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DEVELOPMENT OF APPROACHES TO STANDARDIZATION OF BLACK WALNUT BARK

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▪ ***Significance.*** Black Walnut (*Juglans nigra* L.) is a species of trees of the Walnut family (*Juglandaceae*). This plant of the genus Walnut (*Juglans* L.) has not been sufficiently studied unlike other species e.g. *Juglans regia* L. This medicinal plant raw material is quite perspective, its preparations have antimicrobial, general tonic effect. We can use its leaves, unripe fruit, and the bark. However they are not widely used in medicine. In order to introduce the plants of the Walnut genus to the State Pharmacopoeia of the Russian Federation (RF State Pharmacopoeia), it is necessary to conduct of pharmacognostic studies, to develop product specification file to confirm the identification and quality of medicinal plant raw materials. ***The aim of this study*** is to develop a method of quantitative determination of flavonoids in the bark of the black walnut (*Juglans nigra* L.). ***Materials and methods.*** Material of the study was black walnut bark, stocked during the sap flow period (April) in 2018. The bark was skived up to 15 cm long and 2–3 cm wide. The bark was air-dried with the protection from direct sun light. The end of the drying was checked by the brittleness of the bark. ***Results.*** The methods of the quantitative determination of flavonoids in walnut bark has been developed. We used the differential spectrophotometry taking into consideration state standard sample of rutin at the analytical wavelength of 416 nm. The error of a single determination with a confidence level of 95% is ±1.20%. ***Conclusion.*** We used the developed technique and analyzed a number of samples of black walnut bark. The content of total flavonoids in the plant raw material is 5.13 ± 0.02% (as calculated on rutin). The flavonoid content should be at least 4.0%.

▪ **Keywords:** black walnut (*Juglans nigra* L.); barks; flavonoids; rutin; spectrophotometry; standardization.

РАЗРАБОТКА ПОДХОДОВ К СТАНДАРТИЗАЦИИ КОРЫ ОРЕХА ЧЕРНОГО

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▪ ***Актуальность.*** Орех черный (*Juglans nigra* L.) — вид деревьев семейства Ореховых (*Juglandaceae*). Данный представитель рода Орех (*Juglans* L.) недостаточно изучен по сравнению с другими видами, в том числе орехом грецким, однако является перспективным видом лекарственного растительного сырья, препараты которого оказывают противомикробное, общеукрепляющее действие. В качестве сырьевого источника представляют интерес листья, незрелые плоды и кора видов рода Орех, которые пока не нашли широкого применения в научной медицине. С целью введения представителей рода Орех в Государственную фармакопею Российской Федерации необходимо проведение фармакогностических исследований с последующей разработкой нормативной документации, подтверждающей подлинность и качество как лекарственного растительного сырья, так и лекарственных фитопрепаратов на их основе. ***Цель исследования*** — разработка методики количественного определения суммы флавоноидов в коре ореха черного (*Juglans nigra* L.). ***Материалы и методы.*** Материалом исследования служила кора ореха черного, заготовленная в период сокодвижения (апрель) в 2018 г. Кора была разрезана на полоски длиной до 15 см и шириной 2–3 см. Сушка коры проводилась естественным способом под навесом без доступа прямых солнечных лучей. Окончание сушки проверяли по ломкости коры. ***Результаты.*** В результате проведенного исследования разработана методика количественного определения суммы флавоноидов в коре ореха черного методом дифференциальной спектрофотометрии с использованием государственного стандартного образца рутина при аналитической длине волны 416 нм. Ошибка единичного

определения с доверительной вероятностью 95 % составляет ±1,20 %. ***Заключение.*** С использованием разработанной методики проанализирован ряд образцов коры ореха черного. Содержание суммы флавоноидов в сырье составляет 5,13 ± 0,02 % (в пересчете на рутин). Это позволяет рекомендовать в качестве нижнего предела содержание суммы флавоноидов не менее 4,0 %.

▪ **Ключевые слова:** орех черный (*Juglans nigra* L.); кора; флавоноиды; рутин; спектрофотометрия; стандартизация.

