Saponins and Flavonoids from an Infusion of
Herniaria hirsuta

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Abstract
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Stone diseases present a major health problem in the Western society, since both urinary and biliary stones occur with a relatively high prevalence of 10–12% and 10–20%, respectively, and demonstrate a high recurrence rate. At the moment treatment is mainly based on interventional procedures, or prophylactic and dissolution therapy. However, many of the current drugs cause severe side effects, and therefore, there is an increasing interest in natural medicines. At the moment no registered herbal medicinal products are available for treatment of gallstones. Since an infusion of *Herniaria hirsuta* L. has a proven efficacy against urolithiasis and cholelithiasis, its phytochemical composition has been investigated. Two previously undescribed triterpene saponins, 28-O-{[$β$-D-xylopyranosyl-(1 → 4)-$α$-L-rhamnopyranosyl-(1 → 2)]-[$β$-D-glucopyranosyl-(1–6)]-$β$-D-glucopyranosyl}-medicagenic acid and 3-O-[$α$-L-rhamnopyranosyl-(1 → 3)-$β$-D-glucuronopyranosyl]-28-O-{[$β$-D-glucopyranosyl-(1 → 3)-$β$-D-xylopyranosyl-(1 → 4)]-[$β$-D-apiofuranosyl-(1 → 3)]-$α$-L-rhamnopyranosyl-(1 → 2)-$β$-D-fucopyranosyl}-medicagenic acid and three known flavonoids, quercetin-3-O-(2″-O-$α$-L-rhamnopyranosyl)-$β$-D-glucuronopyranoside, rutin, and narcissin (isorhamnetin-3-O-rutinoside), were isolated using flash chromatography and successive semi-preparative HPLC and were well characterized by MS and 1D and 2D NMR spectroscopic techniques. These findings could contribute to the development of a standardized extract that can be used in prophylaxis and treatment of gall and kidney stones.

Supporting information available online at http://www.thieme-connect.de/products

Introduction
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Both urinary and biliary stones have a relatively high prevalence of about 10–12% and 10–20%, respectively, in Western society and constitute a major health problem. In addition, the recurrence rate is rather high [1–6]. The treatment of urolithiasis is focused on interventional procedures, such as extracorporeal shock wave lithotripsy (ESWL), ureteroscopy (URS), or percutaneous nephrolithotomy (PNL) on the one hand; and prophylactic, dissolution, and medical expulsion therapy on the other hand. Various drugs are used in these therapies, such as thiazide diuretics, potassium citrate, allopurinol, UroPhos-K (a slow-release potassium phosphate preparation), sodium bicarbonate, D-penicillamine, acetohydroxamic acid, Tham E (an alkalinizing irrigant), alpha-blockers, calcium-channel blockers, and corticosteroids. However, many of these drugs cause severe side effects and up until today no drugs are available for clinical therapy, especially for the prevention and the recurrence of stones [7–9]. The cornerstone of the treatment of gallstone disease consists almost exclusively of cholecystectomy and endoscopic or medical treatment of complications and the use of drugs is still limited [3]. Medical dissolution therapy, mainly based on resolving the cholesterol supersaturation of the bile, is also a potential approach since almost 80% of the gallstones consist mainly of cholesterol and are caused by saturation of the bile with cholesterol [3, 10, 11]. In this way ursodeoxycholic acid (UDCA) is used to treat patients with moderate symptoms. All these treatments have their specific disadvantages: continuation of pain after cholecystectomy (in 20% of patients), a slow effect, low efficacy (40%) and possible stone recurrence for UDCA [12]. At the moment statins and ezetimibe are not yet used against
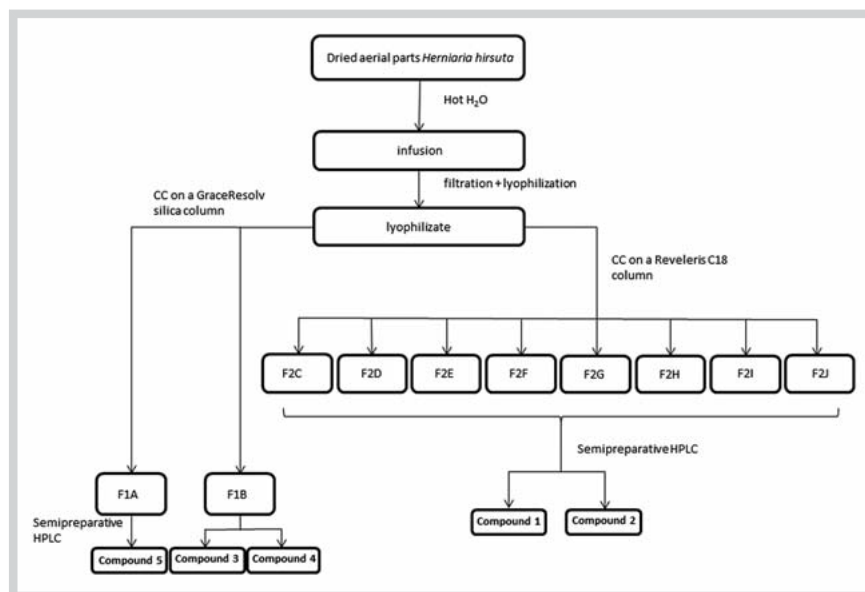
gallstones because their efficacy should still be proven, although they are well known to lower the hepatic synthesis and/or secretion and/or intestinal absorption of cholesterol and could probably influence the formation of cholesterol-based gallstones and promote their dissolution [13, 14].

