Ukrainian Journal of Ecology

Ukrainian Journal of Ecology, 2021, 11(2), 300-305, doi: 10.15421/2021_114

ORIGINAL ARTICLE

Comparative GC-MS study of *Schizonepeta multifida* essential oil from Khakassia Republic shows potentials for nutraceuticals, flavor, and conservation

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Received: 03.03.2021. Accepted 14.04.2021

Schizonepeta multifida (L.) Briq. (Lamiaceae) is among the widely used botanicals in Siberian traditional and modern health and personal care practices, shelf life extension of agricultural produce, antimicrobial, and insect repellent. We investigated the variations or differences in the chemical composition of essential oils in wild accessions harvested from two distinct geographical regions, 'habitat-1' and 'habitat-2', in the Republic of Khakassia, Siberia. The oils were obtained by subjecting the aboveground parts of the plant to hydro-distillation, with subsequent assessment of the chemical composition using GC-FID and GC-MS analyses. The oil composition varied quite a lot according the number of components (61 in samples from 'habitat-1', 45 in sample from 'habitat-2'), the dominant components (pulegone 33.36 %, limonene 20.95 %, *cis-beta*-ocimene 9.96 % and isomenthone 5.31 % from 'habitat-1', limonene 27.98 %, *cis-beta*-ocimene 14.42 %, pulegone 14.20 %, *beta*-myrcene 11.95 %, and terpinolene 6.33 % from 'habitat-2'). In light of the continued overharvesting resulting in genetic erosion, further studies are warranted to establish in-situ and ex-situ conservation strategies of all populations representing different chemotypes/ecotypes and identifying promising genetic lines for sustainable cultivation as a source of renewable raw material for the oil.
Keywords: *Schizonepeta multifida*, Lamiaceae, pulegone, limonene, *cis-beta*-ocimene, nutraceuticals.

Introduction

In Siberia, the Altai Mountain Region is a rich source of numerous indigenous, genetically diverse botanicals, such as *Schizonepeta multifida* (L.) Briq. (Lamiaceae), with unique biochemical compounds that have been used for centuries for human and animal health, food, flavor, beauty, and environmental applications. The genus *Schizonepeta* (Bentham) Briq. includes three aromatic species: *S. multifida* (L.) Briq., *S. annua* (Pallas) Schischkin, and *S. tenuifolia* Briq., where all are distributed across most central and southern Europe, the near East, and central and southern Asia (Ivanov, 1961; Filippova, 2003; Rabzhaeva, 2011). Though there is much to be scientifically authenticated, debated, and revealed, some investigators conveniently considered the three species within the *Nepeta* L. (Li & Hedge, 1994).

For centuries, native Siberians, in many isolated, harsh, depressive, and frozen areas, using locally harvested wild botanicals, such as *S. multifida*, for human and animal health care, food preservation, flavor, cosmetics. However, in the 21st century, native and endemic *Schizonepeta* species in Siberia (Altai Region) tend to be found growing primarily in the mountain steppes (Ivanov, 1961; Filippova, 2003; Rabzhaeva, 2011) and face unprecedented pressure from increasing consumer demand, and overexploitation from wild harvesting, thus resulting in a potential loss of genetic diversity. There has been little effort in the country to establish an effective program to study the biodiversity and genetic conservation of the unique indigenous Siberian species in general and the genus *Schizonepeta* in particular. Chemical screening studies of the genus *Schizonepeta* essential oils made it clear that some established species in this genus are interested in emerging nutraceutical, food preservation, flavor, and environmental applications. *S. multifida* and *S. tenuifolia* are cultivated in some parts of China to prepare various formulations containing *S. tenuifolia* for traditional or folk remedies such as for wound healing (Isohama, 2014), anti-inflammatory and antioxidant (Wang *et al.,* 2012), anti-allergic (Yoo *et al*., 2011), anti influenza virus (H1N1) (Wu *et* al., 2010), antitumor (Kang *et al.,* 2010), anti-osteoporosis, anti-atopic dermatitis, fungal skin diseases (Lee *et al*., 2007; Choi *et al.*, 2013), anti-immunodeficiency (Kang *et al*., 2010), anti-multiple sclerosis and paralysis (Ra *et al*., 2011; Choi *et al*., 2015).

