

# Antibacterial Evaluation of Cuprous Oxide Nanoparticles Synthesized Using Leaf Extract of *Callistemon viminalis*

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## ABSTRACT

**Objectives:** Nanoparticles synthesis via green approach is a scorching theme of research nowadays as it minimizes the use of harmful chemicals. Not much work has been done on bio reduction of copper salts; therefore our present work was focused on green synthesis of copper oxide nanoparticles using leaf extract of *Callistemon viminalis*.

**Methods:** Bio reduction method was used for preparation of copper oxide nanoparticles using *Callistemon viminalis* leaf extract. Synthesis was carried out at three different temperatures namely 4°C, room temperature and 60°C. **Results:** Completion of reaction occurred at 60°C and reduced copper oxide particles were further characterized by ultra violet spectroscopy, dynamic light scattering technique, fourier transform infrared spectroscopy, X-ray diffraction and photoluminescence spectroscopy. The antibacterial evaluation of nanoparticles was done against standard bacterial strains *Acinetobacter baumannii* and *Escherichia coli*. Synthesized copper oxide nanoparticles were found to exhibit antibacterial activity in both pathogenic strains. **Conclusion:** Overall we can conclude that bio reduction is facilitated at 60°C and it is rapid, simple, inexpensive and effective way of producing antibacterial copper oxide nanoparticles.

**Key words:** Bio reduction, Copper oxide, Green synthesis, Cuprous oxide, Metallic nanoparticles, *Callistemon viminalis*.

## INTRODUCTION

Nanotechnology has emerged as being a valuable technology in all fields of science,<sup>1</sup> nanoparticles having size between 1-1000 nm have been used as therapeutic moieties in treatment of diseases.<sup>2</sup> Among various types of nanomaterial, metallic nanoparticles hold a valuable place as these nanoparticles have been found to play important roles in field of cosmetics, electronics and biomedicine. Commonly used metallic nanoparticles are gold, silver, copper oxide and zinc oxide. Copper oxide nanoparticles have been utilized as gas sensors, antimicrobial agents and photo-catalysts.<sup>3</sup> Cuprous oxide, a form of copper oxide is a common pigment used in paint industry. It is used as antifouling compound in place of banned organotins.<sup>4</sup>

Along with these advantages production of nanomaterial poses health as well as environmental hazard risks.<sup>5</sup> Chemical reduction method has limitation of being toxic to environment, whereas lot of energy is required for physical reduction. Therefore researchers have started to focus on developing environment friendly methods for synthesis of nanoparticles. Green synthesis is preferred technique for synthesis as it is not only simple and cost effective technique, but it is nontoxic to environment also.<sup>6-8</sup> Plants act as natural reducing agents as they contain various metabolites which help in reduction of metallic salts to metallic nanoparticles.<sup>9-11</sup>

*Callistemon viminalis* belongs to family Myrtaceae.<sup>12</sup> Zubair M *et al.* 2013 have

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reported that *Callistemon viminalis* leaves contain considerable levels of total phenolic contents.<sup>13</sup> It has been used since ages as medicinal agent in traditional medicines. Almost each and every part of this plant has been utilized for biological applications. This plant has been reported to have insecticidal, antifungal, anti-oxidant,<sup>14</sup> molluscicidal,<sup>15</sup> and antibacterial activity.<sup>16</sup> Among all types of metallic nanoparticles silver and gold nanoparticles have been the particular focus of plant based synthesis, however there are only few reports on synthesis of copper oxide nanoparticles.<sup>17</sup> Therefore our present work was focused on bio reduction of copper salt into cuprous oxide nanoparticles via *Callistemon viminalis* leaf extract. This is the first report based on utilization of this plant extract for production of copper oxide nanoparticles. Synthesized nanoparticles were characterized by UV spectroscopy, dynamic light scattering technique, FTIR spectroscopy, XRD analysis and photoluminescence spectroscopy. Finally synthesized nanoparticles were tested for their antibacterial potential against *A. baumannii* ATCC 19606 and *E. coli* 25922 using Kirby Bauer's disk diffusion method.