During the last decades the interest in natural medicines has increased, because usually fewer side effects are expected with herbal medicinal products compared to many synthetic drugs [15]. At the moment no registered herbal product is available for treatment of gallstones.

The genus *Herniaria* (Caryophyllaceae) contains several species which are widely distributed in Europe, Asia and North-Africa. *H. glabra, H. hirsuta* and *H. fontanesii* are traditionally used in Moroccan folk medicine for the treatment of biliary dyskinesia, (uro)lithiasis or as a diuretic. In Europe Herniariae Herba, which can contain *H. glabra* and *H. hirsuta*, is used as a urological drug [16–20]. Some phytochemical research on these species revealed the presence of saponins, flavonoids, and coumarins. While *H. fontanesii* was reported to contain herniariasaponins A–D [21–23], which are bidesmosidic triterpenoid saponins, two monodesmosidic derivatives of medicagenic acid, herniariasaponins E and F, were isolated from the aerial parts of *H. hirsuta* [19]. Also *H. glabra* contained several mono- and bidesmosidic medicagenic acid derivatives, named herniariasaponins 1–7 [24–27]. Among others, herniarin and umbelliferone were identified as coumarins and quercetin- and isorhamnetin derivatives as flavonoids being present in *Herniaria* species [28–32].

Since an infusion of *H. hirsuta* has a proven efficacy against urolithiasis and cholelithiasis, this work focuses on the phytochemical characterization of this infusion using semi-preparative HPLC, mass spectrometry and NMR, finally aiming at the development of a standardized extract that can be used in the treatment and prophylaxis of stone diseases [16–18, 33].

Results and Discussion
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Normal phase flash chromatography of 7.3 g of the dry residue after lyophilization of the infusion of *H. hirsuta* resulted in two fractions of 78 mg (1 A) and 310 mg (1 B), respectively (○ **Fig. 1**). Semi-preparative HPLC of fraction 1 A resulted in the isolation of

compound **5** (1.36 mg), whereas semi-preparative HPLC of fraction 1B resulted in the isolation of compound **3** (3.5 mg) and **4** (1.9 mg). Fractions 2 C–J (3.0–38.0 mg) yielded compound **1** (9.0 mg) and compound **2** (25.5 mg).

Compound **1** (○ **Fig. 2**) was obtained as a white amorphous powder. The UV spectrum of **1** showed an absorbance maximum at 194 nm. The HR-ESI-MS mass spectrum showed a molecular ion peak at m/z 1103.5289 $[M - H]^-$ and supported a molecular formula of $C_{53}H_{84}O_{24}$. The ESI-MS spectrum of **1** exhibited a pseudo-molecular ion peak at m/z 1103 $[M - H]^-$. The fragmentation patterns are depicted in ○ **Fig. 3**. The m/z 1103 MS^2 product ion spectrum resulted in ion peaks at m/z 1085 $[M - H-18]^-$, 971 $[M - H-132]^-$, 941 $[M - H-162]^-$, and 923 $[M - H-180]^-$ due to the loss of a molecule of water (18 u), a pentose unit (132 u), a hexose unit (162 u), and a hexose unit together with a molecule of water (180 u), respectively. The product ion at m/z 501 $[M - H-162-132-162-146]^-$ attributed to the loss of two hexoses, a pentose unit, and a deoxyhexose unit (602 u) together with product ions formed at m/z 483 $[Aglycon-H-18]^-$ and 439 $[Aglycon-H-18-44]^-$ was characteristic for medicagenic acid as an aglycon [34]. The MS^3 product ion spectrum of m/z 971 $[M - H-132]^-$ resulted in a peak at m/z 809 $[M - H-162]^-$ due to the loss of a hexose moiety, whereas MS^3 analysis of m/z 941 $[M-H-162]^-$ resulted in a product ion peak at m/z 747 $[M-H-132-18-44]^-$ due to the loss of a pentose moiety together with a water and a carbon dioxide molecule. The formation of these latter MS^3 product ions together with the MS^2 spectra indicated that both residues were in a terminal position, suggesting the presence of a branched sugar chain.

The monosaccharides obtained after acidic hydrolysis of compound **1** were identified as D-glucose, D-xylose, and L-rhamnose by GC-MS [35] (see Materials and Methods).

The ^{1}H-NMR and ^{13}C-NMR spectra of the aglycon part of compound **1** displayed six tertiary methyl signals at δ_H 0.80 (H-26), 0.91 (H-29), 0.94 (H-30), 1.16 (H-27), 1.291 (H-25), and 1.33 (H-24) giving correlations in HSQC with δ_C 18.3 (C-26), 33.7 (C-29), 24.4 (C-30), 26.4 (C-27), 17.9 (C-25), and 13.3 (C-24), an olefinic proton at δ_H 5.29 (H-12), two olefinic carbons at δ_C 123.7 (C-12) and 145.0 (C-13) and two oxygen bearing methine protons at δ_H 3.98 (H-3) and 4.08 (H-2) (○ **Table 1**). Furthermore, a downfield shifted signal at δ_C 182.4 (C-23) and an upfield shifted carbon sig-

