

Antitumoral effects of *Salvia absconditiflora* Greuter & Burdet syn. *Salvia cryptantha* Montbret & Aucher ex Benth. on Breast cancer

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This work aims to investigate the antiproliferative properties of *Salvia cryptantha* on breast cancer. *Salvia cryptantha* (SC) extracts were studied for cytotoxicity against the breast cancer cell lines. *In-vitro* apoptosis studies of breast cancer cells were performed by annexin V staining in flow cytometry analyses. Immunohistochemistry studies for Ki-67 and p16 in the tumoral tissue sections of Dimethyl Benzantracene (DMBA) induced mammary tumor in rats were performed. *In-vivo* anticancer activity testing was carried out by inhibiting the growth of mammary tumor in rats. SC showed cytotoxicity to three cancer cell lines. Annexin-positive cells level in SC treated cell lines were higher than the untreated control cells. The expression of the Ki-67 decreased in treatment groups compared with the control group. The expression of p16 protein was much higher for the rats treated by SC, compared with the untreated control group. *In vivo* studies showed that mean tumor volume inhibition ratio in SC treated group was 38 % compared with the untreated rats. These results indicate that *Salvia cryptantha* has antitumoral potential against breast cancer.

Keywords: *Salvia cryptantha*, Breast cancer, Antitumoral, Extract

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Salvia is the largest genus of plants in the mint family, Lamiaceae, with approximately 700-900 species of shrubs, herbaceous perennials, and annuals¹. The genus is distributed throughout the Old World and the Americas, with three distinct regions of diversity: Central and South America (approx. 500 species); Central Asia and Mediterranean (250 species); Eastern Asia (90 species)². This genus is represented in Turkey by 89 species with a total of 94 taxa, of which 45 are endemic in Turkey. The ratio of endemism in the genus *Salvia* in Turkey is about 45%³. Some members of the genus are of economic importance since they have been used as flavouring agents and in perfumery and cosmetics.

“*Tanshen*”, the rhizome of *Salvia miltiorrhiza*, has been used in traditional Chinese medicine for the treatment of coronary heart diseases, particularly angina pectoris and myocardial infarction⁴. The herb has also been applied for hemorrhage, dismenorrhea, miscarriage, swelling, and insomnia⁴. Numerous tanshinones were isolated from *S. miltiorrhiza*, and

diverse medicinal actions were reported, including antitumor, antioxidant and antimicrobial activities⁴⁻⁶.

In recent years, an upsurge of interest in the use of natural substances as phytomedicines has resulted in a more thorough investigation of plant resources. *Salvia cryptantha* is an aromatic herb belonging to Lamiaceae. The wild growing species is endemic to the rocky lands and chalky hills of Turkey, widely distributed from 700 to 2500 meter.³ *S. cryptantha* is a perennial bush branching in a disorderly fashion on the ground. Its older branches are brittle and rough while the younger ones are soft and covered by fine fuzz.⁷ Its flowers in May up to late August. *S. cryptantha* plants are generally propagated through cuttings. Yigit *et al.* reported the antimicrobial activity of *Salvia cryptantha*.⁸ Suntar *et al.* reported the wound healing promoting and antioxidant activities of the ethanol extracts of SC.⁹

As far as our literature survey could ascertain, antitumoral activity of *Salvia cryptantha* (SC) has not been previously reported elsewhere. In this study, we investigated the antitumoral effects of *Salvia cryptantha* on breast cancer.

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Materials and methods

Collection of the plant material

The herbal parts of SC was collected from Ziyarettepe district, Ulas, Sivas-Turkey, in 18.07.2009. Aerial parts (especially leafs and flowers) of the plant species were used. The taxonomic identification of plant materials was confirmed by a senior plant taxonomist, Dr. H. Askin AKPULAT, in Department of Biology, Cumhuriyet University, Sivas, Turkey. The voucher specimens have been deposited at the Herbarium of the Department of Biology, Cumhuriyet University, Sivas-Turkey (AA 4402).

