Biological testing of powders based on Fe and Zn obtained in parsley extract

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Abstract. A number of metal oxide nanopowders (Fe2O3, ZnO, CoFe2O4, ZnFe2O4) were synthesized in an aqueous extract from the leaves of Petroselinum crispum by a biological method. Physicochemical certification of synthesized nanomaterials was carried out. The data on plant cell viability of the reductases enzyme activity (WST test) were obtained against the background of seed treatment with obtained nanopowders in doses from 10^{-3} to 10^{-5} M per 4 hours and further short-term action (4, 12 and 24 hours) oxidizing agents (50 μmol H2O2). A following noticeable protective effect was exhibited by CoFe2O4 NPs (pH = 2) and CoFe2O4 (pH=10): cell viability averaged 103, 93, and 92 % respectively. Particles Fe2O3 (84 %) and zinc ferrite ZnFe2O4 both synthesized in an acidic medium (79 %) and alkaline (87 %) had a slightly smaller effect. The remaining nanometals (ZnO, Co3O4) did not have such a strong effect and their reductase activity index was no more than 73 %. The data obtained show the likelihood of using these components in improving the agricultural performance of plants in crop production, improving their growth characteristics and plant productivity.

1. Introduction

Metal and metal oxide nanoparticles (NPs) have received substantial research attention due to their exceptional electrical, optical, magnetic and catalytic properties. These have enabled their broad use in diverse industrial, medical, agricultural and environmental applications with further uses [1]. Traditional synthesis methods for pristine metal and metal oxide NPs include reducing and stabilizing chemical agents that are toxic to humans and to other species in different trophic levels [2]. In response, researchers are now looking for alternative “green synthesis” approaches in an effort to reduce or eliminate harmful chemicals during the production of NPs. Green chemistry is a set of principles or practices that encourage the design of products and processes that reduce or eliminate the use and generation of hazardous substances [3].

Currently, the number of reports on green-synthesized NPs is increasing substantially. Extracts derived from diverse plant species, their organs, and isolated compounds are being successfully used in the green synthesis of NPs. In addition to being eco-friendly, NPs can be synthesized using agricultural and industrial waste to make the process more sustainable [4]. Green-synthesized nanoparticles often possess better bioactivities and catalytic characteristics compared to their counterparts, which are synthesized by other methods [5].

The ability of plant secondary metabolites to chelate metal ions in the production of stable complexes and their potential to conjugate with NPs has opened a new opportunity for NPs’ use in harvesting these natural products.

Plant extract-mediated, in vitro green synthesis of NPs has gained popularity due to its simplicity, low cost, eco-friendly nature, and easiness to scale-up [6–9]. In [10], ZnFe2O4 zinc ferrite NPs were obtained by the green method using Limonia acidissima juice (wood apple). ZnFe2O4 NPs demonstrated effective photodegradation for Evans blue and methylene blue when exposed to visible light. Researchers [11] obtained a semiconductor from ZnO-CoFe2O4 composites using rambutan peel extract (Nephelium lappaceum L.). Another article describes the successful synthesis of iron-based NPs using extracts of Nephrolepisauriculata [12].

2. Materials and Methods

Extracts preparation. Water extracts were prepared from plants according to the procedure [13]. To prepare the extract, fresh leaves of Petroselinumcrispum plants were washed under running water, then twice with distilled water, dried on filter paper until moisture was removed from the surface of the leaves. Next, the raw material was crushed in liquid nitrogen to a powder state, distilled water was added in a ratio of 1:2.5 (by weight) and the mixture was stirred and boiled for 30 min at 90 °C. Next, the extract was filtered through 2 layers of gauze to remove plant residue and centrifuged for 15 min at 15,000g. The supernatant was passed through a 0.45 μm Millipore filter and used during the synthesis.

Synthesis of zinc oxide NPs. The initial plant extract was diluted 2 times (20 ml) and 2 g of zinc nitrate Zn (NO3) 2 6H2O was added, dissolved in 20 ml of extract. Synthesis with constant stirring at 60–80 °C for 8 hours was implemented until precipitation formed and the liquid evaporated. Annealing was carried out in a muffle furnace at 500 ℃ for 2 hours.

