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ORIGINAL ARTICLE

Synthesis and characterization of biocompatibility of tenorite nanoparticles and potential property against biofilm formation

Davoodbasha MubarakAli [a,c,d,*], Jegatheesan Arunkumar [b], Pratheesh Pooja [c], Gopalakrishnan Subramanian [c], Nooruddin Thajuddin [d,e], Naiyf S Alharbi [e]

[a] *Division of Bioengineering, School of Lifescience and Bioengineering, Incheon National University, Republic of Korea*
[b] *Department of Microbiology and Biotechnology, Presidency College, Chennai, India*
[c] *Central Inter-Disciplinary Research Facility (CIDRF), Mahatma Gandhi Medical College and Research Institute Campus, Puducherry 607402, India*
[d] *Department of Microbiology, Bharathidasan University, Tiruchirappalli, India*
[e] *Department of Botany and Microbiology, College of Science, King Saud University, Riyadh-11451, Kingdom of Saudi Arabia*

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Abstract Aim is to assess the anti-biofilm property of tenorite nanoparticles and to study their suitability as a possible coating material for medical implants. Tenorite (CuO) nanoparticles were synthesized by the optimized thermal decomposition method and characterized using TEM, XRD, FTIR and UV–Vis analysis. Their influence on biofilm formation of microbes was studied by growing multi drug resistant bacterial strains in the presence or absence of these nanoparticles at various concentrations. The cytotoxicity of nanoparticles on mammalian cells was studied at the corresponding concentrations. The nanoparticles were found to be uniformly dispersed, spherical shaped and < 50 nm in size. They showed various degrees of anti-biofilm property against clinically isolated, biofilm forming multi drug resistant microorganisms such as *Staphylococcus aureus*, *Pseudomonas fluorescens*, *Burkholderia mallei*, *Klebsiella pneumoniae*, and *Escherichia coli*. Furthermore, Hep-2 cells showed excellent viability at tenorite nanoparticles concentration toxic to microbial

* Corresponding author at: Division of Bioengineering, School of Lifescience and Bioengineering, Incheon National University, Republic of Korea. Tel.: +82 32 835 4609; fax: +82 32 835 0763.
E-mail address: mubinano@gmail.com (D. MubarakAli).
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growth. These results indicate that tenorite nanoparticles may be ideal candidates for being utilized as coating on medical implants in general and dental implants in particular.

1. Introduction

Nanotechnology is an emerging field in science and technology and is poised to bring in revolutionary changes across all spheres of science, especially medicinal science. The field of nanomedicine is the technology of diagnosing, treating, and preventing the disease and traumatic injury, and of preserving and improving human health, using nanostructured materials. Synthesis of nanoparticles that have the right size, shape, desired characteristics and functionality is at the core of their use in nanomedicine (MubarakAli et al., 2013). Nanoparticles are attractive for medical purposes due to their important and unique features, such as quantum properties and large surface to mass ratio which lead to better binding and adsorption of compounds like drugs and probes. Other than their roles in diagnostics and drug delivery, there are recent reports on the inhibitory effect of metallic nanoparticles on biofilm forming microorganisms (Nithya et al., 2011). But there are no modalities available to combat biofilm forming, multi drug resistant microorganisms (MDRs) (Gilbert et al., 2002). Thus there is great scope in developing nanoparticles that target biofilm forming and multi drug resistant microbes (MubarakAli et al., 2011, 2012; Gopinath et al., 2012). Copper nanoparticles have shown much promise in the area of nanotechnology and nanomedicine in the last few decades because of their excellent catalytic, optical, electrical and antibacterial properties (Ponce and Klabunde, 2005; Huang et al., 2008). Most of the enzymes and proteins are dependent on copper for their functionality and the human body has an efficient system to metabolize copper (Jose et al., 2011).

Nanoparticles are synthesized by chemical, physical and biological methods. These methods not only require expensive chemicals and complex steps, but some of the synthesis methods lead to impure preparations (MubarakAli et al., 2013). Thus, it is desirable to choose a method that synthesizes nanoparticles without affecting their physical, chemical and biological properties, and is cost effective at the same time. Copper nanoparticles have been prepared using methods such as thermal reduction (Dhas et al., 1998); vacuum vapor deposition (Liu et al., 2003); microwave irradiation (Zhao et al., 2004); chemical reduction (Yang and Zhu, 2003); laser ablation (Yeh et al., 1999) and Polyol method (Park et al., 2007). CuO, as a *p*-type semiconductor exhibiting a narrow band gap (1.2 eV), is ideal to synthesize nanoparticles through inexpensive methods without using organic solvents, expensive raw materials, and complicated equipments.

