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ORIGINAL ARTICLE

Cholinesterase and Tyrosinase Inhibitory Potential and Antioxidant Capacity of *Lysimachia verticillaris* L. and Isolation of the Major Compounds

Lysimachia verticillaris L.'nin Kolinesteraz ve Tirozinaz İnhibitör Etki Potansiyeli ve Antioksidan Kapasitesi ile Ana Bileşiklerinin İzolasyonu

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ABSTRACT

Objectives: The scope of the present study was to specify the therapeutic potential for neurodegenerative diseases through evaluating cholinesterase and tyrosinase (TYR) inhibitory and antioxidant activity of *Lysimachia verticillaris* (LV), and to isolate the major compounds considering the most active fraction.

Materials and Methods: The methanol extract (ME) of LV and the chloroform, ethyl acetate (EtOAC), and aqueous fractions obtained from it were used for biological activity and isolation studies. The ME and all fractions were tested for their acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and TYR inhibitory and antioxidant potentials using ELISA microtiter assays. Seven major compounds were isolated from the active EtOAC fraction by semi-preparative high performance liquid chromatography. The structures of the compounds were elucidated by several spectroscopic methods.

Results: Marked AChE inhibitory activity was observed in the EtOAC fraction (6337±1.74%), BChE inhibitory activity in the ME and EtOAC fraction (85.84±3.01% and 83.82±3.93%), total phenol content in the EtOAC fraction (261.59±3.95 mg equivalent of gallic acid/1 g of extract) and total flavonoid contents in the EtOAC fraction (515.54±2.80 mg equivalent of quercetin/1 g of extract), and 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity and ferric-reducing antioxidant power values in the aqueous and EtOAC fractions (92.54±0.67%, 92.11±0.30%; 2.318±0.054, 2.224±0.091, respectively). Accordingly, the isolation studies were carried out on the EtOAC fractions. Compounds 1-7 (gallic acid, (+)-catechin, myricetin 3-O-arabinofuranoside, myricetin 3-O-α-rhamnopyranoside, quercetin 3-O-β-glucopyranoside, quercetin 3-O-arabinofuranoside, and quercetin 3-O-α-rhamnopyranoside, respectively) were isolated from the active EtOAC fraction.

Conclusion: LV may be a potential herbal source for treatment of neurodegenerative diseases based on its strong antioxidant activity and significant cholinesterase inhibition similar to that of the reference.

Key words: Anticholinesterase, HPLC, isolation, *Lysimachia*, tyrosinase

ÖZ

Amaç: Bu çalışmanın amacı, *Lysimachia verticillaris*'in (LV) kolinesteraz, tirozinaz inhibitör etkisini ve antioksidan aktivitesini değerlendirerek nörodejeneratif hastalıklar için terapötik potansiyelini belirlemek ve en etkili fraksiyondan hareketle ana bileşiklerini izole etmektir.

Gereç ve Yöntemler: Biyolojik aktivite ve izolasyon çalışmaları için LV'nin metanol ekstresinden hareketle kloroform, EtOAC ve sulu fraksiyonları elde edilmiştir. Etkili EtOAC fraksiyonundan, yarı preparatif yüksek performanslı sıvı kromatografisi (YBSK) yöntemi ile 7 ana bileşik izole edilmiştir. İzole edilen bileşiklerin yapıları çeşitli spektroskopik yöntemler kullanılarak aydınlatılmıştır. Metanol ekstresi (ME) ve tüm fraksiyonların asetilkolinesteraz (AChE), butirilkolinesteraz (BChE), tirozinaz inhibitor etkileri ve antioksidan potansiyelleri ELISA yöntemleri kullanılarak belirlenmiştir.

Bulgular: En yüksek AChE inhibitor etki EtOAc fraksiyonunda (%63,37±1,74), en yüksek BChE inhibitör etki ME'de ve etil asetat fraksiyonunda

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(%85,84±3,01 ve %83,82±3,93), en yüksek total fenolik içeriği etil asetat fraksiyonunda (261,59±3,95 mg gallik asit eşdeğeri/g ekstre), en yüksek total flavonoit içeriği etil asetat fraksiyonunda (515,54±2,80 mg kersetin eşdeğeri/1 g ekstre) gözlenmiştir. En yüksek 2,2-difenil-1-pikrilhidrazil radikal süpürücü etki ve demir azaltıcı antioksidan güç değerleri ise su ve EtOAC fraksiyonlarında sırasıyla %92,54±0.67, %92,11±0,30 ve 2,318±0,054 2,224±0,091 olarak belirlenmiştir. Aktivite sonuçlarına dayanarak izolasyon çalışmalarının etil asetat fraksiyonunda yürütülmesine karar verilmiştir. Etil asetat fraksiyonundan, gallik asit (1), (+) kateşin (2), mirsetin 3-O-arabinofuranozit (3), mirsetin 3-O-α-ramnopiranozit (4), kersetin 3-O-β-glukopiranozit (5), kersetin 3-O-arabinofuranozit (6) ve kersetin 3-O-α-ramnopiranozit (7) ana bileşikleri izole edilmiştir.

