

Polyphenols of *Perilla frutescens* of the family Lamiaceae identified by tandem mass spectrometry


M.P. Razgonova^{1, 2}, N.G. Kon'kova¹ , A.M. Zakharenko^{3, 4}, K.S. Golokhvast^{1, 2, 3, 4}

¹ Federal Research Center the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR), St. Petersburg, Russia

² Far Eastern Federal University, Vladivostok, Russia

³ Siberian Federal Scientific Centre of Agro-BioTechnology of the Russian Academy of Sciences, Krasnoobsk, Novosibirsk Region, Russia

⁴ Tomsk State University, Tomsk, Russia

 ninakonkova.1@mail.ru

Abstract. *Perilla frutescens* is mainly cultivated as an oilseed crop. *Perilla* seeds contain 40–53 % of oil, 28 % of protein. The growing season is 100–150 days. In Russia, *perilla* is grown in the Far East, where the yield is 0.8–1.2 t/ha. *Perilla* of different geographical origin has its own special, sharply different features that characterize two geographical groups: Japanese and Korean-Chinese. These groups differ from each other in the length of the growing season, the height of plants, the color of the stem, the surface and the size of the leaves, the shape of the bush, the shape and size of the inflorescences, the size of the cups, the size and color of the seeds. *P. frutescens* contains a large number of polyphenolic compounds that are biologically active components. The purpose of this research was a metabolomic study of extracts from leaves of *P. frutescens* obtained from the collection of Federal Research Center the N.I. Vavilov All-Russian Institute of Plant Genetic Resources, grown on the fields of the Far East Experiment Station – Branch of Federal Research Center (Primorsky Krai, Russia). To identify target analytes in extracts, HPLC was used in combination with an ion trap. Preliminary results showed the presence of 23 biologically active compounds corresponding to *P. frutescens*. In addition to the reported metabolites, a number of metabolites were newly annotated in *P. frutescens*. There were hydroxycoumarin Umbelliferone; triterpene Squalene; omega-3 fatty acid Stearidonic [Moroctic] acid; higher-molecular-weight carboxylic acid: Tetracosenoic acid and Salvianic acid C; lignan Syringaresinol and cyclobutane lignan Sagerinic acid, etc. A wide range of biologically active compounds opens up rich opportunities for the creation of new drugs and dietary supplements based on extracts of *perilla* of the family Lamiaceae, subfamily Lamioideae, tribe Satureji and subtribe Perillinae. Key words: *Perilla frutescens*; HPLC–MS/MS; tandem mass spectrometry; phenolic compounds; triterpene acids; lignans.

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Полифенолы *Perilla frutescens* семейства Lamiaceae, идентифицированные с помощью тандемной масс-спектрометрии


М.П. Разгонова^{1, 2}, Н.Г. Конькова¹ , А.М. Захаренко^{3, 4}, К.С. Голохваст^{1, 2, 3, 4}

¹ Федеральный исследовательский центр Всероссийский институт генетических ресурсов растений им. Н.И. Вавилова (ВИР), Санкт-Петербург, Россия

² Дальневосточный федеральный университет, Владивосток, Россия

³ Сибирский федеральный научный центр агробиотехнологий Российской академии наук, р.п. Краснообск, Новосибирская область, Россия

