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Phenolic compounds in *Geranium dalmaticum* (Beck) Rech. f. and G. *macrorrhizum* L. (Geraniaceae) growing in Croatia

Dario Kremer[1], Iztok Jože Košir[2], Marina Šimunić[1], Ksenija Karlović[3]†,
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Abstract

The quantitative analysis of bioactive phenolic compounds was carried out for the first time in above-ground plant parts of *Geranium dalmaticum* (Beck) Rech. f. and *G. macrorrhizum* L. (Geraniaceae) from Croatia by means of UV-Vis spectrophotometric methods and HPLC analysis. Both species had similar contents of polyphenolic substances expressed as mg of standard equivalent /g of dried herbal sample: total polyphenols (217.60 ± 1.08 and 215.53 ± 1.10 mg/g), tannins (155.83 ± 0.60 and 157.73 ± 0.61 mg/g), total flavonoids (5.10 ± 0.10 and 5.53 ± 0.10 mg/g), and total phenolic acids (13.27 ± 1.34 and 15.33 ± 0.45 mg/g), respectively for *G. dalmaticum* and *G. macrorrhizum*. Only the content of phenolic acids was somewhat higher in *G. macrorrhizum* but it was not statistically significant ($p > 0.05$). HPLC analysis showed that, among seven tested phenolic compounds, only quercetin was quantified in *G. dalmaticum* (0.23 % of dry weight), and rutin in *G. macrorrhizum* (1.12 % of dry weight). The obtained results represent a useful basis for further research of phytochemicals and biological activities of *G. dalmaticum* and *G. macrorrhizum* extracts.

Key words: *Geranium*, eastern Adriatic, polyphenols, flavonoids, phenolic acids.

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Introduction

Even today, domestic species still represent a significant source from which specimens can be selected for planting for decorative purposes, especially in urban areas. In the light of current climate changes, species that tolerate climatic extremes, especially drought, are particularly interesting. Domestic species are also important as a food source for many organisms, such as pollinating insects. Furthermore, some of these species are also interesting because of their phytochemical traits. *Geranium* L. species (family Geraniaceae) are aromatic and decorative species, and some of them are used in horticulture as well as in traditional medicine (Şöhretoğlu et al., 2017). One of the most interesting *Geranium* species in Croatia is *Geranium dalmaticum* (Beck) Rech. f. (Fig. 1a–c). It is a perennial herb up to 15 cm high which grows in Mediterranean-mountainous area on screes, at the edges and on the sides of the sinkhole and scrapes, in crevices of limestone and calcareous rocks at altitudes from 200 m to 960 m. *G. dalmaticum* occurs within the eastern Adriatic scrub (*Cisto-Ericetalia* Horvatić, 1958) and black pine forests on dolomite (*Erico-Fraxinion orni* Horvat 1959) at different exposures. It forms small, dense, pure populations in shady or semi-shady places (Šilić, 1990; Nikolić, 2015). The only known population of this species in Croatia is on St. Ilija Mountain, Pelješac peninsula in south Dalmatia. Other several populations are in Montenegro, Kosovo, and Albania (Nikolić, 2015). *Geranium dalmaticum* have small leaves up to 2.5 (– 4.5) cm wide and beautiful dull purplish-red flowers with petals up to 18 mm long (Nikolić, 2015). The chemical composition of the essential oil and the content of macroelements and trace elements in *G. dalmaticum* were investigated by Kremer et al. (2013). *G. macrorrhizum* L. is similar, but larger perennial herb up to 50 cm high with dull purplish-red flowers (Fig. 1d and 1e). *G. macrorrhizum* grows in mountainous area on shallow, calcareous soils from montane to subalpine vegetation belt (Webb and Ferguson, 1978). It inhabits edges of the trail, sinkhole, scrapes, scree, terraces and crevices in limestone and calcareous rocks in shady or semi-shady places.

