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Original Article

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Characterization of *Trametes versicolor*: Medicinal Mushroom with Important Health Benefits

Raluca M. POP[1], Ion Cosmin PUIA[2,3], Aida PUIA[4]*, Veronica S. CHEDEA[5], Nicolae LEOPOLD[6], Ioana C. BOCSAN[1], Anca D. BUZOIANU[1]

[1]*"Iuliu Hatieganu" University of Medicine and Pharmacy, Faculty of Medicine, Department of Pharmacology, Toxicology and Clinical Pharmacology, 23 Marinescu Street, Cluj-Napoca, Romania; raluca_parlog@yahoo.com; corinabocsan@yahoo.com; ancabuzoianu@yahoo.com*

[2]*"Iuliu Hatieganu" University of Medicine and Pharmacy, Faculty of Medicine, Department of Surgery, 19-21 Croitorilor Street, Cluj-Napoca, Romania; cosmin.puia@umfcluj.ro*

[3]*"Octavian Fodor" Regional Institute of Gastroenterology and Hepatology, 3rd General Surgery Clinic, 19-21 Croitorilor Street, Cluj-Napoca, Romania*

[4]*"Iuliu Hatieganu" University of Medicine and Pharmacy, Faculty of Medicine, Department Community Medicine, Discipline of Family Medicine, 19 Moților Street, Cluj-Napoca, Romania; aida.puia@umfcluj.ro (*corresponding author)*

[5]*National Research and Development Institute for Biology and Animal Nutrition, Laboratory of Animal Biology, INCDBNA-IBNA Balotesti, Ilfov, Romania; chedea.veronica@ibna.ro*

[6]*Babeş-Bolyai University, Faculty of Physics, 1 Kogălniceanu Street, Cluj-Napoca, Romania; nicolae.leopold@phys.ubbcluj.ro*

Abstract

Trametes species represents a rich source of nutritive compounds with important pharmacological properties like antioxidant, antiinflammatory and anti-cancer properties. However, factors like genetic background, harvesting period, geographic location, climatic conditions and others are influencing the biosynthesis of bioactive compounds, their fingerprint and their concentration. The aim of this study was to determine the antioxidant capacity, total phenolic compounds and total flavonoids content of two mushroom species, namely *Tramestes versicolor* (TV) and *Trametes gibbosa* (TG), mushrooms with potential health benefits, harvested from north-west part of Romania. In order to determine the phenolic compounds profile, water, methanol, and acetone mushroom extracts were analyzed using UV-Vis spectroscopy, FTIR spectroscopy and LC-MS analysis. In total 28 compounds were tentatively identified as phenolic acids (11 compounds), flavonols (6 compounds), flavones (6 compounds), coumarins (2 compounds), flavanols, isoflavonoids and biflavonoids (1 compound). The highest antioxidant activity was determined for the methanolic extract while the highest total polyphenols content and total flavonoids content were determined for the water extract. The results obtained suggested that *Trametes* species can be considered important sources of bioactive compounds, their phenolics composition and content being influenced by a series of factors like geographic area origin and genetic background.

Keywords: chromatography; flavonoids; FTIR spectroscopy; mass spectrometry; phenolics; polypore mushroom

Introduction

Mushrooms have an important nutritional significance mostly because of their valuable chemical composition like high fibre and low-fat content, proteins, vitamins and, minerals all essential in an equilibrated diet (Alispahić *et al.*, 2015; Yahia *et al.*, 2017). Studies have shown that in addition to these nutrients, mushrooms are rich in phytochemicals with antioxidant properties. Among bioactive compounds, phenolic compounds identified in different mushroom varieties have important antioxidant properties (Cheung and Cheung, 2005; Puttaraju *et al.*, 2006; Barros *et al.*, 2007; Yahia *et al.*, 2017).

