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Determination of metabolic profiling by LC-MS/MS, evaluation of antioxidant activities, and enzyme inhibition effects of *Helichrysum plicatum* subsp. *pseudopliacatum*

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ABSTRACT

Helichrysum species are traditionally used to treat many diseases. In this study, the antioxidant and enzyme inhibition abilities of methanol (MEHP) and water extracts (WEHP) from *Helichrysum plicatum subsp. Pseudopliacatum* was evaluated. Also, the phenolic compounds will be assessed and the quantities of total phenols and flavonoids will be calculated. For evaluation of antioxidant activity of both extracts was assessed using DPPH, ABTS and DMPD radical scavenging, FRAP, CUPRAC, and Fe^{3+} reduction methods were used. The inhibition effects of extracts were spectrophotometrically evaluated on the α-glucosidase (α-Gly), acetylcholinesterase (AChE), and carbonic anhydrase II (CA II) enzymes. These inhibition effects were compared to the standard inhibitors The qualitative and quantitative phenolic components in extracts were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), employing both methods. The IC_{50} value, which is the inhibitor concentration that causes 50% inhibition of MEHP and WEHP, is found as 2.39 and 2.97 µg mL^{-1} for α-gly, 2.48 and 3.59 µg mL^{-1} for AChE and 11.95 and 25.67 µg $mL^{-1}$for CA II. The quantity of phenols in MEHP and WEHP were 46.00 and 33.50 µg GAE mg^{-1} extract, 62.13 and 103.93 µg QE mg^{-1} extract flavonoids, respectively. IC_{50} values of MEHP and WEHP were calculated as 40.77 and 49.51 µg mL^{-1} for DPPH radical scavenging, 10.50 and 17.77 µg mL^{-1} ABTS radical scavenging and 99.02 and 77.01 µg mL^{-1} DMPD radical scavenging. The absorbance values of reducing abilities of MEHP and WEHP were found as 0.555 and 0.495 Fe^{3+} reducing ability, 0.577 and 0.286 for FRAP reducing ability and 0.402 and 0.315 for Cu^{2+} reducing ability. As a result of the LC-MS/MS analysis, the levels of phenolic compounds such as chlorogenic acid, cyanidin-3-O-glucoside and quinic acid were found to be high in MEHP and WEHP. In this study, it was observed that MEHP and WEHP have strong antioxidant properties and effectively inhibited the enzymes activities. The findings clearly reveal the antioxidant and enzyme inhibition potential of Helichrysum species, which have been used in traditional medicine for a long time. *Helichrysum plicatum* extracts used in this study show that they are very effective against diabetes, glaucoma and Alzheimer's disease, which are global and common diseases.

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Helichrysum plicatum subsp. *pseudopliacatum*'un LC-MS/MS ile metabolik profilinin belirlenmesi, antioksidan aktivitelerinin ve enzim inhibisyon etkilerinin değerlendirilmesi

ÖZET

Helichrysum türleri geleneksel olarak birçok hastalık tedavisinde kullanılmaktadır. Bu çalışmada, *Helichrysum plicatum* subsp. *pseudopliacatum*'den elde edilen metanol (MEHP) ve su (WEHP) ekstrelerinin antioksidan ve enzim inhibisyon etkilerinin belirlenmesi amaçlanmıştır. Ayrıca her iki ekstrede bulunan fenolik bileşiklerin belirlenmesi ve toplam fenolik ve flavonoit miktarlarının hesaplanması da amaçlanmıştır. Her iki ekstrenin antioksidan aktivitesini

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değerlendirmek için DPPH, ABTS ve DMPD radikal giderme, FRAP, Cu^{2+} ve Fe^{3+} indirgeme metotları kullanılmıştır. Ekstrelerin *α*-glikozidaz (α-Gly), asetilkolinesteraz (AChE) ve karbonik anhidraz II (CA II) enzimleri üzerinde inhibisyon etkileri spektrofotometrik olarak değerlendirildi ve standart inhibitörler ile mukayese edildi. Ekstrelerde bulunan fenolik maddelerin kalitatif ve kantitatif analizi ise sıvı kromatografisi tandem kütle spektrometresi (LC-MS/MS) ile belirlendi. MEHP ve WEHP'ye ait %50 inhibisyona sebep olan inhibitör konsantrasyonu olan IC_{50} değeri, α-Gly için 2.39 ve 2.97 µg mL^{-1}, AChE için 2.48 ve 3.59 µg mL^{-1} ve CA II için ise 11.95 ve 25.67 µg mL^{-1} olarak bulundu. MEHP ve WEHP'deki toplam fenolik miktarı sırasıyla 46.00 ve 33.50 µg GAE mg^{-1} ekstre; toplam flavonoit ise 62.13 ve 103.93 µg QE mg^{-1} ekstre olarak hesaplandı. MEHP ve WEHP'nin sırasıyla DPPH radikal gidermeye ait IC_{50} değerleri 40.77 ve 49.51 µg/mL, ABTS radikal gidermeye ait IC_{50} değerleri ise 10.50 ve 17.77 µg mL^{-1} ve DMPD radikal gidermeye ait IC_{50} değerleri IC_{50}= 99.02 ve 77.01 µg mL^{-1} olarak belirlenmiştir. MEHP ve WEHP'in Fe^{3+} indirgeme kapasitesine ait absorbans değerleri sırasıyla indirgeme 0.555 ve 0.495, FRAP indirgeme kapasitesine ait absorbans değerleri 0.577 ve 0.286 ve Cu^{2+} indirgeme kapasitesine ait absorbans değerleri ise 0.402 ve 0.315 olarak bulundu. LC-MS/MS analizi sonucunda MEHP ve WEHP'de klorojenik asit, siyanidin-3-*O*-glikozit ve kinik asidin en fazla bulunduğu belirlendi. Bu çalışmada, MEHP ve WEHP'in etkili antioksidan etkiye sahip olduğunu ve α-Gly, AChE ve CA II enzimlerini etkili bir şekilde inhibe ettiği gözlendi. Elde edilen bulgular, geleneksel tıpta uzun süredir kullanılan *Helichrysum* türlerinin antioksidan ve enzim inhibisyon potansiyelini açık bir şekilde ortaya koymaktadır. Bu çalışmada kullanılan *Helichrysum plicatum* ekstreleri global ve yaygın birer hastalık olan diyabet, glokom ve Alzheimer hastalığına karşı oldukça etkili olduklarını gösterdi.

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INTRODUCTION

Helichrysum genus (Asteraceae), a Mediterranean region plant, has more than 600 species (Kherbache et al., 2020). *Helichrysum* species are known by names such as immortelle, everlasting flower in Europe, and "altın otu, ölmez çiçek" in Turkiye (Miloglu et al., 2023). When the botanical characteristics of *H. plicatum* subsp. *pseudopliacatum* are examined, it is seen that it is generally hairless, straight stem, narrow linear leaves and small capitula (4-6 pieces). Compared to *H. plicatum* subsp. it has denser indumentum, slightly more curved stems, wider leaves and larger capitula (6-8 mm). Except for the difference in color, the taxa are clearly indistinguishable, there are intermediate forms with yellow capitula (Davis & Kupicha, 1975). *Helichrysum plicatum* is traditionally used in the treatment of diseases such as heart, liver and kidney diseases, kidney stone, jaundice, cough, stomach and head AChE, high blood pressure, hemorrhoids, diarrhea, hair care complaints (growth, loss). The plant has also wound healing properties (Miloglu et al., 2023). It is generally used for infusion, decoction and soaking in oil or milk (Kazancı et al., 2020). In biological effect studies on *Helichrysum* species, antimicrobial (Smirnov et al., 1982), antioxidant (Tepe et al., 2004), antidiabetic (Aslan et al., 2007), antihyperlipidemia (Farris et al., 2017), hepatoprotective (Pljevljakusic et al., 2018), and kidney stone-reducing effects (Bayir et al., 2011) have been reported. *Helichrysum* species contain phenolic acids (Carini et al., 2001), flavonoid derivative compounds (Albayrak, et al., 2010a), steroidal compounds (Aydin, 2020), chalcones (Al-Rehaily et al., 2008), sesquiterpenes (Benelli et al., 2018), coumarin and pyrones (Pljevljakusic et al., 2018) and phloroglucinol (Popoola et al., 2015) derivatives.

Medicinal plants have antioxidant capability with their reducing, free radical scavenging and quenching the formation of singlet oxygen. These properties are due to phenolic substances, also known as secondary compounds, in their structure (Guven et al., 2022).

Oxidative stress is a result of an imbalance between reactive oxygen species (ROS) and antioxidant defenses (Gulcin 2020). ROS causes cell and DNA damage and is linked to neurodegenerative diseases. Phenolic compounds in plants protect cells against the harmful effects of ROS by donating electrons, chelating metal ions and stimulating antioxidant enzymes (Sezer Senol et al., 2016; Durmaz et al., 2022). Antioxidants are substances that have a protective role in food and pharmaceutical products against oxidative degradation and against pathological processes mediated by oxidative stress in the body (Gulcin 2020). According to some studies, plants with antioxidant effects are suitable for the treatment of Alzheimer's disease, diabetes mellitus, and glaucoma. Some of the drugs used in such diseases act inhibition effects on some metabolic enzymes such as AChE, α-Gly, and CA II. In addition, the interest in herbal products is increasing day by day due to the various side effects of the drugs used in the treatment (Güven et al., 2023).

