Synthesis, characterization and evaluation of antibacterial activity of copper oxide nanoparticles against clinical strains of *Staphylococcus aureus*

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Abstract: Bacterial resistance is spreading globally due to excessive use of antibiotics, making it one of our times biggest challenges. To address this issue present study was conducted to evaluate the antibacterial activity of copper oxide nanoparticles against methicillin-resistant *S. aureus* (MRSA). Copper oxide nanoparticles were synthesized by chemical precipitation method and were characterized by UV-Visible, FT-IR spectroscopy, X-ray diffraction (XRD) and Scanning Electron Microscopy. These nanoparticles of 27nm were assessed for antibacterial activity using disc diffusion method. Our results showed superb inhibitory effects of CuO nanoparticles with increase in concentration and complete inhibition was recorded against tested strains of *S. aureus* at 100µl/ml and 125µl/ml concentration. The study concludes that the drugs which do not show any inhibitory effects against resistant bugs could be augmented with CuO nanoparticles to achieve the treatment goal.

Keywords: Methicillin-resistant *S. aureus,* UV-Visible spectroscopy, copper nanoparticles, inhibition of *S. aureus.*

INTRODUCTION

The ability of bacterial pathogens to evolve quickly is one of the biggest clinical challenges around the world including Pakistan. Among various species of *Staphylococci, Staphylococcus aureus* has the highest pathogenic potential because of the ability to resist various traditional antibiotics commonly used to cure *Staphylococcal* infections. The frequency of MRSA incidence in Asia has been shown to be high as compared to European countries (Laxminarayan and Malani, 2007)., the prevalence of MRSA in India and Pakistan has also shown to be elevated as compared to Europe (Anwar *et al.,* 2004).Keeping in view the high mortality and morbidity of MRSA, many antibiotics have been searched upon such as synercid, linezolid (anoxazolidinone) (Dailey *et al.*, 2001) and daptomycin (Richter *et al.,* 2003) to kill *S. aureus* strains. But unfortunately, few *S. aureus* strains have been emerged to develop resistance to these newly discovered agents. Hence, there is a dire need to develop alternative antimicrobial agents (Cho *et al.,* 2005). In an attempt to develop innovative antimicrobial drugs nanotechnology has played its role and the controlled fabrication of nanoparticles has impelled nanotechnology into today's most promising and hopeful area of research **(**Shenmar *et al.,* 2005). Of the variety of nanoparticles choices available, CuO nanoparticles are strikingly important because of their exceptional properties and low cost as compared to other metallic NPs (Anzlovar *et al.,* 2007). In the past copper has been used to kill bacteria and fungi for many years (Ren *et al.,* 2009). Cu^{2+} ions generate reactive oxygen species (ROS) which lead to oxidative stress and bacterial cell membrane damage (Pramanik *et al.,* 2012; Weitz *et al.,* 2015). The aim of present study was to synthesize and characterize copper oxide nanoparticles and to assess the anti-MRSA activity of synthesized nanoparticles.

MATERIALS AND METHODS

Synthesis of copper oxide nanoparticles

Copper (II) sulfate penta hydrate as precursor salt was used to synthesize CuO nanoparticles by chemical precipitation process. Synthesis began by drop wise addition of prepared sodium hydroxide solution in aqueous $CuSO_4.5H_2O$ solution with constant stirring for about 2 hours at room temperature. Afterwards the solution was heated at 50^{o}C for 60 minutes, which resulted in precipitates formation. The resultant solution was centrifuged 3-4 times at 1600 rpm for 15 minutes, washed 2-3 times with deionized water and ethanol. Finally the material was heated and the copper oxide formed was scratched off the glass beaker. The obtained samples were dried in air for 24 hours. Annealing at

