"Green" Synthesis of Cerium Oxide Particles in Water Extracts

Petroselinum crispum

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Abstract: Background: Synthesis of metal oxides nanoparticles with specific morphology and size has become the subject of many experimental protocols. Biosynthesis of the nanoparticles using plants is more preferable than physical and chemical methods because of its environmental friendliness.

Objective: The purpose of this study was to report the potential for green synthesis of cerium oxide nanoparticles using plant extracts with a high content of phenolic metabolites.

Methods: We have synthesized the CeO2 nano- and microparticles using Petroselinum crispum aqueous extract. The particles were characterized by UV-visible spectroscopy, IR spectroscopy, X-ray diffraction (XRD), scanning electron microscopy (SEM), transmission electron microscope (TEM) and dynamic light scattering analysis. For detection the reduction capacity of the extract the evaluation of total phenolic and flavonoid content as well as high-performance liquid chromatography-mass spectrometry (HPLC-MS) were performed. Biological activity of the particles was identified by bioluminescent tests and bio tests with Triticum vulgare.

Results: Testing on T. vulgare showed that biogenic cerium dioxide powders stimulated the growth of up to 5-11,4% relative to intact samples, significantly increased the length of the leaves of seedlings and the root length. When seedings were exposed to the biosynthesized CeO2 particles, the level of chlorophylls was more stable and even slightly higher than control. Noticeable protective properties of the biosynthesized CeO2 powder under oxidation conditions were observed on the plant T. vulgare after a brief exposure (from 4 to 24 h).

Conclusion: Thus, biogenic CeO2 can be potentially utilized in oxidative damage protection of agricultural plants.

Keywords: Cerium oxide nanoparticles, green synthesis, morphology, antiradical activity, cell viability, seed germination energy, bioluminescence.

1. INTRODUCTION

A promising direction in the field of nanotechnology has emerged recently, it is a new, simple, cost-effective and environmentally friendly strategy for the synthesis of nanomaterials using biological tools (plants, fungi, microorganisms), the so-called "green" synthesis, or biosynthesis. Since plants have great potential for the super cascade and biological reduction of metal ions [1], the prospect of their use in the synthesis of biocompatible metal oxides and semiconductor nanostructures is obvious [2]. The presence of natural metabolites of reducing agents (polyphenols, flavonoids,
alkaloids, terpenoids, phenolic acids, sugars and proteins) in extracts not only promotes the recovery of metal ions to metal micro- and nanoparticles (NPs) of various sizes and shapes [3-6] but also increases the stability and bioavailability of the latter [7].

Nanoparticles of cerium oxide (IV) (CeO₂) are a promising antioxidant (AO) [8, 9]. CeO₂ absorbs UV radiation well and can be used in cosmetics as an alternative to ZnO or TiO₂. Earlier it was reported that due to the transition of Ce⁴⁺ to Ce³⁺ CeO₂ NPs exhibit mimetic properties with respect to a number of enzymes, including superoxidase, catalase and oxidase [10]. Ce⁴⁺/Ce³⁺ surface ratio depends on the microenvironment, which plays an important role in determining the biological activity and toxicity of CeO₂. The manipulation of the surface ratio of Ce⁴⁺/Ce³⁺ can be achieved by controlling the synthesis method in the direction of biological applications [11]. Research in the field of “green” synthesis of CeO₂ NPs in plant extracts from the standpoint of enhancing their antioxidant and protective properties is quite relevant. Table 1 presents in more detail the options for the biological synthesis of CeO₂ in plant extracts.

2. MATERIALS AND METHODS

2.1. Selection of Plants for Further Biological Synthesis

The following commercially available plants were selected as substrates for the synthesis: curly parsley Petroselium crispum, dill odorous Anethum graveolens, sowing salad Lactua sativa, cabbage Brassica oleracea, pea seed Pisum sativum, agastache fennel Agastache foeniculum.

The water extracts from plants were prepared according to Makarov et al. [21]. The fresh leaves of the plants were crushed in liquid nitrogen to a powdery state, distilled water was added in a ratio of 1:10 (weight/volume), the mixture was stirred and boiled for 30 min. Next, filter the extract through 2 layers of gauze and centrifuged for 15 min at 15000 g. The supernatant was passed through a 0.45 μm Milipore filter and used for further analysis and synthesis.

The phytochemical screening of the obtained extracts was carried out for the presence of various biological compounds (tannins and phenolic compounds, alkaloids, flavonoids, saponins, glycosides, proteins, terpenoids, and sterols, reducing sugars) in accordance with standard methods [22-24]. The results of the qualitative analysis are presented in Table 2.

