

Anti-tumor activity of the methanolic extracts of *Salvia menthifolia*

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Abstract: In the present research we investigated the anti-proliferative activity of *Salvia menthifolia* Ten. (formerly *Salvia menthaefolia*), Lamiaceae, on a glioblastoma cell line, since up to date poor therapeutic results have been reported for treatment of malignant glioblastoma. Methanol extracts from different anatomical parts of *S. menthifolia* were tested on DBTRG-05MG cell line by MTT assay. The most active primary stems extract was also evaluated for apoptosis induction. Results confirmed the anti-tumor property of all the organs and demonstrated that the primary stems extract induced apoptosis after 4 h with the highest values of DNA fragmentation after 6 to 24 h. Some extracts were also HPLC analyzed for polyphenols, although activities could be due also to other constituents and to synergistic interactions. Rosmarinic acid, caffeic acid, luteolin-7-*O*-glucoside and quercitrin were found in all the extracts. The good performance revealed for *S. menthifolia* towards this extremely aggressive human glioblastoma cell line confirms that the genus *Salvia* is a natural source of anti-tumor agents though there are great differences among the various species.

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Introduction

The genus *Salvia*, Lamiaceae, includes 900-1100 species, distributed in tropical and temperate zones of the Old and New World; plant habit is herbaceous, perennial or, occasionally, annual or biennial; in other cases plants are subshrubs or shrubs. A large percentage of them are cultivated as ornamental flowering plants, others are used for medicinal purposes or as culinary herbs (Huxley et al., 1992; Hsi-wen & Hedge, 1994; Seidemann, 2005). The presence of terpenic compounds and volatile oils are on the basis of the use of some sage species in Western medicine and their inclusion in pharmacopoeias as *Salvia officinalis* L. [leaf and tincture], *S. triloba* L.f. = *S. fruticosa* Mill. [leaf], *S. lavandulifolia* Vahl [essential oil], *S. sclarea* L. [essential oil] which are reported in the seventh edition of the European Pharmacopoeia (Ph. Eur., 2010). Another species, the Mexican *S. divinorum* Epling et Jativa, is illegal in some countries for its hallucinogenic properties attributed to diterpenoids as Salvinorine A (Giroud et al., 2000; FU, 2008). Many other species are used in traditional medicine for different purposes, and their activities have been demonstrated in the last two decades and in most cases attributed to the presence of

terpenoids (Lu & Foo, 2002; Topçu, 2006). In her recent review Topçu (2006) lists the following main activities: antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, antiallergic, antiplasmodial, cytotoxic, cardiovascular and hypoglycaemic. In particular cytotoxicity was tested with crude extracts of many sages or isolated constituents against many tumoral cell-lines, with promising results.

Among these species, the most investigated is probably the Chinese *Salvia miltiorrhiza* Bunge: its root, known as "dan shen", is prescribed in Chinese Traditional Medicine (Tang & Eisenbrand, 1992). Following the identification of its chemical constituents, mainly diterpenoids and phenolics, pharmacological tests assessed many biological activities of *S. miltiorrhiza* root, including the anticancer property (Qing et al., 2001). One of these natural products was structurally modified to give a novel compound, named salvicine, that displayed a marked anti-tumor activity on human solid tumor cell lines and has been chosen for preclinical studies (Qing et al., 2001; Lu & Foo, 2002; Du & Zhan, 2004).

Within the genus, *Salvia menthifolia* Ten., a shrub with ovate leaves and scarlet flowers, is a little known species. It was established by Tenore (1854), who based the diagnosis on plants cultivated *ex situ*

at the Naples Botanical Garden (Italy), without data on their geographic origin. This species was originally named by Tenore *Salvia menthaefolia*. According to the rules of botanical nomenclature, the specific epithet was emended to *menthifolia* since 1897, following the so-called “Berlin Rules” by Engler, successively enclosed in the International Code of Botanical Nomenclature and still valid. At present this rule is in Chapter VII, sect. 1 (Orthography) art. 60 recommendation 60G.1. (Nicolson, 1991; McNeill et al., 2006). Accordingly, this species is included in almost all plant data bases with the right name (IPNI, 2011; Theplantlist, 2011; Tropicos, 2011). Notwithstanding, this rule is very often ignored (there are also proposals to delete it – cf. Manara, 1990), and in consequence all research papers published on this species, before the present one, refer to it as *Salvia menthaefolia*. It is also noteworthy that in some databases (e.g. Theplantlist, 2011) it is suggested that this name could be a synonym of *Salvia chamaedryoides* Cav., but in our opinion this hypothesis should be rejected, since for instance the Mexican *S. chamaedryoides* has sky-blue flowers (Cavanilles, 1792), while *S. menthifolia* has scarlet ones (Tenore, 1854).

