



Biosynthesis of copper oxide nanoparticles using leaves extract of *Leucaena leucocephala* L. and their promising upshot against the selected human pathogens

Yogesh B. Aher¹, Gotan H. Jain¹, Ganesh E. Patil¹, Ajay R. Savale², Suresh K. Ghotekar^{2*}, Dattaprasad M. Pore³, Shreyas S. Pansambal⁴, Keshav K. Deshmukh⁴

1. Department of Physics, KKHA Arts, SMGL Comm. and SPHJ Science College, Chandwad, University of Pune, Maharashtra 423 101, India.

2. Department of Chemistry, KKHA Arts, SMGL Comm. and SPHJ Science College, Chandwad, University of Pune, Maharashtra 423 101, India.

3. Department of Chemistry, Shivaji University, Kolhapur 416; fax: +91 231 2692333

4. Department of Chemistry, S.N. Arts, D.J.M. Comm. and B.N.S. Science College, Sangamner, Savitribai Phule Pune University, Maharashtra 422 605, India.

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ABSTRACT

An ecological and benevolent route for the fabrication of copper oxide nanoparticles (CuONPs) using *Leucaena leucocephala* L. leaves extracts at room temperature is reported. Phytochemical screening of the fresh aqueous leaves extract revealed the presence of tannins, saponins, coumarins, flavonoids, cardiac glycosides, steroids, phenols, carbohydrates and amino acids. The prepared copper oxide nanoparticles are in Nano scale and their morphology and size is characterized by using field emission scanning electron microscopy, energy-dispersive X-ray spectroscopy, transmission electron microscopy, X-ray diffraction, Fourier transform Infra-red spectroscopy, Brunauer-Emmett-Teller, Barrett-Joyner-Halenda and Photoluminescence analysis. Furthermore, CuO-NPs evinced remarkable antimicrobial, antimalarial and antimycobacterial activity against the selected human pathogens.

1. Introduction

Nanomaterials have been a great attraction for the scientific and technological world in the contemporary time due to their unique properties and significant applications in diverse fields (Mallick et al., 2009). Among all metal nanoparticles, CuONPs have attracted significant attention because of their wide range of application such as catalytic (Bhosale & Bhanage, 2014), sensors (Jiang & Zhang, 2010), optical (El-trass et al., 2012), electrical (Yabuki & Arriffin, 2010), gas sensors (Yang et al,

2011), solar energy transformation and preparation of organic-inorganic nanostructure composites (Anandan et al., 2005). Moreover, it can be used as an antimicrobial, antifungal and antibiotic agent when incorporated in plastics, textiles, coating (Borkow & Gabbay, 2009), etc. CuONPs possess competent biological properties having applications in pesticidal formulations (Kiaune & Singhasemanon, 2011).

For the fabrication of CuONPs, several methods are reported such as sonochemical

*Corresponding author: Suresh K. Ghotekar
E-mail address: ghotekarsuresh7@gmail.com

(Kumar & Gedanken, 2002), microwave irradiations (Wang & Zhu, 2002), sol-gel technique (Eliseev et al., 2000), electrochemical methods (Borgohain & Mahamuni, 2000), pyrolysis (Fan et al., 2004), and thermal decomposition of precursor (Nisari, 2009). However, these methods are suffered with some disadvantages like the use of toxic chemicals, need of special instruments, long reaction time and requirement of external additives during the reaction. In this context, green synthesis protocol is economically affordable, environmentally benign, energy efficient and free from use of toxic chemicals (Sadeghi & Gholamhoseinpoor, 2015). Several methods for metal nanoparticles synthesis using the microorganisms (Bansal et al., 2012), fungi (Vigneshwaran, 2007), enzyme (Willner et al., 2006), plant extracts (Pande et al., 2015; Ghotekar, 2016; Sadeghi, 2015), etc. are reported, amongst which the method comprising use of plant extracts has received much attention. The plant extracts are safe to handle, easily available and have a broad variability of phytochemicals. The phytoconstituents such as tannins, carbohydrates, flavonoids, saponins, coumarins, proteins, amino acids, and terpenoids present in the plant extracts play an important role in the synthesis of nanoparticles (Amooaghaie et al., 2015). In literature, biofabrication of CuONPs using *Gloriosa superba* (Ghosh & Jha, 2002), *Tinospora cordifolia* (Udayabhanu et al., 2015), *Calotropis gigantean* (Sharma et al., 2015), *Aloe barbadensis* (Gunalan & Sivaraj, 2012), *Carica papaya* (Sankar & Ravikumar, 2014) and *Ziziphus mauritiana* (Ghotekar, 2016) has been already reported.

