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Cytotoxicity of Some Plants of the Asteraceae Family: Antiproliferative Activity of *Psiadia punctulata* Root Sesquiterpenes

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Abstract: According to the World Health Organization Cancer is the second cause of death globally. The methanol extracts of fourteen Middle-Eastern plants of the family Asteraceae were screened for antiproliferative activity on five cancer cell lines (A2780, MCF7, HeLa, RKO and Jurkat) by using MTT assay. *Psiadia punctulata* (DC.) Vatke was selected for isolation and elucidation of the bioactive constituents by 1D- and 2D-NMR, and MS analyses. Flow cytometry was used to evaluate cell cycle analysis, apoptotic hallmarks and reactive oxygen species. *P. punctulata* yielded a new sesquiterpene characterized as 7-hydroxy eudesman-3,5-dien-2-one (punctulin) **(3)** and three known sesquiterpenes: **1**, **2** and **4**. The antiproliferative activity of all sesquiterpenes was evaluated in Jurkat (T-cell leukemia) and HeLa cancer cell lines. 1β-hydroxy-8-oxo-cyperone (**1**) has induced a significant growth inhibition in Jurkat and HeLa cells (IC50 =12 and 18μM respectively). Flow cytometry of compound **1** has elucidated the mechanism of action by showing its ability to induce cell cycle arrest in Jurkat cells mainly in G0/G1 and, less markedly, in G2/M. Compound 1 also expressed strong antioxidant activity by reducing the basal level of peroxides DHFDA-load in Jurkat cells. Antioxidant activity of compound **1** may have contributed to the observed cell cycle arrest.

Keywords: Cytotoxic activity; *Psiadia punctulata*; Asteraceae; leukemia; cell cycle; sesquiterpenes. © 2019 ACG Publications. All rights reserved.

1. Introduction

Natural products are of vital importance in efforts to defeat diseases. These products were a principal source of early medicines, and they continue to be important today, even considering the recent advances in drug discovery technologies, including high throughput screening, combinatorial chemistry and rational drug design. The molecular and structural diversity of natural products is considered unreachable by benchtop chemistry techniques, so natural materials are recognized as important sources for lead compounds and new opportunities for drug discovery [1]. In particular, the number of natural

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anticancer agents developed is increasing constantly due to the efforts spent in this field. New anticancer entities include alkaloids, polyphenols and terpenoids [2].

The Asteraceae family contains different classes of sesquiterpenes, showing a vast range of biological activities including cytotoxic and anticancer ones [3]. Plants belonging to Asteraceae family, are also considered important sources of chemo-preventive agents due to their content of different classes of antioxidant compounds [4,5].

Sesquiterpenes are good candidates for anticancer drug development due to their cytotoxic properties, some of them are being tested in clinical trials as an anticancer agent such as artemisinin, thapsigargin, and parthenolide [6].

The flora of Saudi Arabia and Jordan are very rich due to their ecological diversity. Recently the flora of the Middle-East has attracted the attention of several groups of scientists to screen and detect the most promising plants as a source of antitumor agents. The objective of this study is the screening of some medicinal plants for their cytotoxic effect and to isolate the active constituents to determine the probable mechanism of cytotoxicity and anti-proliferative activity. Fourteen plants that belong to the Asteraceae family collected in Saudi Arabia and Jordan and screened for this purpose.

