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Phenolic acids in extracts obtained from the flowering herbs of *Cirsium vulgare* (Savi) Ten. growing in Poland

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Abstract

In this work the phenolic acids in the methanol extract from the flowering herbs of *Cirsium vulgare* (Savi) Ten. growing in Poland were isolated and identified. The samples containing free phenolic acids and those released after acid and alkaline hydrolyses were investigated by 2D TLC on cellulose. After purification by SPE, samples were also analyzed by RP-HPLC. Six phenolic acids such as gallic, protokatechuic, gentisic, hydrobenzoic, vanillic and caffeic acids were detected in the fraction of free phenolic acids of the methanol extract, irrespectively of the method used.

Keywords: *Cirsium vulgare* (Savi) Ten., phenolic acids

Introduction

Cirsium vulgare (Savi) Ten. belongs the Asteraceae represent one of the largest plant families. The composites are famous for their use in both traditional and conventional medicine. *Cirsium* species are very common and widespread plants in Poland that grow on pastures or in thickets, and prefer calcareous soils with a large content of nitrogen. In Polish folk medicine these plants are used in the treatment of numerous diseases due to their diuretic, astringent, antiphlogistic or anxiolitic activities [1]. Moreover, the extracts from *Cirsium* species were also shown to possess antioxidant and antibacterial activity [1–6].

The main secondary metabolites of *Cirsium* species were reported to be flavonoids, tannins, sterols, triterpens and also phenolic acids [7–11]. It is well documented that phenolic acids widely occur in plants. They are a predominant group of substances that play an important role in plant physiology, as stimulations of the plant growth. Besides, phenolic acids are known to have various biological activities, especially fungistatic, bacteriostatic, choleretic, potential sedative – hypnotic, antianxiety and anticonvulsant activity [1,3,12,13].

The objective of this work was to analyze chromatographically and to identify the phenolic acids occurring in the methanol extract obtained from flowering herbs of *C. vulgare* (Savi) Ten.

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Material and methods

Plant material

The investigation was performed on dried and powdered flowering herbs of *C. vulgare* (Savi) Ten. (90 g) collected in the Medicinal Plant Garden, Department of Pharmacognosy, Lublin, Poland in September.

Extraction and chromatographic analysis

Plant material was dried at room temperature, powdered, macerated (24 h) and extracted exhaustively for 48 h in a Soxhlet apparatus with methanol. The obtained extracts were concentrated under reduced pressure and analyzed by the procedure described elsewhere [14,15]. Fractions containing free phenolic acids or those after acidic or alkaline hydrolyses were analyzed. Several standards of phenolic acids were used: ferulic, vanillic, protokatechuic, *p*-hydroxybenzoic, *p*-coumaric, caffeic, gallic, chlorogenic, syryngic, gentisic, rosmarinic, elagic acid.

One-dimensional TLC (1D-TLC) was performed on 200 × 200 × 0.1 mm cellulose plates; two-dimensional TLC (2D-TLC) was performed on 100 × 100 × 0.1 mm cellulose plates (E. Merck, Darmstadt, Germany). Each fraction and standards were spotted on 1D TLC plates and the plates were developed in horizontal DS chambers (CHROMDES, Lublin, Poland) using the following mobile phases: toluene-ethyl formate-formic acid (5:4:1) v/v/v; 15% aqueous acetic acid; sodium formate-formic acid-water (10:1:200) w/v/v; chloroform-ethyl acetate-acetic acid (50:50:1) v/v/v; toluene-acetonitryle-formic acid (70:30:1) v/v/v; chloroform-methanol-acetic acid (90:10:1) v/v/v; methanol-water (8:2) + 1% acetic acid v/v/v; methanol-water (8:2) + 3% acetic acid v/v/v. 2D-TLC was also performed in horizontal DS chambers (CHROMDES). Before the development, the plates spotted with standards and fractions were conditioned in the chamber for about 5 min in the vapors above benzene-methanol-acetic acid (94:1:5) and then developed with

Tab. 1 Phenolic acids in the crude methanol extracts of *Cirsium vulgare* (Savi) Ten. identified by 2D TLC.

