Supporting Information

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Phenolic Acid Analogues from *Balanophora laxiflora* Inhibit Proliferation of *In Vitro* Acute Myeloid Leukemia Cells

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S1. Experimental and Chemistry

S1.1. General Experimental

S1.1.1. Chemical Methods

ESI MS spectrum was obtained on an Agilent 1100 LC-MSD Trap spectrometer. FT-ICR-MS model 910 TQ- FTMS, Agilent USA. HPLC-MS Agilent 1100 USA. ^{1}H NMR (500.13 MHz) and ^{13}C NMR (125.77 MHz) spectral data were measured on a Bruker Avance III NMR spectrometer, Switzerland. Chemical shifts were expressed in δ (ppm) downfield from TMS as an internal standard and coupling constants were reported in Hertz. Solvents $CDCl_3$ ($^{1}H = \delta_H 7.26$; $^{13}C = \delta_C 77.0$) or CD_3OD ($^{1}H = \delta_H 3.33$; $^{13}C = \delta_C 49.0$). Silica gel 60 F-254 (0.25mm, Merck); CC: Silica gel 60 (230-400 mesh, Merck) for the first column, silica gel 60, 40-63 μm (Merck) and Sephadex LH-20 for the following columns.

The purity of compound MC was determined by HPLC-DAD (HPCHEM) using XDB-C18 column (150 × 4.6 mm I.D, 5 μm) - CUD-4, flowrate 1.0 mL/min, isocratic elution (30% methanol in distilled water + 0.1% formic acid, MeOH-H_2O, 30:70), UV spectrum at 225, 290, 330 nm. temp. 25 °C. Detection was carried over 35 min at a flow rate of 1 mL/min. Detection was carried out at 230 and 280 nm. The injection volume was 5μL.

S1.1.2. Extraction and Isolation

The air-dried powder of *B. laxiflora* (6.0 kg) were extracted five times (each in one a day) with ethanol at room temperature. Evaporation of the solvent under reduced pressure gave an ethanol crude (1.8 kg), which was suspended in water and extracted sequentially with *n*-hexane, dichloromethane, ethyl acetate and ethanol. After evaporating solvent to give *n*-hexane (BLH, 800 g), dichloromethane (BLD, 41 g), ethyl acetate (BLE, 194 g) and ethanol (BLEt, 665 g) residues, respectively. The dichloromethane fraction (BLD, 41 g) was subjected to a silica gel column chromatography (CC) using a gradient of chloroform-methanol (100:0 → 50:1 → 40:1 → 20:1 → 15:1) to yield ten fractions (BLD1→BLD10). Fraction BLD1 was separated on silica gel column eluting with CH_2Cl_2-MeOH (50:1) to obtain **1** (63 mg). Similarly, fraction BLD2 was repeated column chromatography (CC) on silica gel eluting with CH_2Cl_2-MeOH (30:1) to give **2** (40 mg). Compound **3** (53 mg) was isolated from BLD3 by using a silica gel CC with CH_2Cl_2-MeOH (20:1). Compound **4** (210 mg) was purified from BLD5 by Sephadex LH-20 (MeOH).

*S1.1.3. Spectra Data of Compounds **1-4***

*Methyl gallate (methyl 3,4,5-trihydroxybenzoate, **1**).* HR-ESI-MS: *m/z* 185.0468 $[M + H]^+$ (calc. $C_8H_9O_5$, 185.0450). ^{1}H-NMR (500 MHz, CD_3OD, *δ* ppm): 7.06 (2H, *s*, H-2/H-6); 3.83 (3H, s, OCH_3). ^{13}C-NMR (125 MHz, CD_3OD, *δ* ppm): 121.5 (C-1), 110.1 (C-2/C-6), 146.5 (C-3/C-5), 139.8 (C-4), 169.0 (C-7).