2. Materials and Methods

2.1. Instruments and Chemicals

Optical rotations were measured on JASCO DIP-1000 polarimeter equipped with a sodium lamp (589 nm) and a 1 cm microcell working at 25 °C and using MeOH or $CHCl_3$. NMR experiments were performed on a Bruker DRX-600 spectrometer (Bruker, Germany) equipped with 5 mm TCI CryoProbe (Bruker) at 300 K. All 2D NMR spectra were acquired in CD_3OD (Sigma-Aldrich), and standard pulse sequences and phase cycling were used for DQF-COSY, HSQC, and HMBC spectra. FTIR spectra were recorded as films on a Perkin Elmer System 2000 instrument. ESI-MS were obtained using a Finnigan LC-Q Advantage Termoquest spectrometer. HR-ESIMS spectra were acquired in positive ion mode on a with nano-electrospray ion source (Waters-Milford, MA, USA). TLC was performed on Kieselgel 60 F254 plates (Merck); Compounds on TLC were revealed by using the spray reagent of $Ce(SO_4)2/H_2SO_4$ solution. Column chromatography 100 × 5 cm, was performed over silica gel 60, 0.063-0.200 mm(70–230 mesh, ASTM, Merck); reversed-phase (RP) HPLC separations were performed on a Shimadzu LC-20AT series pumping system equipped with a Shimadzu RID10A and Rheodyne injector, using a C18μ-Bondapak column (30 cm × 7.8 mm, 10 μm, Waters-Milford) and a mobile phase consisting of MeOH-H_2O mixtures at a flow rate of 2 mL/min. Solvents for extraction n-hexane, $CHCl_3$, MeOH (Sigma Aldrich), Solvents for HPLC-MS, MS acetonitrile, MeOH (Baker Mallinckrodt, Phillipsburg, NJ), formic acid, trifluoroacetic acid (Merck), nanopure HPLC water prepared with Millipore Milli-Q.

2.2. Plant Material and Extraction

The plants were collected from Saudi Arabia and Jordan and were identified by Prof. A. Bader (Pharmacognosy Department, Umm Al-Qura University, Saudi Arabia), voucher specimens are preserved in the Herbarium of Pharmacognosy Lab at the Faculty of Pharmacy, Umm Al-Qura University (Table1.). The plant material was dried in shade then ground finely, 2 g of powdered material was macerated in 20 mL of methanol and sonicated for 8 min, then left for 24 h prior filtration, the filtrate was dried in Petri dishes under ventilation, the dry crude extract collected in vials and preserved in freezer until required.

As mentioned in Table 1, the methanol extract of *P. punctulata* (DC.) Vatke roots had a Moderate activity in the MTT assay in three cancer cell lines; the IC50 in μg/mL was 65.7 ±14.7 for MCF7; 77.5 ±6.2 for HeLa and 79.5 ±7.4 for Jurkat cell lines. A further phytochemical investigation was performed in order to isolate the active constituents. 400 g *P. punctulata* powdered roots were exhaustively extracted by maceration with solvents with increasing polarity starting with n-hexane then with CHCl3, afterward with CHCl3: MeOH (9:1), and finally with Methanol to yield 1.25, 4.77, 1.4, and 7.97 g respectively.

Table 1. Methanolic extracts of the plants screened for cytotoxicity in MTT test*

