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UV-PROTECTIVE PROPERTIES OF EXTRACTS OBTAINED FROM TRANSFORMED AND NON-TRANSFORMED ROOTS OF *ARTEMISIA* GENUS PLANTS

Background. ***Medicinal plant extracts are widely used in traditional and non-conventional medicine. Special interest is given to extracts obtained from transgenic so-called "hairy" roots. Usually, such roots are characterized by a high content of biologically active components. However, such plant transformation can lead to the appearance of both undesirable effects of the obtained extracts (e.g., a genotoxic effect) and positive effects (e.g., antioxidant and UV-protective properties). In this work the content of flavonoids in extracts of roots of three species of the Artemisia genus, as well as their potential genotoxic and UV-protective properties, were studied.***

Methods. ***Transgenic roots of Artemisia annua, A. vulgaris and A. tilesii with the inserted roll gene were obtained using Agrobacterium rhizogenes A4 mediated transformation. Water extracts were prepared according to a standard procedure. Total flavonoid amount was estimated spectrophotometrically. Comet assay was used as an approach to evaluate extracts genotoxicity and UV-protective properties.***

Results. ***It was shown that the content of flavonoids in extracts obtained from the transgenic plants is more than 2 times higher than that for control plants. Extracts obtained from A. vulgaris and A. annua had no genotoxic effect, while extracts obtained from A. tilesii had a weak but statistically significant mutagenic effect. A. vulgaris extracts had pronounced UV-protective properties, which were correlated with the content of flavonoids in these extracts.***

Conclusions. ***Extracts of plants studied mostly did not show a genotoxic effect, but had UV-protective properties. The ability of extracts to reduce the negative effects of UV-irradiation depends on the concentration of flavonoids: the increased content of these compounds in transgenic plants led to almost 3-fold decrease in the relative amount of DNA in the comet tails.***

Keywords: ***"hairy" root extracts, Artemisia spp., flavonoids, UV-protective properties, genotoxicity, comet assay.***

Background

Extracts of medicinal plants are widely used as biologically active additives, components of drugs and cosmetics. The extracts contain biologically active compounds that belong to different classes: polyphenols, essential oils, carbohydrates, etc. These compounds show a wide spectrum of biological activity, including antioxidant and UV-protective properties that can protect DNA from damage (Jomová et al., 2019; Kim et al., 2018). It can be assumed that plants that will be used as a material to obtain biologically active compounds potentially have protective properties. Representatives of the *Artemisia* genus, which have long been used both in folk and evidence-based medicine can be used for this purpose (Abiri et al., 2018). Recently, extracts obtained from the roots of these plants grown *in vitro*, especially from transgenic so-called "hairy" roots, in the genome of which some bacterial genes are inserted, have gained great popularity (Bora, & Sharma, 2011). Such genetic modifications can lead to changes in the plant biochemical processes and this, on the one hand, can cause an increase in the content of useful compounds in the extracts obtained from these plants, and on the other, cause an accumulation of harmful substances or their complex activity (Bisht et al., 2021; Bordean et al., 2023; Lang et al., 2019). That is why it is necessary not only to study the protective properties of extracts in relation to physical and chemical factors (pollutants), but also their genotoxicity.

A large number of different approaches have been developed to study DNA damages, one of which is single-cell gel electrophoresis (the comet assay). This method has proved itself as a convenient approach for the assessment of single- and double-strand DNA breaks at the level of individual cells; its simplicity, availability, and sensitivity made this technique indispensable in genotoxicological studies for testing the mutagenic/antimutagenic activity of various compounds (Cordelli, Bignami, & Pacchierotti, 2021; Prylutska et al., 2017). The principle of the method is that cells are immobilized in a layer of a low-melting point agarose on the surface of a glass slide and then are lysed at high-salt conditions. Obtained lysed cells (nucleoids) are subjected to electrophoresis. The migration of broken DNA fragments to the anode is observed, and an electrophoretic track called the comet tail is formed (Møller, 2018; Olive, & Banáth, 2006). The efficiency of DNA migration from nucleoids during electrophoresis reflects the relative number of DNA breaks.

In this work, we were focused on study of the content of flavonoids in extracts obtained from *A. annua, A. vulgaris,* and *A. tilesii "*hairy" roots and roots of the non-transformed plants, as well as of the genotoxicity and potential UV-protective properties of the extracts obtained from the roots.

