Fitoterapia 128 (2018) 198–203

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Oleanane-type glycosides from the roots of *Weigela florida* “rumba” and evaluation of their antibody recognition

Anne-Sophie Champy-Tixier[a,b,c], Anne-Claire Mitaine-Offer[a], Feliciana Real Fernández[b,d], Tomofumi Miyamoto[e], Chiaki Tanaka[e], Anna-Maria Papini[b,c,f], Marie-Aleth Lacaille-Dubois[a,*]

[a] PEPITE EA 4267, Laboratoire de Pharmacognosie, UFR Sciences de Santé, Université de Bourgogne Franche-Comté, BP 87900, 21079 Dijon Cedex, France
[b] Interdepartmental Laboratory of Peptide and Protein Chemistry and Biology, University of Florence, 50019 Sesto Fiorentino, Italy
[c] Department of Chemistry “Ugo Schiff”, University of Florence, 50019 Sesto Fiorentino, Italy
[d] Department of Neurosciences, Psychology, Drug Research and Child Health (NeuroFarBa) - Section of Pharmaceutical Sciences and Nutraceutics, University of Florence, 50019 Sesto Fiorentino, Italy
[e] Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, 812-8582, Japan
[f] Laboratory of Chemical Biology EA 4505 & PeptLab@UCP, University of Cergy-Pontoise, 95031 Cergy-Pontoise Cedex, France

ARTICLE INFO

Keywords:
Weigela florida “rumba”
Triterpene glycosides
NMR
ELISA
Multiple sclerosis

ABSTRACT

Three triterpene glycosides were isolated from the roots of *Weigela florida* “rumba” (Bunge) A. DC.: two previously undescribed 3-*O*-β-D-xylopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→4)]-β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyloleanolic acid (**1**) and 3-*O*-β-D-xylopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyloleanolic acid (**2**), and one isolated for the first time from a natural source 3-*O*-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyloleanolic acid (**3**). Their structures were elucidated mainly by 2D NMR spectroscopic analysis (COSY, TOCSY, NOESY, HSQC, HMBC) and mass spectrometry. Compounds **2** and **3** were further evaluated as antigens in enzyme-linked immunosorbent assay (ELISA) to recognize IgM antibodies in multiple sclerosis (MS) patients' sera.

1. Introduction

In Multiple Sclerosis (MS), the disease pathogenesis is still unclear and the main hypothesis includes the role of viral, immunological, and vascular factors [1]. Pathophysiological post-translational modifications of native antigens, mainly myelin glycoproteins, can trigger the immune response generating antibodies [2]. In fact, autoantibodies against aberrant glycosylated forms are proposed as biomarkers, which are helpful in MS diagnosis [3–4]. The glucosylated peptide CSF114(Glc) is able to evaluate MS-related autoAbs associated with clinical assessment of MS activity and brain lesions MRI profile [3]. In any case, despite the role of glycosylation has been previously investigated in MS pathogenesis [5], the nature of the native glycosylated antigen has not been yet clarified. The interaction between glycosides, as possible native antigens, and autoantibodies was previously evaluated in ELISA [6]. Natural triterpene glycosides showed good capacity to recognize IgMs and can be good candidates as native glycoconjugates. A deep study of anti-saponin antibodies in MS can be helpful to understand the role of carbohydrate structures in antibody recognition. In this context, the Caprifoliaceae family is a rich source of triterpene glycosides, especially the genus *Weigela*. Saponins from three species, *Weigela hortensis* [7], *W. subsessilis* [8], and *W. stelzneri* [9] were already described in literature. The phytochemical study of a horticultural cultivar of *Weigela florida* (Bunge) A. DC. was thus realized. Herein we report the isolation, purification, and characterization of natural glycosides from *Weigela florida* “rumba”. The further immunochemical evaluation of two isolated compounds was performed by enzyme linked immunosorbent assay (ELISA) and results were compared with the CSF114(Glc)-based ELISA.

2. Experimental

2.1. General experimental procedures

Optical rotation values were recorded on an AA-10R automatic polarimeter. NMR spectra were performed using a Varian INOVA 600 (Agilent Technologies) at the operating frequency of 600 MHz. The operating conditions were as follows: ^{1}H: frequency, 600 MHz; sweep width, 8 kHz; sampling point, 66 k; spectral width, 7804 Hz, accumulation, 32 pulses; temperature, 304 K. ^{13}C: frequency, 150 MHz; sweep width, 32 kHz; sampling point, 160 k;

* Corresponding author.
E-mail address: m-a.lacaille-dubois@u-bourgogne.fr (M.-A. Lacaille-Dubois).

https://doi.org/10.1016/j.fitote.2018.04.017
Received 12 February 2018; Received in revised form 22 April 2018; Accepted 27 April 2018
Available online 30 April 2018
0367-326X/

Table 1
^{13}C NMR and ^{1}H NMR data of the aglycone of compounds **1–3** in Pyridine-d_5 (δ in ppm, J in Hz).

