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Chemical Components of *Thymbra spicata* subsp. *spicata* L. Essential Oil and Its *In Vitro* Physiological Effects on Human Origin Cell Lines

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Abstract: *Thymbra spicata* subsp. *spicata* L. is a member of the Lamiaceae family, which is used in Türkiye against various health issues among other uses. The main components of *T. spicata* essential oil (TE) were identified as carvacrol (52.3%) and p-cymene (21.1%) using GC-FID and GC/MS. The in vitro effects of TE on Miapaca-2 and HUVEC cell lines were reported for the first time. The initial results showed that TE applied to Miapaca-2 cell lines at concentrations of ≥62.18 g/mL for 48 hours could reduce cell growth and induce apoptosis. The application of relatively high concentrations of TE (≥82.91 g/mL) significantly (P<0.001) suppressed cell growth. Administration of relatively high TE concentrations (≥82.91 g/mL) significantly (P<0.001) suppressed cell growth. Concentrations of TE lower than 20.73 g/mL did not affect the viability of the HUVEC cell line in 48 hours. The IC_{50} value for Miapaca-2 cells was 62.18 µg/mL, and HUVEC cells' IC_{50} value was 263.97 µg/mL for 48h. The number of apoptotic cells in Miapaca-2 (55±5%) and HUVEC (38.33±6%) were significantly higher (P<0.001). Significantly lower migration rates (P<0.001) were seen for Miapaca-2 (52±5%) and HUVEC cell lines (64±1.67%).

Keywords: *Thymbra spicata* subsp. *spicata*; cancer; apoptosis; wound-healing; cellular-migration; proliferation

1. Introduction

After heart disease, cancer is the second most common cause of death, and it is becoming a global health problem [1]. Recently, more than 19.3 million new cases of cancer were identified and reported; based on the provided data, in 2020, 10 million people would die from cancer [2]. Pancreatic cancer is a serious disease with high morbidity and mortality. The initial symptoms are few, which means that diagnosis is usually delayed. At the time of diagnosis, the majority of individuals have either locally advanced disease or metastatic disease [3]. At this stage, surgery or other treatments are

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impossible. Without treatment, the prognosis is very poor. The median overall survival (OS) is less than 6 months, and the 5-year survival rate is less than 5% [4]. Interfering with the regulatory steps (initiation, ascent, and progression) and associated signal transduction pathways is the most appropriate way to affect carcinogenesis [5]. Many physiologic and biochemical carcinogens exist, for example; Zheng et al. [6] revealed that smoking, alcohol consumption, and dietary factors affected pancreatic cancer. There is no highly effective drug on the market to treat most cancers. New natural products provide opportunities for drug discovery innovation [7]. Natural ingredients play a critical role in cancer prevention and care. Since the 1940s, natural compounds isolated from herbs have been used as a rich source of new anticancer drugs [8]. Nutrition, according to sources, can protect against cancer by inducing cell defense mechanisms such as detoxification, and antioxidant enzyme systems also stop the cell cycle and/or result in cell death [9].

Thymbra L. is a member of the Lamiaceae family. There are three species of *Thymbra* in Türkiye and seven worldwide. *T. spicata* L. is a small glandular-punctate shrub. Leaves entire, inflorescence spicate, usually dense. Verticillasters with 6-l0 subsessile flowers. Bracts like upper leaves but pointed. It is called Havai kekik, Bayır kekiği, Karabaş otu, Kaya kekiği, Karakülef, Kekik, Şeker otu, Mercanköşk in Kepsut and Savaştepe, Balıkesir, Türkiye [10]. According to ethnobotanical studies, *T. spicata* is commonly used in different regions of Türkiye among local people, for the treatment of stomach ache the aerial parts of the plant is brewed into tea in Mount Arat, Şanlıurfa [11], for kidney stones in the Mediterranean region of Türkiye [12], for cold and abdominal pain in Turgutlu (Manisa) [13], for diabetes, hypercholesterolemia, constipation and intestinal spasm, headache, respiratory tract diseases, and atherosclerosis in Alaşehir (Manisa) [14], for gastrointestinal disorders, as an anthelmintic in Dalaman (Muğla) [15], for cardiac diseases, hemorrhoids, eczema in İzmir [16], and for cardiac deficiency, arteriosclerosis, insomnia, and as a sedative in Gönen (Balıkesir) [17]. *T. spicata* is considered both a herbal tea and spice in Bodrum (Muğla) [18].

In current studies, it was found that TE had an antibacterial effect [19] and an antioxidant effect [20]. *T. spicata* oil is used as a natural antioxidant in sausages to enhance quality and produce safe products [21]. The essential oil (EO) of *T. spicata* has acaricidal activity against adult carmine mites [22]. *T. spicata* extracts improved DNA tolerance to $HgCl_2$-mediated genetic damage in human lymphocytes, according to Dirican et al. [23].

A wide range of bioactivities of EO components has attracted great attention. Carvacrol, γ-terpinene, p-cymene, myrcene, β-caryophyllene, and thymol are the EO's primary ingredients of *T. spicata* from Türkiye, their percentages are given respectively: 75.74%, 9.28%, 7.17%, 1.39%, 1.13%, and 0.15% [24]. Numerous EOs, including the phenolic monoterpene carvacrol, have gained popularity due to their biologically beneficial properties. In the literature, carvacrol and thymol are often referred ~~to~~ as phenols. This is incorrect, according to KHC Baser [25], because they are not biosynthetically derived from phenol. Phenol is a non-living compound that can only be found in fossil fuels. Carvacrol and thymol have monoterpene structure having a methyl and an isopropyl groups, with a hydroxyl group replaced in opposite positions on a phenol ring. Aromatic plants and EOs contain these volatile compounds. Thymol and phenol are crystalline, whereas carvacrol is frequently liquid, even though their only difference in formula is the position of the hydroxyl group [26]. Thymol and carvacrol were discovered to be the most important constituents of TE [27].

In vitro studies showed that carvacrol could prevent the growth of human laryngeal cancer [28], mouse melanomas [29], and non-small cell lung cancer cells [30]. Researchers also discovered that cytotoxicity in different mammalian cells (V79, HepG2, and Caco-2 cells) [31] had an antiproliferative effect on MDA-MB 231 human metastatic breast cancer cells [32] and Hep-G2 cells [33]. In primary rat neurons and N2a neuroblastoma cell cultures, carvacrol 400 and 200 mg/L concentrations dramatically decreased cell viability [34]. Also in another cell culture study, A549 lung cancer cells showed dose-dependently reduced cell viability and hindered cell proliferation at 24 hours [35].

Although the exact mechanism of carvacrol's cytotoxicity is unknown, oxidative stress is thought to be its primary cause. Other factors linked to the cytotoxicity of plant products include oxidative stress, proteasome inhibition, fatty acid synthesis inhibition, topo-isomerase inhibition, accumulation of p53, phosphatidyl-inositol 3-kinase inhibition, triggering cell cycle arrest, or increased expression of c-fos and c-myc [34].

In vitro cell culture models are helpful for biocompatibility and medication toxicity research before using animal models [36]. To our knowledge, there are only a few reports published, however, regarding thymol and carvacrol's potential synergistic effects as the EO of *T. spicata* with other phytochemicals against cancer cell lines. Thymol and carvacrol, two components of TE, were more effective than the oils of other components at killing MCF-7 cancer cell lines [37].

Systemic TE administration, on the other hand, has the potential to affect a wide range of cell types. This EO's effects on endothelial cell lines or pancreatic cancer cell lines have not been thoroughly investigated. Accordingly, we aimed to investigate the effect of TE on Miapaca-2 pancreatic cancer and human umbilical vein endothelial cell (HUVEC) lines and compared the changes in viability, the rate of cell migration, and the ratio of apoptotic cells. The relationship between the detection of cellular reactive oxygen species (ROS) and the ratio of apoptotic cells was discovered.

