

## Research Article



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## Antioxidant capacity, anti-acetylcholinesterase activity and inhibitory effect on lipid peroxidation in mice brain homogenate of *Achillea millefolium*

*Alchillea millefolium*'un antioksidan kapasitesi, anti-asetilkolinesteraz aktivitesi ve fare beyin homojenatında lipid peroksidasyonu üzerine inhibitör etkisi

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### Abstract

**Objective:** *Achillea millefolium* (*A. millefolium*) is a traditional herbal medicine that contains natural compounds with antioxidant activities and is used for a wide range of conditions among people. The aim of our study was to investigate antioxidant and anti-acetylcholinesterase activities of *A. millefolium* extracts to determine its potential therapeutic usage in Alzheimer's disease (AD).

**Methods:** Methanol extracts (ME) and ethanol extracts (EE) of *A. millefolium* were prepared to determine (a) *in vitro* antioxidant activities, (b) the effects on acetylcholinesterase kinetics (by using a colorimetric spectroscopic method) and (c) the effects on sodium nitroprusside (SNP)-induced lipid peroxidation in mice brain homogenate.

**Results:** ME had higher antioxidant activities compared to EE. Both extracts displayed a competitive inhibition of acetylcholinesterase; however, the inhibitory activity of ME was higher than EE. The inhibitory constant ( $K_i$ ) values of ME and EE were found to be 28.43 and 68.47  $\mu\text{g/mL}$ , respectively. Both extracts caused a significant concentration-dependent decrease in malondialdehyde (MDA) contents in mice brain homogenate, indicating a strong inhibition of lipid peroxidation.

**Conclusions:** Our results showed that *A. millefolium* has a high antioxidant capacity and anti-acetylcholinesterase activity due to its phenolic compounds, suggesting a potential use as adjuvant therapy in neurodegenerative conditions such as AD.

**Keywords:** Acetylcholinesterase; Alzheimer's disease; Brain; Lipid peroxidation; Sodium nitroprusside.

### Özet

**Amaç:** *Achillea millefolium* (*A. millefolium*), antioksidan aktiviteye sahip doğal bileşikler içeren ve halk arasında çeşitli amaçlar için kullanılan geleneksel tıbbi bir bitkidir. Çalışmamızın amacı, *A. millefolium* ekstrelerinin Alzheimer hastalığının (AD) tedavisinde kullanılma potansiyelini ortaya koymak için gerekli antioksidan ve anti-asetilkolinesteraz aktivitelerini araştırmaktır.

**Metod:** *A. millefolium*'un metanol (ME) ve etanol ekstraktları (EE) hazırlanarak, (a) *in vitro* antioksidan aktiviteleri, (b) Asetilkolinesteraz kinetiği üzerindeki etkileri (kolorimetrik spektroskopik yöntem kullanılarak) ve (c) Fare beyin homojenatında sodyum nitroprusiyat (SNP) ile indüklenen lipid peroksidasyona etkisi incelenmiştir.

**Bulgular:** ME, EE'den daha yüksek antioksidan aktiviteye sahiptir. Her iki ekstrakt da yarışmalı asetilkolinesteraz inhibisyonuna neden olmaktadır; ancak ME'nin bu

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inhibitor etkisi EE'den daha yüksektir. ME ve EE'nin inhibitor sabitleri ( $K_i$ ) sırasıyla 28.43 ve 68.47  $\mu\text{g/mL}$  olarak bulunmuştur. Her iki ekstrakt da fare beyin homojenatındaki malondialdehit (MDA) düzeylerinde konsantrasyona bağlı olarak anlamlı bir azalmaya neden olmuş ve bu durum güçlü bir lipid peroksidasyonunun inhibisyonunu göstermektedir.

**Sonuç:** Bu sonuçlar *A. millefolium*'un sahip olduğu yüksek antioksidan kapasite ve anti-asetilkolinesteraz aktivitesinin taşıdığı fenolik bileşiklerden kaynaklandığı ve bu sebeple AD gibi nörodejeneratif hastalıklarda destekleyici tedavi yaklaşımında doğal terapötik bir ajan olarak kullanılabilirliği gösterilmiştir.

**Anahtar kelimeler:** Asetilkolinesteraz; Alzheimer hastalığı; Beyin; Lipid peroksidasyon; Sodyum nitroprusiyat.

## Introduction

*Achillea millefolium*, known as yarrow, is a member of the Asteraceae family and it has been commonly used as a folk medicine for external bleeding, hemorrhoids, headaches, diabetes, eczema, influenza, menstrual disorders and inflammation [1, 2]. This plant contains tocopherols and ascorbic acid and is also rich in flavonoids, apigenin and quercetin, and phenolic acids [3]. Previous studies have revealed that *A. millefolium* has an antiulcerogenic and anti-inflammatory potential due to its antioxidant capacity [4, 5].

Alzheimer's disease (AD) is a progressive neurodegenerative disorder which is characterized by defects in memory and cognitive functions [6]. Accumulation of extracellular senile plaques, intracellular neurofibrillary tangles and acetylcholine (ACh) deficiency are considered to underlie the AD pathophysiology [7]. Also, it has been shown that an AD brain is under excessive oxidative stress leading to lipid peroxidation and accumulation of free radicals with a consequent decrease in cholinergic neurotransmission. These processes are considered to be major contributing factors in early stages of AD [8, 9].

