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Phenolic acids in the flowering herbs of Cirsium oleraceum (L.) Scop. growing in Poland

Kwasy fenolowe w kwitnącym zielu *Cirsium oleraceum* (L.) Scop. rosnącym w Polsce

INTRODUCTION

Cirsium oleraceum (L.) Scop. belongs to the *Asteraceae* family – very common and wide-spread 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, adstringent, antiphogistic or anxiolitic activity [19]. A decoction of the whole plant is used as a poultice to sore jaws or can be applied both internally and externally to treat bleeding piles. Moreover, hot infusion of the whole plant is used as a herbal steam for treating rheumatic joints. The extracts from *Cirsium* species were also shown to possess antioxidant and antibacterial activity [1, 10–13, 20].

The main secondary metabolites of *Cirsium* species were reported to be flavonoids, tannins, sterols, triterpens and also phenolic acids [5, 7, 9]. 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 anticolvulsant activity [10–12, 20, 22].

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. oleraceum* (L.) Scop. growing in Poland. In addition, the *in vitro* activity of the extracts against a panel of reference strains of Gram-positive or Gram-negative bacteria was determined.

MATERIAL AND METHODS

Plant material. The investigation was performed on dried and powdered flowering herbs of *C. oleraceum* (L.) Scop. (200 g) collected in the Medicinal Plant Garden, Department of Pharmacognosy, Lublin, Poland, in August and September, respectively.

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 extract was concentrated under reduced pressure and analyzed by the procedure described elsewhere [6, 17]. 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 x 200 x 0.1 mm cellulose plates; two-dimensional TLC (2D-TLC) was performed on 100 x 100 x 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 vapours above benzene-methanol-acetic acid (94:1:5) and then developed with 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 [9, 28]. 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 vapour. 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, 30:80, under reduced pressure (SPE -12G chamber, Baker USA). Samples containing phenolic acids purified by SPE were analyzed by RP-HPLC on a 250 x 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; 45min 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 25^0C 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).

Bacterial strains. Antibacterial activity tests were carried out against six strains of Gram-positive bacteria (*Staphylococcus epidermidis* ATCC 12228, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 10876, *Bacillus subtilis* ATCC 6633, *Micrococcus luteus* ATCC 10240) and four Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 9027, *Proteus mirabilis* ATCC 12453). All strains were obtained from the American Type Culture Collection. Stock cultures were maintained at –70°C in containing 16% (v/v) glycerol of Nutrient broth (Biocorp).

Agar well diffusion method. Mueller-Hinton agar (BioRad) was used to determine antibacterial activity of water, methanol, dichloromethane, ethyl acetate and acetone extracts obtained from C. *oleraceum* (L.) Scop. Inocula of density 0.5 McFarland standard scale, approximately 150 x 10^6 colony forming units (CFU) per ml, were prepared with fresh bacterial cultures (on nutrient agar, 35°C, 24 h) with sterile physiological saline. 20 ml of the molten Mueller-Hinton agar were poured into sterile Petri dishes. After cooling the agar media were inoculated - the sterile swabs were used to spread the bacterial suspensions onto the agar surface. Next, wells (d = 6 mm) were made on the agar media with sterile cork borer. 50 µl of plant extracts (20 mg/ml dissolved in DMSO) were dropped into wells. In order to accelerate diffusion of the extracts into agar, the plates were preincubated at room temperature for 1 h and then incubated at 37°C for 24 h. After the incubation period, the growth of bacteria around the wells was observed and the zones of growth inhibition were measured and recorded (including the diameter of the well); the average values were calculated. The well containing DMSO without test compounds served as control and no growth inhibition was observed. Experiments were performed in triplicate.

RESULTS

As shown in Table 1, using 2D TLC only three free phenolic acids were identified – p-coumaric, caffeic and protokatechuic acids in crude methanol extract obtained from flowering herbs of *C. oleraceum* (L.) Scop. Moreover, after acid or alkaline hydrolyses additional phenolic acids were detected – after acid hydrolysis: rosmarinic and syringic acids; after alkaline hydrolysis – ferulic, ellagic and syringic acids. HPLC confirmed the presence of the above mentioned free phenolic acids or those after acid or alkaline hydrolyses in the extracts obtained from *C. oleraceum* (L.) Scop. (Table 2); only slight differences in the profiles of phenolic acids were observed using two chromatographic methods. Caffeic acid was detected in free phenolic acid fractions as well as in those after hydrolyses, irrespective of the method used.