In the present study, tenorite nanoparticles were synthesized by the thermal decomposition method with optimized conditions. It was evaluated for anti-biofilm property. Cell viability of Hep-2 cells was assessed with synthesized nanoparticles at different concentrations. Based on our results we perceive that these nanoparticles can be used for coating on implants.

2. Materials and methods

2.1. Materials

Copper sulfate, Sodium hydroxide, Hydrochloric acid, LB broth, MEMS Media and all other chemicals and reagents were purchased from Sigma, Qiagen, HiMedia.

2.2. Culture and cell lines

All the multi drug resistant pathogenic strains used in this experiment were procured from Department of Microbiology, Bharathidasan University, Tiruchirappalli and maintained in nutrient agar slants and broth.

2.3. Synthesis of tenorite nanoparticles

Hep-2 cells were obtained from National Centre for Cell Science (NCCS), Pune, India. The Hep-2 cells were grown as monolayer in MEM; supplemented with 10% FBS, 1% glutamine, and 100 U/mL penicillin–streptomycin solutions. Cells were incubated at 37 °C in 5% CO_2 atmosphere at 95% humidity.

Tenorite nanoparticles were synthesized by a modified thermal decomposition method (Xu et al., 2002; Kim et al., 2011). Different concentrations of Copper sulfate (0.001, 0.01, 0.1, 1 M) and Sodium hydroxide (0.1–1.0 M) were prepared for synthesis and optimization study. Briefly, 200 mL of Copper sulfate solution was taken in 500 mL of the Erlenmeyer flask and stirred at 50 °C for 10 min. Sodium hydroxide solution was then added drop-wise till the pH was achieved at 6.0 and also observed for color changes. The solution was then filtered and dried, and then washed twice with Milli Q water. The particles were transferred onto a borosil glass plate and heated at 200 °C for 2 days and then cooled at room temperature. CuO nanoparticles obtained as such were used for further characterization.

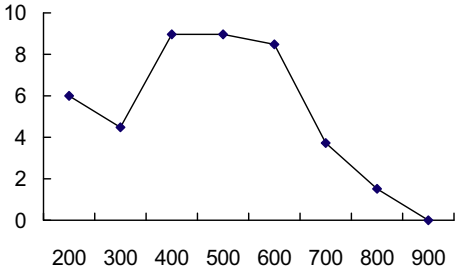

Figure 1 UV–Vis spectrum of tenorite nanoparticles showed Plasmon resonance at 550 nm.

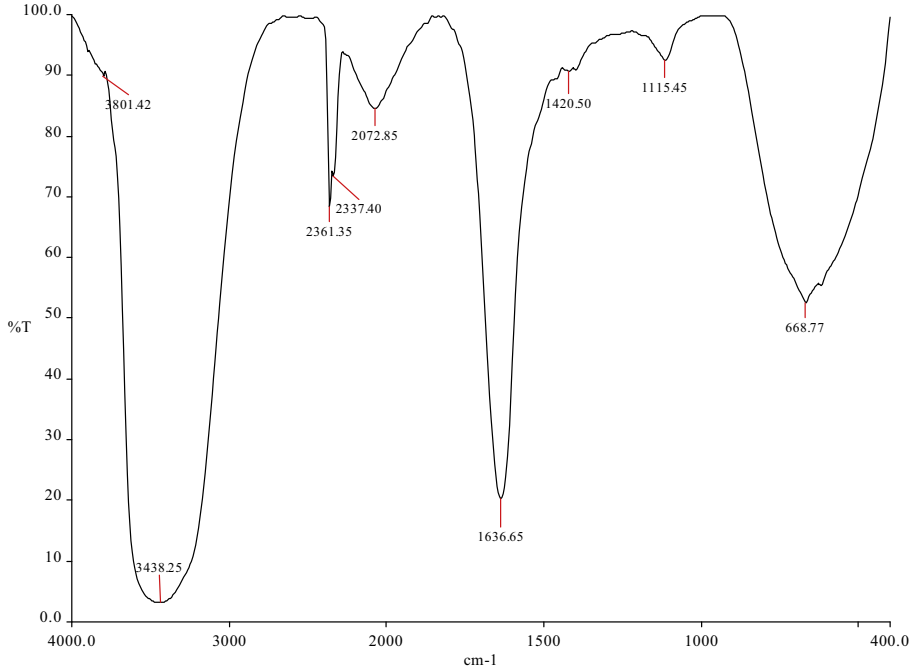

Figure 2 FTIR spectrum recorded for tenorite nanoparticle, the bands seen at 1115.4 and 668.77 cm^{-1} were assigned to the metallic and O stretching vibrations of the metallic oxides respectively.

