Original Article

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Chemical Composition and *In Vitro* Cytotoxicity of Endemic *Thymus brachychilus* Jalas Against Human Breast Adenocarcinoma (MCF-7, HTB-22), Human Lung Adenocarcinoma (A549, CRM-CCL-185), and Human Glioblastoma Cells (U-118 MG, HTB-15)

Endemik *Thymus brachychilus* Jalas'ın Kimyasal Bileşimi ve İnsan Meme Adenokarsinomu (MCF-7, HTB-22), İnsan Akciğer Adenokarsinomu (A549, CRM-CCL-185), İnsan Glioblastoma Hücrelerine (U-118 MG, HTB-15) *İn Vitro* Sitotoksisitesi

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ABSTRACT

Objective: *Thymus* have 341 species in the world and 46 species in Türkiye, 19 of them are endemic. *Thymus* species are used for stomachache, cold, shortness of breath, cough, bronchitis, diabetes in Aladağlar (Niğde). In this study, essential oil (EO) analysis of *Thymus brachychilus*, liquid chromatography/mass spectrometry (LC/MS) analysis of ethanol extract, and its effects on the cell lines mentioned in the title were investigated for the first time.

Methods: The endemic *Thymus brachychilus* Jalas was collected from Aladağlar Mountains, from 3,290 meters. Voucher specimens were prepared after the plant species were identified and kept at the herbarium (HERA 1029). 100 g of dried and powdered aerial part of the plant material was distilled for three hours, yielding 0.42 mL of volatile oil using a Clevenger-style apparatus. The 3.28 g ethanol extract was obtained from 100 g plant. An Agilent GC-FID/MS

ÖZ

Amaç: *Thymus*'un dünyada 341, Türkiye'de 46 türü bulunmakta olup bunlardan 19'u endemiktir. Aladağlar'da (Niğde) *Thymus* türleri mide ağrısı, soğuk algınlığı, nefes darlığı, öksürük, bronşit, şeker hastalığında kullanılmaktadır. Bu çalışma ile, *Thymus brachychilus*'un uçucu yağ analizi, etanol ekstraktının (EE) LC/MS analizi ve bu hücre hatları üzerindeki etkileri ilk kez araştırıldı.

Yöntemler: Aladağlar'ın 3.290 metre yüksekliğinden toplanan endemik *Thymus brachychilus* Jalas'ın tür tayini yapıldıktan sonra ve herbaryum örneği haline getirilip, herbaryum numarası verilerek (HERA 1029) saklanmaktadır. 100 g kurutulmuş ve toz haline getirilmiş toprak üstü kısımlardan oluşan bitki materyali, Clevenger aparatı kullanılarak 3 saat süreyle distile edildi ve 0,42 mL uçucu yağ elde edildi. Yüz g bitkiden 3,28 g etanol ekstresi elde edildi. Uçucu yağ numunelerini analiz etmek için bir Agilent GC-FID/MS sistemi

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ABSTRACT

system was used to analyze EO samples. Phenolic compounds of the extract were analyzed using LC-HRMS. MTT assay was used to evaluate the cytotoxicity of EO and ethanol extract.

Results: Thymol (48.11%), *p*-cymene (12.92%), carvacrol (11.14%) were the major components of the EO. For the extract, the IC50 values were calculated to be between 1.64-15.76 µg/mL, while the values of IC50 were calculated between 68.94-101.2 µg/mL for the essential oil.

Conclusion: Both the ethanol extract and the EO caused cell death in the tested cell lines. However, the extract appeared to be more effective compared to the essential oil. Besides that, a difference between the cell types could be seen, where the U118 MG neuronal cells appeared to be the most sensitive cell line.

Keywords: *Thymus brachychilus*, Aladağlar, essential oil, thymol, GC-MS, cytotoxic activity

ÖZ

kullanıldı. Ekstrenin fenolik bileşikleri, LC-HRMS kullanılarak analiz edildi. Uçucu yağ ve etanol ekstrelerinin sitotoksisitesini değerlendirmek için MTT testi kullanıldı.

Bulgular: Thymol (%48,11), p-cymene (%12,92), carvacrol (%11,14) uçucu yağın ana bileşenleriydi. Ekstre için IC50 değerleri 1,64-15,76 µg/mL arasında hesaplandı. Uçucu yağ için ise IC50 değerleri 68,94-101,2 µg/mL arasında hesaplandı.

Sonuç: Hem EE, hem de uçucu yağ test edilen hücre hatlarında hücre ölümüne neden oldu. Bununla birlikte, ekstrenin uçucu yağa kıyasla daha etkili olduğu görüldü. Bunun yanı sıra, hücre tipleri arasında da bir fark görüldü, U118 MG nöronal hücreleri en hassas hücre hattı olarak tespit edildi.

