New insights into the cytotoxic effects of *Thymus vulgaris* essential oil on the human triple-negative breast cancer cell line MDA-MB-231.

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Abstract

Essential oils (EOs) are natural products that have gained wide interest due to their biological activities and anticancer properties through various mechanisms. The present study aimed to test the cytotoxicity of *Thymus vulgaris* L. (thyme) EO of Italian origin, rich in thymol (49.6%) and p-cymene (18.8%), towards the triple-negative breast cancer cell line MDA-MB-231 and to investigate the biochemical mechanisms underlying its antitumor activity. Thyme EO reduced cancer cell viability in a dose-dependent manner after 24 h treatment, with an IC_{50} value equal to 75.1±15.2 μg/ml; simultaneously, the inhibition of cancer cell migration and colony formation capacity was evidenced. Thyme EO antiproliferative effects were related to the induction of apoptosis as demonstrated by the increased expression of the pro-apoptotic proteins Bax, cleaved caspase-3, phospho-p53, and SMAC/Diablo and by the reduction of the anti-apoptotic proteins Bcl-2, cIAP-1, cIAP-2, HIF-1α, survivin, and XIAP. Thyme EO administration led to the early formation of intracellular ROS, followed by the increment of MDA as an index of lipid peroxidation and by the decreased expression of the antioxidant enzymes catalase and PON2. The upregulation of Nrf2 mRNA expression and the strong induction of HO-1 sustained the activation of the Nrf2 pathway by thyme EO. These data showed that the EO from *Thymus vulgaris* L. might inhibit the malignant phenotype of MDA-MB-231, thus suggesting potential benefits against human triple-negative breast cancer.

Keywords

Essential oils; *Thymus vulgaris*; triple-negative breast cancer cells; apoptosis; oxidative stress; Nrf2 pathway.

1. Introduction

Breast cancer (BC) is women's most frequently diagnosed cancer, the second leading cause of cancer death (Fahad Ullah, 2019). In particular, triple-negative breast cancer (TNBC) [estrogen receptor-negative (ER-), progesterone receptor-negative (PR-), human EGF receptor 2-negative (HER2-)] accounts for 15-20% of BC cases, and it is characterized by an aggressive phenotype and worse outcomes (Marra et al., 2020). Chemotherapy remains the standard of care for TNBC treatment, but poor prognosis and drug resistance represent relevant issues for developing new therapeutic strategies (Nedeljković and Damjanović, 2019).

In this context, increasing attention has been recently paid to naturally occurring compounds as new potential candidates characterized by extreme structural diversity and elevated biochemical specificity (Chen et al., 2021). Natural extracts are complex product mixtures with multi-target effects and synergistic actions, often displaying broad application prospects in cancer prevention and treatment (Danciu, 2020). Among these natural mixtures, essential oils (EOs) from aromatic plants are secondary metabolites consisting primarily of terpenes with a volatile nature (Blowman et al., 2018), which exhibit a wide variety of well-documented bioactivities, including antimicrobial, antioxidant, anti-inflammatory, and anti-tumor capacity (Ramsey et al., 2020). EOs exert their anticancer properties through various mechanisms of action, including induction of apoptosis, modulation of cell signaling, and inhibition of tumor metastasis and angiogenesis (Ishfaq et al., 2018; Sharma et al., 2022). At the same time, they can increase the efficacy of commonly used chemotherapy drugs, sustaining their therapeutic potential in integrated oncology (Lesgards et al., 2014; Russo et al., 2015). Accordingly, we previously demonstrated the capability of the EO from *Pistacia Lentiscus* aerial parts to reduce tumor cell viability through the intracellular accumulation of reactive oxygen species and the induction of apoptotic cell death and to enhance the cytotoxic effects of conventional antineoplastic drugs (Catalani et al., 2017a).

To extend our investigations in this field and to consider those unique ethnobotanical traditions that exist in the Italian region Sardinia (Loi et al., 2004), we tested the effects of different Sardinian EOs (cypress, fennel, laurel, lentiscus, myrtle, rosemary, and thyme) on the human TNBC cell line MDA-MB-231. This initial screening was for identifying promising candidates in contrasting can-

cer cell proliferation (Table S1-S2 and Fig. S1, Supplementary Material). The biochemical mechanisms underlying the antiproliferative activity of the most effective EO (the one from *Thymus vulgaris* L.) were then investigated and herein reported.

2. Methods

2.1. Essential oil

Thyme EO from *Thymus vulgaris* L. flowering tops (site of collection Ogliastra, Sardinia, Italy) was produced through steam distillation by SSA Mediflora (Cagliari, Italy) and provided by the Onlus RUOTA (Rapallo, GE, Italy). The same batch (OE31801) was used for all the experiments on cultured cells.

2.2. Cell culture conditions

The human TNBC cell line MDA-MB-231 was obtained from Interlab Cell Line Collection (ICLC, Genova, Italy) and grown in DMEM medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin 100 U/ml, 1% L-glutamine, and 1% non-essential amino acids (Sigma-Aldrich, Milan, Italy). EndoGRO^{TM} human umbilical vein endothelial cells (HUVEC) were obtained from Merck (Milan, Italy) and cultured in EndoGRO-LS Complete Culture Media Kit (Merck, Milan, Italy). Cells were maintained in a CO_2 incubator at 37 °C and 5% CO_2.

2.3. Cytotoxicity assays

The cytotoxic effects of thyme EO on MDA-MB-231 cells were analyzed by WST-8 and sulforhodamine B (SRB) assays, which evaluate cell metabolic activity and protein content, respectively (Adan et al., 2016). Etoposide (Sigma-Aldrich, Milan, Italy) was used as a positive control (Baldwin and Osheroff, 2005), having been proven to be the most effective among different anticancer drugs in suppressing MDA-MB-231 cell viability (Fig. S2, Supplementary Material). Primary human HUVEC cells were also employed in toxicity studies as a non-cancer cell line (Dinç et al., 2020). In detail, cells ($5x10^3$/well) were seeded in 96-well plates and treated with increasing concentrations of thyme EO (25-200 µg/ml) or vehicle (0.1% DMSO). After 24 h of incubation, thyme EO was removed, and a fresh medium containing WST-8 (Sigma-Aldrich) was added to each well

(Catalani et al., 2017b). Cells were further incubated at 37 °C up to 4 h, and color development was monitored at 450 nm in a multiwell plate reader (Multiskan FC, Thermo Scientific). As previously published, the SRB test was then performed in the same 96-well plate (Benedetti et al., 2022). Briefly, cells were fixed with cold 50% trichloroacetic acid and stained with 0.4% SRB (Sigma-Aldrich) dissolved in 1% acetic acid. Bound stains were subsequently solubilized with 10 mM Tris, and the absorbance was read at 570 nm in a microplate reader (Multiskan FC, Thermo Scientific). Data were expressed as a percentage (%) versus non-treated cells (controls), and the concentration that caused 50% growth inhibition (IC_{50}) was then calculated.

