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Influence of different wild-garlic (*Allium ursinum*) extracts on the gastrointestinal system: spasmolytic, antimicrobial and antioxidant properties

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Keywords
Allium ursinum; enteropathogenic strains; qNMR of alk(en)yl cysteine sulphoxides; rat ileum; spasmolytic activity

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Abstract

Objectives As there are no previous studies of the European wild-garlic (*Allium ursinum*) effects on the gastrointestinal system, despite its traditional applications in gastrointestinal disorders' treatment and regular use in the human diet, we have quantified and compared spasmolytic, antimicrobial and antioxidant activities of its different leaf extracts.

Methods Wild-garlic extracts were tested for spasmolytic activity on isolated rat ileum, antimicrobial activity on selected Gram-positive and Gram-negative bacteria and fungi by microdilution method and antioxidant capacity by DPPH radical-scavenging assay.

Key findings Wild-garlic extracts were found to decrease ileal basal tone. As the relaxation of K^+-induced contractions was similar to one caused by papaverin, the observed spasmolytic effect was most likely mediated through Ca^{2+}-channel inhibition. Ethanolic extract (with the highest phenolic and high alk(en)yl cysteine sulphoxides' levels) produced the strongest spasmolytic activity. In case of acetylcholine-induced contractions, only hydromethanolic extract showed no statistical difference in comparison with positive control. All samples exhibited certain antioxidant potential and strong antimicrobial activity against tested enteropathogenic strains (*Salmonella enteritidis* was the most sensitive, followed by *Escherichia coli*, *Proteus mirabilis* and *Enterococcus faecalis*).

Conclusion Besides other already established health-promoting effects, wild garlic could be useful in treatment of mild gastrointestinal disturbances.

Introduction

Allium ursinum L. (Liliaceae), European wild garlic (wood garlic, ramsons or bear's garlic) is a perennial plant species widespread through Europe and Asia, but absent from different areas in Russia, and rare in the Mediterranean region.[1] *Allium ursinum* has its own, unique aroma and fresh leaves or a dried herb are used in local cuisines of Europe.[2] It has been harvested for centuries for food and also as a natural remedy for cardiovascular, respiratory and gastrointestinal (GI) disorders, and externally for skin diseases.[3,4] Wild garlic is believed to have most of the *Allium sativum* benefits, although it is weaker in action and consequently has to be administered in higher doses.[3] Organosulphur compounds are the secondary metabolites usually related to health benefits claimed for *Allium* species. The leaves of wild garlic are rich in alliins (in particular methylalliin, i.e. methiin, and allylalliin, i.e. alliin) and consequently other sulphur-containing molecules, for example allyl methyl disulphide and allicin; other volatile compounds, saponins, lecithin, bound and free phenolic and

polyphenolic compounds (predominantly flavonoids) are also reported.[2,3,5–7]

It has been shown that *A. ursinum* possesses antiplatelet,[8] cardioprotective and blood pressure-lowering activity,[9] antimicrobial,[6,10,11] anti-inflammatory[12] and antioxidant properties.[5–7,13] Xu *et al.*[14] demonstrated that a wild-garlic extract (extraction solvent: methanol acidified with 1% acetic acid) afterwards dissolved in water possesses antitumor effects towards human AGS gastric cancer cells.

As there are no previous studies of wild-garlic effects on GI system, our research began with an investigation of the traditional use of wild garlic for GI disorders and resulted in the determination of its effect on isolated rat ileum contractility. Antimicrobial activity of different wild-garlic extracts against enteropathogenic bacterial and fungal strains, alongside *in vitro* radical-scavenging potentials, were the second objective of the study. Additionally, as it is well documented that the extraction procedure significantly influences the type of bioactive compounds extracted,[15] we evaluated five different extracts obtained by the same extraction procedure using five types of solvents. Total phenols, tannins and flavonoids quantification was also performed and followed by HPLC analysis of phenolic compounds, as besides well-documented sulphuric active compounds in *Allium* species, these compounds should not be neglected in its health-promoting activities.[16] The content of six different alk(en)yl cysteine sulphoxides (alliin, *allo*-alliin, *trans*-isoalliin, *trans-allo*-isoalliin, methiin and *allo*-methiin) was determined in the five different extracts of *A. ursinum* leaves by a quantitative ^{1}H nuclear magnetic resonance methodology (qNMR) developed for this occasion.

Materials and Methods

All reagents and solvents used in this investigation were of analytical grade or better and were obtained from Sigma-Aldrich (St. Louis, MO, USA) or Carl Roth (Karlsruhe, Germany). As all experimental procedures were carried out in quadruplicate, results are presented as means values ± standard deviation.

Plant material and plant extracts preparation

Leaves of *A. ursinum* subsp. *ucrainicum* Kleopow & Oxner, wild-growing, were collected in the blossoming phase on Kamenica hill in the vicinity of the city of Niš, Serbia (Pavlović D. and Veljković M. collected plant material on April 2016). Taxonomic identification was performed by Professor B. Zlatković, and voucher specimens were deposited in the Herbarium Moesiacum at the University of Niš (HMN No. 11983). Dry plant material was reduced to a fine powder and extracted with ethanol (70%, v/v) = A1, ethanol (96%, v/v) = A2, distilled water = A3, methanol (80%, v/v) = A4 or absolute methanol = A5 by percolation, as described in the European Pharmacopoeia 7.0,[17] in order to obtain dry extracts (A1, A2, A3, A4 and A5) after evaporation of the solvents at room temperature.

Chemical profiling

The content of total flavonoids was determined using an aluminium chloride assay,[18] and the results were expressed as mg rutin (Ru)/g of dry extract after quantification on the basis of a standard curve with rutin.

The total phenolic and tannin contents of the extracts were estimated using the Folin–Ciocalteu colorimetric procedure.[19] Quantification was performed using a standard curve with gallic acid (concentration span 0.1–1.0 mg/ml). The results were expressed as gallic acid equivalents (GE) per gram of sample.

