PHYTOFABRICATION OF ZINC OXIDE NANOPARTICLES USING CYPRUS *MENTHA PIPERITA* AND EVALUATION OF ITS ANTICANCER AND ANTIMICROBIAL ACTIVITY

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Abstract

Mentha piperita was used as a chelating agent to synthesize zinc oxide nanoparticles (ZnO-AE-NPs). The anti-proliferative, cytotoxic, and anti-microbial potential of the biosynthesized ZnO-AE-NPs were studied using 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), trypan blue, and disc diffusion, respectively. A comprehensive analysis of the ZnO-AE-NPs was conducted utilizing various techniques, X-ray diffraction (XRD) analysis, UV-vis (UV-vis), Fourier-transform infrared (FTIR), Scanning electron microscopy (SEM and energy-dispersive X-ray (EDX)), and Zeta sizer. The results of the analysis showcase the ZnO-AE-NPs to have average particle size and zeta potential of 23 nm and +42 mV, respectively, ascertained by the Zeta sizer. The FTIR, XRD, and EDX spectra ascertained the formation of ZnO-AE-NPs. UV-vis spectra revealed an absorption peak at 368 nm due to a colour change from light green to brown, confirming the synthesis and good stability of the nanoparticle, and spherical morphology was observed from our SEM analysis. Furthermore, The ZnO-AE-NPs revealed cytotoxic and anti-proliferative potential against MDA-MB 231 cells with increased concentration. A robust antimicrobial potential was observed in Escherichia coli and Staphylococcus aureus. ZnO NPs AE revealed anti-microbial, anti-proliferative potential on E. coli and S. aureus, and MDA-MB 231 cells.

Keywords: Mentha piperita; anti-proliferative; cytotoxic; anti-microbial; zeta potential

1. Introduction

Nanotechnology is the latest area of study that is growing rapidly, and it utilizes technologies that are environmentally friendly for the purpose of synthesizing biosynthetic nanoparticles (i.e., Na, Au, Ag, ZnO, nanoparticles), which are biocompatible, chemically stable, nontoxic, and can be used as anticancer agents [1], anti-microbial [2], antidiabetics [3], drug carriers [4], cell imaging [5], biosensors [6], and cosmetics [7], due to their unique physiochemical

characteristics. Before the advance of green synthesis, the metal oxide nano-particles (MO NPs) are synthesized using chemical methods involving antagonistic chemicals, making them tedious, expensive, and toxic [8]. MO NPs modifications are usually achieved by substituting some metal ions with specific atoms that will change and enhance the materials' electrical, optical, and mechanical properties by substituting their surface chemical properties [9].

Green synthesis (GS) is a plant-mediated synthetic method that is being used to synthesize nanoparticles (NPs), and it's a green chemistry method that connects plants with nanotechnology [10]. GS method is basically the most novel method for synthesizing NPs because it has always been reported to take place at neutral pH and ambient temperatures, and it's environmentally friendly [10,11]. Before the advance of green synthesis, MO NPs were synthesized using conventional methods (involving chemicals), which made the process involve antagonistic chemicals, making it tedious, expensive, and toxic [9].

In this current study, we reported the synthesis of stable ZnO nanoparticles (ZnO-AE-NPs) using an aqueous extract of *Mentha piperita* obtained from the Turkish Republic of Northern Cyprus, characterized with spectroscopic and microscopic standard methods. We evaluated its anti-proliferative and cytotoxic potentials on MDA-MB 231 cells using MTT and tryphan blue assay, respectively. In addition, we assessed its antimicrobial potential on in *Escherichia coli* and *Staphylococcus aureus* using standard methods.