Plant name	Plant Part	Specimen number and plant site	A2780 IC_{50}	MCF7 IC_{50}	HeLa IC_{50}	RKO IC_{50}	Jurkat IC_{50}
Aaronsohnia factorovskyi Warb. &Eig	AP	UQU-JO-1 Rusayfah	>200	>200	>200	107.1	NA
Achillea membranacea (Labill.) DC.	AP	UQU-JO-3 Dabaa	>200	>200	>200	>200	60 ±5.1
Calendula arvensis M. Bieb.	AP	UQU-JO-4 Juwaidah	>200	>200	>200	>200	80±5.6
Felicia dentata (A.Rich.) Dandy	AP	UQU-SA-1 Taif	>200	151.6	>200	131.9	90±6.8
Helichrysum sanguineum (L.) Kostel	AP	UQU-JO-5 Subaihi	>200	>200	>200	>200	NA
Helichrysum glumaceum D C.	AP	UQU-SA2 Taif	>200	>200	>200	>200	200±18
Dittrichia viscosa (L.) Greuter)	RO	UQU-JO-6 Abu Nusair	>200	>200	>200	>200	>200
Launaea nudicaulis (L.) Hook.f.	AP	UQU-JO-7 Madaba	94.2± 21.5	69.1 ±10.8	>200	116 ±32.3	76±6.2
Launaea nudicaulis (L.) Hook.f.	Ro	UQU-JO-7 Madaba	>200	110.4	>200	152 ±4.9	60 ±3.3
Onopordum ambiguum Fresen.	AP	UQU-JO-8 Tabarbour	79.3 ±8.3	74.2 ±18.9	87.6 ±14.7	72.9 ±15.4	70±4.8
Pallenis spinosa (L.) Cass.	AP	UQU-JO-9 Subaihi	>200	>200	>200	>200	88±7.6
Picnomon acarna (L.) Cass	AP	UQU-JO-10 Tabarbour	>200	>200	>200	>200	84±6.9
Psiadia punctulata (DC.) Vatke	Ro	UQU-SA-3 Taif	157.2 ±35.7	65.7 ±14.7	77.5 ±6.2	200±19.3	79.5 ±7.4
Chiliadenus iphionoides (Boiss. & Blanche) Brullo	Ro	UQU-JO-11 Subaihi	>200	68.2 ±6.6	186.1	80 ±12.2	200±14.1
Xanthium spinosum L.	RO	UQU-JO-12 Tabarbour	125.7 ± 24.3	72.2 ±20.9	131.4±17.1	118.9±22.2	52±2.5

NA: Not applicable; AP = Aerial Parts; Ro = Roots; JO=Jordanian plant, SA = Saudi Plant. ±SD: Standard deviation calculated from three independent experiments. * IC_{50} values are given as in µg/mL

2.3. Purification and Structure Elucidation

All extracts of *P. puntulata* were retested using MTT assay for two cell lines in order to determine the most active fraction, the *n*-hexane extract was the most significant (HeLa 71±3.1 µg/mL , Jurkat 73±2.5 µg/mL) and $CHCl_3$ extract also (HeLa 120±9.6 µg/mL, Jurkat 135±8.4 µg/mL). Part of *n*-hexane extract (1.1 g) was subjected to column chromatography using silica gel (150 × 3 cm) packed with 250 g of silica gel, starting the elution with *n*-hexane followed by increasing the % of chloroform in *n*- hexane (between 10% and 100%) and increasing percentage of methanol in chloroform (between 1% and 100%). Fractions of 25 mL were collected and analysed by TLC using $CHCl_3$: MeOH with ratios 99:1, 49:1, 97:3 and 4:1as mobile phases. Spots were visualized using UV with wavelength 254 and 366 nm and by using cerium (IV) sulphate spray reagent, then heated up to 120 °C for 15 min. These were collected into seven main fractions (A–G). Fraction B (130.3 mg) was purified by semi-preparative RP-HPLC with MeOH: H_2O (35:15) to yield compound **1** (2.5 mg, t_R =8 min) and compound **2** (1.3 mg, t_R =13 min). Fraction E (200.7 mg) was purified by RP-HPLC with MeOH: H_2O (3:2) yielding compound **3** (1 mg, t_R = 25 min). Fraction C (186.3 mg) was separated by RP-HPLC with CH_3OH:H_2O (55:45) yielding compound **1** (2.2 mg, t_R 28 min,) and compound **2** (4.3 mg, t_R 42 min). Fraction D (598.1 mg) was separated by RP-HPLC with CH_3OH:H_2O (1:1), as eluent, yielding; compound **1** (2.3 mg, t_R 52 min). Fraction F (106.7 mg) was subjected to RP-HPLC with CH_3OH:H_2O (1:1) yielding compound **4** (1.1 mg, t_R 32 min) and compound **3** (4.3 mg, t_R 60 min).

