

ANTIBACTERIAL PROPERTIES OF
WURTZITE-HYDROZINCITE MIXED-PHASE
PHYTO-SYNTHESIZED ZnO PARTICLES

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Abstract

In this study, wurtzite-hydrozincite mixed-phase nanoparticles were prepared using a *Mentha arvensis* leaves extract as a stabilizing/reducing agent, followed by hydrothermal treatment at 180 °C. The resulting powder samples were characterized by X-ray diffraction (XRD) phase analysis and by Transmission Electron Microscopy (TEM). The existence of biphasic zinc oxide nanoparticles with hydrozincite and wurtzite crystallographic phases was proven. The particles sizes of biosynthesized oxide powders decreased upon the increase in the *Mentha arvensis* extract concentration. The antibacterial properties of the nanoparticles were investigated under dynamic contact conditions and the resazurin microplate method, using test strains with diverse structural and biochemical features. The effect of mixed-phase ZnO nanoparticles on the animal pathogens *Erysipelothrix rhusiopathiae* and *Aeromonas caviae* is studied for the first time. It was established, that the three powders exert significant antibacterial activity, which is higher against Gram-negative test microorganisms, whereupon the sample with the smallest particle sizes (G-Z3) exhibits the most effective inhibition. It could be supposed that the combination of factors: the

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presence of two phases, reactive oxygen species formation; small particles sizes and surface defects have beneficial effect on enhancement of their antibacterial properties.

Key words: green synthesis, wurtzite-hydrozincite nanoparticles, antibacterial activity, *Aeromonas caviae*, *Erysipelothrix rhusiopathiae*

Introduction. ZnO is a well-known useful and strategic II-VI n-type semiconductor having a wide band gap in the UV spectrum, with application in pharmaceutical and chemical industries, medicine, energy harvesting, electronics, etc. [1]. It is well known that ZnO exhibits significant antimicrobial activities, when the particle size is reduced down to the nano-scale level [2]. These properties, together with the good biocompatibility, make zinc oxide nanoparticles particularly suitable for applications in medicine and in the food industry. Development of nanotechnology opens an up-to-date approach for control of multi-drug resistant pathogenic microorganisms [3]. The bio-synthesis of metal or metal oxide nanoparticles is based on utilization of different kinds of biological systems (plants, microbes, algae), which can act as stabilizing and reducing agents. It has been applied by many research groups, due to its advantages, compared with other physicochemical methods such as: energy efficiency, low cost, protection of human health, non-toxic final products of reaction, etc. [4]. It is important to note that the green synthesis offers possibility to obtain oxide nanoparticles with unique physicochemical characteristics. Various leaf plant extracts can be used in the phyto-synthesis of ZnO nanoparticles: *Corymbia citriodora* [5], *Eucalyptus globulus* [6], and *Vitex trifolia* L. [7]. The effect of ZnO nanoparticles synthesized by means of plant extracts has some specificity depending on the type of microorganisms [8].

The study of the influence of nanoparticles on microorganisms of diverse structure and metabolism provides information both on the mechanism of action of the particles and on the way in which the microorganisms respond to this specific type of stress. In this regard, data on bacteria with pathogenic potential are scarce, especially for animal pathogens. Therefore, our study set out to verify the impact of wurtzite-hydrozincite mixed-phase nanoparticles prepared by green-synthesis method on selected apathogenic and pathogenic representatives of Gram-positive (G+) and Gram-negative (G-) bacteria, for some of which similar data are missing in the scientific literature.

