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Investigation of Phenolic Content, Antioxidant Capacities, Anthelmintic and Cytotoxic Activities of *Thymus zygioides* Griseb.

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Authors' contributions

This work was carried out in collaboration between all authors. Author AK designed the study and interpreted the results. Author ND helped with the development of the experiments. Author MÇ collected the plant samples and assisted in the diagnosis of the species. Author RM supervised the entire study and contributed to the interpretation of the results. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study was designed to evaluate the total phenolic, flavonoid content and antioxidant properties of various extracts (ethanol, methanol, acetone, water) of *Thymus zygioides* and the ethanol extract was also investigated for phenolic components, anthelmintic and cytotoxic activities.
Materials and Methods: The antioxidant capacities of the extracts were examined for radical scavenging activities (DPPH, ABTS), antioxidant activities (β-carotene/linoleic acid test system, phosphomolybdenum method), power reducing and metal chelating activities. The phenolic content of the ethanol extract was determined using HPLC. The brine shrimp lethality test was used to screen for possible cytotoxic activity. Anthelmintic activity was evaluated using the aquarium worm, *Tubifex tubifex*.
Results: Among the four different extracts of *T. zygioides* evaluated, the methanol extract showed

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the highest amount of free radical scavenging (DPPH, 20.82µg/mL and ABTS, 11.44µg/mL) and antioxidant activities (phosphomolybdenum, 45.63µg/mg). In the metal chelating activity, acetone extract was statistically different from the other extracts (54.26%, $P<0.05$). The water extract exhibited the highest antioxidant capacity (β-carotene/linoleic acid test system, 82.20%) and the ethanol extract showed the highest amount of ferric reducing power activity (0.141 mg/mL). The HPLC analysis of the ethanolic extract showed the presence of some phenolics, such as caffeic acid, quercetin, and epicatechin. In addition, the ethanol extract exhibited cytotoxic activity with LC_{50}, 436.25 µg/mL. Dose-dependent activity was observed in the anthelmintic properties of the ethanol extract and all doses of the ethanol extract showed better anthelmintic activity than the standard.

Conclusion: The results will help us to understand the importance and usage of this medicinal species in the food industry, traditional medicine, and pharmaceutical applications.

Keywords: Thymus zygioides Griseb.; antioxidant capacity; cytotoxic activity; medicinal plant; HPLC.

1. INTRODUCTION

Humans have always used plants, not merely as a source of food but also for pharmaceutical applications and the production of chemicals. Studies have shown that many plants possess a wide range of pharmacological and therapeutic properties. In recent years, there has been increasing interest in research on the isolation and identification of these compounds, which are medically and biologically important in plants. Many of these investigations are related to the identification of the antioxidant capacities of these compounds, which can prevent diseases such as cancer, heart and neurodegenerative disorders caused by reactive oxygen species. For this reason, studies on the screening of medicinal plants containing functional compounds, which provide antioxidant and cytotoxic properties, in order to find new and effective sources, are very important [1]. The *Thymus* is one of the most frequently used medicinal plant's genus [2]. The genus *Thymus* belongs to the *Lamiaceae* family, which comprises more than 220 taxa worldwide. Turkish flora includes 38 species, of which 20 are endemic [3]. The essential oils and extracts of many *Thymus* plants are widely used in the pharmaceutical industry and for the flavoring and preservation of food [4,5]. Analyses of various extracts of *Thymus* plants have indicated that they contain many phenolic acids (i.e., rosmarinic, caffeic, chlorogenic acids) and a number of flavonoids [6]. These phenolic compounds are closely associated with the strong antioxidant activities of *Thymus* plants [5,7]. Indeed, the essential oils of *Thymus* plants have demonstrated the presence of different components; mainly, high phenolic monoterpenes such as carvacrol and thymol [4]. These essential oil components have exhibited antioxidant, antifungal, antibacterial and anthelmintic activities [6,8]. Therefore, members of this genus are among the most popular plants in the world due to their antioxidant capacity, cytotoxic potential, significant amounts of phenolic and flavonoid compounds. Due to their biological and pharmacological properties, they are commonly used as herbal teas and spices, and also as aromatic and medicinal plants [9].

Thymus zygioides Griseb. is a medicinal plant species belonging to the genus *Thymus,* and it is used in the treatment of several diseases, such as gastrointestinal and respiratory tract problems [2]. Various *Thymus* species have also previously been studied for the antioxidant activities of their essential oils [10,11] and a variety of extracts [7,10-14]. Nevertheless, to our best knowledge, there are no scientific reports concerning the antioxidant capacity, total phenolic content, the cytotoxic and anthelmintic activity of *Thymus zygioides* extracts. Within this context, we consider that the *T. zygioides* plant merits investigation and this study has been designed to investigate the antioxidant potentials and total phenolic and flavonoid contents of extracts isolated from *T. zygioides* using different polarity solvents (methanol, ethanol, acetone, and water), to evaluate the cytotoxic and anthelmintic activities of the ethanol extract and to determine its chemical composition. The antioxidant potential, as well as the significant amounts of phenolic and flavonoid compounds of *Thymus* plants and their consumption as part of our diet, is acknowledged and research on the use of Thymus plants will continue to be of great interest to the food and pharmaceutical industry. In the near future, *T. zygioides* from the *Thymus* genus could be used in the pharmaceutical industry and for the human diet, as an aromatic and medicinal plant.

