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RESEARCH ARTICLE

SYNTHESIS AND CHARACTERIZATION OF HMTA ASSISTED CuO NANOPARTICLES WITH ITS POTENTIAL ANTIBACTERIAL APPLICATION

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ABSTRACT

Pure CuO nanoparticles and Hexa Methylene Tetra Amine (HMTA) used as a structure directing template added Cuo nanocrystal with diverse weights (0.1, 0.2, 0.3, 0.4, 0.5 gm) were anatomized for structural and morphological research using X-ray diffraction (XRD), Fourier Transform Infrared Spectroscopy (FT-IR), Field Emission Scanning Electron Microscopy (FE-SEM) Transmission Electron Microscope (TEM). The XRD analysis manifested monoclinic cry stallinity in pure and HMTA-added CuO nanocrystals, with an average crystallite size of 21.63 nm and 12-17 nm respectively. The FE-SEM analysis incarnated their nanoflower conformation. The FT-IR spectrum affirmed the presence of Cu-O bonds. The change of structure from nanoflower to nanorods was confirmed by TEM. The antibacterial properties of the as-prepared nanostructures investigated for various human pathogens using disc diffusion method. The result showed the remarkable antibacterial activity both gram positive and gram negative bacteria.

INTRODUCTION

Recent research has shown that the properties of materials depend largely on their particle size, morphology and structure. Among various 3d transition metals and their derivatives, copper oxide (CuO) is a unique monoxide compound (in monoclinic phase, different from normal rock-salt type structure) for both fundamental investigations and practical applications (Zheng *et al*., 2000). The excellent properties of CuO made it as a fantastic material for the diverse application which includes heterogeneous catalysts, gas sensors, optical switch, magnetic storage media, lithium–ion electrode materials, field emission devices, solar cells, etc. (Switzer *et al*., 2003; Chowdhuri *et al*., 2004; Gao *et al*., 2004; Hsieh *et al*., 2003; Chen *et al*., 2003). Nano-sized metallic copper and its oxides also possess good potential in bio-related fields including fouling control and nanotoxicology (Ren *et al*., 2009). Thus nanoparticles of copper and copper oxide have been extensively investigated for enhancing antibacterial property also investigated results suggested that these high quality nanometer-size materials could be easily obtained and scaled up for industrial applications because of their antibacterial activity (Anita Sagadevan Ethiraj and Dae Joon Kang, 2012; Li *et al*., 2002). The surfactants play an essential role in controlling morphology of nanostructure because of their soft - template effect, their ability to modify the chemical kinetics and simple maneuverability. The growth of a nanostructure is due to two processes namely Ostwald ripening and oriented attachment (OA). Oriented attachment mechanism comprises of the direct self organization of two particles into a single crystal by sharing a common crystallographic orientation. This process is dominant at nanometre level. It is reported that the capping agents, as they directly modify the nanoparticle surface can largely influence the OA processes. The molecular weight of the capping ligand also makes a remarkable contribution in the assembly behaviours of the nanoparticles.

Therefore, in the present study, a systematic effort has been made to synthesize CuO nanostructures by a simple and inexpensive chemical precipitation method using copper acetate and NaOH as the precursor material in the presence of structure directing template HMTA (Condorelli *et al*., 2003), In order to facilitate the nanoparticles loading and reduce health risks. The formation of copper oxide nanoparticles were confirmed by XRD, FTIR, FE-SEM and TEM analysis techniques. Also we investigated their antibacterial activities using human pathogens using gram positive and gram negative bacteria. The activity was studied against gram +ve bacteria strains namely Bacillus subtilis and Staphylococcus aureus and gram –ve bacteria strains namely Proteus vulgaris, Escherichia coli and Vibrio cholarae and the MIC values of all the strains has been investigated.

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EXPERIMENTAL METHODS

Chemicals

Copper acetate monohydrate (Cu $(CH_3COO)_2.H_2O$), sodium hydroxide (NaOH) pellet and Hexa Methylene tetra amine $[(CH_2)_6N_4]$ (HMTA, MW: 140.186g/mol) were purchased from Merck, India with 99% purity. De-ionized water was used all the way through the synthesis and ethanol was used for the washing purpose.

