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ent-Kaurane diterpenes from the stem bark of *Annona vepretorum* (Annonaceae) and cytotoxic evaluation

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ABSTRACT

This work describes a novel *ent*-kaurane diterpene, *ent*-3β-hydroxy-kaur-16-en-19-al along with five known *ent*-kaurane diterpenes, *ent*-3β,19-dihydroxy-kaur-16-eno, *ent*-3β-hydroxy-kaur-16-eno, *ent*-3β-acetoxy-kaur-16-eno, *ent*-3β-hydroxy-kaurenoic acid and kaurenoic acid, as well as caryophyllene oxide, humulene epoxide II, β-sitosterol, stigmasterol and campesterol from the stem bark of *Annona vepretorum* Mart. (Annonaceae). Cytotoxic activities towards tumor B16-F10, HepG2, K562 and HL60 and non-tumor PBMC cell lines were evaluated for *ent*-kaurane diterpenes. Among them, *ent*-3β-hydroxy-kaur-16-en-19-al was the most active compound with higher cytotoxic effect over K562 cell line (IC_{50} of 2.49 μg/mL) and lower over B16-F10 cell line (IC_{50} of 21.02 μg/mL).

Annona vepretorum Mart. (Annonaceae), popularly known as 'bruteira', is a shrub or tree of 2.5–10 m tall native from the Brazilian biome Caatinga. Its fruits are consumed raw or in juice form as a nutritional source.[1] When softened, its roots present popular medicinal indication to bite of bees and snakes, inflammatory conditions and pains in the heart, while the leaves (decoction) are used in bath to allergies, skin diseases, yeast and bacteria infection (oral communications received from the local population). Previous phytochemical and pharmacological investigations on this species described the chemical composition of essential oil from the leaves that showed trypanocidal, antifungal and antioxidant properties, and revealed mainly the presence of bicyclogermacrene, spathulenol and α-phellandrene.[2] Moreover, Diniz et al.[3] described that ethanolic extract from the leaves has sedative effect. In our continuous research for bioactive compounds from Annonaceous plants a novel *ent*-kaurane diterpene, ent-3β-hydroxy-kaur-16-en-19-al (**1**), along with five known *ent*-kaurane diterpenes, *ent*-3β,19-dihydroxy-kaur-16-eno (**2**), *ent*-3β-hydroxy-kaur-16-eno (**3**), *ent*-3β-acetoxy-kaur-16-eno (**4**), *ent*-3β-hydroxy-kaurenoic acid (**5**) and kaurenoic acid (**6**), as well as caryophyllene oxide (**7**), humulene epoxide II (**8**), β-sitosterol (**9**), stigmasterol (**10**) and campesterol (**11**) were found in the stem bark of *A. vepretorum* (Fig. 1).[4] Cytotoxic activities towards tumor and non-tumor cells lines were investigated for compounds **1**–**6**. This is the first phytochemical and biological investigation of the stem bark of *A. vepretorum*.

Compound **1** was obtained as an white amorphous powder with the molecular formula, $C_{20}H_{30}O_2$, as determined by HR-ESIMS (observed *m*/*z* 325.2140 $[M+Na]^+$) and NMR data.[5,6] LR-MS tandem analysis showed a fragment at *m*/*z* 285 indicating a lost of H_2O $[M+H-H_2O]^+$. Its infrared (IR) spectrum showed absorptions bands at 3267, 2726 and 1712 cm^{-1} typical of hydroxyl and aldehyde groups. The low frequency of the carbonyl group of the aldehyde, as well as hydroxyl group is due to the hydrogen bonding. The ^1H NMR spectrum showed signals for two tertiary methyl groups at δ 0.94 and 1.27 (3H each), that are typical of axial C-20 and equatorial C-18 methyl groups of *ent*-kaurane diterpenes with a axial C-19 aldehyde group (Fig. 2). The signal for this aldehyde group was observed at δ 9.76 (1H). Additionally, two signals were observed at δ 4.75 and 4.81 (1H each) typical of hydrogens from an exocyclic double bond, as well as a signal to a carbinolic hydrogen at δ 3.16 (1H). The ^1H–^1H COSY NMR experiment revealed that the aldehyde hydrogen was coupling with H-3 at δ 3.16 (1H), probably due to a 'W' orientation (Fig. 2), supporting the equatorial C-18 (methyl group) and axial C-19 (aldehyde group) configurations in the structure of **1**.[7] The $^{13}C\{^1H\}$ and DEPT135 NMR spectra, as well as one-bond and long-range ^1H–^{13}C correlation maps from HSQC and HMBC NMR experiments indicated a total of 20 carbons

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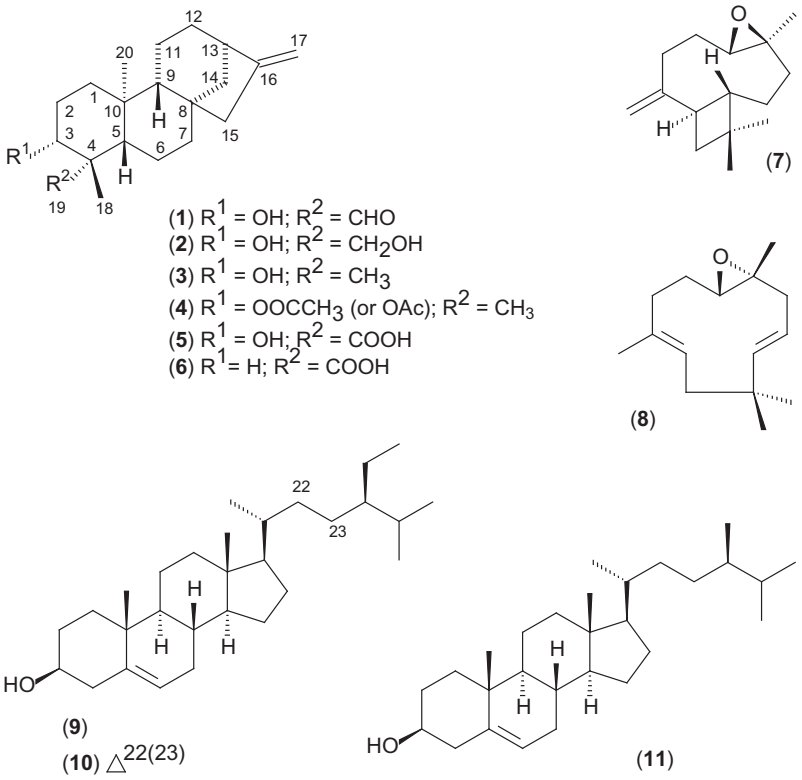

Figure 1. Terpenes found from the stem bark of *Annona vepretorum*.