Sonuç: LV, güçlü antioksidan aktivitesiye sahip olması ve referans bileşiklerle karşılaştırıldığında benzer kolinesteraz inhibitor etki göstermesi nedeniyle nörodejeneratif hastalıkların tedavisi için potansiyel bir bitkisel ilaç kaynağı olabilir.

Anahtar kelimeler: Antikolinesteraz, YBSK, izolasyon, *Lysimachia*, tirozinaz

INTRODUCTION

Neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD), common in the elderly population over the age of 65, have become serious health problems especially in industrialized countries. Hence, a huge amount of research is being conducted to find new drugs and treatment strategies for these diseases. In this sense, natural products and medicinal herb extracts are attractive sources in the search for novel anti-AD and anti-PD drug candidates. Deficiency in acetylcholine, hydrolyzed by acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), has been proved in the brains of AD patients.[1] On the other hand, the function of tyrosinase (TYR) is to catalyze the oxidation reaction of tyrosine to melanin, which is linked to hyperpigmentation of skin, occurrence of melanoma, unwanted browning of fruits and vegetables, and dopamine toxicity in PD.[2]

The genus *Lysimachia* (Primulaceae) is represented by 8 taxa in the Turkish flora.[3] *Lysimachia* species, locally known as "*karga otu*", "*adi karga otu*", and "*altın kamış*" in Turkey, have been recorded to be used for expectorant, antipyretic, and wound healing purposes as well as against cough and bronchitis in Anatolian folk medicine.[4] *Lysimachia* species contain assorted secondary metabolites including flavonoids, triterpenes, phenolic acids, etc.[5-7] Moreover, several *Lysimachia* species have many desirable biological activities such as cytotoxic, hepatoprotective, and vasorelaxant.[8-10]

Based on the information that *Lysimachia monnieri* is a synonym of *Bacopa monnieri*, the reputed plant called "brahmi" in Ayurvedic medicine for its strong memory-enhancing effect, we aimed to study the memory-enhancing effect of another species, *L. verticillaris*. For this purpose, in the current study, AChE, BChE, and TYR inhibitory activity and antioxidant potential including 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and ferric-reducing antioxidant power (FRAP) were studied in the methanol extract (ME) and all fractions. The EtOAC fraction that showed remarkable anticholinesterase and antioxidant effect was subjected to various chromatographic methods, which gave seven pure compounds (1-7). The structures of the compounds were identified by means of ^{1}H-NMR and ^{13}C-NMR. In addition, the total phenol and flavonoid quantities in the samples were measured spectrophotometrically.

MATERIALS AND METHODS

Instruments and chemicals

NMR spectra were obtained on a Varian-600 spectrometer at 600 MHz for ^{1}H-NMR and 150 MHz for ^{13}C-NMR using CD_3OD as solvent. An Agilent-1100 series was used for high-performance liquid chromatography (HPLC) studies (Germany). A Unico 4802 ultraviolet (UV)-visible spectrophotometer (USA) was used for antioxidant activity, total phenol, and flavonoid contents studies. A Supelco Ascentis® RP-amide (250x10 mm, 5 µm) column, HPLC grade acetonitrile and methanol (Scharlau Chemie S.A., Spain), formic acid (Lachema, Brno, CZ), Sephadex LH-20 (Sigma-Aldrich), silica gel 60 (Merck 7734 and Merck 9385), LiChroprep RP-18 (Merck 9303), and silica gel 60 F254 (Merck 5554) were used for the isolation and chromatographic studies.

To measure the enzyme inhibition assays, a 96-well microplate reader (VersaMax Molecular Devices, USA), electric eel AChE (Type-VI-S, EC 3.1.1.7, Sigma), horse serum BChE (EC 3.1.1.8, Sigma), acetylthiocholine iodide and butyrylthiocholine chloride (Sigma, USA), 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB, Sigma, USA), galantamine (Sigma, USA), TYR (EC 1.14.1.8.1, 30 U, mushroom tyrosinase, Sigma), and L-DOPA were used.