Geranium species have been used as anti-infective agents (Radulović, 2010). Antioxidant activity, hepatoprotective effect, and antimicrobial properties have also been demonstrated (Miliauskas, 2006; Radulović, 2012). *G. macrorrhizum* is known to be rich in tannins and its extracts were reported to possess a broad spectrum of antimicrobial activities. It also has strong hypotensive and astringent activity, as well as cardiotonic and sedative properties (Bate-Smith, 1981; Ivancheva et al., 1992). It is increasingly cultivated in Europe for its ornamental flowers, as well as for use in aromatherapy and traditional herbal medicine due to its broad spectrum of biological activities. Essential oil of *G. macrorrhizum* is highly valued in perfumery and is also used as a food-flavouring agent (Ćavar Zeljković et al., 2020).

This study aims to get insight into the content of phenolic compounds of *G. dalmaticum* and *G. macrorrhizum* from Croatia, as well as to compare the content of different polyphenolic substances between these two *Geranium* species as a contribution to investigations of their phytotherapeutic potential.

Figure. 1. *Geranium dalmaticum (a, b, c), and G. macrorrhizum (d, e). (Photo: D. Kremer).*

Materials and methods

Plant material

The samples of *Geranium dalmaticum* and *G. macrorrhizum* were collected by K. Karlović and D. Kremer during the blooming period in June 2015 on Sv. Ilija Mountain, Pelješac peninsula (*G. dalmaticum*: 43 00'03.1" N, 17 10'58.9' E, 942 m a.s.l.), and on Velebit Mountain (*G. macrorrhizum*: 44 37'01.2" N, 15 01'45.8' E, 1139 m a.s.l.). Above-ground plant parts of several dozen plants were collected along hiking trails, on a dry day, by random selection. The collected material was mixed to obtain the randomly selected sample. Herbal material was air-dried at 22ºC and 60 % relative humidity in a well-ventilated room and protected from direct sunlight for 20 days. The dried plant material of each plant species was milled with laboratory mill Foss CT 193 Cyclotec (Foss Analytica Co., Ltd., Suzhou, China) into powder and properly stored until phytochemical analysis. Voucher specimens of herbal material were deposited in the "Fran Kušan" Herbarium of the Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia.

Chemicals and apparatus

Folin-Ciocalteu reagent (FCR), naringenin, quercetin, rutin, caffeic acid, coumaric acid, ferulic acid, sinapic acid, tannic acid (Sigma-Aldrich Chemical Co., St. Louis, USA); methanol, ethanol, and HNO_3 (Kemika, Zagreb, Croatia) were used. Absorbance measurements were performed using Agilent 8453 E UV/Vis spectrophotometer equipped with the PC-HP 845x UV-Visible System (both Hewlett Packard, Boeblingen, Germany) and 1 cm quartz cells. HPLC analysis was performed using a C_{18} reversed-phase packing column (Zorbax Eclipse XDB-C_{18}, 150 mm × 4.6 mm, 5 µm; Agilent, Santa Clara, USA) and Agilent 1100 Series HPLC system (Agilent, Santa Clara, USA).

Extract preparation

For spectrophotometric determination of polyphenolic substances, three extractions were made for each polyphenol determination method, i.e., three extractions of total polyphenols/tannins, three of flavonoids, and three extractions of phenolic acids from both *G. dalmaticum* and *G. macrorrhizum*.

Extraction of total polyphenols and tannins

The mass of 0.250 g of powdered plant material (the above ground parts) was extracted with 80 mL of 30 % (V/V) methanol (70°C, water bath, 15 min). After cooling and filtration, each extract was diluted to 100.0 mL with 30 % methanol (basic sample solution, BSS). BSS (2.0 mL) was mixed with 8.0 mL of water and 10.0 mL of acetate buffer (solution TP, S_{TP}). S_{TP} (10.0 mL) was shaken with 50.0 mg of casein during 45 min to allow adsorption of tannins, and then filtrated (solution T, S_T). Solutions S_{TP} and S_T were subjected to quantitative analysis of total polyphenols and tannins.

