Characterization and Antibacterial Activity of Biogenic Iron Nanoparticles using *Proteus mirabilis*

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Abstract

Background: The green production of iron oxide nanoparticles (FeONPs) due to its numerous biotechnological uses has attracted a lot of attention and clean and eco-friendly approaches in the medical field. **Objectives:** The objectives of this study are to demonstrate the biogenic creation of FeONPs. The search for alternative antimicrobial medicines has been prompted by growing worries about multidrug resistance. **Materials and Methods:** The purpose of this work was to investigate if *Proteus mirabilis* was the source of green-manufactured iron nanoparticles (INPs). The Fourier transform infrared, attenuated total reflectance, scanning electron microscopy, transmission electron microscopy, energy dispersive X-ray spectroscopy, ultraviolet–visible, and dynamic light scattering measurements were used to characterize the green synthesized nanoparticles (NPs). Using the agar-well diffusion method, the antibacterial investigation's efficacy was evaluated. **Results:** The results found that at 128 µg/mL minimum inhibitory concentration, we have demonstrated the potent antibacterial properties of FeONPs produced through biological procedures against Gram-positive and Gram-negative bacteria that are resistant to many drugs. These NPs, including their antimicrobial qualities, how they work, how they affect drug-resistant microbes, the risks involved with using them, the unique qualities of NPs, their mode of action as antibacterial properties, and the elements that influence how well they function in a therapeutic setting all are thoroughly investigated. **Conclusion:** Further research on biogenic INPs was necessary to enhance their nanopharmacological importance for biological applications, as they have demonstrated promising biomedical capabilities were successfully synthesized in a friendly environment with few costs, and the highly INPs product showed exceptional activity against bacterial strains.

Keywords: Antibacterial activity, characterization, iron nanoparticles, *Proteus mirabilis*

Introduction

A science driven by technology, like nanotechnology, has garnered a lot of interest due to its capacity to design practical systems, such as drug delivery systems and molecular-scale diagnostic necessities, which also serve as the cornerstones of nanomedicine and prosperous drug delivery systems for humankind.[1] Nowadays, many alternative substances are used against multidrug-resistant bacteria, such as *Citrus limon*.[2] Precision medicine has benefited from the successful application of nanotechnology to create novel medication delivery methods using nanoparticles (NPs). Advances in NP design have made it possible for NPs to efficiently improve efficacy while overcoming heterogeneous delivery barriers.[3] Furthermore, NPs have a complicated three-layer structure made of metal ions, surfactants, or polymers, a shell layer, and the NP's.[4] NPs use the already-existing cellular machinery to help them carry out their intended actions and are scaled similarly to the macromolecules found inside the human body. NPs have also effectively dropped drug toxicity and side effects due to high efficacy delivery, which supports continued drug release and alters drug delivery in addition to clearance.[5] Biological approaches minimize energy and chemical usage and are stable, affordable, and environmentally friendly, such as zinc oxide nanoparticles (ZnONPs) and

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less toxicity ZnONPs.[6,7] Bacterial and fungal species are among the many microorganisms that have shown diverse biological potentials for NP synthesis. They also provide advantages for wastewater bioremediation, the production of bioenergy, and the large-scale manufacturing of commercial goods like pigments and pharmaceuticals.[8]

The medicinal value of plant materials, their accessible availability, and a dependable, sustainable, and environmentally friendly technique for synthesizing NPs have made the green synthesis of iron nanoparticles (INPs) an intriguing field of study. Biological materials, such as microbes and plants, are thought to be less expensive and environmentally friendlier than physical and chemical syntheses.[9] The application of inorganic nanoparticles in molecular targeted cancer therapy. The iron oxide nanoparticles (FeONPs) have confirmed safety and significant effectiveness for treating hyperthermia and diagnosing cancer, among those that the food and drug administration has approved.[10] In other words, plants have been used as "biofactories" to make metal NPs since they are inexpensive and easily obtainable locally. Important phytochemical components (terpenoids and flavonoids) containing functional groups like amine, hydroxyl, and carboxylic are used as reducing agents in the manufacture of FeONPs.[11]

