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RESEARCH ARTICLE

Effect of Various Parameters on Bio-Synthesis of Copper Nanoparticles Using Citrus Medica Linn (Lemon) Extract and Its Antibacterial Activity

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Highlights

- Bio-synthesis of CuNPs using lemon extract.
- Effect of volume of extract, concentration and temperature on biosynthesis
- Antibacterial activity of CuNPs was studied via agar well diffusion method.

Abstract

This research is focused on bio-synthesis of Copper nanoparticles (CuNPs) using lemon extract to study the effect of various parameters on synthesis and to explore antibacterial activity. The biomlecules present in lemon extract act as self reducing and stabilizing agent. The synthesis of CuNPs was found to be affected by various parameters like volume of the lemon extract, concentration of the precursor and the temperature etc. Preliminary characterization of formation of nanoparticles were done by color change and UV-visible (UV-vis) spectroscopy. Elemental composition of the prepared sample was determined via Energy Dispersive X-ray (EDX) Spectroscopy. Presence of important functional groups associated with biomolecules is well characterized by Fourier Transform Infrared spectroscopy (FTIR). Scanning Electron Microscopy (SEM) revealed the formation agglomerated CuNPs of different shape and sizes and the X-ray diffraction pattern showed the formation of purely crystalline nature of CuNPs. Finally, agar well diffusion method showed that CuNPs have potential antibacterial activity against Gram-ve bacteria compared to Gram +ve bacteria.

***Keywords**: Bio-synthesis, CuNps, Precursor, XRD, FTIR, Agar well diffusion*

Introduction

People have been much aware of the problem of environmental degradation since last decade of the 20th century. Considering, the problem of environment, most of the research has been focused on environmentally benign method employing, green materials. Metal nanoparticle is thought to be one of the current topics of research in the field of nano-science for their wide scope of applications such as in the magnetic recording media or microelectronics, nano-medicine, optoelectronic, nanoelectronics, nanosensors, photonics, catalysis and information storage devices (Ashtaputrey *et al*. 2017; Prathna *et al.* 2016).

Among various types of metallic nanoparticles, copper is the most widely used material in the field of nanoscience due to its electrical, optical, catalytic, biomedical, antifungal, and antibacterial properties. Copper nanoparticles (CuNPs) have been extremely employed in various sectors such as water treatment, pharmacology, medical fields, targeted drug delivery, cancer treatment, gene therapy, DNA analysis, antibacterial agents, biosensors, enhancing reaction etc (Kulkarni & Kulkarni 2013;

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Padma et al. 2018; Dinda et al. 2015). Copper-based compounds have been used in electronics since ancient times in electronic circuits due to its excellent electrical conductivity and low cost (Suramwar et al. 2016). As a consequence of possessing vital conducting properties, good compatibility, and surface-enhanced raman scattering activity, Cu and Cu based compounds will receive much demand in future nanodevices (Suramwar et al. 2016; Mittu 2016). The more important aspect is that Copper nanoparticles (CuNPs) are cheaper compared to gold, silver nanoparticles, and their properties can be controlled depending on the synthesis method and are affected by different parameters while synthesizing the materials (Karthik & Geetha 2013; Padma et al. 2018). Hence, the synthesis and exploration of different properties of CuNPs is still of high demand.

Various methods such as vapour deposition, radiolysis reduction, heat evaporation, electrochemical reduction (Siddiqi *et al.* 2018), chemical reduction (Karthik & Geetha 2013), thermal decomposition (Joshi *et al*. 1998) *etc* have been extensively used for the synthesis of NPs in the nanotechnology. Out of various possible methods, chemical approaches are the most popular methods for the fabrication of nanoparticles. However, the chemical synthesis induces toxicity directly or indirectly to the environment. Hence, the developments of clean, biocompatible, non-toxic and eco-friendly methods for the synthesis of nanoparticles are extensively studied these days (Joseph *et al*. 2016; Aruoma et al. 2012).

Citrus medica Linn (commonly called as Lemon) is a species of small evergreen tree comprising a flowering plant of family *Rutaceae*, native to Asia. Lemon, a yellow fruit is well known for citric acid and ascorbic acid. It has been well known that lemon is of considerable significance from a medicinal point of view. Many of fatal diseases like cerebrovascular, cardiovascular, inflammation, cancer, problems of skin, throat, and asthma have been cured of by regular use of lemon water because of its antibacterial properties (Aruoma *et al.* 2012). Main objective of the present research is to carry out bio-synthesis copper nanoparticles (CuNPs) using locally available lemon extract to study effect of various parameters on synthesis of CuNPs and to evaluate its antibacterial activity against different strains.

