

# Green Synthesis of Copper Nanoparticles Using Leaf Extract of *Ocimum sanctum* and its Antimicrobial Activity

Pradnya Zambare<sup>1,2</sup>, Avinash Survase<sup>1,2</sup>, Shivangi Kanase<sup>1,2,\*</sup>

<sup>1</sup>Rayat Institute of Research and Development, Satara, Maharashtra, INDIA.

<sup>2</sup>Department of Microbiology, Yashwantrao Chavan Institute of Science, Satara, Maharashtra, INDIA.

## ABSTRACT

**Objectives:** We reported a non-toxic, low-cost and environmentally friendly green synthesis technique for the manufacture of copper nanoparticles (Cu NPs) utilizing *Ocimum sanctum* (*O. sanctum*) leaf extract to prevent hospital-acquired and Methicillin-resistant pathogenic bacterial infections strains. **Materials and Methods:** The biogenic synthesis of Cu NPs via chemical precipitation method using *O. sanctum* phytochemical extract as stabilizing and reducing agent. Cu NPs syntheses are characterized using UV-visible spectroscopy (UV-vis spectroscopy), X-ray diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM) to analyze and confirm size and nature of Cu NPs. **Results:** UV-vis spectroscopy of Cu NPs showed an absorption peak at 528 nm with calculated band gap 2.1 eV. XRD analysis indicates crystallite nature of Cu NPs having an average size 48 nm. FTIR showed involvement of *O. sanctum* extract biomolecules in capping process Cu NPs. The hexagonal morphology structure of Cu NPs nanoparticles is observed by SEM analysis. Cu NPs showed antibacterial activity against *Staphylococcus aureus* ( $38.66 \pm 1.15$  mm), *Escherichia coli* ( $43.33 \pm 1.15$  mm), *Pseudomonas aeruginosa* ( $21.66 \pm 0.57$  mm) and *Proteus vulgaris* ( $42.66 \pm 0.57$  mm). **Conclusion:** We report synthesis of simple, inexpensive and eco-friendly *O. sanctum* mediated Cu NPs. The biophysical characterization techniques used for analyze size, shape and nature of nanoparticles. The 32 ug/ml Cu NPs concentration showed excellent antimicrobial activity against Gram positive and Gram-negative bacteria. Cu NPs efficiently used to prevent *Proteus vulgaris* mediated infection.

**Keywords:** Antimicrobial activity, Green nanotechnology, *Ocimum sanctum*, Cu NPs, XRD, SEM.

## Correspondence

**Dr. Shivangi Kanase**

Associate Professor, Department of Microbiology, Yashwantrao Chavan Institute of Science, Satara, Maharashtra, INDIA.

Email id: shivangikanase7@gmail.com

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## INTRODUCTION

Due to ever increasing demand there is continuous increase in the production of nanoparticles and 25 %-fold increase in the production was already reported between 2005 and 2010.<sup>1</sup> Nanoparticles (NPs) are ultra-small units in the size range of 1 to 100 nm with increased surface area and are very active. Copper (Cu) is an essential trace element for plants, animals and human beings. It is a cofactor for numerous enzymes involved in the synthesis of neuropeptides, a cell signalling pathway regulator, antioxidant defence, and engaged in human immune cell activities that are responsible for pathogen eradication.<sup>2</sup> Hence recently synthesis of Cu NPs gained much attention and importance.

The peculiar properties of CuO (Copper oxide) NPs, like high electrical conductivity, low electrochemical migration behaviour high melting point and after all its low cost, there are many applications in different areas such as biomedical (antimicrobial,

antibiotics, antifungal, drug delivery, antioxidants, and anticancer), textile industries, gas sensors, catalytic, synthesis of inorganic-organic nanosized composites, thermo sensing and conducting materials, environmental remediation, magneto resistant materials, high-temperature superconductor.<sup>1-6</sup> Cu NPs act as alternatives for noble metals such as in inkjet printing, for organic transformations, gas phase catalysis, photocatalysis and electrocatalysis.<sup>7,8</sup>

