Eco-friendly synthesis of zinc oxide nanoparticle using *Centella asiatica*: phytochemical analysis, characterization and antimicrobial activity assessment

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Abstract: Zinc oxide nanoparticles (ZnO NPs) were synthesized using an eco-friendly approach, utilizing the aqueous extracts of *Centella asiatica* (*C. asiatica*), a perennial herbaceous plant with important therapeutic uses, locally called 'Ghodtapre' in Nepali. In this work, plant extracts from *C. asiatica* were used to produce ZnO NPs in an environmentally friendly manner. ZnO NPs were characterized through X-ray diffraction (XRD), UV-visible spectroscopy, and Fourier transform infrared (FTIR) spectroscopy. A noticeable absorption peak at 375 nm, which agrees with a band gap of 2.80 eV, was determined by UV analysis. The FTIR analysis confirmed the existence of functional groups that serve as capping and stabilizing agents. XRD analysis estimated 11.74 nm of the crystallite size of the ZnO NPs. According to phytochemical studies, flavonoids, tannins, quinine, terpenoids, and alkaloids are all present. Antimicrobial testing confirms the nanoparticles' effectiveness against both bacteria and fungi.

Keywords: Antifungal; Antimicrobial; *C. asiatica*; Nanoparticles; Phytochemicals.

Introduction

Nanotechnology enables advancements in medicine, electronics, and sustainability by leveraging the unique physicochemical properties of nanoscale materials (1–100 nm)[1-2]. These properties are applied in areas such as drug delivery, antimicrobial solutions, and environmental remediation[3-5].

ZnO NPs are widely recognized for their remarkable antibacterial, antifungal, and UV-filtering capabilities. These properties make ZnO NPs pivotal in cosmetics, electronics, and pharmaceuticals[6-8]. The eco-friendly synthesis of metallic nanoparticles has emerged as a sustainable alternative to conventional chemical and physical methods. Traditional techniques often involve harmful chemicals and generate toxic waste, whereas green synthesis focuses on environmentally responsible processes that minimize ecological impact and reduce toxicity. This method is becoming increasingly favoured for its minimal ecological footprint[9-11].

Renowned for its rich cultural heritage, Nepal has long been a repository of traditional medicinal practices that utilize the diverse plants within its landscapes. This wealth of medicinal plants, particularly in the Himalayan region, offers profound benefits due to their unique bioactive properties[12].With the advancement of science, these traditional practices are being synergized with cutting-edge technologies like nanotechnology, which provide

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novel approaches to enhancing the therapeutic potential of medicinal plants. Furthermore, integrating traditional Plants that are medicinal like *C. asiatica* known as "Ghodtapre" in Nepal, with green-synthesized nanoparticles opens newfrontiers in nanobiotechnology. *C. asiatica,* a perennial herbaceous plant belonging to the Apiaceae family was selected for the green synthesis of ZnO NPs due to its rich phytochemical composition, including flavonoids, triterpenoids, and phenolic compounds. These bioactive constituents not only act as reducing and capping agents during nanoparticle formation but also enhance the biological properties of the synthesized ZnO NPs. *C. asiatica* is widely available, cost-effective, and traditionally used in herbal medicine for its neuroprotective, anti-inflammatory, and antioxidant properties[13-15]. The use of *C. asiatica* for nanoparticle synthesis aligns with the goal of developing biocompatible and sustainable nanomaterials.

This manuscript explores the preparation of ZnO NPs and their potential utilization, with an emphasis on their green synthesis using plant extract of *C. asiatica*. It emphasizes the antimicrobial properties of ZnO NPs, especially their effectiveness against Gram-positive and Gram-negative bacteria, and their potential applications across various fields, including medicine and industry. The study aims to bridge traditional knowledge with modern nanotechnology, contributing to sustainable, effective, and environmentally benign therapeutic solutions.

Materials and Methods

Chemicals

All the chemicals employed in this study were of analytical grade and were used as received, without any additional purification. Zinc nitrate hexahydrate ($Zn(NO_3)_2 \cdot 6H_2O$) with 99.5% purity was obtained from Loba Chemical Pvt. Ltd., India. Sodium hydroxide (NaOH) with 97% purity was supplied by Thermo Fisher Scientific India Pvt. Ltd. Ethanol with 99.9% purity was sourced from RCP Distilleries India Pvt. Ltd., Meerut, India.

