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Biogenic Copper Oxide Nanoparticles from *Acacia caesia*: A study on Antifungal, *In vitro* Antioxidant, and Cytotoxic properties

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ABSTRACT

Biogenic synthesis of nanoparticles using plant extracts has garnered significant interest in recent years. Copper oxide (CuO) nanoparticles (NPs), synthesized via Bio methods, have found extensive applications in biomedical fields. In this study, CuO NPs were synthesized using an *Acacia caesia* (L) aqueous leaf extract and characterized by UV-visible spectroscopy, FTIR, XRD, SEM-EDAX, and HRTEM. The results confirmed the successful formation of monoclinic crystalline structures with nearly spherical shapes and an average particle size of 7.09 nm. FTIR analysis identified functional groups of phytochemicals responsible for the reduction and stabilization of CuO NPs. The Bio-synthesized CuO NPs demonstrated potent antifungal activity against *Candida albicans*, with a zone of inhibition (ZOI) of 21mm and a minimum inhibitory concentration (MIC) of 250 µg/mL. Additionally, they exhibited strong antioxidant activity, with a low IC_{50} value of 37.18 µg/mL, and notable cytotoxicity against human cervical cancer cell lines, achieving an IC_{50} value of 69.58 µg/mL. These findings suggest that Bio-synthesized CuO NPs hold promise as therapeutic agents for treating fungal infections and oxidative stress-induced diseases.

Keywords: Biogenic Synthesis, *Acacia caesia* (L.), phytochemicals, Antifungal activity, Antioxidant activity ,

1. INTRODUCTION:

Nanotechnology focuses on manipulating materials at the atomic scale to create nanostructured materials with unique structural, optical, electrical, magnetic, and mechanical properties. These materials are vital in fields such as medicine, drug delivery, biosensing, energy, electronics, catalysis, food, agriculture, textiles, and polymers [1]. Among various nanoparticles, metal oxide nanoparticles stand out due to their versatile surface properties. Copper oxide (CuO) nanoparticles are widely studied because of their cost-effectiveness, chemical inertness, thermal stability, catalytic activity, narrow bandgap, and role as a p-type semiconductor [2]. Their properties are influenced by their size, shape, and composition, which makes them important for a range of applications [3].

CuO nanoparticles show strong antimicrobial properties and biocompatibility, making them useful for drug delivery, wound healing, antioxidant therapies, and pharmaceutical applications [4]. These nanoparticles are also used in dye-sensitized solar cells, filters, paints, polymers, and textiles. Industrial applications include gas sensors,

solar cells, and high-temperature superconductors [5]. Most synthesis methods, such as sol-gel, precipitation, hydrothermal, microwave irradiation, and vacuum-free spin coating, rely on toxic chemicals, require high energy, and produce harmful waste, raising concerns about their environmental impact [6].

Green synthesis has become a sustainable alternative for producing CuO nanoparticles, relying on natural and renewable resources such as bacteria, fungi, algae, yeast, and plant extracts [7]. Plant-mediated synthesis is particularly advantageous, as it uses biomolecules like flavonoids, proteins, tannins, phenols, and terpenoids as reducing and stabilizing agents [8]. Various plants, including *Piper nigrum* [9], *Balanites aegyptiaca* [10], *Eucalyptus globulus* [11], *Ephedra alata* [12], *Aerva javanica* [13], and *Juglans regia* [14] , have been explored for this purpose.

Acacia caesia (L.), a woody shrub native to the Western Ghats of India, has therapeutic uses for gastrointestinal issues, skin disorders, wound healing, asthma, and menstrual problems [15]. Its leaves, stem, and bark contain bioactive compounds such as alkaloids, flavonoids, terpenoids, phenolic compounds, and tannins [16]. Previous studies on *Acacia caesia* (L.) leaves have shown their potential in synthesizing iron oxide nanoparticles with antioxidant and anti-inflammatory activities [17] and silver nanoparticles with insecticidal properties [18]. Research on using *Acacia caesia* (L.) aqueous leaf extract for CuO nanoparticle synthesis has not been conducted.

This study aims to produce CuO nanoparticles from *Acacia caesia* (L.) aqueous leaf extract and assess their antifungal, *in vitro* antioxidant, and cytotoxic properties. The approach focuses on combining environmentally friendly synthesis methods with the bioactive potential of *Acacia caesia* (L.) for biomedical and industrial applications.