Materials and Method

Preparation of lemon extract

The lemon extract was prepared just by squeezing the fresh, washed lemons into a clean container for the required amount and then filtered with muslin cloths first and then with Whatman No.1 filer paper.

Bio-synthesis of copper nanoparticles (CuNPs)

CuNPs were synthesized by simple and non-toxic, green methodology with slight modification using the protocol (Jayandran et al. 2015). 100 mL of 1 mM $CuSO_4$ solution was allowed to react with 10 mL of lemon extract in a conical flask. Here, biomolecules present in lemon extract is used as reducing and self-stabilizing agents for the CuNPs. The reaction mixture was stirred at 60 °C for 1 hour and the color changed from light blue to reddish dark brown color. Then after, the obtained precipitate was centrifuged at 4000 rpm for 30 min and the supernatant liquid was decanted off. Finally, residue, CuNPs was washed with distilled water many times to remove impurities and collected after oven-dried at less than 80 °C. The process was repeated to study the effect of various parameters like temperature, concentration of precursor solution, and volume of the lemon extract in the synthesis process of CuNPs.

Characterization techniques

Preliminarily, the formation of nanoparticles was analyzed by visual observation of the changes in the color of the reacting solution. Then, the fabrication of CuNPs was confirmed using double beam UV-visible Spectrophotometer (LABTRONIC, Model LT-2802) in a range of wavelengths from 200 to 800 nm at interval of 5 nm. Similarly, the Energy Dispersive X-ray (EDX) spectroscopic studies were carried out by using EDX-8000 SHIMADZU to explore the elemental compositions of the sample. FTIR analysis was carried by using FTIR –Tracer-100 to identify the functional groups of the biomolecules responsible for reduction of the precursor. The FTIR spectrum was recorded at the wave number range of 500 - 4000 cm^{-1} at resolution of 4 cm^{-1}. Crystal morphology of the prepared sample was studied using XRD (Bruker D2 Phaser), at diffraction angle (2θ) range of 10^o to 80^o at the rate of 2^o/min. Surface morphology of the sample was investigated using Field Emission Scanning Electron Microscopy (FE SEM, Carl Zeiss, SUPRA 40 VP, Oberkochen, Geramny).

Agar well diffusion method

Antibacterial assay was performed by agar well diffusion method which measures mainly, zone of inhibition (ZOI) of the CuNPS against pathogenic bacteria. Firstly, a 2/3 colony of bacteria was transferred and cultured into a Nutrient Broth (NB) and incubated at 37 °C for 24 hours. After incubation, each stain was diluted with sterile distilled water. The turbidity of dilution was checked by comparing with 0.5 McFarland standards.

Then after, Muller Hilton Agar (MHA) plates were taken and labeled for each bacteria; *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Salmonella typhi*. Then the sterile cotton swab was dipped into the test tube containing diluted bacterial stain and swabbed in-to the sterile Muller Hilton Agar (MHA) plates. The cotton swab was swabbed uniformly into the MHA plates. It was allowed to diffuse for about 20 minutes at the room temperatures. 5 wells of 4 mm were bored in the inoculated media with the help of sterile cork-borer (4 mm). The liquid sample was divided into three parts, which of them include 100% crude, 50% diluted solution, and 25% diluted solution. Each well was loaded with different extracts, positive control (Ofloxacin) 50µL, negative control (DMSO) 50 µL, Crude liquid (100%) 50µL, liquid (50%) 50µL and liquid (25%) 50µL. Then after, extract was set for diffusion for about 30 minutes at ordinary condition. Finally, the disc was incubated for 24 hours.

Results and Discussion

Visual observation

Upon visual observation, blue color was found to be changed to a shiny reddish-brown color indicating the reduction of Cu^{2+} to metallic copper of zerovalent (Cu^0). The preliminary result was found to be in good agreement with that of Kotval *et al.* (2018). It was further characterized by UV-visible spectroscopy for the confirmation of nanoparticles.