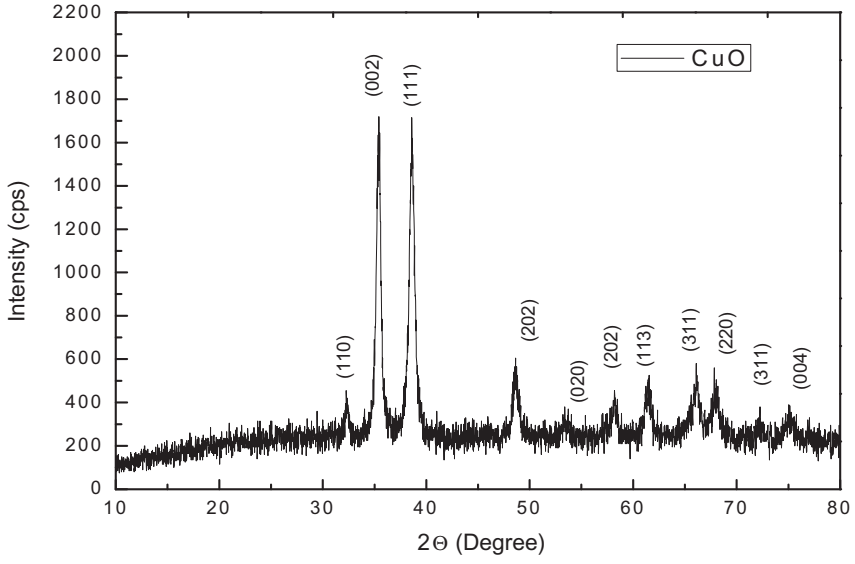

Figure 3 XRD analysis of synthesized tenorite nanoparticles showed (1 1 1), (0 0 2), (1 1 0) planes corresponding to the crystalline nanoparticles of about 17 nm.

2.4. Characterization of tenorite nanoparticles

Morphology and size of tenorite nanoparticles were investigated by transmission electron microscopy (JEOL-1200 EX (TEM with tungsten electron source)) with an accelerating voltage of 120 kV. Tenorite nanoparticles were analyzed by X-ray diffraction (XRD) (Phillips PW1710, Holland) with CuK radiation = 1.5405 Å over a wide range of Bragg angles by the following formula,

$$\text{Crystallite size : } D = 0.09\lambda/\beta\cos\Theta$$

where,

λ – Wavelength of X-ray (1.54 Å)
β – Full Width Half Maximum (FWHM) value
Θ – Bragg's angle

Reduced tenorite nanoparticles were also confirmed with the bond pattern using Fourier transform infrared (FTIR)

spectroscopy and UV–Vis spectroscopy for the measurements of Surface Plasmon Resonance (SPR).

2.5. Antibiofilm property of tenorite nanoparticles

Study of biofilm formation and antibiofilm property assay has been previously described (Nithya et al., 2011). The antibiofilm effect of tenorite nanoparticles was measured by direct visualization by light microscopy. Briefly, cover-slips (1 × 1 cm) were placed in 24 well polystyrene plates and media were added to the wells. Tenorite nanoparticles were added to the media at varying concentrations of 1, 10, 50, and 100 μg/mL. The biofilm forming bacteria were then inoculated and allowed to grow for the formation of biofilm on cover-slips for 24 h at 37 °C inside an incubator. For control, biofilm forming bacteria were grown in a similar set-up but without any tenorite nanoparticles in the growth medium. Each experiment was run in triplicate. Next day the biofilms were stained with methylene blue and the effect of tenorite nanoparticles on biofilm formation was observed under a light microscope (Carl Zeiss, Germany) at the magnification of 40× and images were obtained.

