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Phenolic, Polysaccharidic, and Lipidic Fractions of Mushrooms from Northeastern Portugal: Chemical Compounds with Antioxidant Properties

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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 northeastern Portugal were studied for their chemical composition and antioxidant properties. Protocatechuic, *p*-hydroxybenzoic, *p*-coumaric, and cinnamic acids were found in the phenolic fraction; rhamnose, xylose, fucose, arabinose, fructose, glucose, manose, mannitol, sucrose, maltose, and trehalose were quantified in polysaccharidic fraction; and 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 in agreement 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 due 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 antiallergenic, antiatherogenic, anti-inflammatory, antimicrobial, antithrombotic, 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]

Mushroom 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 radical scavengers, reacting with peroxyl radicals produced from polyunsaturated fatty acids in membrane phospholipids or lipoproteins to yield stable lipid hydroperoxides.[19] Several studies 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, triglyceride levels, blood pressure, and arthritis. LA is present in wild mushrooms' lipidic fraction,[7,8] and it is also a precursor of

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Table 1. Information about the Wild Species Analyzed

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

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 northeastern 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.

MATERIALS AND METHODS

Mushroom Species. Samples of *C. atramentaria* (Bull.: Fr.) Redhead, Vilgalys & Moncalvo, *L. bertillonii* (Neuhoff ex Z. Schaef.) Bon, *L. vellereus* (Fr.) Fr., *R. palmatus* (Bull.:Fr.) Maire, and *X. chrysenteron* (Bull.) Quél. were collected in Bragança (northeastern 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 lyophilized (FreeZone 4.5 model 7750031, Labconco, Kansas City, MO, 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 acid methyl esters (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 (Pleasant Gap, 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).

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

Quantification of Total Phenolics. The extract solutions (1 mL) were mixed with Folin−Ciocalteu reagent (5 mL, previously diluted with water 1:10, v/v) and sodium carbonate (75 g/L, 4 mL). The tubes were vortex mixed for 15 s and allowed to stand for 30 min at 40 °C for color development. Absorbance was then measured at 765 nm (Analytikjena spectrophotometer). Gallic acid was used to obtain the standard curve (0.0094−0.15 mg/mL), and the results were expressed as milligrams of gallic acid equivalents (GAE) per gram of extract.

Analysis of Phenolic Compounds. The extract solutions (1 mL) were filtered through a 0.22 μm disposable LC filter disk for HPLC analysis. The analysis was performed using a Hewlett-Packard 1100 series liquid chromatograph (Agilent Technologies) as previously described.[15] Separation was achieved on a Spherisorb S3 ODS-2 (Waters) reverse phase C_{18} column (3 μm, 150 × 4.6 mm) thermostated at 25 °C. The solvents used were (A) 0.1% formic acid in water and (B) acetonitrile. The elution gradient established was from 10 to 15% B over 5 min, from 15 to 25% B over 5 min, from 25 to 35% B over 10 min, isocratic 50% B for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Detection was carried out in a diode array detector (DAD), using 280 nm as the preferred wavelength. The phenolic compounds were characterized according to their UV and retention times compared with commercial standards. For the quantitative analysis of phenolic compounds, a calibration curve was obtained by injection of known concentrations (5−100 μg/mL) of different standard compounds. The results were expressed as milligrams per 100 g of dry weight (dw).

Characterization of the Polysaccharidic Fraction. *Preparation of Polysaccharidic Extracts.* The lyophilized mushrooms (∼1.5 g) were extracted with water at boiling temperature (50 mL) for 2 h under agitation (150 rpm; Velp Are magnetic stirrer) and subsequently filtered through Whatman no. 4 paper. The residue was then extracted with two more portions of boiling water, in a total of 6 h of extraction. The combined extracts were lyophilized, and then 95% ethanol (10 mL) was added and polysaccharides were precipitated overnight at 4 °C. The precipitated polysaccharides were collected after centrifugation (Centorion K24OR refrigerated centrifuge) at 3100*g* for 40 min followed by filtration and then were lyophilized, resulting in a crude polysaccharidic sample.[27] The crude polysaccharidic samples were redissolved in water for the antioxidant activity assays and further hydrolyzed for chemical characterization.