Extraction of total flavonoids

Powdered plant material (0.20 g) was extracted with 20 mL of acetone, 2 mL of 25 % HCl and 1 mL of 0.5 % hexamethylenetetramine (boiling water bath, 30 min). Each extract was filtered, and extraction of the same herbal material was repeated three times with 20 mL of acetone (boiling water bath, 10 min). After cooling and filtration, each extract was made up to 100.0 mL with acetone (basic sample solution, BSS). 20 mL of BSS was mixed with 20 mL of distilled water and then extracted with ethyl acetate (first with 15 mL and then three times with 10 mL). Ethyl acetate extracts were rinsed two times with distilled water then filtered and made up to 50.0 mL with ethyl acetate (Solution TF, S_{TF}).

Solution S_{TF} was subjected to quantitative analysis of total flavonoids.

Extraction of total phenolic acids

The extraction is performed as follows: to 0.200 g of the powdered drug 80 mL of ethanol (50 %, V/V) was added and boiled in a water-bath under a reflux condenser for 30 min. After cooling and filtration, the filter was rinsed with 10 mL of ethanol (50 %, V/V). The filtrate and the rinsing were combined in a volumetric flask and diluted to 100.0 mL with ethanol (50 %, V/V). Test solutions prepared in the described manner were subjected to quantitative analysis of total phenolic acids.

Phenolic compounds analyses

Total polyphenols and tannins contents (FCR procedure)

Determination of total polyphenols (TP) and tannins (T) was performed by using the prevalidated FCR procedure for polyphenols analysis according to Jurišić Grubešić et al. (2005). This method is based on a reaction with Folin–Ciocalteu's phenol reagent (FCR) and spectrophotometric determination of TP and T (after precipitating with casein) at 720 nm. The standard substance was tannic acid. The contents of TP and T in previously described extracts were evaluated in three independent analyses.

S_{TP} (1.0 mL) was mixed with 0.5 mL of FCR and diluted to 10.0 mL with 33 % $Na_2CO_3 \times 10H_2O$. The same procedure was performed with S_T. After filtration, the absorbance at 720 nm of the final blue solution was measured. Absorbance values obtained for S_{TP} correspond to total polyphenol content. The difference between the absorbance of S_{TP} and S_T corresponds to the concentration of casein-adsorbed tannins in plant samples.

The results were expressed as mg of standard equivalent / g of dried plant sample according to following equations:

$$TP\ (mg/g) = 10A_{TP} / 0{,}025 \quad (1)$$

$$T\ (mg/g) = 10A_{T} / 0{,}025 \quad (2)$$

A_{TP} – absorbance related to TP content; A_T – absorbance related to T content.

Total flavonoids contents (F–Al procedure)

The content of total flavonoids (TF) was obtained by using a prevalidated colorimetric assay with $AlCl_3$ (F–Al procedure) according to Jurišić Grubešić et al. (2007). This method is based on the hydrolysis of glycosides, extraction with ethyl acetate of TF aglycones and complex formation with $AlCl_3$ at 425 nm. The contents of TF in previously described extracts were evaluated in three independent analyses.

In 10 mL of S_{TF} 0.5 mL of 0.5 % solution of sodium citrate and 2 mL of $AlCl_3$ (2 g of $AlCl_3$ in 100,0 mL of 5 % acetic acid solution in methanol) were added and then made up to 25.0 mL with 5 % methanolic solution of acetic acid. After 45 min, yellow solutions were filtered, and the absorbance of the developed complex was measured at 425 nm.

TF concentration was expressed as mg of standard equivalent / g of dried herbal material and calculated as quercetin using the following expression:

$$TF\ (mg/g) = 10A \times 0.772 / m \quad (3)$$

A – absorbance; m – mass of the dry plant material (g); 0.772 – conversion factor related to specific absorbance of quercetin at 425 nm (i.e., 810).

