Composition of terpenes as the dominant part of juniper berry essential oils

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Summary

The genus *Juniperus* contains plants that are primarily used in the food industry for distillate production, with notable examples being gin and a specific Slovak traditional spirit borovička. During the production process of borovička, traditionally manufactured from the berries of *Juniperus communis* L., or nowadays from *Juniperus oxycedrus* L., juniper berry essential oil is produced as a by-product. It is subsequently used for further applications in food industry and for certain pharmaceutical purposes. Gas chromatographic methods were involved in analysis and evaluation of commercially produced juniper berry essential oils together with oils obtained in laboratory. Generally, juniper berry essential oil is comprised primarily of monoterpenes, sesquiterpenes and their oxidized derivatives. In our study, depending on the juniper species, berry essential oils were dominantly represented by α-pinene (23.5–36.0 %), myrcene (11.8–18.9 %), limonene (1.1–13.5 %), terpinen-4-ol (0.5–6.2 %), β-caryophyllene (3.4–5.3 %), α-humulene (1.8–4.2 %) and germacrene D (1.0–9.7 %). This work is an introductory study focusing on qualitative and quantitative differences between the terpenic profiles of juniper berry essential oils gained from *J. communis* and *J. oxycedrus* in laboratory, and juniper essential oils gained as a by-product from borovička production.

Keywords

juniper berry; essential oil; terpene; gas chromatography; distillate

The genus *Juniperus* (family *Cupressaceae*) is an evergreen shrub or tree, mostly distributed in the cool and temperate regions of the northern hemisphere. Occurrences have also been reported in South Africa and Australia but in limited quantities. The genus consists of approximately 75 species and it is divided into three sections: *Coriocesrus* section containing 1 species, the *Juniperus* section containing 14 species and the *Sabina* section containing approximately 60 species. The most important species belonging to the *Juniperus* section are *Juniperus communis* L. and *Juniperus oxycedrus* L. *J. communis* commonly grows in the entire northern hemisphere and *J. oxycedrus* is most widespread in the Mediterranean area [1].

J. communis spreads spontaneously in Slovakia on pastures and meadows, gradually giving way to the growth of forests. In the past, the plant was often used in many aspects of life as traditional medicine. Berries were used as a diuretic, for treating gastrointestinal problems, rheumatism, arthritis and gout. They were presumed to have anti-inflammatory and analgetic effects as

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well. Native Americans used *J. communis* berries as a contraceptive and to treat diabetes [2]. Hypoglycemic, antidiabetic, urinary and antitumor effects were demonstrated in several scientific studies [3–8], but reliable clinical data confirming beneficial therapeutic effects in human studies are still lacking. Several studies reported strong antioxidant activity of juniper berry essential oil [9–13]. Long-term use of drugs made from juniper plants were proven to lead to kidney damage and these drugs are unsuitable for pregnant women [14]. Further studies analysed the inhibitory activity of *J. communis* berry essential oils on bacterial strains *Bacillus cereus, Escherichia coli, Listeria monocytogenes, Cornybacterium* spp. and *Staphylococcus aureus* [15–18]. Juniper essential oil was shown to inhibit the growth of *Staphylococcus aureus* (ATCC 29853) with minimum inhibitory concentration (*MIC*) of 4.8 $\mu l \cdot cm^{-3}$ and *Escherichia coli* (ATCC 10536) with *MIC* of 16.8 $\mu l \cdot cm^{-3}$. It was also shown to contribute positively to the formation of anti-inflammatory factors. The oil has antifungal, antiviral and antioxidant properties as well [13, 19].

Juniper berries are used more often in the food industry than in the pharmaceutical industry. The astringent berries are too bitter to be eaten raw and therefore, they are dried for culinary applications. They are used as a seasoning for meat, soups, sauces, stuffings and to preserve food. Tomović et al. [20] reported satisfactory sensory properties, increased oxidative and microbiological stability of low fat and low sodium processed meat products treated with juniper oil. Nevertheless, distillery industry occupies a significant share in the field of juniper processing. The best-known spirit using juniper berries is gin, which is flavoured not only with said berry extract, but it also contains extracts of other fruits and herbs such as coriander, cardamom, licorice, orange peel, lemon peel, anise and other aromatic herbs [21].

