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Bioactive Compounds of Endemic Species *Sideritis raeseri* subsp. *raeseri* Grown in National Park Galičica

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Abstract: Collection of *Sideritis raeseri* subsp. *raeseri* has a long tradition in local communities in the Ohrid-Prespa region. The aim of the present study was the analysis of bioactive compounds especially those with anti-inflammatory activity. Combination of the UV and MS data allowed the characterization of 17 compounds, which could be classified into flavonoid glycosides or hydroxycynnamic acid derivatives. Six of them were isolated using preparative HPLC: isoscutellarein 7-*O*-[6‴-*O*-acetyl-*β*-D-allopyranosyl-(1→2)]-*β*-D-glucopyranoside, 4′-*O*-methylhypolaetin 7-*O*-[6‴-*O*-acetyl-*β*-D-allopyranosyl-(1→2)]-*β*-D-glucopyranoside, hypolaetin 7-*O*-[6‴-*O*-acetyl-*β*-D-allopyranosyl-(1→2)]-6″-*O*-acetyl-*β*-D-glucopyranoside, 4′-*O*-methylisoscutellarein 7-*O*-[6‴-*O*-acetyl-*β*-D-allopyranosyl-(1→2)]-*β*-D-glucopyranoside, isoscutellarein 7-*O*-[6‴-*O*-acetyl-*β*-D-allopyranosyl-(1→2)]-6″-*O*-acetyl-*β*-D-glucopyranoside and 4′-*O*-methylhypolaetin 7-*O*-[6‴-*O*-acetyl-*β*-D-allopyranosyl-(1→2)]-6″-*O*-acetyl-*β*-D-glucopyranoside. The presence of phenylpropanoid glycoside martynoside was reported for the first time in the wild growing *S. raeseri* subsp. *raeseri.* Hypolaetin derivatives, known for their antiinflammatory activity, dominated and were more abundant in *S. raeseri* subsp. *raeseri* grown in NPG in comparison with *S. scardica* grown nearby the NPG. The type of solvent affected distribution and the amount of bioactive compounds and the advantage was given to less polar extracts which were richer in hypolaetin derivatives.

Keywords: *Sideritis*; polar extracts; polyphenols; LC/MS.

1. Introduction

Aerial parts of plants from *Sideritis* genus are known as a 'mountain tea' which is widely used in Mediterranean folk medicine as very popular tea [1] due to its antiinflammatory, spasmolitic, gastroprotective and antimicrobial properties [2-4].

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In previous studies flavonoids, phenolic acids, diterpenoids, fatty acids and iridoid glycosides have been reported in *Sideritis* species [5-9]. Also, the composition of the essential oil has been widely analyzed [10,11].

Significant role of *Sideritis* species in phytotherapy pointed out the need of their detail investigation, especially its chemical composition and biological activity. Also, important role in therapy and endemic status of some species of the genus *Sideritis* has imposed the need of their cultivation. According to the literature data, there are only a few reports on cultivation of *S. scardica*, hybrid *S. scardica x S. syriaca* [12,13] and *S. raeseri* subsp. *raeseri* [11].

Collection of endemic plant species *S. raeseri* subsp. *raeseri* has a long tradition in local communities in the Ohrid-Prespa region which includes Former Yugoslav Republic of Macedonia (FYROM), Albania and Greece. It is generally picked to meet personal needs but more recently, the frequent examples of collection for resale occurred. As the very wide part of that region belongs to the National park Galičica (NPG), broad investigations started to determine yield and size of *S. raeseri* subsp. *raeseri* population in NPG, quantities that could be collected to keep stabile wild population in the zone of sustainable use and also to evaluate bioactive compounds of plant material.

The aim of the present study was to investigate the effects of locality and altitude on secondary metabolites production in endemic species *S. raeseri* subsp. *raeseri* grown in NPG aiming to choose optimal locality and population for further cultivation as a way of plant protection. Special attention was pointed to compounds possessing anti-inflammatory activity. Comparison was done with species *S. scardica*, also used in traditional medicine of FYROM.

As *Sideritis* species are dominantly used as water extracts, another objective was dedicated to investigation of the effect of different solvents on the extraction of bioactive compounds leading to wider usage of *Sideritis* species in pharmaceutical and cosmetic industry.