The cholinergic hypothesis is a well-accepted strategy for the symptomatic treatment of AD [10]. Thus, inhibition of the acetylcholinesterase (AChE) enzyme prevents degradation of ACh and increases the cholinergic transmission in cholinergic neurons which are exposed to the most severe stage of damage throughout the progression of AD. Curative research for AD, including cholinergic therapies, prevents memory loss and cognitive function [11]. This phenomenon provides the basis for a generation of AChE inhibitors such as donepezil, galantamine, rivastigmine and tacrine that

increase the ACh levels in an AD brain and possibly provide a symptomatic treatment [12–16]. Since the precise etiology is still unknown and current drugs are only partially effective in slowing the progression of AD, there is an increasing need for the development of new anti-AD drugs.

AChE inhibition kinetics of *A. millefolium* and its effects in biological fluid/tissue are unknown. Moreover, the potential therapeutic usage of *A. millefolium* in neurodegenerative disorders, like AD, that is characterized by ACh deficit and lipid peroxidation in the brain due to its combined antioxidant and anti-AChE activities have not been well investigated. The aim of this work is to investigate the antioxidant capacity, kinetics of AChE inhibition of the *A. millefolium* on SNP-induced lipid peroxidation in mice brain in vitro and phenolic compounds using HPLC-DAD to exhibit its potential therapeutic usage in neurodegenerative diseases such as AD.

## Materials and methods

### Plant material and sample preparation

Samples of *A. millefolium* were collected from Çambaşı, Ordu-Turkey, in September 2015. Taxonomic identification of the plant material was confirmed by Dr. Kamil Coşkunçelebi. The voucher specimen was deposited at the Karadeniz Technical University Faculty of Biology Herbarium [Voucher No: kcoskuncelebi-1214 (KTUB)].

Plants were dried at room temperature and pulverized using an automatic herbal grinder. Pulverized plants (~15 g) were extracted with 50 mL of the solvent (methanol or ethanol) in the shaker for 4 h  $\times$  3, then filtered and the solvent was evaporated under reduced pressure using a rotary evaporator. Crude extracts were kept at +4°C until further use.

### Chemicals and reagents

Abscisic acid, acetonitrile, acetic acid, AChE from an electric eel, acetylthiocholine iodide (AChI), aluminum nitrate ( $\text{Al}(\text{NO}_3)_3$ ), ammonium acetate ( $\text{NH}_4\text{CH}_3\text{COO}$ ), ascorbic acid (AA), benzoic acid, butylated hydroxyanisole (BHA), caffeic acid, 5,5-dithio-bis(2-nitrobenzoic) acid (DTNB), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ethanol, ethylenediaminetetraacetic acid (EDTA), ferulic acid, ferrozine, Folin-Ciocalteu reagent, gallic acid (GA), galantamine, iron(II) chloride ( $\text{FeCl}_2$ ), 2-hydroxycinnamic acid, iron(III) chloride ( $\text{FeCl}_3$ ), L-methionine, methanol, nitroblue tetrazolium



(NBT), thiobarbituric acid (TBA), *trans*-cinnamic acid, trichloroacetic acid (TCA), trisma-base, phosphomolybdic acid, *p*-coumaric acid, potassium ferricyanide  $K_3[Fe(CN)_6]$ , riboflavin, rutin, sodium acetate ( $NaCH_3COO$ ), sodium carbonate ( $Na_2CO_3$ ), sodium dodecyl sulfate (SDS), sodium nitroprusside (SNP) and quercetin (QE) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chlorogenic acid, 4-hydroxybenzoic acid, syringic acid and vanillic acid were obtained from Acros Organic. Protocatechuic acid was purchased from Hwi Analytik GmbH.

## Antioxidant activities

### Total phenolic content

The Folin–Ciocalteu reagent was used to determine the total phenolic content of the extracts according to the method described by Dalar [17]. GA was used as the standard. The extracts were separately mixed with 0.5 N of the Folin–Ciocalteu reagent, distilled water and 10% (w/v)  $Na_2CO_3$ . The mixtures were incubated for 2 h at room temperature in the dark. After incubation, the absorbance was measured at 760 nm. The total phenolic contents of extracts were expressed as mg gallic acid equivalents (GAE) per g dry weight of the sample.

### Total flavonoid content

The total flavonoid content of extracts was determined utilizing an  $Al(NO_3)_3$  assay [18]. QE was used as the standard. The extracts, methanol, 10%  $Al(NO_3)_3$  and 1 M  $NH_4CH_3COO$  were added. The mixtures were incubated for 40 min at room temperature. After incubation, the absorbance was measured at 415 nm. The total flavonoid content of extracts was expressed as mg quercetin equivalents (QEE) per g dry weight of the sample.

### DPPH radical scavenging assay

The DPPH radical scavenging activities of extracts were determined using the method described by Blois and compared to AA and BHA as the standard [19]. The total volume of assay mixture was 1 mL containing a methanolic DPPH solution (0.4 mM) and various concentrations of the extracts. The mixtures were incubated for 30 min at room temperature in the dark. After incubation, the absorbance of the extract ( $A_{\text{extract}}$ ) was measured at 517 nm. The assay mixture without any extract was

used as a control ( $A_{\text{control}}$ ). The inhibition percentage was calculated using formula (1). The scavenging concentrations of 50% of DPPH values ( $SC_{50}$ ) were calculated from the graph of the percentage inhibition against extract concentrations.

$$\text{Scavenging effects (\%)} = \left[ \frac{(A_{\text{control}} - A_{\text{extract}})}{A_{\text{control}}} \right] \times 100. \quad (1)$$

### Superoxide dismutase (SOD) radical scavenging assay

SOD radical scavenging activities of the extracts were determined using a modified spectrophotometrical NBT photoreduction method and comparing to AA and BHA as the standard [20]. The total volume of the assay mixture was 1 mL and contained riboflavin, *L*-methionine, NBT, EDTA and the extracts. After illuminating with a fluorescent lamp at 30°C for 10 min, the absorbance of the extract ( $A_{\text{extract}}$ ) was measured at 560 nm. The assay mixture without the extract was used as a control ( $A_{\text{control}}$ ). The free  $O_2^{\cdot-}$  radical scavenging effect was calculated using formula (1).

