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# Biogenic Copper Nanoparticles as a Nanoscale Solution to Address Multiple Drug Resistance in Bacteria

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**Authors' Contribution**
**AAK conceived and designed the study. AT, WA, KNM and MS performed the experimental work and wrote the article with the help of MAG, MB and AAK. MD helped in UV-Vis spectroscopy and SEM imaging. MAG collected fruiting bodies of *F. synchomorus* form Islamabad. AAK and MAG supervised the work.**

**Key words**
**Copper nanoparticles, Green synthesis, Multidrug resistant, *Ficus sycomorus***

## ABSTRACT

Resistance towards a number of antibiotics in a variety of microorganisms is expanding at an alarming pace. There is a dire need to devise novel tactics to deal with these minute yet evolving ever clever microscopic entities. One of the possible ways to fight them is through nanoparticles. Green synthesis is an easy way to synthesize nanoparticles by using biological resources as it is cost effective, eco-friendly and large-scale production possibilities exist. Copper nanoparticles (CuNPs) have been synthesized from the aqueous fruit extracts of *Ficus sycomorus* and their physico-chemical properties as well as antimicrobial activity against Multidrug Resistant (MDR) bacteria is evaluated. The total formation of the Cu nanoparticles was observed visually with a color change and confirmed by the appearance of peak through UV-Vis spectroscopy analysis. The electron microscopy imaging revealed that the biogenic Cu nanoparticles were spherical in shape and had a diameter of 30-40 nm. The synthesized nanoparticles showed promising antibacterial activity against MDR clinical isolates of bacteria.

## INTRODUCTION

Antibiotics is a term described by an American microbiologist Selman Waksman in 1941 but the era of antibiotics started with the discovery of penicillin by Sir Alexander Fleming in 1928 (Sengupta *et al*., 2013; Farley, 2017). The era from 1950 to 1960 is well known as the 'golden age' of antibiotics in which millions of antibiotics had been identified, isolated, purified and recruited for various purposes (Davies and Davies, 2010). However, shortly after prescription many of successful antibiotics had encountered resistance against them and it became a serious clinical threat by 1950s (Ventola, 2015). The antibiotic resistant bacterial strains emerged rather rapidly; penicillin-resistant *Staphylococcus aureus* was discovered within just a few years of penicillin clinical prescription (Davies and Davies, 2010). While methicillin resistant *S. aureus* (MRSA), which is among the leading causes of infectious deaths worldwide, was reported within two years of the induction of methicillin in 1961 (Moxnes *et al*., 2013). After resistance to methicillin, vancomycin became an authentic therapeutic drug against MRSA infections (Hasan *et al*., 2016). However, first vancomycin-resistant *S. aureus* (VRSA) strain appeared in 2002 from Michigan due to transfer of Van gene complex and has become an endemic worldwide (Shekarabi *et al*., 2017).

Treatment of bacterial infections has become difficult due to antibiotic resistant bacteria. These superbugs utilize various natural mechanism to develop resistance against the antibiotics which includes alteration of drugs, reduce binding capacity of drugs, modification of metabolic pathways, decrease permeability and increase efflux pumps. Lately, nanotechnology is progressively exploited for treating the infections caused by these MDR bacteria. Nanoparticles (NPs) are used to target bacteria and have proven affective nanoscale antimicrobials (Brown *et al*., 2012). Metallic nanoparticles with large surface area that enables high synergy and multivalent interaction makes them good candidates to overcome the resistance pathways (Gupta *et al*., 2017). They do not penetrate into the bacterial cells and rather directly interact with the cell wall of bacteria (Wang *et al*., 2017).

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Biosynthesis of metallic NPs using reducing powers of plant extracts is an ecofriendly, nontoxic, rapid and economical approach as compared to other physiochemical methods. Plant extracts contain reducing agents (amino acid, citric acid, flavonoids etc.) and stabilizing (capping) agents (protein, citric acid, amines etc.) to reduce the metallic salt ions into stable metallic (zero valent) nanoparticles with a define size and morphology (Agarwal *et al*., 2017). Copper nanoparticles (CuNPs) have gained much attention over past few years due to their wide range of applications in diverse fields and also because of their cost effectiveness over Au and AgNPs (Bogdanović *et al*., 2014). Different physiochemical methods had been reported in literature for CuNPs synthesis but recently green biosynthesis has proven to be safe, simple and in high demand. The present study reported the synthesis of CuNPs using aqueous fruit extract of *F. sycomorus*. The plant has many applications ranging from medicinal to agroforestry (Salem *et al*., 2013). CuNPs were synthesized, characterized and subsequently evaluated for their antimicrobial activity.

