

EVALUATION OF THE POLYPHENOLIC PROFILE AND THE *IN VITRO* ANTIOXIDANT POTENTIAL OF SEVEN *GERANIUM* SPECIES FROM THE ROMANIAN FLORA

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Abstract

The aim of this study was the qualitative and quantitative analysis of the active compounds and the *in vitro* evaluation of the antioxidant potential of preparations obtained from the aerial parts of seven *Geranium* species of the Romanian flora. Each was processed into a hot hydroalcoholic extract and a tincture, characterised by the total polyphenol (TPC), total flavonoid (TFC) and total caffeic acid derivative (TCADC) content, by HPLC-UV-MS quantification of 31 phenolic compounds and by the DPPH and FRAP tests. TPC ranged from 1.87 to 5.70, TFC from 0.65 to 3.53 and TCADC from 0.23 to 1.87 g equivalents/100 g plant material. The profiles were species-dependent, and caftaric acid, vitexin, epigallocatechin gallate and gallic acid are proposed as candidate chemotaxonomic markers. The reducing power (1186 - 4613 μ M Trolox equivalents/100 mL) was predicted by TPC ($r = 0.916$), whereas radical scavenging (IC_{50} 15.4 - 79.4 μ g/mL) followed the sum of the quantified compounds ($r = -0.783$). The best results were obtained for *G. macrorrhizum*, *G. robertianum* and *G. pyrenaicum*, and hot extraction recovered twice as much flavonoid material as maceration ($p = 0.017$).

Rezumat

Scopul studiului a fost analiza calitativă și cantitativă a principiilor active și evaluarea *in vitro* a potențialului antioxidant pentru preparatele obținute din părțile aeriene a șapte specii de *Geranium* din flora României. Fiecare probă a fost prelucrată sub formă de extract hidroalcoolic la cald și de tinctură, caracterizate prin conținutul total de polifenoli (PT), flavonoide (FT) și derivați de acid cafeic (DACT), prin cuantificarea HPLC-UV-MS a 31 de compuși fenolici și prin testele DPPH și FRAP. PT au variat între 1,87 și 5,70, FT între 0,65 și 3,53, iar DACT între 0,23 și 1,87 g echivalenți/100 g produs vegetal. Profilurile au fost dependente de specie, iar acidul caftaric, vitexina, epigallocatechin galatul și acidul galic sunt propuși ca markeri chemotaxonomici. Puterea reducătoare (1186 - 4613 μ M echivalenți Trolox/100 mL) a fost corelată cu PT ($r = 0,916$), iar IC_{50} DPPH (15,4 - 79,4 μ g/mL) a urmat suma compușilor cuantificați ($r = -0,783$). Cele mai bune rezultate s-au obținut pentru extractele de *G. macrorrhizum*, *G. robertianum* și *G. pyrenaicum*, iar extracția la cald a recuperat de două ori mai multe flavonoide decât macerarea ($p = 0,017$).

Keywords: *Geranium* sp., polyphenols, HPLC-UV-MS, antioxidant activity

Introduction

The genus *Geranium* L. is the largest of the family *Geraniaceae*, with about 400 species distributed over the temperate regions of both hemispheres and the tropical mountains. Several have a long history of use in European and Asian traditional medicine as astringent, anti-inflammatory, anti-haemorrhagic and wound-healing remedies, administered as infusions, decoctions or hydroalcoholic preparations (1, 2). *Geranium robertianum* L. (herb Robert) and *Geranium macrorrhizum* L. are the best documented species, the former also widely marketed as a food supplement and the subject of several recent reviews

(3-5) and primary studies (6). Comprehensive surveys of the genus as a whole have appeared in the last few years (1, 2).

Chemically the genus is characterised by a dense and diversified polyphenolic fraction. Hydrolysable tannins and particularly the ellagitannin geraniin, are regarded as its chemical hallmark and may reach several percent of the dry weight; they are accompanied by proanthocyanidins, flavonol glycosides (rutin, hyperoside, isoquercitrin, quercitrin), free flavonoid aglycones and hydroxycinnamic and hydroxybenzoic acids (4). This composition accounts for the pronounced radical-scavenging and reducing

capacity repeatedly reported for *Geranium* extracts, which in several assays exceeds that of reference antioxidants such as Trolox or ascorbic acid (7, 8).

Comparative studies covering more than one or two species remain uncommon. Ilić *et al.* (7) examined eight Serbian species and found 170.64 - 636.32 mg gallic acid equivalents/g dry extract; Książkiewicz *et al.* (8) compared three species and reported 31.94 - 102.65 mg/g, with chlorogenic and neochlorogenic acids restricted to *G. macrorrhizum*. For the Romanian flora the only systematic comparison published so far is that of Bejenaru *et al.* (9), on four species from Oltenia, Romania, with *G. robertianum* ranking first in both; an earlier contribution, Fodorea *et al.* (10), analysed *Geranii pyrenaici herba* from Cluj-Napoca, Romania.

Among the species examined here, *Geranium lucidum* L. is the one on which least is known. Ilić *et al.* reported for its aerial parts 413.45 mg gallic acid equivalents/g dry extract, 62% of it tannins, and a DPPH SC₅₀ (the concentration scavenging half of the radical, equivalent to the IC₅₀ used here) of 7.76 µg/mL (7), and later geraniin at 69.99 mg/g and the highest ellagic acid content of the eight taxa examined, 21.25 mg/g, with corilagin recorded in the species for the first time (11); for the Romanian flora it ranked third of four (9), and it retains an ethnomedicinal use in the Côa Valley of Portugal (12). What is still missing is a profile of its flavonoid glycosides and procyanidins, the published chromatographic work having concentrated on ellagitannins and phenolic acids.

Three gaps therefore remain: no study has covered a broad set of *Geranium* species from the central and north-western part of Romania; published work almost invariably uses a single extraction protocol, and where several extracts of one species have been

compared the comparison concerned analytical solvents rather than galenical preparations (13), so the influence of the galenical procedure on the recovered polyphenolic classes remains unassessed although officinal tinctures and hot hydroalcoholic extracts are not interchangeable; and the spectrophotometric determination of caffeic acid derivatives, a routine pharmacopoeial assay for other polyphenol-bearing drugs, has not been applied to *Geranium* sp. preparations. The present study was designed to characterise in parallel the polyphenolic fraction of seven growing *Geranium* species from the Romanian flora, each processed into a hot hydroalcoholic extract and a tincture, by three spectrophotometric assays (total polyphenols, total flavonoids and caffeic acid derivatives), HPLC-UV-MS quantification of 31 individual compounds, and *in vitro* evaluation of the antioxidant capacity by the DPPH and FRAP methods.

