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Natural daucane sesquiterpenes with antiproliferative and proapoptotic activity against human tumor cells

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ABSTRACT

Plants of the genera *Ferula* and *Ferulago* are known for their complex content in bioactive secondary metabolites such as coumarins, phenylpropanoids, and sesquiterpenes. We used the ground parts of *Ferula communis* subsp. *communis*, *Ferula glauca* subsp. *glauca* and *Ferulago campestris* as natural sources for the isolation of four coumarins (**CU-1 to CU-4**), two phenylpropanoids (**PE-1** and **PE-2**), one polyacetylene (**PA-1**) and 16 daucane esters (**DE-1 to DE-16**). The cytotoxic activity of the isolated compounds was evaluated against a panel of seven human tumor cell lines. Fourteen of the daucane derivatives showed antiproliferative activity at least against one of the human tumor cell lines tested, four compounds (**DE-5**, **DE-8**, **DE-11**, and **DE-16**) were active against all the tested cell lines. Among them **DE-11** was the most cytotoxic compound against HeLa (4.4 ± 0.7 μM), A549 (2.8 ± 1.4 μM), HL-60 (2.6 ± 0.4 μM), K562 (26.5 ± 6.0 μM) RS 4;11 (1.7 ± 0.3 μM) and SEM (2.4 ± 0.1 μM) cell lines, while **DE-8** was the most active against Jurkat (3.3 ± 0.8 μM). Preliminary structure–activity relationship suggests that the most active compounds in the daucane series present the *trans* fusion of the penta- and hepta-atomic cycles, and lipophylic ester groups linked to position 6. Isomeric derivatives such as **DE-8** and **DE-9** or **DE-3**, **DE-4**, and **DE-5** exhibited significant differences in their IC_{50} supporting that the β orientation for the ester group in the position 2 enhances the cytotoxic activity. Furthermore, the pro-apoptotic effect of the most active compounds evaluated in Jurkat cell line showed that these compounds are able to induce apoptosis in a time and concentration-dependent manner. Our findings suggest the potential role of daucane derivatives as models for the development of proapoptotic compounds.

1. Introduction

The plants of the genus *Ferula* (Apiaceae) are well known for the production of sesquiterpene and coumarin derivatives.[1–11] Morphologically and phytochemically related to the *Ferula* species are the plants belonging to the genus *Ferulago*.[12]

Ferula and *Ferulago* coumarins have been evaluated for antimicrobial,[13,14] in vitro antileismanial,[15] and cancer chemiopreventive activities.[16,17] Essential oils from *Ferula communis* (FCO), *Ferula glauca* L. (FGL) and *Ferulago campestris* (Besser) Grecescu (FGO) have been studied for their antimicrobial activity[18–20] and non alkaloidal acetylcholinesterase inhibitors were reported from FGO roots.[12] The daucane ester ferutinin and some related compounds, isolated from different *Ferula* species, possess estrogenic activity.[21] Furthermore, some of the *Ferula* sesquiterpenes have been studied for their antiviral, cytotoxic, and proapoptotic properties.[22,23] Ionophoretic and apoptotic properties of the daucane ferutinin and related derivatives were studied and showed that ferutinin-induced DNA fragmentation was mediated by a caspase-3-dependent pathway.[24] Further studies about antiproliferative activity of daucane derivatives were reported on MCF-7[25] and colon cancer cells.[11] Suzuki et al. reported that sesquiterpene lactones from *Ferula varia* act as cytotoxic agents against KB (human epidermoid carcinoma of the nasopharynx), KB-C2 (multidrug-resistant KB), K562 (leukemia) and K562/Adr (multidrug-resistant K562) cells.[26] Alkhatib et al. showed an inhibitory effect against two chronic myeloid leukemia (CML) cell lines,[27] by sesquiterpene esters isolated from *Ferula elaeochytris* Korovin and recently humulane and germacrane sesquiterpene from *Ferula lycia* Boiss. have been studied for their

Abbreviations: FCO, *Ferula communis*; FGL, *Ferula glauca*; FGO, *Ferulago campestris* (Besser) Grecescu.

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activity against tyrosine kinase inhibitor-resistant cell lines, K562R and DA1-3b/M2BCR-ABL.[28] Therefore, among the various isolated *Ferula* sesquiterpenes, the daucane derivatives can be considered as attractive molecules especially as potential antiproliferative and anticancer compounds. In the continuation of our work on cytotoxic natural products[29–32] we have selected FCO, FGL, and FGO from Italian populations as sources of bioactive natural products. In this Letter we describe the isolation of two new daucane derivatives (**DE-4** and **DE-3**) together with 21 known compounds. The structures of all the isolated compounds were obtained on the basis of 1D and 2D NMR techniques as well as MS spectrometry. Moreover, the cytotoxic activity of all the isolated compounds was measured against seven different human tumor cell lines and a preliminary investigation on the mode of cell death was also carried out.

2. Results and discussion

2.1. Structures of the isolated compounds

The isolated compounds were obtained from the dichloromethane soluble fractions of FCO, FGL, and FGO on the basis of extensive chromatographic separations, as described in Section 4. Namely compounds **CU-4, DE-1 to DE-5**, **DE-7 to DE-9**, **DE-11**, and **DE-12** were isolated from FCO, **DE-11**, **DE-13**: **DE-16** were isolated from FGL, while compounds **CU-1**, **CU-2**, **CU-3**, **PR-1**, **PR-2**, **PA-1**, **DE-6**, **DE-7**, and **DE-10** were isolated from FGO. Structures of isolated compounds were determined on the basis of 1D and 2D NMR (HMQC, HMBC, COSY, NOESY) experiments as well as on MS experiments (see Supplementary data).

Compound **DE-4** was isolated as clear amorphous solid. The HR-MS spectrum showed a pseudomolecular ion $[M+Na]^+$ at *m/z* 469.2120 (calculated 469.2202 for $C_{25}H_{34}NaO_7$). The ^{1}H-NMR spectrum showed signals due to a *p*-anysate group namely two *ortho* coupled doublets at δ 7.96 (H2′–6′) and 6.93 (H3′–5′) (J = 7.8 Hz) integrating two proton each, and a singlet at δ 3.87 (H-7) integrating for three protons. Signals ascribable to oxymethyne were detected at δ 4.96 (H-2) (dd 1.1; 6.4), at δ 5.52 (H-6) (J = 10.2; 6.00; 3.70) and a doublet at δ 4.10 (H-10) (J = 7.2) integrating one proton each. Doublets at δ 0.79 (H-12) (J = 6.7) and 0.94 (H-13) (J = 6.5) integrating for three proton each, suggest the presence of an isopropyl group while the three singlets at δ 1.11 (H-15), 1.82 (H-14) and 2.12 (3H, each) support the presence of two different quaternary methyl groups and one acetyl residue, respectively. The structure of **DE-4** was established mainly on the basis of 2D NMR experiments. Three oxygenated non quaternary positions were detected in the HMQC spectrum with δ_H 5.52 (ddd, J = 10.2; 6.0; 3.7) and δ_C 70.4, δ_H 4.96 (dd, J = 6.4; 1.1), δ_C 84.6 and δ_H 4.10 (d J = 7.2) δ_C 71.8. Diagnostic HMBC correlations were observed from the methyl group 15 (δ 1.11) with carbon resonances at δ 52.0 (C-1), 71.8 (C-10), 84.6 (C-2) and 47.8 (C-5), from the methyl group 14 (δ 1.82) and the carbon resonances at δ 136.9 (C-8), 126.5 (C-9) and 39.2 (C-7), and from the methyne proton signal at δ 3.20 (H-5) with carbons C-7, C-10, C-1, C-3 and C-2. The comparison with the literature[5,33] allows to establish the presence of a daucane derivative formerly related to a *trans* fused dauc-8-ene as 10-hydroxy-jaeschkeanadiol.[5] HMBC correlations allowed also to establish the esterification position due to the HMBC correlation observed from H-6 to C-1′ (δ 167.0) and due to the HMBC correlation observed from H-2 (δ 5.52) and C-1″ (δ 170.0). The comparison of coupling constants of H-2 (J = 6.4, 1.1), H-6 (J = 10.5, 6.0, 3.7), and H-10 (J = 7.10) with literature[5] and the data obtained with NOESY spectrum allowed to establish the orientation of proton 2, 6, and 10 as β. Thus it has been determined as 2α-acetoxy-6α-*p*-methoxybenzoyl-10α-hydroxy-jaeschkeanadiol. NMR data and assignments are reported in Table 1. This derivative was not previously isolated from natural sources, albeit the epimer derivative 2α-acetoxy-6α-*p*-methoxybenzoyl-10β-hydroxy-jaeschkeanadiol was previously isolated from *F. communis* subsp. *communis*.[5]