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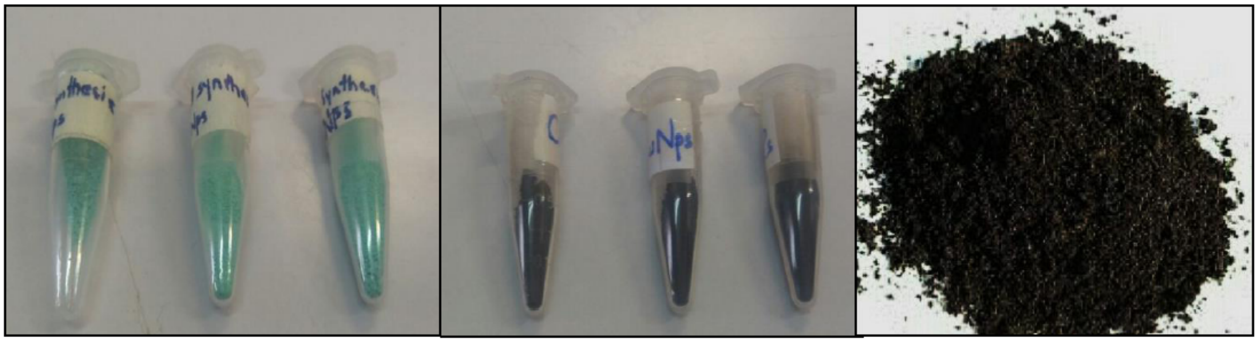

Fig. 1: Precipitates obtained after centrifugation of reaction mixture; black CuO NPs obtained after annealing at 400^{o}C

400^{o}C for 3 hours was done to stabilize the nanoparticles then crystalline CuO-NPs were stored for further analysis and antimicrobial activity (fig. 1).

Characterization of copper oxide nanoparticles

Various characterization techniques were used. Nanoparticles were visualized by UV-Visible spectroscopy. The chemical and structural properties of the CuO-NPs were determined by FT-IR spectroscopy particularly in the region between 500 - 3500 cm^{-1}. The X-ray diffraction (XRD) was done to discover nature of the synthesized copper NPs using X-ray diffractometer (XRD). The XRD patterns with diffraction intensity versus 2θ from 20˚ to 80˚ were recorded. Scanning Electron Microscopy (MIRA3-TESCAN) was done to find shape and size of synthesized nanoparticles.

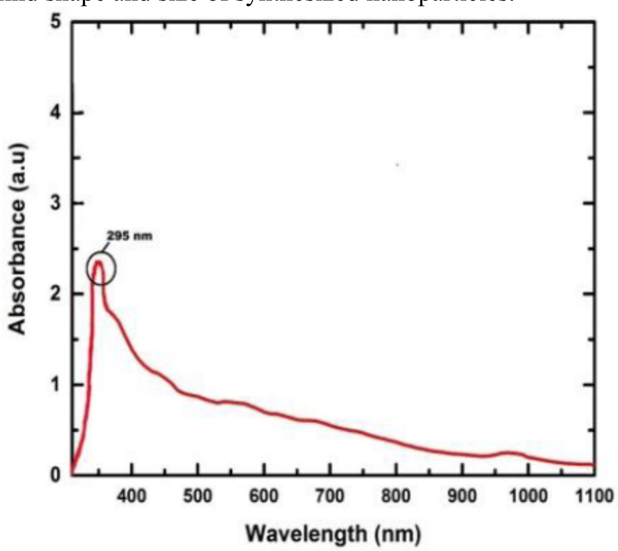

Fig. 2: U.V visible spectra of CuO nanoparticles showing the absorption peak at around 295nm

Antibacterial activity of synthesized copper oxide Nanoparticles

Antibacterial activity of copper oxide nanoparticles against methicillin-resistant *S. aureus* was assayed using standard disc diffusion method. A serial dilution of 25µl, 50µl, 75µl, 100µl and 125µl per ml from 1mg/ml stock solution were used against the tested MRSA strain. The MRSA strain was cultured on MH agar plates. After culturing, autoclaved filter paper discs of 5mm in diameter, instilled with CuO-NPs containing various concentrations, were applied on petri plates inoculated with tested strain and were incubated overnight at 37^{o}C. The next day zones of inhibitions around the discs were measured in mm.