Leucaena leucocephala L. is a thornless long-lived shrub (miracle tree) which may grow up to heights of 5-20 m. The leaves of this plant are internationally marketed as animal feed. Moreover, brown and black dyes are extracted from the leaves and bark of *Leucaena leucocephala* L. The plant can provide timber, green manure, firewood, resin, reclamation, erosion control and shade. Other uses include the young leaves and seeds as vegetables for human consumption. However, only small amounts can be eaten in this way because of the presence of toxic amino acid mimosine in seed and young growth. In the present stage, *Leucaena leucocephala* L. has spread to most

nations of the tropical world where it is utilized as a shade plant for plantation crops. Herein, we report the economically affordable and green synthesis of CuO-NPs using plant extracts of *Leucaena leucocephala* L. and their antibacterial, antimalarial and antimycobacterial activity screening. The results would help to effectively utilize as synthesized CuONPs in future biomedical concerns.

2. Materials and Methods

2.1. Materials

Copper acetate monohydrate [Cu(CH₃COO)₂.H₂O, 98%, LR grade, Sigma-Aldrich), sodium bicarbonate (NaHCO₃, Analytical grade, 99.7%, Sigma-Aldrich) and dimethyl sulfoxide (DMSO, ACS reagent, 99.9%, Sigma-Aldrich) were used. All chemicals were used as such without any further purification. All the solutions were prepared using deionized water during the synthesis. The fresh leaves of *Leucaena leucocephala* L. were sourced from Chandwad college campus, Nashik, Maharashtra, India. The collected leaves were washed with deionized water, snicked into small pieces. All glassware's are washed with distilled water and acetone and dried in oven before use.

2.2. Biogenic synthesis of CuONPs

10g small dried pieces of *Leucaena leucocephala* L. leaves were transferred into 250 mL beaker containing 100 mL deionized water. The mixture was refluxed at 100°C for 20 minutes and cooled at room temperature followed by filtered through ordinary filter paper. Then, resultant filtrate was again filtered through Whatmann No. 1. The filtered extract was stored in refrigerator at 4°C and used for synthesis of CuONPs. The aqueous solution of 0.01 M copper acetate monohydrate was prepared in deionized water. *Leucaena leucocephala* L. leaf extract was mixed to 0.01 M aqueous copper acetate solution in 1:8 ratios in a 250 ml beaker with constant stirring on magnetic stirrer at 500 rpm/25 min. After time of period the color of solution turns to dark yellow. The mixture was kept in a muffle furnace at 400°C (Naika et al., 2014) and subjected for combustion. The reaction was completed within 5 min. A fine black colored

material was obtained and this was carefully collected and packed for characterization purposes (Naika et al, 2014).

2.3. Characterization techniques

The morphology and composition of the synthesized CuONPs were examined by field emission scanning electron microscopy (FESEM, FEI, Nova Nano SEM 450), FESEM coupled energy-dispersive X-ray spectroscopy (EDX, Bruker, XFlash 6130). Find the exact morphological structures and size of the CuONPs using transmission electron microscopic (TEM) analysis is done by using a PHILIPS, CM200 with an accelerating voltage of 200 kV in order to. The crystallinity and crystal phases were characterized by X-ray diffraction (XRD, Bruker, D8-Advanced Diffractometer) pattern measured with Cu- $K\alpha$ Radiation ($\lambda = 1.5406 \text{ \AA}$) in the range of 5–80°. The Fourier transform Infrared (FTIR) spectrum was recorded by JASCO 4100 in the range of 4000–400 cm^{-1} . Photoluminescence studies were evaluated by using fluorescence spectrophotometer (JOBIN YVON FLUOROLOG-3-11, Spectrofluorimeter). The specific surface area and porosity were characterized by Brunauer-Emmett-Teller (BET) and Barrett-Joyner-Halenda (BJH) analysis method at 77.40 deg. K (NOVA-100 Ver. 3.70).

2.4. Phytochemical Screening

The fresh aqueous extract of *Leucaena leucocephala* L. leaves were investigated for the presence of phytochemicals viz. tannins, saponins, coumarins, flavonoids, cardial glycosides, steroids, phenols, carbohydrates and amino acids by standard biochemical method (Fransworth, 1996).

2.5. Antibacterial Activity of Synthesized CuONPs

The antimicrobial activities of synthesized CuONPs were examined by using Disc diffusion method. This method was employed against human pathogens i.e. *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Staphylococcus aureus* and *Escherichia coli* obtained from Institute of Microbial Technology, Chandigarh, India. The nutrient agar medium (g/l) plates were prepared,

well sterilized and solidified. After solidification, bacterial cultures spread over the plate, and then various concentration of CuONPs solution was poured into each plate. These plates were incubated in incubator at 37°C for 24 hrs and zone of inhibition against bacterial strains was measured.

