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Article

Three New Labdanes Isolated from *Eragrostis viscosa*

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Três novos labdanos com anel 8α,15-epoxi [8α,15-epoxilabdan-16β-oato de metila, 8α,15-epoxilabdan-16β-ol e 8α,15-epoxi-16-norlabdan-13β-ol] e cinco compostos conhecidos [8α,15-epoxi-16-norlabdan-13-ona, ácido 8α,15-epoxilabdan-16β-oico, β-(3'',4''-di-hidroxi)-(*E*)-cinamoiloxilup-20(29)-eno, 3-(2',3',4',6'-tetra-*O*-acetil-β-D-glucopiranosiloxi)-β-sitosterol e 16-acetoxi-8α,15-epoxilabdano] foram isolados dos extratos de tolueno e diclorometano de partes aéreas da *Eragrostis viscosa*. A estrutura dos compostos foi atribuída a partir dos seus dados espectroscópicos e da análise de difração de raios X do 8α,15-epoxilabdan-16β-ol. Os compostos 16-acetóxi-8α,15-epoxilabdano, 8α,15-epoxi-16-norlabdan-13-ona e 8α,15-epoxilabdan-16β-ol não apresentam genotoxicidade e mutagenicidade, as quais foram avaliadas usando o teste dos micronúcleos e o teste de Ames, respetivamente. A citotoxicidade dos mesmos compostos foi avaliada usando o teste do MTT sendo o 8α,15-epoxilabdan-16β-ol o mais citotóxico de todos os compostos analisados. Foram também avaliadas a capacidade antioxidante, o valor peróxido e o conteúdo em fenóis totais de extratos de *E. viscosa*.

Three new labdanes with 8α,15-epoxy ring [methyl 8α,15-epoxylabdan-16β-oate, 8α,15-epoxylabdan-16β-ol and 8α,15-epoxy-16-norlabdan-13β-ol] and five known compounds [8α,15-epoxy-16-norlabdan-13-one, 8α,15-epoxylabdan-16β-oic acid, 3β-(3'',4''-dihydroxy)-(*E*)-cinnamoyloxylup-20(29)-ene, 3-(2',3',4',6'-tetra-*O*-acetyl-β-D-glucopyranosyloxy)-β-sitosterol and 16-acetoxy-8α,15-epoxylabdane] were isolated from toluene and dichloromethane extracts of aerial parts of *Eragrostis viscosa*. The structures of all the compounds were established based on their spectroscopic data and X-ray diffraction analysis of 8α,15-epoxylabdan-16β-ol. It was also studied the genotoxicity of *E. viscosa*, particularly compounds 16-acetoxy-8α,15-epoxylabdane, 8α,15-epoxy-16-norlabdan-13-one and 8α,15-epoxilabdan-16β-ol, using a cytokinesis-block micronucleus assay and the Ames test to assess mutagenicity. Both assays were negative. Cytotoxicity was also analyzed using an MTT assay, and 8α,15-epoxy-16β-ol was shown to be the most cytotoxic of the compounds tested. *E. viscosa* extracts were also tested to determine their antioxidant capacities, peroxide values and total phenolic contents.

Keywords: *Eragrostis viscosa*, 8α,15-epoxylabdanes, mutagenic activity, cytotoxic activity, genotoxicity

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Introduction

Eragrostis species (Poaceae, Eragrostoideae tribe) are well known for their nutritive value.[1,2] Nevertheless, cattle refuse to eat *Eragrostis viscosa* (Retz.) Trin., which is used in folk medicine as a poison against snakes.

Some of the most important fodders from this genus include *E. nigra*, *E. tef* and *E. curvula*. However, studies conducted on these plants have primarily addressed their nutritive values, not their chemical compositions.[1,3,4] In our research on Angolan plants,[5-7] our group previously reported the isolation of labdanes containing a novel 8,15-epoxide.[6] Now, it is reported the isolation of new 8α,15-epoxylabdanes **6** and **7** along with known compounds 16-acetoxy-8α,15-epoxylabdane (**2**),[6] 8α,15-epoxy-16-norlabdan-13-one (**3**)[6] and 8α,15-epoxylabdan-16β-oic acid (**4**) (Figure 1).[6] These compounds were isolated by a maceration of the aerial parts of *E. viscosa* at room temperature in dichloromethane, and were characterized by the techniques Fourier transform IR spectroscopy, ^{1}H and ^{13}C nuclear magnetic resonance (NMR), 2D NMR, electron ionization mass spectrometry (HREIMS) and X-ray diffractometry (XRD) of **6**. A new 8α,15-epoxylabdane **1** was isolated from the toluene extract along with the known compounds 8α,15-epoxy-16-norlaban-13-one (**3**),[6] 8α,15-epoxylabdan-16β-oic acid (**4**),[6] 3β-(3'',4''-dihydroxy)-(*E*)-cinnamoyloxylup-20(29)-ene (**5**)[8] and 3-(2',3',4',6'-tetra-*O*-acetyl-β-D-glucopyranosyloxy)-β-sitosterol (**8**) (Figure 1).[8] The known compounds were identified on the basis of their spectral data and comparison with appropriate reported data.

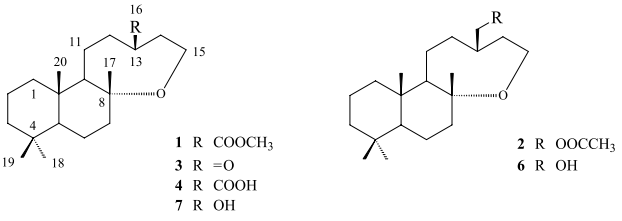

Figure 1. Chemical structures of the isolated compounds.

Our group tested the genotoxicity of *E. viscosa*, particularly compounds **2**, **3** and **6**, using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay as a biomarker of cytotoxicity, the cytokinesis-block micronucleus (MN) assay in hamster V79 cells as a biomarker of cytogenetic damage, and the Ames test to detect potential mutagenic damage was tested. The antioxidant capacities, peroxide values and total phenolic contents of some extracts of *E. viscosa* are also presented.

Experimental

General experimental procedures

Optical rotations were obtained using a Bellingham + Stanley Ltd ADP 220 polarimeter. HREIMS measurements were conducted on a VG Autospec M and recorded at 70 eV. IR spectra were measured in a Unicam Mattson 5000 FTIR. X-ray diffraction analyses were performed in a Bruker SMART CCD 1000 with Mo K_{α} radiation (λ = 0.71073 Å). NMR spectra were recorded in a Bruker Avance II at 600 MHz (^{1}H NMR) or 150.9 MHz (^{13}C NMR) in $CDCl_3$. Chemical shifts (δ) are given in ppm and are referenced to the residual $CHCl_3$, using 7.26 ppm for the ^{1}H peak and 77.0 ppm for the ^{13}C peak. Two-dimensional experiments were performed with standard Bruker software. Column chromatography was performed on silica gel 60 (70-230 mesh, Merck, Darmstadt, Germany) using a hexane-EtOAc gradient.

