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Anticancer activity of *Origanum vulgare* on lung cancer: Antiproliferative, morphological, and apoptotic effects

Onder Yumrutas[1]*, Pınar Yumrutas[2], Mustafa Pehlivan[3], Demet Kahraman[4], Murat Korkmaz[5] and Muhittin Doğan[3]

[1]Department of Medical Biology, Faculty of Medicine, Adıyaman University, 02200 Adıyaman, Turkey
[2]Department of Respiratory Disease and Cancer Biology, Faculty of Medicine; [3]Department of Biology, Faculty of Science;
[4]Department of Medical Biochemistry, Faculty of Medicine, Gaziantep University, 27410, Gaziantep, Turkey
[5]Department of Medical Biology, Faculty of Medicine, Gaziantep Islam Science and Technology University, 27000, Gaziantep, Turkey

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The study was designed to determine the antiproliferation and apoptosis induction activities of *Origanum vulgare* hexane extract (OVHE) on lung cancer cells A549. For this purpose, the viability and proliferation of A549 cells were determined in lung cells after treatment of OVHE. The morphological changes of A549 cells were observed by a phase inverter microscope. Then, the percentage of apoptotic cells was scanned by FACS staining AnnexinV/Propodium iodide. Additionally, proapoptotic Bax and antiapoptotic Bcl-2 mRNA levels were determined by Real-time PCR. It was observed that the viability and proliferation of A549 cells decreased after the treatment of different concentrations of OVHE. Significant changes at the low concentration were observed in Bax and Bcl-2 mRNA levels. After the application of high concentrations of OVHE, it was determined that A549 cells died by necrotic pathway instead of apoptosis. In conclusion, this is thought to be the first report showing the antiproliferation and apoptotic effects of OVHE on lung cancer cells.

Keywords: Antiproliferation, Apoptosis, BAX, BCL2, *Origanum vulgare*

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Introduction

The genus *Origanum* is one of the most important genera of Lamiaceae. The majority of the species of the genus *Origanum* are distributed in the mountainous areas of Mediterranean coastal regions and at altitudes up to 1,500 meters. There are 43 species in the natural flora. The 75% of this genus, which has a wide distribution area in the Eastern Mediterranean Region[1], is currently reported to have 10 sections, 38 species, 6 subspecies and 17 hybrids based on morphological criteria[2]. Moreover, 23 of these species are found in Turkey, and 15 of them are endemic[3]. *Origanum* species have been used for many years in the food industry as teas and spices and as pharmaceuticals, especially in ethnomedicine[4]. These species have antioxidant[5], antimicrobial[6], antifungal[7], antidiabetic[8], anti-inflammatory[9], antispasmodic[10], antinociceptive[11], and anticancer[12] bioactivities.

Origanum vulgare L. is an aromatic perennial plant with white or purple flowers and a height of approximately 50 cm, generally distributed in the Mediterranean region and western Eurasia[3]. *O. vulgare* is also used in traditional medicine for its health effects, such as respiratory diseases, antiseptic, treatment of cold, indigestion, and stomach disorders[13]. Studies of the essential oils of this plant have reported many activities, including potent antimicrobial[14] and antioxidant[15], antiproliferation[16], and anti-inflammatory[17] activities. Moreover, *O. vulgare* contains many phytochemicals related to the biological activities it exhibits. Especially in its essential oils, compounds such as carvacrol, β-fenchyl alcohol, thymol, γ-terpinene, spathulenol, β-caryophyllene, α-terpineol, 1,8-cineol have been identified as main compounds[18,19]. In addition, phenolics such as rosmarinic acid, chlorogenic acid, quercitrin, rutin, apigenin, and acitenin derivatives have been reported[20].

The estimated annual incidence of diagnosis of lung cancer has been thought to be 2.21 million. Each year, 1.79 million lung cancer patients die from cancer[21]. In the United States alone, lung cancer ranks second in terms of incidence after prostate cancer in men and breast cancer in women. As a percentage of mortality, however, it ranks first in both sexes[22]. Due to the high mortality rate, there is a growing interest

*Correspondent author
Email: yumrutasonder@gmail.com;oyumrutas@adiyaman.edu.tr

in new agents for the treatment of lung cancer. Due to the insufficient efficacy and excessive side effects of synthetic drugs, many studies have focused on natural compounds. Most of these studies have sought to induce apoptosis, which is a programmed cell death involved in eliminating cancer cells. Physiologically, apoptosis is involved in the elimination of old and damaged cells. Cancer cells find a way to escape apoptosis and proliferate rapidly. Therefore, induction of apoptosis by the anticancer agents or the extracts containing these agents is one of the most desired properties. In this context, in this study, the effects of OVHE on antiproliferation activity, morphological changes and apoptosis induction of A549 cells were tested.

