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SYNTHESIS, CHARACTERIZATION, AND MULTIFACETED ANTIBACTERIAL ASSESSMENT OF CIPROFLOXACIN-LOADED IRON OXIDE NANOPARTICLES AGAINST *STAPHYLOCOCCUS AUREUS* AND *PSEUDOMONAS AERUGINOSA*

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ABSTRACT
The rapid emergence of multidrug-resistant (MDR) bacterial pathogens has necessitated the development of novel antimicrobial strategies to improve the therapeutic efficacy of conventional antibiotics. In the present study, ciprofloxacin-loaded iron oxide nanoparticles (Cip-IONPs) were chemically synthesized using a co-precipitation method and comprehensively characterized by scanning electron microscopy (SEM), transmission electron microscopy (TEM), UV–Visible spectroscopy, Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), X-ray photoelectron spectroscopy (XPS), and ^1H nuclear magnetic resonance (^1H NMR). The synthesized nanoparticles exhibited predominantly spherical morphology with an average particle size of approximately 10 ± 10 nm, while spectroscopic and structural analyses confirmed the successful loading of ciprofloxacin onto the iron oxide nanoparticle. The antibacterial efficacy of Cip-IONPs was evaluated against *Staphylococcus aureus* and *Pseudomonas aeruginosa* using minimum inhibitory concentration (MIC), antibiofilm assay, acridine orange/ethidium bromide (AO/EtBr) fluorescence staining, scanning electron microscopy, and quantitative real-time PCR (qRT-PCR). The Cip-IONPs exhibited potent antibacterial activity with a MIC of 0.9 µg/ml against both bacterial strains and demonstrated concentration-dependent inhibition of biofilm formation, achieving inhibition rates exceeding 88% at 500 µg/ml. Fluorescence microscopy and SEM analyses revealed severe membrane disruption and extensive bacterial cell death following nanoparticle treatment. Furthermore, qRT-PCR analysis demonstrated significant downregulation of the biofilm-associated *pslA* gene in *P. aeruginosa* (0.337-fold) and the DNA gyrase gene *gyrB* in *S. aureus* (0.137-fold), indicating effective suppression of bacterial virulence and DNA replication. Collectively, these findings demonstrate that Cip-IONPs exhibit enhanced antibacterial activities through synergistic interactions between ciprofloxacin and iron oxide nanoparticles, highlighting their potential as an effective nanotherapeutic platform for combating multidrug-resistant bacterial infections.

KEYWORDS: Ciprofloxacin-loaded iron oxide nanoparticles; antimicrobial resistance; *Staphylococcus aureus*; *Pseudomonas aeruginosa*; antibiofilm activity; qRT-PCR; nanomedicine.

INTRODUCTION
Antimicrobial resistance (AMR) represents a major global health challenge, with pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* exhibiting increasing resistance to conventional antibiotics through mechanisms including biofilm formation, efflux pump activation, and genetic mutations. Ciprofloxacin, a broad-spectrum fluoroquinolone, exerts its antibacterial activity by inhibiting DNA gyrase and topoisomerase IV; however, the emergence of ciprofloxacin-resistant strains has limited its clinical effectiveness. Consequently, nanotechnology-based drug delivery systems have gained considerable attention for improving antibiotic efficacy and reducing resistance development (Fareed *et al.,* 2026).
Among various nanomaterials, iron oxide nanoparticles (IONPs) are particularly attractive owing to their biocompatibility, magnetic properties, chemical stability, and ease of surface modification. In addition to serving as efficient drug carriers, IONPs possess intrinsic antibacterial properties through the induction of oxidative stress and disruption of bacterial membranes. Previous studies have demonstrated that nanoparticle-mediated ciprofloxacin delivery significantly enhances antibacterial and antibiofilm activities against multidrug-resistant pathogens (Fareed *et al.,* (2026) and Harouni *et al.,* (2026)). Furthermore, ciprofloxacin-loaded nanocarriers have shown improved drug stability, sustained release, and enhanced penetration into bacterial biofilms, making them promising candidates for antimicrobial applications (Ashagrachew *et al.,* 2022).
Ciprofloxacin, a second-generation fluoroquinolone antibiotic, has long been employed for the treatment of Gram-positive and Gram-negative bacterial infections owing to its broad-spectrum antibacterial activity and favourable

pharmacokinetic properties. The antibiotic exerts its bactericidal effect by inhibiting bacterial DNA gyrase and topoisomerase IV, thereby preventing DNA replication and transcription, ultimately leading to bacterial cell death. However, the widespread and often inappropriate use of ciprofloxacin has accelerated the emergence of resistant bacterial strains through chromosomal mutations in target enzymes, activation of multidrug efflux pumps, decreased outer membrane permeability, and acquisition of plasmid-mediated quinolone resistance genes (Urban-Chmiel *et al.,* 2022; Jian *et al.,* 2021; Abdallah *et al.,* 2026). Consequently, the clinical efficacy of ciprofloxacin has progressively declined against multidrug resistant (MDR) *S. aureus* and *P. aeruginosa*, creating an urgent demand for advanced drug delivery systems capable of restoring antibiotic effectiveness.

Therefore, the present study aimed to synthesize and characterize Ciprofloxacin-loaded Iron Oxide Nanoparticles (Cip-IONPs) and evaluate their antibacterial efficacy against two multidrug-resistant bacteria: *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The antimicrobial potential of the synthesized nanocomposite was assessed using microbiological and molecular approaches, including Minimum Inhibitory Concentration (MIC), Antibiofilm assays, Scanning Electron Microscopy (SEM), Fluorescence staining, DNA fragmentation, and quantitative real-time PCR (qRT-PCR). It is anticipated that Cip-IONPs will exhibit enhanced antibacterial activity through the synergistic effects of iron oxide nanoparticles and ciprofloxacin, thereby offering a promising strategy for the treatment of multidrug-resistant bacterial infections.

MATERIALS AND METHODS

Chemically synthesized Ciprofloxacin-loaded IONPs were employed in this research against two bacterial strains namely *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Synthesis of Iron Oxide Nanoparticles

Iron oxide nanoparticles (IONPs) were synthesized using a modified chemical co-precipitation method adapted from the protocol described by Justin *et al.,* (2017). Ferric chloride ($FeCl_3$) and Ferrous Sulfate ($FeSO_4$) served as the iron precursors, while Tetramethylammonium hydroxide (TMAH; $C_4H_{13}NO$) was employed as the precipitating/reducing agent. Briefly, 0.1 g of $FeCl_3$ and $FeSO_4$ were individually dissolved in 1 ml of double-distilled water and vortex-mixed for 2 min until clear homogeneous solutions were obtained. The two precursor solutions were then combined to form the iron salt mixture. This mixture was added dropwise into TMAH at volume ratios of 500 µl:100 µl, 500 µl:200 µl, 500 µl:300 µl, 500 µl:400 µl, and 500 µl:500 µl under continuous vortex mixing for 5 min. The reaction resulted in the formation of a black precipitate, which was allowed to stand undisturbed for an additional 5 min to facilitate complete particle formation. Among the different reaction ratios evaluated, the 500 µl:500 µl formulation produced iron oxide nanoparticles exhibiting the strongest magnetic response. It was selected for the microbial application. The nanoparticle suspension was separated from the reaction mixture using a 50 Gauss permanent bar magnet. The collected nanoparticles were washed three successive times with double-distilled water to remove residual reactants and impurities prior to the loading of antibiotic.

A ciprofloxacin stock solution was prepared by dissolving 0.01 g (10 mg) of ciprofloxacin in an acidified aqueous medium, the solution was prepared according to an optimized laboratory protocol, containing 9.9 ml of distilled water and 0.83 µl of hydrochloric acid (HCl). The HCl was added dropwise to the distilled water under continuous vortex mixing to ensure uniform acidification. Ciprofloxacin was then added to the solution and vortexed thoroughly until completely dissolved. To prepare ciprofloxacin-loaded iron oxide nanoparticles (Cip-IONPs), 1 ml of the ciprofloxacin solution was added to the previously washed iron oxide nanoparticle pellet contained in sterile microcentrifuge tubes (2ml). The mixture was incubated in an orbital shaking incubator at 200 rpm for 24 h to facilitate adsorption of ciprofloxacin onto the functional groups present on the surface of the iron oxide nanoparticles.

Following incubation, the nanoparticle suspension was centrifuged to separate the drug-loaded nanoparticles from the unbound drug (Hussan *et al.,* 2024). The pellet containing the ciprofloxacin-loaded iron oxide nanoparticles was wrapped in aluminium foil to protect it from light and stored at 4°C for further characterization and microbiological experiments.

