Effect of Silybum marianum (L.) Gaertn. seeds hydrostatic pressure pretreatment on biological properties of the extract by Drosophila melanogaster example

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Abstract. The article analyzes the feeding intensity, chemotaxis of larvae and changes in the expression of the *sqh* gene in *D. melanogaster* individuals cultivated on a nutrient medium with the introduction of ethanol extract from *S. marianum* seeds under pretreatment with hydrostatic pressure of 5, 10, 15 and 20 MPa. The preferred chemotaxis of larvae with respect to seed extract without prior pressure exposure was revealed. Changes in the intensity of nutrition in individuals of the experimental groups were not registered. A hypo-expressive direction of changes in the activity of the *sqh* gene was found when growing individuals on a medium with the introduction of an extract from seeds treated with pressure levels of 15 and 20 MPa. Variants of further experiments are proposed.

1 Introduction

Various methods of preparation of extracts are aimed at a greater yield of biologically active substances during extraction. In this connection, additional techniques are used, such as the concentration of the extractant, the temperature conditions of extraction, and one of the options is the use of high hydrostatic pressure [1-2]. A joint regime of high-temperature exposure and pressure on the betulin yield is shown as a modification of the birch bark extraction method [3]. At the same time, a pressure of 3.4 MPa is used, which intensifies the output of both betulin and suberin. But the high temperature during the preparation of the extract can lead to the loss of biologically active properties of thermolabile compounds [4]. Therefore, non-thermal effects of high hydrostatic pressure on samples are often used. The optimal mode is selected, which reduces the extraction time and the maximum output of the target component. Such a method of using cold extraction leads to an increase in the

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fraction of phenolic antioxidants and, accordingly, the spectrum of biologically active properties expands. One of the main advantages of the method, the authors suggest a reduction in extraction time. When treated with high pressure, high differences occur between the outer and inner parts of the cell membrane, which leads to an acceleration of concentration equalization [4].

Using of high hydrostatic pressure is the most common method for extraction, but at the same time, data have been obtained that, depending on the selected target component, the optimal mode should be specifically selected [5]. For example, the authors analyzed the efficiency of extraction of two-year-old oenothera fruits in a dried state (extractant 70% ethanol) with pressure screening from 25 MPa to 300 MPa (room temperature, duration 20 minutes). A pressure of 300 MPa reduces the output of fatty acids and 100 MPa is optimal. But at the 100 MPa regime and the maximum output of fatty acids, the yield of flavonoids decreases as a compensatory process, i.e. the fraction of flavonoids decreases due to an increase in the fraction of fatty acids. This, in turn, indicates that a possibly greater yield of flavonoids can occur at pressures less than 100 MPa. The pressure of 100 MPa is characterized by almost complete destruction of cell membranes with the exception of only the smallest cells due to the fact that the hydrostatic pressure in this case is higher than the turgor pressure, which reaches 5-10 MPa. In addition, the use of high pressure leads to an increase in the viscosity of ethanol used as an extractant and its penetration into the matrix of particles worsens and, accordingly, the extraction of substances also decreases [5]. But the yield of bioflavonoids can increase when treated with a high hydrostatic pressure of 100-200 MPa, if a preliminary scheme of pressure treatment of vegetable raw materials is used [6].

Holy thistle is a representative of the Asteraceae family introduced in the Ural region [7]. Holy thistle fruit extract has detoxifying properties after or during chemotherapy, protective for hepatotoxicity after chemotherapy, and also as an enhancer of antiproliferative properties as an additional treatment. Preliminary studies suggest its use as an independent chemotherapy drug and, possibly, even directly for the treatment of cancer [8].

In this connection, the aim of the study was to determine the change in the biologically active properties of the ethanol extract of *S. marianum* seeds during their pretreatment with hydrostatic pressure of 5, 10, 15 and 20 MPa on the example of a model object *Drosophila melanogaster*.

2 Materials and methods

The study used seeds of *Silybum marianum* (L.) Gaertn., introduced plants in the conditions of the Ural region (Botanical Garden of the Ural Branch of the Russian Academy of Sciences, 2021). The seeds were treated with pressure in the strength laboratory of the M.N. Mikheev Institute of Metal Physics of the Ural Branch of the Russian Academy of Sciences at the following levels: 5 MPa, 10 MPa, 15 MPa, 20 MPa in an aqueous medium. After drying, the seeds were crushed to particles less than 1 mm and extracted with 70% ethanol, at the rate of 5 g of biological material per 100 ml of extractant (5% extract). The following lines of flies were used:

- Oregon – R (Isogenic line of wild type).
- Gal 4 – *da (daughterless).*
- *Sqh (spaghetti squash)* – EYFP – Mito.

The flies were kept at a temperature of 24 ºC and humidity of at least 60%. For the experiment on chemotaxis and feeding activity, third-instar larvae were used. The larvae were kept in Petri dishes with an experimental medium: agar medium lubricated with yeast paste with the addition of 5% holy thistle seed extract treated with appropriate pressure.

