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PHENOLIC ACIDS IN THE FLOWERS AND LEAVES OF *GRINDELIA ROBUSTA* NUTT. AND *GRINDELIA SQUARROSA* DUN.(ASTERACEAE)

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Abstract: 2D-TLC and RP-HPLC methods were applied to qualitatively determinate free phenolic acids and those liberated by acid and alkaline hydrolysis in the flowers and leaves of *G. robusta* and *G. squarrosa*. The presence of eleven phenolic acids, namely: caffeic, chlorogenic, *p*-coumaric, *p*-hydroxybenzoic, ferulic, gallic, protocatechuic, vanillic salicylic, *p*-hydroxyphenylacetic and ellagic acids was determined. Quantitative estimate of phenolic acids, expressed as caffeic acid, has been analyzed by the method described in the Polish Pharmacopoeia VIII. The content of phenolic acids in *G. robusta* reached 7.33 mg/g and 6.23 mg/g for flowers and leaves, respectively. The flowers and leaves of *G.squarrosa* were characterized by similar level of phenolic acids, namely 6.81 mg/g and 6.59 mg/g, respectively.

Keywords: *Grindelia robusta* Nutt., *Grindelia squarrosa* Dun., Asteraceae, phenolic acids, qualitative and quantitative methods, 2D-TLC, RP HPLC

Grindelia robusta Nutt. and *Grindelia squarrosa* (Pursh.) Dun. (Asteraceae) are perennial medical plants grown naturally in North and South America (1, 2). They are rarely cultivated in Europe, including Poland (3, 4). Herbs of this two Grindelia species have been used in traditional medicine as expectorant and antiasthmatic drugs due to their antispasmodic and antiinflammatory activities (5–7). Tinctures from these herbs are used in homeopathy (4, 6). Herbs of *G. robusta* and *G. squarrosa* have been previously investigated and diterpenes, methylated flavonoids and essential oils have been isolated and identified. Typical of Grindelia species is the presence of resin reaching 20%, but only 6% in the herb of *G. robusta* (4, 8). Phytochemical investigation of the resin showed that the major components were bicyclic labdane diterpene acids termed grindelanes. From the herb of *G. robusta* and *G. squarrosa* grindelic acid and above twenty of its derivatives were isolated and identified (9, 10). Methylated flavonoids (7, 8, 11) and essential oils (12–14) were also identified as main components in the aerial parts of *G. robusta*. The presence of triterpenoid sapogenins (15), polyacetylenes (16, 17) and tannins (5, 6) was also reported. The occurrence of phenolic acids in mentioned species was known only in part. In the flowers of *G. robusta* and *G. squarrosa* vanillic acid, ferulic acid, *p*-hydroxybenzoic acid and p-coumaric acid were identified (11). Chlorogenic acid and caffeic acid were determined in the herb of *G. robusta* and *G. squarrosa*. (5, 7)

The aim of the present study was to determine the composition and content of phenolic acids in the flowers and leaves of two species of Grindelia genus: *Grindelia robusta* Nutt. and *Grindelia squarrosa* (Pursh.) Dun., cultivated in the Garden of Medicinal Plants in Łódź.

EXPERIMENTAL

Plant material

The material for studies were flowers and leaves of two species from Grindelia Wall.: *Grindelia robusta* Nutt. and *Grindelia squarrosa* (Pursh.) Dun. These plants were cultivated in the Garden of Medicinal Plants, Department of Pharmacognosy at the Medical University of Łódź. The raw materials were collected (July, August 2008) from sixth-year-old plants. The seeds for the beginning of this collection were supported by the Botanical Garden of the University in Bern (Switzerland) (*G. robusta*) and Gottingen (Germany) (*G. squarrosa*). The raw materials were dried in natural conditions and then pulverized and sieved (sieves 0.315 mm).

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Table 1. Identification of free phenolic acids in the investigated raw materials (t_R – retention time, RSD – relative standard deviation; n = 6 analyses for standards, n = 3 analyses for extracts).