(Table 1). These carbons comprised two methyl, nine methylenes, four methines, four quaternary carbons, and one aldehyde at δ 208.1. The hydrogen at δ 3.16 and shown one-bond $^{1}H–^{13}C$ correlation with the carbon at δ 77.4 (C-3) and long-range $^{1}H–^{13}C$ correlation with the carbons at δ 52.6 (C-4) and δ 208.1 (C-19), supporting the presence of a hydroxyl group at C-3 and the aldehyde at C-19 in the structure of **1** (Fig. 2). Moreover, the hydrogen at δ 3.16 (H-3) showed only two additional $^{1}H–^{1}H$ correlation from COSY NMR experiment, with hydrogens at δ 1.86 and δ 1.89 (H-2), supporting the substitution at C-3. The aldehyde group at C-19 was supported on the basis of HMBC NMR experiment, since the aldehyde hydrogen at δ 9.76 (H-19) shown long-range $^{1}H–^{13}C$ correlation with the carbons at δ 52.6 (C-4) and δ 77.4 (C-3) (Fig. 2). The β-orientation of the hydroxyl group at C-3 was established by comparing its NMR data with those described in the literature for 3-hydroxy-kauranoic acids.[7,8] Hydrogens at C-3 in α (axial OH) and β (equatorial OH) isomers are described as having ^{1}H NMR chemical shifts at δ 4.11 and δ 3.14, respectively. Therefore, the ^{1}H NMR chemical shifts found in this work to H-3 at δ 3.16 are in accordance with an β orientation. This fact was also supported by 1D NOE NMR selective experiments. In these, the selective irradiation of the resonance frequency of H-3 at δ 3.16 caused a NOE enhancement in the signals at δ 1.27 (H-18), 1.03 (H-5) and 0.95 (H-1ax) (Fig. 2). Moreover, the selective irradiation of the resonance frequency of the methyl hydrogens H-18 at δ 1.27 showed a NOE intensification of the signals at δ 9.76 (HCO), δ 3.16 (H-3), δ 1.88 (H-6 eq), and δ 1.03 (H-5), although any enhancement of the signal of H-20 at δ 0.94 (Fig. 2). On the other hand, the selective irradiation of the resonance frequency of the hydrogen H-19 at δ 9.76 showed a NOE intensification of the signals at δ 1.27 (H-18), δ 0.94 (H-20) and δ 1.59 (H-6ax), although no enhancement on the signal of H-5 at δ 1.03 (Fig. 2). The overall analysis of 1D and 2D NMR experiments enabled us to fully establish the structure and to completely assign the ^{1}H and ^{13}C NMR chemical shifts of **1** (Table 1).[9] Therefore, compound **1** was identified as a new *ent*-kaurane diterpene named as *ent*-3β-hydroxy-kaur-16-en-19-al.

Compounds **2**–**11** were identified by comparing their spectrometric data[6] with those reported in the literature which were in accordance with *ent*-3β,19-dihydroxy-kaur-16-ene (**2**), *ent*-3β-hydroxy-kaur-16-ene (**3**), *ent*-3β-acetoxy-kaur-16-ene (**4**), *ent*-3β-hydroxy-kaur-16-en-19-oic acid (**5**), *ent*-kaur-16-en-19-oic acid (**6**), mixture of caryophyllene oxide (**7**) and humulene epoxide II (**8**), and a mixture of β-sitosterol (**9**), stigmasterol (**10**) and campesterol (**11**).[7,10–18] Nevertheless, compounds **2**–**4** have been described a long time ago and its NMR data are incomplete as well as have some ambiguities. Therefore, the complete and unequivocal NMR data for these diterpenes were reviewed according to 1D and 2D NMR experiments (Table 1).

The absolute configurations of the diterpenoids *ent* and normal series can be established on the basis of its negative and positive specific rotation ($[\alpha]^{D}$), since they can be correlated to similar kauranoid diterpenes with defined absolute configurations. Therefore, those that divert the light polarized to the left (−) belong to the *ent* series, while those that divert to the right (+) belong to the normal series.[8] In this work, all diterpenes diverted the light polarized to the left (−) according to *ent* series.[6]

The *ent*-kaurane diterpenes are common in Annonaceae plants, particularly in species of *Annona* and *Xylopia*.[19–22] In *Annona*, this class of compound is well represented and considered as chemotaxonomic markers. Among them, compound **6** is the most representative within the family Annonaceae, mainly in *Annona* such as *Annona cherimolia*, *Annona glabra*, *Annona senegalensis* and *Annona squamosa*, and *Xylopia* such as *Xylopia frutescens*, *Xylopia laevigata*, *Xylopia sericeae*.[7,19–23] Compound **5** has been described in *Xylopia laevigata* (Annonaceae), although it was described in species of the family Asteraceae.[7,24,25] Therefore, the presence of *ent*-kaurane diterpenes in *A. vepretorum* supports that this is a typical species of the family Annonaceae. Compound **2** was found

