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PHYTOCHEMICAL STUDIES ON SOME INDIGENOUS *GERANIUM* SPECIES (GERANIACEAE)

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Abstract: In order to continue our previous studies, a phytochemical study was performed on five indigenous Geranium species. Following results were obtained by means of spectrophotometrical methods: *G. phaeum* L.: 0.16-0.43% flavonoids, 0.43-0.60% polyphenolic acids, 3.06-3.44% tannins, $25.29 \cdot 10^{-3}$ % anthocyanins, 0.57 % proantocyanins; *G. columbinum* L.: 1.41% flavonoids, 0.37% polyphenolic acids, 9.27% tannins. Following results were obtained by HPLC methods (expressed in µg compound/100 g vegetal product): *G. phaeum* L.: 23.91 hyperoside, 932.76 ellagic acid, 8.52 rutoside, 60.51 sinapic acid, 17.63 cichoric acid; *G. pratense* L.: 180.37 hyperoside, 7195.45 ellagic acid, 75.09 isoquercitrine; *G. palustre* Torner: 4578.97 ellagic acid, 69.15 isoquercitrine, 67.66 caftaric acid, 67.41 rutoside; *G. columbinum* L.: 3620.24 ellagic acid, 59.05 isoquercitrine, 19.74 caftaric acid, 159.30 quercitrine; *G. dissectum* Jusl.: 29.41 isoquercitrine, 51.32 rutoside. Analysing by HPLC means the hydrolised samples we have obtained the following results (expressed in µg compound/100 g vegetal product): quercetol (48.72 in *G. phaeum* L., 165.76 in *G. pratense* L., 64.87 in *G. palustre* L., 222.76 in *G. columbinum* L. and 88.82 in *G. dissectum* Jusl.) and kaempherol (11.02 in *G. phaeum* L., 36.83 in *G. pratense* L., 77.31 in *G. palustre* L., 12.23 in *G. columbinum* L. and 9.90 in *G. dissectum* Jusl.). We have also demonstrated indirectly the presence of ellagic tannins (the amount of ellagic acid increases after hydrolise) and we have showed the probable presence in *G. dissectum* of polycaffeoil compounds (caffeic acid was identified in amount of 4.27 µg/100 mg only in the hydrolised sample).

Introduction

In our previous papers concerning the Romanian Geranium species we have initiated a phytochemical study using methods based on spectrophotometry, identity reactions and HPLC [1, 2, 3, 4, 5]. We continue our work by analysing some polyphenols using spectrophotometrical methods and an original HPLC method [5]. This HPLC method is of particular interest when the amounts of polyphenols in plant are too small to allow investigation by TLC means. It also allows measuring the amount for every identified active principle, while the quantitative spectrophotometrical methods do not allow measuring each particular polyphenolic compound.

Material and Methods

We have analysed the air dried aerial part of the following species (harvested from different regions from Transylvania): *Geranium phaeum* L., *G. pratense* L., *G. palustre* Torner, *G. columbinum* L. and *G. dissectum* Jusl. Species were collected as wild plants in Cluj-Napoca (district of Cluj) and were identified in the Department of Pharmaceutical Botany, Faculty of Pharmacy, „Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, where herbarium specimens are identified.

Spectrophotometric determinations:

The quantitative analyse of tannins was made using the method described in the Romanian Pharmacopeea Xth Edition [8].

The quantitative analyse of flavonoids and phenolic acids were made using the methods described in the Romanian Pharmacopoeia IXth Edition for the drug *Cynarae folium* [9].

The quantitative determination of anthocyanins was made using the Lees & Francis technique (1972), described by Markakis [7].

The quantitative determination of proanthocyanins was made using the Lebreton technique [6].