Gram-negative bacteria in the genus *Proteus* are members of those belonging to the enter family of bacteria. *Proteus* species can be identified from the majority of other genera by their capacity to swarm over an agar surface.[12] ZnONPs that are greenly made and can be used as an anti-biofilm agent against *Proteus mirabilis* by generating reductive enzymes and bacterial cells were able to catalyze the biosynthesis process. The NPs were created using *P. mirabilis* cell-free extract.[13,14]

Various methods were employed to enhance the characterization of the biogenic FeONPs. Additionally, a wide range of bioactivities have been investigated, such as assays for protein kinase inhibition, alpha-amylase, cytotoxicity, antifungal activity, and biocompatibility. We made it possible for the created NPs to be utilized safely in a variety of industries by assessing their effectiveness.

Material and Methods

Samples collection

Urine samples from 100 patients with urinary tract infections who were hospitalized at Al-Yarmouk Teaching Hospital, Baghdad, Iraq, were collected. The two main techniques used to identify *P. mirabilis* were the cultivation on MacConkey agar and bood agar at 37°C for 24 h as well as the VITEK 2 system Test Card (ASTC) AST-N222 (Netherlands).[15]

Antibiotic susceptibility testing

The antibiotic susceptibility testing was done using the disk diffusion method on Mueller–Hinton agar plates following CLSI 2021.[16] The following antibiotics were used: 5 µg of levofloxacin, 15 µg of ciprofloxacin, 25 µg of ampicillin, 10 µg of gentamicin, 30 µg of cefoxitin, 85 µg of ticarcillin + clavulanic acid, 100/10 µg of piperacillin–tazobactam, 1 µg of cefepime, and 300 µg of nitrofurnation.[17]

Iron oxide nanoparticle preparation

Fifty milliliters of distilled water was used to prepare the molar stock solution of iron (III) chloride hexahydrate ($FeCl_3.6H_2O$), A stock solution of 9 mM was made according to Qassim *et al*.[18]

Bacterial suspension preparation

P. mirabilis was cultured in 250 mL of brain heart infusion broth for 24 h at 37°C in a non-shaking incubator. The bacterial broth was then centrifuged, the sediment was disposed of, and the supernatant was collected.[19]

Synthesis of iron nanoparticles

Iron nanoparticles (INPs) were created using the aqueous precipitation method. An aliquot of 200 mL of *P. mirabilis* broth filtrate and 20 g of $FeCl_3.6H_2O$ were added, sealed, and covered with dark material at 37°C. The mixture was then incubated for 24 h in a shaker incubator. The mixture was centrifuged, the precipitate was removed, and deionized water was used three times to wash the mixture. Metal ions were trapped on the surface as part of the extracellular production pathway, which was followed by a decrease in the amount of reducing enzymes. The synthesis of NPs was then evaluated by changing the color of the solution. After air drying, the precipitant was stored in a dry location for additional analysis.[20]

Characterization of iron nanoparticles

Characterization ZnONPs: This examination was held in the Department of Chemistry/College of Sciences laboratories at the University of Al-Nahrain included:

Ultraviolet–visible spectra analysis; atomic force microscopy (AFM) analysis; X-ray diffraction (XRD) method analysis; field emission scanning electron microscopy (FESEM) analysis; and transmission electron microscopy (TEM) analysis.[21,22]

Minimum inhibitory concentration determination

The antibacterial activity of the INP was assessed by determining the minimum inhibitory concentration (MIC) using Eppendorf tubes and the broth microdilution method. After being prepared in Muller Hinton Broth, the concentration of the bacterial inoculum was adjusted to 100 UFC/µL. Copper oxide nanoparticles (CuONPs; 8, 16, 32, 64, and 128 µg/µL) were serially diluted twice in six tubes using

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Mueller–Hinton broth. After that, the tubes were incubated for a full day at 37°C. Subsequently, the sub-MIC might be computed by observing any indications of bacterial growth in every tube.[23]

