TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2021) 45: 158-171
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doi:10.3906/bot-2010-40

In vitro physiological effects of *Origanum onites* L. (Lamiaceae) essential oil treatment on human origin cell lines

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Received: 26.10.2020 • **Accepted/Published Online:** 08.03.2021 • **Final Version:** 30.03.2021

Abstract: *Origanum onites* L. belongs to the Lamiaceae family which is commonly used for symptomatic treatment of the diseases of the gastrointestinal, endocrine, and respiratory systems in Turkish ethnobotanical studies and public. In this study our aim was to investigate the physiological effects of *O. onites* essential oil (OE) on A549 human lung cancer, MIA PaCa human pancreas cancer and HUVEC human endothelial cell lines. Our findings showed that *O. onites* OE treatment at all concentrations (4.79–191.6 µg/mL, 24 h) can inhibit cell proliferation and induce apoptosis in A549 and MIA PaCa cell lines ($P < 0.0001$). Although ≤19.16 µg/mL OE treatment for 24 h did not inhibit cell proliferation in the HUVEC cell lines, higher concentrations of OE (≥38.32 µg/mL) significantly inhibited cell proliferation. Apoptotic cell counts were significantly ($P < 0.0001$) higher in all cell lines at both incubation times (24 h 56.3 ± 7%, 48 h 53 ± 5% for A549, 24 h 44 ± 7%, 48 h 44.7 ± 7% for MIA PaCa, 24 h 48.7 ± 6%, 48 h 49.3 ± 5% for HUVEC). Migration rates were decreased significantly ($P < 0.0001$) (24 h 64 ± 6%, 48 h 67.2 ± 4% for A549, 24 h 49 ± 5%, 48 h 69.35 ± 5% for MIA PaCa, 24 h 47 ± 5%, 48 h 69.6 ± 2% for HUVEC). Based on these results, OE has been shown to have promising anticancer effects.

Key words: *Origanum onites*, cellular-migration, apoptosis, proliferation, cancer, wound healing

1. Introduction

Origanum L. belongs to the Lamiaceae family. The genus *Origanum* includes approximately 70 taxa in the world and 15 of them are endemic to Turkey, represented by 29 species and 32 taxa in Turkey.[1,2] The endemic rate of Turkish *Origanum* taxa exceeds 44% suggested that this high rate shows the gene center of *Origanum* is Turkey. The Turkish names of *Origanum onites* L. are; Kırkbaş kekik, Tokalı kekik, Bilya kekik, Arıkekiği, İncir kekiği, Yemiş kekiği (Bulut and Tuzlaci, 2013; Sargin et al., 2013). *O. onites* is a stenoendemic taxon of the eastern Mediterranean area. Its distribution is not very wide (Vokou et al., 1988). *O. onites* is a subshrub plant up to 65 cm. Flowering times are 4–8 months. The habitat is rocky hills and rocky slopes, usually on limestone, sometimes in partial shade (Ietswaart, 1982). For centuries, it is known that Turkish people have used medicinal plants for the treatment of some minor diseases. *O. onites* is commonly prescribed as a herbal tea prepared by infusion technique for symptomatic treatment of stomach diseases, digestion problems (Bulut and Tuzlaci, 2013), diabetes, cough, cholesterol, headache, toothache (Polat and Satil, 2012), tachycardia, gastrointestinal diseases, hypertension, bronchitis, flu, respiratory tract diseases, (Sargin et al., 2013), immunogenic, antiemetic, carminative (Sağıroğlu et al., 2013) in Turkish ethnobotanical studies.

When the sample obtained from *O. onites* was investigated by gas chromatography (GC), it was determined that it has 78.2% carvacrol main component and the other important components were myrcene (% 0.5), gamma-terpinene (% 4.3), linalyl acetate (% 1.0), borneol (% 8.1), beta pinene (% 1.1), and cineole (% 1.5) (Andoğan et al., 2002). Recently few studies have investigated the cytotoxic effect of *Origanum* species such as *O. majorana* L. extract on human breast cancer cell line (MCF-7) (Jssim and Abdul-Halim, 2020) or oregano essential oil from *Origanum minutiflorum* O. Schwarz & P.H. Davis on NB2a neuroblastoma cells (Sari et al., 2020). The antimicrobial effect of *O. onites* against *P. aeruginosa, E. coli*, and *S. aureus* strains were investigated and it was found to have a strong antimicrobial effect (Andoğan et al., 2002). It has been reported that cineole (4.3%) and 7-terpinene (1.5%) of *O. onites* components did not show antimicrobial activ-

[1] Sadıkoğlu N (2012). *Origanum* [online]. Website http://www.bizimbitkiler.org.tr [accessed 04 January 2021].

[2] The Plant List (2013). Summary Statistics [online]. Website http://www.theplantlist.org/1.1/browse/A/Lamiaceae/Origanum/ [accessed 04 January 2021].

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ity alone (Andoğan et al., 2002). Carvacrol has been reported to act synergistically against various parasitic organisms, with other compounds found in the essential oil of *O. onites* (Novato et al., 2015). While the possibility of carvacrol's interaction with anticancer drugs is unknown, the synergistic effect of carvacrol and *Cymbopogon citratus* essential oil on breast cancer in female rats was recently studied (Rojas-Armas et al., 2020).

Anticancer, antiproliferative and apoptotic effects of essential oil and several extracts from *Origanum* species on various cell types including leukemic cell, platelets and breast adenocarcinoma cells were also reported by others (Abdel-Massih et al., 2010; Al-Kalaldeh et al., 2010). For example, hepatocellular carcinoma cell line HepG2 has been widely studied in toxicology tests (Özkan and Erdoğan, 2011). Few studies are investigating the possible in vitro physiological effects of *O. onites*. Bostancıoğlu et al. (2012) proved that *O. onites* essential oil inhibits proliferation by in vitro in RATEC which are rat adipose tissue endothelial cells and 5RP7 which are rat embryonic fibroblasts, depending on concentration and time.

The lowest concentration (62.5 µg/mL) tested in RATEC and 5RP7 cell lines were reported to be nontoxic for 4 days. They stated that the studied concentrations of 125, 250, and 500 µg/mL OE had a toxic effect on the cancer cell line, 5RP7. In the same study, 24 h IC_{50} values were determined as approximately 125 µg/mL in 5RP7 cells and 250 µg/mL in RATEC cells. In another report, it has been reported that ethanolic extracts from the *O. onites* plant inhibit the growth of breast cancer (MDA-MB231) and human glioblastoma (U87) cells (Baranauskaite et al., 2017). Spyridopoulou et al. (2019) detected that *O. onites* essential oil in CT26 (IC_{50} values for 48 h by 71.70 µg/mL for 72 h by 1.10 µg/mL) and HT-29 (IC_{50} values for 48 h by 58.00 µg/mL for 72 h by 0.35 µg/mL) cells. Also stated that growth was inhibited depending on the concentration and time. According to the results of the study, it was revealed that human HT-29 cells are more sensitive to OE than murine CT26 cells.

However, the possible effects of OE on other cell types (such as lung and pancreatic cancer or endothelial cell lines) have not been widely studied. It may have different effects on different cell types. Before studying its systemic effect, it should be tested in different cell lines in cell culture studies, which are model systems. For this reason, we purposed to explore the effect of OE on HUVEC umbilical vein endothelial, MIA PaCa pancreas cancer, A549 lung cancer cell lines. With this aim, the viability, apoptotic/necrotic cell ratio, and migration rates of cells treated with OE were examined. The correlation between the ratio of apoptotic cells and cellular reactive oxygen species (ROS) detection was determined.

2. Material and methods

2.1. Cell culture and chemicals

High glucose DMEM (5546; Sigma-Aldrich, St. Louis, MO, USA) was used for culturing the cell lines below. Pulmonary adenocarcinoma human alveolar epithelial (A549 ATCC CCL-185); human pancreas carcinoma epithelial cell [MIA PaCa-2 (ATCC CRL-1420)] human umbilical vein endothelial cell [HUV-EC-C (HUVEC) (ATCC CRL-1730)] lines were purchased from ATCC (USA).

