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# Green Synthesis and Characterization of Zinc Oxide Nanoparticles Using *Hedysarum varium* Wild. and Its Antibacterial Activities

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***Abstract***

The increasing interest in nanomaterials has accelerated the search for sustainable and eco-friendly synthesis routes. In this study, zinc oxide nanoparticles (ZnO NPs) were synthesized for the first time using *Hedysarum varium* extracts through a green synthesis approach. The plant material was extracted using a methanol–water mixture, and the resulting extract was incorporated into a zinc nitrate solution, followed by incubation at 60 °C for 4 hours under pH 10. After purification and drying at 90 °C, pure ZnO NPs were obtained. Characterization studies confirming the formation of crystalline ZnO NPs with sizes ranging from 10–20 nm, while optimization studies revealed that a 25 mg extract concentration provided the stable and controlled nanoparticle formation. Antibacterial activity was evaluated against test bacteria, and the nanoparticles exhibited the strongest effect against *Staphylococcus aureus* (MIC: 0.25 mg/mL), indicating potential for biomedical applications. Compared with previously reported green synthesis methods using *Aloe vera* or *Thymbra spicata*, the use of *H. varium* extract offers a novel, cost-effective, and sustainable alternative with antibacterial properties. This study contributes to the literature by presenting the first report of ZnO NP synthesis mediated by *H. varium*, highlighting its promising role as a green source for nanomaterial production.

***Keywords:*** Green synthesis, *Hedysarum varium,* Zinc oxide, Nanoparticles, Plants extract

## 1. Introduction

Nanotechnology is a rapidly developing technology that can lead to radical changes in different fields of science. Nanomaterials have become an important research topic in both basic and applied sciences due to their size, structure and high surface-to-volume ratio properties, which has led to increased interest in nanoscale semiconductors in recent years. These materials are widely used, especially in optoelectronic applications [1, 2].

There are many methods for the synthesis of nanoparticles, which have a wide range of uses in biomedical and medical fields in recent years. The green synthesis method, which utilizes living materials such as microorganisms, algae, and plants in the biosynthesis of nanoparticles, serves as a cost-effective, biocompatible, environmentally friendly, and reliable alternative to conventional methods, enabling large-scale production of high-purity ZnO nanoparticles with desirable properties and no additional impurities, while also offering significant environmental and economic advantages by reducing the use of toxic and expensive chemicals and enhancing catalytic activity through a biomimetic approach. [3-5] .

The Fabaceae family comprises approximately 224 species, predominantly distributed across the Northern Hemisphere, with primary centers of diversity in North America, Europe, the Mediterranean region, and Central Asia; in Turkey, the genus *Hedysarum L.* is represented by around 24 species [6][7]. Species within this genus exhibit a remarkable ecological adaptability, occurring in a wide range of habitats, from mountainous regions to polar areas, and from coastal environments to grasslands [8, 9]. The majority of *Hedysarum* species are perennial herbaceous plants, although some may exhibit semi-bushy growth forms [10].

Zengin et al. investigated three Fabaceae species, including *Hedysarum varium* Willd., which are utilized in traditional medicine in Turkey. They prepared ethyl acetate, methanol, and aqueous extracts of the plants and evaluated their antioxidant activity as well as their phenolic and flavonoid contents. The methanol extract exhibited the highest concentrations of both total phenols and flavonoids, with values of 45.11 ± 1.399 mg gallic acid equivalent (GAE)/g extract and 39.71 ± 0.092 mg rutin equivalent (RE)/g extract, respectively. Consistent with these findings, the methanol extract of *H. varium* demonstrated superior radical scavenging activity (IC50: 0.30 ± 0.005 mg/mL) compared to the positive control, trolox (IC50: 0.31 ± 0.003 mg/mL) [11].

