Green synthesis, characterization and antibacterial activity of copper nanoparticles using L-ascorbic acid as a reducing agent

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ABSTRACT

Synthesis of Cu nanoparticles using chemical route offers a competitive alternative approach over the common biological and physical procedures. In this study, simple, economical, convenient and environmentally-friendly chemical reduction technique was used for the production of Cu nanoparticles from CuCl2.2H2O solution using L-ascorbic acid as reducing and capping agent. The effects of concentration of precursor salt and ascorbic acid, reaction time and reaction temperature on the synthesis of Cu nanoparticles were studied. The optical properties of the synthesized Cu nanoparticles were characterized by UV-Vis Spectroscopy while the crystallinity of synthesized Cu nanoparticles was verified with the help of X-ray diffraction analysis. The antimicrobial activity of Cu nanoparticles was determined by Agar disc diffusion method against some selected species of bacteria: two gram positive (Staphylococcus aureus, Streptococcus pyogenes) and two gram negative (Escherichia coli, Pseudomonas aeruginosa). The UV-Vis spectrum of solution of Cu nanoparticles showed a characteristic peak at 423 nm that confirms the preparation of Copper nanoparticles. Moreover, FT-IR spectroscopy was performed to detect the binding effect of ascorbic acid on Cu nanoparticles, and its result indicated that ascorbic acid could prevent oxidation and agglomeration. The findings revealed that Cu nanoparticles formed at a concentration of 3mM CuCl2.2H2O solution and 4 mM ascorbic acid exhibited an excellent zone of growth inhibition for both gram-negative and gram-positive bacteria, 16.83±0.42 and 15.50±0.89mm, respectively.

Keywords: - Green synthesis, Copper nanoparticles, Chemical reduction, Optical properties, Antibacterial activity

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INTRODUCTION

In recent years, metal nanomaterials have received particular attention for their positive impact on improving many economy sectors including consumer products, energy, transportation, cosmetics, pharmaceuticals, antimicrobial agents and agriculture (Shobha et al., 2014). Moreover, the controlled fabrication of metal nanoparticles has impelled nanotechnology into its advancement in today’s most promising and popular field of scientific research (Aysha et al., 2015). Among the metal nanoparticles, copper nanoparticles have been paid great interest because of their excellent physical and chemical properties, versatile applications and low cost of preparation (Kruis et al., 1998; Gopinath et al., 2014; Heera et al., 2015). Currently, copper nanoparticles greatly attracted researchers pertaining to their enormous applications, particularly in flexible electronics,
printing (as a copper ink), nanocircuits due to their good electrical conductivity, catalytic properties along with high biocompatibility and antibacterial properties (Nadejda et al., 2017). Copper nanoparticles have also more applications such as in the form of heat transfer systems, antimicrobial materials, sensors and catalysts (Eastman et al., 2001; Ispa and Nayak, 2013; Aysha et al., 2015).

In the synthesis of copper nanoparticles, different methods or approaches such as chemical reduction in aqueous solutions, colloidal synthesis with reduction and extraction steps, evaporation and condensation of metal vapor on a cold surface have been employed (Liu et al., 2012; Kathad and Gajera, 2014). Most of these synthesis methods involved with elevated temperatures, inert atmospheres, large amount of surfactants and organic solvents (Appu and Muthukrishnan, 2015). In addition, the preparation and storage of the synthesized copper nanoparticles is quiet challenging due to its high tendency for oxidation (Appu and Muthukrishnan, 2015; Sudhir et al., 2015).

Thus, designing a suitable synthesis strategy can help researchers synthesize high quality copper nanoparticles having controlled properties. In this study, we designed a synthesis method which is simple, economical, temperature controlled, convenient and environmentally friendly for the production of copper nanoparticles from CuCl$_2$·2H$_2$O solution using L-ascorbic acid for both a reducing and capping agent. The developed synthesis strategy used simple and nontoxic chemicals and reagents. The synthesized copper nanoparticles were characterized by XRD, UV-Vis spectroscopy and FT-IR spectroscopy. The antibacterial activity of the synthesized copper nanoparticles for the selected bacterial strains was also investigated using Agar disc diffusion method.

