Available online at www.sciencedirect.com
SCIENCE DIRECT®
PERGAMON
Phytochemistry 64 (2003) 1125–1131
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Diterpenes from *Alomia myriadenia* (Asteraceae) with cytotoxic and trypanocidal activity

Elita Scio[a,b], Antônia Ribeiro[a], Tânia M.A. Alves[a], Alvaro J. Romanha[c], José Dias de Souza Filho[d], Geoffrey A. Cordell[e], Carlos L. Zani[a,*]

[a]*Laboratório de Química de Produtos Naturais, CPqRR-FIOCRUZ, Av. Augusto de Lima, 1715, Belo Horizonte, MG 30190-002, Brazil*
[b]*Departamento de Bioquímica, ICB, Universidade Federal de Juiz de Fora, Juiz de Fora, MG 36036-030, Brazil*
[c]*Laboratório de Parasitologia Celular e Molecular, CPqRR-FIOCRUZ, Av. Augusto de Lima, 1715, Belo Horizonte, MG 30190-002, Brazil*
[d]*Departamento de Química, ICEx, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil*
[e]*Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, IL 60612-7231, USA*

Received 6 January 2003; received in revised form 17 July 2003

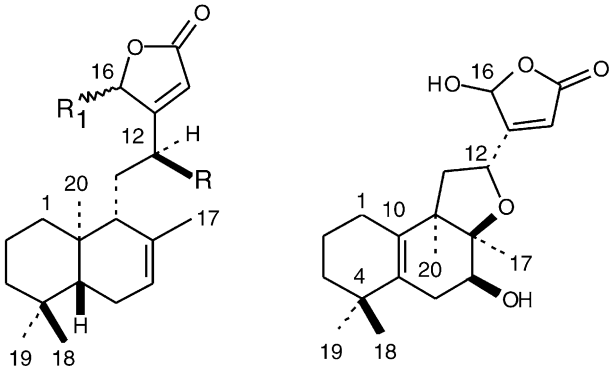
Abstract

Further investigation of the aerial parts of *Alomia myriadenia* revealed an halimane diterpene identified as *ent*-8*S*,12*S*-epoxy-7*R*,16-dihydroxyhalima-5(10),13-dien-15,16-olide along with the known *ent*-16-hydroxylabda-7,13-dien-15,16-olide, *ent*-12*R*-hydroxylabda-7,13-dien-15,16-olide, 6,7-methylenedioxycoumarin (ayapin), and kaempferol-7-methylether (rhamnocitrin). Evaluated in a panel of human cancer cell lines, the 16-hydroxylabade diterpene was the most active, showing an ED_{50} value of 0.3 μg/ml against Lu1 (human lung cancer) cells. Tested in vitro against *Trypanosoma cruzi* in infected murine blood, this compound caused lysis of 100% of the parasites at 250 μg/ml.

Keywords: *Ageratum myriadenium*; *Alomia myriadenia*; Compositae; Halimane; Labdane; *Trypanosoma cruzi*; Human cancer cell line

1. Introduction

In an ongoing program to detect new bioactive natural products, several plants of the Brazilian flora were selected for study after a survey of the NAPRALERT database for the most promising candidates. The ethanol extract of the aerial parts of *Alomia myriadenia* Sch.Bip ex Baker (*syn*. *Ageratum myriadenium* (Baker) R.M. King & H. Rob) showed activity in a preliminary screening with human cancer cell lines, and the labdane diterpene *ent*-12R,16-dihydroxylabda-7,13-dien-15,16-olide (**1**) was found to be the major cytotoxic compound in this extract (Zani et al., 2000). This diterpene was previously described in *Acritopappus hagei* R.M. King & H. Rob (Bohlmann et al., 1980) and *Ageratum fastigiatum* (Gardner) R.M. King & H. Rob (Bohlmann et al., 1981), both from the Asteraceae family. It was found recently that **1** also inhibits the phytohemaglutinin (PHA) stimulated proliferation of human lymphocytes present in peripheral blood mononuclear cells (PBMC) (Souza-Fagundes et al., 2002) via induction of monocyte apoptosis (Souza-Fagundes et al., 2003).



1: R = R_1 = OH
3: R = H, R_1 = OH
4: R = OH, R_1 = H
2

In the present work we report the isolation, using a bioassay-guided fractionation protocol based on

* Corresponding author. Tel.: +55-31-3295-3566; fax: +55-31-3295-3115.
E-mail address: zani@cpqrr.fiocruz.br (C.L. Zani).

