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Comparative Study of Balkan *Sideritis* Species from Albania, Bulgaria and Macedonia

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MS, KS, and VB designed the study, performed the statistical analysis and wrote the first draft of the manuscript. Authors MT and SK managed the literature searches. Authors AI, IA, GS, LE and VP-T collected the plant material and determined the plant species. Authors BQ, DS, VG performed extraction of plant material and spectrophotometric analysis of total phenolics and total flavonoids authors GZ, IP, NM determined antiradical activity. Authors PD and MP performed the ORAC tests. Authors JP, KA and EB performed the chemical profiling of phenolics by HPLC-DAD-ESI-MSn analysis. Authors AT, BA, DG, MJ worked on the profiling and quantification of diterpenes. All authors read and approved the final manuscript.

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ABSTRACT

Background: *Sideritis scardica* and *S. raeseri* are very popular medicinal plants in Albania, Macedonia, and Bulgaria.
Objective: Comparison of the phenolic, flavonoid, and diterpene contents and detailed chemical profiles, as well as data on antioxidant activity, comprising plant material from different locations in the Balkan Peninsula.
Materials and Methods: Total phenolic and flavonoid contents for the studied *Sideritis* samples were determined spectrophotometrically. By HPLC-DAD-ESI-MSn analysis, 48 individual phenolic constituents were completely or tentatively identified. Chemical profiling of *Sideritis* major diterpenes and their quantification was performed by HPLC-ESI-MSD TOF analysis. A combination of two methods (ORAC and DPPH) was applied for evaluation of the antioxidant capacity of *Sideritis* extracts.
Results: The results obtained demonstrated that *Sideritis* samples (*S. scardica* and *S. raeseri*) collected from Albania, Bulgaria and Macedonia are similar in their chemical profiles (they all contain phenylethanoid glycosides, flavonoid 7-O-diglycosides and acetylated flavonoid 7-O-diglycosides, and diterpenes: main components siderol and sideridiol); and antioxidant activity. Although some differences exist, they are not essential.
Conclusion: This fact is a good basis for the formulation of range for parameters for quality control of *Sideritis* extracts and plant material. The studied cultivated plant material is similar in its chemical composition, profile and antioxidant activity to the wild growing samples.

Keywords: Sideritis; chemical profiling; phenolics; flavonoids; diterpenes.

1. INTRODUCTION

The genus *Sideritis* (Lamiaceae) comprises over 150 species distributed across the Mediterranean countries, the Balkan Peninsula and the Middle East. Among them, *Sideritis scardica* Gris. (a Balkan endemic species) and *S. raeseri* Boiss & Heldr are very popular medicinal plants in Albania, Bulgaria, Greece and Macedonia under the names Mountain tea, Ironwort, Shepard's tea. The tea prepared from these plants is widely used in Balkan folk medicine to alleviate the symptoms of common colds including fever, flu, sore throat, and bronchitis, against gastrointestinal disorders such as stomach ache, indigestion and flatulence, as well as a tonic and diuretic remedy [1-3]. Due to its popularity, there is a vast, not controlled, collection of wild growing populations, and *S. scardica* is declared as an endangered species: it is included in Red Book of Bulgaria and is protected by the National Protected Low [4]. On the other hand, this problem has initiated cultivation of *S. scardica* and *S. raeseri* in the region and studies on the chemical composition have demonstrated that cultivation does not affect significantly the chemical composition [4,5].

In the last decade, a large number of studies dealing with the chemistry and biological activity of both species have been published, including data summarized in several review articles on the genus *Sideritis* [6-8]. Many individual compounds: phenolics, terpenoids, hydrocarbons and essential oil components have been identified [3]. It has been suggested that phenolic constituents (phenylpropanoids and flavonoids) are mainly responsible for the antioxidant, antiulcer, antibacterial and anti-inflammatory activity of the extracts [9,10]; while terpene components from the essential oil and diterpenoids contribute to the antibacterial, cytotoxic, and antitumor activities [3,11]. In a recent study, it has been demonstrated that a bioactive-rich extract of the mountain tea plant (*S. scardica*) performs as well in improving cellular antioxidant status as a catechin-rich extract of *C. sinensis* [12]. Nevertheless, a

comparative study of phenolic, flavonoid, and diterpene content and detailed chemical profiles, as well as data on antioxidant activity, comprising plant material from different locations in the Balkan peninsula is yet lacking. In this article, we report the results of the direct phytochemical comparison of samples of *S. scardica* and *S. raeseri* from different locations in Albania, Bulgaria and Macedonia with regards to the polyphenolic and flavonoids total content and antioxidant activity, a detailed polyphenolic profile and diterpenes content.

