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**Original Article**

# The cytotoxic and apoptotic effects of *Thymus vulgaris* extracts on human breast cancer cell lines

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**ABSTRACT**

**Background and Aims:** Plant extracts are an important source of cytotoxic compounds and have consistently been an interesting field of research. The aim of this study is to investigate the cytotoxic and apoptotic effects of *Thymus vulgaris (T. vulgaris)* extracts on human breast cancer cell lines.

**Methods:** This study was carried out using human breast cancer cell lines (MCF-7 and MDA-MB-231) as experimental groups and the healthy human fibroblast cell line (PCS-201-012) as the control group. Petroleum ether and ethanol extracts were obtained from *T. vulgaris*. The extracts were applied to MCF-7 and MDA-MB-231 human breast cancer cell lines and human breast cancer stem cells. Cytotoxicity studies were performed using the RTCA iCELLigence system (Agilent Technologies), and apoptosis studies were performed using terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) and 4',6-diamidino-2 phenylindole (DAPI) methods.

**Results:** The *T. vulgaris* extracts were found to have concentration-dependent cytotoxic effects on human breast cancer cells. The growth of breast cancer stem cells was also determined to be inhibited when an effective concentration (45 µg/mL) of the extracts was applied. Lastly, specific morphological changes related to apoptosis were detected in the cells that had been treated with the effective concentration.

**Conclusion:** The *T. vulgaris* extracts were found to inhibit the proliferation of human breast cancer cells and human breast cancer stem cells selectively and concentration-dependently via an apoptosis-dependent pathway. The results suggest that the extracts may make promising sources for developing drugs for breast cancer therapy.

**Keywords:** DAPI, iCELLigence, MCF-7, MDA-MB-231, *Thymus vulgaris*, TUNEL

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## INTRODUCTION

Cancer involves genetic changes that lead to the transformation of a normal cell into a malignant cell by escaping cell death (Hanahan & Weinberg, 2000). The following are some of the pathways for eliminating apoptosis or the apoptosis resistance of malignant cells: (i) degradation of apoptosis precursors and anti-apoptosis proteins, (ii) suppression of caspase functions, and (iii) disruption of cell death receptors. Therefore, one of the strategies for treating cancer is to induce apoptosis (Wong, 2011).

Anticancer drugs today are derived from natural products or derivatives of natural products (Cragg & Pezzuto, 2016; Thomford et al., 2018). The *Thymus* (thyme) plant is one of the most important genera in the Lamiaceae family and belongs to the Angiosperm phyla (Kuete, 2017). *Thymus vulgaris* (*T. vulgaris*), also known as thyme, is an ornamental plant that grows in the ground and is endemic to the Western Mediterranean coastline (Bone & Simon, 2012; Hosseinzadeh, Jafarikukhdan, Hosseini, & Armand, 2015). Various studies have shown thyme to have antimicrobial (Nikolić et al., 2014; Hosseinzadeh et al., 2015), antioxidant (Roby, Sarhan, Selim, & Khalel, 2013; Nikolić et al., 2014), and anticancer (Berrington & Lall, 2012; Nikolić et al., 2014; Hosseinzadeh et al., 2015) properties. Natural phenolic compounds (e.g., carvacrol) found in *T. vulgaris* are known to have anti-proliferative and apoptotic effects on cancer cells (Yin et al., 2012).

Cancer stem cells occur as a hidden group within cancer cells and are also referred to as the group from which cancer arises (Zheng, Xin, Liang, & Fu, 2013; Batlle & Clevers, 2017). In samples obtained from breast cancer tissues, the cells with the phenotype cluster of differentiation (CD)$24^-$/$CD44^+$ have been identified as breast cancer stem cells (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003; Albeniz & Alkanlı, 2020). Breast cancer treatment failure and treatment resistance are well known for being associated with breast cancer stem cells (Bozorgi, Khazaei, & Khazaei, 2015).

This study hypothesizes *T. vulgaris* extracts to be able to possess anticancer and antiapoptotic properties regarding human breast cancer cell lines due to *T. vulgaris* being rich in active compounds such as thymol and carvacrol. Therefore, the study evaluates *T. vulgaris* extracts for their cytotoxic activity against human breast cancer cell lines (MCF-7 and MDA-MB-231) and human breast cancer stem cells. The study also identified the apoptotic induction of the extracts using the terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay and 4',6-diamidino-2 phenylindole (DAPI) staining methods.

