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Standardization of Dry Extracts from Large Cranberry Leaves

Abstract

Aim. To determine the parameters of standardization of a dry extract from large cranberry (*Oxycoccus macrocarpus* (Ait.)) leaves and a dry extract modified with arginine and develop projects of Drug Quality Control Methods (DQCM) for these substances.

Materials and methods. The study object was dry extracts from large cranberry leaves. Leaves were harvested in October 2021 in the Zhytomyr region (Kostivtsi village, 50.326862437345945, 29.54310845594284). The extracts were obtained with a 50 % solution of ethyl alcohol in the ratio of 1:30 by double maceration. Half of the combined extract was dried to a dry extract (Extract 1), and the other half was modified with arginine in the threefold equimolar amount relative to the total amount of phenolic compounds and evaporated to a dry extract (Extract 2). Standard pharmacopoeial methods were used to determine standardization parameters. The quantitative determination was carried out using the spectrophotometric method by the content of flavonoids calculated with reference to hyperoside and hydroxycinnamic acids calculated with reference to chlorogenic acid on an Evolution 60S spectrophotometer (Thermo Scientific Spectronic, USA).

Results and discussion. The parameters of standardization of dry extracts from large cranberry leaves were determined. The project of DQCM was proposed according to the following indicators: description, solubility, identification using the thin-layer chromatography method (by the content of flavonoids, hydroxycinnic acids and arginine), loss on drying, the residual amount of organic solvents (ethanol), microbiological purity, and the content of heavy metals. The assay was carried out using spectrophotometry by the content of flavonoids and derivatives of hydroxycinnic acids. Three batches of the extracts obtained, which fully corresponded to the projects of DQCM developed, were analyzed.

Conclusions. The parameters of standardization of dry extracts from large cranberry leaves have been determined, and projects of DQCM for the substances obtained have been developed. It is the basis for creating new medicines for the correction of insulin-resistant conditions in Type 2 diabetes mellitus.

Keywords: cranberry; leaves; dry extract; standardization

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Стандартизація журавлини великоплодої листя сухих екстрактів

Анотація

Мета. Визначити параметри стандартизації екстракту сухого з листя журавлини великоплодої (*Oxycoccus macrocarpus* (Ait.)) і сухого екстракту, модифікованого аргініном, та розробити проєкти методів контролю якості (МКЯ) на ці субстанції.

Матеріали та методи. Об'єктом дослідження були сухі екстракти з листя журавлини великоплодої. Листя було заготовлено в жовтні 2021 р. в Житомирській області (с. Костівці, 50.326862437345945, 29.54310845594284). Екстракти одержано 50 % розчином спирту етилового у співвідношенні 1:30, методом двократної мацерації. Половину об'єднаного витягу було висушено до сухого екстракту (екстракт 1), а іншу половину модифіковано аргініном у трикратній еквімолярній кількості щодо суми фенольних сполук та упарено до сухого екстракту (екстракт 2). Визначаючи параметри стандартизації, використовували стандартні фармакопейні методики. Кількісне визначення здійснювали спектрофотометричним методом за вмістом флавоноїдів у перерахунку на гіперозид та гідроксикоричних кислот у перерахунку на хлорогенову кислоту на спектрофотометрі Evolution 60S (Thermo Scientific Spectronic, США).

Результати та їх обговорення. Визначено параметри стандартизації сухих екстрактів з журавлини великоплодої листя. Запропоновано проєкт методів контролю якості за такими показниками: опис, розчинність, ідентифікація за допомогою методу тонкошарової хроматографії (за вмістом флавоноїдів, гідроксикоричних кислот і аргініну), втрата в масі під час висушування, залишкова кількість органічних розчинників (етанолу), мікробіологічна чистота, вміст важких

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металів, а кількісне визначення рекомендовано виконувати за допомогою спектрофотометрії за вмістом флавоноїдів та похідних гідроксикоричних кислот. Проаналізовано три серії одержаних екстрактів, які цілком відповідали розробленим проєктам МКЯ.

Висновки. Визначено параметри стандартизації сухих екстрактів журавлини великоплодої листя та розроблено проєкти МКЯ на отримані субстанції, що є основою для створення нових лікарських засобів для корекції інсулінорезистентних станів у разі цукрового діабету 2 типу.

