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Variability of flavonoid content, reducing and antioxidant activity in *Althaea officinalis* L. hairy roots

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Althaea officinalis L. is widely used as a medicinal plant due to its antiseptic, antioxidant, antimicrobial, anti-inflammatory, and gastroprotective properties. *A. officinalis* roots contain a great number of secondary metabolites including flavonoids which exert antioxidant and chelating abilities. Flavonoids possess protective effects against several chronic diseases, in particular neurodegeneration and cancer; they have also neuroprotective, hepatoprotective, anti-bacterial, anti-inflammatory, anti-viral, and anti-cancer effects. Tissue cultures of different plant species are a promising source of secondary metabolites with pharmacological activities, and hairy roots are one of the types. Hairy roots are known as fast-growing, genetically stable cultures, effective producers of both biomass and specialized plant metabolites including flavonoids. *A. officinalis* hairy roots and roots of *in vitro* cultured control (initial) plants were used in this research to study flavonoid content and some biochemical characteristics (antioxidant activity and ability to reduce iron ions Fe^{3+} to Fe^{2+}) of their ethanolic extracts. Two groups of hairy root lines were studied. Hairy roots of one group were obtained as the result of transformation with A4 wild *Agrobacterium rhizogenes* strain while the second group was initiated by transformation with *A. rhizogenes* strain carrying human interferon-α2b gene under the control of the sugarbeet root-specific Mll promoter. Among the two groups of hairy root lines no significant differences were detected that could suggest the role of additional genes in the antioxidant status of the hairy roots: in both groups, there were lines with low, medium and high values of the studied parameters. The total flavonoid content correlated with DPPH scavenging activity and reducing capacity. The results of study confirm flavonoid participation in antiradical reactions in *A. officinalis* hairy root cells.

Keywords: *Althaea officinalis*, hairy roots culture, bioactivity, rol genes, flavonoids

Introduction

Althaea officinalis L. is a perennial medicinal plant of the family Malvaceae Juss. It originates from the temperate regions of India (Ross, 2001), but is now widespread in temperate and subtropical regions of Europe, America, Asia, and North Africa. In Ukraine, it grows near lakes and rivers.

A. officinalis has been used for a long time as a medicinal plant because of its antiseptic, antioxidant, antimicrobial, anti-inflammatory, and gastroprotective properties (Xue et al., 2023). Marshmallow preparations (powder, aqueous infusion, liquid extract, syrup) are used as an expectorant for catarrhal conditions of the respiratory tract, as well as for diarrhea, acute gastritis

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and enterocolitis. It is also used for treating asthma and diseases of the upper respiratory tract. Marshmallow roots contain up to 35% of mucilagious substances, which determine the healing properties of the plant, as well as starch (up to 37%), sucrose (10.2%), betaine (up to 4%) and fatty oil (up to 1.7%), astragalin, mucopolysaccharides, arabinofuranan, caffeic acid, chicorin, coumarin and coumarin acid, diosmetin, kaempferol, luteolin, quercetin, and scopolin (Xue et al., 2022). Polysaccharides are well-known bioactive compounds naturally synthesized in these plants (Karimi et al., 2021). Flavonoid content correlated with this parameter of antioxidant activity of the plants was studied (Sadighara et al., 2012).

Great attention is paid now to the study of bioactivity of such plant-derived chemicals as flavonoids. Their chemical structure, classification and therapeutic properties are deeply analyzed in some publications (Heim et al., 2002; Kumar and Pandy, 2013; Ahn-Jarvis et al., 2019; Atala et al., 2017; Hussain et al., 2020).

Flavonoids are plant secondary metabolites, polyphenol compounds of low molecular weight that have a three-ring structure in the C6–C3–C6 form. Over 4,000 flavonoids have been identified by now (Heim et al., 2002). They originate from various plant sources (fruits, vegetables, wines, teas and cocoa) and can be sub-classified into six different types (flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones, and isoflavones) (Heim et al., 2002; Shahidi and Yeo, 2018; Hussain et al., 2020).

