

# New Tirucallane-Type Triterpenoids from *Guarea guidonia*

## Authors

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## ABSTRACT

The aerial parts of *Guarea guidonia* afforded three new tirucallane-type triterpenoids: 3,4-*seco*-tirucalla-4(28),8(9),24(25)-trien-7 $\alpha$ ,11 $\alpha$ -dihydroxy-21,23-epoxy-3,11-olide, named guareolide (1), 3,4-*seco*-tirucalla-4(28),7(8),24(25)-trien-21-hydroxy-21,23-epoxy-3-oic acid, named guareoic acid A (2), and 3,4-*seco*-tirucalla-4(28),7(8),24(25)-trien-21,23-epoxy-3-oic acid, named guareoic acid B (3), of which 1 possessed an unusual seven-membered lactone ring. Seven known terpenes were also isolated and characterized as flindissone, 7-acetyldihydronomilin, picroquassin E, boscartol C, and neo-rubins A, B, and X. Their structures were determined by spectroscopic methods including one-dimensional and two-dimensional nuclear magnetic resonance analysis and high-resolution mass spectrometry. The isolates were investigated for their potential cytotoxic activity on Jurkat, HeLa, and MCF7 cancer cell lines. Flindissone and compound 2 showed an antiproliferative activity in all cell lines. Further studies revealed that flindissone, the most active compound, induced in Jurkat and HeLa cells both cytostatic and cytotoxic responses.

## Introduction

*Guarea* is a genus of the Meliaceae family, which is among the richest and most diverse source of secondary metabolites in the Angiospermae, with 50 genera and more than 1400 species. The *Guarea* genus comprised both trees or small shrubs with about 80 species widespread in African and American countries; eight species of this genus were classified in the wet forest of Venezuela [1]. *Guarea guidonia* (L.) Sleumer is a tree typical of Brazil, but it is also found in Venezuela, particularly in the Sierra Parima of the Estado Bolivar, where the wood bark is traditionally used as a febrifugal agent and an abortive, while the leaves and the fruits are known to be toxic for the cattle [2, 3].

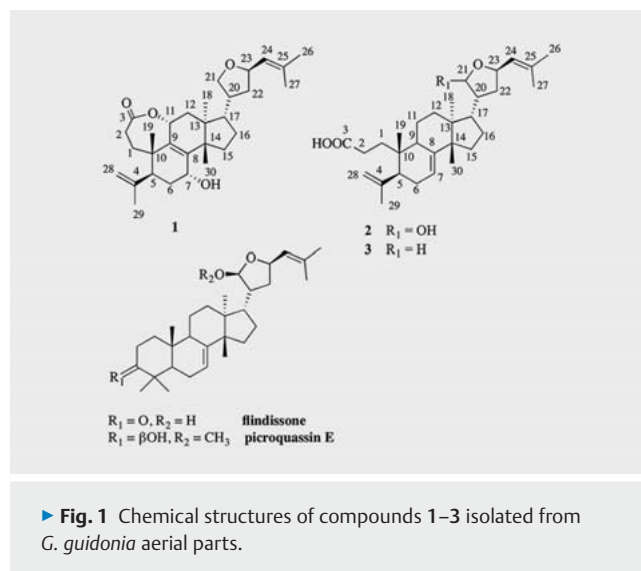
Limonoids, triterpenes, steroids, sesquiterpenes, diterpenes, and coumarins are typical constituents of this species [3]. The aerial parts of this plant were already investigated and different compositions have been found for specimens collected in different regions [4]. In our continuing investigations on Venezuelan Meliaceae plants [5, 6], the phytochemical study of *G. guidonia* aerial parts was carried out, and three new tirucallane-type triterpenoids (1–3) (► Fig. 1) were isolated and characterized, together with seven known compounds. In the framework of a research project aimed to investigate the cytotoxic activity of plant small molecules, all the isolates were assayed.

## Results and Discussion

The *n*-hexane and chloroform aerial parts extract of *G. guidonia* were repeatedly subjected to different chromatographies to afford three new tirucallane-type triterpenes (1–3) (► Fig. 1), along with seven known compounds. NMR spectra of compounds 1–3 and data for the EC<sub>50</sub> determination of cytotoxic activity are available as Supporting Information.

