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Original article

OPTICAL CHARACTERISTICS OF SILVER NANOPARTICLES OBTAINED USING *Artemisia tilesii* Ledeb. "HAIRY" ROOT EXTRACTS WITH HIGH FLAVONOID CONTENT

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Background. "Hairy" roots can be used for production of plant-derived secondary metabolites, such as flavonoids, which have antioxidant and reducing properties. It seems promising to use the process of silver nanoparticles formation as a method of determining the level of reducing power. This approach allows simultaneously to obtain nanoparticles with different biological activity and evaluate the reducing potential of different plants.

Objective. The aim of the study was to determine the dependence of optical properties (ultraviolet/visible spectroscopy, UV-Vis spectra) of solutions of silver nanoparticles obtained using wormwood "hairy" root extracts on the content of flavonoids as compounds with reducing activity.

Methods. Four *Artemisia tilesii* “hairy” root lines from the collection of the Institute of Cell Biology and Genetic Engineering NAS of Ukraine were grown for five weeks in liquid 1/2 MS medium with 20 g/l sucrose. "Hairy" roots were homogenized in 70% ethanol, the extracts were centrifuged, total flavonoid content was determined, and extracts were used for the silver nanoparticles preparation via reduction of Ag^+ to Ag^0 from $AgNO_3$. UV-Vis spectra at 300–600 nm range were recorded right after colloid solution preparation, in five, and in nine days.

Results. Total flavonoid content varied from 4.01 ± 0.39 to 15.37 ± 1.08 mg RE/g FW. The UV-Vis spectra curves of absorption increased with the course of time, mostly from day 0 to day 5. At this period, absorption at 370–500 nm wavelength increased almost twofold. The peak absorption of all samples was detected at 440 nm, and the maximum values at the wavelength of 440 nm correlated with the content of flavonoids. This correlation did not change over time.

Conclusions. The optical properties of silver nanoparticles colloid solutions obtained using *A. tilesii* "hairy" root extracts correlated with the total flavonoid content of the samples. Even though the absorbance of the colloid solutions increased with time, those increases correlated with flavonoid content as well. The determination of the optical properties of AgNPs colloid solution can be used as a convenient way of quickly comparing the reducing ability of extracts both right after the formation of a colloidal solution and after some time of its storage.

Keywords: silver nanoparticles; "hairy" roots; *Artemisia tilesii* Ledeb.; flavonoids; reducing power.

Introduction

"Hairy" roots are cultures of plant organs that arise due to infection by *Agrobacterium rhizogenes* – a Gram-negative aerobic soil bacterium that incorporates its Ri (root inducing) plasmid carrying *rol* genes into the plant genome. Those *rol* genes are known to be plant secondary metabolism activators. So, incorporation of *rol* genes in plant genome may result in boosted antioxidant and reducing activity [1–5].

"Hairy" root lines serve as *in vitro* systems with different applications, mainly for high value production of plant-derived secondary metabolites [6], recombinant proteins, biotransformation and phytoremediation [7]. Many of those metabolites are of special interest for pharmacological industry due to various medicinal properties. Moreover, “hairy” roots are a great alternative to the whole plant system not only by ability of year-round controlled synthesis, but also by the capacity to intensify the production of metabolites using optimal *A. rhizogenes* strains [8]. With the help of transformation, dozens of new lines can be obtained and then selected due to desired qualities: fast growth, high content of polyphenolic compounds, increased antioxidant activity and reducing power etc. Such lines-overproducers can have better commercial application in contrast to extremely expensive organic synthesis. Besides, plant cultures produce a whole spectrum of diverse bioactive compounds at once, which is more valuable and reasonable then producing chemically-derived medicine with one or two active compounds. Furthermore, with the help of new advances in metabolic engineering and synthetic biology, "hairy" root lines with unique complex of compounds that is not characteristic to the same non-transformed species can be obtained [9].

