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Enzyme inhibitory and antioxidant activities and HPLC quantification of chlorogenic acid in *Helichrysum stoechas* (L.) Moench and *H. stoechas* subsp. *barrelieri* (Ten) Nyman

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Abstract

The inhibitory effects of ethanol (80%) and aqueous extracts of *Helichrysum stoechas* (L.) Moench and *H. stoechas* subsp. *barrelieri* (Ten) Nyman on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), two sister enzymes associated with the pathogenesis of Alzheimer's disease (AD), as well as on elastase and collagenase, linked to inflammation and skin aging, were investigated. Simultaneously, the antioxidant activity of the extracts was assessed through DPPH radical scavenging activity, ferric reducing antioxidant power (FRAP), and metal-chelating activity assays since oxidative damage plays a critical role in AD pathophysiology and skin aging. Total phenol and flavonoid contents in the extracts were spectrophotometrically determined. The highest AChE inhibitory activity (44.60 ± 4.4% at 2000 µg/mL) was found in the ethanol extract of *H. stoechas* subsp. *barrelieri* collected from Hatay, and the uppermost BChE inhibitory activity at same concentration was found in the aqueous extract of *H. stoechas* subsp. *barrelieri* collected from Izmir (80.24 ± 2.63%, IC_{50}: 38.52 ± 1.41 µg/mL). Both of them inhibited AChE and BChE in a concentration-dependent manner. Nevertheless, none of the extracts from the two plants inhibited elastase and collagenase. Although both ethanolic and aqueous extracts had significant antioxidant activity in DPPH radical scavenging and FRAP assays, they demonstrated inadequate antioxidant activity in the metal-chelating assay. Chlorogenic acid was quantified in the extracts using HPLC. The mentioned two extracts with strong cholinesterase (ChE) inhibition also had the highest chlorogenic acid content. The ethanol extract of *H. stoechas* (Hatay sample) and the aqueous extract of *H. stoechas* (Izmir sample) seem to contain promising ChE inhibitors, which deserve further investigation.

Key words: *Helichrysum stoechas*, enzyme inhibition, Alzheimer's disease, antioxidant activity, chlorogenic acid.

1. Introduction

Helichrysum stoechas (L.) Moench, a member of Asteraceae family, is a perennial medicinal plant that grows naturally in the Western and Eastern regions of Türkiye. Aside from Türkiye, it grows naturally in Italy, the Balkans, Cyprus, and Lebanon, where it is cultivated in many parts of the world due to its medicinal importance. This plant is called by many different names such as "altın çiçeği, guddeme çiçeği, kudama, ölmez çiçek, altın otu" in diverse parts of Anatolia. *Helichrysum stoechas* subsp. *barrelieri* (Ten) is a sub-taxon of *H. stoechas* that grows in the southern

Marmara, Aegean, and Mediterranean regions of Türkiye as well as Albania, Cyprus, Egypt, Greece, and Italy (Aksoy et al., 2011; Aslan, 1994; Eroğlu, 2018). The ethnopharmacological records of *H. stoechas* indicate that the plant's capitulum is used for its diuretic, emmenagogue, anticoagulant, kidney stone-lowering, appetite-raising, and anthelmintic properties along with its utilization in the treatment of dermatological illnesses such as dermatitis, burn-wound treatment, and body crack healing (Gras et al., 2017; Memariani et al., 2018; Tsioutsiou et al., 2022).

Alzheimer's disease (AD) is an incurable and progressive neurological disease that deprives people of their memory and cognitive abilities, primarily affecting the elderly population. The specific cause of the illness is unknown, while there is no proven therapy yet, to seize the disease. According to the cholinergic theory, levels of two neurotransmitters, *i.e.* acetylcholine and butyrylcholine, have been low in the brains of AD patients, which break down by two sister enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), respectively. Inhibition of cholinesterases (ChE) is critical for the treatment and thus, ChE inhibitors are currently used to treat AD symptoms. Relevantly, current FDA-approved pharmacotherapy for AD includes ChE inhibitors, *e.g.* rivastigmine, galantamine, tacrine, and donepezil, along with *N*-methyl D-aspartate (NMDA) receptor antagonists (*e.g.* memantine) (Terry & Buccafusco, 2003). In a study, which inspired us to carry out the present work, inhibition by the extract prepared from *H. stoechas* capitula on AChE (IC_{50} value of 260.7 µg/mL) shown by *in vitro* and *in silico* molecular docking methods was determined, where chlorogenic acid, cynarin, and arzanol were reported to be responsible for the inhibitory activity of the extract (Silva et al., 2017). On the other hand, skin aging ranks as one of the most widespread dermatologic concerns; it is a multifaceted and unavoidable process of human existence. Elastin and collagen, two key proteins that make up connective tissue, are responsible for the skin's resilience and suppleness. With the skin's natural aging process, their breakdown by elastase and collagenase, which free oxygen radicals would activate, promotes wrinkle development. Many researchers working on skin aging have turned their attention to plant extracts after discovering that cosmetic products containing synthetic agents, including such sodium lauryl sulphate and triethanolamine as active ingredients cause side effects such as allergic or irritant contact dermatitis, phototoxicity, and photoallergic responses. Natural skin care goods are acknowledged to be also effortlessly received through into the epidermal layer and to be typically hypoallergenic. Although so called """natural beauty products" "" appear to be remarkably popular amongst consumers throughout the globe, yet, the overwhelming of them lack scientific evidence to back up their putative aesthetic effects (Mukherjee et al., 2011).

