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Ultrasound-Assisted Synthesis of Iron Oxide Nanoparticles: Application in Cytotoxicity and Antibacterial Activity

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Abstract

Background The rapid emergence of antibiotic resistance among various bacterial species has necessitated the development of alternative therapeutic options. Nanotechnology, particularly the production and surface modification of metal nanoparticles (MNPs), has gained significant attention as a promising approach in combating antibiotic resistance.

Objective To explore a safe and cost-effective method for synthesizing iron oxide nanoparticles (IONPs) with unique shapes through ultrasound-assisted co-precipitation and to use it as a potential solution to the problem of antimicrobial-resistant bacteria especially multidrug-resistant bacteria.

Methods IONPs were synthesized using an ultrasound-assisted method and characterized with techniques such ultraviolet-visible spectrophotometry (UV-Vis), energy dispersive of X-ray spectroscopy (EDX), X-ray diffraction (XRD), atomic force microscopy (AFM), zeta potential analysis, transmission electron microscopy (TEM), and scanning electron microscopy (SEM). Antimicrobial activity was assessed using minimum inhibitory concentration (MIC) tests at 16 µg/ml and a well diffusion assay against Gram-negative bacteria and Gram-positive bacteria.

Results IONPs had an average diameter size of 30 nm, displaying a spherical shape. At concentration of 16 µg/ml, the NPs was found to exhibit a MIC and displayed potent antimicrobial activity against wild-type multidrug-resistant strains of both Gram-positive and Gram-negative bacteria in vitro. Moreover, in vivo experiments showed that the IONPs led to a significant decrease in urothelial bladder cancer (UBC)-40 cell.

Conclusion The US-assisted method utilized in this research for the synthesis of IONPs has proven to be suitable for producing IONPs with precise particle size and high product homogeneity. This efficient synthesis approach holds great promise, particularly in light of the alarming prevalence of multidrug-resistant bacterial strains.

Keywords Fe_2O_3 NPs, UBC-40 cells, MRS.

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List of abbreviations: AFM = Atomic force microscopy, EDX = Energy dispersive X-ray spectroscopy, IC50 = Half maximal inhibitory concentration, IONPs = Iron oxide nanoparticles, MNPs = Metal nanoparticles, MIC = Minimum Inhibitory Concentration, MTT= Medical training therapy, RPMI = Roswell Park Memorial Institute, SEM = Scanning electron microscopy, TEM = Transmission electron microscopy, UBC = Urothelial bladder cancer, UV-Vis = Ultraviolet-visible spectrophotometry, XRD = X-ray diffraction

Introduction

The use of extremely small particles of materials to produce new large-scale materials is known as nanotechnology. It is the process of creating materials and devices by manipulating matter at the levels of atoms,

molecules, and supramolecular (nanoscale) structures. Better comprehend the differences between the various scales [1].

Antibiotics used to treat infectious illnesses are manufactured worldwide at around 100,000 tons per year. Antibiotic overuse has caused pathogenic strains, particularly bacteria, to become multidrug resistant [2].

Iron oxide nanoparticles (IONPs) have drawn a lot of interest because of their distinctive characteristics; greater surface area, super paramagnetism, surface-to-volume ratio, and simple separation techniques. It was found that different physical, chemical, or biological techniques had been used to create magnetic NPs with the right surface chemistry. In the areas of life sciences like biomedicine, agriculture, and the environment, IO have a lot of potential. Applications of magnetic NPs can be made more biocompatible and nontoxic by covering a specific surface with inorganic or organic molecules, like nonionic detergents, polyelectrolytes, proteins, starches, antibodies, enzymes, surfactants, medications, and enzymes [3].