2.6. Cell viability assay

The viability of Hep-2 cells was evaluated by direct observation of cells by an Inverted Phase contrast microscope and followed by the MTT assay method. 5×10^4cells in 100 μl of MEM medium were seeded per well in a 96 well tissue culture plate and incubated at 37 °C in a humidified 5% CO_2 incubator. After 24 h the medium was removed, and fresh MEM containing tenorite nanoparticles at different concentrations with dilutions ranging from 1.56 to 200 μg/100 μl, were added to the wells and incubated at 37 °C in a humidified 5% CO_2 incubator. In control cells, fresh medium was added without any tenorite particles. The cells were observed after 24 h and up to 72 h for any changes in morphology and density of the cells by inverted phase contrast microscope and images were acquired. After 72 h, the media were removed from the wells and 10 μl of MTT solution was added to all test and control wells. The plate was shaken gently and incubated at 37 °C in a humidified 5% CO_2 incubator for 4 h. After the incubation period, the supernatant was removed and 100 μl of the MTT solubilizing solution was added to solubilize the formazan crystals. The absorbance values were measured at the wavelength of 570 nm (Moshmann, 1983; Jeyaraj et al., 2013).

3. Results

Tenorite nanoparticles were synthesized by a modified thermal decomposition method. It was found that 0.5 M of Copper sulfate reacted with 0.1 M Sodium hydroxide among tested concentrations at 50 °C for 10 min showed the formation of nanoparticles. The formation of nanoparticles was indicated when the color of the mixture changed from pale blue to brown. Tenorite nanoparticles were formed as a result of thermal events that included thermal dehydration, *in situ* crystallization of dehydrated amorphous compound, and thermal desulferation (Darezereshki and Min, 2011). The concentration optimization was done for the generation of CuO based on the UV–Vis spectral analysis. The synthesized tenorite nanoparticles exhibited Surface Plasmon Resonance at 550 nm (Fig. 1). FTIR spectra showed the nature of synthesized tenorite nanoparticles (Fig. 2). The absorption bands at 668 cm^{-1} and 1115 cm^{-1} were due to the SO_4 bending and stretching vibration, respectively. There is also a minute dip in the spectra at 2361 cm^{-1} which is attributed to the presence of atmospheric CO_2. The adsorption at 1636 cm^{-1} and

