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THE CHEMICAL COMPOSITION OF *SEDUM RUPESTRE* L. SSP. *RUPESTRE* EPICUTICULAR WAXES: HORTICULTURAL VERSUS THE NATURAL PLANT HABITAT[†]

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Abstract. *The aim of this study was to mutually compare the chemical compositions of epicuticular waxes of two different* Sedum rupestre *ssp.* rupestre *plant material samples. These were collected during the post fructification vegetative stage from the wild-growing (NH) and cultivated populations (HH). Epicuticular waxes (isolated in the form of hexane washings of leaves and stems) were analyzed using GC-MS, GC-FID and 1D- (^{1}H, ^{13}C) and 2D-NMR analyses. The epicuticular wax of both samples consisted of only two alkanes and one triterpene: hentriacontane (2.9 and 4.7% in NH and HH samples, respectively), tritriacontane (31.8 and 41.3% in NH and HH samples, respectively) and germanicyl formate (61.1 and 50.5% in NH and HH samples, respectively). Based on the obtained results, it seems that the type of habitat (natural or horticultural) does not affect the qualitative but only the quantitative composition of* S. rupestre *ssp.* rupestre *epicuticular waxes.*

Key words: Sedum rupestre *ssp.* rupestre, *epicuticular waxes, habitat, n-alkanes, germanicyl formate.*

1. INTRODUCTION

As a hydrophobic layer consisting mostly of non-polar compounds (alkanes, alcohols, aldehydes, ketones, fatty acids, esters, and other related compounds), the epicuticular waxes cover the outer surface of aerial plant tissues providing a sort of protection and a defensive barrier against climate factors and pathogens. The level of their production may be affected by different external factors making them suitable indicators of the

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Dedicated to Professor Radosav Palić on the happy occasion of his 70th birthday.
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extent of climate changes (Marin, 2003). Furthermore, similarities/dissimilarities of the wax profiles (wax constituents are considered to be chemotaxonomic markers) of different species provide insights into plant evolution.

Sedum L. is one of the most common and widely distributed genera of the Crassulaceae family (‘T Hart, 1991; Tutin et al., 2010). By optimization of growing conditions, numerous species of Crassulaceae may be cultivated as valuable decorative or/and curative plants (Gontcharova and Gontcharov, 2009; Mori et al., 2009).

According to morphological and embryological characteristics, flavonoids and triterpenes pattern, *Sedum* series *Rupestria* Berger is distinguished among other European *Sedum* species (Thiede and Eggli, 2007). It comprises nine species and subspecies, as well as several natural and experimental hybrids (Gallo, 2012). Members of this series are characterized as glaucous plants with yellowish flowers. The glaucousness, as a noticeable feature, is the most distinct within members of series *Rupestria*, which is, among other characteristics, associated with triterpene content in epicuticular waxes (Stevens et al., 1994a). Previous studies reported germanicyl formate, taraxeryl acetate and fernenyl formate as ubiquitously present triterpenes (Stevens et al., 1994b), while *S. rupestre* L. ssp. *erectum* epicuticular waxes contained germanicyl formate as the most dominant triterpene, followed by taraxeryl acetate and taraxerone in smaller amounts (Stevens et al., 1994a).

The present study aims to compare the composition and abundances of epicuticular waxes constituents, washed from leaves of two different *S. rupestre* samples (hexane extracts). Differences are reflected in the fact that one sample was collected from its natural habitat while the other was cultivated in adopted conditions (horticultural habitat). In order to define and compare the chemical composition of sampled waxes, hexane extracts were analyzed by GC-MS, GC-FID, ^{1}H, ^{13}C NMR and 2D NMR. To the best of our knowledge, previously published papers did not include the Balkan (Serbian) *S. rupestre* L. ssp. *rupestre*.

2. EXPERIMENTAL

Plant material. This survey includes two plant samples of *Sedum rupestre* L. ssp. *rupestre*. The plant material was collected in October 2013 during the post fructification vegetative stage at two locations in Serbia: Sićevo Gorge (natural habitat, NH) and a garden pot (Niš, horticultural habitat, HH) (Table 1). According to European flora (Tutin et al., 2010) the plant material was identified and the voucher specimens were deposited in the Herbarium collection of the Faculty of Sciences and Mathematics, University of Niš (HMN).

