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NATURAL DRUGS

FREE AND CHEMICALLY BONDED PHENOLIC ACIDS IN BARKS OF *VIBURNUM OPULUS* L. AND *SAMBUCUS NIGRA* L.

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Abstract: Liquid column chromatography, planar chromatography (TLC) on modified and unmodified silica layers, reversed-phase high-pressure liquid chromatography (HPLC), as well as ESI-TOF MS and ^{1}H-NMR have been used for separation, purification and identification of phenolic acids in the barks of *Sambucus nigra* and *Viburnum opulus* (*Caprifoliaceae*). By the use of these procedures three cinnamic acid derivatives: caffeic acid, *p*-coumaric, and ferulic acid, four benzoic acid derivatives: gallic acid, protocatechuic acid, syringic acid, 3,4,5-trimethoxybenzoic acid, two phenylacetic acid derivatives: 3,4-dihydroxyphenylacetic acid, homogentisic acid, and two depsides: chlorogenic acid and ellagic acid were detected and identified in the bark of *Viburnum opulus*. Caffeic acid, *p*-coumaric acid, ferulic acid, gallic acid, syringic acid, 3,4,5-trimethoxybenzoic acid and chlorogenic acid were also detected and identified in the bark of *Sambucus nigra*. Except for chlorogenic acid, this is the first time these phenolic acids have been isolated, detected, and identified in the bark of *V. opulus* and *S. nigra*.

Keywords: phenolic acids, *Viburnum opulus* L., *Sambucus nigra* L., HPTLC, RP HPLC

Plants containing phenolic acids are widely used in phytotherapy. These compounds have varied pharmacological activity, e.g. anti-inflammatory (1), cholagogue (2), antioxidant (3), antibacterial (4), and antivirus efficacy (5). Some research showed also their immunostimulating and anti-cancerogenic properties (6). Phenolic acids inside the plant perform defensive functions against pathogenic agents (7).

Sambucus nigra and *Viburnum opulus* are two species from *Caprifoliaceae* family, widely distributed in Poland. However, their belongings to the same botanical family is strongly discussed nowadays because of many differences also in the field of phytochemistry (8). Previous chemical investigations of this plants were concentrated on the following chemical groups: flavonoids, cyanogene glycosides, terpenes, lectins in different parts of *S. nigra* (9) and iridoides, triterpens, condensed tannins in *V. opulus* (10-12). Anthocyanes were examined many times in the fruits of both species. Investigation of phenolic acids in the bark of this plants has not yet been performed. In this work the presence of phenolic acids in the bark of *Sambucus nigra* and *Viburnum opulus* were investigated by liquid column chromatography, MGD-TLC and RP-HPLC as well as ESI-TOF MS and ^{1}H-NMR.

EXPERIMENTAL

Plant material

Viburnum opulus bark was from "Kawon-Hurt" Nowak Sp.j., Krajewice, Poland. *Sambucus nigra* bark was collected in October 2005 from three years plant grown in the Medicinal Plants Garden at the Wroclaw Medical University.

Chromatography

TLC chromatography

TLC glass plates coated with 0.25 mm layers of silica gel Si 60F_{254} (10 × 20 cm, Merck, Darmstadt, Germany) were used. Samples of extracts of the analyzed plants and standard solutions were spotted and developed with mobile phases: S_1 = ethyl acetate - formic acid - glacial acetic acid - water (100:11:11:26, v/v/v/v), S_2 = benzene - ethyl acetate - formic acid (80:20:10, v/v/v).

MGD-TLC was performed with HPTLC Si60F_{254} (0.20 mm, 10 × 20 cm, Merck, Darmstadt, Germany) with four-step solvent gradient described in Table 1 and HPTLC NH_2 glass plates (0.20 mm, 10 × 20 cm, Merck, Darmstadt, Germany) with two-step solvent gradient described in Table 2. Chromatograms were developed in a horizontal Teflon DS chamber (Chromdes, Lublin, Poland) and plates were dried between developments.