Plant sample collection site

The plant samples of *C. asiatica* were collected from their natural habitat in Dharmadevi municipality -5, Sankhuwasabha district, Koshi Province, Nepal (27.490°-27.510° N, 87.210°-87.260° E) between February and March 2024.

Plant extract preparation

For three weeks, the harvested plants were dried in a cool, shaded area. After drying, the leaves were ground into a fine powder and stored in clean plastic bags. For the extraction process, 5 g of the powdered plant material was soaked in methanol to isolate the phytochemicals for analysis. Plant material is soaked in methanol for phytochemical extraction because methanol is a polar solvent that efficiently dissolves a wide range of bioactive compounds, including alkaloids, flavonoids, tannins, phenolics, and glycosides. Another 5 g of the same powder was mixed with 100 mL of distilled water and agitated for about 70 minutes at 80 °C. The mixture was then cooled to room temperature and undisturbed for 72 hours to ensure efficient extraction of the target compounds. After filtration to remove any solid particles, a clear extract was obtained. The aqueous extract was chosen for nanoparticle synthesis because water-soluble phytochemicals, particularly polyphenols and flavonoids, play a crucial role as reducing and stabilizing agents during nanoparticle formation. Aqueous extraction avoids the interference of organic solvents, ensuring a greener and eco-friendly synthesis process for nanoparticles. The extract was then concentrated and stored in suitable containers for further testing.

ZnO NPs synthesis

To 100 mL of the aqueous extract of *C. asiatica*, 4 g of $Zn(NO_3)_2 \cdot 6H_2O$ was added, and the pH of the solution was adjusted to 10 using a 0.1 M NaOH solution. The solution was cooled and centrifuged for 30 minutes at a speed of 8000 rpm. The resulting product was washed twice with ethanol and water. After that, the particles were dried for 6 hours at 60 °C in an oven. ZnO NPs were then obtained by calcining the dry material for two hours at around 400 °C

in a muffle furnace. Figure 1 displays the schematic diagram for ZnO NPs synthesis. From C. asiatica extract.

Phytochemical analysis

The methanol extract of *C. asiatica* was tested for second-

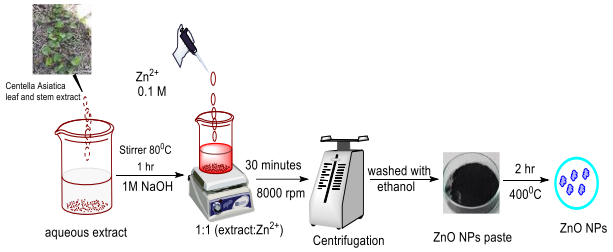

Figure 1: Representation of synthesis of ZnO NPs from *C. asiatica* extract.

-ary metabolites using standard qualitative phytochemical screening methods[16-17].

UV–vis analysis

UV-vis spectroscopy was performed using a double-beam spectrophotometer (SPECORD 200 PLUS, Analytik Jena, Germany). The spectra were recorded in the wavelength range of 200–600 nm. The distinct absorbance peaks verified the successful synthesis and stability of ZnO NPs using green extracts.

FTIR analysis

Dried ZnO NPs and plant extract were subjected to FTIR analysis using a Perkin Elmer Spectrometer, version 10.6.2. With the resolution of 4 cm^{-1}, spectral data were gathered in the range of 450–4000 cm^{-1}.

XRD analysis

X-ray diffraction was used to study the crystal structure and nanoparticles' crystallite size. This technique involves directing X-rays at the sample, where they interact with the periodic atomic planes within the crystal lattice, resulting in diffraction. The angles and intensities of these diffracted beams are recorded to produce a unique diffraction pattern of the nanoparticle. The crystal structure was studied by X-ray diffractometer (Bruker D2 Phaser) with Cu Kα radiation in the 2θ range of 20°-80°. Debye-Scherrer equation was used to calculate the average crystallite size (D)[18-19].

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

Where, D is crystallite size (nm), K is Scherrer constant (0.9), λ is the Wavelength of an incident X-ray (1.5406 Å), β is Full width at half maximum of peak (FWHM) in radians, and θ is diffraction angle (radians).

