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Tyrosinase, Acetyl- and Butyryl-Cholinesterase Inhibitory Activity of *Stachys lavandulifolia* Vahl (Lamiaceae) and Its Major Constituents

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Abstract: The *n*-hexane (HE), dichloromethane (DC), methanol (ME), ethanol 70% (ET), and methanol with Soxlhet apparatus (MS) extracts of *Stachys lavandulifolia* aerial parts were screened for their potential tyrosinase, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activity. ET and MS inhibited tyrosinase with IC_{50} values of 33.4 and 42.8 μg/mL, respectively. The phytochemical investigation of these extracts resulted in the isolation of the known compounds monomelittoside (**1**), melittoside (**2**), 5-allosyloxy-aucubin (**3**), acteoside (**4**) and arbutin (**5**). The HE extract, characterized by germacrene D, β-pinene, β-myrcene, and *trans*-caryophyllene as main constituents, showed the highest AChE inhibitory activity with an IC_{50} value of 13.7 μg/mL while DC extract was the most active against BChE (IC_{50} value of 143.9 μg/mL). The diterpene stachysolone (**6**) was isolated from this extract. The antioxidant properties were also investigated by four *in vitro* methods (DPPH, ABTS, FRAP and β-carotene bleaching tests).

Keywords: *Stachys lavandulifolia*; iridoids; tyrosinase; cholinesterase; antioxidant.

1. Introduction

The genus *Stachys* (Lamiaceae) is comprised of about 300 species distributed in temperate and tropical regions of the world, with the exception of Australasia, and it is widespread in mountainous and moist places. The name is derived from the Greek word σταξυζ (*Stachys*), meaning "an ear of grain". *Stachys* species are used as herbal medicines and wild tea in many local regions of the world. Several studies have demonstrated antitoxic, antiproliferative, antimicrobial, anti-inflammatory, and antioxidant activities of *Stachys* species [1].

S. lavandulifolia Vahl is widely distributed in different regions of Iran where it is known as "chaie koohi" and it has been used as an anxiolytic and sedative in folk medicine [2]. The aerial parts are used as antipyretic, anti-inflammatory, spasmolytic, and sedative medicament [2]. Pharmacological properties of this plant such as anti-inflammatory, anti-anxiety, antibacterial, antinephritic, anticancer, anti-*Helicobacter pylori*, and antioxidant activities have also been reported [3-5]. Phytochemical studies demonstrated the presence of monoterpenes, sesquiterpenes, phenylethanoid glycosides and iridoids as main constituents [6, 7].

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There is general interest among researchers in the two most common neurodegenerative disorders, Alzheimer's disease (AD) and Parkinson's disease (PD). Though AD and PD seem to be caused by different factors, they appear to involve several similar pathophysiological events, including apoptosis, excitotoxicity, toxic proteins, polymeric insoluble inclusions, inflammation, and oxidative damage. Brain aging is associated with the accumulation of the oxidative-induced damage, likely due to the imbalance between antioxidant defence mechanisms and intracellular generation of reactive oxygen species (ROS). The brain contains high levels of unsaturated fatty acids, which are vulnerable to oxidation, and consumes large amounts of oxygen. In PD several pathways have been identified for the oxidative stress induced by the oxidation of dopamine, including the production of ROS or the formation of highly reactive quinone species [8]. Tyrosinase, a multifunctional enzyme that catalyses the *o*-hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine or DOPA and the oxidation of DOPA to dopaquinone, has been proposed to take part in the oxidative chemistry related to PD. Tyrosinase is the major enzyme required for the synthesis of melanin in skin and hair and may contribute to neuromelanin formation that occur after the first 2-3 years of life and accumulates with aging. In mammals, tyrosinase is specifically expressed in differentiated melanin-producing cells such as melanocytes that are distributed in the skin and retinal pigment epithelium. In the mammalian central nervous system (SNC), neuromelanin is found in nigral dopaminergic cells [9]. Neuromelanin differs from the peripheral melanin as it predominates in catecholaminergic neurons of the nervous system and the locus coeruleus. These areas in the human brain undergo degeneration in PD progression [9]. The existence of tyrosinase in SNC is still controversial, although several studies have shown the expression of tyrosinase gene, its protein, and its catalytic activity in the nigral neurons [10]. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are important biological targets responsible for regulation of cholinergic transmission [11]. AChE inhibitors are used as drugs for correcting neurotransmitter disturbances or symptomatic treatment and many more drug candidates are under development at various stages. All the known AChE inhibitor drugs suffer from shortcomings such as low bioavailability, short duration of biological action, narrow therapeutic effects and high toxicity. Hence discovery and development of new drug candidates as AChE inhibitors with enhanced potency and diminished toxicity is imperative. In late AD stage BChE, that cleaves acetylcholine in a manner similar to AChE to terminate its physiological action, represents the predominant cholinesterase in brain. Taking into account these considerations, BChE may be considered a new approach to intercede in the progression of AD. Much research is currently being conducted to identify natural products which can be used to ameliorate the AD and PD conditions.

