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Phytochemical studies of *Helichrysum armenium* subsp. *armenium* and its antioxidant, acetylcholinesterase, and antimicrobial activities

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ABSTRACT: *Helichrysum armenium* subsp. *armenium* plant was extracted with the Soxhlet apparatus. The obtained extract was fractionated and, respectively, AHB (petroleum ether–60% ethanol-chloroform), AHC (ethanol-toluene), AHD (ethanol-chloroform), and AHE (ethanol-ethylacetate) extracts were obtained. Then, antimicrobial, antioxidant, and acetylcholinesterase activity studies were performed on these extracts. In this study, with the using the LC-HRMS method from the aerial parts of *Helichrysum armenium* subsp. *armenium*, the flavonoids: apigenin, apigenin 7-glucoside, apigenin 7-methylate, luteolin 7-glucoside, kaempferol, quercetin, quercitrin, (+)-trans taxifolin, (-)-epicatechin gallate, sinensetin, rutin, 3'-o-methyl quercetin, hispidulin, chrysin, acacetin, genkwanin, chrysoeriol; the phenolic acids: chlorogenic acid, ascorbic acid, fumaric acid, caffeic acid, salicylic acid, caffeic acid phenethyl ester, emodin, vanilic acid; the alkaloid: caffeine and the saponoside; glycyrrhizic acid were obtained.

KEYWORDS: *Helichrysum armenium* subsp. *armenium*; phenolic compounds; antioxidant; acetylcolinesterase; antimicrobial; LC-HRMS.

1. INTRODUCTION

The genus *Helichrysum* Gaertner, belonging to the Asteraceae family, is notably widespread comprising approximately 500 species globally. There is great morphological diversity among the species and therefore they are divided into numerous subspecies. *Helichrysum* species are distributed in Southern Europe, South-West Asia, South India, South Africa, Sri Lanka, Australia, and Madagascar. In the context of Turkish flora, the genus *Helichrysum* is represented by a total of 26 taxa, encompassing 20 distinct species. Remarkably, 14 of these species are exhibiting endemism within Turkey [1-4].

H. armenium DC. is a plant originating from Europe, the Middle East, and the Caucasus, it is also widely known in Anatolia. It has been used for healing purposes by many different cultures and peoples. *H. armenium* is an herbaceous plant that can grow up to 50-60 cm and blooms with yellow flowers. *Helichrysum* species are also known by different names among the public in Turkey: ölmez çiçek, alay çiçeği, altın otu, arı çiçeği, bozoğlan, herdemtaze, sarı savran [5]. *H. armenium* is popularly known as altın otu in Turkey [6].

The medicinal uses of plants of the *Helichrysum* genus date back to the 3rd century BC, and in the work "Historia Plantarum" by the Greek Theophrastus of Eresos, it is reported that *Helichrysum* genus plants can be used in the treatment of burns and stings of poisonous animals. Moreover, in the work "De Materia Medica" written by the Greek Pedanius Dioscorides, it was explained that the decoction of *Helichrysum* flowers, macerated in wine, has diuretic properties and is useful in the treatment of urinary disorders and snake bites. It has continued to be used in traditional medicine for burns, poisonous animal stings, and as a diuretic. Early descriptions of the medicinal uses of plants of this genus often treat *Helichrysum* as a whole, without specifying a specific species [7]. *Helichrysum arenarium* (L.) Moench has a long history of being recognized as a medicinal plant in Europe. The capitulums of this plant, known as Helichrysi flos, are incorporated into medicinal herbal mixtures or added to tea blends for aesthetic enhancement [8]. In Europe,

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the public utilizes preparations made from the capitula of both *H. arenarium* and *H. italicum* in medicinal teas due to their cholagogue and diuretic effects, particularly for gallbladder-related ailments. Additionally, *H. arenarium* is employed to assist in the treatment of chronic gallbladder inflammation [8-9]. In Anatolia, naturally occurring *Helichrysum* species including *H. plicatum* DC., *H. graveolens* (Bieb.) Sweet and *H. orientale* (L.) Gaertner. are traditionally used to prepare infusions, known among the public for their properties as diuretics, gallbladder tonics, and remedies for kidney stones [10].

The studies on *Helichrysum* species have shown that they have choleretic [11], cholagogue [11, 12], diuretic [11], antidiabetic [13], antioxidant [14, 15], anti-inflammatory [14], antimicrobial [15], antiviral [16], antituberculosis [17], antimalarial [18], antispasmodic [19] and anticarcinogenic effects [20].

