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RESEARCH ARTICLE

ANTIBACTERIAL ACTIVITY OF BIO-SYNTHESISED COPPER NANOPARTICLES AND SYNERGICIMPACT WITH TETRACYCLINE

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ABSTRACT

The increasing problem of antibiotic resistance, there is a need to develop effective and eco-friendly antimicrobial strategies. In the present study, copper nanoparticles (CuNPs) were synthesized through a green, fungal-mediated approach using *Aspergillusniger* isolated from onion peel. The biosynthesis of CuNPswere characterized using UV- Visible spectroscopy to confirm nanoparticle formation. Bacterial strains were isolated from sewage water and identified using standard morphological and biochemical tests. The antimicrobial activity of the synthesized CuNPs was evaluated against the identified bacterial isolates using well diffused method. Furthermore, the synergistic antimicrobial effect of CuNPs in combination with antibiotic tetracycline was assessed. The results demonstrated that fungal-derived CuNPsexhibited significant antimicrobial activity, which was markedly enhanced when used in combination with tetracycline, indicating a synergistic interaction. This study highlights the potential of *Aspergillusniger*-mediated CuNPs as effective antimicrobial agents and supports their application in combination therapy to combat antibiotic-resistant microorganism.

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INTRODUCTION

Nanoscience is a relatively new research science, including Technology, Engineering and Mathematics, disciplines, are involved in nanomaterial synthesis of around 1–100 nm. At the nanoscale level, the materials have unique chemical, physical, optical, magnetic and electrical properties due to their greater area to volume ratio. Owing to extensive applications in biomedical field such as antimicrobials, drug delivery (in molecular imaging, target drug therapy, oligonucleotide, and gene delivery etc.), in industries like sensors, optical fibers, catalysts, paints, cosmetics, electronic systems, biolabeling in agriculture and in diverse fields, nanoparticles research had central position(Kumar *et al.,* 2024;Khan *et al.,* 2025). Among the nanoparticles, copper nanoparticles dragattention due to their optical, catalytic, non-toxicity, antibacterial, antifungal and high conductivity properties.They are also significant due to their cost-effectivenessand potential heat transfer properties. Copper nanoparticlescan be synthesized easily and modified into therequired size, shape and dimensions. Their biologicalactivities are also noticeable, making them a usefulsource for antibiotics production (Akinteluet *al.,* 2021).Copper-based nanomaterials can be applied in agriculture, livestock, water treatment, wood preservation, and textile production. In addition, copper nanomaterials can serve asalternative to the other noble metal nanomaterials in solar energy conversion, batteries, and electrochemical sensors due to their conductive capacity and lower production costs(Geng *et al.,* 2023). The bactericidal effect of metal nanoparticles has been attributed to their small size, and high surface to volume ratio, which allow them to interact closely with microbial membranes and it is not merely due to the release of metal ions in solutions. The antibacterial properties of the metal nanoparticles find applications in various fields such as medical instrument, and devices, water treatment and food processing (Tavakoli *et al.,* 2019). Cu NPs are observed as particles for antimicrobial applications as an antibiotic treatment and biocides and find applications in skin remodulation, wound healing and anti-inflammatory therapies (Truong*et al.,* 2023). These have been exhibited to stop the growth of bacteria such as Bacillus subtilis and *E. coli*, *S. aureus*, *P. aeruginosa, C. albicans, A. falvus* Fungi, due to their ability to tolerate and bioaccumulate metals, effortless synthesis method, comparatively economic, simple downstream processing and handling, are gaining the central stage of studies on the generation of biological metal nanoparticles (Ma *et al.,* 2022). *Aspergillusniger* is a fungal strain which can cause allergies and asthma, particularly when exposed to indoors for longer periods of time. This fungus is also called as black mold. *A. niger* is a common soil filamentous fungus with a large range of oxidative and hydrolytic enzymes. A range of these enzymes are crucial in

biotechnological industry. The objectives of this study are to synthesize and characterize copper nanoparticles from *Aspergillusniger*and assessing their biological activities such as antibacterial activities.We synthesized CuNPs via fungal (*Aspergillusniger*) filtrate due to its capability to bear and bioaccumulate metals, comparatively economic, simple handling and downstream processing.

