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## Triterpenoid saponins from the roots of *Gypsophila trichotoma* Wender.

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## Abstract

Eleven triterpenoid saponins were isolated from the roots of *Gypsophila trichotoma* Wender. (*G. trichotoma* Wender. var. *trichotoma*) (Caryophyllaceae), together with one known compound. The structures were established on the basis of extensive NMR analysis ( $^1\text{H}$ ,  $^{13}\text{C}$  NMR, COSY, TOCSY, ROESY, HSQC, and HMBC), completed by analysis of HR-ESI-MS and ESI-MS<sup>n</sup>. The saponins have the commonly found gypsogenin as the aglycone substituted at C-3 with trisaccharide and at C-28 with oligosaccharide through a fucose residue, as saponins isolated from *G. perfoliata* L. originated from China. The oligosaccharide attached to C-28 is substituted with acetyl and (or) sulfate groups.

The cytotoxicity of the saponin extract from *G. trichotoma* was evaluated against a rat alveolar macrophage-like cell line NR8383 and human leukemia cell lines U937 and BV - 173. The synergistic effect of the aminoacyl saponins, previously isolated from *G. trichotoma*, was tested for its ability to enhance the cytotoxicity of the targeted toxin in HER14 cells.

**Keywords:**

*Gypsophila trichotoma*

*Gypsophila perfoliata*

Caryophyllaceae

Triterpenoid saponins

Cytotoxic activity

## 1. Introduction

Triterpenoid saponins from the genus *Gypsophila*, belonging to the Caryophyllaceae family, are exploited commercially for a variety of purposes including as medicines, detergents, adjuvants, cosmetics (Tschesche and Wulff, 1973; Hostettmann and Marston, 1995). A large number of the *Gypsophila* mono- and bidesmosides have been isolated and characterized (Frechet et al., 1991; Henry et al., 1991; Kim et al., 1992; Liu et al., 1995; Elgamal et al., 1995; Acebes et al., 1998; Luo and Kong, 2006; Luo et al., 2006; Bai et al., 2007; Zheng et al., 2007; Elbandy et al., 2007a; Elbandy et al., 2007b; Cevrimli et al. 2007; Luo et al., 2008; De Abreu et al., 2008; Nie et al., 2009; Weng et al., 2009; Weng et al., 2010; Yao et al., 2010; Chen et al., 2010; Chen et al., 2011). The most common basic structures are gypsogenin, gypsogenic acid and quillaic acid (Bottger and Melzig, 2010), as well as vaccaric acid (Luo et al., 2006), segetalic acid (Luo et al., 2008) and the new saponin neogypsogenin A and B (Luo and Kong, 2006). Saponins are considered as the major bioactive components of the drugs, mainly used for their anti-inflammatory, spermicidal (El Bary and Nour, 1979; Primorac et al., 1985), hypocholesterolaemic (Velieva et al., 1988), and antiviral (Dourmashkin et al., 1962; Frechet et al., 1990) activities. Some saponins from *Gypsophila* species have been reported to exert immunomodulant activities (Luo et al., 2006). Also, saponins with an aldehyde function at C-4 from *Gypsophila oldhamiana* Miq. exhibited cytotoxic activity against different human cancer cell lines (Bai et al., 2007). *Gypsophila* saponins are of interest in terms of their applications as immunological adjuvants in vaccines (Marciani, 1999). It was shown that Saponinum album (Merck) enhanced 100 000-folds the cytotoxicity of the type I ribosome – inactivating protein saporin from *Saponaria officinalis* L. (Hebestreit et al., 2006). Individually isolated *Gypsophila paniculata* L. saponins showed also toxicity enhancing properties on saporin without causing toxicity by themselves up to 20 µg/ml (Weng et al., 2009; Weng et al., 2010). The principle of the synergistic cytotoxicity was adopted for saporin-based targeted toxins which are used in tumor therapy (Bachran et

al., 2010; Bottger and Melzig, 2010). Especially saponins of *G. oldhamiana* Miq. and *G. paniculata* L. showed inhibitory activity against  $\alpha$ -glucosidase (Luo et al., 2008). This effect might offer the use of saponins found in some species as an antidiabetic remedy. Besides, saponins from *G. oldhamiana* have been reported to exert inhibitory activity of pancreatic lipase (Zheng et al., 2007).

The medicinal and commercial importance attached to *Gypsophila* prompted us to investigate the saponins in *G. trichotoma* Wender. (synonym of *Gypsophila trichotoma* Wender. var. *trichotoma*) (Valev, 1966). This perennial herb is native to Black Sea region in Bulgaria. Previous phytochemical studies on this species have reported the occurrence of saponins, sterols and flavonoids (Luchanskaya et al., 1971a; Luchanskaya et al., 1971b; Luchanskaya et al., 1971c; Luchanskaya et al., 1972; Krasteva et al., 2009). In order to find reliable source of haemolytic saponins and vaccine adjuvants, we previously carried out an investigation on *G. trichotoma* Wender., which resulted in the isolation of two new aminoacyl triterpene saponins (Gevrenova et al., 2006). 3-*O*- $\beta$ -sulfated glycosides were already reported from the roots of *G. bermejoi* G. Lopez, *G. repens* L. and *G. trichotoma* Wender (Acebes et al., 1998; Elbandy et al., 2007a; Krasteva et al., 2009). Recently, new triterpenoid saponins with an acetyl group or a cinnamoyl group were isolated from the roots of *Gypsophila altissima* L. and *G. perfoliata* L. (Chen et al., 2010; Chen et al., 2011). The taxonomy of *G. trichotoma* is ambiguous and this species is synonym of *Gypsophila perfoliata* L. in Flora Europaea (Barkoudah and Chater, 1996) and Flora of China (Dequan and Turland, 2001). According to Flora Bulgaricae, only *G. trichotoma* Wender. var. *pubescens* Fenzl. is synonym of *G. perfoliata* L. (Valev, 1966). The present study describes the isolation and structural elucidation of eleven new *Gypsophila* saponin (**1-11**) from the roots of *G. trichotoma* Wender. var. *trichotoma*, as well as the cytotoxicity of the saponin extract against a rat alveolar macrophage – like cell line NR8383 and human leukemic cell lines U937 and BV – 173. The synergistic effect of the aminoacyl saponins, previously isolated from *G. trichotoma*, was also tested for its ability to enhance the cytotoxicity of the targeted toxin in HER14 cell cultures.

## 2. Results and discussion

### 2.1. Isolation and identification of new compounds 1–11

The hydroalcoholic extracts of *Gypsophila trichotoma* roots were purified by multiple chromatographic steps over Silicagel, Reversed phase C<sub>8</sub>, Reversed phase C<sub>18</sub> and finally purified by semi-preparative HPLC over C<sub>18</sub>, yielding eleven new saponins (**1–11**, Fig 1) with a known saponin (**12**), the 3-*O*-β-D-xylopyranosyl-(1→3)-[β-D-galactopyranosyl-(1→2)]-β-D-glucuronopyranosylgypsogenin-28-*O*-β-D-xylopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→3)]-α-L-rhamnopyranosyl-(1→2)-β-D-4-*O*-acetyl-fucopyranoside already reported in the seeds of *Agrostemma githago* L. var. *githago* (Siepmann et al., 1998). Among them, saponins **9** and **11** were obtained in a poor quantity to obtain <sup>13</sup>C NMR data.

Acid hydrolysis of crude saponin mixture afforded an aglycone identified, after purification, as gypsogenin from <sup>1</sup>H and <sup>13</sup>C NMR spectra referring to the reported data (Mahato and Kundo, 1994) and a prosaponin identified as 3-*O*-β-D-6-*O*-methylglucuronopyranosyl-gypsogenin (**13**), previously isolated after hydrolysis of *Momordica* saponin (Iwamoto et al. 1985). This prosaponin may be considered as a hydrolysis artefact of prosaponin gypsogenin 3-*O*-β-D-glucuronopyranoside (Henry et al. 1989). Seven sugar moieties were identified in the aqueous layer by co-TLC with authentic samples to glucuronic acid (GlcA), glucose (Glc), galactose (Gal), fucose (Fuc), arabinose (Ara), xylose (Xyl) and rhamnose (Rha). The common D configuration for GlcA, Glc, Gal, Xyl and Fuc and the L configuration for Rha and Ara were assumed after measurement of their optical rotation after purification of each sugar by preparative TLC, and corresponded to the D and L configurations previously characterized in the structural elucidation of trichoside D (Luchanskaya et al., 1972).

Compound **1** was obtained as a white amorphous powder. The HR-ESI-MS (negative ion mode) exhibited pseudomolecular ion peaks at *m/z* 1647.6368 [M-H]<sup>-</sup>, (calcd for C<sub>72</sub>H<sub>111</sub>O<sub>40</sub>S, 1647.6372) indicating a molecular mass of 1648 Da, compatible with the molecular formula

$C_{72}H_{112}O_{40}S$ . This pseudomolecular ion was observed in ESI-MS<sup>-</sup> at  $m/z$  1647 with other fragments ions peaks at  $m/z$  1568 [M-HSO<sub>3</sub>]<sup>-</sup>, 1177 [M-H-470]<sup>-</sup> and 939 [M-H-708]<sup>-</sup>, due to the loss of one sulphated group and two glycosidic chains, respectively. The chain with 470 *uma* was composed with hexose, pentose and hexosuronic acid. Another fragment ion peaks was observed at  $m/z$  707 corresponding to the ion fragment of the ester chain (707 *uma*). These ions suggest that the ester glycosidic chain was constituted by four sugars, two desoxyhexoses, one hexose and one pentose substituted by one sulphated group and one acetyl group. The proton and carbon resonances of the aglycon moiety of saponin **1** were unambiguously assigned to the gypsogenin from the analysis of COSY, HSQC *J*-modulate and HMBC NMR spectra (Table 1), and by comparison with literature data (Mahato and Kundo, 1994). The chemical shift of the carbonyl C-28 ( $\delta_C$  178.2) and the deshielded carbon C-3 ( $\delta_C$  86.7) of gypsogenin indicated a bidesmosidic saponin. Seven anomeric proton signals were observed at  $\delta_H$  5.42 (1H, *d*,  $J=8.1$  Hz), 5.18 (1H, *d*,  $J=1.1$  Hz), 4.84 (1H, *d*,  $J=7.4$  Hz), 4.70 (1H, *d*,  $J=8.0$  Hz), 4.68 (1H, *d*,  $J=8.0$ Hz), 4.59 (1H, *d*,  $J=7.4$  Hz), and 4.49 (1H, *d*,  $J=7.7$  Hz), displaying correlations with anomeric carbon signals at  $\delta_C$  95.3, 102.1, 103.7, 105.2, 105.0, 104.8, and 104.6, respectively (Table 2). Analysis of COSY, TOCSY, and HSQC *J*-modulate experiments and spin-spin couplings in <sup>1</sup>H NMR of **1** allowed the identification of a  $\beta$ -D-glucuronopyranosyl (GlcA), a  $\beta$ -D-galactopyranosyl (Gal), a  $\beta$ -D-glucopyranosyl (Glc), a  $\beta$ -D-fucopyranosyl (Fuc), a  $\alpha$ -L-rhamnopyranosyl (Rha), an  $\alpha$ -L-arabinopyranosyl (Ara), and a  $\beta$ -D-xylopyranosyl (Xyl) moieties (Table 2). The  $\beta$ -anomeric configuration for the D-glucuronopyranosyl, D-galactopyranosyl, D-glucopyranosyl, D-fucopyranosyl, and D-xylopyranosyl moieties and the  $\alpha$ -anomeric configuration for the L-arabinopyranosyl unit were determined by their large <sup>3</sup> $J_{H1,H2}$  coupling constants of 7-8 Hz (Table 2). The  $\beta$ -D-xylopyranosyl unit, identified starting from anomeric signals at  $\delta_H$  4.70 (Xyl-H1) and  $\delta_C$  105.2 (Xyl-C1), the  $\alpha$ -L-arabinopyranosyl unit ( $\delta_H$  4.59 and  $\delta_C$  104.8) and the  $\beta$ -D-galactopyranosyl unit ( $\delta_H$  4.84 and  $\delta_C$  103.7) were identified to be in terminal



position, as observed by their  $^{13}\text{C}$  NMR chemical shifts (Melchior, 1964) (Table 2), suggesting most ramifications in the compound. The methyl doublet at  $\delta_{\text{H}}$  1.27 ( $J = 6.2$  Hz) and the typical small doublet of anomeric proton at  $\delta_{\text{H}}$  5.18 ( $J = 1.1$  Hz) were characteristic of a rhamnopyranosyl unit. It was identified as  $\alpha$  anomeric configuration by the  $^{13}\text{C}$  NMR chemical shift of its C-5 ( $\delta_{\text{C}}$  69.2) (Bock and Pedersen, 1983) and ROE effects observed between Rha H-1 and Rha H-2 and between Rha H-3 and Rha H-5. The deshielding of both Rha C-3 ( $\delta_{\text{C}}$  82.7) and Rha C-4 ( $\delta_{\text{C}}$  79.1) indicated a disubstitution of this rhamnopyranosyl moiety. The  $\beta$ -D-glucopyranosyl unit was identified starting from anomeric signals at  $\delta_{\text{H}}$  4.68 and  $\delta_{\text{C}}$  105.0, characterized by its large coupling constants up to 8 Hz. The deshielding of both proton H-3 ( $\delta_{\text{H}}$  4.25,  $t$ ,  $J = 9.0$  Hz) and carbon C-3 ( $\delta_{\text{C}}$  85.7) of this glucose suggested a substitution at this position. The  $\beta$ -D-fucopyranosyl unit was identified starting from the deshielded anomeric proton at  $\delta_{\text{H}}$  5.42 ( $J = 8.1$  Hz) and characterized by its methyl doublet at  $\delta_{\text{H}}$  1.10 ( $J = 6.4$  Hz) and its equatorial proton H-4 at  $\delta_{\text{H}}$  5.13 ( $d$ ,  $J = 3.4$  Hz). The deshielding of anomeric proton and the chemical shift of anomeric carbon at  $\delta_{\text{C}}$  95.3 suggested that this fucose was attached by an ester linkage to the C-28 carboxylic group of the aglycon. This was confirmed by the long-range correlation observed in the HMBC experiment between signals at  $\delta_{\text{H}}$  5.42 (Fuc H-1) and  $\delta_{\text{C}}$  178.2 (C-28 of gypsogenin). The fucose was substituted at position 2 as observed by its deshielded Fuc C-2 ( $\delta_{\text{C}}$  76.2). Starting from the anomeric proton at  $\delta_{\text{H}}$  4.49 ( $d$ ,  $J = 7.5$  Hz), a  $\beta$ -D-glucuronopyranosyl unit was identified with its carbonyl C-6 at  $\delta_{\text{C}}$  175.0. The deshielding of carbons C-2 and C-3 indicated a disubstitution of glucuronic acid (Table 2). Observation of long-range proton-carbon coupling in the HMBC spectrum between anomeric signal at  $\delta_{\text{H}}$  4.49 of the glucuronic acid and C-3 of gypsogenin, H-1 ( $\delta_{\text{H}}$  4.84) of the terminal galactose and C-2 ( $\delta_{\text{C}}$  78.0) of glucuronic acid, and between H-1 ( $\delta_{\text{H}}$  4.59) of the terminal arabinose and C-3 ( $\delta_{\text{C}}$  86.2) of glucuronic acid, indicated a trisaccharide chain attached at C-3 of gypsogenin (Fig 2), the

$\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 3)-[ $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucuronopyranoside. The sequencing of the ester chain was obtained by analysis of HMBC experiment which showed cross-peaks between H-1 ( $\delta_{\text{H}}$  5.18) of the rhamnose and C-2 ( $\delta_{\text{C}}$  76.1) of the fucose, H-3 ( $\delta_{\text{H}}$  4.00) of the rhamnose and C-1 ( $\delta_{\text{C}}$  105.0) of the glucose, and between H-4 ( $\delta_{\text{H}}$  3.68) of the rhamnose and C-1 ( $\delta_{\text{C}}$  105.2) of the terminal xylose unit (Fig 2). This sequence was confirmed by the observation of ROE connectivities between Rha H-1 and Fuc H-2, Rha H-3 and Glc H-1 and Rha H-4 and Xyl H-1. Thus the ester chain was at least tetrasaccharidic. In the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra, supplementary signals were observed attributed to an acetyl group ( $\delta_{\text{C}}$  21.0 and 173.1, Table 2) which esterify the hydroxyl group at position 4 of the fucose unit as suggested by the deshielding of its proton H-4 and confirmed by the long range correlation observed in HMBC experiment between Fuc H-4 ( $\delta_{\text{H}}$  5.13) and the carbonyl carbon ( $\delta_{\text{C}}$  173.1) of the acetyl group. The presence of a sulphated group in compound **1** was indicated by the ESI-MS spectra and the ion fragment at  $m/z$  1568 [ $\text{M-HSO}_3$ ] $^-$ . This sulphated group was attached to the hydroxyl group in position 3 of glucose as indicated by its deshielding proton H-3 ( $\delta_{\text{H}}$  4.25,  $t$ ,  $J = 9.0$  Hz) and carbon C-3 ( $\delta_{\text{C}}$  85.7) (Acebes et al., 1998). Thus saponin **1** was identified as

3-*O*-( $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 3)-[ $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucuronopyranosyl)-28-*O*-(3-*O*-sulphate- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3)-[ $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 4)]- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-4-*O*-acetyl- $\beta$ -D-fucopyranosyl)-gypsogenin.

