

RESEARCH ARTICLE

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The lignicolous fungus *Trametes versicolor* (L.) Lloyd (1920): a promising natural source of antiradical and AChE inhibitory agents

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ABSTRACT

This study aimed to determine antiradical (DPPH[•] and [•]OH) and acetylcholinesterase (AChE) inhibitory activities along with chemical composition of autochthonous fungal species *Trametes versicolor* (Serbia). A total of 38 phenolic compounds with notable presence of phenolic acids were identified using HPLC/MS-MS. Its water extract exhibited the highest antiradical activity against [•]OH (3.21 μg/mL), among the rest due to the presence of gallic, *p*-coumaric and caffeic acids. At the concentration of 100 μg/mL, the same extract displayed a profound AChE inhibitory activity (60.53%) *in liquid*, compared to donepezil (89.05%), a drug in clinical practice used as positive control. The flavonoids baicalein and quercetin may be responsible compounds for the AChE inhibitory activity observed. These findings have demonstrated considerable potential of *T. versicolor* water extract as a natural source of antioxidant(s) and/or AChE inhibitor(s) to be eventually used as drug-like compounds or food supplements in the treatment of Alzheimer's disease.

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Introduction

It is well-known that fungal organisms possess a tremendous biochemical potential and produce a range of secondary metabolites thanks to their sessile style of life, mainly phenolic compounds¹, such as phenolic acids^{2–6} and flavonoids³ as well as terpenoids^{4,7,8}. In recent years, polysaccharides and polysaccharide–protein complexes found in medicinal mushrooms have attracted a great attention of the scientific audience worldwide, due to their numerous bioactivities, especially antioxidant and antiradical^{5,9,10}. The presence and composition of these compounds in fungi mainly depend not only on genetic determinants, but also on ecological factors, including special environmental conditions in specific habitats and the life style (saprotrophic, parasitic and symbiotic/e.g. mycorrhizal)¹¹.

Trametes versicolor (L.) Lloyd (1920), commonly called Turkey tail, is a widespread white-rot lignicolous fungal species that grows on many deciduous trees (oak, *Prunus*) and some conifers (fir and pine trees), with basidiums mostly appearing on stumps and trunks throughout a year. It is an inedible species; however, it is one of the most known medicinal mushrooms in the world¹². Recent *in vitro* and *in vivo* studies have shown that *T. versicolor* possesses many medicinal properties, such as antitumour¹³, antimicrobial¹⁴, anti-diabetic¹⁵, immunostimulant¹⁶, AChE inhibitory¹⁷ and antioxidant activities^{5,9,10,12}. Bioactive compounds detected in this species mostly belong to a group of proteins and polysaccharides, with the polysaccharide krestin and polysaccharopeptide PSP being the most studied ones¹⁸. The fact that large number of diseases and medical conditions are associated with oxidative stress

has provoked numerous investigations of new sources of natural antioxidants, in particular microorganisms and mushrooms^{2,6}. Their advantage lies in the fact that they could be easily used as functional foods. A higher consumption of mushrooms in a regular diet, which is considered useful in the prevention of many diseases caused by oxidative stress, represents an alternative treatment based on natural products.

Reactive oxygen species (ROS) are formed as by-products in the processes of cellular metabolism including a vast number of free radicals, such as superoxide anion (O^{2-•}), hydroxyl radical ([•]OH) and hydrogen peroxide (H₂O₂). An excessive amount of ROS leads to a process known as oxidative stress, which can be provoked by tissue inflammation caused by extreme exercise or environmental factors such as radiation, xenobiotics, smoking and drugs⁴. Uncontrolled production of free radicals leads to damage of the cellular components such as lipids, proteins and DNA¹⁰, which may cause more than 100 diseases, including several forms of cancer¹⁹, diabetes²⁰, cardiovascular²¹, neurodegenerative diseases²², with stress on Alzheimer's disease (AD), as one of the most progressive (leading) health problems in the world^{16,17}.

The etiology of AD is poorly understood. The oldest hypothesis links its cause with reduced synthesis of the neurotransmitters acetylcholine, noradrenaline and serotonin in the brain²³. Deficiency in acetylcholine, which breaks down into inactive metabolites choline and acetate, is directly connected with the enzyme acetylcholinesterase (AChE) activity. AChE inhibitors (AChEi) are actually compounds that are used in the treatment of AD. Although these inhibitors do not stop the progression of disease, they can

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alleviate certain symptoms²⁴. However, considering that common AChEi-based drugs (galanthamine, tacrine, donepezil and rivastigmine) have limited effectiveness and numerous side effects, there is a real need for novel AD therapeutics²⁵. In the fungal kingdom, *Flammulina velutipes*, *Lentinus edodes* and many *Pleurotus* species have stood out for their ability to prevent or slow down the progression of neurodegenerative diseases²⁴.

