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ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 7355–7360
Bioorganic & Medicinal Chemistry

Natural triterpenoids from *Cecropia lyratiloba* are cytotoxic to both sensitive and multidrug resistant leukemia cell lines

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Received 24 May 2007; revised 24 July 2007; accepted 31 July 2007
Available online 22 August 2007

Abstract—The cytotoxicity of four triterpenoids, euscaphic acid (**1**), tormentic acid (**2**), 2α-acetyl tormentic acid (**3**), and 3β-acetyl tormentic acid (**4**), isolated from the roots of *Cecropia lyratiloba* (Moraceae) by countercurrent chromatography, was evaluated in vitro in sensitive and multidrug resistant leukemia cell lines. A structure/activity relationship analysis of the compounds was performed. Acetylation of compound **2** at C2 increased its activity by a factor of 2 while acetylation at C3 had a smaller effect. Compound **1** induces death by activation of caspase-3, dependent apoptotic pathway. Furthermore, the four triterpenoids were also active toward a multidrug resistant (MDR) leukemia cell line, overexpressing glycoprotein-P (P-gp). These results reveal the potential of the terpenoids as source for the development of new anti-neoplastic and anti-MDR drugs.
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1. Introduction

Cancer is the leading cause of death in the world. Despite technological advances, the index of cancer cure is very low and its treatment is still a challenge. The ability of cancer cells to evade death and the expression of natural or chemically induced multidrug resistance (MDR) are important drawbacks in cancer chemotherapy. Clinically MDR is characterized by the lack of response to several structurally unrelated drugs that do not have a common mechanism of action.[1] The complexity and versatility of cellular MDR mechanisms[2,3] hampers the use of more efficient and clinically applicable therapies leading to a bad prognostic of patients with this kind of tumor.[4–6] Thus, great efforts are being dedicated to the search of compounds able to bypass or to reverse MDR. Nowadays, natural products are the main source of new substances for anti-neoplastic drug development.[7] Indeed, about 60% of the anti-tumoral drugs approved for use by regulatory agencies were shown to be of natural origin.[8]

Plants of *Cecropia* genus are used in traditional medicine for the treatment of several diseases such as asthma,[9] high blood pressure,[10,11] diabetes,[12,13] and anxiety.[14] Recently the anti-hypertensive properties of methanol extract from *Cecropia lyratiloba* were shown.[15] However, the pharmacological properties *C. lyratiloba* are poorly investigated.

As part of a study program to evaluate the therapeutic properties of Brazilian plants, the phytochemical and pharmacological properties of *C. lyratiloba* were investigated. Phytochemical studies of *C. lyratiloba* lead to the identification of four pentacyclic triterpenoids: euscaphic acid (**1**), tormentic acid (**2**), and 2α- and 3β-acetyl tormentic acid (**3** and **4**) Although the anti-tumoral activity of the compounds **1** and **2** had been reported,[16] nothing is known about their mechanism of action. Moreover, 2α- and 3β-acetyl tormentic acid are new structures. This paper evaluated the structure/activity relationship of the triterpenoids obtained from *C. lyratiloba* and investigated their anti-tumoral activities on K562 (leukemia cell line) and on Lucena-1, a VCR-derivative of K562 that overexpresses P-gp and presents

Keywords: *Cecropia lyratiloba*; Triterpenes; Cytotoxic activity; Cancer; Multidrug resistance.
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doi:10.1016/j.bmc.2007.07.020

several characteristics of multidrug resistance.[17] It also studied the cell death mechanism of the compound **1** and its cytotoxicity to different human tumor cell lines: lung (A549), colon (Caco-2), and larynx (HEp-2).

2. Results and discussion

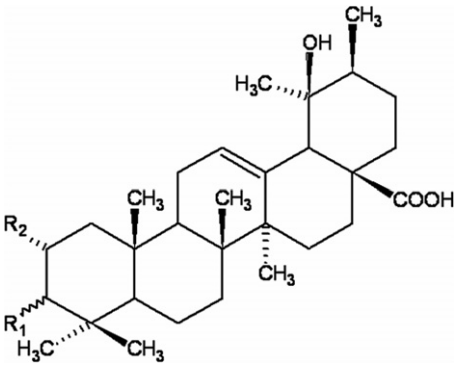
2.1. Purification of triterpenes

Cecropia lyratiloba Miquel was collected in the Serra do Mendanha, Rio de Janeiro, RJ, Brazil and it was identified by the botanist Dr. Jorge Pedro P. Carauta. A voucher specimen was deposited at the Alberto Castellanos Herbarium, FEEMA (Fundação Estadual de Engenharia do Meio Ambiente), Rio de Janeiro, RJ, under the register number (GUA 47028).