The current study was undertaken to explore *S. lavandulifolia* aerial parts as a source of compounds as tyrosinase, AChE and BChE inhibitors for the treatment of neurodegenerative disorders. The antioxidative activities were also examined.

2. Materials and Methods

2.1. Chemicals and reagents

Methanol, ethanol, dichloromethane, *n*-hexane, DMSO, Na_2CO_3, $AlCl_3$, NaOH, acetic acid, $CuSO_4$, HCl, silica gel for column chromatography and thin layer chromatography (TLC) plates were purchased from VWR (Milan, Italy). Mushroom tyrosinase, L-tyrosine, Folin-Ciocalteau reagent, quercetin, aucubin, chlorogenic acid, kojic acid, β-carotene, anhydrous sodium sulfate, ascorbic acid, propyl gallate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), linoleic acid, Tween 20, sodium phosphate buffer, sodium potassium tartrate tetrahydrate, acetylthiocholine iodide (ATCI), butyrylthiocholine iodide (BTCI), 5,5'-dithiobis (2-nitrobenzoic-acid) (DTNB), physostigmine, AChE from *Electrophorus electricus* (EC 3.1.1.7, Type VI-S), BChE from equine serum (EC 3.1.1.8), ABTS solution, Trolox were purchased from Sigma-Aldrich S.p.a. (Milan, Italy).

2.2 Plant materials

The aerial parts of *S. lavandulifolia* were collected during June 2010 in Iran (Binalud Mountains, Dowlat Abad, latitude 36°18°N; longitude: 58°5°E) and authenticated by dr. F. Nadjafi, President of Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, GC, Tehran, Iran. A voucher specimen (no. MPH-211) was deposited at the Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran.

2.3 Extraction and isolation

The aerial parts of *S. lavandulifolia* were subjected to extraction by maceration with *n*-hexane (HE, yield of 0.7%), dichloromethane (DC, yield of 1.6%), methanol (ME, yield of 16.2%), methanol with Soxhlet apparatus (MS, yield of 27.0%) and ethanol 70% (ET, yield of 23.0%). The ET extract was applied to preparative medium pressure liquid chromatography (MPLC) using CH_2Cl_2/MeOH mixtures of increasing polarity (95:5 to 0:100) as eluents. MPLC analysis was carried out on a Buchi instrument consisted of a Complete Flash System, column, Specifications Pump Manager C-615, 2 Pump Modules C-605, Detector UV C-630, Fraction Collector Buchi C-660, and Sepacore Record 1.0 Chromatography Software. A total of 300 fractions were collected. Column fractions were assayed according to their thin-layer chromatography (TLC) profile on silica gel and detected by UV light at 254 and 365 nm, sulphuric vanillin, polyethylene glycol reagent, and H_2SO_4 50% v/v. According to their TLC profile, similar fractions were combined, which resulted into 15 fractions. Fractions 4, 6 and 8 were further purified by flash column chromatography (CC) for the isolation and identification of bioactive compounds 1-5 using silica gel (0.040-0.063 mm) and a CH_2Cl_2/MeOH gradient system.

ME and MS extracts were subjected to the same separation procedure by MPLC and flash CC affording compounds **1**-**5**.

The DC extract was subjected to MPLC using *n*-hexane-ethyl acetate mixtures of increasing polarity as eluent. A total of 95 fractions were collected. Similar fractions were combined, according to thin-layer (TLC) profile, which resulted into 10 fractions. Faction 4, subjected to flash CC over silica gel (0.04-0.06 mm) using an *n*-hexane-ethyl acetate gradient system, yielded compound **6**.

The structures of isolated compounds were elucidated by analysis of their spectral data and by comparison with literature data. All the compounds are known and identified as monomelittoside (**1**), melittoside (**2**), 5-allosyloxy-aucubin (**3**), acteoside (**4**), arbutin (**5**), and stachysolone (**6**) [12-14]. The chemical structures of these six secondary metabolites isolated from *S. lavandulifolia* aerial parts extracts are displayed in Figure 1.

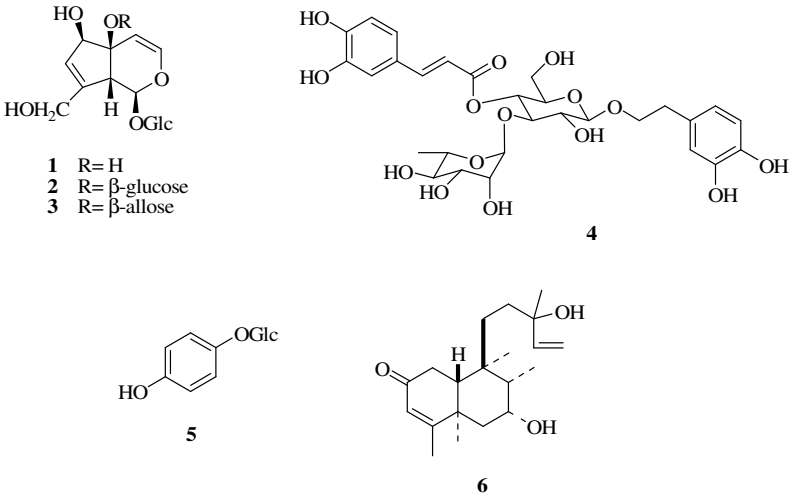

Figure 1. Main constituents of *S. lavandulifolia*.**1**, Monomelittoside; **2**, Melittoside; **3**, 5-Allosyloxy-aucubin; **4**, Acteoside; **5**, Arbutin; **6**, Stachisolone