### Ferrous ion-chelating assay

Ferrous ion-chelating effects of the extracts were determined by the method of Chua and compared to AA, BHA and EDTA as the standard [21]. The extracts were incubated with a 2 mM  $FeCl_2$  solution. The reaction was initiated by the addition of 5 mM ferrozine into the mixture and left to standing at room temperature for 10 min. The absorbance of the reaction mixture was measured at 562 nm. The ferrous ion chelating capacities were calculated using formula (1).

### Ferric reducing antioxidant power (FRAP) assay

FRAP of extracts was determined using the method described by Oyaizu [22]. Various concentrations of the extracts and BHA as the standard were added to a phosphate buffer (pH 6.6) and 10% (w/v)  $K_3[Fe(CN)_6]$ . The mixtures were incubated at 50°C for 20 min and then 10% TCA was added. After vigorous shaking, the solutions were mixed with distilled water and 0.15%  $FeCl_3$ . The mixtures were incubated for 30 min at room temperature in the dark. After incubation, the absorbance was measured at 700 nm. The FRAP of the extracts was expressed as butylated hydroxyanisole equivalents (BHA-E) per g dry weight of the sample.



### Phosphomolibdenum-reducing antioxidant power (PRAP) assay

PRAP assay of the extracts was determined using phosphomolybdic acid [23]. The total volume of the assay mixture was 1 mL containing 10% phosphomolybdic acid solution in ethanol (w/v) and at various concentrations of the extract. The mixtures were incubated for 30 min at 80°C. After incubation, the absorbance was measured at 600 nm. The PRAP of the extracts was expressed as mg QEE per g dry weight of the sample.

## Enzyme inhibition

### AChE inhibition assay

AChE inhibition was determined using the method described by Ingkaninan et al. [24]. Galantamine was used as the standard. Fifty millimolar Tris-HCl buffer (pH 8.00), 3 mM DTNB (in buffer), 0.2 U/mL AChE and various concentrations of the extracts were added in a 96-well microplate. The mixtures were incubated for 15 min at 25°C. After incubation, 15 mM AChI was added to a microplate and incubated for 5 min at room temperature. The hydrolysis of AChI was recorded spectrophotometrically by the formation of a yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine and the absorbance was measured at 412 nm using a 96-well microplate reader. Inhibition of AChE was calculated by using formula (2).  $A_{\text{control}}$  is the activity of the enzyme without the extract (solvent in buffer pH = 8) and  $A_{\text{extract}}$  is the activity of enzyme with various concentrations of the extracts. The inhibitory concentrations of 50% of AChE values ( $IC_{50}$ ) were calculated from the graph of the percentage inhibition against extract concentrations.

$$\text{Inhibition (\%)} = \left[ \frac{(A_{\text{control}} - A_{\text{extract}})}{A_{\text{control}}} \right] \times 100. \quad (2)$$

### Kinetics analysis of AChE inhibition

The kinetics analysis of the AChE enzyme for the extracts was carried out according to the method by Ingkaninan et al. using different concentrations of AChI (10, 15, 20 and 25 mM) as the substrate and extracts to determine the type of inhibition. The data obtained were plotted as 1/activity (1/V) against 1/substrate concentration (1/[S]), according to Lineweaver-Burke graph [24]. The enzyme activity was calculated from the linear portion of the curve. Furthermore,  $K_i$  was deduced from the Dixon plot which is a

graphical method [plot of 1/enzyme velocity (1/v)] versus the inhibitor concentration [I] with varying concentrations of the substrate (10, 15, 20 and 25 mM) for each inhibitor.

## Lipid peroxidation experiment

### Animals

Adult male BALB/c mice (n=6) weighing 30–40 g were used. They were maintained at room temperature on a 12 h light/12 h dark cycle, with free access to food and water. This study was approved by the Animal Research Local Ethics Committee of Karadeniz Technical University (approval no: 13.07.2016-2016/25).

### Preparation of brain homogenates

Mice were decapitated under ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) anesthesia and whole brains were rapidly isolated. The brains were weighed and placed on ice. Whole brain tissues were homogenated in cold saline (1:10, w/v) with a Teflon-glass homogenizer at 1200 rev/min for 10 min. The homogenates were then centrifuged for 10 min at 4°C 3000×g to yield a pellet that was discarded and the low-speed supernatant fraction that was used for the lipid peroxidation assay [25].

### Lipid peroxidation assay

The lipid peroxidation assay was performed according to Ohkawa et al. [26]. The supernatant fraction was mixed with a reaction mixture containing 0.1 M Tris-HCl buffer (pH 7.4), extracts (250, 500 and 1000 µg/mL) and 7 mM SNP. These mixtures were incubated at 37°C for 1 h. After incubation, 8.1% SDS, acetic acid/HCl (pH 3.4) and 0.8% TBA were added and incubated at 100°C for 1 h. Thiobarbituric acid reactive substances (TBARS) production was measured at 532 nm, and the level of TBARS was expressed as MDA% production with that of a standard MDA curve [27].

