PHENOLIC, POLYSACCHARIDIC AND LIPIDIC FRACTIONS OF MUSHROOMS FROM NORTHEAST PORTUGAL: CHEMICAL COMPOUNDS WITH ANTIOXIDANT PROPERTIES

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Running title: Mushrooms phenolic, polysaccharidic and lipidic fractions

ABSTRACT

Mushrooms do not constitute a significant portion of the human diet, but their consumption continues to increase due to their functional benefits and presence of bioactive compounds. Some of those compounds can be found in the phenolic, polysaccharidic and lipidic fractions of edible and inedible species. Herein, those fractions of five wild mushrooms (*Coprinopsis atramentaria*, *Lactarius bertillonii*, *Lactarius vellereus*, *Rhodotus palmatus* and *Xerocomus chrysenteron*) from Northeast Portugal were studied for their chemical composition and antioxidant properties. Protocatechuic, *p*-hydroxybenzoic, *p*-coumaric and cinnamic acids were found in the phenolic fraction, ramnose, xylose, fucose, arabinose, fructose, glucose, manose, mannitol, sucrose, maltose and trehalose were quantified in polysaccharidic fraction, linoleic and stearic (only in *Lactarius* sp.) acids, and β- and γ-tocopherols were the main compounds in the lipidic fraction. *C. atramentaria and X. chrysenteron* phenolic fractions gave the highest free radical scavenging activity, reducing properties and lipid peroxidation inhibition in brain homogenates, which is in agreement with its highest content in total phenolics. Furthermore, among the polysaccharidic fractions *C. atramentaria* also gave the highest antioxidant activity, which is accordingly with its highest total polysaccharides content and sugars obtained after hydrolysis.

KEYWORDS: Wild mushrooms; Phenolic fraction; Polysaccharidic fraction; Lipidic fraction; Antioxidant properties

INTRODUCTION

Wild mushrooms contain a huge diversity of biomolecules with nutritional (*1*) and/or medicinal properties (*2,3*). From a nutritional point of view, it is well established that wild mushrooms are rich in water, minerals, proteins, fibers and carbohydrates, as well as low caloric foods due to their low content in fat (*4-9*). Mushrooms do not constitute a significant portion of the human diet; however, their consumption continues to increase in many countries somehow related to the functional benefits and presence of compounds with bioactive properties. Some of those compounds can be found in the phenolic, polysaccharidic and lipidic fractions.

Phenolic compounds are secondary metabolites that are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways. These compounds possess an aromatic ring bearing one or more hydroxyl groups and their structures may range from that of a simple phenolic molecule to that of a complex high-molecular weight polymer (*10*). Phenolic compounds exhibit a wide range of physiological properties, such as anti-allergenic, anti-atherogenic, anti-inflammatory, anti-microbial, anti-thrombotic, cardioprotective and vasodilatory effects (*11-13*), which have been in part related to their antioxidant activity. They can act as reducing agents (electron donators), free radical-scavengers (donating hydrogen to free radicals involved in oxidative or nitrosative stress), singlet oxygen quenchers or metal ion chelators (*10,14*), and have been identified in different mushrooms species (*14,15*).

Mushrooms polysaccharides and polysaccharide conjugates have been approved in some countries for the clinical treatment of cancer patients, including “Lentinan” from *Lentinus edodes*, “Sonifilan” from *Schizophyllum commune*, “Krestin” from *Trametes versicolor*, “Grifolan” from *Grifola frondosa* and “Pleuran” from *Pleurotus ostreatus*.

Their biological activity has been related to their immunomodulating properties. Data on mushroom polysaccharides, with most belonging to the group of β-glucans, have been collected from hundreds of different species of higher Basidiomycetes (*16-18*).

In the lipidic fraction, tocopherols are important natural antioxidants due to their role as free radicals scavengers, reacting with peroxyl radicals produced from polyunsaturated fatty acids in membrane phospholipids or lipoproteins to yield stable lipid hydroperoxides (*19*). Several reports attributed high biological activity related to protection against degenerative malfunctions, mainly cancer and cardiovascular diseases, to different vitamin E isoforms such as α- or γ-tocopherols (*20*), both found in different wild mushroom species (*14,21*). Linoleic acid (LA), an essential fatty acid to mammals, is the biosynthetic precursor of arachidonic acid and prostaglandins, which take part in a wide range of physiological functions, producing effects in cardiovascular diseases, triglycerides levels, blood pressure and arthritis. LA is present in wild mushrooms lipidic fraction (*7,8*) and it is also precursor of 1-octen-3-ol, known as "fungi alcohol", the main volatile associated with certain mushroom species (*22*).

In the present work, the phenolic, polysaccharidic and lipidic fractions of five wild mushrooms (*Coprinopsis atramentaria*, *Lactarius bertillonii*, *Lactarius vellereus*, *Rhodotus palmatus* and *Xerocomus chrysenteron*) from Northeast Portugal were characterized for the first time. Furthermore, the chemical compounds found in each fraction were related to their antioxidant properties, measured as free radical scavenging activity, reducing properties and lipid peroxidation inhibition in brain homogenates.

MATERIAL AND METHODS

Mushroom species

Samples of *Coprinopsis atramentaria* (Bull.: Fr.) Redhead, Vilgalys & Moncalvo, *Lactarius bertillonii* (Neuhoff ex Z. Schaef.) Bon, *Lactarius vellereus* (Fr.) Fr., *Rhodotus palmatus* (Bull.:Fr.) Maire and *Xerocomus chrysenteron* (Bull.) Quél. were collected in Bragança (Northeast Portugal); their characteristics are shown in **Table 1**. Taxonomic identification of sporocarps was made according to other authors (*23-26*) and online keys (http://www.mycokey.com/), and representative voucher specimens were deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança. All samples were lyophilised (FreeZone 4.5 model 7750031, Labconco, Kansas, USA) and reduced to a fine dried powder (20 mesh).

