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Preliminary biological assay on cerebroside mixture from *Euphorbia nicaeensis* All. Isolation and structure determination of five glucocerebrosides

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Abstract

Preliminary studies of in vitro cytostatic activity on less polar fraction of the MeOH extract of the plant *Euphorbia nicaeensis* All., carried out on KB cells, have evinced a relatively low ability of extract to inhibit cell growth. Successive, five glucocerebrosides were isolated from the cerebroside molecular species obtained from this extract using normal and reversed phase column 'flash-chromatography'. The structures of these cerebrosides 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.

Keywords: Glycosphingolipid; Plant; *Euphorbia nicaeensis* All.; Cerebroside; Cytostatic activity

1. Introduction

The genus *Euphorbia* is the largest genus of the plant family Euphorbiaceae. *Euphorbia nicaeensis* All. has been investigated and phytochemical studies have been reported previously dealing with epicuticular wax constituents and with the isolation and structure elucidation of tetracyclic triterpenoids of the roots and aerial parts [1,2]. Extracts of the roots of *E. nicaeensis* All. have showed significant cytotoxic activity, whereas extracts of the aerial parts showed only moderate activity [2].

As a part of our search for biologically active compounds from Euphorbiaceae the latex of *Euphorbia biglandulosa* Desf. [3], *Euphorbia characias* L. [4] and *Euphorbia wulfenii* Hoppe ex Koch [5], were investigated and complex mixtures of cerebrosides were isolated and identified.

Sphingolipid breakdown products, such as sphingosine, inhibit protein kinase C (PKC), a pivotal enzyme in cell regulation and signal transduction. Sphingolipids affect significant cellular responses and exhibit antitumor promoter activities in various mammalian cells. These molecules may function as endogenous modulators of cell function and possibly as second messengers [6].

In this paper we report on the isolation and structure elucidation of five cerebrosides **1**–**5** (Fig. 1), obtained from the less polar fraction of the MeOH extract of the aerial parts of *E. nicaeensis* All. which was separated by reversed phase 'flash chromatography' (Scheme 1). The complex mixture of the five glucocerebrosides **1**–**5** has shown a single spot on normal-phase silica gel TLC.

The less polar fraction of the MeOH extract of *E. nicaeensis* All. containing the cerebroside molecular species **1**–**5** was shown to possess a significant in vitro cytostatic activity.

2. Experimental

NMR-spectroscopy: nuclear magnetic resonance spectra were recorded with a Varian Unity 400 spectro-

