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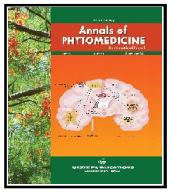

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# Antibacterial activity of green synthesized copper nanoparticles (CuNPs) on seafood spoiling biofilm producing bacterial pathogens

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**Abstract**

In this study, green synthesized copper nanoparticle was synthesized using *Erythrina indica* plant extract as reducing agent. The synthesized copper nanoparticle was confirmed visually and evaluated for their antibacterial activity against seafood spoiling biofilm forming organisms such as *Staphylococcus aureus*, *Enterococcus faecalis* and *Escherichia coli*. The antibacterial activity was achieved by agar diffusion test and their minimum inhibitory concentration was found to be 30 µg/ml. The antibiofilm activity of copper nanoparticle was studied using polystyrene surface and effectively inhibited the biofilm formation as 91%, 94% and 96% against *S. aureus*, *E. faecalis* and *E. coli*. Overall, the copper nanoparticle can be used as antibiofilm agent against biofilm forming organisms.

## 1. Introduction

Antimicrobial agents are biomolecules which will kill or inhibit the growth of microbes without affecting the neighbouring tissues. The antibacterial agent has been used wide variety of fields such as water disinfection, medicine, food packaging and textile industries. Nowadays, over dose of antibiotics may lead to the high change for antibiotic resistance occurrence in different microbial species. Unfortunately, the resistance was developed to most of the currently used antimicrobials. Therefore, an immense effect was performed to tackle the present problem with new antimicrobials. Hence, the nanoparticles have gained much attention due to the particle size ranging from 1 to 100 µM which exhibit various potential application in many fields and also it has the ability to target the drug of choice into the site of action followed by reduction in side effects resulting increased drug uptake (Singh *et al*., 2020; Shafey *et al*., 2020; Lalit *et al*., 2022). The occurrence of nanotechnology has gained huge interest for antimicrobial activity evaluations of nanoscale metals with use of decreased concentration gives increased antimicrobial activity. The antibacterial activity of different metal-based nanoparticle has widely evaluated against various pathogenic microbes present in various fields particularly sea food spoilage.

Sea food spoilage is important and complicated processes in which a greater number of foods are wasted due to microbial contamination even advanced techniques have been used. The microbial contamination may occur during the storage of the sea foods processing conditions such as atmosphere, pH, temperature, *etc*. In particular, sea food underwent fast microbial contamination when it forced into improper storage and handling. Globally, one fourth of sea food supply is lost due to the microbial contamination itself. Consequently, more microbiological analysis is needed on the specific product to predict the original spoilage organisms and also physical and chemical parameters also important for spoilage organisms' predictions. Microbial contamination starts from poor hygienic conditions in food processing resulting serious threat to human health. Furthermore, the complete microbial removal from sea food processing is a difficult task because the biofilm forming ability of microbes can attach the surface of the processing unit wherein it can survive still after disinfection and cleaning.

Biofilms are developed by cluster of microbes which can survive as multicellularorganisms with different biochemical characterisation from planktonic cells (Bernardi *et al*., 2019; Muhammad *et al.,* 2022). These biofilms are formed by a complex material that encase the bacteria inside self-assembled extracellular polymeric substance (EPS) (Seviour *et al.,* 2019), that helps the bacteria to colonize the living body surfaces like lungs, heart and epithelium and non- living surfaces such asmedical device, intrauterine devices, prosthetic heart valves and catheters (Donlan and Costerton, 2002). Biofilms are mainly surrounded by water and composed of variety of biochemical molecule such as carbohydrates, DNA and proteins (Zippel *et al*., 2011). This complex material provides the slimy structure which protects the bacteria from external sources like entry of chemotherapeutic agents resulting bacterial recalcitrance (Yonezawa *et al.,* 2019). Bacteria survive within the biofilms are resistant to