Preparation of the SC extracts

Extracts of air-dried and ground plant materials were prepared by using water as solvent. A portion (100 gm) of dried plant material from SC was extracted with deionized water (yield; 5.72% w/w), in a Soxhlet apparatus during 6 hr. After this period, solvent-extract mixture was filtrated and filtrate was freeze-dried under vacuum. The extract obtained as powder was kept at room temperature until tested. It was dissolved in deionized water at 1 mg/mL concentration. Further dilutions were made in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, St Louis, MO, USA), as 5, 10, 15, 20, 25, 30, 40, 50, 60, 80, 100 µg/mL. According to the results exact Inhibitor Concentration 50 values (IC₅₀) of the SC extract for each breast cancer cell line was found. Inhibitory concentration (IC₅₀) represents the concentration of the extract that is required for 50% inhibition of the cell population *in vitro*.

Reagents and drugs

Dulbecco's Modified Eagle's Medium (DMEM), Fetal bovine serum (FBS), and 2.5% trypsin were purchased from Sigma-Aldrich St. Louis, MO, USA. FITC Annexin V Apoptosis Detection Kit I, was purchased from BD Pharmingen (San Diego, CA)."

Cell cultures

Breast cancer cell line (MCF) was purchased from Sap Institute, Ankara, Turkey; MDA-MB-231 and MDA-MB-468 were kindly donated by Dr. Uygur Tazebay Bilkent University, Ankara, Turkey and Dr. Saban Tekin Gaziosmanpaşa University Tokat, Turkey. All of these cells were maintained in DMEM supplemented with 1 mM SP, 2 mM L-glutamine, 2-fold MEM vitamins and 10% heat-inactivated FBS (56°C for 30 min). The monolayer cultures were

maintained in 75-cm² tissue culture flasks (Corning-Sigma-Aldrich St. Louis, MO, USA) at 37°C in a humidified 5% CO₂ incubator.

Cytotoxicity assay

The level of cytotoxicity SC extracts on MCF-7, MDA-MB-468 and MDA-MB-231 cells was determined using the trypan blue dye exclusion test¹⁰ and 3-(4,5-dimethylthiazol-2-yl)-2-diphenyltetrazolium bromide (MTT) proliferation assay¹¹. For MTT assay; first, tumor cells were seeded into 96- well tissue culture plates. After an attachment period of 6 hr at 37°C in a 5% CO₂ humidified incubator, the cells were treated with fresh medium alone or medium containing SC extracts for 48 hr. During the last 2 hr of incubation, 40 mL MTT (2.5 mg/mL; Sigma) was added into each well (0.42 mg/mL). At the end of the incubation, the MTT was removed and the cells were lysed with dimethylsulfoxide. Metabolically viable cells were monitored for conversion of MTT to formazan using a Multiskan FC 96-well microtiter plate reader at 570-nm (Thermo Scientific, MA, USA). The level of cytotoxicity was calculated using the following formula: cytotoxicity (%) = (A-B/ A) × 100, in which A is the 570-nm absorbance of cells treated with medium alone and B is the 570-nm absorbance of cells treated with SC extracts .

Every experiment also included one set of positive control (paclitaxel). All experiments were performed in triplicate and repeated at least three times.

Apoptosis assay of in-vitro studies of MCF-7, MDA-MB-468 and MDA-MB-231 cells

SC extracts induced apoptosis of MCF-7, MDA-MB-468 and MDA-MB-231 cells was studied by flow cytometry using annexin-V staining. In early stages of apoptosis, phosphatidyl serine is exposed at the external surface of the cell and can be detected by annexin-V. Late apoptotic cells and necrotic cells will also show Propidium Iodide (PI) positivity. Living cells, however, will show neither annexin-V nor PI positivity.

Briefly, MCF-7, MDA-MB-468 and MDA-MB-231 cells were untreated or treated for 24 hr with SC extracts at different dilutions. As a positive control, MCF-7, MDA-MB-468 and MDA-MB-231 cells were incubated in the presence of paclitaxel.

