

Antitumor effects of *Origanum acutidens* extracts on human breast cancer

E. Tuncer¹, S. Unver-Saraydin², B. Tepe³, S. Karadayi⁴, H.Ozer¹, M. Sen⁵, K. Karadayi⁵, D. Inan², S. Elagoz¹, Z. Polat⁶, M. Duman⁷, M. Turan⁵

¹Department of Pathology, ²Department of Histology and Embryology, ³Department of Molecular Biology and Genetics, Cumhuriyet University Faculty of Medicine, Sivas; ⁴Department of Thoracic Surgery, Baskent University Faculty of Medicine, Sivas; ⁵Department of General Surgery, ⁶Department of Parasitology, Cumhuriyet University Faculty of Medicine, Sivas; ⁷Kosuyolu Education and Research Hospital, General Surgery, Istanbul, Turkey

Summary

Purpose: There has been a long-standing interest in the identification of medicinal plants and derived natural products for developing anticancer agents. This work aimed at investigating the antiproliferative properties of *Origanum acutidens* (OA) on breast cancer.

Methods: OA water extracts were studied for cytotoxicity against the breast cancer cell lines MCF-7, MDA-MB-468 and MDA-MB-231. *In vitro* apoptosis studies of these cancer cell lines were performed by annexin V staining in flow cytometry analyses. Immunohistochemistry studies for Ki-67 and caspase-7 of tumor tissue sections of dimethylbenzanthracene (DMBA) -induced mammary cancer in rats were also performed. TUNEL assay was used to detect apoptotic cells of tumor tissue. *In vivo* anticancer activity testing was carried out by inhibiting the growth of DMBA-induced mammary cancer in rats.

Results: OA showed cytotoxicity on all 3 cancer cell lines. Annexin-positive cells level in OA-treated cell lines were significantly higher compared with untreated control cells ($p=0.002$). The expressions of caspase-7 protein and TUNEL-positive cells were much higher for the rats treated by OA, compared with the untreated control group ($p<0.05$). The expressions of the Ki-67 decreased in the treated groups compared with the control group ($p<0.05$). *In vivo* studies showed that the mean tumor volume inhibition ratio in OA-treated group was 41 % compared with the untreated rats ($p<0.05$).

Conclusion: These results indicate that OA has antitumor activity against breast cancer cell lines.

Key words: antitumor effect, breast cancer, *origanum acutidens*

Introduction

Plants have been used as food, spices and medicines for thousands of years. There are at least 250 000 species of vascular plants existing worldwide out of which more than 1,000 plants have been found to possess anticancer properties [1,2]. Various active compounds derived from medicinal plants have been assessed for their efficacy and tolerability in the treatment of human breast cancer. Some of these plant species, including *Taxus brevifolia*, *Taxus baccata*, *Podophyllum peltatum*, *Camptotheca acuminata*, and *Vinca rosea* have well-recognized anticancer activity in breast and other human malignancies, and several isolated pure compounds and their semisynthetic derivatives have been evaluated in clinical trials and marketed [3].

The genus *Origanum* (*oregano*), an important genus of the *Lamiaceae* family, is widespread throughout the world, comprising about 900 species. About 20 species of this genus exist in the Turkish flora [4]. *Origanum* species have traditionally been used to substitute for thyme as a spicy food additive. This genus is rich in essential oils and bitter substances [4]. The best known species of *Origanum* genus in Anatolia are “Yalancikkekik”, “Kekik”, “Istanbul kekigi” and “Keklik otu”. OA is an endemic species found in northeastern Turkey. The essential oil composition of this species has been previously reported [5,6].

OA was analyzed by gas chromatography/mass spectrometry (GC-MS). Carvacrol (87.0%), p-cymene (2.0%), linalool acetate (1.7%), borneol (1.6%) and β -caryophyllene (1.3%) were found to be the main constituents of oil component. In this study we mainly focused on the extracts of this species rather than the essential oil [5]. The phytochemical composition of this plant species has not previously been reported.

As far as our literature survey could ascertain, antitumor activity of OA has not previously been reported elsewhere. In this study, we investigated the antitumor effects of OA (*Lamiaceae*) on breast cancer cell lines *in vitro* and *in vivo*.

