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# Phytosynthesis of Copper Nanoparticles Using Wild *Ceropegia juncea* and its Therapeutic Activities

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## A B S T R A C T

In this study, we have fabricated copper nanoparticles (CuNPs) from the plant of aqueous extracts of wild (WCJ) plants of *Ceropegia juncea* an endangered medicinal plant as bioreducing agents. The aim of present study was to investigate the antimicrobial, antiangiogenesis and anticancer activities of WCJ copper nanoparticles. Crystalline CuNPs of WCJ synthesis was confirmed by different physicochemical analytical techniques such as UV–Visible Spectroscopy (UV-Vis); Fourier Transform Infrared Spectroscopy (FTIR); X-ray diffraction analysis (XRD); Scanning Electron Microscopy (SEM); and Energy dispersive X ray analysis (EDX). The nanoparticles size was found to be ranging from 177.9, 246.3, 263.5, and 346.1 nm in WCJCuNPs the SEM. Further, the synthesized nanoparticles were tested their antibacterial activity. The maximum zone of inhibition 9.0±0.25 and 8.0±0.25 mm, and 5.0±0.10 and 5.0±0.25 mm was observed in *Salmonella paratypi* and *Escherichia coli* in WCJCuNPs. In antiangiogenic activity the percentage of inhibition in WCJCuNPs at the concentration of 500 µg/ml. The best cytotoxicity activity of WCJCuNPs against HT-29 cell was found in 250 µg/ml concentration with 22.84 percent of cell viability. Finally, our study confirmed the CuNPs synthesis from WCJ extract, which is environmentally friendly with antimicrobial, antiangiogenesis and anticancer activities.

***Key words*:** *Ceropegia juncea*, Antiangiogenesis, Copper nanoparticles, FTIR, SEM

Research on the development of new biosynthesis processes for producing different types of nanoparticles with the required shape and size has been a research area for scientists working in nanotechnology and biotechnology [1]. Nanomaterials are a new and exciting area of research in medicine and other biomedical applications, and the biosynthesis of metallic nanoparticles for other therapeutic purposes is a research area that is actively being explored [2]. In recent years due to their characteristics like chemical, physical, biological, electronic, electrical, mechanical, magnetic, thermal, dielectric, and optical properties. Recently, copper nanoparticles have been attracted particular attention for their availability, low cost, and novel optical, catalytic, mechanical, electrical, and thermal conduction properties. The usefulness of copper as antimicrobial agents has been known for a long time [3].

Nano science has been recognized as a remedy for environmental pollution [4]. It has been reported that nanoscience can be used for tumor therapy, cancer therapy, antioxidants, biosensors, drug delivery, catalytic activities, antibacterial, anti-inflammatory, and antifungal activities which can be beneficial to biomedical and other industries [5-6]. As a result of nanoparticles' unique properties, such as surface plasmon resonance (SPR), biological, optical, and electrical properties, they have been in high demand and have also attracted wide popularity among scientists and researchers Worldwide. Metal nanoparticles such as gold, nickel, silver, platinum, zinc and copper nanoparticles had been synthesized [8-13]. Easy production and flexible modification into desired shape and dimension of nano-sized are other exceptional quality of CuNPs [14]. The biological activities of CuNPs had rendered it a good source for the production of antibiotics [15]. Several methods have been adopted for the of synthesis of CuNPs, the physical and chemical methods that encompass the use of sonochemical preparation approach [16], chemical precipitation methods [17], laser ablation methods [18] solidstate reaction procedure [19] irradiation via gamma ray [20] and sol–gel methods [21]. However, the limitation of the aforementioned methods are; production of toxic by-products, high capital intensiveness, painstaking and hectic process [22]. Interestingly, biosynthesis of CuNPs from plants extract had been reported as a remedy to most of the limitations associated with the physical and chemical method of CuNPs production because eco-friendly reducing and stabilizing agents are been used. CuNPs produced via this method revealed good morphological properties and biological activities [23]. There have been several reports that plant extracts can be used to reduce and stabilize CuNPs, including *Asparagus adscendens* Roxb. root [24], *Rosa canina* fruit [25], *Punica granatum* peel

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[26], *Syzygium alternifolium* fruit [27], *Nerium oleander* leaf [28], and *Ziziphus zizyphus* leaf [29].

