

## Comparative Phytochemical Analysis of the Aerial Parts of *Empetrum nigrum* L. Samples, Collected in Various Regions of the Russian Federation

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Received: 20.10.2021      Revised: 03.12.2021      Published: 27.12.2021

### Abstract

**Introduction.** A number of studies have shown that various genetic and environmental factors can affect the biosynthesis and accumulation of secondary metabolites. In particular, it is known that the local geoclimate, seasonal changes, external conditions such as light, temperature, moisture and soil fertility can affect the chemical composition and, as a result, the therapeutic properties of plants used in the pharmaceutical and food industries. *Empetrum nigrum* L. is a rich source of various pharmacologically active secondary metabolites – chalcones, dihydrochalcones, bibenzyls, 9,10-dihydrophenanthrenes, flavonoids, and proanthocyanidins. In the scientific literature, there is no data on the variation in the chemical composition of *E. nigrum* depending on the growing area. The obtained data are necessary for a reasonable choice of the collecting location for the plant, with the aim of its further chemical and pharmacological research for the development of promising drug candidates.

**Aim.** To carry out a comparative analysis of secondary metabolites composition in the aerial parts of *Empetrum nigrum* growing in different regions of the Russian Federation.

**Materials and methods.** Samples collected in three different areas were used to compare HPLC profiles: sample 1 was collected next to SPCPU nursery garden of medicinal plants (Leningrad region, Vsevolozhsky district, Priozerskoe highway, 38 km) in July 2020, sample 2 was collected on the Kola Peninsula, around the Khibiny mountains in July 2020, sample 3 was collected on the Kamchatka Peninsula, next to Khalaktyrsky beach (Pacific Ocean coast) in July 2020. Extracts were analyzed by analytical high performance liquid chromatography (HPLC) using a Prominence LC-20 device (Shimadzu, Japan) equipped with a diode array detector.

**Results and discussion.** As a result of the research, for the first time, a significant variation in the qualitative chemical composition in the aerial parts of *Empetrum nigrum* growing in different regions of Russian Federation was established. Sample 3, collected on the Kamchatka Peninsula, in comparison with samples 1 and 2, contain the greatest variety of polar secondary metabolites belonging to the classes of flavonoids, tannins, and phenol carboxylic acids, while in the shoots collected in the Leningrad region, the major metabolites were weakly polar compounds belonging to the classes of chalcones, dihydrochalcones, bibenzyls, and 9,10-dihydrophenanthrenes, and in sample 2, collected in the Khibiny mountains, the lowest qualitative content of secondary metabolites was found. This variation may be caused by various environmental factors (biotic and abiotic).

**Conclusion.** For the first time, the comparison of HPLC profiles of aerial part samples of *E. nigrum*, collected in different regions of the Russia Federation has been carried out. As a result, significant variations in the secondary metabolites composition of the studied samples were established, depending on the regions and growing conditions of the plants. The data obtained can be used for a reasonable choice of the collection location for the plant, with the aim of its further chemical and pharmacological research for the development of promising drug candidates.

**Keywords:** black crowberry, *Empetrum nigrum*, HPLC-profiling, 9,10-dihydrophenanthrene, bibenzyls, chalcones, dihydrochalcones, flavonoids, secondary metabolites

**Conflict of interest.** The authors declare that they have no obvious and potential conflicts of interest related to the publication of this article.

**Contribution of the authors.** Anastasiia O. Ponkratova, Andrei K. Whaley and Ekaterina A. Bezverkhniaia performed the experimental part and processing of the results. Elena V. Zhokhova took part in the collecting of the researched samples. Vladimir G. Luzhanin supervised the research. All authors took part in the discussion of the results.

**Funding.** Research results were obtained using the equipment of the Center for Collective Use "Analytical Center of Saint-Petersburg State Chemical and Pharmaceutical University" within the framework of agreement No. 075-15-2021-685 dated July 26, 2021 with the financial support of the Ministry of Education and Science of Russia.

