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Research Article

EFFECTS OF GREEN IRON NANOPARTICLES ON BIOFILM-FORMING BACTERIA

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ABSTRACT

Objective: The objective of this study was to observe the effects of iron nanoparticles (FeNP) synthesized from plant source of biofilm-forming bacteria.

Methods: FeNP were synthesized from *Pongamia pinnata* leave extracts and it was characterized using Ultraviolet-Visible Spectrophotometer, scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy, and energy dispersive X-ray Analysis. The synthesized FeNP were evaluated against bio film-forming Gram-negative *Pseudomonas*, Sewage organisms, and Gram-positive hay bacillus *Bacillus subtilis*. These biofilm-forming microorganisms were evaluated for antibiotic sensitivity. The extracellular and intracellular proteins of biofilm-forming bacteria were estimated in the presence of FeNP.

Results: All these biofilm-forming microorganisms were found to be antibiotic-resistant. The green FeNP showed potential antimicrobial effectiveness against hay *Bacillus,* followed by *Pseudomonas* and sewage bacteria. These NPs inhibited the intracellular protein formation more than extracellular proteins of biofilm-forming micro-organisms.

Conclusions: It can be concluded that the FeNP synthesized from plant sources were effectively inhibited the biofilm-forming microorganisms by obstructing the intracellular protein synthesis. These NPs can be used as an eco-friendly, cost-effective, and alternative molecule to treat the antibiotic-resistant biofilm- forming micro-organisms.

Keywords: Biofilm, Iron nanoparticles, Scanning electron microscopy, Fourier-transform infrared, Energy dispersive X-ray.

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INTRODUCTION

Nanotechnology has emerged rapidly during the past few years in a broad range of product domains. Metal nanoparticles (NP) are attained great importance due to their features such as catalytic, magnetic optical, and electrical properties [1]. Several NPs such as silver, copper, iron, and gold have been explored so far. These metals are applied as antimicrobial agents for a long period, but antibiotics supersede them [2]. The application of metals of their nanoparticulate form is currently considered to resolve bacterial infections but has attracted scientific attention only over the past decade. Most NPs are popular due to their characteristics high surface to volume ratio which makes these NPs are effective against several microbes [3,4]. A high surface to volume ratio is generally accompanied by increased production of reactive oxygen species, including free radicals. These characteristics allow NPs to interact closely with microbial cell walls and membranes, damage their internal structures, and inactivate bacteria [5,6]. Iron as metal is as reactive in the air as in water and in the form of NPs it is more active. Moreover, the iron NPs (FeNP) are non-toxic.

The microbes when aggregated together and attached to the surfaces tightly it form the biofilm. These biofilms are strengthened further by extracellular polysaccharides release by the microbes. Biofilm- forming microorganisms are highly pathogenic and in the environment, it causes several health-related hazards [7,8]. Researchers have shown that 60–80% of microbial infections are caused by bacteria grown as biofilm than free-floating bacteria [9].

Drug resistance microorganisms are a serious and increasing public health problem. New strategies for controlling bacterial activities are urgently needed and NPs can be a very promising approach [10]. It is well established that metallic compounds can have antimicrobial activity. A research work had taken on biosynthesis of plant-based FeNP, isolation, and assessment of biofilm-producing micro-organisms and to monitor the effect of FeNP on these micro-organisms [11]. This study is an attempt to evaluate the action of green FeNP on biofilm-forming bacteria. It gives an insight into applications of FeNP as an alternative therapeutic tool against biofilm-forming micro-organisms.

METHODS

Isolation of the biofilm bacteria

The three different biofilm-forming bacteria, namely, *Pseudomonas aeruginosa*, *Bacillus subtilis,* and a consortium of Sewage bacteria were collected and inoculated in the nutrient broth and incubated at room temperature for 48 h. After microscopic observation, the bacteria were subculture in the nutrient broth throughout the experiments [12].

Biofilm formation assay

The sterilized coverslips were dipped into the respective bacterial culture media and then stained with one drop of crystal violet (CV) and observed for the biofilm formation under the microscope [13].

To determine the antibiotic resistance of biofilm-forming bacteria

The three bacterial samples were inoculated to the Muller- Hinton agar (MH Agar). The multiple antibiotic disks were placed on the MH agar containing *P. aeruginosa*, *B. subtilis,* and sewage bacteria. The plates are incubated at 37°C for 48 h and measured the zone of inhibition.