## MATERIALS AND METHODS

### Chemicals

DAPI, dimethyl sulfoxide (DMSO), ethanol (EtOH), fetal bovine serum (FBS), phosphate buffered saline (PBS), and TUNEL kit were acquired from Sigma (St. Louis, MO, USA). Dulbecco's modified eagle medium-12 (DMEM-F12) was acquired from Lonza (Basel, Switzerland). Penicillin-streptomycin and $CD24^-$/$CD44^+$ stem cell markers were purchased from Thermo Fisher Scientific Life Sciences (Rockford, IL, USA). Petroleum ether (PE) was purchased from Honeywell (Charlotte, NC, USA). All other chemicals were of analytical grade.

### Plant material

The aerial parts of *T. vulgaris* were collected during the flowering stage of growth from Nezahat Gökyiğit Botanical Garden (NGGB) in Istanbul in the northwestern part of Turkey at an altitude of 81 m and registered with the NGBB Herbarium as 20070071. Botanist MSc. Burçin Çıngay was responsible for NGBB Herbarium and performed the species identification.

### Extract preparations

The aerial parts of *T. vulgaris* (90 g) were dried in the shade and then powdered in a mill. The powdered plant was first macerated in PE once for 3 days, and then extracted by acetone twice for 3 days, before finally being extracted by EtOH twice for 3 days. The obtained mixtures were then filtered. The PE and EtOH extracts were obtained by evaporating the solvents in a rotary evaporator then stored at -20°C for use in the experiments.

The extracts were prepared at different concentrations (360 µg/mL, 180 µg/mL, 90 µg/mL, 45 µg/mL, 20 µg/mL, and 10 µg/mL) by dissolving with DMSO (Esmaeili-Mahani, Falahi, & Yaghoobi, 2014; Nikolić et al., 2014).

### Cell culture conditions

MCF-7 and MDA-MB-231 human breast cancer cell lines, as well as the PCS-201-012 healthy human fibroblast cells, were obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in a culture medium containing DMEM-F12, FBS (10%, v/v), and penicillin-streptomycin (1%, v/v). The cells were maintained in 25 $cm^2$ and 75 $cm^2$ cell culture dishes in an incubator (SANYO, Osaka, Japan) with 5% $CO_2$ at 37°C. When the cells reached a density of 75% in the culture dishes, they were sub-cultured.

### Real-time cytotoxicity assay (RTCA)

The RTCA was performed using the iCELLegince system (ACEA Biosciences Inc., CA, USA). The iCELLigence system offers several significant benefits, including the elimination of the need to label cells, real-time monitoring capabilities, reduced potential for human error, and the generation of more accurate results (Düzgün et al., 2017; Türker Şener, Albeniz, Dinç, & Albeniz, 2017).

The cells ($1.5x10^4$ cells/well) were seeded in each well of the E-plates (ACEA Biosciences Inc., CA, USA), and the cell index (i.e., cell-electrode impedance of the E-plate well) was checked using an iPad device (Apple, Cupertino, CA, USA) containing the RTCA iCELLigence software. The extracts were then applied to the cells. The cell index values were measured every 15 min using the RTCA iCELLigence system for 96 h once the extracts were applied. Results are expressed as $IC_{50}$ values (the concentration required to inhibit 50% of cell growth), with $IC_{50}$ values being calculated using the cell index values obtained from the RTCA iCELLigence software.

In order to determine the cytotoxic effects of extracts on breast cancer stem cells and apoptosis studies, the effective concentrations of the extracts were determined according to the results from the cytotoxicity analysis using the RTCA iCELLigence system. The effective concentration was applied to breast cancer stem cells and breast cancer cells for the TUNEL assay and DAPI staining.

