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Research paper

Multimetallic Fe-Cr-Ni Nanocomposites with Propitious Antibacterial Effects on Hospital Acquired Bacterial Pathogens: A Promising Substitute to the Conventional Antibiotics

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ABSTRACT

Present study was aimed to synthesize, characterize, and explore antimicrobial potential of multi-metallic nanocomposites as an alternative of existing antimicrobial agents to deal with the emergence of drug resistance and superbugs. Fe-Cr-Ni nanocomposites were prepared by a wet chemical method i.e., sol-gel technique. Characterization of formulated nanocomposite was then made based on Fourier Transformed Infrared (FTIR) Spectroscopy, Energy Dispersive X-Ray Spectroscopy (EDS), X-Ray Diffraction (XRD) and Scanning Electron Microscopy (SEM) analysis. Antibacterial effect of Fe-Cr-Ni nanocomposite was evaluated against clinically significant bacterial cultures using agar well diffusion assay. Moreover, Minimum inhibitory concentration was also estimated by tube dilution method and then bactericidal concentrations was also determined. Oxygen, Iron, Nickel and Chromium bonded with metal oxide interactions were the major constituents of nanocomposite with an average size of 86.0 nm as affirmed by SEM. The XRD analysis revealed cubic spinel structure of nanocomposites with Debye-Scherrer crystallite size of 48.76nm. Antibacterial effect of Fe-Cr-Ni nanocomposite was observed against distinguished multi drug resistant nosocomial pathogens i.e., *S. aureus* and *P. aeruginosa*. Furthermore, 150µg/ml of Fe-Cr-Ni nanocomposites was established as the optimized inhibitory concentration for both sensitive strains. The MIC was found as 0.5 mg/ml whereas concentration of > 0.5 mg/ml i.e., 1.0 mg/ml was identified as MBC for both sensitive strains. Hence, Fe-Cr-Ni nanocomposites can be employed in topical formulations as an inexpensive and easily maintainable therapeutic agent in the prevention as well as treatment of various nosocomial infections caused by bacterial resistant mutants or superbugs.

KEYWORDS Agar well diffusion assay, antimicrobial activity, Minimum Bactericidal Concentration, Nanocomposites, Sol-gel technique.

INTRODUCTION

During the last two decades the emergence and re-emergence of multidrug resistant pathogens attributed to haphazard, continual, and inappropriate consumption of conventional antibiotics, antivirals, antiseptics, and biocides has become a serious hazard for well-being of community worldwide [1,2]. The drug resistant microorganisms, which are also known as superbugs are likely to grow and endure the presence of antimicrobials and pose an ever-growing challenge in the search for viable antimicrobial treatments. As indicated by a research report, around 700 000 people lost their lives in the course of 2017 by reason of infections caused by multidrug resistant pathogens and number of mortalities due to antimicrobial resistance has been estimated to reach beyond 10 million deaths in the following next 35 years [3]. Therefore, the prerequisite to develop new antimicrobial agents has become imperative owing to medical as well as economical concerns. Amongst several bacterial strains, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* has been recognized as notorious multiple-drug resistant opportunistic pathogens, accounting for 80-87 % of critical nosocomial infections such as pneumonia, urinary tract, septicemia and has also been associated with the formation of biofilms on medical instruments [4,5].

Over the last few years, expansion in the field of nanotechnology with the potential applications of numerous synthetic nano-scale materials (nanoparticles, nano powders, nanocomposites, nanofibers, nanotubes etc.) has been viewed as prime way out to combat issue concerning pathogen cessation. Among various advanced functional nanomaterials, nanocomposites i.e., solid multi-element substances characterized of having at least one of the elements with a dimension of less than 100 nanometers, has gained attraction among the researchers as a prospective option over conventional antimicrobial agents in consequence of their relatively extended shelf-life and stability at elevated ranges of temperature and pressure. Moreover, enhanced synergistic effect of combination of metals in the form of nanocomposites, e.g., CdO-NiO-ZnO, Pt-Bi-FeO_3, CuO-TiO_2, Cd-Ag-ZnO and α-Fe_2O_3-CO_3O_4 have also been reported for enhanced antibacterial properties against various bacterial species [6,7]. Due to relatively smaller size as compared to bacterial cells, nanoparticles may easily penetrate within the cellular structures to inhibit bacterial growth and metabolism [8]. Therefore, it is crucial to find some novel nanocomposites with enhanced antibacterial activity. A thorough review of literature indicated an information gap related to the antibacterial action of trimetallic-nanocomposites.

