

Biosynthesis of copper nanoparticles mediated by *Staphylococcus aureus* and evaluation of their antibacterial effects

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Abstract: Biological synthesis of nanomaterials offers numerous advantages over chemical synthesis. This study was aimed at the synthesis of biosynthesized copper nanoparticles using *Staphylococcus aureus*. Biosynthesis of CuSO₄ nanoparticles using *Staphylococcus aureus* isolated from poultry droppings was carried out. Effect of CuSO₄ concentration and cell free supernatant on CuNP production was determined. Antimicrobial, and synergistic studies of conventional antibiotics, and CuSO₄ mediated samples were carried out also. The CuSO₄ nanoparticles was characterized using Energy dispersive X-ray fluorescence. *Staphylococcus aureus* mediated CuNPs was assessed for antimicrobial properties using some selected bacterial cultures. CuSO₄ nanoparticles was produced at varying levels of copper concentration with absorbance highest at 400nm at 750ppm. Inhibition of *Streptococcus cohnii*, *Enterococcus raffinose*, *Serratia marcescens* and *Salmonella enteritidis* was achieved at the tested strengths (318.4ppm, 750ppm, 1000ppm) of CuNPs produced by the *Staphylococcus aureus*. Significant increase in the activities of tetracycline, gentamycin, vancomycin ranged between 1.9-100% as recorded in this study. Energy dispersive X-ray fluorescence showed Cu as the predominant element. *Staphylococcus aureus* mediated CuNP showed significant antimicrobial activities.

Keywords: Copper Nanoparticles; Antimicrobial activities, *Staphylococcus aureus*, UV-VIS spectroscopy.

Introduction

Nanoparticles (NPs) belong to a group of materials at the nanoscale (1-100nm) point with potential applications in industry, technology and medicine (Rónavári et al., 2021). Their particularly unique dimensions coupled with the immense surface area to volume ratio, has imparted innovative physicochemical attributes (John et al., 2021a; Lateef et al., 2015).

Unlike their heavier minerals, nanoparticles have attracted increasing deployed, across areas revolving around conductivity, electrical resistivity, oxidation-reduction reactions, and but not limited to their physiological roles in natural processes, albeit living organisms (Grigore et al., 2016; John et al., 2021b; Katwal et al., 2015).

A considerable number of methods exist in the literature and these include physical methods such as ionization radiation, dissociation, targeted heating methods, photoablation, chemical methods ("Metal Nanoparticles in Microbiology," 2011). These protocols are expensive and hazardous to the environment because toxic chemicals are used and have become a matter of great concern due to health and environmental reasons. Research findings have revealed that a significant number of microorganisms possess the potency to initiate inorganic nanoparticles within their living systems, or in their metabolic environment.

Although, several metals have attracted keen attention based on their inherent nanoparticulate attributes and the numerous potential applications (John et al., 2021b).

Copper mediated nanoparticles is of particular interest owing to the potential uses arising from its chemical-physical changes. Expressly, some copper nanoparticles can be blended through some other methods such as an amalgam of oxides of copper (cupric and cuprous oxides) (John et al., 2021b; Sagadevan et al., 2017). Cupric oxide nanoparticles are p-type oxide semiconductors widely utilized in various sectors due to their catalytic, optical, magnetic, and electrical properties. They are commonly employed as catalysts, sensors, and photocatalysts, as well as in optoelectronics. Additionally, they play a significant role in antibacterial, antifungal, antiviral, anticancer, antioxidant, and drug delivery applications, among others (John et al., 2021b; Waris et al., 2021; Zhang et al., 2014).

Copper nanoparticles are also blended/produced through different processes such as chemical and electrochemical synthesis, thermal evaporation, the solvothermal route, and vapor-liquid-solid growth (Zayyoun et al., 2016). Although, several drawbacks associated with physical processes, range from the need for costly and complex vacuum techniques. Similarly, chemical synthesis restricts nanoparticle applications in clinical areas, attributed to the presence of toxic chemicals with inherent environmental and biological risks. In contrast, green approach of CuNPs is an eco-friendly and cost-effective approach that avoids harmful chemicals,

making them particularly appealing for mainstream applications (John et al., 2021b).

Copper oxide (CuO) is the simplest compound (with crystalline lattice) in the copper family, it possesses various beneficial physical properties, including superconductivity at extremes of temperatures, electron correlation effects, and pivoting dynamics, making it valuable for diverse applications (Allaker, 2012). CuO crystal also has photocatalytic/photovoltaic characteristics and photoconductive usefulness. Owing to the existence of limited data detailing the antimicrobial activity of nano CuO, coupled with its affordability unlike silver based nano, hence it is widely desired (Koper et al., 2005). The nanoparticles have interesting attributes which can be explored for machinations and desired applications.

