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DNA PROTECTIVE ACTIVITY OF *Artemisia spp.* HAIRY ROOT EXTRACTS

Shakhovsky A.M.[1]
Duplij V.P.[1]
Afanasieva K.S.[2]
Chopei M.I.[2]
Matvieieva N.A.[1]

[1]Institute of Cell Biology and Genetic Engineering of the National Academy of Sciences of Ukraine, Kyiv, Ukraine

[2]Taras Shevchenko National University of Kyiv, Ukraine

E-mail: duplijv@icbge.org.ua

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Artemisia spp. plants are known as producers of bioactive compounds and used both in folk and traditional medicine. They possess antitumor, antiproliferative, antidiabetic, antimicrobial, antiviral, antioxidant, and anti-inflammatory activity.

Aim. Because these plants exhibit antioxidant activity, it is of interest to investigate the possibility of using extracts from mugwort to prevent DNA damage initiated by some reactive oxygen species.

Methods. In this work, extracts from transformed roots of *Artemisia vulgaris* and *A. tilesii* were used to study their DNA protective activity. The extracts were prepared according to standard procedure. Total flavonoid content was quantified by the modified spectrophotometric method in rutin equivalent using the calibration curve. The antioxidant activity of the extracts was determined using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). It was evaluated by the half maximal effective concentration (EC_{50}) calculated as the dry root weight needed for scavenging 50% of DPPH in the sample and expressed as mg DW. To calculate this value, linear regression was applied to the linear interval of radical scavenging activity. DNA protective activity was studied by the Fenton reaction assay.

Results. Differences in the content of flavonoids in *A. vulgaris* "hairy" roots and control roots were found. For hairy roots this parameter ranged from 75.89 ± 2.32 to 126.04 ± 5.37 mg RE/g DW, which is 1.45–2.41 times more than in the control roots. Flavonoid content in *A. tilesii* hairy root line also differed from the control. It was 74.52 ± 0.96 ... 107.8 ± 5.98 mg RE/g DW in root lines and 28,6 ± 2,11 mg/g DW in *A. tilesii* control roots. The level of antioxidant activity studied in the reaction with DPPH (EC_{50}, effective concentration) was more significant in the extracts of hairy roots of both plant species. It varied from 0.16 to 0.33 and 0.17 to 0.31 in hairy root lines of *A. vulgaris* and *A. tilesii*, respectively. In comparison, this parameter reached 0.44 and 0.65 in the control roots. Adding the extracts to the reaction mixture in the Fenton reaction has some protective effects. At the same time, there were no significant differences in the degree of protection of plasmid DNA from damage (percentage of supercoiled DNA) when extracts from hairy root lines of *A. vulgaris* and *A. tilesii* were added to the reaction mixture. However, these extracts differed in the content of flavonoids and had a higher ability to scavenge DPPH radicals.

Conclusions. The extracts of *A. vulgaris* and *A. tilesii* hairy roots had contained a higher content of flavonoids and had higher antioxidant activity compared to the extracts from the control roots. However, they differed little in their ability to protect DNA from damage in the Fenton reaction. Likely, that not only flavonoids, but also other components of extracts from wormwood hairy roots are involved in this process.

Key words: antioxidant activity, flavonoids, Fenton reaction, oxidative stress, *Artemisia vulgaris*, *Artemisia tilesii*, hairy roots.

As a result of the cell's vital activity as well as under stress conditions, both biotic and abiotic, active forms of oxygen are formed in organisms constantly. Therefore, in order to maintain homeostasis, there are antioxidant enzyme systems capable of deactivating them. However, a certain amount of reactive oxygen species (ROS) bypasses the protective mechanisms and exerts a negative influence on the cell's components, including DNA [1]. In certain pathological conditions, the amount of ROS increases as a result of homeostasis disruption, which can be critical for normal cell function.