Plant material

The aerial parts of *E. viscosa* were collected in the outskirts of Lubango (Huíla), Angola, in July 2003 and were identified by the staff of the Lubango Herbarium. A voucher specimen (No. 3666) was deposited at the Lubango Herbarium, Angola.

Extraction and isolation

The dried aerial parts (400 g) that were collected in 2003 were successively extracted with hexane (hexane 2 extract), toluene (producing 8.4 g of toluene extract) and ethyl acetate in a Soxhlet apparatus for 24 h. The hexane 1 extract was obtained from plants collected in 2001.[6] A total of 200 g of the dried aerial parts collected in 2003 was macerated in dichloromethane for one week to yield 30 g of crude extract. The dichloromethane crude extract afforded 18 g of material after being dewaxed with methanol. The toluene extract was separated into 5 fractions (hexane/EtOAc 95:5, 8:2, 6:4, 1:1 and ethyl acetate). The fraction eluted with 95:5 hexane/EtOAc was separated on a silica gel column with a hexane/EtOAc gradient to yield methyl 8α,15-epoxylabdan-16β-oate (**1**) (8 mg) and 8α,15-epoxy-16-norladban-13-one (**3**) (20 mg). The fraction eluted with 8:2 hexane/EtOAc was separated on a silica gel column with a hexane/EtOAc gradient to yield 8α,15-epoxylabdan-16β-oic acid (**4**) (70 mg). The fraction eluted with 1:1 hexane/EtOAc was separated on a silica gel column with a hexane/EtOAc gradient to yield 3β-(3'',4''-dihydroxy)-(*E*)-cinnamoyloxylup-

20(29)-ene (**5**) (12 mg). The fraction eluted with EtOAc was separated on a silica gel column with a hexane/EtOAc gradient to yield 3-(2',3',4',6'-tetra-*O*-acetyl-β-D-glucopyranosyloxy)-β-sitosterol (**8**) (35 mg). A sample of the dichloromethane extract (8.5 g), obtained from maceration was separated into 7 fractions (hexane/EtOAc 95:5, 9:1, 8:2, 7:3, 6:4, 1:1 and ethyl acetate). The fraction eluted with 8:2 hexane/EtOAc was separated on a silica gel column with a hexane/EtOAc gradient to yield 16-acetoxy-8α,15-epoxylabdane (**2**) (470 mg) and 8α,15-epoxy-16-norlabdan-13-one (**3**) (17 mg). The fraction eluted with 6:4 hexane/EtOAc was separated on a silica gel column with a hexane/EtOAc gradient to yield 8α,15-epoxylabdan-16β-oic acid (**4**) (720 mg). The fraction eluted with 1:1 hexane/EtOAc was separated on a silica gel column with a hexane/EtOAc gradient to yield 8α,15-epoxylabdan-16β-ol (**6**) (23 mg) and 8α,15-epoxy-16-norlabdan-13β-ol (**7**) (55 mg).

Methyl 8α,15-epoxylabdan-16β-oate (**1**)

Colorless oil; $[\alpha]_D^{25}$ +55.2° (*c* 0.12, $CHCl_3$); IR ν_{max}/cm^{-1} 2926, 2868, 1738, 1452, 1385, 1271, 1165, 1084, 756; ^{1}H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150.9 MHz): see Table 1; HR-TOF-EIMS (pos.) *m/z* 336.2664 $[M]^+$ (calcd. for $C_{21}H_{36}O_3$, 336.2664).

8α,15-Epoxylabdan-16β-ol (**6**)

Colorless oil; $[\alpha]_D^{25}$ +21.2° (*c* 0.5, $CHCl_3$); IR ν_{max}/cm^{-1} 3422, 2940, 1461, 1388, 1076, 1024, 757; ^{1}H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150.9 MHz): see Table 1; HR-TOF-EIMS (pos.) *m/z* 308.2714 $[M]^+$ (calcd. for $C_{20}H_{36}O_2$, 308.2715).

8α,15-Epoxy-16-norlabdan-13β-ol (**7**)

Colorless oil; $[\alpha]_D^{25}$ +25.2° (*c* 0.16, $CHCl_3$); IR ν_{max}/cm^{-1}: 3426, 2936, 1462, 1389, 1072, 756 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150.9 MHz): see Table 1;

Table 1. ^{1}H and ^{13}C NMR data and HMBC correlations of compounds **1** and **6**[a]

