Growth Inhibitory Effect of Oven Dried Copper Nanoparticles (CuNPs) on Drug Resistant Clinical Isolates

U. Rasool, S. Kumar Sah and S. Hemalatha *

* *hemalatha.sls@bsauniv.ac.in*

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[1] School of Life Sciences, B. S. Abdur Rahman Crescent University, Chennai, India.

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Abstract: *Drug resistant pathogenic microbes have been causing serious health issues resulting in the substantial increase of death rates and morbidity paving the way for nanoparticles to be utilized as antimicrobial agents. This study was performed to evaluate the effectiveness of CuNPs on the growth of drug resistant clinical isolates of Streptococcus pyogenes, Enterococcus faecium and Enterococcus faecalis. Minimum inhibitory concentration of CuNPs against Streptococcus pyogenes, Enterococcus faecium and Enterococcus faecalis was found to be 1.25. 1.25 and 0.625 mg/ml and minimum bactericidal concentration against the same isolates was found to be 2.5, 2.5 and 5 mg/ml respectively. The ratio of MBC/MIC, referred to as tolerance level, was calculated for all the isolates which signifies the bactericidal or bacteriostatic effect of any antimicrobial agent. For Streptococcus pyogenes and Enterococcus faecium, the tolerance level was 2 while as for Enterococcus faecalis, it was 8. Antibiotic susceptibility results were calculated which showed that the isolates were resistant to Ampicillin (10 µg), Amoxycillin (30 µg) and Aztreonam (30 µg). Susceptibility results were followed by calculating multiple antibiotic resistance indices (MARI). MARI is an important tool which gives an idea about the bacterial resistance in a given population. For all the three isolates, MARI results were equivalent to 1 because of their resistance towards all the three antibiotics used. Antimicrobial activity through well-plate method was carried out and inhibitory effect of CuNPs on biofilm formation was evaluated.*

Keywords: *Clinical isolates, Drug resistance, Metal nanoparticles, CuNPs, Antimicrobial, Biofilm.*

1. INTRODUCTION

Resistance to antibiotics, particularly in hospitals, is a global and an intense clinical complication which is further intensified by the scarcity of new therapeutic agents that block resistance mechanisms [1]. Continuous investigations and research to find novel, harmless and effective antibiotic formulations as alternative agents to substitute the less effective ones will result as a means of decreasing the rate of antibiotic resistance [2, 3]. Multi-drug resistant species of bacteria have become a serious threat and a major health issue. As the evidence of bacterial resistance and the cost of advanced antimicrobial drugs is increasing day by day, it has boosted the interest of scientists towards the search for an effective and economic source to cope with the problem, hence novel antimicrobial formulations to combat resistant pathogens is urgently needed [4].

An important and effective approach of nanotechnology is its ability of modifying metals into their nanosize [3] which further present unique and diverse approaches to investigate and regulate a wide range of biological and medical processes that take place at nanometer scale. Nanoparticles offer a potential approach in comparison to the conventional materials in controlling the bacterial growth due to their high reactivity and large surface to volume ratio [5, 6]. The past few years have been dedicated to noble metal nanoparticles because of their diverse range of applications in various biological fields. Compared to other noble metals such as platinum, gold and silver, copper is an economical and easily available metal. This study provided an insight towards the antibacterial effect of copper nanoparticles (CuNPs) synthesized via the assistance of marine actinomycetes on three drug resistant isolates. Nanoparticles synthesized using copper as a metal are of great interest to scientists because of their unique biological applications particularly as antimicrobial agents and the low cost of production. There are certain restrictions that limit the applications of nanoparticles from copper, such as the rapid oxidation upon

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exposure to the air [7, 8]. There have been reports about the synthesis of nanoparticles via actinomycetes such as the synthesis of gold nanoparticles by a novel extremophilic actinomycete, *Thermomonospora* and alkalotolerant actinomycete, *Rhodococcucs* species [9, 10]. One more study has reported the biological synthesis of zinc nanoparticles using actinomycetes for antibacterial food packaging [11].

