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Green synthesis of CuO nanoparticles using *Ligustrum lucidum* extract, and the antioxidant and antifungal evaluation

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Keywords: antioxidant, antifungal, nanoparticles, biosynthesis, green plants, metal oxide

Abstract

Biosynthesis of metal oxide nanoparticles using plant extract is an inexpensive, simple, rapid, and environmentally friendly approach to obtaining nanoparticles for biological applications. Herein, copper oxide nanoparticles (CuO-NPs) were successfully synthesized using an aqueous extract from *Ligustrum lucidum* leaves. The structural, optical, and morphological characteristics of the nanoparticles were assessed using x-ray diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy, UV-visible spectrophotometer, transmission and scanning electron microscopy (TEM and SEM), and energy-dispersive x-ray (EDX). Nanocrystalline CuO with an average crystalline size of 22.0 nm and a band gap energy of 1.4 eV were confirmed from the XRD and UV-vis spectrophotometer, respectively. Morphological studies showed spherical nanoparticles, whose particle size estimation (30 ± 5 nm) agrees with the crystalline size deduced from the XRD pattern. A free radical scavenging activity of the CuO nanoparticles, evaluated using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay, showed that it exhibited high antioxidant activity (IC_{50} : $63.35 \mu\text{g ml}^{-1}$) that is concentration dependent. Antifungal evaluation using four different fungal strains (*Aspergillus flavus*, *Aspergillus niger*, *Fusarium oxysporum*, and *Trichoderma harzianum*) indicated a direct relationship between the potency of the particles and their concentration, with 1 ppm solution exhibiting the highest potency. The green synthesized CuO-NPs using *Ligustrum lucidum* may be potentially used as an antioxidant and antifungal agent for therapeutic applications.

1. Introduction

Nanoparticles have recently gained significant attention in various scientific disciplines due to their unique physicochemical properties and potential applications in different areas such as medicine, agriculture, electronics, and environmental remediation [1]. This has necessitated a quest for different synthesis routes to meet the increasing demand. The conventional methods of synthesis often involve the utilization of hazardous chemicals and high-energy processes, that pose significant environmental and health risks [2]. Consequently, there has been a growing interest in developing eco-friendly and sustainable approaches for nanoparticle synthesis in order to overcome these challenges.

One of the eco-friendly methods is the green synthesis of nanoparticles using extracts from different parts of plants, such as flowers, leaves, and roots. These plant extracts contain biomolecules such as phenolic acids, terpenoids, polyphenols, and alkaloids, which could reduce or oxidize the metal ions and stabilize the dispersible nanoparticles after the synthesis process [3]. Also, plant extract-mediated synthesis has the advantage of cost

efficiency due to the availability of plants and the absence of additional purification processes compared to chemical or microbial-mediated synthesis [4]. Additionally, this strategy is sustainable and safe for nanoparticle preparation because the crystal growth and dispersion of the nanoparticles could easily be controlled [5]. Several studies have reported the green synthesis of nanoparticles using plant extracts for improved biological properties (bioavailability, biocompatibility, cell internalization, antioxidant activity) of both metal and metal oxide nanoparticles [6–8]. Hence, plants continue to play important roles in green-mediated synthesis, leading to the use of several plants in synthesis processes.

Ligustrum lucidum, commonly known as glossy privet, possesses a rich reserve of bioactive compounds, including phenols, flavonoids, and triterpenoids, which exhibit diverse biological activities [9, 10]. This medicinal plant known for its potent antibacterial properties, has emerged as a potential candidate for the synthesis of silver nanoparticles [10]. The utilization of *Ligustrum lucidum* extract for nanoparticle synthesis enhances the eco-friendliness of the process [9]. The phytochemical constituent of *Ligustrum lucidum* also acts as a bio-reducing agent and stabilizer for forming metallic NPs such as Ag [11].

Among the different metal oxides, CuO nanoparticles are of special interest due to their abundant availability, low cost, and biological activity [12]. It has also been reported to be non-toxic in nature, chemically stable, cost-effective, biocompatible, and biodegradable [13]. CuO NPs have been reported for the treatment of cardiovascular diseases [8, 14], oxidative stress [15], and cancer [16]. It is also a potential material for applications in fields such as photocatalysis, energy storage, and biosensing [17, 18]. CuO is one of the most useful and essential substances for the human body, providing balance and stability in tissues. It has been introduced by the US Environmental Protection Agency (EPA) as an effective antibacterial agent [13]. Accordingly, CuO can be used as a drug carrier (chemotherapeutics) for antioxidant and antimicrobial applications.

The remarkable versatility and exceptional biocompatibility exhibited by CuO-NPs obtained through the green synthesis route make them highly sought after across various scientific and industrial domains. Therefore, in this work, CuO-NPs obtained through the green synthesis route using an aqueous extract of *Ligustrum lucidum* is reported for the first time to the best of the authors' knowledge. The nanoparticles were characterized for its structural, morphological, and optical properties. The antioxidant and antifungal activity of the CuO-NPs nanoparticles was further studied to evaluate their biological activity.

2. Materials and methods

2.1. Materials

Fresh *Ligustrum lucidum* fruits were collected from Mahikeng Local Municipality, North-West Province, South Africa (coordinates: 25.8255° S, 25.6110° E). Copper (II) nitrate hemi (pentahydrate), 1, 1-diphenyl-2-picrylhydrazyl (DPPH), and sodium hydroxide (NaOH) were purchased from (Merck (Pty) Ltd., Merck, Darmstadt, Germany) and used without any purification.

