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Scientific paper

Chemical Composition and In Vitro Biological Activity of Two *Thymus* L. Varieties Growing in Turkey

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Abstract

Thymus kotschyanus var. *kotschyanus* (TKK) and *T. kotschyanus* var. *glabrescens* (TKG) varieties were used as both spice and medicine by the people in Turkey. It was determined that plants' methanol extracts had the strongest antioxidant, anticholinesterase, antiurease activity and high total phenolic contents. The ethyl acetate and methanol extracts were found to have strong antimicrobial activity. Methanol extracts showed low hemolytic effect against human erythrocytes. It was determined that TKG extract showed higher anti-proliferative effect compared to TKK extract. Both plants extract significantly decreased reactive oxygen species (ROS) generation in cancer cells. It was determined that amounts of chlorogenic and rosmarinic acid compounds were similar in both plants, but apigenin 7-O-neohesperidoside compound was found in higher amounts in TKK. The findings obtained in this study suggest that methanol and ethyl acetate extracts obtained from these two species can be used as antioxidant, anticholinesterase, antimicrobial and antiurease agents. The findings support the traditional use of these species.

Keywords: *Thymus* species, biological activity, HPLC-DAD

1. Introduction

For many years, human beings have used plants not only as a source of food, but also as fuel, clothing raw material, building material and medicine to prevent and cure diseases. These folk remedies, which have been used by people for centuries, also shed light on the development of modern medicines. In addition, today's research on drug active ingredients with natural origin enriches our current knowledge in this field.[1–4]

Alzheimer's disease is a neurodegenerative condition that progresses over time and is caused by the death of neurons and synapses in many regions of the central nervous system. It is marked by a decline in cognitive abilities, difficulties with self-care, and a variety of neuropsychiatric and behavioral abnormalities. More than half of all cases of dementia have been linked to Alzheimer's disease. One of the early stages of Alzheimer's disease, oxidative stress plays a pathogenic function in the illness. [5,6]

Stone formation in the kidney and urinary system is a significant medical illness that affects 4–20% of the population and can result in renal failure if left untreated.[7] The nucleation, growth, and aggregation of crystals in saturated urine and epithelial cells of the renal papilla are steps of kidney stone development. Among the other forms (calcium oxalate dihydrate, calcium oxalate trihydrate), calcium oxalate monohydrate is the most thermodynamically stable form and has a strong affinity for renal tubular cells. As a result, more research has been done in recent years on the inhibition of calcium oxalate monohydrate crystals.[8–12]

Antimicrobial resistance against bacteria is increasing rapidly around the world, making the treatment of many bacterial diseases, especially infectious diseases, more difficult day. Scientists are working on the discovery of new antimicrobial compounds due to antibiotics that have become highly resistant to bacteria. Plants have significant potential for the discovery of these new compounds.[13] *Helicobacter pylori* is the first bacterium to be identified as a carcinogen and is a pathogen that colonizes the stomach in more than half of the world's population. One of *H. pylori* 's most significant virulence factors, the urease enzyme converts urea to ammonia and carbon dioxide, creating an alkaline environment for the bacteria to thrive in. *H. pylori* establishes a dwelling space in the tissue in this manner without being harmed by stomach acid. Finding substances that specifically affect this bacterium is crucial due to the harmful side effects of the medications used in treatment and the resistance of *H. pylori* to conventional antibiotics.[14] It is predicted that more successful results can be obtained in the treatment, especially by using anti-urease effective compounds. Due to the need for new drug active substances in the treatment of infectious diseases, it is emphasized that plants or plant extracts used in natural traditional medicine can be potential antimicrobial agents that can be used against infections.[15]

Toxicity of the active ingredients in plant extracts on healthy cells is an important factor in terms of drug efficacy and their clinical applications. In this respect, hemolytic activity is an important parameter to be investigated. It provides primary information on the interaction between active ingredients and cell membranes at the cellular level. Many plant extracts contain active ingredients that have potential to cause hemolytic activity on human erythrocytes.[16,17] Reactive oxygen species (ROS) production is elevated in tumor cells because of increased metabolic rate, mutations, and relative hypoxia.[18] In order to damage proteins, nucleic acids, lipids, membranes, and organelles, several cancer therapeutic strategies attempt to raise cellular ROS levels. This can activate cell death processes such apoptosis at the tumor microenvironment. ROS generation capacity of studied plant extracts on cancer cells was evaluated. ROS formation in HT-29 cells was analyzed with the cell-permeable reagent 2',7'-dichlorofluorescein (DCFDA). DCFDA is oxidized by ROS and forms a fluorescent compound. The increased fluorescent emission proves the presence of anticancer activity[18].

Lamiaceae (Labiatae) family has a very intense distribution throughout the world, especially in the Mediterranean basin. It is represented by 224 genera and about 5600 species worldwide. The Lamiaceae family is represented by 45 genera and more than 735 taxa in the flora of Turkey. The *Thymus* genus is a member of Lamiaceae family, with about 300–400 species. Turkey's vegetation includes 37 species and more than 57 taxa, 27 of which are endemic.[19] *Thymus* species were used internally in traditional medicine for indigestion and other gastrointestinal disorders; in the treatment of colds, bronchitis, and pertussis; It was used as a mouthwash against laryngitis and tonsillitis. *Thymus* essential oil and thymol were used internally for the treatment of respiratory ailments, and externally they were included in the composition of wound-healing ointments and antiseptic drugs, syrups, and inhalation preparations. *Thymus kotschyanus* Boiss. Et Hohen. species is a member of the *Thymus* genus, which is widely distributed in nature. This species was used in folk medicine to treat digestive and respiratory ailments, along with other medicinal uses.[20]

Therefore, the aim of this study was to evaluate the biological activities of two varieties of *Thymus kotshyanus* (i), to analyze the phytochemical content of the effective extract qualitatively and quantitatively (iii) and to evaluate the toxicity of the taxa (iii).