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Methods

Plant material and extracts preparation. "Hairy" roots of *A. annua, A. vulgaris,* and *A. tilesii* were obtained using *Agrobacterium rhizogenes* A4 mediated transformation by the method described in (Matvieieva et. al., 2019). "Hairy" root cultures from the collection of the Laboratory of Adaptational Biotechnology of the Institute of Cell Biology and Genetic Engineering of NAS of Ukraine were grown under sterile conditions on Petri dishes using a Murashige and Skoog solid nutrient medium (Duchefa, Netherlands) with twice reduced concentration (½MS). The roots were lyophilized and ground to a uniform degree of grinding (Retsch MM400, Germany); 6 ml of deionized water was added to 50 mg of each sample to do extraction for 3 days at +28 °C and 180 rpm (Clim-J-Shake System Kuhner IRC-1-U). After extraction, the samples were centrifuged for 10 min (Eppendorf Centrifuge 5415C) at 10 000 rpm, and the supernatant was collected. Some of the water extracts after preparation were heated in a water bath at 80 °C for 1 h for protein denaturation**.**

Flavonoid content assay. Total flavonoid content was quantified by a modified spectrophotometric method (Pekal, & Pyrzynska, 2014). 0.25 ml sample of each extract was mixed with 1 ml of double-distilled water and 0.075 ml of 5 % $NaNO_2$ and allowed to react for 5 min at room temperature. After that, 0.075 ml of 10 % $AlCl_3$ was added. After 5 min of incubation, 0.5 ml of 1 M NaOH and 0.6 ml of deionized water were added to the reaction mixture, and the absorbance of the sample was measured at 510 nm. The total flavonoid content was expressed as milligrams per gram of dry root weight in rutin equivalent (mg RE/g DW). The concentration of the flavonoid compounds was calculated by using the equation obtained in the range from 50 to 500 µg/ml with calibration curve: $y = 1.0358x - 0.03$ ($R^2 = 0.9532$).

Comet assay. Human peripheral blood lymphocytes were isolated according to a standard protocol (Afanasieva et al., 2017). A 1 : 1 mixture of blood and Hanks' solution was applied to the Histopaque density gradient (Sigma, USA) and centrifuged for 40 min at 1500 rpm. Isolated lymphocytes were collected, washed with Hanks' solution, and immediately used for further manipulations. Cell treatment with extracts was carried out according to the following algorithm: an aliquot of cells with added 100 µl or 200 µl of an extract and RPMI 1640 culture medium with antibiotics (Gibco, USA) to a final volume of 1.5 ml was incubated for 2 h at 37 °C. Lymphocytes incubated in the culture medium without the extracts were used as a control.

After the incubation, samples were irradiated with ultraviolet C at 40 J/m^2 to induce the formation of thymine dimers and then the cells were again incubated at 37 °C for 15 min to induce breaks in the sites of the formed thymine dimers. Aliquots of suspensions of irradiated or non-irradiated cells were mixed with 1 % low-melting point agarose (Sigma, USA) in a ratio of 1:2 and applied to a glass slide. After agarose polymerization, cell lysis was performed in the following buffer: 2.5 M NaCl, 0.01 M Tris-HCl,0.1 M Na_2EDTA, and 1 % Triton X-100, pH 7.5 for 2 h at 4 °C. After lysis, the cells were electrophoresed for 20 min under neutral conditions in TBE buffer (89 mM Tris-HCl, 89 mM H_3BO_3, 2 mM Na_2EDTA, pH 7.5) at 4 °C and constant electric field V/cm.

After electrophoresis, the slides were stained with the fluorescent dye DAPI (4',6-diamidino-2-phenylindole, Sigma, USA) and analyzed with a fluorescent microscope. 100–150 cells were photographed with a Canon EOS 1000 D camera connected to a microscope and analyzed using CometScore software (TriTek, USA). We evaluated the parameter that reflects the relative level of single- and double-strand DNA breaks, the percent of DNA in the comet tail. This parameter is calculated as the ratio of the fluorescence intensity of the tail of the comet (electrophoretic track) to the entire comet (cells after electrophoresis).

Statistical analysis. All experiments were performed in triplicate. All results are presented as the mean ± standard deviation. For the experimental data the Shapiro-Wilk normality test was performed. In case of the normal distribution of data, the reliability of the difference between them was assessed by the Student's test. Differences at the level of $p < 0.05$ were considered statistically significant.