Trametes versicolor (L.) (TV) known as Turkey tail and *Trametes gibbosa* (TG) known as Lumpy bracket are widespread lignicolous fungal species with white-rot that grows on different trees like oak and *Prunus* and, on different conifers like fir or pine trees (Janjušević *et al.*, 2017). Even though it is an inedible species, it was used in

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the folk medicine of ancient China because of it multiple biological activities (Kamiyama *et al*., 2013; Janjušević *et al*., 2017). Among biological activities, antioxidant and antiinflammatory activity (Kamiyama *et al*., 2013), immune-enhancing activity (Ferreira *et al*., 2010; Li *et al*., 2011), anticancer activity (Standish *et al*., 2008; Ferreira *et al*., 2010; Cruz *et al*., 2016), antiviral effects (Teplyakova *et al*., 2012; Kamiyama *et al*., 2013; Cruz *et al*., 2016), antimicrobial (Özgör *et al*., 2016), prebiotic activity (Zhuo-Teng *et al*., 2013; *Cruz et al*., 2016) anti-diabetic (Shokrzadeh *et al*., 2017) and, AChE inhibitory effect (Janjušević *et al*., 2017) have been mostly studied. Regarding the bioactive compounds responsible for the biological activities, most of the studies published so far are describing the polysaccharides fraction containing β-glucans, polymers of D-glucose in combination or not with units of glucuronic acids, arabinose, mannose, fucose, galactose and xylose (Cruz *et al*., 2016). Beside polysaccharides, 18 types of amino acids like aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, and leucine were identified (Ng, 1998; Cui and Chisti, 2003; Cruz *et al*., 2016). However, there are few studies that analyzed TV phenolic compounds composition, important for their antioxidant properties.

Therefore, in the present study, the total phenolic compounds, total flavonoids content and the antioxidant activity of three types of extracts from TV mushroom were investigated. The identification of phenolic compounds was performed using UV-Vis and FTIR spectroscopy and liquid chromatography coupled to mass spectrometry (LC-MS).

Materials and Methods

Plant material

Trametes versicolor (TV) and *Trametes gibbosa* (TG) were collected in November of 2016, from a deciduous forest in the north-west part of Romania. The botanical identification was performed at the University of Agricultural Science and Veterinary Medicine Cluj-Napoca, Romania.

Extraction method

Fresh material was grounded to a fine powder and subject to extraction procedure immediately. *Trametes versicolor* (TV) (5 g) and *Trametes gibbosa* (TG) (5 g) fresh dried fruiting bodies were separately mixed with 50 mL of water, 50 mL of methanol and 50 mL of acetone, respectively. The mixtures were sonicated for 40 min at 80 °C in case of water extract and at room temperature in case of methanol and acetone extracts. Next, they were subject to solid-liquid extraction for 24 h at room temperature, in dark condition, on a magnetic stirrer (540 rpm). In the end, they were centrifuged (5000 g for 10 min) and filtered through a 0.22 μm nylon syringe filters. The extraction protocol is described in Fig. 1.

Quantitative analysis

Total polyphenol content

The content of total polyphenols (TPC) was determined by the Folin-Ciocalteu method. Shortly, 25 μL sample extract was mixed with 125 μL of Folin-Ciocalteu

