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In vitro antimicrobial and antioxidant activity of acetone and methanol extracts from *Thymus leucotrichius* (*Lamiaceae*)

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Abstract. – *Background and Objectives:* **Thymus species has been used as tonic and herbal tea, antiseptic, antitussive, carminative, antioxidant, anti-inflammatory and antimicrobial activities. The acetone and methanol extracts of *Thymus (T.) leucotrichius (Labiatae/Lamiaceae*) was examined for antimicrobial and antioxidant properties.**

Materials and Methods: **The antioxidant properties of acetone and methanol extracts of *Thymus leucotrichius* were investigated using 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitric oxide (NO) radical scavenging activity, reducing power and total phenolic substance analysis. Antibacterial, antiyeast and antifungal activity of the plant extracts were tested using the disc diffusion method.**

Results and Discussion: **Results showed that IC_{50} of *Thymus leucotrichius* acetone and methanol extracts that scavenged 50% of the DPPH radical in the medium was found to be 109.72 µg/ml, 43.53 µg/ml, respectively. It was found that IC_{50} of *Thymus leucotrichius* acetone and methanol extracts which scavenged 50% of the NO radical in the medium was 180.56 µg/ml, and 67.34 µg/ml, respectively. In the *Thymus leucotrichius* acetone and methanol extracts (1 mg), 35.64 µg and 51.78 µg pyrocatechol equivalents of phenols were detected, respectively. Neither acetone nor methanol extract possessed activity towards *Proteus vulgaris*, *Rhodotorula rubra*, *Candida albicans*, *Aspergillus parasiticus* and *Aspergillus niger*. Acetone extract was the most active on *Bacillus cereus* and *Bacillus megaterium*. The sentivity was also observed against towards *Escherichia coli* H7:O157, *Kluvyeromyces fragilis* and *Fusarium proliferatum* when acetone extract used. The methanol extract also displayed more or less similar inhibitory activity towards the test microorganisms. *Kluvyeromyces fragilis* was resistant to methanol extract of the species unlike acetone extracts of the species. However, the fungus *Fusarium proliferatum* was markedly inhibited by the methanol extract of test species at 1000 µg and above. Significant inhibitory activities of the two extracts were based upon the increasing dose-dependent level.**

Key Words:

Thymus leucotrichius, Antibacterial, Antiyeast, Antifungal, Antioxidant activity.

Introduction

The use of herbal medicine has been dated back to the ancient times. Since that time, there has been increasing focus of interest in the treatment and/or alleviation of several diseases in modern life of the world[1-5]. Lamiaceae (Labiatae) is a well known family and represented by approximately 250 genera and 3000 species within the flowering plants. Flora of Turkey is rich and diverse with well over 11,000 flowering taxa recorded. The species of Thymus is a polymorphic genus with 60 taxa belonging to 39 species in Turkey. The ratio of endemism is 45%[6]. Thymus has been known in several countries as a spice and food preservative as well as protective and curative remedy for many ailments[7]. In traditional medicine, leaves and flowering parts of Thymus species are widely used as tonic and herbal tea, antiseptic, antitussive and carminative as well as treating colds. Thymus oils and extracts are widely used in pharmaceutical, cosmetic and perfume industry also for flavoring and preservation of several food products[8].

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The antioxidant[9-16], immunological[17,18], inflammatory[19] activities of many Thymus species have been reported earlier. Furthermore, the antimicrobial activities of essential oils of many Thymus species have also been investigated and revealed to be active against a number of microorganisms using various assays[8,15,20-28].

Even though the antimicrobial activity of Thymus oil has been reported several times as stated above, the search on the crude solvent extracts of Thymus sp. is limited[15,19,20,29,30]. The antimicrobial activity of the *Thymus leucotrichius* solvent extracts against microorganisms was also scarcely investigated[31]. In addition, their antioxidant activities have not also been reported. Therefore, this paper reports the first attempt to study the antioxidant and antibacterial, antiyeast and antifungal properties when used the acetone and methanol extracts from the aerial parts of *Thymus leucotrichius*.