There is a few literatures on green analysis for ZnO. Santhoshkumar et al., zinc oxide nanoparticles (ZnO NPs) were successfully synthesized and characterized using the extract obtained from the leaves of the *Passiflora caerulea* L. (Passifloraceae). The antimicrobial activity of these nanoparticles was investigated [12]. Sangeetha et al., zinc oxide nanoparticles (ZnO NPs) were synthesized by a biological method using the *Aloe vera* plant. However, the synthesized nanoparticles were found to have a spherical structure and high stability, and their average size varied between 25-40 nm [13]. Gür and his colleagues, zinc oxide nanoparticles (ZnO NPs) were synthesized using the *Thymbra spicata* L. plant. The synthesized nanoparticles were effective against microbiological pathogens and showed protective effects in preventing DNA damage. The DNA damage prevention effect of ZnO NPs increased depending on the concentration [14].

In this research, we synthesized ZnO NPs from *H. varium*, a species not previously synthesized as a nanomaterial, using simple, rapid, inexpensive, greenness and sustainable chemical methods. The characterization of the synthesized ZnO NPs using advanced imaging

techniques was carried out using UV-Visible spectroscopy, Raman spectroscopy, SEM, EDX and optical microscope image analyses. Antimicrobial activities of the NPs against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25923 were investigated.

## 2. Material and Method

### *2.1. Plants and Chemicals*

The examined specimens of *H. varium* were collected during the field trips on 30 May 2024 from the natural environment in Ankara- Aysantı pass. A part of collected specimens was prepared as herbarium samples for use in diagnostic and subsequent research purposes. These dried specimens are currently stored in the Herbarium of Gazi University (GAZI). The rest of collected individuals were dried after cleaning by natural air-drying method.

Zinc nitrate hexahydrate ($Zn(NO_3)_2$ $6H_2O$) was supplied in high purity from Sigma-Aldrich (Hamburg, Germany). Sodium hydroxide (NaOH) and methanol ($CH_3OH$) were purchased from Isolab Chemicals (Eschau, Germany) for use in laboratory-scale syntheses. Purified distilled water used in the experiments was provided by Ankara Medipol University. All chemicals were of analytical grade and were used directly without any additional purification.

### *2.2. Instruments*

The optical properties of the sample were analyzed using Shimadzu 1601 double beam path spectrophotometer (Kyoto, Japan). Spectra were analyzed and evaluated at the appropriate absorbance value using UV-probe software. SEM (ZEISS, Supra 50 V) was utilized to obtain high-resolution images of the surface morphology. EDX (Oxford Instruments, INCA Energy) was used to determine the composition and elemental distribution of the sample. Optical microscopy and Raman spectroscopy (WITec alpha-300R instrument) was employed to investigate molecular vibrational modes, offering valuable insights into the interactions of chemical bonds within the material. Laboratory Comparison SIF500R (Seoul, South Korea) model incubator shaker was used for incubation. ISOLAB Laborgeräte GmbH (Eschau, Germany) digital pH meter was used for pH parameters and magnetic stirrer. Nüve NF200 (Ankara, Turkey) model centrifuge was used for centrifugation. Weighing was carried out using Shimadzu ATX224 model weighing device (Kyoto, Japan).

Condalab Mueller Hinton Broth and Mueller Hinton Agar media were used for culturing bacteria, and Faithful-DH500B11 brand incubator was used for incubation. Automatic pipettes used in inoculation processes were Microlit-MicrolitSLP and Thermo Fischer Scientific-FinnPipette. BioSan DEN-1 spectrophotometer and Velp Scientifica ZX3 vortex were used in the preparation of bacterial suspensions. The microplates used were Cellstar 96 well. TTC used for testing the viability of bacteria was AmBeed. Ildam- NC-75 brand autoclave was used for sterilization processes. All experiments were performed in an Ildam- BSC1100IIA2-X brand biological safety cabinet.

### *2.3. Collection Area and Organic Extract Preparation from H. varium*

In this study, *H. varium* was collected from Ankara, Aysantı Pass, Güdül, stony and marly limestone slopes (1100-1200 m) on 30.05.2024, Taeb 3059 (GAZI), dried appropriately and herbarium specimen was prepared. The stem, leaves and flowers of the dried plant were ground

and used in the preparation of the extract. Preparation of Methanolic Extract: The collected plant material was first washed once with tap water and then twice with distilled water. 5 g of the cleaned sample was taken and cut into small pieces with a stainless-steel knife and added to 250 mL of methanol:water (1:4) mixture. It was mixed in a magnetic stirrer at 300 rpm for 30 minutes to obtain a homogeneous mixture. Then, the extract was filtered using Whatman No:2 filter paper. All prepared extracts were stored in the refrigerator at +4°C until used [15].

