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Short communication

A convenient method for saponin isolation in tumour therapy

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ABSTRACT

Saponinum album (Merck), which is a crude mixture of saponins from *Gypsophila paniculata* L., was shown to improve the anti cancer therapy when used *in vivo* in combination with saporin-based targeted toxins. Unfortunately saponinum album cannot be used for further development since Merck has ceased its production in the 1990s. As pure saponins are mandatory for use in medical purposes we developed a convenient method for saponin isolation directly from the roots of *Gypsophila paniculata* L. The developed method is rapid, cheap and scaling up is also possible. By combining dialysis and HPLC three saponins were isolated in a one-step procedure. Chemical structures of the purified saponins were characterized by extensive one and two-dimensional NMR-spectroscopy and by using ESI-TOF-MS. The biological activities of the purified saponins were also investigated. The method presented herein enabled a rapid and cheap isolation of saponins for tumour therapy.

1. Introduction

Saponins are a widespread class of natural compounds which are found in a lot of plant species. Saponins consist of a hydrophobic triterpenoidal C30 or steroidal C27 backbone and one or two hydrophilic glycoside moieties attached to the backbone. Due to this structural composition, saponins are amphiphilic glycoconjugates which give soap-like foams in water.

Saponins can be further divided in monodesmosidic saponins with one glycoside moiety and bisdesmosidic saponins with two glycoside moieties. With an increasing number of single saccharid units of the glycoside moieties, saponins gain a high structural complexity. According to Oleszek and Bialy [1], the separation of individual saponins is still complicated and time consuming.

There are only few medical applications for saponins. However, there are some prominent saponins like *Quillaja* saponins from *Quillaja saponaria* MOL. which are used as adjuvants for vaccination. As part of the ISCOM (Immune stimulating complex) *Quillaja* saponins were shown to promote the antibody and immune response [2]. Another example for a medical application of saponins is saponinum album (Merck). Saponinum album (Merck) is a complex mixture of triterpenoid saponins from *Gypsophila paniculata* L. which used to be commercially available. Saponins from saponinum album (Merck) consist of a hydrophobic C30 backbone agylcon, attached to one or two glycosylated chain units. It was shown that saponinum album (Merck) enhanced the cytotoxicity of the type I ribosome-inactivating protein saporin from *Saponaria officinalis* L. 100,000-fold [3]. The interaction between saporin and saponinum album (Merck) was coined as the "synergistic cytotoxicity-principle" [3]. The principle of the synergistic cytotoxicity was successfully adapted for saporin-based targeted toxins which are used in tumour therapy. The combination of the saporin-based chimeric anti-tumour toxin (SA2E) with saponinum album (Merck) (used for the combinatorial anti cancer therapy) resulted in 94% tumour regression in mice, despite the fact that the amount of chimeric toxin, was drastically reduced, compared to treatment with pure SA2E [4]. This represents an enormous improvement of the tumour therapy with SA2E since the side effects and the costs for such a therapy could be drastically lowered [4].

As mentioned earlier saponinum album is a crude mixture of triterpenoid saponins. These saponins consist of the hydrophobic C30 backbone aglycon with a formyl group linked to the C4 of the aglycon. At C3 und C28, the aglycon is further attached to branched carbohydrate chains.

We have recently isolated a pure saponin (saponin-1641) from saponinum album (Merck) which showed strong enhancing, cyto-

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toxic properties for the saporin-based chimeric toxin SE [5]. In principle saponin-1641 is a promising candidate for the combinatorial anti cancer therapy. However as in 1990 Merck has ceased the production of saponinum album, saponin-1641 was isolated from older batches, still in our possession in our laboratory.

For the successful development of a combinatorial anti cancer therapy in humans the saponins have to be isolated directly from the roots of *Gypsophila paniculata* L. In contrast to the conventional isolation of *Gypsophila* saponins, involving several chromatographic steps [6], the desired isolation method should be cheap, time saving and the scaling up to preparative mode should be possible. All these requirements were realized by using chemical degradation of the plant raw extracts, followed by extensive dialysis and HPLC purification.

