Phenolic Constituents from *Alchemilla vulgaris* L. and *Alchemilla mollis* (Buser) Rothm. at Different Dates of Harvest

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Acetone/water extracts from the leaves, including stalks, of *Alchemilla vulgaris* L. and *A. mollis* (Buser) Rothm. were investigated for their phenolic composition by liquid chromatography-tandem mass spectrometry (LC-MS/MS). A total of 24 and 27 compounds were detected for *A. vulgaris* and *A. mollis*, respectively. Pedunculagin and agrimoniin, as described in earlier reports for *A. vulgaris*, as well as other monomeric and oligomeric ellagitannins such as sanguiin H-10, castalagin/vescalagin, and galloyl-bis-hexahydroxydiphenoyl (HHDP) hexose constituted the major phenolic fraction of both plant species. Also, gallic and chlorogenic acids were found in both extracts. Interestingly, catechin and a procyanidin trimer were detected only in *A. mollis*. The flavonoid fraction comprised quercetin glucuronide as major compound in addition to several other quercetin glycosides. Most interestingly, a tentatively identified kaempferol glucuronide and a methylated quercetin glucuronide were exclusively found in *A. mollis*. Finally, the overall phenolic fingerprints of both *Alchemilla* species, harvested in May and August, *i.e.* at the beginning and the end of the flowering period, were compared. A general accumulation of phenolic constituents was observed later in the year, especially with regard to the ellagitannins.

Key words: Alchemilla, Rosaceae, Ellagitannins, Flavonols

Introduction

Alchemilla L. (Rosaceae) species are herbaceous plants that grow on wet meadows in Europe, western Asia and North America. The genus *Alchemilla* L. comprises various species (Ayaz and Hayirlioglu-Ayaz, 2001), including the common lady´s mantle *A. vulgaris* L. (*A. xanthochlora* Rothm.) and *A. mollis* (Buser) Rothm. In folk medicine, both lady´s mantle species have been used for the treatment of wounds, eczema, and inflamed mucosa (Eggensperger, 2006; Evstatieva *et al*., 2007; Ghafoor *et al*., 2011). The dried leaves, "Alchemillae herba", are officially recognized as a pharmaceutical drug in the European Pharmacopoeia (2008) and assigned for the therapy of mild diarrhoea. The drug contains considerable amounts of tannins and possesses astringent, antioxidative, and antimutagenic properties. Additionally, a variety of cosmetic applications are reported (Eggensperger, 2006). For dermatologic and cosmetic purposes, the *Alchemilla* herb is collected during the flowering period (Eggensperger, 2006) which usually lasts from May until August.

To date, detailed investigations on the phenolic constituents in the leaves of the plant have only been performed for few *Alchemilla* species including *A. vulgaris* (D´Agostino *et al*., 1998; Fraisse *et al*., 1999, 2000; Geiger *et al*., 1994; Lamaison *et al*., 1991). The phenolic compound pattern was found to comprise the ellagitannins agrimoniin, pedunculagin, and laevigatin F (Geiger *et al*., 1994) as well as several quercetin glycosides including quercetin glucuronide (D´Agostino *et al*., 1998; Fraisse *et al*., 1999, 2000; Lamaison *et al*., 1991). Notably, data on the phenolic constituents of *A. mollis* are hardly existent (Ayaz and Hayirlioglu-Ayaz, 2001; Trendafilova *et al*., 2011). Finally, scattered reports on the variation of the phenolic compound patterns during summer in tannin- and flavonoid-containing plants (Hatano *et al*., 1986; Salminen *et al*., 2004) were available, including *Alchemilla vulgaris* (Fraisse *et al*., 1999).

Therefore, the aim of this study was a thorough characterization of the phenolic constituents of the leaves (including stalks) from *A. vulgaris* and *A. mollis*. In addition, the characteristic compound patterns of both plant species were investigated at the beginning and the end of the flowering period.

Material and Methods

Plant material and harvest dates

Leaves and stalks of *A. mollis* (Buser) Rothm. were collected from the medicinal herb garden of WALA Heilmittel (Bad Boll/Eckwälden, Germany) in May (start of flowering period) and August 2010 (end of flowering period). The *A. vulgaris* L. herb was purchased from Heilkräuter-Gorges (Buch, Germany); it was also harvested in May and August 2010 (place of growth: Blaufelden, Germany). The fresh plant material was immediately sorted and stored frozen at –80 °C until analyses. The plant material was identified by Prof. O. Spring (Department of Botany, Hohenheim University, Stuttgart, Germany) and deposited as voucher specimens (HOH-011275, HOH-011276, HOH-011277, and HOH-011278) at the Department of Botany.

Chemicals

Acetonitrile (HPLC grade; J. T. Baker, Deventer, Netherlands) and formic acid (98–100%; Merck, Darmstadt, Germany) were used for high-performance liquid chromatography-diode array detection (HPLC-DAD) analyses. Liquid chromatography-mass spectrometry (LC-MS) analyses were carried out with acetonitrile (LC-MS grade) and formic acid (98%, eluent additive for LC-MS) from Sigma-Aldrich (Steinheim, Germany). For extraction purposes, diatomaceous earth (kieselgur, calcined, purified; Sigma-Aldrich) and acetone (Merck) were chosen. Purified water (0.055 μS/cm) from a Purelab Option-Q system (Elga Berkefeld, Celle, Germany) was used throughout all analyses. Gallic acid monohydrate and (+)-catechin (Roth, Karslruhe, Germany), rutoside trihydrate (Ph. Eur. reference standard, France), as well as ellagic acid (Fluka, Buchs, Switzerland) served as qualitative reference standards.

