

Biogenesis of Copper Nanoparticles using aqueous extract of Terminalia Chebula fruit and the Study of Antioxidant activity against DPPH.

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ABSTRACT

In the present study, phyto-fabricated Copper nanoparticles (CuNPs) were prepared from aqueous fruit extract of *Emblica officinalis* by a facile, economic, and eco-friendly approach. The facile phyto fabrication of EO-CuNPs was confirmed by UV-visible spectroscopy with characteristic peak around λ_{max} of 560-570nm, FTIR spectroscopic analysis and Zetapotential analysis confirmed that EO-Cu NPs were synthesized by phyto constituents of *Emblica officinalis* aqueous fruit extract, negative charge (-29.1 mV) revealed the formation stable EO-Cu NPs. The XRD pattern showed that the synthesized EO-Cu NPs were crystalline with an FCC structure. The SEM images showed that EO-Cu NPs were irregularly shaped. The TEM analysis determined that Cu NPs have an average diameter of 11-44 nm and are spherical in shape. The Cu NPs has offered fascinating antioxidant property of EC 50% as 78.73 μ g/ml, as highest activity as 68.36 at 100 μ g/ml with dose-dependent activity and photo-catalytic activity against DPPH, with in 26 mins DPPH was completely reduced.

Keywords: *Emblica Officinalis* (EO), Metabolites, Biosynthesis, Copper Nanoparticles, Antioxidant

INTRODUCTION

Currently, Nanotechnology has captivated the interest of researchers from various fields including Material Science, Medicinal Science, Life science, Biotechnology, Chemistry, Physics, Pharmaceuticals, medical, and various industries, and it has become an inevitable and prospective area of the scientific and technological development for forthcoming days (Krishna *et al.*, 2020)

The term nanotechnology, a catchphrase of the present-day Science and Technology to owe its fascinating properties. The concept of nanotechnology was first presented by Richard Feynman through his famous lecture, entitled "There's a plenty of room at the bottom" at the American Institute of Technology.

The nano word originated from the Greek word 'nanos' literally meaning 'dwarf', which refers to things of one billionth of a meter (1nm=10⁻⁹m) (Sharma *et al.* 2019). Nanotechnology represents the design, production, and application of materials at atomic, molecular, and macromolecular scales to produce new nanosized material (Rajan, 2004). The subject of nanotechnology deals with manufacturing, study, and manipulation of matter at the nanoscale in the size range of 1-100 nm which may be called nanoparticles (Usha *et al.*, 2017). Indeed, the properties of NPs, which may considerably differ from those observed for bulk material, are higher specific surface area, specific optical properties, catalytic property, lower melting points, specific magnetizations, mechanical strength, and numerous industrial applications (Kulkarni and Muddapur, 2014).

Metal nanoparticles (NPS) is one of the emerging fields in nanoscience and nanotechnology. The nanoparticle size and shapes strongly impact the physical, chemical, electrical, and optical properties of nanomaterials. Nowadays, Metal nanoparticles are used in a wide range of bio-medical, drug delivery, catalytic, agriculture, antioxidant, anticancer, antibacterial, antifungal, antibiofilm, entomological, and parasitological applications (Gopinath *et al.*, 2016). The copper nanoparticles are mostly found their applications in the field of medical, electronic devices, heat transfer systems, biosensors, pharmaceutical industries for drug-eluting, and reagents in various reactions, lubricants, Food package industry, antibiotic, antimicrobial agents, as catalysts in many reactions and many more (Khare and Verma (2014). The antibacterial activity, catalytic potency, magnetic and optical characteristics are higher for copper when compared to other metals like gold and silver (Parthasarathy *et al.*, 2014). The applications of copper nanoparticles are wide, as it is used in many fields like cosmetics for nano pigment purpose and pharmaceutical industries for drug-eluting (Raigond *et al.*, 2017). In food packages, to reinforce the antibacterial system, laser-generated copper nanoparticles were used (Ruparelia *et al.*, 2008)