## MATERIALS AND METHODS

### Chemicals

Fehling's solution 1 and Fehling's solution 2 were purchased from Lobachemie, India.

### Microbiological culture media

Mac Conkey agar, Mueller Hinton agar, Mueller Hinton broth, Luria broth, antibiotic discs of cotrimoxazole and meropenem were purchased from Hi-Media Pvt. Ltd. (India). The standard reference strains *Escherichia coli* ATCC 25922 and *Acinetobacter baumannii* ATCC 19606 utilized in antimicrobial susceptibility tests were received as a gift from Dr. Arti Kapil, Head of Bacteriology division, Department of Microbiology, AIIMS (Delhi, India).

### Plant material

Leaves of *Callistemon viminalis* were collected from campus of Jaypee University of Information Technology, Waknaghat and their authentication was done at Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni.

### Preparation of leaf extract

Fresh leaves of *Callistemon viminalis* were collected; washed with distilled water several times and shade dried. About 20 g of leaves were taken and boiled with 80 ml of distilled water for 30 min. This extract was filtered through whatman filter paper and centrifuged to

remove insoluble particles. Supernatant was collected in a fresh vial and used as such for bio reduction.

### Bio-synthesis of Cu<sub>2</sub>O nanoparticles and temperature optimization

Method described by Mariselvam R *et al.* 2014<sup>18</sup> was used after modifications for bio reduction of copper sulphate to copper oxide. Mixture of fehling's solution was taken in a conical flask. 30 ml of freshly prepared aqueous extract of leaves of *Callistemon viminalis* was added drop wise to 10 ml of this mixture and stirred at different temperatures till color change was obtained from greenish blue to reddish brown. Synthesis was carried out at 4°C, room temperature and 60°C. No reduction occurred at 4°C and room temperature, whereas reduction occurred at 60°C; therefore 60°C was optimized as temperature required for green synthesis. Obtained reddish brown precipitates were centrifuged and washed 4 times with distilled water and finally with methanol. Obtained pellet was dried in hot air oven at 60° for 24 h; this powder was taken as such for characterization.

### Characterization of Cu<sub>2</sub>O nanoparticles

Cu<sub>2</sub>O nanoparticles were characterized by UV spectroscopy using UV spectrophotometer (ELICO), zeta-potential and diameter of nanoparticles was determined by dynamic light scattering technique (DLS) using zetasizer (Malvern instruments), FTIR spectrum and XRD spectrum of prepared nanoparticles was recorded using FTIR spectrophotometer (Agilent technologies) and XRD diffractometer (X'Pert Pro XRD) respectively to confirm formation of copper oxide and luminescent property of Cu<sub>2</sub>O NPs was recorded by photoluminescence spectroscopy using fluorescence spectrophotometer (Perkin Elmer LS55). Photoluminescence spectrum of Cu<sub>2</sub>O NPs was determined at room temperature taking distilled water as blank.

### Antibacterial activity

Antibacterial activity of synthesized nanoparticles was determined against *Escherichia coli* ATCC 25922 and *Acinetobacter baumannii* ATCC 19606. All the protocols followed were adopted from the CLSI 2015 guidelines (Clinical laboratory standard institute) and ICMR (Indian Council of Medical Research) guidelines. Pure colony of bacteria was inoculated in Luria broth and incubated overnight at 37°. Bacterial culture was adjusted to 0.5 McFarland standards and swabbed onto Muller Hinton agar plates. Disks of standard antibiotics and Cu<sub>2</sub>O nanoparticles were placed on agar plates and incubated at 37° for 16-18 h. Circular zones of inhibition around disk were interpreted according to CLSI and ICMR guidelines.<sup>19,20,21</sup>

