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Characterization and Content of Flavonol Derivatives of *Allium ursinum* L. Plant

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ABSTRACT: The phenolic compounds were extracted from green and yellow leaves, stalks, and seeds of garlic (*Allium ursinum* L.). The extracts were analyzed by liquid chromatography−photodiode array detector−electrospray ionization−tandem mass spectrometry (LC−PDA−ESI−MS/MS). In total, 21 compounds were detected. The flavonol derivatives were identified on the basis of their ultraviolet (UV) spectra and fragmentation patterns in collision-induced dissociation experiments. On the basis of accurate MS and MS/MS data, six compounds were newly identified in bear's garlic, mainly the kaempferol derivatives. As far as the investigated parts of garlic are concerned, the kaempferol derivatives were found to be predominant in yellow leaves [2362.96 mg/100 g of dry matter (dm)], followed by green leaves (1856.31 mg/100 g of dm). Seeds contained the minimal phenolic compounds, less than stalks. The yellow leaves of *A. ursinum* possessed a much larger content of compounds acylated with *p*-coumaric acid than green leaves (1299.97 versus 855.67 mg/100 g of dm, respectively). The stalks and seeds contained much more non-acetylated than acetylated flavonoid glycosides with *p*-coumaric acid compounds (162.4 versus 62.82 mg/100 g of dm and 105.49 versus 24.18 mg/100 g of dm, respectively).

KEYWORDS: *Garlic, green and yellow leaves, stalks, seeds, LC−MS, kaempferol derivatives*

INTRODUCTION

Allium ursinum L., which is known as "wild garlic", is a wild-growing *Allium* species found in the forests of Europe. It is also called ramson bear's garlic. The herbaceous plants grow to a height of 50 cm and have white flowers. The bulbs do not exceed 6 cm in size.[1] The ramson bear's garlic formed trichotomic capsules with black seeds as fruits. Its vegetation cycle starts during the vernal period and ends at the beginning of summer. The whole vegetation period is from March to June. During this interval of time, big forest complexes expressed in tens of hectares are covered with this species. The other forest species are either sparse or completely absent in dense populations where *A. ursinum* is dominant, thus pointing to its inhibitory allelopathic effects.[2]

Because *A. ursinum* has not yet been cultivated, it did not gain any importance until several years ago when people started to look for it as a natural plant. The fresh leaves or dried herb is used in local cuisines of Europe. The leaves are edible and can be used as a salad or a spice, or they can be boiled as a vegetable. The bear's garlic is a common "wild" vegetable in Ukraine and Russia. It is sold on local markets as fresh, pickled, or salted and is becoming increasingly popular in the Czech Republic and Germany.[3] Consequently, attempts are currently undertaken to cultivate *A. ursinum*. Some studies have shown that *A. ursinum* can be a substitute for garlic.[4] It is supposed that *A. ursinum*-manufactured products might have more advantages over those of *Allium sativum* for several reasons. They are odorless; the *A. ursinum* contains considerable amounts of chlorophyll, which, during digestion, binds nitrogen and prevents the development of the smell associated with garlic breakdown products.[5]

A. ursinum has more active substances: more amounts of ajoene (a degraded form of allicin), γ-glutamyl peptides (GLUT), and adenosine than *A. sativum*. Some of the active substances present in the *A. ursinum* products are not found in *A. sativum* or are found in little quantities. Preuss et al.[6] have even achieved a stronger therapeutic effect of *A. ursinum* when compared to *A. sativum*.

A. ursinum is widely used as a spice as well as a traditional medicine. Folk medicine recommends the use of bear's garlic as an antiscorbutic, fever-fighting, hunger-provoking agent, also recommended in problems with intestines.[7] In medieval medicine, the leaves of *A. ursinum* were used as a therapy for cardiovascular diseases.[8] More recently, in our days, a cardioprotective action of *A. ursinum* was described *in vitro*.[9] It has been reported that wild garlic has a greater effect on lowering the blood pressure of rats than regular garlic.[10] Several biological activities of *A. ursinum* plants, such as antioxidative,[11] cytostatic,[12] and antimicrobial,[12,13] were reported. In recent years, the potential health benefits of ramson bear's garlic have been attributed mainly to the sulfur-containing compounds.[14] High amounts of volatile compounds, such as sulfides and disulfides, which had been identified in ramson bear's garlic, have a direct impact on the quality of *A. ursinum* as a medicinal plant and as a spice.[14] Because of the content of allin, allicin, and other sulfuric compounds, the plant possesses parasite-killing, fungicidal, and antibacterial properties.[12,15,16]

Other components, such as lectins and flavonoids, have been found in *A. ursinum*.[17,18] Flavonoids were found to be responsible for the inhibition of platelet aggregation in humans[17] and to have antioxidant activity.[19] *A. ursinum* is found to be more beneficial than *A. sativum* in *in vivo* and *in vitro* studies.[6,20] Thus, *A. ursinum* showed a higher effect in increasing high-density lipoprotein (HDL)

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and decreasing total cholesterol, as well as lowering the systemic blood pressure.[6] While all portions of *A. ursinum* were found to exhibit antioxidant property, the leaves were found to have the highest activity.[19] This activity could be caused by the high content of flavonoids. *In vitro* tests have proven that the presence of bear's garlic flavonoids, β-glucoside and β-neohesperidose of kaempferol, acts as a slowing agent in the aggregation of human blood thrombocytes.[17] However, the chemical profile of flavonoids in the leaves, stalks, and seeds of *A. ursinum* has not been fully studied. There are only five flavonoids isolated from *A. ursinum* by Carotenuto et al.[17] Wu et al.[11] described the isolation and structure elucidation of two novel acetylated flavonoid glycosides as well as five known flavonoid glucosides from this plant. They were kaempferol-3,7-di-*O*-β-D-glucopyranoside, 7-*O*-β-D-glucopyranosyl-kaempferol-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside, kaempferol 3-*O*-β-D-glucopyranoside, kaempferol-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside, kaempferol-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-[3-*O*-acetyl]-β-D-glucopyranoside, kaempferol-3-*O*-α-L-rhamnopyronosyl-(1 → 2)-[6-*O*-acetyl]-β-D-glucopyranoside, kaempferol-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside-7-*O*-[2-*O*-(*trans*-*p*-coumaroyl)]-β-D-glucopyranoside, and 6′-*O*-acetyl-kaempferol-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside-7-*O*-[2-*O*-(*trans*-*p*-coumaroyl)]-β-D-glucopyranoside.