### *2.4. Synthesis of H. varium-Mediated Zinc Nanoparticles*

A 50 mmol zinc nitrate solution was prepared in pure distilled water (200 mL total volume). Different amounts of plant extract (25 mL, 50 mL, and 100 mL) were added to this solution for the optimization study, and the volume was completed to 200 mL with distilled water. The pH value of the solution was adjusted to 10 using NaOH. Homogeneity of the solution was ensured by using a magnetic stirrer. The mixture was then incubated at 60 °C for 4 hours, and a light yellow precipitate was obtained. This precipitate was centrifuged 3 times at 5000 rpm for 15 minutes, and the supernatant liquid was discarded after each centrifugation. To further increase the purity of the precipitate, an additional centrifugation step was performed with distilled water. As the final step, the precipitate was dried in a porcelain crucible in an oven at 90 °C for 24 hours, resulting in the desired dry and pure zinc oxide (ZnO). Among the tested amounts of extract, the 25 mL extract showed the highest absorbance value, indicating optimum conditions. The obtained ZnO nanoparticles were stored in a desiccator at room temperature to preserve their purity and stability.

### *2.5. Antimicrobial Activity of ZnO Nanoparticles*

**Minimum Inhibition Concentration Test**

The antibacterial activity of ZnO nanoparticles (ZnONPs) was evaluated by determining the minimum inhibitory concentration (MIC) using the broth microdilution method. The test microorganisms used in this study were *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 as Gram-negative bacteria, and *Staphylococcus aureus* ATCC 25923 as a Gram-positive bacterium. ZnONPs were dissolved in Mueller Hinton Broth (MHB) and subjected to two-fold serial dilutions in the concentration range of 8–0.015 mg/mL. Bacterial suspensions were prepared from overnight cultures of the test microorganisms, adjusted to a final concentration of $5 \times 10^5$ CFU/mL according to the McFarland 0.5 standard. Wells containing only the culture medium served as the negative control. Chloramphenicol was used as the standard drug. The microplates were incubated at 35 ± 1°C for 18–24 hours. After incubation, 0.1% TTC (2,3,5-triphenyl tetrazolium chloride) solution was added to each well, and bacterial growth was assessed based on color change. The color change in the wells (to red or pink) was visually detected. The lowest concentration at which no color change was observed was recorded as the MIC value (mg/mL) [16].

**Minimum Bactericidal Concentration Test**

Following the MIC test, the minimum bactericidal concentration (MBC), defined as the lowest concentration that eliminates 99.9% of the test bacteria, was determined by inoculating samples from each well onto Mueller Hinton Agar. The inoculation procedure was carried out in three replicates. After incubation at 35 ± 1°C for 24 hours, the lowest concentration at which no bacterial growth was observed was recorded as the MBC value (mg/mL) [16].

## 3. Results

### *3.1. Txonomic status*

***Hedysarum varium* Willd.** Sp. Pl., ed. 4, 3(2): 1206 (1802)

Typus: Armenia, Tournefort (B, Hb. Willd.)

### *3.2. Morphology*

Perennial, caulescent. Stem procumbent, branched, 15-40 cm. Stipules brown, scarious. Leaves imparipinnate; leaflets 4-9 pairs, oblong or oblong-elliptic, 5-20 x 2-8 mm, glabrous or subglabrous above, below with an adpressed pilose indumentum of varying density; Peduncles longer than leaves. Racemes up to 20-flowered, lax or dense, ovate or oblong, elongating in fruit. Calyx 4-9 mm, with teeth equal to, shorter or longer than tube. Corolla yellow or purplish-yellow; standard (12-)14-23 mm, glabrous or slightly pilose; wings 11-18 mm; keel 11-19 mm. Ovary glabrous, sparsely or clearly pilose. Lomentum with 2-3 round or ovate segments, rugose, with bristly setae, pilose or subglabrous. (Figure 1).