2. Materials and Methods

2.1. Investigated Area

Investigated area was within the National Park Galičica (NPG) in the area of sustainable use. It is located in the alpine zone of pastures dominated by the massif Galičica. This zone is represented by the communities *Stipo-Festucetum* and *Juniperus communis.* The floristic composition of alpine pastures is an absolute dominance of thermal xerophile sub-Mediterranean plant species adapted to very unfavorable growing conditions. Participation of mesophile and acidophil species is quite small.

2.2. Plant Material

Aerial parts of *Sideritis raeseri* subsp. *raeseri* and *S. scardica* were collected in the phase of full flowering (July, 2010) on five different localities. *S. raeseri* subsp. *raeseri:* **S1 -** locality Mažon, NPG, maximum altitude 1571 m above sea level; **S2** - locality Sreden Vrv, NPG, maximum altitude 1667 m above sea level; **S3** - locality Visok Vrv, NPG, maximum altitude 1643 m above sea level. *S. scardica*: **S4** – Ilinska Mountain, maximum altitude 1450 m above sea level; **S5** – Ilinska Mountain, maximum altitude 1620 m above sea level. Distance between localities was minimum 3 km. Voucher specimens No. S25/10 – S29/10 have been deposited at the Institute for Medicinal Plants Research, Belgrade, Serbia.

2.3. Preparation of Extracts

Air-dried and powdered aerial parts (10 g each) were separately extracted with 150 mL 96% ethanol in Soxhlet apparatus for 24 h. The ethanol extracts were filtered and evaporated in vacuum at 40°C to yield residues (S1 – 3.1 g; S2 – 2.8 g; S3 – 3.4 g; S4 – 2.2 g; S5 – 2.6 g). Obtained extracts were used for LC/MS analysis and quantification of active compounds.

Sample S2 was used for the isolation of active compounds using column chromatography (CC) and preparative HPLC. Also, 1kg of sample S2 was extracted according to the scheme presented on Figure 1 to investigate the amount of bioactive compounds in different type of extracts. Three

different dry extracts were investigated: dry ethanolic extract (DEE – 148 g), dry water extract (DWE – 86 g) and dry ethyl acetate extract (DEAE – 31 g).

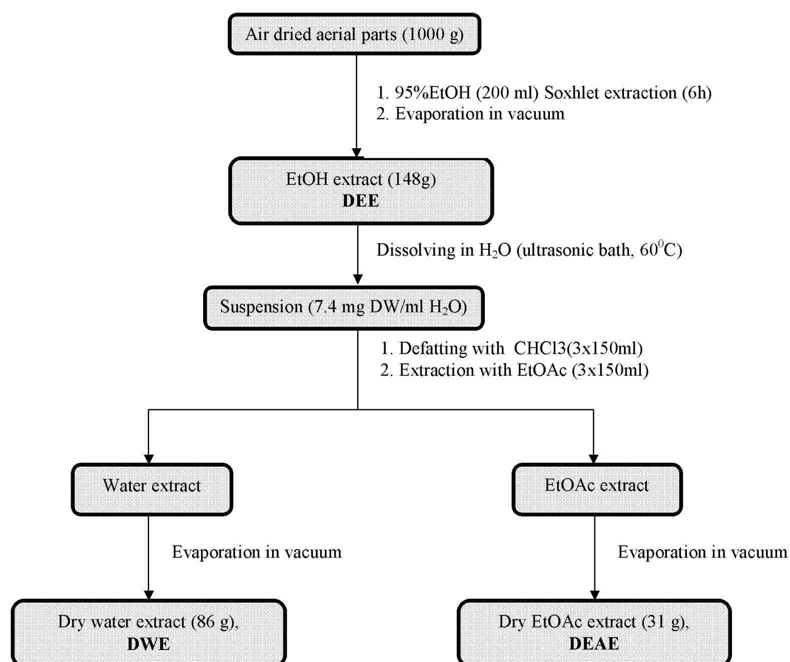

Figure 1. Extraction procedures

2.4. Total Phenolics

The total phenolic content was estimated by Folin-Ciocalteu method with slight modifications [14]. Two hundred microliters of extracts (2 mg of dry extracts/mL MeOH) were added to 1 mL of 1:10 diluted Folin-Ciocalteu reagent (FCR). After 4 min., 800 µL of sodium carbonate (75 g/L) were added. After 2 h of incubation at room temperature, the absorbance at 765 nm was measured. The results were expressed as milligrams of gallic acid equivalents per gram of dry weight of plant extract (mg GAE/g DW). Triplicate measurements were taken and data are presented as mean ± standard deviation (SD).

