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Triterpenoid saponins from *Albizia lebbeck* (L.) Benth and their inhibitory effect on the survival of high grade human brain tumor cells

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ABSTRACT

As part of our search of new bioactive triterpenoid saponins from Cameroonian Mimosaceae plants, phytochemical investigation of the roots of *Albizia lebbeck* led to the isolation of two new oleanane-type saponins, named lebbeckosides A–B (**1**–**2**). Their structures were established on the basis of extensive 1D and 2D NMR (^{1}H, ^{13}C NMR, DEPT, COSY, TOCSY, ROESY, HSQC, and HMBC) and HRESIMS studies, and by chemical evidence. Compounds **1**–**2** were evaluated for their inhibitory effect on the metabolism of high grade human brain tumor cells, the human glioblastoma U-87 MG cell lines and the glioblastoma stem-like TG1 cells isolated from a patient tumor, and known to be particularly resistant to standard therapies. The isolated saponins showed significant cytotoxic activity against U-87 MG and TG1 cancer cells with IC_{50} values of 3.46 μM and 1.36 μM for **1**, and 2.10 μM and 2.24 μM for **2**, respectively.

1. Introduction

The genus *Albizia* comprises about 150 species widely distributed in the tropics, with the greatest diversity in Africa and South America.[1] Triterpenoid saponins are commonly described in this genus. Adianthifoliosides, grandibracteosides, gummiferaosides, julibrosides, coriariosides, and albizosides are complex triterpenoid saponins isolated from the *Albizia* genus.[2] These glycosides, represent a class of very complex glycosides possessing a common aglycon unit, acacic acid, having various oligosaccharide moieties at C-3 and C-28 and an acyl group at C-21. They have been reported to inhibit the growth of tumor cells, and thus appear as a new potential class of anticancer natural triterpenoid saponins.[2] In order to discover new bioactive acacic acid glycosides, we screened the saponin content of Cameroonian Mimosaceae including *Albizia*, *Acacia*, and *Entada* genera. Three *Albizia* species, among which *Albizia lebbeck*, were selected on the basis of their HPLC-DAD, LC-NMR, and LC–MS profiles.

Albizia lebbeck (L.) Benth is a pantropical species distributed in Africa, Asia, America, and Australia. In West Africa, it is traditionally used against diarrhea, dysentery, hemorrhoids, bronchitis, asthma, eczema, and leprosy.[3] In South-East Asia and Australia, the stem bark is used as a folk remedy to treat abdominal tumors, boils, cough, eye disorders, and lung ailments. It is also reported to be astringent, pectoral, rejuvenating, and tonic.[4] Nootropic and anxiolytic activities of a saponin fraction isolated from *A. lebbeck* leaves have been reported.[5] Oral administration of the saponin fraction isolated from *A. lebbeck* bark to male rats has been reported to significantly reduce fertility through reduction of sperm mobility and density.[6] Previous phytochemical studies of *A. lebbeck* stem bark reported the presence of glycosides of acacic acid lactones.[7]

In the present investigation on *A. lebbeck* roots, we report the isolation and structural characterization of two new acacic acid glycosides, named lebbeckosides A–B (**1**–**2**). The isolated compounds were evaluated for their inhibitory effect on the

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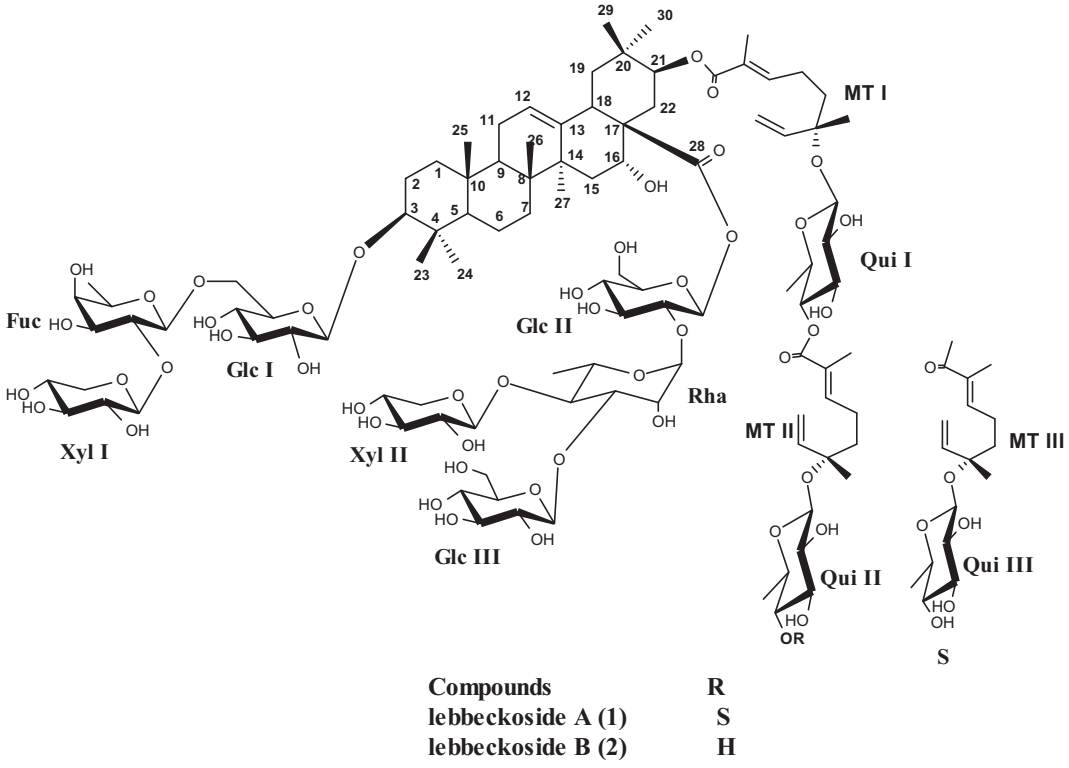
metabolism of high grade human brain tumor cells, namely the human glioblastoma U-87 MG cell lines and temozolomide-resistant glioblastoma stem-like cells isolated from a patient tumor, results are reported herein.

