GC-MS and q-NMR based chemotaxonomic evaluation of two *Leonurus* species

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ABSTRACT:

Introduction - The genus *Leonurus* L. (fam: Lamiaceae) is represented in Uzbekistan by two species, *L. panzerioides* Popov. and *L. turkestanicus* V. I. Krecz. & Kuprian, which are used to treat nervous disorders and also as sedative and hypotensive agents.

Objectives – To establish the taxonomic status of *L. panzerioides* and *L. turkestanicus* based on their chemical constituents analyzed by GC-MS and q-NMR.

Materials and Methods – Quantitative ^{1}H NMR (q-NMR) was used to identify and quantify known major components in the methanol extracts of these two species. Additionally, the chemical composition of the essential oils obtained from the aerial parts of these plants were analyzed by GC-MS.

Results – The q-NMR analyses of *L. panzerioides* and *L. turkestanicus* revealed the presence 8-acetylharpagide, harpagide, leonurine and stachydrine as major components. Using the GC-MS method, overall 24 and 39 constituents were identified, respectively, from *L. panzerioides* and *L. turkestanicus* oils. The major constituents of the essential oil of *L. panzerioides* were eugenol (30.9%) and *p*-vinyl guaiacol (15.8%), whereas thymol (40.1%) and octen-3-ol (13.1%) were the principal compounds in the essential oil of *L. turkestanicus*.

Conclusion – The major components in *L. panzerioides* and *L. turkestanicus* as identified by the GC-MS and q-NMR analyses, were similar to those present in other *Leonurus* species and thus provided chemotaxonomic evidence for the placement of these species under the genus *Leonurus*.

Keywords: *Leonurus turkestanicus; Leonurus panzerioides*; Lamiaceae; chemotaxonomy; q-NMR; GC-MS, essential oil; iridoid

Introduction

The genus *Leonurus* L. (family: Lamiaceae; subfamily: Lamioideae) comprises 25 species (Huang et al., 2015). The Lamioideae are characterized by tricolpate and binucleate pollen, albuminous seeds, spatulate embryos, the presence of iridoid glycosides and lower content of essential oils (Erdtman, 1945). The alkaloid leonurin seems to be the major bioactive principle of the genus *Leonurus* (Kuchta et al., 2012). The most investigated species of the this genus, *L. cardiaca* L., contains terpenes such as monoterpenes (iridoids), diterpenes (clerodane, furanolabdane and labdane types), triterpenes (ursolic and oleanolic acids), nitrogen-containing compounds (leonurine and stachydrine), phenylpropanoids (lavandulifolioside), as well as flavonoids, phenolic acids, volatile oils, sterols and tannins (Wojtyniak et al., 2013; Shang et al., 2014).

The genus *Leonurus* is represented in Uzbekistan by *L. panzerioides* Popov. and *L. turkestanicus* V. I. Krecz. & Kuprian, which are the endemic perennial shrubs that grow in Tien Shan and Pamir-Alay mountains on stony, shallow-soiled slopes, floodplains, streamsides, among trees and shrubs (Vvedenskiy 1961; Eisenman et al., 2013). Previous phytochemical investigations of the aerial parts of *L. turkestanicus* (local name 'Arslonquyruq') identified genkwanin, 6-deoxy-8-acetylharpagide, 8-acetylharpagide and harpagide (Isaev et al., 2011). Quantitative determination carried out by neutral alumina column chromatography showed 1.5% stachydrine in *L. turkestanicus* (Pulatova, 1969). The seed oil of *L. turkestanicus* was found to contain mixtures of fatty acids, *e.g.,* eicos-11-enoic acid (Gusakova and Umarov, 1972). In Uzbekistan, a tea and an infusion of the aboveground parts of *L. turkestanicus* are used to treat nervous disorders, hypertension, hysteria, epilepsy, tachycardia, gastrointestinal, female diseases and used also as soporific, anti-inflammatory, diaphoretic and laxative remedies (Khalmatov 1964; Eisenman et al., 2013). The tincture of *L. panzerioides* is used as a sedative and a hypotensive agent in Central Asian traditional medicine (Mamadalieva et al., 2014), and studies (Eisenman et al., 2013) have shown that a tincture of the herb has a sedative effect which is twice as strong as the effect of a valerian tincture. Therefore, the objective of the current study was to define and compare *L. panzerioides* and *L. turkestanicus* aerial parts on the basis of their major alkaloids and iridoid glucosides using q-NMR, and also the components of their volatile oils by GC-MS to provide further chemotaxonomic evidence in support of their placement in the genus *Leonurus*.

