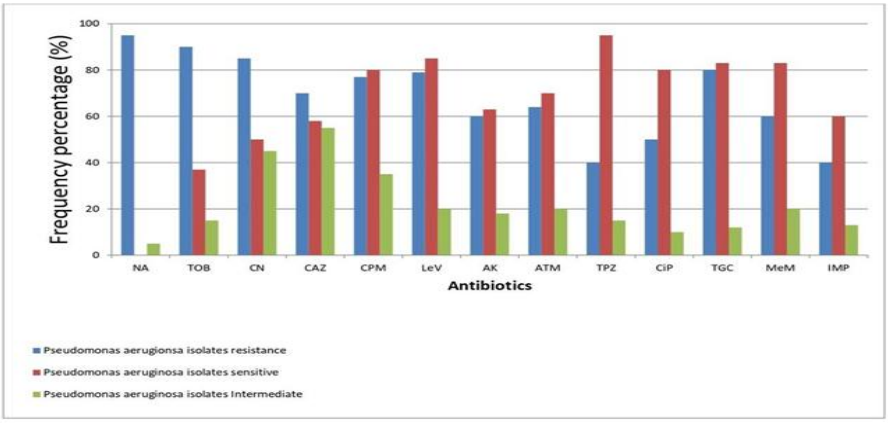

Figure (1): The result of antibiotic susceptibility test of *P. aeruginosa*.

Analysis of biological synthesis Fe_2O_3 nanoparticles

Analysis of the UV-Visible spectrum of Fe_2O_3 obtained from the extracellular environment.

The nanoparticles' optical characteristics were examined by using a UV-visible spectrometer. Ultraviolet-visible spectro-scopy is a type of spectroscopy that involves the measurement of absorption or reflection of light in the ultraviolet-visible range of the electromagnetic spectrum. This indicates that it utilizes light within the visible spectrum and the surrounding wavelengths. The absorption or reflection of light within the visual range at room temperature has a direct impact on the apparent color of the substances of interest. (Figures 2 and 3) show that the nanoparticle exhibited a Sharp absorption peak at a wavelength of 290 nm. The sample was analyzed using UV–vis spectroscopy in the wavelength range of 280 to 780 nm. The extracellular *E. coli* exhibited a distinct emission peak at 300 nm and a broad emission range from 250 to 850 nm. UV irradiation was observed in the emitted light. (21, 25-27).

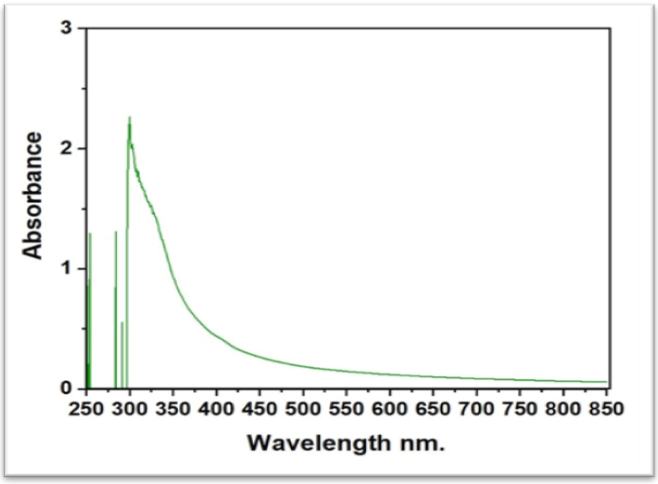

Figure (2) The ultraviolet-visible spectra of extracellular *E. coli*.