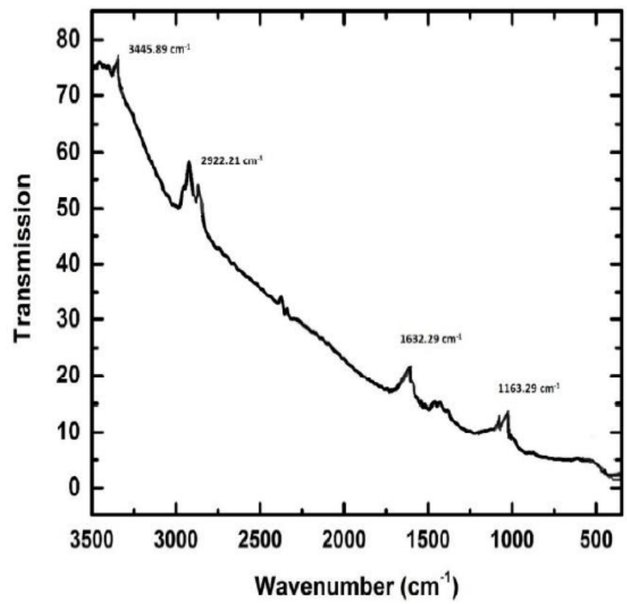

Fig. 3: FTIR spectra of copper oxide nanoparticles

Comparison of antibacterial activity of copper oxide nanoparticles with standard antibiotics

Antibacterial activity of synthesized copper oxide nanoparticles was compared with antibacterial activity of standard antibiotics (methicillin and ceftriaxone) by disc diffusion assay. *Staphylococcus aureus* was cultured on Mueller-Hinton agar and filter paper discs containing 30µg methicillin and 30µg ceftriaxone were used. Antibiotic discs and disc containing synthesized copper oxide nanoparticles (125µl/ml) were placed on the surface of a Mueller-Hinton agar that has been pre inoculated with *Staphylococcus aureus*. After 18-24 h the diameter of zone of inhibition were measured according to reference table (Hammonds 1995).

RESULTS

Copper oxide nanoparticles appeared black-brown which indicated the synthesis of copper oxide nanoparticles as shown in fig. 1. The obvious change in color was observed after annealing at 400°C which is due to reduction of copper ions.