2. Materials and Methods

2.1. Cell Culture and Chemicals

Human pancreatic carcinoma epithelial cells (Miapaca-2 (ATCC® CRL-1420™) and human umbilical vein endothelial (HUV-EC-C [HUVEC] (ATCC® CRL-1730™) were used. The cell lines were grown in high glucose DMEM (Sigma, 5546) with 1% 2mM L-glutamine, P/S (50 U/mL penicillin and 50 g/mL streptomycin; Biological Industries, 03-031-1B), and 10% fetal bovine serum (FBS) as supplements (Biowest, S1810-500). A 10-cm plate was seeded with 1.5 x 10^6 cells from each cell line, which were then split 72 hours later. For each test, a freshly generated 0.1% dimethyl sulfoxide (DMSO) aqueous solution that had been sterilized using a 0.45-m membrane filter and contained TE was used [38]. Cells were subjected to 0.1% DMSO (the TE's solvent) in DMEM as the negative control.

2.2. Plant Material and Essential Oil Isolation

The aerial parts of *T. spicata* samples were collected from Kepsut (Balıkesir) in June 2020. Voucher specimens were prepared after the plant species were identified by Dr. Ebru Özdemir Nath. These voucher specimens were kept at the Pharmacy Faculty's Herbarium at Altınbaş University (HERA 252).

EO was extracted from air-dried and powdered 100 g plant material through hydro-distillation for 3 hours in a Clevenger-type apparatus with a 2.5 mL yield. Anhydrous Na_2SO_4 was used to dry the isolated EOs, and samples were stored at 4°C in amber vials pending analysis.

2.3. Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

EO solutions in n-hexane at a concentration of 10% (v/v) were analyzed using GC-FID/MS. An Agilent 7890B GC-FID (Santa Clara, CA, USA) coupled to an Agilent 5977E electron impact mass spectrometer (Santa Clara, CA, USA) by a two-way capillary splitter provided for the identification and measurement of the components of EOs. An Agilent G4513A (Santa Clara, CA, USA) auto-injector was used to inject 1 µL of sample solutions. The following temperature program was used to operate the DB WAX column (60 m, 0.25 mm, 0.25 m): 70°C for 15 minutes, then 180°C at a rate of 2°C/min. The column temperature was raised to 230°C at a rate of 5°C/min following a 5-minute period of maintaining an isothermal temperature of 180°C. Finally, isothermal column temperatures of 230°C for 15 minutes were set. One hundred minutes were allotted for the analysis. As a carrier gas, helium was employed at a constant flow rate of 1.5 mL/min. A split ratio of 1:50 was chosen. Temperatures for the injector port, quadrupole, MSD transfer line, ion source, and FID were 250°C, 230°C, 150°C, 250°C, and 220°C, respectively. H_2 flow was adjusted to 30 mL/min and FID air flow was 400 mL/min. The 45-450 m/z mass detector scan range was used.

The EO components were identified by comparing the mass spectra of compounds obtained from the Wiley Registry of Mass Spectral Data, 9th edition (April 2011) with the NIST 11 Mass Spectral Library (NIST11/2011/EPA/NIH). Relative retention indices (RRI) were calculated from the co-injected saturated n-alkane series, and the results were evaluated with previous studies and the NIST online webbook. Three separate simultaneous auto-injections were conducted for the same operational conditions on GC-FID/MS. Relative percentage amounts of the separated compounds were calculated from FID chromatograms.

2.4. MTT Assay

MTT analyses were performed 24, 48, and 72 hours after the given concentration of TE. A 12-mM stock solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide; Neofrox 3580 MTT) was prepared according to Mosmann's instructions [39]. Each well of a 96-well plate had about 10^4 cells seeded onto it in a volume of 100 mL. This is how the MTT assay was performed: Each well received 10 L (from a 12 mM stock) of the excess MTT solution, which was then incubated at 37°C (4 hours). One hundred microliters of medium alone in a cell-free well was included as a negative control. Seventy-five microliters of the medium were taken out of the wells after the incubation period of 4 hours. Then, 50 mL of DMSO was used to dissolve the formazan crystals. There were a further 10 minutes of incubation at 37°C, followed by the measurement of 540 nm absorbance.

2.5. Acridine Orange/Ethidium Bromide Double Staining

The acridine orange/ethidium bromide (AO/EtBr) dual staining procedure was used. Each well of a 96-well plate had 10^4 cells seeded in it. Forty-eight hours of incubation with TE followed, and the cells were then trypsinized. The cell suspension (10-25 μL) was transferred onto glass slides. One microliter of an AO/EtBr dye mixture (a staining solution with 100 μg/mL AO and 100 μg/mL EtBr) was used on cell suspensions, then a coverslip was placed over the samples. Under a fluorescence microscope, the morphology of the cells was assessed after the addition of the Ao/EtBr dye mixture (Carl-Zeiss/Axio observer 3., Zen 2.3 Blue Edition software) within 20 minutes. The number of cells needed to be at least 200 for the statistical analysis. The results were from at least three different experiments and were presented as mean values. An examination of the preparations was made considering the situations explained by Liu et al. [40]. Ethidium bromide exclusively stained dead cells that had lost membrane integrity, whereas AO stained both living and dead cells. The nucleus of early apoptotic cells displayed green patches, whereas the color of living cells was already green. Late apoptotic cells displayed constricted and/or often shattered nuclei and were stained orange. The necrotic cells had an orange stain and resembled live cells in terms of their nuclear shape, but they lacked condensed chromatin.

2.6. Detection of ROS

ROS was measured using fluorescence microscopy using the particular identifier DCF-DA (DCF-DH Sigma-Aldrich (D6883) 50 mg). When the substance enters the cell, intracellular esterase cleaves it, and because the hydrophobic portion is lost, the chemical remains inside the cell. Fluorescence microscopy can be used to detect the fluorescein component when it is oxidized by ROS because it is not covered. The amount of ROS present in the cell directly relates to the amount of fluorescence generated. This method is frequently used to evaluate ROS in living cells [39]. Reactive oxygen generation was measured using human endothelial cell lines HUVEC, Miapaca-2 human pancreatic cancer, and A549 human lung cancer. The cells were either treated with TE at their respective IC^{50} levels for 24 hours or left untreated (negative control) before being trypsinized. Cells treated with H_2O_2 (0.1 and 0.2 mM) for 24 hours were used as a positive control. A stock solution of DCF-DA (20 mM) in dimethyl sulfoxide (DMSO) was diluted to a workable concentration of 0.1 mM using a growth medium. The cells were trypsinized, rinsed in ice-cold 1PBS, and then treated for 20 min at 37°C with 0.1 M (working solution) of DCF-DA.

The cells were photographed using a fluorescent microscope equipped with a Carl-Zeiss/Axio observer 3 after being rinsed three times with cold PBS (Zen 2.3 Blue Edition software). Following a single 300 ms exposure for fluorescence photos, DIC photographs were taken from the same field of view. The fluorescence intensity of each cell in a 6060-pixel box was measured using the Zen 2.3 Blue Edition program, and the average of at least 100 cells per cover glass was taken (n = 6) [41].

2.7. In Vitro Scratch Assay

The Liang technique was used to evaluate migration rates in the *in vitro* scratch experiment [42]. A quick straight scratch was produced on the surface of the wells in 6-well plates using a 10 µL sterile pipette tip. Scratch photos were taken under the microscope at 10x magnification after a gentle wash with the culture medium, first at 0 hours, and subsequently at various intervals up to 24 hours (Carl-Zeiss/Axio observer 3). Using Image-J software to examine the gap's dimensions, by comparing the non-scratched region at 0 hours after injury to the non-scratched region at 24 hours, the rate of cell migration was estimated. The average of three replicated experiments was used to express the results.

2.8. Statistical Analysis

For the statistical analysis, GraphPad (Prism 5) software was used. Tukey's technique was used for multiple comparisons. Statistics were judged significant at $P<0.05$. A study of variance was used to conduct a statistical analysis of the differences in apoptosis indexes between each group.