Plants' biologically active compounds attract more and more attention because of their easy accessibility by extraction from plant raw materials and significantly fewer side effects when they are used as potential drugs. It is known that plants produce such bioactive compounds as polyphenols, carbohydrates, essential oils, etc. These compounds show a broad spectrum of bioactivity, including antioxidant, so they can protect DNA from damage caused by ROS. Such plant extracts may be helpful in the development of human-friendly therapies against diseases associated with oxidative stress or irradiation.

Recently, research aimed at identifying compounds with antioxidant properties has attracted increasing attention. The relevance of such studies is due to the fact that the presence of free radicals in cells provokes the development of a number of dangerous diseases, including carcinogenesis, inflammation, Alzheimer's and Parkinson's disease, mutagenesis, and also accelerates the aging process [2–4]. Oxidative stress occurs when the balance between antioxidants and prooxidants is disturbed, which leads to an increase in reactive oxygen species content [5].

Various compounds are characterized by the ability to protect cells from damage caused by oxidative stress and the negative effect of free radicals. Such a group of compounds with antioxidant activity includes, in particular, flavonoids, which, due to the peculiarities of their chemical structure, are potent antioxidants [6]. The use of compounds of natural origin, synthesized in plants, as antioxidants has the advantage due to their low toxicity and a small number of side effects [7].

Artemisia spp. plants are known as medicinal one and used both in folk and traditional medicine. That's why these plants can be used for the production of compounds with high bioactivity [8]. *Artemisia* spp. plants synthesize carbohydrates, flavonoids, essential oils, sesquiterpene lactones, and amino acids [9–13]. The plants of *Artemisia* spp. are used to treat a variety of diseases. Wormwood plants demonstrated antitumor and antiproliferative, antidiabetic, antimicrobial, antiviral, antioxidant, and anti-inflammatory activity [14–19].

Flavonoids, derivatives of phenolic compounds, are among the components synthesized by *Artemisia* spp. plants. For example, it has been found that common wormwood synthesizes apigenin, chrysoberyl, eupafolinisorhamnetin, diosmetin, luteolin, kaempferol 3-, quercetin 3-, rutin, and other compounds [20]. It is worth noting that the qualitative and quantitative composition of flavonoids varies depending on the region where the plant grows, the extraction method, and the process of determining these compounds in the resulting extract. [21]. These bioactive substances are able to inactivate free radicals and reduce oxidative stress, which is the cause of cancer, neurodegenerative, and cardiovascular diseases [22].

There are a number of methods for determining the antioxidant activity of compounds, including such activity of plant extracts. Of these, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay is the most widely used method due to its high availability and reproducibility, sensitivity, rapidness, and the possibility of adaptation for the analysis of the activity of different extracts [23]. The use of this method makes it possible to quantitatively assess the ability of plant extracts to scavenge radicals and to compare the bioactivity of different samples [24].

The Fenton reaction is a reaction of hydroxyl radical generation involving hydrogen peroxide and iron ions. Although it was first described in 1894 by Henry Fenton [25], it was not widely used, including in biology, until the 1960s. This reaction was proposed to be used for the evaluation of oxidative DNA damage [26]. The chemical basis of the Fenton reaction consists of the interaction of iron ions (Fe^{2+}) with hydrogen peroxide (H_2O_2), which is accompanied by the formation of hydroxyl radicals ($\cdot OH$). This method is widely used now to find compounds that are able to prevent DNA damage [27].

Our work was aimed at determining the antioxidant activity and ability of extracts from hairy roots of *Artemisia vulgaris* L. and *Artemisia tilesii* Ledeb to protect against DNA

damaging effects in oxidative stress by Fenton reaction. In the work, we used hairy roots of our *in vitro* collection because of studied earlier [28] increasing of total flavonoid content and antioxidant activity in these roots due to the introduction to the plant genome of *Agrobacterium* (*Rhizobium*) rhizogenes *rol* genes after the genetic transformation.