HPLC analysis of phenolic compounds was performed using the Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump G1312A, a Zorbax Eclipse XDB-C18 column (4.6 × 250 mm with 5 μm particle size) and a photodiode array (DAD) detector G1315B. For HPLC analysis, dry extracts were dissolved in methanol (10 mg/ml). Samples were gradiently eluted with a two phase system, phase A = water/phosphoric acid (99.97 : 0.03, v/v), pH 2.75, and phase B = 10% A in acetonitrile, flow rate of 0.8 ml/min, at 25 °C. Gradient profile was 0 min 90% A, 10% B; 5–15 min 75% A, 25% B; 20 min 70% A, 30% B; 25 min 50% A, 50% B; 30 min 30% A, 70% B and 35 min 90% A, 10% B. Injection volume was 50 μl, temperature 25 °C and pressure 80 bar. Chromatograms of the extracts and standards (1 mg/ml in acetonitrile/water 1 : 1, v/v) were recorded under the same conditions. Identification of phenolic compounds in the extracts was carried out by means of comparison of the retention times of the standards with those observed in the chromatograms of the extracts. They were also characterized by their UV spectra using the photodiode array detector and by line spectral comparisons to the standards.[20]

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III spectrometer (Bruker, Germany) operating at 400 and 100.6 MHz, respectively. Two-dimensional gradient experiments (^{1}H–^{1}H COSY, HSQC, HMBC, ROESY and NOESY) and two multipulse 1D (DEPT-90 and DEPT-135) were run on the same instrument with the usual (built-in) pulse sequences. All NMR spectra were measured at 25 °C in DMSO-d_6 or a mixture of DMSO-d_6 and D_2O (8 : 1, v/v; used for qNMR, see below) with tetramethylsilane (TMS) as internal standard.

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Quantitative nuclear magnetic resonance

Quantitative NMR experiments were performed according to a procedure described in Radulović *et al.*[21] Samples of the corresponding extracts were weighted, dissolved in a solution (a mixture of DMSO-d_6 and D_2O, 8 : 1, v/v, was used to ensure a complete solubility of both the extract constituents and the standard salt, formate, used) containing a known amount of sodium formate which served as an internal standard (no changes to the appearance of the spectra were noted due to the presence of the standard compound). ^{1}H NMR spectra with ^{13}C decoupling and a large data set (10 points per Hz digital resolution) were recorded. A satisfying signal to noise ratio was obtained for all recordings. Parameters were as follows: number of points in the time domain = 32k, spectral width = 10 ppm, O1 = 6.0 ppm, p1 = 45° ^{1}H transmitter pulse, acquisition time = 5 s and number of scans = 1024. After zero-filling and phase and baseline corrections, integration of the following signals was performed: 8.45 ppm (singlet) for the formate; 6.63 ppm (dq, J = 15.2, 1.5 Hz, proton 1′ of the 1-propenyl group) for isomer I of *trans*-isoalliin, 6.57 ppm (dq, J = 15.1, 1.6 Hz, proton 1′ of the 1-propenyl group) for isomer II of *trans*-isoalliin, multiplet at 5.85–5.98 ppm (protons 2′ of the allyl group) for isomers I and II of alliin, 2.67 ppm (singlet, methyl group) for isomer I of methiin, and 2.60 ppm (singlet, methyl group) for isomer II of methiin. The multiplet corresponding to overlapped signals of alliin isomers was further analysed (spectral simulation available in MestReNova 6.0, Mestrelab Research S.L.) to extract the specific ratio of the two isomers. This also allowed to determine the exact values of the chemical shifts and coupling constants for the isomers (isomer I: 5.9267 ppm, dddd, J = 17.3, 10.5, 7.9, 6.7 Hz, and isomer II: 5.9069 ppm, dddd, J = 17.3, 10.7, 6.7, 6.7 Hz). Assignment of signals used in qNMR experiments was performed in two ways: the signals were chosen based on their correspondence with literature values,[22–25] and these were verified by a careful inspection of their correlations in 2D spectra recorded in DMSO-d_6. The ratio of the signal integrals (analytes to standard) was used to calculate the amount of the six *S*-alk(en)yl cysteine sulphoxides in the extract samples. The results are expressed as the weight in mg per g of the extract. The experiments were repeated at least five times per sample.

Determination of spasmolytic activity

Adult male Wistar rats (200–250 g) used in this study were housed under standard laboratory conditions in the Animal Research Centre of the Medical Faculty, University of Niš. The animals received a standard rodent diet and had free access to food and water. All experimental procedures with animals were in compliance with the rules of the European Union Normative (86/609/EEC) and approved by the Animal Ethics Board of the Medical Faculty in Niš (No. 01-6481-8).

Tissue segments' isolation and ileal spasmolytic activity estimation were performed according to previously described methods.[26] Rats were fasted overnight and, after cervical dislocation, the terminal ileum was dissected out and stored in Tyrode's solution (136.89 mM NaCl, 2.68 mM KCl, 1.05 mM $MgCl_2$, 1.80 mM $CaCl_2$, 0.42 mM NaH_2PO_4, 11.09 mM $NaHCO_3$ and 5.55 mM glucose; pH 7.4) where the mesenteries were removed. Each 2-cm-long segment was suspended in a 10-ml tissue bath containing Tyrode's solution maintained at 37 °C and constantly aerated with 5% (v/v) carbon dioxide in oxygen. One end of the isolated ileum preparation was attached to the bath bottom, while the other one to an isotonic force transducer (TSZ-04-E, Experimetria Ltd., Budapest, Hungary). Intestinal responses were recorded and analysed with a SPEL Advanced ISOSYS Data Acquisition System (Experimetria Ltd.). The segments were suspended under 1 g tensions and allowed to equilibrate for 30 min before the experiment. After each assay, the tissue was washed with a fresh portion of Tyrode's solution and equilibrated for around 10 min.

After the stabilization period, individual tested extracts (0.01–3 mg/ml) were alone added to the organ bath and the effects on the ileum basal resting tone was measured, and the results were presented as a percentage change of the basal tone compared with baseline values (% of inhibition of ileal contractility). In the second set of experiments, the effects of the extracts on acetylcholine (Ach)-induced contractions of the isolated rat ileum were investigated. In the presence of wild-garlic extracts (0.3–3 mg/ml) in the organ bath, increasing concentrations of Ach were added cumulatively. Papaverine (1–100 μM) was used as a control substance in the spontaneous contractions assay. The contraction inhibition was expressed as the percentage change of the control response mediated by Ach alone. Atropine was used as the positive control in the Ach-induced contractions assay.