2. Materials and Methods:

2.1. Collection and Preparation of Acacia caesia Leaf Aqueous Extract

Fresh leaves of *Acacia caesia* were collected from Diwancheruvu, Andhra Pradesh, India. Their morphology was confirmed based on established references. The leaves were washed thoroughly with tap water, followed by double-distilled water, and then air-dried. The dried leaves were powdered and used for aqueous extraction. A 1% solution was prepared by dissolving the powdered leaves in distilled water and heating the mixture at 60°C for 20 minutes with constant stirring. After cooling to room temperature, the extract was filtered using muslin cloth and Whatman No. 1 filter paper. The aqueous extract was stored at 4°C for further experiments.

2.2. Phytochemical Screening and Total Polyphenol Content

2.2.1. Qualitative Phytochemical Analysis: The extract was screened for flavonoids, phenols, tannins, alkaloids (Mayer's and Wagner's tests), terpenoids, anthraquinones, saponins, quinones, coumarins, glycosides, and steroids using standard protocols.

2.2.2. Total Polyphenol Content: The total polyphenol content was estimated by mixing 10 mg of dried extract with 5% DMSO, filtering it through Whatman No. 1 paper, and adding 10% Folin-Ciocalteu reagent and 7.5% sodium carbonate. After a two-hour incubation at room temperature, absorbance was measured at 765 nm. Gallic acid was used as a standard.

2.3. Biogenic Synthesis and Characterization of AC-CuO Nanoparticles

The synthesis of AC-CuO nanoparticles was carried out using the co-precipitation method, 1 g of copper(II) nitrate trihydrate was dissolved in 90 mL of distilled water under

continuous stirring. A 10 mL portion of the *Acacia caesia* aqueous extract was added to this solution, and the temperature was increased to 80°C. The reaction mixture changed color from blue to deep green, and sodium hydroxide (1 M) was added dropwise until the solution turned black, indicating the formation of AC-CuO nanoparticles. The precipitate was centrifuged, washed with distilled water and 70% ethanol, and dried at 70°C in a hot air oven.

The UV-Vis spectrophotometer measured the absorbance of the extract and nanoparticles at 200–800 nm. FTIR spectroscopy analyzed functional groups involved in the capping and reduction of nanoparticles. XRD was employed to determine the crystalline size and structure, while FESEM-EDX provided details about morphology and elemental composition. HRTEM confirmed nanoparticle size and distribution.

2.4. Antifungal Activity:

Antifungal activity was carried out using two methods microdilution broth method and disc diffusion method. In microdilution broth method is used to determine the Minimum Inhibitory Concentration (MIC) against *Aspergillus niger* (MTCC 282) and *Candida albicans* (MTCC 183) was determined using a 96-well microtiter plate. Fungal suspensions (10 CFU/mL) in LB broth were treated with AC-CuO nanoparticles at concentrations of 1000–3.9 µg/mL. Wells containing 1% TTC solution served as controls. Plates were incubated at 37°C for 24 hours, and growth inhibition was evaluated visually. In disc diffusion assay fungal suspensions (10 CFU/mL) were inoculated on MHA containing 2% glucose. Discs soaked in 100 µg amphotericin B, AC-CuO nanoparticles, and *Acacia caesia* extract were placed on the agar. Inhibition zones were measured after 24 hours of incubation at 37°C.

2.5. *In vitro* antioxidant activity:

The antioxidant potential of AC-CuO nanoparticles was assessed using three standard methods: DPPH free radical scavenging, Ferric Reducing Antioxidant Power (FRAP), and ABTS cation radical scavenging assays. Nanoparticle concentrations ranging from 500 to 2000 µg/mL were tested in each assay. Ascorbic acid was used as the standard antioxidant reference for comparison.

