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Evaluation of possible *in vitro* neurobiological effects of two varieties of *Cupressus sempervirens* (Mediterranean cypress) through their antioxidant and enzyme inhibition actions

[*Cupressus sempervirens*'in iki varyetesinin (Akdeniz sediri) muhtemel *in vitro* nörobiyolojik etkilerinin antioksidan ve enzim inhibisyon aksiyonları yoluyla değerlendirilmesi]

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ABSTRACT

Objective: The dichloromethane, acetone, ethyl acetate, and methanol extracts of the cones and leaves of *Cupressus sempervirens* var. *horizantalis* (CSH) and var. *pyramidalis* (CSP) were investigated for their *in vitro* neurobiological effects.

Methods: The extracts were screened for their inhibitory activity against acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and tyrosinase (TYRO) enzymes using microtiter plate assays. Antioxidant activity of the extracts was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and *N*,*N*-dimethyl-*p*-phenylendiamine (DMPD) radical scavenging activity, metal-chelation capacity along with ferric- (FRAP) and phosphomolibdenum-reducing antioxidant power (PRAP) tests. Total phenol and flavonoid contents of the extracts were calculated spectrophotometrically.

Results: The extracts displayed weak to moderate cholinesterase inhibition at 200 µg mL^{-1}. The cone dichloromethane extract of CSP showed the highest inhibition (36.10±1.45%) against AChE, while the best inhibition (40.01±0.77%) against BChE was caused by the leaf acetone extract of CSH.

Conclusion: Antioxidant activity of the extracts was observed to vary according to the method used. This is the first study describing anticholinesterase and antityrosinase effects of the varieties of *C. sempervirens*.

Key Words: *Cupressus sempervirens*, cypress, cholinesterase, tyrosinase, antioxidant activity

Conflict of Interest

There is no conflict of interest among the authors who contributed to the present study.

ÖZET

Amaç: *Cupressus sempervirens* var. *horizantalis* (CSH) and var. *pyramidalis*'in (CSP) kozalakları ve yapraklarının diklorometan, aseton, etil asetat ve methanol ekstreleri *in vitro* nörobiyolojik etkileri yönünden incelenmiştir.

Yöntem: Ekstreler, inhibitor etkileri açısından asetilkolinesterase (AChE), bütirilkolinesteraz (BChE) ve tirozinaz (TYRO) enzimlerine karşı mikrotiter plak yöntemleri kullanılarak taranmıştır. Ekstrelerin antioksidan aktivitesi, 2,2-difenil-1-pikrilhidrazil (DPPH) ve *N*,*N*-dimetil-*p*-fenilendiamin (DMPD) radikal süpürücü aktivite, metal-şelasyon kapasite, demir- (FRAP) ve fosfomolibdenyum-indirgeyici (PRAP) antioksidant gücü testleri kullanılarak tayin edilmiştir. Ekstrelerin toplam fenol ve flavonoit içerikleri spektrofotometrik olarak hesaplanmıştır.

Bulgular: Ekstreler 200 µg mL^{-1}'de zayıf – orta derecede kolinesteraz inhibisyonu göstermiştir. AChE'a karşı en yüksek inhibisyonu (36.10±1.45%) CSP'nin kozalak diklorometan ekstresi gösterirken, BChE'a karşı en iyi inhibisyona (40.01±0.77%), CSH'ın yaprak aseton ekstresi sebep olmuştur.

Sonuçlar: Ekstrelerin antioksidan aktivitesinin kullanılan yönteme gore değiştiği gözlenmiştir. Bu, C. sempervirens'in varyetelerinin antikolinesteraz ve antitirozinaz etkilerini açıklayan ilk çalışmadır.

Anahtar Kelimeler: *Cupressus sempervirens*, sedir, kolinesteraz, tirozinaz, antioksidan aktivite

Çıkar Çatışması: Katkıda bulunan yazarların hiçbir çıkar çatışması yoktur.

