PHYTOCHEMICAL RESEARCH AND ANTI-INFLAMMATORY ACTIVITY OF THE DRY EXTRACTS FROM NORTHERN HIGHBUSH BLUEBERRY LEAVES

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All over the world, non-steroidal anti-inflammatory drugs (NSAIDs) are taken annually by about three hundred million people and this figure is constantly increasing. At the same time, NSAIDs are also one of the most common causes of side effects of drug therapy. The development and implementation of new anti-inflammatory drugs, including those of plant origin, with minimal side effects is an urgent task of modern pharmaceutical science. Vaccinium corymbosum L. (family Ericaceae), which is gaining more and more popularity among berry crops and is successfully cultivated in Ukraine, is promising in this direction for research.

The aim: phytochemical analysis of dry extracts from blueberry leaves to establish the possibility of creating new drugs with anti-inflammatory activity.

Materials and methods. The objects of the study were dry extracts of northern highbush blueberry leaves. The content of amino acids and phenolic compounds was determined by HPLC and spectrophotometry. The prototypal activity was studied in vivo and in vitro.

Research results. 4 dry extracts were obtained from northern highbush blueberry leaves. In the extracts obtained by HPLC, 7 amino acids were identified, including 3 essential ones: arginine, histidine, and phenylalanine. As a result of the HPLC study, 7 phenolic compounds were identified in extracts from the leaves of northern highbush blueberry: 5 flavonoids – rutin, quercetin-3-O-glucoside, kaempferol-3-O-glucoside, quercetin and kaempferol and 2 hydroxycinnamic acids, chlorogenic and caffeic acid. For the first time, the anti-inflammatory effect of extracts from blueberry leaves was investigated. It was revealed that extract 1 at a dose of 50 mg/kg and extract 4 modified with arginine at a dose of 25 mg/kg have the highest anti-inflammatory activity.

Conclusions. The results of the conducted studies indicate that extracts from the leaves of northern highbush blueberry in terms of the content of biologically active substances are promising sources for the creation of new drugs and dietary supplements with anti-inflammatory activity.

Keywords: northern highbush blueberry, dry extract, phenolic compounds, amino acid, anti-inflammatory activity

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1. Introduction

Inflammation is a typical pathological process that underlies many diseases, is widespread, is accompanied by severe clinical manifestations and is the cause of partial or complete disability of large groups. Worldwide, non-steroidal anti-inflammatory drugs (NSAIDs) are taken by about three hundred million people annually and this number is constantly increasing [1]. At the same time, NSAIDs are also one of the most common causes of side effects of drug therapy. In the United States, NSAIDs are the 15th most common cause of death. Thus, in the United States alone, about 16,500 patients die each year from NSAID gastropathies, and in the United Kingdom - about 2,000 [2, 3]. Development and introduction of new anti-inflammatory drugs, including herbal ones, with minimal side effects is an urgent task of modern pharmaceutical science.

Vaccinium corymbosum L. (Ericaceae family), which is gaining more and more popularity among berry crops and is successfully cultivated in Ukraine, is promising in this direction for research. Northern highbush blueberry is a deciduous plant [4, 5]. During the collection of fruits and pruning of bushes, a large volume of leaves remains, so the study of BAS of this raw material is relevant [6].

The main active ingredients of Northern highbush blueberry are phenols and their glycosides (hydroquinone, arbutin and methylarbutin) [7], hydroxycinnamic acids, coumarins, flavonoids (kaempferol, quercetin, astragalin, hyperoside, herbacetin, 8), luteolin, tannins and their metabolites and terpenes (α-pinene and β-pinene) [10, 11]. Such a rich composition of phenolic compounds creates the preconditions for the development of new raw materials from this raw material with anti-inflammatory activity.

The aim of the work was to conduct a phytochemical analysis of dry extracts from the leaves of Northern highbush blueberry to establish the possibility of creating new drugs with anti-inflammatory activity.
2. Planning (methodology) of research
Given the previous experience of creating extracts from the leaves of plants of the family Ericaceae, it is planned to obtain extracts, modify them, investigate the phytochemical profile and anti-inflammatory activity to establish the possibility of using them as anti-inflammatory agents (Fig. 1).

