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Quantification of Secondary Metabolites of *Satureja pilosa* (Lamiaceae) by LC-HRMS and Evaluation of Antioxidant and Cholinergic Activities

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Abstract: In this study, secondary metabolite profiles of dichloromethane, acetone and methanol extracts of leaves and branches of *Satureja pilosa* species were determined by LC-HRMS. *In-vitro* inhibition activities of the obtained extracts against Alzheimer's disease (AD) which is a neurodegenerative disease were performed, and structure-activity relationships were discussed. While rosmarinic acid was found to be at the highest rate among the reported secondary metabolites in acetone and methanol extracts of the plant, penduletin was the secondary metabolite with the highest concentration in dichloromethane extract of the plant. The highest AChE inhibition was determined in leaf methanol extract with IC_{50}: 41.2 ± 5.6 µg/mL, and the highest BChE inhibition was detected in leaf dichloromethane extract of the species with IC_{50}: 52.3 ± 8.6 µg/mL concentration. Herein, total antioxidant capacities of the extracts were determined and reported by DPPH and ABTS radical scavenging and CUPRAC reducing ability methods.

Keywords: *Satureja pilosa*; secondary metabolite; LC-HRMS; AChE; BChE; antioxidant.

1. Introduction

One of the most obvious problems of the aging world population is neurodegenerative diseases. Developed countries with increasing elderly populations are more affected by these diseases than developing or more developed countries. The phenomenon of an aging world population has led humanity to new searches to combat this situation. In the case of neuronal oxidation, natural products are always the first source to be used for this purpose, as it is known that polyphenolic secondary metabolites with antioxidant properties cross the blood-brain barrier and show critical neuroprotective effects *in vitro* [1-4].

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Although the negative effects of oxidative stress, exogenous and endogenous free radicals are effective in Alzheimer's disease (AD), one of the accepted theories about AD has been the "cholinergic hypothesis". Because it has been determined that the neurotransmitters acetylcholine (ACh) and butyrylcholine (BCh) are less than they should be in the brain cells of Alzheimer's patients. For this reason, inhibition of acetylcholinesterase (AChE), the main enzymes that hydrolyze ACh and BCh, has become an important treatment option for AD [5]. The genus *Satureja*, belonging to the Lamiaceae family, has approximately 200 species worldwide, most of which are distributed in the Mediterranean (Southern Europe, North Africa, Morocco, Libya, Spain, Greece, and Türkiye), Saudi Arabia, Balkans, Southern Russia, the Caucasus, Iraq and Iran. Perennial plants make up the genus *Satureja*. Because glandular trichomes create essential oils, Lamiaceae are classified as aromatic. One of the Lamiaceae species that is most prevalent in Turkey is *Satureja*. Known as "pointed thyme," the *Satureja* genus is widely distributed throughout Turkey, particularly in the eastern Mediterranean and Aegean regions. These aromatic semi-shrub plants, which can be either annual or perennial, thrive in stony, rocky, arid environments. Because of their potent aromatic qualities, *Satureja* species are used by many people as a diuretic, digestive aid, and spice to improve the flavor of food or tea. [6-10]. In the past, several studies have been reported on the chemical contents of *S. pilosa* collected from Anatolia and nearby geographies. Some of these studies are related to the volatile oil content of the species and the DPPH radical scavenging capacity and AChE inhibition [11-16]. Recently, reports on the wound healing potential, anti-inflammatory and antibacterial activity of *Satureja* species and secondary metabolite profiles based on HPLC and NMR have begun to be published. However, there is insufficient data on the cholinergic and antioxidant capacity assessment and secondary metabolite profile of *S. pilosa*. In a study conducted with *S. pilosa* collected in Greece, the flavonoid contents of the species were reported by HPLC-PDA-MS method in polar solvents. In the results, flavonoid glycosides as well as rosmarinic acid and clinopodic acid were reported [17]. In another study conducted in Greece, caffeic acid, quercetin, coumaric acid phenolic substances were reported in *S. pilosa* solvent extracts by HPLC method [18].

