Antimicrobial and Antioxidant activities for medicinal applications of *Azadirachta indica* mediated biosynthesis of Copper oxide nanoparticles

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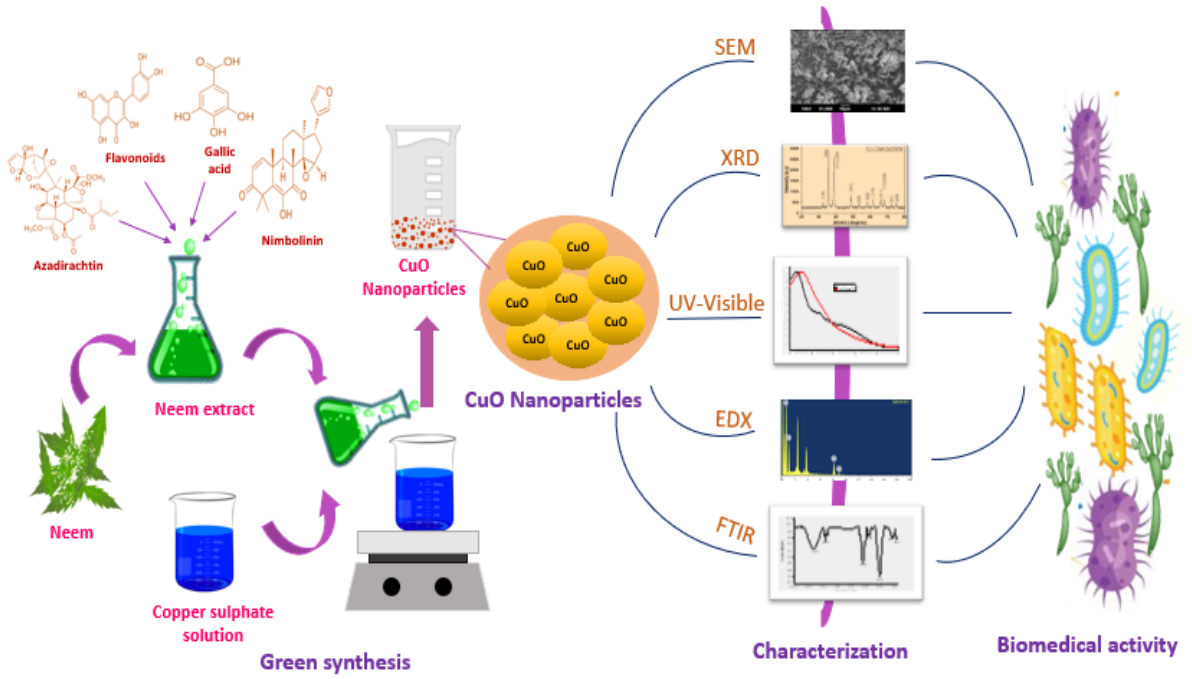
Abstract
During the last few decades, researchers have published plethora of reports on green synthesis techniques. Green production techniques are quick, inexpensive, simple and environmentally benign. Here we report synthesis of copper oxide nanoparticles (CuO NPs) by using the leaves of Azadirachta indica (neem). Neem leaves are rich in several phytochemicals which may serve as capping, stabilizing and size reducing agent. UV-visible spectrophotometer, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), Scanning electron microscopy (SEM) and Energy dispersive X-ray spectroscopy (EDX) were used to characterize the synthesized nanoparticles.

XRD details about the average particle size and phase of synthesized nanoparticles, found to be 21.29 ± 5 nm and have a monoclinic structure. Further, synthesised CuO NPs were tested for the biological activities and antioxidant property. Results obtained were found promising.

Keywords: *Azadirachta indica* (neem) leaves, Green synthesis, Copper oxide nanoparticles, Characterization, Antimicrobial and Antioxidant activity.

Introduction

Nanotechnology is becoming inevitable in almost all fields of applications due to the special physical, chemical and biological characteristics of nanoparticles[6,12,18]. These properties include hardness, tensile strength, catalytic capabilities, potential for sensing applications and antimicrobial and anticancer properties etc[45]. Nanoparticles have also emerged as a promising topic for exploration and development. Nanocrystalline oxides have proven to be especially useful to chemists in the laboratories and industries due to their efficient engagement in adsorption of chemical species, increasing reaction rate, selectivity, ease of use, recyclable nature and environment friendly reaction conditions[20].