Nanotoxicology is a developing area that focuses on establishing the hazard of NPs and the result of their potential risk in light of their growing use and exposure likelihood [4]. The study of nanotoxicology is becoming a more important branch of nanotechnology. The term "nanotoxicology" refers to the study of how nanostructures interact with biological systems. The focus is on determining how the size, shape, chemistry of the surface, composition, and aggregation of nanostructures (as well as other physical and chemical properties) are related to the induction of hazardous biological reactions [5]. Nanomaterials applications in many fields, such as environments and public health, have been grabbing considerable attention. Experiments on animal models have shown many toxic effects, like inflammation, decreased growth rate, and neurobehavioral changes. Great surface-to-volume ratio, chemical composition, size, dosage, and retention in the body represent the major factors that affect nanoparticle toxicity [6].

The physico-chemical characteristics of nanoparticles have recently been demonstrated to contradict earlier theories about IONPs' lack of cytotoxicity [7]. Nevertheless, the toxicity of nanostructured materials is an open issue due to several factors: high reactivity, intrinsic toxicity of the material, and non-specific interactions with biological objects, that are determined by particle shape, size and structure. Biocompatibility, toxicity and ability to penetrate into cells are the main criteria that determine the effectiveness of nanoparticles in medicine [8]. Despite the numerous advantageous applications of IONPs in various medical fields, limited research has been conducted on their potential effects on the reproductive system. Hence, it is crucial to explore and understand their impact on reproductive health. Therefore, the objective of this study was to investigate the influence of IONPs on cell lines and their efficacy against bacterial strains.

Methods

Preparation of IONPs by Sono chemical method

IONPs were synthesized by the sono-chemical method [9] at room temperature using iron (III) chloride ($FeCl_3$) anhydrous. Iron sulfate heptahydrate ($FeSO_4.7H_2O$). An aqueous solution of 0.1 mmol of $FeCl_3$ anhydrous was prepared by dissolving in 250 ml of deionized water, while 0.05 mmol of $FeSO_4.7H_2O$ was prepared by dissolving in 250 ml of deionized water to maintain the molar ratio of 2:1. An ultrasonocator (30 KHz, 20 W) was placed in both salts' aqueous solution in a 100-ml round bottom flask with heating at 60-70°C. An aqueous solution of 4M NaOH was prepared in 150 ml deionized water, which was continuously added to the mixture dropwise until the pH rose to 11-13 and there was the formation of a black precipitate at the bottom of the flask. Otherwise, the flask was covered with a cork, and the reaction was allowed to run at the

specified settings for one hour. After the reaction, the mixture was centrifuged at 6000 rpm for 5 min before being allowed to cool to room temperature. However, the supernatant was thrown away, leaving the black precipitate. The obtained precipitate was washed 2-3 times each with deionized water and ethanol. Finally, the precipitate was dried overnight at 40°C in an oven to get the dry powder. Figure (1) shows the $FeCl_3$, $FeSO_4.7H_2O$, preparation of IONPs, precipitation of IONPs, and powder of IONPs.

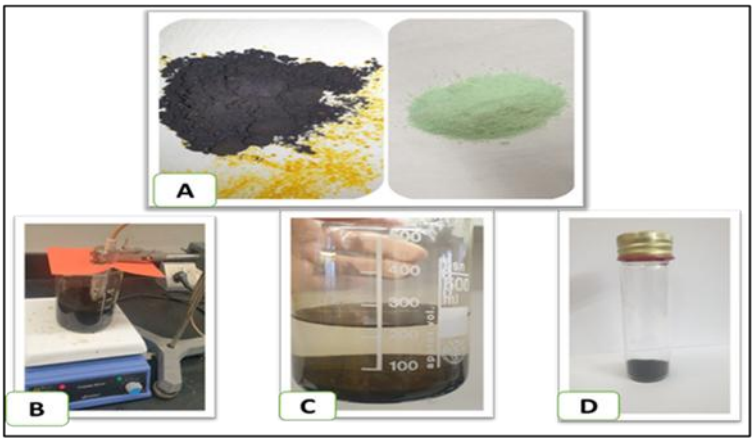

Figure 1. (A) $FeCl_3$, $FeSO_4.7H_2O$ (B) Synthesis of IONPs by Sonochemical method (C) Precipitation of IONPs (D) Powder of IONPs