## RESULTS AND DISCUSSIONS

### Green synthesis of Cu<sub>2</sub>O nanoparticles

Green synthesis is a preferred route of nanoparticle synthesis as natural reducing agents present in plants have the ability to reduce copper salt (CuSO<sub>4</sub>·5H<sub>2</sub>O) into red colored Cu<sub>2</sub>O NPs.<sup>22</sup> In case of green synthesis precise mechanism behind bio reduction has not been well elucidated till date as plant extracts contain diverse metabolites, but a study by Kasthuri J *et al.* 2009 suggested that it proceeded by mechanism of nucleation/aggregation.<sup>23</sup> Hence we evaluated the potential of *Callistemon viminalis* leaf extract for bio reduction of copper sulphate to copper oxide nanoparticles. Entire process of bio reduction is depicted in Figure 1. Completion of bio reduction was determined physically by change in color from bluish solution to reddish brown. Change in color from blue to reddish brown copper oxide nanoparticles indicates the potential of *Callistemon viminalis* leaf extract as natural bio-reducing agent. Cu<sub>2</sub>O NPs synthesis was successfully accomplished using *Callistemon viminalis* leaves extract at 60°C.

### Characterization of Cu<sub>2</sub>O nanoparticles

#### UV Spectroscopy

Reduction of copper salts to copper oxide nanoparticles is monitored via UV spectroscopy.<sup>24,25</sup> Copper oxide gives broad peak between 285-320 nm.<sup>26</sup> Completion of reaction was monitored by taking UV spectrum between 200 to 800 nm. Reddish brown particles gave a broad peak Figure 2 between 285-320 nm; this may be attributed to reduction of copper salt to copper oxide.

#### Zeta-potential

Zeta-potential values are used to describe stability of a system; particles having zeta-potential values between -30 mV to +30 mV are known to have good stability.<sup>27</sup> Advantage of green synthesis lies in the fact that no special stabilizing agents are required for developing nanoparticles, as plant metabolites themselves act as stabilizing agents in nanoparticle synthesis.<sup>28</sup> DLS result show an average zeta-potential value of -18.7 mV Figure 3, this stability may be attributed to natural stabilizing agents present in plant extract.

#### Size distribution

Green synthesis is a “bottom up approach”;<sup>29</sup> study by Wang H *et al.* has shown that bottom up approach yield large size units.<sup>30</sup> Diameter of nanoparticles was determined using DLS technique, Figure 4 shows the size distribution analysis result of synthesized NPs. Average particle size was found to be 423 nm; this may have

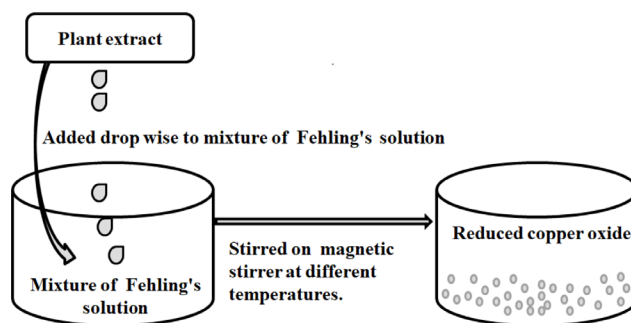


Figure 1: Green synthesis by bio-reduction method.

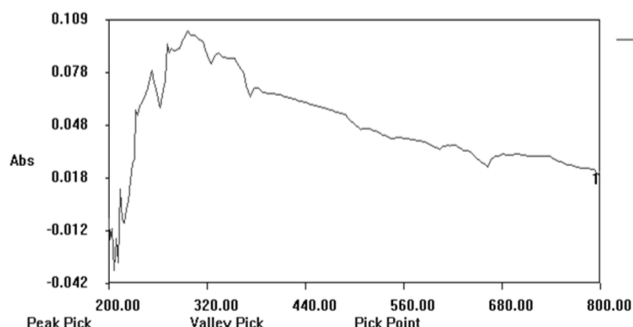


Figure 2: UV spectrum of obtained Cu<sub>2</sub>O NPs.

