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Terpenic subfraction of *Pterodon pubescens* induces apoptosis of K562 leukemic cells by modulating gene expression

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Abstract. Deregulation of cell proliferation and apoptosis is linked to malignant cell development. Leukemia is the most frequent cancer in children, and plants are important sources for new potential anti-cancer agents. Although anti-tumoral effects have been shown for *Pterodon pubescens* extracts, the mechanisms are still obscure. This study describes in *Pterodon pubescens* a furane diterpene only reported in *Pterodon polygalaeiflorus*, the methyl-6 α -acetoxy-7 β -hydroxyvouacapan-17 β -oate, indicated by HRMS and ¹³C-NMR analysis, and demonstrates some mechanisms of the anti-leukemia action of its terpene subfraction SF5. SF5 induced cytotoxic and anti-proliferative effects on K562 cells. Increased sub-G1 nuclei and Annexin V⁺-FITC cells confirmed apoptosis of leukemic cells by treatment of these cells with SF5. Down-regulation of DNMT1 gene transcription and over-expression of Apaf-1 mRNA suggested that SF5 may be inducing apoptosis of K562 cells by epigenetic up-regulation of pro-apoptotic proteins involved in the mitochondrial intrinsic pathway.

Introduction

Apoptosis appears to be an essential mechanism used by biological organisms to maintain cell number homeostasis but it is pivotal in the pathological process of several diseases

(1). The development of a malignant cell is linked to deregulation of the balance between cell proliferation and apoptosis (2). Leukemia is the most frequent malignant disease in children. In spite of the great progress in this disease treatment, 20-30% of the patients still die of the disease.

DNA methylation is crucial for epigenetic control of gene transcription and the cell death by apoptosis involves complex processes under genetic and epigenetic regulation (3). An increased DNA methyltransferase 1 (DNMT1) expression has been reported in a certain amount of human cancers, which can be associated with the silencing of cell cycle inhibitors or pro-apoptotic mediators expression (4). Apoptosis is well characterized by features such as cell shrinking, chromatin condensation and membrane phosphatidylserine exposure (4,5). There are many studies suggesting that the loss of mitochondrial transmembrane potential occurs earlier in the commitment phase of apoptosis which results in cytochrome c release, apoptosome assembly by interaction of cytochrome c with the apoptotic peptidase activating factor 1 (Apaf-1) in the cytosol, in the presence of ATP, and caspase 9 activation. Apaf-1 is a central component of the intrinsic pathway of apoptosis and caspase 9 activates the executioner caspase 3 and 7, responsible for degradation of many cellular substrates (6). Since apoptosis occurs by a conserved and genetically programmed mechanism, it has generated a hope that selective intervention on this modulated process may influence the course of the major degenerative diseases and cancer. Many types of antitumor drug therapies exert their effect by activating apoptosis (2).

Natural products are important sources of new bioactive molecules with promising chemotherapeutic properties (7). A number of low molecular weight compounds are currently being investigated for their pharmacological properties in regulating apoptosis. *Pterodon pubescens* Benth., Leguminosae, popularly known as 'Sucupira branca', is found distributed throughout the central region of Brazil and is used in folk medicine to treat rheumatic and inflammatory diseases (8-10). Both pharmacological activities have already been scientifically demonstrated (11-13). Phytochemical studies

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of *Pterodon pubescens* described linear diterpenes geranylgeraniol and 14, 15-epoxygeranylgeraniol in the seed oil with topic protection against *Schistosoma mansoni* invasion in mice by the latter (14). In 1970, two cyclic diterpenes with vouacapan skeletal were described (15). Later, several new diterpenes were described (13,16,17). Anti-inflammatory effects have been reported for fractions derived from the ethanolic extract of *Pterodon pubescens* containing the diterpenes methyl-dihydroxy-vouacapan-oate, farnesol and geranylgeraniol (13). Growth inhibition and apoptosis of melanoma cells has also been demonstrated for the diterpene methyl-dihydroxy-vouacapan-oate (17). The present study describes important molecular mechanisms related to anti-leukemic effects of a terpenic subfraction from *Pterodon pubescens* (Pp) and reports the major components in this mixture based on ^{13}C -NMR and mass spectra (HRMS) analysis.

