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Article

Comparison of Volatile Constituents, Antioxidant and Antimicrobial Activities of *Thymus leucotrichus* (Lamiaceae) Stem and Leaves Essential Oils from Both Natural Resources and *In vitro* Derived Shoots

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Abstract: This study aimed to compare the volatile constituents, antioxidant and antimicrobial potentials of *Thymus leucotrichus* essential oils (EOs) obtained from natural plants and *in vitro* derived shoots. MS medium fortified 0.5 mg/L kinetin was preferred for an efficient micropropagation protocol and was produced *T. leucotrichus* shoots. The most efficient antioxidant activity was up to IC_{50} 0.823 µg/ml in DPPH, 275.021 mg TE/ml in TEAC, and 25.531 mg GAE/ml in reducing power were found. Total phenolics were determined as 69.249 mg/ml GAE in micropropagated samples while 63.377 mg/ml GAE in natural resources. Thymol was determined as the main volatile compound of both natural plants and *in vitro* derived shoots. The EOs showed a strong antimicrobial effect on selected seven gram-positive, nine gram-negative bacteria, seven gram-negative aquatic bacteria and a fungus. The highest disc diffusion value was obtained as 44.3 mm for *P. damselae* subsp. *damselae* from *in vitro* derived shoots. In both groups of tested EOs, the most effective MICs were obtained with 12.21 µg/ml against *V. parahaemolyticus.* The novelty of this report is the first comprehensive and comparative study on volatile constituents, antioxidant, and antimicrobial properties of naturally growing plants and *in vitro* derived shoots of *T. leucotrichus*.

Key words: Antimicrobial, biological activity, essential oil, micropropagation, *Thymus leucotrichus.*

Introduction

Some plants have significant amounts of essential oils (EOs) include valuable phytochemicals. EOs are rich in antimicrobial and antioxidant substances that can be used to improve the functional properties of foods or they can be an alternative to synthetic preservatives traditionally applied in food [1]. Due to the antimicrobial and antioxidant properties, there is an increasing interest to the plants in their use as alternative medicine. Among these uses, antibacterial, antifungal, antiviral, antioxidant, anticancer, antiparasitic, muscle relaxant, expectorant and aphrodisiac effects are the most prominent [2-4].

However, despite all these benefits, unconscious use of the plants causes dramatic problems related to the contraction in their populations and the survival of their generations. To avoid these problems, scientists have adopted alternative production methods such as tissue culture techniques. Among these techniques, micropropagation is the most effective method for *ex situ* conservation and rapid multiplication of valuable medicinal and aromatic species [5,6].

Micropropagation is preferred for the biosynthesis of active phytochemicals during *in vitro* culture, and this method increases the levels of some crucial metabolites at a significant level [7-9]. Moreover, it is possible to protect natural populations of plants by not using the natural

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resources and so keep nature's beauty intact thanks to this technique [10,11].

Lamiaceae family is represented with 7886 species belonging to 245 genera all over the world [12]. This family is the fourth largest family based on the species number and is characterized by 45 genera, 565 species and 735 taxa in the flora of Turkey [13,14]. The genus of *Thymus* is represented with 59 taxa in Turkey. Among these taxa, the rate of endemism is 53 %[15]. *Thymus leucotrichus* Hal. is a medicinal and aromatic plant in the form of a perennial semi-shrub belonging to this genus and commonly called "Dag kekigi" in Turkey. This species is widely preferred as herbal tea, flavoring agents, reducing high cholesterol agents, relieving abdominal and throat pains because of the high phytochemical contents [7,16,17]. The high rate of EOs of this species increases the pharmacological effects mentioned above and therefore causes more ethnobotanical consumption. Especially in the populations of this species consumed as herbal tea, there has been a serious decrease in recent years due to uncontrolled collection and grazing pressure.

EOs of *T. leucotrichus* are obtained by steam distillation from stems and leaves segments. The four most used antioxidant methods, volatile constituents, and antimicrobial activity of *T. leucotrichus* EOs in two different grown samples as natural grown and *in vitro* derived shoots have been comparatively revealed in this study. Also, this study is the first report on marine bacterial strains with this plant species. There is only one study on the *in vitro* antimicrobial activity of leaf extracts of this important species, which is evaluated ethnobotanically [18]. To the best of our knowledge, this report is the first comprehensive and comparative study on volatile constituents, antioxidant potentials, and antimicrobial properties of naturally growing plants and *in vitro* derived shoots of *T. leucotrichus*. The data to be obtained from this study will also constitute the basis of preserving the continuity of the species evaluated in terms of biological activity.

Material and methods

Plant material

About 100 seeds of *T. leucotrichus* were collected from the indigenous populations of Toplukonak village -Sebinkarahisar (40° 232 08.393 N, 38° 352 06.353 E; 1978 m), Giresun, Turkey, between August and September 2019 and authenticated by Dr. Mutlu Gultepe, and deposited (Herbarium number: KTUB, CUCE & GULTEPE 798) at the Department of Biology, Karadeniz Technical University (Trabzon-Turkey). The seeds were stored in the dark at 4°C until transferred to the culture medium. The tested combined aerial above-ground parts including stem and leaves of the *T. leucotrichus* were randomly collected in the same locality and at the same time of the indigenous natural population during 2019 and 2020. For this purpose, approximately 250 g of fresh samples were collected each year. The collected fresh parts and *in vitro* derived shoots of the *T. leucotrichus* were put dry in a well-ventilated place without direct sunlight to prevent bioactive components from being affected during the drying phase. The dry weight amounts obtained per fresh weight of the samples were determined and stored until isolation processes were performed.