No.	Phenolic acids	2D-TLC		RP-HPLC t_R (min) ± RSD				
		R_f		Standards	*G. robusta*		*G. squarrosa*	
		S1	S2		flowers	leaves	flowers	leaves
1	Gallic	0.00	0.00	5.17 ± 0.0464	5.08 ± 0.030	5.19 ± 0.017	5.10 ± 0.015	-
2	Protocatechuic	0.17	0.54	9.19 ± 0.1336	9.05 ± 0.043	9.29 ± 0.078	9.15 ± 0.030	9.05 ± 0.019
3	*p*-Hydroxybenzoic	0.52	0.61	14.30 ± 0.1598	14.09 ± 0.081	14.37 ± 0.022	14.24 ± 0.054	14.16 ± 0.091
4	*p*-Hydroxyphenylacetic	0.67	0.82	15.12 ± 0.1408	14.54 ± 0.085	-	15.21 ± 0.031	-
5	Chlorogenic	0.02	0.23, 0.58	15.73 ± 0.1268	15.44 ± 0.087	15.78 ± 0.055	15.69 ± 0.023	15.49 ± 0.070
6	Vanillic	0.72	0.51	16.71 ± 0.1320	16.34 ± 0.039	16.77 ± 0.034	16.67 ± 0.028	16.56 ± 0.036
7	*trans*-Caffeic	0.18	0.20	17.87 ± 0.1620	17.57 ± 0.066	17.77 ± 0.091	17.78 ± 0.095	17.66 ± 0.027
7’	*cis*-Caffeic	0.18	0.63	18.39 ± 0.1137	18.09 ± 0.072	18.12 ± 0.086	18.44 ± 0.075	18.60 ± 0.022
8	*p*-Coumaric	0.56, 0.6	0.34, 0.78	24.05 ± 0.1760	23.68 ± 0.067	24.07 ± 0.082	23.97 ± 0.049	23.86 ± 0.102
9	*trans*-Ferulic	0.82	0.16	26.38 ± 0.1401	26.26 ± 0.053	26.50 ± 0.059	26.35 ± 0.098	26.166 ± 0.020
9’	*cis*-Ferulic	0.85	0.53	27.19 ± 0.1227	27.08 ± 0.091	27.25 ± 0.047	27.12 ± 0.049	27.010 ± 0.017
10	Ellagic	0.00	0.00	28.27 ± 0.1330	28.15 ± 0.036	28.33 ± 0.017	28.18 ± 0.046	28.40 ± 0.066
11	Salicylic	0.90	0.33	33.15 ± 0.1145	33.03 ± 0.071	33.04 ± 0.094	33.05 ± 0.045	32.86 ± 0.065

Voucher specimens were deposited at the Department of Pharmacognosy, Medical University of Łódź.

Extraction and isolation of free of phenolic acids fraction

The known method for isolation of phenolic acids fractions was used (18, 19). The air-dried and powdered flowers and leaves (samples 10 g each) were extracted with petroleum ether, next with chloroform in Soxhlet apparatus, exhaustively, to remove lipid constituents and chlorophyll. After drying, the raw materials were heated with boiling methanol (4 × 150 mL) under reflux condenser. The methanol extracts were combined, respectively, concentrated under reduced pressure to dryness and hot water was poured into them. After 24 h, the ballast substances were filtered off and solutions were successively extracted with diethyl ether (8 × 40 mL). The combined ether extracts were concentrated to about 100 mL and extracted with 5% sodium bicarbonate solution (5 × 20 mL). The aqueous fractions were acidified with 10% HCl to pH 3 and re-extracted with diethyl ether (5 × 20 mL). The obtained ether extracts were dried with anhydrous sodium sulfate. Next, the solvents were evaporated and remains were dissolved in 10 mL of methanol. As a result, fractions of free phenolic acids were obtained and they were examined by 2D-TLC and RP-HPLC. Identification of free phenolic acids by comparison of R_f values and retention times with standards are presented in Table 1.

Release of phenolic acids by acid and alkaline hydrolysis

The aqueous fractions (with $NaHCO_3$) were divided in two parts. The first part was acidified by 36% HCl to pH 2, heated for 1 h at 100°C and then extracted with diethyl ether (10 × 10 mL). Part two was submitted to alkaline conditions with $NaBH_4$ and $Ba(OH)_2$ at pH 12. The solution was heated for 15 min and finally, after neutralization and acidification to pH 2, re-extracted using diethyl ether (10 × 10 mL). The ether extracts were evaporated to dryness under reduced pressure and dissolved in methanol (5 mL) yielding fractions of free phenolic acids liberated by acid hydrolysis and alkaline hydrolysis.(18, 19) The presence of liberated phenolic acids is shown in Table 2.