Up to now, only the volatile compound contents and patterns in different organs of *A. ursinum* during the vegetation period were subject to investigation; however, no information was obtained on the accumulation of flavonoids during the period when green leaves of ramson bear's garlic change their color to yellow. In this research, we attempted to quantify and characterize phenolic compounds from green and yellow leaves, stalks, and seeds of *A. ursinum*. A sensitive, accurate, and specific method of ultra-performance liquid chromatography (UPLC) was applied in combination with a photodiode array detector (PDA) and a quadrupole/time-of-flight mass spectrometer (Q/TOF−MS), which offers high mass accuracy. The fragmentation behavior of flavonoid glycosides was investigated with the use of mass spectrometry (MS) in negative mode. The MS, tandem mass spectrometry (MS/MS), and ultraviolet (UV) data together with UPLC retention time (tr) of flavonoids allowed for structural characterization of these compounds.

MATERIALS AND METHODS

Reagent and Standard. Acetonitrile, formic acid, and methanol were purchased from Sigma-Aldrich (Steinheim, Germany). Kaempferol-3-*O*-glucoside was purchased from Extrasynthese (Lyon, France).

Plant Materials. The samples (whole plants of *A. ursinum*) were collected in June 2012 in the forest area near Wroclaw (Poland). The various parts of the plants, such as fruits, stalks, fully developed green leaves, and those that turned yellow, were selected in the amount of 50 plants. Afterward, the samples were frozen and shortly dried with the use of freeze dryer Alpha 1-4 LSC (Christ, Osterode, Germany). The homogeneous powders were obtained by crushing the dried tissues using a closed laboratory mill to avoid hydration (IKA A.11, Germany), and the powder was passed through a strainer (1 mm). The powders were kept in a refrigerator (−80 °C) until 24 h before extract preparation.

Extraction Procedure. The powder samples (1 g) were extracted with 25 mL of methanol acidified with 1% acetic acid. The extraction was performed twice by incubation for 20 min under sonication (Sonic 6D, Polsonic, Warsaw, Poland) and with occasional shaking. Next, the slurry was centrifuged at 19000*g* for 10 min, and the supernatant was filtered through a Hydropilic PTFE 0.20 μm membrane (Millex Samplicity Filter, Merck) and used for analysis. The content of polyphenols in individual extracts was determined by means of the ultra-performance liquid chromatography−photodiode array detector−mass spectrometry (UPLC−PDA−MS) method. All extractions were carried out in triplicate.

Identification and Quantification of Polyphenols by the UPLC−PDA−MS Method. Identification and quantification of polyphenol of garlic extracts was carried out with the use of an ACQUITY Ultra Performance LC system equipped with a photodiode array detector with a binary solvent manager (Waters Corporation, Milford, MA) series with a mass detector G2 Q/TOF Micro mass spectrometer (Waters, Manchester, U.K.) equipped with an electrospray ionization (ESI) source operating in negative modes. Separations of individual polyphenols were carried out using a UPLC BEH C18 column (1.7 μm, 2.1 × 50 mm, Waters Corporation, Milford, MA) at 30 °C. The samples (10 μL) were injected, and the elution was completed in 15 min with a sequence of linear gradients and isocratic flow rates of 0.45 mL min^{-1}. The mobile phase consisted of solvent A (4.5% formic acid, v/v) and solvent B (100% of acetonitrile). The program began with isocratic elution with 99% solvent A (0−1 min), and then a linear gradient was used until 12 min, lowering solvent A to 0%; from 12.5 to 13.5 min, the gradient returned to the initial composition (99% A), and then it was held constant to re-equilibrate the column. The analysis was carried out using full-scan, data-dependent MS scanning from *m*/*z* 100 to 1500. Leucine enkephalin was used as the reference compound at a concentration of 500 pg/μL, at a flow rate of 2 μL/min, and the $[M - H]^-$ ion at 554.2615 Da was detected. The $[M - H]^-$ ion was detected during 15 min analysis performed within ESI−MS accurate mass experiments, which were permanently introduced via the LockSpray channel using a Hamilton pump. The lock mass correction was ±1.000 for the mass window. The mass spectrometer was operated in negative-ion mode, set to the base peak intensity (BPI) chromatograms, and scaled to 12 400 counts per second (cps) (100%). The optimized MS conditions were as follows: capillary voltage of 2500 V, cone voltage of 30 V, source temperature of 100 °C, desolvation temperature of 300 °C, and desolvation gas (nitrogen) flow rate of 300 L/h. Collision-induced fragmentation experiments were performed using argon as collision gas, with voltage ramping cycles from 0.3 to 2 V. The characterization of the single components was carried out via the retention time and the accurate molecular masses. Each compound was optimized to its estimated molecular mass $[M - H]^-$ in the negative mode before and after fragmentation. The data obtained from UPLC−MS were subsequently entered into the MassLynx 4.0 ChromaLynx Application Manager software. On the basis of these data, the software is able to scan different samples for the characterized substances.

The runs were monitored at the following wavelength: flavonol glycosides at 360 nm. The PDA spectra were measured over the wavelength range of 200−800 nm in steps of 2 nm. The retention times and spectra were compared to those of the pure standard. The calibration curves were run at 360 nm for the standard kaempferol-3-*O*-glucoside at concentrations ranging from 0.05 to 5 mg/mL (r^2 = 0.9998).