Antibacterial activity of iron oxide nanoparticles

Antibacterial activity INPs was tested *in vitro* on a variety of bacterial strains.[24] Different Gram-positive and Gram-negative bacteria were obtained from the Department of Biology, College of Science, Baghdad University, Baghdad, Iraq. The culture was brought back to life, proliferated, and put into test tubes with nutrient broth media before the antibacterial activities. After that, the glass test tubes were placed in a shaking incubator set to 200 rpm and 37°C for a full day. The bacterial isolates spread across the medium plates using cotton swabs. The antibacterial potentials were evaluated using the well diffusion agar technique. A hole with a diameter of 5 mm is punched aseptically with a sterile cork borer, and a sterile 0.5 mL of different concentrations of FeONP µg/mL is added. At the end, Petri Plates were kept in an incubator at 37°C for 24 h, and the MIC was estimated along with the periodic monitoring of the formation of a zone of inhibition.[25]

Statistical analysis

Every experiment was run three times, and the mean and standard deviation of the findings were reported. The *t* test was used to determine how the research factors affected the biofilm. The statistical analysis was conducted utilizing SPSS version 26 (IBM Corporation, New York, NY, USA). When $P < 0.05$, the differences were deemed significant.

Ethical approval

The Helsinki Declaration's moral precepts were followed when conducting the study. The patient's verbal and analytical consent was acquired before the sample collection. The study protocol, subject information, and permission form were reviewed and authorized by a local ethics committee in compliance with document number 30, which bears the date and number 25/11/2022.

Results

Isolation and identification

The VITEK 2 system was used to validate the identity of 64 isolates of *P. mirabilis*. Additionally, colony morphology on MacConkey agar and swarming on blood agar were also investigated.

Antibiotic susceptibility test

All tested *P. mirabilis* isolates showed resistance to piperacillin–tazobactam after 24 h of incubation at 37°C. Six isolates showed resistance to ciprofloxacin; 15 isolates showed resistance to ampicillin; 6 isolates were resistant to cefoxitin; and 17 isolates showed resistance to nitrofurantoin. However, two isolates showed resistance to gentamicin, and only one isolate showed resistance to levofloxacin. The results also found that 12 isolates of *P. mirabilis* were multidrug resistant.

Biosynthesis of iron oxide nanoparticles

FeONPs were biosynthesized from *P. mirabilis*. The production of NPs was indicated by a color change from yellow to black as well as the formation of dark precipitates. The precipitate, after centrifugation and following microwave drying, produced a glossy blue powder as illustrated in Figure 1. The synthesis of INPs utilizing bacterial supernatant offers an economical, quick, easy, and environmentally friendly way to create valuable nanomaterials for use in nanomedicine.

Characterization of iron nanoparticles

Ultraviolet–visible spectral analysis

The results showed that the biosynthesized INPs exhibited the highest peak (absorption peaks at 275) nm are shown in Figure 2. Consistent with the data, a sharp peak at 270 nm suggests that the particles are small in size.

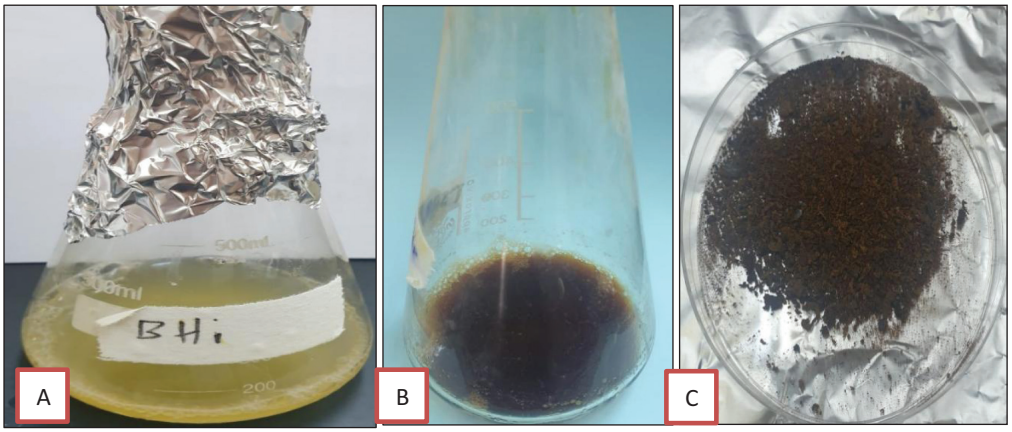

Figure 1: Biosynthesis of iron oxide nanoparticles powder. (A) Bacteria suspension. (B) After 72 h. Perception of iron oxide nanoparticles. (C) Powdered iron oxide nanoparticles

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Atomic force microscopy analysis

Atomic force microscopy investigation of INPs was used to identify and characterize the NP distributions. The predicted particle size and mean square roughness were computed. The three-dimensional profile of CuONPs is depicted in Figure 3. The three-dimensional (3D) picture of the INPs NPs revealed that the grains are dispersed between 13 and 19 nm.