Antimicrobial activity of ZnO nanoparticles

An antimicrobial activity was evaluated using the agar well diffusion method. The microbial strains from American Type Culture (ATCC) used in this study were *Escherichia coli*-ATCC 8739 (*E. coli*), a Gram-negative bacterium, and *Staphylococcus aureus* -ATCC 6538P (*S. aureus*) a Gram-positive bacterium, *Candida albicans*-ATCC 2091 (*C. albicans*), a fungal species. The antimicrobial activity of ZnO NPs and plant extracts was tested against selected strains to determine their effectiveness in comparison to the standard. The cultures were incubated under ideal conditions, enabling accurate measurement of zones of inhibition and clear distinction among the microorganisms. It involved preparing microbial culture media and Mueller-Hinton agar (MHA) plates. Liquid broth (LB) medium was prepared by dissolving 13 grams of LB powder (Sisco Research Laboratories Pvt. Ltd., India) in 1 L distilled water. Then the solution was autoclaved at 121 °C and 15 psi for 25 minutes and cooled to 50 °C. Once cooled, the media was aseptically transferred into sterilized 15 mL Falcon tubes, with 5 mL aliquots per tube. Each tube was then inoculated with a bacterial culture and incubated for 1 day for bacterial cell growth. To ensure accuracy and reproducibility, it is crucial to maintain aseptic techniques throughout the process and to verify that the incubation conditions are optimal for the specific bacterial strains being studied.

Mueller-Hinton Agar (MHA) plates were prepared by dissolving 39 grams of MHA powder (Sisco Research Laboratories Pvt. Ltd., India) in 1 L distilled water. Then sterilizing at 121 °C and 15 psi for 25 minutes was performed. After autoclaving, the media was cooled to 50 °C and stored in sterile petri dishes, with 25 mL media per

dish. Before the antimicrobial assay, each plate (stored in the refrigerator) was labelled with the corresponding sample name. By using a sterile cotton swab, 150 µL aliquot of bacterial suspension was spread evenly on the surface of the agar. Wells were then created in the agar to accommodate the test samples and standards. For plant extracts, 100 µL samples were loaded at a 100 mg/mL concentration dissolved in Dimethyl sulphoxide (DMSO)

Table 1: Results of phytochemical analysis.

S.N	Phytochemicals	Presence/Absence
1	Flavonoids	Present
2	Alkaloids	Present
3	Saponins	Absent
4	Terpenoids	Present
5	Quinine	Present
6	Tannins	Present
7	Phenolic compounds	Present

while nanoparticles were added at approximately 30 mg per well and the final volume was adjusted to 100 µL. A standard kanamycin (Sigma Aldrich-USA, Purity: 95%) solution of 5 mg/mL was also added, with 10 µL loaded in the appropriate wells. Standard itraconazole (Deurali-Janta Pharmaceuticals, Nepal) (20 mg/mL; 10 µL) solution was used for fungal strain as positive controls. Then plates were incubated at 37 °C for 24 hours then antimicrobial effects were evaluated. After the 24-hour incubation period, the diameters of the zones of inhibition were measured.

Results and Discussion

Phytochemicals analysis

The plant leaf extract reveals the presence of several key compounds. The phytochemical analysis of the plant extracts used for ZnO NPs synthesis was carried out, and the findings are summarized in Table 1. The extract of *C. asiatica* revealed the presence of diverse phytochemicals, such as flavonoids, alkaloids, terpenoids, quinones, tannins, and phenols. Phenols and flavonoids are crucial natural agents in promoting the eco-friendly synthesis of ZnO NPs, serving reducing and stabilizing components[20]. Phytochemicals hold substantial promise for antimicrobial and photocatalytic uses. Compounds such as flavonoids and tannins exhibit natural antimicrobial activity, which can synergistically improve the bactericidal efficiency of ZnO NPs against pathogenic microbes[21].

UV- vis analysis

The UV-visible spectrum of the ZnO NPs exhibits absorbance in 200–600 nm, with a distinct peak at 375 nm, indicating their successful formation (Figure 2). Previous studies have also documented similar absorption characteristics for ZnO NPs[22]. The spectrum displayed a single prominent peak, indicating that the ZnO NPs synthesized using plant extract are of high purity. The phenomenon of quantum confinement, a key characteristic of ZnO NPs, was observed. This effect leads to an increase in the band gap of the particles, causing a shift of the absorption edge toward shorter wavelengths as the particle size decreases.