To test the possible Ca^{2+}-channel blocking effects of the samples, a solution containing K^+ (80 mM) was added to the bath with rat ileum in order to produce a sustained contraction. Wild-garlic extracts (0.01–3 mg/ml) were cumulatively added to the tissue bath. The relaxation of ileum precontracted with K^+ was expressed as the percentage change in the control response produced by 80 mM K^+.

Mean and SD values were calculated for each group of results ($n \geq 4$ for each set of experiments), and the comparison between the groups was performed using Kruskal–Wallis test, where the significance of differences

between the means was determined by Dunn's *post hoc* test. A probability value of $P \leq 0.05$ was deemed of significance.

Determination of antioxidant capacity

For a preliminary antioxidant screening of wild-garlic extracts, a stable 1,1-diphenyl-2-picrylhydrazyl free radical ($DPPH^{\cdot}$) was used according to the method described by Cuendet *et al.*[27] with our slight modifications (absorbance was read on an ELISA microplate reader at 540 nm). Results were expressed as IC_{50} values in mg/ml (concentration of sample required for scavenging 50% of the free radical, calculated from dose–response curves). BHT and BHA were used as reference compounds.

Assessment of antimicrobial activity

For the antimicrobial bioassays, one fungal and seven bacterial enteropathogenic strains (all from the American Type Culture Collection – ATCC) were used: *Candida albicans* (ATCC 10031), *Staphylococcus aureus* (ATCC 6538), *Enterococcus faecalis* (ATCC 19433), *Escherichia coli* (ATCC 9863), *Pseudomonas aeruginosa* (ATCC 9027), *Enterobacter aerogenes* (ATCC 13048), *Proteus mirabilis* (ATCC12453) and *Salmonella enteritidis* (ATCC 13076). MIC (minimum inhibitory concentration) and MBC/MFC (minimum bactericidal/fungicidal concentration) were determined by a microwell dilution method according to the recommendations of the National Committee for Clinical Laboratory Standards.[28] Ciprofloxacin and nystatin were used as positive controls for bacterial and fungal strains, respectively, whereas 10% (v/v) DMSO solution, the solvent used for the dissolution of the dry extracts, was used as the negative control.

Results

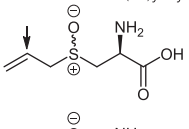
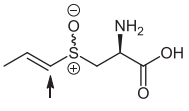
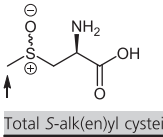
Plant extraction and active constituents' determination

The extraction yields for wild-garlic extracts A1, A2, A3, A4 and A5 were 40.12%, 22.00%, 43.06%, 33.50% and 26.60%, (w/w), respectively. Extraction with water led to the highest extraction yield, followed by the hydro-alcoholic solvents (ethanol, 70%, v/v, and methanol, 80%, v/v).

The total phenolic, tannin and flavonoid contents alongside with IC_{50} values in the applied antioxidant assay are presented in Table 1. The total phenolic content varied from 24.32 to 43.97 mg of GE/g of extract and the total flavonoid content from 7.46 to 38.56 mg of Ru/g of extract. Methanol and aqueous methanol extracted similar amounts of total phenolics and tannins, while ethanol 96% (v/v), followed by absolute methanol, were very efficient in flavonoids extraction.

According to RP-HPLC coupled with DAD detection, flavonoids 3,7-kempferol-diglucoside and kempferol 3-glucoside, and phenolic acid ferulic acid (in trace amounts)

Table 1 Phenolic contents and radical-scavenging IC_{50} values, as well as the content of *S*-alk(en)yl cysteine sulphoxides in the extracts of wild garlic (*Allium ursinum*)

		A1	A2	A3	A4	A5
Total polyphenols (mg GA/g)		30.0 ± 0.8	44.0 ± 0.6	29.8 ± 0.7	24.3 ± 0.6	24.2 ± 0.3
Tannins (mg GA/g)		11.5 ± 0.8	20.3 ± 0.7	16.3 ± 0.9	7.6 ± 0.7	6.1 ± 0.4
Flavonoids (mg Ru/g)		15.5 ± 0.1	38.6 ± 0.7	7.5 ± 0.3	11.0 ± 0.2	28.0 ± 0.1
Radical-scavenging activity, IC_{50} (mg/ml)		1.03 ± 0.03	0.85 ± 0.01	1.40 ± 0.04	1.1 ± 0.1	0.63 ± 0.01
Content of *S*-alk(en)yl cysteines (mg/g)						
	alliin (Isomer I)	10.3 ± 0.1	26.6 ± 0.4	15.5 ± 0.4	27.6 ± 0.8	27.4 ± 0.3
	allo-alliin (Isomer II)	20.1 ± 0.2	29.2 ± 0.2	28.8 ± 0.2	24.7 ± 0.6	22.1 ± 0.5
	Total alliins	30.4 ± 0.3	55.8 ± 0.6	44.3 ± 0.6	52.3 ± 1.4	49.5 ± 0.8
	*trans*-isoalliin (Isomer I)	2.6 ± 0.8	15.8 ± 0.2	11.4 ± 0.7	12.5 ± 0.2	10.3 ± 0.8
	trans-allo-isoalliin (Isomer II)	1.0 ± 0.1	9.5 ± 0.3	1.1 ± 0.1	9.1 ± 0.3	6.0 ± 0.5
	Total isoalliins	3.6 ± 0.9	25.3 ± 0.5	12.5 ± 0.8	21.6 ± 0.5	16.3 ± 1.3
	methiin (Isomer I)	22.4 ± 0.3	13.7 ± 0.3	13.0 ± 0.5	31.0 ± 0.9	23.0 ± 0.1
	allo-methiin (Isomer II)	29.0 ± 0.2	23.6 ± 0.1	23.2 ± 0.2	29.1 ± 0.2	32.0 ± 0.4
	Total methiins	51.4 ± 0.5	37.3 ± 0.4	36.2 ± 0.7	60.1 ± 1.1	55.0 ± 0.5
Total *S*-alk(en)yl cysteines		85.4 ± 1.7	118.4 ± 1.5	93.0 ± 2.1	134.0 ± 3.0	120.8 ± 2.6

Allium ursinum extracts: A1 – 70% ethanol (v/v); A2 – 96% ethanol (v/v); A3 – distilled water; A4 – aqueous methanol 80% (v/v); A5 – absolute methanol. The arrows point to the protons whose ^1H signals that were used for the quantification (qNMR) of the specific *S*-alk(en)yl cysteine sulphoxide isomer.