By this method we were able to isolate three saponins in a one-step separation procedure. All structures were elucidated by using extensive one and two-dimensional NMR-spectroscopy and by ESI-TOF-MS. A new saponin (*Gypsophila* saponin **3**) was further characterized by FT-IR-spectroscopy and polarimetry. All the isolated saponins showed toxicity enhancing properties on saporin at a non-cytotoxic saponin concentration.

2. Materials and methods

2.1. Plant material

Roots of *Gypsophila paniculata* L. (Saponariae alb. Rad. Conc., batch number: 6184) were purchased from Galke (Gittelde, Germany).

2.2. General experimental procedures

HPLC was performed with a Wellchrom HPLC pump K-1001, using a Wellchrom solvent organizer K-1500 and a Wellchrom DAD K-2700, (Knauer, Berlin) diode detector. The optical rotations were measured with a Perkin Elmer 241 MC-Polarimeter. The IR spectra were recorded with a Perkin Elmer 100 FT-IR Spectrometer. For measuring the $^{1}H/^{13}C$ NMR analyses, the samples were prepared by dissolving 8 mg of *Gypsophila* saponin **1**, **2** or **3** in 600 μL pyridine-d_5 to yield a final concentration of about 8×10^{-3} mol/L. All measurements were performed using 5 mm sample tubes. The NMR spectra were recorded on a Bruker DRX600 NMR spectrometer and a Bruker AV600 NMR spectrometer using a 5 mm inverse quadruple resonance probe equipped with a one-axis self shielded gradient coil and a 5 mm cryogenic inverse triple resonance probe equipped with a one-axis self shielded gradient coil, respectively. XWINNMR and topspin were used as the acquisition software. The assignments were based on DQF-COSY, TOCSY, HSQC-TOCSY, and HMBC experiments at 600 MHz/150 MHz. For all electrospray ionization mass spectrometry (ESI-MS) analyses, an ESI-TOF-MS instrument Agilent 6210 (Santo Clara, USA) was used in the negative ion mode. All parameters were adjusted for producing maximum abundance of the deprotonated molecule $[M\text{-}H]^-$.

2.3. Extraction and isolation of the saponins

Roots of *Gypsophila paniculata* L. (500 g) were crushed and powdered. The saponins were extracted by stirring the suspension in 90% methanol (5 L) at room temperature. The methanol solution was evaporated at 100 mbar, 40 °C. The remaining syrup was kept at 4 °C for 24 h. The suspension was then filtered to remove water-insoluble tannins and other insoluble compounds. Thereafter, the solution was fractionated (10×50 mL) and kept in the freezer. In order to hydrolyse crufts like water soluble tannins each 50 mL-fraction was adjusted with 30 mM sodium hydroxide to pH ~ 11. The chemical degradation with NaOH reduced the matrix complexity of the plant raw extract which facilitated an increased separation efficiency of the *Gypsophila* saponins by HPLC.

The solution was swirled 24 h at 40 °C and filtered through a Nalgene™ Disposable Filterware (0.45 μm). To remove the low molecular compounds the solution was dialyzed (MWCO 1000) against distilled water and finally freeze dried. A pre-purified saponin mixture (~300 mg) was obtained.

After dissolving in 20% methanol, the saponins (0.5 mL, 40 mg) were subjected to an UltraSep ES PHARM RP18E (7 μm, 250×8 mm) column from SepServ (Berlin, Germany). Elution was performed with a gradient of methanol (A)/trifluoroacetic acid in water (0.01%) (B) starting with 20% A to 70% A over 60 min. The flow rate was 1.5 mL/min. The separated saponins, which had a retention time of 37 min (*Gypsophila* saponin **1**), 39 min (*Gypsophila* saponin **2**) and 40 min (*Gypsophila* saponin **3**), were collected and analysed by ESI-TOF-MS. Finally methanol was evaporated at 100 mbar, 40 °C and the saponins were freeze dried. For further experiments ~30 mg of *Gypsophila* saponin **1** and ~10 mg each of *Gypsophila* saponins **2** and **3** were isolated.