Due to their special properties, they have been used for several purposes. They can act as an antibiotic, as an antimicrobial and antifungal agent when added to plastics [30-31], as nanometal lubricant additives, used in medical device coatings, optical sensors, in the pharmaceutical industry, in diagnostics and orthopedics. According to research, copper is highly toxic to microorganisms like bacteria (*E. Coli* and *Staphylococcus aureus*) and nontoxic to animal cells, making it an effective bactericidal metal [31-33]. As embryogenesis and development progress, angiogenesis occurs as pre-existing vessels sprout into new ones. In contrast, pathological angiogenesis is observed in several diseases, including rheumatoid arthritis, atherosclerosis, diabetic retinopathy, psoriasis, and cancer [34].

The World Health Organization (WHO) report says that cancer is a leading reason for death in developing and developed countries [35]. In spite of tremendous progress in clinical oncology, the underlying pathological conditions remain a major concern to public health [35]. Several studies have shown that free radical accumulation, chronic inflammatory ailment and angiogenesis play important roles in primary tumor formation, tumor progression and metastasis [36-37]. Reactive oxygen species plays a major role in the maintenance of cell oxidative homeostasis, activation and regulation of cellular signalling pathways [38]. Chronic oxidative stress can induce carcinogenesis, neurodegenerative, diabetes, and cardiovascular diseases [39]. It is the main focus of oncology research to find novel plant-based anticancer drugs.

Considering the above aspects in this study we synthesized CuNPs using wild *C. juncea* plant extract as reducing and stabilizing agent and evaluated its antimicrobial, antiangiogenesis and anticancer activities.

## MATERIALS AND METHODS

*Plant collection and authentication*

The wild plants of *C. juncea* were collected from Coimbatore, Tamil Nadu, India and it was validated by Botanical Survey of India (BSI) Coimbatore, India. (BSI/SRC/5/23/2014-15/TECH-919).

*Plant extracts preparation*

100 g of wild *Ceropegia juncea* (WCJ) powder is added to 100 ml of deionized water in a 250 ml conical flask, and the mixture was heated on a magnetic heater that would repeat for 6–8 min at a temperature of 50 to 60° Celsius. We notice that the entire amount is dissolved for a period not exceeding 10 min. The last step is to filter the solution using Whatman filter paper; here the plant extract has been prepared and the solution was stored in refrigerator for nanoparticles biosynthesis.

*Biogenesis of copper nanoparticles*

WCJ copper nanoparticles were synthesized by mixing 100 ml of copper sulfate solution (5 mM) with 10 ml of aqueous plant extract [40]. By adding NaOH (1N) solution, the pH value of the mixture is adjusted to 7.0. Moreover, green color is obtained after centrifuging and pellets are collected. The pellets are dried overnight at 60°C in a hot air oven. The resulting powder has a dark green color and should be stored at room temperature for later use.

*Characterization of techniques*

The CuNPs was evaluated using by UV-Vis spectrophotometer (Shimadzu UV-1700) and spectra in the range 200-800 nm wavelength, Fourier-Transform Infrared Spectrophotometer (FT-IR Spectrophotometer) between 4000 and 400 $cm^{-1}$ and characterize the morphology and size of nanoparticles produced by X-ray diffraction (XRD), objects are inspected by electrons through a microscope that manipulates electrons (particles with a negative charge) instead of light by the technique Field Emission Scanning Electron Microscope (FE-SEM) and for analyzing the chemical structures of molecules using energy dispersive X-ray analysis (EDX).

*Bioactive studies of copper nanoparticles*

*Antimicrobial activity*

The modified agar well diffusion method was employed to determine the antimicrobial activities [41]. About 0.2 ml of the standardized 24-hour old culture of the tested organisms in nutrients broth was spread onto sterile prepared Muller Hinton Agar plates. Different types of the extracts chloroform, ethanol and methanol (30 µg/ml) were prepared and compared with tetracycline (30 µg /ml,) as a standard. With the aid of a sterile cork borer, wells of about 6 mm in diameter were bored onto the plates. About 0.5 ml of each concentration of the extracts was dispensed into the wells and then allowed to stand for about 15 minutes for pre-diffusion of the extracts to occur. These were then incubated at 37°C for 24 hours. At the end of the incubation period, inhibition zones formed on the agar were evaluated in millimeter (mm). The diameter of the zones of inhibition in the plates was measured by calculating the diameters of inhibition [42]. Four bacterial species viz., *Escherichia coli, Bacillus subtilis, Salmonella paratyphi* and *Klebsiella pnemoniae*, and two fungal pathogens like *Aspergillus fumigatus* and *Verticillum lecanii* were used for antimicrobial bioassay.