Introduction

Black walnut (*Juglans nigra* L.), a plant object, is a representative of the walnut (*Juglans* L.) genus of the Juglandaceae family. The walnut genus is known to include more than 20 species of woody plants growing in warm temperate regions of Eurasia and North America [2, 6].

The presence of various naphthoquinones in the black walnut's aerial part that has antibacterial activity (juglone, hydrojuglone, and hydrojuglone glucoside) is what is interesting about the nut. In addition, the plant also contributes to the pharmacological action because it contains valuable biologically active compounds (BAC), such as lipid substances, nitrogenous substances, carbohydrates, organic acids, flavonoids, and other phenolic compounds [1, 3, 8].

It is believed that flavonoids also contribute to the main antimicrobial effect together with naphthoquinones. The raw material reassigned for the production of aqueous, ethanolic, aqueous-ethanolic extracts, and extracts, active substances of a hydrophilic nature including the flavonoids of this plant, is necessary to consider and should be determined [7].

Earlier, a comparative phytochemical study on the genus walnut's types of medicinal plant raw material (MPRM) was conducted. The study samples' electronic spectra show an absorption maximum at a wavelength of 270 nm, indicating the contribution of flavonoids to the ultraviolet (UV) spectra absorption curve. The comparative study of the aqueous-ethanolic extracts electronic spectra on various types of raw materials of genus walnut representatives revealed that using black walnut bark (*Juglans nigra* L.) as a target MPRM is advisable.

A literature review has shown that in terms of juglone, standardizing black walnut leaves is possible by determining the amount of naphthoquinones using photocolorimetry [4, 5]. The extraction was obtained by double extraction method with 20% ethyl alcohol, followed by evaporation and triple extraction with diethyl ether [4, 5]. In terms of juglone, the content of naphthoquinones in black walnut leaves was determined to reach 0.24% ± 0.01% [4, 5]. Research continuation in this field is urgent because of the sufficient laboriousness of sample preparation, the complexity of the analysis for naphthoquinones as the target group of BAC, and the lack of literature data on black walnut bark (*Juglans nigra* L.) standardization.

Materials and methods

Black walnut bark, harvested during the sap flow period (April 2018 and 2019) in the botanical garden of Samara State University, was used as a material for this study. The bark was cut into strips of up to 15 cm long and 2–3 cm wide. It was naturally dried under shelter without access to direct sunlight. The end of drying was checked by bark brittleness.

Study results and discussion

The UV spectra of aqueous-ethanolic extracts solutions from black walnut bark shown in Figs. 1 and 2 were studied to develop a method for quantitative determination of the amount of flavonoid. A bathochromic shift of the flavonoids' long wavelength band was registered in the UV spectrum of the aqueous-ethanolic extract of black walnut shown in Fig. 1, which is the same as in the case of rutin shown in Fig. 3. UV spectra of the state standard reference sample (SSRS) of rutin showed that in the presence of aluminum chloride, a solution of this standard has an absorption maximum at a wavelength of 412 nm (Fig. 3). However, UV spectrum of aqueous-ethanolic extract from the black walnut bark in the differential version, which is presented in Fig. 4, shows an absorption maximum at a wavelength of 416 nm, which practically corresponded with the maximum of rutin's ethanolic solution.

To develop a method for quantitative determination of the amount of flavonoids, optimal conditions in extracting flavonoids from black walnut bark was determined. The extractant was 80% ethyl alcohol, had a 1:30 ratio of raw material to extractant, an extraction time of 60 min in a boiling water bath, and a 2-mm degree of grinding of raw materials (Table 1).

Method of quantitative determination of the amount of flavonoids in black walnut bark. Raw materials were grounded to particle size capable of passing through a sieve with holes of 2 mm in

