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ORIGINAL ARTICLE

Copper nanoparticles synthesized using Echinops sp. root extract for antimicrobial applications

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Abstract

Metallic nanoparticles synthesized using a green synthetic route has been found to be harmful to pathogens. An attempt was made to synthesize copper nanoparticles (*EcS*-Cu NPs) using the root extract of *Echinops sp.*, Ethiopian medicinal plant. The most advanced techniques were employed to characterize NPs. The presence of the highest absorbance at λ_{max}=454 nm confirms the formation of *EcS*-Cu NPs. The role of biomolecules as capping agents for EcS-Cu NPs was authenticated by FT-IR spectra. The presence of a single weak peak in the XRD pattern of NPs confirmed the amorphous nature of the NPs. The purity of the NPs was corroborated by the SEM-EDAX analysis. TEM-HRTEM-SAED analysis authenticated the presence of partially crystalline natured copper NPs with the appearance of weak concentric SAED rings. The *EcS*-Cu NPs showed significant synergistic antibacterial influence verses *S. aureus*, *E. coli*, *P. aeruginosa, and E. aerogenes.* The uppermost inhibition zone of 13 mm was inscribed against *S. aureus* bacteria. *EcS*-Cu NPs exhibited better antibacterial activities against gram positive and gram negative bacteria.

Keywords: *Amorphous; Biomolecules; Green Synthesis; Medicinal Plants: Pathogens.*

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INTRODUCTION

Materials chemists have been attempting to develop novel metal nanoparticles (NPs) with superior properties, better functionality, and lower cost than the existing ones. The synthesis of metal NPs is the dynamic area of scientific research and more significantly application research in the nanomaterials world. A large number of physical, chemical, mechanical, and biological methods have been developed to enhance the efficiency of nanomaterials displaying improved properties with the aim to have a better control over the particle size, distribution. This biogenic reduction of metal ion to base metal is quite rapid, readily conducted at room temperature and pressure, and easily scaled up. Synthesis mediated by plant extracts is environmentally benign.

The research on the fabrication of plant-mediated Cu NPs for antimicrobial applications has gained significance in recent years. Green Cu NPs have been used for photocatalytic, electrocatalytic, and industrial dye degradation, nano medicinal, environmental, and catalytic applications for many decades. These copper nanomaterials in the form of nanoparticles, nanocrystals, nanorods, nanotubes, and nanosheets exhibit versatile properties. The application of various medicinal plant parts as traditional medicine for varieties of ailments of man has been very common among many nations of the world since for centuries [1-5]. In recent days, little work has been executed, especially with the utility of medicinal plant extracts to reduce and cap copper ions towards the synthesis of Copper NPs [6-7] for the biomedical, photocatalytic, electrochemical sensor, and antibacterial applications [8-13].

In recent years, numerous Ethiopian medicinal plants have been validated in a scientific empirical framework through phytochemical

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analysis and subsequent bioassays. It is understood that 25% of modern medicine enter the market utilizing either directly or indirectly traditional medicinal plant parts. However, as many as 60% of these local plants have been used in the manufacturing of pharmaceuticals, the medicinal plants species were used to treat the number of diseases [14]. *Echinops Sp.,* a 2nd ranked medicinal vegetation in Ethiopia, is valued primarily for its root parts and its medicinal uses documented in the ancient medico-religious pharmacopeia [15]. The roots of *Echinops Sp.,* have been used in the preparation of medicines against migraine, mental illness, heart pain, leprosy, kidney disease, malaria, and syphilis. It grows up to a height of 1.2 M with a leafy stem. To explore the influence of biomolecules of medicinal plant on the eco-friendly synthesis of Cu NPs, *Echinops Sp.* plant has been chosen for our work. Cu NPs were found to exhibit inhibitory activity against many microorganisms and bacterial strains [16]. A very few medicinal plants such as *Barleria prionitis* [17], *Dioscorea bulbifera* [18], *Caesalpinia bonducella* [19]*, Hagenia abyssinica* [20], and *Jatropha curcas* [21] have been used to synthesize silver [22], gold, copper, and their oxides [11-12] in the recent past for antimicrobial applications [25].