Materials and Methods

Plant Material and the Preparation of the Extract

The aerial parts of *Thymus leucotrichius* were collected from its wild habitat in Kars region at an altitude of 1750 m, east of Turkey, during the flowering stage. Voucher specimen (A. ILCIM KSUH 762) was deposited at the Herbarium of Kahramanmaras Sutcu Imam University, Kahramanmaras, Turkey. The aerial parts were dried in the shade at room temperature, powdered using a grinder. Two extracts (acetone and methanol) were prepared from powdered plant. The material was Soxhlet extracted for 8 to 10 h with purified solvents at room temperature. The resulting mixture was then filtered and the filtrate was dried under vacuum at 40ºC (Buchi, Rotavapor R-210, Labortechnik, AG, Flavil, Switzerland).

In Vitro Antioxidant Assays

Chemicals

1,1-Diphenly-2-picrylhydrazyl (DPPH), buthylated hydroxytoluene (BHT), Rutin were purchased from Sigma (Sigma, Aldrich Chemie GmbH, Steinheim, Germany). Pyrocatechole, Folin-Ciocalteu's phenol reagent, sodium carbonate, ethanol, chloroform and the other chemicals and reagents were purchased from Merck (Darmstadt, Germany). All other chemicals and reagents were of analytical grade.

Reducing Power

The reducing power of acetone and methanol extract was measured using the method of Oyaizu[32]. Extract or standard solution (250 µl) was initially dissolved in methanol. Test compound or standard solution was mixed with 2.25 ml of 0.2 mol/L phosphate buffer (pH 6.6), 2.5 ml of potassium ferricyanide solution (1%) and incubated for 20 minute. After incubation, a 2.5 ml of trichloroacetic acid (10%) was transferred into the mixture and centrifuged at 1000 x g for 10 min. Supernatant was mixed with an equal volume of distilled water and 0.5 ml of ferric chloride (0.1%). The absorbance was read at 700 nm at spectrophotometer (Shimadzu, Kyoto, Japan). Butylated hydroxy toluene (BHT) was used as positive control.

Total Phenolics Compounds

Total phenolic compounds were determined according to the method of Slinkard and Singleton[33] using pyrocatechol as a standard phenolic compound. Extract solution was mixed with distilled water and Folin-Ciocalteu's reagent (1:45:1). After 3 min, the reaction mixture was treated with 3 ml of Na_2CO_3 (2%) and left to stand for 2 h. The absorbance was measured at 760 nm in a spectrophotometer (Shimadzu, Kyoto, Japan). The concentration of total phenolic compounds of sample was calculated according to the formula:

Absorbance = [0.00257 × µg pyrocatechol equivalent] – 0.0121 (R^2: 0.993)

DPPH Radical Scavenging Activity

$DPPH^{\cdot}$ free radical scavenging activity was measured using the method of Mokbel and Hashinaga[34]. Extract was dissolved in methanol. The solution was mixed with 2 ml of 0.05 M acetate buffer (pH 5.5) and 1.9 ml of methanol and 1 ml of 0.3 mM $DPPH^{\cdot}$ in methanol. The test tubes were mixed immediately and incubated at room temperature. The decrease in absorbance at 517 nm was measured after 30 min. The inhibition 50% of $DPPH^{\cdot}$ radical value for *Thymus leucotrichius* acetone and methanol extracts was calculated from the following calibration curve, determined by linear regression:

For Acetone Extract

Inhibition = [0.196 × amount of sample (µg)] + 28.494 (R^2: 0.786)

For Methanol Extract

Inhibition = [0.155 × amount of sample (μg)] + 43.252 (R^2: 0.542)

Assay of Nitric Oxide ($NO^{\bullet}$) Scavenging Activity

Nitric oxide scavenging activity was determined by the method of Kumaran and Karanukaran[35]. 2 ml of sodium nitroprusside (10 mM) and 0.5 ml of phosphate buffered saline (PBS) (0.1 M, pH 7.4) was mixed with different concentration of acetone and methanol extracts of *Thymus leucotrichius* or standard solution were incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction mixture was treated with 0.5 ml of Greiss reagent (1% sulfanilamide, 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 5% H_3PO_4) and incubated for 30 min at room temperature. The absorbance of chromophore was read at 548 nm in a spectrophotometer. Rutin was used as positive control. The inhibition 50% of $NO^{\cdot}$ radical value for *Thymus leucotrichius* acetone and methanol extract was calculated from the following calibration curve, determined by linear regression:

For Acetone Extract

Inhibition = [0.325 × amount of sample (μg)] + 14.717 (R^2: 0.799)

For Methanol Extract

Inhibition = [0.385 × amount of sample (μg)] + 24.071 (R^2: 0.709)

Antimicrobial Activity

The antimicrobial activities of crude acetone and methanol extracts were screened against the following microorganisms: *Bacillus cereus* FM 19, *Bacillus megaterium* DSM 32, *Kluvyeromyces fragilis*, *Rhodotorula rubra*, *Aspergillus niger* (soil isolate) were kindly provided by Prof. M. Digrak (Department of Biology, Kahramanmaras Sutcu Imam University, Turkey). *Proteus vulgaris* ATCC 13315 and *Candida albicans* ATCC 10231 were kindly provided by Prof. Z. Demirbag (Department of Biology, Karadeniz Technical University). *Fusarium proliferatum* NRRL 26517 and *Aspergillus parasiticus* NRRL 2999 were kindly provided by Dr. M. Tuzcu (Institute of Adana Veterinary Control Research, Turkey). *Escherichia coli* H7:O157 were purchased from Institute of Refik Saydam, Ankara, Turkey.

Antibacterial and antifungal activity of the plant extracts were employed using to the disc diffusion method. All bacterial species were transferred into 10 ml of Mueller Hinton Broth (Oxoid) and incubated for 24 h at 37ºC before being used for testing. The yeast studied was incubated in Sabouraud dextrose broth (Oxoid) for 48 h. Bacterial and yeast suspension prepared in sterile 0.85% saline corresponding in an optical density of 0.5 McFarland standards corresponding to 10^8 cfu/ml. A 100 μl from each culture was transferred onto the Mueller Hinton agar (Oxoid) for bacteria and Sabouraud Dextrose agar (Oxoid) for the yeast and mixed well with the agar media.

Suspension of spores from fungal strains was prepared by washing the slant culture with sterile saline solution at a known inoculum density (10^4 ml fungal spore). For each fungal culture, total counts were determined in a Thoma haemocytometer. Fungal suspensions were pipetted onto the centre of the Petri dish and then mixed well with the Sabouraud dextrose agar. Sterilized antibiotic assay discs (6 mm in diameter, Oxoid) were loaded with various concentrations of filter sterilized test material and placed onto agar media. Antibiotic assay discs including only solvent were used as controls. The antimicrobial activity was evaluated by measuring the zone expressed as mm of inhibition against test organism.

Statistical Analysis

All assays were conducted in duplicate and the data were reported as the mean ± SD. Different concentration of *Thymus leucotrichius* acetone and methanol extract were analyzed and then half-maximal inhibitory concentration (IC_{50}) values for all the experiments were calculated by linear regression analysis.

Results

The acetone and methanol extracts of *Thymus leucotrichius* displayed different activities. As shown in Figure 1, acetone extract was the most active on *Bacillus cereus* and *Bacillus megaterium.* In addition, *Escherichia coli* H7:O157, *Kluvyeromyces fragilis* and *Fusarium proliferatum* were also sensitive to acetone extract. Whereas, the acetone extract possessed no activity towards *Proteus vulgaris*, *Rhodotorula rubra*, *Candida albicans*, *Aspergillus parasiticus* and *Aspergillus niger* (data were not shown in Figure 1).