Materials and Methods

Plant material. The transformed roots of *A. vulgaris* and *A. tilesii* plants (Fig. 1) from the collection of the Laboratory of Adaptational Biotechnology of the Institute of Cell Biology and Genetic Engineering NAS of Ukraine were obtained by us earlier using *Agrobacterium rhizogenes* A4 — mediated transformation. Hairy roots were cultivated *in vitro* using Murashige, and Skoog solidified nutrient medium (Duchefa, Netherlands) with twice reduced concentration (1/2 MS).

Root extracts preparation. Hairy roots of different lines and roots of the control plants were used for extracts obtaining. The roots were lyophilized, ground by a Retsch MM400 (Germany), and extracted by 70% ethanol in the ratio of 50 mg/6 ml. In 3 days of the extraction (Clim-J-Shake System Kuhner IRC-1-U, 180 rpm, 28 °C), the samples were centrifuged for 10 min (Eppendorf Centrifuge 5415C) at 10000 rpm, and the supernatants were collected.

Flavonoid content assay Total flavonoid content was quantified by a modified spectrophotometric method [29]. For this purpose, 0.25 ml of each extract was added to the mixture of 1 ml of deionized water and 0.075 ml of 5% $NaNO_2$ solution. In 5 min, 0.075 ml of 10% $AlCl_3$ solution was added, and the mixture was incubated for 5 min. Then 0.5 ml of 1 M NaOH solution and 0.6 ml of deionized water were added to the reaction mixture, and the absorbance of the sample was measured at 510 nm using a Panorama spectrofluorometer. The total flavonoid content was expressed as milligrams per gram of dry root weight in rutin equivalent (mg RE/g DW). The calculation was done using the equation of the calibration curve: $y = 1.0358x$ ($R_2 = 0.9532$).

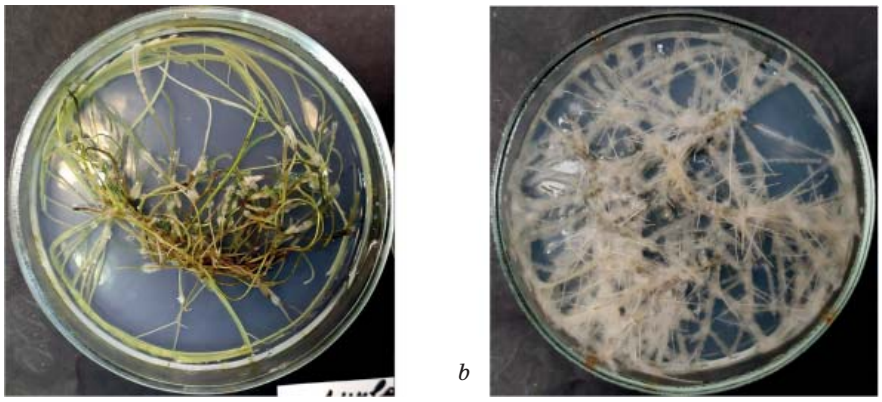
DPPH scavenging activity assay. The antioxidant activity of the extracts was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a free radical. The reaction for scavenging DPPH* radical was performed as described in [28]. The activity was firstly expressed as the inhibition percentage and was calculated using the following formula:

$$\% \text{ radical scavenging activity (RSA)} = \\ = ((\text{control OD} - \text{sample OD})/\text{control OD}) \times 100.$$

Then it was evaluated by the half maximal effective concentration (EC_{50}) calculated as the dry root weight needed for scavenging 50% of DPPH in the sample and expressed as mg DW. To calculate this value, linear regression was applied to the linear interval of RSA.

Fenton reaction assay. As a preliminary preparation, 1 ml of each ethanol extract was dried on a rotary evaporator (Automatic Environmental SpeedVac System AES2010, Savant) for 2 h. Then 50 µl of dimethyl sulfoxide (Sigma) was added as a solvent.