Extraction of epicuticular waxes. Each plant material sample was cleaned (freed from dust particles, soil dirt and remains of fruiting plant parts) and measured prior to any further treatment. Plant material (leaves and stems) was soaked in hexane for 30 seconds (Jenks et al., 1995; Bojović et al., 2012; Jovanović et al., 2015); after filtration, solution was dried over anhydrous magnesium sulphate and solvent removed under *vacuum*. The yields (w/w) of dry residues are given in Table 1 (for both samples, extraction was done in triplicate). It was observed that the yield of cultivated plant wax is two times higher than the yield of native plant sample. Dry residue was measured and dissolved in hexane for further GC-MS and GC-FID analyses (concentration 10 mg mL^{-1}). Before injection each wax sample was filtrated through 0.45 μm membrane PTFE filter.

Table 1 Location of collection and waxes yields of studied samples

Sample	Location	Sample label	Wax yield±SD (%, w/w)
S. rupestre ssp. *rupestre*.	Sićevačka klisura	NH	0.4±0.01
S. rupestre ssp. *rupestre*	Niš	HH	1.0±0.02

NH – natural habitat; HH – horticulture habitat; probes were done in triplicate.

GC-MS and GC-FID analyses. Hexane extracts were recorded immediately after their isolation from the plant material. Their chemical compositions were determined by GC-FID and GC-MS analyses. All samples were analyzed (three repetitions for each sample) on a 7890/7000B GC-MS/MS triple quadrupole system (Agilent Technologies, USA, equipped with a Combi PAL auto sampler). The fused silica capillary column HP-5MS (5% phenylmethylsiloxane, 30 m x 0.25 mm, film thickness 0.25 μm) was used. The injector and interface operated at 250 and 300 °C respectively. The temperature program: 150 °C for 2.25 min isothermal, then 5 °C min^{-1} to 300 °C, and again isothermal at 300 °C for 10 min. The carrier gas was helium with a flow of 1.0 mL min^{-1}. The 5 μL of samples were injected – splitless. Post run: back flash for 1.89 min, at 280 °C, with helium at 50 psi. MS conditions were as follows: ionization voltage of 70 eV, acquisition mass range 50-650, scan time 0.32 s. The GC-FID analyses were carried out under the same experimental conditions as described for the GC-MS analysis. The percentage composition was computed from the GC-FID peak areas without the use of correction factors. Constituents were identified by comparison of their linear retention indices (relative to C8-C40 alkanes on the HP-5MS column) with literature values (Van Den Dool and Kratz, 1963; Adams, 2007; Stein 1990) and their MS with those from Wiley 6, NIST02 and Mass Finder 2.3, by the application of the AMDIS software (the Automated Mass Spectral Deconvolution and Identification System, Ver. 2.1, DTRA/NIST, 2011).

NMR analysis. All NMR spectra were recorded at 25 °C in $CDCl_3$ with TMS as an internal standard (10 mg of a compound was dissolved in 1 ml of the deuterated solvent, and 0.7 ml of the solution transferred into a 5 mm Wilmad, 528-TR-7 NMR tube). Chemical shifts are reported in ppm (δ) and referenced to TMS (δ_H = 0 ppm) in ^1H NMR spectra and to $^{13}CDCl_3$ (δ_C = 77.16 ppm) in ^{13}C spectra. The ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance III 400 MHz NMR spectrometer (^1H at 400 MHz, ^{13}C at 101 MHz), equipped with a 5-mm dual $^{13}C/^1H$ probe head. The ^1H spectra were recorded with 16 scans, 1 s relaxation delay, 4 s acquisition time, 0.125 Hz digital FID resolution, 51 280 FID size, with 6410 Hz spectral width, and an overall data point resolution of 0.0003 ppm. The ^{13}C spectra were recorded with Waltz 161H broadband decoupling, 12 000 scans, 0.5 s relaxation delay, 1 s acquisition time, 0.5 Hz digital FID resolution, 65 536 FID size, 31 850 Hz spectral width, and an overall data point resolution of 0.005 ppm.