3. Materials and methods
3.1. Plant material
Plant raw material, V. corymbosum L. leaves, were collected in growing season, June-July, 2020, from Kharkov region, Ukraine (GPS: 49°27’41.1” N, 36°50’42.7” E). Species identification was carried out by prof. Gontova T.M. at the Department of Botany, the National University of Pharmacy, Ukraine. Plant raw material was air-dried in darkness at room temperature. For chemical analysis, the raw material was ground to a particle size of 2–3 mm.

3.2. Obtaining extracts
Four extracts were obtained from Northern highbush blueberry leaves as follows: 200.0 g of Northern highbush blueberry leaves, crushed to a particle size of 2–3 mm, were placed in a flask, filled with 1000.0 ml of 50 % ethanol solution, extracted overnight at room temperature. The extraction was repeated three times with new portions of the extractant of 600.0 ml. The resulting extracts were combined, settled and filtered. The filtrate was evaporated using a rotary vacuum evaporator “Heidolph 2 Wbeco, Laborata 400 efficient” (Germany) to a dry extract (Extract 1).

3.3. Analysis of the chemical composition of extracts
3.3.1. Investigation of phenolic compounds by HPLC
Separation of the sum of phenolic compounds was carried out by HPLC [14, 15] on a high-performance liquid chromatograph Agilent Technologies (model 1100), which is equipped with a flow-through vacuum degasser G1379A, a four-channel low-pressure gradient pump G1311A, an automatic injector G1313A, a column thermostat G13116A and a diode-matrix detector G1316A. A 2.1×150 mm chromatographic column filled with an octadecylysil sorbent with a grain size of 3.5 μm “ZORBAX-SB C-18” was used for analysis. The analysis was performed under the following conditions: thermostat temperature – 35 °C; the flow rate of the mobile phase – 0.25 ml/min; as the mobile phase used solution A (0.1 % phosphoric acid, 180 μl/l triethylen-
mine, 3 ml/l tetrahydrofuran in water) and solution B (MeOH) in a ratio of 90:10 (first 8 min), 70:30 (8 to 24 min), and from 24 min used only solution B; the working pressure of the eluent is 240–300 kPa. The following detection parameters were set during the analysis: measurement scale – 1.0; scan time – 0.5 s; spectrum removal parameters – each peak 190–600 nm [14]. Sample preparation: 50.0 mg (exact portion) of the extract was weighed in a 5.0 ml test tube and adjusted to the mark with methanol. After 30 min in an ultrasonic bath, the sample was insisted at room temperature for 3–4 hours, then the tube was again placed on an ultrasonic bath for 15 min, then the solution was filtered through a Teflon filter with a pore size of 0.45 μm in a vial for analysis. The sample volume is 2 μl. Identification of phenolic compounds was performed by the retention time of hydroxycinnamic acid and flavonoid standards and their spectral characteristics [16, 17].

3.3.2. Determination of the quantitative content of phenolic compounds

Total polyphenols. Quantitative determination of total polyphenols was carried out by spectrophotometry in terms of pyrogallol according to the pharmacopoeial method (SPHU 2.0) [18–20].

Total flavonoids. Determination of total flavonoids was carried out by absorption spectrophotometry in terms of rutine, which is present in the leaves in the predominant amount. (SPHU 2.0) [19, 20, 22].

Hydroxycinnamic acids. Quantitative determination of hydroxycinnamic acids was carried out by the modified method of absorption spectrophotometry in terms of chlorogenic acid (SPHU 2.0) [19, 22, 23].