The current study is based on the facile synthesis of Fe-Cr-Ni nanocomposites by sol-gel technology method (wet chemical method) that entails the dissolution of molecular precursor (usually metal alkoxide) in water or alcohol and its ultimate conversion into gel by hydrolysis/alcoholysis. In comparison with other methods of nano-synthesis, it is relatively convenient, inexpensive also beneficial for fabricating high quality materials with greater homogeneity and purity at lower temperatures followed by its characterization and evaluation of antimicrobial action on clinically significant microbial strains. This research will be helpful in dealing with bacterial and fungal contamination in various materials providing new strategies towards a healthier environment for human beings.

MATERIALS AND METHODS

Transition metal precursors, sodium hydroxide, hydrochloric acid with ferric nitrate, nickel nitrate and chromium nitrate

were purchased from Merck and used as received. Nutrient Broth (Oxoid) and Sabouraud's dextrose broth (SDB, Oxoid) were used as cultivation media for bacterial and fungal strains, respectively. Technical agar (Oxoid) was also used to solidify the broth medium, when required in the experiments.

For the typical synthesis of Fe-Ni-Cr nanocomposites, 5 millimoles of Fe $(NO_3)_2$, Ni $(NO_3)_2$, and Cr $(NO_3)_2$ were mixed in 100 mL of deionized water. After dissolving all the precursor salts, drop wise addition of 1.5M sodium hydroxide was carried out until pH of the reaction mixture turned to 12. Following that, mixture was subjected to magnetic stirring at 85^{o}C with 200 rpm for 45 minutes. The particles were then separated from the solvent, dried at 100 ^{o}C and kept in desiccators for the further studies [9].

Their characterization was performed by Scanning Electron Microscopy (SEM), X-Ray Diffraction (XRD), Energy Dispersive X-Ray Spectroscopy (EDS) and Fourier Transformed Infrared Spectroscopy (FTIR). The characterization results were observed in Instrumental laboratory of the Department of Chemistry and the Centralized Science Laboratories (CSL), University of Karachi, Pakistan.

To prepare Potassium bromide (KBr) disc for the Fourier Transformed Infrared Spectroscopy (FTIR), the nanocomposites were subjected to 50^{o}C for 6 h to have a completely dried sample. KBr disc was concocted by mixing nanocomposites with KBr and pressing it with bench press. This KBr disc was used to extract qualitative information about the nanocomposites in the range of 4000cm^{-1}-400cm^{-1}.

Scanning Electron Microscopy (SEM) is one of the most widely used characterization techniques to extract information regarding the topography of nanocomposites. The nanocomposites were thoroughly dried, and discs were prepared for SEM analysis. Surface information was acquired by Jeol JSM 6380A Japan [10].

Qualitative and quantitative information about the nanocomposites was also acquired by Energy Dispersive X-Ray spectrum (EDS) which was obtained by Jeol JSM 6380A Japan microscope. The samples were inserted in vacuum chamber after mounting and coating with a conducting film of gold and EDS spectrum was recorded. EDS revealed the elemental composition of the synthesized nanocomposites [10].

X-Ray Diffraction (XRD) analysis was performed by Jeol JDX-3532, Japan. The nanocomposites samples were placed on the sample holder. Flat surface was ensured by pressing the nanoparticles. The diffraction peaks were observed within 2θ values of 50-70^o [11].

Pure cultures of three Gram-positive bacteria i.e., *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus subtilis* and seven strains of Gram-negative bacteria i.e. *Pseudomonas aeruginosa*, *Escherichia coli*, *Vibrio cholera*, *Klebsiella pneumonia*, *Proteus mirabilis*, *Acinetobacter spp.* and *Salmonella typhi* were used as indicator strains to evaluate antibacterial effect of synthesized nanocomposites. Pure cultures of four saprophytic fungi (molds) i.e., *Aspergillus terreus*, *Aspergillus niger*, *Aspergillus flavus*, *Penciillium spp.* and three strains of yeast fungi namely *Candida albicans*, *Candida tropicalis* and *Candida kefyr* were also used to determine the antifungal nature of test compound.