The mechanisms of flavonoid action are diverse and multiple. Flavonoids possess health-promoting properties either directly or indirectly. Most of their health effects are attributed to the flavonoid antioxidant and chelating abilities (Heim et al., 2002). The bioavailability, metabolism, and biological activity of flavonoids depend on the specific chemical structure, total number of hydroxyl groups, and substitution of functional groups about their nuclear structure (Heim et al., 2002; Kumar and Pandy, 2013). Flavonoids possess protective effects against several chronic diseases, including neurodegeneration and cancer; neuroprotective and hepatoprotective activities; anti-bacterial, anti-inflammation and anti-virus effects as well as reducing cardiovascular diseases and type-2 diabetes (Kumar and Pandy, 2013; Rodriguez-Mateos et al., 2014; Costa et al., 2016; Shahidi and Yeo, 2018; Hussain et al., 2020).

Plant hairy roots can be a promising source of flavonoids. They are known as fast-growing, genetically stable cultures, and effective producers of both biomass and specialized plant metabolites. Detailed analysis of the published data concerning the use of hairy root cultures as a source of polyphenolic antioxidants has been summarized by Malarz et al. (2022). The number of plant species of different families studied for this purpose include *Lotus corniculatus* L., *Fagopyrum tataricum* (L.) Gaertn., *Leontopodium alpinum* Cass, *Ipomea batatas* (L.) Lam., *Antirrhinum majus* L., *Medicago truncatula* Gaertn., *Vitis vinifera* L., *Camellia sinensis* (L.) Kuntze, *Panax ginseng* C.A. Meyer, *Isatis tinctoria* L., *Scutellaria baicalensis* Georgi, *Psoralea corylifolia* L., *Raphanus sativus* L., *Cichorium intybus* L. and a number of others (Park et al., 2016; Balasubramanian et al., 2018; Malarz et al., 2022, Matvieieva et al., 2023). Different types of elicitation were studied to increase flavonoid production in hairy root cultures (Park et al., 2016).

In this work, we compared the content of flavonoids and bioactivity in extracts from *A. officinalis* hairy roots obtained as the result of genetic transformation with different strains of *Agrobacterium rhizogenes* (*Rhizobium rhizogenes*) (NCBI Taxonomy Database, 2023). They carried only transferred *A. rhizogenes* genes (obtained by transformation with *Agrobacterium rhizogenes* A4 wild strain) and those that had an additional human interferon-α2b gene (transformed with *A. rhizogenes* carrying human interferon-α2b target gene under the control of the sugar beet root-specific Mll promoter, pCB161).

Material and methodology

Plant material

Althaea officinalis hairy roots and the control plants from the collection of the Laboratory of Adaptional Biotechnology of the Institute of Cell Biology and Genetic Engineering of the National Academy of Sciences of Ukraine were used in the work (Figure 1). Hairy roots were obtained earlier after cocultivation of *A. officinalis* leaves cocultivation with suspension of *Agrobacterium rhizogenes* A4 according to the method described in the article (Matvieieva et al., 2013). Plants and roots were cultivated in Petri dishes on 1/2 Murashige and Skoog (Duchefa, Netherland) solidified medium at 24 °C. Two groups of hairy roots were studied. The roots from the first one were obtained by transformation with *Agrobacterium rhizogenes* A4 wild strain and carried only transferred *A. rhizogenes* genes. The roots of the second group were transformed with *A. rhizogenes* carrying the human interferon-α2b target gene under the control