**EXPERIMENTAL**

**Chemicals and Instruments**

CuCl$_2$·2H$_2$O (99.5%), L-ascorbic acid (99.9%) and Paraffin Oil were brought from Blulux laboratories Priv. Ltd, (INDIA-121005). Muller Hinton Agar was brought from Shimakys pure chemicals. All the chemicals were used without further purification. FT-IR spectrometer (AVATAR 330 FT-IR, Thermo Nicolet), double beam Uv-Vis spectrometer (Sanyo Sp 65), XRD (Philips X-ray diffractometer with the scanning $2\theta$ ranges from 10° to 80°) were used.

**Preparation of copper chloride dihydrate solutions:** A 0.08 M stock solution of CuCl$_2$·2H$_2$O with distilled water was prepared. Different concentrations of the salt solution (1 mM, 2 mM, 3 mM and 4 mM) were also prepared from the stock solution (Vasudeo et al., 2015).

**Preparation of L-ascorbic acid solution:** A 0.08 M stock solution of L-ascorbic acid ($C_6H_8O_6$) was prepared by dissolving 14.08 g of the L-ascorbic acid in 1000 ml of distilled water. Different concentrations (2 mM, 4 mM, 6 mM and 8 mM) of L-ascorbic acid were prepared from the stock solution of L-ascorbic acid.

**Synthesis of copper nanoparticles:** The method reported by Umer et al. (2014) with slight modification was used in this study in order to synthesize Cu nanoparticles. A specified concentration of the solution of L-ascorbic acid was mixed drop wise with a specified concentration of the solution of CuCl$_2$·2H$_2$O salts by heating and stirring at different reaction temperature and time (45-90°C and 0-12 hrs, respectively), until Cu
nanoparticles were formed. In order to study the effects of temperature and concentration of metal salt and L-ascorbic acid on the formation copper nanoparticles, the experiment was conducted at different reaction temperature and concentration of the precursors. The as-synthesized precipitates of copper nanoparticles were formed when the reaction was completed. The precipitate of copper nanoparticles was subjected to centrifugation so as to isolate it from the supernatant solution, and then the precipitate was washed twice with distilled water. Finally, the sample was dried, and then it was kept under desiccators to protect it from moisture and apparently from oxidation (Liu et al., 2012).

**Antibacterial susceptibility test:** The antibacterial assays were performed by standard Agar disc diffusion method against some selected species of bacteria: two gram positive (*Staphylococcus aureus, Streptococcus pyogenes*) and two gram negative (*Escherichia coli, Pseudomonas aeruginosa*). Nutrient broth/agar (1g beef extract, 1g peptone, 0.5 g NaCl) dissolved in 100 mL of distilled water was used to cultivate the bacteria. The media was autoclaved, cooled and poured in the petri dishes and kept for 30 minutes for solidification. Four different fresh cultures of inoculums (100 μL) were spread on to solidified nutrient agar plates. Sterile paper discs made of Whatman filter paper and the paper discs (6 mm in diameter) impregnated with antibiotic solution were placed on the surface of each Mueller Hinton Agar (MHA) plate using a sterile pair of forceps. Sterile paper discs made of Whatman filter paper, 6 mm diameter, were dipped into 50 μL solution of copper nanoparticles. Solution of copper chloride and solution of L-ascorbic acid were placed in each plate including positive control. The cultured agar plates were incubated at 37°C for 24 hrs. After 24 hrs of incubation, the zone of inhibition growth was investigated. Finally, the plates were incubated aerobically. The bigger the diameter of the inhibition zone, the more susceptible is the microorganism to the antimicrobial agent (Espinel-Ingroff and Pfaller, 2007).

**Characterization:** The optical properties of copper nanoparticles were studied by UV-visible spectroscopy using a double beam spectrophotometer (Sanyo Sp 65 UV/Vis spectrophotometer) in the spectral range from 200 nm to 800 nm. The UV-Vis absorption spectra of all the samples were recorded, and numerical data were plotted in the “Origin 8” software.

The XRD patterns of copper nanoparticles were recorded using Philips X-ray diffractometer with the scanning 2θ ranges from 10° to 80°, wherein the radiation employed was copper Kα with a wavelength of 1.54056 Å. The average particle size was determined from the XRD diffractograms of the sample using the Debye-Scherer equation by taking (111) phase of the XRD patterns (Langford and Wilson, 1978; Monshi et al., 2012).