After incubation, cells were washed twice with cold PBS and then resuspended in 1× Binding Buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM CaCl₂,

pH [7.4]) at a concentration of 1×10^6 cells/mL. Then 100 μ L of the solution (1×10^5 cells) transferred to a 5 mL culture tube and 5 μ L of annexin V-FITC and 5 μ L Propidium Iodide added and incubated for 15 minutes at room temperature (25°C) in the dark. Later 400 μ L of 1 $\times$ Binding Buffer was added to each tube and analyzed by flow cytometry (Becton Dickinson FACS Calibur, Heidelberg, Germany) within 1 hr. All analyses were performed with CellQuest software (Becton Dickinson).

***In-vivo* tumor volume studies**

A total of 21 female albino Wistar rats (160 $\pm$ 10 gm body weight) 50-59 days old maintained in Animal Laboratory of Cumhuriyet University Faculty of Medicine, at temperature 24 $\pm$ 2°C with a 12 hr light/dark cycle and 60% $\pm$ 5% humidity. They were provided with standard pellet diet and water *ad libitum*. The experiment was carried out as per the guidelines of Ethical Committee for the Purpose of Control and Supervision of Experiments on Animals, of Cumhuriyet University Faculty of Medicine.

All rats were divided into three groups of 7 rats each. Rats in Groups I and II were induced mammary carcinogenesis by providing single subcutaneous injection in right pectoral area of 25 mg Dimethyl Benzantracene (DMBA) in 1 mL emulsion of sunflower oil (0.75 mL) and physiological saline (0.25 mL) to each rat. During the experimental period, animals were weighed weekly. Animals were observed daily to assess their general health.

After DMBA administration, right pectoral area of all rats were followed up for the tumoral development. Palpation of mammary tumors began 4 week after animals received DMBA. The volume of every tumor was measured weekly using calipers. Tumor volume was calculated using the formula: Tumor volume (cc) $(D \times d^2 \times \pi) / 6$ (D = big diameter, d = small diameter). The results are expressed as mean $\pm$ Standard Error (SE).

When the nodule reached to a mean volume of 250 $\pm$ 4.3 mm³ fine needle aspiration biopsy was performed from the nodules. Nodules reached to that size in a mean of 8 $\pm$ 2 weeks. Histopathological examination was performed from that biopsy for each nodule.

After histopathological examination revealed the breast cancer, treatments with SC extract was started in group II (Treatment Group). In Group I: control animals received no drug. In Groups II and III, rats

were given SC extract (100 mg/kg, body weight) daily through an oral gavage. Groups III, rats did not had DMBA induced mammary carcinogenesis, they had been used as control group for the side effects of SC extract treatment.

After 4 weeks of treatment, rats were sacrificed and tumors were removed from the animals of Groups I and II. The tumor volume inhibition ratio (%) was calculated by the following formula: Inhibition Ratio (%) = [(A - B) / A] $\times$ 100, where A is the average tumor volume of the control group, and B is the tumor volume of the treated group.

Histopathology and morphological observations

Each tumor tissues were sampled macroscopically and fixed in 10% formaldehyde solution for 24 hr. Tumor tissues then processed in autotechnicon device later embedded in paraffin blocks and cut sections with 3-5 μ m thickness were obtained and stained with Haematoxylin-eosin stain for routine histopathological examination by light microscopy.

Immunohistochemistry

For immunohistochemical staining, the deparaffinized and rehydrated tissue sections were inactivated the endogenous peroxidase by an incubation with 3% H₂O₂ for 10 minutes. To recover antigen, these sections were put into EDTA solution (pH 8.5) and heated in the microwave oven twice and subsequently cooled for 20 min. Next, the slides were washed in Phosphate Buffer Solution (PBS; pH 7.2) twice. Non-specific binding sites were blocked with Ultra VBlock (ScyTek Laboratories, Logan, Utah, USA) for 20 minutes. After the redundant liquid was discarded, the sections were incubated with primary antibody Ki-67 (ScyTek Laboratories, Logan, Utah, USA) and primary antibody p16 (Abcam, MA, USA) at room temperature for 1.5 hour and washed with PBS. Then the slides were incubated with biotinylated secondary antibody (ScyTek Laboratories, Logan, Utah, USA) for 20 minutes and washed with PBS, followed by incubation with streptavidin-HRP (ScyTek Laboratories, Logan, Utah, USA) for 20 minutes and washed with PBS. The antibody binding sites were visualized by incubation with a AEC chromogen (ScyTek Laboratories, Logan, Utah, USA) solution. The slides were counterstained for 1 min. with hematoxylin and then dehydrated with sequential ethanol for sealing and microscope observation.