## MATERIALS AND METHODS

*Bacterial strains collection*

We had obtained already characterized and identified test strains (*Eschericha coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, Methicillin Resistant *Staphylococcus aureus* (MRSA) and Vancomycin Resistant *Staphylococcus aureus* (VRSA) from the preserved collection at the Microbiology and Public Health lab (CUI, Islamabad).

*Preparation of aqueous* F. sycomorus *fruit extract*

Ripened fruits of *F. sycomorus* were amassed from Trail 5, Margalla Hills, Islamabad Pakistan. The plant itself as well as were identified through a certified Botanist. All the gathered fruits were cleaned with sterile distilled water and stored in clean containers. To prepare the fruit extract, 350 g fruits were blended with 650 mL of water and the solution was filtered through a filter paper to remove the suspended particles. The fruit extract was concentrated in rotatory evaporator for 15-20 min at 70 °C. The concentrated fruit extract was stored at 4 °C for further use and/or analysis.

*Green synthesis of copper nanoparticles*

CuNPs were synthesized by using the aqueous fruit extracts of *F. sycomorus*. A solution of Cu salt and extracts was prepared by dissolving 12.45 g of $CuSO_4$. $5H_2O$ in 50 mL of *F. sycomorus* fruit extract. The solution was kept in a shaking incubator at 37 °C with 150 rpm for 24 h followed by centrifugation at 14,000 *g* for 30 min. Nanoparticles settled down in pellet, supernatant was removed and pellet was washed with buffer solution, weighed and stored for further use.

*Chemical and physical characterization of CuNPs*

Synthesis of CuNPs i.e., reduction of copper ions was monitored and confirmed by UV-visible spectroscopy (IMPLEN nano-spectrophotometer, Germany). UV-Vis spectra of nanoparticles were obtained periodically at 270 nm-500 nm. The morphology and mean particle size of copper nanoparticles were determined by scanning electron microscopy (SEM) (JEOL-JSM910, Japan). The FTIR evaluation was done to get a clear picture of the different (functional groups of the) biochemicals present in the fruit extract of the plant that were involved to play a role as reducing and capping agents. We had collected all the measured spectra through FTIR RX1 (Perkin Elmer) in the range of 500 – 4000 $cm^{-1}$.

*Antimicrobial activity of CuNPs by well diffusion method*

*E. coli*, *K. pneumoniae*, *A. baumannii*, MRSA and VRSA were tested in this study. Antimicrobial susceptibility testing was performed prior to evaluate the antimicrobial activity of nanoparticles by disc diffusion method with recommended standards of National Committee for Clinical Laboratory Standards (NCCLS), to assess whether the bacterial strains are sensitive or resistant. Antibacterial activity of synthesized CuNPs was analyzed by two methods, i.e. broth culture and well diffusion assays. The microbial cultures were adjusted to 0.5 McFarland turbidity standards and inoculated on Muller Hinton (MH) agar plates. A volume 30 µL, 40 µL, 50 µL of 30 mM of CuNPs were poured in the wells (6 mm) and incubated overnight at 37 °C. Antimicrobial activity was determined by zone of inhibition around each well for bacterial strains by copper nanoparticles. Similarly, broth culture assay was performed by adding 30 mM CuNPs in 1 mL (presterilized) tubes followed by shaking incubation at 37 °C overnight. The next day the cultures were evaluated through spectrophotometric measurements at wavelength of 600 nm.

*Determination of minimum inhibitory concentration (MIC) of CuNPs*

MIC was determined by monitoring the growth of bacteria in a microplate reader at 600 nm. Bacterial strains were grown in nutrient broth medium at 37 °C and 150 rpm over 9 hours, before they were diluted in fresh nutrient broth liquid medium using $5 \times 10^8$ colony forming units CFU/mL. Different concentrations of CuNPs (1, 5, 10, 20, 50, 70 and 100 mM) were then added to the culture

medium. Bacteria-CuNPs mixed cultures were tested evaluated using a microplate reader (Biorad Hercules, CA, USA) at 37 °C and optical density (OD) at 600 nm was determined for the microbial cell growth.

## RESULTS

*Characterization of NPs*

CuNPs synthesized by *F. sycomorus* fruit extracts were confirmed and analyzed by UV Vis. Spectrometry and scanning electron microscopy.