3.3.3. Investigation of amino acids compounds by HPLC

Determination of amino acid content was performed using Agilent 1260 Infinity HPLC System (degasser, binary pump, autosampler; single-quadrupole mass spectrometer Agilent 6120 with electrospray ionization (ESI); OpenLAB CDS Software. Zorbax RX-SIL column (1.8 μm, 46 mm × 50 mm, Agilent) with protective filter. Under the conditions of the HPLC study used a gradient mode using a buffer solution: A – H2O (HCOOH 0.1 %) and a solution of organic modifier: B - CH3CN (HCOOH 0.1 %). The flow rate is 0.4 ml/min. Injection volume was 10 μl. Column temperature 40 °C. MS detection conditions: ion source: API-ES; ion scan mode 50–300 m/z; mode of extraction of chromatograms by individual ions depending on molecular weight, EIS; positive polarity. The work used acetonitrile “HPLC Super gradient” (Avantor performance materials inc, Poland) and formic acid (pure, ApplChem GmbH, Darmstadt). Highly purified water (18 MΩ at 25 °C) was prepared using the Direct Q 3 UV water purification system (Millipore, Molsheim, France). Amino acids (QDA qualifications) – glycine, alanine, serine, valine, threonine, methionine, histidine, phenylalanine, arginine, tryptophan [19, 24, 25].

3.4. Study of anti-inflammatory activity of extracts

3.4.1. In vivo study in a model of carrageenan edema

The study of anti-inflammatory activity of highbush blueberry extracts was conducted at the Clinical-diagnostic center of NUPh under the guidance of prof. Kireev I. V. The experimental work was performed in the scope of simple pharmacological screening. Animals were standardized for physiological and biochemical parameters and were in vivarium in accordance with sanitary and hygienic standards on a standard diet. During the experiment with animals, they behaved in accordance with the International principles of the european convention for the protection of vertebrate animals used for experiments and other scientific purposes [26].

The study of anti-inflammatory activity of highbush blueberry extracts was carried out in accordance with the guidelines “Preclinical studies of drugs” [27]. The experiments were performed on male rats weighing 180–220 g, which were divided into 6 groups of 6 animals each. The first group – control, the sixth group – intact animals. To study the effect of the obtained blueberry extracts on the course of the exudative phase of inflammation, a model of rat paw edema caused by subplanar administration of a phlogogenic agent – carrageenan solution was used. The paw volume was measured before and every hour until the time of the greatest development of edema (4 hours). For 2 h and immediately after the introduction of the phlogogenic agent, the animals were injected intraperitoneally with blueberry extracts at doses of 15, 25, 50, 75 and 100 mg/kg. The effect of blueberry extracts was evaluated on the ability to suppress swelling of the paws of rats. As a drug – a comparison with the known anti-inflammatory effect used diclofenac sodium.

3.4.2. Assessment of anti-inflammatory activity using in vitro assay

Blood was taken from healthy human donors using a protocol approved by the Chang Gung Memorial Hospital review board. Neutrophils were isolated according to the standard procedure described before [28]. The inhibition of superoxide anion generation was measured by the reduction of ferricytochrome C as previously described [29]. Elastase release representing the degranulation from azurophilic granules was evaluated as described before [30]. Experimental conditions have been described in detail earlier [31].

3.5. Statistics

Student’s t-test was used to statistically test the hypothesis of the probability of differences between the indicators of different groups. Statistical processing of the results was done by calculating the arithmetic mean, the average error of the arithmetic value, the reliability of the differences between results by the methods of variation statistics (SPHU 2.0, Vol.1– 5.3, 5.3.N1) using Statistica 6.0 program and Word Excel. The number of repetitions of experiments (n) equals 5 [19].
4. Research results

The obtained extracts are brownish-yellow powders with a characteristic specific odor. The yield of dry extract 1 from the leaves of blueberries was 27.35%. Extract 2 was brownish-yellow in color, but sometimes had white patches of arginine, while extracts 3 and 4 were uniformly light brown in color.

Qualitative composition and quantitative content of free amino acids in blueberry leaf extracts were performed by HPLC (Table 1).