As above mentioned, "hairy" root lines can be valuable not only due to high antioxidant activity, but due to exceptional reducing power as well. Most methods of reducing power evaluation are based on the ability of plant extracts to reduce radicals and metals [10–17]. DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) and NO (nitric oxide) tests are the most common in the first group, and FRAP (ferric reducing antioxidant power), FTC (ferric thiocyanate) and CUPRAC (cupric ions reducing assay) methods in the second. At the same time, such reactions with metals associated with reducing activity of plant extracts can also be used for the so-called "green" synthesis of nanoparticles. For instance, it is possible to initiate silver nanoparticles (AgNPs) formation by Ag^+ to Ag^0 reduction using "hairy" root extracts due to the presence of flavonoids, which are characterized by high reducing activity.

Obtaining AgNPs is of interest not only as a method and assessment of the level of reducing power, but also as an independent biotechnological process of creating a product (nanoparticles), which can find wide practical applications due to having anticancer effect, antibacterial, antioxidant, anti-inflammatory and anti-hyperglycemic activity [18–25], as well as anti-quorum sensing potential [26]. Advances of such "green" silver, zink, gold, platinum, nickel, aluminum and iron nanoparticles are widely researched [27–32].

It is of both fundamental and practical interest to determine the relationship between the total content of flavonoids, known as a chemicals with reducing activity, and the characteristics of silver nanoparticles, in particular, according to the dynamics of changes in the Ultraviolet/Visible (UV-Vis) spectrum. UV/Vis spectroscopy technique is used for quantifying the extinction of solutions (the sum of absorbed and scattered light). Silver nanoparticles have specific optical properties which depend to size, shape, concentration, and agglomeration state. The use of these spectra is possible, since silver nanoparticles are characterized by the presence of a specific peak in the range of about 420–440 nm. This makes UV/Vis spectroscopy a tool for studying different colloidal AgNPs solutions [33].

Therefore, the purpose of the work was to study optical properties of AgNPs obtained using extracts from some *Artemisia tilesii* "hairy" root lines, which differ significantly in flavonoid content. Due to the reducing activity of flavonoids, such a comparison allows for a relative evaluation of the reducing capacity of the extracts without carrying out specific reactions with the simultaneous production of silver nanoparticles.

Materials and Methods

"Hairy" roots cultivation. A. tilesii "hairy" root lines obtained earlier [34] were taken from the collection of the Institute of Cell Biology and Genetic Engineering NAS of Ukraine. Prior to the experiment, all root cultures were grown for five weeks with constant stirring in thermostatic conditions at +24 °C and 16 hours lightening. Liquid half-strength Murashige and Scoog medium (Duchefa, Netherland) with 20 g/l sucrose as carbon source was used for this purpose.

Extracts preparation. After the harvesting of biomass, all "hairy" roots were dried using filter paper, weighted by 0.3 g each and homogenized in 3 ml of 70% ethanol. Then the samples were centrifuged in an Eppendorf Centrifuge 5415C at 15 000 g for 10 min. Supernatant was collected and used in the experiments.

Total flavonoid content determination. The content of flavonoids in the supernatant was determined using the standard method with $AlCl_3$ [35] via spectrophotometry at 510 nm (Fluorat-02-Panorama spectrofluorimeter). The calculation was carried out according to the calibration curve ($y = 0.7969x$, $R^2 = 0.9608$) in mg/g fresh weight (FW) in rutin equivalent (RE).

Preparation of nanoparticles colloid solutions. The same ethanol extracts were used to obtain colloid solutions of silver nanoparticles (AgNPs). Namely, 0.3 ml of the extracts and 3 ml of 1 mM $AgNO_3$ were mixed thoroughly. After that all the solutions were incubated for one hour in a water bath at +80 °C to reduce Ag^+ to Ag^0.