UV Vis. Spectroscopy is a fast and sensitive method to detect the synthesis and stability of nanoparticles (Anandalakshmi *et al*., 2016). Figure 1 illustrates the absorption peak of CuNPs, with the Cu salt solution as a control. The characteristic absorption peak for CuNPs is 330 nm. The absorption peak indicates that nanoparticles absorb visible electromagnetic waves by the oscillation of conducting band electrons; phenomenon known as surface plasmon resonance (SPR) (Tomaszewska *et al*., 2013). SPR is a size dependent phenomenon, decrease in size of nanoparticles produces increase bandwidth of resonance known as red shift (Wang *et al*., 2016). Ahmed *et al*. (2015) reported surface plasmon resonance for CuNPs at the 350 nm. An absorption peak for copper nanoparticles at 330 nm indicates the formation of non-oxidized nanoparticles while the Cu salt spectrum returned a flat line. This further confirmed that the Cu ions had been reduced to CuNPs by the reducing powers present in the aqueous fruit extract of the plant.

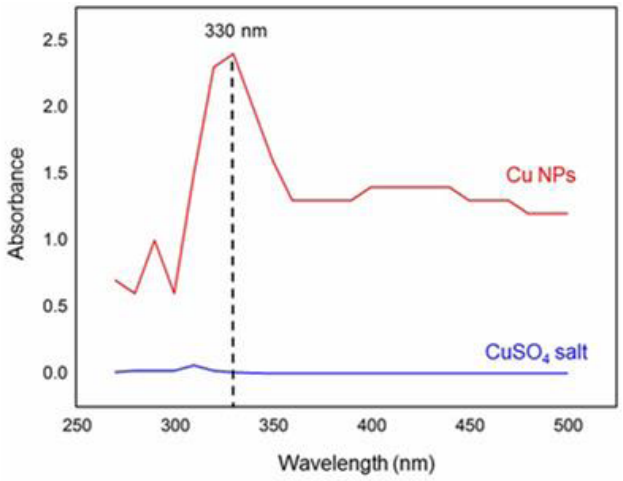


Fig. 1. UV Vis. spectroscopy demonstrating the absorption peak at 330 nm. Copper salt solution was used as control.

Scanning electron microscopy image is shown in Figure 2 which confirmed that CuNPs are in nanoscale range. The biogenic CuNPs were in spherical shape with a diameter of 25-30 nm. These nanoparticles are well dispersed with low agglomeration.

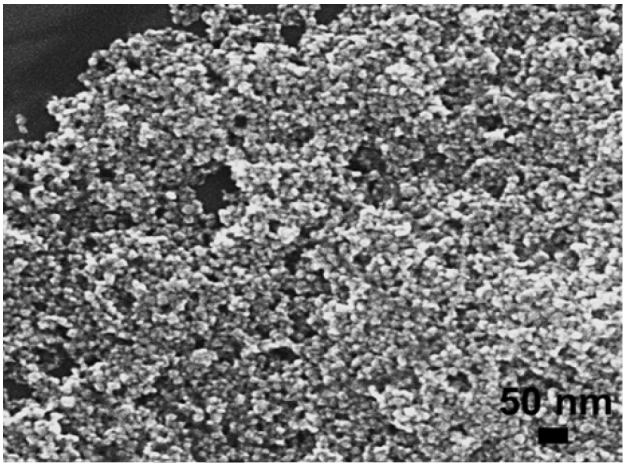


Fig. 2. SEM image of Cu nanoparticles. The nanoparticles appear as (grey coloured) sphereical nanoparticles and the purified concentrated sample still show no signs of aggregation.

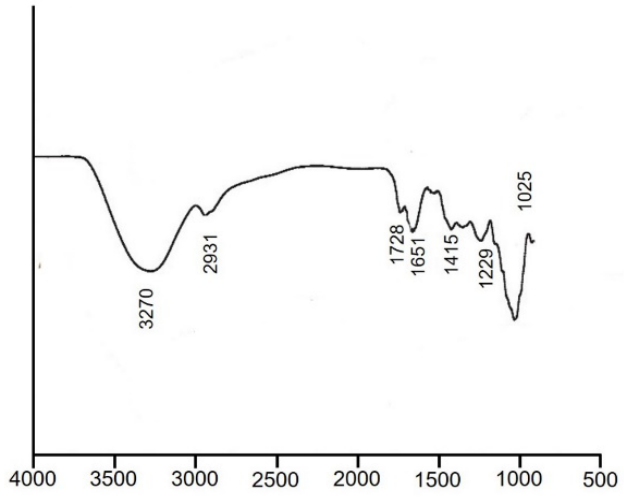


Fig. 3. FTIR spectrum obtained for the biogenic CuNPs.