2.4. Cytotoxicity experiments

The ECV-304 cells (DSMZ-No. ACC 304) were purchased from the German Cell Culture Collection (Braunschweig, Germany). Saporin, XTT and phenazine methosulfate (PMS) were purchased from Sigma–Aldrich (Steinheim, Germany).

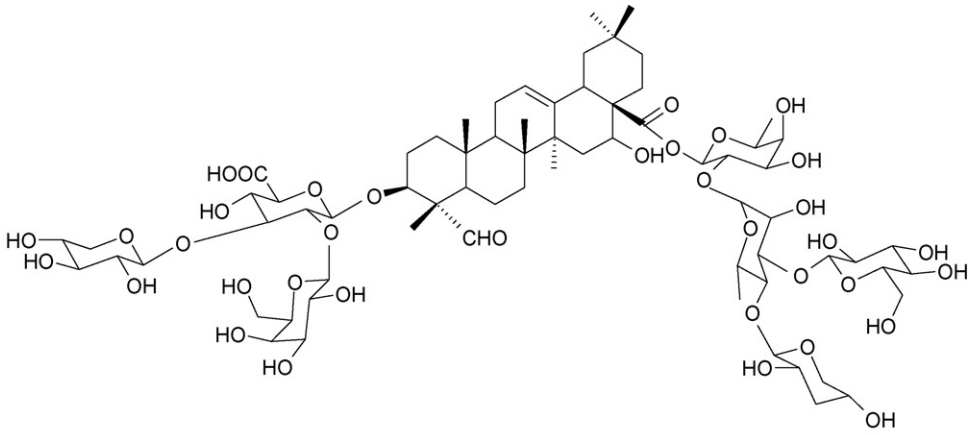
Cytotoxicity was determined in a cell viability assay using the XTT reduction test [7]. In brief, the ECV-304 cells were plated in a 96-well plate at a density of 2000 cells per well in 100 μL modified Eagle's medium (MEM) without phenol red supplemented with 15% fetal bovine serum (FBS).

Saporin (6 nM) with or without *Gypsophila* saponins **1**–**3** (20 μg/mL) was added and cells were incubated for 72 h. For the analysis of cytotoxicity 50 μL (1 mg/mL) XTT solution containing 8 μg/mL PMS, was added to each well and the cells were incubated for 3 h at 37 °C. The absorbance of the produced formazan was measured at 580 nm in a microplate reader (Tecan Spectra Fluor, Germany).