Characterization of Iron Oxide Nanoparticles

Scanning Electron Microscopy (SEM)

The surface morphology of the synthesized Ciprofloxacin-loaded iron oxide nanoparticles (Cip-IONPs) was examined using a Carl Zeiss EVO 18 scanning electron microscope at the Central Laboratory for Instrumentation and Facilitation (CLIF), University of Kerala. Prior to imaging, the analytes were deposited onto 10×10 mm silicon wafer and allowed to dry under ambient conditions. The dried sample was then subjected to SEM analysis to evaluate particle morphology and surface characteristics.

Transmission Electron Microscopy (TEM)

The size and internal morphology of the Ciprofloxacin-loaded IONPs were further characterized by transmission electron microscopy (TEM) using a JEOL JEM-200 instrument at the CSIR–National Institute for Interdisciplinary Science and Technology (NIIST), Thiruvananthapuram. A small volume of the drug loaded nanoparticle suspension was placed onto a 3 mm carbon-coated copper grid and dried. Subsequently analysed to determine particle size and structural features.

UV–Visible Spectroscopy (UV-Vis)

The optical properties of the synthesized drug-loaded nanoparticles were investigated using a Thermo Scientific Nicolet iS50 UV–Visible spectrophotometer at CLIF, University of Kerala. Absorption spectra were recorded over the wavelength range of 200–800 nm to identify the characteristic absorption profile of the Ciprofloxacin-loaded iron oxide nanoparticles.

Fourier Transform Infrared Spectroscopy (FTIR)

The functional groups present on the surface of the Ciprofloxacin-loaded iron oxide nanoparticles were identified using a Thermo Scientific Nicolet iS50 FT-IR spectrometer at CLIF, University of Kerala. A drop of the nanoparticle suspension was applied to a clean glass slide and allowed to dry before analysis. Infrared spectra were acquired in the range of 4000–500 cm^{-1} using transmission mode to determine the chemical bonds and surface functional groups associated with the synthesized drug-loaded nanoparticles.

X-ray Diffraction (XRD)

The crystalline structure and phase composition of the synthesized Cip-IONPs were analysed using a Bruker D8 ADVANCE diffractometer equipped with the DAVINCI system at CLIF, University of Kerala. A thin film of the nanoparticle sample was prepared on a clean glass slide and dried prior to analysis. The diffraction pattern obtained was used to evaluate the crystalline nature and phase purity of the nanoparticles.

X-ray Photoelectron Spectroscopy (XPS)

The elemental composition and surface chemical states of the synthesized drug-loaded nanoparticles were determined using a Thermo Scientific ESCALAB Xi+ X-ray Photoelectron Spectrometer at CLIF, University of Kerala. High-resolution spectra corresponding to iron (Fe), oxygen (O), carbon (C), and nitrogen (N) were collected to confirm the elemental constituents and investigate the surface chemistry of the Ciprofloxacin-loaded iron oxide nanoparticles. The analysis also aided in identifying any residual or incorporated elements originating from the chemical synthesis process.

Nuclear Magnetic Resonance (NMR) Spectroscopy

The chemical environment of the synthesized Ciprofloxacin-loaded iron oxide nanoparticles was investigated by ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy using a Bruker Avance 400 MHz FT-NMR spectrometer at CLIF, University of Kerala. Dimethyl sulfoxide (DMSO) was used as the solvent, and the resulting spectra were analysed to evaluate the proton environment and verify the chemical characteristics of the synthesized drug-loaded nanoparticles.

Antibiofilm Assay

The antibiofilm activity of Cip-IONPs against the test bacterial strains was determined using the microtiter plate (MTP) crystal violet assay as described by Christensen *et al.,* (1985) with minor modifications. Sterile, flat-bottom 96-well polystyrene microplates were employed for biofilm formation.

Briefly, 180 µl of Brain Heart Infusion (BHI) broth was dispensed into each well, followed by the addition of 10 µl of an overnight bacterial culture. Subsequently, 10 µl of Cip-IONPs stock suspension was added to obtain final concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9, and 1.9 µg/ml. Wells containing bacterial culture without Cip-IONPs served as the untreated control. The plates were incubated statically at 37°C for 24 h to facilitate biofilm development.

After incubation, the culture medium containing planktonic cells was carefully aspirated, and the wells were gently washed twice with 200 µl of phosphate-buffered saline (PBS; pH 7.2) to remove loosely attached bacteria. The adherent biofilms were fixed with 2% sodium acetate for 15 min and subsequently stained with 0.1% (w/v) crystal violet for 15 min at room temperature. Excess stain was removed by rinsing the wells thoroughly with deionized water, and the plates were allowed to air-dry completely.

The retained crystal violet bound to the biofilm biomass was dissolved in 95% ethanol, and the absorbance was measured at 600 nm using a Thermo Scientific microplate reader. The percentage inhibition of biofilm formation was calculated according to Wang *et al.,* (2010) using the following equation:

$$\text{Biofilm inhibition} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100 \%$$

Determination of Minimum Inhibitory Concentration (MIC)

The antibacterial efficacy of ciprofloxacin-loaded iron oxide nanoparticles (Cip-IONPs) was evaluated by determining the minimum inhibitory concentration (MIC) using the broth microdilution technique as described by Gang *et al.,* (2021). The study was carried out against *Staphylococcus aureus* MTCC 902 and *Pseudomonas aeruginosa* MTCC 424 obtained from the Microbial Type Culture Collection (MTCC), Chandigarh, India.

Fresh overnight bacterial cultures were standardized to a turbidity equivalent to 0.5 McFarland standard and further diluted 1:100 in sterile nutrient broth to prepare the working inoculum. In sterile 96-well flat-bottom microtiter plates, 50 µl of the bacterial suspension was mixed with 50 µl of serially diluted Cip-IONPs suspension to obtain final concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9, and 1.9 µg/ml. Wells containing bacterial inoculum without Cip-IONPs served as the positive (growth) control, whereas wells containing sterile nutrient broth alone served as the negative (sterility) control.

The microplates were incubated at 37°C for 18–24 h under aerobic conditions. Following incubation, bacterial growth was assessed by measuring the optical density at 600 nm using a microplate reader, and the results were

confirmed by visual examination for turbidity. The MIC was defined as the lowest concentration of Cip-IONPs that completely inhibited visible bacterial growth. The experiment was performed in triplicate, and the results are presented as the mean of three independent experiments.

EtBr /AO STAINING

Acridine Orange/Ethidium Bromide (AO/EtBr) Staining

Briefly, 5 x 10^6 cells/ml of *P. aeruginosa* and *S. aureus* were plated on a coverslip in a 24 well culture plate and treated with 11.59 µg/ml and 14.77 µg/ml respectively of Cip-IONPs sample in a nutrient broth. The plates were incubated at 37°C at a bacteriological incubator for 48 hours. After incubation, 50 µl of 1 mg/ml acridine orange and ethidium bromide were added to the wells and mixed gently. Finally, the plates were centrifuged at 800 rpm for 2 minutes and evaluated immediately within an hour and examined at least 100 cells by a fluorescence microscope using a fluorescent filter.

Scanning electron microscope (SEM)

The morphological changes induced by Cip–IONPs treatment in bacterial cells were examined by scanning electron microscopy using an EVO-18 scanning electron microscope (CAREL ZEISS), with untreated cells serving as the control.

Quantitative Reverse Transcription Polymerase Chain Reaction (QRT-PCR)

RNA Isolation and Quantitative Real-Time PCR Analysis

Overnight culture of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were adjusted to 0.5 McFarland standard and treated with the MIC_{50} concentration of ciprofloxacin-loaded iron oxide nanoparticles (Cip-IONPs), 14.77 µg/ml and 11.59 µg/ml respectively. Untreated bacterial cultures served as controls. Following incubation at 37°C for 18–24 h, total RNA was isolated using TRIzol reagent according to the manufacturer's instructions.

Briefly, bacterial pellets were obtained by centrifugation at 5,000 rpm for 10 min and lysed with 700 µL TRIzol reagent. RNA extraction was performed using chloroform phase separation, followed by isopropanol precipitation and washing with 70% ethanol. The purified RNA was dissolved in nuclease-free water and stored at −80°C. RNA quality and concentration were assessed using a UV–Visible spectrophotometer and agarose gel electrophoresis. Residual genomic DNA was removed by DNase I treatment prior to cDNA synthesis.

