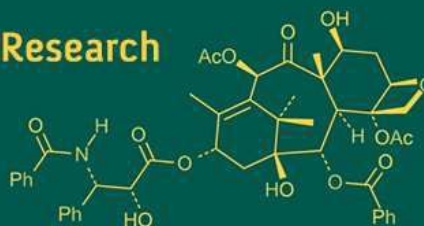


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One-pot synthesis of bimetallic Cu/ZnO nanoparticles using tea leaves (*Camellia sinensis*) for anticancer applications

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Abstract

Nanoparticles are extensively researched across various domains for their unique attributes and broad application potential, with bimetallic nanoparticles receiving particular focus due to their superior catalytic activities and stability over monometallic types. Traditional methods for synthesizing bimetallic nanoparticles often rely on toxic reducing agents, are energy-intensive, and require multiple procedural steps, underscoring the need for more sustainable and straightforward approaches. This study introduces a one-pot synthesis strategy for creating bimetallic copper/zinc oxide nanoparticles (Cu/ZnO NPs) using *Camellia sinensis* (Tea leaves) as a natural reducing and capping agent. This method is eco-friendly and cost-effective, aiming to overcome the drawbacks of conventional synthesis techniques. The nanoparticles produced were thoroughly analysed using scanning electron microscopy (SEM), transmission electron microscopy (TEM), UV-visible spectroscopy (UV-Vis), and X-ray diffraction (XRD), confirming the successful formation of bimetallic nanoparticles.

The Cu/ZnO nanoparticles exhibited a consistent particle size of about 6 to 8 nm. UV-Vis spectroscopy helped elucidate their optical properties, affirming their bimetallic composition. SEM and TEM investigations showed the nanoparticles' spherical shape and crystalline structure. These nanoparticles demonstrated significant biological activity, with a 91% effectiveness in inhibiting cancer cell growth, highlighting their potential in anticancer applications. They also displayed notable antimicrobial properties, enhancing their applicability in a wider range of biomedical contexts. Overall, this green synthesis route not only provides a viable alternative for producing bimetallic nanoparticles but also expands their possible uses in medical treatments and devices.

Keywords: Bimetallic nanoparticles, Cu-ZnO, green synthesis, *Camellia sinensis*, anticancer activity, antimicrobial activity

1. Introduction

Nanotechnology has become a transformative force in various sectors, particularly in the biomedical field, where it has introduced revolutionary methods for diagnosing and treating severe medical conditions such as cancer. The unique physiochemical, electrical, catalytic, and optical properties of nanomaterials have significantly expanded their utility, garnering substantial interest across multiple disciplines including sewage treatment, food technology, optoelectronics, and cosmetics [1-8]. Within the diverse array of nanoparticles, bimetallic nanoparticles (NPs) stand out due to their unique structural and morphological characteristics that enhance their performance in various applications compared to their monometallic counterparts [9, 10].

Historically, the synthesis of nanoparticles has predominantly relied on chemical methods that use various hazardous chemical precursors. These methods not only pose significant environmental risks due to their toxicity but also often lead to colloidal instability in the nanoparticles produced. This instability can severely restrict the usability of nanoparticles, especially in sensitive applications like those in clinical and biomedical settings [11, 12]. As a response to these challenges, biological or green synthesis methods have been developed. These methods leverage natural reducing agents, such as plant extracts, to facilitate the synthesis process, making it more sustainable, rapid, and environmentally friendly. Such green synthesis approaches offer a less toxic, more cost-effective alternative to conventional chemical synthesis methods [13-15].

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In the process of green synthesis, two aqueous metal precursor solutions are typically mixed with a plant extract containing phytochemicals, which act as reducing and stabilizing agents. These phytochemicals efficiently convert metal ions into metal nanoparticles. The reduction potential of the metal ions plays a critical role, with ions having a stronger reduction potential being reduced faster than others, which can lead to the formation of a core-shell structure within the nanoparticles [1, 16]. This research utilizes *Camellia sinensis*, commonly known as tea leaves, revered for its antitumor, antimicrobial, and antioxidant properties. The presence of potent phytochemicals like polyphenols, which include flavonoids and catechins, underscores the plant's potential for nanoparticle synthesis. The efficacy of tea leaves in the synthesis of bimetallic nanoparticles has

been underexplored, making this study particularly novel [17-19].

This research paper presents a novel one-pot synthesis approach using *Camellia sinensis* tea leaves as both a reducing and capping agent to produce biologically active bimetallic Cu/ZnO nanoparticles. This methodology not only highlights the potential of environmentally benign processes in nanoparticle synthesis but also probes the viability of these nanoparticles as a safe, effective treatment for cancer. The Cu/ZnO nanoparticles synthesized in this study exhibited promising biological activity, suggesting their potential applications in a wide array of fields. A schematic representation of the synthesis method is illustrated in Figure 1, providing a visual guide to this innovative synthesis process.