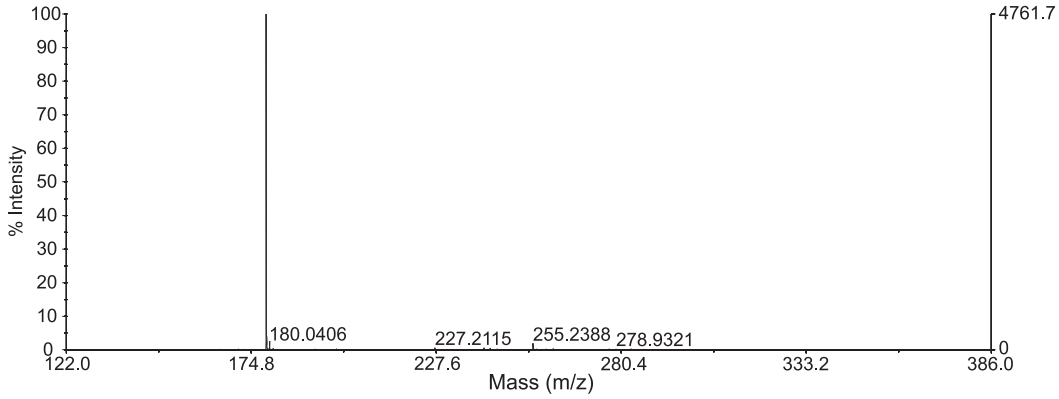

Figure 1. ESI-TOF MS spectra of caffeic acid.

