Research Article

Synergistic Antibacterial Evaluation of Commercial Antibiotics Combined with Nanoiron against Human Pathogens

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Accepted on: 06-12-2012; **Finalized on:** 31-12-2012.

ABSTRACT

The worldwide escalation of bacterial resistance to conventional medical antibiotics is a serious concern for modern medicine. These concerns have led to discover alternative strategies for the treatment of various bacterial infections. In the current scenario, one of the most promising and novel therapeutic agents are the nanoparticles. Nanoparticles have unique and well defined physical and chemical properties which can be manipulated suitably for desired applications. This report would be focused to synthesize and evaluate the bactericidal effect of zerovalent iron nanoparticles (Fe^o). Chemically synthesized zerovalent iron (Fe^o) nanoparticles were obtained by reducing aqueous solution of ferrous sulfate heptahydrate ($FeSO_4.7H_2O$) with sodium borohydride ($NaBH_4$). The synthesized particles were further characterized by X-Ray Diffractogram (XRD), Scanning Electron Microscopy (SEM), and Energy Dispersive Spectroscopy (EDS) techniques to analyze size, morphology of the nanoparticles, and quantitative information of elemental iron (Fe) respectively. Average crystalline size of the particle was found to be 44.87 nm. Bactericidal effect of Fe^o nanoparticles was examined by agar well diffusion technique. Bacterial sensitivity to nanoparticles was found to vary depending on the microbial species. *Bacillus cereus* exhibited highest antibacterial sensitivity (24 mm) than other bacterial strains used. The synergistic inhibitory effect of Fe^o nanoparticles impregnated with commercial antibiotics was evaluated by agar disc diffusion assay. It has been observed that an enhanced antibacterial activity of commercial antibiotics when it combined with Fe^o nanoparticles. Therefore, it could be concluded that Fe^o nanoparticles alone or their formulations in combination with commonly used antibiotics can be used as effective bactericidal agents.

Keywords: Zerovalent iron nanoparticles, Antibacterial activity, Antibiotics, Synergistic effect, Fold increase.

INTRODUCTION

Resistance to antibiotics is a ubiquitous and relentless clinical problem that is compounded by a dearth of new therapeutic agents[1]. Therefore, there is an immediate need to develop new approaches to handle this problem. The emergence of nanoscience and nanotechnology in the last decade presents opportunities for exploring the bactericidal effect of metal nanoparticles[2]. In recent scenario, much attention has been paid to metal nanoparticles which exhibit novel chemical and physical properties owing to their extremely small size and high surface area to volume ratio[3]. It is evident that metal based nanoparticles due to their biological and physicochemical properties are promising as antimicrobials and therapeutic agents. Antimicrobial activity of the nanoparticles is known to be a function of the surface area in contact with the microorganisms. Fe^o nanoparticles have several advantages, such as low cost, easy preparation, and high reactivity compared to other metal nanoparticles. Nanoscale zero-valent iron (nZVI) has been used increasingly over the last decade to clean up polluted waters, soils and sediments[4] but little is known about the antimicrobial activity of nano-Fe^o. Typically, Fe(0) based nanoparticles are prepared by reducing Fe(II) or Fe(III) in an aqueous phase using sodium borohydride appears most suitable for environmental applications because of its minimal use of environmentally harmful solvents or chemicals[5]. You *et al.*[6] reported that nano-Fe^o have shown promise as strong antimicrobial agents against a broad spectrum of bacteria and viruses.

The antibacterial effect of Fe^o has been revealed to involve the generation of intracellular oxidants (eg. HO^o and Fe^{IV}) produced by the reaction with hydrogen peroxide or other species, as well as a direct interaction of Fe^o with cell membrane components[7]. nZVI exhibited a stronger antibacterial activity than other iron-based nanoparticles (*e.g.,* maghemite and magnetite). Inactivation of *E. coli* and *S. aureus* by nZVI was greater under deaerated than air-saturated conditions causing serious damage to the integrity of the cell membrane and to respiratory activity[8]. The ions released by the nanoparticles may attach to the negatively charged bacterial cell wall and rupture it, thereby leading to protein denaturation and cell death[9]. Xiu *et al.*[10] found that the anaerobic dechlorinating bacteria *Dehalococcoides* sp. was sensitive to nZVI exposure when they studied the bioremediation of trichloroethylene using a mixture of bacterial species. Increasing concentration of Fe^o nanoparticles substantially inhibited the growth of *E. coli* and *S. aureus*[11]. Therefore, the present study has been focused to synthesize and assess the antibacterial activity of zerovalent iron nanoparticles and to evaluate the interaction of these nanoparticles and antibiotics on bacterial strains.