Bacterial used

The antibacterial activity of prepared nanoparticles were investigated against five strains of Gram positive bacterial strain namely Staphylococcus aureus (NCIM 2901), Bacillus subtilis (NCIM 2063) and gram negative bacterial strains such as Proteus vulgaris (NCIM 2027), Escherichia coli (NCIM 2256) and Vibrio cholera (ATTC 14033) was obtained from National Collecting Industrial Microorganism (NCIM), Biochemical Sciences Division, National Chemical laboratory, Pune. The stock cultures were maintained on nutrient agar medium at 7 °C for 24 h, antibacterial activity was determined by using Muller Hinton Agar (MHA) and Muller Hinton Broth (MHB) obtained from Himedia Ltd, Mumbai.

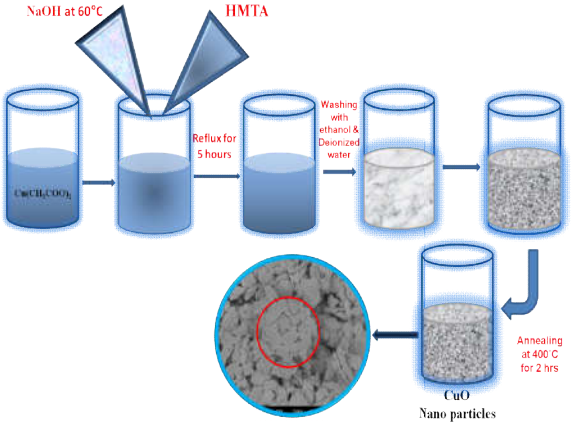

Figure 1 Preparation process of Cuo nanoparticles

Synthesis of CuO nanostructures

For the synthesis of pure CuO nanocrystals, 1.99 g (0.2 M) of copper acetate in 50 ml of deionised water was allowed to stirrer and then 1.2 g (0.6 M) of sodium hydroxide (NaOH) pellet were mixed drop by drop to the above prepared solution. The entire mixture was constantly stirred for 5 h at 60 °C. Then the precipitate was filtered and washed alternately with deionized water and ethanol to remove the impurities and then it was dried in oven at 80 °C, finally annealed for 2 h at temperature of 400 °C to obtain the highly crystalline CuO nanoparticles. For the structure directing template of HMTA added CuO nanoparticles, 0.2 M of copper acetate in 50ml deionised water was stirring in magnetic stirrer. Then diverse concentrations (0.1, 0.2, 0.3, 0.4, 0.5 gm) of HMTA were added to the above solution in a different experiment. The mixture was stirred magnetically at 60 °C until a homogeneous solution was obtained. After that 0.6 M of NaOH pellet was added drop by drop to the above mixture and followed the same procedure as above for washing and drying purpose. Figure 1 shows the flow chart of the preparation process of CuO nanoparticles.

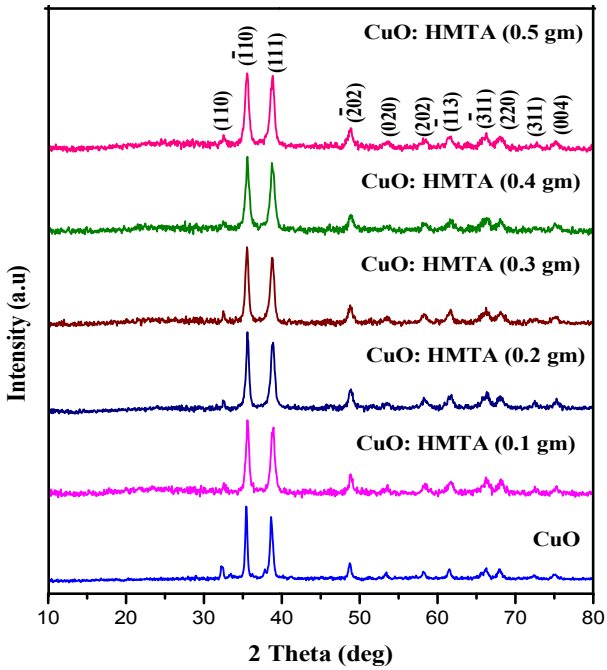

Figure 2 XRD spectrum of Pure and Various concentrations of HMTA capped CuO nanocrystals

