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OBTAINING AND BIOCHEMICAL ANALYSIS OF TISSUE CULTURE *Scutellaria baicalensis* Georgi.

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Aim. To obtain a tissue culture of *S. baicalensis* as a possible source of biologically active compounds (BAC) with a wide range of pharmacological action.

Methods. Photocolorimetric method, reversed-phase high performance liquid chromatography (HPLC) method.

Results. Two stably productive tissue culture strains (16SB3 and 20SB4) of *S. baicalensis* were obtained from fragments of roots seedling on a specially developed agar 5C01 nutrient medium. The yield of dry biomass from 1 liter of this medium per passage (21st day of growth) for strain 16SB3 is 25–30 g, for strain 20SB4 — 30–40 g. The total content of flavonoids in dry biomass was in terms of routine for strains 16SB3 and 20SB4 — 0.6–0.9 and 0.7–0.9 mg/g, respectively, and the yield of flavonoids — 18–27 and 21–36 mg/l of nutrient medium, respectively. BAC, typical for plants in nature, in particular, flavonoids vogonin, baikalein, neobaikalein, skulkapfavon and their derivatives, were found in the studied biomass of both strains.

Conclusions. It was found that the biomass of the two strains of *S. baicalensis* tissue culture accumulated the same BAC, in particular, flavonoids, as do plants in natural conditions. The resulting tissue culture is promising as a possible source of Baikal skullcap BAC.

Key words: *Scutellaria baicalensis* Georgi., plant tissue culture, flavonoids, strains — producers of biologically active compounds.

Scutellaria baicalensis Georgi. is a Daurian endemic, a popular medicinal plant. Its roots are used in traditional Chinese medicine, which is recorded in Chinese, European and British pharmacopoeias. It is a natural source of flavonoids, flavonoid glycosides, polysaccharides — substances with sedative, hemostimulating, antimutagenic, antitumor, neuroprotective, antihypoxic, nootropic, anxiolytic, antineurotic, hepatoprotective and chondroprotective properties [1, 2].

However, the natural sources of raw materials of *S. baicalensis* are almost exhausted, and the natural raw materials themselves are not always high quality and stable in their composition. An alternative source of biologically active compounds (BAC) of plant origin may be tissue and cell culture, as already shown for a number of valuable and rare medicinal plants [3, 4]. That is, the culture of Baikal sagebrush tissues can be an alternative source of practically important BAC. In Russia, Japan and some other countries, tissue cultures of *S. baicalensis* have been obtained, which are of interest as sources of the corresponding compounds [5, 6]. There is no such tissue culture in Ukraine.

We introduced into culture *in vitro* *S. baicalensis* and identified two strains of tissue culture, biochemical analysis of which showed that they were promising as a source of raw materials for flavonoids, flavonoid glycosides, polysaccharides.

Materials and Methods

Seed. Seeds of *S. baicalensis* from the 2015 harvest from the Tartu University Botanical Garden in Estonia were used for the research.

Preparation and disinfection. The seeds of Baikal skullcap were treated with 1% gibberellin solution for 22 h, then the seeds were treated with detergent (2 g of household soap was dissolved in 100 ml of cold water) for 30 min, washed with running water for 60 min, sterilization of seeds was performed under sterile conditions. The seeds were treated with 96% ethyl alcohol for 10 seconds, sterilized in 15% hydrogen peroxide solution for 20 min, washed three times in sterile distilled water and planted one seed to germinate in test tubes on a specially designed nutrient medium 5C01 with mineral base described in [7].

Cultivation. Plant tissue culture was obtained from fragments of roots seedling on the same composition of the nutrient medium 5C01. Seeds were germinated and callus was initiated in 20 ml and 1 cm diameter tubes containing 1 ml of agar medium. The resulting callus (tissue culture) was grown in glass vessels (jars) with a volume of 250 ml, containing 40 ml of medium, at a temperature of 24–26 °C without lighting at a relative humidity of 70–80%. All materials and components of nutrient media were domestically produced.

Materials, methods and equipment for biochemical research. The total content of flavonoids was determined on a photoelectrocalorimeter Photometer KFK3 according to the method [8]. The used reagents were of domestic production.