*In vitro anti-angiogenesis activity*

The chick chorioallantoic membrane (CAM) method was used for the *in vitro* anti-angiogenic activity of copper nanoparticles synthesized sample of WCJ [43]. The obtained fertilized eggs were incubated at 37°C with 60-70% humidity. At the $7^{th}$ day of incubation, a small hole was made in the shell, and 0.5-1 mL of albumin was syringed through an 18-gauge hypodermic needle. The sterile discs were soaked with different concentrations of CuNPs (62.5-1000 µg/mL) and implanted on the embryo blood vessels by using sterile forceps. Cellophane tape is used to close the window after the photograph is taken. On $8^{th}$ day after the treatment of CuNPs photograph was taken. For each experiment, six eggs were used. Calculating the inhibition percentage was done by using the formula:

% inhibition = [(vessel number of untreated CAM-vessel number of CAM treated with plant extract) / vessel number of untreated CAM] × 100.

*In vitro anticancer activity*

*MTT assay*

MTT assay was used to measure cytotoxicity. Cells reduce tetrazolium salt because of their ability to reduce it. The principle is the mitochondrial enzyme succinate dehydrogenase cleaved the tetrazolium salt and changed into blue coloured formazan [44].

For this assay 100 µL of various concentration of wild CuNPs sample were added per well and the plates were incubated for 48 hours at 37°C, 5% $CO_2$ environment. The medium with CuNPs extracts were removed after 48 hours and 20 µL of MTT (2 mg/mL) in MEM-PR was used for solubilization. The plates were kept for 3 hours 37°C in 5% $CO_2$ atmosphere. In order to solubilize the formazon formed during the formation process, 50 µL of isopropanol was added. By

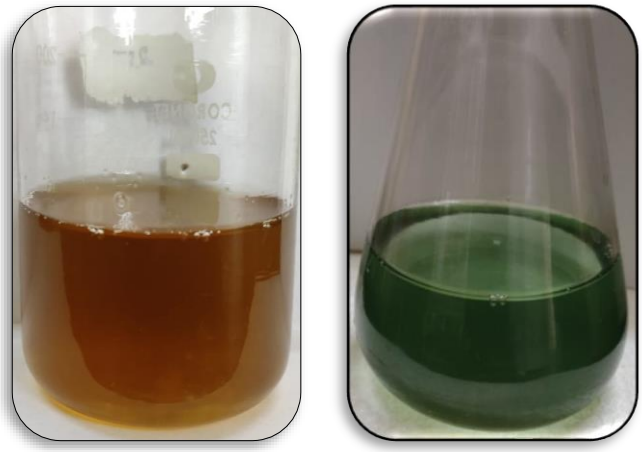
using microplate reader, the absorbance was measured at 540 nm wavelength. In living cells, cellular enzymes reduce the tetrazolium salt MTT to the colored formazon compound. The number of viable cells in the culture directly correlated with the amount of formazon dye formation, whose absorbance was measured spectrophotometrically. The formula for cell viability is:

$$\% \text{ Cell viability} = \frac{\text{Mean OD of treated cells}}{\text{Mean OD of untreated cells}} \times 100$$

## RESULTS AND DISCUSSION

*Synthesis of copper nanoparticles*

Using green route method, copper nanoparticles were made from an aqueous extract of WCJ. Copper nanoparticles are formed in the reaction medium when the colour changes before and after incubation. Copper nitrate ($CuNO_3$) and extract from WCJ used for synthesis of CuNPs. The change in colour of the mixture from yellowish brown to dark green colour in WCJ and light brown to light green colour was absorbed. The change in color caused by the surface plasma resonance (SPR) and the metal ions reduction by plant extracts [45]. The colour change was occurred after the solution was incubated for 12 hours at room temperature. The colour change was occurred after the solution was incubated for 12 h at room temperature (Fig 1). After the color formation, no significant change was observed. CuNPs exhibit characteristic surface plasmon resonance (SPR), which results in this changing process.



A - Aqueous extract of WCJ with $CuNO_3$ B - Aqueous extract of WCJ with $CuNO_3$ after 24 hours

Fig 1 Copper nanoparticles synthesis of WCJCuNPs

*Characterization of copper nanoparticles*

The characterization of CuNPs were carried out by UV-vis spectroscopy, Fourier Transform infrared spectroscopy (FTIR), X-ray diffraction analysis (XRD), Scanning Electron microscope (SEM) and Energy Dispersive X- ray analysis (EDX).

