

ECO-FRIENDLY SYNTHESIS OF COPPER OXIDE NANOPARTICLES USING *Ayapana triplinervis* : A STUDY ON ANTIBACTERIAL EFFICACY AND PHYTOCHEMICALS

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ABSTRACT

Our research explored an environmentally sustainable synthesis method for producing copper oxide nanoparticles (CuO NPs) using leaf extract from *Ayapana triplinervis*. This study was carried out in December 2023 at the Department of Chemistry, Carmel College (Autonomous), Mala, with assistance from the institution's Biochemistry Lab for conducting antibacterial studies. The synthesis involved mixing *Ayapana triplinervis* leaf extract with a copper precursor solution, resulting in the formation of CuO nanoparticles. Characterization using UV-Vis spectroscopy and X-ray diffraction (XRD) confirmed the successful formation and morphology of the CuO nanoparticles. XRD analysis specifically confirmed the monoclinic structure of the synthesized CuO nanoparticles, with an average crystallite size of 28 nm determined by the Debye-Scherrer formula. Our research highlights the role of various phytochemicals present in the leaf extract—saponins, flavonoids, phenols, starch, coumarins, and anthocyanins—in facilitating the formation of CuO nanoparticles. These bioactive metabolites act as reducing and capping agents, aiding in the synthesis and stabilization of the nanoparticles. The antibacterial efficacy of the synthesized CuO NPs was evaluated against two common pathogenic bacteria, *Escherichia coli* and *Staphylococcus aureus*, using the agar well diffusion method. The results demonstrated significant antibacterial activity, with the CuO NPs producing a 4 mm zone of inhibition against *E. coli* at a concentration of 10 mg ml⁻¹. In contrast, a more substantial antibacterial effect was observed against *S. aureus*, with a 10 mm zone of inhibition at the same concentration. These findings suggest that *Ayapana triplinervis* leaf extract can serve as a viable and green alternative for synthesizing CuO nanoparticles with notable antibacterial properties. This method stands out for its simplicity, affordability, and avoidance of toxic chemicals. Future research should focus on elucidating the detailed mechanisms by which these phytochemicals facilitate nanoparticle formation, as well as exploring the broader spectrum of antibacterial activities and potential biomedical applications of the synthesized CuO NPs.

(Key words: Copper Oxide, nanoparticles, zone of inhibition, monoclinic, disc diffusion method, antibacterial challenges, crystalline size)

INTRODUCTION

Ayapana triplinervis, belonging to the Asteraceae family, is a perennial herbaceous plant indigenous to South America, notably present in countries like Brazil, Argentina, and Paraguay. Recognizable by its aromatic leaves and clustered small flowers, this plant thrives in its natural habitat. Its widespread presence in the region underscores its ecological significance and potential for various uses. Its medicinal value stems from the presence of bioactive compounds which contribute to its pharmacological effects (Dev *et al.*, 2018).

Copper oxide (CuO) nanoparticles have garnered significant interest in various fields due to their unique physicochemical properties and versatile applications. They hold promise in diverse areas such as catalysis, electronics, energy storage, environmental remediation, and biomedicine (Zhang *et al.*, 2006; Gou *et al.*, 2008; Wijesundera, 2010; Gawande *et al.*, 2016; Dahoumane *et al.*, 2017).

Traditional synthesis routes include chemical precipitation, hydrothermal synthesis, sol-gel methods, and thermal decomposition (Zhou *et al.*, 2006; Mittal *et al.*, 2013; Renuga *et al.*, 2020). However, these methods often involve the use of hazardous chemicals, high temperatures, and energy-intensive processes, raising concerns about environmental sustainability and biocompatibility. In recent years, there has been growing interest in green synthesis approaches for the production of CuO nanoparticles, aiming to address the limitations associated with conventional methods. Green synthesis methods utilize natural sources such as plants, microorganisms, and bio-waste materials as reducing and stabilizing agents, offering several advantages including eco-friendliness, cost-effectiveness, scalability, and biocompatibility (Iravani, 2011; Ahmed *et al.*, 2019). Among these green synthesis approaches, the use of plant extracts has emerged as a promising strategy due to the abundance of phytochemicals with reducing and capping capabilities

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(Sutradhar *et al.*, 2014; Jayarambabu *et al.*, 2020). *Ayapana triplinervis*, presents an intriguing source for the green synthesis of CuO nanoparticles. The plant's phytochemical profile, including flavonoids, terpenoids, and phenolic compounds, suggests its potential as a reducing agent for nanoparticle synthesis. Furthermore, the eco-friendly nature of *Ayapana triplinervis* aligns with the principles of sustainable nanotechnology, making it an attractive candidate for green synthesis approaches.

