

STUDY OF OUTWARD SIGNS, MICROSCOPY AND CHEMICAL COMPOSITION OF WALNUT

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Received: 29 June 2018, Revised and Accepted: 06 September 2018

ABSTRACT

Objective: Walnut is a medicinal plant that is widely used in traditional medicine. We believe that the study of the medicinal plant material of walnut-fruit in the stage of milky-wax maturity and walnut bark is relevant. We described outward signs and microscopy, the diagnostic features of both whole and grinded raw materials, powder bark of walnut and fruits of walnut in the stage of milky-wax maturity.

Methods: Alcohol extraction which was derived from walnut bark is a transparent liquid of brown color and alcohol extraction which was derived from walnut fruits in the stage of milky-waxy maturity is a transparent, green-brown liquid with a fragrant smell. We performed thin-layer chromatography for the walnut cortex and identified gallic acid.

Results: A method of chromatography-mass spectrometry in alcohol extraction from walnut fruit in the stage of milky-waxy maturity allowed to identify 17 compounds belonging to different classes of biologically active substances. We identified sugars, flavonoids, phenolic compounds of coumarins and organic acids. In alcohol extraction from the walnut bark we identified marker substances.

Keywords: Walnut, Walnut bark, Walnut fruit.

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INTRODUCTION

At present, an impressive number of scientists around the world is engaged in the search for new natural biologically active substances; thorough study of the pharmacological action of known BAS, i.e., actions on individual systems and the body as a whole; is engaged in the search for new sources of biologically active substances, more economically advantageous. These sources are plants. Scientists have given nature the widest choice of objects for research. One of them is a powerful plant with a majestic crown - a walnut (*Juglans regia* L.), one of the popular names of which is the royal nut [1,2].

Many researchers mentioned this medicinal plant in their literary works, having previously conducted many experiments to study the chemical composition - these are qualitative reactions to the main groups of BAS, the paper chromatography method, and the thin-layer chromatography (TLC) method. Quantitative determination of ultraviolet (UV) spectrophotometry and high-performance liquid chromatography [3-6] was carried out.

Qualitative reactions determined the rich chemical composition. In the medicinal plant, there are quinones (naphthoquinone juglone and α and β -hydrogens), flavonoids (hyperoside and kaempferol), Vitamin B, ascorbic acid, tannins, carotenoids, phenolic acids, lilac aldehyde, and juglone and this is only in the leaves [4,7-10].

Green pericarp contains hydrocoals and tannins. In the kernels of walnut fruit identified fatty oils, protein substances, Vitamins K and P, and amino acids [11-13]. The raw material of walnut is widely used in folk medicine and homeopathy [7,14]. A number of researchers indicate a wide pharmacological activity of various types of walnut raw materials. Hence, according to a number of authors [7,14], walnut has a pronounced antitumor effect and antiproliferative activity [15]. The extract from walnut fruit shows high antifungal, antimicrobial, and antioxidant activity [16-19,20]. The antifungal action of the

ethanol extract from the roots of walnut has been revealed. Petroleum extraction from walnut fruit showed high anthelmintic activity and antiparasitic action [21,22].

The aim of the study is to study external features, microscopy of walnut bark raw materials and walnut fruit in the stage of milky-wax maturity and chemical composition of alcohol extraction from these types of raw materials.

METHODS

The object of the study was the medicinal plant raw materials of walnut fruit in the stage of milky-wax maturity and the walnut bark, as well as alcohol extraction from this type of raw material. The material of the study was samples of the bark and fruit of walnut (*J. regia* L.), collected in the Moscow region, air-shade dried, and used as medicinal plant material.

Fruits were harvested in June in the stage of milky-wax maturity.

To perform the cuts, the dried bark was soaked in a mixture of 95% ethanol-glycerol-water (1:1:1). Cross-sections were examined using a light microscope.

Photographs were performed using a Canon Digital IXUS 80 IS digital camera. Alcohol extraction was prepared using 96% alcohol by the method of obtaining a tincture of the matrix homeopathic [23].