In this study, antioxidant, antidiabetic, anti-Alzheimer's disease and antiglaucoma properties of the traditionally used plant *H. plicatum* subsp. *pseudoplicatum* were revealed. The phenolic compounds that may be responsible for these effects were determined by LC-MS/MS.

MATERIAL and METHOD

Plant materials

Helichrysum plicatum subsp. *pseudopliacatum* DC. was collected by Dr. Leyla Güven from Erzurum Köşk village in July 2019 at an altitude of 1950 meters and identified by Prof. Dr. Yusuf Kaya. The plant sample is stored at the Biodiversity Application and Research Center of Atatürk University Erzurum, Turkey (AEF 1374) (Figure 1).

Figure 1. Helichrysum plicatum subsp. *pseudopliacatum*
Şekil 1. Helichrysum plicatum subsp. *pseudopliacatum*

Extraction

To prepare the aerial part of *Helichrysum plicatum* subsp. *pseudopliacatum* methanol extract (MEHP), 500 mL of methanol was added to 20 g of dried and powdered plant. The mixture was then extracted with a mechanical stirrer at 40 ºC overnight. The mixture was then filtered and the filtrate was evaporated to dryness in the rotaryevaporator. In order to prepare the aerial part of *Helichrysum plicatum* subsp. *pseudopliacatum* water extract (WEHP), 20 g of powdered plant was poured with boiling water at 100 °C and extracted with a mixer at 50 °C for 4 hours and filtered while hot. The separated filtrate was evaporated to dryness in a lyophilizer. The obtained extracts were stored at -18 ºC until the day of the experiment. The yield of methanol and water extracts were found respectively as 27.02 % and 20.53%

Liquid chromatography-electrospray tandem mass spectrometry (LC-MS /MS) Method

Phenolic compound analyzes were determined according to the method of Güven et al. (2023). Standard stock preparation process, validation study, quality control solutions and chromatography/mass spectroscopy conditions for phenolic compounds are explained in detail in the study of Güven et al. (2023). Liquid chromatography coupled to mass spectrometer (LC-MS/MS) is used in the analysis of phenolic compounds. Agilent Technologies 1290 Infinity LC system (Palo Alto, USA) with an autosampler (Agilent 1260 Infinity G1329B ALS), dual pump (Agilent 1260 Infinity G1312B (600 bar), degasser (Agilent 1260

Infinity G4225A HiP), and thermostated column (Agilent 1290 Infinity TCC G1316C) was used for the liquid chromatography component. On a Zorbax SB-C18 column with dimensions of 3.5 m, 100 mm 4.6 mm, kept at 30°C, the phenolic compounds were separated. By gradient elution of a mobile phase combination consisting of water with (A) 0.1% formic acid and (B) acetonitrile with 0.1% formic acid, the phenolic compounds were separated by chromatography.

An Agilent 6460 mass spectrometer (Palo Alto, USA) with an electrospray ionization (ESI) source was used for the mass spectrometric detection. The nebulizing gas (N_2) pressure was 55 psi; the capillary voltage was 3.5 kV in negative mode; the drying gas (N_2) temperature and flow were 350°C and 12 L min^{-1}, respectively. To each quadrupole, a resolution of one unit was applied. To improve the MRM conditions, flow injections of each particular chemical were employed. The Agilent MassHunter Workstation (Agilent) software performed this automatically for the majority of metabolites, although the ideal cone voltages and collision energies for select compounds were found during collision-induced dissociation (CID) studies and manually configured. The minimum dwell period for each MRM transition was 25 milliseconds.

Reducing potentials

The Fe^{3+} reducing capacity method of *Helichrysum* extracts was developed by Oyaizu (1986) as described in detail (Gulcin, 2020). In this method, reducing activity was detected by the straight reduction of $Fe^{3+}(CN^{-})_6$. Then, addition of excess ferric ions (Fe^{3+}) resulted the formation of the Perl's Prussian blue complex. For this purpose, 0.75 mL *Helichrysum* extracts, which including different concentrations of the (15-45 µg mL^{-1}) were added with $K_3Fe(CN)_6$ (1 %, 1.25 mL) and buffer (1.25 mL, 0.2 M, pH 6.6) solutions. Then, the mixture was incubated at 50°C for half hour. Then, 1.25 mL of trichloroacetic acid (TCA, 10 %) and $FeCl_3$ (0.5 mL, 0.1 %) was added to the mixture and the absorbance was read at 700 nm (Kiziltas et al., 2021).

Cu^{2+} reducing capacity (CUPRAC) of *Helichrysum* extracts was determined according to Gülçin's method (2008). In this method, neocuproine was used as a chromogenic oxidizing agent. Firstly, acetate buffer (1.0 M), $CuCl_2$ solution (10 mM), and neocuproine solution (7.5 mM) were added to each tube, as 1 mL and each tube were vortexed. The concentrations of the all samples were between 15-45 µg $mL^{-1}$and were added to tubes. The volumes of those tubes were completed to 4 mL with distilled water. The samples were kept at 25 °C for half hour. The absorbance was measured at 450 nm. The increased absorbance was defined as a rate of reducing ability (Apak et al., 2022; Ak & Gülçin, 2008; Koçyiğit et al., 2022).

Reduction method of TPTZ-Fe^{3+} complex of *Helichrysum* extracts, also known as FRAP method. FRAP method is based on reduction of TPTZ-Fe^{3+} complex at acidic conditions. For this purpose, fresh TPTZ solution (10 mM) was prepared and mixed to buffer solution (pH 3.6, 0.3 M) and 20 mM $FeCl_3$ solution in water. Different concentrations (15-45 µg mL^{-1}) of *Helichrysum* extracts were dissolved in 5 mL of appropriate buffer, stirred and left at 37°C for half hour. Finally, the absorbances were recorded at 593 nm (Göçer and Gülçin 2011).

Radical scavenging activities

The $DPPH^{\cdot}$ radical scavenging capacity of *Helichrysum* extracts was determined by modifying the Blois method (1958) as given in a previous study (Gulcin & Alwasel, 2023). The method is rest on the removal of DPPH free radicals through antioxidants. The concentration of both extracts and standards were prepared as 15-45 µg mL^{-1}. 1 mL of $DPPH^{\cdot}$ (0.1 mM) was added to tubes of each sample. These tubes were left in the dark at 25 °C for 30 min. The measurements were made at 517 nm. The potentials of samples on $DPPH^{\cdot}$ were determined and compared to the standards. In the end, IC_{50} values of all samples were calculated. The reduction in absorbance shows the DPPH free radical scavenging of samples capability.

The determination of $DMPD^{\cdot+}$ radical scavenging activity was made according to the method of Fogliona et al (1999). For this aim, 0.2 mL of 0.05 M $FeCl_3$ and 1 mL of DMPD solution were added to of buffer (100 mL, pH 5.3, 100 mM). The concentrations of the all samples were prepared as 15-45 µg mL^{-1}. The total volume was adjusted to 0.5 mL by water. An aliquot (1 mL) of $DMPD^{\cdot+}$ solution was transferred and absorbance was recorded at 505 nm after incubation for an hour (Gülçin et al., 2010).

As the last radical scavenging technique, the $ABTS^{\cdot+}$ free radical scavenging capacity of *Helichrysum* extracts was developed and applied according to the method of Re et al. (1999) as give priorly Köse et al. (2015). According to this assay firstly, ABTS radical cation was generated. Thus, ABTS (7.0 mM) and $K_2S_2O_8$ (2.45 mM) were reacted. Before measurement, absorbance of the solution was adjusted to 0.750±0.025 at 734 nm with buffer solution (pH 7.4, 0.1 M). Then, to 1 mL of $ABTS^{\cdot+}$ solution, 3 mL solution of *Helichrysum* extracts at different concentrations (15-45 µg mL^{-1}) was added. After half hour, the percent inhibition of $ABTS^{\cdot+}$ at 734 nm was measured for all samples. The reduction in absorbance shows the $ABTS^{\cdot+}$ free radical scavenging of samples capability.

In addition, the Fe^{2+} chelating effect of *Helichrysum* extracts was performed by modifying the method described by Re et al. (1999) as given in a recent study (Gulcin & Alwasel, 2022). Different concentrations (15-45 µg mL^{-1}) of *Helichrysum* extracts and standard compounds were transferred to 0.125 mL $FeSO_4$ (2 mM) solution. Then 0.5 mL of Tris-HCl solution (pH 7.4) is added and incubated for 30 minutes. Then, 0.75 mL of 0.2% bipyridyl solution dissolved in HCl (0.2 M), 0.125 mL of ethanol, and 0.595 mL of water are added to the mixture, respectively, and incubated for 15 minutes. Ethanol was used as a blank and absorbances were measured at 522 nm (Çetinkaya et al., 2012).