Preparation of samples. The samples of callus tissue, dried at 54–56 °C, were ground in an agate mortar and filled with methanol (10 ml per 1 g of sample).

Analysis of secondary compounds was carried out by reversed-phase high performance liquid chromatography. Separation of the samples was performed on an Agilent 1100 chromatographic system with 4-channel pump, vacuum degasser, autosampler, column thermostat and diode-matrix detector. It was used a two-eluent scheme (eluent A = 0.05 M aqueous solution of orthophosphoric acid H_3PO_4; B = methanol/all eluents and additives Sigma-Aldrich, gradation of purity HPLC) on a column Poroshell C18, 2.7 µm, 2.1×150 mm with a passport resolution of more than 18,000 t.t. We made use of sample volume 5 µl, column temperature was 20 °C, flow rate — 0.15 ml/min, analysis time up to 70 min. Detection at wavelengths of 206, 254, 300, 350 and 450 nm was carried out to determine the most organic compounds (including terpenoids), most aromatic substances, phenylpropanoids (oxycinnamic acids and lignans), flavonoids (flavones and flavonols), carotenoids and chlorophyll, respectively. Absorption spectra were recorded for all substances in the ultraviolet and visible ranges in order to establish the nature of secondary metabolites and assign chromatographic peaks to certain groups of substances [9]. Vogonin was identified based on chromatographic data of the standard.

Statistical analysis. The calculation of the Student's test was performed using the Origin program.

Results and Discussion

Primary calluses obtaining. Five days later, 4 seeds out of 10 planted germinated (Fig. 1). With a sterile scalpel, the seedlings roots of *S. baicalensis* were cut into pieces 1–3 mm long placed in the test tubes on nutrient medium 5C01, and left in a thermostat at a temperature of 24–28 °C in dark until the formation of callus tissue. A month later, a callus was formed, well visually accessible, the size of the pieces of callus was 3–4 mm in diameter. The frequency of callus formation reached 100%. Pieces of callus were transferred to Petri dishes with a nutrient medium of the same composition. Then callus culture was grown under the same conditions for another month. The selected light, actively growing pieces of callus culture were transferred to 40 ml and 2 cm diameter tubes containing 2 ml of nutrient medium.

Three weeks later, the most productive light yellow and yellow, pink-colored pieces of callus culture were collected and transferred to 250 ml glass jars containing 40–45 ml of nutrient medium of the same composition. The passage duration was reduced to 3 weeks. After several consecutive passages, two strains (variants) of callus culture of tissues 16SB3 and 20SB4 of Baikal skullcap of two genotypes (from two different original seedlings) were obtained.

Cultivation of strains 16SB3 and 20SB4 of *S. baicalensis* plant tissue culture was performed at a temperature of 24–28 °C, relative humidity of 70–80% in dark in a one-step method on agar nutrient medium 5C01, prepared according to the recipe of [5]. The medium was poured into glass jars with a

Fig. 1. **One of the four *S. baicalensis* seedlings**

volume of 250 ml of 40–45 ml, covered with foil, autoclaved for 15 min at a pressure of 1 atmosphere.

The biomass of both strains consisted of cells and cell aggregates of medium density. Strain 16SB3 was homogeneous, medium density, culture color from light yellow to dark yellow with a pink tinge (Fig. 2). Strain 20SB4 was homogeneous, loose, culture color from light yellow to dark yellow (Fig. 3).

The duration of the passage of both strains was 21 days. The weight of the inoculum when transplanted to fresh nutrient medium was 100–150 g of living biomass per 1 liter of nutrient medium.

The type of growth was disorganized, the growth index for strain 16SB3 — 4, for 20SB4 — 5. The yield of dry biomass on the 21st day of growth for strain 16SB3 was 25–30 g/l of medium, and for strain 20SB4 — 30–40 g/l (Table 1).

Biochemical analysis of the total content of flavonoids showed that the dry biomass contained flavonoids in terms of rutin for 16SB3 — 0.6–0.9 mg/g of dry tissue or 18–27 mg/l of nutrient medium on day 21 of growth, for 20SB4 — 0.7–0.9 mg/g of dry tissue or 21–36 mg/l of nutrient medium for 21 days (Table 1).

