Preparation of biotechnological raw materials of Iris sibirica L. with a given content of mangiferin and antiviral activity

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Abstract. The raw materials Iris sibirica L. were obtained by biotechnological methods. The relationship between biomass accumulation and a mangiferin content is peculiar to Siberian iris. On a medium with 5.0 μM BA supplemented with auxin, the xanton content in the phytomass sharply decreased with an increase in the total shoot height. To maintain a balance between biomass accumulation and a mangiferin content in I. sibirica, it is recommended to use media with 2.5 μM BA supplemented with auxin. Water and ethanolic extracts of I. sibirica showed a high antiviral activity against herpes simplex virus of type 1.

1. Introduction
The genus Iris L. is represented by medical and ornamental perennials that synthesize a wide range of biologically active substances. Irises are rich in xanthones, such as neomangifein, nigricaniside (rhizomes and roots of Iris dichotoma Pall.) and mangiferin (rhizomes of Iris rossii Baker) [1, 2].

The main xanton mangiferin (2-C-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxantone) was isolated in yellow iris, which is most often present together with isomangiferin and other O-glycosidam [3]. Mangiferin is a unique substance with anti-inflammatory, antihepatotoxic and antiviral effects. Unlike other xanthones, it has a wide, even if scattered dispersal in nature. It was first identified in Mangifera indica L. (Anacardiaceae), so that it was so named. In scientific works there has been reported of some fern-like and various docotyledonous (28 genera from 19 families) where mangiferin was also found. In monocotyledonous mangiferin was first described as part of the biologically active substances Iris germanica L., as well as in other iris families (Iridaceae and Liliaseae) [4, 5].

Mangiferin may occur in Iridaceae in association with its structutal isomer, isomangiferin. In Iris dichotoma and Iris domestica (L.) Goldblatt & Mabb. mangiferin and neomangiferin, belonging to the class of xanthones were found. In addition to these compounds, isomangiferin and 7-O-methylmangiferin were identified in extracts from I. domestica. Mangiferin is found in the leaves of Iris bungei Maxim. in big quantities [6]. Blinova K F and her collegues [7, 8] isolated xanthon glycosides in Iris ensata Thunb. The content of xanthonic glycosides mangerin (antioxidant, immunomodulator and antiviral drugs) was determined in different representatives of the genus Iris, including Iris lactea Pall., Iris ensata, Iris reichenbachii Heuffel., Iris sibirica [9-12]. Mangiferin is the main component of Alpizarin, manufactured in Russia, an antiviral agent for the treatment of infection caused by the herpes virus. [13, 14].
In the researches of Sladjana B. Jevremovic and her colleagues (2017) the results of using tissue culture as a method for increasing the mangiferin content of the Balkan endemic species Iris reichenbachii Heuffel were shown. The production of mangiferin in the culture of I. reichenbachii is connected with the formation and differentiation of shoots. According to scientists, a smaller amount of mangiferin was determined in the plants cultivated in vitro than in plants cultivated in the fields [15].

Methods of biotechnology allow getting high-quality medicinal plant materials in a short time, in large quantities, without destroying natural resources. Microclonal propagation makes it possible to obtain healthy planting material in the required quantity, regardless of the season of the year, including perennial and difficult to propagate species. The combination of microclonal propagation and cultivation under hydroponic conditions allows developing biotechnology for the production of medicinal plant materials. A team of scientists from Barnaul and Moscow has developed a biotechnology for the production of plant materials based on clonal micropropagation and cultivation under aeroponics [16, 17].

The aim of this research was to study the content of mangiferin and the antiviral activity of the raw material Iris sibirica L., obtained by biotechnology.

2. Materials and methods

The plant material of two varieties of Iris sibirica was used as raw material: Sterkh and regenerated plants are obtained according to generally accepted methods in the Biotechnology Department of the South Siberian Botanical Garden, Altai State University [18]. Intact 6-year-old plants were harvested in the vicinity of Novoaltaysk, Altai region in 2015. The raw materials were dried to an air-dry state and stored in a cool, dry place.