3. RESULTS AND DISCUSSION

The GC-MS, and GC-FID analyses of both samples resulted in the identification and quantification of three components (Fig.1): two *n*-alkanes and one triterpene (hentriacontane, tritriacontane and germanicyl formate). Structural elucidation of germanicyl formate was based on the results of NMR and MS analyses and their comparison with the literature data on the same compound (Stevens et al., 1994a, 1994b).

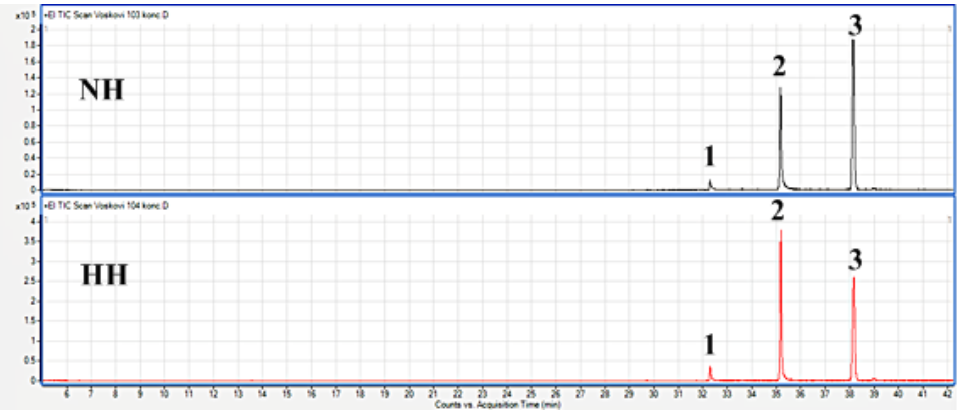

Fig. 1 TIC (total ion chromatogram) of the studied samples: NH (natural habitat) and HH (horticulture habitat). 1 – hentriacontane, 2 – tritriacontane, and 3 – germanicyl formate.

According to Stevens et al. (1994a), ^{1}H NMR spectra (recorded at 300 MHz in $CDCl_3$; δ, ppm) of germanicyl formate was as follows: δ 0.74, 0.87, 0.88, 0.92 (each 3H, s, 4 x Me), 0.94 (6H, s, 2 x Me), 1.02 and 1.09 (each 3H, s, 2 x Me), 4.62 (1H, multiplet, H-3), 4.86 (1H, s, H-19), 8.11 (1H, s, HCOO-); ^{13} C NMR (recorded at 75.4 MHz in $CDCl_3$; δ, ppm): δ 38.6 (C-1), 23.8 (C-2), 81.1 (C-3), 37.7 (C-4), 55.6 (C-5), 18.1 (C-6), 34.5 (C-7), 40.7 (C-8), 51.1(C-9), 37.1 (C-10), 21.1(C-11), 26.1 (C-12), 38.3 (C-13), 43.3 (C-14), 27.5 (C-15), 37.6 (C-16), 34.3 (C-17), 142.0 (C-18), 129.7 (C-19), 32.2 (C-20), 33.3 (C-21), 37.3 (C-22), 27.8 (C-23), 16.4 (C-24), 16.0 (C-25), 16.7 (C-26). 14.5 (C-27), 25.2 (C-28), 31.2 (C-29), 29.1 (C-30), 161.0 (HCOO-). Having these literature data at hands, we were able to additionally corroborate tentative (based on the general agreement of the mass spectra we recorded with that given in Stevens et al. (1994a)) identification of germanicyl formate despite the fact that we performed an NMR analysis (1D and 2D experiments) of the mixture of three compounds (with overlapping of the ^{1}H NMR resonances that appeared under 2 ppm). For example, the value of the NMR chemical shift of the proton from the formyl group at δ 8.16 ppm–this was directly attached to the carbon atom at δ 160.84 (HSQC experiment; direct C-H coupling)–was in excellent agreement with the results of Stevens et al. (1994a). The same was true for the multiplet at 4.68 ppm that showed HSQC correlation with the carbon atom at 81.1 ppm (these were assigned to H-3 and C-3) or singlet at 4.92 ppm that correlated with carbon at 129.81 ppm (H-19/C-19). Signal of C-18 (142.7 ppm) was also easily discernable from all other shifts observable from ^{13}C NMR spectra of the studied sample.