⁴ Томский государственный университет, Томск, Россия

 ninakonkova.1@mail.ru

Аннотация. *Perilla frutescens* получила применение в основном как масличная культура. Семена периллы содержат 40–53 % масла, 28 % белка. Vegetационный период составляет 100–150 дней. В России периллу выращивают на Дальнем Востоке, где урожайность достигает 0.8–1.2 т/га. Это растение короткого дня, поэтому большинство форм не цветет в условиях средней полосы России. Перилла различного географического происхождения имеет свои особенные, резко отличные признаки, характеризующие две географические группы: японскую и корейско-китайскую. Эти группы различаются длиной вегетационного периода, а также по высоте растений, окраске стебля, поверхности и величине листьев, форме куста, форме и размеру соцветий, величине чашечек и цветку семян. *Perilla frutescens* содержит большое количество полифенольных соединений, которые являются биологически активными компонентами. Цель данной работы состояла в метаболомном исследовании экстрактов из листьев *P. frutescens*, полученных из коллекции Всероссийского института генетических ресурсов растений им. Н.И. Вавилова и выращенных на полях его Дальневосточной опытной станции (Приморский край, Россия). Для идентификации целевых аналитов в экстрактах использовали метод высокоэффективной жидкостной хроматографии в сочетании с ионной ловушкой. Предварительные результаты показали наличие 23 биологически активных соединений, соответствующих виду *P. frutescens*. В дополнение к упомянутым метаболитам, в экстрактах

P. frutescens был впервые обнаружен ряд соединений. Это кумарин умбеллиферон; тритерпен сквален; стеарионовая кислота; высокомолекулярные карбоновые кислоты: тетракозановая кислота и сальвиановая кислота С; лигнан сирингарезинол; циклобутановый лигнан сагериновая кислота и др. Широкий спектр биологически активных соединений открывает богатые возможности для создания новых лекарственных средств и биологически активных добавок на основе экстрактов периллы семейства Lamiaceae, подсемейства Lamioideae, трибы Satureji и подтрибы Perillinae.

Ключевые слова: *Perilla frutescens*; ВЭЖХ–МС/МС; тандемная масс-спектрометрия; фенольные соединения; тритерпеновые кислоты; лигнаны.

Introduction

This research presents a detailed study of the metabolomic composition of *Perilla frutescens* leaves. *Perilla frutescens* L. is an annual plant belonging to the mint family Lamiaceae, subfamily Lamioideae, tribe Satureji and subtribe Perillinae (Zhou et al., 2014). *Perilla* is widely cultivated in Asian countries such as China, Japan, South Korea and India for its oils and leaves used in cooking. *Perilla* has also been cultivated in Russia in the Far East since the 1930s to obtain high quality oil.

Perilla is a heat-loving and moisture-loving plant. It requires fertile soils. *Perilla* is a short-day plant, so most forms do not bloom in the conditions of Central Russia or bloom only in late autumn. *Perilla* of different geographical origin has its own special, sharply different features that characterize two geographical groups: Japanese and Korean-Chinese. These groups differ from each other in the length of the growing season, the height of plants, the color of the stem, the surface and the size of the leaves, the shape of the bush, the shape and size of the inflorescences, the size of the cups, the size and color of the seeds. *Perilla* leaves are commonly used for their antioxidant, anti-allergic, antimicrobial, anti-tumor and anti-cancer effects due to the presence of phenolic compounds including rosemary acid, essential oil and vitamins (Ahmed, 2019).

The fatty acid composition of perilla oil is characterized by the presence of five main fatty acids. On average, perilla oil contains (% of the total fatty acids): palmitic acid – 5.9, stearic acid – 1.8, oleic acid – 15.3, linoleic acid – 12.4, α -linolenic acid – 61.9. The increased content of polyunsaturated fatty acids – up to 90 % – indicates a high biological activity of perilla oil. By their properties, these acids are close to vitamins (vitamin F), which are not synthesized in the human organism. In terms of the sum of these acids, perilla oil even exceeds many varieties of flax and hemp. It is important to observe the ratio of ω -3 and ω -6 fatty acids in the diet. The optimal ratio of ω -3 and ω -6 fatty acids is 1:4 (Banno et al., 2004; Gu et al., 2009; Meng et al., 2009). Since unsaturated fatty acids and α -linolenic acid are thought to have various beneficial effects on the human health, such as lowering serum cholesterol and triglyceride levels, reducing the risk of colon cancer, and preventing overgrowth of visceral adipose tissue (Longvah et al., 2000), perilla seed oil is considered to be of high quality.