2. Materials and Methods

2. 1. Plant Material and Preparation of Extracts

Thymus kotschyanus var. *glabrescens* (TKG) (Herbarium No:27) and *Thymus kotschyanus* var. *kotschyanus* (TKK) (Herbarium No:28) species were collected from Adıyaman province and preserved in the Adıyaman University Pharmacy Faculty Herbarium. Prof. Dr. Omer Kılıc, a member of the Pharmaceutical Botany Department of Adıyaman University, made the identification. Plant species were dried in the shade at 25°C. The crude extracts from the plant's aerial parts were obtained using the sequential maceration process with petroleum ether, ethyl acetate, and methanol.

2. 2. Antioxidant Activity Assays

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay: 3.9 mL of DPPH solution (0.1 mM) were added to the 0.1 mL of extracts that had been obtained at various concentration. Before being incubated for 30 minutes at 25 °C, the produced mixtures were stirred for 1 minute. The mixes' absorbance values were measured at 517 nm. Under identical circumstances, the absorbance of the control sample was measured using 0.1 mL of methanol rather than the extract. Using the following formula, the percentage DPPH radical scavenging activity was determined: % DPPH radical scavenging activity= $[(A_0\text{-}A_1)/A_0]x100$ where A_0 is the absorbance of the control, and A_1 is the absorbance of the extractives/standard. Then % of inhibition was plotted against concentration, and from the graph IC_{50} was calculated. The information gathered throughout the investigation is provided as IC_{50} (mg/mL).[21]

CUPRAC (cupric ion reducing/antioxidant power) test: In this assay, 60 µL of Cu(II).$2H_2O$, 60 µL of neocuproine, and 60 µL of 1 M NH_4Ac were mixed, then 60 µL of the extracts were added, and finally, 10 µL of ethanol was added to the mixture. The mixes' absorbance was spectrophotometrically evaluated at 450 nm after 60 min against a reference solution that was made by substituting ethanol for the plant extracts. The extracts' CUPRAC values were provided as mg Trolox equivalent (TE)/mg extract. [21]

***FRAP (iron reducing antioxidant power)*:** Benzie and Strain's (1996) method was used to examine the extracts' ability to reduce ferrous iron. The FRAP reagent was stored at 37 ºC for 30 min. It consisted of 25 mL of 300 mM acetate buffer (pH 3.6), 2.5 mL of TPTZ solution, and 2.5 mL of 20 mM $FeCl_3.6H_2O$. 10 µL of extract were combined with 190 µL of FRAP reagent, and after 4 minutes, the mixture's absorbance at 593 nm was measured. The FRAP values were presented as mM Fe^{2+}/mg extract.[22]

Plants' phenolic contents: The total amount of phenolic compounds in the extracts was obtained using the Folin-Ciocalteu reagent. 0.1 mL of the diluted plant extracts, 4.5 mL of water, and 0.1 mL of the Folin-Ciocalteu reagent were combined, and then 0.3 mL of sodium carbonate solution (2%) was added. One minute of medium-continuous shaking was then performed. After two hours at room temperature, the absorbance at 765 nm was measured using a UV/Vis spectrophotometer. Total phenolic content was calculated as milligrams of Gallic acid equivalent (GAE) per gram of plant extract.[23]

2. 3. Anti-cholinesterase Activity Testing

AChE (20 µL) and extracts (20 µL) were added to a phosphate buffer solution (pH 8, 0.1 M, 40 µL). Ten minutes were spent incubating this mixture at 25°C. Following incubation, the mixture was combined with substrates DTNB (100 µL) and AcI (20 µL). 5-thio-2-nitrobenzoic acid was measured spectrophotometrically at 412 nm.[24]

2. 4. Anti-urease Activity Assay

Plant extracts (100 µL) and an enzyme solution (500 µL) were combined and maintained in an incubator at 37 °C for 30 minutes. This combination was then given 1.100 µL of urea, and it was allowed to sit in an incubator at 37 °C for 30 minutes. After taking it out of the incubator, the mixture was then combined with the reagents R1 (1% phenol, 0.005% sodium nitroprusside) and R2 (0.5% NaOH, 0.1% sodium hypochlorite), and the resultant mixture was then held at 37 °C in an incubator for 2 hours. The mixture's absorbance was determined (635 nm) in comparison to a reference solution that had been made by substituting a buffer solution for the urease enzyme solution.[21]

2. 5. Antimicrobial Activity

Antimicrobial activity analysis of plant extracts determined primarily by the agar well diffusion method. Minimum inhibitory concentration (MIC) determined for extracts showing antimicrobial activity in agar well diffusion method. *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 11228, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC 14153, *Klebsiella pneumoniae* ATCC 4352, *Candida albicans* ATCC 10231 were used in the antimicrobial tests.