In this context, our study aims to explore the green synthesis of CuO nanoparticles using *Ayapana triplinervis* leaf extract and evaluate their potential applications, particularly in the field of antibacterial activity. By harnessing the natural resources offered by *Ayapana triplinervis* and employing green synthesis principles, we can develop CuO nanoparticles with enhanced

biocompatibility and reduced environmental impact.

MATERIALS AND METHODS

Chemicals and materials

All the chemical reagents (analytical grade) were purchased from Sigma–Aldrich Chemicals, India. Test organisms for antibacterial studies, *Staphylococcus aureus* and *Escherichia coli* were collected from Poly labs, Thrissur. Distilled water was used for the preparation of extract and the solutions of metal salt. Fresh leaves of *Ayapana triplinervis* were collected from Carmel College (Autonomous), Mala campus on 10th December 2023. The collected leaf material was tightly packed in polyethene bag and then transferred to the chemistry laboratory.



Figure 1. *Ayapana triplinervis*

Preparation of leaf extract

Fresh leaves of the plant were washed three times under running tap water and then in double distilled water to remove the impurities. It was then dried and cut into fine pieces. The leaf extract was prepared by adding 50 g leaves to 500 ml distilled water and boiling at 100 °C for 30 min. The mixture was allowed to cool to room temperature and filtered using Whatman no.1 filter paper.

Synthesis of CuO nanoparticle

Copper oxide nanoparticles were obtained by combining the prepared leaf extract in a 1:9 ratio with a 1 mM concentrated anhydrous copper sulfate solution. The mixture was stirred at room temperature for two hours. CuO NPs were synthesized, as evidenced by the solution's dark green color. After cooling, the colloidal was centrifuged to obtain residue, which was then purified by washing three to five times with double distilled water. The final residue obtained was calcinated in a muffle furnace at 400 °C to remove the attached organic matter of plant, powdered and stored for further use (Dev *et al.*, 2018).

Phytochemical screening test

The leaf extract of *Ayapana triplinervis* was subjected to phytochemical analysis in accordance with recent methodologies of plant analysis (Yadav and Munin,

1. Test for tannins and phenols

Ferric chloride test : Crude extract was mixed with 2 ml of 2 % solution of FeCl₃. A blue-green or black coloration indicated the presence of phenols and tannins (Lawal *et al.*, 2019 ; Khan *et al.*, 2023).

2. Test for saponins

About 1 ml of the extract was boiled in 20 ml of distilled water in a water bath and filtered. 10 ml of the filtrate was mixed with the 5 ml of distilled water and mixed vigorously for 15 min to form a stable persistent froth. The presence of froth after 5 min was taken as an indication of presence of saponins (Lawal *et al.*, 2019 ; Khan *et al.*, 2023).

3. Test for coumarins

; To 1 ml of extract, 1ml of 10 % NaOH was added. Formation of yellow colour indicated the presence of coumarins (Lawal *et al.*, 2019 ; Khan *et al.*, 2023).

4. Test for flavonoids

Ammonia test: A few drops of 1 % NH₃ solution was added to 1ml of the extract in a test tube. A yellow coloration was observed for the presence of flavonoids (Lawal *et al.*, 2019 ; Khan *et al.*, 2023).

Alkaline reagent test: Few drops of 20 % NaOH solution was added to 1 ml of extract. On addition of HCl, the changed yellow colour of the extract turned to a colourless solution that depicted the presence of flavonoids (Lawal *et al.*, 2019; Khan *et al.*, 2023).

Shinoda test: Crude extract was mixed with few fragments of magnesium ribbon and concentrated HCl was added drop wise. Pink scarlet colour appeared after few minutes which indicated the presence of flavonoids (Lawal *et al.*, 2019 ; Khan *et al.*, 2023).