Experimental

Plant material

The aerial parts (stems, leaves and flowers) of *L. panzerioides* (Accession no. 20108066) and *L. turkestanicus* (Accession no. 20101091) were collected, respectively, from Namangan and Tashkent regions of Uzbekistan, at the flowering stage during the summer. The plant species were identified morphologically and using the herbarium specimens. The authenticated voucher specimens have been maintained at the Department of Herbal Plants (Institute of the Chemistry of Plant Substances, Uzbekistan).

Extraction

The aerial parts of *L. panzerioides* and *L. turkestanicus* were air-dried away from direct sunlight at room temperature and then ground to a fine powder. Powdered plant materials (100 g) were extracted by maceration with methanol (500 mL) for 24 h. The extract was subsequently filtered to remove plant debris and then evaporated to dryness using a rotary evaporator at 40°C. The dried extracts were stored in sealed glass flasks at -20°C.

Isolation of essential oils

The air-dried aerial parts of *L. panzerioides* and *L. turkestanicus* were hydrodistilled separately for 2 h using a Clevenger-type apparatus. The oils were trapped in dichloromethane, dried over anhydrous sodium sulphate and stored at -4°C until use.

GC-MS analysis

The essential oils of both plant species were analyzed by GC-MS using a gas chromatographer HP 5890 II plus equipped with a split-splitless injector maintained at 270°C and a capillary column Restek Rxi-5Sil MS (30 m x 0.25 mm ID, stationary phase film thickness 0.25 mm, Crossbond® similar to 95% diphenyl/5% dimethyl polysiloxane). The carrier gas was helium at a flow rate of 1.0 mL/min, the oven temperature was held at 85°C for 2 min, increased to 270°C at 4°C/min and then held at 270°C for 2 min. A 1% w/v solution of the samples in *n*-hexane were prepared and 1.0 µL injected in splitting mode (50:1). The GC was interfaced, by a transfer line maintained at 280°C, with a quadrupole mass spectrometer HP 5971 MSD. MS

conditions were as follows: ionization energy 70 eV, electron impact ion source temperature 180°C, scan rate 1.6 scan/s, mass range 35–500 amu. The quantitation was done by injecting three individual runs on GC-FID and standard deviations were not exceeding 1%. Identification of the oil components was based on the comparison of their retention indices determined by reference to a homologous series of *n*-alkanes (Kovats RI) and their mass spectral fragmentation patterns with those reported in the literature (Adams, 2007) and stored in the MS library (NIST 08 and WILEY 6).

q-NMR analyses

Quantitative ^{1}H NMR (q-NMR) analyses were carried out on Unity 400 plus spectrometer (Varian, Palo Alto, USA) in DMSO-d_6. 1,2,4,5-Tetrachlorobenzene (100% purity) was used as an internal standard. For every q-NMR measurement, 15-20 mg of dried extract was weighted into NMR glass tube and added of 80-90 mg solution of 1,2,4,5-tetrachlorobenzene in DMSO-d_6 (C=2.026 %). The samples were then diluted with DMSO-d_6 and weighted. The ^{1}H NMR spectra were recorded with 256 scans at the temperature 25^{o} C. Relaxation delay and acquisition times were set at 2s and 4s, respectively. FIDs were Fourier transformed with line broadening 1.0 Hz. NMR processing for all samples included manual phase correction for each replicate and manual baseline correction over the entire spectral range. The resulting spectra referenced to the HMDSO at 0 ppm. For each sample, three replications were analyzed.

Results and Discussion

Extractions

Extraction of the aerial parts of *L. panzerioides* and *L. turkestanicus* with methanol by maceration yielded 8.45% and 1.56% extracts, respectively. The air-dried aerial parts of *L. panzerioides* and *L. turkestanicus* (each 200 g) were hydrodistilled separately for 2 h using a Clevenger-type apparatus to provide 0.2% and 0.12% of oils, respectively.

q-NMR analysis

The q-NMR method is an unique structural tool and quantitative analytical technique for identification and quantification of complex samples, such as medicinal plant extracts, naturally occurring compounds in medicinal plants (Staneva et al., 2011) and medicinal

components in tablets (Zoppi et al., 2005; Bobakulov et al., 2007). Unlike chromatography, q-NMR does not require a high purity reference standard for the accurate quantification of the target compounds of interest. However, the technique provides several advantages, such as simple method development and easy sample preparation (Holzgrabe et al., 2005). Any pure compound can be used as an internal standard, which gives separate NMR resonances from the selected proton signals of the target compounds.