*4-Hydroxy-3-methoxycinnamaldehyde [3-(4-hydroxy-3-methoxyphenyl)-2-propenal, **2**].* HR-ESI-MS: *m/z* 179.0718 $[M+H]^+$ (calc. $C_{10}H_{11}O_3$, 179.0708). ^{1}H-NMR (500 MHz, $CDCl_3$, *δ* ppm): 7.07 (1H, *d, J* = 2.0 Hz, H-2); 6.96 (1H, *d, J* = 8 Hz, H-5); 7.12 (1H, *dd*, *J* = 8.0, 2.0 Hz, H-6); 6.60 (1H, *dd, J*= 7.5, 16.0 Hz, H-8); 7.40 (1H, *d, J* = 16 Hz, H-7), 9.67 (1H, *d*, *J* = 7.5 Hz, H-9); 3.94 (1H, *s*, 3-OCH_3). ^{13}C-

NMR (125 MHz, $CDCl_3$), δ ppm): 56.0 ($O\underline{C}H_3$), 126.6 (C-1), 109.5 (C-2), 149.0 (C-3), 147.0 (C-4), 115.0 (C-5), 124.0 (C-6), 153.2 (C-7), 126.3 (C-8), 193.7 (C-9).

3-Methoxycinnamic acid [3-(3-methoxyphenyl)-2-propenoic acid, ***3****].* HR-ESI-MS: *m/z*: 179.0726 $[M+H]^+$ (calc. $C_{10}H_{11}O_3$, 179.0708). ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 7.43 (2H, *d, J* = 8.5 Hz; H-2/H-6); 6.85 (2H, *d, J* = 8.5 Hz, H-3/H-5), 7.64 (1H, *d, J* = 16.0 Hz, H-7), 6.30 (1H, *d*, *J* = 16.0 Hz, H-8), 3.80 (3H, *s*, OCH_3). ^{13}C-NMR (125 MHz, $CDCl_3$, δ (ppm): 51.6 ($O\underline{C}H_3$), 126.7 (C-1), 130.0 (C-2/C-6), 116.0 (C-3/C-5), 158.5 (C-4), 144.8 (C-7), 114.8 (C-8), 168,0 (C-9).

Methyl caffeate [methyl trans-3-(3,4-dihydroxyphenyl)-2-propenoate, ***4****].* HR-ESI-MS: *m/z* 195.0668 $[M+H]^+$ (calc. 195.0657, $C_{10}H_{11}O_4$,). ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 7.08 (1H, *d, J* = 2.0 Hz, H-2), 6.87 (1H, *d, J* = 8.0 Hz, H-5), 7.01 (1H, *d, J* = 8.0 Hz, H-6); 7.58 (1H, *d, J* = 16.0 Hz, H-7); 6.26 (1H, *d, J* = 16.0 Hz, H-8), 3.80 (3H, *s*, OCH_3). ^{13}C-NMR (125 MHz, $CDCl_3$, δ ppm: 51.7 ($O\underline{C}H_3$), 127.8 (C-1), 114.4 (C-2), 144.7 (C-3), 146.1 (C-4), 115.60 (C-5), 122.6 (C-6), 143.7 (C-7), 115.65 (C-8), 167.8 (C-9). HMBC correlation (H $\rightarrow$C, s: strong, w: weak): H-2/C-6, C-7, H-5/C-1, C-3, H-6/ C-8, C-7, H-7/C-9s, C-8, C-6, H-8/C-9 w, C-1, C-9 s.

S1.1.4. Identification of the Purity of Isolated Compound Methyl Caffeate (MC, ***4****)*

The purity of methyl caffeate (MC) was determined by HPLC (Agilent 1260 infinity) system using a XDB-C18 column (150 × 4.6 mm I.D, 5 µm particle size), an isocratic solvent system of 30% methanol in distilled water + 0.1% formic acid), over 25 min at a flow rate of 1.0 mL/min. UV detection was carried out at 225, 280 and 330 nm. The injection volume was 10µL. The HPLC chromatogram of MC is shown in Figure S2. The purity of MC was determined to be 96.35 % by HPLC.

S2. Biological Activity Assessments

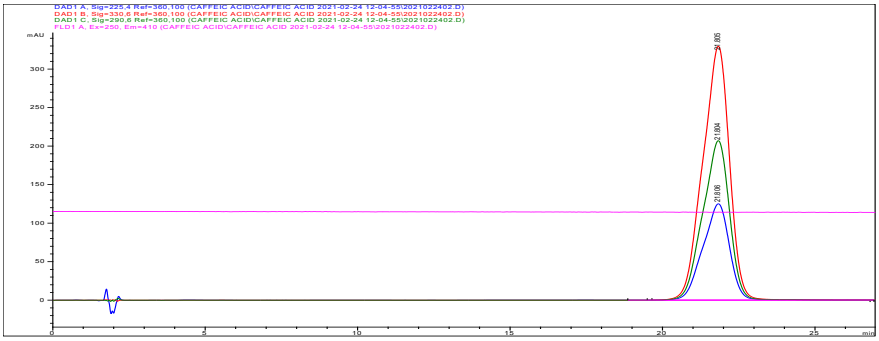
Cell culture: The subtype 3 of OCI-AML3, was obtained from the American Type Culture 97 Collection (ATCC, Manassas, VA, USA). Cell were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in 5% CO_2. The medium was changed every 2 days. In the experiment, cells were seeded into 24-wells of culture plates, kept ad concentration of 2×10^5 cells/mL, and were treated for 24 hours with different concentrations of dimethylsulfoxide (DMSO) (The maximum concentration was 1.5 µl/mL) or the test compounds at the final concentrations (identified after a preliminary screening) reported in the figures. We dissolved the compounds in DMSO, for this reason, we used DMSO as a control.