2. Results and discussion

The air-dried powdered roots of *A. lebbeck* (300 g) were extracted with aq-EtOH 70% using a soxhlet apparatus. After evaporation of the solvent, the resulting brown residue was partitioned between water and water-saturated *n*-BuOH. The *n*-BuOH fraction was then submitted to vacuum-liquid chromatography (VLC) on reversed-phase silica gel yielding three main fractions that were subjected to VLC on silica gel. Purification of the eluated subfractions by Semprep-HPLC afforded two new triterpenoid saponins (Chart 1).

Lebbeckoside A (**1**) was obtained as a white, amorphous powder. Its high-resolution electrospray ionization mass spectrometry (HRESIMS) (positive-ion mode) exhibited a pseudo-molecular ion peak at *m*/*z* 2486.2146 $[M+NH_4]^+$ (calcd 2485.2147), consistent with a molecular formula of $C_{118}H_{186}O_{54}$. Upon acid hydrolysis with 2.0 M HCl, **1** gave an acacic acid lactone unit, which was identified with an authentic sample, and compound **1** also gave glucose (Glc), xylose (Xyl), fucose (Fuc), rhamnose (Rha), arabinose (Ara), and quinovose (Qui), which were identified by co-TLC with authentic samples. The absolute configuration of these sugar residues was determined to be D for Glc, Xyl, and Fuc, and L for Ara and Rha based on GC analysis of their trimethylsilyl thiazolidine derivatives.[8] Its ^{1}H NMR spectrum showed seven angular methyl groups as singlets at δ_H 0.90, 1.01, 1.08, 1.10, 1.12, 1.31, and 1.84 (each 3H, s), one olefinic proton at δ_H 5.64 (1H, br s), and sugar proton signals at δ_H 4.89–6.42. ^{13}C NMR spectrum showed two olefinic carbon signals at δ_C 123.5 and 143.8, suggesting that **1** was an oleanane type triterpenoid saponin. 1D (^{1}H, ^{13}C NMR, DEPT) and 2D (COSY, HSQC and HMBC) NMR techniques permitted assignments of all ^{1}H and ^{13}C NMR signals of the aglycone of **1**. This aglycon was thus recognized to be acacic acid (3β,16α,21β-trihydroxyolean-12-ene-28-oic acid) by comparison of its ^{1}H and ^{13}C NMR signals with those reported in the literature.[9–21] The downfield position of the axial group at C-14 (Me-27, δ 1.84) in the ^{1}H NMR spectrum, implied an additional axial (α) hydroxyl group at C-16. The ROESY correlations observed between H-21 (δ 5.37) and H-29 (δ 1.01, s), suggested an α-axial orientation of H-21, as well as between H-3 (δ 3.49) and H-5 (δ 0.82) indicated the α-axial orientation of the two protons. The 3,21-hydroxy groups and 28-carbonyl group of the aglycon carried a sugar moiety, respectively, as evidenced by the glycosylation- and acylation-induced shifts observed at δ_C 89.0 (deshielded signal for C-3 of the aglycon), 174.9 (shielded signal for C-28 of the aglycon), and 77.6 (deshielded signal for C-21 of the aglycon), indicating that **1** was a 21-acyl 3,28-bidesmosidic acacic acid derivative with sugar chains linked to C-3 and C-28 of the aglycon through an ether and ester bond, respectively, and with an acyl group attached at C-21.

The ^{1}H NMR spectrum of **1** showed 10 anomeric protons at δ_H 4.91 [d, *J* = 8.0 Hz, glucose (Glc I)], 4.97 [d, *J* = 8.0 Hz, fucose (Fuc)], 5.09 [d, *J* = 7.1 Hz, xylose (Xyl I)], 6.16 [d, *J* = 8.0 Hz, glucose (Glc II)], 6.42 [br s, rhamnose (Rha)], 5.42 [d, *J* = 8.1 Hz, glucose (Glc III)], 5.30 [d, *J* = 7.1 Hz, xylose (Xyl II)], 4.90 [d, *J* = 8.0 Hz, quinovose (Qui I)], 4.89 [d, *J* = 8.0 Hz, quinovose (Qui II)], and 4.97 [d, *J* = 8.0 Hz, quinovose (Qui III)], which correlated with ten anomeric carbon atom resonances at δ_C 105.3, 103.7, 107.3, 95.6, 101.6, 106.4, 106.8, 99.7, 99.6, and 97.3, respectively, in the HSQC spectrum (Tables 1 and 2). The ^{1}H and ^{13}C NMR data (Tables 1 and 2) of the monosaccharide residues were assigned starting, either from the readily identifiable anomeric proton of each hexosyl or pentosyl unit, or from the CH_3-proton doublet of each 6-deoxyhexosyl unit, by means of TOCSY, HSQC, and HMBC spectra obtained for this compound. The anomeric centers of the D-glucose, D-fucose, D-quinovose, and D-xylose units were each determined to have a β-configuration based on large $^3J_{H\text{-}1,H\text{-}2}$ values. And the α-anomeric configuration of the L-rhamnose was judged by the broad singlet of the anomeric proton and the chemical shift value of C-5 (δ 68.8).[22] In



Compounds	R
lebbeckoside A (1)	S
lebbeckoside B (2)	H

Chart 1. Structures of triterpene saponins **1**–**2**.

