Comparison of the antiproliferative activity of crude ethanol extracts of nine *salvia* species grown in Jordan against breast cancer cell line models

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Submitted: 07-09-2011 Revised: 21-12-2011 Published: 22-11-2012

ABSTRACT

Background: The antiproliferative activity of *Salvia* species grown in Jordan has not been fully evaluated yet. The aim of this work was to study the antiproliferative activity of crude ethanol extracts from nine *Salvia* species grown in Jordan against a panel of breast cancer cell lines. **Material and Methods:** Cytotoxic activity was evaluated in human tumor models of breast cancer; MCF-7, T47D, ZR-75-1, and BT 474 by the sulforhodamine B assay. In addition, the extracts were evaluated using a non-transformed cell line (Vero) and normal fibroblast cells in order to demonstrate their selectivity and safety. **Results:** From the nice ethanol extracts under investigation, those of *S. dominica* and *S. fruticosa* showed an inhibitory concentration of 50% of cells (IC_{50}) in concentrations less than 30µg/mL against the four cell lines under investigation. *S. syriaca* and *S. hormium* showed an IC_{50} below 30µg/ml for two out of the four cell lines. *S. fruticosa, S. hormium* and *S. syriaca* showed selectivity in their antiproliferative activity against estrogen receptor positive cell lines with minimal toxicity against normal human periodontal fibroblasts. Phytochemical screening using thin layer chromatography indicated the presence of terpenoids, flavonoids and coumarins in all examined extracts. **Conclusion:** Three of the plant extracts under investigation exhibited antiproliferative activity against breast cancer cells and were shown to be safe and selective. These could be considered as a potential source for novel anticancer therapy.

Key words: Antiproliferative activity, Breast cancer cell lines, Jordan, *Salvia* species

Access this article online

Website: www.phcog.com

DOI: 10.4103/0973-1296.103664

Quick Response Code:

INTRODUCTION

The family Labiateae (Lamiaceae) has 180 genera and about 3500 species.[1] *Salvia* is the largest and the most important genus of this family. It is reported that this genus has more than 900 species.[2] Wild growing and cultivated medicinally valuable species as well as ornamentals are found among the representatives of the genus *Salvia*. Most of them grow in temperate regions of the world.[1-3] In Jordan, the reported 19 indigenous species of *Salvia* are found mainly in the Mediterranean and Irano-Turanian biogeographic zones of the country.[3]

Several indigenous *Salvia* species are linked to Jordanian traditional medicine such as the treatment of simple infectious diseases and stomach disorders as well as the common cold.[4-6] Antibiofilm, antiadhesive and anti-MERSA activities of the extracts, volatile oils and some isolated pure compounds of several *Salvia* species have been described in the literature.[7-12] Other researchers investigated the analgesic, anti-inflammatory, antioxidant, antimalarial, antidiabetic, anti-tumor and central nervous system depressant activities for a variety of *Salvia* species.[13-18] In fact, such a big variety of pharmacological effects are expected since the *Salvia* species show high diversity in their secondary metabolites. Flavonoids, diterpenoids, volatile oils and tannins have been reported to occur in these species.[14,19] There are also reports on the cosmetic use of some *Salvia* species.[4,11]

In an attempt to add to the existing pool of data on *Salvia* species, the present study was designed to screen the ethanol extracts of nine indigenous *Salvia* species of Jordan for their cytotoxic activity and compare their safety using Vero cell line and normal periodontal fibroblasts. Furthermore, their potency was assessed in comparison to two standard drugs, namely doxorubicin and cisplatin. Additionally, from a phytochemical point

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of view, simple thin layer chromatography (TLC) analysis was carried out to identify the classes of secondary metabolites.