MATERIALS AND METHODS

Isolation andidentification of *Aspergillusniger (A. niger*)

Isolation of *Aspergillusniger* from onion peel and checked its morphological properties.

Collection of pathogenic isolates: The water sample was collected aseptically from Municipal sewage water of jaipur. Sample was collected in a sterile bottle and brought to lab. This water sample was stored in clean place. 1 mL of water sample was used for isolating the bacteria through serial dilution and agar plate culture technique.

Synthesis of Cu-nanoparticles by *A. niger:* Colonies of the *A. niger* were sub-cultured on Potato Dextrose Agar plates and incubated at 37°C for 2-3 days. It was used to inoculate 100ml of Potato Dextrose broth. The flasks were incubated at ~37 °C for 4 days with shaking at 150 rpm. Mycelial pads were harvested aseptically and washed 3 to 4 times with distilled water. Then, 6 g of biomass was weighed and mix containing 30 ml of sterile deionized water and left in the shaking incubator for 46 hours. The biomass was filtered using sterile filter paper No.1, and 30 ml of supernatant was taken in flask. Fungal supernatant was used for the synthesis of nanoparticles.The fungal filtrate was added in 7mM of $CuSO_4.5H_2O$ with the ratio of 1:1 at room temperature. On addition of $CuSO_4.5H_2O$ solution [pH-7], a colour change was observed and this was monitored under UV visible spectrophotometer.

Characterization of CuNPs

Visual inspection: The bio-reduction of the aqueous solution of copper sulphate using *A.niger* extract was monitored and the appearance of green colour indicates the formation of copper nanoparticles.

pH analysis: The pH of the extract, precursor as well as the resulting mixture after addition ofNaOH was determined using digital pH meter.

UV spectroscopy: The reduction of copper sulphate to copper was monitored by recording UV-Visible spectrum of the reaction mixture after diluting a small aliquot of the sample with deionised water. The measurements are recorded on Shimadzu dual beam spectrometer (model uv-1650pc) operated at resolution of 1nm.

Antibacterial activity of CuNPs against pathogenic isolates: A loop of *Vibrio gazogene*, *Staphylococcus faecalis* was added in respective sterilized nutrient broth tubes and incubated for 24 hr at 37 C. After incubation, Agar well diffusion procedure was used to observe antibacterial action. Bacterial strains were spread on nutrient agar plates using sterilized cotton swab andwells were formed in each plate.The agar well diffusion method was using Mueller Hinton Agar Plate the sensitivity of the bacterial isolates against Cu-NPs Different dilutions were made using sterilised water and various concentrations (20, 40, 60, 80%.) Approximately (0.2) ml were placed in each well. After streaking (0.1) ml of fresh bacterial suspension on plates by sterilized cotton swab, and then the plate was left in the incubator at 37 °C for 24 hours. Next day, the diameter (mm) of the inhibition zone was measured. *A. niger* supernatant was included as control (Ramos-Zúñiga *et al.,* 2023).

Synergistic activity of CuNPs with Tetracycline antibiotic: Well diffusion method was employed in similar fashion as mentioned above to screen the synergistic effect of CuNPs with antibiotics. Tetracycline is the antibiotics used in this study. Each well of petriplates labelled with specific bacterial strain was filled with standard antibiotic solution (20 µg/well) and Cu-NPs solution (60 µl/well). In a similar way synergistic activity was screened for sample 2. All the plates were left to diffuse the sample and kept in an incubator at 37° for 24 h. The assays were performed in doublet and mean inhibition zone diameters were measured.

RESULTS AND DISCUSSION

Results

Isolation and Identification of *Aspergillusniger:* The fungal isolate recovered from onion peel samples exhibited rapid growth on Potato Dextrose Agar, producing black sporulating colonies with white margins. Microscopic examination revealed septate hyphae and characteristic conidial heads typical of *Aspergillusniger*, confirming the identity of the fungal isolate.

