SUPPLEMENTARY MATERIAL

Flavonoid and phenolic acid profile by LC-MS/MS and biological activity of crude extracts from Chenopodium hybridum aerial parts

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Abstract

Extracts from leaves and stems of *Chenopodium hybridum* were characterized for the presence and quantity of flavonoids and phenolic acids by LC-ESI-MS/MS. Five flavonoids and eight phenolic acids were detected for the first time in aerial parts of this plant species, the most abundant compounds being rutin (2.80 µg/g DW), 3-kaempferol rutinoside (2.91 µg/g DW), 4-OH-benzoic (1.86 µg/g DW) and syringic acids (2.31 µg/g DW). Extracts were tested for anti-inflammatory/anti-arthritis, anti-hyaluronidase and cytotoxic activities against human prostate cancer (Du145, PC3) and melanoma cell lines (A375, HTB140, and WM793) of different malignancy. None of the extracts protected bovine serum albumin from heat-induced denaturation. Anti-hyaluronidase effect at the tested concentration was higher than standard naringenin. Cytotoxic activity was generally low with an exception of the extract from the leaves which was found most effective against prostate Du145 cell line with 98.28 ± 1.13 % of dead cells at 100 µg/mL.

Keywords: Chenopodium hybridum; flavonoids; phenolic acids; LC-MS/MS; anti-hyaluronidase; cytotoxic activity; anti-denaturation activity

Experimental

1 Plant material

*Chenopodium hybridum* aerial parts were collected in Cracow (50°04'N 19°56'E), Poland, in August 2013 and identified by Dr. Agnieszka Szewczyk, Department of Pharmaceutical Botany, Pharmaceutical Faculty, Medical College, Jagiellonian University, Cracow, Poland. The Plant List was used as a reference. A voucher specimen (No. KFg/2013011) is deposited at the Department of Pharmacognosy, Pharmaceutical Faculty, Medical College, Jagiellonian University, Cracow, Poland.

2 Extraction

Dried aerial parts (leaves and stems) of *Ch. hybridum* were ground to a fine powder using a laboratory mill. 1 g samples, six from each plant part, were weighed accurately and extracted successively with chloroform and next with methanol (3 x 10 mL for 1 h) on a boiling water bath under reflux. The combined MeOH extracts were then
concentrated under reduced pressure on a rotary evaporator, on a boiling water bath, and kept in the dark at +4 °C until used for quantification of flavonoids and phenolic acids, and for the determination of biological activities.

3 Quantification of flavonoids by LC-ESI-MS/MS

Chemicals and samples
Standards of luteolin 7-glucoside, luteolin 3,7-diglucoside, rutin, hyperoside, isoquercetin, naringin, naringenin 7-glucoside, quercitrin, apigenin 7-glucoside and LC grade acetonitrile were purchased from Sigma–Aldrich Fine Chemicals (St. Louis, MO, USA). Kaempferol 3-rutinoside and astragalain were from Carl Roth (Karlsruhe, Germany). Luteolin 4’-O-glucoside was obtained from LGC Standards (Dziekanów Leśny, Poland). All chemicals were of analytical grade. LC grade water was prepared using a Millipore Direct-Q3 purification system (Bedford, MA, USA).

1 mg of MeOH extracts, prepared as described in 3.2, were accurately weighed, redissolved in 1 mL of MeOH, filtered, and 3 µL aliquots of each sample was injected onto the LC-ESI-MS/MS system in triplicate.

LC-ESI-MS/MS conditions
Analysis of flavonoids was performed using reversed-phase high-performance liquid chromatography and electrospray ionization mass spectrometry (LC-ESI-MS/MS). For this purpose an Agilent 1200 Series HPLC system (Agilent Technologies, USA) equipped with a binary gradient solvent pump, a degasser, an autosampler and column oven connected to 3200 QTRAP Mass spectrometer (AB Sciex, USA) was used.

