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RESEARCH ARTICLE

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Effects of *Astragalus dasyanthus* Extracts on the level of DNA damages in human lymphocytes

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ABSTRACT. The use of bioactive compounds of plant origin in the pharmaceutical and cosmetic industries has become increasingly widespread, and therefore the question of the safety of such raw materials arises, primarily for the genetic material of cells. In addition, an important aspect is the study of the possibility of growing potentially useful plants *in vitro*, which is promising in the context of the scaled-up production of standardized medicinal raw materials. In this work, the potential cytotoxic and mutagenic effects of aqueous and alcoholic extracts of the *Astragalus dasyanthus* Pall. plant, grown *in vitro*, on human lymphocytes were investigated, and their antioxidant activity was also analyzed. The contents of water-soluble sugars in aqueous and flavonoids in alcohol extracts were in sufficiently high concentrations, which indicates the effective accumulation of these substances in plants grown *in vitro*, and the validity of the selected extraction protocols. The content of flavonoids in methanol extracts exceeded this value in ethanol extracts by almost one and a half times. Incubation of lymphocytes with extracts at different concentrations did not lead to an increase in the number of non-viable and apoptotic cells, indicating the absence of cytotoxic effects. Aqueous extracts at a concentration of 6 µg/µl led to a statistically significant increase in the relative number of single- and double-stranded DNA breaks in cells, while lower concentrations did not show a mutagenic effect. Ethanol and methanol extracts at a concentration of 3 µg/µl showed an antimutagenic and/or reparogenic effect, reducing the level of damage present in lymphocytes by almost three times. In addition, the antioxidant activity of methanol extracts was demonstrated: the relative level of single- and double-stranded breaks in cells was significantly reduced when the Fenton reaction was performed in the presence of methanol extracts at a concentration of 3 µg/µl.

Keywords: comet assay, genotoxicity, *in vitro* plants, aqueous and alcohol extracts.

INTRODUCTION

The search for natural bioactive compounds that possess genoprotective properties is a topical issue in biomedical science and pharmacology, since genotoxic processes play an important role in the development of chronic diseases, aging, carcinogenesis and other pathologies [1, 2, 3]. Assessment of the mutagenic and antimutagenic potential of compounds is a critically important part of the pharmacological and toxicological evaluation of medicinal plants and their metabolites, as it ensures the safety of their use.

Natural plant-derived antioxidant compounds are able to neutralize free radicals, reduce oxidative stress level and consequently decrease DNA damage, as confirmed by numerous studies on plant extracts of various species [4, 5, 6]. Among them, plants of the *Astragalus* genus deserve special attention, as their high content of phenolic compounds, flavonoids, saponins and polysaccharides correlates with strong antioxidant and cytoprotective activities *in vitro* through the activation of cellular defense mechanisms [7, 8, 9, 10]. Considering that more than 15 species of this genus are listed in the Red Book of Ukraine, including *Astragalus dasyanthus* Pall., which is studied in this work, it is necessary to develop a strategy for stable and sustainable production of raw materials from these plants for scientific and practical purposes. This cannot be achieved using plants from natural populations, given their protected status and the seasonality of *in vivo* growth.

The *in vitro* plant cultivation technique provides a standardized and controlled source of biomass with reduced variability in the metabolic profile due to constant physiological and chemical cultivation conditions, which is critically important for the correct assessment of the biological effects of extracts, including DNA-protective activity [11]. An additional advantage of *in vitro* plant cultures is the possibility of targeted regulation of secondary metabolite biosynthesis by optimizing the nutrient medium composition and using growth regulators, elicitors and physical factors (light, temperature, osmotic stress). Thus, the study of the DNA-protective properties of *Astragalus dasyanthus*

extracts obtained from *in vitro* cultures is relevant due to the insufficient understanding of cellular protection mechanisms, the potential of this plant as a source of natural antioxidants, as well as the necessity to create a stable raw material base for further biomedical and pharmacological applications. At the same time, the question of the potential mutagenicity of extracts from this plant arises, especially in the context of the use of different extraction protocols, which may result in considerable differences in chemical composition and, consequently, biological properties.

