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Neurobiological evaluation of thirty-one medicinal plant extracts using microtiter enzyme assays

Ilkay Erdogan Orhan[1*], Fatma Sezer Senol[1], Mehmet Zeki Haznedaroglu[2], Halil Koyu[2], Sinem Aslan Erdem[3], Gulderen Yılmaz[4], Mehmet Cicek[5], A. Emre Yaprak[6], Esin Ari[7], Nurgun Kucukboyaci[1] and Gulnur Toker[1]

Abstract

Background: Inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) related to Alzheimer's disease as well as tyrosinase (TYR) relevant to Parkinson's disease is an important approach to find novel drug candidates for these diseases.

Methods: The extracts from fourteen plant species in various polarities were subjected to high-throughput screening against acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and tyrosinase (TYR), the key enzymes related to Alzheimer's and Parkinson's diseases. The extracts were subjected to the microtiter enzyme inhibition assays at 100 μg mL^{-1}. Antioxidant effect of the extracts was tested for their scavenging activity against DPPH, DMPD, and NO radicals as well as their ferric- (FRAP) and phosphomolibdenum-reducing power (PRAP) and metal-chelation capacity. Total phenol and flavonoid quantities in the extracts were determined spectrophotometrically. HPLC analysis was performed on *Atriplex lasiantha*, *Conringia grandiflora*, and *Vaccaria hispanica*.

Results: The active extracts inhibiting AChE over 50 % were *Centaurium erythraea* subsp. *rhodense* (51.33 ± 3.35 %) and *Posidonia oceanica* (61.88 ± 2.23 %), while BChE was inhibited most effectively by the root extract of *P. oceanica* (82.55 ± 2.14 %), followed by *Origanum haussknechtii* (66.88 ± 0.17 %), which also had the highest inhibition toward TYR (35.28 ± 1.90 %). The extracts from *Zostera noltii*, *P. oceanica*, and *Ricotia carnosula* possessed the best DPPH scavenging activity, whereas *Z. noltii* caused the highest NO scavenging activity (70.19 ± 0.43 %) and FRAP (1.326 ± 0.065). *Atriplex lasiantha* and *Ecballium elaterium* had the strongest effect in PRAP and metal-chelation assays, respectively. Besides, *A. lasiantha* was found to be a rich source of rutin.

Conclusion: Among the screened plants, *Centaurium erythraea* subsp. *rhodense* and *Origanum haussknechtii*, and the roots of *Posidonia oceanica* seems to deserve further investigation for their neuroprotective potential.

Keywords: Medicinal plant, Neurobiology, Alzheimer's disease, Enzyme inhibition, Cholinesterase, Tyrosinase

Background

Alzheimer's disease (AD) is a progressive neurodegenerative disorder affecting senior individuals and their families. The evidence has proven that pathophysiology of the disease is quite complex with a multifactorial nature including inflammation, oxidative stress, abnormal protein formation, neurotransmitter deficits [1]. Low levels of acetylcholine (ACh), vital for cerebral functions related to memory, have been found in the brains of AD patients, while butyrylcholinesterase (BChE, EC 3.1.1.8), known as pseudocholinesterase or acylcholine acylhydrolase, may also hydrolyze most of the choline esters including Ach, which is mainly hydrolyzed by acetylcholinesterase (AChE, EC 3.1.1.7). Thus, inhibition of cholinesterases has been an attractive target for treatment of AD [2] and cholinesterase inhibitors have become the extensively used therapeutic tools towards AD.

On the other hand, tyrosinase (TYR; polyphenol oxidase or oxygen oxidoreductase, EC 1.14.18.1) catalyzes the rate-limiting oxidation of tyrosine to melanin, which plays a critical role in pigmentation of skin related to melanoma,

* Correspondence: iorhan@gazi.edu.tr
[1]Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, 06330 Ankara, Turkey
Full list of author information is available at the end of the article

undesirable browning of fruits and vegetables, molting process of insects, and dopamine toxicity in Parkinson's disease (PD) as well as neuronal death in PD and Huntington's disease [3, 4]. Therefore, inhibition of TYR is a new target and quite important for treatment of the aforementioned diseases.

As well-known, screening of medicinal plants and natural products for their pharmacological activities desired for human health is an essential step for discovery of novel drug candidates. Since our extensive studies on discovery of new cholinesterase and TYR inhibitors from herbal sources have been conducted since the year of 2000, the current stage, we have aimed to screen randomly selected fourteen plant species [*Atriplex lasiantha* Boiss. (AL), *Ecballium elaterium* (L.) A. Rich. (EE), *Centaurium erythraea* subsp. *rhodense* (Boiss & Reut.) Melderis (CER), *Centaurium erythraea* subsp. *turcicum* (Velen.) Melderis (CET), *Centaurium maritimum* (L.) Fritsch (CM), *Centaurium spicatum* L., *Centaurium tenuiflorum* (Hoffmans. & Link) Fritsch, *Ricotia carnosula* Boiss. & Heldr. (RC), *Conringia grandiflora* Boiss. & Heldr. (CG), *Vaccaria hispanica* (Mill.) Rauschert (VH), *Origanum haussknechtii* Boiss. (OH), *Zostera noltii* Hornem. (ZN), *Zostera marina* L. (ZM), and *Posidonia oceanica* (L.) Delile (PO) with medicinal and industrial importance for their AChE, BChE, and TYR inhibitory activities by ELISA microtiter assays at 100 μg mL^{-1}. Among the plant species screened, *Atriplex* species in Bulgaria [5], *Ecballium elaterium* (squirting cucumber) in Italy [6], and *Centaurium* species [7] in southern Italy are consumed as food, while *Ricotia carnosula* and *Conringia grandiflora*, the endemic species to Anatolia, along with *Vaccaria hispanica* (cow cockle) have been recently cultivated to be used as ingredients in nutraceutical industry in our country and these three species have also potential to be used for ornamental purposes. The marine seagrass (or Neptun grass), *Posidonia oceanica*, is an endemic species to the Mediterraneaen Sea, which forms wide meadows underwater, while *Zostera* species (eelgrass) forms dense sea grass beds. All these three marine species are considered to be ecologically imperative species making critical habitat and hunting zones for plentiful fish and invertebrates.