Graphical Abstract

Metal oxides such as TiO_2, Al_2O_3, ZnO, CaO, CuO and MgO exhibited catalytic and many other properties due to their high surface to volume ratio, high yields, selectivity and recyclability in a variety of chemical[38,41]. Copper oxide nanoparticles (CuO NPs) have attracted the attention of researchers as in comparison to other metal oxides, they exhibit high electric conductivity, low migratory behaviour, excellent anti-microbial characteristics and low cytotoxicity[36]. CuO NPs are utilized in almost every area of applied sciences and engineering due to their significant characteristics than the bulk particles[19,26,37,40]. CuO nanoparticles were synthesised primarily using top-down and bottom-up approached[12,24]. The size, shape and properties of synthesised nanoparticles depend upon method of synthesis (chemical/physical/biological/green)[1,30,42].

More weightage is given to green methods these days as they are versatile, simple and economic. Bacteria, algae, fungi, plants and their numerous parts, fruits and peels are popularly used in green methods of synthesis of nanoparticles[47]. Dealing with microorganisms may culminate into culture contamination, storage issues and size control of the generated nanoparticles[4]. Plant parts are reported to be convenient, inexpensive, safe, readily available and free of contamination and storage problems[3]. Phytochemicals found in plant parts are reported to serve as capping, reducing and stabilizing agents[21,23].

UV-visible, FTIR, XRD, SEM and EDX etc. were widely reported to analyse the biosynthesized CuO NPs and their potential as bioactive nonmaterial[8,13,28,32]. Antibacterial properties and antifungal properties of CuO NPs were published against Gram-negative, Gram-positive bacteria and against yeast and *Aspergillus*[27]. The most likely mechanism of antibacterial activity is that CuO NPs bound to the cell walls of microorganisms or penetrate it which may harm DNA and other cellular processes finally leading to bacterial cell death[7,43].Anti-oxidant properties of NPs may be due to their electrons to stop the action of free radicals called radical scavenging[5,9]. The current study aims to synthesize and characterize CuO NPs in a benign manner by using Neem leaves.

Material and Methods

Materials: *Azadirachta indica* leaves extract was used as a biological agent in the green synthesis of CuO NPs. Copper sulphate solution was used as precursor for formation of CuO NPs.

Preparation of *Azadirachta indica* Extract: To get rid of contaminants, the gathered leaves were first cleaned with tap water and then again with distilled water. After that, leaves are exposed to the sun light for a day in order to make them dry. Fine powder was made by crushing dried leaves in a pestle and mortar. 100 ml of distilled water was added to one gram of powdered neem leaves. For 15-20 minutes at 50–55°C, the mixture was continuously stirred using a magnetic stirrer. After that, the solution was allowed to cool down at ambient temperature. The colour of the mixture slightly turns yellowish the it is filtered by using Wattmann filter paper[17,46].

Green Synthesis of Copper Oxide Nanoparticles (CuO NPs): With very minor modifications, the green synthesis of CuO NPs was carried out as described by Gemachu et al[16]. Leaf extract was added in a 4:1 ratio to a freshly made copper sulphate solution and the mixture was continuously stirred for 30 minutes at 55°C until the colour changed from blue to green, further brown precipitate was obtained. The solution was stored for 24 hours in a dry and dark place. The colour of solution gets darkened and the dark brown precipitates was obtained after 24 hours. The mixture was then centrifuged for 15 minutes at 2500 rpm. The precipitates obtained was washed and allowed to dry in the sunlight. Further, it was heated in a Muffle furnace to 100°C for one hour which resulted into a fine black copper oxide powder (Scheme 1)[16].

Characterization: UV-Visible spectra of synthesized CuO NPs were recorded on a JASCO V-750, UV-Visible spectrophotometer (Tokyo, Japan) with EHCS-760 ranging from 200-800 nm. FTIR spectroscopy of synthesized CuO NPs was performed on Shimatzu IRAffinity-1S with a 4000-400 cm^{-1} range. XRD of CuO NPs was performed on X- ray diffractometer (generator, Standard Goniometer), equipped with Cu_K-beta_1D (40 kV, 30 mA, with k = 0.15418 nm). The scan of the analysis was run in 120 min. range of 5-90^o with a step width of 0.01^o and step time of 10.00^o/ min. The morphology of synthesized nanoparticles was confirmed by SEM JSM-6390, accelerating voltage was 10 kV. EDX spectra of CuO NPs were recorded on an X-act energy dispersive X-ray spectrometer (Oxford), with 20 keV acceleration voltage, collected for 20 s.

