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Sex-related Differences in the Dioecious Species *Rumex thyrsiflorus* Fingerh. – Analysis of the Content of Phenolic Constituents in Leaf Extracts

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Rumex thyrsiflorus Fingerh. is mentioned as a European folk medicinal plant. This species has also been traditionally used as an edible plant in Eastern Europe because of its nutritional value. During the study, qualitative and quantitative sex-related differences of phenolic constituents in methanolic leaf extracts of *R. thyrsiflorus* were evaluated. The presence of the same substances (nine phenolic acids before, and six phenolic acids after acid hydrolysis, nine flavonoids, and a catechin) was estimated in both female and male specimens, using the HPLC-DAD method. A statistically significant higher content of eleven constituents in female plant extracts (acids: chlorogenic, *p*-coumaric, cryptochlorogenic, gallic, protocatechuic, neochlorogenic, vanillic; flavonoids: quercitrin, rhamnetin, rutoside; and a catechin) was shown. This is the first report concerning the relation between the sex and the content of biologically active phenolic secondary metabolites in leaf extracts of *R. thyrsiflorus*. Female plants of *R. thyrsiflorus* could be useful for pharmaceutical purposes as a preferential source of bioactive phenolic acids, flavonoids and especially catechin.

Keywords: *Rumex thyrsiflorus*, thyrse sorrel, dioecy, sex-related differences, sexual dimorphism in plants, phenolic compounds, phytochemical analysis, HPLC-DAD

INTRODUCTION

Interesting phenomena that can be observed in many dioecious species include a biased sex ratio and sex-related differences between males and females. Despite the theoretical expectation of an equal offspring (primary) sex ratio, aberrations from this assumption are widespread (Hardy, 2002). Additionally, the biased sex ratio occurs at different stages of the life cycle. Various mechanisms (both pre- and postzygotic) are postulated to explain this deviation concerning, e.g., differences in germination, frequency of flowering, sex-related response to different environmental factors, etc. (Che-Castaldo et al., 2015; Korpelainen, 2002). Stehlik and Barret (2005) reported that in dioecious plants, females may incur higher reproductive costs compared to males because of seed production. As a result, the reduced growth and delayed flowering can be observed in the population. Also, Field et al. (2012) revealed that a male bias occurs more often than a female bias in dioecious flowering plants. However, in species with heteromorphic sex chromosomes the female-

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biased sex ratios are more frequent (Lloyd, 1974). Studies on both the primary and secondary sex ratio of natural *Rumex thyrsiflorus* populations show a female bias (Kwolek and Joachimiak, 2011; Rychlewski and Zarzycki, 1981,1986; Zarzycki and Rychlewski, 1972). Moreover, *in vitro* experiments conducted by Ślesak et al. (2017) revealed a tendency to higher ability in morphogenetic reaction in female explants compared to male ones. Sex-related differences in dioecious species are frequently reported. They concern the dynamics of resource allocation, growth rate or carbohydrate storage patterns (DeSoto et al., 2016; Teitel et al., 2016). According to Petry et al. (2016), ecological differences between the sexes in response to climate change can also be observed.

Furthermore, researchers noticed sexual dimorphism in some physiological traits, such as photochemical capacity, activity of antioxidant enzymes, stress tolerance or the content of secondary metabolites (Juvany and Munné-Bosch, 2015; Maldonado-López et al., 2014; Massei et al., 2006; Robakowski et al., 2018). Additionally, Sánchez Vilas et al. (2016) showed evidence of sexually dimorphic responses to growth in metal-polluted soil.