Compound 1 showed a sodiated molecular ion peak at  $m/z$  491.3122 for  $[M + Na]^+$  in the HRESIMS, allowing the assignment of molecular formula C<sub>30</sub>H<sub>44</sub>O<sub>4</sub>. The <sup>1</sup>H NMR of 1 (► Table 1) showed the presence of six methyl singlets, three of which on double bonds ( $\delta$  1.72, 1.73, and 1.82), an exomethylene group ( $\delta$  4.80, 5.01), one resonance for olefinic proton ( $\delta$  5.24), three hydroxymethines ( $\delta$  4.12, 4.67, 5.18), and one hydroxymethylene ( $\delta$  3.24, 4.15). The <sup>13</sup>C NMR (► Table 1) displayed 30 carbon signals that were assigned as six methyls ( $\delta$  18.3, 19.7, 20.2, 24.0, 25.9 double signal), seven methylenes ( $\delta$  28.3, 30.5, 30.8, 35.0, 35.3, 39.1, 39.3), three methines ( $\delta$  43.4, 46.9, 49.7), three quaternary carbons ( $\delta$  41.5, 43.0, 52.4), one methylene double bond ( $\delta$  115.8, 148.0), one trisubstituted double bond ( $\delta$  127.6, 138.0), one tetrasubstituted double bond ( $\delta$  133.2, 145.8), three oxygenated methyne carbons ( $\delta$  64.0, 75.1, 76.0), one oxygenated methylene carbon ( $\delta$  72.8), and one carboxyl group ( $\delta$  178.0). Results obtained from 1D TOCSY and COSY experiments established the correlations of all protons in 1, showing the sequences H-1–H-2, H-5–H-7, H-11–H-12, H-15–H-21, H-15–H-23. Full assignments of all protonated carbon signals were secured by HSQC and HMBC NMR interpretation. The HMBC correlations between  $\delta$  5.18 (H-11) and  $\delta$  133.2 (C-9), 145.8 (C-8), and 178.0 (C-3), and  $\delta$  1.96 (H-1a) and  $\delta$  20.2 (Me-19), 41.5 (C-10), 133.2 (C-9), and 178.0 (C-3) suggested that the hydromethine group at C-11 was involved in an unusual lactone ring with C-3. Other key cross-peaks were observed between  $\delta$  0.93 (Me-30) and  $\delta$  145.8 (C-8), confirming the presence of a C-8/C-9 double bond. The chemical shifts of H<sub>2</sub>-21 and H-23 suggested the presence of an epoxy function at these positions, forming a tetrahydrofuran ring in the side chain, while C-24–C-27 formed an isobutenoyl group [7]. The relative stereochemistry of 1 was determined by 1D ROESY (rotating frame Overhauser effect spectroscopy) experiments and comparison with literature data. The signal characteristic of H-7 (br s) and its 1D ROESY correlation with  $\delta$  0.93 (Me-30) demonstrated an  $\alpha$ -orientation for the 7-OH group. Selective irradiation of H-11 ( $\delta$  5.18) showed significant enhancement of the methyl signal at  $\delta$  1.09 (Me-19) and of H-2 $\beta$ ; similarly, irradiation of  $\delta$  1.09 (Me-19) produced enhancement of H-11 and H-7 signals. In light of these data, the structure of 1 was elucidated as 3,4-*seco*-tirucalla-4(28),8(9),24(25)-trien-7 $\alpha$ ,11 $\alpha$ -dihydroxy-21,23-epoxy-3,11-olide and was named guareolide. To the best of our knowledge, this is the first report of a tirucallane-type triterpene with an unusual seven-membered lactone ring.

The molecular formula of C<sub>30</sub>H<sub>46</sub>O<sub>4</sub> was assigned to compound 2, obtained as white amorphous powder, by its negative HRESIMS spectrum ( $m/z$  469.6838  $[M - H]^-$ ). Analysis of <sup>1</sup>H and <sup>13</sup>C NMR data (► Table 1) suggested a triterpenoid structure. The <sup>13</sup>C, HSQC, and HMBC NMR spectra of 2 showed 30 carbon signals, indicative of six methyls, eight methylenes, four methines,