Antibacterial Activity of Copper Oxide nanoparticles (CuO NPs): CuO NPs antibacterial activity was examined against Gram-negative *Salmonella typhi* and *Escherichia coli* bacteria as well as Gram-positive *Bacillus subtilis* and *Staphylococcus aureus* bacteria. The antibacterial activity of CuO NPs was studied by Kirby Baureri techniques well diffusion method was described by Manogar et al[25]. A 10 mg/ml CuO NPs solution was prepared in methanol and 2% HCl. As a positive control, 10 mg/ml streptomycin was taken and as a negative control, methanol and 2% HCl were taken.

10 ml of Muller Hilton agar media was put into a Petri dish, swabbed colonies of bacteria and left plate for 15 min. for drying. Make holes and use a sterile cork to bore a hole 6 mm in diameter. Wells will load CuO NPs, positive control (streptomycin) and negative control (methanol and 2% HCl). All the plate was incubated at 37°C for 24 hours and after that, the zone of inhibition was calculated[25,35,44].

Antifungal Properties of Copper Oxide Nanoparticles (CuO NPs): Antifungal properties of CuO NPs were detected against *yeast* and *Aspergillus*. The antifungal

activity of CuO NPs was studied by Kirby Baureri techniques well diffusion method described by Manogar et al[25]. CuO NPs were dissolved in 10 mg/ml methanolic solution and 10 mg/ml 2% HCl solution. 15 mg/ml fluconazole was taken as a positive control and methanol and 2% HCl were taken as a negative control. 10 ml of Muller Hilton agar media was put into a Petri dish and swabbed colonies of fungi. Leave the plate for 15 min. for drying. Wells will loaded with CuO NPs, positive control (fluconazole) and negative control (methanol and 2% HCl). All the plates were incubated at 37°C for 48 hours and after which the zone of inhibition was calculated[25,35,44].

Antioxidant Properties of Copper Oxide Nanoparticles (CuO NPs): Antioxidant properties of CuO NPs were analysed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) methods described by Santhosh et al[39] with minor modification. A methanolic DPPH solution was prepared and kept in a dark and cold place. CuO NPs 1 mg/ml stock solution was prepared in methanol and diluted in 20 µl, 40 µl,60 µl, 80µl, 100µl and 200 µl range. Methanolic DPPH was added to methanolic CuO NPs solution having different concentrations. The mixture was evaluated for 517 nm absorbance for 30 min. The DPPH % Inhibition was calculated using these formulas:

$$\% \text{ RSC} = \frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}}$$

Antioxidant properties were found after 30 min. by calculating % inhibition[9,15,31,39].

Results and Discussion

The colour of the copper sulphate solution turns from blue to translucent green when neem extract is added. The initial green colour observed upon adding the neem extract indicates the reduction of copper ions as depicted in figure 1. The colour of the solution gradually darkens with time, confirming the successful synthesis of CuO NPs.

UV-Visible spectroscopy: The optical characteristics of nanoparticles determined absorbance and their ability to absorb different wavelengths in UV visible spectroscopy[33]. As shown in figure 2, CuO nanoparticles exhibits clear peak at 228 nm. However, two distinct broad peaks at 266 nm and 328 nm and one peak at around 214 nm was obtained in the UV spectrum of neem extract. Results obtained are in accordance with previously published literature[29,34].

Fourier Transform Infrared Spectroscopy (FTIR): Results for FTIR are shown in figure 3 and its analysis is summarised in table 1. Peaks at 601 cm^{-1} and 451 cm^{-1} indicate the presence of Cu-O. Many other peaks are obtained mentioned in table, which may be due to the phytochemicals (e.g. polyphenols, amides, flavonoids, terpenoids etc.) present in Neem extract[2,21].

$$Cu^{+2}\,[CuSO_4 \cdot 5H_2O] + \text{Azadirachta (Neem) leaves Extract} \xrightarrow{\Delta} [\text{Cu} - \text{Extract}]^{+2}$$

$$[\text{Cu} - \text{Extract (Polyphenols)}]^{+2} \xrightarrow[\text{Stirring}]{\Delta} [Cu(OH)_2 - \text{Extract}]$$

$$[Cu(OH)_2 - \text{Extract}] \xrightarrow{\text{Stirring}} [\text{CuO}] - \text{precipitate} \xrightarrow[\Delta]{100°C} \text{CuO NPs}$$

