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Short communication

Influence of soil traits on polyphenols level in *Moltkia petraea* (Tratt.) Griseb. (Boraginaceae)

Dario Kremer[1], Renata Jurišić Grubešić[1], Dalibor Ballian[2], Danijela Stešević[3], Ivan Kosalec[1], Jadranka Vuković Rodríguez[1], Marija Vukobratović[4], Siniša Srečec[4]*

[1] Faculty of Pharmacy and Biochemistry, University of Zagreb, A. Kovačića 1, Zagreb HR-10000, Croatia
[2] Faculty of Forestry, University of Sarajevo, Zagrebačka 20, BIH-71000, Bosnia and Herzegovina
[3] Faculty of Natural Sciences and Mathematics, University of Montenegro, Džordža Vašingtona bb, 81000 Podgorica, Montenegro
[4] Križevci College of Agriculture, M. Demerca 1, HR-45260 Križevci, Croatia

Abstract – The Illyric–Balkan endemic species *Moltkia petraea* (Tratt.) Griseb. is very interesting as a potential horticultural and medicinal plant. The aim of this study was to investigate soil conditions of *M. petraea* habitats, the phenolic content in plant parts, and the influence of soil properties on the phenolic contents. The results were evaluated using Spearman rank order correlations. Analyzed soil samples contained very low to intermediate levels of physiologically active phosphorus, but were very rich in potash. Organic matter content of soil was high. Phenolic compound content was higher in leaves than in flowers or stems. The analyses showed that *M. petraea* possesses considerable quantities of phenolic compounds and has no specific demands for particular soil conditions. A negative correlation was found between soil phosphorus content and total phenols content in leaves and stems, and with the total phenolic acids content in flowers. Organic matter in soil also found to have a negative influence on total tannins content in stems. Among the tested geographical locations, the Mljet population showed a higher degree of separation from the remaining locations.

Keywords: endemic, *Moltkia petraea*, phenolic compounds, plant habitats, soil traits

Introduction

The north-western Balkan Peninsula, particularly the area of Dinaric Alps, though poorly investigated, is known to be very rich in endemic plant species. From the biogeographical perspective, the Dinaric Alps mountain complex towards the north and the Mediterranean region towards the south are hotspots of endemicity. This is the meeting point of two large phytogeographic regions: the Euro Siberian – North American and the Alpine – high Nordic regions (Rešić et al. 2011).

Moltkia petraea (Tratt.) Griseb. is a typical representative of an endemic plant with horticultural and medicinal potential. It is an endemic, lithophytic, xerothermic Illyric–Balkan species distributed along the Adriatic Coast in Croatia, Bosnia and Herzegovina, Montenegro, Albania, and Greece, mostly in Mediterranean and sub-Mediterranean regions, at altitudes between near sea level to 2000 m. It is a dense, dwarf shrub that grows up to 40 cm and blooms from May to July with very decorative, deep violet-blue, tubular flowers (Šilić 2005). *M. petraea* is a strictly protected and threatened species in Croatia (Anonymous 2013).

Among biologically active compounds, phenolic compounds have attracted a great deal of public and scientific interest due to their health-promoting effects as antioxidants. The content of biologically active compounds varies greatly among species, including closely related species, making them useful chemotaxonomic markers. The differences between populations of a species could very often be significant (Dunkić et al. 2012). These differences could be associated with the region of origin, growth phase, habitat condition, and seasonal environmental variability, which encompass biotic and abiotic factors (Buchwald et al. 2015, Ramegowda and Senthil-Kumar 2015). According to Dapkevicius et al. (2002), it is possible to increase the content of pharmacologically desirable compounds through agriculture techniques, such as irrigation or using photo bioreactor systems. On the other hand, Buchwald et al. (2015) did not observe a statistically significant influence of mineral fertilization on the level of main active compounds. To date, chemical compound contents in *M. petraea* have only been investigated by Zovko Končić et al. (2010).