**For citation:** Ponkratova A. O., Whaley A. K., Zhokhova E. V., Bezverkhniaia E. A., Luzhanin V. G. Comparative phytochemical analysis of the aerial parts of *Empetrum nigrum* L. samples, collected in various regions of the Russian Federation. *Razrabotka i registratsiya lekarstvennykh sredstv = Drug development & registration*. 2021;10(4–1):138–145. [https://doi.org/10.33380/2305-2066-2021-10-4\(1\)-138-145](https://doi.org/10.33380/2305-2066-2021-10-4(1)-138-145)

## Сравнительный фитохимический анализ образцов надземной части *Empetrum nigrum* L., собранных в различных регионах РФ, как перспективного источника фармакологически активных вторичных метаболитов

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Статья поступила: 20.10.2021

Статья принята в печать: 03.12.2021

Статья опубликована: 27.12.2021

### Резюме

**Введение.** В ряде исследований показано, что различные генетические факторы и факторы окружающей среды могут влиять на биосинтез и накопление вторичных метаболитов. В частности, известно, что местный геоклимат и сезонные его изменения, прочие внешние условия, такие как свет, температура, влажность и химический состав почвы оказывают влияние на химический состав и, как следствие, на терапевтические свойства экстрактивных комплексов растений, используемых в медицинской практике. *Empetrum nigrum* L. (водяника черная) является источником разнообразных фармакологически активных вторичных метаболитов – халконов, дигидрохалконов, бибензилов, 9,10-дигидрофенантронов, флавоноидов, а также проантоцианидинов. В научной литературе отсутствуют данные о вариации химического состава *E. nigrum* в зависимости от регион произрастания. Полученные данные являются необходимыми для обоснованного выбора места заготовки объекта с целью проведения дальнейших химико-фармакологических исследований для разработки перспективных лекарственных кандидатов.

**Цель.** Провести сравнительный анализ состава вторичных метаболитов образцов надземной части *E. nigrum*, собранных в различных регионах Российской Федерации.

**Материалы и методы.** Для изучения состава вторичных метаболитов использовали образцы, собранные в трех различных географических точках: образец 1 собран в окрестностях питомника лекарственных растений СПХФУ (Ленинградская область, Всеволожский район, Приозерское шоссе, 38 км) в июле 2020 года, образец 2 собран на Кольском полуострове, в районе горного массива Хибин в июле 2020 года, образец 3 собран на полуострове Камчатка, в районе Халактырского пляжа (побережье Тихого Океана) в июле 2020 года. Установление состава вторичных метаболитов проводили методом аналитической высокоэффективной жидкостной хроматографии (ВЭЖХ) на приборе Prominence LC-20 (Shimadzu, Япония), оснащенном диодно-матричным детектором.

**Результаты и обсуждение.** В результате данного исследования впервые были установлены значительные вариации качественного химического состава образцов надземной части *E. nigrum*, произрастающих в различных регионах Российской Федерации. Образец 3, собранный на полуострове Камчатка, по сравнению с прочими образцами содержит наибольшее разнообразие полярных вторичных метаболитов, относящихся к классам флавоноидов, танинов и фенолкарбоновых кислот, в то время как в образце 1, собранном в Ленинградской области, мажорными соединениями являются слабополярные соединения, а в образце 2, собранном в горном массиве Хибин, обнаружено наименьшее качественное содержание вторичных метаболитов. Данная вариативность химического состава могут быть обусловлены различным воздействием факторов окружающей среды (биотическими и абиотическими).

**Заключение.** Впервые проведено сравнение ВЭЖХ-профилей образцов надземной части *E. nigrum*, собранных в различных регионах РФ. В результате установлена значительная вариация состава вторичных метаболитов в исследуемых образцах в зависимости от места и условий произрастания. Полученные данные могут быть использованы для обоснованного выбора места заготовки объекта с целью проведения дальнейших химико-фармакологических исследований для разработки перспективных лекарственных кандидатов.