Taking into account relevant literature data and bioactive potential of wild-growing autochthonous medicinal mushrooms, the aim of the present study was to investigate antiradical (DPPH• and •OH) and AChE inhibitory activities of autochthonous fungal species *T. versicolor* originated from Serbia at the same time assessing its total contents of phenolics and polysaccharides. The overall aim was to estimate its potential as a possible source of bioactive compounds to be used as functional foods.

Materials and methods

Biological material and extract preparation

Fruiting bodies of *T. versicolor* were collected from Fruška Gora Mountain (Iriški Venac) in January 2014. Determination and identification of the collected material was carried out in the microbiological laboratory at the Department of Biology and Ecology, University of Novi Sad. Voucher specimens are deposited in the Herbarium of the University of Novi Sad (BUNS), under the number 12–00706.

Fresh material was lyophilized (Alpha 2–4 LDplus, Christ GmbH, Switzerland), grounded to a fine powder (IKA A11 basic, Germany) and kept in dark bottles at room temperature until further use. For the preparation of the H₂O extract, 5 g of freeze-dried fruiting bodies was macerated with 100 mL of boiling water and stirred in a mechanical stirrer at 120 rpm (New Brunswick Scientific, Edison, NY), at room temperature for 24 h.

For the preparation of methanolic (MeOH) and ethanolic (EtOH) extracts, 5 g of fungal material was mixed with 100 mL of 100% methanol (Sigma Aldrich, Darmstadt, Germany) or 96% ethanol (Zorka Pharma, Serbia) and stirred at 120 rpm at room temperature for 72 h. After filtration (Filters FIORONI, France), H₂O extracts were lyophilized, while MeOH and EtOH extracts were evaporated (Büchi R-210, Switzerland) until the dry weight (dw) is reached. Finally, all extracts were redissolved in 100% DMSO (Baker Analyzed Reagent, JT Baker, Netherlands) to achieve the concentration of 20% (w/v). Polysaccharide (PSH) extracts were prepared according to the procedure of Ren et al.²⁷.

Determination of TPhC

TPhC was determined using the Folin-Ciocalteu (FC) reagent²⁸, based on the detection of phenols that form a colored complex with the FC reagent, whose absorbance was read at 760 nm (MultiscanGO, ThermoScientific, Waltham, MA). Gallic acid (GA) was used for the preparation of calibration curve (0.1–1000 µmol/L). The content of TPhC was expressed as mg eq. gallic acid (GAE)/g dw of the extracts.

Determination of TFC

Determination of TFC in extracts was determined spectrophotometrically²⁹. TFC was calculated on the basis of the calibration curve for quercetin (QUE) standard solution. The result was expressed as the mean of three measurements (mg eq. QUE/g dw).

Determination of TPSH

Determination of TPSH was performed by the phenol–sulfuric acid test (PSA), which was modified and adapted to experiment using microtiter plates³⁰. The absorbance was measured at 490 nm. Standard of glucose (Merck Ltd., Stara Pazova, Serbia) was made at an initial concentration of 10 mg/mL, while a standard curve was made by diluting the stock solution in order to get the total sugar content, which was expressed as mg eq. glucose (GLUE)/g dw

HPLC-MS/MS determination of the phenolic compounds

Reference standards for the phenolic compounds were obtained from Sigma-Aldrich Chem (Steinheim, Germany), Fluka Chemie gmbh (Buchs, Switzerland) or from Chromadex (Santa Ana CA). HPLC grade methanol was purchased from J. T. Baker (Deventer, The Netherlands), while p.a. formic acid and DMSO from Merck (Darmstadt, Germany).

