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Testing the antigenotoxicity and anticytotoxicity properties of *Prunella Grandiflora* L. extract using the example of *Drosophila Melanogaster*

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Abstract

Using the *Drosophila melanogaster* model object, the presence of protective properties in 10% of *Prunella grandiflora* L. (Lamiaceae) extract was determined in relation to the toxic and cytotoxic effects of the antitumor drug etoposide in two concentrations of 800 and 8000 μg/kg of nutrient medium. The grass *P. grandiflora* was collected in the flowering phase in Sverdlovsk region (N 56°09'22.0'', E 058°32'19.6'') in 2021. No genotoxic manifestations of the extract were found. Comparative characteristics of wing parameters under the influence of etoposide at a dose of 800 and 8000 μg showed significant differences in linear and two-dimensional parameters of the wing.

Keywords

extract
prunella
etoposide
morphometry
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1. Introduction

Cancer is a disease that causes great harm to human society. There are accepted approaches to the treatment of this disease, but the side effects that occur force us to look for alternative ways to recover or improve the quality of life of each patient. More and more often in scientific circles we hear about personalized medicine, on the threshold of which we are standing. This means that the search for new pharmaceuticals that will be preventive or complementary to existing treatment methods is an important problem in the applied aspect.

One of the promising species in the treatment of cancer is the common blackhead *Prunella vulgaris* L. family Lamiaceae. Using this plant extract, clinical trials were conducted to inhibit cancer cells of the human esophagus, stomach, colon, cervix, liver, which showed positive results [1].

In addition, its chemical composition was studied in the most detail, according to which its high therapeutic effect in the treatment of cancer is explained. Thus, several phytochemicals from *P. vulgaris*, including rosemary acid [2] and caffeic acid [3] cause or promote apoptosis of cancer cells. In the literature, there is mainly information about the medicinal properties of *P. vulgaris* [4], while data on the pharmacological features of the closely related species *P. grandiflora* are extremely scarce. Previously, we conducted a comparative study on some phenol carboxylic acids of these types. As the research results showed, the content of most phenol-carboxylic acids, including those having antitumor properties, in the leaves of *P. grandiflora* was higher than that in the leaves of *P. vulgaris*. We noted a higher content of rosemary acid in *P. grandiflora* (41.77–52.39 mg/g) than in *P. vulgaris* (17.88–31.17 mg/g). In *P. grandiflora* and *P. vulgaris*, caffeic acid contains 1.13–1.60 mg/g and 0.40–0.64 mg/g, respectively. In this regard, the species *P. grandiflora* was chosen as a protector.

One of the tasks in testing protective substances that presumably have antigenotoxic properties is to identify various types of side effects when using a protector and an antitumor preparation together. The protective properties of *Prunella grandiflora* L. extract were studied in 10% concentration relative to the etoposide drug used at a dose of 800μg/kg and 8000μg/kg of nutrient medium.

2. Materials and Methods

The grass of the large-flowered blackhead in an amount of 0.8 g was extracted in 10 ml of 70% alcohol for 24 hours. 2.4 ml of 10% extract was added to a nutrient medium weighing 17.6 ml (Figure 1). The drug etoposide 20 mg /ml (Vero-pharm Ebave) was used at a concentration of

800 μg/kg and 8000 μg/kg of nutrient medium. The Oregon – R laboratory line was used to assess viability and morphometric analysis. To account for fertility, 25 individual pairs were placed in 25 tubes with a hollow lid filled with agar medium and lubricated with yeast, from which laid eggs (F_1) were collected daily and placed on Petri dishes. The percentage of undeveloped eggs at an early stage of development (<6 hours, white color, EEL [early embryonic lethality]), at a late stage of development (>6 hours, brown color, LEL [late embryonic lethality]) was calculated from the total number of eggs laid per day. The genotoxic effect was determined using the SMART (Somatic Mutation and Recombination Test) technique. To do this, females from the mutant yellow line (yellow body color, the yellow gene is localized on the X chromosome) were crossed with males from the mutant white singed 3 line (white eyes and singed bristles on the body, the genes are also localized on the X chromosome), placing them on the test medium for 72 hours. Hybrid females of the wild F_1 phenotype (brown-gray body, straight bristles, red eyes) were used for analysis. In females, the bristles on the body were examined, and the number of bristles not typical for the normal phenotype in color and shape was noted. The area containing a similar bristle was recorded in the table as a single spot y (yellow) or sn (singed) or double y sn.