For the reaction, 3 µl of p133 plasmid (0.04 mg/µl), 2 µl of the sample, 4 µl of 3.5% hydrogen peroxide solution, and 3 µl of 2 mM $FeSO_4$ solution were mixed. For the control reaction, 2 µl of dimethyl sulfoxide was added instead of the sample. The mixture was incubated at 37 °C for 1 hour. Electrophoresis was performed in %0.8 agarose gel in Tris-



a *b*

Fig. 1. ***Artemisia vulgaris* (*a*) and *Artemisia tilesii* (*b*) hairy root cultures**

acetate buffer at 100 V for 45–50 minutes. DNA damage was assessed visually by changes in plasmid shapes (supercoiled, open circular, and linear) detected after electrophoresis. Quantitative evaluation of electrophoresis results (the percentage of supercoiled DNA) was carried out using the Gel Analyzer program.

Statistical analysis

All experiments were performed in triplicate. All results performed as mean ± confidence interval ($P < 0.05$). Calculations and plot building were made using RStudio (version 2024.09.1), R (version 4.4.2), and Microsoft Excel 2003.

Results and Discussion

Previously, we found a correlation between the content of flavonoids in hairy roots and the ability of flavonoid-containing extracts to scavenge the DPPH radical [30]. So, the first stage of the study was aimed at analyzing the content of flavonoids in the roots of different lines to identify the differences in the content of these compounds and the samples with possible high antioxidant activity. According to the results of the analysis of ethanol extracts of *A. vulgaris* roots, differences in the content of flavonoids between control roots and transgenic lines were found (Fig. 2). This parameter in the hairy roots ranged from 75.89 ± 2.32 to 126.04 ± 5.37 mg RE/g DW, which is 1.45–2.41 times more than in control nontransformed roots (52.27 ± 0.86 mg RE/g DW). Flavonoid content in the *A. tilesii* hairy root line also differed from the control (the roots of nontransformed plants cultivated in the same *in vitro* conditions). In particular, flavonoid content in *A. tilesii* hairy root lines varied from 74.52 ± 0.96 to 107.8 ± 5.98 mg RE/g DW. At the same time, the content of flavonoids in the control *A. tilesii* roots was 28,6 ± 2,11 mg/g DW.

The level of antioxidant activity studied in the reaction with DPPH calculated as EC_{50} (effective concentration) was more significant in the extracts of hairy roots of both plant species and varied from 0.16–0.33 mg and 0.17–0.31 mg in hairy root lines of *A. vulgaris* and *A. tilesii*, respectively. In comparison, this parameter reached 0.44 and 0.65 mg in the control roots of *A. vulgaris* and *A. tilesii*, respectively.

Thus, differences in the content of flavonoids and the level of antioxidant activity in extracts from different transgenic root lines of plants of the same species were found. Such differences are associated with the features of genetic transformation using *A. rhizogenes*, namely, the indeterminacy of the place of insertion of bacterial genes (in particular, *rol* genes) transferred to the plant genome [31]. Each of the transgenic lines is an independent transformational event, may differ in the place of insertion of transferred bacterial genes, and, thus, have specific features (for example, differences in morphology, growth rate, and biosynthetic activity). The peculiarities of the accumulation of flavonoids studied in our research make it possible to select such lines that are characterized by a high content of