**Ключевые слова:** *Empetrum nigrum*, водяника черная, ВЭЖХ-профайлинг, 9,10-дигидрофенантроны, бибензилов, халконы, дигидрохалконы, флавоноиды, вторичные метаболиты

**Конфликт интересов.** Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

**Вклад авторов.** А. О. Понкратова, А. К. Уэйли и Е. А. Безверхняя выполняли экспериментальную часть и интерпретировали результаты. Е. В. Жохова принимала участие в заготовке исследуемых образцов. В. Г. Лужанин осуществлял руководство научным исследованием. Все авторы участвовали в обсуждении результатов.

**Финансирование.** Результаты работы получены с использованием оборудования ЦКП «Аналитический центр ФГБОУ ВО СПХФУ Минздрава России» в рамках соглашения № 075-15-2021-685 от 26 июля 2021 года при финансовой поддержке Минобрнауки России.

**Для цитирования:** Понкратова А. О., Уэйли А. К., Жохова Е. В., Безверхняя Е. А., Лужанин В. Г. Сравнительный фитохимический анализ образцов надземной части *Empetrum nigrum* L., собранных в различных регионах РФ, как перспективного источника фармакологически активных вторичных метаболитов. *Разработка и регистрация лекарственных средств*. 2021;10(4–1):138–145. [https://doi.org/10.33380/2305-2066-2021-10-4\(1\)-138-145](https://doi.org/10.33380/2305-2066-2021-10-4(1)-138-145)

## INTRODUCTION

Currently, there is a tendency for the widespread use of herbal medicines, the advantages of which are low toxicity and better tolerance in a wide range of doses [1, 2]. Plants of the genus *Empetrum* widely used in the traditional medicine of Siberia, the Far East and Tibet for the treatment of convulsive conditions and neurodegenerative diseases are, undoubtedly, of interest [3].

The presence of various classes of secondary metabolites in *E. nigrum* determines the uniqueness and complexity of the pharmacological action. Thus, the anticonvulsant, cerebroprotective, antioxidant and antiradical, antifungal, antimicrobial and anti-inflammatory effects of flavanoid-containing and chalcone-containing extractive complexes from black crowberry were experimentally established [4–8].

Black crowberry (*Empetrum nigrum* L.) is an evergreen shrub belonging to the *Ericaceae* family. The habitat of *E. nigrum* covers almost the entire northern part of Russia, Japan, China, as well as, the northern Europe (Scandinavian countries and Great Britain). Typical habitats of this species include sphagnum bogs, moss-lichen tundra, and swampy coniferous forests [9]. Previous studies reported the detection and isolation of various classes of secondary metabolites from *E. nigrum*, among which the most representative classes were chalcones and dihydrochalcones, bibenzyls, 9,10-dihydrophenanthrenes [10–13], as well as type A proanthocyanidins [14, 15] and flavonoids [16].

Secondary metabolites of medicinal plants are the material basis for their pharmacological effects. However, the processes of synthesis and accumulation of secondary metabolites are very complex and influenced by numerous factors. A number of studies have shown that various genetic and environmental factors can affect the biosynthesis and accumulation of secondary metabolites [17–19]. In particular, it is known that the local geoclimate and its seasonal variations including external conditions such as light, temperature, humidity and chemistry of the soil affect the chemical composition and, as a result, the therapeutic properties of plants used in medicine [20]. For example, it has been shown that changes in the amount and seasonality of precipitation affect the concentration of cyanogenic glycosides [21], and an increase in CO<sub>2</sub> concentration often leads to an increase in the concentration of condensed tannins. There is no data in the scientific literature on the variability of the chemical composition of *E. nigrum* depending on the growing area. This data is necessary to select reasonably areas for the collection of *E. nigrum* plant material in order to conduct further chemical and pharmacological studies for the development of promising drug candidates. Thus,

the purpose of this research was the comparative analysis of secondary metabolite composition in samples of the terrestrial parts of *E. nigrum* collected in different regions of the Russian Federation.