Compound **DE-3** was isolated as clear amorphous solid. The HR-MS spectrum showed a pseudomolecular ion $[M+Na]^+$ at *m/z* 469.2165 (calculated 469.2202 for $C_{25}H_{34}NaO_7$), accounting for an isomer of **DE-4**. The ^{1}H NMR spectrum appears similar to that of **DE-4** except for the chemical shift of a oxymethine at δ 3.88 (m) and the broad singlet at δ 5.57. Careful reading of HMQC, HMBC and COSY spectra and comparison with literature[5] allowed

Table 1
NMR data for compounds **DE-3** and **DE-4**

Position	DE-3		DE-4	
	δ_H (mult, J, int)	δ_C	δ_H (mult, J, int)	δ_C
1	—	52.3	—	52.0
2	3.88 (dd, 6.1, 1.1, 1H)	79.0	4.96 (dd 6.4, 1.1, 1H)	84.6
3	1.92-2.23 (m, 2H)	21.1	2.14 (m, 2H)	20.0
4	—	85.2	—	84.8
5	2.74 (d, 10.5, 1H)	53.1	3.20 (d, 10.5, 1H)	47.8
6	5.31 (ddd,10.2, 6.0, 3.3, 1H)	69.9	5.52 (ddd, 10.5, 6.0, 3.7, 1H)	70.4
7	2.53–2.10 (m, 2H)	40.8	3.87–2.10 (m, 2H)	39.2
8	—	132.2	—	136.9
9	5.26 (br s, 1H)	128.2	5.69 (brd, 7.10, 1H)	126.5
10	5.57 (br s, 1H)	76.3	4.10 (d, 7.10, 1H)	71.8
11	1.81 (m, 1H)	37.9	1.77 (m, 1H)	37.2
12	0.87 (d, 6.5, 3H)	17.3	0.79 (d, 6.6, 3H)	17.8
13	1.02 (d, 6.9, 3H)	18.2	0.94 (d, 6.9, 3H)	18.6
14	1.87 (br s, 3H)	26.7	1.82 (br s, 3H)	27.4
15	1.18 (s, 3H)	15.2	1.11 (s, 3H)	20.8
1′	—	122.6	—	122.8
2′–6′	8.00 (d, 7.9, 2H)	131.8	7.97 (d, 8.3, 2H)	131.9
3′–5′	6.97 (d, 7.9, 2H)	114.1	6.93 (d, 8.3, 2H)	114.0
4′	—	165.5	—	164.5
7′	—	168.1	—	167.1
OCH_3	3.91 (s, 3H)	55.8	3.87 (s, 3H)	55.6
CH_3–	2.16 (s, 3H)	21.2	2.10 (s, 3H)	21.8
CH_3C=O	—	171.4	—	170.0

Spectra were recorded in $CDCl_3$ at 300 MHz for ^{1}H and 75 MHz for ^{13}C. Coupling constant are reported in Hz.

to establish the structure of 10-hydroxy-jaeschkeanadiol for the terpene nucleus and to determinate the esterification position as 10-acetyl and 6-p-methoxybenzoyl. The comparison of the coupling constants for H-2 (J = 6.1, 1.1), H-6 (J = 10.2, 6.0, 3.3) and H-10 (br s) and literature data[5] as well as the NOESY correlations observed from methyl group 15 (δ 1.18) with proton signals at δ 3.88 (H-2) and 5.31 (H-6) and the NOESY correlations from proton signal at δ 2.74 (H-5) with proton at δ 5.57 (H-10) allow to establish the relative orientation of the H-2 and H-6 as β while H-5 and H-10 as α.

Thus, the structure **DE-3** was established as 2α-hydroxy-6α-*p*-methoxybenzoyl-10β-acetoxy-jaeschkeanadiol. NMR data and assignments are reported in Table 1. This compound was not previously isolated from natural source; the similar derivative 2α-acetoxy-6α-*p*-methoxybenzoyl-10β-hydroxy-jaeschkeanadiol was isolated from *F. communis* subsp. *communis*[5] and also in this work as **DE-5**.

Structure of compound **DE-12** was established as 8,9-dihydro-8,14-dehydro-9-hydroxyferutinin; obtained by semisinthesys,[34] but never isolated in natural sources.

The comparison of the obtained spectral data with the literature allows to establish the following structures for the other constituents: **DE-1** 14-*p*-methoxybenzoyl-dauc-4,8-diene,[35] **DE-2** 14-*p*-methoxybenzoyl-4,5epoxy-dauc-8-ene,[5] **DE-5** 2α-acetoxy-