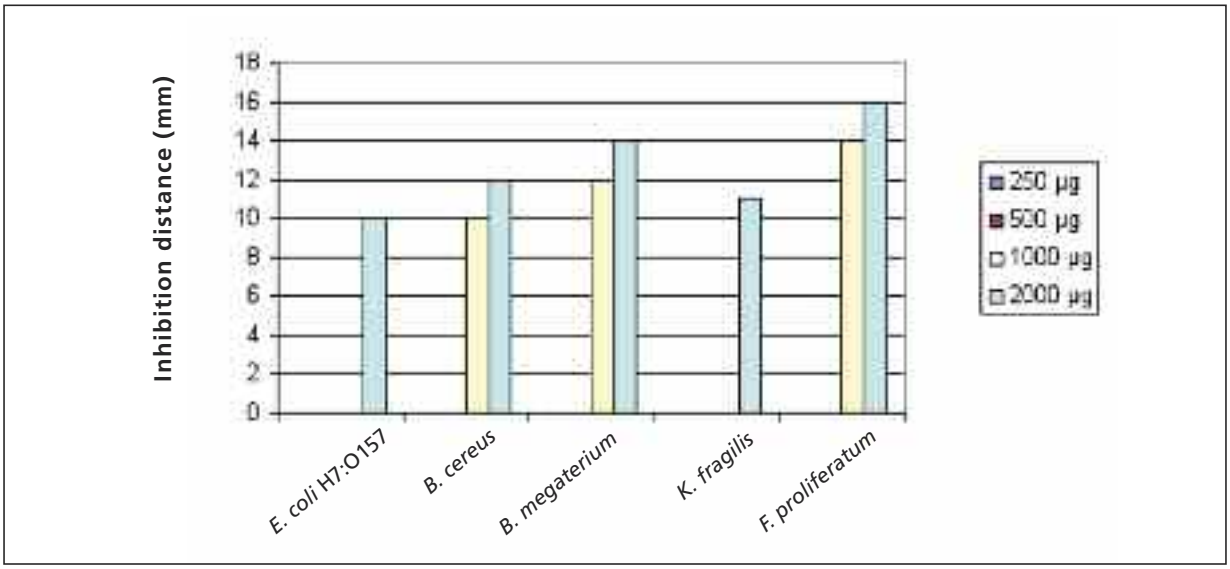

Figure 1. Zones of growth inhibition (mm) revealing antimicrobial activity for acetone extracts of *Thymus leucotrichius*; disc diameter 6.0 mm

As shown in Figure 2, methanol extract compared to acetone extract from the same species used in this work displayed more or less similar inhibitory activity towards the test microorganisms. Unlike acetone extract, no inhibitory activity was observed against *Kluvyeromyces fragilis*. The fungus *Fusarium proliferatum* was markedly inhibited by the methanol extract of test plant at 1000 µg and above. It has been observed that significant inhibitory activities of the two extracts were based upon the increasing dose-dependent level. Methanol extract offered no activity towards *Proteus vulgaris*, *Kluvyeromyces fragilis, Rhodotorula rubra*, *Candida albicans*, *Aspergillus parasiticus* and *Aspergillus niger (*data were not illustrated in Figure 2).

Figure 3 shows reducing capacity of acetone and methanol extract of *Thymus leucotrichius*