2.5.1. DPPH Free Radical Scavenging Assay: A solution of DPPH (3×10^{-3} M in methanol) was mixed with various concentrations of AC-CuO nanoparticles in methanol. A control sample containing only DPPH and methanol was prepared. The mixtures were incubated in the dark for 30 minutes at room temperature, after which the absorbance was recorded at 517 nm. The decrease in absorbance indicated the scavenging of DPPH free radicals. The percentage inhibition of DPPH radicals was calculated using the formula 1 and The IC_{50} value (concentration required to inhibit 50% of free radicals) was calculated from the inhibition plot

2.5.2. FRAP Assay: The FRAP reagent was prepared by mixing 10 mM TPTZ in 40 mM HCl, 20 mM FeCl , and 0.3 M acetate buffer (pH 3.6) in a 1:1:10 ratio. This reagent was mixed with varying concentrations of AC-CuO nanoparticles in acetate buffer. A control sample containing only the FRAP reagent and acetate buffer was included. The reaction mixtures were incubated at room temperature for 30 minutes, and the absorbance was measured at 593 nm. Higher absorbance values indicated greater ferric-reducing antioxidant potential. The percentage inhibition and IC_{50} values were calculated as described earlier.

2.5.3. ABTS Radical Cation Scavenging Assay: The ABTS reagent was prepared by mixing 7 mM ABTS solution with 2.45 mM potassium persulfate in equal volumes and incubating it in the dark for 12-16 hours to form the ABTS radical cation. The reagent was diluted with methanol until the absorbance at 734 nm reached 0.7 ± 0.02. This reagent was then mixed with different concentrations of AC-CuO nanoparticles. The control contained only the ABTS reagent and distilled water. After incubation for 30 minutes at room temperature, the absorbance was measured at 734 nm. The percentage

inhibition and IC_{50} values were calculated using the same formula.

2.6.*In vitro* cytotoxicity: Cytotoxicity was evaluated using the MTT assay on HeLa cell lines. Cells were treated with AC-CuO nanoparticles at concentrations ranging from 0 to 200 µg/mL and incubated for 48 hours at 37°C in a CO incubator. After incubation, MTT reagent was added, and absorbance was measured at 540 nm using an ELISA reader. Cell viability percentage was calculated, and the IC_{50} value was determined based on the plotted graph.

3. Result and Discussion:

3.1. Phytochemical Investigation:

Preliminary phytochemical screening of *Acacia caesia* leaf aqueous extract (ACLAE) revealed the presence of alkaloids, flavonoids, tannins, and phenols, which are critical bioactive compounds. The total polyphenol content (TPC) was determined to be 120 mg of gallic acid equivalent /g of dry extract. Previous studies have demonstrated that plant- derived polyphenols act as effective reducing and capping agents in nanoparticle synthesis, facilitating the stabilization of metal nanoparticles. These bioactive compounds not only contribute to the synthesis process but also enhance the biological activities of the nanoparticles due to their synergistic interactions with metal oxides [19]. Similar phytochemical profiles have been reported for *Acacia species*, which possess potent antioxidant and antimicrobial properties, further validating the current findings [20], [21].

3.2.Characterization of AC-CuO Nanoparticles:

3.2.1. UV-Vis Spectroscopy:

The UV-Vis spectrum of ACLAE exhibited characteristic peaks between 250–400 nm, corresponding to the electronic transitions in phenolic and flavonoid compounds (Fig 1(a)). The addition of ACLAE to the copper precursor resulted in a color change from light green to dark brownish-black, indicating the formation of CuO nanoparticles. A distinct surface plasmon resonance (SPR) peak at 278 nm confirmed the synthesis of CuO nanoparticles represented in Fig 1(b). The shift to shorter wavelengths is consistent with smaller nanoparticle sizes and has been observed in similar green synthesis studies, such as CuO nanoparticles synthesized using *Moringa oleifera* [22] and *Passiflora edulis* [23], which reported SPR peaks in a comparable range. The observed SPR peaks align with the optical properties expected for CuO nanoparticles.

The UV absorbance spectrum of the synthesized AC-CuO nanoparticles reveals the direct bandgap energies, measured at 4.19 eV and 4.69 eV (Fig 1(c)), suggest that the nanoparticles possess strong light absorption capabilities in the UV region. These direct
transitions occur when photons are absorbed, promoting electrons directly from the valence to the conduction band without the need for a phonon. Conversely, the indirect bandgap energies at 3.92 eV and 4.36 eV (Fig 1(d))s indicate transitions where photon absorption is accompanied by a phonon to conserve momentum. This dual bandgap nature suggests that the AC-CuO nanoparticles have potential for various optical applications, where both direct and indirect transitions might be advantageous, such as in photocatalytic processes and optoelectronic devices.