The mangiferin content in Iris sibirica extracts was determined by high performance liquid chromatography [19] in our modification. The differences in the methods used relate mainly to the methods of extraction and elution of substances. 0.5 g (accurately weighed) of the crushed raw material was poured into 50 ml of 60% ethanol and extracted in a water bath for one hour. It was filtered through a paper filter and the extraction was repeated twice more for 30 min. The combined extract was evaporated to dryness and the residue was dissolved in 5 ml of 70% ethanol. The xanthones were separated by the method of thinlayer chromatography. 0.05 ml of ethanolic extract was applied to paper (Sorbfil brand 10 × 10 mm) and dispersed in an ascending manner in 40% acetic acid. Then the dried chromatogram was viewed under a Lenchrome UV irradiator at a wavelength of 254 nm. The resulting spots were eluted with 70% ethyl alcohol and the volume of the solution was adjusted to 25 ml. The mangleferin content was determined by HPLC on Shimadzu instrument with the following instrument parameters: loudspeaker Zorbax SB-C18 (250 mm × 4.6 mm, 5 μm) (Agilent); wavelengths are 260-320 nm; the mobile phase is solution A (phosphoric acid (1%)) and solution B (acetonitrile); gradient is 95-90% solution A - 0-3 min, 90% solution A – 3-7 min, 90-60% solution A – 7-15 min, 60-0% solution A – 15-22 min; the injection volume is 10 μl; flow rate is 1ml/min; temperature is 22-24°C.

A sample of mangiferin from the company “Sigma” was used as a standard. The standard absorption was determined by HPLC at wavelengths of 260 nm, 320 nm and retention time of 16.886 and 16.887, respectively. During the researches, HPLC chromatograms were obtained of a test solution of regenerated plants with a retention time of 16.845 at a wavelength of 260 nm and 16.845 at a wavelength of 320 nm, as well as leaves of intact plants with a retention time of 16.829 at a wavelength of 260 nm and 16.830 at a wavelength 320 nm (figure 1).

The analysis of antiviral activity in vitro was carried out by the methods of measuring the absorption of intravitral dye by cells – neutral red (NR) [20].

The work was performed on plastic tablets to identify and determine the sensitivity of viruses to the resulting drugs. The research was conducted on scattered in tablets transplantable cell culture of the kidney of green monkey VERO. The nutrient medium was removed; herpes simplex virus of type 1 was introduced at a dose of 100 TCID50/ml in a volume of 50 μl. Virus adsorption was carried out at
37°C in the atmosphere of 5% CO₂ for 50-60 minutes. At the end of the incubation, the virus was removed and maintenance medium (MEM medium, 1% FBS, 50 μg/ml gentamicin, Gibco) was added in a volume of 200μl/well. The tablets were placed in CO₂ incubator for 48 hours.

After 48 hours, the activity of anti-herpes drugs was evaluated in a neutral red absorption test. Assessing cell viability by their absorption of neutral red is widely used in the biomedical researches. The method is based on the ability of viable cells to absorb and accumulate a neutral red supravital dye in lysosomes due to electrostatic attraction. Damage of lysosomal membranes leads to a decrease in dye accumulation; therefore, the staining intensity is proportional to the number of viable cells. Then, the optical density of the contents of the wells was measured on a Model 680 microplate reader at a wavelength of 490 nm using Zemfira 2.0 program.

3. Results and discussion

3.1. Quantitive determination of mangeferin in the raw materials Iris sibirica, cultivar Sterkh
Kovalev V N and his colleagues [19] developed the method of HPLC analysis, which can be used to quantify mangiferin in plants of the genus Iris, and is also proposed as standardization. The following results were obtained: Iris hungarica – 0,101 ± 0,003%, Iris sibirica – 0,081 ± 0,001%.

It has been proved that there is a content of mangiferin in regenerated plants of Iris sibirica L., which is an important achievement in solving the problem of developing biotechnology for obtaining plant materials as a source of valuable antiviral compound.

![Figure 1. HPLC chromatograms. A – standard solution, B – leaf extract of intact plants (Iris sibirica cultivar Sterkh).](image)

3.2. Features of accumulation of mangiferin in raw materials Iris sibirica cultivar Sterkh.
In the production of medicinal plant materials, two goals are pursued: obtaining the maximum amount of biomass and the accumulation of biologically active substances. During cultivation of I. Sibirica, the number of formed microprobe shoots and the height of the plant were determined. Multiplying these indicators, the total height of the plant was received. As a result of changes in the hormonal composition of agar culture media during microclonal propagation, a change in the multiplication factor and shoot length, and as a result, the total shoot length was noted. The differences with control are significant for culture media containing 10.0 μM BA, as well as 5.0-10.0 μM BA with auxins (figure 2).

The researches have shown that the maximum biomass accumulation (total shoot length in mm) is determined with a content of 5.0 μM 6-BA supplemented by auxins. In this nutrient medium there was a sharp increase in the indicator due to the multiplication factor, which directly depends on the concentration of the introduced BA, as well as an increase in the average shoot length, which is stimulated by auxin. The selected medium is optimal for growing I. sibirica for several years. Taking into account the high cost of phytohormones, it is recommended to use an environment with 5.0 μM BA supplemented by auxins for I. sibirica upstream.
The accumulation of mangiferin in the biomass of *I. sibirica* has been studied. A sharp decrease in the synthesis of this xanthone on a medium with 5.0 μM BA supplemented by auxins should be noted. Apparently, the more actively plants accumulate biomass, the less mangiferin is synthesized.