HPLC determinations [5]***:***

Apparatus and chromatographic conditions: We used an Agilent 1100 HPLC Series (Agilent, USA) equipped with a degasser G1322A, a quaternary gradient pump G1311A, an autosampler G1311A, a column oven G1316A, a Zorbax SB-C18 reversed-phase analytical column 100 mm x 3,0 mm i.d., 3,5 μm particle (Agilent, USA) and we operated at 48^{o}C. The mobile phase was a binary gradient: methanol and buffer solution. The buffer solution was prepared by dissolving potassium dihydrogen phosphate (40 mM) in water and the pH was adjusted to 2,3 with 85% orthophosphoric acid. The gradient begun with a linear gradient started at 5% methanol and 42% methanol over first 35 minutes, followed by isocratic elution with 42% methanol over the next 3 minutes. The flow rate was 1 ml/min and data were collected at 330 nm. The injection volume was 10 μl.

Samples preparation: 200 mg air dried powdered herba was placed in a 10 ml centrifuge tube; 2 ml water and 2 ml ethanol were added in the centrifuge tube. In order to study the flavonoid aglycones that can be obtained by hydrolise we have prepared a second sample containing 200 mg air dried herba powdered herba, 2 ml hydrochloric acid 2M and 2 ml methanol, all placed in a 10 ml centrifuge tube. In both samples we have added 200 μl ascorbic acid 10% solution (Sicomed Bucharest, Romania) as antioxydant. The mixtures were heated at 80°C for 30 minutes on a water bath, then they were sonicated for 15 minutes and finally heated again for another 30 minutes at 80°C, on the water bath. After extraction the mixtures were centrifuged with 4000 rpm and the remaining solids were extracted two times with additional 5 ml buffer solution using the same procedure. The combined extracts were diluted with buffer solution in a 25 ml volumetric flask and filtered through a 0,45 μm filter before injection.

Detection: detector UV 330 nm. All compounds were identified by external standard addition and comparison of their retention times with those of the standards, in same chromatographic conditions. Quantitative determinations were performed using external standard method.

Standards: caftaric acid, gentisic acid, caffeic acid, chlorogenic acid, p-cumaric acid, ferulic acid, sinapic acid, cicoric acid, hyperoside, ellagic acid, isoquercitrine, rutoside, quercitrine, quercetol, patuletine, luteoline, kaempherol, apigenine.

We present the HPLC chromatograms for the external standards at 330 nm (Fig. 1). We also present the retention times for all used standards (Table 1).

Table 1: Retention times for all standards

Polyphenolic compound	Retention time 330 nm		Polyphenolic compound	Retention time 330 nm
caftaric acid	3.27		ellagic acid	19.90
gentisic acid	3.76		isoquercitrine	20.27
caffeic acid	6.10		Rutoside	20.78
chlorogenic acid	6.80		quercitrine	23.64
p-cumaric acid	9.49		quercetol	27.57
ferulic acid	12.80		patuletine	29.39
sinapic acid	15.01		Luteoline	29.93
cichoric acid	15.83		kaempherol	32.50
hyperoside	19.32		apigenine	33.95