One of the widely used methods for studying the mutagenic or antimutagenic activity of various compounds is the single-cell gel electrophoresis (comet assay) [12, 13]. Its main advantages include high sensitivity to DNA damage detection (primarily single- and double-stranded DNA breaks), high-rate performance, and the ability to detect abnormalities at the level of individual cell. This is especially important from the perspective of discriminating subpopulations of cells with different levels of DNA damage, including apoptotic cells. Briefly, the method is based on the analysis of DNA migration under electric force from cells immobilized in an agarose layer on a slide that have been subjected to low-salt lysis. In case of genomic DNA breaks, migration of DNA fragments from the lysed cell (nucleoid) toward the anode is observed, forming an electrophoretic track, which resembles a comet tail and can be visualized by fluorescent microscopy [14, 15]. The efficiency of such migration directly depends on the number of fragments present. Quantitative assessment of some parameters of this migration, in particular the relative DNA amount in the electrophoretic track (comet tail), allows evaluation of the extent of existing DNA damage.

In this study, the potential mutagenic activity of aqueous and alcohol extracts of *Astragalus dasyanthus* plants grown *in vitro* was analyzed, and their antioxidant effects were also assessed using the comet assay.

MATERIALS AND METHODS

Plant cultivation

Aseptic plants of *Astragalus dasyanthus* Pall. grown *in vitro*, were used as plant raw materials for obtaining extracts. Such plants were obtained by surface sterilization of seeds, which were successively incubated with 70% ethanol (30 sec), 25% sodium hypochlorite solution (commercial preparation "Bilyzna") and washed with sterile distilled water (three times for 10 min). The seeds were germinated on Murashige and Skoog (MS) culture medium, supplemented with 1.0 mg/l benzylaminopurine (BAP) [16].

Extract preparation

Aqueous and alcohol extracts were prepared from dried *Astragalus dasyanthus*Pall. plants. For preparation of aqueous extracts, the plants were homogenized, then obtained material was transferred into centrifuge tubes and filled with distilled water (preheated to temperature of 40 °C) in a weight:volume ratio of 1:40. The mixture was incubated in a water bath for 60 min at 80 °C, then centrifuged at 8000 rpm and the supernatant was collected, which was further used to estimate the content of polyfructans. For methanol and ethanol extracts preparation, 96% methanol or ethanol was added to centrifuge tubes with homogenized plants in a weight:volume ratio of 1:40 and incubated in an ultrasonic bath at 60 °C for 30 min. The obtained supernatant was used to determine the content of flavonoids.

Polyfructans and flavonoids content assay

The content of polyfructans in the obtained aqueous extracts was determined by the McRARY and Slattery method, based on the Selivanov reaction. Total flavonoid content was quantified by spectrophotometric method. For this, 200 µl of 0.1 M aluminum chloride solution and 300 µl of 1 M sodium acetate were successively added to 300 µl of the extract. The absorbance of the sample was measured at 419 nm using Optizen Pop spectrophotometer (South Korea). The calibration curve was constructed using quercetin (Sigma, Germany).

Lymphocyte isolation from whole blood and their incubation with extracts Separation of whole blood elements, obtained from healthy donors, was conducted by rate-zonal centrifugation for 40 minutes at 1500 rpm using density gradient Histopaque-1077 (Sigma, USA). The obtained lymphocyte fraction was washed twice with RPMI 1640 medium and incubated in the same medium with extracts for 1 and 2 hours at 37 °C. As a negative control, cells were incubated under thesame conditions without adding extracts to the medium.

Assessment of cell viability

For all variants of the experiments, cell viability was assessed using methylene blue staining. For this analysis, an aliquot of the freshly obtained cell suspension was mixed with a 0.4% solution of methylene blue, prepared in PBS buffer, in a ratio of 1:1. A small amount of the mixture (5-10 µl) was applied to a hemocytometer and covered with a cover glass. The number of non-viable cells and cell concentration in the suspension were counted according to standard recommendations using a Biorex-3 light microscope (Konus, Italy).