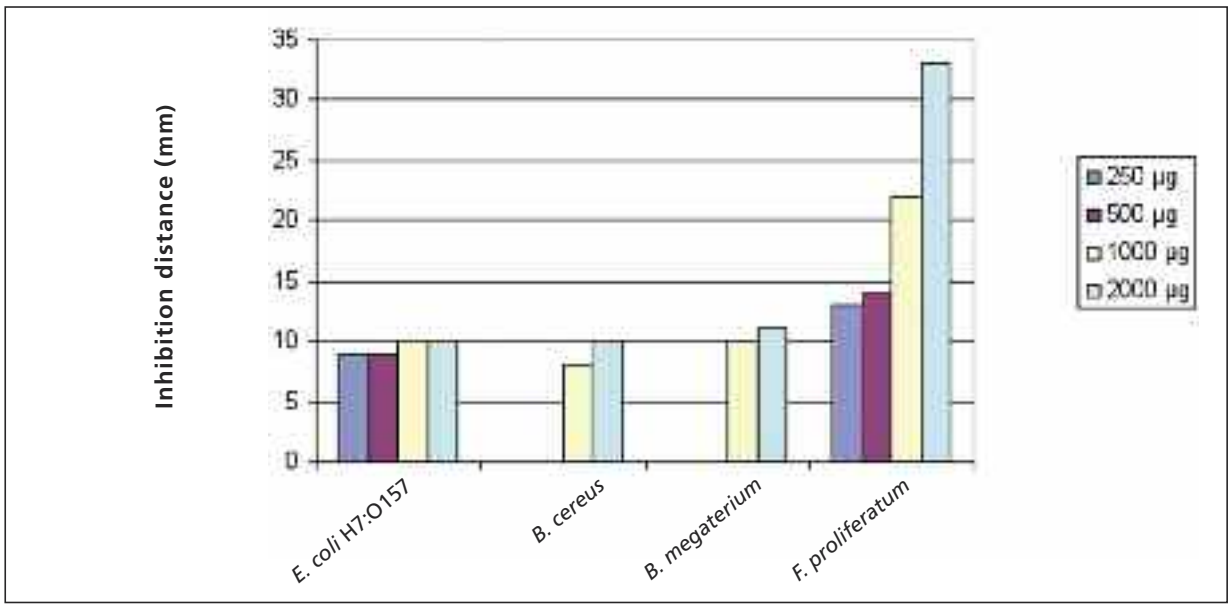

Figure 2. Zones of growth inhibition (mm) revealing antimicrobial activity for methanol extracts of *Thymus leucotrichius*; disc diameter 6.0 mm

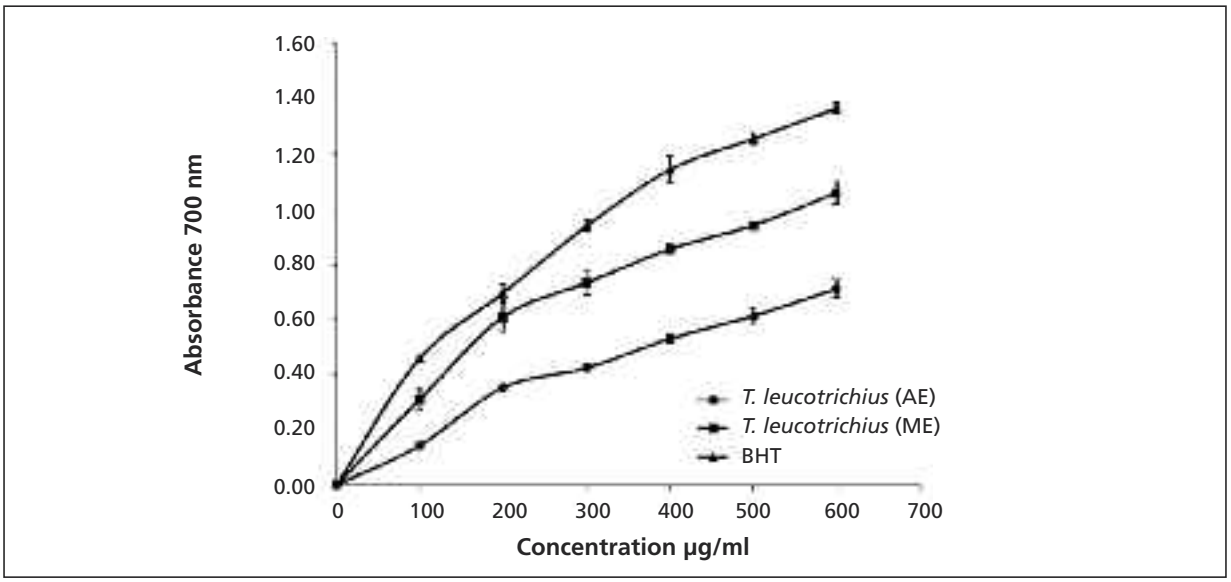

Figure 3. Reducing power of acetone (AE) and methanol (ME) extract of *Thymus leucotrichius* and butylated hydroxy toluene (BHT).

compared with BHT. The reducing power of *Thymus leucotrichius* acetone and methanol extracts increased with increasing amount of sample, respectively (R^2: 0.973, R^2: 0.933).

Thymus leucotrichius acetone and methanol extracts added into 0.3 mM DPPH containing medium at different amounts was compared with Rutin standard for DPPH free radical scavenging activity (Figure 4). Results analyzed by linear regression showed that IC_{50} of *Thymus leucotrichius* acetone and methanol extracts that scavenged 50% of the DPPH radical in the medium was found to be 109.72 µg/ml (R^2=0.786), 43.53 µg/ml (R^2: 0.542), respectively.