Biologically synthesized Cu NPs by using *M. quadrifolia rhizome* extract showed significant antibacterial activity against Streptococcus faecali bacteria with a 15 mm zone of inhibition [26]. Comparatively higher zone of inhibitions of 19 mm and 18 mm were recorded against *E. coli* was observed for doxycycline-capped Cu NPs and *Zingiber officinale* mediated Cu NPs, respectively [27]. In another work, researchers concluded that Cu NPs synthesized from *Cestrum nocturnum* leaf extract were more effective as an antibacterial agent when compared with Ag NPs [28]. Many more plants such as *Eucalyptus camaldulensis, Azadirachta indica, Murraya koenigii, Avicennia marina, Rosa rubiginosa* and *Datura stramonium* were also utilized for the production of copper nanoparticles by other researchers [29]. It was found that the antibacterial effect of each Cu NPs was observed to follow the order *A. indica* > *D. stramonium* > *M. koenigii* > *R. rubiginosa* > *A. marina* > *E. camaldulensis.*

However, not much significant work has been executed, especially with the use of medicinal plant root extracts to reduce and cap copper ions for the synthesis of Cu NPs in Ethiopia for antibacterial application. The present work therefore aims an eco-friendly green synthesis of Cu NPs using medicinal plant *Echinops Sp.* plant root extract at low temperature to investigate the synergistic influence of phytochemicals around the Cu NPs on bacterial strains. The synthesized Cu NPs have been characterized by using many advanced techniques and investigated for antibacterial properties.

MATERIALS AND METHODS

Chemicals and reagents

The chemical compounds and reagents, $Cu(NO_3)_2$, C_2H_5OH, Mueller-Hinton Agar solution, 0.5 McFarland standard, Chloramphenicol discs, Dimethyl sulfoxide, Indigo carmine and Malachite green dyes, used during the experiments were of analytical grade (procured from Merck company).

Collection and authentication of plant materials

Echinops sp. plant roots have been collected from the agricultural plots of Wondo Genet Agricultural Research Centre, Oromia, Ethiopia after accomplishing the sector inspection. The authentication of *Echinops Sp.* (Code EB005) plant roots was conducted at Herbarium, Addis Ababa University.

Preparation of aqueous plant root extract

The roots of *Echinops Sp.* were washed with tap water followed through distilled water and then shade dried for 2 weeks to cast off moisture contents from the roots. The procedure adopted is similar to the synthetic procedure as stated in our earlier work and thus not discussed in detail here [20]. The prepared *Echinops Sp. plant root (EcS-PR) extract* was stored at 4 °C for future experiments.

Green synthesis of EcS-Cu NPs

100 mL of *EcS-PR* extract was blended with $CuNO_3$ (400 mL of 0.5 M solution) in a 500 mL flask, which was incubated for 24 hrs at room temperature. The obtained brownish colored solution was centrifuged for 30 min at 8000 rpm to get black Cu NPs. These *EcS*-Cu NPs (Fig. 1) were washed, dried, ground and stored [30].

Characterization Techniques

The synthesized EcS-Cu NP was characterized for its structural features by an analytical X-ray diffractometer (X'pert pro MPD) equipped with a CuKα sealed tube (λ = 1.5406 Å). The sample was scanned over 2θ ranging from 20º to 80º

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Fig. 1. The scheme of synthesis of *EcS*-Cu NPs.

with a step size of 0.016º. Chemical bonding interactions were explored with the Shimadzu FTIR spectrophotometer (IR Affinity 1S). UV-Visible spectra were recorded in a range of 200–800 nm using Shimadzu's UV-2600 spectrophotometer to evaluate the optical properties. The morphology of the sample was examined by a Zeiss ultra plus FESEM instrument (tungsten filament is used in accelerating voltages up to 20 kV). Microstructural analysis of the samples was conducted using JEOL, JEM-2100 (accelerating voltage up to 200 kV, LaB_6 filament) EDS-1.5 Å TEM resolution [31]. The evaluation of interplanar spacings (IPS) of lattice fringes was carried out using Gatan Digital Micrograph (DM) Software [32].

Method of antimicrobial evaluation

All the antibacterial experiments were performed at Oromia Regional Laboratory, Adama, Ethiopia. The disk-diffusion agar method was used to evaluation the *invitro* antibacterial properties of *EcS*-Cu NPs verses *S. aureus, E. coli, P. aeruginosa* and *E. aerogenes*. The effectively growing bacterial cultures were dispersed on the Mueller-Hinton Agar (MHA) plate (turbidity was adjusted with Tryptone Soy Broth, TSB to match 0.5 McFarland standard). The extract of the NPs was prepared with four different concentrations in Dimethyl Sulfoxide. Four concentrations (6.25, 12.5, 25 and 50 µg/µl) of the synthesized NPs were added to the respective labelled wells.