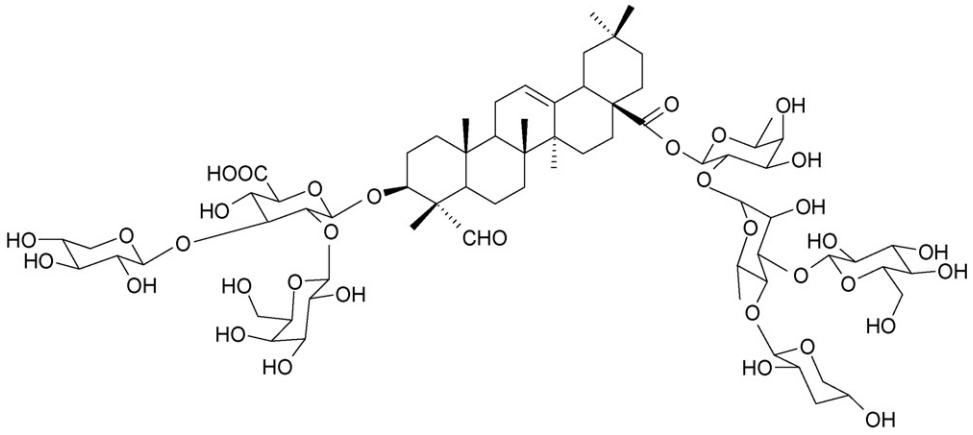
3. Results and discussion

3.1. Separation of saponins

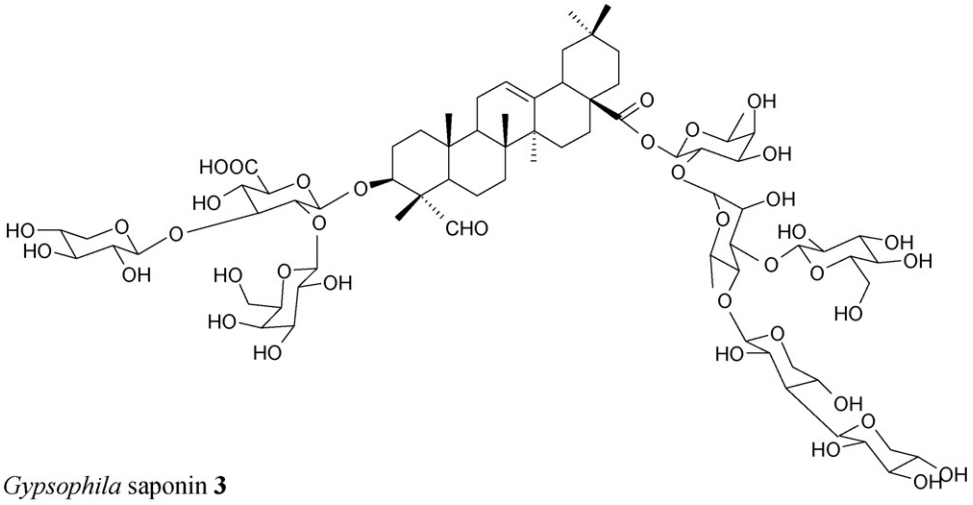
In this study we have isolated three saponins from *Gypsophila paniculata* L. by combining dialysis with HPLC. *Gypsophila* saponin **3** (3-O-β-D-galactopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucuronopyranosyl gypsogenin 28-O-β-D-glucopyranosyl-(1 → 3)-[β-D-xylopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)]-α-L-rhamnopyranosyl-(1 → 2)-β-D-fucopyranoside was isolated for the first time. *Gypsophila* saponin **1** (3-O-β-D-galactopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucuronopyranosyl quillaic acid 28-O-β-D-glucopyranosyl-(1 → 3)-[β-D-xylopyranosyl-(1 → 4)]-α-L-rhamnopyranosyl-(1 → 2)-β-D-fucopyranoside and *Gypsophila* saponin **2** (3-O-β-D-galactopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucuronopyranosyl gypsogenin28-O-β-D-glucopyranosyl-(1 → 3)-[β-D-xylopyranosyl-(1 → 4)]-α-L-rhamnopyranosyl-(1 → 2)-β-D-fucopyranoside) (Fig. 1) were isolated previously, using a time consuming and expensive method [6]: In this method, following solvent extraction of the plant material, the crude extract was separated on a RP-18 column for 8 h followed by HPLC analysis on a Nucleosil 120-C18 column. The obtained fractions were further



Gypsophila saponin **1**



Gypsophila saponin **2**



Gypsophila saponin **3**

Fig. 1. Structures of three isolated *Gypsophila* saponins (**1**, **2** and **3**) from the roots of *Gypsophila paniculata* L.

separated on a RP-8 column. This was followed by semipreparative HPLC with a Nucleosil 100-C18 column. Thereafter the saponins were further separated on a RP-18 column.

