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Chemical Profiling and Assessment of Antineurodegenerative and Antioxidant Properties of *Veronica teucrium* L. and *Veronica jacquinii* Baumg.

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Neuroprotective potential of *V. teucrium* and *V. jacquinii* methanol extracts was analyzed. Chemical analysis of investigated extracts showed the presence of phenolic acid derivatives, flavonoids and one secoiridoid. The detected flavonoids derived from flavones (luteolin and isoscutellarein in *V. jacquinii*; apigenin, isoscutellarein and luteolin in *V. teucrium*) and flavonol (quercetin in *V. jacquinii*). Acteoside was the dominant compound in *V. jacquinii*, while plantamajoside and isoscutellarein 7-*O*-(6‴-*O*-acetyl)-*β*-allosyl (1‴→2″)-*β*-glucoside were the major phenolics in *V. teucrium*. Additionally, the antineurodegenerative activity was tested at concentrations of 25, 50, and 100 μg/ml using acetylcholinesterase (AChE) and tyrosinase (TYR) assays. The inhibition of both enzymes was achieved with the investigated extracts, ranging from 22.78 to 35.40% for AChE and from 9.57 to 16.38% for TYR. There was no statistical difference between the activities of the analyzed extracts. Our data indicate that *V. teucrium* and *V. jacquinii* may have beneficial effects against *Alzheimer*'s and *Parkinson*'s disease.

Keywords: Antineurodegenerative activity, Natural products, Acteoside, Plantamajoside, Antioxidans.

Introduction

Veronica genus (Plantaginaceae) includes around 450 species, which have been used in folk medicine in Europe and Asia for the treatment of certain problems of the nervous and respiratory systems, wound healing, as well as a diuretic.[1] Also, aerial parts of numerous species from genus *Veronica* are edible, either raw or cooked. On the other hand, there is absence of scientific evidence to support their traditional use. A few studies reported that some of them exhibited noticeable antioxidant,[2] anti-inflammatory,[1][3] and neuroprotective activity.[4]

Due to the high utilization of oxygen as well as high content of unsaturated fatty acids, the neurons are very sensitive to oxidative stress.[5] That is why it is considered that the oxidative stress is involved in *Alzheimer*'s and *Parkinson*'s diseases as well as other neurodegenerative diseases. Although the human body produces enzymes and non-enzyme antioxidants that usually maintain ROS levels under control, moderate consumption of exogenous dietary antioxidants is also suggested, especially in neurological diseases, as mammalian brain has lower levels of endogenous antioxidants compared to other organs.[6]

Alzheimer's disease (AD) is a progressive neurological disease connected with reduction of acetylcholine (ACh) and butyrylcholine (BCh) levels in cortex and hippocampus in the brain.[7] Inhibition of cholinesterase enzymes, which break down ACh and BCh, could be a possible therapeutic approach for AD patients.[7] *Parkinson*'s disease (PD) is also a progressive

neurodegenerative disease, connected with dopamine deficiency in the brain. Current treatments include generally use of L-DOPA and dopamine agonists.[8] On the other hand, various studies showed that tyrosinase might be linked with neuromelanin production and damaged neurons typical in PD, marking tyrosinase inhibitors as potential drugs.[9]

In this study, the neuroprotective and antioxidant potentials of *Veronica teucrium* and *V. jacquinii* were investigated while HPLC-DAD/ESI-MS technique was used for qualitative and quantitative analyses of their phenolic compounds.

Results and Discussion

HPLC-DAD/ESI-MS Analysis

The characterization of the phenolic compounds was performed by HPLC-DAD/ESI-MS analysis, and data of the retention time, λ_{max}, pseudomolecular ion, main fragment ions in MS^2, tentative identification and concentration of phenolic acid derivatives and flavonoids are presented in *Tables 1* and *2*. UV and mass spectra obtained by HPLC-DAD/ESI-MS analysis showed that the phenolic composition of these Plantaginaceae species was characterized by the presence of phenolic acid derivatives (*p*-hydroxybenzoyl and hydroxycinnamoyl derivatives) and flavonoids in both species, and also secoiridoids in *V. teucrium*. The analysis of the MS^2 fragments revealed that in glycosylated forms, sugars were always *O*-linked, except for a *C*-glycosylated flavone in *V. teucrium*. The detected flavonoids derived from flavones (luteolin and isoscutellarein in *V. jacquinii*; apigenin, isoscutellarein and luteolin in *V. teucrium*) and a flavonol (quercetin in *V. jacquinii*). Sugar substituents consisted of glucuronides, hexosides, deoxyhexosides, and pentosides, as deduced from the losses in the MS^2 spectra of 176, 162, 146, and 132 Da, respectively.