S. multifida is a representative species, which grows wild in Siberia, the Far East, China, and Mongolia, serving as an essential historical and traditional source of human and animal wellbeing. The wild-harvested aerial part of *S. multifida* formulation is used in traditional Buryat, Chinese, Mongolian, Russian, and Siberian folk medicine for gastroenteritis, endometritis, headache, goiter, nervous system diseases, eye diseases, blood pressure, respiratory tract infections, whooping cough, antiseptic, diarrhea and stomach cancer (Sokolov, 1991). However, there has been no published report on the essential oil profiles of the *S. multifida*, endemic to different parts of the Republic of Khakassia in Siberia.

The objectives of our study were, therefore: a. to investigate the volatile oil content and compositional variations of *S. multifida* accessions collected from two ecologically distinct 'habitats' or locations of Khakassia Republic, b. to develop rational conservation strategies, and identify promising genetic lines for sustainable cultivation and processing as a renewable raw material source of locally produced oil. Our findings reported here for the first time shed some light on the complex ecological and chemotaxonomic diversity of the volatile oils of *S. multifida* from the Khakassia Republic of Siberia and reveals the availability of promising lines with unique chemical profiles warranting further studies for conservation and sustainable cultivation.

Materials and Methods

Harvesting the plant material and description of the growing 'habitats'

Fresh harvested samples of the vegetative tops of *Schizonepeta multifida* (2n=12) (Malyshev, 1997) were collected from 2 different localities during the late phase of flowering and early fruit setting in July. The voucher specimens for each source are authenticated and deposited in the Herbarium collections of P.N. Krylov in the Tomsk State University (TK), under the identification number 'TK-004650'. Subsequently, the plant samples were dried in bunches to 12 % humidity under a forced air blower before distillation. The rest was packaged, protected from humidity, heat, light, and stored in cool storage (4°C) for future reference.

The two different habitats (growing conditions) where we collected the plant samples in the Khakassia Republic, Shira District are described hereunder. Location 1 or 'habitat-1' is the Kuznetsk Alatau, sub-taiga belt, outskirts of Efremkino village (54°27'N 89°27'E), 600–900 m above sea level, characterized by forb steppe. Location 2 or 'habitat-2' is Khakass-Minusinsk intermountain valley, Iyus-Shira steppe, outskirts of Lake Itkul (54°27'N 90°02'E), 550m above sea level, which is characterized by rocky steppe. The positive mean annual temperature varies from 1600–1750°C with an annual rainfall of 500–800mm. 70 % of the annual precipitation is in the form of rain. The climate is sharply continental. The winters are very cold, with little snow in the basins. The average January temperature ranges from -19°C to -21°C (sometimes reaching over -50°C) in the basins and from -15°C to -17°C in the foothills. The total vegetation (growing) days for both ecological regions is 160 days. The soil of the collection area is mainly of chernozems (black) soil type, with patches of solonetzes, having mountain taiga and mountain-tundra-soils. The mean soil pH value varied from 8.5 to 9.25, and humus content from 8.5 to 17.5 % (Polyakova, 2016). It is essential to mention here that this study is part of a series of an international collaborative research program to identify and establish unique and promising essential oil-bearing botanicals for scientific and industrial development under Siberian growing conditions (Peshkova *et al*., 1985; Korolyuk & Tkachev, 2002; Tkachev *et al*., 2002; Letchamo *et al*., 2005; Myadelets *et al.,* 2002). However, the program was slowed down due to unexpected changes in the position of the last author and significant shortages in research funding.