The DNA damage in fly enterocyte cells of the control and experimental groups was evaluated using the alkaline DNA comet method, which allows determining single-strand breaks. The DNA damage analysis was performed as described in [5] with modifications. To do this, 5 flies of all the studied variants were selected and washed three times in PBS. Then the intestine was isolated in the Poel's saline solution (15 mM NaCl, 6.4 mM NaH_2PO_4, 42 mM KCl, 7.9 mM $CaCl_2$, 1.8 mM $KHCO_3$, 20.8 mM $MgSO_4$; pH 6.95). Further, the samples were centrifuged for 5 min (5000 rpm) at 4 °C, a supernatant was selected and applied to 80 μl 0.65% low-melting agarose. After that, the obtained samples were applied to prepared slides coated with 1% agarose. Then, for 1 hour in the dark, the glasses were treated with a lysing buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100; pH 10). Before electrophoresis (15 V/300 mA, 30 min), the samples were incubated in an electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 13) for 15 minutes, after which the glasses were washed 3 times with 20 mM Tris, pH 7.5 and 3 times with distilled water. Further, the samples were treated with an EtBr solution for 20 minutes. The analysis of the finished preparations was performed using a fluorescence microscope Carl Zeiss Axio Imager M2. For each variant of the experiment, three glasses were prepared and 50 cells were counted (150 cells per 1 variant), the DNA comet index (IDC) was determined [6].

The change in the morphology of the wing was determined using morphometric analysis of the wing by 24 indicators (18 linear and 6 two-dimensional (areas of individual wing cells) (Figure 2). The wings of the individuals were fixed in 70% alcohol on a slide in the form of a temporary preparation, photographed and processed using the Universal Dekstop Ruler program.

Statistical analysis was performed using the Statistica Ultimate Academic for Windows program. During comparison and analysis of samples, the Student's criterion, the chi-square criterion together with the Yates correction and discriminant analysis were used.

3. Results and Discussion

3.1. The Average Individual Fecundity (AIF)

The frequency of early and late mortality of offspring at the embryonic stage (up to 6 hours of development – EEL, after – LEL) were evaluated to analyze the viability of each group of *D. melanogaster*. Figure 3 shows an improvement in fertility indicators with the combined use of etoposide at a dose of 800 μg/kg and extract. This indicator increases almost twofold: when using etoposide together with an extract AIF is 16.50; when using a single cytostatic agent, it is 9.03. An increase in the applied dose of etoposide to 8000 μg/kg significantly reduces the fertility rate compared to the control sample. However, the use of the extract together with etoposide at a dose of 8000 μg/kg restores the CPI index to the values of the control sample. Accordingly, the extract has a positive effect on leveling the toxic effect on the fertility potential, increasing the average value of the AIF index twice, regardless of the dose of cytostatic.

Figure 1 *Prunella grandiflora* L. before the drying.

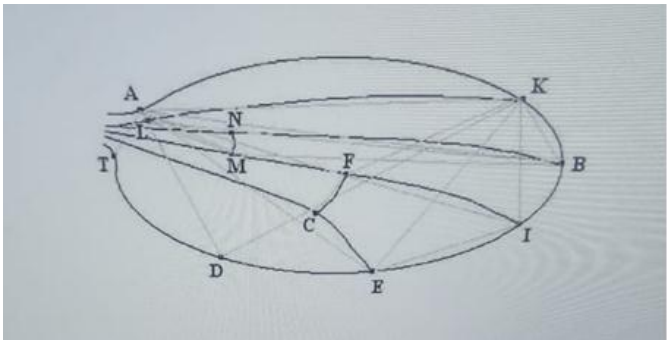

Figure 2 Linear parameters of the wing plate of *Drosophila melanogaster*.

According to the indicators of EEL and LEL, a similar pattern can be noted. The range of LEL, LEL and the average value of EEL, EEL decreases with the use of the extract and etoposide at a dose of 800 μg/kg. The reverse pattern was obtained when using a high dose of etoposide 8000 μg/kg. The maximum mortality value was noted in the early embryonic stage when etoposide was administered at a dose of 8000 μg/kg and is 60. It can be noted that in all groups, mortality is higher in the early embryonic stage.