![Typical chromatogram of phenolic compounds of extract 1 from highbush blueberry leaves](image)

**Table 1**

| Amino acid        | Extract 1 | Extract 2 | Extract 3 | Extract 4 |
|-------------------|-----------|-----------|-----------|-----------|
| Glycine           | 1.02±0.03 | 0.63±0.06 | 0.67±0.06 | 0.81±0.04 |
| Alanine           | 0.66±0.03 | 0.66±0.03 | 0.66±0.03 | 0.65±0.02 |
| Serine            | 1.50±0.04 | 0.98±0.07 | 0.93±0.04 | 0.95±0.06 |
| Valine            | 0.53±0.04 | 0.33±0.05 | 0.35±0.02 | 0.34±0.03 |
| Histidine         | 0.22±0.01 | 0.15±0.01 | 0.15±0.02 | 0.14±0.01 |
| Phenylalanine     | 0.33±0.04 | 0.23±0.02 | 0.19±0.01 | 0.21±0.03 |
| Arginine          | 1.16±0.04 | 35.09±1.18| 32.1±0.95 | 31.82±0.76|

Determination of the qualitative composition and quantitative content of phenolic compounds in the study objects was performed by HPLC (Table 2, Fig. 2).

Quantitative determination of the content of the main groups of phenolic compounds (total polyphenols, flavonoids and hydroxycinnamic acids) in extracts of blueberry leaves was performed by spectrophotometry by pharmacopoeial methods (Table 3).

**Table 2**

| No. | Compound                  | Retention time, min. | Content in the extract, mg/100 g |
|-----|---------------------------|----------------------|----------------------------------|
| 1   | Chlorogenic acid          | 13.14                | 1217.21 799.10 719.19 759.09     |
| 2   | Caffeic acid              | 17.22                | 3124.65 2051.33 1846.20 1948.63  |
| 3   | Routine                   | 19.70                | 474.88 311.76 280.59 296.15      |
| 4   | Quercetin-3-O-glucoside   | 21.17                | 2293.65 1507.05 1356.34 1431.60  |
| 5   | Kaempferol-3-O-glucoside  | 22.82                | 1136.74 746.27 671.65 708.91     |
| 6   | Quercetin                 | 23.27                | 888.60 583.37 525.03 554.16      |
| 7   | Kaempferol                | 25.18                | 206.74 135.73 122.15 128.93      |
| Sum |                           |                      | 9342.47 6134.61 5521.15 5827.48  |
Table 3

Quantitative content of phenolic compounds in extracts of highbush blueberry leaves (M±m, n=5)

| BAS group                                      | The used method                                             | Quantitative content in the extract, % |
|------------------------------------------------|-------------------------------------------------------------|----------------------------------------|
| Derivatives of hydroxycinnamic acids           | Spectrophotometric method in terms of chlorogenic acid      | 2.92±0.12, 1.92±0.11, 1.9±0.12, 1.83±0.12 |
| Flavonoids                                     | Spectrophotometric method in terms of routine               | 3.03±0.11, 1.99±0.10, 1.97±0.11, 1.77±0.11 |
| Phenolic compounds                             | Spectrophotometric method in terms of pyrogalol             | 18.42±0.97, 12.1±0.63, 11.97±0.62, 11.86±0.62 |

The obtained results of experimental studies of anti-inflammatory activity of blueberry extracts in the model of carrageenan edema of the hind limb in rats are given in Table 4. When studying extracts from highbush blueberry leaves in vitro on human blood neutrophils, only extracts 1 and 4 showed activity (Table 5).

