Phytochemistry 71 (2010) 1539–1544

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Sesquiterpene lactones from *Vernonia scorpioides* and their *in vitro* cytotoxicity

Humberto Buskuhl [a], Fabio L. de Oliveira [a], Luise Z. Blind [a], Rilton A. de Freitas [a], Andersson Barison [b], Francinete R. Campos [b], Yuri E. Corilo [c], Marcos N. Eberlin [c], Giovanni F. Caramori [d], Maique W. Biavatti [e,*]

[a] Curso de Farmácia, Centro de Ciências da Saúde, Universidade do Vale do Itajaí (UNIVALI), Itajaí, SC, Brazil
[b] Departamento de Química, Centro Politécnico, Universidade Federal do Paraná (UFPR), Curitiba, PR, Brazil
[c] Instituto de Química, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brazil
[d] Departamento de Química, CFM, Universidade Federal de Santa Catarina (UFSC), Florianópolis, SC, Brazil
[e] Departamento de Ciências Farmacêuticas, CCS, Universidade Federal de Santa Catarina (UFSC), Florianópolis, SC, Brazil

ARTICLE INFO

Article history:
Received 27 May 2009
Received in revised form 5 March 2010
Available online 3 July 2010

Keywords:
Vernonia scorpioides
Asteraceae
Hirsutinolide
Glaucolide
Piptocarphol esters
Luteolin
Apigenin
Ethyl caffeate
Cytotoxicity

ABSTRACT

Fresh leaves of *Vernonia scorpioides* are widely used in Brazil to treat a variety of skin disorders. Previous *in vivo* studies with extracts of this species had also demonstrated a high antitumor potential. This paper reports isolation of four sesquiterpene lactones (hirsutinolides and glaucolides), together with diacetylpiptocarphol, 8-acetyl-13-etoxypiptocarphol, luteolin, apigenin, and ethyl caffeate from fresh leaves and flowers of *Vernonia scorpioides*. The hypothesis that hirsutinolide **3** is formed during extraction was verified theoretically using Density Functional Theory. The effects of isolated compounds on *in vitro* tumor cells were investigated, as well as their genotoxicity by means of an *in vitro* comet assay. The results indicate that glaucolide **2** and hirsutinolide **4** are toxic to HeLa cells. These compounds were genotoxic *in vitro*, a property that appears to be related to the presence of their epoxy groups, which has been a more reliable indication of toxicity than substitution on C-13 or the presence of α,β-unsaturated keto-groups. These results need to be replicated *in vivo* in order to ascertain their toxicity.

1. Introduction

Vernonia scorpioides (Lam.) Pers., Asteraceae, is well known in Brazil as *Piracá*, *Enxuga* or *Erva-de-São-Simão*, and usually grows in poor, deforested, neotropical soils (Cabrera and Klein, 1980). Topical use of the alcohol extract from fresh leaves is widespread for treatment of various skin disorders, including chronic wounds and ulcers. Previous studies using *V. scorpioides* crude extracts, and its chloroform and hexane derived fractions, have shown fungicidal and moderate bactericidal activities (Freire et al., 1996), as well as mild wound healing (Leite et al., 2002) and antitumor effects (Pagno et al., 2006).

Members of the genus *Vernonia* are good sources of sesquiterpene lactones (SLs). These are often members of the highly oxygenated family of germacranolides, such as glaucolides and hirsutinolides, but the cadinanolides have also been reported (Costa et al., 2005). Hirsutinolides have been reported as having cytotoxic (Chen et al., 2005), antibacterial and anti-inflammatory properties (Kos et al., 2006), as well as antiplasmodial (Chea et al., 2006; Pillay et al., 2007) effects. Glaucolides have properties including those of smooth muscle relaxants (Campos et al., 2003) and phytogrowth inhibitors (Barbosa et al., 2004), as well as weak cytotoxic (Williams et al., 2005) and molluscicidal effects (Alarcon et al., 1990). Some SLs from *V. scorpioides* have also been reported (Drew et al., 1980; Jakupovic et al., 1985; Warning et al., 1987), but no bioactivity for any compound isolated from *V. scorpioides* has been reported to date.

Hirsutinolides have also been named piptocarphol esters, and it has been proposed that they are formed by rearrangement of glaucolides when exposed to silica gel in alcohol solution during chromatographic separations (Catalán et al., 1986). However, some authors report that hirsutinolides are probably natural products, as they were detected in fractions before silica gel column chromatography with methanol, and also without the use of silica gel in the absence of either MeOH or EtOH (Kos et al., 2006).

The present paper reports isolation of four new sesquiterpene lactones (hirsutinolides and glaucolides), together with diacetylpiptocarphol, 8-acetyl-13-etoxypiptocarphol, luteolin, apigenin and ethyl caffeate from an ethanol extract of fresh leaves and flowers of *Vernonia scorpioides*. The effect of the isolated compounds on a tumor cell line and their genotoxic properties were investigated.

* Corresponding author. Address: UFSC/CCS/CIF, Campus Universitário Trindade, 88040-900 Florianópolis, SC, Brazil. Tel./fax: +55 48 3721 5075/9542.
E-mail address: maique@ccs.ufsc.br (M.W. Biavatti).

0031-9422/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2010.06.007

2. Results and discussion

The EtOH extract of leaves and flowers of *V. scorpioides* were partitioned with solvents of increasing polarity. The resulting CH_2Cl_2 extract exhibited cytotoxic activity (with an IC_{50} of 0.7 μg/mL; 55.1 μg/mL and >100 μg/mL on HeLa, L929 and B16F10 cells, respectively). This extract was then subjected to SiO_2 flash chromatography and the sesquiterpene lactone-rich fractions obtained were further purified to isolate sesquiterpene lactones **1**–**6** (Fig. 1).