Since oxidative stress, free radical formation, and metal accumulation are strongly associated with pathogenesis of neurodegenerative diseases including AD and PD [8], the extracts obtained from the plants mentioned above were subjected to several antioxidant assays adapted to high-throughput screening methods using microtiter plates.

Methods

Plant materials

The plant species screened in the present study were collected throughout Turkey between 2008-2012 as listed in Table 1. Among the species used herein, the voucher specimens of the *Centaurium* species were kept at Herbarium of Faculty of Pharmacy, Ankara University (Ankara, Turkey), while *Ecballium elaterium*, *Atriplex lasiantha*, and *Origanum haussknechtii* were deposited at Herbarium of Faculty of Pharmacy, Gazi University (Ankara, Turkey). The voucher specimens of *Ricotia carnosula*, *Conringia grandiflora*, and *Vaccaria hispanica* were preserved at the Herbarium of Akdeniz University (Antalya, Turkey), while the samples of the segrass species; *Zostera noltii*, *Zostera marina*, and *Posidonia oceanica* were deposited at the Herbarium of Faculty of Pharmacy, Ege University (Izmir, Turkey). All voucher specimens are available upon request for those who would like to see them.

Extraction procedure

The corresponding parts of the plants screened were dried in shade at room temperature and powdered to a fine grade by using a laboratory scale mill. The corresponding solvents for each species are given in Table 2 [dichloromethane (DCM), ethyl acetate (EtOAc), ethanol (80 %) (EtOH), and methanol (MeOH)]. Each plant species was extracted with its corresponding solvents by maceration, and following filtration, the combined extracts were evaporated to dryness *in vacuo* to give the crude extracts. Preparation of the aqueous extract of OH was as follows: 5 g of the powdered plant material were boiled with 100 mL of distilled water for 30 min. The aqueous extract was filtered when hot, and, then the resultant extract was lyophilized.

Microtiter assays for enzyme inhibition

AChE and BChE inhibitory activity assays

AChE and BChE inhibitory activity of the samples was determined by modified spectrophotometric method of Ellman et al. [9]. Electric eel acetylcholinesterase (Type-VI-S, EC 3.1.1.7, Sigma) and horse serum butyrylcholinesterase (EC 3.1.1.8, Sigma) were used as the enzyme sources, while acetylthiocholine iodide and butyrylthiocholine chloride (Sigma, St. Louis, MO, USA) were employed as substrates of the reaction. 5,5'-Dithio-bis(2-nitrobenzoic)acid (DTNB, Sigma, St. Louis, MO, USA) was used for the measurement of the cholinesterase activity. All the other reagents and conditions were the same as described in our previous publication [10]. In brief, 140 μL of 0.1 mM sodium phosphate buffer (pH 8.0), 20 μL of 0.2 M DTNB, 20 μL of sample solutions and 20 μL of 0.2 M acetylcholinesterase/butyrylcholinesterase solution were added by multichannel automatic pipette (Gilson pipetman, France) in a 96-well microplate and incubated for 15 min at 25 °C. The reaction was then initiated with the addition of 10 μL of 0.2 M acetylthiocholine iodide/butyrylthiocholine chloride. The hydrolysis of

Table 1 Families, collection places, years, and botanical authenticator of the plant species

Species	Family	Collection site	Collection year	Botanical authentication by
AL	Chenopodiaceae	Ankara	2011	Hayri Duman (Gazi Univ.)
EE	Cucurbitaceae	Ankara	2011	Gulnur Toker (Gazi Univ.)
CER	Gentianaceae	Marmaris, Mugla	2010	Mehmet Cicek (Pamukkale Univ.)
CET	Gentianaceae	Acipayam, Denizli	2010	
CM	Gentianaceae	Izmir	2011	
CS	Gentianaceae	Dalyan, Mugla	2010	
CT	Gentianaceae	Demre, Antalya	2010	
RC	Brassicaceae	Antalya	2012	Esin Ari (Akdeniz Univ.)
CG	Brassicaceae	Antalya	2012	
VH	Caryophyllaceae	Antalya	2012	
OH	Lamiaceae	Kemaliye, Erzincan	2008	Mecit Vural (Gazi Univ.)
ZN	Zosteraceae	Urla, Izmir (Agean Sea)	2012	M. Zeki Haznedaroglu (Ege Univ.)
ZM	Zosteraceae	Urla, Izmir (Agean Sea)	2012	
PO	Posidoniaceae	Urla, Izmir (Agean Sea)	2012	

acetylthiocholine iodide/butyrylthiocholine chloride was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at a wavelength of 412 nm utilizing a 96-well microplate reader (VersaMax, Molecular Devices, USA). Galanthamine, the anticholinesterase alkaloid-type of drug isolated from the bulbs of snowdrop (*Galanthus* sp.), was purchased from Sigma (St. Louis, MO, USA) and was employed as reference.

TYR inhibitory activity assay

Inhibition of TYR (EC 1.14.1.8.1, 30 U, mushroom tyrosinase, Sigma) was determined using the modified dopachrome method with L-DOPA as substrate [11]. Assays were conducted in a 96-well microplate using ELISA microplate reader (VersaMax Molecular Devices, USA) and absorbance was measured at 475 nm. An aliquot of the samples dissolved in DMSO with 80 μL of phosphate buffer (pH 6.8), 40 μL of tyrosinase, and 40 μL of L-DOPA was put in each well. Results were compared with control (DMSO) and α-kojic acid (Sigma, St. Louis, MO, USA) employed as the reference for this assay.

Data processing for enzyme inhibition assays

The measurements and calculations were evaluated by using Softmax PRO 4.3.2.LS software. Percentage inhibition of AChE/BChE and TYR was determined by comparison of rates of reaction of test samples relative to blank sample (ethanol in phosphate buffer pH = 8 for AChE/BChE and DMSO for TYR). Extent of the enzymatic reaction was calculated based on the following equation: $E = (C\text{-}T)/C \times 100$, where E is the activity of the enzyme. E value expresses the effect of the test sample or the positive control on the enzyme activity articulated as the percentage of the remaining activity in the presence of test sample or positive control. C value is the absorbance of the control solvent (blank) in the presence of enzyme, where T is the absorbance of the tested sample (plant extract or positive control in the solvent) in the presence of enzyme.