2.9. Assay for Colorimetric Screening of Cyclooxygenase Inhibitors

Using a Colorimetric COX (ovine) Inhibitor Screening Assay Kit, the drug (1)'s inhibitory activity toward COX-1 and COX-2 was measured (Cayman, No. 760111). Peroxidase activity was determined by analyzing the colorimetric behavior of oxidized N,N,N,N-tetramethyl-p-phenediamine (TMPD) at 590 nm. The positive control was aspirin. DMSO was used to dissolve the compound and aspirin at concentrations of 0.625, 0.125, 0.25, 0.5, and 1 mg/mL. The following protocol, which was suggested by the supplier, was followed to conduct the test in a 96-well plate [43]. Heme (10 µL)) and 160 µL of assay buffer were added to three wells as background wells. Assay buffer (150 µL), 10 µL of heme, and 10 µL of COX-1 or COX-2 were placed in three wells as 100% initial activity wells. Assay buffer (150 µL), 10 µL of heme, and 10 µL of COX-1 or COX-2 were used for inhibitor wells. Inhibitor wells were filled with 10 µL of inhibitors and the background and 100% initial activity wells were filled with 10 µL of DMSO, which was used as a solvent. The plate was incubated at 25°C for 5 minutes. After incubation, 20 µL of the TMPD colorimetric substrate solution and 20 µL of arachidonic acid were added to each well, respectively. After a brief shake, the plate was incubated for 5 minutes at 25°C. The absorbance at 590 nm was measured using a microplate reader. All tests were repeated in triplicate, and the average of the experimental results was used to compute the percentage of inhibition using the provided formula:

% inhibition = [(100% initial activity -inhibitor well) / (100% initial activity)] x 100

3. Results and Discussion

3.1. Chemical Composition of EO

TE was found to have a total of 16 different constituents. The elements are listed in Table 1 according to the order of the retention indices. The components of the oil were 71.05% carvacrol as the major compound, 15.68% p-cymene, and 5.40% ɣ-terpinene, respectively. Carvacrol is a phenol that is a natural monoterpene derivative of cymene. The "Oregano" aroma is caused by carvacrol, an oxygen-containing monoterpene with phenol-like behavior that has substantial antioxidant, antibacterial, and other effects. p-cymene is a naturally occurring aromatic organic compound.

According to classification, it is a monoterpene-related hydrocarbon. The group of chemical molecules known as branched unsaturated monoterpenes includes the substance gamma-terpinene, also known as 1,4-p-menthadiene. This subspecies belongs to the carvacrol-chemotype, and other important oregano species such as *Thymbra* and *Origanum* species also exhibit the dominance of the carvacrol-chemotype [44]. Environmental factors such as soil chemical composition, growing season, precipitation, temperature, and vegetative stage affected the chemical composition of plant species in previous studies [45].

Table 1. The essential oil compositions of *Thymbra spicata* subsp. *spicata* growing in Balıkesir

No	Components	KI[a]	RRI[b]	Relative %	Identification Method
1	α-Pinene	1018-1032	1027	0.78±0.04	RRI, MS
2	Camphene	1060-1076	1074	0.10±0.01	RRI, MS
3	β-Pinene	1102-1118	1114	0.09±0.01	RRI, MS
4	Myrcene	1155-1169	1160	1.14±0.06	RRI, MS
5	α-Terpinene	1170-1188	1187	0.64±0.03	RRI, MS
6	Limonene	1190-1204	1204	0.21±0.01	RRI, MS
7	β-Phellandrene	1202-1218	1213	0.14±0.01	RRI, MS
8	ɣ-Terpinene	1238-1255	1249	5.40±0.22	RRI, MS
9	*p*-Cymene	1264-1280	1272	15.68±0.15	RRI, MS
10	1-Octen-3-ol	1437-1454	1456	0.28±0.01	RRI, MS
11	Caryophyllene	1585-1612	1599	0.68±0.01	RRI, MS
12	4-Terpineol	1592-1611	1606	0.64±0.01	RRI, MS
13	Borneol	1690-1719	1706	0.17±0.01	RRI, MS
14	Caryophyllene oxide	1970-2008	1987	1.73±0.04	RRI, MS
15	Thymol	2130-2198	2191	0.40±0.01	RRI, MS
16	Carvacrol	2204-2232	2220	71.05±0.41	RRI, MS
	Total identified			99.12	

RRI[b]: Relative retention indices calculated against n-alkanes; % calculated from FID data.
Identification method based on the relative retention indices (RRI) of compounds on the HP Innowax column; MS, identification was performed based on computer matching of the mass spectra with those of the Wiley and MassFinder libraries and comparison with literature data.
KI[a] were given from literature with confidence intervals of 50% of RI data ranges for each compound [46-52].

3.2. MTT Cytotoxicity Assay

Understanding the biologic and pharmacologic characteristics of medications requires knowing the effective concentration (IC50) at which the concentration inhibiting cell development falls by half. First, the MTT method was used to compare the proliferation rate of Miapaca-2 (human pancreatic cancer epithelial cells) and HUVEC cell lines. The impact of TE's IC_{50} value on cell viability was then investigated. For this target, nine different concentrations (5.18 - 207.28 µg/mL) for Miapaca-2 and HUVEC) of TE [53] were tested for 24, 48, and 72 hours. IC_{50} values for the Miapaca-2 cell line were 88.09 µg/mL for 24 h, 62.18 µg/mL for 48 h, and 20.73 µg/mL for 72 h. Miapaca-2 cell line proliferation rates were decreased at all concentrations of TE treatment (5.18–207.28 g/mL for 72 hours), according to the analysis of data from proliferation assay tests (Figure 1a). Higher TE concentrations (62.18 g/mL for 24 hours, 82.91 g/mL for 48 and 72 hours) significantly decreased cell growth but a 24-hour TE treatment at 51.82 g/mL did not (in the HUVEC cell lines) (Figure 1b). A TE concentration of 5.18 µg/mL did not significantly inhibit proliferation rates at 24 hours (0.19%) and 48 hours (10.4%), but was significant at 72 hours (31.96%) in the Miapaca-2 cell line. However, a 10.36 µg/mL concentration of TE inhibited, not significantly, by 0.08%, significantly by 29.84% and

36.02%. A 20.73 µg/mL concentration inhibited by 19.93%, 40.15% and 50.33%; a 3 µg/mL concentration inhibited by 17.84%, 41.08%, and 61.06%, a 51.82 µg/mL concentration inhibited by 18.04%, 39.33% and 76.96%; a 62.18 µg/mL concentration inhibited proliferation rates by 23.90%, 42.42% and 80.42%; a 82.91 µg/mL concentration inhibited proliferation rates by 40.22%, 56.04% and 81.33%; a 103.64 µg/mL concentration inhibited proliferation rates by 84.02%, 65.05% and 93.24%; and a 207.28 µg/mL concentration inhibited proliferation rates by 83.95%, 78.76% and 94.12%, in the Miapaca-2 cell line at 24 hour, 48 hour and 72 hour, respectively (Figure 1a). Concentrations of TE of 5.18 and 10.36 µg/mL increased proliferation rates by 12% and 8.05% at 24 hours; 5.18, 10.36, and 20.73 µg/mL concentrations increased proliferation rates by 3.6%, 3.3%, and 1.25% at 48 hours; and 5.18, 10.36, 20.73 and 41.46 µg/mL concentrations increased proliferation rates by 2.5%, 4.07%, 6.08%, and 3% at 72 hours, respectively, in the HUVEC cell line.