Phenolic acids	Standards		*Cirsium vulgare* (L.) Scop.		
	R_{f1}	R_{f2}	F_a	F_b	F_c
chlorogenic	0.01	0.62	-	+	-
gallic	0.03	0.25	+	+	+
ellagic	0.04	0.03	-	-	-
rosmarinic	0.05	0.48	-	-	-
caffeic	0.23	0.19	+	(+)	+++
protokatechuic	0.25	0.67	+	+	+
gentisic	0.34	0.68	+	+	+
hydroxybenzoic	0.52	0.63	+	+	+
p-coumaric	0.60	0.16	-	+	+
vanillic	0.72	0.54	+	+	-
ferulic	0.74	0.17	++	++	++
syringic	0.92	0.50	-	-	+++

The plates were conditioned for about 5 min with the vapors above benzene-methanol-acetic acid (94:1:5) and developed benzene-methanol-acetic acid-acetonitryle (80:10:5:5) v/v/v/v in the first direction, and sodium formate-formic acid-water (10:1:200) w/v/v in the second direction; stationary phase: cellulose. F_a – fraction of free phenolic acids; F_b – fraction of phenolic acids after acid hydrolysis; F_c – fraction of phenolic acids after alkaline hydrolysis.

benzene-methanol-acetic acid-acetonitrile (80:10:5:5) v/v/v/v in the first direction, and sodium formate-formic acid-water (10:1:200) v/v/v in the second direction [11,16,17]. Between the developments, the mobile phase was completely evaporated by air. After drying, all chromatograms were observed under UV light (λ = 254 and 366 nm) before and after treatment with ammonia vapor. Derivatization (after both 1D and 2D TLC) was performed by spraying with 3% methanolic solution of iron (III) chloride and diazotized sulfanilic acid in 20% sodium carbonate solution (1:1) v/v. Photographs of the sprayed plates were taken in visible light by the use of VideoScan (Camag, Switzerland). The compounds were identified according to their R_f values comparing with R_f values of the standards.

Samples containing phenolic acids were purified from fatty components and chlorophylls by SPE. Samples were evaporated to dryness, dissolved in 30% aqueous methanol and applied to octadecyl BakerBond SPE microcolums (500 mg, 3 ml, J. T. Baker) previously activated with 10 ml methanol and then 10 ml water. Free phenolic acids were obtained by the elution of the columns with 10 ml water-methanol, 70:30, under reduced pressure (SPE-12G chamber, Baker USA). Samples containing phenolic acids purified by SPE were analyzed by RP-HPLC on a 250 × 4.6 mm i.d.; d_p = 5 µm Hypersil ODS column eluted with gradient mobile phase prepared from 1% aqueous acetic acid (component A) and methanol (component B; v/v). The gradient was: 0 min 10% B in A; 2 min 10% B in A; 8 min 15% B in A; 25 min 40% B in A; 30 min 40% B in A; 45 min 60% B in A, 47 min 65% B in A. A Hewlett-Packard model 1100 liquid chromatograph equipped with a 20-µl sample injector (Rheodyne) and a variable wavelength DAD detector were used. Chromatography was performed at 250°C and the flow rate was 1 ml/min. The identification was performed comparing retention time (t_R) with those of standards, by comparison of UV spectra (λ = 254, 280 and 320 nm).

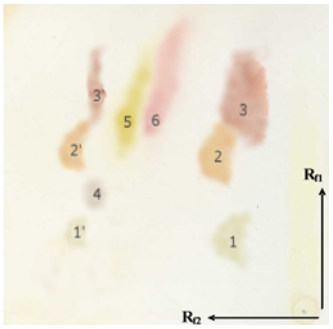

Fig. 1 2D TLC chromatogram of fractions F_a of phenolic acids from the flowering herb *Cirsium vulgare* (Savi) Ten. Direction I: benzene-methanol-acetic acid-acetonitrile (80:10:5:5) v/v. Direction II: sodium formate-formic acid-water (10:1:200) w/v/v. The spots were visualized with diazotized sulfanilic acids (dSa) in 20% sodium carbonate solution 1:1 v/v. 1,1′ – caffeic acid; 2,2′ – ferulic acid; 3,3′ – synapic acid; 4 – gentisic acid; 5 – vanilic acid; 6 – syryngic acid.

