

Mechanistic Studies of Antiproliferative Effects of *Salvia triloba* and *Salvia dominica* (Lamiaceae) on Breast Cancer Cell Lines (MCF7 and T47D)

Rana Abu-Dahab^{a,*}, Maha R. Abdallah^a, Violet Kasabri^a, Nizar M. Mhaidat^b, and Fatma U. Afifi^a

^a Faculty of Pharmacy, The University of Jordan, Amman 11942, Jordan. Fax: +962 6 5300250.

E-mail: abudahab@ju.edu.jo

^b Faculty of Pharmacy, The Jordan University of Science and Technology, Irbid, Jordan

* Author for correspondence and reprint requests

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Ethanol extracts obtained from two *Salvia* species, *S. triloba* and *S. dominica*, collected from the flora of Jordan, were evaluated for their antiproliferative activity against MCF7 and T47D breast cancer cell lines by the sulforhodamine B assay. The ethanol extracts were biologically active with IC₅₀ values of (29.89 ± 0.92) and (38.91 ± 2.44) µg/mL for *S. triloba* against MCF7 and T47D cells, respectively, and (5.83 ± 0.51) and (12.83 ± 0.64) µg/mL for *S. dominica* against MCF7 and T47D cells, respectively.

Flow cytometry analysis and the annexinV-propidium iodide (PI) assay revealed apoptosis-mediated, and to a lesser extent necrosis-induced, cell death by the *S. triloba* and *S. dominica* ethanolic extracts in T47D cells. The mechanism of apoptosis was further investigated by determining the levels of p53, p21/WAF1, FasL (Fas ligand), and sFas (Fas/APO-1). The extract from *S. triloba* induced a more pronounced enrichment in cytoplasmic mono- and oligonucleosomes than that from *S. dominica* ($p < 0.05$) in T47D cells. In response to the extract from *S. dominica*, but not from *S. triloba*, the proapoptotic efficacy was specifically regulated by p21. Extracts from both *Salvia* spp. did not enhance p53 levels, and apoptosis induced by them was not caspase-8- or sFas/FasL-dependent. Thus, our findings indicate that *S. triloba* and *S. dominica* ethanolic extracts may be useful in breast cancer management/treatment via proapoptotic cytotoxic mechanisms.

Key words: *Salvia* spp., Apoptosis, MCF7, T47D

Introduction

Cancer is an important factor in the global burden of disease. The estimated number of new cases each year is expected to rise from 10 million in 2002 to 15 million by 2025, with 60% of those cases occurring in developing countries (WHO Eastern Mediterranean Regional Office, 2006). Cancer is one of the leading causes of morbidity and mortality in Jordan. It is the second cause of death (14%) after cardiovascular disease (35%). Moreover, breast cancer is the most commonly diagnosed cancer among Jordanians with 19.6% incidence frequency (Jordan National Cancer Registry, 2009).

Breast cancer is a heterogeneous disease with various subtypes exhibiting differential susceptibility to

anticancer drugs. Therefore, a therapy targeting the specific breast cancer subtype is recommended for an effective treatment (Harris *et al.*, 2007; Hussain *et al.*, 2007).

The genus *Salvia* (sage) is one of the largest genera of the Lamiaceae family (formerly Labiatae). It encompasses about 900 species, widespread throughout the world, and includes several ornamental, culinary, and medicinal species. Nineteen species of *Salvia* are reported to occur in the flora of Jordan (Al-Eisawi, 1982; Flamini *et al.*, 2007).

Most *Salvia* species are inherently linked to local traditional medicine systems in their country of origin. They are used traditionally to treat various conditions such as colics, diarrhea, common cold, cough, liver sickness, bacterial infections, febrile attacks, and

body sores, and as purgative. Moreover, they also are recommended for alimentary and cosmetic purposes (Ulubelen, 1964; Oran and Al-Eisawi, 1998; Perry *et al.*, 2003; Kamatou *et al.*, 2008).