Results

The first step of this work was to determine the concentration of flavonoids in extracts obtained from the roots of non-transformed (control) plants and "hairy" roots (two clones, C1 and C2, were used, which were obtained in independent transformations) of three species of the *Artemisia* genus (Fig. 1). The highest content was detected in the control roots of *A. annua* (43.34 ± 2.59 mg RE/g DW), the lowest one – in *A. tilesii* (25.18 ± 2.87 mg RE/g DW). At the same time, regardless of the species, for the extracts obtained from the transformed roots a significant increase in the content of flavonoids (about 2 times) was observed, and for *A. tilesia*, this value exceeded the control by as much as 3 times. The highest concentration of flavonoids was found in *A. annua* C1 "hairy" root line (91.18 ± 9.18 mg RE/g DW).

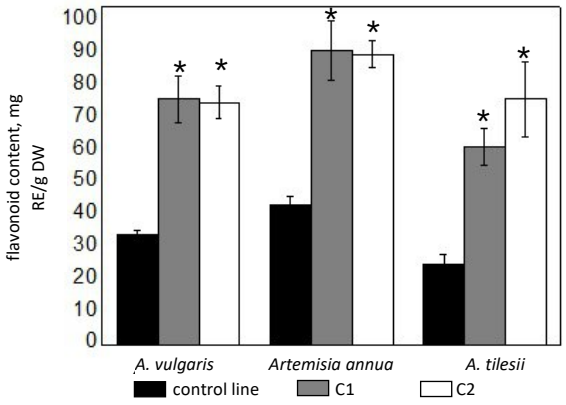

Fig. 1. Flavonoid content in extracts of *A. vulgaris*, *A. annua* and *A. tilesii* control roots (control line) and "hairy" transgenic roots of clones C1 and C2
(* $p < 0.05$ – in comparison with control (non-transformed) lines)

Given the fact that the "hairy" roots carry the bacterial *roll* gene, which is known as an inducer of plants secondary metabolism, the question arises whether, along with flavonoids, the level of other secondary metabolites, including those that may have a mutagenic effect, also increases in the transgenic roots. Therefore, at the next stage of the study, the mutagenic activity of the obtained extracts was assessed using the comet assay. For this, lymphocytes obtained from healthy donors were incubated with extracts of the control (control line) and transgenic plants (clones C1 and C2), as described in the Materials and methods. In order to detect whether protein molecules contribute to a potential mutagenic effect of extracts, lymphocytes were also incubated with extracts preheated to 80 °C. Lymphocytes incubated in a culture medium without extracts served as a control.

The comets appearance was similar for control and treated cells. The relative amount of DNA in the comet tails of control cells (without prior incubation with extracts) was 7.55 ± 0.73 %, which is within the generally accepted norms for the level of DNA damage in lymphocytes (Fig. 2).