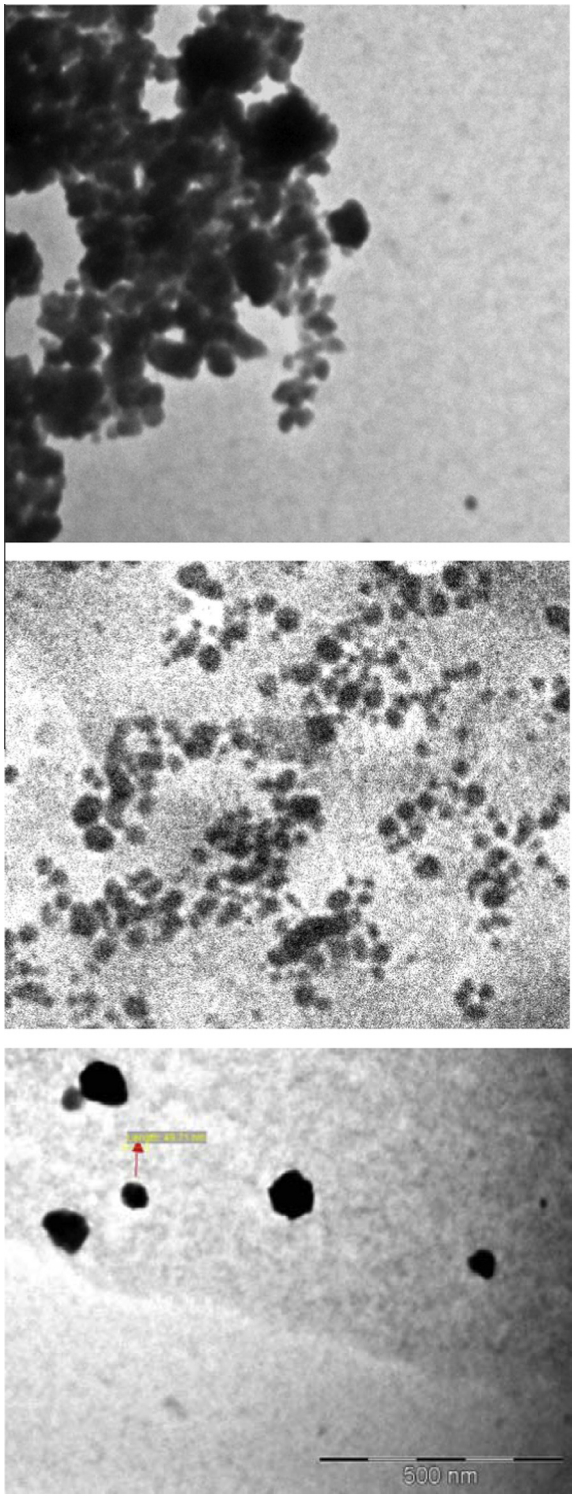

Figure 4 TEM micrograph of tenorite nanoparticles showed the average size of < 50 nm was spherical in shape.

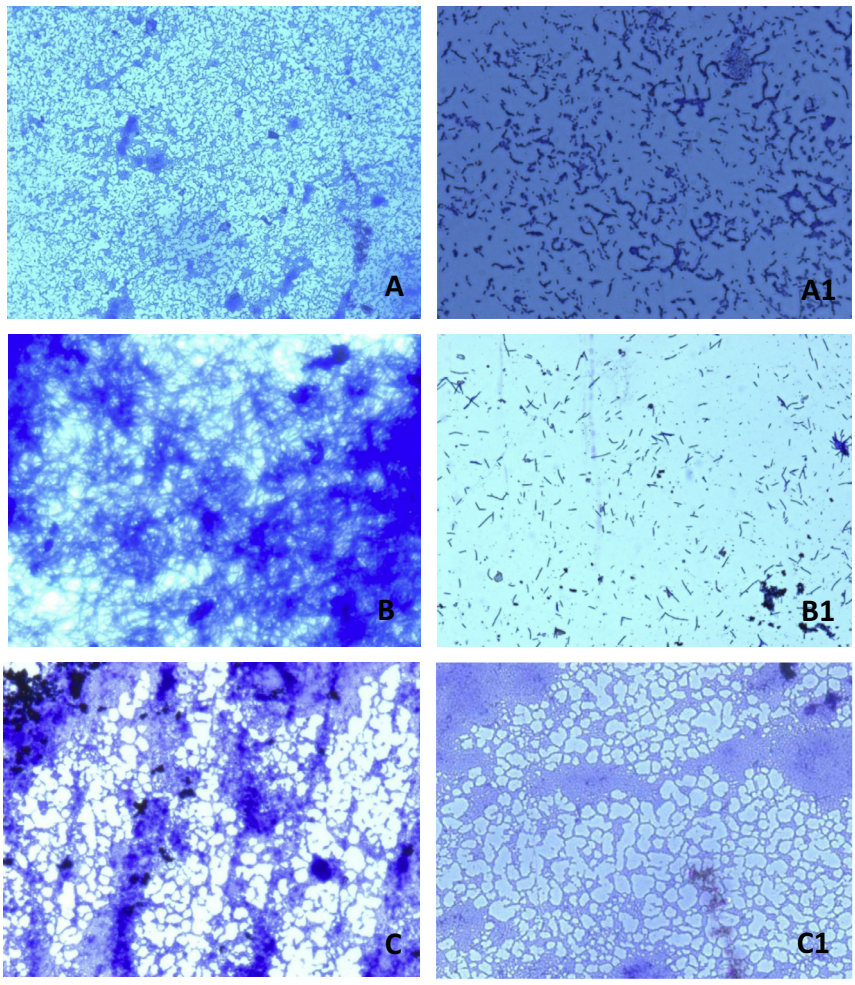

Figure 5 Light microscopic images of biofilm formed by *Klebsiella pneumoniae* (A), *Escherichia coli* (B) and *Staphylococcus aureus* MTCC96 (C) and (A1; B1;C1) treated with tenorite nanoparticles (32 μM).