Many *Helichrysum* species are rich sources of flavonoids, chalcones, phloroglucinol derivatives, essential oils, α-pyrones, and diterpenes [21,22].

Flavonoids are compounds responsible for antioxidant activity. *Helichrysum* species have been examined in various studies in the literature for their various biological properties, including anti-inflammatory [14], antioxidant [23], acetylcholinesterase activity [24], and antimicrobial activity [15].

When the literature is examined, it is seen that the number of studies on the phytochemical content and biological activity of the *Helichrysum armenium* plant is quite limited. There is only one previous phytochemical and biological activity study on the *H. armenium* subsp. a*rmenium* species [25].

In a previous study, Albayrak et al. [25] found that *Helichrysum* plant samples were collected from Sivas, and the methanol extract was subjected to antioxidant and antibacterial activity studies. Also, they used only the DPPH method to evaluate antioxidant activity and used the agar-well diffusion method to analyse antimicrobial activity. In our study, unlike the study of Albayrak et al., DPPH, CUPRAC, and FRAP analysis methods were used to determine antioxidant activity on four different extracts, and the disk dilution method was used for antimicrobial analysis. Different microorganisms were used in antimicrobial analysis in both studies. Additionally, in our study, unlike the study of Albayrak et al. [25], acetylcholinesterase activity was examined and detailed phytochemical analysis by LC-HRMS.

2. RESULTS

2.1. Antioxidant Activity

It was determined that AHE (IC_{50}: 0.022 mg/mL) and AHD (IC_{50} :0.022 mg/mL) extracts showed the highest DPPH radical scavenging activity compared to other extracts. These results demonstrated that both extracts 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 extracts, it was found that all extracts had minimal radical scavenging capacity. The CUPRAC test results showed that AHE (4.314 mM troloxE/mg extract) and AHD (3.723 mM troloxE/mg extract) extracts have a higher Cu(II) to Cu(I) reduction potential than extracts. These results demonstrated that all extracts have lower Cu(II) to Cu(I) reduction potential than the ascorbic acid compound (5.683 mM troloxE/mg extracts). It was discovered that AHD (3.667 mM$FeSO_4$/mg extract) and AHE (3.001 mM$FeSO_4$/mg extract) extracts had more iron-reducing antioxidant power than other extracts. Additionally, it was discovered in this study that all extracts from the plant had lower FRAP values than the BHA compound.

The total phenolic and flavonoid contents of the plant's different extracts were calculated in this investigation. The AHE (79.895 mg GAE/ g extracts) and AHD (78.914 mg GAE/ g extracts) extracts were found to have the greatest concentration of phenolic compounds. Furthermore, it has been shown that, in comparison to other extracts, the AHE (77.601 mg quercetinE/g extract) and AHD (42.250 mg quercetinE/g extracts) extracts contain more flavonoid components. It is well known that phenolic and flavonoid compounds and antioxidant activity often have a linear relationship. This study found that AHE and AHD extracts with rich phenolic and flavonoid components had better antioxidant activity than other extracts, which was in line with the literature (Table 1).

Table 1. Antioxidant activities of *H. armenium* subsp. *armenium* extracts

Extracts/ Standards	DPPH (IC_{50}: mg/mL)	FRAP (mMFeSO4/mg extract)	CUPRAC (mMTE/mg extract)	TFC (mg QE/g extract)	TPC (mg GAE/g extracts)
AHC	0.122± 0.005*	2.015±0.324*	1.542±0.121*	47.168±4.123	67.985±4.279
AHB	NA	1.096± 0.043*	0.500±0.039*	7.341± 0.370	36.257±2.327
AHE	0.022± 0.001*	3.001±0.132*	4.314±0.444*	77.601±0.066	79.895±2.325
AHD	0.022± 0.001*	3.667±0.214*	3.723±0.257*	42.250±3.998	78.914±0.533
HAH	0.071±0.002*	1.925±0.064*	1.952±0.039*	33.534±3.071	67.391±2.117
Ascorbic acid	0.004±0.001		5.683±0.337		
BHA		5.81 ± 0.004			

Ascorbic acid positive control for DPPH and CUPRAC assays; BHA, butylated hydroxyanisole, positive control for FRAP assay; DPPH, 2,2-diphenyl-1-picrylhydrazyl; CUPRAC, cupric ion 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; NA: not activity; GAE: gallic acid equivalents; QE: quercetin equivalents; TFC: total flavonoid contents; TPC: total phenolic contents

2.2. Enzyme inhibition activity of extracts

Findings regarding the comparison of the potential of plant extracts and galantamine compounds to inhibit the acetylcholinesterase enzyme at a concentration of 50 µg/ml are shown in Table 2.

