SUPPLEMENTARY MATERIAL

Chemical Compositions and Antibacterial Activity of Extracts Obtained from the Inflorescences of *Cirsium canum* (L.) All.

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The aim of study was to investigate phenolic acids and flavonoids in methanolic, dichloromethane, acetone and ethyl acetate extracts and fractions from inflorescences of *Cirsium canum* (L.). RP-HPLC analysis enabled identification: chlorogenic acid, caffeic acid, *p*-coumaric acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, syringic acid, trans-cinnamic acid, luteolin-7-glucoside, apigenin-7-glucoside, kaempferol-3-glucoside, linarin, apigenin, rutoside, luteolin, kaempferol. The antimicrobial activity of tested extracts was determined *in vitro* against reference microorganisms, including bacteria or fungi, belonging to yeasts. The our data showed that the tested extracts had no influence on the growth of the reference strains of Gram-negative bacteria and of yeasts belonging to *Candida* spp. Among them, the fractions possessed the highest activity against Gram-positive bacteria, especially *S. aureus* and *S. pneumoniae* belonging to pathogens and *S. epidermidis, B. cereus* and *B. subtilis* belonging to opportunistic microorganisms.

**Keywords:** *Cirsium canum* (L.) All., phenolic acids, flavonoids, antibacterial activity.
Experimental

Plant material

Flowers were collected in the Medicinal Plant Garden, of the Department of Pharmacognosy, (Lublin, Poland). The inflorescences were dried in temperature at 45ºC 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 No Cir 12/12 is deposited in the herbarium of the Department of Pharmacognosy, Medical University of Lublin.

Extraction procedure

Extraction and analysis of 50%v/v, 80%v/v, 100% methanol, dichloromethane, acetone, ethyl acetate extracts and fraction F₁, F₂, F₃, F₄ containing flavonoids and fraction F₅, F₆, F₇ containing phenolic acids were performed. The obtained extracts were concentrated under reduced pressure, dissolved in small portion of methanol, transferred to a 10 mL graduated flask, purified by passed through 0.45 μm PTFE membrane filters (Cronus Syringe Filter PTFE 25mm, 0.2µm) and analyzed by RP-HPLC (Reversed Phase High-Performance Liquid Chromatography). Reagents analytical grade were purchased in (POCh, Gliwice, Poland). Identifications were performed by comparing retention times with those of standards.

Several standards of flavonoids were used: apigenin-7-glucoside, kaempferol-3-glucoside, luteolin-7-glucoside, apigenin, luteolin, linarin, kaempferol and phenolic acids: p-hydroxybenzoic, m-hydroxybenzoic, protocatechuic, gallic, vanillic, syringic acid, trans-cinnamic, p-coumaric, caffeic, ferulic acid, rosmarinic acid, chlorogenic acid, coumaric acid, o-coumaric acid, isovanillic acid, and gentisic acid. All standards were purchased from Sigma – Aldrich (Steinheim, Germany).

Extraction in boiling point: (10 g) milled plant material samples of C. canum were soaked in 100 mL: 50%v/v methanol (M₁), 80%v/v methanol (M₂), 100% methanol (M₃), dichloromethane (M₄), acetone (M₅), ethyl acetate (M₆) in a 250 mL round-bottomed flask, then extracted under reflux in heating point for 30 min. Each extract was carefully filtered through cotton and the plant material was re-extracted twice with fresh portions of solvent.

Soxhlet extraction: (50.0 g) powdered plant material was macerated (24 h) and extracted with chloroform in a Soxhlet apparatus. After chloroform extraction, the material was extracted exhaustively for 48 h with 100% methanol at 78ºC. The obtained methanol extract was evaporated to dryness and dissolved in 100 mL hot water, left in a refrigerator for 24 h, and filtered through paper filter. After the separation of ballast extract was divided in two equals parts and a successive extraction were performed.
One part aqueous solution was extracted with diethyl ether, then washed with 5% aqueous sodium bicarbonate solution. After acidification and extraction with diethyl ether, fractions containing free phenolic acids (fraction F_A) were obtained. Hydrolysis was performed by the Schmidtlein and Herrmann method (Schmidtlein & Hermann, 1975; Świątek, 1997; Polish Pharmacopoeia VI). The fractions containing bonded phenolic acids liberated after acid hydrolysis (fraction F_B) and analysis of bonded phenolic acids liberated by alkaline hydrolysis (fraction F_C).