Flowering time: May-June. Habitate: Steppe, bare slopes, *Quercus* woodland, fallow fields, cultivated ground. Altitude: 300-2100 m.

Distribution: Balkans, Caucasia, widespread in Turkey except in S.E. Anatolia. Irano-Turanian element.

Conservation status: LC



**Figure 1**. General view of *H. varium* (F. Taeb 3059).

## 3.3. Characterization of metal oxide nanoparticles

### *3.3.1. UV-Visible Spectrophotometer Analysis*

Optimization studies were conducted to investigate the effects of extract amount on nanoparticle formation in the synthesis of zinc nanoparticles (ZnO NPs) using H. varium plant extract. The absorbance peak of ZnO NPs was scanned between 200 and 800 nm, reaching a maximum at 255 nm, indicating a Surface Plasmon Resonance characteristic for ZnO NPs. This confirms the formation of ZnO NPs (Figure 2a). In this context, 25 mL, 50 mL, and 100 mL of plant extract were used for the optimization study, respectively (Figure 2b). The obtained nanoparticles were characterized by UV-Visible spectroscopy, and the obtained data were compared. As a result of the analyses, it was observed that, contrary to expectations, the ZnO nanoparticles synthesized with 25 mL of extract had more distinct and characteristic absorption peaks, and particle formation occurred in a more stable and controlled manner. This may be due to the decrease in absorption intensity and spectral shifts in syntheses performed with 50 mL and 100 mL extracts. These results indicate that the use of high amounts of extract may negatively affect the nanoparticle formation process. Furthermore, the synthesized ZnO nanoparticles were determined to exhibit absorption at a wavelength of 360 nm in the UV-Vis spectrum, and this value was found to be consistent with the characteristic absorption maxima of zinc nanoparticles reported in the literature [17].

