Research on Creation of Methods for Determining Betulin in Birch Bark Processing Products Using HPLC

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Abstract. The paper assesses the possibility of creating a method for determining betulin in birch bark processing products using HPLC. The relevance and feasibility of developing an analysis are associated with an increase in the production of various products from birch bark and the need for a reliable and rapid way to assess their quality. The basis of the hypothesis is the reproducibility of the determination results of betulin in birch bark processing products by means of chromatography-mass spectrometry using the method of reversed-phase high-performance liquid chromatography. Initial qualitative and quantitative analyzes of betulin-containing extracts are carried out using gas chromatography-mass spectrometry methods. The reproducibility of the results obtained using HPLC has been verified. HPLC analysis of betulin-containing extracts and agrochemical “Stimulin” have revealed a correlation of the quantitative composition of betulin-containing preparations.

1. Introduction
An important role in industrial agriculture and crop production for crop yields, germination, improvement of structural and biometric indicators, and increase in productivity of fruiting is played by plant growth stimulants [1]. Synthetic drugs of various hazard classes are often toxic to humans, animals, or bees; therefore, stimulants of natural origin, plant, animal, or mineral, that are ecologically safe in agriculture, are topical. To increase crop yields, complex effects are needed both during seed treatment and during the growing season. It is advisable to use natural immunomodulators, stimulants and fungicides. The study of the optimal concentration of the complex of drugs and the time of its impact on various seeds requires a significant investment of time, effort and money from agricultural enterprises [2]. The actual direction in agriculture is the use of substances of plant origin that can positively affect the germination, yield, growth processes, resistance to diseases and pests [3, 4]. In Siberia, in conditions of relatively less soil supply with nutrients, such an organic preparation is Stimulin, developed by the scientific and technical department of the company Altai AGRO Gardens LLC. This suspension is made from birch bark extraction products, the active substance is betulin. To increase the resistance of the seed to fungal and bacterial diseases, it is etched before planting. For a sustainable anti-stress response of plants to adverse conditions (weather, pesticide treatment, etc.), stimulation of growth and accelerated ripening of the crop, the sown areas are treated with Stimulin during the growing season [5].
2. The current state of the issue

The chemical composition of birch bark includes up to 40% betulin, which is a biologically active substance, used, as a rule, in medicine for the production of dietary supplements or the synthesis of drugs [6, 7]. Compositions based on this biologically active substance are developed and tested as plant protection products.

Experimental studies on the extraction of betulin from vegetable raw materials, the synthesis of new compounds from it, the preparation of marketable products must be accompanied by the identification of the compositions of the starting material, intermediate and end products.

To identify the composition of plant materials, qualitative analysis and research of the physicochemical properties of betulin, various methods are used: refractometric, photometric, electrochemical, chromatographic (adsorption, distribution, ion exchange chromatography, etc.).

Researchers A.N. Kislitsyn, S.A. Kuznetsova, V.A. Levdansky, B.N. Kuznetsov, V.I. Roshchin identify several methods for obtaining betulin from birch bark by extraction with various solvents [8-11]. The degree of extraction of the substance, the presence of impurities are due to the different solubility of betulin and other components of birch bark in most organic solvents, therefore reliable rapid analysis of the composition of the mixtures is important to study the process of extracting betulin-containing extract under laboratory conditions [12-15].

The quantitative analysis of the components of extraction of birch bark is a difficult analytical task; there is also no certified method for the quantitative determination of betulin in the Russian Federation. The previously mentioned studies suggest the analysis of dry extract of birch bark, powdered products containing betulin, however, the methods for quantitative determination described in the literature do not affect other formulations of betulin-containing compositions, such as suspensions. Gas chromatography-mass spectrometry is a unified method for analyzing many herbal compositions with high reliability, but such complex and expensive analytical equipment is available only in specialized centers. Effective identification and determination of the content in mixtures of this type of compounds is possible using high-performance chromatography using fluorescent or ultraviolet detectors. Physicochemical studies of products of alkaline hydrolysis of bark and betulin are known in the pharmaceutical composition in powder form using chromatographic analysis techniques, namely gas chromatography-mass spectrometry or high-performance liquid chromatography [14, 15, 16]. The high degree of automation of the process of chromatographic separation of the components of the mixture and the processing of the obtained results makes it possible to single out the method in question as relevant and promising for the quantitative analysis of components in products based not only on birch, but also on other plant materials.

3. Problem statement

Objective: To evaluate the possibility of determining betulin in powder and suspension products of birch bark processing using HPLC to further develop the methodology.

Hypothesis: reproducibility of quantitative analysis of the studied betulin-containing extracts by gas chromatography-mass spectrometry using the method of reversed-phase high-performance liquid chromatography is the basis for developing a method for determining betulin in birch bark processing products using HPLC.