In the last decade, a surge of anticancer drugs has been introduced, with about half of them derived from natural sources (Lee, 1999; Kuo *et al.*, 2007). Phytochemicals obtained from vegetables, fruits, spices, herbs, and medicinal plants, such as terpenoids, carotenoids, flavonoids or other phenolic compounds, have shown promise in suppressing experimental carcinogenesis in various organs. Recent studies have indicated that mechanisms underlying chemo-preventive action may include combinations of antioxidant, anti-inflammatory, immune-enhancing, and antihormone effects. Furthermore, modification of drug-metabolizing enzymes, effects on cell cycle and differentiation, induction of apoptosis, and suppression of proliferation and angiogenesis that play a role in the initiation and secondary modification of neoplastic development have been investigated as their possible modes of action (Rabi and Bishayee, 2009).

The major setbacks of most available chemotherapies is the non-selective cytotoxicity of non-cancerous tissues and severe side effects, and furthermore, the fact that they often become ineffectual due to resistance (Kekre *et al.*, 2005; Griffins *et al.*, 2007; Ma *et al.*, 2009). A cornerstone of cancer therapy is to develop a novel alternative to induce molecular apoptosis in cancer cells with high specificity. Apoptosis is a highly regulated mechanism by which cells undergo cell death in an active way. Thus, there is increasing focus on natural products modulating apoptotic signaling pathways and their emerging molecular targets (Sayers, 2011).

In our cell-based screening of the antiproliferative activity of ethanol extracts of plants endogenous to Jordan, several *Salvia* species showed promising results. In the present study we investigated crude extracts from two *Salvia* species for their effects on *in vitro* cell proliferation and cell cycle regulation, as well as on several markers associated with the signal transduction pathway of apoptosis, such as p53, p21, Fas ligand (FasL), sFas, and caspase-8.

Materials and Methods

Preparation of plant extracts

S. triloba was collected from Amman, Jordan, and *S. dominica* from Jordan Valley in Spring 2009. All

plants were taxonomically identified by direct comparison with authenticated specimens which was confirmed by Professor Dawoud Al-Eisawi (Department of Biological Science, The University of Jordan, Amman, Jordan). Voucher specimens were deposited in the Department of Pharmaceutical Sciences, Faculty of Pharmacy, The University of Jordan. The aerial parts of the plants collected during the flowering stage were dried at room temperature (RT) and then were coarsely powdered.

Ethanol extract preparation

Samples of 2.5 g dried aerial parts of *S. triloba* and *S. dominica* were extracted with 50 mL 70% (v/v) ethanol by boiling for 10 min, then kept overnight. After filtration and rotary evaporation to dryness, 1% (w/v) plant extracts were prepared by dissolving 0.1 g of the respective dried extract in 10 mL dimethylsulfoxide (DMSO) (Abu-Dahab and Afifi, 2007).

Assay for antiproliferative activity

Cells of the MCF7 (ATCC number: HTB 22) and T47D (ATCC number: HTB 133) human breast cancer cell lines were cultured in RPMI 1640 tissue culture medium (supplemented with 10% heated foetal bovine serum, 20 μ M L-glutamine, 50 IU/mL penicillin, and 50 μ g/mL streptomycin). The sulforhodamine B (SRB) colorimetric assay was used for determination of the cell viability as reported earlier (Abu-Dahab *et al.*, 2012). Cells were plated in 96-well plates at a density of 5000 cells/well. Plates were incubated for 24 h before addition of the extracts, then for 72 h with the extracts, after which the SRB assay was performed. The final DMSO concentration in the media did not exceed 1%. The relative cell viability was expressed as the mean percentage of viable cells compared with DMSO-treated cells (control). Both cisplatin and doxorubicin (Ebewe Pharma, Unterach am Attersee, Austria) were used as standard anticancer agents in different concentrations.