Glaucolide **1** was obtained, together with hirsutinolide **3**, in a similar concentration**,** as a white amorphous solid (according to the ^{1}H NMR spectrum). Both substances were identified based on analysis of NMR and MS spectroscopic data. The molecular formula of compounds **1** and **3** was $C_{19}H_{24}O_{10}$, as deduced by HR-ESI(+)-MS, which showed an accurate $[M+H]^+$ ion at *m/z* 413.1350 (calcd. glaucolides 413.14477). All isolated SLs were found to be isomers with the same mass. The ESI(−)-MS spectra of compounds **1**–**4** gave a $[M-H]^-$ species of *m/z* 411. The main fragments observed in the MS spectrum of compounds **1** and **3** were *m/z* 369, *m/z* 351, *m/z* 309 and *m/z* 291, which were confirmed by ESI(−)-MS/MS experiments. These fragments indicated a loss of ketene from the protonated molecule (*m/z* 411) to form the fragment *m/z* 369, followed by loss of acetic acid to form the ion fragment *m/z* 309. In contrast, the molecular ion may lose acetic acid to form the ion fragment *m/z* 351, followed by the loss of ketene to form ion fragment *m/z* 291. The ESI(−)-MS/MS spectra of compounds **2** and **4** were similar, indicating that they dissociate mainly into the ion *m/z* 369 from the loss of ketene, as well as the fragments cited above. The presence of a hydroxyl at C-6 of compound **3** probably facilitates the loss of acetic acid to form the main peak (*m/z* 351) in the MS spectra.

In the ^{1}H NMR spectrum of compounds **1** and **3**, four singlet signals were evident in the range of 2.06–2.15 ppm (3H each), interpreted as the presence of four acetate groups. Two doublet signals in the range of 4.88–5.08 ppm (J = 13.0 Hz), characteristic of geminal methylene hydrogens close to oxygen, were assigned as H-13a and H-13b, and four singlet signals (3H) were inferred to the methyl hydrogens H-14 (1.32 and 1.36 ppm) and H-15 (1.47 and 1.61 ppm). The ^{13}C NMR spectrum presented 38 carbons, showing characteristic signals of α,β-unsaturated γ-lactone rings at 167.2 and 167.5 ppm (C-12), 129.9 and 120.7 ppm (C-11), and 162.0 and 160.8 ppm (C-7) (Table 1). Glaucolide **1** is apparently the precursor of hirsutinolide **3**, which may have been formed during the extraction procedure in acidic silica. The formation of an unusual C1–C5 epoxide bridge in compound **3** could be explained by the strong correlation of H-5 at 3.54 ppm with C-1 at 95.0 ppm, as observed in an HMBC experiment.

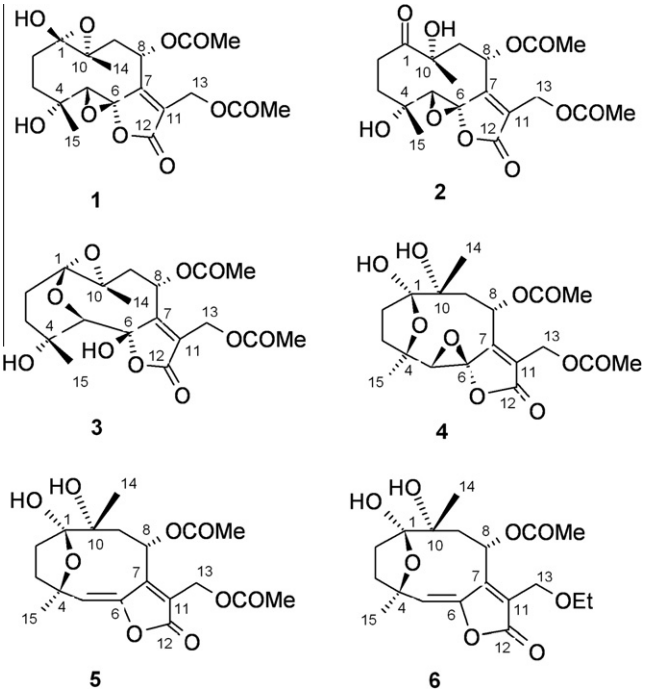

Fig. 1. Structures of compounds **1**–**6**.

In order to evaluate the hypothesis concerning formation of **3** during the extraction, the proposed compound **3** was studied by computational calculations using Density Functional Theory (DFT), in which geometry optimization and stretching frequency calculations were carried out using the B3LYP/6-31+G(d,p) level (Becke, 1993; Rassolov et al., 2001). This study enabled the researcher to determine whether the amount of energy needed to form the compound was prohibitive. The results suggest that the proposed structure **3** corresponded to the minimum level of energy on the potential energy surface and confirmed by harmonic vibration frequency analysis (Fig. 2A). This minimum energy structure showed a short, strong intramolecular hydrogen bond (1.597 Å), which is also in agreement with the O–H bond lengthening with a concomitant red shift in the O–H stretching frequency (1.044 Å and 3436 cm^{-1}) compared with the other O–H (1.028 Å and 3689 cm^{-1}) present in the structure, as depicted in Fig. 2B. This structural feature would give the compound additional electronic stability (Fig. 2A).