three quaternary carbons, an exomethylene group, two trisubstituted double bonds, one hydroxymethine, a carboxyl group, and an emiacetal function. These data led to establish the presence of a *seco*-ring A tirucallane triterpene [8]. COSY and 1D TOCSY experiments, together with HMBC cross-peaks correlations between H-5–C-1, H-5–C-4, H-5–C-28, Me-29–C-4, Me-29–C-5, further confirmed the 3,4-*seco*-A-ring form in 2. The <sup>1</sup>H NMR spectrum of 2 revealed the presence of six singlet methyl groups, of which three were linked to double bonds, two resonances for olefinic hydrogens, two protons for an exocyclic double bond, one hydroxymethine, and one emiacetal function (► Table 1). A spin system, CH(17)–CH(20)–CHOH(21) and CH(17)–CH(20)–CH<sub>2</sub>(22)–CH(23)–CH(24), determined from the COSY spectrum, suggested associations of C-21 with C-20 and C-24, which would require a five-membered ring anchored at C-17. This finding was confirmed by the HMBC correlations of H-17 ( $\delta_H$  2.11) with C-20 ( $\delta_C$  49.0), H-20 ( $\delta_H$  2.02) with C-17 ( $\delta_C$  46.0) and C-21 ( $\delta_C$  104.0), H-21 ( $\delta_H$  4.83) with C-22 ( $\delta_C$  37.0), C-20 ( $\delta_C$  49.0), and C-23 ( $\delta_C$  76.4). The HMBC correlations from the proton signals at  $\delta_H$  1.69 (Me-26) and 1.72 (Me-27) to the carbon signals at  $\delta_C$  128.9 (C-24) and 135.0 (C-25) supported the occurrence of an isobutenyl group at C-23 [7]. The relative stereochemistry of 2 was obtained through 1D ROESY correlations between  $\delta$  1.06 (Me-30) and 0.86 (Me-19) and  $\delta$  0.94 (Me-18) and 2.02 (H-20). Furthermore, similar <sup>1</sup>H and 1D ROESY NMR data showed the relative configuration of the side chain in 2 to be the same as 3-*epi*-flindissol [7]. Therefore, compound 2 was identified as 3,4-*seco*-tirucalla-4(28),7(8),24(25)-trien-21-hydroxy-21,23-epoxy-3-oic acid, named as guareoic acid A.

Compound 3 was purified as a white amorphous powder. Its molecular formula was determined to be C<sub>30</sub>H<sub>46</sub>O<sub>3</sub> from the deprotonated molecular ion  $[M - H]^-$  at  $m/z$  453.3329 in the negative HRESIMS spectrum. Analysis of its NMR spectra (► Table 1) suggested the same 3,4-*seco*-tirucallane skeleton described for 1. Comparison of NMR spectra with those of 2 revealed 3 to differ from 2 only by functionalities of ring E. In fact, in compound 3 the chemical shifts of H<sub>2</sub>-21 at  $\delta$  4.01 (1H, t,  $J$  = 8.3 Hz) and 3.28 (1H, br t,  $J$  = 9.2 Hz), and H-23 at  $\delta$  4.65 (1H, ddd,  $J$  = 13.3, 8.5,

► **Table 1**  $^1\text{H}$  and  $^{13}\text{C}$ -NMR data of compounds 1–3 ( $\text{CD}_3\text{OD}$ , 600 MHz,  $J$  in Hz).