MATERIALS AND METHODS

Materials

Ferrous sulfate heptahydrate ($FeSO_4.7H_2O$), Sodium borohydride ($NaBH_4$), Ethanol and Standard antibiotic discs were purchased from Himedia (P) Ltd, Mumbai were used as starting materials without further purification. Milli-Q water was used for the fabrication of nanoparticles.

Methods

Preparation of Fe^o Nanoparticles

The preparation of Fe^o nanoparticles was followed the method according to He and Zhao[12]. In brief, the preparation was carried out in a 250 ml flask attached to a vacuum line. Before use, deionized (DI) water was purged with purified N_2 gas for 15 min to remove dissolved oxygen (DO). In a typical preparation, a stock solution of 0.21 M $FeSO_4.7H_2O$ was prepared right before use. Fe concentration used in this study was 0.1 g/L. The Fe^{2+} ions were then reduced to Fe^o by adding a stoichiometric amount of $NaBH_4$ aqueous solution at a BH_4^-/Fe^{2+} molar ratio of 2.0 to the mixture with magnetic stirring at 230 rpm under ambient temperature. The ferrous iron was reduced to zero-valent iron according to the following reaction:

$$Fe(H_2O)_5^{2+} + 2BH_4^- \rightarrow Fe^c \downarrow + 2B(OH)_3 + 7H_2 \uparrow \longrightarrow 1$$

The resultant black particles were separated from the solution by centrifugation at 4000 rpm for 5 min and washed with N_2 saturated deionized water and at least three times with 99% absolute ethanol. Finally, the synthesized Fe^o nanoparticles were dried in an oven at 60°C. The dried particles were used for further characterization.

Characterization of Synthesized Fe^o Nanoparticles

Visual Inspection

The reduction of metal ions was roughly monitored by visual inspection of the solution by color change.

X-ray Diffractogram

The crystallographic analysis of the sample was performed by powder X-ray diffraction. The X-ray diffraction patterns of synthesized Fe^o nanoparticles were recorded with an X'pert PROPAN analytical instrument operated at 40 kV and a current of 30 mA with Cu α radiation (λ=1.54060 Å). A continuous scan mode was used to collect 2θ data from 10.02° to 79.92°. The diffraction intensities were compared with the standard JCPDS files. The information of the particle size was obtained from the full width at half maximum (FWHM) of the diffracted beam. Crystalline size of the nanoparticles was calculated from the line broadening of X-ray diffraction peak according to the Debye-Scherer formula[13].

$$D = k\lambda / \beta \cos\vartheta \longrightarrow 2$$

Where 'D' is the thickness of the nanocrystal, 'k' constant, 'λ' wavelength of X-rays, 'β' width at half maxima of reflection at Bragg's angle 2θ, 'θ' Bragg's angle.

Scanning Electron Microscopy

Surface morphology and the size distribution of the particles were observed using Scanning Electron Microscope. For SEM micrograph, the solid samples were sprinkled on the adhesive carbon tape which is supported on a metallic disk. The sample surface images were taken at different magnifications using the JEOL (SU 1510) operated at an accelerating voltage of 5 kV and magnification x10 k.

Energy Dispersive Spectroscopy

The quantitative information and distribution of the elemental Fe was investigated by EDS analysis (JSM 35 CF JEOL) in a resolution of 60 Å, magnification of 5 k. The operating conditions were 15 kV accelerating voltage and 15 mm working distance under high vacuum mode.