Saponin **2** was obtained as a white amorphous powder. It was assigned the molecular formula  $\text{C}_{72}\text{H}_{112}\text{O}_{40}\text{S}$  as in **1** from its negative ion peak in HR-ESI-MS at  $m/z$  1647.6381 [ $\text{M-H}$ ] $^-$  (calcd for  $\text{C}_{72}\text{H}_{111}\text{O}_{40}\text{S}$ , 1647.6372). In ESI-MS $^-$  a pseudomolecular ion peak was observed at  $m/z$  1647 [ $\text{M-H}$ ] $^-$  with other fragments ions peaks at  $m/z$  1568 [ $\text{M-HSO}_3$ ] $^-$ , 1177 [ $\text{M-H-470}$ ] $^-$  and 939 [ $\text{M-H-708}$ ] $^-$  as in **1**, due to the loss of one sulphated group and the two glycosidic chains, respectively. Analysis of the  $^1\text{H}$  NMR spectrum showed signals of seven anomeric proton at  $\delta_{\text{H}}$  5.44 (1H,  $d$ ,  $J=7.6$  Hz), 5.13 (1H,  $brs$ ), 4.82 (1H,  $d$ ,  $J=7.0$  Hz), 4.72 (1H,  $d$ ,  $J=7.8$  Hz),

4.69 (1H, *d*, *J*=8.0 Hz), 4.64 (1H, *d*, *J*=8.2 Hz), and 4.39 (1H, *d*, *J*=6.9 Hz), which were correlated in HSQC *J*-modulate to anomeric carbon signals at  $\delta_C$  95.4, 102.2, 103.7, 105.1, 105.0, 104.4, and 104.4, respectively (Table 2). In addition, signals of one acetyl group at  $\delta_H$  2.21 (3H, *s*) and  $\delta_C$  21.0 was also observed. Analysis of COSY, TOCSY, and HSQC *J*-modulate experiments and spin-spin couplings in  $^1H$  NMR of **2** allowed us to identify a  $\beta$ -D-glucuronopyranosyl, a terminal  $\beta$ -D-galactopyranosyl, a  $\beta$ -D-glucopyranosyl, a  $\beta$ -D-fucopyranosyl, a  $\alpha$ -L-rhamnopyranosyl, and two terminal  $\beta$ -D-xylopyranosyl moieties (Table 2) using the same protocol as in **1**. Observation of HMBC cross-peak between anomeric proton at  $\delta_H$  4.39 of the glucuronic acid and C-3 of gypsogenin, H-1 ( $\delta_H$  4.82) of the terminal galactose and C-2 ( $\delta$  78.4) of glucuronic acid, and between H-1 ( $\delta_H$  4.64) of one xylose (Xyl') unit and C-3 ( $\delta$  86.5) of glucuronic acid, indicated that the trisaccharide chain linked at C-3 of gypsogenin is

$\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 3)-[ $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucuronopyranoside.

This sequence was confirmed by the observation of ROE connectivities between Xyl' H-1 and GlcA H-3 and Gal H-1 and GlcA H-2 and GlcA H-1 and H-3 of gypsogenin. The sequencing of the ester chain was similar to those established in saponin **1** as observed by HMBC cross-peaks between each sugar and between H-4 ( $\delta_H$  5.13) of the fucose and the carbonyl carbon ( $\delta_C$  173.1) of the acetyl group. As in **1**, a sulphated group was attached in compound **2** to the hydroxyl group in position 3 of the glucose unit as suggested by its deshielding proton H-3 ( $\delta_H$  4.25) and carbon C-3 ( $\delta_C$  85.7). Consequently, the chemical structure of saponin **2** was concluded to be 3-*O*-( $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 3)-[ $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucuronopyranosyl)-28-*O*-(3-*O*-sulphate- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3)-[ $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 4)]- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-4-*O*-acetyl- $\beta$ -D-fucopyranosyl)-gypsogenin.

Compound **3** (Fig 1) was obtained as a white amorphous powder. The HR-ESI-MS spectra (negative ion mode) gave a pseudomolecular ion peak [M-H]<sup>-</sup> at *m/z* 1647.6365 (calcd for

C<sub>72</sub>H<sub>111</sub>O<sub>40</sub>S, 1647.6372) indicating the same molecular formula C<sub>72</sub>H<sub>112</sub>O<sub>40</sub>S as saponins **1** and **2**. Analysis of <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra using the same method as in **1** and **2** showed the presence of signals corresponding to the presence of the same seven sugars moieties as in **1**, an acetyl group and a sulphated group. The only difference was the deshielding of the proton H-3 of the fucose unit indeed of H-4, suggesting an isomer position for the acetyl group (Table 2). This was confirmed by the HMBC cross-peak observed between H-3 (δ<sub>H</sub> 4.93) of the fucose and the carbonyl carbon (δ<sub>C</sub> 172.1) of the acetyl group. Consequently, the chemical structure of saponin **3** was concluded to be

3-*O*-(α-L-arabinopyranosyl-(1->3))-[β-D-galactopyranosyl-(1->2)]-β-D-glucuronopyranosyl)-28-*O*-(3-*O*-sulphate-β-D-glucopyranosyl-(1->3))-[β-D-xylopyranosyl-(1->4)]-α-L-rhamnopyranosyl-(1->2)-3-*O*-acetyl-β-D-fucopyranosyl)-gypsogenin.

Compound **4** (Fig 1), obtained as a white amorphous powder, showed a pseudomolecular ion peak [M+Na]<sup>+</sup> at *m/z* 1591.6788 (calcd for C<sub>72</sub>H<sub>112</sub>O<sub>37</sub>Na) indicating a molecular formula of C<sub>72</sub>H<sub>112</sub>O<sub>37</sub> in adequation with a molecular mass of 1568 Da. This formula suggests the loss of the sulphated group comparing to compounds **1-3**. Analysis of COSY, TOCSY, HSQC *J*-modulate, and HMBC experiments allowed us to identify the seven sugars moieties as in **1**, and one acetyl group linked to the hydroxyl in position 4 of the fucose unit as confirmed by the HMBC long range correlation between Fuc H-4 (δ<sub>H</sub> 5.12) and the carbonyl carbone (δ<sub>C</sub> 172.8) (Table 3). The absence of the deshielding signals from H-3 and C-3 of the glucose unit is due to the absence of the sulphated group (Table 3). In addition, the sequencing of the two oligosaccharides chains was identical to those observed for compounds **1** and **3**. Thus, saponin **4** was identified as

3-*O*-(α-L-arabinopyranosyl-(1->3))-[β-D-galactopyranosyl-(1->2)]-β-D-glucuronopyranosyl)-28-*O*-(β-D-glucopyranosyl-(1->3))-[β-D-xylopyranosyl-(1->4)]-α-L-rhamnopyranosyl-(1->2)-4-*O*-acetyl-β-D-fucopyranosyl)-gypsogenin. This saponin was the isomeric form of the known saponin **12**, previously isolated from *Agrostemma githago* L. var. *githago*

(Siepmann et al., 1998), possessing a xylose unit in the place of arabinose attached to the hydroxyl in position 3 of the glucuronic acid.

Compounds **5** and **6** (Fig 1) were obtained as a white amorphous powder. The same molecular formula  $C_{74}H_{114}O_{38}$  was determined separately for the both compounds from the pseudomolecular ion peak observed in HR-ESI-MS at  $m/z$  1609.6896  $[M-H]^-$  in negative ion mode for **5** and at  $m/z$  1633.6909  $[M+Na]^+$  in positive ion mode for **6**. In the ESI-MS spectra, in negative ion mode, the same ion fragments were observed at  $m/z$  1567  $[M-H-Ac]^-$ , 939  $[M-H-670]^-$ , and 469  $[trisaccharidic\ chain-H]^-$ , due to the loss of an acetyl group, the ester chain, and the trisaccharidic chain, respectively. The  $^1H$  NMR and  $^{13}C$  NMR spectra of **5** and **6** showed signals assignable to two acetyl groups ( $\delta_H$  2.11 (3H, *s*) and 2.19 (3H, *s*) and  $\delta_C$  20.8 and 21.2) (Table 3). Analysis of 2D NMR spectra (COSY, TOCSY, HSQC *J*-modulate, and HMBC) indicated that the only difference between **5** and **6** is due to the presence of a terminal arabinopyranose in **5**, or a terminal xylopyranose in **6**, link to the position 3 of the glucuronopyranosyl moiety as in compounds **1** and **2** (Table 3). The HMBC long range correlations in **5** and **6** indicated that the two acetyl groups were attached to the hydroxyl groups in position 4 of the fucose unit ( $\delta_H$  5.12) and in position 6 of the glucose unit ( $\delta_H$  4.23 and 4.44 in **5**, and  $\delta_H$  4.23 and 4.48 in **6**) (Table 3). The sequencing of the two oligosaccharides chains were identical to those observed for compounds **1** and **2**, respectively. Consequently, the chemical structure of saponins **5** and **6** were characterized to be 3-*O*-( $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 3)-[ $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucuronopyranosyl)-28-*O*-(6-*O*-acetyl- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3)-[ $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 4)]- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-4-*O*-acetyl- $\beta$ -D-fucopyranosyl)-gypsogenin, and 3-*O*-( $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 3)-[ $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucuronopyranosyl)-28-*O*-(6-*O*-acetyl- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3)-[ $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 4)]- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-4-*O*-acetyl- $\beta$ -D-fucopyranosyl)-gypsogenin, respectively.

Compound **7** (Fig 1) was obtained as a white amorphous powder and the C<sub>70</sub>H<sub>110</sub>O<sub>36</sub> molecular formula was attributed from their pseudomolecular ion peak [M+Na]<sup>+</sup> observed at *m/z* 1549.6661 (calcd for C<sub>70</sub>H<sub>110</sub>O<sub>36</sub>Na) in HR-ESI-MS (positive ion mode). Analysis of <sup>1</sup>H and <sup>13</sup>C NMR spectra indicated the absence of signals due to acetyl groups in **7**. The 2D NMR spectra allowed us to identify the same oligosaccharidic chain as previous compounds **1**, **3-5** but without deshielding signals due to esterification by acetyl groups. Thus, the structure of saponin **7** was elucidated as

3-*O*-( $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 3)-[ $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucuronopyranosyl)-28-*O*-( $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3)-[ $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 4)]- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-fucopyranosyl)-gypsogenin.

Compounds **8** and **9** (Fig 1) were obtained as white amorphous powder. Their HR-ESI-MS spectra indicated for both a C<sub>79</sub>H<sub>122</sub>O<sub>42</sub> molecular formula by the same pseudomolecular ion peak observed in positive ion mode at *m/z* 1765.7303 [M+Na]<sup>+</sup> for **8** and 1765.7318 [M+Na]<sup>+</sup> for **9**. In the ESI-MS spectra of **8** (negative ion mode) the ion fragments observed at *m/z* 1609 [M-H-132]<sup>-</sup>, 939 [M-H-802]<sup>-</sup> suggest the presence of a supplementary pentose unit in ester chain by comparison with **5**. Analysis of <sup>1</sup>H NMR, COSY, HSQC and <sup>13</sup>C NMR spectra of **8** showed signals assignable to two acetyl groups and eight sugar moieties (Table 4). By comparison with saponin **5** the supplementary pentose unit was identified to an  $\alpha$ -L-arabinopyranosyl moiety in terminal position and the deshielding signals of C-3 of the xylose unit from 79.1 in **5** to 88.3 in **8** suggest a substitution at this position (Table 4). The HMBC long range correlations in **8** confirmed this point of linkage by the cross peak observed between H-1 of Ara' ( $\delta$ <sub>H</sub> 4.58) and C-3 of xylose unit ( $\delta$ <sub>C</sub> 88.3), and indicated that the two acetyl groups were attached to the hydroxyl groups in position 4 of the fucose unit ( $\delta$ <sub>H</sub> 5.12) and in position 6 of the glucose unit ( $\delta$ <sub>H</sub> 4.21 and 4.47) as in **5** (Table 4). Thus, the structure of saponin **8** was established to be

3-*O*-( $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 3)-[ $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucuronopyran

osyl)-28-O-(6-O-acetyl- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3)-[ $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 3)- $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 4)]- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-4-O-acetyl- $\beta$ -D-fucopyranosyl)-gypsogenin. Similarly, the analysis of  $^1\text{H}$  NMR spectrum, COSY and TOCSY experiments of saponin **9** indicated that the only difference between **8** and **9** is due to the presence of a terminal xylopyranosyl moiety in **9** attached to the position 3 of the glucuronopyranosyl moiety, indeed of a terminal arabinopyranosyl moiety in **8**, as in compounds **5** and **6** (Table 4). The fragmentation in positive ion mode of the  $[\text{M}+\text{Na}]^+$  pseudomolecular ion peak of **9** at  $m/z$  1765 gave the ion fragments at  $m/z$  1633  $[\text{M}+\text{Na}-132]^+$ , 1295  $[\text{M}+\text{Na}-470]^+$  and 825 [ester chain+Na] $^+$ , attributed to the loss of a terminal pentose unit, the trisaccharide chain as in other compounds and the ester chain, respectively. The  $\text{MS}^2$  of the ion fragment at  $m/z$  825 [ester chain+Na] $^+$  gave another ion fragments at  $m/z$  765 [ester chain+Na-Ac] $^+$ , 693 [ester chain+Na-132] $^+$ , and 561 [ester chain+Na-132-132] $^+$  attributed to the loss of an acetyl group, a terminal pentose, and two pentoses unit, respectively, suggesting that the supplementary pentose unit was linked to the xylose in the ester chain at 28 position. Consequently, despite no  $^{13}\text{C}$  NMR data are given for compounds **9** because the amount of **9** was not sufficient to give a desirable quality of  $^{13}\text{C}$  NMR spectra, in accordance with MS spectra, the chemical structure of the new saponin **9** could be assignable as

3-O-( $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 3)-[ $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucuronopyranosyl)-28-O-(6-O-acetyl- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3)-[ $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 3)- $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 4)]- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-4-O-acetyl- $\beta$ -D-fucopyranosyl)-gypsogenin.