Materials and Methods

Plant material

The aerial parts (herba) of seven *Geranium* species were collected during the flowering stage in May 2023 and May 2024 from three counties of Romania (Table I). The material was air-dried in a shaded, ventilated room at ambient temperature, ground and stored in paper bags in the dark until analysis. Voucher specimens are deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, “Iuliu Hațieganu” University of Medicine and Pharmacy, Cluj-Napoca, under the accession codes given in Table I. The collection complied with the Convention on Biological Diversity and with CITES; none of the seven species is listed as protected or endangered.

Table I
Origin and coding of the analysed *Geranium* sp. samples

Species	Code/voucher no.	Extract	Tincture	Collection site
<i>Geranium robertianum</i> L.	GR1	E2	T2	Balnaca, Valea Boiului, Bihor County
<i>Geranium pyrenaicum</i> Burm. f.	GP1	E4	T4	Cluj-Napoca, Cluj County
<i>Geranium lucidum</i> L.	GL1	E5	T5	Balnaca, Valea Boiului, Bihor County
<i>Geranium macrorrhizum</i> L.	GM1	E6	T6	Cluj-Napoca, Cluj County
<i>Geranium pusillum</i> Burm. f.	GP2	E12	T12	Cluj-Napoca, Cluj County
<i>Geranium dissectum</i> L.	GD1	E13	T13	Cluj-Napoca, Cluj County
<i>Geranium phaeum</i> L.	GP3	E16	T16	Braşov, Braşov County

Chemicals and reference substances

The reference substances used for HPLC quantification (caftaric, gentisic, caffeic, chlorogenic, 4-*O*-caffeoylquinic, *p*-coumaric, protocatechuic, vanillic and syringic acids; vitexin, hyperoside, isoquercitrin, rutin, quercitrin, quercetin, patuletin, luteolin, kaempferol and apigenin; catechin, epicatechin and epigallocatechin gallate; procyanidins A1, B1, B2, B3, B4, C1 and C2), with purities between 78% and 98%, were obtained from Sigma-Aldrich Chemie

GmbH (Schnelldorf, Germany), except gallic and ferulic acids, from Merck (Darmstadt, Germany) (14, 15). Folin-Ciocalteu reagent and the solvents were purchased from Merck; DPPH and Trolox from Alfa-Aesar (Karlsruhe, Germany); sodium carbonate, sodium acetate trihydrate, anhydrous aluminium chloride, sodium nitrite, sodium molybdate, sodium hydroxide, hydrochloric acid, ferric chloride and 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) from Sigma-Aldrich (15). Methanol and acetonitrile were

of HPLC grade, ethanol and all other reagents of analytical grade, and ultrapure water was used throughout.

Preparation of the extracts and tinctures

Two preparations were obtained from each sample, using about 3 g of plant material (3.039 - 3.405 g, each mass recorded and used in the conversions to dry drug) and 30 mL of 70% (v/v) ethanol, a drug-to-solvent ratio of about 1:10 (m/v), following the procedures previously applied in our laboratory (16). Hydroalcoholic extracts (E) were prepared by hot extraction on a water bath at 60°C for 30 min; tinctures (T) by maceration at room temperature for 10 days with occasional stirring and protection from light, followed by 7 days of rest before filtration, according to the Romanian Pharmacopoeia, 10th edition (17). Both were filtered through paper, brought to 30 mL with the extraction solvent (31 mL for T2) and stored at 4°C in amber vials until analysis.

Determination of total polyphenols, total flavonoids and caffeic acid derivatives

The three classes were determined by validated spectrophotometric methods adapted from the Romanian and the European Pharmacopoeias, as previously described (18). For total polyphenols, 2 mL of the diluted preparation were mixed with 1 mL Folin-Ciocalteu reagent, 10 mL distilled water and 29% sodium carbonate up to 25 mL; after 30 min in the dark the absorbance was read at 760 nm against a gallic acid curve (10 - 200 µg/mL; $R^2 = 0.999$), and the results are expressed as g gallic acid equivalents (GAE)/100 g dry plant material.

For total flavonoids, determined according to the Romanian Pharmacopoeia (17), 5 mL of preparation were combined with 5 mL of 10% sodium acetate and 3 mL of 25% $AlCl_3$ and made up to 25 mL with methanol; the absorbance was recorded at 430 nm against a rutin curve (10 - 80 µg/mL; $R^2 = 0.992$); the results are expressed as g rutin equivalents (RE)/100 g dry plant material.

For caffeic acid derivatives, 1 mL of preparation was reacted with 1 mL 0.5 N hydrochloric acid, 1 mL of freshly prepared Arnow reagent (10 g sodium nitrite and 10 g sodium molybdate in 100 mL water) and 1 mL sodium hydroxide, and made up to 10 mL with distilled water; the absorbance was read at 500 nm against a caffeic acid curve (5 - 100 µg/mL; $R^2 = 0.989$); the results are expressed as g caffeic acid equivalents (CAE)/100 g dry plant material. All spectrophotometric determinations were carried out on a Jasco V-530 UV-VIS spectrophotometer (Jasco International Co., Tokyo, Japan) (18).

HPLC-UV-MS analysis

The individual compounds were separated and quantified on an Agilent 1100 Series HPLC system (Agilent, Santa Clara, CA, USA) with autosampler, column thermostat, binary gradient pump, degasser and UV detector, coupled to an Agilent Ion Trap 1100

SL mass spectrometer (LC/MSD Ion Trap VL), by three previously validated methods (14, 19). All three used a Zorbax SB-C18 column (100 × 3.0 mm i.d., 3.5 µm, Agilent) with methanol and 0.1% acetic acid as mobile phase, 1 mL/min and 5 µL injected; data were processed with DataAnalysis (v5.3) and ChemStation (vB01.03).