The species of the genus *Rumex* (sorrel, dock) are well-known for their traditional uses. Their representatives are the subject of phytochemical and pharmacological research (Simkova and Polesny, 2015; Sõukand et al., 2017). The tissues of sorrel are rich in anthraquinones, naphthalenes, stilbenoids, triterpenoids, and phenolic compounds such as flavonoids, phenolic acids and tannins (Vasas et al., 2015; Wegiera et al., 2007). Its diverse pharmacological activities that include antimicrobial, antiproliferative, antitumor and detoxifying ones are due to the presence of various biologically active substances. The known antioxidant and anti-inflammatory activity is attributed mainly to phenolic compounds (Orbán-Gyapai et al., 2015, 2017b; Page and Schwitzguébel, 2009; Wegiera et al., 2012). Still, while the phytochemical studies focusing on the genus *Rumex* are numerous, data on *R. thyrsiflorus* are scarce.

Rumex thyrsiflorus Fingerh. (thyrse sorrel) is a perennial herb occurring in a temperate climate, in dry and moderately moist areas in Europe, Asia and North America (eFloras, 2008). As one of the few dioecious plant species with heteromorphic sex chromosomes: females (F) 2n = 12A + XX, males (M) 2n = 12A + XY_1Y_2 (Żuk, 1963), it has been the subject of studies on the structure and functions of sex chromosomes in plants (Löve and Kapoor, 1967; Zaborowska, 1969; Żuk, 1969). Nowadays, researchers focus mainly on the evolutionary and molecular aspects of *R. thyrsiflorus* biology (Grabowska-Joachimiak et al., 2012; Ślesak et al., 2015). However, ethnobotanists have categorized this species as a popular wild food plant in Eastern Europe (Łuczaj et al., 2013), and it is also mentioned as a folk medicinal plant (Jędrzejko, 2001).

The phytochemical investigations of extracts from *R. thyrsiflorus* plant raw material demonstrate the presence of anthraquinones, and especially phenols – such as phenolic acids, flavonoids and tanning agents (Litvinenko and Muzychkina, 2008).

Based on these reports and taking into account the above mentioned female-biased sex ratios of *R. thyrsiflorus*, the following question arises: are there qualitative and quantitative phytochemical differences in the content of selected phenolic secondary metabolites between male and female specimens of *R. thyrsiflorus*?

MATERIALS AND METHODS

PLANT MATERIAL

Leaves of *Rumex thyrsiflorus* Fingerh. were collected in Cracow, Poland (50°01'57.6"N, 19°49'45.4"E) during the flowering time (June 2017) from a wasteland. Botanical identification of the material was performed based on voucher specimens (female No. 6088/0191516, male No. 6088/028854) that are deposited at the Herbarium of the Institute of Botany, Jagiellonian University, Cracow, Poland. The plant name was checked according to The Plant List database (2013). The sex of plants was established based on the inflorescence and then confirmed by molecular analysis as described by Ślesak et al. (2017).

HPLC-DAD ANALYSIS

R. thyrsiflorus samples were dried by lyophilization (Labconco, USA), then powdered. Samples (0.2 g dry wt) were extracted with methanol (5 mL) as described before (Szopa et al., 2017b). To estimate the bound phenolic acids, the analyzed material (0.2 g of lyophilized and powdered tissue) was prepared according to the following procedure: 1) boiling in a water bath with 5 mL 2M hydrochloric acid (Avantor Performance Materials, Poland) for 1 hour (Harborne, 1984); 2) cooling, centrifugation –

8 min., 4000 rpm; 3) transfer of the supernatant to new tubes; 4) adding ethyl acetate (Avantor Performance Materials, Poland) to the supernatant in a 1:1 ratio; 5) shaking; 6) centrifugation of samples – 4 min., 4000 rpm; 7) casting the ethyl acetate fraction to the new tubes; 8) repeating points 5–8 three times; 9) evaporation of ethyl acetate (TurboVap® LV, Caliper Life Sciences, USA), 10) adding 2 mL of methanol for preparative chromatography (Merck for liquid chromatography LiChrosolv®); 11) sonification (Sonic 5, Polsonic, Poland) – 10 min.; 12) centrifugation – 4 min., 4000 rpm; 13) purification of the supernatant by syringe filters (pore diameter = 0.22 μm, Equimed, Poland).