2.4. Cell migration

Cancer cell migration after thyme EO administration was evaluated using the wound-healing assay (Grada et al., 2017). Briefly, cells were seeded in 6-well plates ($4x10^5$/well) and grown until confluence; afterward, a straight scratch was made with a sterile P200 pipette tip. Debris was removed, and cells were treated with thyme EO 75 µg/ml (corresponding to the IC_{50} value) or vehicle (DMSO 0.1%). Cell migration was observed after 3, 6, and 24 h under a phase-contrast microscope (Olympus IX50, Segrate, Italy) using the 10x magnification; images were acquired by ToupCam camera equipped with ToupView control software (ToupTek Europe). Wound area calculation was performed by ImageJ (Schneider et al., 2012) and the closure of the wound was expressed as follows: wound closure (%) = (A0 - An)/A0 x 100, where A0 represents the initial wound area and An the remaining area of the wound at the different experimental time points (Nasoni et al., 2022).

2.5. Colony formation

Thyme EO effects on MD-MB-231 colony formation capacity were evaluated through the clonogenic assay (Franken et al., 2006). Briefly, $1x10^3$ viable cells were plated in 6-well plates and treated for 24 h with increasing concentrations of EO (25-100 µg/ml) or vehicle. After EO removal, cells were allowed to grow for 7 days, thus evidencing colony formation. Colonies were then stained for 30 min at room temperature with 0.5% crystal violet in 25% glutaraldehyde (Benedetti et

al., 2022); well digital images were obtained, and the colony area was quantified by ImageJ software. Data were expressed as a percentage of inhibition of colony formation compared to the control.

2.6 Mitochondrial membrane potential evaluation

Changes in mitochondrial membrane potential after thyme EO administration to MDA-MB-231 cells were determined using the fluorescent dye TMRE (tetramethylrhodamine, ethyl ester) (ThermoFisher, Milan, Italy), which readily accumulates in active mitochondria. Depolarized mitochondria have decreased membrane potential and fail to sequester TMRE. Briefly, cells were seeded in black 96-well plates ($5x10^3$/well) and treated with increasing concentrations of thyme EO (25-100 µg/ml) or vehicle (0.1% DMSO) for 24 h. Cells were then incubated with TMRE (200 nM) for 30 min at 37 °C in the dark and, after excess dye removal, 100 µl of PBS was added. TMRE fluorescence was measured at ex/em 549/575 nm by using a microplate reader (FluoStar Optima, BMG Labtech, Germany).

2.7. Gene expression analysis

MDA-MB-231 cells were plated in 6-well plates ($2x10^5$/well) and treated for 24 h with 50 µg/ml thyme EO or vehicle. Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Milan, Italy), and cDNA was prepared using the PrimeScript RT Master Mix (Takara, Japan). Quantitative RT-PCR was performed in triplicate on the QuantStudio1 Real-Time PCR system (Thermo Fisher, Milan, Italy). The mRNA expression of target genes [Bax, Bcl-2, β-catenin, glutathione peroxidase (GPx), heme oxygenase-1 (HO-1), Nrf2, p53, survivin, telomerase, XIAP] was normalized to β-actin, and relative target gene expression levels were calculated using the $2^{\Delta\Delta CT}$ method. The primer sequences are reported in Table S3 (Supplementary Material). The procedure was performed according to the manufacturer's instructions.

2.8. Apoptosis-related protein expression

The Human Apoptosis Array Kit (R&D Systems, Milan, Italy) was used to investigate the expression of different apoptosis activators/suppressors after thyme EO administration, as previously described (Catalani et al., 2017b). Briefly, MDA-MB-231 cells ($1.2x10^6$) were plated in T75 flasks and treated for 24 h with 50 µg/ml thyme EO or vehicle. Afterward, cells were detached and lysed, and protein levels were determined using the Bradford method (Bradford, 1976). Arrays were incubated overnight with 400 µg of cell lysates to detect the expression profile of 35 apoptosis-related proteins exploiting capture and control antibodies spotted on nitrocellulose membranes. After washing to remove unbound proteins, membranes were incubated with a cocktail of biotinylated detection antibodies. Streptavidin-Horseradish peroxidase (HRP) was then applied. Signals were developed using chemiluminescent reagents and detected in a Chemidoc MP imaging system (Bio-Rad, Hercules, CA, USA). Pictures were analyzed using ImageJ software for densitometric analysis. All spots were in duplicate.

2.9. Annexin V-FITC assay

MDA-MB-231 cells ($2x10^5$) were seeded in 6-well plates and treated with 75 µg/ml thyme EO or vehicle. After 24 h incubation, cells were harvested, and apoptotic cells were detected using Tali Apoptosis Kit-Annexin V Alexa Fluor® 488 and propidium iodide (PI) (Life Technologies, USA). Briefly, the cell pellet was resuspended with 100 µL of Annexin V-Binding Buffer (ABB) and incubated with 5 µL of Annexin V for 20 min. Subsequently, cells were centrifuged and resuspended in ABB containing 1 µl of PI and incubated for 5 min at room temperature. Finally, the analysis was carried out using TALI™ image-based cytometer (Life Technologies, USA) and the data were analyzed with Flow Cytometry Analysis Software (Floreada.io).

2.10. Intracellular reactive oxygen species formation

Intracellular reactive oxygen species (ROS) formation upon thyme EO administration to MDA-MB-231 was evaluated using the probe 2,7-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich) both in the short (2h) and long period (24h), as previously reported (Catalani et al., 2017a). Briefly, cells seeded in black 96-well plates ($5x10^3$/well) were loaded with DCFH-DA (5 µM) and then exposed to thyme EO (25-800 µg/ml) up to 2 h both in the absence and presence of 5 mM N-acetyl

cysteine (NAC, Sigma-Aldrich) as a reference antioxidant molecule. Fluorescence was monitored at ex/em 485/520 nm in the multiwell plate reader FluoStar Optima (BMG Labtech, Germany). In the 24-h experiment, cells were seeded in 6-well plates ($2x10^5$/well) and incubated with thyme EO (50 and 75 µg/ml) or vehicle for 24 h. The test compound was then removed, and DCFH-DA (5 µM) was added to each well for 30 min. After excess probe removal, cells were detached and transferred to black 96-well plates to monitor fluorescence emission. Data were normalized to cell protein content (Bradford method) (Bradford, 1976) and expressed as relative oxidation versus non-oxidized cells.

2.11. Lipid peroxidation levels

Malondialdehyde (MDA) levels were evaluated in MDA-MB-231 cells as a marker of lipid peroxidation. Briefly, cells were seeded in T25 flasks ($5x10^5$/flask) and treated with thyme EO (50 and 75 µg/ml) or vehicle for 24 h. Afterward, cells were lysed by CelLytic M reagent (Sigma-Aldrich), and cell lysates were derivatized with thiobarbituric acid (TBA). The MDA-TBA complex was extracted with butanol, and samples were then analyzed by reverse-phase high-performance liquid chromatography as previously described (Agarwal and Chase, 2002). Results were adjusted for protein content in the sample and expressed as nanomoles of MDA per milligram of protein. Proteins were quantified by the Bradford method (Bradford, 1976).

2.12. Chemical composition

Analysis of the chemical composition of thyme EO was carried out by Mérieux NutriSciences (Chelab S.r.l., Treviso, Italy) as described in the Supplementary Material file.