Hydroxycinnamoyl Derivatives

Various hydroxycinnamoyl derivatives were observed, especially caffeic acid derivatives, that were assigned according to their characteristic UV spectra, showing maximum wavelength around 326 – 334 nm, and to the product ion at *m/z* 179 ([caffeic acid – H]⁻) observed in their MS^2 spectra. Peak 2 in *V. teucrium* was identified as 5-*O*-caffeoylquinic acid by comparison with a standard, and also due to its MS^2 fragmentation pattern as reported by *Clifford et al.*[10]

The characteristic fragments at *m/z* 179, 161 and 135 were observed in peak 3 (pseudomolecular ion

[*M* − H]⁻ at *m/z* 539) in *V. jacquinii*, indicating that this corresponded to a caffeic acid derivative, although its precise structure could not be elucidated.

Peak 7, in *V. jacquinii* yielded a pseudomolecular ion [*M* − H]⁻ at *m/z* 755. Its fragmentation was initiated by the initial loss of the caffeoyl unit, producing an ion at *m/z* 593 ([*M* − H − 162]⁻), and the further loss of pentosyl and deoxyhexosyl residues (or both) resulted in the formation of ions at *m/z* 461 ([*M* − H − 162 − 132]⁻), 447 ([*M* − H − 162 − 146]⁻) and 315 ([*M* − H − 162 − 146 − 132]⁻). This fragmentation pattern fits with that of forsythoside B,[11][12] compound that was also described in other Plantaginaceae.[13] Peak 11, in the same species, presented a molecular ion at *m/z* 607, yielding fragments at *m/z* 461 ([*M* − H − 146]⁻) and *m/z* 315 ([*M* − H − 146 − 146]⁻) interpreted as the successive loss of coumaroyl and rhamnosyl moieties. It also presented the minor fragments at *m/z* 161 and *m/z* 135, corresponding to caffeic acid. Considering the data reported by *Sun et al.*,[12] and *Li et al.*,[14] and also the common presence of phenylethanoid glycosides in *Veronica* genus,[15] peak 11 was tentatively identified as forsythenside K.

Peak 12 showed a pseudo-molecular ion at *m/z* 637, producing ions at *m/z* 491 ([*M* − H − 146]⁻), *m/z* 461 ([*M* − H − 176]⁻) and *m/z* 315 ([*M* − H − 176 − 146]⁻), corresponding to the losses of rhamnose, ferulic acid or both, as well as at *m/z* 193 ([ferulic acid − H]⁻) and at *m/z* 175 ([ferulic acid − H − H₂O]⁻) were also observed. These features coincide with those of leukoceptoside A, as also described by *Amessis-Ouchemoukh et al.*[16]

Peaks 8 in *V. jacquinii* and 7 in *V. teucrium* ([*M* − H]⁻ at *m/z* 623) were identified as acteoside (also known as verbascoside) based on the UV spectrum and MS^2 fragmentation pattern,[11] and previously reported in other *Veronica* species.[15] The MS^2 fragment ion at *m/z* 461 could result from the loss of a caffeoyl moiety ([*M* − H − 162]⁻), while the weak ion at *m/z* 315 was consistent with the additional loss of a rhamnose unit, whereas ions at *m/z* 161 and 135 indicated the presence of caffeic acid. Similar characteristics were also observed for peak 8 in *V. teucrium* that was tentatively assigned as isoacteoside, taking into account that it is expected to elute after acteoside in RP-HPLC.[11][15] Peaks 5 and 10 in *V. teucrium* gave the same pseudomolecular ion [*M* − H]⁻ at *m/z* 771, as well as the same MS^2 fragmentation pattern with fragments at *m/z* 609 ([*M* − H − 162]⁻, loss of a possible caffeoyl/hexosyl moiety), *m/z* 477 ([*M* − H − 162 − 132]⁻, further loss of an additional pentosyl moiety) and *m/z* 315 ([*M* − H − 162 −

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Table 1. Retention time (t_R), wavelengths of maximum absorption (λ_{max}), mass spectral data, relative abundances of fragment ions, tentative identification, and quantification of the phenolic compounds *Veronica jacquinii* methanolic extracts

Peak	t_R [min]	λ_{max} [nm]	Molecular ion ([M − H]⁻) [m/z]	MS² [m/z]	Tentative identification	Quantification (mg/g extract)
1	8.6	356	625	463(47), 301(52)	Quercetin-O-hexoside-O-hexoside I	0.622[b] ± 0.002[c]
2	14.1	264	497	335(25), 221(22), 153(29), 109(11)	Protocatechuic acid derivative	0.555 ± 0.002
3	15.1	332	539	377(36), 341(36), 281(18), 251(14), 179(45), 161(18), 135(18)	Caffeic acid derivative	0.28 ± 0.01
4	15.9	344	625	463(8), 301(56)	Quercetin-O-hexoside-O-hexoside II	0.654 ± 0.004
5	16.6	334	639	477(34), 315(4), 179(2), 161(20), 135(4)	Plantamajoside	0.304 ± 0.001
6	17.3	350	623	489(4), 327(22), 285(100)	Luteolin derivative	1.42 ± 0.01
7	17.8	330	755	593(22), 461(14), 447(3), 315(3), 161(2)	Forsythoside B[11][12]	1.71 ± 0.05
8	18.5	330	623	461(28), 315(2), 161(16), 135(3)	Acteoside[11]	5.00 ± 0.01
9	19.7	352	667	625(43), 463(6), 301(51)	Quercetin-O-acetylhexoside-O-hexoside	0.614 ± 0.003
10	21.4	350	463	301(100)	Quercetin-3-O-glucoside[a]	1.34 ± 0.02
11	22.2	330	607	461(59), 315(6), 161(2), 135(4)	Forsythenside K[12][14]	1.075 ± 0.002
12	23.4	330	637	491(4), 461(40), 315(4), 193(3), 175(14)	Leukoceptoside A[16]	1.91 ± 0.01
13	27.1	268/298/328	651	609(6), 429(22), 285(86)	Isoscutellarein-O-acetyl-allosyl-glucoside isomer[24]	0.144 ± 0.004
14	28.5	278/306/326	651	609(4), 447(4), 429(17), 285(80)	Isoscutellarein-7-O-(6‴-O-acetyl)-β-allosyl (1‴→2″)-β-glucoside[23][24]	0.386 ± 0.005