Extraction of the Alchemilla plant material

Samples of 3.5 g of frozen and cut leaves (including stalks) from *A. vulgaris* and *A. mollis* (May and August harvest, respectively) were extracted with 30 ml acetone/water (8/2, v/v) each in a brown round-bottom flask according to an already established gentle extraction method (Duckstein and Stintzing, 2011) with the following slight modifications: After the first extraction step (5 °C, 5 h) and filtration through diatomaceous earth, the solid was re-extracted with 25 ml of the extraction solvent and kept at 5 °C for 17 h. After re-filtration and acetone removal by rotovaporation, the extracts were transferred into a volumetric flask and made up to 50 ml with water. Before analyses, all extracts were centrifuged at 19,000 x *g*. For each plant species and harvest date, three individual batches were processed.

HPLC-DAD analyses

Chromatographic analyses were carried out on a Dionex Ultimate 3000 HPLC system, equipped with a vacuum degasser SRD-3400, a binary pump HPG-3400 A, an autosampler WPS-3000 TSL, a thermostatic column compartment TCC-3000 SD, and a DAD detector DAD-3000 (Dionex, Idstein, Germany). An analytical SunFire™ C18 reversed-phase column (150 x 2.1 mm i.d.; 5 μm particle size; Waters, Eschborn, Germany) was used at 25 °C with detection wavelengths of 280 and 360 nm for identification of tannins and flavonoids, respectively. Eluents were 1% formic acid (eluent A) and acetonitrile/water (9:1, v/v; eluent B), used for the following gradient with a constant flow rate of 0.210 ml/min: 0–5 min, 0% B; 5–40 min, 0–12.5% B; 40–105 min, 12.5–25% B; 105–110 min, 25–100% B; 110–115 min, 100% B; then re-equilibration to starting conditions. The injection volume was 5 μl. For HPLC control and data processing, Chromeleon V 6.8 software (Dionex) was used.

LC-ESI-MS/MS analyses

Further structural analyses were performed by LC-MS using an Agilent 1200 HPLC-system consisting of a degasser G1322A, a binary pump G1312A, an autosampler G1329A, a thermostatic column compartment G1316A, and a diode array detector G1315B (Agilent, Waldbronn, Germany) coupled to an HCTultra ion trap MS detector fitted with an ESI ion source (Bruker Daltonik, Bremen, Germany). For chromatographic separation, the same conditions as described above were applied. MS acquisition was performed under the

following conditions: operation mode, negative; capillary voltage, 4000 V; dry gas flow (N_2), 9 l/min; nebulizer pressure, 35 psi; capillary temperature, 365 °C. Mass spectra were recorded between *m/z* 50 and 2000. MS^n experiments were carried out with a compound stability and trap drive level of 100% in the auto MS/MS mode. Agilent ChemStation B.01.03 (Agilent) and EsquireControl V 6.1 (Bruker Daltonik) software were used for control and data processing of the HPLC system as well as the MS detector, respectively. Peaks were identified according to their specific fragmentation patterns, UV spectra, and retention times in comparison with literature data as well as commercial reference standards.

Results and Discussion

Since hydrolyzable tannins are widely distributed in the Rosaceae family (Okuda *et al.*, 1992) including *A. vulgaris* (Geiger *et al.*, 1994), fresh plant material from both species was extracted using a gentle extraction procedure (Duckstein and Stintzing, 2011) to avoid compositional changes due to the inherent light sensitivity of tannins (Duckstein and Stintzing, 2011; Hümmer and Schreier, 2008).

For investigation of the phenolic compound patterns, HPLC analysis without further sample treatment with the exception of a centrifugation step was performed. This allowed an insight into the unaltered fingerprint of *Alchemilla* extracts. An established HPLC-DAD and LC-MS/MS method (Duckstein and Stintzing, 2011) originally developed for *Hamamelis* and marginally adapted for *Alchemilla* extracts facilitated the compilation of reliable data.

Characterization of the phenolic constituents in A. vulgaris

A representative chromatogram with an overview of the phenolic compound pattern in *A. vulgaris* is depicted in Fig. 1A. A total of 24 compounds were identified using LC-MS/MS. Chromatographic, spectroscopic and mass spectrometric data are summarized in Table I.

Hydrolyzable tannins

Due to the lack of commercially available reference compounds, the composition of the most important and best investigated fraction of *A. vulgaris* preparations (Geiger *et al.*, 1994), represented by 15 compounds, was identified in comparison with chromatographic and specific mass spectrometric literature data (Buendia *et al.*, 2010; Engels *et al.*, 2011; Fernandes *et al.*, 2011; Hager *et al.*, 2008; Hukkanen *et al.*, 2007; Mullen *et al.*, 2003). The majority turned out to be ellagitannins including two pedunculagin isomers with identical MS data, compounds **3** and **5** (Fig. 1A, Table I). Structurally, pedunculagin is a bis-galloyl-HHDP (hexahydroxydiphenoyl) hexopyranose unit that builds up higher molecular weight ellagitannins, *e.g.* sanguiin H-10 or sanguiin H-6 (Hager *et al.*, 2008). A schematic overview of the identified ellagitannin structures is presented in Fig. 2. Further small ellagitannin basic units detected were galloyl-HHDP hexose (**8**) and three galloyl-bis-HHDP hexose isomers, **14**, **15**, and **21** (Figs. 1A, 2, Table I). Peaks 9 and 12 represented isomers of castalagin/vescalagin (Fig. 2), an example of a monomeric ellagitannin consisting of an altered central hexose with an open-ring structure (Hager *et al.*, 2008). The presence of pedunculagin has already been ascertained for *Alchemilla* (Geiger *et al.*, 1994), whereas no literature data was available for the occurrence of the other ellagitannin basic units.