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external factor (Khatoon *et al*., 2018). The biofilm becomes a specific importance due to the ability to attain resistance to existing antibacterial agents which complicate the management of biofilm and also limit the hygienic conditions (Henly *et al.,* 2019). Over many decades, in the sea food spoilage among many organisms, *Staphylococcus aureus*, *Enterococcus faecalis* and *Escherichia coli* have gained much attention owing to its biofilm forming ability on various surfaces (Strickertsson *et al.,* 2013; Rong *et al*., 2017; Xu *et al.,* 2019). These organisms are more resistant to most of the clinically used antibiotics (Arias *et al.,* 2012). Hence, there is a need to search for novel agent which can able to control the bacterial biofilm formation involved in bacterial infections.

Nanoparticles are gaining much attention in various fields like biology and medicine due to their smaller size and large surface area which can freely enter the microbial barriers (Zhou *et al.*, 2019; Mrunal *et al*., 2022). In recent times, metal nanoparticles are used as the supplement for antibiotic efficacy as well as coating material for many medical and non-medical devices (Silva *et al.,* 2019; Thakore *et al.,* 2019). Among them, copper nanoparticles are gained interest due to their efficacy against pathogens and their biocompatibility (Sneha *et al.,* 2022). Copper nanoparticleare synthesized by many methods such as physical, chemical and green synthesis. Green synthesis is a cost-effective eco-friendly approach involves biomolecules action as reducing as well as capping agent without any toxic effects which supports the synthesis process easy than the chemical and physical process wherein the instrument and chemical usage which induces toxic effect (Sundaramurthy and Parthiban, 2015; Sharma, 2019; Ismail *et al*., 2019; Pankaj *et al*., 2022). Additionally, plant materials and their extracts are the best option for nanoparticle synthesis. Consequently, in the study, copper nanoparticle was synthesized using *Erythrina indica* as reducing agent which commonly known as kalyana murungai which has more medicinal values such as antimicrobial, anti-inflammatory, anticancer activity, *etc*., it contains various secondary metabolites like phenolic compounds, alkaloids and sterols. Here, the extract of *E. indica* was used for the synthesis of copper nanoparticle to evaluate the antibacterial and antibiofilm activity against sea food spoilage organisms such as *S. aureus*, *E. faecalis* and *E. coli.*

## 2. Materials and Methods

### 2.1 Reagents

Copper sulphate was procured from Merck, Mueller Hinton agar, ampicillin and rifampicin was purchased from Hi Media, *E. indica* dried powder was purchased from local market. In the study, all the strains were obtained from American Type Culture Collection and maintained in respective medias. In entire study, 5 µg of ampicillin and rifampicin were used as positive control.

### 2.2 Synthesis of copper nanoparticle

For synthesis, 80 ml of 1 mM copper sulphate solution was prepared and stored. 2 g of *E. indica* dry powder was weighed and added into 100 ml of distilled water and keep it for 2 h at $60^0$C. Then, the extract was filtered using Whatman No.1and the filtrate was used for the study. 20 ml of filtrate was added to 80 ml copper sulphate solution and the mixer was keep it at $60^0$C until the solution becomes greenish brown (Ananda Murthy *et al.,* 2020).

### 2.3 Antibacterial activity of copper nanoparticle

The antibacterial activity of copper nanoparticle against *S. aureus*, *E. faecalis* (ATCC 29212) and *E. coli* (ATCC 25922) was evaluated using well diffusion method as described before (Meiyazhagan *et al*., 2020). Prior to the experiments, all the stains were grown in Mueller Hinton broth and Brain Heart infusion broth and overnight culture was adjusted to the cell density of $1 \times 10^6$ CFU/ml. Briefly, the sterile MHA and BHI plates were prepared were swabbed with overnight cultures of bacterial strains. Then, the drills were made and loaded with various concentration of copper nanoparticle (60, 65 and 75 µg/well). Here, ampicillin and rifampicin were used as positive controls. The zone of inhibition around the well indicates the antibacterial activity of copper nanoparticle against above mentioned organisms.