Recent development in the field of nanotechnology leads to production of metal nanoparticles by various methods. Among available methods, biological method is preferable because of its certain advantages like ecofriendly nature, convenient method, generates nontoxic products.<sup>9</sup> The biological approach to metal or metal oxide nanoparticle synthesis emphasises the use of bacteria, fungi, algae, yeast, and plant extracts as reducing agents for nanoparticle formation which support large-scale production and biocompatibility.<sup>10,11</sup> CuO NPs synthesis has gained a lot of attention because of its long-term viability, low cost, and ease of use.<sup>12</sup>

Combining nanotechnology and biology has enormous potential for developing drugs to treat infectious diseases.<sup>13</sup>



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For researchers in the field of bio-nano-science, this peculiar behaviour of nanoparticles in the medical field in comparison to ordinary materials has opened a new doorway. In addition to their use in other fields, nanoparticles have demonstrated their value in the field of medicine and exhibit broad-spectrum antibacterial action against a variety of diseases.<sup>14,15</sup> Nanoparticles with diverse nature, greater stability and appropriate dimensions are synthesised by green synthesis techniques. Moreover, the undesired conditions during chemical synthesis are eliminated and conditions like physiological temperatures, pH, and pressure are only used in green synthesis with biological components acting as reducing and capping agents and that too at a negligible cost.<sup>16,17</sup> Since it is simple to use and can accommodate large-scale manufacturing, the green approach for the synthesis of nanoparticles, which is a developing field, may have advantages over other methods.<sup>18,19</sup> Due to the many benefits it offers, this innovative method for creating nanomaterials, especially Cu NPs, is greatly desired.<sup>20,21</sup> The present study reports green synthesis of Cu NPs using leaf extract of a medicinal plant viz *O. sanctum* and evaluation of its antimicrobial activity.

## MATERIALS AND METHODS

### Preparation of *Ocimum sanctum* Leaf Extract

For the synthesis of Cu NPs, leaves *O. sanctum* (Tulsi) were used. About 25 g of healthy, fresh leaves were collected, washed thoroughly with deionized water to remove dust, dried in oven at 15-20 min at 50°C. Then leaves were cut into small pieces and added to 100 ml of deionized water in 250 ml capacity Erlenmeyer flask the flask was heated in water bath for 30 min at 60°C. The resultant extract was filtered using Whatman filter paper no.1 and stored in refrigerator for further use.

### Green synthesis of copper nanoparticles

For synthesis of Cu NPs, 20 ml of aqueous *O. sanctum* leaf extract was added to 200 mL of copper sulphate solution, and the mixture was magnetically agitated at room temperature for 30 min until the light blue hue turned light green, indicating the preliminary stage of the synthesis of copper nanoparticles.

The green-colored solution was magnetically agitated for three hours and then heated for two minutes at 80°C. The development of water-soluble Cu NPs is indicated by the transformation of the green mixture into a brown precipitate after adding 1 M NaOH drop by drop. To eliminate the contaminants, the brownish-black precipitate was repeatedly washed with deionized water and then ethanol.<sup>22</sup> The resulting brown colored product was subjected for analysis using UV visible spectrophotometer, FTIR, XRD, SEM.

### Physicochemical characterization

**UV Visible spectrophotometry:** In order to confirm the synthesis of copper nanoparticles on preliminary basis, color change was observed visually, and the intensity of colour change

was determined in the range 300-800 nm using double beam Systronics, UV-visible spectrophotometer instrument.

**Fourier transform infrared spectroscopy (FTIR):** The FTIR of dried powder Cu NPs and liquid *O. sanctum* leaves extract samples was analyzed using lambda-FTIR 7600 instrument, for determination involvement of functional groups in Cu NPs formation. 0.5 mg Cu NPs dried sample grind with potassium bromide as a substrate and prepare 2 mm diameter pellet using hydraulic press. Prepared pellet scanned in the range 400-4000  $\text{cm}^{-1}$ .

