

CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITIES OF ESSENTIAL OILS OF ENDEMIC *THYMUS LEUCOSTOMUS* HAUSSKN. *ET VELEN*.

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Abstract

The chemical composition, antimicrobial and antioxidant properties of the essential oils from the leaves of endemic *Thymus leucostomus* naturally grown in Turkey were investigated and chemical differences were discussed by means of chemotaxonomy. Twenty-six components were identified representing 98.8% of the oils. The main compounds in the essential oil of *T. leucostomus* were: o-cymene (30.6%), carvacrol (9.6%), thymol methyl ether (7.2%), limonene (6.8%). Essential oil was screened for their antimicrobial activities against 7 bacteria and 2 yeast species by using disc-diffusion and MIC procedure. The essential oil showed higher effectiveness against all the tested bacteria and yeast. The extract was observed to be much more effective in Gram-positive bacteria (especially, *S. aureus* ATCC 6538). In vitro antioxidant activity based on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was evaluated for the essential oil, and it was found that the essential oil had good antioxidant activity in the range of the $IC_{50} = 5.42 \pm 0.8 \mu\text{g/ml}$.

Introduction

The use of medicinal plants in the treatment of diseases is an ancient tradition that has coexisted with human habitation. Herbal medicines form a significant part of culture and traditions of rural people in developing countries (Njume *et al.* 2009, Berber *et al.* 2013). The Lamiaceae family is comprised of 236 genera and 7172 species in the World (Harley *et al.* 2004, Öztürk 2015). The genus *Thymus* is part of the Lamiaceae family and is among the most important genera with regard to the number of species included. This polymorphic genus is represented by 39 species and 64 taxa in Turkish flora with an endemism ratio of 47% (Baser 2002). *Thymus* species are considered as medicinal plants due to their pharmaceutical properties and have been used broadly as herbal teas, tonics, carminatives, antitussives, and antiseptics, as well as treatments for constipation and as anti-inflammatory, antioxidant, antibacterial and antifungal agents, anthelmintic, hepatoprotective including as a possible means of treatment for neuroblastoma (Pereira *et al.* 2016, Lemos *et al.* 2017).

Contrary to lipids (fixed oils), essential oils (EOs) are volatile and odorous substances. chemotypes of the *Thymus* genus exists in a wide variety, displaying differences in quantity and quality of their compounds present in the same genus (Franz 1993) as well as in various abiotic parameters, such as geographical origin, harvest time, temperature, storage conditions and drying time (Verma *et al.* 2014, Kazemi and Rostami 2015). Particularly, EOs from *Thymus* species are considered as the most active oils due to their high concentration in phenolic compounds (mainly thymol and carvacrol). Their antioxidant and antimicrobial properties provide the basis for many applications in raw and processed food preservation and pharmaceutical products (Ballester-Costa *et al.* 2013). Essential oils were investigated by the chemical analysis of these compounds and their biological activities against several bacteria, yeast and fungi were studied (Touhami *et al.* 2017). The essential oil (EO) and extracts from medicinal and aromatics plants may contain a wide

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variety of free radical scavenging molecules, such as phenolic compounds, nitrogen compounds, vitamins, terpenoids, and some other endogenous metabolites, which are rich in antioxidant activity (Elkiran *et al.* 2018, Pirbalouti *et al.* 2013).

Essential oil compositions of *T. leucostomus* var. *leucostomus* was previously reported (Tümen *et al.* 1997). The present study presents the results of antibacterial, antifungal and antioxidant activities of essential oils of *T. leucostomus* for the first time from Turkey.

Materials and Methods

Thymus leucostomus specimens were collected in August, 2016 from Boyabat (Sinop) of Turkey, at an altitude of 400 m. (41°28'07.8"N 34°45'24.4"E), by O. Elkiran at during flowering time. Plant materials were identified by taxonomist O. Elkiran with volume 7 of Flora of Turkey and East Aegean Islands (Davis1982). They were studied at Sinop University, Scientific and Technological Research Application and Research Center and Çankırı Karatekin University, Faculty of Science, Department of Chemistry, Turkey.

Air-dried aerial parts of the plant materials (100g) were subjected to hydro distillation using a Clevenger-type apparatus for 3 hrs to yield essential oil.

The essential oil was analyzed using HP 6890 GC equipped with a FID detector and an HP-5 MS column (30m×0.25mm i.d., film thickness 0.25 µm) and the capillary column were used. The column and analysis conditions were the same as in GC-MS. The percentage composition of the essential oils was computed from GC-FID peak areas without correction factors.