2.5. Total Flavonoids

The total flavonoid content was determined using aluminum chloride colorimetric method previously described [15]. Briefly, aliquots of 0.1 g of the samples were, respectively, dissolved in 1 mL deionized water. This solution (0.5 mL) was mixed with 1.5 mLof 95% alcohol, 0.1 mL of 10% aluminum chloride hexahydrate, 0.1 mL of 1 M potassium acetate, and 2.8 ml of deionized water. After incubation at room temperature for 40 min, the reaction mixture absorbance was measured at 415 nm against a deionized water blank. Apigenin 7-O-glucoside, detected in our samples, was chosen as a standard. The amount of total flavonoid contents in samples were determined in triplicate, respectively.

2.6. Isolation of Flavonoid Compounds

A part of DEE (10 g) was fractionated on silica gel CC, eluting with EtOAc/MeOH/H_2O (20/5/4), giving five main fractions (I-V). Fraction III was then rechromatographed on silica gel CC,

eluting with EtOAc/MeOH/H_2O (25/4/3) followed by Sephadex LH-20 CC, eluting with MeOH, yielding 225 mg. Further purification of fraction IV was performed on silica gel CC (EtOAc/MeOH/H2O, 25/4/3) followed by Sephadex LH-20 CC (MeOH), to yield 190 mg.

The further purification was performed by preparative HPLC on a Hewlet-Packard Series 1100 equipped with DAD model G1315B, Binary pump model G1312A, autosampler model G1313A, and Zorbax Eclipse XDB-C18 column (5 µm, 250 x 9.4 mm). Mobile phase A was 0.2% formic acid in water, and mobile phase B was acetonitrile. The injection volume was 500 µL (concentration of extract 15 mg/mL), and elution at 4 mL/min with gradient program (0-5 min 5-24% B, 5-10 min 24% B, 10-35 min 24-100 % B). UV-VIS detection was carried out at 280 and 360 nm.

Isolated compounds were identified as **6** (isoscutellarein 7-O-[6‴-O-acetyl-*β*-D-allopyranosyl-(1→2)]-*β*-D-glucopyranoside, 16 mg), **7** (4′-O-methylhypolaetin 7-O-[6‴-O-acetyl-*β*-D-allopyranosyl (1→2)]-*β*-D-glucopyranoside, 34 mg), **10** (hypolaetin 7-O-[6‴-O-acetyl-β-D-allopyranosyl-(1→2)]- 6″-O-acetyl-*β*-D-glucopyranoside, 4 mg), **12** (4′-O-methylisoscutellarein 7-O-[6‴-O-acetyl-*β*-D-allopyranosyl-(1→2)]-*β*-D-glucopyranoside, 8 mg), **13** (isoscutellarein 7-O-[6‴-O-acetyl-*β*-D-allopyranosyl-(1→2)]-6″-O-acetyl-*β*-D-glucopyranoside, 4 mg), and **14** (4′-O-methylhypolaetin 7-O-[6‴-O-acetyl-*β*-D-allopyranosyl-(1→2)]-6″-O-acetyl-*β*-D-glucopyranoside, 28 mg).

Confirmation of structure and purity were done using UV-vis, NMR and IR spectrometry. NMR spectra were recorded on a Bruker Avance 500 operating at 500.26 MHz, using a 5 mm inverse detection z-gradient probe, in DMSO-d_6 as solvent, with TMS as the internal standard. All spectral data of the six isolated compounds were deduced from ^{1}H, ^{13}C NMR, COSY, ROESY, HSQC, and HMBC, and were consistent with those as previously reported [1,11,16].

2.7. LC/MS Analysis

LC/MS analysis was performed on an Agilent MSD TOF coupled to an Agilent 1200 series HPLC, using RR Zorbax Eclipse Plus C18 column (1.8 *μ*m, 150 x 4.6 mm). Mobile phase A was 0.2% formic acid in water, and mobile phase B was acetonitrile. The injection volume was 1 *μ*L, and elution at 0.8 mL/min with gradient program (0-3 min 5-24% B, 3-6 min 24% B, 6-18 min 24-38% B, 18-24 min 38% B, 24-30 min 38-99% B, 30-33 min 99% B, 33-34 min 99-5% B). Mass spectra were acquired using an Agilent ESI-MSD TOF. Drying gas (N_2) flow was 12 L/min; nebulizer pressure was 45 psig; drying gas temperature was 350 ◦C. For ESI analysis, the parameters were: capillary voltage, 4000 V; fragmentor, 140 V; skimmer, 60 V; Oct RF V 250 V, for negative modes. The mass range was from 100 to 2000 *m*/*z*. Processing of data was done with the software Molecular Feature Extractor and Mass Profiler.