2. Methodology

A. ZnO-AE-NPs Synthesis

ZnO-AE-NPs using *Mentha piperita* was achieved with the technique employed by Huzaifa et al. (2019). ZnO-AE-NPs was synthesized with 0.05 M zinc nitrate solution ($Zn(NO_3)_2{\cdot}6H_2O$). Extraction was carried out using water as our extraction solvent in a conical flask for 72 h at 45 ^oC. Then 15% of *Mentha piperita* aqueous extract was slowly added under constant stirring and a temperature of 70 ^oC for 9 hours. NaOH solution was then included into the mixture during shaking to adjust the pH. The mixture was calcined in a muffle furnace, as illustrated in Figure 1. The obtained residue was the ZnO-AE-NPs.

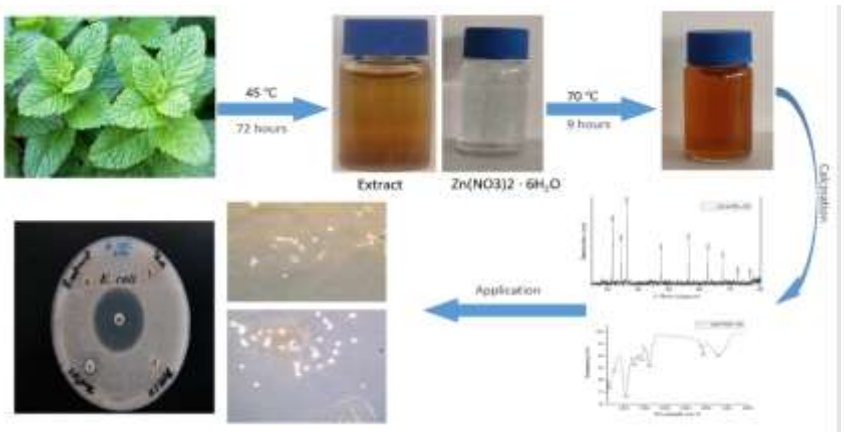

Figure 1: Review of the synthetic process and biological potency of ZnO-AE-NPs.

B. Characterization of the ZnO-AE-NPs

The UV-Vis spectroscopy of the ZnO-AE-NPs was determined with a Shimadzu UV-2450. Biosynthesized ZnO-AE-NPs residue was dissolved in deionised water and mixed using a sonicator. Obtained filtrate taken into a 10 mm cuvette was used, and the spectrum was measured within the wavelength of 300 and 800 nm. The analysis takes place at room temperature. The size e was determined by a Malvern Nano ZS90. The functional groups present in the ZnO-AE-NPs were determined with a Fourier Transform Infrared Spectrophotometer (FTIR) at a frequency of 500-4000 cm^{-1} and absolute resolutions (4 $cm^{-}1$). The crystalline nature of the ZnO-AE-NPs was evaluated using the Rigaku ZSX Primus II, X-ray diffractometer (XRD). Powdered samples were used in an X-ray diffractometer equipped with radiation of CuK to study the ZnO NPs structure. ZnO-AE-NPs morphological structure was analysed using a scanning electron microscope (SEM), and the elemental mapping using energy-dispersive X-ray spectroscopy (EDX).

C. Antimicrobial Activity

The antimicrobial potency of the biosynthesized ZnO-AE-NPs were determined on *Staphylococcus aureus* (6538 P) and *Escherichia coli* (0157:H7) on nutrient agar dishes as revealed by Doga et al. [12]. The organisms were treated with the ZnO-AE-NPs and incubated overnight; zones of inhibition were then evaluated in line with the standard microbiology-based protocols.

D. Tryphan Blue Assay

The toxicity of ZnO-AE-NPs was studied on "MDA-MB 231" cells with the method used by Fraser et al. [13]. Varying concentrations (0 ~ 500 μg/mL) of the synthesized NPs were applied to cells. The medium containing was aspirated, and diluted tryphan blue was added and incubated for 10 mins under the dark; the treatment was then removed, and the dead and live cells were counted. Results were presented as average mean ± standard deviation.