0031-9422/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0031-9422(03)00529-6

cytotoxicity against KB and Col2 cells, of a new halimane diterpene (**2**) and four known compounds. Evaluation of the cytotoxicity of these compounds (**3**–**6**) in a panel of human tumour cell lines and their activity against the protozoan parasite *T. cruzi,* the causative agent of Chagas' disease (American trypanosomiasis) is also described.

2. Results and discussion

The aerial parts of *A. myriadenia* were dried, ground, and macerated with ethanol to produce a crude ethanol extract. This extract was partitioned between immiscible solvents to afford fractions of increasing polarity (Zani et al., 2000). Only the medium polarity fraction (CH_2Cl_2) was cytotoxic in the bioassay with KB and Col2 cell lines showing ED_{50} values 9.2 and 2.9 μg/ml, respectively. Chromatographic separation was monitored with the bioassays using these cell lines, to isolate the active compounds. This procedure yielded compounds **2**–**6**, whose structural elucidation was based on the analysis of their spectral data.

Compound **2** is a mixture of epimers, as deduced by a series of double signals in the ^1H and ^{13}C NMR spectra. Its HRFABMS showed $[M+H]^+$ with *m*/*z* 349.1983, in agreement with the molecular formula $C_{20}H_{28}O_5$ (calculated: 349.2015). The presence of an α,β-unsaturated lactone was indicated by an absorption at ν_{max} 1756 cm^{-1} in the IR spectrum and confirmed by the ^1H NMR signals at δ 6.11 (*s*, H-16) and 6.06 (*s*, H-14) and the corresponding ^{13}C NMR signals (C-13 to C-16 in Table 1). The ^{13}C NMR (Table 1) and DEPT spectra showed signals due to 20 carbons comprising four methyl, five methylene, one olefinic methine, three oxymethine, three quaternary carbons, one of them bearing an oxygen atom, a carbonyl carbon, and three fully substituted olefinic carbons. These fragments account for a partial molecular formula $C_{20}H_{26}O_5$, indicating that the two remaining hydrogen atoms are present in hydroxyl groups.

The α,β-unsaturated γ-lactone moiety is responsible for three of the seven unsaturations calculated for the molecule. Another unsaturation is due to a double bond, as deduced by ^{13}C NMR and DEPT analyses, and the remaining three are due to three ring systems. Furthermore, since the lactone ring and the two hydroxyl groups known to be present sum four oxygen atoms, the last oxygen to complete the molecular formula must be part of a ring. These inferences and a detailed analysis of the ^1H–^1H COSY, HMQC, and HMBC spectra (Table 1) and comparison with literature data suggested a halimane-type diterpene carrying a hydroxylated α,β-unsaturated γ-lactone moiety (Hara et al., 1995; Teresa et al., 1983).

The formation of a tetrahydrofuran ring involving C-8 and C-12 (δ 73.0) was deduced on the basis of the oxygenation of these carbons, their multiplicities and the HMBC and COSY correlations with their neighbours. In the HMBC contour map, the quaternary oxygenated carbon C-8 showed correlations to Me-20, Me-17, and H-11. In the ^1H–^1H COSY contour plot the methine H-12 showed correlations to both H-11α and β. A HMBC correlation between C-12 and H-14 was also observed. The decalin ring system contains a double bond between C-5 and C-10. C-5 showed HMBC correlations to H-3, H-6, Me-18 and Me-19, while C-10 was correlated with H-1, H-2, H-11, and Me-20. In the HMBC contour plot, the C-7 oxymethine showed a correlation to Me-17, while the ^1H–^1H COSY contour map revealed correlations between H-7 and the methylene protons at C-6. All these data are compatible with the structure **2**.

The determination of the absolute stereochemistry of **2** by NMR analysis of its Mosher esters (Latypov et al., 1996) was precluded by the limited amount of this natural product. However, its relative stereochemistry was deduced on the basis of the following analysis. ^1H NMR signals assignments of both 5α- (Elgamal et al., 1999) and 5β-cardenolides (Hanna et al., 1998, 1999) shows clearly that a β-oriented butenolide at C-17 protects the vicinal H-16β hydrogen, that resonates around 1.9 ppm, while its geminal H-16α gives a signal near 2.1 ppm. Using this system as a model, if we assume that the butenolide at C-12 in **2** is β oriented, it can be deduced that the vicinal H-11 resonating at δ 1.85 is also β-oriented. NOESY experiments show the spatial proximity between this hydrogen (H-11β) and the C-20 methyl, which also correlates with the vicinal C-17 methyl that, on the other side, interacts with the oxymethine proton H-7, thus indicating that they are all β-oriented. In this way, except for C-16, the relative stereochemistry of all chiral centers of 2 was established and the molecule was identified as 8*S*,12*S*-epoxy-7*R*,16-dihydroxyhalima-5(10),13-dien-15,16-olide. The absolute stereochemistry was not established but compound **2** probably belongs to the *ent*-series in view of its co-occurrence with *ent*-labdanes. Table 1 shows the proton and carbon assignments, including the signals of each epimer that could be distinguished.

