J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 50(1): 58-69, 2026
Doi: 10.33483/jfpau.1576004
Research Article / Araştırma Makalesi

ANTIDIABETIC AND ANTI-ALZHEIMER POTENTIAL OF THREE *TYMBRA* SPECIES RICH IN HESPERIDIN AND ROSMARINIC ACID

HESPERİDİN VE ROSMARİNİK ASİT AÇISINDAN ZENGİN ÜÇ TYMBRA TÜRÜNÜN ANTİDİYABETİK VE ANTI-ALZHEİMER POTANSİYELİ

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ABSTRACT

Objective: *Lamiaceae is a cosmopolitan family of aromatic and medicinal plants. There are many well-known aromatic, ornamental and weed plants in this family. Many aromatic species in the Lamiaceae family are currently used in traditional medicine, pharmaceutical and food industries due to their biological properties. In this study, it was aimed to examine the ethanol extracts (roots and aerial parts) of Thymbra spicata var. spicata (TS), Thymbra sintenisii Bornm. &Aznav. subsp. sintenisii Bornm. &Aznav (TSS) and Thymbra sintenisii Bornm. & Azn. subsp. isaurica P.H.Davis (TSI) in the terms of their biological activity and chemical content.*
Material and Method: *The phenolic content of the species was analyzed by LC-MS/MS. In addition, cytotoxic, antioxidant, acetylcholinesterase, butyrylcholinesterase, urease, tyrosinase, elastase, collagenase, angiotensin I-converting and α-amylase enzymes activities were investigated.*
Result and Discussion: *It was determined that all ethanol extracts were rich in hesperidin (3900.28-32910.02µg analyte/g extract) and rosmarinic acid (5343.08-11571.54µg analyte/g extract). In addition, the aerial part of TS species showed high α-amylase enzyme inhibition activity (inhibition%: 97.57±2.59 at 50 µg/ml). Due to their high antioxidant, anticholinesterase and α-amylase enzyme inhibition activities and rich phenolic content, tymbra species have potential for use in the pharmaceutical and food industry.*
Keywords: *α-amylase, anticholinesterase, antioxdiant, LC-MS/MS, Thymbra*

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Submitted date: 30.10.2024 **Accepted date:** 11.06.2025 **Published date:** 19.01.2026
Citation: Yigitkan,S., Cavus Kaya, E., Fırat M., Koseoglu Yılmaz, P., Yener, I., Yılmaz, M.A., Irtegun Kandemir, S. Kolak, U., Temel, H., Ertas, A. (2026). Antidiabetic and anti-Alzheimer potential of three *Tymbra* species rich in hesperidin and rosmarinic acid. Journal of Faculty of Pharmacy of Ankara University, 50(1), 58-69.

ÖZ

Amaç: *Lamiaceae aromatik ve tıbbi bitkilerden oluşan kozmopolit bir familyadır. Bu familyada çok iyi bilinen aromatik, süs ve yabani ot bitkileri bulunmaktadır. Lamiaceae familyasındaki birçok aromatik tür, biyolojik özellikleri nedeniyle günümüzde geleneksel tıp, ilaç ve gıda endüstrilerinde kullanılmaktadır. Bu çalışmada, Thymbra spicata var. spicata (TS), Thymbra sintenisii Bornm. &Aznav. subsp. sintenisii Bornm. &Aznav (TSS) ve Thymbra sintenisii Bornm. & Azn. subsp. isaurica P.H.Davis (TSI) türlerinin etanol ekstraktlarının (kök ve toprak üstü kısımları) biyolojik aktivite ve kimyasal içerik açısından incelenmesi amaçlanmıştır.*

Gereç ve Yöntem: *Türlerin fenolik içeriği LC-MS/MS ile analiz edilmiştir. Ayrıca, ekstraktların sitotoksik, antioksidan, asetilkolinesteraz, bütirilkolinesteraz, üreaz, tirozinaz, elastaz, kollajenaz, anjiyotensin I-dönüştürücü ve α-amilaz enzimlerinin inhibisyon aktiviteleri araştırılmıştır.*

Sonuç ve Tartışma: *Tüm etanol ekstraktlarının hesperidin (3900.28-32910.02µg analit/g ekstrakt) ve rosmarinik asit (5343.08-11571.54µg analit/g ekstrakt) bakımından zengin olduğu belirlenmiştir. Ayrıca, TS türlerinin toprak üstü kısmı yüksek α-amilaz enzim inhibisyon aktivitesi göstermiştir (% inhibisyon: 50 µg/ml'de 97.57±2.59). Yüksek antioksidan, antikolinesteraz ve α-amilaz enzim inhibisyon aktiviteleri ve zengin fenolik içerikleri nedeniyle, tymbra türleri ilaç ve gıda endüstrisinde kullanım potansiyeline sahiptir.*

Anahtar Kelimeler: *α-amilaz, antikolinesteraz, antioksidan, LC-MS/MS, Thymbra*

INTRODUCTION

Plant extracts have been utilized by humans for eliminate the public health problems. The Lamiaceae family is a diverse and widely distributed plant group comprising mainly shrubs and herbs, many of which are renowned aromatic, ornamental, or weed species, such as basil, sage, mint, and thyme [1]. The Lamiaceae family contains over 245 genera and approximately 7886 species [2]. Numerous aromatic species from this family are extensively employed in traditional medicine and the pharmaceutical and food industries due to their significant biological properties. Thyme, rosemary, and sage are notably utilized medicinally within this family, primarily for their circulatory, digestive, nervous system-strengthening, expectorant, antispasmodic, antiseptic, diuretic, and carminative effects [3,4].

