Antiproliferative activity of essential oils derived from plants belonging to the Magnoliophyta division

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Abstract. The essential oils obtained from different officinal plants of Lebanon, belonging to the Magnoliophyta division, have been tested for their antiproliferative activity on human erythroleukemic K562 cells. *Satureja montana* showed the most interesting biological activity in inhibiting the cell growth and inducing erythroid differentiation of K562 cells. The essential oil of *Satureja montana* was therefore analyzed using a GC/MS (gas chromatography/mass spectrometry) system in order to identify the major constituents and compare them with analysis performed on *Satureja hortensis*. We demonstrated that the essential oil composition varied with the species, the major constituent of *Satureja hortensis* being carvacrol (50.61%) and that of *Satureja montana* being α-terpineol (12.66%). In order to identify molecules possibly responsible for the biological activity, commercially available derivatives have been assayed on the K562 cell line. *Satureja montana* essential oil displayed different natural derivatives characterized by higher activity than those present in *Satureja hortensis*. The common active principles are α-pinene, γ-terpinene, 4-terpineol, α-terpineol, τ-cadinene, τ-cadinol and caryophyllene. Both caryophyllene and α-terpineol showed important antiproliferative effects on K562 cells.

Introduction

The interest in medicinal plants and their biologically active derivatives has increased in recent years, in relation to the possible development of novel potential drugs for several pathologies of relevant social impact (1,2). In fact, it is well known that medicinal plants are described and used in prenatal care, in obstetrics, in gynaecology, in respiratory disorders, in skin disorders, in cardiac diseases, in nervous and muscular disorders and in mental health (3-7).

With respect to potential anti-tumor activity, possible applications of medicines for cancer prevention have been recently described (8-16). *Aegle marmelos* and *Emblica officinalis*, both medicinal plants derived from Bangladesh and studied by our research group, demonstrated remarkable antiproliferative activity on different human tumor cell lines (17-20).

In order to further identify plants exhibiting antiproliferative activity, we analyzed a variety of officinal plants from Lebanon and belonging to the Magnoliophyta division, including *Satureja hortensis, Satureja montana, Salvia officinalis, Lavandula officinalis, Thymus vulgaris, Calamintha origanifolia, Foeniculum vulgare* and *Mentha arvensis*. From the Mediterranean area, several medicinal plants from Lebabon have been described (21-23). For instance, Barbour *et al* (23) tested the *in vitro* antimicrobial efficacy of extracts derived from different parts of 27 indigenous wild plant species that have been commonly used in Lebanese folk medicine. However, despite these interesting findings, few biological data are available for most medicinal plants from Lebanon.

All obtained essentials oils and related available pure compounds were analyzed for their antiproliferative activity on human K562 cells. Also, their ability to induce erythroid differentiation was determined.

Materials and methods

Essential oils from Satureja hortensis and Satureja montana. The fresh leaves of *S. montana* and *S. hortensis* were collected on Sannine Mountain in Lebanon, in July 2003 when fully flowering, at a 1800-m altitude. The collected species were authenticated according to conventional methods. Then, 200 g of fresh leaves and flowers of each species were submitted to hydro-distillation for 2 h with 2 l of solvent. The essential oils were finally dried over anhydrous sodium sulfate to remove traces of moisture and give a yield of 0.85%. The obtained essential oils were stored at 4˚C.

Essential oils from Salvia officinalis, Lavandula officinalis, Thymus vulgaris, Calamintha origanifolia, Foeniculum vulgare and Mentha arvensis. The fresh leaves (200-300 g) of *Salvia officinalis, Lavandula officinalis, Thymus vulgaris, Calamintha*

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origanifolia and *Mentha arvensis* and the leaves and seeds (200 g) of *Foeniculum vulgare* were collected in Lebanon in March 2003. All of the Lebanese plants were harvested in a flowering vegetative state, except *Salvia officinalis*, which was collected before the flowering season in the Ain-saadeh region (600 m). *Thymus vulgaris* and *Mentha arvensis* came from the Baskinta region (1400 m), *Lavandula officinalis* and *Foeniculum vulgare* were collected in the Kfarakaab and Ain-alkabou regions, respectively (1200 m); and *Calamintha origanifolia* was harvested at Bakish Mount, Lebanon (1800 m). All the collected species were authenticated according to conventional methods. Samples of each species were submitted to hydro-distillation for 2 h with 2 l of solvent. The essential oils were dried over anhydrous sodium sulfate to remove traces of moisture.

