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NATURAL DRUGS

ANALYSIS OF PHENOLIC ACIDS AND ANTIBACTERIAL ACTIVITY OF EXTRACTS OBTAINED FROM THE FLOWERING HERBS OF *CARDUUS ACANTHOIDES* L.

MAŁGORZATA KOZYRA[1]*, ANNA BIERNASIUK[2] and ANNA MALM[2]

[1]Department of Pharmacognosy with Medicinal Plant Laboratory,
[2]Department of Pharmaceutical Microbiology, Medical University, 1 Chodźki St., 20-093 Lublin, Poland

Abstract: In this work the phenolic acids in crude methanol extracts from the flowering herbs of *Carduus acanthoides* L. were 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. Chlorogenic, protocatechuic, *p*- coumaric, caffeic, syringic, *p*-hydroxybenzoic, ferulic, vanillic, gentisic and gallic acids were detected in fractions of the methanolic extract obtained from the flowering herb of *C. acanthoides*. This is the first study concerning the qualitative analysis of phenolic compounds and antibacterial activity of fractions and aqueous, ethyl acetate, dichloromethane, acetone and methanol (50, 80 and 100% v/v) extracts of flowering herbs of *C. acanthoides* L. The antimicrobial activity of tested extracts was determined *in vitro* against reference microorganisms of Gram-positive bacteria, Gram-negative bacteria and fungi belonging to yeasts. The results of this study support the medical usage of *C. acanthoides* L. due to its antimicrobial properties.

Keywords: *Carduus acanthoides* L., 2D TLC, RP-HPLC, phenolic acids, antimicrobial activity, antifungal activity

Carduus acanthoides L. belongs to the *Asteraceae* family. This plant is native to Europe but is also well-known in many other parts of the world.

Phenolic compounds are the best-known group of secondary metabolites in *Carduus* species.

Flavonoids like luteolin, quercetin, luteolin-7-digalactoside, luteolin-7-glucoside, apigenin, apigenin-7-glucoside, kaempferol-3-rhamnoglucoside, kaempferol-3-glucoside, and phenolic acids like chlorogenic acid and *p*-coumaric acid were previously described in *Carduus acanthoides* L. (1-3). Another authors (4) identified neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, kaempferol-3-rhamnoside-7-glucoside, luteolin-7-glucosyl-(1→2)-glucoside, luteolin-7-glucoside, luteolin-7-6’’’-acetyl-glucosyl-(1→2)-glucoside, kaempferol - 3-rhamnoside, and chrysoeriol-7-6’’’-acetyl-glucosyl-(1→2)-glucoside. Second common group of secondary metabolites in *Carduus species* are sterols and triterpenes (β-sitosterol, Δ-5-avenasterol, brassicasterol, campesterol, stigmasterol), and alkaloids (acanthoidine, acanthoine, ruscopine) (1, 2, 5).

Two genus of *Carduus* like *C. crispus* and *C. nutans* were found to be rich in coumarins and flavonoids. Six coumarins (coumarin, umbelliferone, herniarin, esculetin, scopoletin, esculin) and flavonoids (cinaroside, apigenine, luteolin, astragalin) were described in herbs of both species. Additional kaempferol-3-O-α-L-rhamnofuranoside and acacatin-7-β-D-glucopyranoside, isorhamnetin, rutin and tilianin were identified in *C. nutans* (6). The flavonoids isolated from the aerial parts of *C. assoi* were identified as kaempferol, apigenin, tricin, luteolin, hispidulin-7-glucoside, luteolin-3’-rhamnoside, kaempferol-3-O-α-L-rhamnoside, kaempferol-3-O-β-D-glucoside (7). Additionally diosmetin and kaempferol-3-O-α-L-rhamnoside, glucoside palustroide, kaempferol, apigenin, kaempferol 3,4’-dimethyl ether-7-O-glucoside, apigenin-7-O-glucoside, fatty acids and their esters were isolated from aerial parts of *C. pycnocephalus* (8-10).

C. thoermeri were tested in order to determine the lipid composition and oleic, linoleic and palmitic acids were identified as main components.

* Corresponding author: e-mail: mkozyra@pharmacognosy.org; phone +48 81 7423707, +48 81 7423809

Additionally, sinapic acid, ferulic acid, chlorogenic acid, luteolin and hyperoside were identified and quantified by HPLC (11).

Phenolic acids are compounds which are known for their wide range of biological activity like fungistatic, bacteriostatic, choleretic, hypnotic, antianxiety and anticonvulsant activity (13-15). Considering that there are only a few data on antimicrobial activity of *C. acanthoides*, the objective of this study was to investigate the antibacterial and antifungal properties of the extracts from this plant. Phenolic acids in flowering herbs of *C. acanthoides* were qualitatively determined for the first time. Previously, only sum of phenolic acids was determined in plants from the genus *Carduus* and was in the range 0.57-2.43%. The content of flavonoids (1.8-3.2%) and total phenols (1.7-2.3%) was higher in comparison with this of phenolic acids (0.6-2.4%) and anthocyanins (0.5-1.5%) (12).

In this work, the identification of phenolic compounds presented in methanolic extract of flowering herb of *Carduus acanthoides* L. growing in Poland has been performed by use of 2D TLC as well as RP-HPLC methods. Moreover, antimicrobial activity assessment in different extracts and fractions were performed. Those kind of experiments were not performed previously on that species.

MATERIALS AND METHODS

Plant material

Flowering herbs of *Carduus acanthoides* L. (127.0 g) were collected in August in the Medicinal Plant Garden, of the Department of Pharmacognosy, (Lublin, Poland). The herbs were dried in temperature at 45°C and powdered and sieved (sieves 0.315 and 0.074 mm). The procedure of preparation follows the conditions of the Polish Pharmacopoeia VI. Voucher specimen is deposited in the herbarium of the Department of Pharmacognosy, Medical University of Lublin.