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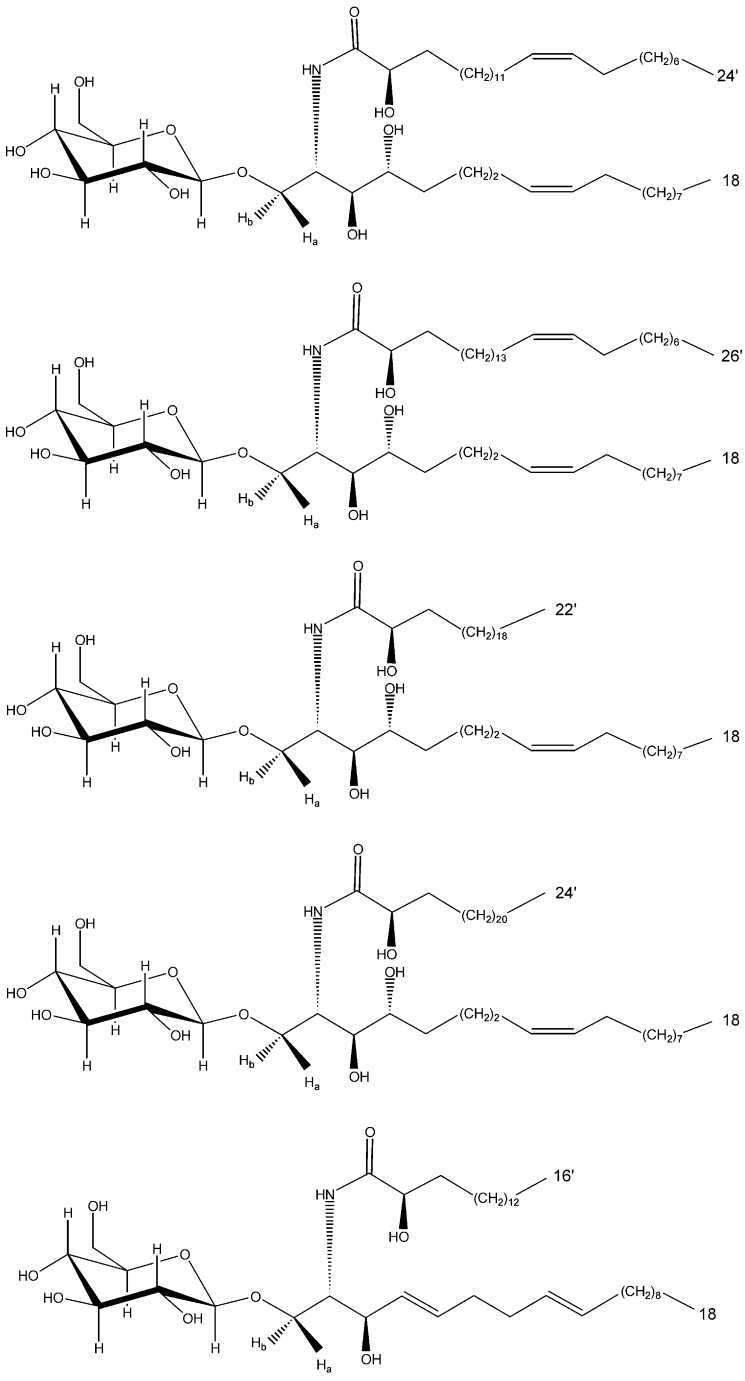
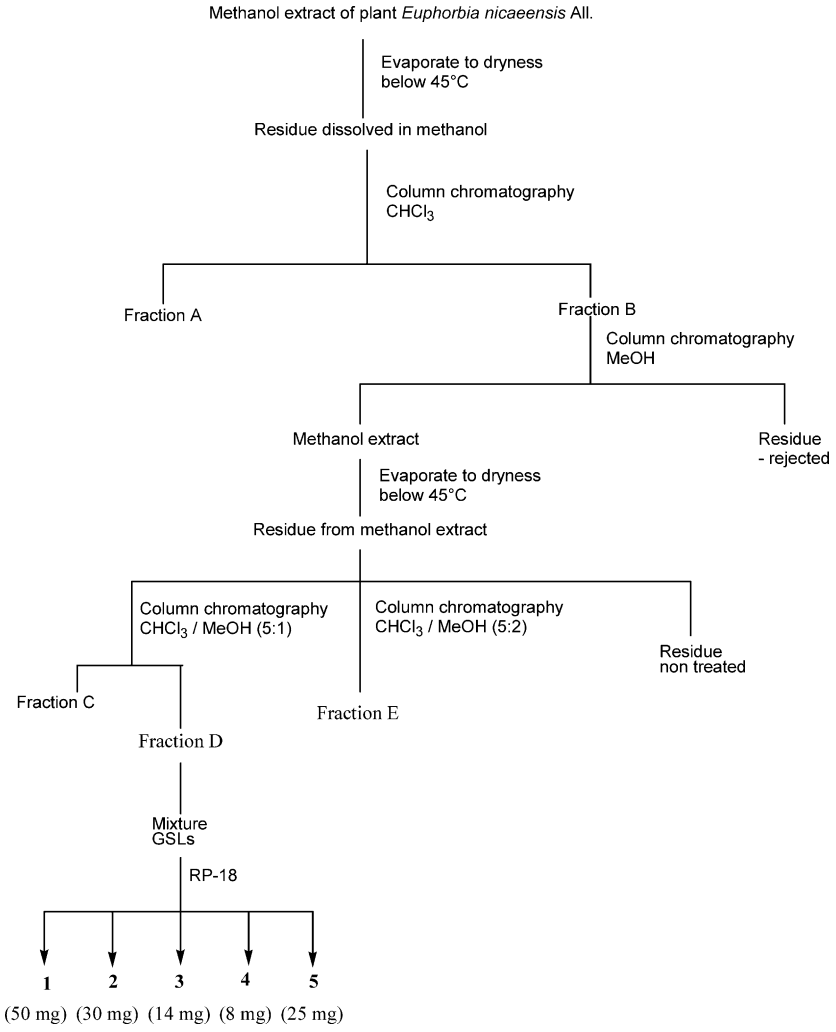


Fig. 1. Chemical structures of cerebrosides **1**–**5**.

meter and a Varian Gemini 200 MHz spectrometer. ^{13}C NMR: 100.4 MHz, Unity 400 spectrometer. NMR spectra were obtained by using C_5D_5N as solvent; chemical shifts are expressed as δ units (ppm) relative to tetramethylsilane (TMS) as internal standard. The abbreviations s, d, dd, t, q, m and br s refer to singlet, doublett, doublett of doublett, triplet, quartet, multiplet and broad singlet respectively. The PI-FD spectra (CH_2Cl_2) were obtained using double-focusing MAT 95 mass spectrometer. FAB MS: Kratos MS 80 RFA. FAB MS: (8 Kv, Xe, methanol as solvent and glycerol matrix + NaCl). Electrospray analysis: API Perkin Elmer (voltage +5600 with orifice 90 and/or 120). Silica gel column chromatography: Kieselgel 60 (230–400 Mesh, 60 Å Merck). IR spectra: Jasco IR-700 infrared spectrophotometer. Flash chromatography reversed-phase: LiChroprep RP-18 (40, 63 μm, Merck). All solvents were distilled before use. TLC: Kieselgel 60 F_{254} (20 × 20 cm; 0.2 mm, Merck). HPTLC: HPTLC-fertigplatten RP-18 F_{254} (10 × 10 cm, Merck).



Scheme 1. Isolation procedure of cerebrosides **1**–**5**.