Ключові слова: журавлина; листя; сухий екстракт; стандартизація

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■ Introduction

Compared to 2011, the number of people suffering from diabetes increased by 31% in 2021 [1]. Complications of diabetes can include kidney failure, heart attacks, strokes, amputation of limbs, and blindness. Type 2 diabetes develops as a result of inefficient use of insulin by the body, namely the development of the insulin resistance state. This type of disease affects more than 90% of diabetic patients. Symptoms may be similar to those of Type 1 diabetes, but less pronounced. Because of this, the disease is often diagnosed only a few years after its occurrence and manifestations of complications. Until recently, this type of diabetes was observed only among adults, but now it is increasingly diagnosed in children [2].

Combating the spread and complications of Type 2 diabetes is one of the most urgent tasks in the world [3]. Despite the sufficient number of synthetic drugs for the treatment of this disease, it is necessary to note a limited range of herbal products although they have a number of advantages, including effectiveness, fewer side effects and the possibility of combined therapy with other groups of drugs [4, 5]. Plants of the *Vaccinium* genus are a promising source for creating hypoglycemic and hypolipidemic agents [6–9]. Earlier studies have already shown that an extract from large cranberry leaves obtained with a 50% solution of ethyl alcohol and its modified extract with arginine have a positive effect on the correction of insulin-resistant conditions [10].

Amino acids are able to form conjugants, complexes, amides and imides with other substances, including phenolic compounds. These interactions lead to changes in the physical and chemical properties, in particular solubility, bioavailability of these substances, potentiation and emergence of new aspects of the pharmacological action. Taking this into account, a dry extract of large cranberry leaves was modified with arginine and showed higher hypoglycemic and hypolipidemic activity [10].

A dry extract of large cranberry can be both a substance for the development of new dosage forms, and for the production of a modified extract; therefore, it needs standardization.

With this in mind, to create new medicines based on large cranberry leaves, it is necessary to standardize and develop projects of Drug Quality Control Methods (DQCM) for both substances obtained since they have proven to be promising agents for correcting insulin resistance.

Therefore, the aim of the work was to determine the parameters of standardization of a dry extract from large cranberry (*Oxycoccus macrocarpus* (Ait.)) leaves and a dry extract modified with arginine and develop projects of DQCM for these substances.

■ Materials and methods

The study objects were a dry extract from large cranberry (*Oxycoccus macrocarpus* (Ait.)) leaves and a dry extract modified with arginine. Leaves were harvested in October 2021 in the Zhytomyr region (Kostivtsi village, 50.326862437345945, 29.54310845594284).

The extracts were obtained with a 50% solution of ethyl alcohol in the ratio of 1:30 by double maceration. Combined extracts were filtered through a paper filter. Half of the combined extract

was dried to a dry extract (Extract 1), and the other half was modified with arginine in the threefold equimolar amount relative to the total amount of phenolic compounds and evaporated to a dry extract (Extract 2).

Weighing was performed using the AN100 digital analytical balances (AXIS, Poland) with d = 0.0001 g. "Merck Silica gel F254" plates were used for chromatography. Solvents for the preparation of chromatographic systems were of "pure for analysis" or "chemically pure" grade; the solvent ratios indicated by numbers were taken in volume units. The optical density was measured on an Evolution 60S spectrophotometer (Thermo Scientific Spectronic, USA).

Various standard pharmacopoeial methods were used as the basis for the development of quality control methods for the extracts obtained [11]. Since large cranberry leaves are not represented in monographs of the State Pharmacopeia of Ukraine (SPhU), the monograph "European blueberry leaves" was taken as the basis.

■ Results and discussion

Taking into account the recommendations of the SPhU and modern approaches to standardization the following indicators were proposed.

Description. Dry extracts of large cranberry leaves are hygroscopic amorphous powders of brown color with a reddish tint with a faint specific odor.

Solubility. Before performing various tests it was necessary to determine the solubility of the extracts. The tests were performed in accordance with the requirements of the SPhU [11]. Extract 1 is easily soluble in 50% ethyl alcohol, moderately soluble in 96% ethyl alcohol, methanol and water, very slightly soluble in chloroform and ether. A modified Extract 2 is moderately soluble in 50, 96% ethanol and methanol and water, readily soluble in ethyl alcohol/water (70:30), slightly soluble in chloroform and ether.

Identification. Large cranberry leaves are not included in the SPhU. Therefore, a modified pharmacopoeial method of thin-layer chromatography for the flavonoid content given in the monograph "European blueberry leaves N" of the SPhU (2.2.27) was used to identify dry extracts from cranberry leaves.