Agar well diffusion method: Bacteria were inoculated on tryptic soy agar and *C. albicans* on Sabouraud dextrose agar, incubated at 37 °C for 24 hours. Microorganism suspensions were prepared from colonies in 0.85% NaCl physiological saline solution (PSS). Bacterial suspensions were adjusted to 10^8 cfu/ml and *C. albicans* suspensions to a concentration of 10^6 cfu/mL according to Mc Farland 0.5 standard turbidity. The microorganism suspensions were spread over the surface of the Mueller Hinton agar for bacteria and SDA for *C. albicans* by sterile swabs under aseptic conditions and then 5 mm diameter wells were made surface of the medium with a sterile punch. The wells were filled with 50 µL (50 mg/mL) of the extracts dissolved in appropriate solvents. In addition, meropenem (10 µg/well) for bacteria, amphotericin B (100 µg/well) as a positive control for the yeast, solvents (DMSO) and PSS were used as negative control. Inoculated petri dishes were incubated at 37°C for 18–24 hours for bacteria, for 24–48 hours at 35 °C for yeast, and at the end of incubation time, inhibition zones were measured in mm. The trials were performed with three replicates and averaged.[25]

Detection of minimal inhibitory concentration for bacteria: Detection of MIC for bacteria were performed in accordance with the standards of the Clinical and Laboratory Standards Institute (CLSI). Cation Adjusted Mueller Hinton Broth (CAMHB) was used as medium. Bacteria suspension was prepared from the colonies in the 24-hour bacterial culture according to McFarland 0.5 turbidity and the final inoculum concentration shall be diluted to $5x10^5$ cfu/ml. The sterile U-based microdilution plates were placed 100 µL of the CAMHB. The extracts were placed 100 µl in the first wells and serial dilutions were made. Then 5 µL of bacterial suspension was added to the wells containing the extract and the plates incubated at 37 °C for 24 hours. At the end of the incubation, the lowest extracting concentrations with no growth was determined as minimal inhibitory concentration (MIC). *Escherichia coli* ATCC 25922 was used as a quality control microorganism. CAMHB, DMSO and PSS were used as negative control. Meropenem was used as positive control.[26]

2. 6. Examination of Phenolic Compounds

The approach we have previously described was used to assess the content of the bioactive extracts using high-performance liquid chromatography with diode-array detection (HPLC-DAD) (Agilent 1260 Infinity). The chemicals were separated using a C18 reverse phase Nova-Pak analytical column (3.9 mm × 150 mm inner diameter, 5 µm). The column temperature was kept at 30 °C. The mobile phases were water (0.05% acetic acid) and (B) acetonitrile. The gradient elution step was used: the mobile phase B was increased from 0% to 20% in 5 minutes, 40% in 10.00 min, 50% in 20.00 min, 60% 30.00 min, 90% B 40.00 min and 45.00 min, 20%. The injection volume was settled as 20 µL.[21]

2. 7. Hemolytic Activity

Preparation of erythrocytes suspension: Blood samples were taken from the arm veins of healthy volunteer individuals and transferred. Transferred into EDTA containing tubes. In order to separate plasma and erythrocytes samples were centrifuged at 3000 rpm for 5 minutes (4 °C). Plasma was discarded and pellets, which contain erythrocytes, were washed 2 times with physiological saline (pH 7.2 ± 0.2) and centrifuged at 3000 rpm and at 4 °C for 5 min. [27]

Hemolytic activity: *In vitro* hemolytic activity of methanol extract of TKK and TKG was performed with spectrophotometer method.[27] The erythrocytes were diluted with 4 mL PBS and incubated on a shaker at 37 °C for 1.5 hours with different concentrations of plant extract at 125, 250, 500 and 1000 µg/mL in phosphate buffer saline at equal volume of erythrocytes. PBS and Triton X-100 were used as controls. The sample was centrifuged at 3000 rpm for 5 min at 4 °C. The amount of free hemoglobin in the supernatant was measured with UV spectrophotometer at 540 nm each experiment was performed in triplicates. The level of hemolysis percentage by the extracts was calculated according to the following formula:

$$\%Hemolysis = \frac{A_c - At}{A_c} x100$$

A_t is the absorbance of plant extract
A_c is the absorbance of Triton X-100

2. 8. Cytotoxic Activity

For the cytotoxic activity of TKK and TKG extracts, HT-29 cells (1 × 10^4) per well were seeded in 96-well plates in fresh complete medium at 37 °C, 5% CO_2 for 24 hours before the test. After, cells were rinsed with phosphate buffered saline (PBS) and incubated for 4 h with a cell culture medium containing increasing concentrations of TKK and TKG extracts (7.5, 75.0, and 750 µg/mL). Control cells were treated with PBS and all samples were studied in triplicate. After incubation of cells with plant extracts, cells were washed with PBS. Cytotoxicity analyzes was performed with XTT (2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5 [(phenylamino)carbonyl]-2H-tetrazolium hydroxide) were conducted by the manufacturer's (Roche Diagnostics Corporation, Indianapolis, IN) protocol. A volume of 20% of the medium XTT mixture was added to cells and allowed to incubate for 4 hours at 37°C, with 5% CO_2. Then absorbance values were measured at 450 nm and 690 nm. Percentage of cell viability was calculated from the absorbance readings and plotted.[24]

2. 9. DCFDA Assay Cells

Oxidative stress that was produced by plant extracts was quantified in HT-29 cells by DCFDA assay. The McCoy's 5A medium, 10% fetal bovine serum (FBS), and 1% antibiotics were used to maintain the HT-29 human colorectal cancer cell line at 37 °C in a 5% CO_2 atmosphere with saturated humidity. Cells (1 × 10^5 cells per well) were seeded into a 96-well plate and grown for 24 hours for the DCFDA test. Plates were then cleaned with PBS before being treated for 24 hours with TKK and TKG extracts at various doses (7.5, 75.0, and 750 g/mL) in medium containing 10% FBS. All samples were examined in triplicate and control cells were treated with PBS. Following a PBS wash, the cells were incubated for an additional 45 min in 100 µL of PBS with a final DCFDA concentration of 10 µM. In a fluorescence microplate reader (Cytation 5, Biotek, CA, USA), fluorescence emission was measured using an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Results are presented as relative fluorescence intensity.[28]

2. 10. Statistical Analysis

The findings were presented as the mean and standard deviations (SD) of three parallel measurements. Following ANOVA procedures, a one-way analysis of variance was conducted. A Tukey multiple comparison test was used to identify significant differences between means, with a p value of 0.05 being regarded as statistically significant.