Analysis of cell number, apoptotic cell death and cell cycle progression: Cells were counted manually using a hemocytometer. Cell viability and cell cycle progression were analyzed by flow cytometry to determine DNA content of cell nuclei stained with propidium iodide (PI), after exclusion of necrotic cells by forward light scatter (FSC). Briefly, cells were collected by centrifuge and washed in PBS (phosphate-buffered saline). DNA was stained by incubating the cells in PBS containing 50 µg/mL PI for 30 minutes at 4^0C. This allows a direct DNA staining in PI hypotonic solution without the requirement of RNase treatment due to removal of RNA by hypotonic shock [1, 2]. Fluorescence was measured and analyzed by flow cytometry using Coulter Epics XL-MCL equipment (Beckman Coulter, Inc., Brea, CA, USA) [3]. In cell cycle analyses, the sub-G1 cells were gated out.

Western Blotting: Cells were pelleted in a conical tube by spinning at 1200 rpm for 5 minat room temperature, after which the media was decanted and the pellet waswashed withPBS and spunat 1200 rpm for 5 min. After the pellet wasplaced in 30 µL RIPA lysis buffer (5 M NaCl, 0.5 M EDTA, pH 8.0, 1 M Tris, pH 8.0, NP-40 (IGEPAL CA-630), 10% sodium deoxycholate, 10% SDS, Deionized H_2O) supplemented with protease (Sigma-Aldrich) and phosphatase (Thermo-Fisher Scientific) inhibitor cocktails. The lysate was incubated in ice for 30 min and then centrifuged at 12,000 rpm for 30 min at 4 °C. The supernatant was collected into new microtubes, and protein concentration was determined by the bicinchoninic acid method. Proteins were separated by 12% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and evaluated by Westernblotting. Primary antibodies were polyclonal anti-caspase-3 (1:1000 dilution, Cell Signaling, Danvers, MA, USA), anti-caspase-8 monoclonal antibody, mAb (1:1000 dilution, clone 12F5, Enzo Life Sciences, Farmingdale, NY, USA), anti-caspase-9 mAb (1:1000 dilution, clone ICE-LAP6, Mch6, Cell Signaling), anti-GAPDH mAb (1:2000 dilution, clone 2D9, OriGene, Rockville, MD, USA), Secondary antibodies were labeled with horseradish peroxidase (1:5000 dilution, Pierce/Thermo-Fisher Scientific, Waltham, MA,USA). Antigen-antibody complexes were detected by enhanced chemiluminescence following the manufacturer'sinstructions (Millipore, Billerica, MA, USA). Western blotting films were scanned, and band signal intensities were determined using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Positive controls from U937 cells treated for 24h with Fusarubin [3].

Real Time PCR: Total RNA was extracted from cells by RNeasy Plus Micro Kit (Qiagen), and generation of cDNA was performed in triplicate using a Quanti Tect Reverse Transcription kit (Qiagen). All reactions were performed using an ABI-7300 Real-Time Cycler, and amplification was performed using TaqMan Assay (Hs00181225 for Fas-L, Hs00174128, Hs00174128 for TNF-α, TGF-β, and eukaryotic 18S rRNA as an endogenous control).

Statistical Analysis: Statistical analysis was performed using GraphPadPrism 6. Differences between groups were evaluated using Mann–Whitney U test. Differences were considered statistically significant according to the following criteria: *p < 0.05; **p <0.01; ***p < 0.001.