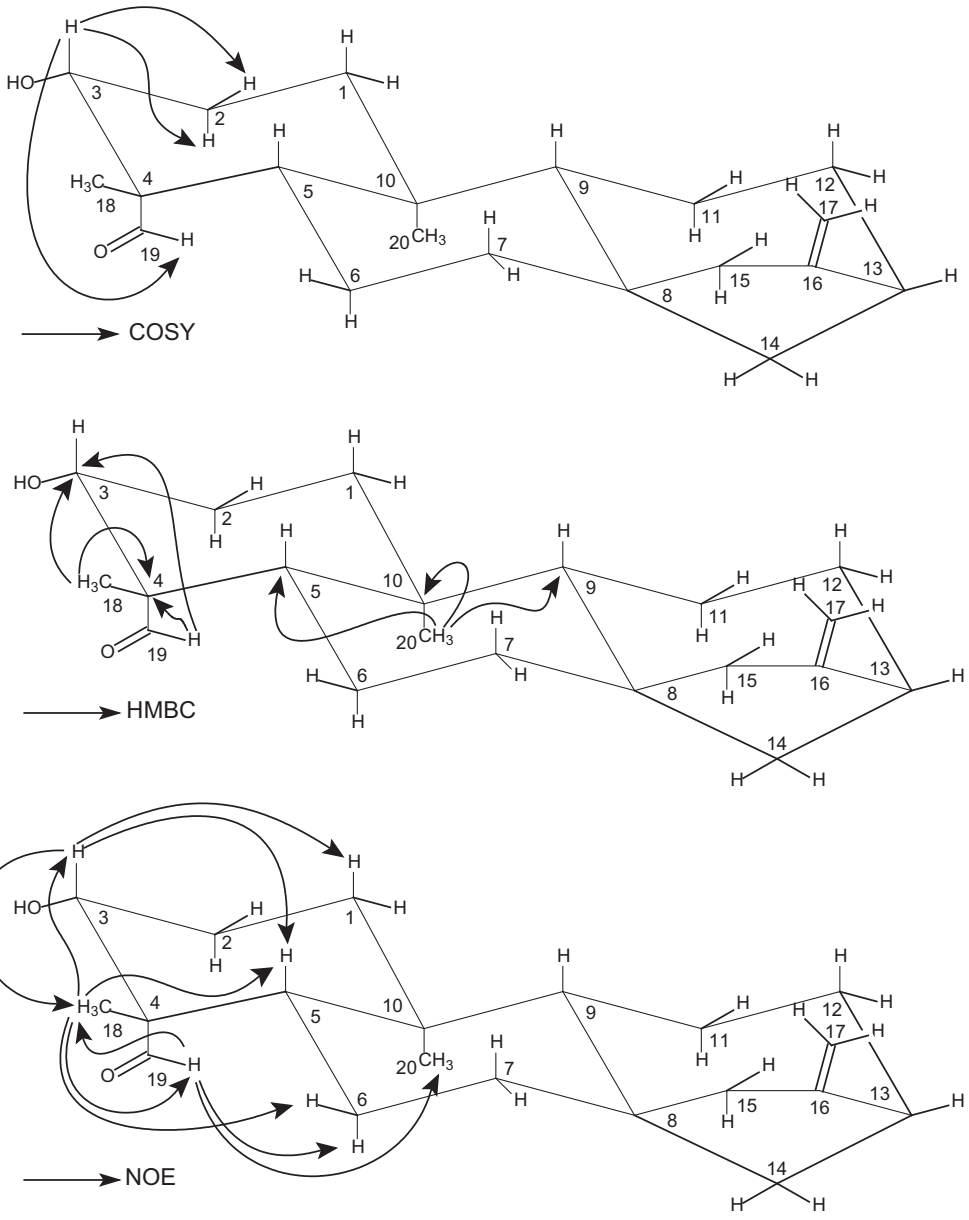

Figure 2. Key COSY, HMBC and NOE correlations for compound **1**.

in *Stachys lanata* (Labiatae) and *Cacalia pilgeriana* (Asteraceae).[10,26] Compound **3** was obtained from *Laetia thamnia* (Flacourtiaceae), *Guarea kunthiana* (Meliaceae) and *Phyllanthus flexuosus* (Phyllanthaceae).[27–29] Compound **4** was observed in *Phyllanthus flexuosus* (Phyllanthaceae).[27] The other compounds (**7**–**11**) are commonly found in species of Annonaceae.[7,30–32]

Compounds **1**–**6** were evaluated for their cytotoxicity on B16-F10 (mouse melanoma), HepG2 (human hepatocellular carcinoma), K562 (human chronic myelocytic leukemia) and HL-60 (human promyelocytic leukemia) tumor cell lines.[33–36] The compounds **1**, **3**, **5** and **6** showed cytotoxic activity, while **2** and **4** were not cytotoxic at the experimental concentration used (IC_{50} >25 μg/mL), in any tumor cell lines tested (Table 2). The cytotoxicity of **2**, **3** and **6** was previously assessed,[28,37,38] whereas the cytotoxic activity of the compounds **1**, **4** and **5** were evaluated for first time in this work.

Regarding structure-cytotoxicity relationship of *ent*-kaurane diterpenes, related compounds with the α-methylene cyclopentanone moiety and/or α,β-unsaturated ketone moiety had been reported to have cytotoxic activity.[39,40] On the other hand, the *ent*-kaurane diterpenes investigated in this work do not present these features and are able to inhibit cell proliferation. Cavalcanti et al.[37] found that the exocyclic double bond ($\Delta^{16(17)}$) is a fundamental pharmacophoric group for the cytotoxic activity of *ent*-kaurane diterpenes. All *ent*-kaurane diterpenes evaluated in this work has exocyclic double bond, although compound **4** was inactive. This fact can be related to the presence of an acetate group at C-3. In this work, compounds **1**, **3** and **6** were higher cytotoxic than the compounds **2**, **4** and **5**, suggesting that acetoxy ($OOCCH_3$) group on C-3 or hydroxymethyl (CH_2OH) group on C-4 decrease the cytotoxic activity of *ent*-kaurane diterpenes. These results may help in the identification of novel *ent*-kaurane diterpene-like structures with optimized cytotoxicity to be tested for cancer treatment.

