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CYTOTOXIC EFFECT OF *Salvia officinalis* L. ESSENTIAL OIL.

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ABSTRACT

The cytotoxicity [as antitumor activity against Ehrlich ascites carcinoma cells (EACC)] of hydrodistilled essential oil which obtained from leaves of *Salvia officinalis* L. cultivated in Egypt was tested *in vitro* and *in vivo*. EACC incubated with different levels of essential oil 25, 50, 100, 500, 1000 ppm for viability test and the percentage of dead cells of EACC after 2h. were 0, 5, 42, 89, 96 % respectively. The antitumor activity of essential oil in mice was assessed using survival time. Intraperitoneal administration of essential oil with single dose 500 ppm after and before EACC injected increased the survival time by 132.69 and 140.38% and decreased the tumor volume by 62.93 and 57.01% respectively compared with untreated tumor control (100%). It could be also observed that the pretreatment with the oil was more effective than the treatment after tumor development. This may recommend the use of *Salvia officinalis* essential oil as preventive agent against tumor. Chemical composition of the essential oil was analyzed by Gas chromatography-mass spectrometry (GC/MS). Forty one compounds were identified from 52 separated compounds. The major components were β-caryophyllene (7.56%), 1,8-cineole (7.27%), 1-terpineol (5.94%) and Camphor (5.40%).

INTRODUCTION

Numerous species of the genus *Salvia* (Labiatae) have been extensively used in popular folk medicine and many pharmacognostic researches intended to identify biologically active compounds responsible for their therapeutic effects. Compounds from *Salvia* essential oil have been shown to exhibit high antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtils*, cytotoxic activity against Vero cells and virucidal activity against herpes simplex virus1 and vesicular stomatitis virus (Sivropoulou *et al.*, 1997 and Tada *et al.*, 1994). According to these studies, the biological properties of *Salvia* essential oil were attributed mainly to 1,8-cineole, camphor, α-thujone and β-thujone. The antioxidant activity of *Salvia* polyphenols, consisting of flavone glycosides and some rosmarinic acid derivatives were also evaluated (Cuvelier *et al.*, 1996 and Lu and Foo, 2001). *Salvia officinalis* essential oil is applied in the treatment of a large range of diseases such as, nervous system, heart and blood circulation, respiratory, digestive, metabolic and endocrine diseases, etc. Although the *Salvia officinalis* infusion is easily obtained and commonly used for the haemostatic, estrogenic, anti-perspiration, anti-neuralgic, antiseptic, hypoglycemic and many other therapeutic effects. Its chemical composition was very little investigated and the scientific literature is poor in information concerning its chemical data (Radulescu *et al.*, 2004).

MATERIALS AND METHODS

Plant material:

The plant material of *Salvia officinalis* was obtained from Experimental station of Medicinal plants, Faculty of Pharmacy, Cairo University, Egypt in summer season.

Essential oil extraction:

Five hundred grams of *Salvia officinalis* leaves were hydrodistilled in a Clevenger type apparatus for 4h. The essential oil was dried over anhydrous sodium sulphate, then stored in a dark glass bottle, and kept at 4 °C until analysis (Radulescu *et al.*, 2004).

GC/MS analysis of essential oil:

The essential oil was analyzed using a Hewelett-Packard gas chromatography-mass spectrometry according to **Adams (1989)**, model 6890 series equipped with selective detector mass spectroscopy model 5973. This equipment was interfaced via HP chemstation version A02.12 software (Hewelett-Packard, Avondale, PA). The gas chromatography was equipped with carbwax capillary column, 80m x 0.53mm i.d. and 0.3µm film thickness (Hewelett-Packard, Avondale, PA) and the operating conditions for gas chromatography were as follows: The injection temperature was 290 °C, the carrier gas (Helium) flow rate was 0.8 ml/min, the oven temperature program was from 40 °C (1 min hold) raised at 3 °C/min to 240 °C for 20 min and the detector temperature was 300 °C. Two microliters of sample were injected. Mass spectroscopy was operated first in scanning mode in mass range from 40 to 650 m/z and identification was based on standard mass library, that National Institute of Standards and Technology (NIST Version 2.0) to detect the possibilities of essential oil components and mass spectroscopy operated in selective ions mode to assure the chemical composition using standard materials.