Nanoparticle characterization

It is necessary to characterize the produced nanoparticles (IONPs) both prior to use and also during the course of a study, because is a significant temporal changes will often depend upon mainly the size and shape of nanoparticles: Field emission scanning electron microscopy (FESEM) and transmission electron microscopic (TEM) to characterize the shapes and sizes of IONPs, Atomic force microscopy (AFM) was used to determine the size and surface roughness, UV-visible spectrophotometers were then used to investigate the optical properties of the sample. X-ray diffraction (XRD) was a method used in material research to identify a material's crystallographic structure. Energy dispersive X-ray spectroscopy (EDX) to get qualitative and quantitative analysis [10,11].

Bacteria isolation and identification

Different gram-positive and negative bacteria (Table 1) where isolated from patient suffer were collected from burn and wound, all isolation identified by biochemical method and preservation in 20% glycerol were obtained from Department of Biology, College of Science, Baghdad University, and confirmation identification was done by Vitek 2 system to choose one strain multidrug resistant antibiotic.

Table 1. Different gram-positive and negative bacteria

Gram-positive bacteria	Gram-negative bacteria
Staphylococcus epidermidis	*Escherichia col*
Staphylococcus aureus	*Salmonella spp.*
	Proteus Mirabilis
	Klebsiella spp.

Determination of minimum inhibitory concentration (MIC)

IONPs bacteriostatic activities were assessed using MIC tests [8]. A sufficient number of bacteria (2 l) in Mueller Hinton broth (MHB). The MHB of two-fold dilution serials was added to (64, 32, 16, 8, 4) µg/ml by using 6 tubes of MHB, respectively. The tubes had been incubated at 37°C for 24 hr. These inoculums of bacteria prepared at 0.1 M concentration demonstrated an antibacterial test and assay. The turbidity was observed in growth, non-turbidity, as well as the absence of growth medium. The lowest concentration was shown by the MIC values.

Well diffusion method (WDA)

The antibacterial activity of IONPs was evaluated using the WDA. All experimental procedures were conducted within a fume hood to ensure safety and sterility. Aseptically, a 5 mm diameter hole was created using a sterile cork borer or tip. A sterile cotton swab was then dipped into the standardized bacterial suspension, and excess inoculum was gently removed by pressing the swab against the tube wall above the liquid level. The Muller-Hinton Agar plate surface was inoculated by streaking with the swab containing the inoculum. Subsequently, different concentrations of IONPs (64, 32, 16, 8, and 4 µg/ml) in 1 ml of distilled water were introduced into the wells created on the agar plate. The plates were incubated for 18 to 24 hr at a temperature of 37°C to allow for bacterial growth and examination of antimicrobial effects [12].

The cytotoxic effect of IONPs

An in vivo method was utilized to investigate the potential cytotoxic effects of physically synthesized IONPs on tumor cell lines (UBC-40) and normal cell line (Hdfn). The IONPs were tested at concentrations ranging from 400 to 25 µg/ml [13].

Cell line maintenance

When the cells in the vessel formed confluent monolayer, the following protocol was performed:

- The growth medium was aspirated and the cell sheet washed with phosphate buffered saline (PBS).
- Two to three ml trypsin/versine solution was added to the cell. The vessel was turned over to cover the monolayer completely with gentle rocking. The vessel allowed incubation at 37°C for 1 to 2 min, until the cells were detached from the vessel.
- Fresh complete Roswell Park Memorial Institute (RPMI) medium (15-20 ml) was added and cells were dispersed from the wedding surface into growth medium by pipetting.

Cells were redistributed at required concentration into culture vessels, flasks or plates whatever needed and incubated at 37°C in 5% CO_2 incubator. Cell concentration was achieved by counting the cells using the hemocytometer and applying the formula.

Total cell count/ml = cell count x dilution factor (sample volume) x 10^4.