Anahtar Sözcükler: *Thymus brachychilus*, Aladağlar, uçucu yağ, timol, GC-MS, sitotoksik aktivite

Introduction

Thymus is a member of Lamiaceae family. It has 341 species in the world (1), 46 species in Turkey, and 19 of them are endemic (2,3). The *Thymus* genus generally has small shrubs, cushion plants, and perennial herbs. *Thymus brachychilus* Jalas is an endemic taxon of the Irano-Turanian phytogeographical region. The upper lip of the calyx is shorter than the lower teeth. Bracts resemble leaves and are 0.8 to 1.5 mm broad, narrowly rhombic, progressively thin into a short petiole. Leaves are narrower, 0.4-0.8 mm wide, and have patent coarse hairs on the stems and leaves. The oil spots are mainly orange to red. Flowering time is between 6th and 8th months. It grows in screes and rocks, 1,800-3,660 meters. *Thymus leucotrichus* Hal. and *Thymus serpylloides* Bory are the closest relatives (3). The common name of *Thymus brachychilus* around Niğde is "Mor kekik" (4). *Thymus* species are used internally in the form of tea prepared as an infusion for stomachache, cold, shortness of breath, cough, bronchitis, diabetes among the people in the villages around Niğde/Aladağlar (5). In a study conducted to determine the plants visited by honeybees in Mersin, beekeepers selected according to the data obtained from Mersin Beekeepers Association and determined criteria were interviewed, and visits were made to the areas where beekeepers regularly put their hives. *Thymus brachychilus* species have been identified among the plants most visited by bees to collect nectar (6). It is used in the production of a special cheese in Erzincan (7). One of the morphological closest species *Thymus leucotrichus* Hal. subsp. *leucotrichus* Hal. Is used for colds, flu, high cholesterol in Giresun (8). Species most similar to *Thymus brachychilus* in terms of chemical content are *Thymus kotschyanus* Boiss. & Hohen. and *Thymus praecox* Opiz in Türkiye. *Thymus kotschyanus* infusion is used as a sedative in Bingöl (9), for abdominal ailments, backache, cancer, colds, diabetes, enteralgia, hypertension, and as anthelmintic in Iğdır (10), for colds in Bingöl and Kahramanmaraş (9,11), for gastritis and to treat shortness of breath in Hakkari (12), to lower high cholesterol in Elazığ (13). *Thymus praecox* is used for the treatment of diabetes (Amasya) (14). The essential oil of *Thymus brachychilus* was first identified in the current study.

Methods

Plant Material

Thymus brachychilus samples were collected from the Maden and Cimbar Valleys in the Aladağlar mountain range, also known as Anti-Taurus Mountains (Niğde-Turkey). The location of the plant was rocky and at the altitude of 3,290 meters. Voucher specimens were prepared after the plant species were identified by authors. The Altınbaş University Pharmacy's Faculty's herbarium housed these voucher specimens (HERA 1029).

Thymus brachychilus Essential Oil Isolation

100 g of dried and powdered plant material was distilled for three hours using a Clevenger apparatus. The essential oils were dried over anhydrous sodium sulfate, and then stored at a temperature of 4 ºC until use.

Extraction of Plant Materials

The aerial parts of *Thymus brachychilus* were air-dried at room temperature in the shade, and then ground into powder. *Thymus brachychilus* powder was macerated with a ratio of 1 part of plant soaked in 10 parts of solvent, by using 96% ethanol solvent, in a tightly closed container for 3 days, and protected from light, while stirring frequently. The solvent was evaporated to dryness under rotary evaporator (Heidolph Hei-VAP Advantage Rotary Evaporator) at a temperature of 40 ºC with a speed of 120 rpm and 3.28 g extract was obtained from 100 g plant.

Analysis of Essential Oil

Essential oil (EO) samples were examined using an Agilent GC-FID/MS system (Santa Clara, California, USA). It included an Agilent 7890B GC-FID and an Agilent 5977E MS detector connected by a capillary column splitter. With the use of an

Agilent G4513A auto injector, 1 µL of sample EO solutions in 10%, v/v, *n*-hexane were injected. The temperature program for the HP-5MS column (30 m, 0.25 mm, 0.25 µm) was as follows: 60 ºC isothermal for 5 minutes, then elevated to 180 ºC at a rate of 3 ºC/min. Further, temperature was kept 5 minutes isothermally. Helium was used as a carrier gas at a constant flow rate of 1.5 mL/min. A split ratio of 1:50 was chosen. The temperatures of the injector port, MSD transfer line, ion source, quadrupole, and FID, among other system components, were maintained at 250 ºC, 250 ºC, 230 ºC, 150 ºC, and 220 ºC, respectively. The FID dry air and H_2 flow were adjusted to 400 mL/min and 30 mL/min, respectively. Mass spectra between 45 and 450 m/z were captured.