Copper nanoparticles were synthesized in many ways such as the biological (green) method, chemical method, and physical method. Among biological method has so many advantages compared to other methods and one of the best methods because of its cost-effectiveness,

simplicity, use of low energy, use of less toxic materials, and eco-friendly (Kotval *et al.*, 2018). The green synthesis techniques to synthesize nano-particles are based on living entities and biological precursors such as plants, actinomycetes, fungus, bacteria, yeast, and viruses (Hulkoti and Taranath, 2014). The Biological entities offer a non-toxic, clean, and environment-friendly approach to reduce Metal ions into Metal NPs with a wide range of sizes, physicochemical properties, shapes, and compositions (Din *et al.*, 2017). Plants contain primary and secondary metabolites such as enzymes, proteins, saponins, tannins, flavonoids, alkaloids, steroids, and terpenoids which impart medicinal properties to them. These metabolites contain reactive groups such as amino (-NH₂), carboxylic (-COOH), hydroxylic (-OH), and amide (-CONH₂) which have high reducing capacities and hence can be used for the synthesis of metal nanoparticles (Balakrishnan *et al.*, 2019).

Emblica Officinalis is also known as *Phyllanthus Emblica*, which belongs to the family *Phyllanthaceae* and genus *Phyllanthus*, whose edible fruits are widely used in Indian Ayurvedic medicine. There are abundant benefits of *E. officinalis*, which include, antibacterial, antifungal, antioxidant, and cardioprotective activities. The *E. officinalis* fruits are a rich source of hydrolyzable phenolics, flavonoids, and tannins that play a key role in reducing and regulating the shape of nanoparticles (Gunti *et al.*, 2019).

The Copper nanoparticles were synthesized and stabilized by using different plants reported in the literature, such as *Solanum Lycopersicon* (Batool and Masood, 2017), Ginger (Subhankari *et al.*, 2013), Oleander leaves (Gopinath, 2014), *Coleus forskolin's* root extract (Baskaran, 2013), *Arevalanata* leaves extract (Seeram, 2016), Eucalyptus plant leaves extract (Kolekar, 2015), *Capparis zeylanica* leaves extract (Saranyaadevi, 2014), *Citrus medica* Linn (Shende *et al.*, 2015), Euphorbia equal leaves (Nasrollahzadeh *et al.*, 2014) and Cloves extract (Subhankari and Nayak, 2013).

MATERIALS AND METHODS

2.1. Material

Fresh *E. officinalis* fruit purchased from local market, Mahabubnagar, Telangana (state). Analytical grade copper sulphate was purchased.

2.2. Preparation of Aqueous extract of *E. officinalis*

Collected *E. officinalis* fruit first washed with tap water then ringed with double distilled water and slice into small pieces followed by dried at room temperature for 15-20 days. After drying it was powdered using grinder and stored in air tight bag for further purpose.

About 1 gm of finely divided powder transferred in to clean 250ml conical flask containing 100ml of double distilled water and stirred for 30 mints at 60 °C using magnetic stirrer. Then after the extract was cooled down at room temperature and filtered with Whatman no.1 filter paper. The filtrate obtained was stored at 4°C. Phytochemical screening determined the presence of secondary metabolites in aqueous extract in order to evaluate its suitability in biogenic production of nanoparticles (Gunti et al., 2019).

Table-1. Details of Phytochemical Screening of *E. officinalis* fruit aqueous extract.

Sl. No.	Phytochemical	Test Result
1	Carbohydrate	+
2	Poly Phenolic compound	+
3	Tannins	+
4	Flavonoids	+
5	Alkaloids	-
6	Anthocyanine	-
7	Terpenoids	-

2.3. Biosynthesis of Copper Nanoparticles:

The reaction mixture prepared by adding 20 ml of *E. officinalis* aqueous extract to 80 ml of 1mM CuSO₄ solution in a clean 250 ml Conical flask and the reaction mixture was heated on a Magnetic stirrer for 60 mints at 80 °C till the color of the reaction mixture turn in to dark brown from light brown. Then the reaction mixture was cool down to room temperature and centrifuged for 20 minutes at 4000rpms followed by discarding supernatant and collected residue. This residue was dispersed twice with double distilled water followed by drying in an oven at 80 °C for 1 hr. Resultant Cu NPs were collected and stored for the further purpose (Altemimi et al., 2019).