The closely related enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are essential for the nervous system and other systems. Acetylcholine (ACh), a neurotransmitter essential for synaptic transmission, especially in cholinergic neurons, is hydrolyzed mainly by AChE [19–21]. AChE guarantees the appropriate termination of brain signals by converting acetylcholine into acetate and choline, preserving the equilibrium required for regular physiological activities like muscle contraction and thought [22–24]. Although BChE and AChE share a similar structure and catalytic activity, BChE is more broadly distributed throughout different organs, such as the liver, plasma, and brain, and it has a wider substrate selectivity [25,26]. Though its exact physiological purpose is unknown, BChE is assumed to function as a backup mechanism for AChE, assisting in acetylcholine control and engaging in the hydrolysis of other ester-based molecules. Recent study has demonstrated the relevance of BChE in a variety of pathological situations [27-29]. The inhibition of these enzymes has received a lot of interest in the disciplines of neuropharmacology and toxicology. AChE inhibitors are commonly employed in the treatment of neurodegenerative illnesses like Alzheimer's because they improve cholinergic transmission by reducing the breakdown of acetylcholine [30]. Similarly, BChE inhibitors have shown promise in the treatment of organophosphate toxicity. This article will provide a complete description of the structural, functional, and therapeutic characteristics of AChE and BChE.

Antioxidants are vital compounds that play a critical role in protecting living organisms from oxidative stress caused by reactive oxygen species (ROS) and free radicals [33-35]. These highly reactive molecules are natural byproducts of cellular metabolism and environmental factors, including pollution, UV radiation, and toxins [36,37]. If left unchecked, ROS can cause significant damage to biomolecules such as DNA, lipids, and proteins, leading to various chronic diseases, including cancer, cardiovascular disorders, and neurodegenerative conditions [38,39]. In recent years, there has been growing interest in the study and application of antioxidants due to their potential health benefits. Found abundantly in fruits, vegetables, herbs, and certain beverages like tea and wine, natural antioxidants, particularly phenolic compounds, flavonoids, and vitamins (C and E), have gained attention for their ability to neutralize free radicals and reduce oxidative damage [40-42]. Synthetic antioxidants are also widely used in food preservation and pharmaceuticals to prevent oxidative degradation. Despite the extensive research into antioxidants, challenges remain in understanding their mechanisms of action, evaluating their efficacy, and standardizing methods to measure their activity [43,44]. This article explores the fundamental principles of

antioxidant activity, their sources, and their implications for health and food technology, shedding light on the current state of antioxidant research and its future potential.

As mentioned above, due to the limited number of studies on the identification of secondary metabolites on the *Satureja* genus and the investigation of their anticholinergic effects and antioxidant activities, this study aimed to determine the secondary metabolites of *S. piolsa* from extracts of different polarities by LC-HRMS and to discuss their AChE, and BChE activities based on structure-activity relationship.

2. Materials and Methods

2.1 Plant Material

Satureja pilosa P.H. Davis species was collected from 1400 m altitude from Gökçeada Island in Çanakkale province by Balıkesir University faculty member Prof. Dr. Tuncay Dirmenci on 25 August 2019. The herbarium sample of this species was recorded and stored in Balıkesir University Necatibey Education Faculty Herbarium with the code TD 5245.

2.2. Chemicals

(-)-Epigallocatechin (>97% TRC Canada), (-)-Epigallocatechingallate (>97% TRC Canada), Chlorogenic acid, Fumaric acid (≥99 % Sigma-Aldrich), Chicoric acid (>97% TRC Canada), Orientin (>97% TRC Canada), Caffeic acid (≥98% Sigma-Aldrich), (+)-*trans* taxifolin (>97% TRC Canada), Luteolin-7-*O*-rutinoside (>97% Carbosynthlimited), Vanilic acid (≥97% Sigma-Aldrich), Naringin (≥90% Sigma-Aldrich), Luteolin 7-*O*-glucoside (>97% TRC Canada), Hesperidin (≥98% J&K), Rosmarinic acid (≥96% Sigma-Aldrich), Hyperoside (>97% TRC Canada), Dihydrokaempferol (>97% Phytolab), Apigenin 7-*O*-glucoside (>97% EDQM CS), Ellagic acid (>97% TRC Canada), Quercitrin (>97% TRC Canada), Quercetin (≥95% Sigma-Aldrich), Naringenin (≥95% Sigma-Aldrich), Luteolin (95% Sigma-Aldrich), Apigenin (>97% TRC Canada), Hispidulin (>97% TRC Canada), Isosakuranetin (>97% Phytolab), Penduletin (>97% Phytolab), CAPE (Caffeic acid phenethyl ester) (≥97% European pharmaco poeiareference standard), Chrysin (≥96% Sigma-Aldrich), Dihydrocapsaicin (≥97 % Sigma-Aldrich), Methanol (≥%99.5 Tekkim), Dichloromethane (≥%99.5 Tekkim), Acetone (≥%99.5 Tekkim).

