SUPPLEMENTARY MATERIAL

Chemical composition and antioxidant, antibacterial activity of *Cirsium rivulare* (Jacq)

All. roots

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Abstract The mixture of three phytosterols (campesterol, stigmasterol and β -sitosterol), β -sitosterol 3-*O*-glucoside and syringin were isolated from hexane and methanol extract of *C. rivulare* roots after chromatographic separation. The main component of the source was syringin which was obtained with yield 0.08% of the dry source. In hexane extract the qualitative and quantitative composition of fatty acids was determined. The predominant component was linoleic acid (23.31 mg/g of extract). The extracts showed antioxidant activity. The ability to scavenge DPPH• free radical was in correlation with appointed total phenol content. The not defatted methanolic extract was the most active. Hexane and defatted methanol extracts showed moderate antibacterial activity against G(+) and G(-) strains with MIC and MBC ranged from 25 to 200 µg/mL.

Keywords: *Cirsium rivulare* roots; fatty acids; phytosterols; syringin; DPPH• scavenge; antibacterial activity

Experimental

Plant material

The underground parts of brook thistle (rhizomes and roots together, further referred to as roots) were collected in June 2013 in the vicinity of Bialystok. The plant was identified by the last author. The voucher specimen, no. CR00006, was deposited in the Department of Pharmacognosy, at the Medical University of Bialystok, Poland. Roots were washed, grinded, and dried at room temperature. Seven hundred g of source were utilized in the experiment.

General procedures

Fatty acids methyl esters (FAME) and derivatized phytosterols were analyzed by GC-MS method. The analysis was performed on a Trace GC Ultra ISQ (Thermo Scientific) apparatus. Operating conditions for FAME: capillary column SLBIL111 (100 m × 0.25 mm i.d., film thickness 0.2 µm). Temperature program 50 °C (0.5 min) -180 °C (15 min) - 260 °C (20 min) at 4 °C/min. Injector and detector temperatures was 250 °C. Carrier gas was helium at the flow rate of 1.0 ml/min.

Operating conditions for phytosterols: the analysis was performed on Pegasus 4D with TOF MS (LECO) spectrometer; RXi-5HT capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm). Temperature program 250 °C (10 min. - 4 °C/min.) - 300 °C (30 min.). Injector and

detector temperatures were 300 °C and 280 °C, respectively. The carrier gas was helium at the flow rate of 1.0 ml/min.

Fatty acids were determined as the FAME, and the phytosterols were analyzed after saponification along with their mass spectra compared with MS spectra of computer library: NIST 2011 and Wiley Registry of Mass Spectral Data 8th edition, and retention time compared with standard data.

Flash chromatography (FC) was carried out on Agilent Technologies 971-FP apparatus, Flash Purification System, on Agilent SF10 – 4g, Si 35, AX 1370-8 column.

Spectrophotometric measurement was performed on UV-Vis SPECORD 40 photometer (Analytic Jena GmbH, Germany).

The ¹H and ¹³C NMR analysis was performed on a Bruker Avance II 400 spectrometer at 400 and 100 MHz, respectively, in Py-d₅ (compound 1) and DMSO-d₆ (compound 2).

Extraction and isolation of compounds

Plant material (700 g) was pulverized and first extracted exhaustively in a Soxhlet apparatus with hexane (fraction from kerosene) to obtain hexane extract, and then, with methanol under reflux and defatting methanolic extract (DME) was obtained. The hexane extract (5,4 g) was subjected to silica gel (35-70 Mesh, Merck) glass column and eluted with solvents of increasing polarity: hexane, hexane - chloroform step gradient, chloroform - ethyl acetate step gradient. Residue of fractions eluted with hexane-chloroform (3:1) was separated with FC on silica gel column, eluent *n*-pentane-diethyl ether step gradient and from fractions eluted with 20-50% diethyl ether 117 mg of white crystalline residue (R1) was obtained. DME (97 g) was chromatographed over silica gel glass column with chloroform and chloroform-methanol in step gradient mode. 47 mg of white residue (R2) were obtained from fractions eluted with chloroform-methanol (9:1), after purification on Sephadex LH-20 and recrystallization from chloroform-ethanol (9:1). 15 mg of pure compound **1** were obtained from fractions eluted with CHCl₃-MeOH (8:2) and 588 mg of compound **2** from further fractions eluted with CHCl₃-MeOH (8:2-7:3).