Table 1
^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of the sugar moieties attached at C-3 and C-28 of compounds **1**–**2** in pyridine-d_5 (δ in ppm)[a]

Position	**1**		**2**	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
3-O-Sugars				
Glc I-1	4.91 d (8.0)	105.3	4.90 d (8.0)	105.4
2	4.03	75.9	4.02	75.8
3	4.12	78.6	4.13	78.7
4	4.18	71.3	4.18	71.4
5	4.02	76.6	4.03	76.7
6	4.36; 4.73	70.2	4.36; 4.74	70.2
Fuc-1	4.97 d (8.0)	103.7	4.99 d (8.1)	103.8
2	4.45	82.6	4.45	82.6
3	4.14	75.7	4.15	75.8
4	4.04	72.2	4.02	72.2
5	3.74	71.8	3.76	71.9
6	1.37 d (6.5)	17.7	1.38 d (6.1)	17.8
Xyl I-1	5.09 d (7.1)	107.3	5.08 d (7.1)	107.3
2	4.08	75.7	4.08	75.8
3	4.03	76.8	4.02	76.7
4	4.10	71.3	4.11	71.3
5	3.60; 4.48	67.5	3.59; 4.46	67.6
28-O-Sugars				
Glc II-1	6.16 d (8.0)	95.6	6.15 d (8.0)	95.6
2	4.31	76.7	4.30	76.8
3	4.07	78.0	4.05	77.9
4	4.16	71.6	4.17	71.3
5	4.00	79.5	4.02	79.5
6	4.30; 4.41	62.4	4.27; 4.39	62.4
Rha-1	6.42 br s	101.6	6.41 br s	101.6
2	4.82	72.2	4.83	72.2
3	4.30	83.6	4.49	83.3
4	4.49	83.4	4.32	83.4
5	4.60	68.8	4.58	68.9
6	1.78 d (6.0)	18.8	1.79 d (6.0)	18.8
Glc III-1	5.42 d (8.1)	106.4	5.43 d (8.1)	106.2
2	4.14	75.5	4.15	75.9
3	4.16	77.7	4.17	77.6
4	4.15	71.7	4.16	71.6
5	3.98	79.4	3.96	79.5
6	4.48; 4.28	63.1	4.49; 4.27	63.1
Xyl II-1	5.30 d (7.1)	106.8	5.28 d (7.1)	106.8
2	4.10	76.3	4.11	76.1
3	4.05	78.0	4.06	77.9
4	4.12	71.3	4.13	71.3
5	3.51; 4.26	67.7	3.50; 4.27	67.7

Overlapped proton NMR signals are reported without designated multiplicity.
[a] Assignments were based on the HMBC, HSQC, COSY, TOCSY, ROESY, and DEPT experiments.

addition, the ^{1}H NMR spectrum of compound **1** exhibited three olefinic proton signals at δ_H 6.89 (d, J = 7.3 Hz), 7.00 (d, J = 7.3 Hz), and 7.08 (d, J = 7.3 Hz), and three groups of one-substituted olefin proton signals, one group at δ_H 6.25 (dd, J = 10.5; 17.5 Hz), 5.28 (d, J = 10.5 Hz) and 5.47 (d, J = 17.5 Hz), another group at δ_H 6.27 (dd, J = 10.5; 17.5 Hz), 5.28 (d, J = 10.5 Hz) and 5.47 (d, J = 17.5 Hz), and the other group at δ_H 5.99 (dd, J = 11.0; 17.2 Hz), 5.30 (d, J = 10.5 Hz) and 5.39 (d, J = 17.2 Hz), indicating that compound **1** had three units of monoterpenoid moieties.[23] Extensive analysis of 1D and 2D NMR spectra of **1**, revealed that the sugar moiety at C-3 was identical to that of julibrosides J_5 and J_8,[17] II and J_{16},[18] and albizoside A,[19] and the monoterpene-quinovosyl moiety at C-21 was identical to that of gummiferaoside C,[10] coriarioside A,[16] and coriariosides C–D.[21] Hence, the units attached at C-3 and C-21 of the aglycon of **1** were established as β-D-xylopyranosyl-(1→2)-β-D-fucopyranosyl-(1→6)-β-D-glucopyranoside, and {(2*E*,6*S*)-6-*O*-{4-*O*-[(2*E*,6*S*)-2,6-dimethyl-6-*O*-(β-D-quinovopyranosyl)octa-2,7-dienoyl]-4-*O*-[(2*E*,6*S*)-2,6-dimethyl-6-*O*-(β-D-quinovopyranosyl)octa-2,7-dienoyl]-β-D-quinovopyranosyl}-2,6-dimethylocta-2,7-dienoyl}, respectively (Tables 1 and 2).