The entire indicator strains were refreshed, subcultured into their respective broth medium and incubated for one hour at 37◦C. After completion of incubation period, the turbidity of inoculated culture tubes was adjusted as per 0.5 McFarland standards (10^6CFU/ml). Broth suspension of bacterial and fungal cultures were spread on to the surface of respective Nutrient agar and Sabouraud's dextrose agar medium by using sterile nontoxic cotton swabs under aseptic conditions. Wells (diameter = 6mm) were punched in the agar medium with the help of sterile cork-borer and dispensed with 100 µl of the nanocomposites as test

samples. Dimethyl Sulfoxide (DMSO) was used as negative control, while chloramphenicol (antibacterial drug) and Nystatin (antifungal) were used as positive controls. The plates were incubated for 24 h at 37 ◦C and 168 h at 28 ◦C for bacterial and fungal growth, respectively [12]. After completion of incubation period, zones of growth inhibition (area where indicator microorganism unable to grow due to susceptibility to the compounds present in sample) around the wells were observed and recorded in millimeter.

To determine the impact of concentrations of Fe-Cr-Ni Nanocomposites on bacterial growth dynamics, different concentrations of Fe-Cr-Ni nanocomposites (0, 50, 100, 150, 200 and 250µg/ ml) were prepared by diluting the stock solution of nanocomposites with pre sterilized nutrient broth. All the flasks were inoculated with 100 µl of overnight broth suspension of sensitive bacterial strains and incubated at ambient temperature under shaking condition (120 rpm) whereas, un-inoculated flasks served as control [13]. Two milliliters of the culture medium from each of the flask was withdrawn after every 24 h and analyzed spectrophotometrically at 600 nm for estimation of growth [13].

Minimum inhibitory concentration (MIC) was evaluated by dilution method. The nanocomposite having potent antimicrobial effect was serially diluted (1.0 mg/ml to 0.15 mg/ml) in sterile nutrient broth, aseptically using micropipette. All the tubes excluding negative control tube were inoculated with 1 ml of the broth suspension of each of bacterial strains i.e., *Ps. aeruginosa* and *S. aureus* and incubated overnight at 37◦C. After incubation period, all the tubes were analyzed for the turbidity of the medium as indication of bacterial growth in medium.

Minimum bactericidal concentration (MBC) was determined by inoculating 0.1 ml of the content of tubes showing no visible turbidity in MIC experiments on the surface of nutrient agar plates. All the plates were subjected for incubation of 24 h at 37◦C and were observed for the bacterial growth.

RESULTS AND DISCUSSION

Characterization of nanocomposite was done by using following techniques:

FTIR spectrum is provided in Figure-1 (a). The peak at 439.77cm^{-1}may be ascribed to Ni-O bond vibration [14]. Absorption at 582.50cm^{-1}corresponds to Fe-O bond vibration [15]. Another maximum at 673.16cm^{-1}corresponds to Cr-O bond vibration. This type of bond presents its another peak which might correspond to its overtone at 1116.78cm^{-1} as described in the literature [16]. The peak at 1384.89cm^{-1} and 1635.64cm^{-1} were due to the presence of adsorbed CO_2 and bending of water molecules present in the sample. The superficial OH groups are responsible for the wide peak at 3450.65cm^{-1}[17].

SEM analysis revealed the homogeneity in size and shape of the nanocomposites. SEM provided the average diameter of the nanocomposites which was 86.0nm. Figure-1 (b) provides the SEM image of the nanocomposites.

EDS analysis indicated that oxygen was the chief constituent of the nanocomposites. It was also indicated by the spectrum that the nanocomposites contain iron, nickel, chromium in significant quantity along with a very little amount of sodium, chlorine, and carbon. These contaminations may be due to the presence of filter paper fibrils and utilization of sodium hydroxide in the preparation of nanocomposites. Figure-1 (c)provides the EDS analysis of Cr-Fe-Ni nanocomposites.

XRD shows 2θ maxima at 24.65, 36.35, 44.9, 50.45, 63.95, 64.95, 30.05, 35.35, 43.0, 43.2, 53.1, 53.7, 57.0, 62.85, 44.4, and 51.55.The corresponding hkl values were (1 1 1), (2 0 0), (2 1 1), (3 0 0), (3 1 0), (2 2 2), (2 2 2), and (2 2 2) which indicated cubic spinel iron oxide structure (JCPDS card number 19-629) [17], (1 1 1), (2 1 1), (3 1 0), (2 2 2), (3 3 0), and (3 3 1) for highly face centered cubic crystalline Cr_2O_3[18]and (1 1 1) and (2 0 0) for face-

centered nickel oxide nanoparticles [19]. Debye-Scherer relation was employed to assess the average crystallite size which was determined to be 48.76nm with a standard deviation of 18.10nm. X-Ray Diffractogram and Debye-Scherer size distribution are provided in Figure-1 (d,e)

