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FEATURES OF QUANTITATIVE ESTIMATION OF FLAVONOID CONTENT IN *JUGLANS NIGRA* L. BARKS PREPARATIONS

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The aim of the research is the development of quantification procedures of flavonoids in *Juglans nigra* L. barks preparations using modern instrumental analytical techniques (spectrophotometry, high performance liquid chromatography).

Materials and methods. The subjects of research were tincture and dry extract of *Juglans nigra* L. bark, the samples of which were prepared in March and April 2020 in the Botanical Garden of Samara State Medical University (Samara); the standard samples (SS) of myricitrin, myricetin. The registration of the electronic spectra was carried out with a spectrophotometer «Specord 40» (Analytik Jena, Germany). The chromatographic analysis was carried out by the method of reversed-phase HPLC on a microcolumn liquid chromatograph "Milichrom-6" (NPAO "Nauchpribor", Russia).

Results. Using differential spectrophotometry, methods for the quantitative determination of the total amount of flavonoids in terms of myricitrin in the tincture and dry extract of *Juglans nigra* L. bark, has been developed. It has been determined that the content of the total amount of flavonoids in terms of myricitrin in the tincture and dry extract of *Juglans nigra* L., is 0.84±0.07% and 12.38±0.24%, respectively. The error of a single determination of the total amount of flavonoids in terms of myricitrin in the tincture and dry extract of *Juglans nigra* L. bark with a confidence probability of 95%, is ± 8.91% and ± 2.10%, respectively. Methods for the quantitative determination of myricitrin in the tincture and dry extract of *Juglans nigra* L. bark by HPLC has been developed. The content of the dominant flavonoid – myricitrin (myricetin-3-O-α-L-rhamnopyranoside) – in the tincture and dry extract of *Juglans nigra* L., was 0.42±0.06% and 8.45±0.24%, respectively. The error of the single determination of myricitrin in the tincture and dry extract of *Juglans nigra* L. with a confidence probability of 95% is ± 15.04% and ±2.96%, respectively.

Conclusion. The developed methods for the quantitative determination of flavonoids in the preparations of *Juglans nigra* L. barks L. can be used in solving the problems of standardization of *Juglans nigra* L. preparations.

Keywords: *Juglans nigra* L.; bark; UV spectrophotometry; HPLC; myricitrin; flavonoids

Abbreviations: MPRM – medicinal plant raw materials; HP – herbal preparation; HPLC – high-performance liquid chromatography; SS – standard sample.

ОСОБЕННОСТИ КОЛИЧЕСТВЕННОЙ ОЦЕНКИ СОДЕРЖАНИЯ ФЛАВОНОИДОВ В ПРЕПАРАТАХ КОРЫ ОРЕХА ЧЕРНОГО

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RESEARCH ARTICLE

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Цель. Разработка методик количественного определения флавоноидов в препаратах коры ореха черного с помощью современных инструментальных методов анализа (спектрофотометрия, микроколоночная высокоэффективная жидкостная хроматография).

Материалы и методы. Объектами исследования являлись настойка и сухой экстракт коры ореха черного (*Juglans nigra* L.), образцы которой были заготовлены в марте-апреле 2020 года на территории Ботанического сада ФГБОУ ВО СамГМУ Минздрава России (г. Самара), стандартные образцы мирицитрина, мирицетина. Регистрацию УФ-спектров проводили с помощью спектрофотометра «Specord®40» (Analytik Jena) методом дифференциальной спектрофотометрии. Хроматографический анализ осуществляли методом обращенно-фазовой ВЭЖХ на микроколоночном жидкостном хроматографе «Милихром-6» (НПАО «Научприбор»).

Результаты. Разработана методика количественного определения суммы флавоноидов в пересчете на мирицитрин в настойке и сухом экстракте коры ореха черного (*Juglans nigra* L.) с помощью метода дифференциальной спектрофотометрии. Установлено, что содержание суммы флавоноидов в настойке и сухом экстракте коры ореха черного составляет 0,84±0,07% и 12,38±0,24% (в пересчете на мирицитрин) соответственно. Ошибка единичного определения суммы флавоноидов в пересчете на мирицитрин в настойке и сухом экстракте коры ореха черного с доверительной вероятностью 95% составляет ±8,34% и ±2,10% соответственно.

Разработана методика количественного определения мирицитрина в настойке и сухом экстракте коры ореха черного (*Juglans nigra* L.) методом ВЭЖХ. Содержание доминирующего флавоноида – мирицитрина (мирицетин-3-О-α-L-рамнопиранозид) в настойке и сухом экстракте коры ореха черного составляет 0,42±0,03% и 8,45±0,24% соответственно. Ошибка единичного определения мирицитрина в настойке и сухом экстракте коры ореха черного с доверительной вероятностью 95% составляет ±7,14% и ±2,96% соответственно.

Заключение. Разработанные методики количественного определения флавоноидов в настойке и сухом экстракте коры ореха черного могут быть использованы для решения вопросов стандартизации препаратов указанного лекарственного растительного сырья.

Ключевые слова: орех черный; *Juglans nigra*; кора; УФ-спектрофотометрия; ВЭЖХ; мирицитрин; флавоноиды

Список сокращений: ЛРС – лекарственное растительное сырье; ЛРП – лекарственный растительный препарат; ВЭЖХ – высокоэффективная жидкостная хроматография; СО – стандартный образец.

INTRODUCTION

At present, the search for herbal preparations with a high content of biologically active compounds and a pharmacological activity is a hot topic in pharmacy. Representatives of the genus *Juglans* L. species of the *Juglandaceae* family have the indicated features and, therefore, are promising species of medicinal plant raw materials to be used in medical practice. Representatives of the *Juglans* genus L. are potential sources of naphthoquinones as an important class of biologically active compounds [1–4]. About eight species of plants of the genus *Juglans L.* tend to be cultivated in the territory of the Russian Federation. Medicinal plants *Juglans regia* L., *Juglans nigra* L. and *Juglans cinerea* L. are very interesting for research [5].

The authors' opinion, the bark of *Juglans nigra* L. can serve as a promising object for the production of new herbal formulations [6, 7]. The previous studies have shown that the bark of *Juglans nigra* L. contains various derivatives of naphthoquinone and other compounds: nitrogenous matters, triterpenes and phenolic compounds, including flavonoids [8–13]. A variety of chemical composition, including the presence of a large number of phenolic compounds, determine a wide range of pharmacological activity of the genus *Juglans* L. representatives (*Juglans nigra* L., *Juglans regia* L. and *Juglans cinerea* L) [14–18]. The known antimicrobial, general tonic, anti-inflammatory and antioxidant activities of *Juglans* L. preparations which are present in the pharmological market, may be due to the substances of a flavonoid identity [19–23]. These data indicate the relevance of studying the flavonoids of the *Juglans nigra* L. bark and preparations based on this medicinal plant.