2. MATERIALS AND METHODS

2.1 Plant Materials

Thymus zygioides Griseb. was collected in June 2016 from Honaz Mountain, Denizli, Turkey. The plant material was identified by Dr. Mehmet Çiçek from the Biology Department of the Faculty of Arts and Science, Pamukkale University, Denizli, Turkey. A voucher specimen (*Thymus zygioides*; Herbarium No: 2016-996) has been deposited in the private herbarium of Dr. M. Çiçek (PAU) in Pamukkale University, Denizli, Turkey.

2.2 Preparation of the Plant Extracts

The aerial parts of *T. zygioides* were dried at room temperature and powdered. Twenty grams of each powdered sample was mixed with 200 mL of solvents with varying polarities (ethanol, methanol, acetone, and water). The extraction was carried out by shaking at 50ºC for 6 h, in a temperature controlled shaker and then the extract was separated from the sample residue by filtration through Whatman No.1 filter paper (repeated twice). The solvent was evaporated at 40-50ºC, using a rotary evaporator (IKA RV10D, Staufen, Germany). The samples were lyophilized (Labconco FreeZone, Kansas City, MO) and stored at -20ºC until tested. We used three-letter abbreviations in the manuscript, the first two referring the species name and the third the solvent used (TZE: Ethanol extract of *T. zygioides;* TZM: Methanol extract of *T. zygioides;* TZA: Acetone extract of *T. zygioides*; TZW: Water extract of *T. zygioides*).

2.3 Chemicals

β-carotene, Linoleic acid, 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH), 2,2'-and-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Quercetin, Sodium phosphate, Gallic acid, methanol, chloroform, ethanol, and acetone were purchased from Sigma-Aldrich. Butylated hydroxy toluene (BHT), Folin-Ciocalteu reagent and Tween 20 were purchased from Merck (Darmstadt, Germany). Albendazole was also purchased (Biopharma Ltd. Istanbul, Turkey). Other chemicals and solvents were analytical grades.

2.4 Total Antioxidant Activity (β-carotene/Linoleic Acid Method)

The method of Amin and Tan [15] was used to determine the antioxidant activity. A stock solution of β-carotene mixture was prepared, as follows: 0.2 mg β-carotene was dissolved in chloroform and 0.02 mL of linoleic acid and 0.2 mL of 100% Tween 20 was added. The chloroform was evaporated using a rotary evaporator, and 100 ml of distilled water was added. The reaction mixture and extracts were dispensed into test tubes and the tubes were placed at 50ºC in a water bath for 2 hours. The absorbance of the mixtures was measured at 470 nm using a spectrophotometer. The same procedure was repeated with synthetic antioxidants (BHT) as the positive control. All the tests were carried out in triplicate. Total antioxidant activity (AA) was calculated based on following equation:

$$AA=[1- (A_0-A_t)/(A^o_0-A^r_t)] \times 100$$

where A_0 and A^o_0 are the absorbance values measured at the initial time of the incubation for samples and control, respectively, While A_t and A^r_t is the absorbance values measured in the samples or standards and control at $_t$=120 min.

2.5 Total Antioxidant Activity (Phospho-molybdenum Method)

In this method, the antioxidant capacities of *T. zygioides* extracts were determined according to the Prieto method [16]. The extracts (0.1-1.0 mg/mL) were mixed with 3 mL reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixtures were incubated at 95ºC for 90 min and then the absorbances of these mixtures were measured at 695 nm against a blank. The antioxidant capacity of the extracts was expressed as equivalents of ascorbic acid. All the tests were carried out in triplicate.

2.6 Free Radical Scavenging Activity (DPPH)

The scavenging activity of *T. zygioides* extracts was determined according to the Meriga method [17]. The different concentration (0.2-1.0 mg/mL) of the extracts were mixed with 4 mL of DPPH (0.004%) solution. The reaction mixture was shaken vigorously and kept for 30 min. The absorbances were measured at 517 nm. Results were expressed as IC_{50} values. BHT was used as a control. The tests were carried out in triplicate.

2.7 ABTS Radical Cation Scavenging Activity

The ABTS method was conducted according to the procedure of Shalaby and Shanab [18], with slight modifications. ABTS (7mM) and potassium persulphate (2.45 mM) were dissolved in distilled water. These two solutions were mixed and the mixture was kept in a dark room for 12-16 h before to use. The absorbance (0.700 ± 0.05) of the diluted reacting mixture and ethanol (1:1) were measured at 734 nm. The ABTS solution and different concentrations of the extracts (100, 200, 400 and 800 µg/mL) were mixed. The absorbances of the solutions were read at 734 nm after 15 min. The tests were carried out in triplicate.