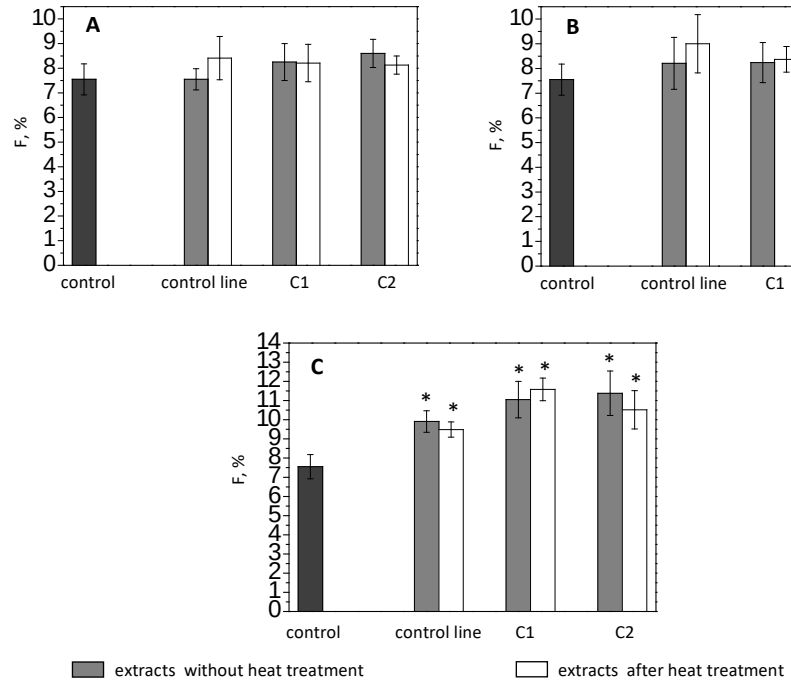

Fig. 2. Effect of root extracts obtained from the control and transgenic (clones C1 and C2) plants *A. vulgaris* (A), *A. annua* (B) and *A. tilesii* (C) on the relative amount of DNA in the comet tails. The relative amount of DNA in the comet tails of untreated lymphocytes is presented as a control
(* $p < 0.05$ – in comparison with control (untreated lymphocytes))

Treatment of cells with extracts of *A. vulgaris* and *A. annua* "hairy" roots did not affect the percent of DNA in comet tails in comparison to the control experiment both for the control and transgenic plant lines (Fig. 2A, B). In contrast, the treatment of lymphocytes with *A. tilesii* root extracts caused a significant increase in the percent of DNA in the comet tails up to ~12 % (Fig. 2C). Thus, one may conclude that the extracts of *A. tilesii* roots have a slight but certain mutagenic effect.

It should be noticed that in all our experiments we didn`t observe any significant differences in DNA fraction in the comet tails (which reflects the relative level of single- and double-strand DNA breaks) between lymphocytes that were incubated with non-preheated and preheated extracts. This indicates that the presence of protein components in the obtained extracts does not affect their genotoxic properties.

It has been shown in previous works on the potential antioxidant properties of extracts obtained from the "hairy" roots of *Artemisia* plants (Matvieieva et. al., 2020) that the ability of flavonoid-containing extracts to scavenge the DPPH radical depends on the content of flavonoids. Therefore, at the next stage, we focused on the analysis of the potential UV-protective properties of the obtained extracts. For this, lymphocytes were incubated for a certain time in a medium with extracts, after which they were exposed to UV-irradiation in order to induce thymine dimers, the main type of DNA damage caused by UV. Irradiated cells were transferred to extract-free medium and incubated some time for thymine dimers to be excised at the first stage of DNA repair, thereby creating DNA breaks. Accordingly, the percentage of DNA that will migrate into the comet tail will reflect the relative number of such breaks.

Extracts of *A. vulgaris* roots were selected to investigate their potential UV protective properties. When cells were irradiated without prior incubation with extracts, the relative amount of DNA in the comet tails was 14.1 ± 1.1 % (Fig. 3), which is significantly higher than that in control cells that were not irradiated. This indicates the presence of a sufficiently large number of breaks that appeared at the sites of thymine dimers after UV exposure. Treatment of cells with non-preheated and preheated extracts before irradiation has led to a significant decrease in the relative amount of DNA content in the comet tails (Fig. 3). This effect was maximally expressed for extracts obtained from the transgenic plants. In particular, clone C1 showed an almost threefold decrease in the percentage of DNA in the comet tails compared to irradiated cells non-treated with the extracts. It should be noted that the flavonoid content was approximately two times higher in the transgenic clones than in the control (non-transformed) plants. Perhaps this explains the more pronounced UV protective effect of these extracts. As with the assessment of the genotoxicity of the extracts studied, their UV-protective properties do not depend on the presence of protein molecules: we did not observe a difference in the values of the fraction of DNA in the comet tails for preheated and non-preheated extracts.

Discussion and conclusions

It was established that transgenic roots of *Artemisia* are characterized by a statistically significant increased content of flavonoids compared to the roots of non-transformed plants. Such increase of flavonoids is most likely related to the fact that "hairy" roots carry an inserted *roll* gene, that can activate the synthesis of plant secondary metabolites. Analysis of the genotoxicity of the obtained extracts indicates that they do not induce DNA breaks (with the exception of the extracts obtained from *A. tilesii*). It should be noted that the content of flavonoids in both control and transformed *A. tilesii* plants was the lowest. At the same time, a significant UV-protective effect was shown, which

was more pronounced the higher the flavonoid content. The results obtained for extracts that were preheated to 80 °C coincide with those obtained for non-preheated extracts, which means that UV-protective properties are provided by non-protein components.

