

RESEARCH COMMUNICATION

Antitumoral Effects of *Melissa officinalis* on Breast Cancer *in Vitro* and *in Vivo*

Serpil Unver Saraydin¹, Ersin Tuncer², Bektaş Tepe³, Sule Karadayi⁴, Hatice Özer², Metin Şen⁵, Kursat Karadayi⁵, Deniz Inan¹, Şahande Elagöz², Zübeyde Polat⁶, Mustafa Duman⁷, Mustafa Turan^{5*}

Abstract

Background: There is a long standing interest in the identification of medicinal plants and derived natural products for developing cancer therapeutics. Here we investigated the antiproliferative properties of *Melissa officinalis* (MO) from Turkey on breast cancer. **Methods:** MO extracts were studied for cytotoxicity against breast cancer cell lines (MCF-7, MDA-MB-468 and MDA-MB-231). *In vitro* apoptosis studies were performed by annexin V staining and flow cytometry analyses. Immunohistochemistry for Ki-67 and caspase 7 in the tumoral tissue sections of DMBA-induced mammary tumors in rats was also performed, along with TUNEL assays to detect apoptotic cells. *In vivo* anticancer activity testing was carried out with reference to inhibition of growth of DMBA induced mammary tumors in rats. **Results:** MO showed cytotoxicity against three cancer cell lines, inducing increase in Annexin-positive cells. Expression of caspase-7 protein and TUNEL positive cells were much higher in rats treated by MO, compared with the untreated control group, while expression of Ki-67 was decreased. Furthermore, *in vivo* studies showed that mean tumor volume inhibition ratio in MO treated group was 40% compared with the untreated rats. **Conclusion:** These results indicated that MO extracts have antitumoral potential against breast cancer.

Keywords: *Melissa officinalis* - breast cancer - antitumoral effects

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Introduction

The plant kingdom provides an enormous potential for discovery of new drugs for the treatment and prevention of diseases. Some of the plant species, including *Taxus baccata*, *Podophyllum peltatum*, *Camptotheca acuminate*, and *Vinca rosea* have well-recognized anticancer activity in breast cancer, and several isolated pure compounds and their semisynthetic derivatives have been evaluated in clinical trials and marketed (Mukherjee et al., 2001).

Lemon balm, *M. officinalis* L. is one of the most used medicinal plants in Europe and the Mediterranean region, as a herbal tea for its aromatic, digestive and antispasmodic properties in nervous disturbance of sleep and functional gastrointestinal disorders (Bisset et al., 1994). The leaves emit a distinct fragrant lemon odour when bruised. The chemical composition is essential oil, polyphenolic compounds: caffeic acid derivatives in large proportions, such as rosmarinic acid, trimeric compounds and also some flavonoids such as luteolin-7-O-glucoside. Some pharmacological properties have been attributed to the principal constituents (De Sousa et al., 2004). Enriched extracts containing rosmarinic acid are used as a virostatic

against herpes viruses, alcohol extracts as sedatives and the essential oil as a digestive aid in pharmaceutical preparations (Kucera et al., 1965; López et al., 2009).

According to the *in-vitro* cytotoxicity analysis, using MTT and NR assays, this plant found as very effective against human colon cancer cell line (HCT-116) (Encalada et al., 2011). *M. officinalis* is a plant, which has shown several types of biological activity. Essential oil from *M. officinalis* has a great economical importance due to its use in industry, especially the pharmaceutical industry. de Sousa et al, demonstrated that *M. officinalis* essential oil inhibited the viability of several tumour cell lines in a concentration-dependent manner. In this study, we investigated the antitumoral effects of *Melissa officinalis* on breast cancer.

Materials and Methods

Collection of the plant material

The herbal parts of *O. acutidens* was collected from Ziyarettepe district, Ulas, Sivas-Turkey, in 18.07.2009. The taxonomic identification of plant materials was confirmed by a senior plant taxonomist, Dr. H. Askin

¹Department of Histology and Embryology, ²Department of Pathology, ³Department of Molecular Biology and Genetics, ⁴Department of Thoracic Surgery, ⁵Department of General Surgery, ⁶Department of Parasitology, Faculty of Medicine Cumhuriyet University, Sivas, ⁷General Surgery, Kosuyolu Education and Research Hospital, Istanbul, Turkey *For correspondence: mturan9@gmail.com

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 *M. officinalis* extracts

Extracts of air-dried and ground plant materials were prepared by using water as solvent. A portion (100 g) of dried plant material from MO was extracted with deionized water (yield; 5.72% w/w), in a Soxhlet apparatus during 6 h. 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 and further dilutions were made in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, St Louis, MO, USA).