An aqueous suspension of the methanol extract from *C. lyratiloba* barks was partitioned successively with hexane, dichloromethane, ethyl acetate, and butanol. The CH_2Cl_2 fraction was submitted to high-speed countercurrent chromatography (HSCCD) using hexane:ethyl acetate:methanol:water (1:2:*X*:1) with *X* = [A (1.25), B (1.50), and C (1.75)] as eluent essentially as described elsewhere.[18] According to the TLC profile, the eluted material resulted in the separation of euscaphic acid (2α-OH, 3α-OH), tormentic (2α-OH, 3β-OH), 2α-acetyl tormentic acid (2α-OAc, 3-βOH), and 3-acetyl tormentic acid (2α-OH, 3β-OAc). The purity and characterization of these compounds (Fig. 1) were determined by measuring their GC/MS, ^1H and ^{13}C NMR, melting points, and optical rotations. All known triterpene acids were identified by comparison with reported physical and spectral data. Usually these compounds were dissolved in dimethylsulfoxide (DMSO, Sigma, St. Louis, MO) then diluted in RPMI for assay.

2.2. Anti-cancer assays

The cytotoxic activity of the triterpenes was assessed in vitro by MTT.[19] Compounds **1**–**4** decreased the viability of the leukemia cell line K562 in a dose-dependent way (Fig. 2). The activity, expressed as IC_{50} values, showed values varying from 38.35 ± 5.29 to 89.36 ± 2.23 μM for **3** and **2**, respectively (Table 1). Analysis of the structure/activity of the compounds indicated that change at C3-OH from the α- to β-position in compound **1** had no effect on the activity of the triterpenoids while acetylation of **2** at C2 increased its activity by a factor of 2. Although the cytotoxic activity to solid tumor cell lines and anti-tumor-promoting activity of **1** and **2** were described previously[16] they have not been tested for leukemia cell lines. This is the first time that the activity of **3** and **4** is reported.



Euscaphic acid: R1= α-OH; R2= α-OH
Tormentic acid: R1=β-OH; R2= α-OH
2α-acetyl tormentic acid: R1=β-OH; R2= α-OAc
3β-acetyl tormentic acid: R1=β-OAc; R2= α-OH

Figure 1.

2.3. Anti-MDR activity

Multidrug resistance (MDR) is a multifactorial phenomenon. One of its most well studied mechanisms is the overexpression of transmembrane proteins that act as efflux pumps, decreasing the intracellular drug concentration and preventing death.[20] Herein, the anti-MDR activity of triterpenes from *C. lyratiloba* was assessed in Lucena-1, a leukemia cell line that overexpresses P-gp and presents cross resistance to several unrelated cytotoxic drugs.[17] Compounds **1**–**4** decreased the viability of this cell line in a dose-dependent manner, indicating their ability to circumvent resistance mediated by P-gp overexpression (Fig. 3). Determination of the IC_{50} revealed that compounds **1**–**4** were as effective in killing a MDR cell line as they were for a sensitive one (Table 1). The molar drug concentration required to cause 50% viability inhibition (IC_{50}) was determined from dose–response curves. Values are expressed as means ± SD. Difference in resistance between K562 and Lucena-1 was seen by using vincristine as control (Table 1).

2.4. Cytotoxic mechanisms

Investigation of the mechanism of cytotoxic activity of the triterpenes was performed with compound **1**. In addition to its activity in leukemia cell lines, this compound also decreased the viability of other human tumor cell lines: HEp-2 (larynx), Caco-2 (colon), and A549 (lung) in a dose-dependent way. The IC_{50} values for HEp-2, Caco-2, and A549 were 105.7 ± 0.39, 104.1 ± 0.68, and 95.58 ± 5.14 μM, respectively.