The component composition of the samples was studied by gas chromatography-mass spectrometry. The study was carried out on an Agilent Technologies instrument consisting of (1) 7890 gas chromatograph (HP-5 column, 50 m \times 320 μ m \times 1.05 μ m) and 2) 5975 C mass-selective detector with quadrupole mass analyzer. Temperature program of chromatography: 40°C, isotherm for 2 mins, further programmed heating to 250°C at a rate of 5°C/min; 250°C -

isotherm for 15 min; further programmed heating to 320°C at a speed of 25°C/min; at 320°C – isotherm for 5 min. Injector with flow division 1:50. Injector temperature is 250°C. The interface temperature is 280°C. The carrier gas is helium; flow rate 1 ml/min. The chromatogram of the samples is based on the total ion current. Mass spectrometric analysis conditions: Ionizing electron energy 70 eV; registration of mass spectra in positive ions in the range (m/z) from 20 to 450 at a rate of 2.5 scan/s. Software - ChemStation E 02.00. Identification of the component composition (qualitative analysis) was carried out from the NIST-05 complete mass spectra library and the corresponding values of chromatographic linear retention indices. The relative content (%) of the components of the mixture (quantitative analysis) was calculated from the ratio of the areas of chromatographic peaks (by the simple normalization method) [24].

External signs of solid raw materials were investigated. The cortex is examined with the naked eye or with a magnifying glass ($\times 10$) or a stereomicroscope ($\times 8$, $\times 16$) in accordance with the section “methods of analysis of medicinal raw materials” [25].

RESULTS AND DISCUSSION

Fresh raw materials are trough-shaped pieces of bark, 5–6 cm long, about 2–3 mm thick. The external surface of the cortex is smooth with transverse wrinkles (Fig. 1). There are lenticels of round shape. The inner surface of the bark is smooth. In the fracture, the edge is fibrous. The color of the bark from the outside is brown and grayish-brown, inside is yellowish flesh. The smell is weak when the cortex is moistened with water it does not increase. The taste is bitterish.

Dried raw materials are trough-shaped pieces of bark, some are curled, a length of 5–6 cm, a thickness of about 2–3 mm. The external surface of the cortex is smooth with transverse wrinkles (Fig. 1). There are lenticels of round shape. The inner surface of the bark is smooth. In the fracture, the edge is fibrous. The color of the bark outside is brown and grayish-brown, inside yellowish fleshy, light brown. The smell is weak when the cortex is moistened with water it does not increase. The taste is bitterish.

External signs of crushed raw materials were researched. Pieces of bark of various shapes, mostly rectangular. They pass through a sieve with apertures 7 mm in diameter. The color of the raw material is from light brown to dark reddish-brown. A dark gray-brown color is possible (Fig. 2). The smell is weak, increasing with soaking. The taste is bitter.

Powder

The powder has grayish brown color, passing through a sieve with holes 0.5 mm in diameter (Fig. 4). The smell is weak, peculiar. The taste is bitter.

The periderm of the bark collected from 2 to 5-year-old branches is represented mainly by a multilayered fallen (cork) consisting of 4–6 layers of thin-walled, cross-sectioned tabular cells colored in brown. The outer layers of the plug consist of colorless, dead, scaly, sloughing cells. Phelloderm cells located under the pigmented layer of the plug are not colored, compressed, and deformed (Fig. 3a and b).

Under the periderms are 3–4 layers of collenchyma cells, slightly transversely tangentially elongated, with thickened walls and greenish-amorphous contents (Fig. 3b).

Parenchyma of the primary cortex consists of isodiametric polygonal or oval cells, the walls of which are often permeated with pores. Some parenchyma cells contain druzes of calcium oxalate, greenish amorphous contents, and starch. Sometimes, intercellular cavities are observed in the parenchyma of the primary cortex (Fig. 3b).

The inner zone of the primary cortex is characterized by the presence of a discontinuous “mechanical belt” consisting of groups of stone cells (sclereids) and mechanical fibers, forming a primary cortex (Fig. 3a).



Fig. 1: (a-d) Fresh and dried whole raw materials, dried shredded raw materials, walnut bark powder

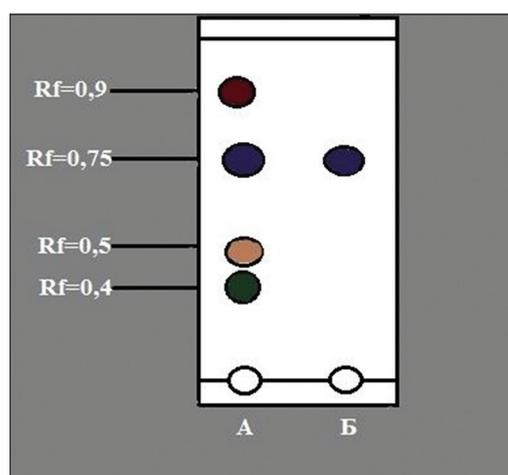


Fig. 2: Chromatogram of alcohol extract from the walnut bark for the detection of gallic acid