Animals:

Female Swiss albino mice, weighing 18-22g, 8-10 weeks old were used. Animals were kept under environmental and nutritional conditions for 2 weeks for adaptation.

Tumor cells:

A line of Ehrlich ascites carcinoma resistant to Endoxan (El-Merzabani and Tawfik, 1976) has been used. The parent line was first supplied through the courtesy of Dr.G.Klein, Amsterdam, Holland. The tumor line was maintained in the National Cancer Institute (NSI) in female Swiss albino mice by weekly intraperitoneal (i.p) transplantation of 2.5×10^6 cells. Similar line was proceeded in our department for the same cells. For *in vivo* and *in vitro* studies, the cells were taken from tumor transplanted animals after ≈ 7 days of transplantation then the number of cells/ml was calculated by using appropriate microscope counting technique (≈$2x10^7$ cells/ml). The cells were centrifuged at 1000 rpm for 5 min, then washed with saline. The number of cells needed to the test was prepared by suspending the cells in the appropriate volume of saline.

In vitro cytotoxicity of essential oil:

The viability percentage of tumor cells were measured by the modified cytotoxic trypan blue-exclusion technique of Bennett *et al.* (1976). The culture medium used was prepared using RPMI 1640 media, 10% fetal bovine serum and 10% L-glutamine. Trypan blue (0.4g) dissolved in 100 ml distilled water to give 0.4%, then kept in brown glass bottle. The viability percentage of tumor cells were measured after incubation with the essential oil (or saline plus tween 80 as control). Two millilitres of cells ($4x10^6$ cells) were transferred into a set of tubes, then different volumes of examined essential oil were added into the appropriate tube as well as control to give final concentrations 25, 50, 100, 500 and 1000 ppm. The tubes were incubated at 37°C for 2h under 5% CO_2, then centrifuged at 1000 rpm for 5 min and the separated cells were suspended in saline. For each examined tube and control, a new clean, dry small test tube was used and 10 µl of cell suspension, 80 µl saline and 10µl trypan blue were added and mixed then the number of living cells was calculated using a haemocytometer slide.

Effect of essential oil on survival time:

After a period of adaptation twenty five mice were divided into five groups (5 mice/group) as follows:

Group I: Mice served as untreated control, they fed the basal diet (negative control).

Group II: Mice injected intraperitoneal with solution of saline and tween 80 (positive control).

Group III: Mice injected intraperitoneal with $2.5x10^6$ EACC/mice (Untreated tumor control).

Group IV: Mice injected intraperitoneal with $2.5x10^6$ EACC/mice and after 24h they were treated with intraperitoneal single dose of essential oil emulsion (500ppm).

Group V: Mice injected intraperitoneal with single dose of essential oil emulsion (500ppm) and after 24h they were treated intraperitoneal with $5x10^6$ EACC/mice.

Antitumor activity of treated groups were evaluated by the increase in life span (ILS) comparing with the untreated tumor control group using the following calculation according to Rajkapoor *et al.*(2004):

$$ILS = \frac{T-C}{C} X\ 100$$

Where T = number of days the treated animals survived and C = number of days untreated tumor control animals survived.

The effect of essential oil on tumor mass:

Tumor mass was measured at the end of mice life span. The volume of tumor mass was calculated using the following calculation according to Ramnath *et al.*, (2002):

$$V = 4/3\ \pi r^2$$

where r is the mean of r^1 and r^2 which are two independent radii of tumor mass.

RESULTS AND DISCUSSION

GC/MS analysis of essential oil:

The hydrodistillation of *Salvia officinalis* leaves gave a colorless oil of a bout 1.6 ml/100g air dried leaves. The essential oil was analyzed by GC/MS for determination of its components and results are given in table (1).