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were present in all investigated samples (Figures S1–S5 presented in supplementary material). The most abundant phenolic compound in all extracts was 3,7-kempferol-diglucoside, with the exception of A3 (water extract of wild-garlic leaves) where kempferol 3-glucoside was the main phenolic compound. According to HPLC chromatograms of *A. ursinum* extracts, methanolic extract (A5) seems to be the most abundant in detected flavonoids and phenolic acids (Figure S5).

A quantitative NMR approach was utilized to assess the content of *S*-alk(en)yl cysteine sulphoxides in the five different extracts. The extensive analyses of the recorded 1D and 2D spectra, in two different NMR solvents, allowed a direct quantitation of six different sulphur-containing compounds (three diastereomeric pairs): alliin and *allo*-alliin, *trans*-isoalliin and *trans-allo*-isoalliin, as well as methiin and *allo*-methiin, differing in the configuration as the sulphur atom. Peak assignment was accomplished through detailed analyses of 2D NMR spectra, alongside a comparison with literature data.[21–25] Specific signals of all mentioned amino acids were clearly resolved from all other signals, except in the case of alliin and its diastereomer, which were quantified from mutually overlapped multiplets, with the aid of spectral simulation (MestReNova 6.0). Typical ^{1}H NMR spectra of A1–A5 are provided in the supplementary material (Figures S6 and S7) file, along with the appropriate expansions. The content of the six amino acids, precursors of the sulphur-containing volatiles of *Allium* species, is presented in Table 1. The total weight percentage of cysteine sulphoxides varied in the range 5.8–13.4% of the dry extracts, with a comparable high relative amounts of alliins (3.0–5.6%; dominant in *A. sativum*) and methiins (3.6–6.0%), and approximately a two times lower amount of *trans*-isoalliins (0.4–2.5%; dominant in *Allium cepa*). Interestingly, NMR signals of propiin(s) and *cis*-isoalliin(s) could not be located in the spectra of the extracts although these could be anticipated to be metabolites of *A. ursinum* based on the volatiles identified in the essential oils of *A. ursinum* samples from the same geographical area.[2] This suggests that these are either present in much lower amounts in the extracts or that the propyl and *cis*-1-propenyl groups form during the enzymatic reactions provoked by mechanical tissue damage.

The lowest total content of cysteine sulphoxides was found in the 70% (v/v) aqueous ethanol extract (A1), while the content gradually increased in the order: A3 < A2 < A5 < A4. Although 80% (v/v) methanol appeared to be the most efficient extracting agent for these amino acids, it did not extract the highest amounts of alliins and isoalliins (96%, v/v, aqueous ethanol turned out to be more efficient). In addition to the polarity of the solvents/solvent mixtures used herein, one should also consider another property of theirs, a denaturing one. Upon plant material damage, these amino acids are brought into contact with alliinases which decrease their content due to the volatilization of their products. Thus, if the solvent system is more efficient in decreasing the activity of the enzymes, one should also expect a higher relative amount of the cysteine sulphoxides. Although, one should then expect the lowest content of alk(en)yl cysteine sulphoxides in the water extract, this is only true for methiins. Thus, a combination of solvent polarity, its denaturing effect upon alliinases, and a different reactivity of the different cysteine sulphoxides, renders a solvent more or less efficient for this extraction. Although not as apparent in the case of these sulphur-containing compounds, the difference in the relative content of sucrose and anomers of glucose reveals the hydrolytic activity of enzymes in the water extract; only the water extract contained no sucrose, but instead both anomers of glucose (Figure S6, signals at 5.22 ppm (d, J = 3.7 Hz; sucrose), 4.95 ppm (d, J = 3.7 Hz; α-glucose) and 4.32 ppm (d, J = 7.8 Hz; β-glucose)). Overall, it appears that methanol, either pure or mixed with 20% water, followed by 96% ethanol are somewhat better in extracting sulphur-containing amino acids, than 70% aqueous ethanol and pure water.

Also, worth mentioning is the relative ratio of the diastereomers within the pairs, that varied in the ranges 0.89–1.95 (isomer II/isomer I of alliin), 1.37–10.4 (isomer I/isomer II of isoalliin) and 0.94–1.78 (isomer II/isomer I of methiin). It appears that isoalliin was the only cysteine sulphoxide to display an order of magnitude variation of the content of the different epimers (sulphur atom configuration). There was no easily observable correlation between the relative ratios of the epimers within the same extract as well, with the water extract being the most fluctuating one as might have been expected if alliinases show a substrate preference of a particular diastereomer within the mentioned pairs.

Effect of *Allium ursinum* extracts on isolated rat ileum contractions

The different solvent extracts of *A. ursinum* caused a concentration-dependent (0.01–3 mg/ml) statistically significant relaxation of the isolated rat ileum (Figure 1). None of the tested extracts was able to induce a 50% of relaxation (Figure 1a–1e), while papaverine, at a concentration of 0.3 mg/ml, produced ≈50% relaxation (Figure 1f). The strongest effect among the tested extracts, on ileum spontaneous contractions, was observed when 3 mg/ml of A2 (ethanolic extract) was applied (≈30%, Figure 1a).

Cumulative application of increasing Ach concentrations (15–1500 nM) produced a concentration-dependent