1β-hydroxy-8-oxo-cyperone (***1***)*:* [α]$_D$: + 12.0 (*c* 0.1, $CHCl_3$)

8-oxo-β-cyperone (***2***)*:* [α]$_D$: + 180.0 (*c* 0.1, $CHCl_3$)

*Compound **3**:* 7(s)-hydroxy eudesman-3,5-dien-2-one (punctulin): colourless oil; [α]$_D$: -15.0 (*c* 0.1, MeOH); UV λmax 295 nm; IR= (v, cm^{-1}) 3400, 3010, 1650, 1220, 115, 870. ESI-MS *m/z* 235 $[M+H]^+$; HR ESIMS *m/z* 235.1690 $[M+H]^+$ calculated as $C_{15}H_{22}O_2H$ 235.1698, ^1H and ^{13}C NMR data Table 2.

*Compound **4***: 2-hydroxy eudesman-1,4,6-trien-3,8-dione colorless oil; [α]$_D$: +123.0 (c 0.1, $CHCl_3$). ESI-MS *m/z* 247 $[M+H]^+$; HR ESIMS m/z 247.1337 $[M+H]^+$ (calculated as $C_{15}H_{18}O_3H$ 247.1334) for NMR data in Table 2, and structure Figure1.

Table 2. 1H and 13C NMR spectroscopic data of compounds **3-4** (CD_3OD, 600 MHz)[a].

Position	**3**		**4**	
	^1H	**^{13}C**	**^1H**	**^{13}C**
1a	2.32 brd (15.0)	52.6	-	149.7
1b	2.45 brd (15.0)			
2		200.6	6.16 s	125.4
3	5.94 brs	127.0	-	195.0
4	-	141.6	-	125.0
5	-	154.8	-	167.0
6	6.08 s	135.2	7.48 s	133.7
7	-	72.0	-	150.0
8a	1.87 m	26.4	-	197.3
8b	1.62 m			
9a	2.03 m	34.2	2.63 d (9.5)	46.5
9b	1.57 m		2.37 d (9.5)	
10	-	36.2	-	41.0
11	1.86 m	38.7	3.08 m	28.0
12	0.94 d, (6.7)	18.0	1.20 d (6.7)	19.0
13	1.02 d, (6.7)	16.5	1.19 d (6.7)	22.0
14	1.07 s	23.0	1.32 s	25.7
15	2.13 s	20.4	2.05 s	13.0

[a] Data assignments were confirmed by, 1D-TOCSY, DQF-COSY, HMBC and HSQC experiments. *J* values are in parentheses and reported in Hz; chemical shifts are given in ppm; assignments were confirmed by DQF-COSY, 1D-TOCSY and HSQC experiments.

2.5. Cells and Treatment

Jurkat (T-cell leukemia) and HeLa (cervical carcinoma) cell lines were obtained from the American Type Cell Culture (ATCC) (USA). A2780, MCF-7, and RKO were obtained as a generous gift from Dr. Ahmad Al-Jada (KSAU-HS, Saudi Arabia). Cells were maintained in DMEM (HeLa, A2780, MCF-7, RKO) or RPMI 1640 (Jurkat), supplemented with 10% FBS, 100 mg/L penicillin, and streptomycin 100 IU/mL at 37 °C in a humidified atmosphere of CO_2 5% other conditions were previously [3].

Table 3. IC50 (μM) of *P. puntulata* sesquiterpenes

Compound	**Jurkat**	**HeLa**
1	12 ± 0.9	18 ± 1.1
2	>50	>50
3	44 ± 2	>50
4	>50	>50
Etoposide	2.5 ± 0.8	4 ± 1.1

All the results are mean values ± SD from triplicate.

2.6. Cell Viability and Cell Cycle

A 96-well plate was used to seed and incubate the cells, with or without the compounds; Etoposide was used for the positive control. 24 h before treatments, cells were seeded at a cell density of 2 x 10^3cells/well. MTT assay was performed as described previously [3,7,8]. Cell cycle was evaluated by propidium iodide (PI) staining according to the protocol of (BD FACSCalibur *flow cytometer*, USA.