2.2. Preparation of the ligustrum lucidum extract

The fruits of *Ligustrum lucidum* were harvested and rinsed several times with distilled water. Thereafter, 120 ml of distilled water was added to 30 g of fruits and then blended to form a pulp. The resulting pulp was then strained and filtered using Whatman No. 1 filter paper to obtain a pure extract, which was stored in a sealed container and placed in a refrigerator at 4 °C for later use.

2.3. Biosynthesis of CuO nanoparticles

For the synthesis of the CuO nanoparticles, the method reported by Gamedze, Mthiyane, Babalola, Singh and Onwudiwe [19] with slight modification was used. In a typical synthesis, about 2.5 g of copper(II) nitrate hemipentahydrate was dissolved in 20 ml of distilled water. Approximately 20 ml of *Ligustrum lucidum* extract was transferred to a beaker, and the pH of the solution was adjusted to 10 by gradually adding NaOH solution in a dropwise manner. Thereafter, the prepared solution (copper nitrate) was added and stirred at 80 °C for 2 h resulting in a deep green precipitate. The obtained precipitate was separated by centrifugation at 4500 rpm for 15 min and the resulting solid product was rinsed with a mixture of water/ethanol three times to remove any impurities. The product was then transferred to a crucible and left overnight to dry, after which it was calcinated at 450 °C for 2 h. Figure 1 illustrates the stages involved in the biosynthesis of CuO-NPs using *Ligustrum lucidum*.

2.4. Characterization of CuO nanoparticles

The structural properties were examined using a D8 advanced diffractometer, with a secondary graphite monochromated CuK α radiation ($\lambda = 1.54060 \text{ \AA}$) at 40 kV/50 mA, in the range of 20°–80° for x-ray

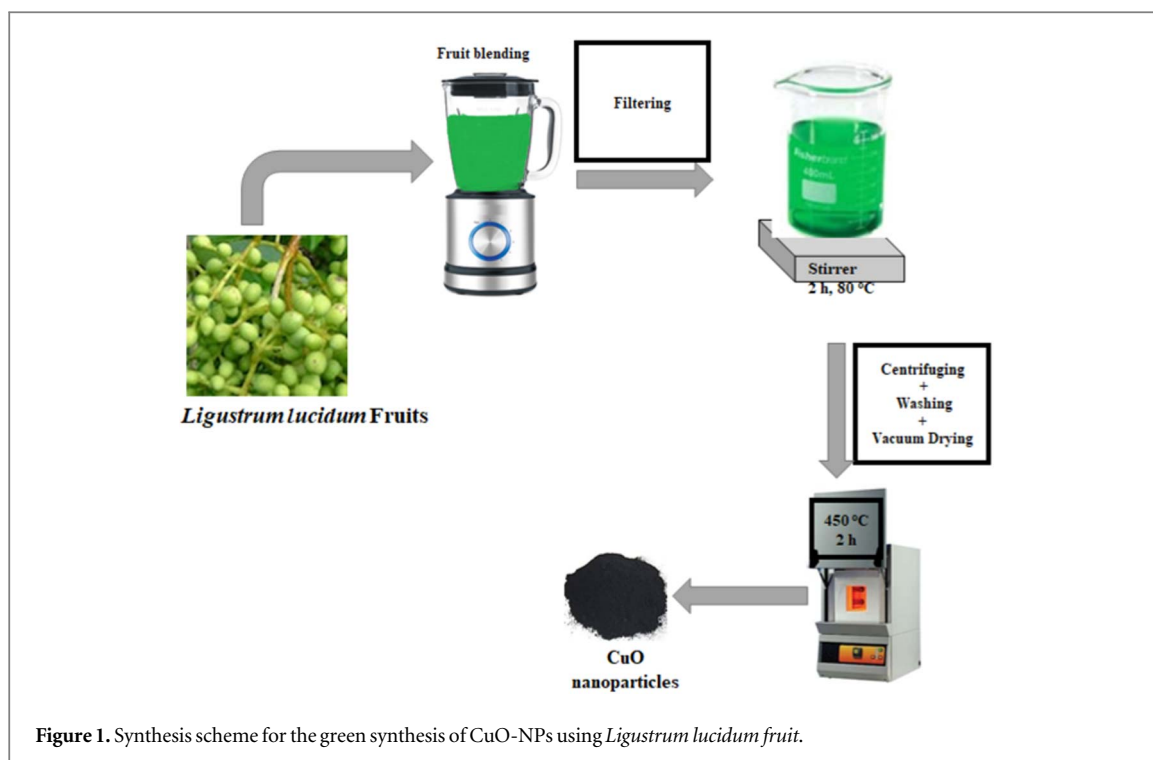


Figure 1. Synthesis scheme for the green synthesis of CuO-NPs using *Ligustrum lucidum* fruit.

diffraction (XRD) spectra. Spectroquant® 300 spectrophotometer with a wavenumber range of 400–5000 cm^{-1} was used for Fourier transform infrared spectroscopy (FTIR) studies. For the examination of the nanoparticles' morphology, FE-SEM FEI 430 Nova NanoSEM system scanning electron microscopy and FEI Tecnai G2 Twin transmission electron microscopy were used, respectively. For the light absorption property of the nanoparticle the Spectroquant® prove 300 spectrophotometer was used.

2.5. Antioxidant activity evaluation

CuO-NPs were assessed for their radical scavenging activity using a modified version of the DPPH assay, which follows the method adopted by Safawo, Sandeep, Pola and Tadesse [20], with slight modification. To prepare the DPPH solution, 12.8 mg of DPPH was dissolved in 50 ml of methanol to achieve a concentration of 0.032 mM. The solution was then incubated in the dark for 30 min. CuO solutions of varying concentrations (50, 25, 12.5, 6.25, 3.13, and 1.56 μg) were dissolved in 1 ml of methanol. Afterwards, 1 ml of the DPPH solution was added to each solution, while the blank solution contained only methanol and DPPH solution. The absorbance was measured at 540 nm against the control of 0.032 mM DPPH, and the experiment was conducted in triplicate. The percentage inhibition was calculated using equation (1);

$$\% \text{ Inhibition} = (A_c - A_s) / A_c \times 100\% \quad (1)$$

where (A_c) represents the absorbance of the control, while (A_s) is the absorbance of the sample.

