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New Cerebrosides from *Euphorbia peplis* L.: Antimicrobial Activity Evaluation

Francesca Cateni,[a,*] Jelena Zilic,[a] Gioacchino Falsone,[a] Giuditta Scialino[b] and Elena Banfi[b]

[a]*Department of Pharmaceutical Sciences, University of Trieste, P.zle Europa 1, 34127 Trieste, Italy*
[b]*Department of Biomedical Sciences, Microbiology sect., University of Trieste, Via A. Fleming 22, 34127 Trieste, Italy*

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Abstract—The less polar fraction of the methanolic extract from the plant *Euphorbia peplis* L. exhibited interesting antifungal and antitubercular activity. A complex mixture of four glucocerebrosides was responsible for this activity. Two new cerebrosides were isolated for the first time from Euphorbiaceae, **4** was assigned as 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*S*,4*E*,8*E*)-2*N*-[(2′*R*)-2′-hydroxy-hexadecanoyl]-4 (*E*), 8 (*E*)-octadecadiene-1,3-diol and **3** as the 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*S*,4*R*,8*Z*)-2*N*-[(2′*R*)-2′-hydroxy-tetracosanoyl]-8 (*Z*)-octadecene-1,3,4-triol. The structures were determined on the basis of chemical and spectroscopic evidences. Mass spectrometry of dimethyl disulfide derivatives was useful for the determination of the double-bond positions in the long-chain bases.

Euphorbia peplis L. (Euphorbiaceae) is a perennial herbaceous plant with a milky juice, distributed mainly in North Italy.

In our previous studies, we reported the isolation and structure elucidation of the biologically active glyceroglycolipids obtained from the less polar fraction of the MeOH extract of the plant *E. peplis* L.[1] In a continuation of those studies, conducted for the considerable interest and importance connected with the determination of the composition of the mixture of glycosphingolipids, we performed the isolation and characterization of the cerebrosides obtained from the less polar fraction in the hope of discovering new medicinal resources from plant natural products.

In this paper, the isolation and structure elucidation of four cerebrosides **1**–**4**, from *E. peplis* L. is reported, together with an interesting antifungal and antitubercular activity of the methanolic extract of the plant, containing the complex mixture of cerebrosides; individual biological activity of the pure cerebrosides is also evaluated.

Compounds **3** and **4** are new cerebrosides and this is the first example of such glycosphingolipids from the Euphorbia genus.

During our investigation on various species of Euphorbiaceae we have reported the presence of different cerebrosides.[2–4]

E. peplis L. was collected in August 2000 in Carso triestino, Italy. After air-drying, the stems and the leaves of the plant were exhaustively extracted with methanol.

The extract was filtered and concentrated in vacuo to yield 10 g of crude material. Chromatography on a silica gel column ($CHCl_3$ to MeOH) of the crude extract led to the active fraction eluted by $CHCl_3$–MeOH (5:1.5).

Repeated chromatography of this active fraction on silica gel column and on a RP column yield 25 mg of pure **1** (MeOH, $R_f = 0.76$), 30 mg of pure **2** (MeOH, $R_f = 0.58$), 20 mg of pure **3** (MeOH, $R_f = 0.63$) and 23 mg of pure **4** (MeOH, $R_f = 0.72$), all were isolated as white amorphous powders.

The IR absorptions of **1**–**4** indicated the presence of hydroxyl groups (ν_{max} 3413 cm^{-1}) and amide functions (ν_{max} 1646, 1540 cm^{-1}).