[a] Compound identified by comparison with standard compound; numbers in square brackets are references used in tentative identification of compounds; [b] Mean value of each constituent in three replicate analyses. [c] Standard deviations based upon three replicates analyses.

Table 2. Retention time (t_R), wavelengths of maximum absorption (λ_{max}), mass spectral data, relative abundances of fragment ions, tentative identification, and quantification of the phenolic compounds *Veronica teucrium* methanolic extracts

Peak	t_R [min]	λ_{max} [nm]	Molecular ion ([M − H]−) [m/z]	MS2 [m/z]	Tentative identification	Quantification (mg/g extract)
1	6.4	258	153	109(100)	Protocatechuic acid[a]	0.17[b] ± 0.01[c]
2	8.7	326	353	191(100), 179(3), 173(3), 161(59),135(4)	5-O-Caffeoylquinic acid[10]	1.20 ± 0.03
3	11.9	330	593	503(4),473(16), 383(11), 353(26)	Apigenin-C-hexoside-C-hexoside[19]	0.040 ± 0.004
4	14.1	264	497	335(38), 21(34), 153(41), 109(17)	Protocatechuic acid derivative	0.57 ± 0.01
5	15.4	328	771	609(50), 477(33), 315(4), 161(7)	Aragoside[17]	1.66 ± 0.01
6	16.4	328	639	477(41), 315(5), 179(2), 161(31), 135(3)	Plantamajoside[11]	10.1 ± 0.3
7	18.5	330	623	461(42), 315(2), 161(25), 135(3)	Acteoside[11]	5.24 ± 0.01
8	19.3	326	623	461(20), 315(2), 161(18)	Isoacteoside[11][15]	0.317 ± 0.004
9	20.4	328	523	361(6), 179(8), 163(13), 135(10)	Verminoside	0.83 ± 0.02
10	21.4	330	771	609(18), 477(14), 315(3), 161(3)	Isoaragoside[17]	0.127 ± 0.001
11	22.6	330	609	447(4), 429(20), 285(100)	Isoscutellarein-7-O-allosyl-glucoside[20]	3.24 ± 0.03
12	26.1	336	445	269(100)	Apigenin-7-O-glucuronide[20]	0.978 ± 0.003
13	28.4	278/306/326	651	609(7), 447(9), 429(33), 285(100)	Isoscutellarein7-O-(6‴-O-acetyl)-β-allosyl (1‴→2‴)-β-glucoside[23][24]	26.3 ± 0.1

[a] Compound identified by comparison with standard compound; numbers in square brackets are references used in tentative identification of compounds. [b] Mean value of each constituent in three replicate analyses. [c] Standard deviations based upon three replicates analyses.

132 − 162]−, additional loss of a caffeoyl/hexosyl moiety); the last fragment at *m/z* 161 is characteristic of the caffeoyl moiety. These characteristics allowed their identification as aragoside and isoaragoside, respectively, compounds previously reported in other *Veronica* species.[17] A related phenylethanoid glycosylated derivative lacking the α-arabinopyranosyl unit present in aragoside was associated with peak 6 ([M − H]− at *m/z* 639) in the same plant and peak 5 in *V. jacquinii*. This compound produced MS2 fragment ions at *m/z* 477, ([M − H − 162]−, loss of a caffeoyl/hexosyl moiety), *m/z* 315 (([M − H − 162 − 162]−, further caffeoyl/hexosyl loss), as well as at *m/z* 179, *m/z* 161, and *m/z* 135, as it is common in caffeic acid derivatives. A compound with similar characteristics was previously identified as plantamajoside in *Veronica fuhsii* Freyn & Sint.[18]

Flavonoids

Peak 3 in *V. teucrium* presented a pseudomolecular ion [M − H]− at *m/z* 593, releasing MS2 fragment ions corresponding to the loss of 90 and 120 u (*m/z* at 503 and 473), characteristic of *C*-hexosyl flavones, and at *m/z* 383 and 353 that might correspond to the apigenin aglycone plus linked sugar residues.[19] According to these characteristics and the fact that no relevant fragments derived from the loss of complete hexosyl residues (−162 u) were detected, suggesting the existence of *O*-linked sugars, the compound was identified as apigenin-*C*-hexoside-*C*-hexoside.