The ^{13}C NMR spectrum of compound **3** showed double peaks in the low field region of the ^1H NMR spectrum, again suggesting a mixture of epimers at C-16. The data listed for **3** in Table 1 are in agreement with those published for the epimeric mixture of *ent*-16-hydroxylabda-7,13-dien-15,16-olides, previously isolated as their acetates from *A. fastigiatum* (Bohlmann et al., 1981). These acetates were dextrorotatory ($[\alpha]_{589}^{20}+7°$, $c=0.5$, $CHCl_3$) and classified as *ent*-labdanes. As our mixture is also dextrorotatory ($[\alpha]_D$ $+40.9°$), we assumed that **3** also belonged to this enantiomeric series.

Compound **4** was isolated as white crystals, and its ^1H and ^{13}C NMR spectra were similar to those of compound **3**, except for the absence of duplicate signals,

Table 1
^{1}H and ^{13}C NMR spectral data (δ/ppm) for compounds **2**, **3** and **4**

Position	**2**				**3**				**4**			
	δ_c[a]	δ_H	HMBC (H to C)	NOESY	δ_c	δ_H	HMBC (H to C)	NOESY	δ_c	δ_H	HMBC (H to C)	NOESY
1	26.4 (2)	β 2.02 (*m*) α 1.90 (*m*)	C-10	Me-20	39.3 (2)	*eq* 1.84 (*m*) *ax* 0.95 (*m*)	C-20	Me-20	39.8 (2)	*eq* 1.65 (*m*) *ax* 0.84 (*m*)		Me-20 H-$3_{ax\xi}$
2	19.9 (2)	1.62 (*m*)	C-4, C-10		18.7 (2)	1.48 (*m*)		H20	19.0 (2)	1.55 (*m*) 1.44 (*m*)		
3	39.5 (2)	α 1.52 (*m*)[b] β 1.45 (*m*)[b]	C-4, C-5, C-18	Me-18	42.1 (2)	*eq* 1.45 (*m*) *ax* 1.17 (*m*)	C-4,C-19		42.3 (2)	*eq* 1.41 (*m*) *ax* 1.14 (*m*)	C-1 C-4, 119	
4	34.4 (0)				32.9 (0)	–			33.3 (0)	–		
5	135.4 (0)				50.0 (1)	1.19 (*m*)	C-4, C-9, C-10, C-18, C-19, C-20	H-6β, Me-18, H-9	50.4 (1)	1.20 (*dd*, 5, 11)	C-4, 6, 9, 10, 11, 18, 19, 20	H-6β, Me-18
6	30.1 (2)	β 2.33 (*dd*, 10.5, 5.4)[b] α 2.13 (*dd*, 11.4, 10.5)[b]	C-5	Me-18	24.4 (2)	β 1.97 (*m*) α 1.85 (*m*)		Me-18, H-5 Me-19, Me-20	24.2 (2)	β 2.01 (*m*) α 1.85 (*m*)		 Me-19, Me-20
7	71.5 (1)	3.56 (*dd*,10.5, 5.4)		Me-17	123.4 (1)	5.45 (br*s*)			124.2 (1)	5.49 (m)		
8	86.3 (0)				133.8 (0)	–			133.6 (0)	–	–	
9	51.7				54.4 (1)	1.69 (*m*)	C-7, C-8, C-9		50.8 (1)	1.72 (*m*)	C-10	
10	128.7 (0)				36.8 (0)				37.2 (0)			
11	41.4 (2)	*Pseudo-eq* (α) 2.43 (*dd*, 12.7, 5.5) [2.31 (*dd*, 12.7, 5.4)][c] *Pseudo-ax* (β) 1.80 (*dd*, 12.7, 10.8) [1.86 (*m*, 12.7, 10.6)][c]	C-10 C-8	 Me-20	24.4 (2)	1.74 (*m*) 1.70 (*m*)	C-9, C-10, C-12		34.4 (2)	1.75 (*m*) 1.73 (*m*)	C-8, C-12, C-13	
12	73.0 (1)	*Pseudo-ax* (α)4.44 (*dd*, 10.8, 5.5) [4.53 (*dd*, 10.6, 5.4)][c]			29.6 (2)	2.55 (*m*) 2.37 (*m*)	C-13, C-14		70.6 (1)	4.76 (*m*)		
13	170.1 (0)				169.5 (0)				171.9 (0)			
14	117.9 (1)	6.06 (*sl*) [6.02 (*s*)][c]	C-12, C-13/15		117.3 (1)	5.87 (*m*)	C-15, C-16	H-12, Me-17, H11	116.2 (1)	5.97, 1H, *s*	C-12, C-15	H-12, Me-17, H11
15	170.1 (0)				171.2 (0)				173.9 (0)			
16	97.4 (1)	6.11 (*s*) [6.06 (*sl*)][c]	C-15		98.6 (1)	6.01 (br*d*, 7.4)	C-15	H-11, H20, H-1, H17	71.2 (2)	4.98 (*dd*, 17, 1.7) 4.90 (*dd*, 17, 1.4)	C-13, C-14, C-13	H-11, H20, H-1, H17
17	22.1 (3)	1.29 (*s*)	C-7, C-8, C-9	H-7, Me-20	22.0 (3)	1.69 (br*s*)	C-7, C-8		23.1 (3)	1.73 (*m*)	C-7,C-8, C-9	
18	28.0 (3)	α 1.04 (*s*)	C-3, C-4, C-5, C-18	H-6α	21.8 (3)	α 0.88 (*s*)	C-18	H-6α	22.2 (3)	α 0.88 (*s*)	C-18	H-6α
19	28.7 (3)	β 1.00 (*s*)	C-3, C-4, C-5, C-19	H-6β	33.0 (3)	β 0.86 (*s*)	C-19, C-3, C-5	H-6β, H-5	33.4 (3)	β 0.86 (*s*)	C-19, C-3, C-5	H-6β, H-5
20	20.8 (3)	β 1.12 (*s*)	C-8, C-9, C-10, C-11	Me-17 Me-18 H11β	13.6 (3)	0.78 [0.77]	C-1, C-5, C-9, C-10	H-1_{eq}, H-6α, H-11, Me-19	14.1 (3)	0.77 (*s*)	C-1,C-9, C-10	H-1_{eq}, H-6α H-11, Me-19
OH	α in C-7 C-16				at C-16	4.11 (*d*, 7.4)	C-13, C-16		at C-12	2.15 (*d*, 4.2)		