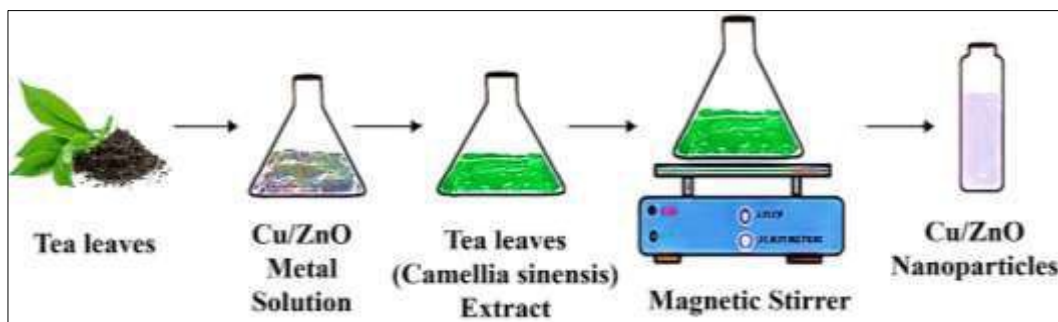


Fig 1: Schematic representation of one-pot synthesis method of bimetallic nanoparticles

2. Materials and Methods

The materials used in this study include *Camellia sinensis* (Tea leaves), Copper Sulphate (CuSO_4) and zinc sulphate (ZnSO_4), ethanol, and deionized water. The Tea leaves were collected from a local source (Mumbai) and washed thoroughly with deionized water to remove any impurities. The leaves were then dried in an oven at 60 °C for 24 hours and ground to a fine powder using a mortar and pestle [20]. DI water was used to extract the phytochemicals from the powdered leaves. Silver nitrate and zinc sulphate were used as precursors for the synthesis of bimetallic Cu-ZnO nanoparticles. Deionized water was used as a solvent for the synthesis process. All the chemicals used in the study were of analytical grade and purchased from Sigma Aldrich, INDIA.

2.1 Preparation of Tea leaves Extract

The preparation of plant extract from *Camellia sinensis* (Tea leaves) was carried out using the following procedure:

1. The dried Tea leaves were ground to a fine powder using a mortar and pestle.
2. 10 g of the powdered leaves were mixed with 100 mL of water in a round-bottom flask.
3. The mixture was refluxed at 70 °C for 1 hours with continuous stirring.
4. The extract was then filtered using Whatman filter paper and the filtrate was collected in a beaker.
5. The filtrate was concentrated using a rotary evaporator under reduced pressure at 40 °C.
6. The resulting concentrated extract was used as a reducing agent for the synthesis of Cu-ZnO nanoparticles.



Fig 2: Plant extract preparation method

Preparation of Bimetallic Cu-ZnO NPs

The synthesis of bimetallic Cu-ZnO nanoparticles using tea leaves extract as a reducing agent was carried out using the following procedure:

1. 8 mM Copper sulphate and 6 mM zinc sulphate were separately prepared in deionized water.
2. 10 mL of each solution were mixed together in a round-bottom flask under magnetic stirring at room temperature.

3. 8 mL of Tea leaves extract was added drop-wise to the above mixture and the reaction mixture was stirred for 24 hours at room temperature.
4. The color of the reaction mixture changed from colourless to dark brown, indicating the formation of Cu-ZnO nanoparticles. The synthesized nanoparticles were centrifuged at 10,000 rpm for 10 minutes to remove any unreacted precursors and plant extract.



Fig 3: Preparation method of bimetallic nanoparticles

2. Anticancer activity: MTT assay

What is MTT?

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow tetrazolium salt commonly used to measure cell viability, proliferation, and cytotoxicity. In live metabolically active cells, water-soluble MTT is reduced in the mitochondria by succinate dehydrogenase to insoluble purple formazan crystals. The amount of formazan produced can be quantified spectrophotometrically (570-590 nm) upon solubilization and is directly proportional to the number of viable cells in the culture. The optimal wavelength for absorbance is 570 nm, but any filter that absorbs between 550 and 600 nm may be used. The MTT assay is a safe, sensitive, and reliable indicator of cell viability and is preferred over other endpoint viability assays, such as the radioactive ³H-thymidine incorporation assay. Besides having a lengthy and tedious protocol, the health hazards of working with radioactive materials are well recognized, as are the accompanying economic considerations required for the appropriate handling and disposal of radioactive waste. MTT is cell-permeable. The net positive charge of MTT allows it to readily penetrate the