Glaucolide **2** was obtained as a colorless gum, with the same molecular formula ($C_{19}H_{24}O_{10}$) as compounds **1** and **3**, as evidenced in its high-resolution MS spectra**.** The ^{13}C NMR spectrum presented nineteen signals, with a typical glaucolide carbonyl at 215.1 ppm (C-1), in addition to the acetate carbonyls at 169.1 and 170.2 ppm (8- and 13-acetate). Its ^{1}H NMR spectrum showed the presence of several singlet signals. The resonances at 4.24 (1H) and 4.97 (2H) ppm were attributed to H-5 and H-13 respectively, whereas the signals at 2.07 and 2.14 ppm were assigned to the methyl groups of the 8- and 13-acetates, respectively. The resonances at 1.31 and 1.36 ppm were related to the methyl hydrogens H-14 and H-15, respectively (Table 2). The correlation of H-15 at 1.36 ppm with C-5 at 67.0 ppm, and the correlations of H-8 at 6.11 and H-13 at 4.97 ppm with the C-6 at 87.6 ppm, as observed in the ^{1}H–^{13}C long-range correlation experiment (HMBC), clearly indicated the presence of an epoxide between C-5 and C-6. Typical glaucolides isolated from *Vernonia* exhibited an epoxide between C-4 and C-5 (Padolina et al., 1974) and are considered precursors of the non-natural hirsutinolides (Jiménez et al., 1995). Glaucolide **2** may, therefore, be the precursor of hirsutinolide **4**.

Hirsutinolide **4**, also an isomer of the previously isolated compounds, gave characteristic signals of *Vernonia* hirsutinolides in the ^{13}C NMR spectrum, such as the hemiacetal carbon at 108.5 ppm (C-1) and the oxygen bearing carbon at 78.1 ppm (C-4) (Table 2). The main differences were found in the ^{1}H NMR spectrum, which showed a singlet at 3.48 ppm (1H) that correlated in the HSQC experiment with the carbonyl carbon at 69.9 ppm (C-5), and in the HMBC with the carbons at 78.1 (C-4) and 90.2 ppm (C-6), suggesting an epoxide between C-5 and C-6 instead of the usual double bond.

The relative configuration of **4** was established through 1D NOE experiments. Selective irradiation of the resonance frequency of H-5 at 3.48 ppm caused a NOE enhancement on the signal of the hydrogen H-15, indicating a β-orientation of the C5–C6 epoxide group (this enhancement was not so clear for the C5–C6 epoxide group of compounds **1** and **2**). Moreover, selective irradiation of the resonance frequency of H-14 at 1.14 ppm showed NOE intensification

Table 1
NMR spectroscopic data for compounds **1** and **3**.[a]

Carbon	**1**			**3**		
	^{1}H NMR mult. (*J*)	^{13}C NMR	HMBC[b]	^{1}H NMR mult. (*J*)	^{13}C NMR	HMBC[b]
1		107.9			95.0	
2	2.16 *m*	36.7	1, 4, 3 and10	1.77 *m*	33.2	1, 3, 4 and 10
	2.23 *m*		1, 4, 3 and10	2.32 *m*		1, 3 and 4
3	2.18 *m*	33.6	1, 2, 4 and 15	1.82 *m*	35.0	2, 4, 5 and 15
	2.51 *m*		1, 4, 5 and 15	2.65 *ddd* (14.2, 7.1, 3.3)		2, 4 and 15
4		80.5			70.2	
5	3.78 *s*	71.4	4, 6 and 15	3.54 *s*	78.7	1, 3, 4, 6, 7 and 15
6		91.5			102.2	
7		160.8			162.0	
8	5.47 *dd* (7.4, 2.7)	63.9	6, 7, 9, 10, 11 and CH_3COO-8	6.73 *dd* (10.4, 7.8)	67.9	7, 9, 11 and CH_3COO-8
9	2.23 *dd* (14.5, 2.7)	47.1	7, 8 and 10	1.56 *dd* (13.3, 10.4)	39.9	1, 8, 10 and 14
	2.61 *dd* (14.5, 7.4)		7, 8, 10 and 15	2.91 *dd* (13.3, 7.8)		7, 8 and 10
10		75.4			82.6	
11		129.9			120.7	
12		167.2			167.5	
13	4.90 *d* (13.0)	55.6	7, 11, 12 and CH_3COO-13	4.88 *dd* (12.3; 0.5)	54.8	7, 11, 12 and CH_3COO-13
	5.09 *d* (13.0)		7, 11, 12 and CH_3COO-13	4.99 *dd* (12.3; 1.2)		7, 11, 12 and CH_3COO-13
14	1.32 *s*	24.8	1, 9 and 10	1.36 *s*	23.0	1, 9 and 10
15	1.61 *s*	29.7	3, 4 and 5	1.47 *s*	26.7	3, 4 and 5
CH_3COO-8	2.06 *s*	20.8	CH_3COO-8	2.15 *s*	20.9	CH_3COO-8
CH_3COO-8		169.5			169.5	
CH_3COO-13	2.07 *s*	20.6	CH_3COO-13	2.13 *s*	20.8	CH_3COO-13
CH_3COO-13		170.4			170.4	

[a] The experiments were recorded in $CDCl_3$ and all NMR chemical shifts are given in ppm related to the TMS signal at 0.00 ppm as internal reference and coupling constants (*J*) are given in Hz. The unambiguous ^{1}H and ^{13}C NMR chemical shift assignments were established by a combination of 1D and 2D NMR including ^{1}H–^{1}H, one-bond and long-range ^{1}H–^{13}C correlation experiments.

[b] Long-range ^{1}H–^{13}C HMBC correlations, optimized for 8 Hz, are from hydrogen(s) stated to the indicated carbon.