2.1. Separation of the cerebrosides *1*–*5*

E. nicaeensis All. (1 kg) was harvested in July 2001 in Carso Triestino (Pesek); Italy. The voucher specimen of the plant material has been deposited at the Herbarium of the Department of Biology (TSB) of the University of Trieste (Italy). The stems and the leaves of the plant were cut and extracted with MeOH (3 l) for 7 days. The extract was filtered, methanol was concentrated in vacuo to give a MeOH extract (30.5 g), which was chromatographed on silica gel ($CHCl_3$, MeOH, $CHCl_3$:MeOH/5:1/v:v and $CHCl_3$:MeOH/5:2/v:v). The fraction eluted with $CHCl_3$:MeOH (5:1/v:v) was concentrated in vacuo and submitted to reversed phase column 'flash chromatography' using MeOH as eluent to afford cerebroside **1** (50 mg), **2** (30 mg), **3** (14 mg), **4** (8 mg) and **5** (25 mg) each showed a single spot on reversed phase TLC (MeOH). $R_f = 0.076$ (**1**), $R_f = 0.15$ (**2**), $R_f = 0.13$ (**3**), $R_f = 0.10$ (**4**) and $R_f = 0.25$ (**5**) (Scheme 1).

2.2. Cerebroside *1*

Amorphous powder. IR (KBr) cm^{-1}: 3415 (hydroxyl), 1647, 1537 (amide). Positive-ion FAB MS: $m/z =$ 842 $(M+H, 28\%)^+$, 824 $(M+H-H_2O, 8\%)^+$, 680 $(M+H-C_6H_{11}O_5, 31\%)^+$, 662 $(M+H-C_6H_{12}O_6, 18\%)^+$, 500 $(M+Na-C_{24}H_{45}O_2)^+$. Negative-ion FAB MS: $m/z = 840$ $(M-H, 62\%)^-$, 678 $(M-H-C_6H_{11}O_5, 14\%)^-$ [7]. ^1H and ^{13}C NMR data are reported in Table 1.

2.3. Cerebroside *2*

Amorphous powder. IR (KBr) cm^{-1}: 3413 (hydroxyl), 1646, 1540 (amide). Positive-ion FAB MS: $m/z =$ 892 $(M+Na, 63\%)^+$, 870 $(M+H, 84\%)^+$, 852 $(M+H-H_2O, 51\%)^+$, 730 $(M+Na-C_6H_{11}O_5, 17\%)^+$, 708 $(M+H-C_6H_{11}O_5, 55\%)^+$, 690 $(M+H-C_6H_{12}O_6, 20\%)^+$, 476 $(M+H-C_{26}H_{50}O_2, 16\%)^+$. Negative-ion FAB MS: $m/z = 868$ $(M-H, 100\%)^-$, 706 $(M-H-C_6H_{11}O_5,$

Table 1
$^{1}H-^{13}C$ NMR spectral data of cerebrosides **1**–**4** (δ value in pyridine-d_5)

