A study on the antimicrobial activity of metal oxide nanoparticles obtained by the method of “green” synthesis

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Abstract. Increasing tolerance of microorganism strains to a range of antibiotics in biomedical practices has led to the need to study preparations from other classes, such as nanoparticles of metals (NPM). Biosynthesized particles with more selective and more distinct activity against both gram-negative and gram-positive strains are especially promising from this point of view. Therefore, it is quite topical to conduct a comparative study on the antimicrobial activity of nanomaterials synthesized biologically. There were synthesized nanopowders of metal oxides \(\text{Co}_3\text{O}_4\) (from 20 to 100 nm) and \(\text{CeO}_2\) (from 14 nm to 500 nm) in the aqueous extract of Petrosellum crispum leaves. The microbiological test of the recombinant strain based on cells of \(S\). typhimurium showed the most pronounced toxic effects were revealed for \(\text{CeO}_2\), which resulted in fewer cells of the test strain and lack of growth. Nanoparticles of \(\text{Co}_3\text{O}_4\) found a moderate antimicrobial action.

1. Introduction
Increasing tolerance of microorganism strains to a range of antibiotics in biomedical practices has led to the need to study preparations from other classes, such as nanoparticles of metals (NPM). Biosynthesized particles with more selective and more distinct activity against both gram-negative and gram-positive strains are especially promising from this point of view \([1, 2]\). There are many conflicting data on the antibacterial activity of biogenic Ag, ZnO and iron-containing nanoparticles \([3]\), and much fewer works are devoted to \(\text{CeO}_2\) and \(\text{Co}_3\text{O}_4\) \([4, 5]\). Therefore, it is quite topical to conduct a comparative study on the antimicrobial activity of nanomaterials synthesized biologically.

2. Materials and Methods
Aqueous extracts from plants were prepared by the methodology of Makarov et al \([6]\). The synthesis of cerium oxide nanoparticles was conducted as follows: 0.862 g \(\text{Ce(NO}_3\text{)}_3\cdot6\text{H}_2\text{O}\) were added to the supernatant of the aqueous extract (20 ml), heated up with stirring for 6 h at 80-90 °C, the sediment was separated by centrifugation at 10000 rpm for 10 min, multiply washed with deionized water. Synthesis of cobalt oxide nanoparticles was conducted according to methodology: the vegetable extract was diluted with distilled water (1:3) and in the amount of 90 ml was mixed with 10 ml of 1 M \(\text{Co(NO}_3\text{)}_3\cdot6\text{H}_2\text{O}\), heated up at 80-90°C until the sediment emerged, then the temperature was lowered to 60 °C and heated for 90 min, settled for 24 h at the room temperature. Next, the residue was washed with alcohol and distilled water by centrifugation at the maximum speed for 10-15 min. The obtained residue of NPM was dried at 60 °C for 6 h and burned in the muffle furnace at 500 °C for 2 h.
The nanoparticles were deposited onto a double-sided adhesive carbon tape (2SPI, USA) and examined with a Zeiss Merlin microscope equipped with Gemini II Electron Optics (Zeiss, Oberkochen, Germany). The measurements were carried out at accelerating voltage of 1-5 kV and probe current 25-80 pA without any conductive coating on the sample surface. The object of the study was a recombinant strain based on parent cells of S. typhimurium LT2 transformed by pACXen plasmids (vectors contain luxCDABE genes of the natural luminous bacterium Photobacterium phosphoreum). To evaluate the bactericidal action, we used the method of serial dilutions with the subsequent measurement of density and dynamics of the death of the microorganisms in a medium with NPM. So the strains were cultivated for 24 h on the LB-agar (by Muller) at 37°C with the addition of the selectivity factors. Next, the cells were suspended in 0.9% NaCl to an optical density of 0.5 relative units at 450 nm, the measurement was carried out in transparent basins on photometer StatFax 303+ (“Awareness”, the United States) [7]. Then the ready-made bacterial suspension was added to LB-broth 500 µl of the bacterial suspension S. typhimurium LT2 pACXen in 1000 µl. In parallel, dilutions of NPM were prepared in the volume of 10 µl, 10 µl of water was added to the control basins, next 100 µl of the bacterial suspension was injected to all the basins of the tablet and 50 µl of the already diluted solution. After this, there was carried out the measurement of optical density at 630 nm after 60 and 120 min of exposure. Then it all was incubated in the thermostat at 37°C for 120 minutes, after which optical density at the same conditions was measured again. NPM were classified by effective concentrations [8]: semilethal (LC50, 50% survived), toxic (Tox, 0-39% survived), LOEC (40-69% survived), and NOEC (70-100% survived). All experiments were performed in three replications. The gained data were processed using Microsoft Excel and Statistica V8.