2.7. Peroxide Depletion Activity of Test Compounds by Cytofluorimetry

The evaluation of intracellular peroxides concentration was performed as reported in literature [9] with some modifications. In details, Jurkat cells were collected by centrifugation and suspended in RPMI containing 5% FBS at a density of 5×10^5 cells/mL. FBS concentration was lowered to 2% to increase the uptake rate of compound **1** and reagents in the short incubation time (1 h.) chosen for the assay. Cell suspensions were incubated with increasing concentrations of compound **1** or vehicle only at 37°C. For each sample duplicate test tubes were prepared. After 30 min of incubation, BHT (550 µM final concentration) or an equal volume of the vehicle were added. This allowed us to monitor the effect of compound **1** on BHT-induced peroxide elevation or the basal levels of peroxides, respectively. In the last 15 min of incubation, cells were loaded with DCFH-DA (8 µM final concentration). Test tubes were gently mixed several times along the incubation period (1 h.). Cells suspension were then washed, re-suspended in an equal volume of medium and 10,000 events were analyzed for DCF-fluorescence by cytofluorimetry (BD FACSCalibur™ instrument, Becton Dickinson, San Jose, CA, USA). DCF green fluorescence was analysed in the FL1 channel (λ_{exc}488 nm; (λ_{em}535 nm). Before the cytofluorimetric analysis, an aliquot of each sample was withdrawn to evaluate cell viability by Tripan-blue exclusion test.

2.8. Statistical Analysis

The reported data represent the mean values ± SD from 3 independent experiments. Student's *t*-test analysis was used to determine the differences between treatment groups. Differences were considered significant when $p \leq 0.05$.

3. Results and Discussion

Among the 14 plants screened for antiproliferative activity, five plant extracts showed various degrees of activity table1. Previous studies on *P. punctulata* aerial parts confirmed the cytotoxic effect of the methanol extract [10]. The phytochemistry of *P. punctulata* roots was not investigated previously, while the aerial parts and the exudate were investigated extensively revealing the presence of flavonoids and diterpenes [11-13].

Further purifications of the active *n*-hexane extracts by silica gel column chromatography and semi-preparative RP-HPLC led to the isolation of four sesquiterpenes one of which (compound **3**) is a new one. The compounds **1** and **3** expressed different antiproliferative potency against HeLa and Jurkat cancer cells. The effect of the most active compound **1** was investigated on cell cycle progression, cell death, and peroxide depletion.

The molecular formula of compound **3** ($C_{15}H_{22}O_2$) was obtained by ^{13}C NMR and HR-ESIMS spectra ($[M+H]^+$ at *m/z* 235.1690, calculated as $C_{15}H_{22}O_2H$ 235.1698).

The ESI MS/MS spectrum displayed two fragments attributable to the loss of one water molecule at *m/z* 217 $[M+H-18]^+$ and at *m/z* 193 $[M+Na-42]^+$. The ^{13}C NMR spectrum (Table 2) exhibited 15 signals, which on the basis of ^{13}C NMR data were assigned to one ketone α,β-unsaturated (200.6, 127.0, 141.6 ppm), one hydroxyl tertiary carbon (72.0 ppm), one quaternary carbon (36.2 ppm), three methylenes (52.6, 26.4, 34.2 ppm), two methines of which one sp2 hybridized (135.2 and 38.7 ppm), and four methyl groups. These evidences suggested the presence of a eudesmane skeleton [14-16]. The ^{1}H NMR data confirmed the presence of α,β-unsaturated carbonyl group (δ 5.94, brs) and four methyl groups at δ 0.94 (d, J = 6.7 Hz), 1.02 (d, J = 6.7 Hz), 1.07 (s), and 2.13 (s). The ^{13}C NMR signals were assigned on the basis of an HSQC spectrum, while HMBC experiment led to locate the substituents.