2.3. Extraction of Plant Material

After the aerial parts of *S. pilosa* were dried thoroughly in the shade, the leaves (188 g) and branches (138 g) were separated from each other and powdered on a spice mill in the laboratory. The powdered leaf plant material was filled into Soxhlet cartridges made of filter paper, approximately 20 grams each, and by performing 3 flushes with dichloromethane, acetone and methanol at each stage, the extracts of the materials in each cartridge were obtained separately. The 9 different extracts obtained at the end of 3 flushes for each solvent type were combined and dried by evaporation in a rotary evaporator. These processes were repeated for a total of 7 cartridges for the branch parts of the powdered plant. The extracts were stored in the refrigerator at +4 °C until the measurements were made. Extract codes are given in Table 1.

Table 1. Codes and amounts of extract *Satureja pilosa* extract

Extracts name	Extract code	Amount of extract (g)
S. pilosa leaf DCM	L-DCM	15.5
S. pilosa leaf Acetone	L-Acetone	2.1
S. pilosa leaf MeOH	L-MeOH	27.1
S. pilosa branch DCM	B-DCM	2.1
S. pilosa branch Acetone	B-Acetone	1.4
S. pilosa branch MeOH	B-MeOH	10.1

2.4. Antioxidant Capacity Assays

2.4.1. Reducing Ability Assay

2.4.1.1. CUPRAC (Cupric Reducing Antioxidant Capacity)

The CUPRAC total antioxidant capacity (TAC) determination method is based on the reduction of the Cu(II)-Nc complex formed by 2.9-dimethyl-1.10-phenanthroline (Nc) with Cu(II) to the Cu(I)-Nc reducing, which gives maximum absorbance at 450 nm in the presence of antioxidant compounds [45,46]. In this method, 1 mL of 10 mM Cu(II) solution, 7.5 mM Nc solution and 1 M NH_4CH_3COO buffer were added to a test tube, respectively. Different concentration (10-30 mg/mL) of plant extracts and in H_2O were added and shaken well. After preparing the solutions to a total volume of 4.1 mL, the tubes were kept in closed caps at room conditions for 30 minutes. At the end of incubation, absorbance values were measured at 450 nm, which is the characteristic wavelength of the color of the formed Cu(I)-Nc chelate, against the reference solution.

2.4.2. Radical Scavenging Ability

2.4.2.1. ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) Assay

This method is a TAC method based on the inhibition of the absorbance of the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) chromogen radical cation by hydrogen donor antioxidants [47]. Unlike color formation monitoring in other techniques, the degree of color loss is monitored in this technique. As stated in the literature, the ready-to-use blue-green $ABTS^{\bullet+}$ solution was diluted 1:10 with EtOH. 4 mL EtOH was added to 1 mL $ABTS^{\bullet+}$ solution and the absorbance values at the end of the 6th minute at 734 nm were determined. Then, (x mL) samples were taken from 1 mg/mL plant extracts and 1 mL $ABTS^{\bullet+}$ solution and (4-x) mL EtOH were added. Then, the absorbance at the same wavelength at the 6^{th} minute was measured. ΔA values were obtained by subtracting the absorbance of the solution containing the plant extract from the initial absorbance value of the radical [48].

2.4.2.2. DPPH (2,2-diphenyl-1-picrylhydrazyl) Assay

This method is based on the principle of measuring the scavenging effects of antioxidants on the stable free radical of $DPPH^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) [49,50] $DPPH^{\bullet}$ radical is a molecule that is easily soluble in methanol and forms a purple solution with a maximum absorbance at 515 nm. When these radical encounters an electron donor or hydrogen donor group in its environment, it is easily reduced to hydrazine. As a result of this reduction, the purple color shifts to a pale-yellow color and this change can be easily followed with a spectrophotometer. This study was performed as follows: 2.65 mL of MeOH and 1 mL of 0.1 mM $DPPH^{\bullet}$ solution was added to a test tube and kept at room conditions for 30 minutes, then the absorbance values of the first $DPPH^{\bullet}$ solution were measured. Plant extract (× mL), 2.65 mL MeOH, and 1 mL 0.1 mM $DPPH^{\bullet}$ solution was added to another test tube and absorbance values were recorded after 30 min. ΔA values were obtained by subtracting the absorbance of the solution containing the sample from the absorbance value of the radical.