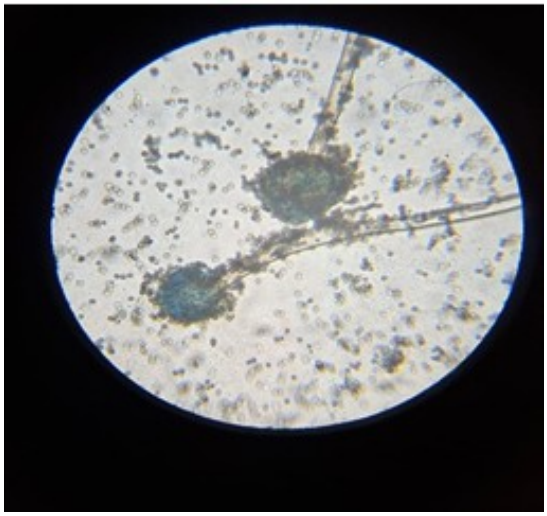

Fig.1. Microscopic view showing septate hyphae and conidial heads characteristic of *A.niger*

Ultraviolet Visible Spectroscopic analysisof fungal-mediated copper nanoparticles: The metal salt reduction to their respective metal ion was carried out by UV-spectroscopic analysis which confirms the stability of nanoparticles in the broth. The wavelength range of filtered broth was between 200- 400 nm, the maximum absorbance was observed at 340 nm. The spectral analysis was performed after every 24 hrs of incubation. The maximum absorbance was observed at the same wavelength for all other incubation periods as shown in

Fig.2 showing wavelength on x-axis and absorbance on the y-axis.

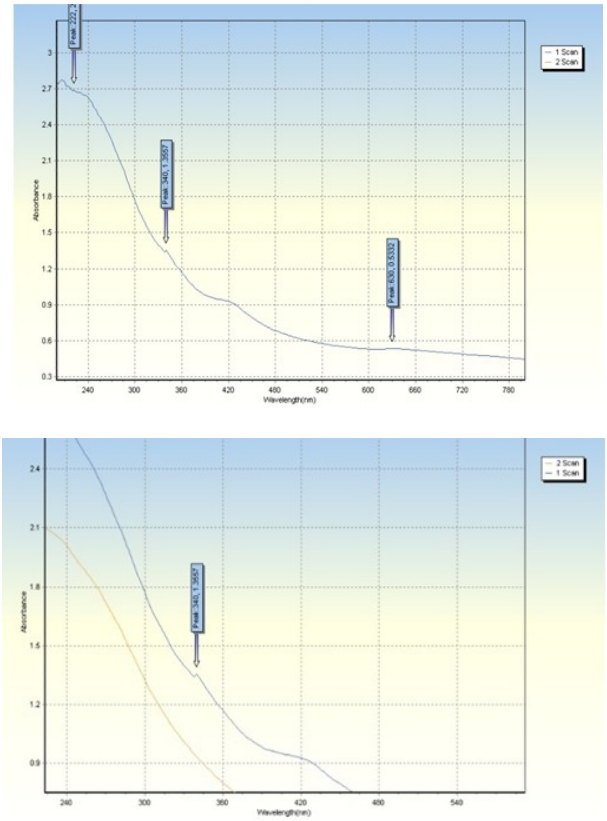

Fig. 2. UV–Visible absorption spectrum of fungal-mediated copper nanoparticles showing a characteristic absorption peak at 340 nm

The UV-Visible spectrum showed an absorption peak at 340 nm with a slight shift from the fungal filtrate peak (351 nm), suggesting the formation of CuNPs. The procedure used in this study has various advantageous features and become first choice above other described physical and chemical methods for CuNPs synthesis as there are no necessities of corrosive, toxic and flammable chemicals as needed for chemical methods (Bhavyasree*et al.,* 2020).

Products obtained through this procedure are nontoxic for human usage like biomedical applications because absorption of toxic chemicals on particles surfaces is unfeasible; but, it is clearly feasible in chemical techniques. More important, it is inexpensive and environmentally safe method and non-toxic product treatments are requisite at mild situations.

Biochemical characterization of municipal sewage water (MSW) isolates: Different biochemical tests were performed for the 2 isolates to know their biochemical characteristics and are noted in (Table 1).