MATERIALS AND METHODS

Plant material

S. ceratophylla L., *S. dominica* L., *S. hormium* L., *S. hierosolimitana* Boiss., *S. indica* L., *S. spinosa* L., *S. syriaca* L., *S. fruticosa* Mill. (syn. *S. triloba* L.) and *S. verbeneca* L. were collected from the Zai, Salt in the Spring of 2009. The plant material was identified by Prof. B. Abu-Irmaileh, Faculty of Agriculture, University of Jordan. Voucher specimens were deposited at the Faculty of Pharmacy, University of Jordan. All plants were purified from extraneous material and dried at room temperature without direct exposure to sunshine.

Extract preparation

For the preparation of the ethanol (EtOH) extracts, 5 g of coarsely powdered plant material was refluxed for 30 minutes with 50 ml 70% EtOH and the extracts were kept overnight. Then the extracts were filtered and the solvent was evaporated at rotavapor. 1 g of the resultant extract was dissolved in 10 ml dimethylsulfoxide (DMSO). This stock solution was further diluted for the cytotoxicity experiments. For the TLC analysis, each 0.1 g extract was dissolved in 5 ml EtOH.

Cell culture

The cell lines under investigation were human breast adenocarcinoma cell line (MCF-7 ATCC Nr: HTB 22), and human breast ductal carcinoma cell lines (T47D, ATCC Nr: HTB 133, ZR-75-1, ATCC Nr. CRL-1500, BT 474, ATCC Nr. HTB-20) and Vero cell line (ATCC Nr. CRL-2783). The properties of the cell lines under investigation as well as their culture conditions are summarized in Table 1.

The tissue culture media used were supplemented with 10% heated foetal bovine serum, 1% of 2mM l-glutamine, 50 IU/ml penicillin and 50μg/ml streptomycin. As positive control Cisplatin and Doxorubicin (both Ebewe Pharma GMBH Nfg. KG, Austria) were used.

Human periodontal fibroblasts (PDL), which are a primary cell culture, were kindly provided by Dr. Suhad Al-Jundi, Dr. Nizar Mhaidat from Jordan University of Science and Technology, Irbid, Jordan. In summary for PDL preparation, under sterile conditions each extracted tooth was held with a sterile forceps and the middle third of the root surface was mechanically scraped to obtain samples of periodontal ligament (PDL) tissue. The PDL tissue was diced into small tissue explants of $1mm^3$. Thereafter, these tissue explants were placed in $25cm^2$ tissue culture flasks. The explants were incubated with culture medium consisting of DMEM, 10% FBS and an antibiotic solution consisting of penicillin (100μg/mL); streptomycin (100μg/mL) and amphotericin B (0.25μg/mL). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air where they grew as monolayer cultures on the bottom of the 25 cm^2 flasks. After 4-5 weeks of incubation, cells reached confluence, and then they were detached after trypsinization (0.25% trypsin with ethylene diaminetetra-acetic acid (EDTA) in PBS) and transferred to 75 cm^2 flasks for continued growth. Culture medium was renewed once needed until cells reached 80-90% confluency. The 7^{th}-15^{th} passage of PDLFs was used in the experiment.

Cytotoxicity assay

Cells were detached, centrifuged and viability determined by trypan blue exclusion and it exceeded 90% as counted in a haemocytometer. The cell suspension was diluted afterwards to give the optimal seeding density and 100μl of the cell suspension was plated in a 96 well plate and

Table 1: Review of cell lines under investigation, their origin, characteristics and culutre conditions

Cell line	ATCC no	Description	Seeding density Cell/well	Growth Media	ER status	P53 status	Her level	Doubling Time (hours)
MCF7	HTB-22	Epithelial adenocarcinoma	5000	RPMI 1640	+	Wt	Very low	29
T-47D	HTB-133	Ductal carcinoma	10000	DMEM/F12	+	Mu	Moderate	32
ZR-75-1	CRL-1500	Ductal carcinoma	10000	DMEM/F12	+		Varying HER expression	80
BT-474	HTB-20	Ductal carcinoma	10000	DMEM/F12	+		High	Slow up to 100
Vero	CCL-81	African Green Monkey Kidney fibroblast	5000	RPMI 1640				18
PDL		Primary Human Peridonal Fibroblasts	1000	RPMI 1640	+			Slow

incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 24hrs the cells were treated with the extracts.