Identification of the compounds was done by co-injecting reference substances and comparing the spectrum data of the compounds to the NIST 11 Mass Spectral Library (NIST11/2011/EPA/NIH). Retention indices were computed using C7-C40 homologous alkane series and then compared to data from the NIST online webbook. Using an external standard method and calibration curves obtained from GC-FID investigations of sample chemicals, quantification was completed.

Quantitative Analysis of Phenolic Compounds Using LC-HRMS

Due to their ability to act as antioxidants, phenolic chemicals were also significant. The aerial part of *Thymus brachychilus* were analyzed using LC-HRMS. In a 6 mL volumetric flask, the dried 60 mg extracts of each species were dissolved in water and ethanol (40:60). Until a clear solution was attained, the flask was held in an ultrasonic bath. The volume was then diluted with mobile phase before 100 µL of dihydrocapsaicin solution (from a 100 ppm stock solution) was added as internal standard. It was mixed and warmly heated to clarify a resolution. The final concentration of the solution (1 mL) was put into a sealed auto sampler vial after being filtered with a 0.45 µm Millipore Millex-HV filter. For each run, 2 µL of the sample was then injected into LC. Throughout the experiment, the samples in the auto sampler were maintained at 15 ºC.

Thermo ORBITRAP Q-EXACTIVE (Bremen, Germany) mass spectrometry-equipped ESI ion source and Dionex LC equipment were used for the LC-HRMS measurements. The scan range was adjusted to m/z 100-900 amu, and the following mass parameters were used: capillary temperature was 320 °C, aux gas heater temperature was 320 °C, gas flow rate was 45, aux gas flow rate was 10, sprey voltage was 3.80 kV, and Slens RF was 50. Compound separation was performed by using a Troyasil C18 column (150x3 mm i.d., 5 µm particle size, İstanbul, Turkey). The mobile phases A and B contained 1% formic acid in water and 1% formic acid in methanol, respectively. The gradient program was 50% A and 50% B for the first 0-1.00 min, 100% B for the next 1.01-6.00 min, and 50% A and 50% B for the final 6.01-15 min. The column temperature was set to 22 ºC, and the mobile phase flow rate was 0.35 mL/min. Compounds were identified by contrasting the HRMS data from the Bezmialem Vakıf University, Drug Application and Research Center Library-ILMER with the retention periods of reference compounds (in the purity range of 95-99%; see section chemicals). Dihydrocapsaicin (purity 95%) was employed as an internal standard for LC-HRMS measurements in order to decrease the repeatability problem brought on by external factors, such as ionization repeatability, in mass spectrometry studies. Table 2 provides the specific mass parameters of each target chemical.

Cytotoxicity Activity Assays

The following chemicals were bought from Sigma Aldrich: dimethyl sulfoxide (DMSO), 2.5-diphenyl-2H-tetrazolium bromide (MTT) (St. Louis, Missouri, USA). The following items were acquired from Wisent INC: Fetal bovine serum (FBS), phosphate buffer solution, high glucose Dulbecco's Modified Eagle Medium (DMEM), F12 cell culture medium, and trypsin/EDTA solution (Quebec, Canada).

The cytotoxicity of the ethanol extract and EO of *Thymus brachychilus* was assessed using the MTT assay. MTT assay is one of the most frequently referred test in cytotoxicity evaluation. In this test, the water-soluble yellow MTT pigment metabolized by the active mitochondrial enzymes in the viable cells to produce a water-insoluble purple formazan. The absorbance of light by formazan after dissolving in DMSO used to calculate the viability and then the cell death ratio. To apply the test, cells were seeded in a 96-well plate ($1x10^4$ cells/100 µL/ well), allowed to be attached overnight, then the medium was removed, and a new fresh medium containing different concentrations of the extract or the EO was added. After 24 hours exposure period, 20 µL/ well of MTT (0.5 mg/mL) were added and incubated for further 3 hours. Then, the supernatants were thrown, and formazan crystals were dissolved in 100 µL/well of DMSO, and the absorbance (OD) was measured by a Thermofisher microplate reader (Massachusetts, USA) at 590 nm. The ratio of the viable and dead cells were calculated compared to the solvent group (1% DMSO), and the results were expressed as half maximal inhibitory concentration (IC50), the concentration caused the death in one-half of the cells.