Flow cytometry (FCM) cell proliferation assay

MCF7 and T47D cells were seeded in 24-well plates in standard growth medium at a density of 500,000 cells/well and allowed to attach for 24 h. Then the medium was refreshed, and extracts or reference standards were added 18, 24, 48, and 72 h after seeding. After exposure, cells were harvested by cen-

trifugation at $314 \times g$ for 10 min at 4°C , washed with phosphate-buffered saline (PBS), and re-centrifuged. Resulting cell pellets were stained with 1.0 mL freshly prepared, hypotonic propidium iodide (PI) staining solution [$50\text{ }\mu\text{g/mL}$ PI, 0.1 % (v/v) Triton X-100, 0.1 % (w/v) sodium citrate]. Cells were stored overnight in the dark at 4°C prior to FCM analysis. Flow cytometric measurements were performed using an LSR II flow cytometer (BD Biosciences, Erembodegem, Belgium), equipped with a 15-mW solid state argon-ion laser emitting at 488 nm. PI fluorescence was collected with a 575/25 nm band pass filter [orange red fluorescence (FL2)] after linear amplification. For each measurement, data from 10,000 to 20,000 single cell events were collected, while cell doublets and aggregates were gated out using a two-parameter histogram of FL2 area versus FL2 width. Cell cycle histograms were analysed using ModFit LT™ 3.0 software packages (Verity Software House, Topsham, ME, USA). Based on this program, only cell cycles with a low variation coefficient of the G_0/G_1 peak ($CV < 5$) and low reduced chi-square (RCS) values ($RCS < 3$), which indicate how well the model describes the observed data, were used for further statistical analysis. The antiproliferative potency of an anticancer agent in this assay is indicated by the change in the percentage of cells in the S phase of the cell cycle. Results are presented as the means of three independent samples per treatment. Normality of data is assumed based on normality of data of solvent control.

AnnexinV-PI double staining

Phosphatidylserine translocation to the outer leaflet of the plasma membrane was assessed by reaction with annexinV-fluorescein isothiocyanate (FITC) and detected by an FAC Scan flow cytometer. After treatment, 500,000 cells were harvested, washed with ice-cold PBS, then collected by centrifugation, re-suspended in $500\text{ }\mu\text{L}$ of 1X binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl_2), and incubated with $5\text{ }\mu\text{L}$ annexinV-FITC ($20\text{ }\mu\text{g/mL}$) and $5\text{ }\mu\text{L}$ PI ($50\text{ }\mu\text{g/mL}$ in water) for 5 min at RT in the dark. FCM at an excitation wavelength of 488 nm and emission wavelength of 530 nm, using an FITC signal detector (FL1), and PI staining by a phycoerythrin emission signal detector (FL2) were carried out to identify the dead cells. The adherent cells were gently harvested and washed once with serum-containing medium prior to incubation with annexinV-FITC. Cells not treated with *Salvia* extracts were used as a negative control.

Tests for apoptosis: reagents, materials, and kits

The Quantum Protein assay kit (EuroClone, Sizzano, Italy), nucleosome ELISA kit (Roche Diagnostics, Mannheim, Germany), ELISA kits for human total p53, total p21, FasL, and sFas, and the caspase-8 colorimetric assay kit (all from R & D Systems Europe, Abingdon, UK) were used.

Assessment of apoptosis by ELISA

Apoptosis of T47D cells was assayed using the nucleosome ELISA kit, which monitors cytoplasmic histone-associated DNA fragments. T47D cells were incubated for 72 h with vehicle alone (1 % DMSO, control) and with the extracts at the respective IC_{50} value.

Assays of apoptosis-related proteins

ELISA assays of p53, p21/WAF1, FasL, and sFas (Fas/APO-1) were performed according to the manufacturer's protocols. T47D cells were treated as above, and cell lysates were prepared using the respective kit-specific lysis buffer. Subsequently, the lysates were placed in 96-well plates coated with monoclonal antibodies against sFas, p53, p21, or FasL, respectively, and incubated for 2 h at RT. After removing the unbound material by washing buffer, a second incubation with sandwich antibodies followed. Thereafter, horseradish peroxidase-conjugated streptavidin was added, and peroxidase activity was determined by measuring the absorbance at 450 nm. The concentrations of p53, p21, FasL, and sFas were directly determined by interpolating from standard curves. Results are presented as the percentage of the change relative to the untreated control.

Assay for caspase-8 activity

As per the manufacturer's instructions, cell lysates were incubated with peptide substrate in assay buffer (100 mM NaCl, 50 mM HEPES, 10 mM dithiothreitol, 1 mM EDTA, 10 % glycerol, 0.1 % CHAPS, pH 7.4) for 2 h at 37°C . The release of *p*-nitroaniline was monitored at 405 nm. Results are presented as the percentage of the change of the activity relative to the untreated control.

