

Plant Extract Mediated Synthesis and Characterization of Copper nanoparticles and their Pharmacological Activities

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ABSTRACT: The development of simple, cost effective and reliable methods for the synthesis of nanomaterials is being emphasized globally. Biosynthetic methods, employing microorganisms, have emerged as an eco-friendly, clean and viable alternative to chemical and physical methods. The present study reports the synthesis of copper nanoparticles using *Triumfetta rotundifolia* plant extract. The synthesized copper nanoparticles were characterized by using UV, FTIR, XRD, SEM-EDX, AFM and CV studies. The SEM analysis reveals that the particles were triangular in shape. The synthesized nanoparticles possess significant anti-inflammatory, antibacterial and antifungal activities.

KEYWORDS: Copper nanoparticles, UV, XRD, SEM-EDX, AFM, CV, Pharmacological activities.

I. INTRODUCTION

Nanotechnology is the most promising technology that deals with understanding and control of matter at the nanoscale, and at dimensions between approximately 1 nm and 100nm. Quantum effects rule the behaviour and properties of particles at nanoscale range. Nanoparticles are considered as building blocks of the next generation of technology with applications in many industrial sectors. Chemical methods are the most popular methods for the production of nanoparticles. However, some chemical methods cannot avoid the use of toxic chemicals in the synthesis protocol. Since metal nanoparticles are widely applied to human contacting areas, there is a growing need to develop environmental friendly process of nanoparticle synthesis that do not use toxic chemicals (Hyo-Jeoung Lee *et al.*, 2011). Green synthesis method was found to be the best method when compared to the other conventional methods such as chemical reduction, photochemical reduction, electrochemical reduction, heat evaporation etc. (Geoprincy *et al.*, 2013). Among the metal nanoparticles, copper nanoparticles are potentially attractive, which may be due to their good optical, electrical and thermal properties, superior strength, and use as sensors and catalysts. Copper nanoparticles are attractive to many researchers due to its low cost compared to noble metals such as Ag, Au and Pt.

Nanoparticle synthesis using plant extracts can be advantageous over other biological processes because of large availability of plants and it eliminates the ease of large scale up and eliminates the process of culture maintaining, and no need to use high pressure, energy, temperature and toxic chemical (Singh *et al.*, 2010, Rajeshkumar *et al.*, 2012). Plant extracts are mostly used in pharmaceutical preparations and they consist of several constituents which act as reducing and capping agent in the reduction of metal ions. Noble metal nanoparticles are most promising as they show good antibacterial properties due to their large surface area to volume ratio, which is coming up as the current interest in the researchers due to the growing microbial resistance against metal ions, antibiotics and the development of resistant strains. These unique properties mainly depend on the size, shape and surface area of nanoparticles (Gupta *et al.*, 1998; Pal *et al.*, 2007).

The current investigation focused on the plant extract of *Triumfetta rotundifolia* used to synthesize copper nanoparticles and thereby improving the importance of plant source and involving green chemistry for the synthesis of other nanoparticles as future research.

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II. MATERIALS AND METHODS

Materials: Copper (II) sulphate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), was purchased from Hi-Media chemicals. Ultra-pure deionised water was used throughout the reactions. During the month of March, the plant of *T. rotundifolia* was collected from Kovilpatti, Thoothukudi District, Tamil Nadu, and India. The plant was identified and authenticated by Botanist Dr. V. Chelladurai, Research Officer Botany (Scientist-C) Central Council for Research in Ayurveda & Siddha, Government of India (Rtd). The collected plant materials were thoroughly washed several times using normal water and then followed by distilled water to remove impurities. The cleaned plant materials were subsequently dried under sunshade to remove moisture completely, powdered by using mechanical grinder and then stored.

METHODS

Preparation of leaf extract: 5g of the collected plant *T. rotundifolia* powder was mixed with 100 mL of double distilled water and refluxed for 30 minutes and cooled down to room temperature. The solution was then filtered through whatman No.1 filter paper and the filtrate obtained was stored then at 4°C for future work.