The oil samples were analyzed by GC-MS, using a Hewlett Packard system. HP-Agilent 5973N GC-MS system with 6890 GC is in Çankırı Karatekin University. HP-5 MS column (30m×0.25mm i.d., film thickness 0.25 µm) was used with helium as the carrier gas. Injector temperature was 250°C, split flow was 1ml/min. The GC oven temperature was kept at 70°C for 2 min and programmed to 150°C at a rate of 10°C/min and then kept constant at 150°C for 15 min to 240°C at a rate of 5°C/min. Alkanes were used as reference points in the calculation of relative retention indices (RRI). MS were taken at 70 eV and at a mass range of 35 - 425. Component identification was carried out using spectrometries electronic libraries (WILEY, NIST) (Elkiran and Avsar 2020). The identified constituents of the essential oils are listed in Table 1. The chromatograms were obtained and they are shown in Fig. 1.

The methods used in antimicrobial and antioxidant tests were described in our previous study (Elkiran and Avsar 2020). The methods are briefly as follows; The test organisms, five Gram-positive bacteria *Bacillus cereus* 7064, *Enterococcus faecalis* ATCC 51299, *Staphylococcus aureus* ATCC 6538, vancomycin resistant *Enterococcus* (VRE), methicillin resistant *Staphylococcus aureus* (MRSA), 2 Gram-negative bacteria *Escherichia coli* ATCC 11293, *Pseudomonas aeruginosa* ATCC27853 and 2 yeast species *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC22019 were collected as pure cultures from the Molecular Biology and Microbiology Laboratory, Department of Biology, Faculty of Arts and Science, Sinop University, Turkey.

The antibacterial and antifungal activity of the essential oil of *Thymus leucostomus* was evaluated by using disc diffusion method (Bauer *et al.* 1966). All the microorganisms were maintained at -80°C in Muller Hinton Agar (MHA) for bacteria and Sabouraud Dextrose Agar (SDA) for fungus (Difco) containing 15% (v/v) glycerol. Before testing, the microorganisms were transferred to Muller Hinton Broth (MHB) for bacteria and Sabouraud Dextrose Broth (SDB) for fungus (Difco) and cultured overnight at 37°C (28°C for fungus). Then, the turbidity was adjusted equivalent to 0.5 McFarland standards (1.5×10^8 cfu/ml). Then, 100 µl of microorganisms were spread over the surface of an agar plate. The filter paper discs (6 mm) were loaded with 50 µl

essential oil and were allowed to dry completely. Next, it was placed on the surface of the freshly inoculated medium. The media were incubated for 24 hrs at 37°C (28°C for fungus). The antimicrobial activity was evaluated by measuring the diameter of inhibition zone (Elkiran and Avsar 2020).

The Minimum Inhibitory Concentration (MIC) was determined by the serial tube dilution method. Essential oil of sample (100 µl) used as initial stock solution. The stock solutions were stirred into 0.9 ml of MHB and SDB in glass tubes in order to adjust to the concentrations of 100-0.7 µl. All tubes were inoculated with 100 µl standardized inoculums of each organism and incubated for 24 hrs at 37°C and 48-72 hrs at 28 ± 1°C. The MIC of the essential oil was taken as the lowest concentration that showed no growth.

The antioxidant potential of the essential oil on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined by Blois (1958), Kumar (2011) method. The essential oil at different concentrations such as 1000, 500, 250, 125 and 62.5 µg/ml was obtained using serial dilution technique in ethanol. 1 ml of an ethanol solution of the essential oil of each concentration was mixed with 4 ml of a DPPH-ethanol solution (0.1 mM). These samples were shaken well and kept in dark for 30 min at room temperature. The absorbance was measured at 517 nm. The scavenging activity on the DPPH radical was calculated by using the following equation:

$$\% \text{ inhibition} = [(A_B - A_S) / A_B] \times 100$$

All experiments were carried out in triplicates and values are expressed as means with standard deviations (±Sd). Graphics were drawn using MS Office Excel 2013.

Results and Discussion

The chemical compositions of volatile metabolites of *Thymus leucostomus* were collected in this study during the flowering period at the Sinop province of Turkey. Essential oils of dried leaves of *T. leucostomus* were analyzed in terms of their chemical composition via GC and GC-MS. The results of the analysis of essential oils of *T. leucostomus* are presented in Table 1 and in Fig. 1. A total of 26 compounds were identified, representing 98.8% of the total oils (Fig. 1). The primary compounds detected in essential oils were O-cymene (30.62%), carvacrol (9.66%), thymol methyl ether (7.22%) and limonene (6.88%). O-cymene is the first major constituent of the *Thymus* essential oils that has been reported for the first time in this article. It can also be indicated that o-cymene is the new chemotype for *Thymus leucostomus*.