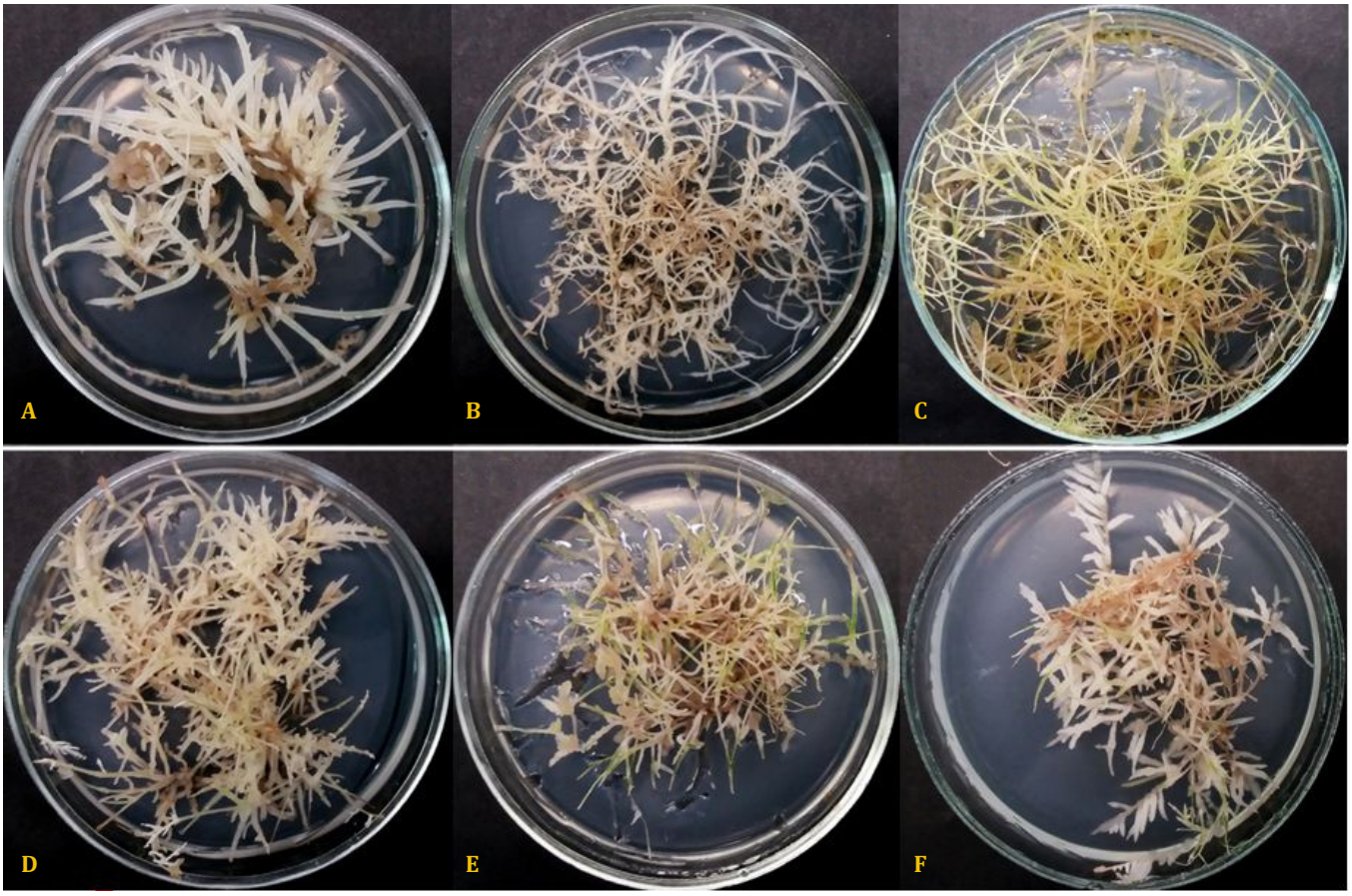

Figure 1 *Althaea officinalis* L. hairy root lines from the collection of the Laboratory of Adaptation Biotechnology in the Institute of Cell Biology and Genetic Engineering National Academy of Sciences of Ukraine
A – line 1; B – line 2; C – line 3; D – line 4; E – line 5; F – line 6; lines 1–3 were obtained as a result of transformation with *Agrobacterium rhizogenes* A4 wild strain; lines 4–6 – with *A. rhizogenes* strain carrying human interferon-α2b target gene under the control of the sugar beet root-specific Mll promoter (pCB161)

of the sugar beet root-specific Mll promoter, pCB161 and had an additional human interferon-α2b gene.