RESULTS AND DISCUSSION

Identification of Flavonols in Garlic. The acidified methanol extracts of garlic's green and yellow leaves, stalk, and seed were analyzed by UPLC−ESI−MS/MS systems. Qualitative analysis obtained by LC−PDA−MS/MS methods and quantitative analysis obtained by UPLC−MS/MS (quantified using PDA and MS detection) are summarized in Tables 1 and 2 and Figure 1. A total of 21 kinds of flavonoids found in garlic's leaf extracts, 19 compounds found in stalk, and 18 compounds found in seed were identified and presented. The structures of compounds **1**, **2**, **7**, **9**, **13**, **15**, and **18** were identified by comparison of their MS data to those reported in the literature.[11,17,20−22] These are kaempferol-3-*O*-glucosyl-(1 → 4)-rhamnosyl-(1 → 2)−glucoside (**1**), kaempferol-3,7-di-*O*-β-D-glucopyranoside (**2**), kaempferol-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside-7-(2″-*p*-coumaryl-3-*O*-β-D-glucopyranosyl-D-glucose (**7**), kaempferol-3-*O*-rhamnosyl-(1 → 2)−galactoside (**9**), kaempferol-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside-7-(2″-*p*-coumarylglucoside)

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Table 1. Characterization of *A. ursinum* Phenolic Compounds Using Their Spectral Characteristic in UPLC−PDA (R_t and λ_{max}) and Negative Ions in UPLC−ESI−MS/MS

peak number	identified compounds	R_t (min)	λ_{max} (nm)	$[M-H]^-$	MS/MS $[M-H]^-$	identification by literature
1	7-*O*-*β*-D-glucopyranosyl-kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranoside	5.31	346	753.2175	593.1556/446.0666/285.0187	2D NMR[11]
2	kaempferol-3,7-di-*O*-*β*-D-glucopyranoside	6.44	346	609.1331	447.0700/285.0187	2D NMR[11]
3	kaempferol-hexosyl-acetyl-deoxyhexose-hexoside derivative	6.58	347	797.2221	635.1520/446.0666/285.0187	
4	kaempferol-hexosyl-acetyl-deoxyhexose-hexoside derivative	6.73	347	797.2221	635.1520/446.0666/285.0187	
5	kaempferol-hexosyl-acetyl-deoxyhexose-hexoside derivative	6.95	347	797.2221	635.1520/446.0666/285.0187	
6	kaempferol-(acetylhexoside)-hexoside	7.21	347	651.1697	489.1113/446.0622/285.0187	
7	kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranoside-7-(2″-*p*-coumaryl-3-*O*-*β*-D-glucopyranosyl-D-glucose	8.84	315	1063.3280	593.1706/447.0838/285.0187	2D NMR[24]
8	kaempferol-deoxyhexose-(1 → 2)-hexoside-(*p*-coumaryl-hexoside-hexoside)	9.02	315	1063.3280	593.1706/447.0838/285.0187	
9	kaempferol-3-*O*-rhamnopyranosyl-(1 → 2)-glucopyranosyl	9.26	346	593.1606	429.0706/285.0187/116.9036	2D NMR[11]
10	kaempferol-hexose-(acetyl-deoxyhexose-(*p*-coumaryl-hexosyl-hexoside)) derivative	9.94	315	1105.2247	635.1520/285.0187	
11	kaempferol-hexose-(acetyl-deoxyhexose-(*p*-coumaryl-hexosyl-hexoside)) derivative	10.03	315	1105.2247	635.1520/285.0187	
12	kaempferol-hexose-(acetyl-deoxyhexose-(*p*-coumaryl-hexosyl-hexoside)) derivative	10.10	315	1105.2247	635.1520/285.0187	
13	kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranoside-7-(2′-*p*-coumarylglucoside)	10.19	315	901.2770	593.1706/446.0838/285.0187	2D NMR[24]
14	kaempferol-deoxyhexose-hexoside-feruloyl-hexoside	10.42	329	931.2077	593.1158/447.1046/285.0187	
15	kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-[3-*O*-acetyl]-*β*-D-glucopyranoside	10.48	347	635.1624	473.0700/285.0187	2D NMR[11]
16	kaempferol-(deoxyhexose-hexoside-(*trans*-*p*-coumaroyl)-hexoside) derivative	10.72	315	901.2647	593.1507/285.0187	
17	kaempferol-(deoxyhexose-hexoside-(*trans*-*p*-coumaroyl)-hexoside) derivative	11.17	315	901.2647	593.1507/285.0187	
18	4′-*O*-acetyl-kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranoside-7-*O*-[2-*O*-(*trans*-*p*-coumaroyl)]-*β*-D-glucopyranoside	11.40	315	943.3172	635.1624/593.1606/447.0700/285.0187	2D NMR[11]
18a	kaempferol-(acetyl-*p*-coumaryl-hexose) (coelution)	11.40	315	635.1624	593.1606/447.0700/285.0187	
19	acetyl-kaempferol-deoxyhexose-hexoside-7-*O*-[2-*O*-(feruloyl)]-hexoside	11.63	329	973.3235	635.1624/593.1606/447.0700/285.0187	
20	acetyl-kaempferol-deoxyhexose-hexoside(*p*-coumaroyl)]-hexoside derivative	12.04	315	943.3172	635.1624/593.1606/447.0700/285.0187	

(**13**) (Figure 5), kaempferol-3-*O*-(6″-acetylgalactoside)-7-rhamnoside (**15**), and 6′-acetyl-kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranoside-7-*O*-[2-*O*-(*trans*-*p*-coumaroyl)]-*β*-D-glucopyranoside (**18**) (Figure 6). Compounds **14**−**17** were identified in plants other than *A. ursinum*.[21]

Compounds **4**−**6**, **8**, **10**−**12**, and **18a**−**20** were found for the first time in *A. ursinum* by us. All 21 identified compounds were kaempferol derivatives. The spectroscopic contour plots (200−400 nm) in combination with the MS/MS fragmentation pattern were used for their identification. The UV spectra of compounds **7**, **8**, **10**−**12**, **16**−**18a**, and **20** revealed a broad band I shifted to a wavelength of 315 nm; the UV spectra of compounds **14** and **19** revealed a broad band I shifted to a wavelength of 329 nm; whereas, all other flavonols exhibited a maximum of band I between 346 and 347 nm (Table 1). This observation indicated that flavonols with band I at 315 and 329 nm were acylated with hydroxycinnamic acid derivatives, such as *p*-coumaric acid and ferulic acid, respectively.[23] The non-acylated kaempferol derivatives (compounds **1**, **2**, and **9**) and those acylated only with an acetyl group (compounds **3**−**6** and **15**) possessed a maximum wavelength at 346 and 347 nm, respectively.