The species is included in the recent book by Seidemann (2005), where it is shortly described as a spicy flavoring plant (parts used: leaf, herb), indicated as distributed in South Europe and Italy, and called with the common name “Italian spring sage” (from the German name “Italienischer Frühlings-Salbei”, commonly used by breeders who sell it as a rare collection sage). This is astonishing, since this species is surely not Italian, and is not included in Italian floras (Pignatti, 1982) or in Flora Europaea (Hedge, 1972), neither is reported in the comprehensive repertory by Guarrera (2006) on popular/ethnobotanical uses of plants in Italy. Also the recent study by Bazan et al. (2005) on the floristic elements of historical Sicilian gardens describes this species as of uncertain origin. Nevertheless, this species is commonly referred to as a food plant of Southern Europe, in particular on the net (Kraeutergaertner, 2011; Pflanzenreich, 2011; Seidemann, 2005).

Also scientific literature on this species is extremely scarce. The only ecologic data refer to the fact that flowers are visited and perforated by *Xylocopa violacea* (L.) (Hymenoptera: Apidae) for pollen robbing. This was firstly reported by Comes (1874) and referred to plants living ex situ at the Naples Botanical Garden; his observation was then retaken by Pammel (1888) and afterwards by other authors (Barrows, 1980; Vicidomini, 2006). *S. menthifolia* was chemically investigated for its content in triterpene acids in aerial parts by Passannanti et al. (1983), who demonstrated the presence of methyl oleanolate, methyl ursolate and methyl micromerate.

They also stated that this species does not contain diterpenes. As it concerns possible pharmacologic activities, methanolic extract of the roots of this plant was evaluated for its affinity for central nervous system receptors, but the extract displayed a moderate affinity only for the 5-HT_{2A} receptors (Cavallo et al., 2006).

Out of the three triterpenes reported for this species by Passannanti et al. (1983), ursolic acid and oleanolic acid are ubiquitous. They have been widely investigated and show a high range of pharmacologic activities, among which a proved anticancer pro-apoptotic ability; this has been considered of possible clinical interest, so that also synthetic triterpenoid derivatives were obtained and are currently tested (Liu, 1995; Petronelli et al., 2009; Xavier et al., 2009a). The third reported triterpene, micrometric acid, is a much more rare molecule, and was tested mainly for its anti-inflammatory properties (Altinier et al., 2007).

In our opinion, a deeper knowledge of *S. menthifolia* chemistry is needed. Since Passannanti et al. (1983) yet analyzed triterpenes and diterpenes, we choiced to search smaller molecules, such as rosmarinic acid, a polyphenolic compound, characteristic of the families Boraginaceae and Lamiaceae, subfam. Nepetoideae, although it is sparingly reported in other families and even in ferns and hornworts. In fact rosmarinic acid shows a good anticancer activity, apoptosis induction being the most often proposed/reported mechanism of action (Petersen & Simmonds, 2003; Osakabe et al., 2004; Lin et al., 2007; Xavier et al., 2009b). Obviously the activity shown by extracts depends on synergistic interaction between many of the present compounds. So, our analytical approach must be intended simply as an example and a contribution to a better knowledge of this species. In this sense it must be underlined that we only analyzed some of the tested extracts, those which seemed more promising or representative, while a better and complete approach to the chemistry of this species is still lacking.

S. menthifolia (root extract) was included in a screening of the antiproliferative activity of the methanol crude extracts of six *Salvia* species in human cancer cell lines from different histological types: brain, colon, prostate, uterus, placenta and peripheral blood (Fiore et al., 2006). This study suggests that the genus *Salvia* could be considered a natural resource of anti-tumor agents though there are great differences among the various species. The good results of *S. menthifolia* extract towards the extremely aggressive human glioblastoma cell line DBTRG-05MG, suggested us the present study, designed to investigate whether the methanol extracts from different anatomical part of plant were cytotoxic and, in the most promising extract, if these effects were mediated via an apoptotic mechanism. Apoptosis, in fact, is now recognized

as an important mode of cell death in response to cytotoxic treatments (Kerr et al., 1994). Indeed, it has been well documented that the administration of many natural compounds with anti-tumor activities triggers the apoptotic death of cancer cells (Smets, 1994; McConkey, 2007). Current literature indicates therefore the use of molecules able to trigger apoptosis in tumor cells as one of the most promising approaches to malignant tumors, as glioblastoma, highly resistant to conventional therapies (Clarke et al., 2010; Kögel et al., 2010).