It was determined that HAH (66.846%) extract exhibited higher acetylcholinesterase enzyme inhibition potential than other extracts. It was determined that the AHB, AHC, and AHD extracts from the plant had very similar enzyme inhibition potential. The acetylcholinesterase enzyme potential of all extracts in the investigation was found to be lower than that of the galantamine compounds**.**

Table 2. Acetylcholinesterase enzyme inhibition potential of the plant' extracts

Extracts	% Inhibition
AHB	52.834±1.223*
AHC	52.315±6.334*
AHD	53.544±1.815*
AHE	49.665±1.506*
HAH	66.846±0.333*
Galantamine	73.805±0.124

Values are mean of triplicate determination (n = 3) ± standard deviation; *P<0.05 compared with the positive control

2.3. Antimicrobial Activity

In this research, the antimicrobial activity study with *H. armenium* subsp. *armenium* was conducted with ten different microorganisms as seen in the table below. According to the results, AHB extract showed a low activity against *E. coli* and also the highest antimicrobial effect against MRSA. While AHC extract shows low antimicrobial activity against *E. faecalis* and *C. tropicalis*; It showed a strong antimicrobial effect against MRSA.

Table 3. Antimicrobial activities of *H. armenium* subsp. *armenium* extracts.

	Microorganisms									
	***S. aureus* ATCC 29213**	**MRSA ATCC 43300**	***S. epidermidis* ATCC 1228**	**E. coli ATCC 25922**	***E. faecalis* ATCC 29212**	***P. aeruginosa* ATCC 27853**	***K. pneumoniae* ATCC 4352**	***C. albicans* ATCC 10231**	***C. parapsilosis* ATCC 22019**	***C. tropicalis* ATCC 750**
Extracts										
AHB	-	312.5	-	625	-	-	-	-	-	-
AHC	-	312.5	-	-	625	-	-	-	-	625
AHD	-	625	625	625	625	-	-	-	-	312.5
AHE	-	-	-	-	625	-	-	-	-	312.5
Cefuroxime-Na	1.2			4.9	4.9					
Amikacin							128			
Ceftazidime						2.4				
Cefuroxime			9.8							
Nystatin									4	
Clotrimazole								4.9		
Fluconazole										0.5
Ciprofloxacin		1								

-No activity
-MIC µg/ml.

2.4. LC-HRMS Analysis

A total of 27 substances were identified in the LC-HRMS determination performed on the methanol extract obtained from the aerial parts of the *H. armenium* subsp. *armenium* plant. These substances are listed in Table 4 with their quantities and uncertainty values. When the results in Table 4 are examined, the methanol extract of the *H. armenium* subsp. *armenium* plant contains chlorogenic acid (45.557 mg/L), fumaric acid (117.739 mg/L), vanilic acid (124.691 mg/L), quercetin (22.76 mg/L), apigenin (3). It was determined to be rich in 6.239 mg/L) and kaempferol (24.326 mg/L). Chromatograms of the four extracts obtained as a result of LC-HRMS analysis are given in Figure 1, 2, 3, 4.

3. DISCUSSION

In this study, antioxidant and antimicrobial activity, acetylcholinesterase activity, and component analysis by LC-HRMS were carried out in four different extract contents of *H. armenium* subsp. *armenium* collected from Turkey, Rize-Beştepe. Antioxidant activities were analyzed by three different methods (DPPH, FRAP, and CUPRAC). According to the results (Table 1), AHB extract did not show DPPH radical scavenging activity. AHC extract showed the strongest effect (0.122±0.005 IC_{50} mg/mL) in DPPH analysis. When Albayrak et al.'s [25] study was examined, it was determined that the free radical scavenging activity of *Helichrysum* species in 16 different extracts varied between 7.95-53.10 lg/mL. In addition, Albayrak et al.'s [25] study found the IC_{50} value in methanol extracts of *H. armenium* subsp. *armenium* to be 7.95 µg/ml in the DPPH method. In our study, using the DPPH, CUPRAC, and FRAP methods, the DPPH IC_{50} value in HAH extract, which is a methanol extract, was found to be 0.071±0.002, FRAP IC_{50} value was 1.925±0.064 and CUPRAC IC_{50} value was 1.952±0.039.

Table 4. Compounds and amounts determined from *H. armenium* subsp. *armenium* methanol extract.