Regardless of the fact that standardization of the bark and *Juglans nigra* L. preparations is carried out according to the content of naphthoquinones (in terms of juglone), has been was found out that the dominant and diagnostically significant compound was flavonoid myricitrin (myricetin-3-O-α-L-rhamnopyranoside). This compound revealed anti-inflammatory, antinociceptive and neurotropic effects [24–26]. Consequently, *Juglans nigra* L. bark preparations are promising for a further study not only in the field of pharmacology, but also in the context of their quality control [27–30].

THE AIM of the research is the application of methods of UV spectrophotometry and microcolumn high performance liquid chromatography (HPLC) to control the content of flavonoids, as well as the analysis of the content of the total amount of flavonoids (UV spectrophotometry) and myricitrin (HPLC) in the obtained tincture and dry extract of the *Juglans nigra* L. bark.

MATERIALS AND METHODS

The objects of research were *Juglans nigra* L. bark tincture and dry extract. These samples were prepared in March-April 2020 in the territory of the Botanical Garden of Samara University (Samara). The tincture and dry extract of *Juglans nigra* L. bark were analyzed using standard samples of myricitrin and myricetin (Fig. 1) by methods of UV spectrophotometry and HPLC.

For HPLC, acetonitrile, c. p. acetic acid ("Component-reactive" LLC, Russia), the water obtained using a

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system for obtaining purified water with a multi-stage purification system (adsorption, reverse osmosis, membrane filtration) and tested for purity under the chromatographic analysis conditions.

The registration of UV spectra was carried out using the spectrophotometry «Specord 40» (Analytik Jena, Germany). The spectral characteristics of these standard samples of myricitrin and myricetin are presented below.

Miricitrin (myricetin-3-O-α-L-rhamnopyranoside) is a yellow with a cream tint crystalline substance with melting points of 203-205 C (aqueous alcohol)[1], UV spectrum (EtOH, λ_{max}, nm): 212, 260, 358; + NaOAc 268, 366; + NaOAc + H_3BO_3 260, 382; + $AlCl_3$ 278, 416; +$AlCl_3$ + HCl 270, 406.

NMR^{1}H (300 MHz, DMSO-d_6, δ, ppm, J/Hz.): 12.68 (1H, s, 5-OH group), 9.23 (2H, br.s , 7-OH-group and 4'-OH-group), 6.88 (2H, s, H-2 'and H-6'), 6.36 (1H , d, 2.5 Hz, H-8), 6.19 (1H, d, 2.5 Hz, H-6), 5.20 (1H, d, 1.5 Hz, H-1'' of rhamnose), 3.1–5.0 (m, 4H of rhamnose), 0.84 (3H, d, 6 Hz, CH_3 of rhamnose).

NMR^{13}C (126.76 MHz, DMSO-d_6, δC, ppm): C-4 (177.85), C-7 (164.24), C-5 (161.37), C-4› (157.57), C-9 (156.49), C-2 and C-3 (145.83), C-3' and C-5' (145.83), C-1 '(119.70), C-2' and C-6 '(108.00), C-10 (104.12), C-1›› of rhamnose (102.00), C-6 (98.41), C-8 (94.30), C-2› (116.21), C-3›› (71.03), C-5 '' (70.62), C-4'' (70.47), C-2'' (70.08), C-6'' (CH_3) (17.57).

Mass spectrum (HR-ESI-MS, 180°C, m/z): m/z 465.1016 [M + H]+, m/z 487.0830 [M + Na]+, m/z 503.0560 [M + K]+.

Myricetin (3,5,7,3',4',5'-hexahydroxyflavone) is a turtle green crystalline substance with melting points of 357°C (aqueous alcohol), UV spectrum (EtOH, λ_{max}, nm): 254, 377; + NaOAc 275, 382; + NaOAc + H_3BO_3 258, 392; + A1C1$_3$ 266, 440; +A1C1$_3$+ HCl 266, 440.

NMR^{1}H (399.78 MHz, DMSO-d_6, δ, ppm, J/Hz): 12.45 (1 H, s, 5-OH group), 10.73 (1H s, 7-OH- group), 9.28 (1H, s, 4'-OH-group), 9.17 (2H, s, 3'-OH-group and 5'-OH-group), 8.75 (1H, s, 3-OH-group), 7.20 (2H, s, H-2 'and H-6'), 6.32 (1H, d, 2.2 Hz, H-8), 6.14 (1H, d, 2.2 Hz, H-6).

NMR^{13}C (100.52 MHz, DMSO-d6, δ_c, ppm): C-4 (176.29), C-7 (164.39), C-5 (161.25), C-9 (156.59), C-4' (147.36), C-3' and C-5' (146.23), C-2 and C-3 (136.38), C-1' (121.30), C-2' and C-6' (107.68), C-10 (103.49), C-8 (93.71), C-6 (98.67).

Based on the spectral data, since the dominant flavonoid myricitrin has an absorption maximum at 360 ± 2 nm in the long-wavelength region of the electronic spectrum, 360 nm wavelength for the detection of analytes during HPLC analysis was chosen.

Preparation of work solutions for analysis by UV spectrophotometry

Juglans nigra L. bark tincture was obtained from the *Juglans nigra* L. bark using a 70% water-ethanolic solution at the ratio of «raw material- extracting solvent» 1:5 using the method of fractional maceration. A part of the *Juglans nigra* L. bark fluid extract 1:1 was used to obtain a solid extract, and then the dry extract of *Juglans nigra* L. bark. The thick extract was obtained by removing the extractant from the liquid extract under vacuum. The thick extract was then dried in a hot-air oven to obtain a dry extract.

Sample processing of *Juglans nigra* L. bark tincture. 1.00 ml of *Juglans nigra* L. bark tincture was placed in a 25 ml capacity measuring flask, the volume of the solution was adjusted by a 70% water-ethanolic solution (sample solution A_1). 1 ml of the sample solution A_1 was placed in a 50 ml capacity measuring flask, 2 ml of 3% ethanolic solutions of aluminum chloride was added and the volume of the solution was adjusted by a 96% ethanolic solution (sample solution B_1). The reference solution was prepared by the following methods: 1 ml of the sample solution A_1 was placed in a 50 ml capacity measuring flask and the volume of the solution was adjusted by a 96% ethanolic solution.

Sample processing of *Juglans nigra* L. bark dry extract. About 0.2 g of the dry *Juglans nigra* L. bark extract (an accurately weighed quantity) was placed in a 25 ml capacity measuring flask, dissolved in 15 ml of a 70% water-ethanolic solution by heating in a water bath and the volume of the solution was adjusted by the same solvent (sample solution A_2). 1 ml of the sample solution A_2 was placed in a 50 ml capacity measuring flask, 2 ml of 3% ethanolic solutions of aluminum chloride was added, and the volume of the solution was adjusted by a 96% ethanolic solution (sample solution B_2). The reference solution was prepared in the following way: 1 ml of the sample solution A_2 was placed in a 50 ml capacity measuring flask and the volume of the solution was adjusted by a 96% ethanolic solution.