It should be mentioned that free radicals and reactive oxygen species (ROS) have been identified as playing a crucial part in both skin and brain aging, *i.e.* AD. The capitula of *H. stocheas* were previously revealed to possess high antioxidant activity (Haddouchi et al., 2014). Furthermore, a single research examining antiaging action of *H. petiolare* has described its ability to prevent skin aging, which should be explored further (Sagbo & Otang-Mbeng, 2020). Based on the aforementioned little data, we were inspired to do research on the skin and brain aging potential of *H. stoechas*. For this purpose, *H. stoechas* and *H. stoechas* subsp. *barrelieri* were subjected to enzyme inhibition assays towards AChE, BChE, collagenase, and elastase, as well as antioxidant assays using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, ferric-reducing antioxidant power (FRAP), and metal-chelating activity as well as HPLC analysis, which will constitute the first study of its kind on this species and sub-taxon.

2. Materials and methods

2.1. Plant materials

The cultivated samples of *H. stoechas* obtained from a farm in Izmir (Türkiye) and naturally growing samples *H. stoechas* subsp. *barrelieri* collected from two provinces of Türkiye (*e.g.* Izmir and Hatay) were used as the plant materials in this study. Firstly, the capitula of these species were manually separated and utilized for extraction and bioassays performed herein. The species were identified by one of us (M.A.) and their herbarium species are preserved at Faculty of Pharmacy, Gazi University (Ankara, Türkiye).

2.2. Preparation of the extracts

The separated capitula of the plant samples were dried in circulating air. The dried plant materials were homogeneously pulverized using a mechanical grinder and each sample was weighed accurately (4.5 g) on a digital scale (Shimadzu, Japan). Each plant material was extracted with ethanol (EtOH, 80%, 150 mL) and hot water (150 mL), sequentially, through maceration for 24 hours with periodic handshaking. The EtOH phases were evaporated under reduced pressure by a rotary evaporator (Büchi, Switzerland) to obtain the crude extracts. A lyophilizer (Telstar Lyo Quest, Japan) was used to freeze-dry the aqueous extracts. A total of six extracts was obtained from three plant samples. The crude extracts were kept in the refrigerator at +4°C until bioassays were performed. Table 1 displays the yields of the extracts obtained from each plant.

Table 1. Yields of the extracts (w/w)

Extracts	Yields (%, w/w)
EtOH extract (80%) cultivated in Izmir (CE)	10.89
Aqueous extract cultivated in Izmir (CW)	14.91
EtOH extract (80%) grown naturally in Izmir (NIE)	9.65
Aqueous extract grown naturally in Izmir (NIW)	13.55
EtOH extract (80%) grown naturally in Hatay (NHE)	7.88
Aqueous extract grown naturally in Hatay (NHW)	14.29

2.3. Determination of total phenol and flavonoid content in the extracts

Total phenolic contents of EtOH (80%) and aqueous extracts were determined using the Folin-Ciocalteau colorimetric technique with some slight modifications (Singleton & Rossi, 1965). To construct the calibration curve, gallic acid (GA) solutions (used as standard) at various concentrations (1, 0.5, 0.25, 0.125, 0.125, 0.0625, and 0.03125 mg/mL) were prepared to calculate total phenol content. The aqueous and EtOH (80%) extracts were dissolved in EtOH (96%). Both samples and varying concentrations of GA were treated with 30 µL of Folin-Ciocalteau reagent (diluted twice, Sigma, USA) and 150 µL of sodium carbonate (3.5%). After half an hour at 40°C incubation, absorbance at 760 nm was measured with an ELISA microplate reader (Molecular Devices, Spectramax i3 microplate reader, USA). The experiment was performed in triplicate. GA equivalents (mg/g extract) were used to express the results. With some slight changes, total flavonoid content of the extracts was determined in the extracts using aluminum chloride colorimetric technique (Woisky & Salatino, 1998). Numerous concentrations of quercetin (1, 0.5, 0.25, 0.125, 0.125, 0.125, 0.0625, and 0.03125 mg/mL) employed as standard were prepared to create the calibration curve. In a 96-well microplate, each sample and quercetin solutions at the given concentrations were mingled with 75 µL of EtOH (96%), 5 µL of $AlCl_3$ (10%), 5 µL of 1 M sodium acetate, and 100 µL of distilled water, respectively. The samples and standard solutions were then incubated for

30 minutes in the dark at room temperature. Absorbance of each sample was measured at 415 nm using an ELISA microplate reader (Molecular Devices, Spectramax i3 Microplate Reader, USA). The experiment was conducted on three parallel assays. The results were represented in milligrams of quercetin equivalent (mg/g extract). Table 2 demonstrates total phenol and flavonoid contents of the extracts.