Concentrations of TE of 20.73, 41.46, and 51.82 µg/mL inhibited, not significantly, proliferation rates by 6.5%, 8.9%, and 13.3%, and 62.18, 82.91, 103.64, and 207.28 µg/mL concentrations significantly inhibited proliferation rates by 23.41%, 28.33%, 40.7%, and 43.05%, respectively, at 24 hour in the HUVEC cell line. At 48 hour incubation time of concentrations of TE of 41.46, 51.82, and 62.18 µg/mL inhibited, not significantly, proliferation rates by 4.52%, 8.21%, and 10.45%, and 82.91, 103.64, and 207.28 µg/mL concentrations significantly inhibited proliferation rates by 20.72%, 33.47%, and 36.33%, respectively. However, at 72 hour incubation, concentrations of TE of 51.82 and 62.18 µg/mL inhibited, not significantly, proliferation rates by 1.80% and 6.65%, whereas 82.91, 103.64, and 207.28 µg/mL concentrations significantly inhibited proliferation rates by 19.70%, 20.30%, and 27.82% in the HUVEC cell line (Figure 1b). TE treatment appears to be less effective on the HUVEC cell line. When it is calculated IC_{50} values-wise, the endothelial cell line needed to be exposed to 2.68, 4.25, and 14.44 times higher concentrations of TE, respectively, than Miapaca-2 in 24, 48, and 72 hours for an inhibitory effect. As shown in Figure 1b, when compared with the nontreated (control) Miapaca-2 cell line at 72 hours, the HUVEC cell line appears to be resistant to concentrations ≥5.18 µg/mL. In the presence of TE, the viability of Miapaca-2 cells was significantly reduced ($P<0.001$). TE had an inhibitory effect on the tested Miapaca-2, not the HUVEC cell line. Thus, depending on the incubation time or test concentration, the effect of the EO can change.

According to ethnobotanical researchers in Türkiye, TE is commonly used for diabetes, kidney stones, hypercholesterolemia, gastrointestinal disorders, hemorrhoids, headache, respiratory tract diseases, insomnia, atherosclerosis, cardiac diseases, and eczema. In different regions of Türkiye, local people use *T. spicata* as tea or as a spice in daily life. Akan et al. [54] revealed that *T. spicata* had dose-dependent protecting and healing effects against the damaging effects of diabetes mellitus. Demiralay et al. [55] revealed that 300 mg/kg of *T. spicata* EO and atorvastatin showed an antihypercholesterolemic effect of approximately the same degree. According to Akdoğan et al.'s results, *T.spicata* had an antihypercholesterolemic effect, but also an anemic effect, and a distinct granular degeneration effect was observed in a group of male adult Wistar albino rats (160 mg/kg) [56]. Duran [57] discovered that *T. spicata* had a strong antiviral effect against the herpes simplex virus at a concentration of 40 µg/mL.

As a result of Asadi et al.'s study, the alcoholic extract of *T. spicata* both improved the lipid profile and could lower blood lipid levels like lovastatin [58]. The potential effectiveness and safety of oral thymol against hepatoprotective effects on the gastric ulcer model were reported in Sprague-Dawley rat studies. A preliminary study [59] was used to compute the concentrations of thymol (75, 150, 250, and 500 mg/kg), based on the concentration patterns of this medication and other investigations. Geyiklioglu et al. [60] discovered that treatment with thymol considerably lessened the hepatic damage brought on by Indo, despite the fact that the livers of rats given a 250 mg/kg dose of the compound had a normal structure. However, other cell types may potentially be impacted by systemic TE treatment.

Little research has been performed on the potential effects of TE on other cell types, like endothelial cell lines or pancreatic cancer. This study looked into some physiologic effects of TE on the pancreatic cancer cell line Miapaca-2 and the endothelial cell line HUVEC.

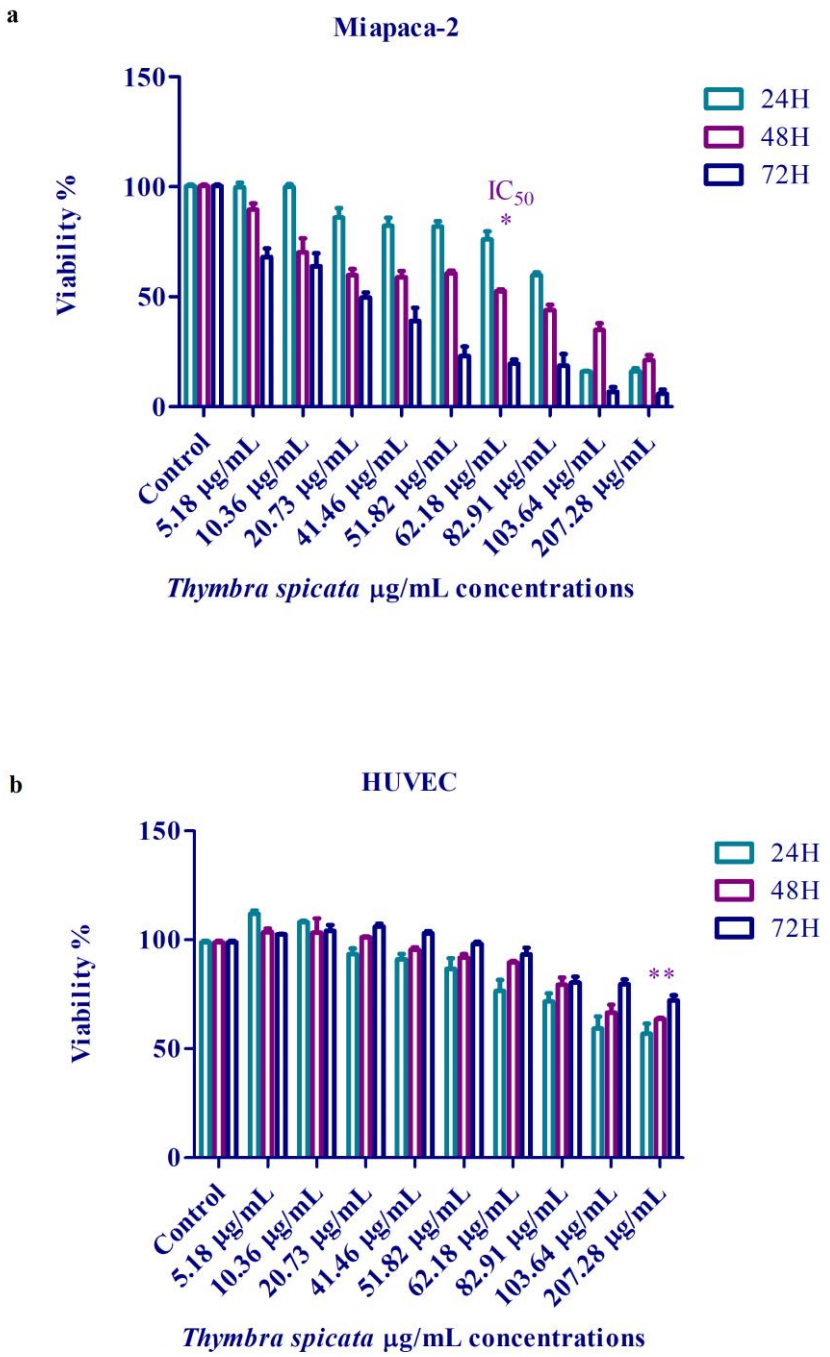

Figure 1. a) Miapaca-2 (pancreatic cancer), **b)** HUVEC (Endothelial cell lines) were treated with *Thymbra spicata* subsp. *spicata* EO for 24, 48, and 72 hours in an incubator.
At 24, 48, and 72 hours following TE treatment with the prescribed concentration, MTT analysis was carried out. One-way ANOVA and the Tukey multiple comparison test were used to perform the statistical significance test and compare the relative percentage proliferation rate to the untreated control group (* P<0.001, n=3).