Complementary DNA (cDNA) was synthesized from 1.5 µg of DNase-treated RNA using M-MLV reverse transcriptase. Quantitative real-time PCR (qRT-PCR) was subsequently performed using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, USA) on a QuantStudio™ 5 Real-Time PCR System. Relative expression of the *gyrB* gene was determined using gene-specific primers (Table 1) and calculated using the 2^−ΔΔCt method, with untreated cells used as the calibrator.

The thermal cycling conditions consisted of UDG activation at 50°C for 2 min, initial denaturation at 95°C for 2 min, followed by amplification cycles comprising denaturation at 95°C for 15s, annealing at 60°C for 1 min, and extension at 72°C for 2 min. Melting curve analysis was performed following amplification to confirm the specificity of the PCR products.

RESULTS AND DISCUSSION

Scanning electron microscope (SEM)

The SEM image (Fig. 1) is shown with a 200 nm scale bar. The image showed tiny round particles. This showed that the size of the chemically synthesized Ciprofloxacin-loaded Iron Oxide Nanoparticles is below 50 nm. Samer *et al.,* (2022) also reported that the Cipro–AgNPs nanocomposites exhibited a predominantly aggregated morphology. In 2025, Ibtissam *et al.,* observed that the SEM image of Cip-loaded Ag nanoparticles revealed noticeable changes in surface morphology compared with bare Ag nanoparticles, it also exhibited a rougher surface texture and a slight tendency toward aggregation, suggesting the successful association of ciprofloxacin with the Ag NP surface. These morphological changes provide evidence of the interaction between ciprofloxacin and the silver nanoparticles.

The surface morphology and elemental composition of the ciprofloxacin-loaded iron oxide nanoparticles (Cip–IONPs) were investigated by scanning electron microscopy coupled with energy-dispersive X-ray spectroscopy (SEM–EDS). The EDS spectrum confirmed the presence of Fe, O, C and N, with weight percentages of 8.43%, 24.28%, 44.96% and 14.46%, respectively. The detection of Fe provides elemental evidence for the iron oxide component, whereas the prominent C, N and O signals are consistent with the organic fraction associated with the ciprofloxacin-loaded formulation.

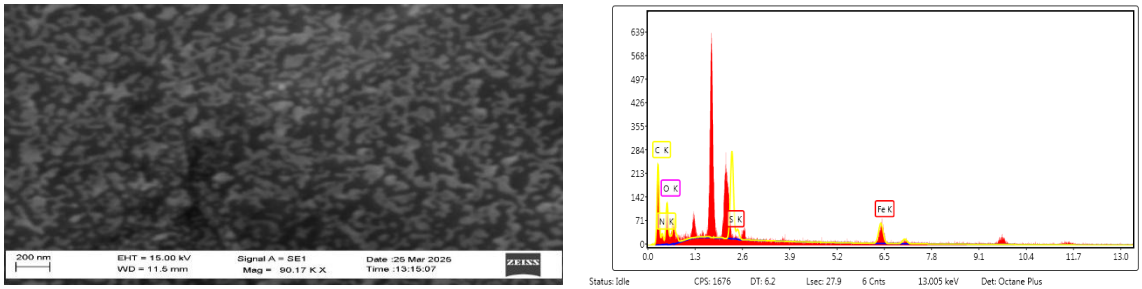

Fig1. SEM and EDX images of chemically synthesized Cip-IONPs.

Transmission electron microscope (TEM)

Transmission electron microscopy (TEM) analysis revealed that the chemically synthesized ciprofloxacin-loaded iron oxide nanoparticles possessed an average particle size of approximately 10 ± 10 nm (Fig. 2). The nanoparticles predominantly exhibited spherical to quasi-spherical morphologies, with a few particles displaying slight irregularities along their boundaries, indicative of minor variations in size and shape. Furthermore, the TEM micrograph (scale bar: 20 µm) demonstrated the presence of loosely aggregated nanoparticle clusters rather than densely fused assemblies, suggesting limited particle agglomeration. In 2022, Samer *et al.,* described that the morphological analysis revealed that the Cipro–AgNPs nanocomposites were predominantly spherical in shape and exhibited good dispersion.

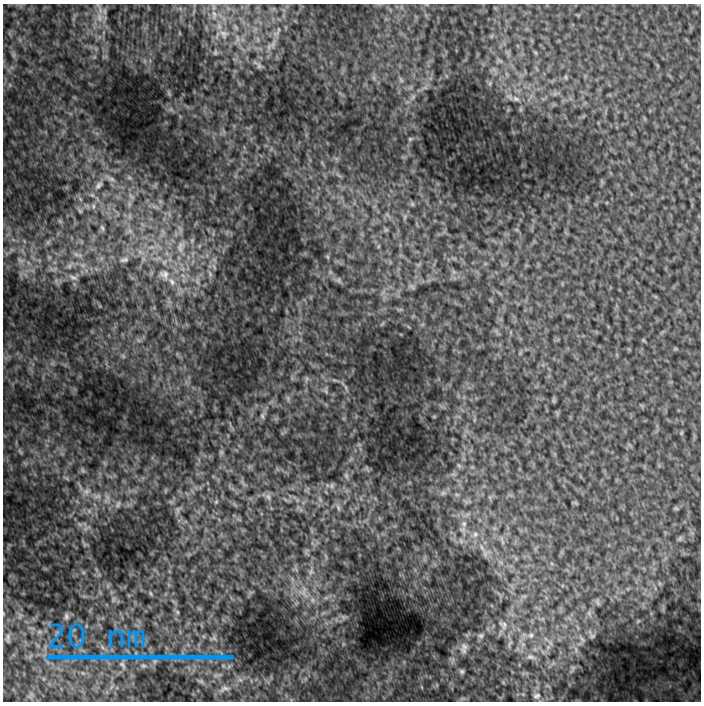

Fig. 2. Ciprofloxacin-loaded iron oxide nanoparticles

UV-Vis Spectroscopy

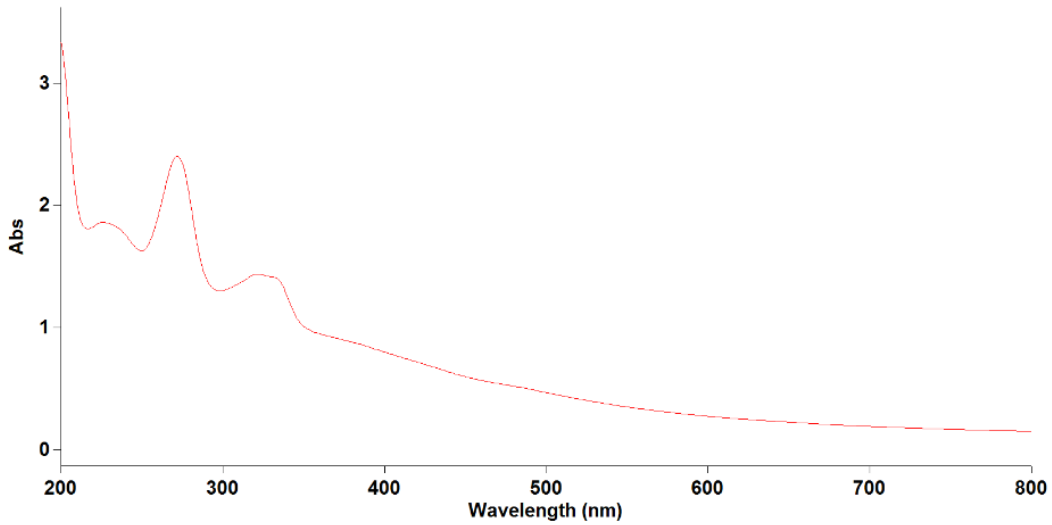

Fig 3. UV-Vis spectrum of chemically synthesized Cip-IONPs.

The UV–Visible spectrum of ciprofloxacin-loaded iron oxide nanoparticles (Cip-IONPs) demonstrated multiple absorption bands in the range of 200–400 nm, indicating the successful formation of the nanoparticle-drug conjugate. A prominent absorption peak was observed at approximately 349 nm, with an additional broad absorption feature extending toward 366 nm. Additional minor peaks were detected at 296, 292, 266, and 248 nm (Fig. 3) These spectral characteristics are distinct from those typically reported for bare iron oxide nanoparticles and suggest the presence of ciprofloxacin molecules associated with the nanoparticle surface. In 2025, Mehwish

et al., observed that the UV–Vis absorption spectrum revealed a characteristic absorption band at 270–300 nm corresponding to ciprofloxacin, thereby confirming its successful incorporation into the iron oxide nanoparticles. Ciprofloxacin is known to exhibit characteristic absorption in the UV region due to π→π* and n→π* electronic transitions of its quinolone ring system, generally around 270–280 nm. The UV–Vis spectrum of free ciprofloxacin (Cip) exhibited a characteristic absorption peak at 274 nm, in agreement with previously reported findings by Halawani *et al.,* (2020). The appearance of broadened and red-shifted absorption bands in the Cip-IONPs compared with free ciprofloxacin indicates interactions between the drug and the iron oxide matrix, likely through adsorption and surface complexation. Furthermore, the broad nature of the spectrum is consistent with nanoscale materials exhibiting heterogeneous surface environments.