Compound **1**–**6** were also cytotoxic to non-tumor PBMC cells, presenting low selectivity (Table 2). Compound **1** shown a selectivity index (SI) of 2.9 for leukemia (K562), while doxorubicin showed a SI of 7.5 for the same tumor cell line. Compound **6**

Table 1
^{1}H and ^{13}C NMR data ($CDCl_3$, 400 MHz) for *ent*-kaurane diterpenes **1**–**4**

Position	1		2		3		4	
	δ_C	δ_H mult. (*J* in Hz)	δ_C	δ_H mult. (*J* in Hz)	δ_C	δ_H mult. (*J* in Hz)	δ_C	δ_H mult. (*J* in Hz)
1	38.6	ax 0.95 *m* eq 1.92 *m*	38.4	ax 0.90 *m* eq 1.87 *m*	38.7	ax 0.90 *ddd* (13.2, 12.4 and 5.2) eq 1.85 *ddd* (13.2. 3.7 and 3.4)	38.4	ax 0.98 *ddd* (13.4, 12.8 and 4.7) eq 1.85 *ddd* (13.4, 3.8 and 3.5)
2	28.2	ax 1.86 *m* eq 1.89 *m*	27.7	ax 1.71 *m* eq 1.84 *m*	27.4	ax 1.60 *m* eq 1.63 *m*	23.7	ax 1.65 *m* eq 1.67 *m*
3	77.4	3.16 *m*	80.9	3.42 *m*	79.1	3.19 *dd* (10.9 and 5.6)	81.1	4.47 *dd* (11.1 and 5.5)
4	52.6		42.9		38.9		37.9	
5	56.3	1.03 *dd* (12.6 and 2.3)	55.8	0.87 *m*	55.2	0.76 *dd* (11.8 and 1.9)	55.4	0.85 *m*
6	20.2	ax 1.59 *m* eq 1.88 *m*	20.1	ax 1.30 *m* eq 1.75 *m*	20.0	ax 1.40 *m* eq 1.55 *m*	19.9	ax 1.36 *m* eq 1.53 *m*
7	41.1	ax 1.51 *m* eq 1.61 *m*	41.3	ax 1.48 *m* eq 1.51 *m*	41.2	ax 1.48 *m* eq 1.52 *m*	41.1	ax 1.51 *m* eq 1.53 *m*
8	43.8		43.9		44.0		44.1	
9	54.5	1.05 *m*	55.9	1.03 *m*	55.9	1.03 *m*	55.9	1.06 *m*
10	39.1		38.7		39.1		39.0	
11	18.5	ax 1.55 *m* eq 1.65 *m*	18.4	ax 1.53 *m* eq 1.63 *m*	18.3	ax 1.53 *m* eq 1.62 *m*	18.3	ax 1.54 *m* eq 1.64 *m*
12	39.8	ax 1.15 *dddd* (11.5, 5.2, 1.8 and 1.5) eq 1.93 *dm* (11.5)	39.6	ax 1.09 *dddd* (11.4, 5.0, 1.8 and 1.5) eq 1.92 *dm* (11.4)	39.8	ax 1.11 *dddd* (11.4, 5.1, 1.8 and 1.6) eq 1.98 *dm* (11.4)	39.8	ax 1.11 *dddd* (11.4, 5.0, 1.8 and 1.6) eq 1.97 *dm* (11.4)
13	43.7	2.66 *m*	43.8	2.64 *m*	43.97	2.64 *m*	44.0	2.64 *m*
14	32.9	pax 1.49 *m* peq 1.59 *m*	33.1	pax 1.48 *m* peq 1.63 *m*	33.2	pax 1.48 *m* peq 1.62 *m*	33.0	pax 1.48 *m* peq 1.64 *m*
15	48.8	pax 2.05 *m* peq 2.06 *m*	48.9	pax 2.05 *m* peq 2.06 *m*	49.0	pax 2.05 *m* peq 2.06 *m*	49.0	pax 2.06 *m* peq 2.07 *m*
16	155.2		155.6		155.8		155.7	
17	103.5	4.75 *m* 4.81 *m*	103.2	4.74 *m* 4.80 *m*	103.0	4.74 *m* 4.80 *m*	103.1	4.74 *m* 4.79 *m*
18	19.2	1.27 *s*	22.7	1.23 *s*	28.4	0.98 *s*	28.4	0.86 *s*
19	208.2	9.76 *s*	64.3	3.32 *d* (11.2) 4.20 *d* (11.2)	15.5	0.78 *s*	16.6	0.85 *s*
20	16.5	0.94 *s*	18.2	0.98 *s*	17.6	1.02 *s*	17.7	1.05 *s*
CH_3**C**OO-**3**							171.0	
C$\mathbf{H_3}$COO-**3**							21.3	2.04 *s*

Table 2
Cytotoxic activity (IC_{50} values)[a] for compounds **1**–**6**

Cell lines	IC_{50} in μg/mL (μM)/compounds						
	1	**2**	**3**	**4**	**5**	**6**	Doxorubicin[b]
Tumor cells[c]							
B16-F10	21.02 (69.55)	>25 (82.17)	19.12 (66.33)	>25 (75.70)	>25 (78.56)	16.56 (54.79)	2.30 (4.23)
HepG2	15.50 (51.29)	>25 (82.17)	19.38 (67.23)	>25 (75.70)	>25 (78.56)	15.33 (50.72)	0.23 (0.42)
HL-60	9.92 (32.82)	>25 (82.17)	9.86 (34.21)	>25 (75.70)	24.21 (76.08)	13.33 (44.11)	0.83 (1.53)
K562	2.49 (8.24)	>25 (82.17)	2.94 (10.20)	>25 (75.70)	20.21 (63.51)	21.92 (72.53)	0.68 (1.25)
Non-tumor cells[d]							
PBMC	7.20 (23.82)	8.93 (29.35)	6.49 (22.52)	>25 (75.70)	>25 (78.56)	24.41 (80.77)	5.09 (9.36)

[a] Data are presented as IC_{50} values in μg/mL (μM) obtained by nonlinear regression from three independent experiments performed in duplicate, measured by Alamar blue assay after 72 h incubation.
[b] Doxorubicin was used as positive control.
[c] Tumor cells: B16-F10 (mouse melanoma), HepG2 (human hepatocellular carcinoma), HL-60 (human promyelocytic leukemia) and K562 (human chronic myelocytic leukemia).
[d] Non-tumor cell: PBMC (human peripheral blood mononuclear cells activated with concanavalin A—human lymphoblast).

has been previously reported as non-selective cytotoxic compound.[37]

Therefore, the stem bark of *A. vepretorum* is an important source for cytotoxic *ent*-kaurane diterpenes.