The MS/MS fragmentation behavior of flavonol glycosides previously identified in *A. ursinum*[11,24] (compounds **1**, **2**, **7**, **9**, **13**, **14**, **15**, and **18**) gave valuable indications for their structural characterization. Compound **1** ($[M-H]^-$ at m/z 753.2175) lost glucose on MS/MS fragmentation, yielding ions at m/z 593.1556 $[M-H-160]^-$, which led to m/z 447.0700 $[M-H-146-162-H_2O]^-$ and m/z 285.0187. Consequently, the structure of compound **1** was 7-*O*-*β*-D-glucopyranosyl-kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranoside. Furthermore, we detected compound **2**, kaempferol-3,7-di-*O*-*β*-D-glucopyranoside ($[M-H]^-$ at m/z 609.1331), which has been shown to be one of the flavonol glycosides in *A. ursinum* identified by Wu et al.[11]

The MS/MS spectra of compound **7** ($[M-H]^-$ at m/z 1063.3280), which showed ions at 593.1706 $[M-H-146-162-162]^-$, corresponded to a loss of *p*-coumaric acid (m/z 146) and the subsequent loss of two glucose molecules. On the basis of UV spectra (λ_{max} = 315 nm), the loss of 146 amu was indicated as *p*-coumaric acid. Consequently, we identified compound **7** as kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranoside-7-(2″-*p*-coumaryl-3-*O*-*β*-D-glucopyranosyl-D-glucose (Figure 4). The MS spectra and a fragmentation pattern similar to that of compound **7** were also observed for compound **8**, not yet identified in the *A. ursinum* plant.

Compound **9** ($[M-H]^-$ at m/z 593.1706) lost glucose (m/z 162) and next rhamnose (m/z 146), showing ions at 431.0706 and 285.0187, respectively, and was identified as kaempferol-3-*O*-rhamnopyranosyl-(1 → 2)-glucopyranosyl in agreement with Wu et al.[11] A fragmentation of compounds **13** and **18** is shown in Figures 2 and 3. The first loss was observed for

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Table 2. Contents of Phenolic Compounds in the *A. ursinum* Leaves, Stalks, and Seeds in mg/100 g of Dry Matter (dm)[a]

peak number	compounds	green leaves	yellow leaves	stalks	seeds
1	7-*O*-*β*-D-glucopyranosyl-kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranoside	176.90 ± 5.49	79.00 ± 2.37	17.26 ± 2.54	13.56 ± 1.55
2	kaempferol-3,7-di-*O*-*β*-D-glucopyranoside	63.07 ± 2.78	30.06 ± 2.97	11.81 ± 2.78	1.81 ± 0.34
3	kaempferol-hexosyl-acetyl-deoxyhexose-hexoside derivative	365.90 ± 10.84	167.94 ± 6.99	1.97 ± 0.11	2.62 ± 0.42
4	kaempferol-hexosyl-acetyl-deoxyhexose-hexoside derivative	128.87 ± 2.07	94.58 ± 2.47	5.88 ± 0.23	5.63 ± 0.14
5	kaempferol-hexosyl-acetyl-deoxyhexose-hexoside derivative	23.88 ± 3.75	17.93 ± 1.99	0.32 ± 0.11	0.37 ± 0.03
6	kaempferol-(acetylhexoside)-hexoside	0.15 ± 0.01	2.62 ± 0.45	3.84 ± 0.14	0.54 ± 0.04
7	kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranoside-7-(2″-*p*-coumaryl-3-*O*-*β*-D-glucopyranosyl-D-glucose	20.46 ± 1.22	18.04 ± 1.41	0.99 ± 0.12	0.10 ± 0.01
8	kaempferol-deoxyhexose-(1 → 2)-hexoside-(*p*-coumaryl-hexoside-hexoside)	44.60 ± 2.77	53.11 ± 2.35	2.31 ± 0.11	1.36 ± 0.12
9	kaempferol-3-*O*-rhamnopyranosyl-(1 → 2)-glucopyranosyl	54.23 ± 6.45	212.51 ± 6.54	57.22 ± 2.15	23.16 ± 2.12
10	kaempferol-hexose-(acetyl-deoxyhexose (*p*-coumaryl-hexosyl-hexoside)) derivative	29.62 ± 2.14	24.29 ± 2.12	1.28 ± 0.32	0.33 ± 0.09
11	kaempferol-hexose-(acetyl-deoxyhexose (*p*-coumaryl-hexosyl-hexoside)) derivative	20.28 ± 2.01	23.11 ± 2.68	2.14 ± 0.45	0.29 ± 0.04
12	kaempferol-hexose-(acetyl-deoxyhexose (*p*-coumaryl-hexosyl-hexoside)) derivative	76.02 ± 1.88	72.18 ± 3.44	2.71 ± 0.07	0.07 ± 0.01
13	kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranoside-7-(2″-*p*-coumarylglucoside)	123.49 ± 5.05	157.40 ± 4.51	nd[b]	0.54 ± 0.00
14	kaempferol-deoxyhexose-hexoside-feruloyl-hexoside	59.37 ± 5.78	61.80 ± 2.11	nd	nd
15	kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-[3-*O*-acetyl]-*β*-D-glucopyranoside	26.64 ± 2.14	277.82 ± 3.89	64.10 ± 1.54	15.13 ± 0.89
16	kaempferol-(deoxyhexose-hexoside (*trans*-*p*-coumaroyl)-hexoside) derivative	38.91 ± 1.69	69.01 ± 2.78	3.28 ± 0.99	0.68 ± 0.14
17	kaempferol-(deoxyhexose-hexoside (*trans*-*p*-coumaroyl)-hexoside) derivative	79.20 ± 3.48	162.81 ± 3.66	2.28 ± 0.54	0.83 ± 0.09
18	4′-*O*-acetyl-kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranoside-7-*O*-[2-*O*-(*trans*-*p*-coumaroyl)]-*β*-D-glucopyranoside	344.77 ± 6.78	630.44 ± 5.98	13.20 ± 1.87	4.85 ± 0.42
18a	kaempferol-(acetyl-*p*-coumaryl-hexose) (coelution)				
19	acetyl-kaempferol-deoxyhexose hexoside-7-*O*-[2-*O*-(feruloyl)]-hexoside	99.66 ± 4.44	118.73 ± 5.01	2.28 ± 0.58	nd
20	acetyl-kaempferol-deoxyhexose hexoside(*p*-coumaroyl)]-hexoside derivative	78.32 ± 2.58	89.58 ± 1.56	13.20 ± 1.11	nd
	total	1856.31	2362.96	206.07	73.14