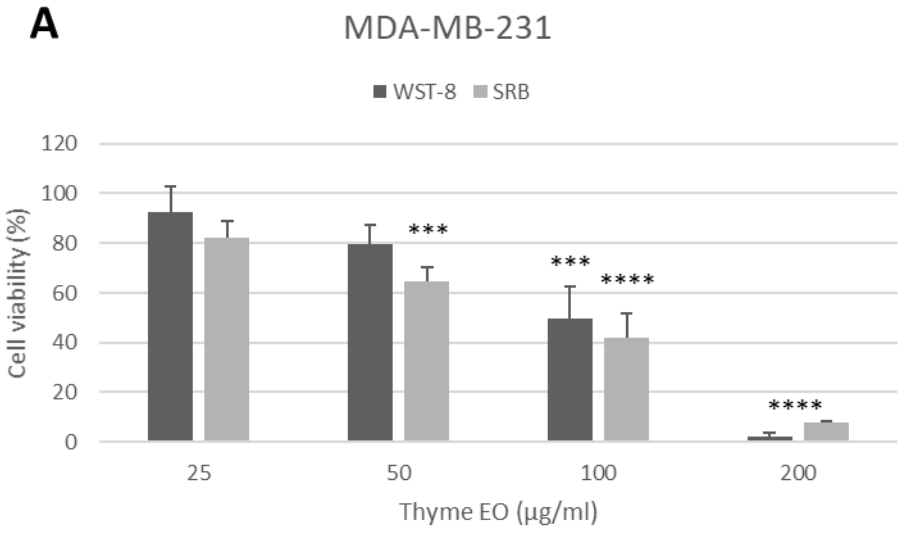
2.13. Statistical analysis

Results were presented as mean ± SD of three independent experiments. Differences between two groups were analyzed by the two-tailed Student's test and those between three or more groups were analyzed by using the one-way analysis of variance test (ANOVA) followed by Tukey's test. A P value <0.05 was considered statistically significant. Statistics were performed using GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Thyme EO cytotoxicity

Thyme EO cytotoxicity towards MDA-MB-231 cells was investigated by WST-8 and SRB assays. Both tests revealed a dose-dependent reduction of cell viability in EO-treated cells after 24 h of incubation (Fig. 1A), with IC_{50} values equal to 101.9±18.8 µg/ml (WST-8 test) and 75.1±15.2 (SRB test). According to validated cytotoxic criteria (Niksic et al., 2021), thyme EO might be considered moderately cytotoxic (IC_{50} between 21 and 200 µg/ml). Etoposide (used as a positive control) showed IC_{50} values equal to 9.8±2.1 µg/ml and 11.5±3.1 µg/ml, respectively, and was considered highly cytotoxic (IC_{50} < 20 µg/ml). Since WST-8 measures cell metabolic activity while SRB quantifies cell protein content (Adan et al., 2016), there were some differences in IC50 values from the two cytotoxicity assays. WST-8 might overestimate cell viability because testing compounds might directly interfere with WST-8 reduction, while SRB staining should not be affected by this type of interference (Vichai and Kirtikara, 2006). Notably, thyme EO displayed lower cytotoxicity towards HUVEC healthy cells as compared to MDA-MB-231 cancer cells, showing an IC_{50} value >200 µg/ml (Fig. 1B). By calculating the selectivity index (a simple ratio of IC_{50} calculated for healthy and cancer cells) (Lica et al., 2021), a favorable value higher than 1.0 was obtained, indicating that the efficacy of thyme EO against tumor cells was greater than its toxicity against normal cells.



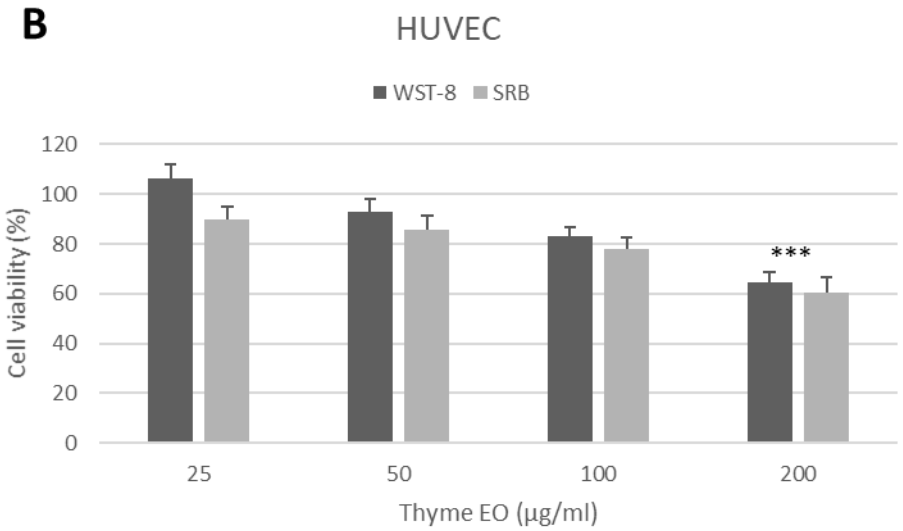
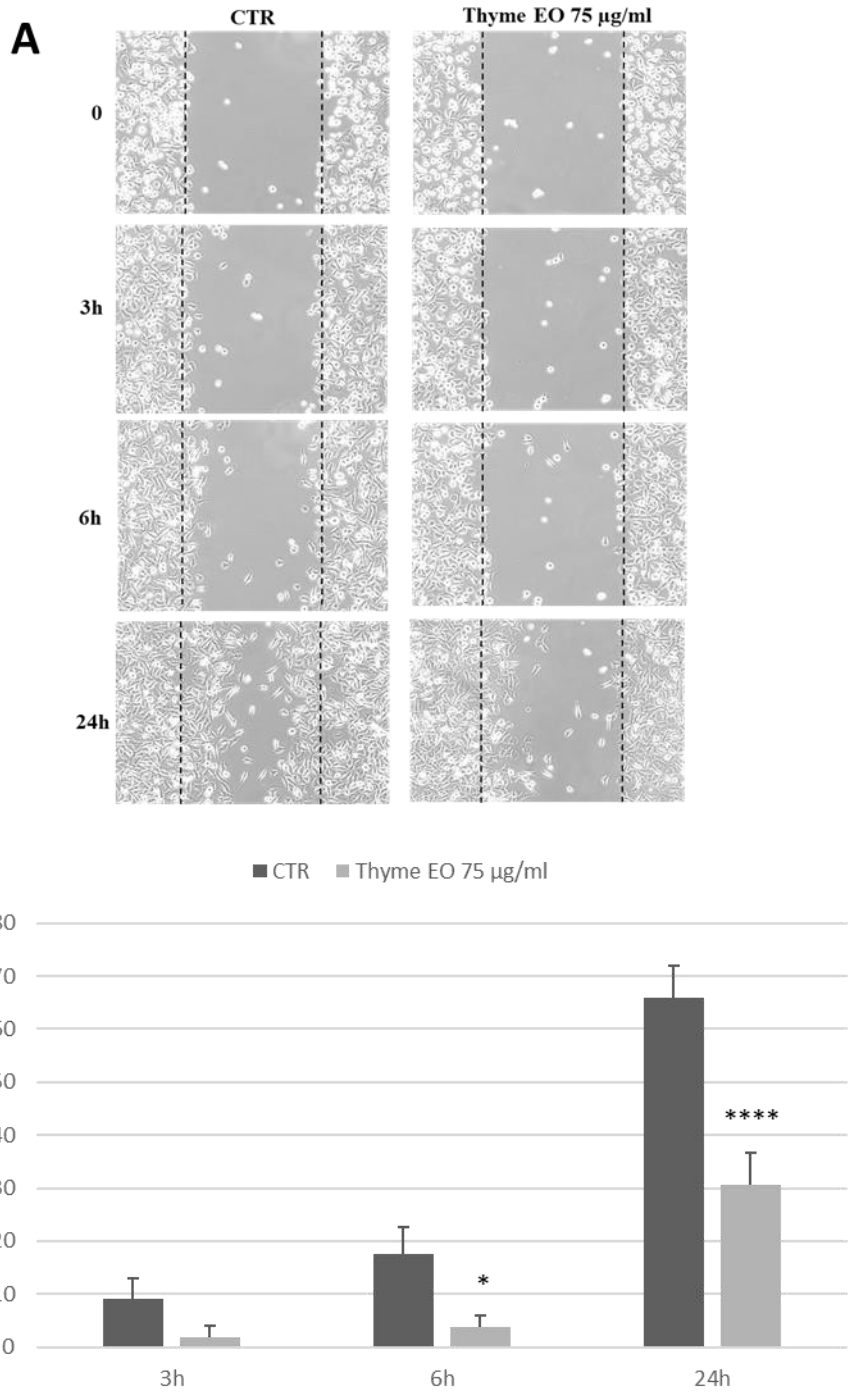


