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IN VITRO ANTIOXIDANT, ANTIMICROBIAL, ANTICANCER ACTIVITIES ASSESSMENT OF *THYMUS PECTINATUS*, SCREENING OF ENZYME INHIBITORY

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ABSTRACT

According to the data obtained in this study, *Thymus pectinatus* has antioxidant and enzyme inhibitory activities while has not showed antimicrobial activities. The results of the current study provide valuable information, showing that the major component of the water extract of *T. pectinatus* is "1,3-Propanediol, 2-methyl-, dipropanoate" (59.34 %). This is the first investigation of the antioxidant and enzyme inhibitory activity of *T. pectinatus*. The results showed that the water extract had high antioxidant, anti-amylase, and anticancer effects, and a higher total phenolic content. Therefore, further phytochemical and bioactivity-guided isolation of *T. pectinatus* water extract could be carried out to identify the active compounds.

KEYWORDS:
Thymus pectinatus, Antimicrobial, Antioxidant, Enzyme inhibitory, Anticancer

INTRODUCTION

Thymus is a perennial plant of the Lamiaceae family [1]. This genus, known as "thyme" in English and "kekik" in Turkish, has 38 species and 64 taxa, 24 of which are endemic in Turkey [2,3]. The leaves of *Thymus* are used as alternative medicine in bronchitis, arthritis, rheumatism, and for the removal of intestinal gas [4]. It has been reported to have antimicrobial and antioxidant activities [4-6].

In recent years, the essential oil or extracts of medicinal and aromatic plants have been the focus of increasing interest in therapy and phytotherapy fields because of the abundant secondary metabolite content [4,7-9]. The use of these components is increased in particular in the treatment of or protection against diseases such as cancer, Alzheimer's disease, diabetes mellitus, cardiovascular disease, and to delay the aging process. The components can show different physiological activity on living [10-12]. The effects may be due to only one compound or the synergistic effect of several compounds. Phenols; it is a chemical component in essential oils that has antioxidant effects and provides the body with protection from reactive oxygen types and damage caused by oxidative stress caused by free radicals [13,14].

Alzheimer's disease and Diabetes mellitus (DM) are major diseases in the general population. Butyrylcholinesterase (BChE) and Acetylcholinesterase (AChE) are effective target enzymes in the treatment of Alzeihmer's disease. In DM treatment, α-amylase and α-glucosidase play an important role in hydrolyzing carbohydrates that lead to an increase in the blood glucose level. Therefore, people have to use such enzyme inhibitors to control and regulate levels of enzymes which are related to Alzheimer's disease and diabetes [15]. Drugs used for such diseases are available, but there are no specific solutions, and they have many side-effects. This has motivated researchers to search for new plants and their active substances for the treatment of such diseases.

Cancer is a disease that has a fatal outcome worldwide and develops due to multiple variable causes. The frequency of different types of cancer in men and women can vary. While breast cancer occurs in 1 out of every 10 women, this rate is much lower in men and the treatment touches are generally not at pleasant levels. Traditional treatments used in the clinic have several serious side effects and can cause damage to non-cancerous tissues [16]. Hereby, the usage of the essential oil or extracts of medicinal and aromatic plants has crucially increased in recent years [17].

The *Thymus* plant is readily available as it is widely consumed by the public. Although the plant is known to be useful, there have been no published studies of the effects of the water extract of the essential oil of *T. pectinatus* on antioxidants and inhib-

itory enzyme activity. In this study, it is aimed to determine the different biological properties of *Thymus pectinatus*, which is an endemic species, such as antioxidant, antimicrobial activity, activity of enzyme and cytotoxicity.

MATERIALS AND METHODS

Plant materials and preparation of extracts. *Thymus pectinatus* plant materials, an endemic region in terms of wild plants in Turkey, which were collected from Sivas. (B6 Sivas: Sivas-Karayün 25 km, roadside, 25 June 2015, Akpulat 4589). Antimicrobial activity and cytotoxicity experiments were conducted at the Faculty of Pharmacy laboratories while *in vitro* antioxidant and enzyme inhibition activity tests were carried out at the Advanced Technology Research and Application Center (CÜTAM), Sivas Cumhuriyet University, Sivas, in 2019.

The Chemical Composition. The plants were dried and ground with a blender (Blue house). 10 g of dried plant sample was taken and soaked in 50 mL of water for 24 hours with intermittent agitation. After the extract was filtered, it was dried in a furnace at 40 ° C. The extracts obtained were analyzed by GC-MS method known as Gas Chromatography-Mass Spectrometry.

