GC/MS Based Metabolite Profiling and Antioxidant Activity of Balkan and Bulgarian Endemic Plants

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Summary

Balkan and Bulgarian endemic plants *Viola rhodopaea* Becker (Violaceae), *Veronica rhodopaea* (Velen.) Degen ex Stoj. & Stefanov (Plantaginaceae), *Silene roemeri* Friv. (Caryophyllaceae), *Jasione bulgarica* Stoj. & Stef and *Campanula lanata* Friv. (Campanulaceae) were examined. Metabolite profiles of methanolic extracts of studied species were analyzed by GC/MS and HPTLC. Total flavonoid and phenol contents were determined by aluminum chloride (AlCl3) and Folin-Ciocalteu's reagent, respectively. Antioxidant potential of the extracts was assayed by DPPH test. Fatty acids and alcohols, phenolic and organic acids, flavonoid aglycones and glycosides, sterols and carbohydrates were identified. *Jasione bulgarica* and *Viola rhodopaea* extracts were determined to have the highest flavonoid and phenol content. Significant radical scavenging activity was estimated for the extracts of *J. bulgarica*, *Veronica rhodopaea* and *Viola rhodopaea*. The present information of chemical composition and antiradical potential of studied species are reported for the first time.

Key words

Viola rhodopae, Jasione bulgarica, Campanula lanata, Veronica rhodopaea, Silene roemeri

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Introduction

Endemic plants are promising source for search of new compounds with new biological activity. Most of them have not been studied due to their limited natural resources and scarce distribution. However, with the development of new techniques it is possible to determine the composition and biological activity using small amounts of plant material.

The Bulgarian flora contains 270 plant species endemic to the Balkan Peninsula and 164 species occurring only in Bulgaria (Petrova and Vlaev, 2006; Petrova and Vladimirov, 2010).

The endemic species represent different plant families and when studying their properties, it is of particular interest to cover wider range of diversity.

The aim of present study was to determine the chemical composition of the methanolic extracts of five endemic plants by GC/MS and HPTLC and to evaluate their antioxidant activity. The five species chosen for the study belong to four families and they are presented briefly below:

Campanula lanata Friv. (Campanulaceae) is endemic to Serbia and west and central Bulgaria. This species prefers meadows, fields, rocky and shady areas at an elevation of 600–1500 metres above sea level. Besides its conservation importance, it is valued also as an ornamental plant.

Jasione bulgarica Stoj. & Stef. (Campanulaceae) is a small perennial species with leaf rosettes at the base of the stem, often forming also sterile rosettes. Its bell-like flowers are light blue, arranged densely in heads up to 2 cm in diameter. It is light demanding plant occurring on the mountain pastures and near *Pinus mugo* scrub between 1800 and 2700 m a.s.l. The natural distribution of this species is limited only to Bulgaria.

Silene roemeri Friv. (Caryophyllaceae) is a perennial dioecious plant up to 50 cm in height, with oblong-lanceolate leaves forming rosette at the base of stem and narrower, almost linear opposite leaves on the stem. Its flowers are white, arranged in dense head-like inflorescences. *S. roemeri* occurs in almost all Bulgarian mountains up to 2700 m. The natural area of distribution includes also Macedonia, Greece and Albania.

Veronica rhodopaea (Velen.) Degen ex Stoj. & Stef. (Plantaginaceae) is a small plant with beautiful dark blue flowers and small glossy opposite leaves. It occurs on high-mountain meadows in Rila, Rhodopes and Slavyanka Mts. in Bulgaria and in the mountainous areas of Albania.

Viola rhodopeia Becker (Violaceae) occurs only in the mountains of the southern part of Bulgaria. The species prefers open and wet meadows and pastures, sometimes in the periphery of *Pinus mugo* stands and in *Juniperus sibirica* plant communities within the altitudinal range 1300-2400 m a.s.l. It is found in Rila and in the western part of Rhodopes. It is a beautiful plant and is easily identifiable by its yellow flowers and very specific deeply dissected leaves with narrow lineate lobes.

Material and methods

Plant material

Aerial parts of the studied species were collected in Rila National Park, except *C. lanata*, where material from cultivated plants (also originating from Rila) was used. This species is protected according to the Biodiversity Act of Bulgaria (2002; Appendix 3). None of the other species is protected.