Method 1 quantified the hydroxycinnamic acids, gentisic acid and the flavonoids, with a binary gradient rising from 5 to 42% methanol over 35 min at 48°C and UV detection at 330 nm for the phenolic acids and 370 nm for the flavonoids, the identities being confirmed by electrospray ionisation mass spectrometry in negative mode. Method 2 quantified the hydroxybenzoic acids and the flavan-3-ols in MS mode, and method 3 the procyanidins in MS/MS mode. The three methods, their gradients, their mass-spectrometric settings and their validation data, including the calibration equations and the limits of detection and of quantification, are described in full in references (19) and (14); the compounds covered by each are listed in Table III and Table IV. Compounds were identified by comparison of retention times and UV and MS spectra with those of the authentic reference substances and quantified from the calibration curves of the individual standards. Values below the limit of quantification are reported as < LOQ and compounds that were not detected as ND.

In vitro antioxidant activity

The radical-scavenging capacity was assessed by the DPPH assay, as previously described (18). After 30 min at 40°C the absorbance was read at 517 nm against a control of 2 mL methanol and 2 mL DPPH solution, and inhibition (%) was calculated as $(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}} \times 100$. The IC_{50} was obtained by linear regression of inhibition against concentration; the IC_{50} values refer to the nominal concentration of the preparation in the reaction mixture. Trolox, assayed under the same conditions as reference antioxidant, gave an IC_{50} of 11.19 ± 0.09 µg/mL.

The ferric reducing antioxidant power was determined by the FRAP assay (18), based on the reduction of Fe^{3+} to Fe^{2+} in the presence of 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ). An aliquot of 0.4 mL of each preparation was diluted to 1.8 mL with distilled water and added to 6 mL of reagent, with water as blank; the absorbance was measured at 450 nm and the reducing capacity expressed as µM Trolox equivalents (TE)/100 mL preparation (Table II).

Statistical analysis

The analyses were performed in GraphPad Prism version 9.1.0 (GraphPad Software, San Diego, CA, USA). The relationships between the spectrophotometric parameters and the chromatographic data were examined by Pearson correlation. Since the fourteen preparations are not mutually independent, each drug having yielded one extract and one

tincture, the principal relationships were recomputed over the seven species means, which are independent. The influence of the extraction procedure was tested on the seven extract–tincture pairs by the paired *t*-test and by the Wilcoxon signed-rank test, both reported since seven pairs are too few for normality to be assumed. A *p*-value below 0.05 was considered significant; no correction for multiple comparisons was applied.

Results and Discussion

Total polyphenols, total flavonoids and caffeic acid derivatives

The content of the three polyphenolic classes determined spectrophotometrically is presented in Table II. Total polyphenols varied more than threefold across the set, from 1.87 g GAE/100 g in the *G. phaeum* extract (E16) to 5.70 g GAE/100 g in the *G. macrorrhizum* tincture (T6). Total flavonoids covered a fivefold range, from 0.65 g RE/100 g (T13, *G. dissectum*) to 3.53 g RE/100 g (E6, *G. macrorrhizum*), while caffeic acid derivatives ranged from 0.23 to 1.87 g CAE/100 g, again with the *G. macrorrhizum* tincture at the upper end.

G. macrorrhizum, *G. robertianum* and *G. pyrenaicum* emerged as the richest species overall: their six

preparations occupied the first six positions for total polyphenols (4.00 - 5.70 g GAE/100 g) and the first three for total flavonoids (3.53, 3.38 and 2.82 g RE/100 g, in the hot extracts). At the opposite end, *G. phaeum* and *G. pusillum* gave the poorest preparations in terms of total polyphenols (1.87 and 1.95 g GAE/100 g in the hot extracts). The relative position of *G. macrorrhizum* agrees with the Serbian data of Ilić *et al.* (7), where the species ranked among the richest of the eight examined, and with Książkiewicz *et al.* (8), who found *G. macrorrhizum* and *G. sanguineum* clearly above *G. phaeum*, an ordering reproduced here.

A direct numerical comparison with the literature is complicated by the reference base: values reported for *Geranium* sp. preparations are most often expressed per gram of dry extract (7, 20), whereas the present results refer to the dry plant material, the basis relevant for a pharmacopoeial-type assay. On that basis the interval found here corresponds to 18.7 - 57.0 mg GAE/g, against 68.17 - 140.14 mg GAE/g reported by Bejenaru *et al.* (9) for four Romanian species – but theirs refer to the extract as prepared, so the gap cannot be read as a difference in extraction efficiency.

Table II

Polyphenolic content and *in vitro* antioxidant activity of the *Geranium* sp. preparations

Sample	Species	Total polyphenols (g GAE/100 g)	Total flavonoids (g RE/100 g)	Caffeic acid derivatives (g CAE/100 g)	FRAP (μM TE/100 mL)	DPPH IC ₅₀ (μg/mL)
E2	<i>G. robertianum</i>	4.81 ± 0.05	3.38 ± 0.03	1.37 ± 0.01	4090.4 ± 10.25	15.41 ± 0.01
T2	<i>G. robertianum</i>	4.31 ± 0.04	1.47 ± 0.02	0.26 ± 0.01	3836.5 ± 8.05	29.30 ± 0.03
E4	<i>G. pyrenaicum</i>	4.50 ± 0.05	2.82 ± 0.02	0.84 ± 0.01	3828.2 ± 12.26	16.98 ± 0.02
T4	<i>G. pyrenaicum</i>	4.00 ± 0.03	0.97 ± 0.01	0.30 ± 0.01	3297.5 ± 20.09	30.25 ± 0.03
E5	<i>G. lucidum</i>	2.93 ± 0.02	1.66 ± 0.02	1.05 ± 0.02	1268.5 ± 10.76	79.35 ± 0.04
T5	<i>G. lucidum</i>	3.34 ± 0.03	0.79 ± 0.01	0.29 ± 0.01	2192.9 ± 21.11	44.57 ± 0.05
E6	<i>G. macrorrhizum</i>	4.76 ± 0.05	3.53 ± 0.02	1.10 ± 0.02	3554.3 ± 10.43	30.02 ± 0.03
T6	<i>G. macrorrhizum</i>	5.70 ± 0.05	1.21 ± 0.01	1.87 ± 0.01	4613.3 ± 21.51	20.33 ± 0.02
E12	<i>G. pusillum</i>	1.95 ± 0.02	0.87 ± 0.01	1.65 ± 0.02	1185.9 ± 11.06	34.10 ± 0.03
T12	<i>G. pusillum</i>	3.04 ± 0.02	0.71 ± 0.01	0.23 ± 0.01	2632.8 ± 10.85	49.70 ± 0.04
E13	<i>G. dissectum</i>	3.02 ± 0.03	1.79 ± 0.02	0.65 ± 0.01	1997.9 ± 9.86	30.58 ± 0.03
T13	<i>G. dissectum</i>	2.51 ± 0.02	0.65 ± 0.01	1.25 ± 0.01	2197.5 ± 23.56	43.49 ± 0.05
E16	<i>G. phaeum</i>	1.87 ± 0.01	0.72 ± 0.01	0.37 ± 0.01	1812.0 ± 9.95	29.06 ± 0.02
T16	<i>G. phaeum</i>	2.98 ± 0.02	0.88 ± 0.01	0.32 ± 0.01	1350.6 ± 11.09	39.50 ± 0.03