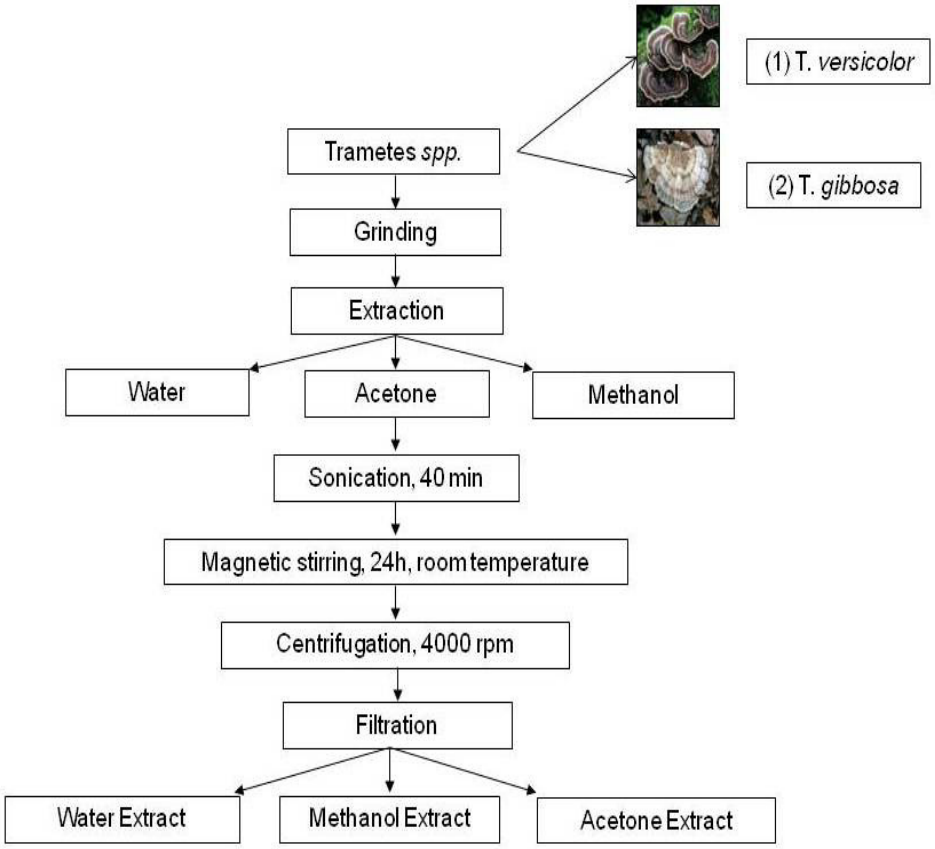

Fig. 1. *Trametes* spp. extraction protocol

reagent (0.2 N), and 100 µL of sodium carbonate (Na_2CO_3) solution (7.5% w/v). The mixture was homogenized and incubated for 2h at room temperature (25 °C), in the dark. The absorbance was measured at 760 nm using a Synergy HT Multi-Detection Microplate Reader with 96-well plates (BioTek Instruments, Inc., Winooski, VT, U.S.A.). Gallic acid was used as standard (r^2=0.9945). The TPC content in the extracts was expressed as gallic acid equivalents (GAE) in mg/g fresh weight (FW) of mushroom. All the experiments were run in triplicate. Results were expressed as mean values with standard deviations.

Total flavonoid content

The flavonoids content (TFC) was determined according to the method based on the complex formation between flavonoids and aluminium trichloride. Quercetin was used as the calibration standard (r^2=0.9914). Shortly, 1 mL of each mushroom extract was mixed with 0.3 mL $NaNO_2$ 5%, 0.3 mL $AlCl_3$ 10% and, 2 ml NaOH solution (1 M). The final volume of the mixture was adjusted to 10 mL with distilled water. After 15 min of incubation, the maximum absorbance was measured at 510 nm. The analysis was performed using a Jasco v530 spectrophotometer. The total flavonoids content was expressed as mg quercetin equivalents (QE) per 100 g fresh weight. All the experiments were run in triplicate. Results were expressed as mean values with standard deviations.

Antioxidant activity (AA) test

The DPPH radical-scavenging activity of mushroom extracts was evaluated using the method of Brand-Williams *et al*. (1995) with modifications (Brand-Williams *et al*., 1995). Briefly, a mixture containing the sample (250 µL) and 1750 µL DPPH solution (0.02 mg/mL in methanol) was incubated at room temperature for 30 min. The absorbance was read at 517 nm using a Synergy HT Multi-Detection Microplate Reader with 96-well plates (BioTek Instruments, Inc., Winooski, VT, U.S.A.). The control was performed with methanol (at the same concentration). The experiments were performed in triplicate. The calibration curve was performed with Trolox (r^2=0.9985). The results were expressed as Trolox equivalents (TE) per 100 g fresh weight. The inhibition percentage was calculated using the equation:

Inhibition (%) = [1 - (test sample absorbance/blank sample absorbance)] × 100.

Qualitative analysis

FTIR analysis

The FTIR spectra of mushroom extracts were recorder with a Shimatzu IR Prestige 21 FTIR spectrometer, equipped with an ZnSe attenuated total reflectance (ATR) accessory. The extracts were applied directly on the ZnSe ATR crystal. Each spectrum was recorded in the spectral range 650-4000 cm^{-1}, by averaging 64 scans. The air spectrum was taken as background. Between measurements, the ATR crystal was carefully cleaned with acetone.