The aim of this study was to investigate the soil traits of *M. petraea* habitats and to evaluate the influence of soil

* Corresponding author, e-mail: ssrecec@vguk.hr

properties on phenolic compound accumulation. The evaluation of soil traits in native populations of *M. petraea* is the first step towards the determination of *M. petraea* as a plant with possible horticultural significance.

Materials and methods

Plant material

Samples of *Moltkia petraea* (Tratt.) Griseb. were collected during the blooming period in June and July of 2011 at ten locations along Croatian Adriatic coast, and in the Dinarides and Durmitor mountain ranges of Montenegro and Bosnia and Herzegovina (On-line Suppl. Fig. 1). Altitude and latitude of each habitat locality were determined by a GPS locator (Tab. 1). Voucher specimens of herbal material were deposited in the Fran Kušan Herbarium of the Department of Pharmaceutical Botany with Fran Kušan Pharmaceutical Botanical Garden at the Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia.

Above-ground parts of several dozen randomly selected plants were harvested from mature plants on a dry day and mixed to obtain the randomly selected sample. Samples were air-dried for three weeks in a well-ventilated room at 60% relative humidity and room temperature (22 °C), single-layered and protected from direct sunlight. Air-dried samples were placed in double paper bags labelled with the sample number, and stored in a dry place at room temperature (22 °C; 60% of humidity) protected from light for five months until analysis.

Soil sampling

Simultaneously with the sampling of plant material, soil was also collected *in situ* from all localities. Soil sampling was provided with a soil probe, from depths of 0–15 cm, due to the very shallow depth of the undeveloped karst soil. Soil samples were prepared for chemical analyses according to the ISO 11464:1994 method (Pernar et al. 2013).

Soil analyses

The pH of soil in H_2O and 1 M solution of KCl were analyzed according to the ISO 10390:1994 method (Pernar et al. 2013), while organic matter content (%) was analyzed according to Tjurin's method (Lal et al. 2002). Total nitrogen (%) was analyzed according to the ISO 11261:1995 method (Pernar et al. 2013), total content of $CaCO_3$ was analyzed according to the Scheibler method (Tatzber et al. 2007), and contents of physiological active phosphorus and potash, calculated in mg of P_2O_5 and K_2O per 100 grams of soil, were analyzed according to the Egner-Riehm-Domingo's ammonium lactate method (Page 1982). All soil analyses were performed in triplicate.

Phenolic compounds analyses

Total polyphenols and tannins contents were determined according to Schneider (1976). This procedure is based on a reaction with Folin-Ciocalteu's phenol reagent (FCR) and spectrophotometric determination of total polyphenols and tannins (indirectly, after precipitation with casein) at 720 nm. Tannin was used as the standard substance.

Tab. 1. Habitats of *Moltkia petraea* and collection data of researched plant and soil samples.

States and locality of sampling	Voucher No.	Latitude; Longitude	Altitude (m)	Abbreviation
Croatia				
Omiš	HFK-HR-113-2011	42°26'17" N; 16°42'01" E	30	Om
Vošac	HFK-HR-117-2011	43°18'46" N; 17°03'07" E	1295	Vo
Mljet	HFK-HR-122-2011	42°42'35" N; 17°40'36" E	225	Ml
Sniježnica	HFK-HR-133-2011	42°34'08" N; 18°21'28" E	1152	Sn
Bosnia and Herzegovina				
Diva Grabovica	HFK-HR-124-2011	43°35'59" N; 17°41'04" E	251	DG
Drežnica	HFK-HR-125-2011	43°31'48" N; 17°42'22" E	208	Dr
Rujišta	HFK-HR-123-2011	43°27'30" N; 17°57'02" E	964	Ru
Rakitnica	HFK-HR-127-2011	43°34'39" N; 18°05'20" E	768	Ra
Montenegro				
Orjen	HFK-HR-142-2011	42°33'45" N; 18°47'47" E	760	Or
Lovćen	HFK-HR-146-2011	42°23'44" N; 18°48'28" E	1365	Lo