*Corresponding author. Tel.: +39-040-558-3720; fax: +39-040-52572; e-mail: cateni@univ.trieste.it

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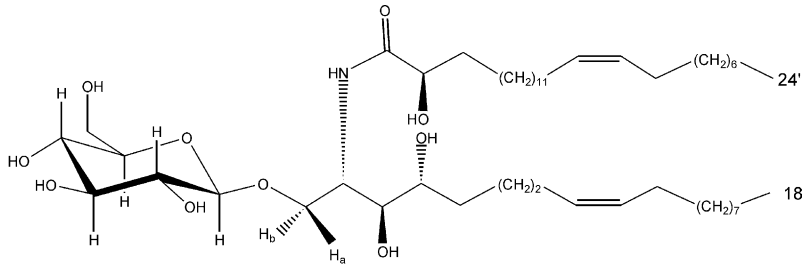

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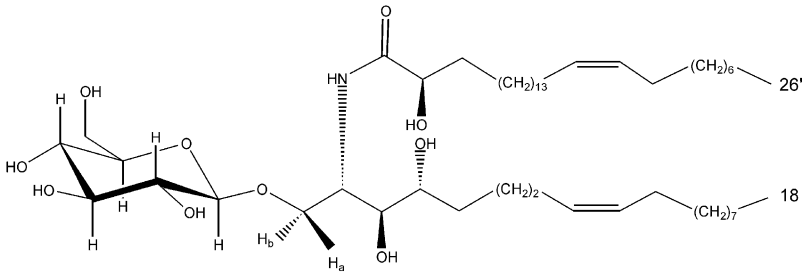

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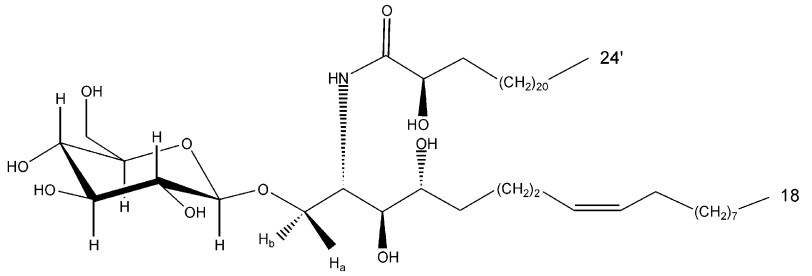

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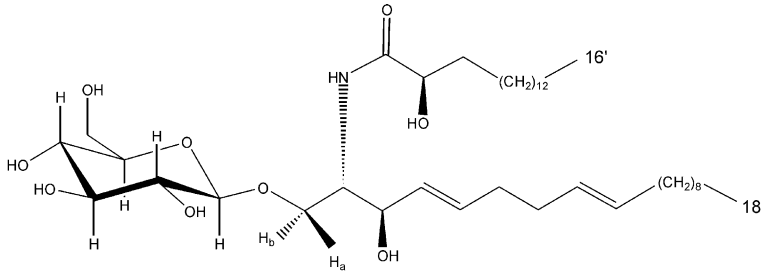

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The positive FAB mass spectra of cerebrosides **1–4** exhibited $(M+H)^+$ ion peaks at *m/z* 842 (**1**), 870 (**2**), 844 (**3**), 714 (**4**), respectively.

The ^1H and ^{13}C NMR spectra were typical of a sphingosine-type cerebroside possessing 2-hydroxy fatty acid and β-glucopyranose moieties (Tables 1 and 2).

Assignments of all protons and carbons in **1–4** can be made by ^1H–^1H COSY, HMQC and HMBC spectra. Owing to the HMBC correlations observed for O<u>H</u>-3 we were able to identify C-3 which in turn showed correlations with H-4 which correlates in the COSY spectrum with O<u>H</u>-4. These long range correlations allowed to clearly deduced the presence of a sphingosine-type cerebroside. The fatty acid linked to C-2 of the sphingosine has been confirmed by the correlations between the NH proton and the carbon C-2. HMBC correlations of the carbonyl at 175.6 with the proton H-2′ which in turn showed correlations with the carbon C-2′ and the proton OH-2′ confirmed the presence of an α-hydroxy fatty acid side chain. In the compound **4** the sphingosine-type cerebroside was assigned by the correlations observed for OH-3 with C-3 which showed correlations with the olefinic protons H-4 and H-5, respectively.

Constituents of the ceramide and sugar moieties of **1–4** were determined as follows.

