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# Antibacterial Application of Copper Nanoparticles Biosynthesized by Water Caltrop Pod.

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**Abstract**

This study investigates the utilization of water caltrop pod, an abundant agricultural waste product, as a green extract for the optimized biosynthesis of copper nanoparticles (CuNPs). To comprehensively characterize the water caltrop pod and the biosynthesized CuNPs by water caltrop pod a sophisticated techniques were employed, including U.V-Vis spectrophotometry for probing their preliminary analysis of copper nanoparticles, FT-IR spectroscopy for elucidating the functional groups present in water caltrop pod, Scanning Electron Microscopy (SEM) for visualizing the morphology, Energy Dispersive X-ray (EDX) for elemental analysis while Energy-Dispersive X-ray (XRD) to determine crystalline structure of copper nanoparticles. Furthermore, the antibacterial application of these biogenic copper nanoparticles was explored. The antibacterial activity of copper nanoparticles (CuNPs) was investigated against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria, revealing their efficacy in combating microbial growth employing a Well Diffusion method. Copper nanoparticles showed greater antibacterial treatment against Gram (+ve) bacteria i.e. *Staphylococcus aureus* as compared to Gram (-ve) bacteria i.e. *Escherichia coli.* This research paves the way for the sustainable production of bio-functional CuNPs from waste biomass, offering promising application in antibacterial therapies.

**Keywords:** Characterization, Concentration, *Escherichia coli*, Penta hydrated copper, sulphate, *Staphylococcus aureus*.

## Introduction

Nanoparticles exhibit unique properties (Ream *et al*., 2024) such as reducing-agent (Giorgio *et al*., 2021), biosensor in reducing pollutants (Harini *et al*., 2023), in reducing pollutant (Chao *et al*., 2024; Vishnu, 2023), composites for removal of pollutants (Xiaoming *et al*., 2024), polymers (Aparana *et al*., 2024). Nanoparticles comprises metals-ion, metal-oxides (Siyuan *et al*., 2023), non-metals (Hanna *et al*., 2024), organic (lignin-based) (Deblina *et al*., 2023). Nanoparticles of varied shape and sizes (Amrita *et al*., 2016) can be incorporate by physical, chemical and biological approaches (Mehrab *et al*., 2024). Physico-chemical mode resulted to low-yield, high-cost and ecological harmful (Uribe-Lopez *et al*., 2021). The biological-mode involves the usage of plants (Fuad *et al*., 2023)/micro-organisms (Katarzyna *et al*., 2021). Microorganism is riskier because of their side effects which may cause other reactions (Katarzyna *et al*., 2021). Methods of nanoparticles by green-material (Ravinra and Vishnu, 2023) comprising utilization of whole-plants (Ravinra and Vishnu, 2023), plants-extracts (Pratibha *et al*., 2024) and phytochemicals (Mehranaz and Mansur, 2024). Plants-extract can be made from leaves (Ahmed *et al*., 2024), from husks (Yee-Shing *et al*., 2018), from stems (Parichehr *et al*., 2022), utilizing roots (Shereen *et al*., 2021), using flowers (Fatima *et al*., 2020; Renata and Jolanta, 2016), from barks (Monisha *et al*., 2024), using fruits (Azza *et al*.,2024), applying seeds (Bijoy *et al*., 2023) and from peels (Khoiriah and Reza, 2018). Biosynthesis of metal nanoparticles was proclaimed by utilizing fruit extracts (Monisha *et al*., 2024), root-extract (Shereen *et al*., 2021) [22] and leaves-extract (Ahmed *et al*., 2024). Biosynthesis of metal nanoparticles is accomplished by reacting a metal precursor with a green waste material (Nervy *et al*., 2018; Kamalpreet *et al*., 2022) under certain parameters, often aiming to produce metal nanoparticles.

The green waste materials have an enriched composition like lignin and cellulose containing the carbohydrates, amino acids. Taking the advantage of this composition, an intention has been created to couple green waste material with the metal nanoparticles to biosynthesize metal nanoparticles of various shapes and sizes (Amrita *et al*., 2016). The green waste material extract (Kamalpreet *et al*., 2022) treating with metal-ions or metal-oxides was carried out. In the present work biosynthesis of copper nanoparticles (CuNPs) involving water caltrop pod (WCP) has been selected as it has gained considerable interest in recent years as a facile, effective, cheap (Kamalpreet *et al*., 2022) and eco-friendly method (Omnia and Deyab, 2023). Copper nanoparticles synthesis depends on several factors like extraction mode, biosynthesis parameters like temperature, pH, contact time and concentration of metal ions or metal-oxides (Siyuan *et al*., 2023). Characterization of water caltrop pod and copper nanoparticles synthesized by water caltrop pod was done using various analytical instruments and copper nanoparticles utilized for antibacterial treatment against Gram (+ve) bacteria i.e. *Staphylococcus aureusas* as compared to Gram (-ve) bacteria i.e. *Escherichia coli* (Ahmed *et al*., 2024; Nervy *et al*., 2018). The objective of this work is to utilize green waste material like water caltrop pod to biosynthesize copper nanoparticles and its applications for treatment of pathogens such as *Staphylococcus aureusas* and *Escherichia coli.*