Extraction procedure

Flowering herbs were macerated (24 h) and extracted with chloroform (Polish Reagents, Gliwice, Poland) at b.p. 61°C by 48 h in a Soxhlet apparatus. After chloroform extraction, the plant material was macerated (24 h) and extracted exhaustively for 48 h with methanol (Polish Reagents, Gliwice, Poland) at b.p. 78°C. The obtained extracts were concentrated to dryness under reduced pressure and analyzed by the procedure described in the literature (16-19). The dry extract was dissolved in 50 mL of hot distilled water and cooled for 24 h. Ballast substances were removed by filtration through quantitative disc filters (Filtrak) and the filtrates were extracted five times with 40 mL of diethyl ether. Ether and aqueous extracts were analyzed separately. Aqueous extract was distributed in two parts.

The ether extract was concentrated and extracted with 6 × 25 mL of 5% aqueous sodium bicarbonate solution. Next, the carbonate extract obtained was acidified to the pH = 3 with 18% hydrochloric acid solution. The carbonate extract was washed 6 × 25 mL of diethyl ether. The ether extract - fraction F_A containing free phenolic acids was washed, dried with anhydrous sodium sulfate and evaporated. Acid and alkaline hydrolysis was carried out on the aqueous solution remaining after fraction F_A.

Acidic hydrolysis was performed on the half volume of water extract which was acidified with 18% solution of hydrochloric acid and heated at 100°C by 1 h and then extracted with 6 × 25 mL of diethyl ether, dried with anhydrous sodium sulfate and evaporated. In this way, fraction F_B containing phenolic acids after acidic hydrolysis from flowering herbs of *C. acanthoides* was obtained.

Alkaline hydrolysis was performed on the half volume of water extract. Sodium borohydride ($NaBH_4$) in the amount of 0.8 g per 100 mL of hydrous aqueous solution was added to water extract and was heated at 100°C under reflux with 1% barium hydroxide – $Ba(OH)_2$ – in pH of 12-13, for 15 min. After cooling, concentrated sulfuric acid was added to the hydrolysate to obtain a solution at pH = 1.5. The received precipitate of barium sulfate was filtered off and the filtrate was extracted 10 × 25 mL with diethyl ether and dried with anhydrous sodium sulfate and evaporated. Fraction F_C of phenolic acids released by alkaline hydrolysis from flowering herbs of *C. acanthoides* was obtained. Fractions containing free phenolic acids (F_A) or those after acidic (F_B) or alkaline (F_C) hydrolysis were evaporated to dryness and then dissolved in 1 mL of methanol and analyzed by two-dimensional thin layer chromatography (2D-TLC) on cellulose plates and by SPE-HPLC.

To test the antimicrobial activity, different types of extract were also prepared. Milled plant material (10 g) samples of *C. acanthoides* were soaked in 50 mL of 50%, v/v methanol, 80%, v/v methanol, 100% methanol, dichloromethane, acetone, ethyl acetate (Polish Reagents, Gliwice, Poland) in a 250 mL round-bottomed flasks, then extracted under reflux at boiling point for 30 min. Each extract was carefully filtered through cotton

and the plant material was re-extracted twice with fresh portions of solvent. Samples containing examined extracts were evaporated to dryness and then dissolved in 1 mL of dimethyl sulfoxide (DMSO) and screened *in vitro* for antibacterial and antifungal activities using the broth microdilution method.

Qualitative analysis

2D-TLC was performed on 100 × 100 × 0.1 mm cellulose plates (E. Merck, Darmstadt, Germany). Several standards of phenolic acids were used: ferulic, vanillic, protocatechuic, p-hydroxybenzoic, p-coumaric, caffeic, gallic, chlorogenic, syryngic, gentisic acids (Sigma, Roth). Each fraction containing phenolic acids and standards was dissolved in methanol and spotted on 2D TLC plates and the plates were developed in horizontal DS chambers (CHROMDES, Lublin, Poland) using the following mobile phases: in the first direction: benzene-methanol-acetic acid-acetonitrile (80 : 10 : 5 : 5, v/v/v/v) and in the second direction: sodium formate-formic acid- water (10 : 1 : 200, w/v/v). 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, v/v/v) Between the developments, the mobile phase was completely evaporated by air (20, 21). After drying, all chromatograms were observed under UV light (λ = 254 and 366 nm). Derivatization (after 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 compared with R_f values of the standards. Their R_{f1} and R_{f2} values from extract F_A, F_B and F_C from the flowering herb are presented in Table 1 and Figures 1-3.

Quantitative analysis

The total amount of phenolic acids in flowering herbs was determined by Arnov's method (Specol Helios b, Unicam, λ = 490 nm, Arnov's reagent (22, 23) and then the contents of phenolic acids (expressed in % of caffeic acid, Sigma, Roth) was calculated using the calibration curve plotted in the same conditions for the standard caffeic acid.

RP-HPLC analysis

Samples containing phenolic acids were purified from fatty components and chlorophylls by SPE. Samples were evaporated to dryness, dissolved in 30% (v/v) aqueous solution of methanol and applied to octadecyl BakerBond SPE microcolums (500 mg, 3 mL, J.T. Baker) previously activated with 10 mL of methanol and then 10 mL of water.

Free phenolic acids were obtained by the elution of the columns with 10 mL water-methanol, 80 : 30 (v/v), under reduced pressure (SPE-12G chamber, Baker USA) (24). Samples F_A, F_B and F_C containing phenolic acids purified by SPE were analyzed by RP-HPLC with an Agilent 1100 chromatograph (Agilent Technologies, USA).

Compounds were separated on 200 × 4.6 mm stainless-steel column packed with 5 µm Hypersil ODS-C18 (Agilent), using a stepwise mobile phase gradient prepared from 1% v/v aqueous acetic acid (component A) and methanol (component B) (v/v). The gradient was as follows: 0 min 10% B in A; 2 min 10% B in A; 8 min 15% B in A; 25 min 35% B in A; 35 min 40% B in A; 45 min 60% B in A; 47 min 60% B in A.

