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Silver nanoparticles initiation using *Calendula officinalis* L. hairy root extracts

Nadiia Matvieieva[1], Volodymyr Duplij*[1], Anatolii Shakhovsky[1], Dmytro Klymchuk[2], Valeriia Belokurova[1], Mykola Kuchuk[1]

[1]Institute of Cell Biology and Genetic Engineering of the National Academy of Sciences of Ukraine, Kyiv, Ukraine
[2]M. Kholodny Institute of Botany of the National Academy of Sciences of Ukraine, Kyiv, Ukraine

Nadiia Matvieieva: https://orcid.org/0000-0002-4877-5222
Volodymyr Duplij: https://orcid.org/0000-0002-7479-7257
Anatolii Shakhovsky: https://orcid.org/0000-0003-4315-300X
Valeriia Belokurova: https://orcid.org/0000-0002-9872-9136
Dmytro Klymchuk: https://orcid.org/0000-0002-7076-8213
Mykola Kuchuk: https://orcid.org/0000-0001-7365-7474

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Nanoparticles (NPs) of various metals, in particular, copper, silver, gold, zinc, and titanium, are now considered promising and multifunctional components for biomedical applications. For the formation of nanoparticles, a "green" synthesis method has been developed based on the use of plant extracts to initiate nanoparticles. This method is considered simple and safe as it involves the use of extracts from well-studied plants. The main condition for the possibility of "green" synthesis of metal nanoparticles is the presence of the similar activity in the extracts. The possibilities of using *Calendula officinalis* L. hairy root extracts for the synthesis of silver nanoparticles (AgNPs) and its dependence on flavonoid concentration in the extracts were studied in this work. The extracts obtained with the use of 70% ethanol had the highest reducing activity in comparison with the aqueous extracts. Reducing activity of the extracts correlated with the concentration of flavonoids. The presence of nanoparticles of different sizes was confirmed by using transmission electron microscopy. Colloid solutions of AgNPs obtained using the extract with a higher content of flavonoids had significantly higher absorption values in the range of 420–440 nm, which is characteristic of AgNPs. An increase in absorption over time (up to two weeks) indicates long-term preservation of reducing activity in the mixture with $AgNO_3$. The smallest AgNPs (0.33… 7 nm) were formed when an aqueous extract was used, and the largest ones (up to 41.83 nm) with an extract obtained with 96% ethanol. Thus, it is the aqueous *C. officinalis* extract that should be chosen if it is necessary to obtain silver nanoparticles of small size.

Keywords: *Calendula officinalis*, rol genes, hairy roots, flavonoids, bioactivity, AgNPs

Introduction

Nanotechnology has made significant progress in the 21st century. Nanoparticles (NPs) of various metals, in particular, copper, silver, gold, zinc, and titanium, are now considered as promising and multifunctional components for biomedical applications (Dos Santos et al., 2014; Adebayo et al., 2019; Akinola et al., 2020). In the recent years, the interest highly increased

***Corresponding Author:** Volodymyr Duplij, Institute of Cell Biology and Genetic Engineering of the National Academy of Sciences of Ukraine, 148 Academika Zabolotnoho St., 03143, Kyiv, Ukraine
duplijv@icbge.org.ua

in the use of nanoparticles in the biomedical field for diagnostics and treatment of diseases, in particular, for the targeted delivery of drugs, vaccines, and genes. Currently, more than 40 plant species have been tested to use plant extracts for the synthesis of silver nanoparticles (Rafique et al., 2017; Vanlalveni et al., 2021; Nie et al., 2023).

"Green" synthesis is a simple and safe method for metal nanoparticle initiation. It is based on the possibility that plant-derived compounds reduce the ions of metals accompanied by NP formation. It is known that plants synthesize a wide spectrum of bioactive metabolites (polyphenols, tannins, polysaccharides, proteins, amino acids, alkaloids, etc.). Due to their chemical activity, these compounds can reduce metal ions, resulting in the formation of metal nanoparticles. In addition, these plant compounds can also act as NP stabilizers, thus providing stability to the newly formed nanoparticles (Asif et al., 2022). In particular, Giri et al. (2022) studied AgNPs synthesized by *Eugenia roxburghii* DC. They evaluated spectrophotometric characteristics, their structure (by X-ray diffraction), together with morphology and size (by high-resolution transmission electron microscope). Liaqat et al. (2022) obtained stable silver nanoparticles using *Eucalyptus camaldulensis* and *Terminalia arjuna* extracts, as well as their combinations.