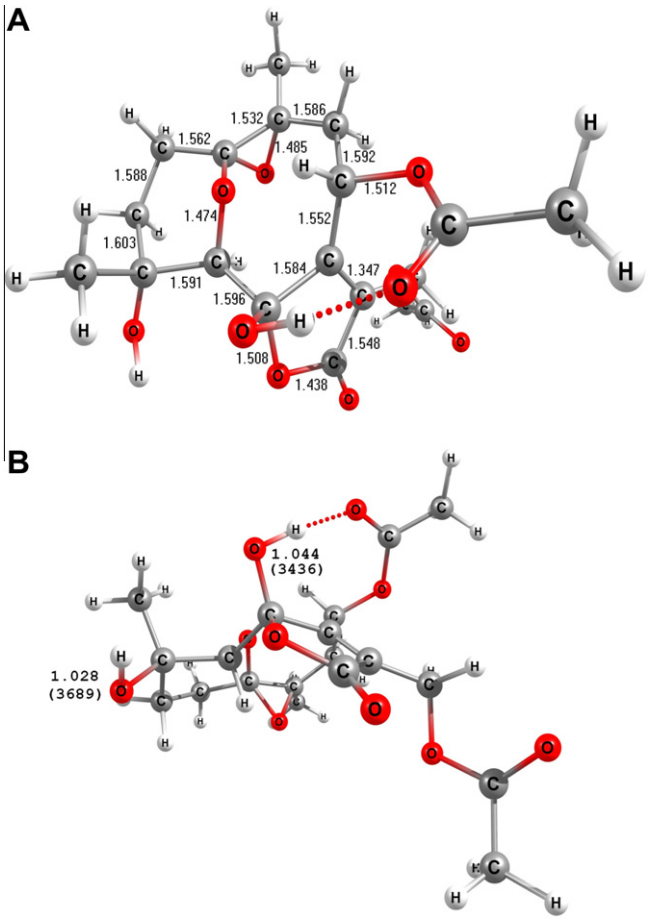

Fig. 2. Optimized structure of hirsutinolide **3** (A), O-H bond lengths (Å) and stretching frequencies (cm^{-1}) (in brackets) of **3** at B3LYP/6-31+G(d,p) level (B).

mainly on the signal of hydrogen H-9 at 2.07 ppm, whereas selective irradiation of the resonance frequency of H-8 at 5.99 ppm caused a strong NOE enhancement on the signals of H-9 at 2.56 ppm and H-13 at 4.78 ppm. The overall analyses of 1D NOE enabled the establishment of **4** as 8α-acetoxy-1α,10α-hydroxy-5,6-epoxy-hirsutinolide-13-*O*-acetate. The relative stereochemistry of compounds **1** and **2** should be in the form presented, but structural confirmation will need to be obtained.

Hirsutinolides **5** and **6** were isolated as amorphous solids and are characteristic compounds found in several *Vernonia* species. Their MS spectra, as well as the 1D and 2D NMR spectroscopic data, were in full agreement with earlier published data for the compounds diacetylpiptocarphol (or 1β,4β-Epoxy-8α,l3-diacetoxy-1α,10α-dihydroxy-germacra-5E,7(11)-dien-6,12-olide) (**5**, Bardón et al., 1988) and 8-acetyl-13-etoxypiptocarphol (or 1β,4β-Epoxy-l3-etoxy-1α,10α-dihydroxy-8-acetoxy-germacra-5E,7(11)-dien-6,12-olide) (**6**, Catalán et al., 1986). The resolution of their ^{1}H NMR spectra was improved by decreasing the temperature to 273 K (see Supplementary data, Fig. S1). Compound **5** has already been described in *V. scorpioides* (Bardón et al., 1988; Catalán et al., 1986), and compound **6** was first published with the H-8 α-orientation (Catalán et al., 1986). After that, structure **6** was commonly represented with an H-8 β-orientation (Borkosky et al., 1996; Valdés et al., 1998).

The configurations of the asymmetric carbon atoms of the sesquiterpene skeleton are assumed to be analogous to those previously established for similar compounds described in the literature (Valdés et al., 1998) because insufficient amount of material hampered attempts to determine the absolute configuration of the molecules.

Some cytotoxicity has been described for SLs from *V. cinerea* (Kuo et al., 2003), *V. lasiopus* (Koul et al., 2003) and *V. amygdalina* (Jisaka et al., 1993).

The results of the *in vitro* tests obtained for the isolated SLs are summarized in Table 3. Hirsutinolide **4** showed significant cytotoxicity only on HeLa cells, with an IC_{50} of 3.3 ± 4.0 μM. On L929 and

Table 2
NMR spectroscopic data for compounds **2** and **4**.[a]