intact plasma membranes of viable cells. Other tetrazolium salts, such as MTS, XTT, and WST-1, are negatively charged compounds and unable to cross the plasma membrane. MTT is a salt, so high levels may be toxic to cells. Should the stock concentration of MTT be too high, cells may be disturbed and reduce viability readings. In such high concentrations, the cytotoxicity of formazan can also pose experimental difficulties when the culture media must be removed from the well-plate. MTT is soluble in water (10 mg/mL), ethanol (20 mg/mL), buffer salt solutions (5 mg/mL), and culture media (5 mg/mL) [21-23].

The principle of the MTT assay

The principle behind the MTT assay is that only viable cells have active metabolism and can convert MTT to purple formazan, which has an absorbance maximum at 590 nm. The amount of formazan produced can be measured using a spectrophotometer and is proportional to the number of viable cells [24-27]. When cells die, they lose their metabolic activity and are unable to convert MTT resulting in no visible color change.

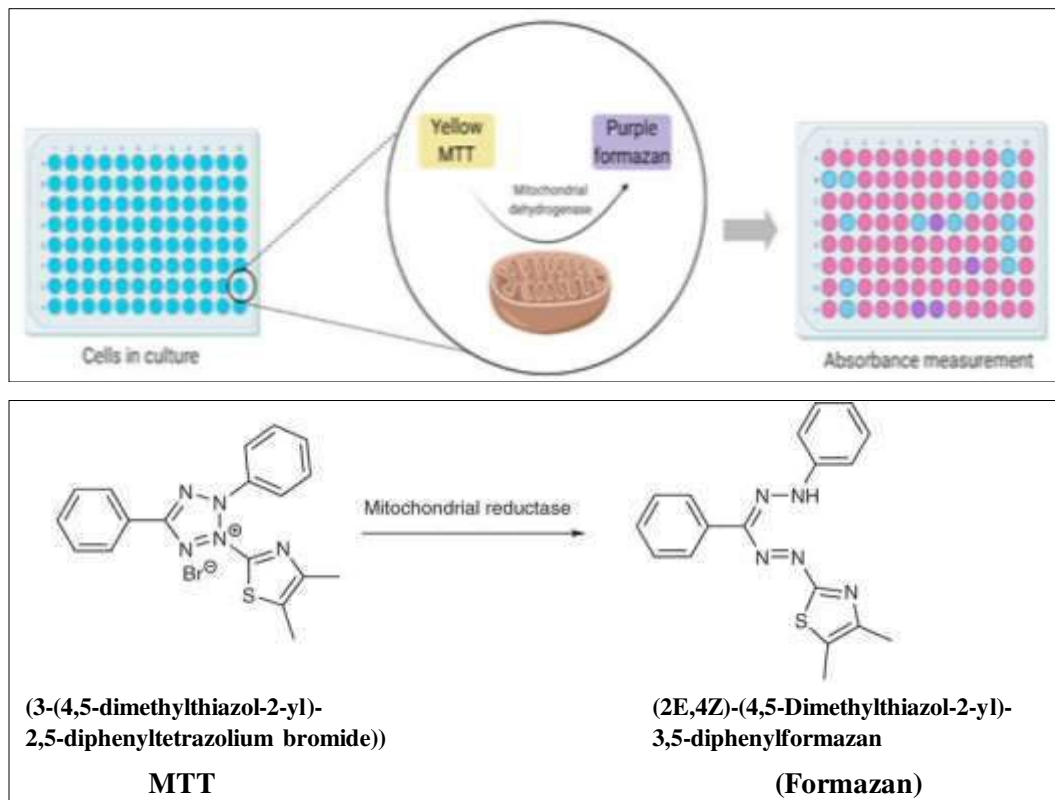


Fig 4: MTT assay of cell viability is based on the principle that MTT is converted to formazan, leading to shift in the emission spectrum from yellow to purple.

Materials & Methods

MTT Powder (The solution is filtered through a 0.2 μm filter and stored at 2-8 $^{\circ}\text{C}$ for frequent use or frozen for extended periods), DMSO, Trypan blue, Phosphate Buffered Saline (PBS), Trypsin, CO_2 incubator, Centrifuge, Hemacytometer

Assay protocol

Cell Preparation: Cervical Cancer cells

HeLa cells (Grown in DMEM media supplemented with 10% Fetal Bovine Serum), harvested using trypsin, and counted using Trypan blue and a hemacytometer.