Compounds **10** and **11** (Fig 1) were obtained as white amorphous powder. It was assigned the molecular formula  $\text{C}_{80}\text{H}_{118}\text{O}_{38}$  from their quasimolecular ion peaks observed in HR-ESI-MS spectra (negative ion mode) at  $m/z$  1685.7211  $[\text{M}-\text{H}]^-$  for **10** and 1685.7230  $[\text{M}-\text{H}]^-$  for **11** (calcd for  $\text{C}_{80}\text{H}_{117}\text{O}_{38}$ , 1685.7223), suggesting that **10** and **11** are isomeric compounds.

Analysis of  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of **10** showed signals corresponding to the same seven

sugar moieties as in compounds **1**, **3-5** and **7** (Table 4). No signals corresponding to the presence of acetyl groups were observed but signals suggesting the presence of *Z-p*-methoxycinnamoyl group at  $\delta_{\text{H}}$  7.78 and 6.92 (2H each, *d*,  $J=8.8$  Hz, H-5'+ H-9' and H-6'+ H-8'), 6.98 and 5.58 (1H each, *d*,  $J=12.9$  Hz, H-3' and H-2'), and 3.85 (3H, *s*). The downfield shifts for the H-4 ( $\delta$  5.21) and C-4 ( $\delta$  74.7) resonances of fucose unit revealed the secondary alcoholic function Fuc 4-OH to be acylated. An HMBC correlation observed between H-4 of fucose ( $\delta_{\text{H}}$  5.21) and the carbonyl C-1' ( $\delta_{\text{C}}$  168.1) confirmed the point of linkage of the *p*-methoxycinnamoyl group to fucose unit at C-4. On the basis of all the evidence, saponin **10** was elucidated as

3-*O*-( $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 3))- $[\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucuronopyranosyl)-28-*O*-( $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3))- $[\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 3)]- $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 4)]- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-4-*O*-cis-methoxycinnamoyl- $\beta$ -D-fucopyranosyl)-gypsogenin. The ESI-MS (negative ion mode) spectra of **11** gave ion fragments at  $m/z$  1553 [M-H-132]<sup>-</sup>, 1523 [M-H-162]<sup>-</sup>, 939 [M-H-746]<sup>-</sup>, and 745 [ester chain-H]<sup>-</sup> attributed to the loss of a terminal pentose unit, a terminal hexose unit, and the ester chain, respectively. The MS<sup>1</sup> of the [M-H]<sup>-</sup> pseudomolecular ion ( $m/z$  1685) gave a ion fragment at  $m/z$  1507 [M-H-178]<sup>-</sup>, and 469 [trisaccharidic chain-H]<sup>-</sup> due to the loss of a methoxycinnamoyl group as in saponin isolated from *Gypsophila perfoliata* L. (Chen et al., 2011) and the trisaccharidic chain, respectively. Analysis of the <sup>1</sup>H NMR spectrum, COSY and TOCSY experiments of compound **11** allowed us to identify the same sugars units as in **10** with signals characteristic of the presence of a trans-methoxycinnamoyl group observed at  $\delta_{\text{H}}$  7.72 and 6.55 (1H each, *d*,  $J=15.8$  Hz, H-3' and H-2'), 7.62 and 7.00 (2H each, *d*,  $J=8.3$  Hz, H-5'+ H-9' and H-6'+ H-8'), and 3.87 (3H, *s*). The downfield shifts resonances for the H-4 ( $\delta$  5.23) of fucose unit are characteristic of the esterification of the Fuc 4 hydroxyl group by the trans-methoxycinnamic acid, as in **10**. Thus, despite absence of <sup>13</sup>C NMR data, saponin **11** was identified as a new compound which could be elucidated as



3-*O*-( $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 3))- [ $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucuronopyranosyl)-28-*O*-( $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3))- [ $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 3)- $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 4)]- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-4-*O*-trans-methoxycinnamoyl- $\beta$ -D-fucopyranosyl)-gypsogenin.

## 2.2. Cytotoxicity results

The cytotoxic activity of the aqueous-methanolic extract of *G. trichotoma* Wender was analyzed in the concentration range from 0.1 to 100  $\mu$ g/ml toward a rat alveolar macrophage NR8383 and chronic myelogenous leukemia (CML) BV – 173 cell lines. Human monocytic U937 cells were cultured with same plant extract in the concentration range from 1 to 20  $\mu$ g/ml.

*G. trichotoma* extract at the range of 0.1 – 10  $\mu$ g/ml was non-toxic on NR8383 macrophage. The largest decrease in viability, compared with the control, was observed in cultures treated with 100  $\mu$ g/ml ( $p < 0.0001$ ) (Table 5). The viability was always above 50% at the studied concentration range. However, this extract showed a stronger toxicity on human leukemic cell lines U937 (IC<sub>50</sub> 10.97  $\mu$ g/ml) and BV-173 1 (IC<sub>50</sub> 40.9  $\mu$ g/ml) compared to the normal macrophages (Table 5).

Recently, triterpenoid saponins from *Gypsophila paniculata* L. in low, non-toxic concentrations, have been reported for their ability to enhance the cytotoxicity of a targeted anti-tumor toxin in a cell culture model (Weng et al., 2009; Weng et al., 2010). The toxin is composed of the plant ribosome-inactivating protein saporin as toxic moiety and human epidermal growth factor (EGF) as a targeting moiety. Previously, we isolate from *G. trichotoma* Wender (Gevrenova et al., 2006) the first aminoacyl saponins in the nature (Fig. 3). This original structure, compare to another *Gypsophila* saponins, prompt us to investigate the aminoacyl saponins (ACS) to their ability to enhance the cytotoxicity of the targeted toxin (SE). Cytotoxicity on EGF receptor (EGFR)-transfected NIH-3T3 cells was analyzed both in presence and absence of saponins. A comparative evaluation of cytotoxic activity of SE alone

and in combination with the ACS in non-toxic concentration (90 µg/ml) shows that the saponins lead to a toxicity increase of SE in HER14 cells (Fig 4). The viability in all cells treated with SE/ACS was significantly decreased ( $p < 0.01$ ) compared with the untreated control. For a parametric assessment of the toxicity enhancement of SE, the toxicity enhancement factor (TEF) was calculated. TEF is defined as the ratio of the  $GI_{50}$  (concentration which lead to 50% reduction of cell growth) of SE without saponins to the  $GI_{50}$  of SE in presence of saponins. The TEF of ACS was calculated to  $1.4 \times 10^6$ . These results corroborate with the recent publications, wherein saponinum album from *Gypsophila paniculata* L. (at a nonpermeabilizing concentration) and targeted toxin enhanced cytotoxicity in an EGFR tumor model (Bachran et al., 2010).

### 3. Conclusions

Recently, new triterpenoid saponins with an acetyl group or a cinnamoyl group were isolated from the roots of *G. oldhamiana* Miq. (Luo et al., 2008), *G. repens* L. (Elbandy et al., 2007b), *G. altissima* L. (Chen et al., 2010) and *G. perfoliata* L. (Chen et al., 2011). 3-*O*-, 4-*O*-diacetylated fucosyl and quinovosyl moieties were identified in the ester chains in *G. repens* (Elbandy et al., 2007b) and *G. perfoliata* (Chen et al., 2011), respectively, and 6-*O*-acetylglycosyl residue - in *G. oldhamiana* (Luo et al., 2008) and *G. altissima* (Chen et al., 2010). Although 3-*O*- $\beta$ -sulfated gypsogenin, oleanolic and quillaic acid monodesmosides were already reported from the roots of *G. bermejoi* G. Lopez and *G. trichotoma* Wender, and one 23-*O*-sulfated hederagenin glycoside from *G. bermejoi* (Acebes et al., 1998; Krasteva et al., 2009), this is the first report of acetylated and sulfated bisdesmosides saponin from the genus *Gypsophila*. Comparison of the spectral data of saponins **7** and **10** with those of related compound isolated from *Arenaria juncea* M. Bieb (Gaidi et al., 2001) and *G. perfoliata* (Chen et al., 2011) revealed that a terminal arabinose, indeed a xylose, was linked to HO-C-3 of glucuronic acid of the trisaccharide moiety at C-3 position of gypsogenin in the case of **7** and **10**. The saponin composition of this species, originated from Bulgaria was very similar to

those observed in *G. perfoliata* L., originated from China (Chen et al., 2011) with differences only in the ester substituents on the tetrasaccharidic chain at C-28 position and the presence of a terminal arabinose linked to HO-C-3 of glucuronic acid in the trisaccharide moiety. Thus, all saponins were isolated from the genus *Gypsophila* for the first time. According to Flora Europaea (Barkoudah and Chater, 1996) and Flora of China (Lu and Turland, 2001), *G. trichotoma* is a synonym of *G. perfoliata*, so based on our chemical structure of saponins, no justification for maintaining separate taxa exists.

The aminoacyl saponins showed a synergistic effect by enhancing the cytotoxicity of the targeted toxin in HER14 cells. The aqueous-methanolic extract has a potential cytotoxic activity toward human leukemic cell lines U937 and BV-173.

## 4. Experimental

### 4.1. General experimental procedures

Optical rotations were measured in MeOH or H<sub>2</sub>O with a Perkin-Elmer 241 polarimeter. <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded in CD<sub>3</sub>OD on a Bruker Avance III - 500 spectrometer (<sup>1</sup>H at 500 MHz and <sup>13</sup>C at 125 MHz), or on a Bruker Avance III - 600 spectrometer equipped with a cryo platform (<sup>1</sup>H at 600 MHz and <sup>13</sup>C at 150 MHz). 2D - NMR experiments were performed using standard Bruker microprograms (XWIN-NMR version 2.1 software). HR-ESI-MS, ESI-MS and MS-MS experiments were performed using a Micromass Q-TOF micro-instrument (Manchester, UK). The samples were introduced by direct infusion in a solution of MeOH at a rate of 5 μM min<sup>-1</sup>. TLC were carried out on precoated silica gel 60 F<sub>254</sub> (Merck), with CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O-HCOOH (61:32:7:1), and spots were visualized by heating after spraying with 50% H<sub>2</sub>SO<sub>4</sub>. Column chromatography (CC) was carried out on Kieselgel 60 (63-200 μm, Merck) or LiChroprep RP-C<sub>18</sub> (40-63 μm, Merck).

### Low-pressure liquid chromatography systems

The low – pressure liquid chromatography (pressure 1-2 bar, pump Duramat CFG, Prominent, Germany) were accomplished on pre-packed column Lobar® size A (240-10), with reversed phase material. Pre-packed Lichroprep RP8, 40-63  $\mu\text{m}$ , Merck, Germany was used with a gradient of  $\text{H}_2\text{O}$ -EtOH and Pre-packed Lichroprep RP18, 40-63  $\mu\text{m}$ , Merck, Germany was used with a gradient of MeOH- $\text{H}_2\text{O}$ .

#### *HPLC system I*

HPLC was performed on a chromatographic system equipped with two Waters Associates Model 510 pumps, a rheodyne injector Model 7010 with 2 ml sample loop, a semi-preparative reversed phase Lichrospher 100 RP18 (end-capped), 10  $\mu\text{m}$ , 10 x 250 mm (Merck, Germany) and a Lambda Max Model 481 LC (Waters Associates) spectrophotometer set at 210 nm. The binary solvent systems consisted of water and methanol, and step-gradients were: 0-5 min 21.5% (MeOH); 5-15 min 25%; 15-20 min 27.5%; 20-25 min 32.5%; 25-35 min 35%; 35-40min 45%; 40-45 min 70% (for subfraction A) and 0-10 min 30% (MeOH); 10-15 min 40%; 15-20 min 46%; 20-25 min 51%; 25-30 min 56%; 30-38min 58.7%; 38-42 min 62.5% ; 42-55 min 70% (for subfraction B). The flow rate was 10  $\text{ml}\cdot\text{min}^{-1}$ .

#### *HPLC system II*

HPLC was performed on a Dionex apparatus equipped with an ASI-100 automated sample injector, a STH 585 column oven, a P580 pump, a diode array detector UVD 340S and the Chromeleon® software. Prepacked  $\text{C}_{18}$  reversed phase columns 201SPTM, 4.6 x 250 mm, 5  $\mu\text{m}$  (Dionex, vydac, France) or a Luna 5u  $\text{C}_{18}$ , 4.6 x 250 mm, 5  $\mu\text{m}$ , 90 Å (Phenomenex, France) were used for analytical HPLC with a binary gradient elution (solvent A:  $\text{H}_2\text{O}$  – TFA, 0.025 %, solvent B: MeCN) and a flow rate of 1  $\text{ml}\cdot\text{min}^{-1}$ . The semi-preparative HPLC were performed with a binary gradient elution (solvent A:  $\text{H}_2\text{O}$  – TFA 0.0025%, solvent B: MeCN) on prepacked  $\text{C}_{18}$  reversed phase columns 201SP510, 10 x 250 mm, 5  $\mu\text{m}$ , 90 Å (Dionex, vydac, France) (system II-A) or a Luna 5u  $\text{C}_{18}$ , 10 x 250mm, 5  $\mu\text{m}$ , 90 Å (Phenomenex, France) (system II-B) with a flow rate of 4  $\text{ml}\cdot\text{min}^{-1}$ . Prepacked  $\text{C}_{18}$  reversed phase column

Luna 5u C<sub>18</sub>, 15 x 250 mm, 5 μm, 90 Å, Phenomenex, France) was used for semi-preparative HPLC (system II-C) with a binary gradient elution (solvent A: H<sub>2</sub>O – TFA 0.0025%, solvent B: MeCN or solvent C: MeOH) and a flow rate of 8 ml.min<sup>-1</sup> with solvents A and B or 6 ml.min<sup>-1</sup> with solvent A and C. The chromatograms were monitored at 205 and 210 nm.

#### 4.2. Plant material

*Gypsophila trichotoma* Wender. (*G. trichotoma* Wender. var. *trichotoma*) roots were collected in 2003, in Bulgaria, Black Sea region, v. Balgarevo, 50 m above sea level. Voucher specimen of plant material was deposited in the Herbarium of the Faculty of Pharmacy, Université Henri Poincaré Nancy 1, France.

