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Lipid metabolites with free-radical scavenging activity from *Euphorbia helioscopia* L.

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ABSTRACT

The methanolic extract of the plant *Euphorbia helioscopia* L. exhibited an interesting free-radical scavenging activity.

From the aerial parts of *Euphorbia helioscopia* L. (Euphorbiaceae), a complex mixture of seven cerebrosides together with glucoclionasterol, a digalactosyldiacylglycerol and a diacylmonogalactosylglycerol were identified. The structures of the cerebrosides were characterized as 1-*O*-β-D-glucosides of phytosphingosines, which comprised (2*S*, 3*S*, 4*E*, 8*E*)-2-amino-4(*E*),8(*E*)-octadecadiene-1,3-diol, (2*S*, 3*S*, 4*E*, 8*Z*)-2-amino-4(*E*),8(*Z*)-octadecadiene-1,3-diol, (2*S*, 3*S*, 4*R*, 8*Z*)-2-amino-8(*Z*)-octadecene-1,3,4-triol as long chain bases with seven 2-hydroxy fatty acids of varying chain lengths (C_{16}, $C_{24:1}$, $C_{26:1}$, C_{24}, C_{26}, $C_{28:1}$) linked to the amino group.

The glycosylglycerides were characterized as (2*S*)-2,3-*O*-di-(9,12,15-octadecatrienoyl)-glyceryl-6-*O*-(α-D-galactopyranosyl)-β-D-galactopyranoside and (2*S*)-2,3-*O*-di-(9,12,15-octadecatrienoyl)-glyceryl-1-*O*-β-D-galactopyranoside. The structures were established on the basis of spectroscopic data and chemical reactions.

1. Introduction

Cellular damage or oxidative injury arising from free radicals or reactive oxygene species (ROS) are involved in various human neurodegenerative disorders, diabetes, inflammation, viral infections, autoimmune pathologies and digestive system disorders.

Over the past two decades, evidences derived from epidemiological and laboratory studies have demonstrated that some edible plants as a whole, or their identified compounds with antioxidant properties have protective effects on human carcinogenesis (Dixit and Ali, 2010).

The genus *Euphorbia* is the largest genus of the plant family Euphorbiaceae, and has been reported to be a rich source of skin-irritating, cytotoxic and tumor-promoting diterpenoids (Singla and Kamala, 1990). *Euphorbia helioscopia* L. is a biennial herb of Euphorbiaceae, which grows wild in Europe, China and Japan. It has been used as a traditional folk medicine for the treatment of malaria, bacillary dysentery and osteomyelitis (Hua et al., 1999). Up to now, more than 30 diterpenoids have been isolated and structurally characterized from *E. helioscopia* (Lu et al., 2008). As a part of our ethnobotanical-directed search for novel bioactive agents, herein we report a phytochemical study of *Euphorbia helioscopia* L. methanolic leaves extract. In detail, bioassay-guided phytochemical study on *E. helioscopia* revealed seven known cerebrosides (**1**–**7**), glucoclionasterol (**8**), a digalactosyldiacylglycerol (**9**) and a monogalactosyldiacylglycerol (**10**), all were isolated from this species for the first time. The isolation and structure elucidation of different lipid metabolites (compounds **1**–**10**) by spectroscopic methods and chemical reactions was described.

Moreover, the free-radical scavenging activity of the crude extract as well as of each isolated fraction was assayed and compared to that one of a commercial standardized antioxidant extract of green the.

2. Materials and methods

2.1. Chemistry

2.1.1. General experimental procedures

NMR-spectroscopy: nuclear magnetic resonance spectra were recorded with a Varian Unity 400 spectrometer. ^{13}C NMR:

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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, doublet, doublet of doublet, 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). FT-IR spectra: Jasco IR-700 infrared spectrophotometer. Flash chromatography reversed-phase: Lichoprep RP-18 (40, 63 μm, Merck). All solvents were distilled before use. TLC: Kieselgel 60 F_{254} (20 cm × 20 cm; 0.2 mm, Merck). HPTLC: HPTLC-fertigplatten RP-18 F_{254} (10 cm × 10 cm, Merck).

The fatty acid composition of was released as methyl esters by the official A.O.A.C. methylation procedure, and analyzed by gas chromatography (GLC) (Helrich, 1990). A Shimadzu GC 14A (Kyoto, Japan) instrument, equipped with a split/splitless injector (1:20) and a flame ionization detector, was used. A SP 2330 fused silica capillary column, 30 m × 0.32 mm I.D., 0.20 μm film thickness (Supelco Inc., Bellefonte, PA) was employed. The chromatographic conditions were: column temperature was programmed from 150 °C (kept for 2 min) to 250 °C at 10 °C/min (maintained for 5 min), injector and detector temperature 280 °C, carrier gas (helium) and flow rate 2.0 mL/min.

2.1.2. Plant material

E. helioscopia (1 kg) was collected from wild stock growing in San Dorligo (Trieste), Italy in May 2010. A voucher specimen of the plant material has been deposited at the Herbarium of the Department of Biology (TSB-11202) of the University of Trieste (Italy).

2.1.3. Extraction and Isolation

The stems and the leaves of the plant were cut and extracted with MeOH (31) for 7 days. The extract was filtered, methanol was concentrated *in vacuo* to give a MeOH extract (24.7 g), which was chromatographed on silica gel (CH_2Cl_2:MeOH/10:1/v:v, CH_2Cl_2:MeOH/10:2/v:v and MeOH) to give five fractions. The fraction **5** eluted with CH_2Cl_2:MeOH (10:2/v:v) was concentrated *in vacuo* and submitted to reversed phase column 'flash chromatography' using MeOH as eluent to afford cerebroside **1** (25 mg), **2** (30 mg), **3** (17 mg), **4** (15 mg), **5** (28 mg), **6** (12 mg), **7** (22 mg) each showed a single spot on reversed phase TLC (MeOH). R_f = 0.25 (**1**), R_f = 0.27 (**2**), R_f = 0.076 (**3**), R_f = 0.15 (**4**), R_f = 0.10 (**5**), R_f = 0.29 (**6**), R_f = 0.14 (**7**). From the fraction **3** eluted with CH_2Cl_2:MeOH (10:1/v:v), the compound **8** (60 mg) was isolated and identified R_f = 0.48 (**8**). From the fraction **4** eluted with CH_2Cl_2:MeOH (10:1/v:v), the compounds **9** and **10** have been isolated with R_f = 0.15 and identified in mixture.