2.6. In Vitro Antimalarial Screening of Synthesized CuONPs

The in vitro antimalarial assay was carried out in 96 well microtitre plates according to the microassay protocol (Riekmann et al., 1978). The cultures of *Plasmodium falciparum* strain were maintained in medium RPMI-1640 supplemented with 25 mM HEPES, 0.23% NaHCO_3 , 1% D-glucose and 10% heat inactivated human serum. The asynchronous parasites of *Plasmodium falciparum* were synchronized after 5% D-sorbitol treatment to obtain only the ring stage parasitized cells. For carrying out the assay, an initial ring stage parasitaemia of 3% haematocrit in a total volume of 200 μl of medium RPMI-1640 was determined by Jaswant Singh Bhattacharya (JSB) staining (Singh, 1956) to assess the percent parasitaemia and uniformly maintained with 50% RBCs (O^{+ve}). The culture plates were incubated at 37°C in a candle jar. After 36 hrs incubation, thin blood smears from each well were prepared and stained with JSB stain. The slides were microscopically observed to record maturation of the ring stage parasites into schizonts and trophozoites in the presence of various concentrations of the synthesized CuONPs. The synthesized CuONPs concentration which inhibited the complete maturation into schizonts was recorded as the minimum inhibitory concentration (MIC). Chloroquine and Quinine were used as the reference drugs.

2.7. In Vitro Antimycobacterial Screening of Synthesized CuONPs

The antimycobacterial screening for synthesized CuONPs was obtained for *Mycobacterium tuberculosis* H₃₇RV, by using L. J. (Lowenstein and Jensen) MIC method (Anargyros, 1990; Patel & Khan, 2010). Stock solutions of primary 1000, 500, 250 and secondary 200, 100, 62.5, 50, 25, 12.5, 6.25, 3.25 $\mu\text{g/ml}$ of CuONPs in

DMSO were added in the liquid L. J. Medium and then media were sterilized. A culture of *Mycobacterium tuberculosis* H₃₇RV growing on L. J. medium was harvested in 0.85% saline in bijou bottles. These tubes were then incubated at 37°C for 24 hrs followed by streaking of *Mycobacterium tuberculosis* H₃₇RV. These tubes were then incubated at 37°C. Growth of bacilli was seen after 12 days, 22 days and finally 28 days of incubation respectively. Tubes having the CuONPs were compared with control tubes where medium alone was incubated with *M. tuberculosis* H₃₇RV. The concentration at which no development of colonies occurred or < 20 colonies was taken as MIC concentration of test compound. The standard strain *M. tuberculosis* H₃₇RV was tested with known drug isoniazid.

3. Results and Discussion

3.1. Phytochemical screening studies

Table 1 describes the qualitative pharmacognostic evaluation of aqueous leaf extract of *Leucaena leucocephala* L, highlighted the presence of tannins, saponins, coumarins, flavonoids, cardial glycosides, steroids, phenols, carbohydrates, amino acids, etc.

Table 1. Phytochemical Screening-Qualitative Analysis

Phytochemical	Test	Phytochemical	Test
Tannin	+	Saponins	+
Coumarins	+	Emodins	-
Proteins	-	Flavonoid	+
Cardial Glycoside	+	Anthraquinone	-
Anthocyanosides	-	Steroid	+
Phenol	+	Amino acids	+
Carbohydrate	+		

3.2. XRD analysis

The CuONPs biosynthesized from *Leucaena leucocephala* L. leaf extract were confirmed by the characteristic peaks observed in the XRD patterns, as shown in figure 1. XRD analysis bared small distinct diffraction peaks at 32.5°, 35.4°, 38.7°, 48.7°, 53.5°, 58.2°, 61.5° and 66.2°, corresponding to (110), (002), (111), (202), (020), (202), (113) and (311) of face-centered-cubic structure of copper oxide nanoparticles with a monoclinic phase (JCPDS No. 45-0937). The XRD pattern exposed that synthesized

copper oxide nanoparticles are crystalline in nature (Ethiraj & Kang, 2012).

3.3. Vibrational studies

The figure 2 describes FTIR spectrum of CuONPs in the plant extract. The broad band seen at 3415 cm⁻¹ reveals the presence of an OH group, resulting from either alcoholic or phenolic stretching, while the peaks around 2918 cm⁻¹ are attributed to an asymmetric stretching vibration of the C-H bond in alkanes. The peaks around 1611 cm⁻¹ may be attributed to C=C in aromatic compounds, and those at 1384 cm⁻¹ correspond to the O-H bend of polyphenol, confirms the presence of an aromatic group (Rao & Paria, 2013). The peak observed at 1080 cm⁻¹ corresponds to secondary -OH of the phenolic group. The FTIR results confirm the presence of the biomolecules in leaf extract of *Leucaena leucocephala* L. i.e. amino acids (Mimosine), phenols, flavonoids (Luteolin-7-o-glucoside) (Hassan and Tawfik, 2014) and enzymes are responsible for the capping and stabilization of CuONPs.