Evaluation of Immunohistochemistry staining results

The percentage of proliferating neoplastic cells was evaluated directly by light microscopy. Quantification of the proliferation was performed by counting, Ki-67 positive cells in 4-6 random fields per slide. For evaluation of p16 immunohistochemistry, the semiquantitative scoring system was used in considering the staining intensity and area extent, which has been widely accepted and used in previous studies¹². Every tumor was given a score according to the intensity of the nuclear or cytoplasmic staining (no staining=0; weak staining=1; moderate staining=2; strong staining=3) and the extent of stained cells (0%=0; 1–10%=1; 11–50%=2; 51–80%=3; 81–100%=4). The final immunoreactive score was determined by multiplying the intensity and extent of positivity scores of stained cells, with the minimum score of 0 and a maximum score of 12.

Statistical analysis

Results are reported as mean $\pm$ Standard Error (SE). We tested the data from the experiments for statistical significance using the Mann-Whitney U Test. A p-value less than 0.05 was considered as significant.

Results

Effect of *Salvia cryptantha* on human breast cancer cells *in-vitro*

These results show that SC induces a significant reduction in the viability of MCF-7, MDA-MB-468, MDA-MB-231 cells in a dose-dependent manner (Fig. 1). Crude extracts of SC showed cytotoxicity to three cancer cell lines with IC₅₀ values 20 ± 1.4 μ g/mL, 22 ± 1.5 μ g/mL, 19 ± 1.2 μ g/mL respectively (Table 1) (Fig. 1).

Detection of cell apoptosis by flow cytometry

We confirmed apoptosis of MCF-7, MDA-MB-468 and MDA-MB-231 cells by flow cytometry analysis using Annexin-V staining. MCF-7, MDA-MB-468, MDA-MB-231 cells were untreated or treated for 24 hr with SC with IC₅₀ values 20 μ g/mL, 22 μ g/mL, 19 μ g/mL, respectively.

For MCF-7 cells percentage of Annexin-positive cells detected in treated cell lines were $20 \pm 2.4\%$, and

percentage of Annexin-positive cells detected in untreated cells were $12 \pm 1.8\%$. (Table 2) (Fig. 2). Results are representative of at least three independent experiments, all of which had similar results. Results were analyzed using the CELL Quest software program. When, these values were compared with each other result was statistically significant ($p < 0.05$). (Table 2) (Fig. 2).

For MDA-MB-468 cells those values were $21 \pm 2.3\%$ versus $13 \pm 1.5\%$, and for MDA-MB-231 cells those values were $19 \pm 2.5\%$ versus $11 \pm 1.4\%$. When, these values were compared with each other results were statistically significant ($p < 0.05$). (Table 2) (Fig. 2). These results showed that SC treatment have apoptosis inducing effect on MCF-7, MDA-MB-468 and MDA-MB-231 cells.

Immunohistochemistry Results

Immunohistochemistry experiments of this study showed that Ki-67 activity was most intensive in Untreated Control Group (Fig. 3a). The expression of Ki-67 in SC Treated Group was lower than the Untreated Control Group (Fig. 3b). When the semiquantitative scoring system results of immunohistochemistry studies were compared, the difference was statistically significant (Table 3)