Table (1):Chemical composition of *Salvia officinalis* leaves essential oil:

No.	Compound name	R_t (min)	Area (%)
1	Tricyclene	3.74	3.56
2	Camphene	3.97	3.85
3	α-Pinene	4.36	4.21
4	α-Terpinene	4.82	0.51
5	p-Cymene	4.93	0.85
6	1,8-Cineole	5.08	7.27
7	1-Terpineol	5.13	5.94
8	δ-Terpinene	5.41	0.93
9	β-*cis*-Ocimene	5.56	0.48
10	α-Terpinolene	5.87	0.41
11	Linalool	5.91	0.53
12	α-Thujone	6.19	1.04
13	β- Thujone	6.34	0.90
14	Pulegone	6.86	2.75
15	Camphor	6.91	5.40
16	Isopinocamphone	7.09	2.49
17	1-Borneol	7.16	2.57
18	4-Terpineol	7.27	1.71
19	Linalyl acetate	7.47	3.97
20	Bronyl acetate	7.56	0.71
21	Carvacrol	8.20	1.01
22	Isobornyl acetate	8.84	2.35
23	α-Terpinenyl acetate	9.68	1.37
24	β-Caryophyllene	10.93	7.56
25	Viridifloene	11.15	2.88
26	α-Humulene	11.34	2.93
27	Aromadendrene	11.49	0.52
28	Germacrene D	12.04	0.54
29	δ-Cadinene	12.10	0.48
30	Valencene	12.92	1.64
31	γ- Cadinene	13.03	3.19
32	Longifolene	13.13	4.07
33	Naphthalene	13.34	0.53
34	α-Guaiene	13.59	0.68
35	β-Selinene	13.82	1.55
36	β-*trans*-Farnesene	14.03	0.65
37	Viridiflorol	14.52	0.42
38	Widdrol	16.76	0.48
39	Caryophilline oxide	16.88	0.40
40	Manool	18.72	5.29
41	α-Santalol acetate	19.22	0.69

*Identified 89.31% from total area. ** R_t = Retention time.

Forty-one compounds were identified, having the total area of 89.31%. The main components found in *Salvia officinalis* essential oil were β-caryophyllene (7.56%), 1,8-cineole (7.27%), 1-terpineol (5.94%) and camphor (5.40%).

The following substances having quantities higher than 1% of the essential oil were evident: tricyclene (3.56%), camphene (3.85%), α-pinene (4.21%), α-thujone (1.04%), pulegone (2.75%), isopinocamphone (2.49%), 1-borneol (2.57%), 4-terpineol (1.71%), linalyl acetate (3.97%), carvacrol (1.01%), α-terpinenyl acetate (1.37%), viridifloene (2.88%), α-humulene (2.93%), valencene (1.64%), γ- cadinene (3.19%), longifolene (4.07%) and manool (5.29%). Eighteen compounds were less than 1%.

These results are in agreement with the percentage of 1,8-cineole reported by Radulescu *et al.* (2004), who indicated that the *Salvia officinalis* essential oil contain 6.72% of 1,8-cineole. On the other hand, results disagree with the same author, who found that the main components of *Salvia officinalis* essential oil were thujone (α and β, 27.36%), camphor (11.25%) and 1-octen-3-ol (8.5%). Also Foray *et al.* (1999), who indicated that the main components of *Salvia officinalis* essential oil were α-thujone (65.5%), β-thujone (15.4%) and 1,8-cineole (11.7%).

***In vitro* cytotoxicity of essential oil:**

The viability percentages tumor cells after incubation with different concentration of *Salvia officinalis* essential oil were recorded in table (2). Data showed that the incubation of tumor cells with essential oil affected the viability of those cells when compared to untreated cells (incubated with saline + tween 80). The dead cells were increased with increasing essential oil concentration. The addition of 0.05 mg (25ppm) of the essential oil did not affect the number of viable cells, but the addition of 0.1 mg (50 ppm) reduced the viability of tumor cells from 100 to 95% and reached to 58% by 0.2 mg (100 ppm). Increasing the essential oil dose to 1 and 2 mg (500 and 1000 ppm) highly affected the viability of tumor cells (the dead cells were 89 and 96% respectively). The IC_{50} was calculated as 473.55 ± 4.86 µg/ml (ppm).