- Collected the cells when they reach about 70-80% confluence
- Checked for the viability and centrifuged the cells

Procedure

- Day 1, seed 2,500-5,000 cells/well (HeLa) in their exponential growth phase in 100 μL volume in a flat-bottomed 96-well polystyrene coated plate (Thermo Scientific Nunc 96-well plate, Nunclon Delta-white color with lid). Incubate overnight at 37 $^{\circ}\text{C}$ with 5% CO_2 to allow cell adhesion.
- Day 2, after 24 hours of seeding, the concentration of Cu/ZnO were diluted in cell culture medium at different concentration (5, 25, 65 and 100 $\mu\text{g}/\text{ml}$) in DMEM without FBS containing a final volume of 100 $\mu\text{L}/\text{well}$. Incubated for 24 hour. Experiments done in triplicate.
- Day 3, after 24 hour of incubation, added 10 μL of MTT solution (5 mg/10 ml of MTT in 1X PBS) into each well. Wrapped the plate in aluminium foil and incubated for 3-4 hours at 37 $^{\circ}\text{C}$, 5% CO_2 incubator, protected from light.

Note: Depending on the individual cell type and concentration used, longer incubation times may be necessary.

- Formazan crystals formed after 3-4 hours in each well were dissolved in 100 μL DMSO solution (solubilizing buffer).
- Read absorbance at 590 nm. Read plate within 1 hour
- The positive control wells contain untreated cells, MTT solution and a solubilizing buffer.
- The blank in the MTT assay is a cell-free medium plus an MTT solution and a solubilizing buffer.
- Experiments were repeated three times for the average calculations.

Viability is defined as a percentage of live cells in a whole population or in other words, cell viability is defined as the number of healthy cells in a solution.

$$\% \text{ Viability} = \text{Sample/Control} \times 100$$

Sample: Mean value of the measured optical density (OD)/Absorbance of the Nanodrugs

Control

Mean value of the measured optical density (OD)/Absorbance of the Control

3. Results and Discussion

Dynamic Light Scattering (DLS)

The size of nanoparticles is measured using DLS, and their stability in suspension over time is also assessed.

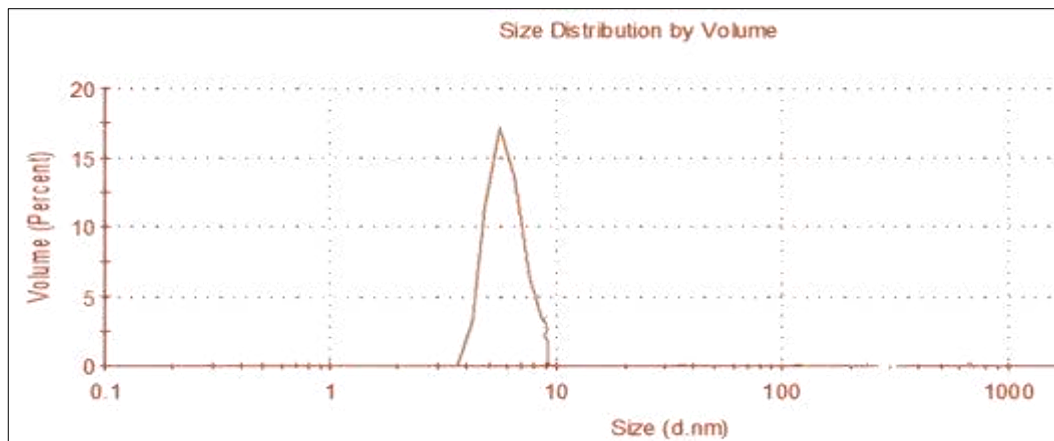


Fig 5: DLS Measurement for Cu-ZnO core-shell

Figure 5 shows the size distribution of the prepared nanoparticles. The size distribution and polydispersity index (PDI) of Cu-ZnO NPs were detected by DLS analysis. The results have indicated the presence of smaller particles of

measures 6-8 nm. Z-average size and PDI for the synthesized nanoparticles were observed to be 12.1 nm and 0.39 respectively ^[35].