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Supplementary data

Supplementary data (spectrometric data, including NMR, MS and IR for the new *ent*-kaurane diterpenoid **1**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.06.005.

References and notes

1. Souza, V. C.; Lorenzi, H. Botânica Sistemática: Guia ilustrado das famílias de Fanerógamas nativas e exóticas no Brasil, baseado em APG I. Instituto Plantarum, 2005, 640.
2. Costa, E. V.; Dutra, L. M.; Nogueira, P. C.; Moraes, V. R.; Salvador, M. J.; Ribeiro, L. H.; Gadelha, F. R. *Nat. Prod. Commun.* **2012**, *7*, 265.

3. Diniz, T. C.; Araújo, C. S.; Silva, J. C.; Oliveira-Júnior, R. G.; Lima-Saraiva, S. R. G.; Quintans-Júnior, L. J.; Nunes, X. P.; Almeida, J. R. G. S. *J. Med. Plants Res.* **2013**, *7*, 2729.

4. *Botanical material:* The stem bark of *A. vepretorum* was collected in 'Serra da Guia', in the city of Poço Redondo [coordinates: 09° 57′ 54″ S, 37° 51′ 46″ W], Sergipe State, Brazil, in April 2010. The identity of the plant was confirmed by Dr. A. P. do N. Prata, a plant taxonomist of Department of Biology from Federal University of Sergipe (UFS), Brazil and a voucher specimen (#15441) has been deposited in the Herbarium of UFS. The authors have authorization from the Chico Mendes Institute for Biodiversity Conservation from Brazilian Ministry of the Environment for plant collection (#25637-1). This work was performed according to the special authorization for access to genetic resources in Brazil # 010240/2013-6, issued by CNPq/MCTI.
5. *Extraction and isolation:* The dried and powdered stem bark of *A. vepretorum* (653 g) was successively extracted with petroleum ether followed by MeOH, to yield petroleum ether (39.03 g) and MeOH (85.00 g) extracts. The petroleum ether extract (5.0 g) was initially subjected to silica gel column chromatography (CC) eluted with increasing concentrations of CH_2Cl_2 in *n*-hexane (100:0 to 10:90, v/v), followed by EtOAc in CH_2Cl_2 (100:0 at 30:70, v/v), and MeOH in EtOAc (100:0 to 70:30, v/v), affording 201 fractions (30 mL each). The eluted fractions were evaluated and pooled according to TLC analysis, to afford 19 groups (GF1 to GF19). Group GF3 (88.3 mg) from *n*-hexane–CH_2Cl_2 (90:10 and 80:20, v/v) was submitted to preparative TLC eluted with *n*-hexane–EtOAc (90:10, v/v, two elution), affording a mixture of **7** and **8** (38.8 mg). Group GF10 (69.0 mg) from *n*-hexane–CH_2Cl_2 (40:60 and 30:70) was submitted to preparative TLC eluted with *n*-hexane–EtOAc (95:05, v/v, three elution) yielding **6** (38.6 mg) and also a mixture of **7** and **8** (15.1 mg), respectively. GF12 (648.9 mg) from *n*-hexane–CH_2Cl_2 (10:90 v/v), CH_2Cl_2 (100%, *v*)), and CH_2Cl_2–EtOAc (95:05, v/v) was also subjected to a preparative TLC eluted with *n*-hexane–EtOAc (90:10, v/v, two elution) giving **4** (15.2 mg) and **6** (346.0 mg), respectively. GF13 (2457.8 mg) from CH_2Cl_2–EtOAc (90:10 and 80:20, v/v) was submitted to a new silica gel CC eluted with the same methodology as describe for initial CC (petroleum ether extract), affording 90 subfractions (30 mL each) that were subsequently pooled into 21 groups (GF13.1 to GF13.21), according to TLC analysis. Group GF13.2 (157.6 mg) was also submitted to preparative TLC eluted with *n*-hexane–EtOAc (90:10, v/v, two elution) again affording **6** (111.6 mg). Group GF13.3 (119.6 mg) was submitted to the same conditions as for GF13.2 also resulting in **6** (49.2 mg). GF13.4 (321.9 mg) was submitted to a new silica gel CC eluted with increasing concentrations of CH_2Cl_2 in *n*-hexane (100:0 to 30:70, v/v), followed by EtOAc in CH_2Cl_2 (100:0 to 50:50, v/v), affording 21 subfractions (30 mL each), that were evaluated and pooled according to TLC analysis, to afford 10 groups (GF13.4.1–GF13.4.10). The groups GF13.4.6 and GF13.4.7 were pooled (270.7 mg) and also submitted to TLC preparative eluted with *n*-hexane–EtOAc (80:20, v/v, three elution), yielding **3** (154.6 mg). Group GF13.5 (183.4 mg) was also submitted to preparative TLC eluted with *n*-hexane–EtOAc (90:10, v/v, three elution) again affording **3** (99.0 mg). Group GF13.8 (85.7 mg) was submitted to preparative TLC eluted with *n*-hexane–EtOAc (80:20, v/v, two elution), again yielding **3** (40.4 mg). Group GF13.9 (325.0 mg) was also subjected to a preparative TLC eluted with *n*-hexane–EtOAc (80:20, v/v, two elution) giving **3** (68.2 mg) and **1** (10.9 mg), respectively. Current fractions of the preparative of GF13.9 were contained and submitted successive CC and preparative TLC eluted in the same conditions that GF13, which resulted in the isolation of the mixture of **9**, **10** and **11** (15.5 