The total flavonoid content (quercetin type) was determined using the method according to Christ and Müller (1960). This procedure includes hydrolysis of glycosides, extraction of total flavonoid aglycones with ethyl acetate and complex formation with $AlCl_3$ at 425 nm. The yield was calculated as quercetin according to the following expression:

$$\text{Total flavonoids (\%)} = A \times 0.772 / b$$

A = absorbance; 0.772 = conversion factor related to specific absorbance of quercetin at 425 nm; b = mass of dry herbal material (g)

Total phenolic acids content was determined according to the monograph of Rosmarini folium in European Pharmacopoeia (2007). Phenolic acids in the extracts were measured spectrophotometrically at 505 nm (three independent analyses), using the nitrite-molybdate reagent of Arnow, in a sodium hydroxide medium**,** and the percent of their content, expressed as rosmarinic acid, was calculated from the expression:

$$\text{Total phenolic acids (\%)} = A \times 2.5 / m$$

A = absorbance; 2.5 = conversion factor related to specific absorbance of rosmarinic acid at 505 nm; m = mass of the substance to be examined (g), taking the specific absorbance of rosmarinic acid to be 400.

The contents of total polyphenols, tannins, total flavonoids, and total phenolic acids were evaluated from three independent analyses and were expressed as the percentages of dry mass of herbal material. A UV/Vis spectrophotometer Agilent 8453 (Agilent, Germany) with PC-HP 845x UV-Visible System (Agilent, Germany) and 1 cm quartz cells was used for all absorbance measurements.

Statistical analysis

Statistical comparisons of phenolic compound contents among investigated populations and between plant organs were conducted using one-way ANOVA followed by Scheffe's post-hoc test at the $P \leq 0.05$ level. Prior to ANOVA, data was transformed using angular (i.e. arcsin) transformation.

The results were evaluated using multivariate analysis. Principal component analysis (PCA) calculation was based on the correlation matrix between the values of the characteristics, meaning that the contribution of each variable was independent of the range of its values. To confirm the results of the PCA, the unweighted pair-group method with arithmetic mean (UPGMA) with Euclidean distance (D_E) was conducted. UPGMA generally yields results that are the most accurate for classification purposes.

Interactions between soil traits and the content of different biologically active compounds were analyzed using the Spearman rank order correlation matrices. Prior to analysis, data were transformed using angular transformation. Statistical analyses were performed using the Statistica 7 software package (StatSoft Inc., Tulsa, OK, USA).

Results and discussion

Soil properties

Chemical properties of soil where native populations of *M. petraea* grow are presented in Tab. 2. Soil samples measured in a 1 M solution of KCl showed a neutral to slightly alkaline reaction. Soils from all sites contained a very low to intermediate amount of physiologically active phosphorus (P_2O_5), but were very rich in potash (K_2O). Soil organic matter content was very high at all localities. Due to the very shallow depth of the undeveloped karst soils, the high organic matter content was related to mulch not humus (Tab. 2). According to descriptive statistics, very high variability of $CaCO_3$, organic matter and potash contents was obtained in soil samples from different sites, indicating that

Tab. 2. Variability of chemical properties of soil in different habitats of *Moltkia petraea*. AL – ammonium-lactate, BLQ – below limit of quantification.