E. Proliferation Assay

Proliferations of the cells following treatment with various doses of ZnO-AE-NPs were assessed on MDA-MB 231 cells with MTT using the steps described by Umar et al. [14]. Cells (3×10^4) were treated with 0, 2.5, 5, 50, 250, and 500 μg/mL of ZnO-AE-NPs. The culture medium was removed, and growth media (600 μL) and a certain volume of MTT (5mg/mL) were added to the cells. The MTT solution was drawn out following the incubation, and Dimethyl sulfoxide (890 μL) and glycine buffer (110 μL) were added. In addition, the absorbance was determined at a wavelength of 570nm with Absorbance Microplate Reader (ELX 800^{TM}). Multiple measurements were carried out, and each experiment was repeated at least three times.

F. Statistical Data Analysis

The results of the experiments were presented as means ± standard deviations (STDs). We analyzed our experimental data using IBM SPSS Statistics version 21, and conducting a one-

way ANOVA and Student's t-test, were necessary. Experiments were repeated at least thrice in triplicates ($n \geq 3$). $P < 0.05$ was considered significant.

3. Results And Discussion

Biosynthesized ZnO-AE-NPs using *Mentha piperita* obtained from the Turkish Republic of Northern Cyprus as a chelating agent was confirmed based on colour shift and characterization response obtained from our microscopic and spectroscopic analysis.

The rapid change in the colour of the extracts resulted from the Surface Plasmon Resonance and UV-vis analysis result of the ZnO-AE-NPs displayed an absorption peak at 368 nm (figure 2a) and is similar to the absorption peak range of zinc oxide nanoparticles as revealed by Kavaz et al. and many more studies [15]. The size determined by the zeta sizer is 23nm in diameter, and the NPs are stable based on the zeta sizer result. FTIR analysis revealed the functional group attached to the synthesized nanoparticles (figure 2b). The absorption bands observed at 3420 cm−1 indisputably indicate the presence of O-H bond due to water absorption during synthesis. Additionally, the bands detected at 1049 and 1450 cm−1 unambiguously signify the existence of C=O of alcohol (saturated) and C=CH of a methyl group, respectively. These remarkable characteristics, including the presence of an alcohol group, confer the ability of the NPs to get attached to metals and impede agglomeration [16]. The X-ray diffraction peaks obtained from our XRD results confirmed the broadening XRD pattern present in zinc oxide nanoparticles, as seen in Figure 2c. Diffraction peaks present at 31.70°, 34.34°, 47.54°, 56.48°, 62.78°, 67.66°, 72.53°, and 76.58 have revealed the spherical phase of zinc oxide NPs, the crystal formation and purity of particle [14]. The diameter of ZnO-AE-NPs crystallites calculated by the Debye–Scherrer formula is 23 nm. SEM results has shown the spherical morphology of nanoparticles with an average size of 23nm, as shown in Figure 1d. Studies reported that morphology size and type played significant role in the biological activity and stability of the NPs [17]. Particles at nano size tend to behave differently with more diverse unique properties and applications [18]. Furthermore, the EDS analysis on the synthesized NPs revealed signals of zinc and oxygen, which confirm that the NPs are zinc oxide NPs and the role played by extract in the synthesis of the NPs.

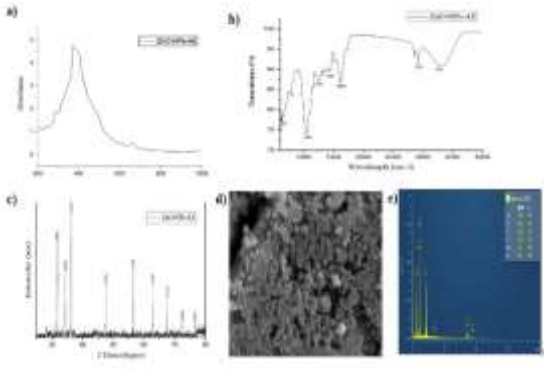

Figure 2. a) Absorption spectra of the biosynthesized ZnO-AE-NPs by UV–visible Spectrophotometer. b) FTIR of the biosynthesized ZnO-AE-NPs using *Mentha piperita*. c) XRD pattern of ZnO-AE-NPs. d) SEM image of ZnO-AE-NPs e) EDS Spectra of the ZnO-AE-NPs.