A quantitative analytical method using q-NMR was developed to analyze the major compounds in the aerial parts of *L. panzerioides* and *L. turkestanicus*. It allowed rapid and simultaneous determination of 8-acetylharpagide (**1**), harpagide (**2**), stachydrine (**3**) and leonurine (**4**) in *L. turkestanicus* and compounds **1** and **3** in *L. panzerioides* (Figure 1). Iridoids **1** and **2** and alkaloids **3** and **4** were determined as the main components of *L. turkestanicus* extract. In *L. panzerioides* extract, **1** and **3** were identified and quantified as the principal components (Figure 2, Table 1).

The chemical constituents of both extracts were identified by comparison of the chemical shifts of pure target compounds and with the literature data. In the ^{1}H NMR spectrum of the *L. turkestanicus* extract, obtained in DMSO-*d*6, there were two intense singlets at δ2.96 and δ3.18 ppm, assignable to the two *N*-Me groups in compound **3** (Figure 3) (Kuchta et al., 2014). The doublets at δ5.50 (J = 1.6 Hz) and δ6.20 ppm (J = 6.4 Hz) were, respectively, typical of the protons of H-1 and H-3 of an iridoid nucleus. Comparative analysis of the chemical shifts of these atoms with the literature data indicated the presence of **2** (Manguro et al., 2011). Similar signals at δ5.82 and δ6.30 ppm could be assigned to the protons of H-1 and H-3 and thus identified the presence of compound **1** (Manguro et al., 2011). In the aromatic region of the ^{1}H NMR spectrum, the singlet at δ7.24 ppm could be assigned to the protons H-2 and H-6 of the molecule **4** (Lin et al., 2007).

In the ^{1}H NMR spectrum of *L. panzerioides* extract, compounds **1** and **3** were identified in a similar way as described above (Figures 2 and 4). For q-NMR measurements the doublets corresponding to the protons H-1 and H-3 (at δ5.82 and δ6.30 ppm, respectively) could be assigned to compound **1**, the doublets corresponding to protons H-1 and H-3 (at δ5.50 and δ6.20 ppm, respectively) identified compound **2**, the singlet for two aromatic protons (at δ7.24 ppm) was for compound **4** and the singlet assignable to *N*-Me was at δ2.96 ppm for compound **3.** As an internal standard 1,2,4,5-tetrachlorobenzene was selected because it

produces simple ^{1}H NMR spectrum consisting of a singlet at δ7.94 ppm. This signal does not overlap with signals of the target compounds that were used in this study. Quantitation was performed by calculating the relative ratio of the selected proton signals peak area of the target compounds to the known amount of the internal standard. The concentration of analyzed compounds was calculated by the following equation

$$C = \frac{C_{st} \cdot K \cdot N_{p-st} \cdot M_x \cdot 100}{N_{p-x} \cdot M_{st} \cdot C_x}$$

where ***C*** is the percentage concentration of analyzed compounds in the extract; $\boldsymbol{C_{st}}$ and $\boldsymbol{C_x}$ are the percentage concentration of internal standard and extract in the measurement solution, $\boldsymbol{N_{p\text{-}st}}$ and $\boldsymbol{N_{p\text{-}x}}$ the number of selected protons for analysis of internal standard and of the analyzed compound respectively, $\boldsymbol{M_{st}}$ and $\boldsymbol{M_x}$ molecular weights of internal and analyzed compounds respectively and ***K*** is the ratio of integral intensities of selected protons of analyzed compounds and internal standard.

The results showed that the content of analyzed extracts varied significantly. The q-NMR analysis of the *L. turkestanicus* extract revealed the presence of large amounts of **1** (17.34%), **3** (14.11%) and **2** (7.65%) (Table 1). The amount of **4** was relatively low (0.58%) in the extract. The *L. panzerioides* extract exhibited low content of **1** (0.86%) and a high concentration of **3** (18.20%).