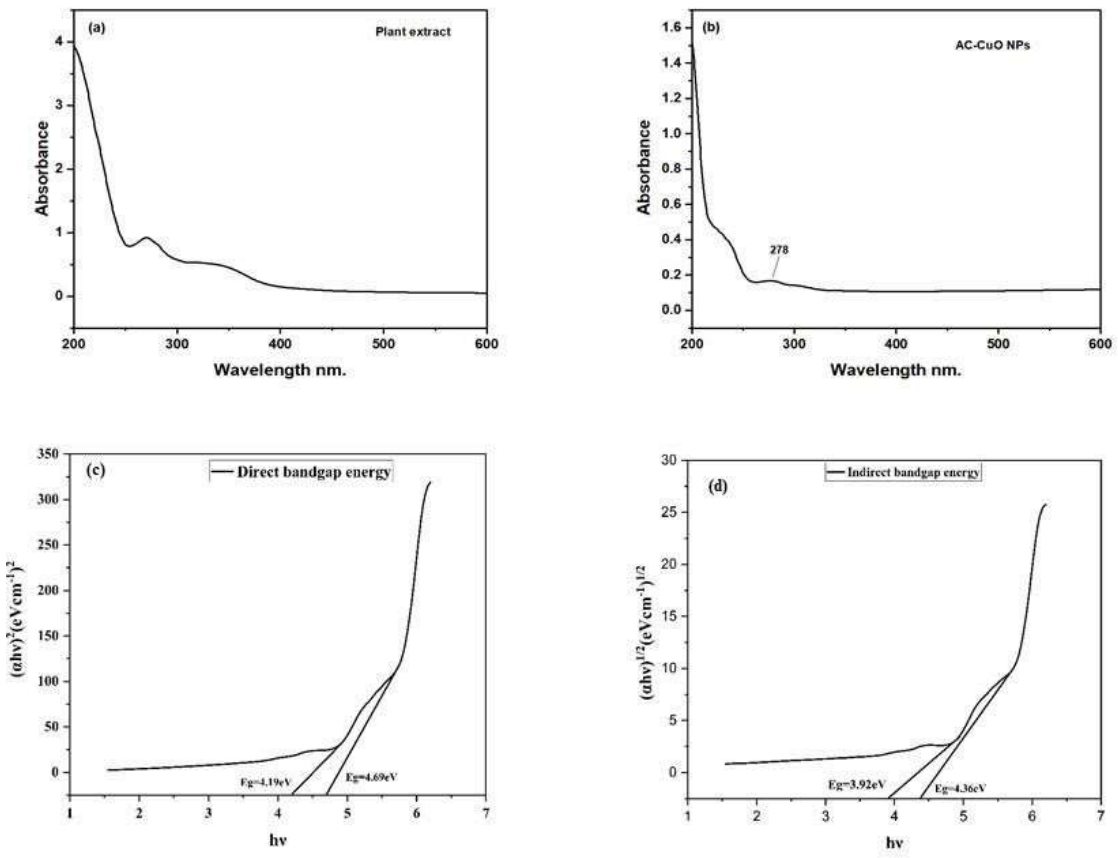

Figure 1: UV and band gap analysis (a) UV absorbance spectrum of ACLAE (b) UV absorbance of AC-CuO NPs (c) Direct bandgap energy of AC-CuO NPs and (d) Indirect bandgap energy of AC-CuO NPs.

3.2.2. FTIR Analysis:

FTIR analysis of ACLAE revealed peaks at 3250–3360 cm ¹ (O-H stretching), 2100 cm ¹ (C≡C stretching), 1630 cm ¹ (C=C stretching), and 509 cm ¹ (C-I stretching). Post- synthesis, AC-CuO nanoparticles exhibited additional peaks at 2899 cm ¹ and 2833 cm ¹ (C- H stretching), 1730 cm ¹ (C=O stretching), 1390 cm ¹ (O-H bending), 1160 cm ¹ (C-
O stretching), and 764 cm ¹ and 659 cm ¹ (metal-oxygen bond vibrations) (Figure 2). The FTIR results indicate the involvement of phenolic and flavonoid compounds in reducing and stabilizing CuO nanoparticles. These findings are similar with earlier studies, such as CuO nanoparticles synthesized using *Eucalyptus globulus* [11], where phenolic groups were shown to play a similar role in capping and stabilization.