This particular study was carried out to study the effect of CuNPs, synthesized via the assistance of marine endophytic actinomycetes, on the growth of three different drug resistant clinical isolates of *S. Pyogenes, E. Faecium and E. Faecalis.*

2. EXPERIMENTAL PROCEDURE

2.1. Microbial Strains and CuNPs

The clinical isolates were obtained from Tagore Medical College and Hospital, Chennai, India after clearing all the ethics. CuNPs used in this study were previously synthesized through the extracellular method with the assistance of marine actinomycetes, characterized and assayed for antibacterial activity against 5 different human pathogenic bacteria [7]. CuNPs used in this study were dried in hot air oven at 60 °C. For the dissolution of CuNPs, distilled water was used as a solvent and different concentrations were prepared.

2. 2. Media Preparation and Autoclaving

The chemicals used in this study were of analytical grade. Luria-Bertani broth was used as a liquid medium and Luria-Bertani agar was used as a solid medium to grow all the clinical isolates. Before proceeding for any inoculation, all glass-wares and media were autoclaved at 121 °C for 15 minutes to avoid any sort of contamination.

2. 3. Culture Maintenance

Cultures used in this study were maintained through slants and glycerol stocks. Continuous sub-culturing was carried out to carry out the experimentations. Slant cultures were stored at 4 °C and were intermittently transferred to fresh media for proper maintenance. Glycerol stocks were maintained at -80 °C [12].

2. 4. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC and MBC were proceeded through the method followed by Dash et al. (2012) with slight modifications [13]. Serial microdilution was employed to determine the MIC values of CuNPs against the clinical isolates of *S. Pyogenes, E. Faecium and E. Faecalis*. CuNPs were serially diluted in triplicates with Luria-Bertani broth in 96-well microplate to make a final of five different concentrations (5, 2.5, 1.25, 0.625 and 0.3125 mg/ml). 10µl fresh culture of all the three clinical isolates (adjusted to 0.5 McFarland standard) was added to each well. Well with Luria-Bertani broth inoculated with isolate was used as a negative control, well with Luria-Bertani broth inoculated with isolate with added antibiotic (Ampicillin, 10 µg) was kept as a positive control and well with Luria-Bertani broth alone was used as a blank. 96-well microplate was incubated at 37 °C and was visually observed after 24 hours. MIC was defined as the lowest concentration of CuNPs required to inhibit the growth of isolates.

MBC of CuNPs was determined by the standard method according to Dash et al. (2012) [13]. 10 µl of aliquots from all dilutions were inoculated at eight different positions on sterile LBA plates using a sterile loop. After inoculation, the plates were incubated at 37 °C for 24 hours. MBC corresponded to the concentration where 100% of the growth was inhibited compared to the positive control. All the above experiments were performed in triplicates.

The antibacterial effect of CuNPs was further confirmed through OD600 by using microplate reader (EnSpireTM Multilabel Reader 2300, S.No. 2300096).

2. 5. Tolerance Level

Tolerance level of each clinical isolate against CuNPs was determined according to the method of May et al. (2006) [14] by applying the formula below:

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Tolerance = (MBC / MIC)

2. 6. Antibiotic Susceptibility Testing

Susceptibility of the clinical isolates to different antibiotics (Ampicillin, Amoxycillin and Aztreonam) was determined via disk diffusion method following the method of Perpetua et al. (2016) with some slight modifications [15]. LBA plates were prepared and an overnight culture of all the clinical isolates (adjusted to 0.5 McFarland standard) were seeded on the prepared LBA plates. Antibiotic discs used for this study included: Ampicillin (10 µg), Amoxycillin (30 µg) and Aztreonam (30 µg). A single antibiotic disk each of Ampicillin (10 µg), Amoxycillin (30 µg) and Aztreonam (30 µg) was placed on each plate (LBA) seeded with *S. Pyogenes, E. Faecium and E. Faecalis*. Plates were incubated at 37 °C for 24 hrs and were later observed for the zone of inhibition.