Extraction procedure for (GC–MS) analysis

A sample of 100 mg of dried plant material as well as internal standards of 50 μg of nonadecanoic acid, 50 μg of ribitol and 50 μg of 3,4 dichloro-4-hidroxy benzoic acid were placed in 2 mL Eppendorf tubes and extracted with 1 mL of MeOH for 24 h at room temperature. Aliquot of 800 μL was transferred into another Ependorf tube, 500 μL H_2O and 500 μL of $CHCl_3$ were added, and after vortexing for 2 min, the mixture was centrifuged. The chloroform fraction was separated, evaporated and transmethylated with 2% of H_2SO_4 in MeOH at 60°C for 18 h, then lipids were extracted with n-hexane (2x500 μL), which was dried with anhydrous Na_2SO_4 and evaporated to obtain lipid fraction. An aliquot of 100 μL from the aqueous fraction was placed in glass vial and evaporated to obtain polar fraction. The rest of aqueous fraction was hydrolyzed with 0.5 mL of 1N NaOH for 18 h at 60°C. After acidification to pH 1-2 with conc. HCl, the phenolic compounds were extracted with EtOAc (2x500 μL), which was dried with anhydrous Na_2SO_4 and evaporated to obtain phenolic fraction. Plant material remaining after methanol extraction was hydrolyzed subsequently first by 2 M NaOH for 4 h at room temperature followed by acid hydrolysis by 6 M HCl 18 h at 60°C resulting in two fractions methanol insoluble bound alkaline and acid hydrolysable phenolic acids, respectively. The obtained extractions and fractions were silylated with 50 μL of N,O-bis-(trimethylsilyl)trifluoro-acetamide (BSTFA) in 50 μL of pyridine for 2 h at 50°C (Berkov et al., 2017).

Gas Chromatography-Mass Spectroscopy (GC–MS) analysis

The methanolic extracts (50 mg) of studies species (50 mg) were silylated with 50 μL of N,O-bis-(trimethylsilyl)trifluoro-acetamide (BSTFA) in 50 μL of pyridine for 2 h at 50°C.

The GC–MS spectra were recorded on a Termo Scientific Focus GC coupled with Termo Scientific DSQ mass detector operating in EI mode at 70 eV. ADB-5MS column (30 m × 0.25 mm x 0.25 μm) was used. The temperature program was: 100-180°C at 15°C x min^{-1}, 180-300°C at 5°C x min^{-1} and 10 min hold at 300°C. The injector temperature was 250°C. The flow rate of carrier gas (Helium) was 0.8 mL × min^{-1}. The split ratio 1:10 1μL of the solution was injected. The metabolites were identified as TMSi derivatives by comparing their mass spectra and Kovats Indexes (RI) with on-line available plant specific database. The measured mass spectra were deconvoluted by the Automated Mass Spectral Deconvolution and Identification System (AMDIS), before comparison with the databases. RI of the compounds were recorded with standard n-hydrocarbon calibration mixture (C9-C36) (Restek, Cat no. 31614, supplied by Teknokroma, Spain) using AMDIS 3.6 software.

Preparation of methanolic extracts

Air-dried, ground plant material (1 g) was extracted with methanol by classical maceration. After evaporation of the solvent the crude extract was subjected to further analysis.

Thin layer chromatographic analysis

The methanolic extracts were examined for polar (glycosides) and apolar (aglycones) flavonoids and by co-TLC with authentic compounds.

Toluene:dioxane:acetic acid (95:25:4, v/v/v), toluene: methylethylketone:methanol (60:25:15, v/v/v) and water:acetic acid (60:40, v/v) were used for the development of the flavonoid aglycones on HPTLC Silica gel 60 F254, DC Alufolien Polyamid 11 F254 and TLC Cellulose F plates, respectively. Ethyl acetate:formic acid:acetic acid:methyl ethyl ketone:water (50:7:3:30:10 v/v/v/v/v) and water:acetic acid (75:15, v/v) were used for the development of the flavonoid glycosides on HPTLC Silica gel 60 F254 and TLC Cellulose F plates respectively. Chromatograms were viewed under UV light at 336 nm before and after spraying with "Naturstoffreagenz A" (1% solution of diphenylboric acid - ethanolamine complex in methanol).

Free radical scavenging activity

The effect of methanolic extracts on DPPH radicals was estimated according to Karabegović et al. (2011). The results were calculated by Software Prizm 3.00. All experiments were carried out in triplicate.

Determination of total flavonoid and phenol content

Total contents of flavonoids and phenols were determined byspectrophotometric methods using aluminum chloride and Folin–Ciocalteu reagent, respectively (Miliauskasa et al., 2004; Giorgi et al., 2009).