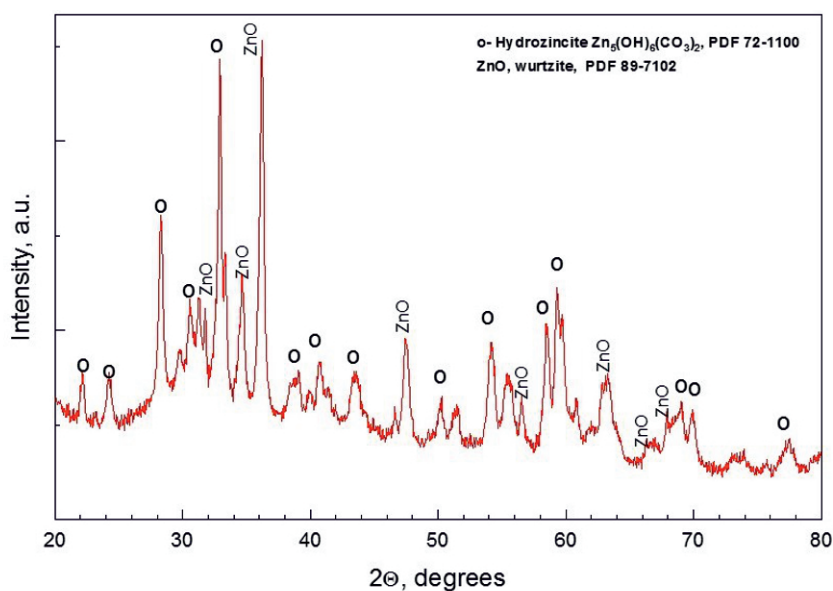
Experimental. Zinc oxide mixed-phase nanoparticles were prepared using green hydrothermal method. The experimental way of the preparation of *Mentha arvensis* extract and the zinc source precursor, as well as the conditions of the hydrothermal synthesis are described in our previous research [9]. The finally obtained precipitates were washed several times and dried up in an oven. Powders containing 10%, 20% and 30% *Mentha arvensis* extract are denoted as G-Z1, G-Z2 and G-Z3, respectively.

The phase composition of the samples was investigated by means of X-ray diffraction (XRD) using a Bruker D2 Phaser diffractometer, Karlsruhe, Germany (Cu K α radiation; $\lambda = 0.154056$ nm at 40 kV). The TEM observations were carried out using a transmission electron microscope, JEOL JEM 1011, at an accelerating voltage of 100 kV. Prior to the TEM measurements, the powders were suspended in ethanol and they were then deposited on a carbon replica and evaporated on a copper grid ($d = 3$ mm). For achieving a better contrast, the grid with the sample was re-coated with porous carbon.

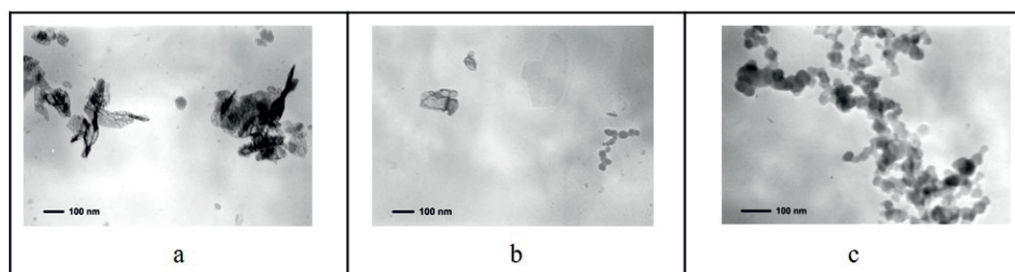
The antimicrobial activities of the biosynthesized powders were tested according to the ASTM Standard Test Method E 2149–10, adapted for 24-well sterile plate. A 0.01 g sample of each material was placed in a well and poured over with 1 mL of a standardized *Escherichia coli* ATCC 25922 cell suspension containing approximately $1.5\text{--}3.0 \times 10^6$ cells in a sterile buffer solution, prepared according to the method requirements. Control wells were set, containing 1 mL of bacterial suspension or only the nanoparticle sample, with 1 mL of sterile buffer poured. Samples and controls were run in duplicate. The plate was placed on a wrist-action shaker in a thermostat at a temperature of 30 °C at 100 rpm speed. The number of viable cells in each well was counted after the following intervals: 1, 24, 48, and 120 h by the modified Koch method of tenfold serial dilutions and Petri plating. Minimum inhibitory concentrations (MICs) of the tested nanoparticles were determined by the serial twofold dilutions microplate method in the presence of resazurin as applied by SARKER et al. [10]. The starting suspensions had the following concentrations: G-Z1 10 mg/mL; G-Z2 15 mg/mL; G-Z3 18 mg/mL. The bactericidal/bacteriostatic effect was evaluated based on the MBC/MIC ratio. The following test microorganisms were used: *Escherichia coli* ATCC 25922, *Aeromonas caviae* 40/02 (NBIMCC 8715), *Bacillus subtilis* ATCC 6633, *Erysipelothrix rhusiopathiae* B40 (Microbial Biochemistry Laboratory collection).