2.8. HPLC Quantification

Analyses were carried out on Agilent series 1200 RR with DAD detector, on reverse phase Lichrospher RP-18 analytical column, 250 x 4 mm i.d., particle size 5 µm (Agilent). Mobile phase A was 1% phosphoric acid in water, mobile phase B was acetonitrile, elution according to the following scheme: 90-80% A 0-5min, 80% A 5-10 min, 80-70% A 10-20 min, 70-30% A 20-30 min, 30-0% A 30-35 min. Detection at 280 and 330 nm. For quantification, two flavonoid compounds, 4′-*O*-methylhypolaetin 7-*O*-[6‴-*O*-acetyl-*β*-D-allopyranosyl-(1→2)]-*β*-D-glucopyranoside (**7**) and 4′-*O*-methylisoscutellarein 7-*O*-[6‴-*O*-acetyl-*β*-D-allopyranosyl-(1→2)]-*β*-D-glucopyranoside (**12**) were used. Data are presented as mean ± standard deviation (SD).

3. Results and Discussion

3.1. Total Phenolics and Total Flavonoids

The amount of total phenolics determined in samples of *S. raeseri* subsp. *raeseri* collected at different localities in NPG (S1 – 18.25 ± 0.25 mgGAE/g DW; S2 – 20.28 ± 0.20 mgGAE/g DW; S3 – 19.11 ± 0.13 mgGAE/g DW) are comparable to the results obtained for *S. scardica* collected on

Ilinska mountain (S4 – 18.42 ± 0.33 mgGAE/g DW; S5 – 20.07 ± 0.43 mgGAE/g DW). It could be noticed that phenolic content was similar in all collected samples ranged from 18.25 – 20.28 mg GAE/g DW. Similar results were obtained in our previous paper where we analysed samples of *S. raeseri* subsp. *raeseri* cultivated in Serbia [11] but in comparison with the other reports for *S. scardica*, *S. amasiaca* and *S. serratifolia* [17] our samples contained lesser amount of total phenolics.

The amount of total flavonoids was higher in the samples of *S. raeseri* subsp. *raeseri* (S1 – 0.20 ± 0.02%; S2 – 0.29 ± 0.02%; S3 – 0.25 ± 0.01%) in comparison with *S. scardica* (S4 – 0.17 ± 0.01%; S5 – 0.21 ± 0.01%) reaching the maximum in sample S2 (0.29%) which was collected at the locality with the highest altitude. It has been reported earlier that light intensity, temperature and altitude could influence the biosynthesis of flavonoids [18].

3.2. LC/MS Analysis

Compounds identification in extracts relied on UV spectra and reasonable molecular formulae calculated from accurate mass measurements, both obtained from LC/UV/MS analyses as well as comparison of these data with isolated compounds and metabolites previously reported from the *Sideritis* species [1,5,11]. Combination of the UV and MS data allowed the characterization of 17 compounds, which could be classified into flavonoid glycosides or hydroxycynnamic acid derivatives (Table 1). In samples S1 – S5 the most numerous flavonoid glycosides were derivatives of hypolaetin (HYP) and isoscutellarein (ISC) which were reported previously in *S. scardica* [1,16] as well as in cultivated *S. raeseri* subsp. *raeseri* [11].

Table 1. LC/UV/MS data of constituents from *S. raeseri* subsp. *raeseri* and *S. scardica.*