Position	1		2		3	
	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$
1a	1.96 br dd (14.7, 8.0)	35.0	1.28 m	32.0	1.40	32.8
1b	1.50 <sup>a</sup>					
2a	3.00 br dd (14.0, 10.0)	30.5	2.00 <sup>a</sup>	30.0	2.38 ddd (15.5, 11.0, 3.8)	29.1
2b	2.44 br dd (14.5, 7.6)		1.80 <sup>a</sup>		2.21 <sup>a</sup>	
3		178.0		178.0		175.0
4		148.0		149.0		148.3
5	2.74 dd (14.5, 2.5)	46.9	2.47 dd (12.0, 6.0)	50.4	2.49 dd (12.0, 6.0)	50.5
6a	2.03 m	28.3	2.24 m	31.3	2.24 <sup>a</sup>	31.2
6b	1.57 <sup>a</sup>		2.03 <sup>a</sup>		2.03 <sup>a</sup>	
7	4.12 br s	64.0	5.26 br d (3.5)	119.1	5.39 br d (3.0)	119.5
8		145.8		147.0		147.9
9		133.2	2.60 m	42.2	2.57 m	42.2
10		41.5		37.9		38.0
11	5.18 br d (5.6)	75.1	1.61 <sup>a</sup>	18.0	1.61 <sup>a</sup>	18.7
12a	2.16 <sup>a</sup>	39.3	1.63 <sup>a</sup>	33.0	1.72 <sup>a</sup>	32.7
12b	1.70 <sup>a</sup>		1.50 <sup>a</sup>		1.63 <sup>a</sup>	
13		43.0		45.0		44.8
14		52.4		52.0		52.0
15a	2.06 ddd (17.0, 13.0, 3.5)	35.3	1.99 <sup>a</sup>	36.7	1.65 <sup>a</sup>	35.5
15b	1.69 <sup>a</sup>		1.50 <sup>a</sup>		1.52 m	
16a	1.50 <sup>a</sup>	30.8	1.70 <sup>a</sup>	34.4	1.72 <sup>a</sup>	32.5
16b	1.37 <sup>a</sup>		1.63 <sup>a</sup>		1.49 <sup>a</sup>	
17	1.78 <sup>a</sup>	49.7	2.11 br dd (15.0, 9.4)	46.0	1.72 <sup>a</sup>	52.9
18	1.07 s	19.7	0.94 s	24.0	0.92 s	23.4
19	1.09 s	20.2	0.86 s	16.0	0.87 s	16.2
20	2.35 m	43.4	2.02 <sup>a</sup>	49.0	2.05 <sup>a</sup>	43.5
21a	4.15 t (8.5)	72.8	4.83 <sup>a</sup>	104.0	4.01 t (8.3)	73.0
21b	3.24 t (8.5)				3.28 br t (9.2)	
22a	2.20 <sup>a</sup>	39.1	1.72 <sup>a</sup>	37.0	1.72 <sup>a</sup>	39.6
22b	1.79 <sup>a</sup>		1.50 <sup>a</sup>			
23	4.67 ddd (13.5, 8.0, 5.0)	76.0	4.76 ddd (13.0, 8.3, 4.6)	76.4	4.65 ddd (13.3, 8.5, 4.6)	75.5
24	5.24 br d (8.3)	127.6	5.18 br d (9.0)	128.9	5.20 br d (8.4)	127.1
25		138.0		135.0		138.0
26	1.73 s	18.3	1.69 s	18.0	1.70 s	17.9
27	1.72 s	25.9	1.72 s	26.0	1.72 s	25.0
28a	5.01 br s	115.8	4.86 <sup>a</sup>	114.4	4.89 <sup>a</sup>	114.6
28b	4.80 br s		4.84 <sup>a</sup>		4.85 <sup>a</sup>	
29	1.82 s	24.0	1.81 s	23.0	1.81 s	22.8
30	0.93 s	25.9	1.06 s	27.0	1.05 s	27.9

Data assignments were confirmed by DQF-COSY, 1D TOCSY, HSQC, and HMBC experiments. <sup>a</sup> Overlapped signal.

4.6 Hz), suggested that the emiacetal function at C-21 was replaced by a hydroxymethylene group [9]. Thus, the structure of 3,4-*seco*-tirucalla-4(28),7(8),24(25)-trien-21,23-epoxy-3-oic acid was determined for compound 3 and was named guareoic acid B.

Flindissone [10], 7-acetyldihydronomilin [11], microquassin E [12], boscartol C [13], and cneurubins A, B, and X [14] were also purified and identified by comparison with published spectroscopic data.

► **Table 2** EC<sub>50</sub> (μM) antiproliferative activity of isolates in different cell lines.

Compound	Jurkat <sup>a</sup>	HeLa <sup>b</sup>	MCF7 <sup>c</sup>	PBMC <sup>d</sup>
1	> 100	> 100	> 100	–
2	39	55	75	> 100
3	> 100	> 100	> 100	–
Flindissone	25	27	50	> 100
Picroquassin E	88	> 100	> 100	–
Etoposide	2.5	4	12	–

<sup>a</sup> T-cell leukemia; <sup>b</sup> cervical carcinoma; <sup>c</sup> breast carcinoma; <sup>d</sup> human peripheral blood mononuclear cells

The antiproliferative activity of the isolates was evaluated in Jurkat (T-cell leukemia), HeLa (cervical carcinoma), and MCF-7 (breast cancer) cancer cell lines by ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]) (MTT) assay. Half maximal effective concentration (EC<sub>50</sub>) values, obtained from dose-response curves, are shown in ► **Table 2**. Compound **2** and flindissone showed an antiproliferative activity in all cell lines. In particular, Jurkat resulted slight more susceptible than HeLa and MCF7 cells. Furthermore, the cytotoxic potential of active compounds was evaluated in human peripheral blood mononuclear cells (PBMC) from healthy donors, chosen as the normal counterpart of leukemia-derived Jurkat cell line. Compound **2** and flindissone did not causes any significant reduction of the number of freshly isolated non-proliferating PBMC, at least in the range of cytotoxic doses in leukemia cells.