2.6. Antifungal evaluation

Potatoes dextrose agar (PDA) medium supplement was used in evaluating the antifungal activity of CuO-NPs. In a typical process, 20 μl of the nanoparticles in 15 ml of PDA media plate was used at each concentration level. The fungal spores of each of the fungal *Aspergillus flavus*, *Aspergillus niger*, *Fusarium oxysporum*, and *Trichoderma harzianum* at the center of each plate. The plates were incubated at $28 \pm 2^\circ\text{C}$, and a media supplemented with corresponding levels of dimethylsulfoxide (DMSO) and sterile distilled water without the nanoparticles were used as control plates. The fungal growth was monitored for 2 weeks at 3-day interval and the percentage inhibition rate was calculated according to equation (2)

$$\% \text{ growth} = \frac{D_c - D_t}{D_c} \times 100, \quad (2)$$

where:

D_t = Diameter of the fungal inoculated in a nanoparticle-supplemented PDA plate

D_c = Diameter of the fungal without nanoparticle-supplemented PDA plate (control)

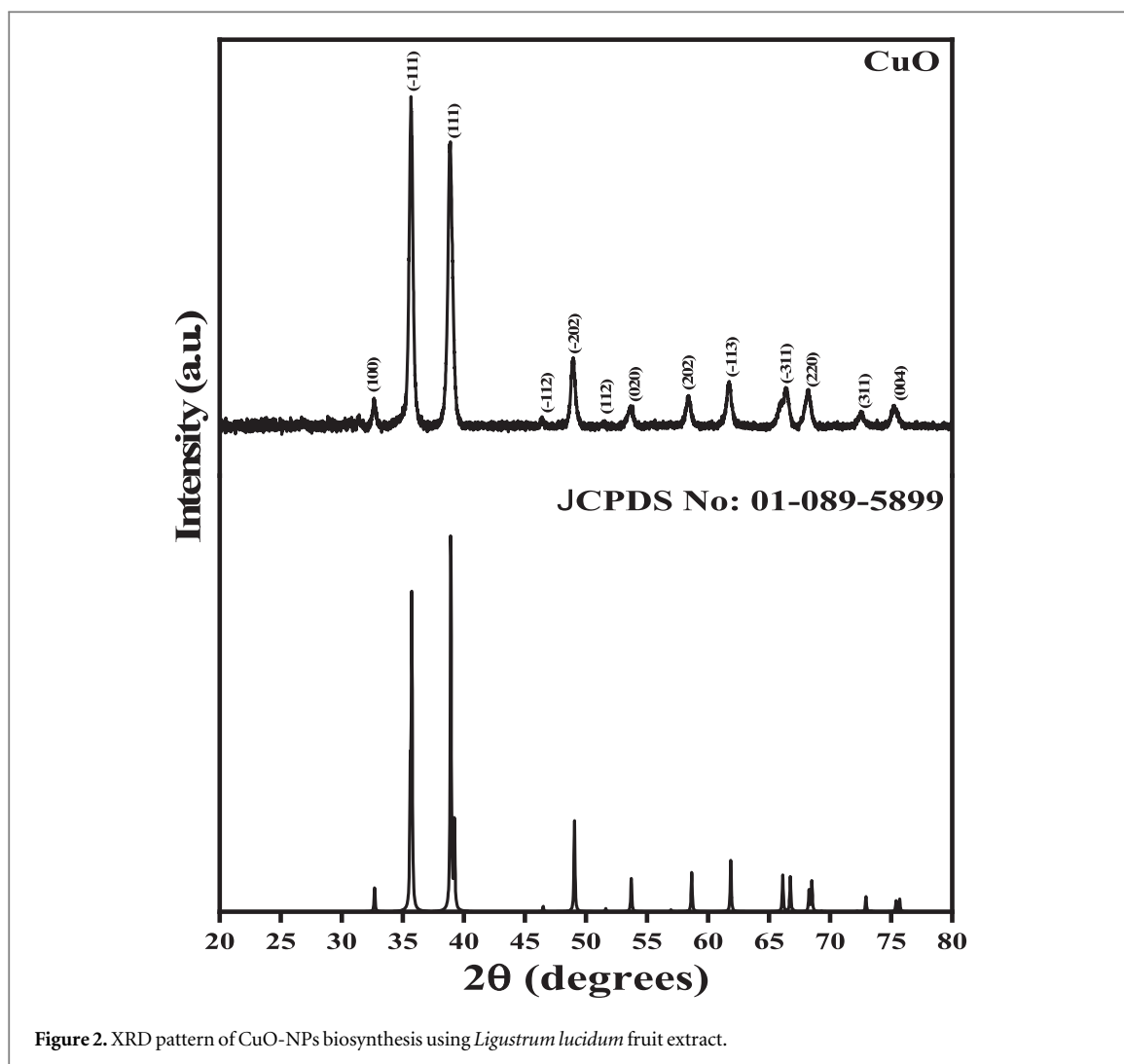


Figure 2. XRD pattern of CuO-NPs biosynthesis using *Ligustrum lucidum* fruit extract.

