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Research Article

Synthesis and Characterization of Biogenic Zinc Oxide Nanoparticles Using *Eugenia uniflora* Extract and its Anticancer Potential

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ABSTRACT

Introduction: Green synthesized nanoparticles have continued to be an important bioresource, exhibiting targeted delivery to diseases' active sites with considerable eco-friendliness and effectiveness. **Objective:** In this study, the medicinally useful *Eugenia uniflora* L. through green synthesis with zinc oxide nanoparticles (ZnONPs), was potentiated for its anticancer activity. **Materials and Methods:** The leaf aqueous extract of *E. uniflora* (EU) was biosynthesized with zinc acetate dihydrate precursor to develop EU-ZnONPs. Characterization was based on field emission scanning electron microscopy (FESEM), high-resolution transmission electron microscopy (HRTEM), ultraviolet-visible (UV-Vis) spectroscopy, and energy-dispersive X-ray (EDX) spectroscopy. The anticancer potential of EU-ZnONPs was based on MTT-based cytotoxicity (CC_{50}) against human cancerous (HepG2 and ACHN) cell lines. **Results:** The FESEM revealed spherical-to-cubical shaped EU-ZnONPs with 40 and 80 nm average size ranges. Further microscopic evaluation by HRTEM showed that the bulk of the nanoparticles (NPs) are spherical, ranging from 5–30 nm in size. The UV-Vis absorption peak at 387 nm agreed with the characteristic 300-400 nm peak range of biogenic ZnONPs. The presence of Zn and O at elemental weight percentages of 73.55 and 23.05% confirmed the successful green synthesis of the Eu-ZnONPs. At 48 h post-treatment, the cytotoxicity against HepG2 and ACHN cancer cell lines was concentration-dependent, with CC_{50} values of 54.21 ± 0.06 µg/mL and 33.36 ± 2.25 µg/mL, respectively. **Conclusion:** This study has shown that EU-ZnONPs possess notable cytotoxicity against HepG2 and ACHN cancer cells, thus suggesting *E. uniflora* extract-based ZnONPs as a promising anticancer bioresource.

Keywords: Anticancer, *Eugenia uniflora,* Green Synthesis, ZnO Nanoparticles.

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INTRODUCTION

One of the worst diseases in the world, cancer is predicted to kill 9.9 million people in 2020 because of unchecked reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) production.[1] ROS can result in base changes, deletions, and strand breaks, which can induce chromosomal rearrangements, unstable genomes, and hyper- and hypo-methylation of DNA, according to Valko *et al.*[2]. If damaged DNA is not repaired, a mutant cell can live and divide abnormally, which can lead to cancer.[3] Apoptosis, also known as programmed cell death, is a crucial regulatory mechanism that causes cells to die if DNA damage is not adequately repaired.[4] Since these processes are essential to neoplastic transformation and metastases, it is believed that the most important therapeutic approaches for the treatment of cancer entail activating apoptotic pathways and negatively regulating the course of the cell cycle in cancer cells.[5]

Recent developments in cancer treatment have favoured the application of nanotechnology in medicine, or nanomedicine, by offering better monitoring, opportunities for regenerative therapies, and targeted medication delivery to the disease-active site. Because nanomedicine targets the cellular and molecular processes that govern cell death directly, it offers a powerful method for causing the death of cancer cells, particularly those that are resistant to conventional therapy.[6] Nanoparticles (NPs) can be created using physical and chemical methods, such as chemical reduction, laser ablation, thermal breakdown, ultrasonication, and electrochemically aided synthesis.[7] In addition to being expensive, several of these techniques contain dangerous chemicals that may be harmful to both the environment and human health. The synthesis of nanoparticles has shifted to green methods in recent years due to their cost-effectiveness, environmental friendliness, and relative safety.[8] Since their size-dependent properties and low toxicity have been widely applied in a variety of industries, including microelectronics, textiles, diagnostics, cosmetics, and medicine, zinc oxide nanoparticles (ZnONPs) have emerged as the most promising nanomaterials in recent years.[9] A vast array of biomolecules and metabolites, including proteins, vitamins, and intermediates based on coenzymes, phenols, flavonoids, terpenoids, saponins, and carbohydrates, are genetically diverse in plants. These plant metabolites contain amine, carbonyl, and hydroxyl functional groups that react with metal ions to reduce them to nanoscale particles.[10]