Peak 12 in *V. teucrium* was identified as an apigenin glucuronide, based on its molecular ion [M − H]− at *m/z* 445 and the loss of a 176 u fragment, releasing the corresponding aglycone at *m/z* 269. The compound was tentatively identified as apigenin-7-*O*-glucuronide based on the previous observations of *Grayer-Barkmeijer*,[20] about the usual presence of such derivatives in *Veronica* species.

Peaks 14 in *V. jacquinii* and 13 in *V. teucrium* (the major compound) showed a pseudomolecular ion [M − H]− at *m/z* 651, releasing MS2 fragments at *m/z* 609 (−42 u, loss of an acetyl residue), *m/z* 447 ([M − H − 42 − 162]−, loss of an acetylhexoside) and at *m/z* 285 ([M − H − 42 − 162 − 162]−, further hexosyl loss). The observation of another intermediate ion at *m/z* 429 ([M − H − 42 − 180]−) suggested that acetyl group was located on the external sugar.[21] The ion at *m/z* 285 corresponding to the aglycone together with the characteristic UV spectra with maxima at 278, 306, and 326 nm were in agreement with those described for isoscutellarein, a flavone found in the same genus.[22] Based on the previous NMR

identification in *Veronica* species by *Albach et al.*,[23] and the fragmentation profile similar to that described by *Pereira et al.*,[24] the compound was identified as isoscutellarein 7-*O*-(6‴-*O*-acetyl)-*β*-allosyl-(1‴→2‴)-*β*-glucoside. Peak 13 in *V. jacquinii*, with the same molecular ion and similar MS^2 fragmentation as peak 14, was assigned as an isomer of this latter. Peak 11 in *V. teucrium* presented also similar UV and mass spectra characteristics, but a molecular weight 42 Da lower, suggesting the lack of the acetyl group, so that it was tentatively identified as isoscutellarein-7-*O*-allosyl-*O*-glucoside.

Quercetin derivatives were also detected in *V. jacquinii*. Peaks 1 and 4 ([M − H]$^-$ at *m/z* 625) were tentatively identified as quercetin di-hexosides. The production of fragment ions at *m/z* 463 ([M − H − 162]$^-$) and *m/z* 301 (([M − H − 162 − 162]$^-$) indicated the alternative loss of each sugar residue suggesting their location on different positions of the aglycone, although their precise identity and location could not be established, so that they were assigned as quercetin-*O*-hexoside-*O*-hexoside I and II. Peak 9, with a molecular weight 42 Da greater, was associated to a quercetin-*O*-acetylhexoside-*O*-hexoside, which was also coherent with its fragmentation profile. One additional quercetin derivative was detected with the pseudomolecular ion at *m/z* 463 (peak 10), which was positively identified as quercetin-3-*O*-glucoside by comparison with a commercial standard. Finally, peak 6 in *V. jacquinii* was associated to a luteolin derivative based on its UV spectrum and the fragment ion observed at *m/z* 285. MS^2 fragmentation yielded ions at *m/z* 489 ([M − H − 134]$^-$), *m/z* 327 ([M − H − 134 − 162]$^-$) and *m/z* 285 ([M − H − 134 − 162 − 42]$^-$), suggesting the presence of acetyl and hexosyl residues, although the precise identity of the compound could not be established.

Other Phenolic Derivatives

Peak 1 in *V. teucrium* was positively identified as protocatechuic acid by comparing its UV spectrum and retention time with those of a commercial standard. Peaks 2 in *V. jacquinii* and 4 in *V. teucrium*, with a pseudomolecular ion at *m/z* 497, were linked to protocatechuic acid derivatives based on their UV spectra similar to this benzoic acid and the characteristic MS^2 fragments observed at *m/z* 153 ([protocatechuic acid − H]$^-$) and 109 ([protocatechuic acid − H − CO_2]$^-$). Despite some of the fragment ions corresponded to common losses such as hexosyl moieties (fragments at *m/z* 335), their precise structures could not be concluded and remain to be identified.