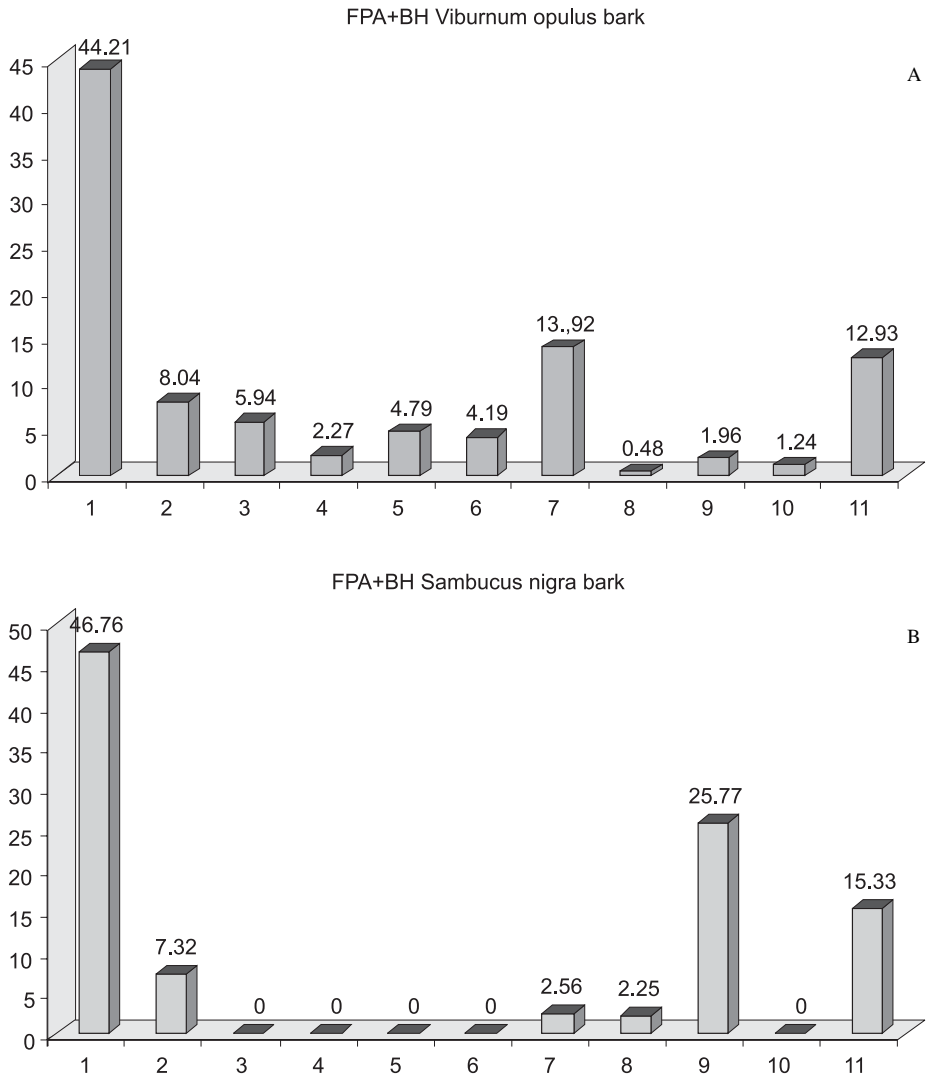

Figure 2. Amounts of phenolic acids FPA + BH, as mean percentage of total phenolic acids found, in the bark of *Viburnum opulus* (2A) and *Sambucus nigra* (2B).1) caffeic acid; 2) gallic acid; 3) protocatechuic acid; 4) ellagic acid; 5) 3,4-dihydroxyphenylacetic acid; 6) homogentisic acid,; 7) syringic acid; 8) *p*-coumaric acid; 9) ferulic acid; 10) 3,4,5-trimethoxybenzoic acid; 11) 4-hydroxybenzoic acid

Table 1. Four step solvent gradient for HPTLC Si60 of phenolic acids.

Step number	Eluent % (v/v)			Volume [mL]	Distance [mm]
	Cyclohexane	Diisopropyl ether	Formic acid		
1.	-	80	20	1	20
2.	20	78	2	5	90
3.	20	78	2	5	90
4.	20	79	1	5	90

Table 2. Two step solvent gradient for HPTLC NH_2 of phenolic acids.

Step number	Eluent % (*v*/*v*)		Volume [mL]	Distance [mm]
	Acetone	Glacial acetic acid		
1.	85	15	5	90
2.	90	10	5	90

Preparative TLC was performed with glass plates coated with 0.5 cm layers of silica gel Kieselgel 60-F_{254}, DC-Fertigplatten (20 × 20 cm, Merck), mobile phase S_2 = benzene - ethyl acetate - formic acid (80:20:10, v/v/v). Developed chromatograms of phenolic acids were analyzed under UV light (λ = 254, 366 nm), before and after exposure to ammonia vapors. Besides, all chromatograms were analyzed after spraying with 2% $FeCl_3$ in water-methanol solution.

Column chromatography

Glass columns packed with polyamide SC-6 (5 × 35 cm, Roth), mobile phase S_3 = methanol - water (1:1, *v*/*v*) and S_4 = methanol-water gradient (5:95 → 30:70, v/v). Final purification was performed on Sephadex LH-20 (5 × 35 cm, Pharmacia, Sweden) with methanol as a mobile phase and preparative TLC.

High performance liquid chromatography (HPLC)

The HPLC system used was from Knauer (Berlin, Germany) and composed of two pumps, Model 64-00, and Model of 890, variable wavelength UV-VIS detector, equipped with a 20 µL sample loop, under computer control (Knauer HPLC, Version 2.21). Phenolic acids were separated on a Supelcosil LC-18 (3 µm) column (150 × 4.6 mm I.D.).

Solvents were of HPLC grade (acetonitrile - Merck, formic acid - Sigma Aldrich). Redistilled water was used. Phenolic acids were separated by gradient elution using mobile phases: A: acetonitrile - formic acid (98.5:1.5, v/v), B: water - formic acid (98,5:1,5, v/v). A step gradient was as follows: 0-2 min. (10% of A isocratic), 2-20 min. (10-15% of A linear gradient), 20-35 min. (15-35% of A linear gradient), 35-37 min. (35-90% of A linear gradient) and 37-40 min. (90% of A isocratic). Elution was carried out at room temperature with a flow rate of 1,0 mL/min and UV detection at 280 nm.