Fenton reaction

To induce DNA damage using free radicals, the Fenton reaction was performed. For this, lymphocyte suspension was mixed with RPMI 1640 medium, 0.75 mM $FeSO_4$ and 0.75 mM H_2O_2. Additionally, an aliquot of lymphocytes was subjected to the Fenton reaction in the presence of alcohol extracts. The mixture was incubated for 2 hours at 37 °C. After incubation, the lymphocytes were washed twice with Hanks' solution and used to prepare slides for comet assay.

Comet assay

To conduct comet assay, cells were immobilized on the surface of a microscope slide within a layer of 1% low-melting agarose and lysed in a high-salt buffer with detergent (2.5 M NaCl, 0.01 M Tris-HCl, 0.1 M Na_2EDTA, 1% Triton X-100, pH 7.5). After lysis, the slides were washed twice in TBE buffer (89 mM Tris-HCl, 89 mM H_3BO_3, 2 mM Na_2EDTA, pH 7.5) and electrophoresis was performed in the same buffer for 10 min at a temperature of 4 °C and an electric field strength of 1 V/cm, using a horizontal electrophoresis system. After electrophoresis, the slides were stained with the fluorescent dye DAPI (4',6-diamidino-2-phenylindole, Sigma, USA) and analyzed with a fluorescence microscope with a Canon EOS 1000 camera attached. The 100–200 randomly selected cells were analyzed using CometScore software (TriTek, USA), determining the relative DNA amount in the comet tail as the ratio of the fluorescence intensity level in the tail to the total fluorescence intensity level of the comet.

Statistical analysis

Statistical analysis was performed by conventional methods of variation statistics. Each experimental variation was performed in five to seven technical replicates to calculate the mean value and standard deviation for all independent experiments. For a number of experimental data, a Shapiro-Wilk test was performed for normality of distribution. Under the condition of normal distribution of data, the significance of the difference between the experimental measurements was estimated within the framework of the Student's t-test using Origin 8.0 software (OriginLab Corporation, USA). The difference between the compared values was considered to be significant at $p<0.05$.

RESULTS AND DISCUSSION

In this work, plant extracts obtained during aqueous and alcohol extraction were used. Water, as a highly polar solvent, effectively extracts hydrophilic low-molecular carbohydrates, in particular fructose-containing sugars, the content of which in our experiment was 47.72±2.08 µg/µl (26.72±2.08 milligrams per gram of dry weight). The values demonstrate a sufficiently high concentration of these soluble monosaccharides in the aqueous extract, which is consistent with the data that carbohydrates are the predominant macromolecules in many specimens of the *Astragalus* genus, where their total content can reach very high values (over 60 g/100 g dry weight in *A. cicer* and *A. glycyphyllos*), as shown by chromatographic analysis of their biochemical composition [17]. Similarly, comparative studies of *Astragalus membranaceus* root extracts have demonstrated that the major water-soluble polysaccharides are composed primarily of common monosaccharides such as arabinose, glucose, galactose and galacturonic acid, with glucose typically being the predominant unit detected in polysaccharide fractions isolated from different geographic origins of the plant. Although the total alcohol-soluble polysaccharide content in cultivated roots was about 1.5-fold higher than in natural roots, the difference was not statistically significant, indicating that both natural and cultivated *A. membranaceus* accumulate substantial amounts of soluble carbohydrates; however, minor monosaccharide components can vary depending on growth conditions [18]. Thus, the *A. dasyanthus* plant raw material, from which the aqueous extracts were obtained, contained a sufficiently high concentration of water-soluble sugars, which reflects the suitability of the chosen extraction method, as well as the effective accumulation of these monosaccharides by plants under *in vitro* conditions.

