Clarification of the Saponin Composition of *Ranunculus ficaria* Tubers

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Six known saponins, glycosides of hederagenin and oleanolic acid, have been isolated from the tubers of *Ranunculus ficaria* L. (Ranunculaceae). Their separation included the use of centrifugal partition chromatography (CPC). Structure determination was achieved on the basis of chemical evidence and extensive spectral studies. This is the first report of these triterpene glycosides from *R. ficaria*.
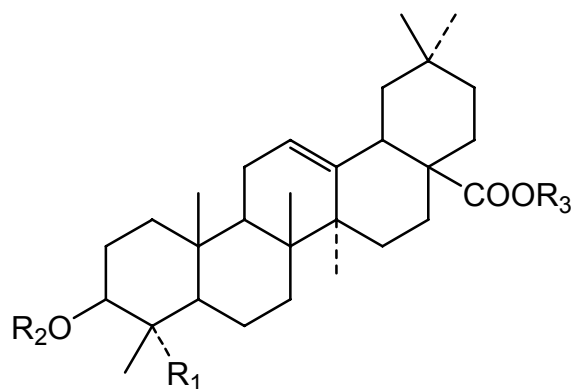
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Ranunculus ficaria L. (syn. *Ficaria ranunculoides* Moench) (Ranunculaceae), commonly known as lesser celandine or pilewort, is a small plant with yellow flowers, distributed widely around Europe and North America. The tubers have a long history of use in traditional medicine for the treatment of hemorrhoids. The origin of this use most probably comes from the Doctrine of Signatures since the tubers resemble hemorrhoids.

It has been shown that saponins from the tubers have local anti-hemorrhoidal activity [1] and patents exist for the preparation of saponin-rich extracts, principally employed for the formulation of antihemorrhoidal ointments, for example [2]. Little is known about the precise structures of these triterpene glycosides and there is some vagueness and uncertainty associated with their content [3-8]. However, hederagenin has been identified as the main triterpene aglycone and oleanolic acid is present in smaller amounts [6]. This paper reports on the isolation and structure determination of the 6 major saponins from the tubers of *R. ficaria*.

The saponin-containing methanol extract of *R. ficaria* tubers was subjected to partition between water and *n*-butanol. The *n*-butanol part was separated by a combination of methods, including silica gel open-column chromatography, CPC, and low-pressure liquid chromatography (LPLC). As a result, six saponins (**1 – 6**) were isolated.

1 R_1 = -CH$_2$OH, R_2 = -Ara, R_3 = -H
2 R_1 = -CH$_3$, R_2 = -Ara^2—Glc, R_3 = -H
3 R_1 = -CH$_2$OH, R_2 = -Ara^4—Glc, R_3 = -H
4 R_1 = -CH$_3$, R_2 = -H, R_3 = -Glc^6—Glc^4—Rha
5 R_1 = -CH$_2$OH, R_2 = -H, R_3 = -Glc^6—Glc^4—Rha
6 R_1 = -CH$_3$, R_2 = -Ara^2—Glc, R_3 = -H
$\quad\quad\quad\quad\quad\quad\quad$ |^3
$\quad\quad\quad\quad\quad\quad\quad$ Glc

The negative APCI-MS of saponin **1** gave a quasi-molecular ion at *m/z* 603 [M-H]⁻ in agreement with a molecular formula of $C_{35}H_{56}O_8$ (MW 604). A MS^2 experiment with the [M-H]⁻ ion gave a negative fragment at *m/z* 471 [M-H-132]⁻ corresponding to the loss of a terminal pentosyl moiety. This was confirmed as arabinose after acid hydrolysis of the saponin. Compound **1** was identified as hederagenin 3-*O*-*α*-L-arabinopyranoside on the basis of ¹H, ¹³C NMR (Table), COSY, HSQC and HMBC spectral data. This saponin has previously been found in several plants, including *Fatsia japonica* (Araliaceae) [9, 10] and *Collinsonia canadensis* (Lamiaceae) [11]. However, the attributions of C-23 of hederagenin and C-5 of arabinose described for **1** in the latter plant must be interchanged, since there is a correlation in the HMBC spectrum between C-23 (δ_C 64.7) and the methyl group at C-24 (δ_H 0.90).