Antibacterial Studies

Bacterial Culture

The following bacterial pathogens namely *Streptococcus epidermis, Bacillus cereus, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae,* and *Staphylococcus aureus* were procured from the Microbial Type Culture Collection (MTCC), Chandigarh, India. All the cultures were grown on nutrient agar plates and maintained in the nutrient agar slants at 4°C. Overnight culture in the nutrient broth was used for the present experimental study.

Assay to Evaluate Antibacterial Activity

The antibacterial activity of the synthesized Fe^o nanoparticles was assessed against above mentioned test strains by agar well diffusion technique. The overnight bacterial cultures grown in nutrient broth was spread evenly over Mueller Hinton agar (MHA) plates with sterile cotton swab. Wells of 6 mm diameter were cut on the MHA plates using sterilize cork borer and 50 µl of nanoparticles suspension was dispensed in each well. The plates were left overnight at 37°C and results were recorded by measuring the diameter of inhibition zone (mm).

Assay to Evaluate Synergistic Effect

Disk diffusion method, to assay the synergistic effect of Fe^o nanoparticles with commonly used antibiotics, was adopted to test the bactericidal efficacy of these nanoparticles alone and in combination with antibiotics. To determine the synergistic effects, each standard antibiotic disc namely *Ampicillin, Amoxicillin, Methicillin, Chloramphenicol, Tetracycline, Amikacin, Kanamycin, Streptomycin, Vancomycin,* and *Erythromycin* was impregnated with 50 µl of freshly prepared Fe^o nanoparticles and was placed onto the MHA medium inoculated with test organisms. Standard antibiotic discs were used as positive control. These plates were

incubated overnight at 37°C. After incubation, results were recorded by measuring the inhibitory zone diameter (mm).

Assessment of Increase in Fold Area

According to Fayaz *et al.*[14], increase in fold area was assessed by calculating the mean surface area of the inhibition zone generated by an antibiotic alone and in combination with Fe^{o} nanoparticles. The fold increase area was calculated by the equation,

Fold increase (%) = (b-a)/a*100 ----→ 3

where a and b refer to the zones of inhibition for antibiotic alone and antibiotic with Fe^{o} nanoparticles.

RESULTS AND DISCUSSION

Visual Inspection

Upon reduction of ferrous ion by $NaBH_4$, the solution color rapidly changed from clear to black in the reaction mixture visually indicating the formation of Fe^{o} nanoparticles (Fig 1).

Figure 1: Solution containing $FeSO_4.7H_2O$ before (left) and after (right) reduction with $NaBH_4$

X-ray Diffractogram

The X-ray diffraction pattern shows that the synthesized Fe^{o} nanoparticles are in amorphous stage and in tetragonal system. The XRD pattern clearly showed the crystalline nature of Fe^{o} nanoparticles. In the respective nanoparticles, the intensive diffraction peaks were observed at a 2θ value of 44.8° from the lattice plane (311) of face-centered cubic (fcc) Fe unequivocally indicates that the particles are made of pure iron (Fig 2).

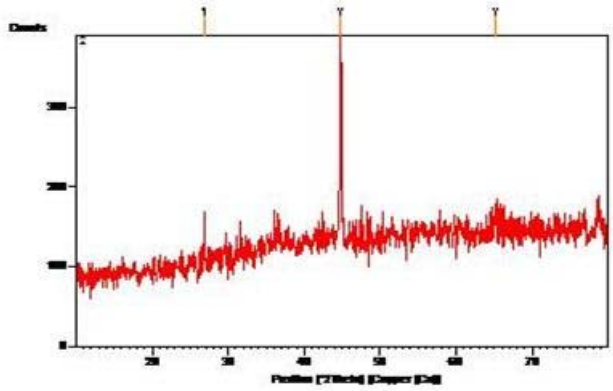

Figure 2: X-Ray diffraction pattern of nanoscale zerovalent iron

Chatterjee *et al.*[15] reported that characteristic peak at 2θ value of 44.7° indicates the crystalline nature of Fe^{o} nanoparticles. In the obtained spectrum, the Bragg's peak position and their intensities were compared with the standard JCPDS files. The size of the particles was found to be 44.87 nm.