Materials and Methods

Collection and extraction of *O. vulgare*

O. vulgare was collected from natural habitat (Adana, Pozanti, Tekir Plateau (Turkiye), 17.06.23, voucher number: MPH2023-3) (Fig. 1), and was identified by Dr Mustafa PEHLIVAN.

For extraction, *O. vulgare* was washed with distilled water and dried on blotting paper in the open air and in a room away from sunlight. Then, it was ground in a mortar before the extraction. *O. vulgare* hexane extraction was performed with a Soxtlet apparatus. Firstly, a cartridge was prepared from powdered aerial parts of *O. vulgare* (50 g), placed in the Soxhlet, and extracted with 250 mL n-hexane for 6 hours at 40°C. The n-hexane solution was filtered and evaporated by a rotary evaporator. The crude n-hexane extract (yield 0.41 %w/w) was stored at +4°C until the assays began.

Fig. 1 — *O. vulgare* in its natural habitat (Gaziantep-Turkey).

Maintenance and growth of A549 cells

A549 cells were grown with 10% fetal bovine serum (FBS; Gibco, USA) and 1% antibiotic (Gibco, USA) supplemented in DMEM. Cells were maintained in an incubator with a 5% CO_2 supply at 37°C. After cultivation, A549 (6 x 10^4 cells/mL) cells were seeded onto 96 well plates and cultured for 24 hours on DMEM medium with supplements. After 24 hours of incubation, the old medium was aspirated and replenished with a new medium containing different concentrations (25, 50, 100, 200 µg/mL) of OVHE with serum-free medium and exposed for 24 hours under specified conditions. OVHE was dissolved in DMSO (at a concentration of 10% of dimethyl sulfoxide).

Morphological evaluation of A549 cells exposed to OVHE

After preserving lung cancer cell cultures in DMEM for 24 hours, 96-well plates containing 70–80% confluent of lung cancer cell cultures were treated for 24 hours with various concentrations of OVHE (25, 50, 100, 200 µg/mL). Then, the cells were examined under a phase-contrast inverted microscope (40X).

Determination of anti-proliferation activity

After preserving lung cancer cell cultures in DMEM for 24 hours, 96-well plates containing 70–80% confluent of lung cancer cell cultures were treated for 24 hours with various dilutions of OVHE (25, 50, 100, 200 µg/mL). MTT was utilised to evaluate cell viability (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide). The culture media was replaced with DMEM containing 1 mg/mL MTT (Sigma) and incubated at 37°C for 15 minutes. The cells were then treated with MTT solution and dimethyl sulfoxide (DMSO, Sigma). The density of the cells will be measured at 550 nm with a colourimetric reader (BioTek instrument, USA).

Determination of apoptosis induction by annexine V and propodium iodide (PI)

For the determination of apoptosis induction activity of OVHE, A549 cells were seeded with a 1x10^6/mL density to 6-well plates and the lowest (25µg/mL) and highest (200 µg/mL) concentrations of OVHE were applied for a period of 24 hours.

Annexin V/PI apoptosis detection kit was used to measure the apoptosis according to the manufacturer's recommended protocol. Results were measured in a Becton-Dickinson flow cytometer.