Table 2. Total phenolic and flavonoid content of the extracts

Extracts	Total phenol content[a] ± S.D[b]	Total flavonoid content[c] ± S.D[b]
CE	118.77 ± 4.88	91.87 ± 0.81
CW	71.50 ± 9.27	47.21 ± 1.92
NIE	113.60 ± 5.37	79.86 ± 1.44
NIW	94.62 ± 1.95	48.09 ± 3.74
NHE	141.55 ± 2.93	75.45 ± 2.05
NHW	108.76 ± 1.46	65.20 ± 2.02

[a]Data expressed in mg equivalent of gallic acid to 1 g of extract, [b]Standard deviation (n: 3),[c]Data expressed in mg equivalent of quercetin to 1 g of extract.

2.4. Antioxidant activity assays

2.4.1. DPPH radical scavenging activity assay

The bleaching characteristic of a violet-colored methanol solution of DPPH (Sigma, USA) was used to determine the antioxidant capacity of the extracts prepared in 80% ethanol and water. Stable DPPH radical scavenging activity was tested utilizing (Hatano et al., 1988) method with mild modifications (Barros et al., 2007). Samples/references (10 µL for each) dissolved in EtOH (96%) were transferred to 96-well plates. After then, using a calibrated multichannel pipette (Eppendorf Research, Germany), 90 µL of DPPH solution (1.5×10^{-4} M) prepared in EtOH was poured *per* each well. Table 3 illustrates DPPH radical scavenging capabilities of the extracts.

Table 3. Antioxidant activities of the extracts

Extracts	Metal-chelating capacity (Capacity % ± S.D.[a])	Ferric-reducing antioxidant power[b] (FRAP) (Absorbance at 700 nm ± S.D.)	DPPH radical scavenging activity (Inhibition % ± S.D.)
CE	NA[c]	0.98 ± 0.01	83.58 ± 1.43
CW	6.58 ± 0.74	0.72 ± 0.02	64.97 ± 1.81
NIE	4.96 ± 0.74	1.23 ± 0.04	83.58 ± 1.72
NIW	22.7 ± 2.35	0.89 ± 0.01	70.68 ± 1.45
NHE	NA*	1.49 ± 0.0	87.39 ± 0.17
NHW	6.29 ± 0.82	0.83 ± 0.09	69.98 ± 0.6
References	94.75 ± 0.13[d]	1.34 ± 0.02[e]	85.51 ± 0.17[f]

[a]Standard deviation (n: 3), [b]Higher absorbance indicates higher antioxidant activity in FRAP, [c]No activity, [d]EDTA (2 mg/mL), [e]Quercetin (1 mg/mL)

2.4.2. FRAP assay

Ferric-reducing potential of the EtOH (80%) and aqueous extracts as well as quercetin (reference) was investigated using Oyaizu's method (Oyaizu, 1986) with minor modifications. The test is based on the diminishing power of the transformation of ferric ion (Fe^{3+}) into ferrous ion (Fe^{2+}), which leads to the creation of a blue complex (Fe^{2+}/TPTZ) that enhances absorbance at 700 nm. In summary, 10 µL of samples and reference in EtOH (96%) were transferred to

a 96-well microplate and pre-incubated at 50°C for 20 minutes with 25 µL phosphate buffer (pH: 6.6) and 25 µL [$K_3Fe(CN)_6$] (1%, w/v, Sigma, USA). FRAP values of the extracts are tabulated in Table 3.

2.4.3. Metal-chelating assay

For evaluating metal-chelating activity, Carter's modified approach was utilized with ethylenediaminetetetraacetic acid (EDTA, Sigma, USA) provided as reference (Carter, 1971). In sum, 20 µL of each extract and reference were incubated for 10 minutes at ambient temperatures with EtOH (96%), 2 mM $FeCl_2$ (Sigma, USA), and 5 mM ferrozine (Sigma, USA). Through the use of an ELISA microplate reader (Molecular Devices, Spectramax i3 microplate reader, USA), absorbance of the formed ferrozine-Fe^{2+} complex was measured at 562 nm. Metal-chelating activity of the extracts is presented in Table 3.

2.4.4. Data analysis for antioxidant activity assays

The subsequent DPPH radical scavenging and metal-chelating results of the extracts and references were determined and expressed in percent inhibition (I%) in accordance to the formula below:

$$\%I = \frac{A_{blank}\ A_{sample}}{A_{blank}}\ x\ 100$$

A_{blank} indicates absorbance of the control reaction (all reagents except the test sample) and A_{sample} represents absorbance of the extracts/references. The bioassays were carried out three times, and the results were expressed as means with standard deviations (S.D.).

