Investigating Anticancer Potency of In Vitro Propagated Endemic Thymus Cilicicus Boiss. & Bal. Extract on Human Lung, Breast, and Prostate Cancer Cell Lines

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Investigating anticancer potency of *in vitro* propagated endemic *Thymus cilicicus* Boiss. & Bal. extract on human lung, breast, and prostate cancer cell lines

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Abstract

Cancer is the leading cause of death in the world, accounting for nearly 10 million deaths in 2020 alone and surpassing all other categories by a large margin. Several different strategies have been and are still being deployed to combat different types of cancers and among the common clinical approaches are the use of synthetic and natural compounds as anticancer agents within chemotherapy regimens. *Thymus cilicicus* Boiss. & Bal is a spice endemic to Turkey, the Northern Aegean Islands, Lebanon, and Syria, while several species of the Thymus genus are known to exhibit different clinically valuable properties, the research on *T. cilicicus* is rather scarce, therefore, in this study, the wound healing properties of *in vitro* propagated *T. cilicicus* ethanolic extracts were investigated on murine fibroblast (NIH-3T3), and its anticancer potency was investigated on the human alveolar basal epithelial adenocarcinoma (A549), human breast adenocarcinoma (MDA-MB-213), and human prostate cancer (DU-145) cell lines via colorimetric MTT assays. The wound healing property assessments didn't lead to any significant results, however, *T. cilicicus* yielded selective and promising anticancer potency on the A549 cell line. Furthermore, molecular docking analyses were on the proteins of 9 genes confirmed to be upregulated in both 3D and 4D A549 cultures against 48 compounds found in the essential oils of *T. cilicicus* were performed and yielded results acquiescent with previous findings in the scientific literature. This study provides *in vitro* evidence to the selective anticancer activity of *T. cilicicus* extracts on A549 cells enhanced with computational evidence on the molecular mechanism involved in this selective activity. This study serves as a precursor for further *in vivo* and clinical research on the constituents of *T. cilicicus* as potent anticancer agents and their potential use in cancer therapies.

Keywords: Cancer; *Thymus cilicicus*; lung cancer; breast cancer ; prostate cancer; anticancer agents; molecular docking

1. Introduction

Cancer is the leading cause of death in the world, accounting for nearly 10 million deaths in 2020 alone, surpassing all other categories by a large margin (WHO 2021). While 30-50% of cancer cases could be prevented by avoiding the risk factors and implementing prevention strategies, the remaining cases require some form of clinical assistance. The oldest record for a cancer case has been reported from breast

cancer in ancient Egypt around 1500 BC, cancer develops in normal cells as they starts proliferating uncontrollably, i.e. when normal healthy cells grow and proliferate without any limitation or restrictions (Sudhakar 2009). DNA damage resulting in tens of thousands of individual molecular lesions per cell per day constantly occur in healthy cells under their regular metabolic activity or when exposed to certain environmental factors, however, under normal circumstances, DNA repair mechanisms recruit several cellular elements through which it identifies and repairs the damage to the DNA molecule, nevertheless, every once in a while depending on the genetic composition of the cell and the environmental factors it's exposed to, certain damage to specific genes aren't properly rectified and hence result in the formation of tumor cells (Lodish et al. 2008). Five major mechanisms involved in this rectification process are, the base excision repair, nucleotide excision repair, mismatch repair, repair by homologous recombination, and repair by non-homologous end joining, different combinations of these pathways are known to be active during different stages of the cell cycle so as to maintain the integrity of the cell's genome and repair any damage, failure of these molecular mechanisms lead to survival and proliferation of the cells with damaged DNA which reflects as tumors (Chatterjee and Walker 2017).

The usage of natural compounds from plants, animals, and microorganisms by humans to treat different medical conditions dates long before recorded history, some paleoanthropological studies done in the Middle East concluded that Neanderthals might have been aware of the medicinal properties of several medicinal plants more than 60 thousand years ago (Solecki 1975). The oldest record mentioning the usage of medicinal plants for their activities comes from ancient Mesopotamia, around 2600 BC, which is not a surprise given that they're a civilization known for keeping written records. The records describe around 1 thousand plants (and their compounds) along with their medicinal application, some of which are still in use nowadays (Newman, Cragg, and Snader 2000). While human ancestors used plant products to relieve pain or improve wound healing without any knowledge of their action mechanisms, recent developments in molecular and cellular biology provide the opportunities to investigate the mechanism of action for these natural products to use them more efficiently and assess their side effects and risks (Schenone et al. 2013). The genus *Thymus* is a member of the *Lamiaceae* family and constitutes of 214 species spread across North Africa, temperate Asia zone, and Europe, several members of this genus have been widely used in food, cosmetics, perfumery, and pharmaceutical industries, they're also known to be widely used in traditional medicine to treat digestive disorders, cough, diarrhea, headache, cold, bronchitis, renal stone, asthma, and many other diseases, their essential oils contain several phenolic derivatives, terpenes, and esters, most of which has been extensively researched (Salehi et al. 2019; Cornara et al. 2009; Alarcón et al. 2015; Rowshan, Bahmanzadegan, and Saharkhiz 2013; X. Li et al. 2019). Several studies have also revealed that extracts from several species of the *Thymus* genus possess antimicrobial, antioxidant, antitumor, anti-inflammatory, analgesic, and other medically valuable properties (X. Li et al. 2019; Nabavi et al. 2015; Sarac and Ugur 2008).