## Material and Method

Water caltrop pod (WCP) Eleocharis-dulcis a Trapa nut is an aquatic annual herb that grows in ponds or marshes with its roots in the muddy soil at the bottom of water and with its upper leaves floating on the water surface. Water caltrop pods purchased from local market in Hyderabad, Sindh province, Pakistan. All the compounds or chemicals utilized throughout the practical work were chemically pure and analytically equivalent-grade. Water caltrop pod, $CuSO_4.5H_2O$, Deionized water, Filter papers, Conc. HCl, Conc. $H_2SO_4$, $HNO_3$, 2-Propanol, NaOH, $CH_3COOH$, $CH_3COONa$, $NH_3$ liquid, Sodium EDTA, Methanol, obtained from Sigma-Aldrich and Merck (Germany).

Water caltrop pods were separated from water caltrop and dried. To obtain water caltrop pod extract, a 20 g water caltrop pod was heated in 250 mL of deionized $H_2O$ for an hour at 90 $^0$C and centrifuged at 500 rpm for 2 hours. Filtered the water caltrop pod extract and utilized it to get biosynthesized copper nanoparticles (Khoiriah and Reza, 2018). The water caltrop pod extract treated with penta hydrated copper sulphate was carried out. Several factors like, concentration of metal precursor, temperature of solution, agitation-time, agitation-speed were reviewed (Siyuan *et al*., 2023). 100 mL water caltrop pod extract equilibrated with 200 mL penta hydrated copper sulphate (0.1 M) in 1:2 at 80 $^0$C and centrifuged at 1000 rpm for 2 hours by using 79-1Magnetic Stirrer with hotplate Jiangsu Jinyi Instrument Technology Company Limited made in China used for biosynthesis of copper nanoparticles. The solution color switched from light blue to dark green indicated the biosynthesis of copper nanoparticles. A peak at 560 nm confirmed successful biosynthesized copper nanoparticles (Figure 1). Then dried the product, copper nanoparticles were put in the oven at 45 $^0$C for 4 h.

The antibacterial activity of these nanoparticles was evaluated by using Gram positive & Gram negative bacteria i.e. *Staphylococcus aureus* and *Escherichia coli* respectively employing a Well Diffusion Mode. Blood samples collected through swab consist of a sterile cotton tipped sticks and prepared media by adding copper nanoparticles. Blood samples then transferred to blood culture bottle. A slide test for bound coagulase and takes several hours to overnight to produce results. Applying MHA (Mueller Hinton Media), 1mg CuNPs + 1L $H_2O$. Heated it then sterilized using steam sterilizer at 121$^0$C for half an hour, nurtured at 37 $^0$C for 1 day then observed the inhibition zone (Ahmed *et al*., 2024; Nervy *et al*., 2018).

## Results

**UV-Visible Analysis:** Preliminary identification of copper nanoparticles (CuNPs) and water caltrop pod (WCP) extract was done using U.V-Vis spectrometer. A peak at 560 nm confirmed successful biosynthesized copper nanoparticles (Figure 1).