Determination of total soluble phenolic and flavonoid contents

MEHP and WEHP (0.5 mL) were added to 1.0 mL of Folin–Ciocalteu reagent (Singleton and Rossi, 1999) as described in detail in Kızıltaş et al. (2021). Afterward, carbonate (0.5 mL, 1%) was added and the mixture was stirred vigorously. Absorbance was measured at 760 nm against a water-containing blank sample after 2 h of incubation in the dark at room temperature. The quantity of phenol in one gram of *Helichrysum* extracts was calculated as mg of gallic acid equivalents (GAE).

The total flavonoid contents were determined using the aluminum chloride ($AlCl_3$) technique (Kızıltaş et al. 2021) Briefly, 0.5 mL of the *Helichrysum* extract solutions were combined with 1.5 mL of 95% methanol, 1.5 mL of 10% $AlCl_3$, 0.5 mL of 1.0 M potassium acetate solution, and 2.3 mL of distilled deionized water. The absorbance was measured at 415 nm after incubation in the dark (25 °C, 40 min). Water was used as the blank sample. The total amount of flavonoids was calculated as mg quercetin equivalents (QE) g^{-1} of *Helichrysum* extracts

Enzym inhibitory capacity assays

The inhibitory capacity of *Helichrysum* extracts against AChE was analyzed according to the Ellman's method (1961). Acetylthiocholine iodide were used as substrates. AChE ability was designated using 5,5′-dithiobis (2-nitrobenzoic) acid (DTNB). The solutions (50–200 µL) and the buffer solution (100 µL, pH 8.0, Tris-HCl, 1.0 M) were mixed to with AChE enzyme solution. After this step, the mixture was waited at 20°C for 10 min. Then, 50 µL substrate and DTNB and were added to the mixtures and finally, the activity was measured at 412 nm (Gulcin et al., 2019, Karagecili et al., 2023)

The inhibitory effect of *Helichrysum* extracts on *α*-glycosidase enzyme inhibitory effect were tested using a *p*-nitrophenyl-*D*-glycopyranoside (*p*-NPG) substrate (Tao et al., 2013). First, 40 µL of the sample solution was mixed with 200 µL phosphate buffer (0.15 EU/mL, pH 7.4). Furthermore, after preincubation, 50 µL p-NPG in phosphate buffer (5 mM, pH 7.4) was added and incubated again at 30 °C. Absorbance was measured spectrophotometrically at 405 nm according to previous studies. (Gülçin et al., 2022; Ozaslan et al., 2022).

The CA II isoenzyme was purified from human erythrocytes using Sepharose-4B-L-Tyrosine sulphonamides affinity column chromatography. The erythrocytes were precipitated by centrifugation, the serum separated and the pH of the serum was then adjusted to 8.7 with solid Tris. The prepared sample was then placed in the affinity chromatography column and subjected to equilibration with Tris-Na_2SO_4/HCl (pH 8.7, 22 mM/25 mM). The enzyme CA II was subjected to a washing procedure with a solution containing sodium acetate/$NaClO_4$. The pH of the solution was adjusted to 5.6 and the temperature was maintained at 25°C. Quantification of the protein content was performed during the purification test using the Bradford method (Taslimi & Gulçin, 2018; Şenol et al., 2023). The standard protein used in the study was bovine serum albumin. The purity of CA II was verified by SDS-PAGE. During the purification and inhibition of CA II, the esterase activities were performed by monitoring the variation in absorbance at 348 nm (Topal & Gulcin, 2014; Huyut et al., 2017; Bayrak et al., 2019)

Statistical analyses

Statistical analyses were performed in the SPSS 25.0 package program (IBM Corp., Armonk, N.Y., USA). Descriptive statistical analyses were performed. p values less than 0.05 at the 95% confidence interval were considered statistically significant. To examine the effect of the intervention on the biochemical parameters, one-way ANOVA and two-way (2x2) analysis of variance (two way between subjects ANOVA) tests were used. Two-way (2x2) ANOVA analysis resulted p values for group differences, for dose differences, and for interaction of group and dose. Post-hoc Tukey test was used to analyze pairwise comparisons.

RESULTS and DISCUSSION

Helichrysum species have attracted the attention of many researchers due to their use among the public. The determination of the active substances responsible for the effect takes an important place in the researches. There is also a need for scientific validation, standardization and safety assessment of herbs used in traditional medicine (Raina et al., 2008). Phytochemical and analytical studies in *Helichrysum* species (Silva et al., 2017) are usually presented together as was done in this study.

Phenolic compounds are produced as secondary metabolites in plants and have beneficial properties

for human health. When consumed regularly for nutritional or health purposes, they play a positive role in preventing or treating many diseases (Gulcin and Beydemir, 2013). Phenolic compounds' effects on health can be attributed to their antioxidant properties, which are mediated by different mechanisms such as the elimination of ROS, the chelation of some metal ions, and oxidative stress It is also used in metabolic and neurodegenerative diseases due to the effects of phenolic compounds on enzymes (Taslimi et al. 2020).

Table 1. Quantitative screening of phenolic compounds in MEHP and WEHP by LC-MS/MS
Çizelge 1. MEHP ve WEHP'deki fenolik bileşiklerin LC-MS/MS ile kantitatif taranması

No	Analytes	RT[a]	M.I. (m/z)[b]	F.I. (m/z)[c]	Ion. mode	MEHP (ng mL^{-1})	WEHP(ng mL^{-1})
1	Quinic acid	2.36	190.9	85.0	Neg	21648.41	5862.96
2	Fumaric acid	3.86	114.9	71.1	Neg	2287.01	695.18
3	Gallic acid	5.48	168.9	79.0	Neg	1.17	ND[d]
4	Pyrogallol	6.16	124.9	96.6	Neg	ND	ND
5	Chlorogenic acid	10.71	352.9	190.9	Neg	30064.34	16801.47
6	Catechin	10.94	289.1	244.9	Neg	ND	ND
7	Peonidin-3-*O*-glucoside	11.02	460.9	298.8	Neg	ND	5.98
8	4-OH-benzoic acid	11.25	137.0	93.1	Neg	37.58	ND
9	Epicatechin	11.36	289.0	244.9	Neg	ND	ND
10	Caffeic acid	11.46	178.8	134.8	Neg	189.62	ND
11	Keracyanin chloride	11.56	592.8	284.7	Neg	ND	118.74
12	Vanillic acid	11.57	166.9	151.9	Neg	ND	ND
13	Syringic acid	11.58	169.9	122.8	Neg	ND	ND
14	Epigallocatechin gallate	11.66	456.8	304.9	Neg	ND	ND
15	Naringin	11.86	579.0	270.8	Neg	ND	764.94
16	Ellagic acid	11.97	300.8	283.4	Neg	ND	ND
17	Hesperidin	11.98	609.0	300.9	Neg	ND	ND
18	Cyanidin-3-*O*-glucoside	12.04	447.1	283.8	Neg	29898.66	31474.77
19	Vitexin	12.11	430.9	310.9	Neg	ND	ND
20	*p*-Coumaric acid	12.22	163.0	118.9	Neg	71.76	ND
21	Sinapic acid	12.34	222.8	163.9	Neg	ND	ND
22	Taxifolin	12.36	302.9	124.7	Neg	ND	ND
23	Rosmarinic acid	12.49	358.8	160.8	Neg	48.17	370.98
24	Ferulic acid	12.50	193.0	134.0	Neg	ND	ND
25	Vanillin	12.59	151.0	135.8	Neg	ND	ND
26	Myricetin	12.71	316.9	150.9	Neg	ND	ND
27	Resveratrol	13.11	226.8	184.8	Neg	ND	ND
28	Luteolin	13.29	284.9	132.9	Neg	1114.82	68.50
29	Quercetin	13.38	300,9	150.7	Neg	8.82	ND
30	Apigenin	13.90	268.9	224.8	Neg	1474.44	135.44
31	Naringenin	13.95	270.9	150.8	Neg	395.12	ND
32	Isorhamnetin	14.10	314.9	299.8	Neg	ND	0.19
33	Galangin	15.54	268.9	168.8	Neg	ND	ND
34	Chrysin	15.72	252.8	208.8	Neg	ND	ND
35	Curcumin	16.13	366.9	148.9	Neg	ND	ND

[a]R.T.: Retention time. [b]MI (m/z): Molecular ions of the standard analytes (m/z ratio). [c]FI (m/z): Fragment ions, [d]ND: Not Detected