3. Results and discussion
SEM-visualization of CeO₂ powder showed that the particles are highly heterogeneous (Figure 1 A), the range of particle sizes was from 14 nm to relatively large particles (larger than 500 nm) consisting of smaller ones that were attached to each other. At the same time, the sample of Co₃O₄ consisted of small cubic particles with sharp edges with a diameter from 20 to 100 nm sticking together into large units (~ 1 µm) (B).

![Figure 1. Scanning electron microscopy of nanoparticles synthesized in an aqueous extract from P. crispum leaves: A – CeO₂, B – Co₃O₄, 100 nm](image)

According to the results of the growth microbiological test, biosynthesized nano-CeO₂ demonstrated a pronounced antibacterial effect throughout the range of the used concentrations, especially at concentrations more than 0.03 M (Figure 2). So 60 minutes and 120 minutes of exposure to the preparation declined the percentage of living cells at a concentration of 1 M by 51.5 and 74% compared to the control, respectively. As the concentration decreased, the percentage of living cells gradually increased but even the minimal dilution (0.0019 M) did not reach the control values and was only 76.4%.
A less distinct antibacterial effect showed preparations of Co₃O₄, which suppressed the growth of culture in the medium by 62.4% compared to the control after the 60-minute exposure at the highest concentration (1 M). After 2-h incubation of Co₃O₄ with microorganism cells, the share of living cells continued to decline by 30% with the 50% barrier in the number of living cells (LC50) was observed only at concentrations of 0.03 M, and at concentrations of more than 0.06 M, the preparations had an expressed toxic effect on the test culture (Figure 2).

Figure 2. The percentage of S. typhimurium surviving cells after exposure to nanoparticles CeO₂ and Co₃O₄, synthesized in the aqueous extract from P. crispum leaves: TOX – 0-39 % survived; LC50 – 50% survived; LOEC – 40-69% survived

The results clearly show that the differences in physicochemical parameters (size, shape, concentration) of the studied NPM play an important role in the realization of their antimicrobial effect. Perhaps, the death of microorganisms is caused by an indirect damage mechanism that generates reactive oxygen species (ROS) and launches oxidative stress [7], which is consistent with data on the antibacterial activity by other authors [9]. Strong oxidative stress in response to Co₃O₄ is possible due to the large variations of oxidation degrees Co²⁺.
Co\textsuperscript{3+}, and Co\textsuperscript{4+} compared to other transient 3d-elements, the emission of ions by the type of Trojan horse and a rapid increase in the pool of ROS [10, 11].

The antibacterial activity of Co\textsubscript{3}O\textsubscript{4} can probably be associated to the form of the particles as particles with a rough surface and with sharp edges (triangle, cubic) cause mechanical damage to cell walls [12], which cannot be ignored for future biological applications of materials.

Despite the widespread distribution of particles (from 14 to 500 nm), CeO\textsubscript{2} showed a marked antibacterial action. It was shown previously that CeO\textsubscript{2} with the particle size of 20 nm (which is larger than the pores of the cell wall) demonstrate a high level of cytotoxicity to bacterial cells [13]. It is likely that non-internalized NPM released ions that interacted with thiol groups (-SH) of protein and resulted in a violation of the cell membrane permeability [14]. Moreover, the antibacterial effect of chemically synthesized CeO\textsubscript{2} could be attributed to the high percentage of Ce\textsuperscript{3+} on the surface of the particles [15], to the subsequent induction of H\textsubscript{2}O\textsubscript{2} generation and cell death [16]. It should be noted that Maqbool et al. [17] revealed a weak and moderate antibacterial activity of CeO\textsubscript{2} (obtained from Olea europaea extract) toward gram-positive strains and a strong activity to gram-negative ones.