Many bioactive compounds from various chemical groups have been identified from the leaves and seeds of extract of *P. frutescens*. *P. frutescens* is used as a spice as well as in medicine and consists of several chemotypes that refer to the essential oils chemical composition. A chemotype containing perillaldehyde is a major component of the essential oil that is most effective as a sedative in China's traditional medicine. Honda et al. (1986) fractionated MeOH extract of *P. frutescens* to presence of stigmaterol and perillaldehyde.

Also, several studies showed the presence of other flavonoids such as apigenin and luteolin, and phenolic compounds such as caffeic acid and rosmarinic acid (Lee et al., 2013; Kauffmann et al., 2016).

Thus, we isolated and investigated the structure of phenolic compounds and triterpenic acids from *P. frutescens* leaves. A total of 23 biologically active compounds: 13 phenolic compounds, omega-3-fatty acids, lignans, sterols and triterpenic acids were identified using tandem ion trap mass spectrometry.

Materials and methods

Perilla frutescens leaves served as the object of the study. The variety 'Novinka' from the collection of Federal Research Center the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR) was grown on the fields of the Far East Experiment Station – Branch of VIR, Primorsky Territory (N 43°21'34", E 132°11'19"; yellow-brown soil). This is the only perilla oilseed variety listed in the State Register of the Russian Federation. The variety 'Novinka' is a medium-ripened variety of the Korean-Chinese ecological group with a growing season length of 106 days and an oil content of 49 %, the yield is 0.8–1.2 t/ha.

The leaves were harvested at the end of August, 2020. Weather conditions were favorable for the perilla growth and development. The average air temperature in August was 20 °C, the amount of precipitation was 250 mm. All samples morphologically corresponded to the pharmacopoeial standards of the Pharmacopoeia of the Eurasian Economic Union (2020).

Chemicals and reagents. HPLC-grade acetonitrile was purchased from Fisher Scientific (Southborough, UK), MS-grade formic acid was obtained from Sigma-Aldrich (Steinheim, Germany). Ultra-pure water was prepared from a SIEMENS ULTRA clear (SIEMENS water technologies, Germany), and all other chemicals were analytical grade.

Fractional maceration. To obtain highly concentrated extracts, fractional maceration was applied. In this case, the total amount of the extractant (ethyl alcohol of reagent grade) was divided into 3 parts and was consistently infused on perilla with the first part, then with the second and third, correspondingly. The infusion time of each part of the extractant was 7 days. Fractional maceration technique was applied to obtain highly concentrated extracts (Azmir et al., 2013). From 300 g of the fresh sample, 50 g of leaves of *P. frutescens* were selected for maceration. The total amount of the extractant (ethyl alcohol of reagent grade) was divided into three parts and consistently infused to the leaves with the first, second and third parts. The solid–solvent ratio was 1:20. The infusion of each part of the extractant lasted 7 days at room temperature.

Liquid chromatography. HPLC was performed using Shimadzu LC-20 Prominence HPLC (Shimadzu, Japan)

equipped with a UV-sensor and a Shodex ODP-40 4E reverse phase column for multicomponent mixtures separation. The gradient elution program was as follows: 0.01–4 min, 100 % C₂H₃N; 4–60 min, 100–25 % C₂H₃N; 60–75 min, 25–0 % C₂H₃N; control washing 75–120 min 0 % C₂H₃N. The entire HPLC analysis was done with a UV detector at wavelengths of 230 and 330 nm; the temperature corresponded to 17 °C. The injection volume was 1 ml.

Mass spectrometry. MS analysis was performed on an ion trap amaZon SL (BRUKER DALTONIKS, Germany) equipped with an ESI source in negative and positive ion mode. The optimized parameters were obtained as follows: ionization source temperature: 70 °C, gas flow: 4 l/min, nebulizer gas (atomizer): 7.3 psi, capillary voltage: 4500 V, end plate bend voltage: 1500 V, fragmentary: 280 V, collision energy: 60 eV. An ion trap was used in the scan range *m/z* 100–1.700 for MS and MS/MS.

Data collection was controlled by Windows software for BRUKER DALTONIKS. All experiments were repeated three times. A four-stage ion separation mode (MS/MS mode) was implemented.