2.4. Characterization of Cu Nanoparticle

The Bio-synthesised Cu NPs was characterized by UV-Visible spectrophotometer (LAB INDIA UV-3000+) in the

wavelength range of 450-750 nm with a resolution of 1 nm After 10-fold diluting the sample with double distilled water against double distilled water as blank. The spectral data recorded were then plotted using Origin 2019b 32 bit. The Fourier transform infrared spectra were identified by an FT-IR spectrophotometer (TENSOR 27, Germany) using KBr. The sample powder was mixed with KBr and prepared pallet scanned at the range of 4000-450 cm⁻¹.

The XRD diffraction (XRD Diffract meter Bruker D8 Advance diffractometer with Cu K α radiation) analysis was used to obtain the crystalline structure and data in the 2 θ range of 10° to 90° after calibrated with Lanthanum hexaboride (LaB₆). The Scherrer equation was applied to calculate the average dimension (D) of crystalline domains and to confirm the formation of biosynthesized Cu nanoparticles.

The Debye Scherrer's formula, $D = k\lambda / \beta \cos \theta$,

Where, D = particle diameter size,

K = constant equals 0.94,

λ = wavelength of X-ray source(1.5406),

β = the full width at half maximum of the diffraction peak and

θ = the Bragg angle was used to calculate the crystallite size.

SEM / EDX

The copper nanoparticle morphology was determined by Field emission scanning electron microscopy. The analysis was performed using an electron microscope (Carl Zeiss model Ultra 55 microscope) to examine the surface morphology. Energy dispersive of X-ray (EDX) analysis was used to determine the composition and distribution of the constituting elements in the biosynthesized Cu NPs with Oxford Instruments X-MaxN SDD (50 mm²) system interfaced at 5 kV and INCA analysis software.

TEM

The structural characterization of biosynthesized Cu NPs determined by TEM. The TEM studies were performed on a JEOL JEM-2100F instrument equipped with a slow-scan CCD camera and an accelerating voltage of the electron beam of 200 kV. The preparation of the samples for TEM analysis involved sonication of each sample in ethanol for 2-5 min, followed by deposition of a drop on the copper TEM grid supporting a perforated carbon film and allowing it to dry.

2.5. Application

Determination of antioxidant activity DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay

Free radical scavenging activity of biosynthesized Cu NPs determined by DPPH assay. The reaction mixture of dilution series (10-100 mg/ml) taking different volumes of biosynthesized Cu NPs (10-100 μ l) incubated with DPPH (3ml; 0.15 mM) solutions in methanol. The solution was allowed to stand for 30 minutes at room temperature. The biosynthesized Cu NPs when reacted with DPPH, a stable purple-colored free radical will convert into colorless compound α - α diphenyl β -picryl hydrazine. The extent of discolorations indicates the amount of DPPH scavenged. The absorbance will be measured at 517nm. Ascorbic acid will be used as a reference antioxidant or standard (Altemimi et al., 2017).

The percent inhibition of DPPH will be calculated using the formula:

$$\text{The percent inhibition of DPPH} = \frac{[(C-T)/C] \times 100}{1}$$

Where C is the absorbance of control and T is the absorbance of the test sample.

RESULTS AND DISCUSSION

3.1. Visual Observation

After the addition of *E. officinalis* aqueous extract to light blue colored 3mM CuSO₄ solution immediately turns to the light brown color shown in Fig-2. During the heating of the reaction mixture, the solution color changed to dark brown which visually indicates the formation of Cu NPs (Kotval et al., 2018).