Statistical analysis

Results were expressed as means (as % of control) \pm S.E.M. (standard error of the mean). Statistical comparisons of the results were made by ANOVA followed by Dunnett's post-test whenever appropriate using Graphpad Prism (version 3.02 for windows; Graph-Pad Software, San Diego, CA, USA). Values of the means of untreated control and treated cells were considered significantly different if $p < 0.05$.

Results and Discussion

Cell viability

The antiproliferative activities of the ethanol extracts of *S. triloba* were characterized by IC_{50} values of $(29.89 \pm 0.92) \mu\text{g/mL}$ and $(38.91 \pm 2.44) \mu\text{g/mL}$ for MCF7 and T47D cells, respectively. The corresponding IC_{50} values of the *S. dominica* extract were $(5.83 \pm 0.51) \mu\text{g/mL}$ and $(12.83 \pm 0.64) \mu\text{g/mL}$.

These values are similar to those reported earlier by our group and decrease over time, where maximum reduction in the cell viability was seen after 72 h of incubation (Abu-Dahab *et al.*, 2012). The IC_{50} values for the control cisplatin were $(7.34 \pm 1.89) \mu\text{g/mL}$ for MCF7 cells and $(21.29 \pm 9.72) \mu\text{g/mL}$ for T47D cells. For doxorubicin the values were $(0.16 \pm 0.02) \mu\text{g/mL}$ for MCF7 cells and $(0.17 \pm 0.03) \mu\text{g/mL}$ for T47D cells.

Mechanism of reduction of cell viability

To examine the involvement of apoptosis in the reduction of cell viability, molecular and cellular events after treatment with the two extracts at their respective IC_{50} concentrations were studied at different time intervals (18, 24, 48, and 72 h).

Cell cycle analysis of both MCF7 and T47D cells was performed by DNA FCM. In general, there was a gradual appearance of hyperploid ($> 4n$ DNA content) cells (sub G_1 population) and a concurrent gradual decrease of cells with $2n$ DNA content (G_1 population)

