

Investigating of Phytochemicals, Antioxidant, Antimicrobial and Proliferative Properties of Different Extracts of *Thymus spathulifolius* Hausskn. and Velen. Endemic Medicinal Plant from Sivas, Turkey

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Abstract: *Thymus* species has been used for antioxidant, antiseptic, antitussive, carminative, anti-inflammatory and antimicrobial activities as well as tonic and herbal teas. The present study was conducted to evaluate antioxidant, antimicrobial and proliferative properties of ethanol crude extract and fractions of *Thymus spathulifolius* (Hausskn. and Velen.) herbes. The antioxidant properties of ethanol extracts and fractions of *Thymus spathulifolius* were investigated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, reducing power, ferrous chelating activity, total flavonoid and total phenolic content analysis. Antimicrobial activity of the plant extracts were tested using the microdilution method, while proliferative activity were evaluated by MTT assay. Results showed that IC₅₀ of *T. spathulifolius* extracts that scavenged 50% of the DPPH radical was found to be ranged from 62.39 to 1000 µg/mL. Hexane extract possessed moderate antimicrobial activity towards gram-positive bacteria of *S. aureus* and fungi of *C. albicans*. *S. aureus* was the most sensitive bacteria than other tested microorganisms. The hexane and water extracts exhibited a good proliferative activity with ED₅₀ of 3.28 µg/mL and 2.77 µg/mL, respectively. The antioxidant and antimicrobial activities, together with the ability of proliferation, provide some support for the *T. spathulifolius*'s traditional use.

Keywords: *Thymus spathulifolius*, antioxidant, antimicrobial, proliferative activity

1. INTRODUCTION

Herbal medicine has been used for the effective treatment of various disorders in various forms including decoction, maceration, powdered sample, oleoresins, crud extracts, fixed oil, essential oil etc. [1]. It is well recognized that free radicals are critically involved in various pathological conditions such as cancer, cardiovascular disorders, arthritis, inflammation and liver diseases [2]. Many studies have shown that natural antioxidants in medicinal plants are closely related the prevention or suppression of aging and many diseases associated with oxidative stress; cancer, cardiovascular diseases, rheumatoid arthritis, autoimmune diseases [3]. These beneficial effects have been partly attributed to antioxidants, which may play important roles in inhibition of free radicals and oxidative chain-reactions within tissues and membranes

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[4]. The majority of the active antioxidant compounds are flavonoids, isoflavones, flavones, anthocyanins, coumarins, lignans, catechins, and isocatechins [5].

The genus *Thymus L.*, belonging to Lamiaceae family, comprises approximately 300 evergreen species of herbaceous perennial and subshrubs, native to southern Europe and Asia [6]. This genus is represented by 38 species and 64 taxa, 24 of which are endemic in Turkey and the East Aegean Islands [7-8]. Various species of *Thymus* is used all over the world as condiments, ornamentals and sources of essential oil [9]. The genus are used in traditional medicine as tonic, antiseptic, antitussive and carminative as well as for treating colds and in pharmaceutical, cosmetic and perfume industry for preservation of several food products or as condiments [10]. Members of this genus are locally known as ‘kekik’ in Turkish and are used as herbal tea and condiments either in the form of fresh or dry [11].

Thymus spathulifolius Hausskn. and Velen. is a dwarf shrub, up to 10 cm high, endemic in inner Anatolia, especially distributed in Sivas, growing in wild in open steppe on gypsous or poor fertile calcareous soils [12]. In terms of Pharmacological and phytochemical studies, many *Thymus* species have been reported for antioxidant, antimicrobial and other biological activities [13-15]. In addition, in the search for phytochemicals, plant parts are usually screened for phytochemicals that may be present. The presence of a phytochemical of interest may lead to its further isolation, purification and characterization. Then it can be used as the basis for a new pharmaceutical product. Successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction process. This therefore underscore the need to try as much solvent as possible in screening plant parts for phytochemicals [16-17]. Nevertheless, to the best of our knowledge, no data on phytochemical studies of the *Thymus* species are available to now. This study aims to evaluate the antioxidant activity by different methods such as DPPH, metal chelating, FRAP, ferric reducing and ABTS tests, the antimicrobial activity against five gram-positive and gram-negative bacteria and fungi, and proliferative effect of different extracts prepared from *T. spathulifolius*. For pharmaceutical application and naturaceutical industry, the present study may supply important information on the phytochemical properties of the *Thymus* species as well as biological activities.