Peak 9 in *V. teucrium* presented a pseudomolecular ion at *m/z* 523 and an MS^2 fragmentation pattern with three fragments at *m/z* 179, 161, and 135 characteristic of caffeic acid derivatives, together with another product ion at *m/z* 361 (−162 u, possible loss of a hexosyl moiety). These characteristics are coherent with the structure of verminoside, consisting of the iridoid catalpol linked to caffeic acid. Actually, iridoids are a type of compounds commonly reported in *Veronica* species, and the presence of various catalpol derivatives in *Veronica* species, including caffeoyl-catalpol, was already reported by *Grayer-Barkmeijer*.[25]

Evaluation of Antioxidant Activity

The role of oxidative stress in the pathogenesis of neurodegenerative diseases has received considerable attention. Accordingly, our first step was evaluating the antioxidant activity of methanol extracts of *V. teucrium* and *V. jacquinii*. Three different tests (FRAP, DPPH, and ABTS), often used to assess the antioxidant activity of medicinal plants, were applied in our study (*Table 3*). The reducing capacity of the extracts may serve as an important indicator of their antioxidant potential. The ability of the same extracts to reduce ferric ions was determined employing FRAP assay. The methanolic extract of *V. teucrium* exhibited significantly stronger activity than the extract of *V. jacquinii* and its activity was similar to the activity of the standard antioxidant BHA. The free radical scavenging activity of *Veronica* extracts was determined using DPPH and ABTS assays. In the DPPH assay, *V. teucrium* extract also showed stronger activity (IC_{50} = 28.49 μg/ml) than *V. jacquinii* extract (IC_{50} = 37.63 μg/ml), although both extracts were less active than the standard antioxidants BHT and BHA. However, in the ABTS assay, the extract of *V. jacquinii* showed significantly higher activity than the extract of *V. teucrium* (1.32 ± 0.04 mg AAE/g for *V. teucrium* and 1.98 ± 0.04 mg AAE/g for *V. jacquinii*), but still lower activity than BHT and BHA.

The antioxidant activity of both extract could be explained by their contents of biologically active compounds. Polyphenols are the most abundant secondary metabolites in plants, which show strong protective effect on cellular oxidative damage. They are also recognized as the most important dietary antioxidants. The dominant group of phenolic compounds in tested *Veronica* species were phenylpropanoid glycosides. The high radical scavenging activity of acteoside and plantamajoside was previously shown,[26] namely in the DPPH assay, with IC_{50} values = 13.0 and 11.8 μM, respectively, compared

Table 3. Antioxidant activities of *Veronica teucrium* and *Veronica jacquinii* methanol extracts

Methanol extracts	DPPH activity (IC_{50} [µg/ml])	ABTS activity (mg AAE/g)*	FRAP assay (µmol Fe(II)/g)**
V. teucrium	28.49 ± 0.6[a]	1.32 ± 0.04[a]	610.47 ± 32.44[ad]
V. jacquinii	37.63 ± 0.6[b]	1.98 ± 0.04[b]	511.85 ± 21.83[b]
BHT	17.94 ± 0.1[c]	2.75 ± 0.02[c]	445.34 ± 7.77[c]
BHA	13.37 ± 0.4[d]	2.82 ± 0.01[c]	583.72 ± 5.26[c]

Data are presented as means ± SD, $n = 3$. Sample concentrations: *0.5 mg/ml, **0.1 mg/ml; means with different letters are significantly different ($P < 0.05$).

with of 302 µM for BHT. The high radical scavenging potential of acteoside and plantamajoside is possibly due to their orto-dihydroxyphenyl moieties. Within the flavonoid family, quercetin and its derivatives, highly present in *V. jacquinii*, are also potent antioxidants. They exert antioxidant effects by various mechanisms (radical scavenging, hydrogen donating, singlet oxygen quenching and metal iron chelating). The biological activity of quercetin derivatives strongly depends on the nature and position of the substituents.[27] Some quercetin derivatives present in *V. jacquinii* showed higher anti-DPPH activity compared to ascorbic acid and quercetin ($IC_{50} = 21.6$ and 27.5 mM for isoquercetin and hyperoside, respectively, and 27.8 and 32.2 mM for ascorbic acid and quercetin, respectively).[28] In general, quercetin glycosides showed smaller reducing capacity in FRAP assay.[29] In this sense, the differences in the antioxidant activity of *V. teucrium* and *V. jacquinii* methanol extracts could be associated to the difference in the detected secondary metabolites and their amounts in the extracts.

Evaluation of Antineurodegenerative Activity

Alzheimer's disease (AD) affects memory and other aspects of human mind and is characterised by the loss of activities relating to the acetylcholine in the cerebral cortex. The most widely used treatment against *Alzheimer*'s disease is the inhibition of acetylcholinesterase (AChE), the enzyme that hydrolyses acetylcholine.[30] Most of the AchE inhibitors such as galanthamine were originally isolated from plants, indicating that herbal medicines are a good source for the search of novel AchE inhibitors.[31] Also, it has been proposed that tyrosinase might be linked with damaged neurons typical for another progressive neurological disease, *Parkinson*'s disease, pointing to tyrosinase inhibitors as potential medications.[9]

In a previous work,[4] our group reported moderate neuroprotective activity of *V. jacquinii* and *V. teucrium* extracts on human neuroblastoma SH-SY5Y cell line by increasing cell survival in cells stressed with sodium nitroprusside and H_2O_2, compared to the non-stressed cells. Accordingly, in this study the antineurodegenerative activity of those extracts was checked more thoroughly by testing them at concentrations of 25, 50, and 100 µg/ml using acetylcholinesterase (AChE) and tyrosinase (TYR) assays. Inhibition of both enzymes was obtained by both extracts and it ranged from 22.78 to 35.40% for AChE and from 9.57 to 16.38% for TYR (*Table 4*). There were no statistical differences between the activities of the extracts, but they showed statistically weaker activity than the standards galanthamine for AChE assay and kojic acid for TYR assay. Also, a statistically significant difference was noticed among tested concentrations for the individual extracts. The methanol extract of *V. teucrium* showed stronger inhibitory effect at the highest tested concentrations in both assays. However, methanolic extracts of *V. jacquinii* exhibited