Cell-cycle analysis was used to evaluate induction of apoptosis in K562 cells treated with 25 μg/mL of **1**. Appearance of the hipo-diploid nuclei population, indicative of DNA fragmentation, demonstrated that **1**-induced DNA fragmentation was dose dependent (Fig. 4, left). Similar results were obtained when the MDR leukemia cell line Lucena-1 was used (Fig. 4, right) corroborating the conclusions drawn from the IC_{50} values obtained for the two cell lines (76.71 ± 8.23 and 83.79 ± 4.17 μM, respectively). The ability of compound **1** to overcome the resistance mediated by P-gp expression is in agreement with other studies from our group showing the anti-MDR activity of other triterpenes.[21–23]

The fragmentation of DNA, a characteristic of the apoptotic process, is mediated by activation of a cascade

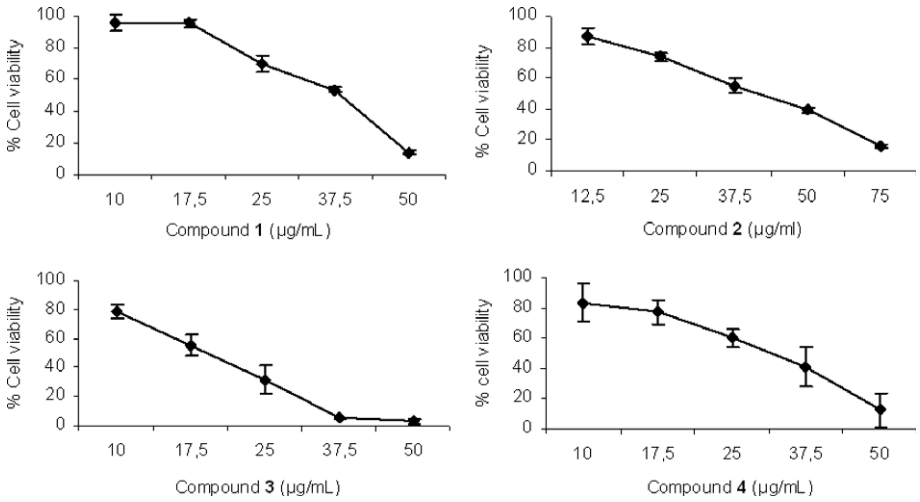


Figure 2. Triterpenes from *C. lyratiloba* decreased the viability of K562. Cells (1×10^4/well) were incubated in the presence of different concentrations of the compounds for 48 h at 37 °C and viability was determined by MTT. Results represent means ± SD of three different experiments performed in triplicate.

Table 1. Cytotoxic activities of triterpenes from *C. lyratiloba*

Triterpenes	IC_{50}[a] (µM)	
	K562	Lucena-1
Compound **1**	76.71 ± 8.23	83.79 ± 4.17
Compound **2**	89.36 ± 2.23	80.25 ± 8.68
Compound **3**	38.35 ± 5.29	41.38;± 4.16
Compound **4**	56.61 ± 9.95	72.87 ± 4.71
Vincristine	0.006 ± 0.0015	>0.1

[a] The molar drug concentration required to cause 50% viability inhibition (IC_{50}) was determined from dose–response curves. Values are expressed as means ± SD.

of cysteine-proteases, named caspases. Activation of initiator caspases (−8 and −9) promotes the activation of effector caspases including caspase-3, with the consequent cleavage of specific substrates and cell death.[24] To confirm the apoptotic nature of cell death induced by compound **1** the kinetics of caspase-3 activation was evaluated by flow cytometry using a commercial assay kit (CaspGlow, Biovision, Mountain View, CA). Analysis of K562 treated with 50 µg/mL of **1** showed a time-dependent increase in the percentage of cells with activated caspase-3 (Fig. 5). These data corroborate studies from our and other groups showing that triterpenes induce a caspase-dependent apoptosis.[25–28]

3. Conclusion

This paper analyzed the in vitro cytotoxic activity of the triterpenes isolated from *C. lyratiloba*. Data obtained with compound **1** confirmed the anti-tumoral activity of this triterpene to different types of cancer and showed that it acts by inducing apoptosis in a caspase-dependent way. The IC_{50} indicated that acetylation of **2** at C2 increases the cytotoxicity of the triterpenoid while