Table 1. ^{1}H and ^{13}C assignments (δ ppm) of **1–2** in C_5D_5N on a Varian Unity 400 at 298 K

Position	**1**			**2**		
	^{1}H (m, *J* Hz)	^{13}C	COSY	^{1}H (m, *J* Hz)	^{13}C	COSY
Ceramide						
NH	8.53 (d, 8)	—	2	8.55 (d, 8)	—	2
1a	4.70 (dd, 12, 7)	70.6	1b, 2	4.71 (dd, 11, 5.5)	70.5	1b, 2
1b	4.52 (m)		1a, 2	4.51 (m)		1a, 2
2	5.22 (m)	51.5	NH, 1a, 1b, 3	5.23 (m)	51.8	NH, 1a, 1b, 3
3	4.28 (dd, 4.5, 3)	76.1	OH, 2, 4	4.27 (dd, 4, 3.5)	76.0	OH, 2, 4
4	4.22 (obs)	72.6	OH, 5a, 5b, 3	4.23 (obs)	72.6	OH, 5a, 5b, 3
5a	2.40 (m)	34.1	5b, 4, 6a, 6b	2.39 (m)	34.1	5b, 4, 6a, 6b
5b	1.88 (m)		5a, 4, 6a, 6b	1.89 (m)		5a, 4, 6a, 6b
6a	1.70 (m)	26.2	5a, 5b, 6b, 7	1.73 (m)	26.0	5a, 5b, 6b, 7
6b	1.65 (m)		5a, 5b, 6a, 7	1.65 (m)		5a, 5b, 6a, 7
7	2.20 (m)	27.8	6a, 6b, 8	2.22 (m)	27.8	6a, 6b, 8
8	5.45 (m)	130.7	7, 9	5.45 (m)	130.6	7, 9
9	5.45 (m)	130.2	8, 10	5.45 (m)	130.4	8, 10
10	2.20 (m)	27.7	9, 11	2.22 (m)	27.7	9, 11
11–15	1.30 (m)	29.4–30.4		1.32 (m)	29.6–30.3	
16	1.25 (m)	32.4		1.26 (m)	32.3	
17	1.25 (m)	23.5		1.26 (m)	23.3	
18	0.85 (t, 7)	14.6		0.85 (t, 7.1)	14.6	
1′	—	175.8		—	175.6	
2′	4.57 (dd, 8, 3.7)	72.6	OH, 3′a, 3′b	4.58 (dd, 8.2, 4)	72.6	OH, 3′a, 3′b
3′a	2.20 (m)	35.7	3′b, 2′, 4′a, 4′b	2.22 (m)	35.7	3′b, 2′, 4′a, 4′b
3′b	2.00 (m)		3′a, 2′, 4′a, 4′b	2.00 (m)		3′a, 2′, 4′a, 4′b
4′a	1.94 (m)	26.7	4′b, 5′, 3′a, 3′b	1.95 (m)	26.8	4′b, 5′, 3′a, 3′b
4′b	1.70 (m)		4′a, 5′, 3′a, 3′b	1.71 (m)		4′a, 5′, 3′a, 3′b
CH_2aliph.	1.25–1.30 (m)	23.5–32.4		1.25–1.30 (m)	23.2–32.4	
14′, 17′	2.20 (m)	27.8		—	—	
16′, 19′	—	—		2.21 (m)	27.9	
15′, 16′	5.45 (m)	130.1		—	—	
17′, 18′	—	—		5.45 (m)	131.3	
CH_3	0.85 (t, 7)	14.6		0.85 (t, 7.1)	14.4	
OH-2′	7.60 (br. s)	—	2′	7.62 (br. s)	—	2′
OH-3	6.82 (br. s)	—	3	6.81 (br. s)	—	3
OH-4	6.00 (br. s)	—	4	6.00 (br. s)	—	4
Glucose						
1″	4.95 (d, 8)	105.3	2″	4.95 (d, 7.9)	105.5	2″
2″	4.00 (t, 8)	75.2	1″, 3″	4.03 (t, 8)	75.1	1″, 3″
3″	4.21 (obs)	78.3	2″, 4″	4.21 (obs)	78.6	2″, 4″
4″	4.19 (obs)	71.2	3″, 5″	4.19 (obs)	71.6	3″, 5″
5″	3.87 (m)	78.6	4″, 6″a, 6″b	3.88 (m)	78.6	4″, 6″a, 6″b
OH-6″	6.40 (br. s)	—	6″a	6.30 (br. s)	—	6″a
6″a	4.48 (dd, 12, 2)	62.2	5″, 6″b	4.48 (dd, 11, 2)	62.7	5″, 6″b
6″b	4.34 (dd, 12, 3)		5″, 6″a	4.36 (dd, 11, 3.5)		5″, 6″a