Determination of BAX and BCL-2 levels by Real-Time PCR

The A549 cells (1x) were exposed to 25 and 200 µg/mL concentrations of OVHE for 24 hours. Then, the supernatant was discarded, and 700 µL of QIAzol Lysis Reagent was added. The total RNA was extracted with TRIpure total RNA extraction reagent (ELK Biotechnology, Wuhan). Then, the total RNA was reverse-translated into cDNA using reverse transcriptase according to manufacturer's protocol. RT-PCR was performed on a Rotor Gene Q (Qiagen, Germany). cDNA template for each primer with SYBR green master mix reacted at 95°C for 15 min, 40 cycles 95°C for 20 sec, 60°C for 30 sec, and 72°C for 30 sec. The following PCR reaction was prepared: 10 µL SYBR Green Master Mix, 1 µL forward primer, 1 µL reverse primer, 5 µL RNase-free water and 3 µL cDNA. Reactions were held in the Rotor-Gene Q instrument using the following cycling conditions: 15 min at 95°C, 40 cycles of 15 sec at 95°C, 30 sec at 60°C, and 30 sec at 72°C. After the reaction, Ct (cycling threshold) values were taken at an appropriate threshold. The relative gene expression for BAX (forward: GTCGCCCTTTTCTACTT TGCC, reverse: GTCGCCCTTTTCTACTTTGCC) and BCL-2 (F: GTCGCCCTTTTCTACTTTGCC, reverse: TCACTTGTGGCCCAGATAGG) genes was determined with normalization to GAPDH (forward: GATCATCAGCAATGCCTCCT, reverse: TGTGG TCATGAGTCCTTCCA). mRNA expression levels are given as $2^{-\Delta\Delta CT}$.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 8.0.2. program. Dunnett's test was used for the statistical evaluation of antiproliferation, apoptosis and mRNA levels. In the evaluation of the statistics, the terms "*", "**", and "***" were meant $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

Results

Possible effects of OVHE on viability and proliferation of A549 cells

The effects of OVHE on the viability of proliferation of A549 cells were tested at concentrations of 25, 50, 100 and 200 µg/mL for 24 hours and given in Fig. 2. As can be seen in Fig. 2, the 200 µg/mL concentration of OVHE decreased significantly the cell viability and proliferation of A549 cells ($p<0.05$). On the other hand, the other concentration did not exhibit any effect on the viability of A549 cells ($p>0.05$).

Evaluation of the morphology of lung cells after treatment of OVHE

The morphology of A549 cells exposed to various concentrations of OVHE for 24 hours was evaluated using a phase-contrast inverted microscope. For the morphological evaluation of A549 cells, 25, 50, 100, 200 µg/mL were used. Control groups (DMEM and DMSO) showed normal morphology, whereas 200 mg/mL concentration of OVHE-treated cells was highly damaged, and almost no intact cell morphology was observed (Fig. 3).

Determination of apoptotic and necrotic cells after the treatment of OVHE

After determining the antiproliferative effect of OVHE on lung cancer cells, A549 cells were exposed to the effective concentration of 200 µg/mL and the lowest concentration, 25 µg/mL, used in the experiment. The percentages of viable, early apoptotic, late apoptotic and necrotic cells were determined by Annexin-V/PI staining. As shown in Fig. 4, the 25 and 200 concentrations of OVHE increased the percentage of A549 cells in the late apoptotic phase at rates of 3.8% and 8.2% compared to control DMSO ($p<0.05$ for both concentrations). On the other hand, 25 µg/mL concentration of OVHE decreased the percentage of viable cells from 99 to 55.2%, whereas the percentage of necrotic cells increased from 0.1 to 40.7% ($p<0.01$). Moreover, the percentage of necrotic cells increased to 85.4% after the treatment of 200 µg/mL concentration of OVHE ($p<0.001$).

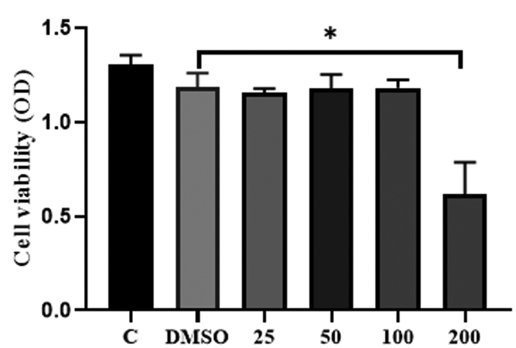

Fig. 2 — Antiproliferative effects of OVHE on lung cancer cells. The antiproliferation assay was repeated four times. According to the results of Dunnett's test, the proliferation of cells decreased significantly in the 200 µg/mL concentration group ($p<0.05$).