mg). GF13.10 (145.9 mg) was submitted to a new silica gel CC eluted with increasing concentrations of CH_2Cl_2 in *n*-hexane (100:0 to 20:80, v/v) and EtOAc in CH_2Cl_2 (100:0 to 80:20, v/v) giving 28 subfractions (30 mL each) that were pooled into 8 groups (GF13.10.1–GF13.10.8), according to TLC analysis. The group GF13.10.1 (47.8 mg) was subjected to a preparative TLC eluted with *n*-hexane–EtOAc (80:20, v/v, two elution), yielding **3** (23.4 mg). Group 13.10.2 (57.1 mg) was submitted to the same conditions as for GF13.10.1 resulting in **1** (32.1 mg). Group 13.11 was also subjected to a preparative TLC eluted with *n*-hexane–EtOAc (80:20, v/v, three elution), again affording **1** (82.7 mg). The group GF16 (238.4 mg) from CH_2Cl_2–EtOAc (70:30, 60:40 and 50:50, v/v) was submitted to a new silica gel CC eluted with the same methodology as describe at the initial CC (petroleum ether extract), affording 69 subfractions (30 mL each) that were subsequently pooled into 7 groups (GF16.1–GF16.7), according to TLC analysis. Group GF16.5 was subjected to a preparative TLC eluted with *n*-hexane–EtOAc (70:30, v/v, four elution) giving **2** (100.4 mg). The group GF17 (76.7 mg) from CH_2Cl_2–EtOAc (40:60 and 30:70, v/v) and EtOAc–MeOH (100:0 and 95:05 v/v) was also submitted to a preparative TLC eluted with *n*-hexane–EtOAc (60:40, v/v, three elution) yielding **2** (6.3 mg). Group GF18 (115.9 mg) from EtOAc–MeOH (90:10 v/v) was submitted to preparative TLC eluted with *n*-hexane–EtOAc (60:40, v/v, two elution), affording **5** (13.0 mg).
6. *General experimental procedures:* Melting points (mp) were measured on a Microquímica MQAPF 301 apparatus. IR spectra were acquired in KBr pellets on a Shimadzu IR Prestige-21 spectrophotometer. Optical rotations were recorded in $CHCl_3$ on a Jasco P-2000 polarimeter. GC-MS analyses were performed on a Shimadzu QP5050A GC-MS system equipped with an AOC-20i auto-injector. The separation of the compounds was achieved employing on RTx®-5SilMS fused capillary chromatography column (30 m × 0.25 mm × 0.25 μm film thickness) coated with 5%-diphenyl-95%-dimethylpolysiloxane. The column temperature program was 200 °C/5 min, a rate of 10 °C/min to 320 °C, and then 320 °C/10 min (27 min total time analysis); carrier gas, He (99.999%; 1.2 mL/min); split ratio, 1:20; injection volume, 0.5 mL of the compound in CH_2Cl_2 (5.0 mg/mL). MS were taken at 70 eV with a scan interval of 0.5 s and fragments from 40–500 Da. Low Resolution Mass Spectra (LRMS) were determined using an ultra-high performance chromatography–mass spectrometry system (Acquity UHPLC-TQD–Waters) with an ESI and APCI source in the positive and negative ion mode. High Resolution Mass Spectra (HR-ESIMS) measurements were performed on a Bruker UltrOTOF-Q MS spectrometer featuring a quadrupole time-of-flight mass analyzer equipped with an electrospray source. 1D and 2D NMR data were recorded at 293 K in $CDCl_3$ on a Bruker Avance III 400 NMR spectrometer, operating at 9.4 Tesla, observing ^1H and ^{13}C at 400.13 and 100.61 MHz, respectively. The spectrometer was equipped with either, a 5-mm multinuclear direct detection probe (1D NMR experiments) or a 5-mm multinuclear inverse detection probe (1D NOE and 2D NMR experiments) both with *z*-gradient. One-bond and long-range ^1H–^{13}C correlation from HSQC and HMBC NMR experiments were optimized for an average coupling constant $^1J_{(C,H)}$ and $^{LR}J_{(C,H)}$ of 140 and 8 Hz, respectively. All ^1H and ^{13}C NMR chemical shifts (δ) are given in ppm related to the TMS signal at 0.00 ppm as an internal reference, and the coupling constants (*J*) in Hz. Silica gel 60 (70–230 mesh) was used for column chromatography, while silica gel 60 F_{254} was used for analytical (0.25 mm), and preparative (1.00 mm) TLC. Compounds were visualized by exposure under $UV_{254/365}$ light and spraying of *p*-anisaldehyde reagent followed by heating on a hot plate.

7. Silva, D. M.; Costa, E. V.; Nogueira, P. C. L.; Moraes, V. R. S.; Cavalcanti, S. C. H.; Salvador, M. J.; Ribeiro, L. H. G.; Gadelha, F. R.; Barison, A.; Ferreira, A. G. *Quím. Nova* **2012**, *35*, 1570.
8. Velandia, J. R.; Carvalho, M. G.; Braz-Filho, R. *Quim. Nova* **1998**, *21*, 397.
9. *ent-3β-Hydroxy-kaur-16-en-19-al* (***1***)*:* White solid (purity 98.5%); mp 117.0–118.1 °C; $[\alpha]_D^{20}$ −93.1° (*c* 0.4, $CHCl_3$); IR(KBr) ν_{max} 3267 (OH), 3078, 2924, 2854, 2726 (HCO), 1712 (C=O), 1642 (C=C), 1479, 1445, 1402, 1350, 1101, 1058, 1007, 869/cm; ^1H and ^{13}C NMR data, see Table 1; EI-MS *m/z* 302 $[M]^{+\cdot}$; LR-APCIMS $[M-H_2O]^+$ *m/z* 285.15; HR-ESIMS *m/z* 325.2140 (calcd for $C_{20}H_{30}O_2$+Na^+, 325.2143).