The crude extracts (initially dissolved in DMSO), were diluted with the medium and passed through a 0.2µm filter. 50µg/ml of each substance was tested initially, and, from the results, the active samples were considered to be those which gave less than 50% survival at exposure time of 72hrs. The IC_{50} of the active extracts was determined through a dose response curve using 8 concentrations (ranging from 0.1 to 100µg/ ml). Incubation for 72hrs followed and at the end of the exposure time, cell growth was analyzed using the sulphorhodamine B assay. Every determination was repeated for at least four times and two replicate plates (representing two cell passages) were used to determine the cytotoxicity of each test substance.

Sulphorhodamine B assay

After 72hrs incubation, adherent cell cultures were fixed *in situ* by adding 50µl of cold 40% (w/v) trichloroacetic acid and incubated for 60min at 4°C. After discarding the supernatant the plates were washed with deionized water and dried. To each well, 50µl of SRB solution (0.4% w/v in 1% acetic acid) was added and incubated for 30min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Then, the plates were air-dried and 100µl of 10 mM Tris base pH 10.5 (Sigma) were added to each well to solubilize the dye. The plates were shaken gently for 20min on a plate shaker and the absorbance of each well was read on an ELISA reader at 570nm. Cell survival was measured as the percentage absorbance compared to that of the control (non-treated cells) with media containing the same concentration of DMSO.

Statistical analysis

The percentage of cell survival was calculated as [Mean (OD test−OD blank)/Mean (OD control−OD blank)] × 100%. The IC_{50} was calculated using the SigmaPlot 9 software programme, obtained by plotting the percentage of cell survival against respective concentrations of extracts used in the assay.

RESULTS AND DISCUSSION

Herbs have been used as food and for medicinal purposes for centuries in all civilizations. Despite competition with other drug discovery methods natural products still play a highly significant role in the drug discovery and development process. Nowadays, medicinal plants are regarded as a potential source for lead compounds which in turn can be further modified, developed or optimized into novel therapeutic agents.

In our search for natural products with anticancer activity, ethanol crude extracts of nine native plants were prepared. Of the nine tested *Salvia* species, four demonstrated substantial antiproliferative effect, inhibiting at least 50% of tumor cell proliferation at 25µg/mL. For these active extracts, complete dose-response curves were generated [Figure 1] and their IC_{50} values were calculated [Table 2] against four breast cancer cell lines (MCF-7, T-47D, ZR-75-1 and BT474).

Out of nine extracts, *S. dominica* and *S. fruticosa* showed IC_{50} values lower than 30µg/mL against the four cell lines under investigation. *S. syriaca* and *S. hormium* exhibited an IC_{50} below 30µg/ml for two out of the four cell lines. Taking into account the criteria of the American National Cancer

Table 2: The antiprolifertive activity of *Salvia* crude ethanol extracts on breast cancer cell lines, Vero and PDL. Results present the IC_{50} (concentration at which 50% of the cells died, in comparison to controls) after 72 hours incubation. Results are the average and standard deviation of two separate experiments done in quadraplets

Plant name	Cell line					
	MCF-7	T47D	ZR-75-1	BT474	Vero	PDL
S. ceratophylla	83. 95±11.96	142.6±25.68	NT	46.45±5.65		
S. dominica	5.83±0.51	12.88±0.64	68.33±8.99	14.03±5.84	5.22±1.4	50.92±3.59
S. fruticosa	25.55±0.92	38.91±2.44	18.38±5.32	17.43±1.08	34.26±16.55	NT
S. hormium	45.36±3.97	25.38±3.89	NT	20.54±2.970	39.38±10.38	NT
S. hierosolymitana	67.82±6.28	147.6±34.70	NT	210.06±29.88		
S. indica	134.8±37.88	NT	182.23±15.05	216.74±74.26		
S. spinosa	154.07±61.88	156.98±13.49	NT	171.67±27.76		
S. syriaca	24.09±5.058	30.13±3.09	52.08±7.88	23.93±12.51	30.18±4.36	NT
S. verbeneca	50.92±8.942	64.40±8.826	122.00±12.13	72.97±3.17		
Cisplatin (µM)	7.34±1.89	21.29±9.72				22.16 ± 4.81
Doxorubicin (µM)	0.16±0.02	0.17±0.03				