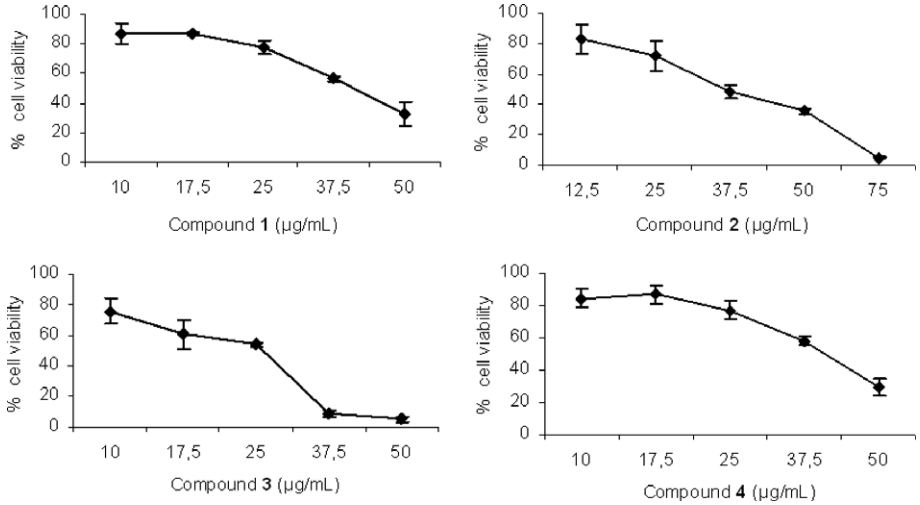


Figure 3. Triterpenes from *C. lyratiloba* decreased the viability of the MDR leukemia cell line, Lucena-1. Cells (1×10^4/well) were incubated in the presence of different concentrations of the compounds for 48 h at 37 °C and viability was determined by MTT. Results represent means ± SD of three different experiments performed in triplicate.

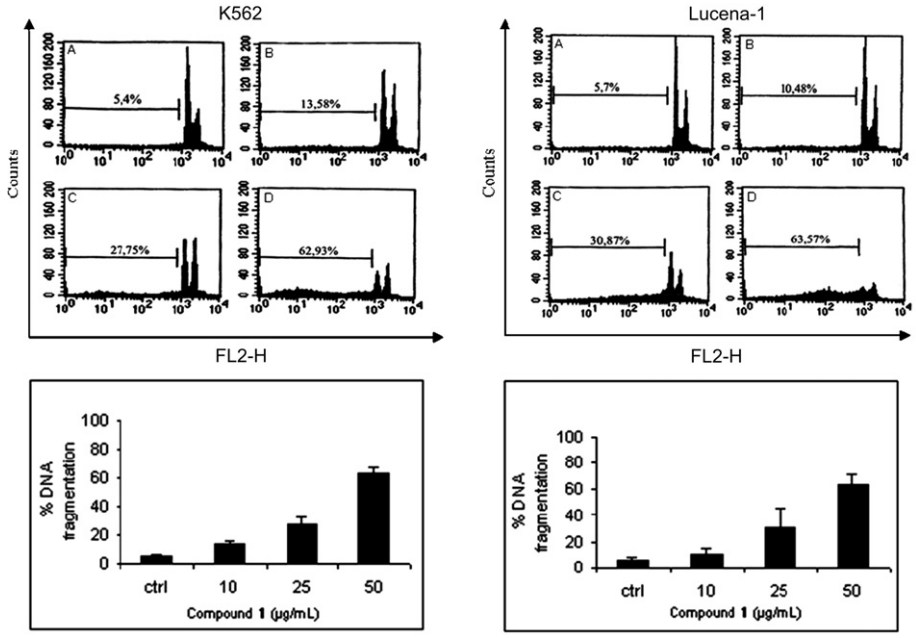


Figure 4. Compound **1** induces apoptosis in both sensitive and MDR leukemia cell lines. K562 (left) and Lucena-1 (right) were treated with medium (A), 10 (B), 25 (C), or 50 (D) μg/mL compound **1** and histogram of the cell cycle (top) and measurement of DNA fragmentation (bottom) were done by FACS.