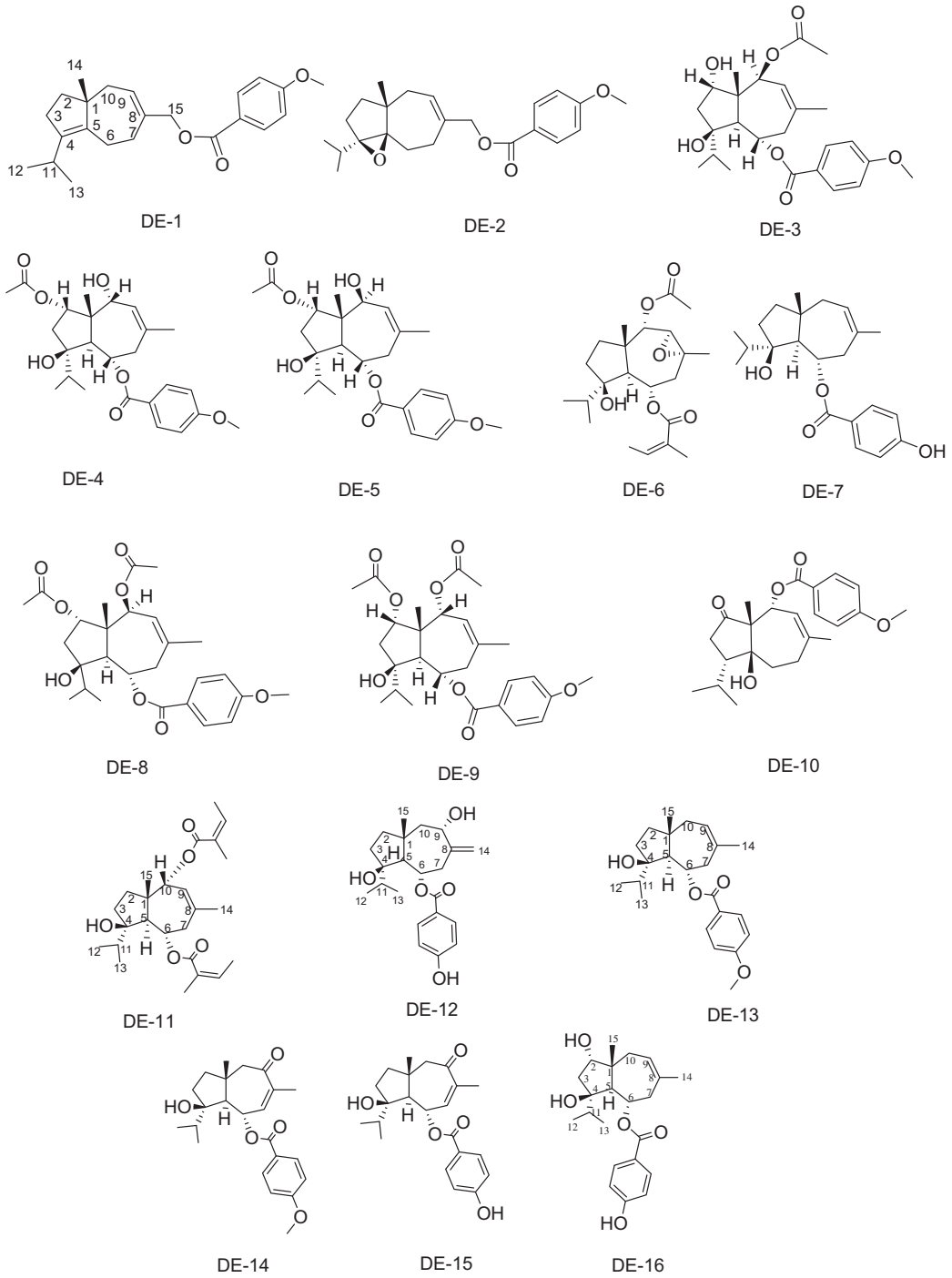

Chart 1. Structures of isolated daucane derivatives.

6α-*p*-methoxybenzoyl-10β-hydroxy-jaeschkeanadiol,[5] **DE-6** lapiferin or 6α-angeloyl-10α-acetyl-8,9-epoxy-jaeschkeanadiol,[36] **DE-7** ferutinin,[21,25] **DE-8** 2α-acetoxy-6α-*p*-methoxybenzoyl-10β-acetoxy-jaeschkeanadiol,[5] **DE-9** 2α-acetoxy-6α-*p*-methoxybenzoyl-10α-acetoxy-jaeschkeanadiol,[5] **DE-10** siol anisate,[5] **DE-11** pallinin or 6α,10α-diangeloyl-jaeschkeanadiol,[37] **DE-12** 8,9-dihydro-8,14-dehydro-9-hydroxyferutinin,[35] **DE-13** ferutidin,[38] **DE-14** lancerodiol *p*-methoxybenzoate,[4] **DE-15** lancerodiol *p*-hydroxybenzoate[4] **DE-16** 2α-hydroxy ferutinin,[39] **CU-1**,[12] **CU-2**,[12] **CU-3**,[40] **CU-4**, **PR-1**,[41] **PR-2**,[42] **PA-1**.[43] Structures of the isolated daucane are reported in Chart 1, while structures of other isolated compounds are reported in Chart 2.

2.2. Antiproliferative activity of isolated compounds

The cytotoxic activity of all the isolated compounds was evaluated against seven different human tumor cell lines, namely HeLa, A549, HL-60, Jurkat, K562, RS 4; 11 and SEM. The four coumarines (**CU-1 to CU-4**), the phenylpropanoid derivatives (**PR-1 to PR-2**) and the polyacetilene derivatives (**PA-1**) were inactive (IC_{50} > 100 μM) in all the cell lines.

Compounds **DE-5**, **DE-8**, **DE-11**, and **DE-16** exhibit cytotoxic effects against all the considered cell lines and the results are reported in Table 2. Compound **DE-8** was the most active against Jurkat (IC_{50} 3.3 μM) while **DE-11** showed the highest activity against all the other tested cell lines.

Some preliminary evaluation of structure–activity relationships can be traced considering the measured IC_{50} values.

Structurally, the daucane derivatives are based on a central bicycle hydrocarbon skeleton, whose geometry depends by the junction stereochemistry influencing the flatness of the molecule, more or less surrounded by a crown of oxygenated functions and a series of acylating moieties, responsible of the differences in polarity of the products.

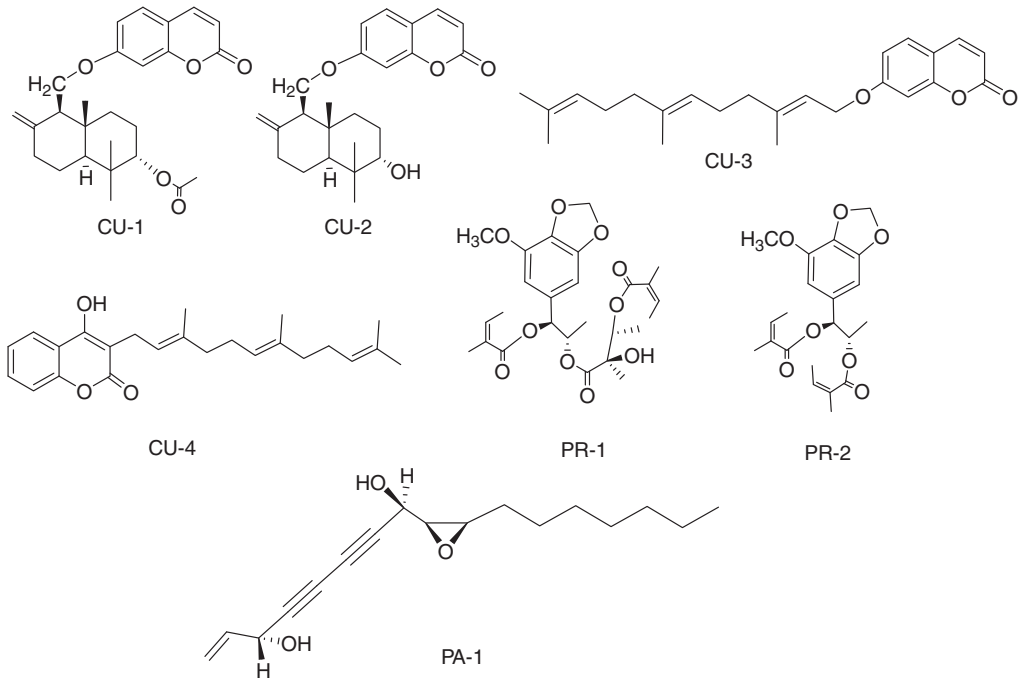

Chart 2. Structures of coumarins, phenylpropanoids, and polyacetilene isolated compounds.