Material and methods

Plant material

Plants of *Salvia menthifolia* Ten., Lamiaceae were propagated from specimens cultivated in open field at the Botanical Garden of Palermo (Italy), and deposited at the Herbarium of the Botanical Garden of Palermo (Fiore et al., 2006). Cuttings, 5-20 cm long, were collected in February and placed in 500 mL plastic pods filled with garden soil, kept in a greenhouse without heating, and watered weekly. Roots appeared within 1-3 weeks and about 80% of cuttings successfully rooted. Rooted cuttings were transferred individually in plastic pots and cultivated in the open. Water was supplied depending on weather condition to avoid complete drying of the soil. After seven months the plants showed well developed roots and stems, and started flowering. Plants were considered suitable for experiments after twelve months from rooting.

Plant material was collected at different vegetative phases, December and July. Leaves, roots, primary stems and secondary stems were cut, quickly washed under tap water, dried in a drying oven at 37 °C (Heraeus Noblelight, Hanau, Germany) to a constant weight, milled with refrigerant sample mill (IKA® Werke, Staufen, Germany), and stored in the dark in a dry place at room temperature until use.

Extraction

The powders of each part of *S. menthifolia* were extracted with methanol. One-step extraction was applied with 1±0.01 g of powder and 15 mL of methanol in an ultrasound bath (Biosonik III, Bronwill Scientific, Rochester, NY, USA) at 60 kHz for 60 min. The extracts obtained were filtered and then dried under vacuum (Rotavapor apparatus, IKA® Werke, Staufen, Germany). The dried methanol extracts obtained were re-dissolved in dimethyl sulfoxide (DMSO) at 50 g L⁻¹ concentration for biological tests.

Cell culture

The DBTRG-05MG cells, human glioblastoma cell line, were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured, according to the manufacturer's protocol of ATCC, in 75 cm² culturing flask in RPMI 1640 medium supplemented with 10 mg L⁻¹ adenine, 1 mg L⁻¹ adenosine triphosphate, 100 mg L⁻¹ L-cystine, 5950 mg L⁻¹ HEPES, 15 mg L⁻¹ hypoxanthine, 50 mg L⁻¹ L-isoleucine, 50 mg L⁻¹ L-proline, 100 mg L⁻¹ sodium pyruvate, 1 mg L⁻¹ thymidine, 100 IU L⁻¹ penicillin, 100 mg L⁻¹ streptomycin and 10% heat-inactivated fetal bovine serum.

For subculturing every two to three days, the medium was removed, 0.25% trypsin was added, rinsed and removed. Fresh medium was added, aspirated and dispensed into new flasks in the ratio of 1:3. DBTRG-05MG cells were maintained at 37 °C in a humidified atmosphere with 5% CO₂ and 95% air.

Assays

For *in vitro* studies, DBTRG-05MG cells were seeded in 96-well flat bottom plates at 5000 cells per well and were allowed to adhere to the wells overnight. The next day, the medium was removed and new culture medium containing *S. menthifolia* methanol extracts at different concentration, 0-500 mg L⁻¹, were added. The final concentration of DMSO used was 1% (vol/vol) at the highest treatment. All biological assays were performed in triplicate.

Antiproliferative assay

The effect of *S. menthifolia* methanol extracts on the cellular viability and proliferation was determined by a tetrazolium dye colorimetric test, MTT (Hansen et al., 1989).

The effect of different *S. menthifolia* methanol extracts on growth inhibition was assessed as percent of cell viability considering DMSO-treated cells as 100% viable. DMSO at the concentrations used had no effects on cell viability. A positive control was also used in each experiment: cells were treated with camptothecin, a DNA topoisomerase I inhibitor, of known activity against glioblastoma cells (Konkimalla & Efferth, 2010). In the present study, camptothecin showed 3.4 µg L⁻¹ as IC₅₀ value, maintained during the 72 h of treatment.

Apoptosis assay

Apoptosis was measured using the Cellular DNA Fragmentation ELISA (Roche Applied Science,

Monza, Italy), a photometric ELISA for the detection of bromo-deoxy-uridine (BrdU)-labeled DNA fragments in cell lysates. The principle of this assay is that cells proliferating *in vitro* are incubated with the non-radioactive thymidine analogue BrdU, which is incorporated into the genomic DNA. BrdU-labeled DNA fragments are released from the cells into the cell cytoplasm during apoptosis (Nag et al., 1996).