## MATERIALS AND METHODS

To study the variation of *E. nigrum* secondary metabolites, samples collected in three different geographical locations were used: sample 1 was collected in the vicinity of the SPCPU medicinal plant nursery (Leningrad region, Vsevolozhsky district, Priozerskoe highway, 38 km) in July 2020, sample 2 was collected on the Kola Peninsula, in the region of the Khibiny mountain range in July 2020, sample 3 was collected on the Kamchatka Peninsula, in the area of Khalaktyrsky beach (Pacific Ocean coast) in July 2020. The composition of secondary metabolites was determined with analytical high performance liquid chromatography (HPLC) on a Prominence LC-20 instrument (Shimadzu, Japan) equipped with a diode array detector at 235 nm. A SUPELCOSIL LC-18 chromatographic column (25 cm × 4.6 mm) with a particle size of 5 μm was used. Sample injection volume was 10 μL with an analysis temperature of 40 °C. Mobile phase: water (component A), acetonitrile (component B) with a TFA content of 0.1 %, by volume. The characteristics of the used HPLC analysis method are shown in Table 1. Method characteristics of HPLC analysis

**Table 1. Method parameters of HPLC analysis**

Time (min)	Flow rate (ml/min)	Concentration of A (%)	Concentration of B (%)
0.01	1.00	95	5
5.00	1.00	95	5
45.75	1.00	0	100
50.00	1.00	0	100
60.00	1.00	95	5
65.00	1.00	95	5

## RESULTS AND DISCUSSION

To obtain HPLC profiles of the collected *E. nigrum* samples, all three samples were extracted with ethanol. Before extraction, the raw material was dried, milled and sieved through a sieve with a diameter of 1 mm. To obtain ethanol extracts, 10 g of each sample was weighed and extracted with 96 % ethanol (100 ml). The extraction was performed in sealed glass flasks at

room temperature for 72 hours. After the extraction, the extracts were filtered through a paper filter, the volume of the obtained extracts was measured, and, if necessary, the volume of the extract was adjusted to 100 ml with an appropriate solvent. Then, 0.5 ml samples of each extract were taken for HPLC analysis. As a result, the following chromatograms were obtained (Figures 1, 2 and 3).

The obtained chromatograms were compared with reference samples (SS) of rutin, quercetin, hyperoside, cinaroside, kaempferol, myricetin, luteolin, veratric, synapic, protocatechuic, chlorogenic, coffee, rosemary, and lilac acids, as well as with chromatograms of individual compounds isolated from *Empetrum nigrum*, the structure of which was established in the previous studies [10, 11, 14]. Peaks were identified by comparing chromatographic parameters (retention time and UV absorption maxima) (Table 2).

Peaks of myricetin and luteolin, as well as of veratric, sinapic, protocatechuic and rosmarinic acids, were not found in the test samples.

The data provided in Table 2 shows a significant variability in the chemical composition of samples collected in different regions of the Russian Federation. The largest amount of secondary metabolites was found in sample 3 collected on the Kamchatka Peninsula; however, most of them are polar compounds (29) belonging mainly to the classes of flavonoids (14), tannins (7) and phenolic acids (5). The presence of 10 less polar compounds with a retention time from 23 to 40 minutes was also established, of which 8 compounds belonging to the classes of chalcones (1), dihydrochalcones (2), 9,10-dihydrophenanthrenes (3) and bibenzyls (2) were identified (table 2, figure 3).

In sample 2, collected on the Kola Peninsula, 23 compounds were observed, of which 5 were identified. The bulk of secondary metabolites (13) are polar compounds belonging mainly to the classes of flavonoids (5), tannins (4), and phenolic acids (2). In addition, 5 less polar compounds with a retention time of 23 to 40 minutes were found, as well as the presence of 5 non-polar compounds with a retention time of more than 40 minutes (Table 2, Figure 2).