For the glycosidic chain at C-28 of the aglycone, a correlation in the HSQC spectrum at δ_C/δ_H 95.6:6.16 (d, J = 8.0 Hz) showed that Glc II is linked to the carboxylic group of the aglycon by an ester linkage. This conclusion was supported by the shielded signal of C-28 at δ_C 174.9, in comparison with the free carboxylic acid, and by the HMBC correlation observed between H-1 of Glc II (δ_H 6.16) and C-28 of the aglycone (δ_C 174.9). Moreover, the MS^2 spectrum of **1** revealed a fragment ion at m/z 1884.0074 $[(M+NH_4)-602]^+$, in which 602 mass units were accounted for the mass of the C-28-linked oligosaccharidic chain consisting of one pentose, one deoxyhexose, and two hexose units. Extensive analysis of 1D and 2D NMR spectra revealed that this oligosaccharide moiety was constituted by two Glc, one Xyl, and one Rha. In addition, the HMBC correlation observed between H-1 of Rha (δ_H 6.42) and C-2 of Glc II (δ_C 76.7), led us to suggest that Rha was attached to C-2 of Glc II. This was supported by the ROESY correlation observed between H-2 of Glc II (δ_H 4.31) and H-1 of Rha (δ_C 6.42). Moreover, in the HMBC spectrum, a correlation observed between H-1 of Glc III (δ_H 5.42) and C-3 of Rha (δ_C 83.6), indicated that Glc III was linked to C-3 of Rha. This was confirmed by the ROESY correlation observed between H-3 of Rha (δ_H 4.30) and H-1 of Glc III (δ_H 5.42). Furthermore, the ROESY correlation observed between H-1 of Xyl II (δ_H 5.30) and H-4 of Rha (δ_H 4.49), showed that Xyl II was linked to C-4 of Rha. Thus, the tetrasaccharide moiety attached at C-28 of the aglycon was determined to be β-D-xylopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→3)]-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl (Fig. 1). Hence, the structure of lebbeckoside A (**1**) was elucidated as 3-*O*-[β-D-xylopyranosyl-(1→2)-β-D-fucopyranosyl-(1→6)-β-D-glucopyranosyl]-21-*O*-{(2*E*,6*S*)-6-*O*-{4-*O*-[(2*E*,6*S*)-2,6-dimethyl-6-*O*-(β-D-quinovopyranosyl)octa-2,7-dienoyl]-4-*O*-[(2*E*,6*S*)-2,6-dimethyl-6-*O*-(β-D-quinovopyranosyl)octa-2,7-dienoyl]-β-D-quinovopyranosyl}-2,6-dimethylocta-2,7-dienoyl}acacic acid 28-*O*-β-D-xylopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→3)]-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl ester.

Lebbeckoside B (**2**), obtained as a white amorphous powder, exhibited in its HRESIMS spectrum (positive-ion mode) a pseudomolecular ion peak at m/z 2174.0665 $[M+NH_4]^+$ (calcd 2173.0574), consistent with a molecular formula of $C_{102}H_{162}O_{48}$. As already observed for **1**, acid hydrolysis of **2** also afforded an acacic acid lactone, identified by co-TLC with an authentic sample, together with D-glucose, D-xylose, D-fucose, L-rhamnose, L-arabinose, and D-quinovose units which were identified by GC analysis of their trimethylsilyl thiazolidine derivatives.[8] Comparison of 1D and 2D NMR data of **2** with those of **1** indicated that **2** had acacic acid as aglycon while the major difference regarding the aglycone moiety was the absence of one monoterpene-quinovosyl (MT-Qui) unit in **2**, as already suggested by its mass spectrum, which displayed 312 mass units less than that of **1**. The observation of glycosylation- and acylation-induced shifts at C-3 (δ_C 89.1), C-21 (δ_C 77.6), and C-28 (δ_C 175.1) in the ^{13}C NMR spectrum of **2** suggested that it should also be a 21-acyl 3,28-bidesmosidic acacic acid derivative with sugar chains linked to C-3 and C-28 through an ether and ester bond, respectively, and with an acyl group attached at C-21. The ^{1}H NMR spectrum of **2** showed nine anomeric protons signals at δ_H 4.90 [d, J = 8.0 Hz, glucose (Glc I)], 4.99 [d, J = 8.1 Hz, fucose (Fuc)], 5.08 [d, J = 7.1 Hz, xylose (Xyl I)], 6.15 [d, J = 8.0 Hz, glucose (Glc II)], 6.41 [br s, rhamnose (Rha)], 5.43 [d, J = 8.1 Hz, glucose (Glc III)], 5.28 [d, J = 7.1 Hz, xylose (Xyl II)], 4.90 [d, J = 7.3 Hz, quinovose (Qui I)], and 4.89 [d, J = 8.2 Hz, quinovose (Qui II)], which gave correlations with nine anomeric carbon resonances at δ_C 105.4, 103.8, 107.3, 95.6, 101.6, 106.2, 106.8, 99.7, and 99.7,

Table 2
^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of the monoterpene-quinovosyl moieties attached at C-21 of compounds **1**–**2** in pyridine-d_5 (δ in ppm)[a]

Position	**1**		**2**	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
(MT)				
MT I-1	–	168.1	–	168.1
2	–	128.9	–	128.9
3	6.89 t (7.3)	142.4	6.91 t (7.1)	142.7
4	2.46	24.0	1.79; 2.45	24.1
5	1.76	40.9	1.78	40.8
6	–	80.1	–	79.9
7	6.25 dd (10.5; 17.5)	144.3	6.21, dd (9.1; 16.9)	144.5
8	5.28 d (10.5); 5.47 d (17.5)	115.4	5.26 d (9.1); 5.46 d (16.9)	115.5
9	1.93 s	13.1	1.91 s	13.1
10	1.51 s	24.2	1.57 s	24.0
MT II-1	–	168.1	–	168.2
2	–	128.5	–	128.3
3	7.00 t (7.3)	143.2	7.06 t (7.3)	143.8
4	2.42	23.8	2.50	24.1
5	1.76	41.4	1.78	40.8
6	–	80.1	–	80.2
7	6.27 dd (10.5; 17.5)	144.4	6.27 dd (10.4; 18.0)	144.3
8	5.28 d (10.5); 5.47(17.5)	115.2	5.31 d (10.4); 5.48 (18.0)	115.3
9	1.85 s	13.1	1.85 s	13.1
10	1.57 s	24.2	1.58 s	24.1
MT III-1	–	167.7		
2	–	128.5		
3	7.08 t (7.3)	143.3		
4	2.46	24.1		
5	1.79	40.9		
6	–	80.2		
7	5.99 dd (11.0; 17.2)	143.5		
8	5.30 d (11.0); 5.39 d (17.2)	115.8		
9	1.96 s	13.1		
10	1.58 s	23.9		
(Qui)				
Qui I-1	4.90 d (8.0)	99.7	4.90 d (7.3)	99.7
2	4.03	75.9	4.02	75.9
3	4.23	76.3	4.23	76.1
4	5.36	77.7	5.37	77.7
5	3.71	70.6	3.71	70.5
6	1.38 d (6.0)	19.0	1.51 d (6.0)	19.1
Qui II-1	4.89 d (8.0)	99.6	4.89 d (8.2)	99.7
2	4.02	75.7	4.00	75.7
3	4.24	76.2	4.13	76.3
4	5.64	75.9	4.22	76.0
5	3.76	72.7	3.70	72.9
6	1.60 d (5.2)	19.2	1.61 d (5.4)	19.2
Qui III-1	4.97 d (8.0)	97.3		
2	4.03	75.9		
3	4.22	76.4		
4	3.73	77.1		
5	3.72	70.6		
6	1.62 d (5.0)	19.5		