The antimicrobial efficacy of synthesized nanocomposites was screened against various Gram-positive and Gram-negative bacterial strains by Well diffusion method involving the dispersal of test compound from the well (region of higher concentration) to the periphery (region of lower concentration), through the agar matrix. Although, this method has been considered as laborious and time consuming, still it is preferred over disc diffusion method due to several reasons, mainly as an ideal technique for the analysis of non-oil-based compounds, as well as, unlike disc diffusion method there is no interaction between molecules and filter paper disc, rendering it an economical and easy way to interpret the results.

The results of agar well diffusion assay (Figure-2 a) clearly indicated that synthesized nanocomposites were found active against two Gram positive strains i.e., *S. aureus* which is usually the flora of human skin and may cause opportunistic infections) [20] and *B. subtilis*, a well-known environmental contaminant and food spoiler. These findings were quite unexpected since genus Bacillus is characterized by the ability to produce highly resistant dormant structures (endospores) in their cytoplasm that enables them to survive even under harsh and unfavorable environmental circumstances.

However, test compounds exhibited no antibacterial effects on *M. luteus*, a dominant flora of human skin and rarely associated with opportunistic infections [21]. In this regard, it is reasonable to state that nanocomposites were unable to interfere with microflora which renders them appropriate to be used in clinical formulations and biomaterials.

The data regarding the antibacterial (Gram negative) screening of synthesized nanocomposites revealed *Ps. aeruginosa* as the most susceptible to Fe-Cr-Ni nanocomposites (Figure-2 b). *Ps. aeruginosa*, a notorious multi drug resistant nosocomial pathogen as well as a well-known biofilm producer, considerably associated with life threatening illnesses such as cystic fibrosis.

However, same compounds were also found to exhibit moderate level of inhibitory effects on *Proteus mirabilis* (causing a wide range of urinary tract and nosocomial infections), *Acinetobacter spp.* (virulent strains are associated with hospital acquired infections), *Vibrio cholera* (causative agent of cholera), *Salmonella typhi* (etiological agent of enteric illnesses such as typhoid).

Though the exact antibacterial mechanism of tri metallic nanocomposites has not yet completely elucidated. Several studies anticipated that antibacterial effect of nanocomposite is the resultant of several phenomena which include alteration in bacterial metabolic system, generation of nanocomposites induced pits in bacterial plasma membrane that influence cell permeability, alteration in cellular electrolyte balance as well as in the expression of genes, formation of DNA lesions and everlasting denaturation of cellular enzymes may eventually result in cell lysis [22].

On the other hand, rest of the tested Gram-negative strains namely, *E. coli* (virulent strain, associated with urinary tract infections, gastroenteritis and nosocomial infections), and *K. pneumonia* (tends to cause pneumonia, septicemia, and biofilm formation) were found insensitive to the nanocomposites examined. It is believed that complexity in cell wall (Gram negative cells) in terms of chemical composition and structural organization enables them to resist the effects of antimicrobials. Zones of growth inhibition of *S. aureus* and *P. aeruginosa* by Fe-Cr-Ni nanocomposites are depicted in Figure-2.

Fe-Cr-Ni nanocomposites were also tested against various fungal species like *A. flavus, A. niger, A. terreus, Penicillium spp.* (mold fungi) and *C. albicans, C. tropicalis, C. kefyr* (yeast fungi) revealing no inhibitory effects.

The effect of different concentrations of Fe-Cr-Ni nanocomposites on the growth kinetics of the most sensitive test bacterial strains i.e., *S. aureus* and *P. aeruginosa* was also estimated. Five different concentrations of nanocomposites were selected i.e., 50, 100, 150, 200 and 250 µg/ml whereas, 0 µg/ml concentration used as a negative control. It was speculated that in any respect the concentrations of Fe-Cr-Ni nanocomposites exhibited bactericidal effect against *S. aureus* at 72 h (Figure-.3). All the concentrations of nanocomposites showed inhibitory effects as compared to control, but the optimum concentration was found to be150 µg/ml (bacterial growth was pent-up at this concentration). Though, in case of *Ps. aeruginosa*, all the concentrations of nanocomposites studied demonstrated inhibitory effects at 72 h (Figure-4), however, 150µg/ml was found as the most favorable inhibitory concentration for the sample of nanocomposites.