Preparation of a standard myricitrin solution for UV spectrophotometry. About 0.0025 g of myricitrin (an accurately weighed quantity) was placed in a 25 ml capacity measuring flask, dissolved in 15 ml of 80% water-ethanolic solutions by heating in a water bath. The contents of the capacity measuring flask was cooled down to room temperature, the volume of the solution was adjusted by a 80% water-ethanolic solution (myricitrin standard solution A_3). 5 ml of myricitrin standard solution A_3 was placed in a 50 ml capacity measuring flask, 2 ml of 3% ethanolic solutions of aluminum chloride was added and the volume of the solution was adjusted by a 96% ethanolic solution (myricitrin standard solution B_3).

Method of quantitative determination of total amount of flavonoids in *Juglans nigra* L. bark tincture

About 1.00 ml of *Juglans nigra* L. bark tincture (an accurately weighed quantity) was placed in a 25 ml capacity measuring flask, the volume of the solution was

[1] USA National Library of Medicine National Institutes of Health. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Myricitrin.

adjusted by a 70% water-ethanolic solution (sample solution A_1). 1 ml of sample solution A_1 was placed in a 50 ml capacity measuring flask, 2 ml of 3% ethanolic solutions of aluminum chloride was added and the volume of the solution was adjusted by a 96% ethanolic solution (sample solution B_1). The reference solution was prepared in the following way: 1 ml of the sample solution A_1 was placed in a 50 ml capacity measuring flask and the volume of the solution was adjusted by a 96% ethanolic solution.

The content of the total amount of flavonoids in terms of myricitrin and absolutely dry raw materials in percent (X) was calculated by the formula:

$$x = \frac{A * m_0 * 25 * 50 * 5 * 100}{A_0 * V * 25 * 1 * 25},$$

where: A – the absorption of the test solution; A_0 – the absorption of the myricitrin standard solution; V – the volume of the tincture for analysis, ml; m_0 – mass of the myricitrin standard sample, g.

If a standard sample of myricitrin is absent, the theoretical value of the specific absorbance – 432 at the wavelength of 416 nm – can be used.

$$x = \frac{A * 25 * 50}{V * 432},$$

where: A – the absorption of the test solution; V – the volume of the tincture for the analysis, ml; 432 – specific absorbance ($E^{1\%}_{1cm}$) of myricitrin at 416 nm.

Method of quantitative determination of total amount of flavonoids in *Juglans nigra* L. bark dry extract

About 0.2 g of dry *Juglans nigra* L. bark extract (an accurately weighed quantity) was placed in a 25 ml capacity measuring flask, dissolved in 15 ml of a 70% water-ethanolic solution by heating in a water bath and the volume of the solution was adjusted by the same solvent (sample solution A_2). 1 ml of the sample solution A_2 was placed in a 50 ml capacity measuring flask, 2 ml of a 3% ethanolic solution of aluminum chloride was added and the volume of the solution was adjusted by a 96% ethanolic solution (sample solution B_2). The reference solution was prepared in the following way: 1 ml of the sample solution A_2 was placed in a 50 ml capacity measuring flask and the volume of the solution was adjusted by a 96% ethanolic solution.

The content of the total amount of flavonoids in terms of myricitrin and absolutely dry raw materials in percent (X) was calculated by the formula:

$$x = \frac{A * m_0 * 25 * 50 * 5 * 100}{A_0 * m * 25 * 1 * 25},$$

where: A – the absorption of the test solution; A_0 – the absorption of the myricitrin standard solution; m – mass of the dry extract, g; m_0 – mass of the myricitrin standard sample, g.

If a standard sample of myricitrin is absent, the theoretical value of the specific absorbance – 432 at the wavelength of 416 nm can be used.

$$x = \frac{A * 25 * 50}{m * 432},$$

where: A – the absorption of the test solution; m – mass of the dry extract, g; 432 – the specific absorbance ($E^{1\%}_{1cm}$) of myricitrin at 416 nm.

Preparation of sample solutions for HPLC analysis

Sample processing for *Juglans nigra* L. bark tincture. 5.00 ml of *Juglans nigra* L. bark tincture (an accurately weighed quantity) was placed in a 25 ml capacity measuring flask. The volume of the solution was adjusted by a 96% ethanolic solution (sample solution A_4). The sample solution A_4 was decanted using a Milipore membrane filter (0.45 µm).

Sample processing of dry *Juglans nigra* L. bark extract. About 0.2 g of dry *Juglans nigra* L. bark extract (an accurately weighed quantity) was placed in a 25 ml capacity measuring flask, dissolved in 15 ml of a 70% water-ethanolic solution by heating in the water bath and the volume of the solution was adjusted by the same solvent (sample solution A_5). 5 ml of the sample solution A_5 was placed in a 25 ml capacity measuring flask. The volume of the solution was adjusted by a 96% ethanolic solution (sample solution B_5). The sample solution A_5 was decanted using a Milipore's membrane filter (0.45 µm).

Preparation of myricitrin sample solution for HPLC. About 0.02 g of myricitrin (an accurately weighed quantity) was placed in a 50 ml capacity measuring flask, dissolved in a 70% water-ethanolic solution, and the volume of the solution was adjusted by the same solvent.

Preparation of a myricetin standard solution for HPLC. About 0.02 g of myricetin (an accurately weighed quantity) was placed in a 50 ml capacity measuring flask, dissolved in a 70% water-ethanolic solution, and the volume of the solution was adjusted by the same solvent.

Chromatographic conditions

A chromatographic analysis was carried out by reverse-phase high performance liquid chromatography (RP-HPLC) on a microcolumn liquid chromatograph "Milichrom-6" by using the following conditions: isocratic mode, steely column "KAH-6-80-4". The mobile phase was acetonitrile: a 1% solution of acetic acid in water at the ratio of 2:8, the elution rate was 100 µL/min. The volume of the eluent was 2500 µL. The substances were detected at the wavelength of 360 nm. The volumes of the injected samples were 4 µl for the tincture and dry extract of *Juglans nigra* L. bark, myricitrin, myricetin.

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Suitability assessment of the developed chromatographic system

To assess the suitability of the chromatographic system, 5-fold chromatography of the test solution of *Juglans nigra* L. bark tincture was carried out. Subsequently, the following indicators were calculated: column performance, resolution between peaks, and the asymmetry factor. Based on the calculations, the following results were obtained (Table 1).

Quantitative determination method of myricitrin in *Juglans nigra* L. bark tincture

About 5.00 ml of *Juglans nigra* L. bark tincture (an accurately weighed quantity) was placed in a 25 ml capacity measuring flask. The volume of the solution was adjusted by a 96% ethanolic solution (sample solution A_4). The sample solution A_4 was decanted using a Milipore's membrane filter (0.45 μm).