Saponin **3** displayed a quasi-molecular ion [M+Na]⁺ at *m/z* 789.4378 in the positive ion HR-ESI-MS, corresponding to a molecular formula of $C_{41}H_{66}O_{13}Na$. It showed a quasi-molecular ion [M-H]⁻ at *m/z* 765 in the negative ion ESIMS, indicating a molecular weight of 766. The MS^2 analysis with the [M-H]⁻ ion gave a negative fragment at *m/z* 603 [M-H-162]⁻ corresponding to the loss of a terminal hexosyl moiety, while the MS^3 experiment of the [M-H-162]⁻ ion gave a negative fragment at *m/z* 471 [M-H-162-132]⁻ corresponding to the loss of a pentosyl moiety. Acid hydrolysis afforded the aglycone hederagenin, together with glucose and arabinose. Analysis of ¹H, ¹³C NMR (Table), COSY, HSQC and HMBC spectral data allowed attribution of all aglycone and sugar signals. When the ¹³C NMR signals of **1** and **3** are compared, a downfield shift (about 10 ppm) for C-4 of arabinose is observed. Thus the terminal glucosyl moiety is attached at C-4 of arabinose and **3** is hederagenin 3-*O*-*β*-D-glucopyranosyl-(1→4)-*α*-L-arabinopyranoside. The ¹³C NMR data (Table) are virtually identical to those given by Joshi *et al.* [11].

Saponin **2** gave a quasi-molecular ion [M-H]⁻ at *m/z* 749 in the negative ion ESIMS, indicating a molecular weight of 750. In the same way as **3**, MS^n analysis of **2** showed the consecutive loss of a hexosyl moiety, followed by a pentosyl moiety. Acid hydrolysis afforded the aglycone oleanolic acid, together with glucose and arabinose. Examination of the COSY spectrum allowed complete assignment of the glycosidic proton system. However, instead of a 10 ppm downfield shift of C-4 of the arabinosyl

moiety as observed in **3**, saponin **2** gave an 8 ppm downfield shift of C-2. Saponin **2** is therefore oleanolic acid 3-*O*-*β*-D-glucopyranosyl-(1→2)-*α*-L-arabinopyranoside. ¹³C NMR data for this compound are also described by Zhong et al. for the same saponin isolated from *Clematis tangutica* (Ranunculaceae) [12].

Acidic hydrolysis of saponin **4** gave the aglycone oleanolic acid and the sugars glucose and rhamnose. Three anomeric carbons in the ¹³C NMR spectrum were observed at δ 95.6, 104.8 and 102.7, suggesting that one of the sugars was attached at C-28 of the aglycone. As no signal appeared between δ 80 and δ 90, this saponin was monodesmosidic. The negative ESI-MS of saponin **4** gave a quasi-molecular ion at *m/z* 925 [M-H]⁻ in agreement with a molecular formula of $C_{48}H_{78}O_{17}$ (MW 926). Analysis of ¹H, ¹³C NMR (Table), COSY, HSQC and HMBC spectral data allowed attribution of all aglycone and sugar signals. From the HMBC spectrum, it was established that the sugar (glucose) attached to the aglycone was substituted at C-6 by a second glucosyl moiety. The terminal sugar (rhamnose) was attached at C-4 of the middle glucose, as confirmed by the upfield shift of ca. 8 ppm of this signal in the ¹³C NMR spectrum. Saponin **4** is oleanolic acid 28-*O*-[*α*-L-rhamnopyranosyl(1→4)-*β*-D-glucopyranosyl-(1→6)] *β*-D-glucopyranoside [13].