Position	**1**		**2**		**3**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	38.6	0.88 m, 1.43 m	38.5	0.84 m, 1.42 m	38.6	0.88 m, 1.42 m
2	26.3	1.78 m, 2.02 m	26.2	1.74 m, 2.00 m	23.3	1.88 m, 2.04
3	88.5	3.23	88.5	3.22	88.5	3.22
4	39.2	–	39.2	–	39.4	–
5	55.7	0.73 br d (12.2)	55.6	0.71 br d (11.6)	55.7	0.72 br d (12.2)
6	18.2,	1.23, 1.47 m	18.2	1.20, 1.45 m	18.1	1.45, nd
7	32.8,	1.22, 1.43 m	32.9	1.19, 1.40 m	32.8	1.17, 1.38 m
8	39.4	–	39.3	–	39.2	–
9	47.7	1.57 dd (14.1, 8.1)	47.7	1.55 dd (15.0, 9.0)	47.7	1.57 dd (14.0, 8.6)
10	36.6	–	36.7	–	36.7	–
11	23.5	1.83 m, 1.91	23.4	1.82 m, 1.86	23.5	1.82 m, 1.86
12	122.2	5.42	122.2	5.40 br t (3.0)	122.2	5.44 br t (3.1)
13	144.5	–	144.4	–	144.5	–
14	41.8	–	41.8	–	41.8	–
15	28.0	1.14 m, 2.08 m	27.8	1.13 m, 2.03 m	28.0	1.12 m, 2.06 m
16	23.3	1.91, 2.06 m	23.3	1.89 m, 2.05 m	26.3	1.75 m, 2.01 m
17	46.4	–	46.4	–	46.4	–
18	41.7	3.23	41.6	3.20	41.6	3.20
19	46.2	1.22, 1.73	46.1	1.20, 1.72	46.2	1.19, 1.74
20	30.6	–	30.6	–	30.6	–
21	33.9	1.11 m, 1.35 m	nd	nd	33.9	1.12 m, 1.37 m
22	32.8	1.70, 1.89	32.8	1.73, nd	32.8	1.71, 1.78
23	27.9	1.21 s	27.8	1.19 s	27.9	1.22 s
24	16.8	1.03 s	16.7	1.01 s	16.8	1.03 s
25	15.2	0.76 s	15.2	0.75 s	15.2	0.76 s
26	17.1	0.90 s	17.0	0.89 s	17.1	0.90 s
27	25.9	1.23 s	25.8	1.22 s	25.9	1.24 s
28	180.2	–	180.3	–	180.2	–
29	33.0	0.89 s	33.8	0.88 s	33.0	0.88 s
30	23.5	0.93 s	23.4	0.92 s	23.5	0.94 s

Overlapped proton signals are reported without designated multiplicity, nd = not determined.

spectral width, 30,000 Hz, accumulation, 8000 pulses; temperature, 304 K. Each sample was dissolved in C_5D_5N (200 µL) using 5 mm micro-sample tube (SHIGEMI Co., Ltd., Japan). Chemical shifts were referenced to solvent signal (δ_H 7.22, δ_C 123.87). Conventional pulse sequences were used for gMQF-COSY, TOCSY, NOESY, gHSQC, and gHMBC. The mixing time in the NOESY experiment was set to 500 ms. TOCSY spectra were acquired using the standard MLEV17 spin-locking sequence and 60 ms mixing time. TOCSY, NOESY and HSQC spectra were recorded using phase-sensitive mode. The size of the acquisition data matrix was 2048 × 256 words in f2 and f1, respectively, and zero filling up to 2 k in f1 was made prior to Fourier transformation. Sine-bell or Shifted sine-bell window functions, with the corresponding shift optimized for every spectrum, were used for resolution enhancement and baseline correction was applied in both dimensions. HR-ESIMS (positive-ion mode) was carried out on a Bruker micrOTOF mass spectrometer. A R.E.U.S ultrasonic apparatus was used for the extraction. Isolations of compounds were carried out using vacuum liquid chromatography (VLC) with reversed-phase RP-18 silica gel (75–200 µm, Silicycle). Medium-pressure liquid chromatography (MPLC) was performed on silica gel 60 (Merck, 15–40 µm) with a Gilson M 305 pump (25 SC head pump, M 805 manometric module), a Büchi glass column (460 mm × 25 mm and 460 mm × 15 mm), and a Büchi precolumn (110 mm × 15 mm). Thin-layer chromatography (TLC, Silicycle) and high-performance thin-layer chromatography (HPTLC, Merck) were carried out on precoated silica gel plates $60F_{254}$, solvent system $CHCl_3$/MeOH/H_2O/AcOH (70:30:5:1 and 60:32:7:1). The spray reagent for saponins was vanillin reagent (1% vanillin in EtOH/H_2SO_4, 50:1).

2.2. Plant material

Weigela florida "rumba" was provided in 2012 from Jardiland ® (Chenôve, France). The plant was properly identified with a voucher specimen (N° 20121101) deposited in the herbarium of the Laboratory of Pharmacognosy, Université de Bourgogne Franche-Comté, Dijon, France.

2.3. Extraction and isolation

The dried, powdered roots of *W. florida* "rumba" (66.4 g) were submitted to an ultrasound assisted extraction three times with EtOH/H_2O (7:3, 3 × 1 L) for 1 h. After the solvent evaporation under vacuum, the resulting extract (2.9 g) was submitted to VLC (RP-18 silica gel, H_2O, MeOH/H_2O 50:50 and MeOH). The eluted fraction with MeOH (330 mg) was fractionated by MPLC on silica gel 60 ($CHCl_3$/MeOH/H_2O 70:30:5). Ten fractions (F1-F10) with two pure compounds (**1,** 6.1 mg and **2,** 11.1 mg) were obtained. Then, the fraction F2 (37.7 mg) was fractionated again by MPLC on silica gel 60 ($CHCl_3$/MeOH/H_2O 70:30:5) to give compound **3** (20.1 mg).