Experimental

Culture conditions, surface sterilization, seed germination and shoot multiplication

The methods described by Bekircan *et al*.[7] were used in the production of *in vitro* derived shoots with some minor modifications.

Briefly, seeds were treated with 5 % sucrose solution containing a few drops of commercial bleach for 12 h before surface sterilization. The solution was removed, and seeds were sterilized with 36.5 % (v/v) hydrogen peroxide solution (H_2O_2) for 30 min. The seeds were cultured on approximately 35 ml cytokinin-free MS basal media [19] in 98.5 × 59 mm glass containers for germination. Microshoots obtained from germination cultures were cut nearly 10 mm and used as an explant for shoot multiplication trials. MS supplemented with 0.5 mg/L kinetin (KIN) was preferred as a shoot proliferation medium according to the EO content results obtained from the study of Bekircan *et al*.[7]. The all-media pH was adjusted to 5.8 with 1 N NaOH or 1 N HCl before autoclaving at 121 °C, 118 kPa for 20 min.

All cultures were incubated at 24 ± 1°C under cool daylight fluorescent lamps for a 16/8 h photoperiod with a photosynthetic photon flux density of 50 µmol/m^2 s for 4 weeks in acclimatization room conditions. After four weeks, the healthy shoots were removed from the cultures media and were dried in a well-ventilated place without direct sunlight.

Isolation of the essential oil (EO)

Air-dried and ground aerial parts of the *T. leucotrichus* samples were subjected to hydrodistillation for 4 h using a Clevenger-type apparatus (Thermal Laboratory Equipment, Turkey). The extracts dried over anhydrous sodium sulphate. After filtration, EOs were stored at +4°C until tested and analyzed.

Determination of volatile compounds

Gas chromatography-Mass Spectrometry (GC/MS) was used for the identification of EO volatile compounds of thyme samples. The solid-phase microextraction (SPME) method was adopted to extract components. SMPE procedure was needed 100 g of samples had blended for 3 min without water. After blending, 10 g from obtained mush was poured into headspace vials and put septa on the vials. Along 30 min, volatile components of thyme samples were collected through Supelco 57348 2 cm, 50/30 µm DVB/Carboxen/PDMS Stable-Flex. At the end of isolation, SPME was inserted into GC/MS (Shimadzu QP2010) for detection of the peaks. GC apparatus had RTX-5M column (0.25 mm I.D., 0.25 mm film thickness, Restek, USA), FID detector and PAL AOC-5000 plus autosampler. The conditions of analysis were as 1.0 ml/min flow rate of helium for carrier gas and 70 eV ionization voltage. Injector and detector temperatures were as follows: 25°C and 230°C. Column temperatures were ramped from 60°C to 240°C at 5°C/min. Under these conditions, standard volatile compounds were co-injected to GC/MS to confirm the assignment with the samples. Volatile compound peaks of the sample were retained along 62 min and compounds were determined by the mass spectral library of the software. Essential oil compounds quantified by peak area. Quantification was done by the external standard method using calibration curves obtained from GC analysis of representative authentic compounds.

Antioxidant activity

The EO of *T. leucotrichus* was examined in terms of "radical inhibition capacity" by DPPH scavenging antioxidant activity, Trolox equivalent antioxidant capacity, reducing power and total phenolic contents.

Determination of total phenolic contents

The EO extracted from samples was used for investigating total phenolic compounds. Enough amount of sodium carbonate (50 g) (Na_2CO_3) (Sigma-Aldrich, Germany), was dissolved in 250 ml distilled water to prepare %20 Na_2CO_3 solution. Gallic acid (0.1 g) (Sigma-Aldrich, Germany) was dissolved in 2 ml ethanol and added to 98 ml distilled water to prepare 1 mg/ml gallic acid solution. Gallic acid solution was used to obtain a 6-point calibration graph. For dissolving the EOs of two different specimens as natural and *in vitro* derived shoots; 20 µL of EO was mixed with 80 µL dimethyl sulfoxide (DMSO) (Carlo Erba) and 300 µL distilled water. Then it was shaken and 50 µL from this solution was pipetted in a test tube. Folin-ciocalteu (Sigma-Aldrich, Germany) solution (100 µL) and Na_2CO_3 solutions (500 µL) were added into the test tube. A cuvette filled with 100 µL folin-ciocalteu solution and 500 µL Na_2CO_3 solutions to measure the blank sample in a spectrophotometer (Shimadzu UV-1601). Each sample was measured in triplicate compared to the blank sample in the spectrophotometer. The total phenolic content of EO was measured at 760 nm and was expressed as mg gallic acid/ml extract using the equation obtained from the standard graph prepared using different concentrations of gallic acid ($y = 0.0089x + 0.0362$, $R^2 = 0.9924$).

DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity

DPPH (*2,2-diphenyl-1-picrylhydrazyl*) is a reducing compound whose purple color disappears at the end of the reaction. To determine DPPH scavenging activity, 0.039 grams of DPPH

(Sigma-Aldrich, Germany) dissolved with 100 ml methanol in a volumetric flask. Trolox (Sigma-Aldrich, Germany) solution was prepared as a 1 mg/ml standard compound. The control sample was obtained by mixing with 500 µL DPPH solutions and 3500 µL methanol. The EOs (20 µL from each sample) were dissolved with the mixtures of 380 µL DMSO and 600 µL methanol. Then it was shaken. For each sample, mixtures in three different concentrations were prepared to measure in a spectrophotometer. All samples' absorbance was measured spectrophotometrically. The inhibition capacity of radicals by DPPH was measured at 517 nm and was expressed as mg Trolox equivalent (TE)/ml extract.

ABTS scavenging activity

Trolox [*(±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid*] is water-soluble equivalent of vitamin E. For the determination of Trolox equivalent antioxidant capacity (TEAC) by ABTS [*(2,2'-azino bis (3-ethylbenzothiazoline 6-sulfonate)*], the following procedures have been performed. 0.15 g of ABTS dissolved in 100 ml of distilled water and mixed on a magnetic stirrer for 30 min. Meanwhile, 0.04 g of potassium peroxodisulphate was gradually added to the mixture. 20 µL of each EO was mixed with 30 µL DMSO and 950 µL methanol to dissolve the oils from two different samples. As a control, 3000 µL methanol + 1000 µL ABTS were mixed and ABTS solution was obtained. Solution of Trolox (as standard compound) at a concentration of 1 mg/ml was used.

Control and experimental samples were kept in the dark for 30 min. Then, they were measured in triplicate with the spectrophotometer on the wavelength of 734 nm.

Reducing power

The reduction potential of the EOs extracted from samples was quantified by the method of Oyaizu [20]. EOs were dissolved in DMSO at %5 concentration and 50 µl of each prepared solution was mixed with 700 µl distilled water, phosphate buffer (1 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (1 ml, 1 %). The mixtures were incubated at 50°C for 20 min. Then, the reaction was terminated by adding 1 ml of trichloroacetic acid (10 %). These solutions were mixed with $FeCl_3$ (250 µL, 0.1 %), and the absorbance was measured at 700 nm in a spectrophotometer against a blank sample. The reduction capacity of the samples was determined as (GAE/ml) using the equation obtained from the standard graph prepared using different concentrations of gallic acid ($y = 0.0338x + 0.063$, $R^2 = 0.997$).

Antimicrobial activity

Microbial strains

The antimicrobial activities of the natural and micropropagated *T. leucotrichus* EOs were individually tested against seven gram-positive (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Staphylococcus epidermidis* ATCC 12228, *Bacillus thuringiensis* ATCC 10792, *Enterococcus faecalis* ATCC 29212, *Streptococcus faecalis* ATCC 9790, *Listeria monocytogenes* ATCC 43251) and nine gram-negative (*Klebsiella pneumoniae* ATCC 13883, *Escherichia coli* ATCC 25922, *Serratia marcescens* ATCC 27117, *Salmonella typhimurium* ATCC 10708, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATCC 6896, *Salmonella enterica* ATCC 13076, *Aeromonas sobria* ATCC 43979, *Aeromonas hydrophila* ATCC 7966) bacteria and a fungus *(Candida albicans* ATCC 10231).

Additionally, the following species of bacteria which are isolated from naturally infected sea bass (*Dicentrarchus labrax)* were also used in the study; *Vibrio vulnificus* (KF443055), *Vibrio ponticus* (NR029032.1)*, Aeromonas veronii* (KF443052), *Photobacterium damselae* subsp. *damselae* (KF443044) and confirmed previously by Uzun and Ogut [21]; *Vibrio anguillarum* (MH 036330.1), *Vibrio parahaemolyticus* (EF684942.1), *Pseudomonas putida* (KY425616.1) confirmed previously by Uzun [22].

Disc diffusion method

Clinical and Laboratory Standards Institute (CLSI) [23] and The European Committee on Antimicrobial Susceptibility Testing (EUCAST) [24] protocols were preferred to determine the zone diameter of microorganisms on agar. Briefly,

bacterial strains were cultured overnight at 37°C in Mueller Hinton Broth (MHB) on a shaker incubator except for marine ones. Marine strains were cultured at 24°C. Yeasts were cultured overnight at 30°C in Sabouraud Dextrose Broth (SDB). The EOs of natural and *in vitro* derived shoots of *T. leucotrichus* were dissolved in 20 % dimethylsulphoxide (DMSO) to a final concentration of 50 mg/ml and sterilized. Antimicrobial tests were then carried out by the disc diffusion method using 100 µL of suspension containing 1 x 10^8 CFU/ml bacteria, 1 x 10^6 CFU/ml yeast spread on Mueller Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) media, respectively. A suspension of the tested microorganism was spread on the solid media plates and the discs (6 mm in diameter) were impregnated with 50 mg/ml extract (500 µg per disc) placed on the inoculated agar. DMSO (20 %) was tested as a negative control. Ofloxacin (10 µg per disc), sulbactam (30 µg) + cefoperazone (75 µg); (105 µg per disc) and/or netilmicin (30 µg per disc) obtained from Oxoid (Basingstoke, UK) were used as positive reference standards to determine the sensitivity of each strain tested. The inoculated plates were incubated at 37°C for 24 h for pathogenic bacterial strains, 24°C for 24 h marine strains and 30°C for 48 h for yeasts. Antimicrobial activity was calculated by measuring the transparent inhibition zone around the discs against the test organisms. In this experiment, each trial was repeated thrice.