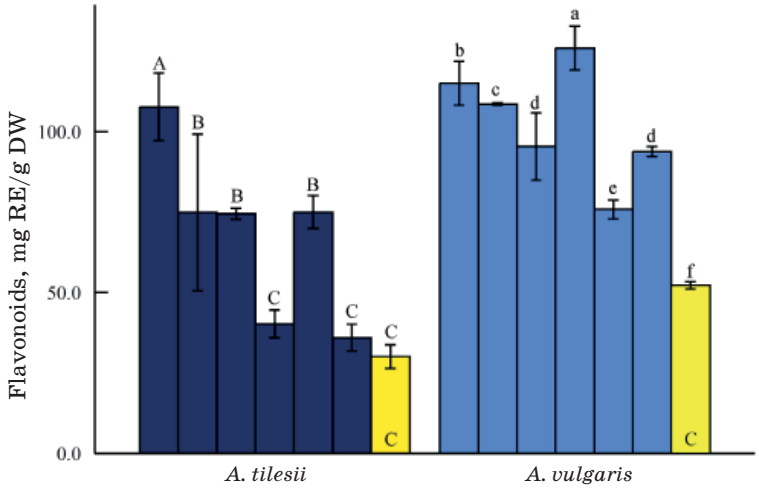


Fig. 2. **Flavonoid content in the extracts of *Artemisia vulgaris* and *Artemisia tilesii* control roots (C, yellow) and hairy root clones (blue) ($P < 0.05$)**

The same letters indicate no significant differences. Uppercase and lowercase letters refer to different comparisons

these compounds and a high level of bioactivity [31].

The content of flavonoids in the hairy roots of plants of all species was significantly higher than the content of these compounds in the roots of the control plants. This effect is also related to the activity of transferred *A. rhizogenes* genes since bacterial *rol* genes are known as inducers of secondary metabolism. Their activity can significantly stimulate flavonoid biosynthesis in transformed cells, which was established earlier for plants of various species, including plants of the *Artemisia* genus [32–34]. An increase in the level of antioxidant activity in transgenic lines of wormwood roots of various species is also a result of the effect of genetic transformation and an increase in the content of flavonoids in the hairy roots compared to the control. Previously, a direct correlation was established between the increase in the content of flavonoids and the ability of the extracts to scavenge the DPPH radical [35].

Polyphenols, as chemicals with specific structures, were evaluated to be o powerful antioxidants. In numerous studies, the correlation between total flavonoid content and antioxidant activity was detected. For example, in five *Artemisia* species (*A. absinthium*, *A. annua*, A. *austriaca, A. pontica, and A. vulgaris*) 15 phenolic acids (mostly hydroxycinnamic acid derivatives) and 26 flavonoids (poly-hydroxylated/poly-methoxylated flavone derivatives, present only in the aerial parts) were identified [36]. The total phenolic contents correlated with the antioxidant effects of the extracts assessed by different methods, including DPPH and ABTS radical scavenging, CUPRAC, and FRAP. Ten A*rtemisia* genus plants were studied for their antioxidant activity and flavonoid content. The extracts from *A. vulgaris, A. campestris, A. annua, A. alba*, and *A. austriaca* were the most active in the reaction with DPPH [37]. *A. copa* extracts contained total flavonoids of 5.5 ± 0.2 mg quercetin equivalents/g and possessed antioxidant activity studied by DPPH, FRAP, and ORAC methods [38]. *A. absinthium, A. dracunculus,* and *A. annua* ethanolic extracts contained different polyphenolic compounds with potent antioxidant activities in the DPPH assay, which was compared to ascorbic acid [39].

Fenton reaction was used in our study for visual evaluation of the possibility of the hairy root extracts to protect DNA from oxidative damage. The p133 plasmid DNA has three forms on agarose gel electrophoresis, namely, supercoiled circular DNA (SC), open circular (OC), and linear (L). In the case of hydrogen peroxide, adding in the reaction without extracting the presence of supercoiled circular form degraded (Fig. 3, line 2). At the same time, the addition in the reaction mixture of the extracts has some protective effect. It should be noted that there were no significant differences (Fig. 4) in the degree of protection of plasmid DNA from damage (the percentage of supercoiled DNA after Fenton reaction in the presence of the extracts) when extracts from different hairy root lines of *A. vulgaris* and *A. tilesii* were added to the reaction mixture. However, these extracts differed in the content of flavonoids and the level of ability to scavenge DPPH radicals. For instance, the percentage of SC DNA evaluated by electrophoresis in the control (without oxidative stress) was 78.30±5.42%. In the Fenton reaction without any protective agents, only 1.08±0.33% of SC DNA was found. In the responses with the extracts