The phenolic profile of extracts was determined by HPLC-MS/MS³¹. The extracts were diluted with mobile phase solvents A (water) and B (methanol) (1:1) to obtain 2 mg/mL. Fifteen working standards (from 1.53 to 25.0 × 10³ ng/mL) were prepared by serial dilutions (1:1) of the standard mixture with solvents A (water) and B (methanol) (1:1). The samples and standards were analyzed using the Agilent Technologies 1200 Series high-performance liquid chromatograph coupled with the Agilent Technologies 6410A Triple Quad tandem mass spectrometer with an electrospray ion source, controlled by the Agilent Technologies MassHunter Workstation software – Data Acquisition (ver. B.03.01). 5 µL of the samples were used, while the compounds were separated on the Zorbax Eclipse XDB-C18 (50 mm × 4.6 mm, 1.8 µm) rapid resolution column held at 50 °C. The mobile phase was delivered at a flow rate of 1 mL/min in gradient mode (0 min 30% B, 6 min 70% B, 9 min 100% B, 12 min 100% B, re-equilibration time 3 min). Eluted compounds were detected by ESI-MS, using the ion source parameters as follows: nebulization gas (N₂) pressure 40 psi, drying gas (N₂) flow 9 L/min and temperature 350 °C, capillary voltage 4 kV, negative polarity. Data were acquired in a dynamic MRM mode, using the optimized compound-specific parameters (retention time, precursor ion, product ion, fragmentor voltage, collision voltage). For all the compounds, peak areas were determined using the Agilent MassHunter Workstation software – Qualitative Analysis (ver. B.04.00). Calibration curves were plotted and the OriginLabs Origin Pro (ver. 8.0) software was used to calculate the concentration of the samples.

Characterization of polysaccharides

Planar Chromatography (PC) – Samples of the PSH extract were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120 °C for 16 h. The resultant hydrolysate was analyzed by descending paper chromatography, with ethyl acetate–pyridine–water as a mobile phase (2.5:1: 2.5, v/v/v). Monosaccharide components were identified by PC chromatography in a hydrolyzed PS extract of the test sample by comparison of chromatographic mobility with authentic monosaccharide standards.

Structured instrumental characterization of PSH – Organic microanalysis of elements was done for PSH. The content of C, H, N and S in the polysaccharide extract was analyzed using the automatic analyzer Vario EL III CHNS/O elemental analyzer (Elementar, Germany).

Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of the PS extract were recorded in a range of 400–4000 cm^{-1} using the KBr disc technique on the camera Nicolet 6700 FT (Thermo Scientific, Waltham, MA) in the technique of shooting ATR-FTIR (Attenuated Total Reflectance – Fourier Transform Infrared Spectroscopy).

Determination of RSC

DPPH[•] assay – DPPH radicals scavenging capacity (RSC) was determined by the DPPH method³², which is based on transformation of purple colored stable DPPH[•] into reduced yellow colored DPPH-H. The color change was measured at 515 nm. The reaction mixture contained 60 μL of DPPH reagent, 10 μL of the extract and 180 μL of MeOH. Butylated hydroxyanisole (BHA, Sigma-Aldrich, Darmstadt, Germany) was used as the standard antioxidant. All tests were performed in triplicate. Free radical scavenging capacity was determined by the following formula:

$$\text{RSC}_{\text{DPPH}} (\%) = (1 - A_{\text{test}}/A_{\text{control}}) \times 100\%$$

where A_{test} and A_{control} are the absorbances of study and control reactions. The result obtained using the regression analysis was expressed as an IC_{50} value (the concentration of the test substance at which 50% of the radicals is neutralized).

[•]OH assay – RSC of the [•]OH was determined by a modified method of Halliwell and Gutteridge³³. [•]OH react with 2-deoxy-D-ribose and oxygen creating malondialdehyde (MDA), which is determined by the TBA (thiobarbituric acid) assay based on the spectrophotometric measurement of pink coloration formed by MDA and TBA. After incubation for 60 min at 37 °C and addition of ethylenediaminetetraacetic acid (EDTA) (Thermo Scientific, Waltham, MA) and TBA reagent, the pink color was developed and measured at 532 nm (Multiscan, Thermo Scientific, Waltham, MA). The RSC value was obtained according to the same formula used for the DPPH[•] assay.

AChE inhibitory activity

In solid

Inhibition of AChE was carried out as described by Marston et al.³⁴, and the H₂O and MeOH extracts were dissolved in MeOH to a stock concentration of 1 mg/mL after lyophilization and evaporation. Dilution series were made from this stock solution as follows: 0.001, 0.01 and 0.1 mg/mL. 10 μL of each solution was applied to TLC plates in order to test 100, 10, 1 and 0.1 μg of samples to detect the minimum concentration which inhibits AChE. Galanthamine (GA, Sigma-Aldrich, Darmstadt, Germany) was used as a positive control at concentrations of 1–0.001 μg . AChE inhibitory activity was detected by a white spot on a purple background after 1–2 min exposure.

In liquid

In vitro determination of AChE inhibitory activity³⁵ adapted for the use in 96-well microplates was done. Acetylcholine iodide (AChI) originating from electric eel (Sigma Aldrich) was used as an artificial substrate for the enzyme AChE, which degrades this compound to acetate and thiocholine. In the next reaction, dithiobenzoate (DTNB) produced the yellow color with thiocholine which was measured at 412 nm at 25 °C. The reaction test wells contained 20 μL of the fungal extract, 150 μL of Ellman's reagent and AChI and 50 μL of AChE (518 U/mL) and phosphate buffer

(PB). All tests were performed in triplicate. Donepezil at 1 mg/mL (Donecept, Zdravlje Leskovac, Serbia) was used as a positive control.