Preparation of FeNPs from plant extracts

The leaves of *Pongamia pinnata* were collected. The leaves were cleaned with water and dried by spreading for 2 days. The dry leaves were crushed in pestle and mortar. 25 g of dry *P. pinnata* leaf powder was taken in 500 ml of distilled water and boiled for 5 min. The extract was filtered with normal filter paper and then with Whatman filters paper. The leaf extract was obtained was used for further experiments.

Various concentrations of $FeSO_4$ salt (0.5 mg/ml, 0.25 mg/ml, and 0.125 mg/ml) were prepared in 10 ml of leaf extract and incubated it at 37°C for 48 h. The solution was centrifuged at 10,000 rpm for 5 min and the supernatant was decanted, the precipitate was washed in distilled water. The precipitate was centrifuged at 10,000 rpm for 5 min and decants the supernatant. The precipitate was dried and stored for analysis. These purified FeNPs were analyzed for Scanning Electron Microscopy (SEM), Energy dispersive X-ray (EDAX), and Fourier-transform infrared (FTIR) analysis [14].

Analysis of FeNP

SEM

SEM gives a morphological examination with direct visualization. For a sampling of SEM, the NPs are dried into a powder. The powder in small quantity was placed on a sample holder and then coated with gold as conductive metal. Next, the sample was scanned with a beam of electrons. The characterization of molecules was done from secondary electrons emitted from the sample surface.

EDAX (energy dispersive analysis of X-ray diffraction spectroscopy)

To gain further insight into the features of the FeNPs, an analysis of the sample was performed using EDAX techniques.

FTIR spectroscopy

The transmission spectra for the NPs are obtained by the formation of thin, transparent potassium bromide (KBr) pellets containing 0.1–1% sample were mixed with 200 –250 mg of KBr. The KBr mixtures were placed in a vacuum line overnight before pellet formation, and the pellets were again placed in the vacuum line before use. The transmission spectra were obtained after purging in dry air and background corrected relative to a reference blank sample (KBr). With the application of modern software tools, quantitative analysis of the NPs can be completed.

Treatment of FeNPs with biofilm forming microorganisms

Overnight culture of biofilm- forming micro-organisms (1 ml) was incubated with FeNPs (100 µl) for 24 h at 37°C. After incubation optical density was determined at 600 nm.

Protein estimation of Biofilm-forming bacteria treated with FeNPs The ELISA plate was inoculated with 100 µl of an overnight culture of biofilm-forming bacteria and 10 µl of NPs. Overnight incubation at 37°C was done. The protein was estimated by Lowry's method in control and treated wells [15].

RESULTS AND DISCUSSION

Isolation of the biofilm- forming bacteria

To observe biofilm production potential of bacterial isolates, the CV assay is commonly used. This assay is preferred due to its simplicity, reliability, and rapidity. With this assay isolates can be categorized as high, moderate, or non-biofilm producers. The formation of biofilm comprises adsorption of macro- and micro- molecules followed by bacterial adhesion to the surface and biofilm maturation and colony formation. The 24 h incubation time helps in biofilm to be maturated and improved adhesion of biofilm on surfaces. [16]. *P. aeruginosa, B. subtilis,* and Sewage bacteria showed the initiation of biofilm formation after 24 h (Fig. 1). After 48 h under ×100 all three isolates showed an aggregate mass (Fig. 2). The biofilm grown toward the center was more than periphery which avoids the false artifacts of the "Edge Effect" phenomenon also [17]. CV is a basic dye that binds non-specifically to negatively charge surface molecules such as polysaccharides and DNA in the extracellular matrix. Because it binds cells as well as matrix components it is generally used to evaluate biofilm biomass in TOTO. Repeated experiments showed that these three microorganisms were biofilm producers.