Moreover, FT-IR spectra (AVATAR 330 FT-IR, Thermo Nicolet) analysis in the region of 4000 cm⁻¹ – 400 cm⁻¹ with a resolution of 0.09 cm⁻¹ was used and recorded. FT-IR (Fourier-transform infrared) spectroscopy provided information about the binding interaction of copper nanoparticles with the reducing agent, L-ascorbic acid. Potassium bromide which is a spectroscopic grade was used as background for IR analysis.
RESULTS AND DISCUSSION

Figure 1 shows the XRD patterns of the prepared copper nanoparticles. The $\theta$ value of synthesized copper nanoparticles was observed at the peaks of 22.4, 31.15, 43.33, 50.27 and 74.18. This agrees with the standard value of face-centered cubic copper (ca. 38.48, 43.73, 51.39 and 73.42 representing Cu (111), (111), (200) and (220) planes of crystal face-centered cubic of copper (JCPDS-04-0836) (Langford and Wilson, 1978; Monshi et al., 2012).

The comparison of $\theta$ obtained in our experimental work with the values of $\theta$ described in JCPDS-040836 for copper nanoparticles are shown in Table 1. The peaks at 43.33, 50.29 and 74.18 correspond to the characteristic peak of the copper nanoparticles. The size of the as-synthesized copper nanoparticles was estimated from Debye - Scherer equation (Langford and Wilson, 1978), and the result revealed that the copper nanoparticles had an average size of 50 nm.

Figure 2 shows the FT-IR spectra of the pure L-ascorbic acid and the as-prepared copper nanoparticles. From the IR spectra of L-ascorbic acid, the peaks in the range of 3215–3520 cm$^{-1}$ correspond to the different hydroxyl groups. The characteristic peak at 1766 cm$^{-1}$ in the spectra of L-ascorbic acid is due to stretching vibrations of the C=O of the five-membered lactone ring, and this band changed after coating on the nanoparticles (Sreeja et al., 2015).

Comparing with the IR spectra of the pure L-ascorbic acid and Cu nanoparticles stabilized with L-ascorbic acid, three major peaks have been disappeared from the later spectra, namely the OH stretching frequency in the range of 3219–3518 cm$^{-1}$, the stretching frequency of the carbonyl group of the oxidized ester at 1724 cm$^{-1}$ and conjugated carbonyl group of the compound at 1629 cm$^{-1}$.

This indicated that the free ascorbic acid had chemisorptions on surface of copper nanoparticles to stabilize the as-prepared nanoparticles. Moreover, most of the peaks between 1000-1800 cm$^{-1}$ of free L-ascorbic acid disappeared after stabilizing the Cu nanoparticles. This is due to the
transformation of ascorbic acid to dehydroascorbic acid after reducing Cu$^{2+}$ to Cu$^0$. Hence, the characteristic peak such as the double bond, which is observed at around 1600 cm$^{-1}$, corresponding to the free L-ascorbic acid disappeared during the reduction process. The difference in the positions of peak results of free ascorbic acid and/or the change in the band width clearly indicated the formation of copper nanoparticles capped with ascorbic acid. According to Razium et al. (2014), the disappearance of other noticeable bands around 623 cm$^{-1}$, 588 cm$^{-1}$, 534 cm$^{-1}$ and 480 cm$^{-1}$ indicates the absence of the possible existence of copper oxides as Cu$_2$O and CuO impurities. Based on the experimental results, it is possible to suggest the advantage of using L-ascorbic acid as reductant and capping agent in the synthesis of copper nanoparticles at various experimental conditions of this work.

In general, the capping agents could prevent the agglomeration of small crystals and influence the structure and crystallinity of the as-synthesized copper nanoparticles. Therefore, we prepared the nanoparticles by changing the dynamics of the surfaces of the reducing and capping agent by controlling the reaction time, temperature and concentration.