4 μl of the sample solution was injected in the liquid chromatograph "Milichrom-6" with a UV detector. Chromatography was carried out by reverse-phase high performance liquid chromatography in the isocratic mode on a steel column "KAH-6-80-4" (No. 2; 2 mm x 80 mm; Separon-C18 7 μm). The eluent system was acetonitrile – water in the ratio of 2:8 with the addition of 1% acetic acid, the elution rate – 100 μl/min, the eluent volume – 2500 μl. The operating wavelength was 360 nm, the sensitivity range – 0.5.

Parallely, 4 μl of the myricitrin standart sample was introduced into the chromatograph and chromatographed as described above. At least 3 parallel determinations for the myricitrin tincture test solution and the myricitrin standard solutions were carried out, as described above. The peak of myricitrin was identified on the chromatograms of the test solution. The average area of the myricitrin peak on the chromatograms of the myricitrin sample solution was calculated according to the results of 3 determinations.

The content of myricitrin in the *Juglans nigra* L. bark tincture was calculated in terms of absolutely dry raw materials in percent (X) by the formula:

$$x = \frac{S * m_0 * 0,98 * V * V_2 * 100}{S_0 * V_t * V_0 * V_1},$$

where: S – the average value of the the myricitrin peak area in the chromatogram of the working solution; S_0 – the average value of the myricitrin peak area in the chromatogram of the standard sample solution; V – the volume of the working solution, ml; V_2 – the volume of the injected sample of the working solution, μl; V_0 – the volume of the sample of the myricitrin standard solution, ml; V_2 – the volume of the injected sample of the myricitrin standard solution, μl; V_t – the volume of the tincture for the analysis, ml; m_0 – mass of the myricitrin standard sample, g; 0,98 – substance assay in 1.0 g of the myricitrin standard sample.

Methods of myricitrin quantitative determination in *Juglans nigra* L. bark dry extract

0.2 g of *Juglans nigra* L. bark dry extract (an accurately weighed quantity) was placed in a 25 ml capacity measuring flask, dissolved in 15 ml of a 70% water-ethanolic solution by heating in a water bath and the volume of the solution was adjusted by the same solvent (sample solution A_5). 5 ml of the sample solution A_5 was placed in a 25 ml capacity measuring flask. The volume of the solution was adjusted by a 96% ethanolic solution (sample solution B_5). The sample solution A_5 was decanted using a Milipore's membrane filter (0.45 μm).

On a microcolumn liquid chromatograph "Milichrom-6" with a UV detector, 4 μl of the sample solution was injected. Chromatography was carried out by reverse-phase high performance liquid chromatography in isocratic mode on a steely column "KAH-6-80-4". The mobile phase was acetonitrile: a solution of 1% acetic acid in water at the ratio of 2:8, elution rate was 100 μL/min. The eluent volume was 2500 μL. The operating wavelength was 360 nm, the sensitivity range was 0.5. 4 μl of the resulting solution was injected in the liquid chromatograph "Milichrome-6" (NPAO "Nauchpribor", Russia) with a UV detector. It was chromatographed under reversed-phase chromatography in the isocratic mode on the steel column "KAH-6-80-4" (No. 2; 2 mm x 80 mm; Separon-C18 7 μm), the eluent system was acetonitrile – water in the ratio of 2: 8 with the addition of 1% acetic acid, the elution rate was 100 μl/min, the eluent volume was 2500 μl. The operating wavelength was 360 nm, the sensitivity range was 0.5.

The content of myricitrin in the *Juglans nigra* L. bark dry extract in terms of absolutely dry raw materials in percent (X) was calculated by the formula:

$$x = \frac{S * m_0 * 0,98 * V * V_2 * 100}{S_0 * m_e * V_0 * V_1},$$

where: S – the average value of the myricitrin peak area in the chromatogram of the working solution; S_0 – the average value of the myricitrin peak area in the chromatogram of the standard sample solution; V – the working solution volume, ml; V_2 – the injected sample volume of the working solution, μl; V_0 – the solution volume of the myricitrin standard sample, ml; V_2 – the injected sample volume of the myricitrin standard sample, μl; m_e – mass of the dry extract, g; m_0 – mass of the myricitrin standard sample, g; 0,98 – the substance assay in 1.0 g of the myricitrin standard sample.

Metrological characteristics of the developed methods

To carry out the calibration procedure, a series of dilutions of myricitrin (250–2500 μg/mL) were chromatographed under the described conditions. Based on the data obtained, a graph was plotted in the coordinates "concentration, μg/mL – peak area" and the linear regression equation ($Y = aX + b$), the value of the coefficient of

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determination (r^2), and the standard deviation were calculated using Microsoft Excel 2013. Statistical processing of the experimental data on the intermediate precision of the developed methods in the analysis of 11 samples of the test solutions of the tincture and dry extract (P = 95%) was carried out. Herewith, Student's t-test to calculate the boundary values of the confidence interval of the average result and determine the error of a single determination (SP RF XIV, GPM 1.1 .0013.15)[2] was used. The stability of the methods was determined by the sample of the *Juglans nigra* L. bark tincture analyzing it 2, 4, 8, 12, 24, 48, and 72 hours after the first analysis. The correctness of the methods was determined at the sample of the *Juglans nigra* L. bark tincture and the standard myricitrin solution in the amount of 25% to 75% of the original content using the standard addition method.

RESULTS AND DISCUSSION

Proceeding from the literature data, there are several approaches to the standardization of medicinal plant materials of the genus *Juglans* species including the *Juglans nigra* L. bark.

The colleagues from the Pyatigorsk Medical and Pharmaceutical Institute, the branch of Volgograd State Medical University, proposed an approach to the standardization of medicinal plant and herbal preparations of the genus *Juglans*. This approach consisted in using naphthoquinones (in particular, juglone) as the analyzed biologically active substance group [4, 28, 29].

To quantify the total amount of naphthoquinones in terms of juglone in the herbal preparations of the genus Juglans species, the developed methods of the photocolorimetric determination was used. In this case, the extraction was obtained by the double extraction method with a 20% water-ethanolic solution, followed by the concentration, and a triple extraction with diethyl ether [4, 28, 29].

In addition to polyphenols, such as phenylpropanoids, flavonoids and tannins, as well as terpenoids, they can also act as the analyzed biologically active substance group for the quantitative determination methods. The analysis of these compounds was carried out by spectrophotometry, HPLC, liquid chromatography, mass spectrometry with an ion trap, GC-MS [29, 31].

Previously, the studies by the Department of Pharmacognosy with Botany and foundations of Phytotherapy of Samara State Medical University showed the possibility of quantifying the total amount of flavonoids in the *Juglans nigra* L. bark by differential spectrophotometry in terms of myricitrin; the analytical wavelength corresponded to 416 nm [7]. Based on the studies carried out, it can be concluded that further research is needed in terms of standardization of medicinal herbal preparations of *Juglans nigra* L.