The second part aqueous solution was extracted sequentially with diethyl ether obtaining fractions: (fraction F_1), ethyl acetate (fraction F_2), n-butanol (fraction F_3). Then the acidic hydrolysis of and total acid hydrolysis of 5% HCl has been performed on diethyl ether extract for 2 h (fraction F_4). Extracts residues were evaporated to dryness under reduced pressure. Afterwards respectively they were investigated for their microbiological activity. For HPLC analysis fractions were dissolved in methanol, passed through 0.45 μm PTFE membrane filters (Cronus Syringe Filter, PTFE 25mm, 0.2μm) and transferred to a 10 mL calibrated vials.

**RP-LC analysis**

LC was performed with an Agilent 1100 (Agilent Technologies, USA) system coupled with an auto-sampler, a column thermostat; a diode – array detector (DAD). Compounds were separated on 250 x 4.6 mm stainless-steel column packed with 5 μm Hypersil XDB- C18 (Zorbax, Eclipse), 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: 0 min, 2% B in A; 8 min 5% B in A; 12 min 10% B in A; 20 min 25% B in A; 35 min 45% B in A; 40 min 60% B in A, 45 min 75% B in A. The mobile phase flow-rate was 1 mL min⁻¹, the sample injection volume was 10 μL, and was performed at 25°C. The LC autosampler, column oven, and DAD were monitored and controlled using of ChemStation rev.10.0 software (Agilent Technologies). Compounds were identified by comparison of retention times and UV spectra with those of appropriate standards analyzed under the same conditions. Qualitative and quantitative determination were performed at the 320 nm wavelength of maximum absorption of flavonoids and for cinnamic acid derivatives (ferulic, chlorogenic, p-coumaric, trans-cinnamic and caffeic acids) and 254 nm for benzoic acid derivatives (protocatechuic, p-hydroxybenzoic, vanillic, and syringic acids).

Each extract was injected in triplicate on the same day. The method of precision was evaluated by use of intra-day tests. Intra-day experiments were performed by replicate
analysis of three aliquots of the same sample on the same day. Peak area of each of the extract components was measured. The RSD (relative standard deviation, %) of peak areas were used. Quantifications were performed by comparing the peak areas of the compounds present in the extracts M1-M6 with those of the external standards.