<table>
<thead>
<tr>
<th>Experimental results of 2θ (degree)</th>
<th>Standard diffraction value of 2θ (degree) from JCPDS-04-0836 [39,40]</th>
<th>The value of hkl (plans) from the value of JCPDS-04-0836</th>
</tr>
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<tbody>
<tr>
<td>24.40</td>
<td>-</td>
<td>(100)</td>
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<tr>
<td>31.26</td>
<td>36.79</td>
<td>(110)</td>
</tr>
<tr>
<td>43.33</td>
<td>43.73</td>
<td>(111)</td>
</tr>
<tr>
<td>50.27</td>
<td>51.39</td>
<td>(200)</td>
</tr>
<tr>
<td>74.18</td>
<td>74.42</td>
<td>(220)</td>
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</tbody>
</table>

Table 1 Experimental and standard diffraction angles of copper nanoparticles.

Figure 2 IR spectra of (a) pure L-ascorbic acid and (b) Cu nanoparticles stabilized with L-ascorbic acid.
When CuCl₂·2H₂O and L-ascorbic acid solutions were mixed at a heating temperature of 80°C with frequent stirring, there was color change that could be used as an indication of the synthesis of copper nanoparticles or of the reduction of Cu²⁺ to Cu⁰ (Shikha et al., 2015). As it is shown in Figure 4, after 12 hrs of the reaction time, the color gets more intense. This phenomenon could occur due to the consumption of higher concentration of L-ascorbic acid to form the copper nanoparticles. As the concentration of L-ascorbic acid increases, the number of dispersed copper nanoparticles will increase resulting in a decreasing agglomeration of the particles (Huixiao et al., 2012).

As noted above, the formation process of Cu nanoparticles can be supported by the color change of the reaction mixture and UV-vis spectra. Thus, the optical properties of the prepared copper nanoparticles were examined using the data obtained from UV-Vis absorption measurement of the as-synthesized copper nanoparticles in distilled water. Figure 3 shows the UV-Visible spectra of the precursor salt, ascorbic acid and the synthesized copper nanoparticles. Moreover, the effect of reaction time and of temperature on the formation of Cu nanoparticles indicated by color changes is explained in Figure 4 and 5, respectively. The color change was due to Surface Plasmon Resonance (SPR).

Nano-sized particles exhibit unique optical properties having an exponential-decay Mie scattering profile with decreasing photon energy (Chen and Sommers, 2001). The collective oscillations of conduction electrons at the surface of nano-sized metal particles absorb visible electromagnetic waves, which is known as Surface Plasmon Resonance (SPR) (Dang et al., 2011). Such phenomenon of SPR of metal nanoparticles is largely size dependent. But the exact position and shape of the Plasmon absorption peaks may depend on several factors including size of particle, type of solvent, capping agent, the particle morphology, dielectric functions of the metal and the surrounding medium as well as surface-absorbed species (George et al., 2007).

![Figure 3 UV-Visible spectra of precursor salt (a), ascorbic acid (b) and Cu NPs (c).](image-url)

As Figure 3 above shows, the absorption edge of the as-prepared copper nanoparticles was determined at around 423 nm, which is closer to the literature value (450 nm) reported for the copper nanoparticles (Arunachalam and Kannappan, 2013). This result, thus, confirms the formation of copper nanoparticles.

In this study, it was realized that the reaction time is one of other important parameters in the synthesis of copper nanoparticles. As time of reaction increases, the formations of copper nanoparticles will increase since the reactants have ample time for completion. In other words,
when the reaction time increases, the copper nanoparticles synthesized by chemical reduction method using L-ascorbic acid as protective agent lead to the formation of well-defined nanoparticles (Dang et al., 2011). Therefore, it is possible to understand that copper nanoparticles could be best synthesized at around 12 hours of reaction time. That is, the color change observation shown in Figure 4 indicates that 12 hours reaction time is the suitable reaction condition for the successful preparation of copper nanoparticles.

Figure 4 UV-VIS spectra of Cu nanoparticles prepared at different intervals of reaction time with its corresponding color change observed.

Reaction temperature is also another important reaction parameter in the synthesis of copper nanoparticles. To elucidate the effect of temperature, 3 mM of copper chloride dihydrate solution and 4 mM L-ascorbic acid solution were heated at different temperature (55°C, 65°C, 75°C and 90°C). The result revealed that temperature below 65°C is not suitable for the complete formation of copper nanoparticles. In addition, reaction temperature above 90°C was also found to be inconvenient as characteristic color and UV-Vis absorption spectrum of copper nanoparticles were not observed. Therefore, it is difficult to control the size of the particles at these levels of temperature, and this may result in agglomeration of particles. This implies that the suitable range of thermal temperature for the formation of copper nanoparticles is between 70°C and 90°C at appropriate concentration (Huixiao et al., 2012). Figure 5 shows UV-Vis absorbance spectra of the as-synthesized copper nanoparticles at different ranges of temperature.