Table 2
Antiproliferative activity of daucane esters in human tumor cells

Compd	IC_{50} [a] (μM)						
	HeLa	A549	HL-60	Jurkat	K562	RS 4;11	SEM
DE-1	>100	>100	30.7 ± 6.2	25.6 ± 7.4	67.0 ± 6.5	44.1 ± 4.0	25.5 ± 1.6
DE-2	>100	>100	>100	>100	>100	60.9 ± 1.8	84.0 ± 9.5
DE-3	52.6 ± 1.8	>100	14.9 ± 5.0	9.1 ± 4.1	70.4 ± 6.2	26.8 ± 4.4	26.5 ± 1.9
DE-4	>100	>100	24.0 ± 4.2	33.6 ± 5.5	85.2 ± 7.9	70.4 ± 4.3	>100
DE-5	52.9 ± 2.6	79.6 ± 0.7	23.0 ± 0.9	5.9 ± 3.4	48.2 ± 7.7	34.4 ± 2.1	18.1 ± 3.0
DE-6	>100	>100	>100	>100	>100	>100	>100
DE-7	>100	>100	>100	>100	>100	>100	>100
DE-8	14.5 ± 3.9	23.3 ± 1.7	11.3 ± 2.4	3.3 ± 0.8	39.6 ± 3.9	2.8 ± 0.2	15.0 ± 1.3
DE-9	>100	>100	83.5 ± 9.8	62.2 ± 8.8	>100	42.9 ± 1.2	32.9 ± 2.5
DE-10	>100	>100	>100	>100	>100	>100	23.9 ± 2.4
DE-11	4.4 ± 0.7	2.8 ± 1.4	2.6 ± 0.4	7.7 ± 1.4	26.5 ± 6.0	1.7 ± 0.3	2.4 ± 0.1
DE-12	>100	>100	43.4 ± 2.8	55.0 ± 12.8	>100	29.0 ± 3.5	34.7 ± 1.8
DE-13	>100	31.9 ± 2.7	20.1 ± 1.8	30.5 ± 7.2	67.8 ± 13.7	17.6 ± 1.4	18.2 ± 2.9
DE-14	29.7 ± 6.8	31.8 ± 4.4	38.9 ± 6.0	40.5 ± 6.5	>100	28.6 ± 3.0	28.4 ± 1.6
DE-15	26.1 ± 3.3	14.5 ± 8.8	82.5 ± 7.5	40.5 ± 12.1	>100	28.2 ± 2.2	24.4 ± 2.0
DE-16	56.5 ± 15.5	83.7 ± 10.2	3.5 ± 6.5	40.3 ± 4.7	95.0 ± 0.7	33.4 ± 1.8	22.4 ± 1.8

Values are the mean ± SEM for four separate experiments.
[a] Compound concentration required to reduce cell growth inhibition by 50%.

Table 3
Cytotoxicity of daucane esters for Human Peripheral Blood Lymphocytes (PBL)

Cell line	IC_{50}[a] (μM)			
	DE-3	**DE-5**	**DE-8**	**DE-11**
$PBL_{resting}$[b]	30.3 ± 3.0	20.5 ± 1.5	6.6 ± 1.4	22.6 ± 2.3
PBL_{PHA}[c]	33.7 ± 1.6	27.2 ± 2.3	9.4 ± 1.0	13.7 ± 1.2

Values are the mean ± SEM for three separate experiments.
[a] Compound concentration required to reduce cell growth inhibition by 50%.
[b] PBL not stimulated with PHA.
[c] PBL stimulated with PHA.

All the most active compounds possess ring fusion with *trans* geometry and the 6(α)-ester linked group (*p*-methoxy benzyl or angeloyl), compounds possessing *cis* geometry (**DE-1** and **DE-10**) or epoxy group in 4 and 5 (**DE-2**) have poor activity. Also the double bond in the hepta-atomic ring appears critical; active compounds present the 8,9 or the 7,8 (**DE-14** and **DE-15**) double bond while the 8,9 epoxy derivative (**DE-6**) is inactive.

Furthermore, significant changes in the cytotoxic effects against all the considered cell lines have been observed for the compounds with different stereochemistry of position 10 (**DE-8** and **DE-9** and **DE-4** and **DE-5**) suggesting that the 10β-hydroxy function or the 10β-acetoxy substituent is important for the cytotoxic activity of these compounds. Notably, for the two isomers **DE-4** and **DE-5** the IC_{50} is lower or at least the same (for HL-60) in all the cell lines for the compound bearing the 10β-hydroxy group (**DE-5**). This is also observed for the two 10-acetyl derivatives **DE-8** and **DE-9** (see Table 2). The 2α-acetyl or 2α-hydroxy group and 10β-hydroxy or acetoxy function as **DE-5** and **DE-8** all present significant cytotoxic activity suggesting that the preferred orientation for hydroxyl or acetoxy functions in such positions are 2α and 10β.

2.3. Cytotoxicity for normal cells

To obtain more insights into the cytotoxic potential of test compounds for normal human cells, the most active compounds were assayed in vitro against peripheral blood lymphocytes (PBL) from