2. MATERIAL and METHODS

2.1. Chemicals

1,1-Diphenyl-2-picryl-hydrazyl (DPPH), 2,2-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid (ABTS), quercetin, gallic acid, 3,5-Di-tert-4-butylhydroxytoluene (BHT), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and trichloroacetic acid (TCA) were obtained from Sigma Aldrich Co., St. Louis, USA. All other chemicals used were of analytical grade.

2.2. Plant Materials

Test plants were collected during the flowering period from natural populations in Sivas province of Turkey. Collected locality is: B6 Sivas: Zara-Divriği road, between Bolucan-Sincan. Plant materials were prepared as herbarium vouchers. These were registered under collector number as M.Tekin 1744. Voucher specimens are conserved at the Cumhuriyet University, Faculty of Science Herbarium (CUFH), Department of Biology, Sivas, Turkey.

2.3. Preparation of the extract

The dried plant materials were powdered using a grinder. The extraction was done at room temperature. 100g of dried and grounded herbs of *T. spathulifolius* were soaked in 80% ethanol (1000 mL) for 48h with intermittent shaking. Then extracts were filtered through Whatman filter paper No.1. In order to increase the yield of extract, the procedure was repeated for three times. The filtrates combined together and concentrated under vacuum on a rotary evaporator

(Buchi R-100 equipped with Vacuum Pump V-300 and Control unit I-300) at 40°C to dryness and dissolved in distilled water. The aqueous extract was fractionated by successive solvent extraction with hexane, chloroform, n-butanol (pre-water-saturated butanol solution). All of the fraction obtained through solvent extractions were then evaporated to dryness and stored at -20°C for further use.

2.4. Antioxidant Activities

2.4.1. DPPH free radical scavenging activity

The antioxidant activity of the plant extracts and the standard was evaluated on the basis of the radical scavenging effect of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH)-free radical activity by the method of Blois [18]. The stock solution of crude extracts (2 mg/mL) was prepared by dissolving a known amount of dry extract in 10% DMSO of methanol. The working solution (1, 10, 50, 100, 250, 500, 1000 µg/mL) of the extracts were prepared from the stock solution using suitable dilution. Ascorbic acid was used as standard in 1-100 µg/mL solution. 0.1mM of DPPH was prepared in methanol and 1 mL of this solution was mixed with 3 mL of sample solution and standard solution in test tubes separately in triplicates. These solution mixtures shaken vigorously, then were allowed to stand at dark for 30 min and optical density was measured at 517 nm using UV-VIS Spectrophotometer. Methanol (3 mL) with DPPH solution (0.1mM, 1 mL) was used as blank. Methanol was used for base line corrections in absorbance of sample. The effective concentration of sample required to scavenge DPPH radical by 50% (IC₅₀ value) was obtained by linear regression analysis of dose-response curve plotting between % inhibition and concentrations. The optical density was recorded and % inhibition was calculated by the formula given below:

Percent (%) inhibition of DPPH activity = (Absorbance of Blank - Absorbance of Test) / Absorbance of Blank × 100

2.4.2. Ferrous ion chelating activity

Ferrozine can form complex by chelates with Fe²⁺. This reaction is restricted in the presence of other chelating agents and results in a decrease of the red color of the ferrozine-Fe²⁺ complexes. Measurement of the color reduction determines the chelating activity to compete with ferrozine for the ferrous ions. The chelation of ferrous ions is estimated using the method of Dinis et al. [19]. 1 mL of the extract is added to a mixture of 3.7mL methanol and a solution of 0.1 mL ferrous chloride (2 mM). The reaction is started by the addition of 0.2 mL of ferrozine (5 mM) and incubated at room temperature for 10 min and then the absorbance is measured at 562 nm. EDTA was used as a positive control.