The q-NMR technique reported here allowed rapid and simultaneous determination of 8-*O*-acetylharpagide (**1**), harpagide (**2**), stachydrine (**3**) and leonurine (**4**) without any pre-cleaning steps from the methanol extracts of *L. panzerioides* and *L. turkestanicus*. The major components could be analyzed by q-NMR within a much shorter time than various chromatographic methods.

GC-MS analysis

In this chemotaxonomical investigation of the genus *Leonurus* from Uzbekistan, the essential oil composition of *L. panzerioides* and *L. turkestanicus* was characterized and determined by GC-MS. The principal constituents of *L. turkestanicus* were found to be oxygenated monoterpenoids: thymol (40.10%), octen-3-ol (13.07%), carvacrol (5.83%) and β-caryophyllene (5.61%). Thirty nine chemical constituents were detected by GC-MS analysis of

L. turkestanicus representing 99.98% of total oil components (Table 2). Main constituents of the essential oil of *L. panzerioides* were eugenol (30.93%), *p*-vinyl guaiacol (15.77%), dihydroactinidiolide (8.95%), phenyl ethyl alcohol (6.51%), verbenone (5.83%) and *p*-cymen-8-ol (5.24%). Twenty four compounds were identified in *L. panzerioides* oil, which accounted for 99.98% of the total oil. In both investigated *Leonurus* species monoterpenes eugenol, spathulenol, caryophyllene oxide, viridiflorol and γ-eudesmol were present.

Chemotaxonomic significance

L. panzerioides and *L. turkestanicus* could be related to non-aromatic group of plants of the Lamiaceae, which are usually characterized by high quantity of iridoid glucosides. Although the compositions of the essential oils of these *Leonurus* species were published (Mockute et al., 2006; Morteza-Semnani et al., 2008; Xiong et al., 2013), very little is known about the significance of the essential oil components for chemotaxonomic purposes for the genus *Leonurus*. Reports on the compositions of the essential oils of *L. cardiaca*, *L. japonicus* (Xiong et al., 2013) and *L. masrubiastrum* (Mockute et al., 2006; Morteza-Semnani et al., 2008) are available. Mockute et al. (2006) analyzed the essential oil of wild *L. cardiaca* in Lithuania and reported that about the half of the oil consisted of sesquiterpene hydrocarbons. The oil of this plant was of the germacrene D (26.6-35.1%) chemotype, whereas the other main constituents were β-caryophyllene (5.8-9.0%) and α-humulene (6.4-9.2%). The major constituents such as *epi*-cedrol (9.7%), α-humulene (9.2%), dehydro-1,8-cineole (8.9%), germacrene D (8.9%) and spathulenol (8.8%) were identified in the oil of the Iranian *L. cardiaca* (Morteza-Semnani et al., 2006). Also, *L. cardiaca*, a fairly common weed in Canada, was found to be oil-poor and β-caryophyllene (39.8%), α-humulene (34.5%) and α-pinene (5.6%) were the major compounds (Morteza-Semnani et al., 2008). Germacrene D (24.0%) dominated in the oil of *L. masrubiastrum* grown in an experimental garden in southern Ontario, Canada (Mockute et. al. 2006). The oil of *L. japonicus* consisted mainly of sesquiterpenes and diterpenes, with phytone (19.02%), phytol (13.75%), caryophyllene oxide (11.49%) and β-caryophyllene (9.89%) being the most significant constituents (Xiong et al., 2013).

Considerable chemical differences (Table 2) among the two *Leonurus* species included in the present study could be observed clearly. The essential oil from the aerial parts of *L. turkestanicus* had oxygenated monoterpenoids (thymol and carvacrol) and sesquiterpenoids

(β-caryophyllene, α-humulene and caryophyllene oxide) as the major components and thus shared characteristics of thymol chemotype. On the other hand, the oil of *L. panzerioides* showed the presence of phenylpropanoids (eugenol and *p*-vinyl guaiacol), diterpenoids (dihydroactinidiolide), and monoterpenoids (verbenone, I-cymen-8-ol and myrtenol) suggesting an eugenol chemotype for *L. panzerioides*.

The major components in *L. panzerioides* and *L. turkestanicus* as identified by the GC-MS and q-NMR analyses, were similar to those present in other *Leonurus* species, and in the family Lamiaceae, and thus provided chemotaxonomic evidence for the placement of these species under the genus *Leonurus*.