Methods

Collection of the plant material

The herbal parts of OA were collected from the Ziyarettepe district, Ulas, Sivas, Turkey, on 18.07.2009. The taxonomic identification of plant materials was confirmed by

a senior plant taxonomist (Dr. H.Askin Akpulat), 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 OA 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 OA was extracted with deionized water (yield 5.72 % w/w) in a Soxhlet apparatus during 6 h. After this period, the solvent-extract mixture was filtrated and filtrate was freeze-dried in vacuum. The extract obtained as powder (5.72g from 100g dried plant) 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's Medium (DMEM) (Sigma, St Louis, MO, USA).

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). All reagents were free of endotoxins as determined using the *Limulus* amoebocyte lysate assay (sensitivity limit 0.025 ng/ml), which was purchased from Sigma (St Louis, MO). FITC Annexin V Apoptosis Detection Kit I, was 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 cell lines were maintained in DMEM supplemented with 1mM Sodium pyruvate (SP), 2mM L-glutamine, 2-fold Minimal Essential medium (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 OA extracts on MCF-7, MDA-MB-468 and MDA-MB-231 cells was deter-

mined using the trypan blue dye exclusion test [7] 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 OA extracts for 48 h. During the last 2 h of incubation, 40 µL MTT (2.5 mg/mL; Sigma) were added into each well (0.42 mg/mL). At the end of 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 OA 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

OA 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 was exposed at the external surface of the cell and could be detected by annexin-V. Late apoptotic cells and necrotic cells would also show propidium iodide (PI) positivity. Living cells, however, would 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 OA extracts with IC₅₀ values 15µg/mL, 13µg/mL, 14µg/mL, respectively. Untreated control MCF-7, MDA-MB-468 and MDA-MB-231 cells were also incubated for 24h.

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) were transferred to a 5 ml culture tube and 5 µl of annexin V-FITC and 5 µl PI was 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 h. 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 the Animal Laboratory of Cumhuriyet University Faculty of Medicine, at temperature 24±2 °C with a 12 h light/dark cycle and 60% ± 5% humidity were used. 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 3 groups of 7 rats each. Rats in groups I and II were induced mammary carcinoma with a single subcutaneous injection in the right pectoral area of 25 mg DMBA in 1 mL emulsion of sunflower oil (0.75 mL) and normal saline (0.25 mL) to each rat. During the experimental period, animals were weighed weekly and were observed daily to assess their general health.

After DMBA administration, the right pectoral area of all rats was observed for tumor development. Palpation of mammary tumors began 4 week after administration of DMBA. The volume of each 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 were expressed as mean ± standard error (SE).

When the nodule reached a mean volume of 250 ± 4.3 mm³, fine needle aspiration biopsy was performed. Nodules reached that size in a mean of 8 ± 2 weeks.

After histopathological examination revealed mammary carcinoma, treatment with OA extract started in group II (treatment group). In group I (control group) animals received no drug. In groups

II and III rats were given OA extract (100 mg/kg, body weight) daily through an oral gavage. Group III rats did not had DMBA-induced mammary carcinoma and had been used as control group for the side effects of OA 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

Tumor tissues were sampled macroscopically and fixed in 10% formaldehyde solution for 24 h and were then processed in autotechnicon device. Later, they were embedded in paraffin blocks, cut in sections 3-5 µm thick and stained with haematoxylin-eosin 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 phosphate buffer solution (PBS) and endogenous peroxidase was blocked with 0.3% H₂O₂ for 15 min. After washing with PBS, the sections were incubated at 27 °C for 1.5 h with caspase-7 antibody (Gene Tex Inc. Cat #15551, San Antonio, Texas, USA). 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 counterstained with a haematoxylin prior to mounting.

Ki-67: For immunohistochemical staining, the endogenous peroxidase of the deparaffinized and rehydrated tissue sections was inactivated by with 3% H₂O₂ for 10 min. To recover antigen, these sections

were put into EDTA solution (pH 8.5) and heated in a 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 min. 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 h and washed with PBS. Then, the slides were incubated with biotinylated secondary antibody (ScyTek Laboratories, Logan, Utah, USA) for 20 min and washed with PBS, followed by incubation with streptavidin-HRP (ScyTek Laboratories, Logan, Utah, USA) for 20 min 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 haematoxylin 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 [8]. 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 semiquantitatively in the cytoplasm of 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) was 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 recombinant DNase I 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) 3 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) 3 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) 3 times. The slides were mounted and analysed by fluorescence microscope (Olympus DP 70, Melville, NY, USA).