Quantification of Total Polysaccharides. The extract solutions (1 mL) were added to 80% phenol (25 μL) and concentrated sulfuric acid (1 mL). The mixture was shaken and allowed to stand at 30 °C for 30 min. The absorbance was measured at 490 nm. Starch (although glycogen is the storage polysaccharide in mushrooms, starch is the most available polysaccharide) was used to obtain the standard curve (0.625−40 mg/mL), and the results were expressed as milligrams of polysaccharides equivalents (PE) per gram of extract.

Analysis of Polysaccharidic Extracts. The polysaccharidic extracts were hydrolyzed with 0.05 M trifluoroacetic acid (TFA, 2 mL), maintained at 90 °C for 16 h, and then centrifuged, following the procedure described by Vaz et al.[27] with some modifications. The supernatant was lyophilized, redissolved in distilled water (1 mL), and filtered through 0.2 μm nylon filters for HPLC-RI analysis.

For a comparison, free sugars were also determined. The lyophilized samples (1 g) were spiked with raffinose as internal standard (IS, 5 mg/mL) and were extracted with 40 mL of 80% aqueous ethanol at 80 °C for 30 min. The resulting suspension was centrifuged at 15000*g* for 10 min. The supernatant was concentrated at 60 °C under reduced pressure and defatted three times with 10 mL of ethyl ether, successively. After concentration at 40 °C, the solid residues were dissolved

in water to a final volume of 5 mL and filtered through 0.2 μm nylon filters for HPLC-RI analysis.

The equipment consisted of an integrated system with a pump (Knauer, Smartline system 1000), degasser system (Smartline manager 5000), autosampler (AS-2057 Jasco), and RI detector (Knauer Smartline 2300). Chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6 × 250 mm, 5 μm, Knauer) operating at 30 °C (7971 R Grace oven). The mobile phase was acetonitrile/deionized water, 7:3 (v/v), at a flow rate of 1 mL/min. Sugar identification was made by comparing the relative retention times of sample peaks with standards (0.5−40 mg/mL). Data were analyzed using Clarity 2.4 software (DataApex). Quantification was made by the IS method, and the results were expressed in grams per 100 g of 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 transesterification procedure: fatty acids (obtained after Soxhlet extraction) were methylated with 5 mL of methanol/sulfuric acid 95%/toluene 2:1:1 (v/v/v) for at least 12 h in a bath at 50 °C and 160 rpm; to obtain phase separation 3 mL of deionized water was added; the FAME were recovered by shaking in a vortex with 3 mL of diethyl ether, and the upper phase was passed through a microcolumn of anhydrous sodium sulfate 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 i.d. × 0.25 μm d_f). The oven temperature program followed was an initial column temperature of 50 °C, held for 2 min, followed by a 10 °C/min ramp to 240 °C for 11 min. The carrier gas (hydrogen) flow rate was 4.0 mL/min (0.61 bar), measured at 50 °C. Split injection (1:40) was carried out at 250 °C. 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. Tocopherol 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. A saturated NaCl aqueous solution (2 mL) was added, the mixture was homogenized (1 min) and centrifuged (5 min, 4000*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 sulfate, 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 °C. 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 were expressed in micrograms 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

Table 2. Extraction Yields, Antioxidant Activity (EC_{50} Values[a]), Total Phenolics, and Total Polysaccharides of the Wild Mushroom Phenolic and Polysaccharidic Fractions[b]

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 ± 0.41f	2.48 ± 0.73g	9.54 ± 0.96d	9.90 ± 0.24d	17.46 ± 0.40a	7.76 ± 0.39e	7.58 ± 0.23e	15.48 ± 0.60b	2.06 ± 0.46g	11.31 ± 0.81c
reducing power (mg/mL)	1.29 ± 0.11er	0.88 ± 0.03g	1.63 ± 0.01d	1.13 ± 0.51gf	3.37 ± 0.05a	2.37 ± 0.17c	1.43 ± 0.29ed	3.36 ± 0.18a	1.28 ± 0.02ef	2.90 ± 0.17b
β-carotene bleaching inhibition (mg/mL)	1.03 ± 0.07e	0.81 ± 0.08e	3.01 ± 0.34c	1.97 ± 0.04d	3.69 ± 0.66b	2.20 ± 0.15d	2.38 ± 0.38d	5.03 ± 0.87a	0.95 ± 0.06e	4.43 ± 0.44a
TBARS inhibition (mg/mL)	1.09 ± 0.18c	1.01 ± 0.11c	1.21 ± 0.17c	1.00 ± 0.03c	3.12 ± 0.49b	1.21 ± 0.03c	1.22 ± 0.68c	4.72 ± 0.13a	0.44 ± 0.07c	4.94 ± 0.16a
total phenolics (mg GAE/g extract)	33.58 ± 0.64a	np	23.09 ± 0.67b	np	12.62 ± 0.18c	np	28.55 ± 0.30b	np	36.28 ± 0.57a	np
total polysaccharides (mg PE/g extract)	np[c]	16.72 ± 0.46a	np	7.91 ± 0.33b	np	6.99 ± 0.19c	np	2.30 ± 0.01e	np	3.42 ± 0.08d

[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.[b]n each row different letters imply significant differences ($p < 0.05$). [c]np, not performed.