Synthesis of copper nanoparticles using plant extract of *T. rotundifolia*: 10 mL of *T. rotundifolia* plant extract was added to 90 mL of 1 mM aqueous copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) solution in a 250 mL Erlenmeyer flask. The colour of the solution changed from blue to pale yellow when the solution of *T. rotundifolia* plant extract and copper sulphate solution were stirred for homogeneous mixing. The flask was kept at room temperature for incubation overnight and the colour of the solution turned into dark brown (Saranyaadev *et al.*, 2014). Copper nanoparticles were separated out which settled at the bottom of this solution. The copper nanoparticle thus obtained was purified by repeated centrifugation method at 10,000 rpm for 30 min followed by dispersion of the pellet in deionized water to remove any unwanted biological materials (Sreemanti Das *et al.*, 2013). Later the copper nanoparticles were dried in an oven at 80°C for 2 hours.

Characterization Techniques: The optical property of nanoparticles was determined by UV-Vis spectrophotometer in 200 to 500 nm range. The absorption spectra of the synthesized nanoparticles were measured by using UV-1800 SHIMADZU UV- spectrophotometer instrument. The possible functional groups involved in the synthesis and stabilization of nanoparticles was identified by performing FTIR analysis in the range 400-4000 cm^{-1} using SHIMADZU-8400S model instrument, and the potassium bromide (KBr) pellet method was applied for spectral analysis. Crystalline size and structure of the synthesized nanoparticles were carried out by XRD using BRUKER, ECO D8 Advance model instrument. Detailed analysis of the morphology, size and distribution of the nanoparticles was documented by Scanning Electron Microscopy (SEM) using CARL ZEISS, EVO 18 model instrument. Elemental analysis of nanoparticles measured by EDX was carried out on BRUKER, X flash 6130 model instrument. In AFM analysis, the sample was analysed using a Veeco di Caliber set to tapping mode with a Veeco TESP silicon tip. Cyclic Voltammetry was carried out by using Chi 650C electrochemical work station, (USA) instrument.

In-vitro Anti-inflammatory activity: The anti-inflammatory activity of the synthesized copper nanoparticles was evaluated using HRBC membrane stabilization method (Sakat *et al.*, 2010). 2 mL of Blood sample was collected from the vein of a healthy human volunteer who did not take any NSAIDS for two weeks prior to the experiment, and then the collected sample was stored in a heparinised tube and washed with PBS, and equal volume of Alsever's solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% NaCl) was added and centrifuged at 3000 rpm for 10 minutes (Centrifuge Make: Eppendorf, Model 5810-R). The packed cells were washed with isosaline (0.85% NaCl) suspension. Various concentrations of the synthesized nanoparticles (200, 400, 600, 800, 1000) using plant extracts were prepared in mg/mL using distilled water, and to each concentration, 1 mL of phosphate buffer, 2 mL hypo saline and 0.5 mL of HRB suspension were added. It was incubated at room temperature for 30 minutes and centrifuged at 1500 rpm for 20 minutes, and the haemoglobin content of the supernatant solution was estimated spectrophotometrically at 560 nm using Micro plate reader (Make: Biotek, Model: Epoch). The experiments were performed thrice, and the mean values were taken for the study. Diclofenac (100 $\mu\text{g/mL}$) was used as reference standard and as a control. The percentage (%) of HRBC membrane stabilization was calculated using the following formula,

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Percentage of Protection (%) = $(100 - \text{OD of drug treated sample} / \text{OD of Control}) \times 100$

Antibacterial activity: The antibacterial screening of the synthesized copper nanoparticles was assessed against clinical bacterial strains such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonia* and *Salmonella typhi*. In order to determine the antibacterial activity of the synthesized copper nanoparticles using *T. rotundifolia* at different concentrations (5 μ l, 10 μ l and 15 μ l) by nutrient agar well diffusion method (Schillenger and Luke (1989)) Sterile nutrient agar medium was inoculated with 0.1ml of fresh overnight sterile nutrient broth culture of bacterial strains (approx. 10^7 CFU/ml) and poured into sterile petriplates. In each plate three wells of 6mm in diameter was punched using a sterile borer and the plates were allowed to dry for 5min. Synthesized copper nanoparticles' extracts at three different concentrations were poured by using sterile pipette and in the middle well a known antibacterial drug Ofloxin (1mg) was poured, and act as a positive control. The same procedure was followed in the above other six strains as given in table 4. After holding the plate at room temperature for 2 hours to allow diffusion of the extracts and controls in to the nutrient agar medium. The plates were incubated at 37°C for 24 – 48h. The sensitivity of the test organisms to each of the extracts were indicated by clear halo around the well. The halos' diameter as an index of the degree of sensitivity, were measured with a transparent plastic ruler. Then they were examined for inhibition of the bacterial growth. The diameters of the zones of inhibition in each case were expressed as mm sensitivity (Bayoub., 2010)