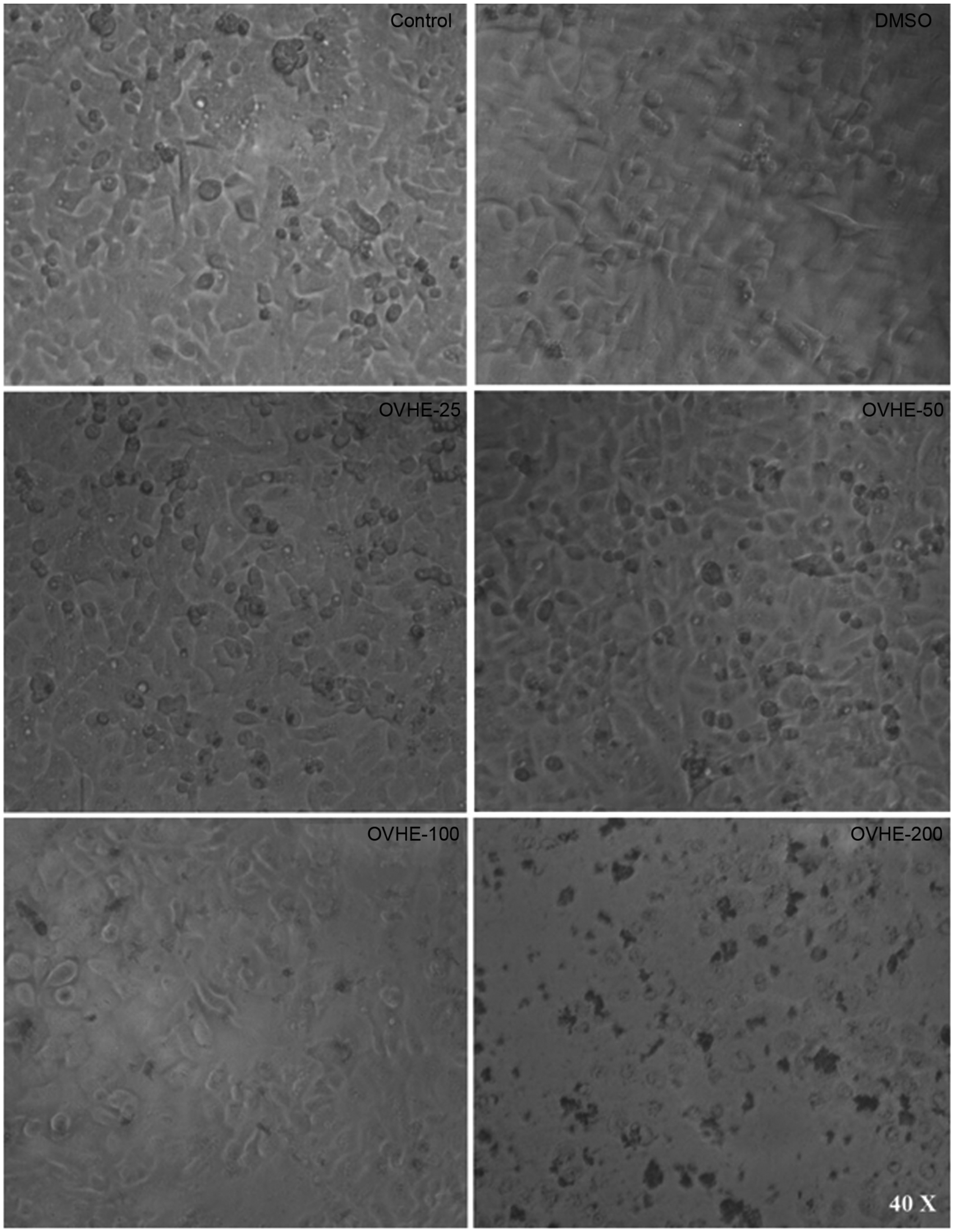

Fig. 3 — Morphological changes in A549 cells after OVHE treatment.

Determination of apoptotic BAX and antiapoptotic BCL-2 mRNA levels after the treatment of OVHE

After the treatment of OVHE for 24 hours, mRNA levels of BAX, a proapoptotic protein, and Bcl-2, an antiapoptotic protein in A549 cells were determined by Real-time PCR method and the results were given in Fig. 5. Both BAX and BCL-2 mRNA levels in A549 cells increased at 25 µg/mL concentration of OVHE when compared to DMSO control (p<0.05). However, at 200 µg/mL concentration of OVHE, BAX and BCL-2 mRNA levels did not change when compared to DMSO control (p<0.05)

Discussion

The role of *O. vulgare* in suppressing the viability and proliferation of cancer cells has been proven in previous studies. The antiproliferation activity of this plant has been specifically attributed to compounds