2.5. Enzyme inhibition assays

2.5.1. ChE Inhibition assays

Inhibitory capacity of the extracts against AChE and BChE was assessed using a slightly altered version of Ellman's procedure (Ellman et al., 1961). Electric eel AChE (Type-VI-S, EC 3.1.1.7, Sigma) and equine serum BChE (EC 3.1.1.8, Sigma) were employed as enzyme sources, whilst also acetylthiocholine iodide and butrylthiocholine chloride were employed as the reaction substrates (Sigma, St. Louis, MO, USA). The activity of ChEs was determined using 5,5'-dithio-bis(2-nitrobenzoic)acid (DTNB, Sigma, St. Louis, MO, USA). Sodium phosphate buffer (0.1 mM, pH 8.0, 140 µL) was added to the 96-well microplate using a multichannel automated pipette (Eppendorf Research, Germany), followed by 20 µL of the samples/EtOH (negative control) at dilutions ranging from 25-200 µg/mL. After that, 0.2 M AChE/BChE solution (20 µL) was incorporated using a multichannel automated pipette (Gilson Pipetman, France). Having followed that, it was incubated for a total of ten minutes at room temperature. As substrates, 0.2 M acetylthiocholine iodide/butyrylthiocholine chloride (10 µL) were supplied to the 96-well microplate to initiate the reaction. Thiocholine is produced by hydrolyzing thiol esters employing AChE or BChE. The yellow byproduct of thiocholine-DTNB reaction is 2-nitro-5-thiobenzoate (TNB). The rate of production and color intensity of the product created as a result of the reaction were analyzed with an ELISA microplate reader (Molecular Devices, Spectramax i3x microplate reader, USA) at 412 nm. Galantamine hydrobromide (Sigma, USA) was utilized as a reference in both investigations. Table 4 displays AChE and BChE inhibition by the extracts.

2.5.2. Elastase inhibition assay

The spectrophotometric approach of Kraunsoe et al. (as modified by Lee et al.) was used to investigate elastase inhibition (Kraunsoe et al., 1996; Lee et al., 2009). The enzyme source was porcine pancreatic elastase (Type IV, Sigma, EC 3.4.21.36), while the substrate was *N*-Suc-(Ala)3-*p*-nitroanilide (Sigma, USA). The reaction is based on determining the quantity of nitroaniline emitted from the substrate at 410 nm. The substrate (1.015 mM) was

prepared in 0.1 M Tris-Cl (pH 8.0) buffer and blended with 10 µL for each extract dissolved in DMSO in a 96-well microplate. After pre-incubating the microplate for 5 minutes at 25°C, 15 µL 0.5 units/mL of the enzyme solution was added. After adding the enzyme, the microplate was incubated at 25°C for 30 minutes, and the quantity of *p*-nitroaniline released from the substrate was measured using an ELISA microplate reader at 410 nm. The reference and control were oleanolic acid (Sigma, USA) and DMSO, respectively. Elastase inhibition percentage of the extracts was estimated using the formula below. The assay was performed in triplicate, and the data are shown as the mean standard deviation of % inhibitions from experimental trials.

Inhibition% = 100 - [(A_1 / A_2) × 100]

In this formula, A_1 represents the absorbance of the sample solutions at 410 nm, and A_2 represents the average absorbance of the control solutions at 410 nm. Table 5 displays the elastase inhibition findings of the extracts tested.

2.5.3. Collagenase inhibition assay

The slightly modified spectrophotometric approach established by Wart and Steinbrink was used to evaluate collagenase inhibition (Barrantes & Guinea, 2003; Van Wart & Steinbrink, 1981). The enzyme source was *Clostridium histolyticum* (Sigma, EC 3.4.23.3), and the substrate was *N*-(3-[2-furyl]acryloyl)-Leu-Gly-Pro Ala (FALGPA) (Sigma, USA). The enzyme was dissolved in a tricine buffer with a pH of 7.5. FALGPA was dissolved in the same buffer at a concentration of 2 mM. Each well in a 96-well microplate was filled with 25 µL of buffer, 25 µL of DMSO or extract, and 25 µL of the enzyme. After 15 minutes of pre-incubation, FALGPA (50 µL) was added. Absorbance was measured at 340 nm in an ELISA microplate reader. The reference was (-)-epigallocatechin gallate (Sigma, USA), while the control was DMSO. Percentage collagenase inhibition of the extracts was estimated using the formula below. Each assay was performed in triplicate and the data is displayed as the mean standard deviation of % inhibitions.

Inhibition % = 100 - [(A_1 / A_2) × 100]

In the formula given above, A_1 represents the absorbance of the sample solutions at 340 nm and A_2 represents the average absorbance of the control solutions at 340 nm. Table 5 displays the collagenase inhibition findings of the samples tested.