Plant material

The aerial parts of *Lysimachia verticillaris* (LV) were gathered in the vicinity of Kafkasör at an altitude of 1300 m (Artvin Province, Turkey). The identification of the plant was performed by Dr. Ufuk Özgen. A voucher specimen (AEF 26311) is deposited at the Herbarium of the Faculty of Pharmacy.

Extraction

Air-dried and finely powdered samples (500 g) were extracted with MeOH (2 Lx8 h, three times). The combined extracts were evaporated to obtain 74 g of the crude residue. The ME (73 g) was suspended in an H_2O:MeOH (9:1) mixture and then partitioned with chloroform (300 mLx2) and EtOAc (300 mLx2) successively. The chloroform and EtOAc fractions were evaporated at reduced pressure at 40 °C and were 15.6 g and 6.6 g, respectively. The aqueous phase was evaporated to give a residue (46.3 g). The ME and all fractions obtained from it were employed in the activity assays performed herein.

AChE and BChE inhibitory activities

The enzyme inhibitory activities were evaluated by a modified version of the method developed by Ellman et al.[11,12]

Tyrosinase inhibitory activity

The modified dopachrome method was used by measuring at 475 nm.[13] The results were compared with a control consisting of 50% dimethyl sulfoxide in place of sample.

DPPH radical scavenging activity

The activities of the samples were detected by Blois' method.[14] Absorbance was measured at 520 nm.

Ferric-reducing antioxidant power assay

The FRAP values were determined by the assay described by Oyaizu.[15] The absorbance was monitored at 700 nm.

Total phenol and flavonoid contents

Phenolic content of the samples was determined using the Folin-Ciocalteu method.[16] Absorbance was read at 760 nm.

The total flavonoid content of the samples was measured by aluminum chloride colorimetric method.[17]

Statistical analysis

The statistical analysis of the enzyme inhibition and antioxidant capacity assays was conducted using Softmax PRO 4.3.2.LS software. The data were expressed as mean ± scanning electron microscopy. Statistical differences between 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 (*$p<0.05$; **$p<0.01$; ***$p<0.001$, ****$p<0.0001$).

Isolation of the major compounds from LV by combined open column chromatography and semi-preparative HPLC

The EtOAC fraction was applied to column chromatography using a silica gel 60 and $CHCl_3$: MeOH (80:20→50:50) solvent system. The subfractions 5-14 combined were subjected to a semi-preparative HPLC column, which yielded seven compounds (1-7) (Figure 1). The flow rate of the solvent was adjusted to 4.0 mL/min. The mobile phase composition was linear gradient; 0 min: 40% MeOH +60% formic acid (0.2% in aqueous solution); 36 min: 65% MeOH +35% formic acid (0.2% in aqueous solution). UV-DAD detection was performed at 280 nm. The column temperature was 50 °C.

Structure elucidation of the isolated compounds

Structure elucidation of the compounds was performed by ^{1}H-NMR, ^{13}C-NMR, and ESI/MS, which was confirmed finally by comparison of the results with the reported data.

RESULTS

AChE, BChE, and TYR inhibitory activity

The ME and all fractions were tested for their enzyme inhibitory activity against AChE, BChE, and TYR. The aqueous fraction was inactive against both AChE and BChE, while the ME and EtOAc and chloroform fractions of LV showed high degrees of inhibition against AChE, having 58.21±3.36%, 63.37±1.74%, and 41.63±0.45% inhibition, respectively. Although the ME (85.84±3.01%) and EtOAC fraction of LV (83.82±3.93%) exhibited very high BChE inhibitory activity, similar to galantamine (86.66±2.72%), the chloroform fraction exhibited lower BChE inhibition (54.65±0.23%). All of the fractions and the ME displayed weak inhibition toward TYR, ranging between 14.11±1.00% and 16.10±2.14% (Table 1).