The phytochemical compositions of the methanol and water extracts of the plant *H. plicatum* subsp. *pseudoplicatum* were determined using the previously validated LC-MS/MS method (Güven et al., 2023). Considering Table 1, a total of 35 phenolic compounds were analyzed in this study, and 13 and 11 phenolic compounds were detected in WEHP and MEHP extracts, respectively. Considering the same table, 22 and 24 phenolic compounds could not be determined in WEHP and MEHP extracts, respectively. As seen in Table 1, phenolic compounds found in MEHP extract were in order to follows: Chlorogenic acid (30064.34 ng mL^{-1}), cyanidin-3-*O*-glycoside (29898.66 ng mL^{-1}), quinic acid (21648.41 ng mL^{-1}), fumaric acid (2287.01 ng mL^{-1}), apigenin (1474.44 ng mL^{-1}), luteolin (1114.82 ng mL^{-1}), naringenin (395.12 ng mL^{-1}), caffeic acid 189.62 (ng mL^{-1}), *p*-coumaric acid (71.76 ng mL^{-1}), rosmarinic acid (48.17 ng mL^{-1}), 4-OH-benzoic acid (37.58 ng mL^{-1}), quercetin (8.82 ng mL^{-1}), and gallic acid (1.17 ng mL^{-1}), and as seen in

the same Table 1 phenolic compounds found in WEHP extract were in order to follows: Cyanidin-3-*O*-glucoside (31474.77 ng mL^{-1}), chlorogenic acid (16801.47 ng mL^{-1}), quinic acid (5862.96 ng mL^{-1}) naringin (764.94 ng mL^{-1}), fumaric acid (695.18 ng mL^{-1}), rosmarinic acid (370.98 ng mL^{-1}), apigenin (135.44 ng mL^{-1}), keracyanin chloride (118.74 ng mL^{-1}) luteolin (68.50 ng mL^{-1}), peonidin-3-*O*-glucoside (5.98 ng mL^{-1}) and isorhamnetin (0.19 ng mL^{-1}). As a result of LC-MS/MS analysis, the sum of phenolic and nonphenolic substances is 87239.92 ng mL^{-1} in methanol extract and 56299.16 ng mL^{-1} in water extract (Table 1).

HPLC is generally preferred method for determining phenolic compounds (Gradinaru et al., 2014). *Helichrysum* species include the major compounds generally chlorogenic acid from phenolic acids and apigenin derivatives from flavonoids (Albayrak, et al., 2010a) . The total amount of phenolic acid in the methanol extract of the plant *H. arenarium* was 160.17 mg gallic acid equivalent g^{-1} extract (Gradinaru et al., 2014).

In two separate studies by Albayrak et al. (2010a, 2010b), phenolic compounds of 20 *Helichrysum* species found in Turkey were determined by HPLC. In 18 plant species, the major compound appears to be chlorogenic acid (32.3-156.2 µg g^{-1} extract), followed by apigenin-7-glycoside (6.4-127.6 µg g^{-1} extract) and apigenin (6.3-44.2 µg g^{-1} extract). The most phenolic compound among these species is *H. plicatum* subsp. *erzincanicum* (340.8 µg g^{-1} extract), and the least phenolic content is in *H. artvinense* (47.52 µg g^{-1} extract).

The reducing capacity of a compound can be an important indicator of its potential antioxidant activity. Antioxidant compounds can donate electrons to reactive radicals, reducing them to more stable and unreactive species (Bursal and Köksal, 2011) The Fe^{2+} reducing potential of the MEHP and WEHP and the standard antioxidants BHT (Butylated hydroxytoluene), BHA (Butylated hydroxyanisole), Trolox and *α*-Tocopherol was determined at the concentrations (15-45 µg mL^{-1}). At 30 µg mL^{-1} concentration the absorbance values of extracts and standards at 700 nm were as follows: BHT (2.018) > *α*-Tocopherol (1.895) > Trolox (1.545) > BHA (1.257) > MEHP (0.555) > WEHP (0.495), as depicted in Figure 2, 3 and Table 2.

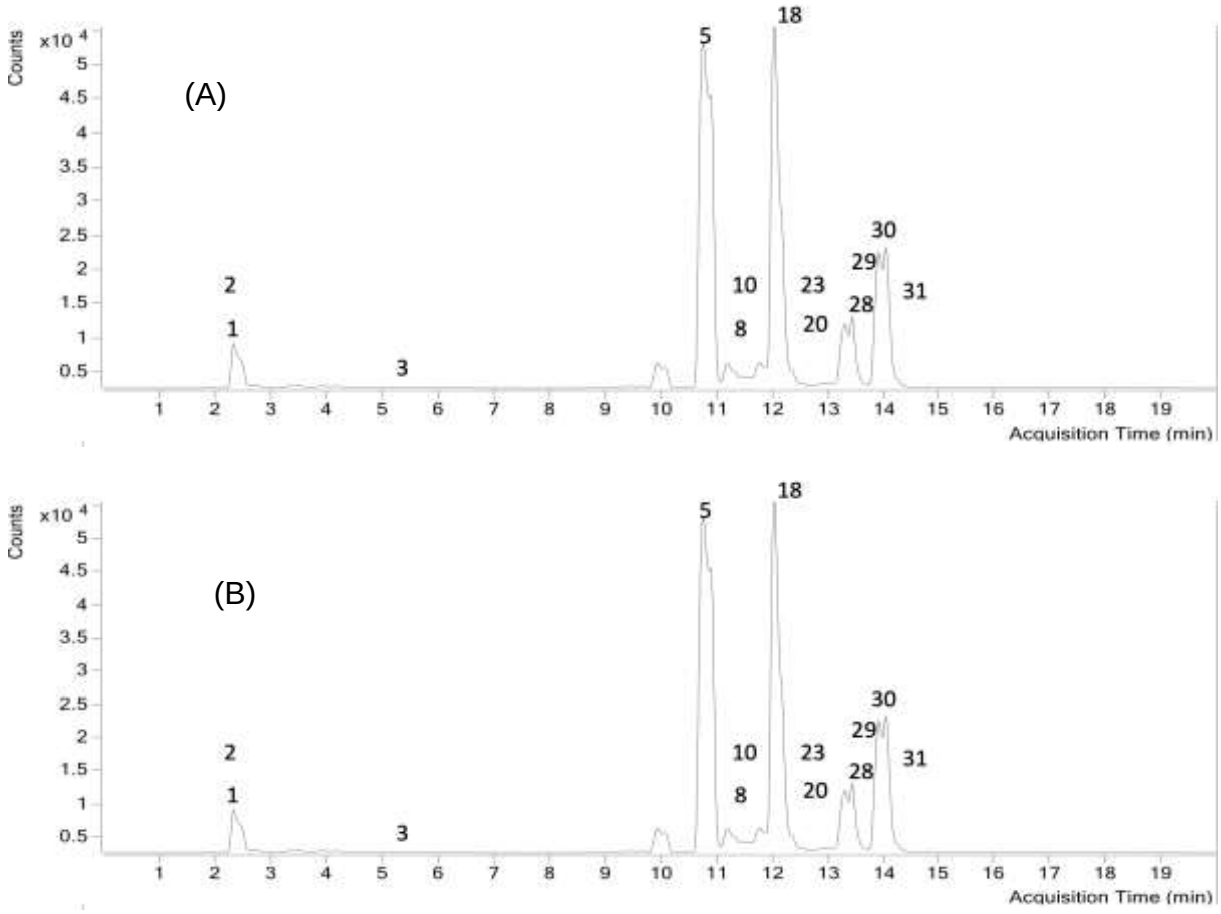


Figure 2. Multiple reaction monitoring chromatograms of MEHP (A), and WEHP (B), analyzed by LC-MS/MS method.

Şekil 2. LC-MS/MS yöntemiyle analiz edilen MEHP (A) ve WEHP (B)'nin çoklu reaksiyon izleme kromatogramları.

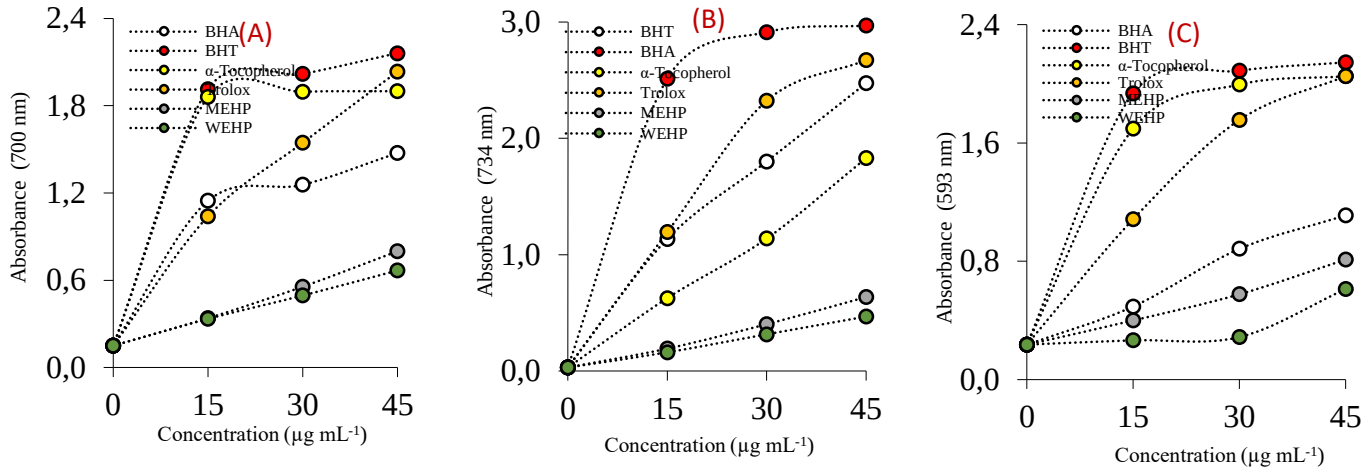


Figure 3. A. Fe^{3+} reducing, B. Cu^{2+} reducing and C. Fe^{3+}-TPTZ reducing capacities of MEHP, WEHP and standards.