Fourier-transform Infrared Spectroscopy

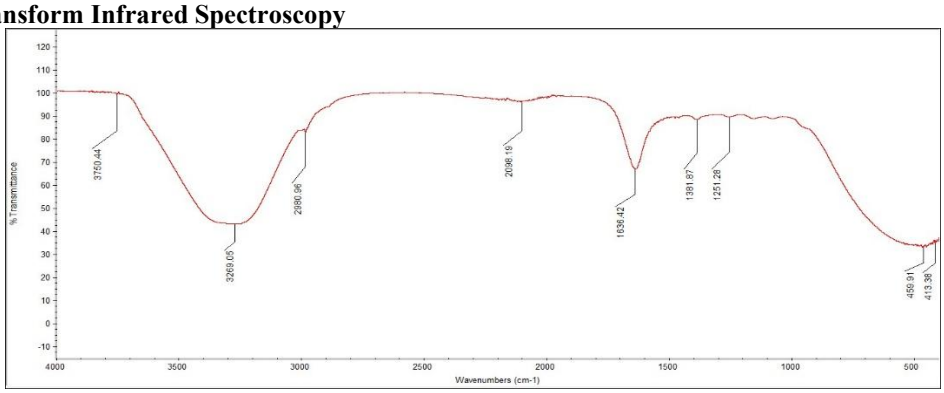

Fig 4. FTIR spectrum of chemically synthesized Cip-IONPs.

FTIR analysis of Cip-IONPs revealed characteristic absorption bands at 3266, 2118, 1636, and ~420 cm^{-1} (Fig.4). The broad peak at 3266 cm^{-1} was assigned to O–H/N–H stretching vibrations, while the band at 1636 cm^{-1} corresponded to the carbonyl and amine functionalities of ciprofloxacin. In 2024, Xin *et al.,* reported that the FTIR spectrum of ciprofloxacin exhibited a distinct absorption band at 1718 cm^{-1}, which is assigned to the C=O stretching vibration of the carbonyl group. Similarly, Samison *et al.,* (2026) stated that the peak at 1694 cm^{-1} is assigned to C—O stretching vibrations, likely originating from ciprofloxacin. The Fe–O stretching vibration observed near 420 cm^{-1} confirmed the formation of iron oxide nanoparticles. Collectively, these findings indicate the successful loading of ciprofloxacin onto the iron oxide nanoparticle surface.

A weak band observed near 2118 cm^{-1} may be associated with overtone or combination vibrations and could also arise from interactions between the nanoparticle surface and the loaded drug molecules. The prominent peak at 1636 cm^{-1} corresponds to C=O stretching and/or N–H bending vibrations of the quinolone ring of ciprofloxacin, suggesting successful conjugation of the antibiotic with the iron oxide nanoparticles. The slight shifts and broadening of the absorption bands relative to free ciprofloxacin indicate possible hydrogen bonding and electrostatic interactions between the drug molecules and the nanoparticle surface, resulting in the formation of a stable Cip-IONPs nanocomposite.

X-Ray Diffraction

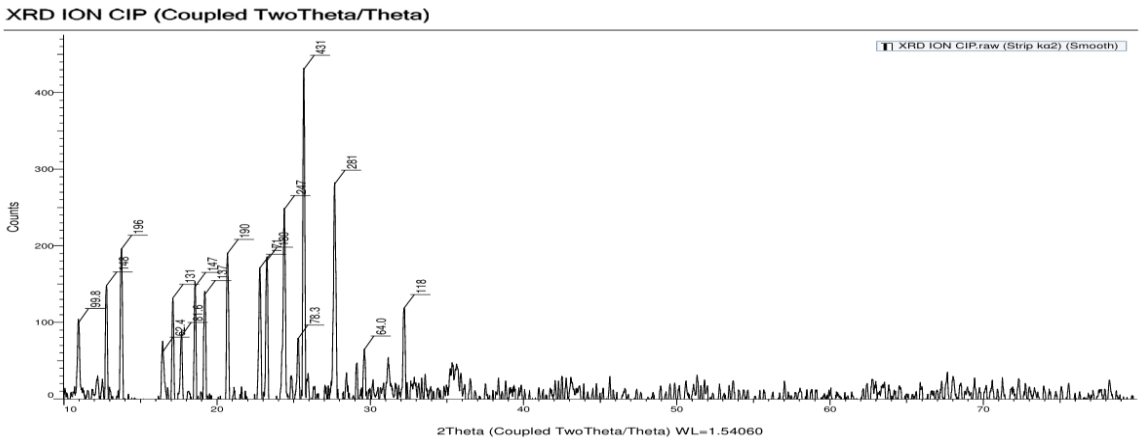

Fig 5. XRD spectrum of chemically synthesized Cip-IONPs.

The XRD profile of Cip-IONPs exhibited diffraction peaks at 25.67°, 27.70°, 29.59°, 31.20°, 32.22°, and 33.18° (2θ) (Fig. 5). The broad nature of the diffraction peaks indicates the nanoscale dimensions of the particles and the partial amorphization of ciprofloxacin following loading onto the iron oxide nanoparticles. In 2024, Tian *et al.,* reported The X-ray diffraction (XRD) pattern of ciprofloxacin exhibited several well-defined Bragg reflections,

with prominent diffraction peaks at 2θ = 14.5°, 20.8°, and 25.4°, confirming the highly crystalline nature of the drug. The observed reduction in crystallinity compared to pure iron oxide nanoparticles suggests successful surface functionalization and the formation of a stable Cip-IONPs nanocomposite. X-ray diffraction (XRD) analysis of ciprofloxacin-loaded iron oxide nanoparticles revealed multiple diffraction peaks in the range of 10–35° (2θ), indicating the formation of a nanostructured material with partially crystalline characteristics. The broad and relatively low-intensity peaks suggest the presence of nanosized particles and reduced crystallinity, which is commonly observed following drug loading onto nanoparticle surfaces.

The absence or attenuation of the characteristic high-intensity peaks of crystalline iron oxide may be attributed to the small particle size, the low concentration of the iron oxide phase in the analyzed sample, and the coating/loading of ciprofloxacin, which can mask or broaden the diffraction signals. Furthermore, ciprofloxacin is known to exhibit semi-crystalline to amorphous behaviour when associated with nanoparticle matrices, contributing to the observed peak broadening.

X-Ray Photoelectron spectroscopy (XPS)