The result expressed as a percentage (%) of AChE inhibition by the extract was calculated according to the following formula:

$$I_{\text{AChE}} (\%) = (1 - A_{\text{test}}/A_{\text{control}}) \times 100\%$$

Statistical analysis

The results were reported as mean values \pm standard deviation (SD). IC_{50} values were determined by the linear regression analysis of RSC (Microsoft Excel program for Windows, v. 2007 and Origin 8). The statistical analysis was performed using the one-way ANOVA (STATISTICA, StatSoft, Inc. (2012), version 10.0, www.statsoft.com). Tukey's test was used to determine significant differences ($p < .01$) between the extracts.

Results and discussion

TPhC, TFC and TPSH contents

It has been previously demonstrated that bioactive compounds derived from fungi, such as phenolics, including phenolic acids and flavonoids^{4,36}, polysaccharides⁹ and proteins³⁷ or polysaccharide–protein complexes⁹ contribute significantly to the antioxidant activity of fungal species.

The results obtained for the contents of TPh, TF and TSH are shown in Table 1. In relation to the values obtained for total contents of detected compounds, the extracts showed activity as follows: **TPhC** – H₂O > EtOH > MeOH, **TFC** – MeOH > EtOH > H₂O, and **TPSH** – H₂O > EtOH > MeOH.

The H₂O extract showed the highest TPhC (142.17 mg eq. GAE/g dw) which was actually twice bigger compared to the EtOH and MeOH extracts. Thus, TPhC may be considered as the possible main components for RSC activity observed (Table 3). Furthermore, the examined MeOH sample had twice higher TPhC (64.76 mg eq. GAE/g dw) than the same extract from China (23.28 mg eq. GAE/g dw)². The TPhC of *T. versicolor* EtOH extract analyzed herein (71.55 mg eq. GAE/g dw) showed a much higher value than the same extract from Turkey (9.58 mg eq. GAE/g extract)¹⁷. The highest content of TFC was recorded in the MeOH extract (5.13 mg eq. QUE/g dw) (Table 1).

HPLC-MS/MS determination

Among 45 investigated phenols, a total of 38 compounds were identified and quantified by HPLC-MS/MS in all the extracts examined (Table 2). A total of 20 flavonoid aglycones and glycosides, 1 biflavonoid (amentoflavone), 2 isoflavonoids (daidzein and

Table 1. The chemical composition of *T. versicolor* extracts.

	TPhC (mg eq. GAE/g dw)	TFC (mg eq. QUE/g dw)	TPSH (mg eq. GLUE/g dw)
H ₂ O	142.17 \pm 3.08*	1.56 \pm 0.48†	58.60 \pm 2.16*
EtOH	71.55 \pm 3.00†	4.13 \pm 0.91*	33.80 \pm 4.45‡
MeOH	64.76 \pm 2.08†	5.13 \pm 0.70*	46.49 \pm 4.96†

Each value is expressed as mean \pm SD.

*, †, ‡Significant differences between extracts were determined by the Tukey HSD test at $p < .01$.

Means with different letters within a column are significantly different.

H₂O: water extract of *T. versicolor*; EtOH: ethanolic extract of *T. versicolor*; MeOH: methanolic extract of *T. versicolor*; TPhC: total phenol content; TFC: total flavonoid content; TPSH: total polysaccharides.

Table 2. Quantification of particular phenolics by HPLC-MS/MS.