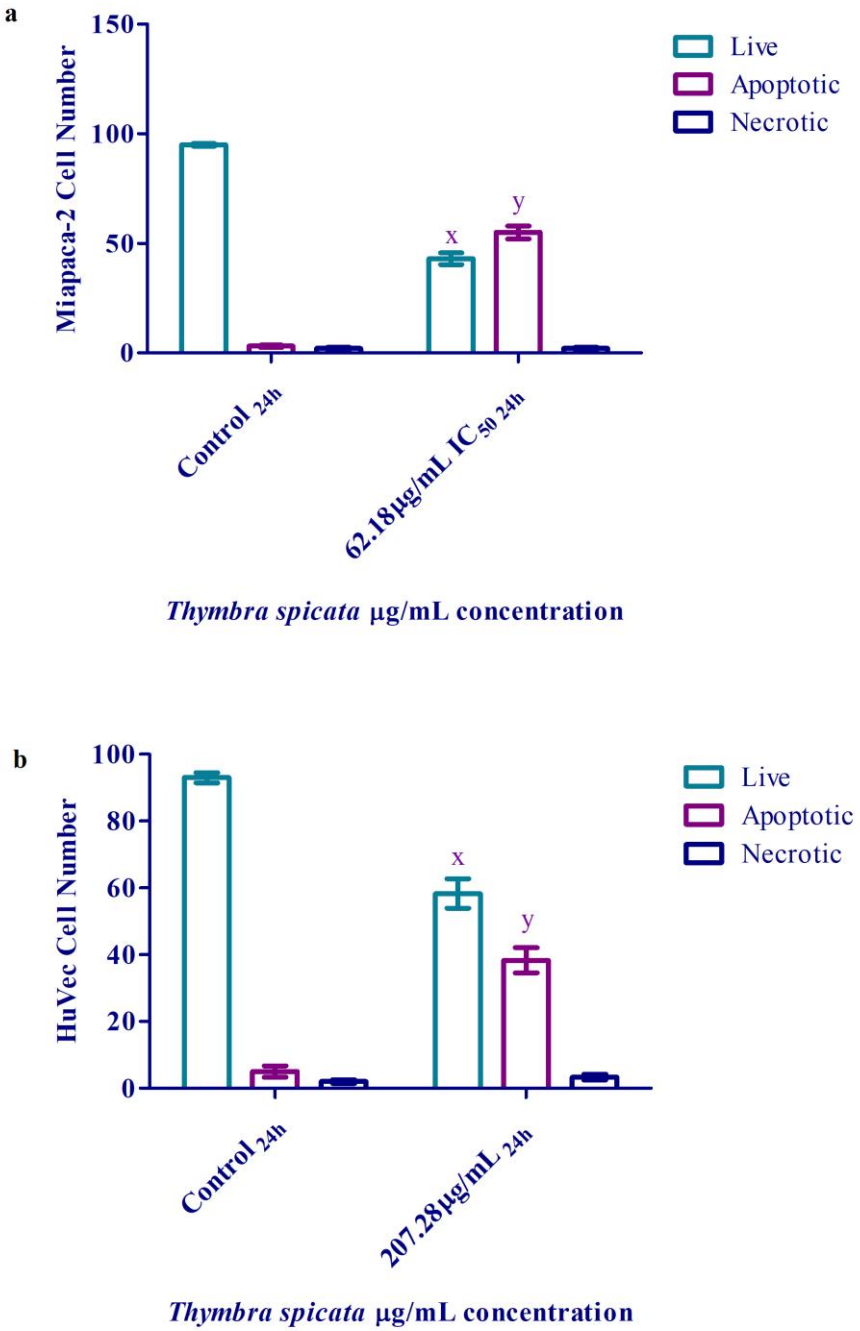

Figure 2. *Thymbra spicata* subsp. *spicata* induces cell apoptosis with IC_{50} value.
a) Miapaca-2 pancreatic cancer, **b)** The HUVEC endothelial cell line was treated with the IC_{50} value of *Thymbra spicata* subsp. *spicata* EO and it was estimated to be 62.18µg/mL and 263.97µg/mL after being placed in the incubator for 48h. After 48 hours of treatment with the indicated concentration of TE, AO/EtBr double staining was performed. Using one-way analysis of variance and Tukey's multiple comparison test, compare the percentage change in the fraction of apoptotic cells with the untreated control group and test for statistical significance (*, **, *** $P<0.001$, n=6).

3.3. Acridine Orange/Ethidium Bromide Double Staining

Various *Origanum* species' extracts and EOs, along with carvacrol, were shown to incite apoptosis in cancer cell lines *in vitro* [61-63]. Apoptosis is a key step in the development of cancer and a common target for new anticancer therapies [64]. Nagoor Meeran established that pre- and co-treatment with thymol (7.5 mg/kg body weight) in the liver of rats with isoproterenol-induced myocardial infraction inhibited apoptosis in the myocardium. Thymol's anti-tachycardia, antihypertrophic, anti-apoptotic, antihyperlipidemic, and free radical scavenging properties are all potential mechanisms for its protective impact [65].

Through the use of "the acridine orange/ethidium bromide" double labeling approach, the variations in the ratio of apoptotic cells were compared. When Miapaca-2 and HUVEC cell lines were exposed to the IC_{50} value of TE, the ratio changes of living cells, apoptotic cells, and necrotic cells were calculated as 62.18 g/mL and 263.97 g/mL for 48 hours. The number of apoptotic cells of the two types of cells was significantly higher over the 48-hour incubation period (P<0.001) (55±5% for Miapaca-2, 38.33±6% for HUVEC). However, as seen in Figures 2a-b, the 62.18 µg/mL concentration of TE over 48 hours triggered necrosis at 2% in Miapaca-2 cells, and the 263.97 µg/mL concentration of TE at 3.3% in HUVEC cells. Miapaca-2 cells were the more sensitive cell line (apoptotic cells 55%), and HUVEC cells (apoptotic cells 38.33%) seemed to be more resistant to TE. The findings support experimental evidence that TE causes cell death primarily through the apoptosis process. Figure 3 shows representative microscope images of AO/EtBr-stained samples. The cytotoxicity of TE against endothelial cells is much lower, but it has a very powerful impact on cancer cells.

In general, natural products are a fantastic source of novel anticancer chemicals [66]. Novel anticancer chemicals can be categorized using EOs, which are combinations of several bioactive substances found in plants, and then used in therapies [67, 68]. The findings of the study indicate that apoptosis-related mechanisms may play a role in the effectiveness of TE against proliferation against Miapaca-2 human pancreatic cancer, making TE a potential for further research.

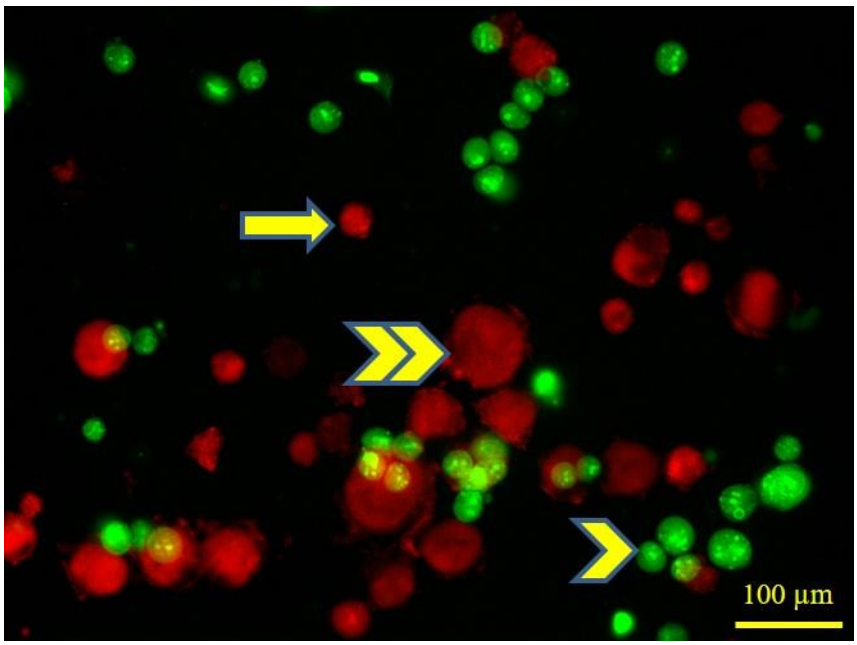

Figure 3. Under the microscope, an example of AO/EtBr dual staining.
40x magnification 62.18 µg/mL for 48 hours of Miapaca-2 cells. The double arrowheads (>>)indicate necrotic cells, the arrow (=>) indicates apoptotic cells, and the arrow (>) indicatesliving cells.