For analyses was applied the HPLC-DAD method according to Ellnain-Wojtaszek and Zgórka (1999), Sulkowska-Ziaja et al. (2017) and Szopa et al. (2017a, 2017b) using a Merck-Hitachi liquid chromatograph (LaChrom Elite) with a DAD detector (L-2455) in a UV range of 200–400 nm (detection wavelength for all compounds was set at 254 nm). The Purospher RP-18e (250 × 4 mm; 5 μm, Merck) column was used, and the temperature was set at 25°C. The mobile phase consisted of A – methanol, B – methanol: 0.5% acetic acid 1:4 (*v*/*v*). The gradient was as follows: 100% B for 0–20 min; 100–80% B for 20–35 min; 80–60% B for 35–55 min; 60–0% B for 55–70 min; 0% B for 70–75 min; 0–100% B for 75–80 min; 100% B for 80–90 min with a flow rate (1 mL/min). The injection volume was 10 μL. The applied HPLC method was previously validated by our group (Sulkowska-Ziaja et al., 2017). The tested parameters were the following: accuracy; precision at three levels of standard substance concentrations in solution, 50%, 100%, and 150%; linearity; limit of detection (LOD); and limit of quantification (LOQ). Identification of compounds was performed either by comparison with UV spectra and retention times of reference substances or using co-chromatography. The compounds were quantified using the calibration curves method.

For analyses were used the following standards – including benzoic acid and related derivatives: ellagic, gallic, 3,4-dihydroxyphenylacetic, protocatechuic, gentisic, *p*-hydroxybenzoic, salicylic, vanillic, syringic acids, in addition to cinnamic acid and related derivatives such as caffeic, coumaric, ferulic, *o*-coumaric, *m*-coumaric, *p*-hydrocaffeic, isoferulic, sinapic acids, and depsides (chlorogenic, rosmarinic and neochlorogenic acids). Catechins included catechin, epicatechin, epigallocatechin gallate, epicatechin gallate and epigallocatechin. Flavonoid standards included aglycones (kaempferol, myricetin, quercetin, luteolin, and rhamnetin) and glycosides (apigetrin, cynaroside, robinin, hyperoside, isoquercetin, quercitrin, rutoside, trifolin, vitexin). The standards were obtained from Sigma-Aldrich, Germany.

STATISTICAL ANALYSIS

Results of 36 measurements (three measurements for each of the twelve individuals of the given sex) were expressed as mean values ± SD. The differences between means were analyzed with a two-tailed t-test. *P* values less than 0.01 were considered statistically significant.

RESULTS AND DISCUSSION

The genus *Rumex* is known to be rich in anthraquinones, naphthalene-1,8-diols, flavonoids and stilbenoids (Vasas et al., 2015). Tissues of *Rumex acetosa*, a dioecious species closely related to *R. thyrsiflorus*, are characterized by the presence of phenolic compounds – resveratrol, vanillic acid, sinapic acid and a catechin (Kucekova et al., 2011), besides anthraquinones (Lee et al., 2005, Wegiera et al., 2007), and oligomeric proanthocyanidins (Gesher et al., 2011).

In the studied *R. thyrsiflorus* leaf extracts, the following constituents were identified: nine free phenolic acids (caffeic, chlorogenic, *p*-coumaric, cryptochlorogenic, ferulic, gallic, protocatechuic, neochlorogenic, vanillic), nine flavonoids (apigenin, hyperoside, isoquercetin, myricetin, quercetin, quercitrin, rhamnetin, rutoside, vitexin) and a catechin. Additionally, in the methanolic extracts after the acid hydrolysis, two phenolic acids: *p*-hydroxybenzoic, syringic, besides caffeic, gallic, protocatechuic and vanillic ones were detected (Table 1, Fig. 1). The amounts of individual phenolic acids ranged from 1.24 mg/100 g dry wt (syringic acid) to 37.80 mg/100 g dry wt (protocatechuic acid, after hydrolysis) and from 1.39 mg/100 g dry wt (syringic acid), to 49.05 mg/100 g dry wt (neochlorogenic acid) in male and female plants, respectively. For flavonoids, their individual amounts ranged from 7.07 mg/100 g dry wt (vitexin) to 181.73 mg/100g dry wt (hyperoside) and from 7.72 mg/100g dry wt (vitexin) to 218.80 mg/100 g dry wt (hyperoside) in male and female plants, respectively (Table 1). The quantita-