Microdilution assay

Minimum inhibitory concentration (MIC) values were determined for the bacterial strains which were sensitive to the extracts in the disc diffusion assay. Inocula of the bacterial strains were prepared from 12 h MHB broth cultures, and suspensions were adjusted to 0.5 McFarland standard turbidity. The extracts dissolved in 20 % dimethylsulphoxide (DMSO), were first diluted to the highest concentration (500 µg/ml) to be tested, then serial two-fold dilutions were made and MIC values of the extracts were determined by the broth microdilution method. A dilution of 1/10 bacterial suspension was prepared from 1x10^8 CFU/ml bacteria and then 5 µL was added to each well from this dilution to make the final concentration 5x10^5 CFU/ml per well. Microbial growth was determined by reading the respective absorbance (Abs) at 600 nm using an ELx 800 universal microplate reader (Biotek Instrument Inc, Highland Park, VT, USA) and confirmed by plating 5 µL samples from clear wells on MHA medium. Wells containing individually only bacterial suspension and EOs were considered as a control group. The extracts were carried out in duplicate to each organism. The MIC was the lowest concentration of extract where no visible growth was seen in the wells.

Statistical analysis

All antimicrobial data were statistically evaluated to the analysis of variance (ANOVA) followed by Duncan multiple range tests by using software version of SPSS 21.0 Inc. (Chicago, IL, USA). LSD tests were performed using IBM SPSS Statistics 22 (for Win, Release 22.0.0.0, 2013) program for statistical analysis of antioxidant results. The statistical significance of differences between treatments was evaluated less than $P < 0.05$ and was specified as mean ± standard deviations (SD) of three applications.

Results

Volatile compositions

As a result of GC/MS analysis, 52 different volatile components were determined in the total ion chromatograms and 16 of them were named (Table 1). Among these volatile components, thymol was the main component with 37.01 % and 36.08 %, both in plants grown in natural environments and shoots obtained by micropropagation. *p*-cymene (21.55 %), γ-terpinene (8.63 %), and myrcene (4.33 %) were the other highest main volatile constituents. In contrast, the other highest volatile components in shoots obtained *in vitro* were γ-terpinene (10.08 %), *p*-cymene (8.74 %), and β-bisabolene (8.91 %). The lowest volatile component of the natural plants was β-bisabolene (0.55 %) followed by α-pinene and eucalyptol (1,8-cineole) with 1.17 % and 1.72 %. The minor volatile component of *in vitro* derived shoots was α-pinene with 2.48 %. Thymol methyl ether, thymoquinone, germacrene-d, and

Table 1. Volatile constituents of naturally grown plants and *in vitro* derived shoots

No.	Compunds	RT	$RI_{(Obs)}$	$RI_{(Lit)}$	Natural plants concentration (%)	*In vitro* derived shoots concentration (%)
1	α-Pinene	8.5	937	939	1.17	2.48
2	3-Octanone	10.8	986	984	2.37	2.49
3	Myrcene	10.8	990	990	4.33	5.44
4	α-Terpinene	11.7	1020	1017	1.80	-
5	Limonene	12.2	1032	1029	3.35	-
6	p-Cymene	12.1	1026	1026	21.55	8.74
7	Eucalyptol (1,8-Cineole)	12.2	1033	1032	1.72	-
8	γ-Terpinene	13.4	1060	1059	8.63	10.08
9	Thymol methyl ether	19.2	1235	1235	-	-
10	Thymoquinone	19.7	1259	1252	-	3.85
11	Thymol	21.2	1292	1290	37.01	36.08
12	Carvacrol	21.3	1299	1299	3.77	3.63
13	β-Caryophyllene	24.6	1421	1419	3.01	4.22
14	Germacrene D	26.3	1486	1485	-	3.74
15	Bicyclogermacrene	26.5	1503	1500	-	-
16	β-Bisabolene	27.0	1508	1511	0.55	8.91
	Monoterpenenes (Sr. No.1, 3-9, 11, 12)				42.13	41.80
	Sesquiterpenes (Sr. No. 13-16)				2.63	16.07
	Ketone (Sr. No. 2)				2.37	2.49
	Quinone (Sr. No. 10)				-	3.85
	Total identified				**136.39**	**153.87**
	Minor and unidentified				10.74	10.34

Given as percentage in 1.0 µL sampling

RT = Retention time, Retention index (RI) values are calculated from retention times relative to that of *n*-alkanes on the RTX-5M column

bicyclogermacrene were not determined in natural plants, followed by α-terpinene, limonene, eucalyptol (1,8-cineole), thymol methyl ether, and bicyclogermacrene in shoots derived *in vitro* (Table 1).

Antioxidant activity

Radical scavenging activities as DPPH and ABTS for EOs of *T. leucotrichus* were represented in Table 2. In general, higher antioxidant activity was obtained in naturally growing plants among the EOs of thyme samples. In this context, the DPPH and ABTS results were agreed with each other. As observed in Table 2, DPPH radical scavenging activity was shown as Trolox equivalent (TE) and as Half-maximal Inhibitory Concentration (IC_{50}) value. The IC_{50} value was the concentration of the antioxidant that yields half-maximal scavenging activity. DPPH value was obtained as 62.528 mg and 24.517 mg for naturally growing plants and *in vitro* derived shoots EOs, respectively in terms of Trolox equivalent. Significant statistical differences have emerged between the DPPH data of these two applications ($P < 0.05$). In connection with DPPH results, IC_{50} values were determined as 0.823 µg and 2.161 µg, respectively for taken from indigenous population plants and *in vitro* derived shoots EOs. This difference was found to be statistically significant (Table 2).