Compounds	HAH (mg/L)	Relative Uncertainty (%) mg/L
Ascorbic acid	4.132	3.94
Chlorogenic acid	45.557	3.58
Fumaric acid	117.739	2.88
(-)-Epicatechin gallate	0.034	3.05
Caffeic acid	3.047	3.74
Caffeine	0.015	3.06
(+)-trans taxifolin	0.092	3.35
Vanilic acid	124.691	3.49
Luteolin 7-glucoside	1.717	4.14
Rutin	0.264	3.07
Apigenin 7-glucoside	1.402	3.59
Quercitrin	9.091	3.78
Quercetin	22.76	2.95
Salicylic acid	0.531	1.89
Kaempferol	24.326	3.56
3'-O-Methyl quercetin	2.531	3.58
Apigenin	6.239	2.87
Hispidulin	2.082	3.41
Glycyrrhizic acid	0.059	2.25
Sinensetin	0.018	3.36
Caffeic asit phenethyl ester	0.001	3.13
Chrysin	0.095	3.24
Acacetin	4.383	3.98
Emodin	0.012	4.27
Genkwanin	0.423	4.44
Chrysoeriol	0.804	2.08
Apigenin 7-metilat	0.568	2.94

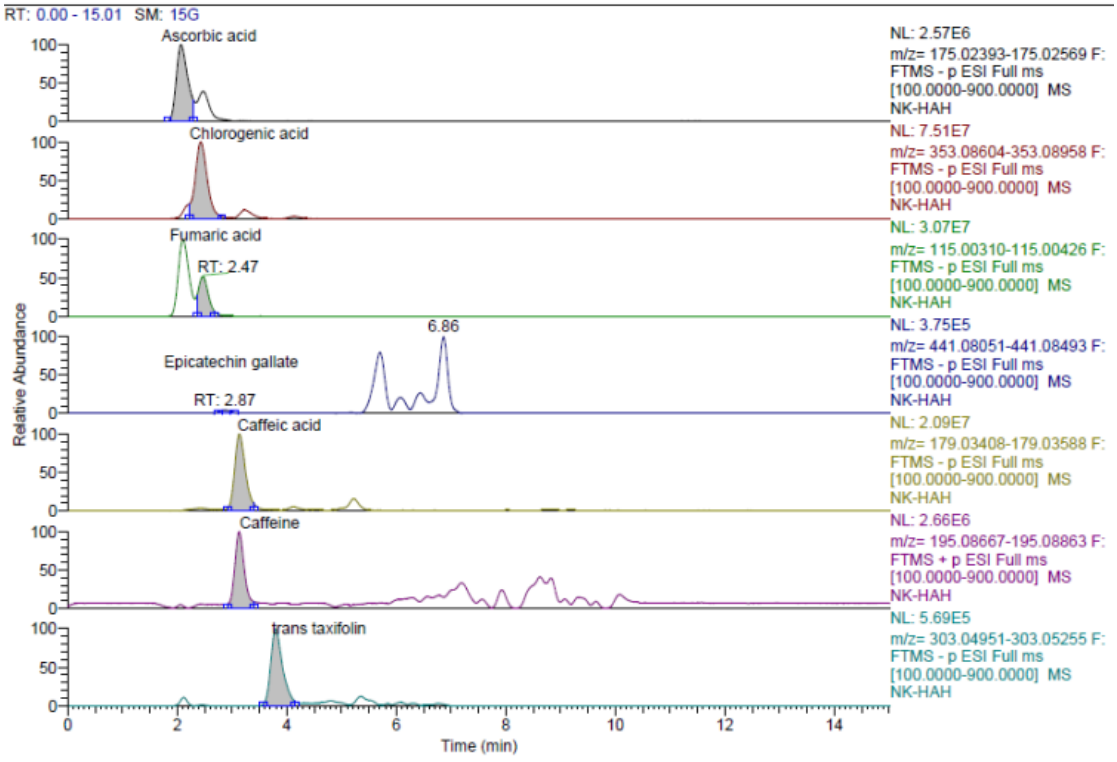

Figure 1. Chromatograms of the methanol extract of the *H. armenium* subsp. *armenium* by LC-HRMS Analysis.