2.1.1. The Determination of the Total Content of Soluble Phenolic Compounds in Plant Extracts

Then a quantitative analysis of the extracts was carried out according to the content of the total amount of soluble phenols and flavonoids. The total content of soluble phenolic compounds in plant extracts was carried out according to the method of Folin-Chocaltelu [25] with some modifications. 2 g of vegetable raw materials were crushed in liquid nitrogen, mixed with 5 ml of 80% ethanol and heated in a water bath for 30 minutes. To separate the plant residues, the extract was filtered through 2 layers of gauze and centrifuged at 13000 rpm for 10 min. The final volume of the extract was adjusted to 5 ml. The analytical mixture consisted of 400 μl of extract, 400 μl of Folin-Chocaltelu reagent and 3200 μl of water. The reference solution contained a similar volume of 80% ethanol instead of extract. Absorbance was measured at 720 nm.

The experiment was carried out in triplicate for each sample. The calculation was made according to a calibration curve constructed from hydroquinone solutions and according to the formula:

\[ C = \frac{(R\cdot V\cdot 0,001\cdot D_k)}{m} \]  

(1)

where C - total content of phenolic compounds, mg/g; R - the value obtained according to the calibration curve, μg/ml; V - extract volume, ml; 0,001 - conversion factor μg in mg; D_k - dilution factor; m - the mass of the plant sample used in the extraction, g. The total content of soluble phenolic compounds was expressed in mg of hydroquinone per 1 g of fresh mass.

2.1.2. The Determination of the Total Content of Flavonoids in Plant Extracts

The total flavonoids content in plant extracts was determined spectrophotometrically based on the complexation reaction with AlCl₃ [6]. For this, a known volume of the extract (about 1 ml) was placed in a 10 ml flask, 3.7 ml of distilled water and 0.3 ml of a 5% aqueous solution of NaNO₂ were added. After 5 min, 0.3 ml of 10% aqueous AlCl₃ solution was added, and after 6 min, 2 ml of 1M NaOH solution. After thorough mixing, the absorbance of the mixture obtained was measured at 510 nm. The experiment was carried out in triplicate. For the quantitative determination of flavonoids used a calibration chart, built on standard solutions of quercetin. The total content of flavonoids in terms of quercitin was determined by the formula:

\[ C = \frac{(R\cdot V\cdot 0,1\cdot D_k)}{m} \]  

(2)

where C - total content of flavonoids, mg/100 g wet weight; R - the value obtained according to the calibration graph, μg/ml; V - extract volume, ml; D_k - dilution factor; m - the mass of the plant sample used in the extraction, g. The total content of flavonoids was expressed in mg per 100 g wet weight in terms of quercetin.

The results of the experiment on the content of phenolic compounds and flavonoids in vegetable raw materials are presented in Fig. (1). The maximum amount of soluble phenolic compounds and flavonoids was recorded in an extract from parsley leaves P. crispum - 1,20±0,35 mg/g wet weight (A) and 0,98±0,06 mg/100 g (B), respectively. Therefore, it was P. crispum extract that was used as a reducing agent for the further synthesis of cerium dioxide nanopowders.

2.1.3. HPLC-MS

The aqueous extract of P. crispum prepared as described above was analyzed by HPLC-MS on a Fluorate instrument with MultiChrom software. For this, 10 μl of the extract was run with a mobile phase - a mixture of acetonitrile and water (1:2 water - at a feed rate of 0.20 ml / min through an Altima C18 column (2.1x150 mm diameter). Selected peaks were identified by comparison with data from the mass spectra library (National Institute of Standard and Technology) and with published data [26, 27].

According to the HPLC-MS analysis, the dominant compounds of the aqueous extract of P. crispum leaves are...
Table 1. Biological synthesis of cerium oxide nanoparticles using plant extracts.