Class	Compound	Extract ($\mu\text{g/g dw}$)			
		PSH	H ₂ O	EtOH	MeOH
Flavones	Apigenin	1.57	1.71	0.93	0.23
	Baicalein	43.00	21.60	8.04	3.63
	Luteolin	2.01	1.47	1.05	1.19
	Chrysoeriol	1.68	1.79	1.21	0.74
	Vitexin	2.06	1.56	1.98	1.19
	Apigenin-7-O-Glucoside	2.37	1.41	1.34	0.54
	Luteolin-7-O-glucoside	0.78	0.91	0.78	0.26
	Apiin	2.86	1.82	2.07	0.86
	Baicalin	9.95	10.7	8.88	6.27
Flavonols	Kaempferol	1.69	2.15	1.96	1.58
	Quercetin	33.70	31.20	29.90	29.30
	Isorhamnetin	21.40	14.60	9.36	8.97
	Quercitrin	0.89	1.62	1.81	1.93
	Kaempferol-3-O-Glucoside	1.68	1.71	1.86	0.80
	Hyperoside	0.85	0.68	0.11	0.43
	Quercetin-3-O-Glucoside	0.96	0.31	1.72	0.59
	Rutin	1.01	1.11	1.34	0.53
Flavanone	Naringenin	1.82	1.70	1.82	1.06
Flavanols	Catechin	nd	17.20	5.91	21.90
	Epicatechin	3.92	nd	nd	nd
Biflavonoid	Amentoflavone	32.40	17.20	7.79	6.60
Isoflavonoids	Daidzein	0.86	0.53	nd	nd
	Genistein	0.51	0.37	0.30	0.21
Hydroxybenzoic acids	<i>p</i> -Hydroxybenzoic acid	10.10	141.00	465.00	184.00
	Protocatechuic acid	2.06	1.82	18.20	8.57
	Gentisic acid	nd	nd	32.10	24.90
	Vanillic acid	nd	nd	45.60	44.70
	Gallic acid	9.30	11.40	16.80	22.00
	Syringic acid	nd	6.02	59.10	30.10
Hydroxycinnamic acids	<i>p</i> -Coumaric acid	1.56	1.21	1.49	1.28
	<i>o</i> -Coumaric acid	0.81	1.16	0.79	0.74
	Ferulic acid	2.12	nd	2.66	2.12
	Caffeic acid	1.72	1.53	3.26	2.33
	Esculetin	nd	0.78	26.20	2.87
Coumarins	Scopoletin	4.22	1.66	0.59	nd
	Umbeliferon	1.40	1.13	1.37	1.13
	Quinic acid	2.72	80.40	8.84	98.70
Cyclohexanecarboxylic acid	5-O-caffeoylquinic acid	2.29	2.93	4.13	2.41
Chlorogenic acid					
Total		206.27	384.39	776.29	514.66

nd – not detected, peak not observed; the concentration is lower than the LOD.

Bold numbers indicate the highest values of the respective compound.

PSH: polysaccharide extract of *T. versicolor*; H₂O: water extract of *T. versicolor*; EtOH: ethanolic extract of *T. versicolor*; MeOH: methanolic extract of *T. versicolor*.

Table 3. Antiradical activity (EC₅₀) and acetylcholinesterase inhibitory activity of PSH, H₂O, EtOH and MeOH extracts of *T. versicolor*.

	DPPH [•] ($\mu\text{g/mL}$)	[•] OH ($\mu\text{g/mL}$)	AChE in solid (μg)	AChE in liquid (%)
PSH	950.16 ± 2.43¶	109.00 ± 1.16¶	–	nd
H ₂ O	14.89 ± 1.36 ^a	3.21 ± 0.10 ^a	10	60.53 ± 2.12
EtOH	155.61 ± 2.62‡	46.52 ± 0.18‡	–	44.35 ± 2.06
MeOH	51.57 ± 3.14†	16.45 ± 0.24†	nd	nd
BHA	8.62 ± 0.50	8.67 ± 0.58	–	–
Galanthamine	–	–	0.001	–
Donepezil	–	–	–	89.05 ± 1.35

Each value is expressed as mean ± SD.

^a, †, ‡, ¶ Means with different letters within a column are significantly different.

(Tukey's HSD test, $p < .01$).

nd: not detected; PSH: polysaccharide extract of *T. versicolor*; H₂O: water extract of *T. versicolor*; EtOH: ethanolic extract of *T. versicolor*; MeOH: methanolic extract of *T. versicolor*.

genisein), 3 coumarins, 6 hydroxybenzoic and 4 hydroxycinnamic acids, quinic acid and 5-O-caffeoylquinic acid were found. Among the investigated samples, the EtOH and MeOH extracts were the richest in phenolics, expressing TPhC of 776.29 and 514.66 $\mu\text{g/g}$, respectively.

The EtOH extract contained most of analyzed phenolic acids, with the highest amount of *p*-hydroxybenzoic acid, syringic acid and vanillic acid. The highest TFC was detected in the H₂O and

PSH extracts. Among flavonoids, the most prevalent were baicalein, catechin, isorahmetin and epicatechin, while epicatechin was detected only in the PSH extract. While several phenolic substances such as gallic, caffeic and protocatechuic acids had already been detected in autochthonous *T. versicolor*⁵, *p*-coumaric acid along with all the flavonoids were detected herein for the first time. More precisely, some flavonoid compounds had been previously found in fungi, such as catechin, naringenin, quercetin, rutin