3.4. FE-SEM microphotographs

From the FESEM image as shown in figure 3(a&b) the synthesized CuONPs are uniform and define spherical morphology. Each CuONPs possesses the average particles size of 10-25 nm. It is noticed that green synthesis of CuONPs produces the small and uniform size of spherical particles.

3.5. TEM images

Figure 4 depicted the TEM images of synthesized CuONPs. The low magnification TEM image [figure 4(a)] reveals almost similar spherical morphology of CuONPs as seen in FESEM image. From TEM image, the average particle size is estimated to be 10-25 nm spherical particles, which is consistent with the FESEM results. From the TEM image of CuONPs as shown in figure 4(b), the particles are aggregated and interconnected to each other, resulting in the less visible lattice fringes. Therefore, the morphological characterizations confirm the spherical morphology of CuONPs biosynthesized employing the leaves of *Leucaena leucocephala* L. plant.

3.6. EDS studies

The composition of synthesized CuONPs was analyzed by investigating the energy-dispersive X-ray spectroscopy (EDS), as shown in figure 5. EDS spectrum displays the peaks relevant to Cu (31.59%) and O (25.91%). Other peaks corresponding to C (38.61%) in the EDS is an artifact of the C-grid on which the sample was coated while peaks for Phosphorous (1.31%), Nitrogen (1.55%), Silicon (0.77%) and Sulphur (0.26%) correspond to the phenols, flavonoids, coumarins and enzymes capping over the synthesized CuONPs. The quantitative data confirms the formation of CuO instead of other copper oxide in the synthesized materials by green synthesis of *Leucaena leucocephala* L. plant.

3.7. Specific surface area and porosity studies

The eventful parameters such as particle size, shape and density are related to the specific surface area measurements ($\text{m}^2\cdot\text{g}^{-1}$) figure 6 exhibit BET plots of CuONPs. The specific surface area of CuO nanopowder calculated using the multipoint BET-equation is $47.54 \text{ m}^2/\text{g}$. Assuming that the particles have solid, spherical shape with smooth surface, and same size, the surface area can be related to the average equivalent particle size by the equation, Where,

$$D_{\text{BET}} = 6000/(\rho \cdot S_w) \dots\dots\dots (\text{in nm})$$

D_{BET} = The average diameter of a spherical particle

S_w = The specific surface area of the powder in m^2/g

P = The theoretical density in g/cm^3

The average crystallite size of the CuONPs calculated from BET data using above equation was 11.27 nm which is in good agreement with the result obtained by TEM. The results of TEM observations and BET methods further confirmed and verified the relevant results obtained by SEM analysis.

Figure 7 represented the typical BJH desorption pore size distribution curves of CuO nanopowder. The pore size estimated from peak position was about 2.13 nm and pore volume was found 0.102 cc/g, indicated relatively

narrow pore size distribution. From the curves, it can be concluded that most of the micropores with a size smaller than 21.32 nm. Moreover, such micropores have not been observed within particles by TEM (Figure 4). Therefore, these particles are actually grain clusters or small polycrystals.

3.8. Photoluminescence study

Biosynthesized CuONPs were tested for fluorescence studies and found to exhibit visible photoluminescence. The fluorescence spectra are shown in Figure 8 (a & b). The optimized CuONPs were found to emit four emissions at 302, 509 and 602 nm for an excitation at 250 nm. When CuONPs were excited at 300 nm, they showed excitation at 352 and 606 nm, the excitation of 300 nm is of high intensity in comparison to other one. The luminescence observed may be due to presence of phytoconstituents or antioxidants present in the plant extract. The AgNPs synthesized using *Azadirachta indica* leaf extract were also reported to exhibit fluorescence emission band at 561 and 600 nm (Ahmed & Ikram, 2015).

3.9. Antimicrobial activity of CuONPs

Literature reports reveal that CuONPs are highly toxic to most of the human pathogens (Sadeghi, 2015). In this context, we decided to investigate antimicrobial activity of biosynthesized CuONPs against various pathogens viz *Pseudomonas aeruginosa*, *Streptococcus pyogenus*, *Staphylococcus aureus* and *Escherichia coli*. These bacterial and fungal strains namely *P. aeruginosa* MTCC 1688, *S. pyogenus* MTCC 442, *S. aureus* MTCC 96 and *E. coli* MTCC 443 were added on nutrient agar plate and spread over the plate with the help of glass spreader and the "well" was made with the help of borer. The various concentrations of synthesized CuONPs (25, 50, 100, 250, 500 $\mu\text{g}/\text{ml}$.) were tested for antimicrobial activity against these pathogen with ampicilline as positive control. The plates were then kept at 4-5°C for 1 hr, followed by incubated in incubator at 37°C for 24 hrs. After 24 hrs, exact zone of inhibition was measured with respect to positive controls (Table 2). Gratifyingly, it was observed that biosynthesized CuONPs exhibited moderate antibacterial activity against the selected strains.