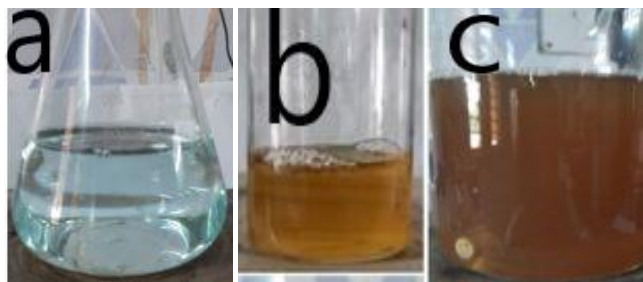


Fig. 2: a) CuSO₄ solution b) EO. Fruit aqueous extract c) after reaction

3.1 UV-Visible Spectroscopy

The UV-visible absorbance spectrum is typically used to confirm the formation of NPS. The Cu NPs exhibited a broad peak around λ_{max} of 560-570 as shown in Figure 2(a). This absorption band arises due to surface plasma resonance by the free electrons of Cu NPs. A similar result was reported for biosynthesized Cu NPs (Kotval et al., 2018). The surface plasmon absorbance depends on the size of NPs, and hence, every researcher reports a different λ_{max} value for NPs synthesized using different plant extracts (Murthy, et al., 2020).

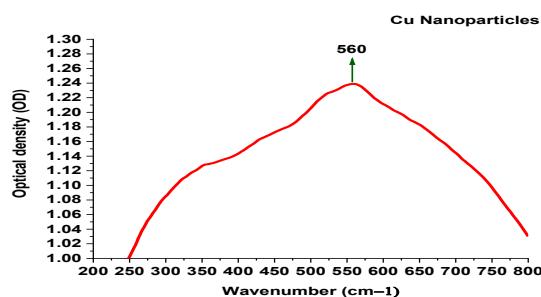


Fig. 3: UV-visible spectra of biosynthesized Cu NPs,

3.2. FTIR Analysis

FTIR measurements were taken for both the aqueous extract of *E. officinalis* fruit and the synthesized dried CuNPs to recognize the possible phytochemicals responsible for the bioreduction, and stabilization of the synthesized CuNPs. The FTIR spectra of the aqueous extract of *E. officinalis* fruit and the synthesized CuNPs are shown in Fig.4. The spectrum was recorded in the wavelength region between 400 and 4000 cm⁻¹.

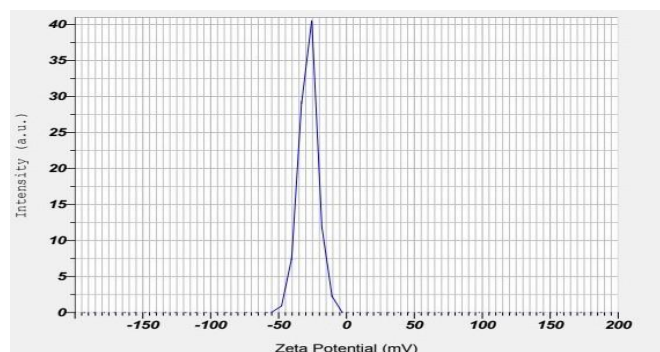


Fig. 4: Zetapotential record of biosynthesized Cu NPs

The spectrum of aqueous extract of *E. officinalis* fruit (Fig. 4) shows the peaks at wave numbers 3382, 2926 cm⁻¹, 12856 cm⁻¹, 1648 cm⁻¹, 1350 cm⁻¹, 1186

cm⁻¹, 1060 cm⁻¹ and 880 cm⁻¹. The peak at 3382 cm⁻¹ showed broad peak O-H stretching of phenolic compound. The peak at 1648 cm⁻¹ was due to the presence of C=O stretching of the Amide functional group. The other peaks obtained at 2926, 2856 cm⁻¹ are of -C-H stretching of -CH₂ & -CH₃ group, 1350 cm⁻¹ of -C-N stretching of Amide group, weak peaks appeared at 1186 cm⁻¹ due to C-O stretching, The bands at the 900–1100 cm⁻¹ region may also indicate C-O-C pyranose ring stretching, beta-glycosidic linkages, and glycosidic ethers bands of some polycarbohydrates, These main bands indicate the presence of Protein and Carbohydrates.

The FTIR spectrum of the CuNPs indicates the lowering of peak intensity for O-H stretch of phenolic compounds and C=O of amide groups, confirming the reduction of CuNPs, which have been possibly proceeded via these groups. This indicates that water-soluble compounds such as polyphenols and terpenoids are present in the extract are responsible for bioreduction of Cu ions to CuNPs and the presence of low intensity of these peaks illustrates that the flavonoids are one of the major reducing and capping agent (Fatma *et al.*, 2017).