2.4. 3-O-β-D-xylopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→4)]-β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyloleanolic acid (1)

White amorphous powder; $[\alpha]_D^{25}$ – 28° (*c* 0.75, MeOH); ^{1}H and ^{13}C NMR data (600 MHz and 150 MHz, pyridine-d_5), Tables 1 and 2; HR-ESIMS *m/z* 1285.5334 $[M + Na]^+$ (Calcd for $C_{61}H_{98}O_{27}Na$, 1285.6193).

2.5. 3-O-β-D-xylopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyloleanolic acid (2)

White amorphous powder; $[\alpha]_D^{25}$ – 24° (*c* 0.75, MeOH); ^{1}H and ^{13}C NMR data (600 MHz and 150 MHz, pyridine-d_5), Tables 1 and 2; HR-ESIMS *m/z* 1315.5336 $[M + Na]^+$ (Calcd for $C_{62}H_{100}O_{28}Na$, 1315.6299).

Table 2
^{13}C NMR and ^{1}H NMR data of the sugar moieties of compounds **1–3** in Pyridine-d_5 (δ in ppm, J in Hz).

No	**1**		**2**		**3**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
Ara-1	104.7	4.80 d (6.2)	104.6	4.79 d (6.2)	104.7	4.79 d (6.0)
2	75.2	4.46 dd (6.7, 6.2)	75.1	4.45 dd (6.9, 6.2)	75.1	4.47 dd (6.9, 6.0)
3	73.7	4.21	73.5	4.20	73.7	4.19
4	68.6	4.24 m	68.5	4.24 m	68.6	4.23 m
5	64.8	3.77 br d (10.7), 4.26	64.7	3.76 br d (9.8), 4.27	64.9	3.75 br d (10.7), 4.25
Rha-1	101.1	6.06 br s	101.0	6.03 br s	101.0	6.08 br s
2	71.4	4.80 br s	71.3	4.79 br s	71.4	4.80 br s
3	82.0	4.62 dd (9.5, 2.3)	82.0	4.59 dd (9.3, 2.7)	82.0	4.63 dd (9.5, 2.9)
4	72.2	4.39 dd (9.5, 9.3)	72.2	4.37	72.3	4.39 t (9.5)
5	69.4	4.53 dq (9.3, 6.0)	69.4	4.51 dq (9.5, 6.0)	69.4	4.53 dq (9.5, 6.2)
6	18.1	1.49 d (6.0)	18.0	1.49 d (6.0)	18.1	1.48 d (6.2)
Xyl I-1	106.3	5.19 d (7.1)	106.1	5.16 d (7.4)	106.6	5.22 d (7.6)
2	74.3	4.01 (dd, 7.8, 7.1)	74.7	3.99	75.0	4.01 dd (8.6, 7.6)
3	75.3	4.07	75.2	4.05	77.6	4.08 dd (8.6, 8.3)
4	76.6	4.10	76.6	4.07	70.5	4.10 m
5	64.4	3.52 t (10.7), 4.28	64.1	3.50 t (10.0), 4.28	66.8	3.58 t (10.5), 4.20
Xyl II-1	102.4	4.78 d (7.8)	102.3	4.76 d (7.4)		
2	73.3	3.93 dd (8.1, 7.8)	73.1	3.92 dd (8.6, 7.4)		
3	71.7	4.12	72.2	4.14		
4	81.2	4.20	82.0	4.23		
5	64.2	3.58, 4.34	64.1	3.56, 4.34		
Xyl III-1	102.1	4.86 d (6.9)	101.8	4.87 d (6.7)		
2	72.2	3.96 dd (7.4, 6.9)	72.2	3.98		
3	76.1	4.09	75.7	4.06		
4	70.1	4.07	69.9	4.07		
5	66.1	3.60, 4.30	65.9	3.58, 4.30		
Xyl IV-1	104.6	5.36 d (6.9)				
2	74.3	4.02				
3	76.6	4.08				
4	70.1	4.07				
5	66.1	3.60, 4.34				
Glc I-1			104.5	5.27 d (7.9)		
2			74.7	3.98		
3			77.6	4.11		
4			70.8	4.01		
5			78.1	3.84 m		
6			62.0	4.14, 4.37		

Overlapped proton signals are reported without designated multiplicity.

2.6. 3-O-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyloleanolic acid (3)

White amorphous powder; $[\alpha]_D^{25}$ – 15° (*c* 0.75, MeOH); ^{1}H and ^{13}C NMR data (600 MHz and 150 MHz, pyridine-d_5), Tables 1 and 2; HR-ESIMS *m/z* 889.4410 $[M + Na]^+$ (Calcd for $C_{46}H_{74}O_{15}Na$, 889.4925).