The structures of the ceramide moieties were examined first. When cerebrosides **1–4** were methanolyzed with methanolic hydrochloric acid, fatty acid methyl esters (FAMs) were obtained together with long-chain bases (LCBs) and methyl glucopyranoside.[4]

On the basis of mass spectrometry analysis, the FAMs were characterized as methyl 2-hydroxytetracosanoate (FAM-1), methyl 2-hydroxyhexacosenoate (FAM-2), methyl 2-hydroxy tetracosenoate (FAM-3) and methyl 2-hydroxyhexadecanoate (FAM-4), respectively. On the other hand, on the basis of mass spectrometry analysis the LCB components were suggested to be 2-amino-1,3,4-trihydroxy-8-octadecene (LCB-1-3) and 2-amino-1,3-dihydroxy-4,8-octadecadiene (LCB-4), respectively.

The EI–MS mass spectra of the dimethyl disulfide (DMDS) derivatives of **1–4** showed remarkable fragments ion peaks at *m*/*z* 221 (**1–3**) and 149, 187 (**4**) respectively, due to cleavage of the bond between the carbons bearing a methylthio group (Fig. 1).[5,6] These data indicate that the double-bonds in the LCB residues of **1–4** are located at C-8 (**1–3**) and at C-4 and C-8 (**4**), respectively. Furthermore, it is known[7] that the geometry of the double-bond in the long-chain alkene can be determined on the basis of the ^{13}C NMR chemical shift of the methylene carbon adjacent to the olefinic carbon, which is observed at $\delta \cong 27$ ppm in (*Z*) isomers and at $\delta \cong 33$ in (*E*) isomers. The proton signals at $\delta = 5.45$ ppm were assigned to the olefin groups based on the ^{1}H–^{1}H correlation spectroscopy (COSY) spectrum of **1–4**.

When the heteronuclear multiple bond connectivity (HMBC) spectra of **1–3** were recordered, significant correlations were observed between the signal of the

Table 2. ^{1}H and ^{13}C assignments (δ ppm) of **3**–**4** in C_5D_5N on a Varian Unity 400 at 298 K