## Determination of phenolic compounds using HPLC

### Quantitative determination of phenolic compounds by HPLC-DAD

The phenolic compounds were determined according to the method described by Yıldırım et al. [28]. The prominence



series of a HPLC system (Shimadzu, Kyoto, Japan) consists of a CBM-20A communications bus module, an LC-20AT pump, a DGU-20A5 online degasser, an SIL-20A autosampler, a CTO-10ASVP column oven and an SPD-M20A diode array detector. The system was controlled by LC Solutions 1.25 software. A Zorbax Eclipse XDB-C8 column (150 mm, 4.6 mm, 5  $\mu$ m) was used for separation. The mobile phase was consisted of A (phosphate buffer, 20 mM, pH: 2.5) and B (100% acetonitrile). The gradient elution was: 0–11 min, linear from 5% to 8% B; 11–15 min, linear from 8% to 20% B; 15–25 min, linear from 20% to 80% B. The mobile phase was pumped at the flow rate of 1.5 mL/min at 40°C. The sample injection volume was 20  $\mu$ L. Phenolic compounds were detected at 280 nm. The column was equilibrated with an initial mobile phase (5% B) for 10 min between injections.

## Statistical analysis

The experiments were done in triplicates and the results were expressed as the mean  $\pm$  standard deviation (SD). The statistical analysis was performed with Microsoft Excel for Windows 10 and GraphPad Prism 5.0. The differences among the extracts were evaluated by a one-way analysis of variance (ANOVA) followed by Bonferroni tests. *p*-Value <0.05 was considered statistically significant.

## Results

In this study, we determined total phenolic and total flavonoid contents of ME and EE and the results were represented in Table 1. The total phenolic contents of ME and EE were  $53.11 \pm 0.35$  mg GAE/g dry weight and  $19.40 \pm 0.13$  mg GAE/g dry weight, respectively. ME ( $35.88 \pm 1.26$  mg QE/g dry weight) had a higher flavonoid content than EE ( $14.62 \pm 0.27$  mg QE/g dry weight).

The results of DPPH radical scavenging activities are shown in Table 1. The extracts showed moderate

DPPH radical scavenging activities with  $SC_{50}$  values of  $0.266 \pm 0.003$  mg/mL for ME and  $0.495 \pm 0.005$  mg/mL for EE, when compared to AA and BHA used as the standard.

The results of SOD radical scavenging activities are presented in Table 1. The extracts indicate moderate SOD radical scavenging activities with  $SC_{50}$  values of  $0.124 \pm 0.004$  mg/mL for ME and  $0.174 \pm 0.006$  mg/mL for EE, when compared to AA and BHA used as the standards.

The results of the ferrous ion-chelating activities of ME and EE are summarized in Table 1.  $SC_{50}$  values of the ferrous-ion chelating activities of extracts were  $0.317 \pm 0.007$  mg/mL for ME and  $0.549 \pm 0.009$  mg/mL for EE. These results showed that ME and EE have higher chelating activities compared to AA ( $0.844 \pm 0.014$  mg/mL) and BHA ( $0.939 \pm 0.003$  mg/mL), whereas the extracts have lower activities than EDTA ( $0.014 \pm 0.005$  mg/mL).

The FRAP results of the extracts are summarized in Table 2. The FRAP of extracts is expressed as  $\mu$ M BHA/g per g of dry weight sample. The ME ( $258.66 \pm 2.48$   $\mu$ M BHA/g dry weight) was similar power as the EE ( $216.44 \pm 1.85$   $\mu$ M BHA/g dry weight).

The PRAP of ME and EE is expressed as mg QEE per g of dry weight sample and the results are summarized in Table 2. PRAP of ME ( $74.50 \pm 1.83$  mg QEE/g dry weight) was higher than that of EE ( $50.50 \pm 0.41$  mg QEE/g dry weight) due to the presence of phenolic and flavonoid compounds.

In this study, the AChE inhibition results of the extracts are expressed as  $IC_{50}$  values and they are presented in Table 3. Lower  $IC_{50}$  values result from the higher inhibition of AChE. The  $IC_{50}$  values of ME and EE were  $105.05 \pm 2.63$  and  $224.33 \pm 3.72$   $\mu$ g/mL, respectively. These results showed ME was about two-fold more potent than EE. In addition, we determined the kinetic inhibitions of extracts by using Lineweaver-Burk plots, as seen in Figure 1. Our results indicate that the inhibition type of ME and EE is competitive. The competitive inhibitors compete with the substrate for the active site of an enzyme. While the inhibitor occupies the active site, it prevents binding of the substrate to the enzyme. As shown in Figure 2, the  $K_i$

**Table 1:** Total phenolic content, total flavonoid content and radical scavenging activities of extract.

Extracts	Total phenolic content (mg GAE/g dry weight)	Total flavonoid content (mg QE/g dry weight)	DPPH (mg/mL)	SOD (mg/mL)	Ferrous ion-chelating (mg/mL)
ME	$53.11 \pm 0.35$	$35.88 \pm 1.26$	$0.266 \pm 0.003$	$0.124 \pm 0.004$	$0.317 \pm 0.007$
EE	$19.40 \pm 0.13$	$14.62 \pm 0.27$	$0.495 \pm 0.005$	$0.174 \pm 0.006$	$0.549 \pm 0.009$
AA	–	–	$0.054 \pm 0.002$	$0.096 \pm 0.003$	$0.844 \pm 0.014$
EDTA	–	–	–	–	$0.014 \pm 0.005$
BHA	–	–	$0.027 \pm 0.02$	$0.073 \pm 0.005$	$0.939 \pm 0.003$