Table (2): In vitro cytotoxicity **effect of *Salvia officinalis* essential oil as viability of EACC:**

Treatment	Essential oil Concentration		% of Viable cells	% of Dead cells
	mg	ppm		
Control: Tumor* + Saline + Tween 80	0	0	100	0
Tumor + Essential oil	0.05	25	100	0
	0.1	50	95	5
	0.2	100	58	42
	1	500	11	89
	2	1000	4	96

*** 2ml of cell solution containing $4x10^5$ cells.**

The effect of essential oil on survival time and tumor mass:

The effect of *Salvia officinalis* essential oil on survival of tumor-bearing mice and the volume of tumor mass are shown in table (3) and fig (1). The mean survival days for the untreated tumor control group was 10.4 ± 0.51 days, whereas it was 24.20 ± 0.10 days and 25.00 ± 1.90 days for the groups treated with 500 ppm essential oil after and before tumor injection respectively . The increase in life span of tumor-bearing mice treated with 500 ppm essential oil after and before tumor injection was found to be 132.69 % and 140.38 % respectively, these results were highly significant compared with untreated tumor control.

Table (3): Effect of *Salvia officinalis* essential oil on the survival of tumor-bearing mice and the volume of tumor mass:

Treatment	Mean of tumor volume (ml)	% of tumor volume	Mean survival day (range)	Increase in life span (%)
Group I Negative control	-	-	>28	> 169.23
Group II Positive control	-	-	>28	> 169.23
Group III Untreated tumor control	3.21^{a} ± 0.11	100	10.40^{b} ±0.51 (9-12)	0
Group IV essential oil (500 ppm) after tumor	2.02^{b*} ± 010	62.93	24.20^{a*} ±0.86 (21-26)	132.69
Group V essential oil (500 ppm) before tumor	1.83^{b*} ± 0.13	57.01	25.00^{a*} ± 1.90 (22-28)	140.38

- **Each value represents the mean of 5 mice (Mean ± SE).**
- **Means in the same column followed by the same letter are not significantly different with untreated tumor control at (P<0.05).**
- ***Means are highly significant compared with untreated tumor control at (P<0.01).**

There was highly significant reduction in the tumor volume of mice treated with essential oil. Tumor volume of untreated tumor control animals was 3.21 ± 0.11 ml, whereas for the essential oil treated groups after and before tumor injection were 2.02 ± 0.10 and 1.83 ± 0.13 ml respectively. It could be also observed that the pretreatment with the oil was more effective than the treatment after tumor development. This may recommend the use of *Salvia officinalis* essential oil as preventive agent against tumor.

These results showed that the increase in life span was correlated with the decrease in tumor volume. Tumor cells killing of *Salvia officinalis* essential oil may be due to the active ingredients terpenes specially 1,8-cineole (7.27%) and camphor (5.40%), these compounds may affect on the activity of some enzymes [lactate dehydrogenase (LDH) and glutathione-s-transferase (GST)] which are related to cell and cell membrane. The essential oil may affect the redox system in the tumor cells and enhanced of cells to enter the cell death (apoptosis), in addition the essential oil may affect the frequencies of chromosomal abnormalities of EACC. These results are in agreement with Sivropoulou *et al.*, (1997), who suggested that the essential

oil of *Salvia fructicosa* and its isolated components thujone and 1,8-cineole exhibited antimicrobial activity against eight bacterial strains.