**Identifying breast cancer stem cells using flow cytometry**

MDA-MB-231 human breast cancer cells were used as the primary culture for sorting the cancer stem cells. The cancer stem cells were identified and isolated using a flow cytometer (Beckman Coulter, CA, USA) based on the CD24 and CD44 markers. The cell concentrations in the test tube and control tube were adjusted to $1.5x10^4$ cells. Anti-human CD24 and anti-human CD44 antibodies were added to the test tube, mixed, and incubated at room temperature in the dark for 30 min. The population of $CD24^-/CD44^+$ cells was sorted by flow cytometry (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003).

**Determination of apoptotic activity**

$15x10^4$ cells/well and $2x10^4$ cells/well were seeded in 24-well plates for the TUNEL assay and DAPI staining, respectively. The cells were then treated with an effective concentration of extracts and incubated for 72 h to determine apoptosis.

DAPI staining was determined by fluorescence microscopy (Leica, Wetzlar, Germany), and the results from the TUNEL assay were obtained using a light microscope (Leica, Wetzlar, Germany). Information about the morphological structure of apoptosis was observed by DAPI staining, and the morphological changes and quantification were determined by the TUNEL assay.

An apoptotic index is a numerical number that counts both the morphological changes of apoptotic nuclei in the cell, which can be referred to as TUNEL (+) cells, and the healthy non-apoptotic nucleating cells, which can be referred to as TUNEL (–) cells. The mathematical calculation of the apoptotic index is as follows:

$$\text{Apoptotic index (\%)} = \left(\frac{\text{Number of apoptotic nuclei}}{\text{Total number of cells}}\right) \times 100$$

**Statistical analysis**

Statistical calculations were performed using the software IBM SPSS Statistics 22 (IBM, NY, USA). When analyzing the data, the suitability of the parameters for normal distribution was tested using the Shapiro-Wilks test. The data were found to not conform to a normal distribution. The Mann Whitney U test was used to compare the quantitative data between the two groups. The Kruskall Wallis test was used for comparisons between more than two groups, while the Mann Whitney U test was used to determine the group that caused the difference. The Wilcoxon signed-rank test was used to evaluate the cell indexes at the 48th, 72nd, and 96th h according to 24th h. Significance was evaluated at the $p < 0.05$ and $p < 0.0001$ levels.

## RESULTS

**Real-time cytotoxicity assay (RTCA)**

The concentrations of the 360 µg/mL, 180 µg/mL, 90 µg/mL and 45 µg/mL of the PE extract had cytotoxic effects on MCF-7 and MDA-MB-231 human breast cancer cell lines, while the concentrations of 20 µg/mL and 10 µg/mL showed no cytotoxicity on the cells ($p < 0.05$) (Figure 1). The PE extract was cytotoxic only at a concentration of 360 µg/mL against the PCS-201-012 cell line, which was used as the control group ($p < 0.05$; Figure 1).

The EtOH extract of *T. vulgaris* had a concentration-dependent cytotoxic effect at all concentrations (360-10 µg/mL) on MCF-7 and MDA-MB-231 human breast cancer cell lines ($p < 0.01$; Figure 2). In contrast, the EtOH extract of *T. vulgaris* was not cytotoxic against the PCS 201-012 cell line used as a control group ($p < 0.05$; Figure 2).

The RTCA software analyzed the results obtained 72 h after the application of the PE and EtOH extracts. $IC_{50}$ was determined using time-dependent impedance values for MCF-7 and MDA-MB-231 human breast cancer cells and for the PCS 201-012 healthy fibroblast cells at 24, 48, and 72 h (Table 1).