Literature search reveals an array of natural sources of bacterial components able to perform reductive and balancing during the formation of copper nanoparticles, they often restrict interfering growth, and accumulation ensuring the production of the target nanoparticles (John et al., 2021b; Waris et al., 2021). In this context, copper oxide nanoparticles are utilized in various applications, including antibacterial, antifungal, antiviral, anticancer, antioxidant, and drug delivery purposes.

The antibacterial properties of metal nanoparticles are primarily linked to their miniscule nanometric sizes and high surface-to-volume ratio, enabling close interaction with microbial membranes, and not singularly the liberation of the metal ions in solution (Ruparelia et al., 2008).

Copper has been regarded as an antimicrobial agent according to The US Environmental Protection Agency (EPA) due to proof of its ability to mitigate lethal infections often caused by microorganisms (European Copper Institute, 2008).

This study investigated the synthesis of copper nanoparticles (CuNPs) using cell-free culture supernatants of *Staphylococcus aureus* isolated from poultry droppings collected from agricultural land in Ekiti State University.

Methodology

Reagents and Culture

Culture used in the biosynthesis and the clinical strains used in antibiotic assays were obtained from the culture collection of the Department of Science Laboratory Technology, Ekiti State University. The strains were grown at 37 °C on Mueller Hinton. Culture media were procured from Oxoid chemicals. Chemicals such as copper (II) sulfate pentahydrate salt, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and others were analytical grade reagents procured from Sigma Aldrich (Sigma Aldrich, St. Louis, MO, USA).

Biosynthesis of CuNPs

Copper nanoparticles were synthesized using the previously isolated bacteria by treating a copper sulfate (CuSO_4) solution with cell-free supernatant. After 24 hours, the culture was centrifuged at 10,000 rpm for 20 minutes at 30°C, and the resulting supernatant was kept for further use. Five milliliters of this supernatant were added to different concentrations of CuSO_4 solution and incubated for 24 hours. The synthesis of CuNPs was then analyzed through visual color changes and UV-Vis spectral analysis (400–800 nm). Additionally, the ability of cell-free spent media to mediate CuNP

synthesis was evaluated. Control experiments were conducted using uninoculated media and CuSO_4 solution.

Effect of CuSO_4 strengths on CuNP formation

Varied strength of CuSO_4 was used (318.4, 750, 1000 ppm) to assess the potential of CuSO_4 on nanoparticles formation. A 5mls of cell-free supernatant was added to 20ml of different CuSO_4 concentrations, it was cultivated at 37°C for 24hrs after which a visual observation shows characteristic colour changes to green which indicates the breakdown of copper ion has taken place to form copper nanoparticles (Shanktkriti et al., 2014). Spectrometry of both the plain inoculated broth and inoculated broth with copper sulphate was done between 400-800nm to view the percentage transformation of copper ion to form copper nanoparticles.

Antimicrobial efficiency of the synthesized CuNP

The antibacterial activity of the synthesized CuNPs was evaluated using the agar well diffusion method (Perez et al., 1990). Strains of clinical bacterial isolates obtained from Federal Teaching Hospital Ido Ekiti and Ekiti State University Ado Ekiti respectively were used (Monica, 2004). The diameters of zone of inhibition of the disc were measured and the result was interpreted with reference to NCCL (2008).

Synergistic study of the CuNPs combined with antibiotics.

Agar diffusion method was used to evaluate the combined efficacy of the synthesized CuNPs in conjunction with conventional antibacterials such as gentamycin, vancomycin, tetracycline, norfloxacin (Lateef et al., 2015). Approximately 100µl of each 1mg/ml antibiotic solution, prepared with sterile distilled water, was dispensed into agar wells on pre-inoculated Mueller-Hinton agar plates containing clinical bacterial isolates. An equal volume (50µl) of CuNPs and antibiotics was mixed and applied against the test isolates. Following incubation as previously described, the inhibition zones were measured and compared to assess the percentage improvement in antibacterial activity due to the synergistic effect between the antibiotics and biosynthesized Copper nanoparticles.

Energy Dispersive X-ray Fluorescence (EDXRF)

It is an analytical technique used for elemental composition determination of a sample. Elemental analysis of biosynthesized CuNPs was performed using EDXRF. This technique is used in order to determine the most prevalent element in the biosynthesized CuNPs. A droplet of CuNPs was placed onto a clean silicon water surface. The droplet was allowed to dry by evaporation at ambient temperature and a thin carbon film was placed on the dry CuNPs to prevent them from charging and the elemental compositions of the samples were obtained. X-ray energy being is a unique elemental attribute of an element is typically used for componential analysis of test material (Poole et al., 2003; Kohler et al., 2004).

Data presented are means of triplicate readings.