Introduction

Alzheimer's disease (AD) is the most common form of dementia, which affects especially elder population over the age of 65 years. AD is characterized by cognitive dysfunction, abnormalities in thinking and behaviors. Since the cholinergic deficit has been described in the brains of AD patients, cholinesterase inhibitors have become a foremost drug class for prescription of AD treatment [1]. As mammalian brain contains two major forms of cholinesterases; acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), it is important to inhibit with both enzyme types. However, depending on the side effects of the present cholinesterase inhibitors, novel alternative drugs in the fight against AD are still in demand. Parkinson's disease (PD) is another type of neurodegenerative diseases, which mainly results from the degeneration of nigrostriatal dopaminergic neurons induced by tyrosinase (TYRO) [2]. TYRO, a copper-containing enzyme, is also known as "monophenol monooxygenase or catechol oxidase" that catalyses the oxidation of phenols (such as tyrosine and dopamine) into melanin. Consequently, inhibition of TYRO is important for the treatment of PD. On the other hand, oxidative stress occurring by different mechanisms is strongly connected with the neurodegenerative diseases including AD and PD [3].

Medicinal and aromatic plants have been always attractive targets for finding new bioactive molecules. Many researchers have also focused on plants in order to discover new drug candidates with neuroprotective activity. *Cupressus* L. species (Cupressaceae), known as "cypress", are native to the Mediterranean basin and its leaves and cones have been used as folk remedy in many countries such as antiseptic, antipyretic, anthelminthic, astringent, antirheumatic, antihemorrhoidal, antidiarrhoeic, and vasoconstrictive [4-6]. The genus is also used for ornamental purpose and known to contain appreciable amounts of essential oil [7-10], which possess mainly antimicrobial [10,11], antiviral [12], and antifungal [13] activity. Besides, *C. sempervirens* was recorded to be used for memory-enhancement in Anatolia, which was described in a very old book written by an Ottoman herbalist-physician at the period of the ruler Sultan Mehmed the fourth (1641–1693) [14].

As a part of our ongoing studies aimed to explore *in vitro* neuroprotective effects of medicinal and aromatic plants; in the current study, the dichloromethane (DCM), acetone (Ace), ethyl acetate (EtOAc), and methanol (MeOH) extracts prepared from the cones and leaves of *Cupressus sempervirens* L. var. *horizantalis* (Mill.) Gord and var. *pyramidalis* Nym. growing in Turkey were screened for their inhibitory potential against acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and tyrosinase (TYRO) using ELISA microtiter plate assay at 200 µg mL^{-1}. Antioxidant activity of the extracts was tested by several *in vitro* methods including 2,2-diphenyl-1-picrylhydrazyl (DPPH) and *N*,*N*-dimethyl-*p*-phenylendiamine (DMPD) radical scavenging activity, metal-chelation capacity, ferric- (FRAP) and phosphomolibdenum-reducing antioxidant power (PRAP) assays. Total phenol and flavonoid contents of the extracts were calculated using Folin-Ciocalteau and $AlCl_3$ reagents as gallic acid and quercetin equivalent, respectively.

Materials and Methods

Plant materials

The cones and leaves of *Cupressus sempervirens* var. *horizantalis* (CSH) and var. *pyramidalis* (CSP) were sampled from Antalya province (Turkey) in October, 2010. The place where the plant materials were collected is under the effect of Mediterranean climate in terms of natural flora. The plant samples were identified by one of us (I.T.) and are preserved at his personal collection, which are readily available for those who would like to see the samples.

Preparation of the extracts

Extractions of the air-dried and powdered cones and leaves of CSH and CSP were performed sequentially with dichloromethane (DCM), acetone (Ace), ethyl acetate (EtOAc), and methanol (MeOH) at 100% concentration by macerating at room temperature. Following filtration, each organic phase was evaporated *in vacuo* to give the crude extracts. Yield% (w/w) of the extracts is listed in Table 1.

Determination of total phenol and flavonoid contents in the extracts

Total phenolic content of the extracts was determined in accordance with Folin-Ciocalteau's method [15]. In brief, a number of dilutions of gallic acid were obtained to prepare a calibration curve. The extracts and gallic acid dilutions were mixed with 750 L of Folin-Ciocalteau's reagent and 600 L of sodium carbonate (10%) 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 [16]. To sum up, a number of dilutions of quercetin were obtained to prepare a calibration curve. Then, the extracts and quercetin dilutions were mixed with ethanol (95%), aluminum chloride reagent (10%), 100 µL of sodium acetate (10%) as well as distilled water. Following incubation for 30 minutes at room temperature, absorbance of the reaction mixtures was measured at wavelength of 415 nm with a Unico 4802 UV-visible double beam spectrophotometer (Dayton, NJ, USA). The total phenol and flavonoid contents of the extracts were expressed as gallic acid and quercetin equivalents (mg g^{-1} extract), respectively.