3.10. Antimalarial activity of CuONPs

The synthesized CuONPs were screened for their significant in vitro antimalarial activity against *Plasmodium falciparum* by measuring the minimum inhibitory concentration ($\mu\text{g/mL}$) against standard Quinine and Chloroquine, as shown in Table 3.

3.11. Antimycobacterial activity of CuONPs

The antimycobacterial screening was performed using Lowenstein-Jensen MIC method (Table 4) and it is worthwhile to note that biosynthesized CuONPs was the only displaying inhibition of *Micobacterium tuberculosis* H₃₇RV completely (99%) at the MIC of 250 $\mu\text{g/ml}$.

Table 2. Zone of inhibition (mm) of biosynthesized CuONPs against bacterial pathogens

Test pathogens	Inhibition zone (mm) of Control CuONPs ($\mu\text{g/ml}$)					
	25	50	100	250	500	
<i>E. coli</i>	09	12	13	14	16	19
<i>P. aeruginosa</i>	08	10	11	12	14	18
<i>S. pyogenus</i>	10	12	14	16	19	14
<i>S. aureus</i>	11	12	15	19	21	16

Table 3. Minimum inhibition concentration (MIC) of biosynthesized CuONPs against *Plasmodium falciparum*

Sr. No	Compound Name	Mean IC ₅₀ values
1)	CuONPs	0.90 $\mu\text{g/ml}$
2)	Chloroquine(Standard)	0.020 $\mu\text{g/ml}$
3)	Quinine (Standard)	0.268 $\mu\text{g/ml}$

Table 4. Minimum inhibition concentration (MIC) of biosynthesized CuONPs against *Micobacterium tuberculosis*

Sr. No	Compound Name	MIC ($\mu\text{g/ml}$)
1)	CuONPs	250 $\mu\text{g/ml}$
2)	Isoniazide (Standard)	0.20 $\mu\text{g/ml}$

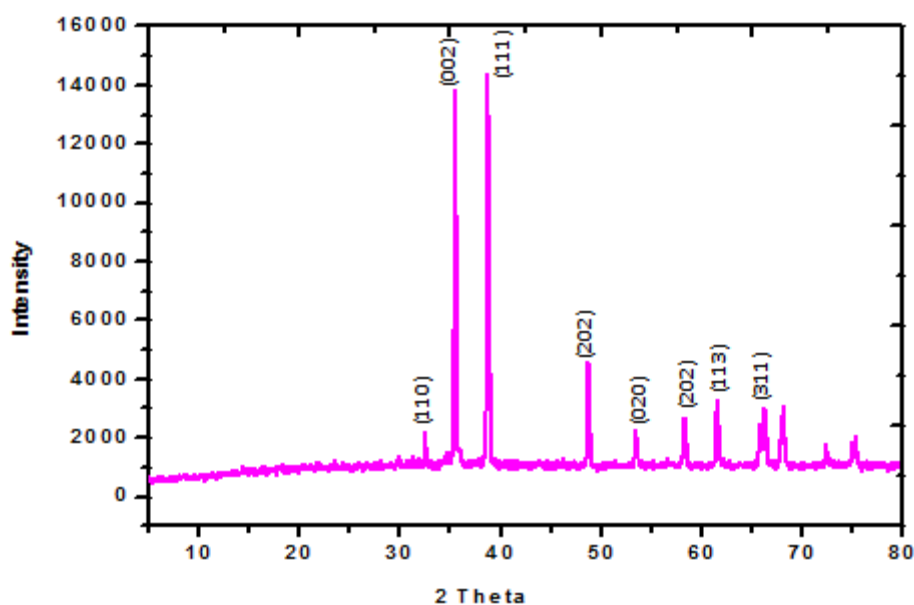


Figure 1. X-ray diffraction pattern of biosynthesized CuONPs.