The mobile phase flow rate was 1 mL/min, the sample injection volume was 20 µL. The analysis was performed at 25°C. The LC column and DAD were monitored and controlled using ChemStation rev.10.0 software (Agilent Technologies). Compounds were identified by comparison of retention times and UV spectra with standards analyzed under the same conditions. Qualitative determination was performed at the 320 nm and 254 nm (Figs. 4-6).

***In vitro* antimicrobial assay**

The obtained extracts and fractions from the flowering herbs of *C. acanthoides* L. were screened *in vitro* for antibacterial and antifungal activities using the broth microdilution method according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) (25) and Clinical and Laboratory Standards Institute guidelines (CLSI) (28).

In this study, were used the reference microbial strains from American Type Culture Collection (ATCC), including Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 10876, *Micrococcus luteus* ATCC 10240), Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Proteus mirabilis* ATCC 12453, *Pseudomonas aeruginosa* ATCC 9027, *Bordetella bronchiseptica* ATCC 4617) and fungi belonging to yeasts (*Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019).

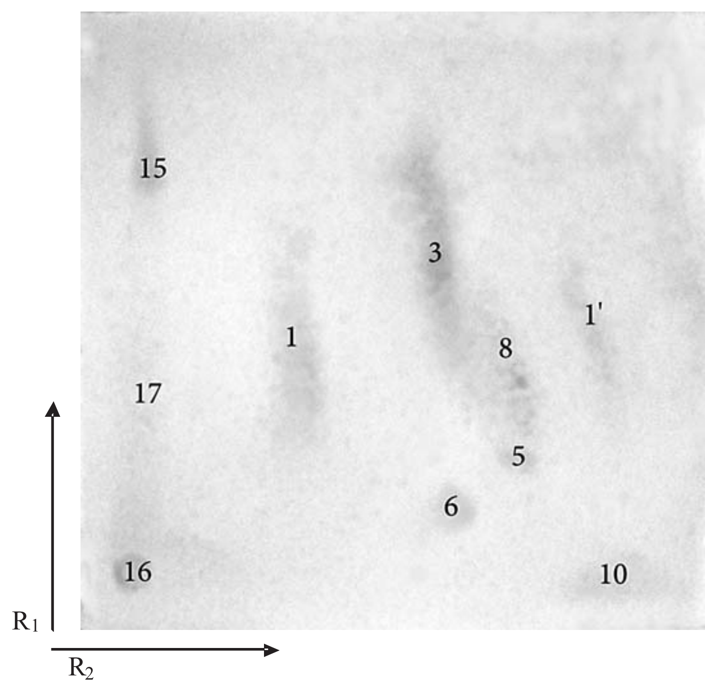

Figure 1. Two-dimensional thin layer chromatogram of phenolic acids – fraction F_A, direction R_1: toluene–methanol–acetic acid–acetonitrile (77.5 : 10 : 5 : 7.5, v/v/v/v); direction R_2: sodium formate–formic acid–water (10 : 1 : 200, w/v/v), conditioning: toluene–methanol–acetic acid (94 : 1 : 5, v/v/v). Adsorbent: cellulose; 1,1' – *p*-coumaric acid, 3 – syringic acid, 5 – *p*-hydroxybenzoic acid, 6 – protocatechuic acid, 8 – vanillic acid, 10 – chlorogenic acid, 15, 16, 17 – unknown compounds

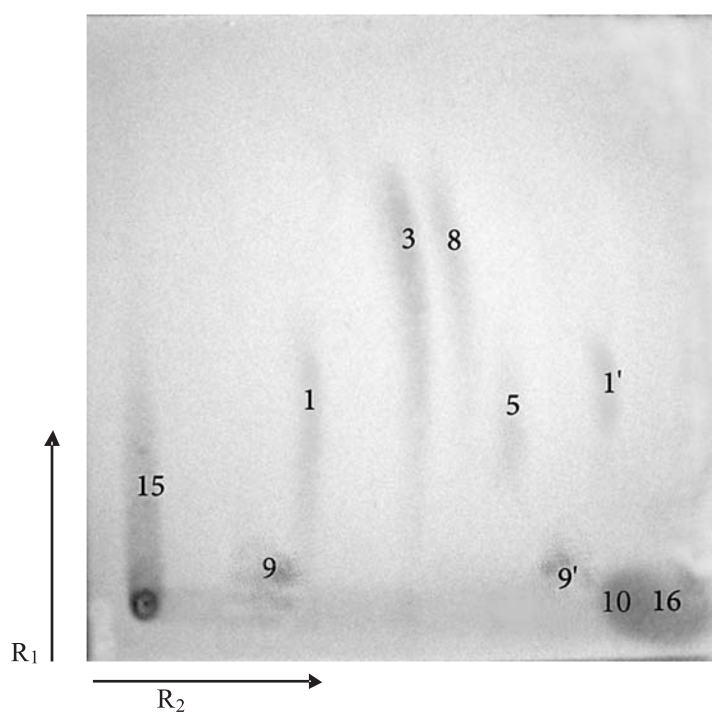

Figure 2. Two-dimensional thin layer chromatogram of phenolic acids – fraction F_B, direction R_1: toluene–methanol–acetic acid–acetonitrile (77.5 : 10 : 5 : 7.5, v/v/v/v); direction R_2: sodium formate–formic acid–water (10 : 1 : 200, w/v/v), conditioning: toluene–methanol–acetic acid (94 : 1 : 5, v/v/v). Adsorbent: cellulose; 1,1' – *p*-coumaric acid, 3 – syringic acid, 5 – *p*-hydroxybenzoic acid, 8 – vanillic acid, 9 – caffeic acid, 10 – chlorogenic acid, 15, 16 – unknown compounds