Results and Discussion

The Biosynthesis of CuNPs was carried out with the cell free supernatant of *Staphylococcus aureus* isolated from poultry waste which mediated the synthesis in the reaction vessel over a period of

24hrs. This agrees with reports of Yu *et al.*, (2011) who detected comparably dark green colour solution on addition of 5 mM CuSO₄ to a reactor with *Morganella* sp. indicating nanoparticles production was reported by Ramanathan, *et al.* (2011).

Figure 1 showed the tolerance of CuSO₄ by *Staphylococcus aureus*. It showed higher absorbance 0.920 at a concentration of 750ppm (400nm). The absorbance was lowest at 1000ppm (0.316)

The cell free extract of CuNP exhibited appreciable antibacterial efficacy against the test clinical bacteria isolates at concentrations of 318ppm, 750ppm, 1000ppm as shown in Table 1. *Enterococcus raffinose* (47) and *Salmonella enteritidis* (24.24) showed higher susceptibility as indicated by the zones of inhibition while *Streptococcus cohnii* and *Serratia marcescens* were lowest. The cell free extract mediate CuNP exhibited notice able antibacterial efficacy against the test clinical bacteria isolates at concentrations of 0.318ppm- 1.000ppm. The antibacterial activities of CuNP obtained from these study is similar to the work of Lateef *et al.* (2015); Salem *et al.*, (2014); Shankar *et al.*, (2014) who reported the antimicrobial activities of AgNP against selected clinical strains of bacteria, they attributed this activities to the interaction of AgNP-sulphur-phosphorous – within the biological systems in the bacterial matrix leading to cell death and Thievasanthi *et al.*, (2014) also attributed it to the interaction between the CuNP and the bacterial cell membranes.

All the clinical isolates showed sensitivity to the test antibiotics, with the highest observed under Norfloxacin (26mm) except for *Streptococcus cohnii* which showed no inhibition to Norfloxacin, and the lowest inhibition was observed under Vancomycin as shown in Table 2.

Synergistic effect of antibiotics with *Staphylococcus aureus* mediated CuNPs as shown in Table 3 revealed a range of mixed results. Gentamycin mediated with *Staphylococcus aureus* copper nanoparticles showed higher inhibitory effect against *Enterococcus raffinose* (31mm), while it was lowest against *Salmonella enteritidis* (13mm). This was followed by Norfloxacin which had higher efficacy *Serratia marcescens*, and - *Enterococcus raffinose* (16.5mm). Across *S. cohnii* showed the most susceptibility to all except for Norfloxacin where no inhibition was observed with the mediated CuNPs. . This is similar to the works of Patil *et al.*, (2009); Devi and Joshi, (2012); Lateef *et al.*, (2015e); Fayaz *et al.*, (2010). Previous studies have showed the combined effects of CuNPs and antibiotics against Gram negative and Gram positive bacteria as reported by Devi and Joshi, (2012); Lateef *et al.*, (2015e); Fayaz *et al.*, (2010). The synergistic activities functions via cytokinesis mediated by most antibiotics, the CuNP is then taken inwards by the bacterial cell resulting in toxicity to the cell.

During EDXRF analysis, the absorption spectroscopy of this solution exhibited a distinct absorption peak in the region of 400-500nm, indicative of Copper Nanoparticles formation. This closely agrees with findings by Shantkriti and Rani (2014). The EDXRF analysis of *Staphylococcus aureus* mediated CuNPs which showing the elemental structure of the nanoparticles and it indicated that copper was the most prevalent element (Cu 98.2%, Zn 1.71%, Sn 0.0%, Pb 0.02%) and the other elements were associated with machine input as shown in Figure 2. This closely agrees with earlier reports of Lateef *et al.* (2015) which described the absorption of Ag-NP between 391 – 600nm. Observation of these broad peaks is an indication that the biosynthesized CuNPs were polydispersed and these are well documented for various metal nanoparticles (Shankar *et al.*, 2003; Mohanpuria *et al.*, 2008; Kalishwaralal *et al.*, 2008).

