Original Article

In vitro Anti-Proliferative Effect of *Salvia officinalis* Essential Oil and its Three Main Components on Human Lung Cancer Cells

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ABSTRACT

Objective: Lung cancer is one of the most common and serious types of cancer. For this reason, novel therapeutic approaches for its treatment are urgently needed. The aim of this study was to investigate the effect of *Salvia officinalis* essential oil and its three main components in human lung cancer A549 and NCI-H226 cells.
Method: A549 and NCI-H226 cells were treated with various concentrations of *Salvia officinalis* essential oil and with a combination of two and three of its main constituents (1,8-cineole, α-thujone and camphor), at a dose of 100 and 200 µg/ml for 48 and 72 hours. The anti-proliferative activity was evaluated by the MTT assay.
Result: We showed that the treatment with *Salvia officinalis* essential oil, at a dose of 200 µg/ml for 72 hours, caused significant growth inhibition on both cell lines, compared with respective controls. The same result was obtained from the treatment with the combination of α-thujone and 1,8-cineole, α-thujone and camphor and 1,8-cineole and camphor, at a dose of 200 µg/ml each for 72 hours, and with the association of α-thujone, 1,8-cineole and camphor at a dose of 100 µg/ml each for 48 hours.
Conclusion: Based on these preliminary results, *Salvia officinalis* could represent an important source of substances with anti-proliferative activity and could improve the treatment of this devastating disease.

Keywords: Lung cancer, *Salvia officinalis* essential oil, thujone, Camphor, 1,8-cineole.

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INTRODUCTION

Lung cancer is one of the most common malignant tumors world-wide[1]. The two major subtypes are small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC)[2]. For early-stage or locally-advanced lung cancer, surgery is the most effective treatment and combined chemotherapy is the standard adjuvant approach. However, over 60% of all NSCLC patients have advanced or metastatic tumors at the time of diagnosis and are often not suited for surgery. The prognosis is poor, with a five-year survival rate of only 14%[3]. Because of the serious side effects of chemo and radiation therapy, many cancer patients seek alternative and/or complementary methods of treatment. Medicinal plants seem to have potential curative effects on cancer and the use of natural dietary agents is becoming appreciated in suppressing cancer growth[4,5] and cancer prevention[6]. Nowadays, 60% of the drugs currently used for cancer treatment (Vinca alkaloids, Taxus diterpenes, Camptotheca alkaloids and Podophyllum lignans), have been isolated from natural sources[7] and others are plant-derived synthetic compounds, for example flavopiridol isolated from the Chinese plant Indigofera tinctoria, which has been shown to exhibit anti-cancer effects with lesser toxicity than conventional drugs[8]. Salvia is the largest genera of the family Lamiaceae being represented by more than 900 species found throughout the world[9,10] and is one of the most appreciated herbs used extensively in folk medicine. The benefits of this herb have been already well-known in Ancient Rome, where it was called the sacred plant[11], as confirmed by the Latin meaning of its name. It was a highly valued herb in that era and is acclaimed for its medicinal values today as well. It is an attractive, shrubby plant that develops upright flowering spikes with tiered clusters of inch-long violet-tinted blue blossoms. The most common use today is as flavoring for meat and poultry stuffing and it is used as a spice in Mediterranean and Near Eastern cooking. Like other medicinal plants used in folk medicine, several studies have been conducted on sage extracts and essential oils, prompted by its reputation as a panacea because of its wide ranging medicinal effects[12]. Most of the biological properties shown by Salvia ssp. are to be ascribed to the polyphenol components present in the extracts and to different volatile compounds of the essential oils[13]. Essential oil is volatile, natural, and complex mixtures of several compounds obtained by a physical process involving steam distillation, hydro or dry distillation, and its chemical composition varies widely[14]. Since *Salvia officinalis* has a wide range of demonstrated biological activities[15,16] a study of the in vitro antiproliferative activity of whole sage essential oil and of a combination of two and three of its main components (1,8-cineole, α-thujone and camphor) on two human lung cancer cell lines (A549 and NCI-H226) by performing MTT assay, was carried out.