2. MATERIAL AND METHODS

2.1 General

Spectrophotometric measurement: Helios Gamma UV-vis spectrophotometer. Fluorescence measurements: FLUOstar OPTIMA (BMG Labtech, Germany). HPLC-DAD-ESI-MS^n of phenolic compounds: a G1312A binary pump, a G1313A autosampler, a G1322A degasser equipped with an Agilent 1100 series diode array detector and mass detector G2445A Ion-Trap Mass Spectrometer equipped with an electrospray ionisation (ESI) system in series (Agilent Technologies, Waldbronn, Germany). LC-MS analysis of diterpenes: Agilent 1200 Series (Agilent Technologies, Waldbronn, Germany) HPLC with ESI-MSD TOF detector. HPLC quantification of diterpenes: Agilent 1200 Series (Agilent Technologies, Waldbronn, Germany) HPLC equipped with degasser, autosampler, column compartment and DAD.

2.2 Plant Material and Chemicals

Samples, upper flowering part of the plants were collected at full flowering phase without traces of soil, dust or parts of other plants. After that, samples were air-dried in place with good ventilation, away from direct sunlight. Dry material was packed in paper bags and closed. The bags were stored at room temperature, on dry and dark place. Year, month and site of collection are given in Table 1 together with the voucher specimen number.

2.3 Chemicals

The chemicals were purchased, as follows: ethanol from Alkaloid (Skopje, Macedonia), hexane, MeOH (Uvasol grade), CH_3CN (Uvasol grade), formic acid, $AlCl_3$, Folin-Chiocalteu's phenolic reagent, Na_2CO_3 were purchased from Merck; DPPH, fluoresceine (FL), Trolox, 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH) – from Sigma-Aldrich.

2.4 Extraction

1 g of the dried and powdered plant is exactly weighted in a 50 mL Erlenmeyer flask and 25 mL 70% EtOH were added and the mixture was immersed in the ultrasonic bath and extracted for 60 min at 50°C. From each sample, three parallel extractions were performed (a1 - a3). From each extract, 1 mL was transferred to a volumetric flask and the volume is made up to 25 mL at 20°C with MeOH (solution b).

2.5 Spectrophotometric Measurement of Total Flavonoids

1 mL from the solution **b** was transferred into a volumetric test tube and 5 mL MeOH were added (solution **c**). Further, 1 mL of this solution (**c**) were added to 10 mL MeOH in a volumetric flask and 0.5 mL 5% $AlCl_3$ solution was added and the volume made up to 25 mL with MeOH. The obtained solution was allowed to stay 30 min. Absorbance was measured at 425 nm (blank prepared in the same way, 1 mL of MeOH instead of plant extract or standard solution). Every sample was measured three times. The concentration of flavones and flavonols was calculated using the regression obtained for rutin, the concentration in μg/mL. Mean of the three measurements was calculated.

2.6 Spectrophotometric Measurement of Total Phenolics

0.5 mL solution **b** was pipetted to 10 mL water in a volumetric flask and 2 mL Folin-Ciocalteau's reagent were added. After 1 min, 3 mL 20% sodium carbonate solution was added, the volume was made up to 25 mL with distilled water and the solution was allowed to stay for 2 h (±3 min). Absorbance was measured at 760 nm (blank prepared in the same way, 1 mL of MeOH instead of plants or standard solution). Every sample was measured three times. The concentration of total phenolics was performed using the regression obtained for caffeic acid standard, the concentration in mg/mL. Mean of the three measurements was calculated.

2.7 Evaluation of the Antiradical Activity of the Extracts (DPPH Assay)

The free radical scavenging activity of extracts on the stable 1,1-diphenyl-2-picrylhydrazyl

(DPPH) radical of each plant extract was evaluated [13] using a dilutions series, in order to obtain a large spectrum of sample concentrations. Properly diluted plant extracts (100 µL) were mixed with 1400 µL of 80 µM freshly prepared methanol solution of DPPH. Absorbance at 517 nm was measured after 20 min of keeping the obtained mixture in the dark. The percentage of scavenging activity was calculated using the following equation:

$$SA = [(A_0-A_i)/A_0] \times 100,$$

Where A_0 is absorbance of the control and *Ai* is absorbance of the samples. EC_{50} values were estimated using a nonlinear regression algorithm. All test analyses were run in triplicate. Caffeic acid was used as a positive control.

2.8 Evaluation of the Antioxidant Activity of the Extracts: Oxygen Radical Absorbance Capacity (ORAC) Assay

One ORAC unit was assigned to the net protection area (S) provided by 1 µM Trolox in final concentration [13]. The ORAC value of the sample was calculated on the basis of a Trolox standard curve. Used solutions: 1. Buffer: Phosphate buffer – 75 mM, pH-7.4 (mixture of NaH_2P0_4 and Na_2HPO_4); 2. Solutions of fluorescein (FL)-diNa: Concentrated FL – $8.37x10^{-4}$ M: dissolve 11.25 mg FL-diNa (Mw=376.28, 70% active substance) in 25 mL phosphate buffer (75 mM, pH-7.4). Durability 4-5 months on dark, –18°C; Stock FL – $4.185 \cdot 10^{-6}$ M: 0.050 mL of the concentrated FL to 10 mL with phoshate buffer (75 mM, pH-7.4). Durability 7 days in dark place, 4-6°C; Working solution – $6.3 \cdot 10^{-8}$ M: 0.48 mL of Stock solution to 32 mL with phoshate buffer (75 mM, pH-7.4). Prepare daily. To be kept in a dark bottle during experiments. 3. AAPH – 0.5151 M: dissolve 0.717 g AAPH (Mw=271.2, purity-97%) in 5 mL phoshate buffer (75 mM, pH-7.4). Prepared prior to every experiment.