Table 1. Yield % (w/w), total phenol and flavonoid contents and DMPD radical scavenging activity of the *Cupressus* extracts

		Extract type	Yield% (w/w)	Total phenol[a] contents±S.E.M.[b]	Total flavonoid[c] contents±S.E.M.	Inhibition %±S.E.M. against DMPD at 2000 mg mL^{-1}
C. sempervirens var. horizontalis	Cone	CH_2Cl_2	5.09	27.28±0.24	7.25±1.32	-
		Ace	1.32	88.62±4.37	9.23±0.23	9.31±0.53
		EtOAc	0.66	124.36±0.49	8.99±0.11	23.69±0.59
		MeOH	9.50	62.16±4.37	0.35±0.07	27.66±0.67
	Leaf	CH_2Cl_2	9.90	41.54±1.94	16.13±1.49	-
		Ace	1.64	100.82±0.24	70.95±0.40	-
		EtOAc	1.12	43.95±3.40	23.96±0.29	-
		MeOH	9.21	68.69±1.46	11.47±0.17	6.06±0.23
C. sempervirens var. pyramidalis	Cone	CH_2Cl_2	2.47	36.73±2.43	8.18±0.34	-
		Ace	1.06	71.09±2.92	6.39±0.69	-
		EtOAc	0.36	76.08±2.19	7.41±1.66	-
		MeOH	8.70	106.49±1.46	0.43±0.06	26.32±1.63
	Leaf	CH_2Cl_2	8.28	34.50±1.70	43.03±1.32	-
		Ace	2.05	85.18±1.94	59.59±2.58	-
		EtOAc	0.95	52.37±1.21	32.36±2.87	-
		MeOH	13.38	75.22±1.46	12.97±1.38	30.34±0.69
Quercetin (Reference for DMPD scavenging activitiy) at 2000 mg mL^{-1}					68.32±0.67	

[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.

Determination of AChE and BChE inhibitory activity

AChE and BChE inhibitory activity of the extracts was determined by the modified Ellman spectrophotometric method [17]. Electric eel AChE (Type-VI-S, EC 3.1.1.7, Sigma) and horse serum BChE (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 same as described in our earlier publication [18]. Briefly, in this method, 140 µL of 0.1 mM sodium phosphate buffer (pH 8.0), 20 µL of DTNB, 20 µL of test solution and 20 µL of AChE/BChE 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 acetylthiocholine iodide/butyrylthiocholine chloride. The hydrolysis of 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). The measurements and calculations were evaluated by using Softmax PRO 4.3.2.LS software. Percentage of inhibition of AChE/BChE was determined by comparison of the reaction rates of the samples relative to blank sample (ethanol in phosphate buffer pH=8) using the formula *(E-S)/E x 100*, where *E* is the activity of enzyme without test sample and *S* is the activity of enzyme with test sample. The experiments were done in triplicate. 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.

Tyrosinase inhibitory activity assay

Inhibition of tyrosinase (EC 1.14.1.8.1, 30 U, mushroom tyrosinase, Sigma) was determined using the modified dopachrome method with L-DOPA as substrate [19]. The assays were conducted in a 96-well microplate using ELISA microplate reader (VersaMax Molecular Devices, USA) to measure absorbance at 475 nm. An aliquot of the extracts dissolved in DMSO with 80 L of phosphate buffer (pH 6.8), 40 L of tyrosinase, and 40 L of L-DOPA were put in each well. Results were compared with control (DMSO). Baicalein (Sigma, St. Louis, MO, USA) was used as the reference. The experiments were performed in triplicate. The percentage tyrosinase inhibition (I%) was calculated as follows:

$I\% = (Absorbance_{control} - Absorbance_{sample}) / Absorbance_{control} \times 100$

Antioxidant activity

DPPH radical scavenging assay

The hydrogen atom or electron donation capacity of the corresponding extracts 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 was determined by the method of Blois [20]. The samples (70 µL) dissolved in ethanol (75%) were mixed with 2700 µL of DPPH solution (1.5×10^{-4} M). Remaining DPPH amount after 30 min reaction time 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. Inhibition of DPPH in percent (I%) was calculated as given below:

$I\% = [(A_{control}-A_{sample}) / A_{control}] \times 100$, where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is the absorbance of the extracts or reference. Experiments were run in triplicate and the results were conveyed as average values with S.E.M. (Standard error mean).