Carbon	**2**			**4**		
	^{1}H NMR mult. (*J*)	^{13}C NMR	HMBC[b]	^{1}H NMR mult. (*J*)	^{13}C NMR	HMBC[b]
1		215.1			108.5	
2	1.89 *m*	27.7	1 and 3	1.44 (*ddd* 12.8, 12.5, 8.6)	32.2	1, 3 and 4
	3.96 *m*		1 and 3	1.91 (*ddd* 12.5, 7.6, 0.5)		
3	1.89 *m*	37.4	1, 2, 4 and 5	2.11 (*ddd* 12.8, 8.6, 0.5)	34.6	1, 2 and 5
	2.21 *m*		2, 4 and 15	2.31 (*ddd* 12.8, 12.8, 7.6)		2, 4, 5 and 15
4		71.2			78.1	
5	4.24 *s*	67.0	4, 6 and 15	3.48 *s*	69.9	4 and 6
6		87.6			90.2	
7		155.1			158.3	
8	6.11 *dd* (5.5, 2.5)	68.0	6, 7, 10 and CH_3COO-8	5.99 *dd* (10.2, 0.7)	68.6	6, 7, 9, 10, 11 and CH_3COO-8
9	2.23 *dd* (15.5, 5.5)	41.8	1, 7, 8, 10 and 14	2.07 *dd* (16.2, 0.7)	40.5	7, 10 and 14
	2.80 *dd* (15.5, 2.5)		10	2.56 *dd* (16.2, 10.2)		1, 7, 8, 10 and 14
10		80.2			77.1	
11		128.2			132.1	
12		166.7			166.0	
13	4.97 *s*	55.0	6, 7, 11, 12 and CH_3COO-13	4.78 *d* (13.5)	55.1	7, 11, 12 and CH_3COO-13
				5.95 *d* (13.5)		7, 11, 12 and CH_3COO-13
14	1.31 *s*	30.3	1, 9 and 10	1.14 *s*	25.6	1, 9 and 10
15	1.36 *s*	23.9	3, 4 and 5	1.52 *s*	26.7	3, 4 and 5
CH$_3$COO-8	2.07 *s*	20.4	CH_3COO-8	2.05 *s*	20.6	CH_3COO-8
CH_3COO-8		169.1			169.6	
CH$_3$COO-13	2.14 *s*	20.6	CH_3COO-13	2.04 *s*	20.6	CH_3COO-13
CH_3COO-13		170.2			170.0	

[a] The experiments were recorded in $CDCl_3$ and all NMR chemical shifts are given in ppm related to the TMS signal at 0.00 ppm as internal reference and coupling constants (*J*) are given in Hz. The unambiguous ^{1}H and ^{13}C NMR chemical shifts assignments were established by a combination of 1D and 2D NMR including ^{1}H–^{1}H, one-bond and long-range ^{1}H–^{13}C correlation experiments.
[b] Long-range ^{1}H–^{13}C HMBC correlations, optimized for 8 Hz, are from hydrogen(s) stated to the indicated carbon.

Table 3
SL cytotoxicity on HeLa, L929 and B16F10 cells, and the Damage Index on bone marrow cells.

Sample	IC_{50} HeLa[a] (μM)	IC_{50} L929[a] (μM)	IC_{50} B16F10[a] (μM)	Damage index[b]
Lactone mixture **1**/**3**	>100	>100	>100	154 ± 23
Glaucolide **2**	2.1 ± 1.8	>100	>100	137 ± 15
Hirsutinolide **4**	3.3 ± 4.0	>100	>100	163 ± 27
Hirsutinolide **5**	>100	>100	>100	128.5 ± 13
Hirsutinolide **6**	58.5 ± 15	>100	>100	132.3 ± 19.5
Paclitaxel	2.5 ± 2.2	8.62 ± 8.8	6.85 ± 5.4	–
MMS	–	–	–	189 ± 1.3

[a] IC_{50} determined after 24-h incubation at 37 °C/5%CO_2.
[b] Damage index determined at concentrations of hirsutinolide **4**, **5** and **6**, glaucolide **2** and lactone mixture **1/3** of 24.3, 25.2, 26.2, 24.3 and 242 μM, respectively. Negative control (1% DMSO) of 14.3 ± 2.1 (maximal possible score 200).

B16F10, the IC_{50} was >100 μM, with a reduction in viability of up to 20%. Hirsutinolide **6** gave an IC_{50} of 58.5 ± 15 μM on HeLa cells and also showed low toxicity on L929 and B16F10 cells (IC_{50} > 100 μM). Hirsutinolide **6** was apparently more cytotoxic than hirsutinolide **5** in HeLa cells (IC_{50} > 100 μM).

Hirsutinolide **6** has an ethoxy group on C-13 and hirsutinolides **4** and **5** presented acetoxy groups. Kuo et al. (2003), comparing hydroxylated with acetylated SLs in C-13, observed lower cytotoxicity for the acetylated SLs. The major difference between hirsutinolide **4** and **5** relates to the presence of an epoxy group (**4**) or a double bond (**5**) across C5–C6. In fact, the presence of epoxy groups (as in glaucolide **2**) could be related to a more significant cytotoxic effect. This hypothesis was confirmed by comparing the cytotoxicity of glaucolide **2** (IC_{50} 2.1 ± 1.8 μM) with that of hirsutinolide **4** (IC_{50} 3.3 ± 4.0 μM) in HeLa cells, with these two cytotoxicities being statistically similar to the IC_{50} of paclitaxel (2.5 ± 2.2 μM) in HeLa cells.

Pillay et al. (2007) observed that a sesquiterpene lactone (4,5α-epoxy-6α-hydroxy-1(10)E,11(13)-germacradien-12,8α-olide) was highly cytotoxic to CHO cells (IC_{50} 2.2 μg/mL). Dirsch et al. (2000), evaluating glaucolide A, suggested that the α-methylene-γ-lactone group also makes a strong contribution to biological activity.

The genotoxicity of the isolated compounds was verified using bone marrow cells. The results obtained from this assay show a tendency of these compounds to damage DNA, but mutagenic properties were not evaluated.

The negative and positive controls used in the genotoxicity test were DMSO (1%) and methyl methane sulfonate (MMS, 40 μM) with respective damage indices (DI) of 14.3 ± 2.1 and 189 ± 1.3. Glaucolide **2**, hirsutinolide **5** and hirsutinolide **6** showed DI values that were statistically lower than MMS ($p < 0.05$), but all the compounds registered DI values when compared with the negative control ($p < 0.01$) indicating genotoxicity (Table 3).

These results cannot be directly related to a mutagenic potential *in vivo*, as mutagenic assays must be performed to confirm the genotoxicity and mutagenic potential. The results obtained here suggest that at least part of the cytotoxicity might be related to genotoxic aspects (Collins and Dusinská, 2002).