Transmission electron microscopy analysis

The imaging of TEM was used to characterize INPs and investigate the morphology and size of NPs. The core–shell structure of the NPs is confirmed by TEM analysis. Before being loaded into a specimen holder, the samples were prepared by drop-coating the INPS solution into a copper grid that had been coated with carbon. Analyzing the prepared grids allowed for the creation of TEM micrographs [Figure 4].

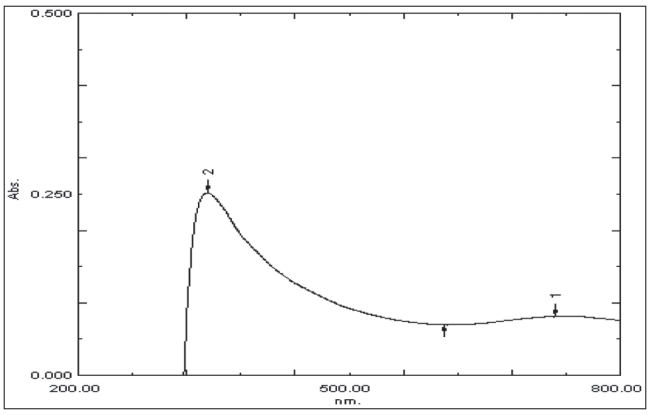

Figure 2: Ultraviolet–visible spectrophotometry of synergistic iron nanoparticles

Field emission scanning electron microscopy

FESEM analysis was performed to understand the morphology and the size of NPs that INPs synthesized by the biological process of field emission scanning electron microscopy as shown in Figure 5. Regarding the shape of INPs, the SEM image of INPs showed that their shape was largely spherical and homogeneous, with a diameter that ranged from 15 to 19 nm. Field emission scanning electron microscopy measures the elemental and structural composition of samples. The FESEM provides for the examination of smaller-area contamination patches at electron-accelerating voltages compared to energy-dispersive X-rays. This method is typically used to identify the presence of different elements.

X-ray diffraction method

The XRD spectra of the INPS powder revealed six unique peaks that conformed with the diffraction peaks (16.414), (18.414), (22.278), (23.983), (26.113), and (43.537), which are characteristic for INPs [Figure 6]. These peaks were attributed to the presence of an INPS shell covering the INPs. Comparing the crystalline phases with conventional crystallographic data from the International Center of Diffraction Data allowed for their identification. The diffractometer's PDXL2 software used the Hader–Wagner method to determine the crystallite size.

Antibacterial activity of iron nanoparticles

The antibacterial effect of INPs was achieved by the well diffusion method. The best inhibitory MIC, which is 32 µg/mL for 24–48 h at 37°C was assessed for their antibacterial activity against Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus pyogenes*) and Gram-negative bacteria (*Escherichia*