DMPD radical scavenging activity

Principal of the assay is based on reduction of the purple-colored radical $DMPD^{+}$ (*N*,*N*-dimethyl-*p*-phenylendiamine) [21]. 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 was mixed up with 50 µL of the extract dilutions and absorbance was taken at 505 nm using a Unico 4802 UV-visible double beam spectrophotometer (Dayton, NJ, USA). Quercetin was employed as the reference and the experiments were done in triplicate. The results were calculated according to the same formula given for DPPH radical scavenging test and expressed as average values with S.E.M. (Standard error mean).

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

The metal-chelation capacity of the extracts was estimated by the method of Chua et al. [22]. Accordingly, 740 L of the samples dissolved in ethanol (75%) were incubated with 2 mM $FeCl_2$ solution. The reaction was initiated by the addition of 40 L of 5 mM ferrozine solution into the mixture 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 follows:

$I\% = [(A_{control}-A_{sample}) / A_{control}] \times 100$. The control contained only $FeCl_2$ and ferrozine and butylated hydroxyanisol was used as reference. Analyses were run in triplicate and expressed as average values with S.E.M. (Standard error mean).

Ferric-reducing antioxidant power (FRAP)

The ferric-reducing antioxidant power (FRAP) of the extracts was tested using the assay of Oyaizu [23]. Various concentrations of the extracts (1000 µL) as well as chlorogenic acid as reference were added to 2500 µL of phosphate buffer (pH 6.6) and 2500 µL of potassium ferricyanide. Later, the mixture was incubated at 50°C for 20 min and then trichloroacetic acid (10%) was added. After the mixture was shaken vigorously, this solution was mixed with distilled water and $FeCl_3$ (0.1%, w/v). After 30 min of incubation, absorbance was read at 700 nm using a Unico 4802 UV-visible double beam spectrophotometer (USA). Analyses were achieved in triplicate. Increase in absorbance of the reaction indicated increase in reducing power of the extracts.

Phosphomolibdenum-reducing antioxidant power (PRAP) assay

In order to perform PRAP assays on the extracts, each dilution (100 µL) was mixed 10% phosphomolibdic acid solution (1000 µL) in ethanol (w/v) [24]. 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 (Dayton, NJ, USA). Analyses were run in triplicate. Increased absorbance of the reaction meant increased reducing power and compared to that of quercetin as the reference.