Burim et al. (1999) observed that glaucolide B, isolated from *V. eremophila,* had an increase in chromosomal aberrations in lymphocytes at 4 and 8 μg/mL, as well as cytotoxicity at a concentration of >8 μg/mL. Glaucolide B at 160–640 mg/kg, however, did not significantly increase the frequency of chromosomal aberrations in mouse bone marrow cells, nor did it affect cell division in *in vivo* experiments using BALBc mice.

3. Experimental

3.1. General experimental procedures

Melting points were determined using a QUIMIS Q-340S23 micromelting point apparatus. All NMR data were recorded in $CDCl_3$ at 295 K for compounds 1–4, and 295 and 273 K for compounds 5 and 6, respectively, using a Bruker AVANCE 400

spectrometer operating at 9.4 T. The ^{1}H and ^{13}C isotopes were observed at 400 and 100 MHz, respectively. One-bond and long-range $^{1}H–^{13}C$ correlation (HSQC and HMBC) experiments were optimized for an average coupling constant ^{1}J(C,H) of 140 Hz and ^{LR}J(C,H) of 8 Hz, respectively. All ^{1}H and ^{13}C NMR chemical shifts (δ) are given in ppm related to TMS as an internal reference at 0.00 ppm, and the coupling constant (J) in Hz. All of the pulse programs employed were supplied by Bruker. Low-resolution ESI-MS and ESI-MS/MS data were acquired in the negative ion mode using a Bruker Esquire 6000 ESI-ion trap instrument, whereas the high-resolution HR-TOF-MS measurements were carried out using a hybrid quadrupole reflector orthogonal time-of-flight high-resolution Bruker Q-TOF mass spectrometer equipped with an electrospray source.

3.2. Plant material

Flowers and leaves of *V. scorpioides* were collected from wild specimens of "restinga" forest (a distinct type of coastal tropical and subtropical moist broadleaf forest) in Navegantes in November 2006, and identified by Dr. Ana Claudia Araújo of the Universidade do Vale do Itajaí. A voucher specimen (M. Biavatti 11) was deposited at the Barbosa Rodrigues Herbarium (HBR), Itajaí, Santa Catarina, Brazil.

3.3. Extraction and isolation

Fresh flowers and leaves of *V. scorpioides* (3 kg) were extracted with EtOH (6 L) at room temperature, in the absence of light, for 7 days. After solvent reduction to 1/6 of the initial volume under reduced pressure and the addition of H_2O (500 mL), the extract was submitted to liquid–liquid fractioning using solvents with increasing polarities. This procedure produced *n*-hexane (1.2 g), CH_2Cl_2 (2.3 g), and EtOAc (1.4 g) fractions. The CH_2Cl_2 fraction was initially subjected to silica gel CC (60–230 mesh) eluted with *n*-hexane (500 mL), followed by CH_2Cl_2 (800 mL), EtOAc (700 mL) and MeOH (400 mL), yielding one subfraction for each of the four solvents. Additional chromatography separation of the CH_2Cl_2 subfractions (420 mg) were carried out by silica gel CC (230–400 mesh), using *n*-hexane with increasing concentrations of EtOAc (0–50% EtOAc) as eluent, and collecting eluent in 30–50 mL portions to furnished 42 fractions. Fractions 17–24 were combined and subjected to silica gel CC using *n*-hexane:ethyl acetate (7:3) to yield compound **6** (4.5 mg, 8-acetyl-13-etoxypiptocarphol (Catalán et al., 1986) and ethyl caffeate (11.86 mg, Su et al., 2008). Fractions 20–26 were combined and subjected to MPLC on a silica gel Lobar B column (Merck) using *n*-hexane:EtOAc (8:2) to yield compounds **1** and **3** (15 mg) as a mixture, as well as compound **5** (6 mg, diacetylpiptocarphol, Bardón et al., 1988).

Chromatographic separation of the EtOAc subfraction (1.7 g) by silica gel CC (230–400 mesh) using *n*-hexane with increasing concentrations of acetone (from 10% to 50%), and collecting eluant fractions of 30–50 mL, furnished 82 fractions. Fractions 20–25 were combined and subjected to silica gel CC as above, yielding a mixture of SLs (163 mg), which was subjected to MPLC on a silica gel Lobar B column (Merck) using *n*-hexane:acetone (1:1) yielding compounds **2** (12 mg) and **4** (25 mg). From fractions 26–61, luteolin (10 mg) and apigenin (5 mg) were obtained after successive silica gel CC, as described above.

3.4. 8α,13-Diacetoxy-1α,10α,-5β,6β-diepoxygermacra-7(11)-en-12-olide (**1**)

Amorphous solid, mp 98–100 °C; for ^{1}H and ^{13}C NMR spectroscopic data, see Table 1; ESI-MS *m/z* 411.5 $[M-H]^-$; ESI-MS/MS (daughter ions, 25%) *m/z* 369 (30), *m/z* 351 $[M-H-AcOH]^-$ (60), 309 (83), 291 (50); HR-TOF-MS (ESI positive) *m/z* 413.1350 $[M+H]^+$ (calcd for $C_{19}H_{24}O_{10}$+H, 413.14477).

3.5. 10α,4α-Dihydroxy-5β,6β-isoglaucolide B (**2**)

Colorless gum; for ^{1}H and ^{13}C NMR spectroscopic data, see Table 1; ESI-MS *m/z* 411.2 $[M-H]^-$; ESI-MS/MS (daughter ions, 25%) *m/z* 369 (80), 351 $[M-H-AcOH]^-$ (30), 309 (20), 291 (40), 247 (70); HR-TOF-MS (ESI positive) *m/z* 413.1350 $[M+H]^+$ (calcd for $C_{19}H_{24}O_{10}$+H, 413.14477).