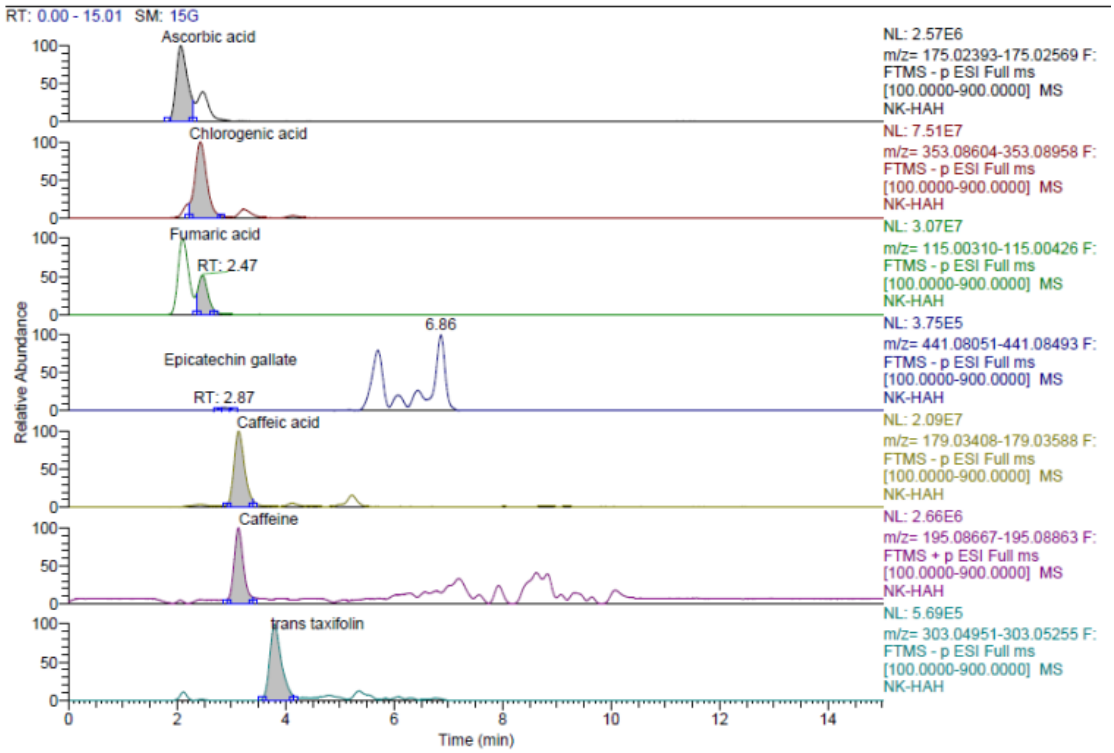

Figure 2. Chromatograms of the methanol extract of the *H. armenium* subsp. *armenium* by LC-HRMS Analysis.