Previous *in vivo* and *in vitro* studies that investigated the extracts from *T. quinquecostatus, T. vulgaris, T. carnosus, T. kotschyanus, T. mastichina, and T. citriodorus* also concluded their anticancer potentials (Martins-Gomes et al. 2019; S. Wu et al. 2013; Martins-Gomes et al. 2018; Sun et al. 2005; Heidari et al. 2018; Doosti, Ahmadi, and Fasihi-Ramandi 2018; Gordo et al. 2012; Abaza et al. 2015). Further in-depth investigations into thymol (2-isopropyl-5-methylphenol, 36.0–55.0 % of essential oils constituent, found in most of the *Thymus* species) had designated that it showed anticancer efficacy against human liver cancer (Bel-7402), breast cancer (MCF-7), acute promyelocytic leukemia (HL-60), bladder cancer (T24, SW780, and J82), immortalized urothelial (SV-HUC-1), colon adenocarcinoma (Caco-2), ovarian cancer (SKOV-3) and colon cancer (HCT116 and LoVo) cell lines (QingHua, YinZhi, and FengXiang 2010; Seresht et al. 2019; Deb et al. 2011; Y. Li et al. 2017; Llana-Ruiz-Cabello et al. 2014; Zeng et al. 2020;

Elbe et al. 2020). Two other extracts, oleanolic acid and ursolic acid isolated from *T. mastichina* L. have also been reported to exert improved synergistic anticancer activity on colon cancer (HCT-116) cell lines (Gordo et al. 2012). Carvacrol, another monoterpene phenol found in the essential oils several *Thymus* species are known to exert anticancer activity on human alveolar basal epithelial adenocarcinoma (A549), hepatocellular carcinoma (HepG-2), breast cancer (MCF-7), and ovarian cancer (SKOV-3) cell lines (Koparal and Zeytinoglu 2003; Elbe et al. 2020; Esmaeili-Mahani, Falahi, and Yaghoobi 2014; Patel, Shah, and Bavadekar 2012; Yin et al. 2012). Rosmarinic acid has also been reported to possess the ability to inhibit the proliferation of human colorectal adenocarcinoma (HT-29) cell lines with synergistic activity on some other cancerous cell lines (Erenler et al. 2016). Similarly, apigenin, a dietary flavonoid found in the former plants is known for its cell-cycle arrest and apoptosis induction activity in different tumor types such breast, cervical, colon, lung, liver, prostate, pancreatic, and stomach (X. Li et al. 2019; Imran et al. 2020; Erenler et al. 2016).

Thymus cilicicus Boiss. & Bal is a member of the *Thymus* genus, it is endemic to Turkey, the Northern Aegean Islands, Lebanon, and Syria and is sold as a spice and herbal tea only in the Çamlı (Chamli) village of Marmaris province in Mugla (Turkey), its locally known as lemon thyme, fish thyme, and cheese thyme and commonly used for stomachache relief, it's also reported in the literature for its use as a tranquilizer and for toothache relief. They can be consumed fresh at any time of the year or harvested when they bloom and distilled in oil or dried for later use (Baser 2002; Gürdal and Kültür 2013; Everest and Ozturk 2005). In a study conducted by Sarac and Ugur (2008), essential oils of *T. cilicicus* collected from various regions of Mugla province were obtained by hydrodistillation and their antimicrobial activity against some microorganisms was investigated, including multi-antibiotic-resistant bacteria, and gram-positive bacterias, and yielded promising results.

In this study, the aerial shoots of *in vitro* propagated *T. cilicicus*. were isolated and dried, ethanol extraction was performed on the dried leaves and the extract was analyzed for their wound healing property on murine fibroblast (NIH-3T3), and their antiproliferative activity on NIH-3T3, human alveolar basal epithelial adenocarcinoma (A549), human breast adenocarcinoma (MDA-MB-231), and human prostate cancer (DU-145) cell lines. Computational analyses such as molecular docking was utilized to elucidate the molecular mechanism underlying the potent antiproliferation activity on selected cell lines.

2. Materials & Methods

2.1. Preparing the plant material

In vitro cultures of *T. cilicicus* Boiss. & Bal. was obtained by following the optimum propagation protocol reported by (Kaya et al. 2021). The aerial shoots of the plant were isolated and left to dry on top of filter papers inside a dry air ventilating incubator at 30 °C for 24 hours, after 24 hours, the dry leaves were weighed and transferred into a mortar. Liquid nitrogen was added until all the dry leaves were immersed and they were milled by grinding until the liquid nitrogen evaporated and fine powders were obtained. The dry powder was weighed and taken into a falcon into which 10 mL ethanol (99.9% pure laboratory-grade) was added, the falcon was vortexed and placed into an ultrasonic bath for 1 hour. After 1 hour, the falcon was vortexed again and the content was filtered into a clear beaker via a filter paper and left in a fume hood overnight to allow the ethanol to evaporate. The dry extract was dissolved in Dimethyl sulfoxide (DMSO, 99.98% pure laboratory-grade) at a final concentration of 0.2 g/mL.

2.2. Cell culturing and extract preparation

A fresh batch of NIH-3T3 (RRID:CVCL_0594) cell line was thawed from a -86 °C stock and seeded into a sterile T75 flask with 15 mL fresh high glucose DMEM (Sigma Dulbecco's Modified Eagle Medium, supplemented with 10% fetal bovine serum, and 1% 10^4 U/mL streptomycin, 10^5 µg/mL penicillin, and 25 µg/mL amphotericin B mix) and left to incubate in a humidified atmosphere with 5% CO_2 at 37 °C until it reached 80% confluency (changing the medium every 24 hours). After reaching 80% confluency, the cells were harvested and seeded into a sterile 96-well cell microplate at a concentration of 10^4 cells per well per 200 µL. The same procedure was followed to prepare the cell microplates for the A549 (RRID:CVCL_0023), MDA-MB-231 (RRID:CVCL_0062), and DU-145 (RRID:CVCL_0105) cell lines. Each cell plate was incubated in a humidified atmosphere with 5% CO_2 at 37 °C for 24 hours.

Before applying the *T. cilicicus* extracts to the cell lines, the stock concentration of the extract (0.2 g/mL) was diluted 1/40-fold in the medium (DMEM) to obtain a working solution with a concentration of 2 mg/mL, further dilutions (with DMEM) were performed to obtain aliquots with concentrations ranging between 1000 to 100 µg/mL. The same dilution ratio was used to dilute DMSO (1/40-fold in DMEM) which was further diluted to obtain aliquots with concentrations ranging between 1000 to 100 µg/mL (normalized control group). 1/40-fold dilution of the initial plant material and DMSO was performed to standardize the toxic effects of DMSO on the cell lines (standardizing the toxicity of the solvents).