Cerebroside ***1***. 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%)$^-$ (Shibuya et al., 1990a,b). ^{1}H and ^{13}C NMR data are reported in Table 1 .

Cerebroside ***2***. 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%)$^-$ (Shibuya et al., 1990a,b). ^{1}H and ^{13}C NMR data are reported in Table 1.

Cerebroside ***3***. 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%)$^-$ (Shibuya et al., 1990a,b). ^{1}H and ^{13}C NMR data are reported in Table 1.

Cerebroside ***4***. 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–$C_6H_{11}O_5$, 31%)$^-$ (Shibuya et al., 1990a,b). ^{1}H and ^{13}C NMR data are reported in Table 1.

Cerebroside ***5***. 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%)$^-$ (Shibuya et al., 1990a,b). ^{1}H and ^{13}C NMR data are reported in Table 1.

Cerebroside ***6***. Amorphous powder. IR (KBr) cm^{-1}: 3417 (hydroxyl), 1645, 1540 (amide). Positive-ion FAB MS: m/z = 920 (M+Na, 87%)$^+$, 898 (M+H, 23%)$^+$, 880 (M+H–H_2O, 34%)$^+$, 735 (M+H–$C_6H_{11}O_5$, 65%)$^+$, 718 (M+H–$C_6H_{12}O_6$, 55%)$^+$. Negative ion FAB MS: m/z = 896 (M–H, 95%)$^-$, 733 (M–H–$C_6H_{11}O_5$, 39%)$^-$, 716 (M–H–$C_6H_{12}O_6$, 23%)$^-$ (Shibuya et al., 1990a,b). ^{1}H and ^{13}C NMR data are reported in Table 1.

Cerebroside ***7***. Amorphous powder. IR (KBr) cm^{-1}: 3413 (hydroxyl), 1647, 1545 (amide). Positive-ion FAB MS: m/z = 894 (M+Na, 95%)$^+$, 872 (M+H, 34%)$^+$, 854 (M+H–H_2O, 42%)$^+$, 709 (M+H–$C_6H_{11}O_5$, 60%)$^+$, 692 (M+H–$C_6H_{12}O_6$, 53%)$^+$, 499 (M+Na–$C_{26}H_{51}O_2$, 15%)$^+$. Negative ion FAB MS: m/z = 870 (M–H, 88%)$^-$, 707 (M–H–$C_6H_{11}O_5$, 31%)$^-$, 690 (M–H–$C_6H_{12}O_6$, 24%)$^-$ (Shibuya et al., 1990a,b). ^{1}H and ^{13}C NMR data are reported in Table 1.

2.1.4. Methanolysis of *1–7*

Compounds **1** (30 mg) was refluxed with 0.9 M HCl in 82% aq. MeOH (10 mL) for 18 h. The mixture was extracted with *n*-hexane and the combined organic phase was washed with water and dried over Na_2SO_4. Removal of the solvent gave a colorless wax which was (19.0 mg) which was chromatographed on silica gel [hexane/EtOAc (5:1)] to yield fatty acid methyl ester as a colorless wax (11.5 mg).

The compounds **2–7** were methanolyzed using the same method described above. The esters were analyzed by GC–MS. The results were as follows: FAMs-1,2 (methyl 2-hydroxypalmitate), EI-MS: m/z = 286 [M]$^+$, 254 [M–CH_3OH]$^+$, 227 [M–CH_3COO]$^+$, FAM-3 (methyl 2-hydroxytetracosenoate), EI-MS: m/z = 396 [M]$^+$, 364 [M–CH_3OH]$^+$, 337 [M–CH_3COO]$^+$, FAM-4 (methyl 2-hydroxyhexacosenoate), EI-MS: m/z = 424 [M]$^+$, 392 [M–CH_3OH]$^+$, 365 [M–CH_3COO]$^+$, FAM-5 (methyl 2-hydroxytetracosanoate), EI-MS: m/z = 398 [M]$^+$, 366 [M–CH_3OH]$^+$, 339 [M–CH_3COO]$^+$, FAM-6 (methyl 2-hydroxyoctacosenoate), EI-MS: m/z = 452 [M]$^+$, 420 [M–CH_3OH]$^+$, 393 [M–CH_3COO]$^+$, and FAM-7 (methyl 2-hydroxyhexacosanoate), EI-MS: m/z = 426 [M]$^+$, 394 [M–CH_3OH]$^+$, 367 [M–CH_3COO]$^+$.

The aq. MeOH layer was neutralized with NH_4OH and extracted with EtOAc. The combined EtOAc extract was washed with H_2O, dried over Na_2SO_4 and evaporated to give the long-chain base (LCB) as a slightly yellow wax (14.5 mg). The aq. MeOH layer was then evaporated to dryness and chromatographed on silica gel

Table 1
^{1}H and ^{13}C NMR data for compounds **1**–**7**.