Following the linear regression analysis of data, it was found that IC_{50} of *Thymus leucotrichius*

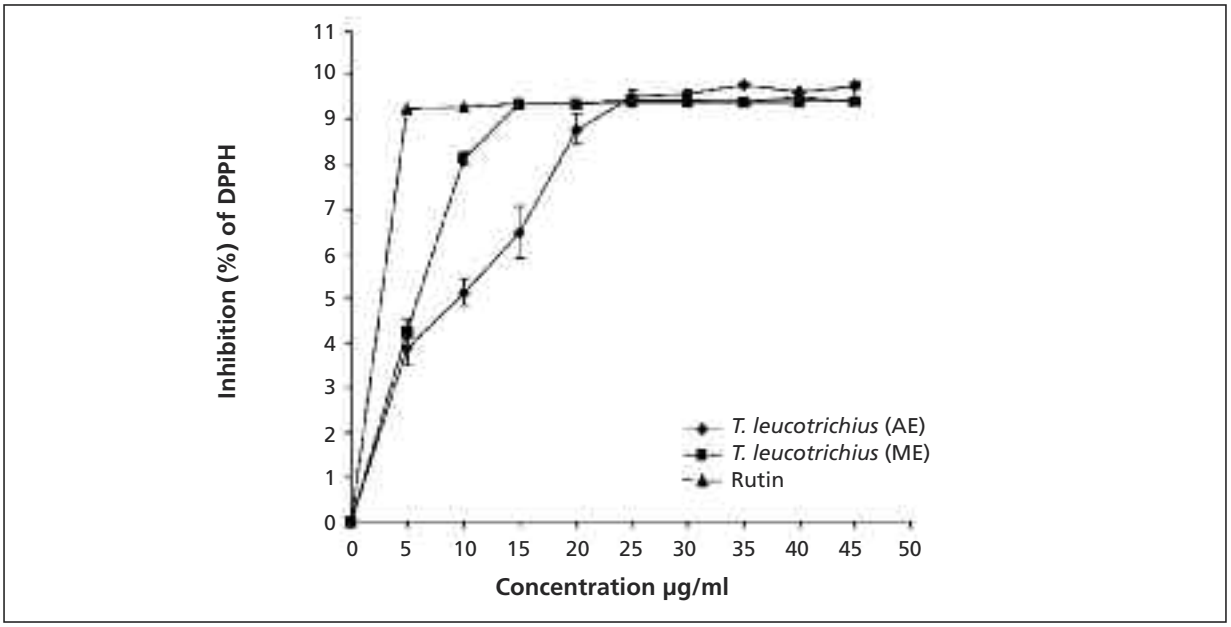

Figure 4. Radical scavenging activities of the *Thymus leucotrichius* acetone (AE) and methanol (ME) extracts and Rutin against DPPH.

acetone and methanol extract which scavenged 50% of the NO radical in the medium was 180.56 µg/ml (R^2: 0.799), 67.34 µg/ml (R^2: 0.709), respectively (Figure 5).

In the *Thymus leucotrichius* acetone and methanol extracts (1 mg) 35.64 µg and 51.78 µg pyrocatechol equivalents of phenols were detected, respectively. This result indicates a strong correlation between phenolic compounds and antioxidant capacity (R^2: 0.993).

Discussion

Bacillus cereus, *Bacillus megaterium, Escherichia coli* H7:O157, *Kluvyeromyces fragilis* and *Fusarium proliferatum* except *Proteus vulgaris*, *Rhodotorula rubra*, *Candida albicans*, *Aspergillus parasiticus* and *Aspergillus niger* revealed sensitivity towards the acetone extract. The similar inhibitory effect on the growth of some microorgansism was also observed using the methanol extract. No activity towards *Kluvyeromyces fragilis* was observed using the methanol extract compared to acetone.