**In vitro antimicrobial assay:**

The obtained extracts from flowering *C. canum*: M1 – M6 and fraction extracted from this plant containing flavonoids: F1 – F4 and free phenolic acids: FA, FB, FC were screened *in vitro* for antibacterial and antifungal activities using the broth microdilution method according to EUCAST (European Committee on Antimicrobial Susceptibility Testing) [EUCAST, 2003] and Clinical and Laboratory Standards Institute guidelines (Wayne & Pa, 2012) against a panel of reference strains of 20 microorganisms, including Gram-positive bacteria (*S. aureus* ATCC 25923, *S. aureus* ATCC 6538, *S. epidermidis* ATCC 12228, *S. pyogenes* ATCC 19615, *S. pneumoniae* ATCC 49619, *S. mutans* ATCC 25175, *B. subtilis* ATCC 6633, *B. cereus* ATCC 10876, *M. luteus* ATCC 10240), Gram-negative bacteria (*E. coli* ATCC 3521, *E. coli* ATCC 25922, *K. pneumoniae* ATCC 13883, *P. mirabilis* ATCC 12453, *B. bronchiseptica* ATCC 4617, *S. typhimurium* ATCC 14028, *P. aeruginosa* ATCC 9027, *P. aeruginosa* ATCC 27853) and fungi, belonging to yeasts (*C. albicans* ATCC 2091, *C. albicans* ATCC 10231, *C. parapsilosis* ATCC 22019). These microorganisms came from American Type Culture Collection (ATCC), routinely used for the evaluation of antimicrobials. All the used microbial cultures were first 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 or Mueller-Hinton agar with sheep blood (for bacteria) and RPMI 1640 with MOPS (for fungi) were inoculated with the suspensions of bacterial or fungal species. Microbial suspensions were prepared in sterile saline (0.85% NaCl) with an optical density of McFarland standard scale 0.5 – approximately 1.5 x 10⁸ CFU (Colony Forming Units)/ml for bacteria and 0.5 McFarland standard scale – approximately 5 x 10⁵ CFU/ml) for fungi. Samples containing 5000 µg/ml, 1000 µg/ml and 500 µg/ml of examined flower extracts from *C. canum*: M1 – M3 and fraction extracted from this plant F1 – F4 and FA, FB, FC were dissolved in DMSO and were dropped into the wells on the mentioned above agar media. The agar plates were preincubated at room temperature for 1 h, next they were incubated under appropriate conditions. The well containing DMSO was used as negative control and ciprofloxacin or fluconazole (Sigma) as positive controls.
Subsequently Minimal Inhibitory Concentration (MIC) of the extracts was examined by the microdilution broth method, using their two-fold dilutions in Mueller-Hinton broth or RPMI 1640 broth with MOPS prepared in 96-well polystyrene plates. Final concentrations of these extracts ranged from 1000 µg/ml to 7.81 µg/ml. Microbial suspensions were prepared in sterile saline (0.85% NaCl) with an optical density of 0.5 McFarland standard. The bacterial or fungal suspension was added per each well containing broth and various concentrations of the examined extracts. After incubation the MIC was assessed spectrophotometric as the lowest concentration of the samples showing complete bacterial or fungal growth inhibition. Appropriate DMSO, growth and sterile controls were carried out. The medium with no tested extracts was used as control.

The MBC (Minimal Bactericidal Concentration) or MFC (Minimal Fungicidal Concentration) are defined as the lowest concentration of the compounds that is required to kill a particular bacterial or fungal species. MBC or MFC was determined by removing 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 repeated three times and representative data are presented (Łączkowski et al., 2013).

In this study, no bioactivity was defined as a 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 (O'Donnell et al., 2010). 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.
Figure. S1. Chromatogram obtained from the inflorescences of *Cirsium canum* (L.) All.
- 100% methanolic extract.

a - chlorogenic acid, b - caffeic acid, 1 - luteolin-7-glucoside, 2 - apigenin-7-glucoside, 3 - kaempferol-3-glucoside, 4 - linarin, 5 - apigenin,

Figure. S2. Chromatogram obtained from the inflorescences of *Cirsium canum* (L.) All.
- diethyl ether extract - fraction F1.

a - chlorogenic acid, b - caffeic acid, c - *p*-coumaric acid, 1 - luteolin-7-glucoside, 3 - kaempferol-3-glucoside, 4 - linarin, 5 - apigenin, 8 – kaempferol
Figure. S3. Chromatogram obtained from the inflorescences of *Cirsium canum* (L.) All.
- ethyl acetate extract - fraction F2.

a - chlorogenic acid, 1 - luteolin-7-glucoside, 3 - kaempferol-3-glucoside, 4 - linarin,

Figure. S4. Chromatogram obtained from the inflorescences of *Cirsium canum* (L.) All.
- n-butanol extract - fraction F3.

a - chlorogenic acid, c - *p*-coumaric acid, 4 - linarin, 6 - rutoside,
Figure. S5. Chromatogram obtained from the inflorescences of *Cirsium canum* (L.) All. - extract after acid hydrolysis - fraction F₄.