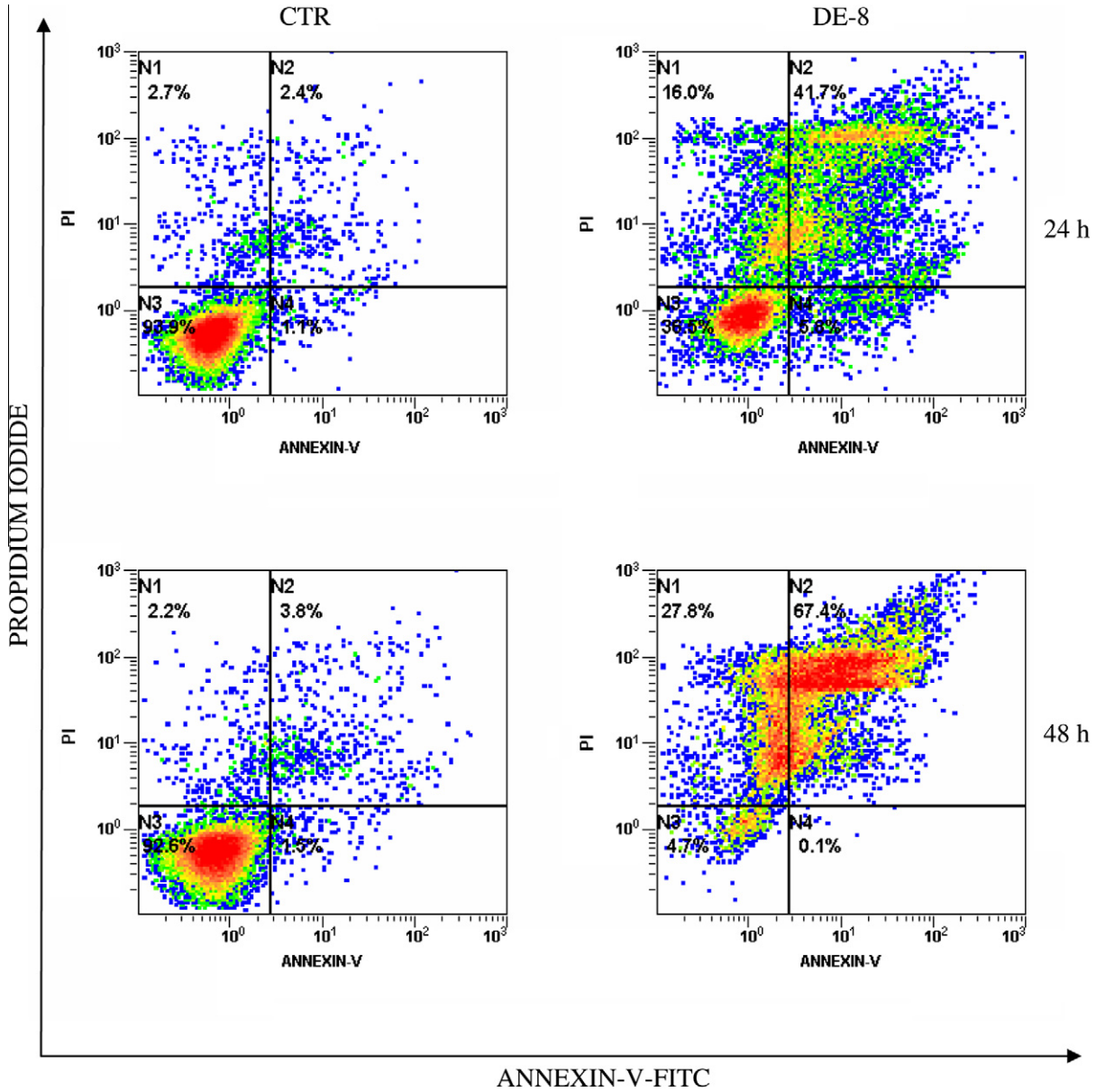

Figure 1. Representative biparametric histograms obtained by flow cytometric analysis. Jurkat cells after 24 and 48 h after incubation in the presence of **DE-8** 50 μM, were collected and stained with Annexin-V-FITC and propidium iodide (PI) and analysed by flow cytometry.

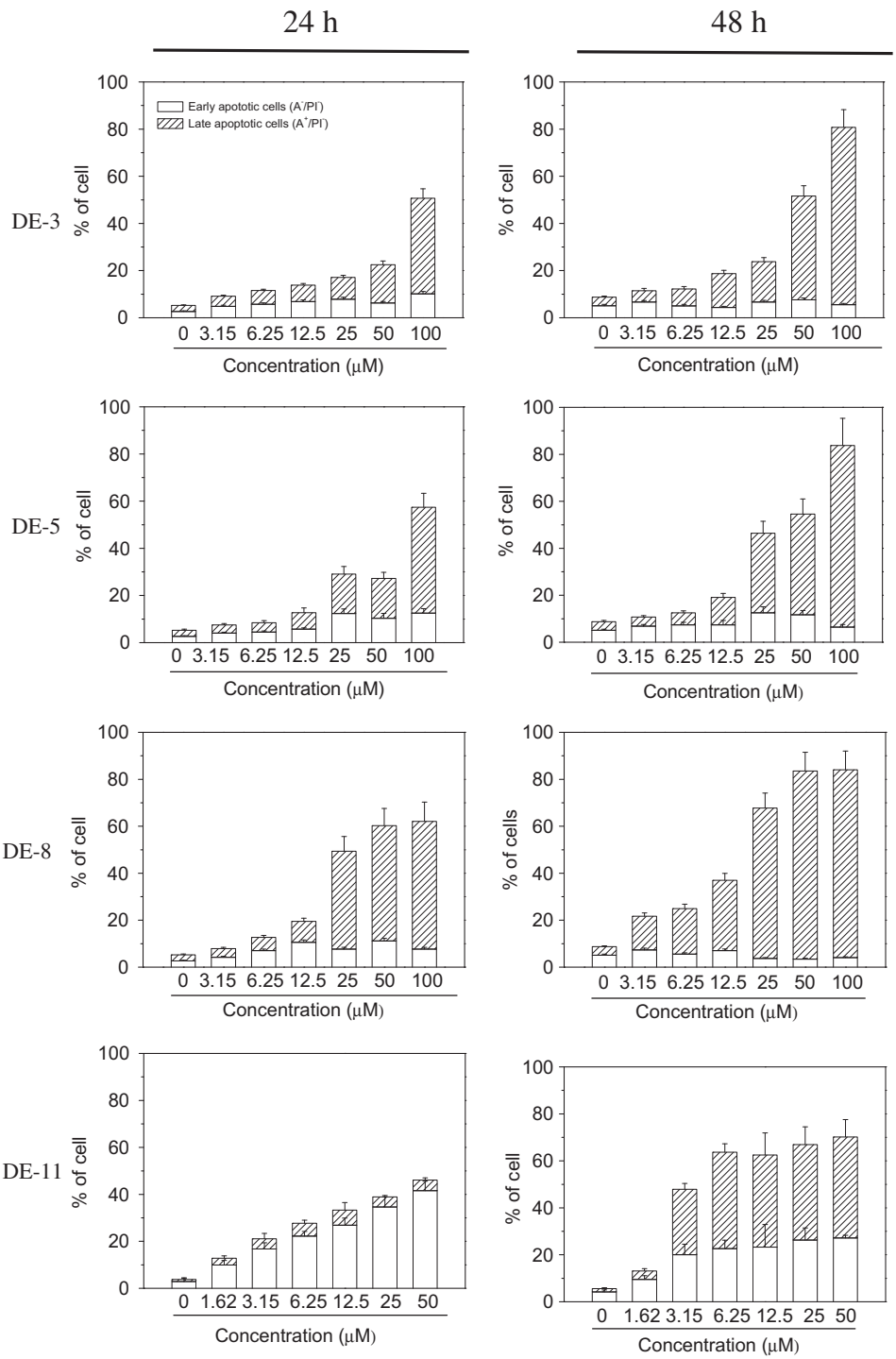

Figure 2. Determination of the mode of cell death using Annexin-V and PI staining and flow cytometric analysis. Jurkat cells were incubated in the presence of DE-3, DE-5, DE-8 and DE-11 at the indicated concentrations and after 24 and 48 h the cells were collected and stained with Annexin-V-FITC and propidium iodide (PI) and analysed by flow cytometry. The results are expressed as percentage of early apoptotic cells (Annexin-V^+/PI^-) and late apoptotic cells (Annexin-V^+/PI^+) as found in the different region of the biparametric histograms showed in Figure 1. Data represent mean ± SEM of three independent experiments.

healthy donors (Table 3). Compounds **DE-3**, **DE-5**, and **DE-8** proved cytotoxic for both PHA-stimulated and resting PBL, but at concentrations higher than those active against the lymphoblastoid cell line Jurkat. Instead, compound **DE-11** is more cytotoxic in PHA-stimulated PBL respect to the unstimulated lymphocytes indicating that this derivative acts preferentially on proliferating cells. Anyway, also in this case it works at concentrations higher respect to Jurkat cells.