Medical training therapy (MTT) Protocol

The cytotoxic effect of IONPs performed by using MTT ready to use kit.

Statistical analysis

A one-way analysis of variance ANOVA and student's t-test were performed to test whether group variance was significant or not. The software used was statistical package for social sciences (SPSS) version 23. P value less than 0.05 was considered the level of significance.

Results

NPs physicochemical characteristics

Ultraviolet (UV-Visible) spectrophotometry

The optical characteristics of specific nanoscale particles are frequently studied using a technique called the UV-visible absorption spectrophotometer. The optical properties of the synthesized IO is shown in figure (2), which illustrates that the peak absorption found at (345 nm).

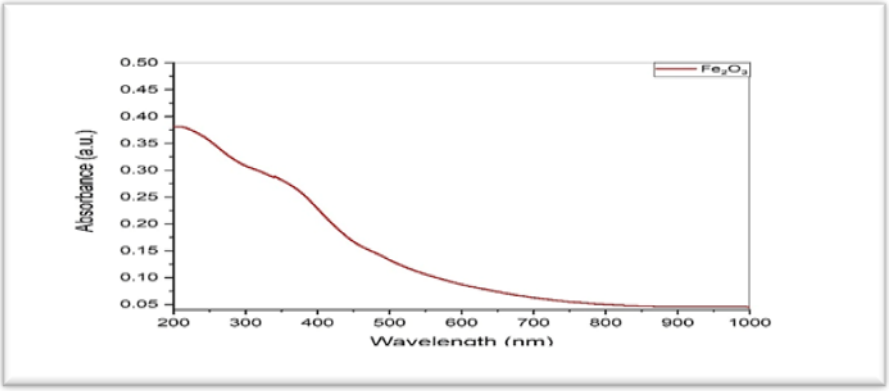

Figure 2. UV–Visible spectrophotometry of IONPs sono chemical

X-ray Diffraction (XRD)

The XRD spectrum of the IONPs powdered sample is shown in figure (3). Confirmed the formation of the IONPs by revealing three prominent peaks that corresponded to the diffraction peaks 31.9450, 35.8371, and 45.6745. The result of the XRD pattern emphasizes that the NPs are crystalline and have an average size of about 33.46 nm by using the Debye-Scherrer equation. IONPs are available in two common phases, namely magnetite Fe_3O_4 and maghemite Fe_2O_3. Upon exposure to oxygen atoms, the magnetite phase readily oxidizes into the maghemite phase, with the partial conversion of ferrous ions into ferric ions.

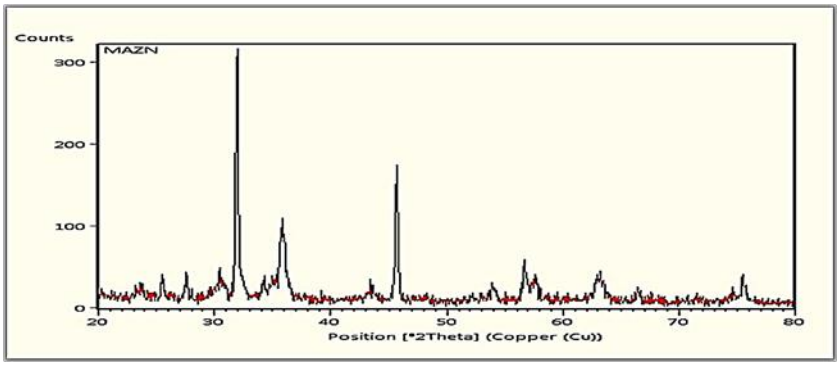

Figure ۳. X-ray Diffraction analysis of synthesized IONPs

AFM analysis

The two- and three-dimensional topography images of the IONPs are shown in figure (4). The results of AFM showed that the average roughness was about 1.27 nm and the average size was about 25 nm. The precipitation method, with the aid of ultrasound, indicated homogeneities and uniform nanoparticles.