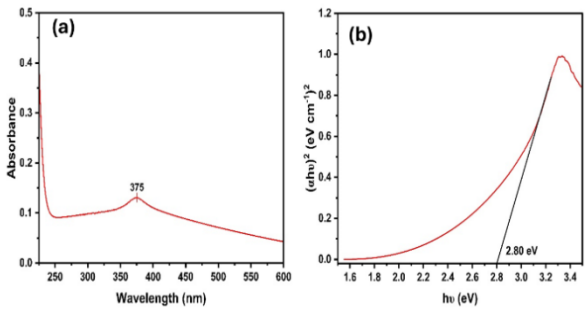

Figure 2: UV-visible spectral analysis of the biosynthesized ZnO NPs using aqueous extract of *C. asiatica:* (a) absorbance spectra and (b) band gap energy.

The optical band gap was determined using a Tauc plot from absorption spectra and found to be 2.80 eV (Figure 2), which closely aligns with previously reported values[23]. The band gap of biogenic ZnO was determined as:

$$(\alpha h\nu)^{\frac{1}{n}} = k(h\nu - E_g)$$

Where α = Absorbance coefficient, h = Plank's constant, v = photon frequency, k = energy independent constant, E_g = optical band gap, n =1/2 for direct band gap, n = 2 for indirect band gap. The sample exhibits semiconductor properties with a band gap of 2.80 eV. This band gap

enables it to primarily absorb UV radiation while likely maintaining transparency in the visible spectrum.

FTIR analysis

FTIR spectra of ZnO NPs (red line) and plant extract (black line), Figure 3, are examined within 450-4000 cm^{-1}. The plant extract spectrum shows that the peaks at 3301 cm^{-1} correspond to O-H stretching indicating the presence of alcohol, phenol or water in the sample [24]. 1592 cm^{-1} is C=C stretching, 1026 cm^{-1} is C-O stretching corresponds to plant biochemicals[24]. These functional groups are commonly associated with phenolic compounds, flavonoids, and terpenoids, suggesting their involvement in nanoparticle stabilization. Phenolic compounds containing hydroxyl can chelate metal ions, leading to nanoparticle formation and capping. The broad O-H stretching band at ~3301 cm^{-1} confirms the presence of these hydroxyl-rich phytochemicals, which contribute to hydrogen bonding and electrostatic interactions, thereby stabilizing the nanoparticles. The observed C=C and C-O stretching bands further suggest the presence of phytochemicals such as flavonoids and phenolics, which can donate electrons to metal ions, facilitating their reduction and contributing to nanoparticle stabilization. The spectrum of ZnO NPs shows the broad O-H stretching at 3465 cm^{-1} corresponding to the water molecules. 551 cm^{-1} is related to Zn-O stretching which confirms the formation of ZnO NPs[25]. Compared to the spectrum of plant extract, there are noticeable shifts and reductions in intensity for peaks in the ZnO spectrum, indicating the biomolecules in the plant extract are crucial during the synthesis of ZnO nanoparticles.

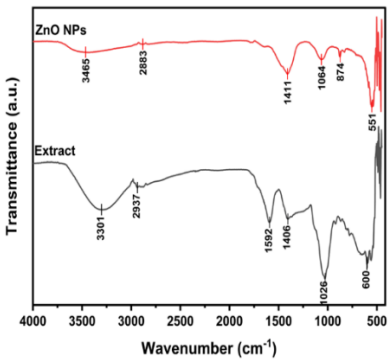

Figure 3: FTIR spectra of ZnO NPs and plant extract.

XRD study

Figure 4 shows that the XRD shows well-defined diffraction peaks within the 2θ range of 20º to 80º range. XRD was analyzed about this standard JCPDS card no: 00-036-1451[26]. The 2θ of XRD were obtained at 31.44º, 34.0º, 35.94º, 47.28º, 56.32º, 62.61º, 66.2 º,67.67º, 68.95º, 72.39º, 76.92º that are allocated to Miller indices namely (100), (002), (101), (102), (110), (103), (200), (112), (201), (004), and (202) respectively.

The average crystalline size of ZnO NPs was determined to be 11.74 nm obtained by using Gaussian fitting on the widths of peaks and the Debye Scherrer formula[27-30]. The size obtained aligns well with the values reported in similar studies, confirming the accuracy of both synthesis and analytical methods [18].

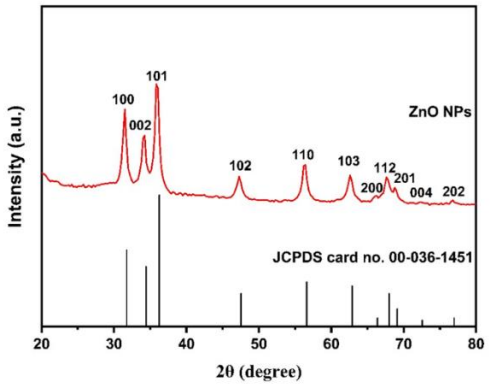

Figure 4: XRD patterns of ZnO NPs.