2.8 Metal Chelating Activity

The metal chelating activity on ferrous ions was evaluated using the Dinis method [19] with slight modifications. One milliliter of the extract was mixed with 2 mM FeCl2 (0.1 mL) solution. The reaction was started by adding 5 mM of ferrozine (0.2 mL), and then reaction mixture was left standing at room temperature for 10 min. The absorbances of the solutions were measured at 562 nm. The metal chelating activity was calculated using the following equation:

$$\text{Chelating ability (\%)} = [(A_0 - A_s) / A_0] \times 100,$$

where A_0 was the absorbance of the control, and A_s was the absorbance of the extract.

2.9 Reducing Power

The reducing power of the extract was estimated using the method described by Oyaizu [20] .Different concentrations of the sample were mixed with 0.2 M phosphate buffer (2.5 mL) and 1% potassium ferricyanide (2.5 mL). The mixture was kept at 50ºC for 20 min. Trichloroacetic acid (2.5 mL,10%) was added to reaction mixture. An aliquot of the upper layer (2.5 mL) was combined with 2.5 mL distilled water and 0.5 mL of a 0.1% ferric chloride. The absorbances were measured after 10 min, at 700 nm. The increased absorbance of the reaction mixture suggests a high reducing power. All the tests were carried out in triplicate.

2.10 Total Phenolic Content

Total phenolic content was evaluated with the Folin-Ciocalteu method [21]. The sample solution (1 mL) was mixed with 1 mL Folin-Ciocalteu reagent and 46 mL distilled water. After 3 min, 3 mL of 2% sodium carbonate (Na_2CO_3) solution was added. The mixture was allowed to stand for 2 h at room temperature and absorbance was measured at 760 nm. All the tests were performed in triplicate. Gallic acid was used for calibration and the results were expressed as mg of gallic acid equivalents (mg GAE g^{-1} extract).

2.11 Total Flavonoid Content

Total flavonoid content of extracts was determined using the method of Arvouet-Grand [22]. Briefly, 1mL of 2% $AlCl_3$ was mixed with the same volume of extract solution (2 mg/mL). The absorbance of the reaction mixtures were measured at 415 nm after 10 min incubation at room temperature. The flavonoid content was calculated from a quercetin standard curve (mg QEs/g extract).

2.12 Total Tannin Content

Tannin content was determined using the vanillin method of Bekir [23] with slight modifications. The extracts (0.5 mL) were mixed with 1.5 mL vanillin reagent (1% vanillin in 7M H_2SO_4) in test tubes that were in an ice bath. Then reaction mixture was left standing for 15 min at room temperature. Absorbances of the solution were measured at 500 nm. Tannin content was expressed as equivalents of catechin (mgCEs/g).

2.13 Quantification of Phenolic Compounds Using HPLC

The phenolic compounds were determined by reversed-phase high performance liquid chromatography (RP-HPLC, Shimadzu Scientific Instruments). Detection and quantification were made with a diode array detector (SPD-M20A), a LC-20AT pump, a CTO-10ASVp column heater, SIL-10ACHT auto sampler, SCL-10Avp system controller and DGU-14A degasser. Separations were carried out on C-18 reversed-phase column (250 x 4.6 mm length, 5 µm particle size). The eluates were determined at 278 nm. The mobile phases were A: 3.0% formic acid in distilled water and B: methanol. The samples dissolved in methanol and then 20 µL of this solution was injected into the column. The phenolic composition of the extract was determined according to the Caponio method [24] with slight modifications. Gallic acid, 3,4 dihydroxybenzoic acid, 4-hydroxybenzoic acid, 2,5 dihydroxybenzoic acid, chlorogenic acid,

epicatechin, vanillic acid, caffeic acid, p-coumaric acid, ferulic acid, cinnamic acid, quercetin and rutin were used as standards. The quantity of individual phenolic compound was determined based on peaks. The quantity of each phenolic compound was expressed as µg/g per gram of the extract.

2.14 Cytotoxic Activity

Possible cytotoxic activity was evaluated using the Brine shrimp lethality test [25]. *Artemia salina* is a simple marine organism. Due to some of its features, such as its ease of culture, low cost, availability of eggs, rapid growth and its commercial availability in dry cysts, *Artemia* can be used to determine toxicity by estimating the medium lethal concentration (LC_{50}). As a result of these properties, this method is an alternative method for screening toxicity that is cheap, effective, simple, rapid and includes procedures that could replace the experiments carried out with animals [26]. The *Artemia salina* eggs were placed into water containing 3.8% sea salt and left to incubate under artificial light for 48 h at 28ºC. Ethanol extract of *T. zygioides* was tested at 1000, 500, 100, 50 and 10 ppm. In each experiment 0.5mL of plant extract was mixed with 4.5 mL of brine solution. After incubation for 48 h, ten brine shrimp larvae were collected and placed into test tubes containing different concentrations of the extracts. After 24 h, the number of dead shrimps were counted in each concentration of the extracts and the control. Larvae were considered dead if no movement of the appendage was observed within 10 sec. To determine the LC50 values, the data was analysed using the EPA Probit Analysis Programme (version 1.5) [27].