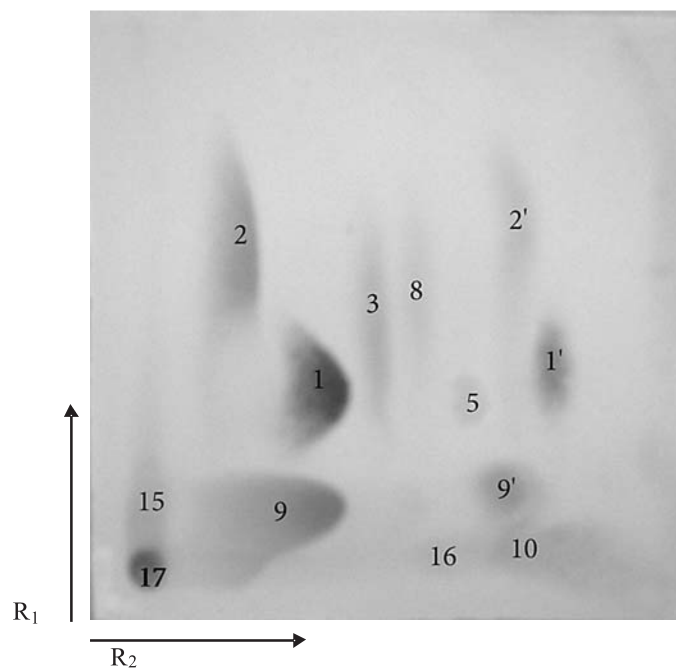

Figure 3. Two-dimensional thin layer chromatogram of phenolic acids – fraction F_C, direction R_1: toluene–methanol–acetic acid–acetonitrile (77.5 : 10 : 5 : 7.5, v/v/v/v); direction R_2: sodium formate–formic acid–water (10 : 1 : 200, w/v/v), conditioning: toluene–methanol–acetic acid (94 : 1 : 5, v/v/v). Adsorbent: cellulose; 1,1' – *p*-coumaric acid, 2 – ferulic acid, 3 – syringic acid, 5 – *p*-hydroxybenzoic acid, 8 – vanillic acid, 9, 9'– caffeic acid, 10- chlorogenic acid

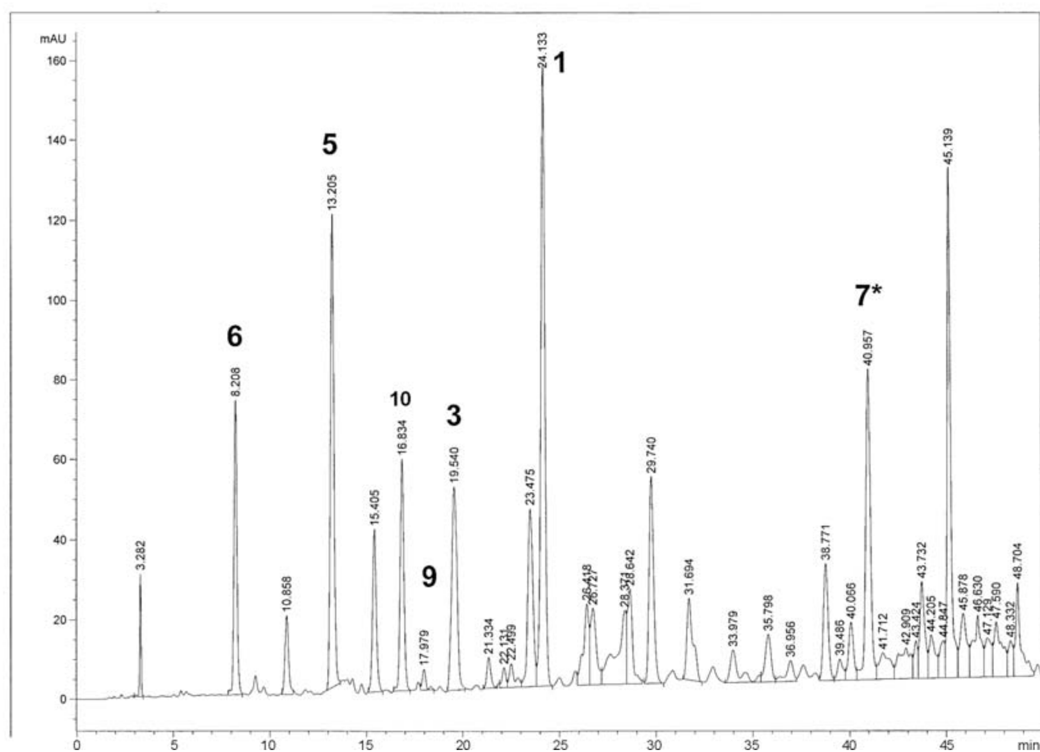

Figure 4. RP-HPLC chromatogram of phenolic acids from the herb fraction F_A. Peaks: 1 – *p*-coumaric acid, 3 – syringic acid, 5 – *p*-hydroxybenzoic acid, 6 – protocatechuic acid, 9 – caffeic acid, 10 – chlorogenic acid, 7* – isorhamnetin-3-rhamnoglucoside

The microbial cultures were subcultured on nutrient agar or Sabouraud agar at 35°C for 18-24 h or 30°C for 24-48 h for bacteria and fungi, respectively. The surface of Mueller-Hinton agar and RPMI 1640 with MOPS (3-(*N*-morpholino)propanesulfonic acid) were inoculated with the suspensions of bacterial or fungal species. Microbial suspensions were prepared in sterile 0.85% NaCl with an optical density of McFarland standard scale 0.5. Samples containing 20 mg of examined extracts and some reference acids: *p*-protocatechuic, vanillic, caffeic and chlorogenic were dissolved in 1 mL of dimethyl sulfoxide (DMSO). Next, 50 µL of the extracts and acids were dropped into the wells (d = 9 mm) on the mentioned above agar media. The agar plates were preincubated at room temperature for 1 h, next, they were incubated at 37°C for 24 h and 30°C for 48 h for bacteria and fungi, respectively. After the incubation period, the zones of growth inhibition were measured and average values were calculated. The wells containing DMSO without the tested compound was used as controls.