2.4.3. ABTS radical cation decolorization assay

In this method, measure the loss of color when an antioxidant is added to the blue-green chromophore ABTS⁺. The antioxidant reduces ABTS⁺ to ABTS and decolorize it. Antioxidant activity can be measured as described by Ree et al. [20]. ABTS radical cations are generated with 2.45 mM potassium persulphate and a 7 mM aqueous ABTS stock solutions. The ABTS cation working solution was obtained by mixing the two stock solutions in equal volumes and incubate them to react for 16h at 25°C in the dark. Before using, this solution was dilute with methanol and adjusted the absorbance 0.70 ± 0.2 units at 734nm by spectrophotometer. Frech solvent was prepared for each assay. Trolox, a water-soluble analog of vitamin E, used as an antioxidant standard. A standard calibration curve is constructed for Trolox at 0, 50, 100, 150, 200, 250, and 500 µM concentrations. 1mL of diluted samples are mixed with 1 mL of ABTS⁺ radical cation solution in test tubes, and absorbance is read (at 734 nm) after 7 min. TEAC values can be calculated from the Trolox standard curve and expressed as Trolox equivalents (in µM).

2.4.4. FRAP Assay

This assay was used to measure the Fe^{3+} ion's reducing power according to the method described by Aksu et al. [21] with slight modifications. 1 mL of samples, BHT and Ascorbic acid at different concentrations in appropriate solvent were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$] (2.5 mL, 1%) and incubated at 50 °C for 20 min. Then, 2.5 mL trichloroacetic acid (10%) was added to the mixture to stop the reaction. 2.5 mL of pure water and 0.5 mL of FeCl_3 (0.1%) were added to 2.5 mL of the reaction mixture, then allowed to stand for 30 min. The increases in the absorbance were spectrophotometrically measured at 700 nm as an indication of reducing capacity.

2.4.5. Determination of total phenolics contents

The determination of total phenolic content was performed by the Foline-Ciocalteu method [22] with slight modifications. The samples were read at 730 nm in spectrophotometer. The total phenolics content was expressed in milligrams equivalents of gallic acid (GAE) per gram of each fraction. The equation obtained for the calibration curve of gallic acid in the range of 0.1–1 mg. mL^{-1} was $Y = 0.7846X + 0.0764$ ($r = 0.9995$).

2.4.6. Determination of total flavonoids contents

The determination of flavonoids was performed according to the method [23] with slight modifications. The absorbance was determined by spectrophotometer at 415 nm. Ethanol was used as a blank. The equation obtained for the calibration curve of quercetin in the range of 0.0625–1.0 mg. mL^{-1} was $Y = 3.1965X + 0.2828$ ($r = 0.9933$). The content of flavonoids was established as quercetin mg/g dry extract. The experiments were conducted in triplicate.

2.4.7. Reducing power

Reducing activity was carried out by using the method of Oyaizu [24]. Different concentration (1000, 500, 250, 100, 10, 1 mg/mL) of extracts and fractions were prepared with methanol and taken in test tube as triplicates. To the test tubes 2.5 mL of sodium phosphate buffer and 2.5 mL of 1% potassium ferric cyanide solution was added. These contents were mixed well and were incubated at 50°C for 20 minutes. After incubation 2.5 mL of 10% TCA was added and were kept for centrifugation at 3000 rpm for 10 minutes. After centrifugation, 5 mL of supernatant were taken and to this 5 mL of distilled water was added. To this about 1 mL of 1% ferric chloride was added and was incubated at 35 °C for 20 minutes. The absorbance measured at 700 nm and the blank was prepared by adding every other solution but without extract and ferric chloride (0.1%) and the control was prepared by adding all other solution but without extract.

2.5. Antimicrobial Activities

2.5.1. Micro-well dilution assay

In order to determine the minimal inhibitory concentration (MIC) of *T. spathulifolius* extracts the broth microdilution method was used [25]. The antimicrobial activity of the plant extracts were tested against bacterial and fungal strains: Gram positive (*Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212)), Gram-negative (*Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922)) and fungal strain (*Candida albicans* (ATCC 10231)). Plant extracts were dissolved in 8% DMSO (20 mg/mL). 50 μL sterile distilled water was added in each well of 96-well microtiter plate. 50 μL of plant extract was added into the first well and a serial 2-fold dilution was performed by transferring 50 μL of the suspension to the subsequent wells up till the 9th well; the final 50 μL of the suspension was discarded. 10th well was added Gentamicin for bacteria and Fluconazole for *Candida*. 11th well was added 50 μL 2% DMSO and 12th well was added 50 μL Mueller Hinton Broth

(sterility control). Concentration of plant extract in wells ranged from 5.00 to 0.02 µg/mL. Final inoculum size was 5×10^5 CFU/mL at bacteria and $0.5-2.5 \times 10^3$ CFU/mL at *Candida* in each well [26-27]. Mueller Hinton Broth and Saboraud Dekstroz Broth was used for dilution bacteria and *Candida* culture's, respectively. Microtiter plates were incubated at 37 °C for bacteria and 35 °C for *Candida* between 16-24 hours. Afterwards, 50 µL 2 mg/mL 2,3,5-Triphenyltetrazolium chloride (TTC) (Meck, Germany) was added to each well to indicate microbial growth. The microtitre plates were further incubated at 37 °C for 2 h. Reduction in density of formazan's red color after incubation was accepted MIC value. The experiment was performed in duplicate and the standard deviation was zero.