The location of the isopropyl group at C-7 was revealed by correlations between Me-12 signal at delta 0.94 with carbon resonances at 72.0 (C-7), 38.7 (C-11), and 16.5 (C-13) ppm, whereas an alfa,beta-unsaturated carbonyl group was positioned between C-2-C-4 by correlations between Me-15 at delta 2.13 and 127.0 (C-3) and 154.8 (C-5) ppm.

Similarly, the attribution of all carbon signals was deduced from long range correlations between proton at δ 1.07 (Me-14) and C-1 (δ_C 52.6) and C-10 (δ_C 36.2); δ 2.03 (H-9a) and C-1 (δ_C 52.6), C-7 (δ_C 72.0), C-10 (δ_C 36.2). The relative configuration at C-10, and C-7, was established by a 1D ROESY experiment showing correlations between δ 1.07 (Me-14) and 1.86 (H-11), indicating that these protons were on the same side. Thus, compound **3** was identified as 7 (S)-hydroxy eudesman-3,5-dien-2-one.

HR-ESIMS of compound **4** (molecular formula $C_{15}H_{18}O_3$) gave an $[M+H]^+$ peak at *m/z* 247.1337. This was further confirmed by ^{13}C NMR and DEPT spectra. The ^{13}C NMR spectrum (Table 2) showed the presence of two carbonyl groups at 195.0 and 197.3 ppm, six further sp^2 carbon atoms at 167.0, 150.0, 149.7, 133.7, 125.0, and 125.4 ppm. The remaining signals were assigned to one methylene, one methine, one quaternary carbon, and four methyl groups.

From the HSQC correlations it was possible to deduce the occurrence of two α,β-unsaturated ketone. The spectral data showed that compound **4** was a eudesmane derivative. Complete elucidation of the structure of **4** was achieved by HMBC experiments, showing correlations between H-1—C-3, H-1—C-10, H-1—C-5; H-6—C-11, H-6—C-4; H_2-9—C-1, H_2-9—C-5; H_2-9—C-7, Me-14—C-1, Me-14—C-9, Me-15—C-4, Me-15—C-6, Me-12—C-7, Me-12—C-11. Full NMR data of **4** is reported for the first time [16]. Thus, compound 4 was elucidated as 2-hydroxy eudesman-1,4,6-trien-3,8-dione. The structure of the following known compounds were established by comparison of spectroscopic data with those previously reported compound **1**: 1β-hydroxy-8-oxo-cyperone and compound **2**: 8-oxo-β-cyperone [14,17].

Figure 1. Chemical structures of the isolated compounds (**1-4**) from *P. punctulata.*

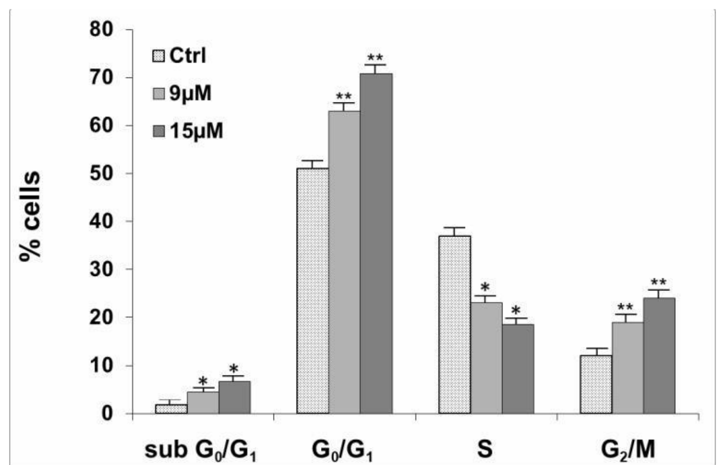

Figure 2. Effect of compound 1 on proliferation of leukaemia cells**.** Flow cytometric evaluation of DNA content in Jurkat cells exposed for 48 h to **1**(9 and 15 μM) or vehicle alone (Ctrl). All results are mean values ± SD from at least three experiments performed in duplicate (*p< 0.05, **p< 0.001).