There were 6 experimental groups in total: reference without extract, with extract without pressure treatment of seeds, with seed extracts from seeds treated with pressures of 5, 10, 15 and 20 MPa and with extractant (ethanol) without seeds.

The assessment of the food preference index (chemotaxis) at the larval stage was performed in a two-sector Petri dish. Larvae were placed in the central part, while 250 ml of water was dug on one side, and 250 ml of the tested extract on the other. After 5 minutes, the distribution of larvae by sectors was analyzed. The index of food preference was calculated by the formula (1):

$$\frac{\text{Larval number in chemical part} - \text{Larval number in water part}}{\text{Whole larvae number used for the experiment}} \times 100 \quad (1)$$

To obtain F_1 hybrid individuals (heterozygous for *sqh*), Gal 4 females were crossed in the da variation no later than 4 hours after departure from the puparium and *sqh* males in a 2:1 sex ratio. After departure, the imago was distributed by gender, the abdominal area was photographed using a camera on a Leica DM500B fluorescence microscope, the intensity of the protein glow was evaluated using the Image J program. Nutrition activity was determined using the bromophenol blue indicator (2%). The indicator was added to a nutrient substrate of 500 µl per 20 ml of substrate and third-instar larvae were placed on substrate. After 24 hours, the larvae were photographed and the blue color was analyzed using the Image J program. Statistical analysis was performed in the Statistica 13.0 program. The Kraskel-Wallis criterion was used to analyze changes in the expression of the *sgh* gene. Pairwise comparisons were carried out according to the Mann-Whitney criterion, adjusted for multiple comparisons using the Holm-Bonferroni method. The intensity of nutrition was analyzed by the Kraskel-Wallis method.

3 Results

According to the analysis of the food preferences (chemotaxis) of the larvae, it was determined that the extract of holy thistle seeds not treated with pressure was the most preferable (table 1).

Table 1. The index of food preference (OPI) in experimental groups of *D. melanogaster*, where extracts from pretreated seeds with different pressure levels were tested.

Experimental group	Chemical part	Water part	Summary	OPI
Reference	7	8	15	-6.66667
EtOH 70%	8	9	17	-5.88235
Extract 0 MPa	10	5	15	33.33333
Extract 5 MPa	6	9	15	-20
Extract 10 MPa	7	8	15	-6.66667
Extract 15 MPa	7	8	15	-6.66667
Extract 20 MPa	7	8	15	-6.66667

At the same time, the least preferred is an extract from seeds treated with a pressure of 5 MPa. With an increase in the pressure level, the extract did not become preferable than in the control sample (figure 1a).

Despite the preference for seed extract without pressure pretreatment, no effect was observed on the intensity (activity) of larval feeding. All experimental groups were fed with the intensity characteristic of the reference sample (figure 1b).

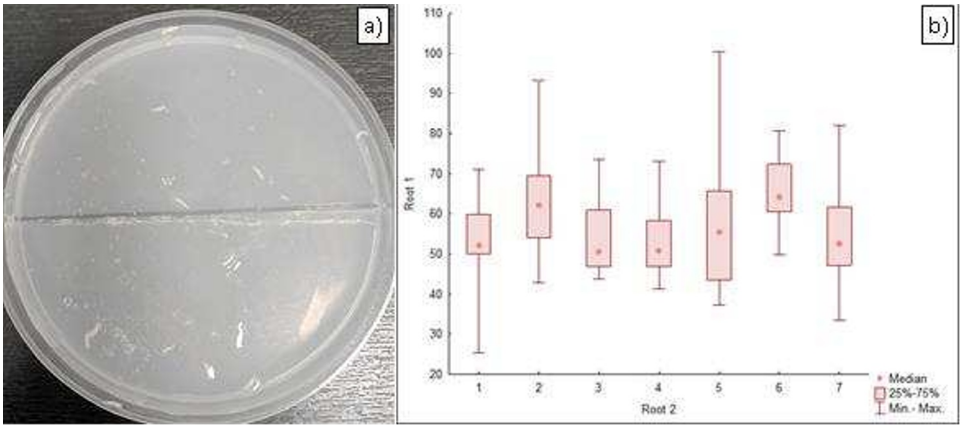

Fig. 1. a) Distribution of larvae by sectors when testing the extract from seeds pretreated with hydrostatic pressure of 20 MPa (water on top, extract on the bottom); b) Feeding intensity in various experimental groups of *D. melanogaster* (1 – Reference, 2 – EtOH 70%, 3 – Extract 0 MPa, 4 – Extract 5 MPa, 5 – Extract 10 MPa, 6 – Extract 15 MPa, 7 – Extract 20 MPa).