GAE – gallic acid equivalents; RE – rutin equivalents; CAE – caffeic acid equivalents, all referred to the dry plant material. Each value is a single determination. E – hot hydroalcoholic extract; T – tincture obtained by cold maceration. The DPPH IC₅₀ was obtained by linear regression of percentage inhibition against concentration; the values are the mean of 3 determinations ± SD

The spectrophotometric determination of caffeic acid derivatives correlated with none of the chromatographic sums ($|r| \leq 0.31$, $p \geq 0.29$), and in every sample the nitrite–molybdate value (231 - 1870 mg/100 g) exceeded the sum of the individually quantified hydroxycinnamic acids (0 - 82 mg/100 g) by a factor of four to more than a thousand. The Arnow reaction is specific for the *ortho*-dihydroxyphenyl (catechol) moiety and therefore responds to every catecholic constituent – the hexahydroxydi-

phenoyl units of the ellagitannins, the catechol-bearing flavonoids and protocatechuic acid included – and not only to esters of caffeic acid. In *Geranium* sp. preparations the assay should consequently be read as a measure of total catecholic polyphenols rather than of caffeic acid derivatives *sensu stricto*; to our knowledge it has not previously been applied to this genus.

Influence of the extraction procedure

Pairing each tincture with the hot extract obtained from the same drug allows the two galenical procedures to be compared directly. Hot extraction recovered about twice as much flavonoid material as cold maceration (mean 2.11 vs. 0.96 g RE/100 g; paired *t*-test *p* = 0.017; Wilcoxon *p* = 0.031), the extract being richer in six of the seven pairs. Total polyphenols behaved differently: the tinctures gave slightly higher values than the extracts (3.70 vs. 3.41 g GAE/100 g), with no significant difference (*p* = 0.360), and caffeic acid derivatives showed no consistent pattern either (1.01 vs. 0.65 g CAE/100 g, *p* = 0.299); with seven pairs these last two comparisons indicate an absence of a detected difference rather than equivalence.

The effect concerns the spectrophotometric flavonoid content but not the chromatographic one: the sum of the nine individually quantified flavonoids was practically identical in the two types of preparation, the hot extract and the tincture (160.3 vs. 164.7 mg/100 g, *p* = 0.641). The aluminium chloride assay and the chromatographic sum therefore do not measure the same thing, the former responding to every flavonoid bearing the required hydroxyl pattern and the latter being restricted to nine identified structures. The dissociation is chemically plausible, bearing in mind that the two procedures differ in temperature and in contact time at once (60°C for 30 min against room temperature for 17 days), so that neither factor can be isolated: heat increases the solubility of the flavonoid material, whereas prolonged contact at room temperature favours the diffusion of the high-molecular-weight hydrolysable tannins to which the Folin-Ciocalteu assay also responds (4, 7). In practical terms the two preparations carry the same Folin-reactive load and the same identified flavonoids, but the hot extract carries about twice the

flavonoid material measured by the aluminium chloride assay, the parameter on which pharmacopoeial specifications are usually written.

Phenolic acids

Eleven phenolic acids were quantified by the first two chromatographic methods (Table III); representative chromatograms are shown in Figure 1. Hydroxybenzoic acids were quantitatively dominant in every species except *G. phaeum*, where the hydroxycinnamic acids prevailed. Gallic acid was the single most abundant compound of the study, reaching 273.5 mg/100 g in the *G. dissectum* extract, followed by *G. pyrenaicum* (119.1 and 182.5 mg/100 g). Protocatechuic acid followed a different ordering, with *G. pyrenaicum* clearly ahead (95.1 and 94.8 mg/100 g) and *G. pusillum* second (46.4 and 48.9 mg/100 g); in *G. phaeum* it did not reach 3 mg/100 g. The prominence of these two hydroxybenzoic acids is consistent with Bejenaru *et al.* (9), who found their concentrations to track the reducing power of Romanian *Geranium* sp. extracts most closely.

Among the hydroxycinnamic acids, chlorogenic acid separated the species most sharply: it reached 53.4 and 53.6 mg/100 g in *G. phaeum* and 49.0 and 48.3 mg/100 g in *G. macrorrhizum*, while remaining below 6 mg/100 g in all the other species. Książkiewicz *et al.* (20) proposed chlorogenic and neochlorogenic acids as markers of *G. macrorrhizum*; the present data confirm its high content but show that *G. phaeum* is equally rich, so the marker is shared rather than exclusive. 4-*O*-Caffeoylquinic acid was detected in all species except *G. dissectum* but remained modest (5.6 - 16.7 mg/100 g), and free caffeic, *p*-coumaric and ferulic acids were minor throughout (below 6 mg/100 g). Caftaric acid was the most discriminating compound of the group, quantified only in *G. phaeum* (16.9 and 8.4 mg/100 g) and *G. pyrenaicum* (10.8 and 3.4 mg/100 g).