Toxicity of the ZnO-AE-NPs was studied on MDA-MB 231 cells *in vitro,* with various concentrations of the extract (0, 2.5, 5, 50, 250, and 500 μg/mL), and the result revealed the cytotoxic effect in a dose-dependent approach (Figure 3a). The synthesized NPs are toxic to the cells with increased concentrations of the NPs, and more effects are observed in the MDA-MB cells treated with 500 μg/mL following 24 hours incubation period. Cytotoxic potentials of ZnO NPs synthesized vis green synthesis on breast cell lines have been reported. The studies revealed the effect in a concentration-dependent manner and agreed with the result we obtained in our studies [19]. In addition, our previous studies on MDA-MB 231 cells showed ZnO NPs synthesized using plant extract to exact cytotoxic effect in a concentration-dependent manner [14].

Lower concentrations of the ZnO-AE-NPs that are not toxic to the cells were used to ascertain the potentials of the NPs on the cells' proliferation, and our result showed a significant decrease in cells treated with 5 μg/mL ($P < 0.001$, $n \geq 5$, Figure 3b). In addition, the 2.5 μg/mL ZnO-AE-NPs did not show any effect on the proliferation of cells following the 24-hour treatment. Studies revealed that metal oxide NPs that are not toxic could potentially inhibit the proliferation of the cells, making them candidates that can inhibit metastasis [20].

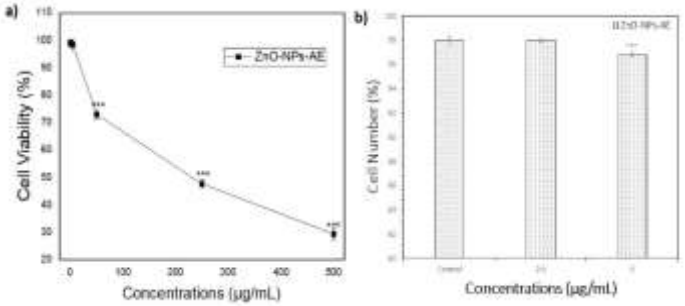

Figure 3. Effect of ZnO-AE-NPs on a) Viability and b) Proliferation of MDA-MB 231 cells.

Antimicrobial potency of the synthesized NPs was determined on *Escherichia coli* and Staphylococcus aureus (Table 1). The ZnO-AE-NPs revealed more antimicrobial potential than Mentha piperitai on both bacteria, and comparing the potential of the ZnO-AE-NPs with that of ciprofloxacin (control) and other groups revealed a significant difference ($P < 0.05$; Table 1). Umar et al. revealed the anti-bacterial potential of ZnO NPs synthesized using *Ficus thonningii* on both bacteria, and the result is in conformity with our findings [21]

Table 1. The Anti-microbial potency of ZnO-AE-NPs using *Mentha piperitai*

	Zone of Inhibition (mm)	
Organisms	*E. coli*	*S. aureus*
Ciprofloxacin (10ug/disc)	11.31 ± 0.39	13.50 ± 0.67
Mentha piperitai extract (500 μg/mL)	$2.86 \pm 0.31^{***}$	$2.65 \pm 0.26^{***}$

ZnO-AE-NPs (500 µg/mL)	$4.52 \pm 0.40^{***}$	$3.96 \pm 0.58^{***}$

4. Conclusion

The aqueous extract of *Mentha piperita* leaves has the potency to be used as a stabilizing agent in the synthesis of ZnO-AE-NPs due to its phytochemical composition. Biosynthesized ZnO-AE-NPs were synthesized and characterized using standard spectroscopic and microscopic methods. It also revealed cytotoxic and anti-proliferative potential against MDA-MB 231 cells with increased concentration. A strong antimicrobial potential was observed in *Escherichia coli* and *Staphylococcus aureus*. ZnO NPs AE revealed anti-microbial, anti-proliferative potential on *E. coli* and *S. aureus* and MDA-MB 231 human breast cancer cells, respectively.

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