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Active caspase-3 detection to evaluate apoptosis induced by *Verbena officinalis* essential oil and citral in chronic lymphocytic leukaemia cells

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Abstract: *Verbena officinalis* L., Verbenaceae, commonly known as vervain, is a plant widely used in medicine. Despite of its widespread use in different traditional practices, the mechanisms of pharmacological actions of the plant and its volatile oil are still unclear. We evaluated the pro-apoptotic activity of *V. officinalis* essential oil and of its main component, citral, on lymphocytes collected from ten patients with chronic lymphocytic leukaemia (CLL), a disease in which a faulty apoptotic mechanism is still retained one of the primary pathogenic events, by adding to treated mononuclear cells, annexin-V, propidium iodide, and CD19. Apoptosis was also evaluated using anti-active-caspase-3 monoclonal antibody after permeabilization of the cells. Both *V. officinalis* essential oil and citral were found able to induce apoptosis in CLL cells and to activate caspase-3, which is considered the way by means they active apoptosis in B neoplastic cells. This data further support evidences that indicate natural compounds as possible lead structure to develop new therapeutic agents for CLL.

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Introduction

Numerous bioactive substances seem to act as cancer-preventing agents by inhibiting the activation of pro-carcinogens, enhancing the detoxification of carcinogens or impeding the progression of carcinogenesis (Hursting et al., 1999; Wattenberg, 1992). Chronic lymphocytic leukaemia (CLL) is the commonest form of leukaemia in Western World and is considered a disease of B-cell in which a faulty apoptotic mechanism is still retained one of the primary pathogenic events (Chiorazzi et al., 2005; Reed & Kitada, 2001). We have recently showed that *Verbena officinalis* L., Verbenaceae, essential oil and its component citral are able to induce *in vitro* apoptosis of CLL cells thus suggesting its hypothetical therapeutic role in this disease (De Martino et al., 2009). However, the molecular mechanism, which underlies this process, is still unclear. Results of some studies are consistent with the hypothesis that some essential oil components in cancer cellular lines act on cell cycle and apoptosis (Carnesecchi et al., 2001; Gu et al., 2010). Dudai and coworkers (2005) suggested that citral displays its proapoptotic activity through a direct procaspase 3 activation in human and mouse leukaemic cell lines.

In this study, we report our data of a flow cytometric study aiming to evaluate the ability of *V. officinalis* essential oil and its component citral to activate caspase-3 of CLL cells *in vitro*.

Materials and Methods

Plant material

Verbena officinalis L., Verbenaceae, aerial parts were collected in July 2008 from plants growing at the Garden of Medicinal and Aromatic Plants on the Campus of Salerno University. The plant was identified by Prof. Vincenzo De Feo. A voucher specimen of the plant is deposited in Herbarium of the Medical Botany Chair, at the University of Salerno, labeled as DF/154/2008.

Oil isolation and analysis

One hundred grams of fresh aerial parts were submitted to hydrodistillation, in agreement with procedures of the *European Pharmacopoeia* (2004). A pale yellow essential oil was recovered in a 0.39% yield. The chemical composition of the oil was obtained by GC and GC-MS. Analytical gas chromatography was carried out on a Perkin-Elmer Sigma 115 gas chromatograph fitted with an HP-5 MS capillary column (30 m x 0.25 mm i.d.; 0.25 µm film thickness). Column temperature was initially kept at 40 °C for 5 min, then gradually increased to 250 °C at 2 °C/min, held for 15 min and finally raised to 270 °C at 10 °C/min. Diluted samples (1/100 v/v, in *n*-hexane) of 1 µL were injected manually at 250 °C, and in splitless mode. Flame ionization detector (FID) was kept at 280 °C. Analysis was also run by using a fused silica HP Innowax polyethylenglycol capillary column (50 m x 0.20 mm, i.d.; 0.25 µm film thickness). In both cases, carrier gas was He, with flow rate of 1 mL/min.

GC-MS analyses were performed on an Agilent 6850 Ser. II apparatus, fitted with a fused silica HP-1 capillary column (30 m x 0.25 mm; 0.33 µm film thickness), coupled to an Agilent Mass Selective Detector MSD 5973; ionization voltage 70 eV; electron multiplier energy 2000 V. Gas chromatographic conditions were as reported above; transfer line temperature, 295 °C. Most constituents were identified through gas chromatography by comparing their retention indices to either those from the literature (Davies, 1990) or with those of authentic compounds available in our laboratories. The retention indices were determined in relation to a homologous series of *n*-alkanes (C_8-C_{24}) under the same operating conditions. Further identification was made by comparing the mass spectra either to those stored in NIST 02 and Wiley 275 libraries or to the mass spectra from literature (Adams, 2007) and our home-made library. Component relative concentrations were calculated based on GC peak areas without using correction factors. Citral was purchased by Sigma-Aldrich Co, Milan, Italy.