2.3. Colorimetric MTT assay

After incubating the 96-well cell microplates seeded with NIH-3T3, A549, MDA-MB-231, and DU-145 cell lines for 24 hours, their mediums was discarded and 200 µL of each of the extract aliquots at concentrations of 1000, 900, 800, 700, 600, 500, 400, and 300 µg/mL was added to 6 wells in each row (i.e. sextuple replicas) and their DMSO controls were added to the remaining 6 wells of the same row at the same concentration (i.e., each row contained 6 wells with extract aliquots at a particular concentration and 6 wells with the DMSO aliquots at the same concentration), each 96-well cell microplate was incubated in a humidified atmosphere with 5% CO_2 at 37 °C for 24 hours. After 24 hours, the content of each plate was discarded and the wells in each plate were rinsed with 100 µL D-PBS (Dulbecco's phosphate-buffered saline, Pan Biotech, Turkey), and 200 µL fresh DMEM and 20 µL MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide prepared in D-PBS at a concentration of 5 mg/mL) was added to each well. The plates were left in a humidified atmosphere with 5% CO_2 at 37 °C for 3 hours after which their content was discarded (without disturbing the formazan crystals at the bottom of the wells) and 100 µL DMSO was added to each well of the cell plates and they were incubated at 37 °C in a shaking incubator at 120 rpm for 20 minutes to dissolve the formazan crystals formed by MTT. After the incubation, the absorbance of each microplate was immediately measured at 540 nm in a spectrophotometer.

To calculate the viability of the cell, the absorbance of the wells incubated with plant extract was divided by the average absorbance of their corresponding DMSO control and converted into a percentage by multiplying by 100 (cell viability percentage). The inhibition percentages were calculated via *Eq.* 1, where $A_{540,X}$ is the absorbance of a well incubated with X concentration of the plant extract at 540 nm, and $A_{540,\Delta CX}$ is the average absorbance of the control group at the same (X) concentration. A logarithmic plot of the concentration against their respective inhibition percentages was plotted and the IC_{50} (i.e., the concentration required for 50% proliferation inhibition) was calculated from the equation of the line by substituting 50 for y. SciPy (RRID:SCR_008058) was used to fit the data and their plots were generated via MatPlotLib (RRID:SCR_008624) and seaborn (RRID:SCR_018132) libraries in Python enviroment (Hunter 2007; Waskom 2021; Virtanen et al. 2020).

$$Inhibtion\ \% = 100 - Cell\ viability\ (\%) \qquad (Eq.\ 1)$$

$$Cell\ viability\ (\%) = \left(\frac{A_{540,X}}{A_{540,\Delta CX}} \times 100\right) \qquad (Eq.2)$$

2.4. Scratch wound assay

A portion of the freshly thawed NIH-3T3 cells was seeded into 6 sterile Petri dishes (2r=60 mm) at a concentration of 1.25 x 10^5 cells per Petri dish and was incubated in a humidified atmosphere with 5% CO_2 at 37 °C until they were completely coated with cells (confluency > 90%, ≈24 hours), after the incubation period, their mediums were discarded, and scratch wound assay (artificial wound drawn across the Petri dish) was performed on each of the 6 Petri dishes. The Petri dishes were rinsed in D-PBS and 2.5 mL of DMEM containing 100, 200, 300, and 400 µg/µL plant extract (1/40 diluted in DMEM as explained above, doses selected were below the IC50 obtained from the MTT assay in the previous step) was applied to the first 4 Petri dishes, 2.5 mL 1/40-fold diluted DMSO was applied to the 5th Petri dish (normalized solvent control) and 2.5 mL fresh DMEM was applied to 6th Petri dish (medium control). The area around the artificial scratch wound was observed under a light microscope (and their snapshots taken) immediately after adding the solutions and after 16 and 24 hours while incubating the Petri dishes in a humidified atmosphere with 5% CO_2 at 37 °C.

2.5. Structure-based molecular docking analysis

To address the molecular mechanisms involved in the antiproliferation effect of *T. cilicicus* on the A549 cell line, gene expression profiles derived from microarray results of A549 cells grown in *ex vivo* (4D) and matrigel (3D) models reported by Mishra *et al.* (2014) was evaluated and the common genes upregulated in both the models was collected (Mishra et al. 2014). The sequences for 9 common genes, namely, *EIF5* (Eukaryotic translation initiation factor 5), *ABCC8* (ATP-binding cassette sub-family C member 8), *GRIK2* (Glutamate receptor ionotropic, kainate 2), *COL4A6* (Collagen alpha-6 chain), *EPHA7* (Ephrin type-A receptor 7), *KIAA1841* (uncharacterized protein), *GALNT5* (Polypeptide N-acetylgalactosaminyltransferase 5), *DOCK4* (Dedicator of cytokinesis protein 4), and *MED31* (Mediator of RNA polymerase II transcription subunit 31) were retrieved from UniProt and their 3D structures were modeled via DeepMind's AlphaFold (The UniProt Consortium 2017; Jumper et al. 2021). A chemical library containing 48 compounds present in the essential oils of *T. cilicicus* was prepared based on the GC and GC/MS study reported by Tümen *et al.* (1994) (Tümen et al. 1994). Structure-based molecular docking was performed with each of the 48 ligands against each of 9 protein structures via AutoDock Vina (v1.2.0) using explicit hydrogens, search exhaustiveness of 64, Gasteiger empirical atomic partial charge model, and continuum solvation models following the blind docking protocol (Trott and Olson 2010). Each protein-ligand complex was evaluated based on their affinity scores (a scoring function based on binding affinity implemented in AutoDock Vina) and the interaction profile of the top ligand for each protein was visualized via Protein-Ligand Interaction Profiler (PLIP v2.2.2) (Salentin et al. 2015).