Identification of free and liberated phenolic acids

The qualitative analysis of free and liberated phenolic acids in the ether extracts from flowers and leaves of *G. robusta* and *G. squarrosa* using 2D-TLC and RP-HPLC methods was undertaken.

2D-TLC methods

Ten mg of each standard were diluted with 50% methanol in 10 mL volumetric flask. 2D-TLC on cellulose (Cellulose DC – Alufolien 20 × 20 cm,

Table 2. RP-HPLC chromatographic comparison of the distribution of liberated phenolic acids in the flowers and leaves of two Grindelia species.

No	Phenolic acids	*Grindelia robusta*				*Grindelia squarrosa*			
		flowers		leaves		flowers		leaves	
		Ac	Al	Ac	Al	Ac	Al	Ac	Al
1	Gallic	tr	–	tr	tr	tr	tr	–	–
2	Protocatechuic	⊕	+	⊕	tr	⊕	+	⊕	+
3	*p*-Hydroxybenzoic	⊕	+	⊕	+	⊕	+	⊕	+
4	*p*-Hydroxyphenylacetic	–	–	tr	–	–	–	tr	–
5	Chlorogenic	tr	–	tr	–	–	–	tr	–
6	Vanillic	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕
7	Caffeic	+	⊕	+	+	+	+	+	+
8	*p*-Coumaric	tr	+	tr	–	–	+	tr	–
9	Ferulic	–	+	tr	–	tr	+	–	+
10	Ellagic	+	–	+	⊕	–	–	–	–
11	Salicylic	–	+	–	–	–	tr	–	–

Ac, Al – fractions containing phenolic acids liberated by acid (Ac) or alkaline (Al) hydrolysis. Amounts: ⊕: dominant, +: present, tr: trace, –: absent.

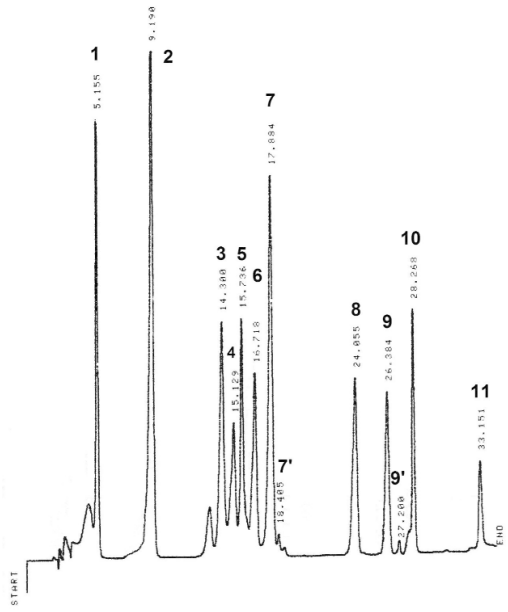

Figure 1. RP-HPLC chromatogram of the standards mixture of phenolic acids (number of compounds as in Table 1)

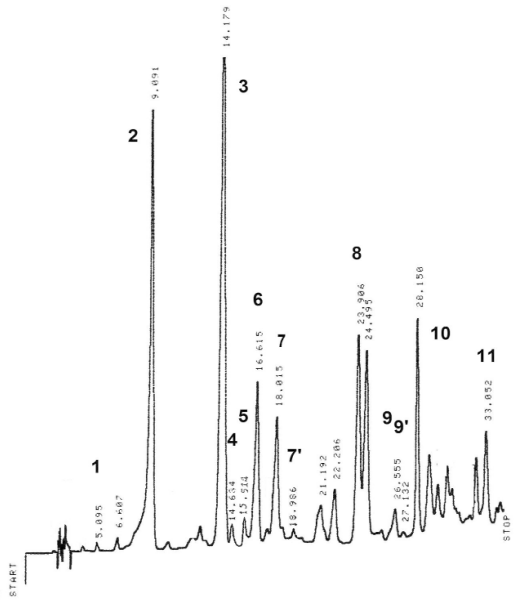

Figure 2. RP-HPLC chromatogram of the ether fraction of free phenolic acids from *G. robusta* flowers