Biological assays

Ten patients with untreated CLL were included in this study. Clinical and biological features of these patients at study entry are given in Table 1. A written informed consent was obtained before sample collection from all subjects. Mononuclear cells were isolated from heparinized peripheral blood by density gradient centrifugation and washed twice with phosphate buffered saline (PBS). Briefly, cells were incubated for up to 24 h at a density of 2.5 x 10^6/µL in RPMI 1640 at 37 °C CO_2, with a mixture of 90 µL of PBS and 0.1 µL of vervain essential oil (A) and 9.9 µL of distilled water (to obtain vervain essential oil diluted 1:100) or 10 µL of pure citral (B) at the concentration of 1.9 mM and with only 100 µL of PBS to also assess spontaneous apoptosis and to use as internal control.

The proapoptotic effect of the compound was evaluated after three different times of incubation (4, 8, 24 h) at room temperature in dark conditions, by adding to treated mononuclear cells, after having been washed twice in PBS, annexin V 5 µL and propidium iodide 5 µL [Annexin V-FITC Apoptosis Detection Kit I (Becton Dickinson Biosciences [BDB] Pharmingen) and CD19-APC-Cy7 (BDB). In addition, anti-active caspase-3-FITC (BDB Pharmingen) and CD19-APC-Cy7 (BDB) were added to 1 x 10^6 treated mononuclear cells, after fixation with 100 µL of Reagent A (Permeabilization Medium, Fix & Perm, Caltag, USA) for 15 min at room temperature in the dark, washing in PBS, permeabilization with 100 µL of Reagent A (Permeabilization Medium), and washing in PBS. Cells were then incubated in the dark for 15 min, finally resuspended in PBS and analyzed by flow cytometry, by acquiring a minimum of 20,000 events for each sample and using an analysis gate on CD19-positive cells to avoid non neoplastic T-cell contamination. As internal control, untreated mononuclear cells were also stained in the similar fashion. A FACSCanto II cytometer equipment (Becton Dickinson) was used. All subsequent analyses were performed using FACS-Diva Software (BDB).

Table 1. Clinical-biological characteristics of CLL patients at study entry.

Characteristics	Patients (no.)
Sex	
Male	6
Female	4
Age (years)	
Mean	64.5 years
Range	46 - 87 years
Rai clinical stage	
0-II	6
III-IV	4
B-cell lymphocytosis (/µL)	
Mean	32,000
Range	9,400-65,000
CD38 expression (cut-off 20%)	
Positive	2
Negative	8
ZAP-70 expression (cut-off 20%)	
Positive	3
Negative	7
IgVH mutational status	
Mutated	7
Unmutated	3
Cytogenetic abnormalities (by FISH)	4
Normal	3
del13q14	3
+12	

CD38: Cluster Differentiation number 38; ZAP-70: Zeta chain associated protein kinase; IgVH: Immunoglobulin heavy chain variable region mutational status; FISH: Fluorescence in situ hybridization.

Statistical analysis

Results were expressed as the mean of three replicates±SD. The values of apoptotic cells were compared by using Student t test as appropriate.

Results and Discussion

Table 2 reports the percentage composition of the essential oil of *Verbena officinalis* L, Verbenaceae. Forty components were identified, accounting for

Table 2. Percentage composition of *Verbena officinalis* essential oil.