[a]Values are means ± standard deviation (n = 3). [b]nd = not detected.

p-coumaroyl-*β*-D-glucopyranoside molecules (m/z 308). Next, the acetyl group (m/z 42) in compound **18** and the rhamnosyl moiety (m/z 146) were lost, followed by glucose (m/z 162) (Figure 6). This fragmentation pattern corresponds to data found in the literature,[25] namely, that in acylated flavonol diglycosides, which were mainly quercetin and kaempferol glycosides. The MS/MS fragmentation follows the pattern: first the sugar with the acyl moiety at the 7-*O* position is split off, followed by diglucoside at the 3-*O* position. This is in agreement with our findings (Table 1 and Figure 6). Compound **13** was identified previously by Wu et al.[11] as kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranoside-7-(2″-*p*-coumarylglucoside ($[M - H]^-$ at m/z 901.2770) (Figure 5). We have found in *A. ursinum* samples that the next compounds (compounds **16** and **17**) possess the same ion $[M - H]^-$ at m/z 901.2770 on the chromatograms in 10.72 and 11.17 min (Table 1 and Figure 1). Also, the same ion and MS/MS fragmentation as in compound **18** were found in compound **20**. For full identification, the exact structures of compounds **16**, **17**, and **20** need nuclear magnetic resonance (NMR) spectra analysis.

Compound **15** ($[M - H]^-$ at m/z 635.1624) lost glucose (m/z 162) and next acetyl-deoxyhexose {$[M - H]^-$ 168 − (146 and 42)} and was identified in line with Wu et al.[11] as kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-[3-*O*-acetyl]-*β*-D-glucopyranoside.

Three compounds **3**, **4**, and **5** were found to be present in the garlic for the first time ($[M - H]^-$ at m/z 797.2221). MS/MS fragmentation of these compounds yielded ions at m/z 635.1520 $[M - H - 162\ (\text{hexose})]^-$, m/z 447 $[M - H - 162 - 188\ (\text{acetyl-deoxyhexose})]^-$, and m/z 285.0187 $[M - H - 162 - 188 - 162\ (\text{hexose})]^-$, which corresponds to kaempferol-hexosyl-*O*-acetyl-deoxyhexose-hexoside. A fragmentation pattern was similar to that of kaempferol-3-*O*-glucosyl-(1 → 3)-(4″-acetylrhamnosyl)-(1 → 6)-galactoside that has been isolated from the leaves of *Colubrina faralaotra* (Rhamnaceae).[26] The identification structure of compounds **3**, **4**, and **5** needs to be further researched with the NMR method. As with all other identified acyl derivatives, compound **14** ($[M - H]^-$ at m/z 932.2077) released deoxyhexose with ferulic acid on MS/MS fragmentation, yielding m/z 593.1706 $[M - H - 146 - 193]^-$ as the most intense ion. MS/MS fragmentation of m/z 932.2077 led to m/z 447.1046 $[M - H - 146 - 193 - 146]^-$ and m/z 285.0187 $[M - H - 146 - 193 - 146 - 162]^-$, which corresponded to a loss of deoxyhexose [$[M - H]^-$ = 146] and subsequent loss of a hexose molecule. Consequently, we identified compound **14** to be kaempferol-deoxyhexose-hexoside-(feruloyl-hexoside) (Figure 3). The presence of feruoyl group in compound **14** was confirmed by the exhibition of a maximum of band I at 329 nm. Carotenuto et al.[17,24] also found compound **14** to occur in the garlic. A fragmentation pattern and exhibition of a maximum of band I at 329 nm similar to that of compound **14** was also observed for compound **19** {($[M - H]^-$ at m/z 973.3235) acetyl-kaempferol-deoxyhexose-hexoside-(feruloyl)-hexoside}. A difference between compounds **14** and **19** was the presence of an acetyl moiety in compound **19**.

Compounds **10**−**12** identified as kaempferol-hexosyl-(acetyl-deoxyhexose-(*p*-coumaryl-hexosyl-hexose)) showed ions at m/z 1105.2247. The MS/MS fragmentation follows the pattern that is in agreement with our previous finding (Table 1): first, 470 amu,

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which corresponds to two hexose with the *p*-coumaroyl moiety, probably at the 7-*O* position, is split off, followed by hexose and acetylrhamnose (350 amu), probably at the 3-*O* position.

To our knowledge, the occurrence of compounds **3**−**6**, **8**, **10**−**12**, **14**, **16**, **17**, and **18a**−**20** in the garlic has never been described before. The exact structures of these compounds could be identified by comparing their NMR and MS data.