Plant extracts were initially grinded in a mechanical mill to obtain a fine powder. Then 0.5 g of the extracts were weighted and 20 mL of acetone/water mixture (50:50, *v/v*) was added. The mixture was shaken on orbital shaker for 1h at room temperature. The extracts were centrifuged at 14000 rpm for 15 min and the supernatant was ready for analysis after appropriate dilution.

2.8.1 Experimental conditions

For 200 µL well the reaction mixture contained: FL – (170 µL, $5.36 \cdot 10^{-8}$ M in final concentration), AAPH – (20 µL, 51.51 mM in final concentration), Sample – 10 µL.

2.8.2 Measurement

170 µL FL working solution and 10 µL sample were pipetted in the wells and incubated at 37°C for 20 min. The gain of the fluorimeter was adjusted and 20 µL AAPH was added. Always add the AAPH in the row with the blank and standard samples last. Adjust the gain on every run. The initial fluorescence was measured. After that the fluorescence readings were taken on every cycle. For blank sample, phosphate buffer solution was used. 3.125; 6.25; 12.5; 25 and 50 µM Trolox solutions were used for the standard curve. The final ORAC values were calculated using a regression equation between the Trolox concentration and the net area under the curve and were expressed as Trolox equivalents as micromole per liter or gram.

2.9 Chemical Profiling of *SIDERITIS* Phenolics by HPLC-DAD-ESI-MS^n Analysis

Chromatographic separations were carried out on 150 mm x 4.6 mm, 5 µm XDB-C18 Eclipse column (Agilent, USA). The mobile phase consisted of two solvents: water-formic acid (1%, *v/v*) (A) and methanol (B). The system was run with the following gradient program:

Time / min	% B (*v/v*)
3	25
7	30
15	30
25	35
30	45
50	50
55	100
60	100

The flow rate was 0.4 mL min^{-1} and the injection volume 10 µL.

Spectral data from all peaks were accumulated in range 190-400 nm and chromatograms were recorded at 290 and 300 nm for glycosides and acylated derivatives and at 330 nm for hydroxycinnamic acids and derivatives.

Nitrogen was used as nebulising gas at pressure of 65 psi and the flow was adjusted to 12 L min^{-1}. The heated capillary and the voltage were maintained at 350°C and 4 kV, respectively. The full scan mass covered the range from *m/z* 100-1200. Collision – induced fragmentation experiments were performed in the ion trap using helium as collision gas, with voltage ramping cycle from 0.3 up to 2V.

Maximum accumulation time of ion trap and the number of MS repetitions to obtain the MS average spectra were set at 300 ms and 5, respectively. MS data were acquired in the negative ionization mode.

2.10 Phenolics Identification and Quantification

The identification and peak assigmentation of all phenolic compounds was based on comparison of their retention times and mass spectral data with those of standards and published dates. Hydroxycinnamic acids were quantified using 5-caffeoylquinic acid external standard at 330 nm, phenylethanoid glycosides were quantified and expressed as verbascoside equivalent at 330 nm, hypolaetin glucosides were quantified with 4'-*O*-methylhypolaetin 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2)glucoside at 290 nm, whereas isoscutellarein glucosides were quantified and expressed as isoscutellarein 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2)glucoside equivalent at 300 nm. The stock solutions of phenolic standards were made up in 70% methanol to a concentration of 1000 µg/mL. The corresponding calibration curves were constructed with five dilutions of the stock solutions.

2.11 Chemical Profiling of *Sideritis* Diterpenes by HPLC-MS Analysis

2.11.1 Extraction of diterpenes

1 g of the dried and powdered plant (flowering tops) is exactly weighted in a 50 mL Erlenmeyer flask and extracted twice with 20 mL hexane in the ultrasonic bath at 25°C. The duration of the extraction is 2 x 30 min. The extracts obtained after filtration are concentrated to dryness under vacuum using rotary evaporator at temperature below 35°C. For each sample, three parallel extractions are performed. The hexane extracts are weighed and stored in fridge prior LC/MS and HPLC analyses. 4-10 mg of the extract is exactly weighted in a vial and dissolved in 1 mL methanol (**SS**) and stored in fridge prior analysis.

2.11.2 LC-MS analysis of diterpenes

For LC-MS analysis Agilent 1200 Series (Agilent Technologies) HPLC with ESI-MSD TOF detector was used, column: Zorbax Eclipse XDB-C18 (RR HT 50 x 4.6 mm, 1.8 µm). Mobile phase: 0.2% ammonium formiate in water (A)/ acetonitrile (B) in combination of isocratic and gradient modes: 0-0.24 min, 5% B; 0.24-14 min, 95% B, 14-20 min, 95% B, 20-21 min 95-5% B and 21-24 min, 5% B. The flow rate was 0.5 mL min^{-1} and the injection volume 2 µL. ESI mode: positive.