ABTS data of EO samples obtained by two different methods demonstrated a similar effect with DPPH results and was given in Table 2. The

EOs of natural plants and *in vitro* derived shoots had different values in terms of the Trolox equivalents of ABTS. The highest ABTS value in terms of Trolox equivalent was calculated from EOs obtained from natural plants to be 275.021 mg. This value was 256.638 mg in EOs of *in vitro* derived shoots. These results created statistically significant differences ($P < 0.05$). In terms of importance, ABTS IC_{50} values of EO samples belonging to natural plants were found to be more effective than the EO results of shoots obtained by micropropagation with 0.1778 µg ($P < 0.05$, Table 2).

Total phenolics

Yield performance (v/w) obtained from natural plants and *in vitro* derived shoots were 3 % and 2 %, respectively (Table 3). Despite that naturally growing plants EOs of *T. leucotrichus* gave higher values in the analyzes for antioxidant activity, *in vitro* derived shoots EOs of *T. leucotrichus* had more effective results in total phenolic content analyzes. Total phenolic contents were found at 63.377 mg GAE and 69.249 mg GAE from EOs of naturally growing plants and *in vitro* derived shoots, respectively (Table 3). As in Table 3, on the contrary of total phenolic data, reducing power by ferric chloride solution resulted in 25.531 mg and 18.880 mg GAE for EOs of naturally growing plants and *in vitro* derived shoots, respectively. As a result of this method, EOs obtained from naturally growing plants are more efficient than EOs obtained from *in vitro* derived shoots ($P < 0.05$).

Antimicrobial activity

Although the effectiveness of EOs against microbial pathogens has been known since ancient times, there are not many reports in the literature regarding the evaluation of the plants from which these valuable phytochemicals are obtained within the scope of microbial control by protecting their natural populations. This study is the first crucial report to investigate the antimicrobial effect of *T. leucotrichus* EOs both grown in the natural populations and produced by tissue culture methods. EOs obtained from natural and *in vitro* derived shoots demonstrated considerable anti-microbial activity against tested all of the clinical and aquatic pathogens as well as fungus according to antimicrobial activity test results. However, disk diffusion and MIC values differed in EOs obtained from natural and *in vitro* derived shoots (Table 4). These data are summarized by the result

Table 2. DPPH and ABTS scavenging activities of *T. leucotrichus* essential oils

Essential oil	DPPH radical (mg TEs/ml)	DPPH IC_{50} (µg/ml)	ABTS (mg TEs/ml)	ABTS IC_{50} (µg)
Natural	62.528±0.49a	0.823±0.007a	275.021±4.99a	0.1778±0.01a
Micropropagated	24.517±0.99b	2.161±0.079b	256.638±4.01b	0.1909±0.02b

Values with different letter(s) in the same column(s) were significantly different ($P < 0.05$)
TEs, trolox equivalents

Table 3. Total phenolic content and reducing power of *T. leucotrichus* essential oils

Essential oil	Yield (% w/w)	Total phenolics (mg GAE/ml)	Reducing power (mg GAE/ml)
Natural	3	63.377±0.81b	25.531±1.03a
Micropropagated	2	69.249±1.64a	18.880±0.36b

Values with different letter(s) in the same column(s) were significantly different ($P < 0.05$)
GAEs, gallic acid equivalent

Table 4. Zones of growth inhibition (mm) showing antimicrobial activity of natural and micropropagated *Thymus leucotrichus* essential oils