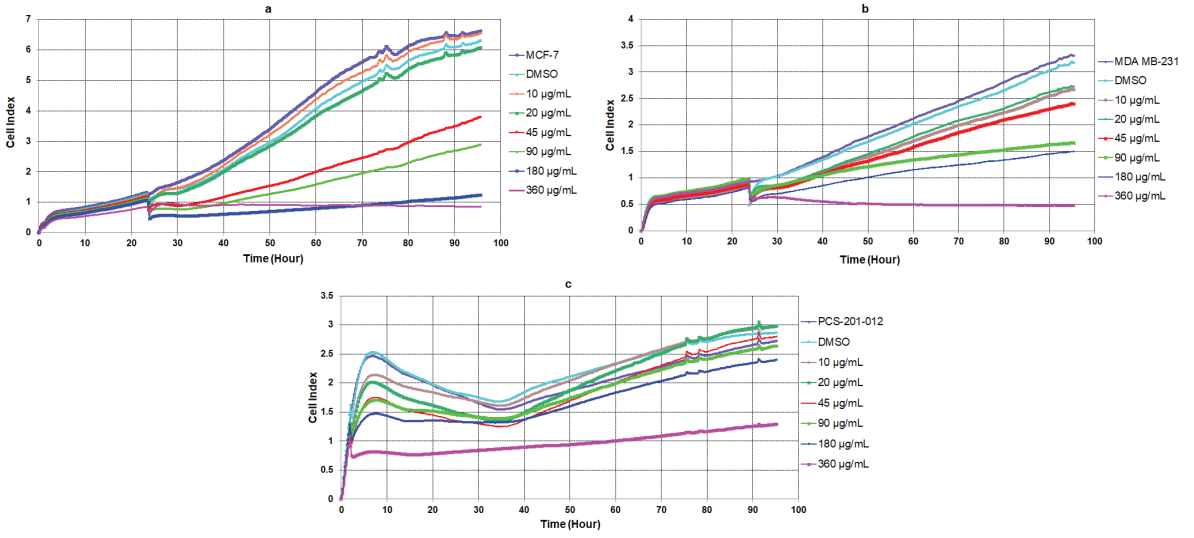


**Figure 1.** The cytotoxic effects of the petroleum ether extract from *T. vulgaris* on the (a) MCF-7 and (b) MDA-MB-231 human breast cancer cells and on the (c) PCS-201-012 healthy fibroblast cells.

Based on the cytotoxicity results (at 24th, 48th, and 72nd h) for both extracts on both breast cancer cell lines, the 10 and 20 µg/mL concentrations exhibited very low cytotoxic effects, while the 90, 180, and 360 µg/mL concentrations showed very high cytotoxic effects (Figures 1 & 2). Since the 45 µg/mL concentration showed a moderate cytotoxic effect on the cells among the concentrations applied in the RTCA (10-360 µg/mL), 45 µg/mL was determined as the effective extract concentration. The effective concentration was then used for determining the cytotoxic effect of extracts on human breast cancer stem cells.

The effective concentration of *T. vulgaris* PE and EtOH extracts was applied by flow cytometry to the $CD24^{-}/CD44^{+}$ cell-specific antigen profile of MDA-MB-231 human breast cancer cell line. A cytotoxic effect was observed at the administered effective concentration (Figure 3).

### Determination of apoptotic activity

The effective concentration was also used for determining apoptotic activity. After incubation with 45 µg/ml of both extracts, morphological alterations in breast cancer cells showed comparisons with the control cells.

### TUNEL assay

Brown-stained cells were noted as a result of the TUNEL assay for detecting cells with apoptotically labeled nuclei. Figure 4 shows the changes in the apoptotic cell index of the PE and EtOH extracts applied to MCF-7 cells compared with the control cells. The PE extract applied to MCF-7 cells was found to have a higher apoptotic cell index than the EtOH extract. The apoptotic cell index was significantly different from that of the control ($p < 0.0001$).

The changes in the apoptotic cell index of the PE and EtOH extracts applied to MDA-MB-231 cells compared with the control cells are shown in Figure 5. The PE and EtOH extracts show MDA-MB-231 cells to have been killed by very high rates of apoptosis. The index of apoptotic cells is significantly different from that of the control cells ($p < 0.0001$).

Table 2 shows the apoptotic index values obtained by applying the *T. vulgaris* extracts to two different cell lines. MCF-7 cells were more resistant to apoptosis than MDA-MB-231 cells and had a slightly lower apoptotic index ($p < 0.0001$).