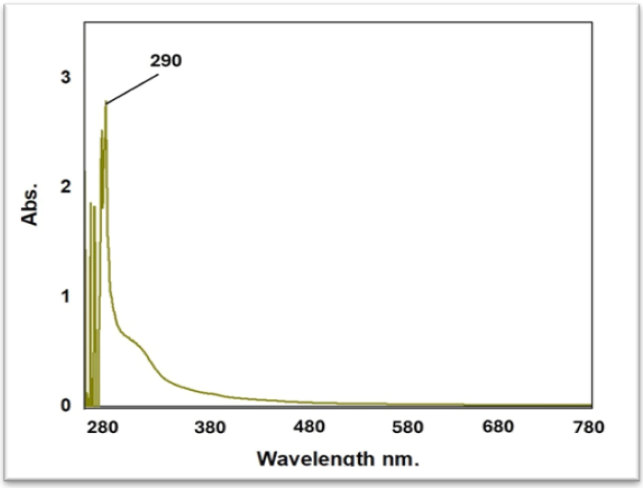

Figure (3): the ultraviolet-visible spectrum of Fe_2O_3 nanoparticles synthesized by *E. coli*.

Analyzing Fe_2O_3 by using atomic force microscopy (AFM) from an extracellular source

The atomic force microscope was used to investigate the formation of the surface shape of the Fe_2O_3 nanoparticles. The results revealed that the Fe_2O_3 nanoparticles exhibited both two-dimensional and three-dimensional structures, as shown in (Figure 4) (28), AFM images demonstrate the spherical shape of the biosynthesized Fe_2O_3 NPs. An AFM was used to quantify the average diameter of 57.94nm (Figure 4).

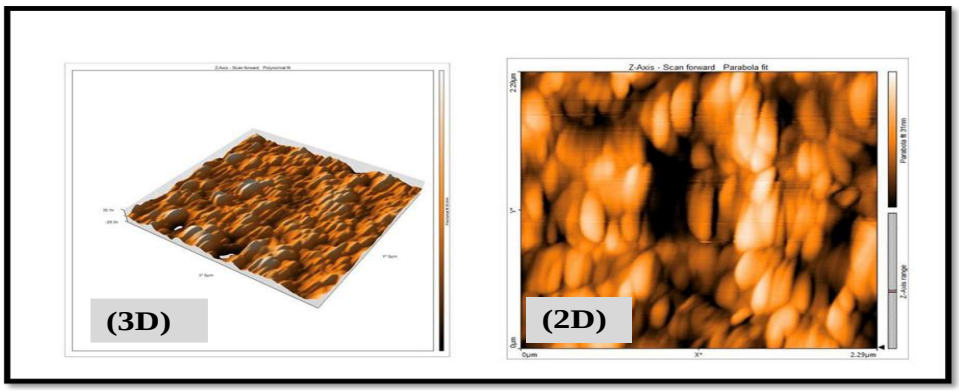

Figure (4): The AFM analysis of iron oxide nanoparticles that *E. coli* biosynthesized.

An extracellular sample of Fe_2O_3 was subjected to Fourier transform infrared (FT-IR) analysis.

The functional groups of nanoparticles have been identified by using FT-IR spectra. The (Figure 5) depicts the absorption spectrum of nanoparticles that have been synthesized through biological means, as observed using the signal observed at 1639 cm^{-1}, which corresponds to the C=O functional group present in tertiary amides, (29) Peaks within the range of 567 to 467 cm^{-1} correspond to the stretching vibration of the Fe-O bond. (30, 31), and The FTIR spectrum of Fe_2O_3-NPs exhibited bands/peak assignments within the range of 3433.29-Br (OH) to 3410 cm^{-1}. (32), is associated with the hydroxyl group that is found in water molecules or ferric hydroxides that are present in the samples (33), bands at 1072 cm^{-1} correspond to the O-H stretching of phenols (O-H stretch) (34).