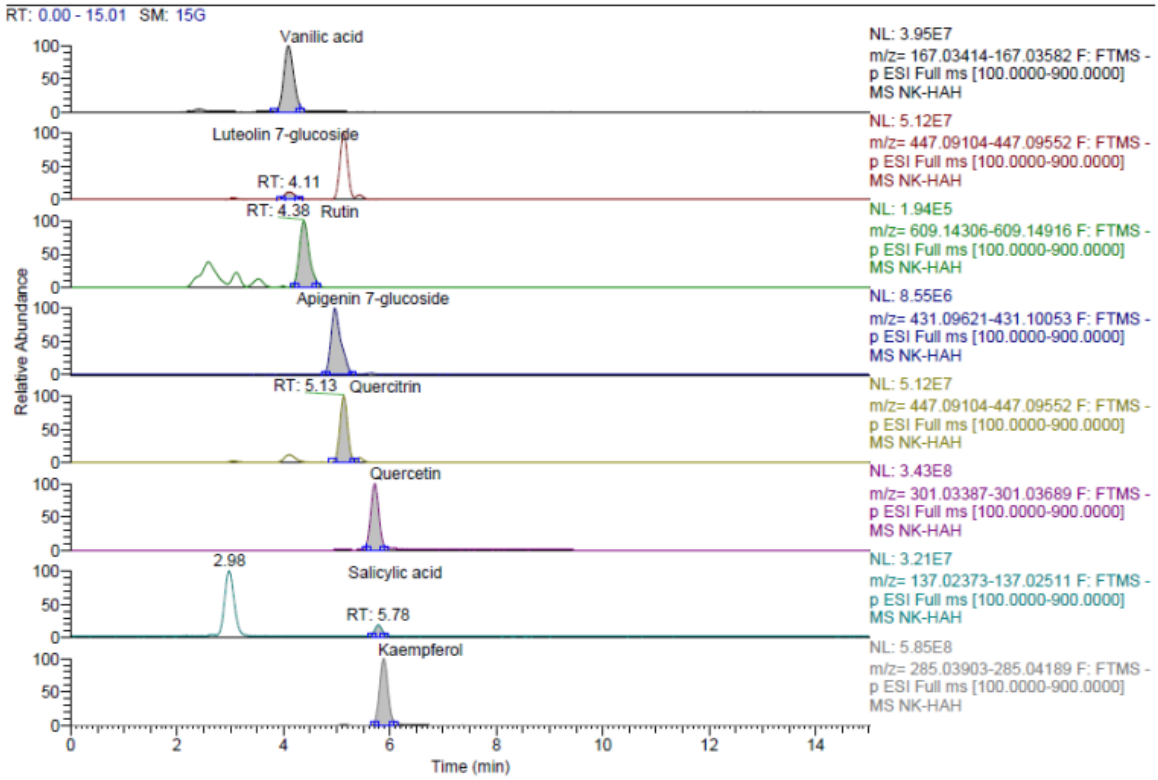

Figure 3. Chromatograms of the methanol extract of the *H. armenium* subsp. *armenium* by LC-HRMS Analysis.

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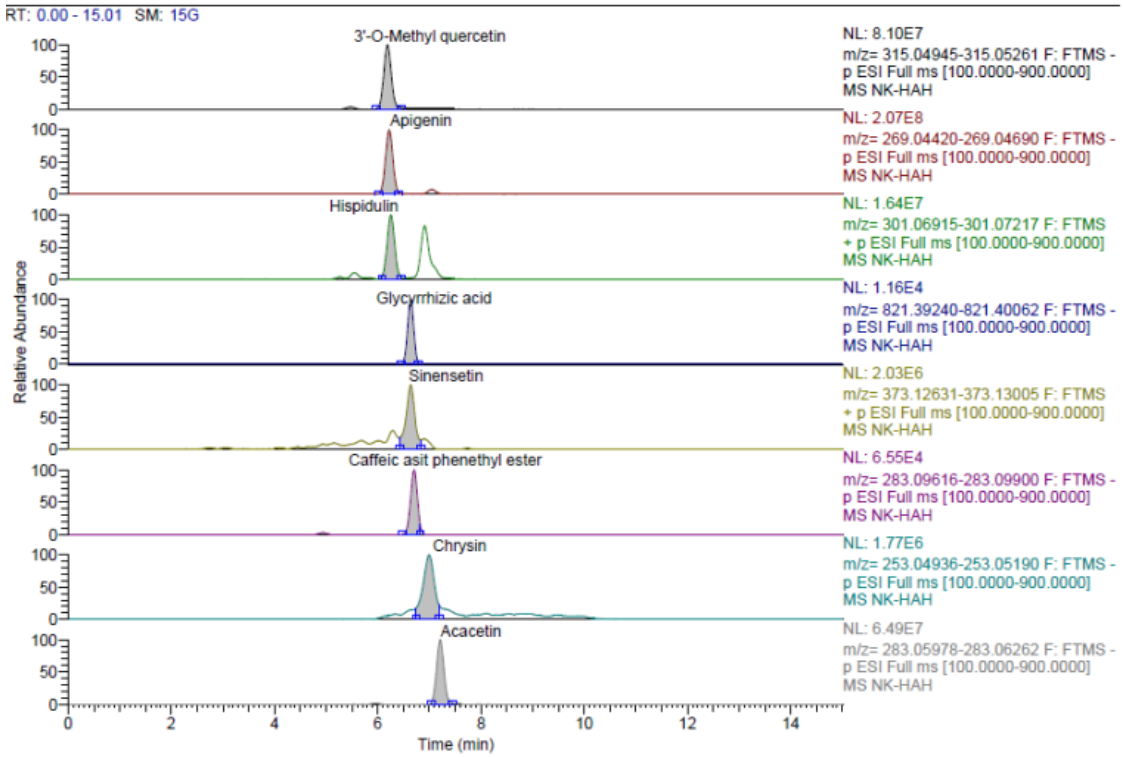

Figure 4. Chromatograms of the methanol extract of the *H. armenium* subsp. *armenium* by LC-HRMS Analysis.