A member of the Myrtaceae family, *Eugenia uniflora* is a tiny, edible fruit-bearing plant. The plant is rich in flavonoids, carotenoids, phenolic compounds, vitamin C, and other minerals like calcium, iron, magnesium, and phosphorus.[11] Several pharmacological properties, including antibacterial, antifungal, antiviral, anti-helminth, insecticidal,

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antidiarrheal, antihypertensive, anticancer, and anti-rheumatic effects, have been associated with the fruit.[12-14] Considering the plant's immense medicinal value, the current endeavour seeks to develop a sustainable process for the biosynthesis of zinc oxide nanoparticles (ZnONPs) using leaf extract from *E. uniflora*, characterize the synthesized ZnONPs using UV, energy-dispersive X-ray spectroscopy (EDX), field emission scanning electron microscopy (FESEM) and high-resolution transmission electron microscopy (HRTEM), and assess their anticancer potential.

MATERIALS AND METHODS

Plant material

The leaves of *Eugenia uniflora* were collected in March 2021 at Obafemi Awolowo University (O.A.U.), Ile-Ife, Nigeria (GPS coordinates: N 7˚3103.799200, E 4˚31034.852800). The collection was authenticated at the Ife Herbarium, O.A.U., Ile-Ife, with a voucher number, IFE 16589. The leaves were separated from their branches and air-dried away from direct sunlight inside the screen house with frequent turning. The dried leaves were powdered and kept in an airtight plastic bag for further use.

Green synthesis of ZnONPs using *Eugenia uniflora* leaf extract

The green synthesis of zinc oxide nanoparticles (ZnONPs) using *Eugenia uniflora* leaf extract (EU) is illustrated in Figure 1. Here, 100 mg of EU was dissolved in 10 mL of distilled water to make an aqueous solution of the extract. This was added to 100 mL of 0.2 M zinc acetate dihydrate solution (4.3898 g in 100 mL distilled water) and heated to 100 °C for 4 h under continuous stirring at 250 rpm. A slightly higher temperature was used to synthesize nanoparticles, as it is proven to produce small-sized, uniform nanoparticles.[15] The colour shift in the solution indicates the formation of zinc oxide nanoparticles. After the incubation, the solution was centrifuged at 10,000 rpm for 10 min. The afforded EU-ZnONPs were washed with distilled water, centrifuged again, and dried in a hot oven set at 60 °C. Completely dried nanoparticles were stored at 4 °C for further analysis.

Characterization of biogenic zinc oxide nanoparticles

UV-Visible spectroscopic analysis

Characterization of biogenically synthesized nanoparticles was done by UV-visible spectroscopy after 24 h of the experiment, and a graph was also plotted.

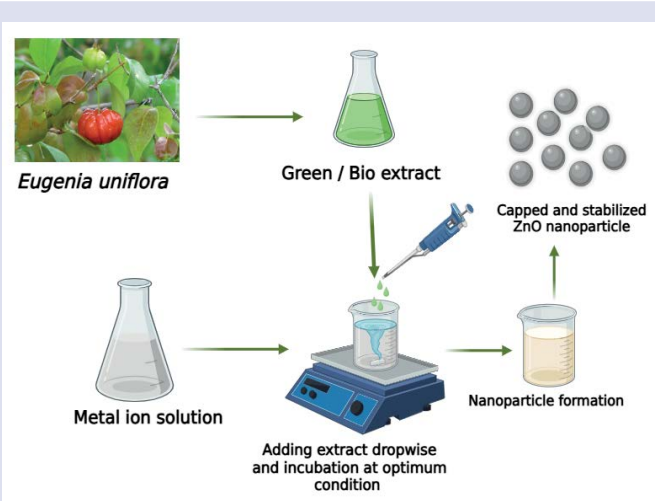

Figure 1: Green synthesis of ZnONPs using *Eugenia uniflora* leaf extract.