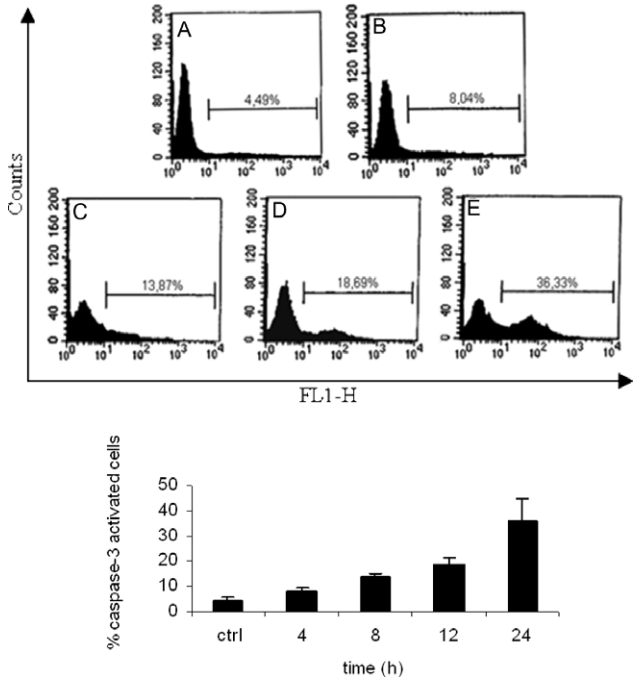


Figure 5. Activation of caspase-3 by compound **1**. Upper panel: histograms of cells treated with medium (A) or 50 μg/mL of **1** for 4 h (B), 8 h (C), 12 h (D), 24 h (E). Lower panel: % of caspase-3 activated cells. Values are expressed as means ± SD.

acetylation of C3 had no effect on the cytotoxicity. Remarkably, the four triterpenes were active to both sensitive (K562) and multidrug resistant (Lucena 1) leukemia cell lines indicating their potential for the treatment of neoplasias including tumors that express the MDR phenotype.

4. Experimental

4.1. Apparatus

The high-speed countercurrent chromatograph used in this work was the CCC-1000 model from Pharma-Tech Research Corp., equipped with three columns connected in series with total capacity of 325 mL. The 2.6 id. Teflon coils show *b* value ranging 0.5–0.75. It was used as a solvent delivery system based on a Pharma-Tech Research Corp constant flow pump; an injection valve with a 5 mL loop; and a Spectra-Chrom CF1 Fraction Collector from Spectrum Chromatography. RMN ^{1}H and ^{13}C spectra were performed in two different types of spectrophotometers: Variant-Gemini, operating at 200 MHz for hydrogen and 50 MHz for carbon, and Bruker model DRX with fields at 400.13 MHz for hydrogen and 100.61 MHz for carbon. All solvents used for solubilization of the substances were deuterated and TMS was used as an internal standard.

4.2. Chemicals and reagents

Methanol, Hexane, CH_2Cl_2, AcOEt, and BuOH, silica gel, and TLC were from Merck (German). RPMI-1640, DMEM, dimethylsulfoxide (DMSO), propidium iodide (PI), MTT (3-4,5-dimethylthiazo-2yl-2,5-diphenyltetrazolium bromide), Triton X-100, penicillin, and streptomycin were from Sigma (St. Louis, MO). FCS was from Gibco (Gaithersburg, MD) and CaspGlow (caspases-3 assay kit, Biovision, Mountain View, CA).

4.3. Separation and identification

Barks (2.5 kg) of *C. lyratiloba* were dried, crushed, and extracted with methanol at room temperature. The methanol extract was dissolved into MeOH/H_2O 50%

mixture and partitioned successively with hexane, dichloromethane, ethyl acetate, and butanol. Separation of dichloromethane fraction (32.0 g) was carried out in gradient elution on countercurrent Chromatography, using the solvent system hexane:ethyl acetate:methanol:water (1:2:*X*:1) with different methanol concentrations {system A ($X = 1.25$) system B ($X = 1.5$) system C ($X = 1.75$)}.[18] The coil was entirely filled with the upper phase of solvent system A. Then, the apparatus was rotated at 900 rpm, while the lower phase of the same solvent system was pumped into the column at a flow rate of 1.0 mL/min in the head to tail direction, resulting 75.8% retention of stationary phase. After the mobile phase front emerged and the hydrodynamic equilibrium was established in the column, the 600 mg of the dichloromethane fraction dissolved in 10 mL of both phases from solvent system A was injected through the injection valve. The mobile phase eluting from the chromatographic column was collected in 4 mL fractions. After 70 fractions were collected, the rotation was stopped and the mobile phase was changed. The lower phase of system B was used as the new mobile phase pumped at 1.0 mL/min in head to tail direction, and the same collection procedure was applied for 92 fractions. The same procedure was done with the lower phase of system C. After 43 fractions were collected, the rotation was stopped and the coil content (upper and lower phase) was collected. Fractions were pooled together according to their TLC profile.

The fractions CLCMC 71-78, CLCMC 98-107, CLCMC 142-146, and CLCMC 158-164 were identified by comparison of ^{13}C NMR data with those from the literature as euscaphic acid[29] and tormentic acid,[30] respectively. 2α-acetyl tormentic acid and 3β-acetyl tormentic acid were identified by spectroscopic data.