A change in the expression of the *sqh* gene was found in relation to all experimental groups (figure 2). Lower expression of this gene was found in males relative to females in the reference sample, when using 70% alcohol and at pressures of 15 and 20 MPa. At the same time, the most pronounced hypo-expression was registered in males when exposed to an extract from seeds treated with a pressure of 20 MPa. Hyperexpression was found in females and males in experimental groups when using an extract from seeds treated with a pressure levels of 5 and 10 MPa. When exposed to the extract without pretreatment of seeds by pressure, a multidirectional change in expression was observed in females (hypo) and males (hyper).

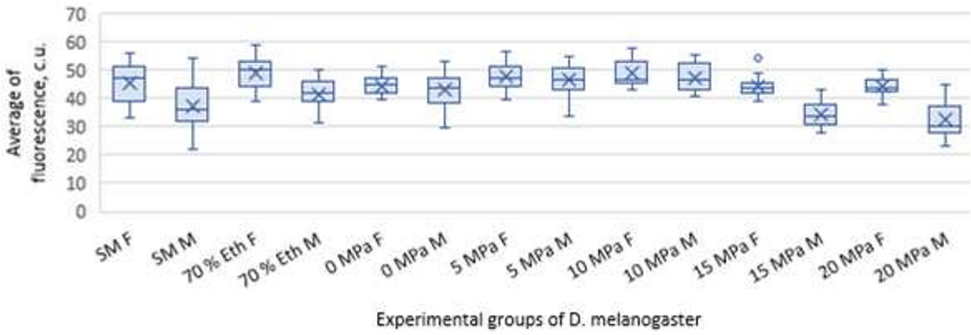

Fig. 2. Average fluorescence of hybrid individuals F_1 (♀*da* x ♂*sqh*) depending on the gender and pretreatment of seeds by pressure (F – female, M – male; SM – reference group, Eth – Ethanol; $p<0.001$).

4 Discussion

According to the data obtained, an extract prepared from seeds not previously treated with pressure is more preferable than extracts prepared from seeds after pressure treatment, which is consistent with the previously obtained results regarding cholesterol levels in larvae feeding on the corresponding holy thistle extracts [9]. It is possible that the pressure level used for seed treatment leads to a change in the ratio in favor of substances that form a greater stress of the nutrient substrate, whereas without pressure treatment, the nutrient

substrate remains comfortable for the larvae. Despite the attractiveness of the substrate containing seed extract without pretreatment by pressure, the intensity of nutrition does not change. According to the literature data, when the nutrient substrate is saturated with certain components, the larvae can either avoid feeding and starve for longer periods of their development, or at the beginning of their feeding, they eat more intensively, but quickly saturate and then eat in their usual mode [10].

An important indicator of the genetic activity of the tested extracts is a change in the expression of the gene involved in such various processes, starting from the earliest stages of ontogenesis: participation in cell proliferation, regulation of morphogenesis, signaling, etc. Excessive hypo-expression (inhibition) can lead to a violation of the above functions and, accordingly, a violation of development. According to the data obtained, with an increase in the pretreatment pressure of seeds (15 and 20 MPa), there is a decrease in the expression of the *sqh* gene, which indicates a more appropriate use of extracts from seeds prepared according to the described scheme and treated with pressure closer to the turgoric or without pretreatment pressure at all. Taking into account the fact that the transmitting medium for pressure treatment of seeds is water, some of the water-soluble substances can be extracted during processing into the working medium and not get into the extract under study. The data in Table 1 show that the extraction into an aqueous solution of substances attractive to larvae successfully occurs at pressures above 5 MPa and does not differ in the quality of the resulting product from 70% alcohol solution and reference sample. At the same time, processing at 5 MPa further aggravates the effect. Thus, it makes sense to investigate the possibility of extraction during pressure treatment and to study the effect of such an extract on the feeding and development of larvae. Both water and alcohol solutions of different concentrations can be used as a working medium. At the same time, it is possible to change the frequency of treatment and the relaxation time necessary to equalize the concentration of soluble components between the solution and the seed material, i.e. apply and remove pressure several times for a more complete extraction of active substances with different pause times between treatments.

5 Conclusion

It is shown that the larvae of *Drosophila melanogaster* of the selected lines show sensitivity to the method of preparation of extracts from *S. marianum*, which can be used to improve the extraction technique and quality control of the extracts obtained. Pretreatment of holy thistle seeds with hydrostatic pressure of 5, 10, 15 or 20 MPa during subsequent preparation of ethanol extract according to the standard method does not lead to intensification of larval nutrition. In addition, when using extracts from seeds treated with a pressure of 15 and 20 MPa, the expression of the *sqh* gene decreases, which indicates the presence of genetic activity of these extracts obtained by a similar method. The chemotaxis of larvae with respect to seed extract without pretreatment by pressure is more pronounced than the chemotaxis of extracts from seeds with pretreatment by pressure, which may indicate partial extraction of active water-soluble substances into the transmitting medium during pressure treatments.

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