In addition, antioxidant activity was evaluated in 4 different extracts other than methanol extract, and the highest activity was reached in AHC extract (0.122± 0.005) in the DPPH method, AHD extract (3.667±0.214) in the FRAP method and AHE extract (4.314±0.444) in the CUPRAC method. Bojilov et al. [26], just like in this study, examined the antioxidant activity in *Helichrysum* species with a total of four different methods, including DPPH, CUPRAC and FRAP methods, and obtained parallel results. Phenolic compounds have an important role as scavengers of free radicals, and it can be said based on previous studies that the antioxidant properties of *Helichrysum* species are due to flavonoids [27, 28]. The results obtained in this study appear to be compatible with previous studies in the literature on the *Helichrysum* genus [29-31]. In light of the results obtained in this study and in the literature, it can be deduced that the compounds responsible for antioxidant activity in *Helichrysum* species obtained from different regions of the world are flavonoids located in the aerial parts. The antimicrobial activity results are given in Table 3. The antimicrobial activity of extracts obtained from *Helichrysum* species has been widely reported in the literature [15, 32, 33]. In our study, AHB extract against *E. coli*; AHC extract showed low activity against *E. faecalis* and *C. tropicalis*. Both extracts showed high levels of activity against MRSA. Among the extract types, AHD extract was the most active against microorganisms and was determined to have moderate activity against MRSA, *S. epidermidis*, *E. coli* and, *E. faecalis*. AHD extract showed strong antifungal activity against *C. tropicalis*. Finally, AHE extract showed moderate antimicrobial activity against *E. faecalis*; It showed the highest antifungal activity against *C. tropicalis*. It has been reported that salicylic acid, which is involved in the defense mechanism against pathogens in plants, plays an important antimicrobial role [34]. Albayrak et al. [25] used the disc diffusion method in their antimicrobial analyses. Differently, in this study, the disc dilution method was used. Albayrak et al. [25] worked with a total of 15 microorganisms in their study, while we performed analyses with 10 microorganisms in our study. The microorganisms studied together were *C. albicans*, *E. coli*, and *K. pneumoniae*. A comparison with this study cannot be made due to the use of different methods. Süzgeç et al. [23] identified the components isolated in the study from the capitula of *Helichrysum compactum*, such as apigenin, kaempferol, and naringenin, and determined that the extracts had antibacterial activities. In the light of this information, it is thought that the components found in *H. armenium* subsp. *armenium* are responsible for the current antimicrobial activity in the study. In addition, there is a lot of data in the literature regarding the microbial activities of *Helichrysum* species, and the number of studies on the activities of *Helichrysum* species grown from the flora of Turkey. Albayrak et al. [25] determined the activities of *Helichrysum* species separately against Gram (+) and Gram (-) bacteria and

yeasts in their study. Similarly, in this study, it was determined that some extracts showed moderate activity against *E. coli*, while the antimicrobial effect on different microorganisms was also revealed in their study.

As a result of the LC-HRMS analysis, a total of 27 components were isolated from the aerial parts of *H. armenium* subsp. *armenium* (Table 4). In this study, a more detailed phytochemical study was carried out than Albayrak et al. While a total of 27 components were analysed with the LC-HRMS method in this study, Albayrak et al. analysed a total of 15 compounds with HPLC in their studies. Components analysed in common in the two studies: chlorogenic acid, caffeic acid, apigenin 7-glucoside, quercetin, and apigenin. Different components have been identified in *Helichrysum* species in the literature.

4. CONCLUSION

As a result, in this study, antioxidant activity (CUPRAC, FRAP, and DPPH), antimicrobial activity (in 10 different microorganisms), acetylcholinesterase enzyme activity analysis of four different extracts obtained from *H. armenium* subsp. *armenium* and component analysis were carried out using the LC-HRMS method of methanol extract. The presence of phenolic compounds is responsible for the activity. In future research, it is planned to conduct isolation studies on AHD and AHE extracts, which have the highest total phenolic substance and flavonoid amounts and have antioxidant activity in all methods. Additionally, pure substances responsible for the activity will be obtained. Further studies are needed to find the active components of the highest extracts in *Helichrysum* species and to confirm the mechanism of action.

5. MATERIALS AND METHODS

5.1. Plant Material

Helichrysum armenium subsp. *armenium* plant was collected from the rocky land in Rize-Beştepe, June 2021, and plant identification was made by Prof. Dr. Şükran KÜLTÜR. A voucher specimen is deposited in the Herbarium of the Faculty of Pharmacy, Istanbul University with ISTE; 118609. The ariel parts of the plants dried in the shade were ground into powder in the plant mill.

5.2. Extract Preparation

Preparation of extracts for biological activity experiments; the dried and powdered material (430 grams) was first extracted with petroleum ether by Soxhlet. Petroleum ether extract (A) was concentrated in a rotavapor and consumed with 60% EtOH. The separated aqueous ethanol fraction was concentrated and consumed with chloroform. AHB extract (petroleum ether–60% ethanol-chloroform) was obtained by condensing the $CHCl_3$ part. The petroleum ether-depleted material was extracted with 96% ethanol in the Soxhlet apparatus. The concentrated extract was diluted with H_2O and consumed with toluene, chloroform, and ethyl acetate in the separatory funnel to obtain AHC (ethanol-toluene), AHD (ethanol-chloroform), and AHE (ethanol-ethyl acetate) extracts, respectively.

