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Comparative analysis of specialized metabolites and antioxidant capacity *in vitro* of different natural populations of *Globularia* spp.

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Abstract – Total phenolic, flavonoid, condensed tannin and iridoid content, as well as antioxidant capacity *in vitro,* were determined spectrophotometrically in methanolic extracts of different plant parts of the Mediterranean medicinal plant *Globularia alypum* L. and three widespread European species of the same genus: *G. cordifolia* L., *G. meridionalis* (Podp.) O. Schwarz and *G. punctata* Lapeyr. In order to consider possible environmental influences on the production of specialized metabolites, each species, except *G. alypum*, was collected from three different natural populations. Great variations in the amounts of specialized metabolites were observed among different plant parts and species. For example, total phenolic content ranged from 10.13 (*G. punctata*, flowers) to 44.90 (*G. cordifolia*, flower stems) mg gallic acid equivalent g^{-1} dry weight. Moreover, great differences, attributed to location-specific environmental factors, were observed among different populations of the same species. For example, a strong positive correlation was observed among mean monthly temperatures and total phenolic contents in the leaves of studied *Globularia* spp. ($r = 0.75$, $p = 0.019$). However, despite these differences, all species were rich in bioactive substances when compared to *G. alypum*, especially in their aerial parts. A very good positive correlation was observed between total phenolic content and DPPH radical scavenging capacity ($r = 0.86$, $p < 0.001$)/ABTS radical scavenging capacity ($r = 0.83$, $p < 0.001$). The results obtained show that *G. cordifolia*, *G. meridionalis* and *G. punctata* are rich in bioactive substances, providing support for their pharmaceutical utilization. Further investigations are needed to verify the possibility of their medicinal use.

Keywords: antioxidant activity, environmental factors, flavonoids, *Globularia*, iridoids, polyphenols, proanthocyanidins, secondary metabolites

Introduction

Plants have been used as healing agents since ancient times; many bioactive compounds isolated from herbal sources have been used as drugs or served as lead compounds in drug development. In Europe, herbal medicines in the crude forms of teas and decoctions are often used as supportive therapy, while standardized herbal preparations are a popular alternative to synthetic drugs. Finally, about 80% of the world population (primarily in developing countries) still uses herbal medicine in the treatment of different diseases and in maintaining health (Gurib-Fakim 2006).

The Old World genus *Globularia* L., recently included in the Plantaginaceae family (Albach et al. 2005), consists of perennials, subshrubs and small shrubs. Some members of the genus, mainly *Globularia alypum* L., *G. arabica* Jaub. & Spach and *G. trichosantha* Fisch. & C.A. Mey., are used in the traditional medicine of countries such as Spain, Italy, Morocco, Algeria, Tunisia, Libya, Egypt and Turkey (Leporatti and Ghedira 2009, Altundag and Ozturk 2011, Carrió and Vallès 2012, De Natale and Pollio 2012, Bouzabata 2013, Eissa et al. 2014, El Abbouyi et al. 2014). They are tradition-

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ally used as hypoglycaemic agents, purgatives, depuratives (De Natale and Pollio 2012, Bouzabata 2013), antiparasitic and antifungic agents (Altundag and Ozturk 2011, De Natale and Pollio 2012), tonics and diuretics, for wound healing and in the treatment of insomnia, fits of epilepsy, gastrointestinal disorders, intermittent fever (Leporatti and Ghedira 2009, De Natale and Pollio 2012, Eissa et al. 2014), arthritis and rheumatism (De Natale and Pollio 2012).

Phenolic compounds and iridoids are the main specialized (secondary) metabolites of *Globularia* species (Kirmizibekmez et al. 2008, Kirmizibekmez et al. 2009, Tundis et al. 2012b). *G. alypum*, the most widely used member of the genus *Globularia*, was shown to be especially rich in phenolic compounds in comparison with some other medicinal plants (Djeridane et al. 2006, Djeridane et al. 2010, Amessis-Ouchemoukh et al. 2014, El Guiche et al. 2015). Phenolic compounds possess a wide range of biological activities and thus may contribute to the healing properties of *Globularia* preparations. Radical scavenging activity, also attributed to plant phenolics, is one of the possible protective mechanisms against cancer, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases (Rice-Evans et al. 1997). It was noted that *G. alypum* extracts possess high antioxidant capacity both *in vitro* (Djeridane et al. 2010, Amessis-Ouchemoukh et al. 2014) and *in vivo* (Taleb-Dida et al. 2011). Antioxidant activity was also recently reported in *G. meridionalis* (Tundis et al. 2012a).