Antibacterial susceptibility test

The antibacterial activity of iron oxide nanoparticles was examined by using Gram-negative bacteria, specifically *P. aeruginosa*. The agar well diffusion method was employed to determine the minimum inhibitory concentration (MIC) of Fe_2O_3 NPs against bacteria. (35, 36). Dispense around 25 mL of aseptic Mueller–Hinton agar onto a sterile plate and let it reach a controlled temperature of room temperature. Wells were formed by applying a culture of the test isolates to the agar, the wells were created by pinching with a sterile cotton swab. Various concentrations of Fe_2O_3 nanoparticles range from extracellular (1000, 500, 250, 125, 62.5, 31.25) mg/mL. Plates containing Fe_2O_3 nanoparticles were placed in the incubator at 37°C overnight. the area of growth inhibition surrounding the well estimated after the period of incubation (37).

This causes an electromagnetic interaction between the metal oxides and the microorganisms, which ultimately leads to oxidation and the eventual death of the microorganisms. The extracellular studied bacteria Fe_2O_3 nanoparticles exhibited minimum inhibitory concentrations (MICs) ranging from 1000 to 31.25 mg/ml; it was determined by using the serial dilution procedures that are outlined in the Clinical and Laboratory Standards Institute (CLSI) guidelines. (37). The bactericidal effect of Fe_2O_3 nanoparticles on bacteria is highly significant because the pathogenic bacteria can integrate with the food chain of an ecosystem. (38).

The bactericidal properties of Fe_2O_3 were verified in a recent scientific study and subsequently disseminated in contemporary research. (38). The results of extracellular antibacterial activity of Fe_2O_3 nanoparticles are shown in (Figure 6). The antibacterial activity was observed to be directly proportional to the concentration of Fe_2O_3 nanoparticles). When the concentration of Fe_2O_3-NPs from extracellular material is 1000 mg/mL, a max inhibition zone of *P. aeruginosa* is 18mm, while a min inhibition zone occurs at a concentration of 250 mg/mL Fe_2O_3 NPs. The results indicate (Table 1) that the differences in inhibitory diameter can be linked to a unique interaction between Fe_2O_3 nanoparticles and the microorganisms, along with the different susceptibility of the bacteria in this study. The primary mode of toxicity of Fe_2O_3 NPs is likely attributed to the positive charge that is carried by the metal oxides despite the negative charges present in the microorganisms.