Results and discussion. All the bio-synthesized samples possess a mixture of two crystallographic phases: wurtzite and zinc hydroxide, as it has been proved by XRD analyses. Figure 1A represents X-ray diffractogram of sample G-Z2.

TEM photographs of the biosynthesized zinc oxide particles are shown in Fig. 1B. The particle size of the samples slightly decreases upon the increasing of the plant extract concentration in the zinc solution. They are within the range of 25–50 nm, 20–30 nm, and 10–25 nm for the samples G-Z1, G-Z2 and G-Z3, respectively. The shapes of the particles are almost identical. In order to determine whether the materials have an antibacterial effect, we applied a contact test method under dynamic conditions using *E. coli* as a test microorganism. We found out that such an action has already been manifested in the first hour of the experiment. After incubating the bacterial suspension for 24 h in the presence of the materials, no more viable cells were detected (Fig. 2). Therefore, at concentrations of 10 mg/mL, all materials were bactericidal for *E. coli* after 24 h of contact.



A



B

Fig. 1. XRD pattern of G-Z2 sample (A); TEM photographs of G-Z1 (a), G-Z2 (b) and G-Z3 (c) samples (B)

This gave us reason to investigate their influence on other microorganisms. For this purpose, we performed a serial *twofold* dilutions microplate experiment in the presence of resazurin, which also allows the calculation of MICs of the materials against the test microorganisms.

The three materials showed significant antibacterial activity, which was higher against the G(−) test microorganisms (Table 1). Regarding MICs, the most sensitive for all three types of nanoparticles was *A. caviae*. To our knowledge, the data on the effect of wurtzite-hydrozincite mixed nanoparticles on this bacterial species (animal pathogen and agent of food spoilage) is new in the scientific literature. For *E. coli* the effect of all nanoparticles was bactericidal.

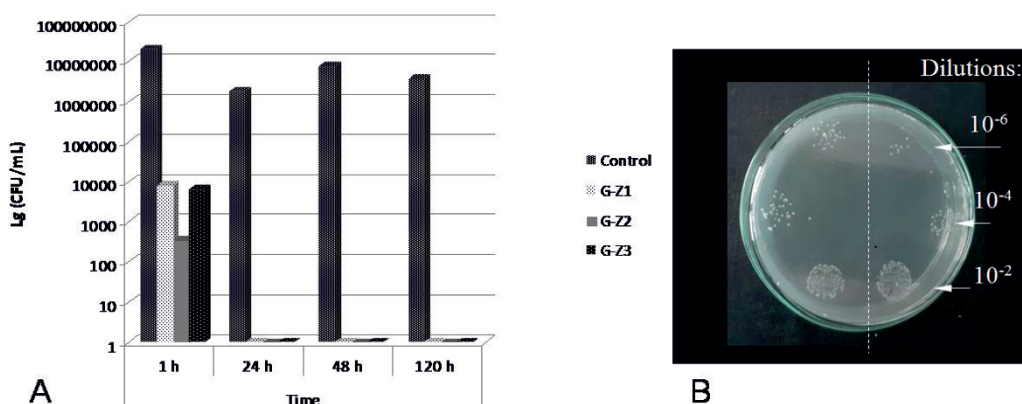


Fig. 2. Effect of G-Z1, G-Z2, and G-Z3 on *E. coli* under dynamic contact conditions. Ten mg from each sample was mixed with 1 mL of cell suspension (1:10 (w/v) ratio). The number of viable cells (CFU/mL) was evaluated by plate counting at the 1st, 24th, 48th and 120th hour and displayed in log (10) values. (A); Example of a Petri dish used for counting of colonies by the modified Koch method (B)

