

Cholinesterase Inhibitory and Antioxidant Properties of *Verbascum mucronatum* Lam. and its Secondary Metabolites

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The aqueous extract of *Verbascum mucronatum* Lam. along with its fractions and secondary metabolites were assessed for their antioxidant, acetylcholinesterase (AChE), and butyrylcholinesterase (BChE) inhibitory activities. The antioxidant activity was evaluated by three methods: as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, ferrous ion-chelating effect, and ferric-reducing antioxidant power (FRAP) tests. The AChE activity was determined by the Ellman method using an ELISA microplate reader. Phytochemical investigations revealed the presence of four iridoid glucosides, ajugol (**1**), aucubin (**2**), lasianthoside I (**3**) and catalpol (**4**), two saponins, ilwensisaponin A (**5**) and C (**6**), and a phenylethanoid glycoside, verbascoside (**7**), in *Verbascum mucronatum*. Their structures were elucidated by spectral techniques. The aqueous extract and fractions including the phenylethanoid glycoside **7** showed DPPH scavenger effect and had the best FRAP. Besides these results, one of the phenylethanoid fractions displayed the highest ferrous ion-chelating effect. While only **7** was found to possess moderate AChE inhibition, the extract, fractions, and all other tested compounds did not inhibit AChE and BChE.

Key words: *Verbascum*, Antioxidant Activity, Cholinesterase Inhibition

Introduction

The generic name of *Verbascum* is believed to be a corruption of *barbascum*, from the Latin *barba*, meaning a beard, referring to the shaggy appearance of the genus. *Verbascum* (mullein) species are valued for their curative properties and are widely employed both in domestic and regular medicine. Historically, mullein has been used as a remedy for the respiratory tract, particularly in cases of irritating coughs with bronchial congestion. Some herbal texts extended its therapeutic uses against pneumonia, tuberculosis, and asthma. The species are also used to treat haemorrhoids, rheumatic pain, superficial fungal infections, wounds and diarrhoea, and have inhibitory activities against the murine lymphocytic leukaemia and influenza viruses A2 and B. A decoction of leaves can be used as heart stimulant and a decoction of roots to alleviate toothache and also to relieve cramps, convulsions, and migraines. The leaves, roots, and flowers have also anodyne,

antiseptic, antispasmodic, astringent, emollient, nerve, vulnerary, analgesic, antihistaminic, anticancer, antioxidant, antiviral, bactericide, cardio-depressant, oestrogenic, fungicide, hypnotic, and sedative activities (Ucar Turker and Gurel, 2005).

The *Verbascum* genus is represented by 232 species, 196 of which are endemic in Turkish flora (Huber-Morath, 1978; Ekim, 2000). Phytochemical studies on these species have revealed the presence of phenylethanoid glycosides (Tatli and Akdemir, 2004). The pharmacological activity of phenylethanoid glycosides in plants, such as their antioxidant, anti-inflammatory, cytotoxic, antitumour, antiulcer, analgesic, antihepatotoxic, and immunosuppressant effects, has been known for many years, while new activities are continually being discovered (Jimenez and Riguera, 1994).

In this study, our aim was to investigate *in vitro* antioxidant and anticholinesterase potentials of the aqueous (H₂O) extract prepared from *V. mucronatum*, which is used as haemostatic in Turkish folk medicine (Cubukcu *et al.*, 1994), along with

those of its major metabolites; four iridoid glucosides [ajugol (**1**), aucubin (**2**), lasianthoside I (**3**), catalpol (**4**)], two saponins [ilwensisaponin A (**5**) and C (**6**)], and a phenylethanoid glycoside [verbascoside (**7**)] (Fig. 1). The antioxidant activity of the extract, fractions, and pure compounds was assessed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging tests (Badami *et al.*, 2003) and ferrous ion-chelating power tests (Chua *et al.*, 2008). The ferric-reducing antioxidant power (FRAP) of the extract, fractions, and pure compounds was also measured (Oyaizu, 1986). Their anticholinesterase inhibitory activity was assessed *in vitro* against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) by the spectrophotometric method of Ellman (Ellman *et al.*, 1961) using an ELISA microplate reader (Orhan *et al.*, 2008).