Euscaphic acid: RMN ^{1}H (200 MHz DMSO-d_6): δ 4.10 (1H, m, H-2); 3.90 (1H, d, H-3); 5.20 (1H, s, H-5); 0.69 (3H, s, H-23); 1.30 (3H, s, H-24); 0.89 (3H, s, H-25); 0.95 (1H, s, H-26); 1.22 (IH, s, H-27); 1.06 (3H, s, H-29); 0.90 (3H, d, H-30). RMN ^{13}C (200 MHz DMSO-d_6): δ 37.2 (C-1); 64.6 (C-2); 77.9 (C-3); 41.6 (C-4); 46.5 (C-5); 17.7 (C-6); 32.6 (C-7); 41.1 (C-8); 47.6 (C-9); 38.1 (C-10); 25.1 (C-11); 126.9 (C-12); 138.6 (C-13); 40.7 (C-14); 25.9 (C-15); 23.1 (C-16); 46.8 (C-17); 53.1 (C-18); 71.6 (C-19); 41.4 (C-20); 29.0 (C-21); 38.0 (C-22); 28.9 (C-23); 21.8 (C-24); 16.3 (C-25); 16.1 (C-26); 24.1 (C-27); 178.9 (C-28); 26.4 (C-29); 16.6 (C-30).

Tormentic acid: RMN ^{1}H (200 MHz DMSO-d_6): δ 4.10 (3H, m, H-2); 3.91 (3H, s, H-3); 5.19 (1H, s, H-5). RMN ^{13}C (200 MHz DMSO-d_6): δ 39.1 (C-1); 67.1 (C-2); 82.3 (C-3); 41.4 (C-4); 53.1 (C-5); 18.1 (C-6); 32.6 (C-7); 42.4 (C-8); 46.7 (C-9); 37.6 (C-10); 25.2 (C-11); 126.7 (C-12); 138.9 (C-13); 40.7 (C-14); 30.4 (C-15); 25.1 (C-16); 47.0 (C-17); 54.8 (C-18); 71.7 (C-19); 41.4 (C-20); 28.1 (C-21); 39.1 (C-22); 28.8 (C-23); 17.1 (C-24); 16.6 (C-25); 23.3 (C-26); 24.0 (C-27); 179.0 (C-28); 26.4 (C-29); 16.5 (C-30).

2α-Acetyl tormentic acid: RMN ^{1}H (200 MHz $CDCl_3$): δ 4.95 (3H, m, H-2); 3.20 (3H, d, H-3); 5.35 (1H, s, H-5); 1.03 (3H, s, H-23); 2.03 (3H, s, H-24); 0.89 (3H, s, H-25); 0.95 (1H, s, H-26); 1.90 (1H, s, H-27); 1.19 (3H, s, H-29); 1.05 (3H, d, H-30); 3.5 (3H, s, CH_3). RMN ^{13}C (200 MHz $CDCl_3$): δ 37.5 (C-1); 73.1 (C-2); 80.7 (C-3); 39.6 (C-4); 55.0 (C-5); 29.8 (C-6); 28.2 (C-7); 41.0 (C-8); 46.4 (C-9); 39.8 (C-10); 43.5 (C-11); 128.8 (C-12); 137.8 (C-13); 40.9 (C-14); 18.5 (C-15); 23.5 (C-16); 47.0 (C-17); 41.0 (C-18); 73.0 (C-19); 52.7 (C-20); 25.0 (C-21); 32.5 (C-22); 28.5 (C-23); 21.2 (C-24); 16.1 (C-25); 15.9 (C-26); 23.5 (C-27); 183.5 (C-28); 27.2 (C-29); 16.5 (C-30); 171.6 (CO); 51.9 (CH_3).