Table 3. Negative ions (M-1) values of phenolic acid isolated from *Sambucus nigra* and *Viburnum opulus* bark.

Phenolic acid	**M-1**
Caffeic	179.0426
Gallic	169.0201
Homogentisic	167.0593
Protocatechuic	153.1012
Ellagic	301.0143
Chlorogenic	353.2931
Syringic	197.0207
Ferulic	193.0058
4-Hydroxybenzoic	138.0712

All standards of phenolic acids were purchased from Extrasynthese, Koch-Light Laboratoires, Laboratory BDH Reagent, and Fluka.

Extraction and isolation.

Dried and finely powdered plant material (500 g) was extracted with petroleum ether on a water bath for 6 h to remove lipid constituents. TLC analysis showed that these extracts contained no phenolic acids. After draining and drying, the plant material was extracted with 70% methanol for 12 h on a water bath. The crude extracts were concentrated

Table 4. TLC analysis of phenolic acids isolated from bark of *V. opulus* and *S. nigra*.

Comp. no.	Phenolic acids	R_f (×100) values in solvent system			Spot color	
		S2	HPTLC NH_2 MGD-TLC	HPTLC Si60 MGD-TLC	VIS	UV 254nm
I	Caffeic	68	81	50	br	ybr
II	Gallic	24	36.6	41	dbr	dbr
III	Homogentisic	42	59.4	44	y	pl
IV	Protocatechuic	48	74	56.7	ybr	pl
V	Ellagic	14	9.4	0	dbr	dbr
VI	Chlorogenic	16	5.8	4.4	br	br
VII	Syringic	49	95.3	42.2	y	v
VIII, IX	Ferulic	80	92	54.4	dy	bv
X, XI	4-Hydroxybenzoic	72	93	67	y	d

Explanations: d – dark, y – yellow, br – brown, v – violet, pl – plum

under reduced pressure at 40°C and cooled under refrigeration for 12 h. After that, 36% HCl was added until pH = 2 was reached, then the solutions were heated for 2 h on a water bath and finally cooled. The precipitate, which appeared after that time, was filtered off and the aqueous solutions were successively re-extracted with diethyl ether (5 × 250 mL) and ethyl acetate (5 × 250 mL). Extracts were then concentrated to dryness and combined to give 22 g (*V. opulus*) and 17 g (*S. nigra*) of sirupy masses. They were applied on prepared polyamide column and eluted with S_3 mobile phase to separate phenolic compounds from other plant metabolites. Fractions rich in phenolic acids were then applied on other polyamide column and compounds were separated with gradient solvent system S_4. Final separations for some acids were achieved by column chromatography on Sephadex LH-20 with methanol as a mobile phase and preparative TLC with S_2. From the bark of *V. opulus* 8 pure phenolic acid were obtained (**I –** 112 mg, **II** – 14 mg, **III** – 10 mg, **IV** – 21 mg, **V** – 8 mg, **VI** – 92 mg, **VII** – 61 mg). From the bark of *S. nigra* 3 pure phenolic acids were obtained (**VIII** – 30 mg, **IX** – 7 mg, **X** – 20 mg). Acids obtained in this way were crystallized from absolute methanol and identified by ESI-TOF MS and NMR. An example of MS spectra for caffeic acid is depicted in Figure 1. Negative ions (M-1) values of isolated phenolic acids are presented in Table 3.

Release of phenolic acids by alkaline and acid hydrolysis

The procedure used here was described elsewhere (13). Briefly: 10 g of each bark were extracted with petroleum ether and then 70% methanol as described in the extraction and isolation part. These aqueous extracts were then divided into two equal parts.