Determination of antibiotic resistance of biofilm-forming bacteria

Biofilms protect the micro-organism by blocking the access of bacterial biofilm communities from antibiotics. Sewage bacteria were found to be more antibiotic-resistant than *Pseudomonas* (Table 1). The sewage

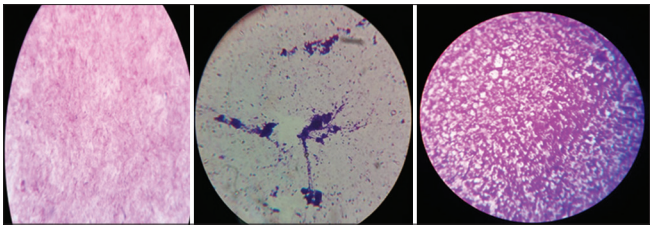

Fig. 1: Biofilm formation after 24 h under ×100

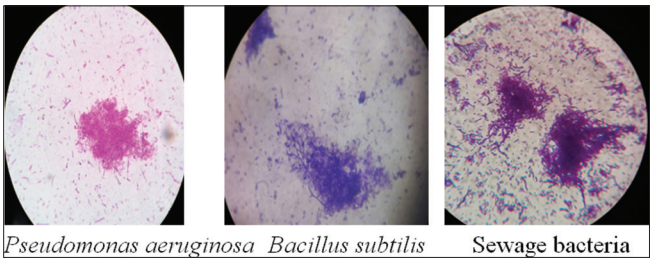

Fig. 2: Biofilm formation after 48 h under ×100

bacteria revealed resistance to 67% of the antibiotics used in this experiment whereas *Pseudomonas* bacteria showed 33% resistance. Biofilms are associated with the emergence of antibiotic-resistant bacteria probably because the extracellular polysaccharides released by biofilm bacteria act as a shield to prevent the entry of antibiotics. The literature said that the classes of antibiotics that are hydrophilic and positively charged, such as aminoglycosides, are more obstructed than others [18]. *Pseudomonas* was found to be sensitive to the antibiotics amikacin, ciprofloxacin, gentamicin, cefoperazone, lomefloxacin, and ceftazidime. Chemically, amikacin and gentamycin are aminoglycosides, ciprofloxacin and lomefloxacin are fluoroquinolones whereas cefoperazone and ceftazidime are cephalosporins class of antibiotics. The sewage bacteria showed sensitivity toward ciprofloxacin, gentamicin, lomefloxacin, and ceftazidime. Sewage bacteria being consortia of micro-organisms showed greater resistance than *Pseudomonas*. These bacteria when present in a group were great competitors and therefore showed more resistance than individuals.

B. subtilis showed resistance toward the majority of antibiotics (Table 2). This biofilm-forming bacterium has gained resistance for most of the antibiotics except cefuroxime, roxithromycin, and cefadroxil. Cefuroxime is second generation and cefadroxil is first generation cephalosporin antibiotic. Roxithromycin is a semisynthetic advanced generation macrolide antibiotic. The Gram-positive, spore former motile bacterium is a model organism to study biofilm formation. These bacteria are aerobes and form a white pellicle on the surface of a liquid medium. *B. subtilis* produces a wide array of antibiotics. It was reported that some of these antibiotics are nonribosomal peptides such as surfactin, bacillaene, fengycin, iturin, and bacilysin which these bacteria use it for their survival in a natural environment. *B. subtilis* produces some ribosomally synthesized peptide antibiotics, such as bacteriocins and other protein-derived toxins, which are generally effective against genetically similar bacteria and present in similar ecological niches [19]. *B. subtilis* showed 62% antibiotic resistance and intermediate toward Cefadroxil and Roxithromycin.

Preparation of FeNPs from plant extracts

After the addition of ferrous sulfate ($FeSO_4$) salt in the leave extracts of *P. pinnata,* the color of the solution changes from faint yellow to green indicating the synthesis of FeNP in the aqueous medium. These solutions were further analyzed for NP production.

Analysis of FeNPs

The EDAX profile of FeNPs showed the strong signal of the Fe atom indicates the crystalline property. The EDAX spectrum showed the elemental profile of FeNPs, primarily composed of C, O, S, and Fe. The C

and O are mainly from the compounds present in plant extracts, while Fe and S from the $FeSO_4$ precursor. The sharp peak showed FeNPs' production and % estimated to be 17.3% (Fig. 3).

FTIR spectrophotometric analysis of FeNPs

FTIR identifies various groups that involve the reduction and capping of NPs. FTIR spectroscopy measures the spectral peaks of functional groups. FeNPs spectra and absorbance bands have been observed in the region of 3419.28, 2110.30, 1645.46, 1011.64, 951.82, and 788.40 cm^{-1} which confirmed O-H group, alkyne group, amide (C = O), ether, alkene, and alkyl halide, respectively. Furthermore, adsorption bands at around 581 cm^{-1} correspond to the formation of FeNPs. This result indicates that the hydroxyl and phenolic groups are the active sites during the synthesis and hence, the O-H & C=C groups are involved in the reduction of $FeSO_4$ into FeNPs (Fig. 4).