	tR (min)	Compound	DAD λmax (nm)	Mass	Molecular formula
1	4.7	Chlorogenic acid[1,2,4,5]	220, 240, 300sh, 326	354,0947	$C_{16}H_{18}O_9$
2	5.8	Lavandulofolioside[1,3,4]	222, 234sh, 244sh, 292sh, 302sh, 330	756,2334	$C_{34}H_{44}O_{19}$
3	6.1	Verbascoside[1,2,3,4,5]	222, 234, 244sh, 290sh, 302sh, 330	624,2048	$C_{29}H_{36}O_{15}$
4	7.5	Leucosceptoside A[1,2,3,4,5]	220, 232sh, 244sh, 290sh, 330	638,2203	$C_{30}H_{38}O_{15}$
5	7.6	Apigenin 7-O-glucoside[1,2,3,4,5]	268, 334	432,1052	$C_{21}H_{20}O_{10}$
6	8.6	AcO-All-Glc-ISC[1,2,3,4,5]	226, 276, 306, 326, 356sh	652,1646	$C_{29}H_{32}O_{17}$
7	9.5	AcO-All-Glc-HYP-Me[1,2,3,4,5]	222, 256, 276, 300sh, 340	682,1745	$C_{30}H_{34}O_{18}$
8	11.0	Martynoside[1,2,4,5]	220, 282, 308sh, 330	652,2332	$C_{31}H_{40}O_{15}$
9	11.9	AcO-All-Glc-HYP-Me isomer[1,2,3,4,5]	222, 256, 276, 300sh, 340	682,1738	$C_{30}H_{34}O_{18}$
10	12.3	$(AcO)_2$-All-Glc-HYP[1,2,3,4,5]	222, 256, 276, 300sh, 342	710,1686	$C_{31}H_{34}O_{19}$
11	13.1	$(AcO)_2$-All-Glc-HYP-Me[1,2,3,4,5]	220, 256, 276, 396sh, 340	724,1865	$C_{32}H_{36}O_{19}$
12	14.4	AcO-All-Glc-ISC-Me[1,2,3,4,5]	226, 276, 306, 326, 356sh	666,1784	$C_{30}H_{34}O_{17}$
13	14.5	$(AcO)_2$-All-Glc-ISC[1,2,3,4,5]	226, 276, 306, 326, 356sh	694,1739	$C_{31}H_{34}O_{18}$
14	15.3	$(AcO)_2$-All-Glc-HYP-Me[1,2,3,4,5]	220, 256, 276, 298sh, 340	724,1847	$C_{32}H_{36}O_{19}$
15	17.3	Echinacin isomer[1,2,3,4,5]	226, 270, 318	578,1422	$C_{30}H_{26}O_{12}$
16	17.6	Echinacin isomer[1,2,3,4,5]	226, 270, 318	578,1408	$C_{30}H_{26}O_{12}$
17	19.9	$(AcO)_2$-All-Glc-ISC-Me[1,2,3,4,5]	226, 276, 306, 326, 356sh	708,1895	$C_{32}H_{36}O_{18}$

Legend: [1] – detected in samples S1-S3; [2] – detected in samples S4 and S5; [3] – detected in DWE; [4] – detected in DEAE; [5] - DEE

In total, twelve flavonoid compounds have been detected in *S. raeseri* subsp. *raeseri*, mainly in the EtOAc and EtOH extracts. Nine of the identified compounds were *β*-D-allopyranosyl-(1→2)-*β*-D-glucopyranosyl derivatives of 5,8-dihydroxyflavones with different substitution in the ring B (ISC or HYP derivatives). Four of them were monoacetylated, and five were diacetylated in the glycosidic part. The remaining three flavonoid compounds were apigenin 7-*O*-glucoside (**5**) and its *p*-coumaroyl derivatives (**15**,**16**).

Four phenylpropanoid glycosides were detected in the extracts of *S. raeseri* subsp. *raeseri*. The most polar compound among phenylpropanoids, lavandulofolioside (**2**), was detected in DWE and DEAE extracts, whiles the less polar, martynoside (**8**) was detected in DEE and DEAE extracts. Chlorogenic acid was detected in DEE and DEAE extracts, but not in DWE extract.

In extracts of *S. scardica* same compounds, except lavandulofolioside (**2**), were detected. The presence of phenylpropanoid glycoside martynoside, exhibiting anticancer, cytotoxic and antimetastatic activities, previously reported in *S. perfoliata* [2], *S. ozturkii* [19], and *S. lycia* [20] have not been reported in wild growing *S. raeseri* subsp. *raeseri* and *S. scardica* until now.

3.3. HPLC Analysis

Two flavone glycosides 4′-*O*-methylhypolaetin 7-*O*-[6‴-*O*-acetyl-*β*-D-allopyranosyl-(1→2)]-*β*-D-glucopyranoside (**7**) and 4′-*O*-methylisoscutellarein 7-*O*-[6‴-*O*-acetyl-*β*-D-allopyranosyl-(1→2)]-*β*-D-glucopyranoside (**12**), representatives of HYP type and ISC type of compounds, which were isolated using preparative HPLC were quantified in all samples using HPLC (Table 2). Our results showed that *S. raeseri* subsp. *raeseri* was more abundant in both compounds then *S. scardica.* The highest amount of both compounds was detected in sample S2 which also contained the highest quantity of total flavonoids.

Table 2. The amounts (mg/g dw) of flavone glycosides **7** and **12** and total hypolaetin (HYP) and total isoscutellarein (ISC) derivatives in *S. raeseri* subsp. *raeseri* and *S. scardica*.