The MS fragmentation pattern of the structurally related compounds discussed above provided characteristic information which may be found in most types of ellagitannins: neutral mass losses of 302, 180 or 162, and 170 or 152 Da indicate the presence of HHDP, hexose, and gallic acid units, respectively (Fig. 2). The neutral mass loss digit of hexose or gallic acid depends on the position of the structural scission and may vary according to whether an oxygen atom is attached or not. Also, the ion *m/z* 301 representing an ellagic acid moiety (Fig. 2) is often found in the ellagitannins (Fernandes *et al.*, 2011). Finally, the fragmentation order may provide information on the structure of oligomeric ellagitannins.

Peak 4 exhibited a UV characteristic which fits to ellagitannins in general and showed some of the specific fragments discussed above: neutral loss of galloyl (*m/z* 883 → 731) and hexose units (*m/z* 639 → 477) as well as a mass peak at *m/z* 301 which represents an ellagic acid residue (Table I). However, other fragments which are not characteristic for ellagitannins made a structural proposal based on LC-MS difficult. As a result, compound **4** remained assigned as an unknown ellagitannin.

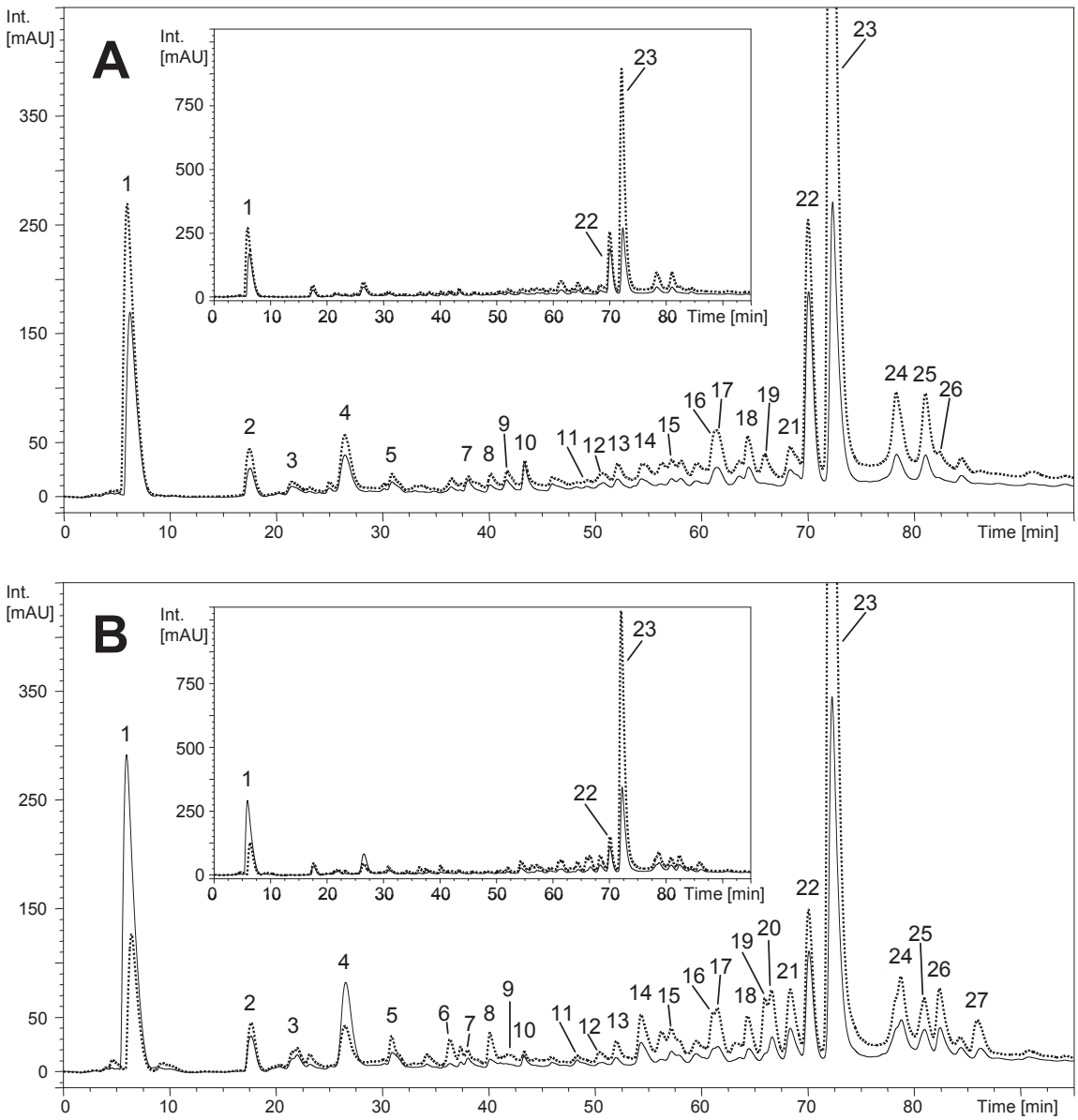

Fig. 1. Enlarged fingerprints (HPLC-DAD, 280 nm) of representative (A) *A. vulgaris* and (B) *A. mollis* extracts with the unzoomed chromatogram (inset) at the two harvest dates May (solid line) and August (dotted line); n = 3 for each experimental setup.