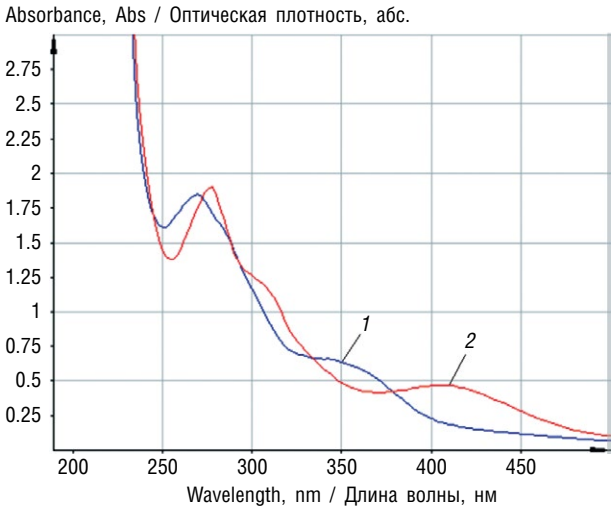

Fig. 1. Electronic spectra of solutions of water-ethanolic extraction from *Juglans nigra* bark: *1* — solution of extraction; *2* — solution of extraction with the addition of aluminum chloride

Рис. 1. Электронные спектры растворов водно-спиртового извлечения из коры ореха черного: *1* — раствор извлечения; *2* — раствор извлечения с добавлением алюминия хлорида

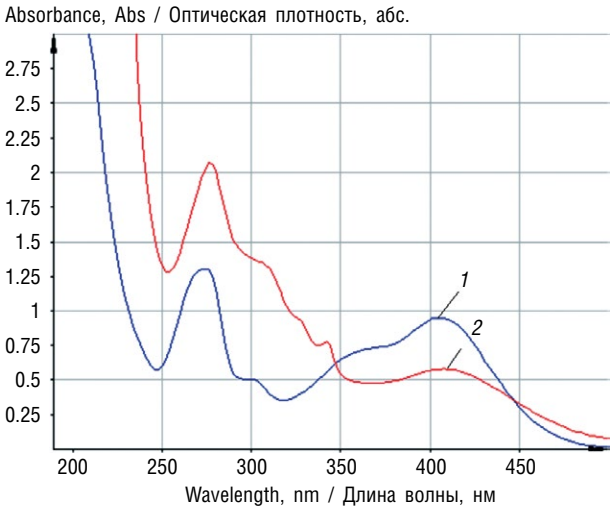

Fig. 2. Electronic spectra of solutions of water-ethanolic extraction from *Juglans nigra* bark: *1* — solution of extraction with the addition of aluminum chloride; *2* — solution of rutin with the addition of aluminum chloride

Рис. 2. Электронные спектры растворов водно-спиртового извлечения из коры ореха черного: *1* — раствор извлечения с добавлением алюминия хлорида; *2* — раствор рутина с добавлением алюминия хлорида

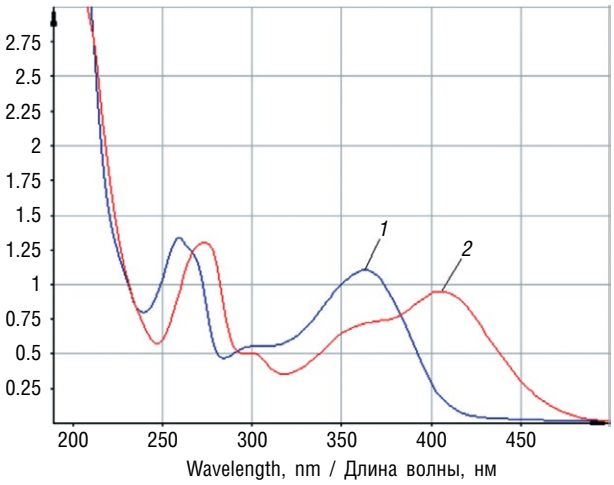

Fig. 3. Electronic spectra of ethanolic solutions of rutin: *1* — initial solution; *2* — solution with the addition of aluminum chloride

Рис. 3. Электронные спектры спиртовых растворов рутина: *1* — исходный раствор; *2* — раствор с добавлением алюминия хлорида