Results and discussion

Five of the most EtOH extracts of *P. frutescens* were selected. All of them had a rich polyphenolic and triterpene composition. High accuracy mass spectrometric data were recorded on an ion trap amaZon SL BRUKER DALTONIKS equipped with an ESI source in the mode of negative/positive ions. The four-stage ion separation mode (MS/MS mode) was implemented. All the chemical profiles of the samples were obtained by the HPLC – ESI – MS/MS method. A total of 300 peaks were detected in the chromatogram (Fig. 1).

The combination of both ionization modes (positive and negative) in MS full scan mode is giving certainty to the molecular mass determination. The negative ion mode provides the highest sensitivity and results in limited fragmentation making it most suited to infer the molecular mass of the separated polyphenols especially in cases where concentration is low. A tentative identification of compounds was carried out using comparisons of the *m/z* values, the RT and the fragmentation patterns with the MS² spectral data taken from the literature (Banno et al., 2004; Vallverdu-Queralt et al., 2012; Zhou et al., 2014; Spinola et al., 2015; Cirlini et al.,

2016; Pandey et al., 2016; Sharma et al., 2016; Marzouk et al., 2018; Sun L. et al., 2019; Goufo et al., 2020; etc.) or the data bases (MS2T, MassBank, HMDB). A unifying system table of the molecular masses of the target analytes isolated from the EtOH-extract of *P. frutescens* was compiled for ease of identification (see the Table). The 23 compounds are shown in the Table. Some of them belong to different polyphenolic families: anthocyanidins, flavones, hydroxycinnamic acids, hydroxybenzoic acids, lignans.

In addition to the reported metabolites, a number of metabolites were newly annotated in *P. frutescens*. The newly annotated metabolites were hydroxycoumarin Umbelliferone; triterpene Squalene; omega-3 fatty acid: Stearidonic [Morotic] acid; higher-molecular-weight carboxylic acids: Tetracosenoic acid and Salvianic acid C; cyclobutane lignan Sagerinic acid; sterol 7-oxo-beta-sitosterol [3-Hydroxystigmast-5-en-7-one]; flavone Vicenin-2 [Apigenin-6,8-Di-C-Glucoside].

A total of 13 polyphenol compounds have been identified (see the Table). The flavones Chrysoeriol, Diosmetin, Apigenin 7-*O*-glucuronide, Scutellarin, Vicenin-2 have already been characterized as a component of *P. frutescens*. This identification was satisfactory according to the studied references in *P. frutescens* (Yamazaki et al., 2003; Gu et al., 2009; Meng et al., 2009; Zhou et al., 2014), *Triticum aestivum* L. (Di Loreto et al., 2018), apple (Sanchez-Rabaneda et al., 2004), rice (Chen W. et al., 2013), *Mentha* (Xu et al., 2017), *Cirsium japonicum* (Zhang et al., 2014), etc.

The CID-spectrum (collision induced dissociation spectrum) in negative ion modes of Apigenin-7-*O*-glucuronide from extracts of *P. frutescens* is shown in Figure 2. The [M–H][–] ion produced three fragment ions at *m/z* 269.02, *m/z* 341.00, *m/z* 175.03 (see Fig. 2). The fragment ion with *m/z* 269.02 yields two daughter ions at *m/z* 225.04, and *m/z* 149.04. The fragment ion with *m/z* 225.04 yields three daughter ions at *m/z* 224.03, *m/z* 183.00, and *m/z* 132.08. It was identified in the bibliography in extracts from *P. frutescens* (Yamazaki et al., 2003), pear (Sun L. et al., 2019), *Hedyotis diffusa* (Chen X. et al., 2018), *Coriandrum* (Hussein et al., 2018), *Thymus vulgaris* (Justesen, 2000).

The anthocyanin Shisonin [Cyanidin 3-*O*-(6-*O*-para-coumaroyl) glucoside-5-*O*-glucoside] was found in extracts of *P. frutescens* (Fig. 3). The Shisonin CID-spectrum in negative ion mode is shown in Figure 3.