Data are expressed as average inhibition ± standard error mean (S.E.M.) and the results were taken from at least three independent experiments performed in triplicate.

Antioxidant activity assays

DPPH radical scavenging assay

The hydrogen atom or electron donation capacity of the corresponding samples was computed from the bleaching property of the purple-colored methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). The stable DPPH radical scavenging activity of the extracts was determined by the method of Blois [12]. The samples (2700 μL) dissolved in ethanol (75 %) were mixed with 300 μL of DPPH solution (1.5×10^{-4} M). Remaining DPPH amount was measured at 520 nm using a Unico 4802 UV-visible double beam spectrophotometer (Dayton, NJ, USA). The results were compared to that of gallic acid employed as the reference.

DMPD radical scavenging assay

Principal of the assay is based on reduction of the purple-colored radical $DMPD^+$ (*N*,*N*-dimethyl-*p*-phenylendiamine) [13]. According to the method, a reagent comprising of 100 mM DMPD, 0.1 M acetate buffer (pH = 5.25), and 0.05 M ferric chloride solution, which led to formation of DMPD radical, was freshly prepared and the reagent was equilibrated to an absorbance of 0.900 ± 0.100 at 505 nm. Then, the reagent (1000 μL) was mixed up

Table 2 AChE, BChE, and TYR inhibitory effects of the extracts at 100 μg mL^{-1}

Species	Plant part	Extract type	Inhibitory effect of the extracts (% ± S.E.M.[a])		
			AChE	BChE	TYR
AL[b]	Fruit	DCM	-[c]	14.23 ± 1.22****	26.55 ± 1.42****
		EtOAc	-	7.56 ± 2.00****	27.21 ± 2.55****
		EtOH	-	-	24.23 ± 1.33****
AL[b]	Aerial	DCM	-	-	21.78 ± 0.91****
		EtOAc	-	-	22.12 ± 0.09****
		EtOH	-	10.19 ± 0.56****	27.30 ± 2.20****
EE	Fruit	Juice	-	2.15 ± 0.78****	1.33 ± 0.68****
	Fruit	EtOH	-	15.90 ± 1.93****	6.18 ± 2.39****
	Aerial	EtOH	-	8.16 ± 1.80****	-
	Root	EtOH	1.71 ± 0.42****	15.98 ± 2.94****	4.92 ± 0.77****
CER	Aerial	EtOAc	51.33 ± 3.35**	15.21 ± 1.32****	8.00 ± 1.65****
	Aerial	MeOH	-	10.42 ± 1.43****	4.38 ± 0.68****
CET	Aerial	EtOAc	-	11.52 ± 3.19****	2.24 ± 0.15****
	Aerial	MeOH	-	11.55 ± 3.27****	1.52 ± 0.14****
CM	Aerial	EtOAc	-	13.19 ± 1.26****	-
	Aerial	MeOH	-	14.48 ± 0.24****	6.24 ± 1.15****
CS	Aerial	EtOAc	4.20 ± 0.04****	10.05 ± 1.46****	-
	Aerial	MeOH	6.55 ± 1.90****	9.85 ± 1.84****	15.10 ± 0.30****
CT	Aerial	EtOAc	12.69 ± 1.70****	13.03 ± 1.64****	-
	Aerial	MeOH	14.67 ± 1.80****	10.32 ± 1.85****	22.14 ± 2.62****
RC (wild)	Aerial	EtOH	-	-	4.36 ± 1.23****
RC (cultivated)	Aerial	EtOH	-	-	-
CG	Aerial	EtOH	-	-	-
VH	Aerial	EtOH	-	-	3.67 ± 0.42****
OH[b]	Aerial	MeOH	22.36 ± 2.93****	66.88 ± 0.17**	35.28 ± 1.90****
	Aerial	Aqueous	-	-	-
ZN	Aerial	EtOH	-	-	-
ZM	Leaf	EtOH	-	5.39 ± 0.40****	6.34 ± 1.21****
PO	Leaf	EtOH	10.03 ± 3.00****	3.18 ± 1.65****	-
	Rhizome	EtOH	15.77 ± 4.68****	25.82 ± 1.32****	5.69 ± 1.35****
	Root	EtOH	61.88 ± 2.23**	82.55 ± 2.14**	17.04 ± 1.31****
Galanthamine[d]			92.13 ± 1.47	89.95 ± 0.87	
Alpha-kojic acid[e]					87.62 ± 0.23

[a]Standard error mean ($n = 3$)
[b]Tested at 200 μg mL^{-1}
[c]No inhibitory activity
[d]Reference for AChE and BChE inhibitory effect at 100 μg mL^{-1}
[e] Reference for TYR inhibitory effect at 200 μg mL^{-1}
[*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$]

with 50 μL of the sample dilutions dissolved in ethanol (75 %) and absorbance was taken at 505 nm using a Unico 4802 UV-visible double beam spectrophotometer (USA). Quercetin was employed as the reference and the experiments were done in triplicate.

Nitric oxide (NO) radical scavenging activity

The scavenging activity of the samples against NO was assessed by the method of Marcocci et al. [14]. Briefly, the extract dilutions were mixed with 5 mM sodium nitroprusside and left to incubation for 2 h at 29 °C. An

aliquot of the solution was removed and diluted with Griess reagent (1 % sulfanilamide in 5 % H_3PO_4 and 0.1 % naphthylethylenediamine dihydrochloride). Absorbance of the occurred chromophore was measured at 550 nm using a Unico 4802 UV-visible double beam spectrophotometer (USA).

Fe^{+2}-ferrozine test system for metal-chelation

The metal-chelating effect of the samples by Fe^{+2}-ferrozine test system was estimated in consistent with Chua et al.'s method [15]. Accordingly, 740 μL of ethanol and 200 μL of the samples dissolved in ethanol (75 %) were incubated with 2 mM $FeCl_2$ solution. The reaction was initiated by the addition of 40 μL of 5 mM ferrozine solution into the mixture, shaken vigorously, and left standing at ambient temperature for 10 min. The absorbance of the reaction mixture was measured at 562 nm. The ratio of inhibition of ferrozine-Fe^{2+} complex formation was calculated as given in "*Data processing for antioxidant activity assays*" and ethylenediaminetetraacetic acid (EDTA) was employed as the reference in this assay.