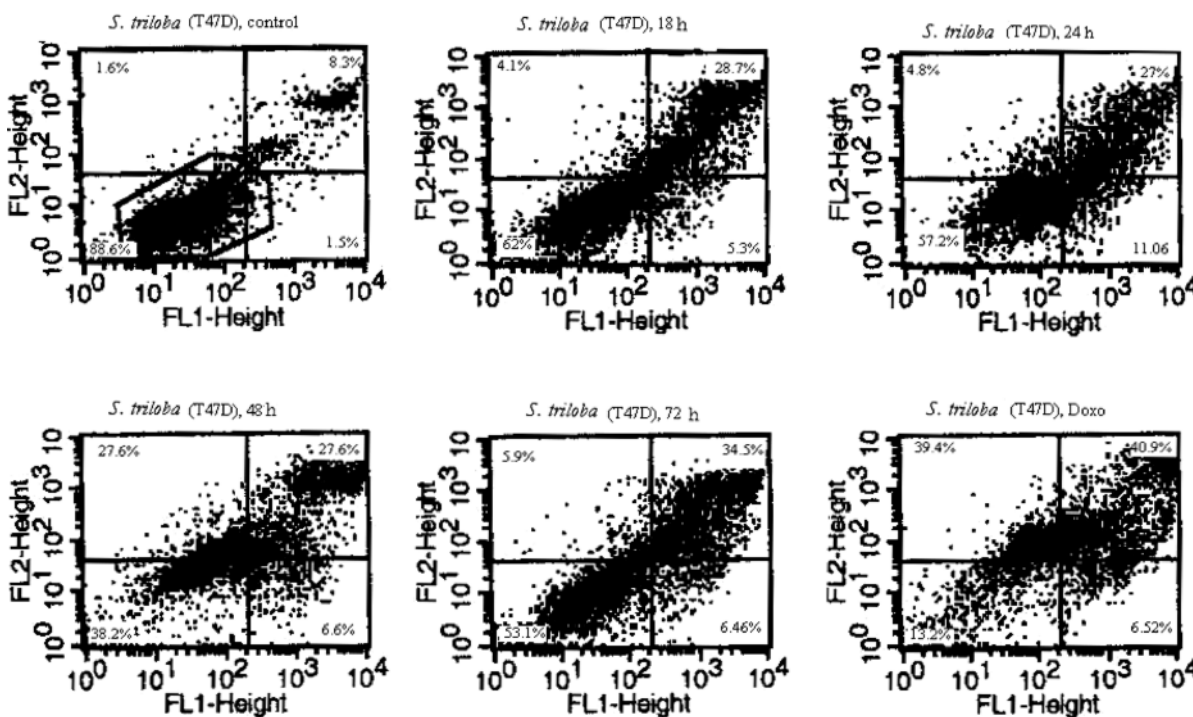


Fig. 1. Cytogram of annexinV binding (FL1) vs. propidium iodide (PI) staining (FL2) in the T47D cell line treated with $39 \mu\text{g/mL}$ of the ethanolic *S. triloba* extract for 0, 18, 24, 48, and 72 h. In each panel, live cells (annexinV⁻-PI⁻) are seen in the lower left quadrant, apoptotic cells (annexinV⁺-PI⁻) in the lower right one, and necrotic or necrotic-like cells (annexinV⁺-PI⁺) in the upper right one. A progressive increase in the incidence of cells in the annexinV⁺-PI⁻ compartment (apoptotic cells) is seen. Doxorubicin (Doxo; $0.25 \mu\text{M}$) was used as positive control.

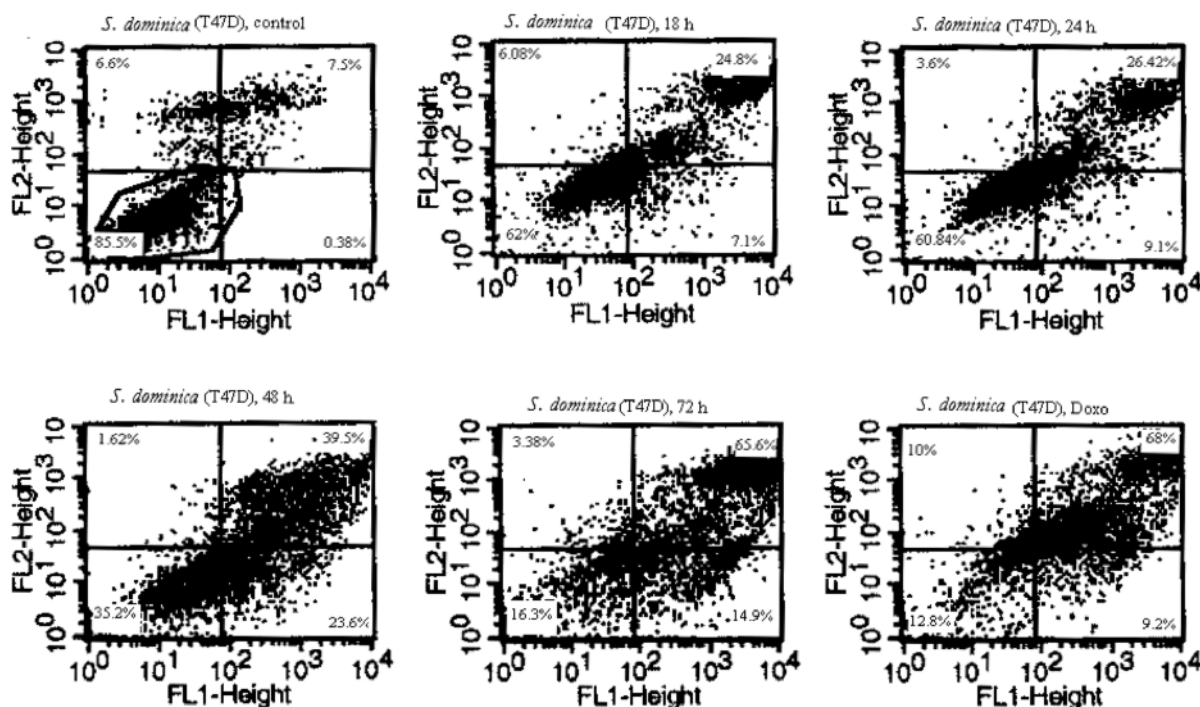


Fig. 2. Cytogram of annexinV binding (FL1) vs. propidium iodide (PI) staining (FL2) in the T47D cell line treated with 13 $\mu\text{g/mL}$ of the ethanolic *S. dominica* extract for 0, 18, 24, 48, and 72 h. See legend to Fig. 1 for further details.

in both cell lines exposed to the extracts, cisplatin or doxorubicin. Such an effect was more pronounced in the T47D cell line and may signify apoptosis.