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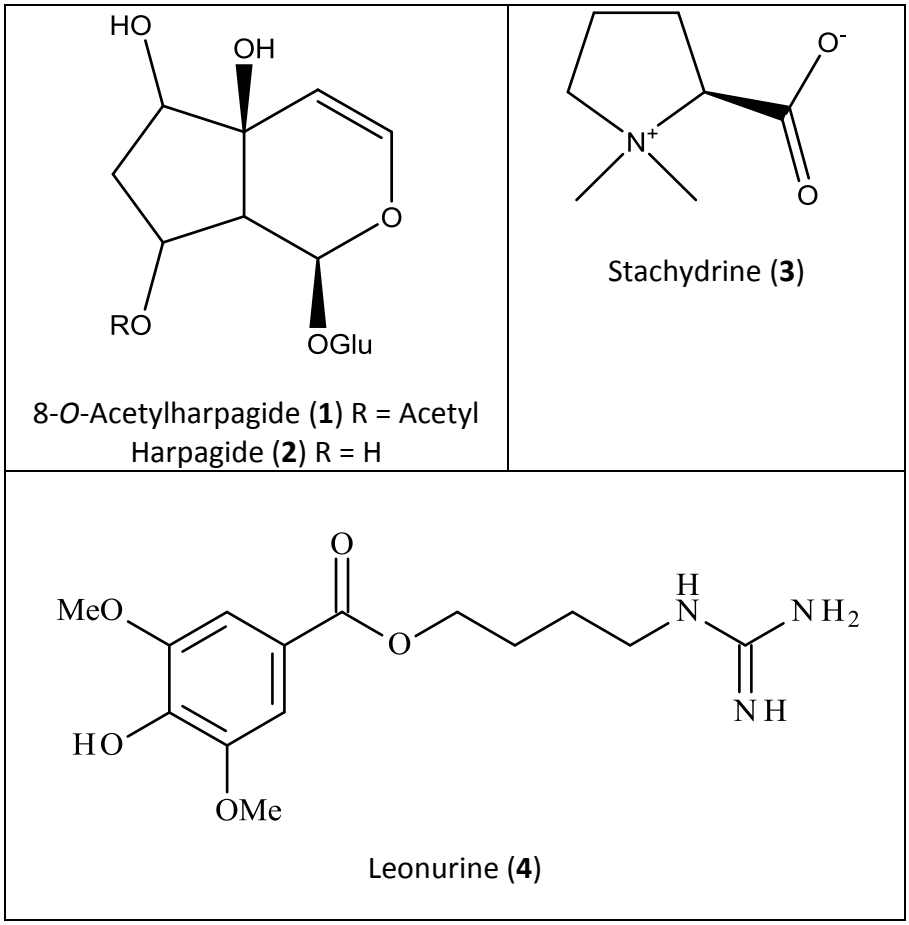

8-*O*-Acetylharpagide (**1**) R = Acetyl
Harpagide (**2**) R = H

Stachydrine (**3**)

Leonurine (**4**)