UV-visible spectroscopy

Result obtained from UV-vis spectroscopy of the bio-synthesized CuNPs and that of lemon extract is presented graphically in Figure 1. There is no any absorption peak in plot (a), UV-vis spectrum of neat lemon extract whereas the plot (b), the UV-vis spectrum of CuNPs (at volume ratio 1:10) shows a maximum absorption peak at around wavelength 565 nm which attributes the presence of CuNPs due to surface plasmon resonance of conducting electrons (Kotval *et al*. 2018; Chung *et al*. 2017).

Similarly, UV-visible spectra were used to study the effect of different parameters such as the volume of lemon extract, the concentration of $CuSO_4$ solution, and temperature on bio-synthesis of CuNPs and the results are shown in next section.

Effect of volume of the lemon extract on the bio-synthesis of CuNPs

The UV-visible spectra of CuNPs synthesized using different volumes 10, 20, 30 and 50 mL of the lemon extract with fixed volume of precursor (1mM) are presented in Figure 2. Each of the plots shows presence of maximum absorption band at wavelength around 565 nm, which is due to surface plasmon resonance of CuNPs. The peaks are shifted slightly towards higher wavelength from 565 to 590 nm and broadened with the increase in volume of the extract from 10 to 50 mL. The higher the volume of extract, more reducing

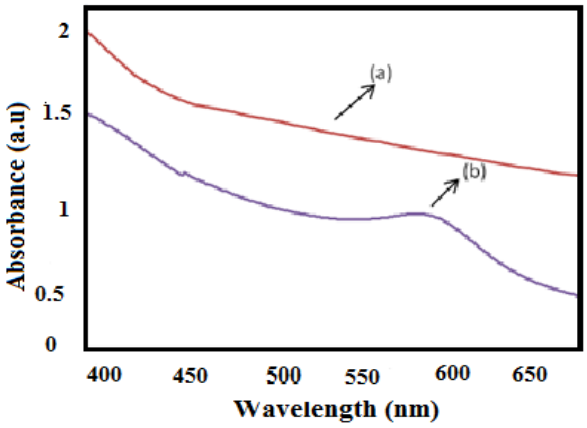

***Fig 1**: UV-visible spectra of (a) Lemon extract (b) as-synthesized CuNPs using lemon extract and $CuSO_4$ (1mM) of ratio (1:10) stirring at 60°C.*

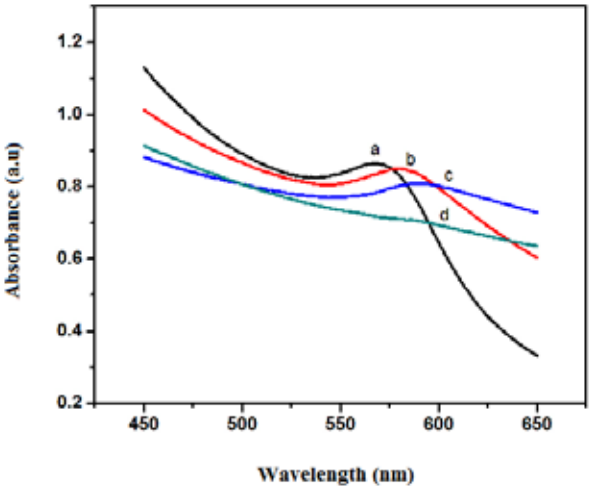

***Fig 2:** UV-visible spectra of Cu NPs prepared with 1mM $CuSO_4$ at different volume of lemon extract (a) 10 mL (b) 20 mL (c) 30 mL (d) 50 mL.*

agent are available hence, more number of CuNPs are formed which soon forming agglomerated nanoparticles at higher volumes (30 & 50 mL). The frequency and width of the absorption peak depend on the size and shape of the metal nanoparticles (Augustine et al. 2013).The absorption peak formed by low volume i.e 10 mL is intense due to formation of nanosized particles. Hence, this volume is found to be the optimum for the bio-synthesis.

Effect of concentration of $CuSO_4$ solution on the bio-synthesis of CuNPs

Result of UV-visible spectra of CuNPs at concentration variation (Figure 3) shows that the absorption peak of minimum concentration i.e 1mM is more intense compared to that of higher concentration (5 mM and 9 mM) because of the formation of smaller sized nanoparticles. With the increase in concentration of $CuSO_4$, the peak position is slightly shifted towards higher wavelength and broadened also as more Cu^{2+} ions take part in reduction reaction and collision between nanoparticles accomplished leading to formation of agglomerated nanoparticles (Sekhar et al. 2016; Surmawar et al. 016). Thus, 1 mM is referred as the optimum concentration for fabrication of CuNPs.