2.4.3. Reporting of Antioxidant Capacity Assays Data

Each experiment was repeated 6 times, and the measurement results are given as Trolox equivalents [51], and the calculation method for each method is given below,

$\varepsilon TR = 1.67x10^4\ Lmol^{-1}cm^{-1}$ (CUPRAC method),

$\varepsilon TR = 2.6x10^4\ Lmol^{-1}cm^{-1}$ (ABTS method),

$\varepsilon TR = 4.65x10^3\ Lmol^{-1}cm^{-1}$ (DPPH method).

Trolox equivalent antioxidant capacities of plant extracts (in mmol TR/g) were calculated according to the following equation:

$$TAC\ (mmol\ TR\ g^{-1}) = (Af / \varepsilon TR)\ (Vf / Vs)\ (1 / c)$$

Where c = plant extract concentration
Vs = Volume of plant extract taken for analysis
Vf = Final volume after reagents were added
Af = Measured absorbance value

2.5. Anticholinergic Assays

2.5.1. Acetylcholinesterase/Butyrylcholinesterase Inbitition Assay

AChE and BChE inhibitions are used within the scope of anticholinergic studies. The AChE/BChE inhibitory effects of *S. pilosa* extracts were determined in accordance with Ellman's method [52] as given in our previous studies and the literature [53-55]. AChE and BChE were purchased and obtained Electrophorus electricus and equine serum, respectively. DTNB and acetylthiocholine iodide/butyrylthiocholine iodide (AChI/BChI) were used as substrates for both cholinergic reactions [56,57].

2.6. LC-HRMS Analysis

2.6.1. Sample preparation for LC-HRMS

LC-HRMS analysis was performed according to our previous studies [58-60]. Each plant extract was weighed into a 5 mL volumetric flask as approximately 200 mg and 3.5 mL of methanol was added and vortexed for 1 minute. The vortexed solution was placed in an ultrasonic bath at 24 ^{o}C and kept until a clear, dark green solution was obtained. In order to prevent the flasks from breaking during the ultrasonic bath process, the flask caps were slightly loosened, and polyurethane foam was placed between the flasks and the ultrasonic bath walls and compressed. Then, 200 µL of 1000 ppm dihydrocapsaicin solution was added to each flask as internal standard and the volumetric flask was completed with methanol and mixed by shaking by hand. The flask was placed in the ultrasonic bath again and kept in the ultrasonic bath for another 10 minutes and then kept at room temperature (24 ± 3^{o} C) for 10 minutes by covering it with aluminum foil. After 10 minutes, the solution was filtered through a 0.45 µm Millipore Millex-HV filter and each sample was placed in 1.5 mL vials and placed on the HPLC tray for LC-HRMS measurements. For each run, 0.35 µL of sample was injected into the LC-HRMS device.

2.6.2. Standard Solutions

Calibration solutions containing all standard substances with known purity values, specified in section 2.2, were prepared with methanol in 10 mL volumetric flasks in the range of 0.01, 0.05, 0.1, 0.3, 0.5, 1, 3, 5, 7 and 10 mg/L. In this study, 1000 mg/L dihydrocapsaicin (purity 97%) solution in methanol was used as internal standard.