Chromatographic separations were carried out at 25°C, on an Eclipse XDB-C18 column (4.6 x 150 mm, 5-µm particle size; Agilent Technologies, USA) with a mobile phase consisting of water containing 0.1% HCOOH (solvent A) and acetonitrile containing 0.1% HCOOH (solvent B), using 5 µL injections. The flow rate was 450 µL min⁻¹ and the gradient was as follows: 0 – 1 min – 18% B; 1.5 – 5.5 min – 20% B; 7 – 10 min – 25 % B; 13 - 15 min – 60% B, 17 – 21 min – 18%B.

The QTRAP-MS system was equipped with electrospray ionisation source (ESI) operated in the negative-ion mode. ESI worked at the following conditions: capillary temperature 500 °C, curtain gas at 25 psi, nebulizer gas at 50 psi, negative ionisation mode source voltage −4500 V. Nitrogen was used as curtain and collision gas. For each compound the optimum conditions of Multiple Reaction Mode (MRM) were determined in the infusion mode. The data was acquired and processed using Analyst.
1.5 software (AB Sciex, USA). Triplicate injections were made for each standard solution and sample. The analytes were identified by comparing retention time and m/z values obtained by MS and MS² with the mass spectra of corresponding standards tested under the same conditions. The calibration curves obtained in MRM mode were used for quantification of all analytes. The identified flavonoids were quantified on the basis of their peak areas and comparison with a calibration curve obtained with the corresponding standards. Linearity ranges for calibration curves were specified. The limits of detection (LOD) and quantification (LOQ) for compounds were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of diluted solutions at known concentrations (Table S3).

3.4 Quantification of phenolic acids by LC-ESI-MS/MS

Chemicals and samples
Standards of gallic, protocatechuic, gentisic, 4-OH-benzoic, vanillic, caffeic, syringic, p-coumaric, ferulic, salicylic, veratric, synapic, 3-OH-cinnamic and rosmarinic acid were purchased from Sigma–Aldrich Fine Chemicals (St. Louis, MO, USA). All chemicals were of analytical grade. LC grade methanol (MeOH) was purchased from J.T. Baker (Phillipsburg, USA). LC grade water was prepared using a Millipore Direct-Q3 purification system (Bedford, MA, USA). 1 mg of extracts, prepared as described in 3.2, were accurately weighed, redissolved in 1 mL of MeOH, filtered, and 3 μL aliquots of each sample was injected onto the LC-ESI-MS/MS system in triplicate.

LC-ESI-MS/MS conditions
The samples were analyzed according to method previously described by Nowacka et al. (Nowacka et al. 2014) with some modifications. Phenolic acids contents were determined by reversed-phase high-performance liquid chromatography and electrospray ionization mass spectrometry (LC-ESI-MS/MS). For this purpose an Agilent 1200 Series HPLC system (Agilent Technologies, USA) equipped with a binary gradient solvent pump, a degasser, an autosampler and column oven connected to 3200 QTRAP Mass spectrometer (AB Sciex, USA) was used. Chromatographic separations were carried out at 25°C, on a Zorbax SB-C18 column (2.1 x 50 mm, 1.8-μm particle size; Agilent Technologies, USA) with a mobile phase consisting of water containing 0.1% HCOOH (solvent A) and methanol containing 0.1% HCOOH (solvent B), using 3
µL injections. The flow rate was 500 µL min⁻¹ and the gradient was as follows: 0 – 0.8 min – 5% B; 2 – 3 min – 20% B; 5 – 7.5 min – 100% B; 8.5 - 11 min – 5% B.

The QTRAP-MS system was equipped with electrospray ionisation source (ESI) operated in the negative-ion mode. ESI worked at the following conditions: capillary temperature 600 °C, curtain gas at 25 psi, nebulizer gas at 60 psi, negative ionisation mode source voltage −4500 V. Nitrogen was used as curtain and collision gas. For each compound the optimum conditions of Multiple Reaction Mode (MRM) were determined. The data was acquired and processed using Analyst 1.5 software (AB Sciex, USA). Triplicate injections were made for each standard solution and sample.

The analytes were identified by comparing retention time and m/z values obtained by MS and MS² with the mass spectra from corresponding standards tested under the same conditions. The calibration curves obtained in MRM mode were used for quantification of all analytes. The limits of detection (LOD) and quantification (LOQ) for phenolic compounds were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions at known concentrations (Table S4).