Fig. 1. Thyme EO cytotoxicity towards (A) MDA-MB-231 cancer cells and (B) HUVEC healthy primary cells after 24 h treatment evaluated by WST-8 and SRB assays. ***p<0.001 and ****p<0.0001 vs. untreated control cells (100% viability). Data are expressed as mean ± SD (n=3).

3.2. Inhibition of cell migration and colony formation by thyme EO

The wound-healing assay revealed that thyme EO-treated cells exhibited diminished migration to close the wound compared to untreated control cells (Fig. 2A). This decreased migration was observed as early as 3 h post-treatment and became significant after 6 h of incubation with the EO. As such, it could not be due to decreased viability since there was no significant change in cell viability after 3 and 6 h of treatment with thyme EO 75 µg/ml (Supplementary Material, Fig. S1, panel H). As regards the clonogenic ability of MDA-MB-231 cells, a dose-dependent inhibition of the colony formation capacity of cancer cells was observed following incubation with increasing concentrations of thyme EO (Fig. 2B).


A
CTR
Thyme EO 75 µg/ml
0
3h
6h
24h
CTR
Thyme EO 75 µg/ml
Wound closure (%)
80
70
60
50
40
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20
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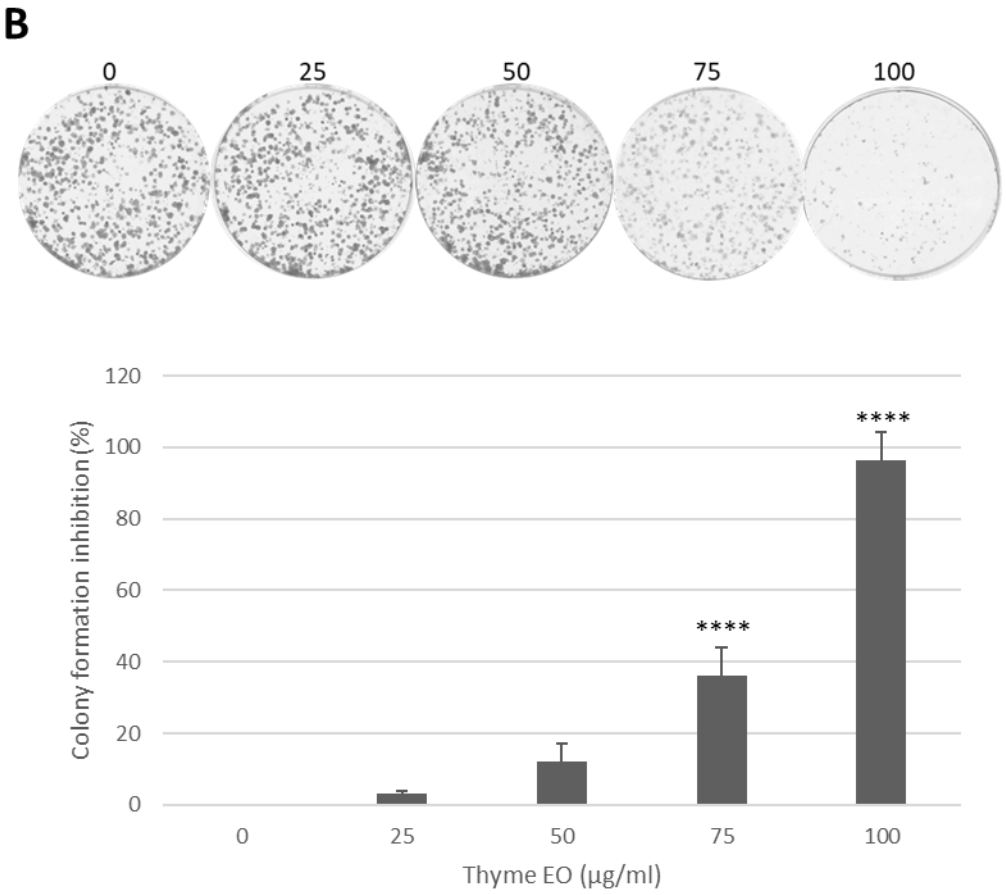
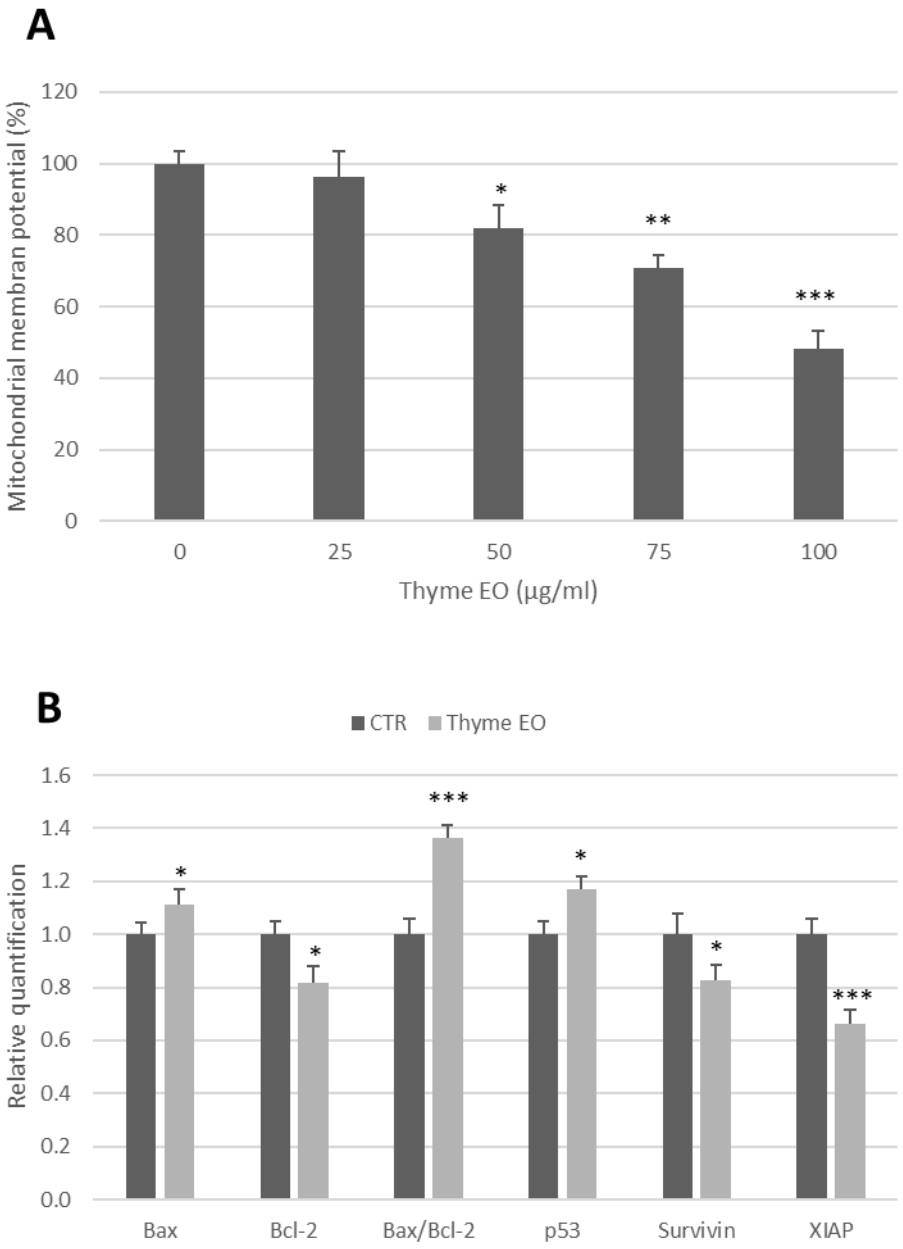