Materials and methods

Plant material. *Pterodon pubescens* seeds were collected by Luciana Pontes Coelho in Goiás, Brazil. The taxonomic identity was confirmed by Haroldo Cavalcante de Lima, at the Departamento de Botânica Sistemática, Jardim Botânico do Estado do Rio de Janeiro, Brazil, where a voucher of the collected specimen was deposited (RB 350279).

Extraction, fractioning and identification of methyl,6 α -acetoxy-7 β -hydroxyvouacapan-17 β -oate in SF5 subfraction. The seeds were powdered and submitted to 100% ethanol extraction (15 g/100 ml) at room temperature for 15 days. The oil (EPP) obtained (50% yield, w/w) after ethanol evaporation was fractionated by liquid-liquid partition chromatography using hexane. The EPP was submitted to successive extractions with hexane. After solvent evaporation, the hexanic fraction (55% yielding) was submitted to a column chromatography (42x2.5 cm) on silica gel (Art. 7733 - Kieselgel 60, 35-70 MESH ASTM, Merck), eluted successively with hexane, hexane/dichloromethane (1:1), hexane/ethyl acetate (1:1), ethyl acetate, ethanol and 2% acetic acid in ethanol. After analysis of sample gas chromatography profiles, they were joined in eight sub-fractions. The most cytotoxic subfraction (SF5, 32.9% yield) is a yellow, viscous and aromatic sample, and showed the presence of a furane diterpene, the methyl-6 α -acetoxy-7 β -hydroxyvouacapan-17 β -oate (compound 1), not described previously in this species. Its identification was based on ^{13}C -NMR spectra recorded on a Bruker spectrometer (^{13}C , 100.6 MHz), using deuterated chloroform (CDCl_3) as solvent and internal standard ($\delta=0$). The chemical shifts are expressed in parts per million (ppm), reported from the central peak of deuteriochloroform (77.16 ppm). Compound 1: δ_c 39.27 (C1), 18.28 (C2), 43.38 (C3), 33.13 (C4), 54.95 (C5), 75.54 (C6), 81.29 (C7), 42.53 (C8), 47.95 (C9), 38.71 (C10), 21.97 (C11), 150.46 (C12), 113.37 (C13), 46.37 (C14), 108.45 (C15), 141.42 (C16), 175.43 (C17), 36.18 (C18), 22.47 (C19), 15.46 (C20), 52.23 (C17-MeO). High resolution mass spectra (HRMS) for SF5 performed on a JEOL JMS-Gcmate II, GC/MS system spectrometer (direct probe; ionization mode; EI $^+$): 404.2205 (measured mass) corresponding to the

molecular formula $\text{C}_{23}\text{H}_{32}\text{O}_6$ (calculated mass 404.2199), confirmed the presence of compound 1. Mass spectral fragmentations and ^{13}C -NMR spectra of SF5 were compared to those of literature.

Biological assays. The human chronic myelogenous leukemia cell line K562 (CCL-243) was purchased from the American Type Culture Collection (ATCC) and was cultured in supplemented medium. The effects of SF5 on cells were evaluated (duplicates) by co-culture of cells with different SF5 concentration at 37°C and 5% CO_2 . The SF5 sample was diluted with ethanol and then with RPMI-1640 medium, supplemented with 10% fetal bovine serum (Cultilab, Brazil), penicillin (70 mg/l) and streptomycin (100 mg/l), until the final concentration of 0.01% ethanol. Control cultures received only 0.01% ethanol in supplemented medium.

MTT cytotoxicity assay. K562 cells ($1 \times 10^5/\text{ml}$) were cultured (triplicates) in 96 flat-bottom-wells plates (Becton Dickinson Labware, USA), either in the absence or presence of different SF5 concentrations in a final volume of 100 μl . The plates were incubated for 34 h, at 37°C, with 5% CO_2 . Thereafter, cell survival was determined by the MTT assay (18) adding 10 $\mu\text{l}/\text{well}$ of a 5 mg/ml stock solution of MTT (Sigma Chemical Co., St. Louis, MO, USA) in phosphate-buffered saline pH 7.4 (PBS). Following incubation for 2 h, 100 $\mu\text{l}/\text{well}$ of SDS 10% solution with 0.01 N HCl were added. The extent of formazan crystal production was measured by absorbance at 570 nm (microplate reader μQuant , Bio-Tek Instruments, Inc.). Control cultures did not receive SF5.