2.7. Acid hydrolysis and GC analysis

Each compound (3 mg) was hydrolyzed with 2 N aq. CF_3COOH (5 mL) for 3 h at 95 °C. After extraction with CH_2Cl_2 (3 × 5 mL), the aqueous layer was repeatedly evaporated to dryness with MeOH until neutral, and then analyzed by TLC over silica gel ($CHCl_3$/MeOH/H_2O 8:5:1) by comparison with authentic samples. Furthermore, the sugars residue was dissolved in anhydrous pyridine (100 μL), and L-cysteine methyl ester hydrochloride (0.06 mol/L) was added. The mixture was stirred at 60 °C for 1 h, then 150 μL of HMDS-TMCS (hexamethyldisilazane/trimethylchlorosilane 3:1) was added, and the mixture was stirred at 60 °C for another 30 min. The precipitate was centrifuged off, and the supernatant was concentrated under a N_2 stream. The residue was partitioned between *n*-hexane and H_2O (0.1 mL each), and the hexane layer (1 μL) was analyzed by GC [10]. The absolute configurations were determined by comparing the retention times with thiazolidine derivatives prepared in a similar way from standard sugars (Sigma–Aldrich).

2.8. Peptides and ursolic acid

The N-glucosylated peptide CSF114(Glc) was prepared by a microwave-assisted solid-phase peptide synthesis as described elsewhere [11]. Peptide was purified by solid-phase extraction and reversed phase high pressure liquid chromatography (HPLC) and further characterized by mass spectrometry and analytical HPLC. Ursolic acid ≥90% was purchased to Sigma Aldrich.

2.9. Immunoenzymatic assay of CSF114(Glc)

Sera from 19 Multiple Sclerosis patients collected at diagnosis and 10 healthy blood donors were obtained for diagnostic purposes and stored at −20 °C until use. Multiple Sclerosis (MS) patient sera samples were collected in two Multiple Sclerosis Centers: The Multiple Sclerosis Clinical Care and Research Centre, Department of Neurosciences, Reproductive Sciences and Odontostomatology, Federico II University (Naples, Italy) and the Azienda Ospedaliera Universitaria Careggi, Clinica Neurologica, University of Florence (Florence, Italy). All patients and healthy donors gave their informed consent. Multiple Sclerosis patients were previously diagnosed after a lumbar puncture, MRI examination, and cerebrospinal analysis. Samples were pre-selected depending on their reactivity to CSF114(Glc). Antibody responses were determined in SP-ELISA.

The glycopeptide CSF114(Glc) was coated on 96-well activated polystyrene ELISA plates (NUNC Maxisorb) using a solution 10 μL/mL

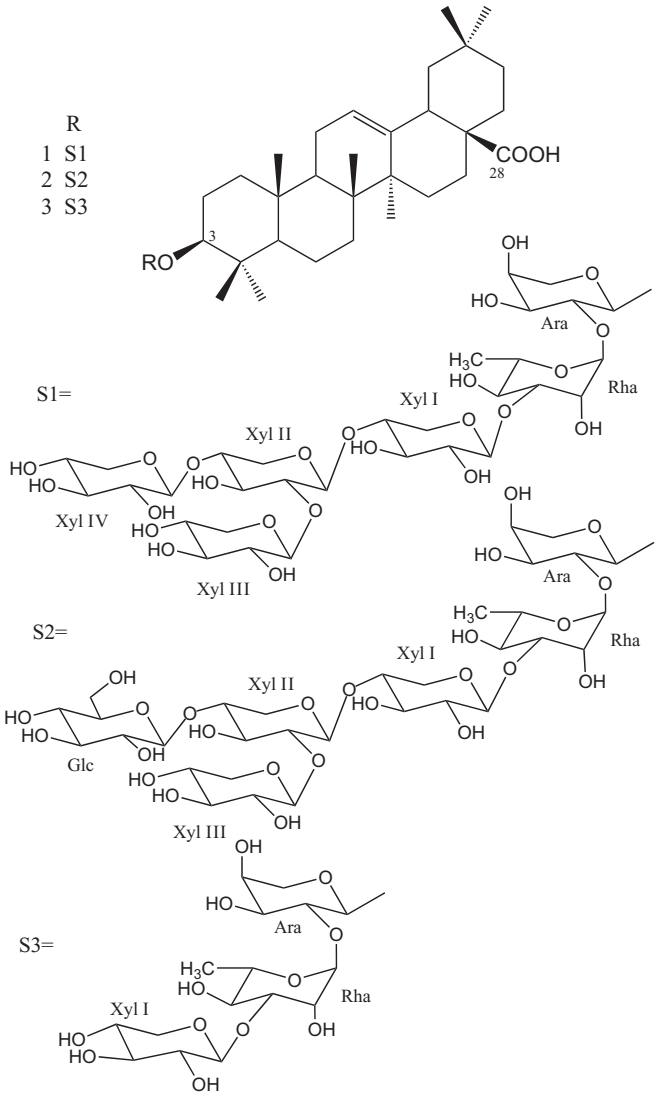

Fig. 1. Structures of saponins **1–3.**

in pure carbonate buffer 0.05 M at pH 9.6 (100 µL/well) and incubated at +4 °C overnight. After three washes with 150 mM NaCl solution containing 0.05% Tween 20 (washing buffer), non-specific binding sites were blocked with 10% fetal bovine serum (FBS) in washing buffer (100 µL/well) at room temperature for 60 min. Sera diluted 1:100 in 10% FBS in washing buffer were incubated at +4 °C overnight. After three washes, 100 µL of alkaline phosphatase conjugated with anti-human IgM (diluted 1:1200 in 10% FBS in washing buffer) were added to each well. After 3 h incubation at room temperature and three washes in washing buffer, 100 µL/well of 1 mg.mL^{-1} *p*-nitrophenylphosphate (Sigma-Aldrich) in carbonate buffer (pH 9.6) were added. After 30 min, the reaction was stopped with 1 M NaOH (50 µL/well), and the absorbance was read in a multichannel ELISA reader (Tecan Sunrise, Männedorf, Switzerland) at 405 nm. Antibody levels are expressed as absorbance in arbitrary units at 405 nm.