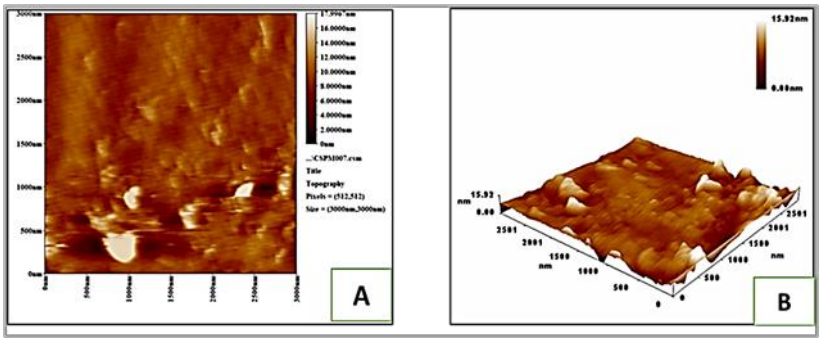

Figure 4. Atomic force microscopy images; (A) Two-dimensional (B) Three-dimensional

Transmission electron microscopy (TEM) analysis

TEM image of IONPs is shown in the figure (5). This image confirms that the IONPs have an average size of 25-30 nm and are grown in a spherical shape, which demonstrates the good quality of the IONPs and their good homogeneity.

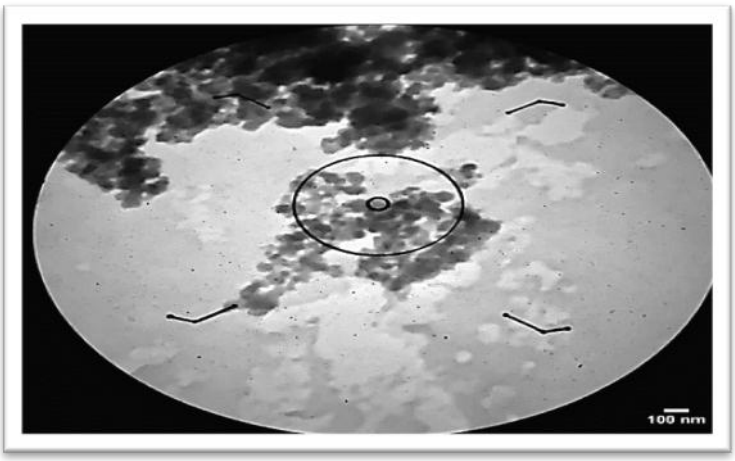

Figure 5. The Transmission Electron Microscopic (TEM) image of the IONPs

Energy dispersive X-ray spectroscopy (EDX)

The IONPs were analyzed using EDX to determine their elemental compositions. The results of the EDX study, presented in figure (6), confirmed the presence and distribution of iron oxide within the NPs. EDX spectra were collected specifically from the core region of the IONPs.

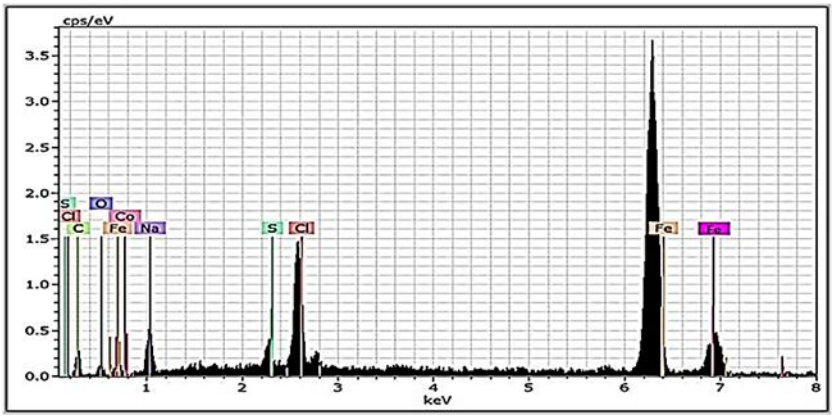

Figure 6. Energy dispersive of X-Ray spectrometry (EDX)