*UV- visible spectroscopy analysis (UV)*

UV-Vis spectroscopic measurements of copper nanoparticles extract from WCJCuNPs showed the characteristic surface plasmon resonance spectrum (SPR) with absorbance between 400 and 800 nm and peak maximum at 310 nm (Figure 2), caused by the formation of copper nanoparticles. Earlier report also analyzed the synthesized CuNPs using the *Ocimum sanctum* leaf extract [46] and *Citrus sinensis* juice extract [47]. The formation of the copper nanoparticles was considered successful by initial change in colour. CuNPs exhibited green colour in the solution due to excitation of surface plasmon vibration in CuNPs. Various particle properties, such as size, shape, and capping agents, can affect where the SPR band is located (Fig 2).

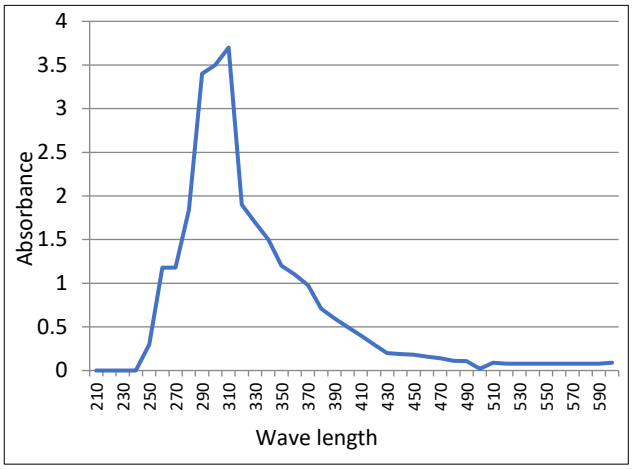



Fig 2 UV–vis absorption spectrum of WCJCuNPs

*FTIR analysis*

FTIR used to investigate the interaction between copper and phytochemicals of WCJ. Using this method of characterization, copper nanoparticles can be reduced, coated, and stabilized thanks to the bioactive compounds present in the reduction, coating, and stabilization processes. Characterization of FTIR is used to find the molecules and their functional group present in the extracts. The FTIR spectrum of WCJCuNPs extracts showed the bands at 3883.88, 3836.60, 3749.61, 3665.06, 3536.80, 3470.59, 3375.03, 3294.13, 2695.40, 1632.36, 1402.13, 1107.42, 743.31, 675.96, 514.74 $cm^{-1}$ (Fig 3). Also reported in previous work where CuO nanoparticles were synthesized using different leaf extracts [48-50].

The FTIR spectra showed the presence of different functional groups like alcohol (O-H stretching), phenols (H bonded), aliphatic primary amine N-H stretching), aldehyde (C-H stretching) 1° amines (N-H band) aromatics (C-C stretch), halo compound (C-Cl stretching) and alkyl halides (C-Br stretch) in WCJCuNPs (Table 1). Copper nanoparticles are formed by using these functional groups. The spectrum obtained from the CuNPs was presented in the (Fig 3, Table 1).

Table 1 FTIR analysis of WCJCuNPs

| Origin | Peak value | Functional group |
|---|---|---|
| O-H stretching | 3883.88 | Alcohol |
| O-H stretching | 3836.60 | Alcohol |
| N-H stretching | 3749.61 | Amides |
| O-H stretching | 3665.06 | Alcohol |
| O-H stretching | 3536.80 | Alcohol |
| H bonded | 3470.59 | Phenols |
| N-H stretching | 3375.03 | Aliphatic primary amine |
| H bonded | 3294.13 | Phenols |
| C-H stretching | 2695.40 | Aldehyde |
| N–H bend | 1632.36 | 1° amines |
| C–C stretch | 1402.13 | Aromatics |
| C–N stretch | 1107.42 | aliphatic amines |
| C-Cl stretching | 743.31 | Halo compound |
| C-Br stretching | 675.96 | Halo compound |
| C–Br stretch | 514.74 | Alkyl halides |