Statistical analysis

The obtained data were subjected to a statistical analysis and the consequent evaluations were analyzed using a variance analysis. Linear regression parameters were computed together with r² indices.

5 Inhibition of albumin denaturation

The anti-inflammatory activity was determined using the inhibition of albumin denaturation technique. The test was performed according to method of Mizushima et al. (Mizushima & Kobayashi 1968) and Williams (Williams et al. 2008) with minor modifications.

An aqueous solution of 0.5 % w/v BSA was prepared and pH was adjusted to 6.0 using 1M HCl. The diclofenac sodium was used as a standard drug. All dry tested extracts were dissolved in DMSO to obtain stock solutions (5000 µg/mL). These solutions were used to produce final concentrations ranging from 1-500 µg/mL of examined substances. Reagent mixtures, were prepared as follows:

The test solutions (0.5 mL) were prepared by combining 450 µL of aqueous solution of bovine serum albumin (0.5% w/v) and solutions of tested substances (50 µL).

The test control (0.5 mL) consisted of 450 µL of 0.5% (w/v) aqueous solution of bovine serum albumin fraction and DMSO (50 µL).

Product control solutions (0.5 mL) consisted of 450 µL of distilled water and solutions of tested substances (50 µL).
All the reaction mixtures were incubated at 25°C for 20 min and then heated in water bath to 70°C for 5 minutes to denature proteins. After cooling the samples, the turbidity was measured spectrophotometrically at 660 nm (Multi-Detection Microplate Reader Synergy™HT – BioTek) The experiment was performed in triplicate. The inhibition of albumin denaturation was expressed as percent of inhibition of protein denaturation, relative to the control, which represents 100% of protein denaturation and was calculated by using the following formula:

\[
\% \text{ inhibition} = 100 - \frac{A_{TS} - A_{PC}}{A_{TC}} \times 100
\]

whereas:
- \(A_{TS}\) - absorbance of the test solution
- \(A_{PC}\) - absorbance of the product control solution
- \(A_{TC}\) - absorbance of the test control

The results were compared with diclofenac sodium, a standard anti-inflammatory drug used for arthritis pain (Table S5).

6 Anti-hyaluronidase assay

The ability of the extracts to inhibit Hyal was determined by the modified, spectrophotometric method of Yus et al. (2012). The activity was assayed on the basis of precipitation of undigested hyaluronic acid (HA) with albumin. The extract concentration was 1.0 mg/mL in 10% water ethanol solution. 50 µL of enzyme (30 U/mL of acetate buffer pH 4.5), 11 µL of plant sample, 50 µL of sodium phosphate buffer (50 mM, pH 7.0; with 77 mM NaCl and 1 mg/mL of albumin) were incubated at 37 °C for 10 min. Next, 50 µL of HA (0.3 mg/mL of acetate buffer pH 4.5) was added and incubated at 37 °C for 45 min. The undigested HA was precipitated with 1 mL acid albumin solution made up 0.1% bovine serum albumin in 24 mM sodium acetate and 85 mM acetic acid. The mixture was kept at room temperature for 10 min., the absorbance of the reaction mixture was measured at 600 nm using the microplate reader (Bio-Tek, USA). Naringenin was used as positive control. All assays were performed in triplicate. The percentage of inhibition was calculated as

\[
\% \text{ inhibition} = \frac{A_B - A_E}{A_S - A_E} \times 100\%
\]
whereas: \( A_B \) - absorbance of the enzyme+substrate+extract sample
\( A_E \) - absorbance of the enzyme+substrate sample
\( A_S \) - absorbance of the enzyme+extract sample