Synthesis of iron oxide NPs. 9 ml of an extract (1:3) diluted with distilled water was mixed with 1 ml of a 0.1 M FeCl3 solution, stirred vigorously at room temperature, and after 24 hours the reaction mixture was centrifuged to sediment detachment. Next, the sediment was washed with alcohol (1 time) and distilled water (2 times) by centrifugation at maximum speed for 10–15 minutes and dried for 24 hours at 50 ºC.

Synthesis of cobalt oxide NPs. 90 ml of initial plant extract was diluted with distilled water (1:3), mixed with 10 ml of a 1 M solution of cobalt nitrate CoNO3 6H2O, followed by stirring and heating at 90 ℃ until a sediment was detached, then the temperature was reduced to 60 ℃ and heated for 90 min, precipitated for 24 hours at room temperature. Next, the sediment was washed with alcohol and distilled water by centrifugation at maximum speed for 10–15 minutes, dried for 24 hours at 60 °C, and annealed at 500 ℃ for 2 hours.

Synthesis of cobalt ferrite NPs. Fresh vegetable raw materials of parsley Petroselinumcrispum were washed under running water, twice with distilled water, and dried on filter paper to remove moisture from the surface of the leaves. Then it was ground to a powder state by means of liquid nitrogen, 2.5 parts of distilled water (by weight) were added to 1 part of the extract. Then it was heated for 30 min at 90 °C. After heating, it was filtered through 2 layers of gauze to remove plant residue. The extract was purified by centrifugation at 15,000 rotations within 15 minutes. The resulting supernatant was filtered through a millipore with a pore diameter of 0.45 μm. The resulting purified extract was diluted with distilled water (1:3) for further use in synthesis.

The synthesis was initiated by pre-dissolving the salts of the precursors, 7 g of $CoCl_2$ $6H_2O$ and 13.9 g of $FeCl_3$ $6H_2O$, in 200 ml of the extract (pH=2) and constant stirring at 100 °C for 6 hours (before xerogel was formed). Then the liquid was evaporated and the resulting gel was washed with distilled water (2 times) and alcohol (1 time) by centrifugation at maximum speed for 10–15 minutes. Then, the gel was dried in a thermostat at 200–300 °C for 3 hours and annealed at 800 ℃ for 1 hour. The synthesis of $CoFe_2O_4$ in an alkaline medium was characterized by the fact that 1 M NH_3 H_2O was added drop by drop to the iron sulfate solution at constant stirring until the titrimetric pH=9 was established.

Synthesis of zinc ferrite NPs. Fresh vegetable raw material of parsley Petroselinumcrispum was washed under running water, then twice with distilled water, dried on filter paper to remove moisture from the surface of the leaves. Then it was ground using liquid nitrogen to a powder state, 2.5 parts of distilled water (by weight) were added to 1 part of the extract. Then it was heated for 30 min at 90 °C. After heating, it was filtered through 2 layers of gauze to remove plant debris. The extract was purified

by centrifugation at 15,000 rotations within 15 minutes. The resulting supernatant was filtered through a millipore with a pore diameter of 0.45 μm. The obtained purified extract was diluted with distilled water (1:3) for further use during the synthesis [13]. Synthesis in an alkaline medium was characterized by the fact that a 10 % solution of NH_3 H_2O was added drop by drop to an iron sulfate solution with constant stirring until the titrimetric pH≥9 was established.

The analysis of the physicochemical parameters of the obtained metal NPs included microanalysis of energy dispersive X-ray spectroscopy (EDX) using SEM at a probe current of 300–350 Pa using a XMaxN 150 silicon drift detector (Oxford Instruments, Abingdon, UK) and AZtecEnergyEDS software (version 3.0).