Since flavonoids are phenolic compounds with moderate polarity, alcohol solutions were used for their extraction. The quantity of flavonoids in methanol and ethanol extracts was 33.57±2.53 µg/µl (18.79±2.53 mg/g dry weight) and 22.15±1.26 µg/µl (12.41±1.26 mg/g dry weight), respectively. These concentrations are completely comparable with those obtained in the works of other authors [19, 20]. The higher extraction efficiency of methanol compared to ethanol is explained by its greater polarity and ability to penetrate the plant matrix more effectively, which contributes to the release of cell wall-bound flavonoids. According to established findings, *A. dasyanthus*is a rich source of phenolic compounds, in particular flavonoids, among which neohesperidin and rutin predominate as the main flavonoid components, which were identified in the herbs of this plant with quantitative values at the level of 1885.06 ± 1.04 and 1390.15 ± 17.41 µg/g, respectively, by chromatographic analysis [19]. This indicates a significant content of flavonoids in methanol extracts of *A. dasyanthus* plant, which generally correlates with the high concentration of flavonoid fraction obtained in our study. Corresponding data for other species of the *Astragalus* genus show a wide range of accumulation of phenolic and flavonoid compounds depending on the species, plant part and extraction conditions. Thus, in several Iranian species of *Astragalus*, the total phenolic content in the aerial plant parts ranged from 2.34 to 10.08 mg/g dry weight, and flavonoids varied significantly between plant species and organs from which the extracts were obtained, indicating significant interspecies variability for this group of compounds [20]. The values obtained for the methanol extracts of *A. dasyanthus* indicate a relatively high level of flavonoids compared to some other *Astragalus* species, especially considering that many studies of *Astragalus* genus show lower concentrations of these bioactive compounds in alcohol extracts. In general, the literature data indicate that hydromethanolic extracts of *Astragalus* species contain higher total phenolic and flavonoid contents than the corresponding water extracts, which confirms the higher efficiency of alcohol-containing extraction systems for the recovery of phenolic compounds from *Astragalus* plant material [21].

Thus, the results of the quantitative analysis of flavonoids in *Astragalus dasyanthus* indicate a high biochemical potency of this medicinal raw material as a source of phenolic compounds, which is consistent with the known pharmacological significance of this species and its use in traditional medicine. Compared with the research data for related *Astragalus* species, the amount of flavonoids in methanol extracts of *A. dasyanthus* can be considered relatively high, especially if we take into account that flavonoid components, such as rutin and neohesperidin, predominate the phenolic fraction of this plant.

To assess the potential mutagenic effect of the extracts of *A. dasyanthus* cells were incubated for 1 and 2 hours in RPMI 1640 medium with them. Since no significant difference in the studied parameters was found depending on the incubation time, the results will be presented for experiments with 1 h of incubation. Fig. 1 shows representative examples of DNA comets for different experiment variants, including the so-called "atypical comets", in which exiting DNA formed a cloud, rather than a tail, which had lost its connection with the comet head. Such comets are usually formed by nucleoids obtained from cells that undergo apoptosis, therefore, have significant DNA fragmentation: small fragments are able to migrate quite quickly through the pores of the agarose gel to sufficiently large distances from the main mass of cellular DNA (comet head) [22].