Cell proliferation assay. The cells ($2.5 \times 10^5/\text{ml}$) were cultured in supplemented medium in 96-well flat-bottom plates (200 μl), either in the absence or presence of different EPP concentrations, for 36 h, at 37°C, in 5% CO_2 (triplicates). To assess DNA synthesis, 25 $\mu\text{l}/\text{well}$ of 10 $\mu\text{Ci}/\text{ml}$ [^3H]-methylthymidine (^3H -Tdr; Amersham Biosciences, Brazil) were added at the beginning of the culture. Then, the cells were harvested on filter paper and processed for ^3H -Tdr radioactivity determination by liquid scintillation.

Flow cytometric analysis of sub-G1 nucleus and cell shrinkage. K562 cells ($2.5 \times 10^5/\text{ml}$) were incubated either in the absence or presence of 30 $\mu\text{g}/\text{ml}$ of SF5 or methotrexate (MTX) for 36 h at 37°C and 5% CO_2 and processed for different analyses. After treatment of cells with PI solution (final concentration 2 $\mu\text{g}/\text{ml}$), cell death was evaluated by high membrane permeability to PI indicated by high fluorescence at 585 nm. The cell shrinking was determined by analyzing cell size with the forward-scatter (FSC) parameter of the cytometer. Plots showing cell distribution accordingly to size (FSC) and granularity (SSC) (not shown) were used to delimit a region to avoid apoptotic bodies and cell debris in the analysis. To determine the sub-G1 nuclei, cells (1×10^6) were centrifuged (400 x g, 5 min), suspended in 500 μl DNA staining solution (0.3% Triton X-100 and 50 $\mu\text{g}/\text{ml}$ PI in 43 mM citrate buffer solution pH 8.2), and maintained for 15 min at room temperature in the dark. Later, the samples were treated with 500 μl ribonuclease A 50 $\mu\text{g}/\text{ml}$ (Sigma Chemical Co.) in 43 mM citrate buffer pH 8.2 for 15 min at room temperature

Table I. Main peaks and respective fragment ions observed in the GC-MS analysis (chemical ionization/ NH_3) of SF5.

R_t (min)	m/z	Compound	Ref.
4.74	238 (M+18)	Epoxyfarnesol isomer	(13)
8.08	272 (M-18) ^a	Geranylgeraniol derivative	(14)
13.50	342 (M); 343 (M+1); 360 (M+18)	7 β -acetoxy vouacapan derivative	(16)
14.23	404 (M); 405 (M+1); 422 (M+18)	Methyl 6 α -acetoxy 7 β -hydroxyvouacapan 17 β -oate	(20)
14.60	464 (M+18) ^b	6 α , 7 β -diacetoxy vouacapan 14 β -oate	(16)

The results are expressed in retention time (R_t min) and m/z. ^aMolecular ion (M=290 m/z) not observed. ^bMolecular ion (M=446 m/z) not observed.

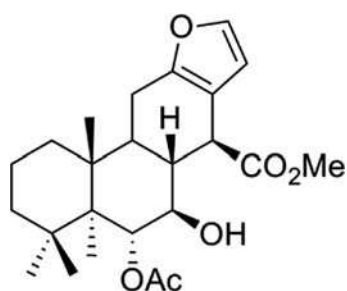


Figure 1. Chemical structure of the methyl-6 α -acetoxy-7 β -hydroxyvouacapan-17 β -oate (compound 1).

and the PI fluorescence measured. One hundred thousand events per sample were evaluated using a FACSCalibur cytometer (Becton Dickinson Labware). The excitation wavelength for PI was 488 nm and the emission was detected at 585 ± 15 nm (19). Analysis was done with WinMdi 2.8 software.