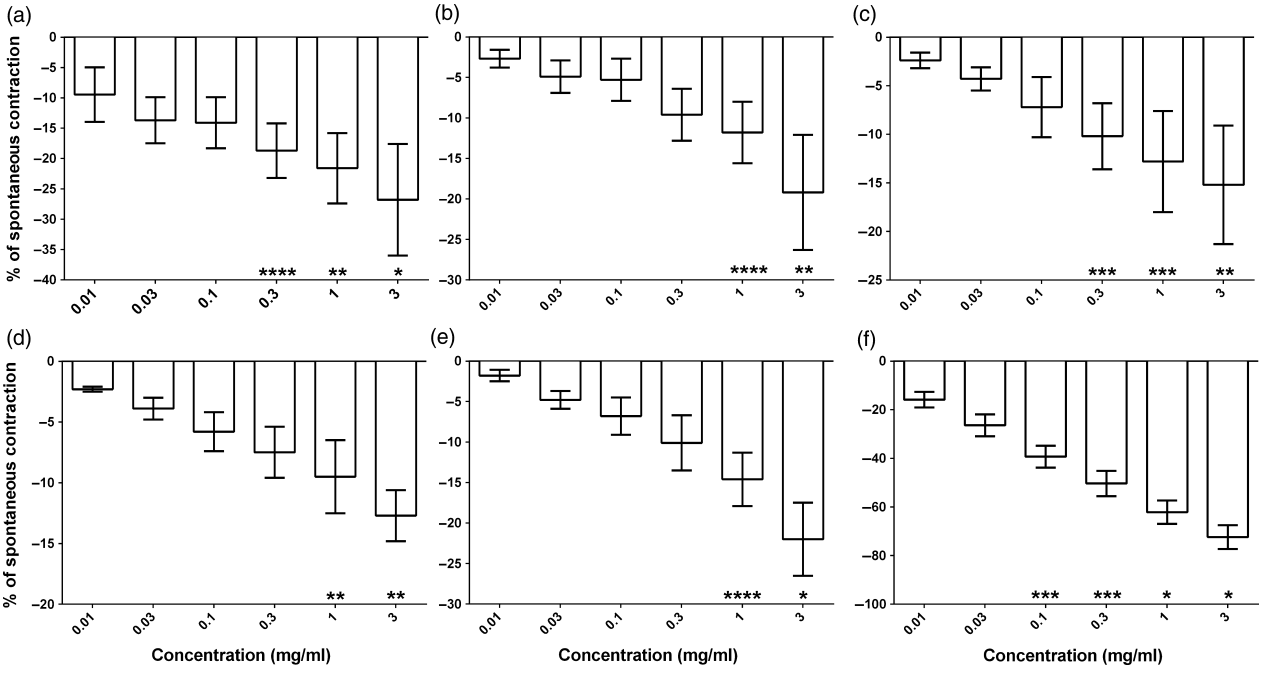

Figure 1 Effects of different concentrations of *Allium ursinum* 96% aqueous ethanol (a), 70% ethanol (b), water (c), absolute methanol (d), 80% methanol extract (e) and papaverine (f) on rat ileum spontaneous contractions. Data are presented as % change from baseline ± SD obtained from four independent experiments. Comparison was performed using Kruskal–Wallis test, followed by Dunn's *post hoc* test. **P* < 0.0001, ***P* < 0.001, ****P* < 0.01, *****P* < 0.05 vs control (baseline).

contraction of the rat ileum, which was taken as the baseline in this set of experiments. Atropine at a concentration of 140 nM produced almost a constant statistically significant ($P < 0.05$) reduction in ileum spasms produced by Ach (Figure 2). The same trend of activity, predominantly less active then atropine, was observed when both 0.1 and 0.3 mg/ml of different solvent extracts were applied (Figure 2a–2e). Only in the case when 0.3 mg/ml of A4 (80% aqueous methanolic extract) was added, the observed activity was similar to that of atropine (Figure 2e).

Potassium (80 mM)-induced contractions of the rat ileum were statistically significantly, in a concentration-dependent manner, reduced by different solvent extracts of wild garlic. However, this inhibition was only a modest one (<30%), where the water *A. ursinum* extract was the most potent one in inhibiting K^+-induced contractions (≈27%) (Figure 3a–3e). Again, papaverine, at concentration of 0.3 mg/ml, produced an almost 50% relaxation of K^+-induced contractions (Figure 3f). Both aqueous methanolic and methanolic extracts (A4 and A5) produced a similar relaxant pattern of activity at all applied concentrations (Figure 3d and 3e).

All recorded ileum relaxing effects were reversible as they disappeared after a washing with Tyrode's solution. Thus, the demonstrated spasmolytic activities are not the consequences of irreversible ileum damage.

Radical-scavenging activity

The results obtained from the radical-scavenging activity assay (IC_{50} values in mg/ml) of the tested extracts are presented in Table 1. The IC_{50} values for the free radical-scavenging assay suggest a solid antiradical potential of the tested extracts. The extracts were able to reduce the stable free radical DPPH to the yellow-coloured 1,1-diphenyl-2-picrylhydrazyl with an IC_{50} ranging from 0.63 to 1.40 mg/ml. As a lower IC_{50} value indicates a stronger antioxidant capacity, the order of antioxidant power of wild-garlic extracts is as follows: A5 > A2 > A1 > A4 > A3. As expected, the synthetic antioxidants BHT and BHA were far stronger radical scavengers than the wild-garlic extracts (IC_{50} values were: 2.82 ± 2.07 μg/ml and 2.44 ± 0.09 μg/ml, respectively).

Antimicrobial activity of wild-garlic extracts

All wild-garlic extracts demonstrated a certain antimicrobial potential against all tested enteropathogenic strains (Table 2). The MIC values were in the range from 1.56 to 25.00 mg/ml, while MBC/MFC values varied from 3.13 to 50.00 mg/ml. The most prominent effect was achieved for A2 extract (MIC/MBC = 1.56/3.13 mg/ml) in the case of *S. enteritidis*. This bacterial strain was the most sensitive of