#### 4.3. Extraction and isolation of new compounds **I-11**

Air-dried powdered roots of the plant (100 g) were extracted with aqueous methanol (25% w/w) to yield 40.87 g of the crude extract (Gevrenova et al., 2006). An aliquot of the crude extract (5 g) was fractionated by a low pressure liquid chromatography over a RP-C<sub>8</sub> column using initial passage of a water (100 ml) and then a linear gradient elution from 100% water (500 g) to 100% ethanol (500 g) to give three enriched saponin fractions (I-III). The fraction I (352 mg) gave a mixture of aminoacyl saponins (Fig 2) (Gevrenova et al., 2006). The saponin fractions II (177mg) and III (140 mg) were fractionated by a repeated semi-preparative HPLC (HPLC system I), to give subfractions A (32.8 mg) and B (58.4 mg). An aliquot of subfraction A (15 mg) was further separate with a semi-preparative HPLC (HPLC system II-A) with the elution program 39% B for 7' and 39% - 45 % B for 22' (flow 4 ml.min<sup>-1</sup>) to give compounds **3** (R<sub>t</sub> = 16.5, 0.6 mg), **1** (R<sub>t</sub> = 18.8, 1.1 mg) and **2** (R<sub>t</sub> 19.2, 4.6 mg). An aliquot of subfraction B (18.5 mg) was further separate by semi-preparative HPLC (HPLC system II-A) with the elution program 35%-40 % B for 6' and 40 % B for 21' (flow 4 ml.min<sup>-1</sup>) to give saponins **5** (R<sub>t</sub> = 17.3, 3.2 mg) and **6** (R<sub>t</sub> 17.5, 6.3 mg). A second aliquot of fraction B (26.3 mg) was further separate by semi-preparative HPLC (HPLC system II-A) using isocratic elution with 39 % B for 15' (flow 4 ml.min<sup>-1</sup>) to give saponins **5** (R<sub>t</sub> = 12.9, 3.0 mg) and **6** (R<sub>t</sub> 13.5, 2.6 mg).

Two samples of air-dried powdered roots of the plant (50 and 60 g) were extracted by lixiviation with aqueous methanol (25% w/w) to give, after concentration 6.59 and 14 g of crude extracts, respectively. The two crude extracts were each dissolved in H<sub>2</sub>O and dialyzed against H<sub>2</sub>O in seamless cellulose tubing with agitation for 48 h. The contents of the tubes were freeze-dried to afford 2.4 g (A) and 4 g (B) of saponin mixtures, respectively.

An aliquot of saponin mixture A (1.5 g) was chromatographed on RP-C<sub>18</sub> column eluted with 40%, 50%, 60%, 70% and 100% MeOH in H<sub>2</sub>O, successively, to give 130 fractions (each 30 ml). Fractions [21-23] (18 mg) eluted with MeOH-H<sub>2</sub>O 4:6, were separated by semi-preparative HPLC (HPLC system II-C) using 31% B for 30' as mobile phase (flow 8 ml.min<sup>-1</sup>) to give saponins **1** (Rt = 12.4, 2.5 mg) and **2** (Rt 12.9, 3.8 mg). Fractions [48-63] (241 mg) eluted with MeOH-H<sub>2</sub>O 5:5, were further purified by silicagel column chromatography (CC) using a gradient of CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O (70:30:4-70:30:5) to give saponins **5** (1.5 mg) and **6** (2 mg). Fractions [86-89] (113 mg) eluted with MeOH-H<sub>2</sub>O 7:3, were further purified by a prepacked RP-C<sub>18</sub> column Lobar® with the binary gradient (MeOH/H<sub>2</sub>O, 55:45-0:1) to afford 65 fractions (each 5 ml). Fractions [42-44] (28 mg) eluted with MeOH-H<sub>2</sub>O 7:3 was purified by semi-preparative HPLC (HPLC system II-C) with 39% B for 20' as mobile phase (flow 8 ml.min<sup>-1</sup>) to give saponins **5** (Rt = 13.2, 3.5 mg) and **6** (Rt 14.2, 1.6 mg). Fractions [47-48] (12.4 mg) eluted with MeOH-H<sub>2</sub>O 7:3 was purified by semi-preparative HPLC (HPLC system II-C) using the same isocratic system to give saponins **8** (Rt = 13.7, 3.5 mg) and **9** (Rt 14.7, 1.3 mg).

An aliquot of saponin mixture B (3.5 g) was solubilized in H<sub>2</sub>O and passed through an ion exchange IRN 77 (H<sup>+</sup>) Amberlite® resin column before purification. This saponin mixture (3 g) was fractionated by chromatography over a silica gel column using a gradient of CHCl<sub>3</sub>/MeOH/0.1% HCOOH in H<sub>2</sub>O (70:30:1-70:30:2) and gave 60 fractions (each 100 ml). Fractions [36-44] (216 mg) eluted with CHCl<sub>3</sub>/MeOH/0.1% HCOOH in H<sub>2</sub>O (70:30:1) were purified by RP-C<sub>18</sub> CC using a binary gradient of MeOH-H<sub>2</sub>O (45:55-0:1) to give 141 fractions (each 6 ml). Fractions [55-80] (19.2 mg), eluted with MeOH-H<sub>2</sub>O (5:5), were further

purified by semi-preparative HPLC (HPLC system II-B), using 36% B for 25' to give saponins **7** (Rt = 6.9, 1.7 mg), **4** (Rt = 19.3, 2.5 mg) and **12** (Rt 19.8, 1.3 mg). Fractions [132-141] (14 mg), eluted with 100% MeOH, were further purified by semi-preparative HPLC (HPLC system II-B) using the elution program from 45% B to 50% B for 20' to give saponins **11** (Rt = 11.6, 1.0 mg) and **10** (Rt = 13.8, 1.0 mg). Fractions [56-60] (461.6 mg) eluted with CHCl<sub>3</sub>/MeOH/0.1% HCOOH in H<sub>2</sub>O (70:30:2) were purified by RP-C<sub>18</sub> CC using a binary gradient of MeOH-H<sub>2</sub>O (55:45-7:3) to give 63 fractions (each 9 ml). Saponin **4** (2.2 mg) was identified in fractions [28-31]. Fractions [9-12] (25.5 mg), eluted with MeOH-H<sub>2</sub>O (55:45), were further purified by semi-preparative HPLC (HPLC system II-C) using 65% C for 20' (flow 6 ml.min<sup>-1</sup>) to give saponins **7** (Rt = 7.5, 2.8 mg) and **4** (Rt = 12.5, 0.5 mg).

#### 4.4.

*3-O-(α-L-arabinopyranosyl-(1->3)-[β-D-galactopyranosyl-(1->2)]-β-D-glucuronopyranosyl)-28-O-(3-O-sulphate-β-D-glucopyranosyl-(1->3)-[β-D-xylopyranosyl-(1->4)]-α-L-rhamnopyranosyl-(1->2)-4-O-acetyl-β-D-fucopyranosyl)-gypsogenin **1***

White powder;  $[\alpha]_D^{20} + 6.5$  (c 0.18, MeOH); <sup>1</sup>H NMR of the aglycon (CD<sub>3</sub>OD, 500 MHz) :  
 $\delta_H$  : 0.83 (3H, s, H-26), 0.94 (3H, s, H-29), 0.96 (3H, s, H-30), 1.01 (1H, (m, H-6a), 1.02 (3H, s, H-25), 1.08 (1H, m, H-1a), 1.16 (1H, m, H-15a), 1.17 (1H, m, H-19a), 1.20 (3H, s, H-27), 1.24 (3H, s, H-24), 1.27 (1H, m, H-21a), 1.33 (1H, m, H-7a), 1.35 (1H, dm, J = 10.2 Hz, H-5), 1.43 (1H, m, H-21b), 1.53 (2H, m, H-6b, H-7b), 1.62 (1H, dm, J = 13.3 Hz, H-22a), 1.63 (1H, m, H-15b), 1.65 (1H, m, H-16a), 1.70 (1H, m, H-9), 1.76 (1H, tm, J = 13.6 Hz, H-19b), 1.77 (1H, m, H-1b), 1.79 (1H, m, H-22b), 1.80 (1H, m, H-2a), 1.94 (1H, m, H-11), 1.95 (1H, m, H-2b), 2.10 (1H, m, H-16b), 2.85 (1H, dd, J = 13.6, 3.8 Hz, H-18), 3.86 (1H, m, H-3), 5.30 (1H, brt, J = 3.2 Hz, H-12), 9.54 (1H, s, H-23); <sup>13</sup>C NMR of the aglycon (CD<sub>3</sub>OD, 125 MHz), see Table 1; <sup>1</sup>H and <sup>13</sup>C NMR of the glycosidic part, see Table 2; HR-ESI-MS (negative ion mode): m/z 1647.6368 [M-H]<sup>-</sup> (calcd for C<sub>72</sub>H<sub>111</sub>O<sub>40</sub>S, 1647.6372); ESI-MS (negative ion

mode):  $m/z$  1647 [M-H]<sup>-</sup>, 1568 [M-HSO<sub>3</sub>]<sup>-</sup>, 1177 [M-H-470]<sup>-</sup>, 940 [M-708]<sup>-</sup>, 707 [ester chain-H]<sup>-</sup>.

4.5.

*3-O-(β-D-xylopyranosyl-(1→3)-[β-D-galactopyranosyl-(1→2)]-β-D-glucuronopyranosyl)-28-O-(3-O-sulphate-β-D-glucopyranosyl-(1→3)-[β-D-xylopyranosyl-(1→4)]-α-L-rhamnopyranosyl-(1→2)-4-O-acetyl-β-D-fucopyranosyl)-gypsogenin 2*

White powder; [α]<sup>20</sup><sub>D</sub> + 3.6 (c 0.11, H<sub>2</sub>O); <sup>1</sup>H NMR of the aglycon (CD<sub>3</sub>OD, 500 MHz) were identical to those described for **1** (± 0.4 ppm) ; <sup>13</sup>C NMR chemical shift values of the aglycon (CD<sub>3</sub>OD, 125 MHz), see Table 1; <sup>1</sup>H and <sup>13</sup>C NMR of the glycosidic part, see Table 2; HR-ESI-MS (negative ion mode):  $m/z$  1647.6381 [M-H]<sup>-</sup> (calcd for C<sub>72</sub>H<sub>111</sub>O<sub>40</sub>S, 1647.6372); ESI-MS (negative ion mode):  $m/z$  1647 [M-H]<sup>-</sup>, 1568 [M-HSO<sub>3</sub>]<sup>-</sup>, 1177 [M-H-470]<sup>-</sup>, 939 [M-708]<sup>-</sup>, 707 [ester chain-H]<sup>-</sup>.

4.6.

*3-O-(α-L-arabinopyranosyl-(1→3)-[β-D-galactopyranosyl-(1→2)]-β-D-glucuronopyranosyl)-28-O-(3-O-sulphate-β-D-glucopyranosyl-(1→3)-[β-D-xylopyranosyl-(1→4)]-α-L-rhamnopyranosyl-(1→2)-3-O-acetyl-β-D-fucopyranosyl)-gypsogenin 3*

White powder; [α]<sup>20</sup><sub>D</sub> + 5.5 (c 0.07, MeOH); <sup>1</sup>H NMR of the aglycon (CD<sub>3</sub>OD, 500 MHz) were identical to those described for **1** (± 0.4 ppm); <sup>13</sup>C NMR of the aglycon (CD<sub>3</sub>OD, 125 MHz), see Table 1; <sup>1</sup>H and <sup>13</sup>C NMR of the glycosidic part, see Table 2; HR-ESI-MS (negative ion mode):  $m/z$  1647.6365 [M-H]<sup>-</sup> (calcd for C<sub>72</sub>H<sub>111</sub>O<sub>40</sub>S, 1647.6372) ; ESI-MS (negative ion mode):  $m/z$  1669 [M-H+Na]<sup>-</sup>, 1647 [M-H]<sup>-</sup>, 1568 [M-HSO<sub>3</sub>]<sup>-</sup>, 1177 [M-H-470]<sup>-</sup>, 939 [M-708]<sup>-</sup>, 707 [ester chain-H]<sup>-</sup>.

4.7.

*3-O-(α-L-arabinopyranosyl-(1→3)-[β-D-galactopyranosyl-(1→2)]-β-D-glucuronopyranosyl)-28-O-(β-D-glucopyranosyl-(1→3)-[β-D-xylopyranosyl-(1→4)]-α-L-rhamnopyranosyl-(1→2)-4-O-acetyl-β-D-fucopyranosyl)-gypsogenin 4*



White powder;  $[\alpha]_{\text{D}}^{20} + 4.2$  ( $c$  0.15, MeOH);  $^1\text{H}$  NMR of the aglycon ( $\text{CD}_3\text{OD}$ , 500 MHz) were identical to those described for **1** ( $\pm 0.4$  ppm);  $^{13}\text{C}$  NMR of the aglycon ( $\text{CD}_3\text{OD}$ , 125 MHz), see Table 1;  $^1\text{H}$  and  $^{13}\text{C}$  NMR of the glycosidic part, see Table 3; HR-ESI-MS (positive ion mode):  $m/z$  1591.6788  $[\text{M}+\text{Na}]^+$  (calcd for  $\text{C}_{72}\text{H}_{112}\text{O}_{37}\text{Na}$ , 1591.6780); ESI-MS (negative ion mode):  $m/z$  1567  $[\text{M}-\text{H}]^-$ ; ESIMS-MS :  $\text{MS}^1$  (1567):  $m/z$  939  $[\text{M}-\text{H}-628]^-$ , 469  $[\text{triaccharidic chain}-\text{H}]^-$ , 337  $[\text{GlcA}-\text{Gal}]^-$ ;  $\text{MS}^2$  (939):  $m/z$  469  $[\text{triaccharidic chain}-\text{H}]^-$ .

4.8.

*3-O-( $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 3))- $[\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucuronopyranosyl)-28-O-(6-O-acetyl- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3))- $[\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 4)]- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-4-O-acetyl- $\beta$ -D-fucopyranosyl)-gypsogenin **5***

White powder;  $[\alpha]_{\text{D}}^{20} + 3.8$  ( $c$  0.29, MeOH);  $^1\text{H}$  NMR of the aglycon ( $\text{CD}_3\text{OD}$ , 500 MHz) were identical to those described for **1** ( $\pm 0.4$  ppm);  $^{13}\text{C}$  NMR of the aglycon ( $\text{CD}_3\text{OD}$ , 125 MHz), see Table 1;  $^1\text{H}$  and  $^{13}\text{C}$  NMR of the glycosidic part, see Table 3; HR-ESI-MS (negative ion mode):  $m/z$  1609.6896  $[\text{M}-\text{H}]^-$  (calcd for  $\text{C}_{74}\text{H}_{113}\text{O}_{38}$ , 1609.6910); ESI-MS (negative ion mode):  $m/z$  1609  $[\text{M}-\text{H}]^-$ , 1567  $[\text{M}-\text{H}-\text{Ac}]^-$ , 939  $[\text{M}-\text{H}-670]^-$ ; ESI-MS-MS :  $\text{MS}^1$  (1609):  $m/z$  939  $[\text{M}-\text{H}-670]^-$ , 469  $[\text{triaccharidic chain}-\text{H}]^-$ .

4.9.

*3-O-( $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 3))- $[\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucuronopyranosyl)-28-O-(6-O-acetyl- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3))- $[\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 4)]- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-4-O-acetyl- $\beta$ -D-fucopyranosyl)-gypsogenin **6***

White powder;  $[\alpha]_{\text{D}}^{20} + 28.0$  ( $c$  0.03, MeOH);  $^1\text{H}$  NMR of the aglycon ( $\text{CD}_3\text{OD}$ , 500 MHz) were identical to those described for **1** ( $\pm 0.4$  ppm);  $^{13}\text{C}$  NMR of the aglycon ( $\text{CD}_3\text{OD}$ , 125 MHz), see Table 1;  $^1\text{H}$  and  $^{13}\text{C}$  NMR of the glycosidic part, see Table 3; HR-ESI-MS (positive ion mode):  $m/z$  1633.6909  $[\text{M}+\text{Na}]^+$  (calcd for  $\text{C}_{74}\text{H}_{114}\text{O}_{38}\text{Na}$ , 1633.6886); ESI-MS (negative ion mode):  $m/z$  1609  $[\text{M}-\text{H}]^-$ , 1567  $[\text{M}-\text{H}-\text{Ac}]^-$ , 939  $[\text{M}-\text{H}-670]^-$ ; ESI-MS-MS :  $\text{MS}^1$

(1609):  $m/z$  939 [M-H-670]<sup>-</sup>, 469 [triaccharidic chain-H]<sup>-</sup>; MS<sup>2</sup> (939):  $m/z$  469 [triaccharidic chain-H]<sup>-</sup>.