Method A. Identification of major flavonoids and hydroxycinnamic acid derivatives [12].

Test solution. Dissolve 50 mg of Extract 1 in 10 mL of 96% ethanol or methanol, filter through a paper filter, distill the solvent and dissolve in 1 mL of methanol; dissolve 50 mg of a modified Extract 2 in 10 mL of 96% ethanol or methanol and water (70:30), then filter through a paper filter, distill the solvent and dissolve in 1 mL of methanol.

Reference solution. Dissolve 1.0 mg of hyperoside *R*, 1.0 mg of chlorogenic acid *R* in 10 mL of methanol *R*.

Plate: TLC plate with silica gel *R* layer.

Mobile phase: ethyl acetate *R* – water *R* – anhydrous formic acid *R* – anhydrous acetic acid *R* (72:14:7:7).

Injection volume: 10 μL, in bands.

Distance that the mobile phase must move: 10 cm from the start.

Drying: at a temperature of 100 °C to 105 °C.

Detection: spray with the solution of 10 g L^{-1} aminoethyl ether of diphenylboric acid *R* in methanol *R*. Then spray the plate with the solution of 50 g L^{-1} macrogol 400 *R* in methanol *R*, dry in the air for 30 min and view in the ultraviolet light at a wavelength of 366 nm.

Results: below is the sequence of zones on the chromatograms of *Test solutions* and *Reference solution* (Figure 1). Other fluorescent zones may also be detected on the chromatograms of *Test solutions*.

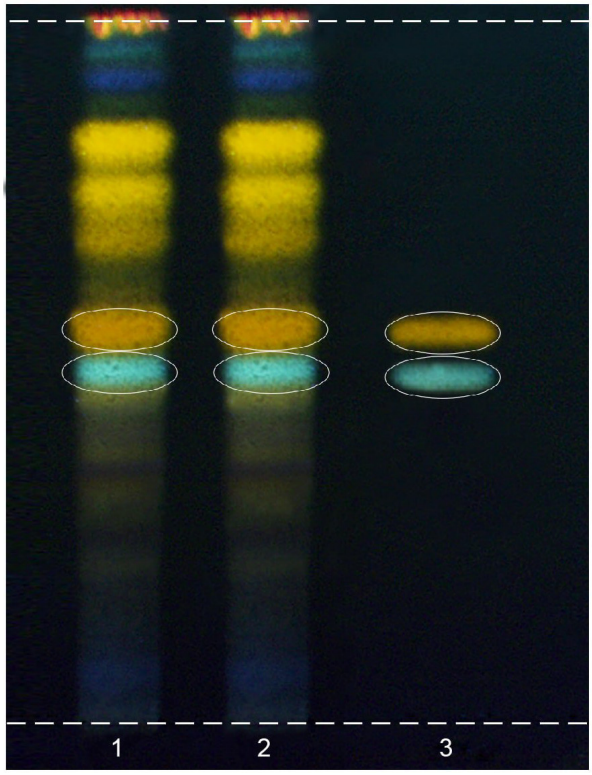

Figure 1. The chromatogram of dry extracts from large cranberry leaves: 1 – Extract 1; 2 – a modified Extract 2; 3 – *Reference solution*: hyperoside and chlorogenic acid

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In the middle part of the chromatogram of *Reference solution*, an orange fluorescent zone corresponding to hyperoside is detected. A blue fluorescent zone corresponds to chlorogenic acid, which is lower than hyperoside. On the chromatogram of *Test solution*, an orange fluorescent zone is detected at the level of *Reference solution* of hyperoside, and a blue fluorescent zone is detected at the level of *Reference solution* of chlorogenic acid. Other fluorescent zones may also be detected on the chromatogram of *Test solution*.

Method B. Identification of arginine.

Test solution. Dissolve 50 mg of the extract in 10 mL of 96% ethanol or methanol, filter through a paper filter, distill the solvent and dissolve in 1 mL of methanol.

Reference solution. Dissolve 10 mg of the reference standard (*RS*) of arginine hydrochloride in water *R* and dilute the volume to 50 mL with the same solvent.

Mobile phase: concentrated concentrated ammonia solution *R* – 2-propanol *R* (30:70).

Plate: TLC plate with silica gel *R* layer.

Injection volume: 10 µL of each solution, in bands.