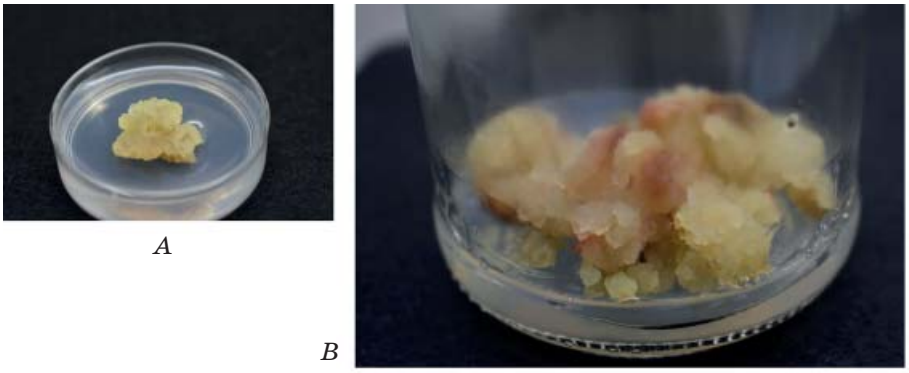
Strains 16SB3 and 20SB4 of Baikal skullcap tissue culture are stored in the collection of plant tissue cultures of the Institute of Molecular Biology and Genetics of the National Academy of Sciences of Ukraine. At the time of writing, both strains were cultivated for 1.5 years, passed 20 passages and were characterized by the stability of the studied characteristics.

Analysis of the spectrum of secondary compounds. Qualitative biochemical analysis of the spectrum of secondary compounds of both strains (16SB3 and 20SB4) of *S. baicalensis* tissue culture by high-performance liquid chromatography revealed substances characteristic of the plant in nature [5, 6], for example, the flavonoids neobaikalein, skulcapflavone, baicalein, wogonin and their derivatives.

Figures 4 and 5 show the chromatographic profiles of dry biomass extract of *S. baicalensis* tissue culture of both strains 16SB3 and 20SB4. Comparison of profiles shows that the strains differ only in the number of secondary compounds accumulated by culture cells (Table 2–5).

Thus, strain 20SB4 predominates in the content of derivatives of benzoic and oxybenzoic acids, flavanes, and the content of individual flavonoids of neobaikalein, baikalein and wogonin, while strain 16SB3 contains more flavonoid skulcapflavone.

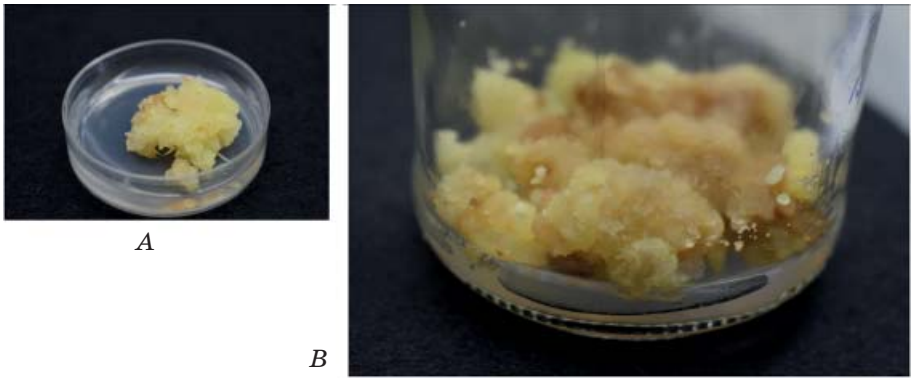
Further study of the obtained plant tissue culture will allow to determine a promising version of the culture.