3.6. 8α,13-Diacetoxy-1α,10α,-1β,5β-diepoxygermacra-7(11)-en-12-olide (**3**)

Amorphous solid, mp 98–100 °C; for ^{1}H and ^{13}C NMR spectroscopic data, see Table 1; ESI-MS *m/z* 411.5 $[M-H]^-$; ESI-MS/MS (daughter ions, 25%) *m/z* 369 (30), *m/z* 351 $[M-H-AcOH]^-$ (60), 309 (83), 291 (50); HR-TOF-MS (ESI positive) *m/z* 413.1350 $[M+H]^+$ (calcd for $C_{19}H_{24}O_{10}$+H, 413.14477).

3.7. 8α,13-Diacetoxy-1α,10α-hydroxy-5,6-epoxy-hirsutinolide (**4**)

Colorless gum; for ^{1}H and ^{13}C NMR spectroscopic data, see Table 1; ESI-MS *m/z* 411.2 $[M-H]^-$; ESI-MS/MS (daughter ions, 25%) *m/z* 369 (100), 351 $[M-H-AcOH]^-$ (40), 309 (38), 291 (50), 247 (45); HR-TOF-MS (ESI positive) *m/z* 413.1350 $[M+H]^+$ (calculated for $C_{19}H_{24}O_{10}$+H, 413.14477).

3.8. Computational methods

All calculations were carried out using exchange–correlation functional B3LYP (Becke, 1993) with 6-31+G(d,p) (Rassolov et al., 2001) using the Gaussian03 suite of programs (Frisch et al., 2003). The structures presented in Fig. 2 were obtained using ChemCraft 1.5 (http://www.chemcraftprog.com).

3.9. Cytotoxicity assay

Cell viability was assessed by 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays to determine IC_{50} concentrations of the studied agents, as described previously (Mosmann, 1983). The full procedure is supplied as Supplementary data.

3.10. Comet assay

In vitro genotoxicity was assessed using bone marrow cells obtained from the femur of *Mus musculus* (Swiss). The UNIVALI protocol 236/07 was used, as described previously (Tice et al., 2000), and the full procedure is supplied as Supplementary data.

3.11. Statistical analysis

The results are expressed as mean values ± SD from three separate experiments. The IC_{50} values, i.e., the concentration necessary for 50% inhibition, were calculated from the dose response curves using non-linear regression analysis that gave a percentage of the inhibition values. Group differences were determined by analysis of variance (ANOVA). When statistically significant differences were indicated by ANOVA, the values were compared by the Tukey test. The differences were considered statistically significant from the controls at $p < 0.05$.

Acknowledgements

The authors are grateful to CNPq and CAPES for their financial support. G.F.C. thanks CENAPAD – SP for the allocated computational time and resources.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2010.06.007.

References

Alarcon, M.C., Callegari Lopes, J.L., Herz, W., 1990. Glaucolide B, a molluscicidal sesquiterpene lactone, and other constituents of *Vernonia eremophila*. Planta Med. 56, 271–273.

Barbosa, L.C., Costa, A.V., Pilo-Veloso, D., Lopes, J.L., Hernandez-Terrones, M.G., King-Diaz, B., Lotina-Hennsen, B., 2004. Phytogrowth-inhibitory lactones derivatives of glaucolide B. Z. Naturforsch. [C] 59, 803–810.

Bardón, A., Catalán, C.A.N., Gutierrez, A.B., Herz, W., 1988. Piptocarphol esters and other constituents from *Vernonia cognata*. Phytochemistry 27, 2989–2990.

Becke, A.D., 1993. Density-functional thermochemistry. III. The role of exact exchange. J. Chem. Phys. 98, 5648–5652.

Borkosky, S., Alvarez Valdés, D., Bardón, A., Díaz, J.G., Herz, W., 1996. Sesquiterpene lactones and other constituents of *Eirmocephala megaphylla* and *Cyrtocymura cincta*. Phytochemistry 42, 1637–1639.

Burim, R.V., Canalle, R., Lopes, J.L.C., Takahashi, C.S., 1999. Genotoxic action of the sesquiterpene lactone glaucolide B on mammalian cells *in vitro* and *in vivo*. Gent. Mol. Biol. 22, 401–406.

Cabrera, A.L., Klein, R.M., 1980. Compostas: 3. Tribo: Vernoniae. Fl. Ilustr. Catarin. 354–355.

Campos, M., Oropeza, M., Ponce, H., Fernandez, J., Jimenez-Estrada, M., Torres, H., Reyes-Chilpa, R., 2003. Relaxation of uterine and aortic smooth muscle by glaucolides D and E from *Vernonia liatroides*. Biol. Pharm. Bull. 26, 112–115.

Catalán, C.A.N., de Iglesias, D.I.A., Kavka, J., Sosa, V.E., Herz, W., 1986. Sesquiterpene lactones and other constituents of *Vernonia mollissima* and *Vernonia squamulosa*. J. Nat. Prod. 49, 351–353.

Chea, A., Hout, S., Long, C., Marcourt, L., Faure, R., Azas, N., Elias, R., 2006. Antimalarial activity of sesquiterpene lactones from *Vernonia cinerea*. Chem. Pharm. Bull. 54, 1437–1439.

Chen, X., Zhan, Z.J., Zhang, X.W., Ding, J., Yue, J.M., 2005. Sesquiterpene lactones with potent cytotoxic activities from *Vernonia chinensis*. Planta Med. 71, 949–954.