The genus *Thymbra* L., belonging to the Lamiaceae family (Labiatae), is economically important and consists primarily of dicotyledon dwarf shrubs typically found in arid and mountainous areas [5]. Globally, *Thymbra* is represented by four species: *Thymbra calostachya* (Rech.f.) Rech.f., *Thymbra capitat*a L. Cav., *Thymbra sintenisii* Born. et Aznav., and *Thymbra spicata* L. var. *spicata* L. [2,4]. In the Mediterranean region of Türkiye, these species are commonly known as Karabaş kekik, Siyah kekik, or Zahter [6]. In Türkiye, the genus *Thymbra* contains five taxa across three species: *T. capitata*, *T. sintenisii* subsp. *sintenisii*, *T. sintenisii* subsp. *isaurica*, *T. spicata* subsp. *intricata*, and *T. spicata* subsp. *spicata*. Among these, *T. sintenisii* subsp. *isaurica* and *T. spicata* subsp. *intricata* are endemic [7].

Previous studies have identified major bioactive components of *Thymbra* species, including hesperidin, rosmarinic acid, syringic acid, carvacrol, protocatechuic acid, rutin, naringenin, quercetin, carnosic acid, and apigenin [8-10]. Literature indicates that *Thymbra* species possess substantial phenolic content, contributing to their potent antioxidant and antimicrobial activities [11-15].

In this study, the phenolic contents of the ethanol extracts of the aerial and root parts of TS, TSS and TSI species which are widely used to eliminate health problems among the public and have economic value, were determined by LC-MS/MS. Additionally, the ethanol extracts of these *Thymbra* species were analyzed for their antioxidant effects using the ABTS, CUPRAC, and DPPH methods. The anticholinesterase activity was assessed through the acetylcholinesterase and butyrylcholinesterase enzyme inhibition assays. In addition, the extracts were analyzed for their inhibitory effects on urease, tyrosinase, elastase, collagenase, ACE (antihypertensive), and α-amylase enzymes. Furthermore, using the MTT method, the toxic effects of these extracts on healthy cell lines (PDF) and their cytotoxic effects on HT-29 (colon cancer) and MCF-7 (breast cancer) cell lines were assessed.

MATERIAL AND METHOD

Plant Material Collection and Identification

TS, TSS and TSI samples from Siirt and Antalya were collected and identified by Dr. Mehmet Firat. The herbarium records of the plant species, the information about collection locations and the extract codes were detailed in Table 1.

Table 1. Information about of studied *Thmbra* species

Samples	Abbreviations	Yield (%)	Collection location	Collection time	Herbarium number
***T. spicata* var. *spicata* root ethanol extract**	TSR	16.77	Siirt	August 2014	M. Fırat (VANF) 31098
***T. spicata* var. *spicata* aerial part ethanol extract**	TSAP	11.87			
***T. sintenisii* subsp. *sintensii* root ethanol extract**	TSSR	13.09	Siirt	August 2014	M. Fırat (VANF) 31112
***T. sintenisii* subsp. *sintensii* aerial part ethanol extract**	TSSAP	15.98			
***T. sintenisii.* subsp. *isaurica* root ethanol extract**	TSIR	11.99	Antalya	July 2014	M.Fırat (VANF) 31082
***T. sintenisii.* subsp. *isaurica* aerial part ethanol extract**	TSIAP	13.75			

Preparation of Ethanol Extracts

The root and aerial parts of the collected *Thymbra* species were dried in the shadow for chemical content analysis and biological activity tests. After the dried species were ground, 5 g of plant materials from each species was weighed. The extracts used for chemical and biological activity tests were prepared by macerating various parts of each species with ethanol (50 ml, 3x8 h). After filtration and evaporation of the solvent medium, the yields of crude extracts were given in Table 1. After dissolving the crude extracts in ethanol, 4 ml solutions were prepared at a concentration of 4000 mg/l. For LC-MS/MS analysis, 1000 mg/l samples were prepared from the prepared ethanol extracts. Before the samples were given to the devices, the extract solutions were transferred to HPLC vials by passing through a 0.22 um syringe tip filter [16].

LC-MS/MS Analysis and Chromatographic Conditions

Qualitative and quantitative analyses of 37 phytochemicals were performed using Shimadzu Nexera UHPLC coupled with triple quadrupole LC-MS/MS 8040 systems, following previously validated methods. Limits of detection (LOD) and quantification (LOQ) ranged between 0.05-25.8 µg/l and 0.17-85.9 µg/l, respectively, with recoveries between 96.9−106.2% [16-18].

Antioxidant Activity Tests

The antioxidant capacities of the TS, TSS, and TSI samples were evaluated using the ABTS, CUPRAC, and DPPH methods [19-21].

DPPH Free and ABTS Cation Radical Scavenging Activity Method

0.1 mM 160 μl of DPPH solution in ethanol was added to 40 μl of sample solutions in ethanol at different concentrations. After 30 min. the absorbance values were read at 517 nm [19].

Seven milimolar ABTS in H_2O was added to 2.45 mM potassium persulfate to produce $ABTS^{\bullet+}$ and solution was stored in the dark at 25ºC for 12 h. The prepared solution was diluted with ethanol to get an absorbance of 0.700±0.025 at 734 nm. $ABTS^{\bullet+}$ solution (160 μl) was added to each sample solution at different concentrations. After 30 min, the percentage inhibition at 734 nm was read for each concentration relative to a blank absorbance (ethanol) [20].