***In vitro* Antioxidant Activity.** The antioxidant activity of the water extract of *Thymus pectinatus* aerial parts was tested using different methods. The DPPH radical scavenging activity of the extract was evaluated according to the Blois method (1958) with slight modification. ABTS radical scavenging activity was evaluated by the method of Re et al. (1999) with minor modifications [18]. Total phenolic content was determined using the Folin-Ciocalteu spectrophotometric method and expressed as milligrams of gallic acid equivalents per gram of the dry weight of the extract [19]. The flavonoid content was determined with the aluminum chloride colorimetric method of Molan and Mahd (2014) [20]. The content of total flavonoids was expressed as milligrams of catechine equivalent per gram of the dry weight of the extract.

***In vitro* enzyme inhibition assay.** The AChE/BChE inhibition assay was carried out according to the Ellman method as described by our previous study [21,33]. The α-glucosidase inhibition method was reported by Kumar et al. (2012) [22]. The α-amylase inhibition activity of the extract was investigated by the method reported by Kumar et al. (2013) [23]. In both of the α-glucosidase and α-amylase inhibition method, acarbose was used as a positive control. In the same time, tyrosinase inhibitory activity was determined using the 96-well plate spectrophotometric method as described by Jeong et al. (2009) with slight modifications [24].

In vitro cytotoxicity assay. The cytotoxicity of the *Thymus pectinatus* water extract was tested against MDA-MB-231 (human breast adenocarcinoma) and L-929 (mouse fibroblast) cell lines. Both cell lines were cultured in DMEM containing 10% FBS, 1% L-glutamine, 100 IU/mL penicillin, and 10 mg/mL streptomycin in 25cm^2 polystyrene flasks and sustained in a humidified atmosphere with 5% CO_2 at 37°C. Cells were passaged when they had reached 85-90% confluence.

Antiproliferative activity of the extract was evaluated on the MDA-MB-231 and L-929 cell lines by the XTT cell proliferation method. Initially, the cells were seeded at a density of 5x10^3 cells per well in 96-well culture plates in 100 µl of culture medium and were allowed to attach overnight before treatment. Then these cells were treated with various concentrations (0,0625, 0,125, 0,25, 0,5, 1 mg/ml) of extract for 24 h. Following treatments, the medium was removed and wells were washed twice with 200 µl phosphate-buffered saline (PBS). At the end of these periods, for determination of living cells, 100 µl DMEM without phenol red and 50 µl XTT labeling mixture were added to each well, and then the plates were incubated for another 4 h. The absorbance of XTT-formazan was measured using a microplate (ELISA) reader at 450 nm against the control (the same cells without any treatment). All experiments were carried out as three different independent experiments and cell viability was expressed as % of control (100% viability).

Antimicrobial Activity. Microdilution broth method. The broth microdilution method was applied to 96-well microtiter plates, and the minimum inhibitory concentration (MIC) of *T. pectinatus* was determined [25]. The bacterial and yeast test strains used in this study were *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus cereus* (ATCC 27853), *Candida albicans* (ATCC10231) and *Candida tropicalis* (DSM11953). Mueller-Hinton broth (Accumix®AM1072) was utilizated as a culture media for bacteria and Sabouraud Dextrose Broth (Himedia ME033) was used for Candida spp. [26,27].

The extract was dissolved in DMSO (50 mg/mL). 90 µl of media were added to the first row of the microtiter plates and 50 µl of the remaining wells. The 11th wells were used as the reproductive controls and 100 µL broth was added. 10 µL extract was added in the first line of the microtiter plate and serial two-fold dilutions were prepared. The extract concentration in the wells was between 2.5 and 0.004 mg/mL. The bacteria and fungi suspensions (50µL) were added to prepared samples. The final inoculum size was 5×10^5 CFU/mL in the bacteria wells and

0.5-2.5×10^3 CFU/mL in the Candida sp. wells (CLSI, 2002, CLSI, 2012). The MIC concentration of the extract was determined as the lowest concentration that prevents visible growth of bacteria and yeast after incubation at 37°C overnight.

RESULTS AND DISCUSSION

The Chemical Composition. In the water extract of *Thymus pectinatus*, eight components were obtained by GS-MS method. 1,3-propanediol, 2-methyl-, dipropanoate (59.34 %) was determined as the major component (Table 1). The following main components are; hexadecanoic acid, methyl ester (CAS) (7.87 %), propanoic acid, pentyl ester (7.00 %), 2-methoxy-4-vinyl phenol (4.20 %), thietane (2.66 %), benzene, 1-(1,1-dimethyl ethoxy)-4-methyl (2.44 %), 2-oxazolidinethione, 4,4-dimethyl (1.81 %), 2-hexenal, (E)- (CAS) (1.10 %). Vardar-Ünlü et al. (2003) reported that the essential oil of *Thymus pectinatus* was analyzed with GC-MS and thymol, γ-terpinene, p-cymene, carvacrol, and borneol were identified as major components [28].

