CHEMISTRY AND TECHNOLOGY OF MEDICINAL COMPOUNDS AND BIOLOGICALLY ACTIVE SUBSTANCES
ХИМИЯ И ТЕХНОЛОГИЯ ЛЕКАРСТВЕННЫХ ПРЕПАРАТОВ И БИОЛОГИЧЕСКИ АКТИВНЫХ СОЕДИНЕНИЙ

ISSN 2686-7575 (Online)

https://doi.org/10.32362/2410-6593-2020-15-4-30-38

UDC 615.32: 547.97 + 543.544

RESEARCH ARTICLE

New approaches for the standardization of the *Monarda fistulosa* herb

Vladimir A. Kurkin[@], Anastasiya S. Tsibina

Samara State Medical University, Samara, 443099 Russia
[@]Corresponding author, e-mail: kurkinvladimir@yandex.ru

Objectives. *The Monarda fistulosa herb of the Lamiaceae family is particularly interesting among essential oils from medicinal plants that have a wide spectrum of pharmacological activities. However, information regarding some of its flavonoids, which are found in the essential oil, is controversial. Inaccuracies in identification of the chemical composition of the herb have led to several different standardization approaches, which are cumbersome. To establish a uniform classification, here, we present confirmation for new approaches for the standardization of the Monarda fistulosa herb.*

Methods. *Silica gel column chromatography was used to extract the flavonoids. Identification was based on ultraviolet spectroscopy, nuclear magnetic resonance spectroscopy, mass spectrometry, and acidic hydrolysis. The quality of the proposed quantitation methodology for total flavonoids was assessed by differential spectrophotometry at 394 nm, in isorhoifolin equivalent.*

Results. *We have verified new approaches for the standardization of the Monarda fistulosa herb. The approaches can determine the authenticity of the herb by detecting monoterpene phenols and flavonoids that have diagnostic value. We also developed a technique for quantitation of the total flavonoids.*

Conclusions. *We investigated the possibility of establishing the authenticity of the Monarda fistulosa herb based on the diagnostically significant flavonoids, isorhoifolin and linarin.*

Keywords: *flavonoids, Monarda fistulosa, isorhoifolin, linarin, spectrophotometry, thin-layer chromatography, standardization.*

For citation: Kurkin V.A., Tsibina A.S. New approaches for the standardization of the *Monarda fistulosa* herb. *Tonk. Khim. Tekhnol. = Fine Chem. Technol.* 2020;15(4):30-38. https://doi.org/10.32362/2410-6593-2020-15-4-30-38

ОРИГИНАЛЬНАЯ СТАТЬЯ

Новые подходы к стандартизации травы монарды дудчатой

В.А. Куркин[@], А.С. Цибина

Самарский государственный медицинский университет, Самара, 443099 Россия
[@]Автор для переписки, e-mail: kurkinvladimir@yandex.ru

Цели. *Среди эфиромасличных лекарственных растений, обладающих широким спектром фармакологической активности, особый интерес представляет трава монарды дудчатой (Monarda fistulosa) семейства Яснотковых. Однако информация о некоторых флавоноидах, содержащихся в ней наряду с эфирным маслом, носит противоречивый характер. Неточности, выявленные в отношении химического состава травы, становятся причиной появления различных подходов к стандартизации, что создает трудности. Для обеспечения единообразия классификации в данной работе представлено обоснование новых, ранее не применявшихся подходов к стандартизации травы монарды дудчатой.*
Методы. *Для экстракции флавоноидов использовали колоночную хроматографию на силикагеле. Идентификацию осуществляли на основании данных ультрафиолетовой спектроскопии, спектроскопии ядерного магнитного резонанса и масс-спектрометрии, а также результатов кислотного гидролиза. Оценку качества предложенной методики количественного определения суммы флавоноидов проводили методом дифференциальной спектрофотометрии при 394 нм в пересчете на изороифолин.*
Результаты. *Обоснованы подходы к стандартизации травы монарды дудчатой, заключающиеся в определении ее подлинности путем обнаружения монотерпеновых фенолов и флавоноидов, имеющих диагностическое значение. Разработана методика количественного определения суммы флавоноидов.*
Выводы. *Изучена возможность определения подлинности травы монарды дудчатой, исходя из диагностически значимых флавоноидов – изороифолина и линарина.*