Statistics

Results were reported as mean \pm SE. Data from the experiments were tested for statistical significance using

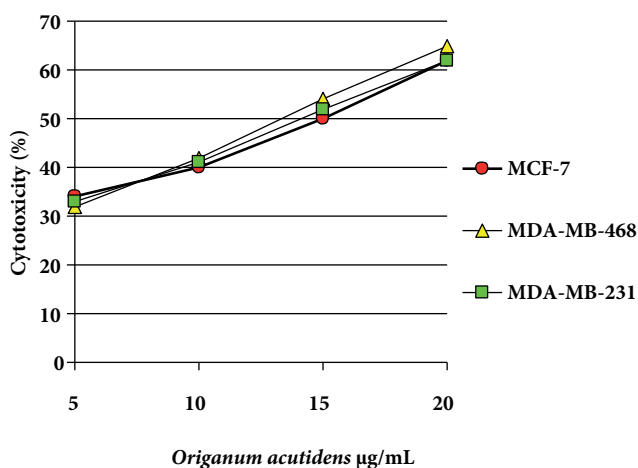


Figure 1. Cytotoxicity of *Origanum acutidens* (OA) against MCF-7 (circles), MDA-MB-468 (triangles), MDA-MB-231 (squares) cells.

Target cells were incubated with OA for 48 h at different concentrations ($\mu\text{g/mL}$).

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 \pm standard error. Experiments were repeated three times.

the Mann-Whitney U test. A p-value less than 0.05 was considered significant.

Results

Effect of *Origanum acutidens* on human breast cancer cells in vitro

The results showed that OA induced a significant reduction ($p=0.002$) in the viability of MCF-7, MDA-MB-468 and MDA-MB-231 cells in a dose-dependent manner (Figure 1). Crude extracts of OA showed cytotoxicity to 3 cancer cell lines with IC₅₀ values $15 \pm 2.0 \mu\text{g/mL}$, $13 \pm 1.7 \mu\text{g/mL}$, and $14 \pm 1.8 \mu\text{g/mL}$, respectively (Table 1, 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. The percentage of annexin-positive cells detected in treated cell lines was $25 \pm 2.5 \%$ in MCF-7 cells, $27 \pm 2.0 \%$ in MDA-MB-468 cells and $26 \pm 2.6 \%$ in MDA-MB-231 cells, compared with $12 \pm 1.8 \%$, $13 \pm 1.5 \%$, $10 \pm 1.4 \%$, of annexin-positive cells detected in untreated cells, respectively (Table 2). The results are representative of at least 3 independent experiments, all of which had similar results. When these values were compared with each other (annexin-positive cells detected in treated cell lines vs annexin-positive cells detected in untreated cells), the results were statistically significant ($p < 0.05$, Table 2). These results showed that treatment with OA displayed apoptosis-inducing effects on MCF-7, MDA-MB-468 and MDA-MB-231 cells.

Immunohistochemistry results

Immunohistochemistry experiments showed that Ki-67 activity was most intense in the untreated control group (Figure 2a). The expression of Ki-67 in the OA-treated group was lower than in the untreated control group (Figure 2b).

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 mean \pm standard error

	MCF-7	MDA-MB-468	MDA-MB-231
Origanum acutidens ($\mu\text{g/mL}$)	15 ± 2.0	13 ± 1.7	14 ± 1.8
Paclitaxel (ng/mL)	12 ± 1.8	13 ± 1.4	10 ± 1.2