Liquid Chromatography-Diode Array Detection–Electro-Spray Ionization Mass Spectrometry (HPLC-DAD-ESI MS)

An Agilent 1200 HPLC equipped with DAD detector, coupled with single quadrupole MS (Agilent 6110) detector was used to characterize the three extracts. Separations were performed on Eclipse column, XDB C18 (4.6 × 150 mm, particle size 5 µm) (Agilent Technologies, U.S.A.) at 25 °C. Samples were analyzed in triplicate. The chromatographic separation was done using the method described by Pop et al. (2013). The mobile phases were 0.1% acetic acid / acetonitrile (99:1) in distilled water (v/v) (A) and 0.1% acetic acid in acetonitrile (v/v) (B). The gradient programme started with 5% B (0-2 min). The percent of mobile phase B was further increased as follows: 5-40% B (2-18 min), 40-90% B (18-20 min), 90% B (20-24 min). After 24 min, the initial conditions were reached by decreasing the percentage of mobile phase B from 90% to 5% in one minute and followed by column equilibration for 5 min (Pop *et al*., 2013). The flow rate was 0.5 mL/min in all separations. The MS fragmentation used ESI source in the (+) mode. The capillary voltage was 3000 V, temperature of 350 °C and a nitrogen flow of 8 L/min. The scan range was in the range of 100-1000 m/z. The eluent was monitored by DAD. The absorbance spectrum was continuously collected in the 200-600 nm interval. The phenolic acids were detected at 280 nm while the flavonols at 340 nm. Data analysis was carried out by Agilent ChemStation Software (Rev B.04.02 SP1, Palo Alto, California, U.S.A.). Phenolic compounds identification was made according to their retention time, additionally by co-chromatography with authentic standards (when available), and mass spectra.

Results

Quantitative analysis

Total polyphenol content, total flavonoids content and the antioxidant activity of TV and TG water, methanol and acetone extracts are presented in the next table (Table 1).

The methanol extract of TG exhibited the highest antioxidant activity, followed by TV water extract and TG water extract. Regarding the total polyphenol content, TV

Table 1. Total polyphenols content, total flavonoids content and antioxidant activity of TV1 and TG2 water, methanol and acetone extracts

Sample	TPC (mg GAE/100 g FW)	TFC (mg QE/ 100 g FW)	AA (mM TE)
TV Water Extract	46.22 ± 0.89	14.77 ± 0.55	0.402 ± 0.01
TG Water Extract	43.79 ± 0.80	2.83 ± 0.30	0.312 ± 0.01
TV Methanol Extract	15.40 ± 0.81	ND	0.213 ± 0.01
TG Methanol Extract	22.22 ± 0.79	11.65 ± 0.40	0.534 ± 0.01
TV Acetone Extract	8.18 ± 0.80	ND	0.192 ± 0.01
TG Acetone Extract	3.19 ± 0.73	ND	0.160 ± 0.02

and TG water extract had the higher content, followed by methanol and acetone extracts. When total flavonoids content was analyzed, TV water extract had the highest content, followed by TG methanol extract. In this study, flavonoids were not detected in TV methanol extract, TV and TG acetone extracts, respectively.

Qualitative analysis

UV-Vis analysis

The results obtained in the quantitative analysis are supported by the UV-Vis spectra as presented in the next figure (Fig. 2). For example, the acetone extract was rich in other bioactive compounds with absorption maxima higher than 340 nm. The phenolics compounds (phenolic acids and flavonoids) are well represented in the first two UV-Vis spectra. The first one (water extract) clearly indicates the presence of phenolic acids by the high-intensity absorption peak at 280 nm while the second one (methanolic extract) shows the presence of flavonoids by the absorption spectra around 340 nm.