Position	**3**			**4**		
	^{1}H (m, *J* Hz)	^{13}C	COSY	^{1}H (m, *J* Hz)	^{13}C	COSY
Ceramide						
NH	8.54 (d, 9)	—	2	8.41 (d, 8)	—	2
1a	4.69 (dd, 13.3, 5)	70.6	1b, 2	4.76 (dd, 12, 6)	70.3	1b, 2
1b	4.56 (m)		1a, 2	4.55 (m)		1a, 2
2	5.20 (m)	51.9	NH, 1a, 1b, 3	4.80 (m)	54.6	NH, 1a, 1b, 3
3	4.29 (dd, 4.7, 3)	76.1	OH, 2, 4	4.80 (m)	72.4	OH, 2, 4
4	4.27 (obs)	72.5	OH, 5a, 5b, 3	6.00 (dd, 15, 6)	132.07	3, 5
5a	2.41 (m)	34.0	5b, 4, 6a, 6b	5.92 (dt, 15, 6)	132.24	4, 6
5b	1.91 (m)		5a, 4, 6a, 6b			
6a	1.75 (m)	26.3	5a, 5b, 6b, 7	2.07 (m)	33.1	5, 7
6b	1.63 (m)		5a, 5b, 6a, 7			
7	2.23 (m)	27.6	6a, 6b, 8	2.09 (m)	32.9	6, 8
8	5.44 (m)	130.2	7, 9	5.45 (t, 4.9)	130.0	7, 9
9	5.44 (m)	129.5	8, 10	5.45 (t, 4.9)	131.2	8, 10
10	2.23 (m)	27.8	9, 11	1.80 (m)	33.14	9, 11
11–15	1.31 (m)	29.6–30.4		1.30–1.38 (m)	29.6–30.3	
16	1.24 (m)	32.3		1.26 (m)	32.3	
17	1.24 (m)	23.1		1.26 (m)	23.2	
18	0.84 (t, 7)	14.7		0.95 (t, 6)	14.5	
1′	—	175.6		—	175.7	
2′	4.55 (dd, 8, 5.2)	72.5	OH, 3′a, 3′b	4.55 (dd, 8, 4.2)	72.6	OH, 3′a, 3′b
3′a	2.19 (m)	35.8	3′b, 2′, 4′a, 4′b	2.10 (m)	35.8	3′b, 2′, 4′a, 4′b
3′b	1.98 (m)		3′a, 2′, 4′a, 4′b	1.90 (m)		3′a, 2′, 4′a, 4′b
4′a	1.93 (m)	26.7	4′b, 5′, 3′a, 3′b	1.92 (m)	26.1	4′b, 5′, 3′a, 3′b
4′b	1.69 (m)		4′a, 5′, 3′a, 3′b	1.70 (m)		4′a, 5′, 3′a, 3′b
CH_2aliph.	1.24–1.31 (m)	23.2–32.3		1.26–1.30 (m)	23.2–32.3	
CH_3	0.84 (t, 7)	14.5		0.95 (t, 6)	14.5	
OH-2′	7.61 (br. s)	—	2′	7.71 (br. s)	—	2′
OH-3	6.83 (br. s)	—	3	6.98 (br. d)	—	3
OH-4	6.00 (br. s)	—	4	—	—	
Glucose						
1″	4.90 (d, 8)	105.7	2″	4.95 (d, 8)	105.7	2″
2″	4.06 (t, 8)	75.4	1″, 3″	4.07 (t, 8)	75.2	1″, 3″
3″	4.23 (obs)	78.5	2″, 4″	4.21 (obs)	78.5	2″, 4″
4″	4.18 (obs)	71.6	3″, 5″	4.20 (obs)	71.6	3″, 5″
5″	3.85 (m)	78.8	4″, 6″a, 6″b	3.90 (m)	78.7	4″, 6″a, 6″b
OH-6″	6.40 (br. s)	—	6″a	6.57 (m)	—	6″a
6″a	4.52 (dd, 11.7, 2)	62.5	5″, 6″b	4.52 (dd, 12, 2)	62.7	5″, 6″b
6″b	4.40 (dd, 10.6, 3)		5″, 6″a	4.36 (dd, 12, 3.5)		5″, 6″a

olefin protons at δ = 5.45 ppm and the methylene carbon atoms at δ = 27.7, 27.8, while in the compound **4** HMBC correlations of the olefin protons H-4 with the carbons C-3 and C-6 and H-8 with C-7 and C-10 clearly confirmed the geometry of the double bonds as shown in Tables 1 and 2. Accordingly, these methylene carbon atoms must be the carbon atoms adjacent to the double-bonds and were thus assigned to C-7 and C-10 (δ = 27.8, 27.7) for the compounds **1**–**3** and to C-6, C-7 and C-10 (δ = 33.1, 32.9, 33.1) for the compound **4**. Thus the olefin groups in the LCB of **1**–**3** were determined to have *cis* (*Z*) geometry (Tables 1 and 2), while in the LCB of **4** *trans* (*E*) geometry has been assigned (Table 2).