Dimeric ellagitannins consisting of the already discussed basic units were also detected. Peaks 13 and 16 (Fig. 1A, Table I) both generated doubly charged negative pseudomolecular ions at *m/z* 783. The type of negative ion charge was proven by zoom scan analysis (Duckstein and Stintzing, 2011; Mullen *et al.*, 2003), whereas the spacing between the isotope peaks was equal to 0.5 Da characterizing the ion as a doubly charged one. At first sight, the molecular weight and the basic unit composition of compounds **13** and **16** (Fig. 1) led to two possible types of dimeric ellagitannin structures. Sanguiin H-10 and laevigatin F sharing the same molecular mass are both formally

Table I. Phenolic constituents in *A. vulgaris* and *A. mollis* extracts (harvest date: May and August) and their characteristic chromatographic and spectrometric data (ESI negative).

Peak and compound no.	t_r [min]	Peak assignment	λ_{max} [nm]	MS data		Reference[a]	Detection[b]			
				Pseudomolecular ion *m/z* [BPI (%)]	Fragments *m/z* [BPI (%)]		*A. vulgaris* May	*A. vulgaris* August	*A. mollis* May	*A. mollis* August
1	6.0	Gallic acid	266	507 (100)[c]	MS^2: 331 (100), 169 (12); MS^3: 169 (100), 125 (12)	ref	+	+	+	+
2	17.5	2-Pyrone-4,6-dicarboxylic acid	234, 315	183 (100), 139 (15)	MS^2: 139 (100), 111 (15), 95 (23); MS^3: 95 (100)	tent	+	+	+	+
3	22.0	Pedunculagin isomer I	232	783 (100)	MS^2: 481 (38), 301 (100), 275 (25); MS^3: 284 (29), 257 (100), 201 (24)	L1	+	+	+	+
4	26.5	Unknown ellagitannin	232	947 (100)	MS^2: 929 (94), 901 (100); MS^3: 883 (100); MS^4: 731 (100), 639 (36), 477 (17), 299 (75), 255 (12)	X	+	+	+	+
5	30.9	Pedunculagin isomer II	233	783 (100)	MS^2: 481 (22), 301 (100), 275 (14); MS^3: 285 (11), 257 (100), 201 (18)	L1	+	+	+	+
6	36.3	Catechin +	232, 279	289 (100)	MS^2: 245 (100), 205 (50); MS^3: 227 (23), 203 (100); MS^4: 188 (89), 175 (100), 160 (40)	ref	–	–	tr	+
		Procyanidin trimer		865 (100)	MS^2: 847 (14), 739 (34), 695 (100), 577 (57), 425 (38), 407 (36), 287 (36)	L2				
7	38.0	Chlorogenic acid	290sh, 329[d]	353 (100)	MS^2: 191 (100), 179 (4); MS^3: 173 (53), 127 (100), 93 (33), 85 (30)	L3	+	+	tr	+
8	40.1	Galloyl-HHDP hexose	231, 272[d]	633 (100)	MS^2: 463 (5), 301 (100); MS^3: 257 (100), 229 (57), 185 (11)	L4	+	+	tr	+
9	41.7	Castalagin/vescalagin isomer I	231, 267[d]	933 (100)	MS^2: 765 (46), 631 (54), 479 (78), 451 (100), 301 (60); MS^3: 433 (41), 407 (91), 327 (58), 301 (100)	L1	+	+	tr	+
10	43.4	Quercetin hexoside-glucuronide	256, 352	639 (100)	MS^2: 463 (100), 301 (67); MS^3: 301 (100); MS^4: 272 (7), 179 (94), 151 (100)	tent	+	+	+	+
11	48.3	Trigalloyl hexose	272	635 (100)	MS^2: 465 (100); MS^3: 447 (4), 313 (100), 169 (36); MS^4: 169 (100), 125 (14)	L5	tr	tr	+	+
12	50.3	Castalagin/vescalagin isomer II +	253, 360[d]	933 (100)	MS^2: 451 (100), 301 (14); MS^3: 433 (87), 407 (77), 351 (100), 285 (81); MS^4: 323 (100), 279 (22)	L1	+	+	–	+
		Quercetin hexoside I		463 (100)	MS^2: 301 (100); MS^3: 257 (53), 229 (33), 185 (23)	L6	+	+	+	+
13	52.0	Sanguiin H-10 isomer I	231, 262[d]	783 (100)[e]	MS^2: 1265 (14), 935 (46), 633 (100), 301 (38); MS^3: 481 (2), 301 (100); MS^4: 257 (100), 229 (66)	L7, L8	+	+	+	+
14	54.3	Galloyl-bis-HHDP hexose I	232, 280sh	935 (100)	MS^2: 633 (100), 301 (63); MS^3: 301 (100); MS^4: 257 (100), 229 (52), 185 (27)	L9	+	+	+	+
15	57.2	Galloyl-bis-HHDP hexose II	230, 281sh	935 (100)	MS^2: 633 (100), 301 (58); MS^3: 301 (100); MS^4: 257 (100), 229 (79), 185 (24)	L9	+	+	+	+

Table I continued.