Cell lines, culture conditions and assays of in vitro anti-proliferative activity. Human erythroleukemia K562 cells (18) were cultured in a humidified atmosphere at 5% CO_2, in RPMI-1640 (Flow laboratories, Irvine, UK) supplemented with 10% fetal bovine serum (FBS; CELBIO, Milano, Italy), 100 units/ml penicillin and 100 mg/ml streptomycin (Aldrich, St. Louis, MO, USA). The *in vitro* antiproliferative activity of essential oils and pure commercially available derivatives from *S. montana* and *S. hortensis* was assayed as follows. Cell number/ml was determined by using a model ZBI Coulter Counter (Coulter Electronics, Hialeah, FL). Cells were seeded at an initial concentration of $3x10^4$ cells/ml and the cell number/ml was determined after 2, 3, 4 and 5 days of cell-culture. The IC_{50} was usually determined after 4 days, when untreated cells are in the log phase of cell growth.

Assays of in vitro induction of erythroid differentiation. Erythroid differentiation of essential oils from *S. montana* and *S. hortensis* and their pure derivatives was determined by counting benzidine positive cells after suspending the cells in a solution containing 0.2% benzidine in 0.5 M glacial acetic acid, 10% H_2O_2, as elsewhere described (24-26). Induction of differentiation was compared with that obtained using well established inducers of differentiation of K562 cells, such as cytosine arabisonide (27), mithramycin (28), angelicin (29), hydroxyurea (30) and butyric acids (31).

Gas chromatography/mass spectrometry (GC/MS) analysis of essential oils from Satureja montana and Satureja hortensis. A Fisons (Thermo Finnigan, San Jose, CA) model GC 8000 gas chromatograph interfaced to a Fisons model MD 800 quadrupole mass spectrometer was used for all measurements. The fused-silica gas chromatographic capillary column was a MEGA SE 54 (methyl phenyl polysiloxanes), 25 m x 0.25 mm I.D. and 0.25 μm film thickness (32). The head pressure of the carrier gas (helium, 99.99% purity) was 50 kPa (7.2 p.s.i.). One μl of sample dissolved into appropriate solvents was injected into the gas chromatograph. The injector and detector temperatures for the gas chromatograph were 250˚C and 300˚C, respectively. The column oven temperature was increased linearly from 40˚C (held for 4 min) to 200˚C (held for 10 min) at 10˚C/min. The mass spectrometer operated at source and interface temperatures of 250˚C. The ionization mode was Electron Impact (E.I.) (70 eV). The 'solvent delay', the time gap of a given analysis in which the mass spectrometer is turned off, was 3 min. The GC/MS system was operated in 'full scan' mode. The software utilized was Excalibur (Fisons) with NIST library to recognize all the derivatives found in plant extracts.