The mechanism(s) underlying the antiproliferative effect of the most active flindissone was further studied in HeLa and Jurkat cells. To investigate whether flindissone reduced cells number by affecting cell cycle progression and/or by inducing cell death, HeLa and Jurkat cells were exposed for 48 h to concentrations (15 and 35 μM) close to the EC<sub>50</sub> value of flindissone. DNA content was evaluated by flow cytometry analysis of propidium iodide stained nuclei. As shown in ► **Fig. 2**, flindissone caused in HeLa (► **Fig. 2A**) and in Jurkat (► **Fig. 2B**) an increase of cell population in G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub>/M phases. Moreover, an increase of cells with subG<sub>0</sub>/G<sub>1</sub> DNA content was also observed, thus indicating the onset of apoptotic events.

## Materials and Methods

### General experimental procedures

Optical rotations were measured on a Autopol IV Automatic Polarimeter (Rudolph) equipped with a sodium lamp (589 nm) and a 1-dm microcell. NMR experiments were performed on a Bruker DRX-600 spectrometer (Bruker BioSpin GmbH) equipped with a Bruker 5-mm TCI CryoProbe at 300 K. Chemical shifts were expressed in δ (parts per million) referred to the solvent peaks δ<sub>H</sub> 3.34 and δ<sub>C</sub> 49.0 for CD<sub>3</sub>OD. The NMR data were processed as reported previously [15]. HRESIMS were acquired in the positive and negative ion mode on a LTQ Orbitrap XL mass spectrometer (Ther-

mo Fisher Scientific). Column chromatographies were performed over silica gel (70–220 mesh, Merck); RP-HPLC separations were conducted on a Waters 590 series pumping system equipped with a Waters R401 refractive index detector, using a C<sub>18</sub>μ-Bondapak column (30 cm × 7.8 mm, 10 μm, Waters-Milford) and a mobile phase consisting of MeOH-H<sub>2</sub>O mixtures at a flow rate of 2 mL/min. TLC were carried out on precoated Kieselgel 60 F<sub>254</sub> plates (Merck); compounds were detected by Ce(SO<sub>4</sub>)<sub>2</sub>/H<sub>2</sub>SO<sub>4</sub> (Sigma-Aldrich) solution.

### Plant material

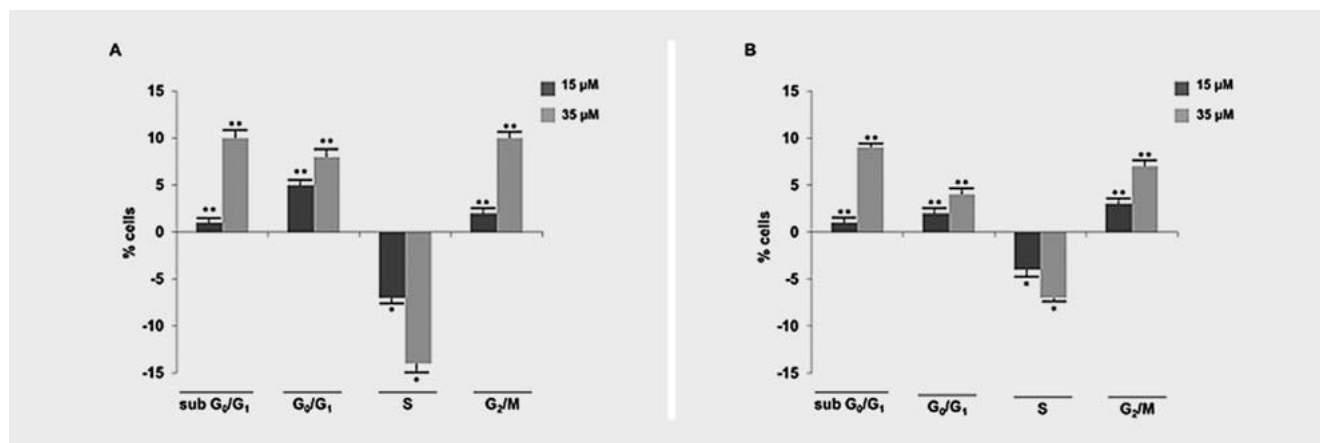
The aerial parts of *G. guidonia* were collected in December 2008, in the Estación Experimental Caparo (7°28' 13" N; 71°03' 16" O), Llanos Occidentales, Suroeste del Estado Barinas, Municipio Ezequile Zamora, Venezuela. The plant was identified by Prof. Dr. Pablo Melendez, and a voucher specimen (n. 625) was deposited in the Herbarium MERF of the Facultad de Farmacia y Bioanálisis, Universidad de Los Andes, Mérida, Venezuela.