Preparation of extracts for phytochemical research; The dried and powdered material (10 grams) was extracted with methanol in a Soxhlet apparatus. HAH extract (methanol) was obtained by concentrating the resulting extract. LC-HRMS method was used to investigate the phytochemical content of HAH extract.

5.3. General Experimental Procedure

Analysis of phenolic compounds in *H. armenium* subsp. *armenium* above-ground methanol extract was performed by Liquid Chromatography/High-Resolution Mass Spectrometry (LC-HRMS) method. HPLC and MS conditions of the method are given below.

HPLC terms (as seen in Table 5); Mobile Phase A: 1% Formic Acid- H_2O; Mobile Phase B: 1% Formic Acid- MeOH; Column: Troyasil C18 HS – 150 x 3 mm 3.5 µ.

Table 5. HPLC Terms.

Gradient Time	Flow (mL/min)	% B
0.00	0.35	50
1.00	0.35	50
3.00	0.35	100
6.00	0.35	100
7.00	0.35	50
15.00	0.35	50

Mass spectrometry terms;

- (Mass Spectrometer) System: Thermo ORBITRAP Q-EXACTIVE
- Ion Source: ESI
- Mass Scan Range: 100-900 *m/z*
- Sheath gas flow rate: 45
- Aux gas flow rate: 10
- Spray voltage (kV): 3.80
- Capillary temp. (°C): 320
- Aux gas heater temp (°C): 320
- S-lens RF level: 50.0
- Descriptions: İLMER Library

Environmental Condition;

- Temperature: (22.0 ± 5.0) °C
- Relative Humidity: (50 ± 15) %rh

5.3. Biological Activities

5.3.1. Antioxidant activity

DPPH assays: 240 µL of DPPH solution (0.1 mM) were added to the 10 µL of extracts that had been obtained at various doses (0.5-3 mg/mL). Before being incubated for 30 minutes at 25 °C, the produced mixtures were stirred for 1 minute. Every day at 517 nm, the mixes' absorbance values were measured. Under identical circumstances, the absorbance of the control sample was measured using 10 µL of methanol rather than the extract. The information gathered throughout the investigation is provided as IC_{50} = mg/mL [35].

CUPRAC assays: The extracts' ability to reduce copper (II) ions while still having antioxidant activity was assessed using a method created by Apak et al. in 2004. In a nutshell, 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/mg extract [36].

FRAP assays: The FRAP reagent was stored at 37 °C 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 extracts' FRAP values were presented as mM Fe^{2+}/mg extract [37].

Assay for total phenolic content: Briefly, 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 mg gallic acid equivalents (GAE) per milligram of plant extract [38].

Total flavonoid contents: Using a colorimetric test with aluminum chloride, the total flavonoid concentration was calculated. In summary, 10 µl of 10% aluminum chloride solution was mixed with 50 µl of extracts, and then 150 µl of 96% ethanol was added. The mixture on a 96-well plate was supplemented with 10 µl of 1 M sodium acetate. The reagent blank was 96% ethanol. All of the ingredients were combined, and they were kept out of the light for 40 minutes at room temperature. A microplate reader was used to determine the absorbance at 415 nm. The total amount of flavonoids in the plant extract was represented as milligrams of Quercetin Equivalents (QE) per gram.

5.3.2. Anticholinesterase activity assay

Briefly, the AchE (20 µL) and different concentrations of extracts (20 µL) were added to the phosphate buffer solution (pH 8.2 0.1 M, 40 µL). This mixture was incubated at 25°C for 10 min. After incubation, DTNB (100 µL) and AcI (20 µL) as substrate were added to the mixture. The same procedure was applied to the galantamine used as standard. 5-thio-2-nitrobenzoic acid was spectrophotometrically measured at 412 nm. Anticholinesterase activity of the extracts was calculated using the following equation 1 as % inhibition relative to control [39].

The % inhibition of acetylcholinesterase was calculated by the formula:

(Eq. 1). $$\%I = (A_{control} - A_{sample}/A_{control}) \times 100$$

5.3.3. Antimicrobial activity studies

Test Microorganisms: Microorganisms to be used in the determination of antimicrobial activity: *Staphylococcus aureus* ATCC 29213, MRSA ATCC 43300, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Enterecoccus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 4352, *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019 ve *Candida tropicalis* ATCC 750.

Method: Antimicrobial activities of the extracts were determined by the microdilution method, Minimum Inhibitory Concentration (MIC) values against standard strains of the above microorganisms, in line with the Clinical Laboratory Standards Institute (CLSI) criteria [40, 41].

5.4. 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.

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