Peak and compound no.	t_r [min]	Peak assignment	λ_{max} [nm]	MS data		Reference[a]	Detection[b]			
				Pseudomolecular ion *m/z* [BPI (%)]	Fragments *m/z* [BPI (%)]		*A. vulgaris* May	*A. vulgaris* August	*A. mollis* May	*A. mollis* August
16	61.0	Sanguiin H-10 isomer II	231, 280[d]	783 (100)[e]	MS^2: 1265 (35), 935 (100), 633 (45), 613 (80), 301 (45); MS^3: 633 (100), 301 (87); MS^4: 301 (100)	L7, L8	+	+	+	+
17	61.5	Quercetin-feruloyl hexose	253, 360	623 (100), 433 (25)	MS^2: 301 (100); MS^3: 273 (14), 179 (100), 151 (63), 107 (6)	tent	+	+	+	+
18	64.3	Ellagic acid	253, 366	301 (100)	MS^2: 257 (100), 229 (52), 185 (25)	ref	+	+	+	+
19	66.0	Quercetin hexoside-deoxyhexoside	232, 278[d]	609 (100), 463 (30)	MS^2: 301 (100); MS^3: 273 (11), 179 (100), 151 (61), 107 (9)	ref	+	+	+	+
20	66.6	Quercetin hexoside	256, 353	463 (100)	MS^2: 301 (100); MS^3: 271 (42), 179 (100), 151 (69), 107 (7)	L6	–	–	+	+
21	68.3	Galloyl-bis-HHDP hexose III	232, 280sh	935 (100)	MS^2: 633 (100), 435 (8), 301 (77); MS^3: 301 (100); MS^4: 257 (100), 229 (44), 185 (19)	L9	+	+	+	+
22	70.1	Quercetin glucuronide	257, 353	477 (100)	MS^2: 301 (100); MS^3: 273 (16), 257 (7), 179 (100), 151 (75)	L6	+	+	+	+
23	72.1	Agrimoniin	233, 280sh	934 (100)[e]	MS^2: 1567 (47), 1265 (20), 1085 (44), 897 (100), 633 (56), 301 (100); MS^3: 273 (9), 257 (100), 229 (82), 185 (39)	L7	+	+	+	+
24	78.7	Sanguiin H-6-like structure	232, 280sh	941 (100)[e]	MS^2: 919 (100)[d], 769 (36)[d]; MS^3: 1537 (12), 1235 (7), 1055 (100), 783 (96), 301 (13); MS^4: 481 (29), 301 (100), 275 (61)	tent	+	+	+	+
25	80.9	Unknown ellagitannin + Kaempferol glucuronide	232, 265, 353	932 (100)[e]	MS^2: 1563 (92), 1261 (9), 1077 (17), 918 (86), 783 (29), 613 (5), 301 (100); MS^3: 284 (79), 258 (69), 229 (100)	tent	+	+	+	+
				461 (100)	MS^2: 285 (100), 175 (9); MS^3: 257 (100), 229 (45), 163 (21)	L6	–	–	+	+
26	82.3	Sanguiin H-6 with an additional gallic acid	232, 280sh	1009 (100)[e]	MS^2: 1717 (20), 1567 (34), 1415 (2), 1265 (23), 1085 (100), 897 (86), 783 (39), 633 (44), 450 (84), 301 (29); MS^3: 633 (100), 451 (25), 301 (8)	tent	+	+	+	+
27	85.9	Methyl-quercetin glucuronide	252, 356	491 (100)	MS^2: 315 (100); MS^3: 300 (100); MS^4: 271 (100), 255 (57), 151 (3)	tent	–	–	+	+

[a] tent, Tentative assignment; ref, reference standard used to verify compound identity; X, no literature data or reference available; L1, Hager *et al.* (2008); L2, Valls *et al.* (2009); L3, Jaiswal *et al.* (2010); L4, Fernandes *et al.* (2011); L5, González *et al.* (2010); L6, Hokkanen *et al.* (2009); L7, Hukkanen *et al.* (2007); L8, Mullen *et al.* (2003); L9, Buendia *et al.* (2010).

[b] +, present; –, not present; tr, trace amounts: compound barely detectable.

[c] Gallic acid cluster; monomer in MS^3.

[d] UV data not unambiguous due to coelution.

[e] $[M-2H]^{2-}$ species.