Material and Methods

Plant material

Verbascum mucronatum Lam. (Scrophulariaceae) was collected from the vicinity of Aksaray province in Turkey, 17 km from Aksaray to Ulukisla, in July 2007. A specimen of the original collection (coded as GAZI 10097) is preserved in the herbarium of Botany Department, Faculty of Science and Art, Gazi University, Ankara, Turkey.

Preparation of the H₂O extract of *V. mucronatum* flowers

The air-dried and powdered flowers of *V. mucronatum* (586.2 g) were extracted with methanol (MeOH) (3 × 2.5 l) at 40 °C for 8 h, and the combined MeOH extracts were concentrated under reduced pressure (yield: 70.4 g; 12.0%, w/w). The resultant residue was dissolved in H₂O (100 ml) and the water-soluble portion was partitioned in CHCl₃ (2 × 100 ml) to remove chlorophyll and other lipophilic constituents (yield: 3.6 g; 0.6%, w/w). The rest of the H₂O phase was 65.8 g (yield: 11.2%, w/w). The H₂O extract was administered for activity assessments.

Fractionation of the H₂O extract

The lyophilized H₂O phase (65.8 g) was fractionated over a polyamide column (ICN, VLC, 150 g), eluted with H₂O, followed by increasing concentrations of MeOH to afford six main fractions: Fraction A, 18.2 g; Fraction B, 9.6 g; Fraction C, 12.5 g; Fraction D, 4.9 g; Fraction E, 5.1 g; Fraction F, 0.8 g.

tion C, 12.5 g; Fraction D, 4.9 g; Fraction E, 5.1 g; Fraction F, 0.8 g.

Chromatographic separation and isolation of the constituents

Fraction D (4.9 g), the most active fraction, was fractionated over a LiChroprep C₁₈ column (Merck, Opfikon-Glattbrugg, Switzerland; C-18, Sepalyte 40 μm, VLC, 150 g). Employment of H₂O and MeOH (0–100% MeOH) afforded ajugol (**1**, 48.6 mg), aucubin (**2**, 103.0 mg), catalpol (**4**, 62.1 mg) and additional four fractions, Fraction D₁ – Fraction D₄. Fraction D₃ (1.2 g) was rechromatographed on a LiChroprep C₁₈ column (MPLC) using H₂O and MeOH/H₂O gradients (30–70% MeOH) to yield lasianthoside I (**3**, 6.7 mg), ilwensisaponin A (**5**, 51.5 mg) and ilwensisaponin C (**6**, 14.7 mg). Purification of fraction D₄ (625.3 mg) by silica gel column chromatography [Merck, 230–400 mesh, 140 g, CHCl₃/MeOH (70:30 → 60:40)] furnished verbascoside (**7**, 14.8 mg).

Structure elucidation of compounds 1–7

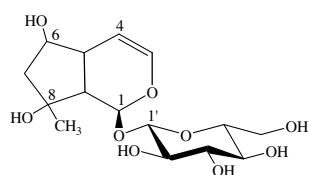
Structure elucidation of the isolated compounds **1–7** from fraction D was carried out by spectral techniques; UV, IR, 1D- and 2D-NMR, and mass spectroscopy (HR-ESIMS), and detailed data were recently submitted elsewhere (Tatli *et al.*, 2004, 2006). The structures of compounds **1–7** were as follows (Fig. 1): ajugol (**1**), aucubin (**2**), lasianthoside I (**3**), catalpol (**4**), ilwensisaponin A (**5**), ilwensisaponin C (**6**), verbascoside (**7**).