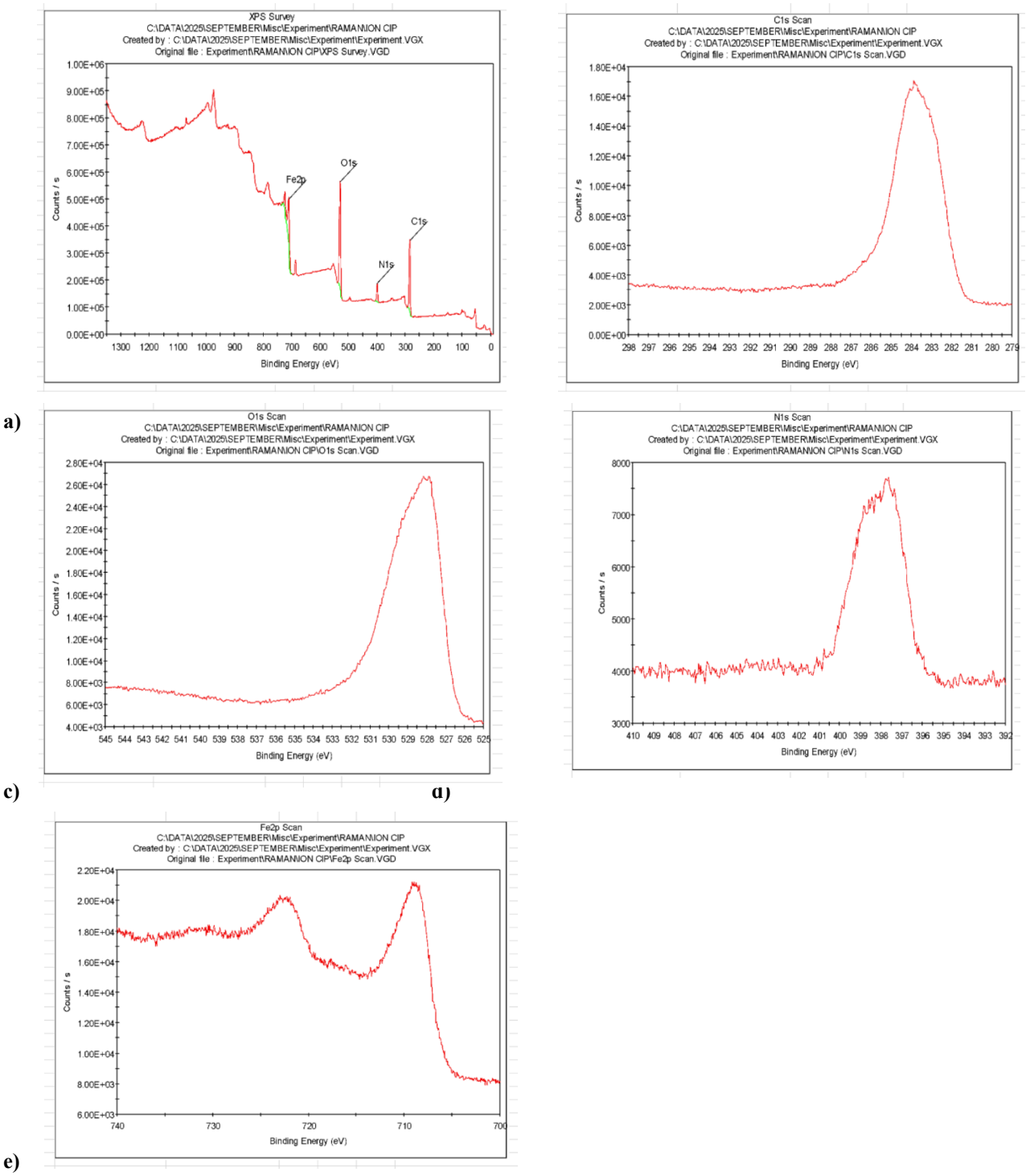

Fig 6. XPS survey spectrum of ciprofloxacin-loaded iron oxide nanoparticles (CIP–IONPs), showing the characteristic C1s (b), O 1s (c), N 1s (d), and Fe2p (e) signals.

The XPS survey spectrum of Cip-IONPs revealed distinct signals corresponding to Fe, O, C, and N, confirming the elemental composition of the synthesized nanocomposite. The absence of additional major impurity peaks indicates the successful synthesis of a relatively pure ciprofloxacin-loaded iron oxide nanoparticle formulation. The Fe 2p spectrum displayed characteristic doublet peaks centered at approximately 710 and 723 eV, corresponding to Fe 2p3/2 and Fe 2p1/2, respectively (Fig. 6). These findings confirm the presence of iron in the oxide state and support the formation of iron oxide nanoparticles. The O 1s spectrum exhibited a prominent peak at approximately 528–530 eV, indicative of oxygen associated with iron oxide lattices and oxygen-containing functional groups originating from ciprofloxacin. The broad profile of the peak suggests contributions from both lattice oxygen and surface-bound species. The presence of a distinct N 1s signal at approximately 398 eV confirms the successful incorporation of ciprofloxacin onto the nanoparticle surface. The detected nitrogen species are attributed to the piperazine moiety and amine functionalities of the antibiotic. The C 1s spectrum exhibited an intense peak at approximately 284 eV, corresponding to carbonaceous species derived from ciprofloxacin. XPS analysis confirmed the results reported by Sergio *et al.,* (2022). The C 1s spectrum showed four peaks: 284.8 eV (C—C/C—H, used as reference), 285.8 eV (C—N), 287.0 eV (C—O and/or C—F), 287.8 eV (C=O), and 288.9 eV (carboxylic (C=O)—OH group). The O 1s spectrum showed three peaks: 531.5 eV (carboxylic (C=O)—OH), 533.1 eV (C—O and Si—O), and 535.3 eV (adsorbed OH groups). The N 1s spectrum showed two peaks: 399.7 eV (C—NH) and 401.1 eV (C—N—C). The F 1s spectrum showed one peak at 687.5 eV, corresponding to C—F bonds.

These results confirm the presence of nitrogen, oxygen, and fluorine functional groups on the surface, consistent with successful surface functionalization. The observed signal confirms the presence of organic functional groups associated with the loaded drug molecules. Collectively, the XPS findings demonstrate the successful formation of a Cip-IONPs nanocomposite and corroborate the results obtained from UV–Vis, FTIR, TEM, SEM and XRD analyses.

Nuclear Magnetic Resonance

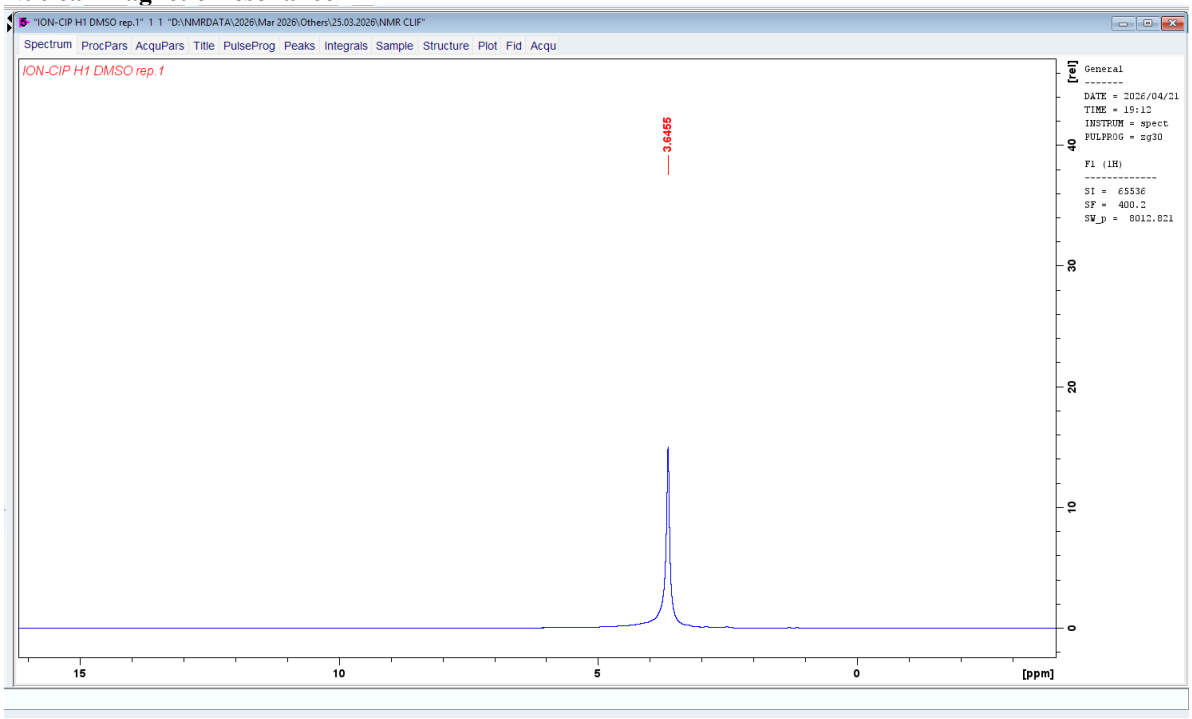

Fig 7. ^1HNMR spectrum of chemically synthesized Cip-IONPs.

^1H NMR analysis of Cip-IONPs revealed a single prominent resonance at δ 3.6455 ppm (Fig.7). The observed signal is likely associated with ciprofloxacin-derived protons, while the absence of additional characteristic peaks may be attributed to the paramagnetic behaviour of iron oxide nanoparticles and restricted mobility of the surface-bound drug molecules. These findings support the successful formation of the ciprofloxacin-loaded iron oxide nanoparticle conjugate. Mesallati *et al.,* in 2016, described that Ciprofloxacin (Cipro; 1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl) quinoline-3-carboxylic acid) is a fluoroquinolone antibiotic characterized by a six-membered nitrogen-containing piperazine ring in a chair conformation and a cyclopropyl substituent). In 2025, Filipe *et al.,* observed in their study that the average theoretical chemical-shift deviations were approximately 1.5 ppm for the cyclopropyl group, 4.5 ppm for the piperazine ring, and 5.5 ppm for the quinolone core.