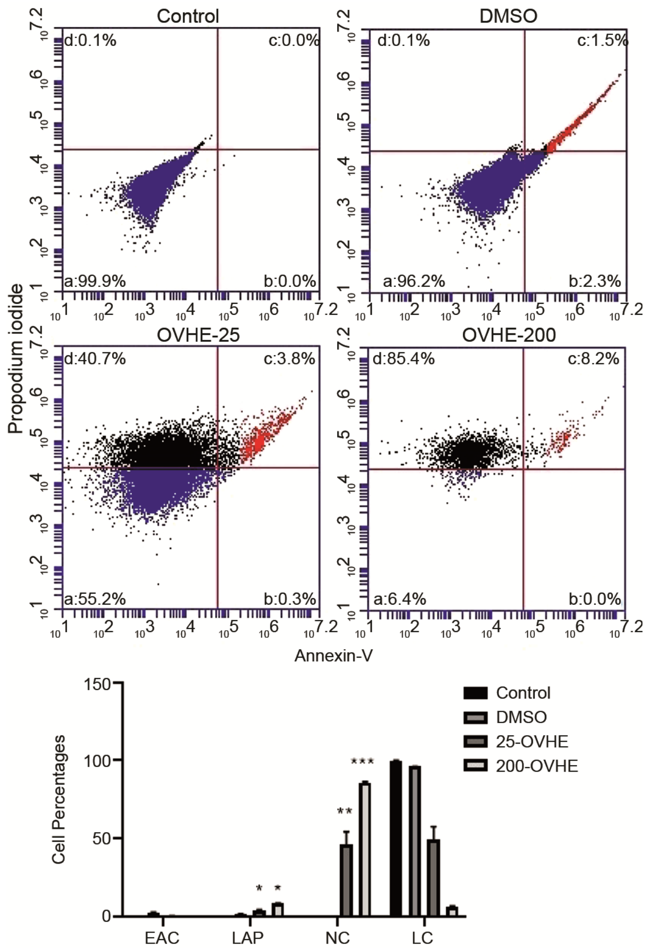

Fig. 4 — Percentages of apoptotic and necrotic cells in lung cancer cells after OVHE treatment. The statistical evaluation was performed according to Dunnett's test. The term "**" indicates $p<0.01$ and "***" indicates $p<0.001$ significance level.

such as rosmarinic acid, carvacrol and thymol[23]. However, as a result of our literature survey, no study on the anticancer activity of OVHE on lung cancer cells was found. Therefore, the present study tested the antiproliferation activity of OVHE on lung cancer cells A549. In light of the findings, it was determined that OVHE inhibited the viability and proliferation of lung cancer cells at high concentrations. In addition, results similar to the antiproliferation activity were obtained in the morphological evaluation. While the antiproliferation activity and morphological changes were not observed at low concentrations, similar results were obtained at high concentrations of OVHE. In previous studies, the antiproliferation effects of OVHE on different cancer cells have been shown. It was determined that *O. vulgare* ethanol extract significantly reduced the proliferation and viability of human colon cancer Caco2 cells at concentrations of 300 and

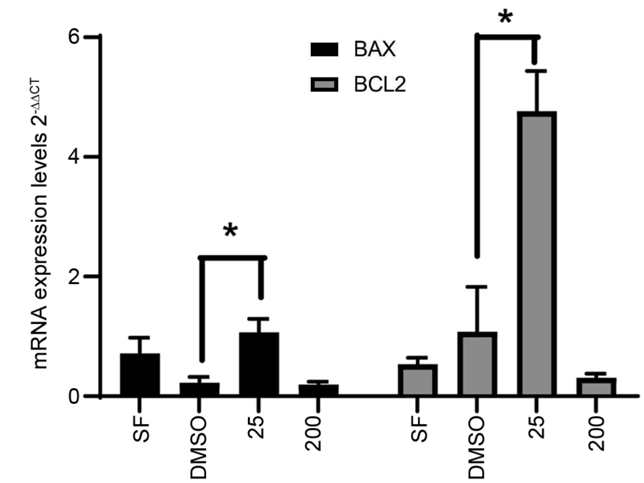

Fig. 5 — Changes in Bax and Bcl-2 expression levels after OVHE treatment. The expression levels were determined in repeated three times. According to the results of Dunnett's test, the expression levels increased significantly in the 25 µg/mL concentration group ($p<0.05$).