Distillation conditions and essential oil isolation

Once obtained, 100g of dried aerial vegetable material (flowers, leaves, buds, and stems) from each accession of *S. multifida* were blended and then subjected to 3 h of hydro-distillation (until there was no significant increase in the volume of oil) replicated twice, based on the standard procedure described in the current European Pharmacopoeia (Council of Europe, 2008), in a modified Clevenger type apparatus (Russian version of mobile stainless still field distillation apparatus). The resulting oil was separated from water by decantation, where the soil samples were dried over anhydrous sodium sulfate to remove the traces of water for 10 min in dried pre-weighed flasks. The flasks with the oil were brought into desiccators to cool and stabilize before subsequent gravimetric (w/w) determination of the oil content, expressed in % as follows. The oil was stored under N2 in a sealed vial until required. We revealed the content of essential oil according to the formula:

$$x = \frac{m_o \times m_s \times 100}{m_s} ,$$

where x – the percentage of oil, m_o – the weight of oil (g), m_s – the weight of the sample (g).

GC-FID and GC-MS analysis of the oil

The oil samples were subjected to analytical GC-FID, and GC-MS for their compositional components. The GC-MS analyses of the oil were performed with a quadrupole MS (Hewlett-Packard MSD 5971 as a detector) coupled to an HP 5890 series II GC, equipped with a split-splitless injector, fitted with an HP-5 fused silica capillary column (30 m×0.25mm i.d, film thickness 0.25μm), 5 % biphenyl, and 95 % dimethylsiloxane copolymer under the following conditions. The inlet pressure was set at 7.81psi. The carrier gas was He, at a constant flow rate of 1 mL/min. The column temperature was programmed from 50 °C (2 min initial hold), then gradually increased to 200°C at 4 °C/min, and finally increased to 280°C (5 min hold) at 20°C/min. Injector and detector (MSD) temperatures were set at 280°C and 170°C, respectively. For GC-MS detection, an electron ionization system was used with ionization energy of 70eV. An inlet pressure was adjusted to 7.81psi. MS interface temperature was set at 170°C; MS mode: EI; detector voltage: 1094.1 V.; scan speed: 2067 Daltons/sec; interval: 0.3sec. Data handling was made through MSD ChemStation D.01.02.16 (Agilent Technologies).

The oil was diluted (1:100v/v) with acetone, where 1μL of 1 % sample solution was automatically injected in split mode (20:1), with a mass range of 30 to 650 Dalton. Identification of the oil components was conducted by combined comparison of retention times of GC, full mass spectra, and linear retention indices of the components with the parameters listed in manual (Adams, 2007; Tkachev, 2008) as well as using Wiley 275 mass spectra library and compared both with authentic compounds and with those published reports. The linear retention indices of the components were determined from GC-MS runs of the oils with the addition of a mixture of the normal hydrocarbons C8, C9, ..., C24 used as the internal standards.
The GC-FID analysis of the oil was performed on a Hewlett-Packard 5890A gas chromatograph (a flame-ionization detector - H_2, air, 270°C) fitted with a 30 m × 0.25mm id., film thickness 0.25 m, (5 % - biphenyl, 95 % - dimethylsiloxane copolymer), under the same chromatographic conditions as GC-MS described above. The GC oven temperature was programmed from 50°C (2 min hold) to 280°C (5 min hold), at 4 °C/min. Percentages of the components were calculated from the GC peak areas (TIC) without using correction factor, as the data are presented in Table 1.

Results

Essential oil content

The existence of a clear difference in the essential oil content of *S. multifida* samples obtained from two different ecological conditions, i.e. 'habitats'. The plant samples harvested from 'habitat-1' yielded a mean liquid volatile oil of 0.11 %, while samples from 'habitat-2' had 0.57 % oil content, having yellowish-green color, with a characteristic floral scent *S. multifida*. This indicates that *the S. multifida population growing in 'habitat-2' of Kuznetsk Alatau contained over 4.5 times more volatile oil than* samples collected from 'habitat 2' of the Khakass-Minusinsk depression (Table 1).