Standards and Reagents

Acetonitrile 99.9%, *n*-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Lab-Scan (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as well as other individual fatty acid isomers, L-ascorbic acid, tocopherols (α-, β-, γ- and δ-tocopherols), sugars (L(+)-arabinose, D(-)-fructose, L(-)-fucose, D(+)-glucose anhydrous, maltose 1-hydrate, D(+)-mannitol, D(+)-mannose, D(+)-melezitose, L(+)-rhamnose monohydrate, D(+)-sucrose, D(+)-trehalose and D(+)-xylose) and phenolic standards (gallic, protocatechuic, *p*-hydroxybenzoic, *p*-coumaric, and cinnamic acids). Racemic tocol, 50 mg/mL, was purchased from Matreya (PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). All other chemicals and solvents were of analytical grade and purchased from usual suppliers. Water used in the studies was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

118

119 **Characterization of the phenolic fraction**

120 *Preparation of phenolic extracts.* The lyophilized samples (~1 g) were extracted with
121 methanol:water (80:20, v/v; 30 mL) at -20 °C for 2h. After sonication for 15 min, the
122 extract was filtered through Whatman nº 4 paper. The residue was then extracted with
123 two additional 30 mL portions of the methanol:water mixture. Combined extracts were
124 evaporated at 40 ºC under reduced pressure (rotary evaporator Büchi R-210) to remove
125 acetone. The aqueous phase was washed with *n*-hexane, and then submitted to a liquid-
126 liquid extraction with diethyl ether (3 × 30 mL) and ethyl acetate (3 × 30 mL). The
127 organic phases were evaporated at 40 ºC to dryness and re-dissolved in water:methanol
128 (80:20, v/v) for the antioxidant activity assays and further chemical characterization.

129

130 *Quantification of total phenolics.* The extracts solutions (1 mL) were mixed with *Folin-*
131 *Ciocalteu* reagent (5 mL, previously diluted with water 1:10, v/v) and sodium carbonate
132 (75 g/L, 4 mL). The tubes were vortex mixed for 15 s and allowed to stand for 30 min at
133 40 °C for colour development. Absorbance was then measured at 765 nm (Analytikjena
134 spectrophotometer). Gallic acid was used to obtain the standard curve (0.0094 – 0.15
135 mg/mL), and the results were expressed as mg of gallic acid equivalents (GAE) per g of
136 extract.

137

138 *Analysis of phenolic compounds.* The extracts solutions (1 mL) were filtered through a
139 0.22 µm disposable LC filter disk for HPLC analysis. The analysis was performed using
140 a Hewlett-Packard 1100 series liquid chromatograph (Agilent Technologies) as
141 previously described (*15*). Separation was achieved on a Spherisorb S3 ODS-2 (Waters)

142 reverse phase C_{18} column (3 µm, 150 × 4.6 mm) thermostated at 25 ºC. The solvents
143 used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient
144 established was 10% B to 15% B over 5 min, 15–25% B over 5 min, 25–35% B over 10
145 min, isocratic 50% B for 10 min, and re-equilibration of the column, using a flow rate of
146 0.5 mL/min. Detection was carried out in a diode array detector (DAD), using 280 nm
147 as the preferred wavelength. The phenolic compounds were characterised according to
148 their UV and retention times compared with commercial standards. For the quantitative
149 analysis of phenolic compounds, a calibration curve was obtained by injection of known
150 concentrations (5-100 µg/mL) of different standard compounds. The results were
151 expressed as mg per 100 g of dry weight (dw).
152
153 **Characterization of the polysaccharidic fraction**
154 *Preparation of polysaccharidic extracts.* The lyophilized mushrooms (~1.5 g) were
155 extracted with water at boiling temperature (50 mL) for 2 h under agitation (150 rpm;
156 Velp Are magnetic stirrer) and subsequently filtered through Whatman No. 4 paper. The
157 residue was then extracted with two more portions of boiling water, in a total of 6 h of
158 extraction. The combined extracts were lyophilized, and then 95% ethanol (10 mL) was
159 added and polysaccharides were precipitated overnight at 4 ºC. The precipitated
160 polysaccharides were collected after centrifugation (Centorion K24OR refrigerated
161 centrifuge) at 3100 × *g* for 40 min followed by filtration, and then were lyophilized,
162 resulting in a crude polysaccharidic sample (*27*). The crude polysaccharidic samples
163 were re-dissolved in water for the antioxidant activity assays, and further hydrolysed for
164 chemical characterization.
165

166 *Quantification of total polysaccharides.* The extracts solutions (1 mL) were added to
167 80% phenol (25 µL) and conc. sulphuric acid (1 mL). The mixture was shaken and
168 allowed to stand at 30 ºC for 30 min. The absorbance was measured at 490 nm. Starch
169 (although being glycogen the storage polysaccharide in mushrooms, starch is the most
170 available polysaccharide) was used to obtain the standard curve (0.625-40 mg/mL), and
171 the results were expressed as mg of polysaccharides equivalents (PE) per g of extract.
172
173 *Analysis of polysaccharidic extracts.* The polysaccharidic extracts were hydrolyzed with
174 0.05 M trifluoroacetic acid (TFA, 2 mL), maintained at 90 ºC for 16 h and then
175 centrifuged, following the procedure described by Vaz et al. (2011) with some
176 modifications. The supernatant was lyophilized, redissolved in distilled water (1 mL)
177 and filtered through 0.2 µm nylon filters for HPLC-RI analysis.
178 For a comparison, free sugars were also determined. The lyophilized samples (1 g) were
179 spiked with raffinose as internal standard (IS, 5 mg/mL) and were extracted with 40 mL
180 of 80% aqueous ethanol at 80 ºC for 30 min. The resulting suspension was centrifuged
181 at 15,000 *g* for 10 min. The supernatant was concentrated at 60 ºC under reduced
182 pressure and defatted three times with 10 mL of ethyl ether, successively. After
183 concentration at 40 ºC, the solid residues were dissolved in water to a final volume of 5
184 mL and filtered through 0.2 µm nylon filters for HPLC-RI analysis.
185 The equipment consisted of an integrated system with a pump (Knauer, Smartline
186 system 1000), degasser system (Smartline manager 5000), auto-sampler (AS-2057
187 Jasco) and RI detector (Knauer Smartline 2300). Chromatographic separation was
188 achieved with a Eurospher 100-5 NH_2 column (4.6 × 250 mm, 5 µm, Knauer) operating
189 at 30 ºC (7971 R Grace oven). The mobile phase was acetonitrile:deionized water, 7:3

(v/v) at a flow rate of 1 mL/min. Sugars identification was made by comparing the relative retention times of sample peaks with standards (0.5-40 mg/mL). Data were analysed using Clarity 2.4 Software (DataApex). Quantification was made by the IS method, and the results expressed in g per 100 g of dry weight (dw).