Fig. 2. (A) Effects of thyme EO administration (75 µg/ml) on MDA-MB-231 cell migration capacity after 3, 6, and 24 h treatment and expressed as wound closure (%). Representative images of the wound healing assay performed at each experimental time point were included (images were obtained with an optical microscope at 10x magnification). (B) Effects of thyme EO administration (25-100 µg/ml) on the clonogenic ability of MDA-MB-231 cells and expressed as colony formation inhibition (%). Representative images of the clonogenic test were included. *$p<0.05$ and ****$p<0.0001$ vs. untreated cells. Data are expressed as mean ± SD (n=3).

3.3. Induction of apoptosis by thyme EO

A dose-dependent decrement of mitochondrial membrane potential was observed by TMRE quantification following thyme EO administration to MDA-MB-231 cells (Fig. 3A), suggesting apoptosis induction via the mitochondrial pathway. The assessment of mRNA expression levels of different apoptosis-related genes evidenced an increased Bax expression and a decreased Bcl-2 expression in thyme-treated cells compared to control cells (Fig. 3B). These changes in gene expression led to a

significant Bax/Bcl-2 ratio increment that sustained a pro-apoptotic effect of thyme EO administration, also confirmed by p53 up-regulation. At the same time, a significant mRNA downregulation of the apoptosis inhibitors survivin and XIAP were observed. β-catenin and telomerase mRNA expression were not significantly modulated by thyme EO (Fig. S3, Supplementary Material). Noteworthy, the proteome profiler array, which determines the relative levels of 35 apoptosis-related proteins, confirmed the increased expression of the pro-apoptotic proteins Bax and phospho-p53, and the decrement of the anti-apoptotic proteins Bcl-2, survivin, and XIAP (Figure 3C and Table 1) (see Fig. S4 and Table S4 of the Supplementary Material file for the complete array profile).



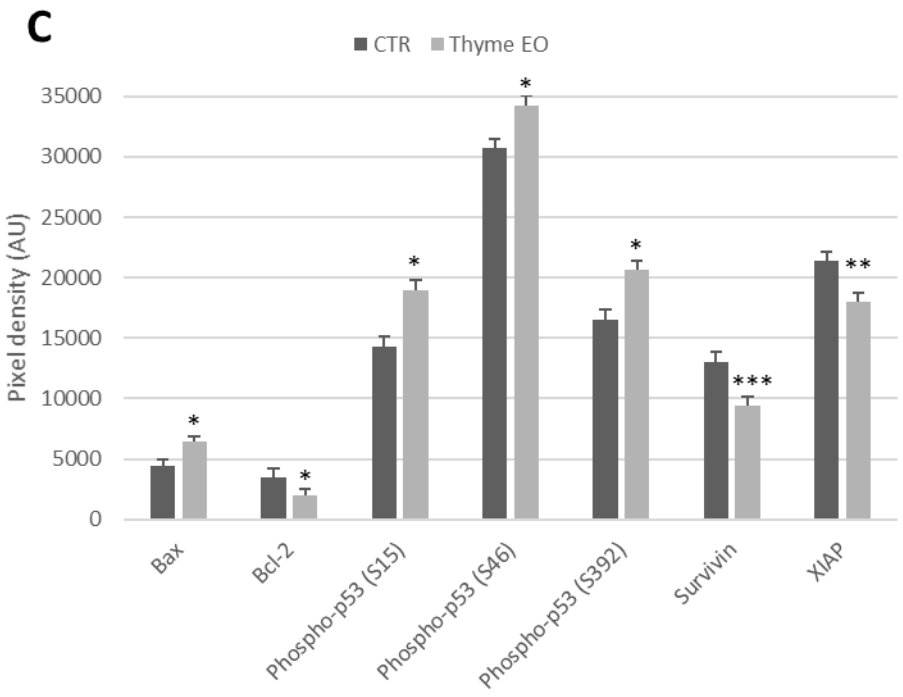


Fig. 3. (A) Mitochondrial membrane potential changes in MDA-MB-231 cells following 24 h treatment with thyme EO (25-100 µg/ml); (B) mRNA expression and (C) protein levels of different apoptosis activators/suppressors after 24 h treatment with thyme EO (50 µg/ml). *p<0.05, **p<0.01, and ***p<0.001 vs. untreated cells (CTR). Data are expressed as mean ± SD of three (A and B) or two (C) independent experiments.

Furthermore, a significant increment of the proteins cleaved caspase-3, SMAC/Diablo, and TNF RI/TNFRSF1A, which are apoptosis activators, was found in thyme-treated cells as compared to control cells (Table 1), while the protein levels of the apoptosis suppressors catalase, cIAP-1, cIAP-2, HIF-1α (hypoxia-inducible factor-1alpha), and PON2 (paraoxonase 2) were significantly reduced.