To study the characteristics of nano- and micro-sized particles in dispersions and solutions (diffusion coefficient, polydispersity index, hydrodynamic diameter), a Litesizer 500 light scattering analyzer (Aurora, AntonPaar) was used. The integral distribution and particle measuring in the liquid phase was carried out on a laser diffractometer PSA 1190 LD (Aurora, AntonPaar) after preliminary dispersion by ultrasound for 1 min. The distribution was estimated by number, volume and surface.

Modified methods were used to assess the viability of model plants [14, 15]. TriticumvulgareVill wheat seeds were preliminarily washed and soaked for 3 hours in distilled water. The seeds were kept in suspensions of metal NPs Fe_2O_3, Co_2O_3, ZnO, $CoFe_2O_4$, $ZnFe_2O_4$ in concentrations from 10^{-3} to 10^{-5} M for 4 hours, then they were treated with H_2O_2 (50 μM) for 4, 12, and 24 hours (KimJae-Hwanetal., 2016). The control was plants grown in distilled water. Then, the enzymatic activity of reductase-colorimetric was determined according to the manufacturer's protocol "Cellcounting kit-8 (CCK-8)" (WST-8 patent No.2.251.850, Canada) using the highly sensitive test "Cellcounting kit-8 (CCK-8)" ("Sigma-Aldrich", USA). For the CCK-8 test, cell suspensions were prepared with regards to the following procedure: 3 roots of 40 mg mass were ground for 10 minutes in 120 μl of FSB, centrifuged for 5 minutes at 10,000 rpm. Next, 100 μl of supernatant was taken and mixed with 10 μl of SKK-8 dye in a 96-well plate. The obtained samples were incubated for 1 hour at 37 °C. After that, every 15 minutes, the optical depth was measured at λ=450 nm using a microplate reader (Tecan, Austria) in the mode of shaking and heating to 37 °C. The number of viable cells (in %) was calculated by the formula based on the ratio of optical depth (OD) of experimental samples to the control values minus the background absorption of the kit reagents (CCM8):

$$\text{Cells viability, \%} = ((OD\text{-}ODc)/(Oc\text{-}CCK8))\ 100\ \%.$$

All the experiments were performed in 3 replicates; the obtained data were processed by the methods of variation statistics with the use of MicrosoftExcel (USA).

3. Results and discussion

According to SEM imaging, Fe2O3 had a lamellar structure (more than 80 nm), Co3O4 was represented by small cubic particles with sharp edges (from 20 to 100 nm), adhered to large aggregates (~ 1 μm), ZnO was characterized by a spherical, oval and hexagonal structure (from 60 to 160 nm). The ZnFe2O4 specimen synthesized in a slightly acidic medium (pH=2) consisted of granular particles with a size of ~250–400 nm, which contained smaller particles with a diameter of ~50 nm, while alkaline powders had a lamellar structure and a larger size. In the synthesis of CoFe2O4 in an alkaline medium (pH≥9), the particles were smaller (from 30 to 100 nm) and had almost no faceting. In turn, large particles with a characteristic facet were formed in a slightly acidic medium (pH=2) and, possibly, were single-crystal.