Figure 1. Structures of compounds **1-4**

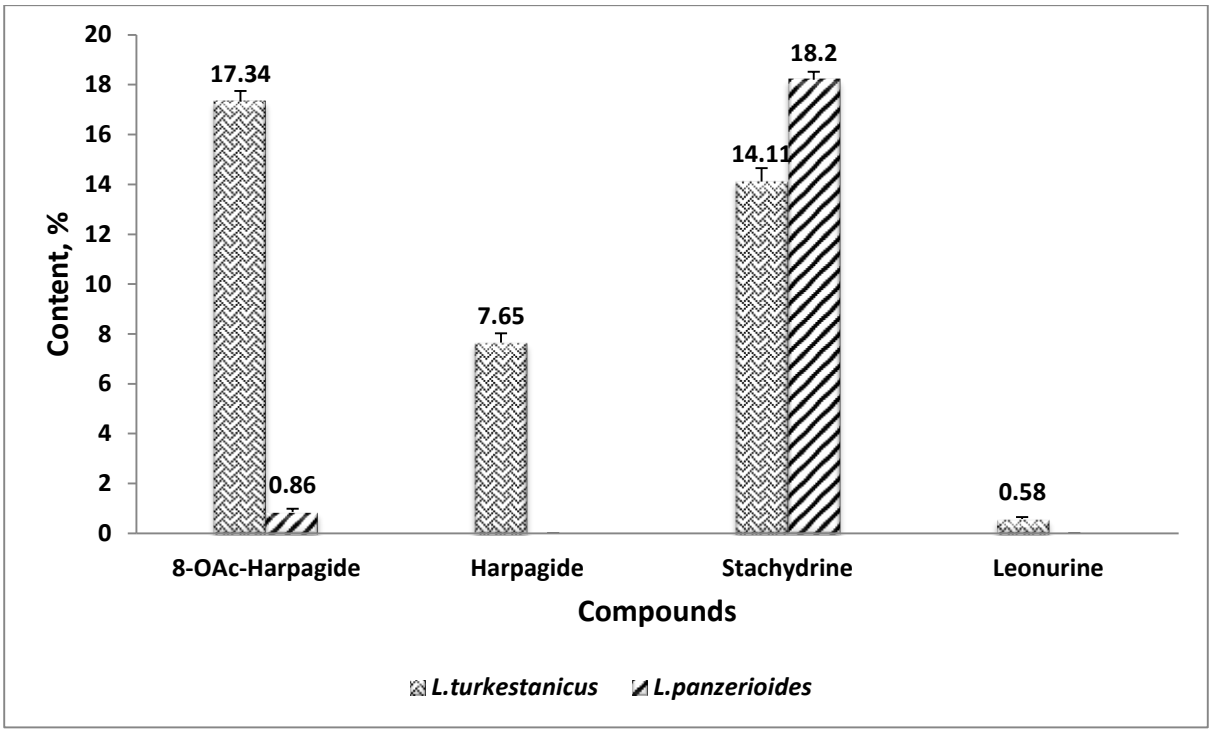

Figure 2. Quantification of compounds found in *L. turkestanicus* and *L. panzerioides* extracts as determined by q-NMR