The aim of the present study was to analyse and compare the total phenolic content, including flavonoid and condensed tannin content, iridoid content and antioxidant capacity in four members of the genus *Globularia* L. Three of these, namely *G. cordifolia* L., *G. meridionalis* (Podp.) O. Schwarz and *G. punctata* Lapeyr., are less-investigated, although they have a relatively wide distribution in Europe (Tutin 1972). In order to examine their therapeutic potential with regard to the content of bioactive substances, their results were compared to those of the well-investigated medicinal plant *G. alypum* L., which is distributed mainly in the Mediterranean area. Since synthesis and distribution of specialized metabolites is complex and differs between tissues and organs (Boudet 2007), the study was focused on different plant parts. The analysis was done on material collected from three different locations in order to consider possible ecological influences on the production of the specialized metabolites investigated. Exceptionally, *G. alypum* was collected from the only location at which it grows wild in Croatia and was sampled without underground parts because of its near threatened status. Obtained phytochemical data were correlated with environmental factors of different habitats, enabling the interpretation of obtained results from both medicinal and ecological perspectives.

Materials and methods

Plant material and extraction

A total of ten samples of four *Globularia* taxa were collected during the phenophase of blooming from seven locations in Croatia and one in Bosnia and Herzegovina (Tab. 1). Voucher specimens are deposited in the Herbarium of the Department of Pharmaceutical Botany, Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia. The identity of plant material was verified by Prof. Kroata Hazler Pilepić. Meteorological data were obtained from the nearest meteorological stations (Meteorological and Hydrological Service

Tab. 1. Collection and geographical data for plant material of the investigated *Globularia* species; *Ga – Globularia alypum*, *Gc – G. cordifolia*, *Gm – G. meridionalis*, *Gp – G. punctata*.

Species (Voucher No.)	Location	Geographical latitude	Geographical longitude	Elevation (m)	Habitat type	Harvest time
Ga (16 020)	Dubrovnik area (Konavle cliffs)	42°30′50″N	18°19′07″E	15	Limestone cliffs by the sea	March 2013
Gc (1) (16 032)	Northern Velebit area (Alan)	44°43′15″N	14°58′05″E	1340	Calcareous grasslands	May 2013
Gc (2) (16 031)	Middle Velebit area (Baške Oštarije)	44°31′41″N	15°08′38″E	917	Calcareous grasslands	May 2012
Gc (3) (16 030)	Mostar area (the Neretva banks)	43°21′41″N	17°48′20″E	57	Rocky grasslands along a river	May 2012
Gm (1) (16 041)	Istrian peninsula (Mala Učka)	45°17′59″N	14°11′27″E	926	Limestone cliffs	May 2012
Gm (2) (16 040)	Rijeka area (Grobničko polje)	45°22′39″N	14°30′53″E	308	Karst field	May 2013
Gm (3) (16 043)	Middle Velebit area (Baške Oštarije)	44°31′41″N	15°08′38″E	917	Calcareous grasslands	May 2012
Gp (1) (16 051)	Istrian peninsula (Vižintini)	45°23′36″N	13°51′20″E	386	Calcareous grasslands	May 2012
Gp (2) (16 056)	Rijeka area (Grobnik field)	45°22′39″N	14°30′53″E	308	Karst field	May 2013
Gp (3) (16 057)	Žumberak area (Slapnica canyon)	45°44′29″N	15°29′26″E	325	Limestone cliffs	May 2013

of Croatia and the Federal Hydrometeorological Institute of Bosnia and Herzegovina) (Tab. 2).

Dried and powdered plant parts (2.5 g) were subjected to ultrasound-assisted extraction (Bandelin Sonorex Super, Germany) at room temperature for 30 min with 25 mL of methanol. The residue after filtration was extracted again for 30 min with 25 mL of methanol, filtered and the final volume was adjusted to 50 mL. Four classes of metabolites were measured spectrophotometrically using a Varian Cary 50 Bio UV-Vis spectrophotometer (USA).

Determination of plant specialized metabolites

The total phenolic content was determined using Folin-Ciocalteu's reagent according to the method of Singleton and Rossi (1965). The reaction mixture was prepared by mixing 0.5 mL of methanolic extract with 2.5 mL of 10% (v/v) Folin-Ciocalteu's reagent (diluted in distilled water). After 5 min, 2 mL of 7.5 g/100 mL sodium carbonate decahydrate solution were added and the mixture was incubated for 1 h at room temperature. After incubation, the absorbance was read at 765 nm against a distilled water blank. Gallic acid was used for the construction of the calibration curve. The total phenolic content was expressed as mg gallic acid equivalent (GAE) g^{-1} dry weight (DW).

The flavonoid content was measured using the Dowd method (Arvouet-Grand et al. 1994). Briefly, 1 mL of appropriately diluted extract was mixed with 1 mL of 2 g/100 mL $AlCl_3$ solution in pure methanol. The absorbance was measured after 15 min at 415 nm against a sample blank which consisted of 1 mL of extract and 1 mL of methanol. A standard calibration curve was plotted using quercetin as a reference standard. The results were expressed as mg quercetin equivalent (QE) g^{-1} dry weight (DW).