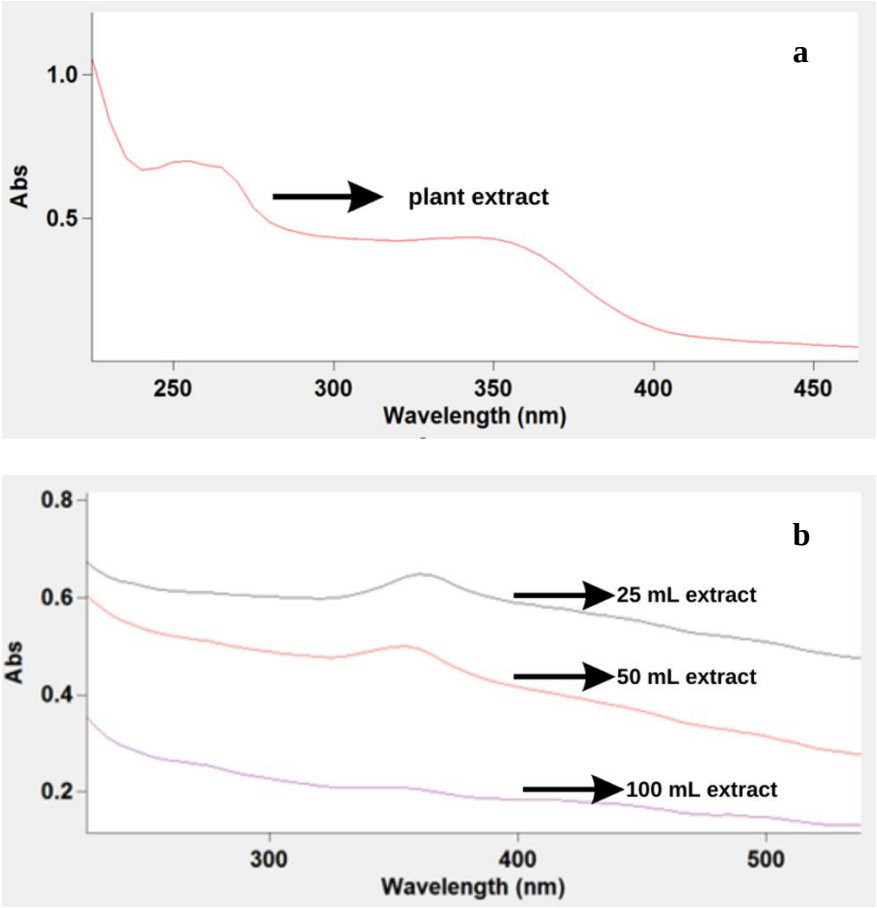



**Figure 2.** UV-Visible spectra of ZnO NP synthesized**, a)** *H. varium* plant extract, **b)** 25 mL, 50 mL and 100 mL *of H. varium* extract.

### *3.3.2. Optical Microscope Images*

To improve the quality of optical microscopy analysis of ZnO NPs, a solution obtained by mixing ZnO NPs with pure water was dropped onto a clean $Si/SiO_2$ substrate using a pipette. This method achieved a homogeneous surface, and the surface was heated to 70°C to evaporate the water in the solution. This method eliminated scattering and shedding problems during characterization of powder samples. Optical microscopy images of ZnO NPs synthesized from Hedysarum Varium extract at 10x, 50x, and 100x magnifications are shown in Figure 3a-c. They provide information about the morphology of ZnO NPs coated on the $Si/SiO_2$ surface.

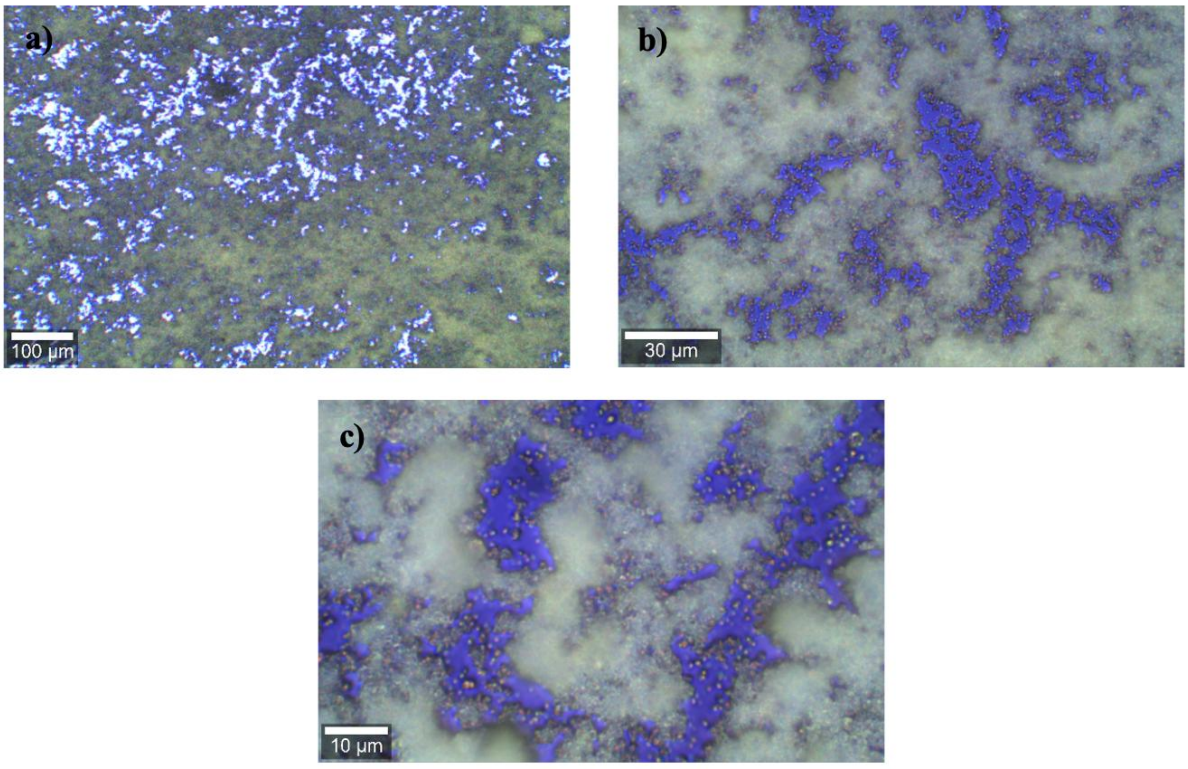



**Figure 3.** Optical microscope images of ZnO NPs synthesized from *H. varium* extract **a)** 10X, **b)** 50X, **c)** 100X.