Saponin **5** gave a quasi-molecular ion [M-H]⁻ at *m/z* 941 in the negative ion ESIMS, indicating a molecular weight of 942. Acid hydrolysis gave the same sugars as **4**, but the aglycone hederagenin instead of oleanolic acid. As in **4**, the chemical shifts of the three anomeric carbon atoms in the ¹³C NMR spectrum (Table) and the absence of a signal between δ 80 and δ 90 suggested the presence of a monodesmosidic saponin with the sugar moiety at C-28 of hederagenin. Attribution of signals in the ¹³C and ¹H NMR spectra by analysis of the 2D spectral data (COSY, HSQC, HMBC) gave a trisaccharide side chain with the same substitution positions as **4**. Thus saponin **5** is hederagenin 28-*O*-[*α*-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranosyl (1→6)]-*β*-D-glucopyranoside first isolated by Kizu *et al.* [14] from *Hedera nepalensis* (Araliaceae). The ¹³C NMR chemical shifts of this glycosidic side chain correspond to those given for the 28-*O*-ester of a hederagenin saponin isolated by Texier *et al.* from *R. ficaria* [4]. However, in the latter report, a branched-chain structure is presented. In light of the present

Table 1: ^{13}C-NMR (125 MHz) data [δ] for glycosides **1 - 6** (pyridine-d_5, TMS).

Carbon	1	2	3	4	5	6
1	38.7	38.7	38.7	38.9	38.8	38.8
2	26.0	26.4	26.1	28.0	27.7	26.6
3	81.8	88.8	82.1	78.1	73.4	89.0
4	43.4	39.5	43.5	39.3	42.9	39.7
5	47.5	55.8	47.6	55.8	48.6	55.9
6	18.1	18.4	18.2	18.8	18.6	18.5
7	32.8	34.2	32.9	33.1	32.9	34.3
8	39.7	39.7	39.8	39.9	39.9	39.8
9	48.1	48.0	48.2	48.1	48.2	48.0
10	36.9	36.9	37.0	37.3	37.2	37.1
11	23.8	23.8	23.9	23.8	23.8	23.8
12	122.4	122.4	122.7	122.9	122.9	122.5
13	144.8	144.8	144.8	144.1	144.1	144.9
14	42.1	42.1	42.2	42.1	42.1	42.2
15	28.2	28.3	28.4	28.2	28.3	28.4
16	23.6	23.7	23.9	23.3	23.3	23.8
17	46.4	46.6	46.8	47.0	47.0	46.7
18	41.9	42.0	42.1	41.7	41.7	42.1
19	46.7	46.5	46.7	46.2	46.2	46.6
20	30.8	30.9	31.0	30.7	30.7	31.0
21	34.1	34.2	34.3	33.9	34.0	34.3
22	33.1	33.1	33.3	32.5	32.5	33.3
23	64.7	28.2	64.5	28.7	67.9	28.1
24	13.5	16.7	13.6	16.5	13.1	16.8
25	16.0	15.4	16.1	15.6	16.1	15.6
26	17.4	17.3	17.6	17.5	17.6	17.4
27	26.0	26.1	26.1	26.0	26.1	26.2
28	180.2	180.2		176.5	176.5	
29	33.1	33.3	33.3	33.1	33.1	33.3
30	23.7	23.6	23.9	23.6	23.7	23.8
Ara-1	106.6	104.8	106.5			105.5
Ara-2	73.0	81.0	73.8			77.5
Ara-3	74.6	73.4	74.7			83.3
Ara-4	69.5	68.3	79.9			68.8
Ara-5	66.9	64.9	66.4			66.0
Glc-1		106.0	106.9	95.6	95.6	104.4
Glc-2		76.3	75.9	73.9	74.0	76.2
Glc-3		78.1	78.4	78.7	78.7	78.6
Glc-4		71.5	71.3	70.8	70.9	72.5
Glc-5		78.0	78.8	78.0	78.0	77.5
Glc-6		62.5	62.6	69.1	69.2	63.2
Glc-1				104.8	104.9	105.0
Glc-2				75.3	75.3	75.3
Glc-3				76.5	76.5	78.7
Glc-4				78.2	78.2	71.6
Glc-5				77.1	77.2	78.3
Glc-6				61.2	61.3	62.6
Rha-1				102.7	102.8	
Rha-2				72.5	72.6	
Rha-3				72.7	72.8	
Rha-4				73.8	73.9	
Rha-5				70.3	70.3	
Rha-6				18.5	18.5	

evidence, supported by 2D NMR data (including HMBC experiments), this saponin is more likely to be 3-*O*-α-L-arabinopyranosyl hederagenin 28-*O*-[α-L-rhamnopyranosyl (1→4)-β-D-glucopyranosyl-(1→6)] β-D-glucopyranoside.