The antibiotic disks with a diameter of 6 mm is applied to the Agar surface using forceps with gentle pressure and then impregnated with dissolved extract. The positive and negative controls taken were Chloramphenicol and DMSO, respectively. The plates were incubated in an ambient air incubator at 35 ±2 °C for 18-24 hours. The zone of inhibition was calibrated to the nearest millimeters (mm) using a ruler and recorded for all samples.

RESULTS AND DISCUSSION

Synthesis of EcS-Cu NPs

The synthesis of *EcS*-Cu NPs was accomplished by the application of *EcS-PR* extract to reduce and cap copper ions to copper. The alkaloids, tannins, flavonoids and terpenoids were identified in the *EcS-PR* extract during the phytochemical analysis. The list of phytochemicals present in the extract is as shown in Table 1.

The three steps involved in the origin of NPs

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Table 1. The details of phytoconstituents screening of *Echinops Sp.* plant root extract.

Sl. No.	Phytoconstituents	Test / Reagent	Result
1	Alkaloids	Wagner's reCuent	+
2	Tannins	KOH	+
3	Flavonoids	Shinoda Test	+
4	Terpenoids	Salkowski Test	+
5	Anthraquinone glycosides	BorntrCuer's Test	-
6	Cardiac glycosides	Keller-Kiliani Test	-
7	Saponins	Frothing Test	-

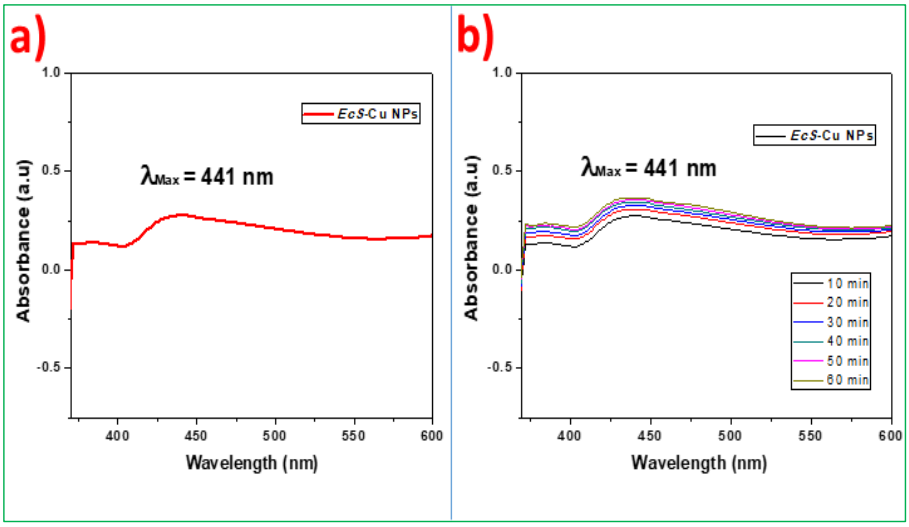

Fig. 2. The absorbance spectra recorded for *EcS*-Cu NPs a) immediately after the formation of NPs and b) at different time intervals varying between 10 min and 60 min.

includes: the reduction of metal ions, the formation of cluster, and the growth of nanoparticles. Essentially, the extract biomolecules act as antioxidants. In addition, *EcS-PL* extract enzymes also help to reduce copper ions and to form protein capped copper NPs [33]. It is also natural to know that phytochemicals act as organic ligand and assist in the reduction of copper ions to form copper NPs [34]. In addition, these compounds also influence the size of the nanoparticle as reported by the earlier researcher [35].

Characterization of EcS-Cu NPs

The *EcS*-Cu NPs is defined using UV-visible, UV-DRS, FT-IR, XRD, SEM, EDXA, TEM, HRTEM and SAED techniques.

The UV-visible absorbance spectra of the samples were recorded in the range of 200–800 nm using the Shimadzu's UV-2600, UV-visible spectrophotometer. The EcS-Cu-NPs is instantaneously synthesized by adding 10 mL of *EcS-PR* extract into 40 ml of 0.5M $CuNO_3$ solution in a 100 mL flask. No additional parameters such as pH and temperature were imposed, but the experiments were performed at room temperature. The UV-visible absorbance spectrum of the instantaneously synthesized *EcS*-Cu NPs revealed a λ_{max} of 441 nm as shown in Fig. 2a, just after 10 min of mixing plant extract with copper nitrate solution. The absorbance spectrum recorded after 20, 30, 40, 50 and 60 minutes of homogeneous mixture formation exhibited similar absorbance bands at λ_{max} of 441 nm (Fig. 2b) but with enhanced absorbance.