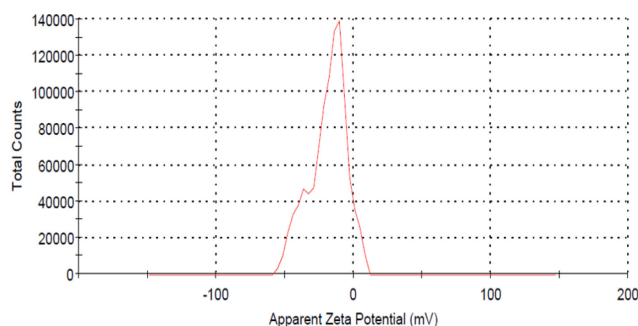


Figure 3: Zeta-potential of Cu<sub>2</sub>O NPs.

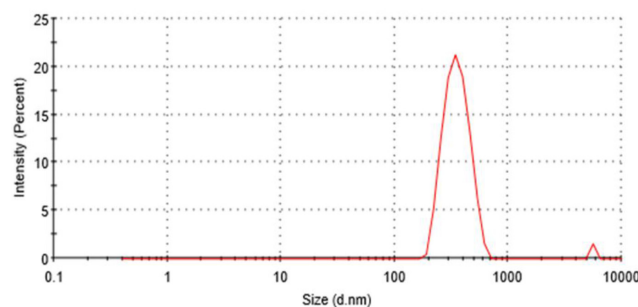


Figure 4: Size distribution of Cu<sub>2</sub>O NPs.

happened as selected method of synthesis is a bottom up approach.

#### FTIR spectroscopy

Infra-red spectroscopy is a non-destructive technique of analysis with high sensitivity being used in research.<sup>31</sup>

FTIR spectra of plant extract and washed copper oxide nanoparticles was recorded. Interpretation of major IR peaks is given in Figure 5 and Figure 6. Vibrations in FTIR spectra of plant extract (Figure 5) could be attributed to (668 and 683  $\text{cm}^{-1}$ ; aromatic C-H bending), (1640  $\text{cm}^{-1}$ ; amide C=O stretching) and (3329 and 3331  $\text{cm}^{-1}$ ; phenol OH stretching). Whereas vibrations in FTIR spectra of copper oxide NPs (Figure 6) at (648  $\text{cm}^{-1}$ ; Cu-O stretching) clearly indicated formation of copper oxide and at (1089  $\text{cm}^{-1}$ ; C-OH stretching) indicated role of plant metabolite in reduction of copper salt.<sup>32,33</sup> Fourier transform infrared spectroscopy supported the role of plant metabolites in reduction.

### Photoluminescence spectroscopy

Substances that exhibit photoluminescence property have the ability to act as biological probes; nano-sized

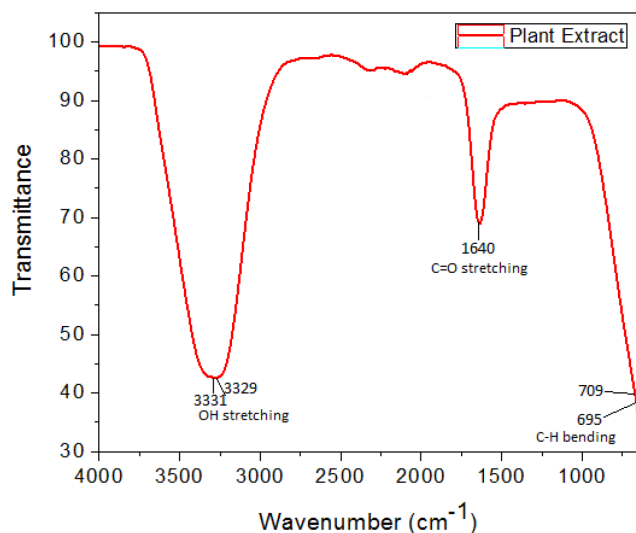


Figure 5: FTIR Spectrum of *Callistemon viminalis* plant extract.