### DAPI staining

For the nuclear morphological analysis, the effective concentration (45 µg/mL) of the *T. vulgaris* PE and EtOH extracts was applied to MCF-7 and MDA-MB-231 human breast cancer

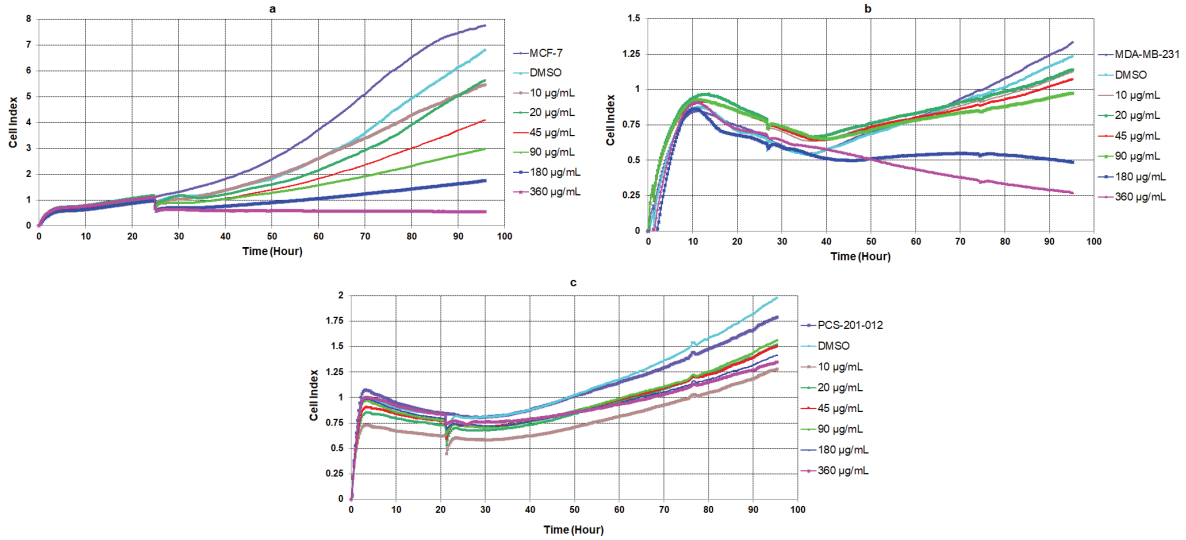


**Figure 2.** The cytotoxic effects of the ethanol extract from *T. vulgaris* on (a) MCF-7 and (b) MDA-MB-231 human breast cancer cells and on the (c) PCS-201-012 healthy fibroblast cells.

**Table 1. $IC_{50}$* values for the *T. vulgaris* extracts on breast cancer cells and healthy cells.**

| | $IC_{50}$ Values (µg/mL) | | | | | |
|---|---|---|---|---|---|---|
| | Petroleum ether extract | | | Ethanol extract | | |
| | 24th h | 48th h | 72nd h | 24th h | 48th h | 72nd h |
| **MCF-7** | 30.155 | 26.622 | 32.549 | 106.32 | 59.915 | 78.043 |
| **MDA-MB-231** | 100.9 | 50.618 | 49.888 | 42.457 | 84.698 | 74.826 |
| **PCS-201-012** | $5.48x10^8$ | $33.3x10^8$ | $26.04x10^8$ | $8.91x10^8$ | $34.75x10^8$ | $15.84x10^8$ |

* $IC_{50}$ is the concentration required to inhibit 50% of cell growth.

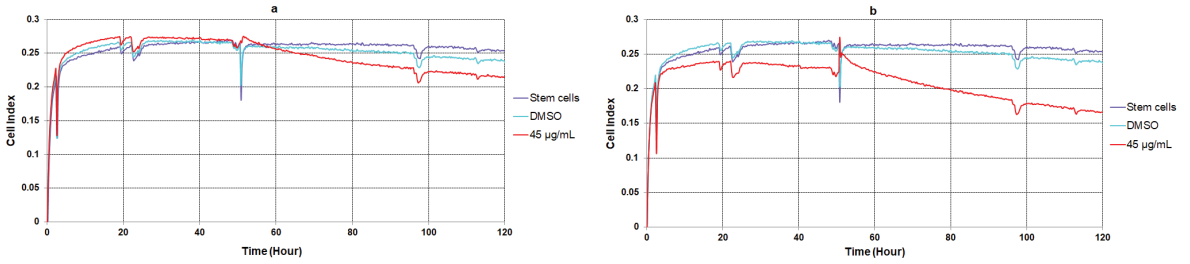


**Figure 3.** The cytotoxic effects of the (a) PE and (b) EtOH extracts from *T. vulgaris* on human breast cancer stem cells.