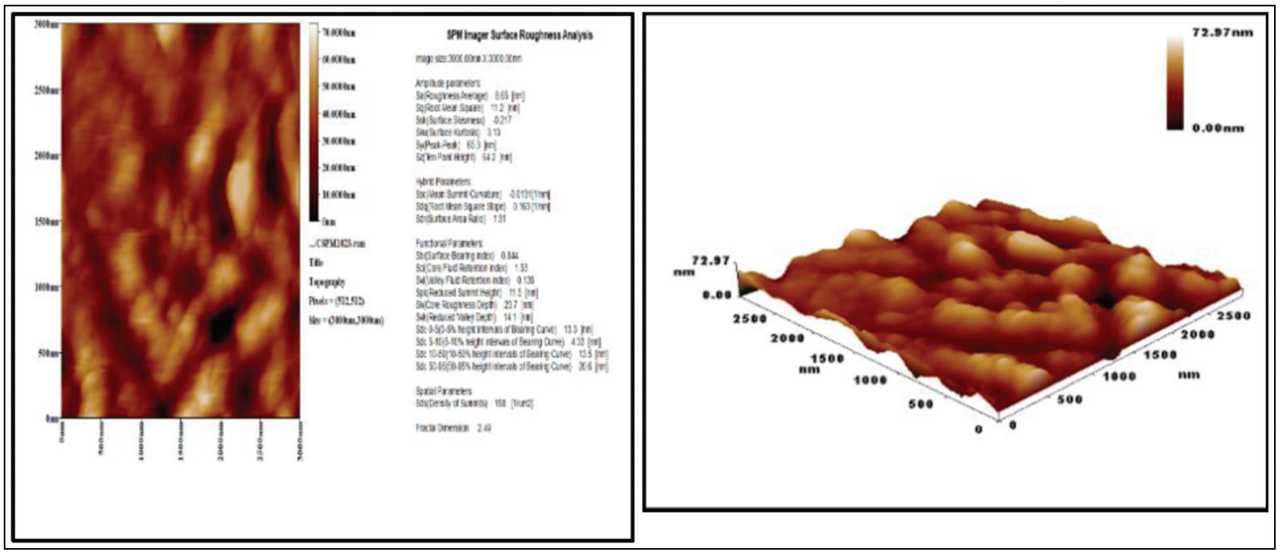

Figure 3: 3D AFM observation for iron nanoparticles (INPs). 3D: three-dimensional, AFM: atomic force microscopy

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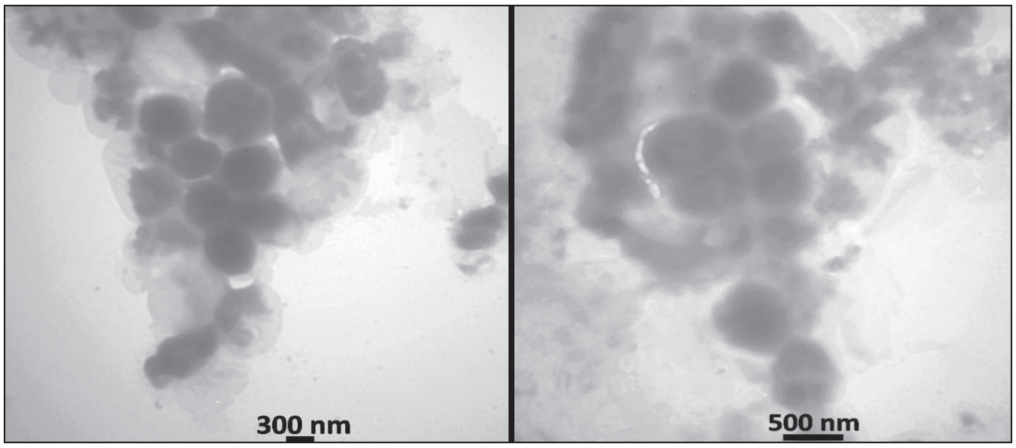

Figure 4: The transmission electron microscopy images of INPs show particles. In spherical shape. INPS: iron nanoparticles