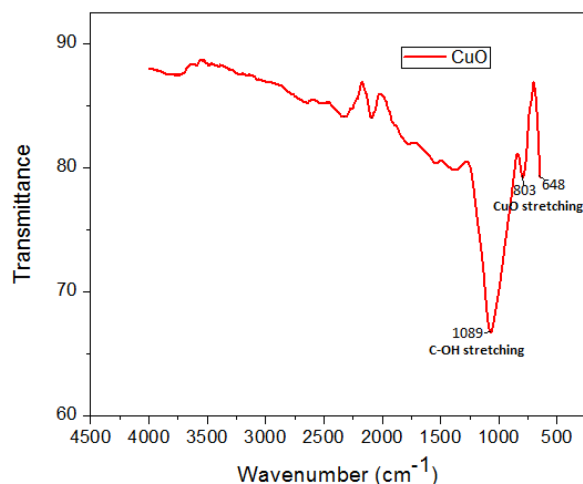


Figure 6: FTIR Spectrum of Cu<sub>2</sub>O NPs.

copper clusters have been found to exhibit luminescence property.<sup>34</sup> Photoluminescence spectrum of Cu<sub>2</sub>O NPs is shown in Figure 7. Results show an emission spectrum at 630 nm; this could be attributed to band edge emission. This indicates that prepared nanoparticles have luminescent property.

### XRD analysis

Formation of CuO Nps was analyzed by XRD diffractometer. Figure 8 shows XRD peaks of synthesized NPs, largest peak obtained at 36.5° and a small peak around 42.5° in 2 $\theta$  unit suggested the diffraction of cuprous oxide NPs. These peaks confirmed the oxidized state of copper and XRD results are in accordance with study by Firmansyah DA *et al.* 2009.<sup>35</sup>

### Antibacterial evaluation

*A. baumannii* is a multidrug resistant pathogen responsible for various nosocomial infections throughout the world; it has the ability to form colonies on abiotic materials,<sup>36</sup> whereas pathogenic variants of *E. coli* are responsible for majority of health problems in humans.<sup>37</sup> Therefore we selected these standard bacterial species and evaluated the effect of copper oxide nanoparticles on these strains. Antibacterial activity of synthesized NPs was evaluated against *A. baumannii* (ATCC 19606) and *E. coli* (ATCC 25922). Kirby Bauer's disk diffusion test showed *E. coli* strain susceptibility zone against copper nanoparticles at concentration of 1024  $\mu\text{g}$  and 512  $\mu\text{g}$ .

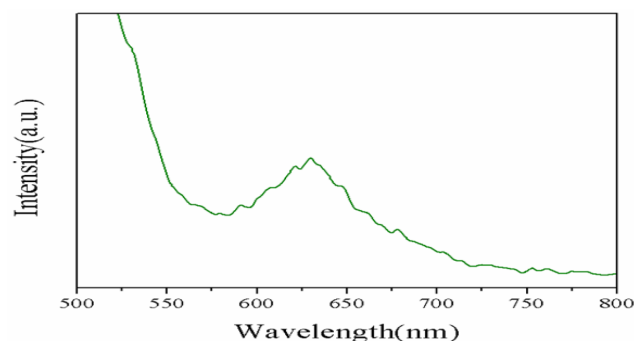


Figure 7: Photoluminescence spectrum of Cu<sub>2</sub>O NPs.

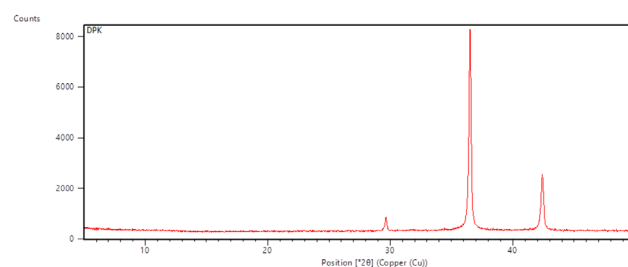
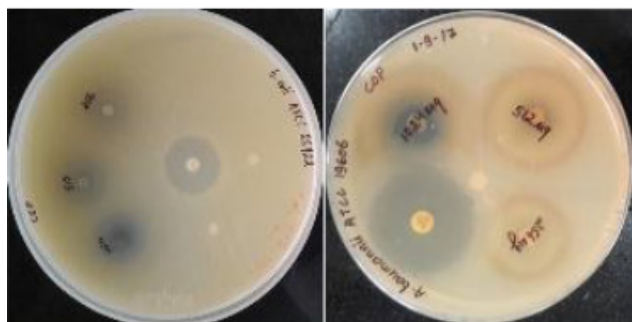


Figure 8: XRD spectrum of Cu<sub>2</sub>O NPs.