Scheme 1: Green synthesis of CuO NPs

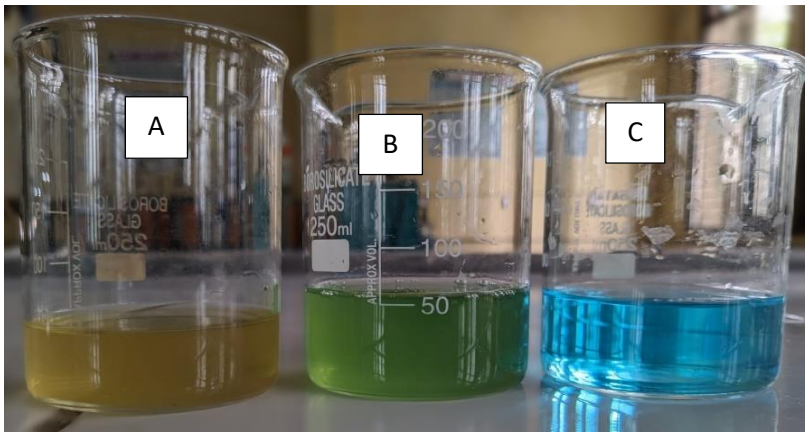


Figure 1: A. Neem extract, B. CuO NPs and C. Copper sulphate

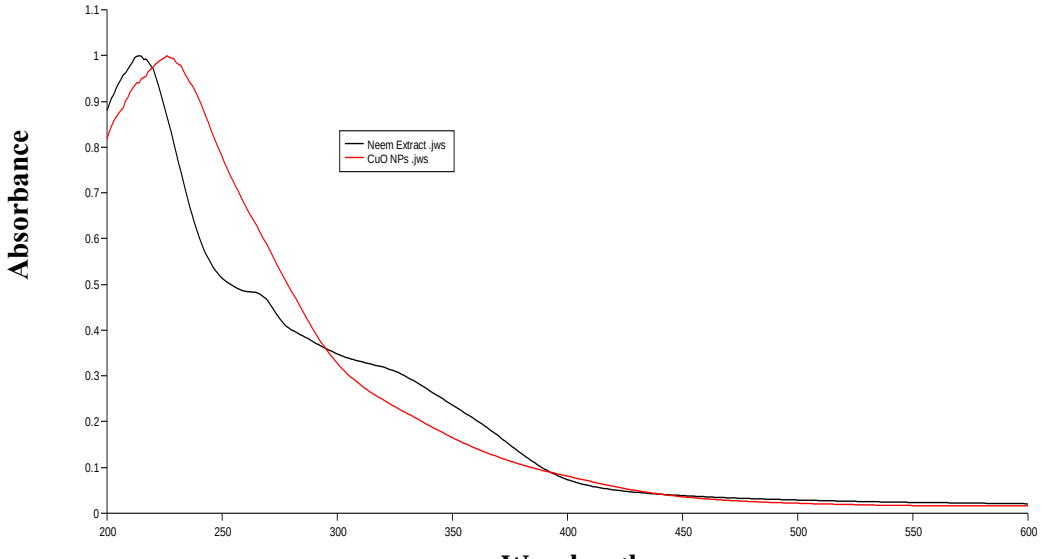


Figure 2: UV-Visible spectrumof CuO NPs

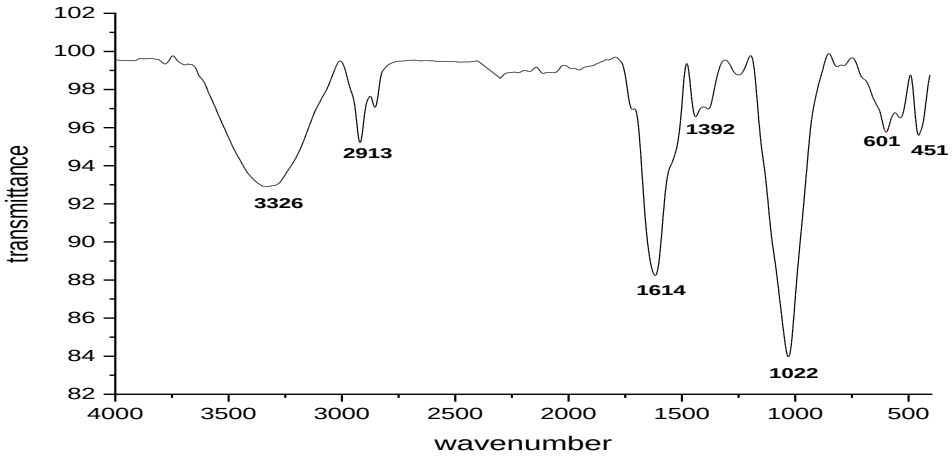


Figure 3: FTIR spectrum of CuO NPs

Table 1
FTIR Spectral analysis of green synthesized CuO NPs

S.N.	Wavenumber (cm^{-1})	Expected Functional Group
1.	3326	O-H stretching vibration (alcoholic or phenolic)
2.	2913	C-H Asymmetric stretching
3.	1614	C=O Stretching vibration of Amide
4.	1392	C-OH Stretching vibration of Flavonoid
5.	1022	C-OH bending Vibration
6.	601	Cu-O stretching vibration (bond-forming)
7.	451	Cu-O Stretching vibration along the direction