MATERIALS & METHODS

Chemicals

α-thujone, 1,8-cineole, camphor, dimethylsulfoxide (DMSO) and alkanes standard solutions C_8-C_{20} were purchased from Sigma-Aldrich (Milan, Italy).

Plant materials

Samples of *Salvia officinalis*, at bloom stage, were collected in Avola, Siracusa province, Sicily, southern Italy (Fig. 1). The plant was dried naturally at the collection point (GPS N 36° 56' 20", E 14° 58' 51). The voucher specimens, after botanical characterization, were deposited in the herbarium of the Agronomy Department of University of Catania.

Essential oil isolation

The essential oil was prepared from the dried aerial part of *Salvia officinalis* (100 g) by hydrodistillation, according to the European Pharmacopoeia 6.0. 2008[17]. After drying over anhydrous sodium sulfate, the oil (2.15 ml) was stored under nitrogen gas in a sealed vial at -20°C, until required.

Gas chromatography and mass spectrometry

The analysis of the essential oil was carried out by Gas Chromatography (GC) and Mass Spectrometry (MS). A Shimadzu GC-17A gas chromatograph, equipped with a flame ionization detector and a fused silica capillary column (Supelco SPBTM-5, 15 m x 0.1 mm x 0.1 µm) was used. The GC program temperature was set at 60°C for 1 minute, from 60°C to 280°C at a rate of 10°C/min and then reprogrammed at 280°C for 1 minute. GC-MS analysis was performed on a Shimadzu GCMS-QP5050A using the same column and the same operative conditions applied in the GC. The operating conditions were as follows: injector and detector temperatures 250°C and 280°C, respectively; carrier gas Helium 1ml/min. Mass spectra in the electron mode were generated at 70 eV and mass spectral data were acquired in the scan mode in m/z range 40-400. Oil solution was injected with the split mode (1:96).

Identification of oil components

The identity of the oil components was established from its GC retention indices, relative to C_9-C_{22} alkanes, by comparing their fragmentation patterns with those reported in the literature and by computer matching with the NIST MS 107, NIST 21 libraries, using the software GCMS solution version 1.02 (Lab solution, Shimatzu), by co-injection with authentic samples. Pure standards were purchased from Sigma-Aldrich (Milan, Italy), Alltech Italia (Milan, Italy) and Extrasynthese (Lyon, France).

Human lung cancer cell lines

Human lung cancer cell lines A549 and NCI-H226 were kindly donated by the Department of Bio-Medical Sciences at the University of Catania, Italy. All cell culture media and supplements were purchased from Life Technologies (Carlsbad, CA 92008, USA) unless otherwise indicated. Dulbecco's Modified Eagle's Medium (DMEM) and Roswell Park Memorial Institute Medium (RPMI) 1640 were used as culture media for A549 and NCI-H226 cells, respectively. Both media were supplemented with 10% foetal bovine serum (FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were maintained under standard cell culture conditions at 37°C and 5% CO_2 in a humid environment. Culture media were refreshed every three days. Cells were routinely grown since 4-5 days, then they were washed with Hank's Buffered Salt Solution (HBSS) and detached from the culture flask by incubating for 3-5 min at 37°C with 0.05% of trypsin-EDTA solution. A549 cells in DMEM/10% FBS and NCI-H226 in RPMI/10% FBS were plated at a density of $2x10^3$ cells/well in 96-well culture plates. Twenty-four hours after plating, the medium was replaced with the respective medium containing 1% FBS and incubation continued at 37°C in a humidified chamber.

Human lung cancer cell treatments

After 24 hours, whole sage essential oil was added at a dose of 200 µg/ml and the cells were incubated for 48 and 72 hours. In other experiments, both cell lines were incubated with couples of two main components of essential oil (α-thujone and 1,8-cineole, α-thujone and camphor, 1,8-cineole and camphor) prepared by dissolving standards in DMSO up to a final concentration of 200 µg/ml each, and separately with a combination of all three main components (α-thujone, 1,8-cineole and

camphor) prepared as reported above up to a final concentration of 100 µg/ml for each. The incubation was continued for 48 and 72 hours. A stock solution of the compounds was prepared in DMEM for A549 cells and in RPMI for NCI-H226 cells. Control cultures received DMSO alone.