2.6.3. Instrumentation and Chromatographic Conditions of High-Resolution Mass Spectrometry (LC-HRMS) Method

The analysis of secondary metabolites of *S. pilosa* extracts was carried out using a method previously developed and validated by our group. In this method, a Troyasil C18 column (150 × 3 mm, 5 µ, Istanbul, Turkey) connected to a Thermo Ultimate 3000 HPLC and a Thermo Orbitrap Q-Exactive mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) were used. In order to optimize the ionization parameters of each analyte, all standards were recorded separately in the mass spectrometry instrument. Then, analyses were performed in the mobile phase system with gradient programs of 50% A and 50% B for 0-1 min, 100% B for 1-6 min, and 50% A and 50% B for 6-10 min. The column oven was used and set at 25 °C, and the flow rate of the mobile phase was 0.35 mL/min. The ambient temperature and humidity of the laboratory were recorded as 22.0 ± 5.0 °C and 50 ± 15%, respectively. The MS conditions were used as follows: sheath gas flow rate 45, auxiliary gas flow rate 10, spray voltage 3.80 kV, capillary temperature 320 °C, auxiliary gas heater temperature 320 °C, S-lens RF level 50. Compounds were identified by comparing the retention times of compounds and target ions in LC-ESI-HRMS [58-60].

2.6.4. Method Validation of LC-HRMS

The LC-HRMS method used in this study was previously validated by our group according to the EURACHEM CITAC Guide and its data have been published [61-63]. The method validation parameters used here were selected as specificity, accuracy, linearity, LOD and LOQ.

2.6.4.1. Specificity

Specificity is the proof that the analytical method used has a selective character against that substance. Specificity is based on the proof that the target analyte is not negatively or positively affected by the matrix or working processes. For this purpose, an LC technique combined with extremely LC-HRMS was used. Thus, it was aimed to provide a high degree of specificity with both retention times and mass spectra of the analytes. The specificity of the developed LC-ESI-HRMS method was successfully determined by direct analysis (blank) of all the different solvents prepared, *S. pilosa* extract and added target analytes.

2.6.4.2. Linearity, Accuracy, LOD and LOQ of the LCHRMS Method

Accuracy is a parameter that expresses the closeness or estimation of the measurement results to the real quantity. The % recovery value of the method for each target analyte is one of the checked parameters for accuracy. This value was calculated based on LC-ESI-HRMS data for each analyte according to the following formula in our previous reports (See Table S1 in supporting information).

$$Recovery\ \% = Recovered\ concentration\ /injected\ concentration\ x\ 100$$

All validation data including recovery, linear regression equation, linearity ranges, the regression coefficients, LOD and LOQ values of all reported secondary metabolites by LC-ESI-HRMS were given in Table S1. Limits of detection (LOD), limit of quantification (LOQ), substances were determined by using following equations:

LOD or LOQ = κSDa/b, where LOQ is 3 and κ = 3 for LOD [64-68]

2.6.5. Measurement Uncertainty Assessment

The uncertainty parameters of the applied method herein were determined as uncertainty from purity of standard (u_{standard}), weighing (u_{weighing}), (u_{volume}), recovery (u_{recovery}) and calibration curve ($u_{calibration\ curve}$) of the applied method. GUM methodology was applied in accordance with the EURACHEM CITAC and ISO Guide 35 for the estimation of the uncertainty measurement [34,67-70]. The combined uncertainty ($u_{combined}$) was calculated as follows:

$$u_{combined} = \sqrt{u_{standard} + u_{weighing} + u_{volume} + u_{recovery} + u_{calibration\ curve}}$$

The expanded uncertainty (uExpanded) calculated using a coverage factor of k=2 giving a confidence level of approximately 95% was calculated as follows:

$$u_{expanded} = u_{combined} \times k$$

3. Results and Discussion

The leaves and branches of *S. pilosa* were separately powdered and extracted by Soxhlet extraction, then analyzed using the LC-HRMS method developed and validated by our group. A total of 28 secondary metabolites were quantitatively determined from all extracts and the results are given in Table 2 and, antioxidant capacities and cholinergic activities of the extracts were discussed herein. Antioxidant capacities of the extracts values of plant extracts were determined in mmol TR/g by applying CUPRAC, ABTS-persulfate and DPPH methods (Table 3). CUPRAC method is a technique based on measuring as spectrophotometric the concentration of copper neocuproine [Cu^{2+}-Nc] complexes to investigate the reducing ability of the pure compounds or plant extracts [71,72].