3. Result

3.1. Scratch wound assay

The dry weight of the extracts obtained was measured as 0.201 g, resulting in a yield of ≈30 .78%. Assessment of *T. cilicicus* wound healing properties was based on scratch wound assay, snapshots taken after the initial application of the extract to the NIH-3T3 coated Petri dishes (t=0), and after 16 hours (t=16), and 24 hours (t=24) of incubation is shown in Figure 1, the wound healing activity was evaluated based on

the migration rate of the NIH-3T3 cells over time towards the artificial wound (i.e. healing of the wound) at each concentration compared to the DMSO (solvent control) and DMEM (medium control) Petri dishes at the same time period. As shown in Figure 1, at any timestamp and across each concentration, the migration rate of the NIH-3T3 cells in the medium and DMSO control Petri dishes are higher than the migration rate in the Petri dishes treated with the plant extract, indicating a poor wound healing property (or a strong cytotoxicity). At the 24 hour timestamp, the artificial wound in the medium control is almost recovered, and the artificial wound in the DMSO control is significantly shrunken whereas the opposite is true for the Petri dishes treated with the plant extract.

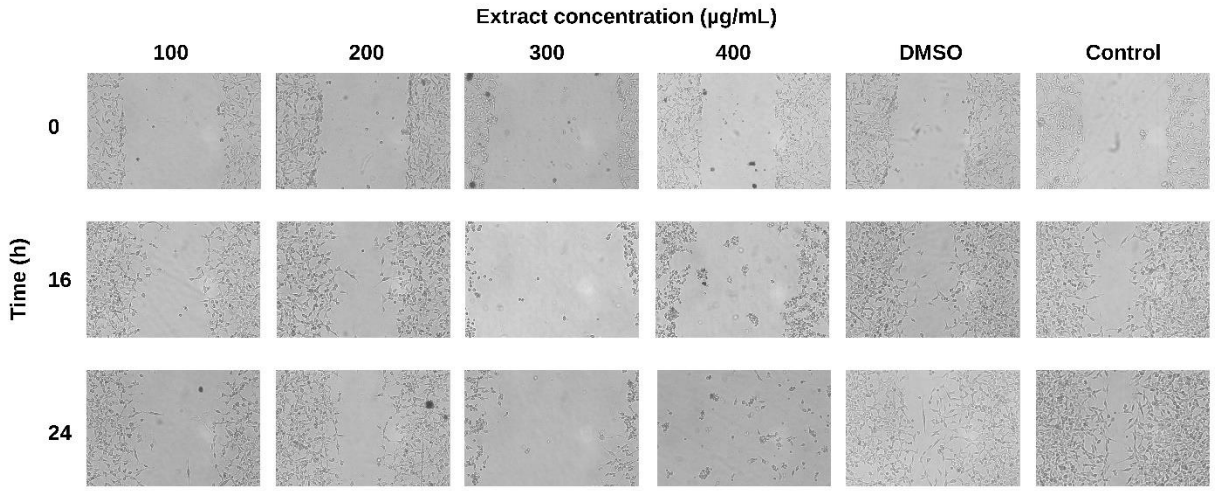

Figure 1. Assessment of *T. cilicicus* Boiss. & Bal. wound healing properties. Snapshots were taken immediately after introducing the artificial wound (t=0), after 16 hours (t=16), and after 24 hours (t=24), the extract concentration investigated were 100, 200, 300, and 400 µg/mL, DMSO Petri contains pure DMSO with DMEM but no plant extract (solvent control) whereas the Control Petri contains no DMSO or extract (medium control).

3.3. Colorimetric MTT assay

The dose-response plots (concentration against inhibition percentage) for each cell line investigated via MTT assay are shown in Figure 2, the results are also summarized in Table 1. By substituting y=50 in the line equations (shown on each plot in Figure 2), the IC_{50} for the NIH-3T3, A549, MDA-MB-231, and DU-145 cell lines was calculated as 556.58, 426.01, 1133.46, and 2219.09 µg/mL, respectively. As the NIH-3T3 cell line is a healthy (not a cancerous) cell line, the relative antiproliferative activity against A549, MDA-MB-231, and DU-145 cell lines were 0.77, 2.04, and 3.99 folds compared to the NIH-3T3 (calculated by dividing cancer cell's IC_{50} with NIH-3T3 cell line's IC_{50}). This results indicates *T. cilicucus* exerts selective antiproliferative activity on the A549 cell line. It's worth noting that the R^2 for the A549 dose-response plot was calculated as 0.928 whereas for MDA-MB-231 and DU-145 plots it was determined as 0.443 and 0.773 respectively (Figure 2), indicating a higher bias in the calculated IC_{50} values for the latter 2 cell lines.