Şekil 3. MEHP, WEHP ve standartların A. Fe^{3+} indirgeme, B. Cu^{2+} indirgeme ve C. Fe^{3+}-TPTZ indirgeme kapasiteleri

Table 2. Fe^{3+} and Cu^{2+} ions reduction capacities of extracts (MEHP, WEHP) and positive controls at different concentrations and with different methods

Çizelge 2. Ekstrelerin (MEHP, WEHP) ve pozitif kontrollerin farklı konsantrasyonlarda ve farklı yöntemlerle Fe^{3+} ve Cu^{2+} iyonlarını indirgeme kapasiteleri

Antioxidants	Fe^{3+} reducing ability			
	15 µg mL^{-1}	30 µg mL^{-1}	45 µg mL^{-1}	r^2
BHA	1.146±0.05	1.257±0.09	1.476±0.06	0.9523
BHT	1.912±0.02	2.018±0.03	2.160±0.07	0.9466
***α*-Tocopherol**	1.860±0.01	1.895±0.01	1.899±0.01	0.9402
Trolox	1.039±0.03	1.545±0.02	2.033±0.02	0.9966
MEHP	0.339±0.02^{ac}	0.555±0.01^{ac}	0.799±0.01^{ac}	0.9968
WEHP	0.335±0.01^{bd}	0.495±0.01^{bde}	0.668±0.01^{bde}	0.9930
	Cu^{2+} reducing (CUPRAC) ability			
	15 µg mL^{-1}	30 µg mL^{-1}	45 µg mL^{-1}	r^2
BHA	2.513±0.02	2.912±0.16	2.969±0.11	0.9742
BHT	1.135±0.05	1.800±0.01	2.473±0.02	0.9969
***α*-Tocopherol**	0.626±0.02	1.139±0.09	1.831±0.05	0.9967
Trolox	1.194±0.05	2.323±0.05	2.672±0.05	0.9980
MEHP	0.192±0.02^{ac}	0.402±0.01^{ac}	0.636±0.01^{ac}	0.9936
WEHP	0.160±0.02^{bde}	0.315±0.01^{bde}	0.467±0.03^{bde}	0.9990
	Fe^{3+}-TPTZ reducing (FRAP) ability			
	15 µg mL^{-1}	30 µg mL^{-1}	45 µg mL^{-1}	r^2
BHA	0.494±0.06	0.884±0.12	1.111±0.07	0.9899
BHT	1.936±0.08	2.089±0.03	2.146±0.01	0.9581
***α*-Tocopherol**	1.696±0.07	1.995±0.02	2.052±0.02	0.9807
Trolox	1.084±0.02	1.755±0.09	2.051±0.08	0.9990
MEHP	0.400±0.01^{ac}	0.577±0.02^{ac}	0.811±0.04^{ac}	0.9926
WEHP	0.265±0.01^{bde}	0.367±0.01^{bde}	0.613±0.01^{bde}	0.9479

^{a,b,c,d,e} show significant differences ($p<0.05$) in post-hoc comparisons between different groups. $^{a:}$ MEHP vs. *α*-Tocopherol, $^{b:}$ WEHP vs. *α*-Tocopherol, $^{c:}$ MEHP vs. Trolox, $^{d:}$ WEHP vs. Trolox, $^{e:}$ MEHP vs. WEHP

The measurement of the reducing capacity of cupric ions (Cu^{2+}) was conducted at various concentrations (15-45 µg mL^{-1}) of MEHP and WEHP. The capacity of the extracts to reduce copper ions varied depending on the concentrations used. As the concentration increased, the capacity of both extracts to reduce copper ions also increased. The copper ions reduction capacities of the standards and extracts (MEHP and

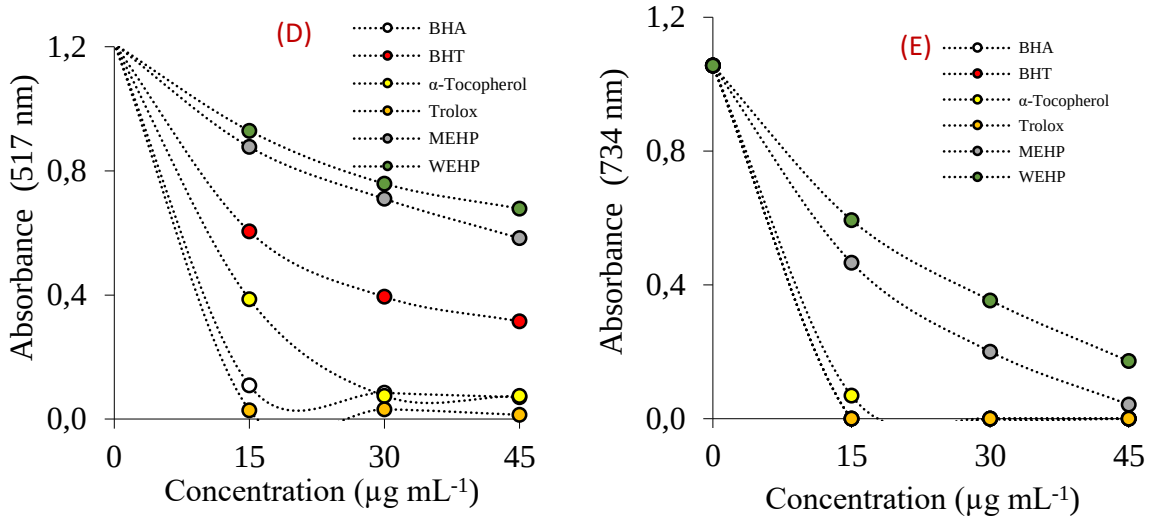
WEHP) at 450 nm and at 30 µg mL^{-1} concentration were as follows, respectively: BHT (2.912) > Trolox (2.323) > BHA (1.800) > α-Tocopherol (1.139) > MEHP (0.402) > WEHP (0.315), as shown in Figure 3 and Table 2. Ferric ions (Fe^{3+}) reduction capacity of the standards and extracts (MEHP and WEHP) was measured spectrophotometrically at 593 nm and the ferric ions (Fe^{3+}) reduction capacity at 30 µg mL^{-1} concentration was found as follows, respectively: BHT (2.089)> α-Tocopherol (1.995)> Trolox (1.755)> BHA (0.884)> MEHP (0.577)> WEHP (0.286) and the results were given in Figure 3 and Table 2. The effects of dose (15, 30, 45 µg mL^{-1}), groups (antioxidants), and dose*x*group interaction were examined by Two-Way ANOVA test (Table 3).

Table 3. The effects of dose, group, and dose*x*group interaction (p)
Çizelge 3. Doz, grup ve dozxgrup etkileşiminin etkileri (p)

	Two-Way ANOVA test			One-Way ANOVA post-hoc test		
Reducing assays	Groups (Antioxidants)	Doses (15-45 µg mL^{-1})	GroupxDose interaction	Dose 1 (15 µg mL^{-1}) vs Dose 2 (30 µg mL^{-1})	Dose 1 (15 µg mL^{-1}) vs Dose 3 (45 µg mL^{-1})	Dose 2 (30 µg mL^{-1}) vs Dose 3 (45 µg mL^{-1})
Fe^{3+} reducing	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
CUPRAC	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
FRAP	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

p values were reported for two-way (2 x 2) ANOVA analysis tests statistics for group, dose, and groupxdose interaction; p values were reported for post-hoc Tukey test after One-Way ANOVA test for pairwise comparisons of different doses

Due to the negative functions played by free radicals in dietary and biological systems, radical scavenging activity is crucial. The oxidation of lipids in food is accelerated and the quality is decreased by excessive free radical generation (Bursal et al. 2013). There was a positive correlation between the concentration of *Helichrysum* extracts and their efficacy in scavenging DPPH radicals. The ranking of IC_{50} values for DPPH scavenging activity of the MEHP, WEHP and standard antioxidants was determined to be as follows: α-Tocopherol (5.92 µg mL^{-1}) < BHA (9.0 µg mL^{-1}) < Trolox (9.63 µg mL^{-1}) < BHT (21.0 µg mL^{-1}) < MEHP (40.77 µg mL^{-1}) < WEHP (49.51 µg mL^{-1}), and the results were given in Figure 4 and Table 4. In a study of the DPPH analysis of the 20 *Helichrysum* extracts, the IC_{50} values are between 53.10-7.95 µg mL^{-1} (Albayrak et al., 2010a, 2010b). The scavenging activity of the MEHP, WEHP towards DMPD radicals exhibited a direct proportionality to the concentration increment. The ranking of IC_{50} values for DMPD scavenging activity of the MEHP, WEHP and standard antioxidants was determined to be as follows: Trolox (23.90 µg mL^{-1}) < BHA (43.32 µg mL^{-1}) < WEHP (77.01µg mL^{-1}) < MEHP (99.02 µg mL^{-1}), as depicted in Figure 4 and Table 4. ABTS radical scavenging activity of the extracts varied depending on the concentrations used. As the concentration of the extracts increased, the ABTS radical scavenging activity also increased. The IC_{50} values for the $ABTS^{+}$ free radical scavenging activity of MEHP, WEHP and standard antioxidants were presented in order to as: Trolox (7.71 µg mL^{-1}) = BHA (7.71 µg mL^{-1}) = BHT (7.71 µg mL^{-1}) < α-Tocopherol (8.10 µg mL^{-1}) < MEHP (10.50 µg mL^{-1}) < WEHP (17.77 µg mL^{-1}) and the results are given in the relevant table (Figure 4 and Table 4).