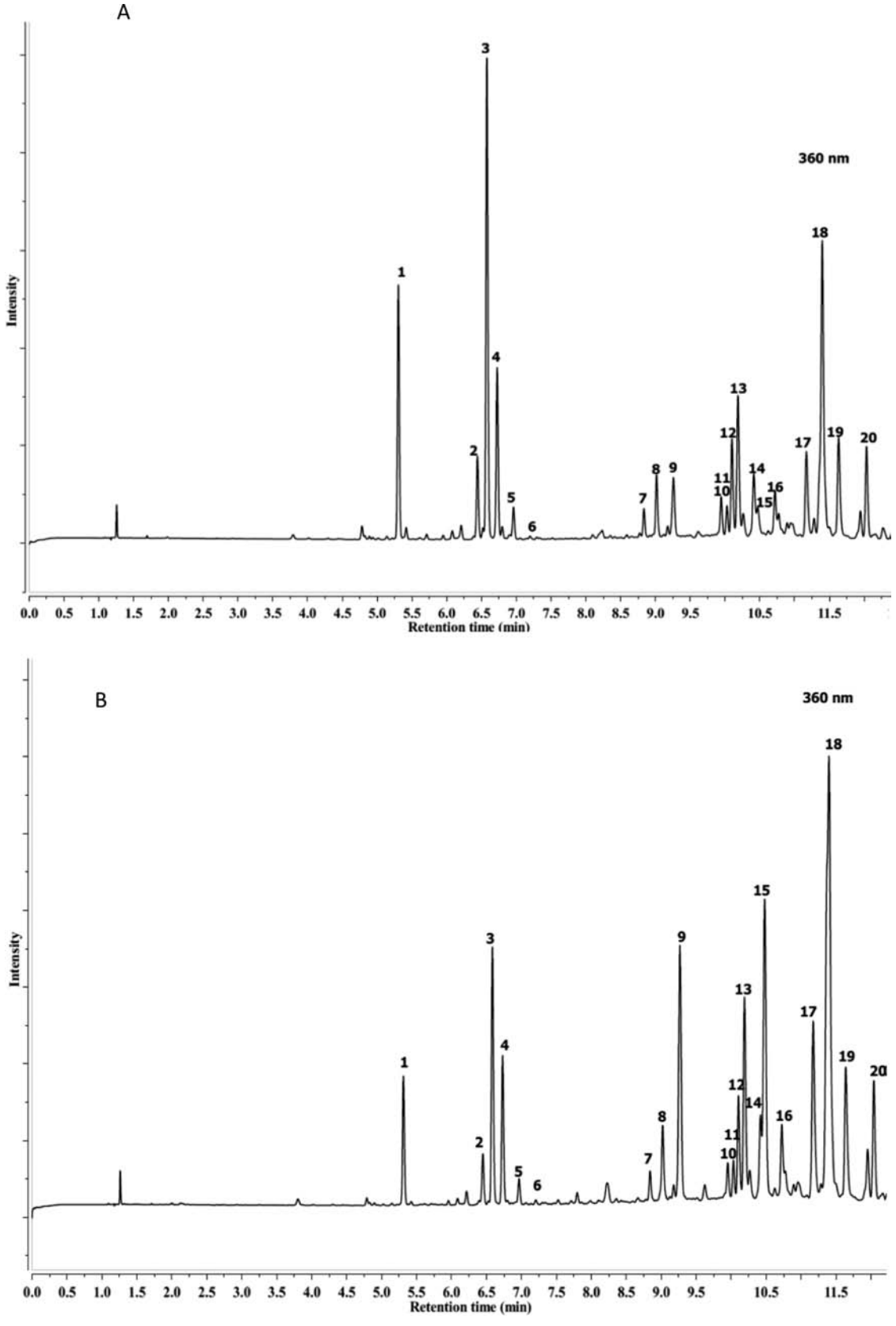

Figure 1. continued

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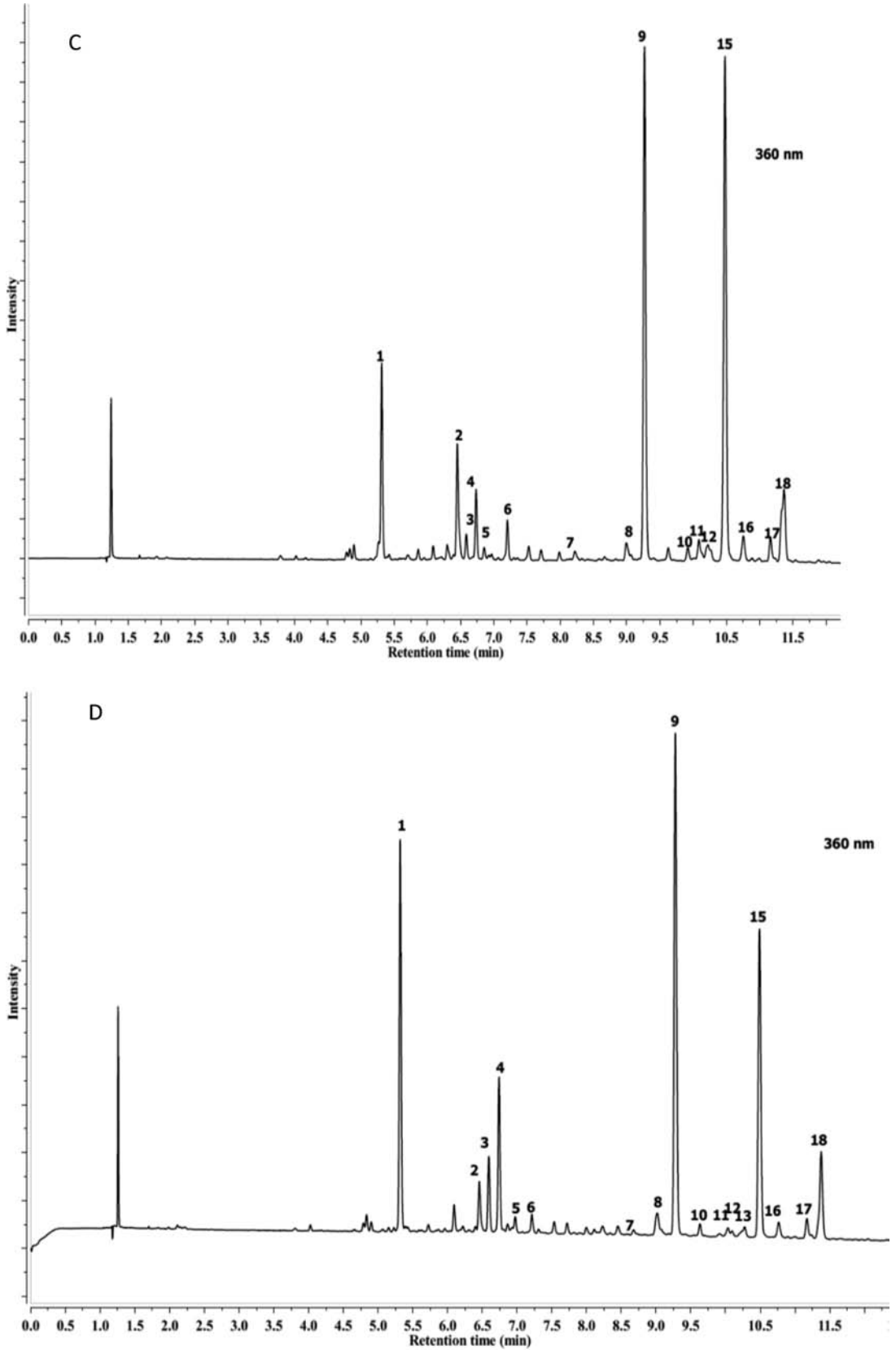

Figure 1. UPLC−PDA−UV at 360 nm chromatograms of phenolic compounds extracted from the green (A) and yellow (B) leaves, stalks (C) and seeds (D) of *A. ursinum* (for abbreviations peaks, see Table 1).