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Plant</th>
<th>Size, nm</th>
<th>Morphology</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ce(NO$_3$)$_3$</td>
<td>Euphorbia tirucalli</td>
<td>37-40</td>
<td>Flaky</td>
<td>[12]</td>
</tr>
<tr>
<td>CeCl$_3$</td>
<td>Acalypha indica</td>
<td>25-30</td>
<td>Agglomerates</td>
<td>[13]</td>
</tr>
<tr>
<td>(CH$_3$CO$_2$)$_3$Ce·xH$_2$O</td>
<td>Cymbopogon flexuosus</td>
<td>10-40</td>
<td>Polycrystalline</td>
<td>[14]</td>
</tr>
<tr>
<td>Ce(NO$_3$)$_3$</td>
<td>Aloe vera</td>
<td>5-10</td>
<td>Highly porous agglomerates</td>
<td>[15]</td>
</tr>
<tr>
<td></td>
<td>Leucas aspera</td>
<td>4-13</td>
<td>Spherical</td>
<td></td>
</tr>
<tr>
<td>CeCl$_3$</td>
<td>Gloriosa superba L.</td>
<td>5</td>
<td>Cubic</td>
<td>[8]</td>
</tr>
<tr>
<td>Ce(NO$_3$)$_3$</td>
<td>Olea europaea</td>
<td>24</td>
<td>Spherical</td>
<td>[16, 17]</td>
</tr>
<tr>
<td>Ce(NO$_3$)$_3$</td>
<td>Azadirachta indica</td>
<td>10-15</td>
<td>Spherical</td>
<td>[18]</td>
</tr>
<tr>
<td>CeCl$_3$</td>
<td>Justicia adhatoda</td>
<td>20-45</td>
<td>Rod-shaped</td>
<td>[19]</td>
</tr>
<tr>
<td>Ce(SO$_4$)$_2$</td>
<td>Euphorbia hirta</td>
<td>4</td>
<td>Monoclinic</td>
<td>[20]</td>
</tr>
</tbody>
</table>

Table 2. Primary phytochemical screening of water plant extracts.

<table>
<thead>
<tr>
<th>Group of Phytochemical Compounds</th>
<th>Brassica oleracea</th>
<th>Petroselinum crispum</th>
<th>Anethum graveolens</th>
<th>Agastache foeniculum</th>
<th>Pisum sativum</th>
<th>Lactuca sativa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolic compounds and tannins</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids (10% NaOH)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids (10% lead acetate)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids (Mayer's test)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Alkaloids (Dragendorf test)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Terpenoids and sterols (Salkovsky reaction)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Reducing sugars</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Protein (Biuret test)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glycosides (Keller-Kiliani Reaction)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Fig. (1). The total content of soluble phenolic compounds in aqueous extracts of test plants (A, mg/g in terms of hydroquinone) and flavonoids (B, mg/100 g wet weight in terms of quercetin): bars display standard deviation from the mean.
2.2. Biological Synthesis of Nanoparticles CeO$_2$

0.862 g of cerium ammonium nitrate (NH$_4$)$_2$Ce(NO$_3$)$_6$ (analytical grade) was added to the supernatant of an aqueous extract of *P. crispum* (20 ml), heated with stirring for 6 h at 80-90°C. The resulting precipitate was separated by centrifugation at 10000 rpm for 10 min, washed several times with deionized water to remove unmatched biomolecules from the extract. Then, they were dried in a furnace with hot air at 60°C for 6 h and annealed in a muffle furnace at 500°C for 2 h. The color change from orange to dull yellow indicated the conversion of Ce$^{4+}$ to Ce$^{3+}$ [28] and a white precipitate of Ce(OH)$_3$ formed. Then, Ce$^{3+}$ reoxidized to CeO$_2$. Ce(OH)$_3$ can be directly oxidized to CeO$_2$ NPs, using a low-temperature thermal treatment under high oxygen level environment [29, 30]. A more detailed synthesis scheme is presented in Fig. (2).

2.3. Characterization of CeO$_2$ Particles

The obtained CeO$_2$ particles were resuspended in distilled water in an amount of 1 g/l, the suspension was treated in an Elmasonic ultrasonic bath for 15 min, and characterized using UV/vis-spectrophotometry, Fourier Infrared (IR) spectroscopy, X-ray analysis, scanning (SEM) and transmission electron microscopy (TEM) and a particle size analyzer using dynamic light scattering (DLS).

2.3.1. UV-spectrophotometry

A suspension of 0.1 M CeO$_2$ and a solution of (NH$_4$)$_2$Ce(NO$_3$)$_6$ were analyzed spectrophotometrically in the wavelength range 200-400 nm in quartz cuvettes with an optical path length of 1 mm. Water was used as a reference solution. The efficiency of the synthesis of CeO$_2$ NPs was analyzed by integrating the UV-spectra of (NH$_4$)$_2$Ce(NO$_3$)$_6$, CeO$_2$ and the supernatant. The supernatant was obtained by mixing the salt and the extract and then filtering the mixture through a filter (0.22 μm).

2.3.2. X-ray Analysis

Powder X-ray diffraction data were collected with a STOE STADI-MP diffractometer equipped with a curved Ge(Kα1) monochromator (Co Kα radiation, λ = 1.78899 Å). The data was collected in a step-by-step mode of overlapping the scanning areas using a position-sensitive linear detector. The capture angle of the detector was 5º by 2θ with a channel with of 0.02º. The phase composition was determined using the WinXPow Software (2002) software package and the Match! Software (2016) and its associated powder database PDF-2 Database Copyright International Center for Diffraction Data (ICDD-2013). As a result of processing the diffractogram, the sample was found to be consistent with ICDD card-index data. The parameters of the unit cell were determined by the formula for cubic syngony:

$$a = d_{hkl} \cdot \sqrt{h^2 + k^2 + l^2},$$  \hspace{1cm} (3)

where \(a\) - length of the unit cell edges, \(d\) - interplanar distance, \(\text{Å}\), \(hkl\) - X-ray scattering plane indices.