A comparative study of the tested solutions electronic spectra of *Juglans nigra* L. bark preparations (tincture and dry extract) made it possible to establish two absorption maxima of about 260 nm and 360 nm, which were characteristic of flavonoids (flavonols) and this was confirmed by the bathochromic shift of the long-wavelength band in the presence of $AlCl_3$, as well as facts of differential spectra with an absorption maximum of 414–416 nm (Fig. 2B-2E).

The authors found out that myricitrin in *Juglans nigra* L. makes a significant contribution to the nature of the absorption spectrum of the hydroalcoholic extract from the *Juglans nigra* L. bark, therefore, this is a dominant and diagnostically significant substance for this type of raw materials. Taking into account the fact that the absorption maxima of the solution of the standard myricitrin sample and the aqueous-alcoholic extract of the *Juglans nigra* L. bark are in the region of 416 nm (differential variant), it is advisable to determine the content of the total amount of flavonoids in terms of myricitrin at the wavelength of 416 nm (Fig. 2E and 2E).

During the development of quantitative determination methods for the *Juglans nigra* L. bark tincture and dry extract, the optimal parameters of the sample processing and the analytical wavelength for the quantitative analysis (416 nm) were determined.

The dependence of the optical density on the myricitrin concentration was described by a linear regression in the concentration range from 10 to 50 μg/mL (Fig. 3).

The metrological characteristics of the quantitative determination methods of the total flavonoids content in the preparations of *Juglans nigra* L. bark are presented in Table 2. The results of the intermediate precision assessment of the results of the experiments carried out, indicate a satisfactory reproducibility of the analysis results. The error of a single determination of the flavonoids amount in the *Juglans nigra* L. bark tincture and dry extract with a confidence level of 95% is ±8.34% and ±2.10%, respectively (Table 2).

The validation assessment of the developed methodology was carried out according to the following indicators: specificity, linearity, correctness. The specificity of the method was determined by the correspondence of the absorption maxima of the *Juglans nigra* L. bark flavonoid complex and the myricitrin standard reference with aluminum chloride. The linearity of the method for a series of the standard myricitrin solutions (with the concentrations ranging from 10 to 25 μg/ml) was determined. The coefficient of determination was 0.99988.

The methods correctness was determined by the standard addition method. A myricitrin solution with a known concentration (25%, 50% and 75%) was added to the test tincture solution. A relative error of the analysis was ±3.19%. The experiments with the addition of myricitrin to the samples of raw materials showed that the analysis error is within the error of a single determination, which indicates the absence of a systematic error in the developed method (Table 3).

[2] State Pharmacopoeia of the Russian Federation. XIV edition. Vol. 4. Moscow, 2018. – 1832 p. Available from: http://resource.rucml.ru/feml/pharmacopia/14_4/HTML/index.html.

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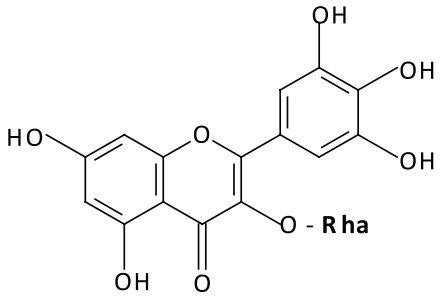

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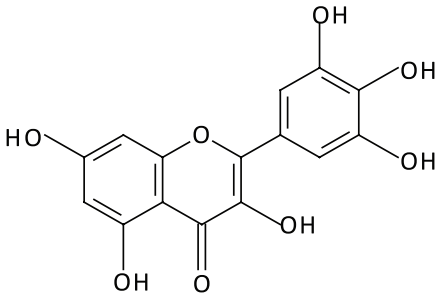

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Table 1 – Determination of chromatographic column suitability

Chromatographic column parameter	Meaning	Standard indicator
Column performance	5115	Not below 2000 theoretically perfect plates
Resolution between peaks	1.65	Not below 1.5
Asymmetry factor	1.35	Not Below 1.5

Table 2 – Metrological characteristics of quantitative determination methods of the flavonoids total amount in *Juglans nigra* L. bark preparations

Sample	n	f	$\overline{X}$	S	$S_{\overline{x}}$	P (%)	T (P, t)	±ΔX	E,%
Tincture of *Juglans nigra* L. bark	11	10	0.84	0.03357	0.01012	95	2.23	± 0.07	± 8.34
Dry extract of *Juglans nigra* L. bark	11	10	12.38	0.10650	0.03211	95	2.23	± 0.24	± 2.10

Table 3 – The content of flavonoids total amount ~~of~~ in the *Juglans nigra* L. bark tincture depending on the addition of myricitrin

Initial content of flavonoids total amount, mg/g	Addition of myricitrin, mg/g	The content of flavonoids total amount, mg/g		Error	
		Injected quantity	Detected quantity	Absolute, mg	Relative, %
5.0	1.25	6.25	6.05	–0.2	–3.2
5.0	2.5	7.5	7.9	+0.4	+5.33
5.0	3.75	8.75	8.45	–0.3	–3.43

Table 4 – The content of the flavonoids total amount in *Juglans nigra* L. bark samples

No.	Sample raw material	Absorbance, A	The content of total of flavonoids calculated on myricitrin and absolutely dry raw materials, %
1	Tincture of *Juglans nigra* L. bark	0.29	0.84 ± 0.07
2	Dry extract of *Juglans nigra* L. bark	0.85	12.38 ± 0.24

Table 5 – Retention times of peaks in *Juglans nigra* L. bark flavonoids

Flavonoid	Retention time on the chromatogram, min		
	Standard sample	Tincture	Dry extract
Miricitrin (1)	7.326	6.951	6.741
Myricetin (2)	14.211	18.909	17.277

Table 6 – Results of determining the methods correctness

Initial content of myricitrin, mg/g	Quantity of added of myricitrin, mg/g	Content of myricitrin, mg/g		Error	
		Injected quantity	Detected quantity	Absolute, mg	Relative, %
4.20	1.05	5.25	4.93	–0.32	–6.09
4.20	2.10	6.30	6.01	+0.29	+4.60
4.20	3.15	7.35	7.12	–0.23	–3.14

Table 7 – The content of myricitrin in the samples of *Juglans nigra* L. bark preparations of determined with HPLC

No.	Sample	Myricitrin content (%)
1	Tincture of *Juglans nigra* L. bark	0.42 ± 0.06
2	Dry extract of *Juglans nigra* L. bark	8.45 ± 0.25

Table 8 – Evaluation of the inter-assay precision of the methods for the quantitative determination of myricitrin in *Juglans nigra* L. bark

Sample	*f*	$\overline{X}$	S^2	*S*	*P,%*	*t (P,f)*	$\Delta\overline{X}$	*E,%*	F (P, f_1, f_2) (table)	$F_{estim.}$
Tincture of *Juglans nigra* L. bark	10	0.42	0.000436	0.02089	95	2.23	±0.04	±8.45	4.80	1.14
Tincture of *Juglans nigra* L. bark	10	0.36	0.000496	0.02228	95	2.23	±0.05	±13.87		
Dry extract of *Juglans nigra* L. bark	10	8.45	0.00377	0.06139	95	2.23	±0.21	±2.06	4.80	2.74
Dry extract of *Juglans nigra* L. bark	10	8.31	0.01035	0.1017	95	2.23	±0.23	±2.73		

The content of the flavonoids total amount in *Juglans nigra* L. bark preparations, determined by the differential spectrophotometry method at the analytical wavelength of 416 nm, is presented in Table 4.