3. Results

3. 1. Antioxidant Activity

It was determined that TKK (IC_{50}: 0.0630 mg/mL) and TKG (IC_{50}: 0.0436 mg/mL) methanol extracts showed the highest DPPH radical scavenging activity compared to other extracts. These results demonstrated that both plants have very similar potential for radical scavenging activity. Comparing the ascorbic acid (IC_{50}: 0.004 mg/mL) employed as a reference to the radical scavenging potentials of the plant extracts, it was found that all extracts had minimal radical scavenging capacity. The CUPRAC test results showed that TKK methanol (6.658 mM TE/mg extract) and ethyl acetate (4.0827 mM

TE/mg extract) extracts have a higher Cu(II) to Cu(I) reduction potential than petroleum ether (0.09443 mM TE/mg extract) extract. The same experiment showed that the CUPRAC values of the TKG methanol (7.097 mM TE/mg extract) and ethyl acetate (5.314 mM TE/mg extract) extracts were higher than petroleum ether extract. These results demonstrated that TKG and TK methanol extracts have higher Cu(II) to Cu(I) reduction potential than the ascorbic acid compound. It was discovered that TKG (17.704 mM TE/mg extract) and TKK (16.907 mM TE/mg extract) methanol extracts had more iron reducing antioxidant power than other extracts. Additionally, it was discovered in this study that TKG methanol extract (FRAP: 17.704) had higher FRAP values than BHA compound (FRAP: 16.91), while TKK (FRAP: 16.907) methanol extract displayed FRAP values to BHA compound (Table 1).

Table 1. Antioxidant activity of extracts from plants.

	DPPH (IC_{50}, mg/mL)		CUPRAC (mM TE/mg extract)		FRAP (mM TE/mg extract	
	TKK	**TKG**	**TKK**	**TKG**	**TKK**	**TKG**
Petroleum ether	0.431±0.033*	1.98±1.40*	0.094±0.108*	2.21±0.20*	7.18±0.29*	6.27±0.55*
Ethyl acetate	0.069±0.003*	0.066±0.003*	4.08±0.36*	5.31±0.16*	NA	1.49±0.30*
Methanol	0.063±0.001*	0.044±0.003	6.66±0.25*	7.10±0.33*	16.91±1.30	17.7±3.5*
Ascorbic acid	0.004±0.007		5.92±0.51			
BHA					16.91±0.02	

TKK: *Thymus kotschyanus* var. *kotschyanus*; TKG: *T. kotschyanus* var. *glabrescens*, ascorbic acid positive control for DPPH and CUPRAC assays; BHA, butylatedhydroxyanisole, positive control for FRAP assay; DPPH, 2,2-diphenyl-1-picrylhydrazyl; CUPRAC, cupricion reducing/antioxidant power; FRAP, ferric reducing antioxidant power; Values are mean of triplicate determination (n = 3) ± standard deviation; *P < 0.05 compared with the positive control

3. 2. Determination of Total Phenolic Content (TPC)

It is well known that phenolic contents and biological activity often have a linear relationship.[29] Based on this knowledge, the total phenolic contents of several plant extracts were spectrophotometrically measured in this investigation. The results, which corroborated the literature, demonstrated that TKG (105.8 mg GAE/g extract) and TKK (81.6 mg GAE/g extract) methanol extracts had higher phenolic component concentrations than other extracts (Table 2).

Table 2. The amount of phenolic compounds contained in the extracts from plants.

	TPC (mg GAE/g extract)	
	TKK	**TKG**
Petroleum ether	25.5±0.2	36.3±0.3
Ethyl acetate	48.1±0.3	60.5±0.2
Methanol	81.6±0.5	105.8±0.7

TKK: *Thymus kotschyanus* var. *kotschyanus*; TKG: *T. kotschyanus* var. *glabrescens*; TPC, total phenolic contents; GAE, gallic acid equivalent; Values are mean of triplicate determination (n = 3) ± standard deviation

3. 3. Antimicrobial Activity

Antimicrobial activities of 6 different extracts obtained from TKK and TKG varieties were investigated. All the extracts used in our study showed antimicrobial activity only against *S. aureus* and *S. epidermidis*. It has been determined that they were more effective against *S. epidermidis*. It is particularly interesting for TKG-EA (ethyl acetate) and TKG-M (methanol) against *S. aureus* and *S. epidermidis*. When we look at our results, it was concluded that our extracts were effective on gram positive strains and among these positive strains, *S. epidermidis* was more effective than *S. aureus*. While the highest

Table 3. Antimicrobial activity of plant extracts on various microorganisms by agar well diffusion method (in mm).