Figure 5 UV-VIS spectra of Cu nanoparticles prepared at (a) 55°C, (b) 65°C, (c) 75°C and (d) 90°C with its corresponding color change observed.

In the formation mechanism of Cu²⁺, the first step, Cu²⁺ is reduced to Cu⁰ by the help of the reducing agent, L-ascorbic acid, so as to form a nucleus. Then the nuclei were stabilized by the capping agent, L-ascorbic acid, for the growth of Cu nanoparticles. Cu²⁺ ions were initially transformed to Cu(OH)₂ as precursor, and then Cu(OH)₂ was reduced to Cu₂O using L-ascorbic acid. Cu₂O was finally reduced to Cu nanoparticles. The reduction process can be rationalized using the equations shown below.
The antibacterial activity of the prepared copper nanoparticles was investigated against both gram-positive and gram-negative bacteria. The growth inhibition of the tested bacterial strains is also indicated in Table 2 and 3.

The results in Table 2 shows that copper nanoparticles formed at a concentration of 3mM cupric chloride dihydrate salt solution and that 4 mM L-ascorbic acid exhibited an excellent zone of growth inhibition of 16.83±0.42 mm for gram-negative bacteria while Table 3 reveals the zone of growth inhibition of 15.50±0.89 mm for gram-positive bacteria.

Table 2 The antibacterial activity of the prepared copper nanoparticles: Zone of inhibition (mm) against *E. coli* and *P. aeruginosa*.

<table>
<thead>
<tr>
<th>Concentration, (mM) (the ratio of salt to AAH)</th>
<th>Zone of inhibition (mm)</th>
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<tbody>
<tr>
<td></td>
<td><em>E. coli (G –Ve)</em></td>
<td><em>P. aeruginosa (G-Ve)</em></td>
<td>Mean± SD</td>
<td>Mean± SD</td>
<td></td>
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<tr>
<td>2CuCl₂.2H₂O</td>
<td>8.2 9.3 8.5</td>
<td>8.67±0.57</td>
<td>8.8 8.7 9.2</td>
<td>8.90±0.26</td>
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<tr>
<td>4AAH</td>
<td>12.4 13.3 12.9</td>
<td>12.87±0.47</td>
<td>13.4 14.5 13.8</td>
<td>13.90±0.55</td>
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<tr>
<td>2 mM salt:4 mM AAH</td>
<td>13.3 14.4 13.9</td>
<td>13.83±0.55</td>
<td>13.5 12.7 13.3</td>
<td>13.17±0.66</td>
<td></td>
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</tr>
<tr>
<td>3 mM salt:4 mM AAH</td>
<td>17.5 16.9 18.4</td>
<td>17.60±0.75</td>
<td>16.7 16.5 17.3</td>
<td>16.83±0.42</td>
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</tr>
<tr>
<td>Tetracycline</td>
<td>19.6 20.4 19.8</td>
<td>19.93±0.42</td>
<td>16.9 17.4 17.5</td>
<td>17.27±0.18</td>
<td></td>
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</tr>
<tr>
<td>Sterile distilled H₂O</td>
<td>0 0 0</td>
<td>0±0</td>
<td>0 0 0</td>
<td>0±0</td>
<td></td>
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<tr>
<td>n = 3; * triplicate measurement</td>
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</table>
Table 3: The antibacterial activity of the prepared copper nanoparticles: Zone of inhibition (mm) against *S. aureus* and *P. pyogenes*.