According to the published data, different plant extracts were used for silver nanoparticle (AgNPs) initiation. Such interest in the development of methods of "green" synthesis of nanoparticles is promoted by their bioactivity and possible practical application. For example, it was shown that AgNPs possessed antibacterial, antifungal (Jalal et al., 2018; Khan et al., 2021; Marinescu et al., 2022), and anticancer (Hemlata et al., 2020; Wang et al., 2021) activities.

Calendula officinalis L. is a medicinal plant with a sufficiently well-studied chemical composition. Secondary metabolites synthesized by the plants are used to treat a number of diseases (Butnariu and Coradini, 2012; Ashwlayan et al., 2018; Ak et al., 2020). In recent years a number of metal nanoparticles were obtained using *C. officinalis* extracts, in particular, titanium (Wei et al., 2021), gold (Zhao et al., 2022), and silver (Zhangabay and Berillo, 2023), and their possible practical application in pharmacology and medicine was studied. Earlier *C. officinalis* hairy roots were used for the production of secondary metabolites, as well as for studying the effect of various factors on their growth and biosynthetic activity (Długosz et al., 2013; Al-Abasi et al., 2022).

In our work, we investigated the possibility of using *C. officinalis* hairy root extracts that differed in flavonoid content for AgNPs synthesis. Special interest in the use of extracts from the hairy roots of medicinal plants is associated with a possible increase in their content of bioactive compounds with reducing properties.

Material and methodology

Preparation of extracts

To prepare the extracts, *C. officinalis* hairy roots from the *in vitro* collection of the Institute of Cell Biology and Genetic Engineering, NAS of Ukraine(Figure 1a) were cultured on solidified Murashige and Skoog medium (Duchefa, Netherland) for 4 weeks. After that, the roots were removed from the medium, washed with deionized water, homogenized in the solvents (deionized water, 40%, 70%, and 96% ethanol), and centrifugated at 14,000 rpm for 10 minutes (Eppendorf Centrifuge 5415C). The supernatants were collected and used for the further studies.

Total flavonoid content assay

To determine the total content of flavonoids, we have used a reaction with aluminum chloride (details in Matvieieva et al., 2019). The optical density of the samples was measured using a Fluorat-02 Panorama spectrofluorimeter at λ = 510 nm. The content of flavonoids was estimated in $mg.g^{-1}$ wet weight (ww) in routine equivalent (RE) using the calibration graph $C = 1.9575x$, $R^2 = 0.9723$.

Nanoparticle initiation

The extracts were added to the silver nitrate solution at a concentration of 1 mM (200 µl of the extract was added to 4 ml of the silver nitrate solution), mixed, and incubated in a water bath at a temperature of 80 °C for 60 min. The presence of reductive activity was determined visually by the formation of a colloidal solution of AgNPs (a change in the color of the solution to yellow-brown). The presence of the formed nanoparticles was confirmed using transmission electron microscopy.

UV-Vis spectra assay

The surface plasmon resonance of the samples (spectra of the obtained AgNPs colloidal solutions) was measured in 1, 7, and 14 days in the wavelength range of 300… 700 nm by a Fluorat-02 Panorama spectrophotometer.

Transmission electron microscopy

The transmission electron microscope (TEM) analysis was performed with a JEM-I230 microscope (JEOL, Tokyo, Japan), with an accelerating voltage of 80 kV. For the sample preparation, 0.05 µl of AgNP colloid solutions were applied to a grid and air-dried. AgNPs spectrometry was performed using an X-MAX N 80T detector (Oxford's instruments, Oxford, UK) integrated with a TEM 1230 transmission electron microscope (Jeol, Tokyo, Japan) operating at 15 kV accelerating voltage. Nanoparticle sizing was conducted using the ImageJ software (https://imagej.net/ij/download.html).