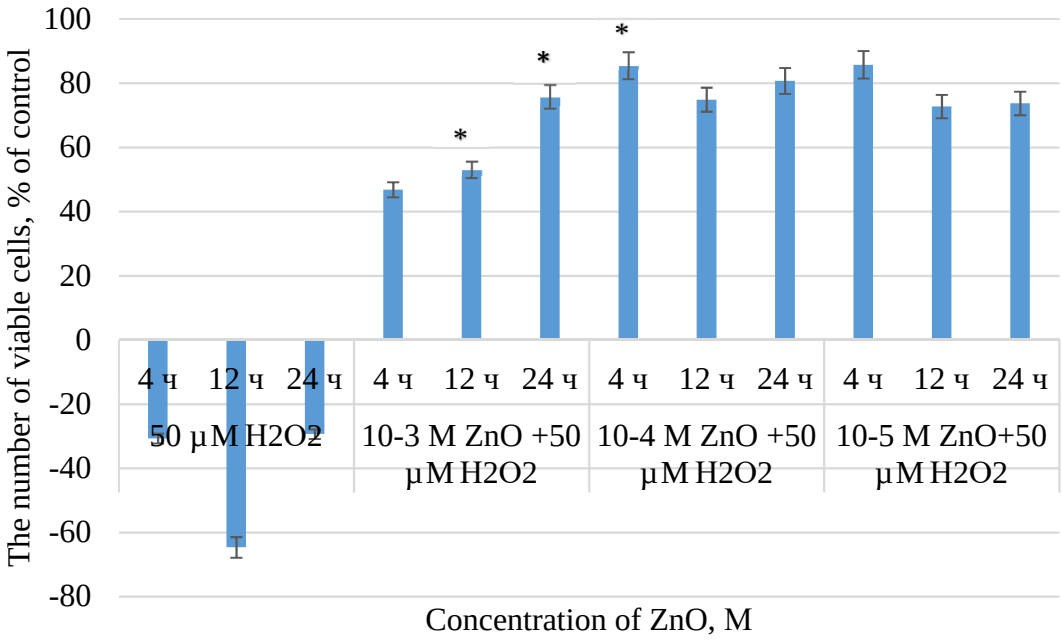

Figure 1. Cell viability of the roots of T. vulgare seedlings after exposure to ZnO particles synthesized in an aqueous extract from P. crispum leaves under oxidation conditions of 50 µM H_2O_2 according to the enzymatic activity of reductases and the yield of formazan; *option significantly different from the control one (value P≤0.05)

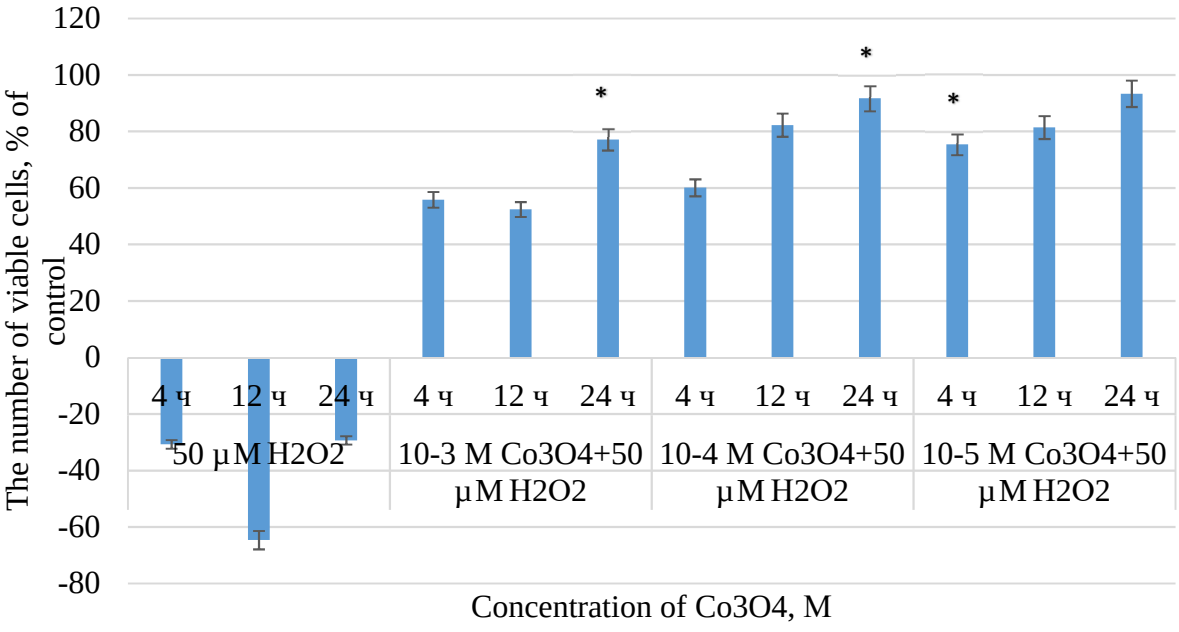

Figure 2. Cell viability of the roots of T. vulgare seedlings after exposure to Co_3O_4 particles synthesized in an aqueous extract from P. crispum leaves under oxidation conditions of 50 µM H2O2 by the enzymatic activity of reductases and the yield of formazan; *option significantly different from the control one (value P≤0.05)