Characterization of the lipidic fraction

Analysis of fatty acids. Fatty acids were determined by gas chromatography with flame ionization detection (GC-FID) as described previously by the authors (*8*) and after the following trans-esterification procedure: fatty acids (obtained after Soxhlet extraction) were methylated with 5 mL of methanol:sulphuric acid 95%:toluene 2:1:1 (v/v/v), for at least 12 h in a bath at 50 ºC and 160 rpm; to obtain phase separation 3 mL of deionised water were added; the fatty acids methyl esters (FAME) were recovered by shaking in a vortex with 3 mL of diethyl ether, and the upper phase was passed through a micro-column of anhydrous sodium sulphate to eliminate the water. The sample was recovered in a vial with Teflon and filtered through a 0.2 µm Whatman nylon filter. The fatty acid profile was analyzed with a DANI model GC 1000 instrument equipped with a split/splitless injector, a flame ionization detector (FID) and a Macherey-Nagel column (30 m × 0.32 mm ID × 0.25 µm d_f). The oven temperature program followed was an initial column temperature of 50 ºC, held for 2 min, followed by a 10 ºC/min ramp to 240 ºC for 11 min. The carrier gas (hydrogen) flow-rate was 4.0 mL/min (0.61 bar), measured at 50 ºC. Split injection (1:40) was carried out at 250 ºC. For each analysis 1 µL of the sample was injected in GC. Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results

were recorded and processed using CSW 1.7 software (DataApex 1.7) and expressed as a relative percentage of each fatty acid.

Analysis of tocopherols. Tocopherols content was determined following a procedure previously described by the authors (*28*). Butylated hydroxytoluene (BHT) solution in hexane (10 mg/mL; 100 µL) and IS solution in hexane (tocol; 50 µg/mL; 400 µL) were added to the sample prior to the extraction procedure. Samples (~500 mg) were homogenized with methanol (4 mL) by vortex mixing (1 min). Subsequently, hexane (4 mL) was added and again vortex mixed for 1 min. Saturated NaCl aqueous solution (2 mL) was added, the mixture was homogenized (1 min), centrifuged (5 min, 4,000 *g*) and the clear upper layer was carefully transferred to a vial. The sample was re-extracted twice with n-hexane. The combined extracts were taken to dryness under a nitrogen stream, redissolved in 2 mL of n-hexane, dehydrated with anhydrous sodium sulphate and filtered through 0.2 µm nylon filters and transferred into a dark injection vial. Analyses were performed by the HPLC system (described above) connected to a fluorescence detector (FP-2020; Jasco) programmed for excitation at 290 nm and emission at 330 nm. Chromatographic separation was achieved with a Polyamide II (250 × 4.6 mm) normal-phase column from YMC Waters operating at 30 ºC. The mobile phase used consisted of a mixture of n-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 mL/min, with an injection volume of 20 µL. The compounds were identified by chromatographic comparisons with authentic standards (0.05-2 µg/mL). Quantification was based on the fluorescence signal response, using the IS method and the results expressed in µg per 100 g of dry sample (dw).

Evaluation of antioxidant properties

DPPH radical-scavenging activity. This assay was performed in 96-well microtiter plates using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc). The reaction mixture in each of the 96-wells of the plate consisted of one of the different concentrations of the extracts (dissolved in water:methanol 80:20 or water for phenolic and polysaccharidic fractions, respectively; 30 µL) and methanolic solution (270 µL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 60 min in the dark. Reduction of the DPPH radical was determined by measuring the absorption at 515 nm (*27*). Radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation: % RSA = $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% of radicals scavenging activity (EC_{50}) was calculated from the graph of RSA percentage against extract concentration. Trolox was used as standard.

Reducing power. This assay was also performed using microtiter plates and the Microplate Reader described above. Different extract concentrations (dissolved in water:methanol 80:20 or water for phenolic and polysaccharidic fractions, respectively; 0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 ml) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50 ºC for 20 min and trichloroacetic acid (10% w/v, 0.5 mL) added. This mixture (0.8 mL) was then poured into the wells of a 48-well microplate, also containing deionised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL) and the absorbance was measured at 690 nm (*27*). The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from

261 the graph of absorbance at 690 nm against extract concentration. Trolox was used as
262 standard.
263
264 *Inhibition of β-carotene bleaching.* A solution of β-carotene was prepared by dissolving
265 β-carotene (2 mg) in chloroform (10 mL). Two millilitres of this solution were pipetted
266 into a round-bottom flask. The chloroform was removed at 40 ºC under vacuum and
267 linoleic acid (40 mg), Tween 80 emulsifier (400 mg) and distilled water (100 ml) were
268 added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were
269 transferred into test tubes containing different concentrations of the extracts (dissolved
270 in water:methanol 80:20 or water for phenolic and polysaccharidic fractions,
271 respectively; 0.2 mL). The tubes were shaken and incubated at 50 ºC in a water bath. As
272 soon as the emulsion was added to each tube, the zero time absorbance was measured at
273 470 nm (*27*). β-Carotene bleaching inhibition was calculated using the following
274 equation: (β-carotene content after 2h of assay/initial β-carotene content) × 100. The
275 extract concentration providing 50% antioxidant activity (EC_{50}) was calculated by
276 interpolation from the graph of β-carotene bleaching inhibition percentage against
277 extract concentration. Trolox was used as standard.
278
279 *Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS).*
280 Porcine (*Sus scrofa*) brains were obtained from official slaughtering animals, dissected
281 and homogenized with a Polytron in ice-cold Tris–HCl buffer (20 mM, pH 7.4) to
282 produce a 1:2 (w/v) brain tissue homogenate which was centrifuged at 3000 *g* for 10
283 min. An aliquot (0.1 mL) of the supernatant was incubated with the different
284 concentrations of the extracts (dissolved in water:methanol 80:20 or water for phenolic
285 and polysaccharidic fractions, respectively; 0.2 mL) in the presence of $FeSO_4$ (10 μM;