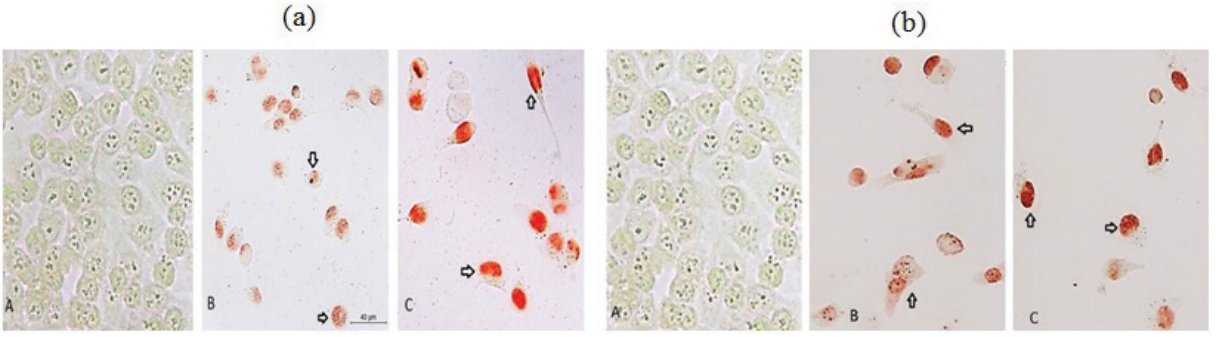


**Figure 4.** The apoptotic effects of the (a) PE and (b) EtOH extracts from *T. vulgaris* on MCF-7 human breast cancer cells. (A) shows non-treated cells; (B) and (C) show cells treated with effective concentration of the extracts (45 µg/mL) after 24 h, and the TUNEL-positive apoptotic cells, respectively (Bar; A = 80 µm, B and C = 40 µm).

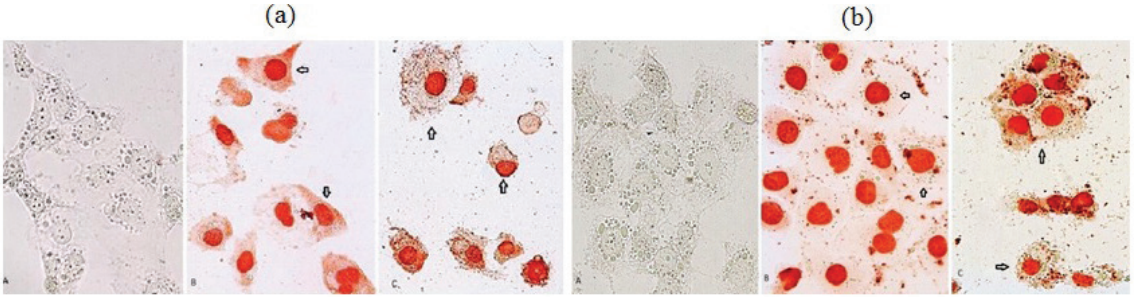


**Figure 5.** The apoptotic effects of (a) PE and (b) EtOH extracts from *T. vulgaris* on MDA-MB-231 human breast cancer cells. (A) shows non-treated cells; (B) and (C) show cells treated with effective concentration of the extracts (45 µg/mL) after 24 h, and TUNEL-positive apoptotic cells, respectively (Bar; A = 80 µm, B and C = 40 µm).

**Table 2. Apoptotic index values (%) of breast cancer cells treated with *T. vulgaris* extracts.**

| | Apoptotic index values (%) | | |
|---|---|---|---|
| | **Control** | **PE** | **EtOH** |
| **MCF-7** | 2.38 ± 0.36 | 76.27 ± 6.33 | 67.18 ± 23.42 |
| **MDA-MB-231** | 1.52 ± 0.23 | 96.88 ± 1.68 | 93.87 ± 1.84 |

EtOH = Ethanol extract; PE = Petroleum ether extract

cells. After 24 h, the cells were stained with DAPI and visualized under fluorescence microscopy. The images of the apoptotic cell nuclei morphology as a result of applying the PE and EtOH extracts to MCF-7 and MDA-MB-231 cells are shown in Figures 6 and 7. The changes in the nuclei of the cells were observed after treatment with the extracts. The treated cancer cells showed condensed chromatin and nuclear fragmentation, which are characteristics of apoptosis compared to the non-treated cells, which showed clear round nuclei. Also, the number of apoptotic cells was observed to have increase in the treated cells compared to the non-treated cells.