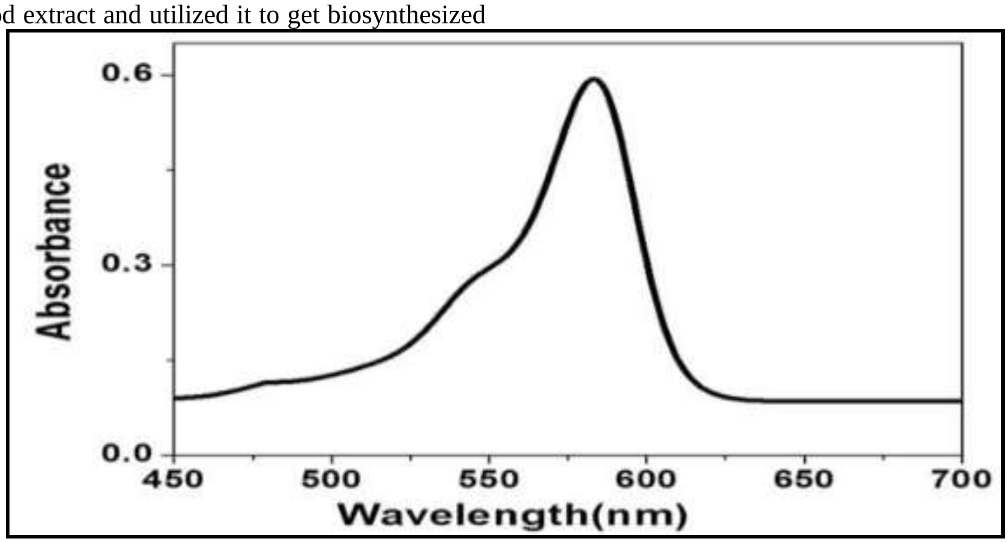

**Figure 1.** Ultraviolet visible Spectra of copper nanoparticles biosynthesized by water caltrop extract treated with 0.1 $mgL^{-1}$ $CuSO_4.5H_2O$ using hot plate with magnetic stirrer. Graph plotted Absorbance ranges 0 to 0.6 vs wavelength ranges 450 to 700 nm. Maximum absorbance was 0.6 at 560 nanometer (nm) wavelength.

**FT-IR** spectra of **CPP:** WCP infrared spectrum was attained to explore the presence of functional groups in water caltrop pod. Peaks appeared were 3341.09, 1594.86, 1340.08, 1129.96 and 945.23 $cm^{-1}$. 3341.49 $cm^{-1}$ for O:H stretches of alcohol. 1594.86 $cm^{-1}$ for carbonyl compounds C=O stretches of RCOOR (ester), ROOH (acid) or RCOR (ketone), 1129.96 and 945.23 $cm^{-1}$ for C:N or N:H deformation of amines respectively were recorded in spectra of water caltrop pod as shown in Figure 2.

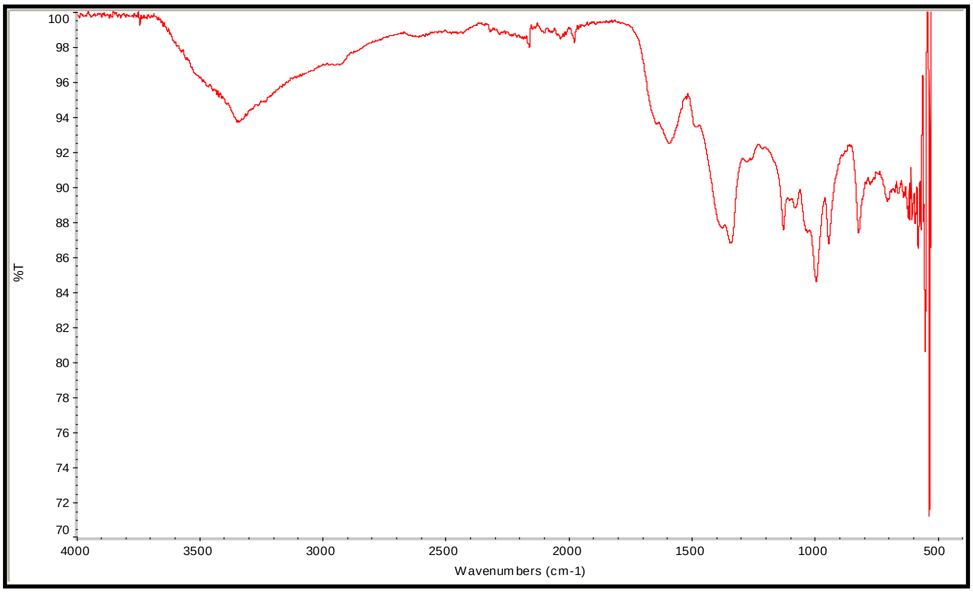


**Figure 2.** FT-IR spectra determines the molecular structure of water caltrop pod (WCP). Graph plotted Transmittance percentage (% T) vs wavelength ranges from 500 to 4000 per centimeter ($cm^{-1}$). Peaks appeared were 3341.09, 1594.86, 1340.08, 1129.96 and 945.23 $cm^{-1}$.

**Sem Analysis**: The surface morphology of water caltrop pod and copper nanoparticles exhibit a micro-porosity at 100x resolution while the images of water caltrop pod have particle size of 10 micro-meter. SEM analysis showed the images of water caltrop pod and copper nanoparticles biosynthesized by water caltrop pod. Figure 3. (A) and (B) SEM image of copper nanoparticles biosynthesized by water caltrop pod.