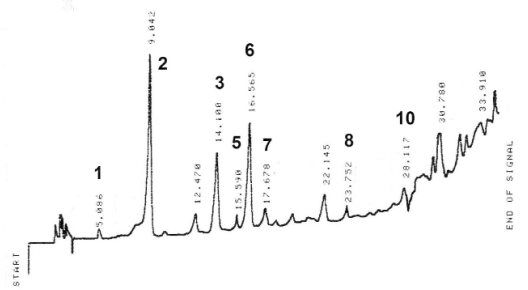

Figure 3. RP-HPLC chromatogram of the ether fraction of phenolic acids liberated by acid hydrolysis from *G. robusta* flowers

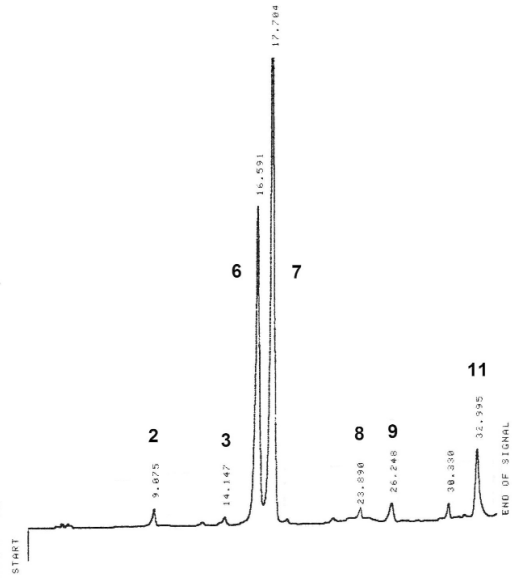

Figure 4. RP-HPLC chromatogram of the ether fraction of free phenolic acids liberated by alkaline hydrolysis from *G. robusta* flowers

layer thickness 0.1 mm, Merck, Germany) was carried out using the following systems: S1 – toluene–acetic acid–water (6:7:3, v/v/v, upper phase), S2 – acetic acid–water (15:85, v/v). The all-glass chambers were conditioned for 30 min with mobile phase vapor. After application of extracts and standard solutions (approx. 50 µL each), plates were developed to a distance of 18 cm in chambers. After development, the mobile phase was evaporated to dryness. Then, the chromatograms were analyzed under UV light (254 nm and 366 nm) before and after treatment with ammonia vapor. Next, all chromatograms were sprayed with 0.5% diazotized sulfanilic acid in 10% sodium carbonate solution and visualized in the daylight.

Table 3. The content of phenolic acids (mg/g of dry weight), expressed as caffeic acid, in the flowers and leaves of *G. robusta* and *G. squarrosa* and their statistical evaluation (n = 6, $t_{\alpha f}$ = 2.571, α = 0.05, f = 5).

Plant material		$\bar{X}$ [mg/g]	S	S_x	VC [%]	μ	$\bar{X} \pm 2S$
G. robusta	Flowers	7.331	0.2349	0.0959	3.21	7.331 ± 0.2465	7.331 ± 0.4698
	Leaves	6.230	0.1663	0.0679	2.67	6.230 ± 0.1744	6.230 ± 0.3326
G. squarrosa	Flowers	6.808	0.0946	0.0386	1.39	6.80 ± 0.0993	6.808 ± 0.1892
	Leaves	6.597	0.1196	0.0488	1.81	6.597 ± 0.1255	6.597 ± 0.2392

$\bar{X}$ – mean content of phenolic acids, S – standard deviation, S_x – average deviation, VC – variation coefficient, μ ($\bar{X} \pm S \cdot t_\alpha$) – confidence interval.