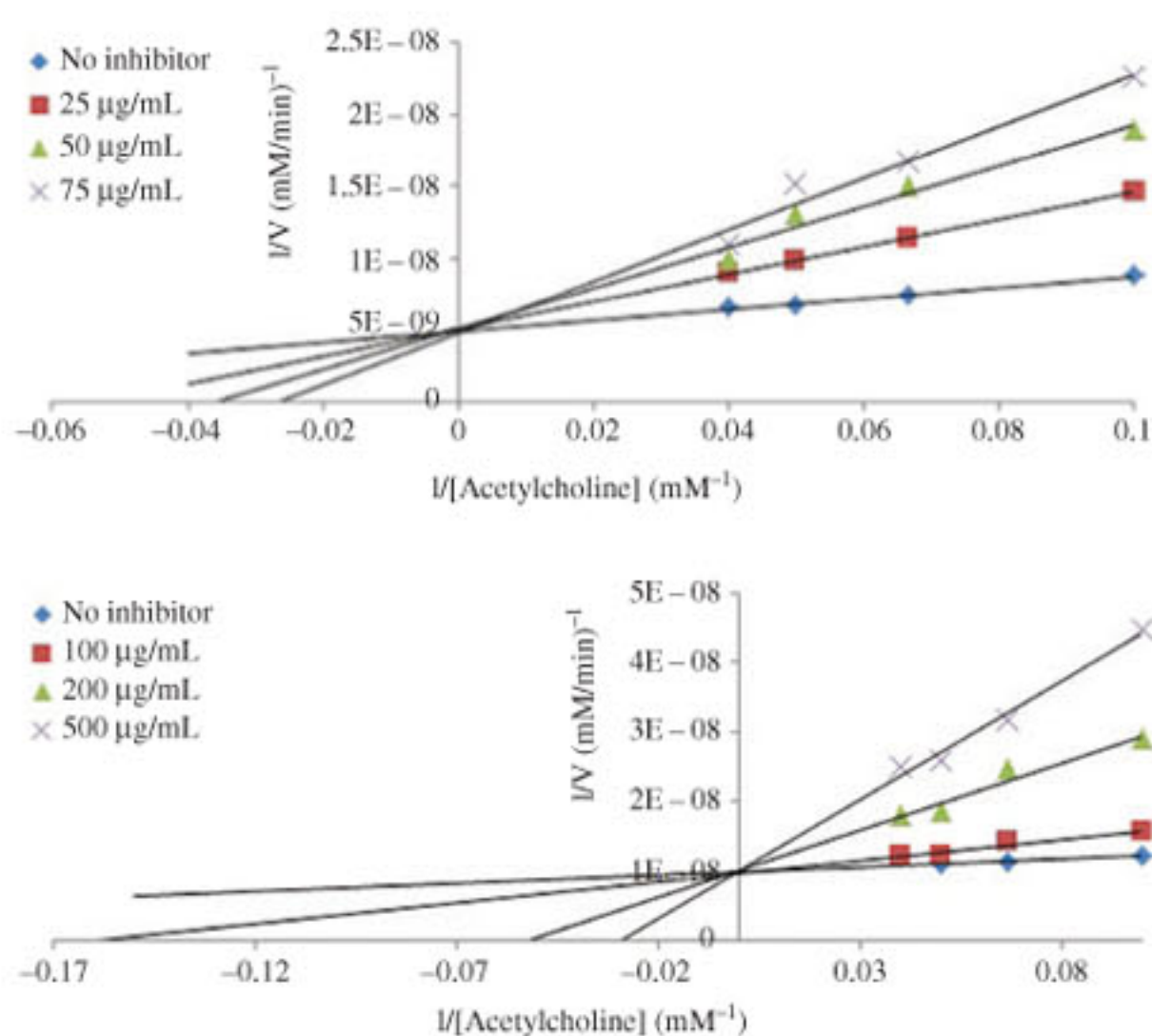


**Table 2:** Reducing antioxidant power of extracts.

Extracts	FRAP ( $\mu\text{M BHA E/g}$ dry weight)	PRAP (mg QE/g dry weight)
ME	$258.66 \pm 2.48$	$74.50 \pm 1.83$
EE	$216.44 \pm 1.85$	$50.50 \pm 0.41$