Habitat (locality abbreviation)	pH		$CaCO_3$ (%)	Organic matter (%)	Nitrogen (%)	AL-method (mg/100 g)	
	H_2O	1 M KCl				P_2O_5	K_2O
Omiš	7.78	7.33	29.93	5.04	0.41	8.50	26.61
Vošac	7.70	7.32	39.84	7.89	0.64	3.25	68.14
Mljet	7.68	7.25	27.10	12.85	0.48	2.79	61.36
Sniježnica	7.45	6.96	2.63	19.89	0.57	3.25	45.42
Diva Grabovica	7.33	6.89	BLQ	35.69	0.68	4.68	46.10
Drežnica	7.66	7.31	50.59	8.35	0.68	2.43	38.72
Rujišta	7.76	7.21	34.10	4.19	0.33	1.22	26.88
Rakitnica	7.04	6.59	BLQ	36.26	0.70	5.29	36.45
Orjen	7.24	6.97	6.45	13.76	0.41	11.09	125.00
Lovćen	7.71	7.17	17.97	9.67	0.40	1.55	27.81
Mean	7.54	7.10	26.08	15.36	0.53	4.41	50.25
Stand. dev.	0.26	0.24	16.35	11.78	0.14	3.16	29.82
Var.	0.07	0.06	267.32	138.68	0.02	9.98	889.28
Coef. of var.	3.39	3.40	62.70	76.67	26.37	71.70	59.35
Stand. error	0.08	0.08	5.78	3.72	0.04	0.10	9.43

M. petraea has no specific demands regarding particular soil conditions.

Phenolic compounds

The contents of total polyphenols (TP), tannins (T), total flavonoids (TF), and total phenolic acids (TPA) in leaves, flowers, and stems of the investigated *M. petraea* populations are presented in Tab. 3. TP, TF and TPA contents were highest in leaves, while T content was highest in flowers in most populations. In general, the concentrations of the analyzed bioactive compounds were lowest in stems. The smallest differences between populations were observed in TF content. It can be concluded that TF content in *M. petraea* is under a lesser influence of habitat conditions than the contents of TP, T and TPA.

The results confirmed that the content of biological active compounds in analyzed plants varied between populations. According to Dunkić et al. (2012), the contents of TP, T and TF in aerial parts of the investigated populations of *Satureja montana* L. and *S. subspicata* Vis. (Lamiaceae) revealed a statistically significant within-species difference, depending on the locality and plant organ used for determination. Variations between locations could be ascribed to biotic (vermin, alleopathy, diseases) and abiotic (climate, soil, fertilization) factors (Young et al. 2005). The content of biologically active compounds also depends on plant age and harvesting time (Kołodziej and Sugier 2013).

With regard to the analyzed phenolic compounds, the PCA and UPGMA separated the investigated *M. petraea* populations as presented in Fig. 1. The first principal component (PC 1) explained 46.1% of the total variance, the second 25.9%, and the third component 13.9%. Thus, the first three components accounted for 85.9% of the variance, emphasizing the usefulness of the PCA. The most similar populations were Vo and Lo, Om and DG, and Sn and Or, respectively (Fig. 1A). The population Ml showed a higher

Tab. 3. Content of total polyphenols, tannins, total flavonoids and total phenolic acids in leaves, flowers, and stems of *Moltkia petraea* expressed as mean ± standard deviation of the three independent analyses. Capital letters and symbols in superscript denote difference between populations for leaves (A, B, D, E, F, G, H, I, J), flowers (K, L, M, N, O, P, R, S, T, U) and stems (V, W, X, Y, Z, ", Γ, Λ, Π, Σ) related to certain investigated trait.