2. 7. Multiple Antibiotic Resistance Indices (MARI)

MARI calculations were carried out by dividing the number of antibiotics to which the isolate showed resistance by the total number of antibiotics to which the isolate was subjected [15]. MARI calculations were carried out for all the three clinical isolates (*S. Pyogenes, E. Faecium and E. Faecalis*).

2. 8. Antimicrobial Activity of CuNPs Against *Streptococcus Pyogenes, Enterococcus Faecium and Enterococcus Faecalis* Through Well-Plate Method

The antimicrobial activity of oven dried CuNPs against three different clinical isolates was performed through well-plate method. All the cultures were adjusted to 0.5 McFarland standard before seeding. LBA plates were prepared, isolates were seeded and the wells were punched. In each plate, two wells were loaded with 10 and 15 mg/ml of CuNPs and one well was loaded with distilled water (blank). Ampicillin (10 µg) was kept as a control antibiotic for all the isolates. Plates were incubated at 37 °C for 24 hours and were later observed for zone of zone of inhibition [16].

2. 9. Biofilm Inhibition Assay

This assay was performed through tube method according to Christensen et al. (1982) with some modifications [17]. Overnight cultures of all three clinical isolates were prepared in Luria-Bertani broth and OD was adjusted to 0.5 McFarland standard. A set of four different test tubes was prepared for each isolate. One with only the clinical isolate (negative control), second with isolate and copper nanoparticles (treatment), third with isolate and antibiotic (positive control) and the final one containing only broth (blank). All the test tubes were incubated at 37 °C for 24-48 hrs. After proper incubation, test tubes were rinsed with PBS, air dried, followed by staining with 0.1% of crystal violet. Excess amount of stain was discarded and the tubes were rinsed with distilled water. All test tubes were photographed to check for biofilm formation followed by the addition of 30% glacial acetic acid in order to prepare the tubes for spectrophotometric measurements to quantify the biofilm.

3. RESULTS AND DISCUSSION

This study was performed to test the efficacy of CuNPs on three different drug resistant clinical isolates of *S. Pyogenes, E. Faecium and E. Faecalis*.

3.1. MIC and MBC Determination

MIC of oven dried CuNPs against *S. Pyogenes, E. Faecium and E. Faecalis* was carried out through microdilution method using 96-well microplate. After proper incubation (37°C for 18-24 hours), the plates were observed for the particular concentration of CuNPs where there was no visible growth of the isolates used. The MIC value for *S. Pyogenes, E. Faecium and E. Faecalis* was found to be 1.25, 1.25 and 0.625 mg/ml respectively. MBC was carried out by culturing the different MIC dilutions on sterile LBA plates. MBC values for *S. Pyogenes, E. Faecium and E. Faecalis* were found to be 2.5, 2.5 and 5 mg/ml respectively (Table 1).

The antimicrobial effect of nanoparticles is

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determined by their size, shape and concentration. The surface area provided by larger nanoparticles to contact bacterial cells will have lesser percentage of interaction compared to the small sized nanoparticles. The toxicity that is displayed by smaller nanoparticles is comparatively higher than larger ones because the smaller nanoparticles are likely to diffuse easily compared to the larger ones. The toxic effects of nanoparticles even vary with different species which has been observed by various researchers. According to a study by Yoon et al. 2007, copper nanoparticles were found more effective against gram positive *B. Subtilis* compared to gram negative *E. Coli*. There is a lesser amount of knowledge about whether the bactericidal effect of nanoparticles is due to them or the ions that are released by them [4, 18]. A study by Ruparelia et al. (2008) regarding the effect of CuNPs on bacteria reported a negative correlation between the zone of inhibition through disc diffusion and MIC/MBC through liquid culturing [19]. Generally speaking, and irrespective of the mechanism that is followed by the nanoparticles, gram positive bacterial cell walls are wider as compared to gram negative bacteria and the outer membrane covering the peptidoglycan layer which is absent in gram positive bacteria but is present in gram negative ones makes it easy for harmful agents to penetrate the bacterial cell walls causing cell death [20]. As all the isolates used in this study were gram positive, so the thick cell wall might have posed a hindrance for CuNPs in penetrating the cell, but due to the lesser size of the nanoparticles, they penetrated the cell which caused bacterial cell mortality.