2.4 Gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) analyses

The *S. lavandulifolia n*-hexane extract was analysed by GC-MS using helium as carrier gas. Analyses were carried out using a Hewlett-Packard 6890 gas chromatograph equipped with an HP-5 non polar capillary column (30 m length, 0.25 mm i.d., 0.25 μm film thickness) and interfaced with a Hewlett Packard 5973 Mass Selective. Ionization of the sample components was performed in electron impact mode (EI, 70 eV). Column temperature was initially kept at 50 °C for 5 min, increased to 280 °C at 13.0 °C/min, then hold at 280 °C for 10 min. The injector and transfer line temperatures were 250 and 280 °C, respectively. Constituents were tentatively identified by gas chromatography by comparison of their retention indices with those of the literature or with those of authentic compounds available in our laboratory [15]. The retention indices were determined in relation to a homologous series of *n*-alkanes (C_8-C_{28}) under the same operating conditions. Further tentative identification was made by comparison of their mass spectra with those stored in Wiley 138 and NIST 98 libraries. GC analyses were performed on a Shimadzu GC17A gas chromatograph equipped with a flame ionization detector (FID) and controlled by Borwin Software, using a fused silica 30 m SE-54 capillary column with an internal diameter of 0.25 μm and a film thickness of 0.25 μm. Nitrogen was used as the gas vector at a constant flow of 1 mL/min. Injector and transfer line temperatures were maintained at 250 and 280 °C, respectively. The oven temperature programming was as described above. The quantitative determinations of each component expressed in percentages were carried out by peak area normalization measurements using an external standard method.

2.5 Total phenol and flavonoid content

The total phenol content of *S. lavandulifolia* polar extracts was determined as previously described [16]. Briefly, 100 μL of the extract (1.5 mg/mL) were mixed with 0.2 mL Folin-Ciocalteau reagent, 2 mL of distilled water and 1 mL of 15% Na_2CO_3. The absorbance was measured at 765 nm using a UV-Vis Jenway 6003 spectrophotometer after 2h incubation at room temperature. Chlorogenic acid was used as a standard and the total phenol content was expressed as mg chlorogenic acid equivalents/g of plant materials.

The total flavonoid content was determined spectrophotometrically using a method based on the formation of a flavonoid-aluminium complex [16]. One mL of the extract (1.5 mg/mL) was added to a 10 mL volumetric flask. Distilled water was added to make a volume of 5 mL. At zero time, 0.3 mL of 5% sodium nitrite was added to the flask. After 5 min, 0.6 mL of 10% $AlCl_3$ was added, and then at 6 min 2 mL of NaOH μM were also added to the mixture, followed by the addition of 2.1 mL distilled water. Absorbance was read at 510 nm. Quercetin was chosen as standard and the levels of total flavonoids were determined in triplicate and expressed as mg quercetin equivalents/g of plant materials.

2.6 Total iridoid content

The content of iridoids was determined according to a colorimetric method based on the Trim and Hill reaction. In this assay 400 μL of extract was mixed with 4.0 mL of Trim-Hill reagent (acetic acid/0.2% $CuSO_4$/HCl 10:1:0.5). After the sample had been heated at 100 °C for 5 min, the absorption was read at 609 nm a blue colour indicating the presence of iridoids. Aucubin was used as standard. The total iridoid content was determined in triplicate and expressed as mg aucubin equivalents/g of plant materials.

2.7 Mushroom tyrosinase inhibition assay

The assay was performed using L-tyrosine as a substrate and kojic acid as positive control [17]. Forty microliters of mushroom tyrosinase solution (100 units/mL), 40 μL of 0.1 mg/ml L-tyrosine

solution in phosphate-buffered saline (PBS) solution (25 mM, pH 6.8), 80 μL of phosphate-buffered saline (PBS) solution (25 mM, pH 6.8), and 40 μL of sample in 20% MeOH solution were added to a 96-well microplate. The assay mixture was incubated at 37 °C for 30 min. A 20% MeOH solution was added to a blank solution. Before and after incubation, the amount of dopachrome produced in the reaction mixture was measured at 492 nm in the microplate reader. The percentage of the inhibition of tyrosinase was calculated by the equation: Inhibition (%)= [(A−B)−(C−D)]/(A−B)×100, where A is the absorbance of blank solution after incubation, B is the absorbance of blank solution before incubation, C is the absorbance of sample solution after incubation, and D is the absorbance of sample solution before incubation.