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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 eq	R^2	linear eq	R^2	linear eq	R^2	linear eq	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[a]

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

[a]In each row different letters imply significant differences ($p < 0.05$). [b]nd, not detected.

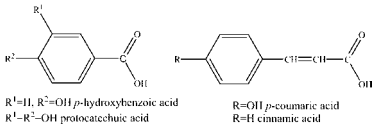

Figure 1. Chemical structure of the phenolic acids and related compounds found in the wild mushrooms phenolic fraction.

(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 discoloration 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 radical-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 °C 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 deionized 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 the graph of absorbance at 690 nm against extract concentration. Trolox was used as standard.

Inhibition of β-Carotene Bleaching. A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two milliliters of this solution was pipetted into a round-bottom flask. The chloroform was removed at 40 °C under vacuum and linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into test tubes containing different concentrations of the extracts (dissolved in water/methanol 80:20 or water for phenolic and polysaccharidic fractions, respectively; 0.2 mL). The tubes were shaken and incubated at 50 °C in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm.[27] β-Carotene bleaching inhibition was calculated using the following equation: (β-carotene content after 2 h of assay/initial β-carotene content) × 100. The extract concentration providing 50% antioxidant activity (EC_{50}) was calculated by interpolation from the graph of β-carotene bleaching inhibition percentage against extract concentration. Trolox was used as standard.

Inhibition of Lipid Peroxidation Using Thiobarbituric Acid Reactive Substances (TBARS). Porcine (*Sus scrofa*) brains were obtained from official slaughtering animals, dissected, and homogenized with a Polytron in ice-cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 1:2 (w/v) brain tissue homogenate, which was centrifuged at 3000*g* for 10 min. An aliquot (0.1 mL) of the supernatant was incubated with the different concentrations of the extracts (dissolved in water/methanol 80:20 or water for phenolic 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 °C 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 °C for 20 min. After centrifugation at 3000*g* for 10 min to remove the precipitated protein, the color 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 (%) = $[(A - B)/A] \times 100\%$, where A and B were the absorbances 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 of 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 the 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), whereas the phenolic fraction (extracts dissolved in water/methanol 80:20) was shown to have highest antioxidant potential in *R. palmatus* and *X. chrysenteron*. *C. atramentaria* and *X. chrysenteron* phenolic extracts revealed the highest content of total phenolics (33.58 and 36.28 mg GAE/g extract, without significant statistical differences, $p < 0.05$). The *C. atramentaria* polysaccharidic extract also gave the highest total polysaccharides content (16.72 mg PE/g extract). Among the phenolic fractions, *C. atramentaria* and *X. chrysenteron* gave the highest antioxidant activity demonstrated by the lowest EC_{50} values obtained (Table 2), which was coherent

Table 5. Composition in Free Sugars and Sugars Obtained after Hydrolysis of the Wild Mushrooms' Polysaccharidic Fraction[a]