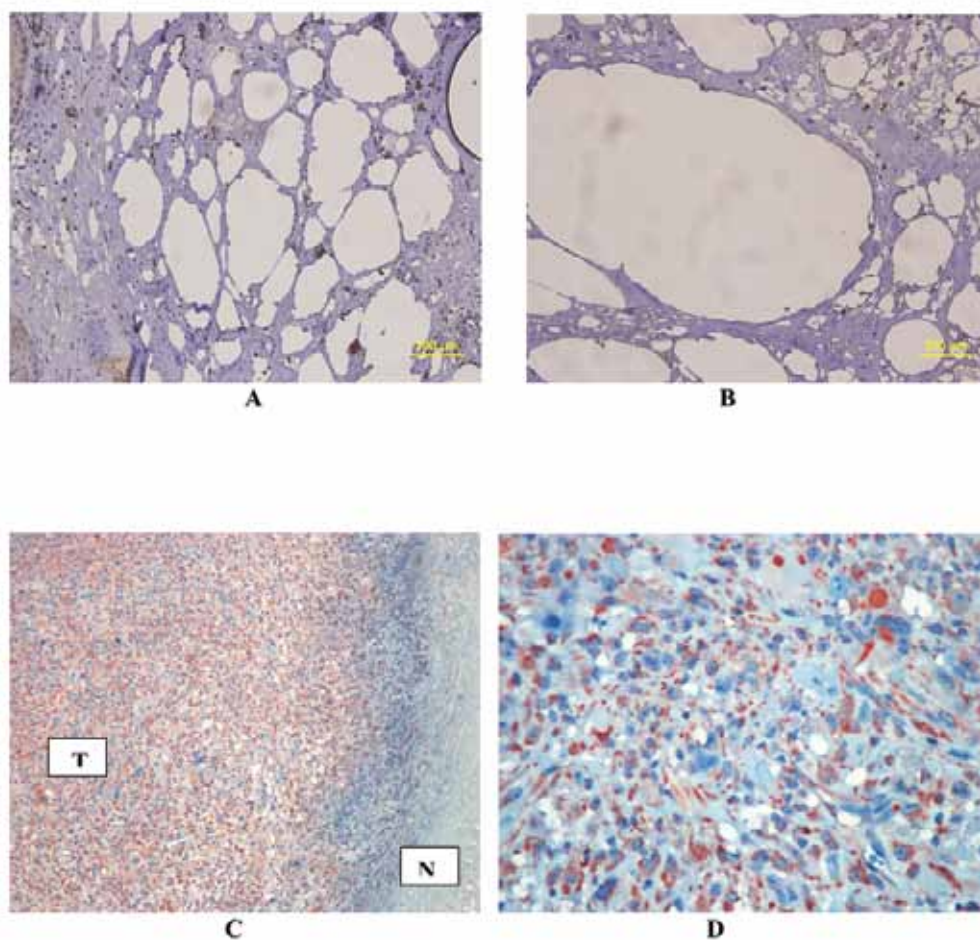


Figure 2. Immunohistochemical experiments of Ki-67 in DMBA induced mammary tumor tissue of rats. **A:** Untreated control group (x40); **B:** *Origanum* treated group (x40). Caspase-7 expression in breast tumor cells (**T**) treated with *Origanum* extract is strong. Note that expression is very weak and almost negligible in the necrotic area (**N**). (**C** x100, **D** x400).

Table 2. Percentage of annexin-positive cells detected in OA treated cell lines. The results are expressed as the means ± standard error

Percentage of annexin-positive cells	MCF-7	MDA-MB-468	MDA-MB-231
In untreated control cell lines	12 ± 1.8	13 ± 1.5	10 ± 1.4
In OA treated cell lines	25 ± 2.5	27 ± 2.0	26 ± 2.6
p-value	0.002	0.002	0.002

Table 3. Results of immunohistochemistry studies according to the semiquantitative scoring system. The results are expressed as the mean ± standard error

Immunohistochemical examination of tumor tissues	Ki-67	Caspase-7	TUNEL-positive apoptotic cells
In untreated control rats	2.45 ± 0.08	0.51 ± 0.14	0.69 ± 0.14
In <i>Origanum acutidens</i> treated rats	1.20 ± 0.21	2.78 ± 0.21	2.51 ± 0.18
p-value	0.003	0.003	0.002

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

In vivo immunohistochemistry experiments showed that treatment with OA extract induced activation of caspase-7 (Figure 2c,d). When the semiquantitative scoring system results of immunohistochemistry studies were compared, the difference was statistically significant ($p < 0.05$; Table 2).