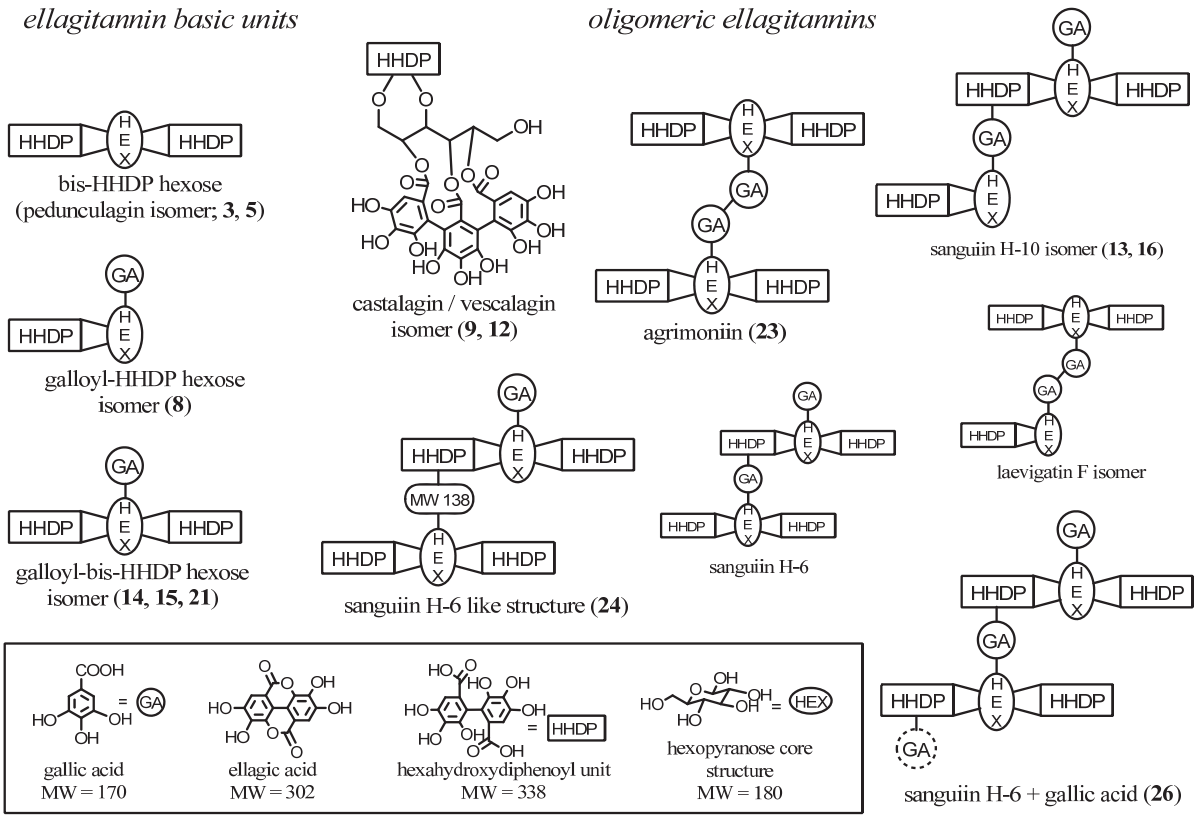

Fig. 2. Schematic overview of ellagitannin structures and their basic units. Numbers in brackets refer to the compounds detected in *A. vulgaris* and *A. mollis* extracts (for additional data, see Table I). For comparative reasons, sanguiin H-6 and a laevigatin F isomer are also depicted. Ellagitannins **24** and **26** are structural proposals, but the position of the gallic acid (dotted circle) in **26** is uncertain.

built up by a galloyl-bis-HHDP hexose attached to a galloyl-HHDP hexose (Fig. 2). However, the type of conjunction between these two basic units differs: sanguiin H-10 consists of a linkage between a galloyl and an HHDP unit (DOG-type) whereas laevigatin F is linked between two galloyl moieties (GOG-type; Hager *et al.*, 2008; Okuda *et al.*, 2009). The characteristic fragmentation scheme of sanguiin H-10 was described by Mullen *et al.* (2003) as follows: the doubly charged mass peak produces singly charged fragments at *m/z* 1265 (loss of HHDP), 1103 (loss of hexose), 933 (loss of gallic acid), 631 (loss of HHDP), and 301 (loss of galloyl hexose). In the present study, the dimeric ellagitannin structure (*m/z* 783) fragmented almost the same way (Table I) allowing the conclusion that the ellagitannin was sanguiin H-10. In contrast to these findings, Geiger *et al.* (1994) identified laevigatin F in *A. xanthochlora*. Due to lack of commercial reference substances, the presence of laevigatin F instead of sanguiin H-6 cannot be completely excluded.

Another dimeric ellagitannin eluting as the very prominent peak 23 (Fig. 1) was represented by *m/z* 934 (doubly charged). The molecular mass (1870 Da) and typical ellagitannin fragments may refer to two possible structures: agrimoniin and sanguiin H-6 (Fig. 2). Both compounds formally consist of two galloyl-bis-HHDP hexose units with varying conjunction to each other. While in sanguiin H-6 the units are connected via DOG-type, agrimoniin is featured by a GOG-type linkage between the two galloyl moieties (Okuda *et al.*, 2009). These structural differences should result in an alternated fragmentation pattern. Typical MS fragments for sanguiin H-6 from the literature (Buendia *et al.*, 2010; Hager *et al.*, 2008; Mullen *et al.*, 2003) are *m/z* 1567 (loss of HHDP),

1265 (loss of HHDP), 933 (loss of galloyl hexose), 633 (loss of HHDP), and 301 (loss of galloyl hexose). Interestingly, peak 23 produced slightly different fragments (Table I): 1567 (loss of HHDP), 1265 (loss of HHDP), 1085 (loss of hexose + O), 915 (loss of galloyl + O), 783 (loss of 132 Da), 481 (loss of HHDP), and 301 (loss of hexose + O). In both cases, the first step of the MS fragmentation is the cleavage of two HHDP units. Then, sanguiin H-6 expells a galloyl hexose unit which represents the connection point of the two ellagitannin basic units, while peak 23 lost a hexose (180 Da) and thereafter a galloyl + O moiety (170 Da) following 132 Da (Fig. 3). The latter fragment has not been previously described for sanguiin H-6 and was therefore regarded characteristic for compound **23**. The neutral loss of 132 Da depicting the residue of a galloyl moiety lacking oxygen after cleavage (Fig. 3) indicated the linkage to be of the GOG-type. This assumption seems to be plausible, since the fragments 180 Da (hexose) and 302 Da (HHDP) corresponded with the residual part (bis-HHDP hexose) of the proposed agrimoniin structure. Interestingly, the cleavage of 132 Da followed by a hexose instead of a whole galloyl hexose moiety as described for sanguiin H-6 seemed to distinguish two GOG- or DOG-type linkages. Of the two suggested structures, agrimoniin possessed the proposed GOG linkage. Therefore, peak 23 was tentatively identified as agrimoniin. This assumption is corroborated by reports of agrimoniin as a constituent in *A. xanthochlora* (Fecka, 2009; Geiger *et al.*, 1994). Also, agrimoniin has been described as a marker compound for the Rosaceae familiy in general (Okuda *et al.*, 1992).