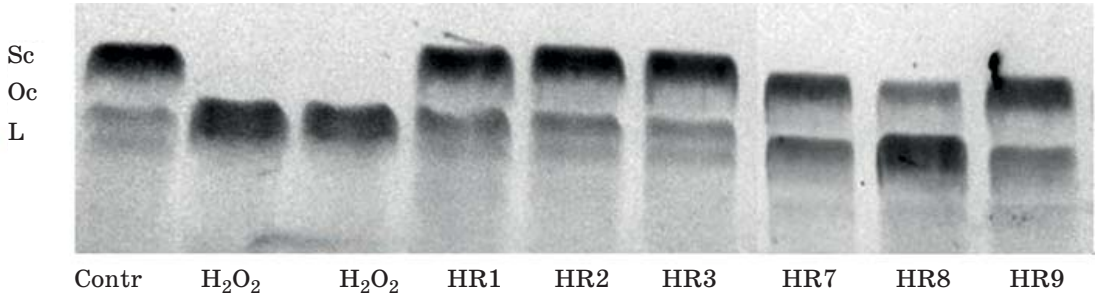


Fig. 3. **Electrophoregram of the results of Fenton reaction of p133 plasmid DNA treated with H_2O_2 in the presence and absence of the extracts:**
Contr — p133 plasmid DNA without treatment; plasmid DNA with H_2O_2; HR — the reaction with the presence of the hairy root extracts (HR1-HR3 — *A. vulgaris*, HR7-HR9 — *A. tilesii*)

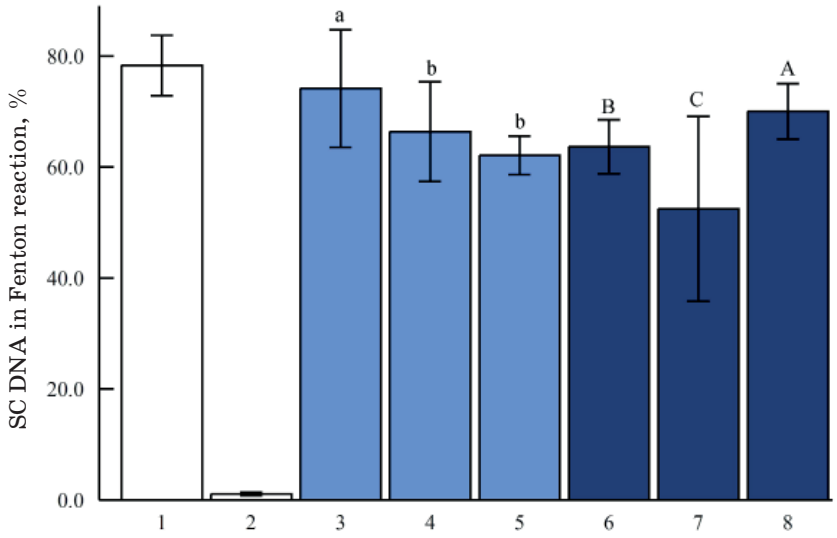


Fig. 4. **Percentage of supercoiled (SC) DNA in Fenton reaction without and with the extracts:**
1 — p133 plasmid DNA without treatment; 2 — plasmid DNA with H_2O_2 and without extract; 3–8 — plasmid DNA with H_2O_2 and with extract (3–5 — *A. vulgaris*, 6–8 — *A. tilesii*) ($P < 0.05$)
The same letters indicate no significant differences. Uppercase and lowercase letters refer to different comparisons

from *A. vulgaris* hairy roots, this parameter was 74.14±10.79%, 66.37±10.59%, and 62.08±3.47%. When the extracts from *A. tilesii* hairy roots were used for the study of the protective activity, the percentage of SC DNA was 63.63±4.84%, 52.45±16.66%, and 70.00±5.03%. Thus, it can be stated that extracts from the hairy roots had DNA protective activity. The best sample differed in the percentage of SC DNA from the control (native DNA) by only 5.3%.