The condensed tannin (proanthocyanidin) content was determined using the vanillin assay (Broadhurst and Jones 1978, Sun et al. 1998) as described previously by Toda (2005), but with a slightly modified reaction temperature. For the preparation of the sample 2 mL of 1 g/100 mL vanillin in 7 M sulfuric acid were added to a test tube containing 1 mL of diluted extract and the mixture was incubated for 15 min in a water bath with the temperature set at 30±1 °C. The reaction was performed in normal laboratory daylight. After the incubation, the absorbance was recorded at 500 nm against a sample blank which consisted of 1 mL of extract and 2 mL of 7 M sulfuric acid, which was incubated under the same conditions as the sample. (+)–catechin was chosen as a standard for the calibration curve. The levels of total condensed tannin content were expressed as mg catechin equivalent (CE) g^{-1} dry weight (DW).

The iridoid content was measured using the Trim-Hill reagent (Trim and Hill 1952) as adapted by Tundis et al. (2012a). To 200 µL of diluted extract 2 mL of Trim-Hill reagent (glacial acetic acid:0.2% copper (II) sulfate pentahydrate:concentrated hydrochloric acid at a ratio of 10:1:0.5, v:v:v) were added and the mixture was heated in a boiling water bath for 5 min. After that, absorbance of the prepared solution was read at 609 nm with methanol used as blank. The concentration of iridoids in the samples was calculated based on an aucubin calibration curve and the results were presented as mg aucubin equivalent (AE) g^{-1} dry weight (DW).

Evaluation of antioxidant capacity

Antioxidant capacity of the extracts was evaluated by the Blois method (1958) using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical solution according to the procedure of Thetsrimuang et al. (2011) with some modifications. A 0.1 mM stock solution of DPPH in methanol was prepared. The working solution was obtained by diluting the stock solution with methanol to obtain an absorbance of 0.7±0.02. To 2 mL of this solution, 10 µL of properly diluted extract were added and the decrease in absorption of the radical was measured at 517 nm after 30 min incubation in the dark against a methanol blank. A calibration curve was obtained by us-

Tab. 2. Records of mean monthly temperatures and monthly precipitation amounts from meteorological stations close to the locations where *Globularia* species were collected (for details see Tab. 1). Records are shown for the month in which the plant material was harvested (M), the three previous months (M-3, M-2, and M-1) as well as their average; *Ga* – *Globularia alypum*, *Gc* – *G. cordifolia*, *Gm* – *G. meridionalis*, *Gp* – *G. punctata*.

Plant species	Mean monthly temperature (°C)					Monthly precipitation amounts (mm)					Meteorological station (Elevation (m))
	M-3	M-2	M-1	M	Average	M-3	M-2	M-1	M	Average	
Ga	9.6	10.1	9.5	11.2	10.1	283.2	205.2	238.1	214.0	235.1	Dubrovnik (52)
Gc (1)	–6.4	–3.1	3.8	5.6	0.0	284.6	293.9	152.7	253.4	246.2	Zavižan (1594)
Gc (2)	–5.0	7.3	9.8	13.4	6.4	58.2	1.0	116.2	102.0	69.4	Gospić (564)
Gc (3)	1.7	13.5	13.2	18.6	11.8	202.7	0.3	266.6	92.3	140.5	Mostar (99)
Gm (1)	0.7	8.9	11.7	15.5	9.2	30.1	1.3	54.2	104.4	47.5	Letaj (120)
Gm (2)	5.1	8.0	14.3	16.4	11.0	210.1	386.9	80.0	228.3	226.3	Rijeka (120)
Gm (3)	–5.0	7.3	9.8	13.4	6.4	58.2	1.0	116.2	102.0	69.4	Gospić (564)
Gp (1)	0.2	8.8	11.4	15.3	8.9	11.9	0.0	61.4	105.6	44.7	Pazin (291)
Gp (2)	5.1	8.0	14.3	16.4	11.0	210.1	386.9	80.0	228.3	226.3	Rijeka (120)
Gp (3)	1.5	4.4	12.9	15.8	8.7	93.7	121.1	50.2	107.0	93.0	Maksimir (128)

ing different concentrations of gallic acid and the results were expressed as mg gallic acid equivalent (GAE) g^{-1} dry weight (DW).

The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging capacity was determined following the method of Re et al. (1999) with some modifications. An activated solution of $ABTS^{\bullet+}$ radical cation was prepared by mixing ABTS and potassium peroxodisulfate solutions so that the final concentrations in the mixture were 7 mM and 2.45 mM, respectively. The solution was held at room temperature (22±2 °C) for at least 16 h before use. The working solution was obtained by diluting the stock solution with distilled water to obtain an absorbance of 0.7±0.02. The fall of absorbance was measured at 734 nm against a distilled water blank 1 min after mixing 10 μL of properly diluted extract with 2 mL of activated radical. ABTS radical scavenging activity was calculated from a gallic acid calibration curve and presented as mg gallic acid equivalent (GAE) g^{-1} dry weight (DW).