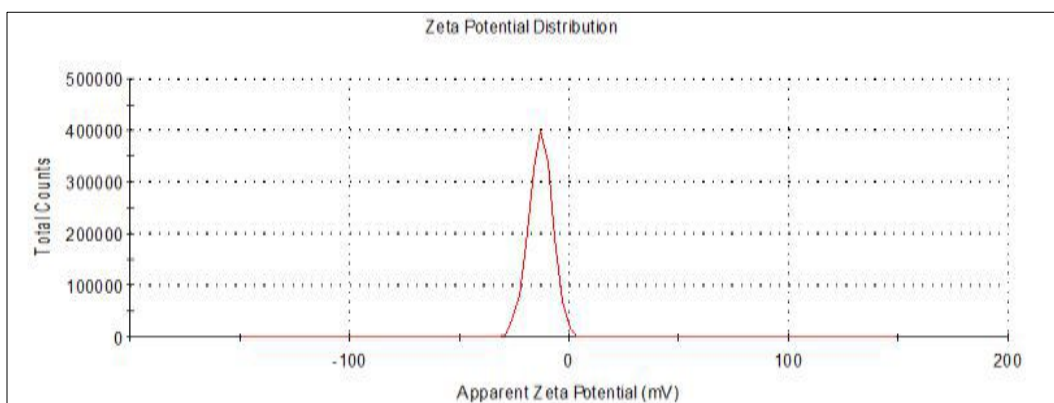


Fig 6: Zeta potential of Cu-ZnO NPs

Figure 6 shows the zeta potential distribution for Cu-ZnO NPs. The zeta potential value for the synthesized nanoparticles was found to be -46 mV.

UV- Visible Spectroscopy

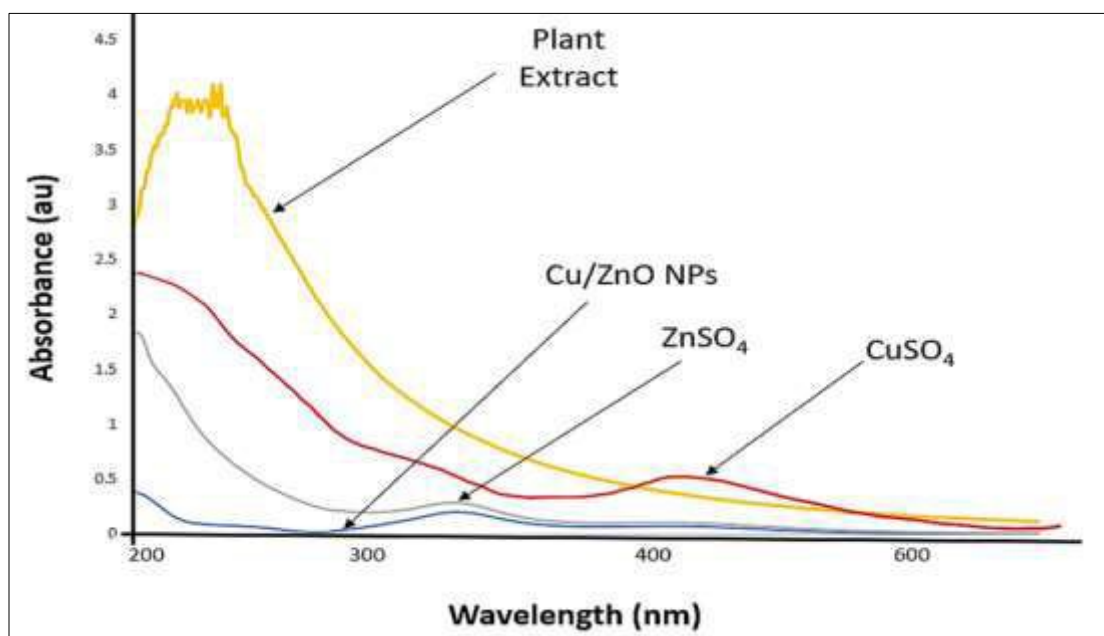


Fig 7: UV-Visible spectrum of bimetallic Cu-ZnO NPs

Copper-zinc oxide nanoparticles (Cu/ZnO NPs) in comparison to plant extracts, zinc sulfate (ZnSO_4), and copper sulfate (CuSO_4) across the wavelength range of 200 nm to 600 nm. The absorption peak observed for the plant extract at approximately 260 nm suggests the presence of phenolic compounds or other UV-absorbing organic molecules, which typically exhibit strong absorption in this region due to π - π^* transitions. The Cu/ZnO nanoparticles demonstrate a broad and intense absorption band from about 200 nm to over 300 nm. This broadening can be attributed to the quantum confinement effects and the interband transitions within the nanoparticles. In contrast, the absorption spectra for ZnSO_4 and CuSO_4 show minimal activity in this region, with the latter displaying a subtle absorption feature near 800 nm, indicative of the d-d transitions of Cu(II) ions in an octahedral coordination.