In the present study we were able to isolate *Gypsophila* saponin **1**, **2** and **3** by simple dialysis followed by one-step semipreparative HPLC. The improvement of the separation of the saponins by HPLC can be seen by comparing the HPLC-chromatograms of the raw extracts after 0 h (Fig 2A), 24 h (Fig. 2B) and 72 h (Fig. 2C) dialysis. With increasing dialysis time the resolution between the peaks increased and the width of the peaks decreased. The saponins with retention time 37 min (*Gypsophila* saponin **1,** Fig. 1), 39 min (*Gypsophila* saponin **2,** Fig. 1) and 40 min (*Gypsophila* saponin **3,** Fig. 1) could be collected in high purity (ESI-TOF-MS spectra of the *Gypsophila* saponins **1**–**3** are shown in Supporting information).

The problem with the conventional method [6] for the isolation of *Gypsophila* saponins was the presence of the undesired high matrix complexity of the raw extract after solvent extraction of the plant material. In order to reduce this matrix complexity, several chromatographic techniques like classical column chromatography were necessary prior to the saponin isolation [6]. This inconve-

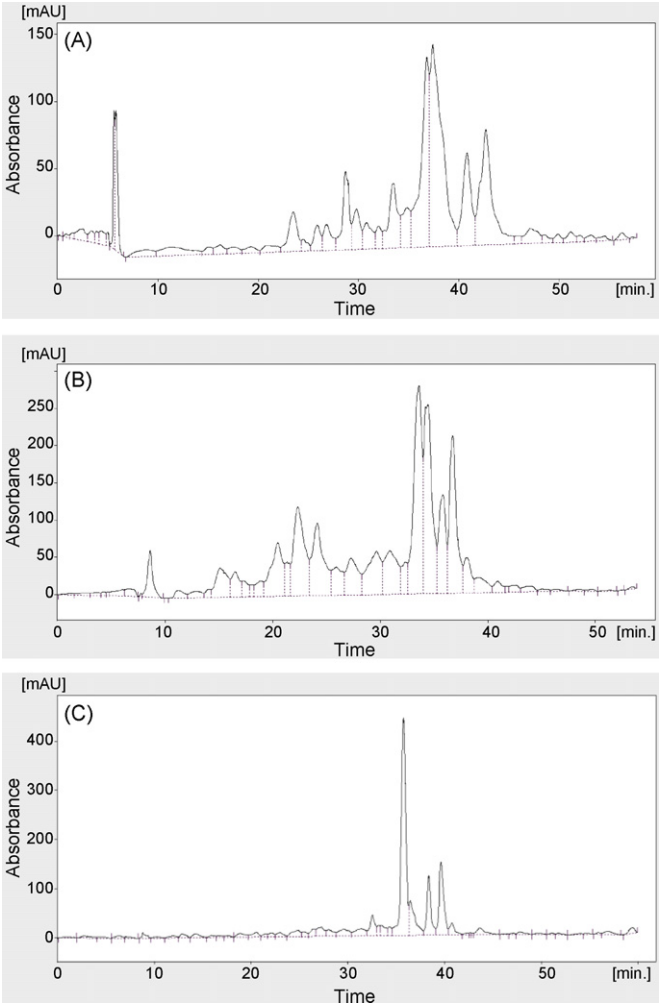


Fig. 2. HPLC-chromatogram of the plant raw extracts after mild basic treatment (30 mM NaOH) followed by 0 h (A), 24 h (B) or 72 h (C) dialysis. The separation efficiency of the saponins was improved by increasing the dialysis time.

nience rendered the method to become a labour-intensive and costly procedure. In view of the joint therapeutic application of anti cancer toxins with saponins, such elaborate purification methods are unfavorable under GLP (Good Laboratory Practice) conditions, which is mandatory for medical purposes.

To circumvent these problems we have first reduced the matrix complexity of the raw extract by mild basic hydrolysis (chemical degradation) and removed the low molecular compounds by extensive dialysis.

Thereafter the saponins were isolated by semipreparative HPLC. It is obvious that the presented method is advantageous compared to the classical isolation of *Gypsophila* saponin **1** and **2** as described above. By using our described method, saponins can be easily isolated under GLP conditions. The method is therefore favorable for the industrial isolation of pure saponins for tumour therapy.