The enhanced absorbance in consecutive bands, clearly confirms the increased concentration of nanoparticles. As time progresses, the reduction of copper ions is followed by the nucleation of small cluster of copper atoms to form nanoparticles in the presence of plant extract biomolecules, likely though to have acted as a reducing agent and stabilizing agent. Almost the same results were observed during the analysis of the synthesized Cu NPs using the *Zingiber and Allium sp.* [27]

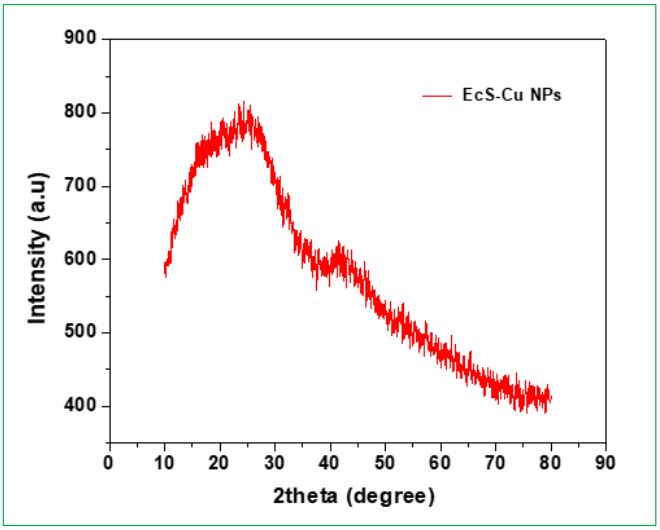

Fig. 3. The XRD pattern of *EcS*-Cu NPs.

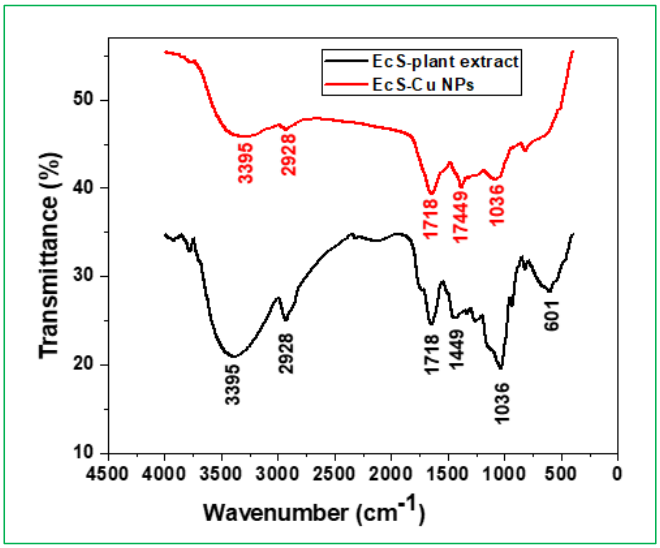

Fig. 4. The FTIR spectra of *EcS* plant extract and *EcS*-Cu NPs.

and *Psidium guajava* leaves extract [36]. Surface plasmon absorbance provides a range of different λ_{max} values for NPs that are synthesized using different plant extracts, likely due to morphological characteristics of the NPs.

The XRD analysis was executed to explore the in-depth details of the crystalline nature of the *EcS* -Cu NPs. The XRD spectrum of *EcS*-Cu NPs (Fig. 3) demonstrates a very weak broad band signifying the amorphous nature of the NPs. However, it can also be concluded that a small percentage of Cu NPs formed may have a crystalline nature otherwise the XRD pattern may not have contained a peak. It can be understood from the XRD results that the obtianed Cu NPs were mostly amorphous, suggesting inefficiency of the bioactive molecules to reduce and rapidly cap the copper ions. This is in consistent with the results published by the researcher in the recent past [26].

The FTIR spectroscopy was helpful in revealing the bonding features of both the *EcS-PL* extract and the *EcS*-Cu NPs. The intense peaks seen in Fig. 4, respectively at 3395 cm^{-1} corresponds to –OH stretching frequencies.