Quantification of Flavonols in Garlic. The content of flavonoids in the leaves, stalks, and seeds of garlic showed great differences, as presented in Table 2. The total content of kaempferol derivatives in seed extract was only 73.14 mg/100 g of dm of seeds. In leaves, it was much higher (1856.31 mg/100 g of dm in green leaves and 2362.96 mg/100 g of dm in yellow leaves).

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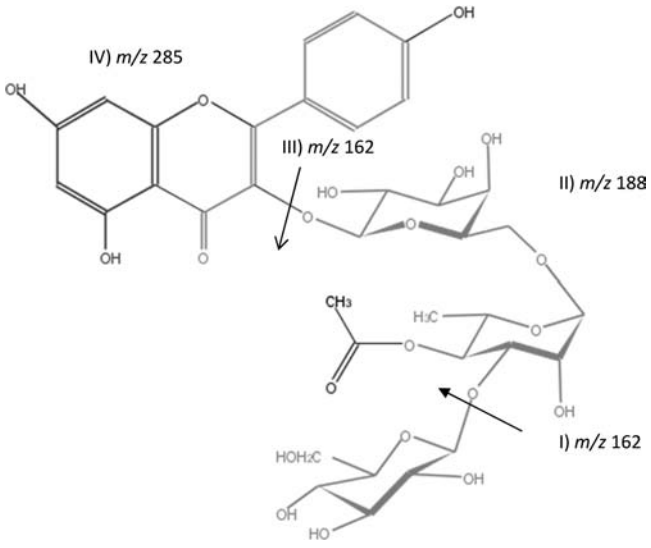

Figure 2. Proposed sructure and fragmentation of kaempferol-[hexosyl-*O*-acetyl-rhamnopyranosyl-hexoside] (compounds **3**, **4**, and **5**) *m*/*z* 797: (I) hexose I, *m*/*z* 162; (II) acetyl-rhamnopyranosyl, *m*/*z* 188; (III) hexose, *m*/*z* 162; and (IV) kaempferol, *m*/*z* 285.

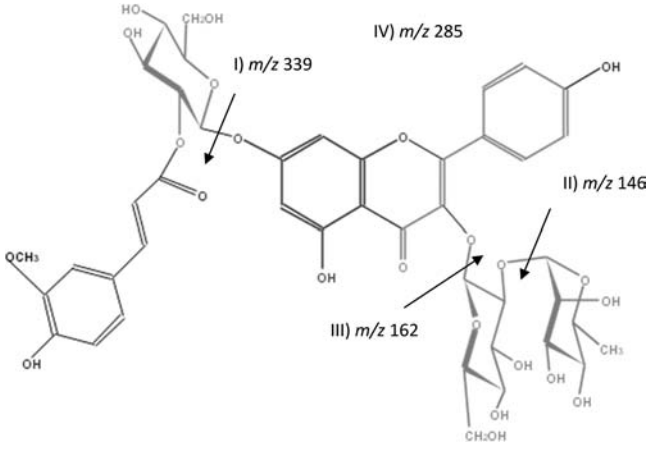

Figure 3. Proposed sructure and fragmentation of kaempferol-deoxyhexose-hexoside-(feruloyl-hexoside) (compound **14**) *m*/*z* 932: (I) feruoyl-hexoside, *m*/*z* 339; (II) deoxyhexose, *m*/*z* 146; (III) hexose, *m*/*z* 162; and (IV) kaempferol, *m*/*z* 285.

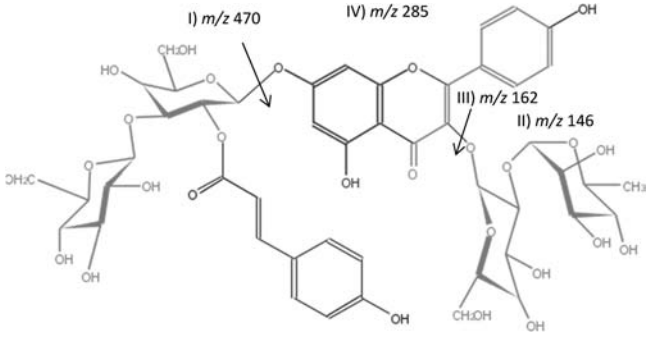

Figure 4. Proposed sructure and fragmentation of kaempferol-deoxyhexose-hexoside-(*p*-coumaryl-hexoside)-hexsoside (compound **8**) *m*/*z* 1063: (I) glucosyl-*p*-coumaryl-hexoside, *m*/*z* 470; (II) deoxyhexose, *m*/*z* 146; (III) hexose, *m*/*z* 162; and (IV) kaempferol, *m*/*z* 285.

According to Błażewicz-Woźniak and Michowska,[3] the leaves of *A. ursinum* contained a significantly lower content of

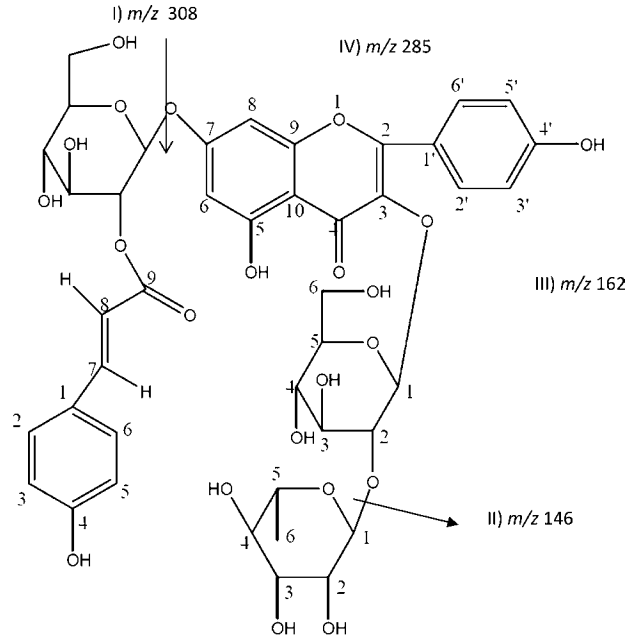

Figure 5. Fragmentation of kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranoside-7-(2″-*p*-coumarylglucoside) (compound **13**) *m*/*z* 901: (I) (*p*-coumaroyl)]-*β*-D-glucopyranoside, *m*/*z* 308; (II) rhamnopyranosyl, *m*/*z* 146; (III) glucopyranoside, *m*/*z* 162; and (IV) kaempferol, *m*/*z* 285.