2.6. Assessment of *In Vitro* proliferative activity

The *In vitro* proliferative activity was evaluated in the colorimetric MTT assay [28]. Exponential growing L929 Mouse fibroblast cells were plated in 96-well micropates at a density of 5×10^3 cells per well in 100 µL of culture medium and were allowed to adhere for 16 h, cultured in a humidified atmosphere at 37 °C in 5% CO₂ before treatment. Increasing concentrations of extract (1–1000 µg/mL) in their respective extraction solvent were then added. The final concentration of ethanol in the culture medium was maintained at 0.5% (volume/volume) in order to avoid solvent toxicity. The cells were incubated for 24 h in the presence or absence of extract or fractions. After incubation, 100 µL of MTT [5 mg/ml in PBS: medium (1:3)] was added per well, and the plate incubated for 4 h to allow reaction of MTT by cellular mitochondrial dehydrogenases. The excess MTT was aspirated and the formazan crystals formed were dissolved with 100 ml of dimethyl sulfoxide (DMSO). The absorbance of purple formazan, proportional to the number of viable cells, was measured at 595 nm using a microplate reader (Epoch, USA). The experiments were carried out in triplicate. The potency of cell proliferation for each extract was expressed as ED₅₀ value, defined as the concentration that caused a 50% of maximum proliferation of cells.

2.7. Qualitative phytochemical screening

Chemical test for the preliminary phytochemical screening and identification of bioactive chemical constituents in the different extracts obtained from *T. spathulifoliosus* were performed using the standard procedures as described by Trease and Evans [29], Sofowara [30], and Ugochukwu [31].

3. RESULTS and DISCUSSIONS

The yields of crude ethanol extract (TSE), and n-hexane (TSH), chloroform (TSC), n-butanol (TSB) and water (TSW) fractions obtained from crude extract were calculated according to dry weight basis. The highest yield was obtained with TSW (25.0%), followed by TSB (23.3%), TSC (12.31%), TSE (11.88%) and TSH (10.27%) fractions, respectively.

Scavenging activity for free radicals of DPPH has been widely used to evaluate the antioxidant activity of natural products from plant and microbial sources. Free radical scavenging activity of ethanolic extract and fractions prepared from the herbs of *T. spathulifoliosus* was quantitatively determined using DPPH assay. Figure 1 shows the results of DPPH radical scavenging assay.

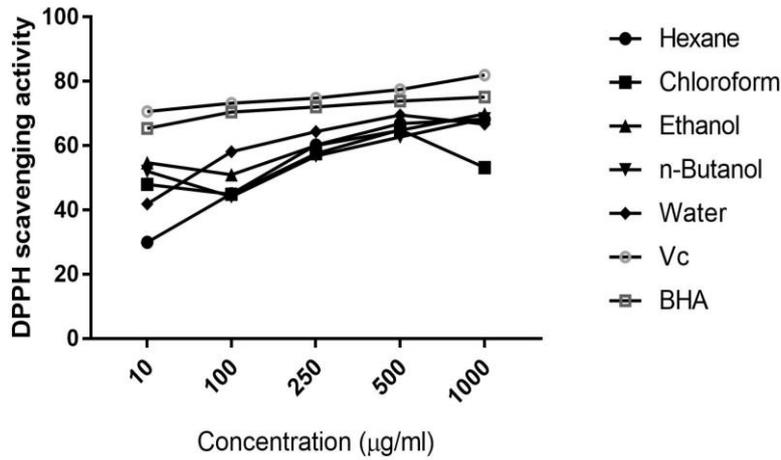


Figure 1. DPPH free radical scavenging activity of different extracts from herbs of *T. spathulifolius*