Compound	Ki[a]	Ki[b]	%[c]	Identificationd
α-Pinene	938	1032	0.2±0.0	LRI, MS, Co-GC
Sabinene	973	1132	0.5±0.0	LRI, MS, Co-GC
Hepten-3-one	975		0.2±0.1	LRI, MS
β-Pinene	978	1118	T	LRI, MS, Co-GC
α-Terpinene	1012	1188	T	LRI, MS, Co-GC
o-Cymene	1020	1187	0.1±0.0	LRI, MS, Co-GC
β-Phellandrene	1029	1218	0.7±0.2	LRI, MS, Co-GC
Limonene	1030	1203	2.3±0.9	LRI, MS, Co-GC
1,8-Cineole	1034	1213	0.4±0.1	LRI, MS
(*Z*)-β-Ocimene	1038	1246	T	LRI, MS, Co-GC
(*E*)-β-Ocimene	1049	1280	0.3±0.1	LRI, MS, Co-GC
γ-Terpinene	1057	1255	0.1±0.0	LRI, MS, Co-GC
Terpinolene	1086	1265	T	LRI, MS
Linalool	1097	1553	0.1±0.0	LRI, MS, Co-GC
trans-Pinocarveol	1138	1654	T	LRI, MS
iso-Pinocamphone	1153	1566	0.2±0.0	LRI, MS
trans-Pinocamphone	1159		T	LRI, MS
Pinocarvone	1165	1587	T	LRI, MS
Borneol	1167	1719	0.1±0.0	LRI, MS, Co-GC
Terpinen-4-ol	1176	1611	0.2±0.0	LRI, MS, Co-GC
p-Cymen-8-ol	1185	1864	T	LRI, MS
α-Terpineol	1189	1706	0.3±0.1	LRI, MS, Co-GC
Isobornyl formate	1228		44.4±0.9	LRI, MS
cis-Anethole	1262		0.2±0.0	LRI, MS
(*E*)-Citral	1270		45.5±0.9	LRI, MS, Co-GC
Isobornyl acetate	1277		T	LRI, MS
Bornyl acetate	1284	1591	T	LRI, MS
α-Copaene	1377	1497	0.2±0.1	LRI, MS
Isoledene	1382		0.1±0.0	LRI, MS
β-Elemene	1387	1600	0.2±0.1	LRI, MS
Longifolene	1411	1576	T	LRI, MS
β-Caryophyllene	1418	1612	0.1±0.1	LRI, MS
β-Cedrene	1424	1638	0.4±0.1	LRI, MS
α-Humulene	1455	1689	0.2±0.0	LRI, MS
allo-Aromadendrene	1463	1661	0.1±0.0	LRI, MS
γ-Gurjunene	1473	1687	T	LRI, MS
Bicyclogermacrene	1491	1756	0.1±0.0	LRI, MS
cis-Muurola-4(14),5-diene	1510	1675	0.2±0.1	LRI, MS
α-7-*epi*-Selinene	1518	1740	0.2±0.1	LRI, MS
Total			97.6	

Ki[a]: Kovats retention index on HP-5 MS column; Ki[b]: Kovats retention index on HP Innowax; [c]: T trace <0.05; [d]: LRI linear retention index; MS: identification based on comparison of mass spectra; Co-GC: retention time identical to authentic compounds.

97.6% of the total oil. The oil is mainly constituted by monoterpenes (95.4%), of which oxygenated compounds constitute 91.2%; citral is the main constituent (45.5%). The composition of the essential oil differs from others reported in literature. In fact, Ardakani and co-workers (2003) reported 1-octen-3-ol and verbenone as the main constituents of the essential oil from *V. officinalis* collected in Iran. On the other hand, spathulenol, limonene and 1,8-cineole have been reported as principal constituents of an essential oil from Morocco (Chalchat & Garry, 1996).

In all patients with CLL, the number of B-cells was found >85% of all lymphocytes. Table 3 shows the pro-apoptotic activities of *V. officinalis* essential oil and citral, compared to untreated cells, used as internal control to evaluate spontaneous apoptosis, on B-cells collected from CLL patients. The analysis of these ten patients again confirmed our previous observation that *V. officinalis* essential oil and citral are able to induce apoptosis of neoplastic B-cells in CLL at all different times of incubation. As showed, the percentage values of apoptosis increase in 8 h and reduce after 24 h. This is probably due to the fact that the percentage of necroctic cells steadily increased with time peaking at 24 h in all samples. In addition, no difference was found between the activities of both compounds (p ns).

Preliminary literature evidence indicates that isoprenoids, a broad class of mevalonate-derived phytochemicals which are ubiquitous in the plant kingdom, may suppress, with great potency, the proliferation of tumor cells: also in a previous study De Martino and co-workers (2009) demonstrated that both vervain essential oil and citral induced a significant apoptosis in CLL samples compared to controls; Gu and co-workers (2010) demonstrated that linalool, a natural small molecule monoterpene, inhibits growth of leukaemia cells with wt p53 while sparing normal hematopoietic cells.