X-ray Diffraction (XRD): Figure 4 displays the XRD pattern of CuO NPs that were synthesised using neem leaves. The XRD verified the dimensions, form and crystalline phase of synthesized CuO NPs. The lattice plane of CuO NPs was (1 1 0), (1 1 1), (2 0 0), (2 0-2), (1 1 2), (2 0 2), (3 1 0), (1 1 3) and (2 2-2) corresponding to the peak position at 2θ of 26.68°, 36.44°, 38.77°, 48.80°, 58.55°, 61.55°, 66.22°, 68.11° and 75.13° respectively. Results exhibit extremely crystalline nature of CuO NPs.

The crystalline monoclinic phase of green synthesized CuO NPs is confirmed by the XRD data. Green synthesized CuO NPs were found to be pure, as no reflection related impurity was detected up to the detection threshold. Additionally, the high intensity of the XRD pattern verifies the extremely crystalline nature of the produced compounds. Furthermore, by utilizing the (111) peak in Debye-Scherrer's formula, the size of the synthesized nanoparticles can be determined using the equation:

$$D = \frac{k\lambda}{\beta\cos\theta}$$

where D = Average crystalline size, k = Shape factor constant (0.9), λ = wavelength of X-Ray (λ= 0.15405 nm for Cu), ß = Full width of half maxima (Ave. of FWHM) and θ = Diffraction angle in degree. Using these formulas, the synthesized CuO NPs' crystalline size was determined to be 21.29 ± 15 nm. Earlier, size of 80 ± 15 nm was reported by Ahmad et al[2] while using neem leaves[2].

Scanning Electron Microscope (SEM): The green synthesized CuO NPs SEM image is displayed in the figure 5. It can be seen from the image that CuO NPs exhibit an elongated or rod-like structure. A variety of size range is reported in literature for CuO NPs and they are often found in an agglomerated state[2,17].

Energy Dispersive X-ray Spectroscopy (EDS): The energy dispersive X-ray spectroscopy (EDS) revealed the chemical composition of the produced particles (Figure 6).