Slovakian traditional product is a spirit drink called borovička. According to Regulation (EU) 787/2019 [22], borovička may be designated by the name "Wacholder" or "Genebra". The unique processes that distinguish the production of borovička from gin are processing of juniper berries by fermentation and maceration. Berries are mixed with drinking water and subsequently fermented with *Saccharomyces cerevisiae* to achieve 2–3 % ethanol by volume. Current production methods include the adition of refined mild alcohol to the fermented macerate, in order to increase the yield of terpenes. The production process is unique for individual sub-brands, e. g. Slovenská borovička, Spišská borovička or Trenčianska borovička, and the exact procedures are trade secrets of each distillery. After the adition of refined alcohol, the subsequent production step is distillation on an apparatus with a juniper oil separator, in which juniper oil is separated as a secondary product. The primary product is a crude juniperus distillate, which is then rectified. Aroma of juniper distillates is determined by the content of terpenes in the final product which, depending on the extraction method, may have an impact on the final sensory quality of the beverage. Juniper berry oil is a valuable raw material in the production of cosmetics and pharmaceutics. The aromatic character of juniper oil and the products made from it is therefore mainly determined by the composition of terpenes in juniper berries. The amount of essential oil and its quality is variable and is influenced by several factors such as juniper species, soil, climatic conditions, time of harvest, age of the plant and the method of extraction and distillation. In the past *J. communis* berries were used for the production of juniper distillate in Slovakia but at present, due to the reduction of areas where juniper trees or shrubs grow, almost exclusively *J. oxycedrus* berries are used for the production of juniper distillate. Annually, 500 tons of *J. oxycedrus* berries are imported to Slovakia to satisfy the quotas for borovička production.

The aim of the presented work was to evaluate the qualitative and quantitative differences in the composition of terpenes in commercial juniper berry essential oils obtained at the production of juniper spirit drink borovička, and juniper berry essential oils isolated from *J. communis* and *J. oxycedrus* in laboratory conditions using hydrodistillation by Clevenger apparatus.

MATERIAL AND METHODS

Samples

Juniper berry essential oils were obtained from *Juniperus communis* berries gathered from plants in the region of Banská Bystrica, Slovakia (October 2020 harvest). Ripe dark violet berries of 4–10 mm were carefully separated from branches, air-dried at 25 °C and stored at room temperature of 21 ± 2 °C in a dry dark place for a maximum of 1 month until extraction. Berries of *Juniperus oxycedrus* (Plovdiv, Bulgaria, 2019 harvest) were provided by the stock company Prelika (Prešov, Slovakia). Commercial juniper berry oil samples were obtained from three commercial producers of juniper spirit drink borovička from Slovakia.

Samples were stored in the dark and refrigerated at 10 °C and labelled as Juniper oil No. 1, No. 2, and No. 3.

Reference standards

Chemicals used as reference standards to support identification of volatiles (listed in Tab. 1) were obtained from Sigma Aldrich (St. Louis, Missouri, USA). Several standards were gifts donated by Bedoukian Research (Danbury, Connecticut, USA), Graz University of Technology (Graz, Austria) and French National Institute for Agricultural Research (INRA) laboratories (Dijon, France).

Hydrodistillation of juniper berries

Juniper berry essential oils were obtained in laboratory conditions by hydrodistillation on a Clevenger apparatus using a modified version of ISO 6571:2008(E) standard for determining volatile oil content in spices, condiments and herbs [23]. Juniper berries were carefully crushed to avoid breaking of the seeds and to prevent contamination of the extracts with lipids. Subsequently, 25 g of crushed juniper berries were weighed into the extraction flask, submerged in 150 ml of distilled water and hydrodistilled for 2 h at reflux. Total yield of juniper essential oil obtained in laboratory from *J. communis* and *J. oxycedrus* berries reached 1.2 ± 0.1 ml per 100 g and 0.8 ± 0.1 ml per 100 g, respectively. The origin of berries, method of extraction and oil yield of commercial oils were not provided by the producers.