The compounds in the essential oil of *T. leucostomus* may be grouped in four main classes: monoterpene hydrocarbons (58.4%), oxygenated monoterpenes (31%), oxygenated sesquiterpenes (5.3%) and sesquiterpenes hydrocarbons (4.1%) as shown in Table 1.

Tümen *et al.* (1997) reported carvacrol (21.59%) and α-terpinyl acetate (23.80%) as the main compounds of essential oils of *leucostomus* var. *leucostomus* from different regions of Turkey, whereas Lemos *et al.* (2017) reported o-cymene (20.3%) as the primary compound as was the case in the present study. Chemotypes of different *Thymus* species from different regions around the world have been reported. Constituents of the essential oils are influenced by several factors such as genetics, crop area, and parts of the plant, temperature, humidity, and geographical location which make it possible to notice differences in the thyme essential oils from distinct regions around the world (Lemos *et al.* 2017).

Petrovic *et al.* (2016) reported geraniol as the major constituent in Serebria thyme (34.4%), while Carrasco *et al.* (2016) observed that thyme collected in Spain has 1,8-cineole as a major compound. Khadir *et al.* (2016) reported linalool as the main compound in Algeria and Tunis thyme, whereas researches on Iran thyme indicated thymol to be predominant which was in accordance with the results of the present study (Tohidi *et al.* 2017).

Moreover, six chemotypes growing in Lithuania were identified for *T. pulegioides*; the chemotypes consist of linalool, geraniol/geranial/neral, thymol, carvacrol/c-terpinene/p-cymene, thymol/carvacrol/p-cymene/c-terpinene, and a-terpenyl acetate (Loziene and Venskutonis 2005). Mancini *et al.* collected *T. vulgaris* from five different areas of the Campania region in southern Italy (Mancini *et al.* 2015). Thymol (46.2 - 67.5%), carvacrol (5.7 - 7.3%) and caryophyllene oxide (1.7 - .3%) were the most abundant compounds in all oils.

Table 1. Chemical composition of essential oil of *T. leucostomus*.

No.	Compounds	RT	RRI	Percent
1	α -phellandrene	11.52	899	0.7
2	α -pinene	11.80	909	3.2
3	Camphene	12.31	926	3.0
4	β -pinene	13.38	961	1.0
5	Terpinolene	14.39	991	0.4
6	o-cymene	14.66	998	30.6
7	Limonene	14.80	1003	6.8
8	Sabinene hydrate	14.91	1006	5.4
9	α -terpinene	15.28	1018	2.5
10	γ -terpinene	15.78	1033	4.8
11	Camphor	18.96	1124	0.7
12	Borneol	19.66	1145	8.3
13	4-terpineol	20.00	1155	0.5
14	p-cymen-8-ol	20.22	1161	0.2
15	α -terpineol	20.43	1167	0.3
16	Dihydrocarvone	20.96	1181	1.6
17	Thymol methyl ether	22.09	1214	7.2
18	Thymol	23.65	1261	0.6
19	Carvacrol	24.00	1270	9.6
20	β -bourbonene	27.08	1364	1.0
21	Caryophyllene	28.22	1398	2.0
22	β -copaene	30.09	1459	1.0
23	β -bisabolene	30.63	1476	2.1
24	Spathulenol	33.01	1556	1.5
25	Caryophyllene oxide	33.24	1563	3.6
26	γ -himachalene	34.68	1613	0.2
	Monoterpene hydrocarbons		58.4	
	Oxygenated monoterpenes		31	
	Oxygenated sesquiterpenes		5.3	
	Sesquiterpenes hydrocarbons		4.1	
	Total			98.8

RT: Retention time, RRI: Relative retention indices.

The antimicrobial activities of essential oils detected against seven bacteria and two yeasts using disc-diffusion and MIC procedures are presented in Tables 2-3. All essential oils inhibited the growth of tested bacteria and yeast according to the results of the disc-diffusion method. The

essential oils in the present study especially showed higher activity against *S. aureus* ATCC 6538 (21 ± 1.2 mm). The present data also show that essential oils possess a strong antibacterial activity in comparison with some antibiotics as standard (Table 2).