Zeta Potential

Zeta potential is considered to be an important indication of a colloid's internal stability. The stability based on electrokinetic potential is classified as highly unstable when zeta values are in the range of ±0-10 mV, relatively stable at zeta values of ±10-20 mV, moderately stable at values in the ranges of ±20-30 mV, and highly stable when zeta values are greater than ±30 mV [15]. This test describes how to measure the potential for electrostatic charge at the double layer of electricity within a nanoparticle in solution. Since cellular membranes are negatively charged, zeta potential can affect a NPs tendency to permeate membranes, in cationic particles generally displaying more toxicology associated with cell wall disruption. It may be concluded that the IONPs are remarkably stable based on figure (7), which displays the zeta potential of the IONPs in this work to be -19.54 mV, which indicates that the synthesized NPs were relatively stable and well-dispersed.

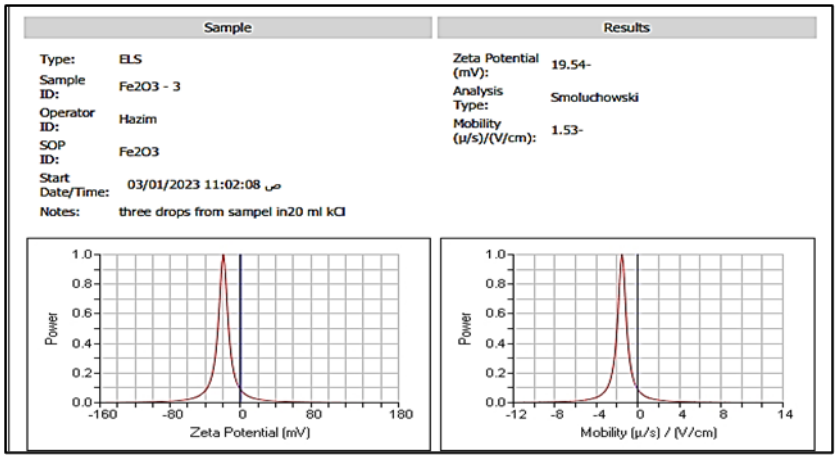

Figure 7. The zeta potential of IONPs

Field emission scanning electron microscopy study (FESEM)

The morphology and size of the NPs were all examined using FESEM analysis. Figure (8) shows the results clearly demonstrating that the majority of the synthesized IONPs were spherical in shape IONPs Synthesized ranged in size from 19 to 30 nm. The agglomeration of NPs produced using the ultrasound method may be caused by their high surface energy.