5. Test for terpenoids

Salkowski test: One ml of each extract was mixed with 0.5 ml of chloroform and 1 ml of concentrated H₂SO₄ was carefully added to form a layer. A reddish-brown colouration of the interface formed showed positive results for the presence of terpenoids (Lawal *et al.*, 2019 ; Khan *et al.*, 2023).

6. Test for anthocyanins ; To 2 ml of extract, 2 ml of 2 N HCl was added followed by the addition of NH₃. Pinkish red to bluish violet coloration indicates the presence of anthocyanins (Lawal *et al.*, 2019 ; Khan *et al.*, 2023).

7. Test for starch : Take 2 ml of clear fruit juice and add a few drops of iodine solution. Formation of blue complex indicates the presence of starch (Lawal *et al.*, 2019 ; Khan *et al.*, 2023).

8. Test for protein

Millon's test: Crude extract when mixed with 2 ml of Millon's reagent, white precipitate appeared which turned red upon gentle heating that confirmed the presence of protein (Lawal *et al.*, 2019 ; Khan *et al.*, 2023).

Ninhydrin test: Crude extract when boiled with 2 ml of 0.2 % solution of Ninhydrin, violet colour appeared suggesting the presence of amino acids and proteins (Lawal *et al.*, 2019, Khan *et al.*, 2023).

RESULTS AND DISCUSSION

Characterization techniques

The crystallinity and crystal phases were characterized by X-ray diffraction (XRD, Bruker D8 Analytical X-ray System) pattern measured with Cu- K α radiation ($k = 1.54060 \text{ \AA}$) in the range of 20 to 80°. UV visible spectra were recorded with Systronics PC based double beam spectrophotometer.

Characterization of copper oxide nanoparticles

1. UV-Visible spectral analysis

UV-Visible absorption spectrum is the preliminary characterization to know the optical property of synthesized nanoparticles. The results obtained from UV-Visible spectroscopy analysis of the nanoparticles sample are presented in Figure 2. UV-visible spectra of the formed CuO nanoparticles dispersed in ammonia solution exhibited the maximum absorption peaks at about 227 nm (Vijay Kumar *et al.*, 2015).

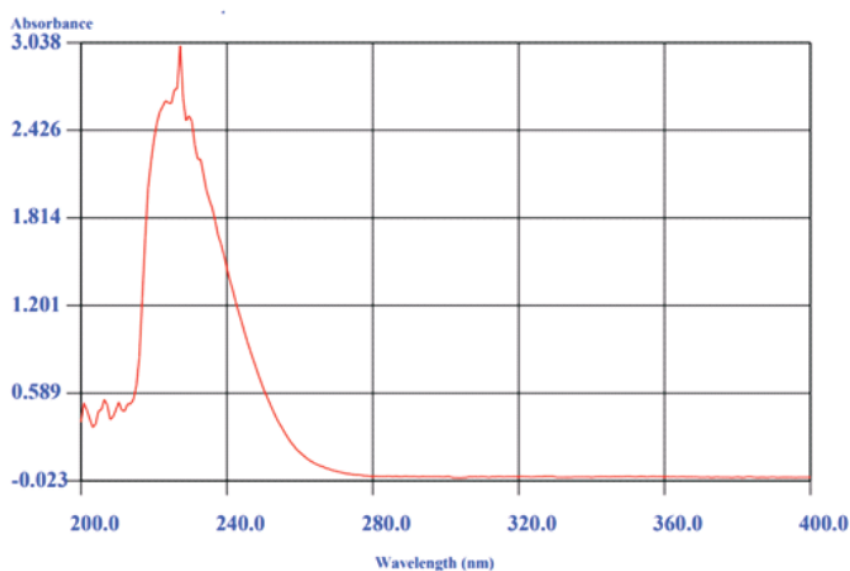


Figure 2. UV-Visible spectra of CuO nanoparticle

1. XRD analysis

The X-ray diffraction study was undertaken to determine and confirm the crystalline structure of synthesized CuO NPs. The reflection lines of monoclinic CuO nanoparticles were identified by the diffraction pattern at $2\theta = 33.97, 35.7, 38.8, 48.8, 58.4, 61.7, 66.25$ and 68.1 which were assigned to the planes (110), (022), (111), (200), (202), (020), (202), (022) respectively of monoclinic phase CuO NPs as in Figure 3. The pattern was the same as that of pure CuO, confirming the creation of monoclinic single-

phase CuO nanoparticle (JCPDS-89-5895). The grain size for different FWHM (\hat{a}) values was calculated using Debye - Scherrer's equation. (Vijayalakshmi and Rajendran, 2012).

