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

Lysimachia verticillaris L.'nin Kolinesteraz ve Tirozinaz İnhibitor Etkisi ve Antioksidan Kapasitesi ve Ana Bileşiklerinin İzolasyonu

Ufuk Özgen[1], SILA OZLEM SENER[1], Karel Šmejkal[2], Jiri Vaclavik[2], FATMA SEZER SENOL DENIZ[3], ILKAY ERDOGAN ORHAN[3], Emil Svajdlenka[2], Ahmet C Goren[4], Milan &381;emli&269;ka[2]

[1]Department of Pharmacognosy, Faculty of Pharmacy, Karadeniz Technical University, Trabzon, Turkey

[2]University of Veterinary and Pharmaceutical Sciences Brno, Faculty of Pharmacy, Department of Natural Drugs, Czechia

[3]Gazi University, Faculty of Pharmacy, Department of Pharmacognosy, Ankara, Turkey

[4]Bezmialem Vakif University, Faculty of Pharmacy, Department of Chemistry, Istanbul, Turkey

ABSTRACT

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

Material and methods: The methanolic extract (ME), and the chloroform, EtOAc, and aqueous fractions obtained from the ME of LV were used for biological activity and isolation studies. The ME and all fractions were tested for their acetylcholinesterase (AChE), butyrylcholinesterase (BChE), tyrosinase (TYR) inhibitory, antioxidant potentials using ELISA microtiter assays. Seven major compounds were isolated from active EtOAc fraction by semi-preparative High Performance Liquid Chromatography (HPLC). The structures of the compounds were elucidated by several spectroscopic methods.

Results: The marked AChE inhibitory activity was observed in the EtOAc fraction (63,37 ± 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), DPPH radical scavenging activity and FRAP values in the aqueous and EtOAC fractions (92,54 ± 0,67%, 92,11 ± 0,30%; 2,318 ± 0,054, 2,224 ± 0, 091, respectively). Point of view, 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.

Keywords: Anticholinesterase, HPLC, isolation, *Lysimachia*, tyrosinase

ÖZET

Amaç: Bu çalışmanın amacı, *Lysimachia verticillaris* (LV)'in 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 Yöntem: 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. ME ve tüm fraksiyonların asetilkolinesteraz (AChE), butirilkolinesteraz (BChE), tirozinaz (TYR) 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 metanol ekstresinde ve etil asetat fraksiyonunda (%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 mg kersetin eşdeğeri/1 g ekstre) gözlenmiştir. En yüksek DPPH radikal süpürücü etki ve FRAP 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*-rhamnopiranozit (**7**) ana bileşikleri izole edilmiştir.

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

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

INTRODUCTION

The neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's (PD), common in the elderly population over the age of 65, have become one of the 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 the attractive sources for search aiming to discover novel anti-AD and anti-PD drug candidates. The deficiency of acetylcholine, caused hydrolyzed by acetylcholinesterase and butyrylcholinesterase, has been proved in the AD patients brain.[1] On the other hand, function of tyrosinase 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 Turkish flora.[3] *Lysimachia* species, locally known as "karga otu, adi karga otu, 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] Besides, several *Lysimachia* species have many desirable biological activities such as cytotoxic, hepatoprotective, and vasorelaxant, etc.[8-10]

Based on the information that *Lysimachia monnieri* is the synonym of *Bacopa monnieri*, the reputed plant called "brahmi" in Ayurvedic medicine for its strong memory-enhancing effect, we have aimed to study on the memory-enhancing effect of another species, *L. verticillaris*. For this purpose, in the current study, AChE, BChE, TYR inhibitory activity studies, and antioxidant potential including DPPH radical scavenging activity and FRAP were performed on the ME and all fractions. The EtOAc fraction which 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. Also, 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. Agilent-1100 series was used for HPLC studies (Germany). Unico 4802 UV-visible spectrophotometer (USA) was used for antioxidant activity, total phenol and flavonoid contents studies. Supelco Ascentis® RP-Amide (250 × 10 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), silica gel 60 F254 (Merck 5554) were used for isolation and chromatographic studies.

To measure the enzyme inhibition assays, 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), galanthamine (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 LV were gathered from Kafkasör vicinity at altitude of 1300 m (Artvin province, Turkey). The identification of the plant was performed by Dr. Ufuk ÖZGEN. Voucher specimen (AEF 26311) is deposited at the Herbarium of Faculty of Pharmacy (AEF).