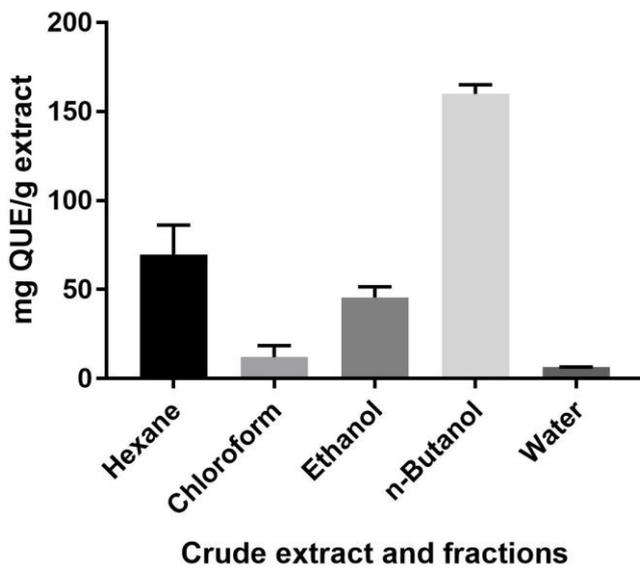


Figure 2. Total flavonoid content of crude ethanol extract and fractions from herbs of *T. spathulifolius*

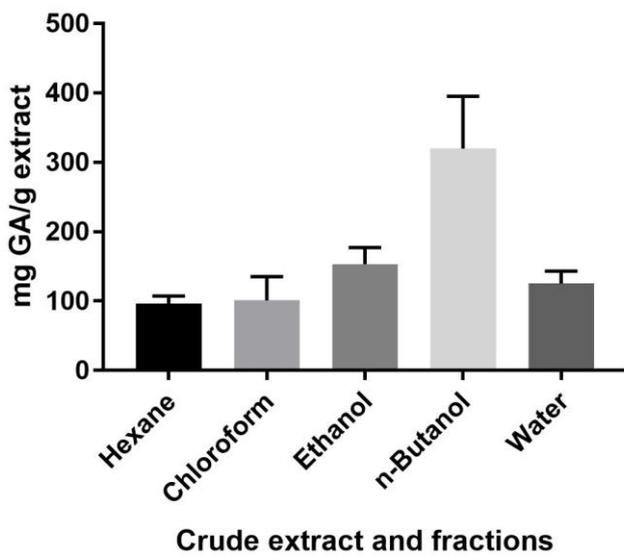


Figure 3. Total phenolic content of extract and fractions from herbs of *T. spathulifolius*

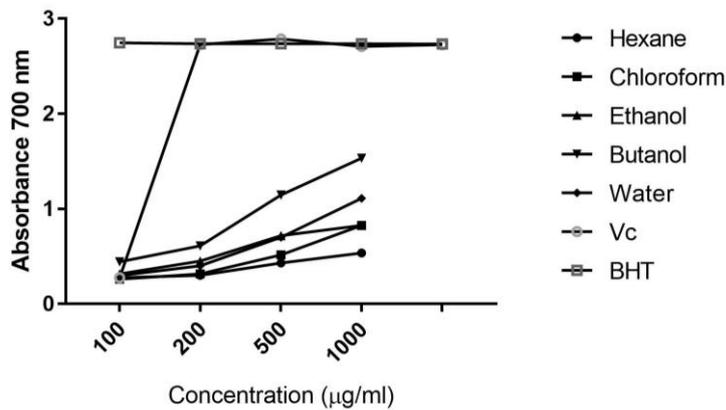


Figure 4. Ferric reducing power of different extracts from herbs of *T. spathulifolius*.

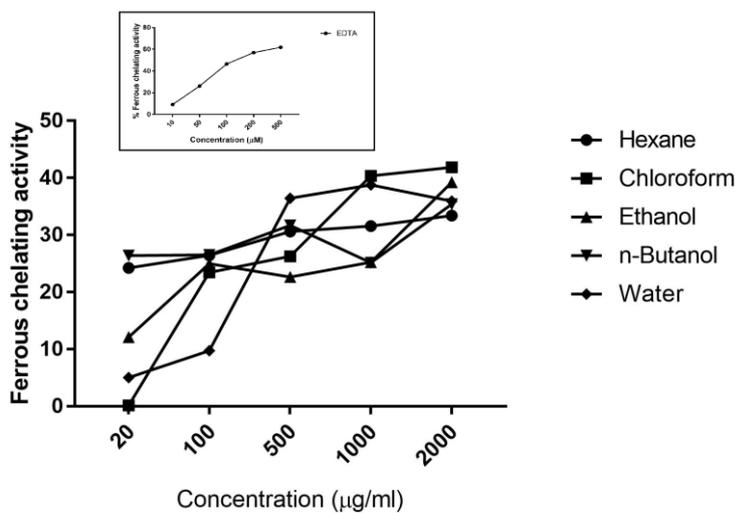


Figure 5. Metal chelating activity of various extracts from herbs of *T. spathulifolius*