This study can help us conclude that the NPM synthesized by means of “green” chemistry have great potential for future antimicrobial therapy. Further, you could find interesting some variation of the antimicrobial activity by changing the ratio of ions (Ce\textsuperscript{3+}/Ce\textsuperscript{4+}) on the surface of particles, as well as a combination of NPM in different proportions among themselves or with preparations of other class [16].

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**Reference**
[1] Agarwal H, Menon S et al 2018 Mechanistic study on antibacterial action of zinc oxide nanoparticles synthesized using green route Chemico-biological interactions 286 60–70
[2] Qidwai A, Pandey A et al 2018 Advances in Biogenic Nanoparticles and the Mechanisms of Antimicrobial Effects Indian J. of Pharmaceutical Sciences 80(4) 592–603
[3] Kareem Z H and Shareef H K 2018 Evaluation of antibacterial activity of Fe\textsubscript{3}O\textsubscript{4} nanoparticles against Shigella dysenteriae L.J. of Pharmaceutical Sci. and Res. 10(8) 1980–82
[4] Maqbool Q 2017 Green-synthesised cerium oxide nanostructures (CeO\textsubscript{2}–NS) show excellent biocompatibility for phyto-cultures as compared to silver nanostructures (Ag–NS) RSC Advances 7 56575–85
[5] Varapradas T, Govindh B et al 2017 Green Synthesized Cobalt Nanoparticles using Asparagus racemosus root Extract & Evaluation of Antibacterial activity Int. J. of Chem. Tech. Res. 10(9) 339–45
[6] Makarov V V, Makarova S S et al 2014 Biosynthesis of stable iron oxide nanoparticles in aqueous extracts of Hordeum vulgare and Rumex acetosa plants ACS J. of surfaces and colloids 30 5982–88 (Langmuir)
[7] Kosyan D B, Ruskova E A et al 2015 Comparative evaluation of the toxicity of iron and its oxides nanoparticles using Stylonichia mytilus AACL Bioflux 8(3) 453–60
[8] Jackson P, Jacobsen N R et al 2013 Bioaccumulation and ecotoxicity of carbon nanotubes Chemistry Central J. 7(1) 154
[9] Alahmadi N S, Betts J et al 2017 Synthesis and antibacterial effects of cobalt–cellulose magnetic nanocomposites RSC Advances 7 (32) 20020–26
[10] Diallo A and Beye A C 2015 Green synthesis of Co\textsubscript{3}O\textsubscript{4} nanoparticles via Aspalathus linearis Physical properties, Green Chemistry Letters and Reviews 8 (3–4) 0–36
[11] Lebedev S, Korotkova A and Osipova E, 2014 Influence of Fe0 nanoparticles, magnetite Fe\textsubscript{3}O\textsubscript{4} nanoparticles, and iron (II) sulfate (FeSO\textsubscript{4}) solutions on the content of photosynthetic pigments in Triticum vulgare Rus. J. of Plant Physiol. 61(4) 564–9
[12] Korotkova A, Lebedev S and Gavrish I 2017 The study of mechanisms of biological activity of copper oxide nanoparticle CuO in the test for seedling roots of Triticum vulgare Environ. Sci. Pollut. Res. 24(11) 10220–33

[13] Priya G S, Kanneganti A et al 2014 Bio synthesis of cerium oxide nanoparticles using Aloe arbadensis Miller Gel Int. J. of Sci. and Res. 4(6) 1–4

[14] Tong G X, Du F F et al 2012 Polymorphous ZnO complex architectures: Selective synthesis, mechanism, surface area and Zn-polar plane-codetermining antibacterial activity J. of Mater. Chemistry B Home-Materials 1(4) 454–63

[15] Pulido-Reyes G, Rodea-Palomares I et al 2015 Untangling the biological effects of cerium oxide nanoparticles: the role of surface valence states Scientific Reports 5 15613

[16] Santos C, Lima C et al 2014 Antimicrobial activity of nano cerium oxide (IV) (CeO₂) against Streptococcus mutans BMC Proc. 8(4) 48

[17] Maqbool Q, Mudassar N et al 2016 Antimicrobial potential of green synthesized CeO₂ nanoparticles from Olea europaea leaf extract Int. J. of Nanomedicine 11 5015–25