10. Piozzi, F.; Savona, G.; Hanson, J. R. *Phytochemistry* **1980**, *19*, 1237.
11. Facundo, V. A.; Polli, A. R.; Rodrigues, R. V.; Militão, J. S. L. T.; Stabelli, R. G.; Cardoso, C. T. *Acta Amazonica* **2008**, *38*, 733.
12. *ent-3β,19-Dihydroxy-kaur-16-ene* (***2***)*:* Yellow crystals (*n*-hexane–EtOAc 7:3) (purity 98.7%); mp 180.9–182.2 °C (lit. 190 °C);[10] $[\alpha]_D^{20}$ −26.4° (*c* 1.5, $CHCl_3$); IR(KBr) ν_{max} 3341 (OH), 3068, 2966, 2923, 2846, 1653 (C=C), 1465, 1431, 1362, 1064, 1004, 859/cm; ^1H and ^{13}C NMR data, see Table 1; EI-MS *m/z* 304 $[M]^{+\cdot}$. LR-APCIMS $[M-H_2O]^+$ *m/z* 287.17.
13. *ent-3β-Hydroxy-kaur-16-ene* (***3***)*:* White solid (*n*-hexane–EtOAc 8:2) (purity 98.7%); mp 161–163.0 °C (lit. 177–178 °C);[27] $[\alpha]_D^{20}$ −149.2° (*c* 0.45, $CHCl_3$); IR(KBr) ν_{max} 3345 (OH), 3070, 2924, 2854, 1651 (C=C), 1487, 1427, 1101, 1041, 989, 869/cm; ^1H and ^{13}C NMR data, see Table 1; EI-MS *m/z* 288 $[M]^{+\cdot}$. LR-APCIMS $[M-H_2O]^+$ *m/z* 271.24.
14. *ent-3β-Acetoxy-kaur-16-ene* (***4***)*:* Yellowish amorphous solid (purity 92.7%); mp 160.3–162.5 °C (lit. 162–164 °C);[27] $[\alpha]_D^{20}$ −28.56° (*c* 0.5, $CHCl_3$); IR(KBr) ν_{max} 3078, 2924, 2846, 1651 (C=C), 1720 (C=O), 1454, 1367, 1238, 1015, 869, 757/cm; ^1H and ^{13}C NMR data, see Table 1; EI-MS *m/z* 330 $[M]^{+\cdot}$. LR-APCIMS $[M-OCOCH_3]^+$ *m/z* 271.24.
15. *ent-3β-Hydroxy-kau-16-en-19-oic acid* (***5***)*:* White crystalline powder (*n*-hexane–EtOAc 6:4) (purity 100.0%); mp 204.4–205.0 °C (lit. 218–219 °C);[7] $[\alpha]_D^{20}$ −107.19° (*c* 0.7, $CHCl_3$); IR(KBr) ν_{max} 3482 (OH), 3371, 3070, 2941, 2854, 2623, 1737 (C=O), 1668 (C=C), 1454, 1350, 1256, 1195, 989, 860, 757, 636, 542/cm; ^1H and ^{13}C NMR data in agreement with those from the literature;[7] EI-MS *m/z* 318 $[M]^{+\cdot}$; LR-ESIMS $[M-H]^-$ *m/z* 317.27.
16. *ent-Kaur-16-en-19-oic acid* (***6***)*:* White needles (*n*-hexane–EtOAc 9:1) (purity 96.8%); mp 160.5–162.2 °C (lit. 160–162 °C);[7] $[\alpha]_D^{20}$ −93.56° (*c* 1.0, $CHCl_3$); IR(KBr) ν_{max} 3448 (OH), 3379, 3070, 2924, 2846, 2588, 2356, 1695 (C=O), 1642 (C=C), 1462, 1402, 1264, 1170, 955, 869, 782, 636/cm; ^1H and ^{13}C NMR data in agreement with those from the literature;[23] EI-MS *m/z* 302 $[M]^{+\cdot}$. LR-ESIMS $[M-H]^-$ *m/z* 301.28.
17. *Mixture of caryophyllene oxide* (***7***) *(rate 75.3%) and humulene epoxide II* (***8***) *(rate 24.7%):* Colorless oil; ^1H and ^{13}C NMR data in agreement with those from the literature; EI-MS *m/z* 220 and 218 $[M]^{+\cdot}$.
18. *Mixture of β-sitosterol* (***9***) *(rate 50,1%), stigmasterol* (***10***) *(rate 20.9%) and campesterol* (***11***) *(rate 19.8%):* White needles (*n*-hexane–EtOAc 8:2); mp 135.3–136.7 °C (lit. 138–140 °C);[31] ^1H and ^{13}C NMR data in agreement with those from the literature;[7,11] EI-MS *m/z* 414, 412 and 400 $[M]^{+\cdot}$.

19. Leboeuf, M.; Cavé, A.; Bhaumik, P. K.; Mukherjee, B.; Mukherjee, R. *Phytochemistry* **1982**, *21*, 2783.
20. Takahashi, J. Á.; Boaventura, M. A. D.; Bayma, J. C.; Oliveira, A. B. *Phytochemistry* **1995**, *40*, 607.
21. Takahashi, J. A.; Vieira, H. S.; Boaventura, M. A. D.; Hanson, J. R.; Hitchcock, P. B.; Oliveira, A. B. *Quim. Nova* **2001**, *24*, 616.
22. Chen, C. Y.; Chang, F. R.; Wu, Y. C. *J. Chin. Chem. Soc.* **1997**, *44*, 313.
23. Vieira, H. S.; Takahashi, J. A.; Boaventura, M. A. *J. Agric. Food Chem.* **2002**, *50*, 3704.
24. Barrero, A. F.; Oltra, J. E.; Cabrera, E.; Reyes, F.; Álvarez, M. *Phytochemistry* **1999**, *50*, 1133.
25. Rezende, M. C.; Urzua, A.; Bortoluzzi, A. J.; Vásquez, L. *J. Ethnopharmacol.* **2000**, *72*, 459.
26. Li, E. W.; Gao, K.; Jia, Z. J. *J. Asian Nat. Prod. Res.* **2007**, *9*, 191.
27. Tanaka, R.; Matsunaga, S. *Phytochemistry* **1988**, *27*, 2273.
28. Henry, G. E.; Adams, L. S.; Rosales, J. C.; Jacobs, H.; Heber, D.; Seenam, N. P. *Cancer Lett.* **2006**, *244*, 190.
29. Garcez, F. R.; Garcez, W. S.; Silva, A. F. G.; Bazzo, R. C.; Resende, U. M. *J. Braz. Chem. Soc.* **2004**, *15*, 767.