2.11.3 HPLC quantification of diterpenes

For HPLC quantification, Agilent 1200 Series Agilent Technologies (Waldbronn, Germany) HPLC equipped with degasser, autosampler, column compartment and DAD was applied, column: Zorbax Eclipse XDB-C18 (RR HT 50 x 4.6 mm, 1.8 µm); mobile phase A (water) and B (acetonitrile) in combination of isocratic and gradient modes: 0-0.24 min, 5% B; 0.24-14 min, 95% B, 14-20 min, 95% B, 20-21 min 95-5% B and 21-24 min, 5% B., flow rate - 0.5 mL min^{-1}, injection volume - 2 µL, UV detection at 210 nm. For quantification, siderol and sideridiol dissolved in methanol were used. These compounds were previously isolated in our laboratory. The confirmation of the structure and purity was done using NMR and LC–MS. No additional peaks in any of the chromatographic/spectroscopic techniques were found except those of the diteprenoids used for quantification. The concentrations of siderol and sideridiol in extracts were determined from the peak areas by using the equation for linear regression obtained from the calibration curves ($r > 0.9998$). The data are presented as the mean value and standard deviation of three determinations.

3. RESULTS AND DISCUSSION

3.1 Total Phenolics and Total Flavonoids

The phenolics (mainly phenolic acids, phenylethanoids and flavonoids) are considered to be responsible for some of the important biological activities of *Sideritis* “mountain tea” infusions. Total phenolics and total flavonoids were measured spectrophotometrically. For the quantification of total phenolics by the Folin-Ciocalteu method, caffeic acid was used as calibration standard, because of the structural

similarity with the phenolics in the studied plants. The results obtained are presented in Table 1.

The results are similar to those reported in the literature [14-16]. From the available data it is clear that the mean values for total phenolics and total flavonoids content in *S. scardica* and *S. raeseri* are not significantly different ($p<0.05$), although the mean values for *S. raeseri* are slightly higher. The values vary from 14.7 to 23.4 mg caffeic acid equivalents/g dry plant material (DM) for phenolics and between 6.8 and 13.9 mg rutin equivalents/g dry plant material for flavonoids. Moreover, the concentration of biologically active substances in the cultivated plants is very close to the mean values for *S. scardica* (18±3 mg/g for phenolics and 8±1 mg/g for flavonoids), confirming that cultivation has no negative influence on the quality of the plant material with regards to the content of these bioactive constituents.

3.2 Evaluation of the Antioxidant Activity of the Extract

A combination of two methods was applied for evaluation of the antioxidant capacity of *Sideritis* extracts. The oxygen radical absorbance capacity (ORAC) represents a hydrogen atom transfer-based method, since it employs a competitive reaction scheme between antioxidants and a fluorescence probe (fluorescein) for a peroxyl radical generated by 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH). On the other hand, DPPH is an electron transfer based method. In this case, antioxidant capacity in the reduction of the oxidant radical DPPH is monitored by measuring the colour changes of the radical. Both methods together give a good estimate of the antioxidant potential of the studied material, acting by different mechanisms. The results of these tests are represented in Fig. 1.

Table 1. Total phenolic and total flavonoids content of *Sideritis* samples

Plant species	Sample	Region, period of colection	Voucher speciemen	Total phenolics[a]	Total flavonoids[b]
Sideritis raeseri	SR1	Gramozi Mt (Kolonja) Albania, July 2013	SOM 13-06	19.2±0.7	6.8 ± 0.6
	SR2	Qafa e Thanasit (Llogara) Albania, Augist 2013	SOM 13-08	23.4±2.0	13.9 ± 0.7
	SR3	Ostroviza Mt (Korça) Albania, Augist 2013	SOM 13-07	18.9±0.5	9.8 ± 0.5
Sideritis scardica	SS1	Rhodopi Mt Trigrad Bulgaria, July 2013	SOM 13-03	21.9±0.2	9.2±0.2
	SS2	Pirin Mt Peak Orelek Bulgaria, July 2013	SOM 13-04	17.9±0.2	9.2±0.2
	SS3	Slavyanka Mt Peak Shabran Bulgaria, July 2013	SOM 13-05	19.2±0.3	6.9±0.3
	SS4	Shara Mt Macedonia, August 2013	SOM 13-09	14.7±0.3	8.8±0.4
	SS5	SOFIA-2 Cultivated, July 2013 Bulgaria	SOM 13-11	16.2±0.7	6.9±0.2

[a]*mg caffeic acid equivalents/g dry plant material,* [b]*mg rutin equivalents/g dry plant material*