Results

Essential Oil Yield and Composition

0.42 mL of EO was obtained from 100 g dried aerial part with 0,42% (v/w) yield of *Thymus brachychilus*, density of 0.8885 g/cm^3 at 20 °C, and 99.71% of the EO was made up of 20 identified and quantified components. The four main components of the EO were determined to be thymol, which made up 48.11% of the oil, 12.92% *p*-cymene, 11.14% carvacrol, and 9.36% *endo*-borneol. The EO's chemical composition is shown in Table 1 and Figure 1.

Quantitative Analysis of Phenolic Compounds Using LC-HRMS

In this study, a total of 18 phenolic compounds were quantitatively determined by LC-HRMS in the ethanol extracts of the aerial part of *Thymus brachychilus*. According to the

results presented in Table 1, 6-methoxyapigenin-7-glucoside (10.2080±0.0052 mg/g), rosmarinic acid (9.8926±0.0038 mg/g), luteolin-7-glucoside (5.5110±0.0041 mg/g), caffeic acid (2.7753±0.0037 mg/g), and kaempferol (2.0358±0.0036 mg/g) were shown to be the primary ingredients in the aerial section of the *Thymus brachychilus* extract.

Cytotoxicity Activities

Both the ethanol extract and the EO of *Thymus brachychilus* caused cell death in the tested cell lines. However, the extract appeared to be more effective compared to the essential oil. For the extract, the IC50 values were calculated to be between 1.64-15.76 µg/mL. While the values of IC50 were calculated between 68.94-101.2 µg/mL for the essential oil. Besides that, a difference between the cell types could be seen, where the U118 MG neuronal cells appeared to be the most sensitive cell line (Table 3, Figure 2,3).

Table 1. Chemical composition of *Thymus brachychilus* essential oil

No	Components	KI[a]	RRI[b]	Relative %	Identifaction method
1	α-pinene	1012-1039	1027	1.63±0.05	RRI, MS
2	Camphene	1057-1083	1072	2.53±0.08	RRI, MS
3	β-myrcene	1145-1169	1160	0.43±0.03	RRI, MS
4	α-terpinene	1170-1201	1181	0.80±0.04	RRI, MS
5	γ-terpinene	1238-1253	1248	2.92±0.07	RRI, MS
6	*p*-cymene	1261-1290	1274	12.92±0.20	RRI, MS
7	1-octen-3-ol	1430-1460	1452	0.19±0.00	RRI, MS
8	*cis*-sabinene hydrate	1438-1474	1465	1.00±0.01	RRI, MS
9	Camphor	1490-1535	1511	1.26±0.01	RRI, MS
10	*trans*-sabinene hydrate	1542-1556	1548	0.23±0.10	RRI, MS
11	4-terpineol	1590-1635	1600	0.75±0.01	RRI, MS
12	Verbenol	1665-1686	1676	0.26±0.00	RRI, MS
13	*endo*-borneol	1696-1705	1699	9.36±0.02	RRI, MS
14	β-bisabolene	1715-1748	1727	1.11±0.46	RRI, MS
15	Carvacrol acetate	1868-1908	1902	3.61±0.04	RRI, MS
16	Thymoquinone	NA	1918	0.15±0.01	RRI, MS
17	*tau*-cadinol	2036-2184	2100	0.48±0.01	RRI, MS
18	Thymol	2153-2187	2184	48.11±0.66	RRI, MS
19	Carvacrol	2186-2239	2214	11.14±0.24	RRI, MS
20	α-cadinol	2201-2259	2230	0.83±0.02	RRI, MS
	Total identified			99.71	

RRI[b]: Relative retention indices calculated against n-alkanes; % calculated from FID data.
Identification method based on the relative retention indices (RRI) of compounds on the HP innowax column, MS, identification was performed on the basis of computer matching of the mass spectra with those of the Wiley and MassFinder libraries and comparison with literature data (15-20).
[a]KI were given from literature with confidence intervals 50% of RI data ranges for each compound
NA: Not available current literature