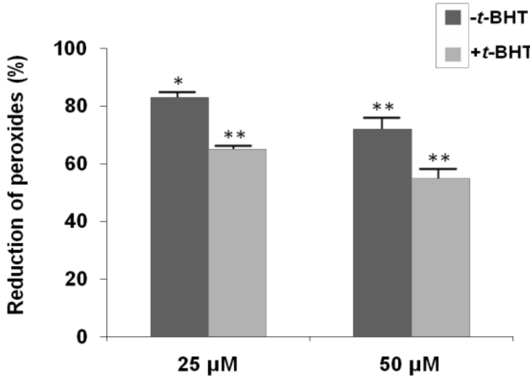

Figure 3. In-cells antioxidant potential of compound **1.** Unstimulated (black bars) and BHP-stimulated (grey bars) Jurkat cells were incubated with compound **1** (25 and 50 μM) or vehicle only. Cellular concentrations of peroxides (DCF fluorescence) were measured by cytofluorimetry. Data shown were obtained using the mean fluorescence values and are the mean values ± SD of at least three experiments performed in duplicate. P values were always <0.01.

Among 14 tested extracts, the MeOH extract of *P. punctulata* root has shown the highest antiproliferative activity against cancer cell lines. Phytochemical fractionation led to the isolation of four sesquiterpenes of which compound **3** is a new sesquiterpene. Compound **1** expressed moderate antiproliferative activity involving cell cycle arrest mainly at G1 phase and induced growth inhibition in Jurkat cells. Compound **1** has also been found to have some antioxidant properties as it strongly inhibited peroxidase activity in Jurkat cells.

The antiproliferative activity of the isolated compounds was evaluated in HeLa and Jurkat cancer cell lines. Cells were exposed to increased concentrations of all isolates and cell viability was evaluated after 48 h exposure time by MTT assay. Half-maximal inhibitory concentration (IC_{50}) values, obtained from dose-response curves, are shown in Table 3. We found that only compound **1** inhibited the growth in a dose-dependent manner for both Jurkat and HeLa cells. Jurkat resulted slightly more susceptible than HeLa cells, being the half maximal inhibitory concentration (IC_{50}) value 12 µM and 18 µM, respectively. These results indicate that Compound **1** is not the only responsible for the antiproliferative activity of the crude methanol extract. Unlike Compound **1**, the methanol extract induced more growth inhibition in HeLa cells compared to a week activity in Jurkat.

The mechanism underlying the antiproliferative effect of Compound**1** was further investigated in Jurkat cells. To investigate whether compound **1** reduced cells number by affecting cell cycle progression and/or by inducing cell death, Jurkat cells were exposed for 48 h at the concentration 9 and

15 µM of compound **1**, these concentrations are close to the IC_{50} value. DNA content was evaluated by flow cytometry analysis of propidium iodide (PI) stained nuclei. As shown in Figure 2, compound **1** reduced Jurkat cell number also by affecting cell cycle progression, with cells accumulated mainly in G_0/G_1 and, less markedly, in G_2/M.

In addition, the ability of compound **1** to reduce the level of peroxides in control and BHP-treated Jurkat cells was measured by cytofluorimetry. Compound **1** was tested at 25 µM and 50 µM concentrations. As shown in Figure 3. Compound **1** has a significant dose-dependent antioxidant activity. In particular, it was able to reduce the rate of basal peroxides of Jurkat cells and to contract the rate of peroxides induced by *t*-BHT treatment. Such an antioxidant activity might contribute to the observed cell cycle arrest since low levels of oxygen reactive species are known to promote cell proliferation [18]. Compound **1** may serve as a good candidate for future drug development studies aiming to synthesize more potent derivatives.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/ records-of-natural-products

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