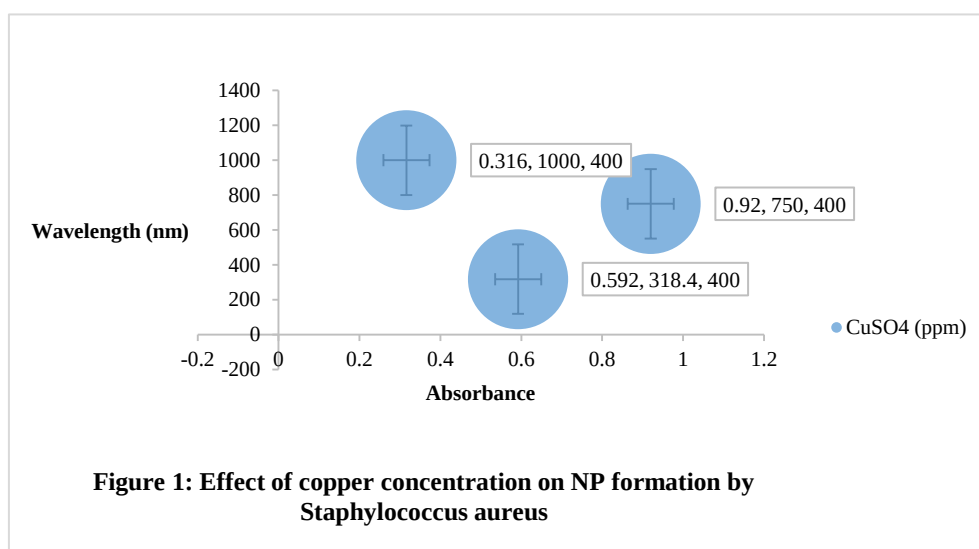


Figure 1: Effect of copper concentration on NP formation by *Staphylococcus aureus*

Table 1: Zones of inhibition of the clinical bacteria isolate to the CuNPs mediated by *Staphylococcus aureus*

Isolates	ZONE OF INHIBITION (mm)		
	318.4ppm	750ppm	1000ppm
<i>Streptococcus cohnii</i>	-	-	-
<i>Enterococcus raffinose</i>	19.5	-	47
<i>Serratia marcescens</i>	-	19	-
<i>Salmonella enteritidis</i>	24.24	-	-

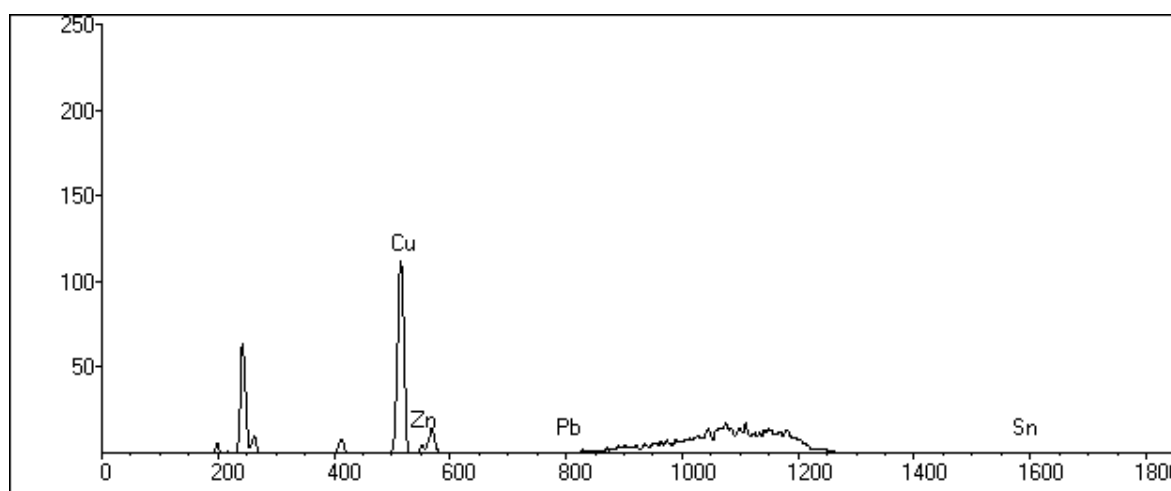
Table 2: The effect of selected antibiotics on the tested clinical bacteria isolates

ISOLATES	ZONE OF INHIBITION (mm)			
	Gentamycin	Vancomycin	Norfloxacin	Tetracycline
<i>Streptococcus cohnii</i>	20	-	-	18
<i>Enterococcus raffinose</i>	19	19	26	24
<i>Serratia marcescens</i>	19	-	26	9
<i>Salmonella enteritidis</i>	13	10	26	-

Table 3: Synergistic effect of antibiotics with *Staphylococcus aureus* mediated CuNPs and their percentage improvement

	Zone of inhibition (mm)			
	Gentamycin	Vancomycin	Norfloxacin	Tetracycline
<i>S. enteritidis</i>	13 (14)	15 (NI)	26 (16)	22 (NI)
<i>S. marcescens</i>	19 (NI)	15 (NI)	26 (16.5)	15 (14.5)
<i>S. cohnii</i>	20 (27.5)	10 (11.5)	NI	18 (16)
<i>E. raffinose</i>	19 (31)	19 (22)	24 (16.5)	NI

NB: The final values are indicated in brackets


Figure 2: The EDXRF analysis of *Staphylococcus aureus* mediated CuNPs

Conclusion

This study demonstrates the propensity of obtaining bio nanomaterials that can be used as antibiotics alternatives against the growing number of drug-resistant pathogens bacteria, which has created serious concern across the globe due to the limited choices in antibiotic treatment.

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Conflict of interest

The authors declare that no conflict of interest exists in the authorship of this article

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