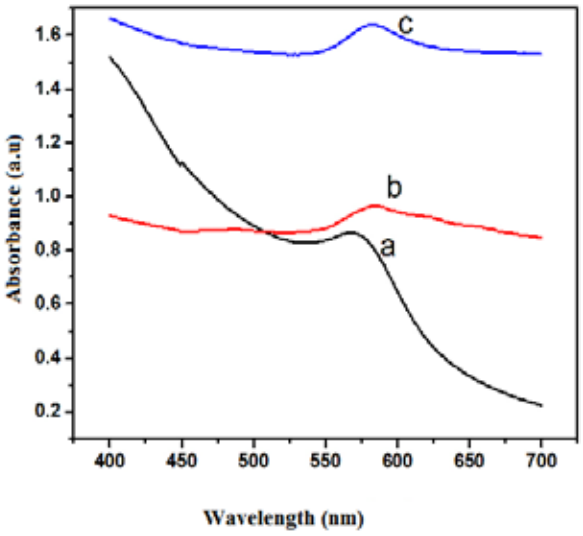

Fig 3: *UV-visible spectra of CuNPs formed at different concentrations of $CuSO_4$ solution (a) 1 mM (b) 5 mM (c) 9 mM*

Effect of temperature on the bio-synthesis of CuNPs

It is a well known fact that temperature is being one of the significant parameters for carrying out any reaction. Bio-synthesis of CuNPs was carried out at different temperatures, i.e. at room temperature, 40°C, 60°C, and 80°C, respectively. Then, results of UV-visible spectroscopy of each sample are shown in Figure 4. The figure depicts that no absorption band appear at low temperatures i.e at room temperature and 40°C indicating low temperature is not favorable for the formation of nanoparticles. When the temperature is increased to 60°C and 80°C, maximum absorption of the CuNPs are appeared at 570 nm and 585 nm, respectively. The values are in the UV range of characteristic values of CuNPs as reported in literatures (Jayandran et al. 2015; Dinda et al.(2015). On comparing two UV peaks, curve c is comparatively sharper than the curve d indicating the presence of nanosized CuNPs. Hence, 60°C is found to be the favorable temperature for fabricating the CuNPs.

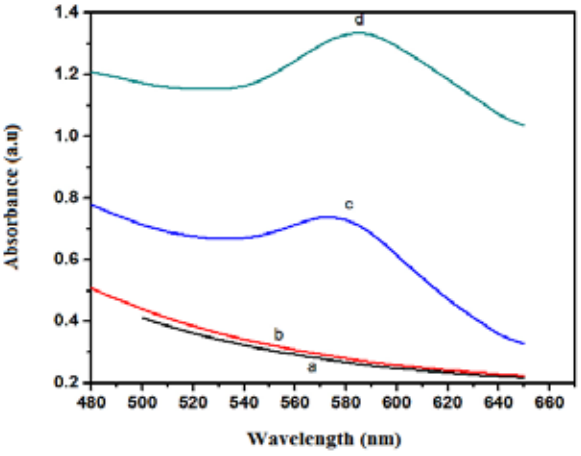

Fig 4: UV-visible spectra of biosynthesized CuNPs using lemon extract and $CuSO_4$ at different temperature (a) room temperature, (b) 40 °C, (c) 60 °C and (d) 80 °C

Energy dispersive X-ray (EDX) spectroscopy

Energy dispersive X-ray (EDX) spectroscopy is essential tool for achieving information about elemental composition. The Figure 5 shows the presence of energy absorption band at 8keV supporting the presence of copper as reported by Caroling *et al*. (2015). From the EDX analysis, the weight percentage of elemental copper is found to be 22.599%. Hence, as-synthesized copper nanoparticles seemed associated with other elements remaining as impurities which may be obtained from the instrument and the sample preparation process.