Cupric Reducing Antioxidant Capacity (CUPRAC) Method

The extracts were dissolved in ethanol, to prepare their stock solution at 1000 μg/ml concentration. Aliquots of 61 ml of 1.0×10^{-2} M copper (II) chloride, 61 μl of NH_4OAc buffer (1 M, pH 7.0), and 61 μl of 7.5×10^{-3} M neocuproine solution were mixed, x µl sample solution (2.5, 6.25, 12.5, and 25 μl) and (67 − x) μL distilled water were added to make the final volume 250 μl. The tubes were stopped, and after 1 h, the absorbance at 450 nm was measured against a reagent blank [21].

Anticholinesterase Inhibition Activity

The anticholinesterase enzyme activities were determined using Ellman et al.'s [22] method. Aliquots of 150 μl of 100 mM sodium phosphate buffer (pH 8.0), 10 μl of sample solution and 20 µl BChE (or AChE) solution were stirred and incubated for 15 min at 25ºC, then DTNB (10 μl) is added to mixture. In the next step, by the addition of butyrylthiocholine iodide (or acetylthiocholine iodide) (10 μl) the reaction was started. At the end, final concentration of the tested solutions was 200 μg/ml. BioTek Power Wave XS at 412 nm was used to monitor the hydrolysis of these substrates [22].

Urease and Tyrosinase Inhibition Activities

Urease enzyme activity and tyrosinase enzyme activity in the ethanol extracts of the species were assessed using the methods described by Zahid et al. [23] and Hearing and Jimenez [24], respectively.

Twenty-five microliters of urease (Jack bean) solution was mixed with 10 μl of each samples (4000 μg/ml) and incubated at 30°C for 15 min. Aliquots were taken and immediately transferred to assay mixtures containing urea (100 mM) in buffer (50 μl) and reincubated for 30 min in 96-well plates. Forty-five microliters from each of phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and 70 μl of alkali reagent (0.5% w/v sodium hydroxide and 0.1% sodium hypochlorite) were added to wells. Increase in absorbance was measured after 50 min at 630 nm against blank. All reactions were performed in triplicates. Thiourea was used as a positive control.

Briefly, the samples were screened for the o-diphenolase inhibitory activity of tyrosinase using L-DOPA as substrate. All samples were dissolved in ethanol to reach to a concentration of 4000 µg/ml. One hundred fifty microliters of phosphate buffer (pH = 6.8), 10 µl of the samples and 20 µl of the enzyme solution were added to the wells in the microplate, and the initial absorbance at 475 nm was read after stirring for 3 min. This solution was then incubated for 10 minutes at 37°C, after 10 minutes 20 µl of L-DOPA was added and incubated again at 37°C, after 10 minutes the final absorbance at 475 nm was read in the Microplate ELISA reader. Tyrosinase activity (inhibition%) was calculated using the following equation. Kojic acid was used as a standard inhibitor for tyrosinase inhibition.

Elastase and Collagenase Inhibition Activities

The ethanol extracts of the samples were used to determine elastase inhibitory activity and collagenase enzyme activity. The methods used were developed by Kraunsoe et al. [25] and Thring et al. [26] respectively, with minor modifications.

For the elastase inhibition activity of the extracts; the method developed by Kraunsoe et al. was applied with minor modifications. 10 μl of sample and 20 μl of elastase enzyme solutions were added to 40 μl (0.1 M Tris-Cl, pH=8) buffer solution and incubated for 10 minutes (37ºC). Afterwards, 30 μl of 1.015 mM substrate (N-succinyl-(Ala)-3-nitroanilide) solution prepared with buffer solution (0.1 M Tris-Cl, pH=8) was added and incubated at 37ºC for 20 minutes. Then absorbance values were measured at 410 nm. Oleanolic acid was used as standard reference material.

For the collagenase inhibition activity of the extracts; the method was performed with minor modifications to the protocol developed by Thring et al. 20 μl of the sample solution prepared in DMSO, 10 μl of collagenase enzyme solution (0.8 U/ml) and 50 μl of phosphate buffer (pH: 7.5) were added to the microwells and incubated at 25ºC for 15 min. Then, 20 μl of substrate solution (N-(3-[2-Furyl]acryloyl)-Leu-Gly-Pro-Ala) was added and incubated at 25ºC for 20 min and absorbance values were measured at 340 nm. Epicatechin gallate was used as standard reference material.

ACE Enzyme Inhibition Activity

The modified method described by Kwon et al. [27] was employed to determine the ACE (Angiotensin I-converting Enzyme) activity of the the species. 50 microliters of sample solution were

incubated with 200 μl of NaCl-borate buffer solution (0.3 M NaCl, pH 8.3) containing 2 mU of ACE at 25°C for 10 min. After incubation, 100 μl of 5.0 mM substrate (hippuryl-histidyl-leucine) was added to the reaction solution and the solution was incubated at 37°C for 1 h. Blank sample (containing buffer solution instead of enzyme and substrate), control (containing distilled water instead of sample extract) and blank (containing sample extract and buffer instead of enzyme) were also analyzed. The reaction was terminated by adding 150 of 0.5 N HCl and the determination of the resulting hippuric acid was performed by high-performance liquid chromatography with an ultraviolet detector (HPLC-UV) at 228 nm. Lisinopril was used as standard reference molecule [27].