Overlapped proton NMR signals are reported without designated multiplicity.
[a] Assignments were based on the HMBC, HSQC, COSY, TOCSY, ROESY, and DEPT experiments.

respectively, in the HSQC spectrum (Tables 1 and 2). In addition, the ^{1}H NMR spectrum of **2** exhibited two olefinic proton signals at δ_H 6.91 (d, J = 7.1 Hz) and 7.06 (d, J = 7.3 Hz), and two groups of one-substituted olefin proton signals, one group at δ_H 6.21 (dd, J = 9.1; 16.9 Hz), 5.26 (d, J = 9.1 Hz) and 5.46 (d, J = 16.9 Hz), the other group at δ_H 6.27 (dd, J = 10.4; 18.0 Hz), 5.31 (d, J = 10.4 Hz) and 5.48 (d, J = 18.0 Hz), indicating that compound **2** had two units of monoterpenoid moieties.[23] Hence, the units attached at C-3 and C-28 of the aglycon of **2** were established as β-D-xylopyranosyl-(1→2)-β-D-fucopyranosyl-(1→6)-β-D-glucopyranoside, and β-D-xylopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→3)]-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside, respectively.

Extensive analysis of 1D and 2D NMR spectra of **2** allowed to identify the unit attached at C-21 as {(2*E*,6*S*)-6-*O*-{4-*O*-[(2*E*,6*S*)-2,6-dimethyl-6-*O*-(β-D-quinovopyranosyl)octa-2,7-dienoyl]-β-D-quinovopyranosyl}-2,6-dimethylocta-2,7-dienoyl}, a unit identical to that of gummiferaosides A and B,[10] and coriarioside E.[21] Accordingly, the structure of Lebbeckoside B (**2**) was established as 3-*O*-[β-D-xylopyranosyl-(1→2)-β-D-fucopyranosyl-(1→6)-β-D-glucopyranosyl]-21-*O*-{(2*E*,6*S*)-6-*O*-{4-*O*-[(2*E*,6*S*)-2,6-dimethyl-6-*O*-(β-D-quinovopyranosyl)octa-2,7-dienoyl]-β-D-quinovopyranosyl}octa-2,7-dienoyl}acacic acid 28-*O*-β-D-xylopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→3)]-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl ester.

Acacic acid-type saponins have been reported to exhibit cytotoxic activity on various tumor cell types, namely human Jurkat leukemia cells, cutaneous lymphoma cells, breast, ovarian and pancreatic tumors.[2] To address the cytotoxic activity of these our

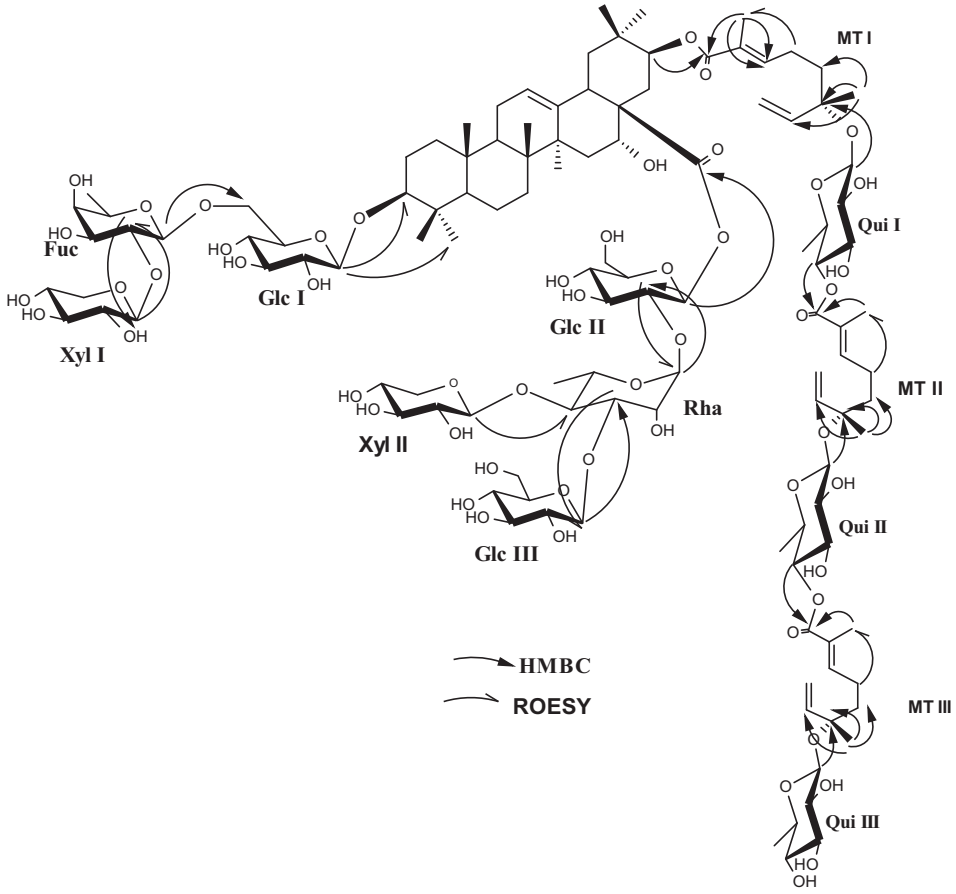


Figure 1. Key HMBC and ROESY correlations for compound **1**.