A

B

Fig. 2. **Strain 16SB3 of *S. baicalensis* plant tissue culture for cultivation in Petri dishes (*A*) and in glass vessels with a volume of 250 ml (*B*)**



A

B

Fig. 3. **Strain 20SB4 *S. baicalensis* plant tissue culture for cultivation in Petri dishes (*A*) and in glass vessels with a volume of 250 ml (*B*)**

Table 1. **Productivity of strains 16SB3 and 20SB4 of *S. baicalensis* plant tissue culture**

Strain	Passage	Yield of dry weight, g/l environment	Yield of dry weight, % from the living	Growth index	The content of flavonoids, mg/g of dry tissue
16SB3	5 9 16	35.0 ± 0.07 30.6 ± 0.10 30.9 ± 0.05	4.9 ± 0.21 5.5 ± 0.09 6.2 ± 0.06	4 4 4	0.73 ± 0.01 1.46 ± 0.01 1.15 ± 0.03
20SB4	5 9 16	33.6 ± 0.09 27.3 ± 0.08 32.0 ± 0.06	4.8 ± 0.12 4.6 ± 0.10 4.2 ± 0.08	5 5 5	0.75 ± 0.01 0.94 ± 0.07 1.04 ± 0.01

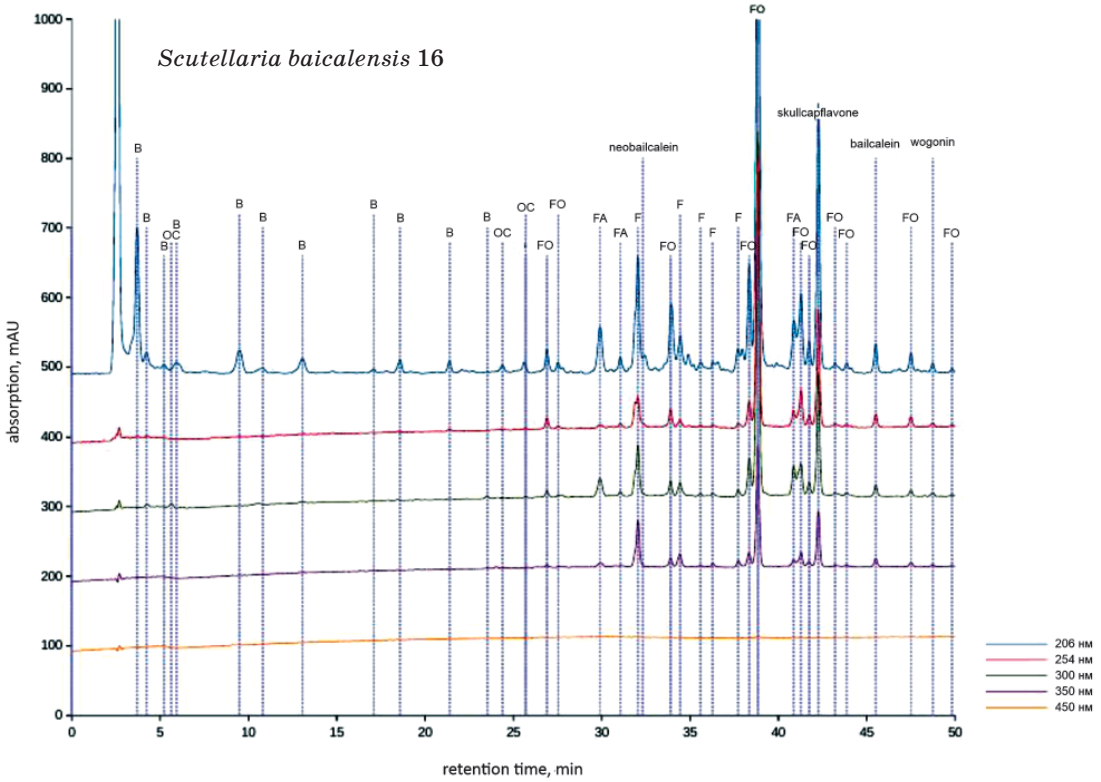


Fig. 4. **Chromatographic profiles of dry biomass extract of strain 16SB3 of *S. baicalensis* plant tissue culture**
Labels of substances: A — unrestrained pool of hydrophilic substances (free organic acids, amino acids, etc.) + solvent; B — derivatives of benzoic and oxybenzoic acids; OS — derivatives of oxycinnamic acid: F — flavans; FA — flavones; FA — flavones similar to neobaikalein (there are hydroxyls in the phenyl moiety); FO — flavones similar to baicalein and vogonin (without hydroxy fragments); F — other flavonoids; flavones: neobaicalein, skullcapflavone, baicalein, wogonin