DPPH radical scavenging activity of *V. mucronatum* extract, fractions, and pure compounds

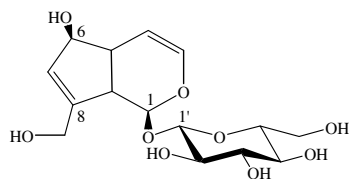
The DPPH radical scavenging activity at 12.5, 25, 50, 100, and 200 μM concentrations was determined according to Badami *et al.* (2003). The samples and references dissolved in MeOH were mixed with DPPH solution (1 mM). The remaining DPPH amount was measured at 517 nm using a Unico 4802 UV-visible double beam spectrophotometer (Unico, Dayton, NJ, USA). Ascorbic acid and green tea extract were employed as the references. Inhibition of DPPH in percent (*I*%) was calculated as follows:

$$I\% = [(A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}] \cdot 100,$$

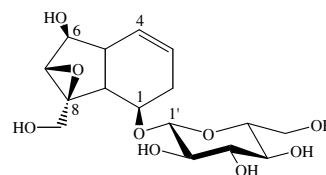
where A_{blank} is the absorbance of the control reaction (containing all reagents except the test sam-



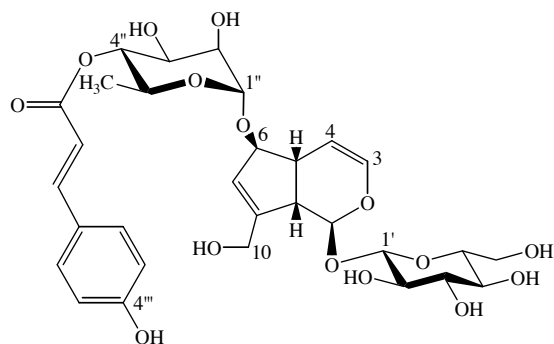
Ajugol (1)



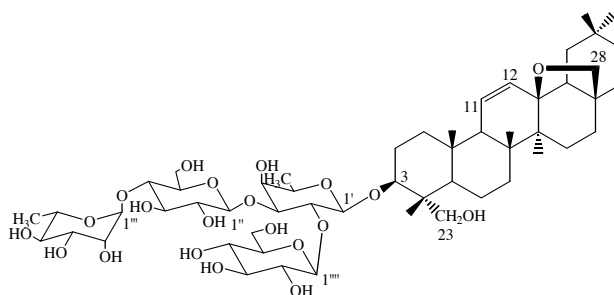
Aucubin (2)



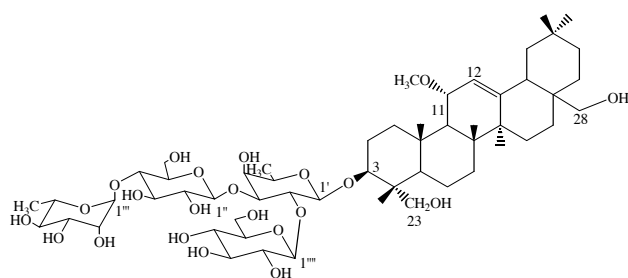
Catalpol (4)



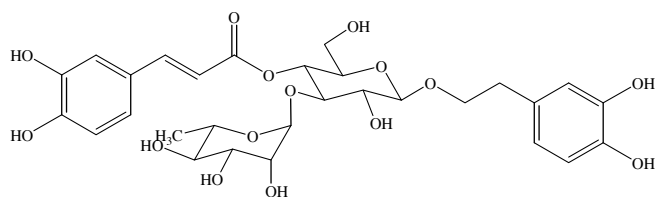
Lasianthoside I (3)



Ilwensisaponin A (5)



Ilwensisaponin C (6)



Verbascoside (7)

Fig. 1. Chemical structures of the isolated compounds 1–7.

ple), and A_{sample} is the absorbance of the extracts/reference. Analyses were run in three replicates.