Ferric-reducing antioxidant power (FRAP) assay

The ferric-reducing antioxidant power (FRAP) of the extracts and reference was tested using the assay of Oyaizu [16] based on the chemical reaction of Fe(III) = > Fe(II). Different concentrations of the extracts dissolved in ethanol (75 %) were added into 2500 μL of phosphate buffer (pH 6.6) and 2500 μL of potassium ferricyanide [$K_3Fe(CN)_6$] (1 %, w/v). Later, the mixture was incubated at 50 °C for 20 min and then 2500 μL of trichloroacetic acid (10 %) was added. After the mixture was shaken vigorously, this solution was mixed with 2500 μL of distilled water and $FeCl_3$ (100 μL, 0.1 %, w/v). After 30 min incubation, absorbance was read at 700 nm using a Unico 4802 UV-visible double beam spectrophotometer (Dayton, NJ, USA). Analyses were achieved in triplicate. Chlorogenic acid was the reference in this assay.

Phosphomolibdenum-reducing antioxidant power (PRAP) assay

In order to perform PRAP assays on the extracts, each dilution of the samples was mixed with 10 % phosphomolybdic acid solution in ethanol (w/v) [17]. The solution was subsequently subjected to incubation at 80 °C for 30 min and the absorbance was read at 600 nm using a Unico 4802 UV-visible double beam spectrophotometer (USA) and compared to that of quercetin as the reference.

Data processing for antioxidant activity assays

Inhibition of DPPH, DMPD, and nitric oxide (NO) radicals and metal-chelation capacity was calculated as given below and the results were expressed as percent inhibition (I%):

$I\% = [(A_{blank}\text{-}A_{sample})/A_{blank}] \times 100$, where A_{blank} is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is the absorbance of the extracts. Analyses were run in triplicate and the results were expressed as average values with S.E.M. (Standard error of the mean). For FRAP and PRAP assays, the analyses were also achieved in triplicate and increased absorbance of the reaction meant increased reducing power in both assays.

Statistical analysis of data

Data obtained from in vitro enzyme inhibition and antioxidant experiments were expressed as the mean standard error (±SEM). Statistical differences between the reference and the sample groups were evaluated by ANOVA (one way). Dunnett's multiple comparison tests were used as *post hoc* tests. $p < 0.05$ was considered to be significant [$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, $^{****}p < 0.0001$].

Determination of total phenol and flavonoid contents in the extracts

Phenolic amount of the extracts was determined in accordance with Folin-Ciocalteau's method [18]. In brief, a number of dilutions of gallic acid dissolved in ethanol (75 %) were obtained to prepare a calibration curve. The extracts and gallic acid dilutions were mixed with 750 μL of Folin-Ciocalteau's reagent and 600 μL of sodium carbonate in test tubes. The tubes were then vortexed and incubated at 40 °C for 30 min. afterward, absorption was measured at 760 nm at a Unico 4802 UV-visible double beam spectrophotometer (USA). Total flavonoid content of the extracts was calculated by aluminum chloride colorimetric method [19]. To sum up, a number of dilutions of quercetin dissolved in ethanol (75 %) were obtained to prepare a calibration curve. Then, the extracts and quercetin dilutions were mixed with 95 % ethanol, aluminum chloride reagent, 100 μL of sodium acetate as well as distilled water. Following incubation for 30 min at room temperature, absorbance of the reaction mixtures was measured at wavelength of 415 nm with a Unico 4802 UV-visible double beam spectrophotometer (USA). The total phenol and flavonoid contents of the extracts were expressed as gallic acid and quercetin equivalents (mg g^{-1} extract), respectively.

Quantification of rutin in the extracts of AL, CG, and VH by HPLC

Analyses were performed using an Agilent Technologies 1200 Series high pressure liquid chromatography (HPLC), including a binary pump, a vacuum degasser, an autosampler, and a diode array detector. Chromatographic separations were performed on ACE-5-C18 column (150 mm ×

4.6 mm, 5 μm). A mobile phase consisting of two eluents (acetonitrile and 40 mM formic acid) was used for separation with a gradient elution at a flow rate of 1 mL min^{-1}. All solvents were filtered through a 0.45 μm Millipore filter before use. Detection wavelength was set at 254 nm for rutin and 330 nm for vitexin. The injection volume was 20 μL for each sample and standard solutions. Identification of rutin and vitexin was made by comparing their

Table 3 Radical scavenging activity of the extracts against DPPH, DMPD, and NO at 1000 μg mL^{-1}

Species	Plant part	Extract type	Radical scavenging effect (% ± S.E.M.[a])		
			DPPH	DMPD	**NO**
AL[b]	Fruit	DCM	-[b]	-	33.74 ± 2.70****
		EtOAc	-	-	51.65 ± 4.80***
		EtOH	9.51 ± 1.32****	9.51 ± 1.32****	47.96 ± 3.25****
	Aerial	DCM	-	-	24.49 ± 2.28****
		EtOAc	3.25 ± 0.35****	3.25 ± 0.35****	46.01 ± 1.84****
		EtOH	10.88 ± 1.49****	10.88 ± 1.49****	48.16 ± 4.66***
EE	Fruit	Juice	5.98 ± 0.60****	15.18 ± 3.07****	34.05 ± 0.53****
	Fruit	EtOH	12.21 ± 2.34****	-	40.26 ± 2.21****
	Aerial	EtOH	8.37 ± 2.81****	-	36.17 ± 2.05****
	Root	EtOH	5.67 ± 2.44****	-	21.19 ± 2.52****
CER	Aerial	EtOAc	6.02 ± 0.18****	-	19.76 ± 0.85****
		MeOH	11.96 ± 3.03****	-	24.75 ± 2.24****
CET	Aerial	EtOAc	9.85 ± 1.37****	-	7.24 ± 1.67****
		MeOH	12.27 ± 2.40****	-	20.23 ± 1.50****
CM	Aerial	EtOAc	10.55 ± 2.10****	-	-
		MeOH	23.44 ± 2.27****	-	28.34 ± 0.87****
CS	Aerial	EtOAc	4.88 ± 0.79****	-	-
		MeOH	14.26 ± 0.44****	-	30.19 ± 1.06****
CT	Aerial	EtOAc	6.33 ± 0.54****	-	-
		MeOH	16.18 ± 1.66****	-	27.64 ± 3.29****
RC (wild)	Aerial	EtOH	84.99 ± 1.09**	8.47 ± 1.79****	33.72 ± 3.15****
RC (Cultivated)	Aerial	EtOH	19.38 ± 1.34****	-	22.57 ± 2.02****
CG	Aerial	EtOH	15.34 ± 2.84****	-	28.21 ± 0.62****
VH	Aerial	EtOH	11.18 ± 2.33****	-	30.85 ± 2.83****
OH[b]	Aerial	MeOH	41.19 ± 1.19****	-	§[c]
		Aqueous	30.22 ± 1.66****	-	§
ZN	Aerial	EtOH	84.96 ± 0.35**	-	70.19 ± 0.43***
ZM	Leaf	EtOH	41.66 ± 2.02***	-	41.82 ± 1.97****
PO	Leaf	EtOH	8.43 ± 0.93****	-	47.24 ± 1.61***
	Rhizome	EtOH	34.45 ± 1.32****	4.47 ± 1.77****	33.62 ± 0.03****
	Root	EtOH	82.77 ± 0.77**	15.92 ± 1.98****	46.45 ± 1.61***
Quercetin[e]			90.13 ± 0.31		
EDTA[f]				96.21 ± 0.13	
Gallic acid[g]					93.12 ± 3.11