$$D = K\lambda / \beta \cos \theta$$

Where, K is a constant representing shape factor which is about 0.89, λ is the X-ray wavelength used which is 1.54060\AA , while \hat{a} is the full width half maximum (FWHM) of the diffraction angle. Average crystallite size of CuO NPs was found to be 28 nm.

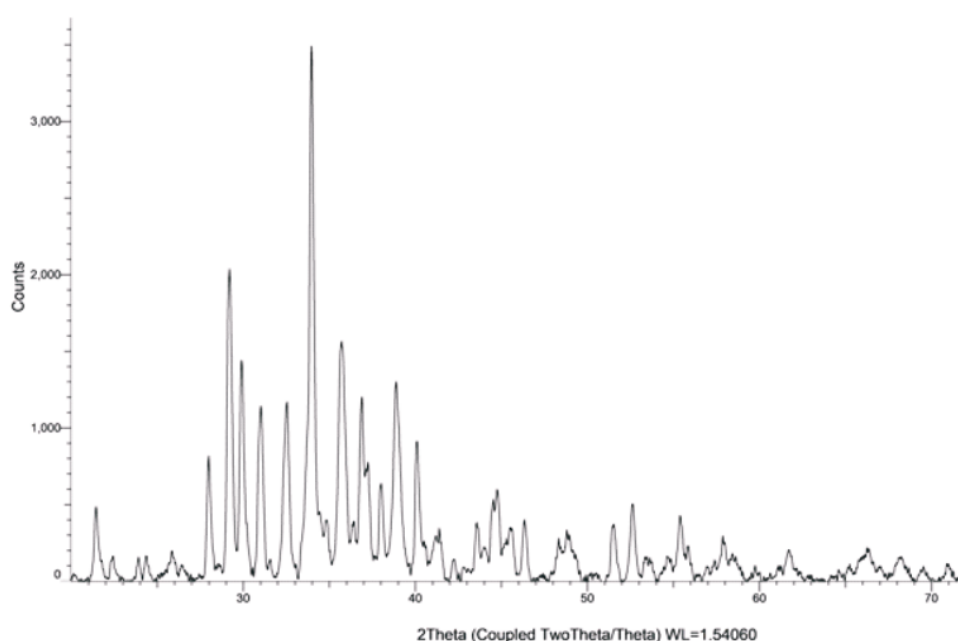


Figure 3. XRD analysis of CuO NPs

Antibacterial activity

Preparation of cultural media for bacteria

The antibacterial activity of the synthesized green CuO nanoparticles against bacterial strains namely, *E. coli* and *Staphylococcus aureus* were analysed by using disk diffusion method (Renuga *et al.*, 2020). The disc diffusion method is a simple yet effective technique for determining the susceptibility of bacteria to various antimicrobial agents. It provides valuable information to clinicians for selecting appropriate antibiotic therapy and monitoring the emergence of antimicrobial resistance.

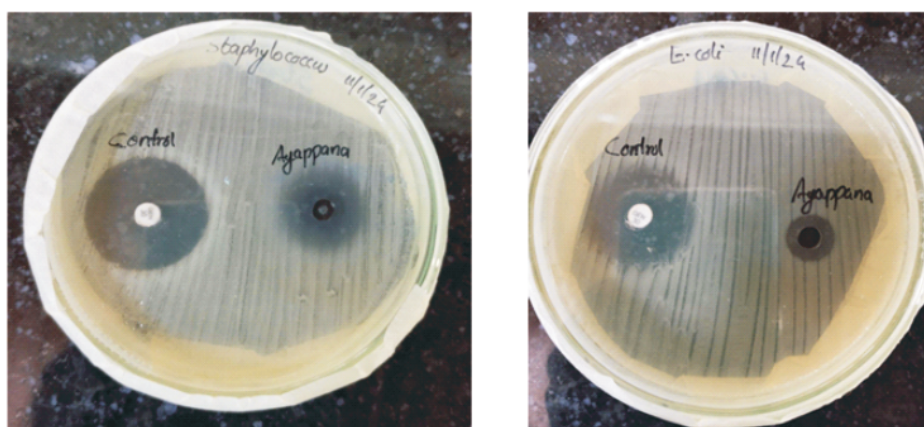
Fresh overnight grown cultures of the bacteria were spread on nutrient agar containing petri plates. Different concentrations of CuO nanoparticles (6 mg ml^{-1} , 8 mg ml^{-1} , 10 mg ml^{-1} and 12 mg ml^{-1}) were added to the agar plate. On the clean disks, synthesized CuO NPs was poured. As a positive control, Gentamycin (10 mg ml^{-1}) disks were maintained, and all plates were incubated overnight at 37°C