α-Amylase Inhibition Activity

The modified Caraway-Somogyi iodine/potassium iodide (IKI) method was applied for α-Amylase inhibitory activity determination [28]. Sample solution mixed with α-amylase solution in buffer and incubated at 37°C for 10 min. incubated. Starch was used as a substrate in the method and the last absorbances were measured at 565 nm.

Cytotoxic Inhibition Activity

For this study, human healthy and cancerous cell lines, including PDF, MCF-7, and HT-29, were selected. These cell lines were obtained from ATCC and cultured to enable adequate cell growth for subsequent molecular and biochemical investigations [29]. Twenty-two thousand cells for MCF-7, 20.000 cells for HT-29, and 12.000 cells for PDF were added into the 96-well plates. After incubation for 24 hours, the cells were treated with 10 µl of extracts prepared at different concentrations (10, 25, 50, 100, 200, μg/ml) for 48 h. After addition of 10 µl of MTT reagent, and then incubation for 4 h., purple precipitate occured. A hundred microliters of detergent reagent were added. The plate was incubated in the dark for overnight in humidified atmosphere (37°C, 5% CO_2). The absorbances of the samples were measured using a microplate (ELISA) reader at 570 and 690 nm. Measurements at 690 nm were used as reference absorbances. MTT assay was performed in 3 parallel for each concentration and each MTT assay was repeated 3 times. Ethanol, which was used as the extraction solvent, was used in the same volume as the control sample.

RESULT AND DISCUSSION

LC-MS/MS Results of Ethanol Extracts of The Studied *Thymbra* Species

The phenolic content of the ethanol extracts from the three *Thymbra* species under study was ascertained using the LCMS/MS. The results of an LC-MS/MS study of the extracts are shown in Table 2. Chromatograms are provided in Figure 1. The LC-MS/MS results revealed a varying number of components across the different samples: 16 for TSR, 18 for TSAP, 15 for TSSR, 18 for TSSAP, 17 for TSIR, and 18 for TSIAP. When the results were analyzed, the following were determined: The first three main components for TSR were hesperidin (27174.64 μg analytes/g extract), rosmarinic acid (9875.75) and malic acid (2862.41); for TSAP, hesperidin (9151.19), rosmarinic acid (5735.78) and naringenin (3228.28); for TSSR, hesperidin (20297.30), rosmarinic acid (11571.54) and malic acid (1079.38); For TSSAP, hesperidin (32910.02 μg), nicotiflorin (6723.16) and rosmarinic acid (6223.42); For TSIR, hesperidin (24169.69), rosmarinic acid (5538.63) and malic acid (2692.59); for TSIAP, rosmarinic acid (5343.08), hesperidin (3900.28) and naringenin (2342.18). According to the results, it was seen that all three species contained high levels of hesperidin, rosmarinic acid and malic acid. It was determined that TSSAP sample had the highest hesperidin content, TSSR had the highest rosmarinic acid content and TSR had the highest malic acid content (Table 2).

Table 2. Phenolic compound identification and quantification in ethanol extracts from three *Thymbra* species using LC-MS/MS

No	Analytes	RT[a]	Motherion (*m/z*)[b]	Fragmentions	TSR	TSAP	TSSR	TSSAP	TSIR	TSIAP
					Quantification (µg analyte/g extract)[c]					
1	Quinic acid	1.13	190.95	85.3-93.3	92.99	48.53	99.25	174.58	73.01	113.64
2	Malic acid	1.23	133.00	115.2-71.3	2862.41	246.29	1079.38	507.35	2692.59	597.11
4	Gallic acid	3.00	168.85	125.2-79.2	10.95	43.39	10.13	16.17	8.64	18.78
5	Protocatechuic acid	4.93	152.95	108.3	16.21	7.61	28.13	57.03	12.82	27.30
7	Chlorogenic acid	7.13	353.15	191.2	N.D.	N.D.	N.D.	N.D.	N.D.	11.73
9	Vanillic acid	8.57	166.90	152.3-108.3	112.97	198.39	230.55	411.38	189.12	237.31
10	Caffeic acid	8.80	178.95	135.2-134.3	129.10	111	215.61	327.63	114.72	82.21
11	Syringic acid	9.02	196.95	182.2-167.3	9.40	281.14	2.78	258.31	111.01	265.57
12	Vanillin	10.87	151.00	136.3-92.2	48.04	21.28	71.56	45.29	33.87	12.1
13	Salicylic acid	11.16	136.95	93.3-65.3	7.93	16.88	3.52	15.39	8.14	39.68
14	*p*-Coumaric acid	11.53	162.95	119.3-93.3	11.77	58.3	19.92	62.06	19.83	47.61
18	Hesperidin	12.67	610.90	303.1-465.1	27174.64	9151.19	20297.3	32910.02	24169.69	3900.28
20	Rosmarinic acid	14.54	359.00	161.2-197.2	9875.75	5735.78	11571.54	6223.42	5538.63	5343.08
21	Nicotiflorin	14.68	593.05	285.1-255.2	51.08	61.7	29.76	6723.16	158.76	20.61
23	Rhoifolin	16.11	577.05	269.2-211.1	6.11	36.66	N.D.	255.61	10.53	7.53
25	Apigetrin	16.59	431.00	268.2-239.2	N.D.	12.54	N.D.	137.07	N.D.	N.D.
33	Naringenin	30.68	284.75	151.2-119.3	9.80	3228.28	23.45	1283.39	270.84	2342.18
34	Apigenin	31.43	270.95	117.3-151.2	N.D.	43.94	N.D.	29.94	4.73	56.03
35	Hesperetin	31.76	268.95	164.2-136.2	241.26	532.63	536.67	3919.57	594.47	1960.14