In the positive control, *i.e.* cisplatin-treated cells, there was a small increase in hyperploid ($> 4n$ DNA content) cells (subG₁ population) that was observable at 24 h and reached a maximum after 48 h in both cell lines.

Based on our findings, as well as on the previous observation that in T47D cells apoptosis biomarkers are differentially and preferentially expressed in comparison to MCF7 cells (Aka and Lin, 2012), we selected T47D over MCF7 cells in the studies on the mechanism of the antiproliferative action of *Salvia* spp.

T47D cells were subjected to annexinV-PI staining followed by FCM analysis in order to differentiate between apoptotic and necrotic events. Results revealed time-dependent apoptosis for both plant extracts, similar to the positive control doxorubicin (Figs. 1 and 2).

Although MCF7 cells were more sensitive to the *Salvia* extracts than T47D cells, the onset of apoptotic events started later in MCF7 cells. Measurements at earlier incubation times should be considered for accurate assessment. Doxorubicin is known to induce apop-

tosis in T47D cells (Crawford and Bowen, 2002) and was therefore used as a positive control (0.25 μM for 72 h).

Effects of *Salvia* spp. on DNA fragmentation

A quantitative evaluation of apoptosis was sought using a nucleosome ELISA assay to detect the degree of DNA fragmentation. Compared with untreated control cells, the *S. triloba* extract, but not the *S. dominica* extract (at IC₅₀ value) provoked a pronounced enhancement of cytoplasmic mono- and oligonucleosomes [*S. dominica*: (123.2 \pm 28.6) % ($p > 0.05$); *S. triloba*: (176.5 \pm 15.3) % ($p < 0.05$); control: (98.7 \pm 12.3) %, $n = 4$; Fig. 3].

Effects of *Salvia* spp. on receptor-mediated apoptosis target molecules

We examined the effects of the two extracts on the levels of p53 as well as of p21, which acts downstream of p53. Figure 4 demonstrates that *S. dominica* and *S. triloba* extracts at their respective IC₅₀ values did not affect the level of p53 indicating that

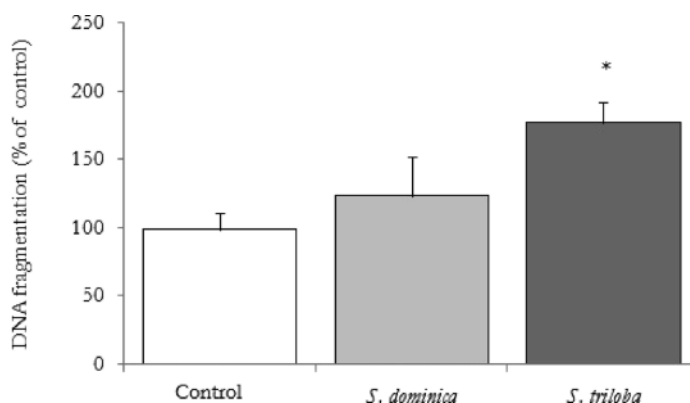


Fig. 3. Effects of antiproliferative *Salvia* spp. extracts on proapoptotic DNA fragmentation. Oligonucleosomes in lysates of apoptotic T47D cells were analysed by ELISA. Results, expressed as % of control, are means \pm S.E.M. ($n = 4$ independent determinations). * Statistically different from control at $p < 0.05$.