7 Cytotoxicity testing
Human malignant melanoma cell lines: HTB140, A375, WM 793 and also human prostate cancer cell lines of low metastatic potential DU-145 and highly metastatic PC-3 were used in the experiment. Cells were grown at 37°C in a 5% CO₂ atmosphere, with relative humidity, using as culture medium DMEM/F12 (WM793, PC3), DMEM low glucose (DU145) or DMEM high glucose (A375, HTB140), supplemented with 10% fetal bovine serum (FBS) and antibiotics. Before the experiment, cells were seeded onto 24-well plates (1.5 x 10⁴ cells/well) for 24 hours. Then the culture medium was replaced with the same medium containing different concentration of the tested substances from 10 to 100 µg/ml. The controls were incubated in the culture medium (to achieve spontaneous LDH release) or with the addition of Triton X100 (for maximum LDH release after cell lysis). The cells were incubated for 24 hours. LDH release was measured by using the assay kit provided by Clontech. Briefly, after the incubation with tested substances, the supernatant from each well were transferred to a new 24-well plate, and a proper quantity of a reagent mixture was added. Then the mixture was incubated in room temperature for 30 minutes and the absorbance was measured at 490 nm (the reference wavelength 600 nm) with Biotek Synergy microplate reader. Cytotoxicity of the samples was measured as follows:

\[
\% \text{ cytotoxicity} = \left( \frac{A_{\text{sample}} - A_{\text{spont}}}{A_{\text{max}} - A_{\text{spont}}} \right) \times 100
\]

where \( A_{\text{sample}} \) is the absorbance value for the cells treated with the tested substances, \( A_{\text{spont}} \) is the value for the spontaneous LDH release and \( A_{\text{max}} \) the value in lysed cells in the presence of Triton X100. Results are presented in Table S6.

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**Table S1.** LC-ESI-MS/MS identification and concentrations of phenolic acids (µg/g DW) in leaves and stems of *Chenopodium hybridum* \(^1\)

| compound name       | \(T_r\) (min.) | \([M-H]\) | \([M-H]\) products | conc. in leaves | conc. in stems |
|---------------------|----------------|-----------|---------------------|----------------|---------------|
| gentisic acid       | 2.70           | 152.8     | 107.9 81            | 1.65 ± 0.03    | ND            |
| 4-OH-benzoic acid   | 3.26           | 136.8     | 92.9               | 1.86 ± 0.28    | 0.92 ± 0.10   |
| vanillic acid       | 4.49           | 166.8     | 107.9 123          | 1.21 ± 0.03    | 1.34 ± 0.14   |
| caffeic acid        | 4.65           | 178.7     | 134.9 88.9         | 0.59 ± 0.30    | ND            |
| syringic acid       | 5.26           | 196.9     | 181.9 122.8        | 2.31 ± 0.19    | 2.13 ± 0.19   |
| \(p\)-coumaric acid | 5.60           | 162.7     | 119 93             | 0.39 ± 0.07    | 0.11 ± 0.01   |
| ferulic acid        | 5.77           | 192.8     | 177.9 133.9        | 0.51 ± 0.06    | 0.15 ± 0.02   |
| salicylic acid      | 5.80           | 136.8     | 93 75              | 0.17 ± 0.02    | 0.20 ± 0.04   |

\(^1\)values are mean ± SD, n=6 (six independent extractions), injections in triplicate. ND = not detected; BQL = concentration below LOQ; gallic, protocatechuic, veratric, synapic, 3-OH-cinnamic, and rosmarinic acid were not detected in any sample.
Table S2 LC-ESI-MS/MS identification and concentrations of flavonoids (µg/g DW) in leaves and stems of *Chenopodium hybridum* ¹

| Compound Name                  | T<sub>r</sub> (min.) | [M-H]  | [M-H] Products | Conc. in Leaves | Conc. in Stems |
|--------------------------------|----------------------|--------|----------------|-----------------|----------------|
| Rutin                          | 8.10                 | 608.7  | 299.6, 270.9   | 2.80 ± 0.39     | 1.95 ± 0.36    |
| Isoquercetin                   | 9.52                 | 462.7  | 299.7, 270.7   | BQL             | ND             |
| Kaempferol 3-O-rutinoside      | 9.90                 | 592.7  | 284.8, 226.7   | 2.91 ± 0.97     | 1.80 ± 0.43    |
| Astragaline                    | 11.90                | 446.7  | 254.8, 226.8   | BQL             | BQL            |
| Apigenin 7-O-glucoside         | 12.70                | 430.7  | 267.7, 116.9   | BQL             | BQL            |