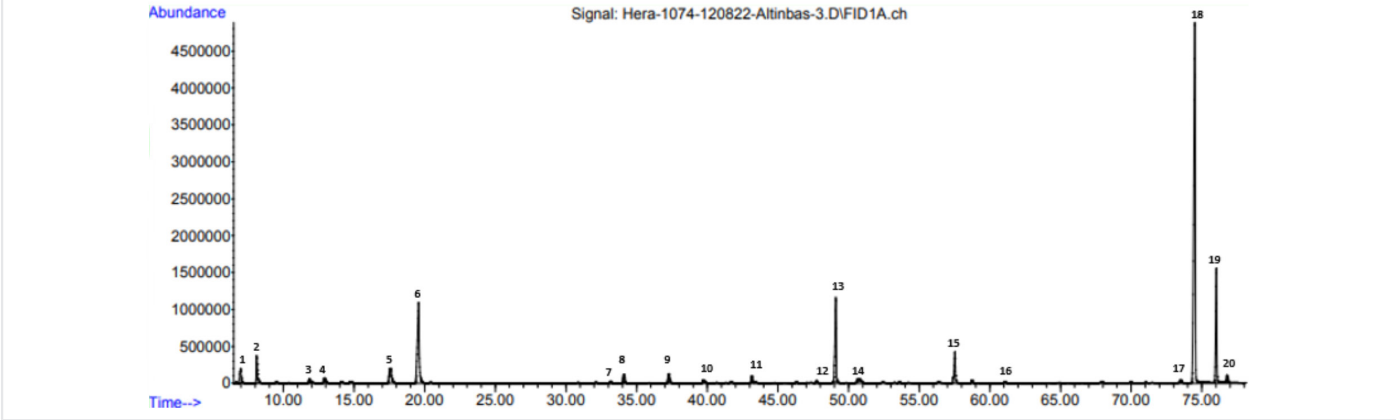

Figure 1. GC-FID chromatogram of Thymus brachychilus essential oil major compounds

Discussion

The chemical content of *Thymus* species, besides their ethnobotanical and folk medicine applications, encourage the researchers to evaluate their biological activity in general and cytotoxic effects in particular. Apigenin, chrysin, and lutein have promising futures as potent antitumor medicines for cervical cancer. For the treatment of gastric and ovarian cancer, respectively, kaempferol, lutein, and apigenin could be viewed as viable candidate medicines. The cells from the colon and liver, which are the primary sites of flavonoid metabolism, exhibit quite considerable swings in anticancer activity, which is likely caused by exposure to numerous metabolites with varied actions. The same appears to be true for chrysin, and compared to melanoma and lung cancer cells, apigenin is possibly more sensitive and effective at killing cervical cancer cells. Both cervical cancer and melanoma cell lines exhibit high levels of luteolin activity, demonstrating that these flavones may have promising futures as the active ingredients in potent anticancer drugs for the specified target areas (21). According to studies, flavonoids such chrysin,

Table 2. Quantitative analysis of phenolic compounds using LC-HRMS

No	Analyte	RT[a]	Quantification (mg/g)[b]	Polarity (ESI)	m/z
1	Ascorbic acid	2.19	0.3444±0.0039	Negative	175.0248
2	Chlorogenic acid	2.46	0.1626±0.0036	Negative	353.0878
3	Fumaric acid	2.48	0.3608±0.0029	Negative	115.0037
4	Caffeic acid	3.07	2.7753±0.0037	Negative	179.0350
5	Luteolin-7-glucoside	3.58	5.5110±0.0041	Negative	447.0933
6	Luteolin-7-rutinoside	3.62	0.4101±0.0031	Negative	593.1511
7	Hyperoside	4.40	0.0546±0.0035	Negative	463.0882
8	Rosmarinic acid	4.52	9.8926±0.0038	Negative	359.0772
9	6-Methoxyapigenin-7-glucoside	4.79	10.2080±0.0052	Negative	461.1089
10	Apigenin-7-glucoside	4.83	0.0413±0.0036	Negative	431.0984
11	Quercetin	5.66	0.0236±0.0029	Negative	301.0354
12	Salicylic acid	5.66	0.5360±0.0019	Negative	137.02442
13	Naringenin	5.70	0.0988± 0.0042	Negative	271.0612
14	Luteolin	5.81	0.9749±0.0034	Negative	285.0405
15	Kaempferol	5.81	2.0358±0.0036	Negative	285.04046
16	Apigenin	6.15	0.2724±0.0029	Negative	269.0456
17	Chrysoeriol	6.18	0.3542±0.0061	Negative	299.0561
18	Acacetin	7.07	0.0401±0.0040	Negative	283.0612

[a]RT: Retention time, [b]Values in mg/g (w/w) of plant

Table 3. The cytotoxic effects of *Thymus brachychilus* extract and essential oil in different cell lines

Cell line	IC_{50}	
	Extract (µg/mL)	Essential oil (µg/mL)
MCF-7	2.02	101.2
A549	15.76	79.86
U-118 MG	1.64	68.94