Table 4. Cholinesterase inhibitory activities of the extracts and their IC_{50} values

Extracts	**(Inhibition % ± S.D.[a]) at 200 µg/mL[b]**	
	AChE	**BChE**
CE	7.36 ± 4.87	16.08 ± 4.98
CW	31.33 ± 4.79	12.36 ± 0.65
NIE	13.18 ± 6.07	25.67 ± 1.76
NIW	23.12 ± 5.86	80.24 ± 2.63 (IC_{50}: 38.52 ± 1.41 µg/mL)
NHE	44.60 ± 4.41	67.24 ± 3.74 (IC_{50} =157.3 ± 6.93 µg/mL)
NHW	30.14 ± 3.01	32.39 ± 2.17
Reference[c]	97.57 ± 2.59 (IC_{50}= 0.67 ± 0.02 µg/mL)	89.56 ± 0.8 (IC_{50} = 92.34 ± 5.05 µg/mL)

[a]Standard deviation (n: 4), [b]Final concentration, [c]Galantamine hydrobromide (200 µg/mL)

Table 5. Elastase and collagenase inhibitory activities of the extracts

Sample	Collagenase Inhibition (Inhibition % ± S.D.[a]) 133 µg/mL[b]	Elastase Inhibition (Inhibition % ± S.D.[a]) 133 µg/mL
CE	NA*	6.47 ± 4.4
CW	NA*	2.44 ± 2.1
NIE	NA*	1.79 ± 3.49
NIW	NA*	2.4 ± 1.5
NHE	NA*	8.3 ± 3.11
NHW	28.8 ± 1.48	NA*
Reference	80.83 ± 11.53[c]	99.65 ± 0.08[d]

[a]Standard deviation (n: 4), [b] Final concentration,*No activity, [c] Epigallocatechin gallate (1 mM), [d]Oleanolic acid (1 mM)

2.6. RP-HPLC analysis of chlorogenic acid in crude extracts

Chlorogenic acid is utilized to standardize *Helichrysum* crude extracts according to Turkish Pharmacopoeia as an approved standard. Chlorogenic acid analysis was carried out in crude extracts prepared with 80% EtOH and water using the technique defined for RP-HPLC (Gök et al., 2022). Each extract solution was prepared with 25% (v/v) acetonitrile at a concentration of 1 mg/mL and transferred to vials through membrane filters. Chlorogenic acid (Sigma, USA) was prepared at various concentrations using 25% aqueous acetonitrile, including 100, 50, 20, 5, and 1 ppm. An HP Agilent 1260 series LC System and an ACE_5 C18 (5 m, 150 mm x 4.6 mm) column with a diode array detector (DAD) was employed for the analysis. Temperature inside the column was adjusted to 25°C. Solvent A (acetonitrile:H_2O:formic acid/80:20:0.1) and solvent B (H_2O:formic acid/100:0.5) were used as the mobile phase. A gradient method has been used to separate the peaks appropriately,. The flow chart was adjusted as follows; from 0-minute 5% A, to 10 minutes 15% A, to 17 minutes 15% A, to 22 minutes 20% A, to 32 minutes 30% A, to 50 minutes 100% A, to 53 minutes 100% A. Duration of the analysis was set at 53 minutes. Injection volume was 20 µL, and the flow rate was 0.8 mL/min. The calibration equation and correlation coefficient for chlorogenic acid were determined as y = 124.09x - 9.988 and r2 = 0.9999, respectively. Table 6 and Figure 1 demonstrate chlorogenic acid quantities in each extract.

Table 6. Amount (%, w/w) of chlorogenic acid in the extracts

Extracts	Amount (%, w/w)
CE	1.14 ± 0.000
CW	0.15 ± 0.000
NIE	0.81 ± 0.000
NIW	1.36 ± 0.000
NHE	1.95 ± 0.000
NHW	1.22 ± 0.000

3. Results and discussion

3.1. Enzyme inhibition findings

AChE and BChE inhibitory activities of aqueous and EtOH extracts prepared from *H. stoechas* subsp. *barrelieri* (Izmir and Hatay samples) and *H. stoechas* (Izmir sample) are shown in Table 4. The outcomes revealed that AChE inhibitory activity was low in the extracts, whereas the highest activity was found in EtOH extract of the plant collected from Hatay (NHE) with moderate inhibitory activity (44.60 ± 4.41 %). The inhibitory activity was nearly half that of galantamine, the reference (97.57 ± 2.59 %). It was determined that the most effective extract against BChE was the aqueous extract of the plant collected from Izmir (NIW, IC_{50}: 38.52±1.41 µg/mL), and the extract demonstrated more activity than galantamine (IC_{50}: 92.34 ± 5.05 µg/mL). In addition, NHE was also found to exhibit a high inhibition (IC_{50}: 157.3 ± 6.93 µg/mL) against BChE.