¹Values are mean ± SD, n=6 (six independent extractions), injections in triplicate. ND = not detected; BQL = concentration below LOQ; luteolin 7-O-glucoside, luteolin 3,7-diglucoside, luteolin 4’-O-glucoside, hyperoside, naringin, naringenin 7-O-glucoside, and quercitrin were not detected in any sample.
Table S3. Regression equations (calibration curves), linear ranges, LODs, LOQs for quantitative LC-MS/MS analysis of flavonoids

| Analyte                  | Calibration curve\(^a\)          | \(r^2\)  | LOD [ng mL\(^{-1}\)] | LOQ [ng mL\(^{-1}\)] | Linearity range [ng mL\(^{-1}\)] |
|--------------------------|----------------------------------|----------|-----------------------|-----------------------|-----------------------------------|
| Luteolin 3,7-diglucoside| \(y = 656 x + 1.85e+03\)        | 0.9999   | 0.02                  | 0.05                  | 0.5 - 2500                        |
| Rutin                    | \(y = 245x - 3.09e+02\)         | 0.9999   | 2.5                   | 5                     | 5 - 5000                          |
| Hyperoside               | \(y = 295 x + 6.52e+03\)        | 0.9988   | 10                    | 20                    | 20 - 2500                         |
| Luteolin 7-glucoside     | \(y = 701 x + 6.65e+03\)        | 0.9998   | 2.5                   | 5                     | 5 - 2500                          |
| Isoquercetin             | \(y = 308 x + 5.35e+03\)        | 0.9993   | 10                    | 20                    | 20 - 2500                         |
| Kaeempferol 3-rutinoside | \(y = 266 x + 1.27e+02\)        | 0.9999   | 5                     | 10                    | 10 - 5000                         |
| Naringin                 | \(y = 730 x - 1.92e+02\)        | 0.9999   | 2                     | 5                     | 5 - 2500                          |
| Astragalin               | \(y = 385 x + 1.58e+03\)        | 0.9999   | 3                     | 7.5                   | 10 - 2500                         |
| Quercetin                | \(y = 453 x + 5.05e+03\)        | 0.9998   | 5                     | 10                    | 10 - 5000                         |
| Apigenin 7-glucoside     | \(y = 902 x + 4.77e+03\)        | 0.9996   | 2                     | 4                     | 5 - 1000                          |
| Luteolin 4-glucoside     | \(y = 1320 x + 2.0e+04\)        | 0.9996   | 1                     | 2.5                   | 8 - 2500                          |
| Naringenin 7-glucoside   | \(y = 402 x + 7.72e+03\)        | 0.9996   | 0.01                  | 0.04                  | 0.1 - 1000                        |

\(^a\)in the calibration curve \(y = mx + b\), \(x\) refers to the concentration of standards, \(y\) the peak area

\(r^2\) is the correlation coefficient; LOD limit of detection; LOQ limit of quantification
Table S4. Regression equations (calibration curves), linear ranges, LODs, LOQs for quantitative LC-MS/MS analysis of phenolic acids