Extraction

Air-dried and finely powdered sample (500 g) were extracted with MeOH (2 L × 8 h, three times). The combined extracts were evaporated to obtain 74 g of the crude residue. The methanol extract (ME) (73 g) was suspended in H_2O:MeOH (9:1) mixture, then, partitioned with chloroform (300 mL × 2) and EtOAc (300 mL × 2), 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 the ME were employed in the activity assays studied herein.

AChE and BChE inhibitory activities

The evaluation of the enzymes inhibitory activities was performed by modified method developed by Ellman et al.[11-12]

TYR inhibitory activity

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

DPPH radical scavenging activity

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

Ferric-reducing antioxidant power assay (FRAP)

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

Total Phenol and Flavonoid Contents

Phenolic content of the samples was determined using Folin-Ciocalteau's method.[16] Absorbances were read at 760 nm.

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

Data processing for assays

The statistical analysis of enzyme inhibition and antioxidant capacity assays was obtained using by Softmax PRO 4.3.2.LS software. Data were stated as mean ± SEM. 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 Silica gel 60 and $CHCl_3$:MeOH (80:20→50:50) solvent system. The 5-14 subfractions combined were subjected to semi-preparative HPLC column which yielded seven compounds (**1-7**) (Figure 1). Flow rate of the solvent was adjusted to 4.0 mL/min. 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. 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, EtOAc and chloroform fractions of LV showed high degree of inhibition against AChE having 58.21±3.36%, 63.37±1.74%, and 41.63±0.45% of 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 galanthamine (86.66±2.72%), the chloroform fraction exhibited relatively 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 DPPH radical quenching activity test, the chloroform fraction of LV had moderate activity (63.66±0.57%), whereas occurrence of high activity against DPPH radical was observed in aqueous (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

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), which have been isolated from LV for the first time. 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**

Greyish 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 data for gallic acid.[18]

Compound **2**

Greyish 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 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 data for myricetin 3-*O*-*α*-arabinofuranoside.[20]

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 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 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 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 data for quercetin 3-*O*-*α*-rhamnopyranoside.[18-21]

DISCUSSION

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

It have been approved the asset of many desirable biological activities such as analgesic, anticholecystitis, cholagogic, cytotoxic, hepatoprotective, vasorelaxant

activity of *Lysimachia* species, used for expectorant, antipyretic, and wound healing purposes in Turkey, traditionally.[4-10]

Taking the folkloric and modern use of *L. monnieri* for memory enhancement into account, we designed the current study which has been the first one on neuroprotective effect of any *Lysimachia* species ever. Confirming its folkloric use, the ME as well as the EtOAc and chloroform fractions of LV inhibited AChE and BChE effectively. Among them, we have chosen the EtOAc fraction due to its high cholinesterase inhibitory and antioxidant effects for further study. Our phytochemical studies in order to identify substances found in the fraction led to 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 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, isovitexin, etc.[5-7] Phenolic compounds are known to be generally responsible for 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 the 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 proven by Ramzi et al.[26] DPPH assay, which determines the scavenging ability of antioxidants against stable radical DPPH·, is applied as a valid and practical assay, while FRAP assay is based on the determination of 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 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 to 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, keampferol 7-*O*-gentiobioside, keampferol 3-*O*-rutinoside, myricetin 3-*O*-rhamnoside, and quercitrin exhibited strong AChE inhibition over 90% in dose-dependent manner.[30]

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

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

Actually, it should be also noted that occurrence of the marked cholinesterase inhibitory activity as well as antioxidant activity might be also possibly due to 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] Because antioxidant activity of green tea, grape seed, and lettuce extracts was shown to increase after 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 this study as well.[37-38]

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

The results of the study have showed that EtOAc fraction has the highest total phenol and flavonoid content. It may explain the highest enzyme inhibitor effect was observed on EtOAc fraction. The total flavonoid content of ME extract were found high level. High flavonoid content and other minor compounds of ME extract may be responsible for similar enzyme inhibitor effect compared with the EtOAc fraction. Although aqueous fraction has high antioxidant activity, the enzyme inhibitor effect of aqueous fraction was not observed. Also, total phenol and flavonoid content of aqueous fraction were not detected. The high antioxidant activity of the aqueous fraction may be based on the other compounds which have no cholinesterase inhbitory effect.