4.10.

*3-O-( $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 3))- $[\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucuronopyranosyl)-28-O-( $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3))- $[\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 4)]- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-fucopyranosyl)-gypsogenin 7*

White powder;  $[\alpha]_D^{20} + 2.7$  ( $c$  0.08, MeOH); <sup>1</sup>H NMR of the aglycon (CD<sub>3</sub>OD, 500 MHz) were identical to those described for **1** ( $\pm$  0.4 ppm); <sup>13</sup>C NMR of the aglycon (CD<sub>3</sub>OD, 125 MHz), see Table 1; <sup>1</sup>H and <sup>13</sup>C NMR of the glycosidic part, see Table 3; HR-ESI-MS (positive ion mode):  $m/z$  1549.6661 [M+Na]<sup>+</sup> (calcd for C<sub>70</sub>H<sub>110</sub>O<sub>36</sub>Na, 1549.6675); ESI-MS (negative ion mode):  $m/z$  1525 [M-H]<sup>-</sup>; ESI-MS-MS : MS<sup>1</sup> (1525):  $m/z$  939 [M-H-628]<sup>-</sup>, 469 [triaccharidic chain-H]<sup>-</sup>; MS<sup>2</sup> (939):  $m/z$  469 [triaccharidic chain-H]<sup>-</sup>.

4.11.

*3-O-( $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 3))- $[\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucuronopyranosyl)-28-O-(6-O-acetyl- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3))- $[\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 3)]- $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 4)]- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-4-O-acetyl- $\beta$ -D-fucopyranosyl)-gypsogenin 8*

White powder;  $[\alpha]_D^{20} + 2.1$  ( $c$  0.29, MeOH); <sup>1</sup>H NMR of the aglycon (CD<sub>3</sub>OD, 500 MHz) were identical to those described for **1** ( $\pm$  0.4 ppm); <sup>13</sup>C NMR of the aglycon (CD<sub>3</sub>OD, 125 MHz), see Table 1; <sup>1</sup>H and <sup>13</sup>C NMR of the glycosidic part, see Table 4; HR-ESI-MS (positive ion mode):  $m/z$  1765.7303 [M+Na]<sup>+</sup> (calcd for C<sub>79</sub>H<sub>122</sub>O<sub>42</sub>Na, 1765.7308); ESI-MS (negative ion mode):  $m/z$  1741 [M-H]<sup>-</sup>, 1639 [M-H-Ac]<sup>-</sup>, 1609 [M-H-Ara]<sup>-</sup>, 939 [M-H-802]<sup>-</sup>, 469 [triaccharidic chain-H]<sup>-</sup>; ESI-MS-MS: MS<sup>1</sup> (1741):  $m/z$  1639 [M-H-Ac]<sup>-</sup>, 1609 [M-H-Ara]<sup>-</sup>, 939 [M-H-802]<sup>-</sup>, 469 [triaccharidic chain-H]<sup>-</sup>; MS<sup>3</sup> (939):  $m/z$  807 [M-H-802-Ara]<sup>-</sup>, 469 [triaccharidic chain-H]<sup>-</sup>.

4.12.

3-O-( $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 3))- [ $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucuronopyranosyl)-28-O-(6-O-acetyl- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3))- [ $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 3))- $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 4)]- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-4-O-acetyl- $\beta$ -D-fucopyranosyl)-gypsogenin **9**

White powder;  $[\alpha]_D^{20} + 3.7$  (*c* 0.11, MeOH);  $^1\text{H}$  NMR of the aglycon (CD<sub>3</sub>OD, 500 MHz) were identical to those described for **1** ( $\pm 0.4$  ppm);  $^1\text{H}$  NMR of the glycosidic part, see Table 4; HR-ESI-MS (positive ion mode): *m/z* 1765.7318 [M+Na]<sup>+</sup> (calcd for C<sub>79</sub>H<sub>122</sub>O<sub>42</sub>Na, 1765.7308); ESI-MS (positive ion mode) : *m/z* 1765 [M+Na]<sup>+</sup>, 1633 [M+Na-Ara]<sup>+</sup>, 1295 [M+Na-470]<sup>+</sup>, 825 [ester chain+Na]<sup>+</sup>, 493 [trisaccharidic chain+Na]<sup>+</sup>; ESI-MS-MS (positive ion mode) : MS<sup>1</sup> (1765): *m/z* 1295 [M+Na-470]<sup>+</sup>, 825 [ester chain+Na]<sup>+</sup>; MS<sup>2</sup> (1633): *m/z* 1163 [M+Na-Ara-470]<sup>+</sup>, 693 [ester chain+Na-Ara]<sup>+</sup>; MS<sup>2</sup> (825): *m/z* 765 [ester chain+Na-CH<sub>3</sub>COOH]<sup>+</sup>, 693 [ester chain+Na-Ara]<sup>+</sup>, 561 [ester chain+Na-Ara-Xyl]<sup>+</sup>, 501 [ester chain+Na-Ara-Xyl-CH<sub>3</sub>COOH]<sup>+</sup>; ESI-MS-MS (negative ion mode) : MS<sup>1</sup> (1741): *m/z* 1741 [M-H]<sup>-</sup>, 939 [M-H-802]<sup>-</sup>, 469 [trisaccharidic chain-H]<sup>-</sup>.

4.13.

3-O-( $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 3))- [ $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucuronopyranosyl)-28-O-( $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3))- [ $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 3))- $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 4)]- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-4-O-cis-methoxycinnamoyl- $\beta$ -D-fucopyranosyl)-gypsogenin **10**

White powder;  $[\alpha]_D^{20} + 3.7$  (*c* 0.11, MeOH);  $^1\text{H}$  NMR of the aglycon (CD<sub>3</sub>OD, 500 MHz) were identical to those described for **1** ( $\pm 0.4$  ppm);  $^{13}\text{C}$  NMR of the aglycon (CD<sub>3</sub>OD, 125 MHz), see Table 1;  $^1\text{H}$  and  $^{13}\text{C}$  NMR of the glycosidic part, see Table 4; HR-ESI-MS (negative ion mode): *m/z* 1685.7211[M-H]<sup>-</sup> (calcd for C<sub>80</sub>H<sub>117</sub>O<sub>38</sub>, 1685.7223).

4.14.

3-O-( $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 3))- [ $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucuronopyra

*nosyl)-28-O-(β-D-glucopyranosyl-(1→3)-[α-L-arabinopyranosyl-(1→3)-β-D-xylopyranosyl-(1→4)]-α-L-rhamnopyranosyl-(1→2)-4-O-trans-methoxycinnamoyl-β-D-fucopyranosyl)-gypsogenin **11***

White powder;  $[\alpha]_D^{20} + 0$  (*c* 0.06, MeOH);  $^1\text{H}$  NMR of the aglycon (CD<sub>3</sub>OD, 500 MHz) were identical to those described for **1** ( $\pm 0.4$  ppm);  $^{13}\text{C}$  NMR of the aglycon (CD<sub>3</sub>OD, 125 MHz), see Table 1;  $^1\text{H}$  NMR of the glycosidic part, see Table 4; HR-ESI-MS (negative ion mode): *m/z* 1685.7230 [M-H]<sup>-</sup> (calcd for C<sub>80</sub>H<sub>117</sub>O<sub>38</sub>, 1685.7223); ESI-MS (negative ion mode): *m/z* 1785 [M-H]<sup>-</sup>, 1553 [M-H-Ara ou Xyl]<sup>-</sup>, 1523 [M-H-Gal]<sup>-</sup>, 939 [M-H-746]<sup>-</sup>, 745 [ester chain-H]<sup>-</sup>; 469 [triaccharidic chain-H]<sup>-</sup>; ESI-MS-MS : MS<sup>1</sup> (1685): *m/z* 1507 [M-H-transMCin]<sup>-</sup>, 1609 [M-H-Ara ou Xyl]<sup>-</sup>, 939 [M-H-746]<sup>-</sup>, 469 [triaccharidic chain-H]<sup>-</sup>; MS<sup>2</sup> (939): *m/z* 807 [M-H-746-Ara]<sup>-</sup>, 469 [triaccharidic chain-H]<sup>-</sup>.

#### 4.16. Acid hydrolysis of saponin mixture

An aliquot of the crude saponin mixture (100 mg) was refluxed with 6 ml of 1 N HCl for 3 h. The saponin mixture (27.9 mg) was extracted with EtOAc (3 x 6 ml), washed with H<sub>2</sub>O, and evaporated to dryness. The saponin mixture was purified by silica gel column using a gradient of CHCl<sub>3</sub>/MeOH (1:0-95:5) to give gypsogenin (5.3 mg) and gypsogenin-3-*O*-(6-*O*-methyl)-glucoside **13** (6.6 mg). The acid aqueous layer was neutralized with 1N NaOH and freeze-dried. Seven sugars were identified and compared with authentic samples by TLC using MeCOEt-isoPrOH-Me<sub>2</sub>CO-H<sub>2</sub>O (20:10:7:6) as rhamnose, xylose, fucose, glucose, arabinose, galactose and glucuronic acid. After preparative TLC of the sugar mixture (60 mg) in this solvent (20 mg on each TLC plate (20x20 cm)), each isolated sugar was filtered to eliminate SiOH residue. The optical rotation of each purified sugar was measured to afford rhamnose (2.9 mg; *R<sub>f</sub>* 0.66;  $[\alpha]_D^{20} -14 \rightarrow +19$  (H<sub>2</sub>O; *c* 0.24)), xylose (3,5 mg; *R<sub>f</sub>* 0.62;  $[\alpha]_D^{20} + 18$  (H<sub>2</sub>O; *c* 0.13)), fucose (1.6 mg; *R<sub>f</sub>* 0.59;  $[\alpha]_D^{20} + 78$  (H<sub>2</sub>O; *c* 0.12)), arabinose (0.7 mg; *R<sub>f</sub>* 0.48;  $[\alpha]_D^{20} + 41$  (H<sub>2</sub>O; *c* 0.06)), glucose (1.7 mg; *R<sub>f</sub>* 0.42;  $[\alpha]_D^{20} + 18$  (H<sub>2</sub>O; *c*

0.14)), galactose (4.3 mg; *R<sub>f</sub>* 0.35;  $[\alpha]_D^{20} + 45^\circ$  (H<sub>2</sub>O; *c* 0.36)), and glucuronic acid (4 mg; *R<sub>f</sub>* 0.06;  $[\alpha]_D^{20} + 8^\circ$ ; (H<sub>2</sub>O; *c* 0.2)).

#### 4.17. *Gypsogenin-3-O-(6-O-methyl)-glucoside* **13**

White powder; <sup>13</sup>C NMR of the aglycon (CD<sub>3</sub>OD, 125 MHz), see Table 1; <sup>1</sup>H NMR of the glycosidic part (CD<sub>3</sub>OD, 500 MHz): 3.32 (1H, *dd*, 8.7, 8.1, H-2'), 3.50 (1H, *t*, 9.1, H-3'), 3.70 (1H, *t*, 9.1, H-4'), 3.84 (3H, *s*, H-7'), 3.85 (1H, *m*, H-5'), 4.24 (1H, *d*, 8.1, H-1'); <sup>13</sup>C NMR of the glycosidic part (CD<sub>3</sub>OD, 125 MHz):  $\delta_C$ : 52.7 (C-7'), 71.1 (C-4'), 73.2 (C-2'), 74.5 (C-5'), 75.3 (C-3'), 104.0 (C-1'), 169.6 (C-6'); HR-ESI-MS (positive ion mode): *m/z* 683.3777 [M+Na]<sup>+</sup> (calcd for C<sub>37</sub>H<sub>56</sub>O<sub>10</sub>Na, 683.3771).

#### 4.18. *Cytotoxicity experiments*

##### *Cytotoxicity toward normal and leukemic cells in culture*

Lyophilized crude extract (10% aqueous methanol) from the roots of *G. trichotoma* was dissolved at a concentration of 10 mg/ml in phosphate-buffered saline (PBS) (as a stock solution), filtered through a filter 0.22  $\mu$ m (Millipore, Bedford, MA, USA) and stored at 4° C. The stock solution was diluted to 1mg/ml, 100  $\mu$ g/ml, 10  $\mu$ g/ml before the cell viability assay. The rat alveolar macrophage-like cell line NR8383, and the human leukemic U937 and BV – 173 cell lines were purchased from the American Type Culture Collection (CRL-2192 and CRL-1593.2, ATCC, Manassas, VA) and the Leibnitz Institute-DSMZ German Collection for Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), respectively.

NR8383 and U937 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplied with 200 mM L-glutamine, supplemented with 15 % of heat-inactivated foetal calf serum and a mixture of antibiotic/antimycotic compounds (100 U/mL of penicillin, 100  $\mu$ g/mL of streptomycin and 0.25  $\mu$ g/mL of amphotericin B ). BV – 173 cells were maintained in RPMI-1640 medium supplied with 200 mM L-glutamine and 10% of heat-inactivated fetal

calf serum. Cells were incubated by standard conditions (37°C under 5 % CO<sub>2</sub> and humidified atmosphere).

The cellular viability was determined by Trypan blue exclusion test (NR8383 and U937) (Liu et al., 1999) and the colorimetric MTT-assay (BV – 173) (Mosmann, 1983). NR8383 and U937 cells were dispensed into 24-well microplates (at 5x10<sup>5</sup> cells/ mL), in 1 ml of DMEM medium/well and incubated overnight at 37° C, 5 % CO<sub>2</sub> and 95 % air. Cells are then treated with different concentrations of plant extract (final concentration 100, 10, 1 and 0.1 µg/ml in the cultures with NR8383 cells and 20, 10 and 1 µg/ml in those with U937 cells) for 24 hours. Unexposed control cultures were maintained under the same conditions. In the Tripan blue exclusion assays the enumeration is carried out by immediate microscopic observation using a Malassez counting cell. Seven numerations per sample were performed to limit the risk of error. The percentage of viability corresponds to the percentage of living cells resistant to any adverse effect of the tested extracts.

Logarithmic-phase growing BV-173 cells (10<sup>4</sup> cells/ml) were treated in 96-well with different concentrations of plant extract (final concentration 1000, 100, 10, 1 and 0.1 µg/ml). Briefly, after 24 h incubation with the plant extract, MTT-solution (10 mg/ml in PBS) was added (10 µl/well) to tissue culture medium and cells were further incubated for 3.5 h at 37°C. Formed formazan crystals were dissolved by adding 110 µl/well of 0.04 N HCl in isopropanol.

Absorption was measured by an ELISA reader (Labexim LMR1) at 540 nm. The reference filter was set on 690 nm. For each concentration, at least 8 wells were used. RPMI-1640 medium (100 µM) with 10 µl MTT stock-solution and 110 µl 0.04 N HCl in 2-propanol was used as blank solution. Data obtained were presented as percentage of untreated control (% of untreated control =  $E_{\text{(extinction of viable cells in treated samples)}} \times 100 / E_0_{\text{(extinction of viable cells in untreated control)}}$ ).

#### *Synergistic effect of aminoacylsaponins (ACS) and targeted toxin (SE) on cytotoxicity*

HER14 cells which are Swiss mouse embryo NIH-3T3 cells transfected with human EGFR

were cultured in MEM medium supplemented with 10 % fetal bovine serum (BioChrom KG, Berlin, Germany), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were cultivated at 37° C, 5% CO<sub>2</sub>, and 95% humidity.