The Fenton reaction is a chemical reaction that involves the generation of hydroxyl radicals (·OH) from hydrogen peroxide (H_2O_2) in the presence of ferrous ions (Fe^{2+}). These hydroxyl radicals are highly reactive and can cause damage to biomolecules such as DNA by inducing oxidative stress. In the context of evaluating the DNA protective activity of plant extracts, the Fenton reaction assesses the ability of the plant extract to protect DNA from oxidative damage. The extract with different bioactive compounds can protect DNA molecules from this damage. Such protective activity may be attributed to their high content of polyphenols demonstrated antioxidant properties. For example, Asadi with coauthors [40] studied *Berberis vulgaris* protective effect using pBR322 plasmid and lymphocyte genomic DNA cleavage induced by the Fenton reaction. The extract really inhibited Fenton reaction-induced DNA cleavage in a small concentration, but it possessed pro-oxidant properties in higher concentrations. Pilaisangsuree et al. [41] studied the dose-dependent effect of peanut hairy root extract: its low concentration exhibited DNA-protective activity; the pro-oxidant activity increased in a concentration-dependent manner.

To a certain extent, the protective effect of the extracts may be due to the presence of flavonoids in them. In particular, the possibility of increasing the percentage of intact supercoiled DNA in the Fenton reaction with the addition of gallic acid was shown previously [42]. The authors observed the same effect when using extracts from *Emblica officinalis, Spondias dulcis,* and *Terminalia chebula* plants. DNA protection assay of *Artemisia absinthium* flavonoid containing methanolic extracts showed a concentration-dependent protective effect in the Fenton reaction conducted using pUC19 plasmid DNA treated with 30 mM hydrogen peroxide [43].

In our studies, flavonoid-containing extracts, which showed the ability to scavenge the DPPH radical, also protected plasmid DNA from damage by the OH radical, which is formed in the Fenton reaction. The best sample (the extract from *A. vulgaris* hairy roots) differed in the percentage of SC DNA from the control (native DNA) by only 5.3%. However, significant differences in the degree of protection of extracts from different lines of transgenic roots were not detected.

This may be due to the various chemical composition of the extracts (for example, the ratio of other polyphenolic compounds) and, thus, their different activity in the Fenton reaction.

Conclusions

Therefore, a comparative study of the content of flavonoids in the transgenic roots of two species of wormwood, *A. vulgaris* and *A. tilesii*, showed an increase in the concentration of these compounds in the hairy roots of plants of all species compared to the corresponding roots of non-transformed plants. Extracts with a high content of flavonoids had a greater ability to scavenge the DPPH radical, and this effect was observed in the study of extracts from the hairy roots. It is obvious that this effect is related to the specifics of genetic transformation using *A. rhizogenes* and the transfer of bacterial *rol* genes to the plant genome. Despite differences in flavonoid content in identically prepared extracts from different lines of hairy roots, their ability to protect plasmid DNA from damage in the Fenton reaction did not differ significantly.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Shakhovsky A.M. — Fenton reaction assay, manuscript preparing; Duplij V.P. — statistical analyses, manuscript preparing; Afanasieva K.S. — Fenton reaction assay; Chopei M.I. — biochemical study; Matvieieva N.A. — idea of the study, manuscript preparing.