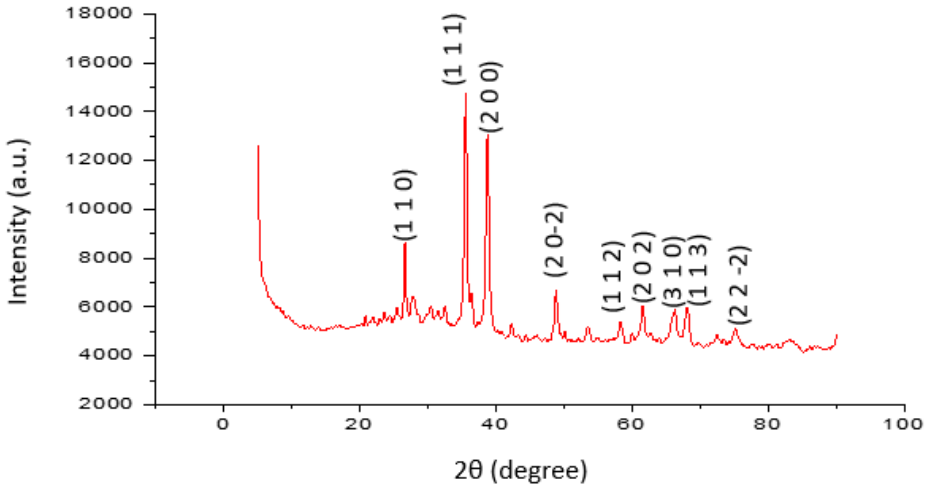


Figure 4: X-ray diffraction pattern of CuO NPs

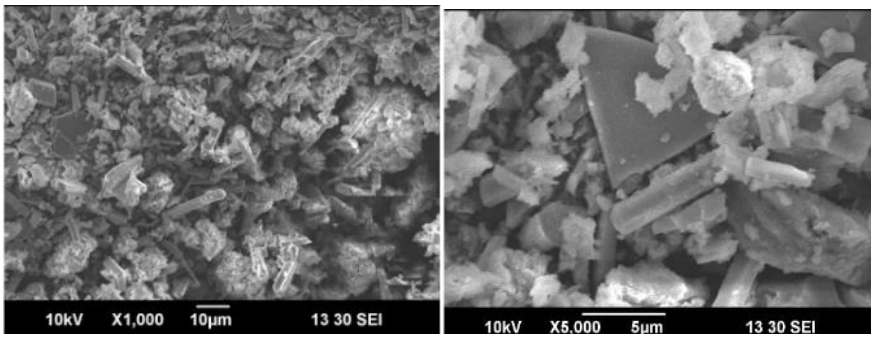


Figure 5: SEM image of CuO NPs

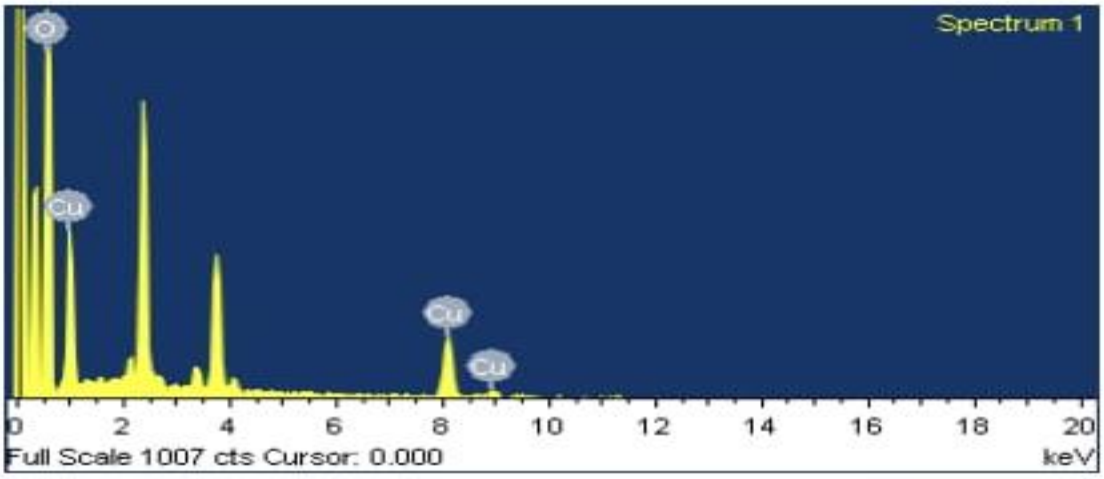


Figure 6: EDS image of CuO NPs

Cu and O with weight percentages of 35.15% and 64.85% with chemical composition CuO were validated by the EDS spectrum. The sharp signal close to 1 keV, 8 keV and 9 keV verified the presence of nanocrystalline Cu and 0.7 keV signal of oxygen. There are also some weak signals found in the EDS spectrum, which are likely due to other components such as polyphenols, saponins and flavonoids present in the neem extracts.These outcomes are consistent with previously reported data[17].

Antibacterial Activity of Copper Oxide Nanoparticles (CuO NPs): The antibacterial effect of CuO NPs was analysed on the basis of the zone of inhibition. Antibacterial activity of CuO NPs is shown in figure 7 and table 2. CuO NPs were dissolved in two different solvents to analyse the effect of the solvent. After 24 hours, incubation zone of inhibition was calculated. There is no zone of inhibition found in *Salmonella typhi* and *Staphylococcus aureus*. There is no antibacterial effect of CuO NPs against *S. typhi* and *S. aureus*.

The zone of inhibition against *E. Coli* was 39±5 mm and was 35±5 mm against *B. subtilis* when methanol was used as solvent. Acidic solution of CuO NPs shows no zone of inhibition for *E. coli* and 16± 5 mm for *B. Subtilis*. The positive control streptomycin shows 0 mm in *E. coli* and 18 ± 5mm in *B. subtilis***.** The methanolic solvent alone has no zone of inhibition against bacteria, but CuO NPs methanolic solution has a great effect against bacteria. CuO NPs methanolic solution shows effective results rather than acidic solution[25,35,44].

Antifungal Activity of Copper Oxide Nanoparticles (CuO NPs): Antifungal properties of CuO NPs were examined against yeast and *Aspergillus.* CuO NPs were dissolved in two different solvents to analys the solvent effect. After 48 hours of incubation, zone of inhibition was calculated. Zone of inhibition of methanolic CuO NPs in Yeast was 40 ± 5mm and in *Aspergillus*, it was 20± 5 mm. Zone of inhibition of acidic CuO NPs was 17± 5 mm in yeast and in *Aspergillus*, it was 37 ± 5mm. Zone of inhibition in fluconazole in yeast was 18± 5 mm and in *Aspergillus,* 20± 5 mm. Methanolic solution of yeast has greater antifungal properties than the acidic solution. But in the case of *Aspergillus* acidic solution shows great results[25].