Table III

Phenolic acids quantified by HPLC-UV-MS (mg/100 g dry plant material)

Sample	Species	Caf	Gen	Cff	Chl	4-CQ	Cou	Fer	Gal	Pro	Van	Syr
E2	<i>G. robertianum</i>	ND	2.46	ND	1.89	9.83	<LOQ	ND	27.82	23.82	1.10	0.03
T2	<i>G. robertianum</i>	ND	1.73	ND	1.22	12.99	3.67	ND	42.20	27.24	1.21	0.05
E4	<i>G. pyrenaicum</i>	10.79	< LOQ	1.45	3.80	16.68	<LOQ	2.61	119.11	95.13	0.41	0.04
T4	<i>G. pyrenaicum</i>	3.43	1.60	2.20	5.36	12.33	<LOQ	1.83	182.50	94.82	0.53	ND
E5	<i>G. lucidum</i>	ND	< LOQ	0.59	2.68	9.21	<LOQ	ND	28.86	5.35	0.68	ND
T5	<i>G. lucidum</i>	ND	< LOQ	< LOQ	2.73	8.65	3.25	ND	53.07	5.47	0.72	ND
E6	<i>G. macrorrhizum</i>	ND	< LOQ	2.54	49.00	6.51	5.12	ND	88.03	6.29	0.66	0.11
T6	<i>G. macrorrhizum</i>	ND	< LOQ	2.60	48.26	6.22	<LOQ	ND	82.30	5.95	0.80	0.12
E12	<i>G. pusillum</i>	ND	< LOQ	1.38	2.97	5.56	1.73	2.23	93.37	46.42	0.52	ND
T12	<i>G. pusillum</i>	ND	1.96	< LOQ	2.78	6.74	1.64	3.18	102.63	48.91	0.58	ND
E13	<i>G. dissectum</i>	ND	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	273.55	18.21	0.14	ND
T13	<i>G. dissectum</i>	ND	0.85	ND	0.86	ND	ND	ND	211.29	16.53	0.25	ND
E16	<i>G. phaeum</i>	16.87	< LOQ	1.13	53.39	7.42	3.05	< LOQ	49.25	2.93	0.36	0.34
T16	<i>G. phaeum</i>	8.40	< LOQ	1.37	53.62	7.56	3.71	ND	42.50	2.87	0.48	0.19

Caf – caftaric acid; Gen – gentisic acid; Cff – caffeic acid; Chl – chlorogenic acid; 4-CQ – 4-*O*-caffeoylquinic acid; Cou – *p*-coumaric acid; Fer – ferulic acid; Gal – gallic acid; Pro – protocatechuic acid; Van – vanillic acid; Syr – syringic acid. ND – not detected; < LOQ – below the limit of quantification

Flavonoids

The flavonoid profiles (Table IV) were strongly species-specific and, unlike the phenolic acids, dominated by glycosides. Isoquercitrin led in *G. macrorrhizum* (103.8 and 105.6 mg/100 g) and *G. phaeum* (94.1 and 94.9 mg/100 g), and rutin reached its highest values in *G. robertianum* and *G. macrorrhizum* (56.6 - 65.6 mg/100 g). Hyperoside showed the opposite distribution, most abundant in *G. pyrenaicum* (72.8 and 65.7 mg/100 g; Fodorea *et al.* (10) reported 21.6 mg/100 g for the same species at Cluj-Napoca), *G. macrorrhizum* and *G. pusillum* (40 - 45 mg/100 g) and almost absent from *G. robertianum* and *G. lucidum* (below 4 mg/100 g).

Among the aglycones, kaempferol was by far the most characteristic, reaching 135.5 and 118.8

mg/100 g in *G. robertianum* and 42.2 and 44.2 mg/100 g in *G. lucidum*, whereas in the remaining five species it never exceeded 16 mg/100 g; *G. robertianum* was also the richest in free quercetin (57.4 and 49.4 mg/100 g). This kaempferol- and quercetin-rich, hyperoside-poor pattern sets the two species apart from the rest of the genus in the present material. Vitexin, the only C-glycosylflavone screened, was detected in a single sample, the *G. phaeum* tincture (10.1 mg/100 g); Książkiewicz *et al.* (8) similarly reported *G. phaeum* as the species distinguished by C-glycosyl flavonoids. Luteolin and apigenin were present only at trace level, and patuletin was not detected in any sample.

Table IV

Flavonoids quantified by HPLC-UV-MS (mg/100 g dry plant material)

Sample	Species	Vit	Hyp	Isq	Rut	Qct	Que	Lut	Kae	Api	Cat	Epi	EGCG	B3	B1	B4	B2
E2	<i>G. robertianum</i>	ND	2.32	54.91	65.06	23.21	57.44	ND	135.53	2.01	0.07	1.34	ND	1.18	0.41	ND	0.06
T2	<i>G. robertianum</i>	ND	3.00	42.67	56.56	25.81	49.41	ND	118.79	1.89	ND	1.03	ND	0.79	0.17	ND	ND
E4	<i>G. pyrenaicum</i>	ND	72.78	< LOQ	< LOQ	23.07	7.53	0.48	1.75	0.53	0.09	0.97	ND	0.25	0.03	ND	ND
T4	<i>G. pyrenaicum</i>	ND	65.68	< LOQ	8.52	22.26	8.07	1.72	2.67	0.69	0.08	0.64	ND	0.14	ND	ND	ND
E5	<i>G. lucidum</i>	ND	3.59	< LOQ	34.39	5.04	12.05	ND	42.25	0.74	0.07	1.20	ND	0.32	0.68	ND	ND
T5	<i>G. lucidum</i>	ND	3.25	12.25	21.62	6.26	11.97	ND	44.17	< LOQ	0.03	1.97	ND	0.27	0.22	ND	ND
E6	<i>G. macrorrhizum</i>	ND	44.91	103.81	57.23	14.81	38.72	< LOQ	13.59	1.02	2.14	0.74	ND	0.12	0.47	ND	0.07
T6	<i>G. macrorrhizum</i>	ND	45.44	105.62	65.62	17.96	40.16	0.37	15.09	1.30	1.36	1.07	ND	0.26	0.61	ND	ND
E12	<i>G. pusillum</i>	ND	41.44	54.11	ND	8.22	2.73	< LOQ	1.55	ND	ND	1.24	0.66	ND	ND	ND	0.10
T12	<i>G. pusillum</i>	ND	40.24	60.53	< LOQ	8.88	7.25	< LOQ	1.42	ND	ND	1.52	0.66	ND	ND	0.10	0.28
E13	<i>G. dissectum</i>	ND	4.58	< LOQ	29.47	6.84	5.65	0.64	1.83	ND	0.01	0.81	0.95	0.21	ND	ND	ND
T13	<i>G. dissectum</i>	ND	0.54	76.80	4.24	< LOQ	0.81	ND	ND	ND	0.07	0.98	0.80	0.57	0.18	0.21	0.57
E16	<i>G. phaeum</i>	ND	15.74	94.11	5.17	28.76	0.32	0.74	0.77	0.89	0.18	0.14	ND	0.14	0.05	ND	ND
T16	<i>G. phaeum</i>	10.15	13.63	94.91	3.44	26.08	1.42	1.05	1.21	1.14	0.15	ND	ND	0.18	ND	ND	0.03