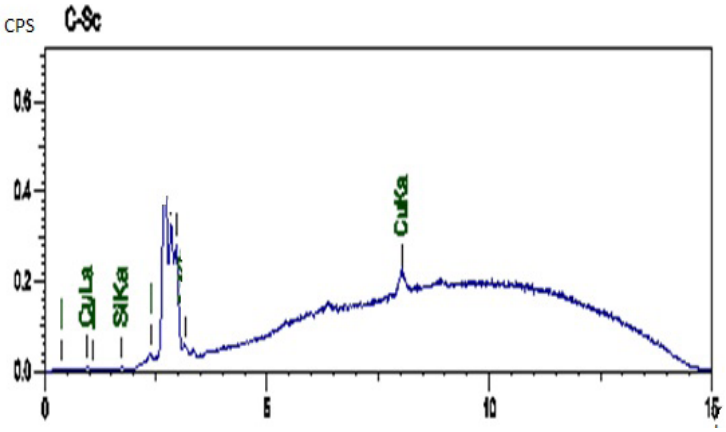

Fig 5: *Energy Dispersive X-ray (EDX) Spectrum of as-synthesized CuNPs at 60°C*

Fourier transform infrared spectroscopy (FTIR)

The results of FTIR spectroscopy of bio-synthesized CuNPs using lemon extract is shown in Figure 6, which

illustrates the presence of various kinds of biomolecules surrounding the CuNPs. The absorption peak located at 3293 cm^{-1} corresponds to stretching vibrations of hydrogen bonded -OH groups. Further, peaks at 2900 cm^{-1} are due to symmetric & asymmetric C-H stretching of phenolic (Kumar *et al*. 2017), peaks observed at 2093 cm^{-1}, 1639 cm^{-1} , 1300 cm^{-1}, 1450 cm^{-1} and 1022 cm^{-1} refers to -CN stretching, -C=C relation, –C=O stretching,–OH bending of phenolic group and –C-H stretching characteristic of aromatic nucleus, respectively (Kumar *et al*. 2017; Trifunschi *et al*. 2015). While the undesirable peak observed 2300 cm^{-1} is due to instrumental error according to technician.

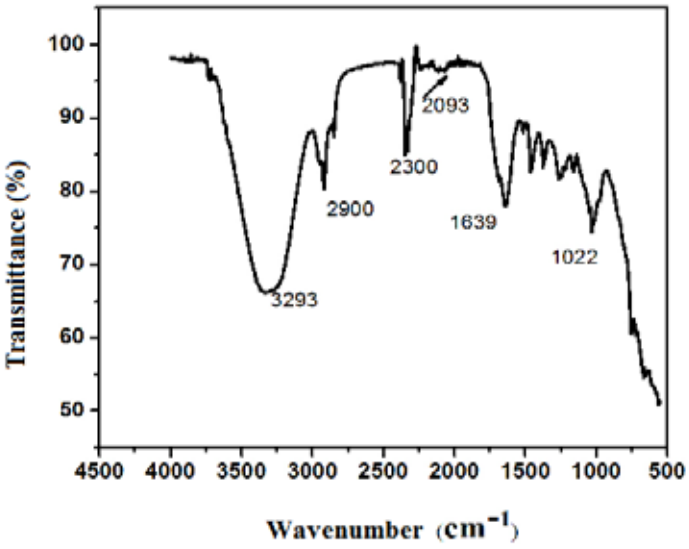

Fig6: *FTIR Spectra of CuNPs synthesized from the lemon extract and $CuSO_4$ solution (1:10)*

X-Ray diffraction (XRD)

X-ray diffraction pattern of CuNPs is shown in Figure 7. The figure shows sharp peaks at diffraction angles 43.5°, 50.6°, and 74.0° which are equivalent to the reflections revealed by different literatures. It depicts the presence of (111), (200) and (220) planes indicating formation of face-centered cubic (fcc) crystals of CuNPs which follows Joint Committee of Powder Diffraction Standards (File No.089-2838) (Dinda *et al*. 2015; Suramwar *et al*. 2016; Samar *et al.* 2017; Kumar *et al*. 2017). Various other small peaks observed at 26°, 31° and 36° may be due to formation of oxidized copper because sample was oven dried at less than 80°C.

The average size of the crystallite is estimated by using the Debye-Scherrer equation (Carolling *et al*. 2015; Mittu 2016).

$$D = \frac{0.9\lambda}{\beta \cos\theta} \quad \ldots (1)$$

Where 'λ' is wavelength of X-ray (0.1541 nm), 'β' is FWHM (full width at half maximum), 'θ' is the diffraction angle, and 'D' is crystallite size. Substituting the corresponding values on the Scherrer equation, the average crystallite size was estimated to be 17.7 nm.