**Figure 9: Antibacterial evaluation results for standard reference strains: *E. coli* ATCC 25922 and *A. baumannii* ATCC 19606. [1= Meropenem (10 µg), 2= Cotrimoxazole (25 µg)].**

**Table 1: Antimicrobial susceptibility test results.**

Concentration of copper oxide nanoparticles in µg (microgram)	Zone of inhibition in mm (millimeter)	
	<i>E. coli</i>	<i>Baumannii</i>
1024	14	12
512	11	No zone
256	9	No zone
Cotrimoxazole (30 µg)	32	-
Meropenem (10 µg)	-	33

where – stands for not applied.

*A. baumannii* showed 12 mm zone of lysis at 1024 µg and no zone of lysis was seen at 512 µg concentrations (Figure 9, Table 1).

## CONCLUSION

Overall we can conclude that these green synthesized  $\text{Cu}_2\text{O}$  NPs are cost effective molecules; as synthesis process did not require any chemical reducing agents. These NPs have the ability to serve as potential antibacterial agents for the treatment of infections caused by *A. baumannii* ATCC 19606 and *E. coli* 25922 pathogens. Along with antibacterial activity, results of PL study shows that these green synthesized nanoparticles may be utilized as bio sensing material. This study suggests that  $\text{Cu}_2\text{O}$  nanoparticles can be rapidly synthesized using natural reducing agents present in leaves of *Callistemon viminalis*. However, for future studies further size reduction of these particles may be done by milling techniques to determine their potential in other bio-physical applications.

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for providing facilities to carry out research. D. Sharma would like to thank ICMR (Indian Council for Medical Research), Delhi for providing fellowship.

## CONFLICT OF INTEREST

There is no conflict of interest.

## ABBREVIATIONS

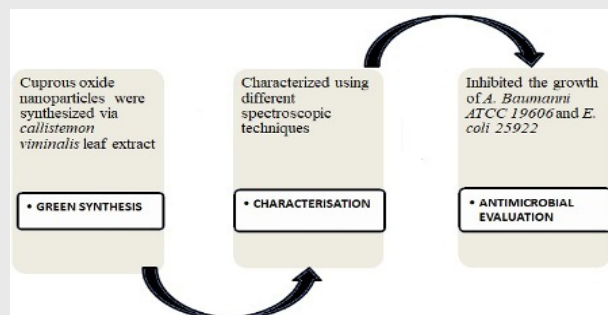
ATCC- American type culture collection, nm-nanometer, NPs- nanoparticles, UV- Ultra violet, FTIR- Fourier Transform Infra-Red, *E. coli*- *Escherichia coli*, *A. baumannii*- *Acinetobacter baumannii*. AIIMS- All India Institutes Of Medical Sciences, mV- millivolts, CLSI- Clinical laboratory standard institute and ICMR- Indian Council Of Medical Research,  $\text{Cu}_2\text{O}$ - Cuprous oxide,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ - Copper sulphate pentahydrate, DLS- Dynamic Light Scattering Technique, JUIT: Jaypee University of Information Technology.

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## PICTORIAL ABSTRACT



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## SUMMARY

Our present work was focused on reduction of copper sulfate to copper oxide utilizing *callistemon viminalis* leaf extract by bio reduction method. Synthesized copper oxide nanoparticles were characterized utilizing various physical methods. Later on, these copper oxide nanoparticles were evaluated for their antimicrobial activity against pathogenic strains of *Acinetobacter baumannii* and *Escherichia coli*. These nanoparticles were successfully prepared by green synthesis method and inhibited the growth of bacteria, signifying their antimicrobial potential.



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