In the next phase, minimum inhibitory concentration (MIC) of the potent antibacterial nanocomposites sample was also determined against *Ps. aeruginosa* and *S. aureus*. MIC is generally defined as the highest dilution of an antimicrobial (mg/ml) that arrests the visible microbial growth and reproduction but does not necessarily exert biocidal effect after overnight incubation. It is regarded as a monetary standard to evaluate the susceptibility of microbes to the active concentrations of antimicrobial agents. Fe-Cr-Ninanocomposite sample at MIC value of 0.5 mg/ml and 1 mg/ml could cease the growth of *S. aureus* and *P. aeruginosa*, respectively, in vitro conditions.

Minimum bactericidal concentration (lower most concentration of antimicrobial that will cease the growth of *P. aeruginosa* / *S. aureus* after sub culturing into antimicrobial free medium) was also determined and registered as > 0.5 mg/ml i.e., 1.0 mg/ml for both tested strains.

CONCLUSION

In the current study, Fe-Cr-Ni nanocomposites were prepared and then characterized by FTIR, SEM, EDS and XRD. These techniques affirmed the presence of iron, chromium and nickel oxide bonds. The nanocomposites were found homogeneous in nature with an average diameter of 86.0nm. They had cubic spinel and face center cubic structure with an average Debye-Scherrer crystallite size of 48.76nm with standard deviation of 18. 10nm.These synthesized nanocomposites showed tremendous efficacy against the eminent nosocomial pathogens and well-known biofilm producers like *S. aureus* and *Ps. aeruginosa*. In future, this research would be facilitating in preventing the adhesion and colonization of pathogens on medical devices such as syringes, catheters, infusion pumps, endotracheal tubes, and prosthetics through the application of Fe-Cr-Ni nanocomposites based antimicrobial coatings or its incorporation the materials that will results in the release of biocides into the biofilm or in contact killing.

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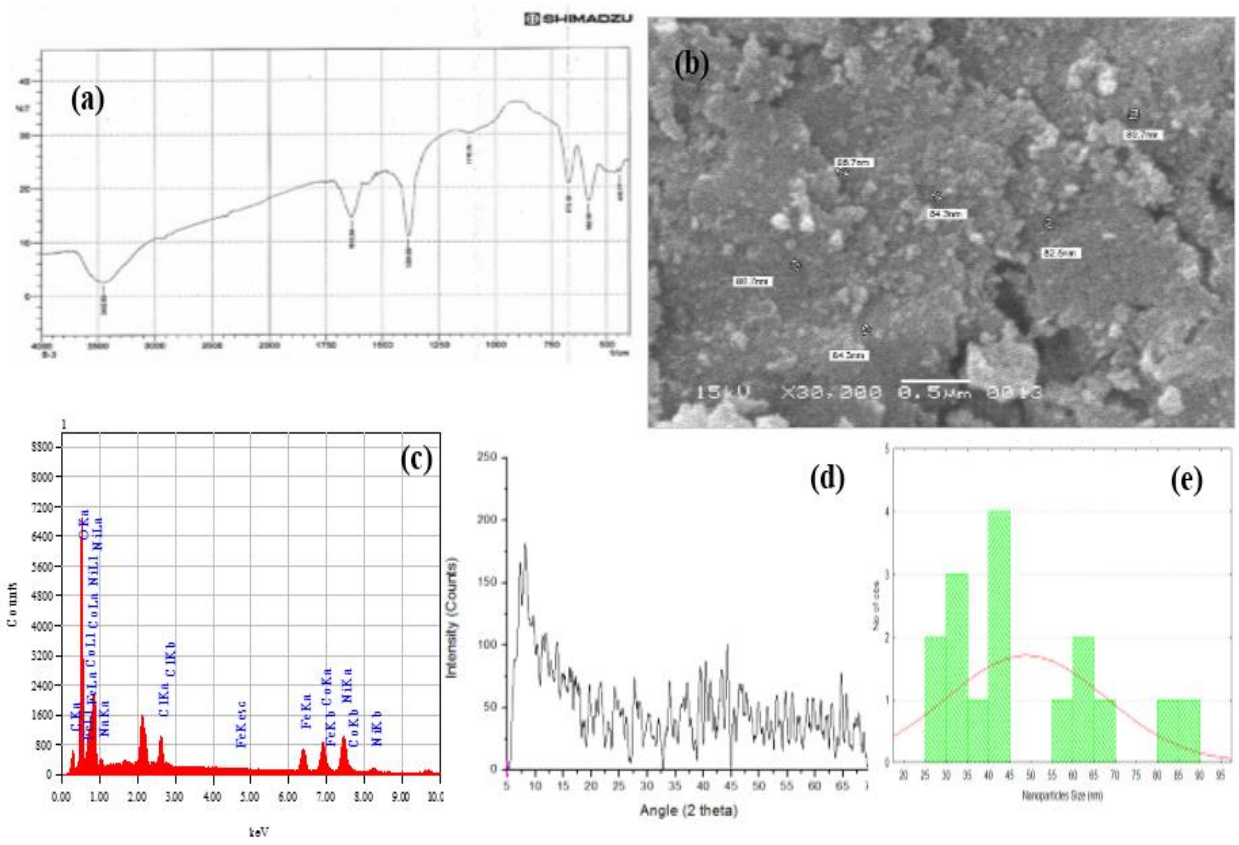