[a]RT: Retention time (min), [b]Mother ion (*m/z)*: Molecular ions of the standard compounds (*m/z* ratio). [c]Values in µg/g (w/w) of plant ethanol extract. ND: Not detected. 3: fumaric acid, 6: pyrocatechol, 8: 4-OH-benzoic acid, 15: rutin, 16: ferulic acid, 17: sinapinic acid, 19: isoquercitrin, 22: *o*-coumaric acid, 24: quercitrin, 26: coumarin, 27: myricetin, 28: fisetin, 29: cinnamic acid, 30: liquiritigenin, 31: quercetin, 32: luteolin, 36: kaempferol, 37: chrysin were not dedected. TSR: *T. spicata* var. *spicata* root ethanol extract, TSAP: *T. spicata* var. *spicata* aerial part ethanol extract, TSSR: *T. sintenisii* subsp. *sintensii* root ethanol extract, TSSAP: *T. sintenisii* subsp. *sintensii* aerial part ethanol extract, TSIR: *T. sintenisii.* subsp. *isaurica* root ethanol extract, TSIAP: *T. sintenisii.* subsp. *isaurica* aerial part ethanol extract

When the phenolic content studies in the literature were examined, the following determinations were made: Bener [9] determined the main component of TS ethanol extract as rosmarinic acid (1089.2±10.9 mg/100 g-DS); Khalil et al. [10] reported that the top three main components for aqueous extract of TS were rosmarinic acid (38.60%), salvalonic acid I (10.17%) and rutin (7.17%), while the top three main components of ethanolic extract were carvacrol (36.84%), thymusin (20.25%) and eriodictyol (9.45%); Askun et al. [8] determined the first three main components of the methanol extract of TS as carvacrol (81.481 μg/ml), rosmarinic acid (26.644 μg/ml), hesperidin (9.428 μg/ml). When the studies were analysed, it was found that the results of the present study showed similarities with the previous studies [8-10].