Statistical analysis

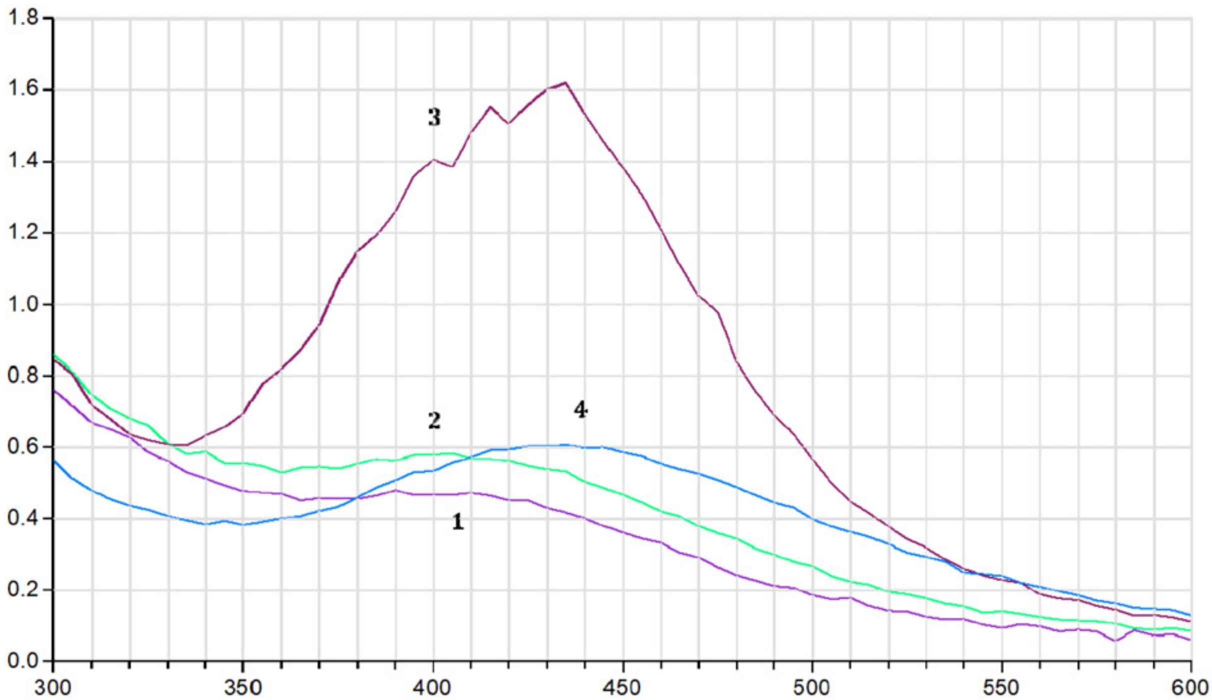
All analyses were performed in triplicate. Data were analyzed with ANOVA followed by Tukey's test using R version 4.2.2 software. Differences at the level of $p < 0.05$ were considered statistically significant.

Results and discussion

The samples obtained by the extraction with different solvents were studied for the total flavonoid content. This content differed depending on the used solvent. It was the lowest in the case of using water for the extraction (3.40 ±0.12 mg RE.g^{-1} ww) and increased when ethanol in the concentrations of 40%, 70%, and 96% was used (5.75 ±0.22, 6.68 ±0.48, and 5.12 ±0.26 mg RE.g^{-1} ww, respectively).