Annexin V⁺-FITC flow cytometric assay. K562 cells ($2.5 \times 10^5/\text{ml}$) were incubated either in the absence or presence with 50 $\mu\text{g}/\text{ml}$ of SF5 or MTX for 36 h at 37°C and 5% CO_2 . Phosphatidylserine (PS) exposure on plasmatic membrane of apoptotic cells was determined by Annexin-V labeling, according to the manufacturer's instructions (Annexin V-FITC apoptosis kit, BD Pharmingen, CA, USA). Briefly, cells (1×10^6) were washed twice with cold PBS and suspended in 1 ml binding buffer. Cells (1×10^5 , 100 μl) were treated with Annexin V (5 μl) and 50 $\mu\text{g}/\text{ml}$ PI solution (10 μl) for 15 min at room temperature. After addition of 400 μl of binding buffer, cells (5×10^4) were acquired by Cell QuestPRO software and analyzed using WinMdi 2.8 software. Annexin V-FITC emission was determined by FITC fluorescence detection at 535 ± 15 nm and PI at >670 nm by flow cytometry. Annexin V positive cells were considered apoptotic cells. Apoptotic bodies and cell debris were avoided as possible from analysis.

mRNA expression analysis by RT-PCR. Total RNA was extracted from various cell samples using TRIzol reagent according to the manufacturer's instructions (Invitrogen). The RNA concentration was determined by optical density at 260 nm. Samples were treated with DNase I and RNA integrity was confirmed by agarose gel electrophoresis.

Reverse transcription reaction was performed using MMLV reverse transcriptase (Invitrogen) with 2 μg of total RNA in a 30 μl reaction mixture containing random hexamers as primers. The cDNA was brought into 25 μl mixture of PCR buffer, 0.2 mM of each dNTP, 1.5 mM MgCl_2 , 3.2 μg of each primer, and 2.0 units of Taq DNA polymerase platinum (Invitrogen). The primers, annealing temperature of each primer and cycles used were: β -actin forward primer: 5'-TCC TGTGGCATCCACGAAACT-3', reverse primer: 5'-GAA GCATTTGCGGTGGACGAT-3' (59°C, 30 cycles, 314 pb); Apaf-1 reverse primer: 5'-TTGCTGCCCTTCTCCATGAT-3', reverse primer: 5'-TCCCAACTGAAACCCAATGC-3' (55°C, 30 cycles, 334 pb); DNMT1 reverse primer: 5'-GAT TTGTCCTTGGAGAACGG-3', reverse primer: TTGGG TGTGGTTCTTTGGTT-3' (57°C, 30 cycles, 499 pb). All genes examined were normalized to a housekeeping gene encoding β -actin. PCR was performed in a Perkin Elmer GeneAmp PCR system 9600. Each PCR cycle consisted of denaturation at 94°C for 30 sec, annealing at specific annealing temperature for 30 sec, and extension at 72°C for 1 min. Under the conditions used, the cDNAs were exponentially amplified, and thus a semiquantitative estimation of the products was allowed by 2% agarose gel electrophoresis. Band intensities were quantified by using Lab Image software (Germany).

Statistical analysis. Significant differences between pairs of groups were accessed using Student's t-test, with a significance level set at $p < 0.05$.

Results

¹³C-NMR and HRMS analysis. The major components of SF5, listed in Table I, were determined with basis on spectral fragmentation analysis and its comparison to data described in literature (13,14,16,20). The presence of compound 1, the methyl-6 α -acetoxy-7 β -hydroxyvouacapan-17 β -oate ($\text{C}_{23}\text{H}_{32}\text{O}_6$) was confirmed in SF5 by ¹³C-NMR analysis comparing to data from *Pterodon polygalaeflorus* (16). This compound is described here for the first time in *Pterodon pubescens*. Its chemical structure is shown in Fig. 1.

SF5 effects on cell proliferation and cytotoxicity. SF5 inhibited the incorporation of DNA synthesis in all tested concentrations (Fig. 2A). The inhibition index ($p < 0.0001$) ranged from 40.3 to