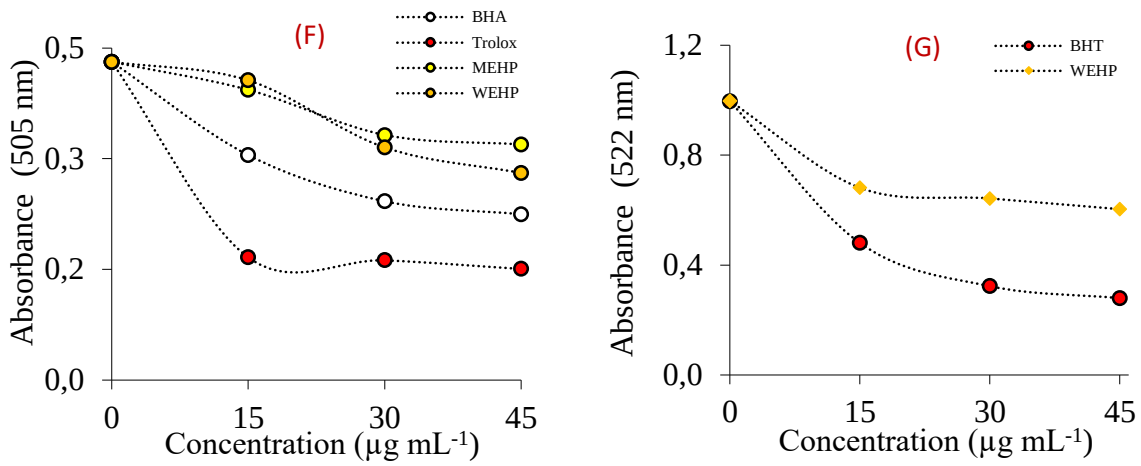


Figure 4. Radical scavenging effects of MEHP, WEHP, and positive controls. D. DPPH· scavenging capacity, E. ABTS·+ scavenging capacity, F. DMPD·+ scavenging capacity, and G. Ferrous chelating activity

Şekil 4. MEHP, WEHP ve pozitif kontrollerin radikal giderme etkileri. D. DPPH· giderme kapasitesi E. ABTS·+ giderme kapasitesi, F. DMPD·+ giderme kapasitesi ve G. Demir şelatlama aktivitesi

Table 4. IC_{50} values (µg mL^{-1}) of extracts (MEHP, WEHP) and positive controls for DPPH·, ABTS·+, and DMPD·+ and metal chelation

Çizelge 4. DPPH·, ABTS·+ ve DMPD·+ ve metal şelasyonu için ekstraktların (MEHP, WEHP) ve pozitif kontrollerin IC_{50} (µg mL^{-1}) değerleri

Antioxidants	DPPH· scavenging		ABTS·+ scavenging		DMPD·+ scavenging		Ferrous chelating	
	IC_{50}	r^2	IC_{50}	r^2	IC_{50}	r^2	IC_{50}	r^2
BHA	9.00	0.9399	7.71	0.9330	43.32	0.9993	-	-
BHT	21.00	0.9668	7.71	0.9330	-	-	21.66	0.9908
α-Tocopherol	5.92	0.9770	8.10	0.9550	-	-	-	-
Trolox	9.63	0.9947	7.71	0.9330	23.90	0.9349	-	-
MEHP	40.77	0.9965	10.50	0.9916	99.02	0.9689	-	-
WEHP	49.51	0.9999	17.77	0.9984	77.01	0.9510	53.32	0.9606

Total phenolic compound amounts in MEHP and WEHP were determined using the standard gallic acid equation (y= 0.002x, r^2: 0.9983) and were found to be 46.00 and 33.50 µg GAE mg^{-1} extract, respectively. In addition, MEHP and WEHP were determined to contain 62.13 and 103.93 µg QE mg^{-1} extract total flavonoids quantities, using the standard quercetin equation (y=0.0061x r^2 = 0.9980), respectively.

In a study by Aslan et al. (2007), the total phenolic and flavonoid compound amounts of ethanol and water extracts of *H. plicatum* were found as 113.5 and 75.9 mg GAE g^{-1} extract and 50.9 and 31.5 QE g^{-1} extract respectively. In a study, it has been stated that *Helichrysum* species (*H. arenarium* subsp. *erzincanicum, H. arenarium* subsp. *rubicundum*, *H. armenium* subsp. *araxinum*, *H. plicatum* subsp. *pseudoplicatum*) collected from Eastern Anatolia show strong antioxidant and radical scavenging activities. The study showed that *H. arenarium* subsp. *erzincanicum* had the highest level of free radical scavenging activity with an IC_{50} value of 23.03 µg mL^{-1}. It has been reported at the same study that the total phenolic contents of the extracts ranged between 71.81-144.50 mg gallic acid g^{-1} extract (Albayrak, et al., 2010b).

In the ferrous chelating activity experiment, only BHT from the standards and WEHP from the extracts reacted. This may be caused by different phenolic substances in the plant extract (Table 4). The inhibition effects of MEHP, WEHP extracts and acarbose, tacrine and acetazolamide used as standard controls against α-glycosidase, acetylcholinesterase and carbonic anhydrase II enzymes are shown in Table 5. In general, methanol extracts have a better inhibition ability than water extracts. However, chemical substances used as standard also have a better inhibitory effect than extracts. The inhibition effects of MEHP and WEHP extracts, and standards (acarbose, tacrine and acetazolamide) against α-glycosidase, acetylcholinesterase (AChE) and carbonic anhydrase II (CA II) enzymes are summarized at the Table 5. In general, the methanol extracts exhibited strong inhibition effect against enzymes compared to water extracts. Against CA II and AChE enzymes, acetazolamide and tacrine were found powerful inhibitors, respectively. As expected, both drugs were determined to be much stronger inhibitors compared

with the extracts. The inhibition capacities of MEHP and WEHP extracts at different doses (1-6 µg mL^{-1}) against α-glycosidase enzyme were determined. The IC_{50} values of MEHP and WEHP extracts against α-glycosidase enzyme were found as 2.39 and 2.97 µg mL^{-1}, respectively (Table 5).

There are many methods to prevent diabetes. Thanks to the phenolic and non-phenolic compounds found in plants, it has positive effects on diabetes with its enzyme inhibiting effects. Inhibition of digestive enzymes may delay carbohydrate absorption in diabetic patients (Karageçili et al. 2023). *H. plicatum* subsp. *plicatum* was reported to have significant antihyperglycemic effect in diabetic rats (Aslan et al., 2007). In a study, the α-glycosidase enzyme inhibitory effects of *H. graveolens* and *H. plicatum* subsp *pseudoplicatum* alcohol and water extract flowers were tested the IC_{50} values were determined as 0.71 and 0.86 mg mL^{-1} and 2.2 mg mL^{-1} and 5.1 mg mL^{-1}, respectively (Orhan et al., 2014). In this study, IC_{50} values of MEHP and WEHP extracts were as 2.39 and 2.97 µg mL^{-1}, respectively. IC_{50} values of water extracts are compatible with this study. Current results show that WEHP extract has an antidiabetic effect and can be preferred as an antidiabetic agent. Alzheimer's disease (AD) is currently one of the most important global health problems. Inhibiting AChE is one of the different treatment strategies for AD. By inhibiting AChE, the acetylcholine level in the synaptic pathway increases, which causes increased neural transmission (Türkan et al. 2020). The inhibitory effects of different doses of MEHP and WEHP extracts on the AChE enzyme were examined and IC_{50} values were calculated. The results showed that the methanol extract inhibit the AChE enzyme better than the aqueous extract and the IC_{50} values were found as 2.48 and 3.59 µg mL^{-1}, respectively. Both extracts also exhibited a strong inhibitory effect against the AChE enzyme (Table 5). The anti-Alzheimer's disease effect of *Helichrysum* species is also noted in another study by Silva et al. (2017). In the AChE inhibition experiment of plant extracts, it was reported that the flowers had IC_{50} inhibition values of 260.7 µg mL^{-1} and the roots/leaves 654.8 µg mL^{-1}. The inhibitory effect of MEHP and WEHP at different concentrations (15-45 µg mL^{-1}) on the CA II isozyme was examined and compared with acetazolamide. IC_{50} values for MEHP and WEHP were determined as 11.95 µg mL^{-1} and 25.67 µg mL^{-1} respectively. The results showed that both extracts were weaker inhibitors of the CA II enzyme (IC_{50} = 1.85 µg mL^{-1}) compared to standard (acetazolamide).