Antibiofilm

S.No	Tested sample concentration (µg/ml)	Mean OD Value at 600 nm	Mean value of inhibition (%)
1.	Control	1.260	-
2.	Antibiotic	0.099	92.1206
3.	0.9 µg/ml	0.986	21.7612
4.	1.9 µg/ml	0.937	25.6744
5.	3.9 µg/ml	0.823	34.7173
6.	7.8 µg/ml	0.629	50.1059
7.	15.6 µg/ml	0.548	56.5575
8.	31.25 µg/ml	0.474	62.3745
9.	62.5 µg/ml	0.323	74.3523
10.	125 µg/ml	0.294	76.7055
11.	250 µg/ml	0.247	80.4337
12.	500 µg/ml	0.143	88.6833

Table 1. Mean OD values and percentage inhibition of P. aeruginosa treated with chemically synthesized Cip-IONPs.

S.No	Tested sample concentration (µg/ml)	Mean OD Value at 600 nm	Mean value of inhibition (%)
1.	Control	1.375	-
2.	Antibiotic	0.085	93.8439
3.	0.9 µg/ml	0.89	91.7838
4.	1.9 µg/ml	0.755	88.1483
5.	3.9 µg/ml	0.671	80.9985
6.	7.8 µg/ml	0.538	74.2365
7.	15.6 µg/ml	0.465	71.11
8.	31.25 µg/ml	0.397	66.1657
9.	62.5 µg/ml	0.354	60.8579
10.	125 µg/ml	0.261	51.2359
11.	250 µg/ml	0.163	45.1283
12.	500 µg/ml	0.113	35.2883

Table 2. Mean OD values and percentage inhibition of S. aureus treated with chemically synthesized Cip-IONPs.

Ciprofloxacin-loaded iron oxide nanoparticles (Cip-IONPs) exhibited significant, concentration-dependent antibiofilm activity against both *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The highest biofilm inhibition was observed at 500 µg/ml, with mean inhibition values of 88.68% and 91.78% against *P. aeruginosa* and *S. aureus*, respectively (Table 1 and 2), while lower concentrations showed a gradual reduction in antibiofilm efficacy. Furthermore, the IC_{50} values were determined to be 16.36 µg/ml for *P. aeruginosa* and 14.12 µg/ml for *S. aureus*, indicating a comparatively greater susceptibility of *S. aureus* to Cip-IONPs treatment. The pronounced inhibition of biofilm formation may be attributed to the synergistic effects of ciprofloxacin and iron oxide nanoparticles, which likely interfere with bacterial adhesion, extracellular polymeric substance production, and biofilm maturation. These findings demonstrate the potential of chemically synthesized Cip-IONPs as an effective antibiofilm agent against clinically relevant bacterial pathogens.

Minimum inhibition concentration (MIC)

Bacterial strain	Type	MIC (µg/ml)	IC50 (µg/ml)	Inhibition at 0.9 µg/ml (%)	Inhibition at 500 µg/ml (%)
Staphylococcus aureus	Gram positive	0.9	14.77	55.06	90.39
Pseudomonas aeruginosa	Gram negative	0.9	11.59	48.22	90.49

Table 3. Antibacterial activity of ciprofloxacin-loaded iron oxide nanoparticles against selected bacterial pathogens based on MIC, IC_{50}, and percentage inhibition.

Ciprofloxacin-loaded iron oxide nanoparticles (Cip-IONPs) exhibited potent antibacterial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* demonstrating a concentration-dependent reduction in bacterial growth. The nanoparticles showed a minimum inhibitory concentration (MIC) of 0.9 µg/ml and an MIC_{50} value of 11.59 µg/ml for P. aeruginosa and MIC value of 0.9 µg/ml l and MIC_{50} value of 14.77 µg/ml., indicating high antibacterial efficacy even at low concentrations (Table 3). Increasing concentrations of Cip-IONPs resulted

in progressively lower optical density values and higher percentages of bacterial inhibition, with a maximum inhibition of approximately 90.49% observed at 500 µg/ml. de Juan Mora *et al.,* in 2021 reported that the smallest IC50 values for Ciprofloxacin against Staphylococcus aureus is 29.16 µg/ml. These findings suggest that the synergistic interaction between ciprofloxacin and iron oxide nanoparticles effectively suppresses bacterial proliferation and highlights the potential of Cip-IONPs as a promising nano-antibiotic formulation for the treatment of drug-resistant *P. aeruginosa* infections.

AO/EtBr fluorescence staining

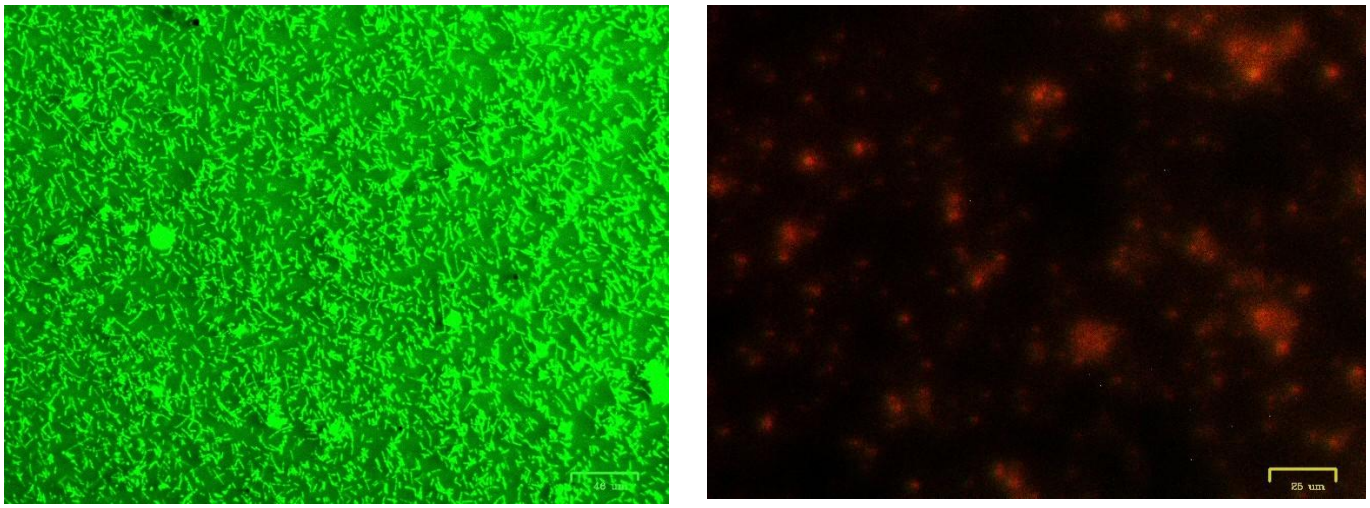

Fig 8. Fluorescent staining of P. aeruginosa: Control (25 µl) and treated with Cip-IONPs.

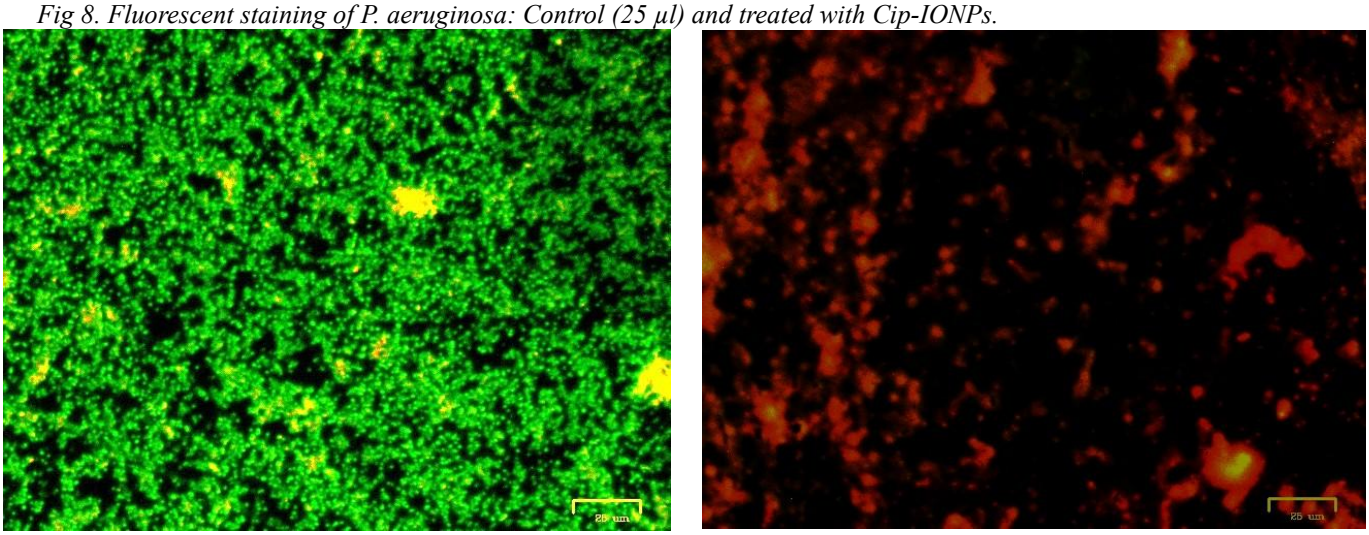

Fig 9. Fluorescent staining of S. aureus: Control (25 µl) and treated with Cip-IONPs.