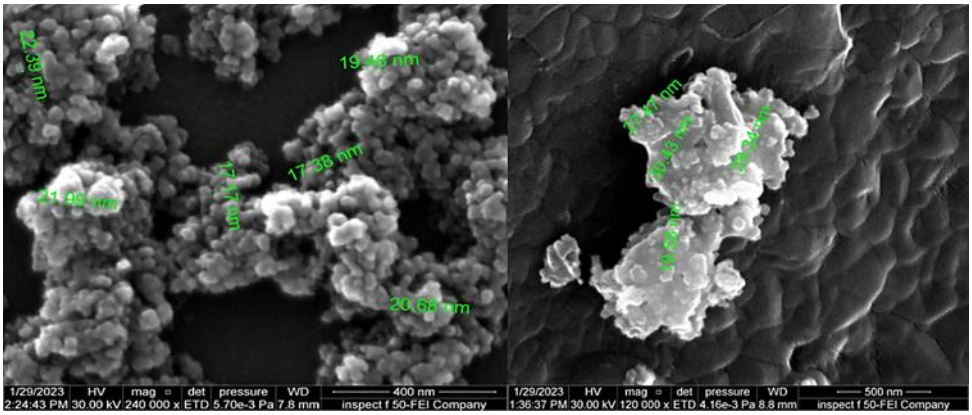

Figure 8. FESEM images and size of IONPs

MIC of IONPs Antibacterial activity

The prepared IONPs at different concentrations (4, 8, 16, 32, and 64 µg/ml) were assessed for their antibacterial activity against gram-positive bacteria (*Staphylococcus aureus* "*S. aureus*" and *Staphylococcus epidermidis* "*S. epidermidis*") and gram-negative bacteria (*Escherichia coli* "*E. coli*", *Klebsiella spp.*, *Pseudomonas aeruginosa* "*P. aeruginosa*", and *Proteus mirabilis* "*P. mirabilis* "). The inhibition zone was measured between 3 mm and 16 mm by using the well diffusion assay method and measuring the zones of inhibition after incubation at 37°C for 24 hr. The antibacterial capacity of novel preparations of IONPs by the sonicater method was evaluated first against *S. aureus* and *P. aeruginosa* by the well diffusion method. The plates were incubated at 37°C and the inhibition zone diameter was evaluated, which proved the antibacterial assay. The results showed the effectiveness of IONPs with high efficiency against different types of positive and negative bacteria that are resistant to antibiotics, as shown in tables (2). This experiment demonstrated that a higher concentration (64 µg/ml) had the highest inhibition zone diameter value. In this case, a basic concentration of 8 µg/ml was employed to measure the MIC assay. Where the lowest concentration of IONPs against *P. aeruginosa* and *S. aureus*, the resistant isolates was observed, the inhibition diameter reached 7 mm and 8 mm, respectively. The antibacterial activity of IONPs prepared at various concentrations (4, 8, 16, 32, and 64 µg/ml) was investigated against gram-positive bacteria including *S. aureus* and *S. epidermidis*, as well as gram-negative bacteria such as *E. coli*, *Klebsiella spp.*, *P. aeruginosa*, and *P. mirabilis*. The well diffusion assay method was employed to measure the zones of inhibition after incubating the plates at 37°C for 24 hr. The antibacterial efficacy of the novel IONPs preparations synthesized using the sonicater method was initially evaluated against *S. aureus* and *P. aeruginosa* using the well diffusion method. Following incubation at 37 °C, the diameter of the inhibition zone was measured to assess antibacterial activity. The results demonstrated that IONPs exhibited high effectiveness against various antibiotic-

resistant strains of both gram-positive and gram-negative bacteria, as shown in table (2). Furthermore, this experiment revealed that a higher concentration (64 µg/ml) resulted in a greater diameter value for the inhibition zone. A baseline concentration of 8 µg/ml was selected to determine the minimum.

Table 2. Mean (±SD) Zone of bacterial Inhibition in mm treated with different concentrations (in µg/ml) of IONPs

Bacterial Isolate	Mean ± SD Zone of Bacterial Inhibition in mm Treated with Different Concentrations (in µg/ml) of IONPs					*P* value
	4	8	16	32	64	
S. aureus	5.2±0.2	8.23±0.21	10.23±0.3	13.13±1.2	15.2±0.8	<0.0001
S. epidermidis	4.2±0.2	8.45±0.3	9.3±0.3	11.91±0.8	12.17±0.21	<0.0001
Klebsiella Sp.	3.3±0.3	6.3±0.25	9.3±0.31	11.27±0.4	13.6±0.79	<0.0001
P. aeruginosa	4.16±0.14	7.13±0.16	10.27±0.6	14.3±0.4	16.23±0.5	<0.0001
E. coli	3.2±0.15	4.7±0.31	8.63±0.31	10.28±0.67	12.11±0.3	<0.0001
P. mirabilis	3.17±0.19	6.4±0.35	10.2±0.21	11.27±0.45	12.2±0.2	<0.0001

P value is calculated by one way ANOVA test

The cytotoxic effect of IONPs on tumor cell lines

The cytotoxic activity of IONPs on the UBC-40 cell line was studied, as seen in figure (9) and table (3). The results obtained showed that the cell viability decreased as the concentration of IONPs increased. Use the cytotoxicity effect of IONPs synthesized by the physical method in normal cell lines (HdFn) and tumor cell lines (UBC-40) at 400 mg/ml. The Half maximal inhibitory concentration (IC50) values of the HdFn (367.6), while the IC50 values of the UBC-40 (131.3) cells viability, were increasing the concentration of the IONPs and displayed a dose-dependent sequence of progressive cytotoxicity, beginning at a lower concentration to its maximum inhibition (20%) of HdFn cells and (55%) inhibition of UBC-40 cells.