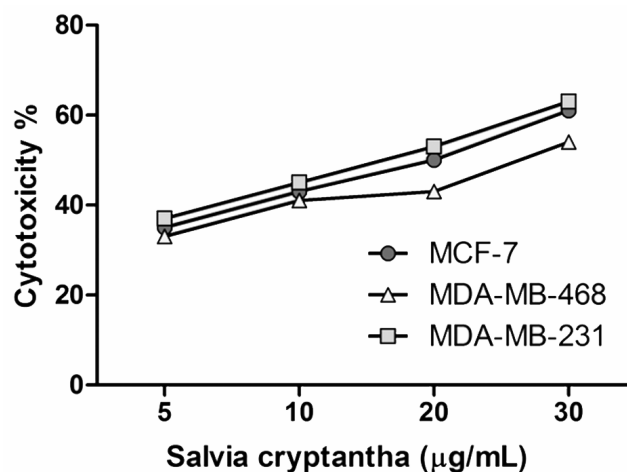


Fig. 1—Cytotoxicity of *Salvia cryptantha* against MCF-7, (circles), MDA-MB-468 (triangles), MDA-MB-231 (squares) cells.

Table 1—IC₅₀ values of *Salvia cryptantha* on MCF-7, MDA-MB-468, MDA-MB-231 breast cancer cells. Paclitaxel is positive control. The results are expressed as the mean $\pm$ S. E.

	MCF-7	MDA-MB-468	MDA-MB-231
<i>Salvia cryptantha</i>	20 ± 1.4 μ g/mL	22 ± 1.5 μ g/mL	19 ± 1.2 μ g/mL
Paclitaxel	12 ± 1.5 ng/mL	13 ± 1.4 ng/mL	10 ± 1.2 ng/mL

Table 2—Percentage of Annexin-positive cells detected in treated cell lines. The results are expressed as the means $\pm$ Standard Error. Statistical significance was set at $p < 0.05$. (Mann-Whitney U Test)

Percentage of annexin-positive cells	MCF-7	MDA-MB-468	MDA-MB-231
In untreated control cell lines	12 $\pm$ 1.8 %	13 $\pm$ 1.5 %	11 $\pm$ 1.4 %
In treated cell lines	20 $\pm$ 2.4 % ($p = 0.002$)	21 $\pm$ 2.3 % ($p = 0.002$)	19 $\pm$ 2.5 % ($p = 0.002$)

Table 3—Results of immunohistochemistry studies according to semiquantitative scoring system. Statistical significance was set at $p < 0.05$.

Immunohistochemical examination of tumoral tissues	Ki-67	p16
In untreated control rats	2.45 $\pm$ 0.08	1.21 $\pm$ 0.14
In <i>Salvia cryptantha</i> treated rats	1.30 $\pm$ 0.21	2.34 $\pm$ 0.21
Statistical results	($p = 0.003$)	($p = 0.004$)

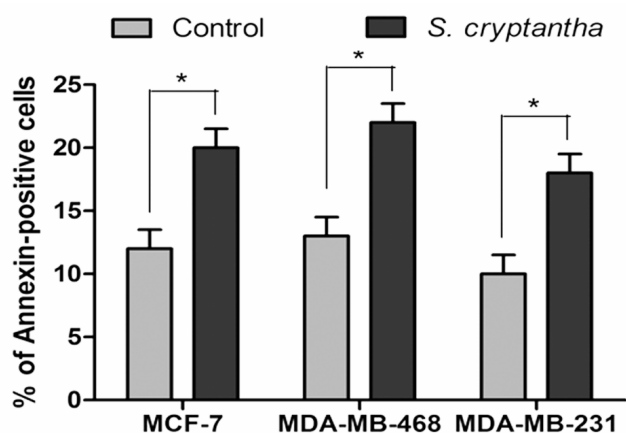


Fig. 2—When these values were compared with each other (Annexin-positive cells detected in treated cell lines versus annexin-positive cells detected in untreated cells), results (*) were statistically significant with each other ($p < 0.05$). These results showed that *Salvia cryptantha* treatment have apoptosis inducing effect on MCF-7, MDA-MB-468 and MDA-MB-231 cells.

($p < 0.05$). These results showed that SC has antitumoral potential.

In-vivo immunohistochemistry experiments of this study showed that the SC extract treatment induced the activation of p16 (Fig. 4a,b). When, the semiquantitative scoring system results of immunohistochemistry studies were compared, the difference was statistically significant (Table 3) ($p < 0.05$).