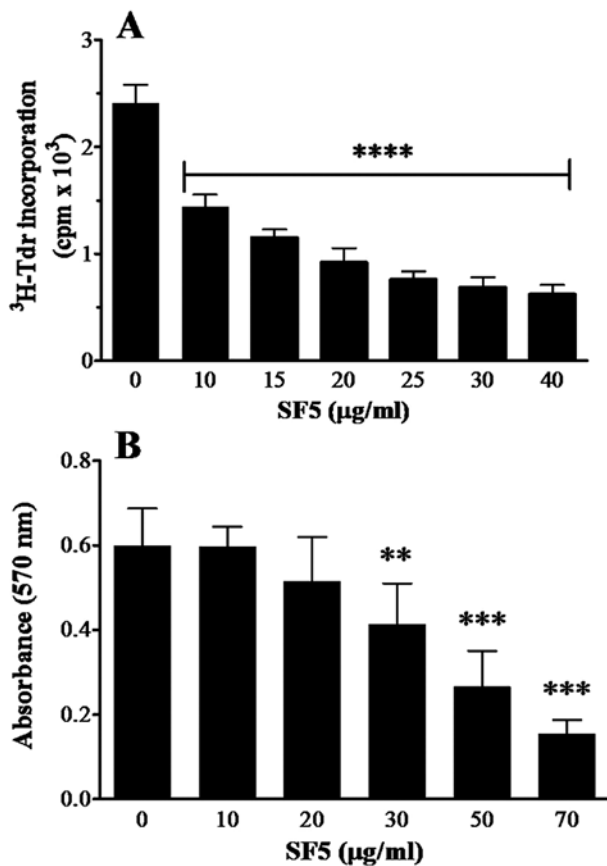


Figure 2. Effect of hexanic subfraction SF5 on K562 cell proliferation. (A) DNA synthesis; (B) mitochondrial reduction activity. Cells were treated either in the absence (control) or presence of different SF5 concentrations for 36 h. Cell proliferation was determined by ³H-Tdr incorporation into DNA. Cell survival was determined by the MTT assay. Results represent the mean \pm SD of three and two independent experiments, respectively, in triplicate. ***p*<0.01, ****p*<0.001, and *****p*<0.0001 related to control by Student's *t*-test.

74.2% when cells were treated with 10 and 40 μ g/ml of SF5, respectively. SF5 cytotoxicity to leukemic cells was concentration dependent (Fig. 2B). Cell viability was significantly reduced to 70, 44 and 26%, when treated with SF5 at 30, 50 and 70 μ g/ml, respectively.

SF5 effects on leukemic cell death. Treatment of K562 cells for 36 h with 30 μ g/ml SF5 increased from 3.9% (control culture) to 29.5% (treated cells) the percentage of cells with high PI fluorescence (Fig. 3A, right marker), meaning high membrane permeability to PI and cell commitment to death. The analysis of cell size histograms shows that SF5 at 30 μ g/ml increased the number of cells with reduced size (shrunken), a morphologic feature of cell apoptosis, compared to the control culture (Fig. 3B). The relative number of shrinking cells (apoptotic) after treatment with SF5 increased from 6.9% (control) to 26.7. Methotrexate, a positive drug control, showed lower effects than SF5.

SF5 effects on the leukemia cell apoptosis. To confirm the apoptotic status, the percentage of nuclei with sub-G1 DNA content was determined by flow cytometry after treatment with SF5 (30 μ g/ml) for 36 h. SF5 increased from 3.8%