In our study, other reference acids like: ferulic, *p*-coumaric, syringic, *p*-hydroxybenzoic, gentisic and gallic (Sigma, Roth) were used to determine their antimicrobial activity. Samples containing 20 mg of all examined reference acids were dissolved in 1 mL DMSO. Furthermore, bacterial and fungal suspensions were put onto Petri dishes with solid media containing 1000 µg/mL of the tested compounds followed incubation under similar conditions as previously. The inhibition of microbial growth was judged by comparison with a control culture prepared without any sample tested.

Subsequently MIC, defined as the lowest concentration of the samples showing complete bacterial or fungal growth inhibition, was examined by the microdilution broth method using their two-fold dilutions from 1000 to 0.488 µg/mL, in Mueller-Hinton broth (for bacteria) and RPMI 1640 broth with MOPS (for fungi) prepared in microtiter plates. Next, 2 µL of each bacterial or fungal suspension, was added per each well containing 200 µL broth with various concentrations of the examined compounds. The microplates were incubated at suitable conditions (37°C or 30°C, 24 h for bacteria or fungi, respectively) and then MIC was assessed spectrophotometrically. Appropriate DMSO, growth and

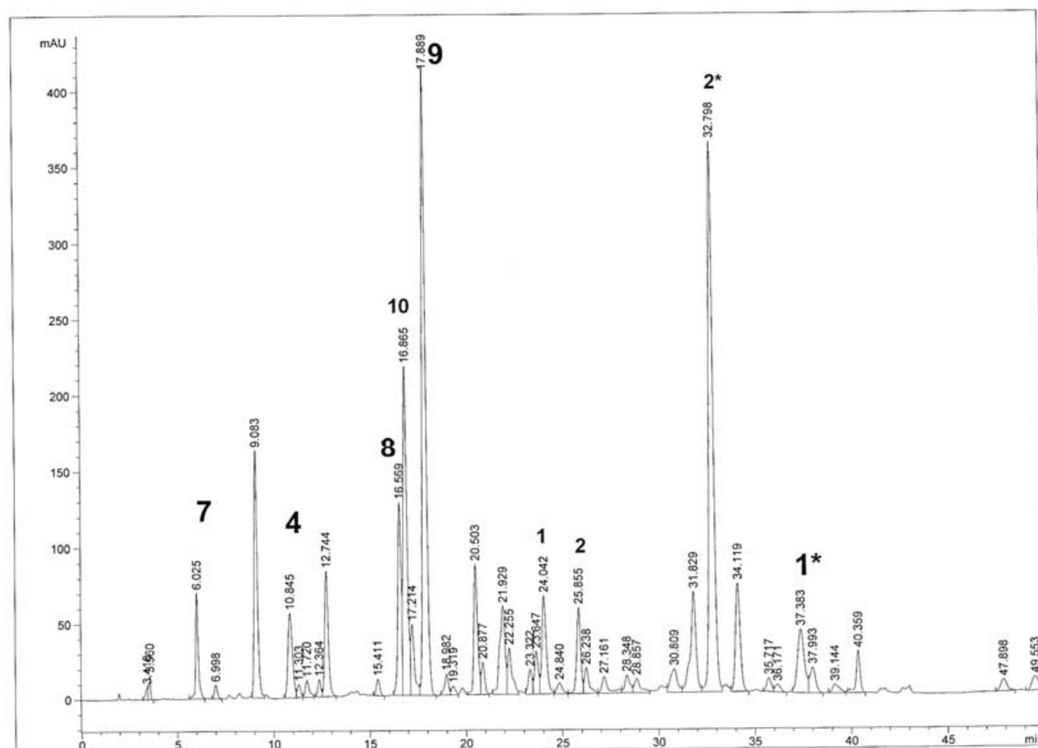

Figure 5. RP-HPLC chromatogram of phenolic acids from the herb fraction F_B. Peaks: 1 – *p*-coumaric acid, 2 – ferulic acid, 4 – gentisic acid, 7 – gallic acid, 8 – vanillic acid, 9 – caffeic acid, 10 – chlorogenic acid, 1* – apigenin-7-glucoside, 2* – luteolin-7-glucoside