Further oligomeric ellagitannins in *A. vulgaris* were **24**, **25**, and **26** which were tentatively identified according to their specific UV and MS fragmentation data. Peak 24 showed characteristic ellagitannin fragments: *m/z* 1839 → 1537 (loss of HHDP), 1235 (loss of HHDP); *m/z* 935 → 633 (loss of HHDP), 301 (loss of galloyl hexose; Table I), but the first neutral mass loss of 44 Da (*m/z* 941 → 919, both doubly charged) which may refer to a CO_2 moiety and later 120 Da (*m/z* 1055 → 935) cannot be explained yet. On the basis of this information, a sanguiin H-6-like structure was supposed (Fig. 2). A proposal for the ellagitannin structure of peak 25 was not possible because of additional fragments which could not be assigned. However, some typical transitions were observed [*m/z* 1865 → 1563 (loss of HHDP), 1261 (loss of HHDP) and *m/z* 301] which allowed the assertion that the compound exerts structural features of an ellagitannin. Peak 26 exhibited a doubly charged mass peak at *m/z* 1009 which also produced typical ellagitannin fragments: *m/z* 1009 (doubly charged) → 1717 (loss of HHDP), 1415 (loss of HHDP), 1265 (loss of hexose), 1085 (loss of galloyl), 633 (loss of HHDP), 301 (loss of galloyl hexose). The last neutral loss of a galloyl hexose indicated the units to be DOG-type linked as discussed above. Further structural inspection led to the proposal that the structure is sanguiin H-6-like with an additional galloyl group attached to any HHDP unit (Table I, Fig. 2).

In the present study, the presence of pedunculagin and agrimoniin as important ellagitannins of *A. vulgaris* and marker compounds for the Rosaceae family was confirmed as described by earlier studies (Fecka, 2009; Geiger *et al.*, 1994; Okuda *et al.*, 1992). In contrast to this, the occurrence of leavigatin F could not be corroborated, although this assumption cannot be completely excluded. In addition, sanguiin H-10 was also considered as a marker compound for the Rosaceae family (Okuda *et al.*, 1992). Several other monomeric ellagitannin units and oligomeric structures which cannot be found in the *Alchemilla* literature contributed to the picture of lady´s mantle being a plant with a considerable spectrum of ellagitannins.

Within the group of the hydrolyzable tannins, a minor gallotannin component was detected as well. Peak 11 revealed a pseudomolecular ion peak at *m/z* 635 and the typical fragment pattern of a trigalloyl hexose (Table I) (Duckstein and Stintzing, 2011; González *et al.*, 2010). For *A.*

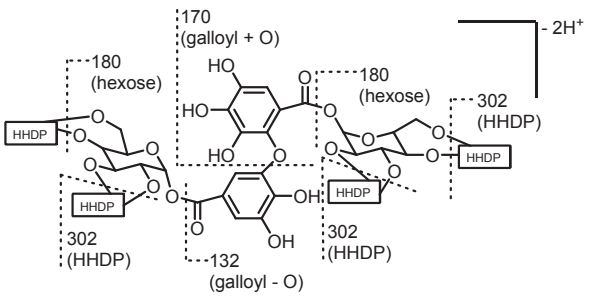

Fig. 3. Proposed fragmentation characteristic of agrimoniin (**23**; ESI negative); HHDP, hexahydroxydiphenoyl unit.

vulgaris, this constituent has hitherto not been described in the literature, but its occurrence is plausible in the biosynthetic context of ellagitannins and their basic units as discussed for other plants (Niemetz *et al*., 2003; Niemetz and Gross, 2001).

Phenolic acids

This fraction included low-molecular weight compounds that may partly occur in connection with the already discussed ellagitannins. Gallic acid (**1**) and ellagic acid (**18**) (Table I) were identified as a major and minor constituent of the phenolic spectrum, respectively (Fig. 1). There are only scant reports on gallic and ellagic acids in *A. vulgaris* (Fecka, 2009). Since these structures represent starting compounds or intermediates in the ellagitannin biosynthesis (Niemetz *et al*., 2003; Niemetz and Gross, 2001; Quideau and Feldman, 1996), the two acids may appear as accompanying substances of the tannin fraction.

The hydroxycinnamic acid derivative chlorogenic acid (**7**) was identified (Jaiswal *et al*., 2010) as a minute peak in *A. vulgaris* (Table I, Fig. 1). To our knowledge, no reports on chlorogenic acid in *A. vulgaris* exist, but Okuda and co-workers (1992) reported the compound to occur in many plants of the Rosaceae family.

Another low-molecular weight phenolic acid, compound **2** (Fig. 1, Table I) exhibited a UV maximum at 316 nm and was tentatively identified according to its fragments *m/z* 183 → 139 (loss of CO_2), 111 (loss of CO), and 95 (loss of another CO_2) as 2-pyrone-4,6-dicarboxylic acid. Exactly this phenolic compound was suggested earlier as chemotaxonomic marker substance for plants of the Rosaceae family including *A. xanthochlora* (syn. *A. vulgaris*; Wilkes and Glasl, 2001).

Flavonol glycosides

In addition to ellagitannins and phenolic acids, flavonol glycosides represent another important class of phenolic compounds in *A. vulgaris*. In this study, five flavonol derivatives were detected and identified in comparison with MS data from the literature (Hokkanen *et al*., 2009) or, failing this, tentatively. Peaks 10 and 12 included two minor quercetin glycosides: quercetin hexoside-glucuronide (**10**) and quercetin hexoside (**12**). Other flavonol glycosides detected were quercetin-feruloyl hexose (**17**) and quercetin hexoside-deoxyhexoside (**19**). Compound **22** represented the main flavonol peak in the chromatogram (Fig. 1; Table I) and was identified as quercetin glucuronide in accordance with previous literature (Hokkanen *et al*., 2009). A former study also reported quercetin glucuronide to be the major flavonoid glycoside in *A. vulgaris* (Lamaison *et al*., 1991). Generally, all of the flavonols detected in *A. vulgaris* were quercetin derivatives and in most cases attached to a hexose or glucuronic acid moiety. These findings are consistent with earlier reports (Condrat *et al.,* 2009; D´Agostino *et al*., 1998; Felser and Schimmer, 1999; Fraisse *et al*., 2000).