HPLC (Agilent 1260 infinity) system:

[Column XDB-C18 column (150 ×4.6 mm I.D, 5 µm)- CUD-4, flowrate 1.0 mL/min, isocratic elution (30% methanol in distilled water + 0.1% formic acid, MeOH-H_2O, 30:70), UV spectrum at 225, 290, 330 nm. Temp. 25^{O}C]

Figure S1: Analytical HPLC choromatogram of compound **4** (methyl caffeate) (t_R: 21.81 min, purity 96.4%)

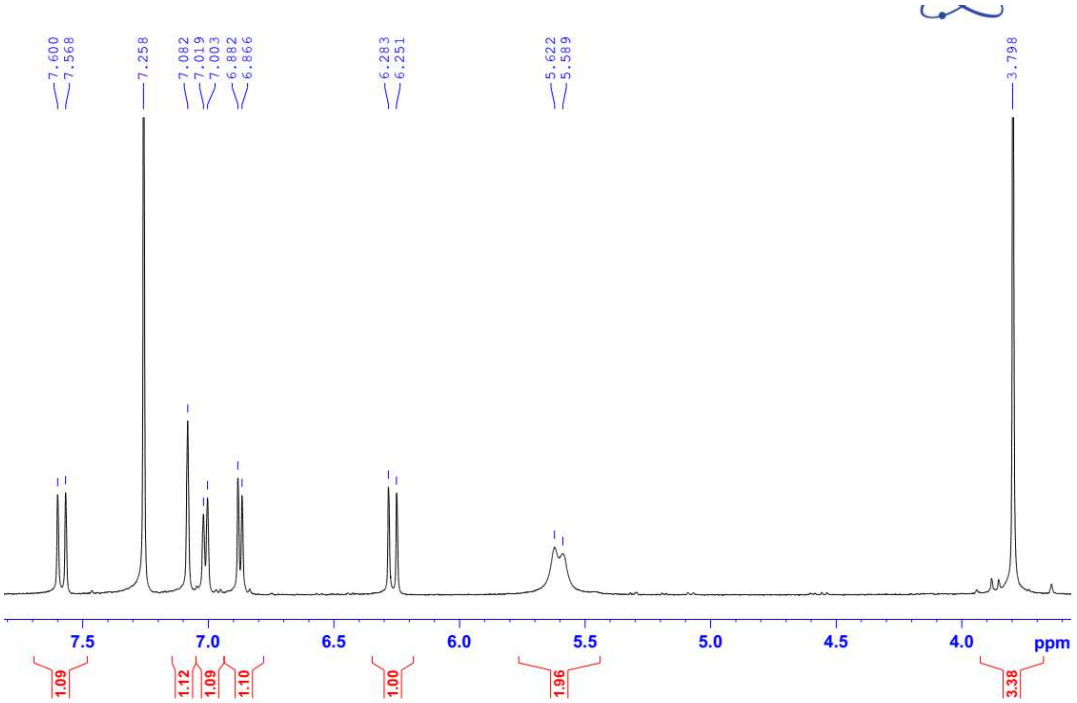


Figure S2 : ^{1}H-NMR (500 MHz, $CDCl_3$) spectrum of compound **4** (methyl caffeate)