	Staphylococcus aureus	***Staphylococcus epidermidis***	***Escherichia coli***	***Pseudomonas aeruginosa***	***Proteus mirabilis***	***Klebsiella pneumoniae***	***Candida albicans***
TKK Petroleum ether	18.10±0.27	14.30±0.17	0	0	0	0	0
TKK Ethyl acetate	8.82±0.08	13.61±0.13	0	0	0	0	0
TKK Methanol	9.37 ±0.48	17.88±0.04	0	0	0	0	0
TKG Petroleum ether	16.34±0.16	24.71±0.13	0	0	0	0	0
TKG Ethyl acetate	24.10±0.84	41.46±0.23	0	0	0	0	0
TKG Methanol	18.09±0.14	21.10±0.15	0	0	0	0	0
Meropenem	33.90±0.02	47.09±0.21	29.40±0.22	32.81±0.18	31.61±0.12	30.98±0.21	–
Amphotericin B	–	–	–	–	–	–	16.42±0.13

TKK: *Thymus kotschyanus* var. *kotschyanus*; TKG: *T. kotschyanus* var. *glabrescens*, –: not done.

antimicrobial activity was observed against *S. epidermidis* strain of ethyl acetate extract of TKG species, the lowest antimicrobial activity was determined as the activity of ethyl acetate extract of TKK species against *S. aureus* strain. The diameter of the inhibition zones and the minimum inhibitory concentration (MIC) values formed by the extracts against the tested strains are shown in Tables 3 and 4, respectively.

Table 4. MIC values of plant extracts (mg/mL).

	Staphylococcus aureus	Staphylococcus epidermidis	Escherichia coli
TKK Petroleum Ether	6.25	6.25	–
TKK Ethyl Acetate	1.56	3.12	–
TKK Methanol	3.125	3.12	–
TKG Petroleum Ether	1.25	1.25	–
TKG Ethyl Acetate	0.78	0.78	–
TKG Methanol	0.78	0.78	–
Meropenem	–	–	0.06 µg/mL

TKK: *Thymus kotschyanus* var. *kotschyanus*; TKG: *T. kotschyanus* var. *glabrescens*, –: not done.

3. 4. Enzyme Inhibition Activity of Extracts

The findings of comparing the potentials of plant extracts (200 µg/mL) to inhibit the enzymes acetylcholinesterase and urease are shown in Table 5. The results showed that the acetylcholinesterase enzyme inhibition potentials of various extracts of both plants were extremely like one another. In comparison to the other extracts and the galantamine molecule, the TKG methanol (98.33%) extract was found to have a larger potential for acetylcholinesterase activity. Plant extracts and thiourea compound at a concentration of 12.5 µg/mL were examined for their anti-urease properties. The urease enzyme inhibitory potential of the TKK (34.404%) and TKG (17.726%) methanol extracts was found to be greater than that of other extracts. In this investigation, it was shown that all extracts had lower capacity to inhibit enzymes than the thiourea (70.05%) molecule employed as a reference (Table 5).

3. 5. Hemolytic Activity

There was no report on the hemolytic activity of these *Thymus* species in the literature. In this study for the first-time hemolytic activity of methanol extracts of *Thymus kotschyanus* var. *kotschyanus* and *T. kotschyanus*

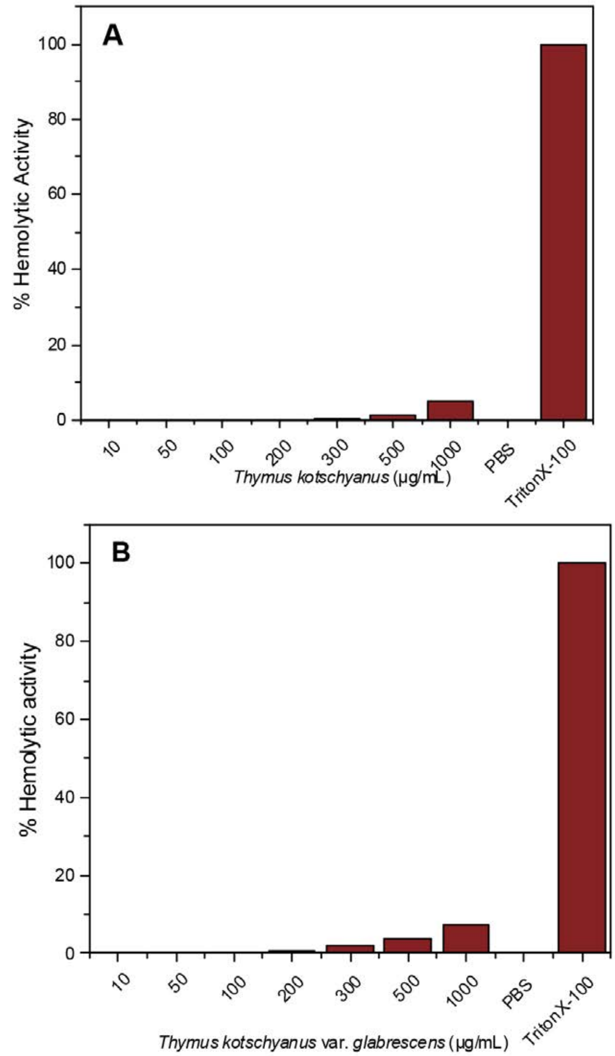

Figure 1. Hemolytic activity of methanol extracts from plants. **A** *Thymus kotschyanus* var. *kotschyanus*, **B** *T. kotschyanus* var. *glabrescens.*

Table 5. Enzyme inhibition potential of different extracts from plants.

	Inhibition of the acetylcholinesterase enzyme (%)		Inhibition of the urease enzyme (%)	
	TKK	TKG	TKK	TKG
Petroleum ether	95.12 ± 0.60*	90.91 ± 1.87*	12.346 ± 1.623*	6.995 ± 1.138*
Ethyl acetate	93.20 ± 0.33	91.41 ± 0.57*	19.846 ± 0.218*	9.834± 0.197*
Methanol	95.18± 0.95*	98.33 ±0.66*	34.404 ± 3.869*	17.726± 8.356*
Galantamine	97.14 ±0.14			
Thiourea			70.05 ±0.01	

var. *glabrescens* species in the selected dose range against human erythrocytes were examined and compared to Triton X-100 and PBS. The hemolytic activity results of these extracts are shown in Figures 1. The methanol extract of both plant extracts presented low hemolytic activity. However, the hemolytic activity of both extracts increased in a dose-dependent manner as expected. As no significant difference was observed, TKK methanol extract presented rather higher hemolytic activity than TKG at 300–1000 µg/mL.