Figure 1: (a) FTIR Spectrum (b) SEM Image (c) EDS Spectrum (d) X-Ray diffractogram (e) Debye-Scherer size distribution of Fe-Cr-Ni Nanocomposites.

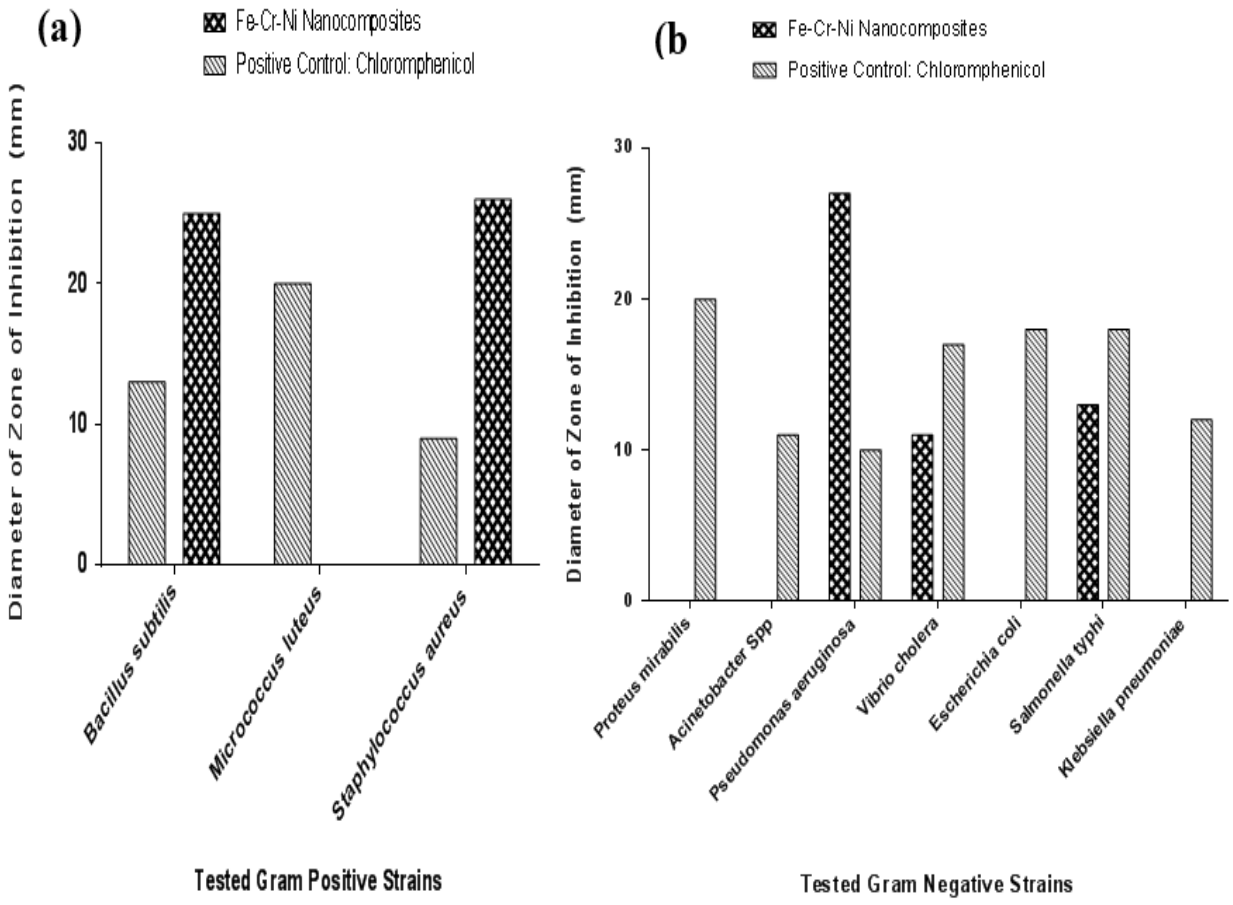

Figure 2: Antimicrobial Potential of Fe-Cr-Ni Nanocomposites against Tested (a) Gram Positive and (b) Gram Negative Strains