Antifungal activity: The synthesized nanoparticles were screened for antifungal activity by agar well diffusion method (Perez *et al.*, 1990) with sterile cork borer of size 6.0mm. The cultures of 48 hours old grown on potato dextrose agar (PDA) were used for inoculation of fungal strains such as *Candida albicans*, *Candida tropicalis*, *Aspergillus fumigatus*, *Aspergillus niger* on PDA plates. An aliquot (0.02mL) of inoculum was introduced to molten PDA and poured into a petridish by pour plate technique. After solidification, the appropriate wells were made on agar plate by using cork borer. Ketoconazole was used as control after holding the plate at room temperature for 2 hours to allow diffusion of the synthesized copper nanoparticles and controls in to the nutrient agar medium. Incubation period of 24-48 hours at 28°C was maintained for observation of antifungal activity of the synthesized copper nanoparticles. The antifungal activity was evaluated by measuring zones of inhibition of fungal growth surrounding the synthesized copper nanoparticles' extracts. The complete antifungal analysis was carried out under strict aseptic conditions. The zones of inhibition were measured with antibiotic zone scale in mm, and the experiment was carried out in triplicates.

III. RESULTS AND DISCUSSION

UV-Visible spectroscopy: The optical property of synthesized copper nanoparticles using *T. rotundifolia* was determined by UV-Visible spectroscopy. It is the most important method of analysis to detect the surface plasmon resonance property of copper nanoparticles (Curtis *et al.*, 1988). Reduction of copper sulphate to copper ions is identified by the colour change from light yellow of the extract to dark brown as given in Figure 1. The synthesized copper nanoparticle formation was confirmed from the peak at 321 nm for using the plant extract of *T. rotundifolia* Figure 2. Absorbance peaks nearly around 300 nm may be due to the presence of proteins, enzymes and flavanoid-like biomolecules in the extract that might be responsible for the reduction of copper sulphate (Jain *et al.*, 2009). This will confirm the formation of copper nanoparticles. Copper SPR effects decrease with time because of the oxidation of the synthesized copper nanoparticles (Tilakiet *et al.*, 2007).

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A) Copper sulphate solution B) *T. rotundifolia* plant extract C) Copper sulphate and *T. rotundifolia* plant extract solution

Figure 1 Colour change photographs of copper nanoparticles using *T. rotundifolia* plant extract

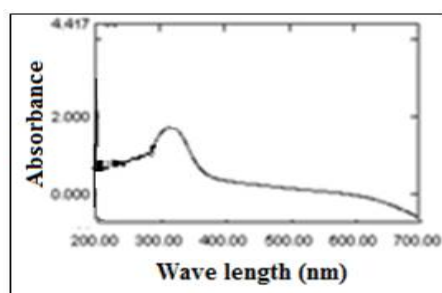


Figure 2 UV-Visible spectrum of synthesized copper nanoparticles using *T. rotundifolia* plant extract

Fourier Transform Infrared Spectroscopy: The FTIR spectrum of synthesized copper nanoparticles using extract of *T. rotundifolia* is shown in Figure 3. The FTIR analysis is used to identify the capping, reducing and stabilizing capacity of the plant extract. Figure shows the peak at 3414 cm^{-1} corresponding to O-H stretching of phenolic group. The peak at 1628 cm^{-1} is due to the presence of C=O stretching of aldehydes (Rastogi *et al.*, 2011). The peak at 1382 cm^{-1} shows N=O bend of nitro groups. The peak at 1142 cm^{-1} is due to the presence of C-N stretching of amines and the peak range at 1097 cm^{-1} is due to C-O stretching of alcohols. The peak value at 983 cm^{-1} shows the presence of =C-H bend of alkenes. The peak at 617 cm^{-1} is due to C-H stretching of alkynes. The peak at 457 cm^{-1} reveals that copper binds to oxygen moiety indicated by M-O stretching of synthesized copper nanoparticles.