[a] Obtained in $CDCl_3$ at 300 MHz for ^{1}H and 75 MHz for ^{13}C. Chemical shifts are indicated in δ (ppm) and coupling constants (*J* in Hz) are in parenthesis. NOESY experiments were run in $CDCl_3$ at 400 MHz. Carbon type, as determined from DEPT spectra, are represented in parenthesis at the right of their respective chemical shift: 0 = quaternary, 1 = methine, 2 = methylene, 3 = methyl.

[b] Proton assignments are interchangeable within the same carbon.

[c] Values for the chemical shifts and *J* for the second epimer present in the mixture that presented distinct signal.

indicating that the center of epimerization no longer exists in **4**. All the data (Table 1), including NOE correlations observed in the NOESY experiment, are compatible with the structure *ent*-12-hydroxylabda-7,13-dien-15,16-olide, previously isolated from *Acritopappus confertus* (Bohlmann et al., 1983). As the coupling constants of H-12 are similar to those of the corresponding proton in the previously isolated labdane **2**, it is plausible to assume that the configuration at this center is also *S*. On the basis of this analysis the diterpene **4** was identified as *ent*-12*R*-hydroxylabda-7,13-dien-15,16-olide. The ^{13}C NMR spectroscopic assignments for **3** and **4** are reported here for the first time. No study concerning the biological activities of these diterpenes was found in the literature.

Based on spectral analysis and comparison with reported data, compound **5** was characterised as ayapin (Debenedetti et al., 1998) and compound **6** as rhamnocitrin (Lin et al., 1991). The presence of a methoxyl group at C-7 in the latter compound was confirmed by the absence of bathochromic shift of band II after the addition of NaOAc, and by the fragments of *m*/*z* 121 ($C_7H_5O_2$) and 167 ($C_8H_7O_4$) recorded in the EIMS spectrum. Ayapin 5 is also known to occur in *A. fastigiata* Benth (Pozetti, 1966). This coumarin was shown to decrease the blood coagulation time in guinea pigs and dogs (Souza and Pozetti, 1974), and to display trypanocidal (Lopes et al., 1997), antifungal (Tal and Robeson, 1986) and antibacterial (Kashihara et al., 1986) activities. Rhamnocitrin 6 is reported to show several biological activities, including strong inhibition of platelet aggregation induced by arachidonic acid (Okada et al., 1995), and anti-inflammatory and antispasmodic activities (Ram et al., 1989).