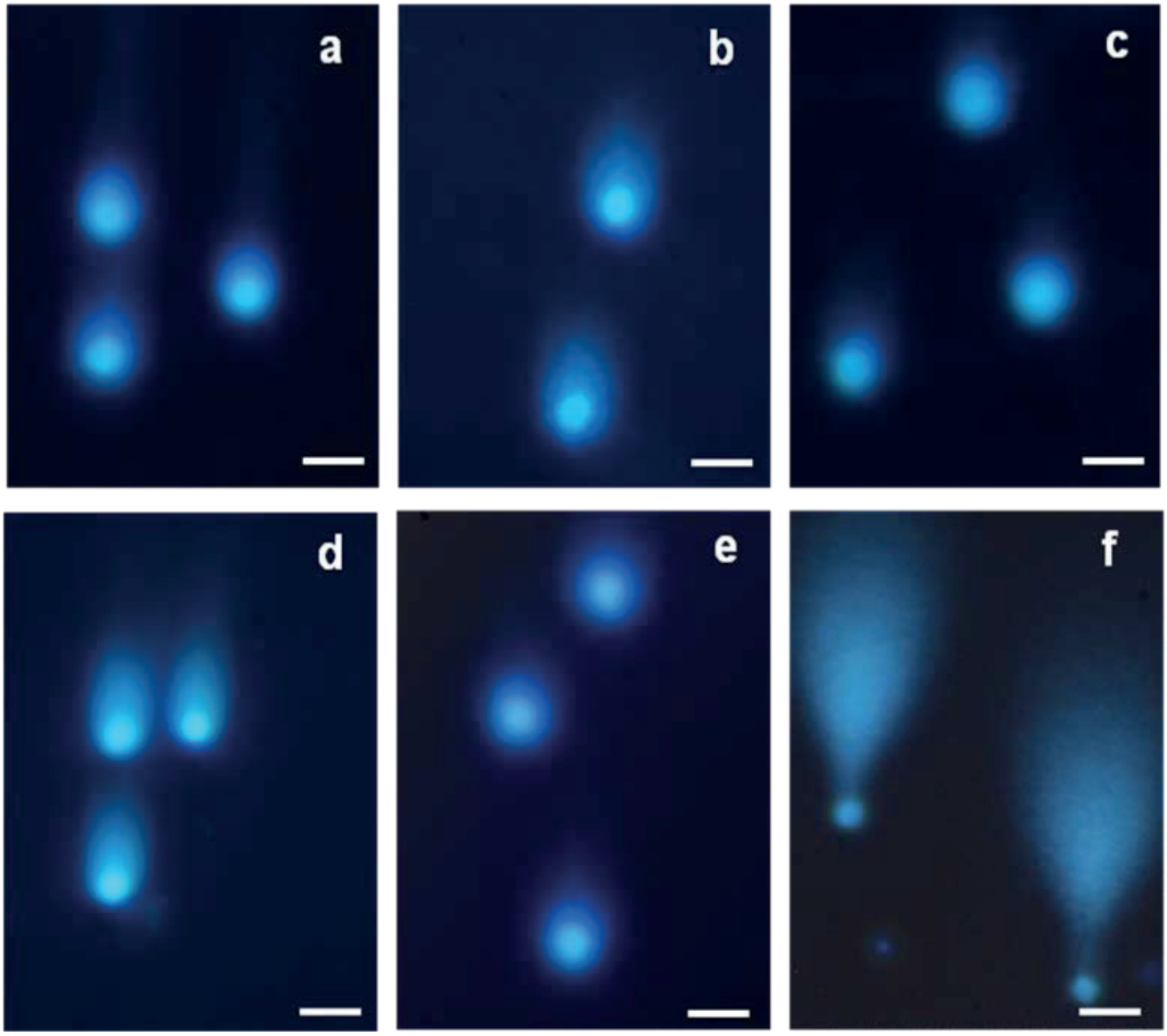

Fig. 1. Typical images of comets obtained in different experiments: (a) – control nucleoids, (b) – nucleoids obtained from cells incubated with aqueous extracts, (c) – nucleoids obtained from cells incubated with alcohol extracts, (d) – nucleoids obtained from cells after Fenton reaction, (e) – nucleoids obtained from cells after Fenton reaction with incubation with alcohol extracts, (f) – atypical comets (apoptotic cells). Bar – 10 μm.

Fig. 2 shows the results of cell viability assessment, determined by methylene blue staining (non-viable cells appear on the preparations stained blue) and the number of apoptotic cells, estimated by comet assay. In all experiments, the level of both parameters was low and ranged within 1-3%, which corresponds to the total number of such cells in control experiments (the figure shows data for the maximum concentrations of extracts studied). No statistically significant difference was found between the experiments, although there was a slight tendency for increasing the number of apoptotic and dead lymphocytes incubated with aqueous extracts. Therefore, none of the studied extracts had a pronounced cytotoxic effect.