β-Sitosterol 3-*O*-*β*-glucoside (**1**): white crystalline powder from EtOH. ¹H NMR (400 MHz), ¹³C NMR (100 MHz), Py-d₅: value of shifts in accordance with literature data (Faizi et al. 2001).

Syringin or sinapyl alcohol 4-*O*-*β*-glucopyranoside (**2**): white crystalline powder from MeOH, UV λ_{max} (MeOH) 221, 264; ¹H NMR (400 Hz, δ, DMSO, TMS internal standard) 6.72 2H, s (H-2, H-6), 6.46 1H, dt, J=1.5, 15.92 (H-7), 6.33 1H, dt, J=4.88, 15.88 (H-8), 4.9 1H, d,

$J=7.32$ (H-1', glc), 4.1 2H, dd, $J=1.5$, 4.88 (H-9), 3.77 6H, s (2 x -OCH₃); ¹³C NMR (100 Hz, δ , DMSO, TMS internal standard) 152.7 (C-3,5), 133.9 (C-1), 132.59 (C-4), 130.16 (C-7), 128.43 (C-8), 104.5 (C-2,6), 102.58 (C-1', glc), 77.18 (C-3', glc), 76.54 (C-5', glc), 74.17 (C-2', glc), 69.95 (C-4', glc), 61.44 (C-9), 60.9 (C-6', glc), 56.35 (-OCH₃).

Additional methanolic extract (5g of source/100mL of methanol, 1h under reflux) was prepared for DPPH free radical scavenging and total phenol content determination.

Fatty acids methyl esters (FAME) preparation

The FAME were obtained by heating 100 mg root hexane extract with 3 ml of 2 % NaOH (methanolic solution) under reflux. After alkaline hydrolysis, 3 ml of anhydrous 14 % boron trifluoride in methanol were added. Samples were heated 30 min. in water bath under reflux, and then 4 ml of heptane and 10 μ L ethyl oleate (inner standard) were placed. After that, the solution was cooled down and 5 ml of saturated solution of NaCl were added. The amount of 2 ml of heptane solution was placed in screw-capped tube and dried with anhydrous MgSO₄. A solution was analyzed by GC-MS.

The results of the analysis are presented in Table 1.

Table 1. The composition and content of fatty acids in the hexane extract of *C. rivulare* (n=3).

Compound	Content (mg/g)
Hexanoic acid	0.26 $\pm$ 0.09
Oct-3-enoic acid	1.44 $\pm$ 0.12
Miristic acid	0.68 $\pm$ 0.08
Palmitic acid	13.72 $\pm$ 0.31
Palmitoleic acid	4.2 $\pm$ 0.06
Palmitelaidic acid	1.25 $\pm$ 0.1
Stearic acid	0.19 $\pm$ 0.04
Oleic acid	1.21 $\pm$ 0.07
Elaidic acid	0.63 $\pm$ 0.02
Linoleic acid	23.31 $\pm$ 0.15
α -Linolenic acid	4.54 $\pm$ 0.04

Phytosterols sample preparation

The experiment was performed according to Thanh et al., 2006. The quantity of 100 mg of hexane extract was heated for 2 h under reflux with 3 ml of 2 % NaOH (methanolic solution)

and 100 μL of cholesterol in hexane (inner standard). The reactive mixture was cooled and 5 ml of saturated solution of NaCl and 4 ml of heptane were added. After shaking, 1 ml of organic layer was placed in screw-capped tube and 150 μL of BSTFA were added. GC-MS analysis was made after 30 min.

Total phenolic compound analysis

The concentration of total phenolic compounds in methanolic extracts (without and after defatting with hexane) was determined with the Folin-Ciocalteu colorimetric method according to Djeriddane et al. (2006). The results as the mean value $\pm$ standard deviation of three independent measurements were expressed in mg of gallic acid per g of dry weight of extract.

DPPH radical scavenging assay

The method previously described by Brand-Williams et al. (1995) with small modifications was used to measure free radical scavenging properties of extracts from the roots of *C. rivulare*. A 2.5 mL of 6×10^{-5} mol/L methanol DPPH $\bullet$ (2,2-diphenyl-1-picryl-hydrazyl) solution was added to the 0.070 mL of methanol solution of extract (1 mg/mL). The absorbance was measured after 30 min. at 517 nm. As positive control caffeic acid in concentration of 1 mg/mL was used. The determination was made in triplicate and the mean value $\pm$ standard deviation was counted.

The results of these two experiments are presented in Table 2.