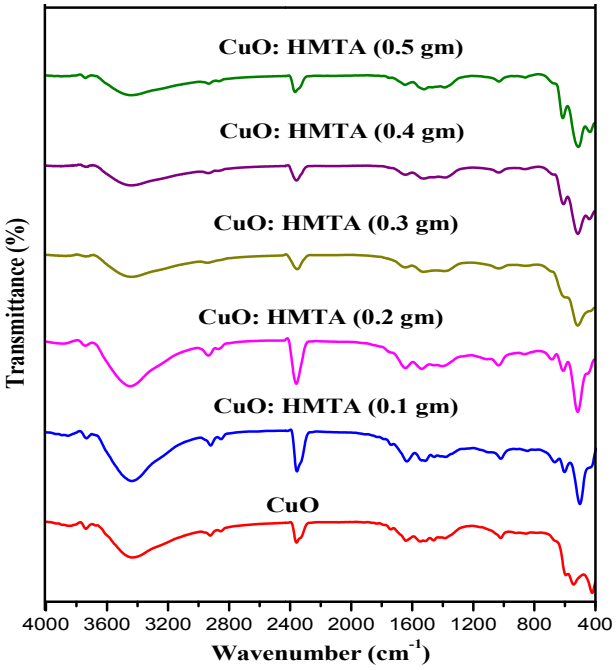

Figure 3 FTIR spectrum of Pure and various concentrations of HMTA capped nanorods

Characterization

The crystal structure and phase purity of the as synthesized products were investigated by X-ray diffractometer (X'PERT PRO) with CuKα radiation (λ=1.5406 Å). FT-IR has been employed to find the presence of functional groups in the range of 4000-400 cm^{-1} and it was recorded using SHIMADZU-8400 with a resolution of 4 cm^{-1}. Measurements were performed with pressed pellets which were made using KBr powder as

diluents. The morphology of the products was examined using Philips Field Emission Scanning Electron microscopy (FESEM). Further investigation on the morphology of prepared sample of TEM and SAED patterns was performed using Transmission Electron Microscope (PHILIPS) and the model is CM200 with an operating voltage of 20-200 kV with resolution 2.4 Å. The antibacterial activity was done using disc diffusion method.

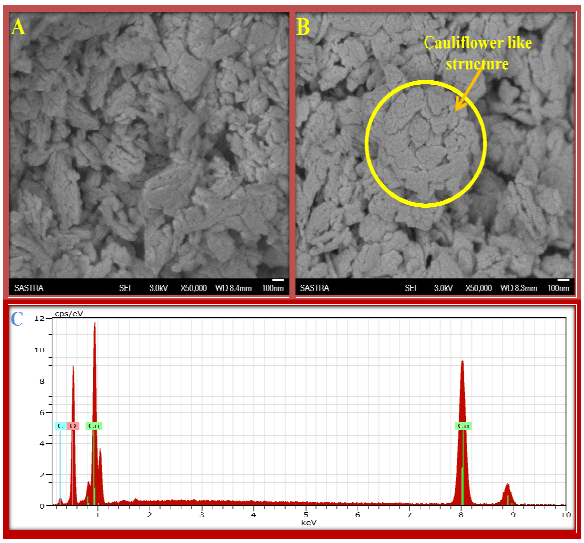

Figure 4 FE-SEM images for uncapped and 0.4 gm HMTA capped nanorods

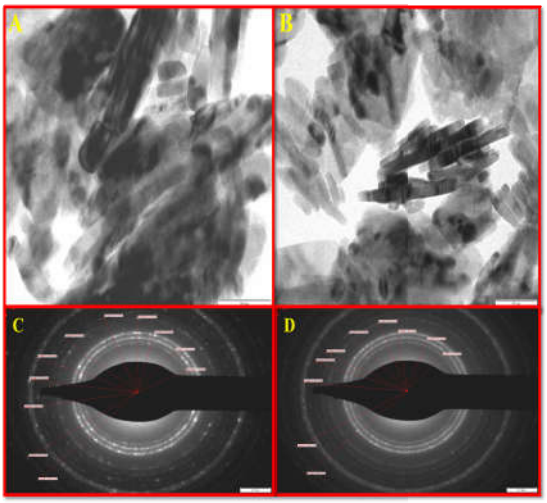

Figure 5 TEM images of A) Pure and B) 0.4 gm HMTA capped CuO C) and D) Corresponding SAED pattern