Gas chromatography-mass spectrometry

Juniper berry essential oils were analysed by gas chromatography-mass spectrometry (GC-MS), performed on a gas chromatograph Agilent 6890N (Agilent Technologies, Palo Alto, California, USA), hyphenated to a mass spectrometer 5973 inert (Agilent Technologies). The analytical system was equipped with an Agilent 7683B auto-sampler (Agilent Technologies). The column used for separation was HP-INNOWax (30 m × 0.25 mm × 0.5 μm; Agilent Technologies) with a polyethylene glycol (PEG) polar stationary phase. Helium was used as the carrier gas with a linear velocity of 45 cm·s^{-1} (measured at 143 °C). Oven was operated with a program 40 °C (1 min), 5 °C·min^{-1} and 240 °C (2 min). Injector and detector temperatures were held at 250 °C. The injector operated in split mode with a split ratio of 20 : 1. Electron ionization (*EI*) energy was 70 eV.

Gas chromatography-flame ionization detection

In order to determine the relative contents of individual terpenic compounds of juniper berry essential oils, in paralel with GC-MS, quantitative data (peak area percentage) of the compounds were obtained using a gas chromatograph 7890A (Agilent Technologies) paired with a flame-ionization detector (FID). The chromatographic column used for separation was DB-WAX (30 m × 0.32 mm × 0.5 μm; Agilent Technologies) with a PEG polar stationary phase. The inlet was heated up to 250 °C and operated in the split mode with a split ratio of 20 : 1. Oven temperature program was 40 °C (1 min), 5 °C·min^{-1}, 240 °C (2 min). Helium was used as a carrier gas at a linear velocity of 45 cm·s^{-1} (measured at 143 °C). Samples of juniper berry essential oils were analysed in triplicate.

For validation of the method, limit of detection and limit of quantification (0.03 % relative content and 0.1 % relative content, respectively) as well as precision were assessed. The precision was evaluated as intra-day repeatability (n = 6) using commercial essential oil No. 1 as reference. The obtained relative standard deviation for individual terpenic compounds ranged from 0.5 % to 4.6 %.

Identification of terpenic compounds

Individual terpenic compounds of juniper berry essential oils were identified on the basis of comparison of their linear retention indices (*LRI*), mass spectra, analysis of standard compounds, data on occurrence in literature [1] and in our in-house *LRI* database. *LRI* were calculated using a homologous n-alkane mixture of C10–C23 (Sigma-Aldrich) as reference compounds using the equation of Van den Dool and Kratz [24]. *LRI* values were compared and confirmed with *LRI* data obtained by measurement of relevant standard compounds. For this purpose, our in-house database of *LRI* values was used. Identification of compounds by comparison of their mass spectras was done using Automated Mass Spectral Deconvolution and Identification System (AMDIS) software (National Institute of Standards and Technology, Gaithersburg, Maryland, USA), Mass Spectral Library NIST 20 (National Institute of Standards and Technology) and mass spectra found in literature [25].

Statistical analysis

Statistical analysis was performed using Unistat v. 6.0 (Unistat, London, United Kingdom). Principal component analysis (PCA) and principal component factoring (PCF) with varimax rotation was used to define and visualize differences between commercial juniper berry essential oils and oils isolated from *J. communis* or *J. oxycedrus*.

Tab. 1. Composition of terpenes in juniper berry essential oils.