FTIR analysis

Fig. 3 represents the comparative FTIR spectra (3500-600 cm^{-1}) of water, methanol and acetone extracts for TV (Fig. 3A) and TG (Fig. 3B). The mushroom extracts (Fig. 3) indicated several strong broad absorption bands. The polysaccharide chains are represented by the absorption band in the range 3500-3000 cm^{-1} due to O-H stretching vibration (Janjušević *et al*., 2017). The high-intensity bands at 2923 cm^{-1} and 2850 cm^{-1} were assigned to CH_2 asymmetric and symmetric lipids stretching vibrations. The presence of lipids was also indicated by the presence of alkyl-esters, through the carbonyl stretching vibration band at 1730 cm^{-1}. Further, the high-intensity bands at 1640 cm^{-1} (C=O stretching), 1560 cm^{-1} (N–H bending) and 1350 cm^{-1} (C-N stretching) were tentatively assigned to the amide I, amide II, and amide III vibrations, respectively from the structure of the protein. Also, the presence of polysaccharides was confirmed through the C-C and C-O stretching vibrations in glycosidic bonds and pyranoid rings, which showed high-intensity bands within 1200-800 cm^{-1}.

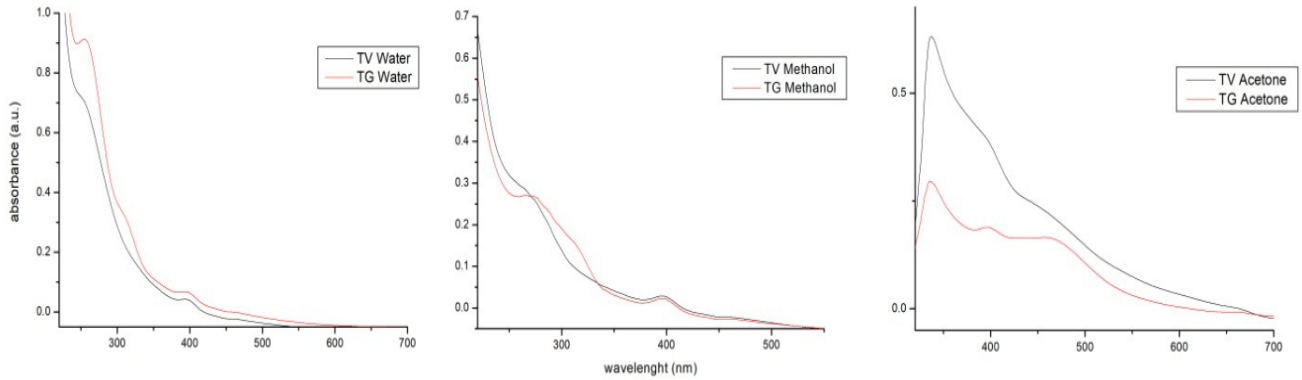

Fig. 2. Comparative UV-Vis spectra of the *Trametes versicolor* and *Trametes gibbosa* water, methanol and acetone extracts