Sample	Plant part	Total polyphenols (%)	Tannins (%)	Total flavonoids (%)	Total phenolic acids (%)
Omiš	leaves	4.29±0.10^{A}	1.04±0.05^{A}	0.42±0.00^{A}	2.06±0.06^{A}
Omiš	flowers	3.91±0.02^{K}	1.06±0.02^{K}	0.19±0.01^{K}	1.72±0.02^{K}
Omiš	stems	3.33±0.09^{V}	1.01±0.05^{V}	0.04±0.01^{V}	1.76±0.03^{V}
Vošac	leaves	5.78±0.09^{AB}	1.95±0.01^{AB}	0.44±0.00^{AB}	3.43±0.02^{AB}
Vošac	flowers	6.07±0.07^{KL}	2.45±0.05^{KL}	0.28±0.00^{KL}	3.12±0.08^{KL}
Vošac	stems	4.44±0.03^{VW}	1.53±0.07^{VW}	0.15±0.00^{VW}	2.20±0.02^{VW}
Mljet	leaves	5.88±0.11^{AD}	0.39±0.09^{ABD}	0.37±0.01^{ABD}	3.00±0.02^{ABD}
Mljet	flowers	5.53±0.07^{KLN}	1.17±0.02^{LMN}	0.21±0.00^{LMN}	2.58±0.04^{KLMN}
Mljet	stems	5.53±0.06^{VWXY}	0.16±0.02^{VWXY}	0.12±0.00^{VWXY}	2.92±0.04^{VWXY}
Sniježnica	leaves	4.62±0.02^{BDE}	1.07±0.02^{BDE}	0.39±0.00^{ABDE}	1.89±0.06^{ABDE}
Sniježnica	flowers	4.64±0.07^{KMNO}	1.20±0.02^{LMO}	0.18±0.00^{LMO}	1.71±0.06^{LMNO}
Sniježnica	stems	3.26±0.03^{WXYZ}	0.53±0.04^{VXYZ}	0.31±0.00^{VWXYZ}	1.56±0.02^{VWXYZ}
Diva Grabovica	leaves	3.97±0.02^{BDF}	0.14±0.03^{ABEF}	0.84±0.01^{ABDEF}	2.01±0.05^{BDF}
Diva Grabovica	flowers	4.46±0.02^{KLMNP}	0.81±0.09^{LMNOP}	0.43±0.02^{KLMNOP}	2.20±0.02^{KLMNOP}
Diva Grabovica	stems	3.37±0.05$^{WX"}$	0.88±0.05$^{WXYZ"}$	0.09±0.02$^{VWXYZ"}$	1.70±0.06$^{WY"}$
Drežnica	leaves	6.01±0.05^{AEFG}	1.93±0.03^{ADEFG}	0.81±0.01^{ABDEFG}	2.59±0.02^{ABDEFG}
Drežnica	flowers	5.19±0.03^{KLMOPR}	1.48±0.02^{KLPR}	0.45±0.01^{LMNOR}	2.49±0.03^{KLMOPR}
Drežnica	stems	5.26±0.00$^{VWXZ"T}$	1.83±0.00$^{VXYZ"T}$	0.16±0.01$^{VYZ"T}$	2.15±0.09$^{VXYZ"T}$
Rujišta	leaves	6.28±0.45^{AEFGH}	3.00±0.43^{ABDEFGH}	0.91±0.00A^{BDEFGH}	1.98±0.06^{BDGH}
Rujišta	flowers	4.30±0.01^{LMNRS}	1.48±0.06^{KLPS}	0.52±0.01^{KLMNOPRS}	2.12±0.03^{KLMNORS}
Rujišta	stems	5.09±0.22$^{VWXZ"\Lambda}$	2.55±0.20$^{VWYZ"\Gamma\Lambda}$	0.18±0.01$^{VYZ"}$	1.72±0.03$^{WYZ\Gamma\Lambda}$
Rakitnica	leaves	5.31±0.28^{AFGHI}	1.61±0.26^{DFH}	1.13±0.01^{ABDEFGHI}	2.57±0.05^{ABEFHI}
Rakitnica	flowers	4.65±0.21^{KLMNRT}	1.11±0.12^{LMPRST}	0.48±0.01^{KLMNOPRST}	1.92±0.05^{KLMNOPRST}
Rakitnica	stems	3.87±0.15$^{VWYZ"\Gamma\Lambda\Pi}$	1.00±0.11$^{WXYZ\Gamma\Lambda\Pi}$	0.19±0.01$^{VWYZ"T}$	2.20±0.05$^{VXYZ"\Lambda\Pi}$
Orjen	leaves	5.42±0.10^{AFGJ}	1.24±0.08^{BDFGH}	0.50±0.00^{ABDEFGHIJ}	1.58±0.03^{ABDEFGHIJ}
Orjen	flowers	5.55±0.04^{KLMOPSTU}	1.51±0.02^{KLPT}	0.27±0.00^{KMNOPRSTU}	2.00±0.05^{KLMNOPRU}
Orjen	stems	3.88±0.04$^{VWXZ"\Gamma\Lambda\Sigma}$	0.30±0.02$^{VWXZ"\Gamma\Lambda\Pi\Sigma}$	0.20±0.00$^{VWYZ"T}$	1.50±0.02$^{VWXY"\Gamma\Lambda\Pi\Sigma}$
Lovćen	leaves	6.61±0.04^{AEFGIJ}	1.71±0.02^{ADFH}	0.37±0.00^{ABDEFGHIJ}	2.53±0.03^{ABDEFHJ}
Lovćen	flowers	5.90±0.06^{KLMOPRST}	1.77±0.01^{KLNOPT}	0.21±0.00^{LMOPRSTU}	3.26±0.03^{KMNOPRSTU}
Lovćen	stems	5.32±0.07$^{VWXYZ"\Pi\Sigma}$	1.19±0.04$^{XYZ\Gamma\Lambda\Pi\Sigma}$	0.19±0.00$^{VWYZ"T}$	2.44±0.04$^{VWXYZ"\Gamma\Lambda\Pi\Sigma}$