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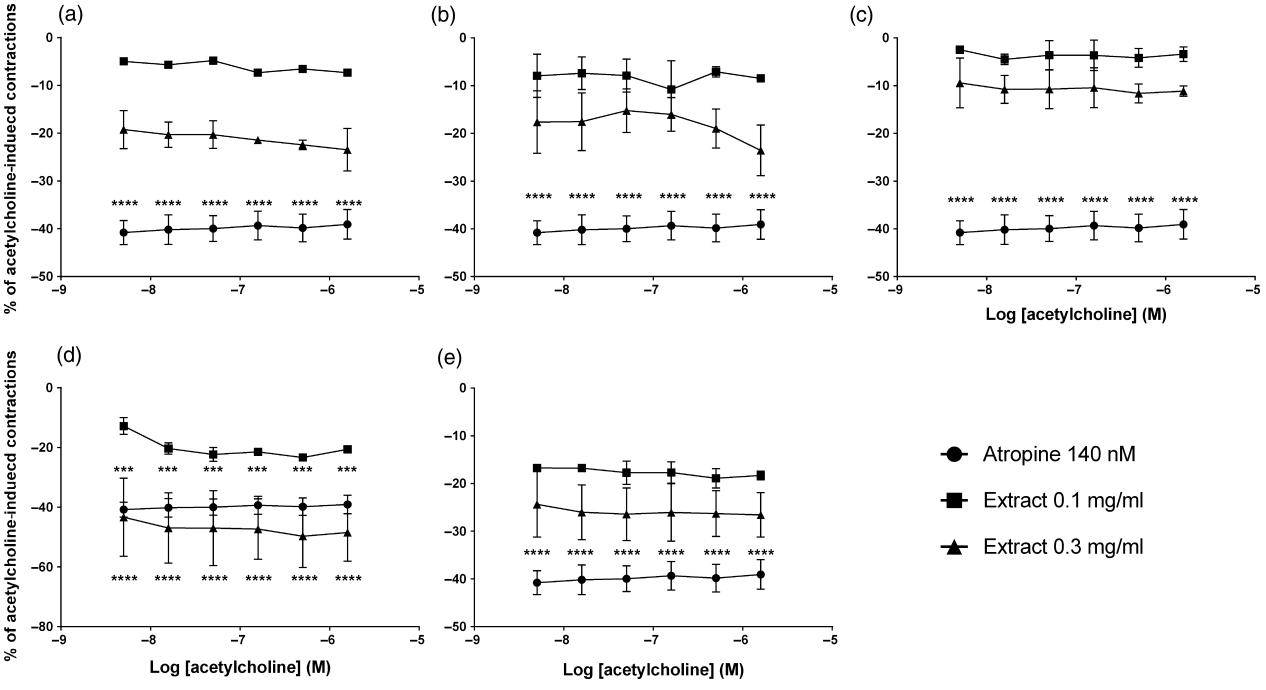

Figure 2 Effects of atropine and different concentrations (0.1 and 0.3 mg/ml) of *Allium ursinum* 96% aqueous ethanol (a), 70% ethanol (b), water (c), absolute methanol (d) and 80% methanol extract (e) on acetylcholine-induced contractions of rat ileum. Data are presented as % change from baseline ± SD obtained from four independent experiments. Comparison was performed using Kruskal–Wallis test, followed by Dunn's *post hoc* test. ***$P < 0.01$, ****$P < 0.05$ vs baseline contraction produced by Ach.

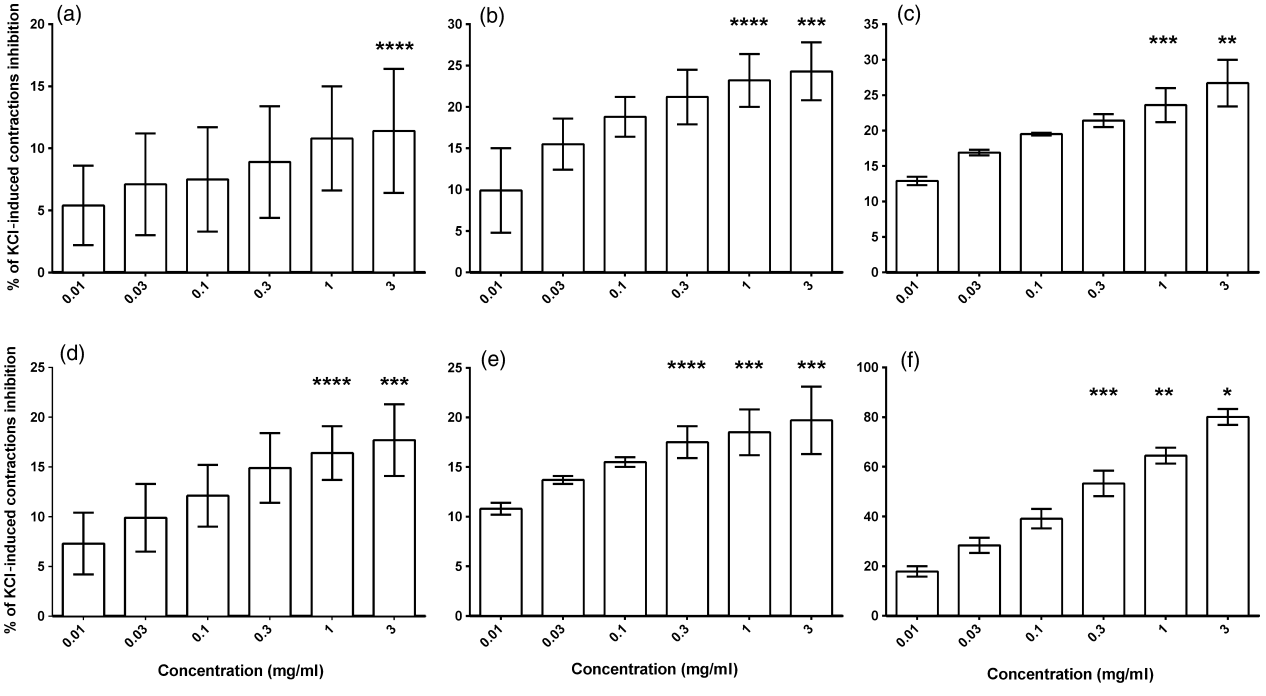

Figure 3 Effects of different concentrations of *Allium ursinum* 96% aqueous ethanol (a), 70% ethanol (b), water (c), absolute methanol (d), 80% methanol extract (e) and papaverine (f) on K^+ (80 mM)-induced contractions of rat ileum. Data are presented as % change from baseline ± SD obtained from four independent experiments. Comparison was performed using Kruskal–Wallis test, followed by Dunn's *post hoc* test. *$P < 0.0001$, **$P < 0.001$, ***$P < 0.01$, ****$P < 0.05$ vs maximal contraction induced by K^+.