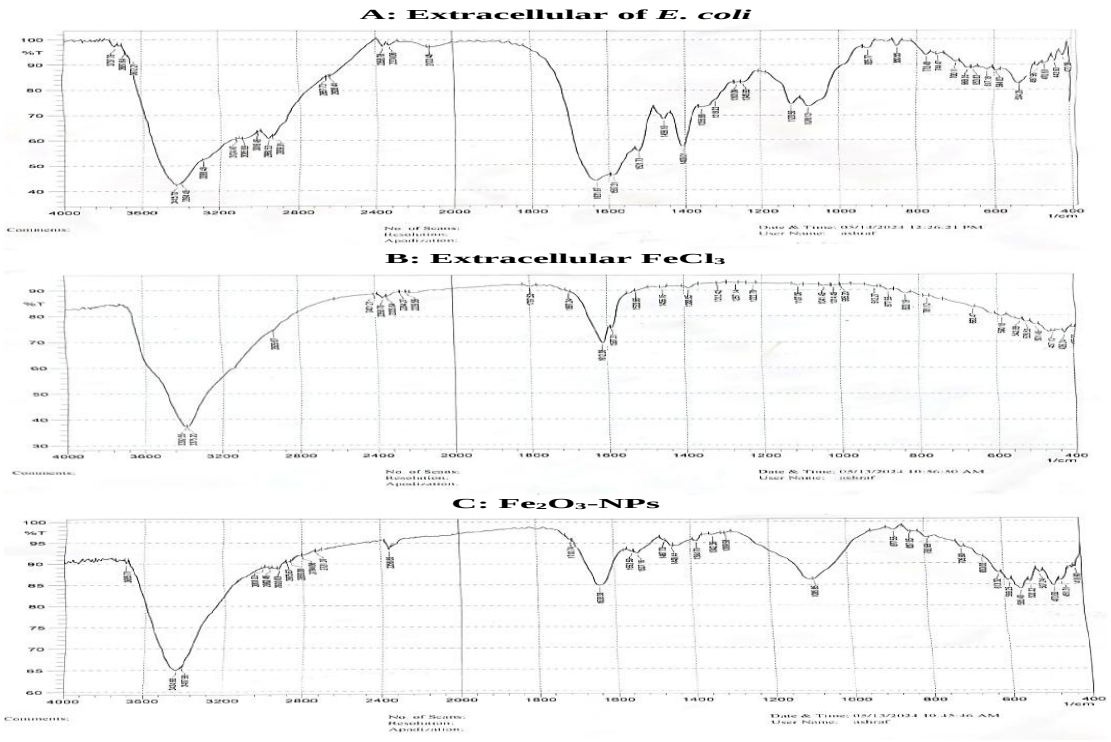

Figure (5): The FT-IR spectroscopy measurement of A: Extracellular of *E. coli*, B: Extracellular $FeCl_3$, C: Fe_2O_3-NPs.

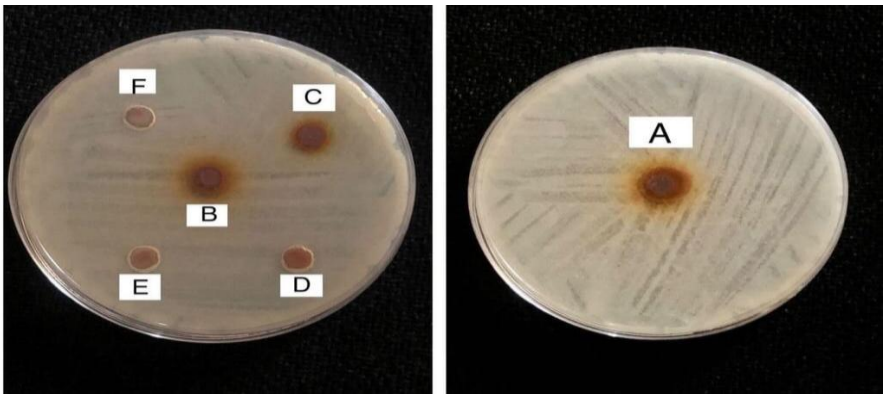

Figure (6): Antibacterial action of Fe_2O_3 NPs from extracellular on *P. aeruginosa*.

Table (1): The inhibitory zone of the antibacterial activity of extracellular Fe_2O_3 on *P. aeruginosa*.

ID	Fe_2O_3 concentration (mg/mL)	Inhibition zone (mm)
A	1000	18
B	500	15
C	250	10
D	125	Nil
E	62.5	Nill
F	31.25	Nill

Discussion

The identification of 70 P. aeruginosa isolates indicates that this bacterium represents a significant cause of burn wound infections. The relatively high prevalence may be attributed to the organism's ability to survive in hospital environments and its resistance to multiple antimicrobial agents, making it a common pathogen in burn units. Biosynthesized iron oxide nanoparticles (IONPs) produced from ferric chloride show considerable promise as antibacterial agents against Multidrug-Resistant *Pseudomonas aeruginosa*. This work shows the process of producing Fe_2O_3 nanoparticles by the use of extracellular *E. coli* as a reducing agent. In addition to that, the obtained Fe_2O_3 nanoparticles were characterized by UV-vis, AFM, and FT-IR techniques. Simultaneously, the AFM disclosed the diameter range of the nanoparticles. The antibacterial activity test revealed that the bio-synthesized substance exhibits potent antibacterial activity against the targeted bacterium. The highest level of inhibition was seen with an inhibition zone of 18 mm at a dosage of 1000mg/mL.

Conclusion

The production of IONPs by utilizing ferric chloride not only relates to sustainable and environmentally friendly chemistry principles but also offers a hopeful resolution to combat the increasing problem of antibiotic resistance in bacterial pathogens. Continued studies in this subject are needed because they will lead to groundbreaking uses of antimicrobial therapies.

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