Position	**1**		**2**		**3**		**4**	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
Ceramide								
NH	8.53 (d, $J = 7.9$ Hz)		8.55 (d, $J = 7.8$ Hz)		8.55 (d, $J = 7.9$ Hz)		8.55 (d, $J = 7.8$ Hz)	
1a	4.70 (dd)	70.5	4.71 (dd)	70.5	4.70 (dd)	70.6	4.70 (dd)	70.5
1b	4.52 (m)		4.51 (m)		4.52 (m)		4.52 (m)	
2	5.22 (m)	51.8	5.23 (m)	51.8	5.22 (m)	51.8	5.22 (m)	51.8
3	4.28 (dd)	76.0	4.27 (dd)	76.0	4.28 (dd)	76.0	4.28 (dd)	76.0
4	4.22 (obs)	72.6	4.23 (obs)	72.6	4.21 (obs)	72.5	4.23 (obs)	72.6
5a	1.87 (m)	34.0	1.89 (m)	34.1	1.87 (m)	34.0	1.87 (m)	33.7
5b	2.38 (m)		2.39 (m)		2.39 (m)		2.39 (m)	
6a	1.65 (m)	26.0	1.65 (m)	26.0	1.65 (m)	26.2	1.65 (m)	26.3
6b	1.72 (m)		1.73 (m)		1.73 (m)		1.73 (m)	
7	2.20 (m)	27.8	2.22 (m)	27.8	2.20 (m)	27.9	2.21 (m)	27.8
8	5.45 (m)	130.6	5.45 (m)	130.6	5.45 (m)	130.6	5.45 (m)	130.2
9	5.45 (m)	130.4	5.45 (m)	130.4	5.45 (m)	129.6	5.45 (m)	129.5
10	2.20 (m)	27.7	2.20 (m)	27.7	2.19 (m)	27.7	2.19 (m)	27.7
11–15	1.30 (m)	29.7–30.3	1.32 (m)	29.6–30.3	1.32 (m)	29.9–30.3	1.31 (m)	29.7–30.3
16	1.25 (m)	32.4	1.26 (m)	32.3	1.25 (m)	32.4	1.25 (m)	32.3
17	1.25 (m)	23.2	1.26 (m)	23.3	1.25 (m)	23.2	1.25 (m)	23.1
18	0.85 (m)	14.5	0.85 (m)	14.6	0.86 (m)	14.6	0.84 (m)	14.5
1′		175.8		175.6		175.9		175.8
2′	4.57 (dd)	72.5	4.58 (dd)	72.6	4.57 (dd)	72.5	4.57 (dd)	72.5
3′a	2.00 (m)	35.7	2.00 (m)	35.7	2.02 (m)	35.8	2.00 (m)	35.7
3′b	2.20 (m)		2.22 (m)		2.21 (m)		2.21 (m)	
4′a	1.70 (m)	26.7	1.71 (m)	26.8	1.71 (m)	26.4	1.71 (m)	26.7
4′b	1.94 (m)		1.95 (m)		1.96 (m)		1.95 (m)	
CH_2 aliph.	1.25–1.30 (m)	23.7–32.3	1.26–1.30 (m)	23.1–32.3	1.32 (m)	23.1–32.0	1.30 (m)	23.2–32.3
CH_2CH=	2.20 (m)	27.8	2.21 (m)	27.9				
CH=CH	5.45 (m)	130.2	5.45 (m)	131.3				
CH_3	0.85 (m)	14.5	0.85 (m)	14.4	0.86 (t)	14.6	0.84 (m)	14.5
OH-2′	7.62 (br.s)		7.62 (br. s)		7.60 (br. s)		7.61 (br. s)	
OH-3	6.82 (br. s)		6.81 (br. s)		6.82 (br. s)		6.83 (br. s)	
OH-4	6.00 (br. s)		6.00 (br. s)		6.00 (br. s)		6.00 (br. s)	
Glucose								
1″	4.95 (d, $J = 7.6$ Hz)	105.6	4.95 (d, $J = 7.6$ Hz)	105.6	4.95 (d, $J = 7.7$ Hz)	105.7	4.95 (d, $J = 7.6$ Hz)	105.6
2″	4.00 (t)	75.3	4.03 (t)	75.3	4.05 (t)	75.4	4.06 (t)	75.3
3″	4.21 (obs)	78.5	4.21 (obs)	78.6	4.21 (obs)	78.6	4.21 (obs)	78.5
4″	4.19 (obs)	71.6	4.19 (obs)	71.6	4.20 (obs)	71.7	4.20 (obs)	71.6
5″	3.87 (m)	78.7	3.88 (m)	78.6	3.88 (m)	78.8	3.90 (m)	78.7
6″a	4.48 (dd, $J = 11.8$, 5.4 Hz)	62.7	4.48 (dd, $J = 11.9$, 5.3 Hz)	62.7	4.48 (dd, $J = 11.8$, 5.4 Hz)	62.8	4.48 (dd, $J = 11.9$, 5.3 Hz)	62.7
6″b	4.36 (dd, $J = 11.8$, 2.0 Hz)		4.36 (dd, $J = 11.9$, 2.0 Hz)		4.38 (dd, $J = 11.8$, 2.1 Hz)		4.40 (dd, $J = 11.9$, 2.0 Hz)	
OH-6″	6.30 (br. s)		6.30 (br.s)		6.35 (br. s)		6.40 (br. s)	

31%)$^-$ [7]. ^{1}H and ^{13}C NMR data are reported in Table 1.

2.4. Cerebroside **3**

Amorphous powder. IR (KBr) cm^{-1}: 3413 (hydroxyl), 1646, 1540 (amide). Positive-ion FAB MS: $m/z =$ 816 ($M+H$, 83%)$^+$, 798 ($M+H-H_2O$, 35%)$^+$, 654 ($M+H-C_6H_{11}O_5$, 95%)$^+$, 636 ($M+H-C_6H_{12}O_6$, 40%)$^+$, 476 ($M+H-340$, 10%)$^+$, 500 ($M+Na-C_{22}H_{43}O_2$, 100%)$^+$. Negative-ion FAB MS: $m/z = 814$ ($M-H$, 100%)$^-$, 652 ($M-H-C_6H_{11}O_5$, 23%)$^-$ [7]. ESI MS: $m/z = 838$ ($M+Na$, 50%)$^+$, 658 ($M+Na-C_6H_{12}O_6$, 40%)$^+$ ^{1}H and ^{13}C NMR data are reported in Table 1.

2.5. Cerebroside **4**

Amorphous powder. IR (KBr) cm^{-1}: 3415 (hydroxyl), 1646, 1542 (amide). Positive-ion FAB MS: $m/z =$ 866 ($M+Na$, 90%)$^+$, 844 ($M+H$, 21%)$^+$, 826 ($M+H-H_2O$, 29%)$^+$, 682 ($M+H-C_6H_{11}O_5$, 78%)$^+$, 664 ($M+$

H–$C_6H_{12}O_6$, 53%)$^+$, 500 (M+Na–$C_{24}H_{47}O_2$, 10%)$^+$. Negative-ion FAB MS: m/z = 842 (M–H, 96%)$^-$, 680 (M–H–$C_6H_{11}O_5$, 33%)$^-$, 663 (M–H–$C_6H_{12}O_6$, 19%)$^-$ [7]. ^{1}H and ^{13}C NMR data are reported in Table 1.