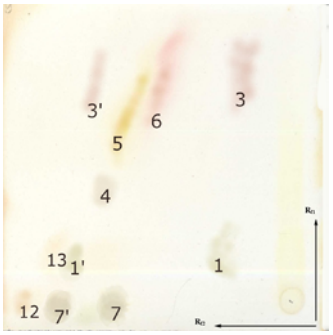

Fig. 2 2D TLC chromatogram of fractions F_b of phenolic acids from the flowering herb *Cirsium vulgare* (Savi) Ten. Direction I: benzene-methanol-acetic acid-acetonitrile (80:10:5:5) v/v. Direction II: sodium formate-formic acid-water (10:1:200) w/v/v. The spots were visualized with diazotized sulfanilic acids (dSa) in 20% sodium carbonate solution 1:1 v/v. 1,1′ – caffeic acid; 3,3′ – synapic acid; 4 – gentisic acid; 5 – vanilic acid; 6 – syryngic acid; 7 – chlorogenic acid; 12 – unknown compounds; 13 – unknown compounds.

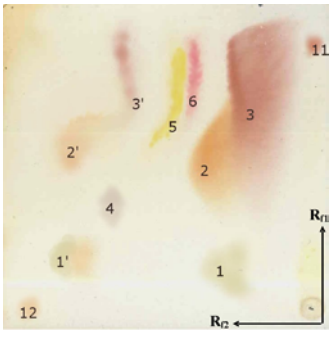

Fig. 3 2D TLC chromatogram of fractions F_c of phenolic acids from the flowering herb *Cirsium vulgare* (Savi) Ten. Direction I: benzene-methanol-acetic acid-acetonitrile (80:10:5:5) v/v. Direction II: sodium formate-formic acid-water (10:1:200) w/v/v. The spots were visualized with diazotized sulfanilic acids (dSa) in 20% sodium carbonate solution 1:1 v/v. 1,1′ – caffeic acid; 2,2′ – ferulic acid; 3,3′ – synapic acid; 4 – gentisic acid; 5 – vanilic acid; 6 – syryngic acid; 11 – unknown compounds; 12 – unknown compounds.

Tab. 2 The retention times (t_r) of phenolic acids identified by RP-HPLC.

Phenolic acids	Standards	*Cirsium vulgare* (L.) Scop.		
		F_a	F_b	F_c
gallic	4.437	4.479	-	-
protokatechuic	9.026	8.140	-	-
gentisic	10.949	10.950	-	-
hydroxybenzoic	12.086	13.070	-	-
chlorogenic	16.006	15.270	16.107	-
vanillic	16.296	16.438	-	-
caffeic	17.254	17.333	17.271	17.117
syringic	18.653	18.751	18.819	-
p-coumaric	22.553	-	22.576	22.499
ferulic	24.093	-	24.366	24.307
rosmarinic	30.650	-	8.905	-

F_a – fraction of free phenolic acids; F_b – fraction of phenolic acids after acid hydrolysis; F_c – fraction of phenolic acids after alkaline hydrolysis.

Results

As shown in Tab. 1 and Fig. 1–Fig. 3, the presence of ten phenolic acids was found in the extract obtained from flowering herbs from *C. vulgare* (Savi) Ten. using 2D TLC.

HPLC confirmed the presence of the phenolic acids mentioned above or those after acid or alkaline hydrolyses in the extracts obtained from *C. vulgare* Tab. 2 and Fig. 4–Fig. 6, only slight differences in the profiles of phenolic acids were observed using two chromatographic methods. Fraction F_a is richer in phenolic acids then other fractions F_b and F_c. Using RP-HPLC, the presence of free phenolic acids mentioned above, that is gallic, protokatechuic, gentisic, hydroxybenzoic, vanillic and caffeic acids was found and additionally chlorogenic and syringic acids were identified. After acid hydrolysis F_a additional, *p*-coumaric, ferulic acids were detected, while after alkaline hydrolysis – caffeic, ferulic and *p*-coumaric acids.

Discussion

The isolation and separation of natural compounds, including phenolic acids is a very important analytical problem in