3. 6. Cytotoxicity Activity

The cytotoxic activity of methanol extracts of *Thymus kotschyanus* var. *kotschyanus* and *T. kotschyanus* var. *glabrescens* species were tested on HT-29 colorectal cancer cells. Methanol extract obtained from TKK did not show cytotoxic activity against HT-29 cells. *T. kotschyanus* var. *glabrescens* showed relative cytotoxic activity at concentrations of 750 µg/ml (Figure 2).

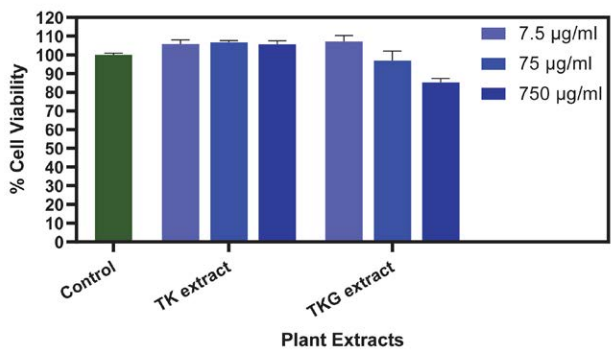

Figure 2. Cytotoxicity analysis of methanol extracts of *Thymus kotschyanus* var. *kotschyanus* (TK) and *T. kotschyanus* var. *glabrescens (*TKG*)* in HT-29 cells. The cytotoxicity effect of plant extracts was analysed with *in vitro* XTT assay. Cells were incubated with 7.5, 75, and 750 µg/mL plant extract for 4 h, and the medium was replaced with fresh complete medium. Cells were then cultured for an additional 24 h before the XTT assay. Control cells were treated with PBS.

3. 7. DCFDA Assay

Both plants extract significantly decreased ROS generation in cancer cells. Such dramatic changes in ROS generation in cancer cells are expected to affect cell proliferation profile of cancer cells.[30] However, we did not observe a significant difference in terms of cell proliferation with control groups (Figure 3).

3. 8. Phenolic Compounds Analysis

The composition of these extracts was examined both qualitatively and quantitatively since methanol extracts from plants show considerable biological activity

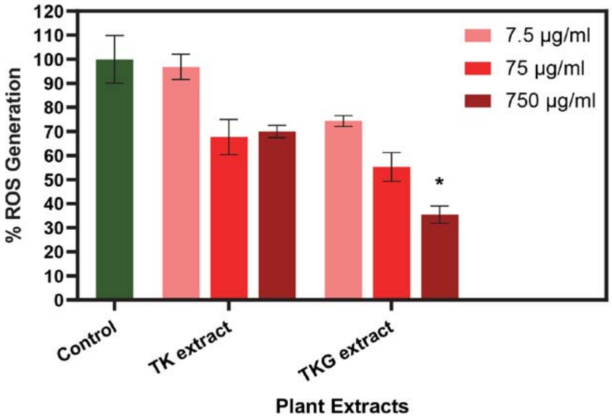

Figure 3. ROS generation analysis of methanol extracts of *Thymus kotschyanus* var. *kotschyanus* (TK) and *T. kotschyanus* var. *glabrescens* (TKG) in HT-29 cells. The ROS effect of plant extracts was analysed with *in vitro* DCDFA assay. Cells were incubated with 7.5, 75 and 750 µg/mL plant extract for 45 min and medium was replaced with fresh complete medium. * $p < 0.05$

when compared to other extracts. Compounds of apigenin 7-O-neohesperidoside, chlorogenic acid and rosmarinic acid in both plants were examined and it was shown that apigenin 7-O-neohesperidoside molecule (TKK: 7.250 µg analyte/mg extract; TKG: 6.103 µg analyte/mg extract was dominant in both plant extracts. In addition, chlorogenic acid and rosmarinic acid compounds were found similar amounts in both plant extracts (Figure 4, Table 6).

Table 6. Quantitative determination of compounds in methanol extracts of plants.

	µg analyte/mg extract	
	TKK	**TKG**
Apigenin 7-O-neohesperidoside	7.25±0.09	6.10±0.04
Chlorogenic acid	3.60±0.01	3.73±0.01
Rosmarinic acid	2.93±0.37	2.26±0.11

TKK: *Thymus kotschyanus* var. *kotschyanus;* TKG: *T. kotschyanus* var. *glabrescens*

4. Discussion

Egyptians laid the foundation for the widespread usage of *Thymus* species for medicinal purposes today. Because of its antibacterial and antiviral qualities, they employed it as an antiseptic. Today, it is employed as a mouthwash or a tea to treat inflammation of the upper respiratory tract and cough. *Thymus* species' flowering stems are used as diuretics, carminatives, and nausea remedies. It is applied to eliminate gastrointestinal parasites. It is utilized in several gynecological disorders, including breast cancer, breast swelling, breast inflammation, sick and inflamed uterine treatments, and as a miscarriage