Reagents and drugs

Dulbeccos modified Eagles medium (DMEM), Fetal bovine serum (FBS), and 2.5% trypsin were purchased from Sigma-Aldrich St. Louis, MO, USA. All reagents were free of endotoxins as determined using the Limulus ameocyte lysate assay (sensitivity limit, 0.025 ng/ml), which was purchased from Sigma (St Louis, MO). FITC Annexin V Apoptosis Detection Kit I, were purchased from BD Pharmingen (San Diego, CA).

Cell cultures

Two human breast cancer cell lines (MCF-7, MDA-MB-468) were purchased from Sap Institute, Ankara, Turkey) and MDA-MB-231 was kindly donated by Dr. Uygur Tazebay Bilkent University, Ankara, Turkey. All of these cells were maintained in Dulbeccos modified Eagles medium (DMEM) supplemented with 1mM SP, 2mM 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 MO extracts on MCF-7, MDA-MB-468 and MDA-MB-231 cells was determined using the trypan blue dye exclusion test (Roomi et al., 2011) and 3-(4,5-dimethylthiazol-2-yl)-2, diphenyltetrazolium bromide (MTT) proliferation assay. For MTT assay; first, tumor cells were seeded into 96-well tissue culture plates. After an attachment period of 6 h at 37 °C in a 5% CO₂ humidified incubator, the cells were treated with fresh medium alone or medium containing MO extracts for 48 h. During the last 2 h 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) x 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 MO extracts.

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

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

MO 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 h with MO 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 1X Binding Buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM CaCl₂, pH [7.4]) at a concentration of 1 x 10⁶ cells/mL. Then 100 µl of the solution (1 x 10⁵ cells) transferred to a 5 ml culture tube and 5 µl of annexin V-FITC and 5 µl Propidium Iodide added and incubated for 15 min at room temperature (25°C) in the dark. Later 400 µl of 1X 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±10 g body weight) 50-59 days old maintained in Animal Laboratory of Cumhuriyet University Faculty of Medicine, at temperature 24±2 °C with a 12 hour light/dark cycle and 60%±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 DMBA in 1 mL emulsion of sunflower oil (0.75 mL) and physiological saline (0.25 mL) to each rat (Chidambaram et al., 1996). 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) (Dx d² x π) / 6 (D= big diameter, d= small diameter). The results are expressed as the mean±Standart Error.

When the nodule reached to a mean volume of 250±4.3

mm³ (± S. E.), fine needle aspiration biopsy was performed from the nodules. Nodules reached that size in a mean of 8±2 weeks. Histopathological examination was performed from that biopsy for each nodule.

After histopathological examination revealed the breast cancer, treatments with MO extract was started in group II (Treatment Group). In Group I: control animals received no drug. In Groups II and III, rats were given MO 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 MO 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] x 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 hours. Tumor tissues then processed in autotechnicon device later embedded in paraffin blocks and cut sections with 3-5 µm thickness were obtained and stained with Haematoxylin-Eosin stain for routine histopathological examination by light microscopy.

Immunohistochemistry

Caspase 7: Tumor sections were deparaffinized with xylene and dehydrated with ethanol. The slides were then immersed in water for 10 min. For antigen retrieval, the slides were boiled in EDTA buffer, pH 8.2, for 15 min. in a microwave oven and subsequently cooled for 20 min. Next, the slides were washed in PBS and endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 15 min. After washing with Phosphate Buffer Solution (PBS), the sections were incubated at 27 °C for one and a half hour for Caspase 7 antibody. After antibody reaction, the slides were washed and incubated with a multilink antibody for 20 min., washed in PBS and incubated for 20 min with the avidin-biotin-peroxidase complex. After washing with PBS, the slides were incubated for 3 min with AEC chromogen, and finally counter-stained with hematoxylin prior to mounting.

Ki -67: 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. The slides were then washed with PBS (pH 7.2-7.6) twice. Non-specific binding sites were blocked with Ultra V Block (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) 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 kromogen (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.