The peak at 1718 cm^{-1} arises due to the C=O vibration of the ketone groups. The small peak

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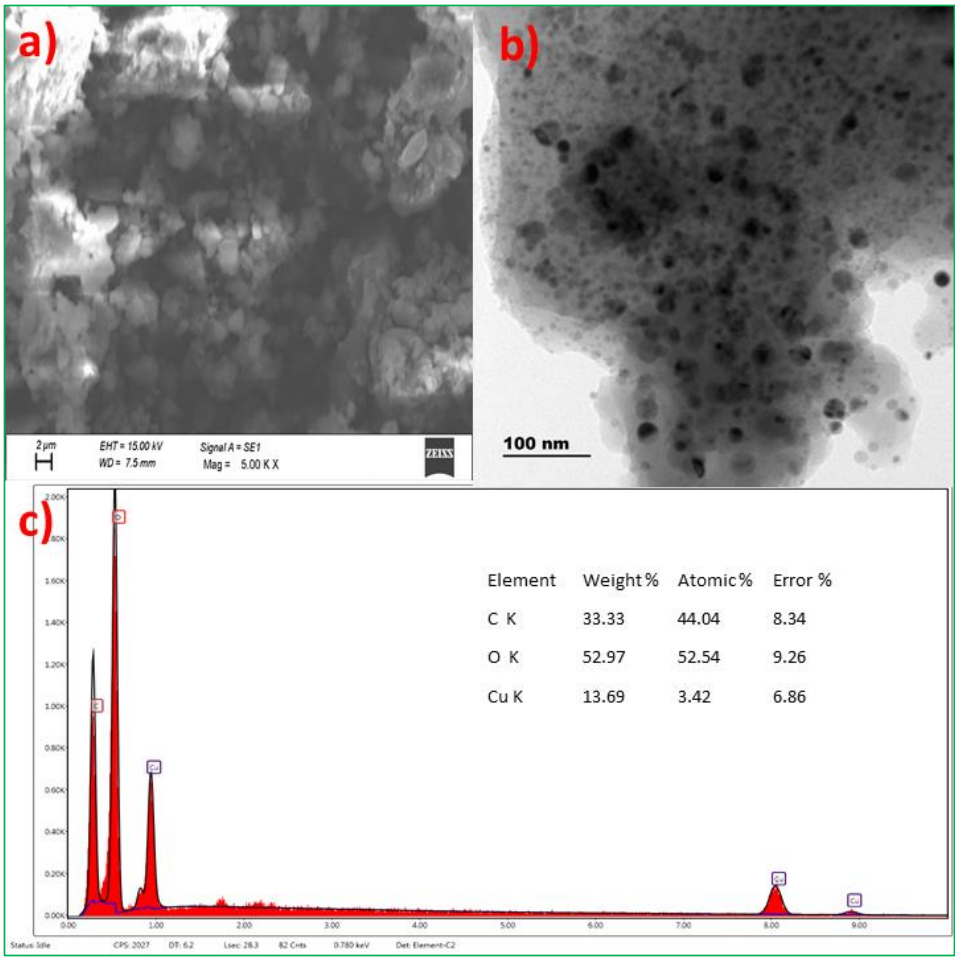

Fig. 5. (a) SEM micrograph and (b) TEM micrograph (c) EDAX spectrum of *EcS*-Cu NPs.

at 2925 cm^{-1} was correlated to the alkane C−H stretching mode. The vibration of the −COO carboxylic acid group was found to appear at 1449 cm^{-1}. A moderately intense band around 1035 cm^{-1} confirms the stretching of the C-O-C bond. The presence of prominent peaks at 3395 cm^{-1}, 1718 cm^{-1}, 1449 cm^{-1}, 1035 cm^{-1} and 601 cm^{-1} in the FTIR spectra of both plant root extract and NPs, clearly indicate the presence of bioactive molecules around NPs [37]. These bioactive molecules have been confirmed to have played a significant role in the nucleation and growth of *EcS*-Cu NPs. The bending vibrations of the Cu–O–H bonds resulted in a small peak at 601 cm^{-1}, probably due to the Cu–O bond. FTIR spectral inspection confirmed the presence of phytochemicals (phenolics, tannins, glycosides and proteins) in *EcS-PR* extract and their roles as reducing agent and stabilizing agent during the synthesis of *EcS-Cu* NPs. Especially the phenolic were reported to be good candidates for binding to copper NPs [38].

The electron microscopy was applied to analyze the morphological characteristics of the *EcS*-Cu NPs. The SEM micrographs of NPs are presented as Fig. 5 (a and b) representing nanoparticles in agglomerated form [39].