3.3. Zetapotential

Zeta potential determines the surface charge indicating the stability of CuNPs. It was stated that the particles with zeta potential values greater than ±25 mV are considered more stable (Parthasarathy, 2020). The phytomolecules based stabilization of metallic NPs typically renders a negative charge on the surface of NP (Khan *et al.*, 2020). The mean zeta potential of CuNPs in the present study was -29.1 mV (Fig. 5). The Particles that have negative charges tend to have a strong force of repulsion between them. This indicates the properties of stability and quality.

3.4. X-Ray Diffraction

In the X-ray diffraction study, three peaks at 2θ values of 43.640, 50.800, and 74.420 deg corresponding to (111),

(200), and (220) planes of copper were observed (Fig-6). and compared with the standard powder diffraction card of JCPDS, copper file No. 04-0836. The obtained 2θ values of three peaks are in accordance with the standard card of JCPDS (Table -2) (Thirugnanasambandan and Alagar, 2010). The XRD study confirms that the resultant particles have an (FCC) structure. Apart from three peaks a very small peak with low intensity observed at 36.5451 indicates the formation of a minute amount of cubic Cuprite (I) oxide (Fatma *et al.*, 2017).

The mean crystalline size (D) of the Cu NPs was calculated by using the Debye Scherrer's formula,

$$D = k \lambda / \beta \cos \theta,$$

Where,

D = particle diameter size,

K = constant (equals 0.9 to 1),

λ = λ' is the wavelength of X-Ray (0.1541 nm),

β = the full width at half maximum of the diffraction peak and

θ = the Bragg angle.

The mean crystalline size (D) of the Cu NPs has been estimated at 18.133 nm (Table -3). Which was related to the results of the TEM results.

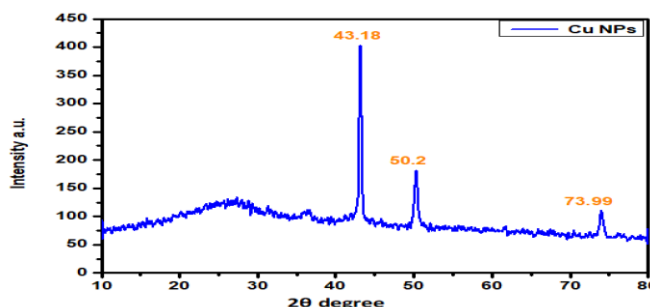


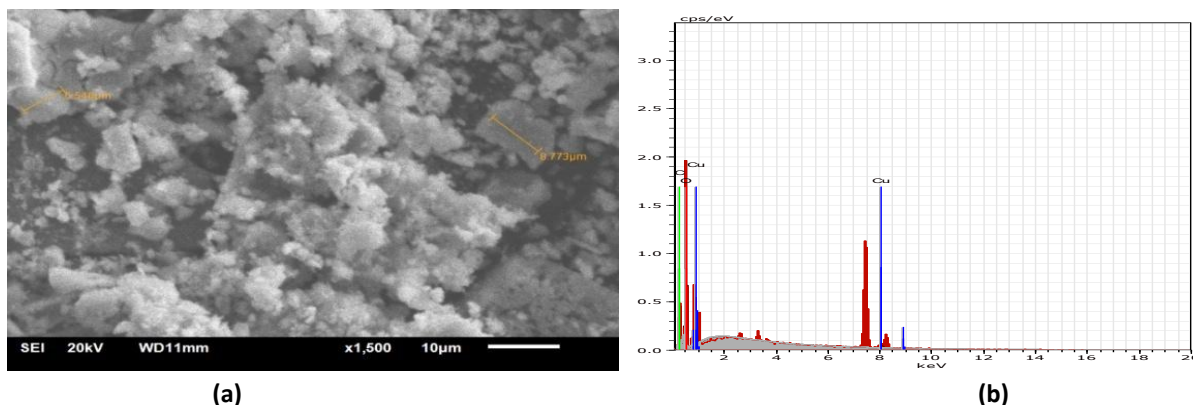
Fig-5: XRD Diffraction patterns of Cu NPs indicating the growth of particles in monoclinic Cristal structure.