Ключевые слова: *флавоноиды, монарда дудчатая, Monarda fistulosa, изороифолин, линарин, спектрофотомерия, тонкослойная хроматография, стандартизация.*

Для цитирования: Куркин В.А., Цибина А.С. Новые подходы к стандартизации травы монарды дудчатой. *Тонкие химические технологии.* 2020;15(4):30-38. https://doi.org/10.32362/2410-6593-2020-15-4-30-38

INTRODUCTION

Currently, essential oils of medicinal plants that have a wide range of pharmacological activity are widely used in medicine owing to the chemical variety of the biologically active compounds (terpenoids, aromatic compounds, etc.). Of particular interest are plants containing monoterpene phenols, for example, thymol and carvacrol. In this regard, creeping thyme (*Thymus serpyllum* L.), common thyme (*Thymus vulgaris* L.) and oregano (*Origanum vulgare* L.), which belong to the *Lamiaceae* herb family, are the most well-known[1].

One of the promising plants for study is the *Monarda fistulosa* herb of the *Lamiaceae* family. This herb grows in North America [1]. The *Monarda fistulosa* herb contains over 3% essential oil, the components of which (thymol, carvacrol, etc.) determine the high bactericidal, fungicidal, anti-inflammatory and anthelmintic activity [1–3]. Interestingly, the dominant component in the essential oil of the *Monarda fistulosa* herb is not thymol, as in the case of creeping and common thyme, but carvacrol, for which a more pronounced antimicrobial activity has been shown [4].

To determine the authenticity of the *Monarda fistulosa* herb, thin layer chromatography (TLC) using a standard thymol sample was previously proposed. In this case, the chromatogram of the analyzed extract at the level of a standard sample of thymol should contain an orange spot with an R_f coefficient of

[1] The State Pharmacopeia of the Russian Federation. 14th edition. Moscow: Ministry of Health of the Russian Federation; 2018. (In Russ.). Available from: http://www.femb.ru/femb/pharmacopea.php (Accessed September 09, 2019).

approximately 0.9 [4]. Taking into account the current trends in pharmacopoeia analysis, which are typically used to determine the authenticity of anthocyanins and the quality of medicinal plant raw materials via two (or more) groups of biologically active substances[2], the study of the flavonoid composition of the *Monarda fistulosa* herb seems relevant.

In addition to the essential oil, this plant also contains other valuable biologically active compounds, in particular, flavonoids (flavones, flavonols, flavonones, anthocyanins), which also contribute to the pharmacological activity [5, 6]. The literature contains information on the content of the following flavonoids in the *Monarda fistulosa* herb: hesperidin, diosmin, quercetin, luteolin, rutin, naringenin, cynaroside (luteolin-7-O-glucoside), hyperoside, and catechin [5, 6]; however, these data are controversial. The controversy also applies to methodical and methodological approaches for standardization of the *Monarda fistulosa* herb. Thus, for quantitative determination of the total flavonoid content, differential spectrophotometry at a wavelength of 390 nm calculated for rutin (0.48 ± 0.01%) has been used [5, 6]. For this technique, the extrication of substances from the raw material was performed by triple extraction with 70% ethyl alcohol.

According to the results of previously published studies, the total flavonoid content in the *Monarda fistulosa* herb is 2.14% in terms of luteolin [5, 6]. The authors used double extraction with 70% ethyl alcohol in the ratio of "raw materials to extractant" of 1 : 30 as well as differential spectrophotometry. Additionally, a method was proposed for the quantitative determination of flavonoids in the *Monarda fistulosa* herb by double (30 min each) extraction of raw materials (1 : 30) with 50% ethanol (analytical wavelength of 398 nm). The content of flavonoids in terms of luteolin was in this case 1.57 ± 0.02% [5, 6].

Previously, we developed approaches for standardization of the *Monarda fistulosa* herb that involved determining the amount of flavonoids in terms of cynaroside and the content of essential oil [7–9]. The choice of cynaroside as a standard sample was because this flavon, according the literature, is present in the *Monarda fistulosa* herb, has a pharmacopoeial status, and is widely used to standardize medicinal plant raw materials[3]. Additionally, the maximum in the absorption spectrum for the aqueous–alcoholic extract of the *Monarda fistulosa* herb (394 nm) was close to the maximum absorption of the cynaroside solution (approximately 400 nm). However, in further studies, we did not confirm the presence of cynaroside in the plant.