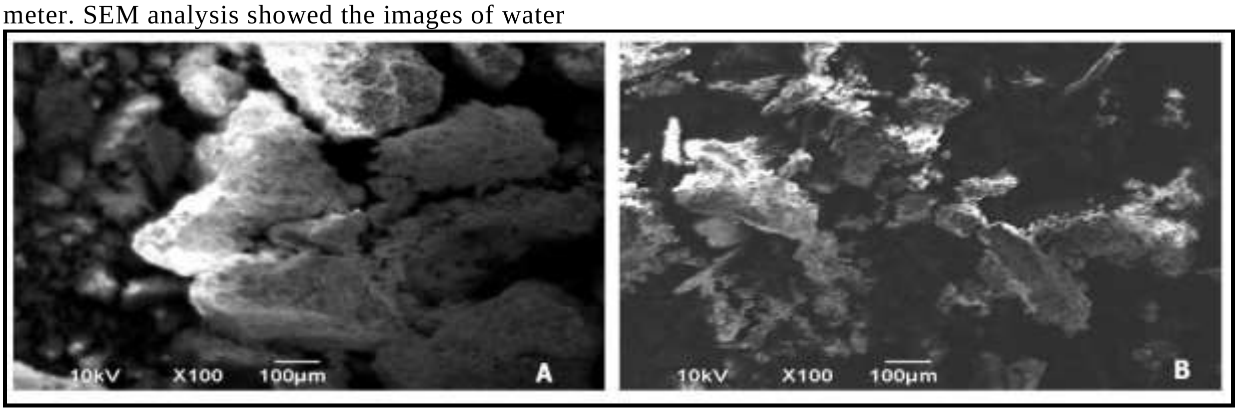


**Figure 3.** (A) and (B) scanning electron microscope (SEM) image of water caltrop pod and copper nanoparticles respectively. The surface morphology of water caltrop pod and copper nanoparticles was elaborated by Hitachi S-2300 SEM with 10 kV at x100 resolution and 100 micro meter (μm). The SEM was equipped with EDX, which elaborated the elemental composition of WCP and CuNPs surfaces. The samples were carbon-coated using Edwards-Scan coat.

**EDX analysis of CPP:** Water caltrop pod elemental composition was explored by EDX analysis. The EDX analysis of water caltrop pod appeared in Figure 4. (A), elaborate the presence of different elements comprises maximum quantity of K. EDX spectrum of CuNPs biosynthesized by water caltrop pod shown in Figure 4. (B).