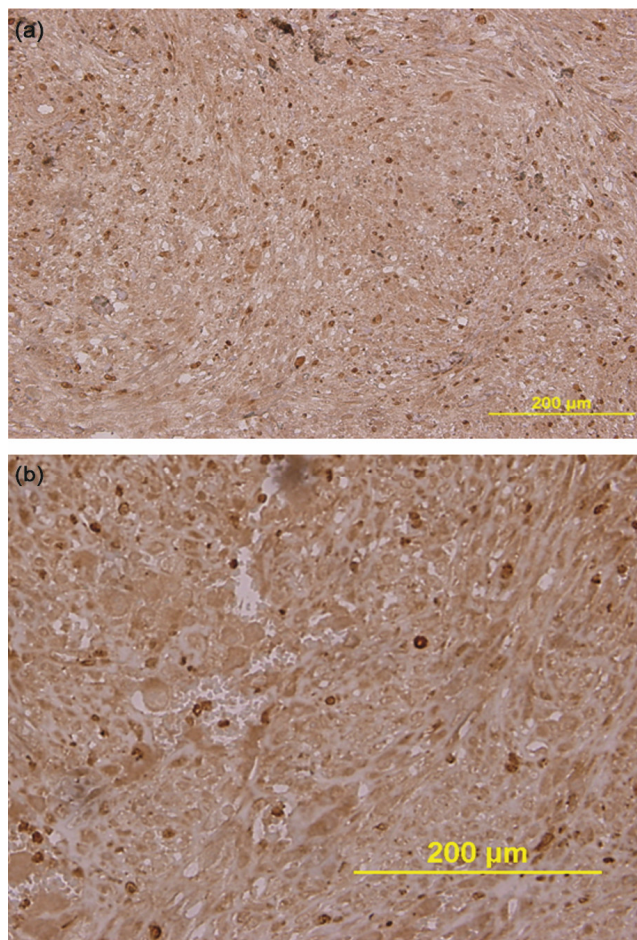


Fig. 3—Immunohistochemical experiments of Ki-67 in DMBA induced mammary tumor tissue of rats. a) Untreated Control Group (Group I) b) *Salvia cryptantha* treated Group (Group II).

Tumor volume studies

After 4 weeks of treatment, the mean tumor volume inhibition ratio in SC treated group (Group II) was 38% compared with the untreated rats (Group I) (Table 4). Tumor volume inhibition ratio differences between the treatment and control groups were all statistically significant ($p < 0.05$) (Table 4, Figs 5 & 6). No signs of toxicity (weight loss, ruffled fur and

Table 4—When the tumor volume reached to $250 \pm 4.3 \text{ mm}^3$, treatment with SC started in (Group II). Control group (Group I) received no treatment. The tumor volume inhibition ratio (%) was calculated by the following formula: inhibition ratio (%) = $[(A - B)/A] \times 100$, where A is the average tumor volume of the control group, and B is the tumor volume of the treated group. The results represent the mean tumor volume $\pm$ Standard Error (mm^3). Statistical significance was set at $p < 0.05$. (Mann-Whitney U Test)

	Beginning	1 week	2 week	3 week	4 week
Control (Group I)	250 ± 5.3	370 ± 7.3	640 ± 8.4	980 ± 10.1	1250 ± 24.3
Treated (Group II)	250 ± 6.4	303 ± 6.4	499 ± 7.2	714 ± 8.1	775 ± 9.1
Tumor Volume Inhibition ratio (%)	0	18	22	27	38
	-	($p = 0.002$)	($p = 0.002$)	($p = 0.002$)	($p = 0.002$)