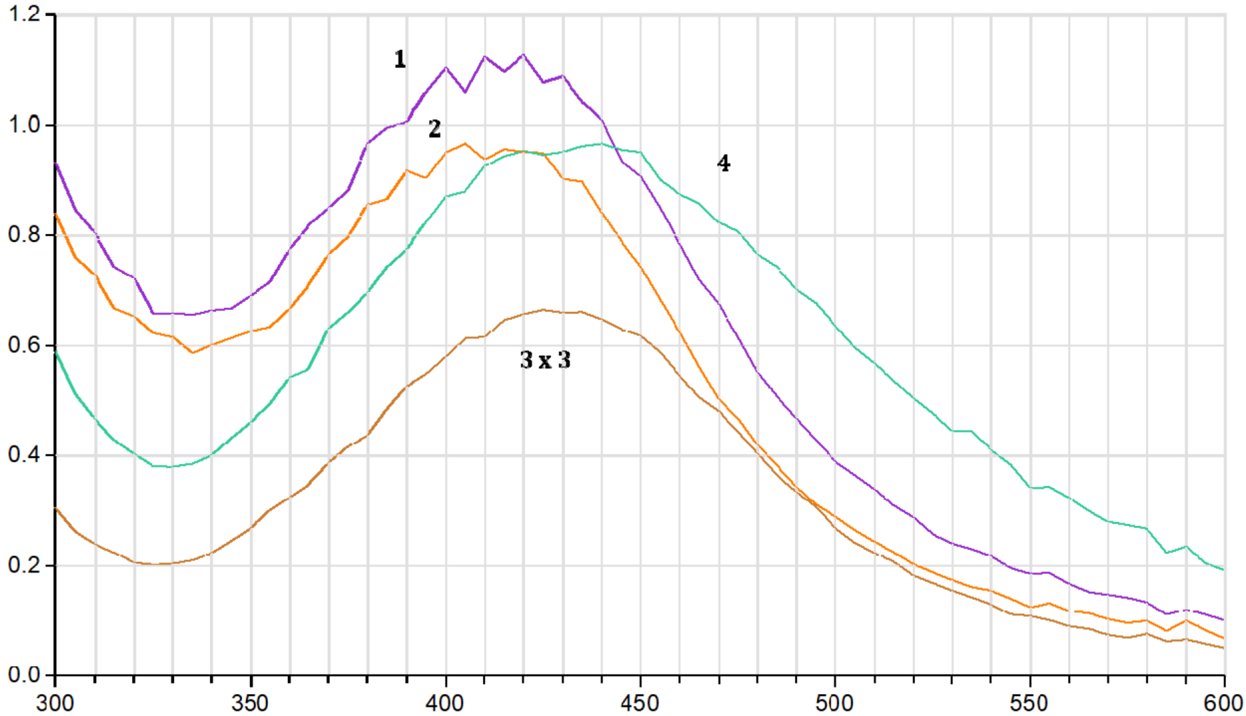
After adding the extracts to the silver nitrate solution and heating the mixture, the appearance of a yellow-brown color was observed. This is an evidence of the formation of silver nanoparticles. Analysis of the UV-Vis spectra of the obtained colored solutions carried out in the range of λ = 300… 600 nm, revealed a characteristic peak at a wavelength of about 420–430 nm that confirms the possessed reducing activity of the extracts. According to the spectra shown in Figure 1, the extract obtained with the use of 70% ethanol had the highest activity (the optical density changed from 1.60 units in one day to 2.43 units in 14 days). The lowest activity was detected in the case of aqueous extract (0.50 units in one day and 1.25 units in 14 days). Therefore, the extract, which had the highest concentration of flavonoids, showed the highest reducing activity in the reaction with silver nitrate. It should be noted that the height of the peaks increased during two weeks (Figure 1A–B a, b, c). This indicates the stability of the reducing activity of the extracts in the reaction mixture during this period.

Earlier (Bohdanovych and Matvieieva, 2022) we suggested using the process of obtaining a colloidal