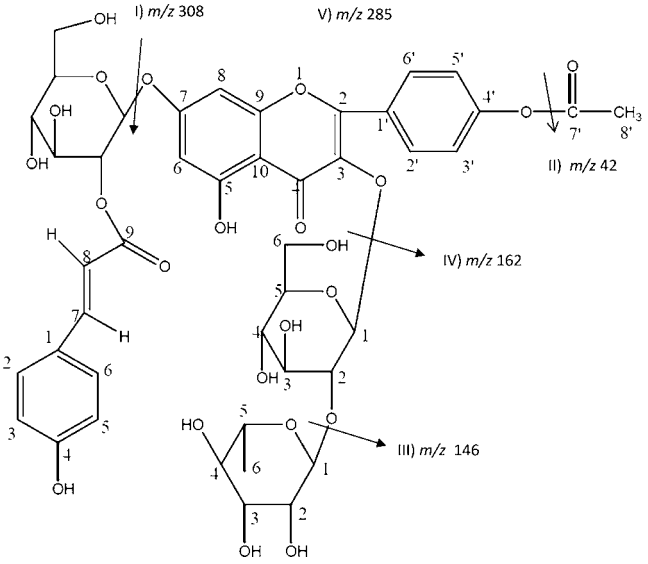

Figure 6. Fragmentation of 4′-*O*-acetyl-kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranoside-7-*O*-[2-*O*-(*trans*-*p*-coumaroyl)]-*β*-D-glucopyranoside (compound **18**) *m*/*z* 943: (I) (*p*-coumaroyl)]-*β*-D-glucopyranoside, *m*/*z* 308; (II) acetyl, *m*/*z* 42; (III) rhamnopyranosyl, *m*/*z* 146; (IV) glucopyranoside, *m*/*z* 162; and (V) kaempferol, *m*/*z* 285.

flavonoids than found during our experiment, Dependent upon the ecotype, the sum of all flavonoids and *O*-dihydroxyphenyls varied from 318.5 and 788.20 mg/100 g of leaf dm of 'Dukla' ecotype to 342.9 and 709.00 mg/100 g of leaf dm in 'Roztocze' ecotype, respectively. Błażewicz-Woźniak and Michowska[3] showed that the ecotypes of bear's garlic significantly differ in their content of biologically active agents. The differences in the values of these compounds reported in both works were probably the result of different methods of quantification.

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Błażewicz-Woźniak et al.[3] used spectrophotometric methods for the analysis of flavonoids, and their results were expressed as quercetin equivalents. The *O*-dihydroxyphenyls (sum) were analyzed by the non-specific old method, and they were expressed as caffeic acid equivalents using spectrophotometry by the Singleton and Rossi method.[3] Our results were obtained by the chromatographic method and expressed as kaempferol equivalents.

The yellow leaves of *A. ursinum* possessed a much larger content of compounds acylated with *p*-coumaric acid than green leaves (1299.97 versus 855.67 mg/100 g of dm, respectively) (Table 2). The stalks and seeds contained much more non-acetylated than acetylated flavonoid glycosides with *p*-coumaric acid compounds (162.4 versus 62.82 mg/100 g of dm and 105.49 versus 24.18 mg/100 g of dm, respectively). Compounds **3**, **18**, **15**, and **9** were the most abundant in kaempferol derivatives found in green leaves, yellow leaves, stalks, and seeds, respectively (Table 2 and Figure 1).

Rice-Evans et al.[27] have explained that the differences in activity among and within various classes of polyphenols result from their chemical structure and individual ability to transfer a hydrogen atom to a radical. The highest activities among flavonols correspond to those with an *ortho*-dihydroxy structure on the B ring and a −OH group at position 3, as occurs with quercetin. Kaempferol with one OH group accounted for lower antioxidant activity than quercetin. Glycosylation or acylation of the hydroxyl substituent on C3 causes the drop of antioxidant activity.[27,28] However, epidemiological studies have found a positive association with the consumption of foods containing kaempferol. Many edible plants contain kaempferol, and it has been estimated that the human dietary intake of this polyphenol may be up to approximately 10 mg/day.[30,31] Numerous *in vitro* and *in vivo* studies support a role of kaempferol and their glycosides having a wide range of pharmacological activities, including anti-inflammatory,[32−34] antimicrobial,[35] anticancer,[36−38] cardioprotective and neuroprotective,[39−42] antidiabetic,[43] antiallergic, and antiasthmatic activities.[44]

These properties stem mainly from the antioxidant activity of these compounds. Several studies have shown that the presence of a double bond at C2−C3 in conjugation with an oxo group at C4 and the presence of hydroxyl groups at C3, C5, and C4′ or an acylated structure are important structural features involved in the antioxidant activity of kaempferol.[28,29]

This study clearly demonstrates that LC−MS/MS is a powerful tool for the determination of kaempferol derivatives in the *A. ursinum* plant. The 11 phenolic compounds were characterized and quantified in leaves, seeds, and stalks for the first time. The main phenolic derivatives of kaempferol profiles were significantly affected by the part of the plant where they originated. The yellow leaves of *A. ursinum* are a very good source of kaempferol derivatives, better than green leaves, stalks, and seeds. These findings may help to further elucidate the health-promoting potential of garlic and products derived from them, such as medical preparations, and to standardize such products based on the contents of their biologically active substances.

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Notes

The authors declare no competing financial interest.

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