Antioxidant activity. DPPH and ABTS radical scavenging activity. *In vitro* antioxidant activities were determined by comparing the total phenolic and flavonoid contents of *Thymus pectinatus* water extract with DPPH and ABTS radical scavenging activity with standard antioxidant components, BHT and BHA. The lower the IC_{50} value, the higher the radical scavenging activity of the extract. The water extract showed DPPH radical scavenging activity with the IC_{50} value of 120.52 ± 0.36 µg/mL and ABTS radical scavenging activity with the IC_{50} value of 59.39 ± 1.69 µg/mL, although these values were lower than the reference BHT and BHA (8.68 ± 0.16 and 6.93 ± 0.65 µg/mL, respectively) (Table 2). Other investigators have confirmed that *T. pectinatus* has powerful antioxidant activity [4;28]. As a result of DPPH analysis, it was determined that methanol extract (IC50 = 76.24 ± 1.84 µg / mL) had better activity than water extract (168.64 ± 0.91 µg / mL)

Total phenol and flavonoid content. As shown in Table 2, the total phenolic content values for the water extract of *T. pectinatus* was 57.47 ± 4.07 mg gallic acid equivalents (GAE)/g dried extract, while total flavonoid content value was 4.06 ± 0.87 mg catechin equivalents (CE)/g dry weight of the extract. Phenolic compounds have antioxidant properties and protect living organisms from oxidative damage [29-31].

Enzyme Inhibiton Activity. Anti-cholinesterase Activity. Both AChE and BChE play important role in controlling against Alzheimer's disease and related dementia. When the inhibitory activity of AChE and BChE of the water extract of *T. pectinatus* was examined, the AChE inhibitory activity was 50.92 ± 4.85 %, and no BChE inhibition activity was observed (Table 3). But the AChE inhibitory activity was lower than that of the reference drug Galanthamine (93.87 ± 0.56).

TABLE 1
The chemical composition of the water extract of *Thymus pectinatus*

No	RT	Components	Relative percentage (%)Water Extract
1	4.740	Thietane	2.66
2	5.925	Propanoic acid, pentyl ester	7.00
3	6.102	1,3-Propanediol, 2-methyl-, dipropanoate	59.34
4	6.755	2-Oxazolidinethione, 4,4-dimethyl	1.81
5	18.714	2-Hexenal, (E)- (CAS)	1.10
6	22.633	2-Methoxy-4-vinylphenol	4.20
7	32.601	Benzene, 1-(1,1-dimethylethoxy)-4-methyl	2.44
8	37.739	Hexadecanoic acid, methyl ester (CAS)	7.87
Total			**86.42**

TABLE 2
***In vitro* antioxidant activities of the water extract of *T. pectinatus* (IC_{50} value in µg/mL)**

Extracts	DPPH Radical Scavenging Activity	ABTS Radical Scavenging Activity	Total Phenolic Content	Total Flavonoid Content
Water	120.52 ± 0.36	59.39 ± 1.69	57.47 ± 4.07	4.06 ± 0.87
Reference Drugs				
BHT	8.68 ± 0.16	-		
BHA	-	6.93 0.65		

TABLE 3
In vitro* enzyme inhibitory activities of the water extract of *Thymus pectinatus

Extracts	*Anticholinesterase Activity*		*Antidiabetic Activity*		*Skin Whitening*
	AChE	*BChE*	*a-Glucosidase*	*a-Amylase*	*Tyrosinase*
Water	50.92± 4.85	-	49.41±1.94	17.34±1.50	78.55±0.30
Reference Drugs					
Galanthamine Hydrobromide	93.87± 0.56	89.89± 0.01			
Acarbose			57.56±0.52	58.40±0.63	
Kojic Acid					56.42 ±1.59

α-Glucosidase and α-Amylase Inhibition Activity. α-Glucosidase and α-Amylase are catalyzing the hydrolysis of polysaccharides and disaccharides to monosaccharides in digestive organs, therefore, they can be a therapeutical approach to treat diabetes mellitus by reducing postprandial hyperglycemia [21]. The inhibitory activity of the water extract of *T. pectinatus* was evaluated against α-glucosidase and α-amylase enzyme (Table 3) in comparison with the positive control drug acarbose. According to the data, the water extract demonstrated vigorous inhibitory activity both against α-glucosidase (49.41 ± 1.94%) and α-amylase (17.34 ± 1.50%) compared to the reference drug acarbose (57.56 ± 0.52% and 58.40 ± 0.63%, respectively) at the same concentration.