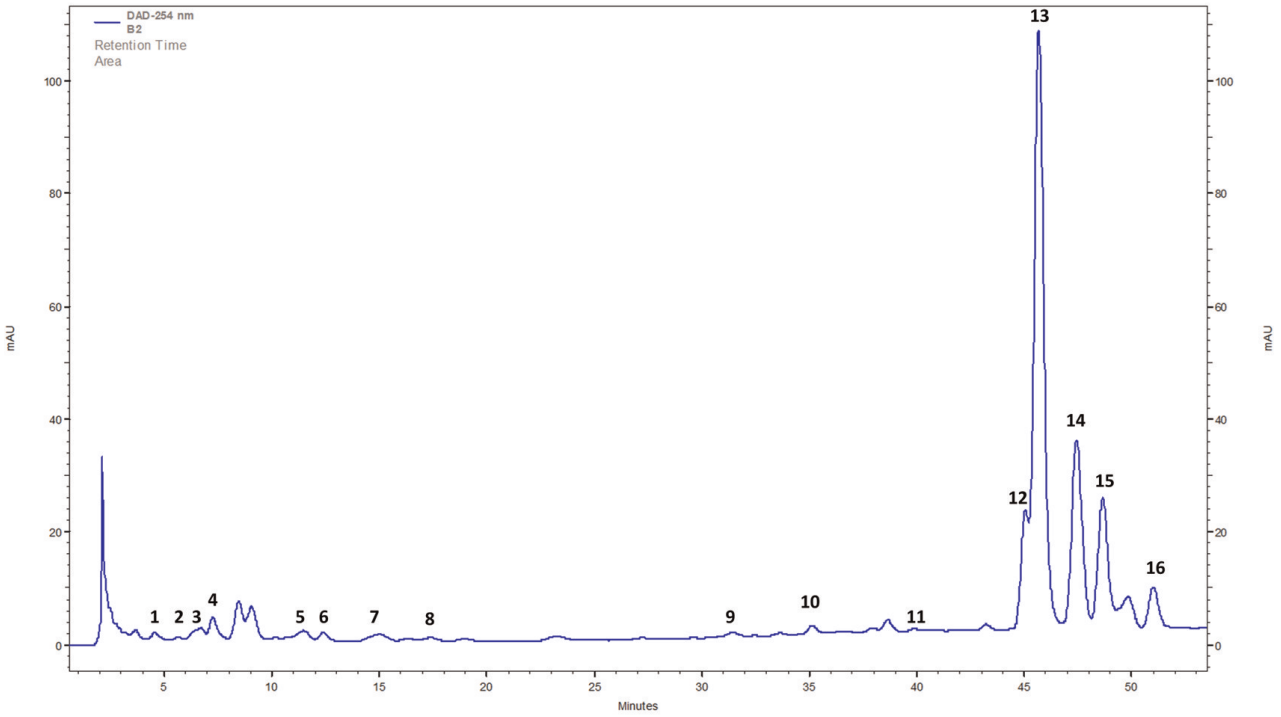

Fig. 1. HPLC-DAD chromatogram of investigated phenolic compounds – an example. Methanolic extract from leaves of male *Rumex thyrsiflorus* plant: 1 – gallic acid, 2 – neochlorogenic acid, 3 – protocatechuic acid, 4 – catechin, 5 – chlorogenic acid, 6 – cryptochlorogenic acid, 7 – vanillic acid, 8 – caffeic acid, 9 – p–coumaric acid, 10 – ferulic acid, 11 – vitexin, 12 – rutoside,13 – hyperoside, 14 – isoquercetin, 15 – myricetin, 16 – quercitrin.