Scanning Electron Microscopy

The scanning electron microscopy of synthesized Fe^{o} nanoparticles reveals that the particles are spherical in nature (Fig 3). The micrograph shows that the synthesized particles did not appear as discrete particles but form much larger dendritic flocs. The aggregation is attributed due to the vander waals forces and magnetic interactions among the particles. This finding is very much closer to the earliest report[16].

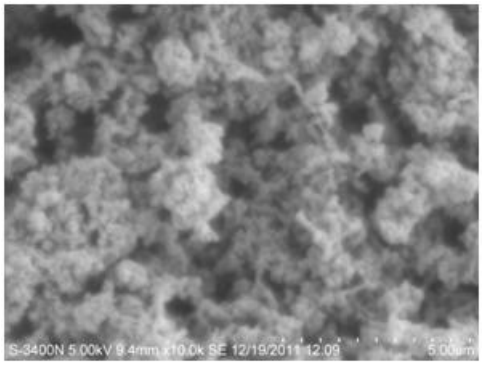

Figure 3: Scanning electron micrograph of nanoscale zerovalent iron

Energy Dispersive Spectroscopy

EDS micrograph explains the surface atomic distribution and chemical composition of Fe^{o} nanoparticles. In our analysis, we confirmed the presence of elemental iron signal (Fig 4).

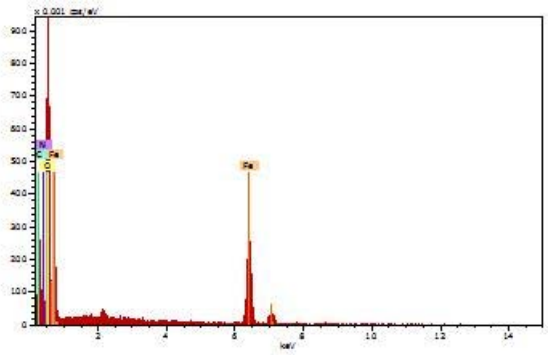

Figure 4: Energy dispersive spectroscopy of nanoscale zerovalent iron

Strong signals from the iron atoms are observed (72.11%), while weaker signal from N (7.23%) and O (20.66%) are also recorded. Our result corroborate as per the EDS report of Shih *et al.*[17].

Antibacterial Activity of Fe^{o} Nanoparticles

Due to overuse of antibiotics and a growing problem of antibiotic resistance, nanoparticles are being researched

as an alternative antibacterial agent. The inhibitory activity of the Fe^{o} nanoparticles was evaluated against pathogenic bacteria and their potency was assessed qualitatively by the presence of inhibition zones (Fig 5). Different classes of bacteria exhibit different susceptibilities to nanoparticles. Fe^{o} nanoparticles showed excellent antibacterial activity against the bacterial pathogens. Among the tested strains, Fe^{o} nanoparticles were found to be highly effective against *Bacillus cereus* with 24 mm zone of inhibition.