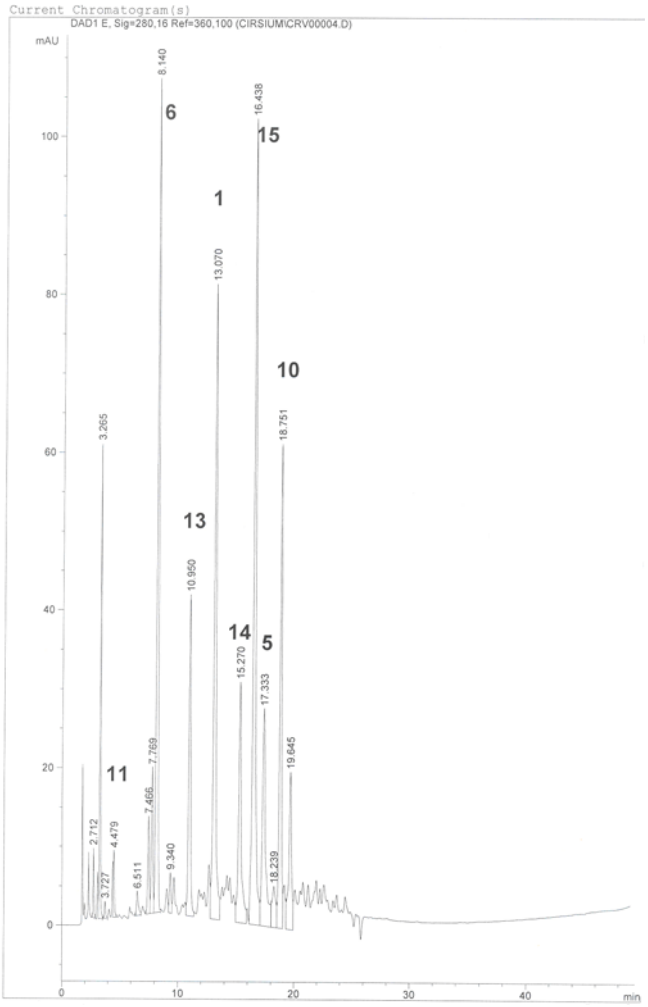

Fig. 4 Chromatogram of fractions F_a of phenolic acids from the flowering herb *Cirsium vulgare* (Savi) Ten. 1 – hydroxybenzoic acid, 5 – caffeic acid; 6 – protokatechuic acid; 10 – syryngic acid; 11 – gallic acid; 13 – gentisic acid; 14 – chlorogenic acid; 15 – vanillic acid.

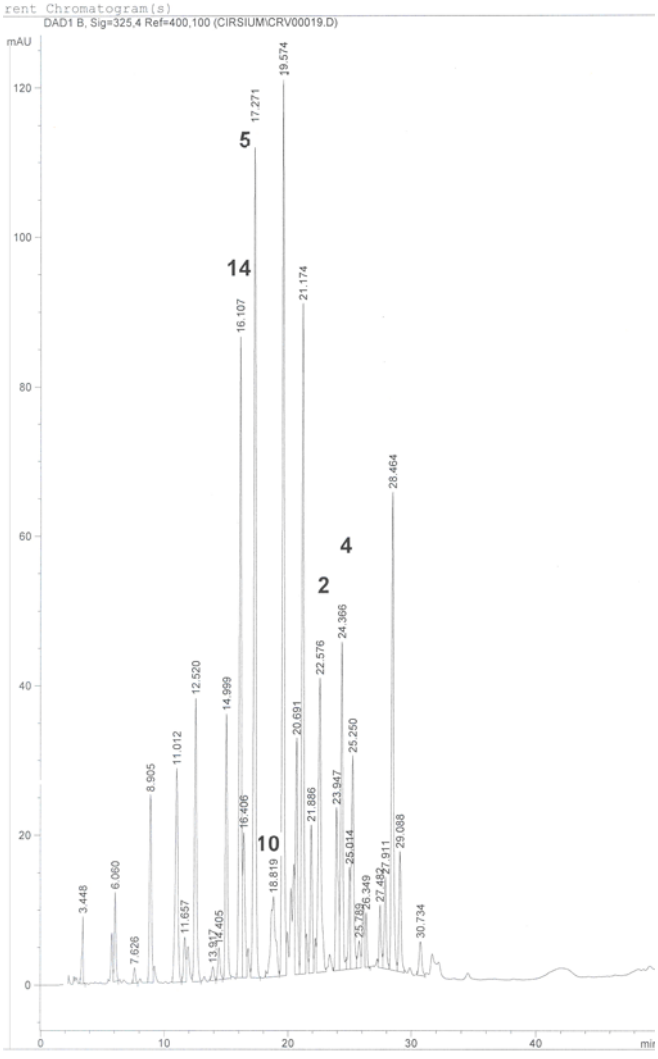

Fig. 5 Chromatogram of fractions F_b of phenolic acids after acid hydrolysis from the flowering herb *Cirsium vulgare* (Savi) Ten. 2 – *p*-coumaric acid; 4 – ferulic acid; 5 – caffeic acid; 10 – syringic acid; 14 – chlorogenic acid.