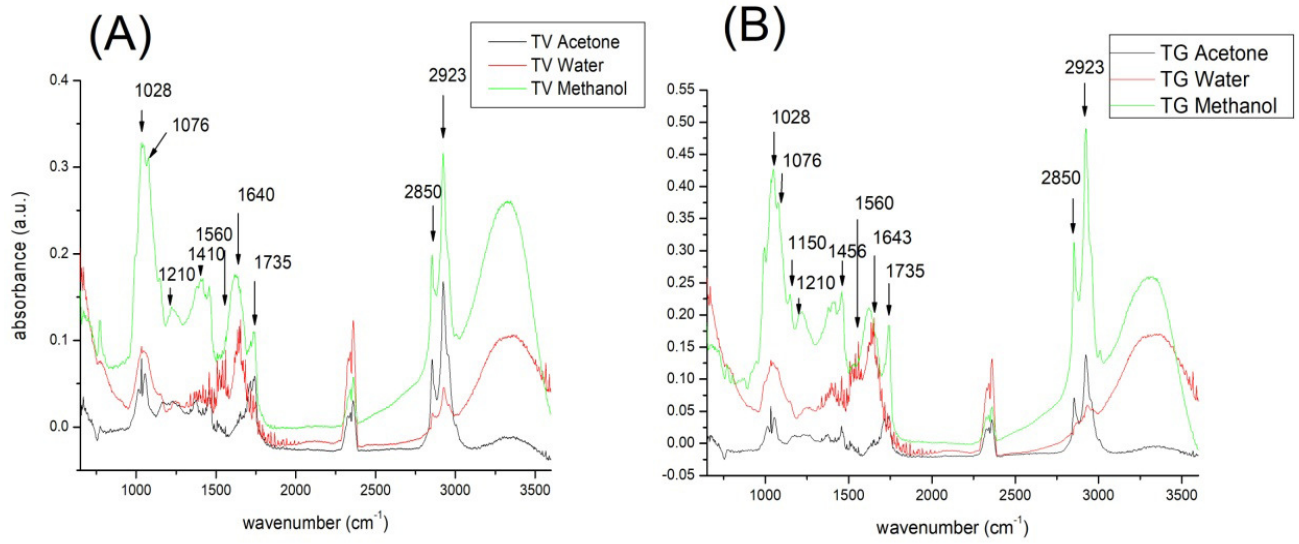

Fig. 3. Comparative FTIR spectra of (A) *Trametes versicolor* and (B) *Trametes gibbosa* water, methanol and acetone extracts

Table 2. Phenolic compound tentative identification from *Trametes versicolor*

No	Compound class	M / $[M+H]^+$	Tentative identification	Extract type	Reference
1	Phenolic acids	192	Quinic acid	m	(Janjušević *et al*., 2017)
2		164	Coumaric acid	a	(Janjušević *et al*., 2017)
3		134	Malic acid	m	(Yahia *et al*., 2017)
4		522	Dimmer of caffeic acid hexoside	m	(Yahia *et al*., 2017)
5		138	*p*-Hydroxybenzoic acid	w	(Janjušević *et al*., 2017)
6		168	Vanilic acid	w	(Janjušević *et al*., 2017)
7		198	Syringic acid	w	(Janjušević *et al*., 2017)
8		170	Gallic acid	w	(Janjušević *et al*., 2017)
9		180	Caffeic acid	m	(Janjušević *et al*., 2017)
10		164	*p*-Coumaric	w	(Janjušević *et al*., 2017)
11		194	Ferulic acid	a	(Janjušević *et al*., 2017)
12	Flavonols	480	Quercetin - glucuronide	a, w	(Flamini, 2013)
13		418	Kaempherol - arabinoside	a, m	
14		610	Rutin	a	(Janjušević *et al*., 2017)
15		448	Kaempherol - glucoside	m	(Janjušević *et al*., 2017)
16		463	Quercetin - glucoside	w	(Janjušević *et al*., 2017)
17		303	Quercetin	w, m	(Janjušević *et al*., 2017)
18	Flavones	305	Apigenin derivative	a	(Zhu *et al*., 2015)
19		270	Baicalein	m,a	(Janjušević *et al*., 2017)
20		565	Apiin	w	(Janjušević *et al*., 2017)
21		432	Vitexin	w, m	(Janjušević *et al*., 2017)
22		433	Apigenin-glucoside	m	(Janjušević *et al*., 2017)
23		270	Apigenin	a	(Janjušević *et al*., 2017)
24	Coumarins	162	Umbeliferon	w, m	(Janjušević *et al*., 2017)
25		178	Esculetin	a	(Janjušević *et al*., 2017)
26	Flavanols	291	Catechin	a	(Janjušević *et al*., 2017)
27	Isoflavonoid	255	Daidzein	w	(Janjušević *et al*., 2017)
28	Biflavonoid	538	Amentoflavone	w	(Janjušević *et al*., 2017)

Thus, as expected, lipids, proteins and polysaccharides were extracted in different ratios in the mushroom extracts. The bands identified in the methanolic extract had a higher intensity as compared with water and acetone extracts.

HPLC-DAD ESI (+) MS analysis

In total 28 compounds were tentatively identified as presented in Table 2. Among them, 11 compounds belonged to phenolic acids class, 6 were flavonols, 6 flavones, 2 coumarins and one compound from flavanols, isoflavonoids and biflavonoid class, respectively. The compound identification was performed according to their retention time, their protonated molecules $[M+H]^+$ as eluted in the Total Ion Chromatogram (TIC) and literature data. The amount of compounds identified in the three types of extracts contained approximately the same number as follows: 13, 11 and 10 for water, methanol and acetone extract, respectively.