Statistical analysis

All measurements were performed in triplicate and the results are presented as means ± standard deviations. A two-way analysis of variance (ANOVA) followed by the Bonferroni *post-hoc* test was carried out on averaged results for plant parts of each species, to determine significant differences among the same plant parts of different species and different plant parts of the same species. Pearson's correlation coefficient (r) was used to determine the association among parameters, more precisely, among the contents of particular groups of specialized metabolites (total phenolics, flavonoids, condensed tannins and iridoids) in all samples (altogether 48 samples) and their antioxidant capacities evaluated by two different assays (DPPH and ABTS) or among the contents of particular groups of specialized metabolites in specific plant parts and averages of mean monthly temperatures and monthly precipitation amounts of individual locations (maximum of ten samples per analysis), with a significance level $\alpha = 0.05$. The statistical analysis was carried out using GraphPad Prism 5.03 for Windows (GraphPad Software, San Diego, USA).

Results

Content of plant specialized metabolites

All four investigated species were found to be rich in phenolic compounds, with observed differences among plant parts (Tab. 3). The content of total phenolics ranged from 10.13 (*G. punctata* (3), flowers) to 44.90 (*G. cordifolia* (1), flower stems) mg GAE g^{-1} DW. High amounts of polyphenols were observed in leaves of all species, with no significant differences noticed between *G. alypum* and related species. Leaves and flowers of *G. alypum* were the richest plant parts for this species. On the other hand, it was observed that flower stems of *G. cordifolia* and *G. meridionalis*, as well as woody stems and underground parts of *G. punctata*, also contained high amounts of phenolic compounds. Flowers of *G. punctata* were the poorest of all the tested samples ($p < 0.05$).

The flavonoid content ranged from 0.84 (*G. cordifolia* (3), woody stems) to 17.77 (*G. punctata* (2), leaves) mg QE g^{-1} DW (Tab. 3). When different plant parts between species were compared, it was observed that the leaves contained the highest amounts of flavonoids. *G. punctata* leaves and flower stems contained more flavonoids than those of other species ($p < 0.05$), while there were no significant differences among the species for flowers and underground parts. The lowest amounts of flavonoids were observed for woody stems and underground parts.

Condensed tannin (proanthocyanidin) content varied from 0.19 (*G. meridionalis* (2), underground parts) to 9.77 (*G. cordifolia* (3), flower stems) mg CE g^{-1} DW (Tab. 3). Taking into account all plant parts, condensed tannin content was especially high in the population collected from Mostar. Green aerial parts contained more tannins than woody stems and underground parts. In *G. cordifolia*, leaves and flower stems contained the highest amounts of tannins ($p < 0.05$), while for other species no significant differences were observed among plant parts.

The content of iridoids varied from 3.94 (*G. punctata* (3), underground parts) to 143.29 (*G. punctata* (1), leaves) mg aucubin equivalent (AE) g^{-1} DW (Tab. 3). High amounts of iridoids were found in *G. cordifolia, G. meridionalis* and *G. punctata*, especially in their leaves and flower stems ($p < 0.05$). No significant differences in iridoid content were noticed between different organs of *G. alypum*.

Correlations between phenolic/iridoid content and environmental factors

In order to verify the influence of specific environmental factors on the variability of the compounds determined, results obtained for each plant part were correlated with mean monthly temperatures and monthly precipitation amounts of different stands and seasons (Tab. 2). Averages for the month in which the plant material was collected and the three previous months were correlated with the amounts of investigated compounds. During evaluation of the influence of temperature, the population of Mala Učka had to be excluded due to the great elevation difference between the location of sampling (926 m) and the nearest meteorological station (120 m), from which meteorological data were available, which made it impossible to accurately predict the mean monthly temperatures for this location. Taking this into account, however, a strong positive correlation was observed between mean monthly temperatures and total phenolic contents in the leaves of investigated species ($r = 0.75$, $p = 0.019$), together with a strong negative correlation in the flower stems ($r = -0.88$, $p = 0.002$). In spite of the significant variability discovered in the iridoid contents among populations, no correlation with mean monthly temperatures ($p > 0.05$) was found. Also, no significant correlation between precipitation and amounts of bioactive substances was observed ($p > 0.05$).

Tab. 3. Total phenolic, flavonoid, condensed tannin and iridoid contents as well as radical scavenging capacities obtained by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays, in different plant parts of four *Globularia* species collected from different locations (for details see Tab. 1). Values are means ± SD, n = 3; statistically significant differences ($p < 0.05$) between the same plant parts of different species are indicated by different superscript lower case letters (a > b > c > d) and between different plant parts of the same species in different superscript capital letters (A > B > C > D); * – flowers of the plant are carried by woody stems; *Ga* – *Globularia alypum*, *Gc* – *G. cordifolia*, *Gm* – *G. meridionalis*, *Gp* – *G. punctata*; L – leaves, F – flowers, FS – flower stems, WS – woody stems, UP – underground parts; n.m. – not measured; GAE – gallic acid equivalent, DW – dry weight, QE – quercetin equivalent, CE – catechin equivalent, AE – aucubin equivalent.