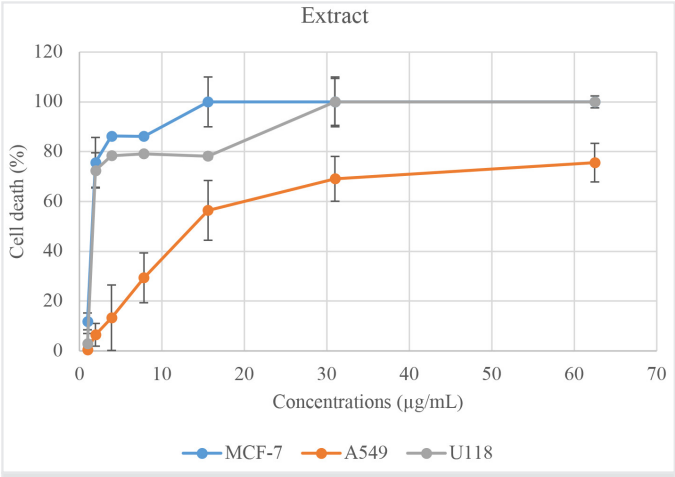

Figure 2. Cytotoxic effect of *Thymus brachychilus* extract in different cell lines

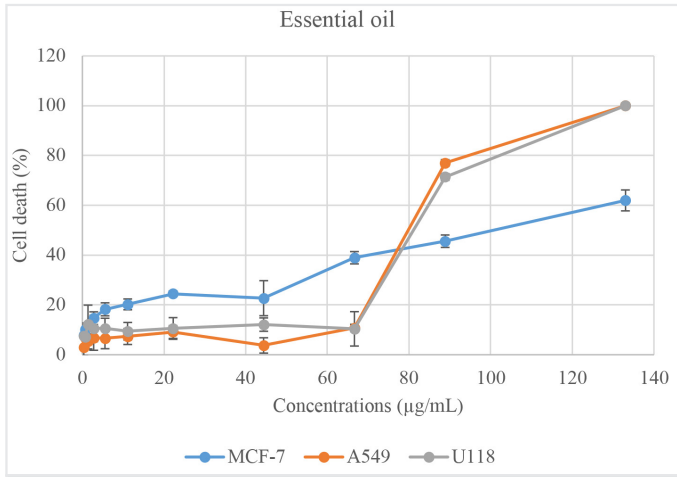

Figure 3. Cytotoxic effect of *Thymus brachychilus* essential oil in different cell lines

epigallocatechin-3-gallate (EGCG), formononetin, hispidulin, icariin, quercetin, rutin, and silibinin work in concert to boost the effectiveness of conventional chemotherapeutics. The regulation of intracellular signaling pathways involved in apoptosis, proliferation, autophagy, motility, and chemoresistance mediates these favorable effects. In light of this, flavonoids show potential in enhancing current therapeutic approaches and ultimately overcoming medication resistance in glioblastoma (GBM) (22). The chance of tumor development in numerous human organs, including stomach, colon, liver, breast, and leukemia cells, has also been found to be reduced by rosmarinic acid and some isolated chemicals from rosemary extract, such as carnosic and ursolic acids and carnosol (23).

The EO's five primary constituents were identified as being thymol, which made up 48.11% of the oil, 12.92% *p*-cymene, 11.14% carvacrol, and 9.36% *endo*-borneol, respectively. *Thymus* species are known for their rich EO content. There are also different chemotypes of the same species. When we compared our study with the studies on EO contents of other *Thymus* species, it was observed that they had similar properties with *Thymus* species, especially those with high thymol content. *Thymus ciliatus* Desf. has 79.1% thymol, 4.4% carvacrol (24), *Thymus kotschyanus* Boiss. has 22.75% carvacrol, 16.52% thymol, 11,39% thymoquinone, 4.52% borneol (25), *Thymus praecox* subsp. *scorpilii* (Velen) Jalas var. *laniger* (Borbas) Jalas has 69.09% thymol, 5.54% borneol, 3.08% carvacrol (26), *Thymus daenensis* Čelak. subsp. *daenensis* has 74.7% thymol, 1.3% carvacrol (27), *Thymus pulegioides* L. has 63.2% carvacrol, 15.55% thymol (28), *Thymus serpyllum* L. has 46.24% thymol, 9.43% thymoquinone, 1.34% borneol (29) in their EO.