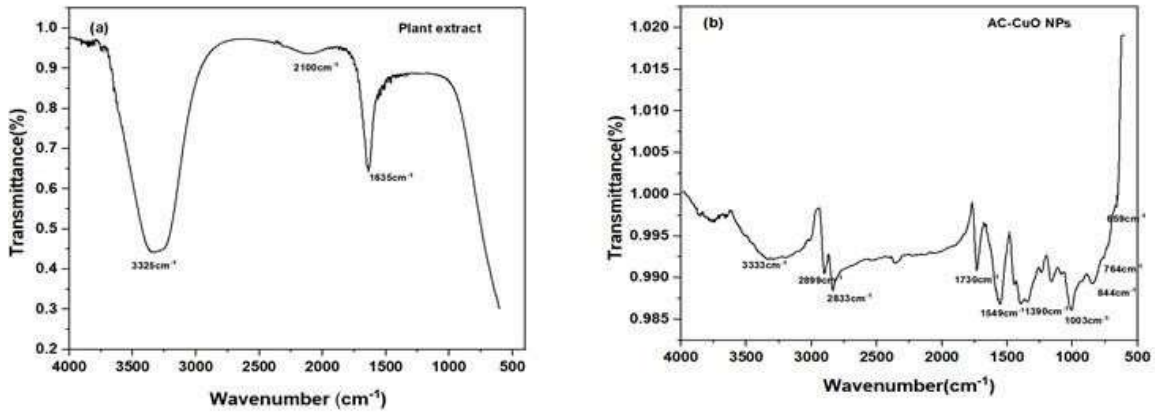

Figure 2: FTIR transmittance peaks (a) ACLA extract and (b) AC-CuO NPs.

3.2.3. XRD Analysis:

XRD analysis confirmed the crystalline structure of AC-CuO nanoparticles, with peaks at 2θ values of 35.43°, 35.65°, 38.67°, 48.90°, 53.43°, and 61.63°, corresponding to crystal planes
002, -111, 111, -202, 020, and -113, respectively (Fig 3). These results align with JCPDS card no. 01-089-2529, indicating a monoclinic tenorite phase. The average crystallite size, calculated as 12.60 nm using the Debye-Scherrer equation, is similar in results of other green synthesis methods, such as CuO nanoparticles synthesized from *Balanites aegyptiaca* leaves [10].

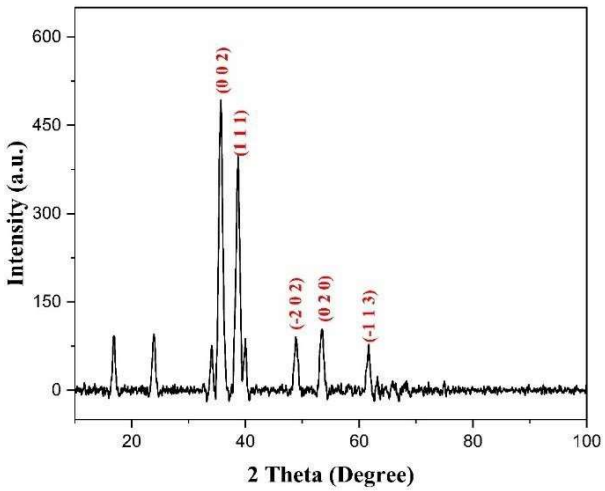

Figure 3: X-ray diffractogram of AC-CuO NPs.

3.2.4. FESEM and EDX:

FESEM images showed partly spherical AC-CuO nanoparticles with moderate agglomeration, attributed to high surface energy. Similar morphologies have been reported in CuO nanoparticles synthesized from *Aerva javanica*

[13]. EDX analysis confirmed the elemental composition, showing Cu and O as major components with weight percentages of 66.6% and 33.4%, respectively (Fig 4). The purity of the nanoparticles was validated, with minor peaks likely due to residual phytochemicals from the plant extract.