Table 1. Phenolic acids in the crude methanol extract of *Cirsium oleraceum* (L.) Scop. identified by 2D TLC*

Phenolic acids	Standards		*Cirsium oleraceum*		
	R_{f1}	R_{f2}	Fa	Fb	Fc
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	-	+++	+++

*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; stationary phase: cellulose. Fa – fraction of free phenolic acids; Fb – fraction of phenolic acids after acid hydrolysis; Fc – fraction of phenolic acids after alkaline hydrolysis

Table 2. Retentions times of phenolic acids identified by RP-HPLC

Phenolic acids	Standards	*Cirsium oleraceum*		
		Fa	Fb	Fc
Gallic	4.437	-	-	-
Protokatechuic	9.026	-	-	7.907
Gentisic	10.949	-	-	-
Hydroxybenzoic	12.086	-	-	-
Chlorogenic	16.006	-	-	-
Vanillic	16.296	-	-	-
Caffeic	17.254	16.779	16.856	16.613
Syringic	18.653	-	18.48322.358	18.46622.323
p-coumaric	22.553	22.265	22.809	-
Ferulic	24.093	24.115	30.808	-
Rosmarinic	30.650	-		

Fa – of free phenolic acids; Fb – fraction of phenolic acids after acid hydrolysis; Fc – fraction of phenolic acids after alkaline hydrolysis

The methanol extract obtained from *C. oleraceum* (L.) Scop. was screened for the spectrum of antibacterial activity using agar well diffusion method. No influence on the growth of bacterial strains was found at the extract concentration of 20 mg/ml.

DISCUSSION

The isolation and separation of natural compounds, including phenolic acids, from plants is a very important analytical problem in phytochemistry [11]. Standard procedures based on TLC still play a major role in the isolation and purification of phenolic compounds [17–19, 21]. 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 [6, 17, 19]. Therefore, Solid Phase extraction, a popular procedure used for isolation, purification and preconcentration of organic compounds present in biological material was used prior HPLC [4, 21]. In the present paper phenolic acids from the flowering herbs of *C. oleraceum* (L.) Scop. 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 an inexpensive but also very suitable method for rapid separation and identification of phenolic acids present in the *Cirsium* species extracts. The phenolic acids in the methanol extract from the flowering herbs of *Cirsium oleraceum* (L.) Scop. were isolated and identified for the first time.

Nazaruk *et al.* [12] performed studies concerning detection of phenolic acids by HPLC in the aqueous extracts from the leaves of several *Cirsium* species growing in Poland, including *C. oleraceum* (L.) Scop.; the leaves were collected from plants in the period of full flowering. In *C. oleraceum* (L.) Scop. the following free phenolic acids were detected: protokatechuic, chlorogenic, caffeic, p-coumaric and ferulic acids. Chlorogenic acid was detected in the leaves from several *Cirsium* species. According to our data, the methanol extracts obtained from the flowering herbs of *C. oleraceum* (L.) Scop. contained less free phenolic acids (p-coumaric, ferulic, and caffeic acids) detected by HPLC as compared to data obtained by Nazaruk et al. [14].

Plants contain numerous biologically active compounds and many of these display potential antimicrobial properties. The phenolic compounds are expected to be responsible for biological activity of *Cirsium* spp., including antimicrobial activity [2, 8,15, 16]. Due to therapeutic limitations of conventionally used drugs, drug-related toxicity, hazardous drug interactions, insufficient bioavailability or the alarming incidence of drug resistance among microorganisms, it is important to look for effective new antimicrobial agents. However, the results obtained in this study indicate that the methanol extract obtained from *C. oleraceum* (L.) Scop. had no antibacterial activity as determined by agar well diffusion method, most probably due to the insufficient content of phenolic acids to exert inhibitory effect.

CONCLUSIONS

Phenolic acids in the methanol extract from the flowering herbs of *Cirsium oleraceum* (L.) Scop. were isolated and identified for the first time. 2D TLC method is inexpensive and very suitable for rapid separation and identification of phenolic acids present in the *Cirsium* species extracts.

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SUMMARY

In this work phenolic acids in the methanol extract from the flowering herbs of *Cirsium oleraceum* (L.) Scop. were isolated and identified for the first time. 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. Three free phenolic acids were identified – p-coumaric, caffeic and protokatechuic acids. Moreover, after acid hydrolysis rosmarinic and syringic acids were detected, while after alkaline hydrolysis – ferulic, ellagic and syringic acids. Using agar well diffusion method, no activity of the extract (20 mg/ml) against the panel of ten reference strains of Gram-positive and Gram-negative bacteria was found.

Keywords: *Cirsium oleraceum* (L). Scop., phenolic acids**,** antibacterial activity

STRESZCZENIE

W pracy przedstawiono po raz pierwszy izolację i analizę chromatograficzną kwasów fenolowych w ekstrakcie metanolowym uzyskanym z kwitnącego ziela *Cirsium oleraceum* (L). Scop. przy użyciu 2D TLC i RP- HPLC. W badanym ekstrakcie stwierdzono obecność kwasów: p-kumarowego, kawowego, protokatechowego. Po hydrolizie kwasowej zidentyfikowano kwasy rozmarynowy i syryngowy, natomiast po hydrolizie zasadowej – kwasy ferulowy, ellagowy i syryngowy. Stosując metodę dyfuzji w agarze wykazano, że badany ekstrakt metanolowy w zastosowanym stężeniu (20 mg/ml) nie hamował namnażania referencyjnych szczepów bakterii Gram-dodatnich i Gram-ujemnych.

Słowa kluczowe: *Cirsium oleraceum* (L). Scop., kwasy fenolowe, aktywność przeciwbakteryjna