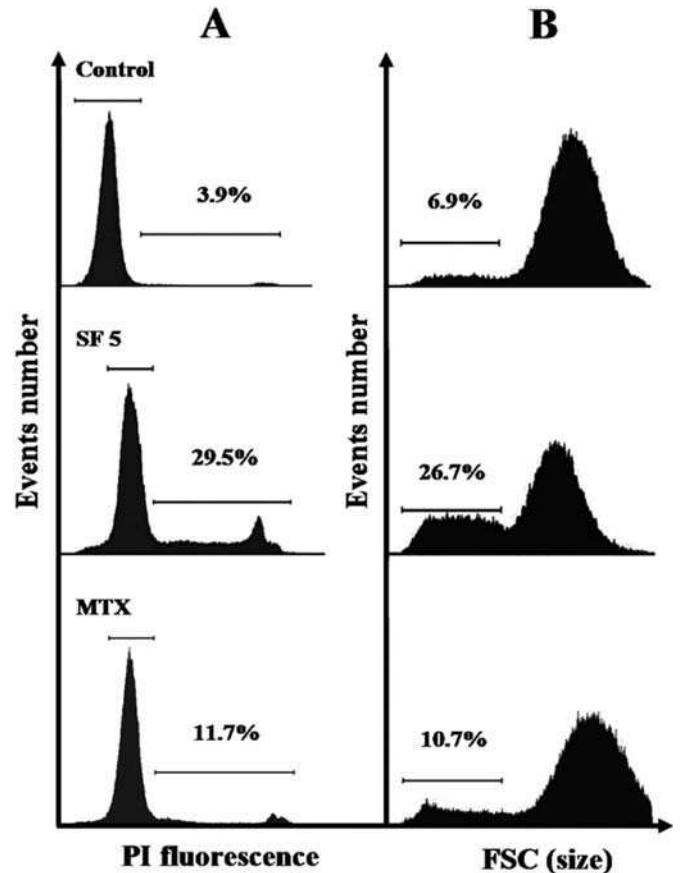


Figure 3. Effects of SF5 hexanic subfraction on K562 cell size and membrane permeability by flow cytometry. (A) PI fluorescence histograms; (B) Cell size histograms. Cells (2.5×10^5 /ml) were cultured in the absence (control) or presence of 30 μ g/ml SF5 or methotrexate (MTX) for 36 h and processed as described in Materials and methods. Representative results from three independent experiments.

(untreated cells, Fig. 4A) to 16.3% (Fig. 4B) the number of cells with sub-G1 DNA content. Methotrexate (30 μ g/ml) induced effect similar to SF5 (18.9%, Fig. 4C). Results from three independent experiments (Fig. 4D) showed increased number (by 14- or 9-fold) of sub-G1 nucleus following treatment with SF5 or MTX, respectively. Induction of apoptosis by SF5 treatment was also confirmed by PS exposure on the outer side of plasma membrane of K562 cell, determined by Annexin V labeling. Cells treated with SF5 (50 μ g/ml) for 36 h showed considerable increase in Annexin V-positive cells (Fig. 5B), compared to untreated ones (Fig. 5A). The number of Annexin V-positive cells after treatment with MTX was lower than that of SF5. Results from three experiments confirm the K562 cell apoptosis by treatment with SF5 (Fig. 5D).

SF5 effects on Apaf-1 and DNMT1 gene expression by RT-PCR. In order to approach some molecular mechanisms involved in this process cells were incubated in the absence or presence of SF5 50 μ g/ml for different times and then the total mRNA was isolated for RT-PCR analysis of regulatory molecules. Apaf-1 is a protein involved in the mitochondrial pathway of apoptosis activation (intrinsic pathway) and its promoter gene is regulated by methylation. DNA methyl-

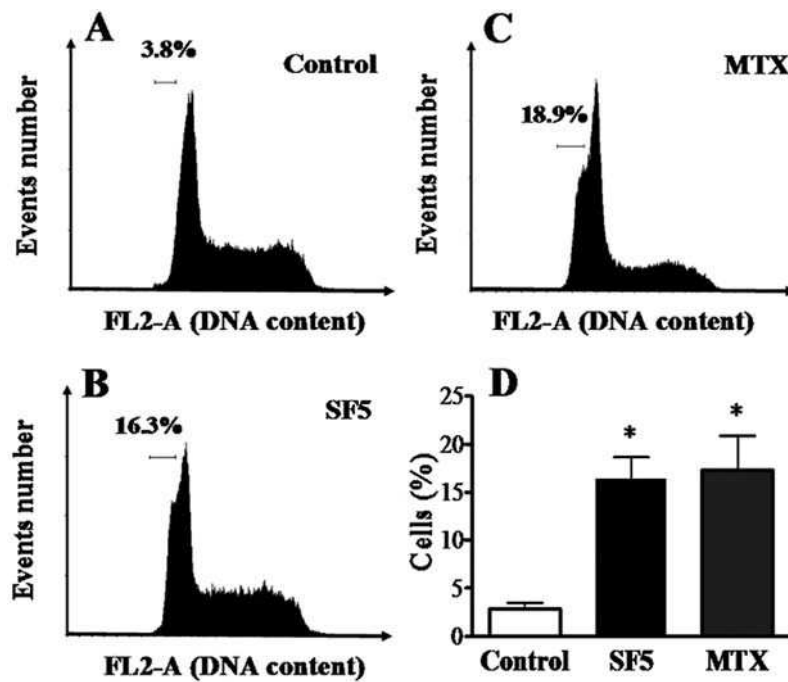


Figure 4. K562 cells apoptosis by flow cytometric analysis of sub-G1 nucleus of K562 cells treated with SF5 hexanic subfraction. Histograms of DNA content: (A) Control, (B) SF5, and (C) MTX treated cells; (D) relative number of sub-G1 nuclei fraction (mean \pm SD of three independent experiments). Cells (2.5×10^5 /ml) were cultured in the absence (control) or presence of $30 \mu\text{g/ml}$ SF5 or methotrexate (MTX) for 36 h and processed as described in Materials and methods. * $p < 0.05$ and related to control by Student's t-test.