P/S (50 U/mL penicillin and 50 µg/mL streptomycin; Biological Industries, Beit-Haemek, Israel; 03-031-1B), 1% 2 mM L-glutamine (Biological Industries, BI03-020-1B) was added to DMEM with %10 FBS (Biowest, Nuaillé, France; S1810-500). 1.5×10^6 cells from each cell line were seeded into a 10 cm plate and divided 72 h later. *O. onites* essential oil was dissolved in 0.1% dimethyl sulfoxide (DMSO) aqueous solution. It was prepared freshly for each test and filtered and sterilized through a 0.45 µm membrane filter (Özkan and Erdoğan, 2011). Negative control (0 µg/mL OE) indicated treatments of cells with 0.1% DMSO (the solvent of OE) containing DMEM.

2.2. Plant material

Aerial parts of *O. onites* were collected during the flowering stage from Assos (Çanakkale) the northwestern part of Turkey in July 2015 at altitudes of 500 m. The plant material was confirmed and the voucher specimen was prepared. Specimens of these vouchers are kept in the Herbarium (HERA 249) of the Altınbaş University of Pharmacy.

***Origanum onites* essential oil preparation**

The dried and powdered plant material (100 g) was distilled for 3 h using Clevenger type equipment. The essential oil was dried with anhydrous sodium sulfate, filtered and stored at –20 °C until use in 2016.

Gas chromatography/mass spectrometry (GC/MS) analysis

The oil was analyzed by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) techniques using Agilent 7890B system, fitted with a Agilent DB-wax (60 m × 0.25 mm × 0.25 µm) capillary column. Detector and injector temperatures were set at 220 °C. The temperature program for the column was from 70 °C (15 min), 2 °C/min to 180 °C (5 min), 5 °C/min and then held at 230 °C (15 min), total 100 min. Helium was used as a carrier gas at a flow 1.5 mL/min and injection volume of each sample was 1 µL. Split ratio was adjusted as 40:1. MS were taken at 70 eV. Mass range was from m/z 35 to 450 (This analysis was performed in Bezmialem University Phytotherapy Education Research and Application Center-BİTEM, İstanbul, Turkey).

2.3. MTT assay

12 mM stock solution of MTT (3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide) (Neofrox 3580

MTT) was prepared as described by Mosmann (1983). Cells were seeded into a 96-well plate at a volume of 100 µL (about 104 cells per well). The MTT assay was performed as follows: Add 10 µL of 12 mM MTT stock solution for each well and incubate at 37 °C for 4 h. Only 100 µL of medium is included as a negative control. After incubating with MTT for 4 h, remove 75 µL of medium from the well, and then dissolve formazan crystals in 50 µL of DMSO by mixing well with a pipette. After incubating for another 10 min at 37 °C, mix the samples briefly again and record the absorbance at 540 nm.

2.4. Acridine orange/ethidium bromide double staining

Acridine orange/ethidium bromide (AO/EtBr) dual staining technique was performed as described by Liu et al. (2015). In short, cells were seeded in 96-well plates at a density of approximately 10^4 cells/well. After 48 h of incubation with OE, the cells were trypsinized and 10–25 µL of the cell suspension was transferred to a glass slide. One microliter of AO/EtBr staining solution was added to the cell suspension and then the sample was covered with a coverslip.

Within 20 min after adding AO/EtBr dye (a mixture containing 100 µg/mL AO and 100 µg/mL EtBr), the cell morphology was checked under a fluorescence microscope (Carl-Zeiss/Axio Observer 3, Zen 2.3 Blue Edition software; Carl Zeiss Microscopy, Oberkochen, Germany). For statistical analysis, at least 200 cells were counted and the results expressed as the average of at least three independent experiments. Both live and dead cells were stained with AO, while ethidium bromide stains only dead cells that have lost membrane integrity. Living cells were uniformly green, while early apoptotic cells show green dots in their nuclei. Late apoptotic cells were stained orange and show nuclear condensation or fragmentation. Necrotic cells were stained orange, nuclear morphology is similar to living cells, but chromatin is not concentrated (Liu et al., 2015).

2.5. Detection of ROS

Reactive oxygen species (ROS) was measured by the fluorescence microscopy method by using a specific probe, DCF-DA [DCF-DA (D6883) 50mg; Sigma-Aldrich]. Once the compound enters the cell, it is cleaved by the intracellular esterase and remains in the cell due to the loss of its hydrophobic part. After being oxidized by ROS, the fluorescein part is not covered and can be detected by a fluorescence microscope. The amount of fluorescence emitted is related to the amount of ROS in the cell. This method has been widely used to measure ROS in living cells (Larsen et al., 2002). A549 human lung cancer, MIA PaCa human pancreas cancer, and HUVEC human endothelial cell lines were used for the determination of reactive oxygen production. The cells were treated or nontreated (negative control) with *O. onites* essential oil at their respective IC_{50} doses for 24 h and then trypsinized. H_2O_2 (0.1 mM and 0.2 mM) treated cells for 24 h were taken as the positive control. Briefly, prepared a stock solution of DCF-DA (20 mM) in DMSO (dimethyl sulfoxide) and further diluted it with culture medium to a working concentration of 0.1 mM. The trypsinized cells were washed with ice-cold 1×PBS and incubated with 0.1 µM (working solution) DCF-DA at 37 °C for 20 min. After washing 3 times in cold PBS, the cells were imaged under a fluorescence microscope (Carl-Zeiss/Axio viewer 3, Zen 2.3 Blue Edition software). Fluorescence images were acquired through a single 300 ms exposure and then DIC images of the same field of view were acquired. Using Zen 2.3 Blue Edition software, measured the fluorescence intensity of each cell in a 60 × 60 pixel box, and took the average of at least 100 cells per cover glass (n = 6) (Jiang et al., 2004).

2.6. In vitro scratch assay

In vitro scratch assay for evaluating migration rates were carried out according to the protocol described by Liang et al. (2007): Briefly, scrape the surface of the wells in a 6-well plate with a 10 µL sterile pipette tip. After gently washing with the culture medium, take pictures of the scratches (gap) at different time points (0–15–24 hours) at 10 times magnification (Carl-Zeiss/Axio Observer 3) under a microscope and use image analysis. The J software and cell migration rate are calculated by comparing the scratch-free area at 15 and 24 h after injury with the scratch area at 0 h. The results are expressed as the average of three experiments.

2.7. Statistical analysis

GraphPad (Prism 5) software was used for statistical analysis. Multiple comparisons were made using Tukey's procedure. $P < 0.05$ was considered statistically significant. Statistical analysis was performed on the apoptosis index between each group using an analysis of variance.

3. Results

Results of GC analysis of *O. onites* OE obtained by steam distillation are given in Table 1. Carvacrol was found to be a major component of the oil as 68.071%, and then followed by cymene (11.150%), γ-terpinene (6.177%) and borneol (2.624%), respectively (Table 1).

Determining the effective concentration that causes the cell growth inhibitory concentration (IC_{50}) to decrease by 50% is essential for understanding the biological and pharmacological properties of the drug. First, we compared the proliferation rate of A549 (human lung adenocarcinoma basal epithelial cells) MIA PaCa (human pancreas carcinoma epithelial cell) and HUVEC (human umbilical vein endothelial) cell lines to study the IC_{50} value of *O. onites* essential oil and its effect on cell viability by MTT assay. For this target eleven different concentrations (4.79 µg/mL, 9.58 µg/mL, 19.16 µg/mL, 38.32 µg/mL, 47.9 µg/mL, 57.48

Table 1. Main components of *O. onites* OE.