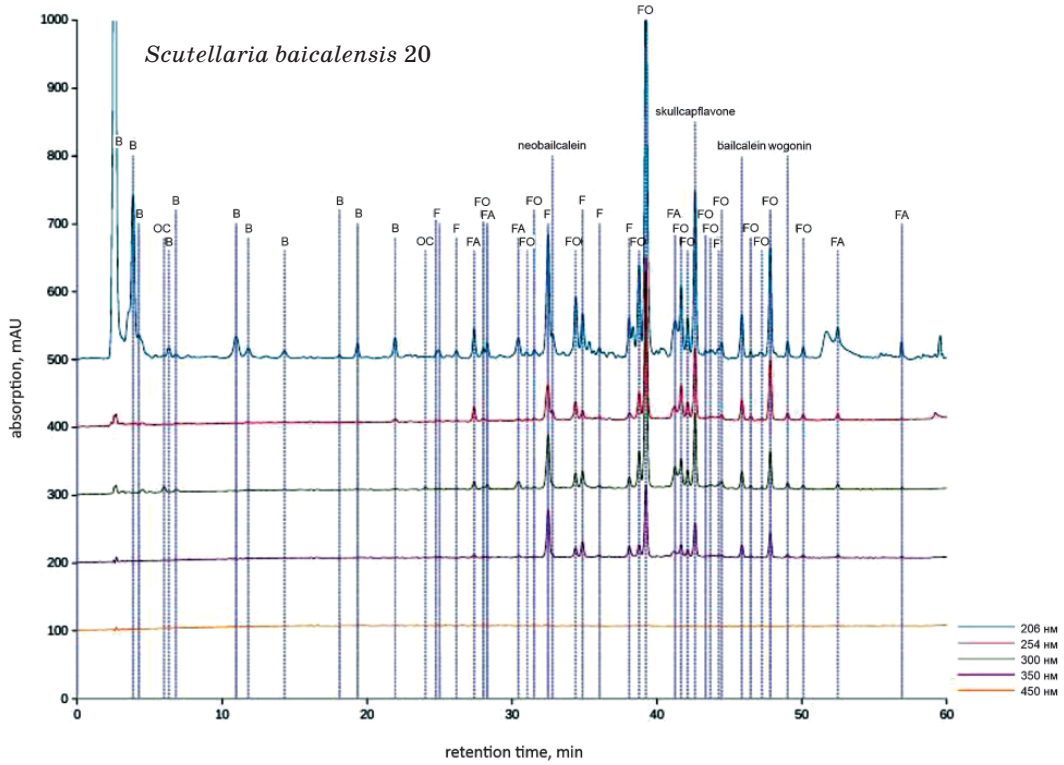


Fig. 5. **Chromatographic profiles of dry biomass extract of strain 20SB4 of *S. baicalensis* plant tissue culture**

Labels of substances: A — unrestrained pool of hydrophilic substances (free organic acids, amino acids, etc.) + solvent; B — derivatives of benzoic and oxybenzoic acids; OS — derivatives of oxycinnamic acid; F — flavans; FA — flavones; FA — flavones similar to neobaikalein (there are hydroxyls in the phenyl moiety); FO — flavones similar to baicalein and vogonin (without hydroxy fragments); F — other flavonoids; flavones: neobaicalein, skullcapflavone, baicalein, wogonin

Table 2. **The content of certain classes of organic substances in the dry biomass of strain 16SB3 of *S. baicalensis* plant tissue culture, (μg/g), the measurement error of the device ≤ 10%**

Substances*	Content, μg/g of biomass
B	2630
OC	174
F	890
FA	527
FO	1510

Note: * B — derivatives of benzoic and oxybenzoic acids; OS — derivatives of oxycinnamic acid; F — flavans; FA — flavones; FA — flavones similar to neobaikalein (there are hydroxyls in the phenyl moiety); FO — flavones similar to baicalein and vogonin (without hydroxyl fragments); F — other flavonoids.

Table 3. **The content of individual flavones in the dry biomass of strain 16SB3 plant tissue culture *S. baicalensis* (μg/g), the measurement error of the device ≤ 10%**

Flavon	Content, μg/g of biomass
Neobaicalein	5
Skullcapflavone	354
Baicalein	36
Wogonin	17

The peaks in the chromatograms were assigned to specific compounds based on the literature UV spectra. Vogonin was identified based on chromatographic data of the standard.