AgNPs spectrophotometry assay. Absorbance of the samples (colloid solutions of AgNPs) was measured automatically in the wavelength range of 300–600 nm (Fluorat-02-Panorama spectrofluorimeter) right after the incubation in a water bath, then in five and nine days to observe the dynamics of spectra changing. UV-Vis spectra of the samples were obtained using the PanoramaPro software.

Statistical analysis. All the experiments were performed in triplicate. Necessary calculations were performed in MS Excel ($P \leq 0.05$) and results were presented as the mean value ± confidence interval. The data were analyzed for statistical significance using ANOVA followed by Tukey HSD test using R software version 4.0.4. The difference between mean values was considered statistically significant at $P < 0.05$.

Results

Total flavonoids content values in the extracts obtained using different "hairy" root lines were scattered from 4.01 ± 0.39 to 15.37 ± 1.08 mg RE/g FW (Fig. 1). Therefore, four root lines differed significantly in total flavonoid content. Such differences correlated with the visual results of AgNPs formation in the samples. It was studied the process of formation of nanoparticles is characterized by a change in the color of the solution – from transparent and colorless to yellow-brown, and the degree of color intensity corresponds to the number of nanoparticles formed [36]. As it turned out, higher flavonoid content resulted in deeper coloration of the colloid solutions from light to dark brown (Fig. 2). Moreover, coloration occurred very rapidly right after addition of "hairy" root extracts to $AgNO_3$ (Fig. 2b), even before samples treatment in the hot water bath. After the treatment, all the samples darkened even more (Fig. 2c), so that in order to analyze the UV-Vis spectra, it was necessary to dilute the obtained NPs solutions. Samples № 2 and 3 were diluted 1:4, and sample № 4 was diluted 1:2 with deionized water.

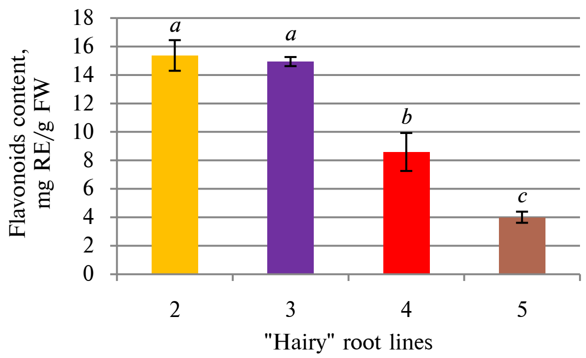

Figure 1: Total content of flavonoids in the extracts of *Artemisia tilesii* "hairy" root lines. Error bars with different small letters denote significant differences in values among four samples at $P < 0.05$

The UV-Vis spectra of these samples were recorded right after the NPs initiation, in five and nine days (Fig. 3). As it is seen from the figures, the curves of absorption drastically increased with the course of time, mostly from day 0 to day 5. At this period, absorption at $\lambda = 370–500$ nm increased almost twofold. The peak absorption of all the samples remained at $\lambda = 440$ nm. The presence of such a peak is the evidence that AgNPs were present in the analyzed solutions.

These peaks were recorded at each time point for all samples without dilution in order to better analyze the dynamics of changes that occur during short-term storage of the solutions (Fig. 4). The absorption peaks at each point of spectrum analysis fully correlated with the flavonoid content in the extracts (Fig. 5). The correlation slightly changed with time, and coefficient of determination stayed in the reliable range (R^2 from 0.9223 to 0.8815). In addition, as it was seen from the spectra, bigger increase in absorption was from day 0 to day 5 (up to 81%), and almost didn't change from day 5 to day 9. Such features indicate that the process of AgNPs formation does not occur instantly and continues mainly for five days. The value of increase fully correlated with flavonoid content as well: the significant increase corresponded to the high content of flavonoids in the extracts. Hereby, the higher was the content of flavonoids, the more absorption increased from day 0 to day 5 and from day 5 to day 9. For example, the highest flavonoid content was in the line №2 (15.37 ± 1.08 mg RE/g FW). Its peak absorption ($\lambda = 440$ nm) at day 0 was 3.68 a.u., in five days it increased by 2.32 a.u., and in nine days it increased by 0.4 a.u. At the same time, the lowest flavonoid content was in the line №5 (4.01 ± 0.39 mg RE/g FW). Its peak absorption at day 0 was 1.21 a.u., in five days and nine days it increased only by 0.51 and 0.07 a.u., respectively.