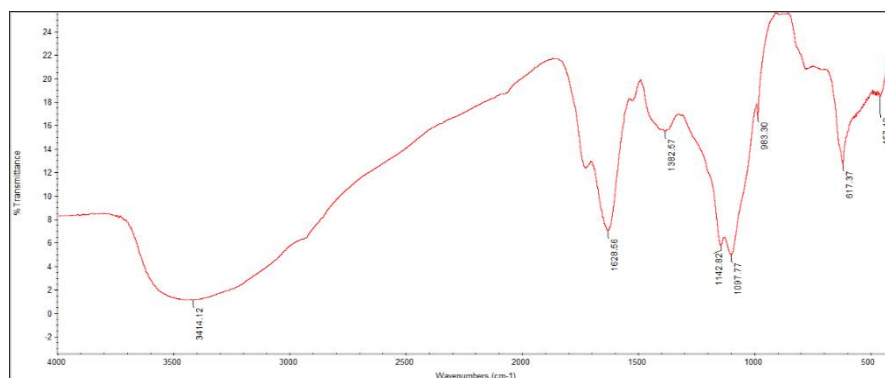


Figure 3 FTIR spectrum of synthesized copper nanoparticles using *T. rotundifolia* plant extract

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X-Ray Diffraction analysis: The particle size and nature of the synthesized copper nanoparticles are determined by using XRD analysis in the angle ranging from 20° to 70° . A thin film of the copper nanoparticle is made by dipping a glass plate in the solution and carried out the X-ray diffraction studies. The diffraction peaks at 43.9° , 50.5° , and 74.20° for synthesized copper nanoparticles using *T. rotundifolia* as shown in Figure 4. The number of Bragg reflections of the synthesized copper nanoparticles using the plant extract of *T. rotundifolia* corresponding to (111), (200) and (220) are the sets of lattice planes observed, which can be indexed to face-centred cubic copper. Theivasanthi *et al.* (2010) the peaks match with the Joint Committee of powder Diffraction Standards (File No.089-2838), which further proves the formation of crystals of copper nanoparticles. The crystalline size of copper nanoparticle synthesized using *T. rotundifolia* is calculated from the width of the XRD peaks and the average size of 12.46 nm is shown in Table 1.

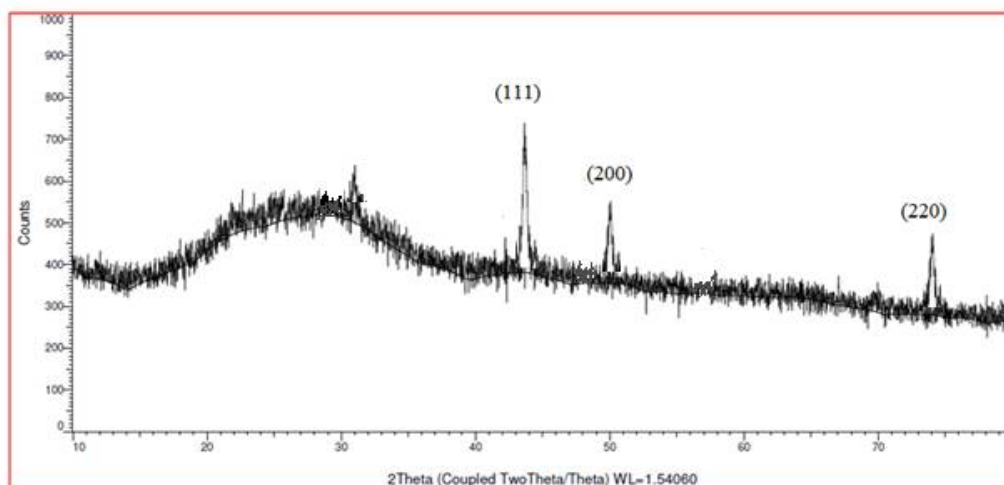


Figure 4 XRD Spectrum of synthesized copper nanoparticles using *T. rotundifolia* plant extract

Table 1 XRD parameters of synthesized copper nanoparticles using *T. rotundifolia* plant extract

$2\theta(^{\circ})$	FWHM(β)	Crystalline Size(nm)	Plane
43.9	1.2	7.4	111
50.5	1.0	9.18	200
74.2	0.5	20.81	220

Scanning Electron Microscopy: Scanning Electron Microscopy provides further insight into the shape and the size details of the copper nanoparticles synthesized using *T. rotundifolia*. The experimental results show that the prepared copper nanoparticles using the plant extract of *T. rotundifolia* is in the range 20-100 nm, and the shape is found like triangle as shown in Figure 5.