new acacic acid glycosides, lebbeckosides A (**1**) and B (**2**), we evaluated their activity on cancer stem-like cells isolated from glioblastoma which correspond to high grade human brain tumors, with very poor prognosis and for which there is an unmet medical need. Brain tumors, like many other tumors contain cancer stem-like cells,[24,25] known for their implication in tumor fueling, resistance to chemotherapies and tumor recurrence after therapy.[26,27] To increase therapy efficacy, new chemical compounds targeting those cells need to be found.[26–28] We therefore tested our newly isolated molecules on cancer stem-like cells isolated from patients' glioblastoma.[29] These cells were characterized and were shown to exhibit the typical properties of cancer stem-like cells, including resistance to chemotherapeutic agents and namely against temozolomide (TMZ) the standard of care for glioblastoma.[29] Finding molecules with cytotoxic activity toward such cells is of prime importance to improve cancer control and therapy. Activity of the lebbeckosides was also evaluated on U-87 MG the commercially available glioblastoma cell line. Compounds **1** and **2** inhibited U87 glioblastoma cell survival, assessed by ATP level measurements, with IC_{50} values of 3.16 and 2.10 μM (Fig. 2 and Table 3). Lebbeckosides A (**1**) and B (**2**) were also active on the highly aggressive, drug resistant TG1 cells with an IC_{50} in the same range as that determined for the U-87 MG cell lines (Fig. 2 and Table 3). Their cytotoxic effect on both cell types was confirmed by trypan blue staining. Lebbeckoside A (**1**) showed a slightly higher activity toward TG1 than lebbeckoside B (**2**) as indicated by their IC_{50} values (Table 3). Also, the level of cell death observed for **1** and **2** after 24 h of treatment pointed out a larger efficacy of the molecules toward TG1 compared to U-87 MG cells. Hill coefficients values (n_H) determined from curve fitting suggested the presence of several binding sites for the different compounds with binding cooperativity. Non avicin-type saponins, such as glycosides of oleanolic acid, had no effect on glioblastoma cell survival (*data not shown*). Under the experimental conditions used to test the lebbeckosides, known anticancer agents such as etoposide, topotecan, paclitaxel, and daunorubicin did not show any activity on TG1 cells' viability at concentration up to 50 μM. Only tamoxifen, an estrogen antagonist used to treat estrogen receptor positive breast cancers, induced cell death within the 24 h of the experiment. Tamoxifen has already been shown to act on cancer cells different from breast cancer cells and to be active on glioma and glioblastoma cell lines.[30,31] In addition, tamoxifen alone in association with other drugs, namely TMZ has been used in clinical trials to treat glioblastoma.[32–34] In comparison to tamoxifen (Table 3) lebbeckosides induced cell death of both glioblastoma cell types and more particularly the glioblastoma stem-like cells with a 10 fold higher activity.

3. Conclusion

This study unveiled the presence in *Albizia lebbeck* of new acacic acid-type saponins, molecules cytotoxic at a micromolar concentration toward high-grade glioma cancer cells including those with stem-like cell properties, known to be resistant to standard treatments. The cytotoxic effect of the two major avicins (D and G) isolated from *Acacia victoriae*, another Mimosaceae species from Australia, on cancer cells, has been reported to occur through various mechanisms. Indeed, avicin D-mediated cell death has been reported to be associated with the activation of the death receptor-caspase-8 pathway.[35] Avicins appear also to affect phospholipid-dependent processes,[36] and trigger caspase-independent autophagic cell death by the regulation of the AMPK-TSC_2-mTor

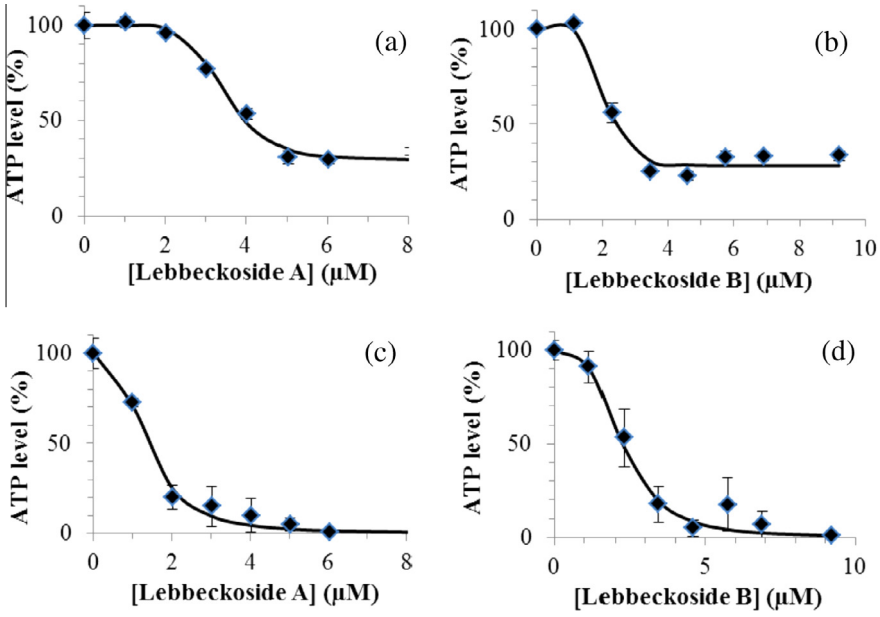


Figure 2. Effect of lebbeckosides A and B on (a and b) glioblastoma U-87 MG cell lines and (c and d) TG1 glioblastoma stem-like cells. Cells were treated for 24 h at the given lebbeckoside concentration. The same number of cells is treated with either lebbeckoside or vehicle (control). Ordinate represents percent ATP in lebbeckoside treated versus vehicle treated cells. Experiments were performed in triplicate. Data points were fitted as indicated under materials and methods. IC_{50} and n_H values obtained from curve fitting are given in Table 3.