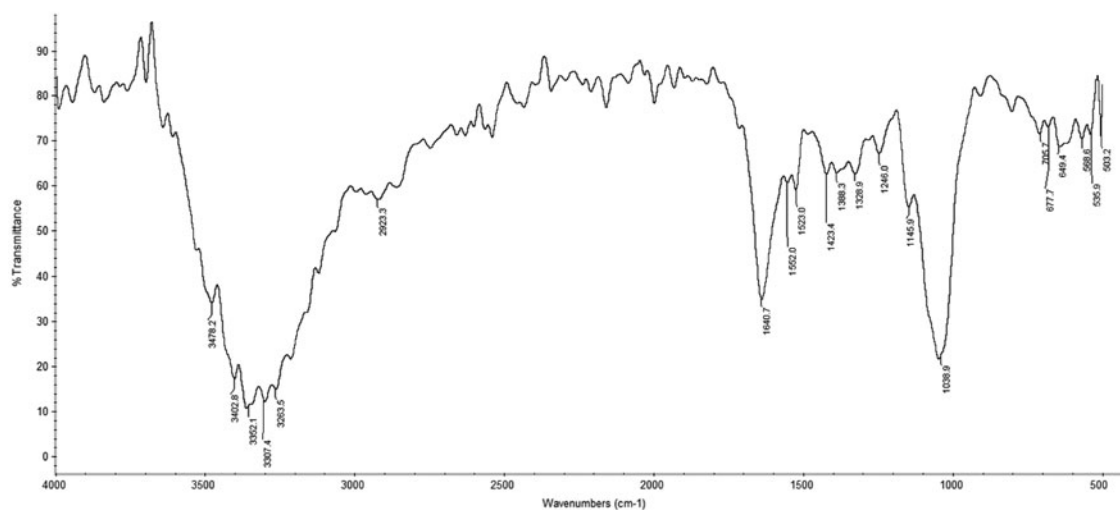


Figure 1. FTIR spectrum of the polysaccharide extract of *Trametes versicolor*. FTIR assignments, wave number (cm^{-1}): $3000\text{--}3500\text{ cm}^{-1}$ stretching vibration O-H and N-H; $2920\text{--}2950\text{ cm}^{-1}$ C-H stretching vibration; $1630\text{--}1650\text{ cm}^{-1}$ is characteristic of the absorbed water; $1200\text{--}1500\text{ cm}^{-1}$ C-H deformation vibrations; $1030\text{--}1070\text{ cm}^{-1}$ and 1150 cm^{-1} stretching vibrations of glycosidic linkage (C-O-C) and C-O-H, respectively; 850 cm^{-1} and 950 cm^{-1} indicate the presence of α -glycoside bond; 890 cm^{-1} indicates the presence of β -glycoside bond; $1630\text{--}1655\text{ cm}^{-1}$ corresponds to the amide I band while the peak at about 1550 cm^{-1} ($1500\text{--}1600\text{ cm}^{-1}$) refers to the amide II band. The presence of pigments is characterized with frequencies in the range ($1600\text{--}1650\text{ cm}^{-1}$) indicative for the aromatic C=C double bond conjugated with the C=O and/or -COO groups; the area of $1310\text{--}1410\text{ cm}^{-1}$ is specific to OH groups of the phenolic compounds.

and kaempferol³, a more fruitful detection of phenolic compounds proceeded in this work. Indeed, flavonoids (eight flavones, six flavonols, flavanone, flavanols, biflavonoid and isoflavonoids), phenolic acids (hydroxybenzoic, hydroxycinnamic, cyclohexanecarboxylic and chlorogenic acids) and coumarins of this species have not been reported so far. Although some authors claim that mushrooms do not contain flavonoids, since they are not plants and cannot synthesize them³⁸, we have to point out that we have detected a variety of these compounds in the fruiting bodies of *T. versicolor*. The possibility for the accumulation of these compounds from the substrate may be strictly strain-specific and influenced by specific environmental conditions in the fungal habitat (soil and wood)³⁹. Furthermore, some polyphenols detected herein (e.g. quercetin and kaempferol) together with some others found in plants (huperzine A, tri- and tetra-hydroxyflavones) have shown to express acetylcholinesterase inhibitory activity^{40,41}. Similarly, the AChE inhibition had also been previously observed in the case of flavonoids, such as catechin and amentoflavone^{26,42}. In the present study, both these flavonoid compounds were identified in the extracts examined (Table 2). Hence, we can assume that they may be responsible molecules for AChE inhibitory activity potential of *T. versicolor*.