Drying: dry at a temperature of 100 °C to 105 °C until the smell of ammonia disappears and spray with ninhydrin solution *R*. Heat the plate at a temperature of 100 °C to 105 °C for 15 min.

Apply 10 µL of each solution to the start line of the chromatographic plate. Dry the plate in the air and place in a chamber with a mixture of solvents, including the concentrated solution of ammonia *R* – 2-propanol *R* (30:70). When the solvent front passes 15 cm from the start line, remove the plate from the chamber, dry at a temperature of 100 °C to 105 °C until the smell of ammonia disappears and spray with ninhydrin solution *R*. Heat the plate at a temperature of 100 °C to 105 °C for 15 min. On the chromatogram of *Test solution* a spot at the level with arginine *RS* of *Reference solution* is identified.

Tests

Heavy metals. For the purpose of toxicological safety, it is necessary to control the content of heavy metals in extracts according to the requirements of the SPhU *(2.4.8)*. Their content should not exceed 100 ppm [11].

Microbiological purity. It is necessary to control the total aerobic bacteria and fungi count in the extract since phytochemicals are characterized by microbial contamination. Tests were carried out in accordance with the requirements of the SPhU, *2.6.12, 2.6.13*.

The *loss on drying* should not exceed 20.0 %. It is determined according to the SPhU 2.0 *(2.8.17)*. Place 0.50 g of the extract in a weighing bottle and dry in a drying cabinet at a temperature of 100 °C to 105 °C for 3 h. Cool in a desiccator and weigh [11].

Residual amount of organic solvents (ethanol). The content of ethyl alcohol in extracts of large cranberry leaves should not exceed 1.0 %. Place approximately 1.0 g (accurate weight) of the extract to a 10 mL measuring flask, dissolve in 7 mL of water, add 1 mL of acetone and 1,2-dichloroethane (internal standards), dilute the solution to the volume with water and mix.

Chromatograph 1 µL of the resulting solution and *Reference solution* of ethyl alcohol *RS* on a gas chromatograph with a flame-ionization detector, obtaining at least 5 chromatograms.

The results of the analysis are considered reliable if the requirements of the Chromatographic System Suitability Test are met.

Quantification. The flavonoid content in extracts of large cranberry calculated with reference to hyperoside was determined in accordance with the monograph "European blueberry leaves N" of the SPhU by the spectrophotometric method [11–13].

Place 200 mg (accurate weight) of large cranberry leaf extracts in a 100 mL round-bottomed flask, add 1 mL of a 5 g L^{-1} hexamethylenetetramine solution *R*, 20 mL of acetone *R*, 2 mL of hydrochloric acid *R*, boil at reflux for 30 min, and filter through a cotton swab into a 100 mL flask. Add a cotton swab to the residue in a round-bottomed flask, and extract in 2 portions of acetone *R*, 20 mL each; each time boil at reflux for 10 min, cool to room temperature, filter each extract through a cotton swab into the flask. Filter the resulting cooled combined acetone extracts through a paper filter into a measuring flask, dilute the volume of the solution to 100 mL with acetone *R*, rinsing the flask and the paper filter. Place 20.0 mL of the solution obtained into a separation funnel, add 20 mL of water *R,* extract the mixture with 15 mL, and then with 3 portions of ethyl acetate *R*, 10 mL each. Combine the resulting ethyl acetate extracts in a separation funnel, wash with 2 portions of water *R*, 50 mL each, filter over 10 g of anhydrous sodium sulfate *R* into a 50 mL measuring flask, and dilute the volume of the solution to 50.0 mL with ethyl acetate *R* (Solution A and B, respectively).

Test solution. Add 1 mL of aluminum chloride reagent *R* to 10.0 mL of Solution A (B) and

dilute the volume to 25.0 mL with the solution of 5 % (*v*/*v*) glacial acetic acid *R* in methanol *R*.

Compensation solution. Dilute 10.0 mL of the initial solution to 25.0 mL with the solution of 5 % (*v*/*v*) glacial acetic acid *R* in methanol *R*. Measure the optical density (2.2.25) of *Test solution* in 30 min compared to *Compensation solution* at a wavelength of 425 nm.

The content of flavonoids calculated with reference to hyperoside was calculated as a percentage by the formula:

$$X = \frac{A \times 1.25}{m}$$

where: *A* – is the optical density of *Test solution* at a wavelength of 425 nm;
m – is the weighed sample of the extract, g.

The specific index of hyperoside absorption equal to 500 was used.