Table 3
IC_{50} and Hill coefficients (n_H) of lebbeckosides A (**1**) and B (**2**) on U87 MG and TG1 glioblastoma stem-like cells (TG1 GSCs)

Compound	U87 MG		TG1 GSC	
	IC_{50} (μM)	n_H	IC_{50} (μM)	n_H
1	3.46 ± 0.10	6.6	1.36 ± 0.07	2.8
2	2.10 ± 0.05	6.6	2.24 ± 0.18	3.3
Tamoxifen	31 ± 2	5.9	14 ± 2	4.4

pathway.[35] Moreover, it has recently been shown that some avicins may increase the level of ubiquitinated proteins in human Jurkat T cells possibly through an up-regulation of ubiquitin ligase activity.[36] However, avicins seem to have other, and yet only partially identified, molecular targets.[37] Lebbeckosides A–B, two new avicin analogs isolated from *Albizia lebbeck*, might therefore be highly useful to further decipher the mechanism of action of this triterpene saponin and obtain new structure–activity relationship data. It will be also of high interest to compare the effect of lebbeckosides A and B on other types of cancer cells, as well as to use these molecules as tools to investigate the pathophysiology of cancer cells with stem properties versus non stem cancer cells. In addition, on the chemotaxonomic point of view, this study represents a valuable contribution to the chemotaxonomy of the *Albizia* genus, which is known to be a rich source of triterpenoid saponins. Furthermore, the findings, especially for the monoterpene-quinovosyl units, established in compounds **1** and **2**, and identical to those found in the related compounds isolated from *Albizia coriaria* and *Albizia gummifera* may indicate a close relationship between the three species of *Albizia* genus.

4. Experimental section

4.1. General methods

Optical rotations were measured on a Jasco P-200 polarimeter. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded at room temperature in pyridine-d_5 using a Bruker AVANCE III 500 spectrometer. Chemical shifts are given in δ (ppm) value relative to TMS as internal standard. HRESIMS spectra were recorded on a microTOF ESI-TOF mass spectrometer (Bruker) operating in positive mode. Analytical HPLC was performed on Varian 920-LC apparatus equipped with an autosampler, a pump, a diode array detector, and Galaxie software. Semipreparative HPLC was performed on a Gilson apparatus equipped with Trilution LC software using a Nucleodur 100-5 C18ec (21 × 250 mm, 5 μm) column purchased from Machery-Nagel (Germany). Thin layer chromatographies (TLC) were performed on precoated silica gel plates (60 F_{254}, Merck) (*n*-BuOH–AcOH–H_2O, 65:15:25). The spots were observed after spray with Komarowsky reagent, a (5:1) mixture of *p*-hydroxybenzaldehyde (2% in MeOH) and (50% H_2SO_4 in EtOH). Vacuum-liquid chromatography (VLC) was carried out using RP-18 silica gel 60 (25–40 μm) and silica gel 60 (15–40 μm and 40–63 μm).

4.2. Plant material

The roots of *Albizia lebbeck* (L.) Benth were harvested at Nkolbisson, Yaoundé peripheral quarter, in Cameroon in October 2011 under the guidance of Mr. Victor Nana, botanist of the National Herbarium of Cameroon (NHC), where a voucher specimen (47196/HNC) was deposited.

4.3. Extraction and isolation

Air-dried and powdered roots of *A. lebbeck* (300 g) were extracted with EtOH 70% in a soxhlet apparatus. The hydroalcoholic solution was then evaporated to dryness under reduce pressure to yield a brown residue (4.83 g). This residue was suspended in 200 mL of water and partitioned between H_2O and saturated *n*-BuOH (3 × 300 mL). The *n*-BuOH-soluble part (3.04 g) which exhibited cytotoxic activity against glioblastoma cancer stem cells (TG1) and human glioblastoma cell line (U-87 MG), with IC_{50} values of 8.52 and 6.03 μg/ml, respectively, was dissolved in a minimum of water (10 mL) and then submitted to VLC using RP-18 (25–40 μm) silica gel and eluting with a gradient of MeOH–H_2O (100% H_2O→100% MeOH) to yield 3 main fractions (L1–L3). The

70–85% MeOH fraction (L3) (484.2 mg) which showed significant cytotoxic activity against the used cancer cells (TG1, IC_{50} 4.87 μg/ml and U-87 MG, IC_{50} 3.18 μg/ml) was subjected to VLC on silica gel 60 (15–40 μm), eluted with $CHCl_3$–MeOH–H_2O (80:20:2, 70:30:5, 60:33:7, and 60:40:10) to give 29 subfractions. The subfractions were combined into 6 main subfractions (L31–L36) based on TLC behaviors. Subfraction L33 (66.6 mg, TG1 IC_{50} 3.31 μg/ml, and U-87 MG IC_{50}, 2.28 μg/ml) was purified by Semipreparative HPLC using a gradient system of CH_3CN–H_2O (20 ml/min) to yield compounds **1** (19.8 mg, t_R 27.07 min) and **2** (8.7 mg, t_R 24.21 min).