CONCLUSIONS

The results obtained from this study have demonstrated that the ME and EtOAc fraction of the aerial parts of LV have strong AChE and BChE inhibitory effect which provides the scientific proof for the folk medicine. The compounds mentioned herein have been isolated from LV for the first time. In the current study, we disclose the very first study on phytochemistry and neuroprotective effect through cholinesterase and TYR inhibitory activity of LV. Phenolic compounds isolated (**1-7**) in this study may be responsible for anticholinesterase and antioxidant activities.

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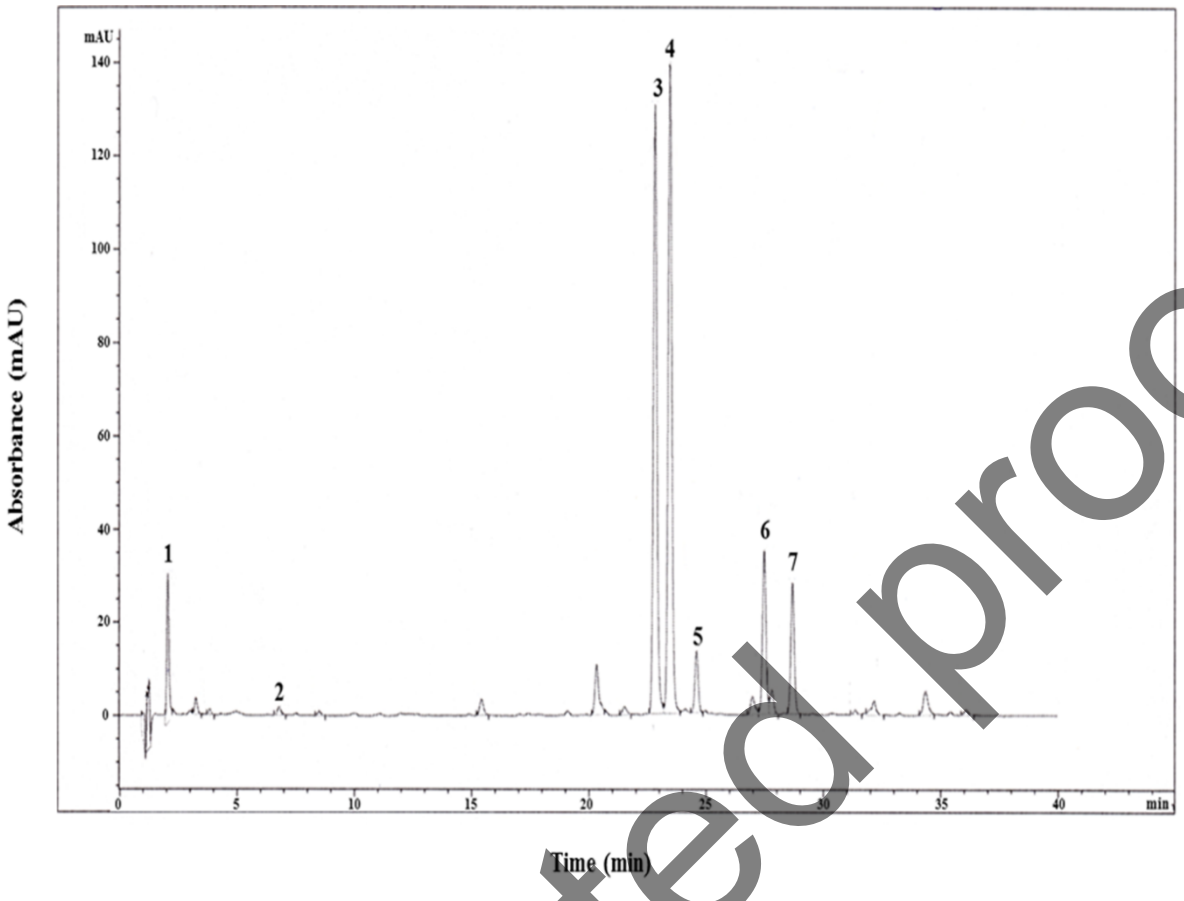