The FAMs obtained from the methanolysis of the cerebrosides **1**,**2** exhibit ^{13}C NMR signals at *about* 173.4, 131.3 and 130.2 expected for monounsaturated fatty acid methyl esters. The EI-MS mass spectra of FAMs **1**–**4** exhibited $(M)^+$ ion peaks at *m*/*z* 396 (FAM-1), 424 (FAM-2), 398 (FAM-3), 286 (FAM-4) and $(M\text{-}59)^+$ fragments at *m*/*z* 337 (FAM-1), 365 (FAM-2), 339 (FAM-3) and 227 (FAM-4), respectively. The resonance at *about* 27.8, 27.9 ppm confirms the *Z* geometry of the double bonds in the long-chain fatty acids (Tables 1 and 2). The position of the double bonds in the monounsaturated fatty acid methyl esters was determined by EI-MS analysis of the corresponding dimethyl disulfide (DMDS) derivatives.[5] The characteristic fragments at *m*/*z* = 317, 345 and 173, obtained after the cleavage of the disulfide bond, indicates the position of the double bonds in **1**,**2** (Fig. 1).

1D and 2D ^{1}H NMR spectroscopy, DQF-COSY and HMQC indicated that the head group consists of a single glucose residue in the β configuration. The glucose configuration was determined by the characteristic chemical shifts, the spin–spin splitting and the multiplicity of the characteristic resonance of the H-4″ proton, as well as by the splittings of the other ring protons. The absolute configuration of the glucopyranose moiety was determined to be the D-form using the Hara method.[7]

The stereochemistry of the ceramide moiety was determined by comparison of the ^{1}H NMR data of

the cerebrosides isolated from *E. peplis* L. with that of synthetic analogues as reported in literature in terms of the signals due to 1-H to 4-H.[8]

Interpretation of NMR experiments (^1H–^1H COSY, HMQC, HMBC) permitted to establish the structures of **1–4** as 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*S*,4*R*,8*Z*)-2*N*-[(2′*R*)-2′-hydroxytetracosenoil]-8 (*Z*)-octadecene-1,3,4-triol (**1**), 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*S*,4*R*,8*Z*)-2*N*-[(2′*R*)-2′-hydroxyhexacosenoil]-8 (*Z*)-octadecene-1,3,4-triol (**2**), 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*S*,4*R*,8*Z*)-2*N*-[(2′*R*)-2′-hydroxytetracosanoil]-8 (*Z*)-octadecene-1,3,4-triol (**3**) and 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*S*,4*E*,8*E*)-2*N*-[(2′*R*)-2′-hydroxyhexadecanoylamino]-4 (*E*), 8 (*E*)-octadecadiene-1,3 diol (**4**), respectively.

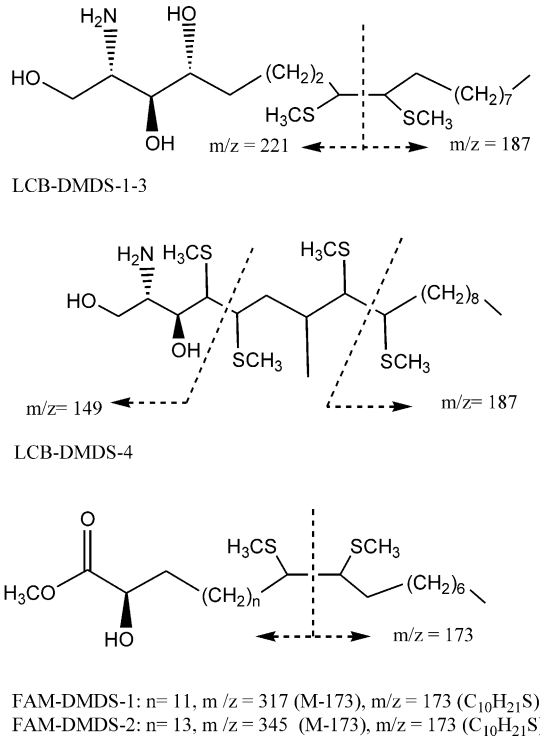

Figure 1. LCB-DMDS and FAM-DMDS derivatives of **1–4**.