TABLE 1. Differences between average content [mg/100 g dry wt] of secondary metabolites in leaf extracts of male and female specimens of *Rumex thyrsiflorus*.

Secondary metabolite		**Average content [mg/100 g dry wt] (standard deviation)**		**Difference male – female (*P*, t-test)**
		Male	**Female**	
Free phenolic acids	Caffeic acid	5.06 (1.19)	6.72 (1.82)	0.0148
	Chlorogenic acid*	12.73 (3.34)	24.96 (9.88)	0.0004
	p-Coumaric acid*	4.55 (1.11)	8.18 (2.74)	0.0003
	Cryptochlorogenic acid*	24.35 (5.64)	34.52 (8.45)	0.0022
	Ferulic acid	5.14 (1.13)	4.44 (1.43)	0.2157
	Gallic acid*	4.03 (1.03)	9.10 (2.39)	0.0001
	Protocatechuic acid*	5.21 (1.24)	10.50 (3.05)	0.0001
	Neochlorogenic acid*	29.49 (7.28)	49.05 (12.91)	0.0001
	Vanillic acid*	4.66 (1.12)	7.31 (1.95)	0.0005

Secondary metabolite		Average content [mg/100 g dry wt] (standard deviation)		Difference male – female (*P*, t-test)
		Male	Female	
Bound phenolic acids	Caffeic acid	2.86 (1.33)	4.16 (1.74)	0.0518
	Gallic acid	5.31 (2.34)	6.16 (2.56)	0.4050
	p-Hydroxybenzoic acid	6.12 (2.36)	5.92 (2.33)	0.8364
	Protocatechuic acid	37.80 (16.32)	39.21 (17.29)	0.8391
	Syringic acid	1.24 (0.60)	1.39 (0.57)	0.5663
	Vanillic acid	4.94 (1.92)	4.58 (1.85)	0.6446
Flavonoids	Apigenin	26.91 (6.31)	29.38 (6.79)	0.3775
	Hyperoside	181.73 (44.85)	218.81 (51.86)	0.0744
	Isoquercetin	44.27 (12.75)	40.44 (10.25)	0.4260
	Myricetin	101.57 (23.96)	101.69 (25.49)	0.9906
	Quercetin	24.43 (6.00)	29.93 (8.30)	0.0763
	Quercitrin*	50.01 (11.81)	69.89 (17.67)	0.0038
	Rhamnetin*	15.77 (3.95)	31.89 (8.72)	0.0001
	Rutoside*	39.76 (10.16)	69.12 (17.54)	0.0001
	Vitexin	7.07 (1.71)	7.72 (2.04)	0.4067
	Catechin*	49.61 (13.62)	162.18 (45.64)	0.0001

* indicates significant differences between means at least at the level of $P < 0.01$

tively dominant metabolite in both sexes was hyperoside (M: 181.73 mg/100g dry wt, F: 218.80 mg/100 g dry wt). Previously, the presence of phenolic compounds such as phenolic acids (caffeic, gallic, *p*-hydroxybenzoic), flavonoids (isorhamnetin, isorhamnetin-3-*O*-rutoside, myricetin, myricetin-3-*O*-rhamnoside, rutoside, quercetin), catechins (catechin, epicatechin gallate) and other phenols (hydroquinone, pyrogallol, phloroglucinol) in the root extracts of *R. thyrsiflorus* was confirmed (Litvinenko and Muzychkina, 2008). However, prior to our work, Orbán-Gyapai et al. (2017a) had researched the n-hexane, chloroform and ethyl acetate soluble fractions of the methanolic extract obtained from the underground parts of this species. In these extracts, epicatechin, palmitoylglycerol, β-sitosterol and procyanidin B5 were barely confirmed. The mentioned author reports that extracts of this species demonstrate considerable cell growth inhibitory activity against two human cancer cell lines: A431 and MCF7 at a concentration of 30 μg/mL, and against four bacterial strains (*Bacillus subtilis, Moraxella catarrhalis, Staphylococcus epidermidis* and *S. aureus*). Herein, xanthine oxidase inhibitory action of the aqueous fraction of both the herb and the roots was notable at IC50 = 78.45 μg/mL and IC50 = 39.25 μg/mL, respectively.