2.15 Anthelmintic Activity

The *in vitro* anthelmintic activity of the ethanol extract of *T. zygioides* was evaluated using the method reported by Dash [28] with slight modifications. The experiment was conducted using *Tubifex tubifex* (Annelida) because they belong to the same group of annelid as the intestinal round worm parasite found in human beings and have an anatomical and physiological similarity. In addition, aquarium worms such as *Tubifex tubifex* have been used widely for the initial evaluation of anthelmintic compounds *in vitro* due to their easy availability [29]. The average size of the *Tubifex tubifex* were 1-2 cm and 6 of these worms were placed in a Petri dish containing 20 mL test solutions of ethanol extract. Test samples of the extracts were prepared in distilled water at concentrations of 2.5, 5, 10 mg/mL. Albendazole (2.5, 5, 10 mg/mL) was used as a reference standard and distilled water was used as a negative control. The worms were observed and the time taken for paralysis and the time taken death were noted in minutes. The mean time for paralysis was noted when movement was lost or no movement could be observed, except when the worm was shaken vigorously; the time of death of each worm was recorded after ascertaining that the worms neither moved when shaken nor when given external stimuli.

2.16 Statistical Analysis

All analyses were performed in triplicate and the results presented as mean ± SE (Standard Error). The results obtained were analysed using the MINITAB Statistical Package programme. The differences between the different extracts were tested with Analysis of Variance (ANOVA) and with Tukey, to see how the groups differed from each other (P<0.05), different groups were shown with different letters in the same column.

3. RESULTS AND DISCUSSION

There is no standard method for determining the antioxidant capacity of a compound due to several parameters, such as the concentration and the structure of the compound to be analysed. The antioxidant activity can also contain different mechanisms, such as free radical scavenging, reducing and chelating activities [30]. For this reason, we applied several antioxidant methods (DPPH, ABTS, Phosphomolybdenum, metal chelating activity etc.) to evaluate true antioxidant potential of the *T. zygioides* extracts.

3.1 β-Carotene/linoleic Acid and Phosphomolybdenum Antioxidant Activity

β-carotene/linoleic acid is used to measure antioxidant activity. Antioxidant activity of the water extracts (82.20±1.06%) from *T. zygioides* were better than the acetone (TZA, 41±3.77%), ethanol (TZE, 52.02±1.35%)) and methanol (TZM, 71.28±0.7%) extracts; however, synthetic antioxidant (BHT) showed highest antioxidant activity (over 90%) (Fig. 1). There were statistically differences among the antioxidant content of different extracts of T. *zygioides*

($F_{4,40}$=129.93, *P* <0.001). Antioxidant activity for the methanol extract (TZM, 71.28%) determined in this study was lower than that reported by Sokmen [31] in the polar and nonpolar subfraction of methanol extract from *T. spathulifolius*. In addition, similar to our findings, Lagouri [7] found that total antioxidant capacity differed, according to the solvents used. The appearance of different antioxidant activities in the plant extracts obtained in different solvents may result from the polarity of the solvents used. In addition, to the best of our knowledge, this is the first study until now on β-carotene/linoleic acid activity of extracts from *T. zygioides*.

The antioxidant activities of the samples were also evaluated using the phosphomolybdenum complex formation, according to the method of Prieto [16]. This method is based on detection at 695 nm of the reduced green molybdenum complex, formed as a by-product of the reduction of Phosphate-Molybdenum (VI) to Phosphate-Molybdenum (V). As can be seen from Table 1, the phosphomolybdenum activity of the extracts was in the range of 26.87-45.63 µg/mg. The methanol (TZM) and water (TZW) extracts performed better activities than the ethanol (TZE) and acetone (TZA) extracts in the phosphomolybdenum assay.