### 2.4 MIC determination of copper nanoparticle

The minimum inhibitory concentrations of copper nanoparticle were evaluated against *S. aureus*, *E. faecalis* and *E. coli* using microdilution method as mentioned earlier (Meiyazhagan *et al.*, 2016). For the experiment, 60 µl of copper nanoparticle was serially diluted up to 0.45 µl in Mueller Hinton Broth (MHB) and brain heart infusion broth using 96 well plates. Finally, 20 µl of overnight cultures ($1 \times 10^6$ CFU/ml) of all the bacterial strains were added and incubated in standard conditions. Then, the optical density of the plates was measured using spectrophotometer at 600 nm. The experiments were repeated thrice.

### 2.5 The effect of copper nanoparticle on biofilm formation

The effect of copper nanoparticle on *S. aureus*, *E. faecalis* and *E. coli* matured biofilm was evaluated using biofilm formation assay as illustrated earlier (Meiyazhagan *et al.*, 2015). The overnight cultures of all the above-mentioned strains were added into in polystyrene microtiter plates and incubated for 96 h for maturation. After that, the matured biofilm was treated with 1X, 2X and 3X MIC for 6 h. Afterwards, the non-adherent cells were removed by PBS wash and the attached cells were fixed with methanol for sometimes followed 0.1% crystal violet staining. The excess stain was removed and the mixture of ethanol and acetone was observed to get purple colour. The plate was measured at 570 nm. Untreated wells served as negative controls for all the strains, whereas ampicillin and rifampicin treated wells served as positive controls. The experiment was repeated twice.

## 3. Results

### 3.1 Synthesis of copper nanoparticle

The copper nanoparticle was synthesized using copper sulphate as precursor and *E. indica* plant extract as reducing agent and the various stages of copper nanoparticle synthesis is presented in Figure 1. Here, the colour change from blue to greenish brown indicates the copper nanoparticle formation.

### 3.2 Antibacterial activity of copper nanoparticle

The antibacterial activity of various concentrations of copper nanoparticle against *S. aureus*, *E. faecalis* and *E. coli* determined is presented in Figure 2 and Table 1. The zone of inhibition around the well indicating the antibacterial activities of copper nanoparticles various concentrations against the selected bacteria. The antibacterial

activities were attained in 60 µl of copper nanoparticle against all the tested organisms. As seen in figure, sizes of the zones of inhibition were increased against all the microbes when the copper nanoparticle concentrations increase.