Plant species	Plant part	Polyphenols (mg GAEg^{-1} DW)	Flavonoids (mg QE g^{-1} DW)	Tannins (mg CE g^{-1} DW)	Iridoids (mg AE g^{-1} DW)	DPPH (mg GAE g^{-1} DW)	ABTS (mg GAE g^{-1} DW)
Ga	L	37.58 ± 0.85^{aA}	9.23 ± 0.39^{bA}	0.76 ± 0.03^{bA}	9.38 ± 0.55^{cA}	18.29 ± 2.64^{aA}	12.35 ± 0.81^{aA}
	F	35.39 ± 0.78^{aA}	5.46 ± 0.04^{aB}	0.77 ± 0.01^{aA}	6.18 ± 0.08^{aA}	20.48 ± 2.03^{aA}	14.41 ± 0.56^{aA}
	FS = WS*	21.16 ± 0.72^{cB}	4.74 ± 0.04^{cB}	0.34 ± 0.03^{bA}	5.06 ± 0.04^{bA}	11.67 ± 0.37^{aB}	8.09 ± 0.37^{bB}
	UP	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
Gc (1)	L	27.67±0.82	9.08±0.36	2.55±0.07	77.34±1.54	9.98±0.97	8.18±0.02
	F	25.68±0.70	7.12±0.08	2.30±0.10	24.67±0.81	7.96±0.68	7.52±1.02
	FS	44.90±1.18	9.56±0.15	4.17±0.07	57.56±2.25	14.70±1.43	12.52±1.98
	WS	22.22±0.51	2.00±0.01	0.28±0.01	8.37±0.48	9.12±0.96	7.91±0.43
	UP	22.15±1.03	1.96±0.07	0.21±0.00	6.03±0.14	9.91±0.22	7.56±0.10
Gc (2)	L	34.42±0.94	9.24±0.15	1.92±0.02	132.43±1.18	11.28±1.90	11.53±0.14
	F	20.06±0.45	4.55±0.02	1.40±0.05	28.74±3.16	7.22±2.27	7.86±0.28
	FS	33.31±0.32	6.73±0.09	3.24±0.08	51.04±0.63	11.45±0.52	8.95±0.19
	WS	14.55±0.10	0.93±0.01	0.33±0.00	6.64±0.01	5.19±0.04	7.27±0.16
	UP	16.54±0.90	1.36±0.13	0.26±0.01	4.92±0.47	5.96±0.26	6.47±0.13
Gc (3)	L	33.33±0.96	9.50±0.27	8.01±0.17	98.25±2.47	10.36±0.49	10.37±0.32
	F	18.88±0.28	3.73±0.09	4.53±0.04	40.56±0.98	5.44±0.44	5.83±0.15
	FS	23.66±0.52	7.59±0.07	9.77±0.20	100.51±1.31	9.80±0.08	8.73±0.33
	WS	13.61±0.56	0.84±0.01	0.75±0.01	10.51±0.04	4.53±1.17	6.24±0.13
	UP	20.37±0.72	1.45±0.02	0.66±0.01	9.95±0.25	8.71±0.91	8.30±0.16
Gc average	L	31.81 ± 3.62^{aAB}	9.27 ± 0.21^{bA}	4.16 ± 3.35^{aA}	102.70 ± 27.81^{abA}	10.54 ± 0.67^{bA}	10.03 ± 1.70^{aA}
	F	21.54 ± 3.63^{bcBC}	5.13 ± 1.77^{aB}	2.74 ± 1.61^{aAB}	31.32 ± 8.25^{aC}	6.87 ± 1.30^{bA}	7.07 ± 1.09^{bA}
	FS	33.96 ± 10.63^{aA}	7.96 ± 1.45^{bA}	5.73 ± 3.53^{aA}	69.70 ± 26.88^{aB}	11.98 ± 2.49^{aA}	10.07 ± 2.13^{bA}
	WS	16.79 ± 4.72^{cC}	1.26 ± 0.65^{dC}	0.45 ± 0.26^{bB}	8.51 ± 1.94^{bC}	6.28 ± 2.48^{bA}	7.14 ± 0.84^{bA}
	UP	19.69 ± 2.87^{bBC}	1.59 ± 0.32^{aC}	0.38 ± 0.25^{aB}	6.97 ± 2.64^{aC}	8.19 ± 2.03^{aA}	7.44 ± 0.92^{bA}
Gm (1)	L	23.03±0.29	6.96±0.08	1.38±0.00	85.89±5.76	10.16±0.22	9.47±0.34
	F	21.69±0.78	5.16±0.03	1.74±0.02	37.65±2.01	9.89±0.08	8.33±0.16
	FS	29.50±0.55	6.07±0.03	2.12±0.02	62.61±1.42	14.07±0.85	11.36±0.11
	WS	17.56±0.04	1.02±0.02	0.34±0.00	8.54±0.12	5.76±0.82	7.68±0.07
	UP	26.05±0.68	2.25±0.04	0.39±0.00	15.98±0.53	9.91±0.18	10.01±0.28
Gm (2)	L	35.18±0.22	11.32±0.17	1.77±0.03	70.50±0.77	12.08±1.01	9.95±0.67
	F	18.28±0.29	5.35±0.07	1.86±0.12	20.50±1.55	7.04±0.61	4.62±0.10
	FS	29.38±0.45	6.37±0.24	2.78±0.09	40.94±3.89	8.19±1.06	8.50±0.31
	WS	20.00±0.17	1.71±0.09	0.24±0.00	9.29±0.49	6.34±0.10	5.67±0.16
	UP	17.13±0.53	1.08±0.01	0.19±0.01	6.20±0.22	5.73±1.20	5.38±0.15
Gm (3)	L	30.81±0.45	8.40±0.10	1.71±0.05	114.08±7.72	9.49±1.08	10.02±0.18
	F	27.25±0.85	4.09±0.03	1.60±0.02	25.19±1.60	6.82±2.20	6.66±0.24
	FS	37.76±1.22	7.86±0.09	3.55±0.11	49.46±4.80	13.19±0.17	9.96±0.92
	WS	16.80±0.54	0.90±0.02	0.41±0.01	8.27±0.13	6.59±0.29	7.99±0.30
	UP	15.86±0.19	1.40±0.09	0.26±0.01	5.04±0.15	5.25±0.25	5.25±0.22
Gm average	L	29.67 ± 6.15^{aAB}	8.89 ± 2.22^{bA}	1.62 ± 0.21^{abA}	90.16 ± 22.10^{bA}	10.58 ± 1.34^{bA}	9.81 ± 0.30^{aA}
	F	22.41 ± 4.53^{bAB}	4.87 ± 0.68^{aB}	1.73 ± 0.13^{aA}	27.78 ± 8.86^{aBC}	7.92 ± 1.71^{bA}	6.54 ± 1.86^{bA}
	FS	32.21 ± 4.80^{abA}	6.77 ± 0.96^{bcAB}	2.82 ± 0.72^{abA}	51.00 ± 10.92^{aB}	11.82 ± 3.17^{aA}	9.94 ± 1.43^{bA}
	WS	18.12 ± 1.67^{cB}	1.21 ± 0.44^{dC}	0.33 ± 0.09^{bA}	8.70 ± 0.53^{bC}	6.23 ± 0.43^{bA}	7.11 ± 1.26^{bA}
	UP	19.68 ± 5.55^{bB}	1.58 ± 0.60^{aC}	0.28 ± 0.10^{aA}	9.07 ± 0.60^{aC}	6.96 ± 2.56^{aA}	6.88 ± 2.71^{bA}
Gp (1)	L	35.31±1.31	14.46±0.20	1.68±0.04	143.29±7.15	15.32±1.04	12.22±0.24
	F	14.02±0.25	6.76±0.05	0.84±0.02	15.26±0.41	4.18±0.42	4.34±0.01
	FS	24.60±0.59	13.93±0.25	0.97±0.04	86.75±1.62	12.91±0.57	7.53±0.29
	WS	34.96±0.57	3.71±0.04	0.58±0.02	8.53±0.18	16.94±1.09	18.95±0.50
	UP	32.68±0.59	2.47±0.02	0.43±0.01	4.37±0.33	11.38±0.77	12.53±0.47