3. Results and discussion

3.1. X-ray diffraction (XRD) studies

The diffraction patterns of the nanoparticles (figure 2) showed characteristics peaks at 2θ values corresponding to the 110, -111 , 111, -112 , -202 , 112, 020, 202, -113 , -311 , 220, 311, 004 diffraction planes. These patterns were consistent with the monoclinic phase of CuO, with lattice parameters $a = 4.653$, $b = 3.41$, $c = 5.108$ (JCPDS No:01-089-5899). The sharpness of the peak indicates highly crystalline nanoparticles were obtained and the absence of impurity peaks confirmed the obtained CuO are of high purity. The crystallite size was calculated using the Debye–Scherrer equation (equation (3)), which showed an average size of 22 nm.

$$D = \frac{K\lambda}{\beta \cos \theta} \quad (3)$$

where, D is the crystallite size (nm), K is constant (0.94), λ is the wavelength of x-ray ($1.5406\text{\AA}$), β is the full width at FWHM of the peak, and θ = Bragg's angle of re- flection.

3.2. Fourier transform infrared (FTIR) studies

FTIR spectrum (figure 3) of the plant extract displayed several absorption bands that corresponded to the functional groups of the biomolecules present in the plant extract. The broad band at 3288 cm^{-1} is attributed to hydrogen-bonded O-H groups of alcohols or phenols or carboxylic group [13, 21]. The band at 1658 cm^{-1} is due to the (C = O) stretching band of the carbonyl bond vibration which might indicate the presence of a carboxylic group [22]. The weak band at 1360 and 1211 cm^{-1} are attributed to C-O stretching and C-O-C stretching asymmetric vibration respectively, while the band at 575 cm^{-1} corresponds to the aromatic (C-H) group [23]. The FTIR spectrum of the synthesized CuO-NPs reflects only two main peaks. The bands situated at $\sim 500\text{ cm}^{-1}$ and $\sim 800\text{ cm}^{-1}$ correspond to Cu-O vibrations, which confirm the formation of CuO-NPs [24], and which is in good agreement with values reported in literature [23, 24].

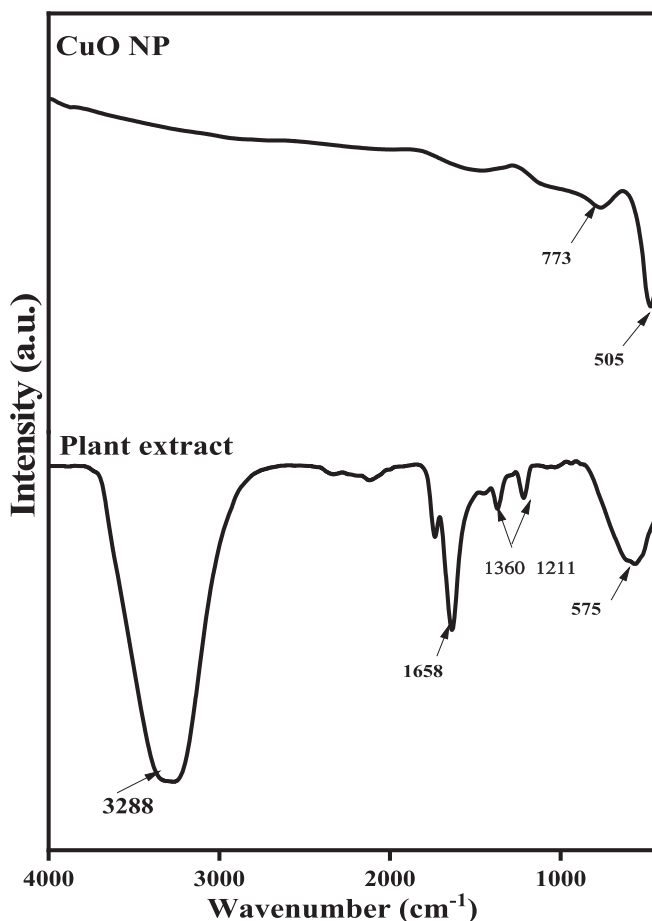


Figure 3. FTIR spectra of *Ligustrum lucidum* plant extract and CuO-NPs.

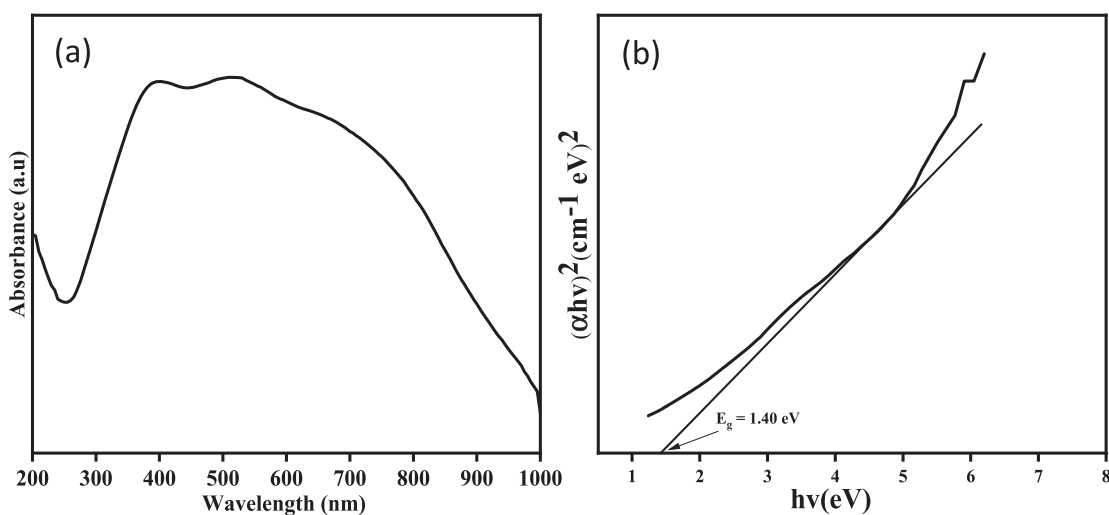


Figure 4. (a) Absorption spectrum and (b) Tauc plot of CuO-NPs synthesized using *Ligustrum lucidum* plant extract.