## DISCUSSION

The present study has shown the effects of the PE and EtOH extracts of *T. vulgaris* on MCF-7 human breast cancer cell line. The results show a dose-dependent cytotoxic effect, with the $IC_{50}$ values of the PE and EtOH extracts after 24 h incubation be-

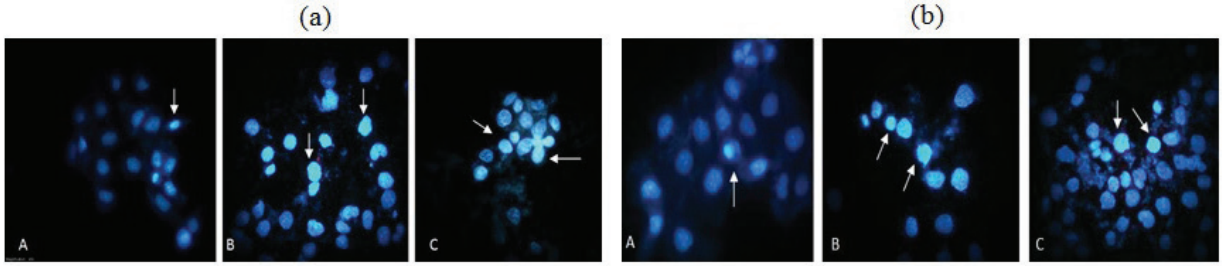


**Figure 6.** Image of nucleus morphologies of apoptotic cells in fluorescent microscopy by DAPI staining of MCF-7 human breast cancer cells treated with the (a) PE and (b) EtOH extracts from *T. vulgaris*. Normal and mitotic nuclei in the (A) non-treated cells and (B & C) apoptotic cells were observed at the end of 24 h in the treated cells. The cells were treated with the effective concentration of the extracts (45 µg/mL; magnification: x40).

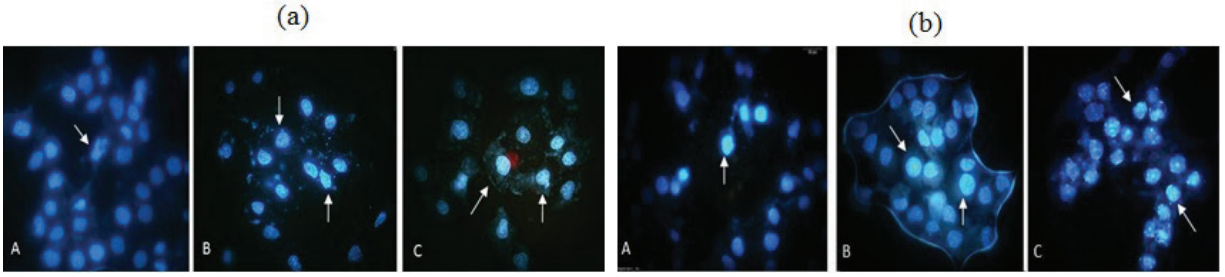


**Figure 7.** Image of nucleus morphologies of apoptotic cells in fluorescent microscopy by DAPI staining of MDA-MB-231 human breast cancer cells treated with the (a) PE and (b) EtOH extracts from *T. vulgaris*. Normal and mitotic nuclei in the (A) non-treated cells and (B & C) apoptotic cells were observed at the end of 24 h in the treated cells. The cells were treated with the effective concentration of the extracts (45 µg/mL; magnification: x40).