Vit – vitexin; Hyp – hyperoside; Isq – isoquercitrin; Rut – rutin; Qct – quercitrin; Que – quercetin; Lut – luteolin; Kae – kaempferol;

Api – apigenin; Cat – catechin; Epi – epicatechin; EGCG – epigallocatechin gallate; B1, B2, B3, B4 – the corresponding procyanidins.

ND – not detected; < LOQ – below the limit of quantification. Patuletin and procyanidins A1, C1 and C2 were not detected in any sample and are omitted

The monomeric and oligomeric flavan-3-ols formed a quantitatively minor fraction (Table IV), which contrasts with the tannin-rich character generally attributed to the genus and indicates that the astringent fraction of these species is made up of hydrolysable rather than condensed tannins – in agreement with the central role assigned to geraniin and related ellagitannins in *Geranium* sp. chemistry (4). Catechin exceeded 1 mg/100 g only in *G. macrorrhizum* (2.14 and 1.36 mg/100 g), and epicatechin remained below 2 mg/100 g in all samples. Epigallocatechin gallate was detected exclusively in *G. pusillum* and *G.*

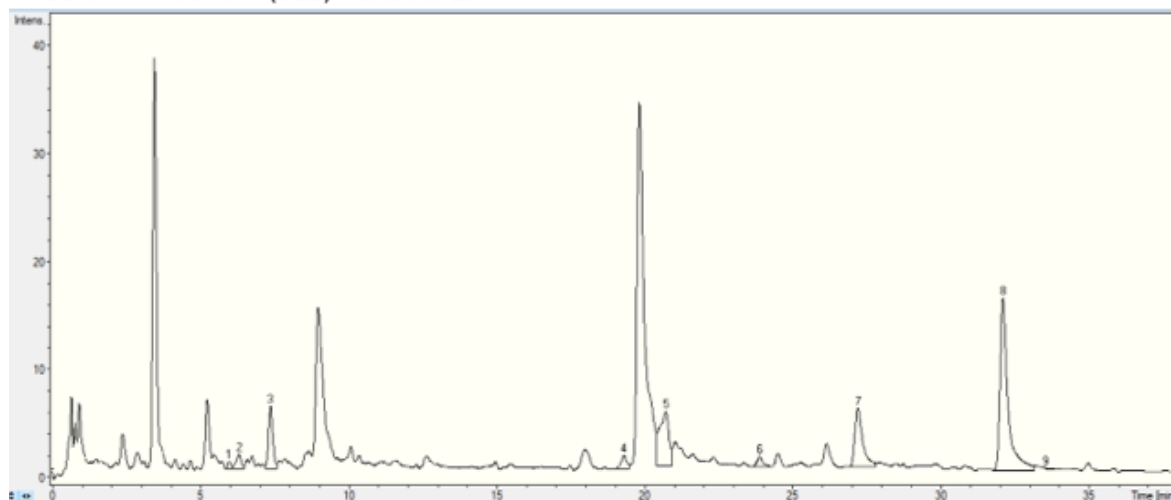
dissectum (0.66 - 0.95 mg/100 g), where it may serve as a discriminating minor marker.

Of the seven procyanidins screened, only the B-type dimers B3, B1, B4 and B2 were detected, all at concentrations below 1 mg/100 g; the A-type dimer A1 and the trimers C1 and C2 were absent from every *Geranium* sp. sample. Procyanidins B3 and B1 were the most widely distributed, and the broadest oligomeric profile was that of the *G. dissectum* tincture (T13), the only sample in which four procyanidins were simultaneously quantified. Unlike the flavonoid fraction, the flavan-3-ols showed no systematic response to the extraction procedure, the hot extract

being the richer preparation in four of the seven pairs; since the whole class remained below 4 mg/100 g,

these differences are close to the resolution of the method and should not be over-interpreted.

A *Geranium lucidum* (GL1) – extract E5



B *Geranium robertianum* (GR1) – extract E2

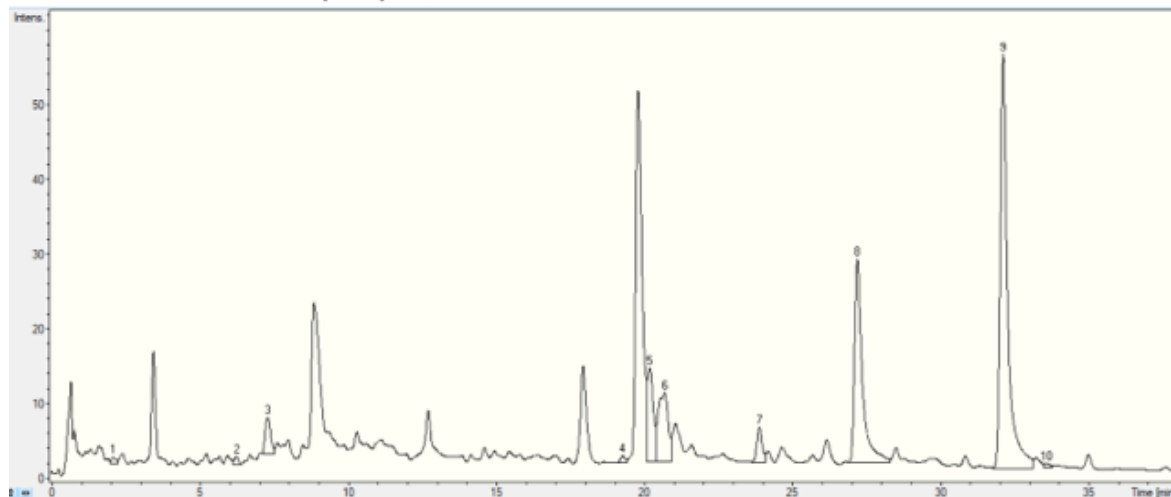


Figure 1.