	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
rhamnose (g/100 g dw)	nd[b]	9.74 ± 0.54a	nd	nd	nd	nd	9.04 ± 0.08a	4.42 ± 0.42b	nd	tr[c]
xylose (g/100 g dw)	nd	6.22 ± 0.76a	nd	nd	nd	nd	nd	nd	nd	0.23 ± 0.04b
fucose (g/100 g dw)	nd	nd	nd	nd	nd	nd	nd	1.40 ± 0.10a	nd	1.39 ± 0.13a
arabinose (g/100 g dw)	nd	0.79 ± 0.08a	nd	nd	nd	nd	nd	nd	nd	0.67 ± 0.11b
fructose (g/100 g dw)	0.26 ± 0.02b	1.10 ± 0.17d	nd	0.20 ± 0.01d	nd	5.08 ± 0.40c	20.30 ± 0.73a	33.61 ± 1.12a	nd	7.80 ± 0.17b
glucose (g/100 g dw)	0.32 ± 0.01a	1.54 ± 0.16a	nd	0.11 ± 0.01bc	nd	nd	nd	nd	nd	0.22 ± 0.07b
mannose (g/100 g dw)	nd	1.06 ± 0.10a	nd	nd	nd	nd	nd	nd	nd	nd
mannitol (g/100 g dw)	nd	nd	11.71 ± 0.37b	11.98 ± 0.17b	24.77 ± 0.32a	24.05 ± 0.77a	2.62 ± 0.02d	5.53 ± 0.68c	5.81 ± 0.35c	6.38 ± 0.35c
sucrose (g/100 g dw)	0.26 ± 0.02a	2.57 ± 0.32a	nd	nd	nd	nd	nd	nd	nd	0.05 ± 0.00b
maltose (g/100 g dw)	nd	20.64 ± 2.21a	nd	nd	nd	nd	nd	nd	nd	nd
trehalose (g/100 g dw)	5.35 ± 0.18a	1.32 ± 0.17c	1.61 ± 0.04d	0.22 ± 0.01d	2.41 ± 0.17c	3.32 ± 0.05b	0.90 ± 0.18e	nd	4.16 ± 0.27b	9.71 ± 0.53a
total sugars (g/100 g dw)	6.19 ± 0.06e	44.98 ± 1.77a	13.32 ± 0.23c	12.51 ± 0.16d	27.18 ± 0.50b	32.45 ± 0.42b	32.86 ± 1.01a	44.96 ± 0.97a	9.98 ± 0.28d	26.45 ± 1.25c

[a]In each row, and independently for free sugar and polysaccharide hydrolyses, different letters imply significant differences ($p<0.05$). [b]nd, not detected. [c]tr, traces.

Figure 2. (A) Free sugars (- - -) and sugars obtained after polysaccharide hydrolysis (—) in *Rhodotus palmatus*. Peaks: 1, rhamnose; 2, fucose; 3, fructose; 4, mannitol; 5, trehalose. (B) Fatty acids in *Lactarius vellereus*. Peaks: 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*. Peaks: 1, α-tocopherol; 2, BHT; 3, β-tocopherol; 4, γ-tocopherol; 5, δ-tocopherol; 6, tocol (IS).

with their higher contents of total phenolics. In all cases, good correlations between total phenolic or total polysaccharides and DPPH scavenging activity, reducing power, and β-carotene bleaching inhibition were observed ($R^2 > 0.72$, Table 3). However, TBARS inhibition seemed not to be correlated with those compounds, which might be due to other antioxidant molecules, probably with less polar characteristics, involved in that activity.

As far as we know, this is the first study on the antioxidant activity of phenolic extracts of the studied species, with the exception of *X. chrysenteron*.[29] These authors reported a lower content in phenolics (17.91 mg GAE/g extract) and a lower reducing power but a higher DPPH scavenging activity and β-carotene bleaching inhibition in a sample from Turkey.

The composition of the phenolic fractions of the studied samples is shown in Table 4. Phenolic acids (protocatechuic, *p*-hydroxybenzoic, and *p*-coumaric acids) and a related compound (cinnamic acid) (Figure 1) were found in the studied species. It has been reported that the antioxidant activity of phenolic acids (ArOH) is related to the presence of hydroxyl

Table 6. Extraction Yield and Composition in Fatty Acids and Tocopherols of the Wild Mushrooms' Lipidic Fraction[a]

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

[a]In each row different letters imply significant differences ($p < 0.05$). 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 from 100% corresponds to other 21 less abundant fatty acids (data not shown).

groups in the molecule, through transfer mechanisms of (i) hydrogen atoms ($LOO^{\bullet} + ArOH \rightarrow LOOH + ArO^{\bullet}$; the $ArO^{\bullet}$ radical must be stable so that it can slowly react with the substrate, LH, and quickly with the $LOO^{\bullet}$ interrupting the chain reactions) or (ii) electrons ($LOO^{\bullet} + ArOH \rightarrow LOO^{-} + ArOH^{+}$; $ArOH^{+} + H_2O \leftrightarrow ArO^{\bullet} + H_3O^{+}$; $LOO^{-} + H_3O^{+} \leftrightarrow LOOH + H_2O$).[30] The introduction of a second hydroxyl group in the ortho or para position seems to increase the antioxidant activity; therefore, the *o*-diphenol protocatechuic acid should be more efficient than the corresponding monophenol, *p*-hydroxybenzoic acid.[31]