Components	Composition (%)
Alpha pinene	1.070
Camphene	0.556
Delta 3-carene	0.114
Myrcene	1.744
Alpha terpinene	1.927
Limonene	0.451
Beta phellandrene	0.170
Gamma terpinene	6.177
Cymene	11.150
Alpha terpinolene	0.334
1-octen-3-ol	0.121
Linalool	0.090
Linalyl acetate	0.127
Caryophyllene	0.747
4-terpineol	1.428
Alpha terpineol	0.220
Borneol	2.624
Beta bisabolene	0.728
Carvone	0.302
Carvacryl acetate	0.147
Caryophyllene oxide	0.367
Spathulenol	0.159
Thymol	0.408
Carvacrol	68.071
Others	0.765

μg/mL, 67.06 μg/mL, 76.64 μg/mL, 86.22 μg/mL, 95.8 μg/mL, 191.6 μg/mL for A549 and MIA PaCa, 4.79 μg/mL, 9.58 μg/mL, 19.16 μg/mL, 38.32 μg/mL, 47.9 μg/mL, 67.06 μg/mL, 86.22 μg/mL, 95.8 μg/mL, 191.6 μg/mL, 287.4 μg/mL and 383.2 μg/mL for HUVEC) of *O. onites* essential oil according to Sivas and Tomsuk (2011) were tested for 24 and 48 h. IC_{50} values for A549, MIA PaCa and HUVEC were found as 4.79 μg/mL; 14.37 μg/mL; 67.06 μg/mL for 24 h and 14.37 μg/mL; 1.916 μg/mL; 306.56 μg/mL for 48 h in turn. Analysis of data obtained from proliferation studies for A549 and MIA PaCa as shown in Figures 1a–1d, all levels of *O. onites* essential oil treatment (for 24 and 48 h: 4.79 μg/mL, 9.58 μg/mL, 19.16 μg/mL, 38.32 μg/mL, 47.9 μg/mL, 57.48 μg/mL, 67.06 μg/mL, 76.64 μg/mL, 86.22 μg/mL, 95.8 μg/mL, 191.6 μg/mL) inhibited the proliferation rate of the two test cell lines. Although as shown in Figures 1e and 1f, ≤19.16 μg/mL OE treatment for 24 h did not inhibit cell proliferation in the HUVEC cell lines, higher concentrations of OE (≥38.32 μg/mL) significantly inhibited cell proliferation. *O. onites* essential oil' 4.79 μg/mL concentration inhibited proliferation rate by 52.5% in the A549 cell line at 24 h and increased proliferation rate by 32% at 48 h. However as shown in Figures 1a and 1b, 9.58 μg/mL concentration of OE inhibited significantly by 54.4% and not significantly 24.4%, 19.16 μg/mL concentration inhibited by 68.8% and 61.1%, 38.32 μg/mL concentration inhibited by 80.7% and 64.8%, 47.9 μg/mL concentration inhibited by 80.6% and 75.1%, 57.48 μg/mL concentration inhibited proliferation rate by 84.9% and 76.8%, 67.06 μg/mL concentration inhibited proliferation rate by 84.8% and 77.4%, 76.64 μg/mL concentration inhibited proliferation rate by 85.1% and 76.4%, 86.22 μg/mL concentration inhibited proliferation rate by 87.3% and 81.5%, 95.8 μg/mL concentration inhibited proliferation rate by 85.8% and 74.2%, 191.6 μg/mL concentration inhibited proliferation rate by 93.1% and 78.7% in the A549 cell line at 24 h and 48 h. *O. onites* essential oil' 4.79 μg/mL concentration increased proliferation rate by 5.1% in the MIA PaCa cell line at 24 h and inhibited proliferation rate by 71.4% at 48 h. As shown in Figures 1c and 1d, 9.58 μg/mL concentration of OE inhibited significantly by 22.1% and 80.3%, 19.16 μg/mL concentration inhibited by 56.4% and 94.7%, 38.32 μg/mL concentration inhibited by 71.6% and 92.9%, 47.9 μg/mL concentration inhibited by 90.6% and 87.5%, 57.48 μg/mL concentration inhibits the rate of proliferation by 91.8% and 98%, 67.06 μg/mL concentration inhibits proliferation rate by 98.1% and 87.9%, 76.64 μg/mL concentration inhibited proliferation rate by 99.2% and 95.6%, 86.22 μg/mL concentration inhibited proliferation rate by 99.6% and 98.3%, 95.8 μg/mL concentration inhibited proliferation rate by 98.7% and 97.9%, 191.6 μg/mL concentration inhibited proliferation rate by 98.2% and 96% in the MIA PaCa cell line at 24 h and 48 h, while HUVEC cell line seems to be more resistant to *O. onites* essential oil treatment. Endothelial cell line to be exposed 20 times higher doses of OE than MIA PaCa, while respectively 27 times higher doses of OE than A549 in 24 h for inhibitory effect.

As shown in Figures 1e and 1f, *O. onites* essential oil treatment has a significant inhibitory effect on the proliferation rate of HUVEC cell line by 39.3% and 5% in the concentration of 38.32 μg/mL, by 45.4% and 11.9% in the concentration of 47.9 μg/mL, by 47.8% and 23.3% in the concentration of 67.06 μg/mL, by 50.1% and 26.5% in the concentration of 86.22 μg/mL, by 52.5% and 28.7% in the concentration of 95.8 μg/mL, by 52.7% and 38.6% in the concentration of 191.6 μg/mL, by 54.3% and 41% in the concentration of 287.4 μg/mL and by 57.2% and 54.1% in the concentration of 383.2 μg/mL for 24 and 48 h. As shown in Figure 1e, the HUVEC cell line seemed to be resistive to 19.16 μg/mL compared with the untreated

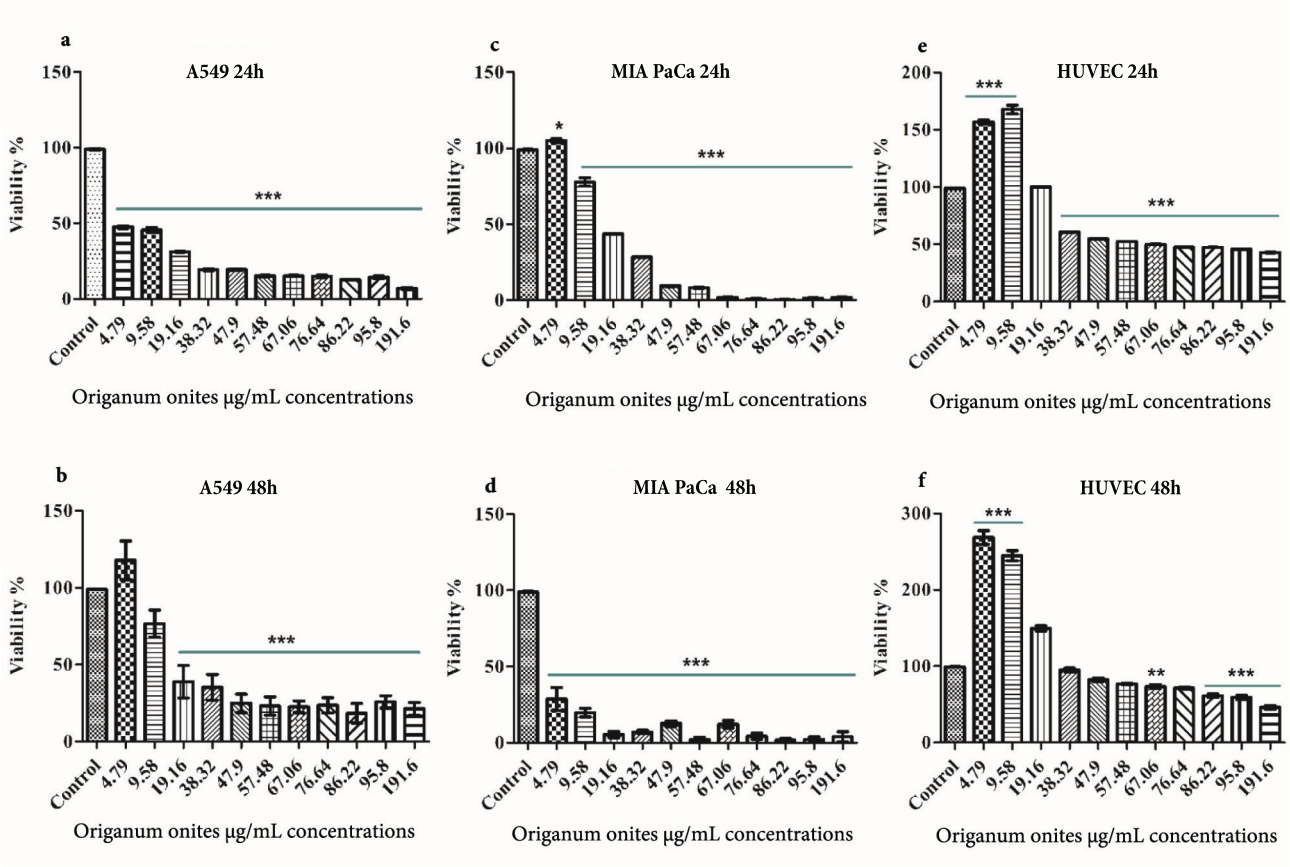

Figure 1. a–b. A549 lung cancer, c–d. MIA PaCa pancreas cancer, e–f. HUVEC Endothelial cell lines were treated with *O. onites* essential oil placed in the incubator for 24 and 48 h. MTT analysis was performed 24 and 48 h after treatment with the specified dose of *O. onites*, the relative change percentage of the proliferation rate was compared with the untreated control group, and the statistical significance was determined by one-way ANOVA and Tukey multiple comparison tests was performed (*,**,*** $P < 0.0001$, n = 3).