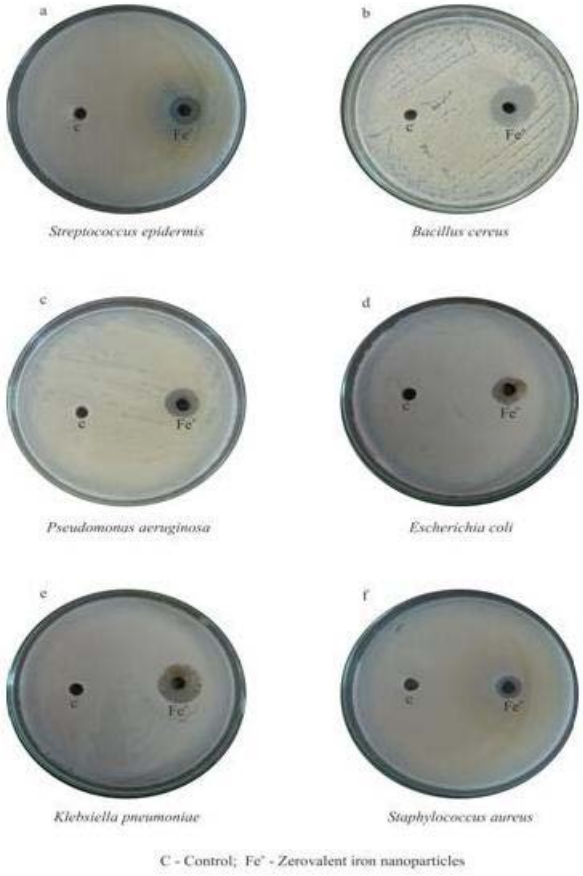

Figure 5: Inhibitory effect of zerovalent iron nanoparticles against bacterial pathogens

On the other hand, weaker activity was observed against *Staphylococcus aureus* with 12 mm zone of inhibition (Fig 6).

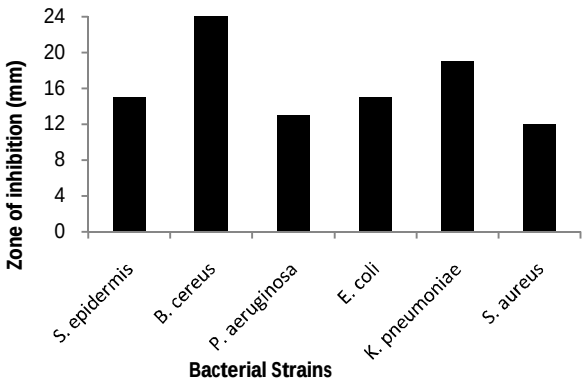

Figure 6: Zone of inhibition (mm) of nanoiron against bacterial strains by agar well diffusion method

Zone of inhibition reflects the magnitude of microbial susceptibility. The strains susceptible to nanoparticles exhibit larger zone, whereas resistant strains exhibit smaller zone. Specific modes of action for the bactericidal properties of nZVI have been postulated to be reductive decomposition of protein functional groups in the cell membrane due to strong reducing conditions at the nZVI surface[8]. Zhang[18] suggested that Redox-active Fe^{o} reacts with oxygen or water and releases Fe^{2+}. Fe^{2+} ions further generate Reactive Oxygen Species (ROS) *via* Fenton chemistry[19] and the elevated concentrations of ROS in a cell can result in a situation known as oxidative stress[20]. Cells under severe oxidative stress show various dysfunctions of membrane lipids, proteins and DNA which could end in apoptosis or death of microbes[21]. Zorov *et al.*[22] suggested that nZVI might indirectly generate ROS that damage iron-sulfur groups, cofactors in many enzymes, leading to Fenton chemistry that catalyzes the production of more ROS. The generated ROS can be released into the cytosol and trigger ROS-induced ROS-release in other mitochondria, potentially leading to cellular injury and death.