2.8 Cholinesterase inhibitory activity assay

AChE and BChE inhibitory activity was measured by a spectrophotometric method based on the reaction of released thiocholine to give a coloured product with a chromogenic reagent [11]. AChE or BChE and samples (20 μL) at final concentrations in the range 20-500 μg/mL were added to 2 mL of buffer and pre-incubated in an ice bath at 4 °C for 30 min. The reaction was started by adding DNTB solution and either ATCI or BTCI, and the tubes were placed in a water bath at 37 °C for 20 min. The reaction was halted by placing the assay solution tubes in an ice bath and adding physostigmine. The hydrolysis of acetylthiocholine and butyrylthiocholine was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released during enzymatic hydrolysis, and recorded at 405 nm. Physostigmine was used as positive control.

2.9 Determination of DPPH radical scavenging capacity

The assay was carried out as previously described [16]. In an ethanol solution of DPPH radical, samples at different concentrations were added. The reaction mixtures were shaken and kept in the dark for 30 min. The absorbance of the resulting solutions was measured at 517 nm against blank without DPPH. A decrease in the absorbance of the DPPH solution indicates an increase of DPPH radical scavenging activity. Ascorbic acid was used as positive control.

2.10 Determination of ABTS radical scavenging capacity

ABTS assay was based on the method previously reported with slight modifications [16]. Ascorbic acid was used as positive control. The scavenging ability of samples was calculated according to the equation: ABTS scavenging activity (%)= $[(A_0–A)/A_0] \times 100$, where A_0 is the absorbance of the control reaction and A is the absorbance in the presence of samples.

2.11 Ferric Reducing Antioxidant power (FRAP) assay

This assay measures the absorption change when the TPTZ-Fe^{3+} complex is reduced to the TPTZ–Fe^{2+} form in the presence of antioxidants. Briefly, 0.2 mL of sample solution was mixed with 1.8 mL of FRAP reagent, and the absorption was measured at 595 nm. Ethanol solutions of known Fe (II) concentration, in the range of 50-500 μM $FeSO_4$, were used to obtain the calibration curve. The FRAP value represents the ratio between the slope of the linear plot for reducing the Fe^{3+}-TPTZ reagent by sample compared with the slope of the plot for $FeSO_4$. BHT was used as positive control.

2.12 β-Carotene bleaching test

One mL of a β-carotene solution (0.2 mg/mL in chloroform) was added to 0.02 mL of linoleic acid and 0.2 mL of 100% Tween 20. After evaporation of chloroform and dilution with water (100 mL), 5 mL of the emulsion were transferred into different test tubes containing 0.2 mL of samples.

The tubes were shaken and placed at 45 °C in a water bath for 60 min. Absorbance was measured at 470 nm against a blank consisting of an emulsion without β-carotene. The measurement was carried out at t= 0, t= 30 and 60 minutes. Propyl gallate was used as standard.

2.13 Statistical analysis

The inhibitory concentration 50% (IC_{50}) was calculated from the Prism concentration-response curve (GraphPad Software, San Diego, CA, USA) obtained by plotting the percentage of inhibition versus the concentrations. Differences within and between groups were evaluated by one-way analysis of variance test (ANOVA) followed by a multicomparison Dunnett's test compared with the positive controls.

3. Results and Discussion

3.1. Compositional analysis

The phytochemical investigation of ME, MS and ET extracts from *S. lavandulifolia* led to the isolation of three iridoids (**1**-**3**), the phenylethanoid glycoside acteoside (**4**) and the hydroquinone glucoside arbutin (**5**) (Table 1). These compounds are known and were identified in other *Stachys* species [7, 13, 14, 18-24].

Table 1. Total phenol, flavonoid and iridoid content and yield (%) of isolated constituents of *S. lavandulifolia*.

	ME	MS	ET
Total phenols[a]	36.20	50.8	39.13
Total flavonoids[b]	7.13	10.4	8.61
Total iridoids[c]	21.72	30.9	26.14
Monomelittoside[*] (**1**)	1.35	0.2	0.33
Melittoside[*] (**2**)	0.28	0.2	0.13
5-Allosyloxy-aucubin[*] (**3**)	0.85	1.0	2.33
Acteoside[*] (**4**)	0.83	0.2	0.37
Arbutin[*] (**5**)	0.13	0.1	0.34

ME: methanol extract; MS: methanol Soxhlet apparatus; ET: ethanol 70% extract. [a]mg equivalents of chlorogenic acid/g plant materials. [b]mg equivalent of quercetin/g plant materials. [c]mg equivalents of aucubin/g plant materials. [*]Data represent the percentage calculated respect to plant materials.