Antioxidant activity

In the DPPH radical quenching activity test, the chloroform fraction of LV had moderate activity (63.66±0.57%), whereas high activity against DPPH radical was observed in the aqueous

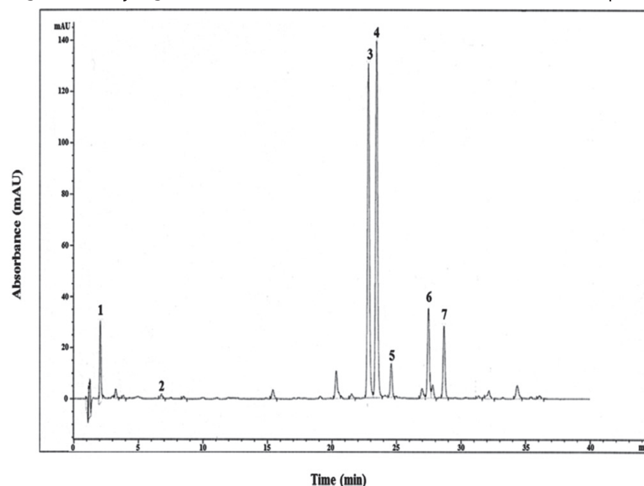

Figure 1. HPLC chromatogram of the EtOAc fraction
HPLC: High-performance liquid chromatography, EtOAc: Ethyl acetate, Min: Minute

Table 1. TYR, AChE, and BChE inhibitory activity of the methanol extract and EtOAC, chloroform, and aqueous fractions

Samples	Inhibitory level (% ± SEM[a]) at 100 µg/mL		
	TYR	AChE	BChE
MeOH Extract	14.11±1.00****	58.21±3.36**	85.84±3.01
EtOAc Fr.	16.10±2.14****	63.37±1.74**	83.82±3.93
Chloroform Fr.	15.33±0.97****	41.63±0.45****	54.65±0.23***
Aqueous Fr.	14.31±0.98****	-[b]	-[b]
Galantamine[c]		94.48±3.81	86.66±2.72
Kojic acid[d]	85.44±0.14		

[a]Standard error mean, [b]No inhibition, [c]Reference for inhibitory activity against AChE and BChE, [d]Reference for inhibitory activity against tyrosinase, (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$), TYR: Tyrosinase, AChE: Acetylcholinesterase, BChE: Butyrylcholinesterase, EtOAc: Ethyl acetate, SEM: Scanning electron microscopy

(92.54±0.67%) and EtOAc fractions (92.11±0.30%). On the other hand, the aqueous and EtOAc fractions possessed the highest FRAP values, which are higher than that of quercetin used as the reference (Table 2).

Total phenol and flavonoid contents

The total phenol contents of the chloroform, EtOAC, and aqueous fractions and ME were stated as gallic acid equivalent (GAE, mg/g extract), while their total flavonoid contents were stated as quercetin equivalent (QUE, mg/g extract). The richest total phenol content belonged to the EtOAC fraction (261.59±3.95 mg/g extract). Similarly, the EtOAc fraction was also found to have the most abundant total flavonoid content (515.54±2.80 mg/g extract) (Table 3).

Identification of the compounds isolated from the active EtOAC fraction

Compounds 1-7 were isolated from the active EtOAC fraction (Figure 2); they were isolated from LV for the first time. The NMR data for all compounds are given as ^{1}H-NMR (600 MHz, CD_3OD) and ^{13}C-NMR (150 MHz, CD_3OD):

Compound 1

Grayish powder. ^{1}H-NMR: δ 6.94 (*s*, 2H, H-2 and H-6). ^{13}C-NMR: δ 169.45 (-COOH), 144.91 (2C, C-3 and C-5), 137.90 (C-4), 121.25 (C-1), 108.84 (2C, C-2 and C-6). NMR data are in total agreement with the data for gallic acid.[18]

Compound 2

Grayish powder. ^{1}H-NMR: δ 6,73 (*d*, 1H, *J*=2.0 Hz, H-2′), 6.65 (*d*, 1H, *J*=8.1 Hz, H-5′), 6.62 (*dd*, 1H, J_1=2.0, J_2=8.1 Hz, H-6′), 5.82 (*d*, 1H, *J*=2.4 Hz, H-8), 5.75 (*d*, 1H, *J*=2.4 Hz, H-6), 4.46 (*d*, 1H, *J*=7.6 Hz, H-2), 3.87 (*td*, 1H, H-3), 2.75 (*dd*, 1H, J_1=5.3, J_2=15.9 Hz, H-4a), 2.40 (*dd*, 1H, J_1=8.2, J_2=15.8 Hz, H-4b). ^{13}C-NMR: δ 156.42 (C-7), 156.16 (C-5), 155.49 (C-9), 144.83 (C-3′ or C-4′), 144.80 (C-3′ or C-4′), 130.79 (C-1′), 118.59 (C-6′), 114.63 (C-5′), 113.82 (C-2′), 99.37 (C-10), 94.83 (C-8), 94.05 (C-6), 81.43 (C-2), 67.39 (C-3), 27.10 (C-4). NMR data are in total agreement with the data for (+)-catechin.[19]