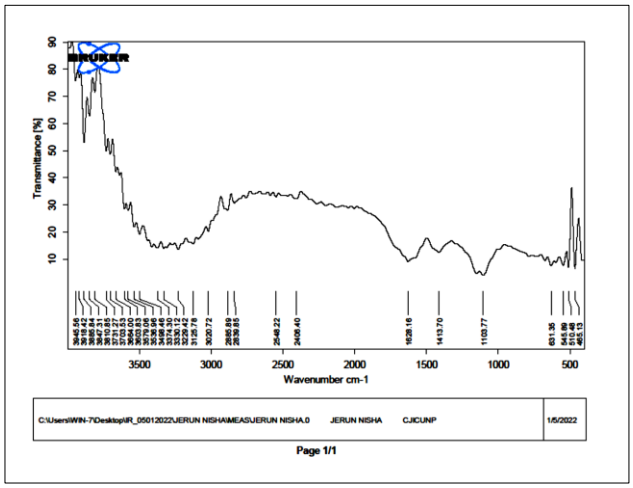



Fig 3 FTIR analysis of WCJCuNPs

*XRD analysis*

The XRD diffraction pattern of WCJCuNPs is presented in (Fig 4). The peaks observed in the pattern at 2θ values = 11°, 21°, 65° and 73.61° corresponds to (100), (110), (200) and (622) lattice planes of face centered cubic structure of CuNPs. This is similar to the synthesis of CuNPs using leaves from *Eclipta prostrate* as the natural reagent [3]. Crystallinity can be determined from the XRD spectrum of nanoparticles. The copper nanoparticles synthesized using WCJCuNPs was confirmed by X-ray diffraction analysis (Fig 4).

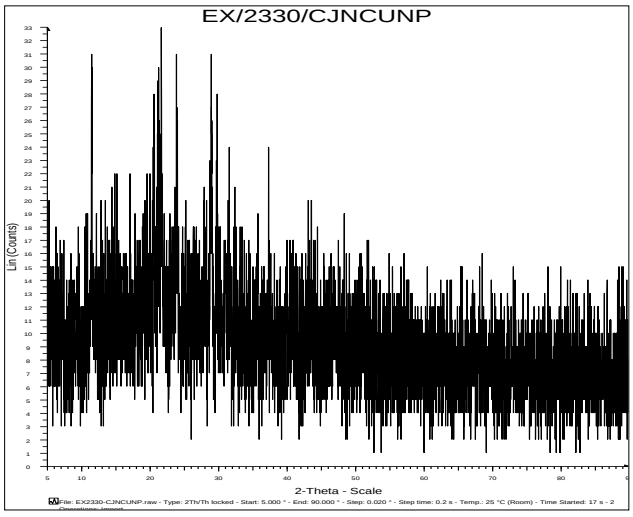



Fig 4 XRD analysis of WCJCuNPS

*SEM analysis*

Scanning Electron Microscopy (SEM) was used to analyze the surface morphology and size of the nanoparticles. The (Fig 5) shows the CuNPs synthesized by the plant extract of WCJCuNPs. SEM images were taken under normal atmospheric conditions in order to visualize the texture and diameter of CuNPs. The SEM images visibly displayed that the CuNPs were in a spherical form with a smooth surface (Fig 5). The average size of CuNPs was determined from WCJ SEM is 177.9, 246.3, 263.5, and 346.1 nm. A similar finding on *Punica granatum* extract with an average diameter of 12.5 mm has been observed from the SEM image that CuO nanoparticles were relatively spherical and in a well-defined shape and size [51-52]. The effect of viscous properties of plant extracts is clearly visible on SEM micrographs when the nanoparticles clump together.