**X-ray diffraction (XRD):** In order to determine crystal phases and nature of nanoparticles determine by using powder X-ray diffraction technique. Bruker D8 advance, INDIA with Cu K $\alpha$  ( $\lambda = 1.54 \text{ \AA}$ ) radiation, X ray diffractometer used in the scanning range of 5 to 90°C.

**Scanning Electron Microscopy (SEM):** The JEOL, JSM IT 200, 20kv voltage Scanning Electron Microscopy (SEM) was used for analyze morphological characteristics of green synthesized Cu NPs. Dried powder of Cu NPs coated on carbon grid.

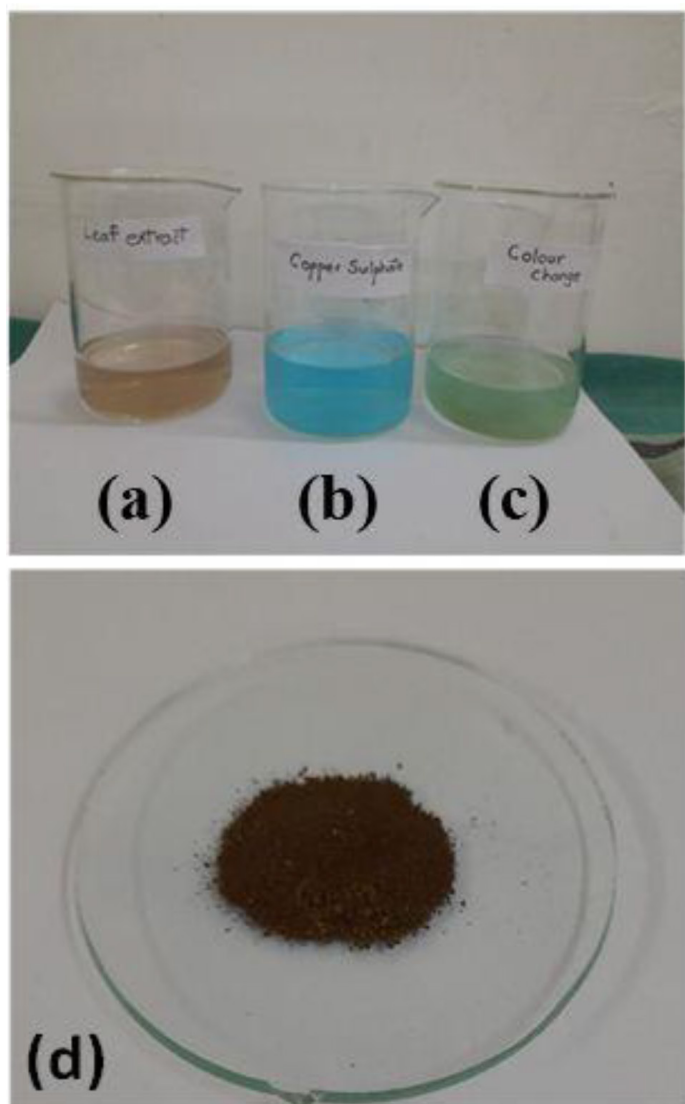
**Antibacterial activity of Cu NPs:** The antibacterial activity of synthesized Cu NPs was investigated by Kirby Baur agar diffusion method against selected Gram positive and Gram negative bacterial pathogenic strains such as *Staphylococcus aureus* NCIM-2654, *Pseudomonas aeruginosa* NCIM-5032, *Proteus vulgaris* NCIM-2813 and *E. coli* NCIM-2832. Cell density of 24 hr old culture of this test organism was adjusted using McFarland standard number 0.5. The nutrient agar plates were swab inoculated with individual bacterial strains separately using a sterile glass rod. Powder of Cu NPs was added in DMSO solvent separately and four different concentrations such as 4  $\mu\text{g/ml}$ , 8  $\mu\text{g/ml}$ , 16 $\mu\text{g/ml}$  and 32 $\mu\text{g/ml}$  were obtained separately. The wells were prepared on nutrient agar plates using sterile cork borer. Prepared solutions of different concentrations were added to wells separately and allowed to diffuse followed by incubation at 37°C for 24 hr. Antibacterial activity was then measured in terms of zone diameter of inhibition in millimetres.<sup>23</sup>

The experiments were carried out in triplicates. The diameter of the zone of inhibition was expressed as mean  $\pm$  standard deviation.