SEM of FeNPs

FeNP were examined through SEM analysis to evaluate their morphology and their degree of dispersion. It indicated that FeNPs were agglomerated because of the adhesive nature. The morphology of SEM found to be irregular spherical structures. The average diameter of FeNP was found to be about 85 nm (Fig. 5a and b).

Treatment of FeNPs with biofilm- forming microorganisms

The positively charged NPs easily get attached to the surface of negatively charged bacterial cells that result in rupture of cell wall followed by cell death [20]. The lowest growth of *P. aeruginosa* biofilm bacteria was observed in the presence of FeNPs produced from 0.25 mg/ml of $FeSO_4$ salt whereas FeNPs of 0.5 mg/ml of $FeSO_4$ showed marginal inhibition when compared with control and FeNPs of 0.125 mg/ml of $FeSO_4$ had a lesser effect on the growth of these micro-organisms (Fig. 6). The antimicrobial

Table 1: Antibiotic sensitivity of *Pseudomonas* and Sewage bacteria

Antibiotic	Zone of inhibition (mm)	Zone of inhibition (mm)	S/I/R	Strength (µg)	Reference antibiotic (Zone of inhibition)		
	Pseudomonas aeruginosa	*Sewage Bacteria*			R	I	S
Amikacin (An)	30±0.02(S)	08±0.01(R)	S	30	≤14	15–16	≥17
Netilmicin(Net)	9±0.03(R)	0±0.01(R)	R	30	≤12	13–14	≥15
Cefadroxil (Cd)	0±0.0(R)	3±0.01(R)	R	30	≤14	15–17	≥18
Sparfloxacin(Sf)	20±0.02(I)	0±0.0(R)	I/R	5	≤15	16–20	≥21
Ceftriaxone (Ctx)	19±0.01(R)	13±0.01(R)	R	30	≤13	14–20	≥21
Ciprofloxacin (Cip)	27±0.03(S)	24±0.02(S)	S	5	≤15	16–20	≥21
Gentamycin(G)	20±0.02(S)	20±0.02(S)	S	10	≤12	13–14	≥15
Cefotaxime (Cf)	0±0.00(R)	0±0.0(R)	R	30	≤14	15–22	≥23
Cefoperazone(Cfp)	24±0.03(S)	7±0.01(R)	S/R	75	≤15	16–20	≥21
Lomefloxacin (Lm)	24±0.02(S)	26±0.02(S)	S	5	≤18	19–21	≥22
Ampicillin+Sulbactam (Slb)	0±0.0(R)	0±0.0(R)	R	25	≤13	14–16	≥17
Ceftazidime (Cpz)	24±0.04(S)	20±0.03(S)	S	20	≤14	15–17	≥18

*Antibiotic disk-diffusion method on MH agar and the zone of clearance was measured after the incubation period. Values are presented as mean±SD of the three triplicates of the experiments. SD: Standard deviation, S: Sensitive, I: Intermediate, R: Resistance

Table 2: Antibiotic sensitivity of *Bacillus subtilis*

Antibiotic	Zone of inhibition (mm)	S/I/R	Strength (µg)	Reference antibiotic (Zone of inhibition)		
				R	I	S
Amikacin (An)	23±0.03	S	30	≤14	15–16	≥17
Ciprofloxacin (Cip)	28±0.03	S	5	≤15	16–20	≥21
Clarithromycin(CLR)	0±0.00	R	15	≤13	14–17	≥18
Cefotaxime (Cf)	0±0.00	R	30	≤14	15–22	≥23
Sparfloxacin(Sf)	10±0.01	R	5	≤15	16–20	≥21
Cefuroxime(CR)	8±0.01	R	30	≤13	14–17	≥18
Cefoperazone(Cfp)	7±0.01	R	75	≤15	16–20	≥21
Ampiclox(ACX)	0±0.00	R	20	≤23	24–27	≥28
Cefadroxil (Cd)	15±0.03	I	30	≤14	15–17	≥18
Roxithromycin (RX)	15±0.03	I	15	≤13	14–17	≥18
Gentamycin(G)	0±0.00	R	10	≤12	13–14	≥15
Azithromycin (AZ)	0±0.00	R	15	≤13	14–17	≥18