Antimicrobial test

ZnO NPs synthesized using *C. asiatica* leaf extract were examined against two bacterial strains and a fungal strain using the agar well diffusion method. The findings are presented in Table 2. The ZnO NPs showed moderate antimicrobial properties as shown in Figure 5. The ZnO NPs produced an inhibition zone of 1.4 cm against the Gram-negative bacterium, *E. coli* and the fungus, *C. albicans*. In contrast, for *S. aureus*, a Gram-positive bacterium, the inhibition zone was slightly smaller at 1.3 cm. These moderate inhibition zones suggest that the ZnO NPs exhibited some antimicrobial efficacy. However, the observed activity is not as pronounced as that of stronger antimicrobial agents. The moderate inhibition could be attributed to factors such as the concentration of the nanoparticles, the bioavailability of active compounds

from the plant extracts, and the specific resistance mechanisms of the microbial strains tested. It is important to note that while the ZnO NPs demonstrated activity, further optimization of the concentration and the synthesis method could enhance their efficacy. In comparison, the plant extract showed no antimicrobial activity, as no inhibition zones were observed. Kanamycin was used as the positive control (C^+) for bacterial strains, while Itraconazole served as the positive control (C^+) for fungal strains. DMSO was utilized as the negative control (C−) in both cases to assess the antimicrobial activity. As expected, the negative control showed no antimicrobial activity.

There is ongoing debate over the precise mechanism behind the antimicrobial activity of ZnO NPs. Among the various mechanisms, the direct interaction of ZnO NPs

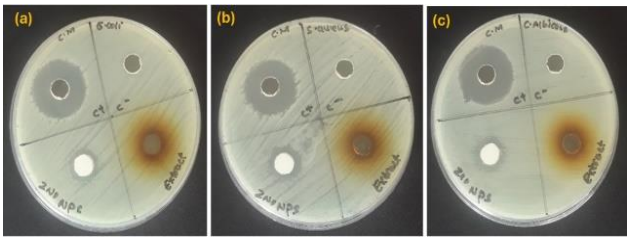

Figure 5: Zones of inhibition displayed by the antimicrobial activity of ZnO NPs and plant extracts against test microbial strains (a) *E. coli* (b) *S. aureus* with kanamycin (C^+) and DMSO (C^-) and (c) *C. albicans* with itraconazole (C^+) and DMSO (C^-).

with cell walls causes the integrity of the cell membrane to be damaged, the generation of reactive oxygen species (ROS) and the release of Zn^{2+} ions, are considered to be the most accepted mechanisms[31-34]. There are various factors such as medium influence the physicochemical properties of ZnO NPs, altering their mechanisms of toxicity[35].

Table 2: Antimicrobial activity of ZnO NPs and plant extracts.

Strain	Reference culture	Type	Zone of inhibition (cm)		
			Positive control (c^+)	ZnO NPs	Plant extract
E. coli	ATCC 8739	Gram -ve	2.6	1.4	0
S. aureus	ATCC 6538P	Gram +ve	2.3	1.3	0
C. albicans	ATCC 2091	Fungi	2.5	1.4	0

Conclusion

C. asiatica has proven to be an excellent source of bioactive phytochemicals, including flavonoids, alkaloids, terpenoids, quinones, phenolics, and tannins. This study successfully employed a biosynthesis approach to produce ZnO NPs using *C. asiatica* extracts followed by characterization using XRD, FTIR, and UV-visible spectroscopy. From XRD analysis, the size of NPs was 11.74 nm, consistent with JCPDS card number: 00-036-1451, while UV-visible spectroscopy estimated a 2.80 eV band gap. FTIR spectroscopy of ZnO NPs identified a significant shift in the peaks compared to the *C. asiatica* extract suggesting the growth of NPs. The synthesized nanoparticles demonstrated significant antibacterial activity against Gram-negative *E. coli*, and Gram-positive *S. aureus*, and antifungal properties against *C. albicans*. These findings highlight the potential of *C. asiatica*-mediated ZnO NPs as effective antimicrobial agents and support further exploration of their applications in nanomedicine and environmental remediation.

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