2.6. Cerebroside 5

Amorphous powder. IR (KBr) cm^{-1}: 3415 (hydroxyl), 1645, 1543 (amide). Positive-ion FAB MS: m/z = 736 (M+Na, 46%)$^+$, 714 (M+H, 7%)$^+$, 696 (M+H–H_2O, 52%)$^+$, 534 (M+H–$C_6H_{12}O_6$, 100%)$^+$, 516 (M+H–$C_6H_{12}O_6$–H_2O, 28%)$^+$, 482 (M+Na–$C_{16}H_{31}O_2$, 15%)$^+$, 336 (M+Na–$C_6H_{11}O_5$–$C_{16}H_{29}O$)$^+$, 319 (M+Na–$C_6H_{12}O_6$–$C_{16}H_{29}O$)$^+$. Negative-ion FAB MS: m/z = 712 (M–H, 30%)$^-$, 533 (M–H–$C_6H_{12}O_6$, 25%)$^-$ [7]. ^{1}H and ^{13}C NMR data are reported in Table 2.

2.7. Methanolysis of cerebroside 1

Compound **1** (1.0 mg) was heated with 0.9 N HCl in 82% MeOH (1 ml) at 70 °C for 12 h in a sealed small-volume vial. The reaction mixture was extracted with *n*-hexane and the hexane layer was concentrated to give the FAM. The MeOH layer was concentrated in vacuo to give a mixture of LCB, glycosphingoside and methyl glycoside (Fig. 2).

The compounds **2**–**5** were methanolyzed using the same method described above (Fig. 2).

Table 2
^{1}H–^{13}C NMR spectral data of cerebroside **5** (δ value in pyridine-d_5)

Position	5	
	^{1}H	^{13}C
Ceramide		
NH	8.41 (d, J = 8.9 Hz)	
1a	4.76 (dd)	70.3
1b	4.55 (m)	
2	4.80 (m)	54.7
3	4.80 (m)	72.4
4	6.00 (dd)	132.1
5	5.92 (dt)	132.2
6	2.07 (m)	33.1
7	2.09 (m)	32.9
8	5.45 (t)	130.0
9	5.45 (t)	131.2
10	1.80 (m)	33.1
CH_2 aliph.	1.30–1.38 (m)	23.1–32.3
18	0.95 (t)	14.5
1′		175.8
2′	4.55 (dd)	72.6
3′	1.90 (m)	35.8
4′	1.70 (m)	26.1
CH_3	0.95 (t)	14.5
OH-2′	7.71 (br. s)	
OH-3	6.98 (br. s)	
Glucose		
1″	4.95 (d, J = 7.7 Hz)	105.7
2″	4.07 (t)	75.2
3″	4.21 (obs)	78.5
4″	4.20 (obs)	71.6
5″	3.90 (m)	78.7
6″a	4.48 (dd, J = 11.8, 5.4 Hz)	62.7
6″b	4.40 (dd, J = 11.8, 2.1 Hz)	
OH-6″	6.57 (m)	

2.8. MS analysis of FAMs from cerebrosides 1–5

The FAMs of cerebrosides **1**–**5** were subjected to MS analysis. The results were as follows: FAM-1 (*M*ethyl 2-hydroxy-tetracosenoate), EI MS: m/z = 396 (M)$^+$, 337 (M−59)$^+$. FAM-2 (methyl 2-hydroxy-hexacosenoate), EI MS: m/z = 424 (M)$^+$, 365 (M−59)$^+$. FAM-3 (methyl 2-hydroxy-docosanoate), EI MS: m/z = 370 (M)$^+$, 311 (M−59)$^+$. FAM-4 (methyl 2-hydroxy-tetracosanoate), EI MS: m/z = 398 (M)$^+$, 339 (M−59)$^+$. FAM-5 (*M*ethyl 2-hydroxy-hexadecanoate), EI MS: m/z = 286 (M)$^+$, 227 (M−59)$^+$.

2.9. DMDS derivatives of FAMs from cerebrosides 1–2

FAM-1 (1.0 mg) was dissolved in carbon disulfide (DMDS, 0.2 ml) and iodine (1 mg) was added to the solution. The resulting mixture was stored at 60 °C for 40 h in a small-volume sealed vial. The reaction was subsequently quenched with aqueous $Na_2S_2O_3$ (5%) and the mixture was extracted with *n*-hexane (3 ml). The extract was concentrated to give the FAM-1 DMDS derivative. The compound **2** was subjected to the procedure described above. The FAMs DMDS derivatives of cerebrosides **1**–**2** were subjected to MS analysis. The results were as follows: FAM-1 DMDS derivative, EI MS: m/z = 317, 173. FAM-2 DMDS derivative, EI MS: m/z = 344, 173 (Fig. 3).

2.10. MS analysis of LCBs from cerebrosides 1–5

The LCBs of cerebrosides **1**–**5** were subjected to MS analysis. LCB-1 (2-amino-1, 3, 4-trihydroxy-octadeca-8-ene), ESI MS: m/z = 339 (M+Na)$^+$. LCB-2 (2-amino-1, 3, 4-trihydroxy-octadeca-8-ene), ESI MS: m/z = 339 (M+Na)$^+$. LCB-3 (2-amino-1, 3, 4-trihydroxy-octadeca-8-ene), ESI MS: m/z = 339 (M+Na)$^+$. LCB-4 (2-amino-1, 3, 4-trihydroxy-octadeca-8-ene), ESI MS: m/z = 339 (M+Na)$^+$. LCB-5 (2-amino-1, 3-dihydroxy-octadeca-4, 8-diene), ES MS: m/z = 320 (M+Na)$^+$.