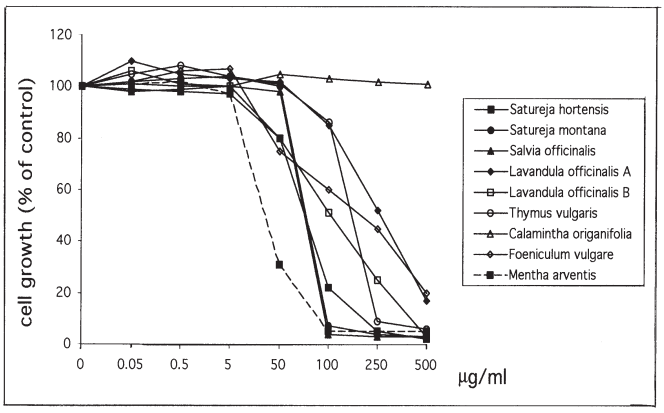


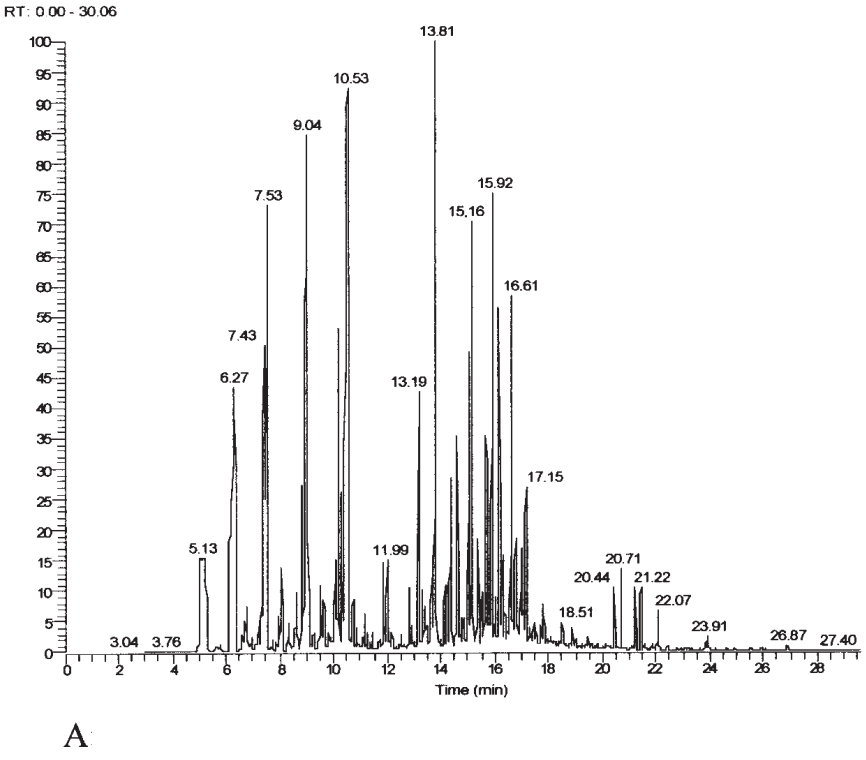
Figure 1. Effects of increasing concentrations of essential oils from Lebanon on K562 cell growth. *L. officinalis A*, plant cultivated in silice land; *L. officinalis B*, plant cultivated in argile land. Cells were cultured for 4 days and the cell number/ml was determined and compared to the value obtained using control untreated K562 cells.

Table I. Effects of essential oils from Lebanon on K562 cell growth (IC_{50}) and differentiation (% of benzidine-positive cells after 5 days of culture at the indicated concentrations).

Essential oil	IC_{50} (μg/ml)	% Differentiation (μg/ml)
Satureja hortensis	85.4±2.4	16 (0.5-5)
Satureja montana	56.15±6.15	30 (0.05-50)
Salvia officinalis	217.7±0.5	23 (0.5-50)
Lavandula officinalis (silice land)	256.1±0.3	18 (0.05-50)
Lavandula officinalis (argile land)	111.6±24.6	11 (0.05-5)
Thymus vulgaris	136.6±25	25 (0.05-0.5)
Calamintha origanifolia	>500	34 (5-250)
Foeniculum vulgare	190.8±0.5	32 (5-50)
Mentha arvensis	40.6±3.5	13 (0.5)

Results

Effects of essential oils on in vitro proliferation of human leukemic K562 cells. Fig. 1 shows the effects of increasing amounts of essential oils derived from *Satureja hortensis, Satureja montana, Salvia officinalis, Lavandula officinalis, Thymus vulgaris, Calamintha origanifolia, Foeniculum vulgare* and *Mentha arvensis* on cell proliferation of K562 cells. We analyzed two different essential oils derived from



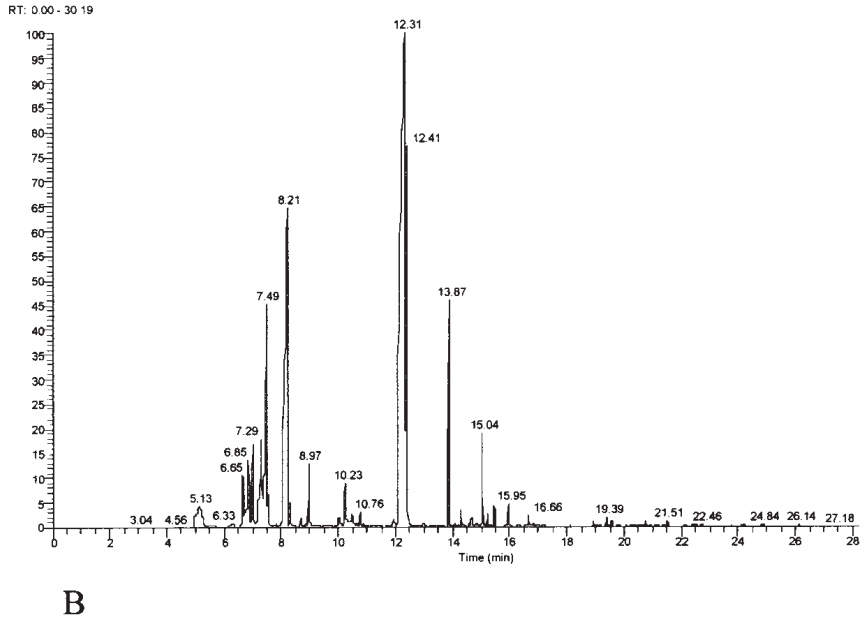


Figure 2. Chromatograms obtained with GC/MS of *Satureja montana* (A) and *Satureja hortensis* (B) essential oils.