Table 4. Antineurodegenerative activities of *Veronica teucrium* and *Veronica jacquinii* methanol extracts

Concentration [µg/ml]	AChE inhibition [%]			TYR inhibition [%]		
	V. teucrium	*V. jacquinii*	Galanthamine	*V. teucrium*	*V. jacquinii*	Kojic acid
25	22.78 ± 0.70[a]	32.76 ± 1.50[a]	42.38 ± 0.74[a]	9.57 ± 1.03[a]	32.76 ± 1.50[a]	35.73 ± 5.46[a]
50	35.40 ± 3.14[b]	30.42 ± 1.76[a]	50.56 ± 0.51[b]	10.85 ± 0.49[a]	30.42 ± 1.76[a]	33.93 ± 3.78[a]
100	35.12 ± 1.94[b]	26.58 ± 1.77[b]	57.11 ± 1.68[c]	16.38 ± 0.84[b]	26.58 ± 1.77[b]	51.81 ± 2.55[b]

Data are presented as means ± SD, $n = 3$. Means with different letters are significantly different ($P < 0.05$).

maximum inhibition at the lowest tested concentrations (25 and 50 mg/ml) in AChE assay, while the activity against TYR was not dose dependent.

As for the antioxidant activity, antineurodegenerative activity of *V. teucrium* and *V. jacquinii* methanol extracts could be attributed to the presence of a variety of biologically active secondary metabolites. A number of polyphenols isolated from plants were identified as AChE and TYR inhibitors, such as quercetin, kaempferol and caffeic acid,[32] which were detected in higher amounts in *V. jacquinii*. Moreover, similar findings were reported for some iridoids.[33] *Bae et al.*,[34] showed that acteoside, which is among the predominant compounds in both *V. teucrium* and *V. jacquinii* extracts, exhibited effective AchE inhibitory activity. This phenylpropanoid has been reported to protect human neuroblastoma SH-SY5Y cells against Aβ cell injury by protecting ROS production and modulating the apoptotic signal pathway.[35] Also, acteoside inhibited the aggregation of Aβ42 in a dose dependent manner.[36] These latter authors also reported that a catechol moiety in the phenylpropanoid glucoside is essential for the exhibited activity. Therefore, the observed inhibitory activity in our study could be, at least partially, explained by the presence of acteoside.

On the other hand, there are no data regarding neuroprotective activity of other dominant compounds in *V. teucrium*, like plantamajoside and isoscutellarein7-*O*-(6'''-*O*-acetyl)-β-allosyl-(1'''→2''')-β-glucoside, and consequently their activity should be determined in some further investigation and compared with the activities of crude extract.

Conclusions

This is the first study that reports the *in vitro* activity of *V. teucrium* and *V. jacquinii* methanol extracts against enzymes involved in neurodegenerative diseases in relation to their metabolites profile. Obtained data provided evidence that *V. teucrium* and *V. jacquinii* methanol extracts inhibit enzymes connected with progression of AD and PD and also possess significant antioxidant activity. Such activities could be connected to the presence of a variety of secondary metabolites; *i.e.* flavonoids, iridoids, and phenylpropanoid glycosides. Although activities of the extracts were weaker than usual standards for those activities, the results of the present study suggest that *V. teucrium* and *V. jacquinii* could be of interest for the development of supplements that could manage neurodegenerative disorders.

Experimental Section

Plant Material

The aerial flowering parts of *V. jacquinii* and *V. teucrium* were collected in May 2014 from the mountain Goč in central Serbia. Plant material was taxonomically determined and deposited with the Herbarium collection of the Institute for Medicinal Plants Research "Dr. Josif Pančić", Belgrade (voucher specimen numbers were VG 421 and VG 428 for *V. jacquinii* and *V. teucrium*, resp.). Plants were air dried and reduced to a fine powder before extraction procedure.

Chemicals

Methanol, ethanol, distilled water, glacial acetic acid, hydrochloric acid, and ethyl acetate were purchased from *Zorka Pharma*, Šabac (Serbia). HPLC-grade acetonitrile was obtained from *Merck KgaA* (Darmstadt, Germany). Formic acid was purchased from *Prolabo* (*VWR International*, France). The phenolic compound standards (quercetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, *p*-hydroxybenzoic acid, caffeic acid, protocatechuic acid, luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, and apigenin-6-*O*-glucoside) were from *Extrasynthese* (Genay, France). Gallic acid, quercetin, ascorbic acid, 2(3)-tert-butyl-4-hydroxyanisole (BHA), 3,5-di-tert-butyl-4 hydroxytoluene (BHT) 2,2-dyphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,4,6-tripyridyl-s-triazine (TPTZ), *Folin–Ciocalteu* reagent, acetylcholinesterase from Electrophorus electricus (electriceel) (AChE), acetylcholine iodide, galanthamine hydrobromide from Lycoris sp., kojic acid, tyrosinase from mushroom and 3,4-dihydroxy-L-phenylalanine (L-DOPA) were purchased from *Sigma Chemicals Co.* (USA). All other reagents were of analytical grade. Water was treated in a *Milli-Q* water purification system (*TGl Pure Water Systems*, USA).