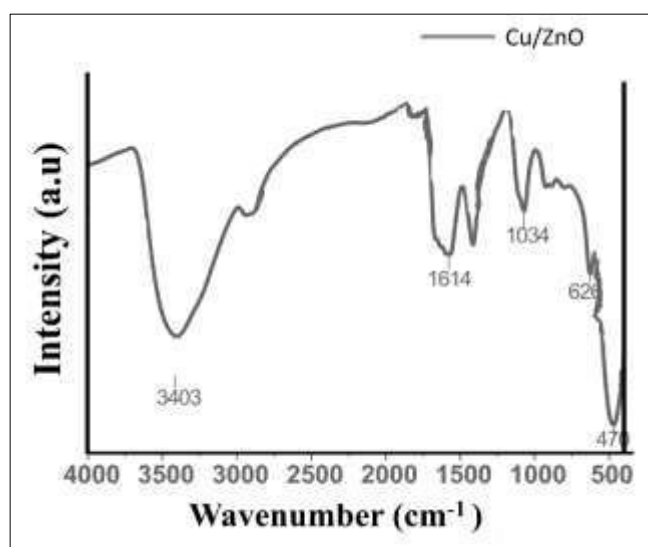


Fig 8: IR Spectrum of bimetallic Cu-ZnO Nanoparticles

Figure 8 represents FT-IR spectrum of Cu/ZnO nanoparticles synthesized using tea leaf extract, several characteristic peaks are evident, each associated with different components of the composite material. The broad peak at 3403 cm^{-1} is indicative of O-H stretching vibrations, typical of hydroxyl groups present in water molecules or organic compounds such as phenols, which are abundant in tea extracts. This suggests the presence of residual moisture or bonded hydroxyl groups from the biological material used in synthesis.

The peak at 1614 cm^{-1} can be attributed to the C=O stretching vibrations commonly found in carboxylic acids or amides, or it could also represent N-H bending in primary amines. This band may also reflect the interaction of organic components from the tea extract with the metal oxide surface, possibly indicating the binding or coordination of organic molecules to the metal sites. The 1034 cm^{-1} band is likely associated with C-O stretching vibrations in alcohols, ethers, or esters, which are typical functional groups in plant extracts. This peak highlights the incorporation of organic moieties from the tea leaf extract into the nanoparticle matrix.

For the inorganic components, the sharp peak at 470 cm^{-1} is characteristic of Zn-O stretching vibrations, a fundamental feature of zinc oxide nanoparticles. This peak confirms the formation of ZnO in the nanocomposite. Although copper itself does not exhibit distinct IR-active peaks due to its metallic nature, the presence of copper may influence the electronic environment of the zinc oxide, potentially affecting the intensities and positions of the zinc oxide peaks. These spectral features collectively provide evidence of the successful synthesis of Cu/ZnO nanoparticles using tea leaf extract, where the extract not only acts as a reducing agent but also imparts functional organic groups that are integrated into the nanoparticle structure, potentially influencing their physicochemical properties.

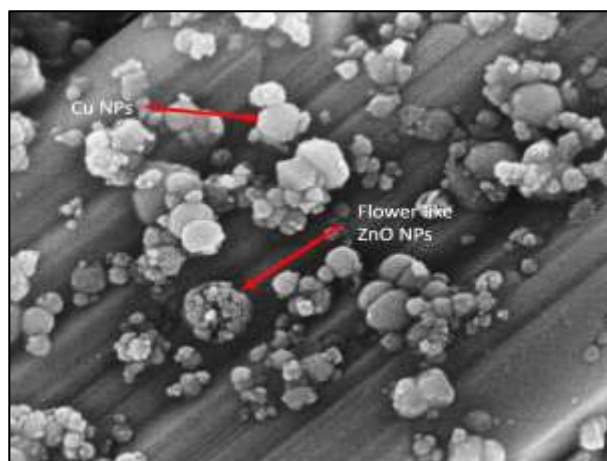


Fig 9: SEM image of synthesized bimetallic Cu-ZnO NPs

The scanning electron microscopy (SEM) (Fig-9) image reveals distinct morphologies of copper (Cu) and zinc oxide (ZnO) nanoparticles. The Cu nanoparticles, identifiable by their dense, spherical appearance, are uniformly dispersed across the substrate. These particles exhibit the typical characteristics of metallic nanoparticles with their compact and homogenous structure. In contrast, the ZnO nanoparticles display a unique flower-like morphology, with multiple nanostructures resembling petals emanating from a central point. This intricate configuration enhances the

surface area of the ZnO nanoparticles, which is advantageous for applications that require high surface reactivity [38].