Table 2 summarises the results of the bioassays of the CH_2Cl_2 fraction and the isolated compounds. Compound **3** was the most active in a panel of human cancer cell lines, displaying strong activity against the Lu1 cell line (ED_{50} 0.3 μg/ml). All other compounds showed ED_{50} values higher than 10 μg/ml in this test system. After the bioassay-guided fractionation using the cancer cell lines Col2 and KB was completed, in vitro assay with the trypomastigote form of *T. cruz*i was next performed. Tested in this assay at 250 μg/ml and 24 h contact at 4 °C, compounds **3** and **4** caused the lysis of 100 and 99% of the parasites, respectively. This potency is comparable to that of gentian violet, used at 250 μg/ml to treat infected-banked blood in order to clear it from the parasites. However, as shown above, these compounds, especially **3**, are cytotoxic at much lower concentrations, a fact that poses a serious obstacle to their potential use to disinfect human blood before transfusion.

Table 3 summarises the results of the bioassays for the three labdane diterpenes isolated from *A. myriadenia* (**2–4**). The only differences between the compounds are the number and positions of the hydroxyl groups. Although the number of compounds considered is small, it seems that the presence of a 16-OH group is a relevant feature for their cytotoxic activity. In the case of anti-*T. cruzi* activity, compounds **3** and **4** were active, with each having only one hydroxyl at different positions.

This work disclosed a new halimane type diterpene as well the strong and selective activity of **3** on Lu1 cell line, suggesting that this class of compound is an interesting system for further development. Preliminary investigations on its mode of action are underway. Studies on this species aiming at further minor components, as well as new biological activities of the isolated compounds, are in progress.

3. Experimental section

3.1. General experimental procedures

Melting points were determined using a Fisher-Johns MP apparatus and are uncorrected. The UV spectra

Table 2
Activity of the ethanol extract and pure compounds from *Alomia myriadenia* against human cancer cell lines, and *Trypanosoma cruzi*

Sample	Cell line[a]							*T. cruzi*[b]
	Lu1	Col2	KB	LN CaP	KB VI$^-$	KB VI$^+$	BC1	% Lysis at 250 μg/ml
CH_2Cl_2 fraction	NT	2.9	9.2	NT	NT	NT	NT	10
1	3.9	3.7	2.4	3.0	9.1	8.6	4.4	25
2	15.4	14.8	16.9	13.7	NT	NT	NT	NT
3	0.3	1.2	1.7	4.2	1.4	9.9	2.6	100
4	–	–	–	–	–	–	–	99
5	–	–	–	–	–	–	–	NT
6	–	–	–	–	–	–	–	NT
Ellipticine	0.02	0.3	0.04	0.8	0.3	0.2	0.5	NT

[a] Lu1 (lung), Col2 (colon), KB (oral epidermoid), LNCaP (prostate). KB VI$^+$ (oral epidermoid in presence of 1 μg/ml vinblastine) e KB VI$^-$ (oral epidermoid in absence of vinblastine). Results expressed as ED_{50} (effective dose to inhibit cell proliferation in 50%). The minus (–) sign means inactive at the highest dose used (20 μg/ml). NT means not tested.

[b] Trypomastigote form of *T. cruzi* Y strain in infected murine blood. Results are expressed as percent of lysis of parasites after 24 h contact at 4 °C. Gentian violet at 7.5 μg/ml was used as positive control (50% lysis).

Table 3
Structure activity relationships of labdane diterpenes isolated from *A. myriadenia*

Diterpene	12-OH	16-OH	Cytotoxic activity[a]	Trypanocidal activity[b]
1	+	+	+	−
3	−	+	+	+
4	+	−	−	+

[a] ED50 < 4 μg/ml for at least one cell line.
[b] Lysis of > 90% of the parasites at 250 μg/ml.

were obtained on a Beckman DU-7 spectrophotometer. NMR spectra were recorded with a Bruker Avance DPX-300 spectrometer at 300 MHz with TMS as the internal standard. NOESY spectra were obtained at 400 MHz. IR spectra were recorded on a Mattson-Galaxy series FTIR 3000, and optical rotations were measured on a Perkin-Elmer 241 polarimeter. EIMS (70eV) and HRFABMS were recorded on a Finnigan MAT 90 spectrometer, ESI-MS was recorded on a Hewlett Packard 5989B single quadrupole mass spectrometer coupled with a 59987A electrospray interface and a Hitachi HPLC L-7100 system. Analytical and semi-preparative HPLC were run on a Shimadzu chromatograph equipped with a LC-6AD pump and a UV detector at 210 and 254 nm. Analytical (4.6×250 mm) and semi-prep (20×250 mm) RP-18 columns (Shimpack prep-ODS kit) were used.