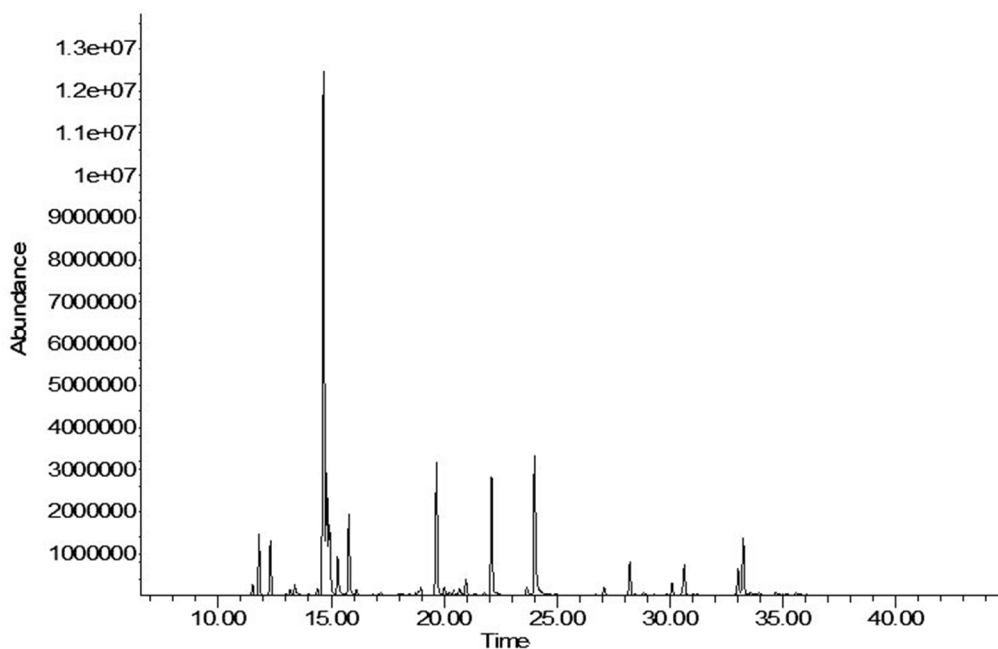


Fig. 1. GC chromatogram of essentials oil of *T. leucostomus*.

In addition, the present results demonstrated a good correlation between the disc-diffusion and the MIC test. The MIC values of essential oils against *S. aureus*, MRSA, *B. cereus*, *P. aeruginosa* and *E. coli* were found to be in the range of 100 - 50 μ l. In addition, the MIC values of essential oils against the yeasts used in the test varied above a value of >100 μ l (Table 3).

These results are in agreement with previous reports indicating that *Thymus* species essential oil have an antimicrobial effect. For example, Ozcan *et al.* 2008 and Vural *et al.* 2008 reported that the essential oils of *Thymus sipyleus* Boiss subsp. *rosulans* and *Thymus argaeus* exhibit antibacterial and antifungal activity against some Gram (+) and Gram (-) bacteria and *Candida albicans*, respectively. Faleiro *et al.* (2003) determined that the *Thymus* species essential oil put forth an antibacterial and antifungal effect against *S. aureus*, *E. coli*, *Listeria monocytogenes*, *Proteus mirabilis*, *Salmonella* spp. and *Candida albicans*. Boruga *et al.* (2014) demonstrated that the *Thymus vulgaris* essential oil has strong antimicrobial properties. Karaman *et al.* (2001) put forth that the *Thymus revolutus* essential oil exhibits a significant antibacterial and antifungal activity.

The IC_{50} values of the essential oils are presented in Fig. 2. The IC_{50} values of essential oils were calculated as 5.42 ± 0.8 μ g/ml and that for the control (ascorbic acid) was determined to be 3.84 ± 0.4 μ g/ml. These results are in agreement with previous reports by Youdim *et al.* (2002), indicating that *Thymus zygis* oil possesses useful antioxidant properties which may be utilized in the food industry and as a dietary supplement. Amiri found that essential oils and methanol extracts of three *Thymus* species showed similar or nearly similar antioxidant activities in comparison with standard BHT (Amiri 2012).

Table 2. Inhibition zones (mm) of the *T. leucostomus* essential oil extracts against tested microorganisms using disc diffusion method.