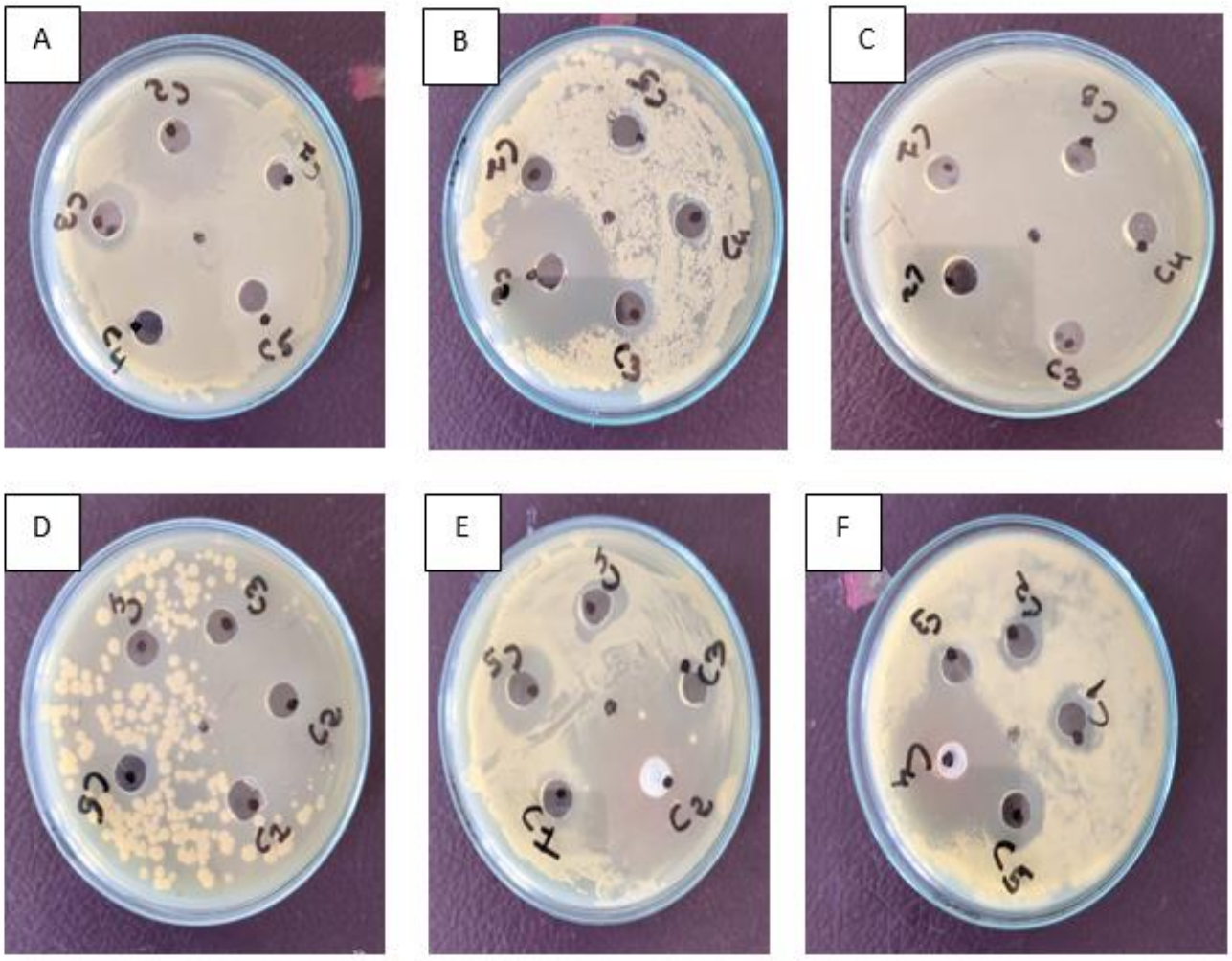


Figure 7: Antimicrobial activity of CuO NPs against A. *E. coli,* B. *B. subtilis,* C. *S. typhi,* D. *S. aureus,* E. *Yeast,* F. *Aspergillus*