C, H	**1**		**2**		**3**		**4**	
	^{1}H NMR δ (m, J in Hz)	^{13}C NMR δ	^{1}H NMR δ (m, J in Hz)	^{13}C NMR δ	^{1}H NMR δ (m, J in Hz)	^{13}C NMR δ	^{1}H NMR δ (m, J in Hz)	^{13}C NMR δ
NH	8.42 (d, 8)	–	8.41 (d, 8)	–	8.53 (d, 8)	–	8.55 (d, 8)	–
1a	4.76 (dd, 12, 6)	70.23	4.76 (dd, 12, 6)	70.21	4.70 (dd, 12, 7)	70.61	4.73 (dd, 11, 5.5)	70.53
1b	4.59 (m)		4.55 (m)		4.52 (m)		4.50 (m)	
2	4.80 (m)	54.60	4.80 (m)	54.63	5.22 (m)	51.49	5.22 (m)	51.19
3	4.80 (m)	72.43	4.80 (m)	72.32	4.28 (dd, 4.5, 3)	76.13	4.26 (dd, 4, 3.5)	76.25
4	6.00 (dd, 15, 6)	132.10	6.00 (dd, 15, 6)	132.06	4.22 (obs)	72.63	4.23 (obs)	72.60
5	5.92 (dt, 15, 6)	132.34	5.92 (dt, 15, 6)	132.19				
5a					2.40 (m)	34.15	2.41 (m)	34.10
5b					1.88 (m)		1.91 (m)	
6	2.08 (m)	33.17	2.07 (m)	32.93				
6a					1.70 (m)	26.17	1.75 (m)	26.05
6b					1.65 (m)		1.65 (m)	
7	2.10 (m)	32.83	2.09 (m)	27.63	2.20 (m)	27.81	2.21 (m)	27.81
8	5.45 (t, 4.9)	130.06	5.39 (t, 4.9)	130.64	5.40 (dt, 10.8, 7.1)	130.67	5.45 (m)	130.47
9	5.45 (t, 4.9)	131.28	5.39 (t, 4.9)	129.42	5.45 (dt, 10.8, 6.8)	130.25	5.45 (m)	130.22
10	1.80 (m)	33.20	1.90 (m)	27.36	2.20 (m)	27.75	2.21 (m)	27.70
11	1.40 (m)	29.65–30.40	1.40 (m)	29.65–30.40	1.40 (m)	29.65–30.40		
11–15	–	–	1.30 (m)	29.61–30.05	1.30 (br s)	29.43–30.42	1.32 (br s)	29.31–30.75
12–15	1.30 (m)	29.65–30.40	–	–	–	–	–	–
16	1.25 (br s)	32.40	1.26 (br s)	32.15	1.25 (br s)	32.45	1.25 (br s)	32.35
17	1.25 (br s)	23.16	1.26 (br s)	22.97	1.25 (br s)	23.47	1.25 (br s)	23.42
18	0.90 (t, 6)	14.53	0.95 (t, 6)	14.31	0.85 (t, 7)	14.64	0.90 (t, 7.1)	14.21
1′	–	175.85	–	175.69	–	175.78	–	175.29
2′	4.55 (dd, 8, 4.2)	72.58	4.55 (dd, 8, 4.2)	72.50	4.57 (dd, 8, 3.7)	72.57	4.60 (dd, 8, 4.3)	72.43
3a′	2.12 (m)	35.88	2.10 (m)	35.68	2.20 (m)	35.69	2.22 (m)	35.92
3b′	1.94 (m)		1.90 (m)		2.00 (m)		2.00 (m)	
4a′	1.95 (m)	26.10	1.96 (m)	25.95	1.94 (m)	26.75	1.94 (m)	26.48
4b′	1.70 (m)		1.70 (m)		1.70 (m)		1.73 (m)	
5′–13′	1.30 (m)	29.65–30.40	1.30 (m)	29.61–30.0	1.30 (m)	29.43–30.42		
5′–15′							1.32 (m)	29.31–30.75
16′–19′							2.21 (m)	27.93
14′	1.25 (m)	32.40	1.26 (m)	32.15	1.26 (m)	32.15		
14′, 17′					2.20 (m)	27.81		
15′	1.25 (m)	23.16	1.26 (m)	22.97				
15′, 16′					5.45 (m)	130.14		
16′	0.90 (t, 6)	14.53	0.95 (t, 6)	14.31				
17′, 18′							5.45 (m)	131.34
20′, 23′							1.32 (m)	29.31–30.75
18′, 21′					1.30 (m)	29.43–30.42		
22′					1.25 (m)	32.45		
23′					1.25 (m)	23.47		
24′					0.85 (t, 7)	14.64	1.25 (m)	32.41
25′							1.25 (m)	23.22
26′							0.90 (t, 7.1)	14.17
OH-2′	7.72 (br s)	–	7.71 (br s)	–	7.60 (br s)	–	7.62 (br s)	–
OH-3	6.98 (br d)	–	6.98 (br d)	–	6.82 (br s)	–	6.81 (br s)	–
OH-4					6.00 (br s)	–	6.00 (br s)	–
OH-6″	6.57 (m)	–	6.57 (m)	–	6.40 (br s)	–	6.30 (br s)	–
1″	4.95 (d, 8)	105.73 (β)	4.95 (d, 8)	105.69 (β)	4.95 (d, 8)	105.31 (β)	4.95 (d, 7.9)	105.55 (β)
2″	4.07 (t, 8)	75.26	4.07 (t, 8)	75.14	4.00 (t, 8)	75.18	4.02 (t, 8)	75.23
3″	4.21 (obs)	78.57	4.21 (obs)	78.47	4.21 (obs)	78.34	4.22 (obs)	78.26
4″	4.20 (obs)	71.64	4.20 (obs)	71.50	4.19 (obs)	71.25	4.19 (obs)	71.72
5″	3.90 (m)	78.70	3.90 (m)	78.62	3.87 (m)	78.63	3.88 (m)	78.31
6a″	4.52 (dd, 12, 2)	62.45	4.52 (dd, 12, 2)	62.64	4.48 (dd, 12, 2)	62.21	4.48 (dd, 11, 2)	62.54
6b″	4.36 (dd, 12, 5)		4.36 (dd, 12, 5)		4.34 (dd, 12, 5)		4.36 (dd, 11, 4)	

C, H	**5**		**6**		**7**	
	^{1}H NMR δ (m, J in Hz)	^{13}C NMR δ	^{1}H NMR δ (m, J in Hz)	^{13}C NMR δ	^{1}H NMR δ (m, J in Hz)	^{13}C NMR δ
NH	8.54 (d, 9)	–	8.53 (d, 8)	–	8.54 (d, 9)	–
1a	4.69 (dd, 13.3, 5)	70.57	4.70 (dd, 12, 7)	70.61	4.69 (dd, 13.3, 5)	70.57
1b	4.56 (m)		4.52 (m)		4.56 (m)	
2	5.20 (m)	51.90	5.22 (m)	51.49	5.20 (m)	51.90
3	4.29 (dd, 3, 4.7)	76.09	4.28 (dd, 4.5, 3)	76.13	4.29 (dd, 3, 4.7)	76.09
4	4.27 (obs)	72.52	4.22 (obs)	72.63	4.27 (obs)	72.52
5a	2.41 (m)	33.91	2.40 (m)	34.15	2.41 (m)	33.91
5b	1.91 (m)		1.88 (m)		1.91 (m)	
6a	1.75 (m)	26.27	1.70 (m)	26.17	1.75 (m)	26.27
6b	1.63 (m)		1.65 (m)		1.63 (m)	
7	2.23 (m)	27.65	2.20 (m)	27.81	2.23 (m)	27.65
8	5.44 (m)	130.23	5.45 (m)	130.67	5.44 (m)	130.23
9	5.44 (m)	129.51	5.45 (m)	130.25	5.44 (m)	129.51
10	2.23 (m)	27.80	2.20 (m)	27.75	2.23 (m)	27.80