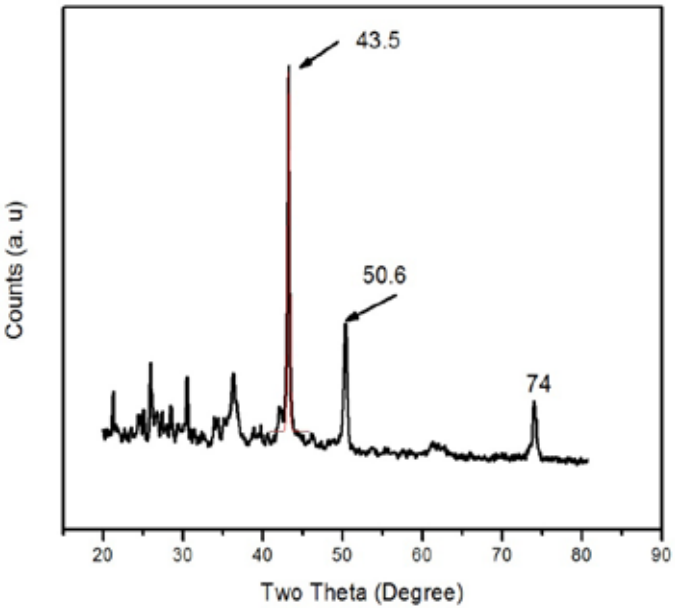

Fig7: *X-ray diffraction pattern of bio-synthesized Copper nanoparticles at 2θ range from 10-80°.*

Field emission scanning electron microscopy (FE-SEM)

Field Emission Scanning Electron Microscopy (FE-SEM) was employed to visualize the surface morphology of CuNPs. SEM images of CuNPs at different magnification are shown in Figure 8**.** SEM image Figure 8(a), lower magnification showed presence of white small grains of spherical and rod shape of CuNPs. Similarly, Figure 8(b), the higher magnification reveals clearly the presence of agglomerated nanoparticles distributed unevenly in a different direction. But it is difficult to calculate the size of these NPs. The size of NPs can be calculated using Transmission Electron Microscope (TEM).

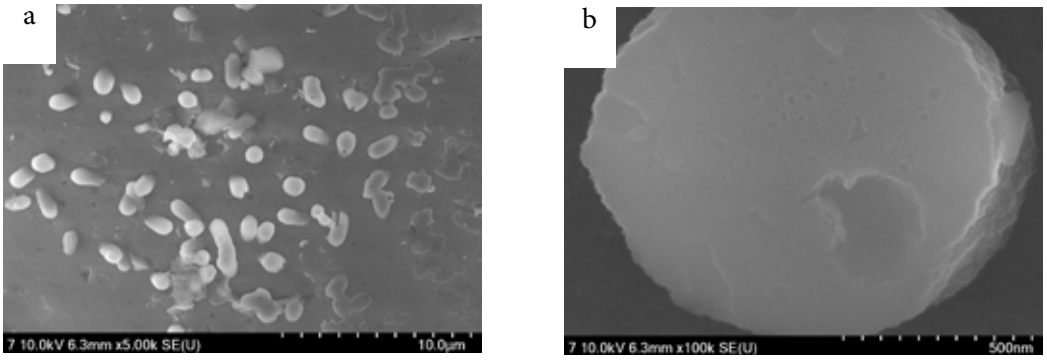

Fig 8: *FE-SEM images of CuNPs at (a) lower magnification, (b) at higher magnification*

Antibacterial activity of copper nanoparticles

The antibacterial activity of bio-synthesized CuNPs was evaluated against *Staphylococcus aureus* (Gram positive bacteria), *Salmonella typhi* and *Escherichia coli* (Gram negative bacteria), using agar well diffusion method. Commercial antibiotic, ofloxacin was used as positive control and Dimethyl sulfoxide (DMSO) was as negative control. Figure 9 presents snaps of zone of inhibition produced by *S. aureus, S. typhi* and *E. coli* at various concentrations, and result obtained is tabulated in Table 1.