2.11. DMDS derivatives of LCBs from cerebrosides 1–5

LCBs DMDS derivatives from cerebrosides **1**–**5** were synthesized according to the procedure described above. The LCBs DMDS derivatives of cerebrosides **1**–**5** were subjected to MS analysis. The results were as follows: LCB-1 DMDS derivative, EI MS: m/z = 221, 187. LCB-2 DMDS derivative, EI MS: m/z = 221, 187. LCB-3

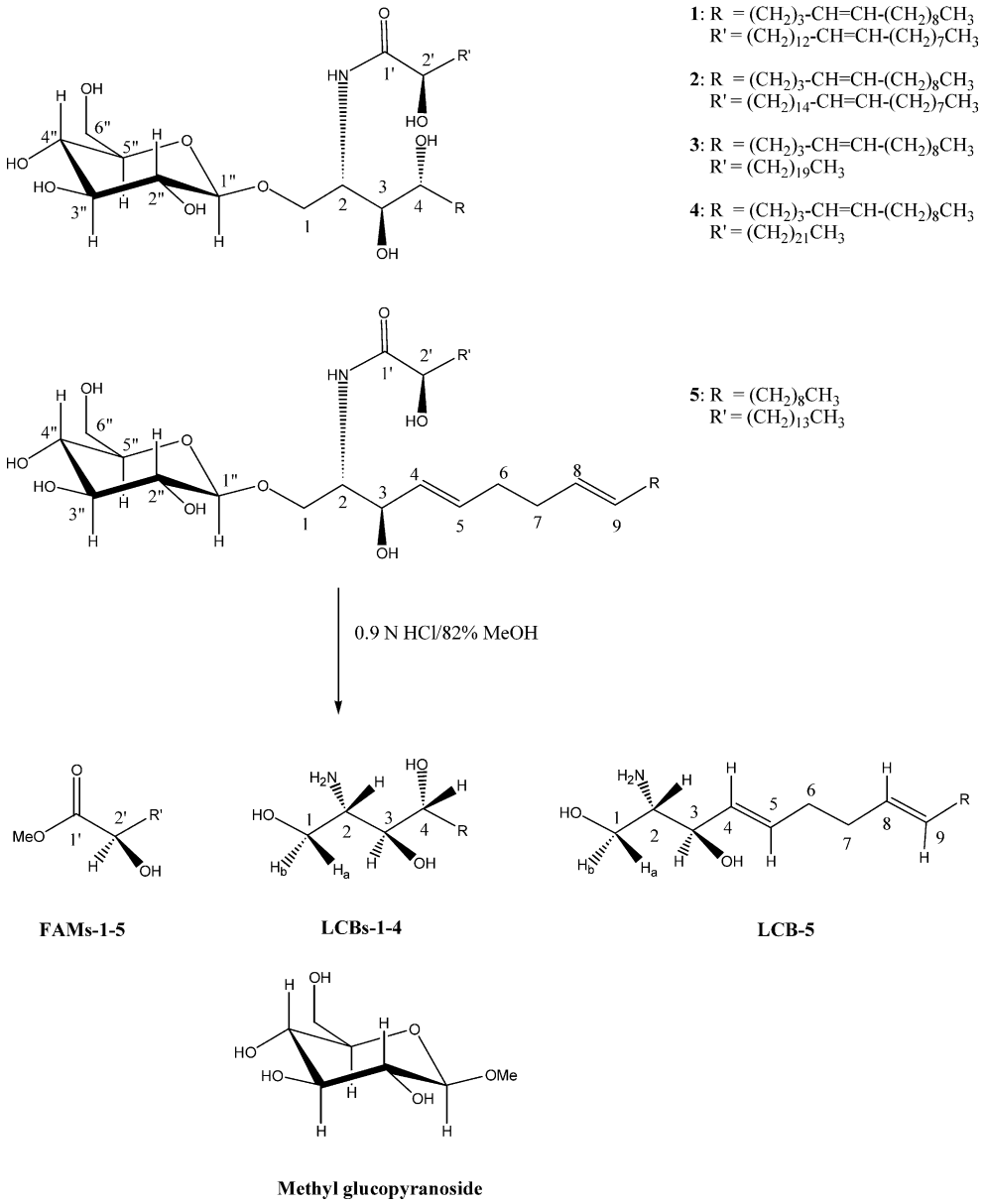


Fig. 2. Methanolysis of cerebrosides **1**–**5**.

DMDS derivative, EI MS: $m/z = 221$, 187. LCB-4 DMDS derivative, EI MS: $m/z = 221$, 187. LCB-5 DMDS derivative, EI MS: $m/z = 149$, 187 (Fig. 3).