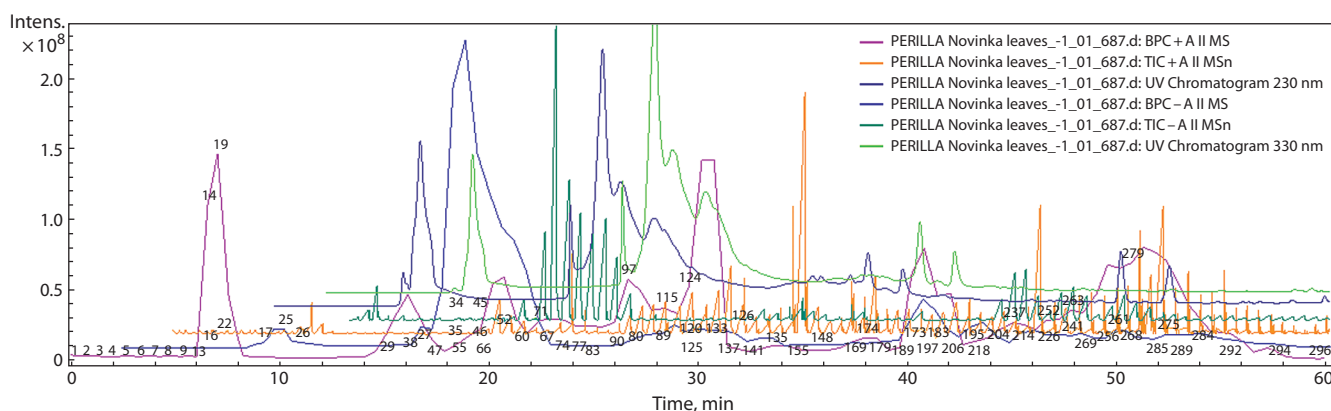


Fig. 1. Chemical profiles of the *P. frutescens* sample (Primorsky Territory, Russia) represented in a total ion chromatogram from EtOH-extract.

Biologically active substances identified from the EtOH-extracts of *P. frutescens*