Representative HPLC-UV-MS chromatograms of the hydroalcoholic extracts: (A) *Geranium lucidum* (E5), the least investigated species of the genus; (B) *G. robertianum* (E2), the most studied species of the genus and the one with the highest flavonoid aglycone content in this set. Both traces were recorded with analytical method 1 (UV detection at 330 nm up to 17 min and at 370 nm thereafter); the numbered peaks are the compounds quantified in each sample, in order of elution. (A) 1 – caffeic acid, 2 – chlorogenic acid, 3 – 4-*O*-caffeoylquinic acid, 4 – hyperoside, 5 – rutin, 6 – quercitrin, 7 – quercetin, 8 – kaempferol, 9 – apigenin. (B) 1 – gentisic acid, 2 – chlorogenic acid, 3 – 4-*O*-caffeoylquinic acid, 4 – hyperoside, 5 – isoquercitrin, 6 – rutin, 7 – quercitrin, 8 – quercetin, 9 – kaempferol, 10 – apigenin

One limitation of the panel should be stated here. The three methods quantify 31 compounds but include neither ellagic acid nor the ellagitannins, geraniin foremost among them, which the literature identifies as the dominant phenolics of the genus (4, 11) and to which a broad multi-target pharmacology has been attributed (21). Fodorea *et al.* (10) found ellagic acid at 1810 mg/100 g of *G. pyrenaicum* drug, five times the sum of all 31 compounds quantified here in the same species (357 mg/100 g), and Ilić *et al.* (11) 69.99 mg/g of geraniin in *G. lucidum*. The class sums quoted above therefore describe the fraction accessible to this panel, not the total phenolic

content, the gap being largely filled by hydrolysable tannins – which is also why the antioxidant response cannot be attributed to the individually quantified compounds alone.

Chemotaxonomic considerations

Taken together, the chromatographic data define a distinct profile for each species. Over the fourteen preparations the class sums ranged from 47.4 to 304.6 mg/100 g for the phenolic acids, from 49.0 to 340.5 mg/100 g for the flavonoids and from 0.3 to 3.5 mg/100 g for the flavan-3-ols and procyanidins, the total varying between 147.7 mg/100 g in the *G. lucidum* extract and 441.1 mg/100 g in the *G.*

macrorrhizum tincture. Three patterns emerge. In the first, flavonoids account for roughly two thirds of the quantified material and free kaempferol and quercetin dominate: *G. robertianum* (76 - 83% flavonoids) and *G. lucidum* (57 - 66%). In the second, phenolic acids dominate, driven by the gallic–protocatechuic pair: *G. dissectum* (73 - 85%), *G. pyrenaicum* (70 - 73%) and, less markedly, *G. pusillum* (58%). The third is defined qualitatively, by the joint prominence of chlorogenic acid and isoquercitrin: *G. macrorrhizum* and *G. phaeum*, which together show the highest hydroxycinnamic acid sums of the study (57 - 82 mg/100 g, against 0 - 35 mg/100 g elsewhere).

Each of these species has been examined individually elsewhere (6, 13, 20, 23, 24), but none of those studies applied one analytical panel across several taxa, which is what makes the direct comparison here possible; the multi-species surveys that did so (7-9, 11) used panels and reference bases different from ours.

Four compounds behaved as candidate markers, with the reservation that each rests on single determinations from one population per species and that vitexin was quantified in only one of the fourteen preparations: caftaric acid, restricted to *G. phaeum* and *G. pyrenaicum*; vitexin, found only in *G. phaeum*; epigallocatechin gallate, confined to *G. pusillum* and *G. dissectum*; and gallic acid, whose concentration in *G. dissectum* exceeded that of the five low-gallic species by a factor of at least five, although it was only about 1.5 times that of *G. pyrenaicum*. This supports the chemotaxonomic approach of Bejenaru *et al.* (9), who likewise assigned individual phenolic acids to particular species, although the markers they proposed for the *Olenia* material differ from those found here – a discrepancy that underlines the need to validate such markers across provenances before using them for identification.

Intraspecific variability is indeed substantial. A second *G. robertianum* population collected from Cluj-Napoca and processed identically gave 2.28 g GAE/100 g total polyphenols and 1.45 g RE/100 g total flavonoids, against 4.81 and 3.38 g/100 g for the Bihor population reported in Table II – a more than twofold difference within the same species, which would have moved *G. robertianum* from first to fifth place in the ranking of the seven hot extracts by total polyphenol content. This is a reminder that the ranking established here applies to the sampled provenances and should be confirmed on material from several populations and harvest years.

In vitro antioxidant activity

The results of the two antioxidant assays are given in Table II. The reducing power spanned a fourfold range, from 1186 μ M TE/100 mL in the *G. pusillum* extract (E12) to 4613 μ M TE/100 mL in the *G. macrorrhizum* tincture (T6). Averaged over the two

preparations the species ranked *G. macrorrhizum* (4085) > *G. robertianum* (3965) > *G. pyrenaicum* (3564) > *G. dissectum* (2099) > *G. pusillum* (1910) > *G. lucidum* (1732) > *G. phaeum* (1582); the three species richest in polyphenols are also the three most active, and in the same order. As with total polyphenols, the extraction procedure had no systematic effect on reducing power (2534 vs. 2874, $p = 0.304$). The DPPH assay gave a consistent picture. The inhibition–concentration relationship was linear for every preparation ($R^2 = 0.905 - 0.997$) and the IC_{50} values covered a fivefold range, from 15.4 μ g/mL for the *G. robertianum* extract (E2) to 79.4 μ g/mL for the *G. lucidum* extract (E5); Trolox, run in parallel, gave 11.19 ± 0.09 μ g/mL, a figure not on the same reference base and therefore not to be compared as a ratio. Ranked by mean IC_{50} the species order was *G. robertianum* (22.4 μ g/mL) < *G. pyrenaicum* (23.6) < *G. macrorrhizum* (25.2) < *G. phaeum* (34.3) < *G. dissectum* (37.0) < *G. pusillum* (41.9) < *G. lucidum* (62.0). The same three species head both assays, although *G. macrorrhizum* leads on reducing power and *G. robertianum* on radical scavenging, a divergence compatible with the different chemistry of the two reactions.