Position	**1**			**6**		
	δ ^{13}C	δ ^{1}H (mult, nH, *J* / Hz)	HMBC	δ ^{13}C	δ ^{1}H (mult, nH, *J* / Hz)	HMBC
1	40.7	0.91 (dd, 1H, 12.8, 3.7) 1.75 (m, 1H)	2, 3, 10, 20	40.0	0.88 (m, 1H) 1.78 (m, 1H)	2, 3, 5, 10, 20
2	18.7	1.42 (m, 1H) 1.60 (m, 1H)	1, 3, 4, 10	18.6	1.30 (m, 1H) 1.57 (m, 1H)	1, 3, 4, 10
3	41.9	1.13 (td, 1H, 13.2, 4.0) 1.38 (dt, 1H, 13.2, 3.6)	1, 2, 4, 5, 18, 19	42.2	1.13 (dd, 1H, 14.0, 4.0) 1.39 (m, 1H)	1, 2, 4, 5, 18, 19
4	33.3			33.5		
5	56.4	0.85 (dd, 1H, 5.2, 3.3)	4, 6, 10, 18, 19, 20	56.5	0.82 (m, 1H)	4, 6, 7, 9, 18, 19, 20
6	20.1	1.65 (m, 2H)	5, 7, 8	20.5	1.23 (m, 1H) 1.69 (m, 1H)	4, 5, 7, 8, 10, 18
7	38.2	1.52 (ddd, 1H, 10.0, 7.3, 3.9) 1.75 (m, 1H)	5, 6, 8, 9, 17	37.1	1.49 (m, 1H) 1.78 (m, 1H)	5, 6, 8, 9, 17
8	78.5			78.5		
9	50.2	1.45 (m, 1H)	1, 5, 8, 11, 12, 17, 20	57.1	1.52 (m, 1H)	5, 7, 8, 10, 11, 12, 17, 20
10	38.5			39.4		
11	22.8	1.46 (m, 1H) 1.66 (m, 1H)	8, 9, 10, 12, 13	24.2	1.18 (m, 1H) 1.62 (ddd, 1H, 14.4, 11.0, 5.6)	8, 9, 10, 12, 13
12	29.5	1.92 (m, 1H) 2.02 (ddt, 1H, 8.6, 5.0, 4.1)	9, 11, 13, 14, 16	33.5	0.86 (m, 1H) 1.93 (dd, 1H, 13.8, 7.2)	9, 11, 13, 14, 16
13	43.5	2.46 (m, 1H)	12, 14	40.7	1.83 (m, 1H)	12, 15, 16
14	33.5	1.78 (m, 2H)	12, 13, 14	32.8	1.32 (m, 1H) 1.79 (m, 1H)	12, 13, 15, 16
15	60.1	3.65 (m, 1H)[b] 3.71 (td, 1H, 13.1, 2.7)	8, 13, 14	57.4	3.47 (m, 1H) 3.81 (ddd, 1H, 13.6, 10.7, 6.7)	8, 13, 14
16	177.5			68.8	3.41 (dd, 1H, 10.4, 6.5) 3.43 (dd, 1H, 10.4, 6.2)	12, 13, 14
17	23.5	1.15 (s, 3H)	7, 8, 9	24.3	1.16 (s, 3H)	7, 8, 9
18	33.4	0.88 (s, 3H)	3, 4, 5, 19	33.4	0.88 (s, 3H)	3, 4, 5, 19
19	21.6	0.79 (s, 3H)	3, 4, 5, 18	21.4	0.79 (s, 3H)	3, 4, 5, 18
20	15.5	0.83 (s, 3H)	1, 5, 9, 10	15.8	0.76 (s, 3H)	1, 5, 9, 10
16-COO$\underline{CH_3}$	51.7	3.65 (s, 3H)[b]	16			

[a]Spectra were recorded at 600 and 150.9 MHz for ^{1}H and ^{13}C NMR, respectively; 2D NMR experiments recorded in accordance; coupling constants are in parenthesis; [b]overlapped signal.

HR-TOF-EIMS (pos.) *m/z* 294.2561 $[M]^+$ (calcd. for $C_{19}H_{34}O_2$, 294.2559).

Hydrolysis of **2**

First, 15 mL of a 4% solution of KOH/MeOH were added to 150 mg of **2**. The mixture was left for 2 h at room temperature, diluted with water (15 mL) and left for an additional 2 h at room temperature. MeOH was evaporated under vacuum and the aqueous solution was extracted with Et_2O. The organic layer was washed with H_2O until a pH 7 was obtained, dried over Na_2SO_4, filtered and evaporated under vacuum to yield **6** (111 mg), whose NMR spectra and optical rotation data were identical to those of the natural alcohol.

Determination of total phenolic contents

The total phenolic contents of extracts from *E. viscosa* were determined using the Folin-Ciocalteu reagent according to the method previously reported by Matkowski and Piotrowska[9] with minor modifications. A total of 400 µL of a 0.05 mg L^{-1} methanolic extract solution was reacted with 2 mL of the Folin-Ciocalteu reagent (0.2 mol L^{-1}) for 8 min at room temperature. The reaction was then neutralized with 7.5% sodium carbonate and allowed to stand for 90 min at room temperature. Absorbance was measured at 765 nm with a Pharmacia Biotech spectrophotometer Ultrospec U/V 3000. Quantification was done based on a standard curve with gallic acid. Results were expressed as mg of gallic acid equivalents (GAE) *per* g of dry extract. The absorbance *versus* concentration curve is described by the equation $y = 0.012x - 0.018$ ($R^2 = 0.9922$), in which *y*: absorbance and *x*: concentration.

ABTS assay

The assay was performed with a Pharmacia Biotech spectrophotometer Ultrospec U/V 3000 using the ABTS (total antioxidant capacity) method described elsewhere[10] with minor modifications. $ABTS^{\bullet+}$ radical cations were generated by reacting 7 mmol L^{-1} ABTS with 140 mmol L^{-1} potassium persulfate. The reaction mixture was allowed to stand in the dark for 16 h at room temperature and was used within 1 day. The $ABTS^{\bullet+}$ solution was diluted with 50% ethanol to an absorbance of 0.700 ± 0.050 at 734 nm. Measurements were taken on a mixture of 3 mL of the $ABTS^{\bullet+}$ solution and 30 µL of the 0.05 mg L^{-1} methanolic extract solutions over a period of 15 min (reading times 1 s, 5 s, 1, 3, 5, 10 and 15 min), and each set of measurements was taken at least three times and in triplicate. Inhibition (I, %) was calculated as follows:

$$I = 100\left(\frac{A_0 - A_t}{A_0}\right) \quad (1)$$

where A_0 is the absorbance of the control (methanol), and A_t is the absorbance of the mixture. A 0.05 mg L^{-1} Trolox® solution was used as a reference standard.

DPPH assay

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay was performed according to the method reported by Villaño *et al.*[11] with some modifications. Briefly, 200 µL of either the 0.05 mg L^{-1} methanolic extract solutions or methanol (control) were added to 2.7 mL of a 40 µmol L^{-1} solution of DPPH in methanol. Mixtures were shaken vigorously, and the absorbance was measured using a Pharmacia Biotech spectrophotometer Ultrospec U/V 3000 over a period of 15 min (reading times 1 s, 5 s, 1, 3, 5, 10 and 15 min). Antiradical activity was expressed as an inhibition percentage (I, %) and calculated using the equation (1).

Oil oxidation

To determine the peroxide values, 20 mL of sunflower oil and 2 mL of the extracts (0.01 g mL^{-1}) were oxidized by heating at 80 ºC for eight days. The control sample was made from 20 mL of sunflower oil and 2 mL of methanol.

Peroxide value assay

The peroxide value assay was conducted as described in Method Cd 8-53 of the AOCS Official Methods and Recommended Practices of the American Oil Chemists' Society[12] with minor modifications. Briefly, 2.5 g of sample were swirled in 15 mL of a 3:2 acetic acid-chloroform solution until dissolved. At this point, 0.25 mL of a saturated KI solution was added, the mixture was shaken for 1 min and an additional 15 mL of distilled water were added. The mixture was then titrated with 0.1 mol L^{-1} sodium thiosulfate standardized with a fresh solution of I_3^- prepared from KIO_3 plus KI.