A comprehensive analysis of the *T. vulgare* seedlings cells viability against the background of peroxide (50 µM) and biosynthesized nanopowders from 4 to 24 hours showed the protective properties

of some drugs. According to the data, a direct dependence of the enzymatic activity of reductases on the content of metal oxides and the incubation time was established. Namely, the cell viability after treatment with ZnO powder at a maximum dose (10^{-3} M) was 55 and 75 % after 12 and 24 hours, and after exposure to a dilution of 10^{-4} M (4 hours) –85 % (P≤0, 05) (Fig. 1). In general, the indicator varied from 47 to 76 %.

Bio-Co_3O_4 led to a significant decrease in the enzymatic activity of reductases as compared to the control after 24 hours of treatment with doses of 10^{-3} and 10^{-4} M (up to 77 and 91 %, respectively) and 10^{-5} M (75 %) (Fig. 2), while the indicator ranged from 52 to 93 %.

Fe_2O_3 powders had a less significant impact on the wheat seedling cells viability. Thus, a statistically significant decrease was at concentrations of 10^{-4} and 10^{-5} M (91 %) (82 to 96 %, respectively) (Fig. 3). Cells viability changed widely from 49 to 102 %.

It is interesting to note that nanopowders, consisting of two metals, had softer properties and contributed to an increase in the number of viable cells in the oxidizing medium. Moreover, the synthesis conditions (in an acidic or alkaline environment) of these metal particles also influenced the indicator. In particular, zinc ferrite ZnFe2O4 at pH=2 led to an increase in cells viability from 56 to 101 %, while in alkaline the indicator was even higher, specifically, from 43 to 114 % (Fig. 4). Cobalt ferrite, on the contrary, increased viability to a greater extent when the synthesis took place in an acidic medium (from 56 to 116 %) than in an alkaline medium (from 40 to 115 %) (Fig. 5).