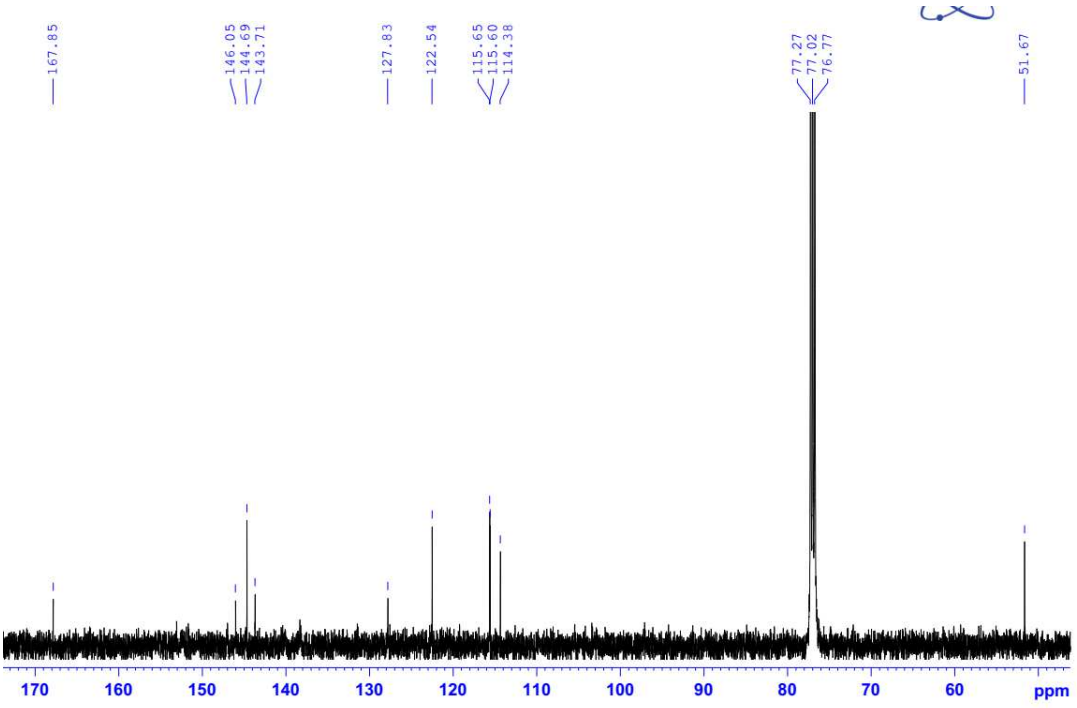


Figure S3 : ^{13}C-NMR (125 MHz, $CDCl_3$) spectrum of compound **4** (methyl caffeate)

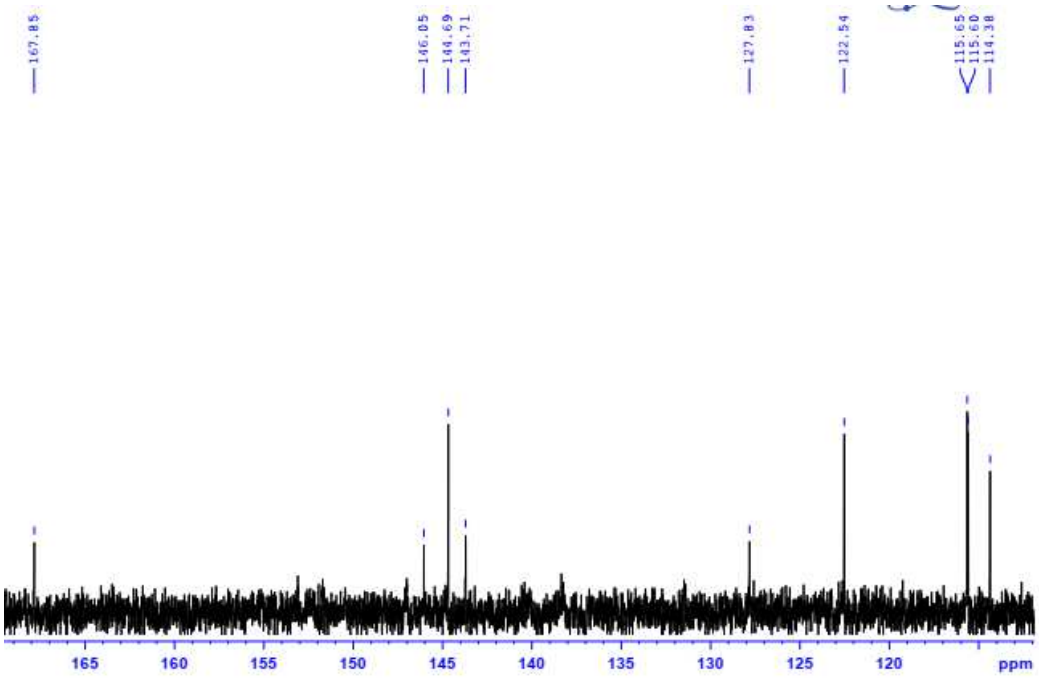


Figure S3a : ^{13}C-NMR spectrum of compound **4** (from 114 to 168 ppm)