Table 1. Biochemical test list for two Bacterial isolates

Serial no.	Tests name	Bacteria no. 1	Bacteria no.2
1.	Amylase test	+	+
2.	Catalase test	+	_
3.	Citrate test	+	+
4.	Indole test	_	_
5.	Methyl red test	_	_
6.	Motility test		
7.	Sugar fermentation test	+	+
8.	VogesProskauer test	+	+

Antibacterial activity of copper nanoparticles

Antibacterial activity of CuNPs against *Vibrio gazogenes* and *Staphylococcus faecalis* using the agar well diffusion method: The synthesized copper nanoparticles demonstrated significant antibacterial activity against both tested bacterial strains. The inhibitory effect increased with increasing nanoparticle concentration, indicating a concentration-dependent antimicrobial response (Martinez-Corona*et al.,* 2025). Among the tested concentrations, the highest antibacterial activity was observed at 80% CuNP concentration, whereas the lowest activity was recorded at 20% concentration. Vibrio gazogenes exhibited greater sensitivity towards CuNP treatment compared with *Staphylococcus faecalis*, as evidenced by larger inhibition zones.

Table 2. Zone of Inhibition Produced by CuNPs

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CuNP concentration	Vibrio (mm)	Streptococcus (mm)
20µL	No / very less zone	No / very less zone
40µL	No zone	11.23mm
60µL	9.18mm	11.69mm
80µL	10.16mm	13.49mm

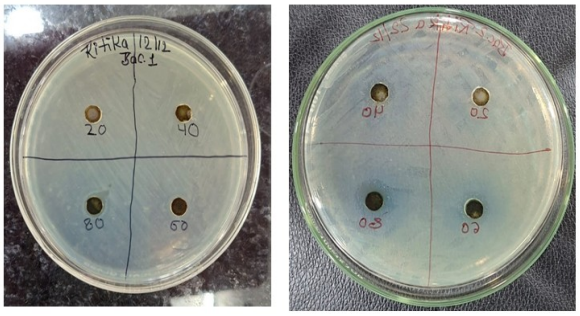

Fig.-3 Zone of Inhibition measured in mm by synthesised CuNPs

Synergistic Activity of CuNPs and Tetracycline: Drug resistance to antibiotics has immense impact on human and animal's health. In present decades, incidence rate of drug resistance has been increased due to excess utilization of antibiotics. The antimicrobial action of CuNPs was quantitatively observed by absence or presence of microbial growth inhibition or clear zones. The result showed in Table I and Figure 2 revealed that CuNPs , CuNps + tetracycline had antimicrobial activity against v. gazogenese , S. faecalis by showing 16.09 mm, 16.31mm in the only presence of CuNps and 32.7 mm and 17.85 mm clear zone with CuNps + tetracycline. Hance prove that CuNps in the presence of tetracycline provides more effective antimicrobial activity. The combination of copper nanoparticles and tetracycline exhibited enhanced antibacterial activity compared with either treatment alone. The inhibition zones produced by the combined treatment were significantly larger than those produced by tetracycline or CuNPs individually.The synergistic effect was observed against both bacterial isolates, suggesting that CuNPs enhanced the efficacy of tetracycline. The increase in antibacterial activity was more pronounced in *Vibrio gazogenes* than in *Staphylococcus faecalis*.

Table 3. Comparative analysis of two different bacterial strains

S. No.	strains	Cu-Nps (mm)	Cu-Nps + Tetracycline
1.	*V. gazogenenes*	16.09mm	32.7mm
2.	*Streptococcus faecalis*	16.31mm	17.85mm

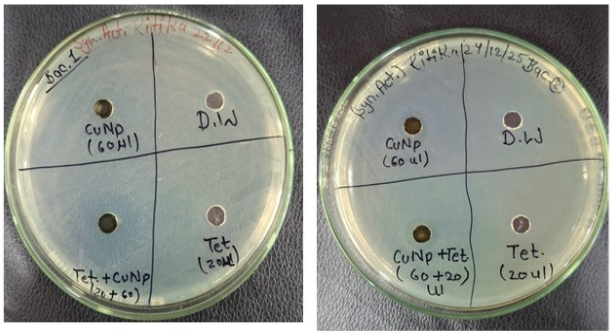

Fig. 4. Comparative antibacterial activity of CuNPs, tetracycline and CuNP–tetracycline combination against bacterial isolates