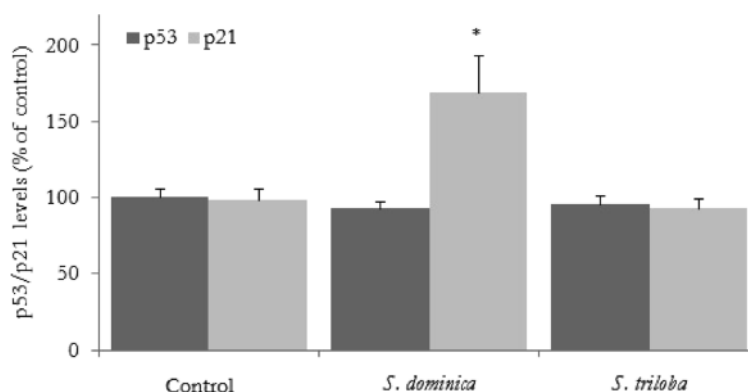


Fig. 4. Effects of antiproliferative *Salvia* spp. extracts on protein expression of p53/p21 T47D cells. Results, expressed as % of control, are means \pm S.E.M. ($n = 4$ independent replicates). * Statistically different from control at $p < 0.05$.

their proapoptotic propensities are p53-independent [93.39 ± 4.11 % and 96.22 ± 5.68 % of the control, respectively ($p > 0.05$), $n = 4$]. Conversely, the *S. dominica* extract markedly enhanced the level of p21 [169.45 ± 23.86 % ($p < 0.05$), $n = 4$], while the *S. triloba* extract did not affect p21 [92.80 ± 6.93 % vs. the non-induced control T47D cells ($p > 0.05$); Fig. 4].

Thus, the extracts from both *Salvia* spp. inhibit T47D cell growth and appear to promote apoptosis regardless of the p53 status. Distinctly, *S. triloba*, but not *S. dominica*, substantially enhances DNA disintegration. Comparably, retigeric acid B has been shown to inhibit human prostate cancer cell proliferation, cause S phase arrest and activate apoptosis independent of p53 (Lui *et al.*, 2010). The tumour suppressor protein p53 is a key cell cycle regulator which responds to DNA damage. Tumours defective in p53

are considered resistant to apoptosis. Targeting signalling pathways and cell cycle checkpoints is considered an effective anticancer therapeutic option (Lee and Houghton, 2005), as shown for some natural chemicals (Agarwal, 2000; Kan *et al.*, 2007). The apoptosis-inducing cytotoxicity of *S. dominica* correlated with an increased level of p21, a cyclin-dependent kinase (CDK) inhibitor. Similar results were obtained for extracts from the basidiomycete *Ganoderma lucidum* that induces cell cycle arrest and apoptosis in MCF7 cells (Hu *et al.*, 2002). Likewise, antiproliferative effects of berberine against rheumatoid arthritis fibroblast-like synoviocytes have recently been reported, most likely through deregulation of numerous cell cycle regulators, including up-regulation of p21 and modulation of the mitochondrial pathway of apoptosis (Wang *et al.*, 2011).