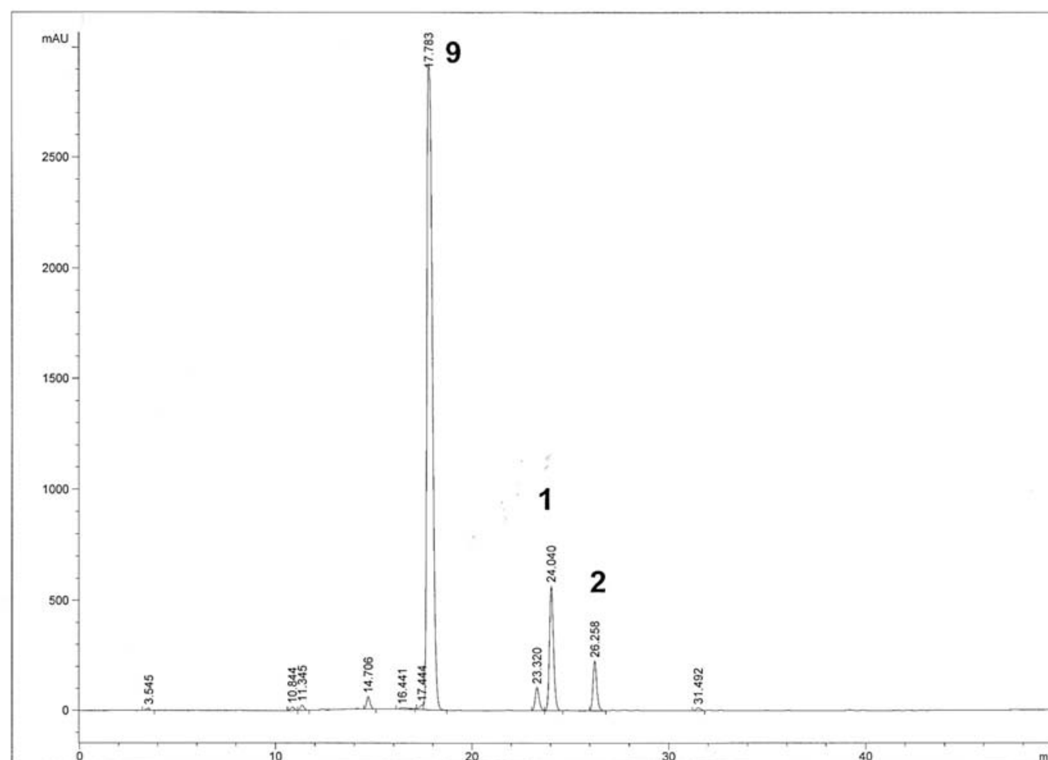

Figure 6. RP-HPLC chromatogram of phenolic acids from the herb fraction F_C. Peaks: 1 – *p*-coumaric acid, 2 – ferulic acid, 9 – caffeic acid

sterile controls were carried out. The medium with no tested substances was used as control.

The MBC or MFC are described as the lowest concentration of the compounds that is required to kill a particular bacterial or fungal species. MBC/MFC was determined by removing 20 µL of the culture using for MIC determinations from each well and spotting onto appropriate agar medium. After incubation, the lowest compounds concentrations with no visible growth observed was assessed as a bactericidal/fungicidal concentration. All the experiments were performed three times and representative data are presented (8, 27).

In this study, no bioactivity was defined as MIC > 1000 µg/mL, mild bioactivity as a MIC in the range 501–1000 µg/mL, moderate bioactivity with MIC from 126 to 500 µg/mL, good bioactivity as a MIC in the range 26–125 µg/mL, strong bioactivity with MIC between 10 and 25 µg/mL and very strong bioactivity as a MIC < 10 µg/mL. The MBC/MIC or MFC/MIC ratios were calculated in order to determine bactericidal/fungicidal (MBC/MIC = 4, MFC/MIC = 4) or bacteriostatic/fungistatic (MBC/MIC > 4, MFC/MIC > 4) effect of the tested compounds (28).

RESULTS

The total content of phenolic acids calculated as caffeic acid in methanolic extract from flowering herbs determined by Arnov's method was equal to 0.69%. The results are in agreement with Zhelev et al. (29).

In the present paper, phenolic acids from the flowering herbs of *C. acanthoides* L. were analyzed by 2D TLC and RP-HPLC. As shown in Table 1 and Figures 1-6, the presence of ten phenolic acids like chlorogenic, protocatechuic, *p*-coumaric, caffeic, syringic, *p*-hydroxybenzoic, ferulic, vanillic, gentisic and gallic were identified in the extracts as well as in fractions obtained after acid (F_B) or alkaline hydrolyses (F_C) in flowering herb of *C. acanthoides* L. Slight differences in the concentrations of phenolic acids were observed using two chromatographic methods. Fractions F_A and F_B are richer in phenolic acids than fraction F_C. Using RP-HPLC, the pres-

ence of free phenolic acids, such like chlorogenic, protocatechuic, *p*-coumaric, caffeic, syringic and *p*-hydroxybenzoic, was confirmed. Moreover, after acid or alkaline hydrolyses additional phenolic acids were detected - after acid hydrolysis: ferulic, gentisic, gallic, vanillic acids while after alkaline hydrolysis: *p*-coumaric, ferulic and caffeic acids. In fraction F_A isorhamnetin-3-rhamnoglucoside and in fraction F_B apigenin-7-glucoside and luteolin-7-glucoside were also identified. These flavonoids were described previously (3).

Our results indicated that the tested extracts at concentration 20 mg/mL, obtained from the flowering herbs of *C. acanthoides* had different activity against the reference strains of microorganisms. Using agar plate method, it was showed that some of these extracts, i.e., aqueous, ethyl acetate and dichloromethane, had no bioactivity against the reference strains of bacteria or fungi, whereas methanol and acetone extracts indicated some antimicrobial effect.

Methanol (50, 80 and 100%) and acetone extracts showed similar activity only against *Bacillus subtilis* ATCC 6633 and *Bacillus cereus* ATCC 10876 belonging to Gram-positive bacteria. The diameters of growth inhibition of these bacteria were 12 mm and 10–14 mm for acetone and methanol extracts, respectively.

The fraction of free phenolic acids (F_A) showed some antibacterial activity against the reference strains of Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 10876, *Micrococcus luteus* ATCC 10240) with diameter of the growth inhibition of 13–16 mm. *Staphylococcus epidermidis* ATCC 12228 was resistant to this fraction. It was shown also antifungal activity against yeasts (*Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019) with the zones of growth inhibition 11–12 mm (Table 2).

The fraction after acid hydrolysis (F_B) had the highest influence on the growth of the reference strains of microorganisms. Among them, staphylococci and micrococci belonging to Gram-positive bacteria, were especially sensitive to this fraction (with 20–25 mm diameters of growth inhibition). In turn, the zones of growth inhibition for *Bacillus subtilis* ATCC 6633 and *Bacillus cereus* ATCC 10876 were 16–17 mm. This fraction showed also bioactivity against reference Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Proteus mirabilis* ATCC 12453, *Pseudomonas aeruginosa* ATCC 9027), giving slight inhibition zones (11–12 mm). In addition, fraction after acid hydrolysis indicated activity against yeasts belonging to *Candida* spp. with 11–14 mm diameters of growth inhibition (Table 2).

Similar results were obtained for the fraction of phenolic acids after alkaline hydrolysis (F_C): the diameters of growth inhibition were 10–20 mm. This

Table 1. Phenolic acids in the crude methanol extracts of *Carduus acanthoides* 2D TLC.