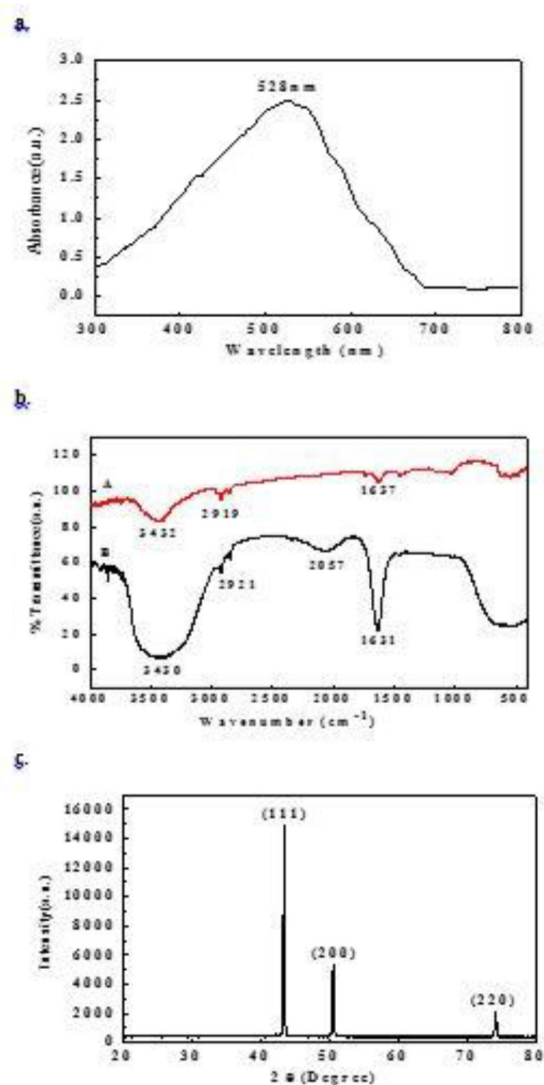
## RESULTS

### Biosynthesis of Cu NPs

Cu NPs were successfully synthesized using an easy, safe, straightforward and less downstream processing *O. sanctum*-based approach. Figure 1 (a) shows brown colour *O. sanctum* leaf extract. Figure 1 (b) show bluish colour copper sulphate solution and Figure 1 (c) show transformation brownish black precipitate Cu NPs after microwave heating irradiation. Figure 1 (d) indicates brownish-black powder Cu NPs.



**Figure 1:** (a)- *O. sanctum* leaf extract, (b)-Copper sulphate, (c)- Cu NPs, (d) Dry powdered Cu NPs.



**Figure 2:** a. UV Visible spectrum of Cu NPs b. FTIR spectrum of *Ocimum sanctum* leaf extract and Cu NPs c. XRD of Cu NPs.

### Biophysical Characterization of Cu NPs

**Optical properties:** Analysis of optical properties of green synthesized copper nanoparticles is presented in graph obtained from UV-visible spectrophotometry. UV-visible spectral analyses of copper nanoparticles was done to characterize the Cu NPs formed at a range of 300 nm to 700 nm. The maximum absorption can be seen at about 528 nm spectra exhibited strong absorption peak in Figure 2 (a). The broadening of peak indicates that the particles are poly-dispersed.<sup>24,25</sup>

**Fourier transform infrared spectroscopy (FTIR):** The FTIR analysis is done in a range 4000 to 500 cm<sup>-1</sup>. Figure 2 (b) shows four different peaks functional groups at 3432 cm<sup>-1</sup>, 2919 cm<sup>-1</sup>, 1637 cm<sup>-1</sup> and 611 cm<sup>-1</sup> involving in formation of Cu NPs shown in Figure 2 b (A). This indicates that functional group plays a significant helping role in the capping and stabilizing copper nanoparticle synthesis.