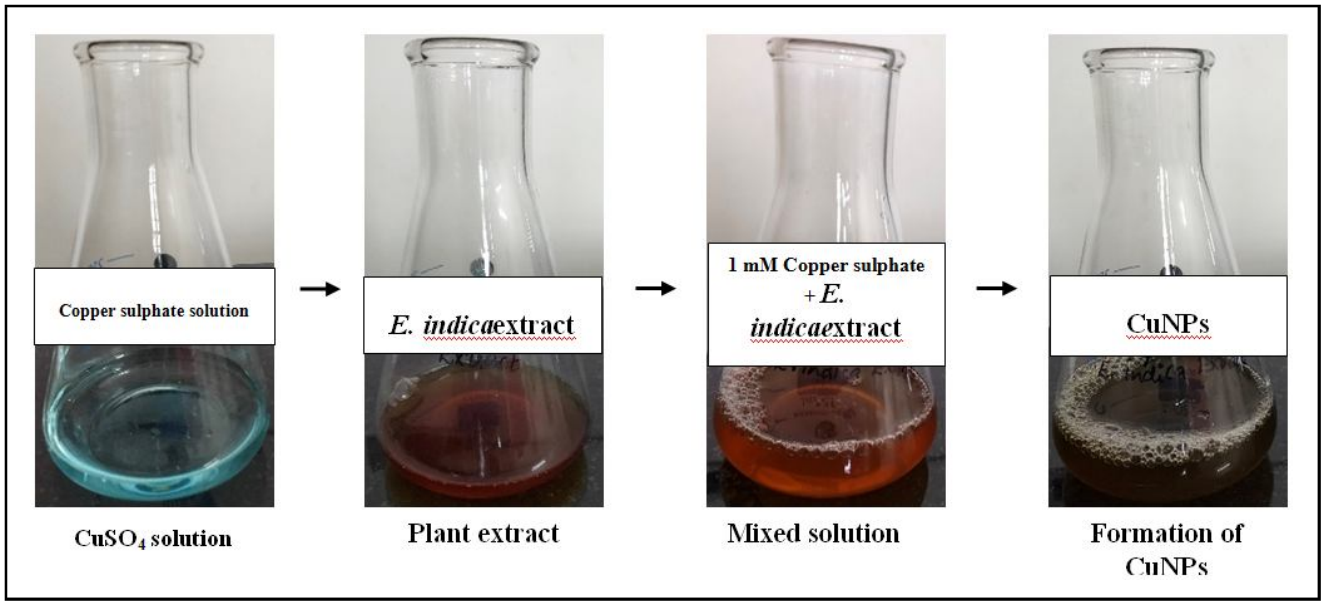


**Figure 1: Various steps involved in copper nanoparticle synthesis.**

**Table 1: Copper nanoparticle antibacterial activity against biofilm forming microbes**

| Organisms | Zone of inhibition in mm | | |
|---|---|---|---|
| | 60 µl | 65 µl | 70 µl |
| *S. aureus* | 24 | 24 | 25 |
| *E. faecalis* | 22 | 23 | 24 |
| *E. coli* | 23 | 23 | 24 |