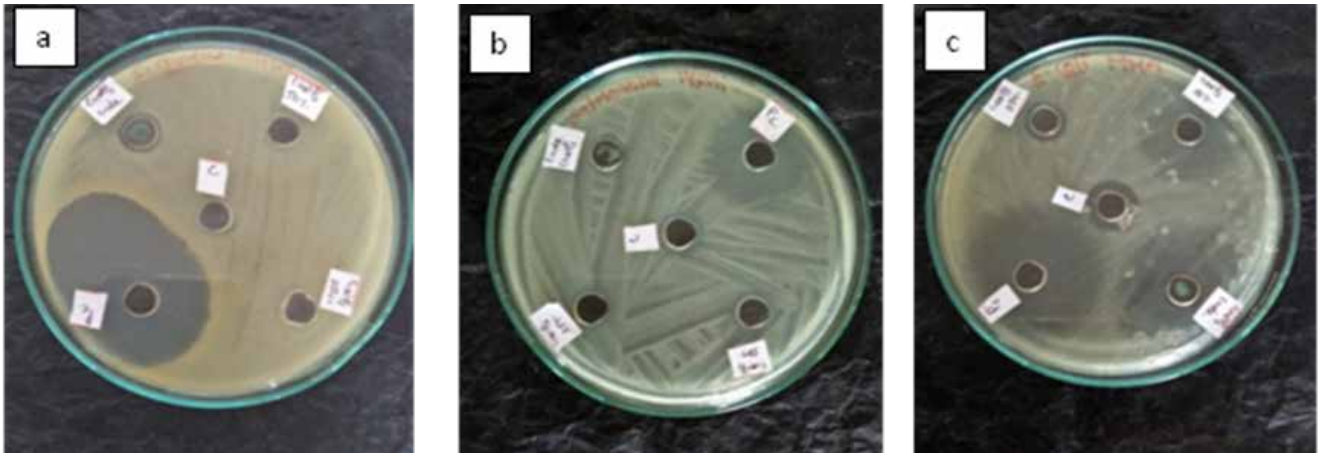

Fig 9: *Zone of inhibition (ZOI) produced by agar well diffusion of bio-synthesized CuNPs against bacteria: (a) Staphylococcus. aureus (b) Salmonella typhi and (c) Escherichia coli*

From the antibacterial test, ZOI of CuNPs of different concentrations is found greater for Gram negative bacteria than Gram positive bacteria due to differences in thickness of peptidoglycan layers (Theivasavthi & Alagar 2011).

Table 1*: Zone of inhibition (ZOI) of as-synthesized CuNPs and positive control ofloxacin*

S.N	Test organisms	Zone of Inhibition				
		CuNPs				Ofloxacin
		Crude	50%	25%	Control (DMSO)	
1	*Staphylococcus aureus*	12 mm	0 mm	0 mm	0 mm	35 mm
2	*Salmonella typhi*	11 mm	11 mm	0 mm	0 mm	28 mm
3	*Escherichia coli*	28.5 mm	24 mm	11 mm	0 mm	39 mm

Conclusions

Bio-synthesis of Copper nanoparticles (CuNPs) was carried out using lemon extract relatively at lower cost. The formation of CuNPs was confirmed by presence of characteristic UV-vis peak at 565 nm. Bio-synthesis of CuNPs was found to be much affected by the various parameters such as volume of lemon extract, concentration of the precursor, temperature *etc*. From the experiment, optimum volume of lemon extract and concentration of precursor is found to be 10 mL and 1 mM, respectively. At the same time, the favorable temperature is referred to be 60°C. Further, EDX spectroscopic analysis confirmed the presence of metallic copper with 22.59% by weight. Likewise, FTIR spectrum confirmed the presence of various biomolecules such as alkaloids, flavonoids, proteins at the surface of nanoparticles. Various reflections observed in XRD pattern revealed CuNPs are of purely crystalline nature and the average crystallite size is found to be of 17.7 nm. Finally, agar well diffusion assay revealed distinct zone of inhibitions (ZOI) by CuNPs against different bacterial strains suggesting that CuNPs have potential antibacterial activity. Regarding future perspective, bio-synthesis using micro-organisms, study of effect of time, pH on synthesis, exploration of optical and catalytic properties of CuNPs are still of interesting topics for research in future. Additionally, the surface of CuNPs can also be decorated with other metal or metallic oxide nanoparticles for enhancement of properties of CuNPs.

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