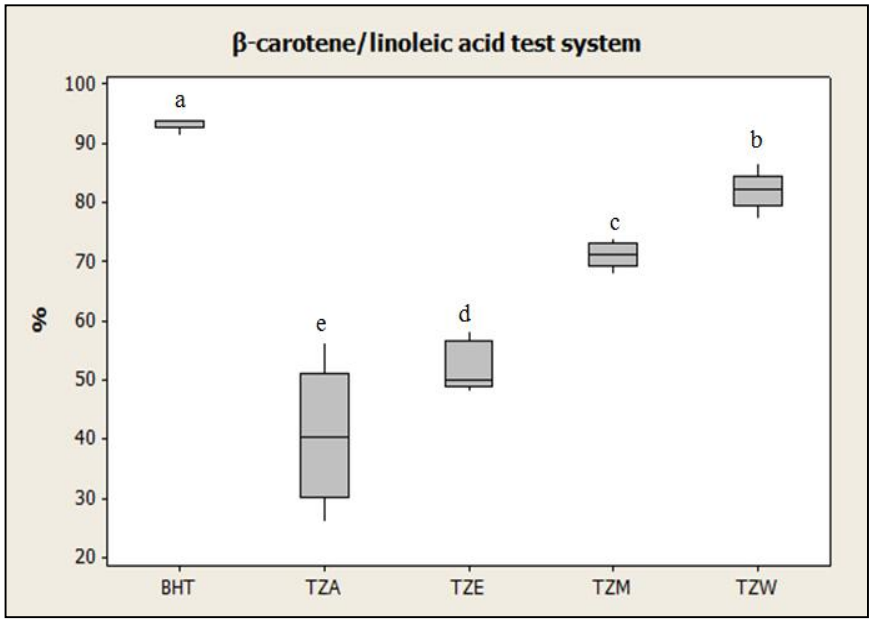

Fig. 1. Total antioxidant activity of different extracts of *T. zygioides*

TZE: Ethanol extract of T. zygioides; TZM: Methanol extract of T. zygioides; TZA: Acetone extract of T. zygioides TZW: Water extract of T. zygioides; BHT: Standard antioxidant (different groups were shown with different letters on each boxplot)

Table 1. Antioxidant activities of *T. zygioides*

Sample	DPPH (IC_{50}, µg/mL)*	ABTS (IC_{50}, µg/mL)*	Phosphomolybdenum (µg/mg)*	Power reducing (mg/mL)*
TZE	420.49 ± 10.9a	300.67 ± 19.5a	30.45 ± 1.42b	0.141 ± 0a
TZA	138.91 ± 6.01b	111.41 ± 6.85b	26.87 ± 0.47b	0.137 ± 0ab
TZM	20.82 ± 5.71d	11.44 ± 2.73c	45.63 ± 2.44a	0.132 ± 0c
TZW	60.81 ± 7.97c	12.53 ± 1.89c	43.27 ± 0.22a	0.134 ± 0bc
BHT	20.91 ± 2.20d	77.86 ± 4.12b	nt	nt

TZE: Ethanol extract of T. zygioides; TZM: Methanol extract of T. zygioides; TZA: Acetone extract of T. zygioides; TZW: Water extract of T. zygioides; BHT: Standard antioxidant; nt: not tested

**Values are mean of three replicate determinations (n=3) ± standard error. Mean values followed by different letters in a column are significantly different (p<0.05).*

Our results showed that the high antioxidant activities of the methanol and water extracts of *T. zygioides* are also due to the presence of high total phenolic contents, as has been previously reported [9]. The Phosphomolybdenum method has been used by Dessalegn [5] for the evaluation of antioxidant capacity in *T. schimperi Ronniger* and similar to our findings, they found that the antioxidant activities differed according to the solvents used and the methanolic extracts had higher antioxidant activities than the water extracts.

3.2 Radical Scavenging Activity (DPPH and ABTS)

The extract concentration that provided 50% inhibition (IC_{50}) and was expressed in µg/mL was calculated from the graph plotted inhibition percentages against the tested samples extracts. The lower the IC_{50} the higher the antioxidant activity of the examined sample. Among the studied extracts, the methanol extract (TZM) showed the strongest radical scavenging effect with IC_{50} values of 20.82 µg/mL (Table 1). There were significant differences in terms of the radical scavenging activity among the extracts($F_{4,40}$=553.94, P <0.001). According to the literature, some *Thymus* species have previously been investigated for their scavenging activity [12,13,32]. Radical scavenging activity for the methanol extract (TZM, 20.82 µg/mL) determined in this study was lower than that reported by Soares [32] (methanol extract of *T. zygis*) and Ismaili [12] (*T. saturcioides*). In addition, our findings are in accordance with the results of Barros [13], in which DPPH activity was found to be highest in the methanol extract. The methanol extract of *T. zygioides* showed a potency as effective as BHT in the scavenging of DPPH free radicals. This may be related to the high amount of total flavonoid and phenolic compounds in the methanol extract.

The ABTS scavenging capacity of the plant extracts was determined and the results are shown in Table 1. The values of IC_{50} were in the following order: TZM < TZW < BHT < TZA < TZE. Similar to DPPH, the methanol extract from *T. zygioides* showed the strongest radical scavenging effect with IC_{50} values of 11.44 µg/mL in the ABTS method. This activity was followed by TZW, TZA, and TZE. Although the difference between the TZW and TZM extracts was not statistically significant, with these extracts there was a statistical difference between TZE and TZA ($F_{4,40}$=154.77, P <0.001). These results are in agreement with Zengin and Aktümsek [33], who found a strong correlation between the DPPH and ABTS assays. Likewise, these findings are in line with previous reports [33] that showed a relatively strong correlation between total phenolic contents and free radical scavenging activities. A potent scavenger of free radicals may serve as a possible preventative for the disease. This is because exposure to free radicals from a variety of sources may increase the risk of disease such as cancer, heart diseases, diabetes, and infections [34]. For this reason, the present study revealed that the *T. zygioides* extracts could serve as free radical inhibitors or scavengers, and they may, therefore, gain pharmacological importance as powerful free radical scavengers.