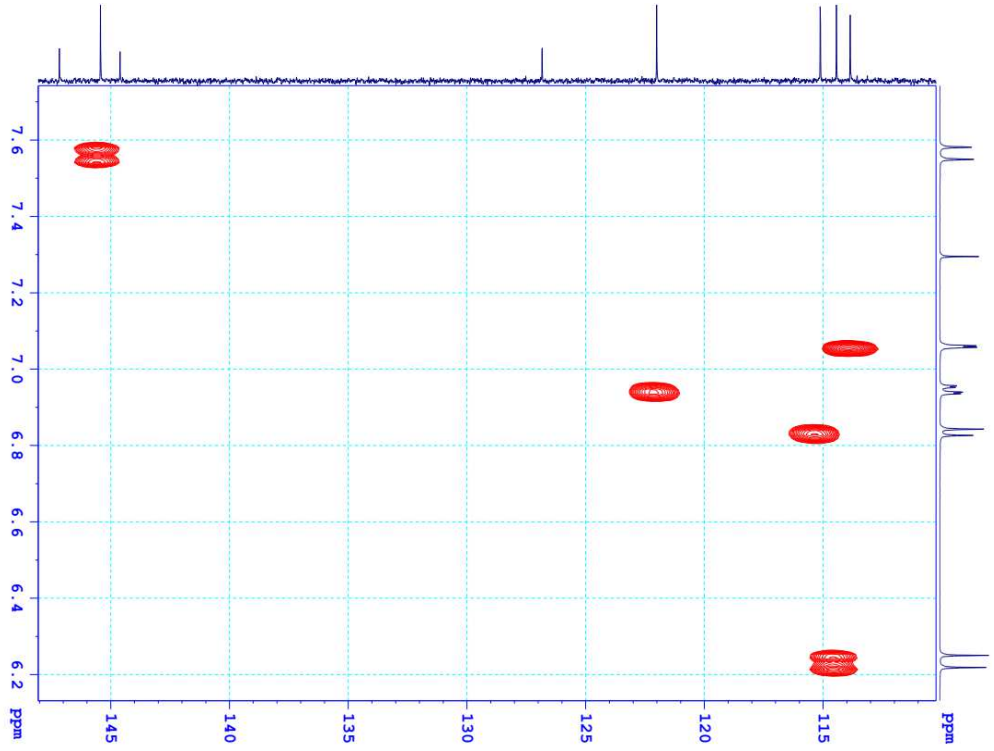


Figure S4 : HSQC Spectrum of compound **4** (methyl caffeate)