Results

AChE and BChE inhibitory activity of the extracts

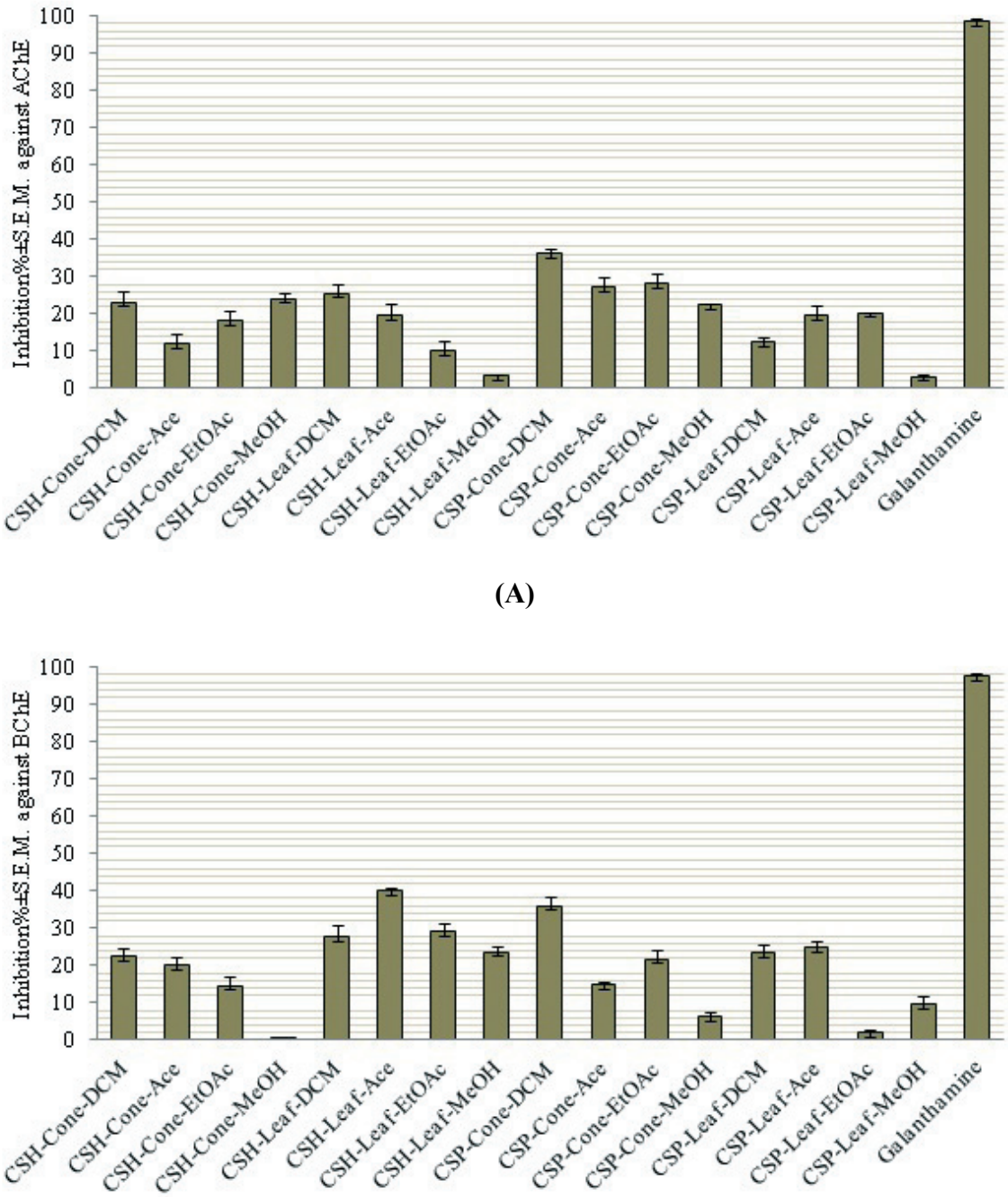
Inhibitory effects of the extracts of CSH and CSP are illustrated in Fig. 1. According to the results we obtained at 200 µg mL^{-1}, the cone DCM extract of CSP showed the highest inhibition (36.10±1.45%) against AChE among the extracts, whereas the leaf Ace extract of CSH inhibited BChE most effectively (40.01±0.77%).

TYRO inhibitory activity of the extracts

As shown in Fig. 2; the cone and leaf extracts of CSH and CSP exerted weak to mild inhibitory effects ranging between 6.28±1.62% and 32.06±2.88% towards TYRO at 200 µg mL^{-1}. Occurrence of the highest TYRO inhibition was observed in the cone Ace extract of CSP.

Antioxidant activity of the extracts

Antioxidant activity of the extracts was screened in five *in vitro* test models at 2000 µg mL^{-1}. In general, antioxidant activity of the extracts was observed to show a discrepancy according to the method used. For instance; the cone EtOAc extract of CSH displayed the highest DPPH radical scavenging activity (87.53±0.17%) (Fig. 3), while only six of the extracts had ability to scavenge DMPD radical varying from 6.06±0.23 to 30.34±0.69% (Table 1). In the FRAP assay, the cone



(A)

Figure 1. AChE **(A)** and BChE **(B)** inhibitory activity (inhibition±S.E.M.%) of the dichloromethane (DCM), acetone (Ace), ethyl acetate (EtOAc), and methanol (MeOH) extracts of the *Cupressus* speciesand the reference (galanthamine) at 200 µg mL^{-1}
(CSH: *Cupressus sempervirens* var. *horizantalis*, CSP: *Cupressus sempervirens* var. *pyramidalis*)

Ace extract belonging to CSH exhibited the highest absorbance value, which is indicative of the highest antioxidant activity in this assay (Table 2). Although the extracts had generally low activity in the PRAP assay, the leaf MeOH extract of CSH was the most active one (Table 1). Concerning the results obtained from the metal-chelation assay, the cone and leaf MeOH extracts of both varieties did not possess metal-chelation capacity (Table 1). However, the leaf EtOAc extracts of CSH (75.86±0.33%) and CSP (77.07±3.22%) showed the highest activity in this assay.