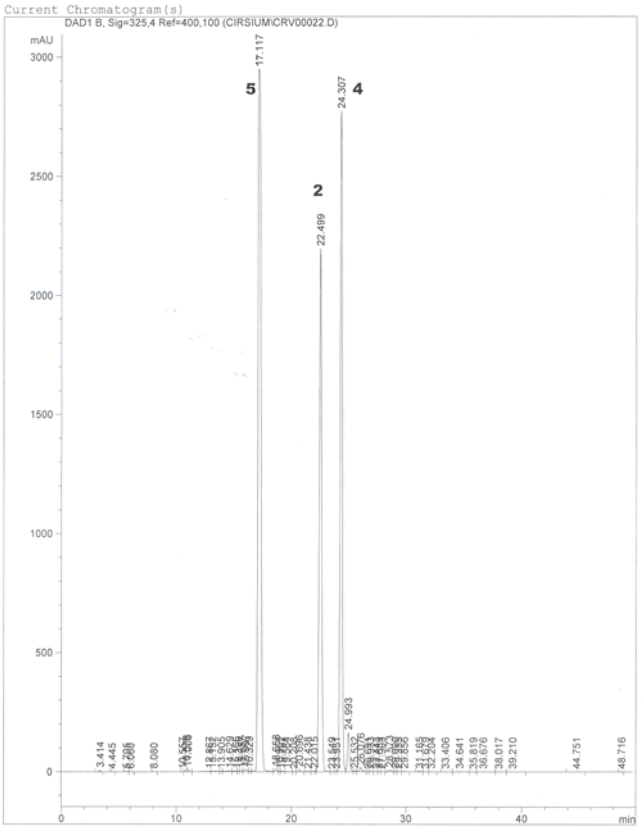

Fig. 6 Chromatogram of fractions F_c of phenolic acids after alkaline hydrolysis from the flowering herb *Cirsium vulgare* (Savi) Ten. 2 – *p*-coumaric acid; 4 – ferulic acid; 5 – caffeic acid.

phytochemistry [16]. Standard procedures based on TLC still play a major role in the isolation and purification of phenolic compounds [17–22]. The extraction of phenolic acids from plant material and their further purification for HPLC analysis is usually a complex procedure because of the presence of various nonpolar ballast compounds in biological extracts (e.g. chlorophylls, oils, sterols etc.), which can cause damage of analytical columns and interfere with the process of chromatographic determination [23]. Therefore, solid phase extraction, a popular procedure used for isolation, purification and preconcentration of organic compounds present in biological material was used prior HPLC [24]. In the present paper phenolic acids from the flowering herbs of *C. vulgare* (Savi) Ten. were analyzed by 2D TLC and RP HPLC. For the optimization of separation, several mobile phases were used. All obtained results were satisfactory, but 2D TLC proved to be the most suitable for the separation of phenolic acids from the extracts. The results were confirmed by RP HPLC analysis. It should be stressed that 2D TLC is not only inexpensive but also a very suitable method for rapid separation and identification of phenolic acids present in the *Cirsium* species extracts. Only some differences in the profiles of phenolic acids were observed using two chromatographic methods

Nazaruk et al. [3] performed studies concerning detection of phenolic acids by HPLC in the aqueous extracts from leaves of several *Cirsium* species growing in Poland, including *C. vulgare* (Savi) Ten.; leaves were collected from plants in the period of full flowering. In *C. vulgare* (Savi) Ten. only the trace of protokatechuic, chlorogenic, caffeic, *p*-coumaric and vanilic acids was found. According to our data, the methanol extracts obtained from the flowering herbs of *C. vulgare* (Savi) Ten., containing eight phenolic acids (vanillic, protokatechuic, hydroxybenzoic, caffeic, gallic, chlorogenic, syryngic and gentisic acids) was found to be richer in the respect of phenolic compound content compared to the water extract from the leaves [3]. The phenolic compounds are expected to be responsible for biological activity of the examined plant, including antimicrobial activity.

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Authors' contributions

The following declarations about authors' contributions to the research have been made: conducting experiments, interpretation of data, and writing the manuscript: MK; revising article critically and final approval of the version to be published: KG.

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