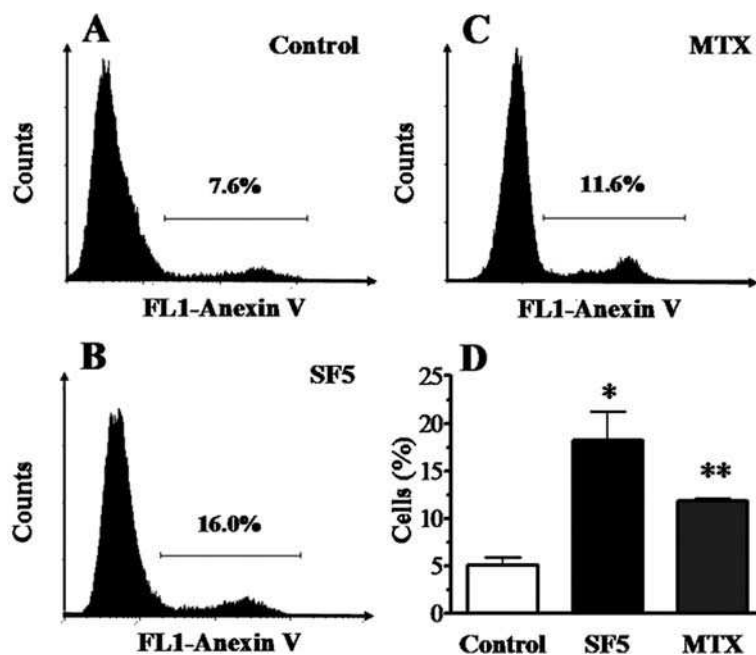


Figure 5. K562 cells apoptosis by flow cytometric analysis of phosphatidylserine exposure on cell membrane by Annexin V-FITC labeling, after treatment with SF5 hexanic subfraction. Histograms of Annexin V-FITC labeled cells: (A) Control, (B) SF5, and (C) MTX treated cells; (D) relative number of Annexin V-FITC positive cells (mean \pm SD from three independent experiments). Cells (2.5×10^5 /ml) were cultured in the absence (control) or presence of $50 \mu\text{g/ml}$ SF5 or methotrexate (MTX) for 36 h and processed as described in Materials and methods. * $p < 0.05$ and ** $p < 0.01$ related to control by Student's t-test.

transferases (DNMTs) hypermethylate the DNA resulting in silencing of gene expression. Treatment with SF5 for 12, 24 and 36 h caused a progressive increase of Apaf-1 and decrease of DNMT1 mRNA level, as can be observed by agarose gel electrophoresis and densitometric analysis using the β -actin as the housekeeping gene (Fig. 6).

Discussion

Chemotherapeutic drugs induce apoptosis of tumor cells through different mechanisms, in which signaling events play an important role depending upon the cell type and stimulus (2), but there are few studies on natural compounds. As SF5

vouacapan, 6 α -7 β -diacetoxyvouacapan-17 β -oate and methyl-6 α -acetoxy-7 β -hydroxyvouacapan-17 β -oate (Table I). These compounds were previously reported for *Pterodon pubescens* (14,16, 31). Both linear or cyclic diterpenes may be involved in the apoptotic effect of SF5 on leukemic cells since similar vouacapan diterpenes have induced cytotoxicity and apoptosis of melanoma cells and prostate cancer cell, respectively (17,31), and apoptosis of leukemia U937 cells has been reported to geranylgeraniol (32).

In conclusion, this study provides important insights into molecular mechanisms of *in vitro* anti-leukemia effects of a terpenic fraction from *Pterodon pubescens*. The DNMT1 gene down-regulation and the overexpression of Apaf-1 mRNA, induced by SF5, suggest that it may induce apoptosis of leukemic cells by epigenetic regulation of pro-apoptotic proteins involved in the mitochondrial intrinsic pathway. A further challenge will be the evaluation of *in vivo* antitumor potency to confirm the potential chemotherapeutic action of SF5.

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