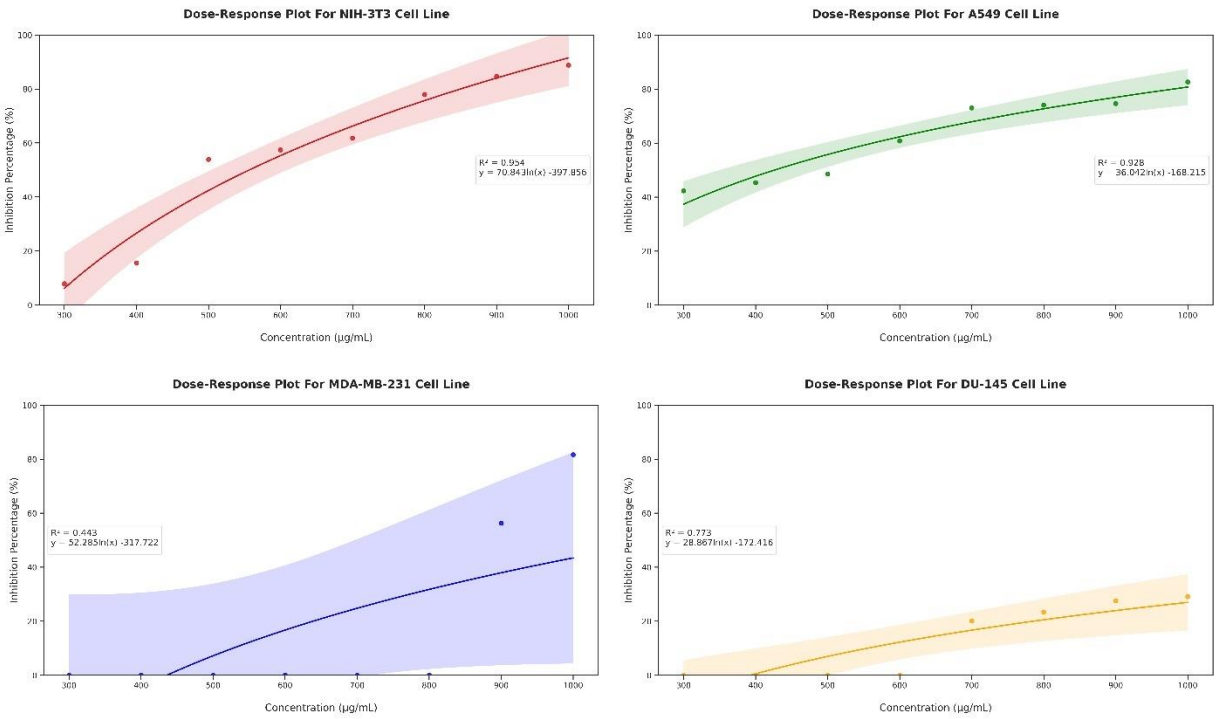

Figure 2. Dose-response plots for NIH-3T3, A549, MDA-MB-231, and DU-145 cell lines. Concentration percentages were calculated based on Eq.1 after 24-hour incubation with *T. cilicicus* extracts.

Table 1. Results from MTT assay for NIH-3T3, A549, MDA-MB-231, and DU-145 cell lines 24 hours post-treatment with *T. cilicicus'* extracts.

Concentration (µg/mL)	1000	900	800	700	600	500	400	300
				NIH-3T3				
Cell viability (%)	11.19±0.90	15.36±0.22	22.06±2.25	38.20±5.09	42.55±2.13	46.10±6.00	84.4±12.83	92.0±27.25
Inhibition (%)	88.81	84.64	77.94	61.8	57.45	53.90	15.59	7.93
IC50[I]	**556.58 µg/mL**							
				A549				
Cell viability (%)	17.37±0.63	25.37±0.88	25.94±0.63	26.94±1.14	39.19±5.23	51.44±5.06	54.6±8.46	57.65±6.29
Inhibition (%)	82.63	74.63	74.06	73.06	60.81	48.56	45.40	42.35
IC50[I]	**426.01 µg/mL**							
				MDA-MD-231				
Cell viability (%)	18.32±5.70	43.69±12.60	134.91±4.03	138.19±0.44	110.35±2.55	109.95±0.65	114.56±12.2	100.66±6.02
Inhibition (%)*[L]	81.68	56.31	0	0	0	0	0	0
IC50[I]	**<1133.46 µg/mL**							
				DU-145				
Cell viability (%)	70.91±7.55	72.42±1.31	76.60±7.82	79.90±12.05	140.29±40.5	115.45±20.8	116.36±45.2	100.76±2.15

Inhibition (%)*	29.09	27.58	23.40	20.10	0	0	0	0
IC50ı	**2219.09 µg/mL**							

* inhibitions percentages below 0% (i.e., no inhibition compared to their respective controls) were capped at 0%.
± indicates standard deviation.
ı Calculated based on the equations derived in Figure 2 by solving for y=50.
L The pattern of narrow IC_{50} range between 800-1000 µg/mL for MDA-MB-231 cell line persisted upon 3 independent repetitions of the experiment.

240

241 **3.4. Structure-based molecular docking**

242 The affinities calculated from docking the 9 upregulated proteins in A549 cell lines against the 48
243 components of *T. cilicicus* essential oils are summarized in Table 2. The raw results from the docking
244 experiment including the structure files are provided in the Supplementary Data 1 (S1).

245 **Table 2.** Results for structure-based molecular docking of 48 compounds found in *T. cilicicus* essential oils
246 against *EIF5, ABCC8, GRIK2, COL4A6, EPHA7, KIAA1841, GALNT5, DOCK4,* and *MED31* proteins.