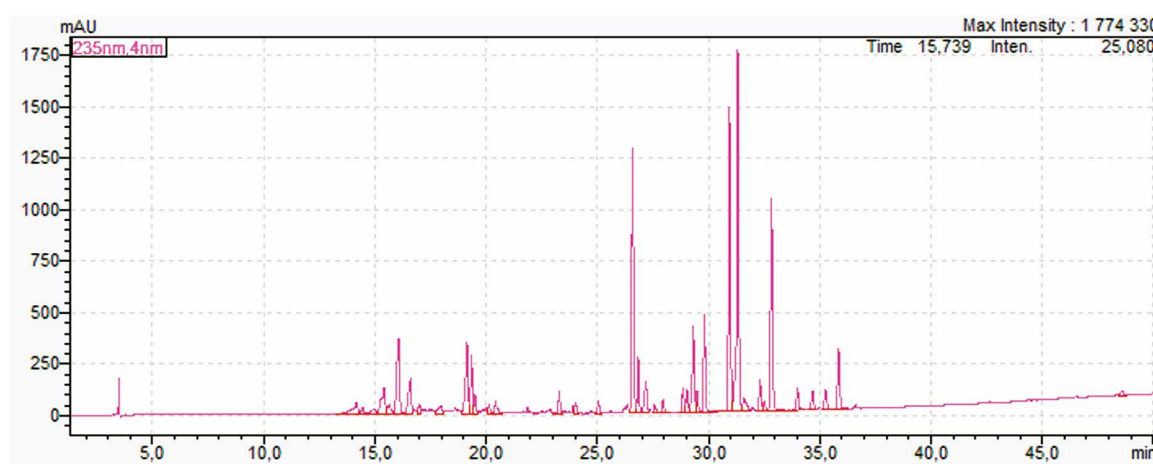


Figure 1. HPLC-UV data of ethanolic extract obtained from shoots of *Empetrum nigrum* growing in Leningrad region (Sample 1)

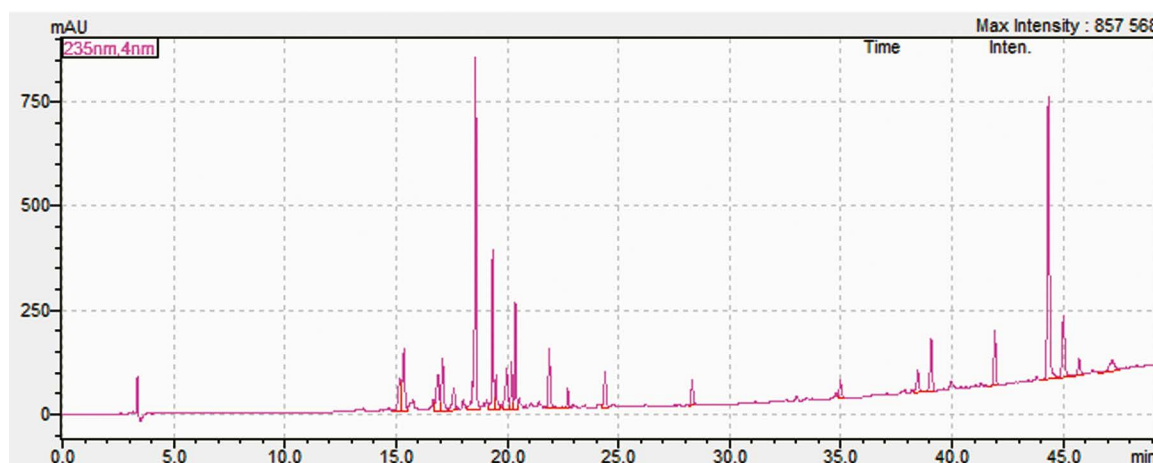


Figure 2. HPLC-UV data of ethanolic extract obtained from shoots of *Empetrum nigrum* growing in Kola Peninsula (Sample 2)