Antibacterial Assays

Disc diffusion method

The antibacterial activity of compounds was determined by disc diffusion method according to Bauer *et al*., (1966) with modification. Petri plates were prepared by pouring 20 mL of MHA for bacteria and then the plates were allowed to solidify and used in susceptibility test. The standard inoculum using bacterial suspensions containing 10^8 CFU per mL was swabbed on the top of the solidified respective media and allowed to dry for 10 minutes. The prepared nanoparticles were dissolved in 10% Di-Methyl Sulfoxide (DMSO) and under aseptic conditions, sterile discs were impregnated with compounds of 200 µg/disc. The discs with compounds were placed on the surface of the medium with sterile forceps and gently pressed to ensure contact with inoculated agar surface. Ciprofloxacin (5 µg/disc) for bacteria was used as positive control and 10% DMSO was used as blind control. Finally, the inoculated plates were incubated at 37 ℃ for 24 h for all the bacterial strains. The zones of inhibitions were observed and measured in millimeters. The assay in this experiment was repeated three times.

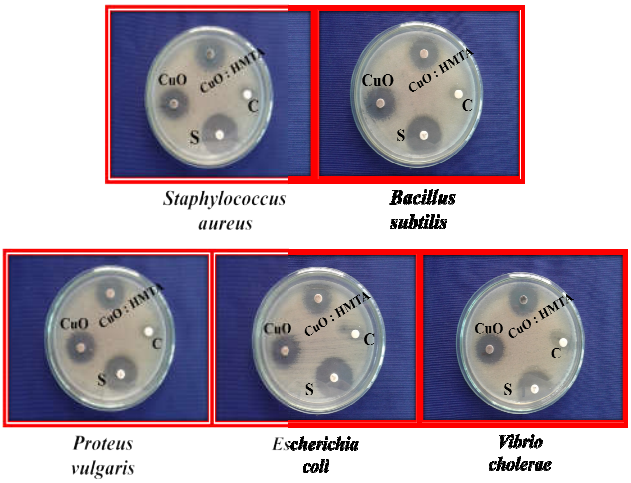

Figure 6 Antibacterial activity photograph of different bacteria for CuO nanoparticles with 0.4 gm HMTA capped nanoparticles

Micro Dilution Broth Assay

Determination of the minimum inhibitory concentration (MIC) for bacteria

The MIC of prepared nanoparticles were determined in MHB by using a microtitre plate assay as reported by Hammond and Lambert (1978) 100 µL of Sterile MHB for bacteria were transferred in to each well of sterile 96 micro titer well plate. The both samples were dissolved in 10 percent DMSO to obtain 400 µg/mL (compounds) stock solutions respectively.

A volume of 100 µL of prepared nanoparticles stock solution was added into the first well. After fine mixing of the compounds and broth 50 µL of solution was transferred to the second well and in this way, the serial dilution procedure was continued to a twofold dilution to obtain concentrations like 200 to 7. 81 µg/mL (compounds) of each well. Finally, 5 µL of bacterial suspension was added to each well to achieve concentrations of approximately 5 $\times 10^5$ CFU/mL. Each plate was a setup positive control (bacterial suspension adding 10 µL of MHB) and negative control (10 % DMSO and bacterial culture). The plates were incubated at 37 ℃ for 24 h for all the bacterial strains. The lowest concentration occurred was taken as the MIC value.

RESULT AND DISCUSSION

Structural analysis

The XRD patterns of uncapped and diverse weights of structure directing template of HMTA added (0.1, 0.2, 0.3, 0.4 and 0.5

Table 1 Zone of inhibition table for pure and HMTA capped CuO nanoparticles

Name of the bacterial strains	Ciprofloxacin (mm)	Zone of inhibition (mm)			MIC (µg /disc)	
		Cuo	Cuo: HMTA (0.4 gm)	Control (DMSO)	Cuo	Cuo: HMTA (0.4 gm)
Bacillus subtilis	30.1±0.28	28.6±0.76	29.6±0.50	8.1±0.28	25	12.5
Staphylococcus aureus	28.6±0.76	26.3±0.57	27.3±0.57	7.5±0.50	25	25
Proteus vulgaris	28.5±0.80	25.1±0.28	27.1±0.28	9.0±0.50	25	25
Escherichia coli	28.7±0.76	25.6±0.75	26.8±0.78	7.1±0.28	25	25
Vibrio cholerae	30.0±0.50	21.4±0.50	22.3±0.57	7.0±0.50	25	25