LRI	Compound	Peak area [%]				
		Commercial essential oils			Laboratory-obtained essential oils	
		No. 1	No. 2	No. 3	*J. communis*	*J. oxycedrus*
Monoterpenes						
1030	α-Pinene	23.5	27.1	28.9	36.0	29.8
1074	Camphene	0.9	0.6	0.7	0.2	0.7
1117	β-Pinene	3.2	3.1	2.7	2.5	3.2
1130	Sabinene	0.1	0.7	0.1	8.2	1.3
1158	3-Carene	0.9	1.5	0.3	0.7	0.3
1171	Myrcene	16.6	18.9	12.9	11.8	18.7
1210	Limonene	12.3	9.9	13.5	1.1	10.3
1257	γ-Terpinene	0.1	1.8	–	0.8	–
1282	*p*-Cymene	1.7	3.5	2.5	0.1	1.0
Oxidized monoterpenes						
1480	4-Thujanol	0.4	0.2	0.1	–	–
1560	Linalool	0.4	0.1	0.3	0.2	0.5
1601	Bornyl acetate	1.0	0.3	0.8	0.3	0.8
1622	Terpinen-4-ol	0.5	2.2	1.0	6.2	1.1
1664	Pinocarveol	0.3	0.1	0.2	–	0.7
1716	α-Terpineol	0.6	0.5	1.0	0.6	0.4
1725	Borneol	0.3	0.2	0.2	0.2	0.5
1734	Carvone	0.2	–	0.2	–	0.3
1779	Citronellol	0.1	0.1	0.1	0.1	0.3
1858	*p*-Cymen-8-ol	0.2	0.1	0.4	–	0.3
Sesquiterpenes						
1471	α-Cubebene	0.6	0.6	0.6	–	0.8
1509	α-Copaene	0.6	0.6	0.5	0.2	0.5
1607	β-Elemene	0.2	0.6	–	–	–
1618	β-Caryophyllene	5.2	3.8	5.3	3.4	3.7
1656	γ-Elemene	0.3	0.2	–	0.8	0.8
1676	β-Farnesene	0.2	0.3	0.1	0.5	0.3
1693	α-Humulene	4.1	2.9	4.2	1.8	2.6
1710	γ-Muurolene (unknown isomere)	1.4	1.0	1.1	–	–
1733	Germacrene D	1.9	3.1	1.0	9.7	3.0
1745	α-Muurolene (unknown isomere)	1.6	1.3	1.5	0.6	1.3
1749	α-Selinene	0.2	0.4	0.2	–	–
1779	δ-Cadinene	1.0	0.9	0.8	0.2	0.8
1783	γ-Cadinene	3.0	2.5	2.2	1.9	1.8
1858	Germacrene B	0.1	0.6	0.2	4.7	–
1955	α-Calacorene	0.6	0.5	0.5	–	0.3
Oxidised sesquiterpenes						
2020	Caryophyllene oxide	1.4	0.6	1.4	0.2	1.6
2154	Spathulenol	0.1	0.2	0.1	0.2	0.3
2264	α-Cadinol	0.8	0.7	1.3	1.2	0.5
	Sum of monoterpenes	59.2	66.9	61.6	61.4	65.4
	Sum of oxidised monoterpenes	4.1	3.9	4.3	7.7	5.2
	Sum of sesquiterpenes	21.1	19.3	18.2	23.8	15.9
	Sum of oxidised sesquiterpenes	2.2	1.4	2.8	1.6	2.4

LRI – linear retention index, calculated from retention data obtained on DB-WAX gas chromatographic column.

RESULTS AND DISCUSSION

The most common method used for the isolation of essential oils is hydrodistillation on a Clevenger-type apparatus. In this study, the composition of terpenes in juniper berry essential oils obtained from the production process of juniper distillate borovička as a byproduct and oils obtained by hydrodistillation on a Clevenger-type apparatus were compared. As follows from the results obtained (Tab. 1), the major terpene constituents in juniper essential oils of both species *J. communis* and *J. oxycedrus* were α-pinene, myrcene, limonene, terpinen-4-ol, α-humulene, β-caryophyllene, germacrene D and caryophyllene oxide. Typical sensory characteristics of these compounds are described as pine-like, woody and spicy, only in the case of limonene it is citrus. Regarding the juniper variety, significant differences in the levels of sabinene, myrcene and limonene were observed. As shown in Tab. 1, sabinene relative content in *J. communis* essential oil (8.2 %) was more than six times higher than in *J. oxycedrus* essential oil (1.3 %). On the contrary, the essential oil of *J. oxycedrus* contained a higher proportion of myrcene (18.7 %) and limonene (10.3 %) compared to the essential oil of *J. communis* (11.8 % and 1.1 %, respectively). On the other hand, terpene profiles of commercial juniper berry oils No. 1, No. 2 and No. 3 did not differ significantly from each other.

Results presented in this study, obtained from *J. communis* and *J. oxycedrus*, were in a good compliance with the European Pharmacopoeia [26]. It states that the chromatographic profile of *J. communis* essential oil contains volatile components within these ranges: α-pinene (20–50 %), sabinene (maximum 20 %), β-pinene (1–12 %), myrcene (1–35 %), α-phellandrene (maximum 1 %), limonene (2–12 %), terpinen-4-ol (0.5–10 %), bornyl acetate (maximum 2 %) and β-caryophyllene (maximum 7 %). As shown in Tab. 1, the obtained results were within these ranges, except of α-phellandrene, which was not detected in samples under study. Other authors did not report α-phellandrene in juniper berry essential oil extracts [6, 12, 15–17], except for Falcão et al. [18] who found it only in commercial essential oil samples. According to Gonny et al. [27], α-phellandrene was present in juniper needle essential oil compared to juniper berry essential oil. This indicates the potential of α-phellandrene as a marker for differentiation of essential oils extracted from juniper needles and berries.