In immunohistochemistry studies the semiquantitative scoring system was used in considering the staining intensity and area extent, which has been widely accepted and used in previous studies (Chih-Ping et al., 2009). 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 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. Caspase-7 activity was evaluated semi quantitatively in the cytoplasm of the tumor cells either in living or in the necrotic tumor cell areas.

In vivo apoptosis assay

Apoptosis was evaluated by using terminal deoxynucleotidyl transferase dUTP nick and labelling (TUNEL) method. In Situ Cell Death Detection Kit, POD (Roche, Germany) is used for apoptosis. Tumor sections were deparaffinized and dehydrated according to standard protocols. Tissue sections were incubated with Proteinase K working solution for 30 min at 21-37°C. The slides were then washed with PBS (pH 7.2-7.6) twice. Positive control was incubated with DNase I recombinant for 10 min at 15-25 °C. Negative control was incubated with Label solution (without terminal transferase) instead of TUNEL reaction mixture. The slides were then washed with PBS (pH 7.2-7.6) three times. Converter-POD was added on slides and incubated in a humidified chamber for 30 min at 37°C. The slides were then washed with PBS (pH 7.2-7.6) three times. DAB Substrate was added on slides and incubated for 10 min at 15-25°C. The slides were then washed with PBS (pH 7.2-7.6) three times. The slides were mounted and analysed by fluorescence microscope (Olympus DP 70, Melville, NY, USA).

Statistical analysis

Results are reported as mean±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 significant.

Results

Effect of Melissa officinalis on human breast cancer cells in vitro

These results show that MO induces a significant reduction in the viability of MCF-7, MDA-MB-468, MDA-MB-231 cells in a dose-dependent manner (Figure 1). Crude *extracts* of MO showed cytotoxicity to three cancer cell lines with IC₅₀ values 18±2.0 µg/mL, 17±1.4

Table 1. IC₅₀ Values of OA on MCF-7, MDA-MB-468, MDA-MB-231 Breast Cancer Cells. Paclitaxel is Positive Control. The Results are Expressed as the Mean±S. E.

	MCF-7 (µg/mL)	MDA-MB-468 (µg/mL)	MDA-MB-231 (µg/mL)
<i>Melissa officinalis</i>	18±2.0	17±1.4	19±1.8
Paclitaxel	12±1.4	13±2.1	10±1.7

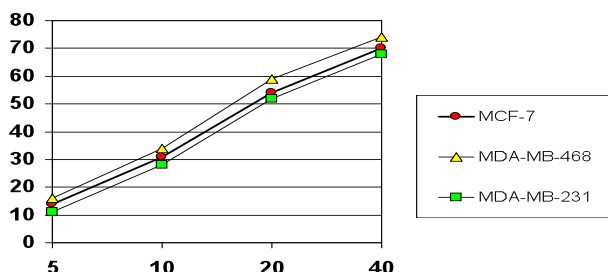


Figure 1. Cytotoxicity of *Melissa officinalis* (MO) against MCF-7, (circles), MDA-MB-468 (triangles), MDA-MB-231 (squares) cells. Target cells were incubated with OA for 48 h at different concentrations (µg/mL). Cytotoxicity was then determined using the trypan blue dye exclusion test and MTT proliferation assay. Triplicate wells were used for each group. Each point represents the mean±SE. One of at least three independent experiments . (p=0.002)

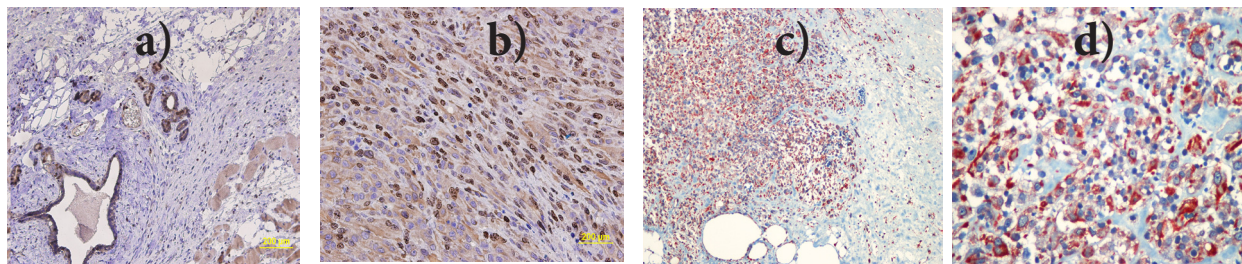


Figure 2. Immunohistochemical Experiments of Ki-67 in DMBA Induced Mammary Tumor Tissue of Rats. a) Untreated Control Group; b) Melissa treated Group, Caspase-7 expression in breast tumor (T) cells in the animal model treated with Melissa extraction. Here the expression is strong and note that expression is very weak and almost negligible in the non-tumoral area (NT). (Figure 2.c ; X100, Figure 2.d X 400)).