3438 cm^{-1} are due to the H–O–H bending and O–H stretching vibration. In the spectrum of copper nanoparticles, the two absorption peaks that appear at 1420 and 1115 cm^{-1} are due to O–H bending and C–O stretch. The peaks observed at 3438 cm^{-1} are characteristics of O–H stretching vibrations. XRD spectra of synthesized tenorite nanoparticles are consistent with the metallic copper (Fig. 3). The peaks at 36.5, 39.5 and 68.2 are corresponding to the indices (0 0 2), (1 1 1), (2 2 0) respectively. The size of tenorite nanoparticles was calculated from the Debye–Scherrer equation and was found to be about 17 nm. Morphology of the tenorite nanoparticles was determined using TEM, which suggested that the size of the synthesized spherical nanoparticles was < 50 nm (Fig. 4). It is worth noting that the TEM imaging was performed along with XRD analysis indicating size distribution (< 50 nm) and crystallite nature of the particles. The antibiofilm property of tenorite nanoparticles was observed at the lowest concentration of 32 μM (Fig. 5). The results show that at the given concentration, the biofilm formation by multi drug resistant pathogens was significantly inhibited. The biofilm formation was reduced by 80% in case of *Klebsiella pneumoniae*, 90% in case of *Staphylococcus aureus* MTCC96 and 70% in case of *Escherichia coli*. This suggests with ample evidence that the synthesized tenorite nanoparticles may be used for coating on the implants to inhibit the biofilm formation. Results of cell viability assay showed an excellent trend with 100% viability observed in the presence of tenorite nanoparticle concentration upto the concentration of 81 μM (Fig. 6). As the tenorite nanoparticles concentration increased (up to 650 μM), the Hep-2 cells viability went down, with just about 20% cells remaining viable at the concentration of 650 μM.

4. Discussion

Previously Cu and CuO nanoparticles have been reported to be synthesized in the ranges of 9–60 nm under supercritical conditions (Shah and Al-Ghamdi, 2011). Copper nanoparticles have also been reported to be synthesized by thermal decomposition of copper oxalate (Salavati-Niasari and Davar, 2009; Salavati-Niasari et al., 2008), copper (I) precursors (Adner et al., 2013) and copper sulfate (Darezereshki and Min, 2011) and the particles sizes obtained were 40 nm, 10–30 nm and 170 nm respectively.

Excitation of Plasmon resonance or inter-band transition is reported to indicate the metallic characteristics of copper nanoparticles. Furthermore, it has been reported that copper