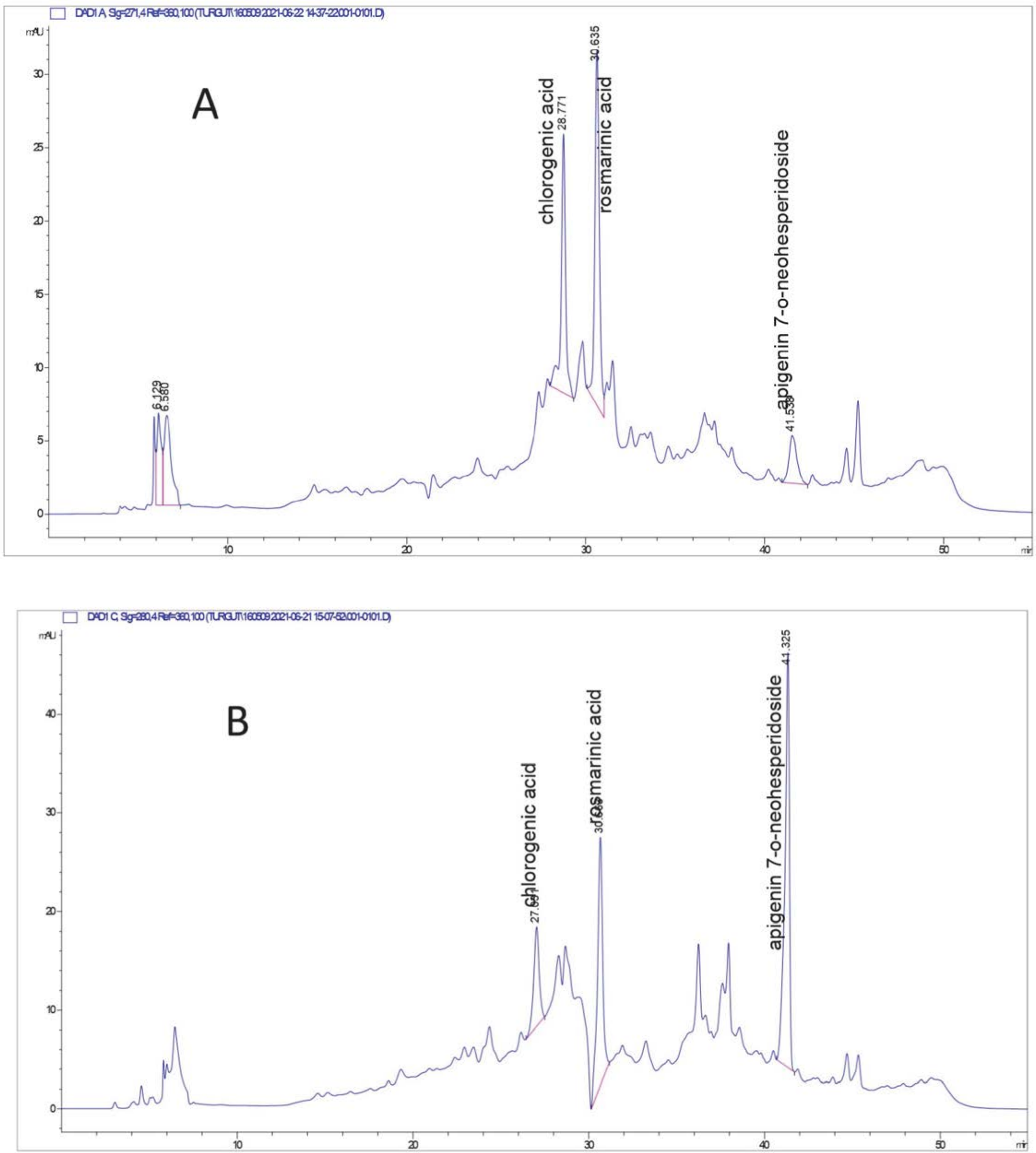

Figure 4. The HPLC-DAD chromatogram of methanol extracts from plants. A: *Thymus kotschyanus* var. *kotschyanus*; B: *T. kotschyanus* var. *glabrescens*

preventative precaution. It is beneficial in controlling excessive menstrual flow.[31] The phenolic components of dichloromethane, methanol and water extracts obtained by using *Thymus argaeus* species were determined. The two substances that were most often identified in methanol extract were chlorogenic acid and rosmarinic acid. In water extract, chlorogenic acid was the most prevalent substance. Sinapic acid and gallic acid were only found in the water extract. Caffeic acid was found to be the most prevalent substance in dichloromethane extract.[32] *Thymus migricus* species phenolic components were examined using LC-MS/MS. Quinic acid, chlorogenic acid, cinaroside, luteolin, and p-coumaric acid are the major substances included in *T. migricus* extract.[33] LC MS/MS chromatography was used to evaluate the phenolic components of the water decoction and water infusion extracts made from aerial portions of the *Thymus sipyleus* species that were collected from the Kaz Mountains within the boundaries of Balkesir province. The main phenolic compounds in the decoction extract are fumaric acid, rosmarinic acid, and quercitin, whereas the main phenolic compounds in the infusion extract are kaempferol, rosmarinic acid, and fumaric acid. The largest flavonoid derivative substance, kaempferol, the major coumaric acid derivative, rosmarinic acid, fumaric acid, have all been identified in the infusion extract.[34]

The antioxidant activity of extracts from the *Thymus argaeus* species in dichloromethane, methanol, and water was evaluated using the DPPH and ABTS radical scavenging assays, as well as the FRAP, phosphomolybdenum and CUPRAC procedures. The water extract of *T. argaeus* obtained by decoction was found to have the most effective ABTS and DPPH radical scavenging activity. The weakest activity belongs to dichloromethane extract. The methanol extract had the highest CUPRAC, while the water extract showed the highest activity in the FRAP test. The dichloromethane extract was found to have the lowest activity in both experiments. While the water extract showed the strongest antioxidant activity in the phosphomolybdenum test, the dichloromethane extract showed the least activity.[32]