Tyrosinase Inhibitory Activity. The tyrosinase inhibitory activities of water extracts of *T. pectinatus* are presented in Table 3 as a percentage. The study results revealed that water extract showed higher tyrosinase inhibitory activity (78.55 ± 0.30%) than the kojic acid, which was used as the positive control and showed an inhibition level of 56.42 ± 1.59% at the same concentration (i.e., 2 mg/mL).

Cell viability. *In vitro* cytotoxicity of the water extract of *T. pectinatus* was appreciated on MDA-MB-231 and L-929 cell lines by XTT assay and results are shown in Figure 1. According to experimental results, in the presence of water extract at all concentrations reduced significantly MDA-MB-231 cell proliferation ($p<0.05$) in a dose-dependent manner when compared with the control group. The IC_{50} of the water extract of *T. pectinatus* in MDA-MB-231 cell lines was calculated as 0.206 mg/mL. Conversely, the water extract did not show evident cytotoxicity on the L929 cell line at the IC_{50} concentrations. In general, the results suggest that *T. pectinatus* extracts may have strong anti-proliferative activity against MDA-MB-231 cell lines and may be a potential anticancer agent. However, in order to evaluate this possibility correctly, the anticancer properties of *T. pectinatus* need to be further investigated.

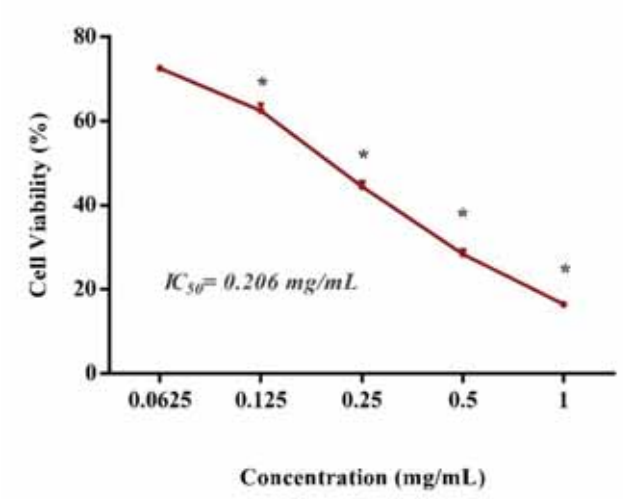

FIGURE 1
Effects of water extract from *T. pectinatus* on viability of MDA-MB-231 cell line, after treatment with different concentrations (range: 0,065-1 mg/mL) for 24 h.

TABLE 4
The antimicrobial activity values of *Thymus pectinatus* water extract

Microorganisms and MIC values (mg/mL)

	E. coli	*S. aureus*	*P. aeruginosa*	*B. cereus*	*C. albicans*	*C. tropicalis*
	ATCC 25922	ATCC 29213	ATCC 27853	ATCC 11778	ATCC 10231	DSM 11953
Thymus	2,5	2,5	>2,5	>2,5	>2,5	2,5

Antimicrobial Activity. The antimicrobial activities of *Thymus pectinatus* water extract against *E. coli, S. aureus, P. aeruginosa, B. cereus, C. albicans and C. tropicalis* were detected using the microdilution technique at the concentration range 2,5 to >2.5mg/mL (Table 4).

In the study conducted by Holetz et al in 2002, antimicrobial activity is good if the MIC of the extract was less than 100 µg/ml; It is reported to be moder at between 100 and 500 µg/ml and weak at between 500 and 1000 µg/ml. Values above 1000 µg/ml are considered inactive [32]. According to these criteria, the water extract of *Thymus pectinatus* not showed antimicrobial activities on tested microorganisms.

CONCLUSION

As far as we know, this study is the first study of the antioxidant, antimicrobial and enzyme inhibitory activity of *T. pectinatus* water extract. In the light of the results obtained, it was seen that water extract has high antioxidant, anti-amylase and antiproliferative effects. The water extract also showed a higher amount of total phenolic content. However, it was determined that the water extract of the plant did not have antimicrobial effect. Therefore, further studies are needed for phytochemical and bioactivity-guided isolation of *T. pectinatus* to identify active compounds.

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