Figure 2: Samples before extracts addition to $AgNO_3$ solution (a), after extracts addition (b), and after incubation in water bath, 80 °C during one hour (c): 1 – $AgNO_3$ only, 2–5 – $AgNO_3$ solution mixed with "hairy" root extracts

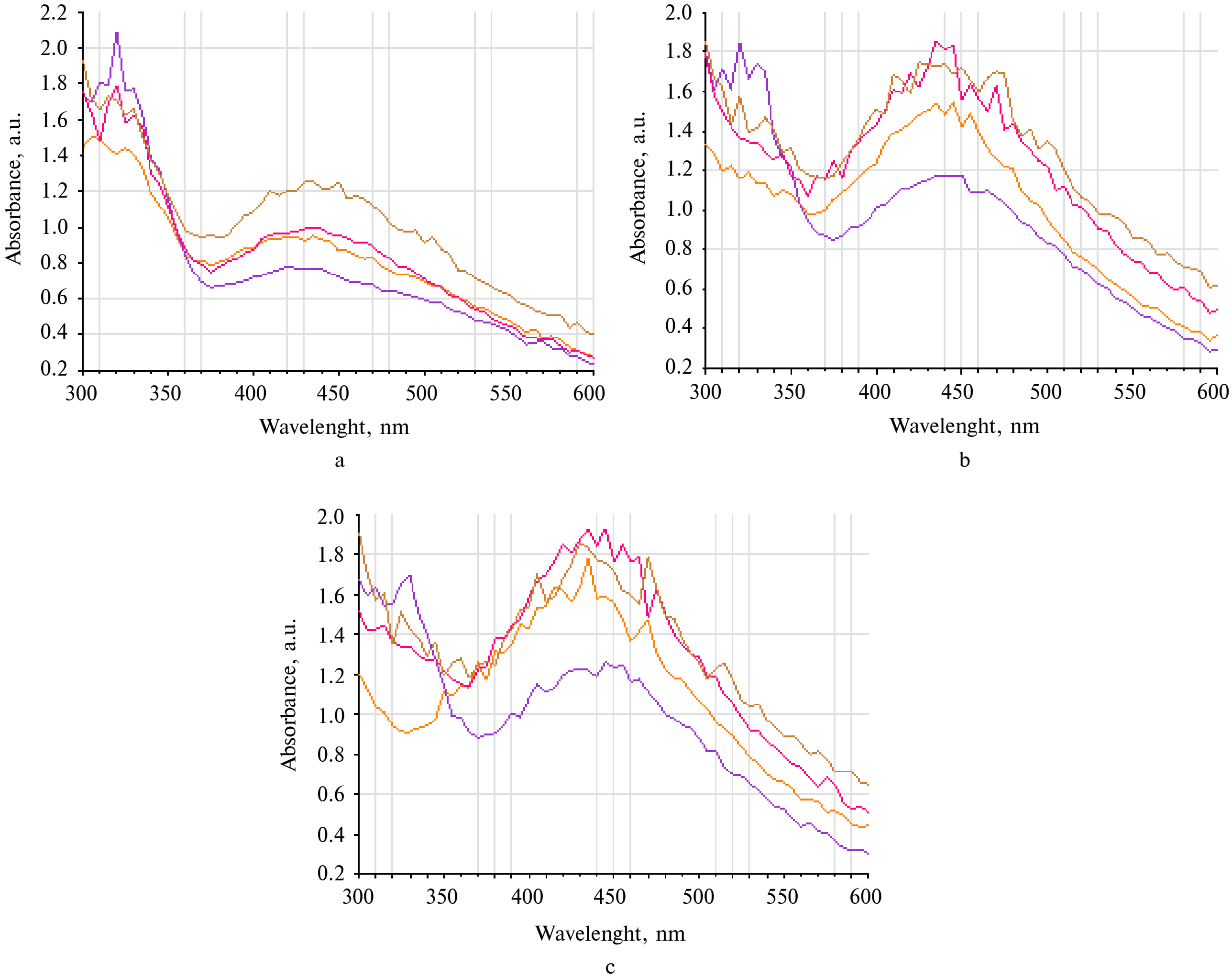

Figure 3: UV-Vis spectra of AgNPs obtained using "green" synthesis with the extracts of *Artemisia tilesii* "hairy" roots right after preparation of the nanoparticles (a), in 5 days (b), and in 9 days (c): 2–5 – "hairy" root lines; — 2 (with dilution 1:4), — 3 (with dilution 1:4), — 4 (with dilution 1:2) — 5