Acid hydrolysis of saponin **6** gave oleanolic acid as aglycone and glucose and arabinose as constituent monosaccharides. The negative ESI-MS of saponin **6** gave a quasi-molecular ion at *m/z* 911 [M-H]⁻, in agreement with a molecular formula of $C_{47}H_{76}O_{17}$ (MW 912). An MS² experiment with the [M-H]⁻ ion gave a negative ion fragment at *m/z* 749 [M-H-162]⁻ corresponding to the loss of a terminal hexosyl moiety. The MS³ experiment of this ion gave a fragment at *m/z* 587 [M-H-162-162]⁻ and MS⁴ gave a fragment at *m/z* 455 [aglycone-H]⁻ corresponding to the loss of a second hexosyl unit and a pentosyl unit, respectively. The anomeric carbons of the three sugar units were detected at δ 104.4, 105.0 and 105.5 and their corresponding anomeric proton doublets at δ 5.51, 5.31 and 4.78 (HSQC). Analysis of 2D experiments (COSY, HSQC) permitted the identification of an arabinose (δ_H 4.78), a terminal glucose (δ_H 5.31) and a second terminal glucose (δ_H 5.51). HMBC correlations were detected between C-3 of oleanolic acid and H-1 of arabinose (δ_H 4.78), between C-2 (δ_C 77.5) and C-3 (δ_C 83.3) of the arabinosyl and H-1 of the two glucosyl moieties. Similarly, HMBC correlations were detected between H-2 (δ_H 4.73) of arabinose and C-1 (δ_C 104.4) of the first glucose and H-3 (δ_H 4.34) of arabinose and C-1 (δ_C 105.0) of the second glucose. Thus, saponin **6** is oleanolic acid 3-*O*-β-D-glucopyranosyl-(1→3)-[(β-D-glucopyranosyl-(1→2)]-α-L-arabinopyranoside. This saponin, first discovered in the aerial parts of *Fagonia arabica* (Zygophyllaceae) [15], has only been subsequently reported in *Akebia trifoliata* (Lardizabalaceae) [16].

The isolation and characterization of saponins **1-6** has been performed in order to clarify the saponin pool of *R. ficaria*. The tubers are known to contain a mixture of saponins, but their complete structure determination until the present study had not been achieved. Brief and rather vague reports were previously given on the presence of hederagenin 28-*O*-β-D-glucopyranoside [5] and the formation of a hederagenin rhamnoglucoside in the presence of an enzyme preparation [8]. Another investigation referred to a polyglucosidic chain attached at the C-3

hydroxyl group of hederagenin [3]. The saponin reported by Texier *et al.* [4], which is more likely to possess a linear instead of a branched 28-*O* ester side chain, was not identified as one of the major *R. ficaria* glycosides in the samples analyzed in this study.

Initial separation of saponins from the crude extracts was successfully achieved by CPC. This all-liquid method has proved of great importance for the isolation of polar, glycosidic compounds and also for the separation of less polar classes of natural products [17]. After the initial fractionation by CPC, final purification of the individual saponins was performed by LPLC and silica gel open-column chromatography. This type of strategy is valuable for the rapid isolation of saponins from plant material.