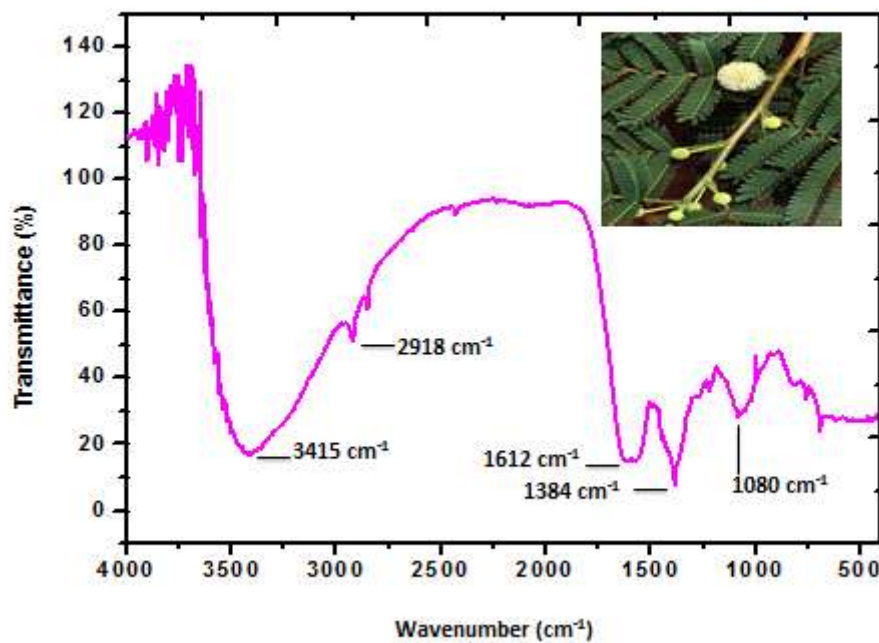


Figure 2. FT-IR spectrum of CuONPs in plant extract.