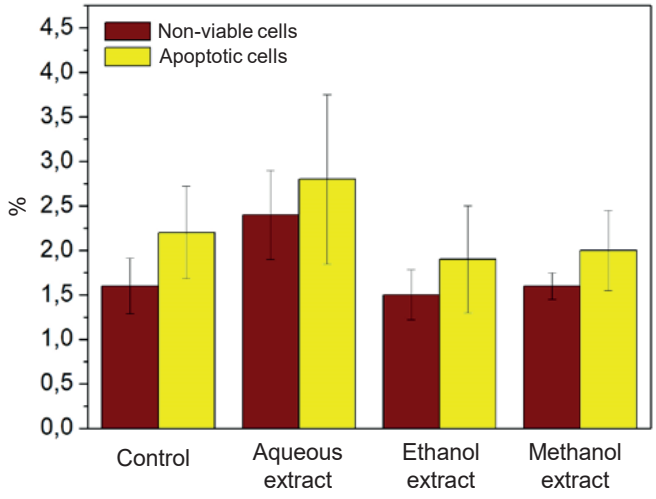

Fig. 2. Percent of non-viable and apoptotic cells in experiments with and without lymphocyte incubation with aqueous and alcohol extracts of A. dasyanthus.

The assessment of the relative level of DNA damage in control experiments without incubation with extracts indicates that this parameter was 5.7±0.47%. This value corresponds to the level of DNA damage in terminally differentiated cells and is typical for lymphocytes. Cell incubation with aqueous extracts at a concentration of 1 µg/µl did not cause any statistically significant differences in the relative DNA amount in comet tails. With a 3-fold increase in concentration, a tendency to an increase in this parameter was observed, which reached statistical significance at 6 µg/µl extract concentration in the RPMI 1640 medium (Fig. 3). Thus, it can be stated that aqueous extracts of *A. dasyanthus* are characterized by a slight, but pronounced genotoxic effect and can cause the accumulation of single- and double-stranded DNA breaks in the nucleus, which are detected by the comet assay. It should be noted that no cells with very high or very low values of this parameter were detected in the experiments, which means that the observed mutagenic effect of the extracts affects all cells, and not only those in which, for some reasons, the cellular defense systems are weakened.

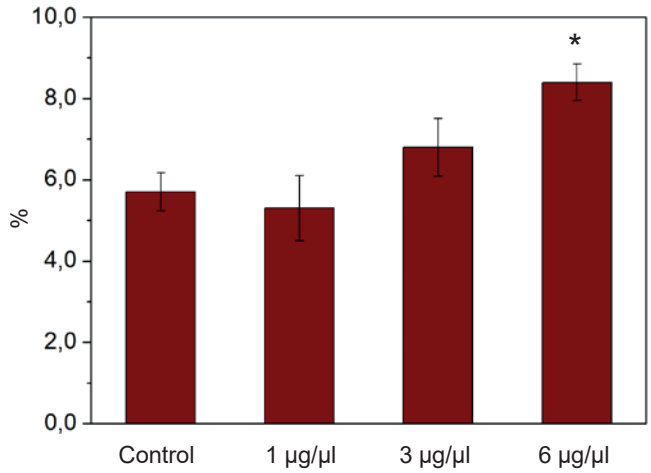

Fig. 3. Effect of different concentrations of aqueous extracts of A. dasyanthus on the level of single- and double-stranded DNA breaks in human lymphocytes, % – relative DNA amount in the comet tail (* – $p<0.05$).