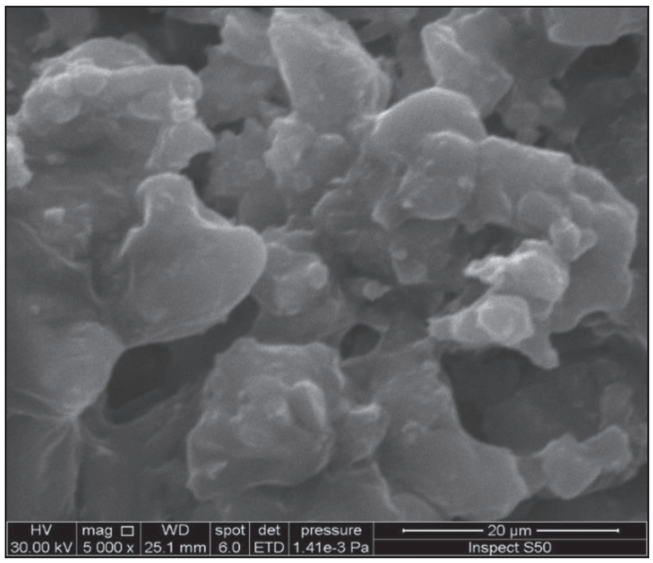

Figure 5: SEM image of iron nanoparticles. SEM: scanning electron microscopy

coli, *Klebsiella*, *Pseudomonas aeruginosa*, and *Salmonella*) is shown in Figures 3 and 4. Where it was observed that the best inhibitory MIC was 32 µg/mL concentration and showed the best efficacy against resistant strains of Gram-positive and Gram-negative bacteria. The inhibition zone was between 3 and 19 mm by using the well diffusion assay method and measuring the zones of inhibition after incubation at 37°C for 24 h. The statistical analysis emphasized a significant difference ($P < 0.05$) in the dissemination mean zone of bacterial inhibition after treated with different concentrations of INPs. There are significant differences against different species of Gram-positive and Gram-negative bacteria [Table 1]. The possible mechanism of the antibacterial capabilities of the Fe_3O_4 NPs may be influenced by the existence of an electrostatic adsorption potential between pathogenic bacteria (negative charge) and magnetic FeONPs (positive charge).

Discussion

The most common bacterial infections are urinary tract infections (UTIs) like *P. mirabilis*, whereas other microbial species can spread through airborne, such as *Mycobacterium tuberculosis* virus and some viruses, coronaviruses, and adenoviruses.[26] *P. mirabilis* was the most common among the other bacterial species isolated from UTIs. This result follows Obaid and Baiee.[27] The patient age ranged from 1 to 83 years, whereas children were aged between 1 and 9 years old. The ceftriaxone resistance rates have increased by three times in inpatient samples than in outpatients.[28]

A study from China revealed that 25 *P. mirabilis* isolates recovered from the samples were taken from both humans and animals. The results of this investigation demonstrated that *P. mirabilis* poses a growing concern to public health. *P. mirabilis* produces Amp β-lactamases and extended-spectrum β-lactamases, and the primary type of resistance is efflux pump resistance, which presents a significant difficulty in the management of infections.

A 3D profile of INPs is depicted in green synthesis NP portraiture; the 3D picture of the IONP NPs indicates that the grains are dispersed between 40 nm, which was consistent with Seifan *et al*.[29] Inorganic nanomaterials with extraordinary properties, primarily zinc, gold, and silver NPs, have been created by bacteria in the last 1o years for the development of *in vitro* antimicrobial activity against some pathogenic bacterial strains. Bacteria are a wonderful candidate for NP synthesis due to their ease of cultivation, rapid proliferation, and extracellular creation of NPs, among other traits.[30] The image of the TEM result demonstrated that the formed INPs were spherical, and were in the range of 13–19 nm with 40 nm resolution power and 77.5 kilopixel (kx) magnifications.[31] Since the bacterial strains resistant to current antibiotics have become a major public health concern, new bactericidal materials are required. Therefore, the development of novel strategies and materials to tackle these pressing

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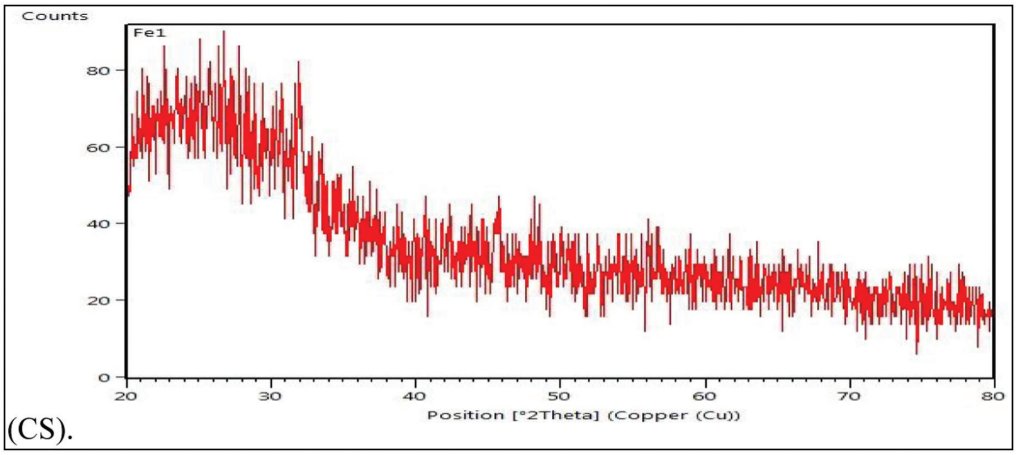