degree of separation. This population is situated on an island in the Adriatic Sea and it is under the stronger influence of the Mediterranean climate than the remaining populations studied. The populations Vo and Lo are situated at similar altitudes on Mt Biokovo and Mt Lovćen, respectively. Both mountains are near the Adriatic Sea and under similar climatic conditions. Consequently, the geographical position of the populations Vo and Lo could explain the similarities obtained in the multivariate analysis. The populations Sn and Or are geographically close and their similarities in the multivariate analysis were expected. The similarity between the populations Om and DG is more difficult to explain. Both populations are found in canyons, Om in the Cetina River Valley, and DG in the Neretva River Valley. It is possible that this environmental factor also played a significant role in the accumulation of phenolic compounds. Although some studies of plant species such as *Tanacetum cinerariifolium* (Trevir.) Sch. Bip. (Grdiša et al. 2014), *Campanula pyramidalis* L. (Lakušić et al. 2013), *Edraianthus tenuifolius* (Waldst. et Kit.) A. DC. (Surina et al. 2011), and *Cardamine maritima* DC. (Kučera et al. 2008) showed a greater or lesser phylogeographical or taxonomical split in the area of the Neretva River Valley, such a split was not confirmed here. Similar results to the PCA were obtained using UPGMA, which separated three groups of populations (Fig. 1B). Similar populations were Vo (Vošac), Lo (Lovćen) and Ml (Mljet) which formed one large group. The remaining populations formed the second group containing two subgroups. The most similar populations were Sn and Or, which formed a single cluster connected at a Euclidean distance of 2.64.