Bacteria	Disc diffusion		MIC		N.C.	Standard antibiotic discs		
	Natural (mm)	Micropropagated (mm)	Natural (µg/mL)	Micropropagated (µg/mL)		OFX	NET 30	SCF 30
Gram negative bacteria								
K. pneumoniae	17.7±0.3bc	15.1±0.6d	195.31	195.31	-	16.7±0.6c	18.1±0.7b	21.6±0.8a
E. coli	20.1±0.5c	17.4±0.5d	48.83	97.66	-	30.6±1.1a	24.6±0.8b	19.0±0.5c
S. marcescens	20.6±0.7c	19.5±0.5c	48.83	195.31	-	26.6±0.7b	20.0±0.6c	28.4±0.9a
S. typhimurium	20.2±0.6c	17.4±0.4d	195.31	390.63	-	29.3±0.2a	27.1±1.5b	19.2±0.0c
P. aeruginosa	20.7±0.6c	19.4±0.0d	97.66	97.66	-	29.8±0.1a	20.6±0.3c	25.4±0.5b
P. vulgaris	17.7±0.7c	14.3±0.3d	390.63	781.25	-	26.1±0.6a	18.6±0.8c	24.2±0.9b
S. enterica	20.6±0.5c	18.2±0.3e	195.31	97.66	-	28.7±0.5a	27.4±0.2b	19.5±0.7d
A. sobria	38.1±0.6b	41.3±0.8a	97.66	195.31	-	33.7±0.8c	30.0±0.0d	26.4±0.4e
A. hydrophila	41.3±1.0a	32.2±0.0c	48.83	97.66	-	35.2±0.0b	31.3±0.4d	26.4±0.8e
Gram negative aquatic bacteria								
V. vulnificus	40.3±0.6a	41.2±0.3a	12.21	48.83	-	35.6±0.4b	32.1±1.0c	26.4±0.5d
V. ponticus	19.1±0.6d	23.3±0.5c	12.21	48.83	-	29.5±0.8a	26.0±0.0b	19.6±0.6d
V. anguillarum	37.2±0.2b	42.7±1.0a	48.83	97.66	-	34.3±0.6c	30.0±1.0d	26.4±0.8e
V. parahaemolyticus	33.3±0.5a	30.8±0.7b	12.21	12.21	-	29.5±0.0c	27.2±0.4d	20.4±0.5e
P. putida	18.6±0.0c	14.2±0.8d	390.63	781.25	-	27.1±0.7a	19.6±0.6c	24.1±0.9b
A. veroni	32.7±0.5b	36.3±1.1a	48.83	48.83	-	28.5±0.0c	25.2±0.5d	19.3±0.3e
P. damselae subsp. *damselae*	43.7±0.6a	44.3±0.8a	97.66	48.83	-	34.8±1.1b	31.0±0.0c	26.7±0.0d
Gram positive bacteria								
S. aureus	21.7±0.3a	20.2±0.4b	48.83	97.66	-	22.1±1.0a	21.6±0.5a	18.1±0.6c
B. subtilis	20.7±0.7b	17.6±0.5c	48.83	97.66	-	28.4±1.4a	27.3±1.5a	20.5±1.5b
S. epidermidis	16.9±1.0c	14.4±0.7d	97.66	195.31	-	29.5±1.5a	24.2±1.1b	25.7±0.0b
B. thrungiensis	17.2±0.9c	15.2±1.0d	97.66	195.31	-	25.4±0.5a	19.6±1.0b	17.6±0.8c
E. faecalis	14.3±1.1c	14.3±0.5c	195.31	390.63	-	19.3±0.5b	21.2±1.3a	14.3±0.7c
S. faecalis	16.2±0.6b	12.8±1.0c	195.31c	390.63	-	19.9±0.5a	13.6±0.0	19.9±0.7a
L. monocytogenes	20.3±1.4bc	19.2±0.6bc	97.66	97.66	-	26.4±1.4a	20.7±0.8b	18.5±0.5c
Fungi								
C. albicans	36.7±0.5a	28.8±0.0b	195.31	390.63	-	20.5±0.5c	19.8±0.6c	18.7±0.0d

Diameter of inhibition zone including disc diameter of 6 mm by the agar dics diffusion method at a concentration of 10 µL of oil/disc. Ofloxacin (10 µg/disc) (OFX), netilmicin (30 µg/disc) (NET30), sulbactam (30 µg) + cefoperazone (75 µg); (105 µg per disc) (SCF) were used as reference antibiotics.Dimethyl sulfoxide (DMSO) (20 %) was used as negative control (N.C.). MIC (minimal inhibition concentration) was calculated as µg/ml. The values are the average ± standard deviation of three determinations ($p < 0.05$)

that EOs of plants obtained from the natural environment is more effective in terms of disk diffusion and MIC values than EOs of plants obtained from tissue cultures. The MIC values of all tested microorganisms were ranging from 12.21 to 781.25 µg/µL. In general, gram-negative aquatic bacteria were more sensitive to the EOs of this species obtained by both methods than other gram-positive and gram-negative bacteria. Both EOs obtained by two different methods showed the same effect against *V. parahaemolyticus* with 12.21 µg/ml in terms of MIC values. The other most effective MICs of EOs obtained from naturally growing plants of *T. leucotrichus* were 12.21 µg/ml for *V. ponticus*, *V. vulnificus* and 48.83 µg/ml for *E. coli*, *S. marcescens*, *A. hydrophila, V. anguillarum, A. veroni, S. aureus, B. subtilis*. Similar MIC values of EOs obtained from shoots produced *in vitro* was 48.83 µg/ml for *V. vulnificus*, *V. ponticus*, *A. veroni*. A surprising result was that EOs obtained from shoots produced *in vitro* were 50 % more effective on *P. damselae* subsp. *damselae* than EOs obtained from naturally grown plants (Table 4). Among the tested microorganisms, *P. vulgaris* and *P. putida* bacterial strains were found to be less sensitive in both treated EOs samples against other bacterial strains. For *C. albicans*, MIC values were calculated as 195.31 and 390.63 for EOs obtained from natural plants and *in vitro* derived shoots, respectively.

Discussion

Volatile compositions

A lot of compounds were identified in our study through GC/MS. The number of compounds (52) was compatible with Bekircan *et al*.[7]. In similar reports, 35 compounds were identified in EO of *Cymbopogon khasianus* Hack.[25], 38 compounds were identified in EO of *Artemisia vulgaris* leaves [26] and 43 compounds were identified in EO of *Citrus limon* L. Burmf peel [27].