The objects of the study are betulin-containing extract and the preparation “Stimulin” made from it.

4. Research

Materials and equipment: birch bark extracts, solvents, double-distilled water, standard laboratory glassware, analytical scales, centrifuge, gas chromatography-mass spectrometer, Lyumakhrom, spectrofluorimetric detector “Lyuhkrom SFLD 2310 Flyuorat-02-Panorama”, column C18 Kromasil 2 , 1x150, 6.2 μm grain, microsyringe per 100 μl.

The first task is to conduct qualitative and quantitative analyzes of birch bark extracts by gas chromatography-mass spectrometry under the following conditions: initial temperature 100 °C, final temperature 295 °C, exposure at final temperature 10 minutes, heating rate 15 °C/min. Analysis of the
chromatograms and mass spectra of the studied samples obtained with the use of various extractants, has led to the conclusion that the isolated extracts are predominantly betulin containing, the identified impurity is lupeol. The results have showed that products isolated from the outer layer of birch bark using various extractants: acetone, isopropyl alcohol, methyl tert-butyl ether, have different concentrations of betulin – 91.2% by weight, 90.9% by weight and 87.9% of mass, respectively [12, 15, 16].

Further studies were devoted to the analysis of betulin-containing extracts by HPLC. When conducting the definitions of betulin, the recommendations of the authors, L. V. Chudinova, N. V. Moskvina are guided. The determination of betulin in the samples is carried out by the method of reversed-phase high-performance liquid chromatography in the isocratic elution mode. The mobile phase is a mixture of acetonitrile and water with a ratio of 9:1, an eluent feed of 0.35 ml/min, a column temperature of 25°C, detection at a wavelength of 210 nm [14, 18]. In the course of the work, solutions of betulin extracts in ethyl alcohol have being prepared. Samples weighing 25 mg are placed in volumetric flasks of 2 ml each and dissolved with ethyl alcohol (1.5 ml) when heated in a water bath with a temperature of 60°C for 1 hour. The resulting solutions are cooled, and then brought up with ethyl alcohol to the required volume.

An example of the analysis is presented in Figure 1 [17, 18, 19].

![chromatogram](image)

**Figure 1.** Chromatogram of a betulin obtained sample by extraction with isopropyl alcohol, performed by HPLC.

The results of HPLC analysis of betulin-containing extracts has showed the reproducibility of the results of analysis of the composition of the same mixtures obtained using gas chromatography-mass spectrometry. Subsequently, the correlation of the study of the quantitative composition of betulin-containing extracts and the content of betulin in the Stimulin agrochemical are tested. In view of the fact that the drug is a betulin glycol suspension, the sample preparation process should have been done differently.

To prepare samples for HPLC – an alcohol solution of betulin, it is initially necessary to extract the substance from the glycolic composition. For this purpose, 0.5 g of Stimulin is mixed with 30 ml of
hot water (50°C), 20 ml of hexane is added to the solution, stirred for 30 minutes, after which the water and hexane layers are separated in a separatory funnel. The aqueous layer is mixed with another 10 ml of hexane and after separation is separated from the organic. Hexane extracts are poured into a 50 ml flask, after that hexane is distilled to a volume of 1.5-2 ml. Next, the solution is placed in a 2 ml volumetric flask, in which it is heated until complete evaporation of hexane. To eliminate the loss of betulin in the distillation flask, it is rinsed with hexane, the resulting liquid is also placed in a volumetric flask. After complete evaporation of hexane, cooling the flask, it is filled with 1.5 ml of ethanol and heated in a water bath at 60°C until the precipitate dissolved and then filled to the mark [16, 19].

![Chromatogram of the sample "Stimulin" by HPLC.](image)

**Figure 2.** Chromatogram of the sample "Stimulin" by HPLC.

The prepared sample of betulin is analyzed by HPLC on a Lumakhrom chromatograph under the same conditions as dry extracts samples. The results of the analysis are presented in Figure 2. The obtained results allowed conducting a correlation between the quantitative determination of betulin in betulin-containing solutions of compositions of different composition.

Thus, the reproducibility of the chromato-mass spectrometry method for quantitative analysis of betulin in solutions has been proved. This makes it possible to study betulin-containing products by HPLC using a spectrofluorimetric detector and develop the technique.

In the future, it is planned to develop and certify the method for determining betulin in dry extracts and suspensions based on them using HPLC in accordance with the general requirements for the development, certification and application of quantitative chemical analysis [20]. The development of an effective and rapid method of analysis of «STIMULIN» allows evaluating the content of betulin, which is an indicator of the quality of the drug in accordance with the technical conditions.

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