Table 2. The quantity of reported secondary metabolites extracts of *S. pilosa* measured by *LC-HRMS (*mg/kg*)*

	Name	L-DCM	B-DCM	L-Acetone	B-Acetone	L-MeOH	B-MEOH
1	(-)-Epigallocatechin	<LOD	<LOD	197.20	<LOD	<LOD	<LOD
2	(-)-Epigallokatechin gallate	<LOD	2.20	<LOD	1.09	37.49	2.80
3	Chlorogenic acid	2.00	10.46	48.55	129.39	208.78	246.52
4	Fumaric acid	<LOD	203.97	237.90	459.82	2922.00	2480.38
5	Chicoric acid	2.49	<LOD	<LOD	<LOD	<LOD	<LOD
6	Orientin	<LOD	0.51	8.62	1.94	18.95	1.70
7	Caffeic acid	11.18	17.83	396.15	1623.05	285.83	488.46
8	(+)-t*rans*-Taxifolin	109.10	3.65	519.45	345.71	61.56	<LOD
9	Luteolin-7- *O*-rutinoside	20.06	227.60	3254.77	6332.72	5117.59	3474.71
10	Vanilic acid	<LOD	82.54	<LOD	<LOD	797.65	1391.24
11	Naringin	4.37	58.94	1817.91	1210.47	5591.66	246.89
12	Luteolin 7-*O*-glucoside	0.93	7.39	231.17	192.60	313.61	89.21
13	Hesperidin	70.98	630.02	4627.72	11800.81	155.67	4951.52
14	**Rosmarinic acid**	**<LOD**	**903.57**	**10144.53**	**51086.79**	**38867.40**	**27245.10**
15	Hyperoside	<LOD	4.85	123.36	191.82	208.50	44.56
16	Dihydrokaempferol	766.82	9.84	546.90	188.57	35.61	3.43
17	Apigenin 7-*O*-glucoside	1.50	1.15	22.60	12.97	23.04	5.56
18	Ellagic acid	<LOD	1.84	2.72	40.92	2.35	3.41
19	Quercitrin	<LOD	0.78	30.15	35.89	43.56	9.95
20	Quercetin	10.34	0.54	64.39	25.49	8.53	1.07
21	Naringenin	7381.01	14.,43	1917.05	1058.32	269.03	5.44
22	Luteolin	64.27	5.56	828.83	367.46	237.03	105.65
23	Apigenin	212.91	3.97	410.88	157.84	104.61	16.44
24	Hispidulin	1562.85	233.99	3078.37	1486.16	824.67	384.77
25	Isosakuranetin	473.29	97.77	181.73	95.25	48.06	1.24
26	Penduletin	21190.16	3590.01	948.25	757.77	136.15	85.40
27	CAPE	45.30	22.58	1.16	1.57	0.13	0.23
28	Chrysin	25.04	7.71	66.76	8.10	1.31	2.73
	TOTAL	**31954.6**	**6129.27**	**29509.92**	**77612.52**	**56320.77**	**41288.41**

Table 3. Trolox equivalent of different antioxidant activities of *S. pilosa* TAC values

Extract	TAC_{CUPRAC} (mmol TR/g)	$TAC_{ABTS\text{-}persulfate}$ (mmol TR/g)	TAC_{DPPH} (mmol TR/g)
L-DCM	1.13±0.04	0.40±0.01	0.78±0.02
L-Acetone	1.18±0.05	1.06±0.05	0.88±0.04
L-MeOH	2.40±0.07	1.13±0.09	1.32±0.11
B-DCM	0.52±0.02	0.24±0.01	0.43±0.01
B-Acetone	3.12±0.11	1.86±0.03	1.34±0.04
B-MeOH	1.90±0.05	1.84±0.06	1.10±0.03

The results showing the Cu^{2+} reducing abilities of dichloromethane, acetone and methanol extracts prepared by Soxhlet extraction of leaves and branches of *S. pilosa* species as Trolox equivalents are given in Table 3. While *S. pilosa* dichloromethane extract had the lowest Cu^{2+} ion reducing capacity among the leaf extracts with a result of 1.13±0.04 mmol/g, leaf methanol extract showed a relatively high Cu^{2+} ion reducing capacity with a TR/g value of 2.40±0.07 mmol. However, while almost 50% decrease in activity was observed in the dichloromethane extract of branches compared to the leaf, the best Cu^{2+} ion reducing capacity was observed in the acetone extract.