Ferrous ion-chelating effect of V. mucronatum extract, fractions, and pure compounds

The ferrous ion-chelating effect of the extract, fractions, and pure compounds at 62.5, 125, and 250 $\mu\text{g ml}^{-1}$ was estimated by the method of Chua *et al.* (2008). Briefly, 740 μl of methanol and the samples (200 μl) were incubated with 20 μl of 2 mM FeCl_2 solution. The reaction was initiated by addition of 40 μl of 5 mM ferrozine and the mixture was 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:

$$I\% = [(A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}] \cdot 100,$$

where A_{blank} is the absorbance of the control reaction (containing only FeCl_2 and ferrozine), and A_{sample} is the absorbance of the extracts, fractions, and pure compounds/reference [butylated hydroxyanisole (BHA)]. Analyses were run in triplicate, and the results were expressed as mean values \pm S.E.M. (standard error mean).

Ferric-reducing antioxidant power (FRAP) assay of V. mucronatum extract, fractions, and pure compounds

The FRAP of the extract, fractions, and pure compounds of *V. mucronatum* was tested using the assay of Oyaizu (1986). 1 ml of different concentrations of the samples as well as chlorogenic acid as reference for comparative purposes were added to 2.5 ml of phosphate buffer (0.1 M, pH 6.6) and 2.5 ml of potassium ferricyanide (1%, w/v). Later, the mixture was incubated at 50 °C for 20 min, and then 2.5 ml of 10% trichloroacetic acid were added. After the mixture was shaken vigorously, 2.5 ml of this solution were mixed with 2.5 ml of distilled water and 0.5 ml of FeCl_3 (0.1%, w/v). After 30 min of incubation, absorbance was read at 700 nm. Analyses were achieved in triplicate. Increased absorbance of the reaction mixture meant increased reducing power.

AChE and BChE inhibition assays

In vitro inhibition of AChE and BChE of the samples at 62.5, 125, and 250 $\mu\text{g ml}^{-1}$ concentrations was assessed by the spectrophotometric

method developed by Ellman *et al.* (1961), while verbascoside (**7**) was tested at 15.625, 31.25, 62.5, 125, 250, and 500 $\mu\text{g ml}^{-1}$ towards both enzymes. Electric eel AChE (Type-VI-S, EC 3.1.1.7; Sigma, St. Louis, MO, USA) was employed as the enzyme source, while acetylthiocholine iodide (Sigma) as substrate and 5,5'-dithio-bis(2-nitrobenzoic)acid (DTNB) were also used for anti-AChE activity determination. Absorbance at 412 nm was measured in a 96-well microtiter plate using an ELISA microplate reader (VersaMax tunable microplate reader with Softmax[®] Pro software; Molecular Devices, CA, USA). All other reagents and conditions were the same as described in one of our previous publications (Orhan *et al.*, 2008). Percentage inhibition of AChE and BChE was determined by comparison of rates of reaction of the samples relative to a blank sample (EtOH in phosphate buffer, pH 8) using the formula $(E - S)/E \cdot 100$, where E is the activity of enzyme without test sample, and S is the activity of enzyme with test sample. All experiments were done in triplicate, and the results were expressed as mean values \pm S.E.M. Galanthamine purchased from Sigma was the reference in this study.

Results and Discussion

Considering the DPPH scavenging potential of the extract, fractions, and compounds **1–7** (Fig. 1) of *V. mucronatum*, the H_2O extract, fractions D, E, and D₄, and verbascoside (**7**) showed the best results (72.51–91.86%) at 200 μM in the DPPH radical scavenging test (Table I), whereas the rest of *V. mucronatum* fractions and compounds **1–6** were not active as compared to the references. Among the extract, fractions, and pure compounds screened in this study, fraction D₃ of *V. mucronatum* displayed the highest ferrous ion-chelating power (19.07, 26.02, and 40.77%) at 62.5, 125, and 250 $\mu\text{g ml}^{-1}$ concentrations as compared to BHA (23.86, 29.91, and 38.99%) (Table II). Considering FRAP tests, the H_2O extract (1.849%), fractions D (3.159%) and D₄ (2.594%), as well as verbascoside (**7**) (1.104%) showed remarkable activity at 250 $\mu\text{g ml}^{-1}$ as compared to chlorogenic acid (3.820%) (Table II), whereas the rest of test materials were found to be not active.