**Figure. S6. Chromatogram obtained from the inflorescences of *Cirsium canum* (L.) All. - fraction F₄ (free phenolic acids).**

a - chlorogenic acid, b - caffeic acid, c - *p*-coumaric acid, e - protocatechuic acid, f - *p*-hydroxybenzoic acid, 1 - luteolin-7-glucoside, 4 - linarin, 5 - apigenin, 7 - luteolin, 8 - kaempferol.
Figure. S7. Chromatogram obtained from the inflorescences of *Cirsium canum* (L.) All.
- fraction FB (the bonded phenolic acids liberated by acid hydrolysis).

a - chlorogenic acid, b - caffeic acid, c - *p*-coumaric acid, d - syringic acid, e - protocatechuic acid, g - *trans*-cinamic acid, 1 - luteolin-7-glucoside, 5 - apigenin, 7 - luteolin, 8 - kaempferol

Figure. S8. Chromatogram obtained from the inflorescences of *Cirsium canum* (L.) All.
- fraction FC (the bonded phenolic acids liberated by alkaline hydrolysis).

d - syringic acid, e - protocatechuic acid, f - *p*-hydroxybenzoic acid, g - *trans*-cinamic acid
Table S1. Flavonoids’ and phenolic acids content (µg/g) compounds in extracts obtained from the inflorescences of *C. canum*

| Flower extracts obtained from *C. canum* | M1   | M2   | M3   | M4   | M5   | M6   |
|----------------------------------------|------|------|------|------|------|------|
| Chlorogenic acids                      | C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %|
|                                        | 93.26| 0.020| 0.021| 95.94| 0.126| 0.13 | 70.76| 0.24 | 0.35 | 0.92 | 0.007| 0.76 | 8.07 | 0.017| 0.21| 0.0002|
|                                        | ±S.D.|      |      | ±S.D.|      |      | ±S.D.|      |      |      | ±S.D.|      |      | ±S.D.|      |      |      |      |      |      |      |      |
|                                        |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Caffeic acids                          | C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %|
|                                        | 1.03 | 0.0035| 0.33 | 1.09 | 0.0095| 0.008| 0.89 | ND   | 0.001| 0.89 | ND   | 0.001| 0.89 | ND   | 0.001| 0.89 | ND   | 0.001| 0.89 | ND   | 0.001|
|                                        | ±S.D.|      |      | ±S.D.|      |      | ±S.D.|      |      |      | ±S.D.|      |      | ±S.D.|      |      |      | ±S.D.|      |      | ±S.D.|      |      | ±S.D.|      |      |
|                                        |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Luteolin-7-glucoside                   | C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %|
|                                        | 1164.46| 2.95 | 0.253| 11728.66| 9.63 | 0.082| 607.75| 0.15 | 0.025| 8.173| 0.057| 0.70 | 54.46| 0.092| 0.17 | 13.33|
|                                        | ±S.D.|      |      | ±S.D.|      |      | ±S.D.|      |      |      | ±S.D.|      |      | ±S.D.|      |      |      | ±S.D.|      |      | ±S.D.|      |      | ±S.D.|      |      |
|                                        |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Linarin                                | C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %|
|                                        | 121.75| 0.18 | 0.14 | 116.12| 0.13 | 0.001| 44.13| 0.04 | 0.09 | 1.94 | 0.006| 0.30 | 10.51| 0.02 | 0.19 | 8.94 |
|                                        | ±S.D.|      |      | ±S.D.|      |      | ±S.D.|      |      |      | ±S.D.|      |      | ±S.D.|      |      |      | ±S.D.|      |      | ±S.D.|      |      | ±S.D.|      |      |
|                                        |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Apigenin                               | C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %| C    | ±S.D.| RSD %|
|                                        | 26.44| 0.096| 0.36 | 23.58| 0.02 | 0.08 | 17.97| ND   | 0.17 | 0.66 |
|                                        | ±S.D.|      |      | ±S.D.|      |      | ±S.D.|      |      |      | ±S.D.|      |      | ±S.D.|      |      |      | ±S.D.|      |      | ±S.D.|      |      | ±S.D.|      |      |

M1 – 50% methanolic extract, M2 – 80% methanolic extract, M3 – 100% methanolic extract, M4 – dichloromethane extract, M5 – acetone extract, M6 – ethyl acetate extract,

Each value is the mean (µg per 1 g dry sample) from three replicate analyses

SD standard deviation,

RSD% Relative Standard Deviation

ND not detected
Table S2. The activity data expressed as MIC and MBC (µg/ml) against the reference strains of Gram-positive bacteria for flower extracts and fraction from *C. canum*. The standard antibiotics used as positive controls: vancomycin (VA)* for *Streptococcus* spp. and ciprofloxacin (CIP) for remaining bacteria.