Experimental

General procedures: All electrospray (ESI) and atmospheric pressure chemical ionization (APCI) mass spectra were acquired on a Finnigan MAT LCQ ion trap mass spectrometer. TOF-MS experiments for accurate mass measurements were conducted on a TOF LCT mass spectrometer (Micromass, Manchester, UK). The ESI conditions were as follows: capillary 2800 V, vaporizer 200°C, nebuliser gas nitrogen, cone voltage 40 V, MS scan time 1s + 0.1s interscan delay. The reference compound used for high resolution molecular weight determination was sulfadimethoxime ([M+H]⁺: 311.0914) (Aldrich, Buchs, Switzerland), added post-column. ¹H- and ¹³C-NMR spectra were obtained in pyridine-d_5 with a Varian Inova Unity 500 spectrometer (499.87 and 125.70 MHz, respectively), using Me₄Si as an internal standard. Complete assignment was achieved on the basis of 2D experiments (DEPT, COSY, HSQC, HMBC), with Varian VNMR software. For gel filtration, Sephadex LH-20 (Amersham Biosciences) with MeOH was used. Silica gel 40-63 µm (Merck) was employed for open-column chromatography. Centrifugal partition chromatography (CPC) was performed on a Pharma-Tech CCC-1000 instrument (Baltimore, Maryland, USA) equipped with a 650 mL coil. For low-pressure liquid chromatography (LPLC), Lobar size B RP-18 columns (Merck) were used.

Plant material: Tubers of *R. ficaria* (batch T0105445) were supplied by Tortay and were collected in the Tourangelle region of France in 2001.

Extraction and isolation: Dried tubers (770 g) of *R. ficaria* were ground and extracted successively with CH_2Cl_2 (3x 3 L) and MeOH (3x 3 L), yielding 3.4 g CH_2Cl_2 extract and 30.0 g of MeOH extract. A first batch of the MeOH extract (14.0 g) was suspended in water and partitioned successively with EtOAc (500 mL) and BuOH (2x 500 mL). The BuOH fraction (5 g) was separated by CPC with the solvent system *n*-hexane-EtOAc-MeOH-H_2O 3:7:5:5 (upper phase as mobile phase) at a flow-rate of 3 mL/min to give five fractions (I-V). Fraction II (450 mg), after silica gel chromatography using an EtOAc-MeOH-H_2O gradient (200:20:1, 800 mL; 160:20:1, 700 mL; 160:40:1, 400 mL), gave 7 fractions (IIa-IIg). Gel filtration of IIb (36 mg) provided saponin **1** (20 mg). Saponin **2** (15 mg) was obtained from fraction IIc (30 mg) after LPLC with MeOH-H_2O 70:30. Silica gel chromatography of fraction V (1.3 g) with an EtOAc-MeOH-H_2O gradient (200:20:1, 1200 mL; 160:20:1, 800 mL; 120:20:2, 1700 mL, 60:20:2, 1200 mL) gave 5 fractions (Va-Ve). LPLC of Vc (60 mg) with a MeOH-H_2O gradient (70:30, 500 mL; 80:20, 300 mL; 90:10, 300 mL) yielded saponin **4** (20 mg). For the separation of the remaining three saponins, a second batch of the MeOH extract (15 g) was fractionated by CPC with the solvent system $CHCl_3$-MeOH-H_2O 5:4:3 (lower phase as mobile phase) at a flow-rate of 3 mL/min to give five fractions (I-V). Fraction II (1.1 g), after silica gel chromatography using a $CHCl_3$-MeOH-H_2O gradient (120:20:1, 400 mL; 80:20:2, 500 mL; 60:20:2, 400 mL; 65:35:5 300 mL), gave 14 fractions (IIa-IIn). LPLC of IIg (60 mg) with MeOH-H_2O 72:28 provided saponin **3** (15 mg). LPLC of IIk (90 mg) with MeOH-H_2O 75:25 provided saponin **6** (25 mg). Silica gel chromatography of fraction IV (700 mg) with an EtOAc-MeOH-H_2O gradient (80:10:1, 500 mL; 70:10:2, 750 mL; 50:10:2, 1100 mL; 30:20:2, 1600 mL) gave 6 fractions (IVa-IVf). LPLC of IVb (62 mg) with a MeOH-H_2O gradient (75:25, 500 mL, 80:20, 300 mL; 90:10, 300 mL) yielded saponin **5** (23 mg).