Table 2 Antimicrobial activity (minimal inhibitory/bactericidal concentrations, MIC/MBC) of wild-garlic extracts (mg/ml) and commercial antibiotics (μg/ml) against enteropathogenic bacterial and fungal strains

Enteropathogenic bacterial strains	A1 MIC/MBC	A2 MIC/MBC	A3 MIC/MBC	A4 MIC/MBC	A5 MIC/MBC	Ciprofloxacin MIC/MBC
Escherichia coli ATCC 9863	6.25/6.25	1.56/6.25	6.25/25.00	12.50/12.50	3.13/6.25	2.50/2.50
Pseudomonas aeruginosa ATCC 9027	6.25/6.25	3.13/6.25	12.50/50.00	12.50/50.00	6.25/6.25	0.62/0.62
Enterobacter aerogenes ATCC 13048	6.25/12.50	6.25/6.25	12.50/50.00	12.50/12.50	6.25/12.50	0.008/0.31
Proteus mirabilis ATCC 12453	6.25/6.25	1.56/6.25	6.25/25.00	12.50/25.00	3.13/6.25	0.62/2.50
Salmonella enteritidis ATCC 13076	3.13/12.50	1.56/3.13	3.13/25.00	12.50/12.50	3.13/6.25	0.008/0.16
Staphylococcus aureus ATCC 6538	6.25/6.25	3.13/3.13	25.00/25.00	6.25/25.50	6.25/6.25	0.008/0.16
Enterococcus faecalis ATCC 19433	3.13/12.50	1.56/6.25	6.25/>100.0	25.00/50.00	3.13/6.25	0.31/0.31
Fungal strain	MIC/MFC	MIC/MFC	MIC/MFC	MIC/MFC	MIC/MFC	Nystatin MIC/MFC
Candida albicans ATCC 10031	12.50/12.50	6.25/12.50	50.00/50.00	50.00/50.00	12.50/12.50	0.008/0.016

Allium ursinum extracts: A1 – 70% ethanol (v/v); A2 – 96% ethanol (v/v); A3 – distilled water; A4 – aqueous methanol 80% (v/v); A5 – absolute methanol.

all tested strains to the applied extracts. The observed antifungal activity was less pronounced: MIC/MFC values against the only tested fungal strain (*C. albicans*) were in the range from 6.25/12.50 mg/ml (obtained for A2) to 50.00/50.00 mg/ml (obtained for A3 and A4).

The solvent used for the preparation of solutions used in this investigation (10%, v/v, aqueous DMSO) showed no activity against the tested microbial strains. As the commercial antimicrobial drugs, ciprofloxacin and nystatin exhibited obviously higher antimicrobial activity than wild-garlic extracts.

Discussion

In order to investigate the traditional use of *A. ursinum* for GI complaints, the first approach was to study the effects of different wild-garlic extracts on the spontaneously contracting isolated rat ileum. All of the tested extracts (0.03–10 mg/ml) produced a decrease in the tone of ileal spontaneous contractions in a concentration-dependent manner (Figure 1), indicating spasmolytic activity. The sample with the highest levels of total phenolics, tannin and flavonoid contents (96% ethanol extract, A2) showed the most prominent spasmolytic activity, although weaker than papaverine. Acetylcholine stimulates the movements of the ileum smooth muscle, acting predominantly *via* muscarinic receptors and increasing intracellular Ca^{2+} concentration, while K^+ activates voltage-dependent Ca^{2+}-channels to trigger this ion influx.[29] These changes in intracellular Ca^{2+} concentration regulate the contractility in gastrointestinal smooth muscles. Papaverine, a smooth muscle relaxant agent, induces relaxation through the inhibition of the voltage-dependent L-type Ca^{2+}-channels. As the relaxation of K^+-induced contractions by the tested extracts was similar to that caused by papaverine, the observed spasmolytic effect might be partially mediated through Ca^{2+}-channel inhibition.[30] According to Sedghi *et al.*,[31] *Allium ampeloprasum* (wild leek) leaf hydroalcoholic extract prevents the increase in cell Ca^{2+} concentration (causing muscle relaxation) and affects rat ileum motor activity via β-adrenergic receptors and voltage-dependent Ca^{2+}-channels. Previously, it has been shown that spasmolytic activity of different medicinal plant constituents is often mediated through a calcium-channel blockade.[32,33] Gaffen *et al.*[34] claimed that garlic constituents, different from allyl sulphide, dimethyl sulphide and diallyl disulphide, are mainly responsible for its spasmolytic effects of on rat gastric fundus. Flavonoids and tannins containing medicinal plants are efficient antidiarrhoeals and could also prevent inflammation as they coat intestinal mucosa.[35] Flavonoids were proved to relax tonic and phasic contractions of isolated guinea pig trachea and rat uterus and blood vessels.[36] To what extent the specific classes of active constituents, which are present in the tested wild-garlic extracts, contribute to the spasmolytic activity has to be evaluated in further

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studies. All recorded ileum relaxing effects were reversible as they disappeared after a washing with Tyrode's solution. Thus, the demonstrated spasmolytic activities were not the consequences of irreversible ileum damage.

Besides the impact of different wild-garlic extracts on the isolated rat ileum, the estimation of *in vitro* antioxidant potentials, alongside antimicrobial activity, was performed, as they could be employed as potential protective agents in microbial and oxidative stress-mediated GI disorders.[37] According to Štajner *et al.*,[16] *A. ursinum* possesses better antioxidant ability compared to other wild *Allium* species from the Balkan flora. Our results also point to a certain ability of wild-garlic extracts to scavenge free radicals, although far less efficient in this when compared to BHT and BHA. The antioxidant activity of *Allium* species was found to be due to a variety of sulphur-containing compounds, polyphenols, dietary fibre and microelements.[12,15] Wild garlic belongs to a methiin/alliin-type *Allium* species, and quantitative profile of sulphur-containing substances strongly depends on the plant organ, time of harvest, plant material processing and storage.[38] Radulović *et al.*[2] analysed the volatile constituents (built enzymatically after harvesting), obtained from fresh leaves of wild garlic harvested at the same locality where the samples for our investigation were collected. The most abundant organosulphur compounds (>3%) identified were allyl methyl disulphide, diallyldisulphide, allyl methyl trisulphide, allyl (*E*)-1-propenyl disulphide, dimethyl trisulphide, methyl (*E*)-1-propenyl disulphide, allyl propyl disulphide and allyl (*Z*)-1-propenyl disulphide. In our study the highest levels of total S-alk(en)yl cysteines (alliins, isoalliins and methiins) were determined in methanolic extracts although their level was also significant in A2. A2 is the ethanolic (96%, v/v) extract that had the highest levels of total polyphenols, tannins and flavonoids. In fact, extracts with the highest levels of flavonoids, A2 and A5, had the lowest IC_{50} values which denote stronger antioxidant activity. Thus, beside the well-documented cysteine sulphoxides compounds ability to scavenge free radicals, stop the reactions of lipid peroxidation and increase the antioxidant enzymes levels (including superoxide dismutase),[5–7,13] this group of secondary metabolites certainly plays an important role in antioxidant capacity of wild-garlic extracts.