Total phenolic acids contents (THD procedure)

The content of total phenolic acids (TPA) in plant extracts was obtained using the official pharmacopoeial method (European Pharmacopoeia 2014) for spectrophotometric determination of hydroxycinnamic derivatives, using the nitrite-molybdate reagent of Arnow, in a sodium hydroxide medium (THD procedure) at 505 nm. The contents of TPA in previously described extracts were evaluated in three independent analyses.

One millilitre of the resulting extract was mixed with 2.0 mL of 0.5 M HCl, 2.0 mL of nitrite-molybdate reagent (10 g of sodium nitrite and 10 g of sodium molybdate was dissolved in 100 mL of distilled water), 2.0 mL of 8.5 % sodium hydroxide solution and with distilled water up to 10 mL. A compensatory solution was made by diluting 1.0 mL of extract with distilled water up to 10 mL.

The absorbance of the test solution was measured immediately at 505 nm.

TPA concentration was expressed as mg of standard equivalent / g of dried herbal material and calculated as rosmarinic acid using the following expression:

$$TPA\ (mg/g) = 10A \times 2.5 / m \quad (4)$$

A – absorbance; m – mass of the dry plant material (g); 2.5 – conversion factor related to specific absorbance of rosmarinic acid at 505 nm (i.e., 400).

HPLC analysis

A powdered herbal material (500 mg) mixed with 20.0 mL of methanol at 25 °C for 25 min was used for the ultrasonic extraction (Čeh et al., 2007). Afterwards, extracts were filtered (filter paper black ribbon, Machery Nagel, Germany), and the obtained filtrates were diluted to 25.0 mL with 80 %

ethanol. Solutions were filtered using a 0.45 µm PTFE 25 mm filter (Restek, Bad Homburg, Germany). Lastly, 5.0 µL of each investigated extract was put into the HPLC instrument for analysis.

The stock solutions of standard compounds (rutin, quercetin, naringenin, sinapic acid, caffeic acid, coumaric acid, ferulic acid) were also prepared according to Čeh et al. (2007). A gradient elution was used for the analysis. The mobile phase was composed of phase A (water, pH = 2.50 adjusted with acetic acid), and phase B (acetonitrile 100 %). Detection was performed by diode array detector at wavelengths of 280 nm (naringenin), 320 nm (sinapic acid, caffeic acid, ferulic acid, coumaric acid), and 370 nm (rutin, quercetin). Individual components were tentatively identified by comparison with retention times of standards and unknown peaks in the samples. The method of standard addition was applied to avoid misinterpretation of the results. HPLC analysis of plant material was performed in triplicate.

Data analysis

All analyses were performed in triplicate and the results were expressed as mean ± SD. The significance of differences between analytical results was checked by the t-test for independent samples using the STATISTICA 7 software (StatSoft Inc., Tulsa, OK, USA).

Results and Discussion

Three different UV-Vis spectrophotometric methods were used for a quantitative analysis of TP, T, TF, and TPA in *G. dalmaticum* and *G. macrorrhizum*, and the results are presented in Table 1. Content of phenolic compounds was similar in both species and according to the t-test there was no statistically significant difference at $p > 0.05$ between species for any group of phenolic compounds. Slightly higher content of TP was recorded in *G. dalmaticum* than in *G. macrorrhizum* (217.60 mg/g dw *vs.* 215.53 mg/g). All other analyzed phenolics showed a tendency of higher concentration in *G. macrorrhizum* than in *G. dalmaticum*, T (157.73 mg/g *vs.* 155.83 mg/g), TF (5.53 mg/g *vs.* 5.10 mg/g), and TPA (15.33 mg/g *vs.* 13.27 mg/g). According to Şöhretoğlu et al. (2017), TP content in two *Geranium L. species from Turkey ranged from* 224.64 to 345.07 mg/g of the dry extract in *G. psilostemon Ledeb.,* and from 208.10 to 389.09 mg/g in *G. stepporum Davis*. In the same study, the TF content was higher in mentioned *Geranium* species compared to our study and ranged from 18.67 to *114.59* mg/g in *G. psilostemon*, and from 7.74 to 116.58 mg/g of the dry extract in *G. stepporum.* According to Radulović et al. (2012), TP content in methanol extracts of *G. macrorrhizum* was 160.2 ± 3.1 mg of gallic acid equivalents (GAE) per g of dry plant material (leaves), which is less than in our study. Ethanol extracts contained even lower, but still significant, amount of phenolics, while extraction with other solvents did not result in high TP values. In the same study, TF content was also the highest for the methanol extracts (44.9 ± 1.1 mg of catechin equivalents per g of dry leaves). TP content in above-ground parts of several *G. macrorrhizum* samples ranged from 35.91 mg/g to 165.95