ing 30.155 µg/mL and 106.32 µg/mL, respectively. Some studies have focused on the biological activity of the *Thymus* species. One of these studies indicated the essential oils of *T. serpyllum* (52.69 µg/mL), *T. algeriensis*, (62.53 µg/mL), and *T. vulgaris* (180.40 µg/mL) to exhibit cytotoxic effects in terms of $IC_{50}$ values on MCF-7 cells (Nikolić et al., 2014). Esmaeili-Mahani et al. (2014) reported the *T. caramanicus* extract to be effective against MCF-7 cells at a concentration of 80 µg/mL and ineffective at a concentration of 40 µg/mL. Another study applied the methanol extracts from *T. serpyllum* and *T. vulgaris* to MCF-7 cells and determined the effective concentrations to be 399.407 µg/mL and 407 µg/mL, respectively (Berdowska et al., 2013). Compared to previous studies on the effects of extracts/essential oils from *T. vulgaris* on MCF-7 cells, the current study shows the cytotoxic effects to have occurred at lower concentrations. The better results obtained in the present study may be due to the different plant extract preparation techniques and the solvents used to prepare the extracts from *T. vulgaris*. In addition, all other previous studies had been performed with classical colorimetric cytotoxicity methods. More sensitive and accurate results may have been obtained compared to the classical methods as a result of the present study's use of the RTCA iCELLigence.

In this study, MDA-MB-231 cells were also treated with both the PE and EtOH extracts of *T. vulgaris*, and the findings show a concentration-dependent cytotoxic effect. After a 24 h incubation, the $IC_{50}$ values of the PE and EtOH extracts were 100.9 µg/mL and 42.457 µg/mL, respectively. These values show *T. vulgaris* to have a cytotoxic effect on MDA-MB-231 cells. A previous study showed the $IC_{50}$ values of the essential oils from *T. vulgaris* on MDA-MB-231 cells to have been 108.71 µg/mL and 71 µg/mL after respective incubations of 24 h and 48 h (Al-Shahrani, Mahfoud, Anvarbatcha, Athar, & Al Asmari, 2017). The literature has had no study on the cytotoxic effects of *T. vulgaris* extracts on MDA-MB-231 cells. In this sense, this is the first study to have investigated the effects of *T. vulgaris* extracts on MDA-MB-231 cells. The study hypothesizes that the *T. vulgaris* extracts could be useful in treating MDA-MB-231 cells, which has a different phenotype than MCF-7 cells.

Stem cells with the $CD24^{-}/CD44^{+}$ antigen profile derived from MDA-MB-231 cells were also treated with the effective concentration of *T. vulgaris*. The cytotoxic effect of the extracts was then observed using the RTCA iCELLigence system. With respect to both the cytotoxic effects of *T. vulgaris* extracts on breast cancer stem cells and using the RTCA iCELLigence system for the determination of the cytotoxicity, no such study is noted to have existed in the literature, and this is the first study to have identified the effect of *T. vulgaris.* Therefore, *T. vulgaris* is thought to be a natural source for preventing the spread of cancer in terms of inhibiting cancer stem cells.

The cytotoxic effective concentration in the human breast cancer cell lines (MCF-7 and MDA-MB-231) induced apoptotic cell death. As a result, the present study has found the PE and EtOH extracts of *T. vulgaris* to have an apoptotic effect on both MCF-7 and MDA-MB-231 cells and to have determined morphological changes specific to apoptosis such as rounding, membrane budding, chromatin condensation, and apoptotic bodies. Compared to the control cells, the nucleus condensation in some TUNEL-positive cells was found to be significantly located along the periphery of the nucleus. Based on these results, *T. vulgaris* extracts are thought to be useful as a natural compound for treating human breast cancer.

## CONCLUSION

This study has revealed the extracts of *T. vulgaris* to show a significant cytotoxic effect on breast cancer cells and breast cancer stem cells compared to healthy cells. In addition, the extracts induced high levels of apoptosis in the breast cancer cells. These extracts may be a new source for breast cancer therapy due to their selective cytotoxic and high apoptosis induction effects. Further studies are needed to determine the molecular effects of the active compounds from *T. vulgaris* on the cytotoxic, anti-proliferative, and apoptotic pathways.

**Peer-review:** Externally peer-reviewed.

**Author Contributions:** Conception/Design of Study- Y.C., I.A.; Data Acquisition- Y.C., L.T.S., B.B.; Data Analysis/Interpretation- Y.C., L.T.S., I.A.; Drafting Manuscript- Y.C., I.A.; Critical Revision of Manuscript- Y.C., I.A.; Final Approval and Accountability- Y.C., L.T.S., B.B., I.A.

**Conflict of Interest:** The authors have no conflict of interest to declare.

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