Figure 1. HPLC chromatogram of the EtOAc fraction

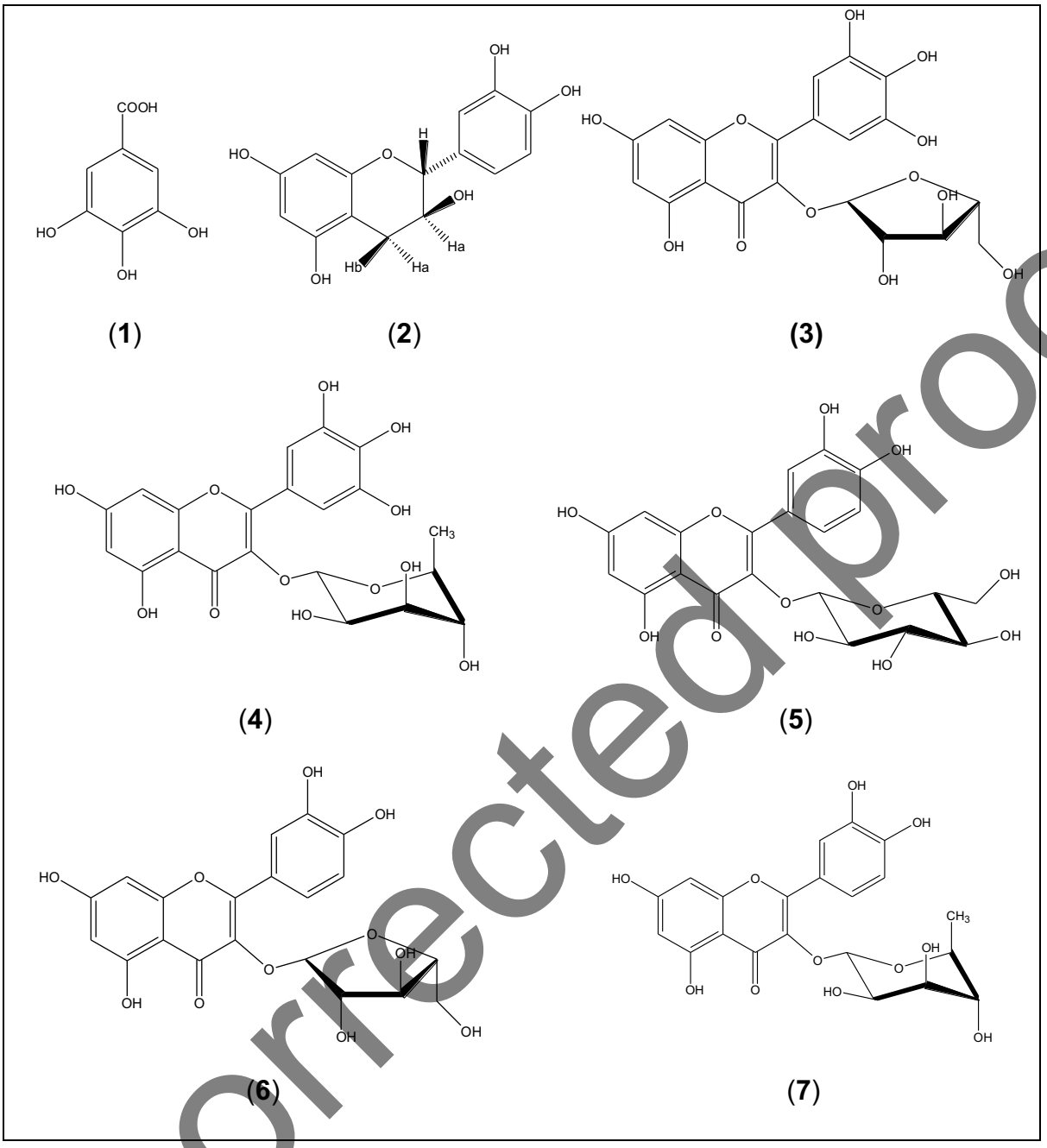

Figure 2. Compounds (**1-7**) isolated from the EtOAc fraction

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]
Galanthamine[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]

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 < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001]

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 mean, [c] Data expressed in mg equivalent of quercetin (QUE) to 1 g of extract, [d] Not determined due to very low solubility.

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