Collagenase and elastase inhibitory activity results of the extracts indicated that only water extract of the plant collected from Hatay (NHW) extract inhibited collagenase moderately. The other extracts possessed low or no inhibition against both of the enzymes (Table 5).

3.2. Antioxidant activity results

Antioxidant activity of the extracts was analyzed *via* determination of metal-chelating capacity, FRAP, and DPPH radical scavenging activity methods (Table 3). The extracts did not exert remarkable activity in metal-chelating capacity and FRAP assays. Only the NIW extract had a moderate level of metal-chelating capacity (22.7 ± 2.35 %). On the other hand, all of the extracts demonstrated high DPPH radical scavenging activity from 64.97 ± 1.81 % to 87.39 ± 0.17 %. Especially, the EtOH extracts exerted high activity nearly as reference quercetin (85.51 ± 0.17 %).

3.3. Total phenol and flavonoid content in the extracts

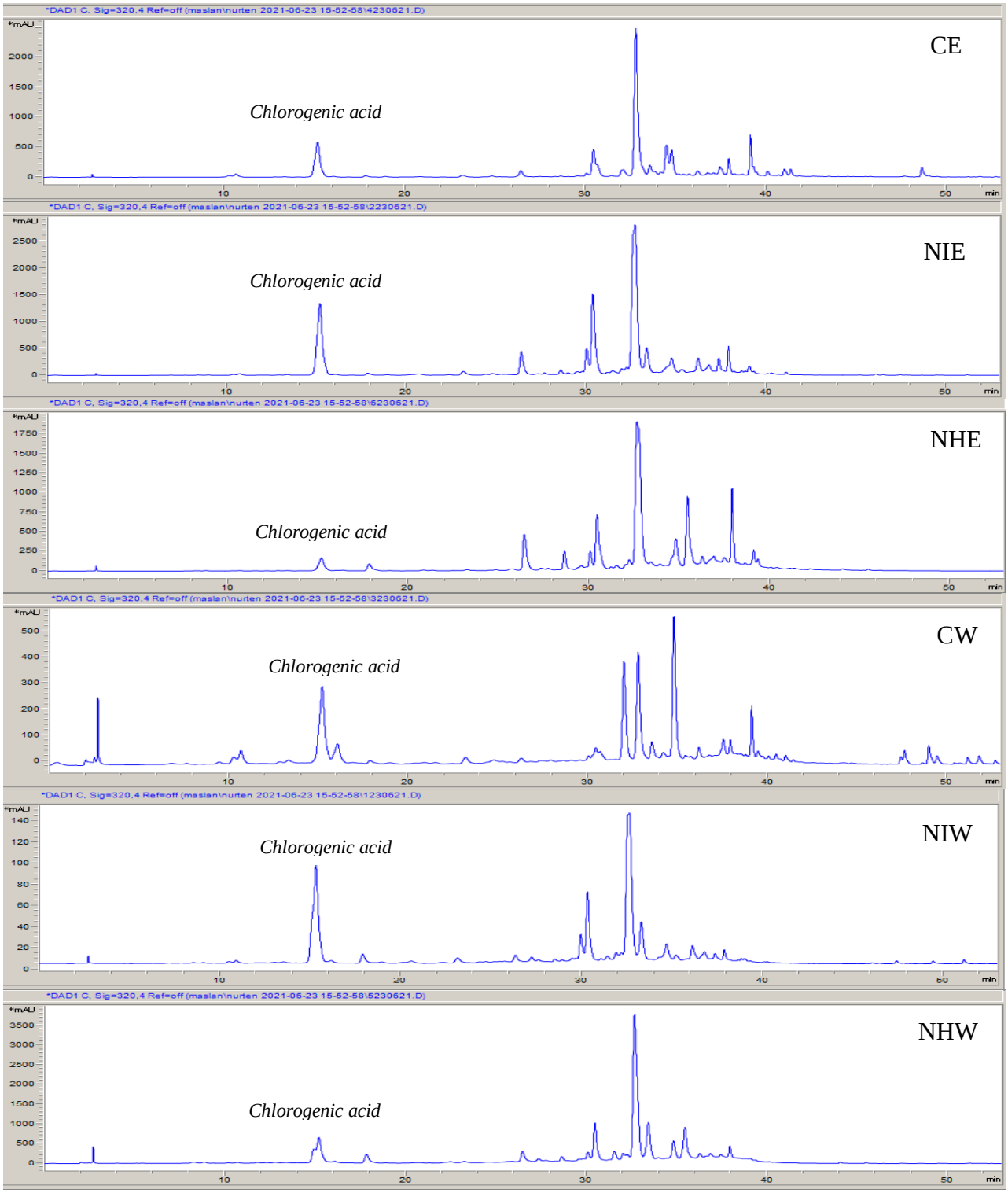
Total phenol and flavonoid contents of the extracts were analyzed spectrophotometrically and expressed in mg equivalent of gallic acid and quercetin, respectively (Table 2). The results demonstrated that the total phenolic and flavonoid contents in the EtOH extracts were found to be higher than those of the aqueous extracts. The highest total phenolic content was found in the NHE extract (141.55 ± 2.93 mg/g gallic acid equivalent, GAE), while the highest total flavonoid content was found in the EtOH extract of the cultivated plant in Izmir (CE) extract (91.87 ± 0.81 mg/g quercetin equivalent, QE). The results were found to be consistent with FRAP and DPPH activity results. The extract with the highest phenolic content was NHE which displayed the highest FRAP (1.49 ± 0.0) and DPPH (87.39 ± 0.17 %) activity. Also, the extracts demonstrated AChE, elastase, and prominent BChE inhibitory activity.