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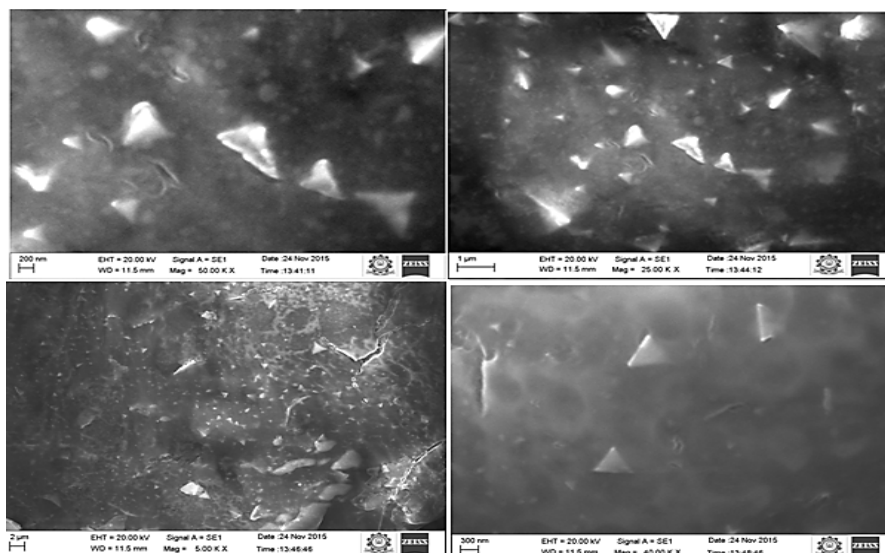


Figure 5 SEM photographs of synthesized copper nanoparticles using *T. rotundifolia* plant extract at different magnification level

Energy Dispersive X-Ray analysis: The elemental composition of the synthesized copper nanoparticles was probed by energy dispersive X-ray (EDAX) analysis. Figure 6 shows the EDAX pattern of copper nanoparticles prepared using copper sulphate and the plant extract *T. rotundifolia* which indicates the presence of Copper and small amount of oxygen. Elemental composition is mentioned in Table 2.

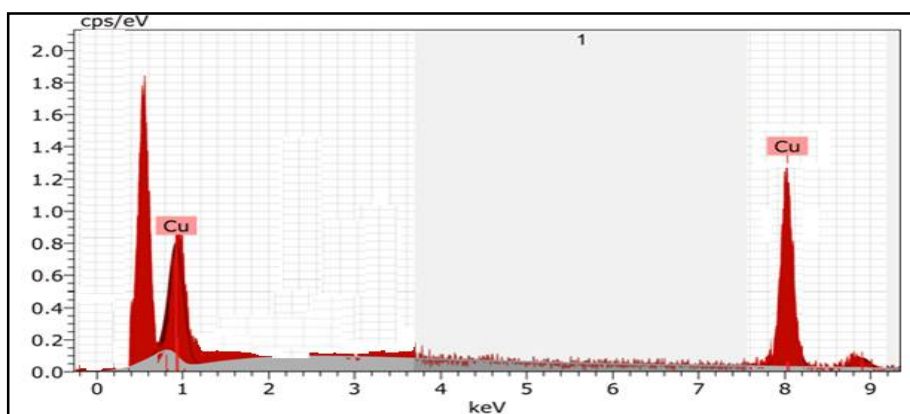


Figure 6EDAX Spectrum of synthesized copper nanoparticles using *T. rotundifolia* plant extract