[a] – diameter of zone of inhibition (mm) including the disc diameter of 6 mm; [b]– mean of three analysis, ± - standard deviation, * - significant at $P < 0.05$.

gm) CuO nanocrystals are shown in Fig. 2.The obtained diffraction planes value is in good co-ordination with the JCPDS card No: 89-2531. It is also confirmed the monoclinic structure of CuO nanoparticles. In addition to this, no other peaks related to other phases and impurities were not found in the XRD pattern. From the X-ray diffraction peak widths, the diameter of the nanocrystals was estimated through Scherrer formula (Birks and Friedman, 1946). The average size of the particles estimated is 21.63 nm for uncapped. However, the estimated sizes of the capped particles are 17.35, 15.35, 13.28, 11.69 and 13.12 nm for capping concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5 gm respectively.

When compared with pure CuO nanocrystals, all the capped particles show reduced size as a result of adding the template. The mechanism behind the formation of size reduced particle is explained as follows. After introducing polymer into their action mixture, Cu^{2+} ions form a complex with the structure directing template of HMTA, ensuing in particle capping in the lead nucleation (Shanmugam Cholan *et al*., 2014).

The equation used for the calculating the average particle size is given as follows:

$$\text{Average particle size } D = K\lambda / \beta\cos\theta$$

Where,

D→ the cry stallite size of CuO NPs,
λ→ the wavelength of Cu Kα source (1.5406 Å),
B→ is the half maximum line-width of the diffraction peak,
K→ the Scherrer constant (shape factor) with a value from 0.89 and,
θ→ Bragg angle (deg).

Hence by considering the particle size 0.4 gm of HMTA has been considered as the optimum level to prepare CuO nanoparticles.

Functional Analysis

To understand the role of structure directing template HMTA on the synthesis of CuO nanoparticles, FT-IR spectra were recorded in the range of 4000-400 cm^{-1}. Fig. 3 illustrates the FT-IR spectra of uncapped and diverse weights of HMTA added CuO nanocrystals. All the samples exhibit a broad absorption band in the range of 3330–3500 cm^{-1} due to Stretching vibration of OH molecules. As shown in Fig. 3, HMTA added samples show absorption bands at 2920 cm^{-1} due to CH asymmetric stretching vibrations. The absorption band positioned at around 2860 cm^{-1} is resulted from characteristic peak of Structure directing template. The band around 2360 cm^{-1} assigned to carboxylic group (COO^-) vibration in which the intensity was increased from uncapped to 0.4 gm of HMTA added nanoparticles and it gets decreases at 0.5 gm of HMTA added CuO nanocrystals which shows the optimum level of o.4 gm. The presence of a peak at 1456 cm^{-1} and around 1650 cm^{-1} corresponding to C=C stretching and C=N stretching respectively. The lower shifting of peak around 1379 Cm^{-1} indicates chelation of the salicylaldehyde group to the metal center (Zahra Shahri *et al*., 2014). The band centred at 1247 cm^{-1} originates from C-O-C bands of adding template (Rahima *et al*., 2012). The absorption bands saw around 420, 505, and 590 cm^{-1} are due to the Cu-O stretching in the monoclinic structure of CuO (Zou *et al*., 2006).

Morphological analysis

Fesem

Figure 4 (A and B) displays the FE-SEM images of CuO nanocrystals for pure and HMTA added CuO nanoparticles respectively. Comparing pure, structure directing template of HMTA added CuO nanostructures shows the better formation of CuO nanostructure. The image illustrates the formation of CuO nanorods of length typically about 100-250 nm for pure. A minimal aggregation of the nanorods has also been noted. Fig. 4B clearly shows the influence of structure directing template by forming the nanoflowers which can be seen as a cauliflower like structure. The petals of the rods are joined together to form a flower like structure.