Total flavonoid content assay

The content of flavonoids was determined spectrophotometrically (Pekal and Pyrzynska, 2014). To prepare the extracts, the roots were separated from the medium, washed with distilled water, dried with filter paper, weighed (0.3 g) and homogenized in 3 ml of 70% ethanol. The resulting homogenate was transferred to test tubes and centrifuged in an EppendorfCentrifuge 5415 C microcentrifuge for 10 min. The reaction mixture in the cuvette contained 0.25 ml of extract supernatant, 1 ml of deionized water, 0.075 ml of 5% $NaNO_2$ solution. After 5 minutes, 0.075 ml of 10% $AlCl_3$ solution was added, then 0.5 ml of 1 M NaOH and 0.6 ml of deionised water were added. Absorption was determined at λ = 510 nm. The content of flavonoids was calculated using the formula $y = 0.8842x$ ($R^2 = 0.9988$).

DPPH scavenging activity assay

For this study, the root samples were homogenized in 70% ethanol, and centrifuged, and the supernatants were used for the analysis. The activity of the hairy root extracts was studied spectrophotometrically on a Fluorate-02-Panorama spectrofluorimeter using the DPPH test (Brand-Williams et al., 1995).

The reaction was carried out in cuvettes with the addition of the extract (0.62, 0.12, 0.25 and 0.5 ml) to the DPPH solution. The cuvettes were kept for 20 minutes in the dark. The optical density of the mixtures was determined at a wavelength of λ = 550 nm. The level of the activity (%) was calculated according to the following formula:

$$AOA = \left[(OD_1 - OD_2)/OD1\right] \times 100\%$$

where: OD_1 was the optical density of the control sample; OD_2 – optical density of the reaction mixture (extract with DPPH)

The ability to radical scavenging was determined by the effective concentration parameter EC_{50}. The effective concentration was calculated as the extract concentration (root wet weight) required to remove 50% DPPH in the sample, expressed as mg FW in rutin equivalent.

Reducing power assay

A study of the ability of root extracts to reduce iron ions Fe^{3+} to Fe^{2+} was carried out spectrophotometrically on a Fluorate-02-Panorama spectrofluorimeter according to the method described in the article (Zhao et al., 2008) with some modifications. The reaction mixture contained 0.312 ml of 0.2 M phosphate buffer (pH 6.6); 0.312 ml of 1% potassium hexacyanoferrate (III) and the root extract. The cuvettes were incubated in a water bath at 50 °C for 30 min. After that, 0.312 ml of 10% trichloroacetic acid, 1.25 ml of deionized water and 0.25 ml of 0.1% iron (III) chloride were added to the reaction mixture. The comparison solution was prepared using the same method, but instead of the extract, 0.25 ml of deionized water was added. The optical density was measured at a wavelength of λ = 700 nm. The activity was evaluated by the effective concentration parameter ($EC_{0.5}$), which corresponds to the amount of wet root mass (mg FW) required to obtain OD = 0.5.

Statistical analysis

All analyses were carried out in triplicate. Three replications of all analyses were carried, and results were processed by Statistical Analysis Software (SAS) (2004) Version 9.2. SAS Institute Inc., Cary. The data were analyzed for statistical significance using Student's *t*-test and Tukey's method. *P* values less than 0.05 were considered significant. The linear regression method was applied and the coefficient of determination (R^2) was calculated for establishing the relationship between the values.

Results and discussion

Some differences were detected in the total flavonoid content in hairy root lines. It is worth noting that variations in this parameter were observed among the lines obtained by the same method (using a wild strain of bacteria or bacteria with an additional plasmid, Figure 2). In particular, the roots of one line obtained by transformation using the wild strain A4 (column 1, Figure 2) contained significantly fewer flavonoids compared to the roots of two other lines obtained with the same method (columns 2 and 3, Figure 2) – 0.16 ±0.04 mg RE.g^{-1} FW, 1.75 ±0.37 mg RE.g^{-1} FW, and 2.29 ±0.21 mg RE.g^{-1} FW, respectively. Differences were also found among the root lines of the second group, but they were not significant. These roots contained flavonoids in concentrations of 1.29 ±0.19 mg RE.g^{-1} FW, 1.56 ±0.02 mg RE.g^{-1} FW, and 1.90 ±0.10 mg RE.g^{-1} FW (columns 4–6, Figure 2). Thus, the variability of flavonoid content in the roots of different lines was revealed. This, perhaps, can be explained by the peculiarities of the plant's transformation by