Antibiotic disk-diffusion method on MH agar and the zone of clearance was measured after the incubation period. Values are presented as mean±SD of the three triplicates of the experiments. SD: Standard deviation, S: Sensitive, I: Intermediate, R: Resistance

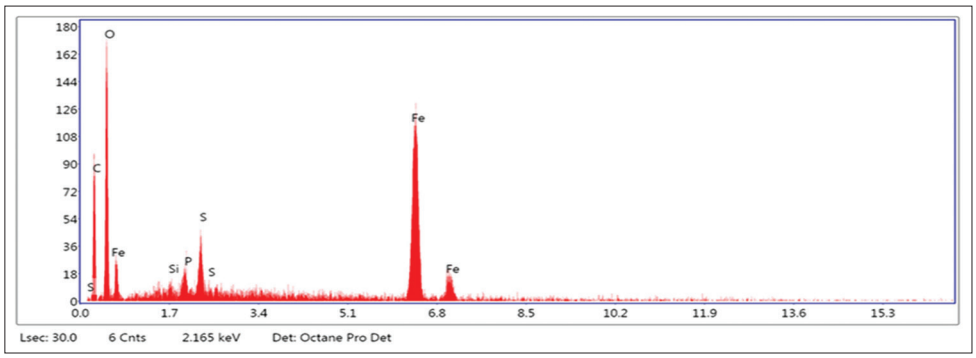

Fig. 3: Energy dispersive analysis of X-ray diffraction spectroscopy of iron nanoparticles

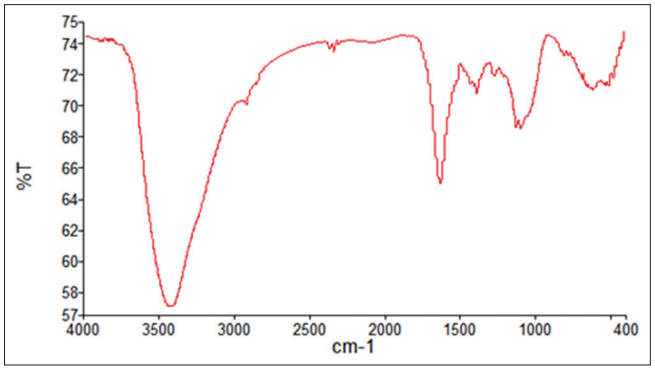

Fig. 4: Fourier transform infrared spectrophotometer of iron nanoparticles

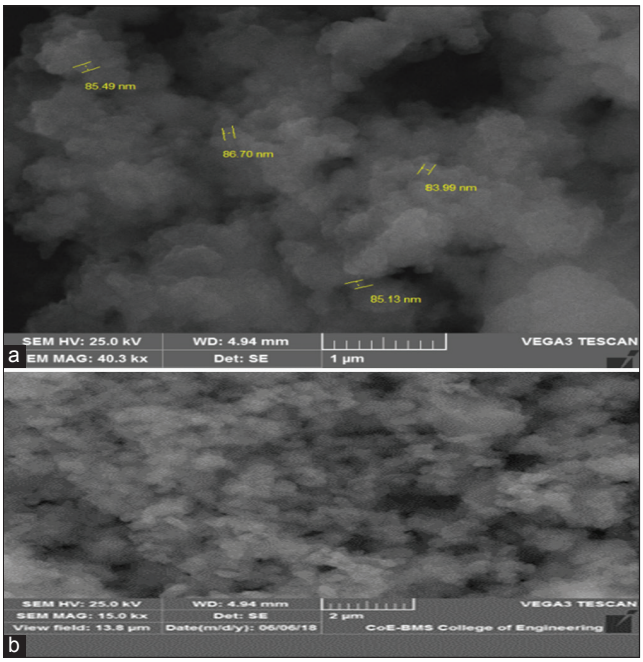

Fig. 5a: Scanning electron microscopy of iron nanoparticles (a) the size ranges of nanoparticles at 40.3 kx. (b) at the magnification of 15.0 kx

activities of FeNPs on *B. subtilis* showed with both 0.25 mg/ml and 0.5 mg/ml $FeSO_4$ salt-producing NPs. The sewage bacteria were not inhibited much with FeNPs to control. Therefore, this study revealed that the FeNPs when produced from aqueous extract of leaves of *P. pinnata* it could effectively inhibit the biofilm-forming *P. aeruginosa* and *B. subtilis*. This study of the antimicrobial effect was according to the report given in the literature [21]. Probably with 0.125 mg/ml of salt concentration could not be converted by leaf extracts into effective NPs and therefore the antimicrobial activity was insignificant to both Gram-positive and Gram-negative bacteria. However, the effect of 0.25 mg/ml of salt concentration FeNPs showed that better inhibitory effect which could be due to smaller NPs has better penetration and accumulation through a bacterial cell wall.