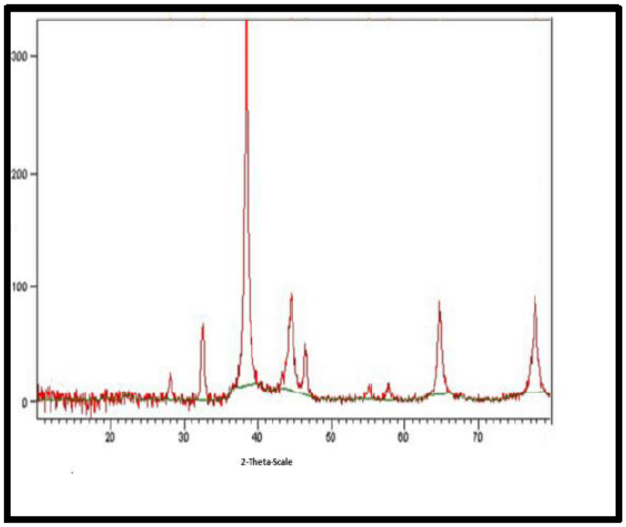

Fig. 4: X-ray diffraction pattern of CuO nanoparticles annealed at 400°C

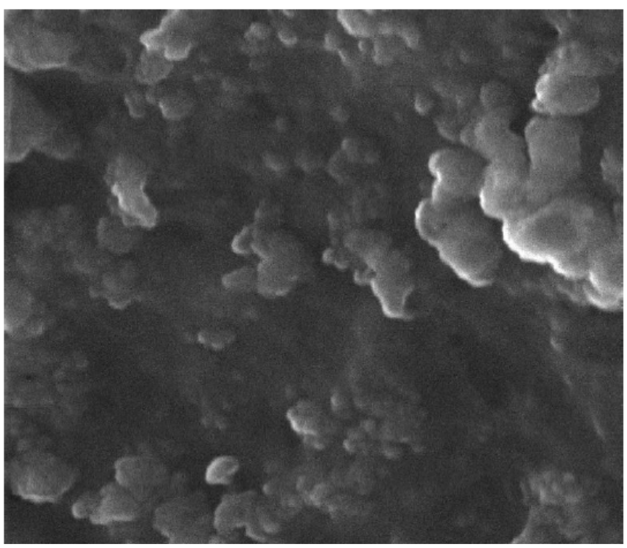

Fig. 5: SEM image of CuO nanoparticles

Characterization of CuO nanoparticles

The reduction of copper oxide nanoparticles was monitored by measuring the UV-Visible absorption spectrum in the range of 200-1000 nm. Results indicated that a broad absorption peak was observed at around 295 nm in 24 h indicative of CuO NPs synthesis (fig. 2).

FTIR spectra of copper oxide nanoparticles which indicated that the broad absorption peak at around 3445.89 cm^{-1} was caused by the adsorbed water molecules (fig. 3) The peak at 2922.21 $cm^{-1}$is attributed to -C-H bond stretching and the peak at 1163.29 cm^{-1} is because of O-H bond deformation. The peaks at 1632.29 is due to Cu-O symmetrical stretchingg.

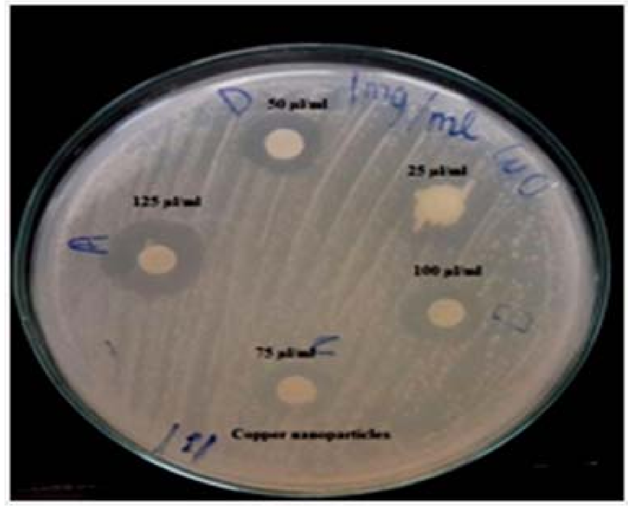

Fig. 6: Antibacterial activity of CuO NPs against *Staphylococcus aureus* (MRSA)

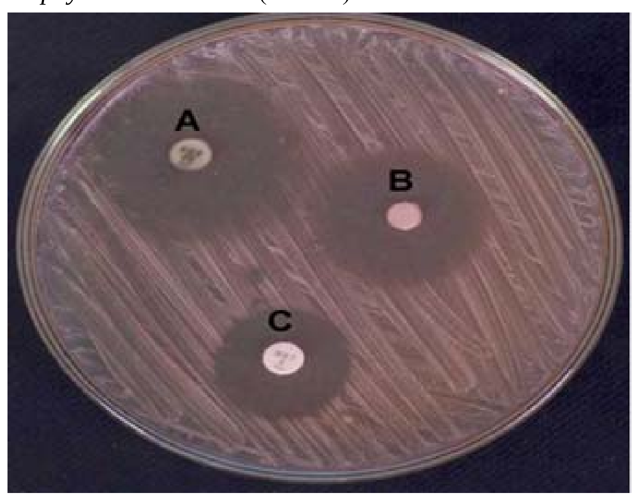

Fig. 7: Comparison of antibacterial activity of copper oxide nanoparticles with standard antibiotics Key, A ceftriaxone disc B, copper oxide nanoparticles C, methicillin disc

The XRD pattern of the synthesized CuO nanoparticles was recorded for the confirmation of crystalline nature of synthesized nanoparticles. Findings of XRD analysis were consistent with SEM analysis which showed average crystalline size of 27 nm. The highest diffraction peak at 38° of 2 theta value is assigned to CuO and the rest of diffraction peaks are well indexed of copper structure (fig. 4).