Table 5. IC_{50} values of extracts and standard drugs
Çizelge 5. Ekstrelerin ve standart ilaçların IC_{50} değerleri

Samples	CA II	r^2	AChE	r^2	α-Gly	r^2
MEHP	11.95	0.9540	2.48	0.9900	2.39	0.9730
WEHP	25.67	0.9880	3.59	0.9925	2.97	0.9772
Acetazolamide	1.85	0.9825	-	-	-	-
Tacrine	-	-	1.18	0.9706	-	-
Acarbose	-	-	-	-	1.47	0.9922

CONCLUSION

With this study, it was shown that the extracts (MEHP and WEHP) of the aerial parts of *H. plicatum* subsp. *pseudoplicatum* were rich in phenolic compounds. By evaluating the antioxidant, antidiabetic, anti-Alzheimer's disease and antiglaucoma activities, major compounds were identified as a result of LC-MS/MS, as well as the remarkable biological activities of the extracts, and it was explained that they could be the compounds responsible for the activity. Considering the promising biological activity of *H. plicatum* subsp. *pseudoplicatum* and its safety with few side effects, knowledge about the effects of the plant with *in vivo* studies should be strengthened in further studies. In future studies, the potential mechanisms of biological effects should be explored and it should be clarified whether the activities are the result of the individual activity of secondary compounds or the synergistic effect of all phenolic and non-phenolic compounds as a whole.

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Conflicts of Interest: The authors declare that there are no conflicts of interest.

REFERENCES

Ak, T., & Gülçin, İ. (2008). Antioxidant and radical scavenging properties of curcumin. *Chemico-Biological Interactions*, *174* (1), 27-37.

Al-Rehaily, A. J., Albishi, O. A., El-Olemy, M. M., & Mossa, J. S. (2008). Flavonoids and terpenoids from *Helichrysum forskahlii*. *Phytochemistry*, *69* (9), 1910-1914.

Albayrak, S., Aksoy, A., Sagdic, O., & Hamzaoglu, E. (2010a). Compositions, antioxidant and antimicrobial activities of *Helichrysum* (Asteraceae) species collected from Turkey. *Food Chemistry*, *119* (1), 114-122.

Albayrak, S., Aksoy, A., Sağdıç, O., & Budak, Ü. (2010b). Phenolic compounds and antioxidant and antimicrobial properties of *Helichrysum* species collected from eastern Anatolia, Turkey. *Turkish*

Journal of Biology, *34* (4), 463-473.

Apak, R., Calokerinos, A., Gorinstein, S., Segundo, M.A., Hibbert, D.B., Gülçin, İ., Demirci Çekiç, S., Güçlü, K., Özyürek, M., Esin Çelik, S., Magalhaes, L.M., & Arancibia-Avila P. (2022). Methods to evaluate the scavenging activity of antioxidants toward reactive oxygen and nitrogen species. *Pure and Applied Chemistry, 94(1)*, 87-144.

Aslan, M., Deliorman, O. D., Orhan, N., Sezik, E., & Yesilada, E. (2007). *In vivo* antidiabetic and antioxidant potential of *Helichrysum plicatum* ssp. *plicatum* capitulums in streptozotocin-induced-diabetic rats. *Journal of Ethnopharmacology*, *109* (1), 54-59.

Aydin, T. (2020). Secondary metabolites of *Helichrysum plicatum* DC. subsp. *plicatum* flowers as strong carbonic anhydrase, cholinesterase and alpha-glycosidase inhibitors. *Zeitschrift Fur Naturforschung Section C- Journal of Biosciences*, *75* (5-6), 153-159.

Bayir, Y., Halici, Z., Keles, M. S., Colak, S., Cakir, A., Kaya, Y., & Akcay, F. (2011). *Helichrysum plicatum* DC. subsp. *plicatum* extract as a preventive agent in experimentally induced urolithiasis model. *Journal of Ethnopharmacology*, *138* (2), 408-414.

Bayrak, C., Taslimi, P., Karaman, H. S., Gulcin, I., & Menzek, A. (2019). The first synthesis, carbonic anhydrase inhibition and anticholinergic activities of some bromophenol derivatives with S including natural products. *Bioorganic Chemistry*, *85*, 128-139.

Benelli, G., Pavela, R., Rakotosaona, R., Randrianarivo, E., Nicoletti, M., & Maggi, F. (2018). Chemical composition and insecticidal activity of the essential oil from *Helichrysum faradifani* endemic to Madagascar. *Natural Product Research*, *32* (14), 1690-1698.

Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature*, *181* (4617), 1199-1200.

Bursal, E., & Köksal, E. (2011). Evaluation of reducing power and radical scavenging activities of water and ethanol extracts from sumac (*Rhus coriaria* L.). *Food Research International*, *44* (7), 2217-2221.

Bursal, E., Köksal, E., Gülçin, İ., Bilsel, G., & Gören, A. C. (2013). Antioxidant activity and polyphenol content of cherry stem (*Cerasus avium* L.) determined by LC–MS/MS. *Food Research International*, *51*(1), 66-74.

Carini, M., Aldini, G., Furlanetto, S., Stefani, R., & Facino, R. M. (2001). LC coupled to ion-trap MS for the rapid screening and detection of polyphenol antioxidants from *Helichrysum stoechas*. *Journal of Pharmaceutical and Biomedical Analysis*, *24* (3), 517-526.

Çetinkaya, Y., Göçer, H., Menzek, A., & Gülçin, İ. (2012). Synthesis and antioxidant properties of (3,4-dihydroxyphenyl)(2,3,4-trihydroxyphenyl)methanone and its derivatives. *Archiv der Pharmazie*, *345* (4), 323-334.

Davis, P., & Kupicha, K. (1975). *Helichrysum*. In P. H. Davis (Ed.), *Flora of Turkey and the East Aegean Islands* (Vol. 5, pp. 80-96). the University Press.

Durmaz, L., Kiziltas, H., Guven, L., Karagecili, H., Alwasel, S., & Gulcin, İ. (2022). Antioxidant, antidiabetic, anticholinergic, and antiglaucoma effects of magnofluorine. *Molecules*, *27* (18), 5902.

Ellman, G. L., Courtney, K. D., Andres, V., & Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology*, *7* (2), 88-95.

Farris, E., Canopoli, L., Cucca, E., Landi, S., Maccioni, A., & Filigheddu, R. (2017). Foxes provide a direct dispersal service to *Phoenician junipers* in Mediterranean coastal environments: ecological and evolutionary implications. *Plant Ecology and Evolution*, *150* (2), 117-128.

Fogliano, V., Verde, V., Randazzo, G., & Ritieni, A. (1999). Method for measuring antioxidant activity and its application to monitoring the antioxidant capacity of wines. *Journal of Agricultural and Food Chemistry*, *47* (3), 1035-1040.

Göçer, H., & Gülçin, İ. (2011). Caffeic acid phenethyl ester (CAPE): correlation of structure and antioxidant properties. *International Journal of Food Sciences and Nutrition*, *62* (8), 821-825.

Gradinaru, A. C., Silion, M., Trifan, A., Miron, A., & Aprotosoaie, A. C. (2014). *Helichrysum arenarium* subsp. *arenarium*: phenolic composition and antibacterial activity against lower respiratory tract pathogens. *Natural Product Research*, *28* (22), 2076-2080.

Gulcin, İ. (2020). Antioxidants and antioxidant methods: an updated overview. *Archives of Toxicology*, *94* (3), 651-715.

Gulcin, İ., & Alwasel, S. H. (2022). Metal ions, metal chelators and metal chelating assay as antioxidant method. *Processes*, *10* (1), 132.

Gulcin, İ., & Alwasel, S. H. (2023). DPPH Radical Scavenging Assay. *Processes*, *11* (8), 2248.

Gulcin, I., Kaya, R., Goren, A. C., Akincioglu, H., Topal, M., Bingol, Z., Çetin Çakmak K., Öztürk Sarıkaya S. B., Durmaz L. & Alwasel, S. (2019). Anticholinergic, antidiabetic and antioxidant activities of cinnamon (*Cinnamomum verum*) bark extracts: polyphenol contents analysis by LC-MS/MS. *International Journal of Food Properties*, *22* (1), 1511-1526.

Gulcin, I., & Beydemir, S. (2013). Phenolic compounds as antioxidants: carbonic anhydrase isoenzymes inhibitors. *Mini Reviews in Medicinal Chemistry*, *13* (3), 408-430.

Guven, L., Ozgen, U., Sevindik, H., Agan, İ., Koca, M., Turan, İ., Demir S., Aliyazicioglu Y. (2022). Evaluation of LC-MS/MS analysis and in vitro biological activities of *Rosa pimpinellifolia* root, pseudo-fruit, and seed extracts. *International Journal of Life Sciences and Biotechnology*, *5* (3), 480-503.

Gülçin, I. (2008). Measurement of antioxidant ability of melatonin and serotonin by the DMPD and CUPRAC methods as trolox equivalent. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *23* (6), 871-876.

Gülçin, İ., Bingöl, Z., Taslimi, P., Gören, A. C., Alwasel, S. H., & Tel, A. Z. (2022). Polyphenol contents, potential antioxidant, anticholinergic and antidiabetic properties of mountain mint (*Cyclotrichium leucotrichum*). *Chemistry & Biodiversity*, *19* (3), e202100775.