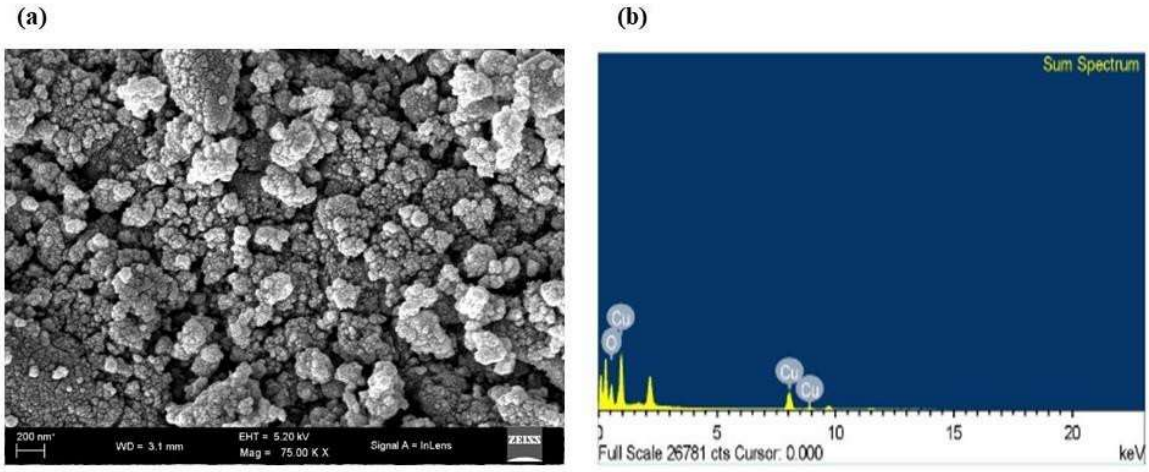

Figure 4: Morphology and elemental composition (a) FESEM Micrograph and (b) EDX spectrum.

3.2.5. HRTEM Analysis:

HRTEM analysis revealed AC-CuO nanoparticles are moderately agglomerated due to capping of plant phytochemicals and fine fringes are small. The polycrystalline nature was observed for AC-CuO nanoparticles using selected area electron diffraction (SAED) pattern and particle size diameter distribution is 0-25 nm with an average particle size diameter of
7.09 nm (Fig 5).

