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Phenolic compounds in two subspecies of *Drypis spinosa* L. (Caryophyllaceae) in Croatia

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Abstract – As a contribution to chemotaxonomic relations, a quantitative analysis of bioactive phenolic compounds was carried out for the first time in *Drypis spinosa* L. subsp. *spinosa* and *D. spinosa* subsp. *jacquiniana* Murb. et Wettst. ex Murb. in Croatia. Total polyphenols (TP), tannins (T) and total flavonoids (TF) were determined in samples of leaves, stems, and roots using UV-Vis spectrophotometric methods. For the subsp. *spinosa*, the highest content of TF was in leaves (0.09%), as was the highest amount of TP (2.36%) and T (1.12%). In the subsp. *jacquiniana*, the highest contents of TF (0.10%), TP (1.96%), and T (0.88%) were measured in stems. Coumaric, ferulic and rosmarinic acid were identified and quantified by HPLC analysis in both subspecies. Quercetin and sinapic acid were identified only in subsp. *spinosa*, while rutin and naringenin were found only in subsp. *jacquiniana*. Among them, ferulic acid was identified only in flowers of both subspecies. The results of this study represent a useful basis for further research of phytochemical and eventually phytotherapeutic potential of *D. spinosa*.

Keywords: *Drypis*, eastern Adriatic, flavonoids, polyphenols, subspecies, tannins

Introduction

The Balkan Peninsula is known to be very rich in endemic plant species. This is the meeting point of two phytogeographic regions: the Alpine – high Nordic regions and the Euro Siberian – North American (Redžić et al. 2011). The genus *Drypis* L. includes one (Wielgorskaya 1995, Erhardt et al. 2014) or two (Euro+Med 2006–2019) species. *Drypis spinosa* L. subsp. *spinosa* (syn. *D. spinosa*, *D. spinosa* subsp. *linneana* Murb. et Wettst.) is distributed in Italy, Slovenia, Croatia, Montenegro, Albania, North Macedonia and Greece, while *D. spinosa* subsp. *jacquiniana* Murb. (syn. *D. jacquiniana* Murb. et Wettst.) is known from Italy, Slovenia and Croatia (Šilić 1990, Stešević and Caković 2013, Euro+Med 2006–2019). Subspecies *spinosa* is a xerothermic, lithophytic species that grows on open, stony or gravelly habitats between mountain and subalpine vegetation belts (Domac 1964, Šilić 1990, Stešević and Caković 2013). On the other hand, subsp. *jacquiniana* is distributed along the eastern Adriatic coast from the close to sea level to 800 m a.s.l. and it grows predominantly on coastal rocks and screes (Domac 1964, Šilić 1990, Jasprica 2015). *Drypis spinosa* is a typical representative of endemic plants with horticultural and possible medicinal potential. Investigation of biologically active compounds in *D. spinosa* is part of our continuous work on Balkan plant species.

Among biologically active compounds, phenolic compounds have attracted a great deal of scientific and public interest due to their health-promoting effects as antimicrobials, antivirals and antioxidants. The content of biologically active compounds varies among species, including closely related species, making them useful chemotaxonomic markers. The plants of the Caryophyllaceae family contain significant amounts of polyphenols, including flavonoids. Ethnopharmacological studies indicate that Caryophyllaceae possess anticancer, antibacterial, antifun-

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gal, antiviral, antioxidant, and anti-inflammatory properties (Chandra and Rawat 2015). This is the reason why the investigations of phenolic compounds were conducted with various species from the Caryophyllaceae family.

The aim of this study is to gain an insight into the content of phenolic compounds in *D. spinosa* subsp. *spinosa* and *D. spinosa* subsp. *jacquiniana* growing in Croatia. To the best of our knowledge this is the first report on the chemical properties of *D. spinosa*.

Materials and methods

Sampling, chemicals and apparatus

The samples of the investigated subspecies were collected during the blooming period in July 2017. Plant material of *Drypis spinosa* subsp. *spinosa* was collected on Mt Velebit at 1200 m a.s.l., while the samples of *D. spinosa* subsp. *jacquiniana* were collected in the town of Rijeka at 70 m a.s.l. Above-ground parts of several randomly selected plants were harvested on a dry day and mixed to obtain a randomly selected sample. The stems, leaves and flowers were collected separately. Herbal material was air-dried in a well-ventilated room at 60% relative humidity and room temperature (22 °C) for three weeks. The plant material was protected from direct sunlight and single-layered during drying. After drying, the plant samples were placed into double paper bags and stored in a dry place at room temperature (22 °C, 60% of humidity), protected from the light until analysis. Voucher specimens of herbal material were deposited in the Fran Kušan Herbarium of the Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia.