Table 1. Fold changes of apoptosis-related protein levels in MDA-MB-231 cells after 24 h treatment with thyme EO (50 µg/ml).[a]

Protein	Anti/Pro-apoptotic	Fold change vs. control
Bax	P	1.45*
Bcl-2	A	0.59*

Cleaved caspase-3	P	1.60*
Catalase	A	0.79*
cIAP-1	A	0.85*
cIAP-2	A	0.85*
HIF-1α	A	0.66*
HO-1/HMOX/HSP32	A/P	4.44***
PON2	A	0.88*
Phospho-p53 (S15)	P	1.33*
Phospho-p53 (S46)	P	1.12*
Phospho-p53 (S392)	P	1.25*
SMAC/Diablo	P	1.15*
Survivin	A	0.72***
TNF RI/TNFRSF1A	P	1.64***
XIAP	A	0.86**

[a] A: anti-apoptotic, P: pro-apoptotic, *p<0.05, **p<0.01, and ***p<0.001 vs. control cells.

Annexin V and PI staining indicated the presence of late apoptotic events (Annexin V^+/PI^+) following 24 h treatment with thyme EO (75 µg/ml) (Fig. 4).

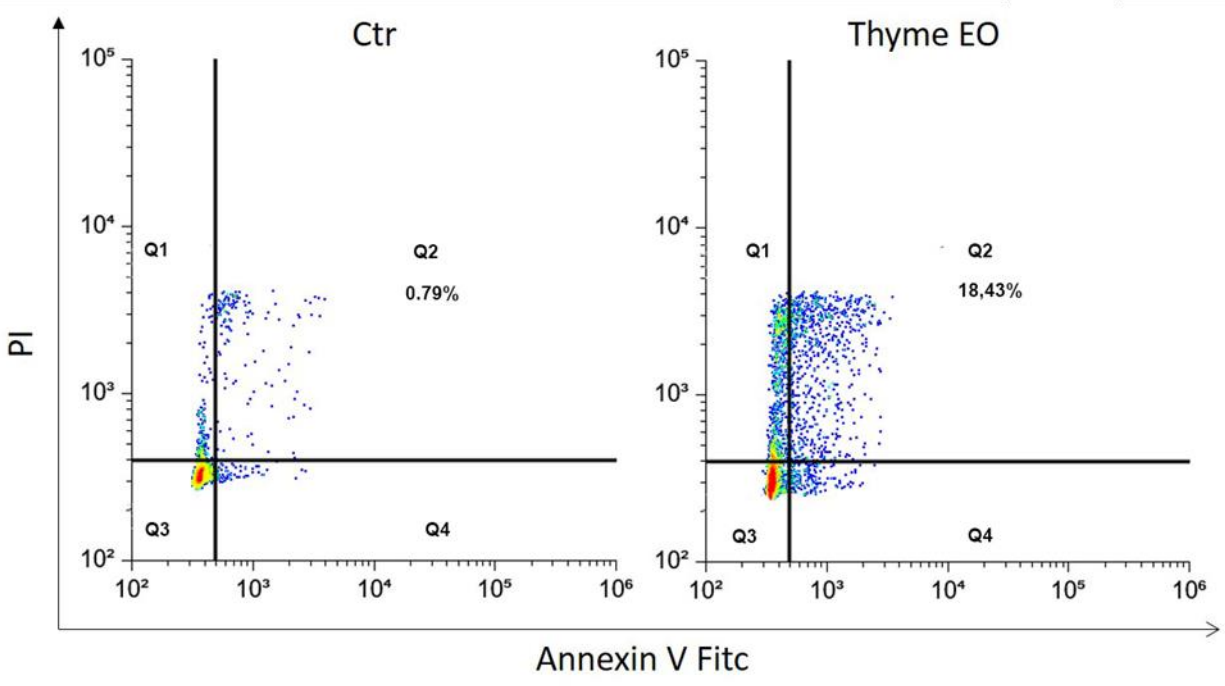
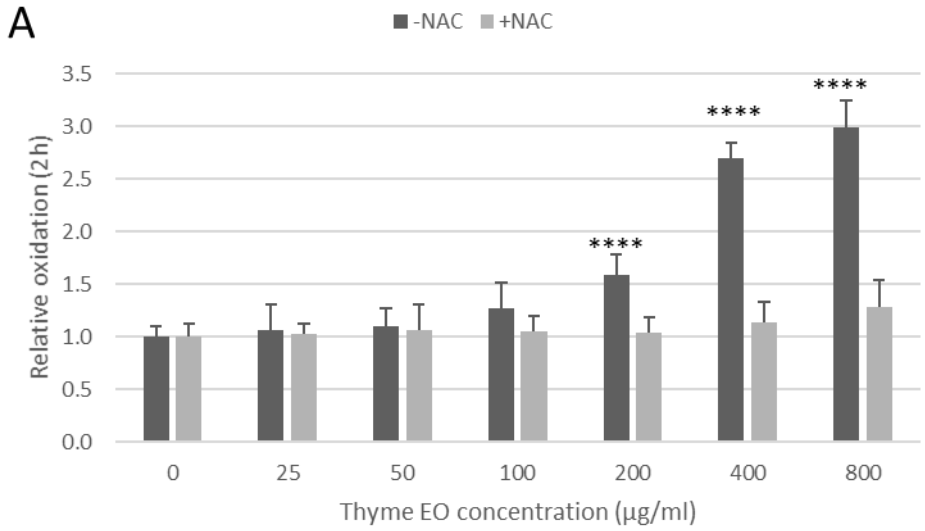


Fig. 4: Representative flow cytometry dot plots using Annexin V-FITC/PI staining for apoptosis assay. The quadrants indicate non-apoptotic (Q3, Annexin V^-/PI^-), early apoptotic (Q4, Annexin V^+/PI^-), late apoptotic (Q2, Annexin V^+/PI^+) and necrotic (Q1, Annexin V^-/PI^+) polupations. Thyme EO treated-cells show a higher percentage of both Annexin-V and PI positivity (Q2, 18.43%) compared to the control condition.

3.5. Increment of intracellular ROS and MDA levels by thyme EO

The DCFH-DA fluorescence assay revealed that thyme EO administration to MDA-MB-231 cells led to the early formation of ROS, as documented by the dose-dependent increment of intracellular oxidation levels after 2 h treatment (Fig. 5A). On the contrary, no significant ROS production was observed within MDA-MB-231 when cells were incubated with thyme EO in the presence of the antioxidant molecule NAC. The evaluation of ROS levels over the long period confirmed the presence of oxidative stress after 24 h upon thyme EO administration (Fig. 5B). Accordingly, the assessment of lipid peroxidation by HPLC analysis evidenced significantly increased intracellular MDA levels in thyme-treated cells compared to control cells (Fig. 5C).