30. Da Silva, F. M. A.; Koolen, H. H. F.; Barisson, A.; De Souza, A. D. L.; Pinheiro, M. L. B. *J. Pharm. Sci.* **2012**, *4*, 522.
31. Costa, E. V.; Marques, F. A.; Pinheiro, M. L. B.; Braga, R. M.; Delarmelina, C.; Duarte, M. C. T.; Ruiz, A. L. T. G.; Carvalho, J. E.; Maia, B. H. L. N. S. *J. Braz. Chem. Soc.* **2011**, *22*, 1111.
32. Trigo, J. R.; Oliveira, J.; Andrade, E. H. A.; Zoghbi, M. G. B. *Rev. Bras. Plants Med.* **2007**, *9*, 113.
33. *Cell lines:* Cytotoxicity was evaluated to tumor cells lines B16-F10 (mouse melanoma), HepG2 (human hepatocellular carcinoma), K562 (human chronic myelocytic leukemia) and HL-60 (human promyelocytic leukemia). All cell lines were donated by Hospital A.C. Camargo, São Paulo, SP, Brazil. Cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 50 μg/mL gentamycin. Adherent cells were harvested by treatment with 0.25% trypsin EDTA solution. All cell lines were cultured in cell culture flasks at 37 °C in 5% CO_2 and sub-cultured every 3–4 days to maintain exponential growth. Cytotoxicity experiments were conducted with cells in exponential growth phase. All cell lines were tested for mycoplasma with a Lookout® Mycoplasma qPCR detection kit and found to be free from contamination.
34. *Primary culture of human lymphoblast:* In order to investigate the selectivity of the compounds toward a non-tumor proliferating cell, human lymphoblast cells were obtained by primary culture. Heparinized blood (from healthy, 20–35 years old, non-smoker donors who had not taken any drug at least 15 days prior to sampling) was collected and peripheral blood mononuclear cells (PBMC) were isolated by a standard protocol using Ficoll density gradient in a GE Ficoll-Paque Plus. PBMC were washed and resuspended at a concentration of 0.3×10^6 cells/mL in RPMI 1640 medium supplemented with 20% fetal bovine serum, 2 mM glutamine, 50 μg/mL gentamycin at 37 °C with 5% CO_2. In addition, concanavalin A was used as a mitogen to trigger cell division in T-lymphocytes. ConA (10 μg/mL) was added at the beginning of culture and, after 24 h, cells were treated with the test drugs. The Research Ethics Committee of the Oswaldo Cruz Foundation (Salvador, Bahia, Brazil) approved the experimental protocol (#031019/2013). All participants signed their written informed consent to participate in the study. For all experiments, cell viability was performed by Trypan blue exclusion (TBE) assay. Over 90% of the cells were viable at the beginning of the culture.
35. *Cell proliferation assay:* Cell growth was quantified by alamar blue assay, as previously described. For all experiments, cells were seeded in 96-well plates (0.7×10^5 cells/mL for adherent cells or 0.3×10^6 cells/mL for suspended cells in 100 μL of medium). After 24 h, the compounds (0.39–25 μg/mL) dissolved in DMSO were added to each well and incubated for 72 h. Doxorubicin (purity ⩾ 95.0%, doxorubicin hydrochloride) was used as positive control (0.08–5 μg/mL). Negative control received the vehicle used for diluting the tested (0.5% DMSO). Four (for cell lines) or 24 (for PBMC) hours before the end of the incubation, 20 μL of stock solution (0.312 mg/mL) of the alamar blue were added to each well. The absorbance was measured using a SpectraMax 190 multiplate reader and the drug effect was quantified as the percentage of control absorbance at 570 nm and 600 nm. The absorbance of alamar blue in culture medium was measured at a higher wavelength and a lower wavelength. The absorbance of the medium was also measured at the higher and lower wavelengths. The absorbance of the medium alone was subtracted from the absorbance of medium plus alamar blue at the higher wavelength. This value was called AO_{HW}. The absorbance of the medium alone was subtracted from the absorbance of medium plus alamar blue at the lower wavelength. This value was called AO_{LW}. A correction factor R_0 was calculated from AO_{HW} and AO_{LW}, where $R_0 = AO_{LW}/AO_{HW}$. The percent alamar blue reduced then was expressed as follows: % reduced = $A_{LW} - (A_{HW} \times R_0) \times 100$.
36. *Statistical analysis:* Data are presented as half maximal inhibitory concentration (IC_{50}) values obtained by nonlinear regression from three independent experiments performed in duplicate. All analyses were carried out using the GRAPHPAD software. SI was determined as IC_{50}[PBMC]/IC_{50}[K562].

37. Cavalcanti, B. C.; Bezerra, D. P.; Magalhães, H. I.; Moraes, M. O.; Lima, M. A.; Silveira, E. R.; Câmara, C. A.; Rao, V. S.; Pessoa, C.; Costa-Lotufo, L. V. *J. Appl. Toxicol.* **2009**, *29*, 560.
38. Fatope, M. O.; Audu, O. T.; Takeda, Y.; Zeng, L.; Shi, G.; Shimada, H.; McLaughlin, J. L. *J. Nat. Prod.* **1996**, *59*, 301.
39. Fujita, E.; Nagao, Y.; Node, M.; Kaaneko, K.; Nakazawa, S.; Kuroda, H. *Experientia* **1976**, *15*, 203.
40. Wang, L.; Li, D.; Wang, C.; Zhang, Y.; Xu, J. *Mini Rev. Med. Chem.* **2011**, *11*, 910.