The MIC values of nanoparticles on E. faecium and *E. Faecalis* and *S. pyogenes* have been reported before. A study by Warnes and Keevil (2011), reported the mechanism of copper ions on *E. Faecalis* involves rapid DNA degradation which is followed by reduction of bacterial respiration. It is also known that there is an inhibition of certain cytochromes that are present in *E. Faecalis* membrane by copper ions [21]. Another comparative study by *A. Alshareef* et al. (2017) reported the lesser activity of silver nanoparticles on *E. Faecium* compared to E. coli and the reason they have suggested is that the gram-positive bacteria have thicker cell wall which makes it difficult for nanoparticles to penetrate [22]. One more comparative study by Ann et al. (2014) reported the reason behind the lesser activity of nanoparticles on gram positive bacteria compared to gram negative bacteria. In their study they have mentioned the thicker cell wall of gram positive bacteria compared to gram negative ones as the main reason behind the lesser penetration of nanoparticles [23]. In the present study, the little higher values of MIC may be as a result of the thick cell wall present in gram-positive bacteria but due to the lesser size of CuNPs, they penetrated the cell causing cell death.

After observing and calculating the MIC and MBC of all the clinical isolates, absorbance (OD600) of all the 96-well microplates was recorded using a multimode reader. The results were graphically plotted as depicted in Fig. 1. It is evident from the figure that with a decrease in the concentration of CuNPs, the OD600 values increased parallelly. Starting with 5 mg/ml of CuNPs, which had a more growth inhibiting effect on all the isolates, 0.3125 mg/ml had a least inhibiting effect along with Ampicillin (10 μg) which was used as a positive control. Fig. 1 shows the inhibitory effect of oven dried CuNPs on Streptococcus pyogenes, Enterococcus faecium and Enterococcus faecalis via OD600.

3. 2. Tolerance Level Determination

From the MIC and MBC values recorded, the tolerance level for each isolate against CuNPs was determined. Tolerance level for *S. Pyogenes, E. Faecium* was found as 2 while as for *E. Faecalis*, it was found as 8. The MBC/MIC ratio is a parameter that gives a clarification about the bactericidal or bacteriostatic effect of an analysed compound. For bacteria, if MBC/MIC ratio is equal to or greater than 16, the antimicrobial agent is believed to be as bacteriostatic while as the ratio of less than 4 makes the agent bactericidal. A bactericidal agent is the one that kills the bacteria, however bacteriostatic agents inhibit the growth of bacteria. Some studies have defined the bacteriostatic activity as the MBC/MIC ratio of greater than 4 [20, 24, 25]. A

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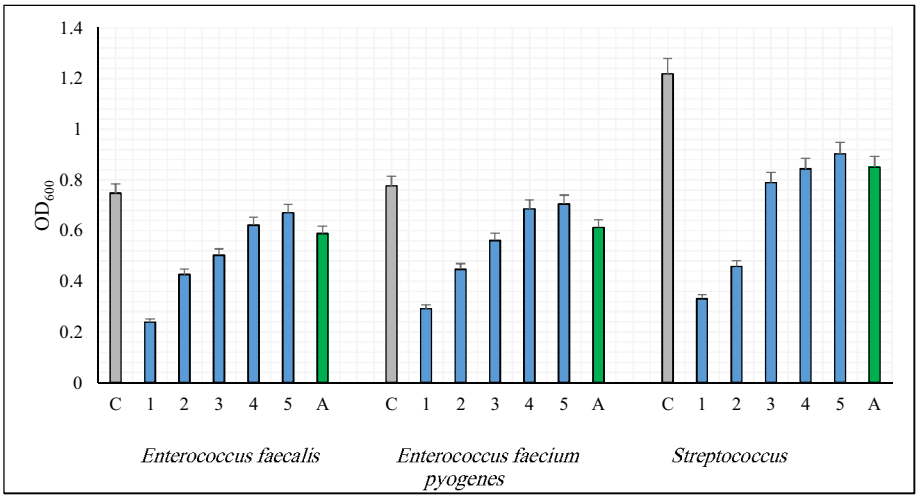