3.3 Metal Chelating Activity

In this method, based on the chelating of Fe^{+2} with ferrozine, the complex is probably disturbed by the other chelating reagents, which results in the reduction of the color. The measurement of the color reduction estimates the chelating activity to compete with ferrozine for the ferrous ions [19]. The metal chelating effect of the samples was determined using Fe^{+2}solution, and the results were compared with EDTA. Among the extracts, the acetone extract (TZA, 54.26±0.71%) showed higher metal chelating activity compared with the methanol (TZM, 24.33±0.87%), water (TZW, 21.53±0.75%) and ethanol (TZE, 19.84±0.71%) extracts (Fig. 2). The TZA extract was statistically different from the other extracts ($F_{4,40}$=452.80, P <0.001).

The most important feature affecting the metal chelating activity is the functional groups found in the structure of the phenolic compounds. The phenolic compounds having at least two of the functional groups -OH, -SH, -COOH, $-PO_3H_2$, -C = O, $-NR_2$, -S- and -O- appear to have better metal chelating properties [35]. For this reason, the results obtained depend on the different functional groups of the samples, the amounts and the position of these groups. Chelating agents are effective as antioxidants because they reduce the redox potential which stabilizes the oxidized form of the metal ion [36]. Although all the extracts of *T. zygioides* had lower values than the standard, they have the potential to act as antioxidants by means of the metal chelating properties. The metal chelating method has been used by Orhan [10] for the evaluation of the

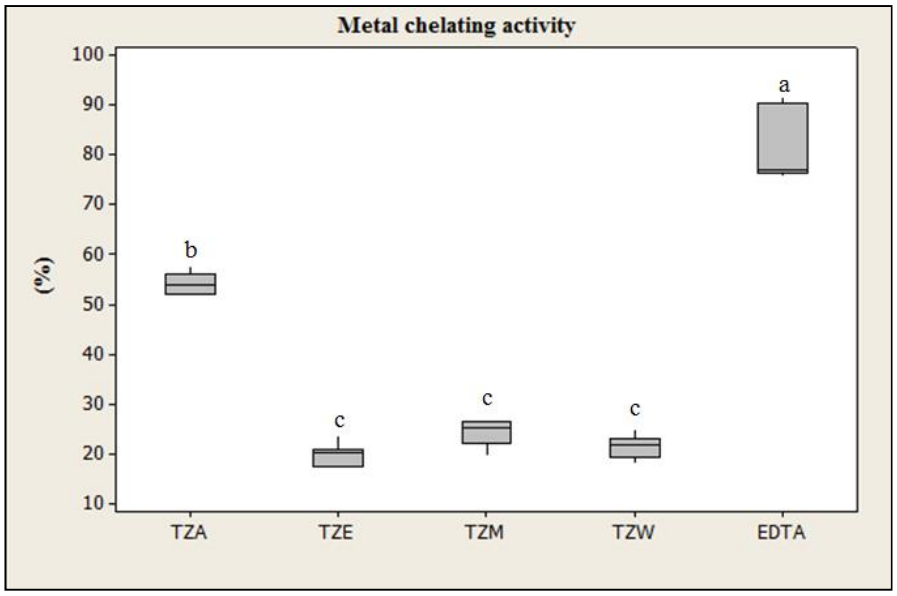

Fig. 2. Metal chelating activity of different extracts of *T. zygioides.*
TZE: Ethanol extract of T. zygioides; TZM: Methanol extract of T. zygioides; TZA: Acetone extract of T. zygioides TZW: Water extract of T. zygioides; EDTA: Standard antioxidant (different groups were shown with different letters on each boxplot)

chelating capacity of essential oil and various extracts *Thymus praecox subsp. caucasicus var. caucasicus*. Similar to our findings, they found that the water extract of this plant had a higher chelating activity than the ethanol extracts. To the best of our knowledge, no data is available concerning the metal chelating activity of *T. zygioides*. Here, for the first time, the chelating capacity determination of *T. zygioides* extracts was performed by measuring the iron-ferrozine complex. For this reason, this data will contribute to the study that will be made of the *Thymus* species and *T. zygioides*.

3.4 Reducing Power

The reducing ability of an extract may serve as an indicator of its potential antioxidant activity [20]. As can be seen from Table 1, the reducing power activity of the extracts was in the range of 0.132 - 0.141 mg/mL. All the *T. zygioides* extracts tested in the present study showed the strongest reducing power abilities. When the reducing power activities were compared for the four tested extracts, among the extracts the ethanol extract of *T. zygioides* showed the highest activity. The reducing power activity for the methanol extract determined in this study was lower than that reported by Jabri-Karoui [30] (*T. capitatus* flowers) and by Barros [13] (methanol extract of *T. mastichina*). These differences in the reducing power activities can be attributed the solvents that were used.

