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Extraction of biologically active substances from Siberian fir and effect of extracts on grain crops germination

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Abstract. This work considers methods of obtaining coniferous extracts containing triterpenic acids (TTAs) as the main active substance. Five coniferous extracts were obtained, the raw materials for their preparation were Siberian fir (*Abies sibirica* L.) wood greens, as well as extracted fir meal and oil-ester complex, both obtained after subcritical carbon dioxide extraction (CO_2-extraction). In order to determine the composition of the obtained extracts, a method of analysis was developed and the study was conducted using high-performance liquid chromatography with mass spectrometric detection (HPLC-MS). The necessity of developing the new analytical method is the fact that the obtained extracts are samples with complex composition. It was found that the composition of the obtained samples includes triterpenic, fatty and resin acids. Seeds of grain crops – spring wheat and spring barley were taken as biological test objects. Laboratory study of the effect of the obtained coniferous extracts on germination, germination energy and morphometric parameters was conducted, for this purpose seeds were treated with aqueous solutions of the obtained coniferous extracts. Samples 1 and 5 showed the greatest stimulating effect, which is manifested, among other, in an increase in the length and mass of sprouts and an increase in mass of 1 plant. The effect of coniferous extracts on seed pathogens of spring wheat in standard phytopathological analysis was also evaluated. The decrease by 1.4 times relatively to the control of total infestation of wheat seeds with seed infection pathogens while using the sample 3 was observed. The obtained data show the perspective of further research in obtaining and studying samples based on Siberian fir extracts for use in agriculture as growth-stimulating and fungicidal preparations.

Keywords: coniferous extracts, triterpenic acids, wheat, barley, initial growth characteristics of seeds, HPLC-MS

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Научная статья
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Извлечение биологически активных веществ из хвойных растений с фунгицидными и росторегулирующими свойствами

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Аннотация. Рассмотрены способы получения растительных экстрактов, содержащих тритерпеновые кислоты (ТТК) в качестве основного действующего вещества, из древесной зелени пихты сибирской. Для определения состава полученных экстрактов было проведено исследование методом высокоэффективной жидкостной хроматографии с масс-спектрометрическим детектированием (ВЭЖХ-МС). Проведено лабораторное исследование влияния хвойных экстрактов на всхожесть, энергию прорастания и морфометрические параметры семян зерновых культур (яровой пшеницы и ярового ячменя), а также оценено влияние хвойных экстрактов на возбудителей семенной инфекции яровой пшеницы в стандартном фитопатологическом анализе.

Ключевые слова: хвойные экстракты, тритерпеновые кислоты, пшеница, ячмень, начальные ростовые показатели семян, ВЭЖХ-МС

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Introduction

Conifers, including Siberian fir (*Abies sibirica* Ledeb.), are known as a large reservoir of biologically active substances, in particular terpenoids [1], as well as carotenoids, fatty acids and their derivatives, triterpenic acids, phenolic compounds, vitamins, etc. Coniferous extracts are widely used in various fields, including medicine when treating the respiratory system diseases, musculoskeletal system diseases, genitourinary system diseases, skin diseases [2, 3] and agriculture due to their fungicidal, bactericidal and growth-promotion properties. However, at the moment, there is a small amount of research on the development of crop protection products with fungicidal and growth-promoting properties obtained from fir.

Using terpenoids in agriculture and forestry is of profound interest. Soviet chemists have begun studying Siberian fir terpenoids and methods of their isolation since 1986 [4]. The extracts had a positive effect on the growth of various crops, for example, that of grain crops. Subsequently, a number of patents were issued on methods of isolating biologically active amounts of terpenic acids [5-8]. Such biological fir extracts contained a mixture of different compounds. Among them, the object of the greatest interest are terpenoids, namely diterpenic acids (so-called "resin" acids) and triterpenic acids (TTAs, so-called "polar" acids); moreover, the main stimulating activity in relation to the cultivated plants is attributed to TTAs [4].

Intensifying technological processes aimed at isolating extractive substances, an integrated approach to processing raw material, the use of new technologies, a detailed study of the chemical composition and biological activity contribute all together to expanding the range of the obtained products [9].

In this regard, a crucial task is to obtain new products containing biologically active substances, including triterpenic acids, as well as to develop a methodology of the quantitative determination of the substances present in the products based on extracts and to conduct the biotesting of different concentrations of the samples in terms of the stimulating activity towards agricultural crops.