Table 1 (*Continued*)

C, H	**5**		**6**		**7**	
	^{1}H NMR δ (m, J in Hz)	^{13}C NMR δ	^{1}H NMR δ (m, J in Hz)	^{13}C NMR δ	^{1}H NMR δ (m, J in Hz)	^{13}C NMR δ
11–15	1.31 (br s)	29.68–30.42	1.31 (br s)	29.43–30.42	1.31 (br s)	29.68–30.42
16	1.24 (br s)	32.26	1.25 (br s)	32.45	1.24 (br s)	32.26
17	1.24 (m)	23.13	1.25 (br s)	23.47	1.24 (br s)	23.13
18	0.84 (t, 7)	14.68	0.85 (t, 7)	14.64	0.84 (t, 7)	14.68
1′	–	175.65	–	175.78	–	175.65
2′	4.55 (dd, 8, 5.2)	72.52	4.57 (dd, 8, 3.7)	72.57	4.55 (dd, 8, 5.2)	72.52
3a′	2.19 (m)	35.82	2.20 (m)	35.69	2.19 (m)	35.82
3b′	1.98 (m)		2.00 (m)		1.98 (m)	
4a′	1.93 (m)	26.73	1.94 (m)	26.75	1.93 (m)	26.73
4b′	1.69 (m)		1.70 (m)		1.69 (m)	
5′–16′			1.30 (m)	29.43–30.42		
5′–21′	1.31 (m)	29.68–30.42				
5′–23′					1.31 (m)	29.68–30.42
17′, 21′			2.20 (m)	27.81		
15′, 16′			5.45 (m)	130.14		
19′, 20′			1.30 (m)	29.43–30.42		
22′	1.24 (m)	32.26	1.25 (m)	32.45		
23′	1.24 (m)	23.19				
23′–27′			1.25 (m)	23.47		
24′	0.84 (t, 7)	14.45			1.24 (m)	32.26
25′					1.24 (m)	23.19
26′					0.84 (t, 7)	14.45
28′			0.85 (t, 7)	14.64		
OH-2′	7.61 (br s)	–	7.60 (br s)	–	7.61 (br s)	–
OH-3	6.83 (br s)	–	6.82 (br s)	–	6.83 (br s)	–
OH-4	6.00 (br s)	–	6.00 (br s)	–	6.00 (br s)	–
OH-6″	6.40 (br s)	–	6.40 (br s)	–	6.40 (br s)	–
1″	4.90 (d, 8)	105.72 (β)	4.95 (d, 8)	105.31 (β)	4.90 (d, 8)	105.72 (β)
2″	4.06 (t, 8)	75.38	4.00 (t, 8)	75.18	4.06 (t, 8)	75.38
3″	4.23 (obs)	78.46	4.21 (obs)	78.34	4.23 (obs)	78.46
4″	4.18 (obs)	71.61	4.19 (obs)	71.25	4.18 (obs)	71.61
5″	3.85 (m)	78.80	3.87 (m)	78.63	3.85 (m)	78.80
6a″	4.52 (dd, 11.7, 2)	62.51	4.48 (dd, 12, 2)	62.21	4.52 (dd, 11.7, 2)	62.51
6b″	4.40 (dd, 10.6, 5)		4.34 (dd, 12, 5)		4.40 (dd, 10.6, 5)	

[CH_2Cl_2/MeOH/H_2O (lower layer) (20:3:1, 10:3:1, 7:3:1)] to give methyl glucopyranoside (β-anomer) as a colorless solid (5. 1 mg). TLC [silica gel, CH_2Cl_2/MeOH/H_2O (lower layer) (10:3:1)] of the resulting methyl glucopyranoside (α- and β-anomer) was identical to that of the standard methyl α-D-glucopyranoside and methyl β-D-glucopyranoside.

2.1.5. GC–MS analysis of TMS ethers of methyl glycosides from *1*–*7*

The mixture of methyl glycosides obtained by column chromatography of the aq. MeOH layer derived from methanolysis of **1**–**7** was converted to their trimethyl derivatives using BSTFA containing 1% TMCS for 3 h at 70 °C. 0.1 μl of silylated mixture was analyzed by gas chromatograph mass spectrometer (GC–MS) system. The chromatographic conditions were: column temperature was programmed from 100 °C to 260 °C at 8 °C/min, with 15 min of final isotherm, injector temperature 280 °C, carrier gas (helium), flow rate 1.5 mL/min. Transfer line temperature was kept at 270 °C. The mass spectrometer scanned from m/z 100 to m/z 600 at 1.0 s cycle time. The ion source was set at 180 °C and spectra were obtained by electron impact (70 eV). Methyl glycosides (GC–MS): methyl α- and β-glucopyranosides were detected. Methyl α-D-glucopyranoside: $C_7H_{14}O_6$, m/z = 194 $[M]^+$, TMS derivative, m/z = 482 $[M]^+$, 204, 191, 217, t_R (min): 12.770. Methyl β-D-glucopyranoside: $C_7H_{14}O_6$, m/z = 194 $[M]^+$, TMS derivative, m/z = 482 $[M]^+$, 204, 191, 217, t_R (min): 14.008.

2.1.6. Dimethyl disulphide derivatives of FAMs 3, 4, 6 and LCBs from cerebrosides *1*–*7*

LCB-1 (10 mg) was dissolved in carbon disulphide (1 mL) and dimethyl disulphide (1 mL) and iodine (20 mg) were added. The reaction mixture was then kept at −60 °C for 48 h in a small sealed vial. The reaction was subsequently quenched with 5% aq. $Na_2S_2O_3$ and the mixture was extracted with *n*-hexane. The *n*-hexane layer was dried over Na_2SO_4, filtered and concentrated to give the dimethyl disulphide derivative of LCB-1 as a light yellow oil (9.4 mg). The LCBs DMDS derivatives of cerebrosides **1**–**7** were subjected to MS analysis. The FAMs 3, 4 and 6 were derivatized using the same method described above. The results are reported in Fig. 3.