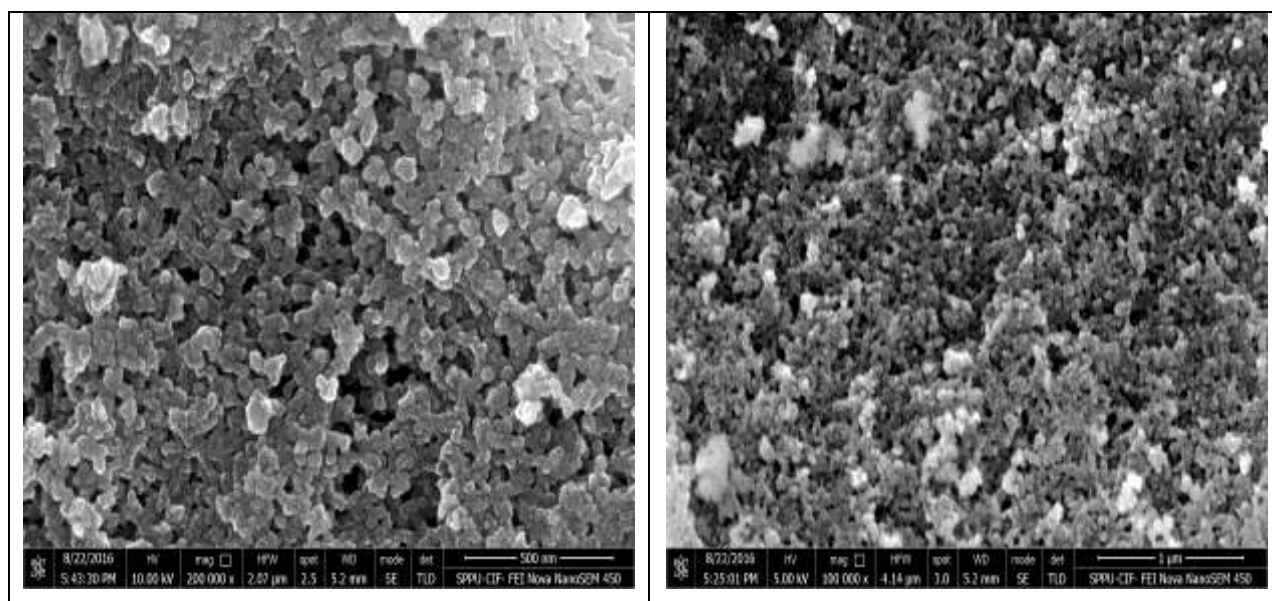


Figure 3. FE-SEM microphotographs of CuONPs deposited on a carbon strip

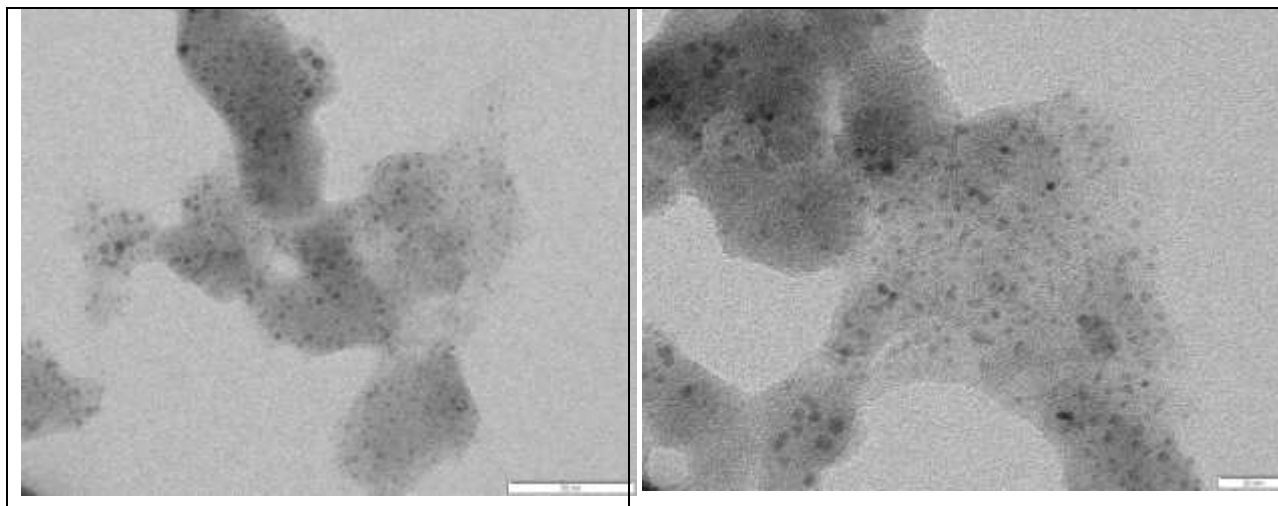


Figure 4. TEM images indicating the presence of spherical CuONPs recorded at various magnifications (a-50 nm and b-20 nm).

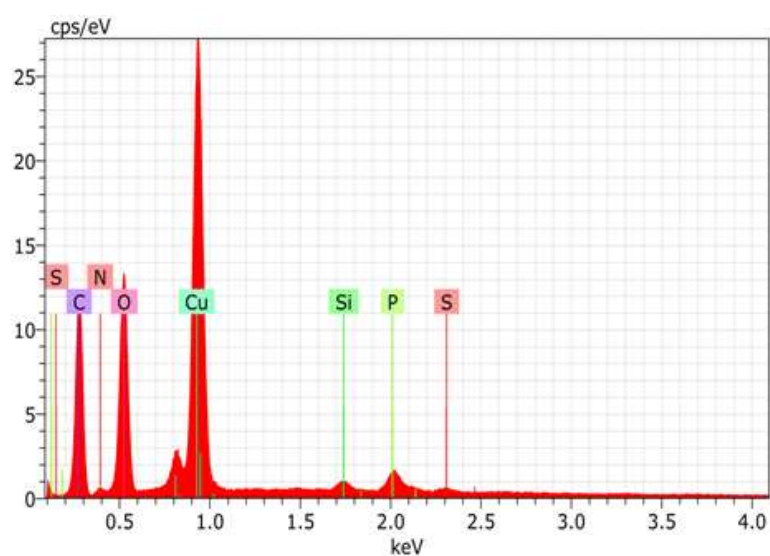


Figure 5. EDS spectrum of synthesized CuONPs.

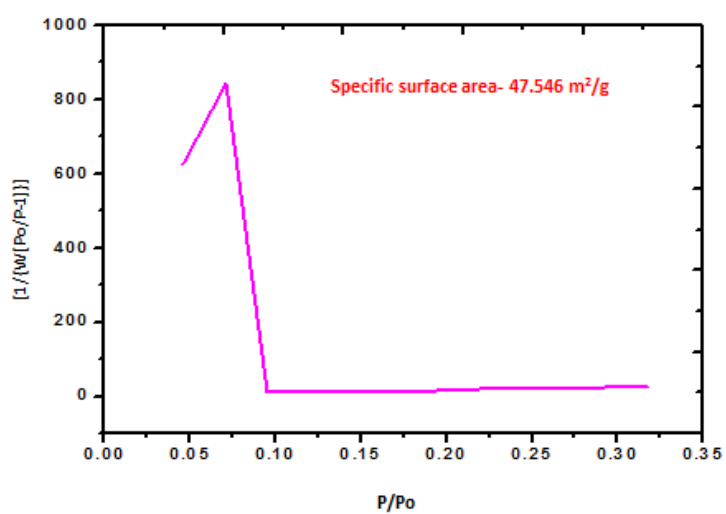


Figure 6. BET plots of biosynthesized CuONPs.