According to the herein performed HPLC analysis results (Figures S1–S5), the methanolic extract (A5) seems to be the richest one in the amount of detected flavonoids and phenolic acids. This is somewhat expected result as the absolute methanol (followed by ethanol) is the most efficient solvent for phenolic compounds isolation.[39] Our chemical profiling of *A. ursinum* leaves extracts is in accordance with Sobolewska *et al.*[38] botanical, phytochemical and pharmacological overview on *A. ursinum*: the most important phenolic compounds are kaempferol derivatives and phenolic acids (including free form of ferulic acid), and wild-garlic leaves are abundant predominantly in alliins and methilalliins.

Antimicrobial activity of wild-garlic samples decreases in the following order A2 > A5 > A1 > A3 > A4. The most pronounced activity of all tested samples was found against *S. enteritidis*. Bearing in mind that the usually applied antibiotic therapy often does not affect *S. enteritidis* infections and increases the risk of antibiotic resistance development,[40] wild garlic could find its place in the prevention and treatment of salmonellosis. Our findings are in accordance with the results of Sapunjieva *et al.*[6]: wild-garlic leaf extract affects *E. coli*, *S. aureus* and *Salmonella enterica* growth. Although Bagiu *et al.*[11] showed that *A. ursinum* extracts inhibited the growth of *Candida* spp. at a concentration span from 0.5 to 4.0 mg/ml, our MIC values were somewhat higher, from 6.25 to 50.00 mg/ml, and i.e. our extracts were less potent (Table 2). The results of the antimicrobial assays could be affected by the applied extraction procedures and the active plants secondary metabolites that are produced during plant development in response to various environmental stressors.[40,41]

Antimicrobial activity of a plant extract depends on the organ of the plant used, the type of the solvent used for extraction, the extraction method and type of microorganisms tested.[10,15] Potential thiosulphinates from *Allium* extracts react with free SH groups of intracellular enzymes and affect intracellular processes and cell communication.[42] The existence of a complex mixture of bioactive compounds in plant extracts and their mutual interactions are common explanations for the overall observed antimicrobial activity (as well as for some other demonstrated pharmacological effects).[42,43] It is possible that some antimicrobial effects may lie in other plant constituents such as tannins (e.g. tannic acid), which were previously demonstrated to possess such properties.[44] Flavonoids could inhibit cytoplasmic membrane function, DNA synthesis and even protein and RNA synthesis.[42] As sample A2 exhibited the strongest antimicrobial potential, the high amounts of polyphenols, tannins and flavonoids present could be, at least partly, responsible for its impact on tested enteropathogenic microorganisms. The same sample, A2, (with the highest phenolic and high alk(en)yl cysteine sulphoxides' levels) produced the strongest spasmolytic activity and after A5 (methanolic extract) it showed the strongest antioxidant effect.

Conclusions

This is the first report that deals with the effects of *A. ursinum* leaf extracts on GI function, despite the fact

that this plant is used traditionally as a food, spice and natural remedy for GI disorders. The herein demonstrated inhibition of both spontaneous and induced ileal contractions (suggesting that the inhibition of Ca^{2+}-channels is a potential mechanism of action), antioxidant and antimicrobial activities, could contribute to the overall action of this *Allium* species on GI system. Further studies are necessary to show more clearly which substances are responsible for the observed effects. In spite of not knowing the exact constituents responsible for the detected pharmacological activities, our study reports for the first time a significant amount of data regarding the impact of wild-garlic extracts on GI system functioning. Besides the other already established health-promoting effects of wild garlic, known and harvested as a functional food, it could also be useful in mild GI disturbances treatment.

Declarations

Conflict of interest

The Authors declare that they have no conflict of interest.

Acknowledgement

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. HPLC chromatogram (DAD detection on 350 nm) of A1 (*Allium ursinum* extract with 70% ethanol, v/v): 1 – 3,7-kempferol-diglucoside, 2 – kempferol 3-glucoside, 3 – ferulic acid; # – phenolic acid, * – kempferol glycoside, ** – flavonoid.

Figure S2. HPLC chromatogram (DAD detection on 350 nm) of A2 (*Allium ursinum* extract with 96% ethanol, v/v): 1 – 3,7-kempferol-diglucoside, 2 – kempferol 3-glucoside, 3 – ferulic acid; # – phenolic acid, * – kempferol glycoside, ** – flavonoid.

Figure S3. HPLC chromatogram (DAD detection on 350 nm) of A3 (*Allium ursinum* extract with water, v/v): 1 – 3,7-kempferol-diglucoside, 2 – kempferol 3-glucoside, 3 – ferulic acid; # – phenolic acid, * – kempferol glycoside, ** – flavonoid.

Figure S4. HPLC chromatogram (DAD detection on 350 nm) of A4 (*Allium ursinum* extract with 80% methanol, v/v): 1 – 3,7-kempferol-diglucoside, 2 – kempferol 3-glucoside, 3 – ferulic acid; # – phenolic acid, * – kempferol glycoside, ** – flavonoid.

Figure S5. HPLC chromatogram (DAD detection on 350 nm) of A5 (*Allium ursinum* extract with absolute methanol): 1 – 3,7-kempferol-diglucoside, 2 – kempferol 3-glucoside, 3 – ferulic acid; # – phenolic acid, * – kempferol glycoside, ** – flavonoid.

Figure S6. Stacked typical ^{1}H NMR (at 400 MHz) spectra of samples A1–A5 recorded in a mixture of DMSO-d_6 and D_2O (8 : 1, v/v). Height of the individual spectra was adjusted to the highest analyte signal (signal of HDO and CHD_2SOCD_3 were not taken into consideration).

Figure S7. Stacked expansions (three regions: 4.30–7.95, 5.75–7.20, and 2.55–3.70) of ^{1}H NMR (at 400 MHz) spectra of samples A1–A5 recorded in a mixture of DMSO-d_6 and D_2O (8 : 1, v/v) showing regions of interest for qNMR (cf. experimental section). Height of the individual spectra was adjusted to the highest analyte signal (signal of HDO and CHD_2SOCD_3 were not taken into consideration).