MTT cytotoxicity assay

The MTT assay was conducted in V79 Chinese hamster cells. Approximately 10^4 cells were grown at 37 °C in a 5% CO_2 atmosphere for 24 h in 96-well plates using 200 µL

of Ham's F-10 medium supplemented with 10% newborn calf serum and 1% penicillin/streptomycin solution. Various doses of compounds **2**, **3** and **6** (25, 50 and 250 μg *per* well) were added, and the cells were incubated for 3 h. The medium was removed, and the cells were incubated for an additional 3 h with MTT (0.5 mg mL^{-1}). The cells were washed carefully with phosphate buffered saline (PBS), then 200 μL of DMSO (dimethyl sulfoxide) was added to each well. The absorbance of the converted dye was measured at 595 nm in a Zenith 3100 microplate reader. Cytotoxicity was assessed by comparing the absorbance values of the treated cells with those of the control cells. Three independent experiments were performed.

Ames assay

Mutagenicity testing was conducted by the plate incorporation assay described by Maron and Ames[13] using *Salmonella typhimurium* strains TA 98, TA 100 and TA 102 in the presence or absence of S9 mix.[13] At least two independent experiments were performed for each assay.

Cytokinesis-block micronucleus assay (CBMN)

Approximately 5 × 10^5 V79 Chinese hamster cells were cultured for 24 h in 25 cm^2 culture flasks and then exposed to compounds **2**, **3** and **6** at concentrations of 10, 25 or 50 μg mL^{-1}. Cyclophosphamide (2.0 μg mL^{-1}) and mitomycin C (2.5 μg mL^{-1}) were used as positive controls for the experiments with and without S9 mix, respectively. At 24 h after the genotoxic treatment, the cells were washed with fresh culture medium, and cytochalasin-B (Cyt-B) was added to produce a final concentration of 4.5 μg mL^{-1}. The cells were incubated for an additional 16 h, harvested by trypsinization, rinsed and submitted to a mild hypotonic treatment as described elsewhere.[14] The centrifuged cells were placed onto dry slides, and smears were made. After air-drying, the slides were fixed with cold methanol for 30 min. One day later, the slides were stained with Giemsa (4% (v/v) in 0.01 mol L^{-1} sodium phosphate buffer, pH 6.8) for 10 min. For each experimental point, 1000 binucleate V79 cells (BN) with well-preserved cytoplasms were scored. Micronuclei were identified under a light microscope using a magnification of 1250 × according to the criteria proposed by Caria *et al.*[15] It was evaluated the MN/BN value, which represents the average number of micronuclei *per* binucleated cell, and the percentage MNBN, which represents the fraction of cytokinesis-blocked (binucleated) cells with micronuclei, regardless of the number of micronuclei *per* BN cell.[16] The decrease in cell proliferation was also measured in these assays by determining the frequency of binucleate cells (BN, %). For this index, the number of nuclei in 1000 cells with well-preserved cytoplasms was determined at a magnification of 500×. At least two independent experiments were performed for each assay.

Statistical analysis

All the values presented throughout the text and tables correspond to mean values and respective standard deviations (SD) from all experiments. The statistical analysis was carried out with student's *t*-test. All analyses were performed with the SPSS statistical package (version 17; Chicago, USA).

Crystal data for **6**

Monoclinic crystals of **6** were formed by a slow crystallization from 4:1 hexane/EtOAc. Operations were performed on a Bruker SMART CCD 1000 diffractometer using graphite-monochromated Mo K_α radiation at 293(2) K with 5287 reflections obtained (2175 independent, R_{int} = 0.0384), measured at θ_{max} = 28.02º from a crystal of size 0.48 × 0.30 × 0.18 mm^3 ($C_{20}H_{36}O_2$), yielding the following parameters: a = 9.5115(18), b = 7.0325(13), c = 14.496(3), α = 90º, β = 104.630(4)º, γ = 90º, V = 938.2(3) $Å^3$, d_{calcd} = 1.092 mg m^{-3}, Z = 2, and spatial group P2(1). The structure was solved by a direct method and refined anisotropically to give R_1 = 0.0619 and wR_2 = 0.01591 for 2175[R(int) = 0.0384] independently observed reflections ($I > 2\sigma(I)$, $\theta \leq 25$º) with a goodness-of-fit = 0.989.

Results and Discussion

Compound **1** was obtained as a colorless oil with an $[\alpha]_D^{25}$ value of +55.2º (*c* 0.012, $CHCl_3$). The HR-TOF-EIMS analysis of **1** revealed a molecular ion peak at *m/z* 336.2664 that corresponds to a molecular formula of $C_{21}H_{36}O_3$ (calculated at 336.2664), which includes four degrees of unsaturation. The IR spectrum showed the presence of a methyl ester (1738 and 1385 cm^{-1}) and an ether (1084 cm^{-1}).

The ^{1}H NMR spectrum of compound **1** showed signals corresponding to four tertiary methyl groups at δ_H 1.15, 0.88, 0.83 and 0.79 (s, each 3H), a methoxy group at δ_H 3.65 (s, 3H) and one diastereotopic oxymethylene at δ_H 3.71 (td, 1H, *J* 13.1, 2.7 Hz) and 3.65 (m, 1H, H-15) that had COSY (correlation spectroscopy) correlations with H-14 and HMBC (heteronuclear multiple bond correlation) correlations from H-15 to C-8 and C-14.

The ^{13}C NMR spectrum showed 21 carbons, which were interpreted as five methyls (one as part of a methyl

ester), nine methylenes, three methines and four quaternary carbons (one as part of a methyl ester) from the DEPT (distortionless enhancement by polarization transfer) spectrum. The ^{13}C NMR chemical shifts of four methyl groups (δ_C 33.4, 23.5, 21.6 and 15.5), one methylene (δ_C 60.1), three methines (δ_C 56.4, 50.2 and 43.5) and three quaternary carbons (δ_C 78.5, 38.5 and 33.3) were very similar to those from an epoxylabdane scaffold. The other ^{13}C and ^{1}H NMR, ^{1}H-^{1}H COSY, HSQC (heteronuclear single quantum coherence) and HMBC data (Table 1, Figure 2) were also consistent with such structure.[17] The H-15/C-8 HMBC correlation, the presence of an ether (1067 cm^{-1}) and the lack of an OH absorption in the IR spectrum, coupled with the four degrees of unsaturation, confirmed the presence of a 8α,15-epoxylabdane[6] in which C-16 is part of a methyl ester (δ_C 177.5, 51.7). The NOESY (nuclear Overhauser effect spectroscopy) correlations (H-5/Me-18, H-9 and H-9/H-13) indicated that H-5, H-9, H-13 and Me-18 were all on the α face of the molecule, which means that C-9 and C-16 were β oriented (Figure 3). Additional NOESY correlations (Me-19/Me-17, Me-20) suggested that these methyl groups were also β oriented (Figure 3). Thus the structure of compound **1** was established as methyl 8α,15-epoxylabdan-16β-oate (Figure 1).