A549 cell line at 24 h. Again, 19.16 µg/mL concentration increased by 49.4% in the HUVEC cell line at 48 h. The viability of both cells was shown to be significantly reduced in the presence of OE ($P < 0.0001$). *O. onites* essential oil showed an inhibitory effect on the growth of all tested cell lines and the effect of essential oil can vary according to the incubation period or test concentration. Analysis of data obtained from MTT analysis shows *O. onites* essential oil treatment was able to inhibit the proliferation of A549 and MIA PaCa cell lines in a dose-dependent (A549 $R^2 = 0.9977$ and MIA PaCa $R^2 = 0.9985$, $P < 0.0001$ for 24 h) (A549 $R^2 = 0.8937$ and MIA PaCa$R^2 = 0.9650$, $P < 0.0001$, for 48 h) manner. In contrast to these cancer cell lines, *O. onites* essential oil treatment inhibited the proliferation of the noncancerous HUVEC cells in high doses. Although, as shown in Figures 1e and 1f, 19.16 µg/mL (24 h) OE treatment did not appear to affect the rate of cell proliferation, higher doses of OE (≥38.32 µg/mL for 24 h and ≥67.06 µg/mL for 48 h) significantly inhibited proliferation of HUVEC cell in a dose-dependent fashion ($R^2 = 0.9977$, $P < 0.0001$ for 24 h) ($R^2 = 0.9924$, $P < 0.0001$, for 48 h).

We then compared the changes in the ratio of apoptotic cells with the "acridine orange/ethidium bromide" double staining protocol. For this reason, the ratio of live cells, apoptotic cells and necrotic cells when exposed to the IC_{50} value of *O. onites* essential oil for A549, MIA PaCa and HUVEC were evaluated as 4.79 µg/mL; 14.37 µg/mL; 67.06 µg/mL for 24 h and 14.37 µg/mL; 1.916 µg/mL; 306.56 µg/mL for 48 h. In the two incubation times, the number of apoptotic cells in both cells was significantly higher ($P < 0.0001$) (24 h 56.3 ± 9%, 48 h 53 ± 5% for A549, 24 h 44 ± 7%, 48 h 44.7 ± 8% for MIA PaCa and 24 h 48.7 ± 6%, 48 h 49.3 ± 4% for HUVEC). However, as shown in Figures 2a–2c, 14.37 µg/mL concentration of *O. onites* essential oil significantly increased the number of necrotic cells of the A549 cell line within 48 h (necrotic cells: 6.7%).

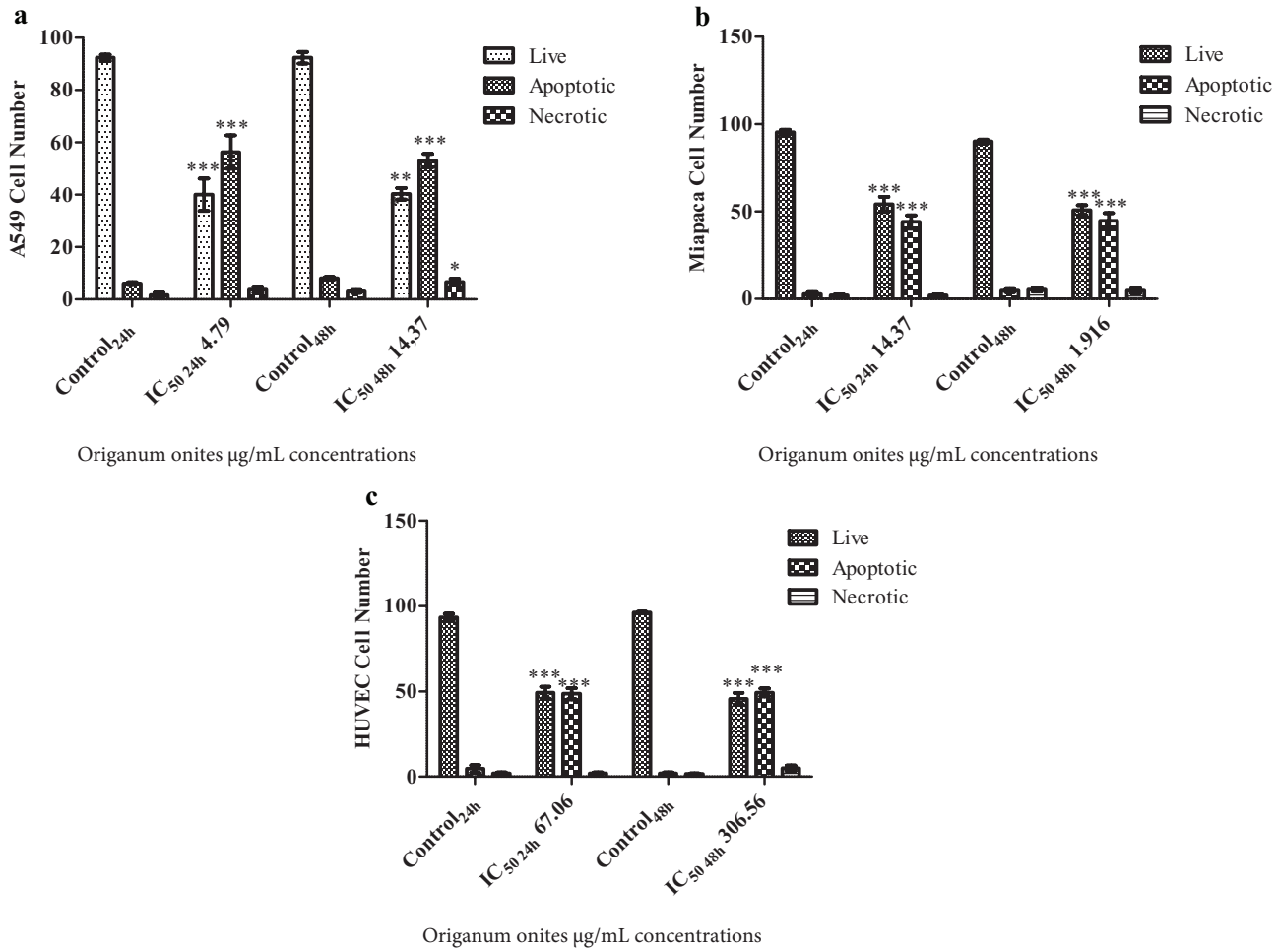

Figure 2. *O. onites* Induce apoptosis with IC_{50} value. a. A549 lung cancer, b. MIA PaCa pancreas cancer, c. HUVEC endothelial cell lines were treated with IC_{50} values of *Origanum onites* essential oil was evaluated as 4.79 µg/mL; 14.37 µg/mL; 67.06 µg/mL for 24 h and 14.37 µg/mL; 1.916 µg/mL; 306.56 µg/mL for 48 h hours in an incubator. AO/EtBr double staining was performed 24 and 48 h after treatment with the indicated concentrations of the essential oil from *O. onites*. The percent change in the ratio of apoptotic cells was compared to the untreated control group and statistical significance was tested using one-way ANOVA followed by Tukey's multiple comparison test (*, **, *** $P < 0.0001$, $n = 6$).