Measurement of cell viability using the MTT assay

Measurement of cell viability was assessed using the MTT assay as previously described[18]. Briefly, at the end of the treatment time, 10 µl of a 0.5 g/ml stock of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT, Sigma Aldrich Inc., St. Louis, MO) was added to each microwell. The reaction was allowed to proceed for 3 to 4 hours at 37°C. The culture medium of each cell line was removed and formazan crystals were dissolved by adding 200 µl of dimethylsulfoxide (DMSO) to each well. The intensity of the color, which is proportional to the number of viable cells, was measured at a wavelength of 570 nm. All values were compared to the corresponding controls. Six wells were assigned to each treatment.

Statistical analysis

All the experiments were repeated at least ten times, and six wells were assigned to each sample. Results are presented as mean ± standard deviation (SD). Data were analyzed by one-way analysis of variance (ANOVA), with $P<0.05$ considered statistically significant.

RESULTS AND DISCUSSION

Essential oil composition

The chemical composition of the *Salvia officinalis* essential oil was characterized by more than 34 compounds (Table 1). The GC analyses revealed the presence of three main components: α-thujone (29.39%), 1,8-cineole (22.8%) and camphor (13.05%) (Fig. 2). Grouping all compounds in terms of chemical families, the oxygenated monoterpenes prove to be the largest group (78.4%), followed by the monoterpene hydrocarbons (15.3%). All the other classes are reported by a percentage lower than 3%.

Antiproliferative activity

Essential oil from *Salvia officinalis* and commercially available samples of some of the identified main compounds (α-thujone, 1,8-cineole and camphor) (Fig. 3) were tested *in vitro* for their antiproliferative activity on two different lung cancer cell lines, namely A549 and NCI-H226. Based on experiments using the MTT assay, the whole essential oil from sage at a dose of 200 µg/ml, caused significant growth inhibition of A549 cells after 72 hours of treatment. A similar behaviour was observed in NCI-H226 cells (Fig. 4). We noticed that the inhibition of cell viability in the treated cells was coupled with morphological changes, which were examined by the inverted light microscope. Cells treated with only DMSO, remained normal in size and shape (data not shown). No activity was observed in both cell lines when they were treated with essential oil at a dose of 100 µg/ml (data not shown). Moreover, the treatment with a mix of α-thujone, 1,8-cineole and camphor, at a dose of 100 µg/ml each, inhibited the growth of both cell lines after only 48 hours of treatment and the A549 cells were more sensitive than H226 cells (Fig. 5). Additional experiments were conducted using a combination of couples with the three main components of sage essential oil (α-thujone and 1,8-cineole; α-thujone and camphor; 1,8-cineole and camphor) at a dose of 200 µg/ml each for 72 hours of treatment. Once again, a significant antiproliferative activity was observed in both cells (Fig. 6).

Many researchers have already reported on the antioxidant, anti-inflammatory, anticancer and antimicrobial properties of sage[16-21]. *Salvia officinalis* has

the highest amount of essential oil compared to the other sage species[22], even if its chemical compositions can be quite different owing to genetic aspects and environmental factors[23]. To the best of our knowledge, in this study, we investigated, for the first time, the anti-proliferative activity of a sample of Sicilian *Salvia officinalis* essential oil and its three major constituents of lung cancer cells. Both lung cancer cells used were treated with whole sage essential oil and with a mix of its two or three main components, in order to verify where the pharmacological activity could be addressed. *Salvia officinalis* essential oil showed an anti-proliferative effect on A549 and H226 lung cancer cell lines. The results obtained from the treatment with a mix of two or three main components of essential oil, which represent more than 65% of oil volume, confirmed that the biological activity of the essential oil would probably be ascribed to the presence in the oil of these compounds. The independence of activity from chemical nature of the active principles as suggested by binary mixtures anti-proliferative data, and the evidence that the combination of the main single components has anti-proliferative effect at a minor dose compared to that of the whole oil, suggests that the anti-proliferative activity is probably due to a synergistic effect. Looking at the results of the treatments with couples of active constituents at a dose of 200 µg/ml each for 72 hours, no difference between both cell lines was observed.