mg/g GAE (Ćavar Zeljković et al., 2020), which is significantly less than the results obtained in our study. According to the abovementioned study, the results for TF content were very different among the samples and ranged from 1.33 mg/g (significantly less than our results) to 32.17 mg/g GAE (higher than in our study). A possible cause of large differences in TF contents may be the use of different methods for TF evaluation. In our study, a more selective method was applied, which is in a slightly modified form used as the official pharmacopoeial method for determining the flavonoid content (European Pharmacopoeia, 2014).

To the best of the authors' knowledge, there are no published results on the content of hydroxycinnamic acid derivatives (TPA content) in *G. dalmaticum* and *G. macrorrhizum*.

Table 1. *Contents (mg/g) of total polyphenols (TP), tannins (T), total flavonoids (TF), and total phenolic acids (TPA) in Geranium dalmaticum and G. macrorrhizum*

Phenolic compound	***G. dalmaticum***	***G. macrorrhizum***
TP	217.60 ± 1.08a	215.53 ± 1.10a
T	155.83 ± 0.60a	157.73 ± 0.61a
TF	5.10 ± 0.10a	5.53 ± 0.10a
TPA	13.27 ± 1.34a	15.33 ± 0.45a

Data represent mean ± SD of three independent analyses; n = 3.

Only two phenolic compounds were tentatively identified and quantified by HPLC analysis in *G. dalmaticum* and *G. macrorrhizum* (Table 2). Quercetin was identified in *G. dalmaticum* (0.230 %, m/m of dry substance) only, while rutin was determined in *G. macrorrhizum* (1.116 %, m/m of dry substance) only. The contents of the other investigated phenolic substances were below the limit of quantification. Leucuta et al. (2005) used HPLC analysis for investigation of phenolic compounds in *G. sanguineum* L. and they determined quercetin (0.82 mg/g) and rutin (1.71 mg/g), as well as caftaric acid, caffeic acid, hyperoside, isoquercitrin, quercitrin, and kaempferol. TPA content in this study was quantified by UV-Vis spectrophotometric method but the applied HPLC method did not manage to individually quantify investigated phenolic acids (i.e., sinapic acid, caffeic acid, coumaric acid, and ferulic acid). The content of individual phenolic acids could not be quantified by the applied HPLC method due to their individual low concentrations, or some other phenolic acids may be present that are not covered by the applied standard substances.

Table 2. *Contents of phenolic compounds (%, m/m of dry substance ± SD) in Geranium dalmaticum and G. macrorrhizum obtained by HPLC analysis*

Phenolic compound	***G. dalmaticum***	***G. macrorrhizum***
Rutin	BLQ	1.116 ± 0.003a
Quercetin	0.230 ± 0.002a	BLQ

BLQ = below the limit of quantification.

Conclusion

This study provides preliminary results regarding the contents of polyphenolic substances in above-ground plant parts in two *Geranium* species (family Geraniaceae) growing in Croatia, *G. dalmaticum* and *G. macrorrhizum*, by using HPLC analysis and UV-Vis spectrophotometric methods. The following research will be focused on further characterization of other phenolic compounds in *G. dalmaticum* and *G. macrorrhizum*, as well as on investigation of their biological activities with the aim of finding new potential phytotherapeutics.

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