Characterization of PSH

The *T. versicolor* acid hydrolyzed PSH extract was identified using PC chromatography. The main polysaccharide component was D-glucose; D-galactose was less prevalent, while D-mannose, L-fucose and D-xylose were found only in traces. This is in agreement with the results obtained by other authors¹⁰. The presence of additional sugar compounds and uronic acid was not observed in the aforementioned sample.

The FTIR spectrum of PSH showed absorption bands typical for a polysaccharide structure (Figure 1). In addition to polysaccharides, the extract contained a mixture of protein and polyphenolic compounds in varying degrees.

In addition to the absorption peaks specific for polysaccharides, the FTIR spectrum included absorption peaks typical for proteins and polyphenolic compounds, taking into account some of the

overlapped absorptions. The bands in the area of $1310\text{--}1410\text{ cm}^{-1}$ were specific for the OH groups belonging to phenolic compounds. The obtained results are in good accordance with published experimental data for the same fungal species⁹.

There is a growing body of evidence that antioxidant activity of polysaccharides depends on their structural features, like molecular weight, monosaccharide composition, configuration and type of glycosyl bonds^{6,9,27}. Polysaccharides with lower molecular weight or those with a β -configuration in the pyranose form show a higher antioxidant activity than α -glycans²⁷. For RSC on DPPH[•], the conformation of *T. versicolor* PSH is of greater importance than the composition of monosaccharides⁹ which is consistent with the assumptions that relation of OH/CH groups is of less importance than structural characteristics based on α - and β -glycoside bonds²⁷.

The total content of macroelements in the PSH extract was as follows: nitrogen (N = 3.36%), carbon (C = 38.85%) and oxygen (H = 6.38%), while microanalysis pointed out that PSH extract did not contain sulfur. The obtained value for N indicated the presence of proteins. The fungal protein content analysis was based on the conversion factor of 4.38^{43} .

RSC on DPPH[•] and [•]OH

The results of antiradical activity of all three crude extracts along with PSH fraction of *T. versicolor* are presented in Table 3. All extracts were arranged in separate statistical groups based on the expressed activities of both the radical species, which is as follows: $\text{H}_2\text{O} > \text{MeOH} > \text{EtOH} > \text{PSH}$.

The highest RSC on [•]OH and DPPH[•] obtained for the H_2O extract ($\text{IC}_{50} = 3.21$ and $14.89\text{ }\mu\text{g/mL}$, respectively) was similar to the activity of the commercial antioxidant BHA ($8.62\text{ }\mu\text{g/mL}$) (Table 3). The MeOH extract was also an efficient scavenger, while the EtOH extract exhibited three times lower RSC. The obtained RSC value for the MeOH extract was lower than previously observed one for 70% MeOH ($\text{IC}_{50} = 239.30\text{ }\mu\text{g/mL}$)⁵. The difference may have been caused by the choice of the solvent concentration used during extraction. As stated in literature till date^{5,9} we found

Table 4. Correlation (R^2) between content of compounds (TF, TPh, TPR and PSH) and antiradical activities.

		TPhC	TFC	TPSH
H ₂ O	DPPH [•]	0.53	0.03	0.66
	•OH	0.50	0.42	0.30
EtOH	DPPH [•]	0.79	0.97*	0.75
	•OH	0.58	0.89	0.75
MeOH	DPPH [•]	0.87	0.48	0.88
	•OH	0.27	0.03	0.07

*Significant correlation coefficient (R^2) ($p < .05$).

Bold numbers indicate the highest values.

TPhC: total phenol content; TFC: total flavonoid content; TPSH: total polysaccharides; H₂O: water extract of *T. versicolor*; EtOH: ethanolic extract of *T. versicolor*; MeOH: methanolic extract of *T. versicolor*.

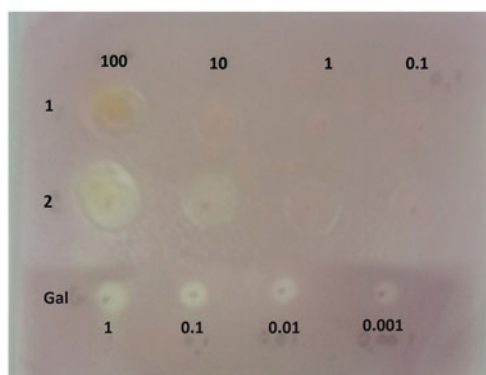


Figure 2. In solid AChE inhibitory activity of selected *T. versicolor* extracts. 1 – methanolic extract (MeOH) of *T. versicolor*; 2 – water extract (H₂O) of *T. versicolor*; Gal – galanthamine, used as a positive control.

that RSC of fungal extracts on DPPH[•] increases with increasing concentrations of extracts.