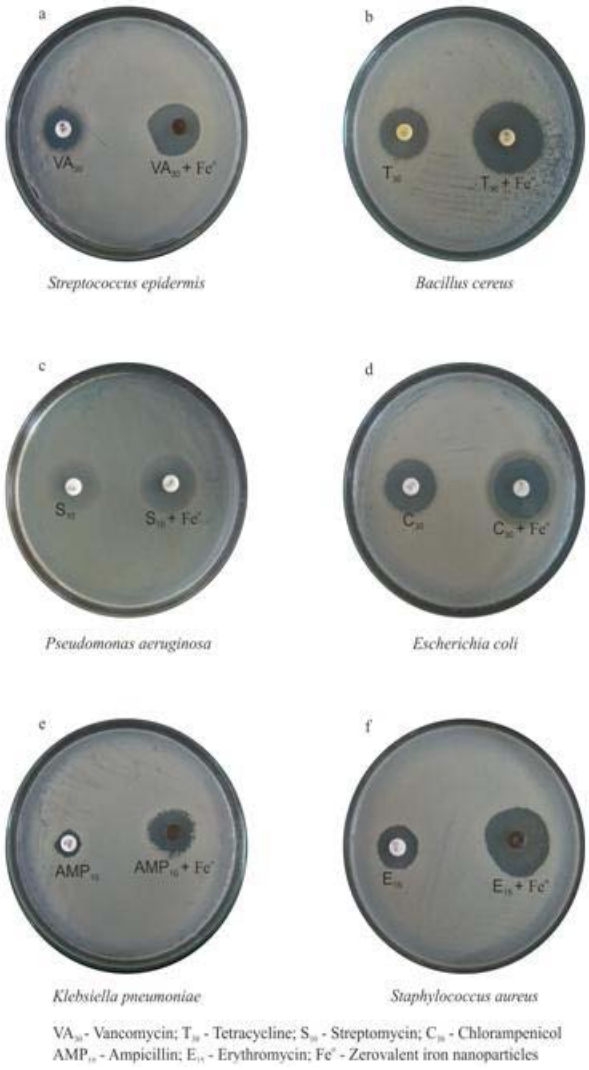

Figure 7: Antibiogram study of bacterial pathogens with and without nanoscale zerovalent iron

Combinatorial Effect of Fe^{o} Nanoparticles with Antibiotics

Synergism has been defined as a phenomenon in which two different compounds are combined to enhance their individual activity. The combined effect of Fe^{o} nanoparticles with standard antibiotic discs was done against the selected human bacterial pathogens (Fig 7).

The diameter of inhibition zones for antibiotics alone and in combination with Fe^{o} nanoparticles showed significant increase in fold area in all the cases (Tables 1-6).

Table 1: Synergistic effect of different antibiotics with and without nanoiron against *Streptococcus epidermis*

Types of antibiotics	Name of the antibiotics	Symbol	Conc. of the disc (µg/disc)	Zone of inhibition (mm)		Increased Zone Size (mm)	Fold increase (%)
				Antibiotic alone	Antibiotic + Fe^{o}NPs		
β-lactams	*Ampicillin*	AMP	10	17	21	4	23.53
	Amoxicillin	AMC	30	16	21	5	31.25
	Methicillin	MET	5	-	9	3	50.0
Sulphonamides	*Chloramphenicol*	C	30	21	24	3	14.29
	Tetracycline	T	30	20	23	3	15.0
Aminoglycosides	*Amikacin*	AK	30	10	15	5	50.0
	Kanamycin	K	30	14	18	4	28.57
	Streptomycin	S	10	12	13	1	8.33
Glycopeptides	*Vancomycin*	VA	30	16	22	6	37.5
Macrolides	*Erythromycin*	E	15	15	20	5	33.33
					Overall synergistic bactericidal effect (%)		**29.18**

Note: In the absence of bacterial growth inhibition zones, the disc diameter (6 mm) were used to calculate the fold increase

Table 2: Synergistic effect of different antibiotics with and without nanoiron against *Bacillus cereus*

Types of antibiotics	Name of the antibiotics	Symbol	Conc. of the disc (µg/disc)	Zone of inhibition (mm)		Increased Zone Size (mm)	Fold Increase (%)
				Antibiotic alone	Antibiotic + Fe^{o}NPs		
β-lactams	*Ampicillin*	AMP	10	10	14	4	40.0
	Amoxicillin	AMC	30	10	12	2	20.0
	Methicillin	MET	5	7	10	3	42.86
Sulphonamides	*Chloramphenicol*	C	30	21	24	3	14.29
	Tetracycline	T	30	15	23	8	53.33
Aminoglycosides	*Amikacin*	AK	30	24	27	3	12.5
	Kanamycin	K	30	19	23	4	21.05
	Streptomycin	S	10	23	26	3	13.04
Glycopeptides	*Vancomycin*	VA	30	16	21	5	31.25
Macrolides	*Erythromycin*	E	15	16	22	6	37.5
					Overall synergistic bactericidal effect (%)		**28.58**

Table 3: Synergistic effect of different antibiotics with and without nanoiron against *Pseudomonas aeruginosa*