For the assay, DBTRG-05MG cells were plated in 96-well round bottom plates at a density of 10000 cells per well, labeled with BrdU to a final concentration of 10 μ M and incubated overnight at 37 °C in a humidified incubator. The next day, the cells were centrifuged for 10 min at 250 x g to remove the BrdU-containing culture medium and fresh culture medium with *S. menthifolia* methanol extracts was added. Apoptosis was measured according to the manufacturer's protocol after 2, 4, 6, 8, 24 h of treatment.

HPLC analysis

Extracts of specimens harvested in December were subjected to a preliminary HPLC analysis, for determining some polyphenolic compounds. This was performed according to Zheng & Wang (2001). The apparatus was a Waters 600E liquid chromatograph (Waters, Vimodrone, MI, Italy) equipped with a Beckman C18 (150 x 4.6 mm, 5 μ m) column coupled with a Nova-Pak C₁₈ 4 μ m, 60 Å pre-column (Beckman Coulter, Cassina de' Pecchi, MI, Italy). The detector was a UV Waters 486 (Waters, Vimodrone, MI, Italy); peaks were revealed at 280 nm. The mobile phase was acetonitrile (A) and acidified water containing 2.5% formic acid (B). The gradient was as follows: 0 min 5% A; 10 min 15% A; 30 min 25% A; 35 min 35% A; 50 min 55% A; 55 min 90% A; 60 min 95% A. Total analysis time 70 min; flow rate 1.0 mL/min. Solvents for the mobile phase (acetonitrile and formic acid) were purchased from J.T. Baker, Deventer, Holland. True standards were purchased from Sigma, St. Louis, MO, USA (rosmarinic acid, caffeic acid, quercetin dihydrate, apigenin) or from Extrasynthèse, Genay, France (rutin, luteolin-7-*O*-glucoside, quercitrin). Standard solutions were prepared in methanol HPLC grade (J.T. Baker) and diluted to obtain calibration standard in the range 1 to 100 μ g/mL. Calibration curves were determined on four levels of concentrations and calculated by linear regression analysis. All the curves had a coefficient of linear correlation >0.998.

Extracts were dissolved in methanol HPLC grade at concentration of 1.25 mg/mL. Each sample was then filtered through a cartridge-type sample filtration unit with 0.45 μ m Ultrafree Millipore (Millipore, Billerica, MA, USA) and immediately injected (injection volume 20 μ L). Analyses were performed

at least in triplicate to calculate the mean percentage content of constituents. Results were expressed as mean \pm standard deviation (SD). Identification of the individual compounds was based on the comparison of the retention time of unknown peaks to those of reference authentic standards.

Statistical analysis

All statistical analysis were carried out with Prism 4 (GraphPad), and *p* values <0.05 were considered significant. The one-way ANOVA method with Bonferroni's Multiple Comparison Test was used to estimate survival and differences among different treatments.

Results

For this study we harvested *Salvia menthifolia* Ten., Lamiaceae, plants in two different vegetative phases, December and July, to evaluate the possible role of the season factor. The freshly cut plants were sorted out and the different anatomical parts were separated and used for the preparation of the methanol extracts. In Table 1, the extract yields (mg/g dried powders) are reported.

Table 1. Extract yields (mg/g dried powders \pm SD) of the different anatomical parts of *Salvia menthifolia*.

Anatomical part	December	July
Roots	57.6 \pm 0.5	33.1 \pm 0.4
Leaves	---	226.6 \pm 1.5
Primary stems	101.4 \pm 0.9	103.3 \pm 1.1
Secondary stems	37.6 \pm 0.5	59.3 \pm 0.6

The dried methanol extracts obtained were dissolved in DMSO at 50 g L⁻¹ concentration and tested at different concentrations to evaluate the effect on the cellular viability and proliferation of cancer cells.

For each anatomical part of the plant and at different vegetative phases, as reported in Table 2, we have obtained a specific IC₅₀ that underlines the anti-tumor property of *S. menthifolia*, with exception of leaves in December due to absence of primary material, which resulted so damaged by meteorological events, that could not be used for the aim of this study.