Characterization of Phenolic Compounds

Extraction procedure and characterization of phenolic compounds were based on our previously published protocol.[15] One gram of powdered plant samples was extracted by stirring with 30 ml of methanol/water 80:20 (*v/v*), at r.t., 150 rpm, for 1 h. After filtration the residue was re-extracted twice with 30 ml of methanol/water 80:20 (*v/v*). The combined extracts were evaporated at 35 °C (rotary evaporator *Büchi R-210*, Flawil, Switzerland) in order to remove methanol. The aqueous phase was lyophilized and then

re-dissolved in 20% aqueous methanol to obtain a final concentration of 5 mg/ml and filtered through a 0.22-µm disposable LC filter disk for high performance liquid chromatography (HPLC-DAD/MS) analysis. The extracts were analysed using a *Hewlett-Packard 1100* chromatograph (*Agilent Technologies*) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. A *Waters Spherisorb S3 ODS-2 C18*, 3 µm (4.6 × 150 mm) column thermostated at 35 °C was used. The solvents used were: (*A*) 0.1% formic acid in water, (*B*) acetonitrile. The elution gradient established was isocratic 15% *B* for 5 min, 15 to 20% *B* over 5 min, 20 – 25% *B* over 10 min, 25 – 35% *B* over 10 min, 35 – 50% for 10 min, and re-equilibration of the column, using a flow rate of 0.5 ml/min. An injection volume of 100 µl was used. Double online detection was carried out in the DAD using 280 and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system *via* the DAD cell outlet.

MS detection was performed in an *API 3200 Qtrap* (*Applied Biosystems*, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 °C, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupols were set at unit resolution. The ion spray voltage was set at −4500 V in the negative mode. The MS detector was programmed to perform a series of two consecutive modes: enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to record full scan spectra to obtain an overview of all of the ions in sample. Settings used were: declustering potential (DP) −450 V, entrance potential (EP) −6 V, collision energy (CE) −10 V. Spectra was recorded in negative ion mode between *m/z* 100 and 1000. Analysis in EPI mode was further performed in order to obtain the fragmentation pattern of the parent ion(s) detected in the previous experiment using the following parameters: DP −50 V, EP −6 V, CE −25 V, and collision energy spread (CES) 0 V. Extraction of phenolic compounds and their characterization were performed according to the previously published procedure.[15] The extracts were analysed using HPLC-DAD/ESI-MS technique and *Hewlett-Packard 1100* chromatograph (*Agilent Technologies*) coupled to an *HP* Chem Station (rev. A.05.04) data-processing station. MS detection was performed in an *API 3200 Qtrap* (*Applied Biosystems*, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software.

Phenolics present in the samples were characterized according to their UV and mass spectra and retention times compared with commercial standards when available. For the quantitative analysis of phenolics, calibration curve was constructed for different standards compounds: caffeic acid, protocatechuic acid, 5-*O*-caffeoylquinic acid, quercetin-3-*O*-glucoside, luteolin-7-*O*-glucoside, and apigenin-7-*O*-glucoside. Quantification was performed based on DAD results, using 280 nm for phenolic acids and 370 nm for flavonoid related compounds.

Evaluation of Antioxidant Activity

The Ferric-reducing ability of plasma (FRAP) assay was determined according to procedure described by *Benzie* and *Strain*,[37] with slight modifications. FRAP reagent was prepared freshly by mixing sodium acetate buffer (300 mmol/l, pH 3.6), 10 mmol/l TPTZ in 40 mmol/l HCl and $FeCl_3 \cdot 6\ H_2O$ solution (20 mmol/l), *i.e.* in proportion 10:1:1 (*v/v/v*), resp. Prior to use FRAP solution was warmed to 37 °C. One hundred µl of test sample (0.5 mg/ml) were added to 3 ml of working FRAP reagent and absorbance was recorded after 4 min at 593 nm (*JENWAY 6305* UV/VIS spectrophotometer). Blank was prepared with distilled water instead of extract. Ascorbic acid, BHA, and BHT (concentration of 0.1 mg/ml) were used as standards. In order to construct calibration curve the same procedure was repeated for standard solution of $FeSO_4 \cdot 7\ H_2O$ (0.2 – 1.6 mmol/l). FRAP values for samples were calculated from standard curve equation and expressed in µmol $FeSO_4 \cdot 7\ H_2O$/g dry extract as average values from three measurements.