Analysis of essential oil, obtained from a Sicilian sample of *S. officinalis*, showed that 1,8-cineole was one of the major components (22.8%). Previous studies have demonstrated that 1,8-cineole increases the penetration of the anticancer drug 5-fluorouracil through the human skin[24] and that the activation of PI3K/Akt and MAPK/ERK pathways and/or antioxidant activity may be involved. It is known that cancer cells produce increased amounts of reactive oxygen species which could inhibit phosphatases and also be associated with signalling events that lead to the activation of redox-sensitive transcription factors, mediating cancer cell proliferation and survival[25-27]. This oxygenated monoterpene may likely contribute to enhancing the anti-proliferative effect of Salvia essential oil in lung cancer cells.

CONCLUSION

Novel therapeutic approaches for the treatment of lung cancer are urgently needed. We believe that *S. officinalis* could be considered a potential source of new active anticancer compounds and its potential use in dietary strategies to stem lung cancer progression may be desirable. Clearly, further evaluation is warranted to clarify the mechanism of action and to appraise the practical value of this possible therapeutic application.

Conflict of interest

None.

REFERENCES

1. Porter AC, Vaillancourt RR. Tyrosine Kinase receptor-activated signal transduction pathways which lead to oncogenesis. *Oncogene* 1998:15:1343-52.
2. Altekruse SF, Kosary CL, Krapcho M et al. In: SEER cancer statistics review 1975-2008. http://www.cancer.gov.
3. Schottenfeld D. The etiology and epidemiology of lung cancer. In *Principles and Practice of Lung Cancer*, Pass HI, Carbone DP, Johnson DH, Minna JD, Scagliotti GV, Turrisi AT (eds). Lippincott Williams & Wilkins: *Philadelphia*, Pa 2000:367-68.
4. Waladkhani AR, Clemens MR. Effect of dietary phytochemicals on cancer development (review). *Int J Mol Med* 1998:1(4):747-53.