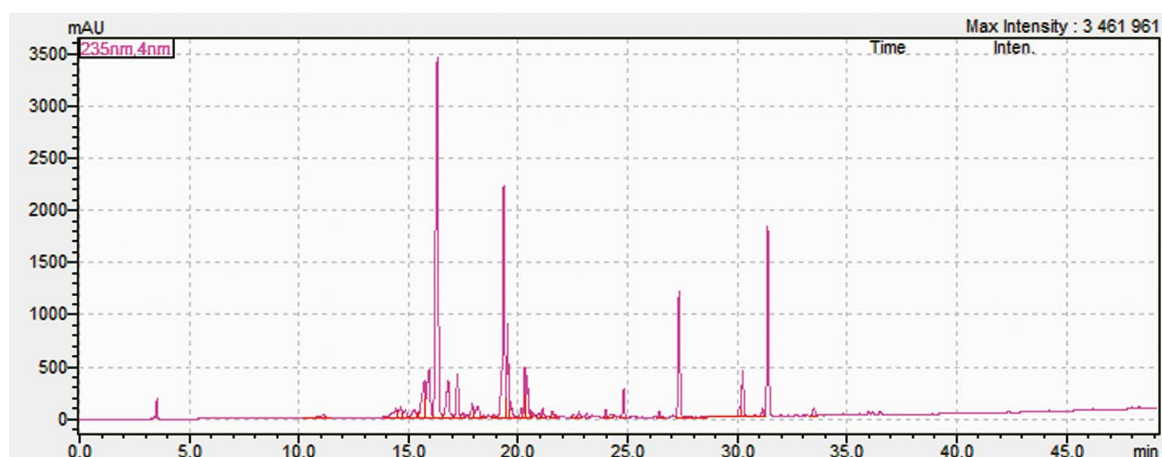


Figure 3. HPLC-UV data of ethanolic extract obtained from shoots of *Empetrum nigrum* growing in Kamchatka Peninsula (Sample 3)

Table 2. Comparison of secondary metabolites composition of the samples 1, 2 and 3

Number	Name of reference compound / Individual compound	Chromatographic parameters (retention time, min; UV-absorption maxima, nm)	Sample 1	Sample 2	Sample 3
1	Rutin	19.1; 254, 351	+	–	+
2	Quercetin	23, 9; 254, 370	+	–	+
3	Kaempferol	20.0; 265, 346	+	–	+
4	Cynaroside	19.6; 253, (266), 347	–	–	+
5	Hyperoside	19.3; 254, 352	+	+	+
6	Syringic acid	16.0; 273	+	–	–
7	Chlorogenic acid	15.1; 232, (284) 326	+	+	+
8	Caffeic acid	15.4; 233, (290), 232	+	+	+
9	Procyanidin A1	18.3; 278	+	–	–
10	Procyanidin A2	19.4; 277	+	+	+
11	Epicatechin-(2 $\beta$ $\rightarrow$ O $\rightarrow$ 5, 4 $\beta$ $\rightarrow$ 6))-catechin	20.2; 278	+	+	–
12	1-(3-hydroxyphenyl)-2-(3-hydroxy-4,5-dimethoxyphenyl) ethane	25.1; 273, 325	+	–	+
13	1-(3,5-dihydroxy-4-methoxyphenyl)-2-phenyl ethane	27.2; 268, (327)	+	–	+
14	1-(3,5-dihydroxy-4-methoxyphenyl)-2-(3-hydroxyphenyl) ethene	23.4; 272, (324)	+	–	–



End of table 2

Number	Name of reference compound / Individual compound	Chromatographic parameters (retention time, min; UV-absorption maxima, nm)	Sample 1	Sample 2	Sample 3
15	2,3,4,7-tetramethoxy-9,10-dihydrophenanthrene	34.9; 283, (300)	+	–	–
16	2,3,4-trimethoxy-5-hydroxy-9,10-di-hydrophenanthrene	33.0; 275, (296)	+	–	+
17	4,7-dihydroxy-2,3-dimethoxy-9,10-dihydrophenanthrene	26.4; (238), 280	+	–	+
18	6-hydroxy-2,3,4-trimethoxy-9,10-dihydrophenanthrene	28.3; 282	+	–	+
19	4'-hydroxy-2'-methoxydihydrochalcone	29.4; 268, 303	+	–	+
20	2'-hydroxy-4'-methoxydihydrochalcone	35.4; 274, 314	+	–	–
21	2',4'-dimethoxydihydrochalcone	34.2; 267, 302	+	–	–
22	2',4'-dihydroxydihydrochalcone	31.2; 276, 313	+	–	+
23	2'-hydroxy-4'-methoxychalcone	36.0; 341	+	–	–
24	2',4'-dihydroxychalcone	31.2; 339	+	–	+
	Identified peaks		24	5	16
	Total peaks:		37	23	39