### *3.3.3. Raman Spectroscopy Analysis*

Figure 4 illustrates the Raman spectroscopy of ZnO NPs synthesized from *H varium* extract. Raman peaks are stated at about 99 $cm^{-1}$ $E_{2L}$ mode, 329 $cm^{-1}$ $3E_{2H}$-$E_{2L}$ mode, 439 $cm^{-1}$ $E_{2H}$ mode. The peak at 520 $cm^{-1}$ is stated 300 nm $SiO_2$/Si substrate in close agreement with previous studies [18, 19].

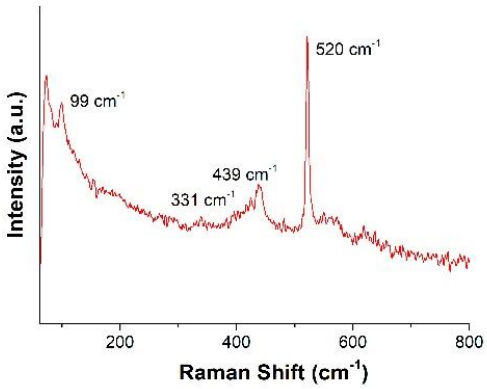



**Figure 4.** Raman spectra of ZnO NPs synthesized from *H. varium* extract.

### *3.3.4. XRD Analysis*

Figure 5 depicts the XRD analysis of ZnO NPs. The XRD peak of ZnO NPs is determined at ~ 70° (201), FWHM is 0.20° and, the average crystallite size was calculated to be 50.68 nm using the Debye-Scherrer formula (eq. 1). Examination of the peak at ~ 70° (201) suggests the formation of mono crystal structure with relatively large grains [20].

$$D = \frac{K\lambda}{\beta Cos\theta} \quad (1)$$

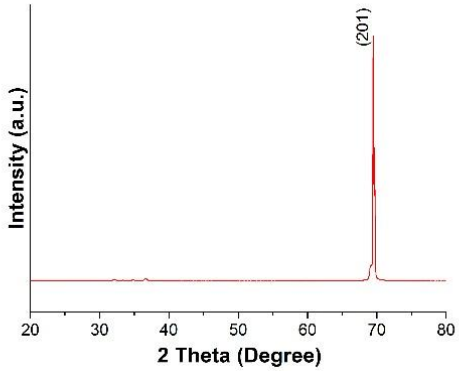



**Figure 5.** XRD diffractograms of ZnO NPs synthesized from *H. varium* extract.

### *3.3.5. Atomic Force Microscopy Analysis*

AFM shows the as-built structures and surface topography of ZnO NPs (Figures 6a and 6b). The measurements reveal that the ZnO NPs are around 300 nm in thickness, and these views are included in Figure 6c. Phase and amplitude measurements made with AFM help to understand the mechanical behavior of these nanoparticles. In particular, the change of amplitude value between 0-2 volts is a critical indicator that determines the elasticity of ZnO NPs (Figure 6d and 6e). In addition, the limited color variations in the phase image indicate that

the nanoparticles have a homogeneous material phase, which increases the structural integrity of ZnO NPs (Figure 6f and 6g).