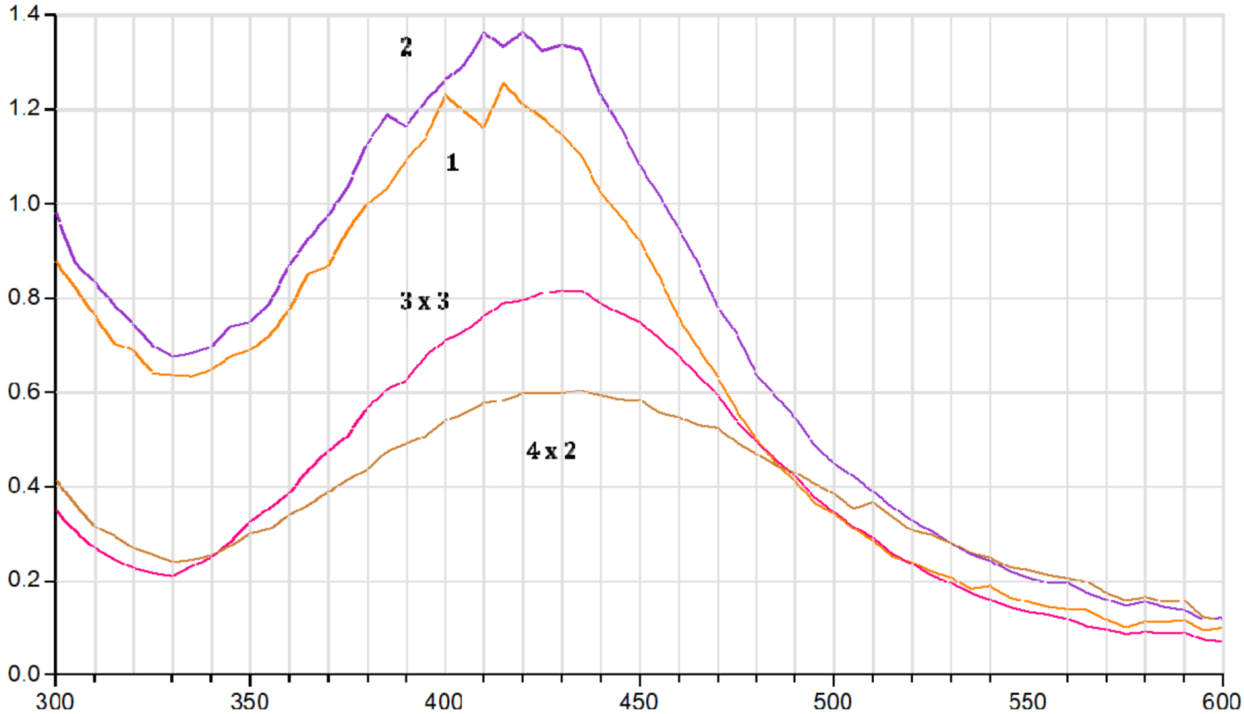


(a)

Figure 1A UV-Vis absorption spectra of colloidal solutions of silver nanoparticles in one (a), 7 (b), and 14 days (c) days after the initiation of their formation
aqueous (1) and ethanol (2 – 40%, 3 – 70%, 4 – 96%) extracts from *Calendula officinalis* L. hairy roots; x3 and x2 – the sample were diluted three or two times



(b)



(c)

Figure 1B UV-Vis absorption spectra of colloidal solutions of silver nanoparticles in one (a), 7 (b), and 14 days (c) days after the initiation of their formation
aqueous (1) and ethanol (2 – 40%, 3 – 70%, 4 – 96%) extracts from *Calendula officinalis* L. hairy roots; x3 and x2 – the sample were diluted three or two times

solution of silver nanoparticles for a comparative assessment of the reducing power of plant extracts added to the $AgNO_3$ solution. This conclusion was based on the analyses of the UV-Vis spectra of colloid solutions obtained by various extracts of hairy wormwood roots. The studied reducing activity correlated with the content of flavonoids in the used extracts. According to these data, the obtained results indicate that the spectrophotometric analysis can indeed be used as an indicator of reducing activity since a correlation of the height of the peaks at $\lambda = 440$ nm with the total content of flavonoids in extracts from wormwood was found. Alim and coauthors (Alim et al., 2020) approved the possibility of using spectra in the UV-Vis range to characterize nanoparticle solutions. Similar results were obtained by us in the study of four extracts from *C. officinalis* hairy roots. Colloid solutions of AgNPs obtained using the extract with a higher content of flavonoids (No 3) had significantly higher absorption values in the range of 420–440 nm, which is characteristic of AgNPs. An increase in absorption over time (up to two weeks) indicates long-term preservation of reducing activity in the mixture with $AgNO_3$.