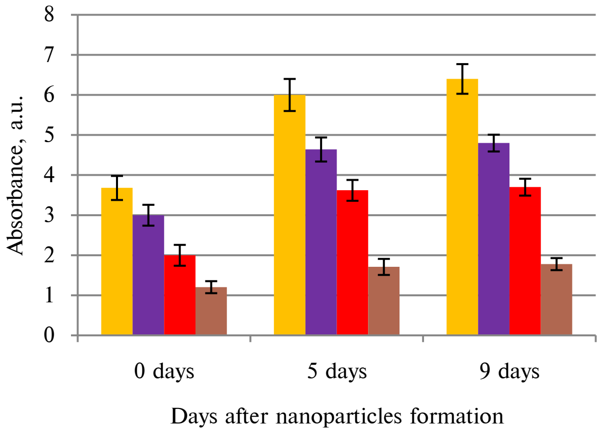

Figure 4: Changes of the absorbance of colloid solutions of AgNPs without dilution at 440 nm: 2–5 – extracts of *Artemisia tilesii* "hairy" root lines: – line 2, – line 3, – line 4, – line 5

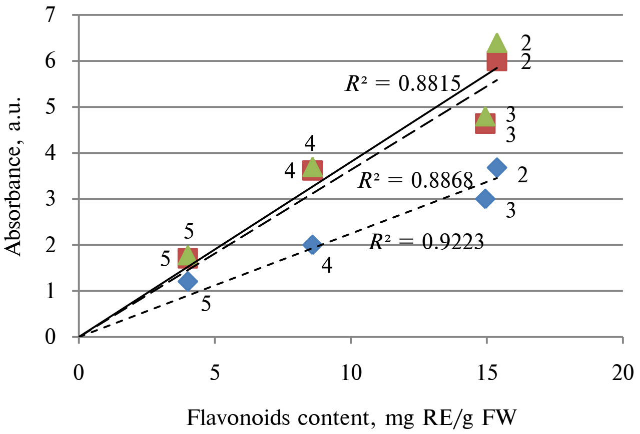

Figure 5: Correlation between the total flavonoid content and peak absorbance of the colloid solutions of AgNPs without dilution at 440 nm: 2–5 – extracts of *Artemisia tilesii* "hairy" root lines: – 0 days, – 5 days, – 9 days, ------ – 0 days, ---- – 5 days, ——— – 9 days