3β-Acetyl tormentic acid: RMN ^{1}H (200 MHz DMSO-d_6): δ 3.20 (1H, m, H-2); 4.40 (1H, d, H-3); 5.20 (1H, s, H-5); 0.69 (3H, s, H-23); 1.30 (3H, s, H-24); 0.89 (3H, s, H-25); 0.88 (1H, s, H-26); 1.10 (1H, s, H-27); 0.96 (3H, s, H-29); 0.80 (3H, d, H-30); 3.60 (3H, s, CH_3). RMN ^{13}C (200 MHz DMSO-d_6): δ 48.8 (C-1); 64.6 (C-2); 83.8 (C-3); 37.3 (C-4); 53.1 (C-5); 17.9 (C-6); 32.4 (C-7); 41.3 (C-8); 46.5 (C-9); 37.1 (C-10); 27.9 (C-11); 126.5 (C-12); 138.6 (C-13); 40.7 (C-14); 28.0 (C-15); 25.8 (C-16); 47.2 (C-17); 54.4 (C-18); 71.6 (C-19); 54.4 (C-20); 25.1 (C-21); 32.4 (C-22); 28.6 (C-23); 20.8 (C-24); 17.5 (C-25); 16.1 (C-26); 23.8 (C-27); 178.7 (C-28); 28.3 (C-29); 16.4 (C-30); 170.1 (CO); 53.1 (CH_3).

4.4. Cell lines and culture conditions

Human cancer cell lines, K562 (erythroleukemia) and Lucena-1, a vincristine-resistant derivative of K562 that displays MDR characteristics,[17] were grown in suspension cultures in RPMI 1640 supplemented with 10% fetal bovine serum, 10 U penicillin, and 100 μg/mL streptomycin at 37 °C in an atmosphere of 5% CO_2. A549 (lung), Caco-2 (colon), and HEp-2 (larynx) were maintained as monolayers in DMEM supplemented with FCS and antibiotics as described above for RPMI. For sub-cultivations, confluent monolayers were gently washed with phosphate-buffered saline (PBS), pH 7.2, and after trypnization the cells were suspended in culture medium. Cell morphology of cell was analyzed under optical phase-contrast microscope (Olympus IX-70, Tokyo, Japan).

4.5. Cell viability assay

Cell viability was assessed by MTT.[19] After 24 h resting, plated cells (1×10^4/well) were treated with medium, the desired concentrations of the triterpenes or DMSO in the same concentrations carried by the drugs and incubated for another 48 h. After 48 h incubation, each well received 2.5 mg/mL of MTT (3-4,5-dimethylthiazo-2yl-2,5-diphenyltetrazolium bromide) and the plates were incubated for an additional 4 h at 37 °C. The medium was removed and the crystals of reduced formazan were dissolved with 150 μL of DMSO. Absorbance was determined at 570 nm with a microplate reader (BenchMark, Bio-Rad, CA). Effects of the drug cell viability were calculated using cells treated with DMSO as control. IC_{50} values, determined in μM, were obtained by a linear regression analysis of the percent of viability inhibition versus the log of the drug concentration. This linear regression was calculated using the software Origin 6.0.

4.6. Apoptosis assay

Apoptosis was evaluated by detection of DNA fragmentation. After 24 h resting, plated cells (1×10^4/well) were treated with medium or different concentrations of the triterpene and incubated for another 48 h. After this time, cells were harvested, centrifuged, and suspended in 300 μL of HFS—Hypotonic Fluorescent Solution [50 μg/mL propidium iodide (PI) and 0.1% Triton X-100 in 0.1% Na citrate buffer]. After 1 h incubation in the dark at 4 °C the DNA content was measured by flow cytometry (FL-2) (FACS Calibur, Becton–Dickinson, San Jose, CA). Data acquisition and analysis were controlled by Cellquest software version 3.1f. Subdiploid populations were considered apoptotic.

4.7. Caspase assay

Caspase-3 activation was assayed using a commercial kit, according to the instructions of the manufacturer (Biovision, Mountain View, CA). In brief, cells treated and untreated for 4, 8, 12, and 24 h with 50 μg/mL of the triterpene were harvested, centrifuged, and suspended in caspase-3 assay solution. This solution contains a potent caspase inhibitor conjugated to FITC that is cell permeable, non-toxic and, irreversibly binds to activated caspase. After 1 h of incubation (37 °C, 5% of CO_2), cells were washed twice with washing buffer. The percentage of caspase-3 activated cells was analyzed by flow cytometry (FL-1).

4.8. Statistical analysis

All experiments were performed in triplicate and repeated at least three times. Results are expressed as means ± SD.

Acknowledgments

G.G.R. and M.S. had a fellowship from CNPq. K.A.L. had a CAPES fellowship. The authors are thankful to A. Esteves-Sousa for helpful discussions and to E.S.J. Santos for the VCR data. This work was supported by grants from Onco/FAF, FAPERJ, and FINEP (NQTN).

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