Thus, despite the literature data on the chemical composition of *Monarda fistulosa*, some contradictions regarding the flavonoid composition have been identified. This, in turn, has led to different approaches for standardization. Ethanol at various concentrations is used as an extractant; there is no single standard for the duration of extraction; there is no consensus on the analytical wavelength or substance used as a standard. Therefore, new steps should be taken to develop methodological approaches for standardization of the *Monarda fistulosa* herb, which was the purpose of this study.

MATERIALS AND METHODS

The object of this study was the *Monarda fistulosa* herb collected during mass flowering in the Botanical Garden of Samara University in July 2016–2018 and in the Nikitsky Botanical Garden (Republic of Crimea) in July 2018.

The flavonoid compounds, isorhoifolin (1) and linarin (2) (Fig. 1), were isolated from the *Monarda fistulosa* herb on a chromatographic column with silica gel L 40/100. Separation of the substances was monitored by TLC analysis on Sorbfil PTLC-AF-A-UV plates (*CHIMMED*, Russia). The eluent was chloroform–ethanol–water (26 : 16 : 3) and *n*-butanol–glacial acetic acid–water (4 : 1 : 2) systems. The extractables were detected by visualizing spots on the TLC plates in ultraviolet at 366 nm, including detection with a solution of aluminum chloride and then with an alkaline solution of diazobenzenesulfonic acid (DSA).

Nuclear magnetic resonance (NMR) spectra were recorded as follows: ^{1}H-spectra on a Bruker AM 300 (*Bruker,* Germany) at a frequency 300 MHz; ^{13}C-spectra on a Bruker DRX 500 (*Bruker*, Germany) at a frequency 126.76 MHz were recorded on a Kratos MS-30 (*Kratos*, United Kingdom), and absorption spectra in the ultraviolet (UV) region on a Specord 40 (*Analytik Jena*, Germany).

Isorhoifolin (7-O-apigenin rutinoside) (**1**). This crystalline substance is a light-yellow color with a composition of $C_{27}H_{30}O_{14}$; m.p. 257–260°C (water alcohol). λ_{max} EtOH 270, 340 nm; + NaOAc 270, 340 nm; + NaOAc + H_3BO_3 270, 405 nm; + $AlCl_3$ 278, 308, 345, 384 nm; + $AlCl_3$ + HCl 278, 308, 345, 384 nm; + NaOMe 254, 269, 400 nm.

[2] The State Pharmacopeia of the Russian Federation. 14th edition. Ministry of Health of the Russian Federation. Moscow; 2018. (In Russ.). Available from: http://www.femb.ru/femb/pharmacopea.php (Accessed September 09, 2019).
[3] The State Pharmacopeia of the Russian Federation. 14th edition. Moscow: Ministry of Health of the Russian Federation; 2018. (In Russ.). Available from: http://www.femb.ru/femb/pharmacopea.php (Accessed September 09, 2019).

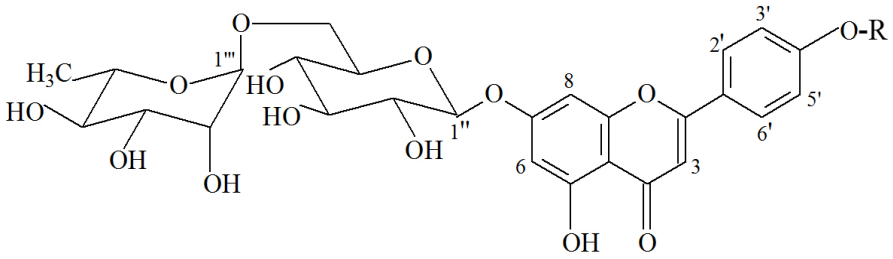

Fig. 1. Flavonoids isolated from *Monarda fistulosa* herb: R = H: isorhoifolin; R = CH_3: linarin.