Comprehensive analysis of mutagenic activity of extracts of another *Astragalus* species, *A. membranaceus* using three genotoxicity assays provided strong overall support that the polysaccharide fraction of these extracts lacks mutagenic potential [23]. However, this work was based on the results of the reverse mutation test, chromosome aberration test and micronucleus test, which allow to detect mainly point mutations, chromosomal rearrangements and aneuploidies, respectively. In our study, we observed the ability of extracts to induce single- and double-stranded DNA breaks in cells, which can potentially be converted into chromosomal aberrations (e.g., intra- and interchromosomal translocations, deletions, inversions, etc.) if the DNA repair systems do not function properly. That means, in fact, that we detect DNA changes that can be attributed to the "pre-mutation state" – those changes in the DNA structure that can be converted into chromosomal mutations. Similarly to the results obtained by us, the absence of cytotoxic effect of aqueous extracts of a number of medicinal plants, including the *Astragalus* genus, was shown in the work of Inami et al [24], however, these extracts were characterized by moderate antimutagenic activity in the analysis of the frequency of revertant colonies of *S. typhimurium*. The mutagenic effect of aqueous extracts was demonstrated for plants of the *Thermopsis* genus, in the extracts of which, along with water-soluble sugars, a small amount of alkaloids was present, which, according to the authors, caused a negative effect [25]. It is possible that a similar situation is typical for extracts obtained from *A. dasyanthus* plants.

Similarly to aqueous extracts, alcohol extracts at a concentration of 1 µg/µl did not stimulate the accumulation of single- and double-stranded DNA breaks in lymphocytes: the percent of DNA in comet tails was ~6%. However,

increasing the concentration to 3 μg/μl significantly reduced this parameter in comparison to the control cells by almost half: the relative DNA amount in comet tails was 2.17±0.39 and 1.86±0.1 for cells incubated with ethanol and methanol extracts, respectively (Fig. 4).

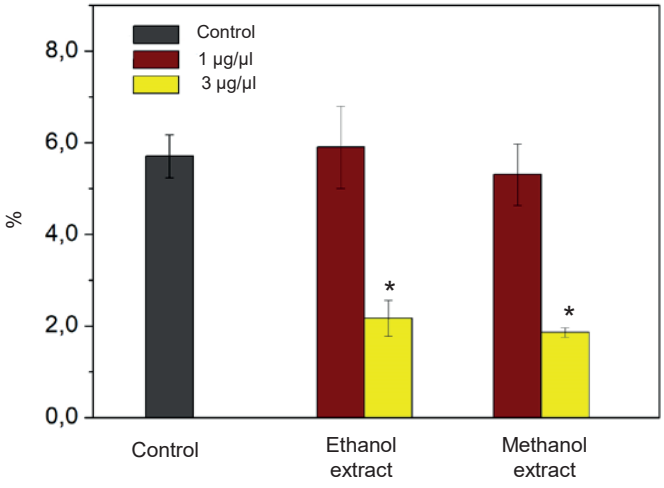

Fig. 4. Effect of different concentrations of alcohol extracts of A. dasyanthus on the level of single- and double-stranded DNA breaks in human lymphocytes, % – relative DNA amount in the comet tail, * – $p<0.05$.

Therefore, the antimutagenic, or rather reparogenic, properties of alcohol extracts of *A. dasyanthus*was demonstrated: incubation of lymphocytes with them led to a decrease in the amount of DNA breaks already present in intact cells. Antimutagenic potency of the methanol, chloroform and ethylacetate extracts of 14 species of *Astragalus* (but not *A. dasyanthus*) against induced mutagenicity of sodium azide (NaN_3), 4-nitroquinoline-1-oxide (4NQO) and 9-aminoacridine (9AC) was also demonstrated in works using the Ames test using *Salmonella typhimurium* mutant strain [26, 27]. It should be noted that the detected effect showed a definitive dose dependence. In these works, a detailed analysis of the chemical composition of the extracts was not investigated, but it is assumed that the presence of phenolic compounds extracted by the described methods, in particular flavonoids, may cause a similar effect.