3.4. Quantification of chlorogenic acid in the extract using RP-HPLC

Chlorogenic acid as one of the major compounds in *H. stoechas* flowers (capitula) was determined in the extracts by using HPLC-DAD (Table 6). As aforementioned, the compound is also used for the standardization of *H. stoechas* subsp. *barrelieri* and *H. plicatum* subsp. *plicatum* flower monographs in Turkish Pharmacopoeia (Pharmacopoeia, 2019). The equation of the chlorogenic acid calibration curve was y = 124.09x – 9.988 (r^2: 0.9999) and the test range was 1-200 ppm. The results indicated that the highest chlorogenic acid content was also found in NHE extract (1.95 ± 0.000 %, Figure 1). It might also be related to the highest AChE, BChE, and elastase inhibitory activity of the extract.

In a study, AChE inhibitory activity of the flower and stem/leaf water extracts of *H. stoechas* was analyzed, and their chemical profiles were detected by using liquid chromatography-mass spectrometry (LC-MS/MS) and HPLC-DAD (Silva et al., 2017). The extracts inhibited AChE with IC_{50} values of 260.7 and 654.8 µg/mL, respectively. The phytochemical analysis revealed presence of chlorogenic acid, myricetin-3-*O*-glucoside, cynarin, dicaffeoylquinic acid, dicaffeoylquinic acid, and arzanol in both of the extracts, whereas malonyl-dicaffeoylquinic acid was found only in the stem/leaf water extracts. Molecular docking studies pointed out that arzanol, chlorogenic acid, and cynarin was compatible with the anti-AChE active site channel and prohibited all access to the catalytic triad. In a similar study, enzyme inhibitory, antioxidant activities, total phenolic and flavonoid contents, and phytochemical analysis using UHPLC/MS of five extracts of *H. stoechas* subsp. *barrelieri* obtained by different extraction techniques were analyzed (Zengin et al., 2020).

Figure 1. HPLC-DAD chromatograms of the extracts



The results demonstrated that the extracts were able to inhibit AChE in a range from 3.56 ± 0.04 to 4.23 ± 0.07 as galantamine equivalent (GAE), BChE from 5.42 ± 0.01 to 6.05 ± 0.03 (GAE), α-glucosidase from 1.59 ± 0.01 to 1.66 ± 0.01 acarbose equivalent (ACE), α-amylase from 0.46 ± 0.01 to 0.63 ± 0.02 ACE and tyrosinase from 174.50 ± 2.71 to 183.32 ± 0.78 kojic acid equivalent (KAE). Besides, the extracts exhibited antioxidant

activity to some extent *via* DPPH in a range from 90.05 ± 0.92 to 219.92 ± 3.21 mg trolox equivalent (TE)/g, ABTS radical scavenging activities from 138.68 ± 1.20 to 313.12 ± 8.42 mg TE/g, FRAP from 285.14 ± 5.25 to 662.87 ± 20.41 mg TE/g, cupric-reducing antioxidant capacity (CUPRAC) from 335.97 ± 9.89 to 927.39 ± 11.19 mg TE/g, metal-chelating capacity from 2.91 ± 0.43 to 17.11 ± 0.69 mg EDTA equivalent/g, and phosphomolybdenum assay from 1.65 ± 0.07 to 2.27 ± 0.20 mmol TE/g. In ultraperformance liquid chromatography-mass spectrometric (UHPLC/MS) characterization, a total of 107 compounds was identified, 40 of which were found as hydroxycinnamic acid derivatives, while 50 of them were flavonoids. Quercetin, 5-O-caffeoyl-quinic acid, and *p*-hydroxybenzoic acid were reported as the major molecules in highest amount.