The total amount of hydroxycinnamic acid derivatives was determined using the spectrophotometric method calculated with reference to chlorogenic acid [14, 15].

Dissolve approximately 100 mg (accurate weight) of dry extracts of large cranberry leaves, constantly stirring, in 5 mL of a 50% solution of ethyl alcohol. Repeat the procedure three times with a new portion of the solvent. After that, combine the solutions, filter through a paper filter, and place quantitatively into a 25.0 mL measuring flask, dilute the solution in the flask to the volume with the same solvent, and mix (Solution C and D).

Add 1.0 mL of Solution C (D) to a 25 mL measuring flask, then dilute to the volume with 50% alcohol, and mix. As *Reference solution,* a 50% ethanol solution is used. Measure the optical density of the resulting solution on an Evolution 60S spectrophotometer (USA) at a wavelength of 327 nm.

As *Reference solution,* the solution of chlorogenic acid is used. To prepare it, weigh 0.05 g (accurate weight) of chlorogenic acid *RS*, place it into a 100 mL measuring flask, dissolve in a 50% ethyl alcohol solution, dilute the solution to the volume with the same solvent, and mix.

Table 1. The results of the analysis of large cranberry leaf extracts according to the projects of DQCM

Quality indicator	Requirements	Extract 1 (batch)			Extract 2 (batch)		
		1101	1701	1902	1309	2001	2102
Description	according to the requirements of the project of DQCM	+[a]	+	+	+	+	+
Identification (TLC)	according to the project of DQCM (Method A)	+	+	+	+	+	+
	according to the project of DQCM (Method B)	–	–	–	+	+	+
Tests							
Loss on drying	Not more than 5 %	4.8	4.3	4.1	4.2	4.6	4.3
Residual amount of organic solvents (ethanol)	Not more than 1.0 %	0.6	0.4	0.7	0.5	0.6	0.8
Heavy metals	Not more than 100 ppm	+	+	+	+	+	+
Microbiological purity	In 1 g of the drug there are no more than 100 CFU (bacteria and fungi in total). The presence of enterobacteria and some other gram-negative bacteria; *Pseudomonas aeruginosa, Staphylococcus aureus* in 1 g is not allowed	+	+	+	+	+	+
Assay							
The content of the total amount of flavonoids calculated with reference to hyperoside	not less than 4 % (for Extract 1); not less than 2 % (for Extract 2)	5.17 ± 0.06	4.62 ± 0.04	5.04 ± 0.05	2.60 ± 0.05	2.22 ± 0.03	2.05 ± 0.04
The content of the total amount of hydroxycinnamic acid derivatives calculated with reference to chlorogenic acid	not less than 10 % (for Extract 1); not less than 3 % (for Extract 2)	11.47 ± 0.07	11.60 ± 0.05	11.56 ± 0.03	3.07 ± 0.04	3.09 ± 0.05	3.15 ± 0.03

Note: [a] «+» – the extract meets the requirements of the projects of DQCM

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After that add 1.0 mL of chlorogenic acid *RS* to a 50.0 mL measuring flask, dilute to the volume with 50% ethyl alcohol, mix, and measure the optical density under the same conditions as *Test solution*. As *Reference solution,* a 50% ethanol solution is used.

The content of the total amount of hydroxycinnamic acid derivatives calculated with reference to chlorogenic acid in the samples studied was calculated as a percentage by the formula:

$$X = \frac{A_1 \times a_0 \times 25 \times 1 \times 25 \times 100 \times 100}{A_0 \times a_1 \times 100 \times 1 \times 50 \times (100 - w)}$$

where: A_1 – is the optical density of *Test solution* of the extract;
A_0 – is the optical density of *Reference solution* of chlorogenic acid *RS*;
a_1 – is the weighed sample of the extract, g;
a_0 – is the weighed sample of chlorogenic acid *RS* (SPhU), g;
w – is the loss on drying, %.

Three batches of each dry extract from large cranberry leaves, which fully corresponded to the projects of DQCM proposed, were analyzed (Table 1).

Based on the results of the analysis, it can be concluded that all dry extracts of large cranberry leaves meet the requirements of the projects of DQCM developed.

■ Conclusions

The parameters of standardization of dry extracts of large cranberry leaves have been determined, and projects of DQCM for the substances obtained have been developed. It is the basis for creating new medicines for the correction of insulin-resistant conditions in Type 2 diabetes mellitus.

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