Saponin 1 (Hederagenin 3-*O*-α-L-arabino-pyranoside)

White amorphous powder.
^{13}C NMR: Table 1.
APCI-MS (negative ion mode): *m/z* 603 [M-H]⁻, $C_{35}H_{56}O_8$.
APCI-MS-MS: MS² (603) *m/z* 471 [M-H-132]⁻.

Saponin 2 (Oleanolic acid 3-*O*-β-D-glucopyranosyl-(1→2)-α-L-arabino pyranoside)

White amorphous powder.
^{13}C NMR: Table 1.
ESI-MS (negative ion mode): *m/z* 749 [M-H]⁻, $C_{41}H_{66}O_{12}$.
ESI-MS-MS: MS² (749) *m/z* 587 [M-H-162]⁻.
MS³ (587) *m/z* 455 [M-H-162-132]⁻.

Saponin 3 (Hederagenin 3-*O*-β-D-glucopyranosyl-(1→4)-α-L-arabinopyranoside)

White amorphous powder.
^{13}C NMR: Table 1.
ESI-MS (negative ion mode): *m/z* 765 [M-H]⁻, $C_{41}H_{66}O_{13}$.
ESI-MS-MS: MS² (765) *m/z* 603 [M-H-162]⁻; MS³ (603) *m/z* 471 [M-H-162-132]⁻.
HR-ESI-MS (positive ion mode): *m/z* 789.4378 [M+Na]⁺ (calcd. for $C_{41}H_{66}O_{13}Na$ 789.4401).

Saponin 4 (Oleanolic acid 28-*O*-[α-L-rhamnopyranosyl (1→4)-β-D-glucopyranosyl-(1→6)] β-D-glucopyranoside)

White amorphous powder.
^{13}C NMR: Table 1.
ESI-MS (negative ion mode): *m/z* 925 [M-H]⁻ $C_{48}H_{78}O_{17}$.

Saponin 5 (Hederagenin 28-*O*-[α-L-rhamno-pyranosyl (1→4)- β-D-glucopyranosyl-(1→6)] β-D-glucopyranoside)

White amorphous powder.
^{13}C NMR: Table1.
ESI-MS (negative ion mode) *m/z* 941 [M-H]⁻ $C_{48}H_{78}O_{18}$.
ESI-MS^n (941) *m/z* 779 [M-H-162]⁻, *m/z* 617 [M-H-162-162]⁻, *m/z* 471 [M-H-162-162-146]⁻.

Saponin 6 (Oleanolic acid 3-*O*-β-D-glucopyranosyl-(1→3)-[(β-D-glucopyranosyl-(1→2)]-α-L-arabinopyranoside)

White amorphous powder.
^{13}C NMR: see Table 1.
ESI-MS (negative ion mode): *m/z* 911 [M-H]⁻ $C_{47}H_{76}O_{17}$.

ESI-MS-MS: MS^2 (911) *m/z* 749 [M-H-162]$^-$; MS^3 (749) *m/z* 587 [M-H-162-162]$^-$; MS^4 (587) *m/z* 455 [M-H-162-162-132]$^-$.

HR-ESI-MS (positive ion mode): *m/z* 935.4905 [M+Na]$^+$ (calcd. for $C_{47}H_{76}O_{17}Na$ 935.4980).

Acid hydrolysis: Saponin (2 mg) was refluxed in 2 N HCl (10 mL) for 3 h. The reaction mixture was extracted with CHCl$_3$. The remaining aqueous layer was neutralised with NaHCO$_3$ and evaporated to dryness. The residue was extracted with pyridine, which was then filtered and evaporated to dryness. Monosaccharides were identified by comparison with authentic samples on TLC (*i*-PrOH-H$_2$O 85:15), after detection with naphthoresorcin.

Saponins **1**, **3** and **5** furnished hederagenin, while saponins **2**, **4** and **6** furnished oleanolic acid. Saponin **1** gave arabinose as monosaccharide, while **2**, **3** and **6** gave arabinose and glucose; **4** and **5** gave glucose and rhamnose.

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