Table 4

The effect of blueberry extracts on the development of inflammation of the hind limb in rats in a model of carrageenan edema

| Group of animals | dose, mg/kg | Limb volume, c.u. | Antiexudative activity, % |
|------------------|------------|-------------------|---------------------------|
|                  | starting point | 1 h | 2 h | 3 h | 4 h | 1 h | 2 h | 3 h | 4 h |
| Control pathology | 42.5±1.47 | 57.5±1.47 | 57.5±1.47 | 62.5±1.47 | 59.5±1.47 |
| Diclofenac sodium | 8 | 36±0.98 | 46±0.98 | 41±0.98 | 47±0.98 | 37±0.98 | 33 | 67 | 45 | 94 |
| Extract 1 | 15 | 35.5±1.47 | 45.5±4.42 | 47.5±4.42 | 46.5±4.42 | 44±6.88 | 33 | 20 | 45 | 50 |
| Extract 2 | 25 | 36±2.95 | 44.5±0.49 | 45.5±1.47 | 46.5±4.42 | 48.5±5.41 | 43 | 37 | 48 | 26 |
| Extract 3 | 50 | 36.5±2.46 | 43±2.95 | 41.5±1.47 | 41.5±1.47 | 41±0.98 | 57 | 67 | 75 | 74 |
| Extract 4 | 75 | 33±0.98 | 50.5±1.47 | 50.5±4.42 | 53.5±5.41 | 48±2.95 | 17 | 17 | 2 | 12 |
| Arginine | 100 | 36±0.98 | 49±4.91 | 49.5±4.42 | 50.5±0.49 | 49.5±0.49 | 13 | 10 | 28 | 21 |

Note: * – p<0.05, in comparison with control pathology
The main active ingredients of highbush blueberry leaves, which determine their pharmacological action, are phenolic compounds. The solubility of these compounds significantly affects their bioavailability and the strength of the pharmacological effect. It was previously shown that the addition of various amino acids to plant extracts led to an increase in the pharmacological effect and the emergence of new activities [12, 13], so taking into account this experience, three ways to modify highbush blueberry extract with arginine: conventional mechanical displacement and modification in acidic alcohol solution, with two acidifying agents acetate and citric acid. The choice of arginine was due to the fact that the key factor regulating the tone of the vascular endothelium is the most important physiological vasodilator—nitrogen monoxide. This mediator is formed from arginine under the action of Ca²⁺ – dependent enzyme NO-synthase (NOS) [34, 35]. 

4 dry extracts with similar organoleptic characteristics were obtained.

In the obtained extracts from highbush blueberry leaves by HPLC revealed 7 amino acids (Table 1), including 3 essential: arginine, histidine and phenylalanine. The dominant amino acids in the original extract 1 were glycine, serine and arginine. Due to the fact that when obtaining extracts 2, 3 and 4 was added arginine in significant quantities, its content increased, but not proportionally: when mechanically mixed, this proportion was maintained, but when added to the solution, the amount of free arginine was lower than added, which can be explained by the formation of conjugates of arginine with other BAS extracts. The ratio of other amino acids also changed.

As a result of HPLC, in extracts of highbush blueberry leaves were found 7 phenolic compounds (Table 2): 5 flavonoids - rutin, quercetin-3-O-glucoside, kaempferol-3-O-glucoside, quercetin and kaempferol and 2 hydroxycinnamic chlorogenic acid and caffeic acid. Among hydroxycinnamic acids, caffeic acid was dominant, the content of which was 2.6 times higher than chlorogenic acid, among flavonoids – quercetin-3-D-glucoside and kaempferol-3-D-glucoside.

In the obtained extracts from highbush blueberry leaves, the quantitative content of the main BAS groups was determined by spectrophotometry according to SPhU methods (Table 3). Because spectrophotometric methods of analysis are more accessible, easy to perform and easy to reproduce and are more convenient and appropriate in the development of methods for standardization of extracts. The results of the analysis showed the same trend as HPLC analysis of the content of the main groups of BAS in the obtained extracts and will be used in the development of regulatory documentation for extracts. The values of spectrophotometric studies differ from HPLC analysis, which can be explained by the lower specificity of the spectrophotometric method and incomplete interpretation of substances in HPLC analysis.

Alteration is the first phase of the inflammatory process, triggers the entire cascade of inflammation, causing destructive changes in the affected tissue [36]. That is why the suppression of inflammation at the stage of its initiation is an important component of the success of anti-inflammatory therapy. The carrageenan edema model is one of the pharmacological models for assessing the anti-inflammatory activity of substances and drugs. This model in rats was used in pharmacological studies [26].