The analysis of the FTIR spectrum obtained for the biogenic CuNPs had clear bands present at 3270, 1651 and 1023 $cm^{-1}$ (Fig. 3). These bands were corresponding well to the O-H, C=O and C-O stretching vibrations and pointing to the presence of alcohols and phenols (Ayo *et al*., 2009), tertiary amines (Selvi *et al*., 2009) and aromatic ethers (Chien *et al*., 2007). Similar to a previous study where we had showed the presence of reducing agents (Jehan *et al*., 2018), these amines and alcohols present in the

plants extract of *F. sychomourus* may be the biochemical agents responsible for the reduction (Cu ions–to–CuNPs) and stabilizing cum capping agents. The capping had an important in determining the diameter of the CuNPs which is quite uniform as seen in the SEM images.

*Drug susceptibility profiling*

*E. coli, K. pneumoniae, A. baumannii, MRSA* and *VRSA* were scrutinized for their susceptibility and resistance towards antibiotics by disc diffusion method. Susceptibility tests were interpreted according to CLSI document M100-S23 (M02-A11) (Fig. 4). Table I illustrates that all the bacterial strains used in this study were resistant to all of selected antibiotics except for *E. coli* which was sensitive to streptomycin and sulphamethoxazole. The susceptibility assay results proved that all bacterial strains used were multidrug resistant.

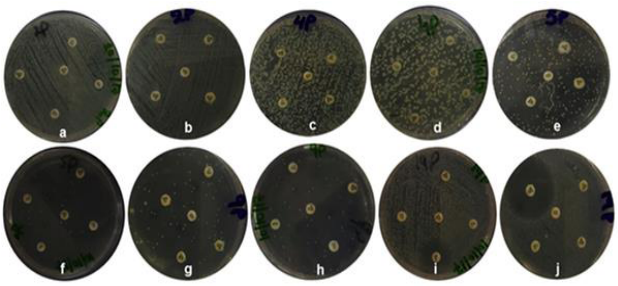


Fig. 4. Antimicrobial susceptibility testing of strains by disc diffusion assay showed no zones of inhibitions (except for *E. coli* against two drugs): a-b) *K. Pneumoniae;* c-d) *A. baumannii;* e-f) MRSA; g-h) VRSA; and i-j) *E. coli*.

*Antimicrobial activity*

Antibacterial activity of synthesized (biogenic) CuNPs was evaluated by broth culture and well diffusion assay against MDR strains. In the broth culture assay CuNPs (30 mM) were added to the selected bacterial cells in nutrient broth and incubated at 37 °C overnight. The next day OD600 reading was obtained using spectrophotometry and it can be seen (Fig. 5) that CuNPs were able to check the growth of the bacterial superbugs. The controls run for every strain had thrived 2-3 times more than the test samples where CuNPs were added too. The results (shown as bar chart; Fig. 6) proved that 30 mM concentration of CuNPs had produced similar effects and all bacterial strains had a (more or less) similar growth inhibition. This result paved the way to confirm that CuNPs could provide an alternate way to kills bacterial superbugs that are otherwise not easy to be tackled with conventional antibiotic drugs.

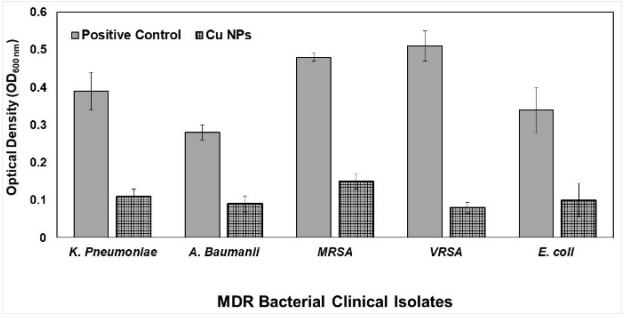


Fig. 5. Antimicrobial susceptibility testing of strains by disc diffusion method. a-b) *K. Pneumoniae;* c-d) *A. baumannii;* e-f) MRSA; g-h) VRSA; and i-j) *E. coli*.