Table-2- Compression of Experimental & Standard diffraction angle of Cu NPs

Experimental 2θ in degree	Diffraction angle	Standard Diffraction angle 2θ in degree JCPDS Copper card No : 04-0836
43.1886		43.297
50.2944		50.433
73.99		74.13

Table-3- Mean average size of Cu Nps

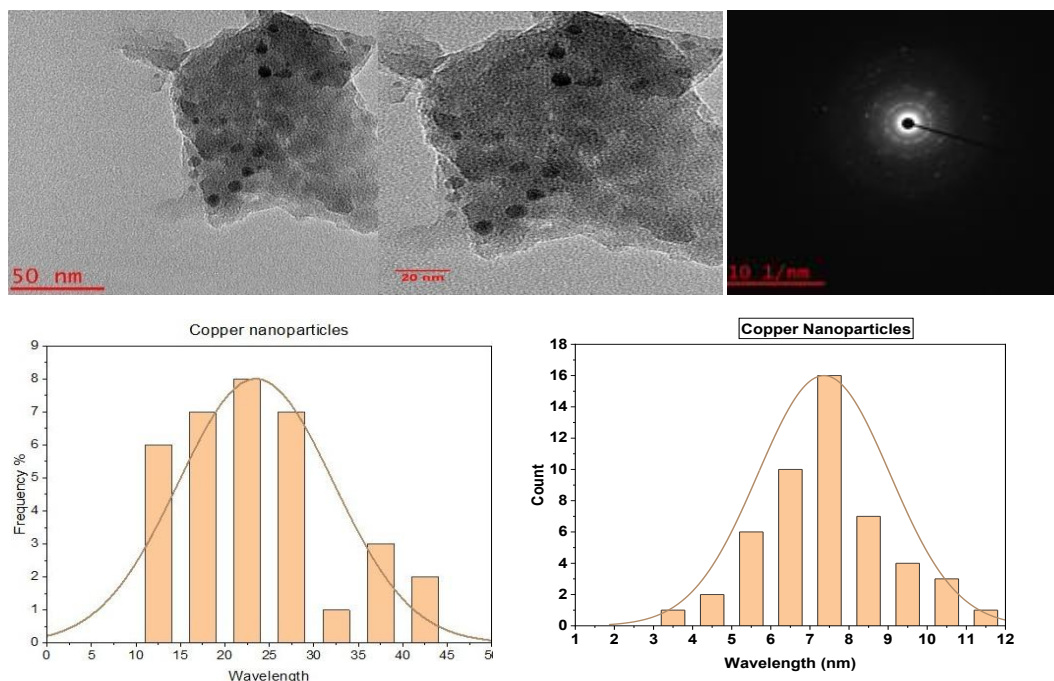
S.No	2 θ in degree	Intensity	FWHM	d- spacing ($\text{\AA}$)	Hkl plane	Particle size in nm
1	43.1886	100	0.9946	2.0982	111	8.98
2	50.2944	54.05	0.8936	1.8142	200	10.26
3	73.99	24.77	0.83	1.280	220	12.43
						10.55nm

**Fig -6) (a) SEM images of CuO NPs, (b) EDAX analysis of CuO NPs**

3.5. Scanning Electron Microscopy (SEM) Analysis

The copper nanoparticle morphology was examined by scanning electron microscope (SEM) image. The image was recorded at 10 μm nanoscale magnitudes. From the surface morphology study, the size of Cu NPs was found

about 0.6-8.733 μm. Fig. 7a shows the existence of symmetrical spherical shape of Cu NPs, and the presence of some large granular particles up to 8.733 μm, which could be due to aggregation of smaller particles and due to nanoparticle oxidation (Karthikeyan and Sang-Jae, 2013).