The structures of the three known iridoids (**1**-**3**) are characterized by the same aglyconic part, with two double bonds at C3-C4 and C7-C8 positions, a hydroxyl function at C6 and a hydroxy methyl group at C8, an a glucose unit at C1. Compounds **2** and **3** showed a second sugar unit at C5. Monomelittoside (**1**) was obtained with a percentage ranged from 0.25% to 1.35% for MS and ME extracts, respectively. Lower percentages were obtained with the iridoid melittoside (**2**) using all the three solvents. To the best of our knowledge, this is the first report of both iridoids in *S. lavandulifolia*. Compounds **1** and **2** were previously recognized in *S. corsica*, *S. grandidentata*, and *S. glutinosa* [13, 20, 21]. 5-Allosyloxy-aucubin (**3**), obtained from ET extract with the major percentage (2.3%), was also identified in *S. lavandulifolia* and *S. glutinosa* [7, 13]. Phenylethanoid glycosides are of common occurrence within the genus *Stachys*. Acteoside (**4**) was previously isolated in *S. byzantina*, *S. lanata*, *S. macrantha*, *S. officinalis*, *S. recta*, and *S. sieboldii* [7, 18-24]. For the first time in *S. lavandulifolia*, we recognized the hydroquinone glucoside arbutin (**5**) that was previously isolated in *S. germanica* subsp. *salviifolia* [14]. Stachysolone (**6**) was identified as major constituent from the dichloromethane extract. This diterpene, previously isolated from *S. annua* and *S. recta* [12, 25-27], was recorded for the first time from *S. lavandulifolia*.

Table 2. Main constituents tentatively identified in *S. lavandulifolia n*-hexane extract.

Compound	*I*[a]	Abundance (%)	Identificati
Monoterpenes			
α-Pinene	936	3.9	A
β-Pinene	978	8.7	A
β-Myrcene	986	8.8	A
β-Phellandrene	1030	5.7	B
(*Z*)-β-Ocimene	1040	4.6	B
(*E*)-β-Ocimene	1047	1.2	B
Carvacrol	1299	0.6	B
Sesquiterpenes			
trans-Caryophyllene	1418	5.3	A
α-Humulene	1454	0.3	B
Germacrene D	1480	10.6	A
γ-Cadinene	1513	0.8	B
δ-Cadinene	1522	1.2	B
Spathulenol	1641	0.4	B
Diterpenes			
Neophytadiene	1830	0.2	B
8,13-Epoxy-15,16-dinorlab-12-ene	1873	0.6	C
trans-Phytol	1950	2.3	B
Manoyl oxide	1989	3.9	B
Fatty acids			
Methyl palmitate	1934	1.8	A
Palmitic acid	1969	3.7	A
Methyl linoleate	1996	2.3	B
Methyl linolenate	1999	1.4	B
Linoleic acid	2156	5.5	A
Aldehydes			
n-Decanal	1205	0.3	B
Tetradecanal	1612	0.9	B
Hexadecanal	1811	0.7	B
Alkanes			
Pentadecane	1500	0.5	A
Eicosane	2000	0.6	A
Tricosane	2300	1.3	A
Tetracosane	2400	0.3	A
Heptacosane	2700	1.4	B
Octacosane	2800	1.5	A
Nonacosane	2900	1.9	A
Triacontane	3000	1.2	A
Sterols			
Campesterol		0.5	C
Stigmasta-5,22-dien-3-ol		2.1	C
β-Sitosterol		3.5	C
Triterpenes			
β-Amyrin		1.7	C
α-Amyrin		1.9	C

[a]*I*, Retention Index on HP-5 column. [b]The reliability of the identification proposal is indicated by the following: A, mass spectrum and retention index agreed with standards; B, mass spectrum and retention index agreed with database or literature; C, mass spectrum agreed with mass spectral database.

The analysis of the chemical composition of the essential oil of the aerial parts of different stages of growth as pre-flowering, flowering and post flowering of *S. lavandulifolia* revealed the presence of α-pinene, myrcene, β-phellandrene, and β-caryophylene as most abundant compounds [30]. More recently, the variation of the oil composition of ten wild populations of *S. lavandulifolia* collected

from different geographical regions of Iran was investigated [6]. The major compounds were myrcene, limonene, germacrene D, bicyclogermacrene, δ-cadinene, pulegone, (*Z*)-hex-3-enyl tiglate, (*E*)-caryophyllene, α-zingiberene, and spathulenol. The results of another recent study confirm that distinct differences in the content of compounds depending on region of sample collection. The main constituents of the oils were α-thujone, α-pinene, myrcene, β-phellandrene, germacrene D, and δ-cadinene (trace to 11.6%) [31].

3.2 Screening for tyrosinase inhibition

The tyrosinase inhibitory effects of samples increased in a linear concentration-dependent manner. ET and MS extracts were the most active with IC_{50} values of 33.4 and 42.8 μg/mL, respectively (Table 3). These extracts were characterized by the presence of known tyrosinase inhibitors, such as acteoside and arbutin [32, 33].

Generally, phenylethanoid glycosides showed moderate tyrosinase inhibitory activity with the exception of diglycosides. The inhibitory activity of phenylethanoid glycosides could be attributed to the presence of *o*-hydroxyls on the phenolic rings, which give them the property to chelate with metals [34].