Plant extract	<i>C. krusei</i> ATCC 6258	<i>C. parapsilosis</i> ATCC 22019	<i>P. aeruginosa</i> ATCC 27853	<i>E. coli</i> ATCC 11293	<i>E. faecalis</i> ATCC 51299	MRSA	<i>B. cereus</i> ATCC7064	<i>S. aureus</i> ATCC 6538	VRE
Extract	14 ± 0.4	13 ± 0.2	14 ± 0.7	14 ± 0.2	10 ± 0.3	15 ± 0.6	15 ± 0.6	21 ± 1.2	10 ± 0.1
DMSO	-	-	-	-	-	-	-	-	-
Bac	*	*	-	-	-	-	8	-	-
Nov	*	*	-	-	15	24	10	29	10
Tet	*	*	17	26	23	10	32	40	13
Amp	*	*	-	-	35	16	*	42	24
Imp	*	*	14	28	34	50	36	50	30
Poly B	*	*	22	11	-	11	9	11	-
Cef	*	*	26	19	20	23	9	25	8
Cyc	43	40	*	*	*	*	*	*	*

-No effect, *Not tested, Antibiotic susceptibility discs including bacitracin (0.04 U), ceftazidime (30 µg), imipenem (10 µg), novobiocin (5 µg), polymyxin B (300 U), tetracycline (30 µg), ampicillin (10 µg) and cycloheximide (50 µg) were used as control, and negative control was to 12.5% DMSO.

Table 3. MIC values (µl/ml) of the *T. leucostomus* essential oil extracts against tested microorganisms using microdilution procedure.

Plant extract	<i>C. krusei</i> ATCC 6258	<i>C. parapsilosis</i> ATCC 22019	<i>P. aeruginosa</i> ATCC 27853	<i>E. coli</i> ATCC 11293	<i>E. faecalis</i> ATCC 51299	MRSA	<i>B. cereus</i> ATCC 7064	<i>S. aureus</i> ATCC 6538	VRE
Extract	-	-	100	100	-	100	100	50	-

-No effect.

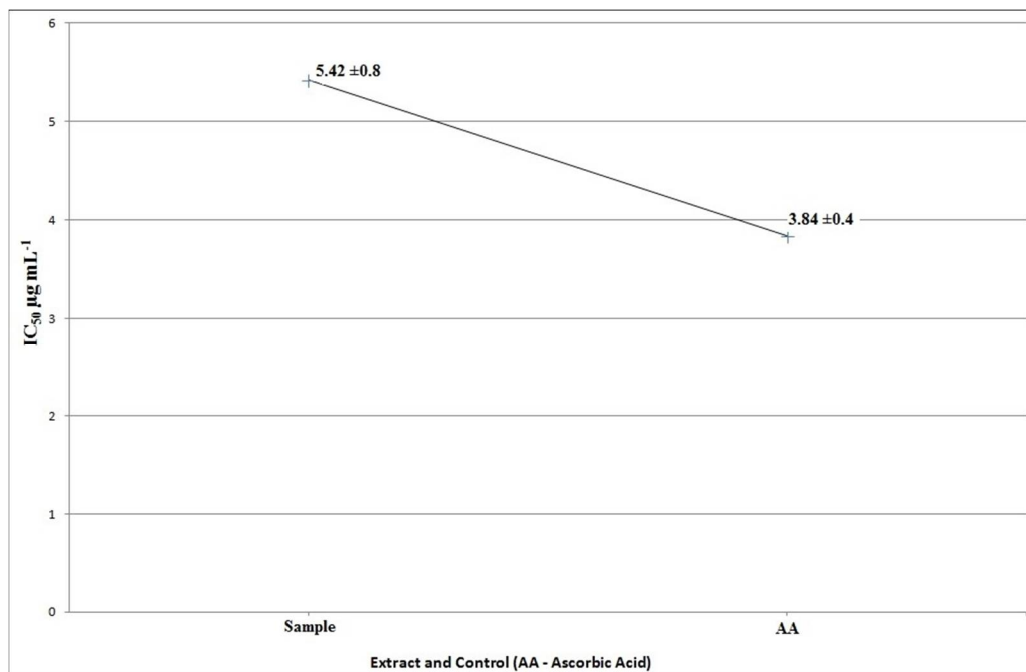


Fig. 2. Antioxidant activity (IC₅₀ value) of the extract of *T. leucostomus*.

In conclusion, the findings indicate that the *Thymus* genus has considerable variations in essential oil composition and this study demonstrates the occurrence of o-cymene, carvacrol, thymol methyl ether and limonene of *leucostomus* in the northern part of Turkey. In addition, this study also indicates that the essential oils of *T. leucostomus* have a potential with regard to antimicrobial, antifungal and antioxidant activities. It has also been determined as a result of the examination of the compositions of the essential oils of *T. leucostomus* samples that they can be used as raw material for medicinal, pharmaceutical purposes, cosmetics industries and as natural products.

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