Essential oils obtained from different plant sources show significant differences in oil composition. While thymol, *p*-cymene and γ-terpinene were the most abundant compounds in EO of *T. leucotrichus* samples, methyl eugenol and myrcene were the most abundant in EO of *Cymbopogon khasianus* Hack.[25], 1,8-cineole and camphor were the most abundant in EO of *Artemisia vulgaris* leaves [28], limonene was the most abundant in EO of *Citrus limon* L. Burmf peel [27]. Limonene is the characteristic compound of citrus peels just as thymol and carvacrol are the characteristic compounds of thyme. In terms of the common compounds, a variety of EOs contains, these substances generally were found to be the higher amount in *T. leucotrichus* samples except for compounds such as limonene, eucalyptol (1,8-cineole) and bicyclogermacrene. A single method reflects the chemical reactivity under specific conditions and it may be misleading to generalize the data as an indicator of "total antioxidant activity". There are more than twenty methods given in the literature for the measurement of antioxidant capacity. It is possible to use all methods to determine the antioxidant capacity though the relationship may not be linear between the results of each method. The molecular diversity of antioxidant substances in the samples may prevent getting linear results between the methods [28]. Determining antioxidant activity is highly dependent on the selected method. Therefore, a "multiple analytical method approach" was adopted in this study; namely *"DPPH scavenging activity", "Trolox equivalent antioxidant capacity (TEAC) by ABTS"* and *"reducing antioxidant power"*.

DPPH scavenging activity

Nowadays, many studies on the antioxidant activity of phenolic compounds and EOs of different plant species are available [1,29,30]. Many scientists have focused on the volatile compounds of *Thymus* species belonging to the Lamiaceae family [31-33]. Based on the literature data, when the DPPH radical scavenging activity of *T. leucotrichus* was compared with the report *T. vulgaris* reported by Aljabeili *et al*.[34], it was determined that *T. leucotrichus* showed a much stronger effect than *T. vulgaris*. Regarding DPPH inhibition, our results agreed with Viuda-Martos *et al*.[35]. *T. vulgaris* in the above-mentioned study were slightly more effective than our micro propagated samples EO and were slightly less effective than the natural sample of *T. leucotrichus* EO.

In another study conducted with *T. vulgaris* leaves using trolox standard material, DPPH antioxidant activity was approximately 30 to 78 times higher than our results [31]. Kulišic *et al*.[36] reported that *T. vulgaris* L. needed the concentration of 300 µg/ml and *T. serpyllum* L. needed the concentration of 450 µg/ml to reach 50 % of DPPH inhibition. The results obtained from above mentioned study differ from the study we conducted. Because the IC_{50} value of EOs obtained from shoots grown *in vitro* was nearly 139 times more effective than the result of Kulišic on *T. vulgaris* (Table 1). This result was not surprising. Because the many researchers who conducted antioxidant studies on the EOs of *Thymus* species obtained less effective results than the EOs of both natural plants and *in vitro* propagated shoots in our study [37-40]. However, the highest IC_{50} value we obtained from *T. leucotrichus* is 56 % less effective than the value obtained from *T. pectinatus* [41]. This can be attributed to the greater effect of *T. pectinatus* species in terms of DPPH radical scavenging activity. Our results in agreement with Nikolic *et al*.[42], in which the DPPH IC_{50} value of *T. serpyllum* was 0.96 mg/ml and the DPPH IC_{50} value of *T. algerensis* was 0.96 mg/ml.

Trolox equivalent antioxidant capacity (TEAC) by ABTS

In terms of ABTS radical scavenging activity, EOs of *T. leucotrichus* in our study compared with reported by Dorman *et al*.[43] and Aljabeili *et al*.[34], EOs of the species in our study showed a very high antioxidative effect from *T. vulgaris* EOs. According to the study of Dorman *et al*.[43], *T. vulgaris* had 10.860 mg/ml ABTS antioxidant activity as Trolox standard equivalent. Compared to this result with our study, *T. leucotrichus* showed at least 25 times more decoloring effect on ABTS for wild-growing (natural) samples and 23 times more for micro-propagated samples than *T. vulgaris.* However, a reduction of ABTS radical cation was found 375 mg TE/ml for *T. daenensis* [44]. This value was nearly 1.4 times higher than natural plants EOs and nearly 1.5 times higher than propagated shoots EOs of *T. leucotrichus* that in our study. It is reported that a significant proportion of thymol and carvacrol compounds in *Thymus* species are associated with the obtained high antioxidant activity [31,34,45]. Based on this information, it was possible that *T. leucotrichus*, which was rich in terms of thymol and carvacrol content, had an effective ABTS IC_{50} value. Many researchers have obtained different IC_{50} values based on tested plant species. ABTS IC_{50} value was determined 0.896 mg/ml for EO of *T. algeriensis* [46] and 0.463 µg/ml for EO of *T. capitata* [47]. These results may differ depending on the species, natural environment in which the species live, environmental factors, flowering stage, collecting time of samples, drying, storage and isolation methods [2,48,49]. Depending on all these factors, obtained results in different studies are likely to differ from each other. The isolation method used in antioxidant studies and the obtained plant extract may cause differences in the results even if the plant species is the same. Ulukanli *et al*.[50] investigated *T. leucotrichius* (Lamiaceae) extracts scavenged 50 % of the DPPH radical. The obtained results were 109.72 µg/ml for acetone extract and 43.53 µg/ml for methanol extract.

Reducing antioxidant power

Antioxidant compounds are oxidized in the course of oxidation-reduction reactions and cause the reduction of some other compounds in the medium. In this respect, they are responsible for the reduction of iron ions. Therefore, it would be possible to determine the antioxidant activity from the reduction level in iron [51]. To the best of our knowledge, there was no other report in the same conditions as our study. Because of this, evaluations were made by making comparisons with similar studies in the literature regarding the reduction potential. In a study BHA was used as a standard antioxidant, the reduction potential of *T. algeriensis* samples was 1.387 µg/ml [46]. For both of our samples, the value we obtained with gallic acid was higher than this value. The reducing power activity related to iron chelation was 0.025 mg ascorbic acid equivalents/mg EO [34]. The fact that this value is quite low compared to our values can be attributed to the difference in the method used by the researchers and the use of ascorbic acid as a standard substance.