As it is known from the literature, the greatest amount of phenolic compounds accumulates in many plants in the aerial part in the phase of budding and flowering [4]. During this period of ontogenesis, the plant reaches the maximum values of the growth of phytomass and goes to the next stage of development – fruiting. Regenerated plants in tissue culture are constantly in the stage of active growth under the influence of phytohormones. The researcher strives to select the hormonal composition of the nutrient medium in such a way as to ensure a high reproduction rate with an optimal shoot length. This is the basis for microclonal propagation. But, as it can be seen from the presented diagram (figure 2), with an increase in the total shoot height by 5.0+A, the mangiferin in the phytomass sharply decreases. In this regard, in order to maintain a balance between biomass accumulation and flavonoid content, it is necessary for *I. sibirica* to use media with 2.5 or 10.5 μM BA supplemented with auxins. But, it’s more economical to use 2.5 μM BA.

![Figure 2. Effect of the hormonal composition of nutrient media on biomass accumulation and mangiferin content in raw materials of regenerated plants *I. sibirica* cultivar Sterkh. Designations: c.d.c. - on a completely dry substance, μM BA, +A – media supplemented with auxins.](image)

### 3.3. The study of antiviral activity against herpes simplex virus of type 1

The study of the biological activity of the extracts we obtained was carried out in the Federal State-Funded Institution of Science, Government Research Centre of Virology and Biotechnology “Vector”, r.s. Koltsovo, Novosibirsk region, Russia.

When studying the composition of water and water-alcohol extracts of *I. sibirica*, cultivar Cambridge, it was revealed that water extracts of regenerated plants and from rhizomes with spring roots were of the least toxicity (CD$_{50}$ equals 1/2 and 1/40 dilutions, respectively). All investigated extracts showed antiviral activity against herpes simplex virus. Ethyl alcohol (95%) extract of rhizomes with spring roots was the most active, ED$_{50}$ was equal to 1/3840 dilution, the selectivity index was 64. The water extract of autumn leaves was also promising; the selectivity index was 24 (table 1). The analyzed water extracts obtained from raw materials of cultivar Sterkh are non-toxic and have antiviral properties. The most powerful inhibitory effect is exerted by water extracts of regenerated plants (table 2).
Table 1. Toxicity and antiviral activity of aqueous extracts of plant materials *I. sibirica*, cultiva Cambridge.

| Test materials          | Collection time | Dissolving agent    | Toxicity, CD$_{50}$ (μg/ml) | Antiviral activity, ED$_{50}$ (μg/ml) | Therapeutic Index, IS |
|-------------------------|-----------------|---------------------|------------------------------|--------------------------------------|-----------------------|
| Rhizomes with roots     | spring          | 95% ethyl alcohol   | 500                          | 7.8                                  | 64                    |
| Rhizomes with roots     | spring          | water               | 3450                         | 431                                  | 8                     |
| Leaves                  | spring          | water               | 375                          | 94                                   | 4                     |
| Leaves                  | autumn          | water               | 725                          | 30                                   | 24                    |
| Regenerated plants      | water           |                     | 44500                        | 2781                                 | 16                    |

Table 2. Toxicity and antiviral activity of aqueous extracts of plant materials *I. sibirica*, cultiva Sterkh.

| Test materials         | Toxicity, CD$_{50}$ (μg/ml) | Efficacy ED$_{50}$ (μg/ml) | Therapeutic Index, IS |
|------------------------|------------------------------|----------------------------|-----------------------|
| Leaves                 | 25100                        | 3188                       | 8                     |
| Rhizomes with roots    | 21000                        | 2625                       | 8                     |
| Regenerated plants     | 25150                        | 1645                       | 16                    |
| Hydroponic leaves      | 52000                        | 6500                       | 8                     |

4. Conclusion
It has been proved that there is a mangiferin content in the regenerated plants of *Iris sibirica* L., which is an important achievement in solving the problem of developing biotechnology for obtaining plant materials as a source of a valuable antiviral compound.

As a result of our experiments, a characteristic relationship between biomass accumulation and mangiferin content was noted for *I. sibirica*. On a medium with 5.0 μM BA supplemented with auxin, the xanton content in the phytomass sharply decreased with an increase in the total shoot height. To maintain a balance between biomass accumulation and mangiferin content in *I. sibirica*, it is recommended to use media with 2.5 μM BA supplemented with auxin.

Water and ethanolic extracts of *I. sibirica* showed a high antiviral activity against herpes simplex virus of type 1. With low toxicity, both intact plants and regenerated plants had a relatively high selectivity index. The data obtained for intact and regenerated plants are comparable: all indicators differ little more than twice, which does not exceed the method error.

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