The analysis using transmission electron microscopy confirmed the presence of nanoparticles (Figure 2). Nanoparticles were mostly round, but varied in size. According to the data obtained, the smallest AgNPs (0.33… 7.0 nm) were formed when an aqueous extract was used, the largest ones (up to 41.83 nm) – when an extract obtained with 96% ethanol was used for NP initiation (Figure 3). Such differences are obviously caused by the different chemical composition of the extracts used for NPs formation (higher content

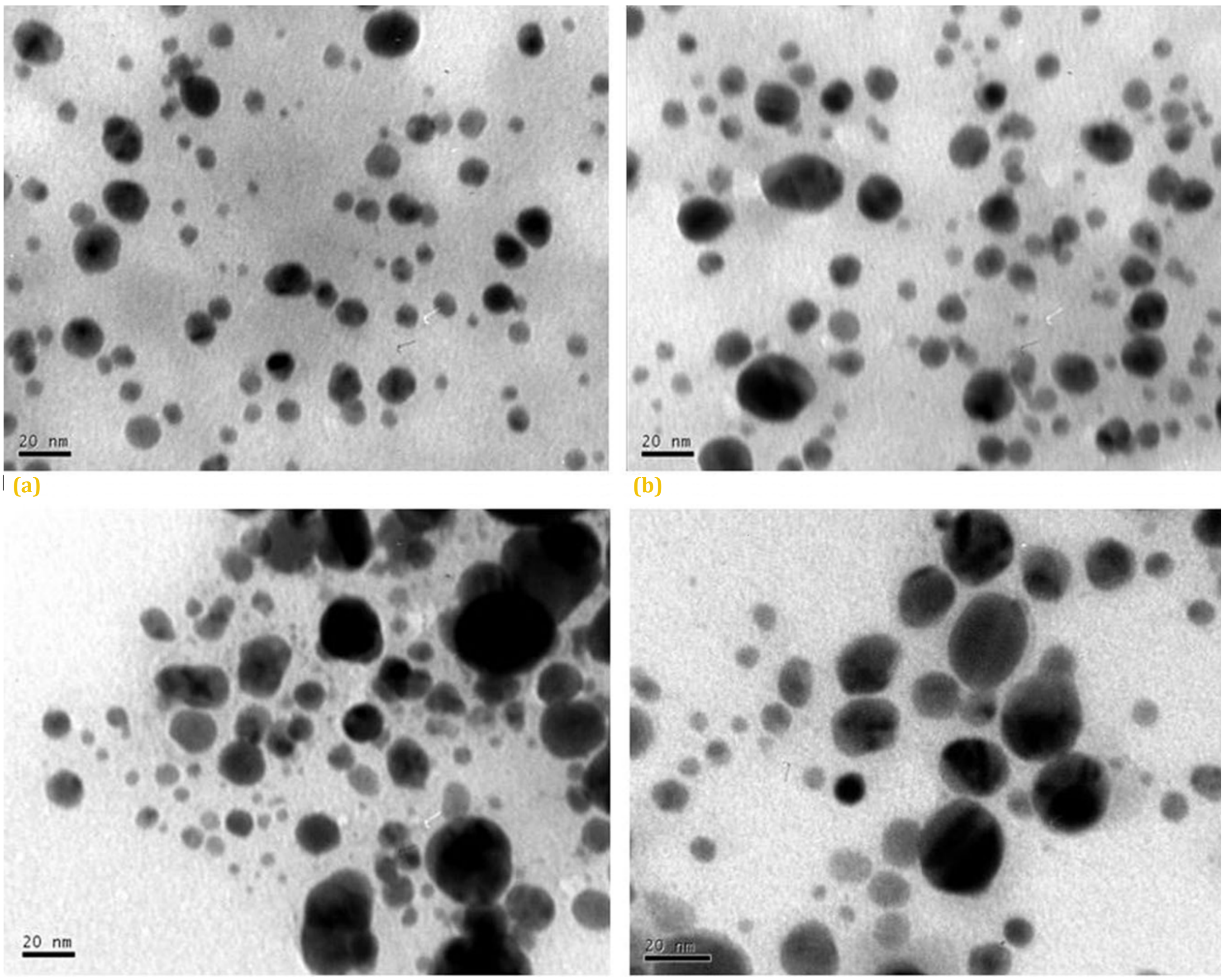


Figure 2 Microphotographs (TEM, JEM-I230) of silver nanoparticles obtained using *Calendula officinalis* L. hairy roots extracts
a – aqueous; b – 40%; c – 0%; d – 96% ethanol