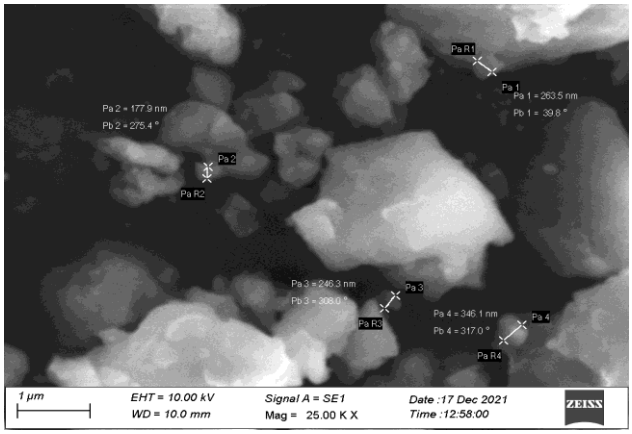



Fig 5 SEM analysis of WCJCuNPs

*EDX analysis*

The energy dispersive X-ray (EDX) analysis confirmed the chemical composition of the synthesized CuO nanoparticles (Fig 6). The EDX analysis of copper nanoparticles synthesized by green method exhibit strong signal from Cu element and weak signals from Cl, C, K, O, Mo and Ca elements in WCJCuNPs. This indicates that the synthesized copper nanoparticles are pure and contains no impurities. The EDX analysis confirmed the presence of copper nanoparticles and mostly showed strong signal energy peaks for copper particles in the range 1-8 keV (Fig 6). Same result was observed in *Punica granatum* extract the copper peak at 1 keV and 8 keV revealed the purity of synthesized CuO nanoparticles [51-52].

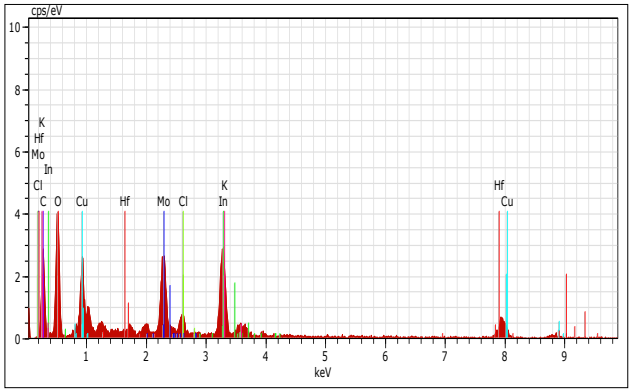



Fig 6 EDX analysis of WCJCuNPs

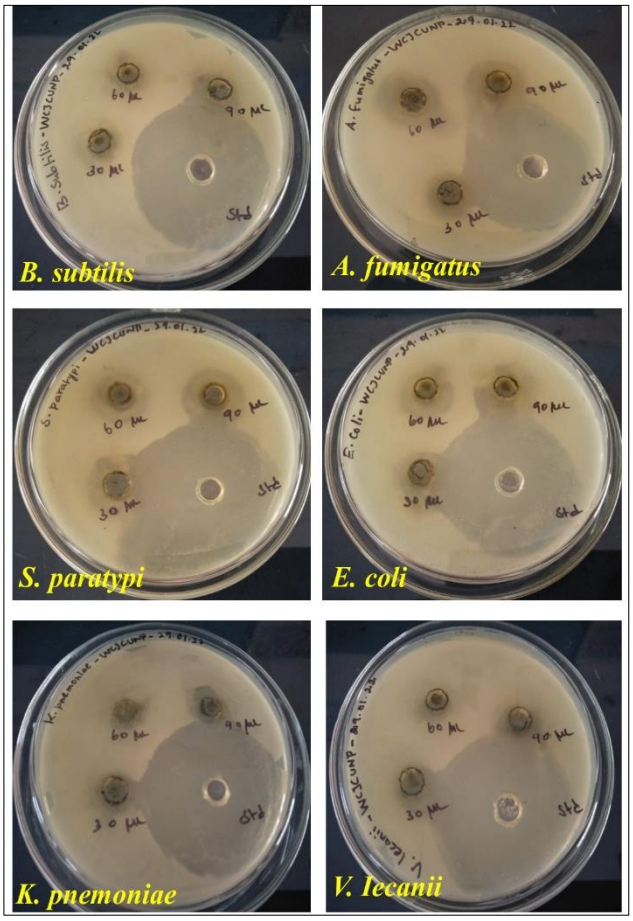
*Antimicrobial activity*

Agar well diffusion method was used to study the antibacterial effects of copper nanoparticles synthesized by biosynthesis against gram positive pathogenic bacteria isolated from clinical samples. The six human pathogenic bacteria species had been used in this assay. Nanoparticles get attached to the surface of bacterial cell membrane that are made up of peptidoglycan and slowly kills bacteria. The maximum zones of inhibition 9.0±0.25 and 8.0±0.25 mm was exhibited by *Salmonella paratypi* and *Escherichia coli* in WCJ CuNPs at the concentration of 90 µL respectively (Table 2). Earlier reports are available for the mechanisms behind antimicrobial activities. In the present case, *E. coli* shows a significant zone of inhibition by CuNPs and releases Cu ions from nanoparticles that attach to the negatively charged bacterial cell wall and rupture it, thereby leading to protein denaturation and cause cell death [53-55]. Synthesized copper nanoparticles from the *Terminalia arjuna* bark aqueous extract, which showed that the nanoparticles had very good antimicrobial activity against *S. aureus*, *E. coli*, *S. typhi*, and *P. aeruginosa* [56].