At the next stage of the work, alcohol extracts were tested (methanol was chosen as the one with the most pronounced antimutagenic/reparogenic activity) for their potential antioxidant activity. For the induction of free hydroxyl radicals in cells, the Fenton reaction was used - an oxidative reaction of hydrogen peroxide with iron ions. To carry out this reaction *in vivo*, a series of experiments were conducted to establish the optimal concentrations of these two reagents, in the presence of which the maximum level of DNA damage was obtained. It was found that the ratio of hydrogen peroxide to iron (II) sulfate 0.75mM:0.75mM is the most effective: the relative level of DNA in comet tails was ~13%, i.e. almost twice as high as this parameter in control cells (Fig. 5). It should be noted that under such conditions, the percent of atypical comets, indicating the induction of apoptosis in cells, was within the control values, i.e. did not exceed 1-2%. Simultaneous Fenton reaction with incubation of cells in methanol extract at a concentration of 3 μg/μl for 2 h. led to a significant decrease in free radical-induced DNA breaks: the percent of DNA in comet tails was 3.74±0.31%.

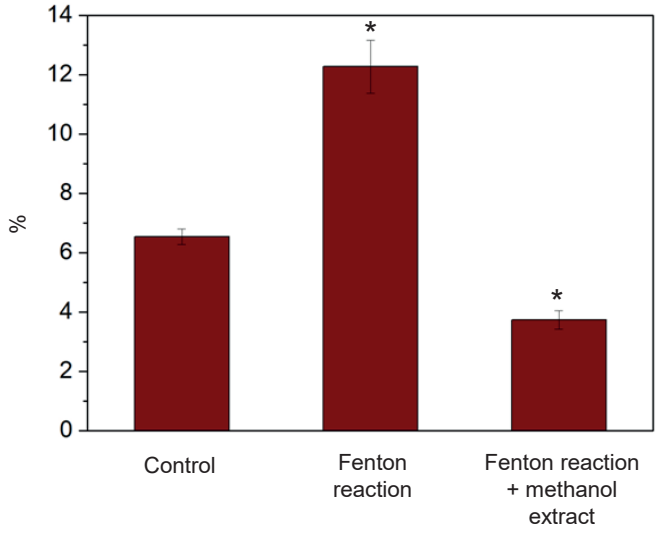

Fig. 5. Antioxidant effect of methanol extracts of A. dasyanthus on human lymphocytes, % – relative DNA amount in the comet tail, * – $p<0.05$.

CONCLUSIONS

We investigated the mutagenic and antioxidant properties of aqueous and alcohol extracts of *A. dasyanthus* Pall. plants grown *in vitro*. It was demonstrated that the amount of fructose-containing sugars in aqueous extracts, as well as the amount of flavonoids in alcohol extracts, corresponds with the values obtained in the works of other authors, which indicates the efficiency of extraction and sufficient accumulation of these substances in plants. At the same time, the content of flavonoids in methanol extracts significantly exceeded this parameter for ethanol extracts. Analysis of the mutagenic potency of the extracts using the comet assay revealed slight mutagenic activity of aqueous extracts at a concentration of 6 μg/μl, while lower concentrations did not cause noticeable effects. At the same time, cell incubation with alcohol extracts was accompanied by a decrease in the relative amount of DNA breaks compared to control (intact) cells, which indicates their antimutagenic/reparogenic properties. In addition to the pronounced reparogenic activity, alcohol extracts were also characterized by antioxidant activity, reducing the relative DNA amount in comet tails during the Fenton reaction.

DECLARATIONS

Author contribution

Concept and experiment design: Katerina Afanasieva, Olena Kvasko; Data collection and their analysis: Mariana Chopei, Oleksandr Manzhura, Maksym Tesliuk; Statistical analysis and literature review: Mariana Chopei, Maksym Tesliuk; Writing: Katerina Afanasieva, Mariana Chopei; Critic and final revision: Katerina Afanasieva, Olena Kvasko.

Conflict of Interest

The authors declare no conflict of interest.

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