µg/mL, 19±1.8 µg/mL, respectively (Table 1 and Figure 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 h with AS with IC₅₀ values 18 µg/mL, 17 µg/mL, 19 µg/mL respectively.

Percentage of Annexin-positive cells detected in treated cell lines were 26±2.1% in MCF-7 cells; 25±2% in MDA-MB-468 cells; 24±1.9% in MDA-MB-231 cells, compared with 12±1.4%, 13±1.2%, 10±1.0%, of Annexin-positive cells detected in untreated cells respectively (Table 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 (Annexin-positive cells detected in treated cell lines versus annexin-positive cells detected in untreated cells) ,

Table 2. Percentage of Annexin-Positive Cells Detected in *Melissa Officinalis* Treated Cell Lines. The Results are Expressed as the Means ±Standart Error. (p<0.05).

	MCF-7	MDA-MB-468	MDA-MB-231
In untreated control cell lines	12±1.4%	13±1.2%	10±1.0%
In <i>Melissa officinalis</i> treated cell lines	26±2.1% (p=0.002)	25±2% (p= 0.002)	24±1.9% (p=0.002)
	(p< 0.05)	(p< 0.05)	(p< 0.05)

Table 3. Results of Immunohistochemistry Studies According to Semiquantitative Scoring System. The Results Represent the Mean Tumor Volume±S.E. (mm³) (p<0.05).

	Ki-67	Caspase-7	TUNEL-Ve+ apoptotic cells
In untreated control rats	2.41±0.08	0.50±0.14	0.64±0.14
In <i>Melissa officinalis</i> treated rats	1.10±0.12 (p=0.003)	2.60±0.20 (p=0.003)	2.41±0.14 (p=0.002)
Statistical results	(p< 0.05)	(p< 0.05)	(p< 0.05)

results were statistically significant with each other. These results showed that MO 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; (Figure 3-a). The expression of Ki-67 in MO treated Group was lower than the Untreated Control Group (Figure 3-b).

When the semiquantitative scoring system results of immunohistochemistry studies were compared, the difference was statistically significant (Table 3) (p< 0.05).

In-vivo immunohistochemistry experiments of this study showed that the MO extract treatment induced the activation of Caspase-7 (Figure 2, 3). When the semiquantitative scoring system results of immunohistochemistry studies were compared, the difference was statistically significant (Table 3) (p< 0.05).

At the result of the Terminal Transferase dUTP Nick

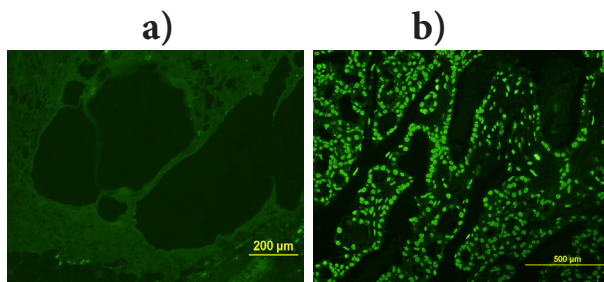


Figure 3. Representative Immunohistochemistry Photomicrographs of TUNEL-Positive Apoptotic Cells in DMBA Induced Rat Mammary Tumor Tissue of Rats. a) Untreated Control Group; b) Melissa treated Group.



Figure 4. DMBA Induced Mammary Tumor Model of Rats. Circles point out the tumoral masses.

Table 3. When the Tumor Volume Reached to 250±4.3 mm³, Treatment with MO 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] x 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±Standard Error (mm³) (p<0.05).