Material and methods

The method of extraction using liquefied carbon dioxide (CO_2 extraction) has become widespread. The small size of CO_2 molecules allows the process to be conducted at a cellular and molecular level, extracting biologically active substances (BAS) in the same composition and ratio as they are naturally present in plant raw material. The final product of the CO_2 extraction method is a superconcentrated substance that includes water part and oil-ether complex. The composition of oil-ether complex obtained through carbon dioxide extraction is different from oil-ether complex obtained through the classical steam distillation method. CO_2 extraction allows to isolate the heavier molecules and has several advantages: a higher quantitative yield of extracts and a richer chemical composition (the composition of Siberian fir CO_2 extract, unlike essential oil, is enriched

with heavy resinous components and oxygen-containing pigments such as carotenoids and xanthophylls) [10]. In this work, 5 samples containing TTAs were obtained from Siberian fir by several ways. The initial stage for 5 samples was CO_2 extraction. Four samples were obtained by treating fir meal after carbon dioxide extraction, 1 sample was obtained by treating the oil-ether complex of Siberian fir. The scheme of 5 samples obtaining from plant raw material is presented in figure 1.

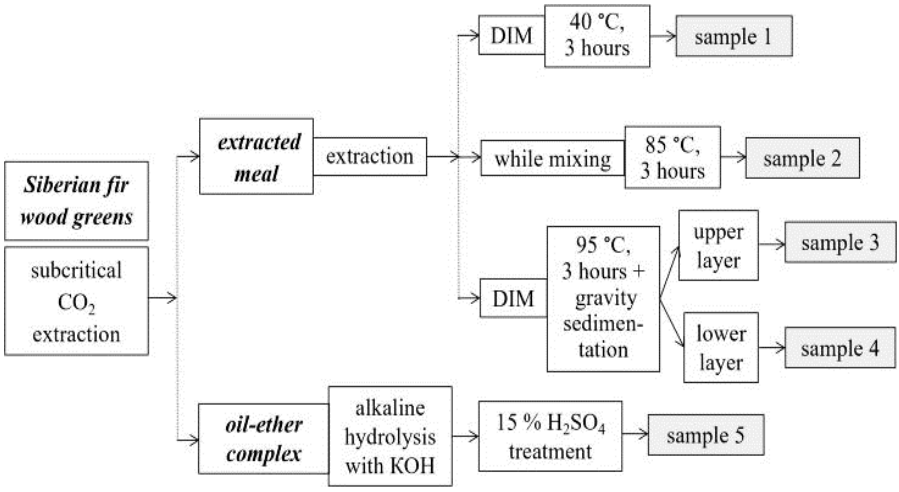

Fig. 1. The scheme of experimental samples obtaining from Siberian fir wood greens

The separation of triterpenic acids was carried out with the high-performance liquid chromatography with mass spectrometric detection (HPLC-MS) in the reverse-phase mode [11]. To purify the samples from mechanical impurities and compounds strongly retained in the reverse-phase mode, solid-phase extraction was performed on the HF Bond Elut LRC-C18 cartridges. The assessment of the total content of triterpenic acids was conducted using the external standard method. As an external standard, ursolic acid was chosen. The chromatogram of sample 3 is shown in figure 2.

In addition to triterpenic acids, fatty and resin acids were found in the composition of the obtained samples. The characteristics of the samples obtained and the results of the HPLC-MS analysis are given in the table 1.

The obtained samples have a complex composition represented by triterpenic acids (including somariyesic, firmanic and cis-sibiraminic), fatty acids (linolenic, oleinic, palmitoleic, arachidonic and eicosenoic) and resin acids (isomers of pimaric and abietinic, lambertianic). It has been established [12] that terpenoid extracts containing a complex of compounds with different structures and pharmacological activities, are stronger and more effective than an individually isolated substances. Therefore, often in certain cases, natural extracted set of terpenoids with complex composition is used.