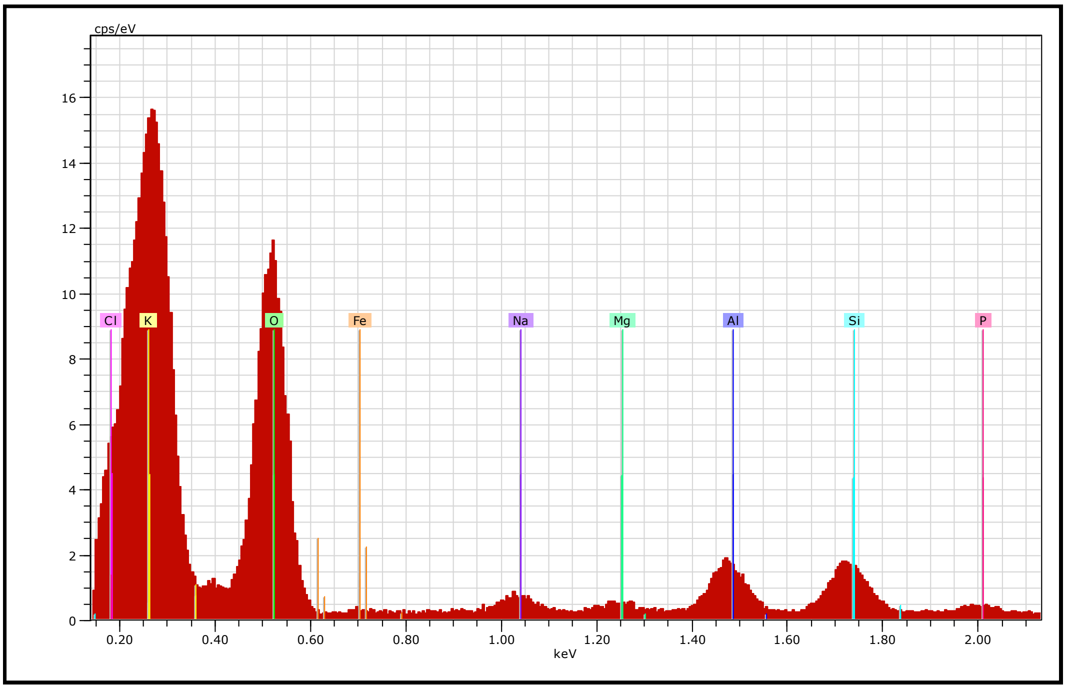


**Figure 4. (A)**

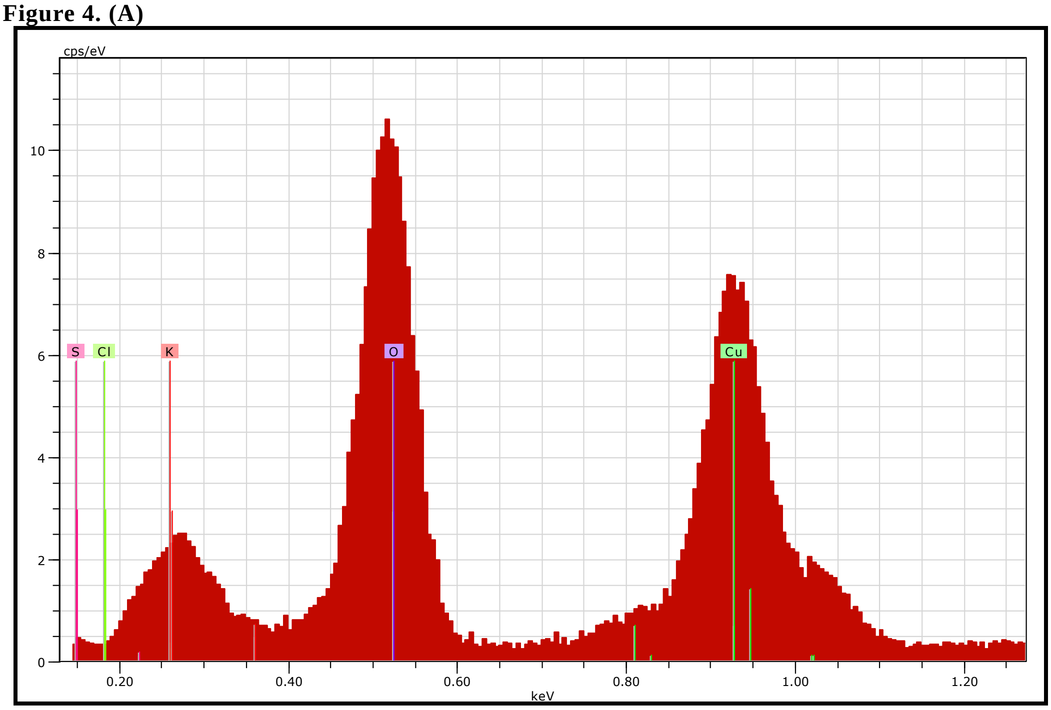


**Figure 4. (A)**

**Figure 4. (A) and (B)** EDX spectra of water caltrop pod (WCP) and copper nanoparticles (CuNPs) respectively. EDX spectra which elaborated the elemental composition of water caltrop pod (WCP) comprises cl (chlorine), K (potassium),(O) oxygen, Na (sodium), Mg (magnesium), Al (aluminum), Si (silicon), P (phosphorus) respectively. While in (B) of copper nanoparticles (CuNPs) S (sulphur), cl (chlorine), K (potassium), O (oxygen) and Cu (copper) respectively

**XRD Analysis of Copper Nanoparticles:** Copper nanoparticles structure and size of the biosynthesized copper nanoparticles were investigated using X-ray diffractometer. The XRD investigation of biosynthesized CuNPs from water caltrop pod (WCP) extract showed peaks at $2\theta$ = $22^0$, $25^0$, $29^0$, $35^0$, $38^0$, $46^0$, $47^0$, $52^0$ and $75^0$ corresponding to Miller indices (46), (73), (40), (70), (35), (15), (17), (22) and (19) respectively represent face centered cubic structure of copper nanoparticles (Roy *et al.*, 2019; Temitayo, 2022). Biosynthesized copper nanoparticles from water caltrop pod (WCP) sizes was found to be 42.5 nm and 46 nm respectively.