Folin-Ciocalteu reagent (FCR), chrysin, quercetin, quercitrin, rutin, chicoric acid, coumaric acid, ferulic acid, protocatechuic acid, rosmarinic acid, syringic acid, tannic acid, linoleic acid, and gallic acid (Sigma-Aldrich Chemical Co., USA); methanol, ethanol, and HNO_3 (Kemika, Croatia) were used. All chemicals and reagents were of analytical grade. Double distilled water was used throughout the study.

Agilent 8453 E UV/Vis spectrophotometer (Hewlett Packard, Germany) equipped with the PC-HP 845x UV-Visible System (Hewlett Packard, Germany) and 1 cm quartz cells were used for absorbance measurements. HPLC analysis was performed using Agilent 1100 Series HPLC system and A C_{18} reversed-phase packing column (Zorbax Eclipse XDB-C_{18}, 150 mm × 4.6 mm, 5 μm; Agilent, USA).

Phenolic compounds analyses

The quantitative analysis of biologically active phenolic compounds was carried out using spectrophotometric methods, following adequate extractions.

Spectrophotometric determination of total polyphenols (TP) and tannins (T) was performed with the use of FCR. Comprehensive prevalidation of this method was carried out by Jurišić Grubešić et al. (2005). This method is based on a reaction with FCR and, after precipitation with casein, spectrophotometric determination of TP and T at 720 nm. Tannic acid was used as the standard substance.

The content of total flavonoids (TF; colorimetric assay with $AlCl_3$) was obtained using spectrophotometric method prevalidated by Jurišić Grubešić et al. (2007). This procedure encloses hydrolysis of glycosides, extraction (with ethyl acetate) of TF aglycones and complex formation with $AlCl_3$ at 425 nm. The yield of TF was calculated as quercetin according to the following expression:

$$TF\ (\%) = A \times 0.772 / b$$

In this expression, A denotes measured absorbance, 0.772 is the conversion factor related to the specific absorbance of quercetin at 425 nm, while b is mass (g) of air-dried herbal material.

TP, T and TF contents were performed in triplicate (technical replicates) and the results were expressed as mean ± SD.

HPLC analysis

The ultrasonic extraction was performed on finely powdered air-dried herbal material (500 mg) with 20 mL of methanol at 25 °C for 60 min, then 20 mL of ethyl-acetate at 25 °C for 60 min, and finally with 20 mL of distilled water at 25 °C and 45 °C for 60 min. After that, samples were filtered (qualitative analytical filter paper) and diluted with the same solvent mixture to a volume of 25.0 mL. An aliquot of 5 μL of each sample solution was injected into the HPLC system for chromatographic analysis. Prior to injection samples were filtered through 0.45 μm PTFE 25 mm filter (Restek Co., USA).

The stock solutions of standard compounds: chrysin, quercetin, quercitrin, rutin, chicoric acid, coumaric acid, ferulic acid, protocatechuic acid, rosmarinic acid, syringic acid and tannic acid were prepared according to Čeh et al. (2007) and Kremer et al. (2013). At first, the stock solutions of standards were diluted separately in the mixture of water and methanol (1:1, V/V) at a concentration of 1.0 mg mL^{-1}. After that, the working standard solutions (at a concentration of 0.01 mg mL^{-1}) were made by the dilution of each stock solution with the same mixture of water and methanol. The mixture of standards was made by the dilution of each stock standard solution with the same solvent mixture at final concentration of each standard of 0.01 mg mL^{-1}.

HPLC analysis was performed according to Čeh et al. (2007) and Kremer et al. (2013). A C_{18} reversed-phase packing column was used for separation at the temperature of 30 °C. A gradient elution type of chromatographic systems was used. The mobile phase for gradient elution was composed of phase A (water, pH = 2.50 adjusted with acetic acid), and phase B (acetonitrile), which was applied according to the following elution scheme: starting with 15% B and 85% A, followed with gradient from 15% B to 22.5% B in 15 min, then from 22.5% B to 40% B in 10 min and kept constant with 40% B and 60% A for another 5 min. In the addition 5 min, the initial conditions were set up. The injec-

Tab. 1. Contents (mean ± SD) of total polyphenols (TP), tannins (T) and flavonoids (TF) in *Drypis spinosa* subsp. *spinosa* and *D. spinosa* subsp. *jacquiniana* obtained by UV-Vis spectrophotometric methods. The asterisk (*) in superscript indicates a significant difference obtained by LSD test for content of TP, T and TF between different plant parts (leaf, flower, stem) of subsp. *spinosa* and subsp. *jacquiniana* at $P < 0.05$. N = 3.