Compound names	EIF5	ABCC8	MED31	EPHA7	GALNT5	COL4A6	KIAA1841	GRIK2	DOCK4
octanol	-3.70	-4.50	-3.80	-4.40	-5.10	-4.30	-3.90	-4.50	-4.50
camphor	-5.20	-6.00	-4.40	-5.00	-5.30	-5.00	-5.80	-5.40	-5.80
1-8-cineole	-4.90	-6.10	-4.50	-4.90	-5.30	-5.00	-5.50	-5.50	-6.00
eugenol	-5.10	-5.90	-4.70	-5.40	-6.80	-5.10	-5.60	-5.90	-5.90
bornyl acetate	-5.30	-6.80	-4.70	-5.30	-5.90	-5.80	-6.50	-5.90	-6.30
3-5-5-trimethyl-2-cyclohexenone	-4.90	-6.00	-4.70	-5.00	-6.10	-5.20	-5.30	-5.20	-6.10
linalool	-4.40	-5.50	-4.70	-4.50	-5.40	-4.90	-5.00	-5.00	-5.40
camphene	-5.00	-5.80	-4.50	-5.00	-5.60	-5.50	-5.30	-5.30	-5.90
α-pinene	-5.00	-6.00	-4.50	-5.20	-5.40	-5.10	-5.40	-5.70	-5.80
carvone	-5.50	-6.50	-4.90	-5.50	-6.80	-5.40	-5.90	-6.10	0.00
α-phellandrene	-5.40	-6.40	-4.90	-5.40	-6.60	-5.20	-5.40	-6.10	0.00
γ-terpinene	-5.00	-6.40	-4.70	-5.50	-6.80	-5.10	-5.40	-5.90	-5.90
α-terpinene	-5.20	-6.30	-5.10	-5.30	-6.80	-5.10	-5.30	-5.60	-6.00
p-cymene	-5.20	-6.20	-5.10	-5.30	-6.80	-5.20	-5.30	-6.30	-6.10
5-methyl-3-heptanone	-4.00	-4.60	-4.10	-4.30	-5.30	-4.10	-4.30	-5.20	-4.80
carvacrol	-5.40	-6.40	-5.40	-5.60	-7.00	-5.50	-5.90	-6.30	-6.20
myrtenol	-5.10	-5.80	-4.70	-4.90	-5.50	-5.50	-5.80	-5.20	-6.20
terpinen-4-ol	-5.20	-6.30	-5.00	-5.40	-5.70	-5.50	-5.50	-5.40	-6.10
terpinolene	-5.20	-6.70	-5.30	-5.40	-7.20	-5.30	-5.40	-6.00	-6.10
docosane	-4.40	-5.70	-4.40	-3.90	-5.30	-4.40	-4.50	-5.40	-4.80
p-cymen-8-ol	-5.40	-6.20	-5.00	-5.60	-6.90	-5.60	-5.80	-5.90	-6.40
β-pinene	-5.10	-6.00	-4.50	-4.90	-5.30	-5.10	-5.50	-5.60	-5.90
α-terpineol	-5.10	-6.10	-4.80	-5.40	-5.70	-5.40	-5.40	-5.70	-6.00
sabinene	-4.80	-6.30	-4.80	-5.20	-6.30	-5.20	-5.70	-5.90	-6.10
1-octen-3-ol	-3.70	-4.60	-4.10	-4.40	-5.20	-4.00	-4.00	-4.90	-4.60
limonene	-4.90	-6.30	-5.10	-5.30	-6.70	-5.20	-5.40	-6.00	-5.90

myrcene	-4.30	-5.60	-4.70	-5.20	-6.00	-4.20	-5.30	-4.90	-5.30
myrtenal	-5.30	-6.00	-4.70	-5.00	-5.50	-5.30	-5.90	-5.30	-6.10
pseudolimonene	-4.90	-6.30	-4.60	-5.00	-6.40	-5.10	-5.20	-6.10	-6.20
perillene	-4.60	-5.80	-4.80	-5.50	-5.90	-4.70	-5.30	-6.00	-5.60
tricyclene	-5.00	-5.90	-4.40	-5.00	-5.20	-5.20	-5.40	-5.40	-5.80
6-methyl-3-heptanol	-3.90	-4.80	-4.10	-4.30	-5.10	-4.30	-4.40	-4.80	-4.70
trans-pinocarveol	-5.20	-6.20	-4.80	-5.30	-5.40	-5.80	-6.10	-6.10	-6.10
spathulenol	-6.40	-7.60	-6.20	-6.30	-7.60	-6.40	-6.90	-6.80	-8.70
trans-campholenic aldehyde	-4.70	-5.90	-4.70	-4.80	-5.50	-5.40	-5.30	-5.30	-5.90
cis-verbenol	-5.20	-6.20	-5.00	-5.30	-5.40	-5.20	-5.80	-5.80	-5.90
β-bourbonene	-6.20	-7.80	-5.90	-6.20	-7.00	-6.20	-7.00	-6.30	-7.20
cis-carveol	-5.20	-6.10	-4.70	-5.20	-5.40	-5.30	-5.40	-5.40	-5.80
β-caryophyllene oxide	-6.20	-7.60	-6.30	-6.40	-7.50	-6.30	-7.00	-7.30	-7.40
z-3-hexenol	-3.90	-4.30	-3.80	-4.10	-4.40	-3.90	-3.70	-4.20	-4.40
β-caryophyllene	-6.70	-9.20	-6.30	-7.10	-7.70	-6.40	-6.80	-6.50	-7.70
germacrene d	-6.20	-7.50	-5.90	-6.30	-8.40	-6.40	-6.60	-7.00	-7.20
isoborneol	-5.00	-5.90	-4.40	-4.80	-5.40	-5.20	-5.70	-5.50	-5.80
cis-linalool oxide	-5.30	-5.80	-4.80	-5.40	-5.60	-5.30	-5.50	-5.70	-5.90
trans-linalool oxide	-5.10	-5.60	-4.60	-4.80	-5.30	-5.30	-5.50	-5.70	-5.70
γ-elemene	-5.70	-7.60	-5.60	-6.20	-7.20	-5.80	-6.10	-5.90	-7.00
trans-sabinene hydrate	-5.00	-6.20	-4.80	-5.70	-5.90	-5.40	-5.70	-6.10	-6.10
cis-sabinene hydrate	-4.90	-6.30	-4.60	-5.10	-6.30	-5.00	-5.40	-5.90	-6.20

*All values are in kcal/mol.

The ligands with the highest affinities for each of the proteins are further visualized in Figure 3. The highest affinities for each protein were *EIF5* with β-caryophyllene (-6.70 kcal/mol), *KIAA1841* with β-bourbonene and B-caryophyllene oxide (-7.00 kcal/mol), *GALNT5* with Germacrene D (-8.40 kcal/mol), *DOCK4* with Spathulenol (-8.70 kcal/mol), *MED31* with β-bourbonene and B-caryophyllene oxide (-6.30 kcal/mol), *ABCC8* with β-caryophyllene (-9.20 kcal/mol), *COL4A6* with Spathulenol, β-caryophyllene, and Germacrene D (-6.40 kcal/mol), *GRIK2* with B-caryophyllene oxide (-7.30 kcal/mol), and *EPHA7* with β-caryophyllene (-7.10 kcal/mol). Both β-caryophyllene and β-caryophyllene oxide show strong affinities toward several proteins overexpressed in A549, highlighting their potential role in causing the selective inhibition of the A549 proliferation.