3.2. Cytotoxicity assay

To scrutinize the toxicity enhancing properties on saporin, *Gypsophila* saponins **1**, **2** and **3** were individually incubated with or without saporin (6 nM). All saponins showed toxicity enhancing properties on saporin without causing toxicity by themselves at 20 μg/mL (Fig. 3) as indicated by the comparison to the toxicity of saporin alone.

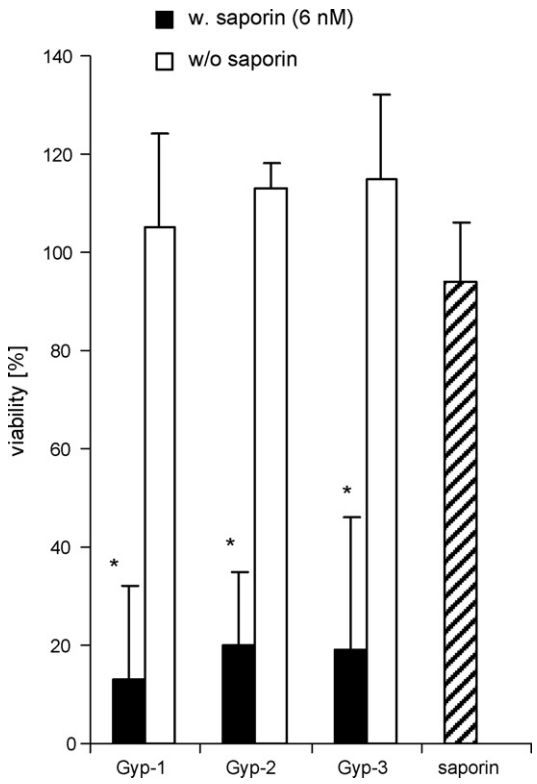


Fig. 3. ECV-304 cells were incubated with *Gypsophila* saponin **1** (Gyp-1, 20 μg/mL)/saporin (6 nM), *Gypsophila* saponin **2** (Gyp-2, 20 μg/mL)/saporin (6 nM) or *Gypsophila* saponin **3** (Gyp-3, 20 μg/mL)/saporin (6 nM) for 72 h. Cells were also incubated with either *Gypsophila* saponin **1**, **2** or **3** alone (each 20 μg/mL), or saporin (6 nM) alone. Cytotoxicity was determined by XTT-assays. Neither saporin nor *Gypsophila* saponin **1**, **2** or **3** caused toxicity on ECV-304 cells. However the combination of both was highly toxic, indicating a cytotoxic synergism. * Significant to control (*U*-test: $p \leq 0.05$).

3.3. Structure elucidation

ESI-TOF-MS of *Gypsophila* saponin **1** (Fig. 1) showed a deprotonated molecule at *m/z* 1541,66 $[M\text{-}H]^-$ and was identified by comparison of its spectroscopic data with literature values as 3-O-β-D-galactopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucuronopyranosyl quillaic acid 28-O-β-D-glucopyranosyl-(1 → 3)-[β-D-xylopyranosyl-(1 → 4)]-α-L-rhamnopyranosyl-(1 → 2)-β-D-fucopyranoside, already obtained previously from *G. paniculata* and *G. arrostii* [6]. *Gypsophila* saponin **2** (Fig. 1) displayed an ESI-TOF-MS spectrum containing the deprotonated molecule *m/z* 1525, 66 $[M\text{-}H]^-$. It was identified as the 3-O-β-D-galactopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucuronopyranosyl gypsogenin 28-O-β-D-glucopyranosyl-(1 → 3)-[β-D-xylopyranosyl-(1 → 4)]-α-L-rhamnopyranosyl-(1 → 2)-β-D-fucopyranoside [6].