[a]Standard error mean ($n = 3$)
[b]Tested at 2000 μg mL^{-1}
[c]No scavenging activity
[d]Not tested
[e]Reference for DPPH radical scavenging activity at 1000 μg mL^{-1}
[f]Reference for DMPD radical scavenging activity at 2000 at 200 μg mL^{-1}
[g]Reference for NO radical scavenging activity at 1000 μg mL^{-1}
[$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, $^{****}p < 0.0001$]

retention times and UV spectra of the peaks of pure standards. Standard solution was then added to the samples; increase in the intensity of the peaks verified the identification. All the calculations concerning the quantitative analyses were performed with external standardization by measurement of peak areas. Each injection was achieved in triplicate to see the reproducibility of the detector response at each concentration level.

Results and discussion

Enzyme inhibitory effect of the extracts

The inhibitory effect of thirty-one extracts obtained from fourteen plant species listed in Table 1 was

Table 4 FRAP, PRAP, and metal-chelation activities (± S.E.M.[a]) of the extracts at 100 μg mL^{-1}

Species	Plant part	Extract type	FRAP (± S.E.M.[a])	PRAP (± S.E.M.)	Metal-chelation (± S.E.M.)
AL[b]	Fruit	DCM	0.448 ± 0.02****	0.444 ± 0.12****	23.19 ± 6.71****
		EtOAc	0.517 ± 0.33****	0.441 ± 0.04****	28.21 ± 5.93****
		EtOH	0.591 ± 0.16****	0.360 ± 0.14****	-[c]
	Aerial	DCM	0.554 ± 0.02****	0.406 ± 0.18****	-
		EtOAc	0.705 ± 0.15****	0.371 ± 0.05****	-
		EtOH	0.441 ± 0.16****	0.349 ± 0.01****	25.42 ± 0.40****
EE	Fruit	Juice	0.173 ± 0.006****	0.233 ± 0.016****	55.86 ± 4.11*
	Fruit	EtOH	0.217 ± 0.005****	0.221 ± 0.004****	13.83 ± 2.19****
	Aerial	EtOH	0.192 ± 0.003****	0.232 ± 0.011****	20.90 ± 1.74****
	Root	EtOH	0.166 ± 0.021****	0.168 ± 0.005****	29.47 ± 3.11****
CER	Aerial	EtOAc	0.209 ± 0.009****	0.207 ± 0.014****	50.96 ± 3.09**
		MeOH	0.185 ± 0.010****	0.211 ± 0.013****	-
CET	Aerial	EtOAc	0.232 ± 0.012****	0.204 ± 0.019****	36.85 ± 1.34****
		MeOH	0.219 ± 0.013****	0.216 ± 0.011****	8.71 ± 2.04****
CM	Aerial	EtOAc	0.245 ± 0.012****	0.194 ± 0.005****	32.98 ± 3.45****
		MeOH	0.294 ± 0.016****	0.226 ± 0.007****	-
CS	Aerial	EtOAc	0.216 ± 0.011****	0.191 ± 0.014****	48.56 ± 2.95****
		MeOH	0.205 ± 0.009****	0.201 ± 0.012****	-
CT	Aerial	EtOAc	0.247 ± 0.025****	0.188 ± 0.007****	13.69 ± 3.79****
		MeOH	0.246 ± 0.020****	0.173 ± 0.008****	-
RC (wild)	Aerial	EtOH	1.036 ± 0.061**	0.310 ± 0.007****	-
RC (Cultivated)	Aerial	EtOH	0.473 ± 0.006****	0.245 ± 0.045****	-
CG	Aerial	EtOH	0.492 ± 0.011****	0.251 ± 0.015****	-
VH	Aerial	EtOH	0.468 ± 0.014****	0.286 ± 0.007****	-
OH[b]	Aerial	MeOH	0.812 ± 0.040***	§[d]	19.86 ± 0.23****
		Aqueous	0.833 ± 0.030****	§	-
ZN	Aerial	EtOH	1.326 ± 0.065**	0.262 ± 0.013****	2.53 ± 1.88****
ZM	Leaf	EtOH	0.495 ± 0.005****	0.141 ± 0.004****	-
PO	Leaf	EtOH	0.346 ± 0.003****	0.160 ± 0.001****	14.79 ± 3.14****
	Rhizome	EtOH	0.532 ± 0.011****	0.124 ± 0.001****	3.43 ± 0.99****
	Root	EtOH	0.965 ± 0.036****	0.189 ± 0.005****	-
Quercetin[e]			1.946 ± 0.038	0.782 ± 0.009	
EDTA[f]					61.87 ± 4.69

[a]Standard error mean ($n = 3$)
[b]Tested at 2000 μg mL^{-1}
[c]No activity
[d]Not tested
[e]Reference for FRAP and PRAP at 1000 μg mL^{-1}
[f]Reference for metal-chelation activity at 1000 μg mL^{-1}
[$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, $^{****}p < 0.0001$]

screened against AChE, BChE, and TYR at 100 μg mL^{-1}. Among them, only two extracts, which are the EtOH extract of the aerial parts of CER (51.33 ± 3.35 %) and the root EtOH extract of PO (61.88 ± 2.23 %), exhibited the highest inhibition against AChE (Table 2). The root EtOH extract of PO (82.55 ± 2.14 %) was also the most effective one in BChE inhibition, followed by the MeOH extract of OH (66.88 ± 0.17 %) that inhibited TYR in the highest rate (35.28 ± 1.90 %), which can be considered as moderate (Table 2). Rest of the extracts was found to be either ineffective or with low inhibition below 50 %.