Part I of each extract was acidified by 36% HCl and extracted with diethyl ether (5 × 25 mL), the extract was evaporated do dryness under reduced pressure and dissolved in methanol (5 mL) followed by TLC and HPLC analysis. This fraction, denoted FPA, contained free phenolic acids. The water wastes were subjected to enzymatic hydrolysis by treatment with β-glucosidase (Koch-Light, Colnbrook, Bucks, UK; 30 mg) at pH = 4,5 adjusted by addition of 2 M NaOH for 10 h on a water bath at 37°C. The enzyme was then inactivated by heating on a hot water bath at 100°C for 30min. After filtration the solution was acidified to pH =2 by addition of 36% HCl and the hydrolysates were extracted with diethyl ether (5 × 25 mL) followed by evaporatation to dryness and finally the residues were dissolved in methanol (5 mL). This fraction, denoted EH, contained free phenolic acids liberated by enzyme hydrolysis. Part II was hydrolyzed under alkaline conditions with $NaBH_4$ (1 g) and NaOH (2 M) at pH = 12 on a water bath. The pH was then adjusted to 2 with 36% HCl and the solution was extracted under reduced pressure and dissolved in methanol. This fraction, denoted FPA + BH, contained free acids and acids liberated by alkaline hydrolysis. Fractions with FPA + BH and EH were also examined by TLC and HPLC.

Identification

The pure isolated compounds were identified by negative ESI-TOF MS (Hewlett-Packard) and ^{1}H-NMR analysis (Bruker MSL 300 MHz in d_6-DMSO; chemical shifts quoted relative to TMS as internal standard). Melting points were determined on a

Table 5. Contents of phenolic acids in the bark of *V. opulus* and *S. nigra* analyzed by HPLC.

Phenolic acid (regression line, correlation coefficient)	***Viburnum opulus* (contents of acids in mg/kg)**						***Sambucus nigra* (contents of acids in mg/kg)**					
	FPA	**SD n = 4**	**FPA+ BH**	**SD n = 4**	**EH**	**SD n = 4**	**FPA**	**SD n = 4**	**FPA+ BH**	**SD n = 4**	**EH**	**SD n = 4**
Caffeic acid (y = 107596.42x + 250.53 r = 0.9993)	-	-	667.2	3.45	7.52	0.022	35.83	0.105	153.28	0.296	7.95	0.02
Gallic acid (y = 53527x + 30.57 r = 0.9991)	-	-	121.32	2.43	6.74	0.026	-	-	23.99	0.29	-	-
Protocatechuic acid (y = 31918x + 277.08 r = 0.9989)	-	-	89.69	3.74	3.64	0.006	-	-	-	-	-	-
Ellagic acid (y = 1353801.45x – 18.71 r = 0.9999)	-	-	34.30	1.30	-	-	-	-	-	-	-	-
3.4-Dihydroxyphenylacetic acid (y = 19016.30x + 2.85 r = 0.9999)	-	-	72.29	2.46	-	-	-	-	-	-	-	-
Homogentisic acid (y = 16748.06x + 9.41 r=0.9995)	-	-	63.21	2.45	-	-	-	-	-	-	-	-
Syringic acid (y = 40529.82x + 30.41 r = 0.9946)	-	-	210.13	1.66	-	-	-	-	8.39	0.28	23.51	0.38
p-Coumaric acid (y = 417772.90x + 1252.53 r = 0.9999)	-	-	7.29	0.075	-	-	trace	-	7.38	0.007	trace	-
Ferulic acid (y = 58176.44x + 4.35 r = 0.9999)	-	-	29.66	1.33	-	-	4.38	0.026	84.48	0.437	5.52	0.13
3.4.5-Trimethoxybenzoic acid (y = 109985.69x – 5.08 r = 0.9999)	-	-	18.76	0.58	-	-	4.01	0.049	-	-	-	-
Chlorogenic acid (y = 31247.70x + 22.08 r = 0.9994)	544.24	14.58	-	-	-	-	31.80	0.188	-	-	-	-
4-Hydroxybenzoic acid (y = 30331.38x + 32.23 r = 0.9992)	-	-	195.14	0.54	5.98	0.11	-	-	50.25	0.78	-	-

Boetius apparatus. The results were in agreement with the literature data for phenolic acids (14, 15).