2.10. Immunoenzymatic assay of glycosides 2 and 3 and ursolic acid

Compounds **2** and **3** were coated on 96-well microplates BD Falcon™ separately using a solution 10 µg/ml in pure ethanol (100 µL/well). The plates were then incubated at +4 °C overnight. The non-specific binding sites were blocked with 10% FBS in 150 mM NaCl solution without surfactant at room temperature for 60 min. Sera diluted 1:100 in 10% FBS in 150 mM NaCl were incubated at +4 °C overnight (100 µL/well). After three washes, 100 µL of alkaline phosphatase conjugated anti-human IgM (Invitrogen) (diluted 1:1500 in 10% FBS washing buffer) were added to each well. After 3 h incubation at room temperature and three washes, 100 µL/well of 1 mg.mL^{-1} *p*-nitrophenyl phosphate (Sigma–Aldrich) in carbonate buffer (pH 9.6) with 10 mM $MgCl_2$ solution was added. After 30 min, the reaction was stopped with 1 M NaOH (50 µL/well), and the absorbance was read at 405 nm. ELISA plates, coating conditions, reagent dilutions, buffers, and incubation times were preliminary tested. Antibody levels are expressed as absorbance in arbitrary units at 405 nm.

2.11. Statistical analysis

Statistical analysis was performed using Graph Pad prism 6. D'agostino-Parson test was employed to evaluate if data assumed a Gaussian distribution. Mann-Whitney *U* tests were used to evaluate the predictive antibody values.

3. Results and discussion

3.1. Structural analysis

Three triterpene glycosides **1–3** (Fig. 1) were isolated from an aqueous–ethanolic extract of the roots of *Weigela florida* "rumba" by various solid/liquid chromatographic methods. Their structures were determined by 2D NMR and mass spectrometry as follow.

The HR-ESIMS (positive-ion mode) spectrum of compound **1** established its molecular formula as $C_{61}H_{98}O_{27}$ with a pseudo-molecular peak at *m/z* 1285.5334 $[M + Na]^+$, indicating a molecular weight of 1262.

The ^{1}H NMR spectrum of the aglycone part of **1** displayed signals assignable to seven angular methyl groups at δ_H 0.76, 0.89, 0.90, 0.93, 1.03, 1.21 and 1.23 (s, each), one olefinic proton at δ_H 5.42 (H-12) and one oxygen-bearing methine protons at δ_H 3.23 (H-3). The HMBC spectrum showed ^2J and ^3J couplings from the methyl protons (23, 24, 25, 26, 27, 29 and 30), which allowed the assignments of most carbons and protons of this aglycone. The deshielded chemical shift of C-28 observed at δ_C 180.2 suggested the presence of a carbonyl group of a carboxylic acid function. According to the literature data, this genin was identified as oleanolic acid, already described in saponins from *Weigela stelzneri* [9] (Table 1, Fig. 1).

The HSQC spectrum of **1** displayed signals of six anomers at δ_H 4.78 (d, J = 7.8 Hz)/δ_C 102.4, δ_H 4.80 (d, J = 6.2 Hz) /δ_C 104.7, δ_H 4.86 (d, J = 6.9 Hz)/δ_C 102.1, δ_H 5.19 (d, J = 7.1 Hz)/δ_C 106.3, δ_H 5.36 (d, J = 6.9 Hz)/δ_C 104.6, and δ_H 6.06 (br s)/δ_C 101.1. The ring protons of the monosaccharide residues were assigned starting from the readily identifiable anomeric protons by means of the ^{1}H–^{1}H COSY, TOCSY, HSQC and HMBC experiments. The monosaccharides obtained by acid hydrolysis of **1** were identified by comparison on TLC with authentic samples as xylose, arabinose and rhamnose. The absolute configurations were determined to be D for xylose, and L for arabinose and rhamnose by GC analysis according to a method previously described [10]. The relatively large $^3J_{H\text{-}1,H\text{-}2}$ value of the Xyl and Ara (6.2–7.8 Hz) in their pyranose form indicated a β anomeric orientation for Xyl, and an α anomeric orientation for Ara. The large $^1J_{H\text{-}1,C\text{-}1}$ value of the Rha (166 Hz) confirmed that the anomeric proton was equatorial (α-pyranoid anomeric form).