Tab. 3. Continued

Plant species	Plant part	Polyphenols (mg GAEg^{-1} DW)	Flavonoids (mg QE g^{-1} DW)	Tannins (mg CE g^{-1} DW)	Iridoids (mg AE g^{-1} DW)	DPPH (mg GAE g^{-1} DW)	ABTS (mg GAE g^{-1} DW)
Gp (2)	L	36.10±0.45	17.77±0.87	1.49±0.04	125.83±1.79	11.71±0.23	10.59±0.15
	F	11.19±0.13	5.52±0.24	1.16±0.07	8.02±0.43	3.44±0.06	2.36±0.12
	FS	19.50±0.27	12.55±0.21	1.55±0.07	72.79±1.47	5.12±0.22	5.50±0.26
	WS	30.79±0.27	2.78±0.00	0.30±0.01	6.84±0.28	9.65±0.57	12.05±0.06
	UP	30.87±0.47	2.71±0.01	0.30±0.00	4.76±0.02	9.90±0.22	12.07±0.18
Gp (3)	L	30.46±1.57	14.46±0.58	2.38±0.02	110.32±1.14	10.33±0.71	13.24±1.40
	F	10.13±0.23	5.57±0.07	0.56±0.03	9.44±0.29	3.38±0.54	2.20±0.14
	FS	22.38±0.69	11.65±0.13	2.11±0.07	75.59±1.03	8.92±0.08	5.45±0.96
	WS	44.77±0.49	2.84±0.11	0.60±0.00	9.95±0.52	18.33±1.05	16.24±1.37
	UP	35.20±0.83	2.75±0.07	0.41±0.01	3.94±0.08	12.88±0.89	9.39±0.50
Gp average	L	33.96±3.05^{aAB}	15.56±1.91^{aA}	1.85±0.47^{abA}	126.50±16.49^{aA}	12.45±2.58^{bAB}	12.02±1.34^{aAB}
	F	11.78±2.01^{cC}	5.95±0.70^{aC}	0.85±0.30^{aA}	10.91±3.84^{aC}	3.67±0.45^{bC}	2.97±1.19^{cC}
	FS	22.16±2.56^{bcBC}	12.71±1.15^{aB}	1.54±0.57^{bA}	78.38±7.39^{aB}	8.98±3.90^{aBC}	6.16±1.19^{cC}
	WS	36.84±7.18^{aA}	3.11±0.52^{cdD}	0.49±0.17^{bA}	8.44±1.56^{bC}	14.97±4.66^{aA}	15.75±3.48^{aA}
	UP	32.92±2.18^{aAB}	2.64±0.15^{aD}	0.38±0.07^{aA}	4.36±0.41^{aC}	11.39±1.49^{aAB}	11.33±1.70^{aB}