RESULTS AND DISCUSSION

The phenolic acid complex was analyzed in dried barks of *S. nigra* and *V. opulus*. Methanolic extacts of these barks after acid hydrolysis, methanol evaporation and re-extraction to ethyl acetate and diethyl ether were applied to series of solid phases for column chromatography. The chromatography on polyamide enabled primary purification which was continued by column chromatography on Sephadex LH-20 and preparative TLC. Finally, the isolated compounds were crystallized from absolute methanol and analyzed by ESI-TOF MS and ^{1}H-NMR spectroscopy. The obtained data were compared with those from literature for phenolic acids (14, 15). On the basis of these data, as well as melting points and TLC R_f value compared with pure standard, compounds were identified as **I** = caffeic acid, **II** = gallic acid, **III** - homogentisic acid, **IV** = protocatechuic acid, **V**= ellagic acid, **VI** = chlorogenic acid, **VII** = syringic acid, **VIII** was identical with **I** (caffeic acid), **IX** was identical with **VI** (chlorogenic acid), **X** = ferulic acid, **XI** = 4-hydroxybenzoic acid. To our best knowledge, only chlorogenic acid was previously detected in the bark of both species. Other acids described in this paper were isolated for the first time from those barks.

Free phenolic acids and those liberated after enzymatic and alkaline hydrolysis were also examined. Qualitative analysis was performed by TLC and HPTLC of samples and standards by one-step as well as multiple development. On the basis of those analyses chlorogenic acid was detected as free acid in *Viburnum opulus* bark. In the bark of *Sambucus nigra*, chlorogenic and caffeic acids were predominant in the free acid fraction. After alkaline hydrolysis a lot of acids were detected for both barks belonging to hydroxybenzoic as well as hydroxycinnamic acids family. R_f values of those phenolic acids on modified and unmodified silica gel for different solvents are presented in Table 4.

Quantitative analysis of free acids and those liberated after enzymatic and alkaline hydrolysis was performed by HPLC. HPLC for a complex of standard phenolic acids revealed the presence of 12 compounds, in both free and bonded forms in the bark of *V. opulus* and *S. nigra*. Chlorogenic acid was present in all free phenolic acids fractions (FPA). The bark of *S. nigra* contained also ferulic, 3,4,5-trimethoxybenzoic and the trace of *p*-coumaric acid in the free form. Caffeic, gallic, syringic, *p*-coumaric, ferulic and 4-hydroxybenzoic acids were present in all free and liberated after alkaline hydrolysis fractions (FPA + BH). The bark of *V. opulus* was richer in acids because it contained also protocatechuic, ellagic, 3,4-dihydroxyphenylacetic and homogentisic acids in the esters form. After enzymatic hydrolysis, caffeic acid was found in all fractions (EH). The bark of *V. opulus* contained also gallic acid and protocatechuic acid and the bark of *S. nigra* contained syringic, ferulic and the trace of *p*-coumaric acids in those fractions.

Concentration of acids calculated from peak areas and properties of the regression equation obtained from the standard calibration plots are given in Table 5. Each sample was determined four times. It was found that the calibration plots for all the phenolic acids were of the type $y = ax + b$, where y is the peak area (mm × AU), x is the concentration of the phenolic acid standard, b is the intercept of the plot on the y-axis, and a is its slope.

Figure 2 A and 2 B show the relative amounts of individual phenolic acids analyzed by HPLC in the materials studied. The predominant phenolic acid in the bark of *V. opulus* and *S. nigra* is caffeic acid (44,21% and 46,76%, respectively).

CONCLUSIONS

As a result of the research, 11 phenolic acids were for the first time isolated from *S. nigra* and *V. opulus* bark. 8 acids were isolated from the plants in the form of amorphous residues and then identified with the use of various chromatographic and spectroscopic techniques. 2 acids were identified on the basis of HPLC analysis. Chlorogenic acid was also isolated from both barks, but that compound was previously described in those barks. In both examined cases, caffeic acid was predominant in the bonded form. Esters and glycosides of caffeic acid are of significant importance (16, 17). The results of this research brings us a bit closer to better understanding of metabolomic aspects of *Viburnum opulus* and *Sambucus nigra* chemistry.

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