DISCUSSION

The present study demonstrated the successful synthesis of copper nanoparticles (CuNPs) using the extracellular filtrate of *Aspergillusniger* isolated from onion peel. The observed colour change of the reaction mixture from blue to greenish-brown following the addition of copper sulphate solution indicated the reduction of Cu^{2+} ions into copper nanoparticles. Fungal-mediated synthesis represents an environmentally friendly and cost-effective alternative to conventional chemical and physical methods of nanoparticle production (Martinez-Corona*et al.,* 2025). The ability of *A. niger* to secrete extracellular enzymes, proteins and other metabolites plays an important role in the reduction and stabilization of metal ions during nanoparticle formation. UV–Visible spectroscopic analysis was employed to characterize the synthesized nanoparticles. The fungal filtrate showed an absorption peak at 351 nm, whereas the nanoparticle-containing solution exhibited a peak at 340 nm. Although UV–Visible spectroscopy alone could not provide complete confirmation of CuNP synthesis, the observed shift in absorption wavelength together with the visible colour change suggested the successful formation of copper nanoparticles. Similar wavelength shifts have been reported in previous studies where fungal metabolites acted as reducing agents for the synthesis of metallic nanoparticles (Pourmadadi*et al.,* 2024). The bacterial isolates obtained from municipal sewage water were identified through morphological and biochemical characterization. The biochemical test results revealed distinct metabolic characteristics that assisted in the identification of the isolates. Sewage water is known to harbour diverse microbial populations, including potentially pathogenic bacteria, making it an appropriate source for evaluating antimicrobial agents.

The synthesized CuNPs exhibited antibacterial activity against both *Vibrio gazogenes* and *Streptococcus faecalis*. The antimicrobial effect increased with increasing nanoparticle concentration, indicating a concentration-dependent response. At lower concentrations (20 µL and 40 µL), little or no inhibition was observed, whereas larger inhibition zones were produced at 60 µL and 80 µL concentrations. *Streptococcus faecalis* showed slightly higher sensitivity to CuNP treatment than *Vibrio gazogenes*, particularly at higher nanoparticle concentrations. This finding suggests that bacterial susceptibility may vary depending on differences in cell wall structure, membrane composition and metabolic activity (Vergara-Llanos*et al.,* 2021). The antimicrobial activity of copper nanoparticles can be attributed to several mechanisms. Due to their small size and large surface area, CuNPs can interact directly with bacterial cell membranes, causing membrane disruption and leakage of intracellular contents. Copper nanoparticles are also capable of generating reactive oxygen species (ROS), which induce oxidative stress and damage cellular proteins, lipids and nucleic acids. Furthermore, released copper ions may interfere with enzyme systems and essential metabolic pathways, ultimately resulting in bacterial cell death. The significant finding of the present study was the enhanced antibacterial activity observed when CuNPs were combined with tetracycline. The inhibition zone against *Vibrio gazogenes* increased from 16.09 mm with CuNPs alone to 32.7 mm when combined with tetracycline, demonstrating a strong synergistic interaction. Similarly, the inhibition zone against *Streptococcus faecalis* increased from 16.31 mm to 17.85 mm in the combined treatment. The greater enhancement observed in Vibrio gazogenes suggests that the synergistic effect may vary among bacterial species. The observed synergy between CuNPs and tetracycline may be explained by multiple mechanisms. Copper nanoparticles can increase bacterial membrane permeability, facilitating greater uptake of tetracycline into bacterial cells. In addition, CuNP-induced cellular stress may weaken bacterial defence systems, thereby enhancing antibiotic effectiveness. Such synergistic interactions are particularly important in addressing the growing problem of antimicrobial resistance, as they may allow lower antibiotic doses to achieve improved antibacterial efficacy.

CONCLUSION

The present findings support previous reports demonstrating that metal nanoparticles can potentiate the activity of conventional antibiotics. The use of biologically synthesized CuNPs in combination with antibiotics may therefore represent a promising strategy for controlling bacterial infections and combating antibiotic-resistant microorganisms.Overall, the results indicate that *Aspergillusniger*-mediated copper nanoparticles possess notable antibacterial properties and exhibit enhanced antimicrobial activity when used in combination with tetracycline. These findings highlight the potential application of fungal-derived CuNPs as eco-friendly antimicrobial agents and antibiotic adjuvants for future biomedical applications.However, additional studies involving advanced nanoparticle characterization techniques such as SEM, TEM, XRD and FTIR, as well as toxicity assessments and evaluation against clinically relevant multidrug-resistant pathogens, are required before clinical or industrial applications can be considered.

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