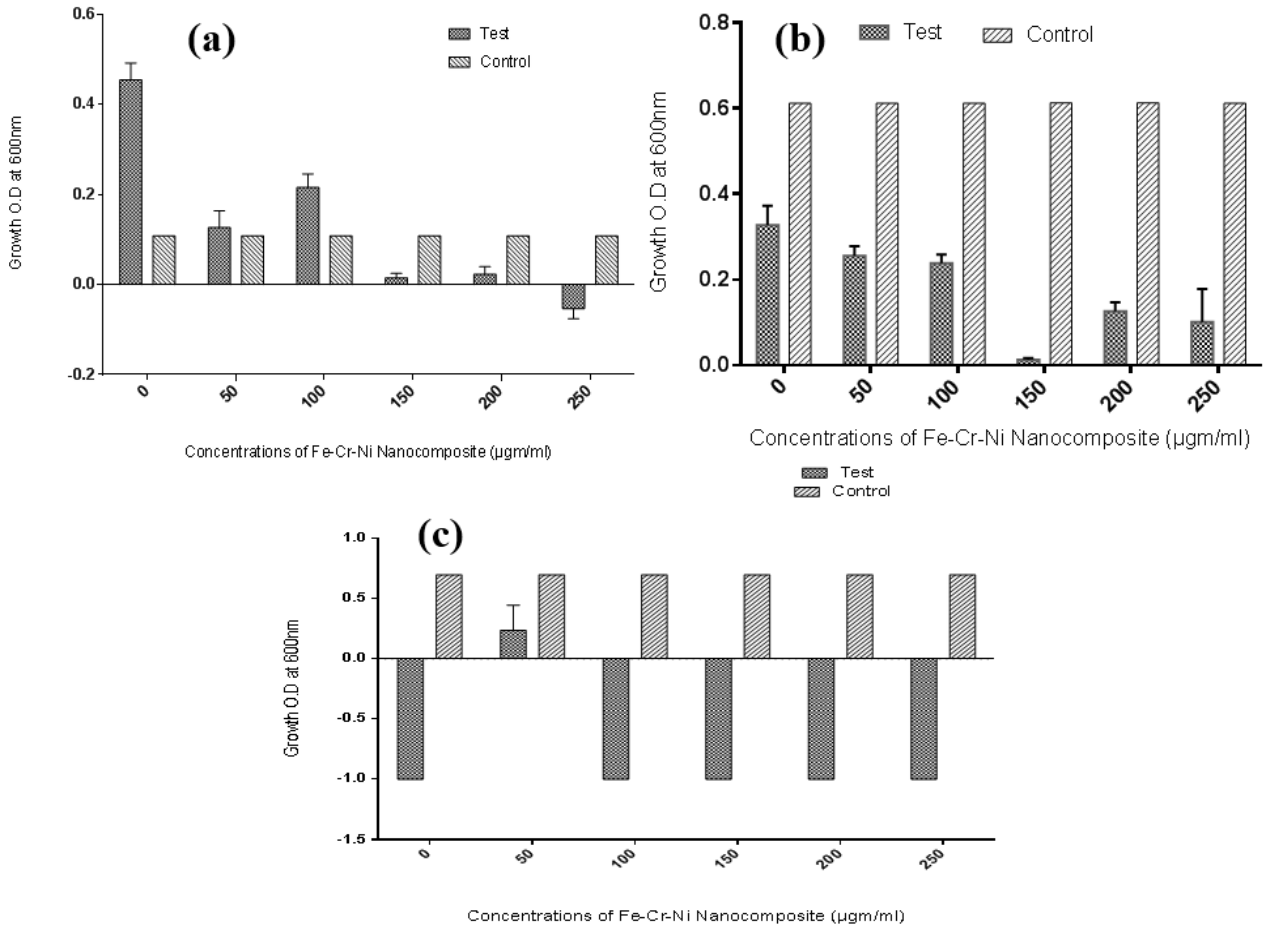

Figure 3: Effect of Different Concentrations of Fe-Cr-Ni Nanocomposites of Growth Kinetics of *S. aureus* at (a) 24(b) 48 and (c) 72 h of Incubation Period