The crystallite size of CeO$_2$ was calculated by the Scherrer equation:

$$d = \frac{0.9 \cdot \lambda}{\beta \cdot \cos \theta},$$  \hspace{1cm} (4)

where \(d\) - average crystal size, \(0.9\) - Scherrer constant for cubic crystals, \(\lambda\) - X-ray wavelength, (for Co - 1.78899 Å), \(\beta\) - half-height peak (FWHM in radians), \(\theta\) - diffraction angle (2theta) or Bragg’s angle.

The average crystal size was calculated by comparing the crystallite sizes from the XRD and SEM spectra using the formula:

$$I = D_1/D_2 \quad (I \geq 1),$$  \hspace{1cm} (5)

where \(I\) - crystallinity index; \(D_1\) - particle size obtained from morphology analysis using TEM or SEM methods; \(D_2\) - particle size calculated by the Scherrer equation.

2.3.3. IR-spectroscopy

To identify the CeO$_2$ particles, Fourier IR-spectroscopy was used. To prepare the samples, 1 mg of CeO$_2$ powder, previously crushed in a mortar, was thoroughly mixed with

Fig. (2). Scheme of biological («green» synthesis) CeO$_2$ particles in an aqueous extract of *P. crispum*. 

KBr (ratio 1:300) and pressed into tablets in a special mold (pressure of 500-1000 kPa). We used pure KBr tablets as reference. The IR-spectra were recorded in a transmission mode at room temperature with a resolution of 1 cm⁻¹ in the wave range from 400 to 4000 cm⁻¹.

2.3.4. SEM

The CeO₂ powder was 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. Energy-dispersive X-ray spectroscopy (EDX) microanalysis was performed by SEM at 0.5-1 nA probe current using the Silicon Drift Detector XMaxN 150 (EDX) microanalysis was performed by SEM at 0.5-1 nA probe current using the Silicon Drift Detector XMaxN 150 (Oxford Instruments, Abingdon, United Kingdom) and AZtecEnergy EDS Software (version 3.0).

2.3.5. TEM

The study of CeO₂ powder was performed using a transmission electron microscope (TEM) H-500 (Hitachi, Japan). Particle size was determined by TEM photographs. To obtain TEM images, the preparations were obtained by applying a drop (100 µl) of a colloidal solution of nanoparticles in solution onto a thin mold-on film-substrate on a copper mesh with subsequent removal of excess moisture and drying [31].

2.3.6. Diffraction Particle Size Analysis (PSA)

The PSA measurements and the zeta potential measurements were carried out using Litesizer 500 (Anton Paar). The measurements were carried out in the Omega-shaped cuvettes at 23±5°C temperature. The CeO₂ powder samples were put in water at 0.05 g/ml concentration and subjected to ultrasound treatment for 30 s at 19 mW.

2.4. Evaluation of the Biological Activity of CeO₂ Powder

2.4.1. DPPH Antiradical Scavenging Activity (ASA)

MPs CeO₂ determined by neutralization of the stable radical 2,2-diphenyl-1-picrylhydrosyl (DPPH) (Sigma-Aldrich, USA). To this end, 1 ml of a solution of DPPH (0.135 mM) in 80% ethanol was mixed with 1 ml of various concentrations of aqueous suspensions of CeO₂. 1 ml of distilled water was added to the control tubes instead of the powders. The reaction mixture was thoroughly stirred and left in the dark at room temperature for 30 min. After the time elapsed, the optical density at 517 nm in a 1.1 cm quartz cell was measured. Based on the data obtained, a calibration curve was plotted in % DPPH inhibition coordinates - Trolox concentration (Acros organics, USA) and the percentage inhibition of DPPH samples was calculated by the formula:

\[
\text{% inhibition of DPPH = } \frac{(C - O)}{C} \times 100,
\]

where C - distilled water; O - aqueous solution of CeO₂.

2.4.2. Bioluminescence Tests

To assess the biological activity of powders CeO₂, bioluminescence tests on microorganism strains, previously tested by the authors on a wide range of nanomaterials, were used [32]. A daily culture of the Salmonella typhimurium soxS::lux strain was used; a suspension was prepared in physiological solution with an optical density of 0.5 relative units (at 450 nm), then 100 µl of the suspension was added to LB-broth (1400 µl) and incubated in a thermostatted shaker for 1.5 h. The studied CeO₂ in various dilutions were excavaed into the plate. 50 µl of water was added to control wells, and 50 µl of paraquat was added to the positive control. After that, 100 µl of LB-broth and 50 µl of bacterial culture were added to all wells. Kept for 15 min and placed in a measuring unit of a microplate reader (Tecan, Austria) and at room temperature, an increase in bacterial luminescence - surface luminescence was recorded after 120 min.