Protein estimation of biofilm- forming bacteria treated with FeNPs

The extracellular and intracellular protein concentration when measured in the presence of FeNPs it was observed that the intracellular protein was greatly reduced in *P. aeruginosa* and *B. subtilis* compared to control (Fig. 7). In both, the situations extracellular protein concentration was increased in the presence of FeNPs. The study of both extracellular and intracellular protein concentration and FeNPs effect was reported the 1st time in this work. Probably, the FeNPs bonded to the cell wall of both Gram-positive and Gram-negative bacteria which, in turn, increased uptake of

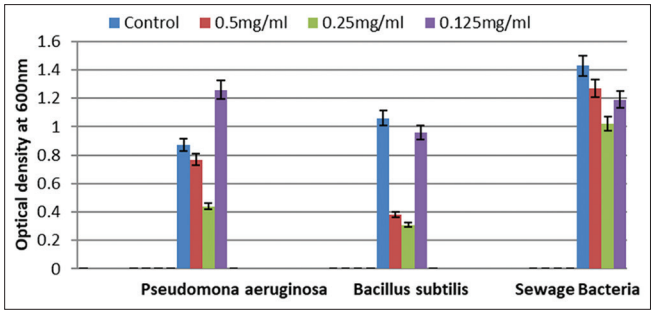

Fig. 6: Effect of iron nanoparticles on Biofilm Bacteria. *All the data were reported as the mean standard error of three replicates (n=3). Control: Only bacteria OD600 of 1.0 is roughly 3×107 cells/ml

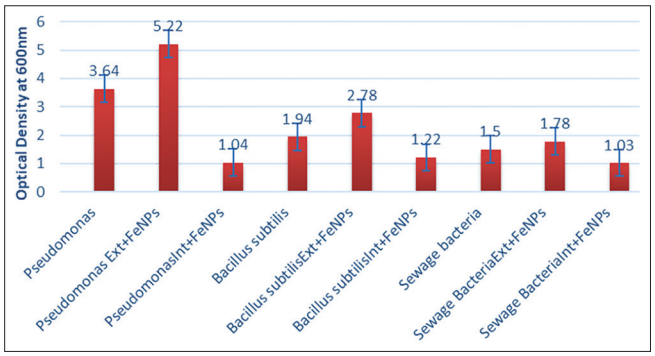

Fig. 7: Effect of iron nanoparticles on protein concentration of biofilm- forming bacteria. *All the data were reported as mean± standard error of three replicates (n=3). Control: Only bacteria OD600 of 1.0 is roughly 3×107 cells/ml

ions leads to intracellular damage. The binding capacity of FeNPs to the Gram-negative cell wall is more due to extra lipopolysaccharide layer and therefore more leakage of extracellular proteins. Although the exact mechanism of action of FeNPs is not known, probably smaller molecules of FeNPs penetrate better through the cell wall and cell membrane of bacteria and inhibit translation process of bacterial cell [22].

CONCLUSIONS

An eco-friendly and economic green FeNP were synthesized from aqueous extracts of *P. pinnata*. In this study, the biofilm-forming bacteria *P. aeruginosa, B. subtilis,* and Sewage bacteria were isolated and identified as resistant to multiple antibiotics. The green FeNP efficiently inhibited the growth of these biofilm-forming bacteria. These NPs showed an inhibitory effect on protein synthesis of bacteria, making these NPs as an effective molecule to treat biofilm forming micro-organisms. The exact mechanism of protein synthesis should be elaborated in the future. Finally, this is a vital area of research that deserves our attention because of its potential application against multidrug- resistant micro-organisms.

AUTHORS' CONTRIBUTIONS

This research work has carried out in the Department of Microbiology Laboratory, Vijaya College, India. The EDAX, FTIR, and SEM work has carried out in BMS Engineering College, Bengaluru.

CONFLICTS OF INTEREST STATEMENT

The authors declared that they have no conflicts of interest.

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Nil.

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