Fibers are narrow, with a diameter of 12–25 μ , with very thick walls, permeated with a few simple pores, with a point (sometimes silicate) cavity. Larger in diameter fibers can be found in small groups of 1–3, fiber size in the bundles is somewhat smaller. Different sclereids of 60–90 μ in size have strongly thickened layered walls with numerous porous tubules (Fig. 3a and c).

The fiber bundles of the “mechanical belt” are powerful, rounded in outline, with them complex groups of stony cells oriented tangentially or radially, which is consistent with the data of other authors [26]. These elements of the stereotype alternate with the areas of the cells of the cortical parenchyma (Fig. 3c). In the bark of the older branches, the stereo is formed as a solid ring.

In the secondary cortex, a typical sequence of alternating layers of soft bast and bundles of mechanical fibers forming a stereo (solid bast) is observed in the form of several tangentially oriented layers, which are penetrated by primary and secondary core rays (Fig. 3a), secondary rays - single-row, small-celled. Cells of primary rays are larger, have porous walls and form multirow radially oriented areas (Fig. 3a and e).

Some cells of the soft bast contain druzes of calcium oxalate, with a diameter of 8–27 μ . On the longitudinal-radial sections, whole strands of the crystal-bearing parenchyma accompanying the bundles of mechanical fibers are visible (Fig. 3d).

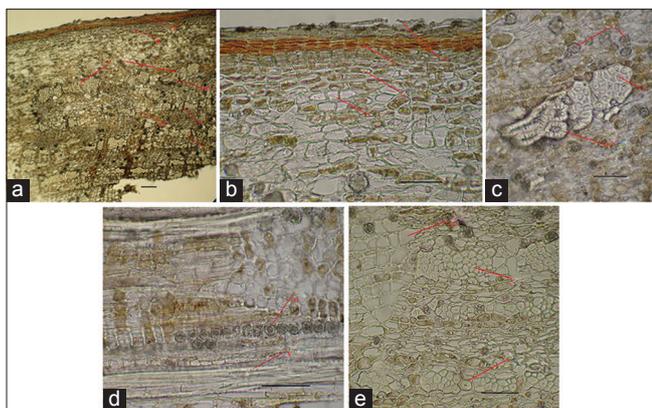


Fig. 3: (a) Is the transverse section of the cortex (scale of 100 μm). c - mechanical fibers; v.l. - secondary beam; plural - cells of the primary ray; with - the stereo of the primary crust ("mechanical belt"); k - collenchyma; p is the periderm; scd - scleraide. (b) Fragment of transverse cut of the cortex, peridermal zone, collenchyma, and primary cortex (scale ruler 50 μm) f - the femme; nf - outer layers of the femme; k - collenchyma. (c) A fragment of the transverse cut of the cortex, the zone of the primary cortex, the section of the "mechanical belt" (scale ruler 50 μm); v - mechanical fibers; d - druzo of calcium oxalate; scd - sclereide. (d) A fragment of the longitudinal-radial cut of the cortex, the zone of the secondary crust (scale ruler 50 μm) c - mechanical fibers; d - druzo calcium oxalate. (e) A fragment of the transverse cut of the cortex, the zone of the secondary crust (scale ruler 50 μm) c - mechanical fibers; p.l. - cells of the primary ray

Thus, to standardize the medicinal plant material - the walnut bark, diagnostic anatomical signs were established. These include the features of the structure of periderm, the stereo of the primary and secondary cortex, the presence of crystalline inclusions (drusen of calcium oxalate).

When wetting the inner surface of the bark with a drop of 1% ferric ammonium alum solution, a greenish-black color is observed (Fig. 4).

Qualitative reactions

The ground bark in the amount of 0.1 g is boiled for 2-3 min with 10 ml of water, cooled and filtered. To 1 ml of the filtrate, 2-3 drops of a 1% solution of iron ammonium alum are added: A black color is observed.

On the bark, 2-3 drops of 5% sodium hydroxide solution are applied: A violet-brown color is observed.