The presence of more agglomerated and amorphous Cu NPs with a high surface area confirms the effective reduction of copper ions to copper atoms. However, the existence of partially crystalline natured amorphous Cu NPs clearly confirms the lack of efficiency of biomolecules of plant extract.

The EDAX analysis revealed the elemental composition of the *EcS*-Cu NPs as depicted in Fig. 5c. The elements, Cu, C and O were identified in the spectrum. This confirms the possible capping of biomolecules over the Cu NPs.

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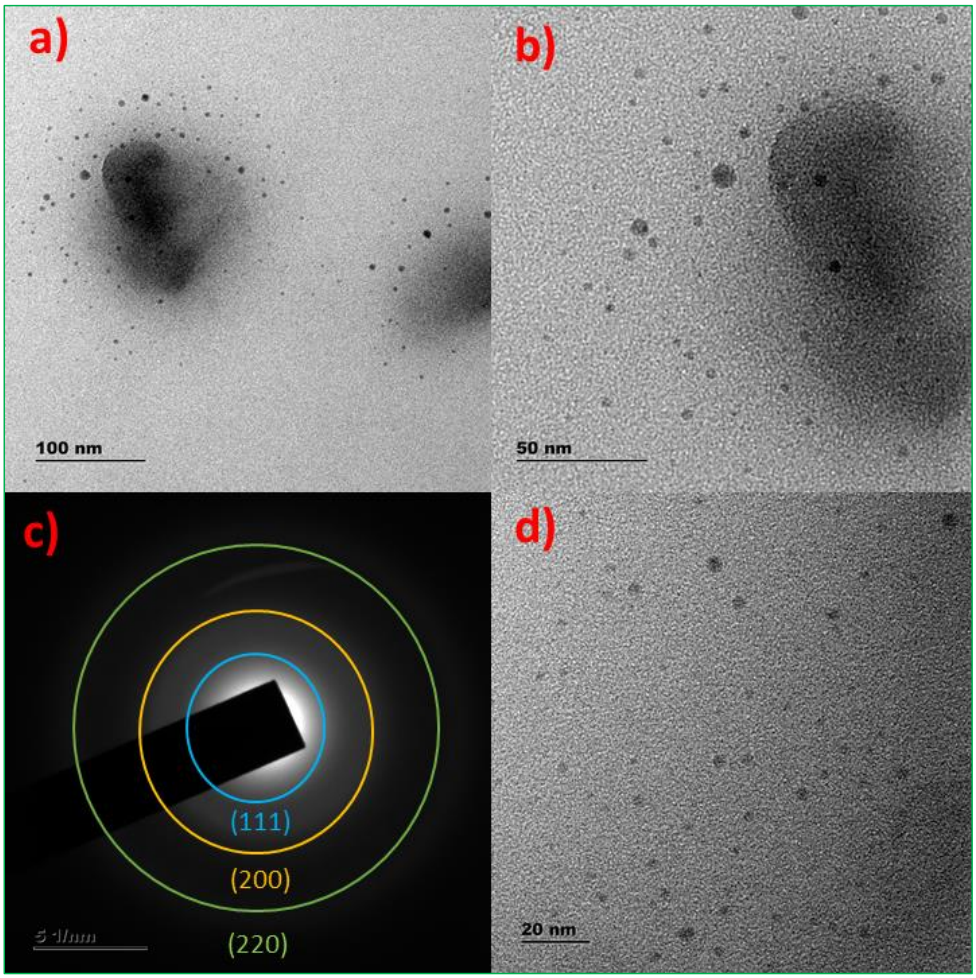

Fig. 6. The TEM images of *EcS*-Cu NPs with nearly spherical shapes at various magnifications a) 100 nm, b) 50 nm d) 20 nm along with SAED pattern (c)).

In order to discover the deep insights on the morphological features of the *EcS*-Cu NPs, TEM-HRTEM-SAED technical micrographs and patterns had been well utilized. The HRTEM images (at different magnifications) as shown in Fig. 6 (a, b and d) affirms that the synthesized *EcS*-Cu NPs were mostly spherical but also irregular geometries.

These TEM images clearly demonstrate the absence of crystalline NPs as there are only spherical particles spread over the wide range of area of examination [40]. It is worth noting that the particle size and shape control still remains a great challenge as since external constraints were imposed during the experiment.

A few brightly dispersed concentric circles appeared in the SAED pattern (Fig. 6c) of the Cu NPs. The most prominent 3 concentric circles represent (111), (200), and (220) planes of crystalline face centered cubic Cu. Although the percentage of crystallinity was found to be less, the SAED rings exhibited obscured concentric patterns for the copper crystal planes [41].