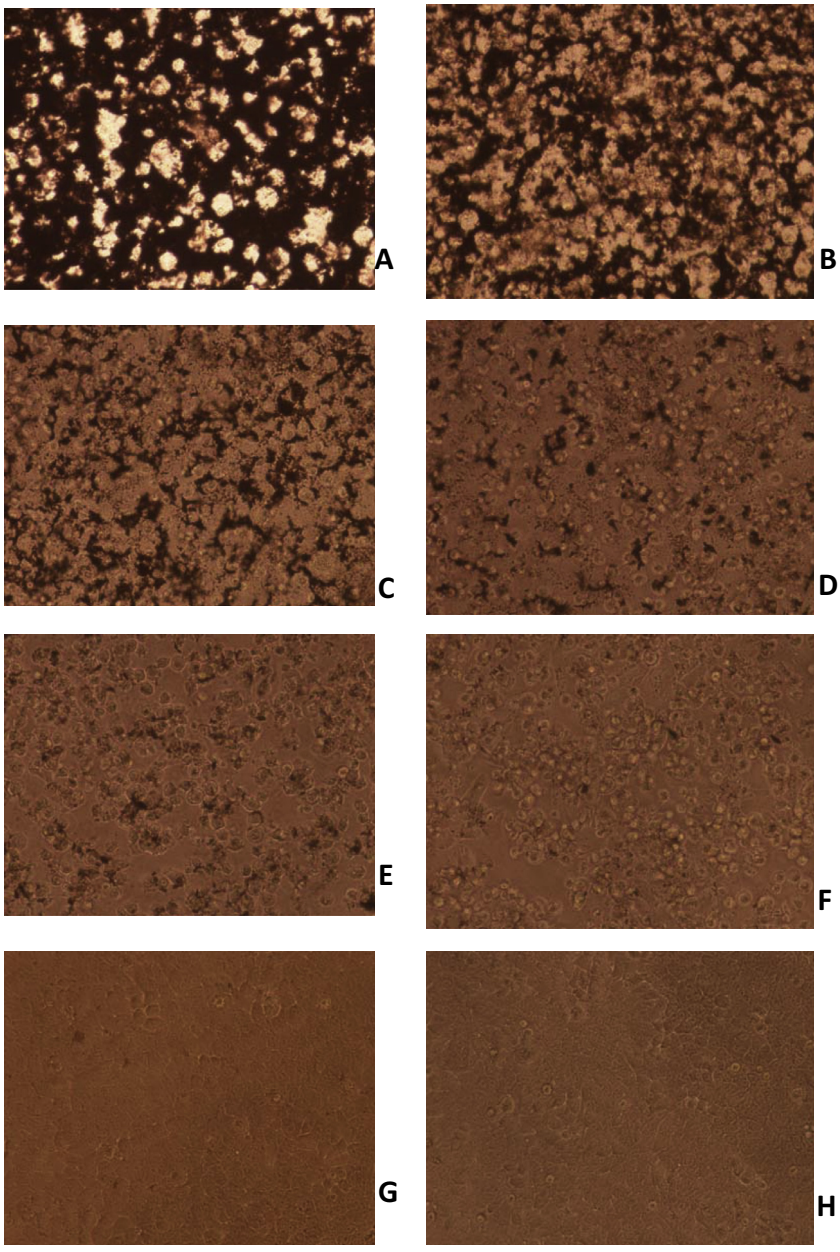

Figure 6 Cytotoxicity of tenorite nanoparticles with Hep-2 cell lines (A–D); cytologically tenorite nanoparticles showed toxicity up to the concentration of (650 μM–162 μM) and viability from 81 μM to 5 μM (E–H).

nanoparticles at a size of 50 nm typically exhibit the surface Plasmon resonance at 560–570 nm (Creghton and Eadon, 1991). Non-oxidized copper nanoparticles show surface plasma resonance at 580 nm while copper nanoparticles exhibit surface plasma resonance at 556–580 nm in general (Zhao et al., 2004; Ramyadevi et al., 2012). The peaks in the range of 670–100 cm^{-1} are shifted to higher wave numbers in the same type. These dramatic differences indicate that the thin layer of starch forms on the surface of copper nanoparticles (Surmawar et al., 2011). The XRD peaks were indexed using JCPDS file (JCPDS card No: 41-0254). The peak-positions are consistent with previous reports (Lo et al., 2005; Harne et al., 2012; Ramyadevi et al., 2012). The CuO nanoparticles, instead of pure copper are produced because of Cu reacting with existing O_2 in dielectric liquid (Lo et al., 2005). The sharp peaks of the XRD pattern indicate the crystalline nature (Ramyadevi et al., 2012).

It has been reported previously that copper nanoparticles display varied antibacterial activities against pathogenic microbes (Ramyadevi et al., 2012). Copper nanoparticles adversely affect microbial pathogens as analyzed by the disk diffusion method (Rupaarelia et al., 2008). Copper nanoparticles are shown to be highly effective against filamentous bacteria. Copper nanoparticles have been shown to be bactericidal against strains like *Bacillus subtilis* and *S. aureus* which are otherwise resistant to antibiotics (Chatterjee et al., 2012).

An earlier report has suggested that HeLa, A549 and BHK21 cell lines show excellent viability in the presence of

synthesized copper nanoparticles up to the concentration of 120 μM (Harne et al., 2012). Other studies have reported that copper nanoparticles induce DNA damage, chromatin condensation, inter-nucleosomal DNA fragmentation, oxidative stress and interact with SH groups on proteins leading to protein denaturation in a dose dependent manner (Yoon et al., 2007). Tenorite nanoparticles are expected to have lower cytotoxicity than ionic copper which was shown to be on Chinese Hamster Ovary cells and Hela cells (Studer et al., 2010). Due to biocompatibility of copper, and effective physiological measures intracellular metal homeostasis and metabolism, tenorite nanoparticles might show lesser toxicity against PC3 and MCF-7 (Kim et al., 2012).

5. Conclusion

We summarize that the first generation method of thermal decomposition was adopted for synthesizing tenorite nanoparticles. These particles were further characterized by techniques like TEM, XRD, FTIR, and UV–Vis for the characterization of nanoparticles. It was found that prepared nanoparticles were spherical in shape and < 50 nm in size. The tenorite nanoparticles (32 μM) displayed anti-biofilm activity against biofilm forming multi drug resistant microorganisms such as *Pseudomonas fluorescens*, *Burkholderia mallei*, *Klebsiella pneumoniae*, *E. coli* and *S. aureus* MTCC96 strain. Cell viability of Hep-2 cells was checked in the presence of tenorite nanoparticles by the MTT assay, which showed that cells had excellent viability up to 81 μM. Given the excellent biocompatibility of copper, combined with tremendous microbicidal properties, copper nanoparticles may be a suitable coating material for the implants.

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