2.4. Determination of the mode of cell death

To characterize the mode of cell death, we performed a biparametric cytofluorimetric analysis using propidium iodide (PI) and AnnexinV-FITC which stain DNA and phosphatidylserine (PS) residues, respectively.[44] Because externalization of PS occurs in the earlier stages of apoptosis, Annexin V staining identifies apoptosis at an earlier stage than sub-G_1 appearance which represents a later

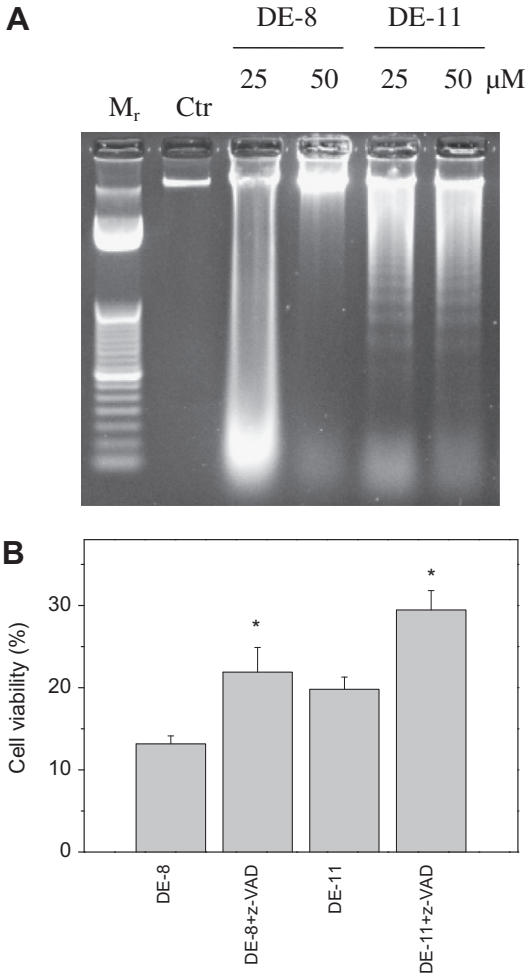

Figure 3. Agarose gel electrophoresis of chromosomal DNA extracted from Jurkat cells after 48 h of incubation in the presence of **DE-8** (50 and 25 μM) and DE-11 (25 and 12.5 μM) Lane 1: Ctr; lane 2: UVA; lane 3: FLV 50 μM; lane 4: FP6 (10 μM) lane M indicated size marker DNAs. Panel B. Percentage of cell viability, determined by MTT test, after 48 h of incubation of Jurkat cells with **DE-8** (50 μM) or DE-11 (25 μM) in the presence or in the absence of z-VAD.fmk (100 μM). Mean ± SEM of three independent experiments. $^{*}p < 0.01$ versus treated cells in the absence of inhibitor.

stage of apoptosis being based on nuclear changes such as DNA fragmentation.

After drug treatment for 24 and 48 h, Jurkat cells were labeled with the two dyes, washed and the resulting red (PI) and green (FITC) fluorescence was monitored by flow cytometry.

Figure 1, shows as representative, four biparametric histograms in which the effect of **DE-8** at 24 and 48 h from the irradiation is depicted. It is quite evident that **DE-8** early induced, an accumulation of both Annexin-V (A^+/PI^-) and Annexin-V/propidium iodide (A^+/PI^+) positive cells in comparison to the non treated cells.

A complete picture of the results is presented in Figure 2 for **DE-3**, **DE-5**, **DE-8**, and **DE-11** evaluated at different concentrations ranging from 1.25 to 100 μM and after 24 and 48 h of incubation. It can be observed an increase of late apoptotic cells (A^+/PI^+) for compounds **DE-3**, **DE-5**, and **DE-8** while for compound **DE-11** we observed the formation of early apoptotic cells (A^+/PI^-) in a concentration-dependent manner at 24 h followed at later times (48 h) by the appearance of A^+/PI^+ cells suggesting that this last derivative may have delayed effect on the induction of apoptosis.

The mode of cell death was also followed by a common endpoint analysis such as analysis of DNA fragmentation.[45] As shown in Figure 3 (panel A), agarose gel electrophoresis of DNA extracted from Jurkat cells treated with the two most active compounds (**DE-8** and **DE-11**) showed a massive DNA fragmentation after 48 h of incubation for **DE-11** while with **DE-8**, we did not observed a clear fragmentation, but only a 'smear' of degraded DNA.

To evaluate if the cell death induced by **DE-8** and **DE-11** is caspase-dependent, Jurkat cells were treated with the two compounds in the absence or presence of the pan-caspase inhibitor z-VAD Inhibition of caspases significantly increased cell viability (Fig. 3, panel B), but this caspase inhibition had only a partial impact on the induced cell death. The caspase inhibitor increased cell survival from about 13–21%, and from 19% to 29% for **DE-8** and **DE-11**, respectively, suggesting that other mechanism(s) may lead to cell death following treatment with daucane esters.

2.5. Analysis of the cell cycle

To investigate the effects of daucane esters on the cell cycle, Jurkat cells were treated with the test compounds at different concentrations, and after 48 h of incubation, the cells were fixed and labeled with propidium iodide. The different phases of the cell cycle were analysed by flow cytometry. Figure 4 (panel A), show the results obtained for compounds **DE-3**, **DE5**, **DE-8**, and **DE-11**. The four compounds showed a different behavior; while **DE-5** and **DE-11** induce, in a concentration-dependent manner, a G2/M arrest along with a decrease of all the other phases of the cell cycle, the other two compounds **DE-3** and **DE-8** induce a slight accumulation of the cells in G1 phase that disappear at higher concentrations (50 μM).

This effect is accompanied by a reduction of the S phase whereas the G2/M remain practically unchanged. It is worthwhile to note the appearance of a remarkable hypodiploid peak (Sub-G1) indicative of apoptosis (Fig. 4, panel B) in well agreement with the above results.

3. Conclusions

This Letter presents the isolation and structural characterization of two new and 14 known daucane esters together with four coumarins, two phenylpropanoids, and one polyacetylene. All the isolated compounds were tested for their antiproliferative activity against a panel of human tumor cell lines and some of the daucane esters present significant activity. On the basis of the cytotoxic effects we could trace some preliminary evaluation of structure–activity relationships for the considered daucane derivatives observing that the *trans* fusion of the penta- and hepta-atomic cycles, and lipophilic ester groups linked to positions 6 are enhancing the cytotoxic activity. Moreover the isomeric derivatives (**DE-8** and **DE-9** or **DE-3**, **DE-4** and **DE-5**) present significant differences in their IC_{50} supporting that the β orientation for the ester group in the position 2 is crucial for the cytotoxic effect. Furthermore we investigated the mode of cell death induced by the two most active compounds showing that apoptosis may be the primary cause of cell death after incubation with **DE-8** and **DE-11**. Interestingly the antiproliferative activity is moderately reduced in the presence of the pancaspase inhibitor z-VAD suggesting that induced apoptosis is partially caspase-dependent. Our results are in good agreement with that reported by Gamal-Eldeen et al.[23] and Macho et al.[24] which found that lapiferin and ferutinin two daucane derivatives induce apoptosis in MCF-7 and Jurkat cell lines respectively. Moreover they also found that apoptosis is mediated by caspase activation.