Field emission scanning electron microscope (FESEM)

The powdered samples were placed on copper mesh for FESEM investigation, and a gold sputtering apparatus was applied with a 3 nm gold coating. FESEM (German manufacturer Carl Zeiss; model: SIGMA-0261) was used to record the surface morphology of the ZnONPs at a 3 kV accelerating voltage at 50,000 × magnification.

Energy-dispersive X-ray spectroscopy (EDX)

To determine the presence of elemental Zn, energy dispersive X-ray spectroscopy (EDX) was done using a Scanning Electron Microscope (JEOL JSM-IT100InTouchScopeTM, Tokyo, Japan) equipped with Oxford-EDX software. The powdered dried nanoparticles were mounted on copper mesh, and a 3 nm gold coating was done by a gold sputtering unit. Eighty mm^2 SDD detectors that detect elements under high resolution were used for the purpose.

High-resolution transmission electron microscopy analysis (HRTEM)

A high-resolution transmission electron microscope (model name FEI TECNAI G2 F300) running at an accelerating voltage of 300 kV was used to determine the size and shape of biogenically produced ZnONPs. The carbon-coated copper stub was covered with a little drop of nanoparticle suspension, allowed to air dry, and then subjected to HR-TEM.[16]

In-vitro anti-cancer activity

Cell culture

The South African company Highveld Biologicals (Pty) Ltd., located in Lyndhurst, provided the Vero, ACHN, and HepG2 cell lines. The cells were maintained using Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham (DMEM/F12). 10% fetal bovine serum (FBS), 100 mg/mL streptomycin, 100 units/mL penicillin, 0.14% sodium bicarbonate, and 0.1 mM sodium pyruvate were added as supplements. For a duration of 24 h, cells were cultured on 35 mm petri dishes at 37 °C, 5% CO_2, and 95% humidity in a CO_2 incubator.

MTT assay

A standard MTT test was used to assess cell viability, following Denizot and Lang's[17] instructions. The effects of different doses of EU-ZnONPs on the inhibition of cancer cell proliferation were examined using the Vero normal cell line, ACHN human renal adenocarcinoma cell line, and HepG2 human liver cancer cell line. The cells were grown to confluence in a DMEM complete medium that was enhanced with 10% FBS and 1% penicillin-streptomycin solution. The medium was kept at 37 °C in an incubator with 5% CO_2, humidity, and a humidified environment. Trypsinized and counted exponentially developing cultured cells were sown at a density of 2×10^4 cells/well in a 96-well plate. The cells were treated with escalating doses of EU-ZnONPs (40, 80, 120, 160, 200 µg/mL) as well as a negative control (water) and a positive control, 5-Fluorouracil (50 µM), for 48 hours following a 24-hour period of adherence. Following the above-mentioned conditions of incubation, 10 µL of MTT solution (5 mg/mL) was applied to each well, and the wells were left in the dark for three hours. After the medium was carefully removed, 50 µL of isopropanol was used to dissolve the formazan that had developed in the wells, and the plates were left on a plate shaker for five minutes. The absorbance was determined at 595 nm with an iMarkTM Microplate Absorbance Reader (Bio-Rad, USA). Every experiment was run in four replicates.

Statistical analysis

To compare the activity of EU-ZnONPs and references, Student's t-test integrated with the KyPlot program (version 5.0) was used for statistical analysis.