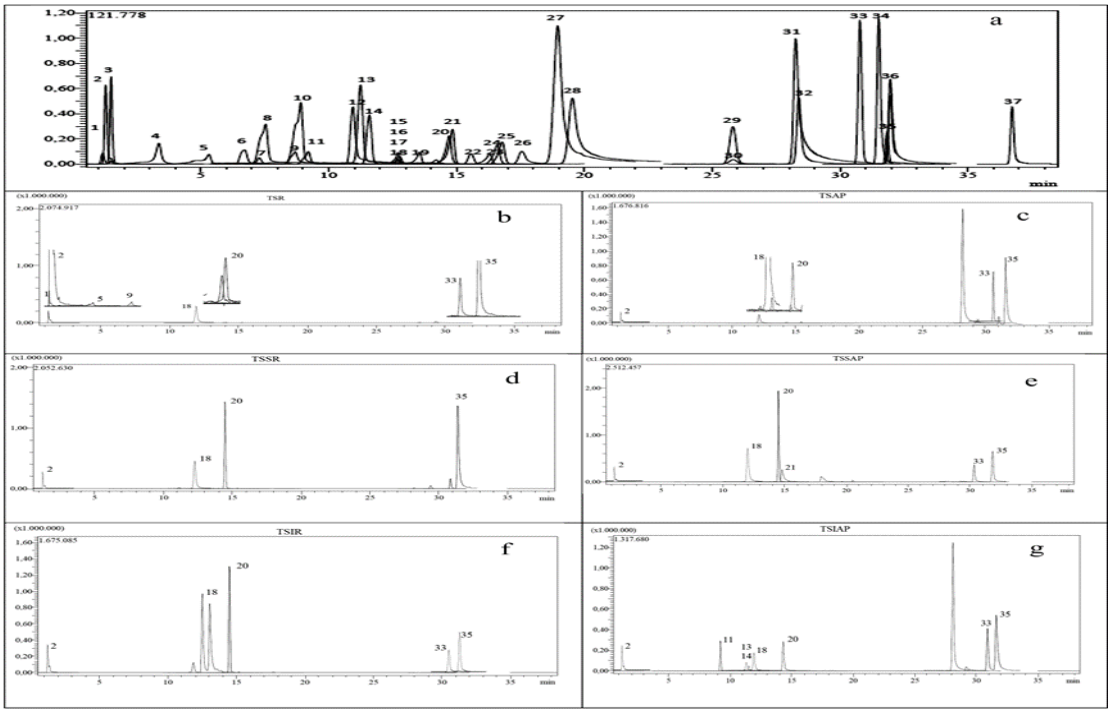


Figure 1. a) TIC chromatogram of the standards mixture (1 μg/ml) analyzed by the LC MS/MS. Legend;1: quinic acid, 2: malic acid, 3: fumaric acid, 4: gallic acid, 5: protocatechic acid, 6: pyrocatechol, 7: chlorogenic acid, 8: 4-OH-benzoic acid, 9: vanillic acid, 10: caffeic acid, 11: syringic acid, 12: vanillin, 13: salicylic acid, 14: *p*-coumaric acid, 15: rutin, 16: ferulic acid, 17: sinapic acid, 18: hesperidin, 19: isoquercitrin, 20: rosmarinic acid, 21: nicotiflorin, 22: α-coumaric acid, 23: rhoifolin, 24: quercitrin, 25: apigetrin, 26: coumarin, 27: myricetin, 28: fisetin, 29: cinnamic acid, 30: liquiritigenin, 31: quercetin, 32: luteolin, 33: naringenin, 34: apigenin, 35: hesperetin, 36: kaempferol, and 37: chrysin. LC-MS/MS chromatograms, b: LC-MS/MS chromatogram of the root part of *T. spicta* ethanol extract, c: LC-MS/MS chromatogram of the aerial parts of *T. spicata* ethanol extract, d: LC-MS/MS chromatogram of the root part of *T. sintenisii* subsp. *sintensii* ethanol extract, e: LC-MS/MS chromatogram of the aerial parts of *T. sintenisii* subsp. *sintensii* ethanol extract, f: LC-MS/MS chromatogram of the root part of *T. sintenisii*. subsp. *isaurica* ethanol extract, g: LC-MS/MS chromatogram of the aerial parts of *T. sintenisii.* subsp. *isaurica* ethanol extract

Antioxidant Activities Results

The ABTS, CUPRAC, and DPPH methods were utilized to ascertain the antioxidant potentials of *Thymbra* species. The findings are presented in Table 3. According to results, TSR extract exhibited the highest antioxidant activity with an IC_{50} value of 24.50±1.32 μg/ml in the DPPH assay. In addition, the samples were found to have high antioxidant activity compared to the standard reference BHT (IC_{50}: 60.43±0.50 μg/ml) and moderate antioxidant activity compared to α-tocopherol (α-TOC) (IC_{50}: 15.98±0.79 μg/ml).

Upon analysis of ABTS results of *Thymbra* species ethanol extracts from aerial parts were more potent than root ethanol extracts. Among the ethanol extracts, TSSAP was found to be the most effective sample with an IC_{50} value of 4.96±0.09 μg/ml as shown in Table 2. It was also found that all ethanol extracts exhibited ABTS activity as high as or higher than the standard references α-TOC (IC_{50}: 9.57±2.50 μg/ml) and BHT (IC_{50}: 11.46±0.12 μg/ml).

In the CUPRAC activity results, it was found that TSAP ($A_{0.5}$: 6.31±0.08 μg/ml) had the highest activity among all samples (Table 3). In general, the aerial parts yielded more potent ethanol extracts than the root parts. It was also found that the ethanol extracts of the species exhibited generally high

activity against BHT ($A_{0.5}$: 13.73±0.24 μg/ml) and α-TOC ($A_{0.5}$: 14.44±0.17 μg/ml). TSAP extract showed high antioxidant activity in the DPPH method, unlike other methods.

In the literature, it was observed that TS, TSS and TSI extracts showed high antioxidant activity (IC_{50}: 16.91, 12.5 and 27.15 μg/ml, respectively) in DPPH method [13,30]. As seen in the literature, TS ethanol extract exhibited a high level of ABTS activity (inhibition%: 98.28) and the present results were in parallel with the data obtained [31]. In addition, there are no studies on the ABTS activity of TSS and TSI in the literature and this study is the first study in this field. When the CUPRAC activity determination studies in the literature were examined, there was no study on the extracts of TS, TSS and TSI therefore, the results from this study were the first entry to the literature.

In general, all three species' ethanol extracts demonstrated high antioxidant capacity in DDPH, ABTS and CUPRAC assays. It was seen that the results obtained were in parallel with the LC-MS/MS content results [16,17].

Table 3. Antioxidant activityies of ethanol extracts of three *Thymbra* species

Samples[a]	DPPH (IC_{50}: μg/ml)	ABTS (IC_{50}: μg/ml)	CUPRAC ($A_{0.5}$: μg/ml)
TSR	24.50±1.32[a]	10.77±0.95[a]	13.43±0.16[a]
TSAP	73.53±1.79[b]	5.25±0.80[b]	6.31±0.008[b]
TSSR	41.99±1.12[c]	11.46±0.12[a]	15.61±0.20[c]
TSSAP	28.66±0.77[d]	7.68±0.06[c]	196.2±2.01[d]
TSIR	48.91±2.66[e]	11.75±0.04[a]	29.28±1.81[e]
TSIAP	46.30±0.66[e]	4.96±0.09[b]	8.066±0.05[f]
BHT[b]	60.43±0.50[f]	11.46±0.12[a]	13.73±0.24[a]
α-TOC[b]	15.98±0.79[g]	9.57±2.50[a]	14.44±0.17[g]

[a]Values expressed are means±S.D. of three parallel measurements and values were calculated according to negative control. Values with different letters in the same column were significantly different ($p<0.05$), [b]Standard compound

Enzyme Inhibition Activity Results

Upon examining the inhibition results of the acetylcholinesterase enzyme, it was found that TSIAP had the highest activity with an inhibition rate of 57.93±0.33% in the ethanol extracts of three *Thymbra* samples. In addition, TSIAP showed moderate acetylcholinesterase enzyme inhibition activity compared to galantamine (inhibition%: 79.13±0.33). Upon analysis of the butyrylcholinesterase enzyme inhibition activity of ethanol extracts from all studied species, it was determined that they exhibited high levels of activity (Table 4). In general, all of the extracts were highly effective in inhibiting the butyrylcholinesterase enzyme compared to galantamine (inhibition%: 76.12±0.83). The study found that the ethanol extracts of these species exhibited moderate activity in the AChE method and high activity in the BChE method. Based on the findings, it is possible to suggest that these three species possess the potential to inhibit anticholinesterase enzyme.