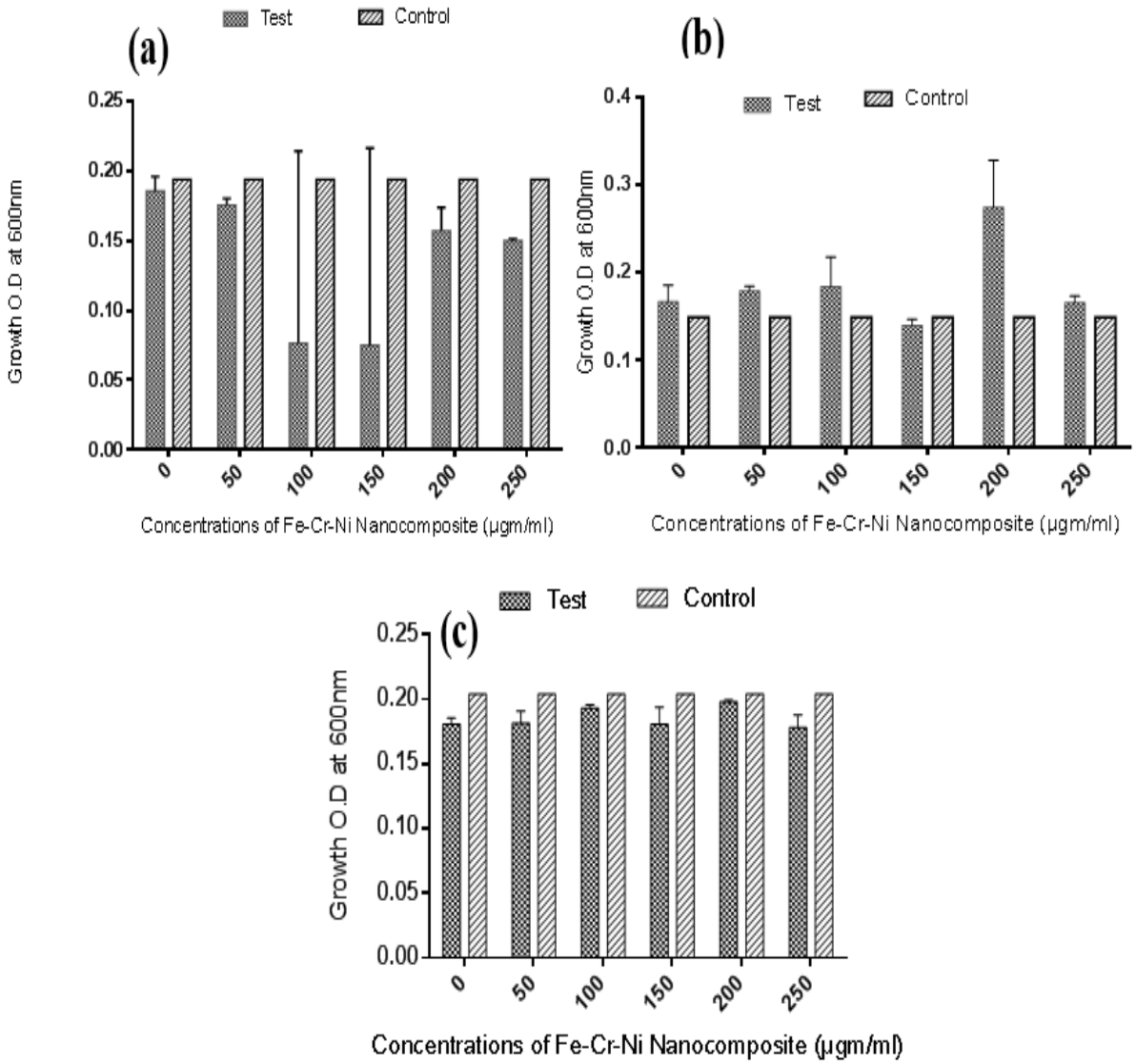

Figure 4: Effect of Different Concentrations of Fe-Cr-Ni Nanocomposites of Growth Kinetics of *Ps. aeruginosa* at (a) 24(b) 48 and (c) 72 h of Incubation Period

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