0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37 ºC for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 0.5 mL), followed by thiobarbituric acid (TBA, 2%, w/v, 0.38 ml), and the mixture was then heated at 80 ºC for 20 min. After centrifugation at 3000 *g* for 10 min to remove the precipitated protein, the colour intensity of the malondialdehyde (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm (*27*). The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = [(A – B)/A] × 100%, where A and B were the absorbance of the control and the compound solution, respectively. The extract concentration providing 50% lipid peroxidation inhibition (EC_{50}) was calculated from the graph of TBARS inhibition percentage against extract concentration. Trolox was used as standard.

Statistical analysis

For each sample assayed three replicates were made and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SPSS v. 18.0 program.

RESULTS AND DISCUSSION

The results for antioxidant activity of the studied wild mushrooms phenolic and polysaccharidic fractions are shown in **Table 2**. For *C. atramentaria*, *L. bertillonii* and *L. vellereus*, the polysaccharidic fraction (extracts dissolved in water) gave the highest antioxidant activity (lowest EC_{50} values), while phenolic fraction (extracts dissolved in water:methanol 80:20) showed to have highest antioxidant potential in *R. palmatus* and

311 *X. chrysenteron*. *C. atramentaria* and *X. chrysenteron* phenolic extracts revealed the
312 highest content of total phenolics (33.58 and 36.28 mg GAE/g extract, without
313 significant statistical differences, $p<0.05$). *C. atramentaria* polysaccharidic extract also
314 gave the highest total polysaccharides content (16.72 mg PE/g extract). Among the
315 phenolic fractions, *C. atramentaria and X. chrysenteron* gave the highest antioxidant
316 activity demonstrated by the lowest EC_{50} values obtained **(Table 2)**, which was
317 coherent with their higher contents of total phenolics. Anyway, in all cases, good
318 correlations between total phenolic or total polysaccharides, and DPPH scavenging
319 activity, reducing power and β-carotene bleaching inhibition were observed ($R^2>0.72$,
320 **Table 3**). However, TBARS inhibition seemed not to be correlated with those
321 compounds, which might be due to other antioxidant molecules, probably with less
322 polar characteristics, involved in that activity.
323 As far as we know, this is the first report on antioxidant activity of phenolic extracts of
324 the studied species, with the exception of *X. chrysenteron* (*29*). These authors reported a
325 lower content in phenolics (17.91 mg GAE/g extract) and a lower reducing power, but a
326 higher DPPH scavenging activity and β-carotene bleaching inhibition in a sample from
327 Turkey.
328 The composition of the phenolic fractions of the studied samples is shown in **Table 4.**
329 Phenolic acids (protocatechuic, *p*-hydroxybenzoic and *p*-coumaric acids) and a related
330 compound (cinnamic acid) (**Figure 1**) were found in the studied species. It has been
331 reported that the antioxidant activity of phenolic acids (ArOH) is related to the presence
332 of hydroxyl groups in the molecule, through transfer mechanisms of *i)* hydrogen atoms:
333 $LOO^{\bullet} + ArOH \rightarrow LOOH + ArO^{\bullet}$; the $ArO^{\bullet}$ radical must be stable so that it can slowly
334 react with the substrate, LH, and quickly with the $LOO^{\bullet}$ interrupting the chain reactions,

335 or *ii)* electrons: $LOO^{\bullet} + ArOH \rightarrow LOO^{-} + ArOH^{+}$; $ArOH^{+} + H_2O \leftrightarrow ArO^{\bullet} + H_3O^{+}$;
336 $LOO^{-} + H_3O^{+} \leftrightarrow LOOH + H_2O$ (*30*). The introduction of a second hydroxyl group in
337 the *ortho* or *para* positions seems to increase the antioxidant activity; therefore, the *o*-
338 diphenol protocatechuic acid should be more efficient than the corresponding
339 monophenol, *p*-hydroxybenzoic (*31*).
340 *R. palmatus* revealed the highest content in total phenolic acids (10.55 mg/100 g dw),
341 and particularly in protocatechuic acid (8.60 mg/100 g dw). Nevertheless, this species
342 showed lower antioxidant activity than *X. chrysenteron* that presented much lower
343 concentrations of the mentioned phenolic acids, but higher total phenolics measured by
344 the *Folin Ciocalteu* assay. Therefore and as it is not expected that fungi present other
345 phenolic compounds than phenolic acids, it should be highlighted that *Folin Ciocalteu*
346 assay measures total antioxidants rather than phenolic compounds.
347 Regarding polysaccharidic fractions, *C. atramentaria* gave the highest antioxidant
348 activity, which was in agreement with its highest content in total polysaccharides
349 (**Table 2**) and in sugars obtained after hydrolysis (44.98 g/100 g dw; **Table 5**). This
350 species also released the greatest diversity of sugars after hydrolysis, including maltose,
351 ramnose, xylose, sucrose, glucose, trehalose, fructose, manose and arabinose. *X.*
352 *chrysenteron* also presented a high diversity of sugars but in lower amounts. *C.*
353 *atramentaria* and *R. palmatus* gave similar (without statistical differences) amounts of
354 total sugars obtained after polysaccharides hydrolysis. Nevertheless, the latter species
355 presented less diversity of sugars, although it showed fucose and mannitol that were not
356 found in *C. atramentaria* (**Figure 2A**). Despite the mentioned observations, it should be
357 highlighted that some of the obtained sugars may not be from polysaccharides
358 hydrolysis but contaminations of free sugars present on the analyzed fraction.