3.4. Detection of ROS

Many anticancer agents depend on ROS generation as a mediator [69]. Previous research has shown that ROS provided by chemotherapy is necessary for the induction of apoptosis in some cancers [70]. Numerous studies demonstrated that ROS could cause apoptosis in a variety of cancer cell types after anticancer medication therapy [71, 72]. Research was conducted to determine if TE-induced apoptosis was associated with elevated ROS levels in Miapaca-2 and HUVEC cells. We found a connection between the rate of apoptosis brought on by this oil and the increase in ROS that occurred with TE treatment (Table 2). Reactive oxygen generation rates in Miapaca-2 and HUVEC cell lines were determined for this purpose using the fluorescence microscopy method and the specific probe, DCF-DA. The effects of IC50 values of TE were evaluated as 62.18 g/mL and 263.97 g/mL for 48 hours on cells (Figure 4). The study findings indicated that TE treatment increased reactive oxygen production significantly in Miapaca-2 but not significantly in HUVEC cell lines (P<0.05). Within 48 hours of TE treatment, ROS generation was seen in the Miapaca-2 and HUVEC cell lines. A concentration of TE of 62.18 µg/mL produced ROS with 32.96±5.1 intensity in the Miapaca-2 cell line, 263.97µg/mL concentration produced ROS with 23.33±6.1 intensity in the HUVEC cell line at 48 hours (Figure 4). These findings suggest a connection between ROS production and TE-induced apoptosis in these cell lines. The material used for the DCF-DA assay is depicted by a representative microscope image in Figure 5.

This is the first study that we are aware of to link the production of ROS and TE-induced apoptosis in HUVEC cell lines. Here, it was discovered that TE-induced ROS buildup had a role in the apoptosis of Miapaca-2 and HUVEC cell lines. TE produced from organic plant material may also destroy cancer cells by affecting several different mechanisms. ROS is one of the main aspects of this research, but there may be other useful targets.

Depending on the duration of incubation or the concentration used to test for a particular component or many components contained in a complex mixture of TE, the effect of EOs may change. Accordingly, we concur with Eruygur et al. in our recommendation [73]. Future studies should concentrate on determining which of its constituent parts is responsible for the antiproliferative activity of TE on cancer cells and whether there is a synergistic interaction between them.

Table 2. ROS intensity values correlation with apoptotic cell rate on cell lines was used to assess the relationship between ROS production and apoptotic cell rates.

Cell line	*Thymbra sp.* IC_{50} concentration	n	ROS Intensity			Apoptosis Rate		
			(+) Control	(-) Control	TSEO	(+) Control	(-) Control	TSEO
MIA paca-2	62.18 µg/mL	6	57.24±1.9	7.49±4.3	a,c 32.96±5.1	97±1%	3±1%	b,d 55±5%
HuVec	263.97 µg/mL	6	57.98±2.8	9.56±2.5	a 23.33±6.2	98±1%	5±3%	b,d* 38.33±6%

Note: (1) n=6 represents the number of samples, and the statistical significance was assessed using the GraphPad (Prism 5) software with Spearman's correlation test. (2) DCF fluorescence compared with the (+) control represents the H_2O_2-treated cells, and (-) control indicates the treatment of cells with 0.1% DMSO (the solvent of TE) containing DMEM, respectively.

[a] $P < 0.05$ indicates a significant difference compared with the (+) control group of ROS intensity.

[b] $P < 0.001$ indicates a significant difference compared with the (+) control group of apoptosis rate.

[c] $P < 0.05$ indicates a significant difference compared with the (-) control group of ROS intensity.

[d] $P < 0.001$ indicates a significant difference compared with the (-) control group of apoptosis rate.

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

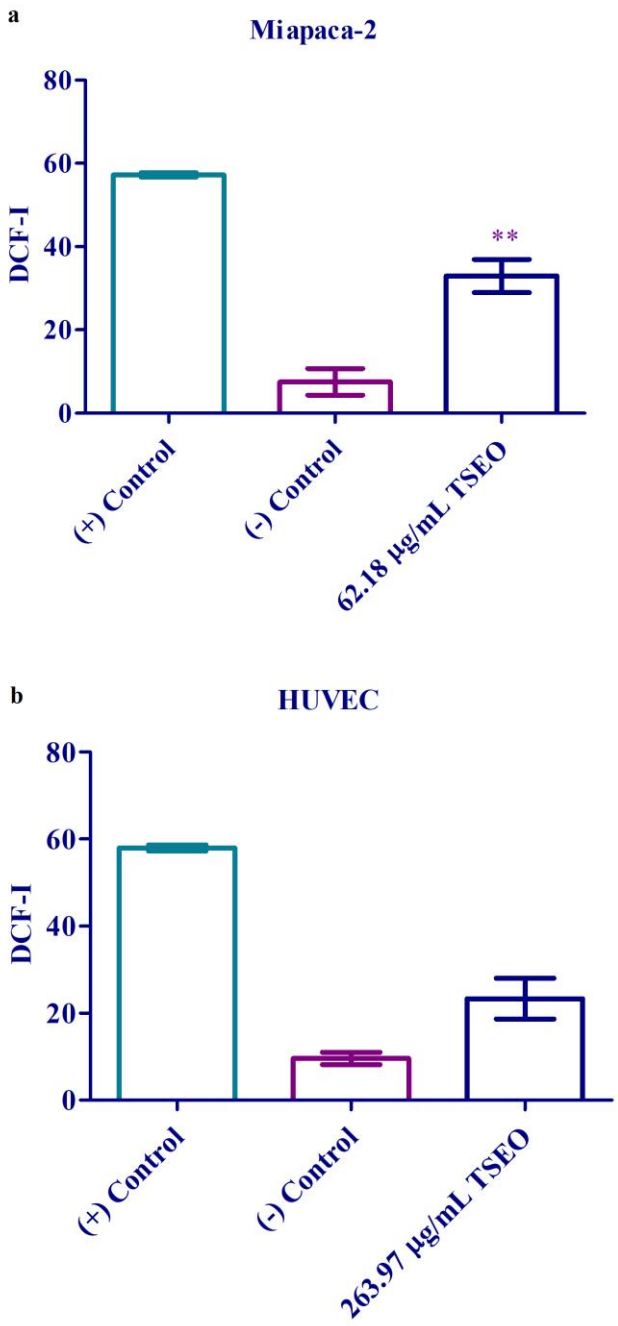

Figure 4. Cellular ROS detection was completed by DCFDA determination
a) The DCF-I value of Miapaca-2 pancreatic cancer **b)** In the incubator, the IC_{50} values of *Thymbra spicata* subsp. *spicata* EO were 62.18 µg/mL and 263.97 µg/mL to treat the DCF-I value of HUVEC endothelial cell line for 48 hours. The DCFDA assay was performed 48 hours after treatment with the indicated concentration of *Thymbra spicata* subsp. *spicata* EO. DCF-1: the typical ROS concentration of each cover glass, which contained at least 100 cells (n = 6). The software Carl-Zeiss / Axio Observer 3 and the ROS intensity of fluorescent cells were both detected. The level of cellular ROS production was compared with the control group, which received no treatment. Tukey's multiple comparison test and one-way analysis of variance were used to determine the statistical significance (** P <0.05 indicates a significant difference compared with the (-) control group, n=6).