Antioxidant effect of the extracts

Radical scavenging activity of the extracts was tested against three radicals *i.e.* DPPH, DMPD, and NO at 1000 μg mL^{-1} (Table 3). The EtOH extracts of the wild sample of RC (84.99 ± 1.09 %), ZN (84.96 ± 0.35 %) as well as the roots of PO (82.77 ± 0.77 %) showed the highest scavenging activity towards DPPH radical (Table 3), whereas the extracts displayed from none to very low scavenging effect (up to 15.18 ± 3.07 %) against DMPD radical. On the other hand, the EtOH extract of ZN (70.19 ± 0.43 %) possessed higher activity than other extracts. According to our findings, this

Table 5 Total phenol and flavonoid amounts (± S.E.M.) of the extracts

Species	Plant part	Extract type	Total phenol content[a] ± S.E.M.[b]	Total flavonoid content[c] ± S.E.M.
AL	Fruit	DCM	9.35 ± 3.82	-[d]
		EtOAc	7.15 ± 3.72	-
		EtOH	5.18 ± 0.93	-
	Aerial	DCM	-	-
		EtOAc	39.17 ± 3.10	-
		EtOH	-	-
EE	Fruit	Juice	8.60 ± 3.48	-
	Fruit	EtOH	8.37 ± 1.82	9.94 ± 1.05
	Aerial	EtOH	2.98 ± 1.16	20.10 ± 1.05
	Root	EtOH	1.80 ± 0.17	-
CER	Aerial	EtOAc	32.86 ± 4.35	40.62 ± 4.76
		MeOH	18.96 ± 0.52	47.95 ± 2.16
CET	Aerial	EtOAc	40.81 ± 5.69	52.63 ± 1.90
		MeOH	27.47 ± 1.27	57.56 ± 5.20
CM	Aerial	EtOAc	44.44 ± 5.56	53.77 ± 2.14
		MeOH	24.53 ± 2.08	66.47 ± 2.06
CS	Aerial	EtOAc	42.62 ± 3.85	44.41 ± 0.84
		MeOH	14.70 ± 0.75	56.10 ± 0.99
CT	Aerial	EtOAc	46.13 ± 3.74	49.66 ± 3.80
		MeOH	16.58 ± 1.06	65.14 ± 1.78
RC (wild)	Aerial	EtOH	73.00 ± 0.013	73.33 ± 1.07
RC (Cultivated)	Aerial	EtOH	23.80 ± 1.23	22.89 ± 5.84
CG	Aerial	EtOH	35.03 ± 2.91	23.92 ± 0.08
VH	Aerial	EtOH	42.89 ± 0.06	20.74 ± 0.61
OH	Aerial	MeOH	§[e]	§
		Aqueous	§	§
ZN	Aerial	EtOH	79.67 ± 2.09	83.63 ± 2.38
ZM	Leaf	EtOH	6.41 ± 0.57	20.22 ± 1.37
PO	Leaf	EtOH	11.02 ± 0.17	11.91 ± 0.23
	Rhizome	EtOH	0.05 ± 0.01	23.96 ± 0.56
	Root	EtOH	1.71 ± 0.21	60.43 ± 3.76

[a]Data expressed in mg equivalent of gallic acid to 1 g of extract
[b]Standard error mean ($n = 3$)
[c]Data expressed in mg equivalent of quercetin to 1 g of extract
[d]Not measured due to very low absorbance of the extracts
[e]Not measured

extract (1.326 ± 0.065) exerted the best FRAP, followed by the EtOH extract of the wild sample of RC (1.036 ± 0.061), while most of the extracts showed a mild level of activity in PRAP assay (Table 4). The fruit juice of EE was found to have the highest metal-chelation capacity (55.86 ± 4.11 %).

Total phenol and flavonoid contents and HPLC analyses of the extracts

The amount of total phenol and flavonoid contents ranged from none to 79.67 ± 2.09 for total phenols as expressed in mg equivalent of gallic acid to 1 g of