^{1}H-NMR spectrum (300 MHz, DMSO-d_6, δ, ppm, J/Hz): 1.07 (3H, d, J = 6, CH_3 rhamnose), 3.0–5.2 (10H routines), 4.56 (1H, br. s, H-1''' rhamnopyranose), 5.07 (1H, d, J = 7, H-1'' glucopyranose), 6.46 (1H, d, J = 2, H-6), 6.77 (1H, d, J = 2, H-8), 6.87 (1H, s, H-3), 6.93 (2H, d, J = 8.5, H-3' and H-5'), 7.94 (2H, d, J = 8.5, H-2' and H-6'), 12.95 (1H, s, 5-OH group).

^{13}C-NMR spectrum (126.76 MHz, DMSO-d_6, δ_C, ppm): C-2 (162.87), C-3 (103.11), C-4 (181.97), C-5 (161.17), C-6 (99.54), C-7 (164.37), C-8 (94.79), C-9 (156.92), C-10 (105.38), C-1' (121.03), C-2' and C-6' (128.62), C-3' and C-5' (116.06), C-4' (161.32), C-1'' glucose (99.91), C-2'' (72.06), C-3'' (75.62), C-4'' (70.74), C-5'' (76.27), C-6'' (66.05), C-1''' rhamnose (100.52), C-2''' (70.32), C-3''' (69.57), C-4''' (73.08), C-5''' (68.31), C-6''' (CH_3 rhamnose) (17.79).

Mass spectrum (ESI-MS, 180°C, *m/z*): *m/z* 579.1739 $[M+H]^+$, *m/z* 601.1554 $[M+Na]^+$, *m/z* 617.1285 $[M+K]^+$.

Linarin (7-O-rutinoside acacetin) (**2**). This crystalline substance is a white color with a composition of $C_{28}H_{32}O_{14}$; m.p. 260°C (dec.) (water alcohol). λ_{max} EtOH 272, 330 nm; + NaOAc 272, 330 nm; + NaOAc + H_3BO_3 272, 330 nm; + $AlCl_3$ 280, 384 nm; + $AlCl_3$ + HCl 280, 384 nm; + NaOMe 287, 372 nm.

^{1}H-NMR spectrum (300 MHz, DMSO-d_6, δ, ppm, J/Hz): 1.07 (3H, d, J = 6, CH_3 rhamnose), 3.0–5.3 (10H routines), 3.83 (s, 3H, CH_3O), 4.54 (1H, br. s, H-1''' rhamnopyranose), 5.07 (1H, d, J = 7, H-1'' glucopyranose), 6.45 (1H, d, J = 2, H-6), 6.78 (1H, d, J = 2, H-8), 6.92 (1H, s, H-3), 7.14 (2H, d, J = 8.5, H-3' and H-5'), 8.04 (2H, d, J = 8.5, H-2' and H-6'), 12.90 (1H, s, 5-OH group).

^{13}C-NMR spectrum (126.76 MHz, DMSO-d_6, δ_C, ppm): C-2 (163.94), C-3 (103.80), C-4 (182.01), C-5 (161.13), C-6 (99.65), C-7 (165.11), C-8 (96.45), C-9 (156.96), C-10 (105.45), C-1' (122.66), C-2' and C-6' (128.44), C-3' and C-5' (114.70), C-4' (162.42), C-1'' glucose (99.94), C-2'' (73.06), C-3'' (75.66), C-4'' (70.34), C-5'' (76.24), C-6'' (68.31), C-1''' rhamnose (100.51), C-2''' (70.74), C-3''' (70.25), C-4''' (72.05), C-5''' (69.60), C-6''' (CH_3 rhamnose) (17.79), CH_3O (55.55).

Mass spectrum (ESI-MS, 180°C, *m/z*): *m/z* 593.1888 $[M+H]^+$, *m/z* 615.1710 $[M+Na]^+$.

Method for the quantitative determination of the total flavonoids in the *Monarda fistulosa* herb. The raw material was crushed so that its particles passed through a sieve with holes 1 mm in diameter. An accurate weighed sample of the ground material (approximately 1 g) was placed in a 100 mL flask, and 50 mL of 60% ethyl alcohol was added. The flask was closed with a stopper and weighed on a balance that is accurate to 0.01 g. The flask was attached to a reflux condenser and heated in a boiling water bath for 60 min. After boiling, the flask was cooled for 30 min, closed with the same stopper and weighed again, and the extractant was added to its original weight. The resulting aqueous–alcoholic extract was filtered through a paper filter (red, grade 589/5).