Units of one α-L-arabinopyranosyl, one α-L-rhamnopyranosyl and four β-D-xylopyranosyl were thus identified (Table 2). The monodesmosidic structure was elucidated by using mainly HMBC and NOESY spectra: the HMBC correlation at δ_H 4.80 (Ara-1)/δ_C 88.5 (C-3) and the NOESY correlation at δ_H 4.80 (Ara-1)/δ_H 3.23 (H-3) confirmed the *O*-heterosidic linkage between Ara and C-3 of the aglycone. The HMBC cross-peak at δ_H 6.06 (Rha-1)/δ_C 75.2 (Ara-2) and the NOESY cross-peak at δ_H 6.06 (Rha-1)/δ_H 4.46 (dd, J = 6.7, 6.2 Hz, Ara-2) proved the

(1→2) linkage between Rha and Ara. The correlation at δ_H 5.19 (Xyl I-1)/δ_C 82.0 (Rha-3) in the HMBC spectrum, and at δ_H 5.19 (Xyl I-1)/δ_H 4.62 (dd, J = 9.5, 2.3 Hz, Rha-3) in the NOESY spectrum, confirmed the (1→3) linkage between Xyl I and Rha. The HMBC cross-peak at δ_H 4.78 (Xyl II-1)/δ_C 76.6 (Xyl I-4) and the NOESY cross-peak at δ_H 4.78 (Xyl II-1)/δ_H 4.10 (Xyl I-4) proved the (1→4) linkage between Xyl II and Xyl I. Finally, the structure analysis of the terminal sequence β-D-xylopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→4)]-β-D-xylopyranosyl was based upon the HMBC correlation at δ_H 5.36 (Xyl IV-1)/δ_C 81.2 (Xyl II-4), the NOESY correlation at δ_H 5.36 (Xyl IV-1)/δ_H 4.20 (Xyl II-4) and the HMBC cross-peak at δ_H 3.93 (dd, J = 8.1, 7.8 Hz, Xyl II-2)/δ_C 102.1 (Xyl III-1) (Table 2, Fig. 1).

On the basis of the above results, the structure of the previously undescribed compound **1** was elucidated as 3-*O*-β-D-xylopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→4)]-β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyloleanolic acid.

The HR-ESIMS (positive-ion mode) spectrum of compound **2** established its molecular formula as $C_{62}H_{100}O_{28}$. It showed a pseudo-molecular peak at m/z 1315.5336 $[M + Na]^+$ indicating a molecular weight of 1292, differing from **1** of 30 uma.

The ^1H and ^{13}C NMR signals of compounds **1** and **2** were almost surimposable excepted for the terminal hexose linked at the C-4 position of Xyl II. The full NMR assignments of this remaining sugar and the GC MS data, led to the identification of a β-D-glucopyranosyl moiety. The NOESY correlation at δ_H 5.27 (Glc-1)/δ_H 4.23 (Xyl II-4) established the structure of the previously undescribed compound **2** as 3-*O*-β-D-xylopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyloleanolic acid.

The HR-ESIMS (positive-ion mode) spectrum of compound **3** established its molecular formula as $C_{46}H_{74}O_{15}$, with a pseudo-molecular peak at m/z 889.4410 $[M + Na]^+$, indicating a molecular weight of 866.

The ^1H NMR spectrum of the aglycone part of **3** displayed signals assignable to seven angular methyl groups at δ_H 0.76, 0.88, 0.90, 0.94, 1.03, 1.22 and 1.24 (s, each), one olefinic proton at δ_H 5.44 (br t, J = 3.1 Hz), and one oxygen-bearing methine protons at δ_H 3.22 (H-3). In the ^{13}C NMR spectrum, a signal at δ_C 180.2 suggested a carboxylic acid function (C-28). As for **1** and **2**, the genin of **3** was identified as oleanolic acid [9] (Table 1, Fig. 1.).

The HSQC spectrum of **3** displayed signals of three anomers at δ_H 4.79 (d, J = 6.0 Hz)/δ_C 104.7, δ_H 5.22 (d, J = 7.6 Hz)/δ_C 106.6, and δ_H 6.08 (br s)/δ_C 101.0. The absolute configurations of these three sugars were determined to be D for xylose, and L for arabinose and rhamnose by GC analysis. After a total assignment by 2D NMR of the protons and carbons of each sugar, units of one α-L-arabinopyranosyl, one α-L-rhamnopyranosyl and one β-D-xylopyranosyl were identified (Table 2). The structural analysis of the oligosaccharidic chain linked at the C-3 of the aglycone was realized using mainly HMBC and NOESY spectra. The HMBC correlation at δ_H 4.79 (Ara-1)/δ_C 88.5 (C-3) and the NOESY correlation at δ_H 4.79 (Ara-1)/δ_H 3.22 (H-3) confirmed the *O*-heterosidic linkage between Ara and C-3. The HMBC cross-peak at δ_H 6.08 (Rha-1)/δ_C 75.1 (Ara-2) and the NOESY cross-peak at δ_H 6.08 (Rha-1)/δ_H 4.47 (dd, J = 6.9, 6.0 Hz, Ara-2) proved the (1→2) linkage between Rha and Ara. The correlation at δ_H 5.22 (Xyl I-1)/δ_C 82.0 (Rha-3) in the HMBC spectrum and at δ_H 5.22 (Xyl I-1)/δ_H 4.63 (dd, J = 9.5, 2.9 Hz, Rha-3) in the NOESY spectrum, confirmed the (1→3) linkage between Xyl I and Rha (Table 2, Fig. 1).