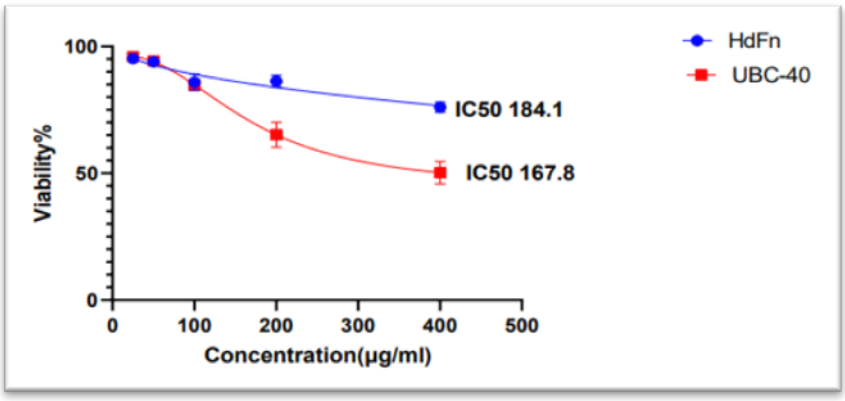

Figure 9. The cytotoxic effect of on HdFn and UBC-40 cell line

Table 3. Comparison study between the cytotoxic effect of IONPs on UBC40 and HdF

Concentrationμg mL^{-1}	Mean viability (%) ± SD	
	HdFn	UBC-40
400	76.04±1.90	50.23±4.42
200	86.26±2.38	65.24±4.94
100	85.96±3.12	84.72±1.33
50	93.94±0.06	94.29±0.82
25	95.22±0.65	95.95±0.90

Discussion

The characterization results of the synthesized IONPs confirmed the unique nature of the ultrasound-assisted synthesis method. UV-visible spectrometry analysis revealed distinctive NPs production, as indicated by a peak value at 345 nm in the absorption spectra [16]. XRD analysis confirmed high crystallinity and purity of the IONPs [17]. FESEM, TEM, and AFM results clearly demonstrated that the majority of the synthesized NPs exhibited a spherical shape and nanoscale size [18]. Zeta potential analysis further demonstrated the stability of these NPs, as their zeta potential fell within the range of -30 mV to +30 mV. This suggests minimal agglomeration potential for these particles [19]. In terms of antibacterial activity, Fe_3O_4 IONPs generated in this study exhibited higher efficiency against Gram-negative bacteria such as *P. mirabilis* compared to Gram-positive bacteria like *S. epidermidis*. This difference in effectiveness can be attributed to variations in cell wall structure, with Gram-positive bacteria possessing a thicker peptidoglycan layer compared to the lipopolysaccharide [20,21].

In conclusion, the ultrasound-assisted method employed in this research proved to be a suitable approach for synthesizing IONPs with desired particle size and product homogeneity. This is of great significance considering the concerning rise of multidrug-resistant bacterial strains. The synthesized IONPs demonstrated exceptional antimicrobial activity against various bacterial strains, highlighting their potential as effective antibacterial agents. These NPs were synthesized using an indirect, eco-friendly, low-cost, and high-yield method, further enhancing their applicability and potential for future antimicrobial applications.

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Author contribution

Abas: performed the clinical isolation of bacteria and prepared the nanoparticles, wrote the article. Dr. Hamid and Dr. Ahmed: designed and performed the experiments.

Conflict of interest

The author declares that there is no conflict of interest.

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