RP-HPLC methods

Equipment

The RP-HPLC system used was the chromatograph Hewlett-Packard 1100 series consisted of a quaternary pump HP 1311A, vacuum degasser HP 1322A, manual injector 20 μL (Rheodyne 7725i, Cotati, CA, USA), variable wavelength UV/VIS detector HP 1314 A, integrator HP series 3395, chart speed 5 mm/min. Phenolic acids were separated using HPLC-cartridge LichroCART (250 × 4 mm) filled up a Lichrosphere 100 RP-18 (5 μm) (Merck, Darmstadt, Germany) with guard pre-column 4 × 4 mm (Hypersil ODS, 5 μm) (HP, Germany). The samples with 25 μL gas tight syringe (Waters, Australia) were injected.

Chromatographic procedure

Phenolic acids were separated by gradient elution using solvents: A: water – phosphoric acid (99.5:0.5), B: acetonitrile. A step gradient was as follows: 0–9 min.: 6–10% B in A, 9–15 min.: 10–14% B in A, 15–17 min.: 14–15% B in A, 17–23 min.: 15–18% B in A, 23–30 min.: 18–25% B in A, 30–34 min.: 25–30% B in A. Elution was carried out at room temperature with a flow rate of 1 mL/min and UV detection at 254 nm (sensitivity 0.05 AUFS). The solvents were of HPLC grade (acetonitrile – Merck, *orto*-phosphoric acid – Sigma Aldrich, redistilled water). Methanol solutions of standards of phenolic acids (0.1 mg/mL) were prepared in 50% MeOH. All standards of phenolic acids were purchased from Fluka AG (Switzerland) or Koch-Light Laboratories (Germany).

Identification of free and liberated phenolic acids by RP-HPLC method was done using external and internal standards.

The results of RP-HPLC analysis are presented in Fig. 1–4.

Quantitative determination of phenolic acids

Air-dried, powdered and sieved flowers (0.5 g) and leaves (1.0 g) of *G. robusta* and *G. squarrosa* were extracted with water (2 × 25 mL) in boiling water bath for 30 min. The combined extracts were filtered to the volumetric flask (50 mL). The content of phenolic acids, calculated as caffeic acids, was determined by the spectroscopic method with the Arnov's reagent (sodium molybdate (10 g) and sodium hydroxide (10 g) dissolved in water in a 100 mL volumetric flask) described in the Polish Pharmacopoeia VIII (20). The reagents: 1 mL of hydrochloric acid solution (18 g/L), 1 mL of the Arnov's reagent and 1 mL of sodium hydroxide solution (40 g/L) were successively added to the exactly measured volumes of the analyzed extracts. In the end, each sample was filled up with water to 10 mL. Extracts and the mixture of the above reagents (without Arnov's reagent) were used as a reference. The absorbance was measured immediately at 490 nm using the spectrophotometer (VIS 6000, Krüss, Germany) and glass cell 1 cm.

Calibration

The stock solution of caffeic acid was prepared by dissolving 10 mg of this compound in methanol in 50 mL volumetric flask. Six different volumes of this solution (0.15, 0.3, 0.45, 0.6, 0.9 and 1.2 mL corresponding to amounts of caffeic acid in the range of 3–24 mg/mL) were pipetted to 10 mL volumetric flask, then 1 mL of hydrochloric acid solution (18 g/L), 1 mL of the Arnov's reagent and 1 mL of sodium hydroxide solution (40 g/L) were added and flasks were filled up with water to the mark. The calibration graph for caffeic acid (Fluka AG, Switzerland) of dependence between absorbance (y) and concentration (x) was described by equation: y = 0.0339x + 0.0224, correlation coefficient r = 0.9989.

The results of quantitative determination and their statistical evaluation are presented in Table 3.

Statistical analysis

The differences between the means were analyzed for statistical significance using the Student's *t*-test.

RESULTS AND CONCLUSIONS

The present investigation is the first trial to reveal the qualitative and quantitative composition of phenolic acids in the flowers and leaves of *Grindelia robusta* and *Grindelia squarrosa,* cultivated in the Garden of Medicinal Plants at the Department of Pharmacognosy in Łódź.