The powder is placed on a slide and 2-3 drops of a 1% solution of iron ammonium alum are dripped: A blue color is observed, which turns into black (hydrolyzed tannins) (Fig. 4).

The powder is placed on a slide and 2-3 drops of a 5% solution of sodium hydroxide drop: Yellow color is observed, which turns into red (phenolic compounds and anthraquinone derivatives) (Fig. 4).

TLC - analysis of alcohol extraction of the walnut bark

To the start line of the finished chromatographic plate with a layer of "Sorbphil" silica gel, 20 μl (0.02 ml) of the test tincture and 5 μl (0.005 ml) of 0.1% solution of gallic acid POO are applied separately and strip chromatographed in an ascending system in a solvent system chloroform:acetic acid ice:ethanol:water (15:8:3:2) to a height of 10 cm. The plate is then removed from the chamber, dried in air to remove traces of solvents, and examined in UV light at a wavelength of 365 nm.

In UV light at a wavelength of 365 nm, a brown zone with a Rf of about 0.75 should be detected on a chromatogram of 0.1% solution of gallic acid.



Fig. 4: Results of histochemical reaction of whole walnut crust and walnut crust powder with 1% ferric ammonium alum solution and the result of reaction of walnut crust powder with 5% NaOH

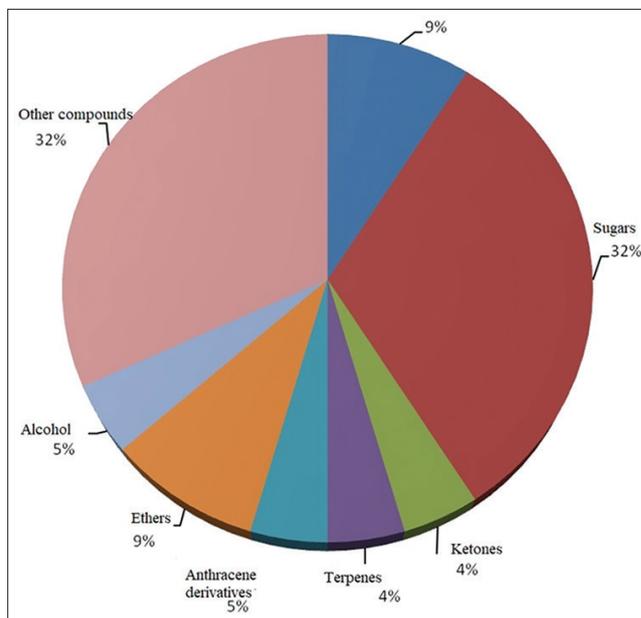


Diagram 1: The relative content of active substances in alcohol extraction from the bark of a walnut

In UV light at a wavelength of 365 nm on the chromatogram, brown zones with Rf about 0.5 and 0.9; in addition, zones can be found: Brown with Rf about 0.4.

In the extraction from the bark of the walnut by TLC, tannic substances, gallic acid, were found (Table 1).

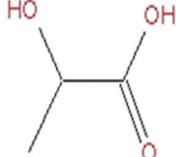
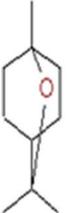
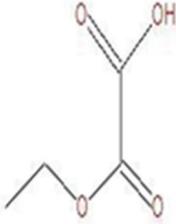
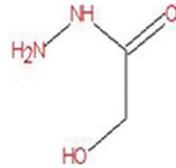
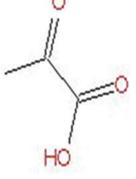
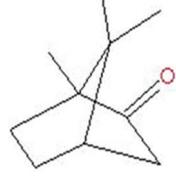
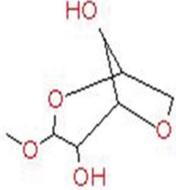
The relative percentage of each component was calculated taking into account unidentified peaks (Fig. 1).

The maximum content falls on sugar, namely for sucrose 25.27%, for ethyl-α-D-glucopyranosyl 29.75%, and for lactose 4.86%.

Terpenic compounds have been identified, the total content of which is almost the secondplace. The content of camphor is 9.63%, the content of cineol is 0.9%, and the content of thujone is 0.4% (Diagram 1). The marker compounds are the derivatives of yuglon 4,5-dihydroxy-3,4-dihydro-1(2H)-naphthalenone and anthracene derivative 4,5-dihydroxy-3,4-dihydro-1(2H)-naphthalenone. With these compounds, the pharmacological activity of medicinal plant material is associated.