Except *H. stoechas*, various *Helichrysum* species also demonstrated ChE inhibitory and antioxidant activities in previous studies. For instance; *H. pallasii* inhibited AChE (IC_{50}: 1.49 mg/mL), BChE (IC_{50}: 1.98 mg/mL), α-amylase (IC_{50}: 2.09 mg/mL), α-glucosidase (IC_{50}: 0.51 mg/mL), tyrosinase (IC_{50}: 0.68 mg/mL), and pancreatic lipase (IC_{50}: 42.5 µg/mL) (Nejmi et al., 2023). Furthermore, antioxidant activity of *H. pallasii* was shown by FRAP (2205 µmol Fe^{2+}/gE), oxygen radical scavenging (ORAC) capacities (2540 µmol Trolox Eq./gE), DPPH (IC_{50}: 0.58 mg/mL), CUPRAC (IC_{50}: 0.37 mg/mL), phosphomolybdenum (IC_{50}: 1.34 mg/mL), and metal-chelation (IC_{50}: 1.42 mg/mL) methods. In another study, *H. plicatum* inhibited AChE by 41.15 ± 1.68 % at 500 µg/mL concentration. At 200 and 100 µg/mL, it is shown similar results, which was indicative of dose-independent activity (Jovanović et al., 2020). In another study, methanol extract from *H. plicatum* DC. subsp. *plicatum* inhibited carbonic anhydrase I (hCAI), hCAII, AChE (IC_{50}: 115.50 mg/mL), BChE (IC_{50}: 117.46 mg/mL), and α-glycosidase (IC_{50}: 81.53 mg/mL) (Aydin, 2020). The study also described isolation of apigenin, β-sitosterol, β-sitosterol-3-*O*-β-D-glucopyranoside, helichrysins A and B, isosalipurposide, and nonacosanoic acid. All of the compounds inhibited AChE and BChE, ranging between IC_{50} values of 1.69–2.90 and 1.09–3.89 µM, respectively. AChE and BChE inhibitory activity of some other species, *e.g. H. chionophilum* and *H. plicatum*, was demonstrated in another report (Acet et al., 2020). In particular, EtOH and ethyl acetate extracts of the flower and stem of *H. chionophilum* and *H. plicatum* inhibited AChE and BChE at moderate levels, where their BChE inhibitory activity was found higher than their AChE inhibition, which is consistent with our data. In the study conducted by Taşkın et al. (Taşkın et al., 2020), anti-ChE, anti-inflammatory, antioxidant, antimicrobial, and anti-urease activities of methanol extracts of *H. plicatum* subsp. *plicatum* by Soxhlet extraction and conventional maceration techniques were investigated. Furthermore, phytochemical analysis of the methanol extracts obtained by maceration and Soxhlet using HPLC-DAD and LC-MS/MS presented 42.10±0.79 % and 58.51±0.85 % of AChE inhibitory activity at 500 µg/mL concentration, which later led to identification of chlorogenic acid, dicaffeoylquinic acid, luteolin, luteolin-7-*O*-glucoside, naringenin-O-hexoside, and isoquercitrin. ChE inhibitory activity of chlorogenic acid described as one of the major molecules in genus *Helichrysum* was demonstrated in former studies. Chlorogenic acid inhibited AChE and BChE with IC_{50} values of 8.01 ± 0.01 and 6.30 ± 0.02 µg/mL, respectively (Oboh et al., 2013), whereas, in another study, it was able to inhibit AChE only IC_{50} value of 98.17 µg/mL (Oboh et al., 2013).

Our literature survey indicated no any previous study relevant to the effect of *H. stoechas* on elastase and collagenase. However, elastase and collagenase inhibitory activity of leaf EtOH extract of *H. petiolare* was weak (Sagbo & Otang-Mbeng, 2020). The

essential oil of aerial parts of *H. italicum* subsp. *italicum* inhibited collagenase and elastase activities with IC_{50} values of 36.99 ± 1.52 and 135.43 ± 6.32 µg/mL, respectively (Fraternale et al., 2019).

4. Conclusion

Our present study revealed water and EtOH (80%) extracts prepared from *H. stoechas* subsp. *barrelieri* capitula growing naturally in two localities (Hatay and Izmir), and water and EtOH (80%) extracts prepared from the capitula of cultivated *H. stoechas* sample inhibited AChE weak to moderate levels. Aqueous extract of the plant collected from Izmir (NIW) and EtOH extract of the plant collected from Hatay (NHE) extracts inhibited BChE strongly with 38.52 ± 1.41 and 157.3 ± 6.93 µg/mL IC_{50} values. The activity of NIW was more than galantamine, the reference drug. The extracts exhibited potent DPPH radical scavenging and FRAP activities; but the metal-chelating capacity was weak except NIW extract (22.7 ± 2.35 %). In addition, amount of chlorogenic acid in the extracts was determined and the highest chlorogenic acid was found in NHE and then in NIW. The findings suggested that chlorogenic acid may be one of the substances responsible for the tested activities.

The current study discloses the first outcomes on AChE, BChE, elastase, and collagenase inhibitory activities and antioxidant data of the capitulum extracts from *H. stoeachas* and *H. stoechas* subsp. *barrelieri* from Türkiye as well as their chlorogenic acid quantification.

Conflict of interest

The authors declare no conflict of interest.

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