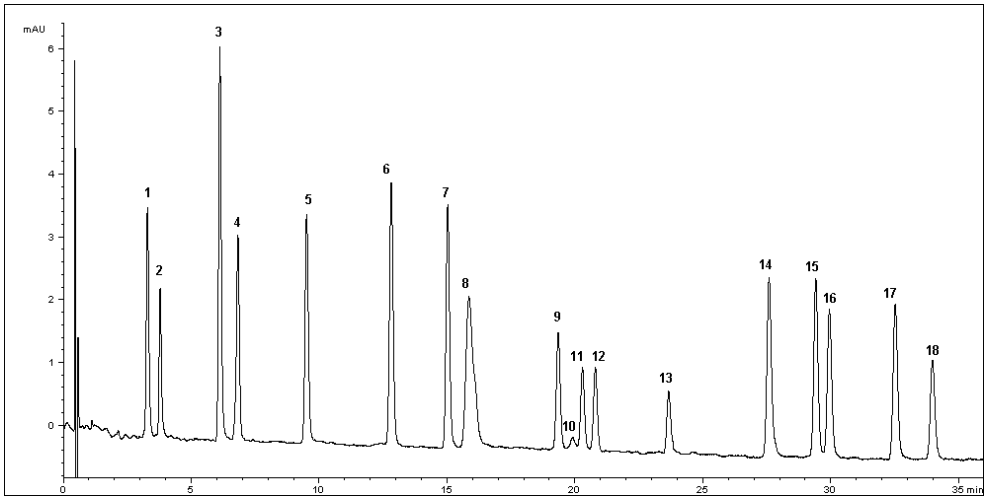

Fig. 1: The HPLC chromatogram for the external standards at 330 nm: 1=caftaric acid; 2=gentisic acid; 3=caffeic acid; 4=chlorogenic acid; 5=p-cumaric acid; 6=ferulic acid; 7=sinapic acid; 8=cichoric acid; 9=hyperoside; 10=ellagic acid; 11=isoquercitrine; 12=rutoside; 13=quercitrine; 14=quercetol; 15=patuletine; 16=luteoline; 17=kaempherol; 18=apigenine.

Results and Discussions

The results of the quantitative spectrophotometric determinations are shown in table 2.

Table 2: Results of the quantitative spectrophotometric determinations

Species	Flavonoids %	Polyphenolic acids %	Tannins %	Anthocyanins %	Proanthocyanins %
G. phaeum	0.16-0.43	0.43-0.60	3.06-3.44	$25.29 \cdot 10^{-3}$	0.57
G. columbinum	1.41	0.37	9.27	-	-

Following these quantitative determinations, the tannins seem to be the main group of active principles.

We have identified and measured by HPLC means the following polyphenolic compounds, working in the conditions described before: hyperoside, ellagic acid, rutoside, sinapic acid, cichoric acid, caftaric acid, isoquercitrine, quercitrine.

We present the under-curve areas and the concentrations (µg polyphenolic compound / 100 mg dried herba) for these compounds (Table 3).

We also present the HPLC chromatograms before hydrolise and after hydrolise (Fig. 2 - 11). After hydrolise, we have also identified kaempherol, quercetol and caffeic acid.

We have confirmed by HPLC means the fact that tannins are the main group of active principles (from all identified compounds, ellagic acid presents the highest concentration) and we have demonstrated indirectly the presence of ellagic tannins (the amount of ellagic acid increases after hydrolise in case of species *G. phaeum* L. and *G. pratense* L.). In acid medium the quantity of ellagic acid identified at a specific moment is the result of two reactions: its formation by ellagic tannins hydrolise and its degradation due to the acid medium. In case of *G. palustre* Torner and *G. columbinum* L. the amount of ellagic acid decreases after hydrolise, suggesting that the ellagic acid exists as free compound in big amount but it is found in smaller amount as ellagic tannins, by comparison with the other analysed species. În case of *G. dissectum* Jusl., determination of ellagic acid was interfered, but its amount seems to be significant.

We have identified and measured 4 flavonoids (hyperoside, isoquercitrine, quercitrine and rutoside) and 3 polyphenolic acids (caftaric acid, sinapic acid, cichoric acid). Identification

of caffeic acid only after hydrolise in *G. dissectum* Jusl. and the increase of sinapic and cichoric acids in *G. phaeum* L. after hydrolise shows that they exists in plant as free compounds (the sinapic and the cichoric acids), as well as bi- or polyderivatives (all of them, e.g. as polycaffeoil derivatives). In exchange, caftaric acid disappears after hydrolise, suggesting that it does not exist as polyderivatives or the its degradation ratio is higher than the hydrolise of polyderivatives ratio.

The flavonoidic aglycones (quercetol and kaempherol) were identified only as part of the molecules of the flavonoids (they can be identified only after hydrolise), they do not exist as free compounds.