The results of qualitative analysis carried out by 2D-TLC and confirmed by RP-HPLC methods showed the composition of eleven free phenolic acids in each examined raw material and several phenolic acids liberated by acid and alkaline hydrolysis. The acids were derivatives of benzoic acid (gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, ellagic, *p*-hydroxyphenylacetic, salicylic) and cinnamic acid (caffeic, *p*-coumaric, ferulic and chlorogenic). Only gallic acid in the leaves of *G. squarrosa* and *p*-hydroxyphenylacetic acid in the leaves of both species were not detected.

In this study, it was observed that the qualitative composition of free phenolic acids in the examined raw materials of two Grindelia species is generally similar, but there are some differences in the composition of phenolic acids liberated by acid and alkaline hydrolysis. The predominant compounds in all four studied materials were vanillic acid after acid as well as alkaline hydrolysis, and protocatechuic acid and *p*-hydroxybenzoic acid after acid hydrolysis only. In the leaves of *G. robusta*, after alkaline hydrolysis, ellagic acid was predominant as well. Caffeic acid was present in all raw materials after both hydrolyses.

The total content of phenolic acids, calculated as caffeic acid, was determined by the spectroscopic method with Arnov's reagent, described in PPh VIII. The flowers of *G. robusta* contain the highest concentration of phenolic acids, namely 7.33 mg/g in the dry raw material, whereas the leaves were characterized by the lowest content of these compounds – only 6.23 mg/g. The concentration of phenolic acids in the flowers and leaves of *G. squarrosa* was similar, 6.81 and 6.59 mg/g, respectively. The results of quantitative determination showed that the investigated raw materials contained relatively similar quantities of phenolic acids, but flowers contain higher amounts of phenolic acids than leaves.

The above described qualitative and quantitative determination of phenolic acids in the fractions obtained from the flowers and leaves of *G. robusta* and *G. squarrosa* has been undertaken for the first time.

Phenolic acids are widespread in medicinal plants and they are responsible for the anti-inflammatory, antibacterial, spasmolytic and antioxidant effect of plant material examined in this work (21).

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REFERENCES

1. Heywood V.H., Harborne J.B., Turner B.L.: The Biology and Chemistry of the Compositae, Vol. 1, Academic Press, London 1997.
2. Bartoli A., Tortosa R.: Flora 198, 106 (2003).
3. Rutkowski L.: Key for identification of vascular plants of lowland Poland (Polish). PWN, Warszawa 2004.
4. Strzelecka H., Kowalski J.: Encyclopedia of herbal medicine and herbal treatment (Polish). PWN, Warszawa 2000.
5. Schimmer O., Egersdörfer S.: Z. Phytother. 9, 86 (1988).
6. Melzig M. F., Gehrmann B.: Dtsch. Apoth. Ztg. 142, 69 (2002).
7. Krenn L., Wollenweber E., Steyrleuthner K., Görick C., Melzig M.F.: Fitoterapia 80, 267, (2009).
8. Timmermann B. N.: Z. Naturforsch. C 49, 395 (1994).
9. Timmermann B. N., Hoffmann J. J., Jolad S. D., Schram K. H.: Phytochemistry 24, 1031 (1985).
10. Adinolfi M., Della Greca M., Mangoni L.: Phytochemistry 27, 1878 (1988).
11. Pinkas M., Didry N., Trock M., Bézanger L.: An. Pharm. Fr. 36, 97 (1978).
12. Kaltenbach G., Schimmer O.: Planta Med. 57, Suppl. A, 82 (1991).
13. Schäfer M., Schimmer O.: J. Essent. Oil Res. 12, 547 (2000).
14. Fraternale D., Giamperi L., Ricci D.: Fitoterapia 78, 443 (2007).
15. Kreutzer S., Schimmer O., Waibel R.: Planta Med. 56, 392 (1990).
16. Schulte K. E., Reisch J., Busch P.: Arch. Pharm. 8, 496 (1964).
17. Bohlmann F., Kleine K. M., Bornowski H.: Chem. Ber. 98, 369, (1965).
18. Schmidtlein H., Herrmann K.: J. Chromatogr. 115, 123 (1975).
19. Świątek L.: Herba Polon. 23, 21 (1977).
20. The Polish Pharmacopoeia VIII, P.T.Farm., Warszawa 2009.
21. Hansel R., Sticher O., Steinegger E.: Pharmakognosie – Phytopharmazie, Springer-Verlag, Berlin 1999.

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