<table>
<thead>
<tr>
<th>Concentration (mM) (the ratio of salt to AAH)</th>
<th>Zone of Inhibition (mm)</th>
<th>Mean ± SD</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>S. aureus (G+Ve)</em></td>
<td></td>
<td><em>P. pyogenes (G+Ve)</em></td>
</tr>
<tr>
<td>2CuCl$_2$·2H$_2$O</td>
<td>6.5, 6.3, 7.4</td>
<td>6.73 ± 0.61</td>
<td>6.9, 7.2, 6.7</td>
</tr>
<tr>
<td>4AAH</td>
<td>9.6, 10.4, 9.6</td>
<td>9.87 ± 0.46</td>
<td>13.4, 13.8, 14.3</td>
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<tr>
<td>2mMsalt:4mMAAH</td>
<td>12.8, 12.9, 13.5</td>
<td>13.07 ± 0.70</td>
<td>14.5, 13.9, 14.5</td>
</tr>
<tr>
<td>3mMsalt:4mMAAH</td>
<td>16.6, 17.2, 16.8</td>
<td>16.87 ± 0.31</td>
<td>14.5, 15.8, 16.2</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>21.65, 21.9, 22.3</td>
<td>21.67 ± 0.34</td>
<td>16.87, 17.7, 17.9</td>
</tr>
<tr>
<td>Sterile distilled H$_2$O</td>
<td>0, 0, 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
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</tbody>
</table>

n = 3; * triplicate measurement

The antibacterial activity study revealed that the concentration of copper nanoparticles has a linear correlation with zone of inhibition growth, i.e. as the concentration of copper nanoparticles increases, the zone of inhibition also increases (Rajesh *et al.*, 2010). This present antibacterial activity investigation showed that the prepared copper nanoparticles exhibited almost similar zone of inhibition growth towards the Gram-positive and Gram-negative tested bacteria strains (Table 2 and 3).

The antibacterial mechanism of metal nanoparticles is not still known clearly. However, it has been reported that when metal nanoparticles are attached to the surface of the cell membrane, the composition of cell wall varies rapidly affecting the wall permeability, the respiratory function and DNA of which it tends to lose its ability to replicate (Pal *et al.*, 2007). The mechanism by which the nanoparticles were able to penetrate the cell wall was due to changes in membrane morphology that significantly increases permeability and affects proper transport through the plasma membrane leaving the bacterial cells incapable of properly regulating transport through the plasma membrane and resulting in cell death (Ekezie *et al.*, 2017).

Furthermore, the enhanced activity of the synthesized nanoparticles could be their improved characteristics and morphological properties of nanoscale materials in terms of specificity and better manipulation, increased...
surface area available for interactions, which enhances bactericidal effect than the large sized particles and thus, they impart cytotoxicity to the microorganisms (Ekezie et al., 2017).

The bactericidal effect of metal nanoparticles has been attributed to their size and high surface to volume ratio, which allows them to interact with microbial membranes and is not merely due to the release of metal ions in solution (Mokae et al., 2017). According to Deryabin et al., the mechanism of antibacterial activity of Cu nanoparticles could be manifested by the disruption of energy metabolism in the target bacterial cells, which causes fast-developing inhibition, resulting in the decrease in transmembrane electrochemical potential of the cytoplasmic membranes (Deryabin et al., 2017).

CONCLUSIONS

A simple, economical, environmentally benign and green as well as temperature controlled synthesis method has been developed for the preparation of copper nanoparticles. The optical properties and crystallinity of the as-synthesized copper nanoparticles were characterized by UV-Vis Spectrophotometer and XRD. The binding effect of L-ascorbic acid was studied by FT-IR spectroscopy. The XRD patterns showed that the structure of the synthesized copper nanoparticles are FCC and their size was calculated using Debby-Scherer equation, and the size was found to be around 50 nm. The UV-Vis absorption spectra show that as the concentration of L-ascorbic acid increases, the formation of copper nanoparticles will increase. The IR stretching frequencies of the hydroxyl group in the range of 3219–3518 cm⁻¹ and carbonyl groups at 1724 cm⁻¹ and 1629 cm⁻¹ of the free ascorbic acid in the synthesized nanoparticles disappeared which indicated that the free ascorbic acid reacted with the cupric chloride dihydrate solution in order to afford the copper nanoparticles. The characterization results of the aforementioned techniques suggested that the copper nanoparticles were prepared successfully. The method will be used as a competitive alternative technique for the preparation of other related nanoparticles. In addition, the antibacterial activities of copper nanoparticles at different concentrations exhibited excellent antibacterial activity against the tested Gram-positive and Gram-negative bacteria. The result also showed the zone of inhibition which depends on the concentration of nanoparticles.

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