Table 4. **The content of certain classes of organic substances in the dry biomass of strain 20SB4 of *S. baicalensis* plant tissue culture, (μg/g), the measurement error of the device ≤ 10%**

Речовини*	Content, μg/g of biomass
B	3080
OC	89
F	1120
FA	499
FO	1180

*Note:** B — derivatives of benzoic and oxybenzoic acids; OS — derivatives of oxycinnamic acid; F — flavans; FA — flavones; FA — flavones similar to neobaikalein (there are hydroxyls in the phenyl moiety); FO — flavones similar to baicalein and vogonin (without hydroxy fragments); F — other flavonoids.

Table 5. **The content of individual flavones in the dry biomass of strain 20SB4 plant tissue culture *S. baicalensis* (μg/g), the measurement error of the device ≤ 10%**

Flavon:	Content, μg/g of biomass
Neobaicalein	12
Skullcapflavone	227
Baicalein	54
Wogonin	21

The peaks in the chromatograms were assigned to specific compounds based on the literature UV spectra. Vogonin was identified based on chromatographic data of the standard.

Conclusions

Thus, Baikal skullcap *Scutellaria baicalensis* Georgi was introduced into *in vitro* tissue culture. Primary calluses were obtained from fragments seed seedlings roots of on a nutrient medium 5C01, specially created for plant cultures of tissues — superproducers.

The promising strains (16SB3 and 20SB4) of *S. baicalensis* tissue cultures were selected and the total content of flavonoids in dry tissue was determined. The yield of dry biomass from 1 liter of medium on the 21st day of growth for strains 16SB3 and 20SB4 is 25–30 g and 30–40 g, respectively. The content of flavonoids in dry biomass was in terms of rutin for strains 16SB3 and 20SB4 — 0.6–0.9 and 0.7–0.9 mg/g of tissue or 18–27 and 21–36 mg/l of nutrient medium for 21 day respectively.

The biochemical spectrum of flavonoids synthesized by cells of the obtained strains was determined. Cultivated callus cells were found to accumulate the same BAC, in particular, wogonin, baicalein, neobaikalein, and skulkapfavone, as *S. baicalensis* plants in nature.

The obtained plant tissue culture is the first stage in the development of cellular biotechnology for the production of practically valuable Baikal skullcap compounds in Ukraine.

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ОТРИМАННЯ ТА БІОХІМІЧНИЙ АНАЛІЗ КУЛЬТУРИ ТКАНИН *Scutellaria baicalensis* Georgi

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Мета. Отримати культуру тканин шоломниці байкальської *S. baicalensis* як можливого джерела біологічно активних сполук (БАС) із широким спектром фармакологічної дії.

Методи. Фотоколориметричний метод, метод обернено-фазової високоефективної рідинної хроматографії (ВЕРХ).

Результати. Отримано два стабільно продуктивні штами культури тканин (16SB3 та 20SB4) *S. baicalensis* із фрагментів корінців насіннєвих проростків на спеціально розробленому агаризованому живильному середовищі 5С01. Вихід сухої біомаси з 1 л цього середовища за пасаж (21-й день росту) для штаму 16SB3 становить 25–30 г, для штаму 20SB4 — 30–40 г. Загальний вміст флавоноїдів у сухій біомасі становив у перерахунку на рутин для штамів 16SB3 та 20SB4 — 0,6–0,9 та 0,7–0,9 мг/г відповідно, а вихід флавоноїдів — 18–27 та 21–36 мг/л живильного середовища відповідно. У дослідженій біомасі обох штамів виявлено БАС, властиві рослинам у природі, зокрема, флавоноїди вогонін, байкалеїн, необайкалеїн, скулкапфавон та їхні похідні.

Висновки. Встановлено, що в біомасі двох отриманих нами штамів культури тканин *S. baicalensis* накопичуються ті самі БАС, зокрема, флавоноїди, що й у рослин у природі. Отримана культура тканин є перспективною як можливе джерело БАС шоломниці байкальської.

Ключові слова: *Scutellaria baicalensis* Georgi., культура тканин рослин, флавоноїди, клітинні штами — продуценти біологічно активних сполук.