Germanicyl formate was the dominant component in both extracts (61.1% and 50.5%), followed by tritriacontane (31.8% and 41.3%) and hentriacontane (2.9% and 4.7%), NH and HH respectively (Table 2).The content of *n*-alkanes is higher in the sample which was cultivated in adopted conditions while the content of germanicyl formate is higher in the sample which was grown in native conditions. The studied extracts did not differ qualitatively, only quantitatively. The obtained results to some level were different from those regarding *S. rupestre* L. ssp. *erectum* 't Hart from Italy, where the total content of alkanes was 27% (among them the two most abundant ones were hentriacontane 5.6% and tritriacontane 16.9%), triterpenes 56% (among them the two most abundant were germanicyl formate 45% and taraxeryl acetate 7%), alcohols 1%, aldehydes 3% and esters 5%, Stevens et al., 1994a). *S. rupestre* ssp. *rupestre* samples collected in France, Netherlands, Sweden and Germany also contained *n*-alkanes (34-55%), triterpenes (1-31%), alkanols (1-2%), aldehydes (7-19%) and esters (9-32%). Triterpene content of the glaucous waxes ranges from 8 to 30% while glossy waxes did not contain triterpenes at all or their relative content was below 1%. Five examined glaucous populations contain germanicyl formate in the range of 2-25% while abundance of taraxeryl acetate was from 3 to 6%. (Stevens et al., 1994b). The observed differences are probably due to different experimental procedures, chloroform extract (Stevens et al., 1994b) *vs.* hexane extract (our samples) or differences in geographic/ environmental conditions or even genotypes.

Table 2 The chemical composition (%) of *S. rupestre* leaf waxes (GC-MS and GC-FID)

RI	Compound name	NH	HH
3099	Hentriacontane	2.9	4.7
3301	Tritriacontane	31.8	41.3
3458	Germanicyl formate	61.1	50.5
		95.8%	**95.5%**

RI – experimental linear retention indices relative to C8-C40 alkanes on the HP-5MS; NH – *S. rupestre* L. ssp. *rupestre* grown at natural habitat, HH – cultivated sample of *S. rupestre* ssp. *rupestre* (horticulture).

4. CONCLUSION

The compositions of two examined samples of *S. rupestre* L. ssp. *rupestre* are similar to each other as well as to the sample of *S. rupestre* ssp. *erectum* from Italy in terms of identity/content of three main components. Taking into account the results obtained through this research, it can be concluded that there are some differences reflected in the composition of epicuticular waxes of the two *S. rupestre* samples grown under different conditions; however, it should be emphasized that these differences are not qualitative, just quantitative. These observations confirm that wax production is susceptible to habitat conditions.

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HEMIJSKI SASTAV EPIKUTIKULARNOG VOSKA BILJNE VRSTE *SEDUM RUPESTRE* L. SSP. *RUPESTRE*: HORTIKULTURNO I PRIRODNO STANIŠTE

Cilj istraživanja je bio određivanje i upoređivanje hemijskog sastava epikutikularnih voskova dva uzorka vrste S.rupestre *ssp.* rupestre *u vegetativnoj fazi nakon plodonošenja sa različitih lokaliteta i različitih uslova staništa (prirodni, NH, i hortikulturni uslovi, HH). GC-MS, GC-FID i 1D- (^{1}H i ^{13}C) i 2D-NMR analize su korišćene radi identifikacije i kvantifikacije sastojaka epikutikularnih voskova izolovanih u obliku heksanskih ispiraka listova i stabla. Oba uzorka su sadržala hentriakontan (NH 2,9% i HH 3,4 %), tritriakontan (NH 31,8% i HH 41,3%) i triterpen germanicil-formijat (NH 61,1% i HH 50,5%). Upoređivanjem dobijenih rezultata opaža se da uslovi staništa ne utiču na kvalitativni sastav, ali da postoji razlika u kvantitativnom sastavu epikutikularnih voskova biljne vrste* S.rupestre *ssp.* rupestre.

Ključne reči: *Sedum rupestre ssp. rupestre, epikutikularni vosak, stanište, n-alkani, germanicil-formijat.*