3.2. Plant material

A. myriadenia was collected near Minduri, Minas Gerais, Brazil, in June, 1997. A voucher specimen (BHCB 42865) was deposited at the Herbarium of the Biological Sciences Institute of the Federal University of Minas Gerais, Belo Horizonte, Brazil.

3.3. Extraction and bioassay-guided fractionation

The aerial parts of *A. myriadenia* (1 kg) were powdered, macerated in EtOH, and partitioned successively against hexane and CH_2Cl_2 (Zani et al., 2000). The CH_2Cl_2 fraction (21.1 g) was cytotoxic to the KB (human oral epidermoid carcinoma, ED_{50} 9.2 μg/ml) and Col2 (human colon cancer, ED_{50} 2.9 μg/ml) cell lines and was subjected to medium pressure chromatography. Sixteen fractions were obtained using a Büchi column (25×450 mm) and elution with mixtures of CH_2Cl_2-EtOAc of increasing polarity. Fractions 4 (152 mg), 7 (365 mg), 8 (331 mg), and 12 (490 mg) were the most active against the above cell lines and were selected for further fractionation by HPLC. A Shimadzu chromatograph equipped with a LC-6AD pump and a UV detector set at 210 and 254 nm was used. Samples were injected into Analytical (4.6×250 mm) or semi-prep (20×250 mm) RP-18 columns (Shimpak prep-ODS kit) and eluted with different proportions of CH_3CN-H_2O. Fraction 4 afforded compound **5** (13 mg); fraction 7 afforded compounds **3** (19.4 mg) and **6** (7.4). Fraction 8 gave compound **4** (30.4 mg) and fraction 12 yielded compound **2** (2 mg).

Compound **2** was isolated as a white amorphous powder; $[\alpha]_D$ −110° ($CHCl_3$, *c* 0.05); UV (MeOH) λ_{max} (log ε) 205 (4.09), 230 (3.70) sh; IR ν_{max} cm^{-1}: 1756; For ^1H NMR ($CDCl_3$; 300 MHz) and ^{13}C NMR (75 MHz), see Table 1; HRFABMS *m*/*z* 349.19826 $[M+H]^+$ (calc. for $C_{20}H_{29}O_5$, 349.20150); 349.2 (6), 331.2 (12), 307.2 (15), 205.2 (20), 154.1 (100), 136.1 (76), 91 (35), 54.8 (42).

Compound **3** was obtained as white crystals from MeOH–H_2O; mp 128–129 °C; $[\alpha]_D$ +40.9° (MeOH, *c* 0.21); UV (MeOH) λ_{max} (log ε) 213 (3.83); IR ν_{max} cm^{-1}: 3395, 2957, 1752; For ^1H NMR ($CDCl_3$; 300 MHz) and ^{13}C NMR (75 MHz), see Table 1; EIMS (70 eV) *m*/*z* 318 $[M]^+$. $C_{20}H_{30}O_3$; 318 (5), 300 (2), 285 (3), 267 (3); 205 (6); 195 (35); 177 (52). ESI-MS (negative mode) $[M–H]^-$ (*m*/*z* 317).

Compound **4** was purified as white crystals from MeOH–H_2O; mp 108–110 °C; $[\alpha]_D$ −5.4° ($CHCl_3$, *c* 0.19); UV (MeOH) λ_{max} (log ε) 208 (4.12); IR ν_{max} cm^{-1}: 3430, 2981, 1780, 1747; For ^1H NMR ($CDCl_3$; 300 MHz) and ^{13}C NMR (75 MHz), see Table 1; EIMS (70 eV) *m*/*z* 318 $[M]^{+\cdot}$. $C_{20}H_{30}O_3$; 318 (0.4), 303 (1), 205 (4), 195 (60). ESI-MS (negative mode) $[M–H]^-$ (*m*/*z* 317).

Compound **5** was obtained as pale yellow crystals from MeOH; mp 222–223 °C; UV (MeOH) λ_{max} (log ε) 205 (4.26), 233 (4.12), 345 (3.99); IR ν_{max} cm^{-1}: 1704; ^1H NMR ($CDCl_3$; 300 MHz) δ 7.58 (1H, *d*, *J* = 9.5 Hz, H-4), 6.83 (1H, *s*, H-5 and H-8), 6.28 (1H, *d*, *J* = 9.5, H-3), 6.07 (2H, *s*, H-9); ^{13}C NMR (75 MHz) δ 161.2 (C-2), 151.2 (C-7), 144.8 (C-8a), 143.4 (C-4 and C-6), 113.4 (C-3), 112.6 (C-4a), 105.0 (C-5), 102.3 (C-9), 98,4 (C-8); EIMS *m*/*z* 190 $[M]^+$. $C_{10}H_6O_4$, 190 (100), 162 (84), 161 (51), 104 (6).