Free radical scavenging activity of extracts was determined using 2,2-dyphenyl-1-picrylhydrazyl (DPPH) assay as previously reported,[38] with BHA and BHT used as positive controls. Results are presented as IC_{50} value (µg/ml). Stock solutions of dry extracts, prepared in concentration of 1000 µg/ml (*w/v*), were diluted with methanolic solution of DPPH (40 µg/ml) to adjust the final volume of reaction mixture of 2000 µl. Methanol was used as a blank, while methanol with DPPH solution was used as a control. BHA and BHT were used as positive controls (standards). Each blank, samples and standards' absorbances were measured in triplicate. Absorbance of the reaction mixture was measured after 30 min in the dark at r.t. at 517 nm using the *JENWAY 6305* UV/VIS spectrophotometer. The decrease of absorption of DPPH radical at 517 nm was calculated using equation:

$$\text{Inhibition of DPPH radical } (\%) = [(A_C - A_S)/A_C] \times 100$$

where A_C is the absorbance of control (without test sample) and A_S is the absorbance of the test samples at different concentrations. The concentrations of the extracts/standard antioxidants providing 50% inhibition of DPPH radicals (IC_{50} values, µg/ml) were calculated from DPPH absorption curve at 517 nm.

The scavenging activity of extracts was evaluated using ABTS assay using procedure of *Miller et al.*[39] Stock $ABTS^+$ solution (7 mM) was prepared $12 - 16$ h before experiment in 2.46 mM potassium-persulfate and stored in the dark at r.t. Stock $ABTS^+$ solution was diluted by distilled water to obtain an absorbance of working solution 0.700 ± 0.020 at 734 nm. 50 µl of extract (0.5 mg/ml) and/or standard solutions (0.1 mg/ml) were mixed with 2 ml of working $ABTS^+$ solution and incubated for 30 min at 30 °C. Absorbance was recorded at 734 nm using *JENWAY 6305* UV/VIS spectrophotometer. Distilled water was used as blank. BHA and BHT dissolved in methanol in concentration 0.1 mg/ml were used as standards. ABTS activity was calculated from calibration curve for ascorbic acid $(0 - 2$ mg/l) and expressed as mg of ascorbic acid equiv. per gram of dry extract (mg AAE/g). All experimental measurements were carried out in triplicate and presented as average $\pm$ standard deviation.

Evaluation of Antineurodegenerative Activity

AChE inhibitory activity assay was performed using 96-well plates according to previous method,[40] with slight modifications. The test reaction mixture (*S*) was prepared by adding 140 µl of sodium phosphate buffer (0.1M, pH 7.0), 20 µl of DTNB, 20 µl of extract-buffer solution containing 5% DMSO (concentration of 25, 50, and 100 µg/ml) and 20 µl of AChE solution (5 units/ml). Blank (*B*) did not contain AChE solution. The mixture without extract was used as the control (*C*), while the commercial anticholinesterase alkaloid-type of drug galanthamine was used as standard. After incubation (15 min, 25 °C), the reaction was initiated with the addition of 10 µl of acetylthiocholine iodide and absorbance was measured at 412 nm using *Tecan Sunrise SN* microplate reader equipped by XFluor4 software. Percentage of inhibition of AChE was determined using the formula $[(C - (S - B))/C] \times 100$.

Tyrosinase inhibitory activity assay was performed using 96-well plates according to slightly modified spectrophotometric method of *Masuda et al.*[41]. Samples and standard compound were dissolved in sodium phosphate buffer (0.1M, pH 7.0) containing 5% DMSO, in concentration 25, 50 and 100 µg/ml. The wells were designed as: *A* (containing 120 µl of sodium buffer and 40 µl of tyrosinase in the same buffer (46 units/l), *B* (containing only buffer), *C* (containing 80 µl of buffer, 40 µl of tyrosinase and 40 µl of sample) and *D* (containing 120 µl of buffer and 40 µl of sample). After addition of 40 µl of L-DOPA and incubation (30 min, 25 °C), absorbance was measured at 475 nm using *Tecan Sunrise SN* microplate reader equipped by XFluor4 software. Percentage of inhibition of tyrosinase was determined using the formula: $[((A - B) - (C - D))/(A - B)] \times 100$.

Statistical Analysis

The results were expressed as mean value of three different trials. Differences between the group means and their significance were verified by one-way ANOVA using the Software package STATISTICA v.7.0. The significance of differences was evaluated using *Bonferroni*'s test and statistical significance was set at $P < 0.05$.

Author Contribution Statement

This work was designed and coordinated by *Isabel Ferreira* and *Jelena Živković*. *João Barreira*, *Maria Inês Dias*, *Celestino Santos-Buelga*, and *Isabel Ferreira* planned and performed chemical analysis of investigated extracts. *Ana Alimpić* and *Sonja Duletić-Laušević* planned and performed antioxidant and antineurodegenerative activity assays. The manuscript was written by *João Barreira*, *Katarina Šavikin*, and *Dejan Stojković* and commented by all authors.

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