For the first time the anti-inflammatory effect of extracts from the leaves of blueberry was studied (Table 4). Extract 1 at a dose of 50 mg/kg and extract 4 modified with arginine at a dose of 25 mg/kg were found to have the highest anti-inflammatory activity. The effect of arginine on alteration was insignificant and, conversely, at a dose of 50 mg/kg and 100 mg/kg led to an increase in edema, while extracts from the leaves of blueberry had an anti-inflammatory effect. In the composition of the extract 4 available arginine, which was added in the preparation, taking into account this, in an effective dose of 25 mg/kg, the content of BAS from the leaves of blueberries is only 16.7 mg/kg, while without the addition of arginine, these BAS are effective at a dose of 50 mg/kg, indicating potentiation of the action of phenolic compounds of Northern highbush blueberry arginine. This dose (16.7 mg/kg) can relieve inflammation at the level of the comparison drug diclofenac at a dose of 8 mg/kg. Given the side effects of diclofenac sodium on the human body, we consider the use of modified extract 4 of the leaves of Northern highbush blueberry to create a new drug.

Anti-inflammatory activity of blueberry extracts against superoxide anion generation and elastase release in human neutrophils is shown in Table 5.

The major function of neutrophils include respiratory burst, degranulation and NETs formation serve as a first line of defense against pathogens and are important processes in the maintenance of human health. However, these processes need to be precisely regulated. Superoxide is a major radical produced by neutrophils while elastase belongs to major components of azurophilic granules [34] and their uncontrolled amount contributes to several acute and chronic diseases, including sepsis, ARDS, lung injury, arthritis or psoriasis [36].
markers of neutrophilic inflammation were shown to be affected by several plant extracts [31].

Blueberry extracts were evaluated in human neutrophils against superoxide anion generation and elastase release triggered by fMLF in CB-primed human neutrophils. According to the results the extracts of blueberry BL1 had a profound effect on superoxide anion generation with IC50 3.96 μg/ml (Table 5). Interestingly, the extracts of blueberry BL1 showed enhancing effects on elastase release by human neutrophils and thus may have immune-promoting effects related to the process of degranulation. BL4 exerted inhibitory effects on elastase release (44% at 10 μg/ml). Samples BL2 and BL3 were not tested due to low solubility in DMSO or water.

Study limitations. The amount of standard substances was limited during the study of plant raw materials by HPLC, so not all compounds of phenolic nature could be identified in the studied extracts. Only one model of inflammation was used in the study, which limits the ability to determine the mechanism of anti-inflammatory activity.

Prospects for further research. The obtained results indicate the possibility of developing drugs based on the leaves of V. corymbosum. The results of research can be further used to develop methods of quality control of raw materials and drugs, developed basic schemes for obtaining extracts can be the basis for the development of technology for obtaining substances in industrial conditions. According to the results of phytochemical studies, it was found that extracts from the leaves of V. corymbosum are promising for the development of drugs with anti-inflammatory activity.

6. Conclusions
From the leaves of highbush blueberry were obtained dry extracts, modified by adding argin in various ways. 7 amino acids and 7 phenolic compounds were found in the obtained extracts: 5 flavonoids - rutin, quercetin-3-O-glucose, kaempferol-3-O-glucose, quercetin and kaempferol 2 hydroxycinnamic acids – chlorogenic and caffeic acids.

For the first time, the anti-inflammatory effect of highbush blueberry leaves extracts was studied in vivo and in vitro. The extract obtained with 50% ethanol solution at a dose of 50 mg/kg and the extract modified with arginine with the addition of citric acid at a dose of 25 mg/kg were found to have the greatest anti-inflammatory activity.

The results of phytochemical and pharmacological studies indicate that extracts of highbush blueberry leaves in terms of BAS are promising sources for the development of new drugs and dietary supplements with anti-inflammatory activity.

Conflict of interests
The authors declare that they have no conflicts of interest.

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