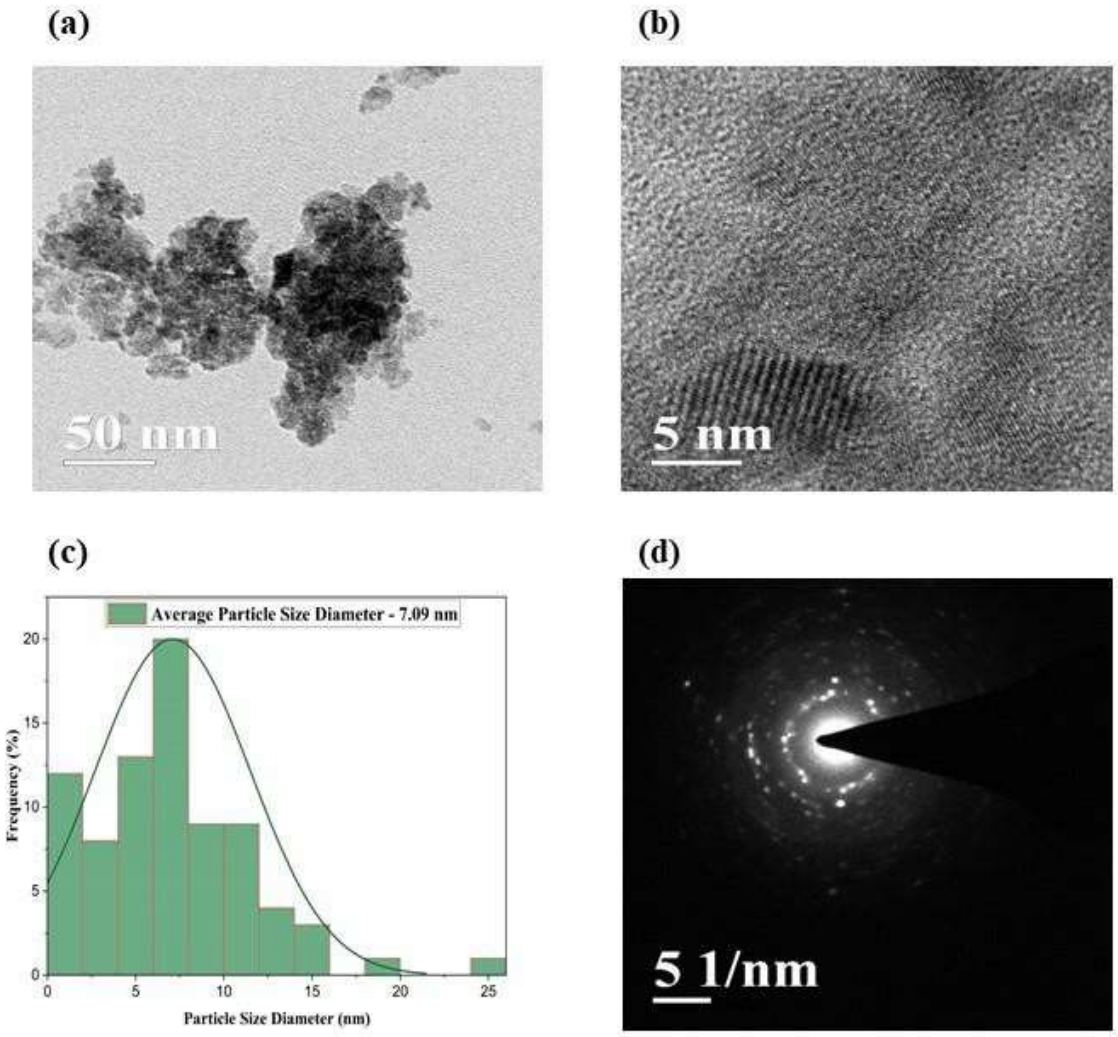

Figure 5: HRTEM analysis (a) HRTEM micrograph (b) Fine fringes (c) Particle size diameter histogram and (d) SAED pattern.

3.3. Antifungal Activity:

AC-CuO nanoparticles exhibited potent antifungal activity against *Candida albicans* and *Aspergillus niger*. The zone of inhibition for *C. albicans* (21 mm) at 100 µg/mL showed higher zone of inhibition than standard Amphotericin B (16 mm), while for *A. niger*, the zone of inhibition was 16 mm. The MIC values were 250 µg/mL and 500 µg/mL for *C. albicans* and *A. niger*, respectively. Mechanisms of action include disruption of cell walls and membranes, generation of ROS, and release of metal ions, which damage fungal proteins and DNA [24]. Studies on CuO nanoparticles synthesized from *Moringa oleifera* leaves showed slightly lower antifungal efficacy, with MIC values of 125 µg/mL for *A. niger* and 62.5 µg/mL for *C. albicans* [22]. It was found that, the ACLAE didn't shown any zone of inhibition but CuO nanoparticles synthesized using ACLAE showed the superior antifungal potential.

3.4. *In vitro* Antioxidant Activity:

The *in vitro* antioxidant activity of AC-CuO nanoparticles was evaluated using DPPH, FRAP, and ABTS assays. The ABTS assay demonstrated the highest scavenging activity (89.33%) at 2000 µg/mL, with an IC_{50} value of 37.18 µg/mL, indicating exceptional antioxidant potential. The DPPH assay showed 73.59% scavenging activity (IC_{50}: 1530 µg/mL), and the FRAP assay exhibited 85.07% reducing power (IC_{50}: 841.1 µg/mL) (Fig 6). These values a those are effective than the CuO nanoparticles synthesized using *Passiflora edulis* leaves [23], which had a DPPH IC_{50} of 1000 µg/mL. The enhanced antioxidant activity is attributed to the high surface area and active functional groups of the nanoparticles.

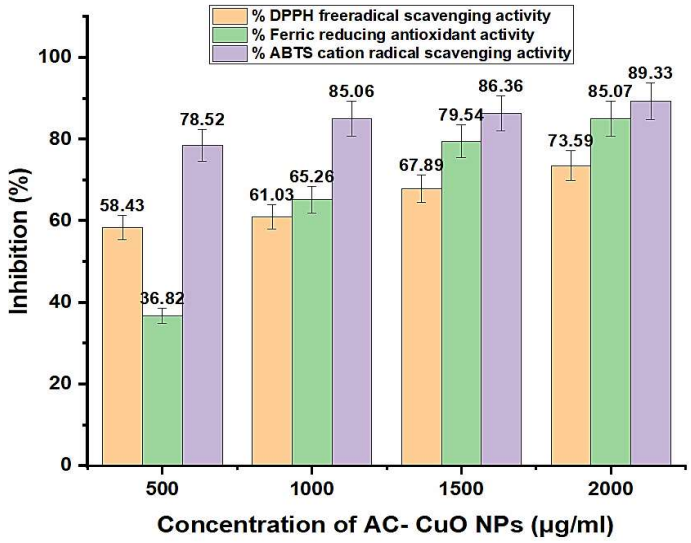

Figure 6: *In vitro* antioxidant activity

3.5. *In vitro* cytotoxicity:

The *in vitro* cytotoxicity of AC-CuO nanoparticles against HeLa cells was assessed using the MTT assay. A significant dose-dependent effect was observed, with cell viability decreasing to 21.78% at 200 µg/mL (Fig 7). The IC_{50} value was calculated as 69.58 µg/mL. The nanoparticles likely induce apoptosis through ROS generation and disruption of cellular pathways [25]. CuO nanoparticles synthesized from *Erythrina variegata* showed a slightly lower IC_{50} of 48 µg/mL [26]. The small size and active surface properties of AC-CuO nanoparticles contribute to their effectiveness as anticancer agents.

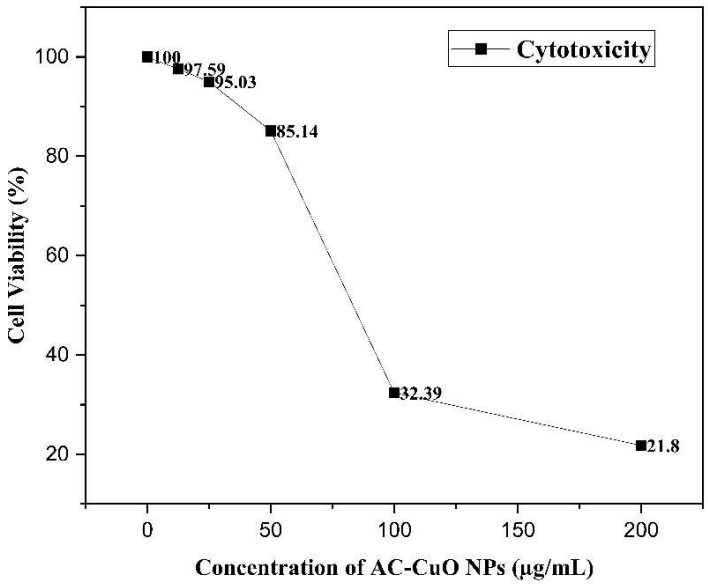

Figure 7*: In vitro* cytotoxicity

4. Conclusion and Future Perspectives:

This study successfully synthesized biogenic copper oxide (CuO) nanoparticles using *Acacia caesia* leaf extract, revealing a robust antifungal, antioxidant, and cytotoxic potential against human cervical cancer cells. Characterization techniques like UV-Vis spectroscopy, FTIR, XRD, SEM-EDX, and HRTEM confirmed the nanoparticles monoclinic crystalline structure and spherical morphology with an average size of 7.09 nm. The nanoparticles exhibited significant antifungal activity against *Candida albicans*, surpassing the efficacy of standard antifungal agents, and demonstrated impressive antioxidant capabilities with a low IC_{50} value, indicating their potential as therapeutic agents. The *in vitro* cytotoxicity results further validated their applicability in biomedical applications, particularly in targeting cancer cells. Future studies should focus on the mechanistic pathways through which these nanoparticles exert their biological effects, particularly their interaction with cellular components and the generation of reactive oxygen species. Finally, *in vivo* studies are essential to assess the therapeutic potential and biocompatibility of these nanoparticles, setting the stage for clinical trials and subsequent pharmaceutical applications.

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Author Contribution:

Shyamala Pulipaka: Supervision, conceptualization, editing and reviewing of manuscript

Manchiraju Padmaja: Methodology, resources, investigation and manuscript writing.

Deva H Puranam: Editing and reviewing of manuscript.

Data Availability:

Addressed to Shyamala Pulipaka

Declarations

Competing and Conflict of interest: The authors declare no competing and conflict of interest.

Ethics Approval and Consent to Participate: Not applicable.
Consent for Publication: Not applicable.
Research Involving Humans and Animals Statement: The article does not contain any studies involving human participants and animals performed by any of the authors.
Informed Consent: Not Applicable.

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