FTIR analysis observed the presence of active phenolic components, such as flavonoids, in the *O. sanctum* leaf extract both before and after the bio-reduction procedure. Five main peaks can be seen in the FTIR spectrum of the aqueous extract of *O. sanctum* Figure 2b (B) and they are located at 3430, 2921, 2057, 1631 cm<sup>-1</sup>, respectively. These peaks correspond to O-H stretching vibrations (alcoholic or phenolic), C-H asymmetric stretching, CO stretching. The components of the *O. sanctum* extract contain carbonyl and hydroxyl linkages, which cause copper ions to be converted to Cu-NPs. The potential interaction between Cu ions and *O. sanctum* is revealed by the FTIR spectra of CuNPs and extract at the point of bioreduction. The other peak obtained at 623 cm<sup>-1</sup> might be due to N-H stretching of primary amine and alkyne.<sup>26</sup>

**X-ray Diffraction (XRD):** Figure. 2 (c) shows XRD pattern of copper nanoparticles exhibited characteristic diffraction peaks at position 43.20, 50.37 and 73.99 corresponding HKL planes values

111,200 and 220 matched with the JCPDS card 04-0836. The sharp diffraction peaks attributed to crystalline nature of copper nanoparticles. Debye-Scherrer Equation estimates the size of the nanoparticles to be roughly 43 nm, which may suggest a high surface area and surface area to volume ratio of the nanoparticles. The following Debye-Scherrer formula is used to determine the crystallite size of nanoparticles:

$$D = \frac{K\lambda}{\beta \cos\theta} \quad (1)$$

where, K is the crystalline shape constant (K=0.89),  $\lambda$  is the wavelength of X-ray (1.54Å for CuK $_{\alpha}$ ),  $\theta$  is the Bragg's diffraction angle and  $\beta$  is the angular full width at half maxima (FWHM) of XRD peaks recorded at diffraction angle. The full reduction of Cu $_2$  ions that led to the creation of metallic copper was strongly supported by XRD data. In order to learn more about the shape and size due to XRD measurement restrictions, SEM were used.<sup>27</sup>

**Scanning electron microscopy (SEM):** The surface morphology of the Cu-NPs was further clarified by scanning electron microscopy (SEM). Monodispersed Cu NPs of size 80-100 nm were clearly visualised from SEM micrograph Figure 3 (a). The particles were observed as smooth and highly crystalline in nature. The

micrograph reveals that the Cu NPs have a high propensity to aggregate, yet several particles with distinct borders could be seen. Further examinations at higher magnification show that the particle having hexagonal morphology shown in Figure 3 (b). The concentration of small particles results in the formation of the big clusters. The estimated particle size from the SEM image ranges from 80 to 110 nm, with an average size of 90 nm.

**Antibacterial activity:** Results of antibacterial activity of copper nanoparticles tested against four different pathogenic bacteria viz *Staphylococcus aureus* (NCIM-2654), *Pseudomonas aeruginosa* (NCIM-5032), *Proteus vulgaris* (NCIM-2813) and *E. coli* (NCIM-2832) are presented in Table 1 and as per Figure 4 (a to d).

The data listed in Table 1 and Figure 4. Graphs shows the antibacterial effect of various concentrations. It is seen that (32 ug/ml) shows largest zone of inhibition is 43.33  $\pm$  1.15 mm in diameter for pathogen. *E. coli*, followed by *Proteus vulgaris* with zone of inhibition 42.66 $\pm$  0.57 mm and *S. aureus*. (38.mm) the antibacterial activity is seen to increase with increase in concentration of NPS.