Preparation of the test solution. The resulting extract (1 mL) was poured into a 50-mL volumetric flask, and 2 mL of a 3% alcohol solution of aluminum chloride was added; then, the solution volume was adjusted to the mark with 96% ethyl alcohol (test solution). The optical density of the test solution was determined 40 min after preparation using a spectrophotometer at a wavelength of 394 nm (to calculate the content of flavonoids). The comparison solution was a solution containing 1 mL of an aqueous–alcoholic extract (1 : 50) with 96% ethyl alcohol, which was adjusted to the mark in a 50-mL flask.

Preparation of the isolated substance solution. An exact weighed portion (approximately 0.02 g) of isorhoifolin was placed in a 50-mL flask and dissolved in 30 mL of 70% ethanol while heating. After cooling to room temperature, the volume of the solution was adjusted to the mark with 70% ethanol (isorhoifolin solution A). Then, 1 mL of isorhoifolin solution A was placed in a 25-mL volumetric flask, 2 mL of a 3% alcohol solution of aluminum chloride was added, and the solution volume was adjusted to the mark with 96% ethyl alcohol (isorhoifolin solution B). The optical density of solution B was determined using a spectrophotometer at a wavelength of 394 nm. The comparison solution was a solution containing 1 mL of isorhoifolin solution A and 96% ethanol, which was adjusted to the mark in a 25-mL flask (comparison solution B of isorhoifolin).

The content of flavonoids in percent (X) in terms of isorhoifolin and absolute dry raw materials was calculated by the formula:

$$X = \frac{D \times m_0 \times 50 \times 50 \times 1 \times 50 \times 100 \times 100}{D_0 \times m \times 50 \times 1 \times 25 \times (100 - W)}$$

where

D – optical density of the test solution;

D_0 – optical density of an isorhoifolin standard sample solution;

m – mass of raw materials, g;

m_0 – the mass of the isorhoifolin standard sample, g;

W – mass loss on drying, %.

In the absence of a standard sample of isorhoifolin in the formula for the calculation, it is advisable to use the theoretical value of the specific absorption index equal to 195:

$$X = \frac{D \times 50 \times 50 \times 100}{m \times 195 \times (100 - W)}$$

where

D – optical density of the test solution;

m – mass of raw materials, g;

195 – specific absorbance ($E^{1\%}_{1sm}$) of isorhoifolin at 394 nm;

W – mass loss on drying, %.

RESULTS AND DISCUSSION

Using column chromatography on silica gel L 40/100, flavonoids 1 and 2, identified as isorhoifolin (apigenin 7-O-rutinoside) [10] and linarin (acacetin 7-O-rutinoside) [11–13], were first isolated from the *Monarda fistulosa* herb. Identification was based on data from UV spectra, ^{1}H-NMR and ^{13}C-NMR spectra, mass spectra, and acid hydrolysis.

Because the isolated substances are the dominant flavonoids in this plant, we studied the possibility of determining the authenticity of the *Monarda fistulosa* herb by the presence of not only thymol and carvacrol but also the diagnostically significant flavonoids, isorhoifolin (**1**) and linarin (**2**). Moreover, in our opinion, it is advisable to carry out TLC analysis using not only thymol [5] but also a standard sample of rutin, which is most widely used in the pharmacopoeial analysis of medicinal plant raw materials[4].

[4] The State Pharmacopeia of the Russian Federation. 14th edition. Moscow: Ministry of Health of the Russian Federation; 2018. (In Russ.). Available from: http://www.femb.ru/femb/pharmacopea.php (Accessed September 09, 2019).

TLC analysis of an aqueous–alcoholic extract obtained using 60% ethanol showed that two flavonoids close to the chromatographic mobility, isorhoifolin (**1**) and linarin (**2**), were found in the chromatogram of the test solution at 366 nm. The values of R_{st} relative to rutin for them are approximately 1.15 and 1.30, respectively (Figs. 2 and 3). Upon subsequent development with an alkaline DSA solution (Fig. 4), an orange-red spot (thymol + carvacrol) was found on the chromatogram at the level of the thymol spot.