### Extraction and isolation

The aerial parts of *G. guidonia* (400 g) were powdered and successively extracted with *n*-hexane, CHCl<sub>3</sub>, and MeOH (3 × 2 L), to give 6.0 g, 12.1 g, and 15.0 g of the respective dried residues. Part of the *n*-hexane extract (5 g) was subjected to column chromatography (5 × 150 cm) over silica gel eluting with *n*-hexane, followed by increasing concentrations of CHCl<sub>3</sub> in *n*-hexane (between 1% and 100%) and increasing concentrations of MeOH in CHCl<sub>3</sub> (between 1% and 100%). Fractions of 30 mL were collected, analyzed by TLC, and grouped into nine major fractions (A<sub>1</sub>–I<sub>1</sub>). Fraction D<sub>1</sub> (241.0 mg) was purified by RP-HPLC using MeOH-H<sub>2</sub>O (4:1) to give cneorubin X (2.0 mg, *t*<sub>R</sub> 42 min). Fraction F<sub>1</sub> (497.2 mg) was chromatographed by RP-HPLC using MeOH-H<sub>2</sub>O (75:25) to yield cneorubin A (3.5 mg, *t*<sub>R</sub> 34 min) and cneorubin B (4.0 mg, *t*<sub>R</sub> 38 min). Fraction H<sub>1</sub> (274.1 mg) was subject to RP-HPLC using MeOH-H<sub>2</sub>O (7:3) to give boscartol C (2.2 mg, *t*<sub>R</sub> 37 min) and cneorubin X (2.3 mg, *t*<sub>R</sub> 47 min). Part of the CHCl<sub>3</sub> extract (5.3 g) was subjected to silica gel column chromatography (5 × 150 cm), eluting with CHCl<sub>3</sub> followed by increasing concentrations of MeOH in CHCl<sub>3</sub> (between 1% and 100%). Fractions of 30 mL were collected, analyzed by TLC, and grouped into 10 major fractions (A<sub>2</sub>–J<sub>2</sub>). Fractions D<sub>2</sub> (990.0 mg) and E<sub>2</sub> (264.5 mg) were purified by RP-HPLC with MeOH-H<sub>2</sub>O (85:15) as eluent to obtain flindissone (8.5 mg, *t*<sub>R</sub> 27 min), compounds **3** (10.0 mg, *t*<sub>R</sub> 42 min) and **2** (6.0 mg, *t*<sub>R</sub> 50 min), from fraction D<sub>2</sub>, and compounds **1** (1.2 mg, *t*<sub>R</sub> 12 min), **3** (2 mg, *t*<sub>R</sub> 42 min), and **2** (2.2 mg, *t*<sub>R</sub> 50 min), from fraction E<sub>2</sub>, respectively. Fraction C<sub>2</sub> (228.3 mg) was purified by RP-HPLC using MeOH-H<sub>2</sub>O (3:2) to give 7-acetyldihydronomilin (1.2 mg, *t*<sub>R</sub> 22 min). Finally, fraction H<sub>2</sub> (145.0 mg) was subjected to RP-HPLC using MeOH-H<sub>2</sub>O (9:1) to give picroquassin E (2.3 mg, *t*<sub>R</sub> 45 min).

*Guareolide (1)*: white amorphous powder; [α]<sub>D</sub><sup>25</sup> – 143 (c 0.1, MeOH); <sup>1</sup>H and <sup>13</sup>C NMR data, see ► **Table 1**; HRESIMS: *m/z* 491.3122 [M + Na]<sup>+</sup> (calcd. for C<sub>30</sub>H<sub>44</sub>NaO<sub>4</sub>, 491.3137).