359 Free sugars were also analyzed in the studied mushrooms species, and *R. palmatus* gave
360 the highest levels (32.86 g/100 g dw; **Table 5**), revealing the presence of fructose,
361 ramnose, mannitol and trehalose. After data observation, it can be concluded that most
362 sugars were included in polysaccharides, which are one of the most abundant bioactive
363 macromolecules in mushrooms (*18*). Mannitol and trehalose are still the most
364 widespread free sugars in the studied species, as it has been described in literature
365 (*1,8,32*).
366 The results for fatty acids and tocopherols composition of the studied wild mushrooms
367 lipidic fraction are shown in **Table 6**. According to the results, linoleic acid (C18:2n6c)
368 was the major fatty acid found in the species *C. atramentaria, R. palmatus* and *X.*
369 *chrysenteron*, while stearic acid (C18:0) was the most abundant in *L. bertillonii* and *L.*
370 *vellereus* (**Figure 2B**). The presence of this fatty acid in high amounts was corroborated
371 in other *Lactarius* sp. previously studied by us, namely *L. deliciosus* and *L. piperatus*
372 (*32*) and *L. salmonicolor* (*8*). Oleic (C18:1n9c), palmitic (C16:0) and α-linolenic
373 (C18:3n3c) acids were also found in not negligible amounts.
374 Besides those fatty acids, twenty-one less abundant fatty acids were also identified (data
375 not shown). PUFA were the main group of fatty acids in *C. atramentaria* (54%), *R.*
376 *palmatus* (51%) and *X. chrysenteron* (46%) due to the high contribution of linoleic acid,
377 while SFA were the main group in *L. bertillonii* (14%) and *L. vellereus* (22%) due to
378 the high amounts of stearic acid.
379 The values obtained in the analysis of the different samples revealed significant
380 differences in what concerns tocopherols composition (**Table 6**). All the isoforms were
381 found in all the studied species, but β- and γ-tocopherols were the major vitamers
382 (**Figure 2C**). *X. chrysenteron* presented the highest content of tocopherols (372.98

383 μg/100g dw) as also the highest levels of γ-tocopherol (220.51 μg/100 g), while *L.*
384 *vellereus* revealed the highest content of β-tocopherol (242.41 μg/100g). The lowest
385 values of tocopherols were found in *C. atramentaria* and *R. palmatus* without statistical
386 differences ($p<0.05$). Tocopherols play an important role in health acting as antioxidants
387 by their capacity to scavenge lipid peroxyl radicals of unsaturated fatty acids, and
388 preventing propagation of lipid peroxidation (*33*). Furthermore, PUFA such as linoleic
389 and α-linolenic acids are essential fatty acids and have been associated with a reduced
390 risk of developing cardiovascular, inflammatory and autoimmune diseases, being
391 biosynthetic precursors of eicosanoids (*34*).
392 Overall, the inclusion of mushrooms in the diet could bring health benefits, considering
393 their antioxidant properties. Furthermore, different fractions (phenolic, polysaccharidic
394 and lipidic) could be separated and purified in order to be included in nutraceutical or
395 pharmaceutical formulations.
396
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405

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Table 1. Information about the wild species analysed.

Scientific name	*Coprinopsis atramentaria* (Bull.) Redhead, Vilgalys & Moncalvo	*Rhodotus palmatus* (Bull.:Fr.) Maire	*Lactarius bertillonii* (Neuhoff ex Z. Schaef.) Bon	*Lactarius vellereus* (Fr.)	*Xerocomus chrysenteron* (Bull.) Quél
English name	Common ink cap or inky cap	Netted Rhodotus	Unknown	Fleecy milk-cap	Red Cracking Bolete
Edibility	Edible	Unknown	Inedible	Inedible	Edible
Habitat	Fields	Decayed wood	Mixed stands	Mixed stands	*Castanea sativa*
Date of collection	November 2010	October 2010	October 2010	October 2010	October 2010
Ecology	Saprotrophic	Saprotrophic	Mycorrhizal	Mycorrhizal	Mycorrhizal

Table 2. Extraction yields, antioxidant activity (EC_{50} values[a]), total phenolics and total polysaccharides of the wild mushrooms phenolic and polysaccharidic fractions.

Species	*Coprinopsis atramentaria*		*Lactarius bertillonii*		*Lactarius vellereus*		*Rhodotus palmatus*		*Xerocomus chrysenteron*	
Fraction	Phenolic	Polysaccharidic	Phenolic	Polysaccharidic	Phenolic	Polysaccharidic	Phenolic	Polysaccharidic	Phenolic	Polysaccharidic
Extraction yield (%)	30.30 ± 3.21	43.22 ± 1.62	21.78 ± 1.08	24.41 ± 1.87	17.40 ± 0.44	19.57 ± 0.65	25.72 ± 1.37	21.65 ± 1.23	12.28 ± 0.63	27.40 ± 0.98
DPPH scavenging activity (mg/mL)	3.87 ± 0.41^{f}	2.48 ± 0.73^{g}	9.54 ± 0.96^{d}	9.90 ± 0.24^{d}	17.46 ± 0.40^{a}	7.76 ± 0.39^{e}	7.58 ± 0.23^{e}	15.48 ± 0.60^{b}	2.06 ± 0.46^{g}	11.31 ± 0.81^{c}
Reducing power (mg/mL)	1.29 ± 0.11^{ef}	0.88 ± 0.03^{g}	1.63 ± 0.01^{d}	1.13 ± 0.51^{gf}	3.37 ± 0.05^{a}	2.37 ± 0.17^{c}	1.43 ± 0.29^{ed}	3.36 ± 0.18^{a}	1.28 ± 0.02^{ef}	2.90 ± 0.17^{b}
β-carotene bleaching inhibition (mg/mL)	1.03 ± 0.07^{e}	0.81 ± 0.08^{e}	3.01 ± 0.34^{c}	1.97 ± 0.04^{d}	3.69 ± 0.66^{b}	2.20 ± 0.15^{d}	2.38 ± 0.38^{d}	5.03 ± 0.87^{a}	0.95 ± 0.06^{e}	4.43 ± 0.44^{a}
TBARS inhibition (mg/mL)	1.09 ± 0.18^{c}	1.01 ± 0.11^{c}	1.21 ± 0.17^{c}	1.00 ± 0.03^{c}	3.12 ± 0.49^{b}	1.21 ± 0.03^{c}	1.22 ± 0.68^{c}	4.72 ± 0.13^{a}	0.44 ± 0.07^{c}	4.94 ± 0.16^{a}
Total phenolics (mg GAE/g extract)	33.58 ± 0.64^{a}	np	23.09 ± 0.67^{b}	np	12.62 ± 0.18^{c}	np	28.55 ± 0.30^{b}	np	36.28 ± 0.57^{a}	np
Total polysaccharides (mg PE/g extract)	np	16.72 ± 0.46^{a}	np	7.91 ± 0.33^{b}	np	6.99 ± 0.19^{c}	np	2.30 ± 0.01^{e}	np	3.42 ± 0.08^{d}