| Analyte              | Calibration curve<sup>a</sup>        | <sup>r</sup><sup>2</sup> | LOQ [ng μl<sup>-1</sup>] | LOD [ng μl<sup>-1</sup>] | Linearity range [ng μl<sup>-1</sup>] |
|----------------------|--------------------------------------|-------------------------|--------------------------|--------------------------|--------------------------------------|
| Gallic acid          | y = 195 x - 6.02e+03                  | 0.9994                  | 0.10                     | 0.05                     | 0.10 – 10.00                        |
| Protocatechuic acid  | y = 82.7 x + 6.08e+03                 | 0.9991                  | 0.02                     | 0.01                     | 0.025 – 3.13                        |
| Gentisic acid        | y = 321 x + 2.16e+04                  | 0.9993                  | 0.015                    | 0.008                    | 0.025 – 25.00                       |
| 4-OH-benzoic acid    | y = 630 x + 1.02e+04                  | 0.9971                  | 0.10                     | 0.05                     | 0.10 – 2.50                         |
| Vanillic acid        | y = 24.4 x -1.14e+03                  | 0.9999                  | 0.20                     | 0.10                     | 0.2 - 50                            |
| Caffeic acid         | y = 999 x + 2.58e+05                  | 0.9972                  | 0.08                     | 0.04                     | 0.08 - 1.25                         |
| Syringic acid        | y = 39.4 x -1.35e+04                  | 0.9997                  | 0.10                     | 0.05                     | 0.1 – 50.00                         |
| p-Coumaric acid      | y = 648 x + 1.5e+04                   | 0.9971                  | 0.061                    | 0.018                    | 0.10 – 10.20                        |
| Ferulic acid         | y = 229 x + 1.82e+03                  | 0.9997                  | 0.025                    | 0.01                     | 0.025 – 5.00                        |
| Salicylic acid       | y = 3.29e+003 x + 1.38e+04           | 0.9986                  | 0.02                     | 0.01                     | 0.02 - 0.50                         |
| Veratric acid        | y = 24.8 x + 7.99e+03                 | 0.9977                  | 0.70                     | 0.40                     | 0.50 – 25.00                        |
| Synaptic acid        | y = 92.2 x -4.17e+03                  | 0.9987                  | 0.025                    | 0.007                    | 0.025 – 5.00                        |
| 3-OH-cinnamic acid   | y = 223 x + 9.07e+03                  | 0.9994                  | 0.05                     | 0.02                     | 0.05 – 2.50                         |
| Rosmarinic acid      | y = 265 x -8.93e+04                   | 0.9985                  | 0.01                     | 0.005                    | 0.025 – 25.00                       |

<sup>a</sup>in the calibration curve <i>y = mx + b</i>, <i>x</i> refers to the concentration of standards, <i>y</i> the peak area

<sup>r</sup><sup>2</sup> is the correlation coefficient; LOD limit of detection; LOQ limit of quantification
Table S5. Anti-denaturation activity of extracts from *Chenopodium hybridum*.

| Final concentration (µg/mL) | Inhibition of protein denaturation (%) | methanolic extract from | methanolic extract from | diclofenac sodium |
|----------------------------|---------------------------------------|-------------------------|-------------------------|-------------------|
|                            |                                       | leaves                  | stems                   |                   |
| 500                        |                                       | -1911.35                | -448.50                 | 99.81             |
| 400                        |                                       | -1913.48                | -451.50                 | 99.44             |
| 300                        |                                       | -1883.69                | -468.36                 | 99.26             |
| 200                        |                                       | -1799.29                | -460.74                 | 98.51             |
| 100                        |                                       | -1728.37                | -429.79                 | 86.83             |
| 50                         |                                       | -1221.99                | -166.05                 | 61.60             |
| 20                         |                                       | -202.13                 | -45.97                  | 32.47             |
| 10                         |                                       | -63.12                  | -28.41                  | 20.04             |
| 5                          |                                       | -14.18                  | -4.85                   | 14.10             |
Table S6. Cytotoxic activity of extracts from leaves and stem of *Chenopodium hybridum*.

| concentration [μg/mL] | % dead cells | DU145 | PC3 | HTB140 | A375 | WM793 |
|-----------------------|--------------|-------|-----|--------|------|-------|
|                       |              | leaf  | stem| leaf   | stem | leaf  | stem |
| 10                    | 8.42±0.82    | na    | na  | na     | na   | 1.03±0.01 | 1.45±0.01 | na |
| 20                    | 26.04±0.78   | na    | na  | na     | na   | 4.21±0.01 | 1.87±0.01 | na |
| 30                    | 63.81±1.66   | na    | na  | na     | na   | 11.26±0.05 | 6.22±0.01 | na |
| 50                    | 89.23±2.54   | na    | na  | na     | na   | 15.30±0.11 | 16.24±0.80 | na |
| 100                   | 98.28±1.13   | na    | na  | na     | na   | 17.01±0.08 | 78.11±2.05 | na |

*na* - not active; leaf = extract from the leaves; stem = extract from the stem; DU145 and PC3 are human prostate cancer cell lines; HTB140, A375 and WM793 are human melanoma cell lines.