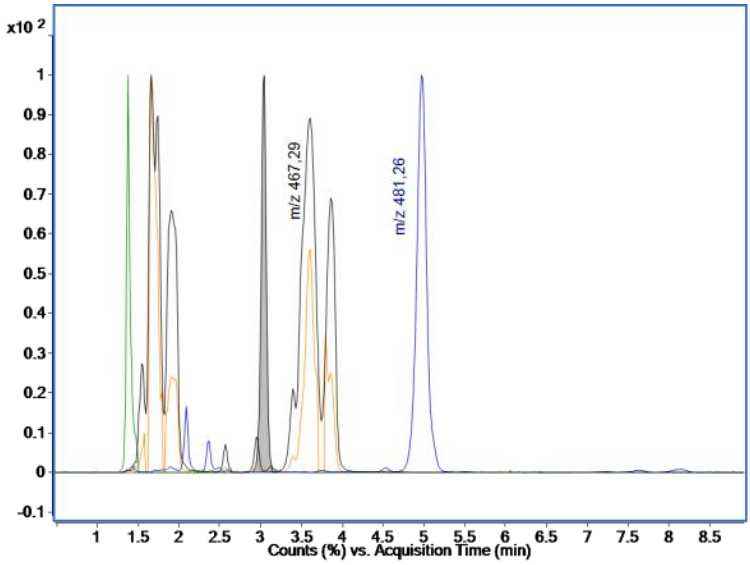

Fig. 2. Extracted ion current (EIC) chromatogram, sample 3

Table 1

Characteristics of the obtained samples

Sample	Humidity, %	Dry residue, %	pH	Content of TTAs, %	Content of fatty acids*, %	Content of resin acids*, %
1	73.20	26.80	9.90	44	1.00	1.00
2	33.50	66.50	9.50	12	2.64	2.15
3	48.30	51.70	8.12	67	0.77	1.08
4	77.20	22.80	8.28	<1%	0.03	0.04
5	29.50	71.50	8.92	64	1.89	2.70

* – relatively to sample 1.

The influence of the coniferous extracts on the germination, germination energy and morphometric parameters of the grain crop sprouts was investigated. The test objects were spring wheat of the “Likamero” variety and spring barley of the “Paustian” variety. In the case of spring wheat, the experiment was carried out in 4 replications and 3 repetitions (50 plants in one Petri dish); in the case of spring barley, there were 4 replications and 1 repetition (50 plants in one Petri dish). The tests were conducted to compare them with the control (distilled water) and the “Biosil” preparation (a growth regulator, an aqueous emulsion of triterpenic acids (LLC “Alsiko-agroprom”, Moscow, Russia). The duration of one repetition was 21 days.

The seeds were treated so that the amount of the preparation introduced per 1 gram of the seeds participating in the experiment would correspond to the amount of the preparation introduced per 1 gram of the seeds treated in production conditions during seed treatment. This process was performed as follows. 1 ml of the

working solution was sprayed on 83.3 g of wheat placed in a container; then the grain was stirred with a plastic stirrer for 1 minute. 50 grains were selected and placed in Petri dishes on the filter paper that was previously moistened with 4.0 ml of distilled water. Throughout the experiment, the humidity of the filter paper was monitored, and the same amount of water was added to all the Petri dishes if required. The Petri dishes were held in darkness at a temperature of 18-20 °C.

After the third day, the germination energy was calculated, and after seven days, the germination was calculated according to [13, 14], the length of the sprouts, the number of roots, the average length of the roots and the total length of the roots were analyzed using the ImageJ program (https://imagej.nih.gov/ij/). The roots and sprouts were separated from the grain and dried separately; then their mass was calculated in an air-dry state. The statistical data were processed using Statistica 10 (http://statsoft.ru/); the nonparametric Mann-Whitney criterion was used to compare the versions.

The spring wheat seeds were also phytopathologically examined. A biological method was used (when the seeds were germinated using filter paper or the method of germination in rolls). Tapes made of filter paper 10x100 cm (±2 cm) in sizes and tracing paper 3x100 cm (±2 cm) in sizes were prepared in accordance with GOST 12038-84 for the examination [4]. The material was packed in envelopes and then sterilized in the autoclaving mode of 1 atm for 20 min. Before starting the phytoexpertise, the surfaces of the desktop, Petri dishes and tweezers were disinfected with alcohol (70%). The filter paper was removed from the sterilized envelopes with the tweezers and immersed into a container filled with hot distilled water (+95 °C) for a few seconds. The wet tape was laid out on the desk surface; the seeds were placed by means of the tweezers on the filter paper with the embryos down at a distance of 2÷3 cm from the upper edge of the sheet at a distance of 2 cm from each other. The wheat seeds were treated with the calculated concentrations of the samples in the specified doses. The control was the seeds treated with sterile water without adding the samples.

The seeds laid out on the paper, were covered with tracing paper moistened in boiling water, after which they were laid in rolls. The rolls were placed vertically into a sterile plastic bag to prevent drying and then were incubated in the thermostat at a temperature of 24±1 °C for 7 days. The germination and the species composition of pathogens of seed infections were determined according to GOST 12044-93 [13]. The infectious agents were determined under a binocular microscope at an 8x2 magnification.

Results

The results of testing the influence of coniferous extracts on the germination, germination energy, and morphometric parameters of spring wheat seeds are presented in figure 4.