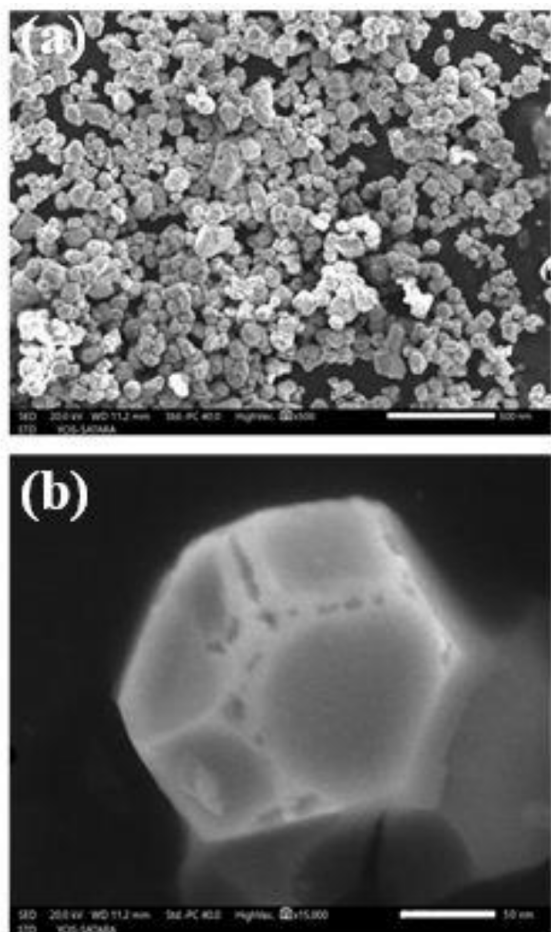
For statistical analysis, each experiment was carried out three times. The outcomes were given as means  $\pm$  standard deviation (SD). One-way ANOVA was employed to evaluate the data's significance levels at  $p < 0.05$ ,

There is a significant difference at four different concentrations for all organisms as the  $p$  value  $< 0.05$ .

## DISCUSSION

Use of plant leaf extract for the synthesis of nanostructural materials is a cost-effective, non-toxic, and environmentally friendly method. In this study, *Ocimum sanctum* leaf extract was used further to investigate.

Its ability to synthesize copper nanoparticles. The extracellular synthesis of copper nanoparticles using CFE was verified by the gradual colour change from blue to green. The color developed within 30 min and the intensity of color increased up to 60 min.