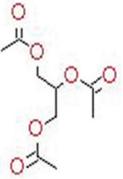
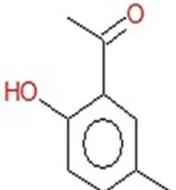
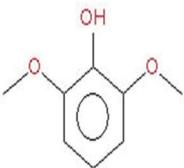
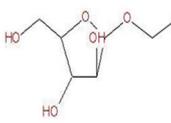
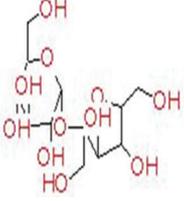
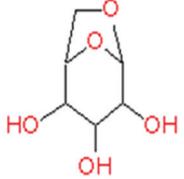
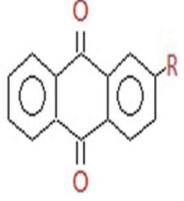
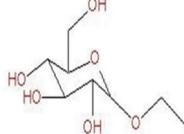
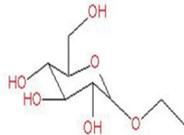
The research task included the study of raw walnut fruit in the stage of milky-wax maturity. Microscopy of the fetus was prepared according to the generally accepted procedure [27,28]. It should be noted the most important diagnostic signs of the fruit: The epidermal cells of the "final" structure; grouped large oval stomata of anomocytous type; hairs simple, unicellular, thick-walled, joined at 2-4 in the base rarely; hairs head with a 1-3- or multicellular single-rowed pedicle and a multicellular glandular head (rarely) and rounded-colored places of attachment of simple and glandular hairs [27-30].

Table 1: Substances contained in alcohol extraction of the walnut bark

No	Name	Formula	Rt, min	ICP	ICTs	Percentage	Major peaks
1	2-hydroxypropanoic acid		19.18	875.44	838	1.84	45, 28, 29, 27, 43, 26, 74, 44, 56, 42
2	Cineol		27.75	1046.95	1059	0.9	43, 81, 71, 108, 111, 41, 69, 84, 93, 55
3	Ethylhydrogenoloxalate		29.38	1000.6	943	0.08	44, 28, 31, 29, 27, 45, 74, 43, 26, 32
4	1,2-hydroxyacetohydrazine		29.47	1198	1106	0.7	31, 32, 29, 28, 30, 44, 90, 62, 43, 42
5	Thujone		30.35	1170.86	1062	0.4	81, 41, 68, 110, 67, 69, 109, 95, 39, 55
6	2-Oxo-propanoic acid		30.96	1152.08	1249	1.03	43, 15, 44, 45, 42, 28, 29, 14, 18, 39
7	Camphor		31.93	1122.45	1121	9.63	95, 81, 69, 55, 108, 83, 67, 109, 68, 152
8	Mannopyranoside		34.56	1238.04	1353	0.34	43, 44, 29, 57, 41, 27, 31, 28, 55, 42

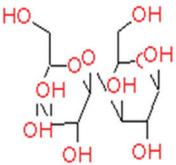
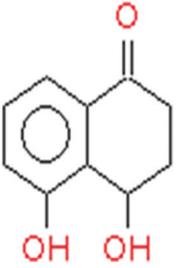
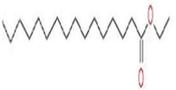
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Table 1: Continued

No	Name	Formula	Rt, min	ICP	ICTs	Percentage	Major peaks
9	Triacetin		36.68	1367	1354	0.81	43, 103, 145, 116, 15, 115, 86, 44, 42, 73
10	O-acetyl-p-cresol		36.90	1359.375	1363	0.47	135, 150, 77, 107, 43, 136, 51, 39, 53, 151
11	1,3-diethyletherofpyrogallol		37.80	1328	1279	2.01	154, 139, 111, 93, 96, 65, 39, 51, 155, 68
12	Ethyl- α -D-ribose		38.657	1498.26	1505	2.03	60, 73, 47, 43, 75, 57, 74, 42, 45, 44
13	Sucrose		39.85	1454.3	3139	25.27	73, 57, 31, 43, 60, 61, 44, 71, 86, 45
14	1,6-anhydro- β -D-talopyranoside		41.38	1597.62	1404	4.23	60, 73, 57, 43, 42, 56, 55, 47, 71, 70
15	2-benzamidoanthraquinone		44.58	1673.37	1705	0.26	207, 105, 133, 151, 134, 132, 104, 77, 106, 18
16	Ethyl- α -D-glucopyranosyl		45.02	1656.28	1813	1.54	60, 42, 43, 73, 57, 47, 75, 45, 74, 71
17	Ethyl- α -D-glucopyranosyl		45.16	1650.7	1813	29.75	60, 42, 43, 73, 57, 47, 75, 45, 74, 71