Compound 3

Yellow powder. ^{1}H-NMR: δ 7.08 (*s*, 2H, H-2′ and H-6′), 6.38 (*d*, 1H, *J*=1.0 Hz, H-8), 6.19 (*d*, 1H, *J*=1.0 Hz, H-6), 5.54 (*d*, 1H, *J*=1.1 Hz, H-1′′), 4.18-3.32 (sugar protons, 5H, *m*, H-2′′, H-3′′, H-4′′, H-5′′). ^{13}C-NMR: δ 177.71 (C-4), 164.64 (C-7), 161.19 (C-5), 157.15 (C-9), 156.38 (C-2), 145.71 (2C, C3′ and C-5′), 136.58 (C-4′), 133.23 (C-3), 119.82 (C-1′), 108.03 (2C, C-2′ and C-6′), 107.57 (C-10), 103.85 (C-1′′), 98.74 (C-6), 93.52 (C-8), 85.33 (C-4′′), 82.00 (C-3′′), 76.74 (C-2′′), 60.40 (C-5′′). NMR data are in total agreement with the data for myricetin 3-*O*-α-arabinofuranoside.[20]

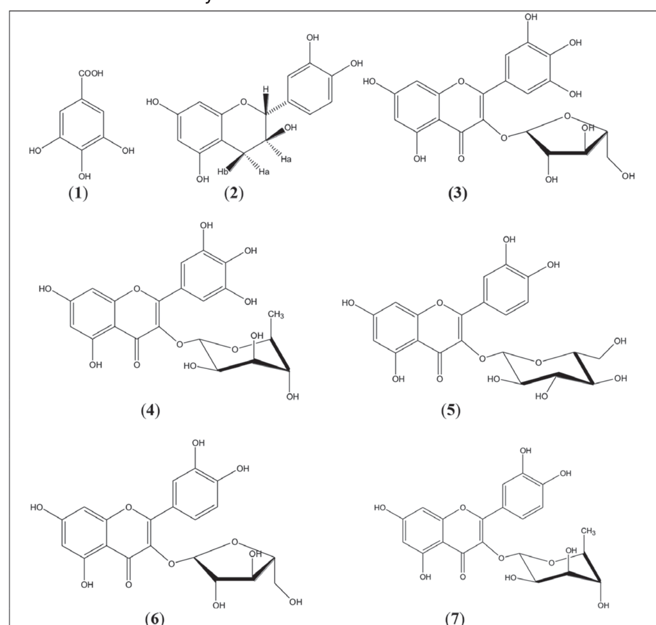

Figure 2. Compounds (1-7) isolated from the EtOAc fraction
EtOAc: Ethyl acetate

Table 2. DPPH radical scavenging activity and FRAP of the methanol extract and EtOAc, chloroform, and aqueous fractions at 1000 µg/mL

Samples	DPPH radical scavenging activity (% ± SEM[a])	FRAP[b]
MeOH Extract	82.20±2.82	0.917±0.030*
EtOAC Fr.	92.11±0.30	2.224±0.091
Chloroform Fr.	63.66±0.57**	0.650±0.007*
Aqueous Fr.	92.54±0.67	2.318±0.054
Quercetin[c]	92.75±0.05	2.090±0.032

[a]Standard error mean, [b]Absorbance at 700 nm ± SEM (greater absorbance indicates greater antioxidant power), [c]Reference, (*p<0.01, **p<0.001), DPPH: 2,2-diphenyl-1-picrylhydrazyl, FRAP: Ferric-reducing antioxidant power, EtOAC: Ethyl acetate, SEM: Scanning electron microscopy

Table 3. Total phenol and total flavonoid contents of the methanol extract and EtOAC, chloroform, and aqueous fractions

Sample	Total phenol content[a] ± SEM[b]	Total flavonoid content[c] ± SEM
MeOH Extract	64.41±2.26	229.45±2.80
EtOAC Fr.	261.59±3.95	515.54±2.80
Chloroform Fr.	20.73±5.53	136.54±2.52
Aqueous Fr.	ND[d]	ND

[a]Data expressed in mg equivalent of gallic acid (GAE) to 1 g of extract, [b]Standard error of the mean, [c]Data expressed in mg equivalent of quercetin (QUE) to 1 g of extract, [d]Not determined due to very low solubility, EtOAC: Ethyl acetate, SEM: Scanning electron microscopy