5. Ranjit PM, Manasa V, Chowdary YA, Kiran Prabhu Das k, Kumar Phani K, Venkateswara Rao1 P, Guntuku G. In vitro Cytotoxic Activity of Abrus precatorius Seed Extracts against MCF-7 Cell Lines. *AJPCT* 2013:1:8:605-10.
6. Pan MH, Ho CT. Chemopreventive effects of natural dietary compounds on cancer development. *Chem Soc Rev* 37 2008: (11):2558-74.
7. Gordaliza M. Natural products as leads to anticancer drugs. *Clin Transl Oncol* 2009:9:767-76.
8. Saklani A, Kutty SK. Plant-derived compounds in clinical trials. *Drug Discov Today* 2008: 13:161-71.
9. Goren AC, Kilic T, Dirmenci T, Bilsel G. Chemotaxonomic evaluation of Turkish species of Salvia: fatty acid compositions of seed oils. *Biochem Syst Ecol* 2006:34:160-64.
10. Cardile V, Russo A, Formisano C et al. Essential oils of Salvia bracteata and Salvia rubifolia from Lebanon: chemical composition, antimicrobial activity and inhibitory effect on human melanoma cells. *J Ethnopharmacol* 2009:126:265-72.
11. Kamatou GP, Makunga NP, Ramogola WP, Viljoen AM. South African Salvia species: a review of biological activities and phytochemistry. *J Ethnopharmacol* 2008:119(3):664-72.
12. Bouaziz M, Yangui T, Sayadi S, Dhouib A. Disinfectant properties of essential oils from *Salvia officinalis* L. cultivated in Tunisia. *Food Chem Toxicol* 2009:47:2755-60.
13. Napoli EM, Ruberto G. In Sicilian Aromatic Plants: From Traditional Heritage to a New Agro-Industrial exploitation. Spices: Types, Uses and Health Benefits. *Kralis JF (ed), NovaPublishers* 2012:1-56.
14. Lucera A, Costa C, Conte A, Del Nobile MA. Food applications of natural antimicrobial compounds. *Front Microbiol* 2012:3:287.
15. Fiore G, Nencini C, Cavallo F et al. In vitro antiproliferative effect of six Salvia species on human tumor cell lines. *Phytother Res* 2006:20(8):701-3.
16. Cavallo F, Nencini C, Capasso A. In vitro binding studies of methanolic extracts from different Salvia species. *Pharmacol Online* 2008:1:1-10.
17. European Pharmacopoeia 6.0. 2008. Determination of essential oils in herbal drugs, 2.8.12:251-52.
18. Luca T, Barresi V, Privitera G, Musso N, Caruso M, Condorelli DF, Castorina S. In vitro combined treatment with cetuximab and trastuzumab inhibits growth of colon cancer cells. *Cell Prolif* 2014:47(5):435-47.
19. Jedinák A, Mucková M, Kost'álová D, Maliar T, Masterova I. Antiprotease and anti-metastatic activity of ursolic acid isolated from *Salvia officinalis*. *Z Naturforsch C* 2006:61(11-12):777-778.
20. Horiuchi K, Shiota S, Hatano T, Yoshida T, Kuroda T, Tsuchiya T. Antimicrobial activity of oleanolic acid from *Salvia officinalis* and related compounds on vancomycin-resistant enterococci (VRE). *Biol Pharm Bull* 2007:30(6):1147-49.
21. Cavallo F, Nencini C, Capasso A. In vitro binding studies of methanolic extracts from different Salvia species. Pharmacol Online 2008:1:1-10.
22. Avato P, Morone Fortunato I, Ruta C, D'Elia R. Glandular hairs and essential oils in micropropagated plants of *Salvia officinalis* L. *Plant Science* 2005:169:29-36.
23. Russo A, Formisano C, Rigano D et al. Mediterranean sage (*Salvia officinalis* L.) grown in different environmental conditions. *Food and Chemical Toxicology* 2013:55:42-47.
24. Moteki H, Hibasami H, Yamada Y, Katsuzaki H, Imai K, Komiya T. Specific induction of apoptosis by 1,8-cineole in two human leukaemia cell lines, but not a in human stomach cancer cell line. *Oncol Rep* 2002: 9(4):757-60.
25. Schottenfeld D. The etiology and epidemiology of lung cancer. In Principles and Practice of Lung Cancer, Pass HI, Carbone DP, Johnson DH, Minna JD, Scagliotti GV, Turrisi AT (eds). Lippincott Williams & Wilkins: *Philadelphia*, Pa 2000:367-68.
26. Waladkhani AR, Clemens MR. Effect of dietary phytochemicals on cancer development (review). *Int J Mol Med* 1998:1(4):747-53.

27. Shobha RI, Andallu B. Oxidative Stress and Cancer: Role of Anti-Carcinogenic Herbs and Spices. *AJPCT* 2013:1:3:351-69.