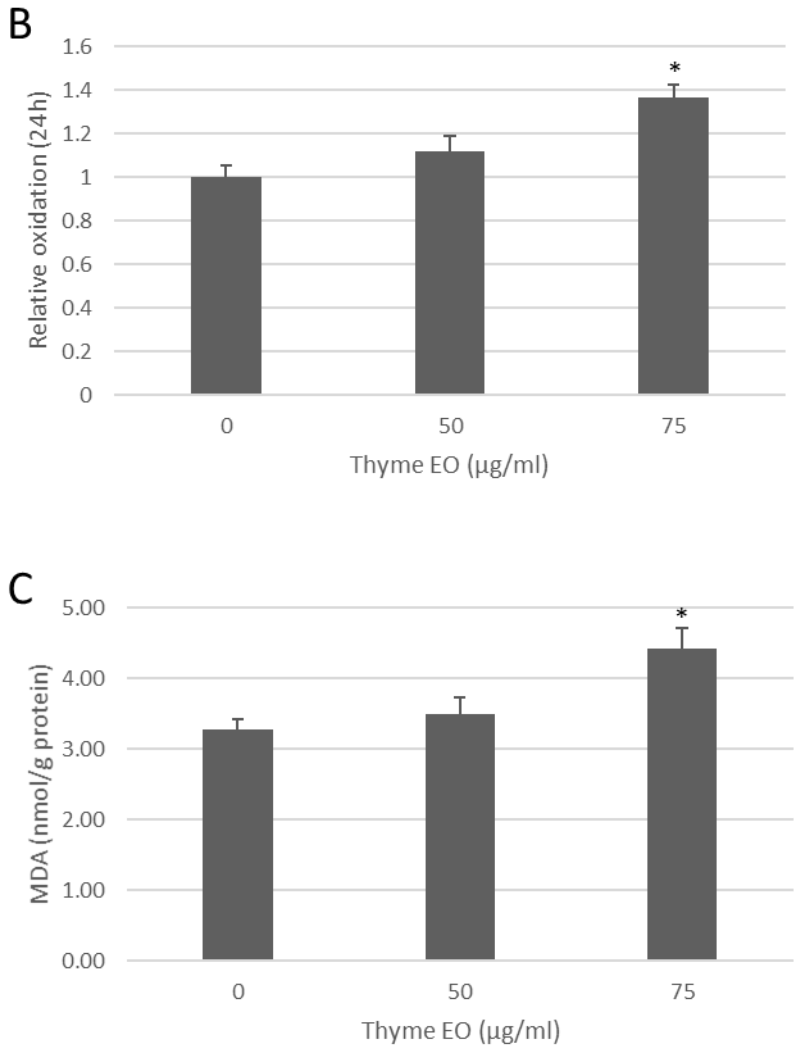


Fig. 5. (A) Intracellular oxidation levels at 2 h after thyme EO administration (25-800 µg/ml) to MDA-MB-231 cells in both the absence and presence of NAC 5 mM. (B) Increment of intracellular ROS and (C) MDA levels after 24 h of cancer cell incubation with thyme EO (50 and 75 µg/ml). *p<0.05 and ****p<0.0001 vs. untreated cells. Data are expressed as mean ± SD (n=3).

3.6. Induction of Nrf2 pathway by thyme EO

The assessment of mRNA expression levels of Nrf2 pathway-related genes after 24 h upon thyme EO administration demonstrated the upregulation of the transcription factor Nrf2 and the downstream inducible enzymes GPx and HO-1 (Fig. 6). The proteome profiler array confirmed the increased level of the protein HO-1 (by 4.4-fold vs. control) in thyme-treated cells after 24 h of incubation (Table 1 and Fig. S4 of the Supplementary Material file).

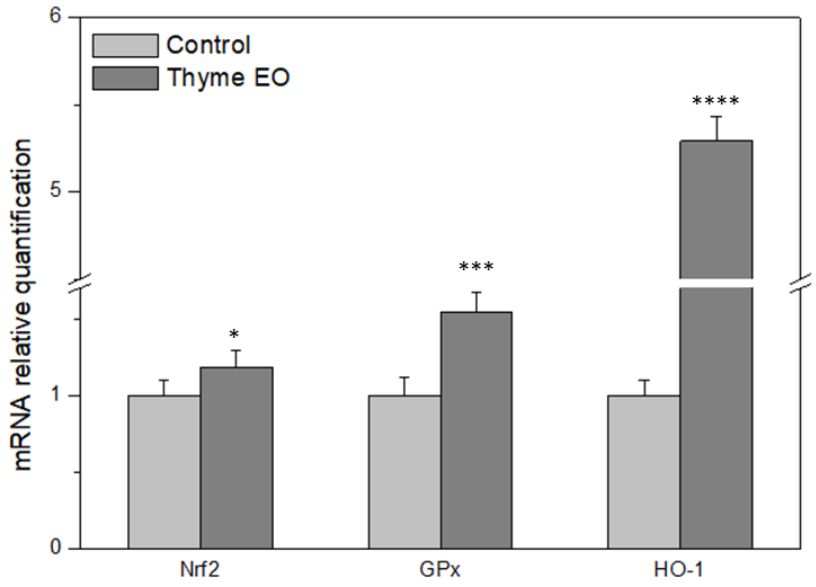


Fig. 6. Relative quantification of Nrf2, GPx, and HO-1 mRNA expression levels after 24 h treatment with thyme EO (50 µg/ml). *p<0.05, ***p<0.001, and ****p<0.0001 vs. untreated cells. Data are expressed as mean ± SD (n=3).

3.7. Chemical composition of thyme EO

Analysis of the chemical composition of thyme EO (Table 2) revealed that the most abundant compound was the monoterpene thymol (49.61%), followed by p-cymene (18.78%) and gamma-terpinene (8.41%).

Table 2. Chemical composition of thyme EO.[a]

Compound	%
thymol	49.61
p-cymene	18.78
gamma-terpinene	8.41
carvacrol	5.24
linalool	3.5
beta-caryophyllene	3
limonene + 1,8-cineole	1.59
beta-myrcene	1.18

borneol	1.14
caryophyllene oxide	0.97
alpha-terpinene	0.77
alpha-pinene	0.67
4-terpineol	0.65
canphor	0.58
alpha-thujene	0.54
canphene	0.47
beta-pinene	0.17
thymol methyl ether	0.09
alpha-humulene	0.09
alpha-phellandrene	0.08
bornyl acetate	0.08
delta-3-carene	0.07
terpinolene	0.06
gamma-muurolene	0.06
alpha-muurolene	0.06
tricyclene	0.02
alpha-copaene	0.02
non-identified sesquiterpenes	0.6
others	1.53

[a]Analysis was carried out by Mérieux NutriSciences (Chelab S.r.l., Treviso, Italy) by gas chromatography/mass spectrometry (GC/MS) and gas chromatography/flame ionization detection (GC/FID) (as reported in Supplementary Material). Chromatographic area percentages (%) of the compounds are reported.