4.3.1. Lebbeckoside A (1)

White amorphous powder; $[\alpha]_D^{25}$ +30.95 (*c* 0.06, MeOH); ^1H NMR (500 MHz, C_5D_5N) and ^{13}C NMR (125 MHz, C_5D_5N) data of the aglycon (Supporting information). ^1H and ^{13}C NMR data of oligosaccharidic chains, see Tables 1 and 2. ^1H and ^{13}C NMR data of monoterpene units, see Table 2. HRESIMS (positive-ion mode) *m/z*: 2486.2146 $[M+NH_4]^+$ (calcd for $C_{118}H_{186}O_{54}$, 2485.2147).

4.3.2. Lebbeckoside B (2)

White amorphous powder; $[\alpha]_D^{25}$ −20.45 (*c* 0.16, MeOH); ^1H NMR (500 MHz, C_5D_5N) and ^{13}C NMR (125 MHz, C_5D_5N) data of the aglycon, (Supporting information). ^1H and ^{13}C NMR data of oligosaccharidic chains, see Tables 1 and 2. ^1H and ^{13}C NMR data of monoterpene units, see Table 2. HRESIMS (positive-ion mode) *m/z*: 2174.0665 $[M+NH_4]^+$ (calcd for $C_{102}H_{162}O_{48}$, 2173.0574).

4.4. Acid hydrolysis of 1–2 and determination of absolute configuration of monosaccharides

Each saponin (2 mg) was hydrolyzed with 2 ml of 2 M HCl at 85 °C during 2 h. After cooling, the solvent was removed under reduced pressure. The sugar mixture was extracted from the aqueous phase (10 ml) and washed with CH_2Cl_2 (3 × 5 ml). The combined CH_2Cl_2 extracts were washed with water to give after evaporation the aglycon moiety. The sugars were first analyzed by TLC over silica gel ($CHCl_3$–MeOH–H_2O, 8:5:1) by comparison with standard samples. The absolute configuration of each monosaccharide was determined from GC–MS analysis of their trimethylsilylated derivatives by comparison with authentic samples using the method previously described.[8] GC analysis was performed with a capillary TR-5MS SQC (15 m × 0.25 mm × 0.25 μm) column. Operating conditions were as follows: carrier gas, helium with a flow rate of 1 ml/min; column temperature, 1 min in 150 °C, 150–220 °C at 4 °C/min; injector temperature, 250 °C; volume injected 1 μl of the trimethylsilylated sugar in methylene chloride (0.1%); split ratio, 1:50. The MS operating parameters were as follows: ionization potential, 70 eV; ion source temperature, 230 °C; solvent delay 4.0 min, mass range 100–700. The following sugars were detected for **1** and **2**: D-glucose, D-quinovose, D-fucose, D-xylose, and L-rhamnose.

4.5. Evaluation of inhibitory effect of saponins

4.5.1. Chemical reagents

Glioblastoma U-87 MG cells were purchased from ATCC. TG1 cancer stem-like cells isolated after surgical resection of a human glioblastoma (WHO grade IV glioma) were obtained from Dr. H. Chneiweiss (Neuroscience Paris). Stock solutions of the chemicals were prepared: 2 mg/mL of lebbeckosides in milliQ water and 10 mM of tamoxifen in DMSO.

4.5.2. Cell cultures

Glioblastoma stem-like cells (TG1) were grown as neurospheres as previously described.[29] Neurospheres were mechanically dissociated twice a week and medium renewed. Glioblastoma U-87 MG cells were expanded in adherent culture according to the supplier's instructions (ATCC). All cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

4.5.3. Effect of chemical compounds on cell viability

Effect of chemical compounds on glioblastoma cell viability was assessed using the CellTiter Glo® luminescent cell viability assay (Promega). Sub-confluent U87-MG cells were trypsinized and resuspended in fresh medium to a density of 300 000 cells/mL. 100 μL of cell suspension were seeded in each well of the 96-well opaque bottom plate (Greiner, Courtaboeuf, France. 30 000 cells/well). Cells in the 96-well plate were incubated at 37 °C, 5% CO_2 for 24 h for cell adherence. The medium in each well was then replaced by fresh medium (100 μL) with the desired concentrations of chemical compounds (or no chemical compound for untreated cells). TG1 cells were mechanically dissociated and resuspended in fresh medium to a density of 600 000 cells/mL. 50 μL of cell suspension was seeded in a 96-well plate (30 000 cells/well). 50 μL of medium with or without 2× the desired concentrations of chemical compounds were added to each well.

Cells were incubated at 37 °C, 5% CO_2 and cell viability was measured 24 h later using the CellTiter Glo® luminescent cell viability assay (Promega) according to the manufacturer's instructions. Luminescence was measured using a Victor™3 plate reader (PerkinElmer). Relative ATP levels in each well were determined by calculating the percentage of luminescent signal in the well with respect to the average signal measured in negative control wells (without compounds). Each compound was tested in triplicate. Compound induced cell death was also assessed using trypan blue exclusion assay.

Curve fitting for IC_{50} calculations: The IC_{50} value was determined for each compound by fitting the data points according to the following equation:

$$y = \frac{S_{max} + S_{min} * \left([\text{compound}] * \frac{1}{IC_{50}}\right)^{n_H}}{1 + \left([\text{compound}] * \frac{1}{IC_{50}}\right)^{n_H}}$$

where y represents the expected response, [compound] is the chemical compound concentration, S_{max} and S_{min}, are the maximum and minimum responses recorded, respectively, and n_H corresponds to the Hill coefficient. Curve fitting was performed using the Microsoft Excel Solver component.

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Supplementary data

Supplementary data (1D, 2D NMR, and HRESIMS of compounds **1**–**2**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carres.2014.12.004.

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