**Fig -8) a) TEM image of Cu NPs b) Particle Size distribution of Cu NPs c) SAED Pattern of Cu NPs**

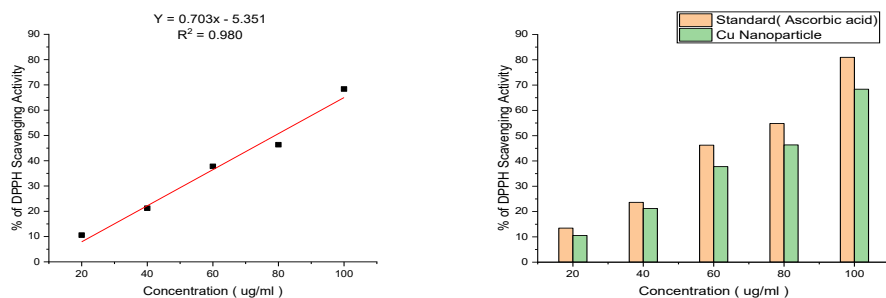


Fig-9) a) Plot of Scavenging activity of Cu NPs, b) Scavenging activity of Cu NPs & Ascorbic acid

Table-4- Scavenging Activity of Ascorbic acid and biosynthesized Cu NPs.

S. No	Volume of DPPH	Concentration of Ascorbic acid	Concentration of Cu NPs	% of Scavenging Activity of Ascorbic acid	% of Scavenging Activity of Cu NPs	IC 50 of Cu NPs	IC 50 of Ascorbic acid
1	3 ml	20 µg/ml	20 µg/ml	13.46	10.57		
2	3 ml	40 µg/ml	40 µg/ml	23.65	21.23		
3	3 ml	60 µg/ml	60 µg/ml	46.25	37.78	78.73	67.49
4	3 ml	80 µg/ml	80 µg/ml	54.8	46.34		
5	3 ml	100 µg/ml	100 µg/ml	80.96	68.36		

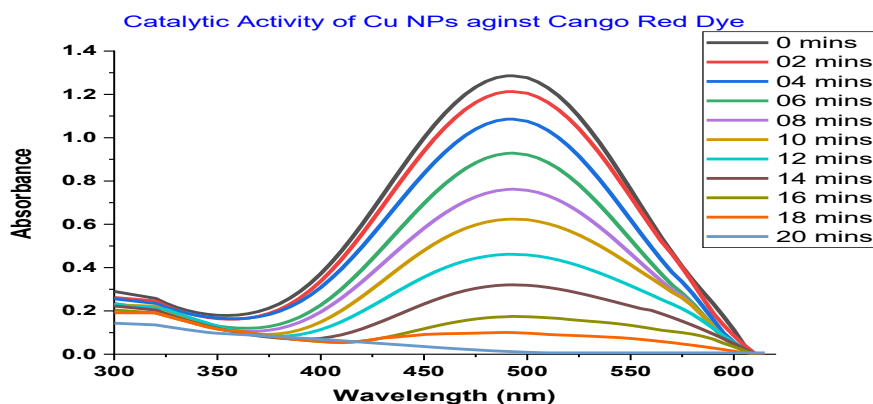


Fig XX:???

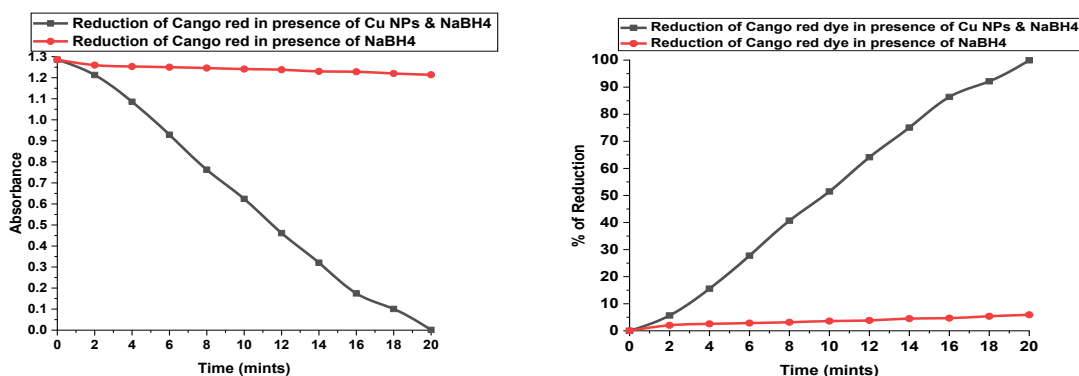


Fig XX:???