3.3. UV-visible studies

The UV-visible absorption spectrum of the CuO-NPs is shown in figure 4. The absorption spectrum showed strong absorption in the visible region, with peaks around 405 and 550 nm, ascribed to surface plasmon resonance (SPR). The phenomenon of SPR occurs in metal oxide, and it is an interesting property found in certain transition metals and transition metal oxide. It is mostly exhibited by particles in the nanometric range and has been described as the manifestation of a resonance effect that occurs due to the interaction of

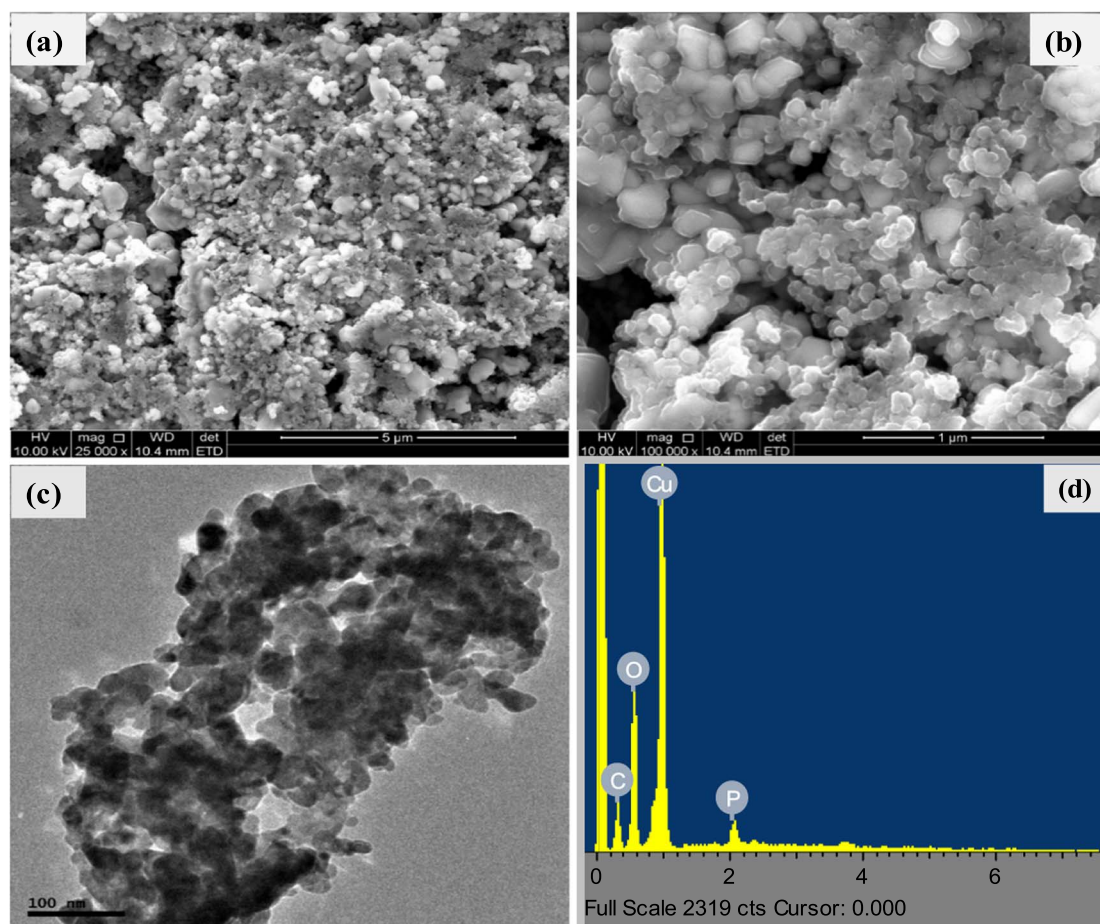


Figure 5. SEM images at (a) low, (b) high magnification, (c) TEM image, and EDX spectrum of CuO-NPs using *Ligustrum lucidum* plant extract.

conduction electrons with incident photons [25]. The degree of this interaction and the dynamics of the process depend on both the size and form of the nanoparticles and the characteristics and composition of the solvent medium [26]. Hence, it gives rise to promising optical applications. In the current study, the wavelength of the SPR closely matches the value reported in previous reports on CuO nanoparticles [27–29].

The bandgap energy was estimated using Tauc's plot given in (equation (4)),

$$\alpha h\nu = A(h\nu - E_g)^n, \quad (4)$$

where α = absorbance coefficient, h = Planck's constant, ν = photon frequency, E_g = optical band gap, and $n = 1/2$ for direct band gap semiconductors. The calculated band gap energy for the CuO-NPs was 1.4 eV, which deviates from the band gap value of bulk CuO (1.24 eV). Khan *et al* [30] reported varying band gaps for different CuO nanostructures, such as ellipsoid-like (1.371 eV), plate-like (1.447 eV), boat-like (1.429 eV), and flower-like (1.425 eV) morphologies, indicating the dependence of optical properties on the morphology of nanoparticles. The larger optical band gap energy of the reported CuO nanoparticles compared to the bulk CuO may also be due to quantum confinement effect [31].