The second highest zone of inhibition was observed *Salmonella paratyphi* (09 mm) in sample of WCJCuNPs at 60 µL concentration. In bacterial test lowest inhibition area was noticed in 30 µL concentrations (Table 2, Fig 7).

Table 2 Antimicrobial activity of WCJCuNPs

| Organisms | Zone of inhibition (mm) | | | |
|---|---|---|---|---|
| | WCJCUNPs | | | |
| | 90 µl | 60 µl | 30 µl | Chloromphenical |
| *Bacillus subtilis* | 5.1±0.26 | 2.03±0.20 | 1.0±0.20 | 25.1±0.30 |
| *Salmonella paratypi* | 9.0±0.25 | 7.00±0.26 | 5.0±0.20 | 26.0±0.15 |
| *Klebsiellapnemoniae* | 5.1±0.30 | 3.0±0.15 | 1.0±0.25 | 23.0±0.25 |
| *Aspergillusfumigatus* | 7.1±0.36 | 5.0±0.20 | 4.0±0.23 | 20.0±0.20 |
| *Escherichia coli* | 8.0±0.25 | 6.0±0.20 | 5.0±0.20 | 22.0±0.25 |
| *Verticillumlecanii* | 6.1±0.26 | 4.0±0.20 | 2.0±0.25 | 23.1±0.17 |

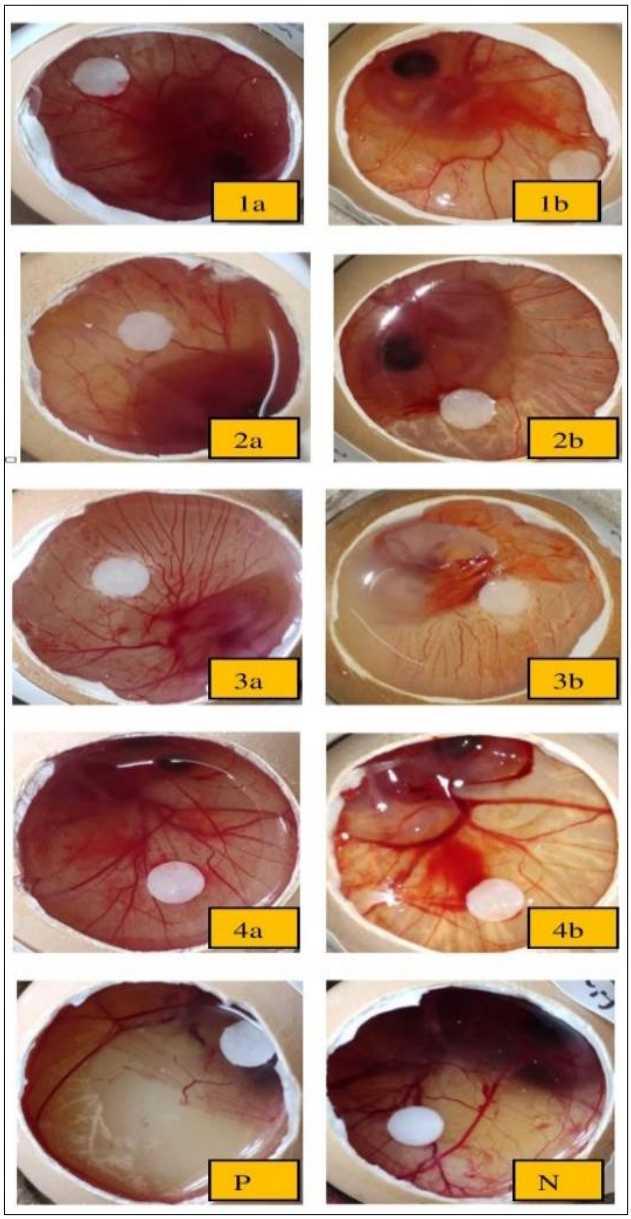



Bacteria: A - *Bacillus subtilis*, C- *Salmonella paratyphi,* D- *Escherichia coli*, E- *Klebsiella pnemoniae*
Fungi: B - *Aspergillus fumigates,* F- *Verticillum lecanii*