**Table 3:** Inhibitory effect and kinetic analysis of extracts on AChE activity.

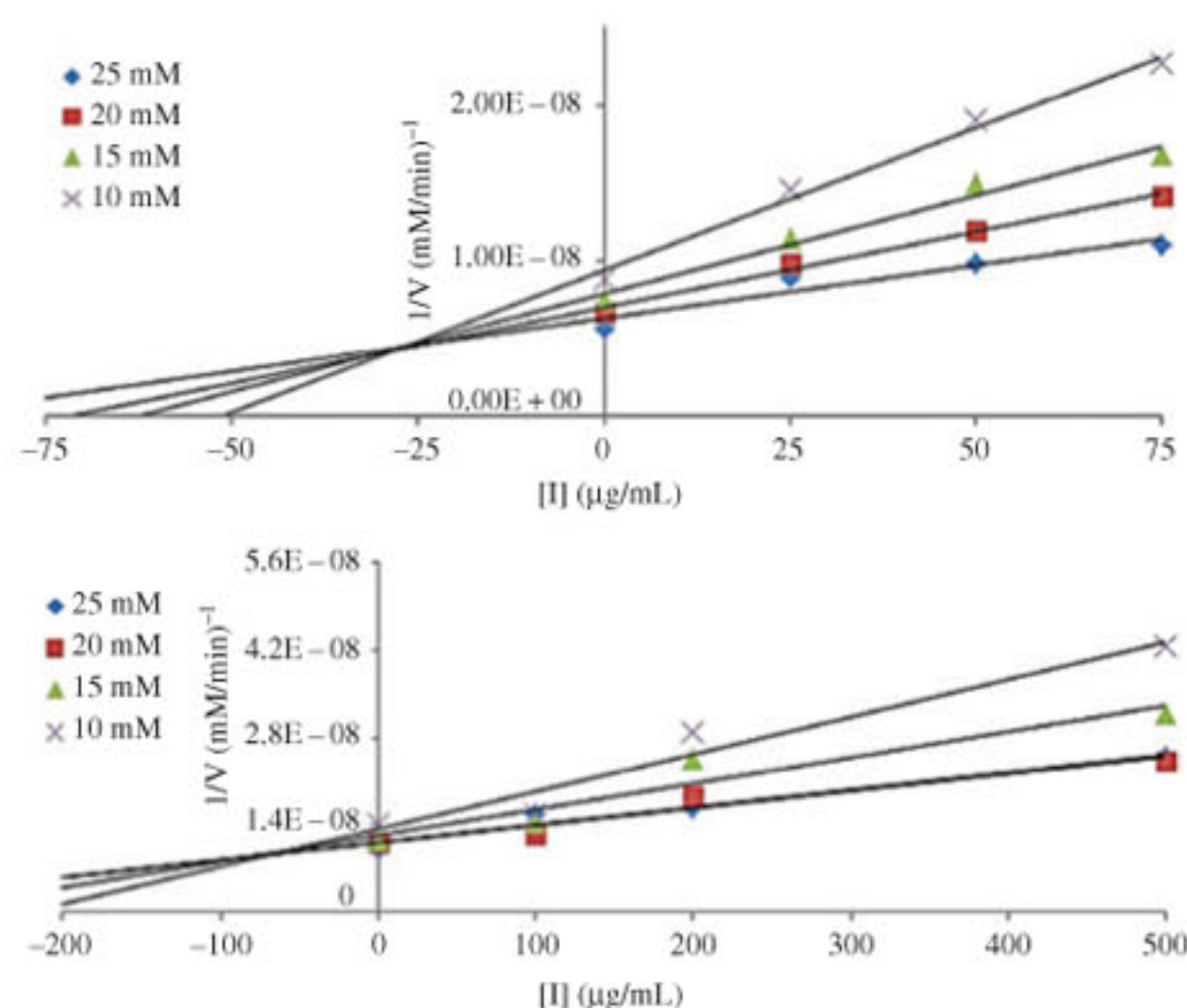
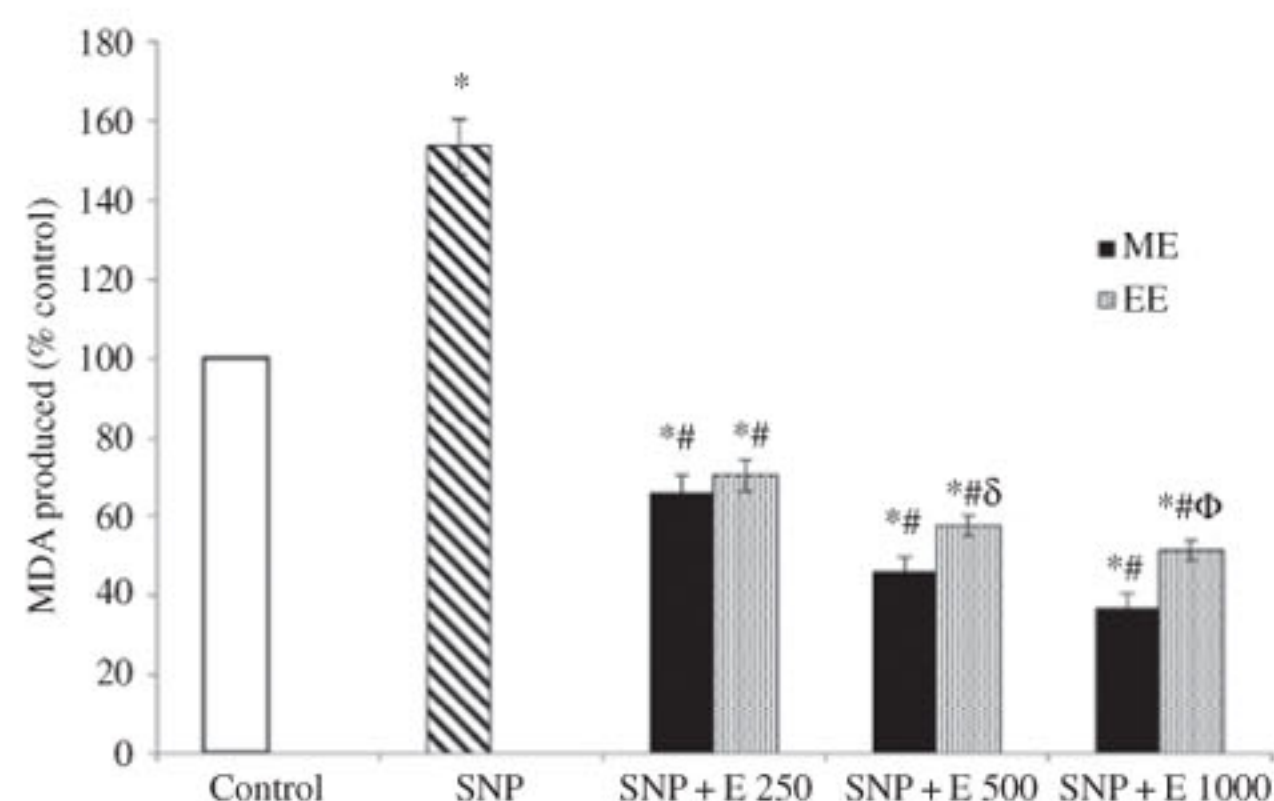
Extracts	$\text{IC}_{50}$ ( $\mu\text{g/mL}$ )	Type of inhibition	$K_i$ ( $\mu\text{g/mL}$ )
ME	$105.05 \pm 2.63$	Competitive	28.43
EE	$224.33 \pm 3.72$	Competitive	68.47
Galantamine	$17.05 \pm 0.01$	Competitive	5.20

**Figure 1:** Lineweaver-Burk plot for inhibition of ME and EE on AChE.

values of the ME, EE and galantamine are 28.43, 68.47 and  $5.20 \mu\text{g/mL}$ .