N	Class of compounds	Identification	Formula	Calculated mass	MS/MS fragmentation			References	
					Observed mass	Stage 1	Stage 2		Stage 3
				[M-H] ⁻	[M+H] ⁺				
1	Hydroxycoumarin	Umbelliferone [Skimmetin; Hydragin]	$C_9H_6O_3$	162.1421	163	145	177	<i>Vitis vinifera</i> (Goufo et al., 2020), <i>Sanguisorba officinalis</i> (Kim et al., 2018), <i>F. glaucescens</i> (Hamed et al., 2021)	
2	Hydroxycinnamic acid	Caffeic acid [(2E)-3-(3,4-Dihydroxyphenyl)acrylic acid]	$C_9H_8O_4$	180.1574	179	135		<i>P. frutescens</i> (Zhou et al., 2014), tomato (Vallverdu-Queralt et al., 2012), <i>Mentha</i> (Chen X. et al., 2017; Marzouk et al., 2018), <i>Vaccinium macrocarpon</i> (Abeywickrama et al., 2016), <i>Rubus occidentalis</i> (Paudel et al., 2013), <i>Rhododendron sichotense</i> (Razgonova et al., 2020)	
3	Trans-cinnamic acid	Ferulic acid	$C_{10}H_{10}O_4$	194.1840	193	161; 134	133	<i>P. frutescens</i> (Peng et al., 2005), <i>Vaccinium macrocarpon</i> (Abeywickrama et al., 2016), triticum (Sharma et al., 2016)	
4	Omega-3 fatty acid; octadecatetraenoic acid	Stearidonic acid [6,9,12,15-Octadecatetraenoic acid; Morortic acid]	$C_{18}H_{28}O_2$	276.4137	277	232; 147	119	<i>Salvia miltiorrhiza</i> (Yang et al., 2015)	
5	Octadec-9-enoic acid	Oleic acid (<i>Cis</i> -9-Octadecenoic acid; <i>Cis</i> -Oleic acid)	$C_{18}H_{34}O_2$	282.4614	281	163; 135	119	<i>P. frutescens</i> (Longvah et al., 2000), <i>Sanguisorba officinalis</i> (Kim et al., 2018)	
6	Monobasic carboxylic acid	Stearic acid (Octadecanoic acid; Stearophanic acid)	$C_{18}H_{36}O_2$	284.4772	285	253; 152	193	<i>P. frutescens</i> (Longvah et al., 2000)	
7	Flavone	Chrysoeriol [Chryseriol]	$C_{16}H_{12}O_6$	300.2629	299	284	227; 137	199	Perilla (Gu et al., 2009), apple (Sanchez-Rabanaleda et al., 2004), rice (Chen W. et al., 2013), <i>Mentha</i> (Xu et al., 2017)
8	Flavone	Diosmetin [Luteolin 4'-Methyl Ether, Salinigriflavonol]	$C_{16}H_{12}O_6$	300.2629	301	286; 211	168; 121	139	<i>Triticum turgidum</i> ssp. <i>durum</i> (Di Loreto, 2018), <i>Cirsium japonicum</i> (Zhang et al., 2014), <i>Mentha</i> (Xu et al., 2017)
9	Phenylpropanoid (cinnamic acid derivative)	Rosmarinic acid	$C_{18}H_{16}O_8$	360.3148	359	161; 197	133	<i>P. frutescens</i> (Yamazaki et al., 2003; Banno et al., 2004; Zhou et al., 2014), <i>Rhodiola rosea</i> (Wang et al., 2007), <i>Mentha</i> (Chen X. et al., 2017; Xu et al., 2017), <i>Salvia miltiorrhiza</i> (Jiang et al., 2005)	
10	Higher-molecular-weight carboxylic acid	Tetracosenoic acid	$C_{24}H_{46}O_2$	366.6208	367	349; 284; 203; 138		<i>A. cordifolia</i> (Hamed et al., 2021)	
11	Higher-molecular-weight carboxylic acid	Salvianic acid C	$C_{18}H_{18}O_9$	378.3301	377	341; 197; 135	179	149	<i>Salvia miltiorrhiza</i> (Jiang et al., 2005)