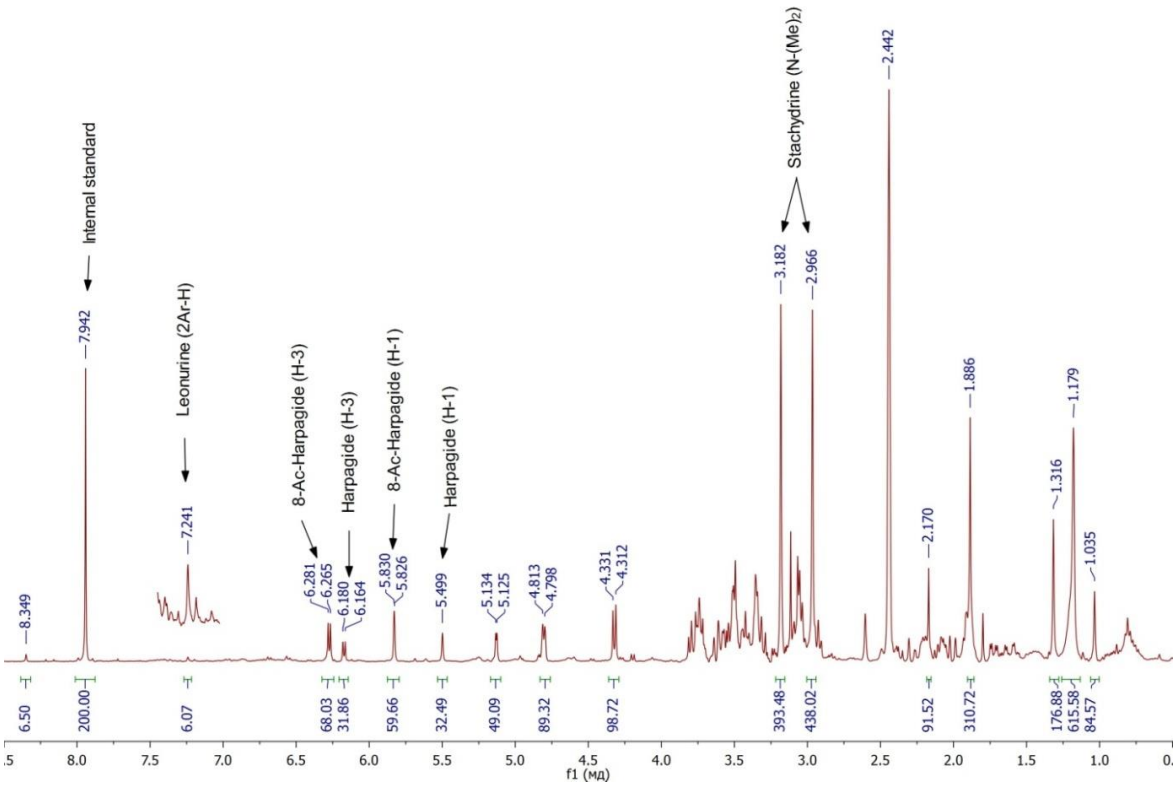

Figure 3. ^{1}H NMR spectrum of *L. turkestanicus* methanol extract in DMSO-d_6

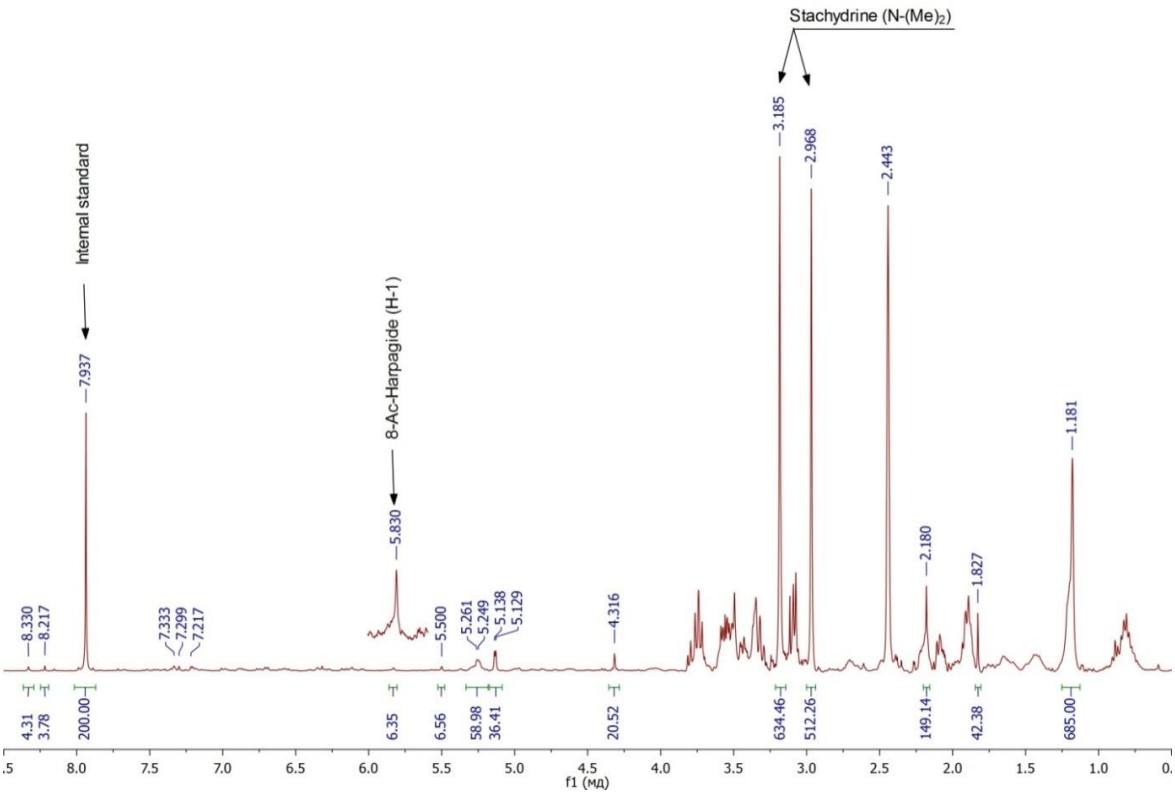

Figure 4. ^{1}H NMR spectrum of *L. panzerioides* methanol extract in DMSO-d_6

Table 1. The content of methanolic extracts of *L. turkestanicus and L. panzerioides* determined by quantitative ^{1}H NMR