L. officinalis cultivated in silice and argile land. K562 cells were seeded at an initial concentration of 30000 cells/ml and then cultured for 7 days in the absence or presence of 0.05-500 μg/ml of all of the hydro-distillate essential oils. The obtained data show that the IC_{50} of *S. hortensis, S. montana* and *M. arvensis* essential oils were 85.4±2.4, 56.15±6.15 and 40.6±3.5 μg/ml, respectively. The essential oil extracted from *C. origanifolia* was completely unable to inhibit K562 cell growth at the used concentrations (IC_{50} >500 μg/ml), and the remaining extracts demonstrated an intermediate activity. The data resulting from three different experiments are summarized in Table I, indicating that the essential oils derived from *S. montana* and *M. arvensis* showed the most interesting biological activity in inhibiting the cell growth of the K562 cell line.

Effects on K562 erythroid differentiation. Since it is well known that many antiproliferative agents are able to exert their anti-tumor effects by the activation of terminal programs of differentiation of targeted tumor cells, we tested the ability

of all essential oils to induce the erythroid differentiation of K562 cells (33). K562 erythroid differentiation was analyzed as reported elsewhere by the benzidine-staining procedure (24-26). *C. origanifolia, F. vulgare* and *S. montana* induced differentiation (30-34%) at concentrations lower than that causing 50% inhibition of K562 cell growth. Also, *Thymus vulgaris* and *Salvia officinalis* induced intermediate levels of differentiation (25%), whereas the percentage of differentiation decreased (13-18%) when *Lavandula officinalis, Mentha arvensis* and *Satureja hortensis* were employed (Table I).

In our opinion, *Satureja montana* differs from the other essential oils because its high antiproliferative effect is associated to the interesting ability to induce erythroid differentiation of K562 cells. Accordingly, *Satureja montana* extracts were further analyzed to identify putative bioactive compounds.

Gas chromatography/mass spectrometry analysis of Satureja montana and Satureja hortensis. In order to identify putative active compounds present within *Satureja montana* essential oil, we employed a gas chromatography/mass spectrometry (GC/MS) system. We decided to analyze and compare *Satureja montana* and *Satureja hortensis*. *S. montana* and *S. hortensis* essential oils were dissolved in acetonitrile (2% V/V) and 1 μl of each solution was injected into the gas chromatograph using an appropriate microsyringe, chromatographed using a fused-silica capillary column and analyzed with a quadrupole mass spectrometric detector. The resulting chromatograms are represented in Fig. 2 (A, *Satureja montana;* B, *Satureja hortensis*) and indicate that, in both assayed essential oils, seven derivatives are present, corresponding to α-pinene, γ-terpinen, 4-terpineol, α-terpineol, caryophyllene, τ-cadinene and τ-cadinol. The other identified molecules differ between the analyzed oils. All the compounds corresponding to the major peaks identified are shown in Tables II and III (*Satureja montana* and *Satureja hortensis,* respectively). The commercially available derivatives (α-pinene, ß-pinene, 1-octen-3-ol, 3-octanol, γ-terpinen, ß-linalool, borneol, 4-terpineol, carvacrol, caryophyllene, α-caryophyllene, caryophyllene oxide, eucalyptol, copaene and ±trans-nerolidol) were tested for their antiproliferative and differentiating activities on K562 cells.

Table II. Composition of *Satureja montana* essential oil.