Table 2. Total phenol content (TPC) and DPPH free radical scavenging ability of extracts from *C. rivulare* roots (n=3).

Extract	TPC (mg/g)	DPPH scavenging (%)
Methanolic not defatted	108 $\pm$ 1.32	81,6 $\pm$ 0.97
Methanolic defatted	81 $\pm$ 2.1	61,7 $\pm$ 2.43
Hexane	30 $\pm$ 1.67	4,3 $\pm$ 0.7
Caffeic acid (1mg/mL) (positive control)	-	94,3 $\pm$ 1.1

Antimicrobial activity determination

Strains were used for the test

The antibacterial activity of hexane and defatted methanolic extracts (DME) from *C. rivulare* roots was assessed against bacteria species: *Staphylococcus aureus* ATCC 25923 (American Type Culture Collection), *Escherichia coli* ATCC 25922, *Moraxella catarrhalis* ATCC 25238, *Streptococcus pyogenes* ATCC 19615, *Streptococcus mutans* ATCC 35668, *Streptococcus salivarius* ATCC 13419, *Enterococcus faecalis* ATCC 29212, *Campylobacter jejuni* ATCC 33560 and *Bacillus subtilis* (environmental strain). Research strains were incubated at BHI broth for 18-24 hours to obtain the logarithmic-phase of growth. After incubation, bacterial suspension (inoculum) was diluted with sterile physiological solution to 10^8 CFU/mL (turbidity corresponding standard 0.5 McFarland barium sulphate).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination

The antibacterial activity of the extracts was determined with microdilution method, following the guidelines of the EUCAST reference body (EUCAST, 2015; Andrews, 2001), which uses two different culture media: cation-adjusted Mueller-Hinton broth (MHB) and MHB supplemented with 5% lysed horse blood and 20 mg/L β -NAD (MH-F broth). MH-F broth is used for testing *Streptococcus* spp., *Moraxella catarrhalis*, *Campylobacter* spp. and several other fastidious organisms. Crude extracts were dissolved in DMSO (12 % of the final volume) and diluted with culture broth to a concentration of 200 μ g/mL. Further (1:2 ratio) serial dilutions were performed by addition of culture broth to reach concentrations ranging from 200 to 0.195 μ g/mL; 100 μ L of each dilution were distributed in 96-well plates, as well as a sterility control and a growth control (containing culture broth with DMSO in addition, without antimicrobial substance). Each test and growth control were inoculated with 5 μ L of a bacterial suspension (10^8 CFU/mL). All experiments were performed in triplicate. Microdilution trays were incubated between 35 - 40°C for 18-24 h. Bacterial growth was formerly detected by optical density (Micro Plate Reader MPR-A4i, Tosoh, Japan). MIC values were defined as the lowest concentration of each extract, which inhibited microbial growth. The results were expressed in micrograms per millilitres.

Similar procedure was done to determine the MIC of amoxicillin against tested bacterial strains.

The MBC test determines the lowest concentration level at which an antimicrobial agent is killing a particular microorganism. The MBC is determined using a series of steps, undertaken after a Minimum Inhibitory Concentration (MIC) test has been completed.

Turbidity indicates growth of the microorganism and the MIC is the lowest concentration where no growth is visually observed. To determine the MBC, the dilution representing the MIC and at least two of the more concentrated test products dilutions are plated and enumerated to determine viable CFU/mL.

The results are presented in Table 3.

Table 3. *In vitro* activity of *C. rivulare* root hexane and defatted methanolic extracts against G(+) and G(–) bacteria (MIC and MBC in µg/mL).

Bacterial strain	Hexane extract		DME ³		Amoxicillin
	MIC ¹	MBC ²	MIC ¹	MBC ²	MIC ¹
<i>Staphylococcus aureus</i>	100	100	50	50	6.25
<i>Escherichia coli</i>	100	100	25	25	25
<i>Bacillus subtilis</i>	100	100	50	50	1.56
<i>Moraxella catarrhalis</i>	50	50	50	100	12.5
<i>Streptococcus pyogenes</i>	100	100	100	100	<0.195
<i>Streptococcus mutans</i>	200	200	100	100	0.195
<i>Streptococcus salivarius</i>	100	100	50	50	0.39
<i>Enterococcus faecalis</i>	100	100	100	100	0.78
<i>Campylobacter jejuni</i>	50	50	50	50	12.5

¹MIC, minimum inhibitory concentration; ²MBC, minimum bactericidal concentration; ³DME, defatted methanol extract

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