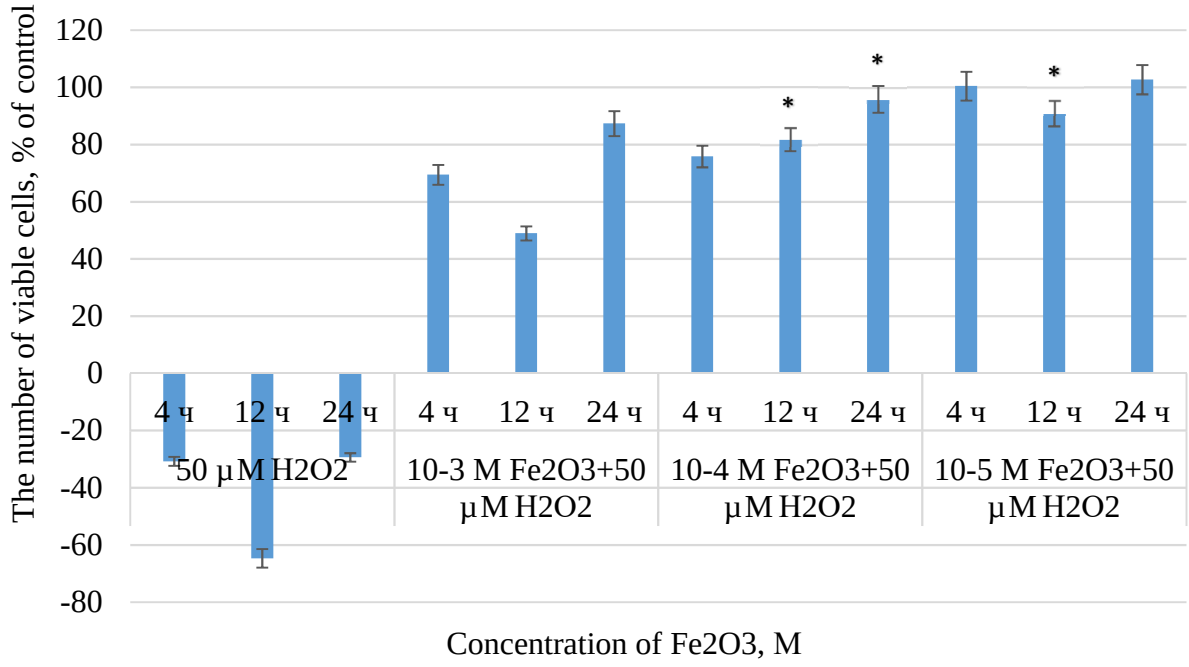

Figure 3. Cell viability of the roots of T. vulgare seedlings after exposure to Fe_2O_3 particles synthesized in an aqueous extract from P. crispum leaves under oxidation conditions of 50 µM H_2O_2 by the enzymatic activity of reductases and the yield of formazan; *option significantly different from the control one (value P≤0.05)

Moreover, a different degree of severity of the cytotoxic effect of metal nanoparticles on plant cells may be stipulated by particle morphology. Since, unlike other nanometals, Co3O4 has a truncated polyhedral triangular shape in the upper basal planes, therefore, it has a high atomic density and, as is known, contribute to greater reactivity (unlike spherical and lamellar) [16].

It is likely that the effects of Co3O4 and ZnO led to an indirect mechanism of cell damage through ROS production and triggering oxidative stress [17]. Strong oxidative stress in response to Co3O4 is possible due to a large variation in the oxidation states of Co2 +, Co3 + and Co4 + compared to other transition 3d elements [18], the release of ions according to the Trojan horse type [19] and the rapid increase in the ROS pool [20].

Moreover, the less negative Zeta potential of Co3O4 (only -8.5 mV compared to ZnO, whose charge was –26 mV) makes strong electrostatic repulsion of negative charges less likely.

Our previous analysis [21] of the biosynthesized powders cytotoxicity was carried out as counting dead cells in the roots of T. vulgare stained with the vital dye Evans blue after 14 days of exposure with a preparation in a wide range of concentrations (from 10^{-1} to 10^{-5} M). The results showed that a cell viability of the seedling roots of T. vulgare after the exposure to the biosynthesized nanomaterials increased as follows: Fe2O3<Co3O4<CeO2<ZnO.