3.4. SEM, TEM, and EDX analysis

Figure 5 shows the morphological and compositional characteristics of CuO-NPs biosynthesized with *Ligustrum lucidum* plant extract. The SEM micrograph (figures 5(a) and (b)) present spherical particles with some degree of agglomeration that results in clusters of particles with no well-defined edges similar to a previous report Peddi, Ptsrk, Rani and Tulasi [32]. The agglomeration indicates a high surface reactivity which could be the result of a high temperature calcination [33, 34]. The TEM images presented in (figure 5(c)) shows spherical morphology, which corroborate the SEM results. Similar to the SEM micrograph, the NPs have close-packed arrangement with significant agglomeration. The EDX spectrograph of CuO-NPs confirms the presence of Cu and O as the main constituent of the nanoparticles which confirms that the NPs formed is CuO (figure 5(d)). The presence of carbon, phosphorous and iron peaks in the EDX spectra could have originated from the bioactive chemical constituents present in the aqueous extract of *Ligustrum lucidum* [35, 36]. Particle size distribution estimation

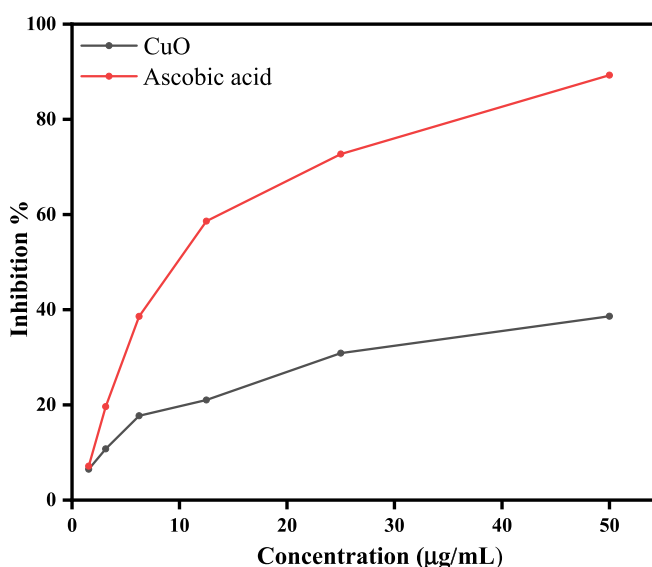


Figure 6. Radical scavenging activity (%) of CuO-NPs and ascorbic acid at various concentrations.

Table 1. Antioxidant activity (%) of CuO-NPs.

Test Samples	Sample Concentration ($\mu\text{g ml}^{-1}$)						IC ₅₀ ($\mu\text{g ml}^{-1}$)
	1.56	3.13	6.26	12.5	25	50	
Ascorbic acid	7.14 ± 0.222	19.63 ± 0.071	38.59 ± 0.07	58.6 ± 0.014	72.7 ± 0.004	89.28 ± 0.015	17.93
CuO-NPs	6.47 ± 0.005	10.76 ± 0.050	17.71 ± 0.006	21.04 ± 0.010	30.89 ± 0.004	38.62 ± 0.236	63.45

Values are expressed as mean inhibition (%) $\pm$ deviation (n = 3). Ascorbic acid is a standard.

obtained from the TEM image of CuO-NPs showed average particle size for CuO NPs was 30 ± 5 nm, which agrees with the value obtained from the Scherrer equation. Furthermore, the particle size distribution shows the CuO-NPs obtained from the aqueous extract of *Ligustrum lucidum* had a narrow size distribution.

3.5. Antioxidant activity analysis of CuO-NPs

Figure 6 and table 1 present the antioxidant properties of CuO-NPs and ascorbic acid as a function of concentration. The radical scavenging activity of CuO-NPs was measured using the DPPH assay and was compared with the standard ascorbic acid. The result shows an increase in the percentage inhibition activity with increasing concentrations of CuO-NPs. The ascorbic acid exhibited a higher scavenging activity than the nanoparticles, with an IC₅₀ value of $17.93 \mu\text{g ml}^{-1}$, indicating its efficiency in capturing the DPPH's free radicals compared to CuO-NPs which has an IC₅₀ value of $63.45 \mu\text{g ml}^{-1}$. These findings align with a previous study by Peddi, Ptsrk, Rani and Tulasi [32] demonstrating that ascorbic acid exhibits a lower IC₅₀ value than CuO-NPs. Comparing the antioxidant activity of the CuO-NPS prepared from the aqueous extract of *Ligustrum lucidum* plant extract showed that it has a higher antioxidant activity than the CuO-NPs obtained from rhizome extract of *Bergenia ciliate* Dulta, Agçeli, Chauhan, Jasrotia, Chauhan and Ighalo [37].

The antioxidant activity of the *Ligustrum lucidum* synthesized CuO-NPs was compared to other CuO nanoparticles as shown in table 2. The CuO-NPs obtained in this study showed comparable antioxidant activity to previously reported CuO-nanoparticles.

3.6. Antifungal analysis of CuO-NPs

Antifungal evaluation of the CuO NPs was carried out on four different pathogenic fungi strains (*A. flavus*, *A. niger*, *F. oxysporum*, and *T. harzianum*). Table 3 shows the percentage inhibition of different concentrations and time intervals of CuO-NPs on the different fungal strain. At day 3 and 5, the percentage inhibition of CuO-NPs in *A. flavus* (29.99, 27.50%), *A. niger* (21.13, 34.52%), and *F. oxysporum* (24.88, 23.31%) respectively, were most significant at 1 ppm concentration. *T. harzianum* on days 5 (19.73%) and 3 (17.87%) produced a higher