2.4.3. Evaluation of Biological Effects of CeO₂ MPs on Plant Organisms

The mechanisms for implementing the biological effects of CeO₂ MPs on plant organisms have been demonstrated on a model plant of wheat Triticum vulgare using previously described methods [33, 34]. To begin with, T. vulgare seeds were pre-disinfected and germinated in the climate chamber (Agilent, USA) under 12-hour light, at a temperature of 22±1 °C and humidity of 80±% for 48 h. Then, the equally germinated seeds (20 pieces) were transferred to individual cups and 5 ml of CeO₂ suspensions were added in concentrations from 10⁻³ M to 10⁻¹ M. Samples were germinated for 4, 7, and 14 days and then measured the length of the leaves and roots of seedlings. The percentage of germination energy (E) was calculated on the 3rd day in the formulation, when the seeds immediately after soaking were watered with 5 ml of suspensions of CeO₂.

To assess the protective and antioxidant properties of CeO₂ in a separate formulation, the plants were germinated in the climatic chamber for 7 days, treated with powder for 4 h, and H₂O₂ was added at a concentration of 50 µM [35]. The samples prepared in this way were incubated for another 4, 12, and 24 h. Next, analysis of the viability (V) of cells by changing the enzymatic activity of reductases was carried out according to the manufacturer's protocol (Cell counting kit-8) [36].

The antiradical and biological activity of biosynthesized CeO₂ powder was compared with the commercial CeO₂ nanopowder obtained by the gas-phase method at the Kazan National Research Technological University named after A.N. Tupolev (hydrodynamic radius 212±62 nm, ζ-potential -43 mV).

2.4.4. The Determination of the Content of Photosynthetic Pigments (PP)

The content of photosynthtic pigments (PP) was determined in the ethanol extract from the leaves by the spectrophotometric method according to the previously described method [34].

3. RESULTS AND DISCUSSION

3.1. Characterization of Biosynthesized Nanoparticles

3.1.1. UV-visible Absorption

It is known that the optical absorption band is sensitive to the nature, size, and shape of the formed particles [37]. Ac-
According to the UV-spectrum, the cerium ammonium nitrate solution had an absorption peak at 285 nm (Fig. 3), in agreement with the previous studies [38]. The CeO₂ suspension had a broad absorption peak with a maximum at 305-310 nm. This is close to the previously reported optical properties of CeO₂ nanoparticles. The previously reported absorbance maxima are 315 nm [16], 285 nm [39], 297 nm [8], from 335 nm to 352 nm [40].

3.1.2 XDR

An X-ray study was used to confirm the crystalline nature of the particles. XRD spectra (taken with Co Kα-radiation, \( \lambda = 1.78899 \) Å) clearly showed that the synthesized low-frequency CeO₂ correspond to the PDF-2 base powder data (ICDD-2013) for a cubic modification of cerium oxide (PDF № 89-8436) (Fig. 4). Additional diffraction peaks in the XRD sample (~19.05°) could be caused by the crystallization of the trace bioorganic phase of the plant extract during calcination of the particles at 500ºC. According to calculations, the average value of the unit cell parameter was 5.4052 Å, and the crystallite size of CeO₂ particles was 16 nm and the corresponding crystallinity index is 5.

3.1.3. IR-spectroscopy

The IR-spectra of the synthesized CeO₂ powder showed the appearance of peaks, which clearly indicates the effective interaction of the bioorganic components of the extract of P. crispum with Ce³⁺ during the synthesis (Fig. 5).

The peaks below 700 cm⁻¹ (from 400 to 600 cm⁻¹) are usually assigned to the Ce-O stretching mode indicating the formation of CeO₂ [41-44]. In our case, Ce-O stretching was observed at ~450 cm⁻¹ in good agreement with the previous studies [8, 45, 46]. The peak at 850 cm⁻¹ can also be assigned to the Ce-O stretching [47]. The peaks at 1384 cm⁻¹ and 1576 cm⁻¹ were explained as the oscillations of the trapped CO₂ [8, 40]. Besides, the peak at 1576 cm⁻¹ could correspond to the bending of H-O-H [48].

The broad peak at 3313 cm⁻¹ can be ascribed to O-H stretching vibrations from surface-adsorbed H₂O molecules or the surface groups -OH or Ce-OH [16]. The IR spectra of the obtained CeO₂ particles were in reasonable agreement with the previous data.