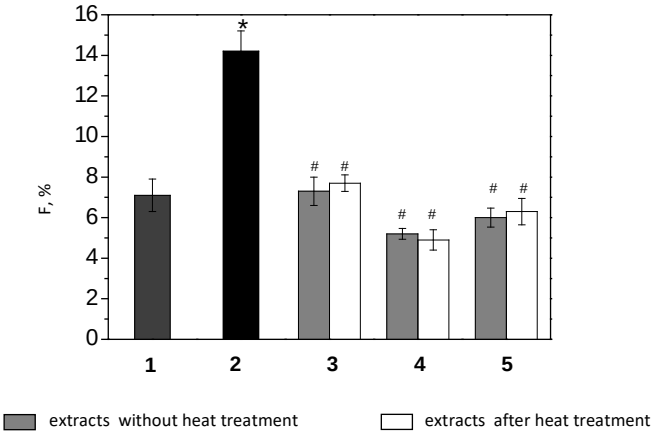

Fig. 3. UV-protective effect of *A. vulgaris* "hairy" root extracts: the relative DNA amounts in the comet tails for control cells (1), UV irradiated cells (2), irradiated cells which were pre-incubated with extracts of the non-transformed plants (3), the transgenic plants of the clone C1 (4), and C2 (5)
(* $p < 0.05$ – in comparison with control cells; # $p < 0.05$ – in comparison with UV irradiated cells)

Authors' contribution: Mariana Chopei and Volodymyr Duplij – performed statistical analysis and literature review; Nadija Matvieieva and Katerina Afanasieva – designed the study. All authors performed the experiments and wrote the paper, discussed the results, commented and approved on the manuscript.

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УФ-ПРОТЕКТОРНІ ВЛАСТИВОСТІ ЕКСТРАКТІВ, ОТРИМАНИХ ІЗ ТРАНСФОРМОВАНИХ І НЕТРАНСФОРМОВАНИХ КОРЕНІВ РОСЛИН РОДУ *ARTEMISIA*

В с т у п . ***Екстракти лікарських рослин широко використовуються у традиційній та нетрадиційній медицині. Особливий інтерес приділяють екстрактам, отриманим із трансгенних, "бородатих", коренів. Зазвичай такі корені характеризуються підвищеним вмістом біологічно активних компонентів. Проте така трансформація рослин може призводити до появи як небажаних ефектів отриманих екстрактів (напр., їхній генотоксичний ефект), так і мати позитивний ефект (напр., антиоксидантні та УФ-протекторні властивості). У цій роботі було досліджено вміст флавоноїдів у екстрактах "бородатих" коренів трьох видів трансформованих і нетрансформованих рослин роду Artemisia, а також їхні потенційні генотоксичні та УФ-протекторні властивості.***

М е т о д и . ***Трансгенні корені Artemisia annua, A. vulgaris і A. tilesii із вбудованим геном rol були отримані за допомогою Agrobacterium rhizogenes A4-опосередкованої трансформації. Водні екстракти готували за стандартною методикою. Загальну кількість флавоноїдів оцінювали спектрофотометрично. Кометний електрофорез використовувався як підхід для оцінювання генотоксичності екстрактів та їхніх УФ-протекторних властивостей.***

Р е з у л ь т а т и . ***Показано, що вміст флавоноїдів в екстрактах, отриманих із трансгенних рослин, більш ніж у 2 рази перевищує їх вміст у контрольних рослин. Екстракти, отримані з A. vulgaris та A. annua, не мали генотоксичної дії, у той час як екстракти, отримані з A. tilesii, мали слабку, але статистично значущу мутагенну дію. Екстракти A. vulgaris мали виражені УФ-протекторні властивості, які були скорельовані з вмістом флавоноїдів у цих екстрактах.***

В и с н о в к и . ***Екстракти дослідних рослин здебільшого не виявляли генотоксичної дії, але мали УФ-протекторні властивості. Здатність екстрактів знижувати негативний вплив УФ-опромінення залежить від концентрації флавоноїдів: підвищення вмісту цих сполук у трансгенних рослинах призвело до зменшення відносної частки ДНК у хвостах комет майже в 3 рази.***

К л ю ч о в і с л о в а : ***екстракти "бородатих" коренів, види Artemisia spp., флавоноїди, УФ-протекторні властивості, генотоксичність, кометний електрофорез.***

Автори заявляють про відсутність конфлікту інтересів. Спонсори не брали участі в розробленні дослідження; у зборі, аналізі чи інтерпретації даних; у написанні рукопису; в рішенні про публікацію результатів.

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; in the decision to publish the results.