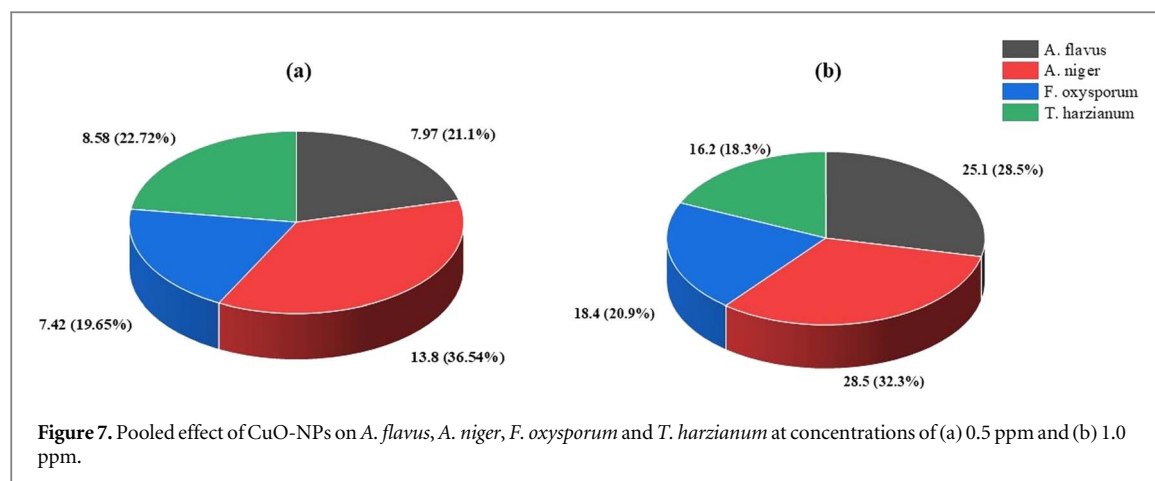


Table 2. Comparison of antioxidant activity of *Ligustrum lucidum* synthesized CuO-NPs to other green synthesized CuO nanoparticles.

Material	Plant extract	IC ₅₀ (μg ml ⁻¹)	References
CuO-S1	<i>A. indica</i>	38.00 ± 2.21	[38]
CuO-S2	<i>H. rosa-sinensis</i>	34.00 ± 1.92	[38]
CuO	<i>Allium sativum</i>	40.52	[39]
CuO	<i>Annona muricata</i>	131.59	[40]
CuO-Mn	<i>Annona muricata</i>	174.81	[40]
CuO-NPs	<i>C. amboinicus</i>	47.13	[41]
CuO-NPs	<i>Ligustrum lucidum</i>	63.45	This study

Table 3. Effect of time interval (days) and concentration of CuO-NPs percentage inhibition on the growth of some pathogenic fungi.

Organism	0.5 ppm	Day 3 1 ppm	0.5 ppm	Day 5 1 ppm	0.5 ppm	Day 7 1 ppm
<i>A. flavus</i>	16.56 ± 4.34a	29.99 ± 6.23a	7.35 ± 3.60b	27.50 ± 7.01a	0.00 ± 0.00b	17.79 ± 8.24a
<i>A. niger</i>	13.79 ± 2.57a	21.13 ± 4.27a	13.44 ± 4.78b	34.52 ± 6.31a	14.27 ± 4.06b	29.81 ± 6.69a
<i>K. oxysporum</i>	3.74 ± 1.47b	24.88 ± 4.41a	16.35 ± 4.08a	23.31 ± 6.98a	2.18 ± 1.23b	7.11 ± 2.94a
<i>T. harzianum</i>	5.35 ± 1.22b	17.87 ± 3.69a	8.01 ± 2.96b	19.73 ± 6.25a	12.39 ± 5.33a	10.86 ± 3.55a

Mean with different letters for each organism between the different concentrations are significantly ($P < 0.05$) different ($n = 4$)

inhibition percentage at 1 ppm concentration compared to day 7 (10.86%). Generally, the percentage inhibition of CuO-NPs against antifungal activity was most significant ($P < 0.05$) at higher concentration (1 ppm). Similar antifungal activity results have been reported [42], for CuO-NPs obtained using leaf the extract of *Zizyphus spina*. The combination of several parameters, including duration of exposure, humidity, and temperature, determines the toxicity of CuO-NPs [43]. According to reports, copper interacts with microorganisms through a variety of mechanisms, including denaturation of nucleic acids, protein modification, membrane lipid peroxidation, and cell membrane permeabilization, all of which can result in cell death [44, 45]. The antifungal efficacy in this study revealed the potential ability of CuO-NPs to provide a surface for killing fungal strain with extensive cell membrane damage through cell vacuole enlargement and disappearance [46, 47].

The pooled mycelium inhibition percentage by CuO-NPs at 0.5 and 1 ppm is shown in figure 7. The pooled mycelium inhibition was higher at 1 ppm, compared to 0.5 ppm for the fungal species. This was similar to the result reported by [45] for *F. oxysporum* mycelium inhibition by CuO-NPs. At both studied concentrations the pooled effect was highest for *A. niger*. At 0.5 ppm a higher pooled effect was observed for *T. harzianum* compared to *F. oxysporum*, however, at 1.0 ppm, the pooled effect of CuO-NPs was higher for *F. oxysporum* compared to *T. harzianum*.