3.5 Total Phenolic, Flavonoid and Tannin Contents

Phenolic acids, flavonoids, and tannins are phenolic compounds that have been extensively studied in recent years. As a result of these studies, the phenolic compounds have proven antioxidant, antibacterial, antiviral, anticancer and antiallergic activities [5,7,34]. The richness of the phenolic compounds in plants indicate their pharmacological properties; hence, analyses of the phenolic compounds in plants are crucial for understanding their medicinal value.

The total phenolic content of the *T. zygioides* extracts ranged from 43.60 to 104.47 mgGAE/g (Table 2) and this was found to be statistically different ($F_{3,31}$=240.28 $p<0.001$). From our results, it was apparent that the amount of phenolic content in the extracts varied according to the type of solvent. In accordance with the previous study of *Thymus* species, phenolic content may vary significantly between the different solvents [11,13,14]. In our results, phenolic content was found to be highest in the methanol extract and these findings are in line with those of Barros [13], Lagouri [7] and Roby [14], who also found that methanol extracts have the highest phenolic content.

Table 2. Total phenolic, flavonoid and tannin content of *T. zygioides* extracts

Sample	Total flavonoid content (mgQEs/g)*	Total phenolic content (mgGAEs/g)*	Total tannin content (mgCEs/g)*
TZE	80.45 ± 2.42b	43.60 ± 0.80d	35.51 ± 2.43b
TZA	89.66 ± 1.08a	59.19 ± 0.34c	51.72 ± 2.68a
TZM	91.86 ± 0.65a	104.47 ± 2.56a	11.91 ± 1.04c
TZW	57.57 ± 1.12c	67.25 ± 1.79b	2.44 ± 0.25d

TZE: Ethanol extract of T. zygioides; TZM: Methanol extract of T. zygioides; TZA: Acetone extract of T. zygioides; TZW: Water extract of T. zygioides

**Values are mean of three replicate determinations (n=3) ± standard error. Mean values followed by different letters in a column are significantly different (p<0.05).*

Flavonoids can scavenge harmful active oxygen species and they are recognized as being beneficial antioxidants. In addition, flavonoids are important components of the human diet [34]. In the present study, the total flavonoids in the *T. zygioides* extracts were determined using the spectrophotometric method with aluminum chloride and the results obtained varied from 57.57 to 91.86 mg/g extract (Table 2). In comparison, the other *Thymus* species, the total flavonoid contents of the methanol, ethanol and water extracts from *T. zygioides* found in the present study are higher than those reported for the methanol, ethanol and water extracts of *T. mastichina* [13] and the methanol, ethanol and water extracts of *T praecox subs. skorpilii var skorpilii* [11]. The flavonoid contents of the ethanol extract of *T. zygioides* are higher than the ethanol extract of *T. diagenesis, T. kotschyanus and T. pubescens* [9] and the methanol, water and acetone extract of *T. zygioides* are higher than those of T. *schimperi Ronniger* [5]. The total amounts of the phenolic and flavonoid components are evidence of the high antioxidant capacity and all extracts of *T. zygioides* tested in the present study were rich in phenolic and flavonoid content.

In this study, tannin content was detected using the vanillin method and these results were evaluated as catechin equivalents. The total tannin content of *T. zygioides* extracts is presented in Table 2. The acetone extract contained the highest value of total tannin content, followed by the ethanol, methanol and water extract. There were significant differences in the means of the total tannin content among the extracts ($F_{3,32}$=140.58 p<0.001). The total tannin content of the methanol extract from *T. zygioides* found in the present study was higher than that reported for the methanol extract of the flowers of *T. capitatus* [30]. In addition, Bekir [23] extracted *Punica granatum* using different solvents and they found that the tannin content differed according to the solvents used. This is in general agreement with our results, which showed that tannin content varied according to the solvent used. There was no report on the total phenolic, flavonoid and tannin content of the extracts from *Thymus zygioides*. For this reason, the data will contribute to future studies on this species.

3.6 Phenolic Composition

Thymus species contain different phenolic compounds, such as caffeic acid, rosmarinic acid, and flavonoids [9]. In this study, the phenolic content of the ethanol extract of *T. zygioides* was identified using the HPLC method. The phenolic compounds that were determined in the ethanol extract are listed in Table 3. Some phenolic compounds determined in the present study, such as ferulic, gallic, caffeic, vanillic, chlorogenic and p-coumaric acids, were obtained from Thymus plants used in previous studies [14,30]. In addition, previously studies have shown that from these phenolics, caffeic acid (3,4-hydroxycinnamic) is one of the hydroxycinnamates. It acts as a carcinogenic inhibitor and is also known to possess antioxidant and antibacterial activity [37]. Phenolic compounds could directly contribute to the antioxidant activity of the plants [38] and therefore the identification and measurement of phenolic compounds in plants have become important tools in understanding the value of plants for human health [39].