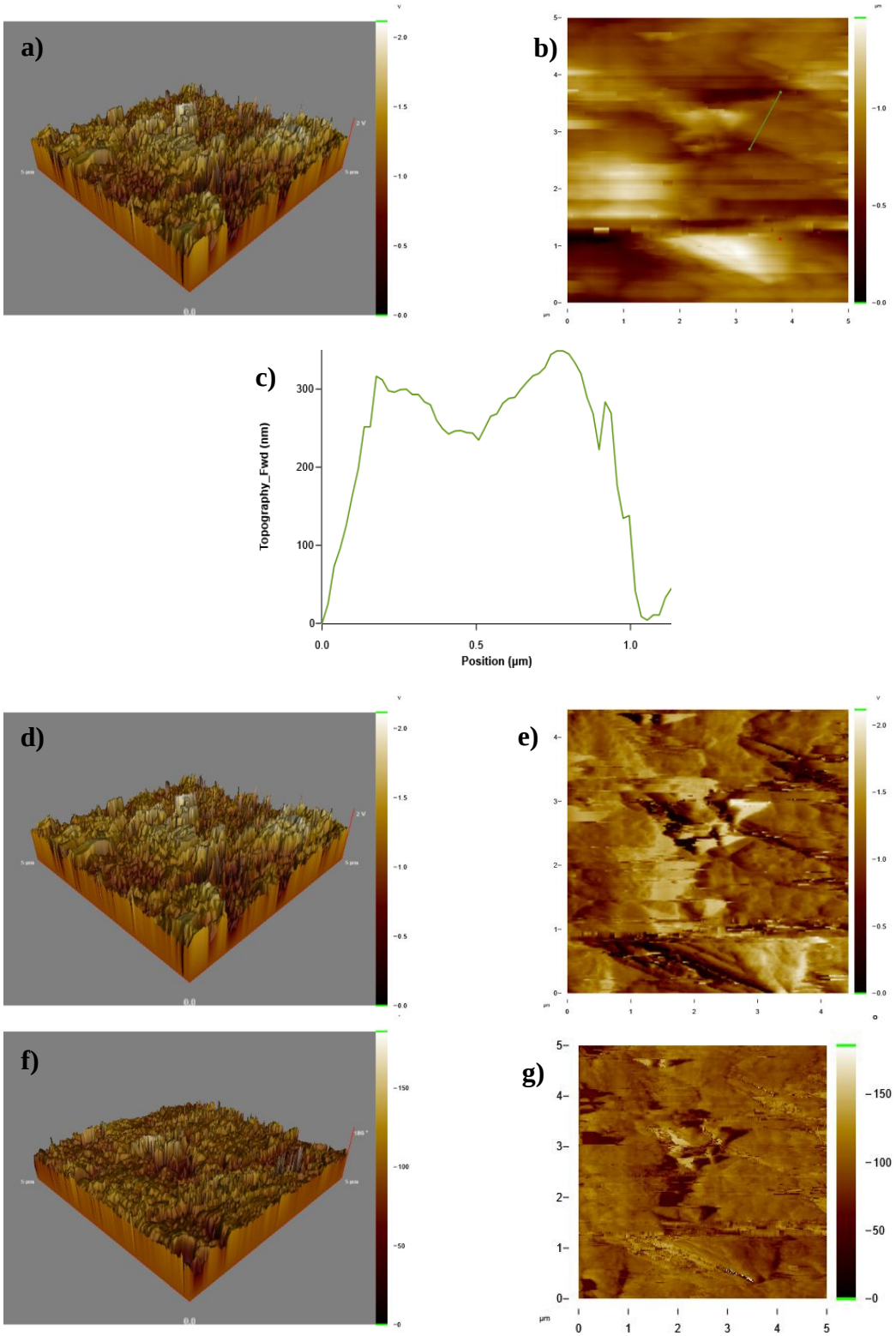



**Figure 6.** AFM results of ZnO NPs synthesized from *H varium* extract; a) 3d view of topography image, b) topography image, c) line profile, d) 3d view of amplitude image, e) amplitude image, f) 3d view of phase image, g) phase image.

### *3.4. Antibacterial Activity*

The MIC and MBC values of the test bacteria are presented in Table 1. In the MBC test, bacterial growth was observed in all inoculated wells after incubation.