R. palmatus revealed the highest content in total phenolic acids (10.55 mg/100 g dw) and, particularly, in protocatechuic acid (8.60 mg/100 g dw). Nevertheless, this species showed lower antioxidant activity than *X. chrysenteron*, which presented much lower concentrations of the mentioned phenolic acids but higher total phenolics measured by the Folin−Ciocalteu assay. Therefore, and as it is not expected that fungi present phenolic compounds other than phenolic acids, it should be highlighted that the Folin−Ciocalteu assay measures total antioxidants rather than phenolic compounds.

With regard to polysaccharidic fractions, *C. atramentaria* gave the highest antioxidant activity, which was in agreement with its highest content in total polysaccharides (Table 2) and in sugars obtained after hydrolysis (44.98 g/100 g dw; Table 5). This species also released the greatest diversity of sugars after hydrolysis, including maltose, rhamnose, xylose, sucrose, glucose, trehalose, fructose, mannose, and arabinose. *X. chrysenteron* also presented a high diversity of sugars but in lower amounts. *C. atramentaria* and *R. palmatus* gave similar (without statistical differences) amounts of total sugars obtained after polysaccharide hydrolysis. Nevertheless, the latter species presented less diversity of sugars, although it showed fucose and mannitol that were not found in *C. atramentaria* (Figure 2A). Despite the mentioned observations, it should be highlighted that some of the obtained sugars may not be from polysaccharides hydrolysis but contaminations of free sugars present on the analyzed fraction.

Free sugars were also analyzed in the studied mushrooms species, and *R. palmatus* gave the highest levels (32.86 g/100 g dw; Table 5), revealing the presence of fructose, rhamnose, mannitol, and trehalose. After data observation, it can be concluded that most sugars were included in polysaccharides, which are one of the most abundant bioactive macromolecules in mushrooms.[18] Mannitol and trehalose are still the most widespread free sugars in the studied species, as has been described in the literature.[1,8,32]

The results for fatty acid and tocopherol compositions of the studied wild mushrooms' lipidic fraction are shown in Table 6. According to the results, linoleic acid (C18:2n6c) was the major fatty acid found in the species *C. atramentaria*, *R. palmatus*, and *X. chrysenteron*, whereas stearic acid (C18:0) was the most abundant in *L. bertillonii* and *L. vellereus* (Figure 2B). The presence of this fatty acid in high amounts was corroborated in other *Lactarius* sp. previously studied by us, namely, *L. deliciosus* and *L. piperatus*[32] and *L. salmonicolor*.[8] Oleic (C18:1n9c), palmitic (C16:0), and α-linolenic (C18:3n3c) acids were also found in not negligible amounts.

Besides those fatty acids, 21 less abundant fatty acids were also identified (data not shown). PUFA were the main group of fatty acids in *C. atramentaria* (54%), *R. palmatus* (51%), and *X. chrysenteron* (46%) due to the high contribution of linoleic acid, whereas SFA were the main group in *L. bertillonii* (14%) and *L. vellereus* (22%) due to the high amounts of stearic acid.

The values obtained in the analysis of the different samples revealed significant differences with regard to tocopherol composition (Table 6). All of the isoforms were found in all of the studied species, but β- and γ-tocopherols were the major vitamers (Figure 2C). *X. chrysenteron* presented the highest content of tocopherols (372.98 μg/100 g dw) and also the highest level of γ-tocopherol (220.51 μg/100 g), whereas *L. vellereus* revealed the highest content of β-tocopherol (242.41 μg/100 g). The lowest values of tocopherols were found in *C. atramentaria* and *R. palmatus* without statistical differences ($p < 0.05$). Tocopherols play an important role in health, acting as antioxidants by their capacity to scavenge lipid peroxyl radicals of unsaturated fatty acids and preventing propagation of lipid peroxidation.[33] Furthermore, PUFA such as linoleic and α-linolenic acids are essential fatty acids and have been associated with a reduced risk of developing cardiovascular, inflammatory, and autoimmune diseases, being biosynthetic precursors of eicosanoids.[34]

Overall, the inclusion of mushrooms in the diet could bring health benefits, considering their antioxidant properties. Furthermore, different fractions (phenolic, polysaccharidic, and lipidic) could be separated and purified to be included in nutraceutical or pharmaceutical formulations.

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Notes

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