The antioxidant activity of 1,2,4 and 8% infusions obtained from aerial parts of *Thymus haussknechtii* species was tested by DPPH radical scavenging test and H_2O_2 scavenging tests. In the DPPH radical scavenging test, 8% infusion was the extract with the highest activity with 77.41% inhibition value. In the H_2O_2 scavenging test, 1% *T. haussknechtii* extract showed the highest H_2O_2 scavenging activity.[35] Only a few studies have been found in the literature on the two *Thymus* L. varieties used in this study. In one of them, the DPPH, FRAP activity of the essential oil of the plant was examined and it was determined that it showed significant antioxidant activity. In addition, the essential oil of the plant was found to be effective against strains *B. cereus*, *L. monocytogenes*, *E. coli* and *S. typhimurium*.[36] In another study, essential oil of *Thymus kotschyanus* var. *kotschyanus* and *T. kotschyanus* var. *glabrescens* were analyzed by HS-SPME/GC-MS. In this study thymol, carvacrol, p-cymene and γ-terpinene were detected the main compounds of *T. kotschyanus* var. *kotschyanus*; thymol, carvacrol and p-cymene were detected the major constituents of *T. kotschyanus* var. *glabrescens*.[37] DPPH radical and iron reducing activity of both essential oil and methanol extract of *T. kotschyanus* species were investigated. It was determined that the DPPH radical scavenging and iron reducing activities of the essential oil were close to the DPPH and FRAP antioxidant activities of the methanol extract.[38] In a recent study, it was emphasized that the water extract prepared from the leaf of the *T. kotschyanus* species showed a significant antibacterial effect on gram-negative bacteria.[39]

For the first time, the biological activities of several extracts from two *Thymus* L. varieties were thoroughly studied in this work. Analyses of the bioactive extracts' composition and toxicity were conducted. In parallel with the literature, methanol extracts of both plants exhibited significant biological activity, and compounds like phenolic compounds previously analyzed from *Thymus* species were also analyzed in these species.

Hemolysis, also known as membrane lipid bilayer lysis, is typically responsible for the destruction of red blood cells and is influenced by the concentration and potency of the extract. Furthermore, the chemical contents of the extracts have a direct impact on the hemolytic activity.[40,41] Many of the natural compounds found in medicinal plants can alter biological membranes. Several authors have reported that flavonoids and the widely distributed polyphenol subgroup have a positive impact on the stability of the erythrocyte membrane.[42] Furthermore, De Freitas et al. report that the flavonoids may be a source of membrane stabilization by intensifying the van der Waals contacts inside the lipid bilayer. [43,44] In this study, using the hemolytic assay, the cytotoxic activity of the extracts was assessed to ascertain the toxicity profile of *Thymus* L. varieties. It seems that the phenolic compounds in the plants' methanol extract affect both the erythrocyte membrane's structural stability and its capacity to stifle free radicals. Nonetheless, the administration of *Thymus* L. varieties extracts results in heightened erythrocyte resistance against hemolysis triggered by free radicals. Our findings demonstrate that these plants in phenolic compounds have a cell membrane-stabilizing and protective function.

5. Conclusion

In this study, the biological activities of two *Thymus kotschyanus* varieties and the chemical content of the active extract were analyzed. It was determined that methanol extract of TKG showed more effective antioxidant and anticholinesterase activity than TKK extracts. According to the anti-urease activity results, the highest urease enzyme inhibition was detected in TKK. It was determined that TKG methanol and ethyl acetate extracts exhibited strong antimicrobial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis* strains. It was determined that both plant methanol extracts showed low hemolytic effect against human erythrocytes. The cytotoxic activity of methanol extract of both species in HT-29 cell line was investigated and it was determined that TKG methanol extract showed anti-proliferative effect compared to TKK methanol extract. In addition, both plants extract significantly decreased ROS generation in cancer cells. It was discovered that apigenin 7-O-neohesperidoside was the most abundant compound in both plants when it was compared to chlorogenic acid and rosmarinic acid. The findings obtained in this study suggest that methanol and ethyl acetate extracts obtained from these two species can be used as antioxidant, anticholinesterase, antimicrobial and antiurease agents.

6. References

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Povzetek

Ljudje v Turčiji so uporabljali sorti *Thymus kotschyanus* var. *kotschyanus* (TKK) in *T. kotschyanus* var. *glabrescens* (TKG) kot začimbo in zdravilo. Ugotovljeno je bilo, da imajo metanolni izvlečki rastlin najmočnejšo antioksidativno, antiholinesterazno in antiureazno aktivnost ter visoko vsebnost skupnih fenolov. Ugotovljeno je bilo tudi, da imajo etil acetatni in metanolni izvlečki močno protimikrobno aktivnost. Metanolni izvlečki so izkazovali majhen hemolitični učinek na človeške eritrocite. Ugotovljeno je bilo, da ima ekstrakt TKG večji antiproliferativni učinek v primerjavi z ekstraktom TKK. Oba rastlinska izvlečka sta pomembno zmanjšala nastajanje reaktivnih kisikovih zvrsti (ROS) v rakavih celicah. Ugotovljeno je bilo, da so bile količine klorogenske in rožmarinske kisline v obeh rastlinah podobne, spojina apigenin 7-O-neohesperidosid pa je bila v večjih količinah ugotovljena v TKK. Ugotovitve, pridobljene v tej študiji, kažejo, da se metanolni in etil acetatni izvlečki, pridobljeni iz teh dveh vrst, lahko uporabljajo kot antioksidanti, zaviralci holinesteraze, protimikrobna sredstva in zaviralci ureaze. Ugotovitve podpirajo tradicionalno uporabo teh vrst.