The practical mechanism at the backside of modify in morphology is explained as follows. While structure directing template is supplementary to the reaction mixture, they primarily join to the surface of growing particles by more over steric or electrostatics repulsion and put off the extra growth of the particles. Here HMTA plays a considerable role in changing the morphology of the CuO nanoparticles and also in controlling the particle (Chun *et al*., 2009). FESEM data established that HMTA addition has a significant effect on the morphology and size distribution of the obtained material. Fig. 4C is the corresponding EDS pattern which confirms the presence of Cu and O without any presence of impurities.

Transmission Electron Microscopy

TEM analysis was done for the further investigations on the morphology of the as-prepared samples. Figure 5 (A, B) shows the TEM images of pure and structure directing template of

HMTA added nanoparticles. As shown in the Figure 5 (A, B), analysis of the TEM micrographs indicates the formation of CuO nanorods with a size of 30-35 nm and 15-22 nm respectively for pristine and HMTA added CuO nanorods respectively. TEM images clearly shows that the petals of the rods shown in TEM are joined together to form Cauliflower like structure which is shown in FE-SEM. The SAED pattern of as-synthesized CuO nanocrystals shows that the particles are poly crystallized as shown in Figure 5 (C and D). The TEM images show that the obtained nanocrystals are rod-like structure (Fig. 5 A, B).

Antibacterial activity

Antibacterial activity of nanoparticles were determined by disc diffusion method as shown in Figure 6. Large and clear zones of inhibition around discs impregnated with test nanoparticles, clearly manifested that nanoparticles are showed a powerful antibacterial impression and activities for Gram-positive and Gram-negative bacteria, respectively . The activity was studied against gram positive bacteria strains namely Bacillus subtilis and Staphylococcus aureus and gram negative bacteria strains namely Proteus vulgaris, Escherichia coli and Vibrio cholerae.

The bacterial inhibition zones for CuO nanorods for both pure and HMTA capped CuO nanoparticles are shown in the Table. 1 and it was compared with standard ciprofloxacin. From the table it was proved that the prepared nanoparticles exhibit a remarkable activity against human pathogens which is near to the value of standard. The highest mean zone of inhibition of 29.6±0.50 mm was developed against B.subtilis for HMTA (0.4 gm) capped CuO nanorods. For all the bacterial strains comparing pristine, HMTA capped nanoparticles are shows the better zone of inhibition.

Several studies have been suggest two possible mechanisms for the interaction between the bacteria and the nanoparticles. The primary reason is the production of increased levels of reactive oxygen species (ROS). The oxygen species are mostly in the form of hydroxyl radicals and singlet oxygen. The next reason is the deposition of the nanoparticles on the surface of bacteria (Heinlaan *et al*., 2008). In addition, the MIC values of nanoparticles against all the five pathogens are listed in Table.2. The results of the lowest MIC values of the CuO nanostructures were ranged between 6.25 and 25 µg/ml. The lowest MIC value of 12.5 µg/ml against B.subtilis. Gram-positive bacteria have thicker peptidoglycan cell membranes compared to the Gram-negative bacteria and it is harder for CuO to penetrate it, still our synthesized nanoparticles of both pure and capped nanoparticles showed the proper response for all the human pathogens. The extent of inhibition of bacterial growth reported in this study clearly showed the effect of capping on CuO nanoparticles.

CONCLUSION

In conclusion, the results evince that a simple chemical precipitation method being a simplistic and skilful route to synthesize structure directing template HMTA-added CuO nanocrystals range of desired grades. At the outset, the X-ray diffraction analysis illustrated the monoclinic phase of the synthesized nanoparticles. However, the size of the nanoparticles were evidently influenced by the percentage of capping, first and foremost proclaiming lessening in size of the nanoparticles as a result of capping when juxtaposed to their pure counterpart. Among experimented concentrations, 0.4 gm of the capping agent yielded smaller sized particles of 11.69 nm. FESEM and TEM studies of HMTA (0.4 gm) capped particles show a considerable effect on the morphology and size distribution of the obtained artefacts. Comparing pristine CuO, the antibacterial activity of HMTA added CuO nanorods developed a highest zone of inhibition for all the human pathogens. Obtained values of MIC for all the strains suggest that the prepared copper oxide nanoparticles shows excellent antibacterial activity and can be used as promising antibacterial agents in wide applications. Upon contemplating the above factors, 0.4 gm of HMTA has been considered as an optimum level for the preparation of CuO nanoparticles in order to control the size of the particle and for the morphology and also for the potent antibacterial applications.

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