Compounds **3** and **4** have been found for the first time in Euphorbiaceae. Compounds **1** and **2** have been found to be identical to the cerebrosides isolated from *E. characias* L.[9]

Biological Activity Evaluation

The fraction containing the complex mixture of cerebrosides and the individual isolated compounds **1–4** were tested for the antimicrobial, antitubercular and antifungal activities (Tables 3 and 4). Reference drugs are reported.

Antibacterial activity was evaluated by a reference agar dilution method[10] against a Gram positive bacterium *Staphylococcus aureus* and a Gram negative bacterium *Escherichia coli*. Antitubercular activity was evaluated by a standard agar dilution method[11] against a *Mycobacterium tuberculosis* reference strain and three different human clinical isolates of *M. tuberculosis* (Table 3). No activity was exhibited against Gram positive nor Gram negative reference strains. We registered an interesting antitubercular activity of the cerebrosides mixture with a MIC of 40 μg/mL against a human clinical *M. tuberculosis* strain and MICs of 80 μg/mL against the reference strain and two additional clinical human isolates. Only compound **2** has shown an inter-

Table 3. Antimicrobial activity of cerebrosides against Gram-positive and Gram-negative and tubercular bacteria

	MIC (μg/mL)					
	S. aureus ATCC 25923	*E. coli* ATCC 25922	*M. tuberculosis* H37Rv	*M. tuberculosis* H331	*M. tuberculosis* H242	*M. tuberculosis* H172
Cerebrosides mixture	>160	>160	80	40	80	80
1	>160	>160	>160	>160	>160	>160
2	>160	>160	40	40	80	40
3	>160	>160	>160	>160	>160	>160
4	>160	>160	>160	>160	>160	>160
Ciprofloxacin	0.5	0.06	0.25	0.125	0.125	0.25

Table 4. Antifungal activity of cerebrosides

	MIC (μg/mL)							
	C. albicans		*C. parapsilosis*		*C. glabrata*		*C. neoformans*	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Cerebrosides mixture	64	128	64	128	64	128	8	32
1	>128	>128	>128	>128	>128	>128	64	128
2	>128	>128	>128	>128	>128	>128	128	>128
3	>128	>128	>128	>128	>128	>128	128	>128
4	>128	>128	>128	>128	>128	>128	128	>128
Miconazole	2.5	40	0.625	1.25	5	10	0.156	0.156

esting antitubercular activity with MICs of 40 μg/mL on reference strain and on two clinical isolates and a MIC of 80 μg/mL against clinical strain H242; all strains being sensitive to ciprofloxacin with MICs ranging from 0.125 to 0.25 μg/mL.

Antifungal activity was evaluated by means of a microdilution method[12] against three *Candida* spp. strains and one *Cryptococcus neoformans* strain. Table 4 reports the interesting results obtained. The complex mixture of cerebrosides had a MIC of 8 μg/mL on *C. neoformans* clinical strain after 24 h incubation and a MIC of 32 μg/mL after 48 h incubation; the cerebrosides mixture showed an interesting inhibiting activity against the three different species of *Candida* studied, having a MIC of 64 μg/mL after 24 h incubation and a MIC of 128 μg/mL after 48 h incubation. No pure cerebroside showed any activity against *Candida* spp.; *C. neoformans* was slightly sensitive after 24 h incubation time to all pure compounds with MICs of 64 μg/mL for compound **1** and MICs of 128 μg/mL for compounds **2**–**4**.

In conclusion, the pure isolated cerebrosides show a synergistic antifungal activity when present in mixture. As concerning antitubercolar activity, only compound **2** is responsible for this effect.

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