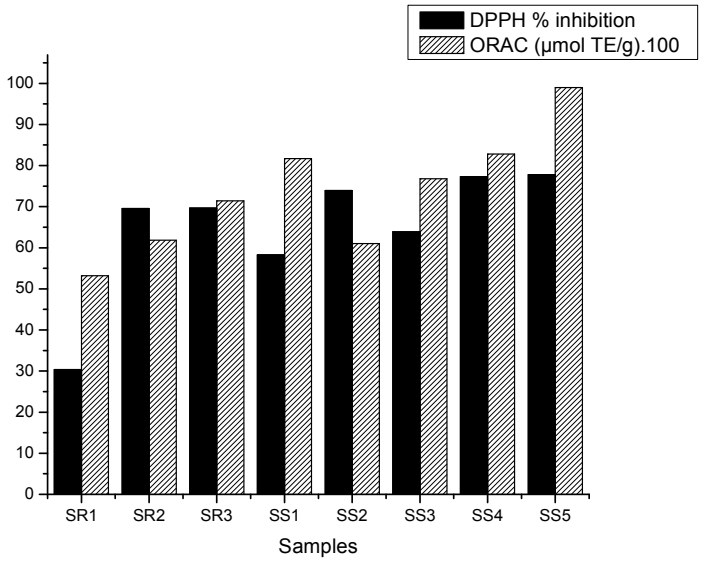


Fig. 1. Antioxidant activity of *Sideritis* samples

No clear correlation could be detected between concentration of active constituents and antioxidant activity, because the number of samples is relatively limited. This observation could also be attributed to some extent to the fact that the concentrations of individual compounds with specific antioxidant activities are varying in different samples, as can be seen further. The antioxidant activity of the cultivated *Sideritis scardica* turned out to be the highest one as evaluated by both methods.

3.3 Chemical Profiling of *Sideritis* Phenolics by HPLC-DAD-ESI-MS^n Analysis

Using HPLC-DAD-ESI-MS^n analysis, a total number of 48 individual phenolic constituents were completely or tentatively identified. The identification and peak assignment of phenolic compounds was based on comparison of their retention times, UV and MS data to those of standards and published data [5,17]. The following phenolics were identified Table 2: six hydroxycinnamic acid derivatives, 13 phenylethanoid glycosides, four flavone 7-*O*-diglucosides, one flavone rutinoside, 21 acetylated flavone 7-*O*-diglucosides, and 3 flavone *p*-coumaroyl glycosides. In individual samples, not all of these 48 compounds were present, but representatives of all above-mentioned compounds groups were detected in every sample. In addition, the presence or absence of individual compounds did not correlate with the plant species.

Typical chromatograms at 280 nm of *S. scardica* and *S. raeseri* extracts are shown in Fig. 2.

The following individual phenolics were detected in all studied samples: 5-caffeoylquinic acid, lavandulfolioside, verbascoside, forsythoside A, isoverbascoside, leucoseptoside A, 3'-*O*-methylhypolaetin 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2)glucoside, 4'-*O*-methylhypolaetin 7-*O*-[6‴-*O*-acetyl]-allosyl-(1→2)-[6″-*O*-acetyl]-glucoside. Looking at all the data, it was obvious that the profiles of both species look similar, with the same major constituent in most samples: isoscutellarein 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2)glucoside. The results suggest that the geographic location where the samples have been collected is at least as important for the phenolic qualitative profile as the species - *S. scardica* or *S. raeseri*. The presence of two types of 8-OH flavones (hypolaetin and isoscutellarein and their methoxy derivatives) and 5,7-OH flavones (apigenin and luteolin) has been confirmed. These results are in accordance with previously published data [5,17,18].

Table 2. Phenolic and flavonoid compounds identified in *Sideritis* samples by HPLC-DAD-ESI-MS^{n}.