Total phenol and flavonoid contents of the extracts

Equations for total phenol and flavonoid contents of the extracts were calculated as y=1.4551x+0.0121 (r^2=0.9930) and y=6.1614x+0.0622 (r^2=0.9983), respectively. The richest extract in terms of total phenol was found to belong to the cone EtOAc extract of CSH (124.36±0.49 mg g^{-1}), while total phenol content was the most abundant in the leaf Ace extract of CSH (Table 1).

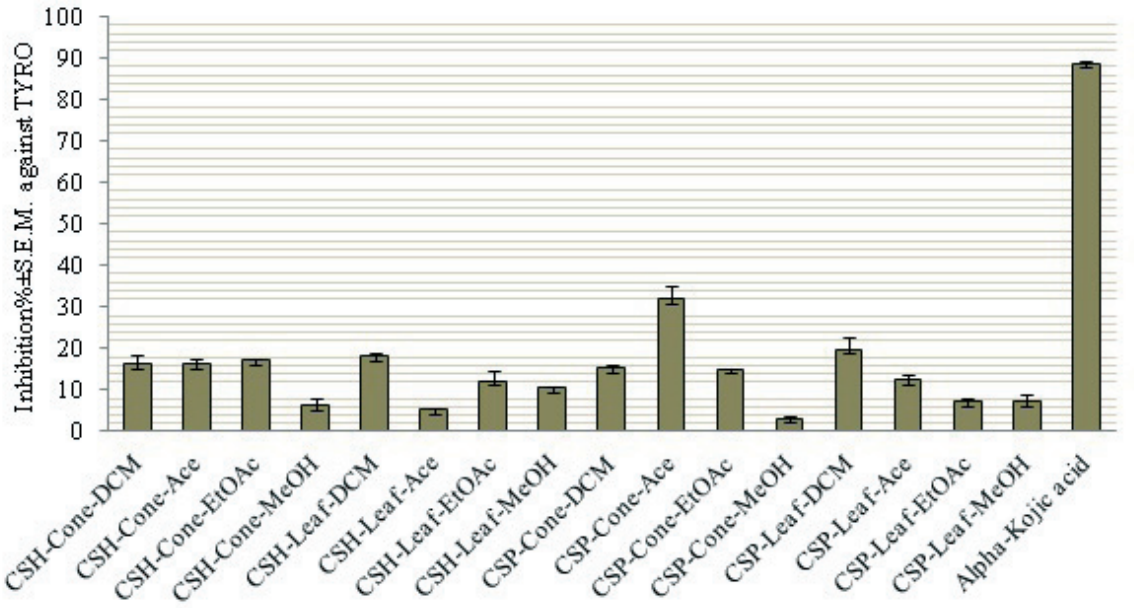


Figure 2. TYRO inhibitory activity (inhibition±S.E.M.%) of the dichloromethane (DCM), acetone (Ace), ethyl acetate (EtOAc), and methanol (MeOH) extracts of the *Cupressus* speciesand the reference (alpha-kojic acid) at 200 µg mL^{-1}
(CSH: *Cupressus sempervirens* var. *horizantalis*, CSP: *Cupressus sempervirens* var. *pyramidalis*)