In sample 1, collected in the Leningrad region, 37 compounds were observed, of which 24 were identified. The predominant group of secondary metabolites (23) are weakly polar compounds with a retention time of 23 to 40 minutes belonging to the classes of bibenzyls, 9,10-dihydrophenanthrenes, chalcones, and dihydrochalcones, while polar compounds with a retention time of up to 23 minutes are represented in a smaller degree (14) and belong to the classes of tannins (6), flavonoids (5) and phenolic acids (3) (table 2, figure 1).

## CONCLUSION

For the first time, the comparison of sample HPLC profiles from the terrestrial parts of *E. nigrum* collected in different regions of the Russian Federation is reported. As a result, a significant variation in the composition of secondary metabolites in the test samples was established, depending on the region and growing conditions.

This variability can be caused by different environmental factors (biotic and abiotic). Most likely, the observed differences can be associated mainly with the temperature factor. In a number of studies examining the effect of temperature on the accumulation of flavonoids, it has been shown that at higher temperatures (30–35 °C) the accumulation of flavonoids decreases. The difference between day and night temperatures is also a factor affecting the accumulation of flavonoids in plants. One study has shown that when the difference between day/night temperatures is greater than 10 degrees or more, the accumulation of flavonoids decreases [22].

The climate of the eastern part of the Kamchatka Peninsula combines maritime and monsoon elements, with an average annual temperature of +2.8 °C, due to the influence of the Kuril-Kamchatka Current and the Pacific Ocean, the temperature in winter rarely drops below –19 °C, at the same time, it rarely rises in summer

above +15 °C, and the day/night temperature difference does not reach 10 °C. Most likely, these temperature conditions determine the greatest variety of polar secondary metabolites in sample 3 belonging to the classes of flavonoids, tannins, and phenolic acids in comparison to other samples.

At the same time, the climate in the Khibiny mountain range has a harsh subarctic character with the average annual temperature at about –3 °C, where July is the hottest month, with an average temperature of 13 °C. Here the day/night temperature difference can reach 15 °C. Presumably, because of the low average annual temperature, a fairly short cold summer, and a large day/night temperature difference in the qualitative composition of the aerial parts of *E. nigrum* is the poorest compared to the rest of the samples, but also, like sample 3, it is predominantly composed by polar metabolites like flavonoids, tannins and phenolic acids.

The Leningrad region has a temperate climate, with an average annual temperature of +5.8 °C, the hottest month is July, with an average daily temperature of +19.5 °C, the coldest month February with an average temperature of –5.8 °C. The day/night temperature difference in the Leningrad region can reach 15 °C in different months which can probably explain the lower qualitative content of flavonoids, tannins and phenolic acids, compared to sample 3. However, sample 1 is rich in weakly polar compounds belonging to the classes of chalcones, dihydrochalcones, bibenzyls, and 9,10-dihydrophenanthrenes, which are mostly absent in samples 2 and 3.

The obtained results can be used to select reasonably sites for *E. nigrum* raw material collection that can be used for further chemical and pharmacological studies aimed at the development of promising drug candidates.

The study results were obtained using the equipment from the Center of Collective Use "Analytical Center of the Federal State Budgetary Educational Institution of Higher Education SPCPU of the Russian Ministry of Health" as part of agreement No. 075-15-2021-685 dated July 26, 2021 with the financial support of the Russian Ministry of Education and Science.

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