The results of a lipid peroxidation assay in the brain are shown in Figure 3. MDA production in the brain increased in the presence of SNP ( $153.79 \pm 6.94\%$ ). However, the addition of ME and EE concentration-dependently inhibited MDA production in the brain homogenates. Both extracts showed a significant ( $p < 0.05$ ) decrease in the MDA content in the brain. At concentrations of 125, 250 and  $500 \mu\text{g/mL}$  of ME, the MDA content was  $66.11\% \pm 4.36\%$ ,  $46.11\% \pm 3.47\%$  and  $36.71\% \pm 3.86\%$ , respectively. At the same concentrations of EE, the MDA contents was  $70.45\% \pm 3.91\%$ ,  $57.72\% \pm 2.65\%$  and  $51.30\% \pm 2.46\%$ , respectively.

In HPLC analysis, phenolic compounds were identified by comparing the retention times and UV-spectra of

**Figure 2:** Dixon plot for the inhibitory effect of ME and EE AChE.**Figure 3:** Inhibition of SNP induced oxidative stress in mice brain by ME and EE of *A. millefolium*.

Values are represented as a mean  $\pm$  standard deviation ( $n = 6$ ).

\* Means  $p < 0.001$  when compared to control group, # means  $p < 0.001$  when compared to the SNP group,  $\delta$  means  $p < 0.05$  when compared to ME500 group and  $\Phi$  means  $p < 0.05$  when compared to ME1000 group according to one-way ANOVA–Bonferroni test. SNP, SNP-induced lipid peroxidation; E 250,  $250 \mu\text{g/mL}$  extracts concentration; E 500,  $500 \mu\text{g/mL}$  extracts concentration; E 1000,  $1000 \mu\text{g/mL}$  extracts concentration.

unidentified peaks with those of standards. If UV-spectra of the solutes did not comply with pure compounds, the peaks were disregarded. The results are presented in Table 4. Protocatechuic acid and chlorogenic acid were determined in the ME at the concentrations of  $4.5 \pm 0.01$  and  $147 \pm 1.05 \mu\text{g/g}$  dry weight, respectively. On the other hand, protocatechuic acid, chlorogenic acid, vanillic acid and caffeic acid were determined in the EE the concentrations of  $11.10 \pm 0.04 \mu\text{g/g}$ ,  $27.59 \pm 0.09 \mu\text{g/g}$ ,  $10.44 \pm 0.05 \mu\text{g/g}$  and  $19.77 \pm 0.1 \mu\text{g/g}$  dry weight, respectively.



**Table 4:** Contents (mean  $\pm$  SD) of protocatechuic acid, chlorogenic acid, vanilic acid and caffeic acid found in ME and EE (results for extracts were expressed in  $\mu\text{g/g}$  dry weight) ( $n=3$ ).

Extracts	Protocatechuic acid	Chlorogenic acid	Vanilic acid	Caffeic acid
EE	4.5 $\pm$ 0.01	147.00 $\pm$ 1.05	ND	ND
ME	11.10 $\pm$ 0.04	27.59 $\pm$ 0.09	10.44 $\pm$ 0.05	19.77 $\pm$ 0.1

## Discussion

Oxidative damage induced by the reactive oxygen species (ROS) such as superoxide anion ( $\text{O}_2^{\cdot-}$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and hydroxyl radical ( $\cdot\text{OH}$ ) have been considered as major causative factors in the pathogenesis of many diseases such as cancer, diabetes, gastrointestinal ulcerogenesis, neurodegenerative and cardiovascular diseases [29, 30].

Plant extracts may have a potential to prevent or control ROS generation due to their content of phenolic and volatile compounds [31]. Phenolic compounds can easily donate hydrogen from hydroxyl groups positioned along the aromatic ring to terminate free radical oxidation of biomolecules, thereby they are able to prevent the harmful effects of ROS [32].

In this study, the results of total phenolic and total flavonoid contents of ME and EE were shown in Table 1. Similar to our findings, Sevindik et al. [1] reported that the total phenolic content of methanol extract of *A. millefolium* was 77.78  $\mu\text{g}$  GAE/mg extract, while Alexandru et al. [33] reported that the total phenolic content of ethanol extract of *A. millefolium* was 12.43 mg chlorogenic acid equivalents (ChAE/g dry extract). The differences in the results of these studies are likely due to differences in the extraction method, standards, geographical location and climate conditions.

DPPH assay, one of the well-established methods to measure the antioxidant activity, is based on the ability of the radical to react with hydrogen donors and generate stable diamagnetic molecules [34]. Candan et al. [35] reported that methanol extracts of *A. millefolium* scavenged DPPH radical with  $\text{SC}_{50}$  values of 45.60  $\pm$  1.30  $\mu\text{g/mL}$ . In addition, Dias et al. [3] reported that  $\text{SC}_{50}$  values of methanol extracts of wild and commercial *A. millefolium* were 0.50  $\pm$  0.01 mg/mL and 0.37  $\pm$  0.01 mg/mL, respectively. Candan et al. [35] reported that  $\text{SC}_{50}$  values of methanol extracts of *A. millefolium* were found to be 304.00  $\pm$  5.10  $\mu\text{g/mL}$  in SOD radical scavenging assay.