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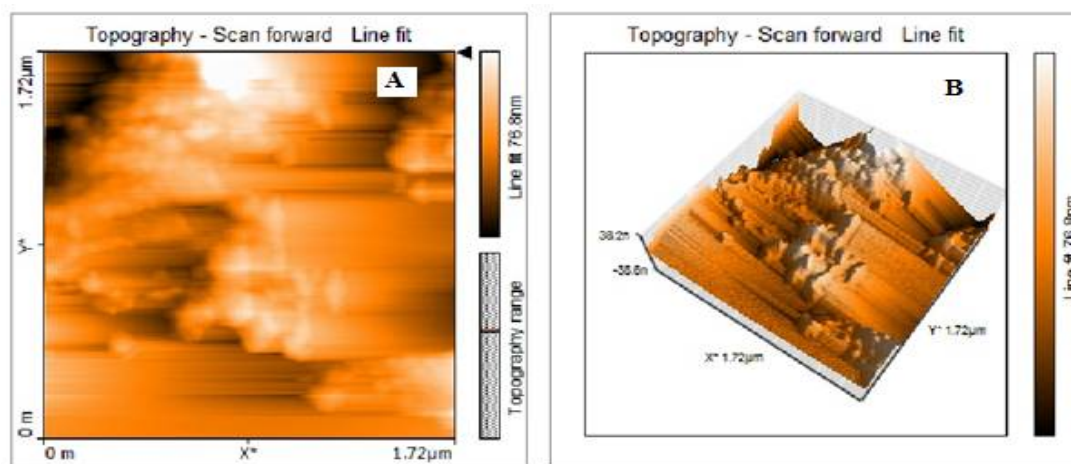
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Table 2 Elemental composition of synthesized copper nanoparticles using *T. rotundifolia* plant extract

Element	Unn.C [wt.%]	Norm.C [wt.%]	Atom.C [at.%]	Error (3 sigma) [wt.%]
Copper	64.02	70.02	37.51	6.42
Oxygen	21.58	13.35	60.06	2.93
Aluminium	7.42	16.63	2.43	1.00
Total	93.02	100	100	

Atomic Fluorescence Microscopy: The AFM images of the Copper nanoparticles in 2D and 3D forms and height distribution are shown in Figure 7. AFM is used to analyse the shape, size and height of the synthesized copper nanoparticles which are formed by irradiation which is coated on the glass plate. The size of the copper nanoparticles is measured in contact mode with silicon cantilevers. For imaging by AFM, the samples are suspended in acetone and spin coated on a silicon wafer. Most of the copper nanoparticles have an average diameter of 10-15 nm range with bar like shape, smooth surface and compacted structure. Scanned area range of synthesized copper nanoparticles is 0 to 1.72 μm . In 3D image, peak height of the synthesized copper nanoparticles is 62.42 nm. The nanoparticles are stable in air and water and did not convert into any other associated compounds. Hence it exists as highly dispersed form. The AFM image reveals high symmetric size of particles with less concentration of copper nanoparticles. Particle profiles show weak agglomeration of particles as a smooth line profile.



(A) Lateral micrographs showing uniformly distributed nanoparticles and some agglomeration (B) 3D image of copper nanoparticle.

Figure 7 AFM image of copper nanoparticles using *T. rotundifolia* plant extract

Cyclic Voltammetry: The cyclic voltammogram of synthesized copper nanoparticle using *T. rotundifolia* is run between the potential range of -0.8 and 1.2V at the scan rate 0.05 V/S for the electrochemical behaviour of copper nanoparticles on Glassy Carbon Electrode. The cyclic voltammogram of copper nanoparticles obtained from *T. rotundifolia* in a solution of pH 7 is shown in Figure 8, which indicates that the cathodic peak at -0.13V. Copper is oxidized to Cu^+ , and Cu^+ is oxidized to Cu^{2+} at 0.4V. The cathodic peaks readily shift towards the negative potential direction which is represented in anodic peaks at -0.5V. It shows that there is oxidation and

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reduction, and the reaction is found to be reversible. Thus, synthesized copper nanoparticles can be used as a redox catalyst.

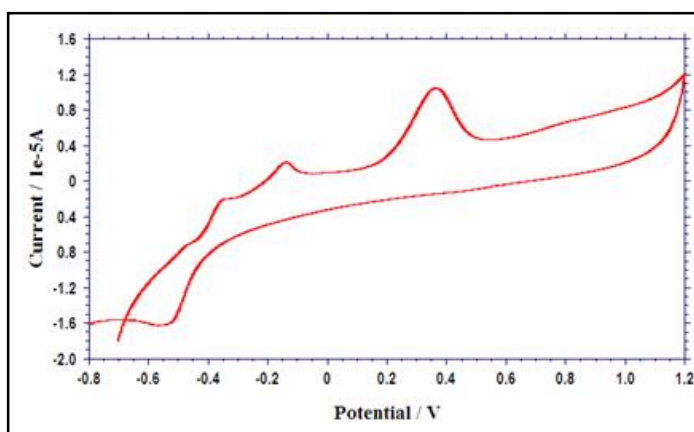


Figure 8 Cyclic Voltammogram of copper nanoparticles synthesized using *T. rotundifolia* plant extract