It has been well established that phenolic substances are strongly associated with antiradical activity^{3–5}. Quercetin and rutin, derivatives of quinic acid, showed high scavenging DPPH[•] activity⁴⁴. Antiradical activity actually depends on the basic structural organization of the phenolic compounds^{44,45}. Both the substituents on the phenyl ring and conjugated carbon skeleton were previously found to be of importance for this particular activity^{45,46}. Consequently, quercetin, kaempferol and myricetin (flavonols with a free 3-hydroxy group) exhibited better antiradical activity compared to flavonoids with glycosylated OH group. Sroka (2014) emphasized potent anti DPPH[•] activity of gallic and caffeic acids, the compounds identified in *T. versicolor* H₂O extract. On the other hand, gallic, *p*-coumaric and caffeic acids are known due to their anti •OH potential⁴⁶.

High correlations between TFC and DPPH[•] ($R^2=0.97$ and 0.96 , respectively) for the EtOH extract were observed. In case of the MeOH extract, the highest correlation was found for TPSH and DPPH[•] ($R^2=0.88$). On the other hand, for the H₂O extract only TPSH displayed a moderate correlation with DPPH[•] ($R^2=0.66$) (Table 4). These correlations point out the roles of flavonoids and polysaccharides in the EtOH and H₂O & MeOH extracts, respectively.

Inhibition of acetylcholinesterase

Qualitative and quantitative inhibition of AChE activity by the aforementioned extracts was examined using thin-layer

chromatography (TLC) and confirmed by the appearance of white halos in places of the enzyme inhibition by the microdilution assay *in vitro*. Among two of the selected crude extracts (H₂O and MeOH), only the H₂O extract exhibited AChE inhibitory activity at 10 µg (Figure 2). The PSH, H₂O and EtOH extracts were analyzed by the quantitative microdilution using Ellman's assay *in vitro* as well. At 100 µg/mL, a strong degree of inhibition (60.53%) was expressed, compared to donepezil (89.05%), which is commonly used as an AChE inhibitor in the treatment of AD. The analyzed EtOH extract (44.35%, at 500 µg/mL) displayed a two times higher AChE inhibitory activity compared with *T. versicolor* EtOH extract previously studied (28.35%, at the same concentration)¹⁷. The values obtained for AChE inhibitory activity of the extracts (H₂O and EtOH) were IC₅₀=78.01 µg/mL and IC₂₅=383.96 µg/mL, respectively. This inhibition was dependent on the concentration, as it was the case of quercetin reported by Zhang et al.⁴⁴ which inhibited AChE 58.80% at the concentration of 40 µg/mL.

Examining AChE inhibitory activity of different phenolic compounds, Balkis et al.⁴⁷ found baicalein as the most potent inhibitor. Indeed, apigenin, baicalein, kaempferol and quercetin expressed inhibitory effect above 90% at 100 µM. In addition to this, daidzein also inhibited the respective enzyme, at the lower extent. Finally, *p*-hydroxybenzoic acid (presented in *T. versicolor* bioactive extract) is known to exhibit the aforementioned bioactivity⁴⁸.

Taken all together, it was found that the flavonoids baicalein (21.60 µg/g dw) and quercetin (31.20 µg/g dw) may be the responsible compounds for the AChE inhibitory activity. While alkaloid compounds represent the main group of AChEi⁴⁹, those not belonging to this structural class include terpenoids, flavonoids and other phenolic substances⁵⁰. The inhibition of AChE activity reported herein may be caused by some complexes. It is well-known that polyphenolic and polysaccharide compounds are linked to each other resulting in, for example, triterpenoids that may be dissolved in H₂O⁷. Indeed, the water soluble triterpenoid compounds in the H₂O extract may also contribute both to the antioxidant activity and synergistic effect of these compounds with polysaccharides, phenols and flavonoids as suggested by other authors in the field⁷.

Conclusions

All the examined extracts exhibited a significant antiradical activity. Among them, the most potent was the H₂O extract. Furthermore, the H₂O extract showed the best AChE inhibitory activity. This is the first record of AChE inhibitory activity of the *T. versicolor* H₂O extract: baicalein and quercetin were responsible for the inhibition of AChE activity. This extract actually contained 35 phenolic compounds. Among the rest, the identified compounds included daidzein (isoflavone molecule not presented in alcoholic extracts) and amentoflavone and catechin (presented in twice bigger concentrations, compared with the EtOH extract). Taken all together, the antiradical and AChE inhibitory activities displayed by phenols and flavonoids may be attributed also to polysaccharides. As a consequence, *T. versicolor* should be considered as an alternative source of bioactive substances to be used in the treatment of AD and other neurodegenerative diseases.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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