nal at δ_C 178.2 (C-28) suggested the presence of a carboxylic acid and an ester function, respectively. Thorough 1D and 2D NMR spectroscopic analysis confirmed the structure of the aglycon to be medicagenic acid (2β,3β-dihydroxyolean-12-ene-23,28-dioic acid). These findings were in accordance with the results obtained with ESI-MS experiments and with previously published data [19,22,24,36]. The presence of an upfield shifted carbon signal (C-28) at δ_C 178.2 suggested compound **1** to be a monodesmosidic saponin. Four anomeric proton signals at δ_H 5.44 (d, J = 1.5 Hz; Rha H-1), 5.38 (d, J = 7.6 Hz; GlcI H-1), 4.42 (d, J = 7.6; Xyl H-1), and 4.34 (d, J = 7.7; GlcII H-1) displaying correlations in the HSQC spectrum with four anomeric carbon signals at δ_C 101.3 (Rha C-1), 95.2 (GlcI C-1), 107.6 (Xyl C-1), and 104.9 (GlcII C-1), respectively, evidenced the presence of four sugar moieties. The complete assignment of the monosaccharides residues was performed by COSY, TOCSY, HSQC, and HMBC experiments and GC-MS analysis. This led to the identification of two β-D-glucopyranose moieties (GlcI and GlcII), a β-D-xylopyranose (Xyl) and a α-L-rhamnopyranose unit (Rha) (○ **Table 1**). The β-anomeric configuration of the glucopyranosyl and xylopyranosyl moiety and the α-anomeric configuration of the rhamnopyranosyl moiety were confirmed by the large $J_{H1, H2}$ coupling constants of 7–8 Hz and 1.5 Hz, respectively. The α-anomeric configuration was confirmed by comparison with the ^{13}C NMR values of methyl α-L-rhamnoside and methyl β-L-rhamnoside published by Agrawal et al. [37].

The HMBC cross peaks at δ_H/δ_C 5.44 (Rha H-1)/76.7 (GlcI C-2), 4.42 (Xyl H-1)/84.9 (Rha C-4), and 4.34 (GlcII H-1)/69.7 (GlcI C-6) suggested the sequence of the oligosaccharide moiety at C-28 to be [Xyl-(1→4)-Rha-(1→2)]-[GlcII-(1→6)]-GlcI-. Therefore, compound **1** could finally be elucidated as 28-O-{[β-D-xylopyranosyl(1→4)-α-L-rhamnopyranosyl(1→2)]-[(β-D-glucopyranoside-(1→6))-β-D-glucopyranosyl}-medicagenic acid, for which the name herniariasaponin G was adopted (○ **Figs. 2** and **3**).

Compound **2** (○ **Fig. 2**) was obtained as a white amorphous powder. The UV spectrum of compound **2** showed an absorbance maximum at 196 nm. The HR-ESI-MS mass spectrum showed a molecular ion peak at m/z 1541.6649 [M – H]$^-$ and supported a molecular formula of $C_{70}H_{110}O_{37}$. ESI-MS of **2** exhibited a pseudo-molecular ion peak at m/z 1541 [M – H]$^-$. The fragmentation patterns are depicted in Fig ○ **4**. The MS^2 product ion spectrum of m/z 1541 resulted in ion peaks at m/z 1523 [M – H-18]$^-$, 1395 [M – H-146]$^-$, and 1379 [M – H-162]$^-$, which indicated the presence of a terminal deoxyhexose and hexose moiety. Other product ions were generated at m/z 1219 [M – H-146–176]$^-$, 1201 [M – H-146–176–18]$^-$, 1157 [M – H-146–176–18–44]$^-$, 1087 [M – H-146–176–132]$^-$, 1057 [M – H-146–176–162]$^-$, 925 [M – H-146–176–162–132]$^-$, 823 [M – H-162+132+132+146+146]$^-$, and 717 [162 + 132 + 132 + 146 + 146-H]$^-$. The MS^3 product ion spectrum of m/z 1395 [M – H-146]$^-$ resulted in ion peaks at m/z 1377 [M – H-H$_2$O]$^-$, 1219 [M – H-176]$^-$, and 1157 [M – H-176–18–44]$^-$, suggesting a hexuronic moiety attached to a terminal deoxyhexose unit. MS^3 analysis of m/z 1219 [M – H-146–176]$^-$ resulted in product ion peaks at m/z 1201 [M – H – H$_2$O]$^-$, 1087 [M – H-132]$^-$, 1057 [M – H-162]$^-$, 925 [M – H-162–132]$^-$, 717 [162 + 132 + 132 + 146 + 146-H]$^-$, 501 [M – H-146–176–162–132–132–146–146]$^-$, and 483 [Aglycon-H-H$_2$O]$^-$. MS^3 analysis of m/z 1087 [M – H-146–176–132]$^-$ resulted in product ion peaks at m/z 1069 [M – H-H$_2$O]$^-$, 925 [M – H-162]$^-$, and 501 [M – H-146–176–162–132–132–146–146]$^-$, whereas MS^3 analysis of m/z 1057 [M – H-146–176–162]$^-$ resulted in product ion peaks at m/z 925 [M – H-132]$^-$, 647 [M – H-146–176–162–132–132–

1 R_1 = H
 R_2 =

2 R_1 =

 R_2 =

Fig. 2 Chemical structure of compounds **1** and **2** including key HMBC correlations.