RESULTS AND DISCUSSION

Characterization of biogenic zinc oxide nanoparticles

UV-Visible spectrum

Figure 2A shows the UV-Vis spectra of the green EU-ZnONPs that were synthesized. The EU-ZnONPs' absorption peaks at 387 nm match the characteristic ZnONP peaks, proving that ZnONPs were produced successfully.[18-20]

Field emission scanning electron microscope (FESEM)

FESEM results revealed that the green synthesis of EU-ZnONPs led to the formation of spherical to cubical ZnONPs, which agglomerate in clusters (Figure 2B). The average size of NPs ranges between 40 and 80 nm.[21]

Energy-dispersive X-ray spectroscopy (EDX)

EDX analysis was used in this investigation to evaluate the elemental composition of the EU-ZnONPs. The spectra revealed the presence of O, Al, Si, Fe, and Zn (Figure 2C). The Zn and O components of the green-produced ZnONPs had corresponding weight percentage values of 73.55 and 23.05%.[22] The surface plasmon resonance effect, which is common for the absorption of ZnO nanostructures, is also responsible for the distinctive Zn peaks in the obtained EDX spectrum at 1.11, 8.55, and 9.23 keV.[23]

High-resolution transmission electron microscopy analysis (HRTEM)

HRTEM analysis revealed that the bulk of the biogenically generated EU-ZnONPs were spherical and varied in size from 5 to 30 nm. Furthermore, Figure 2D demonstrated that the nanoparticles were evenly spaced and did not build up. This result is consistent with Rajput *et al.*[16]. Previous studies have found an inverse relationship between nanoparticle size and bioactivity.[24]

In-vitro anti-cancer activity

The cytotoxicity of the green route-synthesized nanoparticles against the Vero, ACHN, and HepG2 cell lines was assessed using the MTT test. In addition to varying dosages of EU-ZnONPs, the three cell lines used in this investigation—Vero, ACHN, and HepG2—were given comparable amounts of the suspension media. Notably, the EU-ZnONPs showed 75.15 ± 2.34% viability at a dose of 200 µg/ml with 50% cytotoxicity (CC_{50}) values of 261.08 ± 5.59, indicating the likely safety of the EU-ZnONPs. This suggests that the EU-ZnONPs were not cytotoxic to the Vero (non-tumorigenic) cell line (Figure 3A). At a dosage of 200 µg/mL, the EU-ZnONPs demonstrated significant dose-dependent anticancer action against the ACHN and HepG2 cell lines, with 32.67 ± 2.26 % and 35.21 ± 4.24 % viability (Figures 3B, C). After 48 hours of treatment, the CC_{50} values were 54.21 ± 0.06 µg/mL and 33.36 ± 2.25 µg/mL. Furthermore, ACHN and HepG2 were tested with 5-fluorouracil