3.2. Somatic Mutation and Recombination Tests (SMART)

The genotoxic effect of etoposide was also analyzed at a dose of 800 μg/kg and 8000 μg/kg using SMART lines. Table 1 shows that the extract, when used in combination with etoposide at a dose of 800 μg/kg, reduces the frequency of aberrant phenotypes by 3 times in relation to the action of a single cytostatic agent, but does not affect the frequency of occurrence of certain types of spots. Thus, among 873 flies, 14 spots of different types and 3 aberrant phenotypes were found, whereas when exposed to etoposide, 13 spots and 9 aberrant forms were found among 669 individuals. Yellow spots were found in one individual in the control and experimental samples with etoposide at a dose of 800 μg/kg. According to preliminary data, etoposide in both doses mainly caused singed-type spots. In the case of exposure to 8000 μg/kg of etoposide, the use of 10% *P. grandiflora* extract is also effective in reducing the frequency of aberrant types in hybrid females.

3.3. Comet Assay

DNA comet analysis is a fast and sensitive method for detecting DNA damage in individual cells. The size, shape and amount of DNA inside the "comet" determine the severity of DNA damage. This analysis is used to test DNA damage by various chemicals and infectious agents using drosophila as a model system. In drosophila, brain ganglia, midgut cells (enterocytes), and imaginal disc cells are targeted for genotoxicity testing *in vivo* [7]. In the case of invertebrates, enterocytes are used instead of human lymphocytes [8]. Enterocytes are very sensitive to the effects of genotoxic agents, and the treatment methodology is very simple, since the cells come into direct contact with toxic materials that enter the intestines of flies [9].

The data obtained by us showed the absence of genotoxic manifestations in *P. grandiflora* extract in 10% concentration relative to the nutrient substrate ($F = 58.3$; $p<0.05$) (Figure 4). When exposed to 800 μg/kg of etoposide 10% extract, the DNA comet index increased by 18% compared to the standard nutrient substrate ($F = 64.1$; $p<0.05$).

In highly replicating cells, such as hematopoietic stem cells and epithelial cells, DNA mutations resulting from non-repaired DNA damage play a crucial role in malignant transformation and cancer progression [10]. Endogenous agents capable of damaging DNA, such as reactive oxygen species (ROS), lipid peroxidation products, and reactive nitrogen species (RNS) are naturally released during cellular metabolic activity or hydrolytic processes [11].

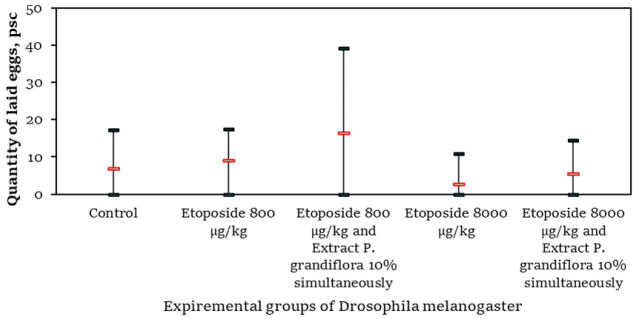

Figure 3 The average individual fertility of flies of individual experimental groups of D. Melanogaster.

Table 2 IDC indicators in different experimental groups of *D. melanogaster.*

Experiment Variant	IDC	One way ANOVA
Control	0.86±0.02	–
10% *P. grandiflora* extract	0.89±0.06	$F = 58.3$; $p<0.05$
10% *P. grandiflora* extract + etoposide	1.05±0.04	$F = 64.1$; $p<0.05$

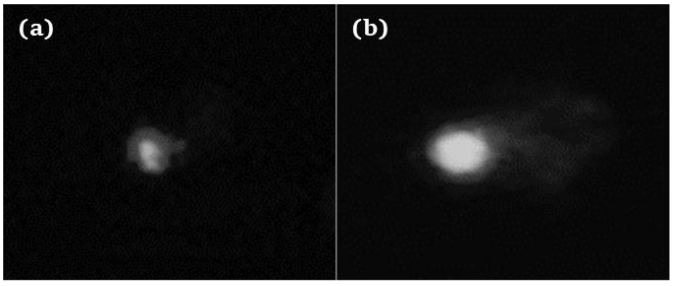

Figure 4 The main types of DNA damage: (a) control, (b) etoposide + extract.

Table 1 The examination of somatic mosaicism in various experimental groups of *D. melanogaster.*

Experimental groups	Number of individuals with mutant spots				Other mutant phenotypes	% fraction of the sample	(χ_2)	(p)
	Samples	y	sn	y sn				
Control	669	1	2	0	0	0.44843	–	–
Etoposide (800 μg/kg)	833	1	12	0	9	2.641056	9.6	0.002
Extract *P. grandiflora* 10%+ etoposide (800 μg/kg)	873	1	12	1	3	1.901141	5.5	0.019
Extract *P. grandiflora* 10%+ etoposide (8000 μg/kg)	591	0	1	0	2	0.507614	0.1	0.797
Etoposide (8000 μg/kg)	263	0	4	0	1	1.901141	3.1	0.077

In addition, activation of the response in DNA damage can be caused by thousands of exogenous agents, including ionizing radiation, chemotherapy, viral infections and chronic inflammation [12].