The pooled effect of CuO-NPs after 3, 5, and 7 days is shown in figure 8. After 3 days, the highest mycelium inhibition was observed for *A. flavus*, followed by *A. niger*, while the pooled effect for *F. oxysporum* and *T. harzianum* were almost equal. However, after 5 days, the order of mycelium inhibition was *A. niger* > *F. oxysporum* > *A. flavus* > *T. harzianum*. After 7 days, the order of pooled effect remains unchanged. This shows the higher susceptibility of *A. flavus* and *A. niger* to CuO-NPs compared to *T. harzianum*. Furthermore, the

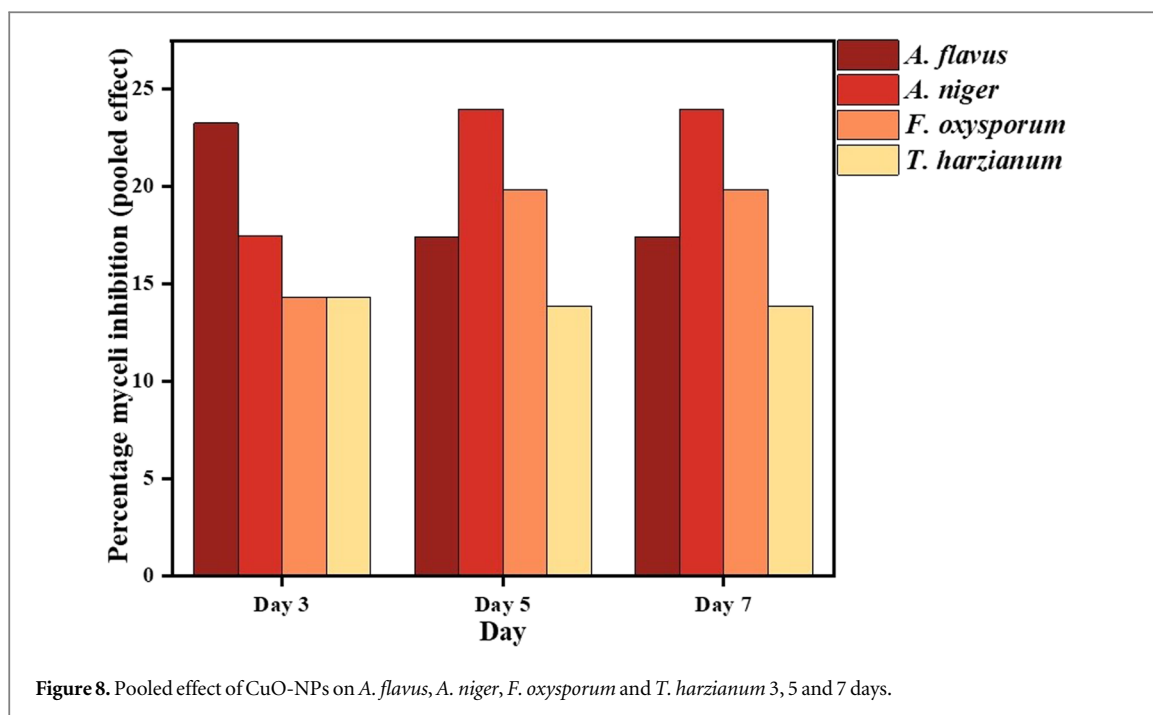


Table 4. Comparison of antifungal activity of *Ligustrum lucidum* synthesized CuO-NPs to other green synthesized CuO nanoparticles.

Test pathogens	NpS used (Concentration/amount)	Method	Fungi toxicity	References
<i>Aspergillus flavus</i>	Cu- NPs (10 μ l/well)	Agar well diffusion	20 mm (Zone of inhibition)	[48]
<i>Aspergillus oryzae</i>			23 mm (Zone of inhibition)	
<i>Aspergillus flavus</i>	CuO NPs 25 μ l	Agar well diffusion	13.1 (Zone of inhibition)	[49]
<i>Aspergillus niger</i>			16.2 (Zone of inhibition)	
<i>Aspergillus niger</i>			19 mm (zone of inhibition)	
	25 μ g ml ⁻¹		19 mm (zone of inhibition)	
	50 μ g ml ⁻¹		21 mm (zone of inhibition)	
	75 μ g ml ⁻¹			
flavus	CuO-NPs 20 μ l	Biochemical assay	27% inhibition rate	This study.
niger			34% inhibition rate	
<i>T. harzianum</i>			19% inhibition rate	

difference in the mycelium inhibition percentage on the studied fungi was only significant after 5 days. The comparison of the antifungal activity of the obtained CuO-NPs was compared to previously reported CuO nanoparticles as shown in table 4.

4. Conclusion

CuO-NPs were successfully synthesized via the green route by using *Ligustrum lucidum* plant extract as a mediating agent and was characterized by different techniques. XRD pattern of the nanoparticles confirmed a monoclinic structure of CuO-NPs with an average size of 22 nm. The maximum absorption peak of the nanoparticles was at 550 nm, with a band gap energy of 1.4 eV. The antioxidant activity of CuO-NPs as assessed through its radical scavenging potential, exhibited a lower scavenging activity than that of ascorbic acid, but can also be used as a potent agent for combating oxidative stress. Furthermore, the CuO-NPs exhibited high antifungal efficacy against *A. flavus*, *A. niger*, *F. oxysporum*, and *T. harzianum* fungal strains, which suggests a novel avenue for addressing fungal-related ailments. Hence, this indicates that green synthesized CuO-NPs can be used as a potent antioxidant and antifungal agent for various treatment purposes. However, a significant limitation of this study is the variation in the synthesis condition that may arise from sourcing plant materials from different places, which could affect the reproducibility of the process.

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Data availability statement

The data cannot be made publicly available upon publication because they are not available in a format that is sufficiently accessible or reusable by other researchers. The data that support the findings of this study are available upon reasonable request from the authors.

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Author contributions

DMNM and DCO conceptualized and designed the work and reviewed the draft. MCO and ASA carried out the study and were involved in the manuscript preparation. OOO provided resources for the study and reviewed the draft. All the authors contributed to the manuscript and agreed to its publication.

Conflict of interest

The authors declare no conflict of interest.

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