4. Discussion

In the present study we demonstrated that thyme EO administration to TNBC MDA-MB-231 cells could significantly reduce cell viability in a dose-dependent manner and inhibit cancer cell migration and clonogenic capacity, which are characteristics of tumor cell metastatic ability (Fares et al., 2020). These results agreed with previous findings evidencing thyme's capacity to inhibit colon cancer cell migration and invasion (Al-Menhali et al., 2015) and thymol's ability to affect single tumor cell survival and suppress cancer cell colonization (de La Chapa et al., 2018; Zeng et al., 2020).
By investigating different apoptosis activators/suppressors, we observed a significant Bax/Bcl-2 ratio increment in thyme-treated cells that indicated the activation of the mitochondrial-mediated apoptotic pathway (Peña-Blanco and García-Sáez, 2018), as also sustained by the presence of a reduced mitochondrial membrane potential following EO administration. The increased expression of p53 as a tumor suppressor further confirmed the induction of apoptotic cell death by thyme EO (Duffy et al., 2014). Among the other pro-apoptotic proteins, we evidenced an increased expression of SMAC/Diablo, a mitochondrial protein that, after releasing into the cytosol, interacts and antagonizes the inhibitors of apoptosis proteins (IAPs), thus allowing the activation of caspases and apoptosis (Martinez-Ruiz et al., 2008; Silke and Vince, 2017). Accordingly, after thyme EO administration, we found increased levels of cleaved caspase-3 and a concomitant downregulation of the negative regulators of apoptosis cIAP-1, cIAP-2, survivin, and XIAP. The overexpression of IAPs is a hallmark of many types of cancer, including breast cancer (Wang et al., 2012). In particular, survivin and XIAP are frequently associated with poor prognoses and drug resistance; consequently, they have been extensively studied as potential therapeutic targets (Abbas and Larisch, 2020; Li et al., 2019; Rathore et al., 2017). To the best of our knowledge, this is the first study showing the negative modulation of survivin and XIAP by thyme EO in cancer cells, suggesting the great potential of this natural mixture for developing new therapeutic strategies against TNBC.
The increased expression of the death receptor TNF R1 after the administration of thyme EO to MDA-MB-231 cells also suggested the involvement of the extrinsic apoptotic pathway in thyme-induced cytotoxic effects. This evidence would agree with previous findings showing that in tumor cells, EOs could activate the apoptotic machinery both intrinsically (mitochondrial pathway) and extrinsically (death receptor signaling) (Catalani et al., 2017a).

To deepen the biochemical mechanisms underlying the cellular effects of thyme EO, we extended our studies to ROS as cytotoxic stimuli able to induce cancer cell apoptosis (Moloney and Cotter, 2018). We found that thyme EO administration led to the early formation of ROS within MDA-MB-231 cells, followed by a significant increment of the reactive aldehyde MDA, an index of lipid peroxidation that forms adducts with DNA and proteins, leading to alterations of cell function and eventually apoptosis (Nam, 2011). At the same time, a decreased expression of the detoxifying and antioxidant enzymes catalase and PON2 was evidenced in thyme-treated cells, which could further sustain the observed condition of oxidative stress within cancer cells. Interestingly, increased levels of catalase and PON2 have been observed in different types of tumors, possibly contributing to stress resistance and apoptotic escape of tumor cells (Bacchetti et al., 2019; Glorieux et al., 2015). Therefore, future approaches aimed at decreasing these enzymes' levels might be crucial for developing pro-oxidant cancer chemotherapy.

The presence of increased ROS levels in thyme-treated cells led us to explore the activation of the Nrf2 pathway in response to oxidative stress since many dietary phytochemicals, and chemopreventive compounds were reported to inhibit tumorigenesis via modulating antioxidant response genes (Keum et al., 2006; Menegon et al., 2016). The gene expression analysis demonstrated the upregulation of the transcription factor Nrf2 and the downstream inducible enzymes GPx and HO-1; the proteome profiler array confirmed the strong expression of HO-1 in thyme-treated cells. It has been demonstrated that in TNBC MDA-MB-231 cells, HO-1 inhibits cancer cell proliferation and invasion through its byproduct, carbon monoxide, and by suppressing the expression of matrix metalloproteinase-9 (Lee et al., 2014; Lin et al., 2008). Moreover, HO-1 may act as a critical mediator in ferroptosis, a newly identified iron- and lipid peroxidation-dependent cell death (Mou et al., 2019). Noteworthy, the induction of ferroptosis has been recently reported by *Thymus vulgaris* extracts in leukemia and multiple myeloma cell lines (Adham et al., 2020), thus encouraging future investigations on ferroptosis as a chemotherapeutic strategy against tumors.

Finally, thyme-related cytotoxic effects might also be ascribed to the decreased expression of HIF-1α levels, a transcriptional activator that promotes angiogenesis, enhances hypoxia tolerance and inhibits apoptosis (Ke and Costa, 2006). It has been demonstrated that the down-regulation of HIF-1α suppresses the malignant biological behavior of TNBC cells and could be a potential means for

treating breast cancer (Wang et al., 2014). Several studies have identified HIF-1α inhibitors from natural products; among them, both terpenes and sesquiterpenes from EOs (Ikeda and Kakeya, 2021; Yun et al., 2021). Herein, we evidenced for the first time the ability of thyme EO to negatively modulate the hypoxic factor within MDA-MB-231 cells.

The chemical analysis showed that thymol was the most abundant metabolite in thyme EO (49.61%) ("thymol" chemotype) (Thompson et al., 2003). Based on previous findings (Islam et al., 2019; Kowalczyk et al., 2020), this molecule could be the main candidate responsible for the anticancer activity of thyme EO towards MDA-MB-231. However, the cytotoxic effect of other minor constituents of thyme EO should not be ruled out. For example, p-cymene (content equal to 18.78%) may prevent tumor invasion and metastasis by inhibiting MMP-9 expression and suppressing ERK1/2 and p38 MAPK signal pathways in tumor cells (Li et al., 2016). At the same time, carvacrol (content equal to 5.24%) may effectively suppress MDA-MB-231 cell growth through apoptotic cell death induction and cell cycle regulation (Arunasree, 2010; Li et al., 2021). Consequently, the observed antitumor activity should be attributed to potential synergies between the components of thyme EO rather than to a single molecule of the phytocomplex.

Noteworthy, thyme and thymol seem to display different mechanisms of action on cancer cells compared to healthy cells (Kowalczyk et al., 2020; Salehi et al., 2018), as also suggested by the selective antiproliferative effect of thyme EO against MDA-MB-231 cells as compared to HUVEC primary cells. As example, thyme and thymol show antioxidant activity and protective effects against oxidative stress in healthy cells (Mapelli et al., 2016) while increasing ROS production within cancer cells (Li et al., 2017). Following this evidence, we observed a strong antioxidant capacity of thyme EO by DPPH radical scavenging assay (Table S5, Supplementary Material) but an increment of ROS and MDA levels after thyme EO administration to TNBC cells.

5. Conclusions

The present study highlights the potential benefits of thyme essential oil (EO) against human triple-negative breast cancer cells. Thyme EO, rich in thymol and p-cymene, was found to have detrimental effects on the MDA-MB-231 cell line, reducing cancer cell viability dose-dependently and inhibiting cancer cell migration and colony formation capacity. The antiproliferative effects of

thyme EO were related to the induction of apoptosis via the mitochondrial pathway, as also demonstrated by the significant changes in the protein levels of different apoptosis activators/suppressors. Thyme EO administration also led to the early formation of intracellular ROS, increment of MDA as an index of lipid peroxidation, and decreased expression of antioxidant enzymes catalase and PON2. The upregulation of Nrf2 mRNA expression and the strong induction of HO-1 sustained the activation of the Nrf2 pathway by thyme EO. These findings suggest that thyme EO from *Thymus vulgaris* L. might be a promising natural product in integrative medicine for triple-negative breast cancer therapy.

Declaration of Competing Interest

The authors declare no conflict of interest.

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