Compound 4

Yellow powder. ^{1}H-NMR: δ 6.94 (*s*, 2H, H-2' and H-6'), 6.35 (*d*, 1H, *J*=2.1 Hz, H-8), 6.18 (*d*, 1H, *J*=2.1 Hz, H-6), 5.30 (*d*, 1H, *J*=1.1 Hz, H-1''), 4.21 (*t*, 1H, *J*=1.5 Hz, H-2''), 3.77 (*dd*, 1H, J_1=3.3, J_2=9.4 Hz, H-3''), 3.50 (*m*, 1H, H-5''), 3.32 (*t*, 1H, *J*=9.6 Hz, H-4''), 0.95 (*d*, 3H, *J*=6.1 Hz, H-6''). ^{13}C-NMR: δ 178.23 (C-4), 164.47 (C-7), 161.77 (C-5), 158.00 (C-2), 157.07 (C-9), 145.42 (2C, C-3' and C-5'), 136.45 (C-4'), 134.87 (C-3), 120.47 (C-1'), 108.12 (2C, C-6' and C-2'), 104.42 (C-10), 102.18 (C-1''), 98.36 (C-6), 93.24 (C-8), 71.91 (C-4''), 70.67 (C-2''), 70.60 (C-3''), 70.44 (C-5''), 16.23 (C-6''). NMR data are in total agreement with the data for myricetin 3-*O*-α-rhamnopyranoside.[21]

Compound 5

Yellow powder. ^{1}H-NMR: δ 7.60 (*d*, 1H, *J*=2.3 Hz, H-2'), 7.49 (*dd*, 1H, *J*=2.3, 8.8 Hz, H-6'), 6.77 (*d,* 1H, *J*=8.8 Hz, H-5'), 6.29 (*d*, 1H, *J*=1.7 Hz, H-8), 6.10 (*d*, 1H, *J*=2.3 Hz), H-6, 5.15 (*d*, 1H, *J*=7.7 Hz, H-1''), 3.61 (*dd*, 1H, J_1=2.4, J_2=11.7 Hz, H-6a''), 3.48 (*dd*, 1H, J_1=5.3, J_2 =11.7 Hz, H-6b''), 3.38 (*dd*, 1H, J_1=7.7, J_2 =8.8 Hz, H-2''), 3.32 (*t*, 1H, *J*=8.8 Hz, H-3''), 3.25 (*dd*, 1H, J_1=8.8, J_2=9.9 Hz, H-4''), 3.12 (*m*, 1H, H-5''). ^{13}C-NMR: δ 178.01 (C-4), 165.11 (C-7), 161.61 (C-5), 157.51 (C-9), 157.08 (C-2), 148.44 (C-3'), 144.49 (C-4'), 134.15 (C-3), 121.73 (C-1'), 121.63 (C-6'), 116.07 (C-2'), 114.56 (C-5'), 104.11 (C-10), 102.87 (C-1''), 98.67 (C-6), 93.42 (C-8), 76.96 (C-5''), 76.69 (C-3''), 74.28 (C-2''), 69.77 (C-4''), 61.12 (C-6''). NMR data are in agreement with the data for quercetin 3-*O*-β-glucopyranoside.[22]

Compound 6

Yellow powder. ^{1}H-NMR: δ 7.43 (*d*, 1H, *J*=1.8 Hz, H-2'), 7.40 (*dd*, 1H, J_1= 2.1, J_2= 8.5 Hz, H-6'), 6.80 (*d*, 1H, *J*=8.3 Hz, H-5'), 6.30 (*s*, 1H, H-8), 6.10 (*d*, 1H, *J*=2.4 Hz, H-6), 5.37 (*s*, 1H, H-1''), 4.23 (*d*, 1H, *J*=2.3 Hz, H-2''), 3.81 (*m,* 1H, H-3''), 3.77 (*m*, 1H, H-4''), 3.40 (*m*, 2H, H-5''). ^{13}C-NMR: δ 178.54 (C-4), 164.80 (C-7), 161.64 (C-5), 157.89 (C-2), 157.15 (C-9), 148.43 (C-4'), 144.94 (C-3'), 133.45 (C-3), 121.65 (C-1'), 121.52 (C-6'), 115.38 (C-2'), 114.99 (C-5'), 108.07(C-1''), 104.12 (C-10), 98.49 (C-6), 93.36 (C-8), 86.57 (C-4''), 81.87 (C-2''), 77.24 (C-3''), 61.09 (C-5''). NMR data are in total agreement with the data for quercetin 3-*O*-α-arabinofuranoside.[20]