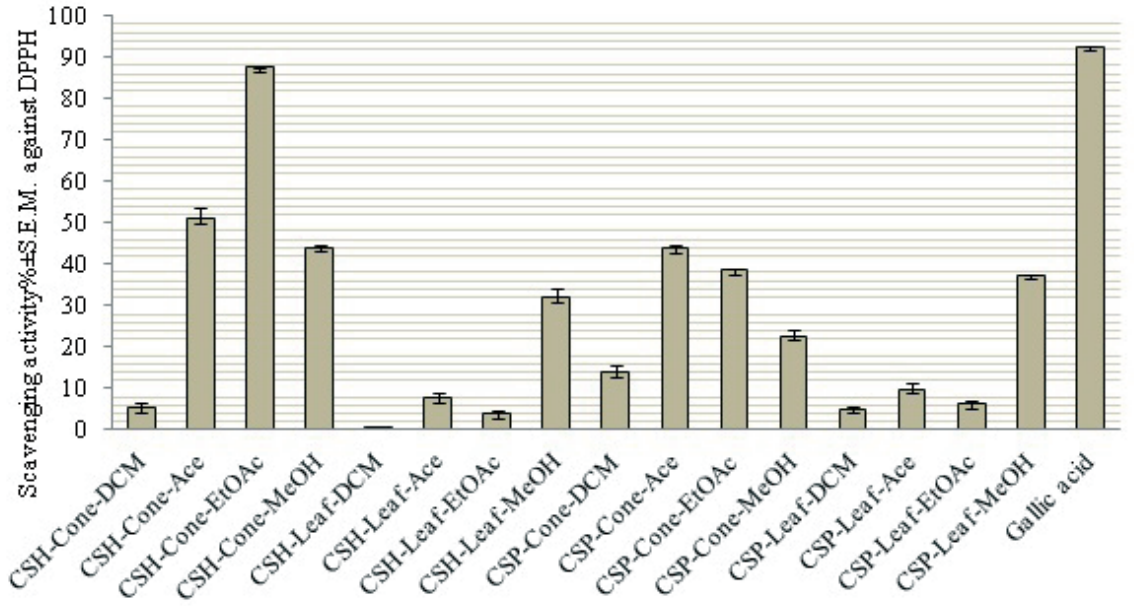


Figure 3. DPPH radical scavenging activity (inhibition±S.E.M.%) of the dichloromethane (DCM), acetone (Ace), ethyl acetate (EtOAc), and methanol (MeOH) extracts of the *Cupressus* speciesand the reference (gallic acid) at 200 µg mL^{-1}
(CSH: *Cupressus sempervirens* var. *horizantalis*, CSP: *Cupressus sempervirens* var. *pyramidalis*)

Discussion

Cupressus sempervirens is a medicinal and aromatic plant with bioactive secondary metabolites and used as a folk remedy. Our literature survey indicated that there has been no study relevant to neuroprotection or memory improvement up to date on this species. Neuroprotection is partly related to antioxidant activity. In fact, a small number of studies, which have evaluated antioxidant potential of *C. sempervirens*, are available in the scientific literature. In two antioxidant screening studies [6,11], the plant was found to display moderate radical scavenging effect against DPPH, which is in accordance with our findings. In Ibrahim et al.'s study [25], the leaf MeOH extract of *C. sempervirens*

Table 2. Ferric- (FRAP) and phosphomolibdenum-reducing antioxidant power (PRAP) and metal-chelation capacity of the *Cupressus* extracts at 2000 μg mL^{-1}

		Extract type	Ferric-reducing antioxidant power[a] (FRAP) (Absorbance at 700 nm±S.E.M.[b])	Phosphomolibdenum-reducing antioxidant power[a] (PRAP) (Absorbance at 600 nm±S.E.M.)	Metal-chelation capacity (Inhibition %±S.E.M.)
C. sempervirens var. horizontalis	Cone	CH_2Cl_2	0.921±0.04	0.307±0.055	64.04±3.01
		Ace	1.445±0.047	0.300±0.006	36.59±0.25
		EtOAc	2.228±0.07	0.329±0.001	24.43±3.30
		MeOH	0.887±0.027	0.376±0.002	-
	Leaf	CH_2Cl_2	0.384±0.019	0.331±0.018	68.99±2.34
		Ace	0.682±0.019	0.326±0.003	64.35±0.38
		EtOAc	0.554±0.03	0.315±0.007	75.86±0.33
		MeOH	0.918±0.015	0.412±0.014	-
C. sempervirens var. pyramidalis	Cone	CH_2Cl_2	0.882±0.063	0.272±0.013	32.32±3.71
		Ace	144±0.231	0.282±0.003	21.65±0.59
		EtOAc	1.141±0.064	0.319±0.087	41.04±1.47
		MeOH	0.599±0.008	0.390±0.004	-
	Leaf	CH_2Cl_2	0.407±0.002	0.276±0.001	66.83±2.16
		Ace	0.703±0.018	0.199±0.007	58.84±1.36
		EtOAc	0.553±0.020	0.325±0.003	77.07±3.22
		MeOH	0.88±0.036	0.291±0.004	-
Chlorogenic acid (Reference for FRAP)				3.547±0.006 at 1000 mg mL^{-1}	
Quercetin (Reference for PRAP)				0.819±0.001 at 1000 mg mL^{-1}	
EDTA (Reference for metal-chelation capacity)				78.35±0.89 at 1000 mg mL^{-1}	