2.12. Tumor line for in vitro test

An established tumor human-derived KB cell line (ECACC no. 86103004), was cultured according to standard procedure [8]. Vials of the original line were maintained in liquid N_2; cells were obtained, routinely subcultured once a week, and used for the experiments reported in the present work. The cell line was maintained in Eagle's minimum essential medium [9] supplemented with 10% newborn calf serum (GIBCO BRL), with 10 ml/l penicillin and streptomycin solution (Sigma Chemical Co., St. Louis, MO) (100 U/ml penicillin G and 100 μg/ml streptomycin) and buffered with 3 mM tris [hydroxymethyl] methyl-2-aminoethane-sulfonic acid, 3 mM N, *N*-bis [2-hydroxyethyl]-2-aminoethane-sulfonic acid, 3 mM *N*-2-hydroxyethyl piperazine-*N*′-2-ethane-sulfonic acid, and 3 mM tricine (Sigma Chemical Co.). The cell population doubling time was ca. 24 h. Cells from confluent monolayers were removed with 2–3 ml of 0.05% trypsin solution (Sigma Chemical Co.).

2.13. Cytostaticity determination assay

For the valuation of cytostatic activity, KB cells were sown at a density of 3×10^4 cells/ml, in 0.2 ml per well in a 96-well plate (Corning Costar Milano, Italy). After 24

h, extract was dissolved in sterile DMSO and solution diluted in culture medium up to obtained opportune concentration (1.25, 2.50, 5.00, 10.00 and 20.00 μg/ml); nutritive medium of every well was substituted with 0.2 ml of extract solution. After 72 h incubation at 37 °C, cellular vitality was valued with a colorimetric assay based on the quantification with sulforhodamine B (SRB-Sigma Chemical Co.) of cellular proteic component [10]. Briefly, adherent cell cultures were fixed in situ by addition of 50 μl of cold 50% (v:v) trichloroacetic acid (TCA) and were kept for 60 min at 4 °C. The supernatant was then discarded and the plates were washed two times with bi-distillated water and air-dried. SRB solution (0.4% w/v in 1% acetic acid) was added and the cells were allowed to stain for 30 min at room temperature. Unbound SRB was removed by washing three times with 1% acetic acid. Then the plates were air-dried. Bound stain was dissolved with unbuffered 10 mM Tris base (tris-hydroxymethyl-aminomethane) (Sigma Chemical Co.) and the optical density was read at 570 nm with an automated microplate reader EL311s spectrophotometer (BIO-TEK Instruments, INC. Winooski, Vermount, USA). Each experiment was performed in quintuplicate and repeated twice. Cytostatic activity was valued as percentage of cellular growth inhibition in culture tracked with extract to respect to the growth observed in control culture.

3. Results and discussion

The MeOH extract of *E. nicaeensis* L. was chromatographed on silica gel and then submitted to reversed phase column 'flash chromatography' to give the cerebrosides **1**–**5** (Scheme 1).

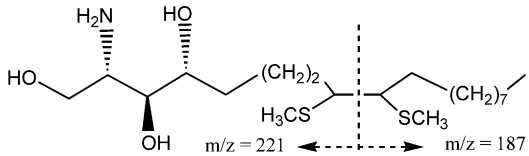
Cerebrosides **1**–**5** showed strong hydroxy (3413 cm^{-1}) and amide (1646, 1540 cm^{-1}) absorptions in the IR spectrum. The positive FAB mass spectra of cerebrosides **1**–**5** exhibited $(M+H)^+$ ion peaks at *m/z* 842 (**1**), 870 (**2**), 816 (**3**), 844 (**4**) 714 (**5**), respectively.

The ^1H and ^{13}C NMR spectra of cerebrosides **1**–**5** exhibited the characteristic signals of a sphingosine-type cerebroside possessing 2-hydroxy fatty acid and β-glucopyranose moieties (Fig. 1, Tables 1 and 2).

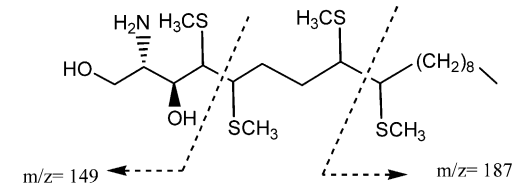
Constituents of the ceramide and sugar moieties of **1**–**5** were determined as follows.

The structures of the ceramide moieties were examined first. When cerebrosides **1**–**5** were methanolyzed with methanolic hydrochloric acid, fatty acid methyl esters (FAMs) were obtained together with long-chain bases (LCBs) and methyl glucopyranoside (Fig. 2).

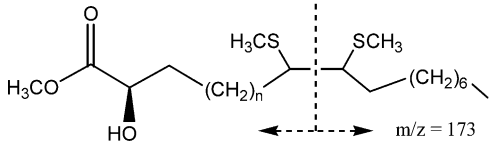
On the basis of mass spectrometry analysis, the FAMs were characterized as methyl 2-hydroxytetracosenoate (FAM-1), methyl 2-hydroxyhexacosenoate (FAM-2), methyl 2-hydroxy docosanoate (FAM-3), methyl 2-hydroxy tetracosaoate (FAM-4) and methyl 2-hydroxyhaxadecanoate (FAM-5) respectively.



LCB-DMDS-1-4



LCB-DMDS-5



FAM-DMDS-1: n= 11, m /z = 317 (M-173), m/z = 173 $(C_{10}H_{21}S)^+$
FAM-DMDS-2: n= 13, m /z = 344 (M-173), m/z = 173 $(C_{10}H_{21}S)^+$

Fig. 3. LCB-DMDS and FAM-DMDS derivatives of cerebroside **1**–**5**.

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-1-4) and 2-amino-1,3-dihydroxy-4,8-octadecadiene (LCB-5), respectively.