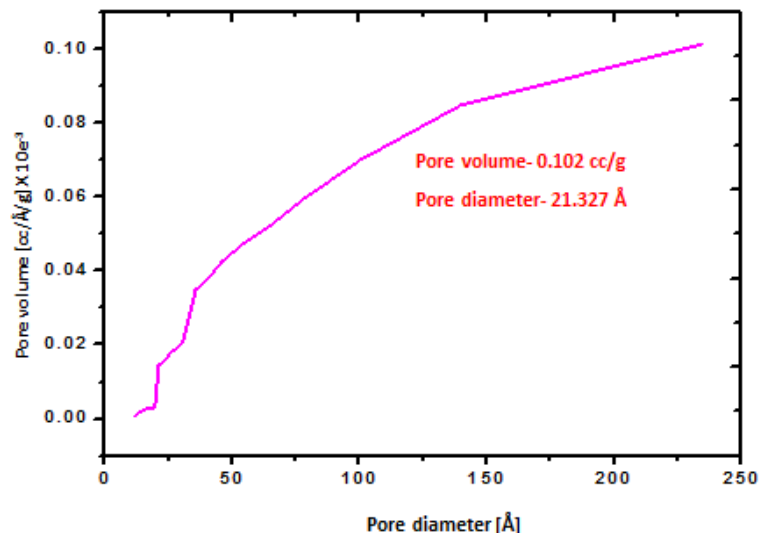


Figure 7. BJH pore size distribution curves plots of biosynthesized CuONPs.

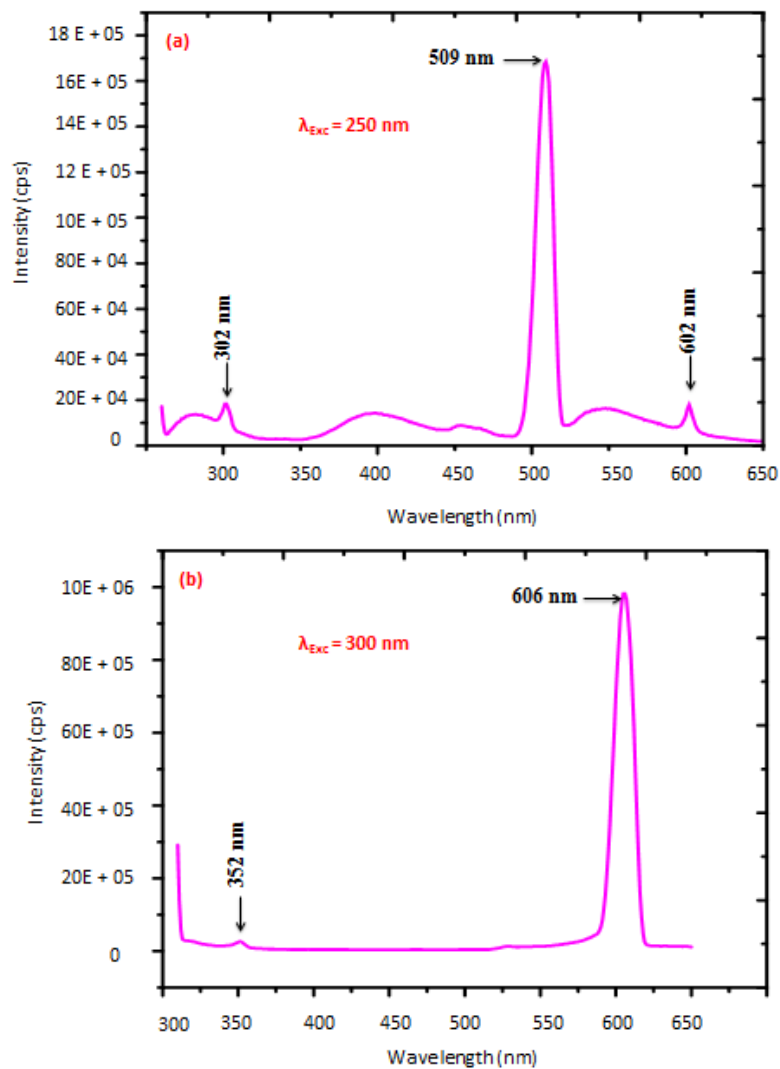


Figure 8. Fluorescence spectra of biogenically synthesized CuONPs at different excitation wavelengths, (a) 250nm and (b) 300 nm.

4. Conclusions

We have demonstrated an environmentally benign, economically affordable and facile biogenic synthesis of stable and spherical CuONPs using aqueous extract of *Leucaena leucocephala* L. The formation of CuONPs was characterized by FESEM, EDS, BET, BJH, TEM, XRD, FTIR and Photoluminescence. The biosynthesized CuONPs were found good antibacterial, antimalarial and antimycobacterial agents and thus can be used as potential candidates for various biomedical applications and will play vital role in medical devices in near future. The plant extract is ascribed to the relative levels of steroids, phenols, carbohydrates, flavonoids and amino acids which act as reducing as well as capping agents CuONPs. Furthermore study successfully demonstrates an easy way of employing underutilized plants for the production of multifunctional CuONPs.

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Disclosure

The authors declare no conflicts of interest in this work.

Refereces

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