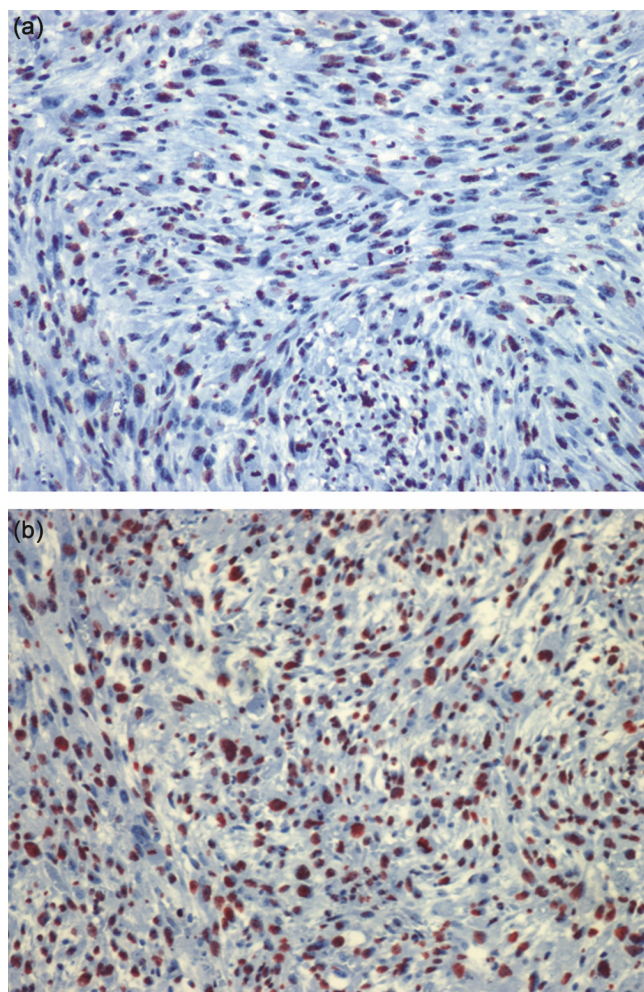


Fig. 4—Immunohistochemical experiments of p16 in DMBA induced mammary tumor tissue of rats. a) Untreated Control Group; b) *Salvia cryptantha* treated Group.

behavioral changes) were observed in any of the treated rats (Group II and III).

Discussion

Medicinal plants are becoming an important research area for novel and bioactive molecules for drug discovery. Origin of the word “*Salvia*” comes



Fig. 5—DMBA induced mammary tumor model of rats. Circles point out the tumoral masses.

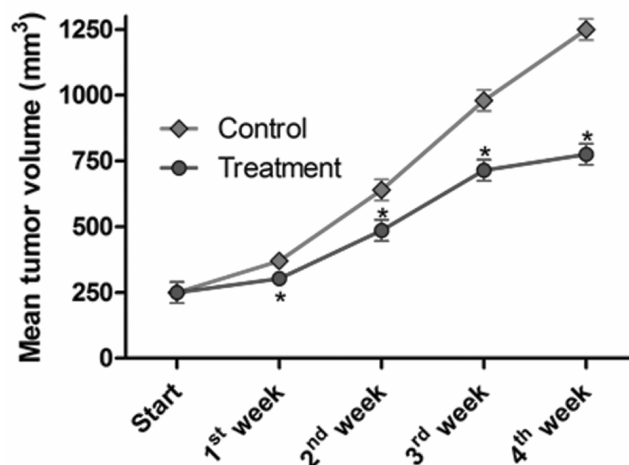


Fig. 6—After 4 weeks of treatment, the mean tumor volume inhibition ratio in SC treated group (Group II) was 38% compared with the untreated rats (Group I). Tumor volume inhibition ratio differences between the treatment and control groups were all statistically significant ($p < 0.05$).

from the Latin “*salvere*” and meaning save, rescue and protect. *Salvia cryptantha* is a plant, which has shown several types of biological activity. Akin *et al.* reported the antibacterial activity of SC and they attributed this activity to 1,8-cineole, camphor and borneol of the *S. cryptantha* oil.¹³