The inhibitory effects of many Thymus spp. against a variety of microorganisms have been reported in earlier studies. Alzoreky and Nakahar[29] revealed that acetone extract of *Thymus. serpyllum* were active on *Staphylococcus aureus* and *Bacillus cereus* except *Escherichia coli*, *S. infantis*, *L. monocytogenes* while methanol extract had no activity against *Escherichia coli* and *S. infantis***.** Ismaili et al.[19] found that chloroform and methanol extracts of *Thymus satureioides* (5 to 0.019 mg ml-1) did not show any activity towards *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus faecalis*. Sokmen et al.[15] tested the methanol extract of *Thymus spathulifolius* (at a rate of 300 µg per disc) against a wide range of microorganisms using disc diffusion assay. They found that the extract had no inhibitory activity towards *Brucella abortus*, *Burkholderia cepacia*, *Enterobacter cloacae, Klebsiella pneumoniae*, *Proteus vulgaris* A1G1 and *Pseudomonas syringae* pv. *tomato*. Whereas, their research also showed that the same extract was active towards *Acinetobacter baumanii* (14 mm), *Bacillus macerans* (12 mm), *Bacillus megaterium* (10 mm), *Bacillus subtilis* (16 mm), *Clavibacter michiganense* (12 mm), *Enterococcus faecalis* (14 mm), *Es-*

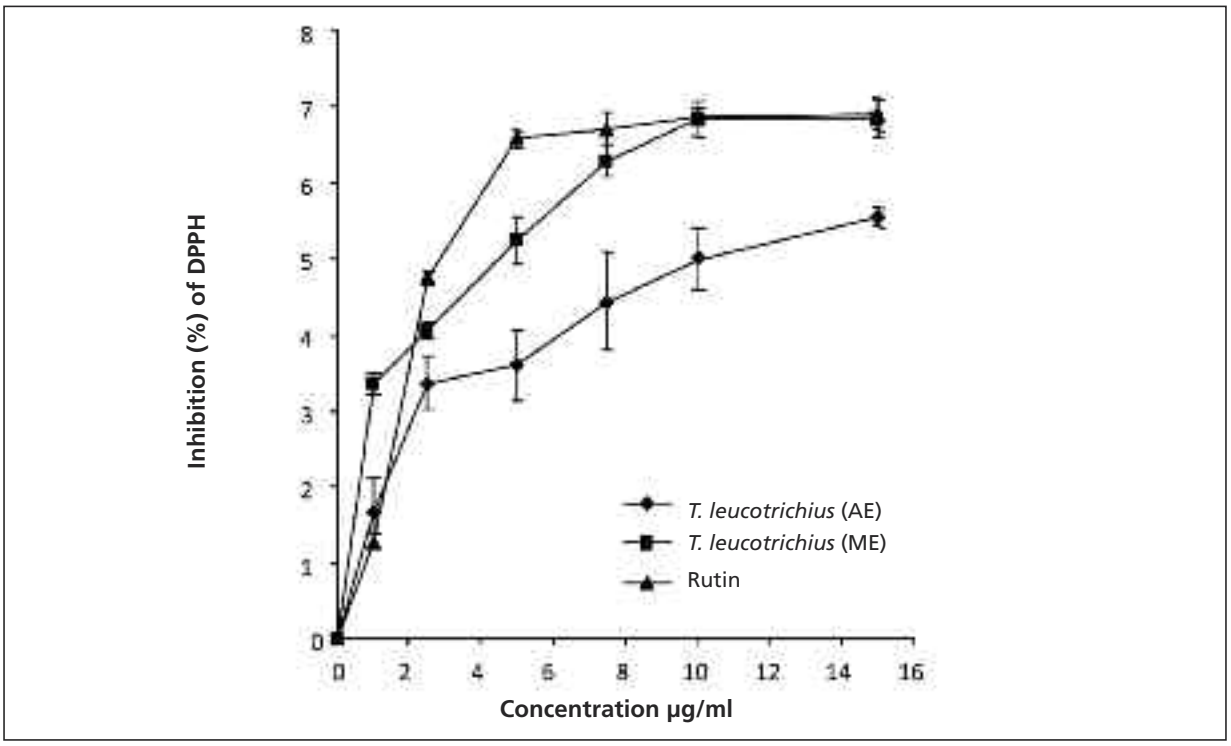

Figure 5. Inhibition of nitric oxide radicals by *Thymus leucotrichius* acetone (AE) and methanol (ME) extracts and Rutin.

cherichia coli A1 (29 mm), *Proteus vulgaris* KÜKEM (12 mm), *Pseudomonas aeruginosa* ATCC 9027 (14 mm), *Salmonella enteritidis* (17 mm), *Staphylococcus aureus* (10 mm), *Staphylococcus epidermidis* (13 mm), *Streptococcus pyogenes* (13 mm), *Streptococcus pyogenes* (12 mm), and *X. campestris* (13 mm). Al Bayati. (2008) reported that, methanol extract of *T. vulgaris* had activity towards *Staphylococcus aureus* (15.6), *Bacillus cereus* (31.2), *Escherichia coli* (250), *Proteus vulgaris* (31.2), *Proteus mirabilis* (62.5), *Salmonella typhi* (62.5), *Salmonella typhimurium* (250), *Klebsiella pneumoniae* (>500), and *Pseudomonas aeruginosa* (>500) using MIC assay (µg/ml).