The content of the flavonoids total amount for the test sample of *Juglans nigra* L. bark tincture was 0.84±0.07%. The content of the flavonoids total amount for the samples of the *Juglans nigra* L. bark dry extract was 12.38±0.24% (in terms of myricitrin).

The analysis by high-performance liquid chromatography showed that under the indicated chromatography conditions and using the "acetonitrile-water" system in the ratio of 2:8 in the tested solutions of the tincture and dry extract, it is possible to identify the analyzed component – myricitrin (Fig. 3B, 3C, 3D).

The retention times of the myricitrin and myricetin peaks on the chromatogram of the myricitrin standard sample, as well as in the working solutions of *Juglans nigra* L. bark tincture and dry extract, are presented in Table 5.

Adding of myricitrin (1) and myricetin (2) solutions into the test solutions of *Juglans nigra* L. bark tincture and dry extract, manifests itself on the chromatogram as the increase in the intensity of myricitrin and myricetin peaks, respectively, compared to that of myricitrin and myricetin in the initial test solution (Fig. 5A and 5B).

Taking into account a low content of myricetin in the extract in comparison with myricitrin, it could be reasonable to carry out a quantitative analysis basing on only myricitrin. The dependence of the chromatographic peak area on the concentration of myricitrin was described by the linear regression equation in the concentration range from 250 to 1500 μg/ml (Fig. 6).

The correctness of the methods was determined by the standard addition method. Solutions of myricitrin with known concentrations (25%, 50% and 75%) were added to the test solution of the tincture (Table 6). A relative error was ±4.19%. A permissible error determined for the samples with additives of standard samples was within the error of a single determination, which indicates the absence of a systematic error.

The content of myricitrin in the samples of *Juglans nigra* L. bark preparations, determined by reversed-phase HPLC, is presented in Table 7.

To assess the index of inter-assay precision, the relative standard deviation, variance, Student's test and Fisher's F-test were calculated (Table 8). The evaluation of the inter-assay precision of the tincture and dry extract samples was carried out on two Milichrom-6 devices. For each sample, the studies were carried out in the amount of eleven experiments (Table 8). The results of calculating the value of the relative standard deviation did not exceed 2%, the error of a single determination of the myricitrin content in the samples of tincture carried out on "Milichrome-61" and "Milichrome-62", were 8.45% and 13.87%, respectively. The errors of a single determination of the myricitrin content in the dry extract samples were 2.06% and 2.73%, respectively (Table 8).

The calculation of the Fisher criterion allows us to state that the average results of the analysis of the tincture samples and of dry extract on different chromatographs are statistically significant (P=95%) and do not differ from each other. Table 8 shows that the calculated value of Fisher's F-test in the analysis of tinctures and the dry extract is less than the table value. Therefore, the variances of the analysis results of both chemists are statistically equivalent (Table 7). Thus, the developed method meets the validation requirements in terms of the intermediate precision.

The results of assessing the inter-assay precision of the developed technique when analyzing 11 trials of the tincture and dry extract samples, indicate a satisfactory reproducibility of the analysis results.

CONCLUSION

So, the results of the performed spectral and chromatographic studies have indicated possibility to standardize the *Juglans nigra* L. bark preparations by determining the total amount of flavonoids calculated in terms of myricitrin and using the method of UV spectrophotometry at the wavelength of 416 nm. Standardization can take place by determining the content of the dominant and diagnostically significant flavonoid – myricitrin – and using the HPLC method at the wavelength of 360 nm too.

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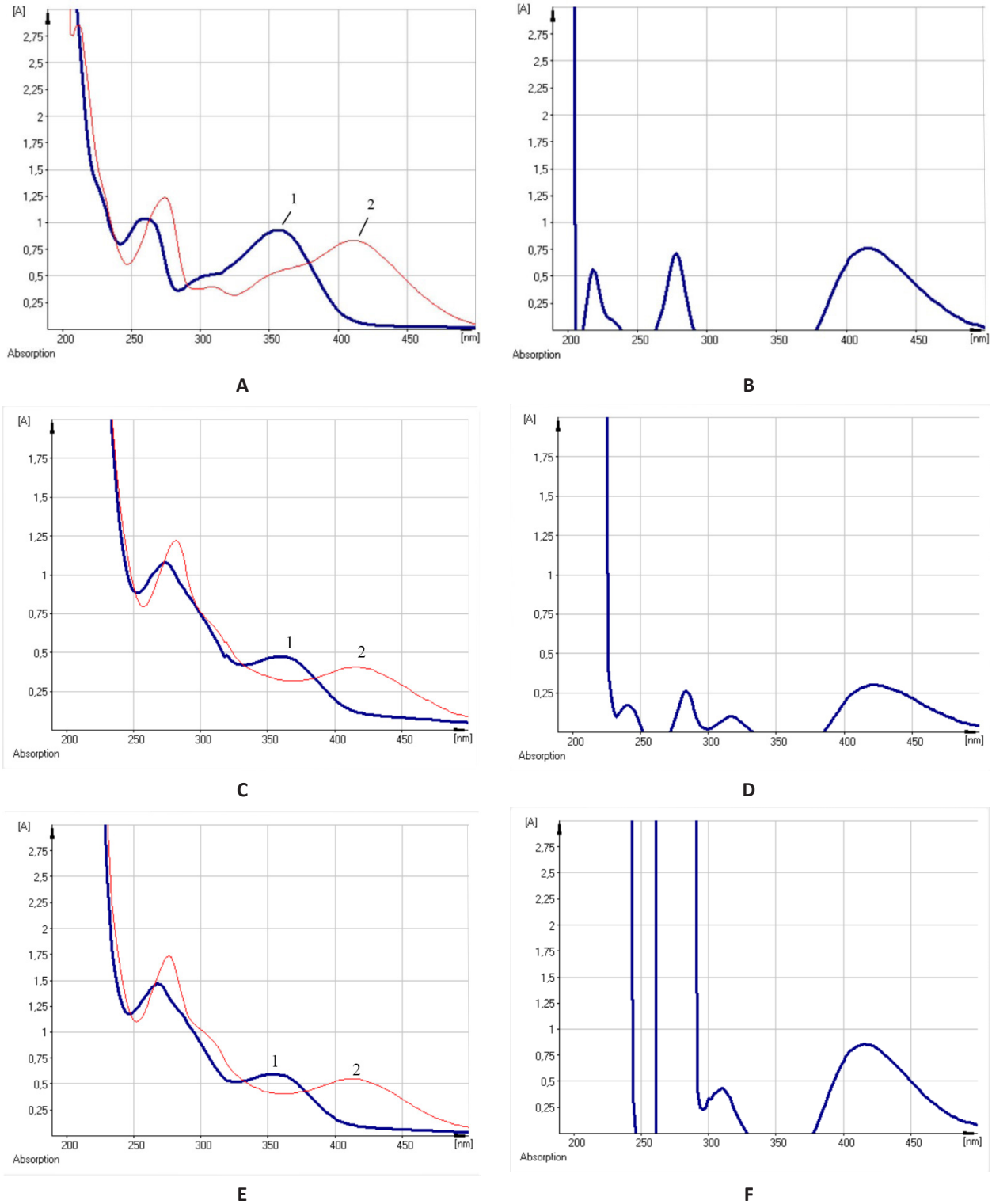