3.4. Morphometry Analysis of Wings

When comparing the wing shape according to the linear parameters of the experimental groups: control, etoposide 800 and etoposide 8000, several discriminating parameters were found (Figure 5). According to discriminant analysis, it was revealed that the wing plate undergoes extensive changes affecting almost all areas of the wing, both its central part and lateral areas.

According to the graph of canonical variables, it can be assumed that when exposed to different concentrations of etoposide, the shape of the wing changes significantly. The samples of control and after exposure to etoposide do not overlap. In etoposide samples 800 and etoposide 8000, there was also a small similarity of the samples in the linear parameters of the wing plate (Figure 6). This suggests that it is possible that with a higher dose of etoposide, changes in the wing occur in a different form than changes at a concentration of 800, at least the RCD (regulated cell death) processes occur more intensively in all 4 compartments [13].

According to the data of discriminant analysis for two-dimensional parameters of the wing, it was found that two of the 6 areas of the wing shown in Figure 7 do not change in area.

These cells are located in the central part of the wing plate respectively, the lateral parts of the wing are more affected by etoposide.

4. Conclusions

According to the preliminary data, etoposide in both tested concentrations have an effect on both generative and somatic cells of individuals of the Oregon-R *Drosophila melanogaster* line. According to the results of SMART analysis, 10% *Prunella grandiflora* L extract does not have genotoxic properties. The use of the extract together with etoposide increases the fertility of individuals. The cytotoxic properties of etoposide of both concentrations are manifested in a change in the shape of the wing plate, both with respect to linear parameters and the size and shape of individual wing cells.

Supplementary materials

No supplementary materials are available.

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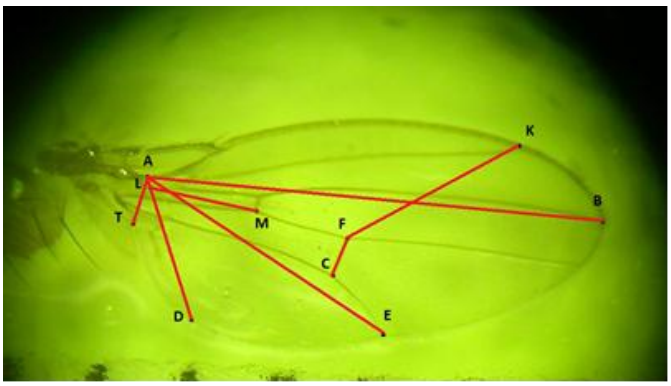

Figure 5 The Discriminating parameters of the wing plate of *D. melanogaster* experimental groups: control, etoposide 800 μg/kg and etoposide 8000 μg/kg.

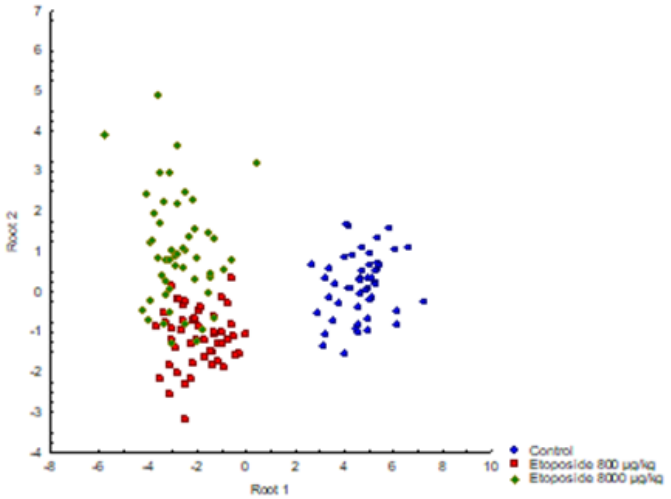

Figure 6 Canonical analysis of morphometric parameters of the wing of experimental groups of *D. melanogaster*.

Figure 7 Non-discriminating two-dimensional wing parameters of *D. melanogaster* experimental groups: control, etoposide 800 μg/kg and etoposide 8000 μg/kg.

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Conflict of interest

The authors declare no conflict of interest.

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