[a] Higher absorbance indicates greater antioxidant activity. [b] Standard error mean (n=3)

of Egyptian origin was revealed to have strong DPPH radical scavenging activity and several phenolic compounds including cosmosiin, caffeic acid, and *p*-coumaric acid, cupressuflavone, amentoflavone, rutin, quercitrin, quercetin, myricitrin were isolated from this extract. As a result, the authors concluded that the strong antioxidant activity of the plant was due to presence of these phenolics. In contrary, the MeOH extracts had a moderate scavenging activity against DPPH in our study (Fig. 3), which might be depending on their phenolic content. On the other hand, the cone EtOAc extract of CSH having the richest total phenol amount displayed the highest DPPH radical scavenging activity, which could be leading to the same statement again that the strong antioxidant activity of the extract is linked to high amount of phenolics as supported by many other reports [26-28]. Needles to say, potency of antioxidant activity of any substance is also correlated with type of the method applied. Consistently, our results agreed well with this statement. In each one of five antioxidant assays applied herein, different extracts were observed to be effective. In another study on the Iranian conifers (29), the leaf and fruit MeOH extract of CSH and *C. sempervirens* var. *sempervirens* were highly effective in ferric thiocyanate (FTC) and thiobarbituric acid (TBA) methods. Nevertheless, activity of these extracts was also changeable according to the method, which is similar to our data The essential oil obtained from *C. sempervirens* from different countries has been reported to contain α-pinene as the major component [7,12,13,25,30]. In fact, the essential oils of the samples of the two varieties of *C. sempervirens* used in this study were analyzed by GC-MS in our previously [9] and α-pinene was the main components as follows; CSH-Cone: 66.75%; CSH-Leaf: 56.91%; CSP-Cone: 52.52%; CSP-Leaf: 37.91%. In many previous studies [32-34], α-pinene has been shown to inhibit strong cholinesterase inhibitory activity and moderate inhibitory activity of the *Cupressus* extracts could be related to their α-pinene content, although they are not supposed to contain α-pinene as much as the essential oil *per se*. Probably, apolar extracts have more possibility to contain this component, where the acetone and dichloromethane extracts were active in our assays.

On the other hand, *C. sempervirens* is known to contain flavonoids such as quercetin, rutin, cupressuflavone, amenoflavone, quercitrin, and myricitrin [35]. Among them, a special importance could be given to quercetin as we previously reported its strong cholinesterase inhibitory effects in competitive manner [36].

Consequently, inhibitory activity of the *Cupressus* extracts, whose total flavonoid amounts were determined by us herein, can be ascribed to presence of quercetin, at least in part. Consistently, the leaf Ace extract of CSH with the highest total flavonoid content as quercetin equivalent exhibited the highest BChE inhibition in our present study.

Conclusion

Taking its use for memory-enhancement in Anatolian folk medicine as the starting point, we evaluated possible effects of the two varieties of *C. sempervirens* of Turkish origin on memory by *in vitro* methods in this work. To conclude, the present study is a contribution to the better knowledge of the neuroprotective effect of the *C. sempervirens* varieties by *in vitro* experimental models and it also needs further evaluation by *in vivo* models. To the best of our knowledge, the current work constitutes the first report describing cholinesterase and tyrosinase inhibitory effects of *C. sempervirens* as well as its antioxidant activity by DMPD radical scavenging, metal-chelation capacity, and PRAP assays.

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Conflict of Interest

There is no conflict of interest among the authors who contributed to the present study.

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