Discussion

Analysis revealed that *A. tilesii* "hairy" root lines differed significantly in flavonoid content (up to 3.84 times). It should be noted that one of the root lines was distinguished by a high content of flavonoids (up to 15.37 ± 1.08 mg RE/g FW). Such differences can be explained by activation of the secondary metabolism by transferred *rol* genes and their undetermined incorporation cite into the plant genome [37–39]. As well, earlier we studied the main components of *A. tilesii* extracts [40]. Many polyphenolic compounds, such as flavonoids, were identified in this extract. Mainly, the most abundant were apigenin, quercetin, kaempferol, luteolin, and rutin. Higher content of such flavonoids in plant cells is beneficial to the plant, as polyphenolic compounds have reducing properties, thus help to withstand the oxidative stress [40]. Moreover, flavonoids were studied to be able to chelate iron and copper ions, and affect the activity of enzymes involved in the oxidation process [41]. Abundance of polyphenolic compounds in *Artemisia* genus results in various biological activities of these plants: antioxidant, anti-inflammatory, anticancer, antimicrobial, antiviral, and enzyme-inhibitory activities [42–53]. That is why *A. tilesii*, perennial arctic herbaceous plant found throughout Alaska, Japan, Siberia, and Canada, has long been used by Native Americans to heal wounds, prevent infectious illnesses and treat diseases of the cardiovascular system [54, 55]. At the same time, as our research showed, ethanolic extracts from the roots of these plants can be effectively used to obtain silver nanoparticles, probably due to the high content of flavonoids with reducing activity.

There are previously known publications on silver nanoparticles formation in such *Artemisia* plants, as *A. absinthium* [56], *A. arborescens* [57], *A. vulgaris* [58], *A. turcomanica* [59], *A. sieberi* [60], *A. afra* [61], *A. capillaris* [62], *A. scoporia* [63], *A. marschalliana* [64], *A. haussknechtii* [65], *A. annua* [66], and *A. tilesii* [36]. However, those studies were focused on the characterization of nanoparticles, namely their yield, concentration and size distribution, as well as the antioxidant and reducing potential of obtained nanoparticles and their biomedical applications. Our research was aimed to study the UV-Vis spectra of colloid solutions obtained using different wormwood "hairy" root extracts, and to compare such features with the content of flavonoids in these extracts as compounds with reducing activity. Probably, such a comparison allows conducting a comparative evaluation of the reducing capacity of the extracts without carrying out specific reactions. The results of the study indicate that the change in the color of the reaction mixture can be detected practically after the initiation of the redox reaction by adding the ethanol extract from the "hairy" roots. These changes as well as spectrum analysis data can be used as an indicator of the reducing activity because of their correlation with the total flavonoid content. It should be noted that previously [67] we found a strong correlation between the content of flavonoids and the reducing power of the extracts from the "hairy" roots of various plant species.

With the course of the reduction of Ag^+ to Ag^0, it could be seen that the reaction proceeded faster and the colloid solution became darker in the samples that had higher flavonoid content (lines № 2 and 3). Such result corresponds to the antioxidant and reducing nature of flavonoids and other polyphenolic compounds extracted by ethanol. Indeed, reducing power of flavanols, flavanonols, flavones, and flavonols has been estimated and confirmed [68–70].

The studied UV-Vis spectra (see Fig. 3) of these samples confirmed this correlation. Higher absorption in the range of 370–500 nm interrelated with the flavonoid content. This relation stayed the same even after the absorption increase in five and nine days after the nanoparticles formation. The increase in the absorption with time indicates the increase of AgNPs content in the solution and may be because of the further proceeding of the redox reaction. This effect may denote the prolonged action of reducing activity of the "hairy" root extracts, and result in the further stabilization of the colloid solution. The increase was proportional to the flavonoid content. It suggests that the process of the colloid silver NPs solution obtaining can in fact be used as comparative evaluation of the reducing capacity of the extracts added to $AgNO_3$ solution. Moreover, it appears to be convenient for analysis no matter when the measurement needs to be done, right after the AgNPs initiation or after some storage. The fact that the peak absorption of all the samples remained at around 440 nm is helpful for the methodology, as it allows to determine the result of the reaction making only one spectrophotometric measurement in spite of obtaining whole UV-Vis spectra. The wavelength of peak absorption at 410–460 nm is characteristic for silver nanoparticles [19, 23, 24], and at 425–440 nm is characteristic for silver nanoparticles synthesised using *Artemisia* plant extracts [36, 60, 61, 64].