This SAED analysis using HRTEM images is in compliance with the previously discussed XRD results for the *EcS*-Cu NPs [42]. It can be concluded that both the XRD and SAED ring analysis confirms the partially crystalline nature of the amorphous Cu NPs.

Antimicrobial activity

The *EcS*-Cu NPs demonstrated superior antibacterial activities verses all the tested pathogens; *S. aureus, E. coli, P. aeruginosa,* and *E. aerogenes.* The present work evaluated synergistic influence of biomolecules with NPs against 4 pathogens. The inhibition zone of (ZoI)

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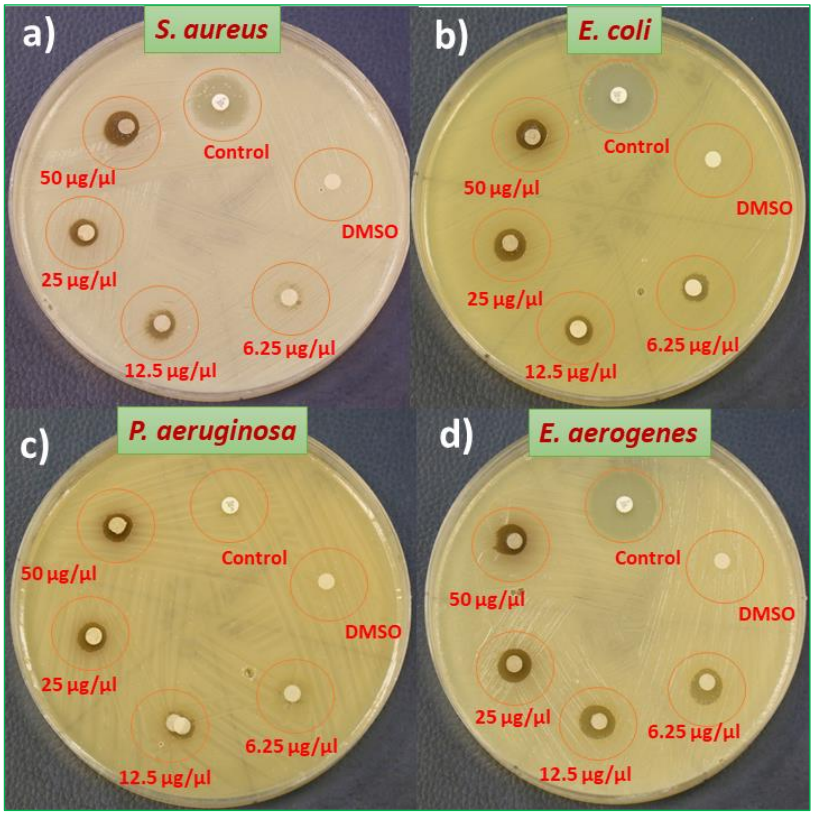

Fig. 7. The antibacterial activity of *EcS*-Cu NPs verses bacteria, *S. aureus* (b) *E. coli* (c) *P. aeruginosa* (d) *E. aerogenes.*

Table 2. The zone of inhibitions for various bacteria by *EcS*-Cu NPs.

Concentration of NPs (µg/µl)	Bacterial strains and Zone of Inhibition in mm			
	S. aureus **ATCC25923**	***E. coli*** **ATCC25992**	***P. aeruginosa*** **ATCC27853**	***E. Aerogenes*** **ATCC13048**
50	13	12	10	12
25	10	12	10	12
12.5	10	10	10	12
6.25	10	10	6	12
DMSO	20	22	6	28
Chloramphenicol	6	6	6	6

for Chloramphenicol, DMSO and NPs at four concentrations (6.25, 12.5, 25 and 50 µg/µl) is shown in Fig. 7a, 7b, 7c and 7d for *S. aureus, E. coli, P. aeruginosa,* and *E. aerogenes* bacterial strains, respectively. *EcS*-Cu NPs were found to show better antimicrobial activity against both Gram negative bacterial strains and Gram positive bacterial strains with slight lower inhibition zone only for *P. aeruginosa* bacterial strains.