Table 3. Tyrosinase inhibitory activity of *S. lavandulifolia* extracts and isolated constituents.

Sample	IC_{50} (μg/mL)
n-Hexane (HE)	272.7 ± 4.2[a]
Dichloromethane (DC)	64.3 ± 1.8[a]
Methanol (ME)	51.8 ± 1.0[a]
Methanol Soxhlet apparatus (MS)	42.8 ± 1.1[a]
Ethanol 70% (ET)	33.4 ± 0.8[a]
Monomelittoside (**1**)	119.6 ± 2.2[a]
Melittoside (**2**)	163.1 ± 3.1[a]
5- Allosyloxy-aucubin (**3**)	22.4%[#]
Acteoside (**4**)	12.9 ± 1.1[b]
Arbutin (**5**)	58.8 ± 2.7[a]
Stachysolone (**6**)	26.2%[#]
Kojic acid	10.8 ± 0.9

Data are expressed as media ± SD (n= 3). Kojic acid was used as positive control. [#]at a concentration of 200 μg/mL. One-way ANOVA ***p< 0.0001. Dunnet's Multiple Comparison Test [a]p< 0.001 (all extracts and compounds *vs* kojic acid, except acteoside), [b]p> 0.05 (kojic acid *vs* acteoside).

There are few studies in the literature on the iridoids as inhibitors of tyrosinase. Herein, monomelittoside (**1**) and melittoside (**2**) showed IC_{50} values of 0.33 and 0.31 mM, respectively, while 5-allosyloxy-aucubin (**3**) inhibited the enzyme with a percentage of 22.4% at a concentration of 200 μg/mL. Iridoids are able to play a role in the neuroprotection of cerebral ischemia and progressive neurodegenerative diseases [35]. Frequent observations show that the iridoid aglycones exhibit a higher activity than the parent glycosides and suggest that glycosides can be reasonably considered as a pro-drug. So, may be of interest the study of these secondary metabolites *in vivo* models.

3.3 Anti-cholinesterase activity

The cholinesterase inhibitory activity of *S. lavandulifolia* extracts and isolated compounds was evaluated according to Ellman's colorimetric method with some modifications. Since physostigmine has been reported as potential anticholinesterase agent, this compound was used as reference standard and positive control. The AChE and BChE inhibitory activity of *S. lavandulifolia* samples is shown in Table 4. The most active extract against AChE was the *n*-hexane extract with an IC_{50} value of 13.7 μg/mL. Against BChE the same extract exhibited an IC_{50} value of 421.9 μg/mL with a Selectively Index (SI, IC_{50} BChE/IC_{50} AChE) of 30.8. This extract was characterized by the presence of

germacrene D, β-myrcene, β-pinene, β-phellandrene, *trans*-caryophyllene, and linoleic acid as main constituents.

Table 4. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activity [IC_{50} μg/mL)] of *S. lavandulifolia* extracts.

Sample	AChE	BChE	SI (Selectively Index)
n-Hexane (HE)	13.7 ± 1.2	421.9 ±	30.8
Dichloromethane (DC)	642.8 ± 6.7	143.9 ±	0.2
Methanol (ME)	211.4 ± 5.1	28.6%[#]	-
Methanol Soxhlet apparat	359.1 ± 4.5	37.9%[#]	-
Ethanol 70% (ET)	391.3 ± 3.7	44.5%[#]	-
Physostigmine	0.2 ± 0.02	2.4 ± 0.(	12

Data are expressed as media ± SD (n= 3). SI: IC_{50} BChE/IC_{50} AChE. [#]at the concentration of 1 mg/mL. One-way ANOVA ***p< 0.0001. Dunnet's Multiple Comparison Test: AChE test p< 0.001 (all extracts *vs* physostigmine). BChE test p< 0.001 (physostigmine *vs* HE, DC, ME).

Previous works reported that essential oils and extracts rich in terpenes exhibited a strong AChE and BChE inhibitory activity. Among identified constituents in HE extract, *trans*-caryophyllene and β-phellandrene were investigated in our previous work for their potential inhibitory activity against AChE and BChE [11]. *trans*-Caryophyllene inhibited BChE with an IC_{50} value of 78.6 μg/mL while β-phellandrene was selective against AChE (IC_{50} value of 120.2 μg/mL). β-Pinene did not inhibit BChE at maximum concentration tested 10 mM. The activity of the extract is presumably related to a synergistic action of the chemical constituents. The most active against BChE was the dichloromethane extract (IC_{50} value of 143.9 μg/mL). Stachysolone (**6**), the main constituent isolated from this extract, was able to inhibit BChE with a percentage of inhibition of 50% at 0.06 mg/mL. All polar extracts namely ME, MS and ET showed a selective inhibitory activity against AChE. The most active was ME with an IC_{50} value of 211.4 μg/mL. Among isolated compounds, arbutin (**5**) and 5-allosyloxy-aucubin (**3**) demonstrated a percentage of inhibition of 50 and 23.1% at 0.06 mg/mL, respectively, against AChE. The other *S. lavandulifolia* constituents resulted inactive at the maximum concentration tested of 0.25 mg/mL.