To develop a method for the quantitative determination of the flavonoids in the *Monarda fistulosa* herb, we studied the absorption spectra of an aqueous–alcoholic extract and solutions of the selected substances, isorhoifolin and linarin. We found that the isolated flavonoids, and in particular isorhoifolin (Figs. 5 and 6), largely determine the nature of the absorption curve of the water–alcohol extract, especially under differential UV spectroscopy (Figs. 7 and 8). In the spectrum, a bathochromic shift of the long-wavelength

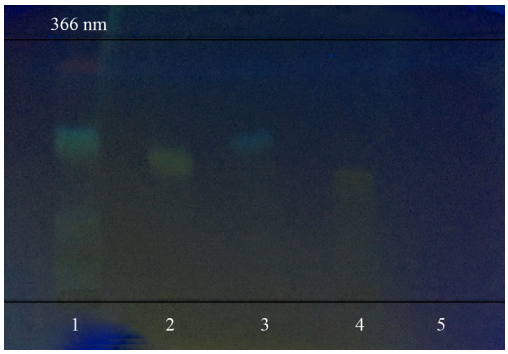

Fig. 2. Chromatogram of the water-alcohol extract from *Monarda fistulosa* in the chloroform–ethanol–water (26 : 16 : 3) system. Detection at 366 nm. Designations: 1 – extract from *Monarda fistulosa*; 2 – isorhoifolin; 3 – linarin; 4 – rutin; 5 – thymol.

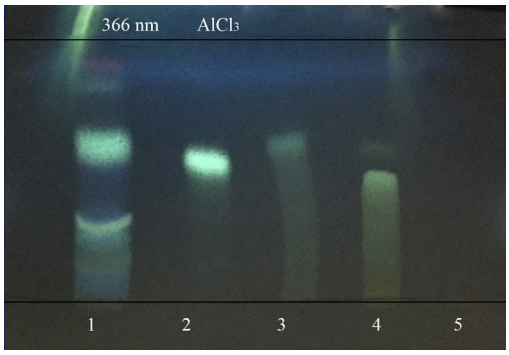

Fig. 3. Chromatogram of the water-alcohol extract from *Monarda fistulosa* in the chloroform–ethanol–water (26 : 16 : 3) system. Detection at 366 nm after treatment with $AlCl_3$ alcohol solution. Designations: 1 – extract from *Monarda fistulosa*; 2 – isorhoifolin; 3 – linarin; 4 – rutin; 5 – thymol.

absorption band of flavonoids was observed (Fig. 7) as in the case of isorhoifolin (Fig. 5). A study of the absorption spectra of isorhoifolin and the test solutionshowed that in both cases in the presence of aluminum chloride (differential spectrophotometry) there is an absorption maximum at 394 nm (Figs. 6 and 8).

Taking into account the fact that the absorption maxima of the solution of the isolated flavonoid and water–alcohol extract from the *Monarda fistulosa* herb are in the region of 394 nm (differential version), it was advisable to determine the flavonoid amount in terms of isorhoifolin at 394 nm. During the development of a method for quantitative determination of the flavonoid amount, we used

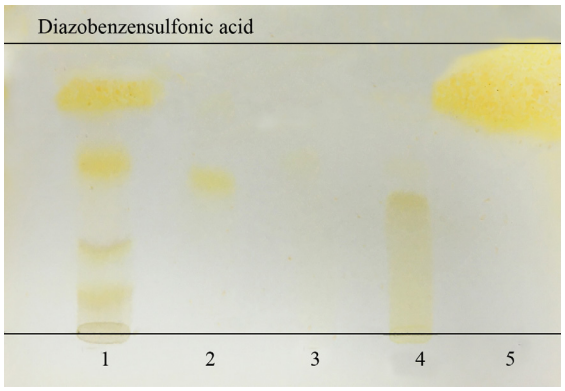

Fig. 4. Chromatogram of the water-alcohol extract from *Monarda fistulosa* in the chloroform–ethanol–water (26 : 16 : 3) system. Detection in visible light after treatment with the alkaline solution of diazobenzenesulfonic acid. Designations: 1 – extract from *Monarda fistulosa*; 2 – isorhoifolin; 3 – linarin; 4 – rutin; 5 – thymol.