HER14 cells (2.000/well) were seeded in 96-well plates and cultured for 24 h in 100 µl cell culture medium per well. The medium was removed and cells were washed (100 µl PBS/well). For determining the cytotoxicity enhancing properties of the saponins 180 µl MEM medium supplemented with 90 µg/ml aminoacylsaponins (Gevrenova et al., 2006) and 20 µl SE was added (final concentration 10<sup>-7</sup> nM – 100 nM) to each well. Control cells were either incubated with PBS, aminoacyl saponins (ACS) or targeted toxine (SE) alone. After 72 h the cellular viability was determined by MTT-assay as described elsewhere (Mosmann, 1983).

Data are expressed as mean ± standard deviation (SD). Student's t test was used to evaluate the differences between various experimental and control groups. P values less than 0.05 were considered significant.

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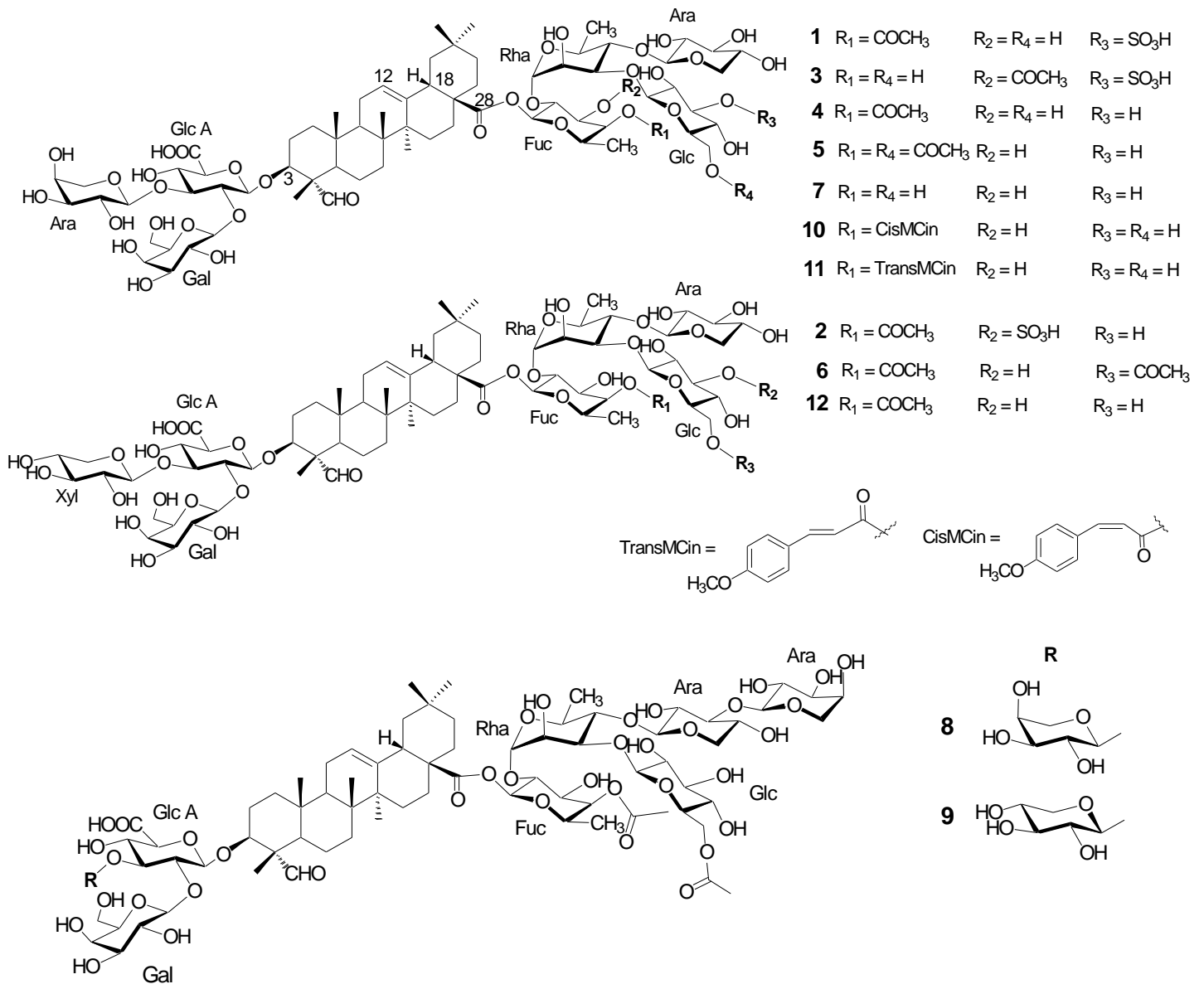
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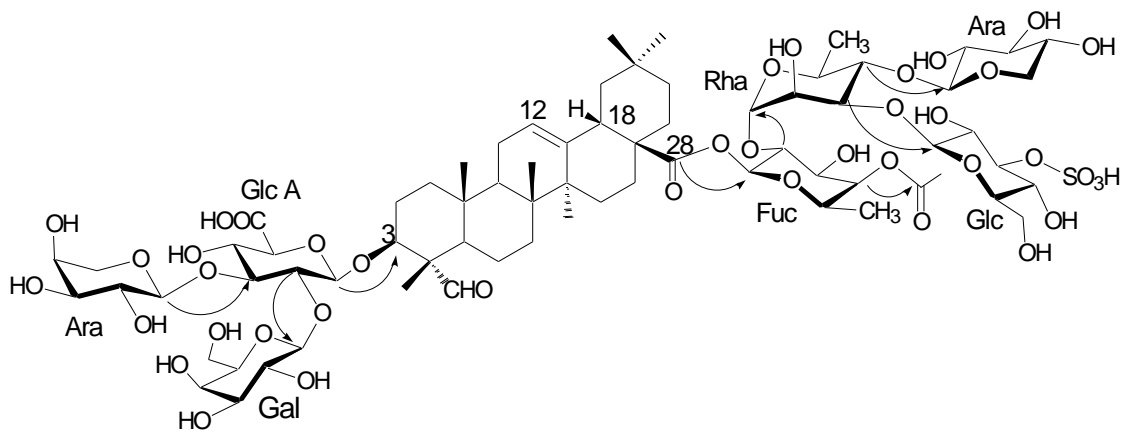
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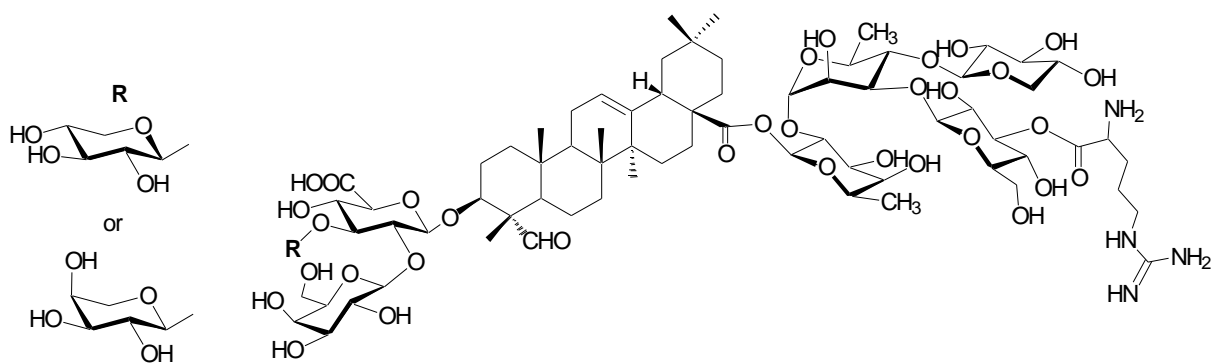
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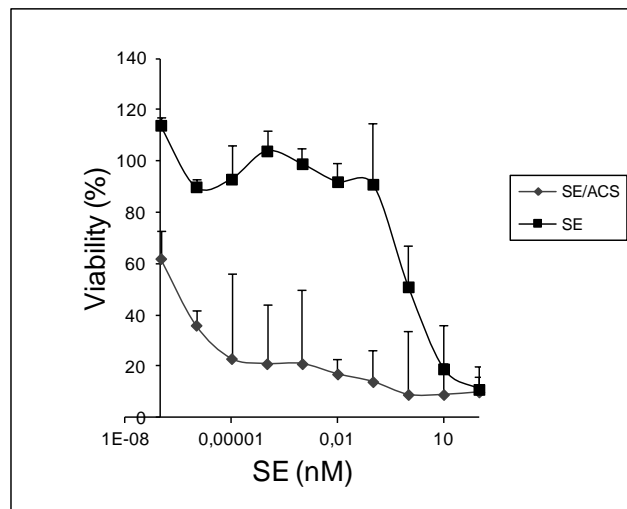
**Fig 1.** Structure of the saponins 1-12



**Fig 2.** HMBC correlations of **1**



**Fig 3.** Structure of the aminoacyl saponins (ACS)



**Fig. 4.** Cytotoxicity enhancement of the targeted toxin SE ( $10^{-7}$  nM – 100 nM) in presence of ACS (90  $\mu\text{g}/\text{ml}$ ) in HER-14 cells (evaluated by MTT assay). All values represents the mean of three individual determinations,  $n=3$ .

**Table 1.**  $^{13}\text{C}$  NMR data ( $\delta$  in ppm) of the aglycon part of **1-3**, **5**, **6**, **8** (125 MHz,  $\text{CD}_3\text{OD}$ ), **4**, **7**, **10** (150 MHz,  $\text{CD}_3\text{OD}$ ) and **13** (125 MHz,  $\text{CDCl}_3$ )

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>10</b>	<b>13</b>
1	39.1	39.2	39.2	39.2	39.2	37.9	39.2	39.2	39.2	37.9
2	25.7	25.7	25.7	25.7	25.7	25.8	25.7	25.7	25.7	24.5
3	86.7	86.3	86.9	86.3	86.4	86.3	86.0	86.4	86.0	82.3
4	56.0	56.3	56.0	56.3	56.4	56.4	56.4	56.6	56.3	55.0
5	49.2	49.2	49.8	48.9	49.2	49.1	48.7	48.7	48.7	48.5
6	21.4	21.4	21.5	21.5	22.0	21.9	21.5	21.4	21.5	20.6
7	33.2	33.2	33.4	33.1	33.8	33.5	33.4	33.8	33.4	31.9
8	41.0	41.1	41.0	41.1	41.0	41.0	41.1	40.9	41.1	39.5
9	48.9	48.9	48.9	48.8	48.9	48.7	48.9	48.9	48.9	47.5
10	37.1	37.1	37.2	37.1	37.1	37.1	37.1	37.1	37.1	35.8
11	24.5	24.5	24.5	24.6	24.6	24.5	24.5	24.5	24.3	23.3
12	123.4	123.5	123.5	123.4	123.4	123.5	123.5	123.4	123.4	121.9
13	144.8	144.7	144.9	144.9	144.8	144.9	144.8	145.0	145.1	143.8
14	43.0	43.0	43.0	42.8	43.0	43.0	43.0	43.0	43.0	41.7
15	28.9	28.9	29.2	29.0	30.8	28.9	29.0	29.7	28.7	27.6
16	24.1	24.2	23.8	24.1	23.8	23.8	24.0	24.2	23.7	22.9
17	48.1	48.1	48.2	48.1	48.1	48.1	48.0	48.1	48.1	46.3
18	42.8	42.8	42.8	43.0	42.9	42.9	42.8	42.8	42.9	41.0
19	47.3	47.3	48.0	47.3	47.2	47.2	47.3	47.2	47.3	45.9
20	31.5	31.5	31.5	31.6	31.5	31.5	31.6	31.5	31.6	30.6
21	34.8	34.8	34.8	34.8	34.9	34.9	34.8	34.8	34.9	33.8
22	33.1	33.1	33.2	33.3	33.1	33.1	33.1	33.1	33.1	32.4
23	212.4	211.8	212.5	211.1	211.3	211.2	211.1	211.8	211.2	207.6
24	11.2	11.0	11.3	10.8	11.0	11.0	10.9	10.9	11.0	9.8
25	16.3	16.3	16.3	16.3	16.3	16.4	16.3	16.4	16.4	15.5
26	17.9	17.9	17.9	17.9	17.9	18.0	17.8	19.9	18.0	16.9
27	26.2	26.2	26.2	26.3	26.2	26.2	26.3	26.2	26.3	25.9
28	178.2	178.2	177.8	178.0	178.2	177.9	178.0	nd	178.1	181.2
29	33.5	33.5	33.5	33.5	33.5	33.5	33.5	33.4	33.5	33.0
30	24.1	24.1	24.1	24.2	24.1	24.1	24.1	24.1	23.7	23.5



**Table 2.** <sup>1</sup>H (500 MHz) and <sup>13</sup>C NMR (125 MHz) data (δ in ppm) of the osidic parts of sulphated saponins **1-3** (CD<sub>3</sub>OD).