For the *G. lucidum* extract (E5) the cleanest regression of the study ($R^2 = 0.997$) and an IC_{50} of 79.4 μ g/mL were obtained; the least active preparation of the set is consistent with *G. lucidum* having by far the lowest sum of quantified compounds.

Both assays correlated with the polyphenolic content, and in the same selective way. Reducing power was very closely predicted by the Folin-Ciocalteu value ($r = 0.916$, $p < 0.0001$; $FRAP = 918.3 \times TPC - 557.0$, $R^2 = 0.838$) and by the sum of all compounds quantified by HPLC-UV-MS ($r = 0.817$, $p = 0.0004$), while the DPPH IC_{50} , an inverse measure of activity, correlated negatively with that sum ($r = -0.783$, $p = 0.0009$) and with FRAP ($r = -0.656$, $p = 0.011$) but only loosely with total polyphenols ($r = -0.498$, $p = 0.070$). Neither assay was related to the sum of the free phenolic acids ($r = 0.092$ and -0.323), which mirrors the Folin-Ciocalteu value itself: it correlated with the sum of all quantified compounds ($r = 0.709$) and with the sum of the individual flavonoids ($r = 0.705$) but not with the phenolic acids ($r = -0.028$), as expected if much of the Folin-reactive material consists of hydrolysable tannins outside the panel. Recalculated over the seven species means, which are independent, both leading relationships were preserved and became stronger ($r = 0.961$, $p = 0.0006$ and $r = -0.944$, $p = 0.0014$).

The two assays are therefore not anchored to the same predictor. Reducing power follows the Folin-Ciocalteu value almost exactly, whereas radical scavenging is better predicted by the sum of the individually quantified compounds ($r = -0.783$) than by the Folin value ($r = -0.498$). The *G. lucidum*

extract is the sample that separates the two: its Folin value is low but not extreme (2.93 g GAE/100 g, eleventh of fourteen) whereas its chromatographic sum is the lowest of the study by a wide margin and it is the least active preparation in both assays. The straightforward reading is that part of the Folin-reactive material contributes to ferric reduction without contributing proportionally to radical scavenging, so the two assays should be reported together rather than treated as interchangeable.

Decoctions of *G. robertianum* have likewise been shown to combine radical scavenging with anti-inflammatory activity (22), so the response measured in vitro is not the whole of the pharmacological picture. The present interpretation also differs from that of Bejenaru *et al.* (9), who concluded from the parallel behaviour of FRAP, total phenolics and the gallic–protocatechuic pair that non-flavonoid phenolics drive reducing capacity. Here *G. dissectum* and *G. pyrenaicum*, by far the richest in those two acids, rank fifth and second for radical scavenging, while *G. macrorrhizum* and *G. robertianum*, the two species with the highest flavonoid sums, lead both rankings – which points to the hydrolysable tannins and the flavonoid glycosides rather than to the free phenolic acids.

A numerical comparison with the literature must remain cautious, because the four data sets use four different reference bases – the dry methanolic extract (11), the extract as prepared (9, 20) and, here, the nominal concentration of the working solution – and recalculating them per unit mass of herbal drug is not possible from the published data. Converted to the same units, the interval found here, 15.4 - 79.4 µg/mL, overlaps the SC₅₀ of 4.24 - 34.52 µg/mL of Ilić *et al.* (11) and lies about an order of magnitude below those of Bejenaru *et al.* (9) and Książkiewicz *et al.* (20). What is comparable is the spread: the fivefold range for DPPH and the fourfold range for FRAP match the interspecific variation reported in those studies.

Limitations

Three limitations should be borne in mind. Each species is represented by one population and one harvest, and the second *G. robertianum* population shows that intraspecific variation can exceed the differences between species, so the ranking established here is valid for the sampled provenances rather than for the taxa in general. The panel does not include ellagic acid or the ellagitannins, so the chromatographic sums describe an accessible fraction rather than the total phenolic content. Finally, the DPPH results are referred to the nominal concentration of the preparation and the FRAP results to a fixed volume of it, which makes both fully comparable within this study but only approximately comparable with published values on other reference bases.

Conclusions

Seven wild-growing *Geranium* species of the Romanian flora were characterised as hot hydroalcoholic extracts and as tinctures. The polyphenolic content varied more than threefold between preparations, *G. macrorrhizum*, *G. robertianum* and *G. pyrenaicum* being the richest and *G. phaeum* and *G. pusillum* the poorest. Hot extraction more than doubled the spectrophotometric flavonoid content, whereas the Folin-reactive polyphenols and the sum of the individually quantified flavonoids were unaffected. HPLC-UV-MS quantification of 31 compounds resolved the species into a flavonoid-dominated group, a phenolic-acid-dominated group and a group marked by chlorogenic acid and isoquercitrin, and identified caftaric acid, vitexin, epigallocatechin gallate and gallic acid as candidate markers. The caffeic acid derivative assay, applied here for the first time to this genus, reflects total catecholic polyphenols rather than caffeic acid esters. The reducing power was closely associated with the total polyphenol content ($r = 0.916$) and followed the flavonoid fraction rather than the free phenolic acids, whereas the DPPH IC₅₀ (15.4 - 79.4 µg/mL) followed the sum of the quantified compounds, so the two assays are complementary. The study adds the first flavonoid and procyanidin profile of *G. lucidum*; *G. macrorrhizum*, *G. robertianum* and *G. pyrenaicum* are the species to take forward for bioactivity-guided fractionation.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Ethics approval and consent to participate

Not applicable.

AI use disclosure

During the preparation of this manuscript, the author(s) used Claude AI for editing assistance and language improvement, specifically to improve spelling, grammar, word choice, readability, and clarity. The author(s) reviewed and revised all outputs from these tools and take(s) full responsibility for the final content of the work.

Conflict of interest

The authors declare no conflict of interest.

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