More recently, Sarıboga and Korkmaz[31] prepared the ethanol:water extract of *Thymus leucotrichius* and subsequently dissolved in carbontetrachloride and water. In their research, two dissolvent revealed only activity towards *Staphylococcus aureus* ATCC 46300, and *Streptococcus pyogenes* KUEN 719 except *Bacillus subtilis* KUEN 16 II D-75, *Escherichia coli* W 3110, *Proteus vulgaris* KUEN1329, *Pseudomonas aeruginosa*ATCC 28753 and the yeast *Candida albicans* KUEN 1475. The three test concentrations of water dissolvent (100, 150 and 200 µg, respectively) revealed inhibition zones of 8, 10 and 17 for *Staphylococcus aureus* and inhibition zones of 0, 7 and 8 mm for *Streptococcus pyogenes*. The inhibition zones of carbontetrachloride dissolvent was 8 mm for *Staphylococcus aureus* at a concentration of 200 µg and 8, 9 and 11 mm at 100, 150 and 200 µg for *Streptococcus pyogenes,* respectively.

Different methodological approach (extracting solvent and dissolvent choice, or bacterial, and yeast strain) used by Sarıboga and Korkmaz[31] appeared to be difficult to compare with our study, which is also hardly comparable due to the plant species grown in different geographical regions and concentration type. The different results offered by different solvent extracts, in fact, may be linked to their phytochemical constituents.

In the present study, we found that *Thymus leucotrichius* acetone and methanol extract has antioxidant properties. The methanol extract has strongly antioxidant capacity compared to acetone extract, and also we observed that *Thymus leucotrichius* acetone and methanol extract includes (1 mg) 35.64 µg and 51.78 µg pyrocatechol equivalents of phenols, respectively.

In earlier studies, the aqueous extracts of *Origanum vulgare* and *Melissa officinalis* exhibited significantly higher antioxidant activity than those of *Thymus vulgaris* and *Agrimonia eupatoria*, using conventional methods and DNA-based biosensor[36]. The *in vitro* methanol extracts of *T. spathulifolius* possessed antioxidant properties when the inhibition of free radical DPPH and b-carotene–linoleic acid methods used[15]. The essential oil of *T. caespititius*, *T. camphoratus* and *T. mastichina* have antioxidant properties[13].

The antioxidant activity of a total of 92 phenolic extracts from edible and nonedible plant materials (berries, fruits, vegetables, herbs, cereals, tree materials, plant sprouts, and seeds) by autoxidation of methyl linoleate was assessed by Kähkönen et al.[37]. They found that 80% aqueous methanol of Thyme has 17.1 ± 0.2 total phenolics (mg of GAE/g dry weights) and possessed strong antioxidant as well as antimicrobial activity as the consequences of thymol and carvacrol in which the basic aroma components of essential oil of thyme possessed. The antioxidant properties of two chemotypes of thyme essential oils in relation to DPPH radical scavenging and by the ferric reducing/antioxidant power methods have been assessed to find any relation to their catalytic transformation[38]. They found that the phenolic chemotype possesses stronger antioxidant properties than the non-phenolic one. Schwarz et al.[39] also noted that p- Cumene-2,3-diol isolated from thyme appeared to be a strong antioxidant capacity. It appeared that phenolic phytocompounds in *Thymus* species exert significant antioxidant and antimicrobial activities. Antioxidant and antimicrobial properties of *T. leucotrichus* found in this work may presumably due to phenolic compounds.

In conclusion, acetone and methanol extract of *T. leucotrichus* possess antioxidant and antimicrobial properties. It could be suggested to use in various pharmaceutical applications.

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