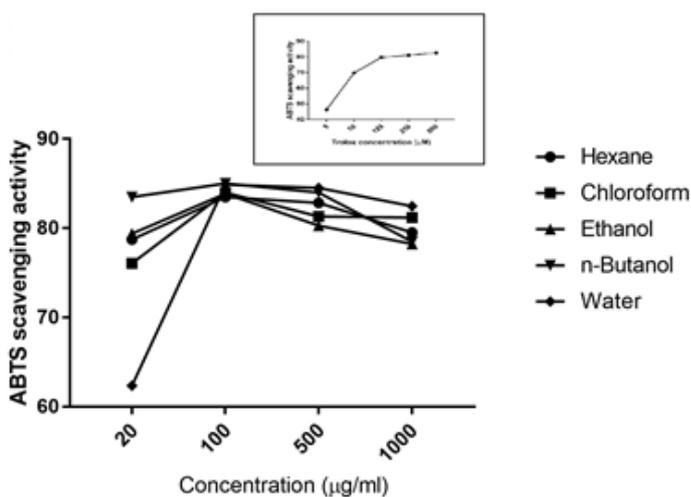


Figure 6. ABTS radical scavenging activity of different extracts from herbs of *T. Spathulifolius*

The total phenolic and total flavonoid contents in different extracts of *T. spathulifolius* are given in Figure 2 and Figure 3. Among studied *T. spathulifolius* extracts, total phenolic content was highest in the butanol extract and lowest in the hexane extract. Total phenolic content in the extracts ranged from 98.31 to 320.29 mg/g GAE. Total flavonoid content was highest in the butanol extract and lowest in water extract, ranged between 6.55 - 160.0 mg

quercetin equivalent flavonoid in g dry weight of extracts. Though the bioactivity of flavonoids appears to be mediated through a variety of mechanisms, particular attention has been focused on their direct and indirect antioxidant actions. The antioxidant properties are conferred on flavonoids by the phenolic hydroxyl groups attached to ring structures and they can act as free radical scavenger, reducing agents and metal chelators [32].

In general, the ABTS radical scavenging activity of the extracts is higher than the DPPH scavenging effect. It was reported that, the capacity of extracts in different test system was effected by solubility of the extract and stereoselectivity of radicals [33]. In this work, all of the tested extracts of *T. spathulifolius* in different polarity showed strong scavenging activities against ABTS radicals. Therefore, they may be valuable therapeutical agents in the treatment of some pathological damage due to free radicals.

3.1. Antimicrobial activity

MIC values of the different extracts of *T. spathulifolius* were detected with broth microdilution assay. As can be seen from the Table 1, plant extracts showed different antimicrobial activity against the test microorganisms. MIC values of the extracts in the range between >5–0.31 mg/mL. According to the results, the most sensitive microorganisms against the Hexane extract of *T. spathulifolius* were *S. aureus* and *C. albicans* that have the lowest MIC values 0.31 and 0.62 mg/mL, respectively.

Table 1. Minimum inhibitory concentrations of different extracts of *T. spathulifolius* herbs

S/No.	Microorganisms	Ethanol extract and partitioned fractions from ethanol extract of <i>T. spathulifolius</i> herbs				
		TSH	TSC	TSE	TSB	TSW
		Concentration (mg/ml)				
1	<i>E. coli</i>	2.5	>5	2.5	2.5	5
2	<i>S. aureus</i>	0.31	0.62	2.5	1.25	5
3	<i>P. aeruginosa</i>	5	>5	5	2.5	>5
4	<i>E. faecalis</i>	5	2.5	5	5	>5
5	<i>C. albicans</i>	0.62	2.5	5	5	5

3.2. Proliferative assay

The proliferative effect of plant extracts on cell growth was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. This colorimetric assay is based on the conversion of the yellow tetrazolium bromide (MTT) to the purple formazan by the action of mitochondrial enzyme succinate dehydrogenase in viable cells. As can be seen from Table 2, the proliferative activities hexane and aqueous fractions with ED₅₀ of 3.28 µg/mL and 2.77 µg/mL, respectively, while ethanolic crude extract showing antiproliferative activity with 12.0 µg/mL. The results in this study suggest that the compounds responsible for proliferative effects may be non-polar or polar compounds. The crude extract has an antiproliferative effect, when fractionated, has the opposite effect, this may be due to the synergistic effect between the compounds in the extract. As can be seen from the study results,

it is worth to evaluating of the active extracts in terms of wound healing activity due to the proliferative effect.