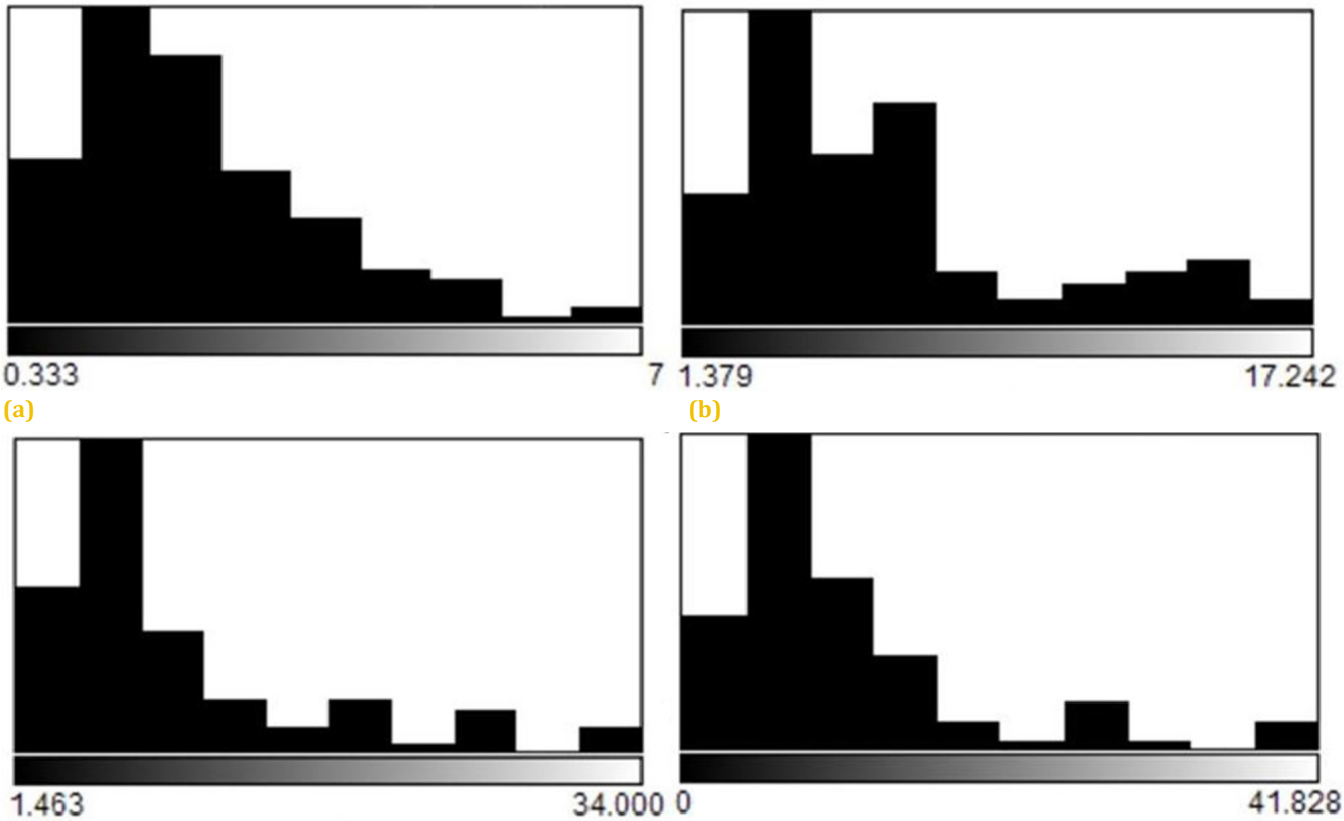


Figure 3 AgNPs size distribution
a – aqueous; b – 40%; c – 0%; d – 96% ethanol extracts were used for nanoparticle initiation

of glycosylated flavonoids in the aqueous extract and higher concentration of aglycones in the extract obtained using high concentration ethanol).