Correlations between phenolic/iridoid content and antioxidant capacity

In this study, all tested samples showed antioxidant activity, which was in very good correlation with observed amounts of total phenolic compounds ($r = 0.86$, $p < 0.001$ for DPPH; $r = 0.83$, $p < 0.001$ for ABTS). Poor correlation was observed between flavonoid content and DPPH radical scavenging capacity ($r = 0.32$, $p < 0.05$) (Tab. 4). *G. alypum* leaves and flowers showed higher antioxidant activity than those of related species in the DPPH assay, while in the ABTS assay only flowers were significantly different ($p < 0.05$) (Tab. 3).

Discussion

Comparison to previous studies of *G. alypum* and evaluation of obtained results

In the present study, the medicinal plant *G. alypum* served as a control species, with which all other investigated species were compared. The reason for this was its broad and well-documented medicinal use together with a number of studies highlighting the high contents of its specialized metabolites and pronounced antioxidant activity. Determination of specialized metabolites was conducted according to the same procedures as those used in previous studies of *G. alypum* and related species (Djeridane et al. 2006, Khlifi et al. 2011, Tundis et al. 2012a, Amessis-Ouchemoukh et al. 2014, Taghzouti et al. 2016, Touaibia and Chaouch 2016) to enable a more reliable comparison with literature data. Amounts of total phenolics and flavonoids observed for *G. alypum* in this study were comparable to those in previously reported studies (Djeridane et al. 2006, Djeridane et al. 2010, Chograni et al. 2012), in which they were expressed in the same manner as in our study, per g dry weight of plant (Djeridane et al. 2006), not per g dry extract, as in some other studies. The latter, unsurprisingly, resulted in several times higher values (Khlifi et al. 2011, Amessis-Ouchemoukh et al. 2014, Taghzouti et al. 2016, Touaibia and Chaouch 2016). The present study also shows that green aerial parts of *Globularia* species contain higher amounts of specialized metabolites, with the exception of total phenolics, which were also observed to be high in woody stems and underground parts of *G. punctata*. Leaves of *Globularia* species were rich in all investigated bioactive substances. This could explain why they are frequently used plant parts in folk medicine (De Natale and Pollio 2012, Bouzabata 2013, El Abbouyi et al. 2014). However, it is important to notice that the use of flowers (Bouzabata 2013) and aerial parts (De Natale and Pollio 2012) is also known, which could be explained by the high amounts of polyphenols found in *G. alypum* flowers and their high antioxidant activity as observed in this study, as well as their recently reported high catalpol content (Sertić et al. 2015) and various biological activities such as antioxidative, anti-inflammatory and acetylcholinesterase inhibitory activity (Amessis-Ouchemoukh et al. 2014).

Two of the most commonly used assays for estimating radical scavenging activity are the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and the 2,2′-azino-bis(3-ethylben-

Tab. 4. Pearson's correlation coefficients between total phenolic, flavonoid, condensed tannin and iridoid content and 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging capacity in different plant parts of four *Globularia* species collected from different locations. n = 48; statistically significant correlations are indicated by an asterisk (*) for $p < 0.05$ and three asterisks (***) for $p < 0.001$.

	DPPH	ABTS
Polyphenols	0.86***	0.83***
Flavonoids	0.32*	0.17
Tannins	0.08	0.05
Iridoids	0.22	0.20

zo (ABTS) assay (Sánchez-Moreno 2002). Both assays were previously used by different authors for estimating the antioxidant capacity of *G. alypum* and showed the species to be a good source of antioxidants (Djeridane et al. 2006, Es-Safi et al. 2007, Djeridane et al. 2010, Khlifi et al. 2011, Chograni et al. 2012). Strong positive correlations between the total phenolic content and antioxidant capacity, similar to those observed in the present study, were also found in earlier studies (Djeridane et al. 2006, Khlifi et al. 2011, Chograni et al. 2012). However, the antioxidant activity observed for *G. alypum* leaves and flowers was not statistically different ($p > 0.05$) from that of previous reports (Chograni et al. 2012, Amessis-Ouchemoukh et al. 2014). Djeridane et al. (2006) noticed higher antioxidant activity in *G. alypum* and other plant extracts in which phenolic acids predominated in comparison to those extracts containing only flavonoids. This could explain why only a poor correlation between flavonoid content and DPPH radical scavenging capacity was found in our study.