Chemicals and reagents

DPPH (2,2-Diphenyl-1-picrylhydrazyl) and Folin-Chiocalteu reagent were purchased from Sigma-Aldrich (Germany). Naturstoffreagenz A (2-aminoethyldiphenylborat) was provided by Merck (Darmstadt, Germany). All the other chemicals used including the solvents were of analytical grade. Flavonoid standards were kindly provided by Prof. E. Wollenweber (Institute of Botany, Darmstadt, Germany).

Results and Discussion

Methanolic extracts of studied species were analyzed for chemical composition by GC/MS and HPTLC. Metabolites from different groups of substances: fatty acids, carbohydrates, sterols, phenolic acids, etc. were identified by using GC/MS. The identification of apolar (aglycones) and polar (glycosides) flavonoid compounds was done by HPTLC. In the TLC screening with twenty reference flavonoid compounds nine were identified in the methanolic extracts of the studied species (Table 1). Four flavonoid aglycones were identified by a direct comparison of their TLC data (R*f* and color) with authentic samples in three TLC systems on three different sorbents: silica gel, cellulose and polyamide. Five flavonoid glycosides and chlorogenic acid were identified on two sorbents: silica gel and cellulose in comparison with authentic samples. Several spots with TLC data of flavonoids of studied extracts remain unidentified. Apigenin, luteolin and kaempferol as flavonoid aglycones and quercetin 3-O-glucoside and luteolin 7-O-glycoside as flavonoid glycosides were identified of *J. bulgarica* extract. Luteolin and luteolin 7-O-glucoside have been isolated from the *J. montana* L. (Zapesochnaya et al., 1972; Jabłonowska et al., 2017). In the methanolic extract of *C. lanata* two flavonol glycosides: quercetin3-O-glucoside and kaempferol 3-O-glucoside were identified by TLC analysis. The obtained result confirms the already observed dependence between the flavonoid content and the taxonomic classification in the subsection level within the genus *Campanula* (Dzhumyrko, 1985; Murai et al., 2014). For the species of *Campanula* s. str. subsection to which *C. lanata* belongs (Roquet et al., 2008) the accumulation of flavonols have been observed unlike the *Rapunculus* subsection, where the species synthesize flavones. Addidionaly in the present study, chlorogenic acid was detected in methanolic extract of *C. lanata*. Presence of chlorogenic acid was reported for other *Campanula* species (Janković et al., 2014). In the methanolic extract of *S. roemeri* kaempferol 3-O-glucoside, luteolin 7-O-glycoside and isovitexin derivative were detected. Presence of isovitexin was reported for other *Silene* species (Van Genderen and Hemert, 1986; Mamadalieva et al., 2014). Quercetin 3-O-rutinoside (rutin) was identified as main flavonoid component in the methanolic extract of *Viola rhodopaea*. Flavonoid glycosides: isorhamnetion 3-O-rutinoside, kaempferol 3-O-rutinoside and orientin as well as flavonoid aglycone kaempferol were detected as well. Rutin was determined as main flavonoid for the extracts of several *Viola* species (Flamini et al., 2007; Vukics et al., 2008). Chlorogenic acid was detected in the methanolic extract of *Veronica rhodopaea*. The TLC analysis showed that methanolic extract of *J. bulgarica* was the richest with flavonoid compounds, but the most of them were not identified. This observation was confirmed by the analysis of total flavonoid content in the studied extracts. The extract of *J. bulgarica* was with the highest content of total flavonoids (Table 2).

Table 1. Flavonoid compounds identified in studied species

Compounds	Methanolic extracts in studied species				
	VR	JB	CL	VeR	SR
Apigenin		•			
Luteolin		•			tr
Kaempferol	•	•			
Quercetin					
Quercetin 3-O-rutinoside (rutin)	•				
Quercetin 3-O-glucoside		tr	•		
Kaempferol 3-O-glucoside			•		•
Kaempferol 3-O-rutinoside	tr		tr		
Isorhamnetion 3-O-rutinoside	•		tr		
Luteolin 7-O-glycoside		tr			•
Isovitexin derivative					•
Orientin	•				
Chlorogenic acid			•	•	

Legend: VR *Viola rhodopaea*, JB *Jasione bulgarica*, CL *Campanula lanata*, VeR *Veronica rhodopaea*, SR *Silene roemeri*; *tr- trace*

Table 2. Free radical scavenging activity and total phenols and flavonoids in the studied species

Plant species	DPPH radical scavenging activity IC_{50} [μg/mL]	Total flavonoids [mgRE/g extract]	Total phenols [mgGAE/g extract]
Viola rhodopaea	66.51	1.23±0.11	9.11±0.28
Jasione bulgarica	42.41	1.44±0.10	11.82±0.21
Campanula lanata	259	0.24±0.02	4.30±0.14
Silene roemeri	171	0.67±0.09	6.91±0.35
Veronica rhodopaea	48.88	0.77±0.07	7.41±0.55
BHT	12.58		
Chlorogenic acid	6.86		
Rutin	5.90		