Total phenolic content

Gülçin *et al*.[29] declared that the high level of total phenolics were related to the high level of antioxidant effect. However; according to Ardag [25], between the observed antioxidant activity and the total phenolic content of the plants, the correlation may not be observed [43,52,53]. It is recommended that folin-ciocalteu has been used in determining the reducing capacity of the antioxidant [54]. In the study carried out with folin-ciocalteu, the total phenol compounds of *T. vulgaris* collected in five different areas of Italy ranged from 0.078-0.165 mg GAE/mg of EO [55]. Again in the total phenolic study on *T. vulgaris* grown in Egypt, the researchers calculated the total phenol value as 0.177 mg GAE/mg EO. While these results show similarities and differences according to *T. leucotrichus* (natural = 0.0715 mg GAE/mg, micro propagated = 0.0785 mg GAE/mg), they show that there may be differences even within the same species.

Antimicrobial Activities

In recent years, antioxidant and antimicrobial studies have focused on plants due to their bioactive metabolites and natural resources [56,57]. Among these valuable bioactive metabolites, EOs are the first to inhibit bacterial and fungal growth [3,58,59]. In addition to revealing the antimicrobial properties of EOs belonging to different plant species [60,61], researchers have especially investigated the antimicrobial effects of members of the genus *Thymus*, which has rich EO content, belonging to the Lamiaceae family [62,63]. However, there is no study on the comparative determination of the antimicrobial activities of the EOs of *T. leucotrichus* grown both in natural and *in vitro*.

The results obtained when evaluated in terms of essential oil components were consistent with the report of Bekircan *et al*.[7]. The only difference was that 2.49 % 3-octanone was obtained in the shoots obtained from the culture media in our study. Bekircan *et al*.[7]. reported that thymol is the main component in essential oil compositions obtained from grown in natural plants and in vitro derived shoots. This finding is supported by the essential oil results of other *Thymus* species obtained from natural and *in vitro* [64,65].

In a study investigating the antibacterial properties of the EOs of *T. longicaulis* and *T. pulegioides*, the researchers tested the EOs of both species on gram-positive and gram-negative bacteria such as *Streptococcus faecalis, Staphylococcus aureus, Bacillus subtilis, Bacillus cereus, Proteus mirabilis, Salmonella typhi* Ty2, *Enterobacter coli* and *Pseudomonas aeruginosa*. It has been reported that EOs of both species at a concentration of 10 mg/ml give inhibition zones of 9 and 18 mm and 11 and 20 mm, respectively [66]. Inhibition zones taken against similar bacterial strains in our study were much higher. EOs obtained from wild and cultivated plants of *T. maroccanus*, MIC values of 460 µg/ml and 960 µg/ml gave on *S. aureus*, respectively [67]. In another study conducted on *T. blecherianus* and *T. riatarum* EOs, the researchers reported that the EOs of both species were more effective on *S aureus* (MIC = 19 µg/ml). The researchers attributed all these differences to the diversity of EO components of the species and the synergistic effect that occurs between the components. The high thymol and carvacrol possessed by the *Thymus* species are considered the main components of these differences [68-70]. This information may be evidence that the EOs of plants grown in the natural population often have a stronger antimicrobial effect than the EOs of shots produced *in vitro*. It is a good example that *Abies holophylla*, *Pinus thunbergii*, *Pinus parviflora*, *Tsuga sieboldii* and *Pinus rigitaeda* EOs show much weaker antibacterial activity (inhibition zones ranged between 8-23 mm) than our study on the gram-negative aquatic fish pathogen *P. damselae* subsp. *damselae* (inhibition zones = 43 and 44 mm)[71].

Conclusions

In this study, the volatile constituents, antioxidant and antimicrobial properties of the *T. leucotrichus* EOs obtained from natural plants and *in vitro* derived shoots were identified for the first time. Multiplication of this species in culture media based on previous literature knowledge was seamless. The results showed that *T. leucotrichus* obtained by two different methods has a high essential oil content. EOs obtained from cultivated

shoots gave effective results in terms of antioxidant and antimicrobial effects like EOs of natural plants. *In vitro* derived shoots had higher total phenolic content than naturally growing plants. The main EO component of samples from natural plants and *in vitro* derived shoots was thymol. Some EOs that could not be detected in naturally grown plants was detected in the shoots obtained from the culture medium. EOs of *T. leucotrichus* was quite effective on pathogenic and aquatic bacteria and fungi in terms of the antimicrobial effect. These results may allow industrial use of this species, especially on aquatic pathogenic bacteria. It is a strong fact that the cultivation of plants with biotechnological methods will also help prevent the unlimited gathering of plants grown in their natural population. In addition, the use of secondary metabolites obtained from these plants instead of natural plant secondary bioactive compounds is an innovative solution to the extinction of plants.

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Author contributions
MC designed the research. MC and OB conducted the experiments, analyzed all data and wrote the manuscript.

Conflicts of interest
The authors declare that they have no conflict of interest.

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