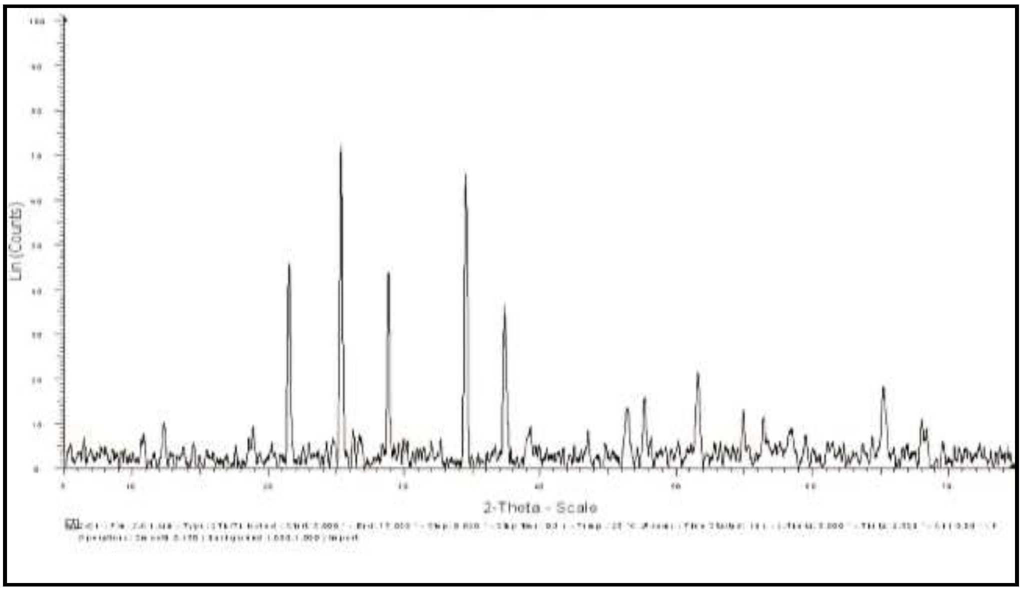


**Figure 5.** The XRD investigation of biosynthesized copper nanoparticles (CuNPs) from water caltrop pod (WCP) extract showed peaks at 2-theta (2θ) = $22^0$, $25^0$, $29^0$, $35^0$, $38^0$, $46^0$, $47^0$, $52^0$ and $75^0$ corresponding to Miller indices (46), (73), (40), (70), (35), (15), (17), (22) and (19) respectively. The CuNPs size evaluated using Scherrer's eq: $D = K\lambda / \beta½.\cos\theta$. D and β are crystal dimension & Line-broadening in Radius (F.W.H.M of peak in Radius). λ and θ are X-Rays wavelength & Bragg's-angle respectively**.**

**Antibacterial Application:** The antibacterial activity of these nanoparticles was evaluated by using Gram positive & negative Bacteria i.e. *Staphylococcus aureus* and *Escherichia coli* respectively employing a Well Diffusion Mode in Figure 6. (A) and 6 (B). After performing experiments, the results showed that copper nanoparticles possess excellent broad-spectrum antibacterial activity against both groups of bacteria by showing very well prominent zones of inhibition for *Staphylococcus aureus* was 15.6 mm and for *Escherichia coli* 13.6 mm.