In details, the treatment with leaves of July shows in absolute the lowest IC₅₀ in comparison to the other parts of the plant, but the primary stems extracts show a lower IC₅₀ in comparison to the roots and the secondary stems extracts, without any significant difference between the period of harvesting.

Since the primary stems give the same IC₅₀ both in the winter and the summer season and cause a

higher effect on inhibition of viability of cancer cells than roots and secondary stems, we have concentrated the study on this organ, to verify if the effect is depending on the exposure time and to better understand the cell death mechanism of the glioblastoma cell line, since cell death may occur by two quite different mechanisms: apoptosis or necrosis.

Table 2. *In vitro* cell growth inhibitory activity (IC₅₀ mg L⁻¹±SD) of crude methanolic extracts from different anatomical parts of *Salvia menthifolia* Ten. Values refer to a 72 h treatment.

Anatomical part	December	July
Roots	425.7±6.3	372±7.8
Leaves	---	112.7±4.5
Primary stems	243.2±7.4	243.6±7.5
Secondary stems	416.3±2.8	347±6.4

Therefore in the second part of the study we evaluated at three different time points the effect of the treatment with different concentrations of primary stem methanol extracts, as reported in Figure 1.

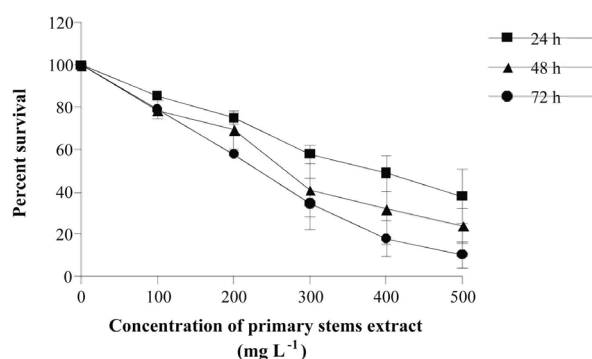


Figure 1. Measuring cell viability and proliferation by MTT assay. DBTRG-05MG cells were cultured in presence of different concentrations of *Salvia menthifolia* primary stems extract for 24, 48 and 72 h at 37 °C. Data are presented as mean±SD of five experiments each one carried out in triplicate.

The results show no significant difference between 24 h vs. 48 h and 48 h vs. 72 h of treatment, but the effect on cell proliferation is statistically significant ($p < 0,01$) after 24 h of incubation vs. 72 h. These results underline a specific activity of *S. menthifolia* extract not only concentration dependent, but also time dependent. The effect on cell viability and proliferation is probably due to molecules which quickly cross cell membranes and act in the first hours of treatment, inducing a cell death process that increases in time.

In this regard, we analyzed if the effect of the primary stem extract of *S. menthifolia* on cell viability was due to a generic toxic effect that leads to necrosis

of cells or if the cells undergo to apoptosis. The cancer cells were exposed to a single concentration of primary stems extract of *S. menthifolia*, 250 mg L⁻¹, at different time points and the apoptosis was measured.

The results obtained and reported in Figure 2 demonstrate that *S. menthifolia* extract induces apoptosis after 4 h with the highest value of DNA fragmentation after 6 to 24 h.

These data on apoptosis seem to remark the presence of one or more small molecules active with anti-tumor properties that interfere on the viability of cancer cells and are able to block their proliferation.

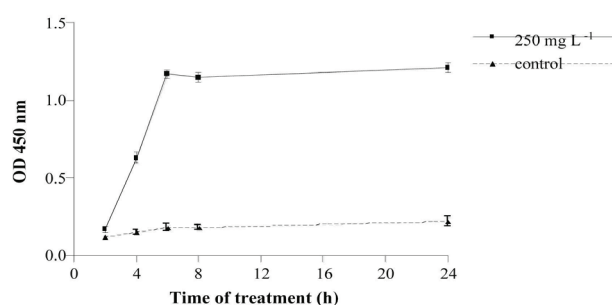


Figure 2. Kinetics of *Salvia menthifolia* primary stems extract-induced apoptotic cell death in DBTRG-05MG cells. 104 BrdU-labeled cells per well were incubated either in the presence of 250 mg L⁻¹ *Salvia menthifolia* Ten. primary stem extract (■) or in the absence (▲) for 1 to 24 h at 37 °C. After the times indicated, 100 µl per well lysates were removed and tested by ELISA.