Thymus vulgaris is one of the famous herbs evaluated for its biological activity. According to the Scopus database (27.11.2022), there are 69 papers containing the words *Thymus vulgaris* and "cytotox" in their abstract section [ABS (*Thymus* AND *vulgaris*) AND ABS (cytotox)], and 144 paper containing the words *Thymus vulgaris* and "cytotox" in their title, abstract or keywords section [TITLE-ABS-KEY (*Thymus* AND *vulgaris*) AND TITLE-ABS-KEY (cytotox)]. These studies reported that *Thymus vulgaris* extracts, essential oils, and its isolated compounds were evaluated in A549 cells, oral cavity squamous cell carcinoma, human breast cancer (SK-Br-3), head and neck squamous cell carcinoma, human breast cancer cell line (MDA-MB-231), human colon adenocarcinoma cell line (Caco-2), human hepatocellular carcinoma cell line (HepG2), THP-1, U937, and K562 leukemia cell lines, human cervical cancer (HeLa) cell line, porcine liver primary cell culture, human breast cancer (MCF-7) cell line, human prostatic adenocarcinoma (PC3) cell line, and other cells (Table 4). Results, in general, indicated the cytotoxicity and anti-cancer activity of *Thymus vulgaris* (30-38). The IC50 was calculated to be 10.50±0.01 µg/mL after 72 h on the A549 cells for *T. vulgaris* L. EO which had thymol (41.33%), 1.8-cineole (24.10%) (34). According to Nikolić et al. (35) *Thymus serpyllum* EO which had thymol (56.02%), carvacrol (14.00%) and *p*-cymene (6.2%) has cytotoxicity with IC50 value 52.69±3.28 µg/mL in MCF7, 17.71±3.23 µg/mL in HeLa, 34.96±2.90 µg/mL in HepG2, *T. algreriensis* EO which had thymol (38.5%), *p*-cymene (8.9%), terpinene (7.1%), bornyl acetate (7.0%), borneol (6.0%) had cytotoxicity with IC50 value 62.53±1.88 µg/mL in MCF7, 64.79±1.51 µg/mL in HeLa, 62.12±3.11 µg/mL in HepG2.

However, the other species of *Thymus*, especially the endemic ones, have different content and so different biological effects. Previous studies in similar species in terms of chemical compositions concluded the cytotoxicity of the tested species (Table 4). The cytotoxicity of *Thymus pulegioides* L. was tested on Caco-2 and HepG2 cell lines. The IC50 was calculated to be 137.4 µg/mL for the aqueous decoctions and 147.4 µg/mL for the hydro-ethanolic extracts in Caco-2 cells and to be more than 500 µg/mL in HepG2 cells (39). *Thymus kotschyanus* extract and essential oils which had carvacrol (27.8±4.68), thymol (16.8±2.10), thymoquinone (5.4±0.40) were evaluated in HeLa and A549 cell lines, data reported the cytotoxicity with IC50 values varied between ≤0.15-≤0.31 µg/mL (33,40). *Thymus daenensis* extracts and essential oils which had 70.12±8.24% thymol, 4.99±0.68% carvacrol were also reported to have cytotoxicity in different cells,

with IC50 equal to 203.6 µg/mL in HepG2 cells, 4.95 µg/mL in HeLa cells and 1455 µg/mL in human normal lymphocytes (40-42). Other species essential oils such as *Thymbra capitata* (L.) Cav. (Carvacrol 71.4%) , *Thymus caespititius* Brot. (carvacrol 45.5 %, thymol 10.3%) (from different five site) reported to have cytotoxicity with IC50 between 200-250 µg/mL in leukemia (THP-1) cells, while *Thymus mastichina* (L.) L. (1.8-cineole 47.4%, thymol 13.7%), *Thymus pulegioides* L. (geraniol 32.8%, carvacrol 12.4%, thymol 12%), and *Thymus villosus* subsp. *lusitanicus* (Boiss.) Cout. (linalool 65.5%) had lower cytotoxic

Table 4. The cytotoxic effects of some *Thymus* species

Species name	Sample type	Cell line	Results	References
Thymus alpestris Tausch ex A.Kern.	Fresh leaves in phosphate Buffer (1:19, w/w)	Human erythrocytes	Mild cytotoxic effect at 5 and 0.5 mg/mL.	(43)
Thymus pannonicus All.				
Thymus porcii Borbás				
Thymus daennesis Čelak.	Essential oils	Human normal lymphocytes	1455 µg	(42)
		HeLa cells	4.95 µg	
Thymus serpyllum L.	Methanol extract	MCF-10A cells	not cytotoxic	(44)
		MCF-7 cells	509 µg/mL (72 hours exposure)	
		MDA-MB-231 cells	276 µg/mL (72 hrs)	
Thymus kotschyanus Boiss & Hohen	Ethanolic extract	HeLa cells	≤0.31 mg/mL after 24 hrs ≤0.08 mg/mL after 48 hrs	(33)
		A549 cells	≤0.15 mg/mL after 24 hrs ≤0.08 mg/mL after 48 hrs	
Thymus caramanicus Jalas	Essential oil	Human Oral Epidermoid Carcinoma KB Cells	0.44 µL/mL	(45)
	Leaves hydro-ethanolic extract		105 µg/mL	
Thymus daenensis Celak	Herbs hydro-methanolic Extracts	HepG2 cell	203.6 µg/mL	(41)
Thymus daenensis Celak	Essential oil, Final exposure concentration 1%	HeLa cells	≤55% cell death after 72 hrs	(40)
Thymus vulgaris L.			≤75% cell death after 72 hrs	
Thymus kotschyanus Boiss & Hohen			≤75% cell death after 72 hrs	
Thymus broussonettii Boiss.	Essential oil	Human ovarian adenocarcinoma IGR-OV1 parental cell line OV1/P	0.4%	(46)
		Its chemoresistant counterparts OV1/adriamycin (OV1/ADR)	0.39%	
		OV1/vincristine (OV1/VCR)	0.94%	
		OV1/cisplatin (OV1/CDDP)	0.65%	
Thymus pulegioides L.	Decoction	HepG2 cells	>500.00 µg/mL	(39)
		Caco-2 cells	137.7 µg/mL	
	Hydroethanolic extracts	HepG2 cells	>500.00 µg/mL	
		Caco-2 cells	148.7 µg/mL	
Thymbra capitata (L.) Cav.	Essential oil	THP-1 cells	IC_{50} btw 200-250 µg/mL	(47)
Thymus caespititius Brot.			IC_{50} btw 200-250 µg/mL (the effect varies according to the site of collection)	
Thymus mastichina L.			≥450 µg/mL	
Thymus pulegioides L.			≥450 µg/mL	
Thymus villosus subsp. *lusitanicus* (Boiss.) Cout.			≥450 µg/mL	