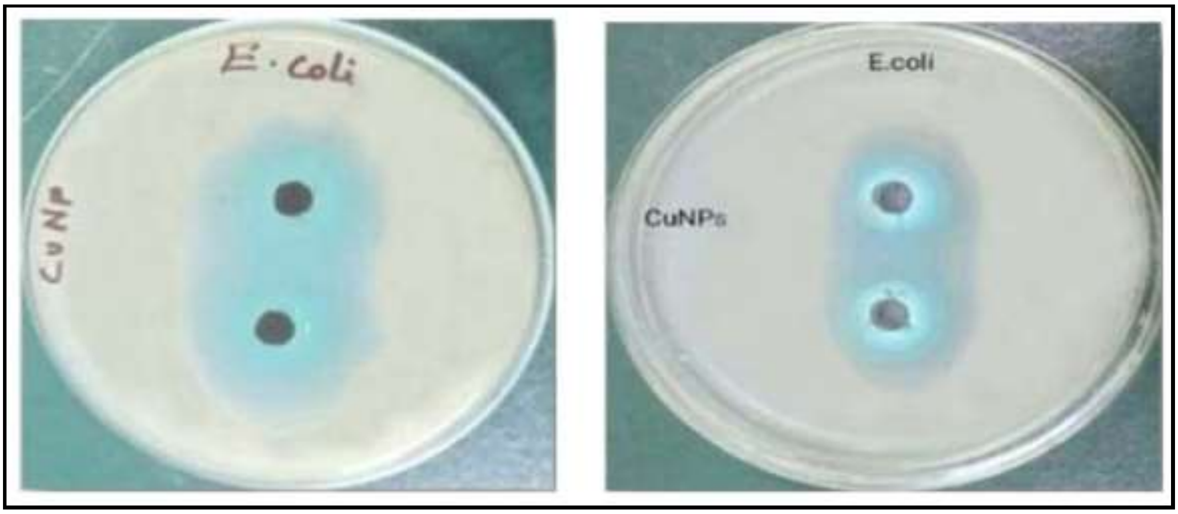


**Figure. 6** (A) zone of inhibition in mili meter (mm) of *Escherichia coli*. The antibacterial activity of copper nanoparticles was evaluated by using Gram-negative Bacteria i.e. *Escherichia-coli* (E-coli) employing a Well Diffusion method. Applying MHA (Mueller Hinton Media) then observed the Inhibition zone.

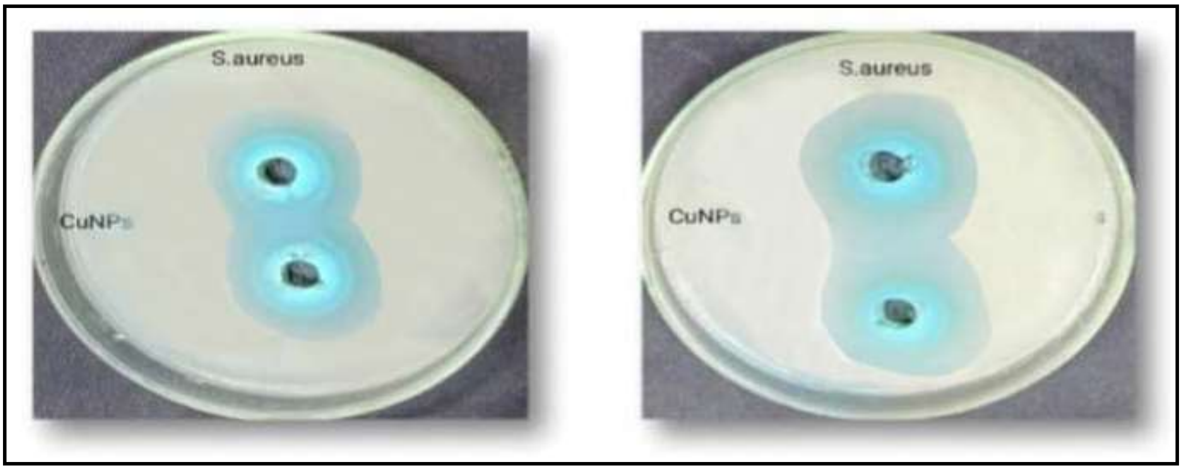


**Figure. 6 (B)** zone of inhibition in mili meter (mm) of *Staphylococcus aureus*. The antibacterial activity of copper nanoparticles was evaluated by using Gram positive Bacteria i.e. *Staphylococcus aureus* employing a Well Diffusion method. Applying MHA (Mueller Hinton Media) then observed the Inhibition zone.

Overall, this data showed that the results are very promising. Copper nanoparticles showed greater anti-bacterial treatment against *Staphylococcus aureus*.

The mechanism of action of copper nanoparticles involve the release of metal ions, which can damage bacterial cell membrane and interfere with cellular processes, ultimately leading to inhibition of bacterial growt.

**Table 1.** Zone of inhibition in mili meter (mm) of *Escherichia coli* (E coli) and *Staphylococcus aureus* (S aureus) for copper nanoparticles (CuNPs).

| | | | | | Zone of Inhibition (mm) | | | |
|---|---|---|---|---|---|---|---|---|
| Nanoparticle Sample | *E. coli* (Test 1) | *E. coli* (Test 2) | *E. coli* (Test3) | Average | *S. aureus* (Test 1) | *S. aureus* (Test 2) | *S. aureus* (Test 3) | Average |
| CuNPs | 14 | 13 | 14 | 13.6 | 16 | 15 | 16 | 15.6 |

In this study, copper nanoparticles (CuNPs) showed zone of inhibition of 13.6 mm zone against *Escherichia coli* while the zone of inhibition of 15.6 mm was observed against *Staphylococcus auerus* (Table 1).