Phenolic compund (%)	Leaf		Flower		Stem	
	subsp. *spinosa*	subsp. *jacquiniana*	subsp. *spinosa*	subsp. *jacquiniana*	subsp. *spinosa*	subsp. *jacquiniana*
TP	2.357 ± 0.094*	1.773 ± 0.032	1.810 ± 0.035	1.808 ± 0.016	1.698 ± 0.137	1.955 ± 0.040
T	1.119 ± 0.081*	0.350 ± 0.113	0.557 ± 0.021	0.468 ± 0.113	0.503 ± 0.144*	0.895 ± 0.050
TF	0.092 ± 0.006*	0.057 ± 0.003	0.073 ± 0.002*	0.056 ± 0.002	0.063 ± 0.005*	0.097 ± 0.006

tion volume was 5 μL, while flow rate was 1.0 mL min^{-1}. Detection was performed by diode array detector at wavelengths 280 nm (chrysin, chicoric acid, syringic acid, protocatechuic acid, tannic acid), 320 nm (ferulic acid, rosmarinic acid, coumaric acid) and 370 nm (quercetin, quercitrin and rutin). Particular compounds were identified by comparing the retention times of standards and unknown peaks in the samples. The method of standard addition was applied in order to avoid misinterpretation of results. Quantification was conducted using the external standards.

Method was previously validated to confirm the linear range, reproducibility (RSD, between 3.8% and 8.6%) and accuracy (R, 76% to 87%) for particular compounds (detailed data not shown here). Limit of quantification (LOQ) was determined at the concentration of 0.001%.

Data analysis

Significance of differences between analytical data was checked by the least significant difference (LSD) test, using the STATISTICA software (Hill and Lewicki 2006).

Results

Total polyphenols (TP), tannins (T) and flavonoids (TF) content

UV-Vis spectrophotometric methods were used for a quantitative analysis of TP, T and TF in *D. spinosa* subsp. *spinosa* and *D. spinosa* subsp. *jacquiniana*. The results of the determinations of TP, T and TF are presented in Tab. 1. For *D. spinosa* subsp. *spinosa*, the highest content of TP (2.36%) was in leaves, as was the highest amount of T (1.12%) and TF (0.09%). In *D. spinosa* subsp. *jacquiniana*, the highest contents of TP (1.96%), T (0.88%) and TF (0.10%) were measured in stems. It is evident that there was no dependence among plant parts and the quantity of investigated phenolic substances.

The largest statistically significant differences in the content of all analyzed polyphenolic substances were observed for leaves of subsp. *spinosa* and subsp. *jacquiniana*, while the least significant difference in the content of polyphenols was noticed among flowers of the investigated plant subspecies (Tab. 1).

HPLC analysis

Seven phenolic compounds were identified and quantified by HPLC analysis in *D. spinosa* subsp. *spinosa* and *D. spinosa* subsp. *jacquiniana* and their concentrations in leaves, flowers and stems are shown in Tab. 2. Among standard compounds only caffeic acid was not identified in the investigated subspecies. Content of phenolic compounds varied between 0.002% (coumaric acid) and 0.839% (naringenin). Among them, quercetin and sinapic acid were identified only in subsp. *spinosa*, while rutin and naringenin were identified only in subsp. *jacquiniana*. On the other hand, coumaric acid, ferulic acid and rosmarinic acid were found in both subspecies. Ferulic acid was identified only in flowers of both subspecies.

Discussion

Quantitative analysis of TP, T and TF was performed with the leaves, flowers and stems of *Drypis spinosa* subsp. *spinosa* and *D. spinosa* subsp. *jacquiniana*. Obtained results showed that there was no dependence among plant parts and the quantity of the phenolic substances investigated.

Tab. 2. Contents of phenolic compounds (%) in dried plant material of *Drypis spinosa* subsp. *spinosa* and *D. spinosa* subsp. *jacquiniana* obtained by HPLC analysis. – : not detected, N = 1.