12	Benzenepropanoic acid	Ethyl rosmarinat	$C_{20}H_{20}O_8$	388.3680	387	341; 207; 163	147	<i>Mentha</i> (Chen X. et al., 2017)	
13	Triterpene	Squalene (Trans-Squalene; Spinacene; Supraene)	$C_{30}H_{50}$	410.7180	411	393; 296; 231; 175	175	Squalene (Toh et al., 2001; Sun S. et al., 2005)	
14	Lignan	Syringaresinol	$C_{22}H_{26}O_8$	418.4436	419	326	298; 254; 218; 174	154	Wheat (Cukelj et al., 2011), <i>Punica granatum</i> (Bonzanini et al., 2009), <i>Xanthium sibiricum</i> (Kan et al., 2011)
15	Sterol	7-oxo-beta-sitosterol [3-Hydroxystigmaster-5-en-7-one]	$C_{29}H_{48}O_3$	444.6896	445	427; 353; 333; 245; 153		<i>C. edulis</i> , <i>F. pottsii</i> (Hamed et al., 2021)	
16	Flavone	Apigenin 7-O-glucuronide	$C_{21}H_{18}O_{11}$	446.3610	445	269; 175	225; 149	224; 183; 132	<i>P. frutescens</i> (Yamazaki et al., 2003), pear (Sun L. et al., 2019), <i>Hedyotis diffusa</i> (Chen X. et al., 2018), <i>Coriandrum</i> (Hussein et al., 2018), <i>Thymus vulgaris</i> (Justesen, 2000)
17	Triterpenic acid	Oleanoic acid	$C_{30}H_{48}O_3$	456.7003	457	439; 369; 277; 203; 145	369; 277; 203	203	Pear (Sun L. et al., 2019), <i>Ocimum</i> (Pandey, Kumar, 2016)
18	Triterpenic acid	Ursolic acid	$C_{30}H_{48}O_3$	456.7003	457	439; 387; 277; 203	207	174	<i>P. frutescens</i> (Banno et al., 2004) <i>Ocimum</i> (Pandey, Kumar, 2016), <i>Hedyotis diffusa</i> (Chen X. et al., 2018), pear (Sun L. et al., 2019), <i>Mentha</i> (Xu et al., 2017)
19	Flavone	Scutellarin [Breviscapin; Scutellarin-7-glucuronide]	$C_{21}H_{18}O_{12}$	462.3604	463	287; 445	269; 241; 169; 123	185; 119	<i>P. frutescens</i> (Yamazaki et al., 2003; Meng et al., 2009)
20	Triterpenoid	Corosolic acid	$C_{30}H_{48}O_4$	472.6997	473	455; 370; 259; 217; 162	437; 358; 323; 264; 233; 193	338	<i>P. frutescens</i> (Banno et al., 2004), pear (Sun L. et al., 2019), <i>Folium erobotryae</i> (Li et al., 2015), <i>Malus domestica</i> (Sut et al., 2019)
21	Flavone	Vicenin-2 [Apigenin-6,8-Di-C-Glucoside]	$C_{27}H_{30}O_{15}$	594.5181	595	577; 541; 457; 427; 355; 271; 219; 163	379; 325	351; 308	<i>Triticum aestivum</i> L. (Dinelli et al., 2011), lemon, passion fruits (Spinola et al., 2015), <i>Mentha</i> (Marzouk et al., 2018), <i>P. aculeata</i> (Hassan et al., 2019)
22	Cyclobutane lignan	Sagerinic acid	$C_{36}H_{32}O_{16}$	720.6297	719	359; 555	161; 197; 133	133	<i>Mentha</i> (Cirilini et al., 2016)
23	Anthocyanidin	Shisonin [Cyanidin 3-O-(6-O-para-coumaroyl) glucoside-5-O-glucoside]	$C_{36}H_{37}O_{18}^+$	757.6789	756	595; 433; 397; 359; 235	217; 179; 205; 134	205; 134	<i>P. frutescens</i> (Yamazaki et al., 2003; He et al., 2015)

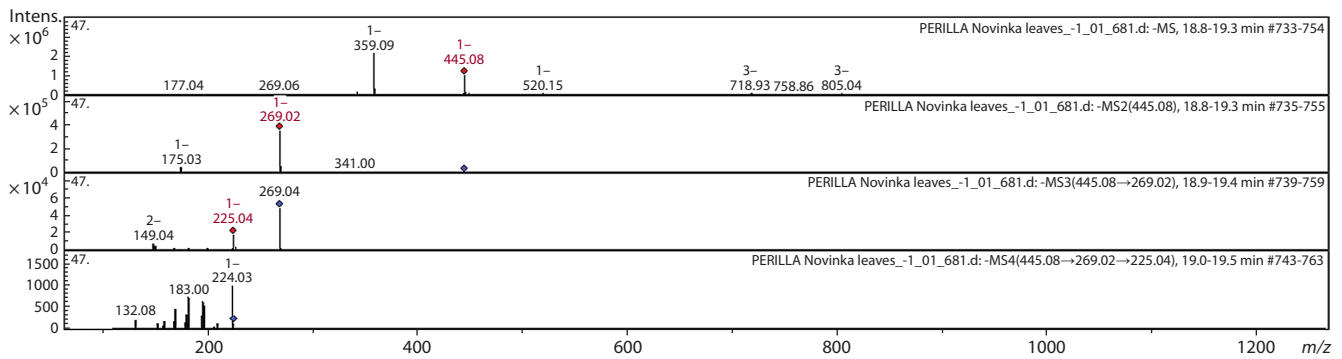


Fig. 2. CID-spectrum of Apigenin-7-O-glucuronide from extracts of *P. frutescens*, m/z 445.08.