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Figure 6: X-ray diffraction analysis of iron nanoparticles

Table 1: Mean ± SD zone of bacterial inhibition in mm treated with different concentrations (in μg/mL) of INPs effects on bacterial

Bacterial isolates	Mean ± SD zone of bacterial inhibition in mm treated with different concentrations (in μg/mL) of INPs					*P* value
	8	16	32	64	128	
Staphylococcus aureus	5.23 ± 0.2^{a}	8.2 ± 0.21^{b}	9.3 ± 0.3^{c}	10.2 ± 0.3^{d}	11.3 ± 0.16^{e}	<0.0001
Streptococcus pyogenes	4.3 ± 0.1^{a}	6.2 ± 0.1^{b}	8.3 ± 0.1^{c}	12.3 ± 0.1^{d}	14.4 ± 0.21^{e}	<0.0001**
Klebsiella Sp.	2.1 ± 0.3^{a}	3.5 ± 0.2^{b}	4.3 ± 0.11^{c}	8.6 ± 0.1^{d}	9.16 ± 0.13^{e}	<0.0001**
Pseudomonas aeruginosa	4.26 ± 0.15^{a}	6.18 ± 0.15^{b}	8.2 ± 0.2^{c}	11.3 ± 0.1^{d}	13.3 ± 0.21^{e}	<0.0001**
Escherichia coli	3.3 ± 0.15^{a}	6.5 ± 0.21^{b}	9.53 ± 0.15^{c}	14.4 ± 0.4^{d}	19.5 ± 0.44^{e}	<0.0001**
Salmonella	3.17 ± 0.12^{a}	4.3 ± 0.18^{b}	6.17 ± 0.15^{c}	7.2 ± 0.1^{d}	9.1 ± 0.2^{e}	<0.0001**

Different letters (a, b, c, d, and e) consider significant differences at $P < 0.05$ between groups in the rows.
**P < 0.05 is consider significant.

issues is imperative. The advancement of nanotechnology has enabled the development of several new antimicrobials. The nanomaterial is a good choice for carrying out antimicrobial biological operations due to its small size. Many types of NPs, such as those composed of metals and organic materials, have demonstrated considerable promise as antibacterial.[32]

Respiratory enzymes are hindered by the reactive oxygen species produced by iron NPs. Activation of NPs interacts with the membrane of the plasma and peptidoglycan cell wall to provide antibacterial effects. They also interfere with protein groups that prevent bacterial deoxyribonucleic acid form replicating. Additionally, the negative charge on bacterial cell walls reacts with the positive charge on the metal to alter the shape of the walls and increase their permeability or leakage.[33]

A local study on the effects of ZnONPs made from green kiwi on *S. aureus* obtained from various cosmetics showed that the reducing agent was kiwi peel extract, and the ions used were zinc acetate with strong antimicrobial activity against methicillin resistance *S. aureus* by well diffusion methods.[34,35] Numerous bacteria (including *Pseudomonas fluoresces*, *Serratia* spp., and *E. coli*) as well as some parasites demonstrated their capacity to decrease certain metal ions and generate NPs.[36,37] On the other hand CuONPs were effectively created using an indirect, cost-effective, environmentally benign, high-yield, and green technique. CuONPs exhibited remarkable antibacterial efficacy against multiple strains of bacteria.[38]

Conclusion

INPs were successfully synthesized using a novel, straightforward, effective, room-temperature, high-yield, and environmentally friendly process. With regards to various bacterial strains, INPs demonstrated excellent antibacterial efficacy. Bacteria are a great option for producing NPs because they are quick to generate and easy to cultivate, thanks to their extracellular reduction enzymes. Its advantages over other processes in terms of both economy and ecology and the green synthesis of metallic NPs are receiving a lot of interest. The NPs are frequently synthesized for solving various kinds of problems in medicine and drug delivery.

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Conflicts of interest

There are no conflicts of interest.

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