The ES MS mass spectra of the dimethyl disulfide (DMDS) derivatives of **1**–**5** showed remarkable fragments ion peaks at *m/z* 221 (**1**–**4**) and 149, 187 (**5**) respectively, due to cleavage of the bond between the carbons bearing a methylthio group (Fig. 3) [11]. These data indicate that the double-bonds in the LCB residues of **1**–**4** are located at C-8 (**1**–**4**) and at C-4 and C-8 (**5**), respectively. Furthermore, it is known [12] 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 ^1H–^1H correlation spectroscopy (COSY) spectrum of **1**–**5**.

When the heteronuclear multiple bond connectivity (HMBC) spectra of **1**–**5** were measured, significant correlations were observed between the signal of the olefin protons at $\delta = 5.45$ ppm and the methylene carbon atoms at $\delta = 27.7$, 27.8 and 32.9, 33.1 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 ($\delta = 27.8$, 27.7) for the compounds **1**–**4** and to C-6, C-7 and C-10 ($\delta = 33.1$, 32.9, 33.1) for the compound **5**. Thus, the olefin groups in the LCB of **1**–**4** were determined to have *cis* (*Z*) geometry (Table 1), while in the LCB of 5 *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 175.9, 131.3 and 130.2 expected for monounsaturated fatty acid methyl esters. The resonance at about 27.8, 27.9 ppm confirms the *Z* geometry of the double bonds in the long-chain fatty acids (Table 1). 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 [11]. The characteristic fragments at $m/z = 317$, 344 and 173, obtained from the cleavage between the sulphide carbons, indicates the position of the double bonds in **1**, **2** (Fig. 3).

1D and 2D ^1H 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 stereochemistry of the ceramide moiety was determined by comparison of the ^1H NMR data of the cerebrosides isolated from *E. nicaeensis* All. with that of synthetic analogs as reported in literature in terms of the signals due to 1-H to 4-H [13]. The absolute configuration of the glucopyranose moiety was determined to be the D-form using the Hara method [14].

Therefore the structures of **1**–**5** were proposed to be 1-*O*-(β-D-glucopyranosyl)-(2*S*, 3*S*, 4*R*, 8*Z*)-2-[(2′*R*)-2′-hydroxytetracosenoilamino]-8 (*Z*)-octadecene-1,3,4-triol (**1**), 1-*O*-(β-D-glucopyranosyl)-(2*S*, 3*S*, 4*R*, 8*Z*)-2-[(2′*R*)-2′-hydroxyhexacosenoilamino]-8 (*Z*)-octadecene-1,3,4-triol (**2**), 1-*O*-(β-D-glucopyranosyl)-(2*S*, 3*S*, 4*R*, 8*Z*)-2-[(2′*R*)-2′-hydroxydocosanoilamino]-8 (Z)-octadecene-1, 3, 4-triol (**3**), 1-*O*-(β-D-glucopyranosyl)-(2S, 3S, 4*R*, 8*Z*)-2-[(2′*R*)-2′-hydroxytetracosanoilamino]-8 (*Z*)-octadecene-1,3,4-triol (**4**) and 1-*O*-(β-D-glucopyranosyl)-(2*S*, 3*S*, 4*E*, 8*E*)-2-[(2′*R*)-2′-hydroxyhexadecanoylamino]-4(*E*),8(*E*)-octadecadiene-1,3 diol (**5**), respectively.

In conclusion, compounds **4** and **5**, have been found for the first time in Euphorbiaceae. Compounds **1**–**3** have been found to be identical to the cerebrosides isolated from *Euphorbia characias* L. [15].

3.1. Antiproliferative activity of the extract containing cerebroside molecular species

The cerebrosides **1**–**5** were isolated from the cerebroside molecular species obtained from the less polar fraction of the MeOH extract of *E. nicaeensis* All.

A preliminary biological assay has been carried out in order to value the antiproliferative activity of the extract containing the complex mixture of cerebrosides **1**–**5** in cell culture; KB cells were exposed at 1.25, 2.50, 5.00, 10.0 and 20.00 μg/ml of product for 72 h at 37 °C.

Results are reported in Table 3 and are expressed as growth inhibition percentage to respect to the control cultures.

It is possible to note that at the high concentration used (20.0 μg/ml) extract appear to be effective to blocking cell proliferation of 26.07 ± 1.91%. Significant cytostatic effect is observed at 2.5 μg/ml of extract ($P < 0.05$) but the entity of reduction in cell proliferation is small.

Table 3
Antiproliferative activity of the extract of *Euphorbia nicaeensis* All.

Concentration (μg/ml)	% Cell growth inhibition versus controls
1.25	0.16 ± 0.04
2.50	3.68 ± 0.13 *
5.00	4.63 ± 0.21 *
10.00	13.64 ± 1.26 **
20.00	26.07 ± 1.91 **

KB cells were seeded on 96 well plastic plate. After 24 h medium of each well was substituted with 200 μl of different extract concentrations and incubated at 37 °C for 72 h. Then, medium was discarded and cells treated as described in Section 2. Data are means ± ES of two experiments assayed in quintuplicate.

* $P < 0.05$.

** $P < 0.01$. Student unpaired *t*-test.

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