Compound type	Individual compound
Hydroxycinnamic acids	5-caffeoylquinic acid[a]
	p-coumaric acid derivative[b] (2 tentatively identified compounds)
	p-coumaroyl 4-*O*-glucoside
	feruloylquinic acid
	feruloylquinic acid (isomer)
Phenylethanoid glycosides	echinacoside
	lavandulfolioside[a]
	verbascoside[a]
	forsythoside A
	echinacoside isomer
	samioside
	allysonoside
	jonaside A1
	allysonoside isomer
	isoverbascoside
	leucoseptoside A[a]
	allysonoside isomer
	leucoseptoside A isomer
Flavone glycosides	apigenin 7-*O*-allosyl(1→2)glucoside
	isoscutellarein 7-*O*-allosyl(1→2)glucoside
	3′-*O*-methylhypolaetin 7-*O*-allosyl(1→2)glucoside
	4'-*O*-methylisoscutellarein 7-*O*-allosyl(1→2)glucoside
	apigenin rutinoside[b]
Flavone acetyl and coumaroyl glycosides	hypolaetin 7-*O*-allosyl-(1→2)-[6″-*O*-acetyl]-glucoside
	isoscutellarein 7-*O*-allosyl-(1→2)-[6″-*O*-acetyl]-glucoside
	hypolaetin 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2)glucoside
	3'-*O*-methylhypolaetin 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2)glucoside
	luteolin 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2)glucoside
	apigenin 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2)glucoside
	luteolin 7-*O*-allosyl-(1→2)-[6″-*O*-acetyl]-glucoside
	4'-*O*-methylluteolin 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2)glucoside
	isoscutellarein 7-*O*-allosyl(1→2)-[6″-*O*-acetyl]-glucoside
	luteolin 7-*O*-[6‴-*O*-acetyl]-allosyl-(1→2)-[6″-*O*-acetyl]-glucoside
	4'-*O*-methylhypolaetin 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2)glucoside[a]
	hypolaetin 7-*O*-[6‴-*O*-acetyl]-allosyl-(1→2)-[6″-*O*-acetyl]-glucoside
	apigenin 7-(6"-*p*-coumaroylglucoside)[a]
	apigenin 7-(4"-*p*-coumaroylglucoside)[a]
	3'-*O*-methylisoscutellarein 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2)glucoside
	4'-*O*-methylisoscutellarein 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2)glucoside[a]
	isoscutellarein 7-*O*-[6‴-*O*-acetyl]-allosyl-(1→2)-[6″-*O*-acetyl]-glucoside[a]
	4'-*O*-methylhypolaetin 7-*O*-[6‴-*O*-acetyl]-allosyl-(1→2)-[6″-*O*-acetyl]-glucoside[a]
	4'-*O*-methylisoscutellarein 7-*O*-[6‴-*O*-acetyl]-allosyl-(1→2)-[6″-*O*-acetyl]-glucoside[a]
	naringenin coumaroylrutinoside[b]
	apigenin coumaroylrutinoside

[a]*the identification of these compounds is performed by comparison with standards;* [b]*tentative identification*

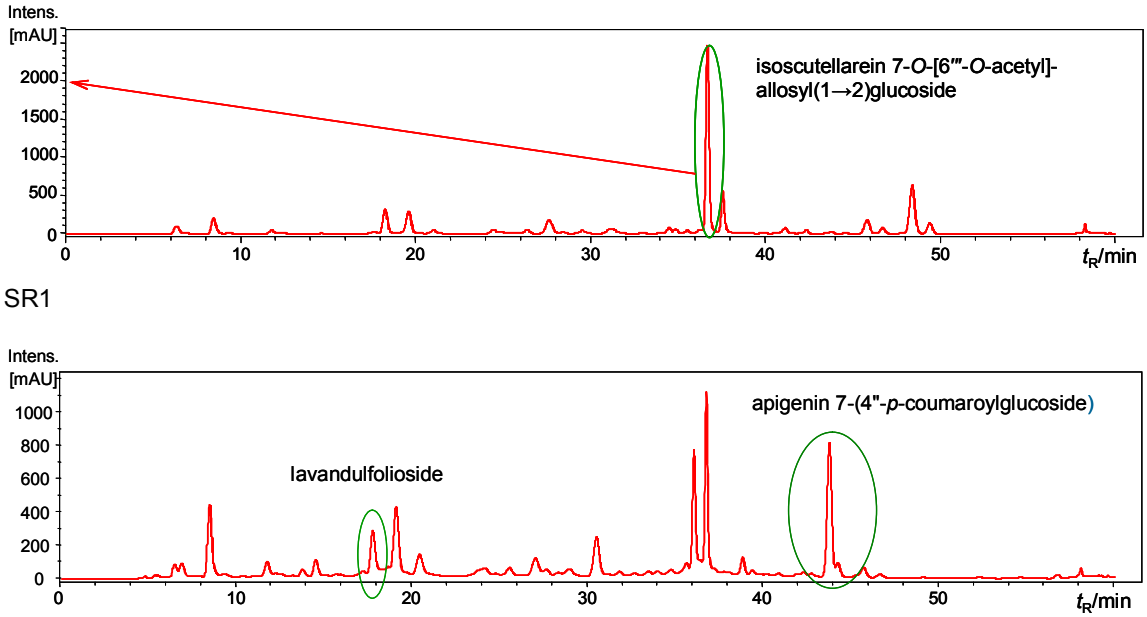


Fig. 2. Phenolic profiles of *S. raeseri* (SR1) and *S. scardica* (SS3)

The quantification of the phenolics of the studied *Sideritis* samples was performed as a sum of hydroxycinnamic acid derivatives, phenylethanoid glycosides, flavonoid 7-*O*-diglycosides and acetylated flavonoid 7-*O*-diglycosides Table 3. They were quantified using peak areas from UV-DAD chromatograms of each peak measured at the corresponding wavelength where an absorption maximum is exhibited. The total phenolic content by HPLC for the studied *Sideritis* samples ranged from 26.2 mg/g dry plant material to 60.5 mg/g dry plant material, hydroxycinnamic acid derivatives from 0.40 to 3.41 mg/g, flavone 7-*O*-diglycosides from 0.44 to 2.83 mg/g, phenylethanoid glycosides from 6.70 to 20.59 mg/g, flavone acetyl diglycosides from 15.55 to 50.45 mg/g.