On the basis of the above results, the structure of compound **3** was elucidated as 3-*O*-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyloleanolic acid. This compound has been already synthetized [12,13] but is isolated here for the first time from a vegetal source.

This sequence was already found in saponins from *Weigela stelzneri* [9]. Other species should be investigated to confirm this sequence as a chemotaxonomic marker of the genus *Weigela*.

3.2. Immunoenzymatic assay

After isolation and purification, triterpene glycosides **2** and **3** were chosen for the length of their oligasaccharidic chain, to be tested as antigens to detect specific IgM antibodies in MS patients' sera and normal blood donors (NBD) by ELISA. In parallel, the synthetic N-glucosylated peptide CSF114(Glc) was employed as positive control antigen. Moreover, the ursolic acid with a similar structure to the aglycone of **2–3,** was tested to evaluate if antibody responses are directed to glycosylated sites or/and the aglycone structure. IgM measurements and statistical analysis were performed separately for saponins **2–3**, ursolic acid and CSF114(Glc). Data distribution of IgM antibody responses to CSF114(Glc), saponins **2–3** and ursolic acid can be observed in Fig. 2.

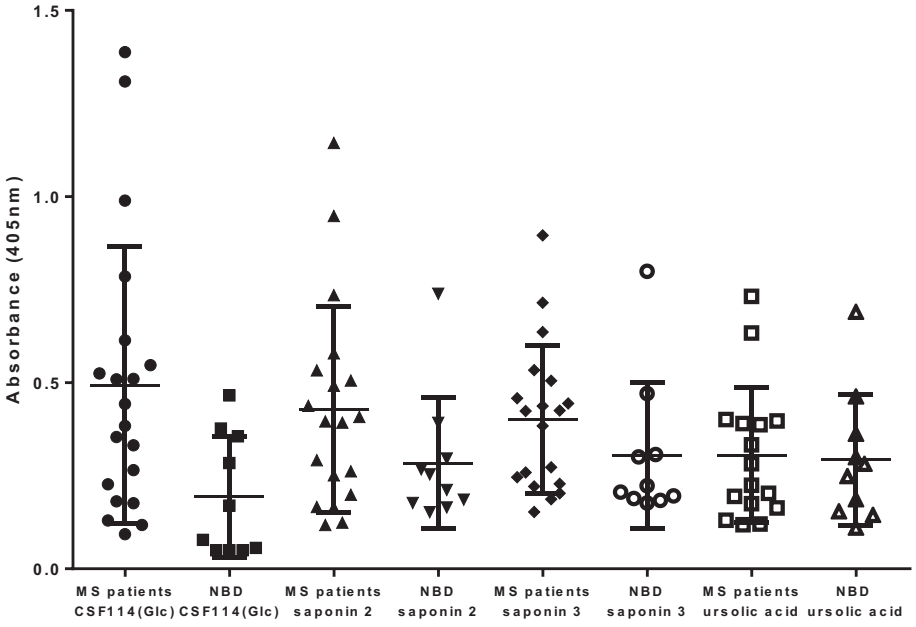

Fig. 2. Data distribution of IgM antibody responses to CSF114(Glc), saponins **2** and **3**, and ursolic acid, in sera of Multiple Sclerosis patients (MS) and normal blood donors (NBD), determined by ELISA. Data are reported as absorbance at 405 nm of sera diluted 1:100. Data are presented as mean values with the corresponding standard deviations.

All compounds presented lower mean values for NBD than for MS populations. The overall data distribution was statistically analyzed using D'agostino-Parson test, and results showed that the ursolic acid was the solely compound that passed the normality test (alpha = 0.05). Then, antibody titers between MS patients and healthy controls were evaluated using the Mann-Whitney *U* test. This cohort of MS patients exhibited significantly greater anti-CSF114(Glc) antibodies when compared to healthy subjects (P value < 0.05, two-tailed). In the other hand, no significant differences in antibody levels against saponins **2** and **3**, and ursolic acid was found between MS patients and healthy controls (P value > 0.1, two tailed).

Indeed, saponins **2** and **3**, monodesmosidic ones with oleanane-type aglycone which differ by the length of their oligosaccharidic chain, are not able to recognize IgMs in MS sera. Besides, ursolic acid, a commercial triterpenic acid which has no sugar moiety also demonstrated same results as saponins **2** and **3**. The aim of this study was to define if antibody responses are directed to glycosylated sites. Results obtained by ursolic acid indicate that terpenoid structure only was no sufficient for antibody recognition.

Peroni et al. [6] have previously tested five natural triterpene glycosides for antibody recognition. From a structural point of view, these compounds were bidesmosidic and monodesmosidic structures with one glycosyl moiety at C-3 position of the aglycone. The glycosidic part was composed of glucopyranosyl, sulfated glucopyranosyl, sulfated quinovopyranosyl and xylopyranosyl moieties. In our study, monodesmosidic structures with three and six glycosyl moieties at C-3 position of the aglycone were tested. The glycosidic part was composed of arabinopyranosyl, rhamnopyranosyl, xylopyranosyl and glucopyranosyl moieties. No relevant results were observed for saponins **2** and **3**, and the length of the oligosaccharidic chain and the type of sugars not allowed the recognition of IgMs in MS sera. But according to previous work [6] and our results, we can confirm the importance of specific carbohydrate structures and hypothesize on the relevant role of the ester carboxyl function at C-28 position in antibody recognition in MS sera. New natural glycosides will be tested to try to establish structure/activity relationships between natural compounds and IgM in sera of patient affected by MS.