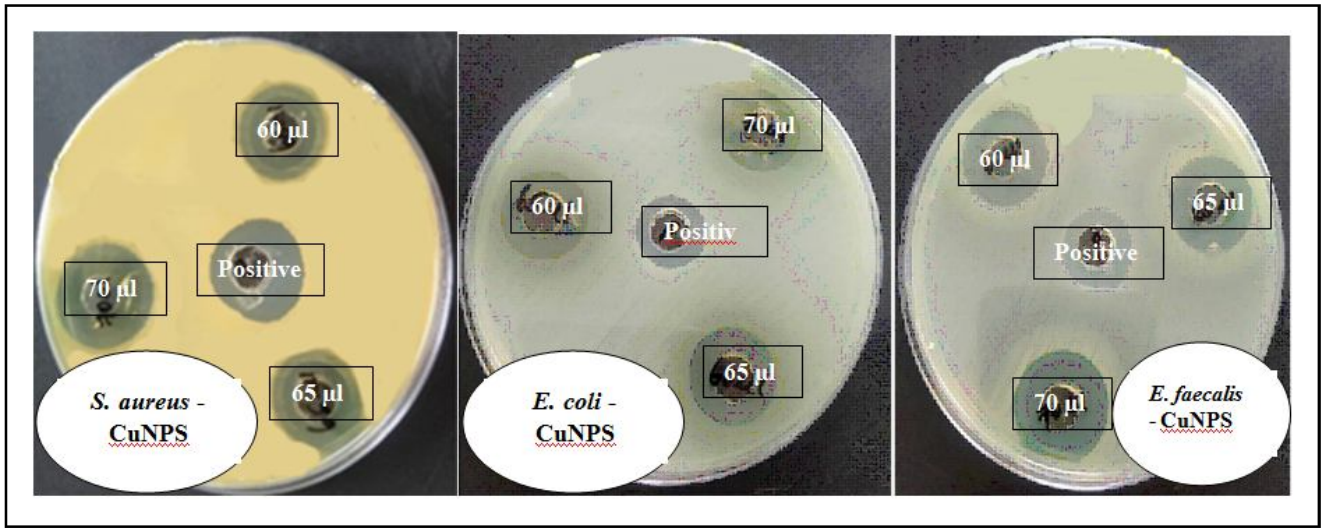


**Figure 2: Antibacterial activity of copper nanoparticle against biofilm forming organisms.**

### 3.3 Copper nanoparticle MIC determinations

The MIC of copper nanoparticle was determined against *S. aureus*, *E. faecalis* and *E. coli* and the calculated MIC which were able to inhibit the growth of all the tested microbes is represented in Figures, 3 and 4. As shown in Figure, 30 µl of copper nanoparticle was needed to inhibit the growth of all the selected microbes.