3. Pharmacology

3.1. Free radical scavenging activity

The free radical scavenging activity (FRS) of MeOH extract as well as each isolated fraction was determined by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Gaggeri et al., 2012; Ancerewicz et al., 1998). A commercially available standardized green tea extract (Green Select®) was used as standard. Briefly, both samples and standard were dissolved in ethanol containing up to 2% DMSO at concentration of 6 mg/mL. Reaction mixture was prepared by adding 100 μl of extract solution (or standard solution) to 3.9 mL of DPPH solution, freshly prepared dissolving DPPH in methanol/KH_2PO_4 and NaOH buffer (50/50, v/v) at a concentration of 6×10^{-5} M, giving test solutions at final concentration of 75, 37.5, 15, and 7.5 μg/mL. After 30 min of incubation at room temperature, the absorbance was measured at 515 nm by a UV-Visible spectrophotometer (Lambda 25 UV/VIS spectrometer, Perkin Elmer instruments, MA, USA).

FRS was expressed as a percentage compared with the control, consisting of 3.9 mL of DPPH solution and 100 μl of methanol. The

percent inhibition of the DPPH radical by the test solution was calculated using the following formula:

$$\text{FRS\%} = \left[\frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}}\right] \times 100.$$

The analyses were carried out in triplicate and results are expressed as mean ± SE. IC50 values were calculated by using Graph Pad Prism 4.0.

4. Results and discussion

A phytochemical study on *E. helioscopia* revealed a complex mixture of cerebrosides (**1**–**7**), together with glucoclionasterol (**8**), a digalactosyl diacyl glycerol (**9**) and a monogalactosyldiacylglycerol (**10**).

The fresh leaves of *E. helioscopia* were extracted with methanol at room temperature. After evaporation of the extract, the residue obtained was subjected to flash column chromatography to give five fractions. Further chromatographic purifications of fraction **5** gave a complex mixture of cerebrosides which was then submitted to reversed phase column 'flash chromatography' to give the cerebrosides (**1**–**7**) (Fig. 1).

The IR spectra of **1**–**7** showed bands at 3315, 1635, 1075, 1036 cm^{-1} indicating the presence of hydroxyl, amide and C—O functional groups.

The ^1H and ^{13}C NMR spectra of **1**–**7** (Table 1) indicated the presence of a sugar moiety (δ_H 4.95–4.90, 1H, d, J = 8.0 Hz, anomeric proton; δ_C 105.7–105.3), an amide function (δ_H 8.4–8.5, 1H, d, J = 8.0, 9.0 Hz, NH; δ_C 175.8–175.3) and a long chain aliphatic and olefinic functions (δ_H 0.90–0.84, t, J = 6.0, 7.0, 7.1 Hz, CH_3; δ_H 1.26–1.24, CH_2, δ_H 5.45–5.39, 1H each, both t, J = 4.9 Hz, δ_c 129.5–132.3). These data are suggestive of a glycosphingolipid structure.

The ^{13}C NMR spectra of **1**–**7** (Table 1) were assigned by a combination of DEPT, HMQC and HMBC experiments. Important long-range correlations were observed between C-1″ and H_{ab}-1; C-1 and H-1″, H-2, H-3; C-2 and NH and C-1′ and NH and H-2′. These results again supported the glycosphingolipid structure (Fig. 2). Methanolysis (Gaver and Sweely, 1965) of **1**–**7** yield methyl glucosides, fatty acid methyl esters (FAMs) and dihydroxy (**1**–**2**) and trihydroxy long chain bases (**3**–**7**) (LCBs). The fatty acid methylesters were identified by EI-MS analysis as methyl 2-hydroxypalmitate (FAMs-1,2), methyl 2-hydroxytetracosenoate (FAM-3), methyl 2-hydroxyhexacosenoate (FAM-4), methyl 2-hydroxytetracosanoate (FAM-5), methyl 2-hydroxyoctacosenoate (FAM-6) and methyl 2-hydroxyhexacosanoate (FAM-7).

The MS spectra of **1**–**7** showed molecular ions $[M+H]^+$ peaks at m/z 714, 842, 870, 844, 898, 872 and fragments ions at m/z 552 [714-glc]$^+$, 680 [842-glc]$^+$, 708 [870-glc]$^+$, 682 [844-glc]$^+$, 736 [898-glc]$^+$ and 710 [872-glc]$^+$. The absolute configuration at C-2 the 2-hydroxy fatty acid was presumed to be *R* from the specific rotation of the fatty acids methyl esters ($[\alpha]_D$-110) (Higuchi et al., 1990; Shibuya et al., 1990a,b; Kang et al., 2001a,b).

The FAMs obtained from the methanolysis of the cerebrosides **3**, **4** and **6** exhibited ^{13}C NMR signals at about 175.8, 131.2 and 130.4 expected for monounsaturated fatty acid methyl esters. The resonance at about 27.7, 27.8 ppm confirms the *Z* geometry of the double bonds in the long-chain fatty acids (Table 1). In order to establish the position of the double bond, the monounsaturated fatty acid methyl esters were treated with dimethyl disulfide (DMDS) and I_2 and the products subjected to electron impact (EI)-MS analysis (Scribe et al., 1988). The characteristic fragments at m/z = 317, 345, 359 and 173, obtained between the cleavage between the sulphide carbons, indicate the position of the double bonds in FAMs moiety of 3, 4 and 6 (Fig. 3).

On the other hand, on the basis of mass spectrometry analysis the LCB components were suggested to be 2-amino-1,3-dihydroxy-4,8-octadecadiene (LCB-1,2) and 2-amino-1,3,4-trihydroxy-8-octadecene (LCB-3–7), respectively.

The ESI-MS mass spectra of the DMDS derivatives of **1**–**7** showed remarkable fragments ion peaks at m/z 149, 187 (**1**, **2**) and 221 (**3**–**7**), respectively, due to cleavage of the bond between the carbons bearing a methylthio group (Fig. 3) (Scribe et al., 1988). The above fragment ions arising from selective fragmentation at the C-4–C-8 (**1**,**2**) and at the C-8–C-9 (**3**–**7**) positions of C18 chain, confirm the position the double bond at C-4–C-8 (**1**, **2**) and C-8–C-9 (**3**–**7**) in the long chain bases. The two double bonds in cerebrosides **1** and **2**, one at C-4/C-5 and another between C-8/C-9 are *trans* oriented in **1** owing to the values of chemical shifts of allylic methylene δ_C > 30 and the J values (Su et al., 1999), while in cerebroside **2** the double bond between C-8/C-9 is *cis* oriented by the upfield shifted carbon chemical shifts of C-7 (δ_C 27.63) and C-10 (δ_C 27.36). The Δ^8 double bond in **3**–**7** was determined to be *cis* (*Z*) by the upfield shifted carbon chemical shifts of C-7 (δ_C 27.81) and C-10 (δ_C 27.70) (Kang et al., 2001a,b) and the relative smart coupling constant of H-8 at δ_H 5.40 (dt, J = 10.8, 7.0 Hz) and H-9 at δ_H 5.45 (dt, J = 10.8, 6.8 Hz). 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 absolute configuration of the glucopyranose moiety was determined to be the D-form using the Hara method (Hara et al., 1987).