Silver nanoparticles were previously obtained by different groups of researchers using extracts from *C. officinalis* plants. Silver nanoparticles are usually obtained by reacting plant extracts with silver nitrate solution (El-Kemary et al., 2016; Xu et al., 2021; Asif et al., 2022; Liaqat et al., 2022). However, it was shown that such NPs can also be obtained using a solution of silver sulfate (Olfati et al., 2021). Dried leaves or flowers (El-Kemary et al., 2016; Olfati et al., 2021; Xu et al., 2021; Balciunaitiene et al., 2022) were used for this purpose. At the same time, until now, the hairy roots of these plants have not been used to obtain silver nanoparticles. However, hairy roots obtained by genetic transformation may have a changed chemical composition. Therefore, nanoparticles obtained using them may also have changed characteristics.

In various studies, the authors present the characteristics of the NPs. Such AgNPs differed in size and other parameters (TEM, Zeta potential, fluorescence spectroscopy, FT-IR spectrum, UV-visible absorption spectra analyses). These features are probably related to differences in the qualitative and quantitative chemical composition of the used extracts, extract/silver nitrate solution ratios, or the use of silver sulfate in the reaction mixture. Unfortunately, it is impossible to make a correlation between the composition of the extracts and the characteristics of the nanoparticles, since the publications lack data on the chemical analysis of the used extracts. At the same time, it is possible to compare the sizes of nanoparticles obtained in different studies. In particular, El-Kemary et al. (2016) mixed dried *C. officinalis* plants with 32% EtOH for extract obtaining. This extract was used for AgNPs initiation after the incubation with $AgNO_3$ solution. Biosynthesized AgNPs ranged from 30 to 50 nm. Analysis of surface plasmon resonance in the UV-visible absorption spectrum of a colloidal solution of NPs revealed the maximum absorption peak at 435 nm. In the experiments of Xu et al. (2021), dried leaves of *C. officinalis* were ground and extracted by boiling water. The nanoparticles were formed in a spherical shape in the range of 38.05 to 75.41 nm. SPR bands were detected at the wavelength of 452 nm. Balciunaitiene et al. (2022) studied the process of NPs initiation by *C. officinalis* aqueous inflorescences extract as a reducing agent. The size of NPs was up to 35 nm and the particles do not tend to form large agglomerates.

A comparison of the results of the above studies shows that, in general, the use of *C. officinalis* water extracts or using of ethanol in lower concentrations for the extraction can result in the production of smaller AgNPs. Such data are consistent with the results of our experiments since nanoparticles of a smaller size were also obtained using the aqueous extract (up to 7 nm). The use of 70% or 96% ethanol to obtain extracts has led to a significant increase in the size of nanoparticles (up to 40 nm). Probably, this may be related to the different chemical compositions of such extracts and the specifics of extracting compounds from *C. officinalis* plants. These results should be taken into account when it is necessary to obtain nanoparticles of different sizes.

In our work, we did not study the bioactivity of the obtained AgNPs, but such work is of interest and will be conducted. The expediency of such an analysis emerges from the results of a number of publications regarding the presence, for example, of antimicrobial (Olfati et al., 2021; Asif et al., 2022; Liaqat et al., 2022; Giri et al., 2022) and antitumor activity (Xu et al., 2021) of silver nanoparticles obtained using *C. officinalis* plants.

Conclusions

The possibilities of using *C. officinalis* hairy root extracts for AgNP synthesis and its dependence on flavonoid concentration in the extracts were studied. The extracts obtained with the use of 70% ethanol had the highest activity in comparison with the aqueous extracts. The reducing activity of the extracts correlated with the concentration of flavonoids. The presence of nanoparticles of different sizes was confirmed by using transmission electron microscopy. The smallest AgNPs (0.33… 7.0 nm) were formed when an aqueous extract was used, and the largest ones (up to 41.83 nm) with an extract obtained with 96% ethanol.

Conflict of interest

The authors have no conflicts of interest to declare.

Ethical statement

This article does not contain any studies that would require an ethical statement.

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