	Beginning	1 week	2 week	3 week	4 week
Control (Group I)	250±4.3	372±7.1	641±9.4	984±12.5	1255±21.3
OA Treated (Group II)	250±4.3	303±7.2	488±9.4	670±10.2	748±14.1
Tumor Volume Inhibition ratio (%)	0	18	24	32	40
	-	(p=0.002)	(p=0.002)	(p=0.002)	(p=0.002)
		(p< 0.05)	(p< 0.05)	(p< 0.05)	(p< 0.05)

End Labeling (TUNEL) Assay of this study while TUNEL positive cells has been detected rarely in the Untreated Control group, numerous TUNEL positive cells were intensively observed in the *Allium Sivasicum* treated group (Figure 4). When the semiquantitative scoring system results of TUNEL Assay studies were compared, the difference was statistically significant (Table 3) (p< 0.05).

Tumor Volume Studies

After 4 weeks of treatment, the mean tumor volume inhibition ratio in AS treated group (Group II) was 40% 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, Figure 5). No signs of

toxicity (weight loss, ruffled fur and behavioral changes) were observed in any of the MO treated rats (Group II and III).

Discussion

The ingestion of plant *extracts* with medicinal properties represents an alternative for the treatment of different pathological states in economically unprivileged countries.

However, in the absence of a scientific basis, such practices may generate serious adverse effects. The analysis of the pharmacological activity of plant *extracts* may therefore make possible the design of less expensive therapies to be used in developing regions.

M. officinalis showed cytotoxicity to three cancer cell lines with IC₅₀ values

18±2.0 μg/ml, 17±1.4 μg/ml and 19±1.8 μg/ml respectively. In this study, those IC₅₀ values were considered as “good” activity on breast cancer cell lines. The antitumour properties of several antioxidants have been described (Keum et al., 2000). Antioxidant activity of Melissa tea and hydro-alcoholic *extracts* have been described previously (Lamiason et al., 1991). Thus our data indicated that the antioxidant properties of *M. Officinalis* essential oil might contribute to its antitumoral activity.

Apoptosis plays an important role in the fight against cancer. The format (Annexin V-FITC) retains its high affinity for PS and thus serves as a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis (Vermes et al., 1995). In this study Annexin-positive cells were detected by flow cytometry in MO treated cell lines at 26±2.1% in MCF-7 cells; 25±2% in MDA-MB-468 cells; 24±1.9% in MDA-MB-231 cells (Table 2), compared with 12%, 13%, 10%, of annexin-positive cells detected in untreated cells respectively. So, results of this assay showed that MO *extracts* treatment induced apoptosis in breast cancer cells.

During apoptosis a group of proteases are activated which cause DNA fragmentation, cytoplasmic shrinkage and membrane blebbing. Here, we therefore examined apoptosis in tumoral tissues by using terminal deoxynucleotidyl transferase dUTP nick and labelling (TUNEL) method. TUNEL Assay results of our *in-vivo* studies demonstrated that MO *extracts* treatment increased the apoptotic cells in tumoral tissues of MO treated rats.

Antigen KI-67 is a protein that in humans is encoded by the MKI67 gene (Bullwinkel et al., 2006). 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 population (Scholzen et al., 2000). In this study, we studied the Ki-67 levels of the DMBA induced tumoral tissues to determine the MO effect on tumoral growth. *In-vivo* immunohistochemistry experiments of this study showed that the MO extract treatment decreased the Ki-67 activity in tumoral tissues of rats.

Caspase-7 is a caspase protein that plays a key role in programmed cell death, or apoptosis. Caspase-7 is activated in the apoptotic cell both by extrinsic (death

ligand) and intrinsic (mitochondrial) pathways (Shin et al., 2001). In caspase cascade pathway, mature caspases-3 and -7 cleave a large set of substrates, ultimately resulting in the characteristic morphological and biochemical hallmarks of apoptosis. *In-vivo* immunohistochemistry experiments of this study showed that the MO extract treatment induced the activation of caspase-7. These results may suggest that MO induced the apoptosis in breast cancer cells via the caspase cascade pathway.

In DMBA induced rat mammary tumor volume experiments, treatment with MO showed a 40% tumor volume inhibition. This difference is statistically significant ($p < 0.05$). This result showed the tumor growth inhibition potential of MO crude extract.

In conclusion, these results showed that natural *Melissa officinalis* extract may constitute a potential antitumor compound against breast cancer. Experiments are necessary to identify which of the components are responsible for these activities.

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