0SEM image 100,000X magnification showed that the particles are spherical in shape and very well-dispersed, It seemed that the particles were agglomerated. In some regions, the competitive large sized nanoparticles were bounded by smaller nanoparticles as shown in fig. 5.

Evaluation of antibacterial activity of CuO-NPs

CuO-NPs were checked for effectiveness against the MRSA strain at five different concentrations i.e., 25µl/ml, 50µl/ml, 75µl/ml, 100µl/ml and 125µl/ml. Results shown in fig. 6 revealed that CuO-NPs at varying concentrations showed significant difference in zone of inhibition. At lowest concentration, zone diameter of 10mm was recorded followed by 50µl/ml and 50µl/ml concentration. There was an incredible increase in inhibition zones with the increase in concentration. Excellent inhibitory results were obtained at 100µl/ml and 125µl/ml respectively. It was recorded that the zones of inhibition were wide and clear at higher concentrations (fig. 6).

Comparison of antibacterial activity of copper oxide nanoparticles with standard antibiotics

Standard antibiotics Methicillin and ceftriaxone were used for evaluation of comparison of antibacterial activity of synthesized copper oxide. The largest zone of inhibition was produced by ceftriaxone that is 26 mm in diameter whereas smallest zone of inhibition 19mm was produced by methicillin, in this experiment copper oxide nanoparticles produced zone of inhibition bigger than methicillin antibiotic which is 23 mm in diameter as evident in fig. 7

DISCUSSION

In the present study it was showed that copper nanoparticles were effective in inhibiting the growth of *S. aureus.* It has been observed that these nanoparticles showed excellent effects with the zone diameter of 10mm at lowest concentration and 19mm at highest concentration copper oxide nanoparticles were found more effective as compare to the antibiotic methicillin as evident by large zone of inhibition generated on agar plates. CuO nanoparticles showed absorption peak at around 3445.89 cm^{-1}. Similar results showing peak at 3434 cm^{-1} in the FTIR spectra of CuO-NPs were documented earlier (Radhakrishnan and Beena, 2014). The diffraction peak at 2922.21 cm^{-1} is assigned to to -C-H bond stretching (Kumar and Rani 2006). The diffraction peak at 1632.29 might be due to the Cu-O symmetrical stretching (Markova-Deneva, 2010). Results of SEM images obtained in the present study were comparable to the previous study conducted in 2014 (Radhakrishnan and Beena, 2014). The experimental results obtained in the present study were found related to the previously reported diffraction peaks of CuO-NPs (Radhakrishnan and Beena, 2014). Present results coincide with the previous studies which reported the significant antibacterial activity of CuONPs against gram positive pathogen such as *Staphylococcus aureus* and gram negative bacteria such as *Escherichia coli* (Padil and Cerník, 2013). Antibacterial and antioxidant activity of CuO nanoparticles has also been reported previously (Das *et al.,* 2013). Previous reports suggest that there is a considerable difference in antimicrobial effects of nanoparticles against Gram positive and Gram negative bacteria as cell wall of gram positive bacteria is thicker and wider (Thiel *et al.,* 2007). Thickening in cell wall is attributed to its varying composition of peptidoglycan, teichoic acids and lipid content compared to gram negative bacterial cell wall. These acids give cell wall a strong negative charge which hinder the killing effect of reactive oxygen species (ROS) produced by copper oxide nanoparticles.

CONCLUSION

As a result copper nanoparticles cannot penetrate cell membrane efficiently and hence reduce their inhibitory effects (Manikandan *et al.,* 2015). But our study showed excellent inhibitory effects against MRSA which may be due to excess production of free ions which may lead to degradation of cell wall of *S. aureus* cells.

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