Table 3: The under-curve areas and the concentrations (µg polyphenolic compound / 100 mg dried herba)

Species / Polyphenolic compound	Under-curve area		Concentration (µg / 100 mg)	
	BH	AH	BH	AH
G. phaeum				
hyperoside	37.58	-	23.918	-
ellagic acid	98.98	134.54	932.760	1268.319
quercetol	-	142.58	-	48.727
kaempherol	-	25.71	-	11.020
rutoside	9.55	-	8.522	-
sinapic acid	182.08	191.01	60.514	63.478
cichoric acid	70.19	80.97	17.639	20.22
G. pratense				
hyperoside	281.3	-	180.373	-
ellagic acid	758.9	994.8	7195.469	9479.462
isoquercitrine	78.47	-	75.092	-
quercetol	-	483	-	165.768
kaempherol	-	88.2	-	36.832
G. palustre				
ellagic acid	487.77	354	4578.979	3372.455
isoquercitrine	72.96	-	69.153	-
quercetol	-	188.32	-	64.873
kaempherol	-	186.16	-	77.316
caftaric acid	138.6	-	67.662	-
rutoside	74.34	-	67.414	-
G. columbinum				
ellagic acid	380.00	355.17	3620.243	3366.855
isoquercitrine	61.35	-	59.058	-
quercetol	-	652.7	-	222.763
kaempherol	-	28.53	-	12.232
caftaric acid	39.15	-	19.476	-
quercitrine	137.06	-	159.300	-
G. dissectum				
ellagic acid	Present in significant amount, but interfered			
isoquercitrine	30.27	-	29.418	-
quercetol	-	260.85	-	88.823
kaempherol	-	22.97	-	9.900
rutoside	55.55	-	51.329	-
caffeic acid	-	14.93	-	4.274
BH = before hydrolise; AH = after hydrolise				