In vitro Anti-inflammatory activity: Anti-inflammatory study of synthesized copper nanoparticles is evaluated by Human Red Blood Cell membrane stabilization method as shown in Figure 9. The medicinal use of *T. rotundifolia* has a good anti-inflammatory activity. As the concentration of the synthesized sample increases, the percentage of inhibition also increases. Synthesized copper nanoparticles using *T. rotundifolia* show moderate anti-inflammatory activity in a concentration-dependent manner. The observed values are noted in table 3. The results are compared with standard Diclofenac. Synthesized copper nanoparticles using the plant extract of *T. rotundifolia* show 57.42% of inhibition in the HRBC membrane stabilization assay.

Table 3 HRBC membrane stabilization activity of synthesized copper nanoparticles using *T. rotundifolia*

Concentration (µg/mL)	% of Inhibition HRBC Membrane Stabilization Mean±S.E.M
31.25	31.16±1.50
62.5	36.31±1.57
125	41.31±1.50
250	47.80±1.82
500	54.17±0.11
1000	57.42±0.29

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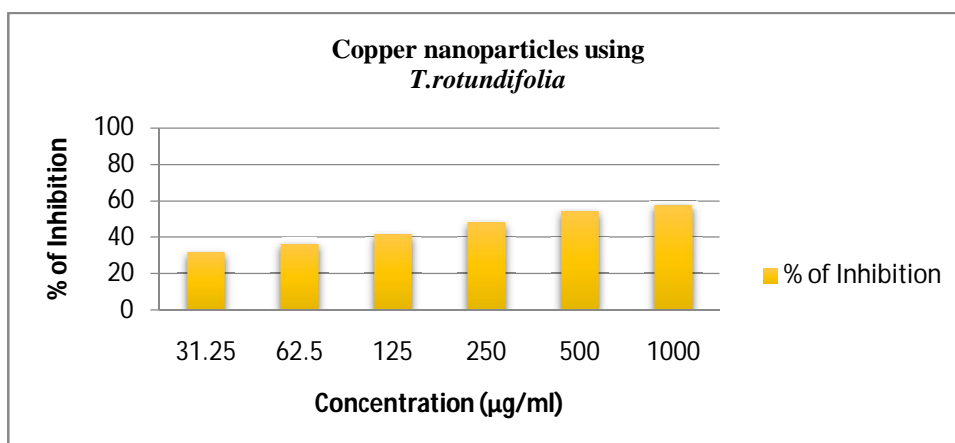


Figure 9 Anti-inflammatory activity of synthesized copper nanoparticles using *T. rotundifolia*

Antibacterial activity: The antibacterial activity of synthesized copper nanoparticles using the extracts of *T. rotundifolia* is determined by agar well diffusion method. Antibacterial activity is carried out on different pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Salmonella typhi*. The zones of inhibition values are noted in Table 4

Table 4. The zones of inhibition values of synthesized copper nanoparticles

Bacterial strains	Zone of inhibition in diameter (mm)			
	Standard Ofloxin (1mg)	Concentration µg/mL		
		5µL	10µL	15µL
<i>Staphylococcus aureus</i>	12	R	8	12
<i>Escherichia coli</i>	16	10	18	22
<i>Pseudomonas aeruginosa</i>	14	10	12	16
<i>Klebsiella pneumoniae</i>	12	12	16	20
<i>Salmonella typhi</i>	16	16	20	22

The synthesized copper nanoparticles using the extract of *T. rotundifolia* showed clear zone of inhibition against different bacterial strains as shown in Figure 10. It is reported that synthesized copper nanoparticles attached to the surface of the cell membrane, disturb its function and penetrate directly with the bacterial outer membrane and release metal ions. Ofloxin 1 mg/mL is used as positive control. If the concentration of the synthesized copper nanoparticles increases, the zone of inhibition values also increases. Synthesized copper nanoparticles show better antibacterial activity against the tested bacterial strains at higher concentration than the standard. The high bactericidal activity of synthesized copper nanoparticles is due to their extremely large surface area, which provides better contact with microorganisms.