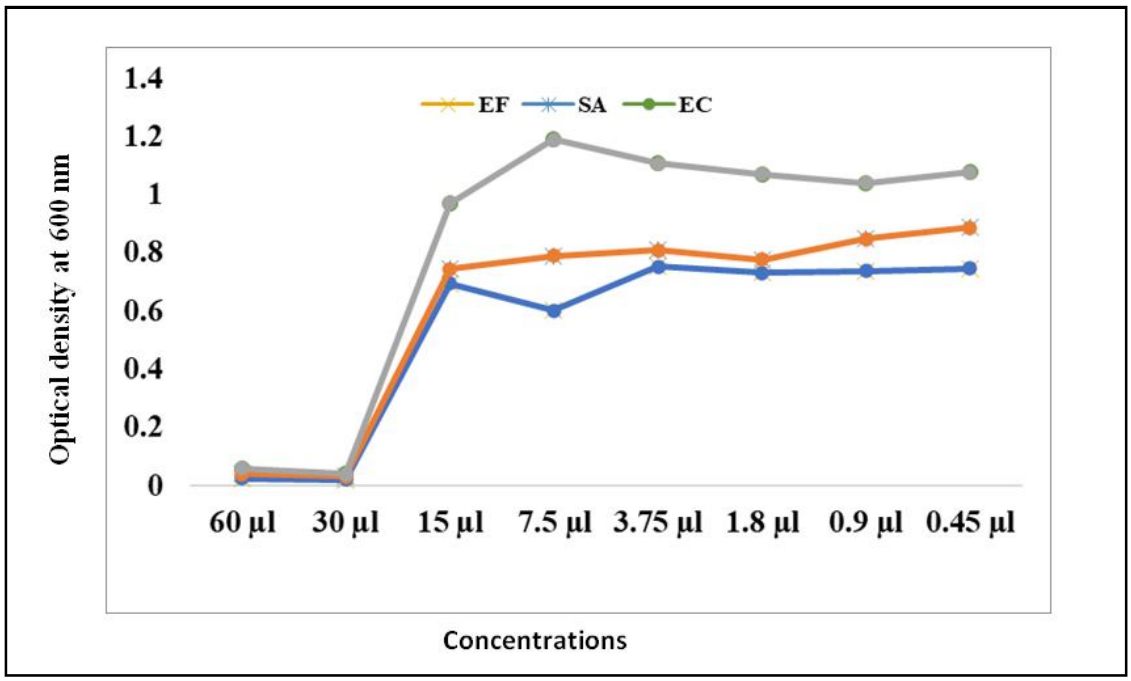


**Figure 3: Graph indicating the MIC of copper nanoparticle.**

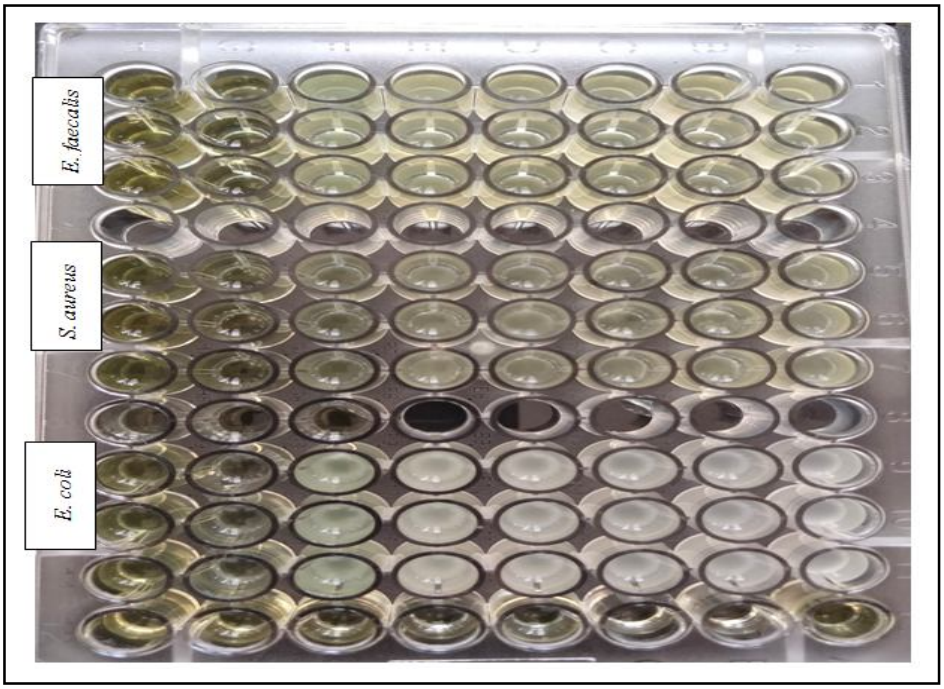


**Figure 4: Visual effect determination of copper nanoparticle MIC against *S. aureus*, *E. faecalis* and *E. coli.***

### 3.4. Copper nanoparticle effect on biofilm formation

The copper nanoparticle effect on biofilm formations of *S. aureus*, *E. faecalis* and *E. coli* was quantified and the calculated percentage of biofilm inhibition is presented in Figures 5 and 6. As seen in figure, the copper nanoparticle effectively inhibited the *S. aureus*, *E. faecalis* and *E. coli* biofilm formation at the tested concentrations in the polystyrene surfaces. The copper nanoparticle effectively reduced 89%, 90% and 91% of biofilm formation of *S. aureus* after treatment with 1 x, 2 x and 3 x MIC. While, the copper nanoparticle 1 x, 2 x and 3 x MIC concentrations effectively reduced each 94% of *E. faecalis* biofilms after treatment. Whereas, copper nanoparticle reduced 91%, 95% and 96% of biofilm when treated with 1 x, 2 x and 3 x MIC, respectively, against *E. coli*. It showed the copper nanoparticle ability in eradicating biofilms of all the selected microbes.