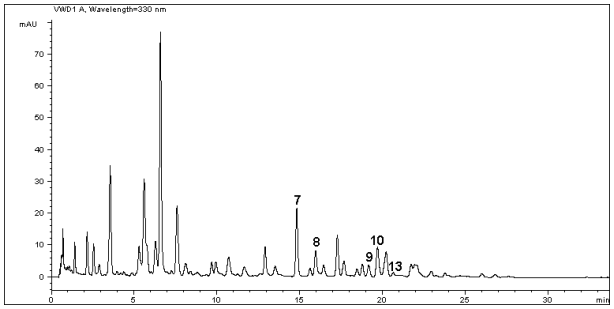

Fig. 2: HPLC chromatogram of *G. phaeum* before hydrolise

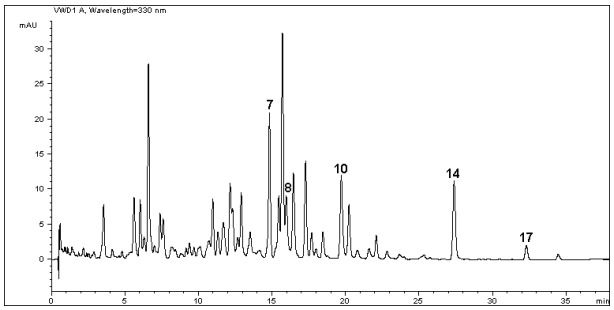

Fig. 3: HPLC chromatogram of *G. phaeum* after hydrolise

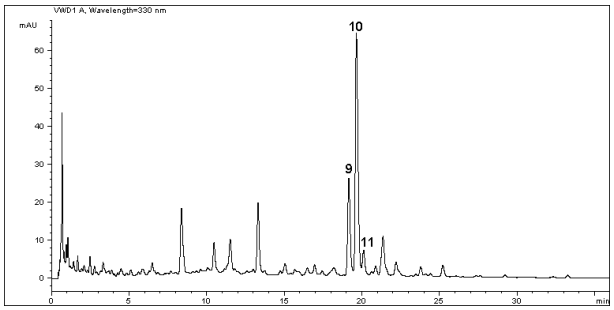

Fig. 4: HPLC chromatogram of *G. pratense* before hydrolise

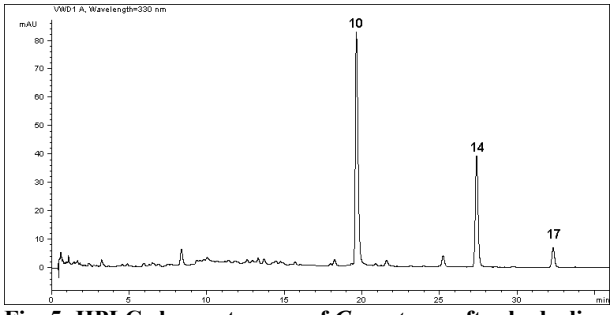

Fig. 5: HPLC chromatogram of *G. pratense* after hydrolise

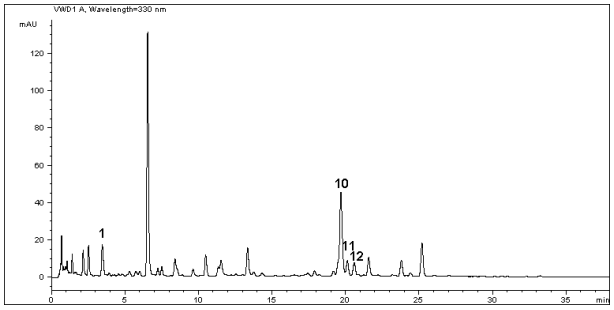

Fig. 6: HPLC chromatogram of *G. palustre* before hydrolise

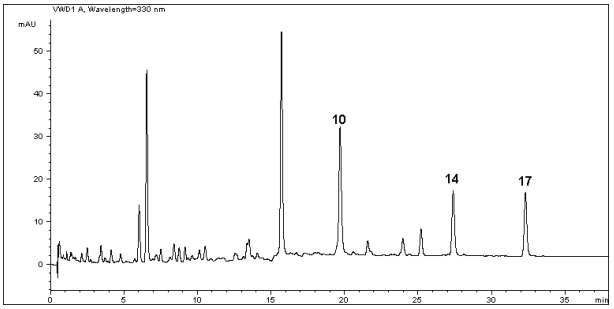

Fig. 7: HPLC chromatogram of *G. palustre* after hydrolise

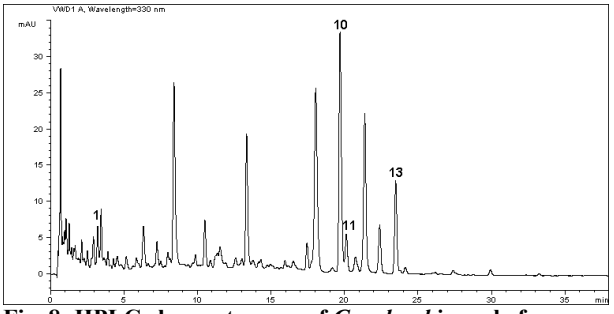

Fig. 8: HPLC chromatogram of *G. columbinum* before hydrolise

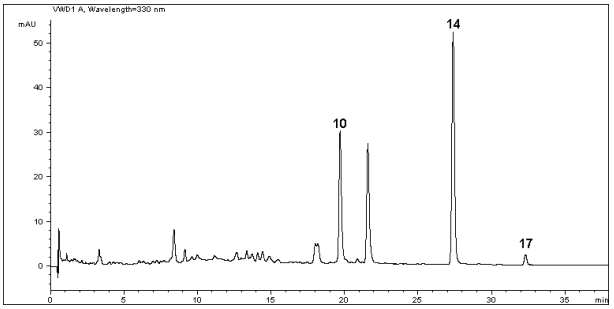

Fig. 9: HPLC chromatogram of *G. columbinum* after hydrolise

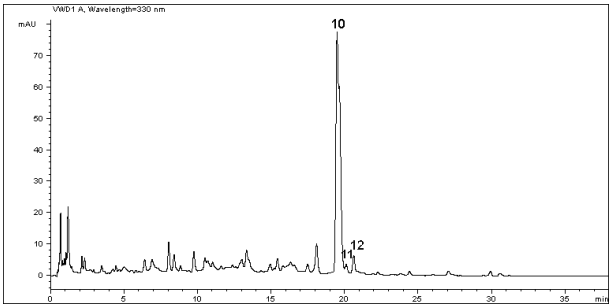

Fig. 10: HPLC chromatogram of *G. dissectum* before hydrolise

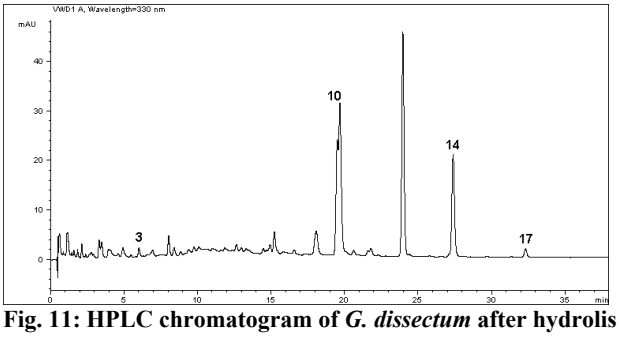

Fig. 11: HPLC chromatogram of *G. dissectum* after hydrolis

Conclusions

We have completed our previous data with new ones, concerning qualitative and quantitative determination for different classes of polyphenolic compounds (performed by spectrophotometrical methods), as well as for individual active principles (using HPLC determinations).

Our results are closely related to the methods that we have used and they do not have absolute value in case of other methods, more performant. They represent basic data for future phytochemical determinations, e.g. HPLC coupled with mass spectrometry. They also represent basic data for future pharmacological determinations, based on phytochemistry.

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STUDII FITOCHIMICE ASUPRA UNOR SPECII INDIGENE DE *GERANIUM* (GERANIACEAE)