Discussion

Literature studies suggest that TV mushrooms possess strong antioxidant properties, extremely useful in the fight of free radical and oxygen reactive species (ROS) production during the development of several diseases like cancer, metabolic syndrome diseases like type 2 diabetes, cardiovascular disorders (CVDs) or neurodegenerative disorders (Uttara *et al*., 2009; Kamiyama *et al*., 2013). Often the endogenous antioxidant system fails in controlling ROS inhibition and free radical elimination process during the pathophysiological processes disease characteristic (Balmus *et al*., 2016). Thus, supplementation with natural antioxidants is very important in reducing oxidative stress, being associated with a decreased risk of associated diseases (Uttara *et al*., 2009). Since high-dose of antioxidant intake can be harmful to human health, the characterization of the potential sources of antioxidants is of primary importance.

Matijašević *et al*. (2016) investigated total polyphenolic content and total flavonoids content of TV, also known as *Coriolus versicolor,* harvested in Belgrade surroundings, in order to establish its antibacterial activity. The concentration of TPC and flavonoids in the mushroom methanolic extract was 25.8 mg GAE /g and 4.3 mg CE /g (Catechin Equivalents), respectively. Their results were almost 100 folds higher than the ones identified in this study. However, the results were in accordance with the ones reported by Vamanu and Voica (2017) which investigated total phenolic analysis and antioxidant activity of several mushrooms harvested from the region of Moldova, Romania (Vamanu and Voica, 2017). Large variations of TPC, flavonoids content and antioxidant capacities were reported across different species of TV

before (Abugri and Mcelhenney, 2013). Generally, these large variations can be explained by the genetic factors (different mushroom species), harvesting place, harvesting time, the solvent type and the extraction conditions. Even though total flavonoids content could not be detected in all extracts by UV-Vis spectroscopy, their presence was further determined by LC-MS technique.

The presence of phenolic compounds was confirmed in the mushroom extracts using both FTIR spectroscopy and LC-MS spectrometry.

Cobaleda-Velasco *et al*. (2018) in their study on the rapid determination of phenolic compounds and flavonoids from two *Physalis* varieties, identified by FTIR spectroscopy three characteristic absorption regions, as follows: 1680-1580 cm^{-1}, 1390-1360 cm^{-1}, and 1090-1030 cm^{-1}. The fingerprint region in the mushroom extracts was also characterized by intense absorption bands within these regions. High-intensity bands at 1735 and 1640 cm^{-1}, which were previously attributed to lipids and proteins, respectively were also assigned to stretching vibrations of C=O and C=C carbonyl groups from phenolic compounds (Preserova *et al*., 2015; Tahir *et al*., 2017). Next, the bands at 1560, 1410, 1210 cm^{-1} were assigned to O-H, C-O, C-H and C=C deformation vibrations of flavonols and phenols (Masek *et al*., 2014; Nickless *et al*., 2014; Tahir *et al*., 2017). Finally, the bands at 1028 and 1076 cm^{-1} were attributed to C-OH, C-C and C-O stretching vibrations in the carbohydrate structure or C-O vibration of phenol (Tahir *et al*., 2017).

The presence of phenolic compounds was further confirmed by the LC-MS analysis. The identification of phenolic compounds was in accordance with literature data (Table 2). The experimental setting allowed the tentative identification of compounds. Using medium values of fragmentation (80 eV), most of the fragments generated from the protonated molecules $[M+H]^+$ were characteristics to water loss (18 Da) (data not shown). Also, several compounds in their nonionized forms, especially phenolic acids, were detected. The selective ionization process was explained by several factors like pH value and the addition of modifiers to the mobile phase, which in some cases can improve column separation but affects HPLC column lifetime and ESI ionization process (Plazonić *et al*., 2009).

Conclusions

The antioxidant capacity, total phenolic and flavonoid content of *Trametes* wild mushroom species harvested in the north-west part of Romania were determined. The total phenolic compounds content showed a complex profile as determined by the LC-MS analysis. Among the 28 compounds identified, the majority were phenolic acids, followed by flavonols, flavones and coumarins. Further studies regarding the content of individuals' compounds and experimental settings to prove their use as functional foods or as sources of phytochemicals with important pharmacological effects will be very helpful in the development and discovery process of new drugs and nutraceuticals.

Acknowledgements

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