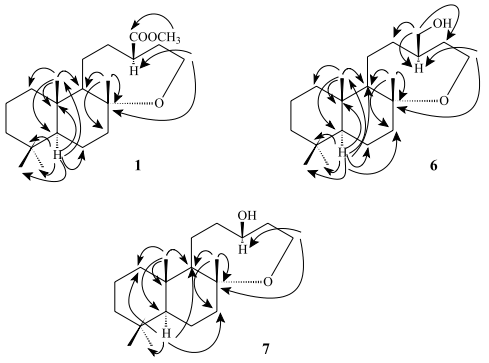

Figure 2. Key HMBC correlations of **1**, **6** and **7**.

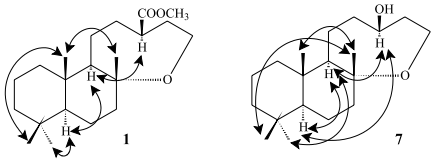

Figure 3. Key NOESY correlations of **1** and **7**.

Compound **6** was obtained as a colorless oil with an $[\alpha]_D^{25}$ value of +21.2º (*c* 0.50, $CHCl_3$). The HR-TOF-EIMS analysis showed a molecular ion peak at *m/z* 308.2714 (calculated at 308.2715), suggesting a tricyclic structure with a molecular formula of $C_{20}H_{36}O_2$. The IR spectrum revealed bands corresponding to a hydroxyl group (3420 cm^{-1}) and an ether (1026 cm^{-1}).

The spectral (^{1}H and ^{13}C NMR) characteristics of compound **6** were very similar to those of **1**, suggesting that **6** is a derivative of **1**. Differences in the ^{1}H NMR spectrum of **6** include the absence of the methyl ester group and the presence of a hydroxyl group. The ^{1}H NMR spectrum of the compound showed signals for four methyl groups at δ_H 1.16, 0.88, 0.79, and 0.76 (each 3H, s) and one diastereotopic oxymethylene at δ_H 3.81 (ddd, 1H, *J* 13.6, 10.7, 6.7 Hz) and 3.47 (m, 1H, H-15) that presented COSY correlations with H-14. The HMBC correlations (H-15/C-8, C-14) were in good agreement with the presence of an 8,15-epoxy ring, and another oxymethylene at δ_H 3.43 (dd, 1H, *J* 10.4, 6.2 Hz) and 3.41 (dd, 1H, *J* 10.4, 6.5 Hz) showed a COSY correlation with H-13 and HMBC correlations (H-16/C-13, C-12, C-14) that established position 16 as the new oxymethylene.

The ^{13}C NMR DEPT spectrum showed 20 carbons, which were interpreted as four methyls, ten methylenes, three methines and three quaternary carbons. The ^{13}C NMR chemical shifts of the four methyls (δ_C 33.4, 24.3, 21.4 and 15.8), two of the methylenes (δ_C 68.8 and 57.4), the three methines (δ_C 57.1, 56.5 and 40.7) and the three quaternary carbons (δ_C 78.5, 39.4 and 33.5), along with the ^{1}H NMR, ^{1}H-^{1}H COSY, HSQC and HMBC data (Table 1, Figure 2), were found to be consistent with an 8,15-epoxylabdane structure similar to compound **1**. The ^{1}H-^{1}H COSY correlations (H-16/H-13) and the HMBC correlations (H-16/C-12, C-13, C-14) established the structure of **6** as an 8α,15-epoxylabdane in which C-16 bears a hydroxyl group. Complete assignments for the NMR spectra of compound **6** were achieved by ^{1}H-^{1}H COSY, HSQC and HMBC (Table 1). The structure of compound **6** was established as 8α,15-epoxylabdan-16-ol (Figure 1).

The hydrolysis of **2** (with a 4% solution of KOH/MeOH, at room temperature, 2 h) led to the isolation of a product whose NMR spectra and optical rotation data were identical with those of the natural alcohol **6**. Colorless crystals of the hydrolysis product (**6**) were obtained by slow crystallization in 4:1 hexane/EtOAc and were found to have a melting point of 119-120 ºC. The relative stereochemistry for compound **6** at C-5, C-8, C-9 and C-16 was established by single crystal X-ray diffraction analysis of the hydrolysis product, which was determined to be 8α,15-epoxylabdan-16β-ol (Figure 4).

Compound **7** was obtained as a colorless oil with an $[\alpha]_D^{25}$ value of +25.2º (*c* 0.16, $CHCl_3$). The HR-TOF-EIMS presented a molecular ion peak at *m/z* 294.2561 (calculated at 294.2559), suggesting a tricyclic structure with a molecular formula of $C_{19}H_{34}O_2$. The IR spectrum revealed bands characteristic of a hydroxyl group (3426 cm^{-1}) and an ether (1072 cm^{-1}).

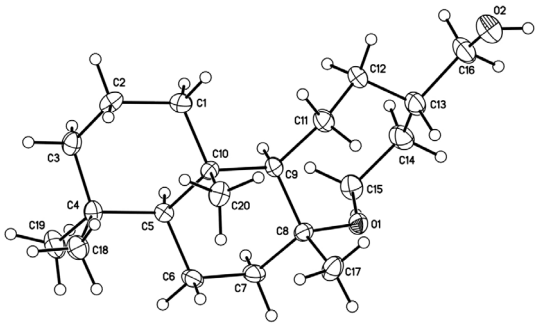

Figure 4. Molecular structure of **6** generated by X-ray diffraction.

The ^{1}H NMR spectrum of **7** displayed features similar to those of **6** (Table 2), except that the H-16 signal was absent and the H-13 signal was 2.00 ppm downfield compared to the same signal in compound **6**. The deshielding of H-13 may be explained by the presence of a geminal hydroxyl group. The concomitant downfield shift of the C-13 signal to δ_C 71.53 ($\Delta\delta$ ca. 31 ppm) is also indicative of an hydroxyl group located at C-13. NOESY correlations (H-9/H-5, Me-18 and H-13/H-9, H-5, Me-18) indicated that H-5, H-9, H-13 and Me-18 were on the α-face of the molecule; consequently, C-9 and C-13-OH were β oriented (Figure 3). Additional NOESY correlations (Me-17/Me-19, Me-20) suggested that these methyl groups were also β oriented. Thus, the structure of compound **7** was established as 8α,15-epoxy-16-norlabdan-13β-ol. Our group has already reported a related 8α,15-epoxy-16-norlabdan-13-one from the same plant.[6]

Earlier studies on this plant[6] were conducted with an extract obtained by Soxleht extraction and doubts remained if the 8,15-epoxy ring was an artifact of the extraction process. However, the same scaffold was isolated from the extract obtained by maceration at room temperature with a different solvent. Therefore, the 8,15-epoxylabdanes can be confirmed as natural products and not artifacts.