3.1.4. SEM and EDX-analysis

SEM imaging revealed that the particles were highly heterogeneous (Fig. 6, the white rectangles in A and B show the zoomed areas). Their size range covered several orders of magnitude from 50 nm to 15 µm. The largest particles (size>500 nm) consisted of the smaller ones, attached to one another. The smallest fraction was also visualized by TEM, as discussed below. The wide size distribution is typical for the particles synthesized by “green” synthesis [42].

We carried out EDX-analysis to verify the elemental composition of the particles. This experiment was carried out with the sample deposited onto the carbon adhesive tape, so the carbon characteristic peaks were always present in the EDX spectra (Fig. 7). We also observed the peaks of Ce, O and K (1% by mass or lower). The ratio of the oxygen and cerium atoms calculated based on the EDX spectra was in the range from 1.9 to 2.1, corresponding to the expected CeO₂ structure.

3.1.5. TEM

The presence of nanoparticles in the samples was verified by TEM. According to the TEM images, both spherical and irregular particles were present in the sample (Fig. 8). Many particles formed-clusters (A-C) or “chains” longer than 200 nm (D).

![Fig. (3). UV-Vis absorption spectra of the CeO₂ particles synthesized in an aqueous extract from P. crispum leaves.](image-url)
Fig. (4). XRD-spectra of CeO₂ particles synthesized in an aqueous extract from P. crispum leaves.

Fig. (5). IR-spectra of CeO₂ particles synthesized in an aqueous extract from P. crispum leaves.

Fig. (6). SEM images of the Ce oxide nanoparticles. The scale bars are 10 µm (A), 1 µm (B) and 200 nm (C).
Fig. (7). A typical EDX spectrum of the studied particles.

Fig. (8). Transmission electron microscopy of CeO2 particles synthesized in an aqueous extract from P. crispum leaves. Scale bar: A - 2 µm, B-D - 100 nm.

3.1.6 PSA

To measure the size distribution of the synthesized particles more accurately, we switched from the single particle-techniques to the ensemble techniques, particularly, PSA (Fig. 9). The particle size distribution had two peaks - the first peak at ~0.15 µm, and the second at ~0.9 µm. Thus, the major fraction of the synthesized CeO2 powder consisted of microparticles, and the fraction of nanoparticles was low. However, they could not be excluded, since they were observed by both SEM and TEM. The light scattering intensity strongly depends on particle size; presumably, the nanoparticles did not produce enough scattering signal to be analyzed in the presence of the larger micro particles.

The zeta-potential of the CeO2 particles in water was found to be -41.3±0.8 mV.

3.2. Biological Activity of Biosynthesized CeO2 Nanoparticles

The next stage of the study was to evaluate the protective and antioxidant properties of the CeO2 powder in comparison with the synthetic analogue.
3.2.1. DPPH Antiradical Scavenging Activity (ASA)

First of all, we analyzed the ability of CeO₂ to neutralize ROS. The results of the DPPH test showed an increase in the anti-radical activity (ARA) of nanomaterials as the concentration of the samples increased, from 10⁻⁹ to 10⁻³ M (Fig. 10). At the same time, the antioxidant potential of biosynthesized CeO₂ powders (in terms of trolox) was 63-68% higher compared to the control, and the synthetic powders were less than the control by 54.5-64.3%, respectively.

3.2.2. Bioluminescence Tests

The bioluminescent testing method based on the biosensor response confirmed the absence of the prooxidant effect of various dilutions of CeO₂ powders synthesized by a biological method. Thus, activation of the oxidative stress promoter was observed only at high concentrations (more than 0.25 M). So, compared with the control luminescence, the difference remained at the level of 30-59% (Fig. 11).

The commercial cerium dioxide synthesized by the gas-phase method in concentrations of 0.002-1 M also did cause changes in the dynamics of the bioluminescence of bacteria, but in concentrations above 0.625 M showed a higher level of luminescence, which differs from the background luminescence test microorganisms on 42-67%. Note that the prooxidant effect of chemically synthesized CeO₂ particles is up to 2 times higher than our synthesized particles of biogenic origin, which characterized biosynthesized CeO₂ powders obtained by various methods as a conditionally safe substance for the living system.

3.2.3. Evaluation of Biological Effects of CeO₂ MPs on Plant Organisms

At the same time, testing on T. vulgare seeds showed that biogenic CeO₂ powders initiated germination processes to a greater extent than their synthetic analog, and even stimulated the seedling to 5-11.4% relative to the intact samples (Fig. 12).