The analysis by GC/MS showed the presence of fatty acids and alcohols, phenolic and organic acids, flavonoids, sterols and carbohydrates. The identified compounds in the studied species are presented in Table 3. Fifteen fatty acids were identified in the methanolic extracts of studied species. Saturated fatty acid palmitic (C16:0) and unsaturated linoleic (C18:2) and linolenic (C18:3) were determined as the major fatty acids. Long-chain fatty acids (C20:0, C22:0, C24:0) were estimated in trace in the individual species. β-Sitosterol was found in the extract of *J. bulgarica*, *Viola rhodopaea* and *C. lanata*. The last both species contain β-amyrin also. Additionally stigmasterol and one unidentified sterol were detected in *Viola rhodopaea* extract. Phytosterols are compounds with important biological effects. There are data that *Viola odorata* contains significant amounts of these biologically active compounds (Jaber and Jasim, 2014). It is important to note that besides the salicylic acid, the presence of an acetylsalicylic acid derivative in the extract of *Viola rhodopaea* was also found. These are compounds with important multiple biological activities in animals and humans (Klessig et al., 2016). Methanolic extractable free and alkaline-hydrolysable phenolic acids were found in the studied extracts; among them the extract of *Viola rhodopaea* has the highest content of this type of phenolic acids. This is consistent with the received results of the analysis of total phenolic content in the studied extracts (Table 2). Methanolic non-extractable bound phenolic acids were better represented qualitatively and quantitatively in the studied extracts than methanolic extractable phenolic acids. The major carbohydrate components were determined as sucrose, fructose, glucose and a variety of unidentified mono- di- and trisaccharides.

For the first time antioxidant activities of methanolic extracts of studied species were evaluated. The data are present at Table 2. Extracts of *J. bulgarica*, *Veronica rhodopaea* and *Viola rhodopaea* displayed significant radical scavenging activity with values of IC_{50} below or around 50 μg/mL. For *J. bulgarica* and *Viola rhodopaea* extracts were determined to have the highest content on flavonoids and phenols.

Conclusion

The obtained results showed that the studied endemic plants, especially extracts of *J. bulgarica* and *Viola rhodopaea*, are rich in flavonoids and phenols and exhibited significant antioxidant activity. In the methanolic extract of *Viola rhodopaea* acetylsalicylic acid derivative was found and a future isolation of the compound and a study of its structure and activity are considered promising. The reported results represent new data on the chemical composition of studied species and will complete the information for the relevant genera.

Table 3. Metabolites identified in extracts of *Viola rhodopaea*, *Jasione bulgarica*, *Campanula lanata*, *Silene roemeri* and *Veronica rhodopaea* by GC/MS

Compounds	RI	Plant species**				
		VR	JB	CL	SR	VeR
Alkanes						
Tetradecane	1400	42.8	1.4	15.1		0.5
Pentadecane (branched)	1488	45.3	2.4	24.2	0.9	0.8
Hexadecane	1600	55.4	9.07	29.6	5.4	
Heptadecane	1700		4.9			3.7
Octadecane	1800	28.7	5.6	25.7	4.7	
Icosane	2000	14.8	2.6	15.1	2.7	
Docosane	2200	8.5	1.2	8.4	1.3.	
Tetracosane	2400	7.9	1	5.9	1	
Heptacosane	2700	36.0	1.6		0.8	
Fatty acids						
Dodecanoic acid C12:0	1521	7.5	2			
Tetradecanoic acid C14:0	1722	23.4	9.6	11.6	3.1	