The ESI-TOF-MS of *Gypsophila* saponin **3,** recorded in the negative mode, gave a deprotonated molecule at *m/z* 1657,71 $[M\text{-}H]^-$ in the HR-ESI-TOF. The difference of 132 mass units compared to *Gypsophila* saponin **2** led to the assumption of an additional pentose moiety. The 2D-NMR study of the aglycon moiety, allowed the assessment of the protonated carbon resonances using the HSQC experiment. In combination with the results obtained from the high-resolution DQF-COSY experiment, which establishes correctly the proton coupling network, all the proton and the protonated carbon resonances were unambiguously assigned. The quaternary carbons were identified using the assigned protonated carbon resonances by means of a long-range proton–carbon correlation experiment (HMBC). By comparison

Table 1
^{1}H and ^{13}C NMR data (δ in ppm) of the aglycon part of compound **3** (pyridine-d_5).

Atom No.	δ_H	δ_C
1	1.34 m 0.80 m	37.8
2	1.79 m 2.06 m	25.1
3	4.02 m	84.5
4	–	55.0
5	1.39 m	48.4
6	1.12 m 1.49 m	20.4
7	1.46 m 1.63 m	32.1
8	–	40.2
9	1.65 m	47.8
10	–	36.2
11	1.77 m 1.83 m	23.2
12	5.33 m	122.1
13	–	144.0
14	–	41.9
15	1.26 m 2.02 m	27.9
16	2.04 m 1.81 m	23.2
17	–	47.5
18	3.08 brd (14 Hz)	41.8
19	1.18 m 1.71 m	46.0
20	–	30.5
21	1.31 m 1.17 m	33.5
22	1.65 m 1.98 m	32.0
23	9.97 s	210.5
24	1.47 s	10.8
25	0.80 s	15.4
26	1.04 s	16.8
27	1.20 s	25.6
28	–	176.1
29	0.85 s	32.8
30	0.86 s	23.2

with literature data [6,8] the aglycon was identified as gypsogenin. The ^{1}H and ^{13}C NMR of the signals of *Gypsophila* saponin **3** are listed in Table 1.

The *Gypsophila* saponin **3** was shown to contain eight glycosyl residues from the HSQC spectrum. The ^{1}H NMR anomeric signals at δ 5.96, 5.79, 5.56, 5.53, 5.37, 5.32, 5.14, and 4.88 ppm were correlated with the ^{13}C NMR signals at δ 94.9, 102.0, 104.0, 104.1, 105.2, 104.8, 105.8, and 103.9 ppm, respectively. The complete assignment of each glycosyl resonance was achieved by measuring the HSQC-TOCSY and ^{1}H–^{1}H COSY spectra. The data showed high similarity to those of *Gypsophila* saponin **2** allowing the identification of one glucuronopyranosyl (GlcA), one xylopyranosyl (Xyl_1) and one galactopyranosyl (Gal) residue (assignments in Table 2) attached to the hydroxyl group in position 3 of gypsogenin aglycone. The cross peaks in the HMBC experiment between H-1 of glucuronic acid (δ 4.88 ppm) and C-3 of the aglycone (δ 84.5 ppm) showed that the glucuronic acid was attached to the aglycone at the C-3 position. Sugar chain was established from the following HMBC-correlations: H-1 of xylose at δ 5.32 ppm and C-3 of glucuronic acid at δ 85.8 ppm, H-1 of galactose at δ 5.56 ppm and C-2 of glucuronic acid at δ 78.2 ppm. Thus the remaining sugars had to be attached to the C-28 position of gypsogenin aglycone, which was supported by

Table 2
^{1}H and ^{13}C NMR data (δ in ppm) of the glycosidic part of compound **3** (pyridine-d_5)[a].