Conclusions

The optical characteristics (UV-Vis spectra) of silver nanoparticles obtained using *A. tilesii* "hairy" root extracts correlated with the total flavonoid content of the samples. The dependence of the intensity of the absorbance in the region of 440 nm on the content of flavonoids was found. Since flavonoids have reducing activity, it is possible to conduct spectrophotometry assay of silver nanoparticles solution at 410–460 nm to evaluate differences in the reducing power of plant extracts. Such method appears to be useful and convenient both right after the colloid solution formation and after its storage.

Interests disclosure

The authors have no conflicts of interest to declare.

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ОПТИЧНІ ХАРАКТЕРИСТИКИ НАНОЧАСТИНОК СРІБЛА, ОТРИМАНИХ ІЗ ВИКОРИСТАННЯМ ЕКСТРАКТІВ “БОРОДАТИХ” КОРЕНІВ *Artemisia tilesii* Ledeb. ІЗ ВИСОКИМ ВМІСТОМ ФЛАВОНОЇДІВ

Проблематика. “Бородаті” корені можна використовувати для виробництва вторинних метаболітів рослинного походження, таких як флавоноїди, які мають антиоксидантні та відновлювальні властивості. Перспективним видається використання процесу утворення наночастинок срібла як методу визначення рівня відновлювальної активності. Такий підхід дає змогу одночасно отримати наночастинки з різною біологічною активністю та оцінити відновлювальний потенціал різних рослин.
Мета. Дослідження залежності оптичних властивостей (ультрафіолетова/видима спектроскопія, спектри UV-Vis) розчинів наночастинок срібла, отриманих із використанням екстрактів “бородатих” коренів полину, від вмісту флавоноїдів як сполук із відновлювальною активністю.
Методика реалізації. Чотири лінії “бородатих” коренів *Artemisia tilesii* з колекції Інституту клітинної біології та генетичної інженерії НАН України вирощували протягом п’яти тижнів у рідкому середовищі 1/2 MS з 20 г/л сахарози. “Бородаті” корені гомогенізували в 70 %-вому етанолі, екстракти центрифугували, визначали вміст флавоноїдів і використовували для приготування наночастинок срібла шляхом відновлення Ag^+ до Ag^0 з $AgNO_3$. УФ-видимі спектри в діапазоні 300–600 нм вимірювали відразу після приготування колоїдного розчину, через 5 і через 9 діб.
Результати. Загальний вміст флавоноїдів варіював від 4,01 ± 0,39 до 15,37 ± 1,08 мг РЕ/г ВМ. Криві поглинання спектрів УФ-видимої області з часом зростали, переважно з 0 до 5-го дня. У цей період поглинання за 370–500 нм зросло майже вдвічі. Пікове поглинання всіх зразків виявлено за 440 нм, причому максимальні значення за довжини хвилі 440 нм корелювали з вмістом флавоноїдів. Ця кореляція не змінювалася з часом.
Висновки. Оптичні характеристики колоїдних розчинів наночасток срібла, отриманих із використанням екстрактів “бородатих” коренів *A. tilesii*, корелювали із загальним вмістом флавоноїдів у зразках. Незважаючи на те що абсорбція колоїдних розчинів збільшувалася з часом, ці зміни також корелювали із вмістом флавоноїдів. Визначення оптичних властивостей колоїдного розчину наночастинок срібла можна використовувати як зручний спосіб швидкого порівняння відновної здатності екстрактів як одразу після утворення колоїдного розчину, так і через деякий час після його зберігання.

Ключові слова: наночастинки срібла; “бородаті” корені; *Artemisia tilesii* Ledeb.; флавоноїди; відновлювальна активність.