At 15.625, 31.25, 62.5, 125, and 250 $\mu\text{g ml}^{-1}$, the H_2O extract, fractions D, D₂, and D₄ along with verbascoside (**7**) were tested for their anticholinesterase activity against AChE and BChE *in*

Table I. DPPH radical scavenging activities of the H₂O extract, fractions, and compounds **1–7**.

Test sample	Percentage of inhibition against DPPH radicals				
	200 μM	100 μM	50 μM	25 μM	12.5 μM
H ₂ O extract	72.51	44.99	30.78	23.08	10.92
Fr. A	23.53	13.64	9.99	-6.21	-5.25
Fr. B	33.19	22.24	13.50	15.25	0.68
Fr. C	61.76	36.39	21.67	15.82	2.48
Fr. D	90.92	71.87	40.89	19.59	8.49
Fr. E	89.98	61.15	28.96	15.42	8.23
Fr. F	48.99	23.55	6.41	4.60	-6.95
Fr. D ₁	2.24	1.19	1.07	0.89	0.65
Fr. D ₂	3.31	0.55	-0.86	0.36	-1.08
Fr. D ₃	46.65	32.45	15.19	14.76	7.01
Fr. D ₄	91.59	49.90	14.56	-15.14	8.09
Ajugol (1)	6.56	4.23	2.10	3.60	3.09
Aucubin (2)	7.53	6.92	3.67	1.45	0.46
Lasianthoside I (3)	12.16	8.80	6.04	-3.39	16.79
Catalpol (4)	4.56	3.21	3.05	1.60	0.39
Ilwensisaponin A (5)	15.68	9.85	8.31	8.99	-3.80
Ilwensisaponin C (6)	6.87	4.06	3.59	4.17	-9.37
Verbascoside (7)	91.86	92.37	91.86	75.32	-39.51
Green tea extract	91.16	87.39	53.72	31.12	17.55
Ascorbic acid	93.62	93.47	93.47	93.39	68.93

Table II. (A) Ferric-reducing antioxidant power (FRAP)^a percentage \pm S.E.M.^b and (B) ferrous ion-chelating power percentage \pm S.E.M. of the H₂O extract, fractions, and compounds **1–7**.