Phenolic acids	Standards					*Carduus acanthoides*		
	R_{f1}	R_{f2}	UV λ = 254 nm and 366 nm	Color reaction		F_A	F_B	F_C
				dSa	3%$FeCl_3$			
Chlorogenic	0.01	0.84	blue	green grey	brown-green	++	-	+
Caffeic	0.08	0.27	blue	light brown	brown-green	-	++	++
Protocatechuic	0.14	0.56	violet	light brown	brown blue	++	-	-
p-Hydroxybenzoic	0.21	0.66	-	yellow	-	+	+	+
p-Coumaric	0.41	0.27	blue red	-	+++	++	++	
Vanillic	0.36	0.55	-	light orange	-	++	++	+
Ferulic	0.59	0.18	violet	violet	-	-	-	++
Syringic	0.61	0.53	light green	purple	-	+++	+	+
Gallic	0.03	0.25	brown	yellow-green	brown blue	-	-	-
Gentisic	0.34	0.68	light blue	light grey	navy blue	-	-	-

Average R_f values of identified phenolic acids separated by 2D TLC with mobile phase; first direction: benzene – methanol – acetic acid – acetonitrile (80 : 10 : 5 : 5, v/v/v/v), and second direction: sodium formate – formic acid – water (10 : 1 : 200, w/v/v); 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.

Table 2. The growth inhibition (mm) in the presence of the tested extracts from *Carduus acanthoides* L. and the standard phenolic acids at concentration 20 mg/mL for the reference strains of microorganisms.

Species		The diameter of growth inhibition (mm)													
		The tested extracts											The reference acids		
		aqueous extract	ethyl acetate extract	dichloromethane extract	acetone extract	50% methanol extract	80% methanol extract	100% methanol extract	free phenolic acid (F_A)	phenolic acids after acid hydrolysis (F_B)	phenolic acids after alkaline hydrolysis (F_C)	chlorogenic acid	caffeic acid	protocatechuic acid	vanillic acid
Gram-positive bacteria	*Staphylococcus epidermidis* ATCC 12228	-	-	-	-	-	-	-	-	20	20	-	38	13	-
	Staphylococcus aureus ATCC 25923	-	-	-	-	-	-	-	16	24	14	-	-	13	-
	Staphylococcus aureus ATCC 6538	-	-	-	-	-	-	-	16	25	15	-	-	13	-
	Bacillus cereus ATCC 10876	-	-	-	12	11	10	10	14	17	14	-	11	12	10
	Bacillus subtilis ATCC 6633	-	-	-	12	14	12	14	13	16	15	-	11	20	-
	Micrococcus luteus ATCC 10240	-	-	-	-	-	-	-	-14	22	18	-	-	13	-
Gram-negative bacteria	*Escherichia coli* ATCC 25922	-	-	-	-	-	-	-	-	11	12	-	-	13	12
	Klebsiella pneumoniae ATCC 13883	-	-	-	-	-	-	-	-	12	10	11	13	14	10
	Proteus mirabilis ATCC 12453	-	-	-	-	-	-	-	-	12	-	-	-	12	-
	Pseudomonas aeruginosa ATCC 9027	-	-	-	-	-	-	-	-	12	11	-	11	10	-
Fungi	*Candida albicans* ATCC 10231	-	-	-	-	-	-	-	11	11	14	-	-	15	-
	Candida parapsilosis ATCC 20019	-	-	-	-	-	-	-	12	14	-	-	-	11	-

- = no diameter of growth inhibition

fraction had no activity only against *Proteus mirabilis* ATCC 12453 belonging to Gram-negative bacteria and *Candida parapsilosis* ATCC 22019 (Table 2).

Our data indicated also that the standard compounds: caffeic, chlorogenic, *p*-protocatechuic and vanillic acids showed some activity against refer-

ence bacteria and yeasts. Among them the widest spectrum of antibacterial activity possessed *p*-protocatechuic acid and the lowest – chlorogenic acid (Table 3).

DISCUSSION

In the present paper, phenolic acids in methanol extract from the flowering herbs of *C. acanthoides* L. were analyzed by 2D TLC and RP HPLC. Fractions F_A and F_B are richer in phenolic acids than fraction F_C. In methanol extract: chlorogenic, protocatechuic, *p*-coumaric, caffeic, syringic and *p*-hydroxybenzoic acids were identified. Additionally: ferulic, gentisic, gallic, vanillic acids were detected after acid hydrolysis, while alkaline hydrolysis let to release of: *p*-coumaric, ferulic and caffeic acids. Previously, only neochlorogenic, chlorogenic and cryptochlorogenic acids were identified by mean of UPLC/DAD/qTOF-MS in methanol extract of *C. acanthoides* grown in Tibet (4).

Research on the quantity of phenolic acids were carried out for the genus *Carduus*. (12). It was shown that content of this group of phenols varies in the range of 0.57-2.43%. Among the investigated plants, high content of phenols (more than 2%) was establish for *C. armatus, C. personata, C. hamulosus* and *C. carduelis*. In comparison, lower values of phenolic acids were reported in *C. acanthoides* (about 0.60%) what is similar to our investigations. In our research, the total amount of phenolic acids

Table 3. The activity data of standard phenolic acids expressed as MIC (MBC/MFC) against the reference microorganisms.