A549 cells were more sensitive cell line (24-h incubation A549: apoptotic cells 56.3%, 48-h incubation A549: apoptotic cells 53%) while MIA PaCa cells seemed to be more resistant to *O. onites* essential oil than A549 (24-h incubation MIA PaCa: apoptotic cells 44%, 48-h incubation MIA PaCa: apoptotic cells 44.7%). As shown in Figures 2a-2c**,** endothelial cell line needed to expose 14 times higher doses of OE than A549 in 24 h as IC_{50} value, while respectively 160 times higher doses of OE than A549 in 48 h as IC_{50} value for inhibitory effect (24-h incubation HUVEC: apoptotic cells 48.7%, 48-h incubation HUVEC: apoptotic cells 49.3%). In general, the results obtained support the experimental basis for reporting that *O. onites* essential oil leads to cell death mainly through the process of apoptosis. Representative microscope images from AO/EtBr stained samples are shown in Figure 3.

Many studies have shown that after treatment with anticancer drugs, ROS can induce apoptosis of various types of cancer cells (Pelicano et al., 2004). Therefore, we next investigated whether OE-induced apoptosis is related to increased ROS levels in A549, MIA PaCa and HUVEC cells. As shown in Table 2, the correlation between reactive oxygen production induced by *O. onites* essential oil treatment and the rate of apoptosis induced by this oil was determined. For this purpose, as shown in Figure 4, we tested the effect of IC_{50} values of *O. onites* essential oil as 4.79 µg/mL; 14.37 µg/mL; 67.06 µg/mL for 24 h on cells for the determination of reactive oxygen production rates

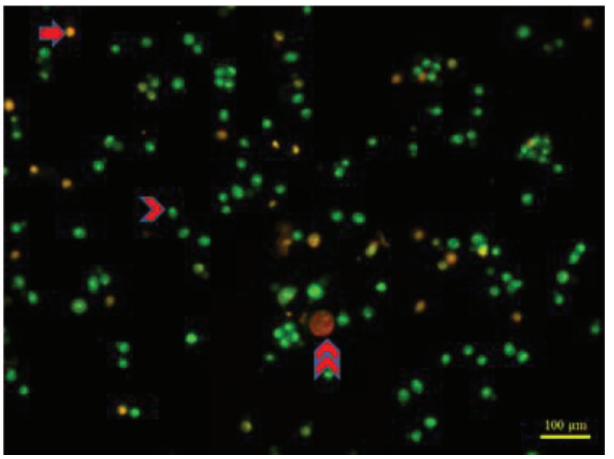

Figure 3. Representative microscope images from AO/EtBr double staining. Magnification: 10× A549 cells (14.37 µg/mL for 48 h). The arrows point to apoptotic cells, the arrowheads point to live cells and the double arrowheads point to necrotic cells.

in A549, MIA PaCa and HUVEC cell lines using the fluorescence microscopy method by using a specific probe, DCF-DA. Our findings indicated that *O. onites* essential oil treatment increased reactive oxygen production significantly ($P < 0.0001$). ROS production was observed within 24 h of OE treatment in A549, MIA PaCa and HUVEC cell lines. As shown in Figure 4, *O. onites* essential oil' 4.79 µg/mL concentration produced ROS by 33.12 ± 1.89 intensity in the A549 cell line, 14.37 µg/mL concentration produced ROS by 25.88 ± 1.56 intensity in the MIA PaCa cell line, 67.06 µg/mL concentration produced ROS by 28.65 ± 1.32 intensity in the HUVEC cell line at 24 h. These results indicated that the OE induced apoptosis in these cell lines was linked to ROS generation. Representative microscope images from the DCF-DA analysis sample is shown in Figure 5.

Then, we investigated whether *O. onites* essential oil treatment can induce changes in cell migration rate. For this target, as shown in Figures 6 and 7, we tested the effect of IC_{50} values of *O. onites* essential oil as 4.79 µg/mL; 14.37 µg/mL; 67.06 µg/mL for 24 h and 14.37 µg/mL; 1.916 µg/mL; 306.56 µg/mL for 48 h on cell migration rates in A549, MIA PaCa and HUVEC cell lines using in vitro scratch assay technique. Our research results show that treatment with *O. onites* essential oil significantly reduces ($P < 0.0001$) the cell migration rate, as shown in Figures 6a and 6b, A549 (24 h 64 ± 6%, 48 h 67 ± 4%), as shown in Figures 6c and 6d, MIA PaCa (24 h multiplied by 49 ± 2%, 48 h 69 ± 4%), as shown in Figures 6e and 6f, HUVEC cell line (24 h multiplied by 47 ± 5%, 48 h 70±1%). A representative microscope image of the scratch experiment is shown in Figure 7.

4. Discussion

Previous studies have shown that environmental factors such as chemical composition of soil, growing season, moisture, temperature, vegetative stage influence the chemical composition of plant species (Loziene and Venskutonis, 2005). According to Bostancıoğlu et al. (2012); *O. onites* EO had carvacrol (64.3%), linalool (13.8%), ρ-cymene (13.8%), γ-terpinene (7.1%). Andoğan et al., (2002) determined that *O. onites* EO had 78.2% carvacrol as a main component and the other important components were borneol (8.1%), γ -terpinene (4.3%), cineole (1.5%). *O. onites* is commonly prescribed for treatment of stomach diseases, digestion problems, diabetes, cough, cholesterol,

Table 2. ROS production correlation with apoptotic cell rate was determined as ROS intensity values correlation with apoptotic cell rate on cell lines.

Cell line	OE IC_{50} concentration (µg/mL)	n	ROS intensity			Apoptosis rate		
			(+) Control	(–) Control	OE IC_{50}	(+) Control	(–) Control	OE IC_{50}
A549	4.79	6	57.65 ± 1.45	10.52 ± 0.8	[a,c,*] 33.12 ± 1.89	98 ± 1%	6 ± 1%	[b,d,] 56.3 ± 7%
MIA PaCa	14.37	6	57.06 ± 2.72	4.73 ± 0.62	[a,c] 25.88 ± 1.56	97 ± 2%	2.7 ± 2%	[b,d] 44 ± 7%
HUVEC	67.06	6	58.24 ± 0.98	8.24 ± 0.7	[a,c,*] 28.65 ± 1.32	99 ± 1%	4.7 ± 4%	[b,d] 48.7 ± 6%

Note: (1) n = 6 represents the number of samples, and the statistical significance was analyzed by the using GraphPad (Prism 5) software spearmann correlation test, (2) DCF fluorescence compared with the (+) control represents the H_2O_2 treated cells and (–) control indicate the treatment of cells with 0.1% DMSO (the solvent of OE) containing DMEM, respectively and averaged values of ROS intensity for at least 100 cells from each coverslip (n = 6).

[a] $P < 0.0001$ indicates a significant difference compared with the (+) control group of Ros intensity.

[b] $P < 0.0001$ indicates a significant difference compared with the (+) control group of apoptosis rate.

[c] $P < 0.0001$ indicates a significant difference compared with the (–) control group of Ros intensity.

[d] $P < 0.0001$ indicates a significant difference compared with the (–) control group of apoptosis rate.

* $P < 0.05$ indicates a significant correlation compared with the OE IC_{50} group of apoptosis rate