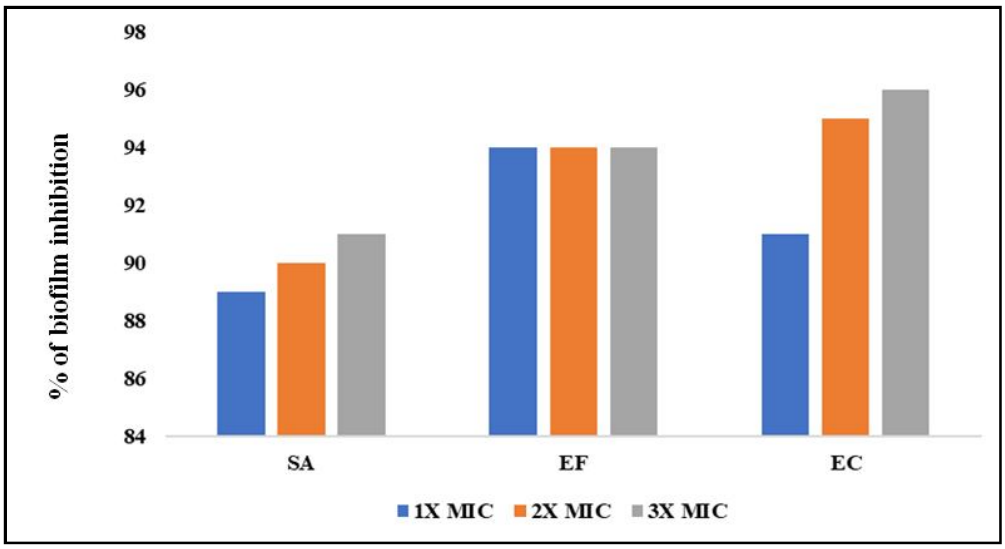


**Figure 5: Graph representing the percentage of biofilm inhibition after treatment with copper nanoparticle.**

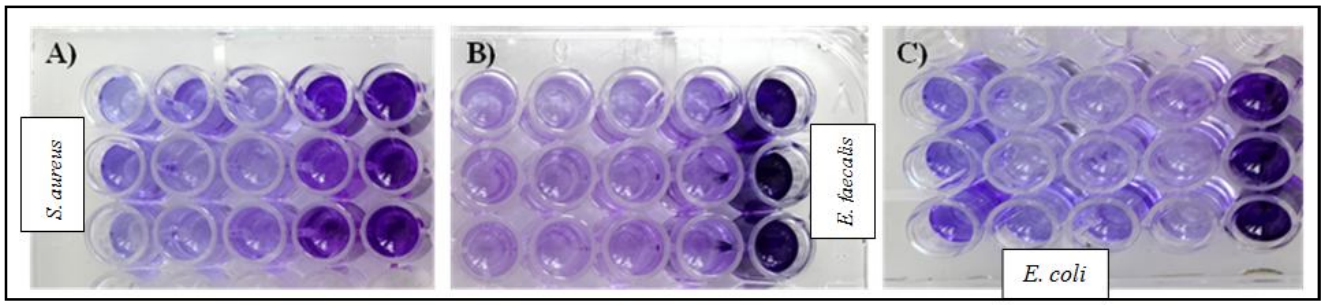


**Figure 6: Visual effect of biofilm inhibition after treatment with copper nanoparticle against. A) B) *E. faecalis* and C) *E. coli.***

## 4. Discussion

Sea food spoilage occurred mainly due to improper food processing and storage resulting microbial contamination leading serious health problem to humans. The removal of microbial contamination in sea food is a complicated process due to the biofilm forming ability on the surface. Consequently, biofilm forming microorganisms such as *S. aureus*, *E. faecalis* and *E. coli* gaining much interest in the sea food spoilage. In the study, antibacterial activity of green synthesized copper nanoparticle was evaluated against biofilm forming organisms such as *S. aureus*, *E. faecalis* and *E. coli*. Here, copper nanoparticle was synthesized using *E. indica* as reducing agent which reduces the salt into metal oxide owing to various biomolecules present in it. The antibacterial activity of synthesized copper nanoparticle evaluated showed 24 mm of zone of inhibition around the well against *S. aureus*, *E. faecalis* and *E. coli*. Similarly, biofabricated copper nanoparticles was synthesized using leaves extract of *Bambusa arundinacea* and exhibited potent antibacterial activity against *Bacillus subtilis, Staphylococcus aureus, Proteus vulgaris and Escherichia coli* (Jayarambabu *et al*., 2022). Likewise, another group studied the green synthesized copper nanoparticle using *Cedrus deodara* as reducing agent, antibacterial activity against *S. aureus and E. coli*. The zone of inhibitions around the wells representing the antibacterial activity with maximum zone size of 29 mm (Ramzan *et al.,* 2021). Same way, a report from recent study showed the green synthesis of titanium oxide nanoparticle using plant extract of *E. variegate* and evaluated for their antibacterial activity against *Streptococcus* sp., *Pseudomonas* sp., *S. aureus*, *E. faecalis* and *E. coli* (Selvi *et al*., 2022). Similarly, very interesting study showed the antibacterial effect of nanoemulsified lemon essential oil was investigated against food borne pathogens such as *E. faecalis, Salmonella paratyphi, A. pneumoniae* sp., *S. aureus* and *Klebsiella* sp. as well as fish spoiling organisms like *Photobacterium damselae* and *Pseudomonas luteola* and showed antibacterial activity indicating that it can be a natural antibacterial agent against sea food spoilage as well as food borne bacteria (Yazgan *et al.,* 2019).