Comparing our results with those obtained in other studies [6, 12, 15–18, 27], some differences in the composition of most abundant terpenes in juniper essential oils derived from *J. communis* were noticed. Nevertheless, α-pinene, sabinene, β-pinene, myrcene, α-phellandrene, limonene, terpinen-4-ol, bornyl acetate and β-caryophyllene remained the most abundant compounds in juniper essential oils. Fierascu et al. [6] used ethanol extraction, which resulted in a lower abundance of major terpenes, but the general composition remained the same with α-pinene, β-pinene and myrcene as most abundant compounds. Authors reported a very low content of limonene, which suggests that the extraction method can affect the final yield of certain compounds [6]. Pepeljnjak et al. [15] reported an increased amount of β-pinene (17.8 %) and significantly lower content of myrcene (0.3 %) in the sample of commercial essential oil, compared to our study. Contrary to our results, Glišić et al. [17] did not detect β-pinene and bornyl acetate. Gonny et al. [27] found that Corsican juniper essential oils were dominated by a high level of limonene and lack of sabinene. The described differences in the quality of juniper essential oils were apparently highly dependent on the properties of used raw materials, which were influenced by various factors such as geographical conditions (altitude, humidity, rainfall, temperature), ripeness, post-harvest treatment, storage conditions as well as by the method of processing.

It is well known that components of essential oils are easily convertible to each other by means of oxidation, isomerization, cyclization or dehydrogenation, which are triggered either by enzymes or chemically at elevated temperatures [28, 29]. Volatile compounds such as *p*-cymene, pinocarveol, *p*-cymen-8-ol, carveol, carvone or caryophyllene oxide can be considered as typical markers of essential oil degradation [28–33].

As follows from Tab. 1, commercial juniper oils contained increased levels of *p*-cymene (1.7–3.5 %) compared to laboratory-prepared essential oils from both types of juniper berries (*J. oxycedrus* 1.1 %; *J. communis* 0.1 %). Elevated levels of *p*-cymene were identified in older essential oils as a result of degradation of limonene, α-terpinene and γ-terpinene caused by improper storage (exposure to air, light, high temperature) [28, 30–33].

Regarding oxidized monoterpenes such as pinocarveol (from 0.1 % to 0.3 %), *p*-cymen-8-ol (from 0.1 % to 0.4 %) and carvone (from 0 % to 0.2 %), they were identified in commercial juniper oils and also in laboratory-obtained *J. oxycedrus* oil, while these compounds were not detected in *J. communis* essential oil. Carveol and its oxidation