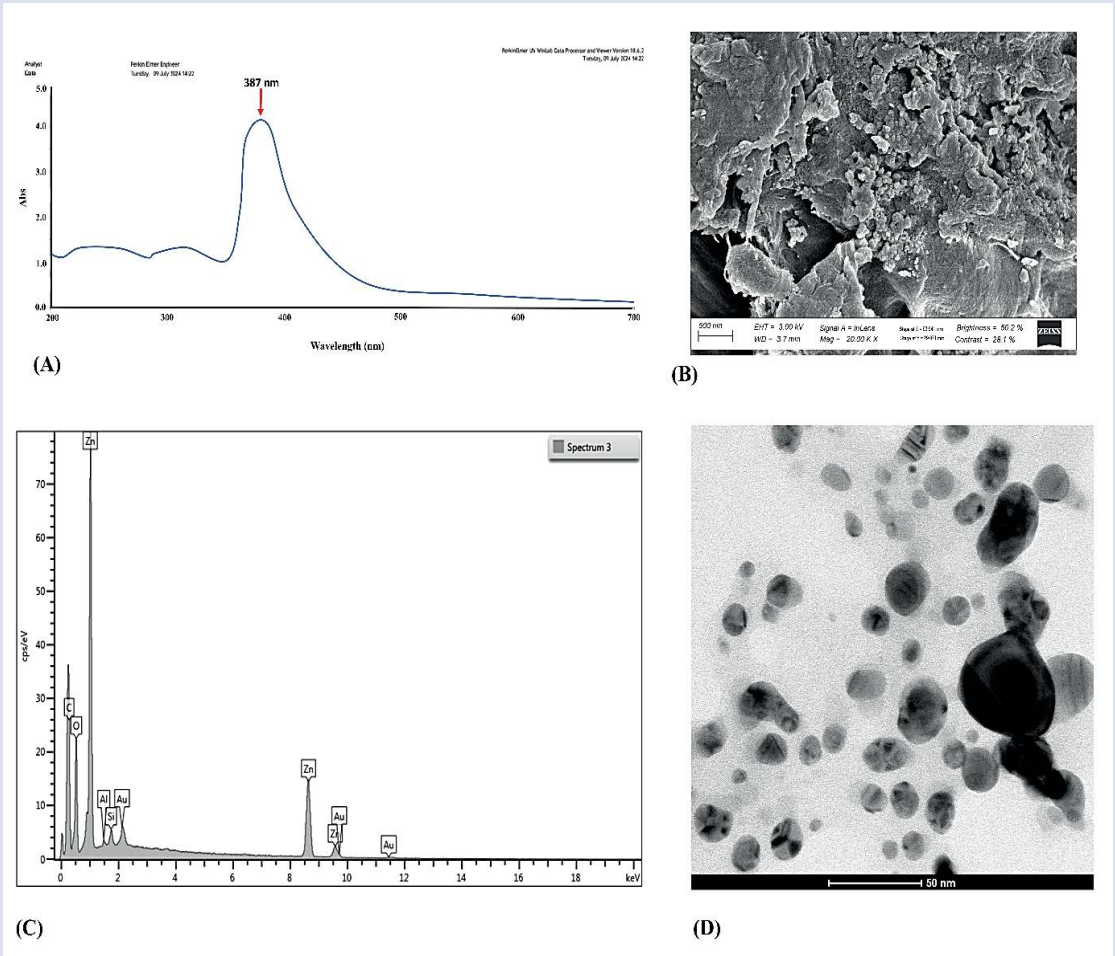

Figure 2: Characterization of biogenically synthesized zinc oxide nanoparticles (ZnONPs): **(A)** UV Spectroscopy showing characteristics peaks at 387 nm. **(B)** Field emission Scanning electron microscopy (FE-SEM) image of EU-ZnONPs. **(C)** EDX mapping and elemental profile of biosynthesized EU-ZnONPs. **(D)** HR-TEM analysis of EU-ZnONPs.