[a]Concentration of extract providing 50% of antioxidant activity in DPPH scavenging activity and β-carotene bleaching inhibition assays, and 0.5 of absorbance in reducing power assay; np- not performed. In each row different letters imply significant differences (p<0.05).

Table 3. Correlations ($p < 0.001$) established between total phenolics, total polysaccharides, and antioxidant activity EC_{50} values.

	DPPH scavenging activity		Reducing power		β-carotene bleaching inhibition		TBARS inhibition	
EC_{50} value (mg/ml)	Linear equation	R^2	Linear equation	R^2	Linear equation	R^2	Linear equation	R^2
Total phenolics (mg GAE/g extract)	Y=-1.5991x+39.921	0.9216	Y=-9.5608x+43.943	0.740	Y=-6.8614x+41.911	0.7675	Y=-4.503x+33.221	0.3848
Total polysaccharides (mg PE/g extract)	Y=-1.1196x+18.042	0.8876	Y=-4.3549x+16.73	0.7245	Y=-2.723x+15.326	0.7702	Y=-2.0827x+12.829	0.5721

Table 4. Composition in phenolic acids and related compounds of the wild mushrooms phenolic fraction.

	Coprinopsis atramentaria	*Lactarius bertillonii*	*Lactarius vellereus*	*Rhodotus palmatus*	*Xerocomus chrysenteron*
Protocatechuic acid (mg/100 g dw)	nd	0.16 ± 0.02^{c}	0.99 ± 0.07^{b}	8.60 ± 0.64^{a}	0.54 ± 0.04^{cb}
p-Hydroxybenzoic acid (mg/100 g dw)	4.71 ± 0.14^{a}	0.20 ± 0.02^{d}	0.16 ± 0.01^{d}	1.96 ± 0.23^{b}	0.98 ± 0.13^{c}
p-Coumaric acid (mg/100 g dw)	0.82 ± 0.04^{a}	0.13 ± 0.01^{d}	0.18 ± 0.01^{c}	nd	0.55 ± 0.01^{b}
Total phenolic acids (mg/100 g dw)	5.53 ± 0.09^{b}	0.50 ± 0.05^{d}	1.33 ± 0.10^{c}	10.55 ± 0.87^{a}	2.06 ± 0.18^{c}
Cinnamic acid (mg/100 g dw)	1.70 ± 0.11^{b}	0.77 ± 0.09^{d}	1.07 ± 0.22^{c}	4.15 ± 0.19^{a}	0.55 ± 0.02^{d}

nd- not detected. In each row different letters imply significant differences ($p<0.05$).

Table 5. Composition in free sugars and sugars obtained after hydrolysis of the wild mushrooms polysaccharidic fraction.

	Coprinopsis atramentaria		*Lactarius bertillonii*		*Lactarius vellereus*		*Rhodotus palmatus*		*Xerocomus chrysenteron*	
	Free sugars	Polysaccharides	Free sugars	Polysaccharides	Free sugars	Polysaccharides	Free sugars	Polysaccharides	Free sugars	Polysaccharides
Ramnose (g/100 g dw)	nd	9.74 ± 0.54^{a}	nd	nd	nd	nd	9.04 ± 0.08^{a}	4.42 ± 0.42^{b}	nd	tr
Xylose (g/100 g dw)	nd	6.22 ± 0.76^{a}	nd	nd	nd	nd	nd	nd	nd	0.23 ± 0.04^{b}
Fucose (g/100 g dw)	nd	nd	nd	nd	nd	nd	nd	1.40 ± 0.10^{a}	nd	1.39 ± 0.13^{a}
Arabinose (g/100 g dw)	nd	0.79 ± 0.08^{a}	nd	nd	nd	nd	nd	nd	nd	0.67 ± 0.11^{b}
Fructose (g/100 g dw)	0.26 ± 0.02^{b}	1.10 ± 0.17^{d}	nd	0.20 ± 0.01^{d}	nd	5.08 ± 0.40^{c}	20.30 ± 0.73^{a}	33.61 ± 1.12^{a}	nd	7.80 ± 0.17^{b}
Glucose (g/100 g dw)	0.32 ± 0.01^{a}	1.54 ± 0.16^{a}	nd	0.11 ± 0.01^{bc}	nd	nd	nd	nd	nd	0.22 ± 0.07^{b}
Manose (g/100 g dw)	nd	1.06 ± 0.10^{a}	nd	nd	nd	nd	nd	nd	nd	nd
Mannitol (g/100 g dw)	nd	nd	11.71 ± 0.37^{b}	11.98 ± 0.17^{b}	24.77 ± 0.32^{a}	24.05 ± 0.77^{a}	2.62 ± 0.02^{d}	5.53 ± 0.68^{c}	5.81 ± 0.35^{c}	6.38 ± 0.35^{c}
Sucrose (g/100 g dw)	0.26 ± 0.02^{a}	2.57 ± 0.32^{a}	nd	nd	nd	nd	nd	nd	nd	0.05 ± 0.00^{b}
Maltose (g/100 g dw)	nd	20.64 ± 2.21^{a}	nd	nd	nd	nd	nd	nd	nd	nd
Trehalose (g/100 g dw)	5.35 ± 0.18^{a}	1.32 ± 0.17^{c}	1.61 ± 0.04^{d}	0.22 ± 0.01^{d}	2.41 ± 0.17^{c}	3.32 ± 0.05^{b}	0.90 ± 0.18^{e}	nd	4.16 ± 0.27^{b}	9.71 ± 0.53^{a}
Total sugars (g/100 g dw)	6.19 ± 0.06^{e}	44.98 ± 1.77^{a}	13.32 ± 0.23^{c}	12.51 ± 0.16^{d}	27.18 ± 0.50^{b}	32.45 ± 0.42^{b}	32.86 ± 1.01^{a}	44.96 ± 0.97^{a}	9.98 ± 0.28^{d}	26.45 ± 1.25^{c}

nd- not detected and tr- traces. In each row, and independently for free sugars and polysaccharides hydrolysis, different letters imply significant differences ($p<0.05$).