Test sample	A		B		A		B	
	62.5 $\mu\text{g ml}^{-1}$		125 $\mu\text{g ml}^{-1}$		250 $\mu\text{g ml}^{-1}$			
	A	B	A	B	A	B	A	B
H ₂ O extract	0.785 \pm 0.01	- ^c	1.257 \pm 0.01	-	1.849 \pm 0.09	-		
Fr. A	0.568 \pm 0.01	-	0.696 \pm 0.01	-	0.929 \pm 0.05	16.10 \pm 1.99		
Fr. B	0.566 \pm 0.01	-	0.691 \pm 0.01	-	0.904 \pm 0.06	-		
Fr. C	0.779 \pm 0.01	-	1.149 \pm 0.01	-	1.627 \pm 0.04	-		
Fr. D	0.704 \pm 0.09	-	1.973 \pm 0.06	-	3.159 \pm 0.14	-		
Fr. E	0.663 \pm 0.01	-	0.550 \pm 0.03	-	0.563 \pm 0.01	-		
Fr. F	0.628 \pm 0.03	-	0.671 \pm 0.04	-	1.343 \pm 0.17	-		
Fr. D ₁	0.418 \pm 0.01	-	0.423 \pm 0.01	-	0.449 \pm 0.02	11.95 \pm 1.84		
Fr. D ₂	0.403 \pm 0.01	-	0.428 \pm 0.01	-	0.437 \pm 0.03	12.88 \pm 1.24		
Fr. D ₃	0.473 \pm 0.01	19.07 \pm 0.75	0.548 \pm 0.01	26.02 \pm 1.47	0.644 \pm 0.03	40.77 \pm 0.14		
Fr. D ₄	1.160 \pm 0.05	-	1.767 \pm 0.03	-	2.594 \pm 0.11	-		
Ajugol (1)	0.428 \pm 0.01	-	0.432 \pm 0.01	-	0.461 \pm 0.01	13.22 \pm 1.38		
Aucubin (2)	0.435 \pm 0.01	-	0.436 \pm 0.01	-	0.481 \pm 0.02	9.96 \pm 0.08		
Lasianthoside I (3)	0.434 \pm 0.01	-	0.439 \pm 0.01	-	0.486 \pm 0.02	11.05 \pm 0.13		
Catalpol (4)	0.426 \pm 0.01	-	0.427 \pm 0.01	-	0.460 \pm 0.01	14.08 \pm 0.56		
Ilwensisaponin A (5)	0.424 \pm 0.01	-	0.431 \pm 0.01	-	0.435 \pm 0.01	12.00 \pm 0.50		
Ilwensisaponin C (6)	0.429 \pm 0.01	-	0.431 \pm 0.01	-	0.452 \pm 0.01	12.04 \pm 1.24		
Verbascoside (7)	0.666 \pm 0.01	-	0.916 \pm 0.01	-	1.104 \pm 0.09	-		
BHA		23.86		29.91			38.99	
Chlorogenic acid	2.729		3.649		3.820			

^a Absorbance at 700 nm. ^b Standard error mean. ^c No activity.

vitro. The data obtained can be commented as follows: fractions D and D₄ and verbascoside (**7**) exerted a moderate inhibition against AChE when

compared to galanthamine (Table III). Nevertheless, verbascoside (**7**) was inactive against BChE (Table IV).

Table III. Anti-AChE activity of the H₂O extract, fractions D, D₂, D₄, and verbascoside (7).

Test sample	Percentage of inhibition ± S.E.M ^a against AChE				
	15.625 µg ml ⁻¹	31.25 µg ml ⁻¹	62.5 µg ml ⁻¹	125 µg ml ⁻¹	250 µg ml ⁻¹
H ₂ O extract	ND ^b	ND	- ^c	-	-
Fr. D	ND	ND	-	28.82 ± 1.62	60.48 ± 1.01
Fr. D ₂	ND	ND	-	6.97 ± 1.01	12.43 ± 0.99
Fr. D ₄	ND	ND	15.29 ± 0.87	31.01 ± 1.23	40.79 ± 1.19
Verbascoside (7)	-	-	-	36.85 ± 1.45	74.12 ± 1.54
Galanthamine	ND	ND	90.45 ± 0.83	98.97 ± 0.24	ND

^a Standard error mean. ^b Not determined. ^c No activity.

Table IV. Anti-BChE activity of verbascoside (7).

Test sample	Percentage of inhibition ± S.E.M ^a against BChE				
	15.625 µg ml ⁻¹	31.25 µg ml ⁻¹	62.5 µg ml ⁻¹	125 µg ml ⁻¹	250 µg ml ⁻¹
Verbascoside (7)	- ^b	-	9.34 ± 0.77	19.37 ± 0.54	37.15 ± 0.89
Galanthamine	ND ^c	ND	90.45 ± 0.83	98.97 ± 0.24	ND

^a Standard error mean. ^b No activity. ^c Not determined.