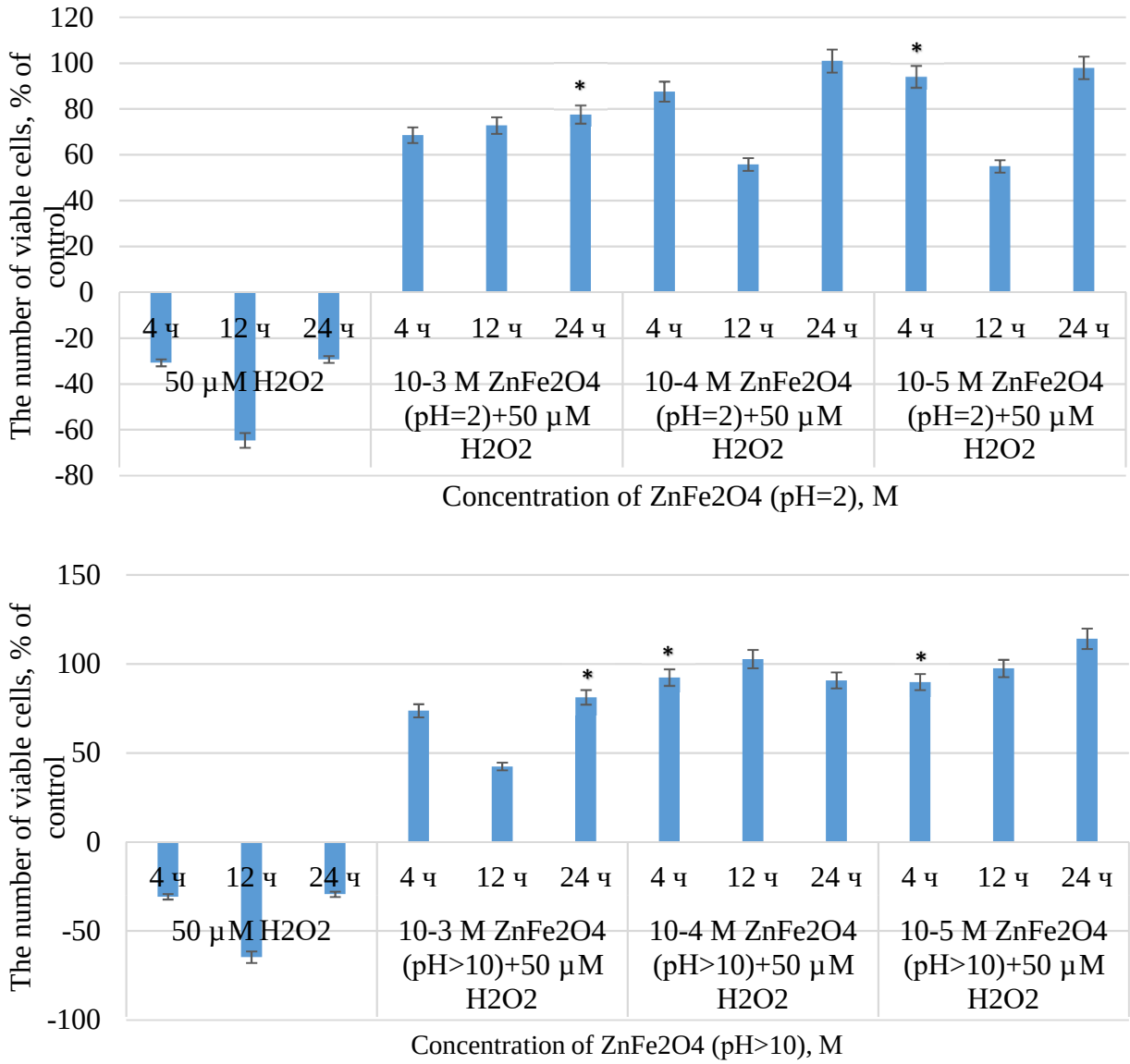

Figure 4. Cell viability of the roots of T. vulgare seedlings after exposure to $ZnFe_2O_4$ particles synthesized in an aqueous extract from P. crispum leaves under oxidation conditions of 50 µM H_2O_2 by the enzymatic activity of reductases and the yield of formazan; *option significantly different from the control one (value P≤0.05)

As for bimetallic powders, the data on their account vary. Thus, studies have shown a dose-dependent cytotoxicity of ZnFe2O4 [22, 23] and the possible use of nano-ZnFe2O4 in cancer therapy. Therefore, the nanoparticles obtained in our study are quite promising and require further testing on other living organisms.

Thus, against the background of the short-term effect of oxidizing agents, the following noticeable protective effect was exhibited by nanoparticles CoFe2O4 (pH=2) and CoFe2O4 (pH=10): cell viability averaged 103, 93, and 92 %, respectively. Particles of Fe2O3 (84 %) and zinc ferrite ZnFe2O4, both synthesized in an acidic medium (79 %) and alkaline medium (87 %), had a slightly smaller effect. The remaining nanometals (ZnO, Co3O4) did not have such a strong effect, specifically, the reductase activity index was no more than 73 %.