Compound 7

Yellow powder. ^{1}H-NMR: δ 7.24 (*d*, 1H, *J*=1.8 Hz, H-2'), 7.21 (*dd*, 1H, J_1=1.8, J_2=8.2 Hz, H-6'), 6.82 (*d*, 1H, *J*=8.2 Hz, H-5'), 6.28 (*s*, 1H, H-8), 6.11 (*d*, 1H, *J*=1.2 Hz, H-6), 5.25 (*d*, 1H, *J*=1.2 Hz, H-1''), 4.12 (*d*, 1H, *J*=1.1 Hz, H-2''), 3.65 (*dd*, 1H, J_1=2.9, J_2=9.4 Hz, H-3''), 3.32 (*m*, 1H, H-5''), 3.24 (*d*, 1H, *J*=9.4 Hz, H-4''), 0.84 (*d*, 3H, *J*=6.4 Hz, H-6''). ^{13}C-NMR: δ 178.21 (C-4), 164.65 (C-7), 161.76 (C-5), 157.89 (C-2), 157.11 (C-9), 148.39 (C-4'), 144.99 (C-3'), 134.78 (C-3), 121.52 (C-1'), 121.42 (C-6'), 115.50 (C-5'), 114.95 (C-2'), 104.41 (C-10), 102.11 (C-1''), 98.45 (C-6), 93.33 (C-8), 71.82 (C-4''), 70.67 (C-3''), 70.61 (C-2''), 70.47 (C-5''), 16.21 (C-6''). NMR data are in total agreement with the data for quercetin 3-*O*-α-rhamnopyranoside.[18-21]

DISCUSSION

Since ancient times, plants have served as one of the most important sources of medicines. Approximately 500 species are known to be used as folk medicine in Anatolia.

Many desirable biological activities such as analgesic, anticholecystitis, cholagogic, cytotoxic, hepatoprotective, and vasorelaxant activity of *Lysimachia* species used traditionally for expectorant, antipyretic, and wound healing purposes in Turkey are reported.[4-10]

Taking the folkloric and modern use of *L. monnieri* for memory enhancement into account, we designed the current study, which was the first on the neuroprotective effect of any *Lysimachia* species. Confirming its folkloric use, the ME as well as the EtOAC and chloroform fractions of LV inhibited AChE and BChE effectively. Among them, we chose the EtOAc fraction for further study due to its high cholinesterase inhibitory and antioxidant effects. Our phytochemical studies in order to identify substances found in the fraction led to the isolation of seven phenolic compounds (1-7) from the plant for the first time. The compounds were characterized as gallic acid (1), (+)-catechin (2), myricetin 3-*O*-α-arabinofuranoside (3), myricetin 3-*O*-α-rhamnopyranoside (4), quercetin 3-*O*-β-glucopyranoside (5), quercetin 3-*O*-α-arabinofuranoside (6), and quercetin 3-*O*-α-rhamnopyranoside (7).

Previous phytochemical studies on other *Lysimachia* species showed the presence of secondary metabolites including flavonoids, triterpenes, phenolic acids, etc.[5-7] According to these studies, *Lysimachia* species have rich phenolic compounds such as gentisic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid, apigenin, luteolin, myricetin, quercetin, kaempferol, isorhamnetin, quercetin 3-*O*-glucoside, quercetin 3-*O*-rutinoside, myricetin 3-*O*-glucoside, myricetin 3-*O*-rhamnoside, eriodictyol 7-*O*-glucoside, vitexin, and isovitexin.[5-7] Phenolic compounds are known to be generally responsible for the antioxidant capacity of plant extract. For instance, among them, gallic acid, (+) -catechin, myricetin-3-*O*-arabinofuranoside, and myricetin-3-*O*-rhamnoside have been demonstrated to be well-known compounds with remarkable antioxidant potential.[23-25] The antioxidant activity, anti-inflammatory, antinociceptive, and antipyretic activities of quercetin 3-*O*-β-arabinopyranoside and quercetin 3-*O*-α-L-rhamnopyranoside have been proven by Ramzi et al.[26] The DPPH assay, which determines the scavenging ability of antioxidants against stable radical DPPH·, is applied as a valid and practical assay, while the FRAP assay is based on the determination of the antioxidant capacity of foods, beverages, and other nutritional supplements rich in polyphenols via their ferric reducing ability.[27,28] It is a simple, automated test to measure antioxidant capacity. It should be noted that the high antioxidant activity of the EtOAC fraction is strongly correlated with its richest total phenol and flavonoid content and the isolated compounds (1-7) are the major contributors to the marked antioxidant activity of the plant.