Fig. 1. Growth of clinical isolates with and without treatment. For every isolate, Number 1, 2, 3, 4 and 5 correspond to 5, 2.5, 1.25. 0.625 and 0.3125 mg/ml of CuNPs respectively, A is the antibiotic (Ampicillin) and C is the control.

study by Gonzalez et al. (2013) used tolerance investigations among MRSA isolates as a means of exploring the antimicrobial activities [26]. In this present study, CuNPs acted as a bactericidal agent for *Streptococcus pyogenes* and Enterococcus faecium because the MBC/MIC ratio was 2. By referring to the table 1, it is clear that MBC/MIC ratio is same for *Streptococcus Pyogenes and Enterococcus Faecium* stating the bactericidal effect of CuNPs while for *E. Faecalis* the MBC/MIC ratio of 8 corresponded to the bacteriostatic activity of CuNPs.

3. 3. Antibiotic Susceptibility Testing

Susceptibility testing revealed that the clinical isolates of *S. Pyogenes, E. Faecium and E. Faecalis* were resistant to Ampicillin (10 µg), Amoxycillin (30 µg) and Aztreonam (30 µg). Fig. 2 and table 2 depicts the results of antibiotic susceptibility of all the clinical isolates.

3. 4. MARI Calculations

MARI analysis for all the clinical isolates showed same results because all three of them were resistant to Ampicillin, Amoxycillin and

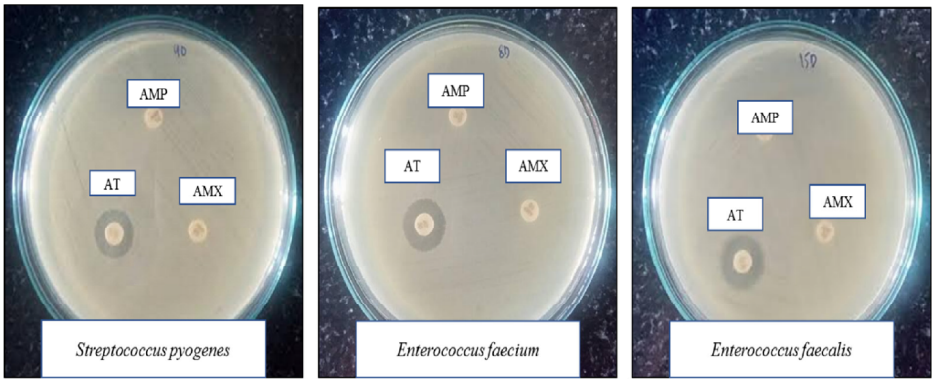

Fig. 2. Antibiotic Susceptibility testing against the clinical isolates (AMP=Ampicillin, AMX=Amoxycillin, AT=Aztreonam, ZOI= Zone of inhibition). The above picture provides an information about the resistance of the particular isolate towards the particular antibiotic.

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Aztreonam (Table 3). MARI defines a tool that unfolds the knowledge about the increasing rate of bacterial resistance in a defined population. MARI values less than 0.20 indicate the use and misuse of several antibiotics in a particular environment from which the isolates have originated. MARI values also give an idea about the exposure of the bacterial isolates to antibiotics which has been a cause of resistance.