		3-O-sugars				28-O-sugars	
		δ_H	δ_C			δ_H	δ_C
GlcA	1	4.88 d (7.2)	103.6	Fuc	1	5.96 d (8.3)	94.9
	2	4.34	78.2		2	4.39	75.8
	3	4.25	85.8		3	4.08	75.0
	4	4.44	71.3		4	3.92	72.5
	5	4.49	77.1		5	3.82	72.2
	6		171.5		6	1.43 d (6.0)	16.9
Gal	1	5.56 d (7.7)	104.0	Rha	1	5.79 brs	102.0
	2	4.48	73.7		2	5.26 brs	70.7
	3	4.14	75.3		3	4.92 d (7.0)	82.3
	4	4.54	70.0		4	4.52	78.2
	5	3.99	76.7		5	4.42	68.9
	6	4.40/4.51	61.2		6	1.65 d (6.0)	19.0
Xyl_1	1	5.32 d (7.7)	104.8	Glc	1	5.37 d (7.0)	105.2
	2	3.92	75.1		2	4.02	75.5
	3	4.10	78.3		3	4.10	78.2
	4	4.11	70.8		4	3.99	72.0
	5	3.64/4.23	67.0		5	3.92	78.2
					6	4.15/4.50	62.7
				Xyl_2	1	5.53 d (7.7)	104.1
					2	3.96	75.0
					3	4.09	87.8
					4	4.05	69.1
					5	3.39/4.16	66.2
				Xyl_3	1	5.14 d (7.7)	105.8
					2	3.93	75.1
					3	4.10	78.0
					4	4.11	70.8
					5	3.42/4.13	66.9

[a] The assignments were based on DQF-COSY, TOCSY, HSQC-TOCSY, and HMBC experiments.

an HMBC-correlation between the anomeric proton resonance at δ 5.96 ppm and C-28 (δ 176.1 ppm). Again the 2D-NMR-data were very similar compared to *Gypsophila* saponin **2**, allowing the identification of one fucopyranosyl (Fuc) residue, one rhamnopyranosyl (Rha) residue, one xylopyranosyl (Xyl_2) residue and one glucopyranosyl (Glc) residue (Table 2). The oligosaccharide chain was established from the following HMBC-correlations: H-1 of glucose unit at δ 5.37 ppm and C-3 of rhamnose unit at δ 82.1 ppm, H-1 of xylose unit at δ 5.53 ppm and C-4 of rhamnose unit at δ 78.8 ppm, H-1 of rhamnose unit at δ 5.91 ppm and C-2 of fucose unit at δ 75.3 ppm. Finally, signals for a third xylopyranosyl moiety were observed. In the HMBC-spectrum, correlations between H-1 of this xylose residue at δ 5.14 ppm and C-3 of Xyl_2 at δ 87.8 ppm were detected thus substantiating *Gypsophila* saponin **3** to be 3-O-β-D-galactopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucuronopyranosyl gypsogenin 28-O-β-D-glucopyranosyl-(1 → 3)-[β-D-xylopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)]-α-L-rhamnopyranosyl-(1 → 2)-β-D-fucopyranoside.

Gypsophila saponin **3**: white amorphous powder; $[\alpha]^{20}_{D}$ +0,51 (c 0.0025, distilled water); UV λ_{max} (70% methanol) 210; FT-IR ν_{max} 3356 cm^{-1} (O–H), 2930 cm^{-1} (C–H), 1720 cm^{-1} (C–O), 1673 cm^{-1} (C=C), 1364 cm^{-1} (C=O), 1025 cm^{-1} (C–C). ESI-TOF-MS (negative ion mode) $[M-H]^-$ at m/z 1657.7106.

For $^{1}H/^{13}C$ NMR data, see Tables 1 and 2.

4. Conclusions

In this study we have developed a very simple, economical and rapid method for the isolation of three *Gypsophila* saponins from the roots of *Gypsophila paniculata* L. which can be used for the combinatorial anti cancer therapy with saporin-based targeted toxins. The

three *Gypsophila* saponins could be isolated in a one-step separation procedure. The simplicity of this method embraces production under GLP conditions, which is a prerequisite for the medical uses of *Gypsophila* saponins.

Conflict of interest

The authors declare that there is no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.01.026.

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