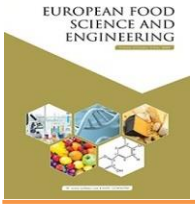


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# Evaluation of *Thymus pseudopulegioides* plant extracts for total phenolic contents, antioxidant and antimicrobial properties

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ABSTRACT

In this study, the ethanolic extracts of leaf and flower of *Thymus pseudopulegioides* Klokov & Des. Shost collected from Sultan Murat Plateau of Trabzon, Turkey was assessed for total phenolic contents and antioxidant activities. In addition, antibacterial activity of extracts against nine different bacterial strains (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomanas aeruginosa*, *Bacillus cereus*, *B. pumilus*, *B. subtilis*, *B. licheniformis*, *Listeria innocua* and *L. monocytogenes*) were determined using the agar-dilution method. The extraction yields from leaf and flower were obtained as 10.95% and 0.94% (w/w), respectively. The total phenolic contents of leaf and flower extracts were determined as 7.84 and 0.39 mg GAE/g, respectively. Antioxidant activities of the ethanolic extracts from leaf and flower were evaluated by using DPPH radical scavenging, and leaf extract showed better antioxidant activity than flower extract. Although, minimum inhibitory concentration (MIC) of leaf extracts was determined as 1.028 mg/mL for all bacteria except *B. subtilis* (0.256 mg/mL), MIC of flower extracts for *B. subtilis* and *B. pumilis* was found as 0.256 mg/mL and for others was 0.512 mg/mL. The extracts of the tested parts of *Thymus pseudopulegioides*, especially the leaves might be valuable for functional food and therapeutic applications.

## 1. Introduction

Since antiquity, spices and herbs have been used for treating common infectious diseases and for flavoring foods and beverages. The use of natural products in food, cosmetic and pharmaceutical industry has considerably increased because of their antioxidant, antibacterial and antiviral effects (Bakkali, Averbeck, Averbeck, & Idaomar, 2008). In these days, there are growing interests in using natural antimicrobial compounds extracted from spices and herbs for the preservation of foods. Additionally, there is a strong consumer demand for more natural foods or food ingredients instead of synthetic additives due to a number of medicinal and ecological problems (Ait-Ouazzou et al., 2012).

Thymus L. (Lamiaceae) is a well-known genus for medicinal and aromatic value, and it comprises about 350 species, 24 of which are endemic in Turkey (Ozen & Demirtas, 2015). For centuries thymus species have been used as medicinal herbs and condiments in folk medicine against asthma, arteriosclerosis, colic, bronchitis, coughs, diarrhea, and rheumatism (Tammar et al., 2018). Besides, they are an aromatic plant and its extracts are used in the food and aroma industries for flavoring (Reyes-Jurado, Cervantes-Rincón, Bach, López-Malo, & Palou, 2019). One member of this genus is called "kekik" in Turkish, which is used as herbal tea, condiments and folk medicine.

"*Thymus pseudopulegioides* Klokov & Des. Shost" is one of the important aromatic species of *Thymus* genus that is an endemic species and has been growing widely in Turkey, especially on the Eastern Black Sea Region. It is known as Anzer tea (Rize) and Anuk (Trabzon) by local people (Günaydin, Laghari, Bektaş, Sökmen, & Sökmen, 2017). The people living in the Eastern Black Sea Region consume this plant as tea. It is also known that extracts of plant materials involve bioactive component that can be beneficial to health such as circulation regulator, diuretic, sedative. There have been limited studies about assessing the chemical constituents and biological activity of *T. pseudopulegioides*. Baser, Kürkçüoglu, Ermin, Tümen, and Malyer (1999) reported that 104 compounds are identified as 97.5-99.5% of the total components detected rich in thymol (50.14%), carvacrol (10.67%), ρ-cymene (10.7%) and γ-terpinene (9.7%). Ozen and Demirtas (2015) found to contain thymol (20.62 %),

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germacrene D (9.84 %) and γ-terpinene (8.36 %) as the major components. However, a survey of the literature showed that *Thymus pseudopulegioides* Klokov & Des. Shost has not yet been properly explored as antibacterial agents. Therefore, objective of present research was to investigate the antibacterial activity of ethanol extracts of both dried fresh leaf and flower of *Thymus pseudopulegioides* Klokov & Des. Shost against nine pathogenic microbial strains. Moreover, the evaluation of total phenolic contents and antioxidant activities was performed.