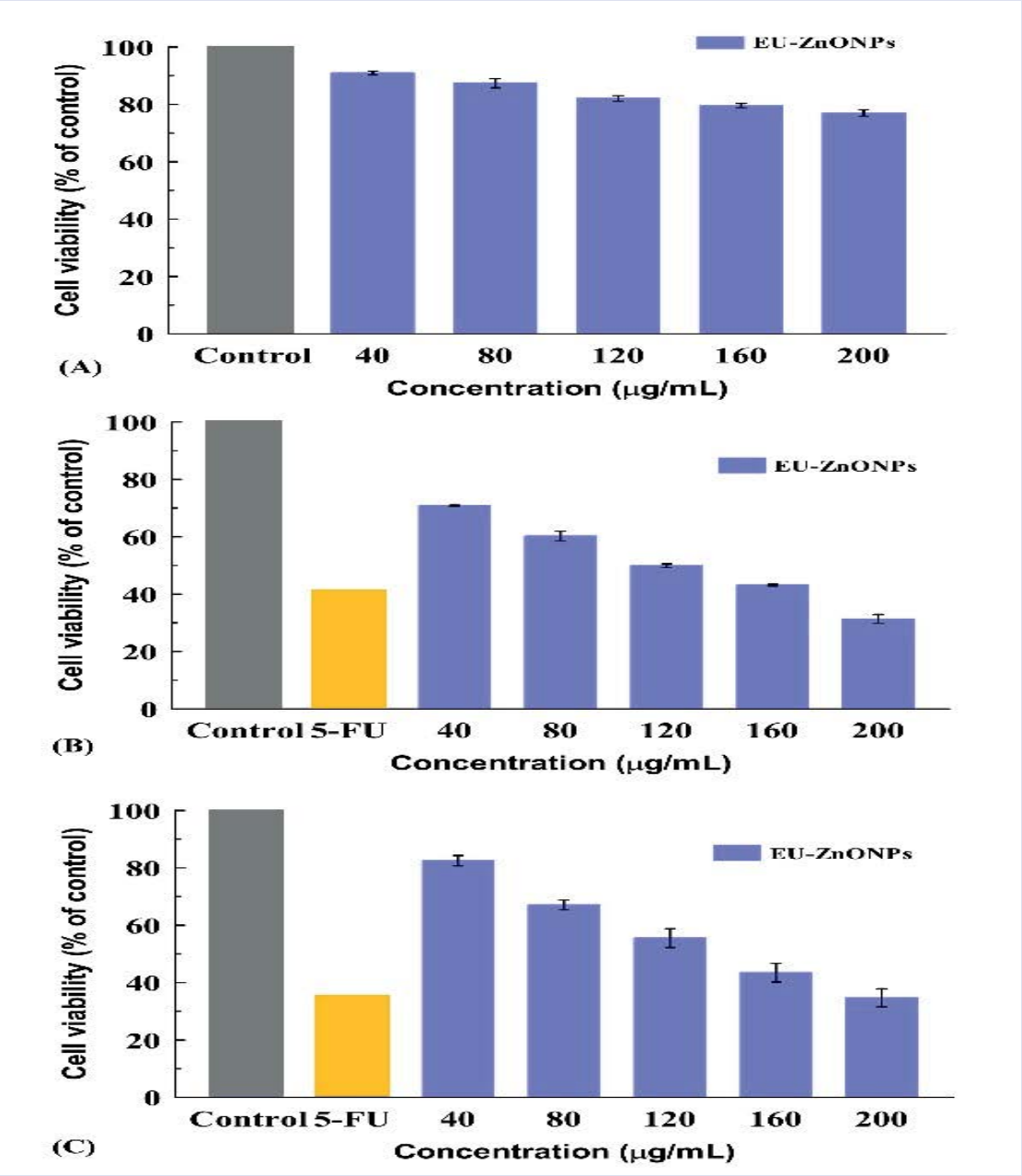

Figure 3: Cell viability (%) of **(A)** Vero normal cell line. **(B)** ACHN. **(C)** HepG2 cell line upon exposure to various concentrations of EU-ZnONPs for 48 h. [Data expressed as mean ± S.D.]

(50 µM) as a positive control. The cells showed approximately 50% viability with CC_{50} values of 3.04 ± 0.07 µg/mL and 2.68 ± 0.04 µg/mL, respectively. Significant differences in the antiproliferative properties of the nanoparticles were observed between the tested normal and cancer cell lines. This shows an interesting variation in the target areas that the nanoparticles may employ to produce cytotoxicity in the tested cells, as mentioned in the text. However, it is crucial to stress that cytotoxicity by itself does not make any therapeutic substance a potential drug or chemical that can be drugged.[25] The potential adverse effects of these more recent therapeutic agents, such as metal nanoparticles and their concentrations on healthy cells, on biological processes *in vitro and in vivo* were unclear until recently. Furthermore, it became necessary to respond to such adverse effects in accordance with the standards set for public health and safety, considering emerging public complaints in this field of study.[25] Overall, the current findings demonstrated experimentally that normal cells did not show appreciable cytotoxic effects when exposed to the same concentration of nanoparticles as cancer cell lines.

CONCLUSION

This study provided evidence through microscopy (FESEM and HRTEM) and spectroscopy (UV-Vis and EDX) for *E. uniflora* leaf aqueous extract (EU)-based ZnONP green synthesis. The EU-ZnONPs showed notable cytotoxicity against the cancerous HepG2 and ACHN cells. Additionally, the normal (Vero) cells did not show appreciable cytotoxic effects when exposed to the same concentrations of biogenic nanoparticles as the cancer cell lines, suggesting EU-ZnONPs to be selectively cytotoxic to the cancer cells only. The study findings have, therefore, shown the potential of *E. uniflora* extract-based ZnONPs as a promising anticancer bioresource. Future studies may include

determining the anticancer mechanism of action of the nanoparticles and evaluating the *in-vivo* anticancer activity using a transgenic mice model.

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