(Rezumat)

În continuarea cercetărilor anterioare, a fost realizat un studiu fitochimic asupra a 5 specii indigene de *Geranium*. Prin dozări spectrofotometrice au fost obţinute următoarele rezultate: *G. phaeum* L.: 0,16-0,43% flavonoide, 0,43-0,60% acizi polifenolici, 3,06-3,44% taninuri, $25,29 \cdot 10^{-3}$ % antociani, 0,57 % proantociani; *G. columbinum* L.: 1,41% flavonoide, 0,37% acizi polifenolici, 9,27% taninuri. Prin HPLC au fost obţinute următoarele rezultate (exprimate în micrograme compus/100 g produs vegetal): *G. phaeum* L.: 23,91 hiperozidă, 932,76 acid elagic, 8,52 rutozidă, 60,51 acid sinapic, 17,63 acid cicoric; *G. pratense* L.: 180,37 hiperozidă, 7195,45 acid elagic, 75,09 izocvercitrin; *G. palustre Torner*: 4578,97 acid elagic, 69,15 izocvercitrin, 67,66 acid caftaric, 67,41 rutozidă; *G. columbinum* L.: 3620,24 acid elagic, 59,05 izocvercitrin, 19,74 acid caftaric, 159,30 cvercitrin; *G. dissectum* Jusl.: 29,41 izocvercitrin, 51,32 rutozidă. Analizând prin HPLC probe supuse hidrolizei acide, am evidenţiat (micrograme compus/100 g produs vegetal) prezenţa cvercetolului (48,72 în *G. phaeum* L., 165,76 în *G. pratense* L., 64,87 în *G. palustre* L., 222,76 în *G. columbinum* L. şi 88,82 în *G. dissectum* Jusl.) şi chemferolului (11,02 în *G. phaeum* L., 36,83 în *G. pratense* L., 77,31 în *G. palustre* L., 12,23 în *G. columbinum* L. şi 9,90 în *G. dissectum* Jusl.), ca agliconi ai flavonoidelor, am evidenţiat indirect prezenţa taninurilor elagice (prin creşterea concentraţiei de acid elagic în urma hidrolizei) şi am evidenţiat existenţa în cazul speciei *G. dissectum* a compuşilor de tip policafeoil (acidul cafeic a fost identificat în cantitate de 4,27 µg/100 mg produs vegetal numai în proba hidrolizată).