Investigation of Antimicrobial Activity

The antibacterial activity of various substances, namely Madagascar periwinkle extract, copper sulphate, and zinc sulphate as well as bimetallic Cu-ZnO NPs was evaluated against both Gram-positive and Gram-negative bacteria. The findings demonstrated that bimetallic Cu-ZnO NPs, at a

concentration of 1000 $\mu\text{g/ml}$, exhibited significant antibacterial effects against *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Enterococcus faecalis*, resulting in inhibition zones measuring 16.4 mm, 15.9 mm, 27.3 mm, 20.7 mm, and 22.8 mm, respectively. Additionally, *Bacillus subtilis* displayed the highest sensitivity to bimetallic Cu-ZnO NPs, with a minimum inhibitory concentration (MIC) of 15.32 $\mu\text{g/ml}$, while *Enterococcus faecalis* exhibited the lowest sensitivity, with an MIC of 250 $\mu\text{g/ml}$. Furthermore, the MIC values of

bimetallic Cu-ZnO NPs against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* were determined as 62.5 $\mu\text{g/ml}$, 125 $\mu\text{g/ml}$, and 62.5 $\mu\text{g/ml}$, respectively.

On the other hand, Madagascar periwinkle extract displayed limited antibacterial activity, specifically against *Escherichia coli* and *Bacillus subtilis*. Neither copper sulphate nor zinc sulphate exhibited any antibacterial effects against the tested bacterial strains.

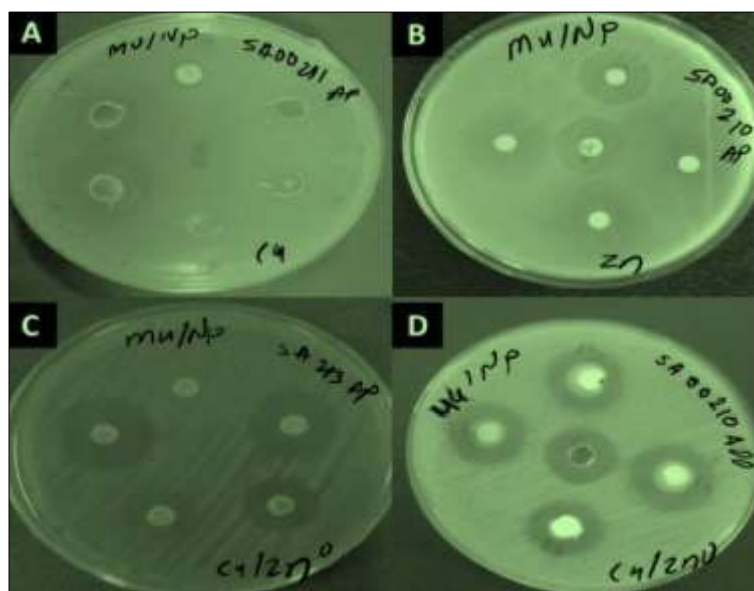
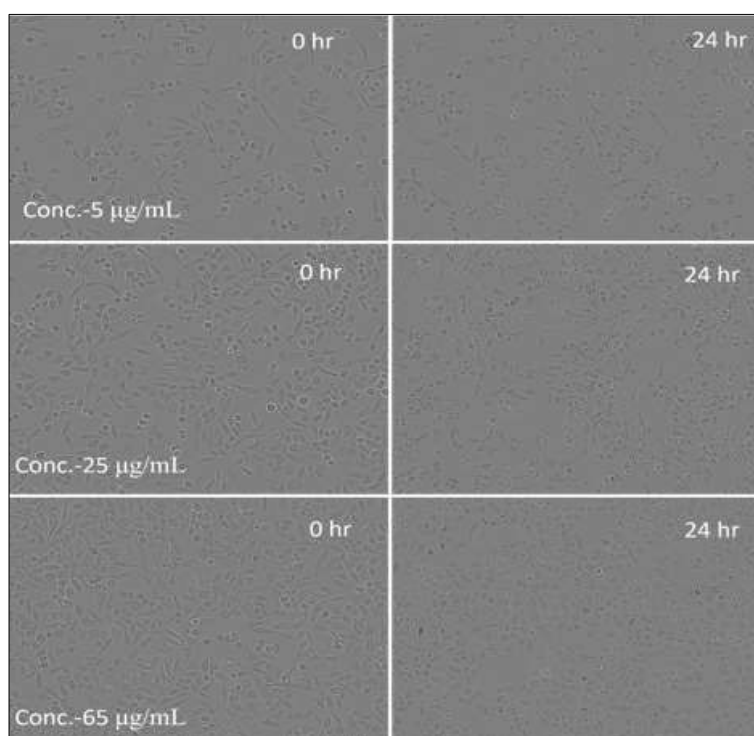


Fig 10. Antimicrobial Activity of Copper, Zinc, plant extract and bimetallic nanoparticles