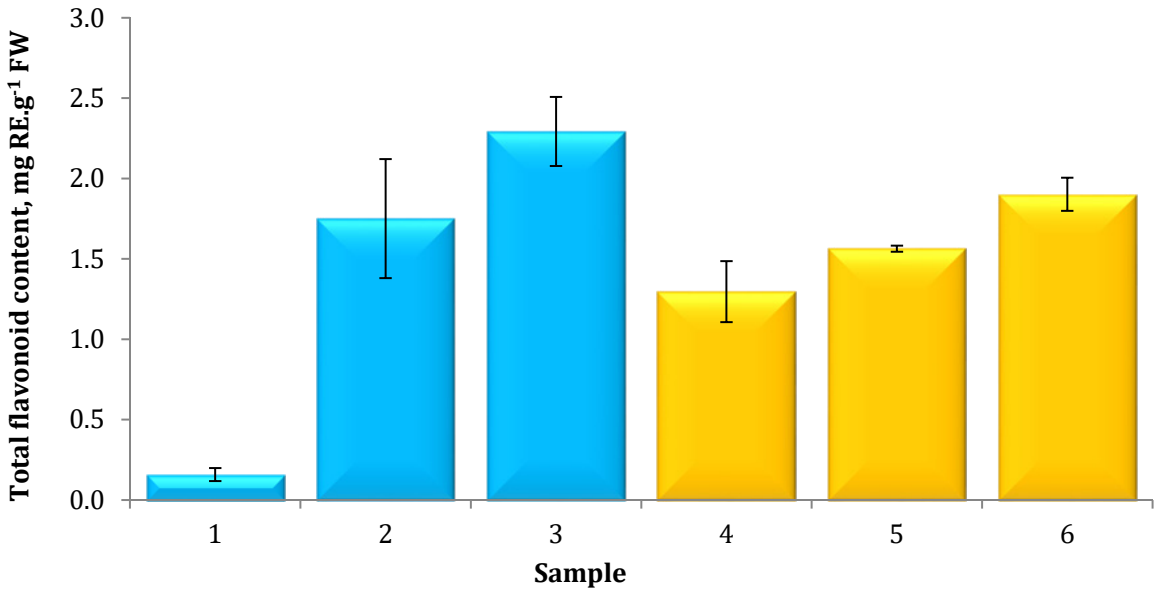

Figure 2 The total content of flavonoids in *Althaea officinalis* L. hairy root lines
lines 1–3 were transformed with *Agrobacterium rhizogenes* A4 wild strain; lines 4–6 were transformed with *A. rhizogenes* strain carrying human interferon-α2b target gene