Table 2. Proliferative effects of various fractions of *Thymus spathulifolius* measured using MTT assay and the determined ED₅₀

Extract	ED ₅₀ (µg/mL)
Crude Ethanol extract	-12.0
Hexane fraction	3.28
Chloroform fraction	1056.44
n-Butanol fraction	58.36
Aqueous fraction	2.77

3.3. Phytochemical screening

The screening of chemical constituents was carried out with different extracts by using standard chemical methods according to the methodology, results were summarized in Table 3.

Table 2. Results of phytochemical screening of various extracts of *T. spathulifolius* herbs

Phytochemicals	Test	TSH	TSC	TSE	TSB	TSW
Alkaloids	Dragendorff's	-	-	-	-	-
	Mayer's	-	-	-	-	-
	Wagner's	-	-	-	-	-
Cardiac glycosides	Legal's	-	+	-	-	-
	Keller-Kiliani's	-	+	-	-	-
Carbohydrate	Molisch's	+	+	+	+	+
	Barfoed's	+	+	+	+	+
	Fehling's	+	+	+	+	+
Flavonoids	Shinoda's	-	+	+	+	+
	FeCl ₃	-	+	+	+	-
	NaOH	-	+	+	+	-
Phenols	FeCl ₃	-	+	+	+	+
Proteins	Ninhydrine's	-	-	-	-	-
Saponins	Foam	-	-	-	-	-
Steroids	L-Buchard's	-	-	-	-	-
Tanins	FeCl ₃	-	+	+	+	+
	Lead acetate	-	+	+	+	+
	Jelatin	-	+	+	+	+
Antraquinones	Borntrager's	-	-	-	-	-
Volatile oil	Sudan III	+	+	+	+	-

4. DISCUSSION AND CONCLUSION

The results of our investigation confirm the use of the studied plants in Turkish ethnomedicine. The results from this study suggest that TSB fractions have stronger antioxidant properties than other fractions, which can be attributed to its high content of total phenolics and flavonoids. Previous study on the antioxidant activities of other species of the genus *Thymus* measured by DPPH, showed an IC₅₀ value of 16.15 µg/mL for methanol extract of *T. spathulifolius* [34], 38.2 and 44.5 µg/mL for methanol and hexane fractions of *T. capitatus* [35].

The results of the antimicrobial activity evaluation of different extracts of *T. spathulifolius* against four bacteria and one fungi are summarized in Table 1. Results demonstrated that TSH fractions displayed moderate antibacterial activities against *S. aureus*

and *C.albicans* with MIC value of 0.31 and 0.62 mg/mL, while TSC shows moderate antibacterial activities against *S.aureus* with MIC value of 0.62 mg/mL. The MIC value of other extracts against all of the tested microorganisms were higher than 1.25 mg/mL. In previous study, the MIC value of *T. capitatus* [35] methanol fractions against *S. aureus* was found to be 0.25 mg/mL, hexane fractions was higher than 1 mg/mL, which are close to our results. Phytochemicals are routinely classified as antimicrobials on the basis of susceptibility tests that produce MIC in the range of 100 to 1000 µg/mL [36]. Activity is considered to be significant if MIC values are below 100 µg/mL for crude extracts and moderate when $100 < \text{MIC} < 625$ µg/mL [37]. Therefore, none of the studied samples could be considered as promising source of antimicrobial activities.

The results of the present study show that the ethanol extract and fractions of *T. spathulifolius* contained a high total phenolics level, and can be a promising source of antioxidant as well as antibacterial agents; therefore, it can be considered potentially useful for medicinal application. However, more detailed *in vivo* studies are required to make firm the safety, bioavailability and quality control of *T. spathulifolius* as well as phytochemical characterization and identification of responsible bioactive compounds.

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Conflict of Interests

Authors declare that there is no conflict of interests.

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