R.t. (min)	Components	Composition (%)
5.13	α-pinene	4.35
6.27	4(10)-thujene	7.00
7.43	p-mentha-1,8-dien-6-ol	1.00
7.53	eucalyptol (1,8-cineole)	8.87
8.08	γ-terpinen	1.03
9.04	ß-linalool	11.41
9.48	(E)-3(10)-caren-4-ol	1.15
9.65	(S)-cis-verbenol	0.18
10.19	4-terpineol	3.98
10.53	α-terpineol (p-menth-1-en-8-ol)	12.66
10.72	2-pinen-4-one	0.35
11.85	p-allyl-anisole	0.13
11.99	nerol acetate	1.58
13.19	copaene	2.77
13.81	caryophyllene	6.66
14.61	germacrene D	1.70
15.01	τ-cadinene	0.44
15.16	cadina-1(10),4-diene	6.05
15.35	4,5,9,10-dehydro-isolongifolene	0.87
15.68	±trans-nerolidol	2.57
15.92	caryophyllene oxide	4.70
16.16	ledol	3.00
16.61	τ-cadinol	2.73
16.76	α-cadinol	0.94
17.15	4-(2-acetyl-5,5'-dimethylcyclopent-2-enylidene)butan-2-one	1.20
20.71	labda-8(20),13(16),14-triene	0.57
21.19	7-isopropyl-1,1,4α,9,10,10α-octahydrophenantrene	0.48
22.07	p-(2,2,4-trimethyl-4-chromanyl)phenol	0.35
		Total: 88.72

Biological activities of pure compounds identified within essential oils from Satureja montana and Satureja hortensis. The commercially available compounds present in *Satureja montana* and *Satureja hortensis* were analyzed for their antiproliferative activity and also for their possible effect on K562 differentiation, in order to identify putative active derivatives. We tested 15 pure compounds, identifying three derivatives present within the plant extracts very active in inhibiting K562 cell growth, including α-terpineol (IC_{50} = 75.0 μM), caryophyllene (IC_{50} = 98.0 μM) and α-caryophyllene (IC_{50} = 98.7 μM). Caryophyllene and α-terpineol are present in both essential oils, but at a different concentration; caryophyllene represents 6.66% of relative composition in *Satureja montana* (Table II) and 3.85% in *Satureja hortensis* (Table III), whereas α-terpineol represents 12.66% in *S. montana* and 0.38% in *S. hortensis*. In Table IV, the IC_{50} is reported for all compounds tested with respect to the ability to inhibit K562 cell growth. From the data shown in Table IV, it is clearly evident that some compounds exhibit no antiproliferative activity, e.g. eucaliptol, 1-octen-3-ol, 3-octanol and m-cymene (IC_{50} >400 μM). Three molecules found in both essential oils (α-pinene, α-terpineol and caryophyllene) exhibit high antiproliferative activity, even if at different concentrations (IC_{50} = 117.3 μM, 75.0 μM and 98.7 μM, respectively). Interestingly, all of these active compounds are more concentrated in *Satureja montana*, which is more active as crude extract than *Satureja hortensis* (IC_{50} = 56.15±6.15 μg/ml and 85.4±2.4 μg/ml, respectively).

Since *Satureja montana* induce erythroid differentiation of K562 cells (30% of induction with concentrations ranging between 0.05 and 50 μg/ml), we also analyzed the effects of the identified bioactive components utilizing the benzidine-staining procedure. No compound showed any detectable

Table III. Composition of *Satureja hortensis* essential oil.

R.t. (min)	Components	Composition (%)
5.13	α-pinene	2.19
6.65	1-octen-3-ol (amyl vinyl carbinol)	1.21
6.85	ß-pinene	2.34
6.99	3-octanol	2.03
7.49	m-cymene	9.07
8.21	γ-terpinene	15.26
8.97	ß-linalool	1.29
10.01	borneol	0.28
10.23	4-terpineol	1.19
10.76	α-terpineol (p-menth-1-en-8-ol)	0.38
11.92	trans-geraniol	0.02
12.31	carvacrol	50.61
12.97	acethyl thymol	0.08
13.87	caryophyllene	3.85
14.29	α-caryophyllene	0.33
15.01	τ-cadinene	0.11
15.04	1-methyl-4-(methyl-1-methylene-4-hexenyl)-cyclohexene	1.49
15.95	caryophyllene oxide	0.43
16.66	τ-cadinol	0.24
		Total: 92.4

Table IV. Effects of pure compounds identified in *Satureja montana* and *Satureja hortensis* on K562 cell growth (IC_{50}) and differentiation (% of benzidine-positive cells after 5 days of culture at the indicated concentrations).