Phenolic profile of A. mollis compared to A. vulgaris

The representative fingerprint of *A. mollis* extracts prepared in the same way as those of *A. vulgaris* is depicted in Fig. 1B. At first sight, very strong similarities between the characteristic peak patterns of the two plant species are obvious. Upon closer inspection, however, distinct differences exist (Table I).

Hydrolyzable tannins

Although most of the ellagitannins in *A. vulgaris* and *A. mollis* showed the same UV and MS fragmentation behaviour, the castalagin/vescalagin isomers (peaks 9 and 12 in *A. vulgaris*) were not detected in *A. mollis* in May. This may be due to differing ellagitannin biosynthethic activities.

Condensed tannins

This compound class was not present in the *A. vulgaris* fingerprint and therefore seems to be an important distinguishing mark. The two constituents catechin (*m/z* 289) and a procyanidin trimer (*m/z* 865) eluted close to each other and were therefore conflated in peak 6 (Fig. 1, Table I). Their MS fragmentation characteristics were clearly identifiable and corroborated by literature data (Valls *et al.*, 2009), and a reference compound in the case of catechin. The occurrence of condensed tannins in several *Alchemilla* species including *A. mollis* was reported earlier (Ayaz and Hayirlioglu-Ayaz, 2001), but information on their structural characteristics had not been available yet.

Flavonol glycosides

Remarkable differences were observed for the flavonol constituent pattern in the two species. Three additional peaks, **20**, **25**, and **27** (Fig. 1),

were detected in the *A. mollis* extracts and identified as follows: compound **20** turned out to be a second quercetin hexose isomer in addition to **12** already discussed for *A. vulgaris* and is in accordance with a very recent report by Trendafilova *et al*. (2011). In contrast to the present investigation, these authors collected above-ground plant parts, including flowers, and only once at full bloom. Therefore, the findings of the present study cannot be directly compared with the data of Trendafilova *et al.* (2011).

Another quercetin derivative only found in *A. mollis* was tentatively identified as methylquercetin glucuronide (**27**). The formation of methyl artefacts upon extraction can be excluded, since no methanol was used. Basically, there are reports on such derivatives naturally occurring in plants, as shown for *Potentilla* (Rosaceae) (Kombal and Glasl, 1995) or *Achillea* (Asteraceae) (Krenn *et al*., 2003). Interestingly, also kaempferol glucuronide (**25**) was detected in *A. mollis*. Reports on kaempferol derivatives in *Alchemilla* are very scant (Condrat *et al*., 2009; D´Agostino *et al*., 1998; Felser and Schimmer, 1999). The detection of kaempferol-3-*O*-*β*-glucuronide in *A. speciosa* (Felser and Schimmer, 1999) is in accordance with the present findings.

Seasonal variations of the phenolic constituents in Alchemilla

For the use in cosmetic or dermatologic preparations, the leaves and stalks of *Alchemilla* are usually collected during the flowering period (Eggensperger, 2006). To recognize possible trends in the phenolic constituent pattern, *A. vulgaris* and *A. mollis* samples were collected in May and August. A representative chromatogram of each plant and harvest date is depicted in Fig. 1. Generally, an enrichment of most of the phenolic constituents was noticeable: in both plant species, agrimoniin (**23**), one of the major constituents, increased from May to August by more than 100%. The same trend was observed for other ellagitannins like **5**, **8**, **9**, **12**–**16**, **21**, **24**–**26**. Quercetin glucuronide (**22**), the major flavonoid, increased almost by 30% during flowering (Fig. 1). One of the exceptions within this general development was gallic acid (**1**). In both *Alchemilla* species, no clear accumulation tendency was observed in all batches prepared. In the same line, the unknown ellagitannin structure **4** showed no trend either. Since gallic acid is one of the precursors of ellagitannin biosynthesis (Niemetz *et al*., 2003; Niemetz and Gross, 2001), its occurrence is plausible. Showing no clear accumulation or depletion, this phenolic acid may also be involved in other branches of metabolism. The general trend towards ellagitannin accumulation is in line with earlier reports (Feeny, 1970; Fraisse *et al*., 1999; Hatano *et al*., 1986) explaining this phenomenon by stepwise biosynthesis of ellagitannin structures during spring and summer (Hatano *et al*., 1986; Salminen *et al*., 2004). Fraisse and co-workers (1999) also reported a slight accumulation of quercetin glucuronide in *A. vulgaris* during early summer, but a drop in August. In the recent study, accumulation was observed in both plant species. Environmental factors such as water stress (Kouki and Manetas, 2002), sunlight (Dudt and Shure, 1994; Olafsdottir *et al*., 2001), and protection against herbivores (Feeny, 1970) might be responsible for the enhanced formation of phenolic constituents during summer.

In conclusion, the present data contribute to broadening our knowledge on the phenolic composition of *A. vulgaris* and represent the first comprehensive report on the phenolic fraction of *A. mollis* with an emphasis on similarities and differences between the two plant species. Furthermore, the accumulation of selected compounds during summer is a valuable finding for the adequate harvest date of the respective plant and preparations derived therefrom.

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