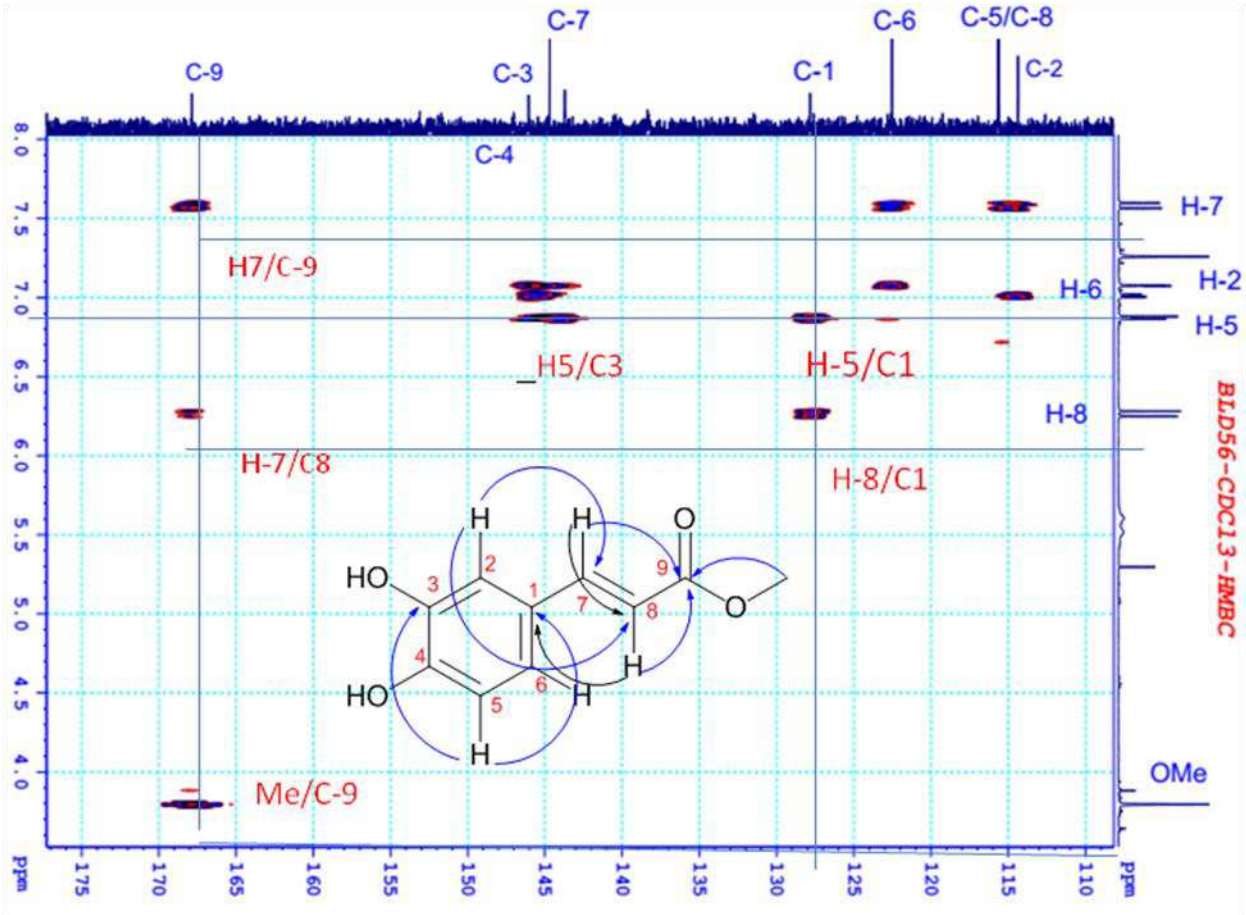


Figure S5 : HMBC spectrum of compound **4** (methyl caffeate)

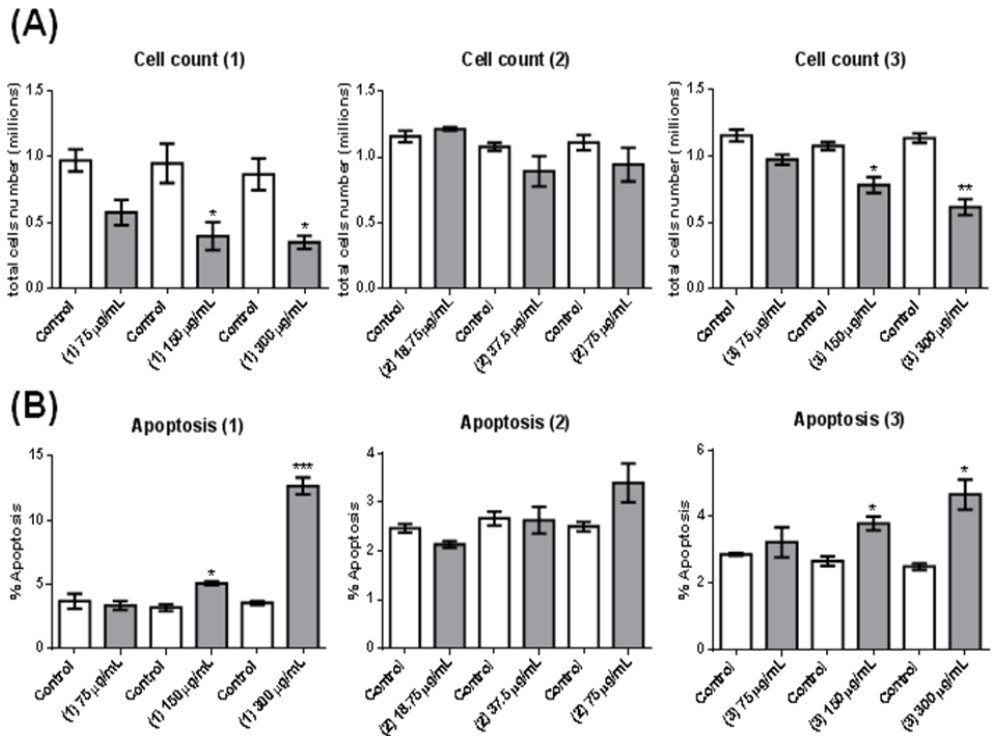


Figure S6 : Effects of compounds (**1**), (**2**) and (**3**) on OCI-AML3 cell number and *apoptotic* cell death. **A.** Bars represent the cell number of cells treated with vehicle (control, white bars) or with different concentrations of substances (**1**), (**2**) or (**3**) (gray bars). **B.** Bars represent the percentage of apoptosis of cells treated with vehicle (control, white bars) or with different concentrations of substances (**1**), (**2**) or (**3**) (gray bars). Data from five independent experiments are reported as mean ± SEM. * $p < 0.05$; **$p< 0.01$; *** $p < 0.001$.