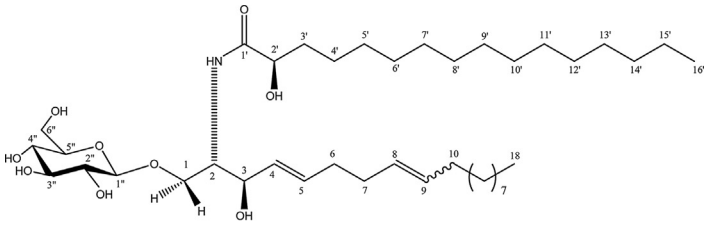
Consideration of the biogenesis and steric hinderance of sphingolipids, generally were acknowledged to determine the absolute stereochemistry of the phytosphingosines moiety. On the basis of the ^{13}C NMR spectral data, the relative stereochemistries at C-2 (δ_C 54.6) and C-3 (δ_C 72.4, 72.3) were deduced to be 2*S* and 3*S* (Tao et al., 2010) for the cerebrosides **1** and **2**. The relative stereochemistry of **3**–**7** at C-2, C-3, C-4 was proposed as 2*S*, 3*S*, 4*R*, identical to that of D-sphingosine on the basis of ^{13}C NMR spectral data, since the chemical shifts of C-2 (δ_C 51.4, 51.2, 51.9, 51.4, 51.9), C-3 (δ_C 76.1, 76.2, 76.09, 76.1) and C-4 (76.2, 72.5) were in agreement with those reported in the literature (Yang et al., 2009).

Therefore the structures of **1**–**7** were proposed to be 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*S*,4*E*,8*E*)-2-[(2′*R*)-2′-hydroxyhexadecanoylamino)-4(*E*),8(*E*)-octadiene-1,3-diol (**1**), 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*S*,4*E*,8*Z*)-2-[(2′*R*)-2′-hydroxyhexadecanoylamino)-4(*E*),8(*Z*)-octadiene-1,3-diol (**2**), 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 (**3**), 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 (**4**), 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*S*,4*R*,8*Z*)-2-[(2′*R*)-2′-hydroxytetracosanoilamino]-8(*Z*)-octadecene-1,3,4-triol (**5**), 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*S*,4*R*,8*Z*)-2-[(2′*R*)-2′-hydroxyoctacosenoilamino]-8(*Z*)-octadecene-1,3,4-triol(**6**) and 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*S*,4*R*,8*Z*)-2-[(2′*R*)-2′-hydroxyhexacosanoilamino]-8(*Z*)-octadecene-1,3,4-triol (**7**). The cerebrosides **1**–**7** isolated and identified are not new. For example, cerebrosides **1** and **2** are identical to soya-cerebrosides I and II isolated from soybean (Shibuya et al., 1990a,b) and cerebroside **5** is identical to pokeweed cerebroside isolated from Phytolaccae Radix (Kang et al., 2001a,b).

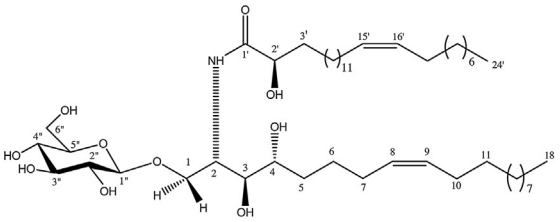
Although, we investigated different species of Euphorbiaceae and complex mixtures of cerebrosides were isolated and identified (Cateni et al., 2003), to our knowledge this is the first report on the isolation and structure elucidation of cerebrosides from *E. helioscopia* L.

From fraction **3** successive chromatographic purifications have led to the isolation and structure elucidation of glucoclionasterol (**8**). Glucoclionasterol was previously isolated from *Euphorbia wulfenii* Hoppe ex Koch (Falsone et al., 1997).

From fraction **4** the compounds **9** and **10** have been isolated and identified (Fig. 1). The spectral data of **9** indicated the presence of a sugar and long-chain unsaturated aliphatic system strongly suggesting a glycolipid. The ^{1}H and ^{13}C NMR spectra of **9** together with ^{1}H–^{1}H COSY, HMQC and HMBC spectra, led to the assignments of all the ^{1}H and ^{13}C NMR signals for the sugar and glycerol moieties. The results suggested that two residues of linolenic acid were connected to C-1 and C-2 and β-D-galactopyranose to C-3 of glycerol, respectively. Compound **9** was thus characterized as (2*S*)-1,2-*O*-di-linolenoyl-3-*O*-β-D-galactopyranosylglycerol (**9**), which



1 (8*E*)
2 (8*Z*)



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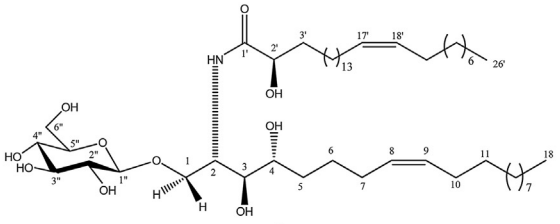

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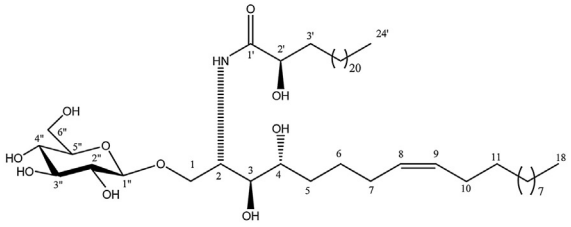