3.4 Antioxidant activity

Biological systems produce ROS which, at high concentrations, can be responsible for lipid oxidation and DNA damage, and are heavily implicated in neurodegenerative disease. Brain aging is associated with the accumulation of this oxidative-induced damage, likely due to the imbalance between antioxidant defence mechanisms and intracellular generation of ROS. The brain contains high levels of unsaturated fatty acids, which are vulnerable to oxidation, and consumes large amounts of oxygen.

Because oxidative stress is a critical event in the pathogenesis of neurodegenerative diseases, the antioxidant properties of *S. lavandulifolia* imply the possibility of its use for therapeutic benefit. Herein, *S. lavandulifolia* extracts and isolated compounds were tested for their antioxidant activity through different methods. The IC_{50} values of all the extracts and compounds are represented in Table 5. All samples were able of scavenging both DPPH and ABTS radicals in a concentration dependent-manner. However, certain variability was observed. Linked to their higher content in phenolic compounds (Table 1), the three most polar extracts of *S. lavandulifolia* are characterized by the best radical scavenging activity. In particular, ethanol 70% and methanol extracts exhibited the highest radical scavenging activity against ABTS radical (IC_{50} values of 19.9 and 22.8 μg/mL, respectively), while methanol Soxhlet apparatus was the most active in the DPPH test (IC_{50} of 25.0 μg/mL).

Table 5. Antioxidant activity of extracts and isolated constituents of *S. lavandulifolia.*

S. lavandulifolia	DPPH test (IC_{50} µg/mL	ABTS (IC_{50} µg	β-Carotene bleaching te (IC_{50} µg/mL)		FRAP test (µM Fe(II)/g)
Extract			**30**	**60 min**	
HE	1393.6 ± 4.2	38%	N	NA	29.9 ± 1.2[g]
DC	121.6 ± 3.2	249.5 ±	N	NA	5.8 ± 0.9 [g]
ME	38.4 ± 1.8 [a]	22.8 ±	29.3	60.3 ± 2.3[f]	37.5 ± 0.7[g]
MS	25.0 ± 1.1 [a]	25.4 ±	95.6	97.1 ± 3.1[f]	44.5 ± 1.0[g]
ET	38.7 ± 2.9 [a]	19.9 ±	33.0	34.6 ± 1.8[f]	35.6 ± 0.6[g]
Compound					
1	118.1 ± 1.7	87.2 ±	8.8 ±	29.8 ± 0.7[a]	8.4 ± 0.8[a]
2	34%[#]	104.3 ±	10.7	43.2 ± 1.2[a]	1.1 ± 0.5[a]
3	32%[#]	156.1 ±	49.3	46%[##]	NA
4	373.5 ± 2.8	75.8 ±	7.5 ±	19.7 ± 0.4[a]	6.8 ± 0.4[a]
5	62.5 ± 0.9[a]	45.7 ±	37.8	61.7 ± 1.5[a]	12.2 ± 0.6[a]
6	30%[#]	38%	20	NA	NA
Positive control					
Ascorbic acid	5.0 ± 0.8	1.7 ±			
Propyl gallate			1.0 ±	1.0 ± 0.03	
BHT					63.2 ± 4.5

Data represent means ± SD (n= 3). NA: not active. [#]at the concentration of 0.3 mg/mL. [##]at the concentration of 1.3 mg/mL. One-way ANOVA ***p< 0.0001. Dunnet's Multiple Comparison Test: DPPH test [a]p< 0.001 (all extracts *vs* ascorbic acid), [a]p< 0.001 (all compounds *vs* ascorbic acid); ABTS test [b]p< 0.001 (all extracts *vs* ascorbic acid), [a]p< 0.001 (all compounds *vs* ascorbic acid); β-Carotene bleaching test at 30 min of incubation [c]p> 0.05 (propyl gallate *vs n*-hexane, dichloromethane), [d]p< 0.001 (propyl gallate *vs* MeOH, MeOH with Soxhlet, EtOH 70%), [a]p< 0.001 (all compounds *vs* propyl gallate except stachysolone); β-Carotene bleaching test at 60 min of incubation [e]p> 0.05 (propyl gallate *vs n*-hexane, dichloromethane), [f]p< 0.001 (propyl gallate *vs* MeOH, MeOH with Soxhlet, EtOH 70%), [a]p< 0.001 (all compounds *vs* propyl gallate except stachysolone and 5-allosyloxy-aucubin); FRAP test [g]p< 0.001 (all extracts *vs* BHT), [a]p< 0.001 (all compounds *vs* BHT).