500 µg/mL[23]. In addition, it was reported that *O.vulgare* essential oil showed the best antiproliferation effect on human stomach cancer cell line (AGS) at a concentration of 100 µg/mL[24]. Moreover, in a study on the HCC-70 breast cancer cell line, *O. vulgare* water extract was shown to concentration-dependently reduce cell proliferation[25]. A study that used hexane, ethyl acetate, ethanol, methanol and ethanol extracts of *O. vulgare* on the human cervical cancer cells (HeLa) reported that all of them exhibited antiproliferative activity except hexane[26]. Elshafie *et al.*[27] showed that *O. vulgare* essential oil and the main compounds of the oil inhibited the viability and proliferation of HepG2 human hepatocarcinoma cells. When evaluated together with both our study and previous studies, it can be said that different *O. vulgare* extracts and essential oils have strong antiproliferative effects on different cancer cell lines. It is also important to show the mechanism that is activated when the viability of the cells decreases for the evaluation of anticancer activity. Therefore, the level of apoptosis and related markers were tested in our study.

Apoptosis is a programmed cell death involving chromatin condensation, DNA fragmentation, and cell membrane shrinkage and is controlled by many proteins. The balance of Pro apoptotic proteins such as Bak, Bax, Bid, and antiapoptotic proteins such as Bcl-2 and Mcl-1 are important for the initiation of apoptosis in the cell. Increased levels of Bax and Bak trigger the intrinsic apoptotic pathway, and factors such as stochrom-C, SMAC/DIABLO, HtrA2/Omi, apoptosis initiation factor, endoG are released from the mitochontri into the cytosol. Afterwards, the caspase cascade is activated, and finally, DNA is broken into fragments by CAD (Caspase-activated DNase). Cells undergoing apoptosis break up into small bodies called apoptotic bodies, which are removed by phagocytosis[28]. Macrophages recognize apoptotic cells as being digested by phosphotidyl serine crossing from the inner membrane to the outer membrane[29]. Previous studies have reported that extracts of *O. vulgare* induce apoptosis by affecting proaoptotic and antiapoptotic protein levels in different cancer cells. It was observed that the ethanol extract of *O. vulgare* suppressed the proliferation-arresting Caco2 cells in the G2 phase and increased the percentage of both apoptosis and necrosis[23]. Additionally, it increased the level of proapoptotic Bax protein and decreased the level of antiapoptotic protein Bcl-2. Balusamy *et al.*[24] reported that *O. vulgare* essential oil inhibited the migration and invasion of human gastric cancer cells and also induced mitochondrial apoptosis by increasing the level of proapoptotic Bax protein and decreasing the level of Bcl-2. In our study, while a small increase in apoptosis percentages of A549 cells exposed to 25 and 200 µg/mL concentration of OVHE was observed, it was determined the percentage of necrotic cells increased significantly at both 25 and 200 µg/mL concentration. In a previous study[30], it was reported that as a result of exposure of adrenocortical tumours SW13 and H295R cells to *O. vulgare* crude extract, percentages of H295R cells decreased in G0/G1 and G2/M phases, while percentages of SW13 cell increased in G2/M phase and decreased in G0/G1, and also killed the cells by necrotic death. As seen in previous studies, different extracts of *O. vulgare* could destroyed different cancer cells through apoptotic or necrotic death pathways. The main reasons for this may be ordered the factors including type of cell, the types and main components of the extracts used and their synergistic effects. Previous studies have shown that the apoptotic effect of *O. vulgare* may be due to carvacrol, thymol and rosmarinic acid. It has also been known that while the compounds such as carvacrol and thymol are in essential oils, rosmarinic acid is in polar solvent extracts. As a limitation of our study, the phytochemical content of OVHE was not determined. Besides, Koldaş *et al.*[26] determined that the main compound of the hexane extract of *O. vulgare* was 1-eicosanol, which is a long-chain fatty compound. Hence, to better assess the necrotic effect of OVHE, the levels of necrotic markers and the chemical composition of OVHE need to be determined in detail.

Conclusion

Consequently, this study demonstrated that OVHE has an antiproliferation effect on non-small lung cancer cells A549 cells, but it has been thought that this effect is due to necrotic death rather than apoptosis. However, one of the limitations of our study is that the levels of necrotic pathway markers such as RIPK1, RIPK2, and MLK were not determined to elucidate the relationship between necrosis and OVHE. Our literature survey did not find any study showing the effect of OVHE extract on the proliferation and death mechanisms of A549 cells. Therefore, the data of this study is thought to be the first report showing the antiproliferation effect of OVHE on A549 cells and its relationship with death pathways like apoptosis and necrosis.

Conflict of interest

The authors declare that there is no conflict of interest.

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