Relatively to the pure control, sample 1 led to an increase in the length of seedling sprout by 4 % and sprout mass by 26 %. The percentage of sprout mass in the total plant mass increased from 45.9 % in the control to 53.8 % in the experiment.

Also, an increase in sprout mass by 26 % and plant mass by 15 % was recorded in the experimental variant relative to the variant with “Biosil”.

Sample 2 decreased wheat sprout length by 4%, increased plant mass by 9% in the experimental variant relative to the variant with “Biosil”.

Sample 3 caused an increase in wheat sprout length relative to the pure control by 6 %, increasing also the percentage of sprout mass in the total plant mass from 45.9 % in the control to 52 % in the experiment.

The use of sample 5 led to an increase in the length of wheat sprouts by 4 % compared to the pure control.

All the variances described are statistically significant.

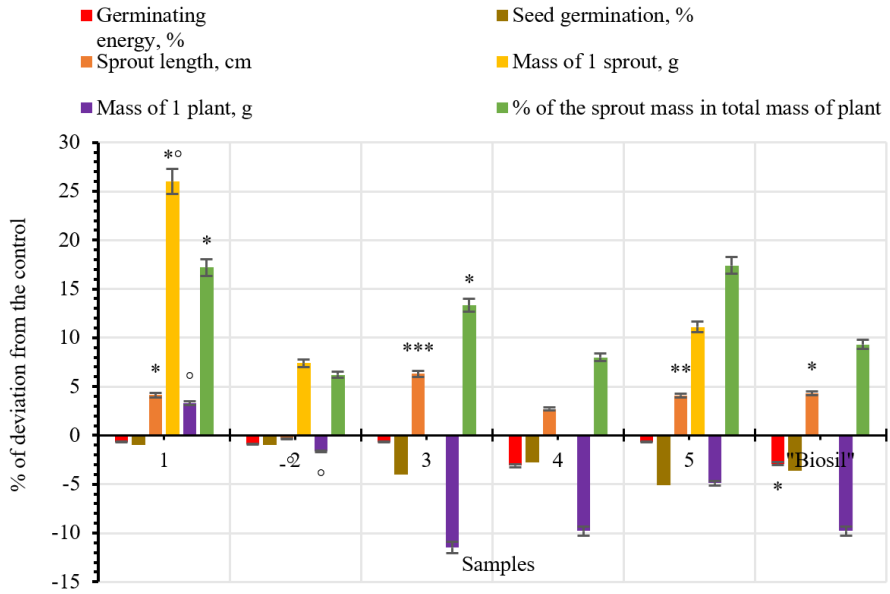

Fig. 4. The results of testing the effect of coniferous extracts on germination of “Licamero” wheat variety in relation to the control in %: *1* – sample 1; *2* – sample 2; *3* – sample 3; *4* – sample 4; *5* – sample 5. * – differences are reliable with $p < 0.05$ compared to control; ** – differences are reliable with $p < 0.01$ compared to control; *** – differences are reliable with $p < 0.001$ compared to control; °° – differences are reliable with $p < 0.05$ in comparison with “Biosil” variant

The results of testing the influence of coniferous extracts on the germination, germination energy, and morphometric parameters of the spring barley germs are presented in figure 5.

Sample 1 caused an increase in barley seed germination by 25 % and an increase in plant mass by 217 % due to sprout mass and root mass.

Sample 2 also caused an increase in mass of 1 plant (by 129 %).

Treatment of barley seeds with sample 3 caused an increase in root mass by 109 % and an increase in plant mass by 201 %.

The use of “Biosil” preparation caused an increase in root mass by 108 % and an increase in total plant mass by 201 %. A tendency to increase sprout mass and

the percentage of sprout mass in the mass of 1 plant was observed with the use of all samples.

All the variances described are statistically significant.

The results of the phytoexpertise (Table 2) of the spring wheat seeds allowed revealing the fact that the studied samples did not influence the seed germination.