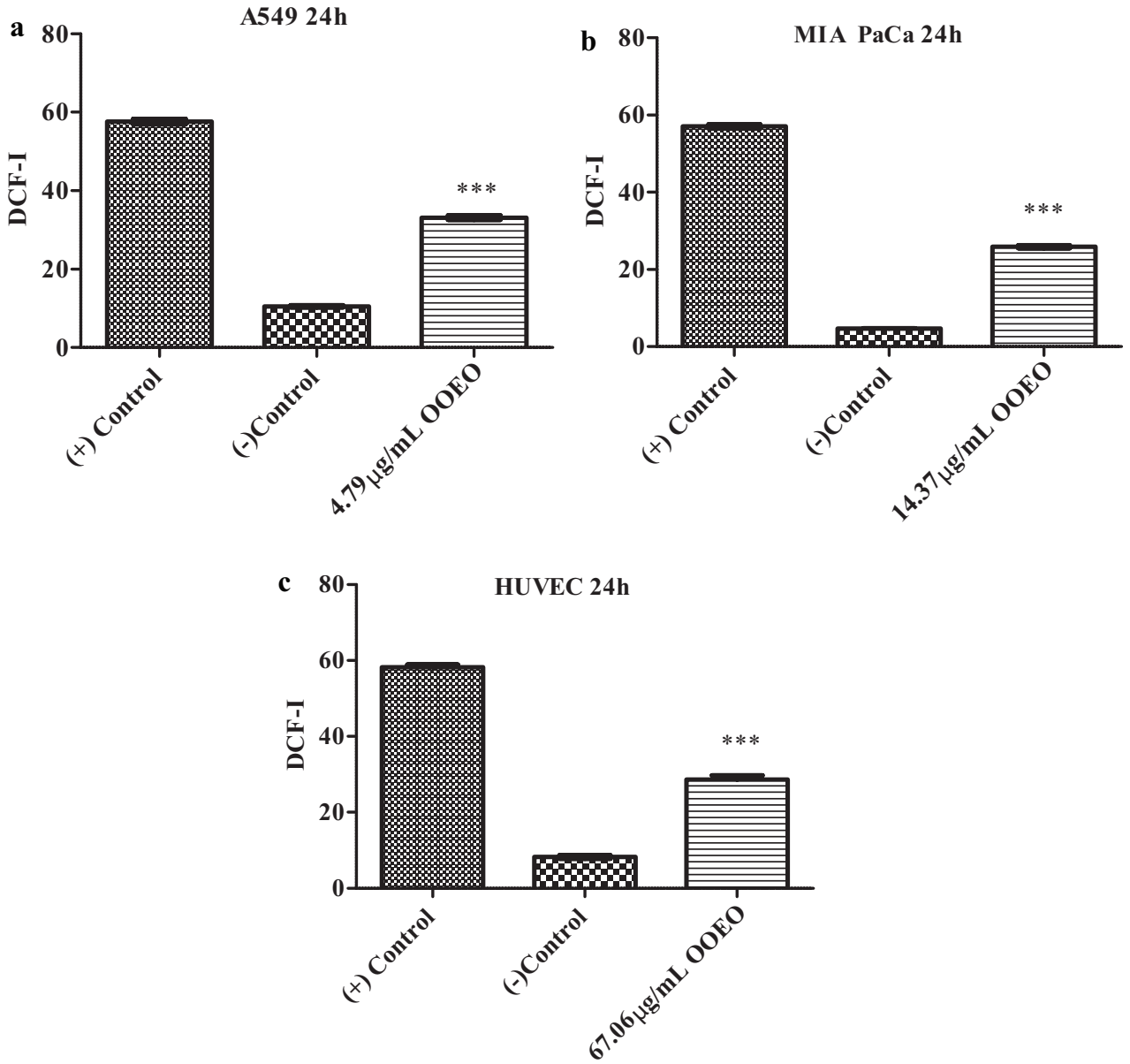

Figure 4. Cellular ROS detection was done by DCFDA assay a. DCF-I values of A549 lung cancer, b. DCF-I values of MIA PaCa pancreas cancer, c. DCF-I values of HUVEC endothelial cell lines were treated with IC_{50} values of *O. onites* essential oil as 4.79 µg/mL; 14.37 µg/mL; 67.06 µg/mL for 24 h on cells in an incubator. Then, DCFDA assay was performed at the indicated concentrations of *O. onites* essential oil. DCF-I: Averaged values of ROS intensity for at least 100 cells from each coverslip (n = 6). Fluorescence cellular ROS intensity was detected by Carl-Zeiss/Axio Observer 3, Zen 2.3 Blue Edition software. Production of cellular ROS intensity was compared against the nontreated control group. Used one-way analysis of variance (ANOVA), and then performed Tukey's multiple comparison test to test statistical significance (*** $P < 0.0001$ indicates a significant difference compared with the (–) control group, n = 6).

headache, toothache, tachycardia, flu, bronchitis, gastrointestinal diseases, respiratory tract diseases, hypertension, immunogenic, antiemetic and carminative (Bulut and Tuzlaci, 2013; Polat and Satil, 2012; Sağıroğlu et al., 2013; Sargin et al., 2013)in Turkish ethnobotanical studies. The antioxidant effects of flavonoids from *O. onites* have been investigated previously. Hazzit et al. (2006) revealed that the *O. onites* distillate has beneficial effects on the antioxidant status, lipid profile and flow- and nitroglycerine-mediated dilatation of the brachial artery in patients with mild hyperlipidemia.

There are reports of orally administered *Origanum* spp. regarding potential safety and efficacy against colorectal cancer. Both Spyridopoulou et al. (2019) and Llana-Ruiz-

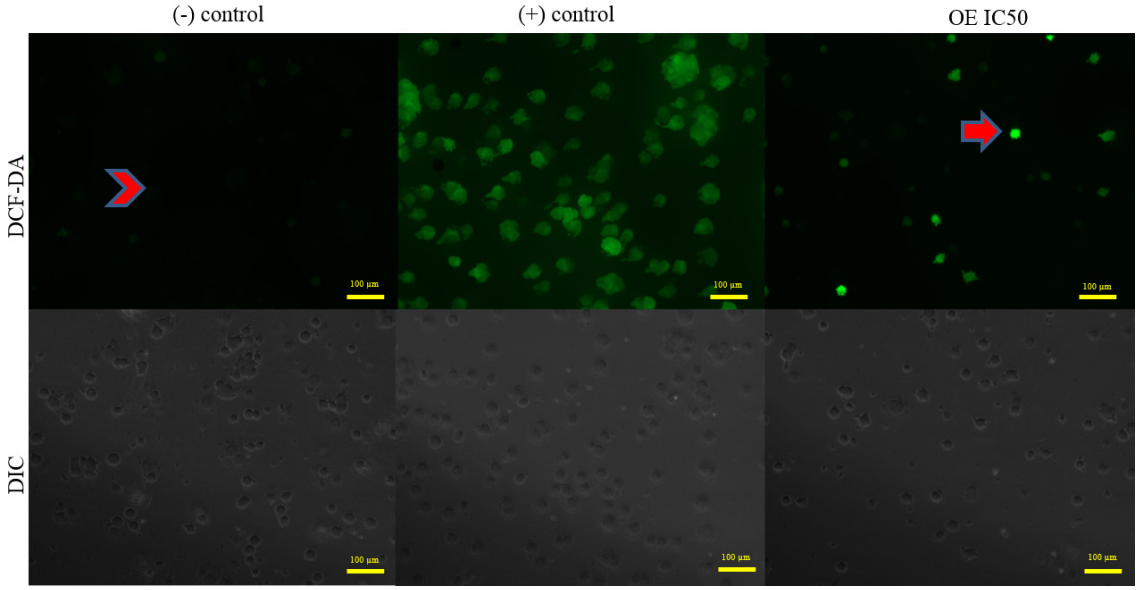

Figure 5. Representative microscope images from DCFDA assay. OE IC_{50} Magnification: 10×, Control Magnification: 40× A549 cells (4.79 µg/mL for 24 h). Arrows point to apoptotic cells, arrowheads point to nonapoptotic cells.

Cabello et al. (2017) reported that oral administration at a dose of 370 mg/kg OE on BALB/c for 13 days mice (Spyridopoulou et al., 2019) and at a dose of 200 mg/kg *O. vulgare* L. subsp. *virens* (Hoffmanns. & Link) Ietsw. in Wistar rats for 90 days did not cause toxic effects (Llana-Ruiz-Cabello et al., 2017). An in vitro study suggested that inhibition of the growth was slightly time-dependent since a reduction in the viability of HepG2 cells from 57.48 µg/mL doses and IC_{50} was around 76.64 µg/mL after 24 h exposure by *O. onites* (Sivas and Tomsuk, 2011). In another report, Özkan and Erdoğan (2011) suggested that the essential oil of *O. onites* decreased HepG2 cell viability at higher concentrations (>149.12 µg/mL).