A genotoxicity analysis of the induction of micronuclei by compounds **2**, **3** and **6** is presented in Table 3, which shows the frequency of micronucleated binucleated cells (MNBN, %), the number of micronuclei *per* binucleated cell (MN/BN) and the decrease in cell proliferation due to the frequency of binucleated cells (%). These compounds showed no statistically significant increase in genotoxicity. No mutagenic activity was observed in the Ames test (Table 4) on all the strains tested in the presence or the absence of S9 mix. The cytotoxicities of compounds **2**, **3** and **6** were analyzed in V79 cells by the MTT assay. The data in Table 5 shows that cytotoxicity was evident at the lowest concentration tested. From the three compounds, **6** was the most cytotoxic, resulting in less than 50% survival at an exposure of 25 μg *per* well. At the highest concentration tested (250 μg *per* well), cell viability for all compounds was below 9%, and compound **6** exhibited the highest overall cytotoxicity in V79 cells. According to the micronucleus assay, none of the three compounds are genotoxic in V79 cells at the concentrations tested, indicating that genotoxic activity is apparently not the main reason for their cytotoxicity.

The hexane (1 and 2), dichloromethane and ethyl acetate extracts of *E. viscosa* were tested by the DPPH and ABTS methods to measure their antioxidant capacities; the peroxide values and the total phenolic contents were also determined. The extracts have a very low antioxidant capacity with respect to both DPPH (Figure 5) and ABTS radicals (Figure 6) when compared to a 0.05 mg mL^{-1} Trolox® solution. The antioxidant capacities of the extracts tested were also expressed in TEAC (Trolox®

Table 2. ^{1}H and ^{13}C NMR data and HMBC correlations of compound **7**[a]

Position	7		
	δ ^{13}C	δ ^{1}H (mult, nH, *J* / Hz)	HMBC
1	40.5	0.90 (m, 1H) 1.77 (dt, 1H, 13.7, 3.4)	2, 3, 5, 9, 10, 20
2	18.7	1.42 (ddd, 1H, 14.3, 7.3, 3.6) 1.59 (m, 1H)	1, 3, 4, 10
3	41.9	1.14 (m, 1H) 1.31 (dt, 1H, 14.4, 4.4)	1, 2, 4, 5, 18, 19
4	33.4		
5	56.3	0.81 (m, 1H)	1, 7, 9, 18
6	20.2	1.38 (m, 1H) 1.58 (m, 1H)	4, 5, 8, 10
7	37.3	1.52 (m, 1H) 1.58 (m, 1H)	5, 6, 8, 9, 17
8	78.8		
9	51.3	1.39 (m, 1H)	1, 8, 10, 11, 12, 17, 20
10	38.7		
11	20.4	1.27 (m, 1H) 1.65 (m, 1H)	8, 9, 10, 13
12	36.5	1.66 (m, 1H) 2.10 (ddd, 1H, 13.4, 9.4, 4.7)	9, 11, 13, 14
13	71.5	3.83 (td, 1H, 10.2, 3.8)	11, 12, 14, 15
14	39.2	1.83 (m, 2H)	12, 13, 15
15	57.5	3.58 (dt, 1H, 13.0, 4.2) 3.72 (ddd, 1H, 13.0, 10.2, 3.2)	8, 13, 14
17	23.7	1.15 (s, 3H)	7, 8, 9
18	33.5	0.86 (s, 3H)	3, 4, 5, 19
19	21.5	0.78 (s, 3H)	3, 4, 5, 18
20	15.3	0.81 (s, 3H)	1, 5, 9, 10

[a]Spectra were recorded at 600 and 150.9 MHz for ^{1}H and ^{13}C NMR, respectively; 2D NMR experiments recorded in accordance; coupling constants are in parenthesis; [b]overlapped signal.

Table 3. Effect of compounds **2**, **3** and **6** on the frequency of micronucleated binucleated cells (MNBN, %) in V79 Chinese hamster cells in the presence (+S9) and absence (−S9) of metabolic activation

Test compound / (μg mL^{-1})	MN/BN		MNBN / %		BN / %	
	−S9	+S9	−S9	+S9	−S9	+S9
2						
0	0.003 ± 0.001	0.002 ± 0.001	0.300 ± 0.071	0.167 ± 0.058	40.950 ± 14.213	37.400 ± 2.263
10	0.005 ± 0.001	0.000 ± 0.000	0.450 ± 0.071	0.000 ± 0.000	34.600 ± 1.980	49.950 ± 5.586
25	0.004 ± 0.000	0.003 ± 0.000	0.400 ± 0.000	0.003 ± 0.000	37.700 ± 2.687	48.950 ± 15.344
3						
10	0.004 ± 0.000	0.001 ± 0.001	0.467 ± 0.115	0.100 ± 0.141	39.250 ± 16.617	35.900 ± 4.243
50	0.006 ± 0.002	0.002 ± 0.001	0.450 ± 0.071	0.133 ± 0.115	45.900 ± 9.617	40.350 ± 7.707
6						
10	0.003 ± 0.001	0.002 ± 0.002	0.250 ± 0.071	0.167 ± 0.153	35.400 ± 1.414	37.600 ± 2.546
25	0.003 ± 0.000	0.001 ± 0.001	0.300 ± 0.000	0.100 ± 0.100	37.600 ± 7.495	33.750 ± 1.626
Mytomicin C						
2.5 μg mL^{-1}	0.154 ± 0.022	–	10.475 ± 1.790	–	25.000 ± 4.243	–
Cyclophosphamide						
2.0 μg mL^{-1}	–	0.036 ± 0.022	–	2.100 ± 0.265	–	38.600 ± 0.141

Results are expressed as mean values ± standard deviations (SD) (n = 2). In each experiment, 1000 binuleated cells were analyzed for the presence of micronuclei. Percentage of binucleated cells (BN) was use as index of cell proliferation. Mytomicin C and cyclophospamide were used as positive controls, dose 0 as negative control. Values are not significant ($p > 0.05$).