146]$^-$, 629 [M – H-146–176–162–132–132–146–18]$^-$, and 501 [M – H-146–176–162–132–132–146–146]$^-$. The formation of product ions at m/z 1087 and m/z 1057, formed by the loss of a pentose and a hexose residue, respectively, together with their MS^3 spectra, showing a loss of a hexose for the ion at m/z 1087 and a loss of a pentose for the ion at m/z 1057, indicated that both residues are in a terminal position, indicating that the sugar chain was most probably branched. The MS^3 product ion spectrum of m/z 925 revealed peaks at m/z 501 [M – H-146–176–162–132–132–146–146]$^-$ and 483 [Aglycon-H-H$_2$O]$^-$.

GC-MS analysis of the trimethylsilylated monosaccharides obtained after acidic hydrolysis of compound **2**, resulted in the identification of D-glucose, D-xylose, D-fucose, L-rhamnose, and D-glucuronic acid (see Materials and Methods).

The ^{1}H-NMR and ^{13}C-NMR chemical shifts of the aglycon part of compound **2** (○ **Table 1**) were similar to those of compound **1** indicating that the same aglycon was present. The presence of an upfield shifted carbon signal and a deshielded carbon signal, respectively, at δ_C 178.2 (C-28) and δ_C 86.7 (C-3) suggested compound **2** to be a bidesmosidic saponin. Seven anomeric proton

signals at δ_H 5.37 (d, J = 1.21 Hz) (RhaII H-1), δ_H 5.35 (d, J = 8.15 Hz) (Fuc H-1), δ_H 5.24 (d, J =3.79) (Api H-1), δ_H 5.19 (d, J = 1.11 Hz) (RhaI H-1), δ_H 4.63 (d, J = 7.74) (Xyl H-1), δ_H 4.58 (d, J = 7.84) (Glc H-1), and δ_H 4.39 (d, J = 7.62 Hz) (GlcA H-1) displaying correlations in the HSQC spectrum with seven anomeric carbon signals at δ_C 101.5 (RhaII C-1), 95.4 (Fuc C-1), 112.0 (Api C-1), 102.4 (RhaI C-1), 105.12 (Xyl C-1), 105.3 (Glc C-1), and 105.09 (GlcA C-1), respectively, evidenced the presence of seven sugar moieties. They were identified, using the same techniques as for compound 1, as β-D-glucopyranosyl (Glc), β-D-xylopyranosyl (Xyl), α-L-rhamnopyranosyl (RhaI and RhaII), β-D-apiofuranosyl (Api), and β-D-fucopyranosyl (Fuc) moieties. The β-anomeric configuration of the Glc, Xyl, Api, Fuc, and GlcA moiety was confirmed by the large $J_{H1, H2}$ coupling constants of 7–8 Hz, respectively. The coupling constants of 1.1–1.2 Hz confirmed the α-anomeric configuration of the rhamnopyranosyl moiety. This α-anomeric configuration was also confirmed by comparison with the ^{13}C NMR values of methyl α-L-rhamnoside and methyl β-L-rhamnoside published by Agrawal et al. [37].

Linkages between both the aglycon and sugar units were mainly achieved by HMBC and are shown in ○ Fig. 2. A cross-peak in the HMBC spectrum from C-3 of the aglycon at δ_C 86.7 to the anomeric proton at δ_H 4.39 (GlcA H-1) indicated the linkage position of the β-D-glucuronic acid moiety. The downfield shifted signal of GlcA C-3 at δ_C 82.7 giving a HMBC correlation with the anomeric proton of RhaI at δ_H 5.19, suggested a substitution at this position. The chemical shifts of RhaI (○ Table 1) were those of a terminal Rha. Thus, the glycan part at C-3 of the aglycon was identified as Rha-(1→3)-GlcA-.

A deshielded signal of the anomeric proton of Fuc at δ_H 5.35 giving a correlation in the HSQC spectrum with a shielded anomeric carbon signal at δ_C 95.4 indicated the linkage of Fuc to the C-28 position of the aglycon through an ester linkage. A HMBC cross peak between δ_H 5.35 (Fuc H-1) and δ_C 178.2 (C-28) confirmed this finding. A cross-peak in COSY from Fuc H-1 (δ_H 5.35) to Fuc H-2 (δ_H 3.82) giving a correlation in the HSQC spectrum to Fuc C-2 (δ_C 74.9) together with long-range peaks in the HMBC spectrum between δ_C 101.5 (RhaII C-1) and δ_H 3.82 (Fuc H-2) and between the anomeric proton signal of RhaII at δ_H 5.37 and Fuc C-2 (δ_C 74.9) suggested a substitution at position 2 of Fuc with RhaII. The presence of two deshielded carbon signals of RhaII at C-3 (δ_C 81.6) and RhaII C-4 (δ_C 79.2) showing HMBC correlations with anomeric protons at δ_H 5.24 (Api H-1) and δ_H 4.63 (Xyl H-1), respectively, revealed a disubstitution of RhaII by Api at C-3 and Xyl at C-4. This was confirmed by a reverse correlation at δ_H/δ_C 3.65 (Rha H-4)/ 105.1 (Xyl C-1). The ^{1}H and ^{13}C NMR signals assigned by 2D NMR spectroscopic analysis were those of a terminal Api and Xyl [38].