| Flower extracts and fractions extracted from *C. canum* (µg/ml) | The reference strains |
|-----------------------------------------------|-------------------------|
|                                              | *Staphylococcus aureus* ATCC 6538 | *Staphylococcus aureus* ATCC 25923 | *Staphylococcus epidermidis* ATCC 12228 | *Streptococcus pyogenes* ATCC 19615 | *Streptococcus pneumoniae* ATCC 49619 | *Streptococcus mutans* ATCC 25175 | *Micrococcus luteus* ATCC 10240 | *Bacillus cereus* ATCC 10876 | *Bacillus subtilis* ATCC 6633 |
| **M1** MIC | 250 | 250 | 500 | 1000 | 500 | >1000 | 500 | 125 | 125 |
| **M1** MBC | 500 | 500 | 1000 | >1000 | >1000 | >1000 | 1000 | 250 | 1000 |
| **M2** MIC | 500 | 1000 | 1000 | 500 | 500 | 1000 | 1000 | 250 | 250 |
| **M2** MBC | >1000 | >1000 | 1000 | >1000 | >1000 | >1000 | 1000 | 1000 | 1000 |
| **M3** MIC | 500 | 500 | 500 | 500 | 250 | 250 | 500 | 250 | 250 |
| **M3** MBC | >1000 | 1000 | 1000 | >1000 | >1000 | >1000 | 1000 | 1000 | 500 |
| **M4** MIC | - | - | - | - | - | - | - | 1000 | 1000 |
| **M4** MBC | - | - | - | - | - | - | - | 1000 | 1000 |
| **M5** MIC | - | - | - | - | - | - | 1000 | 500 | 1000 |
| **M5** MBC | - | - | - | - | - | - | 1000 | 1000 | 1000 |
| **M6** MIC | - | - | - | - | - | - | 1000 | 1000 | 1000 |
| **M6** MBC | - | - | - | - | - | - | 1000 | 1000 | 1000 |
| **F1** MIC | 125 | 125 | 125 | 500 | 125 | 500 | 250 | 62.5 | 125 |
| **F1** MBC | >1000 | >1000 | 1000 | >1000 | >1000 | >1000 | 1000 | 250 | 1000 |
| **F2** MIC | 1000 | 500 | 500 | 1000 | 1000 | >1000 | 500 | 500 | 500 |
| **F2** MBC | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | 1000 | 1000 | 500 |
| **F3** MIC | 125 | 250 | 125 | 500 | 125 | 250 | 62.5 | 125 |
| **F3** MBC | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | 1000 | 250 | 1000 |
| **F4** MIC | - | - | - | - | - | - | - | 1000 | 1000 |
| **F4** MBC | - | - | - | - | - | - | - | 1000 | 1000 |
| **F5** MIC | - | - | - | - | - | - | - | >1000 | >1000 |
| **F5** MBC | - | - | - | - | - | - | - | >1000 | >1000 |
| **F6** MIC | - | - | - | - | - | - | - | 1000 | 1000 |
| **F6** MBC | - | - | - | - | - | - | - | 1000 | 1000 |
M1 – 50% methanol extract, M2 – 80% methanol extract, M3 – 100% methanol extract, M4 – dichloromethane extract, M5 – acetone extract, M6 – ethyl acetate extract, F1 – diethyl ether fraction, F2 – ethyl acetate fraction, F3 – n-butanol fraction, F4 – fraction after acid hydrolysis, FA – free phenolic acids, FB – phenolic acids after acid hydrolysis, FC – phenolic acids after alkaline hydrolysis.

MIC – Minimal Inhibitory Concentration
MBC – Minimal Bactericidal Concentration
– no activity

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|          | CIP/VA* | MIC  | 0.244 | 0.488 | 0.122 | 0.244* | 0.244* | 0.976* | 0.976 | 0.061 | 0.030 |
|----------|---------|------|-------|-------|-------|--------|--------|--------|-------|-------|-------|
| MBC      |         |      |       |       |       |        |        |        |       |       |       |
|          |         |      | >1000 | >1000 | >1000 |        |        |        |       |       |       |