Investigation of Anticancer Activity



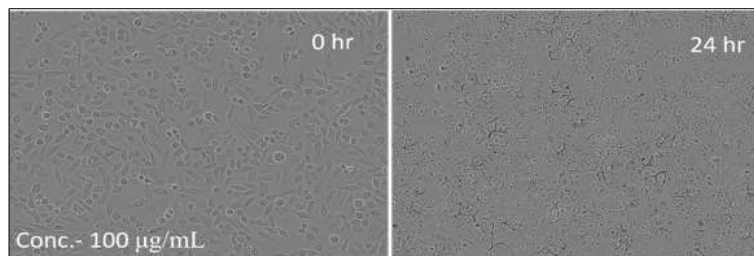


Fig 11: 20x magnified HeLa cells 0 hr and 24 hr after Cu-ZnO treatment with different concentration

Potential anticancer properties of biologically synthesized bimetallic silver-zinc NPs against HeLa cancer cells were evaluated. The results obtained from this study unveiled significant anticancer effects of the bimetallic Cu-ZnO NPs on the cancerous cell lines, with an IC₅₀ value of 56.4 µg/mL.

Furthermore, a detailed assessment of the anticancer activity of the bimetallic Ag-Zn NPs was conducted by exposing HeLa cells to varying concentrations of 5, 25, 65, and 100 µg/mL. The findings demonstrated that the bimetallic Cu-ZnO NPs exhibited high effectiveness in inhibiting the growth of HeLa cells, with observed percentages of inhibition reaching 72%, 75.2%, 82.3%, and 91.2%, respectively, corresponding to the aforementioned concentrations.

These outcomes suggest that the biosynthesized bimetallic Cu-ZnO NPs possess promising potential as an anticancer agent, particularly in combating the growth and proliferation of HeLa cancer cells.

HeLa cells			
Compound name	Conc. µg/ml	OD at 590 nm	% Viability
Control	0	1.590 ± 0.081	100
Cu-ZnO	5	0.494 ± 0.046	28
	25	0.404 ± 0.100	25
	65	0.225 ± 0.055	18
	100	0.133 ± 0.112	9

5. Conclusion

In this study, we have successfully demonstrated the one-pot synthesis of bimetallic Cu-ZnO nanoparticles using tea leaf extract, highlighting its dual role as both a reducing and stabilizing agent. This approach aligns with the growing trend toward utilizing natural plant extracts in nanoparticle synthesis, offering a green and sustainable alternative to traditional chemical synthesis methods. The inherent advantages of using plant extracts include their low toxicity, environmental friendliness, and the availability of a diverse range of naturally occurring reducing agents, which facilitate the reduction of metal ions to their nanoparticulate forms.

The synthesized Cu-ZnO nanoparticles were extensively characterized through several analytical techniques to ascertain their physicochemical properties. Ultraviolet-Visible (UV-Vis) spectroscopy confirmed the successful formation of nanoparticles, while Dynamic Light Scattering (DLS) provided insights into their size distribution, revealing an average particle size of 6-8 nm. Furthermore, Fourier Transform Infrared Spectroscopy (FTIR) was employed to identify functional groups and verify the chemical composition, confirming the presence of characteristic bonds associated with both the metal oxides and organic constituents from the tea leaf extract.

Importantly, the nanoparticles displayed excellent stability and biocompatibility, criteria that are essential for biomedical applications. The biological activity of the Cu-ZnO nanoparticles was evaluated against various microbial and cancer cell lines, demonstrating potent antimicrobial activity against both Gram-positive and Gram-negative bacteria. This broad-spectrum antibacterial effectiveness suggests that Cu-ZnO nanoparticles could be further developed as antibacterial agents in medical and environmental applications. The mechanism of action underlying the antibacterial activity of bimetallic Cu-ZnO NPs appears to involve several factors. Electrostatic interactions may cause damage to the bacterial cell membrane, disrupt proteins and enzymes, generate reactive oxygen species (ROS), and induce oxidative stress. The NPs may also bind to proteins, disrupting cellular homeostasis, including the electron transport chain and signal transduction pathways, potentially leading to genotoxic effects. Moreover, the rough external surface of Cu-ZnO NPs may contribute to cell wall damage, allowing the nanoparticles to penetrate the bacterial plasma membrane and exert toxic effects on the bacteria.

Moreover, the anticancer properties of these nanoparticles were assessed through cytotoxicity studies against cervical cancer (HeLa) cells. The results were promising, showing a significant reduction in cell viability, with only 7% of the cells remaining viable at the highest concentration tested (100 µg/ml). This indicates the potential of Cu-ZnO nanoparticles as effective anticancer agents, particularly in the treatment of cervical cancer.

6. Data availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

7. Conflicts of interest

The authors have no conflicts of interest to declare.

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