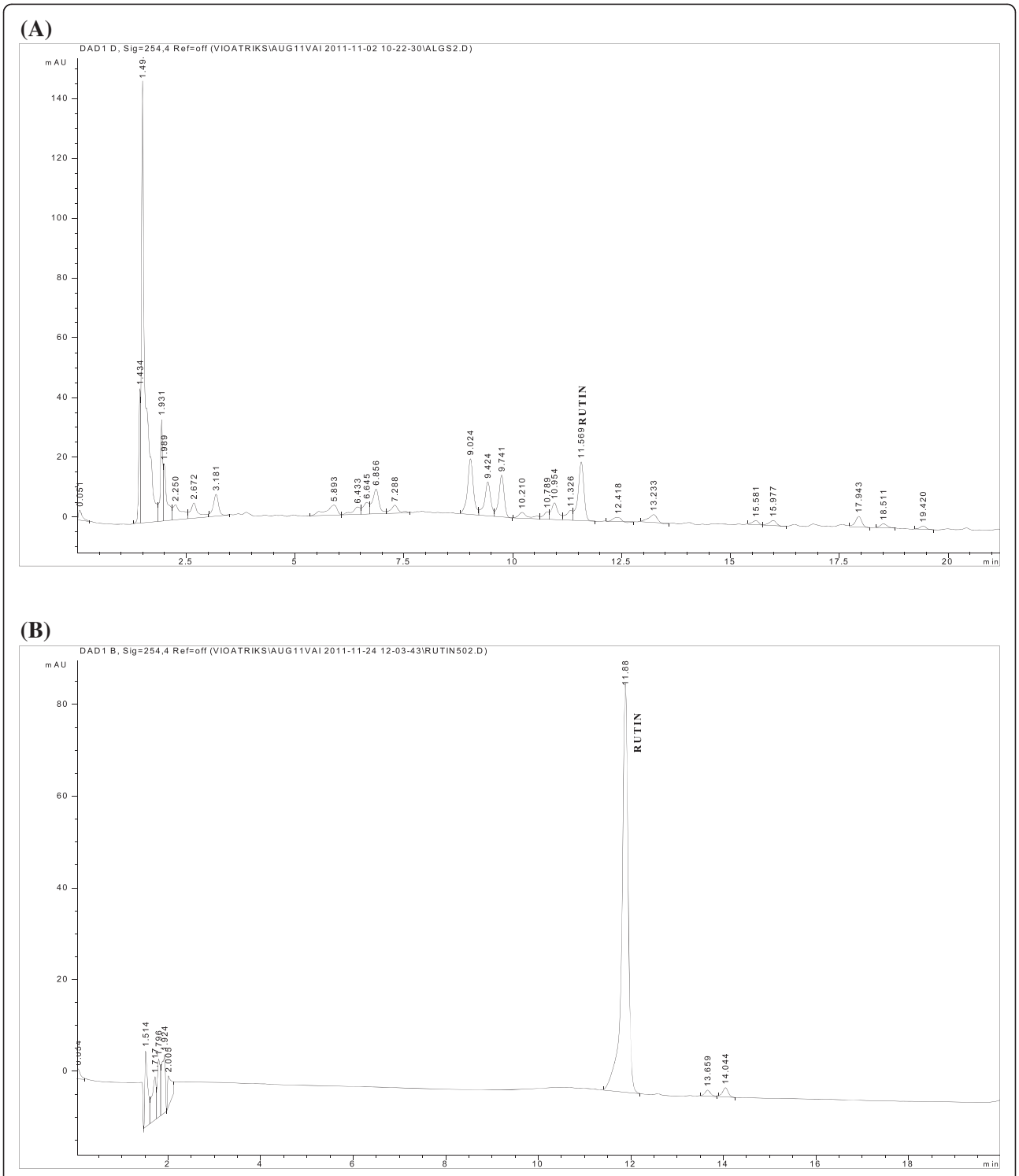

Fig. 1 Representative HPLC chromatograms of the EtOH extract of the aerial parts of AL (**a**) and rutin (**b**)

extract, whilst total flavonoid amount was found to differ from none to 83.63 ± 2.38 as expressed in mg equivalent of quercetin to 1 g of extract (Table 5). As stated by our results, the most abundant total phenol and flavonoid amounts were determined in the EtOH extract of ZN and the EtOH extract of the wild sample of RC.

Our HPLC analysis conducted on the AL, CG, and VH extracts, whose phytochemical contents have been analyzed for the first time herein, indicated that the EtOH extracts of the fruits and aerial parts of the plant contained 0.9522 ± 0.1731 and 1.1094 ± 0.0384 mg g^{-1} of rutin (Fig. 1), whereas DCM and EtOAc extracts of AL did not contain rutin at all (Table 6). On the other hand, CG was found to possess 0.26 % of rutin, while VH had 0.71 % of vitexin. The other flavonoid (quercitrin, luteolin, luteolin-7-glucoside, apigenin, quercetin, and kaempferol) along with some phenolic acid derivatives including rosmarinic acid, gallic acid, and *p*-coumaric acid were also searched in all extracts of AL by HPLC, nevertheless, none of these compounds were found in these plant species.

Screening of plant extracts for their enzyme inhibitory effect is a quite popular pharmacological tool as it gives a preliminary idea about efficiency of a plant towards the targeted disease. On this purpose, we have obtained some promising results out of our screening with thirty-one extracts from fourteen plants species selected for the present study. Besides, our literature survey revealed that none of the plants screened herein has been reported with any of these enzyme inhibitory activities up to date. The genus *Atriplex*, commonly known as saltbush or orache, consists of mostly halophytic species with edible property such as AL. To our knowledge, cholinesterase or TYR inhibitory activity of AL has not been studied so far, whereas another species, *Atriplex halimus* L., was demonstrated to have a significant AChE inhibition by Benamar et al. [20]. Particularly, its chloroform extract had 74.60 ± 1.45 % of inhibition at 125 μg mL^{-1}, while the crude extract of the plant exhibited 66.03 ± 1.10 % of inhibition at 250 μg mL^{-1} which was shown to contain a very low amount of flavonoids consistent with our data. Therefore, the authors stated that some other type of compounds rather than flavonoids may produce the strong AChE-inhibitory effect of *Atriplex halimus*. Consistently, we have found by HPLC analysis that AL lacks of flavonoids except for rutin, which was actually shown to be inactive in AChE/BChE inhibition assays in our earlier publication [21]. Thus, one can make comment that none to very low inhibitory effect of the AL extracts in the enzyme inhibition assays are likely associated with the absence of flavonoid derivatives such as quercetin (Table 2). EE, a medicinal species also called squirting cucumber or exploding cucumber, has been suggested to be used against memory deficiency amongst the local people in Turkey (personal communication). Despite of this information, our experiments revealed that neither the fruit juice, nor the EtOH extracts from different parts of EE, was capable of inhibiting any of these enzymes in a good level (Table 2). This may lead such a comment that EE should be further tested by other mechanisms related to neurodegeneration.