Figure 2 – Electronic spectra of test solutions of *Juglans nigra* L. bark preparations

Notes: A – Electronic spectra of ethanolic solutions of myricitrin; B – Electronic spectra of ethanolic solutions of myricitrin (differential option); C – Electronic spectra of test solution of *Juglans nigra* L. bark tincture; D – Electronic spectra of test solution of tincture of *Juglans nigra* L. bark (differential option); D – Electronic spectra of the test solution of *Juglans nigra* L. bark dry extract; E – Electronic spectra of the test solution of *Juglans nigra* L. bark dry extract (differential option). 1 – initial solution; 2 – solution with the addition of aluminum chloride.

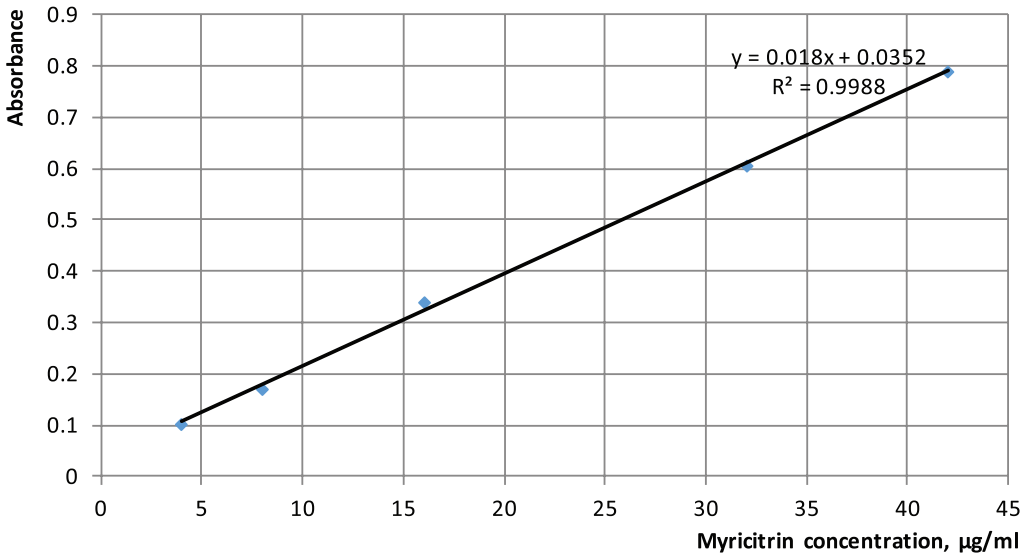

Figure 3 – Graph of the absorbance dependence on the myricitrin concentration in the sample and the linear regression equation

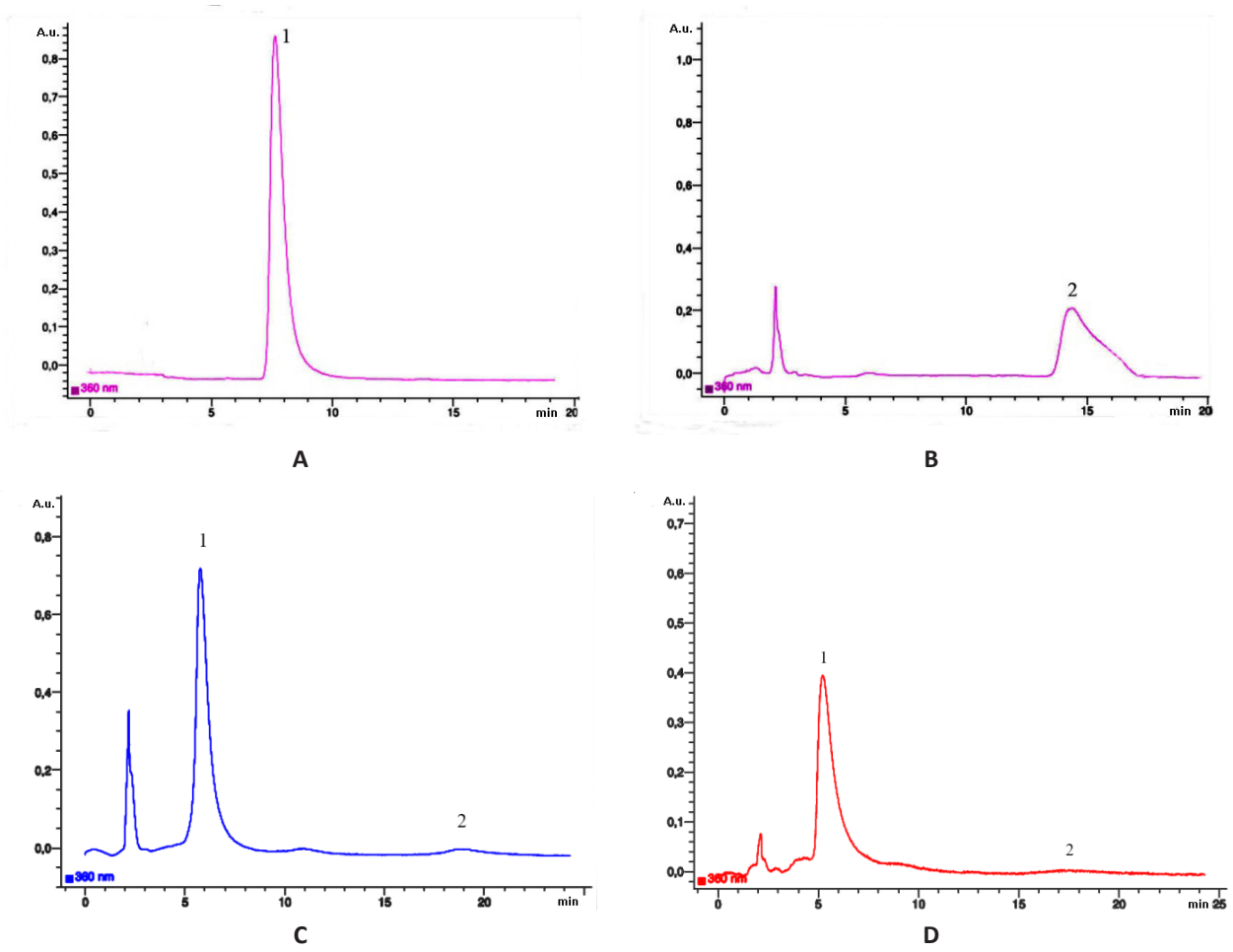

Figure 4 – HPLC chromatograms of test and standard solutions of *Juglans nigra* L. bark preparations

Notes: A – HPLC chromatogram of myricitrin; B – HPLC chromatogram of myricetin; C – HPLC chromatogram of the test solution of *Juglans nigra* L. bark tincture; D – HPLC chromatogram of the test solution of Juglans nigra L. bark dry extract. 1 – myricitrin; 2 – myricetin.