Table 2
Zone of inhibition size in mm

Microorganism	Methanol (C1)	Methanolic CuO NPs (C2)	2% HCl (C3)	Acidic CuO NPs (C4)	Control (C5)
E. coli	-	39± 5	18± 5	-	-
B. subtilis	-	35± 5	17± 5	16± 5	18± 5
S. typhi	-	-	-	-	-
S. auras	-	-	-	-	-
Yeast	15± 5	40± 5	20± 5	17± 5	18± 5
Aspergillus	15± 5	20± 5	16± 5	37± 5	20± 5

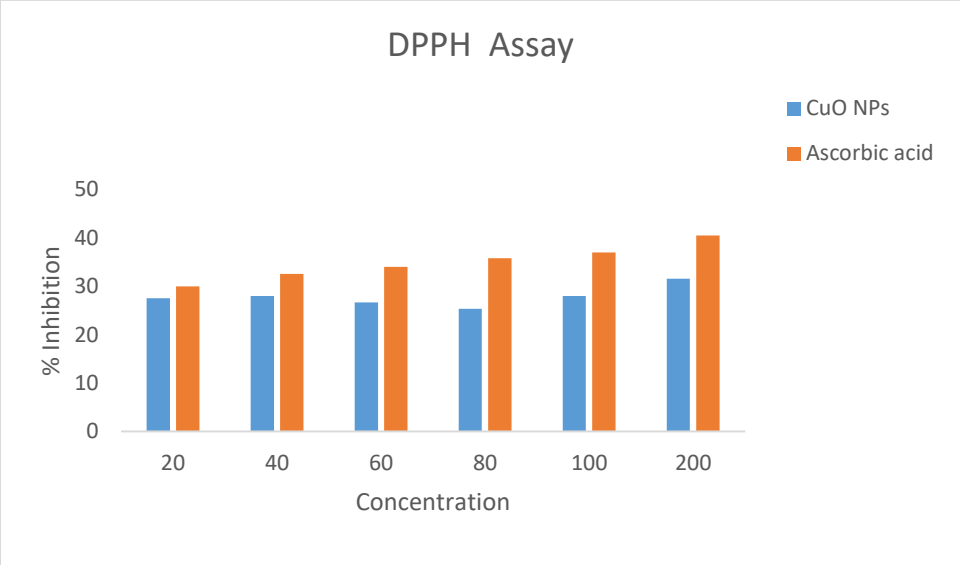


Figure 8: Antioxidant properties of CuO NPs

Table 3
Antioxidant properties of CuO NPs

Concentration (µl)	Absorbance Control	Absorbance of CuO NPs	Absorbance of Ascorbic acid	% RSA of CuO NPs	% RSA of Ascorbic acid
20	0.225	0.163	0.157	27.55	30
40	0.225	0.162	0.151	28	32.55
60	0.225	0.165	0.148	26.67	34.01
80	0.225	0.168	0.144	25.33	35.77
100	0.225	0.162	0.141	28	36.97
200	0.225	0.154	0.133	31.55	40.49

Antioxidant Properties of Copper Oxide Nanoparticles (CuO NPs): Results for antioxidant properties of CuO NPs are shown in figure 8 and table 3. After 30 min, colour of the DPPH solution changed from purple to golden yellow indicating that the compound shows anti-oxidant properties. The percentage inhibition (%RSA) was 27.55%, 28%, 26.67%, 25.33%, 28% and 31.55% at CuO NP doses of 20 µl, 40 µl, 60 µl, 80 µl, 100 µl and 200 µl respectively. The result was compared using standard antioxidant ascorbic acid. The highest anti-oxidant properties 31.55% were found at the highest concentration i.e. 200 µl. Results show that CuO NPs have antioxidant properties which are close to standards[15,31,39].

Conclusion

In this study, CuO nanoparticles (CuO NPs) were synthesised using *Azadirachta indica* (neem leaves). The synthesized CuO NPs were characterized using UV-Visible, FTIR, XRD, SEM and EDX methods. The synthesized CuO nanoparticles in UV-Visible spectroscopy show peaks at 228 nm. The synthesized CuO NPs were 21.29 ± 15 nm in size, highly crystalline and had mono clinic structure confirmed by XRD and SEM. The EDS spectrum verified the existence of Cu and O with weight percentages of 35.15% and 64.85% with chemical composition CuO. Synthesized nanoparticles antibacterial qualities were evaluated against *E. coli, B. subtilis, S. typhi and S. aureus. Aspergillus* and *yeast* were also found to be the targets of antifungal activities of CuO NPs. CuO NPs have also shown antioxidant properties which are close to their standard ascorbic acid. Green synthesis methods of CuO nanoparticles were therefore advocated, not only because they are easy to use and environmentally benign, but also because they improve the biocompatibility and biomedical qualities of CuO NPs.

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