<u>2<i>S</i>-O-</u>	<b>1</b>		<b>2</b>		<b>3</b>	
	δ <sub>H</sub> ( <i>m</i> , <i>J</i> ( Hz))	δ <sub>C</sub>	δ <sub>H</sub> ( <i>m</i> , <i>J</i> ( Hz))	δ <sub>C</sub>	δ <sub>H</sub> ( <i>m</i> , <i>J</i> ( Hz))	δ <sub>C</sub>
<i>β</i> -D-Fuc						
1	5.42 ( <i>d</i> , 8.1)	95.3	5.44 ( <i>d</i> , 7.6)	95.4	5.52 ( <i>d</i> , 8.1)	94.8
2	3.70 ( <i>dd</i> , 9.0, 8.2)	76.1	3.67 ( <i>m</i> )	76.5	4.11 ( <i>t</i> , 8.7)	73.5
3	3.91 ( <i>dd</i> , 9.0, 3.7)	73.7	3.89 ( <i>m</i> )	75.2	4.93 ( <i>m</i> )	78.7
4	5.13 ( <i>dm</i> , 3.4)	75.2	5.13 ( <i>m</i> )	73.4	3.82 ( <i>m</i> )	72.4
5	3.88 ( <i>m</i> )	70.8	3.87 ( <i>m</i> )	71.1	3.84 ( <i>m</i> )	70.4
6	1.10 ( <i>d</i> , 6.4)	16.5	1.10 ( <i>d</i> , 6.4)	16.5	1.24 ( <i>d</i> , 6.4)	16.3
<i>α</i> -L-Rha						
1	5.18 ( <i>d</i> , 1.1)	102.1	5.13 ( <i>brs</i> )	102.2	5.08 ( <i>d</i> , 1.1)	101.7
2	4.32 ( <i>brt</i> , 2.0)	71.3	4.34 ( <i>m</i> )	71.2	4.03 ( <i>brt</i> , 2.0)	71.7
3	4.00 ( <i>dd</i> , 9.0, 3.1)	82.7	4.03 ( <i>dd</i> , 9.2, 3.0)	82.7	3.83 ( <i>m</i> )	82.8
4	3.68 ( <i>t</i> , 9.0)	79.1	3.67 ( <i>t</i> , 9.8)	79.0	3.65 ( <i>t</i> , 9.2)	79.3
5	3.85 ( <i>m</i> )	69.2	3.84 ( <i>m</i> )	69.4	3.83 ( <i>m</i> )	68.9
6	1.27 ( <i>d</i> , 6.2)	18.6	1.26 ( <i>d</i> , 6.2)	18.7	1.28 ( <i>d</i> , 5.9)	18.7
<i>β</i> -D-Glc						
1	4.68 ( <i>d</i> , 8.0)	105.0	4.69 ( <i>d</i> , 8.0)	105.0	4.67 ( <i>d</i> , 7.9)	105.0
2	3.53 ( <i>dd</i> , 9.2, 8.0)	73.8	3.52 ( <i>dd</i> , 9.2, 8.0)	73.8	3.51 ( <i>m</i> )	73.9
3	4.25 ( <i>t</i> , 9.0)	85.7	4.25 ( <i>t</i> , 9.0)	85.7	4.25 ( <i>t</i> , 9.2)	85.6
4	3.61 ( <i>dd</i> , 9.9, 8.7)	69.9	3.60 ( <i>dd</i> , 9.9, 8.7)	70.0	3.68 ( <i>t</i> , 9.6)	69.4
5	3.37 ( <i>m</i> )	77.2	3.37 ( <i>m</i> )	77.2	3.36 ( <i>m</i> )	77.1
6	3.74 ( <i>dd</i> , 12.1, 5.4)	62.0	3.72 ( <i>dd</i> , 12.4, 4.2)	62.0	3.81 ( <i>m</i> )	61.5
	3.89 ( <i>m</i> )		3.88 ( <i>m</i> )		3.87 ( <i>m</i> )	
<i>β</i> -D-Xyl	4.70 ( <i>d</i> , 8.0)	105.2				
1	3.06 ( <i>dd</i> , 8.9, 8.3)	75.8	4.72 ( <i>d</i> , 7.8)	105.1	4.67( <i>d</i> , 8.3)	105.0
2	3.33 ( <i>t</i> , 9.3)	78.6	3.06 ( <i>t</i> , 8.5)	75.9	3.03 ( <i>dd</i> , 9.0, 8.3)	75.9
3	3.50 ( <i>m</i> )	71.1	3.32 ( <i>m</i> )	78.6	3.29 ( <i>t</i> , 9.0)	78.5
4	3.19 ( <i>t</i> , 10.9)	66.9	3.49 ( <i>m</i> )	71.2	3.50 ( <i>m</i> )	71.1
	3.86 ( <i>m</i> )					
5	4.70 ( <i>d</i> , 8.0)	105.2	3.19 ( <i>t</i> , 10.9)	67.0	3.18 ( <i>t</i> , 10.7)	66.9
			3.85 ( <i>m</i> )		3.85 ( <i>m</i> )	
CH <sub>3</sub> CO	2.21(s)	20.9	2.21 (s)	21.0	2.19 (s)	21.2
CH <sub>3</sub> CO	-	173.1	-	173.1	-	172.1
<u>3-O-</u>						
<i>β</i> -D-GlcA						
1	4.49 ( <i>d</i> , 7.7)	104.6	4.39 ( <i>d</i> , 6.9)	104.4	4.48 ( <i>d</i> , 7.5)	104.7
2	3.70 ( <i>dd</i> , 9.0, 8.0)	78.0	3.69 ( <i>m</i> )	78.4	3.69 ( <i>dd</i> , 9.4, 7.5)	78.2
3	3.75 ( <i>m</i> )	86.2	3.72 ( <i>m</i> )	86.5	3.72 ( <i>t</i> , 9.1)	86.3
4	3.61 ( <i>t</i> , 8.7)	71.4	3.57 ( <i>t</i> , 9.3)	72.0	3.60 ( <i>t</i> , 9.1)	71.3
5	3.87 ( <i>d</i> , 9.5)	76.1	3.60 ( <i>d</i> , 9.4)	78.1	3.85 ( <i>m</i> )	76.0
6	-	172.0	-	176.2	-	173.1
<i>β</i> -D-Gal						
1	4.84 ( <i>d</i> , 7.4)	103.7	4.82 ( <i>d</i> , 7.0)	103.7	4.85 ( <i>d</i> , 7.6)	103.8
2	3.50 ( <i>m</i> )	73.5	3.49 ( <i>m</i> )	73.5	3.48 ( <i>m</i> )	73.5
3	3.51 ( <i>m</i> )	75.2	3.51 ( <i>m</i> )	75.2	3.46 ( <i>m</i> )	75.3
4	3.88 ( <i>m</i> )	70.8	3.84 ( <i>m</i> )	70.7	3.88 ( <i>m</i> )	70.8
5	3.55 ( <i>m</i> )	76.6	3.52 ( <i>m</i> )	76.7	3.53 ( <i>m</i> )	76.6
6	3.77 ( <i>dd</i> , 11.6, 5.4)	62.3	3.72 ( <i>m</i> )	62.2	3.78 ( <i>m</i> )	62.2
	3.83 ( <i>dd</i> , 11.6, 5.4)		3.82 ( <i>dd</i> , 12.3, 6.8)		3.81 ( <i>m</i> )	
<i>α</i> -L-Ara						
1	4.59 ( <i>d</i> , 7.4)	104.8			4.58 ( <i>d</i> , 7.4)	104.9
2	3.62 ( <i>m</i> )	72.8			3.62 ( <i>dd</i> , 9.1, 7.4)	72.9
3	3.55 ( <i>dd</i> , 8.0, 3.6)	74.5			3.53 ( <i>dd</i> , 9.0, 3.3)	74.7
4	3.86 ( <i>m</i> )	69.9			3.85 ( <i>m</i> )	70.0
5	3.65 ( <i>brd</i> , 11.0)	67.8			3.63 ( <i>brd</i> , 12.0)	67.8
	3.95 ( <i>dd</i> , 12.8, 2.0)				3.93 ( <i>dd</i> , 12.0, 2.0)	
<i>β</i> -D-Xyl'						
1			4.64 ( <i>d</i> , 8.2)	104.4		
2			3.27 ( <i>dd</i> , 9.1, 8.2)	75.0		
3			3.34 ( <i>m</i> )	77.7		
4			3.54 ( <i>m</i> )	70.8		
5			3.26 ( <i>t</i> , 11.5)	66.7		
			3.92 ( <i>dd</i> , 11.5, 5.9)			

**Table 3.** <sup>1</sup>H and <sup>13</sup>C NMR data (δ in ppm) of the osidic parts of **5**, **6** (500 MHz) and **4**, **7** (600 MHz) in CD<sub>3</sub>OD.

<u>28-O-</u>	<b>4</b>		<b>5</b>		<b>6</b>		<b>7</b>	
	δ <sub>H</sub> ( <i>m</i> , <i>J</i> (Hz))	δ <sub>C</sub>	δ <sub>H</sub> ( <i>m</i> , <i>J</i> (Hz))	δ <sub>C</sub>	δ <sub>H</sub> ( <i>m</i> , <i>J</i> (Hz))	δ <sub>C</sub>	δ <sub>H</sub> ( <i>m</i> , <i>J</i> (Hz))	δ <sub>C</sub>
<i>β-D-Fuc</i>								
1	5.41 ( <i>d</i> , 8.41)	95.3	5.36 ( <i>d</i> , 8.1)	95.2	5.36 ( <i>d</i> , 8.0)	95.2	5.36 ( <i>d</i> , 8.1)	95.4
2	3.70 ( <i>t</i> , 8.9)	76.0	3.83 ( <i>t</i> , 8.6)	74.2	3.83 ( <i>dd</i> , 9.2, 8.3)	73.6	3.76 ( <i>dd</i> , 9.4, 8.1)	75.6
3	3.88 ( <i>m</i> )	73.9	3.91 ( <i>dd</i> , 9.2, 3.5)	74.8	3.91 ( <i>dd</i> , 9.4, 3.5)	74.8	3.60 ( <i>dd</i> , 9.4, 3.4)	75.9
4	5.12 ( <i>dm</i> , 3.5)	75.1	5.12 ( <i>dm</i> , 3.1)	75.3	5.12 ( <i>d</i> , 3.5)	75.2	3.67 ( <i>d</i> , 3.4)	75.3
5	3.87 ( <i>m</i> )	71.2	3.86 ( <i>m</i> )	71.3	3.85 ( <i>m</i> )	70.0	3.70 ( <i>m</i> )	72.7
6	1.09 ( <i>d</i> , 6.4)	16.5	1.11 ( <i>d</i> , 6.3)	16.5	1.10 ( <i>d</i> , 6.4)	16.5	1.24 ( <i>d</i> , 6.4)	16.5
<i>α-L-Rha</i>								
1	5.20 ( <i>d</i> , 1.1)	102.1	5.42 ( <i>brs</i> )	101.6	5.42 ( <i>brs</i> )	102.2	5.54 ( <i>brd</i> , 1.8)	101.9
2	4.29 ( <i>m</i> )	71.3	4.14 ( <i>brt</i> , 3.2)	71.9	4.14 ( <i>brt</i> , 3.2)	71.2	4.31 ( <i>dd</i> , 3.0, 1.8)	71.3
3	3.96 ( <i>m</i> )	82.8	3.84 ( <i>m</i> )	82.5	3.83 ( <i>dd</i> , 9.9, 3.2)	82.7	3.98 ( <i>dd</i> , 9.6, 3.0)	82.8
4	3.66 ( <i>t</i> , 9.9)	79.0	3.65 ( <i>t</i> , 9.5)	79.1	3.65 ( <i>t</i> , 9.9)	79.0	3.66 ( <i>t</i> , 9.5)	79.1
5	3.85 ( <i>m</i> )	69.2	3.91 ( <i>m</i> )	68.7	3.91 ( <i>m</i> )	69.4	3.86 ( <i>m</i> )	69.0
6	1.26 ( <i>d</i> , 6.3)	18.7	1.30 ( <i>d</i> , 6.0)	18.5	1.29 ( <i>d</i> , 6.2)	18.7	1.26 ( <i>d</i> , 6.3)	18.6
<i>β-D-Glc</i>								
1	4.55 ( <i>d</i> , 7.9)	105.4	4.54 ( <i>d</i> , 7.1)	105.2	4.54 ( <i>d</i> , 7.3)	105.2	4.56 ( <i>d</i> , 7.6)	105.4
2	3.31 ( <i>m</i> )	75.3	3.32 ( <i>m</i> )	75.0	3.30 ( <i>m</i> )	75.2	3.31 ( <i>t</i> , 8.3)	75.3
3	3.35 ( <i>m</i> )	78.3	3.34 ( <i>m</i> )	78.1	3.33 ( <i>m</i> )	78.3	3.35 ( <i>m</i> )	78.3
4	3.35 ( <i>m</i> )	71.2	3.30 ( <i>t</i> , 8.9)	71.9	3.28 ( <i>t</i> , 8.9)	71.7	3.35 ( <i>m</i> )	71.3
5	3.28 ( <i>m</i> )	77.8	3.52 ( <i>m</i> )	75.4	3.50 ( <i>m</i> )	75.3	3.31 ( <i>m</i> )	77.8
6	3.71 ( <i>m</i> )	66.4	4.23 ( <i>dd</i> , 11.7, 6.6)	64.4	4.23 ( <i>dd</i> , 11.9, 7.0)	64.5	3.72 ( <i>dd</i> , 11.8, 5.2)	66.5
	3.86 ( <i>m</i> )		4.44 ( <i>dd</i> , 11.7, 1.8)		4.48 ( <i>dd</i> , 11.9, 2.3)		3.88 ( <i>dd</i> , 11.8, 1.9)	
<i>β-D-Xyl</i>								
1	4.67 ( <i>d</i> , 7.9)	105.2	4.67 ( <i>d</i> , 8.0)	105.1	4.67 ( <i>d</i> , 8.0)	105.3	4.67 ( <i>d</i> , 7.9)	105.2
2	3.07 ( <i>dd</i> , 9.0, 8.1)	75.8	3.03 ( <i>dd</i> , 8.7, 8.3)	75.9	3.03 ( <i>dd</i> , 9.2, 8.0)	76.0	3.08 ( <i>dd</i> , 9.1, 7.9)	75.8
3	3.26 ( <i>t</i> , 9.1)	78.9	3.25 ( <i>t</i> , 9.1)	79.1	3.25 ( <i>t</i> , 9.2)	79.2	3.27 ( <i>t</i> , 9.1)	78.9
4	3.48 ( <i>m</i> )	71.4	3.49 ( <i>m</i> )	71.4	3.49 ( <i>m</i> )	71.3	3.48 ( <i>m</i> )	71.4
5	3.17 ( <i>t</i> , 10.9)	66.9	3.18 ( <i>t</i> , 11.0)	66.9	3.18 ( <i>t</i> , 10.7)	66.9	3.18 ( <i>t</i> , 10.9)	66.9
	3.83 ( <i>m</i> )		3.85 ( <i>m</i> )		3.84 ( <i>dd</i> , 10.7, 5.4)		3.83 ( <i>dm</i> , 11.1)	
<u>CH<sub>3</sub>CO</u>	2.19 ( <i>s</i> )	20.9	2.11 ( <i>s</i> )	20.8	2.11 ( <i>s</i> )	20.8		
<u>CH<sub>3</sub>CO</u>	-	172.8	-	172.7	-	172.8		
<u>CH<sub>3</sub>CO</u>			2.19 ( <i>s</i> )	21.2	2.19 ( <i>s</i> )	21.2		
<u>CH<sub>3</sub>CO</u>			-	172.7	-	172.8		
<u>3-O-</u>								
<i>β-D-GlcA</i>								
1	4.46 ( <i>d</i> , 6.8)	104.6	4.48 ( <i>d</i> , 7.7)	104.9	4.46 ( <i>d</i> , 7.3)	104.8	4.39 ( <i>d</i> , 7.3)	104.5
2	3.69 ( <i>m</i> )	78.3	3.68 ( <i>m</i> )	78.1	3.67 ( <i>dd</i> , 9.6, 7.7)	78.1	3.68 ( <i>dd</i> , 9.0, 7.3)	78.5
3	3.71 ( <i>m</i> )	86.4	3.73 ( <i>m</i> )	86.7	3.70 ( <i>t</i> , 9.6)	86.5	3.70 ( <i>t</i> , 9.0)	86.4
4	3.58 ( <i>m</i> )	71.7	3.57 ( <i>m</i> )	71.9	3.60 ( <i>t</i> , 9.8)	72.0	3.57 ( <i>m</i> )	72.1
5	3.68 ( <i>d</i> , 8.8)	77.8	3.68 ( <i>m</i> )	75.8	3.85 ( <i>m</i> )	76.4	3.61 ( <i>m</i> )	77.8
6	-	nd	-	nd	-	nd	-	171.5
<i>β-D-Gal</i>								
1	4.82 ( <i>d</i> , 7.6)	103.8	4.85 ( <i>d</i> , 7.4)	103.8	4.84 ( <i>d</i> , 7.6)	103.8	4.82 ( <i>d</i> , 8.3)	103.8
2	3.47 ( <i>dd</i> , 9.6, 7.4)	73.6	3.49 ( <i>m</i> )	73.6	3.50 ( <i>dd</i> , 9.6, 7.6)	72.9	3.49 ( <i>t</i> , 8.7)	73.7
3	3.45 ( <i>dd</i> , 9.6, 2.4)	75.4	3.47 ( <i>m</i> )	75.4	3.45 ( <i>dd</i> , 9.6, 2.9)	75.3	3.45 ( <i>dd</i> , 8.7, 3.2)	75.4
4	3.84 ( <i>m</i> )	70.8	3.85 ( <i>m</i> )	70.9	3.86 ( <i>m</i> )	70.9	3.84 ( <i>d</i> , 2.8)	70.8
5	3.52 ( <i>m</i> )	76.7	3.52 ( <i>m</i> )	76.6	3.52 ( <i>m</i> )	76.6	3.51 ( <i>m</i> )	76.6
6	3.75 ( <i>dd</i> , 11.5, 6.0)	62.1	3.78 ( <i>m</i> )	62.0	3.67 ( <i>m</i> )	62.1	3.76 ( <i>m</i> )	62.0
	3.79 ( <i>dd</i> , 11.2, 6.7)		3.79 ( <i>m</i> )		3.68 ( <i>dd</i> , 12.3, 6.8)		3.78 ( <i>m</i> )	
<i>α-L-Ara</i>								
1	4.58 ( <i>d</i> , 7.5)	104.9	4.58 ( <i>d</i> , 7.6)	104.9			4.60 ( <i>d</i> , 7.6)	104.8
2	3.61 ( <i>t</i> , 8.8)	72.9	3.62 ( <i>m</i> )	72.9			3.61 ( <i>dd</i> , 9.7, 8.0)	72.9
3	3.52 ( <i>dd</i> , 8.8, 2.7)	74.6	3.55 ( <i>m</i> )	74.6			3.52 ( <i>dd</i> , 9.7, 4.0)	74.7
4	3.83 ( <i>m</i> )	70.0	3.85 ( <i>m</i> )	70.0			3.82 ( <i>m</i> )	70.1
5	3.63 ( <i>brd</i> , 13.6)	67.9	3.65 ( <i>brd</i> , 11.0)	67.9			3.62 ( <i>dd</i> , 13.5, 3.2)	67.8
	3.94 ( <i>dm</i> , 13.5)		3.95 ( <i>dd</i> , 12.8, 2.0)				3.94 ( <i>dd</i> , 13.5, 2.4)	
<i>β-D-Xyl'</i>								
1					4.61 ( <i>d</i> , 7.7)	104.9		
2					3.26 ( <i>dd</i> , 9.2, 7.7)	75.2		
3					3.31 ( <i>t</i> , 8.4)	78.2		
4					3.52 ( <i>m</i> )	70.9		
5					3.27 ( <i>t</i> , 11.1)	67.2		
					3.93 ( <i>dd</i> , 11.1, 5.6)			

**Table 4.** <sup>1</sup>H NMR data (δ in ppm) of the osidic parts of **8**, **9** (500 MHz) and **10**, **11** (600 MHz), and <sup>13</sup>C NMR data (δ in ppm) of the osidic parts of **8** (125 MHz) and **10** (150 MHz) in CD<sub>3</sub>OD.