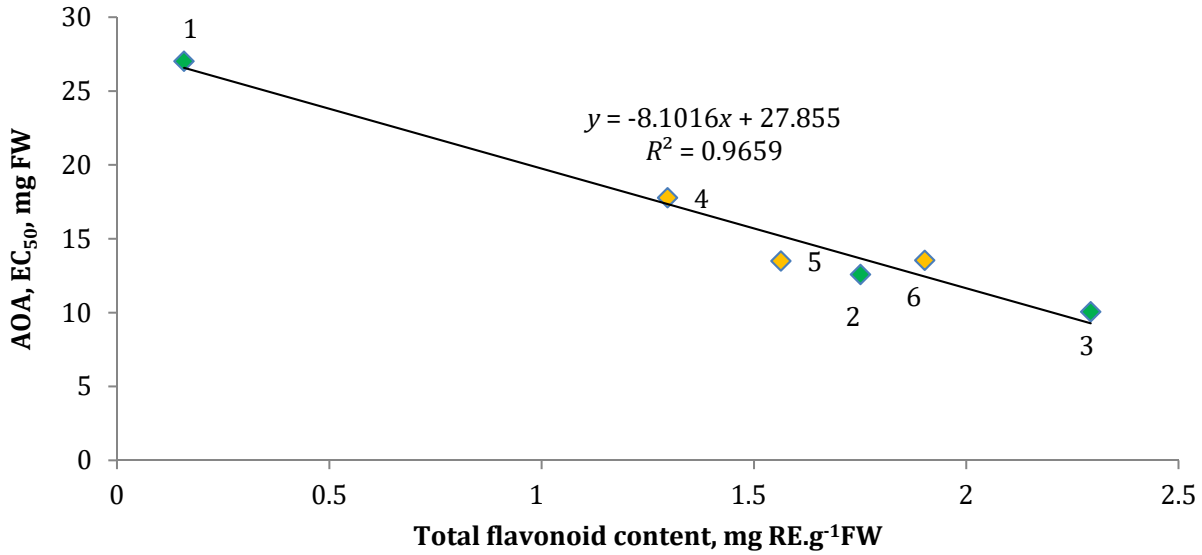

Figure 3 Correlation between the total flavonoid content and DPPH scavenging activity (AOA) in *A. officinalis* hairy root lines
lines 1–3 are the result of transformation with *Agrobacterium rhizogenes* A4 wild strain; lines 4–6 – with *A. rhizogenes* strain carrying human interferon-α2b target gene

A. rhizogenes and the transfer of bacterial rol genes to the plant genome.

DPPH scavenging activity of hairy root extracts initiated via transformation by *A. rhizogenes* wild strain varied from EC_{50} = 27.02 mg (the lowest activity in root line no 1) to the highest activity in root line No3 (EC_{50} = 10.06 mg). The activities of the hairy root extracts obtained using *A. rhizogenes* with an additional *ifn*-α2b gene also differed from each other. Effective concentrations in these samples Nos 4, 5, and 6 were 17.79 mg, 13.51 and 13.55, respectively. A correlation was observed between the total content of flavonoids and the level of DPPH scavenging activity, which is shown in Figure 3. Such a correlation can be explained by the fact that flavonoids are powerful antioxidants and can participate in radical reduction in the test reaction. The antioxidant activity of flavonoids has been studied in some publications. Mechanisms of the reactions with ROS (reactive oxygen species) as well as the activity of flavonoid's metabolites were detected (Hotta et al., 2002; Galleano et al., 2010; Dueñas et al., 2010; Amić et al., 2014; Alov et al., 2015; Atala et al., 2017).