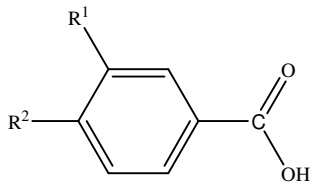
Table 6. Extraction yield and composition in fatty acids and tocopherols of the wild mushrooms lipidic fraction.

	Coprinopsis atramentaria	*Lactarius bertillonii*	*Lactarius vellereus*	*Rhodotus palmatus*	*Xerocomus chrysenteron*
Extraction yield (%)	7.04 ± 0.62^{a}	3.71 ± 0.17^{b}	2.71 ± 0.18^{c}	1.79 ± 0.09^{d}	1.13 ± 0.09^{e}
C16:0	11.11 ± 0.25^{b}	10.07 ± 0.21^{c}	8.86 ± 0.03^{d}	11.38 ± 0.00^{b}	14.96 ± 0.27^{a}
C18:0	1.18 ± 0.02^{d}	65.56 ± 0.07^{a}	58.33 ± 0.23^{b}	1.49 ± 0.01^{d}	4.47 ± 0.54^{c}
C18:1n9c	30.65 ± 0.47^{b}	6.98 ± 0.02^{d}	6.84 ± 0.19^{d}	32.21 ± 0.15^{a}	28.43 ± 0.36^{c}
C18:2n6c	46.69 ± 0.55^{a}	13.23 ± 0.39^{d}	22.13 ± 0.47^{c}	47.28 ± 0.11^{a}	43.95 ± 1.14^{b}
C18:3n3c	7.94 ± 0.11^{a}	0.10 ± 0.01^{d}	0.19 ± 0.01^{d}	3.39 ± 0.08^{b}	2.20 ± 0.07^{c}
SFA (% of total FA)	13.56 ± 0.21^{d}	79.03 ± 0.45^{a}	70.58 ± 0.30^{b}	14.22 ± 0.02^{d}	23.71 ± 0.89^{c}
MUFA (% of total FA)	31.71 ± 0.44^{b}	7.17 ± 0.02^{d}	6.99 ± 0.18^{d}	34.36 ± 0.19^{a}	29.81 ± 0.27^{c}
PUFA (% of total FA)	54.73 ± 0.66^{a}	13.80 ± 0.44^{e}	22.43 ± 0.48^{d}	51.42 ± 0.22^{b}	46.48 ± 1.16^{c}
α-Tocopherol (µg/100 g dw)	4.00 ± 0.40^{dc}	22.08 ± 1.70^{a}	14.55 ± 0.57^{b}	6.48 ± 1.10^{c}	1.77 ± 0.15^{d}
β-Tocopherol (µg/100 g dw)	20.18 ± 1.39^{c}	9.59 ± 1.00^{c}	242.41 ± 15.92^{a}	25.92 ± 0.20^{c}	133.78 ± 5.43^{b}
γ-Tocopherol (µg/100 g dw)	52.66 ± 3.94^{c}	65.43 ± 4.90^{b}	36.86 ± 4.26^{d}	13.66 ± 0.70^{e}	220.51 ± 4.51^{a}
δ-Tocopherol (µg/100 g dw)	1.50 ± 0.30^{b}	17.08 ± 0.70^{a}	22.04 ± 6.60^{a}	6.48 ± 0.10^{b}	16.92 ± 0.25^{a}
Total tocopherols (µg/100 g dw)	78.34 ± 2.46^{d}	114.18 ± 1.51^{c}	315.86 ± 27.35^{b}	52.54 ± 0.31^{d}	372.98 ± 0.82^{a}

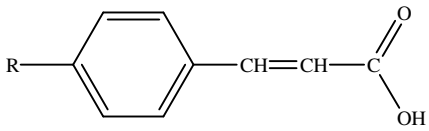
Palmitic acid (C16:0); Stearic acid (C18:0); Oleic acid (C18:1n9c); Linoleic acid (C18:2n6c); α-Linolenic acid (C18:3n3c); SFA- saturated fatty acids; MUFA- monounsaturated fatty acids; PUFA- polyunsaturated fatty acids. The difference to 100% corresponds to other 21 less abundant fatty acids (data not shown). In each row different letters imply significant differences (p<0.05).

Figure 1. Chemical structure of the phenolic acids and related compounds found in the wild mushrooms phenolic fraction.

Figure 2. **(A)** Free sugars (---) and sugars obtained after polysaccharide hydrolysis (⁻) in *Rhodotus palmatus*: 1-rhamnose; 2-fucose; 3-fructose; 4-mannitol; 5-trehalose; **(B)** Fatty acids in *Lactarius vellereus*: 1- C6:0; 2- C8:0; 3- C10:0; 4- C12:0; 5-C13:0; 6- C14:0; 7-C15:0; 8- C16:0; 9- C16:1; 10- C17:0; 11- C18:0; 12- C18:1n9; 13- C18:3n3; 14- C20:0; 15- C20:1; 16- C20:2c; 17- C20:3n3+C21:0; 18- C20:5n3; 19- C22:0; 20-C22:1n9; 21-C23:0; 22- C24:0; 23- C24:1; **(C)** Tocopherols in *Xerocomus chrysenteron*: 1- α-tocopherol; 2-BHT; 3-β-tocopherol; 4-γ-tocopherol; 5- δ-tocopherol; 6- Tocol (IS).