Gülçin, İ., Bursal, E., Şehitoğlu, M. H., Bilsel, M., & Gören, A. C. (2010). Polyphenol contents and antioxidant activity of lyophilized aqueous extract of propolis from Erzurum, Turkey. *Food and Chemical Toxicology*, *48* (8), 2227-2238.

Güven, L., Erturk, A., Miloğlu, F. D., Alwasel, S., & Gulcin, İ. (2023). Screening of antiglaucoma, antidiabetic, anti-alzheimer, and antioxidant activities of *Astragalus alopecurus* Pall-Analysis of phenolics profiles by LC-MS/MS. *Pharmaceuticals* , *16* (5), 659.

Huyut, Z., Beydemir, Ş., & Gülçin, İ. (2017). Inhibition properties of some flavonoids on carbonic anhydrase I and II isoenzymes purified from human erythrocytes. *Journal of Biochemical and Molecular Toxicology*, *31* (9), e21930.

Karagecili, H., Yılmaz, M. A., Ertürk, A., Kiziltas, H., Güven, L., Alwasel, S. H., & Gulcin, İ. (2023). Comprehensive metabolite profiling of berdav propolis using LC-MS/MS: determination of antioxidant, anticholinergic, antiglaucoma, and antidiabetic effects. *Molecules*, *28* (4), 1739.

Karageçili, H., İzol, E., Kireçci, E., & Gülçin, İ. (2023). Antioxidant, antidiabetic, antiglaucoma, and anticholinergic effects of Tayfi grape (*Vitis vinifera*): A phytochemical screening by LC-MS/MS analysis. *Open Chemistry*, *21*(1), 20230120.

Kazancı, C., Oruç, S., & Mosulishvili, M. (2020). Medicinal ethnobotany of wild plants: a cross-cultural comparison around Georgia-Turkey border, the Western Lesser Caucasus. *Journal of Ethnobiology and Ethnomedicine, 16* (1), 71.

Kherbache, A., Senator, A., Laouicha, S., Al-Zoubi, R. M., & Bouriche, H. (2020). Phytochemical analysis, antioxidant and anti-inflammatory activities of *Helichrysum stoechas* (L.) Moench extracts. *Biocatalysis and Agricultural Biotechnology*, *29*, 101826.

Kiziltas, H., Bingol, Z., Goren, A. C., Pinar, S. M., Alwasel, S. H., & Gulcin, I. (2021). LC-HRMS profiling of phytochemicals, antidiabetic, anticholinergic and antioxidant activities of evaporated ethanol extract of *Astragalus brachycalyx* Fischer. *Journal of Chemical Metrology*, *15*(2), 135-151.

Kızıltaş, H., Bingol, Z., Gören, A. C., Kose, L. P., Durmaz, L., Topal, F., Alwasel, S. H., Gulcin, İ. (2021). LC-HRMS profiling and antidiabetic, anticholinergic, and antioxidant activities of aerial parts of kınkor (*Ferulago stellata*). *Molecules*, *26* (9), 2469.

Koçyiğit, Ü.M., Eruygur, N., Ataş, M., Tekin, M., Taslimi, P., Gökalp, F., Gülçin, İ. (2022). Evaluation of anticholinergic, antidiabetic and antioxidant activity of *Astragalus dumanii*, an endemic plant. *KSU Journl of Agriculture and Nature, 25*(S1), 1-10.

Kolaylı, S., Sahin, H., Ulusoy, E., & Tarhan, Ö. (2010). Phenolic composition and antioxidant capacities of *Helichrysum plicatum*. *Hacettepe Journal of Biological Chemistry 38*, 269-276.

Köse, L. P., Gülçin, İ., Gören, A. C., Namiesnik, J., Martinez-Ayala, A. L., & Gorinstein, S. (2015). LC–MS/MS analysis, antioxidant and anticholinergic properties of galanga (*Alpinia officinarum* Hance) rhizomes. *Industrial Crops and Products*, *74*, 712-721.

Miloglu, F. D., Akpınar, A., Güven, L., Demirkaya, A. K., Gundogdu, G., Nalcı, K. A., & Hacımuftuoglu, A. (2023). Evaluation the effects of *Helichrysum plicatum* subsp. *pseudoplicatum* on an *in-vitro* wound model using human dermal fibroblast cells. *The International Journal of Lower Extremity Wounds, 22* (2), 401-408.

Orhan, N., Hocbac, S., Orhan, D. D., Asian, M., & Ergun, F. (2014). Enzyme inhibitory and radical scavenging effects of some antidiabetic plants of Turkey. *Iranian Journal of Basic Medical Sciences, 17* (6), 426-432.

Oyaizu, M. (1986). Studies on products of browning Reaction Antioxidative Activities of Products of browning reaction prepared from glucosamine. *The Japanese Journal of Nutrition and Dietetics*, *44* (6), 307-315.

Ozaslan, M. S., Saglamtas, R., Demir, Y., Genc, Y., Saracoglu, I., & Gulcin, I. (2022) Isolation of some phenolic compounds from *Plantago subulata* L. and determination of their antidiabetic, anticholinesterase, antiepileptic and antioxidant activity. *Chemistry & Biodiversity*, Article e202200280.

Pljevljakusic, D., Bigovic, D., Jankovic, T., Savikin, K., & Jelacic, S. (2018). Sandy everlasting (*Helichrysum arenarium* (L.) Moench): botanical, chemical and biological properties. *Frontiers in Plant Science, 9*, 1123.

Popoola, O. K., Marnewick, J. L., Rautenbach, F., Iwuoha, E. I., & Hussein, A. A. (2015). Acylphloroglucinol derivatives from the South African *Heliehrysum niveum* and their biological activities. *Molecules*, *20* (9), 17309-17324.

Raina, R., Parwez, S., Verma, P. K., & Pankaj, N. K. (2008). Medicinal plants and their role in wound healing. *Online Veterinary Journal,* 3 (1), 21.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, *26* (9), 1231-1237.

Sezer Senol, F., Orhan, I. E., Ozgen, U., Renda, G., Bulut, G., Guven, L., Sezen Karaoglan E., Sevindik H.G., Skalicka-Wozniak K., Koca Caliskan U., Sekeroglu, N. (2016). Memory-vitalizing effect of twenty-five medicinal and edible plants and their isolated compounds. *South African Journal of Botany*, *102*, 102-109.

Silva, L., Rodrigues, A. M., Ciriani, M., Falé, P. L. V., Teixeira, V., Madeira, P Machuqueiro M., Pacheco R., Florencio M. H., Ascensão, L., Marques Serralheiro M. L. (2017). Antiacetylcholinesterase activity and docking studies with chlorogenic acid, cynarin and arzanol from *Helichrysum stoechas* (Lamiaceae). *Medicinal Chemistry Research*, *26* (11), 2942-2950.

Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *In Methods in Enzymology*, 299, 152-178.

Smirnov, V. V., Preobrazhenskaya, N. E., & Kalashnikov, I. D. (1982). Antibacterial properties of *Helichrysum plicatum* DC. *Mikrobiologicheskii Zhurnal, 44* (4), 71-72.

Şenol, H., Çelik Turgut, G., Şen, A., Sağlamtaş, R., Tuncay, S., Gülçin, İ., & Topçu, G. (2023). Synthesis of nitrogen-containing oleanolic acid derivatives as carbonic anhydrase and acetylcholinesterase inhibitors. *Medicinal Chemistry Research*, *32* (4), 694-704.

Tao, Y., Zhang, Y., Cheng, Y., & Wang, Y. (2013). Rapid screening and identification of α-glucosidase inhibitors from mulberry leaves using enzyme-immobilized magnetic beads coupled with HPLC/MS and NMR. *Biomedical Chromatography*, *27* (2), 148-155.

Taslimi, P., & Gulçin, İ. (2018). Antioxidant and anticholinergic properties of olivetol. *Journal of Food Biochemistry*, *42* (3), e12516.

Taslimi, P., Köksal, E., Gören, A. C., Bursal, E., Aras, A., Kılıç, Ö., Alwasel S., & Gülçin, İ. (2020). Anti-Alzheimer, antidiabetic and antioxidant potential of *Satureja cuneifolia* and analysis of its phenolic contents by LC-MS/MS. *Arabian Journal of Chemistry*, *13* (3), 4528-4537.

Tepe, B., Sokmen, M., Askin Akpulat, H., & Sokmen, A. (2004). *In vitro* antioxidant activities of the methanol extracts of four *Helichrysum* species from Turkey. *Food Chemistry*, *90* (4), 685-689.

Topal, M., & Gulcin, I. (2014). Rosmarinic acid: A potent carbonic anhydrase isoenzymes inhibitor. *Turkish Journal of Chemistry*, *38* (5), 894-902.

Türkan, F., Atalar, M. N., Aras, A., Gülçin, İ., & Bursal, E. (2020). ICP-MS and HPLC analyses, enzyme inhibition and antioxidant potential of *Achillea schischkinii* Sosn. *Bioorganic Chemistry*, *94*, 103333.