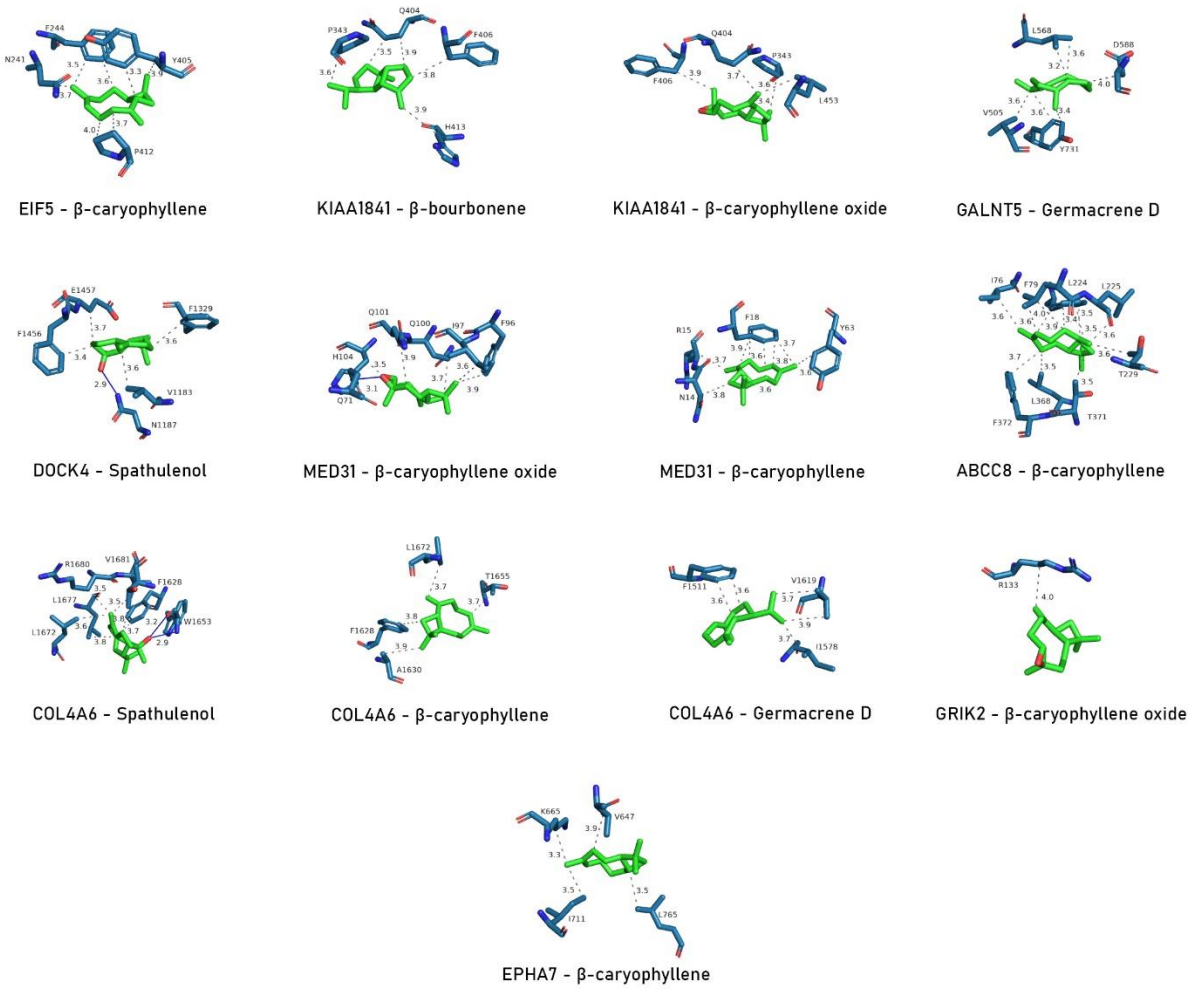

Figure 3. Interaction profiles of *T. cilicicus* essential oil extracts with the highest affinities towards *EIF5, ABCC8, GRIK2, COL4A6, EPHA7, KIAA1841, GALNT5, DOCK4,* and *MED31* proteins. Ligands are shown as green sticks, interacting residues of each protein is shown as blue sticks, hydrophobic interactions are shown in grey dashes, and hydrogen bonds are shown in continuous dark blue lines. All measurements are in Å.

4. Discussion

Cancer alone claims millions of lives annually despite the modern therapies and clinical interventions, the sophisticated molecular mechanism and the enormous variation in the disease's genetics and pathology from patient to patient is among the main reasons why a single cure approach is far from achievable, moreover, therapies that work efficiently in certain patients tend to stop working after some time as the development of drug resistance is common among cancer patients (Gottesman et al. 2016; WHO 2021). Therefore, the research for novel anticancer therapeutics remains an intense area of research despite the availability of several chemotherapy agents since the first introduction of nitrogen mustards and antifolate drugs in chemotherapy regimens back in the 1940s (Chabner and Roberts 2005).