Table 1. Chemical composition of Sicilian wild sage essential oil

	Components	%		Components	%
	Monoterpene hydrocarbons	15.25		Sesquiterpenes	2.5
1	*cis*-Salvene	0.48	33	b-Caryophyllene	0.12
2	*trans*-Salvene	0.08	34	α-Humulene	0.10
3	Santolina triene	0.08	35	Caryophyllene <9-epi-beta>	t
4	Tricyclene	0.10	36	γ-Muurolene	t
5	α-Thujene	0.09	37	δ-Cadinene	t
6	β-Pinene	7.87	38	Caryophyllene oxide	0.17
7	Camphene	3.39	39	Globulol	1.86
8	Thuja-2,4 (10)-diene	t	40	Viridiflorol	t
10	β-Myrcene	1.12	41	Humulene epoxide II	0.25
11	α-Terpinene	0.10	42	*E*-Caryophyllene<14-hydroxy-9-epi>	t
12	*p*-Cymene	1.68		Diterpenes	0.44
15	γ-Terpinene	0.16	43	α-Manool	0.44
16	Terpinolene	0.10		Others	2.79
	Oxygenated Monoterpenes	78.44	9	<1> Octen-3-ol	2.41
13	1,8-Cineole	22.80	14	6-Methyl-5-octen-2-one	t
17	α-Thujone	29.39	30	Hexenyl isovalerate <*2-cis*>	0.38
18	β-Thujone	8.85			
19	*cis-para*-Menth-2-en-1-ol	0.11			
20	α-Campholenal	0.16			
21	Thujanol <-iso-3>	0.18			
22	Champhor	13.05			
23	Thujanol <neo-iso-3>	0.12			
24	Borneol	1.94			
25	Terpinen 4-ol	0.83			
26	*p*-Cimene-8-ol	0.12			
27	α-Terpineol	0.21			
28	Dihydro carveol	0.26			
29	Carveol	t			
31	Thymol	0.24			
32	Carvacrolo	0.18			

The numbering refers to elution order and values (relative peak area percent) represents averages of 3 determinations (t = trace, < 0.05%).

Figure 1. *Salvia officinalis* (http://www.pollicegreen.com/salvia-non-solo-una-spezia/1104/salvia-officinalis/)

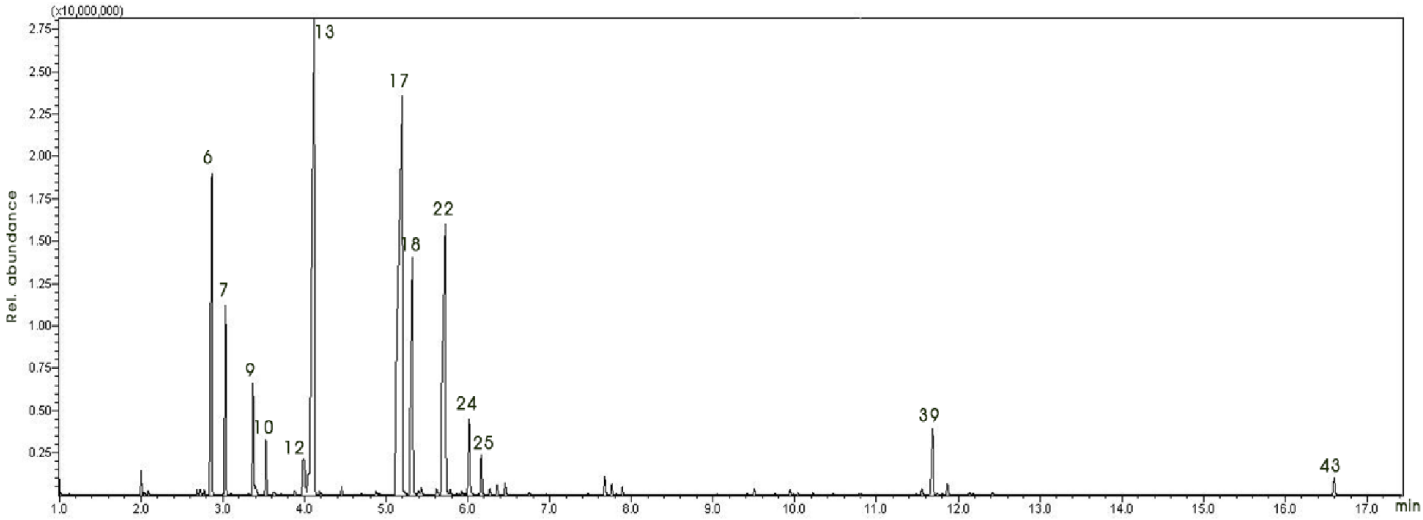

Figure 2. GC analysis of sicilian *Salvia officinalis* essential oil on a fused silica capillary column (Supelco SPBTM-5, 15 m x 0.1 mm x 0.1 µm). Helium was the carrier gas at a flow-rate of 1ml/min. The GC program temperature was set at 60°C for 1 minute, from 60°C to 280°C at a rate of 10°C/min and then reprogrammed at 280°C for 1 minute. Peaks can be identified by references to the numbers in Table 1