$ABTS^{\cdot+}$ and DPPH methods are frequently used methods for the evaluation of radical scavenging capacity of plant extracts [73-75]. As reported in the literature, stable blue/green $ABTS^{\bullet+}$ radicals were produced in a controlled manner in $ABTS/K_2S_2O_8$ medium. The extracts belonging to *S. pilosa* plant were also examined in terms of radical scavenging properties other than CUPRAC method and the results are summarized in Table 3. It was observed that the extracts with the least radical scavenging capacity among *S. pilosa* extracts were dichloromethane extract for leaves and branches, while it was determined that acetone extract had the highest radical scavenging capacity

Total secondary metabolites detected in *S. pilosa* leaf dichloromethane, acetone and methanol extracts were determined as 31.9 g/kg, 29.6 g/kg and 56.3 g/kg, respectively (see Table 2). In all antioxidant capacity experiments-CUPRAC, ABTS and DPPH- leaf methanol extract showed approximately 2 times more antioxidant capacity than leaf acetone and dichloromethane extracts. Whereas, L-DCM extract showed lower antioxidant capacity despite containing high levels of naringenin, penduletin and hispudilin. It is clearly seen that the reason for this is the presence of 38.9 g/kg rosmarinic acid and other vicinal hydroxyl substituted phenolic compounds and high amount of fumaric acid (2.9 g/kg) observed in L-MeOH extract. This relationship can be easily seen from the data of Table 2 and Table 3. Similarly, when the branch extracts were compared, it was determined that branch methanol extract has a higher antioxidant capacity due to its high content of rosmarinic acid (51.1 mg/kg), luteolin-7-*O*-rutinoside (6.3 g/kg), caffeic acid (1.6 g/kg) and other phenolic structures.

3.2. Cholinergic Activities

Inhibition percentages of ACHE and BCHE of plant extracts at four different concentrations were calculated according to the Ellman's method [76]. There is only single research about *S. pilosa*'s cholinergic activity in the literature. According to the assay data, it can clearly be observed that leaf methanol and branch acetone extracts were found to be the most active extracts of studied extracts herein. It showed that plants extracts were found to be significantly active in inhibition of ACHE at low concentrations and activity patterns are similar to antioxidant capacity assays as well. After natural products are extracted, their chemical compositions may sometimes change with artificial products due to oxidation via contact with air. This may cause any extract to be more difficult to dissolve even in the solvent it was obtained from, which is a frequently encountered situation. Since the results were different due to good dissolution or in homogenization of the extract, the experiments were repeated by our group twelve months later from the first data set and the experimental results were given as the average of a total of 12 sets of different experiments with 6 replicates performed at one-year intervals. In this case, the similar trend was found with the experiments one year ago. While the expanded uncertainty budget (U) value calculated according to EURACHEM CITAC Guide and related literatures was calculated as 13.6% for acetylcholinesterase inhibition in methanol extract, the uncertainty budget estimated for butyrylcholinesterase for

dichloromethane extract was calculated as 16.4%. In the light of these results, the highest acetylcholinesterase inhibition was measured in the leaf methanol extract with IC50: 41.2 ± 5.60 µg/mL, while the highest BChE inhibition was determined in the leaf dichloromethane extract with IC50: 52.3 ± 8.6 µg/mL concentration of the species. IC50 values of galanthamine for acetylcholinesterase and butyrylcholinesterase were determines as 4.1 ± 0.2 µg/mL and 12.3 ± 0.3 µg/mL, respectively. Thus we can clearly conclude that, the anticholinergic activity of the species comes from rosmarinic acid and other catecholic phenolic compounds.

In conclusion, where the antioxidant and cholinergic capacities of the leaves and branches of *S. pilosa* species were investigated by dichloromethane, acetone and methanol Soxhlet extractions, it was determined that the methanol extract of the leaf extracts showed the greatest antioxidant capacity, while this effect was highest in the acetone extract in the branch extracts. As a result of the LC-HRMS analysis, it was determined that the extracts rich in high amounts of rosmarinic acid and vicinal hydroxyl substituted phenolic compounds had high antioxidant capacities. Similarly, it was determined that AChE and BChE activities were directly related to the phenolic composition of the extracts. As a result, it was determined that *S. pilosa* species, which has a relatively lower usage rate compared to *Satureja cuneifolia* and *Satureja thymbra* in Anatolia, has potential usage area as both antioxidant and anticholinergic agent.

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