TUNEL-positive cells were detected only rarely in the untreated control group, while numerous TUNEL-positive cells were intensively observed in the OA-treated group (Figure 3). When the semiquantitative scoring system results of TUNEL assay were compared, the difference was statistically significant ($p < 0.05$; Table 3).

Tumor volume studies

After 4 weeks of treatment, the mean tumor volume inhibition ratio in the OA-treated group (group II) was 41% 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 4). No signs of toxicity (weight loss, ruffled fur and behavioral changes) were observed in any of the treated rats (group II and III).

Discussion

Medicinal plants were and still are an important research area for novel and bioactive molecules for drug discovery. *Origanum vulgare* (OV) is a plant which has shown to possess several types of biologi-

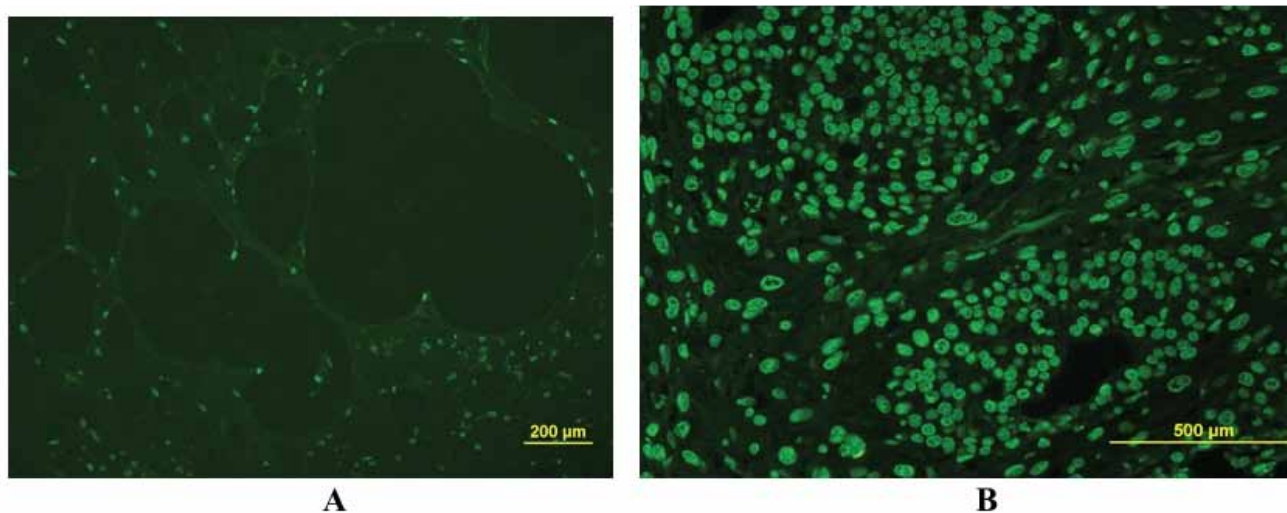


Figure 3. Representative immunohistochemistry photomicrographs of TUNEL-positive apoptotic cells in DMBA-induced mammary tumor tissue of rats. **A:** Untreated control group (x40); **B:** *Origanum* treated group (x100).



Figure 4. DMBA-induced mammary tumor in rats. Circles show the tumor masses.

cal activity. Antioxidant activity of extracts from OV has been reported [9].

OV was shown to possess high activity against cancer in *in vitro* experiments [10]. Al-Kalaldeh et al. detected antiproliferative activity of the ethanol crude extracts of *Origanum syriacum* to MCF-7 human breast adenocarcinoma cell line [11]. Lin et al. showed anti-hepatoma activity of *Origanum majorana* *in vitro* [12]. *Origanum species*, are-among others-rich in carvacrol, a monoterpenic phenol isomeric with thymol [13]. Yin et al. showed that carvacrol inhibited the growth of human hepatocellular carcinoma cell line HepG-2 by inducing apoptosis [14].