The HMBC correlation at δ_H/δ_C 4.58 (Glc H-1)/88.2 (Xyl C-3) and the reverse correlation at δ_H/δ_C 3.47 (Xyl H-3)/105.3 (Glc C-1) indicated a 1→3 linkage between Glc and Xyl. Thus the oligosaccharide moiety at C-28 was identified as [Glc-(1→3)-Xyl-(1→4)]-[Api-(1→3)]-Rha-(1→2)-Fuc-. On the basis of the above results, compound 2 was finally elucidated as 3-O-[α-L-rhamnopyranosyl-(1→3)-β-D-glucuronopyranosyl]-28-O-{[β-D-glucopyranosyl-(1→3)-β-D-xylopyranosyl-(1→4)]-[β-D-apiofuranosyl-(1→3)]-α-L-rhamnopyranosyl-(1→2)-β-D-fucopyranosyl}-medicagenic acid, for which the name herniariasaponin H was adopted (○ Figs. 2 and 4).

Furthermore, three known flavonoids were isolated from the infusion of *H. hirsuta* and identified as quercetin-3-O-(2″-O-α-L-rhamnopyranosyl)-β-D-glucuronopyranoside (3), rutin (4), and narcissin (5) [28, 39].
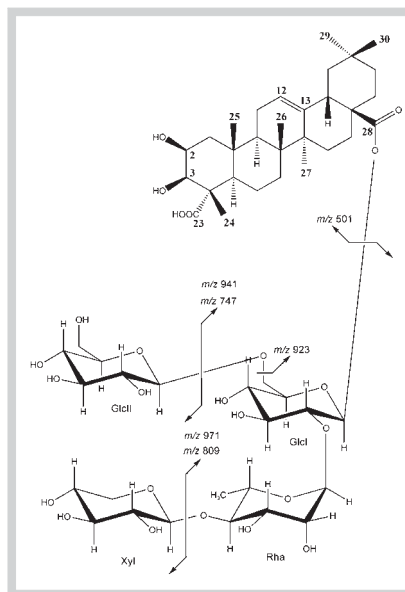

Fig. 3 MS² and MS³ fragmentations of compound 1.

The majority of saponins of the Caryophyllaceae contains as aglycone gypsogenic acid, gypsogenin, or quillaic acid. Saponins containing medicagenic acid as aglycon such as herniariasaponins are less frequently found [40]. To the best of our knowledge, until now thirteen herniariasaponins have been reported, from which only from *Herniaria hirsuta*, i.e. herniariasaponin E and herniariasaponin F [19]. Other herniariasaponins were also found in other species: herniariasaponins A–D were already reported from *Herniaria fontanesii* and herniariasaponins 1–7 were found in *Herniaria glabra* [21–27]. Compounds 1 and 2, for which the name herniariasaponin G and H, respectively, has been adopted, are reported here for the first time from nature. Both compounds 1 and 2 have medicagenic acid as aglycon, which is next to 16-hydroxymedicagenic acid one of the two aglycons found in herniariasaponins. Compound 1 is a monodesmosidic saponin which differs from herniariasaponin E by the absence of an acetylgroup in position 2 of the aglycon. Saponin 2 is a bidesmosidic saponin containing seven sugar moieties, one more than all other herniariasaponins found in nature, and is the largest herniariasaponin found until now. It also contains apiose as a sugar moiety, which is present as well in herniariasaponins 5 and 7.

Herniariae herba was reported to contain quercetin- and isorhamnetin derivatives such as narcissin and rutin [28, 29, 31]. In addition, narcissin (isorhametin-3-O-rutinoside) was isolated from *Herniaria glabra*, isorhamnetin 3-[3′″-feruloylrhamnosyl-(1→6)-galactoside], isorhamnetin 3-robinobioside and catechin were discovered in *Herniaria fontanesii*, and rhamnazin 3-rutinoside in *Herniaria ciliolata* [30, 32]. To the best of our knowledge, this is the first time that quercetin-3-O-(2″-O-α-L-rhamnopyranosyl)-β-D-glucuronopyranoside was isolated from *Herniaria hirsuta*.

Compounds 1–5 isolated during the present work constitute the main peaks in the chromatographic profile of the infusion of *Herniaria hirsuta* (Fig. S1, Supporting Information), proven to have a cholesterol lowering effect in the gall bladder of dogs [33]. Since the infusion is now fully characterized, it will be possible to develop a standardized herbal medicinal product that may be used in the treatment of gall and kidney stones. Other plant species containing medicagenic acid derivatives could possibly have a positive effect on gallstones, on the assumption that the saponins are the active constituents.

Table 1 ^{1}H NMR and ^{13}C NMR assignments of compounds **1** and **2**. [δ (ppm), J in Hz, CD$_3$OD, 400 MHz for ^{1}H and 100 MHz for ^{13}C].