On the other hand, flavonoid derivatives have been known to exert strong cholinesterase inhibitory effects.[29] Many

researchers have so far pointed out various flavonoids to be responsible for the potent cholinesterase inhibitory capacity of plant extracts. For example, the flavonoid fraction obtained from the fern *Dryopteris erythrosora* that contained gliricidin 7-*O*-hexoside, apigenin7-*O*-glucoside, quercetin 7-*O*-rutinoside, quercetin 7-*O*-galactoside, kaempferol 7-*O*-gentiobioside, kaempferol 3-*O*-rutinoside, myricetin 3-*O*-rhamnoside, and quercitrin exhibited strong AChE inhibition over 90% in dose-dependent manner.[30]

Some studies showed that the isolated compounds (+)-catechin, myricetin 3-*O*-arabinofuranoside, quercetin 3-*O*-β-glucopyranoside, quercetin 3-*O*-arabinofuranoside, and quercetin 3-*O*-α-rhamnopyranoside exhibited an active inhibitory effect against the AChE enzyme as well as strong antioxidative activity.[31,32] Moreover, gallic acid is well known as a powerful antioxidant to remedy DNA damage due to oxidative stress.[33]

In our previous study,[17] we reported quercetin with a significant AChE and BChE inhibitory effect in a competitive manner, which was shown to bind with hydrogen bonds to important amino acid residues at the anionic site of AChE. Recently, Ado et al.[34] demonstrated the presence of many flavonoid derivatives, i.e. catechin, quercetin pentoside, quercetin hexoside, etc. in the AChE-inhibiting fraction of *Cynometra cauliflora* leaves. In fact, an AChE inhibitory effect of (+)-catechin isolated from *Canarium patentinervium* was described at low level (>100 µg/mL).[35] Nevertheless, other flavonoid derivatives isolated from LV herein could be suggested to contribute to some extent to the cholinesterase inhibitory effect of the plant as Russo et al.[35] demonstrated a strong correlation between flavonoids and cholinesterase inhibition through calculation o[f] Pearson correlation.

Actually, it should be also noted that the occurrence of marked cholinesterase inhibitory activity as well as antioxidant activity might also be due to a synergistic action of the flavonoids present in the extract, because in many cases flavonoids have been shown to exert synergistic or additive effects, whereas sometimes antagonism occurs.[36-38] This is because the antioxidant activity of green tea, grape seed, and lettuce extracts was shown to increase after the addition of quercetin via acting synergistically, while catechins were proven to be responsible for synergism in green tea regarding its antioxidant activity, which might the case in the present study as well.[37,38]

According to previous bioactivity studies, various *Lysimachia* species such as *Lysimachia laka*, *Lysimachia punctata*, *Lysimachia foenum-graecum*, *Lysimachia clethroides*, and *Lysimachia vulgaris* have potent antioxidant capacity.[39-43] There are limited studies about the cholinesterase inhibitory activity of *Lysimachia* species. One study showed that *Lysimachia christinae* was inactive on AChE.[44]

The results of the present study showed that the EtOAc fraction had the highest total phenol and flavonoid content. This may explain why the highest enzyme inhibitor effect was observed on the EtOAC fraction. The total flavonoid content of ME extract was high. The high flavonoid content and other minor compounds of ME extract may be responsible for the similar enzyme inhibitor effect compared with the EtOAc fraction. Although the aqueous fraction has high antioxidant activity, no enzyme inhibitor effect of the aqueous fraction was observed. Furthermore, no total phenol or flavonoid content of the aqueous fraction was detected. The high antioxidant activity of the aqueous fraction may be based on the other compounds, which have no cholinesterase inhibitory effect.

CONCLUSION

The results obtained from the present study demonstrated that the ME and EtOAc fraction of the aerial parts of LV have strong AChE and BChE inhibitory effects, providing scientific justification for its use in folk medicine. The compounds mentioned herein have been isolated from LV for the first time. The current study is the very first on phytochemistry and neuroprotective effects through the cholinesterase and TYR inhibitory activity of LV. The phenolic compounds we isolated (1-7) may be responsible for the anticholinesterase and antioxidant activities.

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