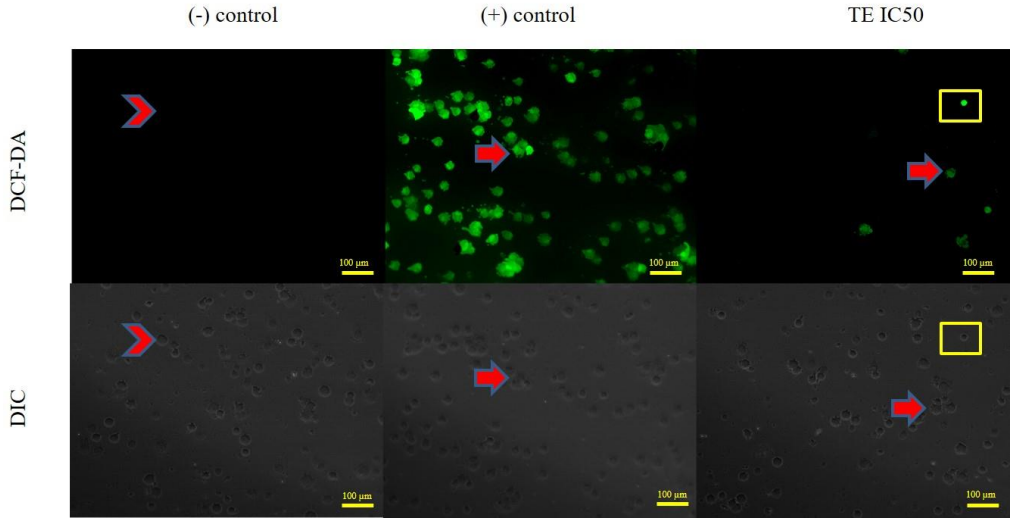

Figure 5. Representative microscope images of Miapaca-2 cells (62.18 µg/mL for 48 hours) from DCFDA assay. TE IC_{50} Magnification: 10×, Control Magnification: 40×. Arrows and arrowheads indicate apoptotic and non-apoptotic cells, respectively.

3.5. In Vitro Scratch Assay

The results of the MTT assay demonstrate that TE administration of at least 10.36 g/mL and 62.18 g/mL (correspondingly for Miapaca-2 and HUVEC) concentrations can decrease cell growth in a cell-type dependent manner in 48 h. These findings suggest that TE treatment may affect the healing of wounds. It was examined whether TE treatments might affect cell migration rates. The effect of TE's IC50 values of 62.18 g/mL and 263.97 g/mL was examined for this target on the cell migration rates in the Miapaca-2 and HUVEC cell lines using the *in vitro* scratch assay technique for 48 hours (Figure 6-7). The study findings showed that in Miapaca-2 (52±5%) (Figure 6a-b) and HUVEC cell lines (64±1.67%) (Figure 6c-d), TE treatment significantly reduced cell migration (P<0.001).

A representative microscope image of the scratch-measuring experiment is shown in Figure 7. Although this topic needs more research, understanding the molecular processes that underlie this discovery could have significant therapeutic and pharmaceutic ramifications. In addition, this study demonstrated that TE at IC_{50} concentrations can slow the migration of all the cell lines (Figure 4). The Miapaca-2 cell line was more sensitive to TE treatment than the HUVEC cell line.

These results imply that TE treatment may affect cancer cell migration processes such as wound healing or cellular invasion. The lower proliferative ability after TE treatment may be the cause of the TE-induced decrease in cell migration rates, though the underlying molecular pathways are yet unclear. There is only one report to our knowledge regarding *T. spicata* extracts. In that study, water (TW) or ethanol (TE) was used as the solvent of the extracts, and rat hepatoma FaO cells and human endothelial HUVEC cells were used for experiments [74]. Khalil et al. discovered that cell migration rates did not change much over a short period (6 h) after the scratch, but that over a longer period (24 h), the control cells reduced the wound width by around 40% in comparison with time 0. Both extracts significantly promoted cell migration at the same time, resulting in wound widths that were 32% and 23% smaller than controls, respectively. The anti-migration activity of TE can also be partly attributed to one or more specific components in the complex mixture of *T. spicata* EOs. Its main ingredient, carvacrol [75], has been shown to inhibit the migration of various human cancer cells *in vitro*, including HCT-116 and LoVo colon cancer cells [76], lung cancer (NSCLC) cells [77], oral squamous cell carcinoma Tca-8113 and SCC-25 cells [78], and PC3 prostate cancer cells [79].

These findings suggest that low-concentration TE therapy may be able to slow the growth and migration of specific cancer cell types. We were unable to locate any studies looking into a potential connection between TE use and pancreatic cancer, however. Additional experimental and clinical investigations are needed to explore this theory.

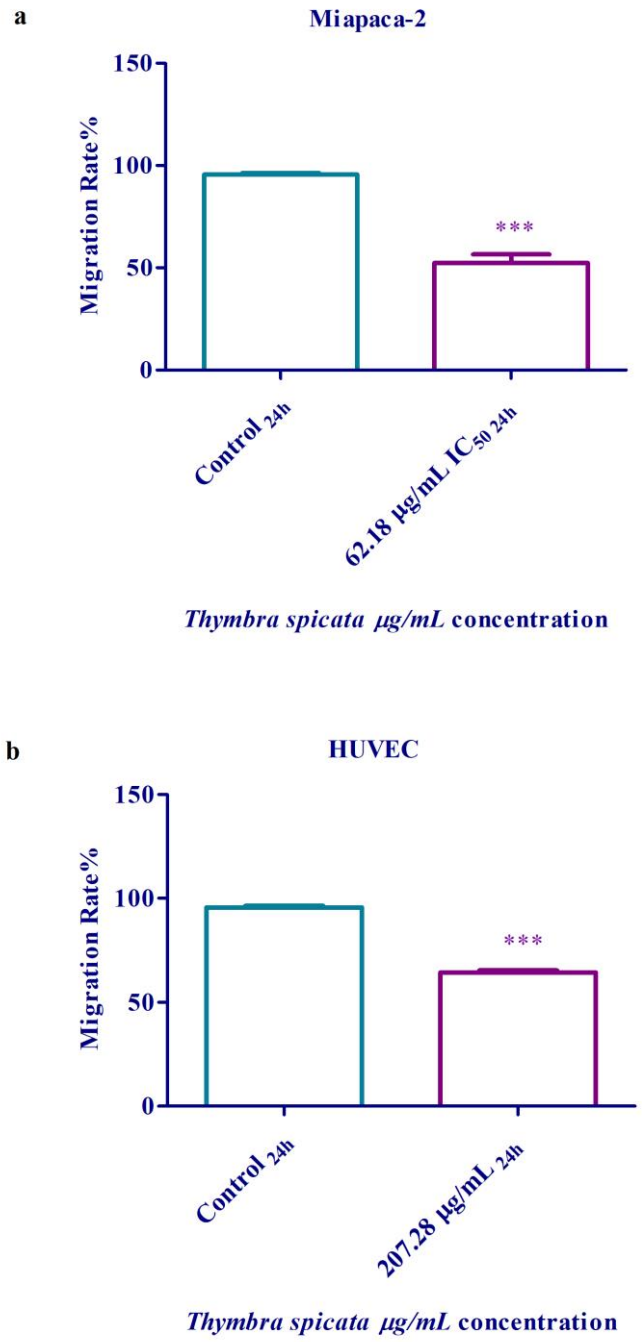

Figure 6. Migration rates were decreased significantly. **a)** Miapaca-2 pancreatic cancer (52±5% for Miapaca-2), **b)** HUVEC endothelial (64±1.67% for HUVEC), cell lines were treated with *Thymbra spicata* subsp. *spicata* EO at IC_{50} concentration for 48 hours in an incubator. Scratch measurement was performed 48 hours after treatment with the indicated concentration of *Thymbra spicata* subsp. *spicata* EO. By measuring the gap at 0 and 48 hours after scraping the plate, the mobility within that period (when the gap was closed) was determined. Using one-way analysis of variance and Tukey's multiple comparison test, the percentage change in migration rate was compared with the untreated control group (*** $P<0.001$, n=3).