for 24–48 h to identify the development of bacterial inhibition zone surrounding the surface of the disk. The experiment was repeated using the best-found concentration of CuO nanoparticle producing maximum inhibition. It was also observed that leaf extract alone has very less inhibition zone. Top of Form

The results revealed that the CuO NPs had showed antibacterial activity against the bacteria, *Escherichia coli*. It has recorded 4 mm zone of inhibition at the concentration of 10 mg ml^{-1} . It also showed antibacterial activity against the bacteria, *Staphylococcus aureus*. It has recorded 10 mm zone of inhibition at the concentration of 10 mg ml^{-1} (Table 1). Factors which affect the sensitivity of bacteria to copper oxide nanoparticle are size of particles, temperature of synthesis of the nanoparticles, structure of bacterial cell wall, and degree of contact of the nanoparticles with bacteria.

Table 1. Antibacterial activity of CuO nanoparticles at various concentrations

	<i>Escherichia coli</i> (Gram-negative bacterium) Zone of inhibition	<i>Staphylococcus aureus</i> (Gram positive bacterium) Zone of inhibition
Control	11mm	14 mm
CuO Nanoparticle (10 mg ml ⁻¹)	4 mm	10 mm
6 mg ml ⁻¹	1.2 mm	6 mm
8 mg ml ⁻¹	3.4 mm	8 mm
12 mg ml ⁻¹	3.6 mm	8.2 mm

**Figure 4. Antibacterial activity of CuO nanoparticles (10 mg ml⁻¹) showing maximum inhibition****Phytochemical analysis**

Preliminary phytochemical analysis for *Ayappana triplinervis* leaf extract was done using standard test procedures to confirm the availability of active

phytochemicals in the aqueous leaf extract (Lawal *et al.*, 2019; Khan *et al.*, 2023). Phytochemical analysis showed the presence of starch, saponins, flavonoids, phenolics, coumarins and anthocyanins (Table 2).

Table 2. Phytochemical analysis

Sl. No.	Phytochemicals	<i>Ayappana triplinervis</i> leaf extract
1	Tannins and phenols	–
2	Protein	+
3	Saponins	+
4	Terpenoids	–
5	Flavonoids	+
7	Anthocyanins	+
8	Coumarins	+
9	Starch	+

(+) shows presence (-) shows absence

The use of *Ayapana triplinervis* leaf extract for green synthesis of copper oxide nanoparticles was indeed fascinating. The fact that the nanoparticles demonstrated significant antibacterial activity against both Gram-positive and Gram-negative bacteria suggests their potential application in various fields, including medicine and environmental science. The presence of various phytochemicals such as saponins, flavonoids, phenols, starch, coumarins, and anthocyanins in the leaf extract likely played a crucial role in facilitating the formation of CuO nanoparticles. These bioactive metabolites can act as reducing and capping agents, aiding in the synthesis and stabilization of the nanoparticles (Jadoun *et al.*, 2021). The greater antibacterial activity observed against Gram-positive bacteria compared to Gram-negative bacteria could be attributed to several factors, including differences in cell wall structure and composition between the two types of bacteria. Overall, our findings highlight the potential of *Ayapana triplinervis* leaf extract as a green and eco-friendly approach for the synthesis of CuO nanoparticles with effective antibacterial properties. This research could pave the way for the development of novel antimicrobial agents and environmentally sustainable nanomaterials.

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