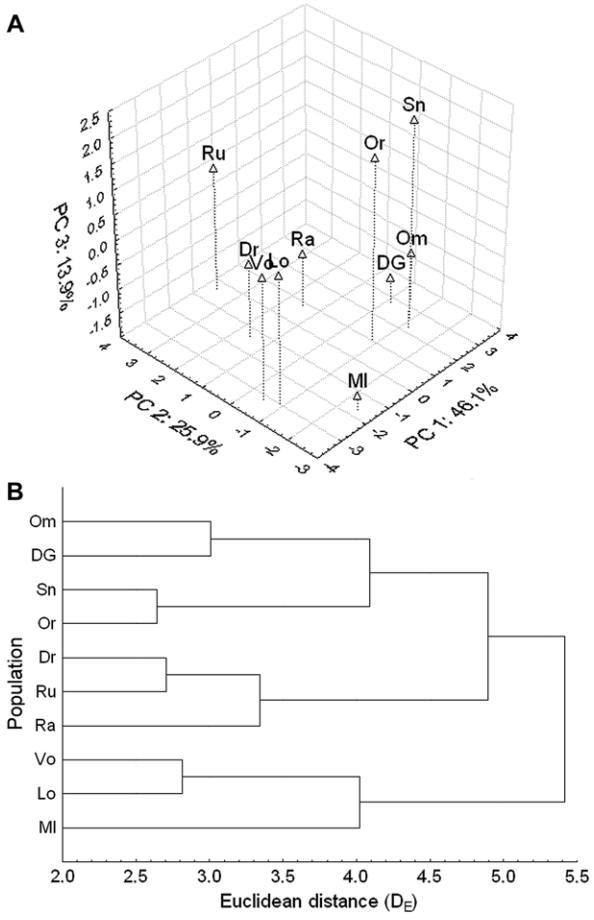

Fig. 1. Principal component analysis (A) and the unweighted pair-group method with arithmetic mean (UPGMA) (B) of the phenolic compounds content in *Moltkia petraea* populations. Om – Omiš, Vo – Vošac, Ml – Mljet, Sn – Sniježnica, DG – Diva Grabovica, Dr – Drežnica, Ru – Rujišta, Ra – Rakitnica, Or – Orjen, Lo – Lovćen.

Correlation between phenolic substances and chemical properties of soil

Spearman rank order correlations between soil reaction (pH in H_2O and in 1 M KCl), nitrogen content, and potash content (expressed as content of K_2O in mg per 100 grams of soil) in soils of all habitats (as independent variables) and biologically active substances (i.e. total polyphenols, tannins, total flavonoids, and total phenolic acids in leaves, flowers and stems as dependent variables) in all plant samples of *M. petraea* were not significant (On-line Suppl. Tab. 1). However, correlations between the content of phosphorus and content of total polyphenols in leaves and stems, as well as the content of total phenolic acids in flowers of *M. petraea* were strongly negative. Spearman rank order correlations between the content of $CaCO_3$ in soil of *M. petraea* habitats and the content of biologically active substances were calculated only for eight (of ten) habitats of *M. petraea*, due to the very low $CaCO_3$ content (below minimal quantities) in soil samples from the two remaining locations. However, only one comparison of $CaCO_3$ content in the soil and tannin content in the stem of *M. petraea* indicated a strong positive correlation. Comparison of the organic matter content in soil of habitats (independent variable) and the content of biologically active substances (dependent variable) showed a moderately negative correlation in comparison with the total tannin content in the stems of *M. petraea*. No other comparisons gave significant correlations (On-line Suppl. Tab. 1).

The results of this study showed negative correlations between the phosphorus content in soil and TP content in leaves and stems. Also, a negative correlation was found between the phosphorus content in soil and TPA content in flowers. These results are in line with results of Gerschenzon (1983) who found that soil deficiencies in phosphorus, sulphur, iron, calcium, and magnesium stimulates the production of phenolic compounds in plant tissues. Higher phenolic contents have also been reported as a response to phosphate starvation in *Phaseolus vulgaris* L. (Juszczuk et al. 2004). According to Tavarini et al. (2015) nitrogen fertilization of soil in the amount of 150 kg per ha will optimize the content of total phenols and flavonoids in leaves of *Stevia rebaudiana* Bertoloni (Asteraceae). Buchwald et al. (2015) showed that mineral fertilization with nitrogen, phosphorus and potassium did not substantially affect the level of phenolic acids in the raw material of *Rhodiola rosea* L. It was also found that using bio-fertilizer significantly increased total flavonoid contents in *Anethum graveolens* L. (Apiaceae) (Said-Al Ahl et al. 2015).

Accordingly, it can be concluded that *M. petraea* has no specific demands regarding particular soil conditions. Two locations (Sn and Or) showing similar phenolic compounds levels, had quite different soil chemical properties. This

suggests that biosynthesis of phenolic compounds in *M. petraea* is largely affected by other factors (genetic factors, topography, and exposition of plant site in relief, possible influence of Aeolian deposits in site, or other soil properties) rather than the investigated soil properties. However, due to the very nice habitus (particularly because of the shape and colour of flowers) and low soil and water demands, *M. petraea* may be considered a potentially valuable horticultural plant in landscape architecture.

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