Table 4. Mutagenic activity of compounds **2**, **3** and **6** in the Ames assay in the presence and absence of metabolic activation (S9) (*Salmonella typhimurium* strains TA 98, 100 and 102)

Dose / (μg *per* plate)	TA 98[a]		TA 100[a]		TA 102[a]	
	−S9	+S9	−S9	+S9	−S9	+S9
2						
0	17.5 ± 3.5	27.5 ± 9.2	130.5 ± 29.0	123.5 ± 20.5	268.0 ± 56.6	342.0 ± 31.1
5	19.05± 2.1	27.0 ± 8.5	125.0 ± 15.6	138.5 ± 0.7	263.5 ± 122.3	375.0 ± 15.6
25	24.0 ± 2.8	22.0 ± 2.8	120.0 ± 22.6	106.5 ± 26.2	266.0 ± 73.5	376.0 ± 36.8
50	19.0 ± 4.2	22.5 ± 3.5	113.0 ± 4.2	114.0 ± 25.5	149.0 ± 103.2	365.0 ± 7.1
250	15.0 ± 4.2	15.5 ± 0.7	116.0 ± 7.1	116.0 ± 28.3	230.5 ± 118.1	342.0 ± 14.1
3						
5	10.5 ± 2.1	23.5 ± 0.7	128.0 ± 11.3	98.0 ± 5.7	254.0 ± 17.0	273.0 ± 52.3
25	16.0 ± 4.2	22.0 ± 8.5	126.5 ± 10.6	120.0 ± 18.4	304.0 ± 50.9	401.0 ± 63.6
50	16.0 ± 2.8	23.0 ± 1.4	121.0 ± 5.7	123.0 ± 7.1	262.0 ± 76.4	348.0 ± 99.0
250	11.0 ± 1.4	20.5 ± 2.1	88.0 ± 11.3	111.5 ± 19.1	212.0 ± 35.4	297.0 ± 9.9
6						
5	20.5 ± 0.7	20.0 ± 2.8	111.5 ± 24.7	127.0 ± 7.1	278.5 ± 33.2	315.5 ± 102.5
25	19.0 ± 1.4	20.0 ± 1.4	123.0 ± 1.4	100.5 ± 14.8	239.5 ± 96.9	276.0 ± 59.4
50	20.5 ± 0.7	19.5 ± 2.1	122.0 ± 1.4	116.5 ± 0.7	244.5 ± 50.2	341.0 ± 89.1
250	12.0 ± 4.2	17.5 ± 2.1	94.0 ± 1.4	45.5 ± 19.1	237.0 ± 91.9	260.5 ± 34.6
Quercetin						
10	284.0 ± 77.7	1314.5 ± 102.5				
4-NQO						
10			1432		2842	

Values are presented as the mean ± standard error (n = 2). Quercetin and 4-NQO were used as positive controls. Values are not significant ($p > 0.05$). [a]Number of revertents; 4-NQO: 4-nitroquinoline-1-oxide.

Table 5. Effect of compounds **2**, **3** and **6** on cell viability of V79 Chinese hamster cells using the MTT assay

Dose / (μg *per* well)	Viability[a] / %		
	2	**3**	**6**
25	88.6 ± 21.4	66.5 ± 26.6	43.0 ± 12.5
50	48.7 ± 39.9	37.6 ± 23.0	35.0 ± 4.1
250	4.2 ± 4.6	10.8 ± 13.0	8.3 ± 5.9

[a]Viability is expressed as percentage values relative to control cells. Results are expressed as mean value viability ± standard deviations (SD) (n = 3). In each independent experiment, four replicate cultures were used.

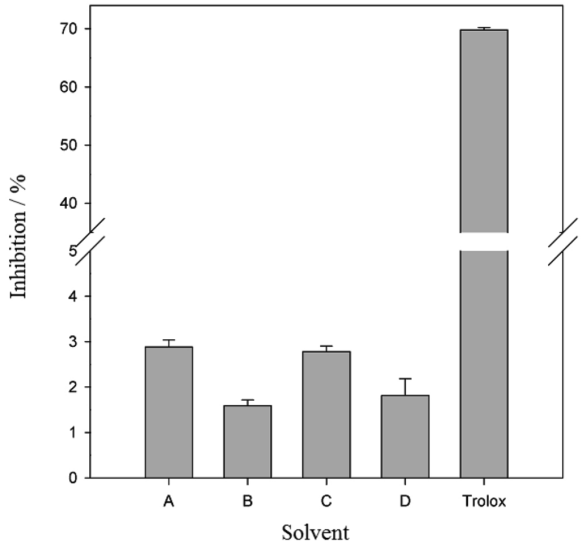

Figure 5. Percentage of inhibition for the DPPH assay (n = 9 ± standard error (SE)) *E. viscosa* extracts in different solvents. A: dichloromethane; B: hexane 1; C: hexane 2; D: ethyl acetate; Trolox® 0.05 mg mL^{-1} as positive control.

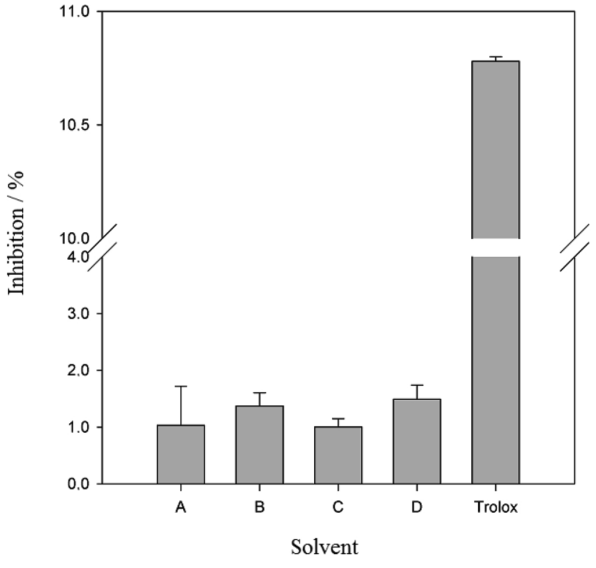

Figure 6. Percentage of inhibition for the ABTS assay (n = 9 ± SE) by *E. viscosa* extracts in different solvents. A: dichloromethane; B: hexane 1; C: hexane 2; D: ethyl acetate; Trolox® 0.05 mg mL^{-1} as positive control.