(1)			(2)		
Aglycon	$δ_C$	$δ_H$	**Aglycon**	$δ_C$	$δ_H$
1	45.8	2.11, 1.221, m	1	45.1	2.10, 1.25, m
2	72.2	4.08, m	2	71.0	4.31, d (J = 2.3) of br s
3	76.6	3.98, d (3.8)	3	86.7	4.07, m
4	54.3		4	53.6	
5	53.0	1.586, m	5	53.3	1.59, m
6	22.2	1.65, 1.17, m	6	21.8	1.57, 1.20, m
7	33.9	1.49, 1.38, m	7	34.0	1.51, 1.38, m
8	41.2		8	41.2	
9	49.9	1.585, m	9	49.8	1.58, m
10	37.6		10	37.6	
11	24.8	2.02, 1.93, m	11	24.1	2.05, 1.62, m
12	123.7	5.29, t (3.2)	12	123.8	5.27, t (3.4)
13	145.0		13	144.8	
14	43.3		14	43.3	
15	29.4	1.55, 1.219, m	15	29.2	1.63, 1.197, m
16	24.1	2.06, 1.63, m	16	24.8	1.99, 1.91, m
17	50.2		17	48.1	
18	42.9	2.82, dd (14.0, 3.8)	18	43.1	2.83, dd (14.0, 4.0)
19	47.5	1.715, 1.14, m	19	47.4	1.73, 1.14, m
20	31.7		20	31.7	
21	35.0	1.40, 1.23, m	21	35.0	1.39, 1.23, m
22	33.2	1.723, 1.57, m	22	33.2	1.76, 1.57, m
23	182.4		23	182.4	
24	13.3	1.33, s	24	13.9	1.39, s
25	17.9	1.291, s	25	17.3	1.27, s
26	18.3	0.80, s	26	17.9	0.80, s
27	26.4	1.16, s	27	26.5	1.17, s
28	178.2		28	178.2	
29	33.7	0.91, s	29	33.6	0.91, s
30	24.4	0.94, s	30	24.3	0.93, s
GlcI			**GlcA**		
1	95.2	5.38, d (7.6)	1	105.09	4.39, d (J = 7.6)
2	76.7	3.62, m	2	75.9	3.39, m
3	79.4	3.56, m	3	82.7	3.55, m
4	71.1	3.47, m	4	72.3	3.51, m
5	77.8	3.49, m	5	76.6	3.71, m
6	69.7	4.10, 3.76 d (10.8); dd (11.8; 4.92)	6	175.7	
Rha			**RhaI**		
1	101.3	5.44, br s (1.5)	1	102.4	5.19, br s (J = 1.1)
2	72.0	3.95, m	2	72.5	3.92, m
3	72.4	3.845, m	3	72.4	3.70, m
4	84.9	3.51, m	4	74.3	3.36, m
5	68.9	3.82, m	5	69.9	4.07, m
6	17.5	1.285, s	6	18.0	1.23, s
Xyl			**Fuc**		
1	107.6	4.42, d (7.6)	1	95.4	5.35, d (J = 8.2)
2	76.4	3.181, m	2	74.9	3.82, m
3	78.5	3.31, m	3	76.6	3.71, m
4	71.3	3.45, m	4	73.8	3.57, m
5	67.4	3.84, 3.178, m	5	72.8	3.69, m
GlcII			6	16.7	1.22, s
1	104.9	4.34, d (7.7)	**RhaII**		
2	75.3	3.20, m	1	101.5	5.37, br s (J = 1.2)
3	78.1	3.37, m	2	72.1	4.088, m
4	71.7	3.30, m	3	81.6	3.84, m
5	78.1	3.24, m	4	79.2	3.65, m
6	62.9	3.850, 3.67, m	5	68.8	3.86, m
			6	18.6	1.24, s
			Api		
			1	112.0	5.24, d (J = 3.8)
			2	78.3	4.03, d (J = 3.9)
			3	80.2	
			4	74.9	4.091, m/3.76, d (J = 9.7)
			5	65.2	3.58, s

continued

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Table 1 *Continued*

(1)			(2)		
Aglycon	**δ_C**	**δ_H**	**Aglycon**	**δ_C**	**δ_H**
			Xyl		
			1	105.12	4.63, d (J = 7.7)
			2	75.3	3.33, m
			3	88.2	3.47, m
			4	70.2	3.59, m
			5	66.7	3.90, m/3.20, m
			Glc		
			1	105.3	4.58, d (J = 7.8)
			2	75.5	3.35, m
			3	77.8	3.46, m
			4	78.3	3.34, m
			5	71.6	3.30, m
			6	62.8	3.88, m/3.66, m

Material and Methods

▼

Reagents and standards

Methanol HPLC grade and acetonitrile Far UV HPLC grade were obtained from Fisher Scientific. Butanol (99%), formic acid (99+ %), acetic acid (99.8%), sulphuric acid 85% (g/g) and ethylacetate HPLC grade were provided by Acros. Hederacoside C (98.19% purity and 97.70% purity) was obtained from Extrasynthese and Carl Roth GmbH, herniarin (> 98% purity) from Carl Roth, α-hederin (99.35% purity) from Extrasynthese, and rutin (99.70% purity) from Extrasynthese and from Sigma (96% purity). *p*-Anisaldehyde,
L-(+) arabinose (min. 99%), L-rhamnose monohydrate (min. 99%), D-ribose (min. 99%), D-(+)-glucose (≥ 99.5%), α-fructose (min. 99%), L-(−)-fucose (≥ 99%), D-(+)-galacturonic acid, and L-(−)-xylose (min. 99%) were obtained from Sigma Aldrich, BSTFA + 1% TMCS from Thermo Scientific, pyridine (99+%), D-(+)-mannose (99+%), D-(+)-galactose, and glucuronic acid (98%) from Acros.