## 2. Materials and methods

### 2.1. Plant material and extraction procedure

The fresh material of the *Thymus pseudopulegioides* Klokov & Des. Shost plucked was collected at an altitude between 2000 and 2200 m a.s.l. from the Sultan Murat Plateau (Çaykara, Trabzon, Turkey) located in the eastern part of the Black Sea region, Turkey. The plants were identified by the Department of Biology, Ondokuz Mayis University, Samsun, Turkey, taxonomically. At first, this plant was air-dried in shade condition and fragmented. Afterwards, the leaf and flower parts of the plants were separated and milled.

The maceration technique was used for extraction. Five grams of the ground sample was extracted with 200 mL of ethanol (99.5%, v/v) at room temperature in the dark with shaking (250 rpm) for 2 days. After 2 days of maceration, the liquid phase was separated from the solid residue by filtering through Whatman No. 4 filter paper and the organic solvent was removed with a rotary evaporator (Buchi Rotavapor, Flawil, switzerland) to obtain a dry extract at low temperature (40 ºC). All of the dried extracts were placed in a glass bottle and stored in the dark at –20 ºC until required for further analysis.

### 2.2. Determination of antibacterial activity

The *in vitro* antibacterial activities of leaf and flower extracts were assessed on nine different bacteria including Gram-positive bacteria: *Staphylococcus aureus* (ATCC 33862), *Bacillus pumilis* (NRRL BD-142), *B. subtilis* (NRRL B-209), *B. licheniformis* (NRRLB-1001), *B. cereus* (NRRL B-3711), *Listeria innocua* (ATCC 33090), *L. monocytogenes* (ATCC 7644), and Gram negative bacteria: *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922). All the test strains belonged to the Biotechnology laboratory, Food Engineering Department, Ondokuz Mayıs University (Samsun, Turkey). Microorganisms were stored in glycerol broth at -80 ºC.

The antibacterial assay was performed by minimum inhibitory concentration (MIC) according to the Clinical and Laboratory Standards Institute guideline (CLSI, 2006). The estimate of the MIC was carried out by agar dilution assay. Each strain was cultured at 30 ºC in Mueller Hinton broth (Merck, Darmstadt, Germany) for 18 h, and suspensions were adjusted to 0.5 McFarland standard turbidity. The leaf and flower extracts were prepared in Mueller Hinton Agar medium (Merck, Darmstadt, Germany) at concentrations ranging from 0.002 mg/mL to 1.028 mg/mL and the plates incubated at room temperature for 6 hours in order to dry the agar surface. The bacterial suspensions were inoculated onto the plant extract supplemented Mueller Hinton Agar plates and incubated at 30 °C for 24 – 48 hours. Plates used as negative control did not contain extract, and those used as a positive control contained plant extracts without bacterial suspension. The MIC is defined as the lowest concentration of extract that will inhibit growth of the microorganism and expressed in mg/mL.

### 2.3. Determination of total phenolic contents

The total phenolic contents of the extracts were determined using the reagent of Folin-Ciocalteau according to modified method described by Singleton and Rossi (1965), with slight modifications. The extracts (0.05 mL) were transferred into test tubes including 2.5 mL distilled water and the mix was combined with 2.5 mL of 0.2 N Folin – Ciocalteu reagent (Sigma Aldrich, Steinheim, Germany). After the mixture was held in dark at room temperature for 5 min, 2 mL of saturated sodium carbonate solution (75 g/L) was added, shaken for 1 min and allowed to stand for 90 min at room temperature for color development. Then the absorbance of the solution was measured using UV–visible spectrophotometer (Shimadzu Scientific Instruments, Japan) at 765 nm against a blank with distilled water and gallic acid as standard. Standard gallic acid solutions at different concentrations were used to construct the calibration curve. The results of total phenolic content were expressed in mg Gallic acid equivalents (GAE).

### 2.4. Determination of antioxidant activity

The free radical scavenging activity of the extracts were monitored using 2,2diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity according to the method described by Brand-Williams, Cuvelier, and Berset (1995) with minor modifications. In this method, 1 mL of a solution of DPPH in methanol (0.06 mM) was mixed with different concentrations of ethanol extracts. After 30 min of incubation at room temperature in darkness, the absorbance was recorded at 515 nm using the UV spectrophotometer against ethanol blank. The capability to scavenge DPPH radicals was calculated as follows:

$$DPPH\ scavenging\ effect\ (\%) = [\frac{(AC-AS)}{AC}] * 100 \qquad (1)$$

where AS refers the absorbance value of the tested extract and AC refers the absorbance of the control reaction. The results were expressed as $IC_{50}$ values that represented the extract concentration providing 50% inhibition of DPPH.