The formation of copper nanoparticle was visually conformed by changing the colour from blue to greenish brown indicating copper nanoparticle formation by reducing the copper salt, though the biomolecules present in the plant extract. Similarly, the green synthesis of copper nanoparticle was achieved by using *Aerva javanica* leaf extract and their characterization was performed and its antibacterial activity was evaluated against *P. aeruginosa, A. baumanii, S. aureus* and *E. coli* and also the MIC was found to be 50 µg/ml (Amin *et al.,* 2021). Our study revealed that, 30 µl of copper nanoparticle was needed to inhibit the growth of all the tested organisms. The antibacterial activity of copper nanoparticle can be achieved in several ways such as contact killing wherein the particle were attached on the surface of bacterium, thereby the killing was mediated and also it has attached on the surface of the bacterial cell and disturb the cell membrane integrity resulting leakage of the internal content leads to cell death. The cell death could be attributed by several factors such as pH and temperature, *etc*. (Chatterjee *et al.,* 2014).

In addition to the antibacterial activity, the copper nanoparticle was evaluated for antibiofilm forming ability against most important biofilm forming organism like *S. aureus*, *E. faecalis* and *E. coli.* The biofilm formation was initiated from the attachment of any living and non-living surfaces and becomes matured biofilm formation (Pelling *et al.,* 2019). Our study effectively reduced the 91%, 94% and 96% of *S. aureus, E. faecalis* and *E. coli* biofilm formation after treatment with various concentrations of copper nanoparticle. In contrast, copper oxide nanoparticle synthesized using fungus *Penicillium chrysogenum* was characterized and evaluated for their antibacterial and antibiofilm activity against *S. aureus* ATCC 25923, *B. cereus* ATCC 11778, *E. coli* ATCC 35,218 and *K. oxytoca* ATCC 51,983. The synthesized nanoparticle sowed potent antibacterial activity against all the tested microbes but the antibiofilm activity was observed only for *K. oxytoca* and *E. coli* not on matured biofilm of *S. aureus* and *B. cereus*. This result suggested that, the copper nanoparticle activity may limit to species specific (Shehabeldine *et al*., 2023). Similarly, the copper nanoparticle was combined with beta lactam antibiotic showed the effective antibiofilm activity against *P. mirabilis* and *S. aureus* and showed synergism with beta lactam antibiotics (Arul *et al*., 2019). A recent study evaluated the anti-bacterial activity of copper nanoparticle coated stainless steel against *Salmonella entertidis* ad also their corrosive capacity and found that copper coated material can be used as supportive agent to limit or control pathogens (Pontin *et al*., 2021). Similarly, to avoid the bacterial contamination on the surfaces, copper alloys was investigated for their antibacterial and antibiofilm property was investigated against *S. aureus* and *pseudomonas* and highlighted the copper alloys importance in eliminating biofilm formation (Colin, 2021). Therefore, the copper nanoparticle effectively inhibited biofilm forming ability of various biofilm forming organisms.

## 5. Conclusion

In the study, copper nanoparticle was synthesized using *E. indica* plant extract and evaluated for their antibacterial activity against various biofilm forming organisms such as *S. aureus, E. faecalis* and *E. coli* and excellent activity was observed against tested pathogens. Also, copper nanoparticle effectively inhibited the biofilm formation. Overall, considering all these facts, the copper nanoparticle can be used as potential anti biofilm agent.

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## Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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