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ДНК-ЗАХИСНА АКТИВНІСТЬ ЕКСТРАКТІВ З БОРОДАТИХ КОРЕНІВ РІЗНИХ ВИДІВ *Artemisia*

Шаховський А.М.[1], *Дуплій В.П.*[1], *Афанасьєва К.С.*[2], *Чопей М.І.*[2], *Матвєєва Н.А.*[1]

[1]Інститут клітинної біології та генетичної інженерії НАН України, Київ
[2]Київський національний університет імені Тараса Шевченка, Україна

E-mail: duplijv@icbge.org.ua

Вступ. Рослини роду *Artemisia* відомі як продуценти біологічно активних сполук і використовуються як у народній, так і в традиційній медицині. Мають протипухлинну, антипроліферативну, протидіабетичну, антимікробну, противірусну, антиоксидантну та протизапальну дію.

Мета. Оскільки ці рослини виявляють антиоксидантну активність, важливим є дослідження можливості використання екстрактів полину для запобігання пошкодженню ДНК, ініційованого деякими активними формами кисню.

Методи. У роботі екстракти трансформованих коренів *A. vulgaris* і *A. tilesii* були використані для вивчення їхньої ДНК-протекторної активності. Екстракти готували за стандартною методикою. Загальний вміст флавоноїдів кількісно визначали модифікованим спектрофотометричним методом в еквіваленті рутина з використанням калібрувальної кривої. Антиоксидантну активність екстрактів визначали за допомогою 2,2-дифеніл-1-пікрилгідразил радикалу (DPPH). Його оцінювали за половинною максимальною ефективною концентрацією (EC_{50}), розрахованою як маса сухих коренів, необхідною для відновлення 50% DPPH у зразку та вираженою в мг сухої речовини (СР). Щоб обчислити це значення була застосована лінійна регресія. ДНК-захисну активність досліджували за допомогою реакції Фентона.

Результати. Виявлено відмінності у вмісті флавоноїдів у «бородатих» коренів *A. vulgaris* та коренях контрольних нетрансформованих рослин. У «бородатих» коренях цей показник коливався від 2,32 ± 75,89 до 5,37 ± 126,04 мг РЕ/г СР, що в 1,45–2,41 рази більше, ніж у контрольних коренях. Вміст флавоноїдів у лініях «бородатих» коренях *A. tilesii* також відрізнявся від контролю. Він становив 74,52 ± 0,96 ... 107,8 ± 5,98 мг РЕ/г СР у трансгенних коренях і 28,6 ± 2,11 мг/г СР у контрольних коренях *A. tilesii*. Рівень антиоксидантної активності, досліджений у реакції з DPPH (EC_{50}, ефективна концентрація), був вищим в екстрактах коренів волосистих обох видів рослин і варіював від 0,16–0,33 та від 0,17–0,31 мг у лініях «бородатих» коренів *A. vulgaris* і *A. tilesii* відповідно (для порівняння: цей параметр досягав 0,44 і 0,65 мг у контрольних коренях *A. vulgaris* і *A. tilesii*). Додавання екстрактів до реакційної суміші в реакції Фентона мало певний захисний ефект. Водночас не було суттєвих відмінностей у ступені захисту плазмідної ДНК від пошкодження (відсоток суперспіралізованої ДНК) при додаванні до реакційної суміші екстрактів з «бородатих» коренів *A. vulgaris* і *A. tilesii*, хоча ці екстракти відрізнялися за вмістом флавоноїдів і мали більш високу здатність відновлювати DPPH радикал.

Висновки. Екстракти з «бородатих» коренів *A. vulgaris* і *A. tilesii* містили більшу кількість флавоноїдів і мали вищу антиоксидантну активність порівняно з екстрактами з контрольних нетрансформованих коренів. Однак вони мало відрізнялися за здатністю захищати ДНК від пошкодження в реакції Фентона. Цілком ймовірно, що в цьому процесі беруть участь не тільки флавоноїди, а й інші компоненти екстрактів з коренів полину.

Ключові слова: антиоксидантна активність, флавоноїди, реакція Фентона, оксидативний стрес, *Artemisia vulgaris*, *Artemisia tilesii*, «бородаті» корені.