### 2.5. Statistical analysis

All experiments were performed in three replicates and the results were reported as means ± standard deviation. The data were analyzed with Student t-test using the SPSS 21.0 for Windows Software Package (SPSS Inc., Chicago, IL). Differences between means were considered significant if $P < 0.05$.

## 3. Results and Discussion

### 3.1. Extracts yield

The extraction yields obtained by maceration technique by using ethanol as an extracting solution for leaves and flowers were determined with means of 10.95% and 0.94%, respectively. These results were in agreement with the previous findings that the highest extraction yield values were mostly obtained in flowers (Krakowska, Rafinska, Walczak, Kowalkowski, & Buszewski, 2017). The extraction yield for *T.*

*pseudopulegioides* flowers were determined higher than that one growing Karagöl Plateau (Dereli-Giresun) located in the eastern part of the Black Sea region, Turkey, which was evaluated to 7.2% (Ozen & Demirtas, 2015).

### 3.2. Antibacterial activity

The antibacterial activity of leaf and flower extracts from *T. pseudopulegioides* with agar-dilution assay was tested against nine different genera of bacteria, including seven Gram-positive (*Staphylococcus aureus*, *Bacillus pumilis*, *B. subtilis*, *B. licheniformis*, *B. cereus*, *Listeria innocua*, *L. monocytogenes*) and two Gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*), and the results were shown in Table 1. The ethanol extracts of leaves were found to be the ineffective antimicrobial activity against tested bacteria, showing the lowest minimum inhibitory concentration (MIC) values as 1.028 mg/mL except *B. subtilis* (0.256 mg/mL). However, flower extracts showed lower MIC values than leaf extracts against the tested bacteria with MIC value ranged from 0.256 mg/mL to 0.512 mg/mL. Among the test microorganisms, *B. subtilis* was found to be the most sensitive bacteria to the plant extracts. Contrary to our findings, Gedikoglu, Sokmen, and Civit (2019) reported that extracts from *Thymus vulgaris* and *Thymbra spicata* did not exhibit any antibacterial effects against the tested bacteria. Considering MIC values of leaf extracts, the tested Gram-positive bacterial strains were more susceptible to the ethanolic extract of *T. pseudopulegioides* compared to Gram-negative bacterial strains. Similar results were obtained for the extracts of *Thymus capitatus* L. (Tammar et al., 2018) and the extracts of *Hypericum* species (Maltas et al., 2013), indicating the Gram-positive bacteria are more sensitive to extracts than Gram-negative one. In general, Gram-positive bacteria are more susceptible to plant extracts than Gram-negative bacteria because Gram-negative bacteria have an outer membrane consisting of lipoproteins and lipopolysaccharides, which is selectively permeable and thus regulates access to the underlying structures (Chan, Lim, & Omar, 2007; Chopra & Greenwood, 2001).

**Table 1.** Antibacterial activity of leaf and flower extracts of *T. pseudopulegioides* against the selected strains of Gram-negative and Gram-positive bacteria

| Strains tested | Extracts (µg/mL) | |
|---|---|---|
| | Leaf | Flower |
| Gram-positive | | |
| *Staphylococcus aureus* | 512 | 512 |
| *Bacillus cereus* | 512 | 512 |
| *B. pumilus* | 512 | 256 |
| *B. subtilis* | 256 | 256 |
| *B. licheniformis* | 512 | 512 |
| *Listeria innocua* | 512 | 512 |
| *L. monocytogenes* | 512 | 512 |
| Gram-negative | | |
| *Escherichia coli* | 1028 | 512 |
| *Pseudomanas aeruginosa* | 1028 | 512 |

### 3.3. Total phenolic content

Figure 1 summaries the total phenolic contents of the extracts determined by using the Folin–Ciocalteu method. Our results point to a higher total phenolic content of leaf extracts (7.84 mg GAE/g) in comparison to the total phenolic content of flower extracts (0.39 mg GAE/g). Although, the extracted amount of total phenolics from leaves in the present study was higher than those reported previously from *T. pseudopulegioides* (Ozen & Demirtas, 2015), total phenolic content in the flower was found lower. According to Rafat, Philip, and Muni (2010), various compounds or different amounts of a particular compound due to their differential gene expression may synthesize and accumulate in the different parts of the same plant. Our total phenolic result obtained from flower is in agreement with the results reported by Fatma, Mouna, Mondher, and Ahmed (2014) who stated that the total phenolic content of *Thymus hirtus sp. algeriensis* from various locations in Tunisia ranged between 7.05 and 8.81 mg GAE/g for dry weight. In contrast, Gedikoglu et al. (2019) stated that the total phenolic content of *T. vulgaris* and *T. spicata* ranged between 13.13 and 15.13 mg GAE/g for dry weight. The difference in the results of studies in the literature might be probably due to the extraction conditions like solvent type, solvent concentration, extraction temperature and time, and plan parts (Sekeroglu, Urlu, Kulak, Gezici, & Dang, 2017; Seyrekoğlu & Temiz, 2020).