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Table 1: Continued

No	Name	Formula	Rt, min	ICP	ICTs	Percentage	Major peaks
18	Lactose		45.67	3116	3131	4.86	73, 43, 60, 31, 61, 57, 85, 71, 29, 103
19	4,5-dihydroxy-3,4-dihydro-1(2H)-naphthalenone		47.14	1776.61	1746	3.71	88, 101, 55, 41, 89, 43, 27, 57, 73, 70
20	Ethyl palmitate		56.42	1907.586	1978	4.61	160, 131, 132, 121, 39, 104, 77, 66, 64, 103

We examined the transverse section of the pericarp. An exocarpus consists of a single-layered small-celled epidermis covered with a layer of yellowish cuticle. A four-layer collenchyma underlies the epidermis. We found a mechanical belt consisting of thick-walled stony cells of various shapes pierced with pores. We also investigated mesocarp, consisting of large thin-walled parenchymal cells with greenish granular contents, druzes of calcium oxalate, randomly scattered conductive bundles with spiral vessels and tracheids and stony cells with porous layered, not very thick walls. We examined endocarp which consists of sclerized porous cells, and parenchymal cells. When examining the micropreparation of the seed coat from the surface, we found polygonal brownish cells of the epidermis; very large stomata with a gaping stomatal gap and kidney-shaped terminal cells; under the epidermis. In the study of the micropreparation of the transverse section of the cotyledons (seed embryo), thin-walled parenchymal cells with granular contents are visible; drops of fatty oil in the immature nucleus are rare [27,28].

Alcohol extraction, obtained from medicinal plant raw materials of walnut fruits in the stage of milky-wax maturity, is a transparent, green-brown liquid with a fragrant smell. Using the chromatography-mass spectrometry method for alcohol extraction from walnut fruit, 17 compounds were identified in the milk-wax stage, related to different classes of BAS.

Sugars, flavonoids, phenolic compounds and coumarins, juglone, and organic acids were identified.

CONCLUSION

1. In describing external signs and microscopy, diagnostic features of both whole and shredded raw materials and powder of the walnut crust, as well as its fruit in the stage of milky-wax maturity, were revealed.
2. Alcohol extraction derived from medicinal plant raw materials, walnut bark is a transparent liquid of brown color. Alcohol extraction, obtained from the medicinal plant raw material of walnut fruits in the stage of milky-waxy maturity, is a transparent, green-brown liquid with a fragrant smell. TLC in the walnut cortex identified gallic acid.
3. The method of chromatography-mass spectrometry in alcohol extraction from walnut fruit in the stage of milk-wax maturity was able to identify 17 compounds belonging to different classes of BAS. Sugars, flavonoids, phenolic compounds of coumarins, and organic

acids were identified. Alcohol extraction from the walnut bark identified marker compounds, the combination of which makes it possible to quickly identify this type of feedstock by chromatography-mass spectrometry marker compounds derivatives of yuglon 4,5-dihydroxy -3,4-dihydro-1 (2H)-naphthalenone and derivative anthracene 4,5-dihydroxy-3,4-dihydro-1 (2H)-naphthalenone. With these compounds, the pharmacological activity of medicinal plant material is associated.

ACKNOWLEDGMENTS

The authors would like to grateful to the Department of Pharmaceutical and Natural Sciences of Sechenov First Moscow State Medical University, Moscow, for providing infrastructure and instrumentation facilities.

AUTHORS' CONTRIBUTION

A.N. Luferov, N.V. Bobkova, and A.V. Strelyaeva conceived of the presented idea. A.V. Strelyaeva developed the theory and performed the computations. N.V. Kartashova and R.M. Kuznetsov verified the analytical methods. All authors discussed the results and contributed to the final manuscript. D.I. Lezhava, N.V. Kartashova, and R.M. Kuznetsov carried out the experiment. D.I. Lezhava wrote the manuscript with support from N.V. Kartashova.

CONFLICTS OF INTEREST

We have no conflicts of interest to declare.

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