Table 6 Rutin and vitexin percentages (w/w) in the ethanol extracts in AL, CG, and VH by HPLC

Plant species	Rutin (%)	Vitexin (%)
AL (Aerial)	1.11	-[a]
AL (Fruit)	0.95	-
CG	0.26	-
VH	-	0.71

[a]Not found

Among the *Centaurium* species, especially *Centaurium erythraea* Rafin., known as small centaury, has been reported to be used for antiflatulent, digestive, gastritis, antidiabetic, and stimulant purposes in Serbia [22] and for treatment of stomach ache and ulcer in Turkish folk medicine [23]. The five species studied herein displayed insignificant enzyme inhibition as well as low to moderate radical scavenging and antioxidant effect. In fact, abundant amount of xanthones and secoiridoids were identified in many *Centaurium* species, which seem to cause little antioxidant activity [24]. Therefore, stumpy level of antioxidant activity of the *Centaurium* species studied herein might be explained by this fact. Conversely, it has been known that higher total phenol content in plant extracts usually causes higher antioxidant activity [25], as described in several *Centaurium* species subjected to some antioxidant assays such as DPPH, hydroxyl, and superoxide radical scavenging and xanthine oxidase inhibitory activity [26]. Hence, this is presumably not the case in our samples of *Centaurium* since they have low to moderate amount of total phenols and flavonoids.

Up to date, no phytochemical or bioactivity data has been reported on any *Ricotia* species and CG. In our study, which is the first one on *Ricotia* sp., the EtOH extracts of the wild and cultivated samples of RC did not exert inhibitory action against the enzymes tested in the present work, whereas the wild sample of the plant was found to exhibit remarkable DPPH radical scavenging activity and FRAP as compared to its cultivated counterpart (Tables 3 and 4). This is an interesting to note that the wild sample of RC may possibly contain more antioxidant substances than that of the cultivated one probably due to some agricultural factors including soil type, rain amount, and other climatic and ecological factors. On the other hand, the only phytochemical study carried out was on the essential oil composition of OH which was shown to contain *p*-cymene (15.56 %) and borneol

(14.24 %) in major quantities [27]. In our study, it was the most active to inhibit BChE and TYR, while several other *Origanum* species have been demonstrated to display inhibitory property on cholinesterases to some extent such as *Origanum syriacum*, *Origanum ehrenbergii* [28, 29], *Origanum vulgare* var. *glandulossum* [30], and *Origanum majorana* [31, 32]. In connection with this data, ursolic acid isolated from *Origanum majorana* was stated to inhibit AChE in competitive/noncompetitive manner [31]. In our previous study [33], we identified selective BChE-inhibiting effect of the essential oils of *Origanum onites*, *Origanum vulgare*, *Origanum munitiflorum*, and *Origanum majorana*, which is in accordance with our current data on OH. In fact, *Origanum* species have been demonstrated to have a rich phenolic content, especially rosmarinic acid as the major constituent [34], which we earlier pointed out to its marked anti-BChE activity [35]. Therefore, it could be speculated that the BChE-inhibiting effect of OH might be related to its rosmarinic acid content, while its antioxidant effects seems to be concomitant with the phenolics found in this plant.

Considering the sea grasses studied, the root EtOH extract of PO exerted notable inhibition towards AChE and BChE (Table 2). In our former study [36], the leaf EtOH extract of PO was found to be ineffective against AChE, which is consistent with our present findings. Although the rich phenolic compounds have been shown to be present in the leaves of the plant such as ferulic acid, phloridzin, phloroglucinol, *p*-anisic acid, acetosyringone, sinapic acid, phenol, *p*-hydroxybenzoic acid, *p*-coumaric acid, and cinnamic acid, 4-hydroxybenzoic acid, 4-coumaric acid, *trans*-cinnamic acid, and caffeic acid, chicoric acid, vanillin, and gentisic acid [37–39], a very little investigation was performed on the roots of this marine plant from the view point of phytochemistry reporting only presence of phenylmethane derivaties [40], which may have some influence on high cholinesterase-inhibiting effect of PO. On the other hand, the copious phenolic content in *Zostera* species such as flavones and sulfated phenols was revealed by McMillan et al. [41], while ferulic, vanillic, *p*-hydroxybenzoic, caffeic, gallic, protocatechuic, and gentisic acids were identified in ZM [41] and rosmarinic acid in ZN [42, 43]. Clearly, the plentiful phenolic contents in two *Zostera* species screened herein prospectively seem to contribute to their antioxidant capacity, which is in accordance with their total phenol and flavonoid amounts determined in our current study.

Conclusions

In summary, our findings obtained from screening of various extracts from fourteen plant species reveal that *Centaurium erythraea* subsp. *rhodense* and *Origanum haussknechtii*, and the roots of *Posidonia oceanica* possess a marked cholinesterase inhibitory activity, which deserve further investigation in order to identify the compound(s) responsible inhibiting cholinesterases. Our results with antioxidant assays pointed out that *Ricotia carnosula*, *Zostera noltii*, and *Posidonia oceanica* have high antioxidant potential acting by different mechanisms. Consequently, we herein disclose the first the study on AChE, BChE, and TYR inhibitory effects of the abovementioned fourteen plant species, three of which may be useful for cognitive impairment by means of enzyme inhibition as well as antioxidant activity. This is also first report on flavonoid and phenolic acid contents of AL, CG, and VH.

Competing interests

No competing interests exist among the authors of this paper.

Authors' contributions

IEO and FSS designed the study, performed the research, collected and analyzed the data and written the manuscript. IEO collected the plant from Chenopodiaceae and prepared the extract. MZH and HK collected and prepared the extracts of three marine species, while SAE carried out all HPLC analyses. GY, MC, and AEY collected the plants from Gentianaceae and prepared their extracts. EA collected the plants from Brassicaceae and Caryophyllaceae and prepared their extracts. NK and GT collected the plant species from Lamiaceae and Cucurbitaceae, respectively. All authors read and approved the final manuscript.

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Author details

[1]Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, 06330 Ankara, Turkey. [2]Department of Pharmaceutical Botany, Faculty of Pharmacy, Ege University, 35100 Izmir, Turkey. [3]Department of Pharmacognosy, Faculty of Pharmacy, Ankara University, 06100 Ankara, Turkey. [4]Department of Pharmaceutical Botany, Faculty of Pharmacy, Ankara University, 06100 Ankara, Turkey. [5]Department of Biology, Faculty of Arts and Science, Pamukkale University, 20070 Denizli, Turkey. [6]Department of Biology, Faculty of Science, Ankara University, 06100 Ankara, Turkey. [7]Department of Agricultural Biotechnology, Faculty of Agriculture, Akdeniz University, 07058 Antalya, Turkey.

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