Compound **6** was isolated as a yellow amorphous powder, mp 225–227 °C; UV (MeOH) λ_{max} (log ε) 364 (3.16), 267 (3.27), 238 (3.30); IR ν_{max} cm^{-1}: 2982; ^1H NMR (DMSO; 300 MHz); δ 12.46 (1H, *s*, OH-5), 10.12 (1H, *s*, OH-3), 9.51 (1H, *s*, OH-4′), 8.09 (2H, *d*, *J* = 8.7 H-2′ and H-6′), 6.92 (2H, *d*, *J* = 8.7, H-3′ and 5′), 6.73 (1H, *d*, *J* = 1.8, H-8), 6.34 (1H, *d*, *J* = 1.8, H-6), 3.85 (3H, *s*, OCH_3-7); EIMS (70 eV) *m*/*z* 300 $[M]^+$. $C_{16}H_{12}O_6$; 300 (100), 257 (19), 167 (5), 121 (46). ESI-MS (negative mode) $[M-H]^-$ (*m*/*z* 299).

3.4. Cytotoxicity activity

The cytotoxicity assays were run according to established protocols (Likhitwitayawuid et al., 1993). To monitor the fractionation process, KB and Col2 tumor

cell lines were used. Tests with pure compounds were performed with BC1 (human breast cancer), Lu1 (human lung cancer), Col2 (human colon cancer), LNCaP (human prostate cancer), KB (human oral epidermoid carcinoma), KB-VI$^-$ (KB in the presence of 1 μg/ml vinblastine) and KB-VI$^+$ (KB in the absence of vinblastine).

3.5. Trypanocidal activity

The assays with *T. cruzi* were carried out using blood from Swiss albino mice collected in the parasitaemia peak (7th day) after infection with the Y strain of *T. cruzi*. The infected blood was diluted with normal murine blood to the concentration of 2×10^6 trypomastigotes/ml. Stock solutions (20 mg/ml) of the compounds were prepared in dimethylsulfoxide (DMSO). A sample (5.0 μl) of each solution was added to 195 μl of infected blood providing a final concentration of 500 μg/ml. Samples of 100 μl were transferred in duplicate to the wells of a microtitre plate (96 wells). To reproduce the blood bank conditions, plates were incubated at 4 °C for 24 h. The experiments were repeated two or three times. The parasite concentration was evaluated according to the procedure described by Brener (1962) DMSO at 2.5% v/v and gentian violet at its IC_{50} concentration (7.5 μg/ml) were used as negative and positive controls, respectively. The trypanocidal activity was expressed in percent reduction of the parasite number (lysis) comparing the wells with the samples to the wells with DMSO alone. DMSO at 2.5% causes no harm to the parasites, erythrocytes, or leukocytes. The samples that caused about 100% reduction in parasite number were tested at lower concentrations.

Acknowledgements

The authors thank to Rosana O. Alves for technical assistance with *T. cruzi*; Dr. J. M. Pezzuto and the staff of the Bioassay Research Facility, PCRPS-UIC for the cytotoxity assays and Ms. Mary Lou Quinn for access to the NAPRALERT database; to CAPES, CNPq, and Fulbright for fellowships; and to Pronex and Fiocruz for their financial support.

References

Bohlmann, F., Ahmed, M., King, R.M., Robinson, H., 1981. Labdane and eudesmane derivatives from *Ageratum fastigiatum*. Phytochemistry 20, 1434–1435.

Bohlmann, F., Zdero, C., Gupta, R.K., King, R.M., Robinson, H., 1980. Diterpenes and tetranorditerpenes from *Acritopappus* species. Phytochemistry 19, 2695–2705.

Bohlmann, F., Zdero, C., Jakupovic, J., King, R.M., Robinson, H., 1983. 459. Diterpenes from *Acritopappus confertus*. Phytochemistry 22, 2243–2252.

Brener, Z., 1962. Therapeutic activity and criterion of cure on mice experimentally infected with *Trypanosoma cruzi*. Rev. Inst. Med. Trop. São Paulo 4, 389–396.

Debenedetti, S.L., Nadinic, E.L., Coussio, J.D., De Kimpe, N., Boeykens, M., 1998. Two 6,7-dioxygenated coumarins from *Pterocaulon virgatum*. Phytochemistry 48, 707–710.

Elgamal, M.H.A., Hanna, A.G., Morsy, N.A.M., Duddeck, H., Simon, A., Gáti, T., Tóth, G., 1999. Complete ^{1}H and ^{13}C signal assignments of 5α-cardenolides isolated from *Calotropis procera* R. Br. J. Mol. Struct. 477, 201–208.