Collins, A.R., Dusinská, M., 2002. Oxidation of cellular DNA, measured with the comet assay. In: Armstrong, D. (Ed.), Oxidative Stress Biomarkers and Antioxidant Protocols. Humana Press, New Jersey, pp. 147–159.

Costa, F.B., Terfloth, L., Gasteiger, J., 2005. Sesquiterpene lactone-based classification of three Asteraceae tribes: a study based on self-organizing neural networks applied to chemosystematics. Phytochemistry 66, 345–353.

Dirsch, V.M., Stuppner, J., Ellmerer-Müller, E.P., Vollmar, A.M., 2000. Structural requirements of sesquiterpene lactones to inhibit LPS-induced nitric oxide synthesis in RAW 264.7 macrophages. Bioorg. Med. Chem. 8, 2747–2753.

Drew, M.G.B., Hitchman, S.P., Mann, J., Lopes, J.L.C., 1980. X-ray crystal structure of the sesquiterpene lactone scorpioidine. J. Chem. Soc., Chem. Commun. 17, 802–803.

Freire, M.F.I., Abreu, H.S., Cruz, L.C.H., Freire, R.B., 1996. Inhibition of fungal growth by extracts of *Vernonia scorpioides* (Lam.) Pers. Microbiology 27, 1–6.

Frisch, M.J. et al., 2003. Gaussian 03, Revision A.1. Gaussian Inc., Pittsburgh, PA.

Jakupovic, J., Baruah, R.N., Chau Thi, T.V., 1985. New vernolepin derivatives from *Vernonia glabra* and glaucolides from *Vernonia scorpioides*. Planta Med. 5, 378–380.

Jiménez, M., Ortega, A., Navarro, A., Maldonado, E., Van Calsteren, M.R., Jankowski, C.K., 1995. Reaction of the molluscicide glaucolide B with bentonite. J. Nat. Prod. 58, 424–427.

Jisaka, M., Ohigashi, H., Takegawa, K., Huffman, M.A., Koshimizu, K., 1993. Antitumoral and antimicrobial activities of bitter sesquiterpene lactones of *Vernonia amygdalina*, a possible medicinal plant used by wild chimpanzees. Biosci. Biotechnol. Biochem. 57, 833–834.

Kos, O., Castro, V., Murillo, R., Poveda, L., Merfort, I., 2006. Ent-kaurane glycosides and sesquiterpene lactones of the hirsutinolide type from *Vernonia triflosculosa*. Phytochemistry 67, 62–69.

Koul, J.L., Koul, S., Singh, C., Taneja, S.C., Shanmugavel, M., Kampasi, H., Saxena, A.K., Qazi, G.N., 2003. *In vitro* cytotoxic elemanolides from *Vernonia lasiopus*. Planta Med. 69, 164–166.

Kuo, Y.H., Kuo, Y.J., Yu, A.S., Wu, M.D., Ong, C.W., Yang Kuo, L.M., Huang, J.T., Chen, C.F., Li, S.Y., 2003. Two novel sesquiterpene lactones, cytotoxic vernolide-A and -B, from *Vernonia cinerea*. Chem. Pharm. Bull. 51, 425–426.

Leite, S.N., Palhano, G., Almeida, S., Biavatti, M.W., 2002. Wound healing activity and systemic effects of *Vernonia scorpioides* extract in guinea pig. Fitoterapia 73, 496–500.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Padolina, W.G., Yoshioka, H., Nakatani, N., Mabry, T.J., Monti, S.A., Davis, R.E., Cox, P.J., Sim, G.A., Watson, W.H., Wu, I.B., 1974. Glaucolide-A and -B, new germacranolide-type sesquiterpene lactones from *Vernonia* (Compositae). Tetrahedron 30, 1161–1170.

Pagno, T., Blind, L.Z., Biavatti, M.W., Kreuger, M.R., 2006. Cytotoxic activity of the dichloromethane fraction from *Vernonia scorpioides* (Lam.) Pers. (Asteraceae) against Ehrlich's tumor cells in mice. Braz. J. Med. Biol. Res 39, 1483–1491.

Pillay, P., Vleggaar, R., Maharaj, V.J., Smith, P.J., Lategan, C.A., Chouteau, F., Chibale, K., 2007. Antiplasmodial hirsutinolides from *Vernonia staehelinoides* and their utilization towards a simplified pharmacophore. Phytochemistry 68, 1200–1205.

Rassolov, V.A., Ratner, M.A., Pople, J.A., Redfern, P.C., Curtiss, L.A., 2001. 6-31G* basis set for third-row atoms. J. Comp. Chem. 22, 976–984.

Su, Y.O., Zhang, W.D., Zhang, C., Liu, R.H., Shen, Y.H., 2008. A new caffeic ester from *Incarvillea mairei* var. *grandiflora* (Wehrhahn) Grierson. Chin. Chem. Lett. 19, 829–831.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environ. Mol. Mutag. 35, 206–221.

Valdés, D.A., Bardón, A., Catalán, C.A.N., Gedris, T.E., Herz, W., 1998. Glaucolides, piptocarphins and cadinanolides from *Lepidaploa remotiflora*. Biochem. Syst. Ecol. 26, 685–689.

Warning, U., Jakupovic, J., Bohlmann, F., 1987. Scorpiolid, ein neuer sesquiterpenlacton-typ aus *Vernonia scorpioides*. Liebigs Ann. Chem., 467.

Williams, R.B., Norris, A., Slebodnick, C., Merola, J., Miller, J.S., Andriantsiferana, R., Rasamison, V.E., Kingston, D.G., 2005. Cytotoxic sesquiterpene lactones from *Vernonia pachyclada* from the Madagascar rainforest. J. Nat. Prod. 68, 1371–1374.