The antimicrobial activity of NPs was highly appreciable against *S. aureus* with ZoI of 13 mm. The maximum ZoI inscribed by *EcS*-Cu NPs against *E. coli, P. Aeruginosa* and *E. aerogenes* bacteria are 12, 10 and 12 mm, respectively (Table 2). In addition, the lowest inhibition region was exhibited by *P. aeruginosa* bacteria which remained at 6 mm with the most diluted solution of NPs. In case of *E. coli,* and *S. aureus*, the ZoI increased from 10 to 13 and 10 to 12 mm on moving from 12.5 to 50 µg/µl concentration, respectively. But *P. aeruginosa* and *E. aerogenes* bacterial strains exhibited almost constant ZoI for concentrations of NPs beyond 12.5 µg/µl. This behaviour is basically due to the structural variations between the two

Table 3. The zone of inhibitions for Cu NPs synthesized by using various plant extracts.

Sl. No.	Plant extract	NSs	Zone of Inhibition (mm)	Tested Pathogens	Reference
1	*Syzygium aromaticum bud*	Cu	7	*E. coli*	[44]
2	*Brassica oleracea var. italic*	CuO	9	*C.albicans*	[45]
3	*Leucaena leucocephala L.*	CuO	11	*P. aeruginosa*	[46]
4	*S. lavandulifolia flower*	Cu/Cu_2O	12	*P. aeruginosa*	[34]
5	*Echinops Sp. root*	Cu	13	*E. coli*	Present work
6	Green and black tea leaves	Cu	14	*S. aureus*	[47]
8	*Hagenia abyssinica (Brace) JF. Gmel.* leaves	$Cu/Cu_2O/CuO$	14.7	*S. aureus*	[20]

types of bacteria, as well as differences in the morphological features of Cu NPs [43].

The antimicrobial resistance recorded for *EcS*-Cu NPs, was found to be superior when compared with few of the earlier results (Table 3) presented by many researchers even though few other workers reported higher inhibitions that could be attributed to higher NP concentrations or higher concentrations of bioactive compounds. The highest inhibition zone (mm) recorded with Cu NPs against bacteria was 13 mm. It can be concluded that the accumulated effect of Cu NPs associated with phytochemicals (alkaloids, tannins, flavonoids and terpenoids) of *EcS-PL* extract has been shown to be toxic for bacterial strains.

While several antimicrobial mechanisms were proposed by earlier researchers, the role of *EcS*-Cu NPs on the bacteria is unknown and yet to be exploited completely. Nanoparticles were found to cause the death of bacteria adopting direct or indirect ways by attacking their cell wall.

It is assumed that the positive copper ions in the NPs get adsorbed directly on to the cell wall of bacteria interacting with negatively charged species. This results in cell wall disruption and damage occurred by entering into the cell through the production of reactive oxygen species (ROS) caused by visible/UV light radiation. It is also assumed that the cumulative effect of *EcS*-Cu NPs and bioactive compounds of *EcS-PL* extract displayed magnificent influence on bacteria as suggested by the recent researcher.

The mechanism of action of NPs is fundamentally different since there are significant structural differences between Gram-negative and Gram-positive bacteria. In addition, electrochemical charge variations across the cell membrane influence interaction with the released Cu^{2+} ion by *EcS*-Cu NPs, which deteriorates the structural stability of the membrane. The maximum antibacterial activity is reported for Gram positive bacteria relative to Gram negative bacteria due to their differences in cell structure and the potentiality of nanoparticles.

CONCLUSION

The application of medicinal plant, *Echinops sp*. root extract towards green copper synthesis (*EcS*-Cu) nanoparticles has been found to be fruitful. The UV-visible spectra, XRD pattern and FTIR spectra substantiated the formation crystalline *EcS*-Cu NPs in the presence of biomolecules (alkaloids, tannins, flavonoids and terpenoids) of *EcS-PL* extract. The absorbance maxima, λ_{max} of 454 nm was confirmed by the formation of EcS-Cu NPs. SEM-TEM-HRTEM-SAED analysis corroborated the existence of partial crystallinity of mostly amorphous copper by the observation of Cu (111), (200) and (220) lattice fringes. The FT-IR peaks at 3395, 1718, 1449, 1035 and 601 cm^{-1} substantiated the effective surface capping of *EcS*-Cu NPs. The synergistic influence of bioactive compounds and *EcS*-Cu NPs proved to exhibit a highly effective antibacterial mechanism against pathogens, *S. aureus,* E. *coli, P. aeruginosa, and E. aerogenes* with 13 mm as a highest zone of inhibition. It can be concluded that the cumulative effect of plant and copper NP biomolecules has the ability to kill the disease-causing bacterial strains.

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CONFLICT OF INTEREST

No potential conflict of interest was reported by the author

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