3. 5. Antimicrobial Activity of CuNPs Against *Streptococcus Pyogenes, Enterococcus Faecium and Enterococcus Faecalis* Through Well-Plate Method

The susceptibility testing of the clinical isolates demonstrated that Streptococcus pyogenes, Enterococcus faecium and Enterococcus faecalis were resistant to 10 µg of Ampicillin, 30 µg of Amoxycillin and 30 µg of Aztreonam. Well-plate method was carried out using two different concentrations of CuNPs (10 mg/ml and 15 mg/ml), keeping Ampicillin (10 µg) as a control antibiotic. After seeding the plates with the respective isolates and loading the wells, plates were incubated at 37 °C for 24 hours and were observed for zone of inhibition. The graphical results of well-plate method are presented in Fig. 3.

3. 6. Biofilm Inhibition

The tube method of biofilm assay revealed that the CuNPs were very much effective in preventing the formation of biofilm by the isolates. Fig. 4 gives a detailed diagrammatic and graphical information on the biofilm inhibition by CuNPs and ampicillin. It is evident from the graph that CuNPs are more effective in inhibiting the biofilm formation when compared to ampicillin. For Streptococcus pyogenes and Enterococcus faecalis, the MBC totally inhibited the formation of biofilm while as for Enterococcus faecium, there was little less inhibition. CuNPs were always ahead in inhibiting the biofilm formation compared to ampicillin. The graph below shows the spectrophotometric measurements that were recorded after the quantification of biofilm.

4. CONCLUSION

This study, as an extension of the previous study on the synthesis of CuNPs with the assistance of marine actinomycetes, provided an insight into the biological application of CuNPs on drug resistant microbes. The clinical isolates (*S. Pyogenes, E. Faecium and E. Faecalis*) used in this study showed a strong resistance towards

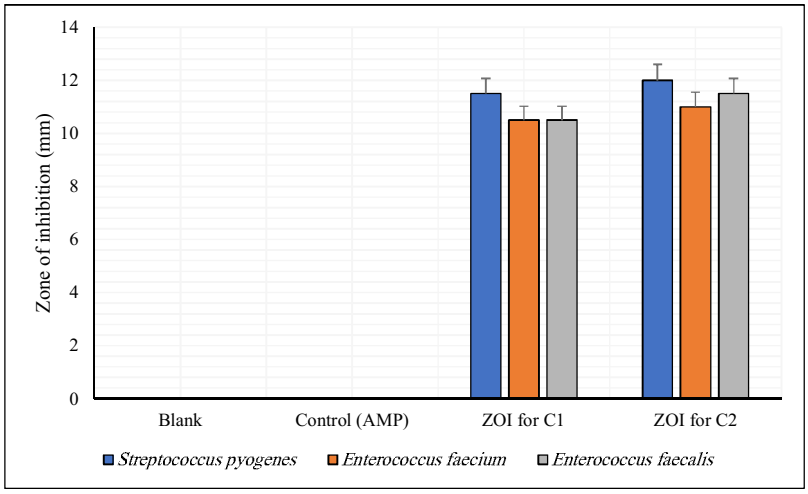

Fig. 3. Zones of inhibition of CuNPs against *Streptococcus Pyogenes, Enterococcus Faecium and Enterococcus Faecalis* where C1 and C2 are 10 and 15 mg/ml of CuNPs, Blank (B) is the Double distilled water and AMP is Ampicillin and ZOI is the zone of inhibition.