The results also showed that TSR (inhibition%: 43.58±0.44) and TSSR (inhibition%: 43.20±1.21) showed the highest tyrosinase enzyme inhibition activity and had moderate activity compared to kojic acid (inhibition%: 91.20±0.73). In contrast, the ethanol extracts of the *Thymbra* species studied showed no inhibition of the urease enzyme (Table 4).

The elastase and collagenase enzyme inhibition activity results of ethanol extracts of the studied *Thymbra* species were given in Table 4. The results showed that TSSR had the highest activity (inhibition%: 15.47±0.46) among all samples and showed moderate activity compared to oleanolic acid (inhibition%: 35.70±0.46). The collagenase enzyme inhibition activity results showed that the root-aerial ethanol extracts had a low level of collagenase enzyme inhibition activity compared to epicatechin gallate (inhibition%: 79.84±1.78). According to the findings, all extracts had weaker effects than the standart compound.

ACE and α-amylase enzyme inhibition activity results of ethanol extracts of three *Thymbra* species were given in Table 4. According to the results, it was determined that the ethanol extracts did

not show any ACE activity, only TSAP (inhibition%: 19.40±0.01) and TSSAP (inhibition%: 10.10±0.06) exhibited a low-level ACE inhibition activity compared to lisinopril (inhibition%: 100.10±0.94). When evaluating α-amylase inhibition activity results, the most active sample among all the extracts was TSAP (inhibition%: 97.57±2.59). It was also determined that TSAP exhibited considerably higher *α*-amylase enzyme inhibition activity than standard reference substance, acarbose (inhibition%: 57.85±0.00).

Table 4. Enzyme activities of ethanol extracts of three *Thymbra* species

Samples[1]	Enzyme inhibition activities (inhibition %: at 50 μg/ml)							
	AChE	BChE	Tyrosinase	Urease	Elastase	Collagenase	ACE	α-Amylase
TSR	25.70±1.58 a	98.71±0.55[a]	43.58±0.44 a	NA[a]	7.31±1.85[a]	1.57±0.90[a]	NA	41.21±0.90 a
TSAP	46.93±0.18 b	96.85±1.22[b]	16.67±1.05 b	NA	7.31±7.38[a]	13.55±0.19[b]	19.40±0.01 4[a]	97.57±2.59 b
TSSR	46.04±1.09 b	99.98 ±0.19[c]	43.20±1.21 a	NA	15.47±0.46[b]	11.31±0.38[c]	NA	34.97±0.37 c
TSSAP	49.49±0.97 c	98.89±1.06[a]	34.78±0.76 c	NA	9.92±0.46[c]	17.36±0.95[d]	10.10±0.06 b	18.59±0.62 d
TSIR	40.41±0.43 d	98.54±0.91[a]	36.54±0.07 d	NA	9.92±0.69[c]	8.96±0.01[e]	NA	72.13±0.04 e
TSIAP	57.93±0.33 e	99.82±0.58[c]	NA	NA	11.23±0.92[e]	8.79±0.24[e]	NA	50.44±3.45[f]
Galantha mine[2]	79.13±0.33[f]	76.12±0.83[d]	-	-	-	-	-	-
Kojic acid[2]	-	-	91.20±0.73 e		-	-	-	-
Thiourea[2]	-	-	-	96.15±0. 78[b]	-	-	-	-
Oleanolic acid[2]	-	-	-	-	35.70±0.46[f]	-	-	-
Epicatechi n gallate[2]	-	-	-	-	-	79.84±1.78[f]	-	-
Lisinopril[2]	-	-	-	-	-	-	100.1±0.94 c	-
Acarbose[2]	-	-	-	-	-	-	-	57.85±0.45 g

[1]Values expressed are means±S.D. of three parallel measurements and values were calculated according to negative control. Values with different letters in the same column were significantly different ($p<0.05$), [2]Standard compound, NA: notactive. TSR: *T. spicata* var. *spicata* root ethanol extract, TSAP: *T. spicata* var. *spicata* aerial part ethanol extract, TSSR: *T. sintenisii* subsp. *sintensii* root ethanol extract, TSSAP: *T. sintenisii* subsp. *sintensii* aerial part ethanol extract, TSIR: *T. sintenisii.* subsp. *isaurica* root ethanol extract, TSIAP: *T. sintenisii*. subsp. *isaurica* aerial part ethanol extract

When the studies in the literature are examined, Eruygur et al. [18] investigated the acetyl and butyrylcholinesterase activities (IC_{50}: 0.23 and IC_{50}: 1.64 μg/ml, respectively) of the ethanol extract of TS. In another study Kirkan et al. [15] determined the tyrosinase enzyme inhibition of TS essential oil (IC_{50}: 19.49 mg/l). There were no studies on the determination of urease, tyrosinase, elastase, collagenase, ACE and α-amylase enzyme inhibition activities with TS, TSS and TSI extracts and these data from our study were the first entry to the literature [15,18].

Cytotoxic Activities Results

The ethanol extracts of *Thymbra* species exhibited low cytotoxicity against MCF-7 and HT-29 cells but were toxic to PDF cells (Table 5). Due to the toxic effects of these species on healthy cells, it is important to consume and use them carefully.