<u>28-O-</u>	<b>8</b>		<b>9</b>		<b>10</b>		<b>11</b>	
	δ <sub>H</sub> ( <i>m</i> , <i>J</i> ( Hz))	δ <sub>C</sub>	δ <sub>H</sub> ( <i>m</i> , <i>J</i> ( Hz))	δ <sub>H</sub> ( <i>m</i> , <i>J</i> ( Hz))	δ <sub>C</sub>	δ <sub>H</sub> ( <i>m</i> , <i>J</i> ( Hz))	δ <sub>C</sub>	δ <sub>H</sub> ( <i>m</i> , <i>J</i> ( Hz))
<i>β</i> -D-Fuc								
1	5.34 ( <i>d</i> , 8.0)	95.1	5.34 ( <i>d</i> , 8.2)	5.42 ( <i>d</i> , 8.1)	95.5	5.45 ( <i>d</i> , 7.2)		
2	3.85 ( <i>t</i> , 8.4)	73.7	3.85 ( <i>t</i> , 8.8)	3.70 ( <i>dd</i> , 9.2, 8.0)	76.3	3.80 ( <i>t</i> , 8.6)		
3	3.92 ( <i>dd</i> , 9.5, 3.5)	75.0	3.92 ( <i>dd</i> , 9.0, 3.1)	3.91 ( <i>dd</i> , 9.8, 3.8)	73.7	3.93 ( <i>dd</i> , 8.6, 3.3)		
4	5.12 ( <i>dm</i> , 3.5)	75.3	5.12 ( <i>brd</i> , 2.7)	5.21 ( <i>dm</i> , 3.6)	74.7	5.23 ( <i>dm</i> , 3.1)		
5	3.85 ( <i>m</i> )	71.1	3.85 ( <i>m</i> )	3.90 ( <i>m</i> )	71.3	3.91 ( <i>m</i> )		
6	1.11 ( <i>d</i> , 6.3)	16.4	1.10 ( <i>d</i> , 6.3)	1.11 ( <i>d</i> , 6.4)	16.7	1.11 ( <i>d</i> , 6.1)		
<i>α</i> -L-Rha								
1	5.52 ( <i>brd</i> , 1.7)	101.5	5.51 ( <i>brs</i> )	5.19 ( <i>d</i> , 1.1)	102.2	5.20 ( <i>brs</i> )		
2	4.12 ( <i>dd</i> , 3.2, 1.4)	72.2	4.12 ( <i>brd</i> , 3.2)	4.31 ( <i>dd</i> , 3.2, 1.7)	71.3	4.33 ( <i>brd</i> , 3.3)		
3	3.80 ( <i>dd</i> , 9.5, 3.4)	82.3	3.79 ( <i>m</i> )	3.98 ( <i>dd</i> , 9.1, 3.4)	82.8	4.00 ( <i>dd</i> , 9.2, 3.3)		
4	3.66 ( <i>t</i> , 9.7)	79.3	3.66 ( <i>t</i> , 9.6)	3.65 ( <i>t</i> , 9.7)	79.6	3.67 ( <i>t</i> , 9.2)		
5	3.90 ( <i>m</i> )	68.4	3.90 ( <i>m</i> )	3.85 ( <i>m</i> )	69.2	3.86 ( <i>m</i> )		
6	1.30 ( <i>d</i> , 6.3)	18.5	1.30 ( <i>d</i> , 6.2)	1.26 ( <i>d</i> , 6.3)	18.7	1.27 ( <i>d</i> , 6.7)		
<i>β</i> -D-Glc								
1	4.53 ( <i>d</i> , 8.0)	105.1	4.53 ( <i>d</i> , 7.5)	4.55 ( <i>d</i> , 7.4)	105.3	4.55 ( <i>d</i> , 8.2)		
2	3.32 ( <i>t</i> , 8.8)	75.0	3.32 ( <i>m</i> )	3.30 ( <i>m</i> )	75.3	3.30 ( <i>m</i> )		
3	3.38 ( <i>t</i> , 9.2)	78.2	3.39 ( <i>m</i> )	3.34 ( <i>m</i> )	78.2	3.32 ( <i>m</i> )		
4	3.30 ( <i>t</i> , 9.1)	71.9	3.28 ( <i>t</i> , 9.4)	3.34 ( <i>m</i> )	71.1	3.36 ( <i>m</i> )		
5	3.52 ( <i>m</i> )	75.5	3.52 ( <i>m</i> )	3.27 ( <i>m</i> )	77.8	3.32 ( <i>m</i> )		
6	4.21 ( <i>dd</i> , 11.9, 7.5)	64.7	4.23 ( <i>dd</i> , 12.3, 4.4)	3.69 ( <i>dd</i> , 12.9, 3.1)	62.3	3.65 ( <i>dd</i> , 12.3, 4.1)		
	4.47 ( <i>dd</i> , 11.8, 2.1)		4.47 ( <i>dd</i> , 11.9, 2.1)	3.84 ( <i>m</i> )		3.83 ( <i>m</i> )		
<i>β</i> -D-Xyl								
1	4.70 ( <i>d</i> , 8.0)	105.1	4.71 ( <i>d</i> , 8.1)	4.68 ( <i>d</i> , 7.7)	105.3	4.68 ( <i>d</i> , 7.8)		
2	3.22 ( <i>dd</i> , 8.2)	75.4	3.22 ( <i>t</i> , 8.6)	3.07 ( <i>dd</i> , 8.7, 7.7)	75.9	3.07 ( <i>dd</i> , 9.0, 7.8)		
3	3.41 ( <i>t</i> , 9.2)	88.3	3.41 ( <i>t</i> , 9.1)	3.27 ( <i>t</i> , 9.2)	79.1	3.27 ( <i>t</i> , 9.0)		
4	3.58 ( <i>ddd</i> , 9.0, 4.4, 3.7)	69.8	3.56 ( <i>m</i> )	3.48 ( <i>m</i> )	71.4	3.48 ( <i>m</i> )		
5	3.22 ( <i>t</i> , 12.3)	66.4	3.22 ( <i>t</i> , 12.3)	3.17 ( <i>t</i> , 11.1)	67.0	3.17 ( <i>t</i> , 10.6)		
	3.90 ( <i>m</i> )		3.90 ( <i>m</i> )	3.84 ( <i>dd</i> , 11.0, 5.7)		3.84 ( <i>dd</i> , 10.6, 5.7)		
<i>α</i> -L-Ara								
1	4.58 ( <i>d</i> , 7.4)	104.8	4.58 ( <i>d</i> , 7.4)	1'	-	168.1	-	
2	3.70 ( <i>m</i> )	73.1	3.71 ( <i>m</i> )	2'	5.98 ( <i>d</i> , 12.9)	117.3	6.55 ( <i>d</i> , 15.8)	
3	3.70 ( <i>m</i> )	74.4	3.71 ( <i>m</i> )	3'	6.98 ( <i>d</i> , 12.9)	166	7.72 ( <i>d</i> , 15.8)	
4	3.93 ( <i>m</i> )	68.8	3.95 ( <i>m</i> )	4'	-	127.9	-	
5	3.71 ( <i>m</i> )	67.9	3.71 ( <i>m</i> )	5'	7.78 ( <i>d</i> , 8.8)	133.7	7.62 ( <i>d</i> , 8.3)	
	3.96 ( <i>m</i> )		3.96 ( <i>m</i> )	6'	6.92 ( <i>d</i> , 8.8)	114.5	7.00 ( <i>d</i> , 8.3)	
CH <sub>3</sub> CO	2.10 ( <i>s</i> )	21.1	2.10 ( <i>s</i> )	7'	-	162.2	-	
CH <sub>3</sub> CO	-	172.7	-	8'	6.92 ( <i>d</i> , 8.8)	114.5	7.00 ( <i>d</i> , 8.3)	
CH <sub>3</sub> CO	2.19 ( <i>s</i> )	20.8	2.19 ( <i>s</i> )	9'	7.78 ( <i>d</i> , 8.8)	133.7	7.62 ( <i>d</i> , 8.3)	
CH <sub>3</sub> CO	-	172.7	-	OCH <sub>3</sub>	3.85 ( <i>s</i> )	55.8	3.87 ( <i>s</i> )	
<i>3-O-</i>								
<i>β</i> -D-GlcA								
1	4.47 ( <i>d</i> , 7.6)	104.8	4.48 ( <i>d</i> , 7.6)	4.40 ( <i>d</i> , 7.5)	104.4	4.39 ( <i>d</i> , 8.2)		
2	3.68 ( <i>m</i> )	78.2	3.65 ( <i>dd</i> , 9.0, 7.6)	3.67 ( <i>m</i> )	78.4	3.67 ( <i>m</i> )		
3	3.74 ( <i>t</i> , 9)	86.5	3.68 ( <i>t</i> , 9.5)	3.71 ( <i>t</i> , 9.2)	86.8	3.69 ( <i>m</i> )		
4	3.61 ( <i>m</i> )	71.9	3.60 ( <i>t</i> , 8.9)	3.56 ( <i>t</i> , 9.2)	71.9	3.58 ( <i>t</i> , 9.4)		
5	3.61 ( <i>m</i> )	nd	3.61 ( <i>m</i> )	3.65 ( <i>d</i> , 9.2)	77.3	3.63 ( <i>m</i> )		
6	-	nd	-	-	171.6	-		
<i>β</i> -D-Gal								
1	4.86 ( <i>d</i> , 7.4)	103.8	4.85 ( <i>d</i> , 7.3)	4.83 ( <i>d</i> , 7.6)	103.7	4.82 ( <i>d</i> , 7.6)		
2	3.53 ( <i>t</i> , 8.5)	73.6	3.53 ( <i>dd</i> , 9.8, 7.3)	3.53 ( <i>dd</i> , 9.3, 7.0)	73.5	3.48 ( <i>dd</i> , 9.4, 7.3)		
3	3.45 ( <i>dd</i> , 9.8, 3.0)	75.4	3.45 ( <i>dd</i> , 9.8, 3.0)	3.45 ( <i>dd</i> , 9.8, 3.4)	75.4	3.45 ( <i>m</i> )		
4	3.86 ( <i>m</i> )	70.8	3.86 ( <i>m</i> )	3.84 ( <i>m</i> )	70.7	3.84 ( <i>m</i> )		
5	3.51 ( <i>m</i> )	76.4	3.50 ( <i>m</i> )	3.51 ( <i>t</i> , 6.6)	76.5	3.51 ( <i>m</i> )		
6	3.78 ( <i>m</i> )	61.9	3.78 ( <i>m</i> )	3.75 ( <i>dd</i> , 12.0, 5.6)	62.0	3.74 ( <i>dd</i> , 10.5, 5.9)		
	3.78 ( <i>m</i> )		3.78 ( <i>m</i> )	3.77 ( <i>dd</i> , 12.0, 6.0)		3.78 ( <i>dd</i> , 11.5, 5.2)		
<i>α</i> -L-Ara'			<i>β</i> -D-Xyl'		<i>α</i> -L-Ara'			
1	4.57 ( <i>d</i> , 7.4)	105.9	4.61 ( <i>d</i> , 8.1)	4.58 ( <i>d</i> , 7.6)	104.9	4.60 ( <i>d</i> , 7.6)		
2	3.62 ( <i>t</i> , 8.3)	72.8	3.26 ( <i>t</i> , 8.5)	3.60 ( <i>t</i> , 9.0, 7.3)	72.9	3.61 ( <i>t</i> , 9.2, 7.3)		
3	3.52 ( <i>dd</i> , 8.3, 1.9)	74.6	3.35 ( <i>m</i> )	3.52 ( <i>dd</i> , 9.7, 2.9)	74.7	3.51 ( <i>dd</i> , 9.7, 2.9)		
4	3.84 ( <i>m</i> )	70.0	3.52 ( <i>m</i> )	3.84 ( <i>m</i> )	70.1	3.82 ( <i>m</i> )		
5	3.65 ( <i>dd</i> , 10.1, 4.4)	67.7	3.29 ( <i>dd</i> , 10.1, 7.4)	3.65 ( <i>dd</i> , 12.2, 6.5)	67.9	3.61 ( <i>dd</i> , 12.9, 6.2)		
	3.95 ( <i>m</i> )		3.94 ( <i>m</i> )	3.95 ( <i>dd</i> , 12.5, 2.4)		3.93 ( <i>dd</i> , 12.9, 4.4)		

**Table 5.** Cell viability assayed by the Trypan blue exclusion test (NR8383 and U937) (n=7) and MTT assay (BV-173) (n=8). Cells are treated with different concentrations of crude plant extract for 24 h. Results are expressed as mean  $\pm$  standard deviation; SE – standard error.

Concentration $\mu\text{g/ml}$	Viability mean $\pm$ SD (%)	NR8383 SE	Viability mean $\pm$ SD (%)	BV-173 SE	Concentration $\mu\text{g/ml}$	Viability mean $\pm$ SD (%)	U937 SE
100	55.9 $\pm$ 6.7	2.4	9.1 $\pm$ 5.4	2.2	20	0	
10	90.8 $\pm$ 4.6	1.5	103.9 $\pm$ 2.2	1.0	10	55.9 $\pm$ 6.7	2.4
1	100		104.0 $\pm$ 2.6	1.1	1	91.5 $\pm$ 4.9	1.5
0.1	100		104.1 $\pm$ 1.7	0.7			