Plant material

Aerial parts from *Herniaria hirsuta* were collected in d'Oujda, Morocco. A voucher specimen of the plant is kept at the Muséum National d'Histoire Naturelle – Institut Scientifique-Université Mohamed V Agdal (Reference number: 5902).

Extraction and isolation

The material was air dried. The aqueous infusions of *Herniaria hirsuta* were prepared from 80 g in 4 L of boiling water as previously described [18]. The infusions were cooled, portions were combined, filtered, and lyophilized. Typically, 100 g of plant material yielded about 15 g lyophilizate.
Column chromatography: To obtain pure compounds of *H. hirsuta*, the infusion was first subjected to column chromatography. The separation was performed using a Gilson 306-pump coupled to a normal phase GraceResolv column (150 g, 40–63 μm) and the sample was applied on top (7.3 g). Elution of the column was performed subsequently with dichloromethane, ethyl acetate and methanol. Fractions were collected in volumes of 30 mL. Subsequently reversed phase flash chromatography was carried out using a Reveleris Flash Chromatography system equipped with a Reveleris C18 column (40 g, 40 μm). Compounds were eluted with water and methanol using the following gradient: Solvent A: H_2O, solvent B: CH_3OH: 5% B for 8 min, from 5% to 100% B in

82 min, 100% B for 5 min; fractions of 25 mL were collected. All fractions were monitored by thin layer chromatography (TLC). Every fraction was applied on a normal phase-TLC plate (NPTLC; 20 × 20 cm, silica gel 60 F$_{254}$, Merck), and subsequently the plate was developed with the organic phase of a *n*-BuOH/CH$_3$COOH/ H$_2$O (13 : 3 : 5) mixture, sprayed with anisaldehyde-sulphuric acid reagent and heated to 105 °C. Based on the observed TLC pattern, similar fractions were combined.
Semi-preparative HPLC: Fraction 1 A (eluted with 90% ethyl acetate and 10% methanol, 78.8 mg), and fraction 1B (eluted with 30% ethyl acetate and 70% methanol, 255.6 mg), obtained by normal phase chromatography, and fractions 2 C–2 J (eluted with a gradient of water and methanol see below), obtained with reversed phase chromatography, were separated by repeated semi-preparative HPLC. An Agilent 1200 Series HPLC with degasser, quaternary pump, automated liquid sampler, thermostatic column compartment, and diode array detector (DAD; Agilent Technologies) and a Gilson 322 HPLC with binary pump, automated liquid sampler, and UV detector (Gilson) were used for the isolation of the flavonoids. Saponins were isolated using an autoPurification system from Waters with a binary gradient module, sample manager, make-up pump, system fluidics organizer, diode array detector, and Micromass Quattro micro TQD-MS analyzer. Fractions were separated using an Apollo column (Grace; 250 × 10 mm, 5 μm). Separation was optimized for all fractions and the following gradients were applied: solvent A: H$_2$O + 0.05% HCOOH; solvent B: CH$_3$OH + 0.05% HCOOH, gradient fraction 1 A: from 50% to 70% B in 20 min, from 70% to 95% in 2 min; gradient fraction 1 B: From 15% to 67% B in 45 min, from 67% to 100% B in 1 min; fractions C–J: 60% B for 5 min, from 60% to 100% B in 30 min, 100% B for 3 min – from 100% to 60% B in 1 min, 60% B for 5 min. Flow rate for all gradients: 3 mL/min; 210 nm and 320 nm detection; injection volume: Fraction 1 A and 1B: 450 μL, fraction 2 C, 2 F, 2 G and 2 H: 600 μL, fraction 2 D, 2 E, 2I and 2 J: 300 μL; concentration, fractions 1 A: 10 mg/ mL and 1B: 30 mg/mL; fractions 2 C-2 J: 1.8–10 mg/mL.
Structure elucidation: LC-MS analysis of the infusion of *H. hirsuta* (10 mg/mL, 80% CH$_3$OH) was performed employing a Surveyor LC system equipped with a diode array detector (Thermo Fisher) and a Grace Smart column (250 × 4 mm, 5 μm). The flow rate was 1.0 mL/min, UV detection was carried out at 210, 254, 300, and 320 nm and the solvent program was as follows: solvent A: H$_2$O + 0.05% HCOOH; solvent B: CH$_3$OH + 0.05% HCOOH; from 5% to 100% B in 60 min, stay at 100% during 2 min. The injection

volume was 20 μL. The LC system was coupled to an LXQ linear ion trap (Thermo Fisher). The experimental data were recorded in the (−)-ESI mode using following conditions: Sheath gas flow: 65 arbitrary units; auxiliary gas flow: 14 arbitrary units; source voltage: 4.0 kV; ion transfer tube temperature: 350°C; and capillary voltage: − 10 V. Mass spectral data were recorded using data dependent scanning in the mass range m/z 150–1800. For MS^n experiments an isolation width of 2 Da was used and normalized collision energy of 35% was applied. All data were acquired and processed using Xcalibur software, version 2.0. (Thermo Fisher). Accurate mass measurements were carried out using a Orbitrap mass spectrometer (Exactive, Thermo Fisher Scientific) equipped with a with an Ion Max ESI source (Thermo Fisher Scientific) in negative ion mode. Chromatographic parameters were used as described in De Paepe et al. [41].