The results reveal both studied *Sideritis* species as a rich source of natural polyphenols with content in the range from 26 - 60 mg/g dry plant material. The diacylated flavone glycosides constitute over 50% of the phenolic compounds in *S. scardica* and *S. raeseri.* This is an important finding, taking into consideration the recently discovered potential of acetylated glycosides of hypolaetin and isoscutellarein to act as acetylcholinesterase inhibitors and neuroprotective agents [19].

In order to see if the differences of the two studied species could be attributed to the presence and content of specific group of polyphenolic compounds, principal component analysis (PCA) was applied using the content of the above-mentioned compound groups.

The obtained two-dimensional plot Fig. 3a covered 90% of the total variation, and revealed two well-defined groups, corresponding to the two species: *S. scardica and S. raeseri.* The only sample that stayed out of the groups originated from the cultivated plant.

The major contribution to this separation, as can be seen in the projection of the variables on the factor plane Fig. 3b, can be attributed to the total sum of phenolic compounds (Var5) that mostly contributes to the PC1 and to the sum of flavone glycosides (un-acylated, Var3) that gives highest contribution in PC2.

3.4 Chemical Profiling of *Sideritis* Major Diterpenes by LC-MS Analysis

The chemical profiling of diterpenes requires a different extraction procedure, due to their low polarity compared to phenolics. The LC-MS profile of the hexane extract of a typical *Sideritis* sample is demonstrated on Fig. 4.

Table 3. Major types of phenolic compounds in *Sideritis* samples (mg/g dry weight), determined by HPLC-DAD

Compound type	SR1	SR2	SR3	SS1	SS2	SS3	SS4	SS5
Phenolic acids	2.2	3.31	2.3	1.4	0.9	1.3	1.7	2.1
Phenylethanoid	12.4	14.8	10.6	11.0	6.7	9.7	6.8	10.1
Flavone gly	1.6	0.6	1.8	1.1	0.4	1.0	1.4	2.8
Flavone acylgly	24.9	34.7	21.4	19.3	18.6	30.5	19.5	18.4
Total	**39.6**	**52.8**	**34.2**	**31.7**	**26.3**	**41.4**	**28.0**	**30.5**

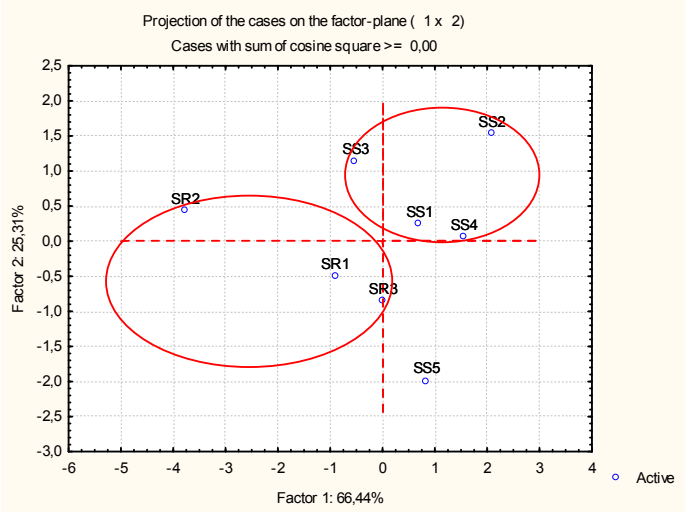


Fig. 3a. PCA of phenolic profiles of *Sideritis* samples (SS 5– cultivated *S. scardica*)

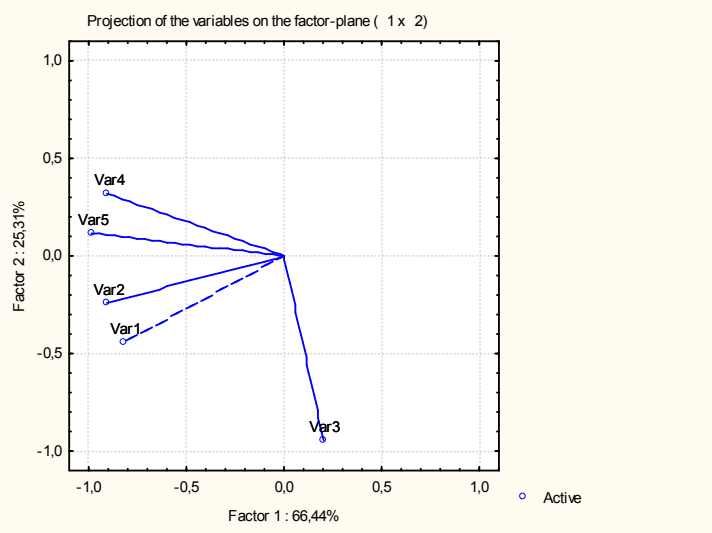


Fig. 3b. Projection of the variables on the factor plain

The qualitative composition turned out to be almost identical in all samples *S. scardica* and *S. raeseri*, the two major constituents being siderol and sideridiol Fig. 4, identified by ESI-TOF LC/MS based on retention times and mass spectra compared with authentic samples.