Phenolic	subsp. *spinosa*			subsp. *jacquiniana*		
compound (%)	leaf	flower	stem	leaf	flower	stem
Rutin	–	–	–	–	–	0.047
Quercetin	–	–	0.008	–	–	–
Naringenin	–	–	–	–	0.839	–
Sinapic acid	0.017	–	0.004	–	–	–
Coumaric acid	–	–	0.004	0.002	0.004	–
Ferulic acid	–	0.060	–	–	0.066	–
Rosmarinic acid	0.041	0.043	0.041	–	0.030	–

This is in accordance with some other investigations. For example, *Satureja montana* L. and *S. subspicata* Vis. (Lamiaceae) had the highest TP content in leaves (Dunkić et al. 2012), while *Moltkia petraea* (Tratt.) Griseb. (Boraginaceae) had the highest TP content in flowers (Vuković Rodríguez et al. 2016). Additionally, investigations of TP content in ten *Moltkia petraea* populations obtained by Kremer et al. (2016) showed that TP content was the highest in leaves in six populations. But in another four populations the TP content was the highest in flowers. Both subspecies of *D. spinosa* contain relatively small amounts of TP, which ranged from 1.70% in the stems to 2.36% in the leaves of *D. spinosa* subsp. *spinosa*. For comparison, TP content ranged from 4.43% in stem to 12.41% in leaves of *Satureja montana*, and from 5.96% in stem to 20.04% in leaves of *S. subspicata* (Dunkić et al. 2012). Additionally, TP content in *Moltkia petraea* ranged from 3.26% in stem to 6.61% in leaves (Kremer et al. 2016).

A similar independence among plant parts and the quantity of investigated phenolic substances was obtained for T content in *Drypis spinosa*. The highest T content was obtained for the leaves and flowers of *D. spinosa* subsp. *spinosa* and *D. spinosa* subsp. *jacquiniana*, respectively. According to Dunkić et al. (2012), leaves of *Satureja montana* and *S. subspicata* generally contained higher concentrations of T than stems and flowers. Content of T varied between 0.08% in stem and 4.90% in leaves of *S. montana*, and between 0.21% in stem and 6.95% in leaves of *S. subspicata*. The lowest T content (0.76%) was also measured in stems and the highest (7.33%) in leaves of *Teucrium arduinii* L. (Kremer et al. 2013). On the other hand, the highest T content was determined in the flowers in most of the ten investigated populations of *Moltkia petraea* (Kremer et al. 2016). The content of catechins in *Stellaria media* (L.) Vill. (Caryophyllaceae), a herb obtained from five Ukrainian populations ranged from 0.67% to 0.90% (Vodoslavskyi 2017).

The content of TF was the lowest among three investigated groups of phenolic compounds. Dunkić et al. (2012) found that the lowest TF content was in the stem of *Satureja montana* (0.06%), and the highest in leaves (0.40%). That investigation also showed that samples of leaves and flowers of *S. montana* have a comparable quantity of TF. The lowest TF content was measured in *S. subspicata* stem (0.07%), and the highest in the leaves (0.57%). Additionally, the highest TF content was identified in leaves in all *Moltkia petraea* populations (Kremer et al. 2016). Although some exceptions exist, the literature data show that stems contain the lowest TF content. The content of TF was also analyzed in five Ukrainian populations of *Stellaria media* herb, ranging from 1.22% to 1.40% (Vodoslavskyi 2017).

The most intensive investigations of the Caryophyllaceae family have been carried out on species with medical properties. These investigations showed that Caryophyllaceae contain a great number of bioactive compounds belonging to several chemical groups. Seven phenolic compounds were identified in *D. spinosa* subsp. *spinosa* and *D. spinosa* subsp. *jacquiniana* using HPLC analysis. The same phenolic compounds were also identified in some other Caryophyllaceae species. Quercetin was identified in *Silene littorea* Brot (Richardson 1978) and *Arenaria serpyllifolia* L. (Zhou and Tu 2013), while naringenin was detected in *Dianthus caryophyllus* L. (Spribille and Forkmann 1982). Teğin at al. (2018) identified naringenin and rutin in *Spergularia rubra* (L.) J. Presl et C. Presl. Rutin, ferulic acid, caffeic acid and p-coumaric acid were detected in *Lychnis flos-cuculi* L. (Ferry and Darbour 1979, Costea et al. 2017). Ferulic acid was also identified in *Stellaria media* (Kitanov 1992), while p-coumaric acid was detected in *Gypsophila paniculata* L. (Chou et al. 2008).

Conclusion

The content of different phenolic compounds was determined in leaves, flowers and stems of *Drypis spinosa* subsp. *spinosa* and *D. spinosa* subsp. *jacquiniana* growing in Croatia. There was no dependence among plant parts and the quantity of investigated phenolic substances. Seven phenolic compounds were identified and quantified by HPLC analysis and their content ranged between 0.002% (coumaric acid) and 0.839% (naringenin). Quercetin and sinapic acid were identified only in subsp. *spinosa*, while rutin and naringenin were detected only in subsp. *jacquiniana*. Further investigations will show if these phenolic compounds really are specific for one or another subspecies and if it is possible to use them as valuable chemotaxonomic markers.

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