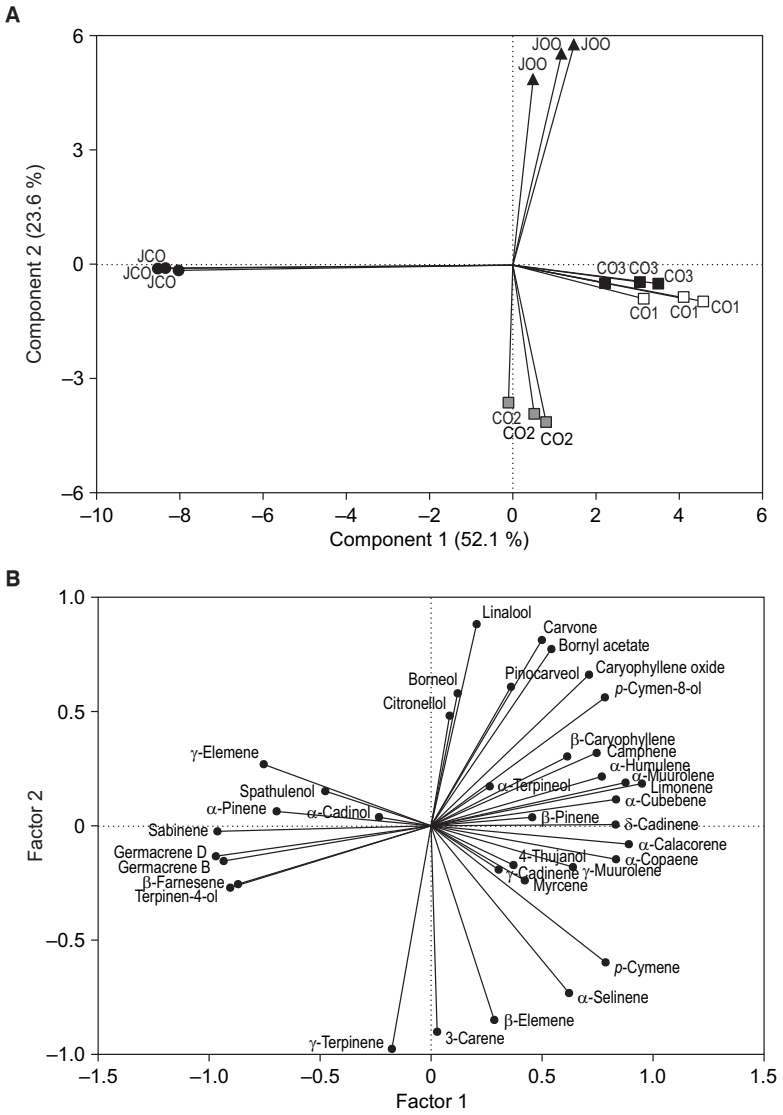

Fig. 1. Principal component analysis and factor analysis of *Juniperus* essential oils.

A – plot of principal components demonstrating differentiation of *Juniperus* essential oils, B – plot of factors (varimax rotation) indicating the importance of individual variables for *Juniperus* essential oils discrimination.
CO1 – commercial essential oil No. 1, CO2 – commercial essential oil No. 2, CO3 - commercial essential oil No. 3, JCO – laboratory obtained essential oil isolated from *J. communis*, JOO – laboratory obtained essential oil isolated from *J. oxycedrus*.

product carvone were described as degradation products derived from peroxides during limonene autoxidation [28]. From the group of oxidized sesquiterpenes, caryophyllene oxide as a stable by-product of caryophyllene oxidation [33], was identifiend in all samples under study.

Regarding the above-mentioned differences in the content of some terpenes (sabinene, myrcene, limonene, pinocarveol, *p*-cymen-8-ol and carvone) between commercial essential oils and laboratory-prepared oil from *J. communis* and *J. oxycedrus*, it can be concluded that the commercial oils were probably obtained by distillation of either *J. oxycedrus* berries or a mixture of berries from both species at an unspecified ratio. This conclusion was partially supported by the results of PCA and PCF. Plot of principal components depicted in Fig. 1 indicated the existence of discriminated groups of eigenvectors belonging to the samples of individual essential oils. As is obvious from Fig. 1, commercial oils and oil from *J. oxycedrus* were located on the right side of the plot, while oil from *J. communis* was located separately on the left side of the plot. As regards the numerical values, the first two principal components (PC) cumulatively explained more than 75.8 %. Eigenvalues indicated that for the construction of the first PC, limonene, terpinene-4-ol and germacrene D had

the most significant weight. Oxidized monoterpenes borneol, linalool, pinocarveol and citronellol revealed the dominant role in the construction of second PC. PCF with varimax rotation resulted in a plot of factor scores corresponding to PCA data projection (data not presented). The plot of factors (Fig. 1B) showed the importance of each descriptor for the purpose of discrimination and also suggested mutual positive or inverse correlations of descriptors. Similarly to PCA, PCF confirmed the importance of limonene, germacene D, γ-terpinene, linalool and sabinene for discrimination of commercial essential oils and essential oils isolated from *J. communis* and *J. oxycedrus*.

CONCLUSION

This work was an introductory study dealing with the differences between commercially produced juniper berry essential oils obtained as a by-product of borovička distillate production, and laboratory isolated essential oils from *J. communis* and *J. oxycedrus* berries. Comparison of terpene profiles of essential oils isolated in laboratory from both juniper species and commercial juniper essential oils No. 1, No. 2, No. 3 suggested that *J. oxycedrus* berries or a mixture of both types of berries was used for the production of borovička spirit. Essential oils obtained by hydrodistillation of *J. communis* and *J. oxycedrus* berries showed significant differences in terpene composition. Notably, the contents of myrcene and limonene were higher in the essential oil of *J. oxycedrus* and this species of juniper had reduced contents of sabinene, compared to the essential oil of *J. communis*. Results of the study suggested that the differences in the composition of commercial and laboratory-isolated juniper oils might be affected by the isolation process as well as by the quality and species of juniper berries used.

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