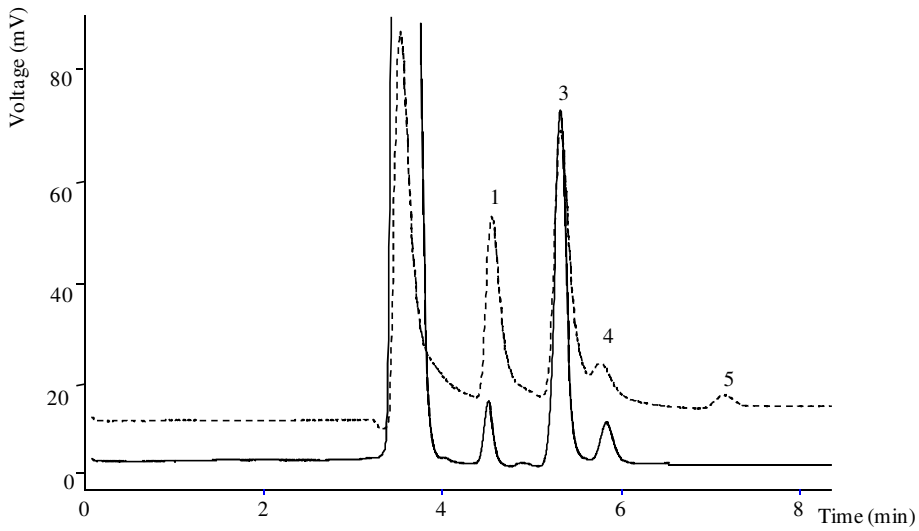


R^1=H, R^2=OH *p*-hydroxybenzoic acid
R^1=R^2=OH protocatechuic acid



R=OH *p*-coumaric acid
R=H cinnamic acid

Figure 1.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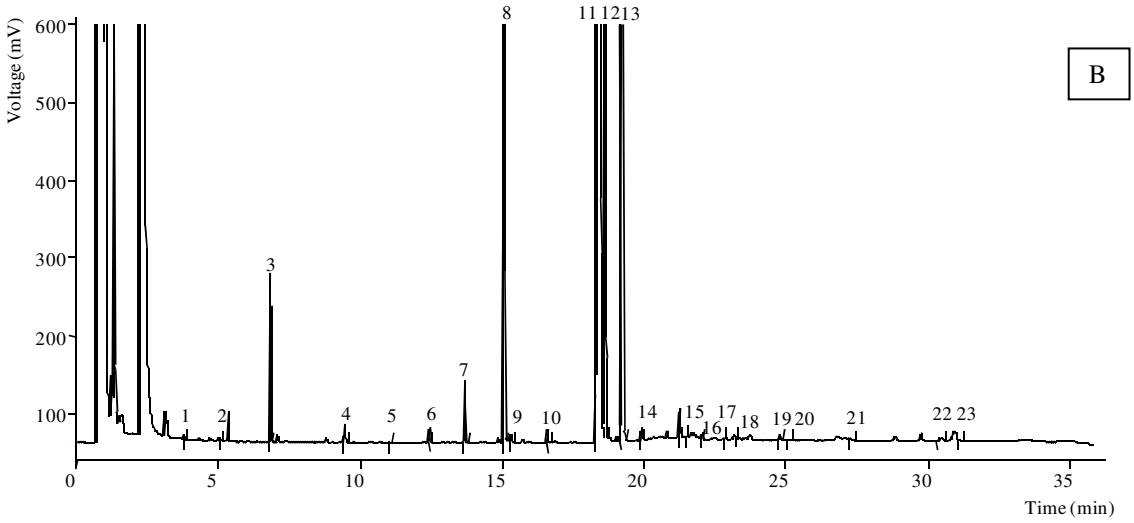
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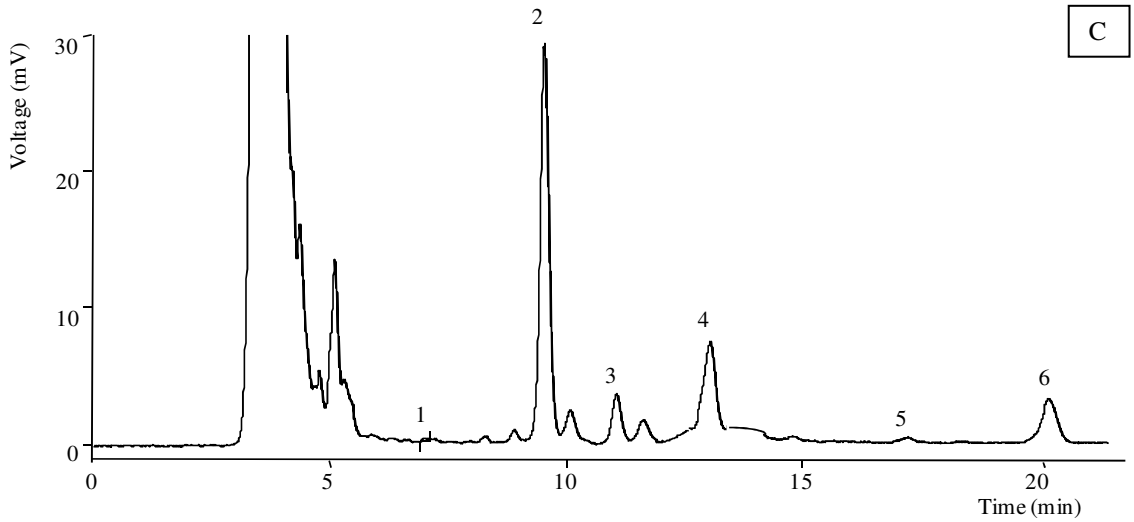
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Figure 2.

TOC Graphic

Phenolic, polysaccharidic and lipidic fractions of mushrooms from northeast Portugal: chemical compounds with antioxidant properties

Sandrina A. Heleno, Lillian Barros, Anabela Martins, Maria João R.P. Queiroz, Celestino Santos-Buelga, Isabel C.F.R. Ferreira