effects with IC50 ≥450 µg/mL (47). *Thymus serpyllum* extracts, essential oils which had 46.24% thymol, 9.43% thymoquinone, 1.34% borneol and isolated compounds were evaluated for the cytotoxic effects in human normal breast (MCF-10A), MCF-7, MDA-MB-231, HepG2, human colon cancer (HCT-116), PC3, and A549 cell lines; the results concluded the cytotoxic and so the anti-cancer effect of the tested herb (44,48,49).

Thymus brachychilus, according to the chemical content, is believed to have effects similar to the mentioned species in Table 4. For that, the cytotoxic effects of the ethanol extract and the EO of *Thymus brachychilus* were evaluated by MTT assay in A549, MCF-7, and U118-MG cancer cell lines. Results indicated the IC50 values were between 1.64-15.76 µg/mL for the extract and between 68.94-101.2 µg/mL for the EO.

Study Limitations

Since there is no previous study on the content of *Thymus brachychilus* EO and extract, the results obtained could not be compared with other studies. However, in the future, it is planned to carry out analysis studies on the chemical content of samples of *Thymus brachychilus* collected in different months.

Conclusion

The conducted study indicates that ethanolic extract and essential oils of *Thymus brachychilus* have potential antiproliferative properties on human breast adenocarcinoma (MCF-7, HTB-22), human lung adenocarcinoma (A549, CRM-CCL-185), and human GBM cells (U-118 MG, HTB-15) cells and may be used as a candidate for further studies. However, the exact molecular mechanism or mechanisms underlying the anticancer effects of *Thymus brachychilus* need to be clarified in further research. *Thymus brachychilus* ethanolic extract contains polyphenols responsible for its observed anticancer effect in this study. As a result of their antioxidative and potential anticarcinogenic properties, dietary phenolics are currently generating a lot of interest. Additionally, phenolic acids and flavonoids serve as reducing agents, free radical scavengers, and inhibitors of the formation of singlet oxygen. Moreover, components like flavonoids and phenolic acids are crucial in the prevention and treatment of cancer as well as other human disorders.

These results confirm the similarity in the activity with the other species and lighten the possibility to be used in the research and development of new anticancer drugs. This study has shown that 6-methoxyapigenin-7-glucoside, rosmarinic acid, luteolin-7-glucoside, caffeic acid, and kaempferol can all be found in *Thymus brachychilus* as good renewable biosources. The EO of *Thymus brachychilus* reduces the viability of a number of tumor cell lines in a concentration-dependent way. The particular oil constituent are typically held responsible for the oil's activity. One key limitation of the study is that it is yet unclear whether thymol acts alone or in concert with other oil constituents to cause the cytotoxicity against tumor cells that has been reported.

Ethics

Ethics Committee Approval: Our study does not require ethics committee approval.

Informed Consent: Our study does not require informed Consent.

Peer-review: Externally peer reviewed.

Authorship Contributions

Concept: E.Ö.N., M.K., Design: E.Ö.N., M.A., M.K., Data Collection or Processing: E.Ö.N., M.B., İ.D., S.S., M.K., Analysis or Interpretation: E.Ö.N., M.B., İ.D., S.S., M.K., Literature Search: E.Ö.N., M.B., İ.D., S.S., M.K., Writing: E.Ö.N., M.B., İ.D., S.S., M.K.

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