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MIC and MBC values of the green-synthesized mixed-phase ZnO nanoparticles, as it was evaluated by the dilution plate method. Where applicable, the bactericidal/bacteriostatic effect was assessed and highlighted in dark grey/grey, respectively

Test bacterium	Gram (-/+)	G-Z1, mg/mL		G-Z2, mg/mL		G-Z3, mg/mL	
		MIC	MBC	MIC	MBC	MIC	MBC
<i>E. coli</i>	-	5.8	5.8	7.5	8.8	2.3	7.5
<i>A. caviae</i>	-	1.5	5.0	4.4	> 15.0	0.14	6.0
<i>E. rhusiopathiae</i>	+	10.0	> 10.0	> 15.0	> 15.0	12.0	> 18.0
<i>B. subtilis</i>	+	3.3	> 10.0	> 15.0	> 15.0	18.0	> 18.0

For *A. caviae*, the action of G-Z1 is bactericidal, while that of G-Z3 is bacteriostatic, and the effect of G-Z2 could not be evaluated. The sample having the smallest particle sizes (G-Z3) exhibits the most pronounced inhibition effect against G(-) test strains. There is a specificity of the antibacterial effect towards the four test microorganisms, indicating that different types of bacteria respond in a characteristic way to the impact of the nanoparticles having identical chemical composition but slight differences in size and shape. Several mechanisms of antimicrobial activity of ZnO nanoparticles share the reactive oxygen species (ROS) formation as a major factor: direct effect due to adhesion on bacterial cell, membrane damage, imbalance in the redox potential of bacterial cells, uptake of toxic dissolved zinc ions [2]. The greater resistance of G(+) bacteria is explained by their rigid cell wall. In *B. subtilis*, the ability to form endospores could also explain the comparatively greater resistance to the tested particles [11]. According to the available literature, up to now *E. rhusiopathiae* has also not been applied

to test the antibacterial efficiency of plant synthesized mixed-phase ZnO particles. Interestingly, it shows remarkably higher resistance to the three types of materials. This G(+) bacterium is a facultative intracellular pathogen, which causes a variety of diseases in many species of birds and mammals, including humans [12]. Among the set of pathogenicity factors which have been identified in it, are the atypical cell wall chemical composition and the large number of genes (9) encoding antioxidant factors [13]. These characteristics are probably responsible for the higher resistance of *E. rhusiopathiae* to the investigated nanoparticles, especially towards the oxidative stress, caused by ROS.

Conclusions. The green synthesis has been successfully applied for preparation of wurtzite-hydrozincite mixed-phase nanosized powders. The particles sizes are within the range 10–50 nm depending on the *Mentha arvensis* extract concentration in the zinc precursor solution. The samples demonstrated a well-defined antibacterial activity, which is more efficient toward Gram-negative bacteria than that against Gram-positive ones. New data were obtained concerning the impact of the mixed-phase ZnO nanoparticles on the animal pathogens *A. caviae* and *E. rhusiopathiae*. Among the group of the microorganisms tested, the most effective inhibition of *A. caviae* in the presence of mixed-phase ZnO was observed. It could be supposed that the combination of factors: ROS production, small particles sizes and the presence of two phases, has beneficial effect on enhancement of their antibacterial properties. The specific differences in the response of microorganisms to the obtained nanopowders provide guidelines for revealing the mechanisms of their action in subsequent studies.

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