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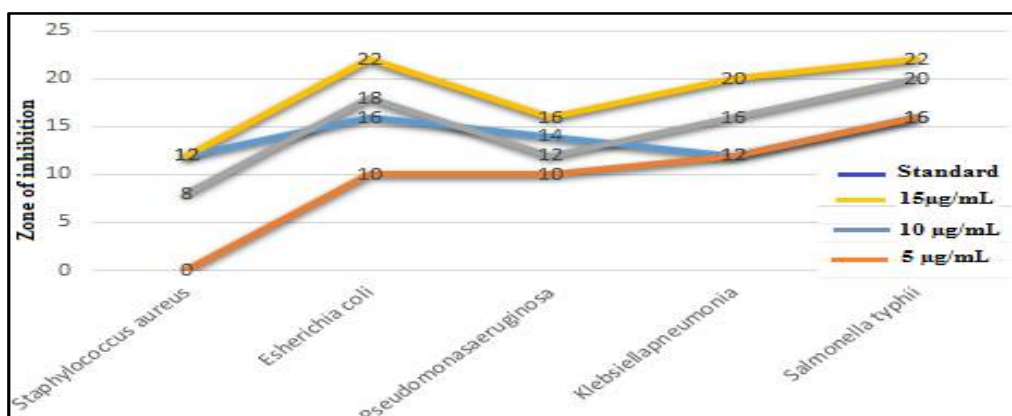


Figure 10 Antibacterial activity of synthesized copper nanoparticles using *T. rotundifolia* plant extract.

Antifungal activity: The antifungal activity of synthesized copper nanoparticles is found among the four tested different fungal strains *Candida albicans*, *Candida tropicalis*, *Aspergillus fumigatus*, *Aspergillus niger* shown in Figure 11. Ketoconazole is used as a standard fungicide. Synthesized copper nanoparticles shows moderate growth against the four different fungal strains. The Zone of inhibition values are noted in Table 5.

Table 5 Zone of inhibition values of synthesized copper nanoparticles against four different fungal strains

Fungal strains	Zone of inhibition in diameter (mm)			
	Standard Ketoconazole (10µg/disc)	Concentration µg/mL		
		5	10	15
<i>Candida albicans</i>	16	8	10	12
<i>Candida tropicalis</i>	14	R	R	5
<i>Aspergillus fumigatus</i>	12	6	8	10
<i>Aspergillus niger</i>	14	R	R	R

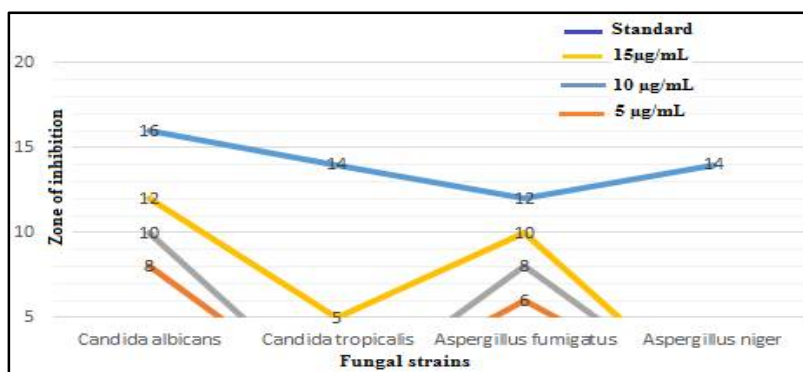


Figure 11 Antifungal activity of synthesized copper nanoparticles using *T. rotundifolia* plant extract

IV. CONCLUSION

In the present work, we have developed a fast, eco-friendly and convenient method for the synthesis of copper nanoparticles using *T. rotundifolia* plant extract by green chemistry method. No chemical reagent or surfactant template

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was required in this method, which consequently enable the bioprocess with the advantage of being environmental friendly. In future enormous number of nanoparticles using various plant extracts should be synthesized and its various medicinal values should be analysed to provide low cost natural products for various ailments. This method shall be valuable in environmental, biotechnological, pharmaceutical and medical applications.

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