Natural compounds are receiving increasing attention as potential antioxidants. To this end, isolation of phenylethanoid glycosides, verbascoside, β -hydroxyacteoside, forsythoside B, angoroside A, and martynoside from the aerial parts of *V. salviifolium*, was reported in our earlier study, and these compounds demonstrated scavenging properties toward the DPPH radical in thin layer chromatography (TLC) autographic assays. They were found to have significant antioxidant properties, based on the experiments with DPPH, which indicated their ability to efficiently scavenge free radicals (Akdemir *et al.*, 2003). Furthermore, free radical scavenging and cell-aggregation inhibitory activities of thirty-six secondary metabolites isolated from the MeOH extracts of *V. cilicicum* Boiss., *V. lasianthum* Boiss. ex Benth., *V. pterocalycinum* var. *mutense* Hub.-Mor., and *V. salviifolium* Boiss. (Scrophulariaceae) were investigated. The isolated compounds, including verbascoside (7), exhibited a dose-dependent inhibition in bioautographic and spectrophotometric DPPH assays. Verbascoside was the most active having an IC₅₀ value of 4.0 µg ml⁻¹ compared to ascorbic acid (IC₅₀ 4.4 µg ml⁻¹) and inhibiting phorbol 12-myristate 13-acetate (PMA)-induced peroxide-catalyzed oxidation of 2',7'-dichlorofluorescein (DCFH) by reactive oxygen species (ROS) within human promyelocytic HL-60 cells (Tatli *et al.*, 2007).

Antioxidant properties of the aerial parts of *V. macrurum* have been determined by monitoring their capacity to scavenge the stable free radical DPPH. They were also evaluated as natural preservatives against oxidative rancidity using the accelerated Rancimat method. Their activities expressed as protection factor (PF_r) indicated that the fractions rich in phenylpropanoid glycosides were more potent compared to α -tocopherol and as potent as butylated hydroxytoluene (BHT), which were used as references. Assessment of their antioxidant activities established that acteoside was the most potent free radical scavenger and showed the highest protection factor against sunflower-oil-induced oxidative rancidity (Aliannis *et al.*, 2003).

Verbascoside is a major phenylethanoid glycoside in the genus *Verbascum*. Antioxidative activity of phenylethanoids might be mainly related to the number of aromatic hydroxy groups, the structure of the acyl moiety and the number of sugar moieties. Structure-activity relationship studies also demonstrated that compounds with phenolic hydroxy groups at the *ortho*-position have a stronger antioxidant effect. There are two phenolic hydroxy groups at the *ortho*-position in each aromatic ring in verbascoside (Liu *et al.*, 2003).

In the present study, our results supported the previous findings. Additionally, in our literature

survey, no reports relating to ferrous ion-chelating power and FRAP of verbascoside have been found so far. This is the first report that verbascoside exhibits notable antioxidant activity according to FRAP tests.

Remarkable ferrous ion-chelating effect and FRAP may be important since Fe²⁺ dysregulation has been also associated with Alzheimer's disease. Because of this reason, we have also herein demonstrated anti-AChE and anti-BChE activities of the extract, fractions, and verbascoside. The results underline that verbascoside possesses a moderate anti-AChE effect. There have been several reports evaluating the neurobiological activity of phenylethanoid glycosides. Kim *et al.* (2003) reported anti-amnesic activities of phenylpropanoids isolated from *Scrophularia buergeriana* roots on memory deficit induced by scopolamine in mice. Moreover, *E-p*-methoxycinnamic acid and other phenylpropanoids significantly suppressed neurotoxicity induced by glutamate in the primary cultures of rat cortical cells (Kim *et al.*, 2002). Additionally, from the qualitative analysis of the flowers of *Syzygium aromaticum*, phenylethanoids, gallic acid, and tannins were detected. The plant is used by indigenous

ethnic populations of different countries for several therapeutic indications because of its tonic and stimulant properties. Its ability to improve learning and memory in studies with rats classify this plant as an adaptogen. The aerial parts of *Ballota nigra* containing cinnamic acids have been used in symptomatic treatment of neurotic states in adults and children, especially in treating mild sleep disturbances and to combat cough in phytotherapy. The root extract of *Polygala tenuifolia* alone could upregulate the choline acetyltransferase (ChAT) activity, and increased nerve growth factor secretion *in vitro* may be due to the cinnamic acid derivative sinapinic acid (Gomes *et al.*, 2009).

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