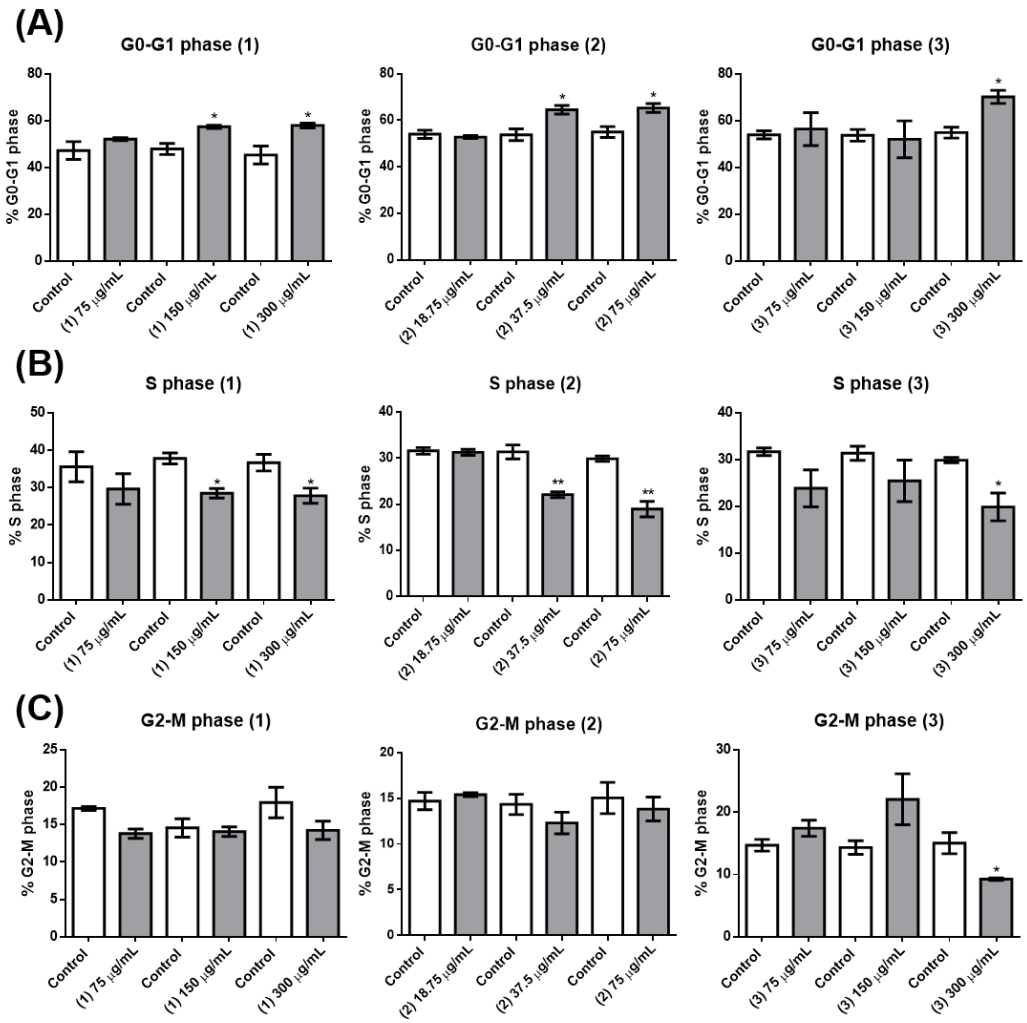


Figure S7 : Effects of cells in G0/G1 (**A**), S (**B**) or G2/M (**C**) phases after 24 h of treatment with**.** Effects of compounds (**1**), (**2**) and (**3**) on OCI-AML3 cell cycle progression. Bars represent the percentage control vehicle (Control, white bars) or (**1**), (**2**) and (**3**) at concentrations reported in the x-axes (Gray bars). Data from five independent experiments are reported as mean ± SEM. *p< 0.05; ** p < 0.01; *** p < 0.001.

References

[1] C.Ricardi and I. Nicoletti (2006). Analysis of apoptosis by propidium iodide staining and flow cytometry, *Nat. Protocol.* **1** (**3**), 1458-1461.

[2] B. T. Cham, N. T. T. Linh, D. T. Thao, N. T. H. Anh, N. T. Tam, B. K. Anh, I. Muscari, S. Adorisio, Sung TV, Thuy TT and D. Delfino (2020). Cell growth inhibition of saponin XII from *Dipsacus japonicas* Miq. on acute myeloid leukemia cells, *Molecules* **25**, 3325.

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