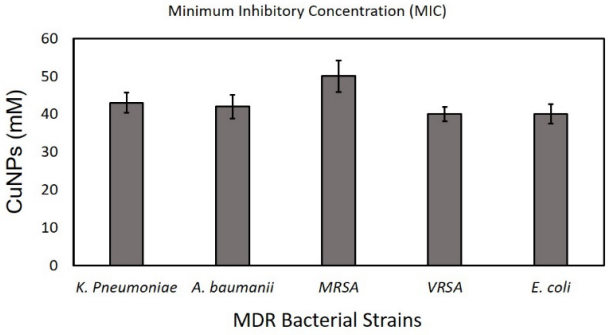


Fig. 6. Minimum inhibitory concentration (MIC) (in mM) of CuNPs against the selected MDR bacterial isolates.

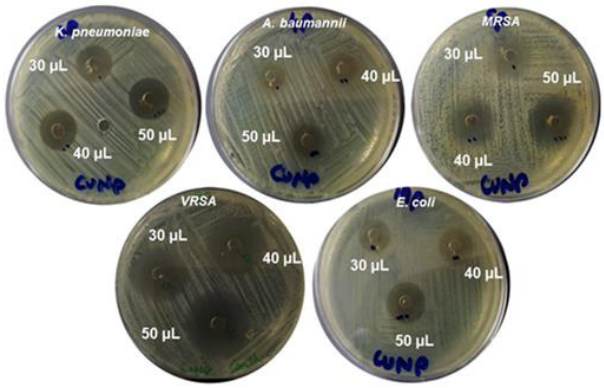


Fig. 7. Bactericidal activity of copper nanoparticles at different concentration of 30 µL, 40 µL and 50 µL.

In case of well dissusion assay, different volumes, i.e. 30, 40, 50 µL of fixed concentration of 30 mM of nanoparticles were used to analyze their bactericidal activity by measuring zone of inhibition showed in Figure 7. The well diffusion assay complemented the results obtained with broth culture assay pointing to the fact that CuNPs effectively checked the growth of MDR bacterial

cells. Similar to broth culture assay, well diffusion assay returned results which proved that 30 mM CuNPs were a good quantity to kill bacterial cells and produce clear zones of inhibition.

**Table I. Antimicrobial susceptibility testing of strains by disc diffusion method. zone of inhibition measured in mm.**

| Antibiotic disc | *K. pneumoniae* | *A. baumannii* | MRSA | VRSA | *E. coli* |
|---|---|---|---|---|---|
| Ceftriaxone (CRO30) | 0 | 0 | 0 | 0 | 0 |
| Ticarcillin (TIC75) | 0 | 0 | 0 | 0 | 0 |
| Cefpodoxime (CPD10) | 0 | 0 | 0 | 0 | 0 |
| Streptomycin (S10) | 0 | 0 | 0 | 0 | 20 mm |
| Sulphamethoxazole (SXT25) | 0 | 0 | 0 | 0 | 35 mm |
| Cefradine CE30) | 0 | 0 | 0 | 0 | 0 |
| Ceftazidime (CAZ30) | 0 | 0 | 0 | 0 | 0 |
| Azteonam (ATM30) | 0 | 0 | 0 | 0 | 0 |
| Ampicillin (AML10) | 0 | 0 | 0 | 0 | 0 |

MRSA, Methicillin resistant *Staphylococcus aureus*; VRSA, Vancomycin resistant *Staphylococcus aureus*.

## DISCUSSION

Cu nanoparticles have large surface area along with enhanced activity and great penetration power. Due to these unique properties Cu nanoparticles are very attractive. They have high catalytic activity and optical properties as well as are of low cost (Rafique *et al*., 2017; Gawande *et al*., 2016). Different physical and chemical methods are used for the synthesis of Cu nanoparticles. Chemical reduction methods are very good as they result in the synthesis of very good CuNPs (that is, monodisperse) but the problem is the hazardous reducing agents and as well as it is costly due to which these methods become less important. (Yeh *et al*., 1999; Molares *et al*., 2001; Lee *et al*., 2008). Thermal and sonochemical reduction, metal vapor synthesis (MVS), electron beam irradiation, microwave irradiation, reverse micelles, pulsed laser ablation, chemical reduction in aqueous solution, and polyol process are multiple methods used for synthesis of Cu NPs. These methods have several limiting factors that limit their use to synthesize the metal nanoparticles (Jardón-Maximino *et al*., 2018).