Summarizing, three new saponins (**1**–**3**) were extracted from the roots of *Weigela florida* “rumba”, an ornamental shrub very common in European gardens. Triterpene glycosides **2** and **3** which differ by their oligosaccharidic chain were tested as antigens to detect specific IgM antibodies in MS patient sera and normal blood donors (NBD) by ELISA. Results showed no significant difference in antibody levels against saponins **2** and **3** between MS patients and healthy controls. New saponins with different structures and sugar moieties will be further tested to confirm the importance of carbohydrate structures in the autoantibodies recognition.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

We acknowledge the Multiple Sclerosis Clinical Care and Research Center, Department of Neurosciences, Reproductive Sciences and Odontostomatology, Federico II University (Naples, Italy) and the Azienda Ospedaleria Universitaria Careggi, Clinica Neurologica, University of Florence (Florence, Italy) for their support.

References

[1] J.S. Alexander, R. Zivadinov, A.H. Maghzi, V.C. Ganta, M.K. Harris, A. Minagar, Multiple sclerosis and cerebral endothelial dysfunction: mechanisms, Pathophysiology 18 (2011) 3–12.
[2] H.A. Doyle, M.J. Mamula, Post-translational protein modification in antigen recognition and autoimmunity, Trends Immunol. 22 (2001) 443–449.
[3] F. Lolli, B. Mulinacci, A. Carotenuto, B. Bonetti, G. Sabatino, B. Mazzanti, A.M. D'Ursi, E. Novellino, M. Pazzagli, L. Lovato, M.C. Alcaro, E. Peroni, M.C. Pozo-Carrero, F. Nuti, L. Battistini, G. Borsellino, M. Chelli, P. Rovero, A.M. Papini, An N-glucosylated peptide detecting disease-specific autoantibodies, biomarkers of multiple sclerosis, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 10273–10278.
[4] C.B. Marta, A.R. Olivier, R.A. Sweet, S.E. Pfeiffer, N.H. Ruddle, Pathogenic myelin oligodendrocyte glycoprotein antibodies recognize glycosylated epitopes and perturb oligodendrocyte physiology, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 13992–13997.
[5] F. Nuti, E. Peroni, F. Real-Fernández, M.A. Bonache, A. Le Chavalier-Isaad, M. Chelli, N. Lubin-Germain, J. Uziel, P. Rovero, F. Lolli, A.M. Papini, Post-translationally modified peptides efficiently mimicking neo-antigens: a challenge for theragnostics of autoimmune diseases, Biopolymers: Pept. Sci. 94 (2010) 791–799.
[6] E. Peroni, F. Real Fernández, C. Gheri, F. Nuti, A.C. Mitaine-Offer, F. Lolli, M.A. Lacaille-Dubois, A.M. Papini, Natural triterpene glycosides for antibody recognition, Planta Med. Lett. 3 (2016) e1–e6.
[7] T. Murayama, A. Kasahara, Y. Shiono, M. Ikeda, Structure elucidation of a triterpene glycoside isolated from *Weigela hortensis*, Nat. Med. 57 (2003) 181–184.
[8] Y.M. Won, Z.K. Seong, J.L. Kim, H.S. Kim, H.H. Song, D.Y. Kim, J.H. Kim, S.R. Oh, H.W. Cho, J.H. Cho, H.K. Lee, Triterpene glycosides with stimulatory activity on melanogenesis from the aerial parts of *Weigela subsessilis*, Arch. Pharm. Res. 38 (2015) 1541–1551.
[9] A. Rezgui, A.C. Mitaine-Offer, T. Miyamoto, C. Tanaka, S. Delemasure, P. Dutartre, M.A. Lacaille-Dubois, Oleanolic acid and hederagenin glycosides from *Weigela stelzneri*, Phytochemistry 123 (2016) 40–47.
[10] S. Hara, H. Okabe, K. Mihashi, Gas-liquid chromatographic separation of aldose enantiomers as trimethylsilyl ethers of methyl 2-(polyhydroxyalkyl)thiazolidine-4(*R*)-carboxylates, Chem. Pharm. Bull. 35 (1987) 501–506.
[11] F. Rizzolo, G. Sabatino, M. Chelli, P. Rovero, A.M. Papini, A convenient microwave-enhanced solid-phase synthesis of difficult peptide sequences: case study of gramicidin a and CSF114(Glc), Int. J. Pept. Res. Ther. 13 (2007) 203–208.
[12] Q. Liu, H. Liu, L. Zhang, T. Guo, P. Wang, M. Geng, Y. Li, Synthesis and antitumor activities of naturally occurring oleanolic acid triterpenoid saponins and their derivatives, Eur. J. Med. Chem. 64 (2013) 1–15.
[13] L. Wang, Z. Wang, S. Su, Y. Xing, Y. Li, M. Li, J. Liu, S. Yang, Synthesis and cytotoxicity of oleanolic acid trisaccharide saponins, Carbohydr. Res. 442 (2017) 9–16.