Compound	IC_{50} (μM)	% Differentiation (μM)
α-pinene	117.3±14.4	5 (10)
ß-pinene	157.4±21.6	4 (50)
eucalyptol	>400	3 (10)
γ-terpinene	329.9±0.5	3 (200)
linalool	150.0±3.5	5 (100)
α-terpineol	75.0±1.5	4 (100)
copaene	136.5±3.2	4 (50)
trans-nerolidol	132.9±4.6	3 (50)
caryophyllene	98.0±0.7	4 (50)
α-caryophyllene	98.7±1.2	4 (5-50)
caryophyllene oxide	136.4±2.5	7 (10)
1-octen-3-ol	>400	4 (400)
3-octanol	>400	3 (50-200)
m-cymene	>400	4 (200-400)
carvacrol	112.5±12.2	7 (400)

activity in inducing erythroid differentiation of K562 cells (Table IV).

The obtained results suggest that the antiproliferative effects found in several active compounds of S*atureja montana*, including α-pinene, γ-terpinen, ß-linalool, terpineol, carvacrol, caryophyllene, caryophyllene oxide, copaene and ±trans-nerolidol, are not associated to induction of differentiation.

The ability of *S. montana* extracts to induce erythroid differentiation could be ascribed to other unidentified or not commercially available pure compound(s) and/or to a combination of singularly inactive molecules.

Discussion

The first objective of our study was to compare the activity of essential oils derived from plants belonging to the Magnoliophyta division on the *in vitro* proliferation and erythroid differentiation of human leukemic K562 cells. The second objective was to analyze the most promising extracts by GC/MS in order to characterize them with respect to composition. The third objective was to determine the biological activity of some identified pure compounds on K562 cells, in order to correlate the biological activity of the plant extracts with specific identified molecules.

To these aims, we determined the effects of essential oils from *Satureja hortensis, Satureja montana, Salvia officinalis, Lavandula officinalis, Thymus vulgaris, Calamintha origanifolia, Foeniculum vulgare* and *Mentha arvensis* on the *in vitro* proliferation and erythroid differentiation of human leukemic K562 cells. We found that the essential oil derived from *Satureja montana* showed the most interesting biological activity in inhibiting the cell growth and in inducing also erythroid differentiation of the K562 cell line. This result is of some importance, since data on the biological activity of *S. montana* extracts are scarce. Among the available data, it was reported that the essential oils of *S. montana* have a broad spectrum of activity against multidrug-resistant pathogens. The highest activity of the savory oil was observed against *Escherichia coli*, methicillin-resistant *Staphylococcus aureus* and yeasts (*Candida albicans*) (34). Furthermore, *S. montana* essential oil is active in inhibiting germination, releasing phytotoxic monoterpenes that hinder the development of herbaceous species (35). Finally, *S. montana* showed also potent anti-HIV-1 activity (36). *S. hortensis* has been used as tea or additive in commercial spice mixtures for food and also as a folk remedy to treat various ailments such as cramps, muscle pains, nausea, indigestion, diarrhea, and infectious diseases. In the literature, it is demonstrated that this medicinal plant shows antispasmodic, antidiarrheal, antioxidant, sedative, antifungal and antimicrobial properties (37). The *in vitro* antimicrobial and antioxidant activities of essential oils from *S. hortensis* have been previously reported, together with the chemical composition obtained by GC/MS, starting from air-dried and ground aerial parts collected in Turkey (37).

Concerning the characterization of bioactive compounds present in the studied plant extracts, we used a GC/MS system and we were able to identify several molecules, some of which were found to be able to inhibit K562 cell growth. Interestingly, α-pinene, α-terpineol and caryophyllene exhibited high anti-proliferative activity, even at different concentrations

(IC_{50} = 117.3 μM, 75.0 μM and 98.0 μM, respectively) and were found to be more concentrated in *Satureja montana*, which is more active as a crude extract than *Satureja hortensis* (compare Tables II and III). Therefore, we found very good correlation between the studies on the unfractionated plant extracts and the analysis of biological effects of isolated compounds on K562 cell growth.

This correlation was not found when erythroid differentiation was determined in K562 cells treated with pure compounds. None of the analyzed compounds showed detectable activity. Therefore, we suggest that the molecule(s) responsible for erythroid induction are not among the compounds analyzed or, alternatively, that erythroid differentiation is induced by a combination of the identified compounds. Further experiments are required to discriminate between these two possibilities.

In any case, our study suggests that α-pinene, ß-pinene, ß-linalool, terpineol, copaene, trans-nerolidol, caryophyllene, caryophyllene oxide and carvacrol, which are structurally related molecules, deserve further evaluation as molecules inhibiting the proliferation of tumor cells *in vitro* and *in vivo*.

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