AO/EtBr fluorescence staining revealed that untreated *P. aeruginosa* cells exhibited strong green fluorescence, confirming high cellular viability and intact membranes. Following treatment with Cip-IONPs (11.59 µg/ml), a substantial increase in orange-red fluorescence, reduced bacterial density, and disrupted cellular morphology were observed, indicating membrane damage and loss of viability. The pronounced shift from green to orange-red fluorescence suggests that Cip-IONPs effectively induce bacterial cell death, corroborating their potent antibacterial activity against *P. aeruginosa* (Fig. 8).

AO/EtBr fluorescence staining revealed a significant reduction in the viability of *Staphylococcus aureus* following treatment with chemically synthesized ciprofloxacin-loaded iron oxide nanoparticles (14.77 µg/ml). The untreated control groups (25 µm and 100 µm) exhibited abundant green fluorescence, indicating a dense population of viable bacterial cells with intact membranes. In contrast, the Cip-IONPs-treated samples demonstrated a pronounced increase in orange/red fluorescence, accompanied by a marked decrease in green fluorescent signals. The treated groups also exhibited reduced bacterial density and disrupted cellular organization, suggesting extensive membrane damage and loss of cellular integrity. At both magnifications, the fluorescence pattern indicated a substantial shift from viable to non-viable cells after nanoparticle treatment (Fig. 9). These observations confirm that Cip-IONPs exert potent bactericidal effects against *S. aureus* and *P. aeruginosa* likely through membrane disruption and subsequent cell death, which is consistent with the MIC, MBC, and antibiofilm findings obtained in this study.

SEM

SEM analysis revealed that untreated *P. aeruginosa* cells retained intact, smooth, and well-defined rod-shaped morphology, whereas Cip–IONPs-treated cells exhibited significant deformation, surface irregularities, aggregation, and cellular damage. These morphological alterations suggest disruption of the bacterial cell envelope and support the antibacterial activity of Cip–IONPs against *P. aeruginosa*. (Figure 10). SEM analysis revealed that untreated *S. aureus* cells exhibited relatively intact and well-defined morphology, whereas Cip–IONPs-treated cells showed pronounced surface irregularities, deformation, aggregation, and structural disruption. SEM analysis revealed intact and relatively smooth cellular morphology in untreated *S. aureus*, whereas Cip-IONPs treated cells exhibited pronounced surface deformation, roughening, aggregation, and structural disruption. These morphological alterations indicate compromised cellular integrity and support the antibacterial activity of Cip-IONPs against *S. aureus*. These morphological alterations indicate compromised cellular integrity and support the antibacterial activity of Cip–IONPs against *S. aureus*. (Figure 11)

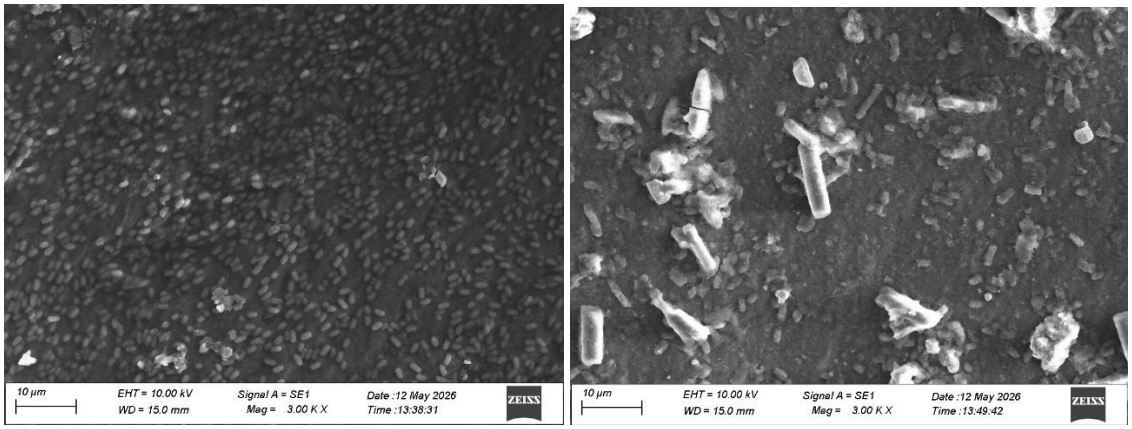

Fig 10. SEM images (Control and treated) of Pseudomonas aeruginosa treated by Cip-IONPs.

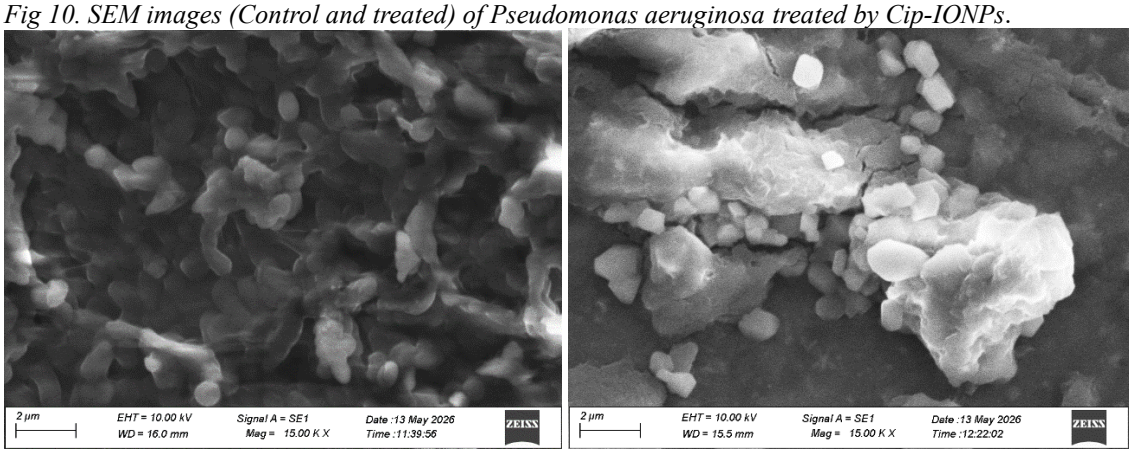

Fig 11. SEM images (Control and treated) of Staphylococcus aureus treated by Cip-IONPs.

Quantitative Reverse Transcription Polymerase Chain Reaction (QRT-PCR)

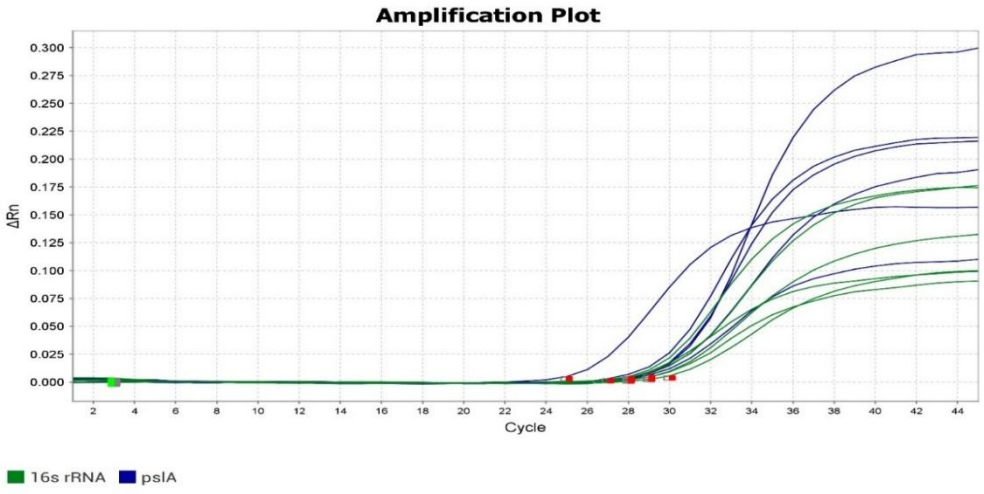

Fig 12. Real-time qPCR amplification curves of psIA and 16S rRNA genes in Pseudomonas aeruginosa.