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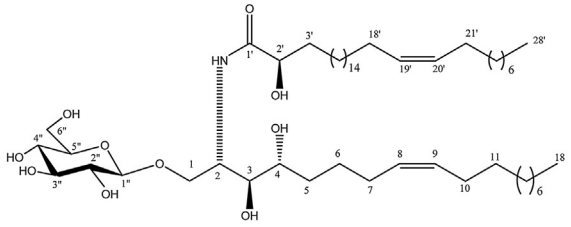

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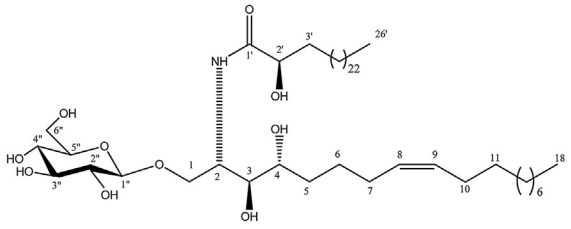

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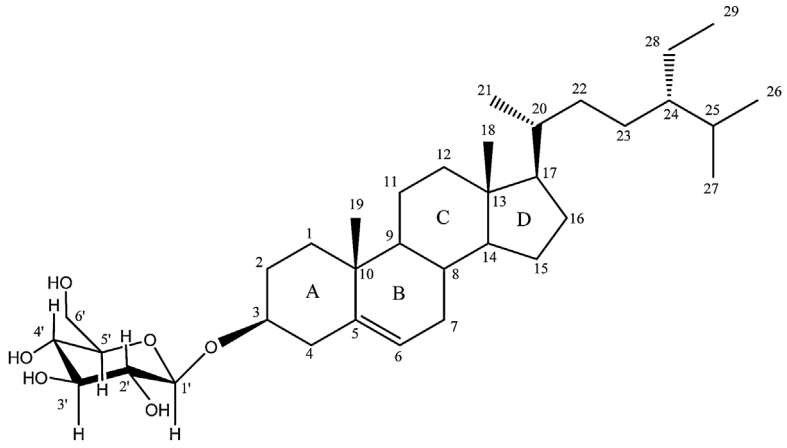
Fig. 1. Chemical structures of compounds **1**–**10** isolated from *E. helioscopia* L.



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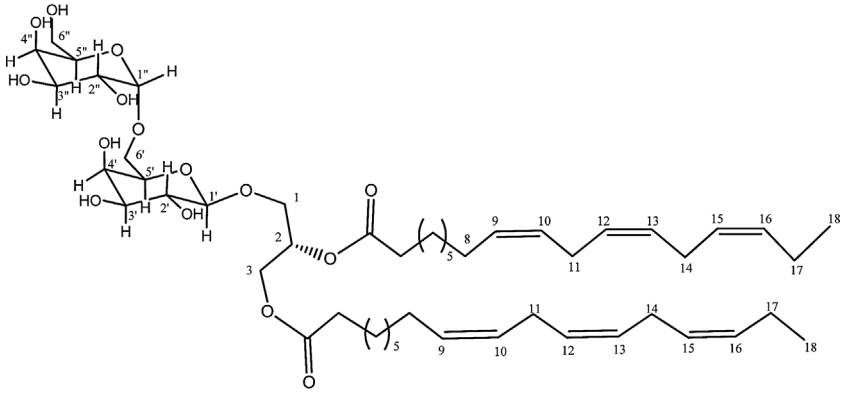

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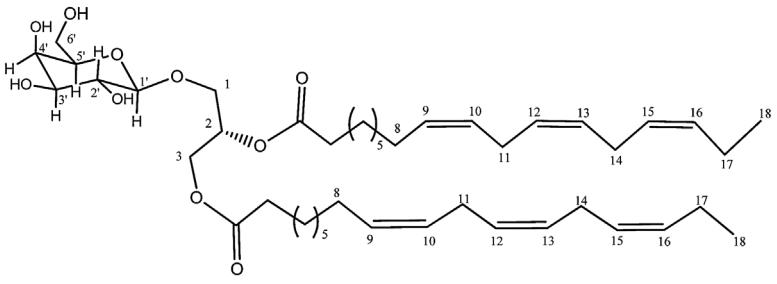


10

Fig. 1. (*Continued*).

was previously isolated from *Euphorbia nicaensis* All. (Cateni et al., 2004). Also compound **10**, identified as (2*S*)-1,2-*O*-di-linolenoyl-glyceryl-6-*O*-(α-D-galactopyranosyl)-β-D-galactopyranoside was proposed in an our preliminary paper (Cateni et al., 2004). The galactose 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. Methyl glycosides obtained from methanolysis of **1**–**7**, **9** and **10** were converted to trimethylsilyl derivatives and the silylated derivatives obtained were analyzed by gas chromatograph–mass spectrometer (GC–MS) system. Identification of galactose was carried out by study of the MS spectra, comparison with members of the NBS library and with retention times of methyl α- and β-D-galactopyranoside and methyl α- and β-D-glucopyranoside standard TMS derivatives (Cateni et al., 2008).

The free-radical scavenging activity (FRS) of *E. helioscopia* methanolic leaves extract, determined by using DPPH assay, was initially evaluated at a stock concentration of 150 μg/mL. Successively, stock solutions were serially diluted into a range of 75–7.5 μg/mL, and their corresponding FRS activity was determined as percentage values. An interesting FRS activity % was evidenced (Fig. 4), since *E. helioscopia* reached a maximum effect (E_{max}) of 60% at 15 μg/mL and with an EC_{50} value of 6,9 μg/mL. This result would be considered of high interest, taking into account that the EC_{50} value of the commercially available standardized green tea extract (Green Select®), used as reference standard, is very close (4.6 μg/mL) to that found.