A detailed analysis of the metric indicators of T. vulgaris seedlings after exposure to CeO₂ of plant and synthetic origin showed high resistance of plants to them. At the same time, cerium dioxide on the 7th day of incubation more stimulated the growth of plant shoots, and on the 14th day - the root system. According to the data (Table 3), when exposed to biogenic particles of cerium oxide at a concentration of from 10⁻³ M to 10⁻² M for seven days there was a significant increase in the length of the leaves of seedlings by 18.6-35.4% relative to control, and after 14th days exposure rate reached 32.6-37%. A similar trend was observed when plants were germinated in the medium of a synthetic analog: there was a significant stimulation of leaf length when exposed to small concentrations of metal (less than 10⁻⁴ M) on day 7 to 22-28% and on 14th day - 26-28%, respectively. It should be noted that on the 14th day of incubation with cerium dioxide powder there was an increase in the root length to 42-44.4% in the case of particles obtained in the plant extract, and up to 31-34% in the treatment of plants with the commercial sample.

Thus, powders obtained by the method of "green" synthesis in the extract of P. crispum, in dilutions of 10⁻⁵ to 10⁻² M, turned out to be the most effective for treating plants.

Integral assessment of the cell viability of T. vulgaris seedlings after a short exposure (4 to 24 h) of the biosynthesized CeO₂ against the background of the action of peroxide (50 μM) showed noticeable protective properties of the powder. According to the data, a direct dependence of the enzymatic activity of reductases on the metal content and incubation time was established (Fig. 13A).

An additional analysis of the T. vulgaris cell viability was carried out by counting the number of cells unstained with a vital dye (Evans blue). We have found showed that at 12 and 24 h of 50 μM H₂O₂ treatment more cells survived at the basal part than at the apical part (Fig. 13B). However, after biosynthesized CeO₂ particles were added to the medium...
(in concentration from $10^{-5}$ to $10^{-3}$ M), a characteristic tendency was observed for an increase in viable cells number to the level of the control and above - to 7-14% ($P \leq 0.05$). Surprisingly, $10^{-5}$ M CeO$_2$ suspension showed better protective properties than the $10^{-4}$ M and $10^{-3}$ M suspensions. This could be explained by the particle aggregation at the higher concentrations.

3.2.4. The Determination of the Content of Photosynthetic Pigments (PP)

Analysis of the photosynthetic pigment content in the leaves of *T. vulgare* after the exposure of CeO$_2$ powders obtained by different methods showed some differences in their content (Fig. 14). So, when exposed to biosynthesized CeO$_2$, ...
Table 3. Growth indicators of *T. vulgare* seedlings after 48 h exposure to particles CeO$_2$.

<table>
<thead>
<tr>
<th>Concentration of CeO$_2$, M</th>
<th>Leaf Length, mm</th>
<th>Root Length, mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Biosynthesized</td>
<td>Commercial</td>
</tr>
<tr>
<td>7 day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>17,6±3,1</td>
<td>23,6±4,3</td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>25,1±1,4*</td>
<td>24,5±2,2</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>27,2±5,8</td>
<td>22,7±6,1</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>24,2±3,9*</td>
<td>27,1±3,9</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>21,6±5,9**</td>
<td>25,3±4,1</td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>32,1±8,9</td>
<td>29,2±3,2</td>
</tr>
<tr>
<td>14 day</td>
<td>45,5±5,7</td>
<td>30,5±5,4</td>
</tr>
<tr>
<td>Control</td>
<td>40,7±3,9</td>
<td>51,4±6,2*</td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>69,4±4,8*</td>
<td>61,7±5,9</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>60,3±3,0</td>
<td>62,5±4,9</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>67,5±2,7*</td>
<td>60,4±5,9</td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>57,2±2,2</td>
<td>53,2±7,1</td>
</tr>
</tbody>
</table>

Note: *, ** option is significantly different from the control (value of P≤0.05 and P≤0.01, respectively).

Fig. (13). Cell viability of the roots of *T. vulgare* seedlings under oxidation conditions (50 µM H$_2$O$_2$) after exposure to CeO$_2$ particles synthesized in an aqueous extract from *P. crispum* leaves: A) on the enzymatic activity of reductase and formazan yield; B) by the number of cells unstained by Evans blue in the basal and apical parts of the root; bars display standard deviation of the mean, * denotes the values significantly different from the control (value P≤0.05).
Fig. (14). The content of photosynthetic pigments in *T. vulgare* leaves after exposure to particles CeO₂: bars display standard deviation from the mean, * option is significantly different from the control (value P≤0.05).

the level of chlorophylls was more stable and even slightly higher than control, namely, by 3.9% for chlorophyll *a* and by 12-37.5% for chlorophyll *b*. A completely different picture was observed in plants with a similar effect of nanoparticles synthesized by the gas-phase method: the indicator decreased even when exposed to low concentrations ($10^{-3}-10^{-5}$ M) by 37.6% (chlorophyll *a*) and 46% (chlorophyll *b*).