However, systemic administration of *O. onites* may also affect other cell types. For example, the possible effects of this drug on other cell types (e.g., cancer, fibroblast, or endothelial cell lines) has not been widely studied. In this study, we investigated some physiological effects of *O. onites* essential oil on A549 lung cancer, MIA PaCa pancreas cancer, and HUVEC endothelial cell lines. Our findings clearly show that *O. onites* treatment at 9.58–191.6 µg/mL concentrations can inhibit cell proliferation in a cell-type (A549 and MIA PaCa) dependent fashion. These observations imply that *O. onites* administration may impact the process of wound healing. Although, this subject has yet to be investigated; clarification of molecular mechanisms underlying this observation can have important clinical and pharmacological implications. As shown in Figure 4, we also demonstrated that *O. onites* at IC_{50} values can exert inhibitory effects on the migration rate of the entire cell lines tested in this study. Especially, the A549 lung cancer cell line was amongst the most sensitive to *O. onites* treatment. These findings suggest that *O. onites* administration may have implications in cancer cellular migratory processes such as wound healing or cellular extravasation. Although underlying molecular mechanisms remain elusive, Spyridopoulou et al. (2019) showed that oral administration of OE significantly inhibited the growth of CT26 tumors in BALB/c mice. The essential oil of *O. vulgare* L has also been tested in vivo tumor models. Misharina et al. (2013) reported that formation and the growth of engrafted Lewis carcinoma tumors were inhibited with *O. vulgare* oil by oral administration to F1 DBA C57 black hybrid mice.

O. onites-induced decrease in cell migration rates might be due to the reduced proliferative capacity upon *O. onites* treatment. To our knowledge, Spyridopoulou et al. (2019) is the only report of the OE-induced cancer cell migration inhibition for now. Bostancıoğlu et al. (2012) described the effects of OE on RATEC cells by antimigratory. It has been reported that different *Origanum* species extracts have antimigration effects in cancer cells. For example methanolic or the ethanolic extract of *Origanum syriacum* L. against LoVo and SW620 human colon cancer (Aldisi et al., 2019) or MDA-MB-231 human breast cancer cell lines (Al Kahlout and Eid, 2014). Also, the ethanolic extract of *Origanum majorana* was reported to reduce the migration rate of the MDA-MB-231 cancer cell line (Al Dhaheri et al., 2013). Carvacrol which is the principal component of *Origanum* spp., Andoğan et al. (2002) reported inhibiting the in vitro migration of various human cancer cells (Dai

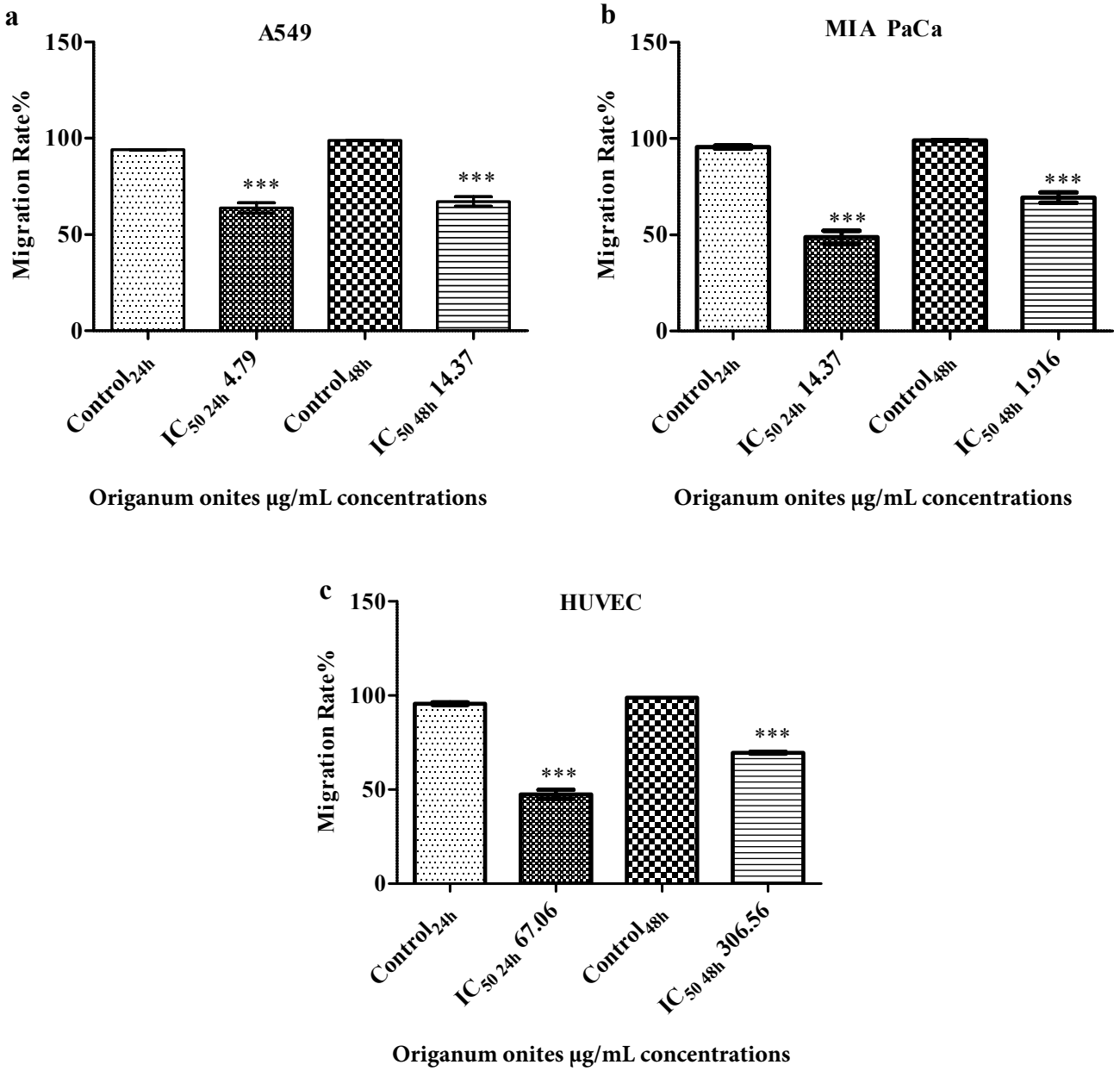

Figure 6. Migration rates were decreased significantly. a. A549 lung cancer (24 h by 64 ± 6%, 48 h 67 ± 4% for A549), b. MIA PaCa pancreas cancer (24 h 49 ± 2%, 48 h 69 ± 4% for MIA PaCa), c. HUVEC endothelial (24 h 47 ± 5%, 48 h 70 ± 1% for HUVEC). In an incubator, the cell line was treated with *O. onites* essential oil at IC50 concentration for 24 and 48 h. Scratch determinations were performed 24 and 48 h after treatment with the indicated concentration of *O. onites* essential oil. The 24- and 48-h mobility (the time the gap was closed) was calculated by measuring the gap at 0 and 24/48 h after scraping the board. The percentage change in the migration rate was compared with the untreated control group, and one-way analysis of variance was used, followed by Tukey's multiple comparison test (*** $P < 0.0001$, $n = 3$) to test for statistical significance.

et al., 2016; Fan et al., 2015; Jung et al., 2018; Trindade et al., 2019).

It has been reported that extracts of different *Origanum* species and carvacrol induce apoptosis and cell death in different colon cancer cell lines in vitro (Fan et al., 2015). While the carvacrol effects were studied by Matluobi et al. (2018) on HUVEC and hMSC (human mesenchymal stem cells), Rajput et al. (2017) studied it on human cancer cell line HCT-12 (colon cancer) and MIA PaCa (pancreatic cancer). Also Khan et al. (2018) (on A549) and Jaafari