Types of antibiotics	Name of the antibiotics	Symbol	Conc. of the disc (µg/disc)	Zone of inhibition (mm)		Increased Zone Size (mm)	Fold Increase (%)
				Antibiotic alone	Antibiotic + Fe^{o}NPs		
β-lactams	*Ampicillin*	AMP	10	14	15	1	7.14
	Amoxicillin	AMC	30	22	22	0	0
	Methicillin	MET	5	-	-	0	0
Sulphonamides	*Chloramphenicol*	C	30	13	15	2	15.38
	Tetracycline	T	30	11	12	1	9.09
Aminoglycosides	*Amikacin*	AK	30	28	30	2	7.14
	Kanamycin	K	30	15	15	0	0
	Streptomycin	S	10	11	18	7	63.64
Glycopeptides	*Vancomycin*	VA	30	-	8	2	33.33
Macrolides	*Erythromycin*	E	15	20	25	5	25.0
					Overall synergistic bactericidal effect (%)		**16.07**

Note: In the absence of bacterial growth inhibition zones, the disc diameter (6 mm) were used to calculate the fold increase

Table 4: Synergistic effect of different antibiotics with and without nanoiron against *Escherichia coli*

Types of antibiotics	Name of the antibiotics	Symbol	Conc. of the disc (µg/disc)	Zone of inhibition (mm)		Increased Zone Size (mm)	Fold Increase (%)
				Antibiotic alone	Antibiotic + Fe^{o}NPs		
β-lactams	*Ampicillin*	AMP	10	11	14	3	27.27
	Amoxicillin	AMC	30	15	17	2	13.33
	Methicillin	MET	5	-	8	2	33.33
Sulphonamides	*Chloramphenicol*	C	30	20	25	5	25.0
	Tetracycline	T	30	13	17	4	30.77
Aminoglycosides	*Amikacin*	AK	30	18	20	2	11.11
	Kanamycin	K	30	22	25	3	13.64
	Streptomycin	S	10	22	24	2	9.09
Glycopeptides	*Vancomycin*	VA	30	-	8	2	33.33
Macrolides	*Erythromycin*	E	15	13	16	3	23.08
Overall synergistic bactericidal effect (%)							**21.99**

Note: In the absence of bacterial growth inhibition zones, the disc diameter (6 mm) were used to calculate the fold increase

Table 5: Synergistic effect of different antibiotics with and without nanoiron against *Klebsiella pneumoniae*

Types of antibiotics	Name of the antibiotics	Symbol	Conc. of the disc (µg/disc)	Zone of inhibition (mm)		Increased Zone Size (mm)	Fold Increase (%)
				Antibiotic alone	Antibiotic + Fe^{o}NPs		
β-lactams	*Ampicillin*	AMP	10	10	18	8	80.0
	Amoxicillin	AMC	30	12	15	3	25.0
	Methicillin	MET	5	8	10	2	25.0
Sulphonamides	*Chloramphenicol*	C	30	18	21	3	16.67
	Tetracycline	T	30	17	22	5	29.41
Aminoglycosides	*Amikacin*	AK	30	21	22	1	4.76
	Kanamycin	K	30	17	19	2	11.76
	Streptomycin	S	10	22	26	4	18.18
Glycopeptides	*Vancomycin*	VA	30	15	21	6	40.0
Macrolides	*Erythromycin*	E	15	15	22	7	46.67
Overall synergistic bactericidal effect (%)							**29.75**

Table 6: Synergistic effect of different antibiotics with and without nanoiron against *Staphylococcus aureus*

Types of antibiotics	Name of the antibiotics	Symbol	Conc. of the disc (µg/disc)	Zone of inhibition (mm)		Increased Zone Size (mm)	Fold Increase (%)
				Antibiotic alone	Antibiotic + Fe^{o}NPs		
β-lactams	*Ampicillin*	AMP	10	10	14	4	40.0
	Amoxicillin	AMC	30	13	19	6	46.15
	Methicillin	MET	5	10	15	5	50.0
Sulphonamides	*Chloramphenicol*	C	30	21	24	3	14.29
	Tetracycline	T	30	20	22	2	10.0
Aminoglycosides	*Amikacin*	AK	30	23	25	3	13.04
	Kanamycin	K	30	17	20	3	17.64
	Streptomycin	S	10	24	26	2	8.33
Glycopeptides	*Vancomycin*	VA	30	13	14	1	7.69
Macrolides	*Erythromycin*	E	15	20	28	8	40.0
Overall synergistic bactericidal effect (%)							**24.71**