## Discussion

The biosynthesis of copper nanoparticles using water caltrop pod was studied for antibacterial application of copper nanoparticles against two bacterial strains *Staphylococcus aureus* and *Escherichia coli* as a positive and negative controls (Mehrab Pourmadadi *et al*., 2024). Biosynthesis of copper nanoparticles using water caltrop pod is an effective, cheap (Kamalpreet *et al*., 2022) and eco-friendly method (Omnia and Deyab, 2023). Copper nanoparticles synthesis depends on several factors like extraction mode, biosynthesis parameters like temperature, pH, contact time and concentration of metal ions or metal-oxides (Siyuan *et al*., 2023).

Characterization of water caltrop pod and copper nanoparticles biosynthesized by water caltrop pod was done using various analytical instruments (Siyuan *et al*., 2023). Preliminary identification of copper nanoparticles and water caltrop pod extract was done using U.V-Vis spectrometer. The CuNPs was formed in a brick brown color during the reaction time was investigated by UV-Vis spectrophotometer Perkins Elmer (Uberlingen, Germany) was used for the analysis of biosynthesized copper nanoparticles. A peak at 560 nm confirmed successful biosynthesized copper nanoparticles (Khoiriah and Reza, 2018). The FT-IR spectra of water caltrop pod (WCP) was attain to elaborate the functional groups present in water caltrop pod (WCP). The FT-IR spectra of water caltrop pod was evaluated in the range of 4000-625 $cm^{-1}$. The surface morphology of water caltrop pod (WCP) and CuNPs exhibit a micro-porosity at 1000x resolution while the images of water caltrop pod (WCP) and particle size of 10 micro-meter. Water caltrop pod (WCP) elemental composition was explored by EDX analysis. XRD measurement for CuNPs synthesized by water caltrop pod extract carried out in scattering range 2θ. The same analysis elaborated by Minakshi (Minakshi A Thakar *et al*., 2022) and indicated the CuNP's crystallinity & cubic geometry.

The antibacterial activity was evaluated by measuring the zone of inhibition surrounding the disc in mm and recorded the results. The antibacterial activity of these nanoparticles was evaluated by using Gram positive & Gram negative bacteria i.e. *Staphylococcus aureus* and *Escherichia coli*. Copper nanoparticles showed greater antibacterial treatment against Gram (+ve) bacteria i.e. *Staphylococcus aureus* as compared to Gram (-ve) bacteria i.e. *Escherichia coli* (Ahmed *et al*., 2024; Nervy *et al*., 2018). From inhibition zone results, biosynthesized copper nanoparticles from water caltrop pod showed higher inhibition activity against *Staphylococcus aureua*. A significant inhibition of zone was shown against gram-positive bacteria. The zone of inhibition against *Staphylococcu aureua* was 15.6 mm and for *Escherichia coli* was 13.6 mm. The antibacterial effect is directly related to the nanoparticles size, minimum inhibitory concentration of nanoparticles, and oxidation degree of surface (Amrita Banerjee *et al*., 2016). The inhibition zone of bacteria decreases as the concentration of nanoparticles decreases. (Pratibha Kaushal *et al*., 2024).

**Conclusion**

Copper nanoparticles biosynthesized by water caltrop pod extract a natural waste material under optimized parameters. The biosynthesized copper nanoparticles were characterized by different analytical instrumental techniques such as FT-IR spectrum displayed a number of peaks appeared in the range 4000-625 $cm^{-1}$. The SEM images revealed the highly porous surface of copper nanoparticles biosynthesized by water caltrop pod. EDS and XRD depicted the confirmation of copper within the surface of material. Overall copper nanoparticles successfully utilized in antibacterial activity against Gram (+ve) bacteria i.e. *Staphylococcus aureus* by showing 15.6 mm zone of inhibition as compared to Gram (-ve) bacteria i.e. *Escherichia coli* having 13.6 mm zones of inhibition. Biosynthesized copper nanoparticles can be applied in number of commercial applications such as medicines, agriculture, optics and environmental remediation.

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**Author Contributions**

Writing-original draft, Farooque Azam Khatri. Visualization, Jamil-ur-Rehman Memon, Imam Bakhsh Solangi and Ghulam Zuhra Memon. Writing-review, Muhammad Imran Khattak and Fayaz Ahmed Keerio. All authors have read and agreed to the published version of the manuscript.

**Conflict of Interest**

The authors have not any conflicts of interest.

**Data availability**

All relevant data generated or analysed are included in this published article.

**Declarations**

Ethics approval and consent to participate All applicable international, national and/or institutional guidelines were followed.

**Competing Interests**

The authors declare no competing interests.

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