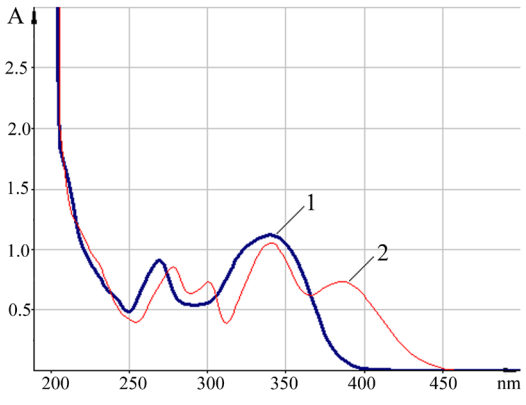

Fig. 5. Absorption spectra of isorhoifolin alcohol solutions. Designations: 1 – initial solution; 2 – solution with added aluminum chloride.

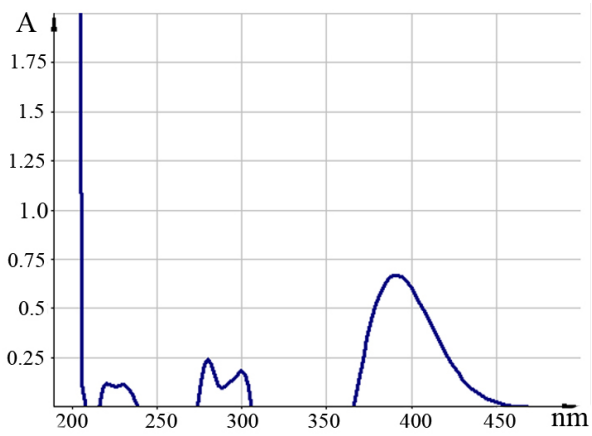

Fig. 6. Differential absorption spectrum of the isorhoifolin solution.

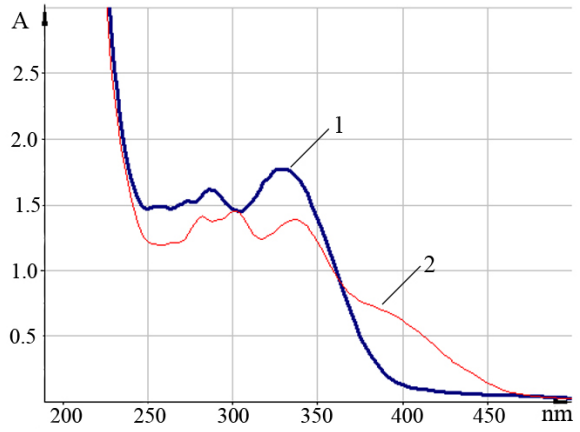

Fig. 7. Absorption spectra of water–alcohol extracts from *Monarda fistulosa*. Designations: 1 – extract solution; 2 – extract solution with added aluminum chloride.

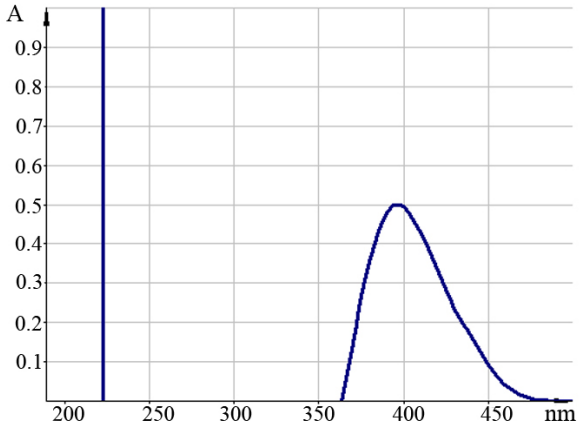

Fig. 8. Differential absorption spectrum of the water-alcohol extract from *Monarda fistulosa.*

previously determined optimal parameters for the extraction of flavonoids from the *Monarda fistulosa* herb: extractant – 60% ethyl alcohol; ratio of raw materials : extractant = 1 : 50; extraction time – 60 min; the extraction was carried out in a boiling water bath [7]. The metrological characteristics of the method for quantitative determination of the total flavonoid content in the *Monarda fistulosa* herb are presented in Table 1.