*Guareoic acid A (2)*: white amorphous powder; [α]<sub>D</sub><sup>25</sup> + 11.5 (c 0.09, MeOH); <sup>1</sup>H and <sup>13</sup>C NMR data, see ► **Table 1**; HRESIMS: *m/z* 469.6838 [M – H]<sup>–</sup> (calcd. for C<sub>30</sub>H<sub>45</sub>O<sub>4</sub>, *m/z* 469.3318).



► **Fig. 2** Effect of flindissone on cell proliferation. Flow cytometric evaluation of DNA content in cells exposed for 48 h to flindissone or vehicle alone (controls). **A** HeLa cells were exposed to 15  $\mu\text{M}$  and 35  $\mu\text{M}$  flindissone; **B** Jurkat cells exposed to 15  $\mu\text{M}$  and 35  $\mu\text{M}$  flindissone. Data in **A** and **B** have been subtracted for the corresponding values in control cells (exposed to vehicle only): HeLa, sub $G_0/G_1 \leq 2\%$ ;  $G_0/G_1$ ,  $53 \pm 1.9\%$ ; S,  $37 \pm 2.2\%$ ;  $G_2/M$ ,  $10 \pm 0.3\%$ ; Jurkat, sub $G_0/G_1 \leq 2.2\%$ ;  $G_0/G_1$ ,  $56 \pm 1.8\%$ ; S,  $37 \pm 2.1\%$ ;  $G_2/M$ ,  $6 \pm 0.9\%$ . Data are the mean values from three experiments; standard deviation (SD) values never exceeded 12% of the means.

*Guareoic acid B* (**3**): white amorphous powder;  $[\alpha]_D^{25} + 139$  (c 0.27, MeOH);  $^1\text{H}$  and  $^{13}\text{C}$  NMR data, see ► **Table 1**; HRESIMS:  $m/z$  453.3329  $[\text{M} - \text{H}]^-$  (calcd. for  $\text{C}_{30}\text{H}_{45}\text{O}_3$ ,  $m/z$  453.3369).

## Cytotoxicity assay

### Cells and treatment

Jurkat, HeLa, and MCF-7 cell lines were obtained from the ATCC. Cells were maintained in DMEM (HeLa and MCF-7) or RPMI 1640 (Jurkat), supplemented with 10% FBS, 100 mg/L streptomycin, and penicillin 100 IU/mL at 37 °C in a humidified atmosphere of 5%  $\text{CO}_2$ . To ensure logarithmic growth, cells were subcultured every 2 d. PBMC were isolated from buffy coats of healthy donors (kindly provided by the Blood Center of the Hospital of Battipaglia, Salerno, Italy) by using a standard Ficoll-Hypaque gradient. Freshly isolated PBMC contained  $90.6 \pm 1.2\%$  live cells as assessed by the manual Trypan Blue exclusion method. Stock solutions (50 mM) of purified compounds in DMSO were stored in the dark at 4 °C. Appropriate dilutions were prepared in culture medium immediately prior to use. In all experiments, the final concentration of DMSO did not exceed 0.15% (v/v).

### Cell viability and cell cycle

Cells were seeded in 96-well plates and incubated for the established times in the absence (vehicle only) and in the presence of different concentrations of compounds. The day before treatments, cells were seeded at a cell density of  $1 \times 10^4$  cells/well. The number of viable cells was quantified by MTT assay. Absorption at 550 nm for each well was assessed using a microplate reader (LabSystems). In some experiments, cell viability was also checked by Trypan Blue exclusion assay using a Bürker counting chamber.  $\text{EC}_{50}$  values were calculated from cell viability dose-response curves and defined as the concentration resulting in 50% inhibition in cell survival as compared to etoposide (etoposide synthetic,  $\geq 98\%$ , Sigma-Aldrich), used as positive control. Each

experimental condition was tested once in quadruplicate.  $\text{EC}_{50}$  values were performed with GraphPad software.

Cell cycle was evaluated by propidium iodide staining of permeabilized cells according to the available protocol and flow cytometry (BD FACSCalibur flow cytometer, Becton Dickinson) [16]. Data from 5000 events per sample were collected. The percentages of the elements in the hypodiploid region and in  $G_0/G_1$ , S and  $G_2/M$  phases of the cell cycle were calculated using the CellQuest or MODFIT software, respectively.