Hanna, A.G., Elgamal, M.H.A., Hassan, A.Z., Duddeck, H., Simon, A., Kovács, J., Tóth, G., 1998. Complete ^{1}H and ^{13}C signal assignments of 5β-cardenolides isolated from *Acokanthera spectabilis* Hook F. Magn. Res. Chem. 36, 936–942.

Hanna, A.G., Elgamal, M.H.A., Morsy, N.A.M., Duddeck, H., Kovács, J., Tóth, G., 1999. Two cardenolides from *Calotropis procera*. Magn. Res. Chem. 37, 754–757.

Hara, N., Asaki, N., Fujimoto, Y., Gupta, Y.K., Singh, A.K., Sahai, M., 1995. Clerodane and *ent*-halimane diterpenes from *Polyalthia longifolia*. Phytochemistry 38, 189–194.

Kashihara, M., Ishiguro, K., Yamaki, M., Takgi, S., 1986. Antimicrobial constituents of *Euphorbia humifusa* Willd. Shoyakugaku Zasshi 40, 427–428.

Latypov, S.K., Seco, J.M., Quiñoá, E., Riguera, R., 1996. MTPA vs MPA in the determination of absolute configuration of chiral alcohols by ^{1}H NMR. J. Org. Chem. 61, 8569–8577.

Likhitwitayawuid, K., Angerhofer, C.K., Cordell, G.A., Pezzuto, J.M., Ruangrungsi, N., 1993. Traditional medicinal-plants of Thailand. 20. Cytotoxic and antimalarial bisbenzylisoquinoline alkaloids from *Stephania erecta*. J. Nat. Prod. 56, 30–38.

Lin, C.N., Chung, M.I., Gan, K.H., Lu, C.M., 1991. Flavonol and anthraquinone glycosides from *Rhamnus formosana*. Phytochemistry 30, 3103–3106.

Lopes, J.L.C., Dias, D.A., Albuquerque, S., Cunha, W.R., 1997. Trypanocidal activity of *Alomia fastigiata*. Fitoterapia 28, 182–183.

Okada, Y., Miyauchi, N., Suzuki, K., Kobayashi, T., Tsutsui, C., Mayuzumi, K., Nishibe, S., Okuyama, T., 1995. Search for naturally-occurring substances to prevent the complications of diabetes. 2. Inhibitory effect of coumarin and flavonoid derivatives on bovine lens aldose reductase and rabbit platelet-aggregation. Chemical & Pharmaceutical Bulletin 43, 1385–1387.

Pozetti, G.L., 1966. Contribuição ao estudo da composição química de *Alomia fatigiata* benth. Rev. Fa. Farm. Odontol. Ribeir.ão Preto USP 4, 137–140.

Ram, S.N., Dwivedi, S.P.D., Pandey, V.B., Rao, Y.V., 1989. Pharmacological actions of kaemperol-7-methyl ether isolated from *Rhamnus triquerta*. Fitoterapia 60, 273–274.

Souza, J.A., Pozetti, G.L., 1974. Hemocoagulant activity of ayapin obtained from *Alomia fatigiata*. Rev. Fac. Farm. Odontol. Ribeirão Preto USP 8, 123–127.

Souza-Fagundes, E.M., Gazzinelli, G., Parreira, G.G., Martins-Filho, O.A., Amarante-Mendes, G.P., Correa-Oliveira, R., Zani, C.L., 2003. In vitro activity of labdane diterpene from *Alomia myriadenia* (Asteraceae): immunosuppression via induction of apoptosis in monocytes. Int. Immunopharmacol. 3, 383–392.

Souza-Fagundes, E.M., Queiroz, A.B., Martins Filho, O.A., Gazzinelli, G., Correa-Oliveira, R., Alves, T.M., Zani, C.L., 2002. Screening and fractionation of plant extracts with antiproliferative activity on human peripheral blood mononuclear cells. Mem. Inst. Oswaldo Cruz 97, 1207–1212.

Tal, B., Robeson, D.J., 1986. The induction, by fungal inoculation, of ayapin and scopoletin biosynthesis in *Helianthus annuus*. Phytochemistry 25, 77–79.

Teresa, J.D., Urones, J.G., Marcos, I.S., Bermejo, F., Basabe, P., 1983. A rearranged labdane; salmantic acid from *Cistus laurifolius*. Phytochemistry 22, 2783–2785.

Zani, C.L., Alves, T.M., Queiroz, R., Fontes, E.S., Shin, Y.G., Cordell, G.A., 2000. A cytotoxic diterpene from *Alomia myriadenia*. Phytochemistry 53, 877–880.