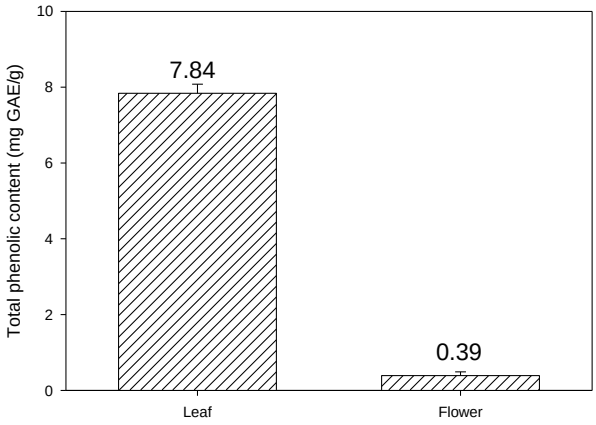


**Figure 1.** Total phenolic content of leaf and flower extracts of *T. pseudopulegioides*

### 3.4. Antioxidant activity

The antioxidant activity of the tested extracts was determined by the DPPH radical scavenging assay that has been widely used to assess the antioxidant ability of various plant extracts, and natural products. The free radical scavenging activity of leaf extract of *T. pseudopulegioides* increased with increasing extract concentration and the leaf extract had stronger DPPH radical scavenging activity ($IC_{50}$ 5.87 µg/mL), however poorer antioxidant capacity was exhibited by flower extract ($IC_{50}$ 1.12 µg/mL) (Figure 2). High DPPH radical scavenging activity of the extracts obtained from the leaf has been mainly due to high phenolic compounds in leaf, which is compatible with the literature (Shabir et al., 2011; Siddhuraju, Mohan, & Becker, 2002). The antioxidant activity of phenolics is mainly originates from their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers (Ozen, Demirtas, & Aksit, 2011). DPPH radical scavenging activity of *T. pseudopulegioides* leaf was found to be in consistent with a previous study reported by Günaydin et al. (2017), who reported that $IC_{50}$ values of natural (wild) *T. pseudopulegioides* collected from Anzer Mountain is 4.89 µg/mL. In another study, higher free radical scavenging activity ($IC_{50}$ value 36.77 µg/mL) of the ethanolic extracts from *Thymus vulgaris* was reported (Gedikoglu et al., 2019). The extraction method and solvents, geographical location and difference in plants within the same family may result in different DPPH radical scavenging activity results (Fatma et al.,

2014; Gedikoglu et al., 2019; Martins et al., 2015).

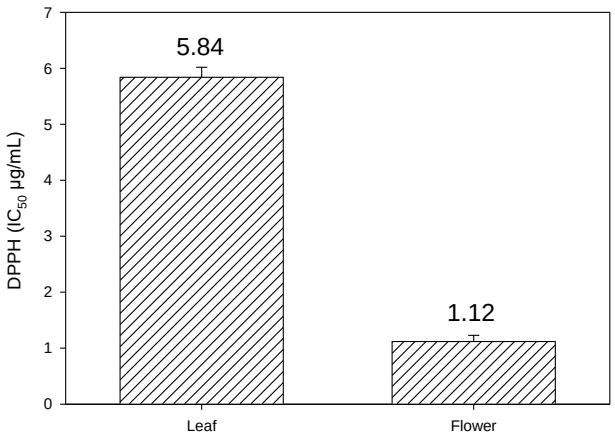


**Figure 2.** Antioxidant activity of leaf and flower extracts of *T. pseudopulegioides*

## 4. Conclusions

According to the results presented in this study, the leaf of *T. pseudopulegioides* Klokov & Des.Shost represents a rich source of phenolic antioxidants. However, the MIC ability of *T. pseudopulegioides* was found to be ineffective for tested microorganisms. Therefore, it has not a potential to be used as natural preservatives and herbal products applicable to the food and nutraceutical industries. Even so, it may be assessable as tea or spice because local people favorably consume it as tea.

## Declaration of Competing Interest

The authors of the submitted manuscript hereby submit that there is no conflict of interest.

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