3.7 Cytotoxic Activity

Brine shrimp (*Artemia salina*) is a convenient preliminary cytotoxicity method (practical and economic) for the investigation of assessments of toxicity, antifungal, antiparasitic, pesticidal effects and antitumor properties. In the toxicity evaluation of plant extracts using the brine shrimp lethality test, IC_{50} values lower than 1000

µgmL are considered bioactive [25]. The lethality of the ethanol extract of *T. zygioides* was 436.25 µg/mL and showed cytotoxic activity against brine shrimp. Thus, the lethality of this extract from *T. zygioides* indicates the presence of a potent cytotoxic component in this species that requires further investigation and this work could serve for further phytochemical research.

3.8 Anthelmintic Activity

The anthelmintic activities of the ethanol extract of *T. zygioides* were evaluated. The results in Table 4 show that the ethanol extracts obtained from *T. zygioides* are active against the tested *Tubifex tubifex*. The present study also shows that the increase in the dose results in an increase in the anthelmintic activity for the ethanol extracts. The ethanol extracts were effective in causing the death of the worms, as well as promoting paralysis. All the doses of the ethanol extracts of *T. zygioides* showed better anthelmintic activity than the standard.

The strong anthelmintic activity of the ethanol extract of *T. zygioides* may be due to the presence of rich polyphenolic compounds in its essential oil, which are monoterpenoid phenols, thymol, and carvacrol. As previous studies have indicated that several terpenes and terpenoids, such as thymol and carvacrol, which are commonly found in essential oils, have been identified as promising anthelmintics. In addition, the researchers have revealed that phenolic monoterpenes (thymol and carvacrol) are present in large amounts in *Thymus* plants [4,8,34]. As a result, many medicinal plants have been used to treat parasitic infections in humans and animals and the search for any anthelmintics derived from plant sources represents economic advantages, as well as advantages related to the pharmacological effectiveness and lower toxicity for animals and humans [40]. For this reason, studies on the screening of medicinal plants containing functional compounds that provide anthelmintic properties, in order to find new and effective sources, are very important. The wormicidal activity of this extract from *T. zygioides,* as described in this study, suggests that it could be effective against the parasitic infection of humans and animals. Therefore, lethality in this species of potent anthelmintic components which require further investigation.

Table 3. Phenolic components in the ethanolic extracts of *T. zygioides*

Phenolic component	Approximate Rt (min)	µg/g extract
Gallic acid	6.8	36.67
2,5 dihydroxybenzoic acid	17.2	55.57
Chlorogenic acid	18.2	61.36
3,4 dihydroxybenzoic acid	10.7	25.33
4-hydroxybenzoic acid	15.7	228.92
Cinnamic acid	71.1	107.19
Quercetin	70.4	1884.28
p-Coumaric acid	26.1	7.57
Ferulic acid	30.1	56.02
Caffeic acid	22.7	3624.69
Vanillic acid	19.2	73.55
Epicatechin	21.3	1196.92
Rutin	45.6	495.79

Table 4. *In vitro* anthelmintic activity of ethanol extract of *T. zygioides*

Type of extract	Concentration used (mg/mL)	Time (min) taken for paralysis (X ± S.E.)*	Time (min) taken for death (X ± S.E.)*
Control (Distilled water)	-	-	-
Ethanol	2.5	20.67 ± 1.12a	31 ± 1.34a
	5	9.5 ± 0.22b	19.5 ± 0.22b
	10	3 ± 0c	6 ± 0c
Albendazole (Standard)	2.5	35 ± 3.16a	40.17 ± 1.90a
	5	23.5 ± 2.08b	32 ± 0.63b
	10	13.67 ± 0.62c	22.33 ± 1.15c

Values are mean ± S.E. of six worms.

**Mean values followed by different letters in a column are significantly different ($p<0.05$).*

4. CONCLUSION

Interest in the antioxidant properties and phenolic compounds of various extracts taken from numerous plants, together with research studies, have considerably increased in recent years. Many *Thymus* species are widely used in the food and pharmaceutical industries [9]. Although the antioxidant activity of several members of genus *Thymus* has been reported [7,10-14], this is the first study on the antioxidant properties, total phenolic, flavonoid, and tannin content of methanol, ethanol, acetone, water extracts and the cytotoxic, anthelmintic activities of the ethanol extract of *T. zygioides*. These results have revealed that all the extracts from this plant have strong antioxidant properties, acting as free radical scavenging, metal chelating and that they also possess rich phenolic and flavonoid content. Moreover, the ethanol extract of *T. zygioides* has cytotoxic and anthelmintic activities. The antioxidant, cytotoxic and anthelmintic properties tested in this study of *T. zygioides* indicates the presence of potential bioactive compounds. These findings of *T. zygioides* will be the basis for future work on the purification and identification of the active compounds, which may be useful for medicinal purposes and on the potential use of this plant for nutrition.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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