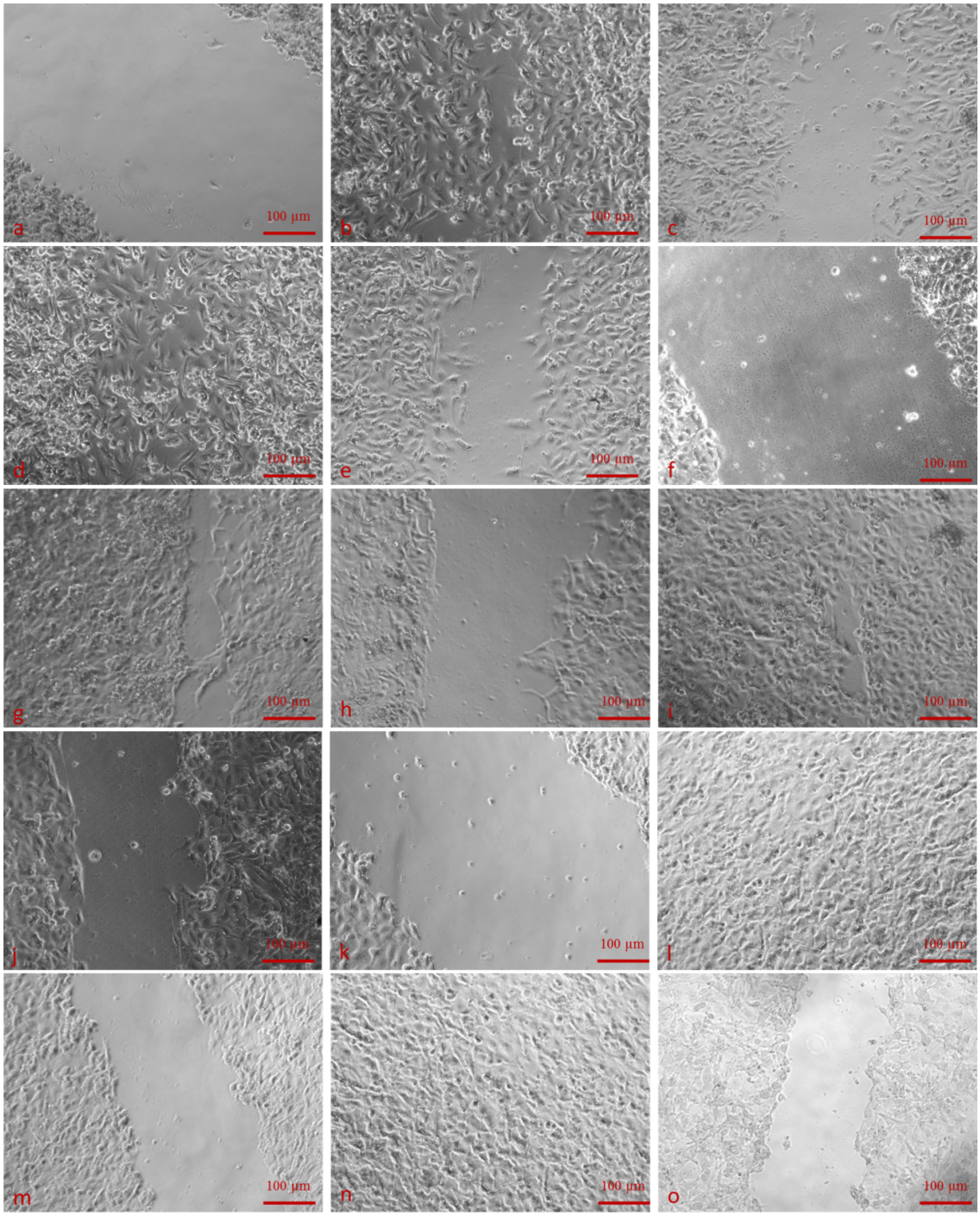

Figure 7. Representative microscope images from in vitro scratch assay (Magnification 10×). a. 0 h A549 cells, b. 24 h 0µg/mL *O. onites* essential oil A549 cells (control for 24 h), c. 24 h 4.79 µg/mL OE A549 cells, d. 48 h 0 µg/mL OE A549 cells (control for 48 h), e. 48 h 14.37 µg/mL OE A549 cells, f. 0 h MIA PaCa cells, g. 24 h 0 µg/mL OE MIA PaCa cells (control for 24 h), h. 24 h 14.37 µg/mL OE MIA PaCa cells, i. 48 h 0 µg/mL OE MIA PaCa cells (control for 48 h), j. 48 h 1.916 µg/mL OE MIA PaCa cells, k. 0 h HUVEC cells, l. 24 h 0 µg/mL OE HUVEC cells (control for 24 h), m. 24 h 67.06 µg/mL OE HUVEC cells, n. 48 h 0 µg/mL OE HUVEC cells (control for 48 h), o. 48 h 306.56 µg/mL OE HUVEC cells.

et al. (2012) (on P-815, K-562, CEM, MCF-7 and MCF-7 gem) have studied the effects of carvacrol, and in addition used anticancer drugs as the known compound to show their anticancer activity in their studies. Spyridopoulou et al. (2019) expressed that OE induces morphological changes usually observed in apoptosis both in the CT26 and HT-29 cells. It was revealed that three concentrations (125, 250, and 500 µg/mL) of OE can significantly inhibit cell viability and induce 5RP7 cell apoptosis, and can also block the formation and migration of rat adipose tissue endothelial cells (RATEC) in vitro. Because this essential oil has much weaker cytotoxicity to endothelial cells, but it has a strong effect on cancer cells (Bostancıoğlu et al., 2012) Natural products are generally a very valuable source of new anticancer compounds (Cragg and Newman, 2005). The essential oils of certain plants are a mixture of certain biologically active compounds and may be the basis for the identification of new anticancer molecules that can be further used in therapeutics (Fitsiou et al., 2016). Our results show that the efficacy of OE against proliferation against A549 human lung cancer, MIA PaCahuman pancreas cancer can be attributed in part to apoptosis-related mechanisms, making OE candidate for further research.

Thus, further tests should be performed to clarify whether or not the effect of *O. onites* on cellular migration is independent of the proliferation rate. These observations point to a possibility that low-concentration *O. onites* treatment can reduce proliferation and cell migration rates in certain types of cancer cells. However, we could not find any reports investigating the possible link between lung or pancreas cancer and *O. onites* treatment. Thus, further experimental and clinical studies are required to investigate this hypothesis.

The effect of drugs on cancer cells can be achieved by inducing cell apoptosis or necrosis or by causing changes in cell morphology.

In different colon cancer cell lines extracts from different *Origanum* species have been reported to induce apoptotic cell death (Fan et al., 2015; Spyridopoulou et al., 2019). In this study, it was found that as the treatment time increased and the concentration of essential oil in *O. onites* oil increased, the cell growth conditions were changed, and the cell growth and apoptosis were inhibited to varying degrees.

The production of ROS is an important mediator of many anticancer drugs (Trachootham et al., 2009). Previous studies have shown that ROS generated by chemotherapy is essential for inducing apoptosis in certain cancers (Simon et al., 2000). To our knowledge, this is the first study of correlating OE induced apoptosis in A549 and HUVEC cell lines also correlated with ROS produce. Here, we found that the ROS accumulation induced by OE is related to the apoptosis of A549 and HUVEC cell lines. Besides, OE made from natural plant materials may have multiple targets that affect cancer cells. According to our current research, ROS is one of the main points, but there may still be other functional goals. The role of essential oils may vary depending on the incubation period or the concentration of specific components or several components tested in a complex mixture of *O. onites* oil essential oils. Therefore, we recommend something like Spyridopoulou et al. (2019). future research should focus on investigating whether the antiproliferative activity of *O. onites* oil on human cancer cells can be attributed to its main components and determine whether there is a synergistic interaction between them.

Based on these results, it is proved that OE has an anticancer effect. The observed cytotoxic effects of OE may be due to the presence of specific components or several components in the complex mixture of OE. These findings may be a prerequisite for the potential development of these bioactive substances as effective antitumor drugs. However, further research is needed to evaluate its toxicity and safety. Also, these data can pave the way for the future development of anticancer treatment opportunities. This preliminary study will be expanded by implementing different cell lines. Even so, to better understand the OE effect, further in vivo studies are still needed.

5. Conclusion

Although further tests are required to confirm our conclusions, our preliminary in vitro observations imply that OE administration may alter processes of cellular proliferation, apoptosis and cellular migration in a cell line- and dose-dependent manner. Therefore, OE treatment can potentially impact cellular processes such as wound healing or cellular extravasation. Moreover, proliferative and metastatic properties of certain cancer cells can also be potentially altered in response to OE treatment in a cell type and dose-dependent fashion. *O. onites* essential oils show promising anticancer effects. The results obtained provide an experimental basis for reporting that OE leads to cell death mainly through the process of apoptosis. The observed cytotoxic effects of OE may be due to the presence of specific components or several components in the complex mixture of OE.

Acknowledgments

The authors thank to DÜAGEM (Altınbaş University Natural Products Research and Development Center) and BİTEM (Bezmialem University Phytotherapy Education Research and Application Center).

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