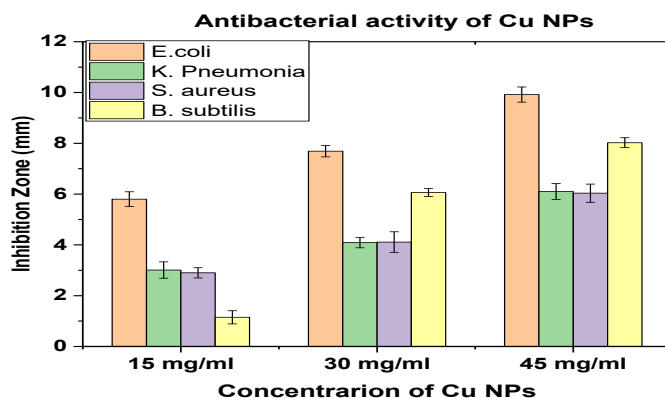


Fig XX:???

It is apparent from Fig. 7b, that the EDX spectrum confirmed the successful formation of Cu NPs with the aqueous fruit extract of *Embllica Officinalis*. The EDX spectrum depicts, the presence of carbon(C), oxygen(O), Nitrogen (N), and copper (Cu). The composition of major constituents was Cu (12%), C (33.54%), and O (54.46%). The presence of oxygen's peak along with the Cu signal, suggested that the Cu NPs were capped by phytoconstituents through oxygen atoms and the presence of carbon peak demonstrated the involvement of plant phytochemical groups in the reduction and capping of the synthesized CuNPs (Khodaie and Ghasemi, 2018). The presence of CuNPs shown strong signal energy peaks for the copper atoms at 1.0 and 8.0 keV. The previous studies carried out for individual spherical-shaped CuNPs also show strong signal peaks at 1.0 and 8.0 keV (Ismail et al. 2019).

3.6. Transmission Electron Microscopy (TEM) analysis

The image of copper nanoparticles synthesized using an aqueous extract of *E. officinalis* fruit is shown in Fig. 8a. The synthesized Cu NPs were spherical and an average diameter of 11-44nm (Fig-8b) (Cuevas et al. 2015).

The selected area electron diffraction (SAED) pattern of CuNPs is shown in Figure-8c. The SAED pattern suggests the polycrystalline nature of the synthesized Cu NPs. The interplanar spacing (d-spacing) was calculated to be 0.209 nm indicating the preferred (110) orientation.

3.7. Scavenging Activity of Cu nanoparticles

Antioxidant activity of EO-Cu NPs was assessed by DPPH free radical scavenging assays. EO-Cu NPS has shown potent DPPH radical scavenging activity and it was exhibited in a dose-dependent manner (Figures 9a.b). It shows that antioxidant activity was directly proportional to the concentration of EO-Cu NPs. The EC50 (effective concentration required to inhibit 50% of free radicals) of EO-Cu NPS was determined as 78.73 ug/mL for DPPH radical scavenging activity and, the EC50 value of ascorbic acid (reference standard) was 67.49 ug/mL for DPPH radical scavenging activity respectively. Though, among the different concentrations of Cu NPs tested, the highest scavenging activity (68.36 %) was obtained for 100 µg/ml and the lowest scavenging effect (10.57%) was observed for 20 µg/ml. Dose-dependent antioxidant activity of CuNPs was observed. Hence the EO-Cu NPs exhibited potent antioxidant activity as like ascorbic acid (Madhuri et al., 2020).

CONCLUSION

The present work acknowledged the eco-friendly synthesis of CuNPs using *E. officinalis* fruit aqueous extract. This method has merits over Physical & Chemical methods. The XRD analysis confirms the crystalline nature (FCC) and means average size (18.133 nm) of CuNPs, which complies with the TEM result. The XRD and EDAX result indicates the presence of Cu zero-valent NPs and FTIR & Zeta potential studies proved that the phytochemicals present in aqueous extract acted as reducing as well as a capping agent to stabilize

synthesized Cu nanoparticle. Therefore, the use of *E. officinalis* fruit aqueous extract enroots facile, cost-effective, non-toxic, eco-friendly, synthesis of Cu NPs. The biologically synthesized CuNPs shown substantial antioxidant activity against DPPH.

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Data Availability Statement: Not applicable.

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