Distinct difference was observed between the inhibitory zones by antibiotics with and without nanoiron. A minimum zone of inhibition was increased from 1 to 8 mm when nanoparticles and the antibiotics are given together. Extend of inhibition depends on the concentration of nanoparticles as well as on the initial bacterial concentration. The highest fold increase in area was observed for *Vancomycin* (6 mm), *Tetracycline* (8 mm), *Streptomycin* (7 mm), *Chloramphenicol* (5 mm), *Ampicillin* (8 mm), and *Erythromycin* (8 mm) against *Sterptococcus epidermis, Bacillus cereus, Pseudomonas aeruginosa, E. coli, Klebsiella pneumoniae,* and *Staphylococcus aureus* respectively. Antibiotics operate by inhibiting crucial life sustaining processes in the organism: the synthesis of cell wall material, the synthesis of DNA, RNA, ribosomes and proteins. Allahverdiyev *et al.*[23] reported that nanoparticles tagged with antibiotics have been shown to increase the concentration of

antibiotics at the site of bacterium-antibiotic interaction, and to facilitate binding of antibiotics to bacteria. Among the tested strains, nanoiron showed highest fold increase (29.75%) on *Klebsiella pneumoniae* is given in Fig 8.

It may be suggested that combined antibiotic therapy produce synergistic effects in the treatment of bacterial infection and has been shown to delay the emergence of antimicrobial resistance[24,25]. The main mechanism by which antibacterial drugs and antibiotics work is *via* oxidative stress generated by ROS including superoxide radicals (O^{2-}), hydroxide radicals (-OH), hydrogen peroxide (H_2O_2) can cause damage to proteins and DNA in bacteria[26].

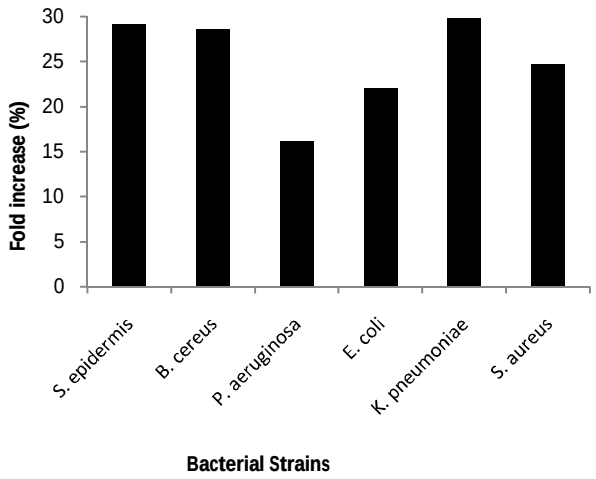

Figure 8: Percentage fold increase of antibiotics with nanoiron against test strains

CONCLUSION

Nanobiotechnology is an upcoming and developing field with potential application for human welfare owing to its potential application to fight against antibiotic resistant pathogens. In the present work, nontoxic nanomaterials which can be prepared in a simple and cost effective manner have great promise as antibacterial agents and it shows an excellent activity against bacterial pathogens. Results of our study show that the combination of nanoiron and antibiotics has a synergistic efficacy on tested strains. This enhancement in the combined effect was preferably due to the difference in the mechanism of inhibition followed by nanoparticles and antibiotics. Hence it is concluded that nanoiron significantly improved antibiotic efficacy against the tested bacterial pathogens when combined with sulphonamides, glycopeptides, aminoglycosides, and beta lactams.

Acknowledgements: The authors are grateful for the financial support provided by Ministry of Science & Technology, DST, Govt. of India for INSPIRE program (Dy.No.100/IFD/10706/) under Assured Opportunity for Research Carrier (AORC), VHNSN College Managing Board, Virudhunagar for providing facilities and Alagappa University, CECRI, Karaikudi for technical assistance.

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Source of Support: Nil, **Conflict of Interest:** None.