Sample	**7**	**12**	**Total HYP**	**Total ISC**
S1	3.37±0.22	1.51±0.09	7.93±0.54	5.69±0.60
S2	5.31±0.32	2.24±0.10	12.87±0.98	7.34±0.33
S3	4.11±0.25	1.40±0.05	10.52±0.45	4.73±0.28
S4	2.89±0.11	0.79±0.01	8.18±0.09	3.75±0.24
S5	3.22±0.21	1.04±0.08	8.54±0.21	4.59±0.08
DEAE	88.42±2.10	45.56±2.22	248.44±5.55	163.53±3.33
DWE	21.36±0.94	0.76±0.03	37.11±2.12	20.06±1.46

Legend: DEAE - dry ethyl acetate extract of *S. raeseri* subsp. *raeseri*; DWE - dry water extract of *S. raeseri* subsp. *raeseri*

Compound **7** and **12** were used for the quantification of total HYP and total ISC derivatives. Similarly as for single compounds, *S. raeseri* subsp. *raeseri* was richer in total HYP and ISC derivatives and among them HYP derivatives dominated (Table 2, Fig. 2). As the *Sideritis* species are widely used in some inflammation conditions and the HYP derivatives are known for their anti-inflammatory activity [21] our results gives an advantage to *S. raeseri* subsp. *raeseri* in comparison to *S. scardica.*

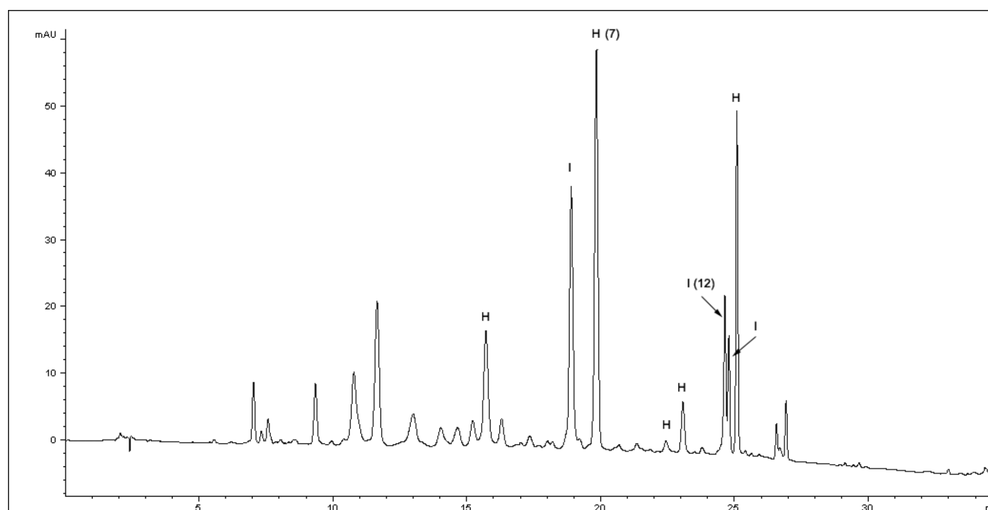

Figure 2. HPLC chromatogram of *S. raeseri* ethanol extract (H-hypolaetin derivatives; I-isoscutellarein derivatives)

We also investigated the effect of different solvents on the amount of bioactive compounds. As it was expected, the amount of both compounds (**7** and **12**) was higher in DEAE than in DWE extract. Also, DEAE contained higher amounts of total HYP and total ISC derivatives than DWE (Table 2). Although *Sideritis* species are mainly used as water extracts (teas) in traditional medicine according to our findings, greater amount of biologically active compounds were found in less polar extracts making them valuable for pharmaceutical and cosmetic industry.

Sideritis raeseri subsp. *raeseri* collected in National Park Galičica (NPG) in the zone of sustainable collection was reach in bioactive compounds, especially hypolaetin derivatives known for their anti-inflammatory activity. Samples collected at the highest altitude were the most abundant in flavonoids and in comparison with *S. scardica* average amount of flavonoids in *S. raeseri* subsp. *raeseri* was greater. The presence of phenylpropanoid glycoside martynoside, exhibiting anticancer, cytotoxic and antimetastatic activities, was reported for the first time in wild growing *S. raeseri* subsp. *raeseri* and *S. scardica.* The type of solvent affected distribution and the amount of bioactive compounds and the advantage was given to less polar extracts.

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