Compounds	*L. turkestanicus*		*L. panzerioides*	
	In extract, %	*In plant, %*	*In extract, %*	*In plant, %*
8-*O*-Acetylharpagide (**1**)	17.34 ± 0.41	0.27	0.86 ± 0.13	0.07
Harpagide (**2**)	7.65 ± 0.38	0.12	-	-
Stachydrine (**3**)	14.11 ± 0.54	0.22	18.20 ± 0.32	1.54
Leonurine (**4**)	0.58 ± 0.07	0.01	-	-

Table 2. Chemical composition of the essential oils of *L. panzerioides* and *L. turkestanicus*

KI*	KI	Compound	RT	Composition, %		KI*	KI	Compound	RT	Composition, %	
				Lt	*Lp*					*Lt*	*Lp*
988	989	Octen-3-ol	9.83	13.07		1366	1349	Decanal dimethyl acetal	21.69	1.77	
1026	1036	1,8-Cineol	11.52	2.12		1356	1360	Eugenol	22.02	0.74	30.93
1036	1052	Benzeneacetaldehyde	12.09	0.71	2.36	1383	1384	*trans*-β-Damascenone	22.71	2.37	
1067						1374		2-(*p*-Methoxyphenyl)			
	1076	*cis*-Linalool oxide	13.04	0.80			1385	ethanol	22.75		3.46
1084	1088	*trans*-Linalool oxide	13.54	0.49		1392	1403	*cis*-Jasmone	23.28		1.99
1082	1094	*p*-Cymenene	13.66		0.20	1392	1413	*trans*-β-Damascone	23.51	0.10	
1095	1101	Linalool	14.08	3.00		1417	1424	β-Caryophyllene	23.79	5.61	
1109		Benzaldehyde dimethyl				1431					
	1108	acetal	14.28	1.64			1432	β-Gurjunene	24.02	0.32	
1114	1119	*α*-Fenchol	14.64	0.32		1453	1452	Geranyl acetone	24.53	0.82	
1106	1123	Phenyl ethyl alcohol	14.75		6.51	1452	1459	α-Humulene	24.73	2.59	
1135	1143	*trans*-Pinocarveol	15.38	0.38		1479	1479	γ-Curcumene	25.27	0.58	
1140	1153	*trans*-Verbenol	15.71		2.29	1481	1481	α-Curcumene	25.34	0.24	
1160	1165	*trans*-Pinocarvone	16.10	0.93		1487	1485	trans-β-Ionone	25.49	0.64	0.86

1165	1175	Borneol	16.42	0.29		1572	1558	Dihydroactinidiolide	27.27		8.95
1186	1183	4-Terpineol	16.71	0.37		1577	1588	Spathulenol	28.06	0.49	1.39
1190	1195	Methyl salicylate	17.14	1.10		1582	1592	Caryophyllene oxide	28.16	2.37	1.60
1196	1197	*p*-Cymen-8-ol	17.20		5.23	1592	1603	Viridiflorol	28.42	0.37	0.57
1194	1205	Myrtenol	17.44		4.54	1602	1614	Ledol	28.67		0.34
	1218	Benzaldehyde diethyl acetal	17.83	0.44		1608	1616	Humulene epoxide	28.72	0.51	
1204	1220	Verbenone	17.87		5.83	1630	1631	γ-Eudesmol	29.07	0.24	0.92
1223	1227	Methyl nonanoate	18.08	0.17		1631	1643	Isospathulenol	29.35		0.21
1226	1231	*cis*-Carveol	18.19		2.35	1648	1651	Methyl jasmonate	29.54		0.33
1235	1233	*trans*-Chrysanthenyl acetate	18.28	3.23		1652	1664	Cadinol	29.85		0.36
1249	1264	Piperitone	19.19		2.55	1685	1692	α-Bisabolol	30.54		0.44
	1278	Octanal dimethyl acetal	19.63	0.40		1845	1844	Hexahydrofarnesyl acetone	33.91	2.21	
1287	1286	Bornyl acetate	19.90	0.20		1927	1924	Methyl palmitate	35.60	0.95	
1289	1303	Thymol	20.42	40.10		1959	1973	Palmitic acid	36.61	1.47	
1298	1309	Carvacrol	20.59	5.83				**Total identified**		**99.98**	**99.98**
1309	1326	*p*-Vinyl guaiacol	21.07		15.77			**Identified compounds**		**39**	**24**

Retention indices on Rxi-%Sil MS used silica capillary column; KI* - KI reported in the literature; *Lp – L. panzerioides, Lt – L. turkestanicus*