Generally, isolated compounds evidenced a low radical scavenging activity. In both DPPH and ABTS tests the most active was arbutin (**5**) with IC_{50} values of 62.5 and 45.7 µg/mL, respectively. Extracts were able to inhibit the discoloration of β-carotene. ME and ET extracts showed the best activity after 30 minutes of incubation with IC_{50} values of 29.3 and 33.0 µg/mL, respectively. It is of interest that this value is about double after 60 minutes of incubation for both ME and ET extracts (IC_{50} values of 60.3 and 34.6 µg/mL, respectively). A lower activity demonstrated MS extract. Acteoside (**4**) and monomelittoside (**1**) demonstrated IC_{50} values of 7.5 and 8.8 µg/mL, respectively.

A concentration-response relationship was observed for all tested samples in FRAP assay. Also using this assay, the most active samples were ME, MS and ET with values in the range 35.6-44.5 µM Fe(II)/g. The reducing ability of these extracts could be related to the phenols content.

Arbutin (**5**), the most active compound as anti-radical scavenger, showed a good activity also in this assay with a value of 12.2 µM Fe(II)/g. If we compare the antioxidant activity of the three isolated iridoids (**1**-**3**), we found that monomelittoside (**1**) was the most active in all used tests. In comparison with compound **1**, melittoside (**2**) and 5-allosyloxy-aucubin (**3**) showed in their structures an additional sugar portion.

The analysis of the literature revealed that plants of the genus *Stachys* may be considered as good sources of natural antioxidants for medicinal uses such as against aging and other diseases related to radical mechanisms. The antioxidant activity of extracts from *S. sylvatica* collected from three different wild populations in Kosovo was recently studied [36]. The extracts exhibited different levels of DPPH radical scavenging capacity. In particular, leaves and inflorescences from Mushtisht and Prevallë displayed significantly higher antioxidant activity compared with the antioxidant activity of the population from Gërmia. The highest FRAP antioxidant capacity was found in leaves collected in Prevallë. FRAP antioxidant activity of inflorescences of *S. sylvatica* exhibited a significantly lower antioxidant activity in Gërmia compared with those of the other populations. A high correlation coefficient was found between phenolic compounds and flavonoids with antioxidant tests in both leaves and inflorescences.

S. iberica water extract showed antioxidant activity with a linoleic acid inhibition capacity of 88.14% at 2 mg/mL concentration, a scavenging activity and chelating effect with a percentage of

46.63% at 1.0 mg/mL and 33.14% at 2 mg/mL, respectively [37]. Methanol, ethanol and dichloromethane extracts of *S. alpina*, *S. officinalis*, *S. palustris*, *S. recta* subsp. *recta*, *S. recta* subsp. *subcrenata*, *S. salviifolia* and *S. sylvatica* showed no inhibitory effects against lipid peroxidation and xanthine oxidase, but they had the ability to scavenge DPPH [38].

In agreement with our results, polar extracts exhibited stronger activity than non-polar extracts. The most effective in the DPPH assay were the methanol extracts of *S. recta* subsp. *recta* and *S. palustris*. Previously, the methanol extract of aerial flowering parts of *S. anisochila*, *S. beckeana*, *S. plumosa* and *S. alpina* ssp. *dinarica* was investigated for total antioxidant activity (TAA), along with DPPH and OH radical scavenging activity, and lipid peroxidation [39]. All extracts, with the exception of *S. plumosa*, exhibited anti-DPPH activity with IC_{50} values < 50 μg/mL. In a concentration range from 6.25 to 50 μg/mL all extracts scavenged OH radical above 40%. As for lipid peroxidation, IC_{50} values for *S. beckeana* and *S. alpina* ssp. *dinarica* extracts were 25.07 and 49.00 μg/mL, respectively, while *S. anisochila* and *S. plumosa* extracts did not reach 50% of lipid peroxidation inhibition. The water extract of *S. byzantina* showed antiradical activity with an IC_{50} value of 0.64 mg/mL [40]. Two flavonoids, chrysoeriol 7-*O*-[6-*O*-acetyl-β-D-allopyranosyl]-(1-->2)-β-D-glucopyranoside and apigenin 7-*O*-β-D-(6-*p*-coumaroyl)-glucopyranoside, isolated from the methanol extract of *S. bombycina*, were assessed by DPPH assay and showed RC_{50} values of 1.25×10^{-2} and 7.69×10^{-4} mg/mL, respectively [41].

S. officinalis demonstrated to reduce the level of lipid peroxidation induced by hydroxyl radical generated by an iron/ascorbate system with a percentage of inhibition of 78% [42]. In DPPH test this species showed EC_{50} values of 3.0, 2.0 and 1.1 μg/mL after 5, 10 and 40 min of incubation, respectively.

4. Conclusions

This is the first study that investigated the *in vitro* activity of five extracts of the aerial parts of *S. lavandulifolia* for the treatment of neurodegenetive diseases in relation to their metabolites profile. Obtained data provided evidence that *S. lavandulifolia* inhibit key enzymes in AD and PD and exert antioxidant effects, suggesting the use of this species for the development of drugs for the management of neurodegenerative disorders.

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