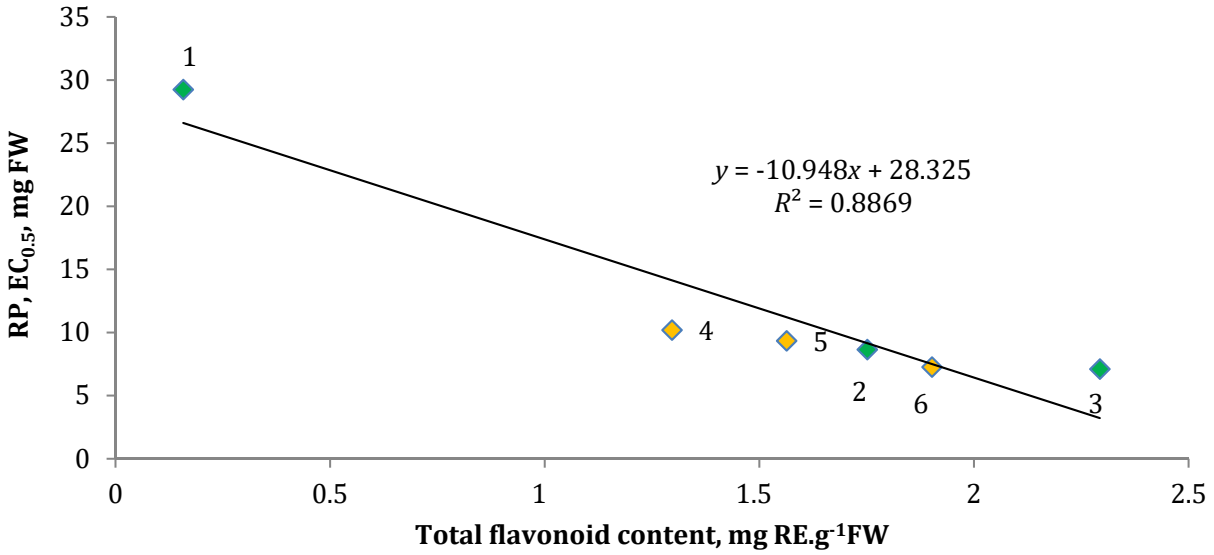

Figure 4 Correlation between the total flavonoid content and reducing power (RP) in *A. officinalis* hairy root lines
lines 1–3 are the result of transformation with *Agrobacterium rhizogenes* A4 wild strain; lines 4–6 – with *A. rhizogenes* strain carrying human interferon-α2b target gene

The revealed antiradical activity of the studied extracts is of considerable practical interest. ROS that can be formed in the human body pose a threat to human health. Oxidative stress is considered a basis for the initiation of many diseases including inflammation-related diseases, neurodegeneration, and cancer. It can be initiated when there is an imbalance between ROS and the activity of the antioxidant defense system of cells. In this case, the presence of a sufficient amount of flavonoids can prevent the negative effect of ROS. So, flavonoids are considered now as dietary supplements with a wide range of bioactivity (Ross and Kasum, 2002; Ahn-Jarvis et al., 2019; Rana et al., 2022).

The reducing power of the extracts varied from $EC_{0.5}$ = 7.10 mg (the highest activity, No 3) to $EC_{0.5}$ = 29.24 mg (the lowest activity, No 1). It also correlated with total flavonoid content (Figure 4). Since a correlation was found between the total content of flavonoids and the reducing activity of extracts of *A. officinalis* hairy roots it can be assumed that this activity is largely due to the presence of flavonoids in the plants of this species.

The effect of the genetic transformation on flavonoid synthesis and bioactivity of different plants was studied earlier. Transformation-induced changes in total phenol contents in transgenic tobacco plants (Seong et al., 2012). Antioxidant activity increased in transgenic *Perilla frutescens* plants which overexpressed the γ-tocopherol methyltransferase gene (Ghimire et al., 2015). Increased antioxidant levels were detected in pRi-transformed *Rehmannia glutinosa* (Piątczak et al., 2016). Variations in flavonoid content and antioxidant activity of *Artemisia vulgaris* hairy roots were studied by us earlier (Matvieieva et al., 2019). Wang et al. (2006) studied a great increase of phenolic compounds in transformed with *Agrobacterium rhizogenes Echinacea purpurea*.

Tavassoli and Safipour Afshar (2018) compared the effect of different *A. rhizogenes* strains (A4, A13, ATCC15834, and ATCC15834$_{(GUS)}$) on the total content of flavonoids and phenols in marshmallow hairy roots in the hormone-free liquid medium after 50 days of cultivation. The highest total phenolic compounds were detected in the roots transformed by A13 strain. At the same time, the highest flavonoid content was studied in hairy roots transformed by A4 strain. The authors concluded that the secondary metabolite of the studied plants depended on the bacterial strain used for transformation.

Conclusions

Studies of *Althaea officinalis* hairy roots have shown that root lines differ both in flavonoid content and in the level of antiradical and reducing activity. This may be due to the specificity of the transformation by *Agrobacterium rhizogenes* when the foreign genes are incorporate in different loci which can lead to the differences in the functions of plant cells. In *A. officinalis* hairy root lines obtained via genetic transformation with *A. rhizogenes* A4 wild type or with *A. rhizogenes* with the additional gene of interest, some variations were found in all studied parameters. This fact suggests the impact of bacterial rol genes presented in all hairy root lines on the antioxidant status of the hairy roots: in both groups regardless of the presence/absence of the interferon-α2b gene there were lines with low, medium and high values of studied parameters. According to the results of the experiments, the total content of flavonoids correlated with DPPH absorption activity and reducing capacity, which confirms the participation of flavonoids in antiradical reactions in hairy root cells.

Conflicts of interest

The authors declare no conflict of interest.

Ethical statement

This article doesn't contain any studies that would require an ethical statement.

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