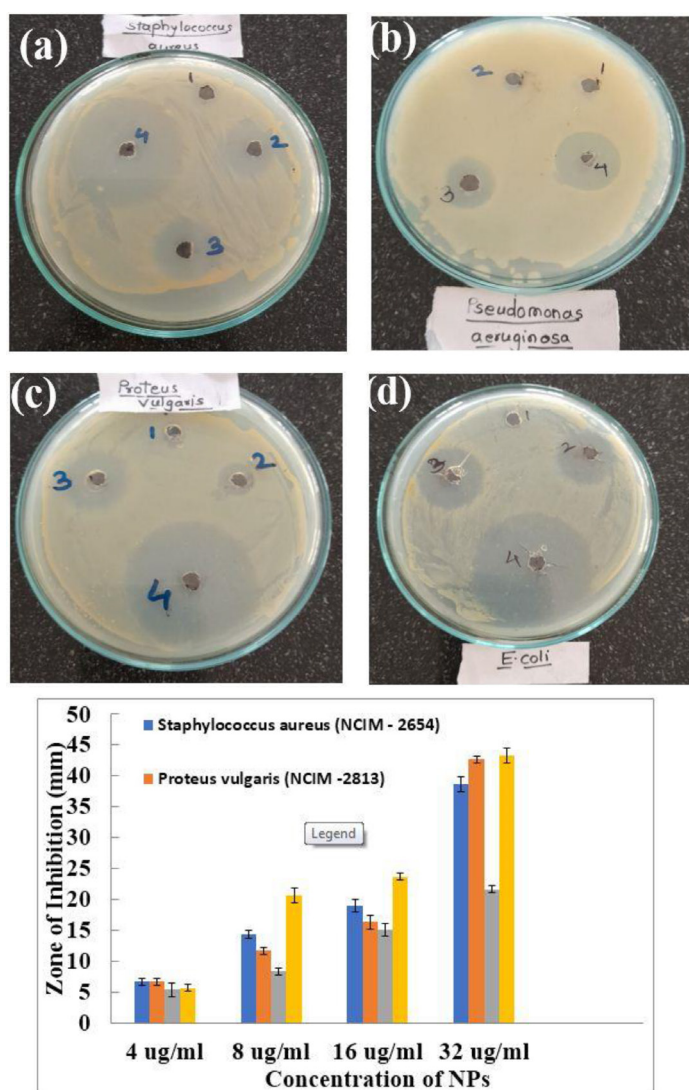


**Figure 3:** (a) Monodispersed CuNPs (b) Hexagonal Cu NP.

**Table 1: Antibacterial activity of *O. sanctum* derived Cu NPs against pathogenic strains.**

Concentration of NP ug/ml	Zone diameter of inhibition (mm) against			
	<i>Staphylococcus aureus</i>	<i>Proteus vulgaris</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>
4	6.66 $\pm$ 0.57	6.66 $\pm$ 0.57	5.66 $\pm$ 0.57	5.33 $\pm$ 1.15
8	14.33 $\pm$ 0.57	11.66 $\pm$ 0.57	20.66 $\pm$ 1.15	26.00 $\pm$ 0.57
16	19 $\pm$ 1.00	16.33 $\pm$ 1.15	23.66 $\pm$ 0.57	15 $\pm$ 1.00
32	38.66 $\pm$ 1.15	42.66 $\pm$ 0.57	43.33 $\pm$ 1.15	21.66 $\pm$ 0.57

Data represents means  $\pm$  standard deviation of three independent measurements.



**Figure 4:** Antibacterial activity of Cu NPs against, (a)- *Staphylococcus aureus*, (b)- *Pseudomonas aeruginosa*, (c)- *Proteus vulgaris*, (d)- *Escherichia coli* e) The statistical analysis of Cu NPs against human pathogens. The data represent means  $\pm$  standard deviation (SD) of three independent measurements.

Similar results were reported by Sumitha *et al.* using plant *Ocimum tenuiflorum*.<sup>22</sup> Colour change from blue to green is primary indication of synthesis of copper nanoparticles. Leaf extract added to copper sulphate solution responsible for reducing activity as it contains biologically active agents which upon drying converted to brownish black powder. UV-visible spectroscopy has maximum absorption at about 528 nm spectra exhibited strong absorption peak the results were in agreement with results reported by Bhukari *et al.* who mentioned that CuONPs exhibited a peak at 550 nm, which corresponds to the surface plasmon resonance of CuONPs.<sup>28</sup> The potential interaction between Cu ions and *O. sanctum* is revealed by the FTIR spectra of Cu NPs. On the basis of peaks obtained in FTIR studies it is established that bio-created copper nanoparticles could be surrounded by any of these biologically active