**Table 1.** MIC and MBC values of test bacteria (mg/mL).

| | **MIC** | | **MBC** | |
|---|---|---|---|---|
| ***Test Bacteria*** | ***ZnONPs*** | ***Chloramphenicol*** | ***ZnONPs*** | ***Chloramphenicol*** |
| ***Escherichia coli ATCC 25922*** | *2.0* | *0.031* | *> 8* | *NT* |
| ***Pseudomonas aeroginosa ATCC 27853*** | *0.5* | *0.125* | *> 8* | *NT* |
| ***Staphylococcus aureus ATCC 25923*** | *0.25* | *0.007* | *> 8* | *NT* |

*NT: not tested.*

## 4. Conclusion

In this study, ZnO NPs were synthesized using aqueous extracts of *H. varium*, representing the first example of such a synthesis. The characterization of the synthesized ZnO NPs was carried out using UV-Visible spectroscopy, optical microscopy, Raman spectroscopy, and AFM. UV-Visible spectroscopy indicated the formation of ZnO NPs with absorption peaks at 255 and 350 nm. Optical microscopy provided insights into homogeneity, growth rate, and physical properties, while Raman spectroscopy investigated molecular vibrations and bond interactions. AFM further confirmed surface topography, roughness, and height profiles.

The antibacterial tests revealed that ZnO NPs were most effective against Gram-positive *S. aureus* ATCC 25923, which showed the lowest MIC value among the tested strains. At the highest tested concentration, ZnO NPs exhibited a bacteriostatic effect. Compared to ZnO NPs, the standard antibiotic chloramphenicol displayed lower MIC values, indicating stronger antibacterial activity. The antimicrobial mechanisms of ZnO NPs are generally attributed to bacterial cell wall disruption, ROS generation, and the release of $Zn^{2+}$ ions. ROS-induced oxidative stress inhibits protein synthesis and DNA replication [21,22].

Previous studies have shown that Gram-positive bacteria are more susceptible to ZnO NPs than Gram-negative bacteria [23]. This difference is primarily associated with cell wall composition: the thick peptidoglycan and teichoic acids of Gram-positive bacteria produce a negatively charged surface that enhances electrostatic interaction with ZnO NPs, leading to cellular damage [22]. Other reports have also documented variations in MIC values for *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853, which are thought to reflect differences in oxidative stress response [24,25]. Our findings are consistent with these observations, suggesting that ZnO NPs synthesized from *H. varium* display higher antibacterial activity against Gram-positive test bacteria. To further understand the limitations of this antibacterial effect, broader testing on both Gram-positive and Gram-negative bacteria will be necessary.

This study successfully demonstrated, for the first time, the green synthesis of ZnO NPs using *H. varium* extracts. The method offers a safe, cost-effective, and environmentally friendly alternative to conventional chemical synthesis. The synthesized ZnO NPs exhibited promising

antibacterial activity, particularly against Gram-positive *S. aureus*, confirming their potential as antimicrobial agents.

The strength of this work lies in the novelty of using *H. varium* as a plant source and in the comprehensive characterization confirming nanoparticle formation. For future perspectives, research should expand antibacterial evaluations to include a wider range of clinically relevant pathogens, while also assessing cytotoxicity and biocompatibility for biomedical applications. Additionally, efforts toward optimizing large-scale synthesis could pave the way for industrial and healthcare applications of plant-based ZnO NPs.

### *Authorship contribution statement*

**E. Ozkan:** Supervision, Conceptualization, Methodology, Writing - Review and Editing. **B. Ozturk:** Methodology, Writing and Review. **G. Arık:** Methodology, Writing - Review and Editing. **F. Taeb Dişli:** Methodology, Writing - Review and Editing. **M. Bay:** Methodology, Writing. **S. Biran Ay:** Methodology, Writing. **N. Kosku Perkgoz:** Methodology, Writing. **İsmail Murat Palabıyık:** Methodology, Writing - Review and Editing.

### *Declaration of competing interest*

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### *Ethics Committee Approval and/or Informed Consent Information*

As the authors of this study, we declare that we do not have any ethics committee approval and/or informed consent statement.

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