It has been demonstrated that EO possesses a variety of cytotoxic characteristics and mechanisms that are effective on cancer cells. It is essential to perform additional research to determine how EO minor components contribute to the overall effect of the EO extract mixture in light of the numerous components present, as well as the mechanism and synergistic properties of the EO mixture. The most potent cytotoxic EO combination composition will be discovered through more *in vitro* and *in vivo* research with cancer cells, allowing for more targeted therapy with improved selectivity to cancer cells over non-cancer tissue. It is interesting that EOs and their components can work through a variety of routes and cellular mechanisms to have such different cytotoxic effects [80]. Through various signaling pathways, carvacrol and thymol showed cytotoxic and antiproliferative action on cancer cells. Carvacrol seems to be more effective than thymol *in vitro*. To define a standard and safe concentration, identify their harmful or side effects, and elucidate their precise mechanisms of action, further *in vivo* research with sound methods is needed [81]. Understanding the half-life in carvacrol is essential for using it effectively in the clinic, as well as the time of intervention with this phytotherapeutic, as the same in certain dosages can decrease cellular division, being thus a potent combatant against pathologies related to bacteria or disorders of excessive cell proliferation. Preclinical research still involves crucial fundamental elements [82]. By focusing on a variety of signaling pathways, such as MAPKs, Notch PI3K, mTOR, and AKT, carvacrol-treated cancer cells demonstrated considerable apoptosis induction, cell cycle arrest, cytotoxicity, antimetastatic activity, and diverse antiproliferative effects. Carvacrol seems to be a highly effective phytoactive chemical against a variety of carcinomas *in vitro*. To clarify safe and standard concentrations, identify their toxic effects, and explain their precise modes of action in order to build a feasible treatment strategy for cancer management, more *in vivo* research with better methodology is still required [83]. Drugs can destroy cancer cells by triggering cell necrosis or apoptosis, or by altering the form of the cells. In this investigation, it was shown that the growth environment of the cells changed and their capacity to develop and undergo apoptosis was prevented to varying degrees as treatment time and TE EO content increased.

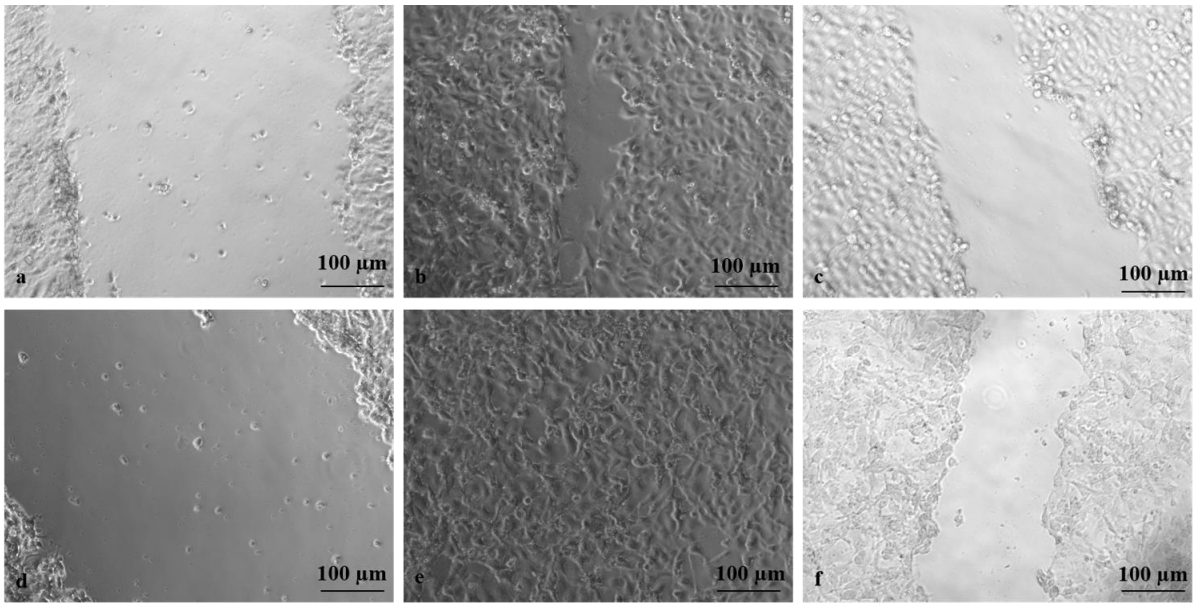

Figure 7. *In vitro* scratch assay representative microscope images.
a) 0 hours Miapaca-2 cells **b)** 48 hours 0% TE Miapaca-2 cells (control for 48h) **c)** 48 hours 62.18 µg/mL TE Miapaca-2 cells **d)** 0 hours HUVEC cells **e**) 48 hours 0% TE HUVEC cells (control for 48h) **f)** 48 hours 263.97 µg/mL TE HUVEC cells (Magnification 10×).

3.6. Effect of Plant Samples on COX Inhibition

The rate-limiting enzyme cyclooxygenase (COX), also known as prostaglandin-endoperoxide synthase, catalyzes the transformation of arachidonic acid into prostaglandin, which subsequently results in the production of other prostanoids (including prostaglandins, thromboxane, and prostacyclin). The COX family has two isozymes, COX-1 and COX-2, and whereas COX-1 is expressed in the majority of tissues and has physiologic functions in the body, COX-2 production can be triggered by a variety of factors, including inflammatory responses.

COX-2 plays a crucial role in facilitating the manufacture of prostaglandins during inflammation, and it also promotes angiogenesis by increasing levels of VEGF. It has been demonstrated that COX-2 is overexpressed in tumor vasculatures and activated in endothelial cells during inflammation. It has been hypothesized that inhibiting COX-2 could stop the progression of cancer at various stages because early cancer and preneoplastic conditions proved to increase COX-2 levels.

The results of the COX inhibition of TE are summarized in Table 3. The average COX-1 and 2 inhibition was calculated by taking the mean of COX inhibition activity. Overall, it was found that the fractions of TE (mean activity COX-1, 10.6% and COX-2, 86.49%) were observed to be significant inhibitors of COX-1 and 2. The results were compared with aspirin showing COX-1 (95.92 ± 3.56%).

Table 3. Effect of *Thymbra spicata* subsp. *spicata* essential oil on COX-1 and COX-2 activity*

Name of the sample	Inhibition of COX-1 (%)	Inhibition of COX-2 (%)
TE	10.6 ± 2.19	86.49 ± 4.24
Aspirin	95.92 ± 3.56	40.25 ± 4.6

*The results summarized are the mean values of n=3 ± SD

4. Conclusion

Other small molecules are likely to influence the behavior of the EO's main components. It is more important to analyze an entire EO rather than any of its components for biological purposes due to the principle of synergism appears to be more important. Although further research is required to support this claim, preliminary *in vitro* findings indicate that TE administration may alter cell proliferation, apoptosis, and migratory processes in a cell line- and dose-dependent manner. As a result, TE treatment may have an impact on cellular functions including cellular extravasation or wound healing. Depending on the cell type and concentration, the proliferative and metastatic characteristics of some cancer cells may also vary in response to TE treatment. According to the results of our investigation, it can be concluded that the EO of *T. spicata* shows anti-proliferative potential, which may be related to the presence of higher content of phenols and flavonoids. These results support Turkish ethnomedicinal use of *T. spicata.* This is the first study to examine the anti-proliferative activity of TE. It was discovered that TE-induced ROS buildup had a role in the apoptosis of the Miapaca-2 and HUVEC cell lines. TE has cytotoxic properties on cancer cells that are promising. The findings support the experimental findings that TE primarily causes cell death via the apoptotic phase. The cytotoxic effects of TE could be caused by a single component or a combination of components in the complex mixture of TE. However, to guarantee safety and bioavailability, additional through *in vivo* studies are required.

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Conflict of interest statement

We declare that we have no conflict of interest.

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