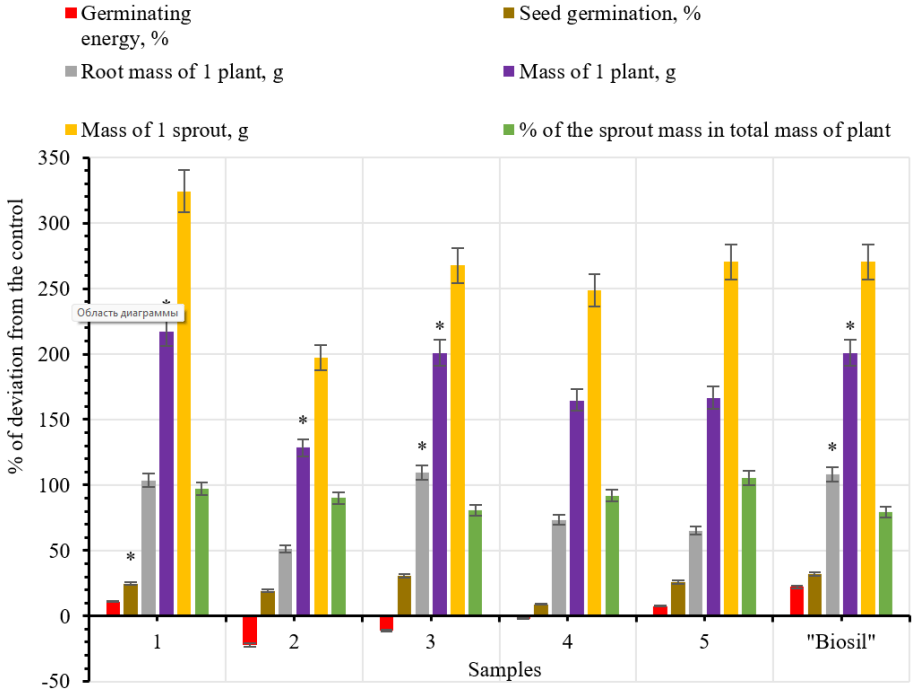

Fig. 5. The results of testing the effect of coniferous extracts on germination of barley seeds of "Paustian" variety in relation to the control in %: *1* – sample 1; *2* – sample 2; *3* – sample 3; *4* – sample 4; *5* – sample 5. * – differences are reliable with $p < 0.05$ compared to control

Table 2

Germination and infectiousness of the wheat seeds with seed infections when treated with the experimental samples (%) (n = 100)

Experiment version	Germination	Infected seeds in total	Including				
			Helmintho-sporiosis	Fusariosis	Alternariosis	Bacteriosis	Molds
Control	$96.0^{+3.0}_{-4.7}$	43.0 ± 9.7	0	$2.0^{+3.7}_{-1.8}$	31.0 ± 9.1	0	$10.0^{+6.6}_{-5.5}$
"Biosil"	$94.0^{+3.8}_{-5.5}$	25.0 ± 8.5*	0	$2.0^{+3.7}_{-1.8}$	$17.0^{+8.0}_{-6.7}$	0	$6.0^{+5.5}_{-3.8}$
1	$96.0^{+3.0}_{-4.7}$	32.0 ± 9.0	0	0	$23.0^{+8.5}_{-7.7}$	0	$9.0^{+6.3}_{-4.8}$
2	$97.0^{+1.7}_{-4.2}$	32.0 ± 9.0	0	0	28.0 ± 8.8	0	$4.0^{+3.7}_{-2.9}$
3	$91.0^{+4.8}_{-6.4}$	30.0 ± 9.0*	0	0	$17.0^{+8.0}_{-6.7}$	$1.0^{+2.8}_{-1.0}$	$12.0^{+7.0}_{-5.6}$
4	$95.0^{+3.4}_{-5.1}$	42.0 ± 9.7	0	0	35.0±9.4	0	$7.0^{+5.8}_{-4.2}$
5	$92.0^{+4.5}_{-6.1}$	39.0±9.6	0	$3.0^{+4.2}_{-2.4}$	$23.0^{+8.5}_{-7.7}$	$1.0^{+2.8}_{-1.0}$	$12.0^{+7.0}_{-5.6}$

Note. n – sample size (pieces); * – obtained data differ reliably from the control ($p < 0.05$).

A decrease in the total infectiousness of the wheat seeds with the pathogens of seed infections when treated with "Biosil" by an average of 1.7-times was observed relatively the control and a 1.4-time decrease was noted when the seeds were treated with sample 3. There was not found the influence on the causative agents of individual diseases.

Conclusion

As a result of studying the influence of the coniferous extracts on the initial growth qualities of the spring wheat seeds of the "Likamero" variety and the spring barley of the "Paustian" variety, a positive effect of using samples 1, 3 and 5 on the length and mass of the wheat sprouts was demonstrated. Their weak depressing influence on the root system of the wheat sprouts, the stimulating influence of samples 1, 2 and 3 on the development of barley germs, was shown. Besides, sample 3 introducing contributed to reducing the overall infectiousness of the wheat seeds with the pathogens of seed infections. Samples 3 and 5 can be recommended for applying in the agricultural production of spring wheat and spring barley after determining the optimal concentrations.

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Contribution of the authors: the authors contributed equally to this article.
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