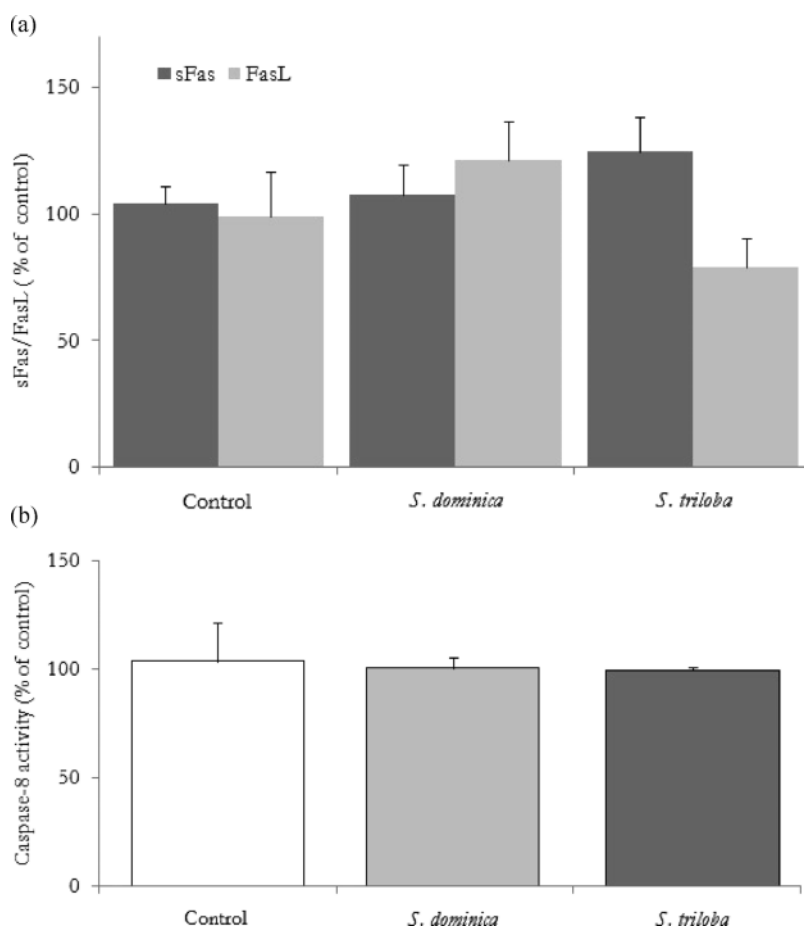


Fig. 5. (a) Non-involvement of the sFas/FasL apoptotic system in *Salvia* spp.-induced apoptosis in T47D cells. (b) Lack of activation of caspase-8 in apoptotic T47D cells. Results, expressed as % of control, are means \pm S.E.M. ($n = 4$ independent determinations). There were no statistically significant differences between controls and extract-treated cells.

Apoptosis usually proceeds by two pathways: the intrinsic pathway and the extrinsic pathway (Walczak *et al.*, 2000). The intrinsic pathway begins with the release of mitochondrial cytochrome c, which is caused by DNA disintegration and damage. It is usually triggered by UV- or γ -irradiation, some chemotherapeutic drugs, reactive oxygen species, and radicals generated by the cell as a by-product of normal metabolism. The intrinsic pathway is regulated by the Bcl family proteins, which comprise proapoptotic and anti-apoptotic proteins. The extrinsic pathway is mediated by the direct interaction between so-called 'death ligands' and 'death receptors', by which caspase-8 is activated. When death ligands, such as tumour necrosis factor α (TNF α), TNF α -related apoptosis-inducing ligand (TRAIL), or Fas, bind to their receptors, a pro-

tein complex called the death-inducing signaling complex (DISC) is formed. DISC formation is followed by the activation of caspase-8, which in turn activates caspase-3, thus promoting apoptosis (Yang *et al.*, 2009).

To further establish the sequence of events involved in the induction of apoptosis by the *Salvia* extracts, the recruitment of sFas/FasL-mediated execution of apoptosis was investigated. Two major distinct extrinsic apoptotic pathways have been described for mammalian cells. Fas receptor-mediated apoptotic signaling is one of the most important extrinsic apoptotic pathways. Binding of Fas to oligomerized FasL activates apoptotic signaling through the death domain that interacts with signaling adaptors, including the Fas-associated protein, with the death domain (FADD)

to activate caspase-8 and caspase-3 to mediate the rapid dismantling of cellular organelles and architecture. Over 72 h, the sFas/FasL system of T47D cells was not significantly up-regulated by either of the extracts ($p > 0.05$; Fig. 5a).

Presently, we sought to identify the apoptotic pathway stimulated by the two *Salvia* spp. by measuring the activity of the downstream caspase-8 of the sFas/FasL system in T47D cells. Neither extract increased the caspase-8 activity at 72 h compared to control cells [$(100.54 \pm 4.89)\%$ and $(99.8 \pm 1.43)\%$, respectively ($p > 0.05$), $n = 4$; Fig. 5b], consistent with the lack of an effect of the *Salvia* extracts on the sFas/FasL system. Therefore, the effects of the extracts are independent of receptor-mediated apoptosis, and alternative pathways are still to be explored.

Conclusions

Antiproliferative activities of ethanolic extracts from two *Salvia* spp. were described for the MCF7 and T47D cell lines and found to be due to both apoptosis and necrosis. Apoptosis was more obvious in T47D cells and does not occur via the receptor-mediated pathway, thus suggesting other multiple mechanisms. Extracts from both *Salvia* spp. may be useful in breast cancer management/treatment.

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