The significant deviation of the level of carotenoids from the control (5 mg/g wet weight) by 5 times in the case of commercially available powder can be considered as one of the adaptation mechanisms, that ensure effective energy absorption and prevent damage to the photosynthetic apparatus and chlorophylls from the excess energy of triplet chlorophyll and $^{1}O_2$ [49].

Changes in the metabolic reactions of the components of the *T. vulgare* pigment-protein complex in response to exposing CeO₂ powder were species-specific, depending on the method of production and the concentration of the metal oxide. When exposed to CeO₂ powder obtained in *P. crispum* extract, the level of chlorophylls in *T. vulgare* seedlings increased relative to the control, which suggests that biogenic metals did not directly affect the processes of chlorophyll biosynthesis or disintegration, and their influence was manifested indirectly. In the case of exposure to synthetic analogues, the primary “target” of the action was both green and yellow pigments of carotenoids. The latest findings are confirmed by many authors, who also did not observe a significant change in the ratio during the processing of nanoforms. Other researchers also noted a general decrease in the number of phenolic pigments after the treatment with synthetic CeO₂ [50].

**CONCLUSION**

The synthesis of homogeneous nanoscale particles with special requirements in terms of size, shape, and physicochemical properties is of great interest. In the present study, we were able to achieve the synthesis of stable and well dispersed CeO₂ particles using an environmentally friendly and cost-effective method using plant extracts. In the current study, the mechanism by which CeO₂ can be synthesized can be explained by a higher total content of phenols in the plant *P. crispum*, which facilitates the oxidation of cerium ions to nanometer-sized cerium particles due to the ability of electron donors to join these phenolic compounds. In addition, quinoid compounds resulting from the oxidation of the phenol group in phenolic compounds can be adsorbed on the surface of the particles, which leads to their stabilization of the suspension [51, 52].

According to the HPLC-MS analysis, the dominant compounds of the aqueous extract of *P. crispum* leaves are flavonols (kaempferol and quercetin) and flavones (apigenin and luteolin), which are found in nature in the glycosidic form and are the main flavonoids found in the extract of *P. crispum*, which is confirmed by literary data [53-56]. *P. crispum* is one of the richest sources of phenolic aglycone, apigenin, which is usually in the form of a conjugate in the form of apiine or its analogs [57] and flavones conjugated with sugars, simple acids (acetyl and malonyl) and cinnamic acid [58-60].

The chemical structure of the particles was confirmed by UV-visible absorbance spectroscopy, IR-spectroscopy and EDX spectroscopy. The particle size distribution had two peaks
(-0.15 μm and ~0.9 μm) identified by particle size analysis (PSA). Microscopic investigation using SEM and TEM indicated that the sample contained not only the microparticles but also the nanoparticles with the size down to 50 nm.

The roentgenogram of the synthesized CeO$_2$ powders shows diffraction peaks at 2θ = 33.38°, 38.75°, 55.81° and 66.6°, that can be indexed as (111), (200), (220) and (311) planes of pure cerium oxide. These data are in good agreement with powder diffraction data (ICDD) №01-089-8436 relating to cerium oxide. The results clearly indicate the presence of cerium oxide in the crystalline cubic form. It can be noted that an additional peak at 2θ = 19.05° may indicate trace amounts of the bioorganic phase in the particles due to the synthesis in the extracts. The particle size calculated by the maximum peak (111) was 16 nm, and the corresponding crystallinity index (I) was 5, which is a sign of a polycrystalline population [61].

Given the radical-absorbing properties of CeO$_2$, the synthesized particles probably act as cellular antioxidants and provide protection against various damages. We assumed that the biosynthesized CeO$_2$ particles will not exhibit a toxic effect on plant cells as compared to chemically synthesized ones, as shown in normal somatic cells [62-64].

Cerium oxide contains the same elements of the core, but does not exhibit similar biological effects due to unstable stoichiometry on the surface and a fairly free Ce$^{3+}\rightarrow$Ce$^{4+}$ transition: with increasing Ce$^{3+}$ fraction, the toxic properties of the metal increase [65-67] and with increasing Ce$^{4+}$ - antioxidant [68].

Since the biosynthesized CeO$_2$ particles showed a more pronounced protective activity with respect to the tested _T. vulgar_ e plants as compared to the synthetic analog, and also had little activity in neutralizing free radicals, an increase in the proportion of CeO$_2$ (Ce$^3+$) as compared to Ce$_2$O$_3$ (Ce$^{3+}$) [69, 70].

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not Applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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