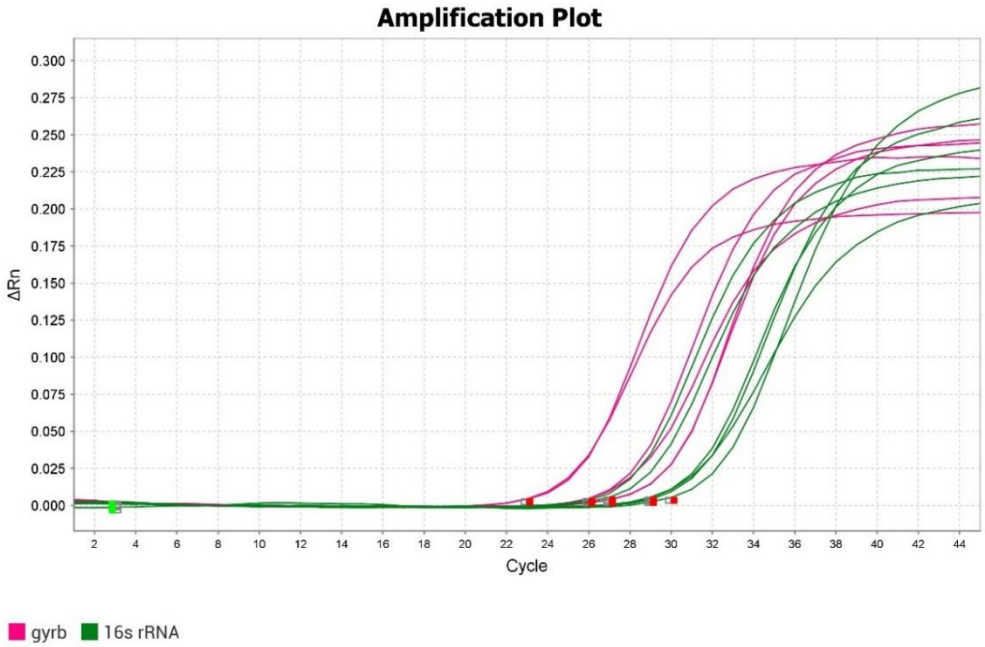

Fig 13. Real-time qPCR amplification curves of gyrB and 16S rRNA genes in Staphylococcus aureus.
Real-time qPCR amplification curves of *gyrB* and 16S rRNA genes in *Staphylococcus aureus*.
Relative quantification (RQ) of *pslA* gene expression in control and Cip-IONPs-treated *Pseudomonas aeruginosa*

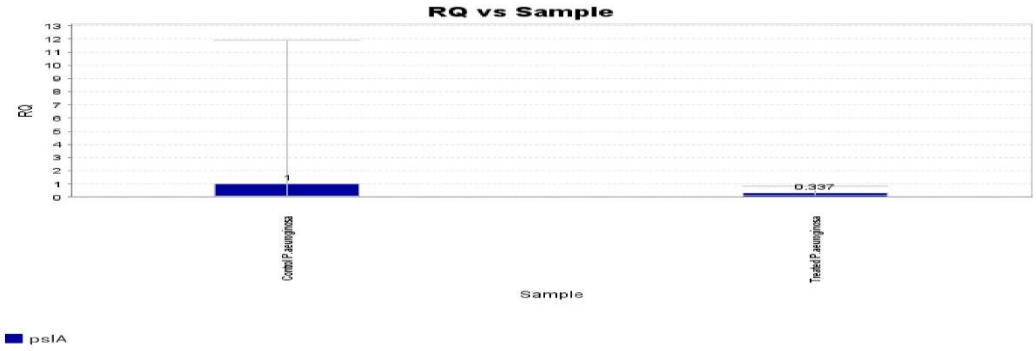

Fig 14. Relative quantification (RQ) of pslA gene expression in control and Cip-IONPs-treated Pseudomonas aeruginosa.

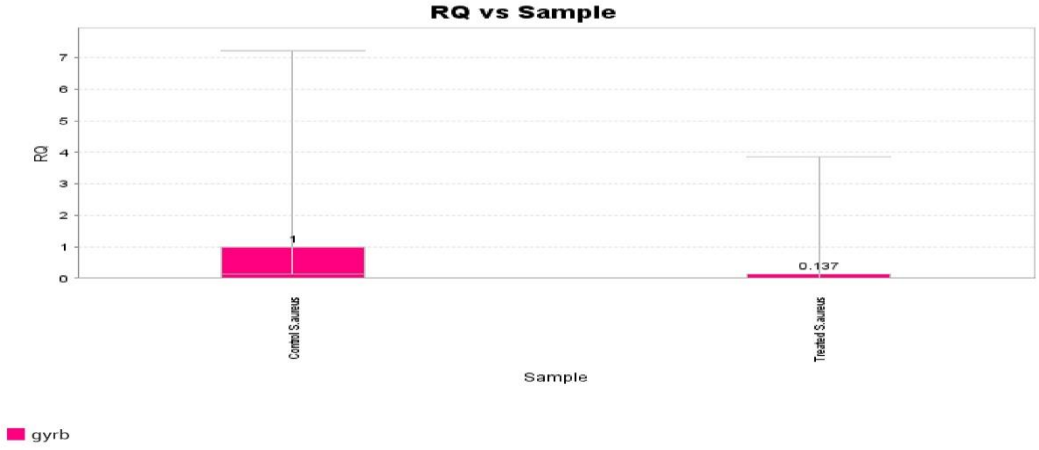

Fig 15. Relative quantification (RQ) of gyrB gene expression in control and Cip-IONPs-treated Staphylococcus aureus.

The qRT-PCR analysis demonstrates that ciprofloxacin-loaded iron oxide nanoparticles effectively suppressed the expression of important bacterial genes in both test organisms. Treatment reduced pslA expression to 0.337-fold in *Pseudomonas aeruginosa* (Fig. 12), indicating inhibition of biofilm formation through decreased exopolysaccharide synthesis. Likewise, gyrB expression in *Staphylococcus aureus* decreased to 0.137-fold, reflecting strong inhibition of DNA gyrase-mediated DNA replication (Fig. 13). These findings suggest that Cip-IONPs enhance the antibacterial activity of ciprofloxacin by simultaneously inhibiting bacterial growth and suppressing virulence-associated mechanisms. The greater reduction observed in gyrB expression compared with pslA suggests that the nanoparticle formulation exerted a stronger inhibitory effect on DNA replication in *S. aureus* than on biofilm-associated gene expression in *P. aeruginosa* under the conditions tested. elative gene expression analysis revealed a marked reduction in target gene expression following treatment. In *S. aureus*, *gyrB* expression decreased from an RQ of 1.0 in the control to 0.205 in the treated group, corresponding to a 79.5% reduction (Fig. 14). Similarly, *pslA* expression in *P. aeruginosa* decreased from 1.0 to 0.283, representing a 71.7% reduction (Fig.15). These findings suggest that the treatment substantially suppressed bacterial gene expression, with reduced *pslA* expression indicating a potential inhibition of biofilm-associated mechanisms in *P. aeruginosa*.

CONCLUSION

The present study successfully demonstrated the synthesis and characterization of ciprofloxacin-loaded iron oxide nanoparticles (Cip-IONPs) using a chemical co-precipitation approach. Comprehensive physicochemical characterization by SEM, TEM, UV–Visible spectroscopy, FTIR, XRD, XPS, and ^1H NMR confirmed the successful formation of stable drug-loaded nanoparticles with nanoscale dimensions and effective ciprofloxacin incorporation. Biological evaluation revealed that the synthesized Cip-IONPs possessed potent antibacterial and antibiofilm activities against both *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The nanoparticle formulation exhibited a low minimum inhibitory concentration (0.9 µg/ml), marked concentration-dependent inhibition of biofilm formation, and extensive membrane damage, as evidenced by fluorescence microscopy and SEM observations. Molecular analysis further confirmed significant downregulation of the *gyrB* gene in *S. aureus* and the biofilm-associated *pslA* gene in *P. aeruginosa*, indicating that the enhanced antibacterial activity of Cip-IONPs involves both inhibition of bacterial DNA replication and suppression of virulence-associated biofilm formation. These results suggest that iron oxide nanoparticles function as an efficient drug-delivery platform that enhances the antimicrobial performance of ciprofloxacin through synergistic mechanisms. Therefore, Cip-IONPs represent a promising nano-antibiotic formulation for the treatment of multidrug-resistant bacterial infections. Nevertheless, further investigations involving drug-release kinetics, cytocompatibility, pharmacokinetics, and in vivo efficacy studies are warranted before clinical translation can be considered.

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