We observed a cell cycle arrest upon incubation with daucane derivatives along with a decrease of the S phase. More importantly, we detected the appearance of a hypodiploid peak (subG1). This

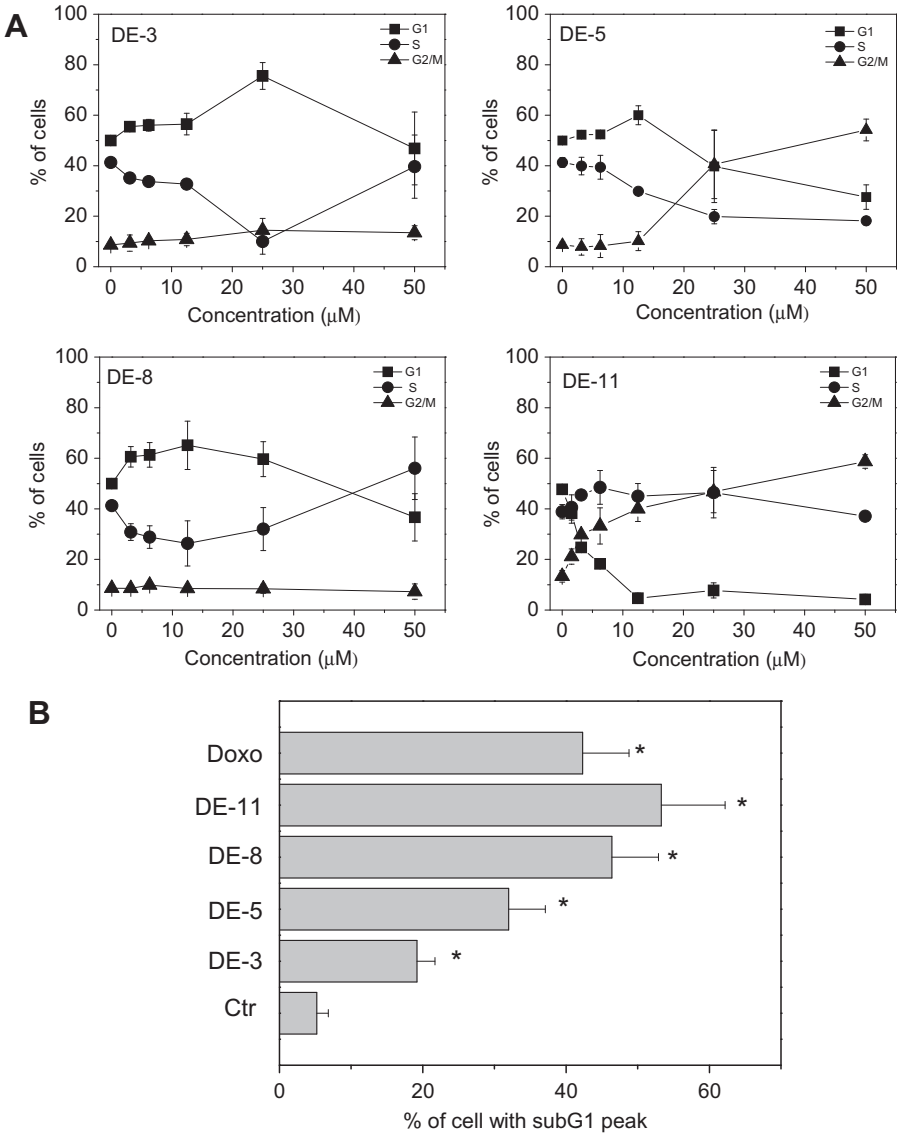

Figure 4. Effects of **DE-3**, **DE-5**, **DE-8**, **and DE-11** on the cell cycle in Jurkat cells (panel A). Cells were treated with different concentrations ranging from 1.25 to 100 μM for 48 h. Then the cells were fixed and stained with PI to analyze DNA content by flow cytometry. Data are presented as mean ± SEM of three independent experiments. Panel B. Percentage of cells presenting a hypodipolid peak (SubG1) after treatment of cells with the indicated compounds at the concentration of 50 μM. Doxorubicin (Doxo) was used as positive control at the concentration of 1 μM. Data are presented as mean ± SEM of three independent experiments. $^{*}p < 0.01$ versus control cells.

peak represents those cells with a DNA content less than G1, usually considered as apoptotic cells, On the contrary, Poli et al.[11] reported that daucane esters induce an antiproliferative activity in three colon cancer cell lines and this effect was preceded by cell cycle arrest in the G1 phase that is not followed by apoptosis.

In conclusion, we have demonstrated the antiproliferative activity of some daucane derivatives isolated from different species of *Ferula* and, although at the present stage is not possible to present a precise mechanism of these compounds, our data suggest that apoptosis may be the major cause of cell death. Further experiments are in progress to evaluate in detail, at molecular level, the mechanism of action of these derivatives.

4. Experimental

4.1. Plant material

Roots of FGL and FGO were collected during the vegetative rest in November 2010 in rocky places sited in Madonna di Val Povera (Camerino, Marche, Central Italy, 900 m above sea level, N 43°06′33″ E 13°00′06″), while FCO was collected in the same period along a speedway in Foligno (Umbria, central Italy, 240 m above sea level, N 42°58′13″ E 12°42′59″). Botanical identification of the plants was confirmed by Dr. Maggi. Voucher specimens were deposited in the Herbarium Camerinensis (included in the online edition of Index Herbariorum: http://sweetgum.nybg.org/ih/) of the School of Environmental Sciences, University of Camerino, Italy, under Accession Nos. CAME 25671 (FGL), CAME 13399 (FGO), and CAME 25672(FCO); they are also available at the following website: http://erbariitaliani.unipg.it.

4.2. Extraction and isolation of phytoconstituents

Silica gel plates (cod 5171 Merck) and silica gel (60 mesh) were from Sigma (Milan, Italy). Solvents from Carlo Erba (Milan). Varian Intelliflash flash chromatograph was used for preparative chromatography. NMR (1D and 2D) spectra were obtained on a Bruker AMX 300 spectrometer; optical rotation power was recorded on

a Yasco 2000 digital polarimeter. ESI-MS measurements were obtained on a Varian 500 MS ion trap spectrometer. HR-MS were measured on an API-TOF spectrometer (Mariner Biosystems). Samples were diluted in a mixture of 1:1 H_2O–AcCN with and directly injected at a flow rate of 10 μL/min.

HPLC-DAD-ELSD was obtained on a Agilent 1100 chromatographic system with Diode Array (1100 series) and a Sedex 60 LT evaporative light scattering detector. Agilent C-8 Zorbax 4.6 × 150 (5 μm) was used as stationary phase. HPLC conditions for FCO and FGL extracts were as follows: acetonitrile (A) and water 0.1% formic acid (B) as eluents; gradient elution started from 50% (A), then in 35 min 75% (A); the flow rate was 1 mL/min and the injection volume was 20 μL. For FGO extracts the HPLC conditions were the following: gradient elution started from 10% (A), then in 2 min 30% (A), and in 25 min 75% (A); the flow rate was 1 mL/min and the injection volume was 20 μL.

Roots of FCO (450 g fresh material) were cleaned, chopped and extracted with methanol (200 mL) at room temperature for 10 min in an Ultrasound bath (ME-extract). Extraction was repeated twice. Solvent was removed under vacuum yielding a semi solid brown residue (12% yield). A part of the solid residue (15 g) was dissolved in a mixture methanol/water (400 mL) and extracted with dichloromethane (DM-extract). The organic layer was anhydrified with sodium sulfate and solvent was removed under vacuum yielding a residue (FCDM-extract, 2.9 g).

FCDM extract (2.9 g) was applied to a silica gel column (500 mL) and eluted with, dichloromethane 1% of methanol (750 mL), dichloromethane 3% of methanol (800 mL), dichloromethane 5% of methanol (240 mL), and then dichloromethane 20% of methanol (400 mL). Seventy five fractions were collected and pooled on the basis of their chromatographic behavior in seven different fractions: A (320 mg), B (220 mg), C (190 mg), D (200 mg), E (250 mg), F (250 mg) and G (300 mg). The purification of fraction A and B by PTLC using cyclohexane ethyl acetate 6:1 yielded compounds **DE-7** (116.7 mg), **DE-1** (75.9 mg); from fraction C by PTLC using cyclohexane ethyl acetate 2:1 the compound **DE-8** (60.7 mg) was isolated. The purification of fraction D and E by PTLC using cyclohexane ethyl acetate 6:1 and then cyclohexane ethyl acetate 2:1 yielded compounds **DE-9** (13.2 mg), **DE-3** (20.1 mg) and **DE-2** (6.7 mg). From fraction F, using PTLC with dichloromethane/methanol 97:3, the compounds **DE-4** (30.1 mg), **DE-5** (21.4 mg), **DE-12** (8.2 mg) were obtained.