Chinou et al. tested the cytotoxic activity of several extracts of *Origanum dictamnus* against murine leukemia (P388) and non small cell lung cancer (NSCLC-N6) cell lines [15]. Both, the initial dichloromethane extract of *Origanum compactum* and the isolated ursolic acid were found to have cytotoxic activity. The anticancer activities for ursolic acid have been reported previously [16]. Ursolic acid showed significant cytotoxicity in the lymphocytic leukemia cell lines P-388 and L-1210, as well as in human lung carcinoma cell line A-549.

OA showed cytotoxicity to 3 cancer cell lines with IC50 values $15 \pm 2.0 \mu\text{g/mL}$, $13 \pm 1.7 \mu\text{g/mL}$, $14 \pm 1.8 \mu\text{g/mL}$, respectively. In this study, these IC50 values were considered as “good” activity on breast cancer cell lines.

Apoptosis is a key biological process in normal and malignant cells. The format (annexin V-FITC) retains its high affinity for membrane phospholipid phosphatidylserine and thus serves as a sensitive probe for flow

cytometric analysis of cells that are undergoing apoptosis [17]. In this study, in OA-treated cell lines annexin-positive cells were detected by flow cytometry in $25 \pm 2.5 \%$ of MCF-7 cells, $27 \pm 2.0 \%$ of MDA-MB-468 cells and $26 \pm 2.6 \%$ of MDA-MB-231 cells (Table 2), compared with 12, 13, 10 %, of annexin-positive cells detected in untreated cells, respectively. Thus, the results of this assay showed that treatment with OA extracts induced apoptosis in breast cancer cells.

During apoptosis a group of proteases are activated which cause DNA fragmentation, cytoplasmic shrinkage and membrane blebbing. In this study we examined apoptosis in tumor tissues by using terminal deoxynucleotidyl transferase dUTP nick and labelling (TUNEL) method . Our *in vivo* results of TUNEL assay demonstrated that treatment with OA extracts increased the apoptotic cells in tumor tissues.

Ki-67 is a protein encoded by the MKI67 gene [18]. It is a nuclear protein associated with cell proliferation and is an excellent marker to determine the growth fraction of a given cell population [19]. In the present study, we studied the Ki-67 levels of DMBA-induced tumor tissues to determine the OA effect on tumor growth. *In vivo* immunohistochemistry showed that treatment with OA extracts decreased the Ki-67 activity in tumor tissues of rats.

Caspase-7 is a protein that plays a key role in apoptosis. Caspase-7 is activated in the apoptotic cell both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways [20]. In the caspase cascade pathway, mature caspases-3 and -7 cleave a large set of substrates, ulti-

Table 4. When the tumor volume reached to $250 \pm 4.3 \text{ mm}^3$, treatment with OA started in group II. The control group (group I) received no OA 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 \pm standard error (mm^3)

	Beginning	1st week	2nd week	3rd week	4th week
Control group (group I)	250 ± 4.3	370 ± 7.3	640 ± 8.4	980 ± 10.1	1250 ± 24.3
OA treated group (group II)	250 ± 4.3	295 ± 6.8	471 ± 7.1	640 ± 8.2	733 ± 9.2
Tumor volume inhibition ratio (%)	0	20	26	34	41
p-value	-	0.002	0.002	0.002	0.002

mately resulting in the characteristic morphological and biochemical hallmarks of apoptosis. *In vivo* immunohistochemistry of this study showed that treatment with OA extracts induced activation of caspase-7. These results may suggest that OA induced apoptosis in breast cancer cells via the caspase cascade pathway.

In DMBA-induced rat mammary tumor volume experiments, treatment with OA showed a 41 % tumor volume inhibition compared with the untreated rats. This difference was statistically significant ($p < 0.05$) and showed that tumor growth inhibition could be attributable to the OA extract.

In conclusion, it is worthwhile screening the commonly used plants from the local flora for different biological activities because some of them might be a source of new bioactive substances. The results of the present study showed that natural OA extract may constitute a potential antitumor compound against breast cancer. However, further phytochemical studies are needed to reach the main anticancer molecule or molecules of this crude extract.

Acknowledgement

This work has been supported by the Scientific Research Project Fund of Cumhuriyet University under the project number T-383.