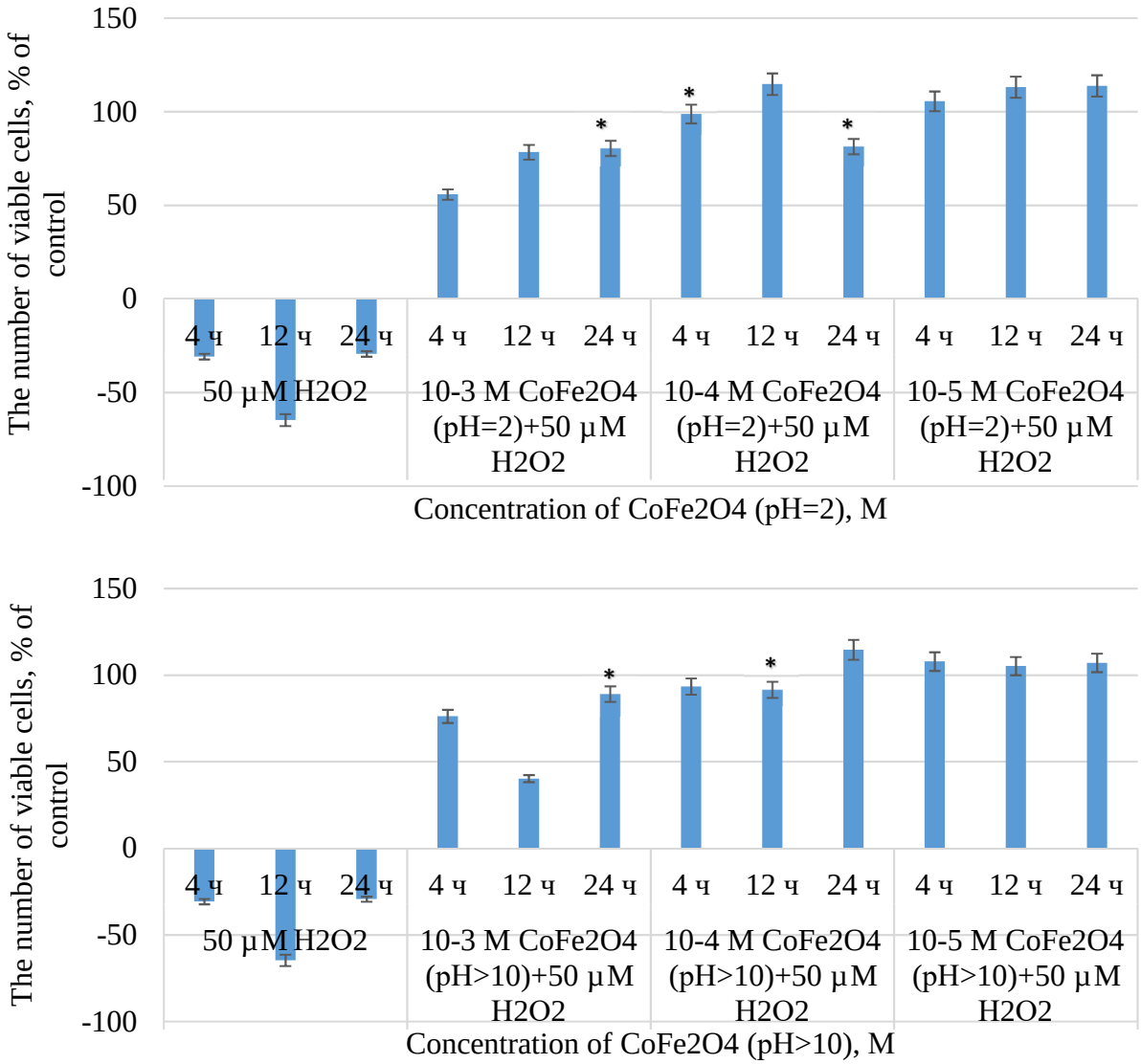

Figure 5. Cell viability of the roots of T. vulgare seedlings after exposure to $CoFe_2O_4$ particles synthesized in an aqueous extract from P. crispum leaves under oxidation conditions of 50 µM H_2O_2 by the enzymatic activity of reductases and the yield of formazan; *option significantly different from the control option (value P≤0,05)

4. Conclusion

Thus, the results of our work are promising for the further implementation of this technological chain in biotechnology and agriculture to increase plant productivity and the introduction of "green" technologies in the field of nanotechnological developments.

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Conflict of Interest: The authors declare that they have no conflict of interest.
Ethical standards: All applicable international, national, and institutional guidelines for animal care and use have been followed.

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