Tutar et al. [14] determined the toxic activity of the ethanol extract of aerial part of TSS on MCF-7 (ATCC HTB-22) and MG63 (human osteosarcoma cell line, ATCC CRL-1427). As a control, they utilized the healthy mouse fibroblast cell line L929. The results of the study showed that the extract exhibited cytotoxic effects in all cell lines at concentrations of 40 μg/ml or lower. The IC_{50} values for MG63, MCF-7, and L929 at 24 hours were 37.28, 44.40, and 44.84 μg/ml, respectively. According to Eruygur et al. [13], the ethanol extract of *TS* demonstrated antiproliferative activity against MCF-7 (IC_{50}: 340 μg/ml), but was ineffective against PC-3 human prostate cancer cells. In this study, the hydro-

alcoholic extract of *TS* exhibited concentration-dependent cytotoxic effects on the SK-Mes-1 lung cancer cell line with an IC_{50} value of 110 μg/ml. According to Hepokur et al. [30], the IC_{50} values of TSI ethanol extract on MCF-7 cell culture were found to be 7.55 and 10.48 μg/ml for 24 and 48 hours, respectively. The IC_{50} values of L929 cell culture were found to be 12.63 and 9.26 μg/ml for 24 and 48 hours, respectively [13,14,30].

Table 5. Cytotoxic activities of ethanol exracts of three *Thymbra* species

Samples	Cytotoxic activity[a] (IC_{50}: μg/ml)		
	HT-29	MCF-7	PDF
***TS*R**	562.40±3.65[a]	99.29±1.52[a]	197.19±3.72[a]
***TS*AP**	455.12±0.76[b]	92.67±0.81[b]	46.46±1.53[b]
***TSS*R**	459.57±1.98[c]	113.36±1.37[c]	47.59±5.01[c]
***TSS*AP**	677.09±5.71[d]	101.66±1.25[d]	190.27±2.79[d]
***TSI*R**	956.87±6.32[e]	91.59±3.67[e]	357.25±4.95[e]
***TSI*AP**	631.54±8.47[f]	96.01±0.93[f]	201.48±1.64[f]

[a]Values expressed are means ± S.D. of three parallel measurements and values were calculated according to negative control. Values with different letters in the same column were significantly different ($p < 0.05$). TSR: *T. spicata* var. *spicata* root ethanol extract, TSAP: *T. spicata* var. *spicata* aerial part ethanol extract, TSSR: *T. sintenisii* subsp. *sintensii* root ethanol extract, TSSAP: *T. sintenisii* subsp. *sintensii* aerial part ethanol extract, TSIR: *T. sintenisii*. subsp. *isaurica* root ethanol extract, TSIAP: *T. sintenisii.* subsp. *isaurica* aerial part ethanol extract

Conclusion

Phenolic compounds present in the ethanol extracts derived from the aerial and root parts of *Thymbra spicata* (TS), *Thymbra sintenisii* subsp. *sintenisii* (TSS), and *Thymbra sintenisii* subsp. *isaurica* (TSI) were thoroughly analyzed and quantified. The results indicated that all the ethanol extracts were notably rich in phenolic substances, particularly hesperidin and rosmarinic acid, highlighting their phytochemical potency. In general, it was determined that the studied samples showed high antioxidant activity in both CUPRAC (excluding TSSAP ($A_{0.5}$: 196.2±2.01 μg/ml) and ABTS methods and moderate activity in the DPPH method. Among the enzyme inhibition tests conducted, all extracts displayed significant inhibitory activity against the butyrylcholinesterase enzyme. Notably, the ethanol extract from the aerial parts of *T. sintenisii* subsp. *sintenisii* exhibited exceptionally potent inhibition of α-amylase, a crucial enzyme involved in carbohydrate metabolism. Additionally, moderate to low inhibitory effects were observed against other enzymes such as tyrosinase, elastase, collagenase, and angiotensin-converting enzyme (ACE). However, none of the evaluated extracts demonstrated inhibitory activity against the urease enzyme. Overall, the findings underscore the promising health-promoting potential of these *Thymbra* species, attributable to their robust antioxidant properties and their capacity to inhibit enzymes such as butyrylcholinesterase and α-amylase. Consequently, these species exhibit considerable potential for pharmaceutical applications, particularly in addressing oxidative stress-related diseases and managing carbohydrate metabolism disorders. Nevertheless, considering the existing literature and the observed toxicity toward healthy human cell lines (PDF), further in-depth toxicological studies are warranted to ascertain the safe dosage parameters, ensuring the responsible utilization of these plant species for therapeutic purposes.

ACKNOWLEDGEMENTS

This work was supported by Dicle University, Scientific Research Projects Coordinator (Project numbers: ECZACILIK. 24.002 and FEN. 24.005).

AUTHOR CONTRIBUTIONS

Concept: S.Y., E.C.K., P.K.Y., I.Y.; Design: S.Y., E.C.K., I.Y., A.E.; Control: P.K.Y., U.K., H.T., A.E.; Sources: S.Y., E.C.K., M.F., A.E.; Materials: S.Y., E.C.K., M.F., A.E.; Data Collection and/or Processing: S.Y., I.Y., A.E.; Analysis and/or Interpretation: P.K.Y., M.A.Y., S.I.K.; Literature Review:

S.Y., E.C.K., M.F., I.Y., M.A.Y., S.I.K.; Manuscript Writing: S.Y., E.C.K., U.K., H.T.; Critical Review: M.A.Y., S.I.K., U.K., H.T., A.E.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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