	Species	MIC (MBC/MFC) [μg/mL] of the tested compounds									
		Protocatechuic acid	p-Hydroxybenzoic acid	Vanillic acid	Gentisic acid	Chlorogenic acid	p-Coumaric acid	Ferulic acid	Syringic acid	Caffeic acid	Gallic acid
Bacteria	*Staphylococcus aureus* ATCC 6538	-	-	-	-	-	-	-	-	-	125 (125)
	Staphylococcus aureus ATCC 25923	-	-	-	-	-	-	-	-	-	31.25 (125)
	Staphylococcus epidermidis ATCC 12228	-	1000 (>1000)	1000 (>1000)	-	250 (500)	1000 (>1000)	-	-	500 (>1000)	31.25 (125)
	Micrococcus luteus ATCC 10240	1000 (>1000)	500 (>1000)	1000 (>1000)	1000 (>1000)	1000 (>1000)	500 (>1000)	500 (>1000)	-	1000 (>1000)	250 (>1000)
	Bacillus subtilis ATCC 6633	1000 (>1000)	1000 (>1000)	1000 (>1000)	1000 (>1000)	1000 (>1000)	1000 (>1000)	1000 (>1000)	1000 (>1000)	1000 (>1000)	1000 (>1000)
	Bacillus cereus ATCC 10876	1000 (>1000)	1000 (>1000)	1000 (>1000)	1000 (>1000)	1000 (>1000)	1000 (>1000)	1000 (>1000)	1000 (>1000)	1000 (>1000)	1000 (>1000)
	Bordetella bronchiseptica ATCC 4617	-	1000 (>1000)	-	-	500 (1000)	-	-	-	500 (>1000)	125 (>1000)
Fungi	*Candida albicans* ATCC 10231	1000 (>1000)	1000 (>1000)	1000 (>1000)	-	-	-	-	-		1000 (>1000)
	Candida parapsilosis ATCC 22019	250 (1000)	1000 (>1000)	1000 (>1000)	-	1000 (>1000)	1000 (>1000)	1000 (>1000)	-	-	500 (>1000)

calculated as caffeic acid in methanolic extract from flowering herbs of *C. acanthoides* determined by Arnov's method was 0.69%.

In recent times, the development of bacterial resistance to currently available antibiotics, high cost of synthetic compounds, as well as undesirable side effects of certain drugs, has necessitated the search for new antibacterial agents from alternative sources including medicinal plants. It has been observed that many plants contain different substances with antimicrobial activities, which can serve as source and template for the synthesis of new antimicrobial drugs (29, 31). Much attention has been directed toward extracts and biologically active compounds isolated from popular plant species. The literature data (29, 31) indicated that the biological activity is due to different chemical agents in the extract, including essential oils, steroids, alkaloids, flavonoids, triterpenoids and phenolic compounds or free hydroxyl groups. In the last few years, increased number of studies have been conducted in different countries to demonstrate such efficacy of antimicrobials (32).

Our results indicated that methanol and acetone extracts at concentration 20 mg/mL obtained from the flowering herbs of *C. acanthoides* showed some activity only against the reference strains of Gram-positive bacteria: *Bacillus subtilis* ATCC 6633 and *Bacillus cereus* ATCC 10876. According to the literature data, some activity of various extracts from *Carduus* spp. has been reported. Rahman S.M.A. at al. (29) found that the extracts from *C. getulus* were shown to be effective against some bacterial species. The plant materials were extracted using two different solvents (methanol and hexane) at two different concentrations (6 and 23 mg/mL). Among them, methanol was quantitatively the best extractant. The methanol extract, especially at concentration of 23 mg/mL, was very active against both bacteria locally isolated by the authors and strains from collection ATTC, giving large zones of growth inhibition. This extract showed significant activity against Gram-positive bacteria, *i.e. Bacillus cereus* and *Staphylococcus aureus* and the reference strains of *Clostridium perfringens* ATTC 13124 and *Listeria innocua* ATCC 33090 (with zone of the growth inhibition of 12–27 mm) and Gram-negative bacteria, especially *Klebsiella pneumoniae, Klebsiella oxytoca, Escherichia coli, Yersinia enterocolitica* ATCC 23715 and *Salmonella enterica* ATCC 25566 (21–35 mm).

In turn, data of other authors (30) indicated that the methanol extracts from the same plant – *C. getulus* at concentrations 23 mg/mL, showed also activity against *Candida albicans* with zone of the growth inhibition of 23 mm. Moreover, this extract (23 mg/mL) possessed antifungal effect against most of the tested mold fungi, i.e.: *Fusarium solani, Fusarium oxysporum, Aspergillus flavus, Alternaria alternate, Rhizoctonia solani, Bipolaris oryzae, Rhizopus* spp., *Chetomium* spp. and *Mucor* spp.

According to investigations of Elita Scio et al. (32), the methanol extract from leaves of *C. marianus* demonstrated anti-*Candida* spp. activity, especially against *C. albicans* ATTC 18804 with MIC = 39 µg/mL; this extract had no inhibitory effect on bacterial growth.

Moreover, our data showed that the fractions of free phenolic acids and phenolic acids after acid or alkaline hydrolysis had the high activity against the examined microorganisms. The diameters of growth inhibition in the presence these fractions for tested microorganisms were 11–25 mm. The fraction after acid hydrolysis indicated the broad spectrum of antimicrobial activity against the reference strains of Gram-positive and Gram-negative bacteria or yeasts. The antimicrobial activity of polyphenolic compounds might be due to their ability to form a complex with bacterial cell wall thus inhibiting the microbial growth (33).

The phenolic acids and antimicrobial activity were for the first time investigated in flowering herbs of *Carduus acanthoides* L.

CONCLUSIONS

Ten phenolic acids (chlorogenic, caffeic, ferulic, protocatechuic *p*-hydroxybenzoic, *p*-coumaric, vanillic, syringic, gentisic and ferulic) were described for the first time by means of SPE-RP HPLC and two-dimentional TLC in the methanolic extract from flowering herbs of *Carduus acanthoides* L. and the bacteriological activity of these extracts was also determined.

Our data showed that the tested fraction of phenolic acids (F_A, F_B, F_C) obtained from the flowering herbs of *C. acanthoides*, had the some activity against the reference microorganisms of Gram-positive bacteria, Gram-negative bacteria and fungi belonging to yeasts. The most active fraction of phenolic acids will be subjected to isolation and further pharmacological evaluation. The results of this study support the medicinal usage of *C. acanthoides* L. due to its antimicrobial properties.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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