Compounds	RI	Plant species**				
		VR	JB	CL	SR	VeR
9-cis-Hexadecenoic acid C16:1	1919	12				
Hexadecanoic acid C16:0	1924	248.7	105.9	234.5	125	44.6
Heptadecenoic acid C17:1	2007		1.5			
Octadecadienoic acid C18:2	2091	139.5	85.3	44.3	163.5	20.5
Octadecatrienoic acid C18:3	2097	159	63.4	101.1	97.1	40.9
Octadecenoic acid. C18:1 (Z)	2098	96.1			71.7	
Octadecenoic acid. C18:1 (E)	2102	9.5			5.4	
Octadecanoic acid C18:0	2124	42.7	35.8	83.2	29.5	
Eicosanoic acid C20:0	2326	3.8	3.7	3.7	1.3	
Docosanoic acid C22:0	2527	14.7				
2-Hydroxydocosanoic acid	2705		1.1			
Tetracosanoic acid C24:0	2728	3			0.9	
2-Hydroxytetracosanoic acid	2900			9.1		
Fatty alcohols						
Dodecanol	1562	815.3	188.3	791.8	223.4	
Tetradecanol	1760	6.0	4.1	11.5	4.1	
Hexadecanol	1957	7.1	14.7	7.8	22.6	
Octadec-9Z-enol	2128	26.2	19.9		21.5	
Octadecanol	2154	7.7	6.7	8.49	10.8	
Hexacosanol	2939		0.2			
Octacosanol	3135		1	1	0.6	
Sterols						
Stigmasterol	3319	5.1				
beta Sitosterol	3338	2.2	19.5	37.2		
β-Amyrin	3366	1.4		31.6		
Sterol	3385	1.9				
*Methanolic extractable phenolic compounds**						
Benzoic acid	1358					30.5
Salicylic acid	1504	39.5				
Acetylsalicylic acid derivative	1330	15.2				
trans-Cinnamic acid	1548		1.4			
4(p)-Hydroxybenzoic acid	1622	36.6	82			7.6
Gentisic acid	1766	4.2				
Vanilic acid	1770	707.3				442
cis(p)-Hydroxycinnamic acid	1784		4.9			256
Protocatechuic acid	1813	258				
Quinic acid	1850	117.4		212.2		
Syringic acid	1890	1.9	1.2			

Compounds	RI	Plant species**				
		VR	JB	CL	SR	VeR
trans(p)-Hydroxycinnamic acid	1933	4.7	68.2			
trans-Ferulic acid	2086		22.7			
trans-Caffeic acid	2130		2.2			17.8
Kaempferol	3060	1.2	9.5			
Apigenin	3126		5.9			
trans-Chlorgenic acid	3110			127.7		
Methanolic non-extractable bound phenolic acid						
Salicylic acid	1504	77.6	14.2		7.9	27.1
4(p)-hydroxybenzoic acid	1623	76	109.4	32.1	64.23	41.2
Gentisic acid	1766	1.2	2.5			
Vanilic acid	1770	884.7		9.2	2.21	946.2
Protocatechuic acid	1813	113.6	8.2	11.4		325.6
cis(p)-Hydroxycinnamic acid	1783		34.9		11.5	
Cis-Ferulic acid	1917				91.2	109.2
trans(p)-Hydroxycinnamic acid	1934	25.6	190.5	203.2	151	52.1
Cis-Caffeic acid	1984					2.2
trans-Ferulic acid	2086	8.7	25.6		299	22
trans-Caffeic acid	2132	1.9	1.7	720	144.5	212
Organic acids						
Succinic acid	1305	30		59.7	30.4	48.8
Glyceric acid	1319	21.9	5.4		1.7	609.5
Malic acid	1474	51.9	10.3	73.5		21.8
Pyroglutamic acid	1515	75.5	18.3		4.7	70.4
Threonic acid	1550			410.6		
2-Hydroxyglutaric acid	1560					26.9
Arabinoic acid 1.4-lactone	1641	6.2	3.2			
Ribonic acid	1750			45.5		36.0
Carbohydrates						
Monosacharide	1615	7.9				19.8
Arabinose	1684	35.6	1		4.8	
Monosacharide	1768				49.5	
Fructose 1	1793	416	103.3		84.2	239.3
Fructose 2	1803	271.5	192			3711
Glucose	1889	412	9.5		1.1	419.5
Galactose	1915	27	4.3	110.1		
Monosacharide 2	1972	41.2	21.7			565
Monosacharide 3	2775			76.4		49.2
Monosacharide 4	2254					31.5

Compounds	RI	Plant species**				
		VR	JB	CL	SR	VeR
Sucrose	2629	83.8	126	345.8	37.3	219
Disacharide 1	2864	56.8				19.3
Disacharide 2	2770					162.1
Disacharide 3	2784					39.8
Trisacharide 1	3263			242.7		
Trisacharide 2	3459			130.7		
Polyols						
Glycerol	1260	37.8	0.5		11.8	113.1
Erythritol	1485	4.1			127.8	41.8
Meso-erythritol	1491	11.8	2.6			
Myo-Inositol	2082	10.5	1	353.4		

Legend: VR *Viola rhodopaea*. JB *Jasione bulgarica*. CL *Campanula lanata*. VeR *Veronica rhodopaea*. SR *Silene roemeri*
* free and alkaline hydrolysable phenolic acids
** data are expressed as percentages of peak area of standard [µg/mL]

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