NT: not toxic within the investigated concentration range (0.1 to 100 µg/ml)

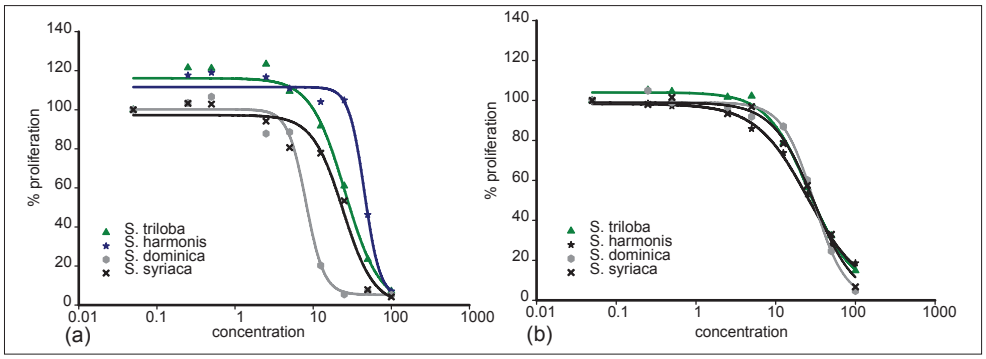

Figure 1: Dose response curves representing the most active *Salvia* species and their effect on proliferation of breast cancer cell lines (a. MCF7 and b. T-47D). Results present the average percent cell proliferation versus extract concentration in µg/ml

Institute which recommends to consider a crude extract promising for further purification, is an IC_{50} value less than 30µg/mL,[20] the above mentioned *Salvia* extracts could present a potential for new anticancer agents. Only scarce published information is available on the cytotoxic activity of *Salvia* extracts grown in Jordan. Talib and Mahasne reported the antiproliferative activities of plants extracts grown in Jordan against a panel of cancer cells, and from those, *S. pinardi* showed a promising activity in the initial screening, nevertheless, the IC_{50} values of chloroform, n-hexane and methanol extract were above 50µg/ml.[21] In another study, six crude methanol extracts of Salvia were also screened in human cancer cell lines with different histological types (none were breast cancer) and from those, *S. spinosa*, *S. sclarea* and *S. dominica* extracts showed a degree cytotoxic activity dependent on the cell line type. It was concluded from this study that *Salvia* species could be considered as a potential natural source of antitumour agents.[22]

With regards to *Salvia* species, grown in countries other than Jordan, Kamatou *et al*, described the biological activities of South African *Salvia*. Using MCF-7 and SRB assay for toxicity assessment, two *Salvia* species (*S. radula* and *S. africanca-caerulae*) were reported active with IC_{50} of 9.69 and 43.65µg/ml. Cell line specificity was also observed.[23]

Even with the relatively long doubling time of ZR-75-1 and BT474 cells (between 80-100 hours), the antiproliferative activity of *S. fruticosa* was maintained. *S. dominica* and *S. syriaca* showed higher IC_{50} values.

All human breast cancer cells used expressed estrogen receptor (ER) on their surface and a varying degree of HER expression. To assess whether the antiproliferative activity was specific to estrogen receptor presence, the antiproliferative activity of the active extracts was estimated using Vero cell line. Here, there was a dose response reduction in the proliferation; the most potent extract with similar potency against the breast cancer cell lines was *S. dominica* (IC_{50} of 5.22µg/ml). *S. fruticosa, S. hormium* and *S. syriaca*, which showed higher IC_{50} values than those of breast cancer cells, namely, 34.23, 39.38 and 30.18µg/ml respectively. This might indicate some selectivity in activity of the active crude extracts against estrogen receptor positive cells.