substances, such as terpenoid, polyphenols, and alkaloids. The results were similar to results reported by Das *et al.* The FTIR spectrum of the synthesized copper nanoparticles depicts a sharp narrow band at 3432  $\text{cm}^{-1}$ , corresponding to the N-H vibration mode and 1631  $\text{cm}^{-1}$  and 1072  $\text{cm}^{-1}$  correspond to N-H bending and C-N bond stretching respectively.<sup>29</sup> The IR spectrum of *Ocimum* leaf extract showed distinct peaks at 3432  $\text{cm}^{-1}$ , 2919  $\text{cm}^{-1}$ , 1637  $\text{cm}^{-1}$ . The copper nanoparticles were analysed for phase crystallinity and structural integrity using the powder X-ray diffraction method which represents peaks at  $2\theta$  value 43.20, 50.37 and 73.99 corresponding hkl planes values 111,200 and 220 matched with the JCPDS card 04-0836. This confirms cubic of lattice of copper. Similar results were reported by Phul *et al.* who synthesized CuNPs by chemical reduction method using L-ascorbic acid as reducing agent and mentioned that diffraction peaks at 43.28°, 50.40° and 74.81° correspond to (111), (200) and (220) planes respectively, confirming the cubic lattice of copper.<sup>30</sup> SEM images of particles revealed that Cu-NPs have a nearly uniform, well-defined, and crystalline structure, with polyhedron shape having size nearly 90 nm. Amjad *et al.* synthesized spherical copper nanoparticles (CuNPs) of the size within range of 51.26–56.66 nm using *Fortunella margarita* leaves' extract.<sup>31</sup> Among Gram Negative Bacteria, the significant anti-microbial activity was reported against *P. vulgaris* and among Gram Positive bacteria, anti-microbial activity of against *S. aureus* was found significant.

Huge surface to volume ratio of Cu NPs and nanoscale size caused them to interact with the bacterial membrane. Gram negative bacteria were found to be more sensitive to the Cu NPs action than Gram positive bacteria. The different cell wall composition of these two kinds of bacteria was the cause of the variation in the antibacterial activity of the Cu NPs. Gram positive bacteria have a thick covering of peptidoglycan in their cell walls, whereas Gram negative bacteria do not. The cell wall's peptidoglycan layer gives it additional strength, making it difficult for entering agents (Cu-NPs) to permeate and diminishing the activity of these nanoparticles.<sup>32</sup> The most significant inhibition zones within the four mentioned bacterial strains were obtained for concentration of nanoparticle 32  $\mu\text{g/ml}$ . the smallest one was reported by 4  $\mu\text{g/ml}$  of CuNPs. By increasing the concentration of CuNPs within the same treatment, the antibacterial impact improved ( $p < 0.05$ ).<sup>33</sup> Results were in agreement with previous studies. Cu NPs showed a significant level of antibacterial activity against *Escherichia coli* and *Bacillus subtilis*, according to Naradala *et al.* Whereas, moderate action is seen for Cu NPs against *Proteus vulgaris* and *Staphylococcus aureus*.<sup>34</sup> Naradala *et al.* and Ghetas *et al.* found that the antibacterial activity found to increase with the increase of concentration of nanoparticle which is in agreement with our study.<sup>33,34</sup>

## CONCLUSION

Cu NPs were synthesized using aqueous leaf extract of *Ocimum sanctum* through green synthesis route. The leaf extract of *Ocimum sanctum* was found efficient for the synthesis of Cu NPs. This approach has advantages over previously reported methods, including simple reaction conditions, easy access to starting materials, ease of laboratory conduct, and a lack of the need for expensive and hazardous reagents. The synthesized Cu NPs were characterized and evaluated using a UV-vis spectrophotometer, FTIR spectroscopy, powdered XRD, SEM. The synthesis of Cu NPs in the reaction mixture is revealed by the UV surface plasmon peak. Leaf extract acting as reducing and capping agents during the production of Cu NPs. FTIR interpretation provides information about potential bioactive compounds. The XRD study confirms that Cu NPs are crystalline (FCC) and that their mean size (43 nm). Cu zero valent NPs are present, according to the results of XRD. Fresh aqueous *Ocimum sanctum* leaf extract contains phenolic groups that have good antioxidant properties. These phenols can react with free radicals to produce phenoxy radicals. As a result, using natural antioxidants to create Cu NPs appears to be a successful method for using green synthesis. Excellent antibacterial activity is displayed by the biologically produced Cu NPs in the reaction mixture.

The antibacterial property of copper nanoparticles against Gram positive and Gram-negative pathogenic bacteria was investigated along with advantages in biosynthesis of nanoparticles such as easily availability of raw material, easy extraction, no use of toxic reagents, pollution free application and moreover ecofriendly nature and ease adaptability.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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