In our study, the sex of plants belonging to species *R. thyrsiflorus* was taken into consideration during the phytochemical analysis. That was done, to the best of our knowledge, for the first time. Overall, in the obtained results, there was no qualitative diversity between male and female specimens. Still, statistically important quantitative differences were confirmed. Our analysis

revealed that female plants have a statistically higher content of eleven natural products, such as free phenolic acids: chlorogenic, *p*-coumaric, cryptochlorogenic, gallic, protocatechuic, neochlorogenic, vanillic; flavonoids: quercitrin, rhamnetin, rutoside, and a catechin (Table 1). In female plants, about two-fold higher contents of the following compounds from phenolic acids were estimated: chlorogenic (M: 12.73 mg/100 g dry wt, F: 24.96 mg/100 g dry wt), *p*-coumaric (M: 4.55 mg/100 g dry wt, F: 8.18 mg/100 g dry wt), gallic (M: 4.03 mg/100 g dry wt, F: 9.10 mg/100 g dry wt) and protocatechuic (M: 5.21 mg/100 g dry wt, F: 10.50 mg/100 g dry wt), and also from flavonoids for rhamnetin (M: 15.77 mg/100 g dry wt, F: 31.89 mg/100 g dry wt). The highest quantitative differences were noticed in the case of catechin; a three-fold higher content in female plants (162.18 mg/100 g dry wt) than in male ones (49.61 mg/100 g dry wt) (Table 1). It is known that catechins have direct antioxidant activities and they can act as free radical scavengers (for review see Bernatoniene and Kopustinskiene, 2018). Ding et al. (2019) revealed, e.g., the involvement of catechins as important non-enzymatic antioxidants in tea plant cold responses. Based on this, it could be hypothesized, that female plants of *R. thyrsiflorus* could be more stress-tolerant compared to males. This is in agreement with reports about the impact of environmental factors on higher male mortality (Zarzycki and Rychlewski, 1972).

The differences in the content of some secondary metabolites between sexes were found in many dioecious plants, for example in *Taxus baccata* (female-biased amount of two taxanes) (Iszkuło et al., 2013) or *Juniperus oxycedrus macrocarpa* (male-biased amount of both phenolics and terpenoids) (Massei et al., 2006). It is postulated, that sexual dimorphism in plants is associated with different costs of reproduction of males and females (Obeso, 2002). Additionally, the amount of resources that are assigned to three functions: vegetative growth, reproduction and defence is finite (Charnov, 1982), which causes trade-offs between the allocation of these resources (Herms and Mattson, 1992).

To conclude, this is the first report concerning the relation between the sex and the content of biologically active phenolic secondary metabolites in leaf extracts of *R. thyrsiflorus*. The results obtained during our research give the opportunity to preferentially use female plants of *R. thyrsiflorus* for pharmaceutical purposes in order to obtain bioactive phenolic acids, flavonoids and especially catechin more economically. These results could be a premise for conducting future research on the sex-related differences in other physiological traits (for example a wider range of secondary metabolites or nutritional quality) and their relationship to biotic and abiotic stress.

An advanced investigation, concerning the phytochemical sex-specific differences under *in vitro* conditions and the influence of the medium composition on biologically active compounds production is in progress.

AUTHORS' CONTRIBUTIONS

KD: design of the study, plant material collection, acquisition of data, HPLC-DAD analysis, data interpretation, writing the manuscript; AS: HPLC--DAD analysis, interpretation of data, writing the manuscript; PW: preparation of samples for HPLC--DAD analysis; HE: proofreading of the manuscript; HŚ: design of the study, plant material collection, acquisition of data, statistical analysis, proofreading of the manuscript

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