This study focused on the traditional method of investigating natural endemic plants used in folk medicine for their potent medicinal property, while several members of the thymus family are known for

their different medicinal properties, *T. cilicicus* has only been investigated for its antimicrobial activity (Sarac and Ugur 2008). Initially, the ethanolic extract of the *T. cilicicus* was investigated for its wound healing properties on the NIH-3T3 cell line which yielded poor results as it inhibited the migration of cells even at the 100 µg/mL dose (Figure 1). This result indicated that *T. cilicicus* could hold a strong cytotoxicity effect even at lower doses which was confirmed by colorimetric antiproliferation assays where *T. cilicicus* extracts inhibited 50% of the NIH-3T3 cell proliferation at a concentration of 556.58 µg/mL (Figure 2 and Table 1). Colorimetric MTT assay performed on A549, MDA-MB-231, and DU-145 cancer cells following their exposure to *T. cilicicus* extract for 24 hours has shown some promising results, *T. cilicicus* extract exhibited the weakest inhibitory activity on the DU-145 cell line with an IC_{50} of 2219.09 µg/mL (4-folds higher than that exhibited on the non-cancerous NIH-3T3 cell line), followed by MDA-MB-231 cell line with an IC_{50} of 1133.46 µg/mL (Table 2), however, the results from MDA-MB-231 cell line was inconclusive as a pattern of no inhibition was observed at below 800 µg/mL even after 3 independent repetitions of the same experiment, nevertheless, in 3 independent replicas of the experiment, the average IC_{50} was calculated to be ≈950 µg/mL, which is still 1.7-folds higher (but still significant) than that of the non-cancerous NIH-3T3 cell line. The most promising result was achieved on the A549 for which the IC_{50} was calculated as 426.01 µg/mL, lower than the IC_{50} calculated on the non-cancerous NIH-3T3 cell line, elucidating into a tumor cell-specific antiproliferative activity.

To further investigate the molecular mechanism involved in the selective inhibition of A549 cell growth by *T. cilicicus* extracts, 9 proteins known for their upregulation in the A549 cell line (both in 3D and 4D cell cultures) were modeled via AlphaFold from their respective sequences and molecular docking was performed with them against 48 compounds found in the essential oils of *T. cilicicus* (Mishra et al. 2014; Tümen et al. 1994; Jumper et al. 2021). β-caryophyllene (which constitutes 1.37% of the essential oils) achieved the highest affinity against *EIF5* (-6.7 kcal/mol), *ABCC8* (-9.2 kcal/mol), *COL4A6* (-6.4 kcal/mol), *EPHA7* (-7.1 kcal/mol), and *MED31* (-6.3 kcal/mol). β-caryophyllene oxide (which constitutes 2.53% of the essential oils) achieved the highest affinity against *GRIK2* (-7.3 kcal/mol), *KIAA1841* (-7.0 kcal/mol), and *MED31* (-6.3 kcal/mol). Germacrene D (which constitutes 1.08% of the essential oils) exhibited a high affinity towards *COL4A6* (-6.4 kcal/mol) and *GALNT5* (-8.4 kcal/mol). Spathulenol (which constitutes 0.29% of the essential oils) exhibited a high affinity towards *DOCK4* (-8.7 kcal/mol) and β-bourbonene (which constitutes 0.32% of the essential oils) exhibited a high affinity towards *KIAA1841* (-7.0 kcal/mol).

Downregulation of *MED31* has been reported to suppress the proliferation of osteosarcoma cells and *in vitro* activation of *EPHA1* has been shown to promote angiogenesis and tumor growth in hepatocellular carcinoma, the calculated affinity of β-caryophyllene on the former proteins indicate the potential of a similar antiproliferation mechanism is involved in tumor suppression on the A549 cell line (Buckens et al. 2020; Jiang et al. 2014). *GRIK2* has been used as an epigenetic target in gastric cancer for its tumor suppressor role, this could hint into the role of β-caryophyllene oxide as a *GRIK2* agonist in the A549 cell line to inhibit its proliferation (C.-S. Wu et al. 2010). *GALNT5* has been reported to be remarkedly upregulated in gastric carcinoma, and *in vivo* hamsters with knocked-down *GALNT5* were reported for low proliferation, migration, and invasion of cholangiocarcinoma cells, Germacrene D affinity towards *GALNT5* in the docking study could result in its downregulation in A549 cell line and synergistically exhibit antiproliferative effect along with β-caryophyllene and β-caryophyllene oxide (Detarya et al. 2020). *DOCK4* is known to mediate cancer cell migration through activation of *RAC1* (by interacting with SH3YL1) in MDA-MB-231 breast cancer and spathulenol's affinity towards it provides some insights into the potency of utilizing spathulenol to block cancer metastasis in *in vivo* models, moreover, *DOCK4*-mediated *RAC1* activation is also involved in MDA-MB-231 breast cancer cell migration, which could explain the uncanonical antiproliferative activity of *T. cilicicus* extracts in the MDA-MB-231 cell line only

at high concentration (as could be derived from the MDA-MB-231 dose-response plot in Figure 2) as the amount of spathulenol in *T. cilicicus* constitutes only 0.29% of its essential oils (Kobayashi et al. 2014).

This study has investigated the medicinal use of endemic *T. cilicicus*, the antiproliferation assays have revealed *T. cilicicus* extracts to possess selective antiproliferative activity on the A549 at significantly low doses, these results were supported with computational analyses that revealed β-caryophyllene, β-caryophyllene oxide, Germacrene D, and spathulenol (all of which are known to be present in *T. cilicicus*' essential oils) to interact with several proteins known for their overexpression in A549 cell line. These results align perfectly with previous studies on the essential oil components of *T. cilicicus*, β-caryophyllene, and β-caryophyllene oxide has been reported for their anticancer and chemo-sensitizing properties (Fidyt et al. 2016; DI GIACOMO et al. 2017; Sultan et al. 2019) and several plants with high levels of Germacrene D has been reported in the literature for their selective cytotoxicity on different cancer cell lines (Zarai et al. 2011; Casiglia et al. 2017; Al-Nemari et al. 2020). Spathulenol has also been reported to possess inhibitory activity on B16-F10, HepG2, K562, and HL-60 cell lines and β-bourbonene has been reported to cause cell cycle arrest at G_0/G_1 phase and induce apoptosis in PC-3M cell line (Bomfim et al. 2016; Wang et al. 2018). This study serves as a precursor for further studies on *T. cilicicus* extracts for their anticancer efficiency and efficacy in vivo and clinically as well as shed the light on its valuable importance to the biopharmaceutical industry.

5. Supplementary Materials

S1: Structure files and raw results from molecular docking.

6. Competing interests

The authors declare no competing interests.

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