Evaluation of medicinal potential of *G. cordifolia*, *G. meridionalis* and *G. punctata*

The high amounts of polyphenols and iridoids found in *G. cordifolia*, *G. meridionalis* and *G. punctata* suggest these species have potentially beneficial health effects similar to those of *G. alypum*. However, it should be noted that the method used for iridoid determination, could only detect the aucubin and asperuloside type iridoids (Trim and Hill 1952), while catalpol derivatives, which are the predominant iridoid glycosides of *G. alypum* (Chaudhuri and Sticher 1981), could not be estimated (Harborne 1998). However, the three species were recently also shown to possess higher amounts of aucubin than *G. alypum*, with *G. punctata* having higher catalpol contents as well (Sertić et al. 2015). High iridoid content in the green aerial parts of *G. cordifolia*, *G. meridionalis* and *G. punctata* observed in this study could be explained by the presence of various aucubin and asperuloside derivatives, which have been previously isolated from these species (Chaudhuri and Sticher 1980, Kirmizibekmez et al. 2003, Tundis et al. 2012b). Indeed, much higher asperuloside amounts have been observed in *G. cordifolia*, *G. meridionalis* and *G. punctata* than in *G. alypum* (Friščić et al. 2016). Similarly to our results, lower amounts of iridoids were previously reported for *G. meridionalis* underground parts in comparison to its aerial parts (Tundis et al. 2012a). Although in most cases, there were no significant differences observed in the amounts of specialized metabolites of the three species, *G. punctata* seems to contain more polyphenols in the woody parts and more flavonoids in the leaves and flower stems. A high catalpol content in the flowers of this species was also recently observed (Sertić et al. 2015). On the other hand, *G. cordifolia* and *G. meridionalis* gave similar results in all performed assays (i.e., no statistically significant differences between the two species were observed). This is not surprising considering their close relationship (Tutin 1972). Although these species could not be distinguished based on the used spectrophotometric assays, it should be noted that the comparison of the proanthocyanidin content indicated a somewhat characteristic chemical composition of the *G. cordifolia* population collected from Mostar. The same phenomenon was observed after an analysis of the essential oil composition of different *Globularia* populations (Crkvenčić et al. 2016).

According to our study, the antioxidant activity of all four species is in good correlation with their total phenolic content. Because *G. cordifolia*, *G. meridionalis* and *G. punctata* are more widely distributed in Europe, it would be interesting to investigate if they have other biological activities that are similar to those of *G. alypum*. Some researches in this field have already yielded promising results. For example, *G. meridionalis* was recently shown to possess inhibitory activity on acetylcholinesterase and butyrylcholinesterase, enzymes representing the targets for the symptomatic treatment of Alzheimer's disease (Tundis et al. 2012a). Iridoid glucoside globularifolin, found both in *G. cordifolia* (Chaudhuri and Sticher 1980) and *G. meridionalis* (Tundis et al. 2012b), was also recently reported to possess immunomodulatory activity (Sipahi et al. 2014).

Production of plant specialized metabolites with respect to environmental factors

Our results confirmed the assumption that phenolic and iridoid concentrations can vary significantly between populations of the same species collected from different locations (Tab. 3) and thus provided a justification for including more populations of each species when comparing different species harvested from natural habitats. As mentioned before, *G. alypum* was presented with only one population. However, the amounts of its specialized metabolites could be compared to numerous literature data.

It is known that environmental factors have a major influence on the synthesis of plant specialized metabolites causing notable differences in the yields of biologically active compounds among different populations. The correlations observed in this study indicate a possible shift in the polyphenolic production and/or translocation of phenolic compounds from the flower stems to the leaves under the influence of increased temperature. On the other hand, the higher tannin content found in all plant parts of *G. cordifolia* from Mostar area could be connected with its location on the humid Neretva river banks, having in mind that all other samples were collected from well-drained areas with full sun exposure. It was previously reported that tannin-rich plants grew mostly on infertile soils with poor drainage (Kraus et al. 2003). Knowing that iridoids play a major role in defending plants against pathogens or herbivores (Dobler 2011), stimuli other than those considered in the present study seem to be more important for their production.

Bearing in mind that, along with the genetically determined variations of metabolite production, plant specialized metabolites represent a way of adaptation to environmental factors (Ghasemzadeh and Ghasemzadeh 2011), the yield of bioactive compounds can be significantly different among

populations. This fact is very important for users of native medicinal plants. Investigations of specialized metabolites with the aim of checking or predicting the medicinal potential of a given plant species should therefore be conducted on several plant populations, when possible. Unfortunately, this is not always the case. The proposed approach could also ensure the selection of the most suitable populations for cultivation and/or the adaptation of growth conditions to those necessary to increase the production of target metabolites. Finally, monitoring the production of plant specialized metabolites could be very important in terms of occurring global climate changes.

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