Among this genus *Salvia miltiorrhiza* is one of the most studied plant. It is also known as Chinese sage, is a perennial plant in the genus. It has been widely used in China and, to a lesser extent, in Japan, the United States, and other European countries for the treatment of cardiovascular and cerebrovascular diseases. In China, the specific clinical use is angina pectoris, hyperlipidemia, and acute ischemic stroke^{14,15}. An antioxidant called salvianolic acid (or salvianolic acid B) isolated from *Salvia miltiorrhiza* is under study for protection against cerebrovascular disorders¹⁶. Tanshinone IIA (Tan IIA) is one of the most abundant constituents of the root of *Salvia miltiorrhiza* which exerts antioxidant and anti-inflammatory actions in many experimental disease models¹⁷. Tan IIA has been widely used for various cardiovascular and cerebrovascular disorder in Asian countries¹⁸. Tan IIA may improve renal dysfunction associated with chronic kidney disease¹⁹. Tan IIA was effective for attenuating the extent of brain edema formation in response to ischemia injury in rats²⁰. The chemicals isolated from *Salvia miltiorrhiza*, Dihydrotanshinone, tanshinone I and Tan IIA are also under study for anti-cancer effects^{21,22}. *Salvia miltiorrhiza* may stop the spread of several different cancer cell types by interrupting the cell division process and also by causing cancer cells to undergo cell death (apoptosis)^{22,23}.

In this study, *Salvia cryptantha* showed cytotoxicity to three cancer cell lines with IC₅₀ values 20 ± 1.4 µg/mL, 22 ± 1.5 µg/mL, 9 ± 1.2 µg/mL, respectively. In this study, those IC₅₀ values were considered as “good” activity on breast cancer cell lines. Apoptosis plays an important role in the fight against cancer. The format (Annexin V-FITC) retains its high affinity for apoptotic cell membrane phospholipid phosphatidylserine and thus serves as a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis. In this study Annexin-positive cells were detected by flow cytometry in SC treated cell lines at 20 ± 2.4% in MCF-7 cells; 21 ± 2.3% in MDA-MB-468 cells; 19 ± 2.5% in MDA-MB-231 cells (Table 2), compared with 12 ± 1.8%, 13 ± 1.5%, 11 ± 1.4%, of annexin-positive cells detected in untreated cells respectively. So, results of this assay showed that SC extracts treatment induced apoptosis in breast cancer cells.

Antigen Ki-67 is a protein that in humans is encoded by the MKI67 gene²⁴. It is a nuclear protein

and associated with cell proliferation and it is an excellent marker to determine the growth fraction of a given cell population²⁵. In this study, we studied the Ki-67 levels of the DMBA induced tumoral tissues to determine the SC effect on tumoral growth. *In-vivo* immunohistochemistry experiments of this study showed that the SC extract treatment decreased the Ki-67 activity in tumoral tissues of rats. So, results of this assay showed that SC extracts has anticancer potential.

Cyclin-dependent kinase inhibitor 2A, (CDKN2A, p16) also known as multiple tumor suppressor 1 (MTS-1), is a tumor suppressor protein, that in humans is encoded by the *CDKN2A* gene²¹. P16 plays an important role in regulating the cell cycle, and mutations in p16 increase the risk of developing a variety of cancers. This gene is frequently mutated or deleted in a wide variety of tumors and is known to be an important tumor suppressor gene²⁶. Homozygous deletion of p16 are frequently found in esophageal cancer and gastric cancer cell lines²⁷. Increased expression of the p16 gene as organisms age reduces the proliferation of stem cells²⁸. *In-vivo* immunohistochemistry experiments of this study showed that the SC extract treatment induced the activation of p16. These results may suggest that increased expression of p16 may have a role in the antitumoral potential of SC.

In DMBA induced rat mammary tumor volume experiments, treatment with SC showed a 38% tumor volume inhibition. This difference is statistically significant ($p < 0.05$). This result showed the tumor growth inhibition potential of SC crude extract.

In conclusion, it is worthwhile to screen the commonly used plants from the local flora for different biological activities because they might present a new alternative source for possible bioactive substances. These results showed that natural *Salvia cryptantha* extract may constitute a potential antitumor compound against breast cancer. Further studies needed to reach the main anticancer molecule or molecules of this crude extract.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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