To determine the possible cytotoxic effect of the active *Salvia* crude ethanol extracts, the cell proliferation assay was done on a normal cell line, PDL fibroblast. The results shown in Table 2 indicated no toxicity for the extracts of *S. fruticosa, S. hormium* and *S. syriaca* on the normal cell line in the concentration between 0.1 and 100µg/ml. For *S. dominica* an IC_{50} of 50.92µg/ml was obtained. As a positive control in these studies, cisplatin and doxorubicin were used as standard reference anticancer agents.

This study, with the best of our knowledge, reports for the first time that *S. fruticosa* grown in Jordan acts as a potent inhibitor of both ER -ve and ER +ve breast cancer cell growth. Still the mechanisms by which growth inhibition occurs must be investigated further; results suggest that *S. fruticosa* contains compounds having anticarcinogenic properties that are likely to act through both ER independent and ER-dependent pathways.

Another important finding in this study is that *S. fruticosa, S. hormium* and *S. syriaca* ethanol extracts display a preferential activity against breast cancer cells. Indeed, comparing the IC_{50} for breast cancer cell lines cells with those obtained in normal mammalian fibroblasts cells tested, breast cells were more susceptible to the extract's action.

From the growth inhibition of the active *Salvia* extracts on slowly growing cells that express high to moderate levels of HER-2 (BT 474 and ZR-75-1); it could be possible that the inhibition is through this pathway as well.

Crude ethanol extract, rather than a single purified compound, was used in this study to benefit from all active compounds present in these plants. In previous studies,[24]

Table 3: Thin layer chromatography results

Plant name	Alkaloids	Terpenoids	Flavonoids	Coumarins
S. ceratophylla	-	++	++	+
S. dominica	-	+	+	++
S. fruticosa	-	++	++	+
S. hormium	-	+	+	+
S. hierosolymitana	-	+	++	+
S. indica	-	+	++	+/-
S. spinosa	-	++	+	+
S. syriaca	-	++	++	+
S. verbeneca	-	++	++	+

the aqueous extract of *S. syriaca, S. fruticosa, S. hormium* and *S. dominica* as well as the crude volatile oils were investigated for their antiproliferative activity using the same tests on MCF-7 and T47D. None of tested extracts or crude essential oils showed biological activity. This is in contrast to the results obtained with crude essential oils reported by Kamatou where the essential oils reported higher toxicities than solvent extracts of 17 *Salvia* species evaluated on kidney epithelial tests.[25]

Phytochemical screening using TLC was conducted for the tested *Salvia* extracts. Development of the TLC plates in appropriate solvent systems and visualization using accepted spraying reagents indicated the presence of terpenoids, flavonoids and coumarins in all examined extracts.[26] The results of the phytochemical analysis are given in Table 3.

CONCLUSION

Nine indigenous *Salvia* species from Jordan were investigated for their antiproliferative activity using a panel of breast cancer cell lines. The results indicated that the extracts of three *Salvia* species (namely; *S. fruticosa*, *S. hormium* and *S. syriaca*, exhibit selective antiproliferative activity against estrogen positive cell lines and with minimum toxicity against PDL. Hence, these *Salvia* species could be considered as promising plant-originated anticancer agents.

ACKNOWLEDGMENTS

This study was carried out during the sabbatical leave of the authors (R. AD and F. U. A.). Mr. Ismail Abaza is thanked for his technical help.

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Cite this article as: Abu-Dahab R, Afifi F, Kasabri V, Majdalawi L, Naffa R. Comparison of the antiproliferative activity of crude ethanol extracts of nine *salvia* species grown in Jordan against breast cancer cell line models. Phcog Mag 2012;8:319-24.

Source of Support: Nil, **Conflict of Interest:** None declared.