Plants extracts are extensively being used as ‘green’ reductants for nanoparticles synthesis from last few years (Ahmad *et al*., 2019). Synthesis of nanoparticles in green environment has many advantages like it is cheap way of synthesizing nanoparticles as well as it is less toxic way as compared to chemical counterparts. These proved very lethal to bacteria due to their increased surface area (Jehan *et al*., 2018; Zain *et al*., 2014). In this reported work, the aqueous fruit extracts of *F. sycomorus* had been used to synthesize CuNPs and then their physico-chemical properties were analyzed along with their antimicrobial activity against (selected) MDR bacteria. The nanoparticles synthesis was firstly detected via color change and later on it was confirmed by the appearance of peak through UV-Vis spectroscopy analysis. A sharp peak obtained at a wavelength around 330 nm versus the flat line retrieved for the $CuSO_4$ salt was a testimony to the fact that CuNPs were synthesized. A peak at 330 nm for nanoparticles synthesis had been already reported and it counter-supported the formulation of our CuNPs (Beltrán-Partida *et al*., 2019). The plant extract are full of biochemical compound; many of which had the reducing powers (Sasidharan *et al*., 2010). It is always necessary to find out the different (functional groups of the) biochemicals present in the extract of the plant that would have acted as the reducing and capping agents. The performed FTIR evaluation had made it cleared in this study that amines and alcohols present in the fruit extract of *F. sychomourus* acted as the reducing agents. The antibacterial assay of synthesized nanoparticles showed that these have very excellent antibacterial activity against clinical isolated of MDR bacteria. The synthesized nanoparticles exhibited antibacterial activity against pathogenic microorganisms with varying degrees measured as zone of inhibition showed in Table II. Bactericidal activity of nanoparticles increases with increase in concentration that suggests nanoparticles follow dose dependent manner to kill pathogenic microorganisms. The pattern of our biosynthesized CuNPs eradicating the MDR bacterial strains had been very consistent in both of the assays, that is, well diffusion and broth dilution assay. This in turn means that the CuNPs had been good enough to penetrate deep into the bacterial cells and disrupt their mechanisms to kill them. The clear zones of inhibitions, low OD values (when cell thriving is attempted in the presence CuNPs) as well as the low MIC values could well be explained from the smaller diameter and (a very fair) monodispersity of the metallic nanoparticles. This fact has been well supported by the electron microscopy imaging where the CuNPs are not only smaller diameters but also of uniform size and shape.

The exact mechanism of bactericidal activity of synthesized nanoparticles in not well-known (Ingle *et al*., 2014; Shaikh *et al*., 2019). CuNPs mostly interacts with bacterial cell membrane which disrupts integrity of membrane and leads to death of bacteria. Toxicity mechanism of CuNPs can be enhanced by altering several

factors like temperature, pH, aeration and concentration of bacteria or NPs. CuNPs also alter several cell functions of bacteria in many ways like these may adhere to Gram negative bacterial cell wall via electrostatic interaction, alter protein structure present in cell membrane, denature intracellular proteins, interact with phosphorus- and sulfur containing compounds e.g. DNA (Chen *et al*., 2019). In one study mechanism of action of CuNPs were investigated over *E. coli* and it was found that CuNPs causes over production of cellular reactive oxygen species (ROSs). And NP mediated increase of ROS leads to protein oxidation, DNA degradation and lipid peroxidation which finally lead to the death of bacterial cells (Mahmoodi *et al*., 2018; Concha-Guerrero *et al*., 2014).

**Table II. Zone of inhibition (mm) through well diffusion assay after 24 hours showing antibacterial activity of Cu nanoparticle.**

| NPs volume | *K. pneumoniae* | *A. baumannii* | MRSA | VRSA | *E. coli* |
|---|---|---|---|---|---|
| W1 (30 µL) | 18 mm | 11 mm | 22 mm | 23 mm | 13 mm |
| W2 (40 µL) | 19 mm | 12 mm | 25 mm | 25 mm | 15 mm |
| W3 (50 µL) | 20 mm | 13 mm | 28 mm | 27 mm | 17 mm |

For abbreviations, see Table I.

## CONCLUSION

Green synthesis of CuNPs using plant extracts is a cost-effective, safe, nontoxic and eco-friendly route of synthesis which can be used to produce the particles at large scale. The synthesized CuNPs exhibited strong bactericidal activity against drug resistant isolates making them a potent source of antibacterial agents. This study would aid in the development of nano-antibiotics as effective agents against MDR microorganisms either alone or in combination with other conventional drugs.

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*Statement of conflict of interest*

The authors have declared no conflict of interest.

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