Table 1. Percentage of essential oil in *Schizonepeta multifida*'s aerial parts in different regions (Russian Federation)

Region of sample collection	Percentage of essential oil, %	Reference
Altai Mountains	0.18–1.7	Polyakova *et al.*, 2016
	0.03–1.8	Peshkova *et al.*, 1985
Khakasia Republic	0.95–1.5	Korolyuk & Tkachev, 2002
	0.58	Tkachev *et al.*, 2002
Irkutsk Oblast	0.84	Letchamo *et al.*, 2005
Novosibirsk Oblast (in cultivation)	0.7–1.3	Myadelets *et al.*, 2012

As can be seen in Table 1, both values obtained from the two habitats under our current study is relatively lower than those published results reported for other ecological conditions (Guskova, 1965; Sobolevskaya, 1972; Peshkova *et al*., 1985; Plennik *et al*., 1989; Korolyuk & Tkachev, 2002; Myadelets *et al*., 2012). Furthermore, in another experiment, we noted that *S. multifida* samples collected from the wild-growing populations from the Altai Mountains showed a higher content of oil during full flowering (1.55–1.70 %), lower during budding (1.33 %), and even less oil during the early growth stage (1.25 %) (Guskova, 1965; Sobolevskaya, 1972; Plennik *et al*., 1989)**.** The content of essential oil in *S. multifida* in Southeast Altai (the aridest region of Altai) has been demonstrated to be almost three times higher than in the northern Altai (Guskova, 1965; Plennik *et al*., 1989).

Composition of volatile components in the oil

Subsequent analyses of the oils of the accessions with GC and GC-MS revealed dramatic differences in the composition of the volatile components (Table 2).
In accessions from 'habitat-1', a total of 61 components (5 unidentified) representing 99.97 % of the oil were detected (Table 2). On the other hand, in accessions analyzed from 'habitat-2', 45 constituents (2 unidentified), representing 99.94 % of the oil, were found. The distinctively principal constituent in the 'habitat-1' oil of *S. multifida* was pulegone 33.36 % (Table 2).
Pulegone is among the most frequently used flavoring agents in food, alcoholic/nonalcoholic beverages, confectionery, chewing gums, nutraceuticals, perfumery, cosmetics, excipient in pharmaceuticals, and aromatherapy products. However, pulegone as a pure synthetic flavoring ingredient in the USA is not permitted and shall not be added to foodstuffs (Food and Drug Administration-DHHS, 2017). Hence, the relatively large concentration of pulegone found in *S. multifida* oil of 'habitat-1' in the Khakassia Republic opens a new opportunity to identify and develop genetically promising *S. multifida* cultivars as a new natural source of pulegone for food, beverage, pharmaceuticals industries. Furthermore, limonene 20.95 %, *cis-beta*-ocimene 9.96 %, and *beta*-myrcene 5.83 % were some of the visible components (Table 2) of *S. multifida* oil from 'habitat-1'. In 'habitat-2' oil, limonene 27.98 %, *cis-beta*-ocimene 14.42 %, pulegone 14.20 %, *beta*-myrcene 11.95 %, and terpinolene 6.33 % were the predominant compounds (Table 2).

Discussion

It is important to note that in our study, two dominant components (pulegone, limonene) were revealed in 'habitat-1', while three dominant factors (limonene, *cis*-β-ocimene, and pulegone) were eminent in the 'habitat-2' samples (Table 2). However, the prevailing eight components, namely limonene, pulegone, β-myrcene, *cis*-β-ocimene, terpinolene, isomenthone,

germacrene D, 1,8-cineole, are similar in the samples of both habitats (Table 2). Therefore, though some apparent variations and differences in the order of dominance and concentrations, *S. multifida*'s essential oil originating from the present study regions (habitats) can be characterized and authenticated by the above-indicated components.
It is important to note that two dominant components, i.e., pulegone and limonene (Table 2) were revealed in 'habitat-1', while in 'habitat-2' oil, four dominant factors, i.e., limonene, *cis*-β-ocimene, pulegone, and *beta*-myrcene were eminent (Table 2).