Transition metals such as iron cause cellular damage by inducing free radical production in a biological system, thus, the ferrous ion-chelating capacity as antioxidant is important to protect against ROS-related diseases [36]. In

the same line as our results, Keser et al. [37] reported that the metal chelating activity of ethanol extract of *A. millefolium* at 100  $\mu\text{g/mL}$  was determined to be 22.64%.

FRAP assay is a fast and simple method to determine the antioxidant activity of samples based on their electron transfer reaction [38]. Consistent with our findings, Navaie et al. [39] reported that the antioxidant activity of the methanol and ethanol extracts of *A. millefolium* flower was measured by FRAP and determined to be 54.79  $\pm$  11.75  $\mu\text{M}$  AA/g dry weight and 37.20  $\pm$  7.06  $\mu\text{M}$  AA/g dry weight, respectively. PRAP assay is based on the reduction of Mo(VI) to Mo(V) by the antioxidants and subsequent production of a green phosphate/Mo(V) complex [40]. To our knowledge, there has no report on the PRAP activities of *A. millefolium* extracts.

AChE inhibition is a well-established theory in AD pathophysiology. Thus, the inhibition of the AChE, an enzyme that hydrolyses the ACh, is a major strategy in the symptomatic treatment of AD. Inhibition of the AChE is a reasonable approach to increase ACh levels in the central nervous system [41, 42]. In the past few decades, many studies have focused on developing drugs that inhibit AChE such as galantamine, rivastigmine and donepezil for AD treatment [43, 44]. However, their therapeutic usages have some limitations due to their side effects including nausea, vomiting, diarrhea, abdominal pain, muscle cramp and hepatotoxicity [10]. This current clinical reality provides the basis for anti-Alzheimer activity with less severe side effects. The results of anti-AChE of ME and EE were shown in Table 3. Sigurdsson and Gudbharnason [45] reported that ethanol extract of *A. millefolium* from Iceland did not show any anti-AChE activity while Mekinić et al. [46] reported that 80% ethanol extract of *A. millefolium* from Croatia showed significant anti-AChE activity. In addition, Sevindik et al. [1] reported that anti-AChE activities of different extracts of *A. millefolium* at 25  $\mu\text{g/mL}$  were found in the range of 29.1%  $\pm$  1.84% to 51.30%  $\pm$  0.11% due to presence of a sugar and catechol moiety.

The kinetics inhibition of *A. millefolium* on AChE has not previously been investigated. Our results suggest that there is a relationship between the total phenolic content and AChE inhibition because there is a structural similarity between natural polyphenolic compounds and AChE



inhibitors in terms of molecular weight, phenolic rings and hydrophobic component [47].

SNP belongs to a class of drugs known as nitric oxide (NO)-releasing agents that are a potent hypotensive agents and it works by releasing NO. It is a universal neuronal messenger in the cardiovascular and central nervous system (CNS). Although SNP does not pass the blood-brain barrier, NO which is released by SNP can react with ROS such as superoxide, generating a powerful oxidant peroxynitrite which initiates lipid peroxidation in CNS and causes neuronal death [27, 48, 49]. Therefore generally SNP has been used to induce lipid peroxidation in vitro experimental AD studies [25, 47]. To our knowledge, there has no report on the inhibitory effects on SNP-induced lipid peroxidation in mice brain homogenate of *A. millefolium*.

To determine the type of phenolic compounds, the ME and EE were subjected to subsequent HPLC analysis. Triplicate analysis of the ME and EE were performed according to the method reported by Yıldırım et al. [28]. Protocatechuic acid and chlorogenic acid were determined in the ME. On the other hand, protocatechuic acid, chlorogenic acid, vanillic acid and caffeic acid were determined in the EE. The obtained results are in agreement with findings reported elsewhere. Tadic et al. [50] determined chlorogenic acid and caffeic acid and different flavonoids in *A. millefolium* extracts. Additionally, Georgieva et al. [51] detected various phenolic acids in *A. millefolium* extracts, including vanillic acid, caffeic acid and chlorogenic acid.

Several studies have suggested that some phenolic compounds have inhibitory effect on AChE [47]. Amin et al. [52] demonstrated that vanillic acid displays a neuroprotective effect against A $\beta$ 1-42-induced neurotoxicity. Thus, vanillic acid can be considered as a neuroprotective agent against AD. Oboh et al. [47] reported chlorogenic acid can be an effective compound in the treatment of AD due to its neuroprotective potential. The higher AChE inhibitory activity of ME can be due to its higher concentration of chlorogenic acid compared to that of the EE.

## Conclusion

In the present study, we showed that ME and EE of *A. millefolium* have antioxidant effects (total phenolic and total flavonoid contents, DPPH, SOD, ferrous ion-chelating effect, FRAP and PRAP), AChE inhibitory and protective effect against SNP-induced lipid peroxidation in mice brains. ME has higher total phenolic, flavonoid content and antioxidant activity compared to that of EE. Both extracts inhibited AChE activity in vitro and ME was

found to be more potent than EE. The inhibition type of ME and EE was determined as competitive. Protocatechuic acid, chlorogenic acid, vanillic acid and caffeic acid were detected in EE whilst protocatechuic acid and chlorogenic acid were detected in ME. Both extracts decreased the SNP-induced lipid peroxidation in mice brain in a concentration-dependent manner. This protective effect could be attributed to its bioactive phenolic compounds. Our results suggest that *A. millefolium* might be effective for prevention or treatment of AD. Further studies are required to clarify the potential therapeutic effect of *A. millefolium* for AD.

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