This study was focalized on only one cancer cell line, DBTRG-05MG glial cells isolated from recurrent glioblastoma, although a previous study demonstrated a good activity of *S. menthifolia* root extract also against other human tumor cell lines (Fiore et al., 2006), because it seems of peculiar interest the seeking for active principle against this aggressive tumor yet without a valid therapy (Hoelzinger et al., 2007; Clarke et al., 2010).

The extract of winter collected primary stems was also used for a preliminary HPLC analysis, limited, as yet said, to some polyphenolic compounds of known activity. This analysis is far from giving a full information on the chemistry of *S. menthifolia*; however it was possible to put in evidence the presence of some molecules which may synergistically interact with one another or with those described by Passannanti et al. (1983), and with others still to be determined, they all contributing to the reported cytotoxic and pro-apoptotic activity of the extracts. It was interesting, for comparison purposes, to analyze also the extracts of the other organs collected in December, while a lacking of material prevented us to analyze also July-collected samples.

The HPLC analysis evidenced the presence of rosmarinic acid, luteolin-7-*O*-glucoside, quercitrin and caffeic acid in all the analyzed extracts, although in different amounts (data are summarized in Table 3). Furthermore, the analysis allowed also to evidence the presence of some other flavonoids; although they are ubiquitous molecules, some of them as luteolin and quercetin were demonstrated to possess a their own anticancer pro-apoptotic activity, and for instance in *Perilla frutescens* it has been hypothesized that rosmarinic acid and luteolin may interact synergistically (Osakabe et al., 2004; Lin et al., 2007; Xavier et al., 2009a).

Table 3. Percentage content (\pm SD) of analyzed samples of *Salvia menthifolia*.

Constituent	Primary stems	Secondary stems	Roots
rosmarinic acid	1.09 \pm 0.032	1.19 \pm 0.041	1.55 \pm 0.059
caffeic acid	0.07 \pm 0.002	0.21 \pm 0.007	0.24 \pm 0.008
rutin	0.08 \pm 0.003	-	-
luteolin-7- <i>O</i> -glc	0.08 \pm 0.002	0.71 \pm 0.023	1.82 \pm 0.068
quercitrin	4.01 \pm 0.135	2.39 \pm 0.089	2.76 \pm 0.109
quercetin dihydrate	0.04 \pm 0.001	-	-
apigenin	0.12 \pm 0.003	-	0.21 \pm 0.008

Primary stems extract seems to have the greatest variability, because of the highest number of revealed components. However, it is not surprising that the activity of this extract cannot be correlated to its richness in the analyzed polyphenolic compounds, since as said other anticancer compounds, as triterpenes, are present in the plant.

Discussion

The present study demonstrates that *Salvia menthifolia*, Lamiaceae, extracts have growth inhibitory capability against a recurrent glioblastoma cell line with higher effect of the extracts obtained by the primary stems and leaves of plants and that this cytotoxic effect is due to induction of apoptosis releasing DNA fragments into the cell cytoplasm. Due to the critical role of apoptosis in tissue homeostasis and cancer development, the modulation of apoptosis has become an interesting target in both therapeutic and preventive approaches in cancer (Kerr et al., 1994; Smets, 1994; McConkey, 2007; Kögel et al., 2010).

These results extend the data of the previous basic screening study on antiproliferative effects of root extracts of different species of *Salvia*, where *S. menthifolia* showed a significantly higher activity than the other tested species (Fiore et al., 2006). Due to the different extraction methods and the various plant

organs extracted, no comparisons are possible with the present study as it concerns obtained values.

Although data from this study demonstrate that *S. menthifolia* extracts were able to inhibit *in vitro* the growth of cancer cells via apoptotic mechanism, the *in vivo* anti-tumor potential remains to be determined.

In our opinion, this species deserves further studies. From the chemical point of view, our HPLC results demonstrate the presence of many molecules possessing cytotoxic and pro-apoptotic properties, but the lacking of clear relationships between demonstrated activities and concentrations of analyzed compounds could be due to further still unknown constituents, as well as to synergistic interaction between the polyphenolic compounds we analyzed (and maybe the triterpenes reported by Passannanti et al. in 1983).

Moreover, we believe that it is necessary also a deeper botanical investigation on *S. menthifolia*. Surely this is not an Italian nor an European species. Our observations show that the good activity of primary stem extracts remains more or less unchanged in organs collected in different times of the year, despite plants were cultivated in an area with seasonal variations. This should suggest that this species comes from a tropical or subequatorial region; the hypothesis is also supported by the fact that plants are continuously in bloom, after a certain size is reached, and by their scarce resistance to low temperature, plants being killed by a slight frost.

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