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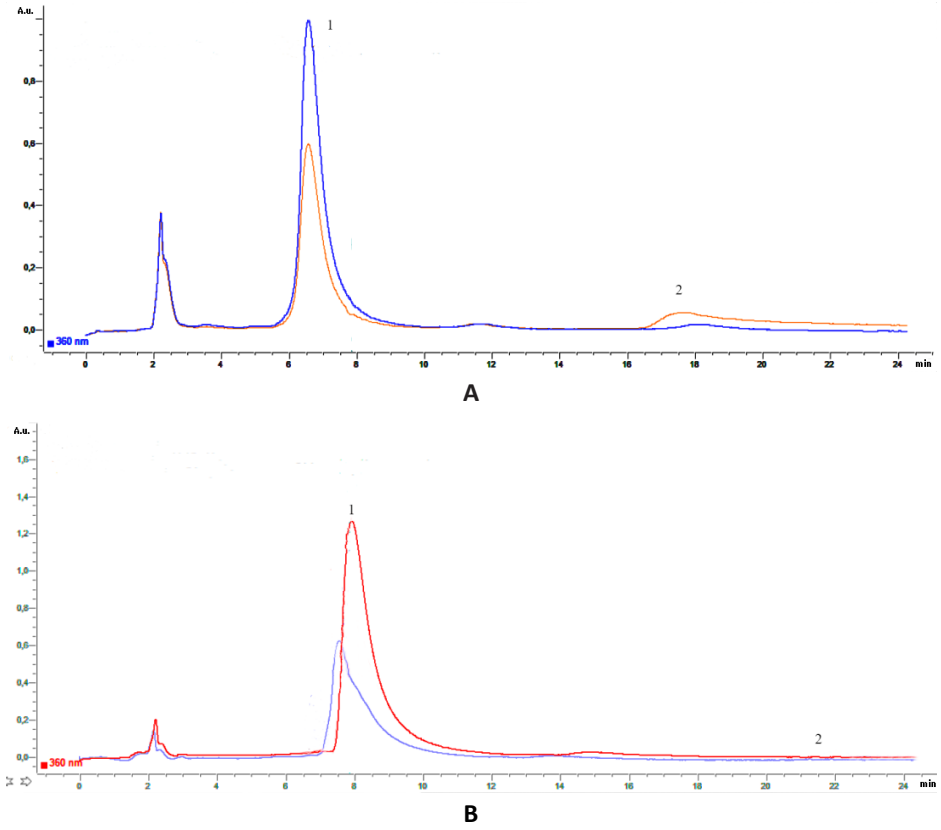

Figure 5 – HPLC chromatogram of test solution of *Juglans nigra* L. bark preparation with addition of myricitrin solution

Notes: A – HPLC chromatogram of the test solution of Juglans nigra L. bark tincture with myricitrin and myricetin addition; B – HPLC chromatogram of the test solution of Juglans nigra L. bark dry extract with myricitrin and myricetin addition. 1 – myricitrin; 2 – myricetin.

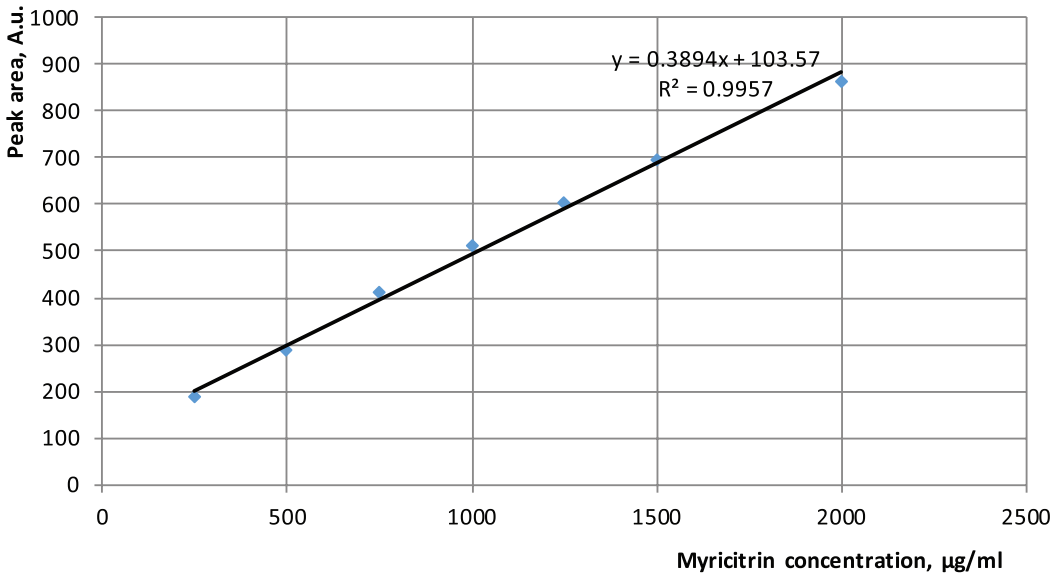

Figure 6 – Graph of the peak area dependence on the concentration of myricitrin in the sample and the linear regression equation

The content of the total amount of flavonoids in terms of myricitrin in the *Juglans nigra* L. bark tincture was 0.84±0.07%. The content of the total amount of flavonoids in terms of myricitrin in the samples of the *Juglans nigra* L. bark dry extract was 12.38±0.24%. The error of a single determination of the total amount of flavonoids in the tincture and dry extract of *Juglans nigra* L. bark by the confidence coefficient of 95% were ±8.34% and ±2.10%, respectively. The content of myricitrin in the samples of the tincture of *Juglans nigra* L. bark was 0.42 ± 0.06%. The content of myricitrin in the samples of the dry extract of *Juglans nigra* L. bark was 8.45±0.21%. The error of a single determination of the total of flavonoids in the tincture and dry extract of *Juglans nigra* L. bark by the confidence coefficient of 95% were ±7.14% and ±2.96%, respectively.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

CONTRIBUTION OF AUTHORS

Vladimir A. Kurkin – concept and design of research, editing; Natalya I. Zimenkina – material collection and processing, text writing and compiling the references, statistical processing of measurement results.

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