## Supporting Information

NMR spectra of compounds **1–3** and data for the  $\text{EC}_{50}$  determination of cytotoxic activity are available as Supporting Information.

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## Conflict of Interest

The authors declare no conflicts of interest.

## References

- [1] Aristeguieta L. Familias y géneros de los árboles de Venezuela. Edición special del Instituto botánico. Caracas: 1973: 841
- [2] Oga S, Sertié JA, Brasile AC, Hanada S. Antiinflammatory effect of crude extract from *Guarea guidonia*. *Planta Med* 1981; 42: 310–312
- [3] Garcez FR, Nuñez CV, Garcez WS, Almeida RM, Roque NF. Sesquiterpenes, limonoid and coumarin from the wood bark of *Guarea guidonia*. *Planta Med* 1998; 64: 79–80
- [4] Lago JHG, Brochini CB, Roque NF. Terpenoids from *Guarea guidonia*. *Phytochemistry* 2002; 60: 333–338

- [5] Dal Piaz F, Malafronte N, Romano A, Gallotta D, Belisario MA, Bifulco G, Gualtieri MJ, Sanogo R, De Tommasi N, Pisano C. Structural characterization of tetranortriterpenes from *Pseudocedrela kotschyi* and *Trichilia emetica* and study of their activity towards the chaperone Hsp90. *Phytochemistry* 2012; 75: 78–89
- [6] Gualtieri MJ, Malafronte N, Vassallo A, Braca A, Cotugno R, Vasaturo M, De Tommasi N, Dal Piaz F. Bioactive limonoids from the leaves of *Azadirachta indica* (Neem). *J Nat Prod* 2014; 77: 596–602
- [7] Kamperdick C, Lien TP, Adam G, Sung TV. Apotirucallane and tirucallane triterpenoids from *Luvunga sarmentosa*. *J Nat Prod* 2003; 66: 675–678
- [8] Zheng Q, Guan B, Qin JJ, Wang CH, Cheng XR, Ren J, Yan SK, Jin HZ, Zhang WD. 2, 3-*seco*- and 3, 4-*seco*-tirucallane triterpenoid derivatives from the stems of *Aphanamixis grandifolia* Blume. *Phytochemistry* 2012; 80: 148–155
- [9] Kim KH, Choi SU, Kim YC, Lee KR. Tirucallane triterpenoids from *Cornus walteri*. *J Nat Prod* 2011; 74: 54–59
- [10] Guang-Yi L, Gray AI, Waterman PG. Chemistry of the Burseraceae. Part 9. Tirucallane and oleanane triterpenes from the resin of *Aucoumea klaineana*. *Phytochemistry* 1988; 27: 2283–2286
- [11] Ahmed FR, Ng AS, Fallis AG. 7 $\alpha$ -Acetoxydihydronomilin: isolation, spectra, and crystal structure. *Can J Chem* 1978; 56: 1020–1025
- [12] Xu J, Xiao D, Lin QH, He JF, Liu WY, Xie N, Feng F, Qu W. Cytotoxic tirucallane and apotirucallane triterpenoids from the stems of *Picrasma quassioides*. *J Nat Prod* 2016; 79: 1899–1910
- [13] Wang YG, Ren J, Wang AG, Yang JB, Ji TF, Ma QG, Tian J, Su YL. Hepatoprotective prenylaromadendrane-type diterpenes from the gum resin of *Boswellia carterii*. *J Nat Prod* 2013; 76: 2074–2079
- [14] Brochini CB, Roque NF. Two new cneurubin related diterpenes from the leaves of *Guarea guidonia* (Meliaceae). *J Braz Chem Soc* 2000; 11: 361–364
- [15] De Leo M, Peruzzi L, Granchi C, Tuccinardi T, Minutolo F, De Tommasi N, Braca A. Constituents of *Polygala flavescens* ssp. *flavescens* and their activity as inhibitors of human lactate dehydrogenase. *J Nat Prod* 2017; 80: 2077–2087
- [16] Jimenez-Usuga N, Malafronte N, Cotugno R, De Leo M, Osorio E, De Tommasi N. New sesquiterpene lactones from *Ambrosia cumanensis* Kunth. *Fitoterapia* 2016; 113: 170–174