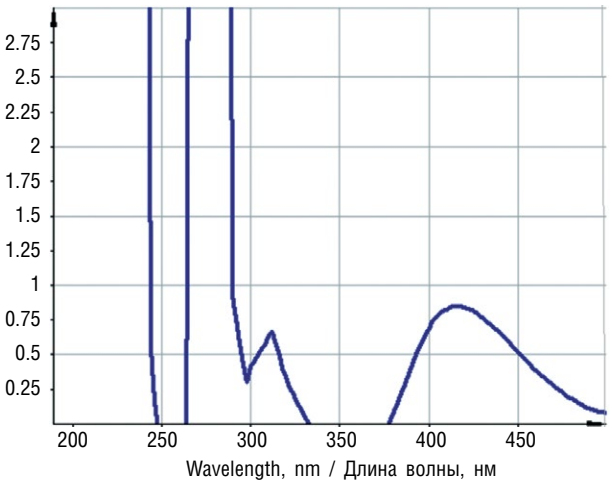

Fig. 4. Electronic spectra of solutions of water-ethanolic extraction from *Juglans nigra* bark (differential type)

Рис. 4. Электронный спектр раствора водно-спиртового извлечения из коры ореха черного (дифференциальный вариант)

diameter. Ground raw material of about 1 g (accurately weighed) is placed in a flask with a thin section, which had a capacity of 100 mL, and 30 mL of 80% ethyl alcohol is added. The flask is closed with a stopper and is weighed on a tared balance to a precision of ±0.01. It is then connected to a reflux condenser and is heated for 60 min in a boiling water bath (moderate boiling). It is then cooled for 30 min, closed with the same stopper, and then weighed again; the missing extractant is replenished to the original weight. A filter paper (red line) is used to filter the extract. The test solution is prepared by placing 1 mL of the resulting extract in a 50 mL volumetric flask; 2 mL of aluminum chloride with a 3% alcohol solution is added; and the solution volume is diluted to the mark with 96% ethyl alcohol (test solution A). A spectrophotometer was used to measure the

Table 1 / Таблица 1

Dependence of the recovery rate of total flavonoids from the *Juglans nigra* bark
Зависимость полноты извлечения суммы флавоноидов из коры ореха черного

No.	Extractant	Ratio of raw material to extractant	Extraction time, min	Degree of grinding of MPRMs, mm	Content of the amount of flavonoids in terms of rutin and absolutely dry raw materials, %
Experiment I					
1	40% ethyl alcohol	1 : 30	60	2	4.48 ± 0.02
2	50% ethyl alcohol	1 : 30	60	2	4.59 ± 0.01
3	60% ethyl alcohol	1 : 30	60	2	4.69 ± 0.02
4	70% ethyl alcohol	1 : 30	60	2	4.78 ± 0.02
5	80% ethyl alcohol	1 : 30	60	2	4.94 ± 0.03
6	90% ethyl alcohol	1 : 30	60	2	4.71 ± 0.01
7	96% ethyl alcohol	1 : 30	60	2	3.92 ± 0.01
Experiment II					
8	80% ethyl alcohol	1 : 30	30	2	4.79 ± 0.03
9	80% ethyl alcohol	1 : 30	45	2	4.88 ± 0.03
10	80% ethyl alcohol	1 : 30	60	2	5.10 ± 0.02
11	80% ethyl alcohol	1 : 30	90	2	5.03 ± 0.02
12	80% ethyl alcohol	1 : 30	120	2	4.76 ± 0.03
Experiment III					
13	80% ethyl alcohol	1 : 20	60	2	4.7 ± 0.01
14	80% ethyl alcohol	1 : 30	60	2	5.10 ± 0.03
15	80% ethyl alcohol	1 : 50	60	2	5.08 ± 0.02
Experiment IV					
16	80% ethyl alcohol	1 : 30	60	1	4.80 ± 0.02
17	80% ethyl alcohol	1 : 30	60	2	5.13 ± 0.02
18	80% ethyl alcohol	1 : 30	60	3	4.55 ± 0.01

optical density of the test solution at a wavelength of 416 nm, 40 min after preparation. The reference solution is prepared by placing 1 mL of the extract (1:30) in a volumetric flask with a capacity of 50 mL and is diluted to the mark with 96% ethyl alcohol.

N o t e: *Preparation of a rutin standard sample solution.* About 0.02 g (accurately weighed) of rutin is placed in a 50-mL volumetric flask, dissolved in 20 ml of 70% ethyl alcohol when heated in a water bath. The volume of the solution is diluted to the mark with 70% ethyl alcohol after cooling to room temperature (rutin solution A). A 25 mL volumetric flask was used to place 2 mL of the rutin solution A and 2 ml of 3% ethanolic solution of aluminum chloride was added. The volume of the solution is diluted to the mark with 96% ethyl alcohol (rutin test solution B). The optical density of the solution B is measured at a wavelength of 416 nm using a spectrophotometer. The reference solution is prepared as follows: 2 mL of rutin solution A is placed in a 25-ml volumetric flask and the volume of the solution is diluted to the mark with 96% ethyl alcohol (rutin reference solution B).