Apoptosis, or programmed cell death, is a highly ordered, genetically controlled process that plays a fundamental role in both normal biological processes and disease status (Kerr et al., 1972). Apoptosis generally occurs as a result of cell insult or activation of death receptors, both of which lead to a cascade of cell signalling and caspase-mediated events culminating in cell death (Cohen, 1997; Danial & Korsemeyer, 2004; Riedl & Shi, 2004). The death receptors include tumor necrosis factor receptor (TNFR), Fas, decoy receptors and death receptors. After ligand binding, these death receptors interact with a variety of death domain adaptor proteins which subsequently activate the caspases and various signalling pathways. The caspase family is involved in a series of cleavage events that results in the initiation and execution of apoptosis. In addition to the death receptors and caspases, members of the Bcl-2 protein family are also critical for the regulation of apoptosis, largely by controlling the permeability of the outer mitochondrial membrane to proteins such as cytochrome c. Bcl-2 and Cbl-xL are the most prominent anti-apoptotic members of this family, and their functions can be regulated through interactions with pro-apoptotic Bcl-2 family members, such as Bad and Bax.

Table 3. *Verbena officinalis* essential oil (A) and citral (B) induced-apoptosis in lymphocytes collected from CLL patients and comparison with spontaneous apoptosis. Data are expressed as mean percentage number of CD19-positive cells (± standard deviation) and range (in brackets).

Hours	A	Spontaneous apoptosis	*p*
4	56.2±10.7 (46-80)	5.4±1.6 (0.9-7.0)	<0.0001
8	68.2±8.9 (58-83)	6.7±2.3 (1.3-10)	<0.0001
24	32.1±6.5 (24-52)	6.9±1.3 (2.6-10)	<0.0001
	B		
4	57.3±9.2 (48-72)	4.3±1.1 (1.0-5.9)	<0.0001
8	65.9±9.6 (50-80)	7.0±1.3 (1.5-9.9)	<0.0001
24	32.2±7.9 (23-47)	7.2±2.0 (2.3-9.8)	<0.0001

Table 4. *Verbena officinalis* essential oil (A) and citral (B) induced-caspase-3 activation in lymphocytes collected from CLL patients and comparison with spontaneous apoptosis. Data are expressed as mean percentage number of CD19-positive cells (± standard deviation) and range (in brackets).

Hours	A	Spontaneous apoptosis	*p*
4	54.3±11.1 (43-78)	2.3±1.1 (1.2-4.5)	<0.0001
8	63.7±8.6 (53-81)	3.9±1.5 (1.9-5.9)	<0.0001
24	32.1±6.5 (22-42)	6.9±1.9 (2.4-8.9)	<0.0001
	B		
4	61±9.8 (47-79)	2.6±1.2 (1.3-5.1)	<0.0001
8	64.5±7.9 (54-79)	4.1±1.6 (2.0-5.2)	<0.0001
24	33.3±9.2 (22-49)	7.8±3.2 (2.0-6.9)	<0.0001

Caspase-3 is an active cell-death protease involved in the execution phase of apoptosis, where cells undergo morphological changes such as DNA fragmentation, chromatin condensation and apoptotic body formation. Caspase-3 is activated in response to serum withdrawal, activation of Fas, treatment with radiation, pharmacological agents, as well as other upstream caspases like caspase-8 and caspase-9.

In this study we showed that active caspase-3 was detected in all samples *in vitro* treated with both *V. officinalis* essential oil and citral, without statistical differences between the two compounds (Table 4). Also in this case, it is probable that the percentage of necrotic cells increased with time picking at 24 h. In Figure 1 a representative experiment is showed. So, this work is a confirmation and a sequel of the previous study (De

Martino et al. 2009): here, we demonstrated that both vervain essential oil and citral induced a significant apoptosis in CLL samples compared to controls and these substances induced a death pathway activating caspase 3, which is considered the way by means they active apoptosis in B neoplastic cells.

This data further support evidences that indicate natural compounds as possible lead structure to develop new therapeutic agents for CLL.

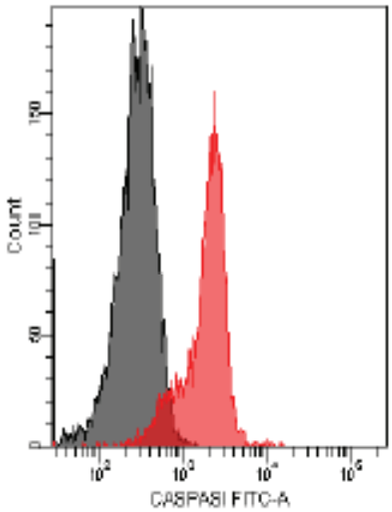

Figure 1. Flow cytometric analysis of activated caspase-3 by *Verbena officinalis* essential oil in a CLL patient sample at 8 h. Untreated cells (grey histogram) and treated cells (red histogram) are shown.

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