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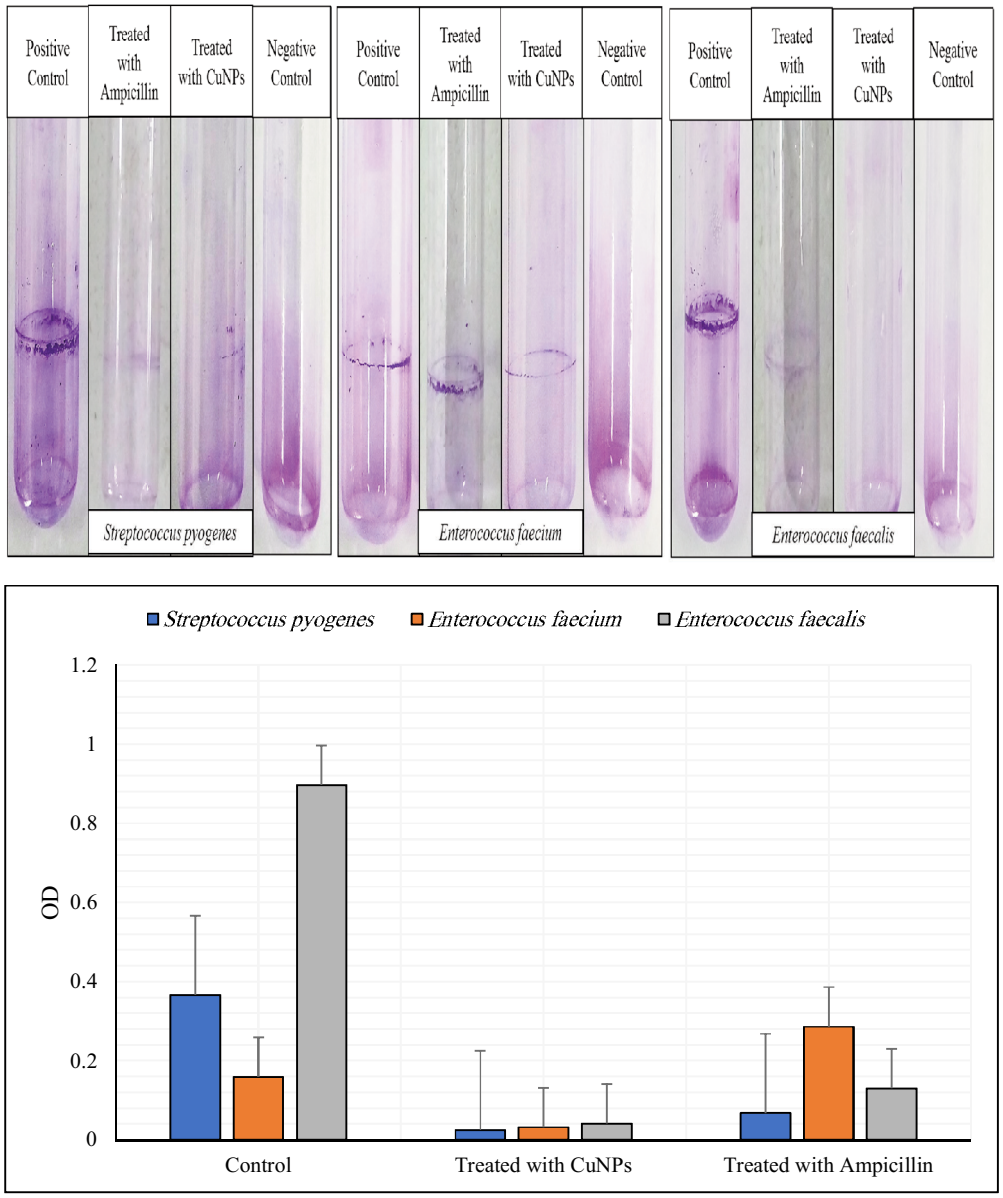

Fig. 4. Inhibition of biofilm formation by CuNPs. The inhibitory effect of CuNPs on biofilm formation was higher than that of ampicillin. The diagrammatic representation provides an insight to the biofilm ring formation on the walls of the test tube while as the graphical representation gives an idea about the biofilm quantification through spectrophotometric measurement.

certain antibiotic formulations like Ampicillin (10 µg), Amoxycillin (30 µg) and Aztreonam (30 µg), but the CuNPs were effective against those resistant isolates. MIC, MBC, tolerance level, antibiotic susceptibility, antibacterial activity through well-plate method and the biofilm inhibition assay were evaluated. Overall, this particular study provided a simple and effective means of growth inhibition of drug resistant clinical isolates with cost effective CuNPs. This study can be executed further to identify the targets and molecular mechanisms to control antibiotic resistance.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest

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