The content of the amount of flavonoids in terms of rutin and absolutely dry raw materials in percentage (x) is calculated by the following equation:

$$x = \frac{D \cdot m_o \cdot 30 \cdot 50 \cdot 2 \cdot 100 \cdot 100}{D_o \cdot m \cdot 50 \cdot 25 \cdot (100 - W)},$$

where D is the test solution's optical density; D_o is the SSRS rutin solution's optical density; m is the weight of raw materials, g; m_o is the weight of the rutin SSRS, g; and W is the weight loss on drying, %.

Table 2 / Таблица 2

Metrological characteristics of the method of quantitative determination of total flavonoids in the *Juglans nigra* bark
Метрологические характеристики методики количественного определения суммы флавоноидов в коре ореха черного

n	f	$\bar{X}$, %	S^2	S	$S_{\bar{X}}$	P, %	$T(P, f)$	ΔX	$\Delta\bar{X}$	E, %
11	10	5.13	0.00044	0.02102	0.006338	95%	2.23	0.05	0.02	1.20

Table 3 / Таблица 3

Content of the flavonoids in the *Juglans nigra* bark
Содержание суммы флавоноидов в образцах коры ореха черного

Sample No.	Characteristics of the raw material sample	Content of the amount of flavonoids in absolutely dry raw material (%) in terms of rutin
1	Botanical Garden of Samara University (March 2018)	5.13 ± 0.02
2	Botanical Garden of Samara University (March 2019)	4.68 ± 0.04

It is advisable to use the calculated value of the specific absorption index at 416 nm in the absence of a standard sample of rutin, namely 238,

$$x = \frac{D \cdot 30 \cdot 50 \cdot 100}{m \cdot 238 \cdot (100 - W)},$$

where D is the test solution's optical density; m is the weight of raw materials, g; m_o is the weight of rutin SSRS, g; 238 is specific absorption index ($E^{1\%}_{1\ cm}$) of rutin SSRS at 416 nm; and W is the weight loss on drying, %.

The method's metrological characteristics for quantitative determination of the amount of flavonoids in black walnut bark are presented in Table 2. The results of statistical processing of the experiments performed indicate that the error of a single determination of the amount of flavonoids in black walnut bark is ±1.20% with a confidence probability of 95% (Table 2).

Validation assessment of the method developed was performed according to specificity, linearity, correctness, and reproducibility. Method specificity was determined by the correspondence of the flavonoid complex's absorption maxima of black walnut bark and rutin with aluminum chloride. A series of rutin solutions were analyzed to determine method linearity (with concentrations ranging from 0.00520 to 0.02080 mg/mL). The correlation coefficient was 0.99996.

The addition method was used to determine method accuracy, which is by adding a rutin solution with a known concentration (25%, 50%, and 75%) to the test solution. Average reduction rate was 98%.

Using the methodology developed, a number of samples of the wild bergamot herb was analyzed (Table 3), and it was determined that the total content of flavonoids varies from 3.92% to 4.28%. Based on the data obtained, we have recommended the lower limit of the content of the amount of flavonoids for the raw material of this plant of not less than 4.0%.

Thus, study results indicate that in terms of rutin, standardizing the black walnut bark is advisable by determining the amount of flavonoids using spectrophotometry at an analytical wavelength of 416 nm.

Conclusions

1. Differential spectrophotometry using rutin SSRS at an analytical wavelength of 416 nm was used to develop a method for the quantitative determination of the amount of flavonoids in the black walnut bark.
2. The content of the amount of flavonoids for the black walnut bark varies from 4.68% to 5.13%. With a confidence level of 95%, the error of a single determination is 1.20%.
3. The study results enable to recommend the content lower limit of not less than 4.0% of the amount of flavonoids for the bark of black walnut.

The authors declare no conflict of interest.

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