Antioxidant potential of *E. helioscopia* whole plant was extensively demonstrated by different authors (Nikolova et al., 2011; Uzair et al., 2009), while only one paper focused on the activity of

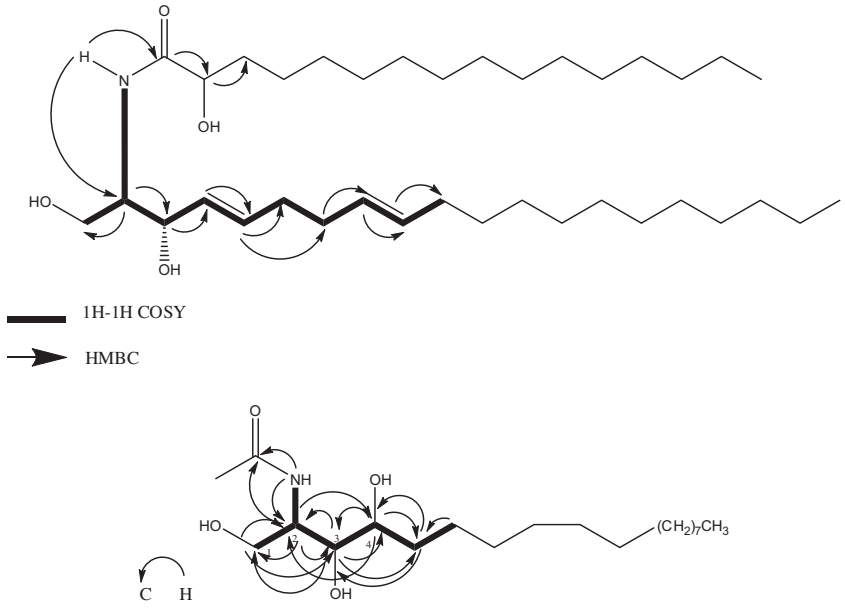
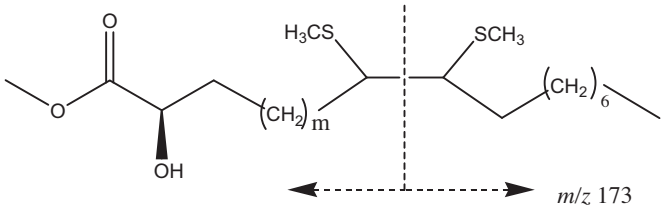
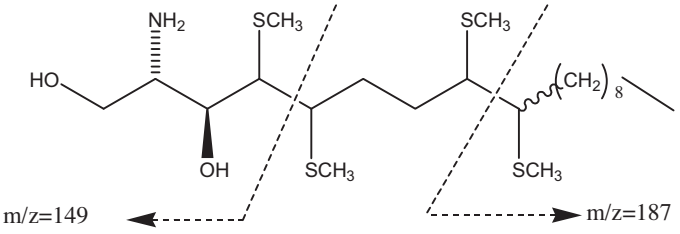


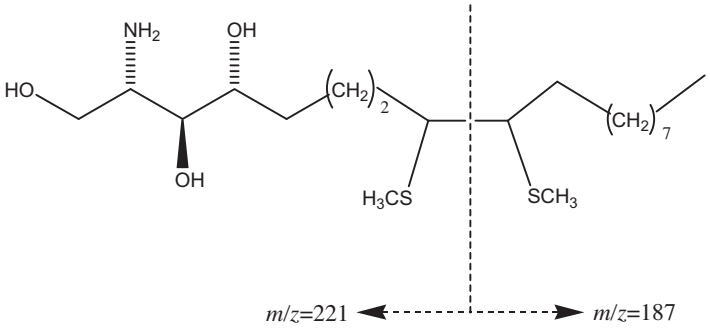
Fig. 2. Selected $^{1}H–^{1}H$ COSY (bold lines) and HMBC (full-line arrows) correlations of ceramide of **1** and sphingosine of **3**.



FAM-DMDS-3: m=11, *m/z*=317 (*M*-173); *m/z*=173 $(C_{10}H_{21}S)^{+}$
FAM-DMDS-4: m=13, *m/z*=345 (*M*-173); *m/z*=173 $(C_{10}H_{21}S)^{+}$
FAM-DMDS-6: m=14, *m/z*=359 (*M*-173); *m/z*=173 $(C_{10}H_{21}S)^{+}$



LCB-DMDS-1,2



LCB-DMDS-3-7

Fig. 3. FAM-DMDS and LCB-DMDS derivatives of cerebrosides **1**–**7**.

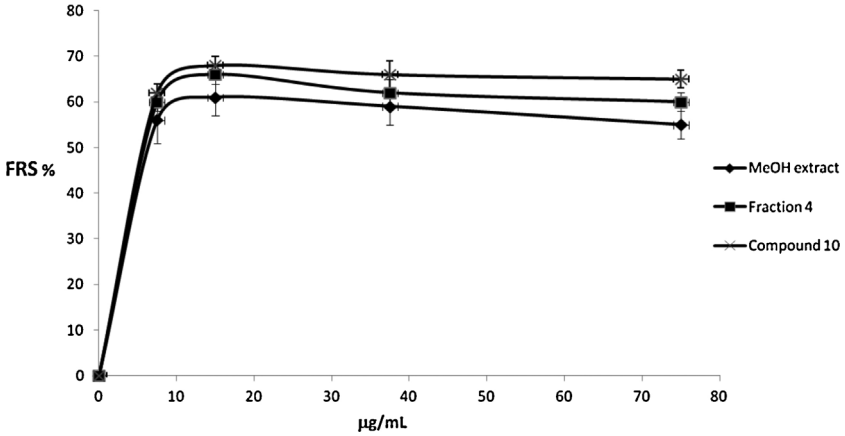


Fig. 4. Free radical scavenging activity % of *E. helioscopia* methanolic extract together with the active fraction (fraction **4**) and compound **10**.

different parts of the plant (flowers, stem and leaves) in correlation with the phenolics and flavonoids content (Nikolova et al., 2011). Our results are in line with those reported by Maoulainine et al. (2012) in terms of free radical potential of the methanolic leaves extract, taking into account the variability related to time and site of collecting plant. Moreover our work focused on the lipid-soluble fraction of the methanolic leaves extract. Basing on the promising results in terms of FRS activity obtained for the crude extract, the FRS screening was extended to the fractions isolated during the purification process, in order to identify the components responsible for the activity observed. In this view, each fraction was assayed by using the DPPH method, in the same conditions described for the crude extract.

Fraction 4 resulted to be the only one active, exerting a FRS effect similar to that of the crude extract, which E_{max} was higher 67 at 15 μg/mL (Ec_{50} = 5.7 μg/mL) (Fig. 4). Since the structural study revealed that this fraction is a mixture of compounds **9** and **10**, we isolated and tested both compounds. Interestingly FRS activity was associated only with compound **10** with an E_{max} of 69% at 15 μg/mL (EC_{50} = 5.2 μg/mL). Therefore, bioactivity-guided fractionation was successful in leading the identification of the compound responsible for the FRS activity of *E. helioscopia* methanolic leaves extract.

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