Fig 7 Antimicrobial activity of WCJCuNPs

*Anti-angiogenesis activity*

The efficacy of the WCJCuNPs against angiogenesis was evaluated by using *in vivo* CAM model. A measurement of the inhibition zone surrounding the applied disc was done on the eighth day after the treatment of the samples. As the CAM is not treated, it has a normal vascularization with primary, secondary and tertiary blood vessels. Whereas the treated CAM showed the contorted vascularization and disordered condition in the present vasculature (Fig 8). This is reported that the CAM model is a widely used *in vivo* model for determining the progress of angiogenesis [57]. Angiogenesis is a fundamental perspective needed for the tumor development in cancer disease [58]. Different concentration 1000, 500, 250, 125 and 61.5 µg/ml were used for this study (Table 3). The degree of inhibition was dose dependent, so as dose concentration increased, the degree of inhibition increased as well. The inhibition percentage is showed in the (Fig 8). The percentage of inhibition in WCJCuNPs was 71.42% at concentration of 500 µg/ml. The second-best inhibition was noticed at 1000 µg/ml with 69.23% in WCJCuNPs against control (85.7 and 11.1) NaOH and DMSO respectively.




1a, 2a, 3a and 4a – WCJCuNPs treatment without inhibition at 0 hr. 1b, 2b, 3b and 4b WCJCuNPs treatment with zone of inhibition after 24 hr. P and N is positive and negative control

Fig 8 Anti-angiogenesis activity of WCJCuNPs

Table 3 Anti-angiogenesis activity of WCJCuNPs

| Sample | Time | 1000 µg/ml | | 500 µg/ml | | 250 µg/ml | | 125 µg/ml | |
|---|---|---|---|---|---|---|---|---|---|
| | | No. of vessel | Percent of inhibition | No. of vessel | Percent of inhibition | No. of vessel | Percent of inhibition | No. of vessel | Percent of inhibition |
| WCJ CuNPs | 0 | 13 | 0 | 14 | 0 | 11 | 0 | 8 | 0 |
| | 24 | 4 | 69.23 | 4 | 71.42 | 5 | 54.55 | 5 | 37.5 |

*Cytotoxicity activity*

Cytotoxicity study of copper nanoparticles with wild *Ceropegia juncea* were tested against HT-29 cell line at various concentrations to decide the $IC_{50}$ values by MTT assay. After that of various concentrations of the sample including 250, 125, 62.5 and 31.25 µg/ml is prepared. MTT test of WCJCuNPs demonstrates huge impact on HT-29 in the concentrations between 250 µg/mL to 31.25 µg/mL (Table 4). Harne e*t al*. synthesized the copper nanoparticles from the aqueous extract of *Calotropis procera* L. latex using the MTT method. They found that these nanoparticles showed excellent anticancer activity [59]. Earlier research, Hashim *et al.* tested the anticancer activity of silver nanoparticles synthesized from *Vitis vinifera* fruit skin extract using the cell proliferation assay. The cytotoxicity results proved the inhibition activity of these nanoparticles against MCF-7 and HeLa cell lines [60].

Table 4 Cytotoxicity activity of WCJCuNPs

| Sample concentration (µg/ml) | Percentage of HT-29 cancer cell viability |
|---|---|
| | WCJCUNPs |
| 250 | 22.84 |
| 125 | 38.85 |
| 62.5 | 59.36 |
| 31.25 | 72.38 |

The best cytotoxicity activity of WCJCuNPs against HT-29 cell was found in 250 µg/ml concentration with 22.84 percent of cell viability. It was discovered that the level of cell passing to increment with expanding convergence of copper nanoparticles engineered tests, and $IC_{50}$ was 92.14 µg/ml (Table 4).

## CONCLUSION

The biogenic synthesis of nanoparticles has attracted a lot of attention in academia and industry due to its easy preparation, economy, and environmental friendliness. An extract of *Ceropegia juncea* was used in this study to prepare copper nanoparticles containing high concentrations of secondary metabolites such as terpenoids and flavonoids, as well as antimicrobial, antiangiogenesis, and cytotoxic properties. As well, the WCJCuNPs extract showed higher antimicrobial, antiangiogenesis, and cytotoxic activities than the crude extract. The plant-mediated synthesis of nanoparticles therefore offers the possibility of developing effective therapeutic agents against human diseases as well as drug delivery systems in the future.

*Conflicts of interests*

*The authors state no conflicts of interests*.

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