Roots of FGL (300 g fresh material) were chopped with methanol (200 mL) and extracted at room temperature for 10 min in an Ultrasound bath (ME-extract). Extraction was repeated twice. Solvent was removed under vacuum yielding a semi solid brown residue (8% yield). A part of the solid residue (9 g) was dissolved in a mixture of methanol/water (250 mL) and extracted with dichloromethane (DM-extract). The organic layer was anhydrified with sodium sulfate and solvent was removed under vacuum yielding a residue (FCDM-extract, 1.2 g). FGDM extract was applied to a silica gel column (200 mL) and eluted with dichloromethane (200 mL), dichloromethane 1% of methanol (300 mL), dichloromethane 5% of methanol (300 mL), and then dichloromethane 10% of methanol (300 mL). Fifty fractions were collected and pooled on the basis of their chromatographic behavior in four different fractions A (380 mg), B (430 mg), C (450 mg) and D (150 mg). Further purifications were obtained with semipreparative HPLC on a Licrosphere 100 C-18 (9.6 × 300 mm) using as mobile phase methanol water (60:40) at a flow of 2.5 mL/min. Compounds were obtained from fraction A **DE-11** (8.0 mg), **DE-16** (10.4 mg), from fraction B **DE-6** (15.5 mg), **DE-14** (4.7 mg), respectively.

Cleaned roots of FGO (350 g fresh material) were chopped with methanol (200 mL) and extracted at room temperature for 10 min in an Ultrasound bath (ME-extract). Extraction was repeated twice. Solvent was removed under vacuum yielding a semi solid brown residue (9% yield). A part of the solid residue (12 g) was dissolved in a mixture methanol/water (400 mL) and extracted with dichloromethane (DM-extract). The organic layer was anhydrified with sodium sulfate and solvent was removed under vacuum yielding a residue (FRDM-extract, 1.6 g). FRDM extract (1.5 g) was applied to a silica gel column (350 mL) and eluted with dichloromethane (350 mL), dichloromethane 1% of methanol (430 mL), dichloromethane 5% of methanol (250 mL), dichloromethane 10% of methanol (640 mL), and then dichloromethane 15% of methanol (300 mL). Eighty fractions were collected and pooled on the basis of their chromatographic behavior in five different fractions A (245 mg), B (330 mg) C (450 mg) and D (500 mg) E (250 mg).

The purification of fraction A and B by repeated PTLC using cyclohexane ethyl acetate 2:1, and chloroform/methanol 95:5 yielded compounds **CU-1** (40.0 mg), **DE-7** (21.0 mg). Further purification of fraction C by PTLC using cyclohexane ethyl acetate 2:1 afforded **PR-2** (10.4 mg), **CU-2** (29.8 mg), **DE-6** (14.4 mg), **DE-10** (6.1 mg). From the fraction E further purifications were obtained by PTLC using chloroform–methanol (95:5) as eluents, and finally by HPLC using as methanol/water 60:40 yielding **PR-1** (20.5 mg), **CU-4** (21.7 mg) and **PA-1** (13.0 mg). Purity of isolated compounds was checked by HPLC analysis and was >97% by software integration.

4.3. Antiproliferative assays

Human T-leukemia (Jurkat), human promyelocytic leukemia (HL-60), human chronic myelogenous leukemia (K562), acute B-lymphoblastic leukemia SEM and RS 4;11 cells, the latter with a t(4;11) translocation cells were grown in RPMI-1640 medium, (Gibco Milano Italy). Human non-small lung carcinoma (A549) and human cervix carcinoma (HeLa) cells were grown in DMEM medium (Gibco, Milano, Italy), all supplemented with 115 units/mL of penicillin G (Gibco, Milano, Italy), 115 μg/mL streptomycin (Invitrogen, Milano, Italy) and 10% fetal bovine serum (Invitrogen, Milano, Italy). Individual wells of a 96-well tissue culture microtiter plate were inoculated with 100 μL of complete medium containing 8×10^3 cells. The plates were incubated at 37 °C in a humidified 5% CO_2 incubator for 18 h prior to the experiments. After medium removal, 100 μL of the drug solution, dissolved in complete medium at different concentrations, was added to each well and incubated at 37 °C for 72 h. Cell viability was assayed by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test as previously described.[46] The IC_{50} was defined as the compound concentration required to inhibit cell proliferation by 50%.

Analogous experiments were performed in the presence of the pancaspase inhibitor z-VAD.fmk (Sigma–Aldrich Milano, Italy).

Peripheral blood lymphocytes (PBL) from healthy donors were obtained by separation on Lymphoprep (Fresenius KABI Norge AS) gradient. After extensive washings, cells were resuspended (1.0×10^6 cells/mL) in RPMI-1640 with 10% FCS and incubated overnight. For cytotoxicity evaluations in proliferating PBL cultures, non-adherent cells were resuspended at 5×10^5 cells/mL in growth medium, containing 2.5 μg/mL phytohematoagglutinin (PHA, Irvine Scientific). Different concentrations of the test compounds were added and viability was determined 72 h later by MTT test. For cytotoxicity evaluations in resting PBL cultures, non-adherent cells were resuspended (5×10^5 cells/mL) and treated for 72 h with the test compounds, as described above.

4.4. Annexin-V assay

Surface exposure of phosphatidylserine on apoptotic cells was measured by flow cytometry with a Coulter Cytomics FC500 (Beckman Coulter) by adding Annexin-V-FITC to cells according to the

manufacturer's instructions (Annexin-V Fluos, Roche Diagnostic). Simultaneously the cells were stained with PI. Excitation was set at 488 nm, and the emission filters were set at 525 and 585 nm, respectively.

4.5. Detection of DNA fragmentation by agarose gel

Total genomic DNA was extracted from treated Jurkat cells by a commonly used salting out protocol. One microgram of DNA obtained was subsequently loaded on a 1.5% agarose gel at 50 V for 6 h in TAE buffer. After staining in ethidium bromide solution, the gel was washed with water and the DNA bands were detected under UV radiation with a ImageQuant 300 transilluminator (GE Healthcare) equipped with a CCD camera.

4.6. Flow cytometric analysis of cell cycle distribution

For flow cytometric analysis of DNA content, 2.5×10^5 Jurkat cells in exponential growth were treated with different concentrations of the test compounds for 24 and 48 h. After an incubation period, the cells were collected, centrifuged and fixed with ice-cold ethanol (70%). The cells were then treated with lysis buffer containing RNAse A and 0.1% Triton X-100, and then stained with PI. Samples were analyzed on a Cytomic FC500 flow cytometer (Beckman Coulter). DNA histograms were analyzed using MultiCycle for Windows (Phoenix Flow Systems).

4.7. Statistical analysis

Unless indicated differently, the results are presented as mean ± SEM. The differences between control and treated were analysed, using the two-sided Student's *t* test.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.08.021.

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