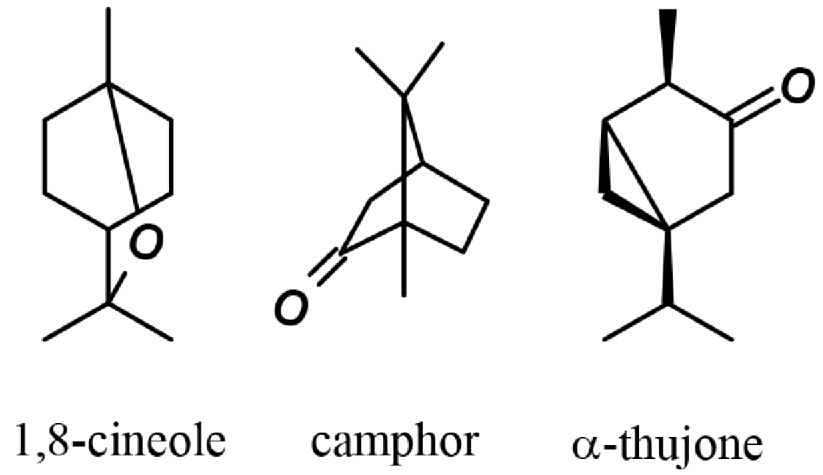

Figure 3. Chemical structure of the main components of *Salvia officinalis* essential oil

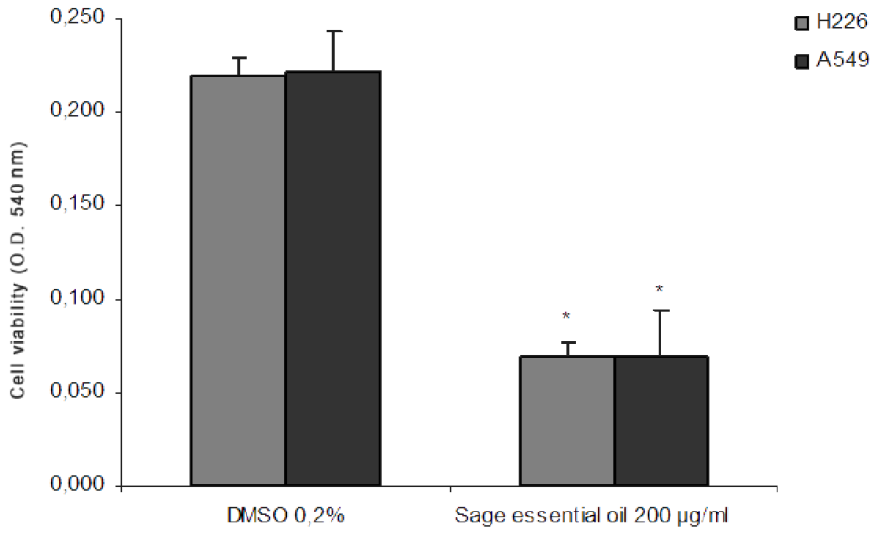

Figure 4. Effect of essential oil of *Salvia officinalis* aerial parts on A549 and H226 cell viability after 72 h of treatment. Results are expressed as a percentage of MTT reduction by control cells maintained in DMSO. Each value represents the mean ± SE from 6 experiments, performed in esaplicate (*** P<0, 01, compared to control)