To obtain information about the identity of the sugar moieties of the two main saponins, GC-MS analysis was performed. Compounds **1** and **2** (1.02 mg) were hydrolyzed using 1 mL TFA (2 M) and samples were kept in an oven at 120°C for 5 h.

Samples obtained from acid hydrolysis and all reference monosaccharides [D-ribose, L-rhamnose monohydrate, L-arabinose, L-xylose, D-glucuronic acid, D-galacturonic acid, D-glucose, D-fructose, L-fucose, D-galactose, and D-mannose (100 μg)] were treated with pyridine and the trimethylsilylation reagent (BSTFA + 1% TMCS) and heated for 1 h at 70°C. All samples were analyzed according to Medeiros and Simoneit [35] employing a Voyager GC-MS with Trace 2000 GC (Thermo Finnigan) and a Alltech Heliflex AT-5 ms capillary column with a length of 30 m, internal diameter of 0.25 mm and film thickness of 0.25 μm (Alltech Associates). The carrier gas He was used at a constant flow rate of 1.3 mL/min, the injector and MS source temperature were maintained at 200°C and 230°C, respectively. The temperature program was as follows: start at 65°C, hold on 65°C for 2 min, temperature increase of 6°C/min till 300°C, hold on 300°C for 15 min. The MS was operated with an ionisation energy of 70 eV. Chromatograms were recorded using selected ion monitoring (SIM), looking for prominent mass peaks at m/z 204 and 217. About 1 μL of every sample was analyzed with a split ratio of 8. All data were recorded and processed using Xcalibur software, version 1.0 (Thermo Fisher). The monosaccharide units present in the hydrolyzed sample were elucidated by means of the retention time of the reference sugars. The absolute configuration was done based on their most abundant configuration in nature. L-rhamnose monohydrate (19.53 min.; 20.72 min.), L-xylose (21.08 min.; 22.02 min.), D-glucuronic acid (25.67 min.; 26.52 min.), D-glucose (24.29 min.; 25.80 min.), and L-fucose (20.24 min.; 20.99 min.).

NMR Spectra were recorded in DMSO-d_6 (flavonoids) and methanol-d_4 (saponins) on a Bruker DRX-400 instrument, operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C. Chemical shifts are expressed in ppm and coupling constants (J) in Hz. Specific rotation was determined on a Jasco P-2000 digital polarimeter.

28-O-{[β-D-xylopyranosyl(1→4)-α-L-rhamnopyranosyl(1→2)]-[(β-D-glucopyranosyl-(1→6)]-β-D-glucopyranosyl}-medicagenic acid (herniariasaponin G) (**1**): White powder; $[α]_D^{20}$ 0.99 (*c* 0.35, MeOH); ^{1}H-NMR and ^{13}C-NMR: See ○ **Table 1**. ESI-MS m/z 1103 [M − H]⁻, MS² m/z 1103 [M − H]⁻ m/z 1085, 971, 941, 923, 501, 483, and 439, MS³ m/z 971 [M − H-132]⁻ m/z 953, 809, 501, 483, 439 MS³ m/z 941 [M − H-162]⁻ m/z 923, 747, 501, 483, 439 HR ESIMS m/z 1103.5289 [M − H]⁻ (calcd. for $C_{53}H_{83}O_{24}$ 1103.5274).

3-O-[α-L-rhamnopyranosyl-(1→3)-β-D-glucuronopyranosyl]-28-O-{[β-D-glucopyranosyl-(1→3)-β-D-xylopyranosyl-(1→4)]-

Fig. 4 MS² and MS³ fragmentations of compound **2**.

[β-D-apiofuranosyl-(1→3)]-α-L-rhamnopyranosyl-(1→2)-β-D-fucopyranosyl}-medicagenic acid (herniariasaponin H) (**2**): White powder; $[α]_D^{20}$ −35.34 (*c* 0.30, MeOH); ^{1}HNMR and ^{13}C-NMR: See ○ **Table 1**. ESI-MS m/z 1541 [M − H]⁻, MS² m/z 1541 [M − H]⁻ m/z 1523, 1395, 1379, 1219, 1201, 1157, 1087, 1057, 925, 823, 717, MS³ m/z 1395 [M − H-146]⁻ m/z 1377, 1219, and 1157, MS³ m/z 1219 [M − H-146−176]⁻ m/z 1201, 1087, 1057, 925, 717, 501, 483. MS³ m/z 1087 [M − H-146−176−132]⁻ m/z 1069, 925, and 501, MS³ m/z 1057 [M − H-146−176−162]⁻ m/z 925, 647, 629, and 501, HR ESIMS m/z 1541.6649 [M − H]⁻ (calcd. for $C_{70}H_{109}O_{37}$ 1541.6648).

Supporting information

All ^{1}H- and ^{13}C-NMR as well as 2D-NMR spectra and a HPLC profile of the infusion of *H. hirsuta* are available as Supporting Information.

Acknowledgements

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Conflict of Interest

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The authors declare they have no conflict of interest.

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