Table 2. Component composition of *Schizonepeta multifida*'s essential oil (Khakasia Republic)

R.I.	Component names	Percentage in essential oil, %	
		sample 1	sample 2
926	3-thujene	0.03	0.12
932	α-pinene	0.17	0.60
973	sabinene	0.51	2.01
975	β-pinene	0.18	0.93
979	1-octene-3-ol	0.22	–
987	3-octanone	0.20	0.19
991	β-mircene	5.83	11.95
1004	α- phellandrene	–	0.05
1005	mentha-1,5,8-triene	0.07	0.06
1024	*p*-cymene	0.06	0.09
1028	limonene	20.95	27.98
1031	1,8-cineol	2.59	3.66
1038	*cis*-β-ocimene	9.96	14.42
1048	*trans*-β-ocimene	1.53	1.93
1079	unidentified(1)	0.08	0.10
1088	terpinolene	4.24	6.33
1098	rosefuran	0.05	0.09
1099	unidentified(2)	0.06	–
1100	linalool	0.13	0.47
1101	perillene	–	0.32
1112	mentha-1,3,8-triene	0.03	–
1113	1-octen-3-yl acetate	0.12	0.13
1121	*trans-p*-mentha-2,8-dien-1-ol	0.09	–
1129	alloocimene	1.41	1.28
1135	*cis-p*-mentha-2,8-dien-1-ol	0.05	–
1136	unidentified(3)	0.08	–
1139	unidentified(4)	0.12	–
1143	(*E*)-myroxide	0.07	0.04
1145	epoxyterpinolene	0.10	–
1154	menthone	0.53	0.77
1165	isomenthone	5.31	2.35
1168	*p*-mentha-1,5-dien-8-ol	0.11	–
1177	*cis*-isopulegone	1.19	0.39
1186	*p*-cymen-8-ol	0.47	0.09
1191	α-terpineol	1.72	0.59
1194	thujenol	0.10	–
1196	dihydro-*neo*-carveol	0.05	–
1196	(*Z*)-4-decenal	0.07	–
1210	2,6-dimethylocta-3,5,7-trien-2-ol	0.08	–
1219	*trans*-carveol	0.21	–
1241	pulegone	33.36	14.20
1245	carvone	0.80	0.25
1273	isopiperitenone	0.07	–
1292	lavandulyl acetate	0.13	0.30
1294	2-undecanone	–	0.13
1338	unidentified(5)	0.38	0.33
1343	piperitenone	0.06	–
1366	neryl acetate	0.08	0.07
1378	α-copaene	0.15	0.24
1387	β-burbonene	0.60	0.80
1392	β-cubebene	0.08	0.18
1392	β-elemene	0.09	–
1422	caryophyllene	0.88	1.12
1432	β-copaene	0.10	0.06
1445	isogermacrene D	0.06	0.03
1456	humulene	0.16	0.15
1458	(*E*)-β-farnesene	–	0.06
1464	*allo*-aromadendrene	0.09	–
1484	germacrene D	2.95	4.18
1496	α-zingiberene	0.69	0.57
1510	(*E,E*)-α-farnesene	0.10	0.08
1527	δ-cadinene	0.04	0.05
1577	germacra-1(10),5-dien-4-ol	0.12	–
1586	caryophyllene oxide	0.15	0.20
1604	β-elemenone	0.06	–
	Total, %:	*99.97*	*99.94*

Comments: (1) R.I.=1079: *m/z* 83 (90%), 67 (100). (2) R.I.=1099: *m/z* 83 (90%), 67 (100). (3) R.I.=1136: *m/z* 134 (100%), 109 (60), 91 (90). (4) R.I.=1139: *m/z* 108 (50%), 94 (90), 81 (70), 79 (60), 67 (60), 43 (100). (5) R.I.=1338: *m/z* 152 (22%), 134 (21), 119 (18), 91 (16), 84 (100), 43 (21).

Though there may be some apparent variations and differences in the order of dominance and concentrations, *S. multifida*'s essential oil originating from the present geographic regions (habitats) can be characterized and authenticated by the above-indicated components.