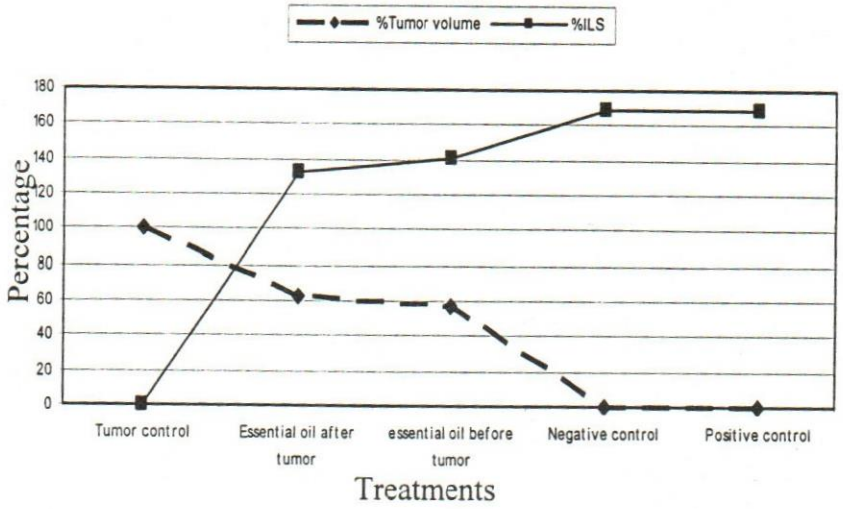

Fig (1): The effect of essential oil on survival time and tumor mass.

In conclusion the present study points to the potential cytotoxicity (as anticancer activity) of the essential oil of *Salvia officinalis*. Further studies are needed to characterize the active principles and elucidate the mechanism of cytotoxicity (antitumor action) of *Salvia officinalis* essential oil although it is believed to be due to more than one compound.

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التأثير السام للزيت الطيار لنبات المرمرية على الخلية

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تمت دراسة التأثير السام للزيت الطيار لنبات المرمرية المنزرع في مصر باختبار تأثيره كعامل مضاد لنمو الخلايا السرطانية معمليا وكذلك على فئران التجارب. تم تحضين الخلايا السرطانية من نوع ارليش اسايتس كارسينوما مع تركيزات مختلفة من الزيت الطيار ٢٥، ٥٠، ١٠٠، ٥٠٠، ١٠٠٠ جزء في المليون لمدة ساعتين وقد أظهرت النتائج أن النسبة المئوية للخلايا السرطانية الميتة كانت صفر، ٥، ٤٢، ٨٩، ٩٦% على التوالي. كما أوضحت دراسة تأثير الزيت الطيار على زيادة فترة حياة فئران التجارب المصابة بالخلايا السرطانية أنه عند حقن الزيت الطيار بتركيز ٥٠٠ جزء في المليون داخل الغشاء البريتوني قبل وبعد حقن الخلايا السرطانية بـ ٢٤ ساعة أدى ذلك إلى زيادة فترة الحياة بمعدل ١٤٠,٣٨ و ١٣٢,٦٩% على التوالي مقارنة بالمجموعة المصابة بالخلايا السرطانية وغير المعاملة كما أدت إلى خفض حجم الورم بحيث أصبح ٥٧,٠١ و ٦٢,٩٣% مقارنة بالمجموعة المصابة بالخلايا السرطانية وغير المعاملة (١٠٠%)، وقد لوحظ أن المعاملة بالزيت الطيار قبل الإصابة بالخلايا السرطانية كانت أفضل من المعاملة بعد الإصابة مما يرشح استخدام الزيت الطيار لنبات المرمرية كوقاية من السرطان. أيضا تم تحليل الزيت الطيار بإستخدام جهاز التفريد الكروماتوجرافى الغازي والمتصل بمطياف الكتلة (GC\MS) وقد أظهرت النتائج وجود ٥٢ مركب تم التعرف منها على ٤١ مركب، وقد وجد أن أعلى المركبات تركيزا هي بيتا-كاريوفيلين (٧,٥٦%)، ٨,١-سينيول (٧,٢٧%)، ١-تربينيول (٥,٩٤%) و كامفور (٥,٤٠%).