References

- Mukherjee AK, Basu S, Sarkar N, Ghosh AC. Advances in cancer therapy with plant based natural products. *Cur Med Chem* 2001; 8 : 1467–1486.
- Rates S. Plants as source of drugs. *Toxicol* 2001; 39: 603–613.
- Mans D, Rocha A, Schwartzmann G. Anticancer drug discovery and development in Brazil: targeted plant collection as a rational strategy to acquire candidate anticancer compounds. *Oncologist* 2000; 5: 185–198.
- Baytop T (Ed): *Therapy with Medicinal Plants in Turkey; Today and in Past* (1st Edn). Istanbul: Istanbul University Press, 1999, pp 8-17.
- Kordali S, Cakir A, Ozer H, Cakmakci R, Kesdek M, Mete E. Antifungal, phytoxic and insecticidal properties of essential oil isolated from Turkish *Origanum acutidens* and its three components, carvacrol, thymol and p-cymene. *Bioresource Technol* 2008; 99 : 8788–8795.
- Baser KHC, Tumen G, Duman H. Essential oil of *Origanum acutidens* (Hund.Murr.) *J Essent Oil Res* 1997; 9: 91–92.
- Roomi MW, Roomi NW, Bhanap B. In vivo and in vitro antitumor effects of nutrient mixture in murine leukemia cell line P-388. *Exp Oncol* 2011; 33: 71–77.
- Chih-Ping H, Lai-Fong Kok, Po-Hui Wang, Tina S Wu, Yeu-Sheng Tyan, Ya-Wen Cheng. Scoring of p16^{INK4a} immunohistochemistry based on independent nuclear staining alone can sufficiently distinguish between endocervical and endometrial adenocarcinomas in a tissue microarray study. *Mod Pathol* 2009; 22: 797–806.
- Lamaison JL, Petitjean-Freytet C, Carnat A. Medicinal Lamiaceae with antioxidant properties, a potential source of rosmarinic acid. *Pharmac Acta Helv* 1991 ;66: 185–188.
- Goun EA, Petrichenko VM, Solodnikov SU et al. Anticancer and antithrombin activity of Russian plants. *J Ethnopharmacol* 2002 ; 81 : 337–342.
- Al-Kalaldehy J, Abu-Dahab R, Afifi F. Volatile oil composition and anti-proliferative activity of *Laurus nobilis*, *Origanum syriacum*, *Origanum vulgare*, and *Salvia triloba* against human breast adenocarcinoma cells. *Nutr Res* 2010 ; 30 : 271–278.
- Lin LT, Liu LT, Chiang LC, Lin CC. In vitro anti-hepatoma activity of fifteen natural medicines from Canada. *Phytother Res* 2002 ; 16 : 440-444.
- Husnu Can Baser . Biological and Pharmacological Activities of Carvacrol and Carvacrol Bearing Essential Oils . *Curr Pharmaceutic Design* 2008 ; 14 : 3106-3119.
- Yin QH, Yan FX, Zu XY et al. Anti-proliferative and pro-apoptotic effect of carvacrol on human hepatocellular carcinoma cell line HepG-2. *Cytotechnology* 2012 ; 64 : 43-51.
- Chinou I, Liolios C, Moreau D, Roussakis C. Cytotoxic activity of *Origanum dictamnus*. *Fitoterapia* 2007; 78 : 342–344.
- Lee KH, Lin YM, Wu TS, Zhang DC, Yamaguchi T, Hayashi T. The cytotoxic principles of *Prunella vulgaris*, *Psychotria serpens*, and *Hypoxis capitata*: ursolic acid and related derivatives. *Planta Med* 1988 ; 54: 308-311.
- Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods* 1995 ;184 : 39-51.
- Bullwinkel J, Baron-Lühr B, Lüdemann A, Wohlenberg C, Gerdes J, Scholzen T . Ki-67 protein is associated with ribosomal RNA transcription in quiescent and proliferating cells. *J Cell Physiol* 2006 ; 206: 624–635.
- Scholzen T, Gerdes J . The Ki-67 protein: from the known and the unknown. *J Cell Physiol* 2000; 182 : 311–322.
- Shin S, Sung B J, Cho Y S et al. An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and -7. *Biochemistry* 2001; 40: 1117–1123.