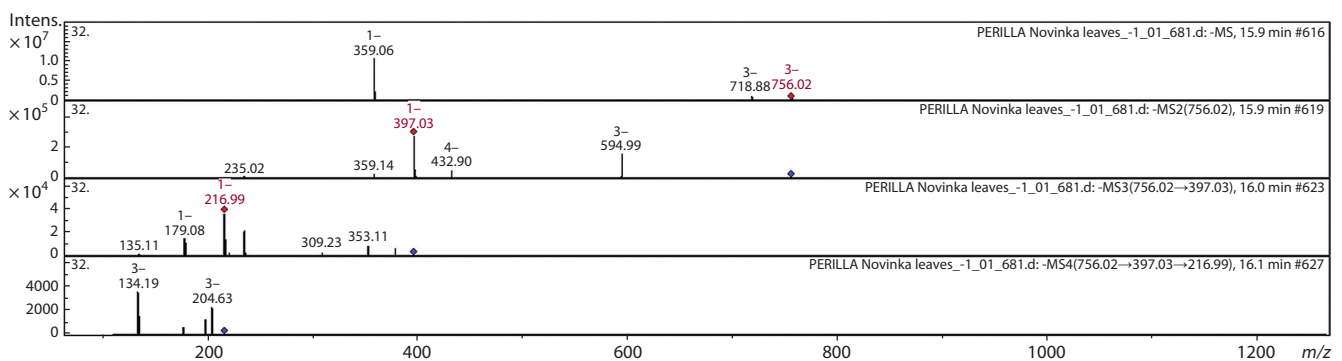


Fig. 3. CID-spectrum of [Cyanidin 3-O-(6-O-para-coumaroyl) glucoside-5-O-glucoside] from extracts of *P. frutescens*, m/z 756.02.

The $[M-H]^-$ ion produced five fragment ions at m/z 397.03, m/z 432.90, m/z 594.99, m/z 359.14, and m/z 235.02 (see Fig. 3). The fragment ion with m/z 397.03 yields five daughter ions at m/z 216.99, m/z 309.23, m/z 353.11, m/z 179.08, and m/z 135.11. The fragment ion with m/z 216.99 yields two daughter ions at m/z 204.63 and m/z 134.19. These results were in agreement with bibliography of *P. frutescens* (Yamazaki et al., 2003; He et al., 2015).

Conclusions

The extracts of *P. frutescens* from the N.I. Vavilov All-Russian Institute of Plant Genetic Resources contain a large number of polyphenolic complexes, which are biologically active compounds. For the most complete and safe extraction, the method of maceration with EtOH was used. To identify target analytes in extracts, tandem mass spectrometry, HPLC and the ion trap were used. The preliminary results showed the presence of 23 bioactive compounds corresponding to *P. frutescens*. In addition to the reported metabolites, a number of metabolites were newly annotated in *P. frutescens* leaves. There were hydroxycoumarin Umbelliferone; triterpene Squalene; omega-3 fatty acid Stearidonic [Morotic] acid; higher-molecular-weight carboxylic acids: Tetracosenoic acid and Salviaric acid C; lignan Syringaresinol and cyclobutane lignan Sagerinic acid; sterol 7-oxo-beta-sitosterol [3-Hydroxystigmast-5-en-7-one]; benzenepropanoic acid Ethyl rosmarinic; flavones Diosmetin and Vicenin-2 [Apigenin-6,8-Di-C-Glucoside].

The findings may support future research into the production of various pharmaceutical and dietary supplements

containing *P. frutescens* extracts. A wide variety of bioactive compounds opens up rich opportunities for the creation of new drugs and bioactive additives based on extracts from mint family Lamiaceae, subfamily Lamioideae, tribe Satureji and subtribe Perillinae. In continuation of the study, we are planning to determine the quantitative content of the identified substances.

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ORCID ID

M.P. Razgonova orcid.org/0000-0002-9732-1649
N.G. Kon'kova orcid.org/0000-0002-4920-3904
A.M. Zakharenko orcid.org/0000-0002-9520-8271
K.S. Golokhvast orcid.org/0000-0002-4873-2281

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