equivalent antioxidant capacity) values (Figure 7), which are defined as the concentration of Trolox® that has the same activity as 1 mmol L^{-1} of the antioxidant substance investigated. Unfortunately, the TEAC values may not correlate exactly with the antioxidant capacities. There may be a high TEAC value for a compound with a relatively low antioxidant capacity because the products of the reaction of $ABTS^{\bullet+}$ with a given compound can also react with $ABTS^{\bullet+}$, contributing to an increase in the TEAC value. This situation is observed in Figure 7. Thus, it may be concluded that the reaction products are better antioxidants than the initial compounds.[18] The *E. viscosa* ethyl acetate extract had the highest phenolic content, 138.66 ± 0.01 gallic acid equivalents (GAE), in mg g^{-1} of dry extract (Figure 8). Sunflower oil that was submitted to heating after the 8th day resulted in a peroxide value of 105.13 ± 6.07 meq O_2 kg^{-1} oil, but in the presence of dichloromethane and ethyl acetate extracts from *E. viscosa*, this value decreased to 83.70 ± 0.12 meq O_2 kg^{-1} oil and 74.13 ± 0.09 meq O_2 kg^{-1} oil, respectively (Figure 9), showing that these extracts are effective for the protection of sunflower oil from oxidation. Two different populations of *E. viscosa* were collected, in Serra do Cristo Rei, Huíla, Angola[6] in July 2001 and the other in the outskirts of Lubango in July of 2003 originating two different hexane extracts, hexane 1 and 2 respectively. Those extracts presented significant differences in the DPPH (Figure 5) ABTS (Figure 6) and total phenolic content assays (Figure 8), although the most significant difference emerges from the TEAC value pointing out to a possible variation in the chemical composition of the phenolic content.

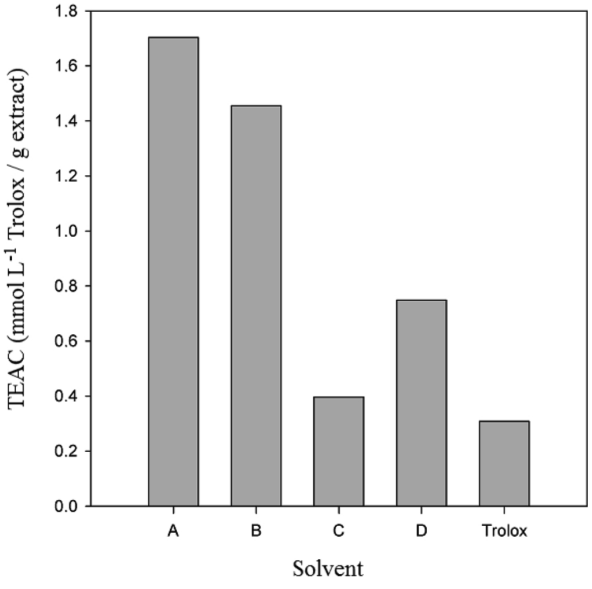

Figure 7. TEAC value for *E. viscosa* extracts in different solvents. A: dichloromethane; B: hexane 1; C: hexane 2; D: ethyl acetate.

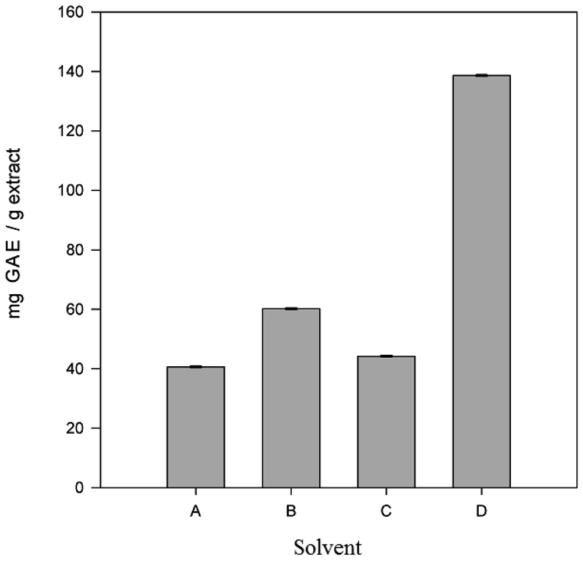

Figure 8. Total phenolic concentration determined by the Folin-Ciocalteau method for *E. viscosa* extracts in different solvents. A: dichloromethane; B: hexane 1; C: hexane 2; D: ethyl acetate.

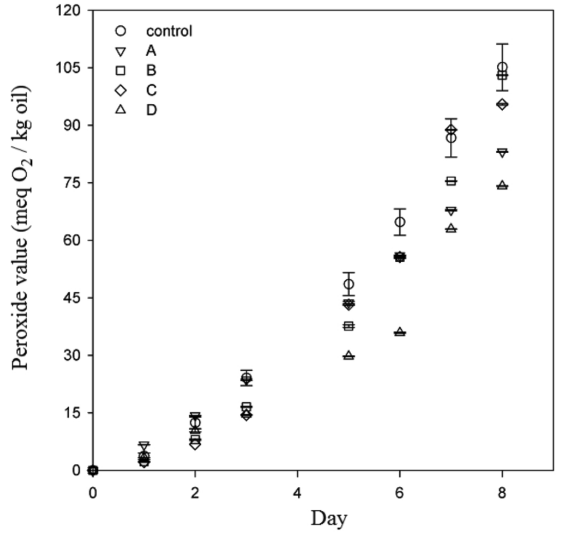

Figure 9. Peroxide value for *E. viscosa* extracts in different solvents, (n = 3 ± SE). A: dichloromethane; B: hexane 1; C: hexane 2; D: ethyl acetate; control: sunflower oil.

Conclusions

The present phytochemical investigation of aerial parts of *E. viscosa* (Retz.) Trin. afforded three new 8α,15-epoxylabdanes **1**, **6** and **7** along with known compounds 8α,15-epoxy-16-norlabdan-13-one (**3**), 8α,15-epoxylabdan-16β-oic acid (**4**), 3β-(3'',4''-dihydroxy)-(*E*)-cinnamoyloxylup-20(29)-ene (**5**), 3-(2',3',4',6'-tetra-*O*-acetyl-β-D-glucopyranosyloxy)-β-sitosterol (**8**) and 16-acetoxy-8,15-epoxylabdane (**2**).

Genotoxicity, mutagenicity and cytotoxicity were tested for compounds **2**, **3** and **6**, genotoxic and mutagenic assays were negative but all compounds proved to be cytotoxic and compound **6** was shown to be the most cytotoxic of the compounds tested.

Antioxidant capacity of the hexane (1 and 2), dichloromethane and ethyl acetate extracts of *E. viscosa* were tested but all extracts presented a very low antioxidant capacity. The ethyl acetate extract had the highest phenolic content of all extracts and dichloromethane and ethyl acetate extracts present the best protection to sunflower oil from oxidation.

Supplementary Information

Crystallographic data (excluding structure factors) for the structure in this work was deposited in the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 860140. 1D and 2D NMR spectra data associated with this article are available free of charge at http://jbcs.sbq.org.br as a PDF file.

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