Besides, oil samples obtained from 'habitat-1' have more diverse sets of components than the samples obtained from 'habitat-2' (Table 2, Figure 1). Hence, it is possible to suggest that the sub-taiga zone of the Kuznetsk Alatau is more favorable for the accumulation of essential oil in the aerial parts of *S. multifida* than the intermountain steppes of the Khakass-Minusinsk depression with more continental ecological conditions. In earlier studies, Plennik *et al.* (1989) reported that the aerial parts of *S. multifida* population growing in corridors of meadow steppe of Nature Reserve 'Kazanovka' in the Khakassia Republic containing essential oil with 63 components of which 51 were identified. The main components of these collections were linalool (19.5 %), β-myrcene (17.6 %), *cis*-β-ocimene (13.8 %), 1.8-cineole (11.7 %), α-terpineol (9.7 %), β-phellandrene (4.8 %), 0.50 % pulegone, where limonene was absent (Korolyuk & Tkachev, 2002; Myadelets *et al.*, 2012). Furthermore, our present results on the qualitative composition of the essential oil in *S. multifida* aerial parts are very different from the results reported for other regions of the Khakassia Republic (Askiz District, Kazanovka river) (Korolyuk & Tkachev, 2002; Myadelets *et al.*, 2012), but similar to those obtained in the analyses of samples from the Altai Mountains (Guskova, 1985). In other studies conducted on collections of *S. multifida* in Khakasia, pulegone (42–44 %) and limonene (27–35 %) dominated the oil (Myadelets *et al.*, 2012). Interestingly, the essential oil of *S. multifida* from the Khakassia Republic revealed similar dominant components (limonene, pulegone, and isomenthone) as in *S. tenuifolia* (Guskova, 1965; Malyshev, 1997; Plennik *et al.*, 1989).

Similarly, the principal constituents of the essential oils from *S. multifida* growing in the Khakassia Republic in south Siberia and a widely used Chinese medicinal species of *S. tenuifolia* in Asia are limonene, pulegone, and isomenthone. This may evidence a somewhat closer biological activity, similar formulation choices of two different species for health, flavor, and fragrances. Applications are practiced in two different geographic locations and cultures.

Furthermore, *S. multifida* of Mongolian origin has been reported to accumulate essential oil, which is dominated by (z)-β-ocimene (38.30 %), 1,8-cineol (28.11 %), and terpinolene (10.52 %) (Sanduin & Altanctetceq, 2003). In a recent study (Liu *et al.*, 2011) reported on Chinese grown *S. multifida*, the main constituents of the essential oil were menthone (40.34 %) and pulegone (26.87 %), followed by D-limonene (5.81 %) and isomenthone (5.14 %), where menthone and pulegone, followed by whole oil were suggested to be effective fumigants or insecticides against major grain storage insects in the tropics and subtropics (Chen *et al.*, 2011).

This finding confirms the traditional use of the whole S. *multifida* dried tops to prevent stored products, such as potatoes, fruits, grain from insect damage, spoilage, and extend their shelf life in many Russian small farms, and 'Datcha™' (summer recreational homes with a small area of land to raise family vegetables and fruits). The long-established traditional uses of *S. multifida* support the emerging interests that botanical pesticides may have the advantage of providing novel modes of action against insects and microbes that can reduce the risk of cross-resistance and offer new leads for the design of target-specific molecules. This indicates the versatile prospects of using *S. multifida* as an effective source of nutraceutical ingredients, shelf life extension of foods, nutraceuticals, cosmetics, and other vital applications in Siberia and other parts of the world.

Our findings reported here for the first time may help to shed light on the complex chemotaxonomic and ecological aspects of the species *Schizonepeta multifida* accessions widely distributed in the Siberian Kuznetsk Alatau sub-taiga belt 'habitat-1', and Khakass-Minusinsk depression 'habitat -2' in the Republic of Khakassia. All the variations and differences in oil yield and volatile composition that are seen under our study and elsewhere could occur due to various factors, such as genetic, eco-physiological, phenological, etc. that lends itself to a broader biodiversity study, genetic conservation, and scientific development that can lead to sustainable cultivation and broader exploitation of essential oil of *S. multifida* from Siberia.

The results obtained under our present investigation and other published reports suggest high diversity in *S. multifida* oil that makes this species increasingly interesting for conservation, genetic selection, and sustainable commercial development for multiple applications (nutraceuticals, beverage, veterinary, environmental, beauty).

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Citation:

Tkachev, A., Nekratova, N., Belousova, N., Shurupova, M., Letchamo, W. (2021). Comparative GC-MS study of *Schizonepeta multifida* essential oil from Khakassia Republic shows potentials for nutraceuticals, flavor and conservation. *Ukrainian Journal of Ecology, 11* (2), 300-305.