The results of statistical processing of the results indicated that the error for single determination of the flavonoid amount in the *Monarda fistulosa* herb with a confidence probability of 95% was ± 4.65%. Additionally, the content of flavonoids in the *Monarda fistulosa* herb varied from 5.96 ± 0.08% to 7.68 ± 0.12% (Table 2).

Validation of the developed methodology was performed according to the indicators of specificity, linearity, correctness, and reproducibility. The specificity of the technique was confirmed by the correspondence of the absorption maxima of the *Monarda fistulosa* herb flavonoid complex and isorhoifolin with the aluminum chloride solution. The linearity of the method was determined for a series of solutions of isorhoifolin in the concentration range of 0.01272 to 0.03816 mg/mL. The correlation coefficient was 0.99974. The correctness of the methodology was established by the method of standard addition via injection of isorhoifolin solutions with known concentrations of 25%, 50%, and 75% to the test solution. The average recovery percentage was 98%.

CONCLUSIONS

The feasibility of determining the authenticity of the *Monarda fistulosa* herb using thin-layer chromatography by detecting monoterpene phenols (thymol and carvacrol) and the flavonoids of isorhoifolin and linarin, the dominant and diagnostically significant components of the plant, was confirmed. To assess the quality of the *Monarda fistulosa* herb, a method for the quantitative determination of the flavonoid contents using differential spectrophotometry at 394 nm in terms of isorhoifolin was proposed.

The authors declare no conflicts of interest.

Table 1. Metrological parameters of the quantitation technique for the total flavonoids in *Monarda fistulosa*

f **(number of degrees of freedom)**	$\overline{X}$ **(sample average)**	***S*,** **(standard deviate)**	***P*, %** **(confidence figure)**	***t* (*P*, *f*)** **(Student t-test)**	**±*X*** **(credible interval)**	***E*, %** **(relative error single determination)**
10	6.51	0.1332	95	2.23	±0.11	±4.65

Table 2. Content of the total flavonoids in the *Monarda fistulosa* samples

No.	Sample origin	Total flavonoid content in an absolutely dry sample in isorhoifolin equivalent, %
1	Samara University Botanical Garden (July 2016)	5.97 ± 0.08
2	Samara University Botanical Garden (July 2017)	7.68 ± 0.12
3	Samara University Botanical Garden (July 2018)	6.61 ± 0.10
4	Nikitsky Botanical Garden, Crimea (July 2018)	5.96 ± 0.10

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About the authors:

Vladimir A. Kurkin, Dr. of Sci. (Pharmaceuticals), Professor, Head of the Department of Pharmacognosy with Botany and Elements of Phytotherapy, Samara State Medical University (89, Chapaevskaya ul., Samara, 443099, Russia). E-mail: kurkinvladimir@yandex.ru. https://orcid.org/0000-0002-7513-9352

Anastasiya S. Tsibina, Postgraduate Student, Department of Pharmacognosy with Botany and Elements of Phytotherapy, Samara State Medical University (89, Chapaevskaya ul., Samara, 443099, Russia). E-mail: nastylapochka@mail.ru. https://orcid.org/0000-0002-0384-5522

Об авторах:

Куркин Владимир Александрович, доктор фармацевтических наук, профессор, заведующий кафедрой фармакогнозии с ботаникой и основами фитотерапии Самарского государственного медицинского университета (443099, Россия, Самара, ул. Чапаевская, д. 89). E-mail: kurkinvladimir@yandex.ru. https://orcid.org/0000-0002-7513-9352

Цибина Анастасия Сергеевна, аспирант кафедры фармакогнозии с ботаникой и основами фитотерапии Самарского государственного медицинского университета (443099, Россия, Самара, ул. Чапаевская, д. 89). E-mail: nastylapochka@mail.ru. https://orcid.org/0000-0002-0384-5522

The article was submitted: January 13, 2020; approved after reviewing: June 29, 2020; accepted for publication: July 25, 2020.

Translated from Russian into English by S. Durakov
Edited for English language and spelling by Enago, an editing brand of Crimson Interactive Inc.