These two diterpenes have been earlier identified in *S. scardica* [20]. It is important to note that

siderol and sideridiol have been found to possess mild antibacterial activity [21] and siderol has demonstrated moderate anti-cholinesterase activity [22]. Quantifcation of the two major diterpenic constituents was performed, using HPLC with UV detection at 210 nm. The results obtained are represented in Table 4.

Analysis of the results demonstrated that the total content of both diterpenes is higher in *S. raeseri,* than in *S. scardica* - the min/max concentration 4.03/13.62 and 1.28/10.95 mg/g dry material in SR2/SR1 and SS3/SS5, respectively. The amounts of siderol in *S. scardica* and *S. raeseri* are not significantly different, while the levels of sideridiol differ significantly ($p<0.05$). The mean value of sideridiol concentration for *S. raeseri* was 4.31 mg/g dry material, while for *S. scardica* it was only 0.99 mg/g dry material. The most distinctive feature of the two species is the siderol/sideridiol ratio, which varied from 0.93 to 1.75 in *S. raeseri* and reached 5.90 in *S. scardica* (SS2) and 7.17 in the cultivated sample SS5. It should be noted that siderol is dominating in all investigated samples of *S. scardica* unlike those of *S. raeseri.* It is important to note also that in the cultivar, the concentration of both diterpenes was higher than the values for the wild populations of *S. scardica.*

Table 4. Content of the two major diterpenes in *Sideritis* samples

Plant species	Sample	Region	Siderol [mg/g DM]	Sideridiol [mg/g DM]	Siderol/sideridiol ratio
Sideritis raeseri	SR1	Gramozi Mt (Kolonja) Albania	6.58±0.01	7.04±0.01	0.93
	SR2	Qafa e Thanasit (Llogara) Albania	2.01±0.06	2.02±0.06	0.99
	SR3	Ostroviza Mt (Korça) Albania	6.77±0.26	3.87±0.15	1.75
Sideritis scardica	SS1	Rhodopi Mt Trigrad Bulgaria	2.64±0.07	0.71±0.02	3.77
	SS2	Pirin Mt Peak Orelek Bulgaria	7.90±0.09	1.34±0.02	5.90
	SS3	Slavyanka Mt Peak Shabran Bulgaria	0.87±0.04	0.41±0.02	2.12
	SS4	Shara Mt Macedonia	3.52±0.16	1.17±0.05	3.01
	SS5	SOFIA-2 Cultivated, Bulgaria	9.43±0.29	1.32±0.02	7.17

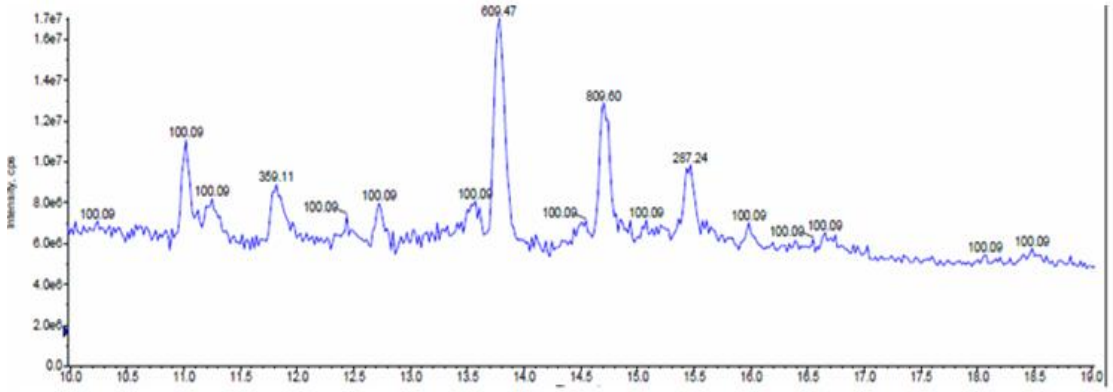
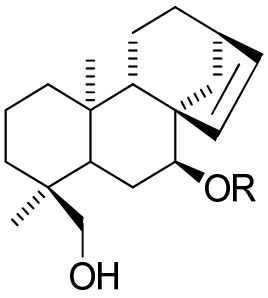


Fig. 4a. HPLC profile of the hexane extract of *Sideritis*



Siderol R = Ac
Sideridiol R = H

Fig. 4b. Major diterpenic constituents in *S. scardica* and *S. raeseri*

4. CONCLUSION

The results obtained demonstrated that *Sideritis* samples (*S. scardica* and *S. raeseri*) collected from Albania, Bulgaria and Macedonia are similar in their chemical profiles (both phenolic compounds and diterpenes); and antioxidant activity. Although some differences exist, they are not essential. This fact is a good basis for the formulation of the range of parameters for quality standard parameters for quality control of *Sideritis* extracts and plant material. The studied cultivated plant material is similar in its chemical composition, profile and antioxidant activity to the wild growing samples; indicating that cultivated *Sideritis* can be successfully used and the wild growing populations protected from vast collection and destruction.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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