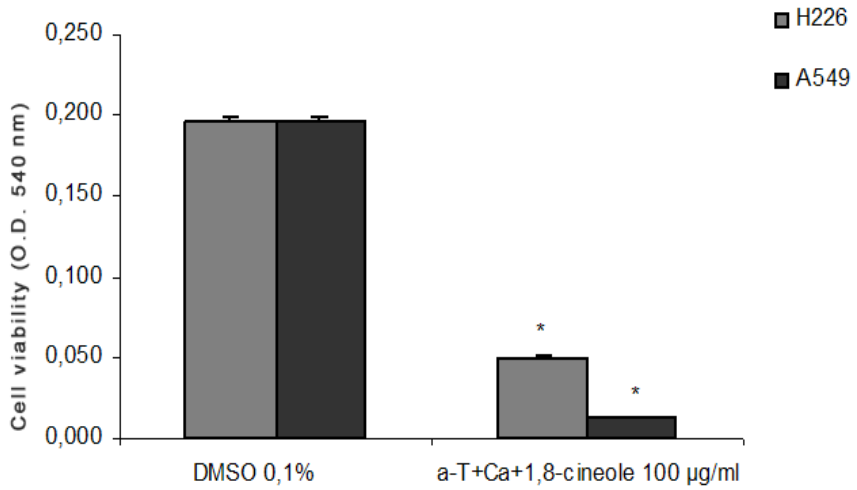

Figure 5. Effect of α-thujone, 1,8-cineole and camphor, at a dose of 100 µg/ml each, on A549 and H226 cell viability after 48 h of treatment. Results are expressed as a percentage of MTT reduction by control cells maintained in DMSO. Each value represents the mean ± SE from 6 experiments, performed in esaplicate (*** P<0, 01, compared to control)

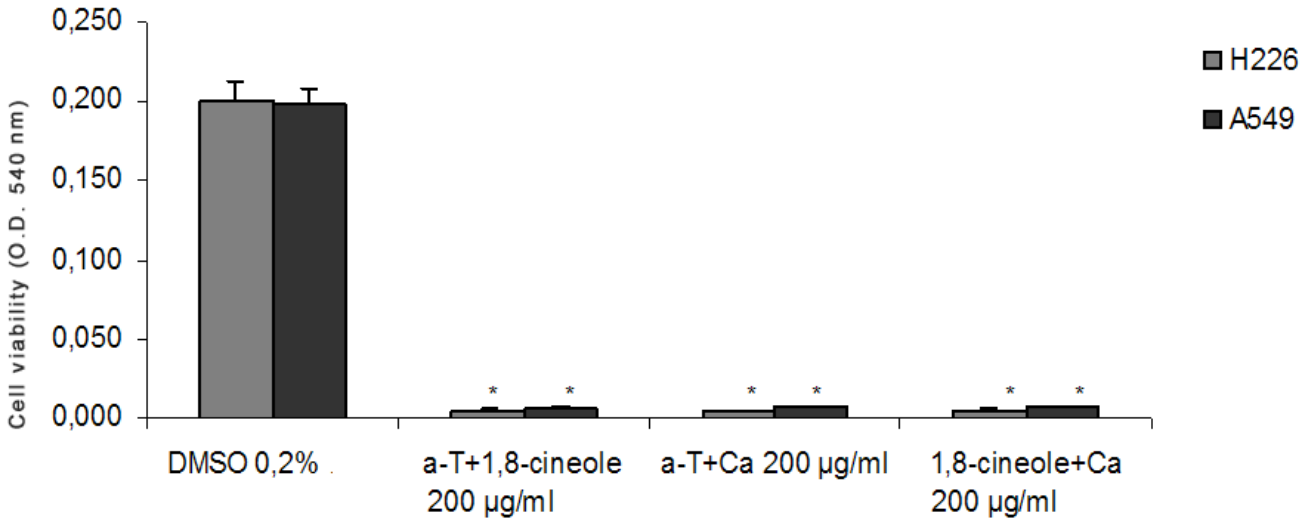

Figure 6. Effect of treatment with couples of three main components of sage essential oil on A549 and H226 cell viability after 72 h of treatment. Results are expressed as a percentage of MTT reduction by control cells maintained in DMSO. Each value represents the mean ± SE from 6 experiments, performed in esaplicate (*** P<0, 01, compared to control)