Towards chemical and nutritional inventory of Portuguese wild edible mushrooms in different habitats

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Abstract

Mushrooms have been valued as highly tasty/nutritional foods and as a source of compounds with medicinal properties. The huge mushrooms reservoir of Northeast Portugal must be chemically and nutritionally characterized for the benefit of populations and for the genetic conservation of wild macrofungi. Herein, a chemical, nutritional and bioactive inventory of potentially interesting species (and not yet characterized in literature) from different habitats (*Castanea sativa*, *Pinus* sp., *Quercus* sp., fields and mixed stands) was performed. Besides macronutrients with a well-balanced proportion, the studied wild mushrooms have also important micronutrients (vitamins) and non-nutrients (phenolics) with bioactive properties such as antioxidant potential. Furthermore, being a source of important antioxidants the wild species, mainly *Suillus variegatus* (*Pinus* sp. habitat), *Boletus armeniacus* (*Castanea sativa* habitat), *Clavariadelphus pistillaris* (Quercus sp. habitat), *Agaricus lutosus* (fields) and *Bovista aestivalis* (mixed stands), can be used in diet as nutraceuticals and/or functional foods maintaining and promoting health, longevity and life quality.

1. Introduction

Mushrooms past and current uses and practices, represent an important cultural heritage as they are used since immemorial times as food and medicine according to traditional ecological knowledge transmitted along generations. Mushrooms have long been valued as highly tasty/nutritional foods. There are available in literature several studies reporting nutrients analysis of mushrooms from Spain (Díez & Alvarez, 2001), Finland (Mattila, Salo-Väänänen, Könkö, Aro, & Jalava, 2002), Greece (Ouzouni, Petridis, Koller, & Riganakos, 2009), Italy (Manzi, Marconi, Aguzzi, & Pizzoferrato, 2004), India (Agahar-Murugkar & Subbulakshmi, 2005), Mexico (Léon-Guzmán, Silva, & López, 1997), Nigeria (Aletor, 1995), Portugal (Heleno, Barros, Sousa, Martins, & Ferreira, 2009), Taiwan (Tsai, Tsai, & Mau, 2008), Tanzania (Mdachi, Nkunya, Nyigo, & Urasa, 2004) and Turkey (Yildiz, Karakaplan, & Aydin, 1998). In Europe, wild mushrooms are collected for consumption being a good source of digestible proteins, carbohydrates, fibres and vitamins (Barros, Baptista, Correia, Casal, Oliveira, & Ferreira, 2007a; Barros, Baptista, Estevinho, & Ferreira, 2007b; Heleno et al., 2009; Kalač, 2009; Ouzouni et al., 2009). Dry matter content is usually about 100 g/kg. Structural polysaccharides and proteins comprise the main components of dry matter, while the lipid content is low. Chitin, glycogen, mannitol and trehalose are typical carbohydrate constituents. The proportion of essential amino acids is nutritionally favourable, while the content of n-3 fatty acid is negligible (Kalač, 2009).

Macrofungi traditionally used in gastronomy are mainly mycorrhizal fungi associated with ecologically/economically important trees such as *Castanea sativa* (Baptista, Martins, Tavares, & Lino-Neto, 2010), *Pinus* (Martín-Pinto et al., 2006) and *Quercus* sp. (Garibay-Orijel et al., 2009). Furthermore, macrofungi have a history of traditional use in oriental therapies and modern clinical practices continue to rely on mushroom-

derived preparations. They accumulate a variety of bioactive metabolites (e.g. phenolic compounds, polyketides, terpenes, steroids and polysaccharides) with immunomodulatory, cardiovascular, liver protective, anti-fibrotic, anti-inflammatory, anti-diabetic, anti-viral, antimicrobial activities, and antitumor properties (Lindequist, Niedermeyer, & Jülich, 2005; Poucheret, Fons, & Rapior, 2006; Zhang, Cui, Cheung, & Wang, 2007; Ferreira, Vaz, Vasconcelos, & Martins, 2010). Purified bioactive compounds derived from medicinal mushrooms are a potentially important new source of natural antioxidants that positively influence oxidative stress related diseases such as cancer (Moradali, Mostafavi, Ghods, & Hedjaroude, 2005; Zaidman, Yassin, Mahajana, & Wasser, 2005; Valko et al., 2007; Ferreira, Barros, & Abreu, 2009).

In some fields like the food and pharmaceutical industries mushrooms are an important and valued commodity. In 2004, the estimated value of wild edible mushroom gathering was $2 billion (Boa, 2004). Therefore, their chemical and biological characteristics attract significant interest as they are natural bioreactors for production of compounds with human interest for biotechnological applications.

The huge mushrooms reservoir of Northeast Portugal must be chemically/nutritionally characterized for practical sustainable applications in biotechnological systems and industries and for the benefit of populations, while contributing for the genetic conservation of wild macrofungi. Following the work carried out by our research group in order to demonstrate the promising health enhancing properties of mushrooms compounds (Barros et al., 2007a: Barros et al., 2007b; Heleno, Barros, Sousa, Martins, & Ferreira, 2010; Vaz et al., 2010; Vaz et al., 2011), a chemical/nutritional/bioactive inventory of potentially interesting species (and not yet characterized in literature) from different habitats (*Castanea sativa*, *Pinus* sp., *Quercus* sp., fields and mixed stands) in Northeast Portugal was performed.

2. Materials and methods

2.1. Samples

Twenty wild edible mushroom species were collected in Bragança (Northeast Portugal) in different habitats (each habitat corresponds to the same local of collection for the different species), in October/November of 2009 and 2010, according to **Table 1**. Three to ten specimens of each mushroom species were collected in the maturity stage recommended for consumption. Taxonomic identification of sporocarps was made according to several authors (Benguría, 1985; Phillips, 1988; Galli, 2001; Frade & Alfonso, 2005; Moreno, 2005), and representative voucher specimens were deposited at the herbarium of School of Agriculture of Polytechnic Institute of Bragança. The specimens of each species were lyophilised (Ly-8-FM-ULE, Snijders, Holland), reduced to a fine dried powder (20 mesh), mixed to obtain an homogenate sample and kept at -20 ºC until further analysis.

2.2. Standards and Reagents

Acetonitrile 99.9%, *n*-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also other individual fatty acid isomers, L-ascorbic acid, tocopherols (α-, β-, γ-, and δ-isoforms), sugars (D(-)-fructose, D(-)-mannitol, D(+)-raffinose pentahydrate, and D(+)-trehalose), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), gallic acid and (+)-catechin standards. 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 common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Macronutrients

2.3.1. Nutritional value. The samples were analysed for chemical composition (moisture, proteins, fat, carbohydrates and ash) using the AOAC procedures (AOAC, 1995). The crude protein content (N × 4.38) of the samples was estimated by the macro-Kjeldahl method; the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600±15 ºC. Total carbohydrates were calculated by difference.

2.3.2. Sugars. Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI) as described by Heleno et al. (2009), using raffinose as internal standard (IS). The equipment consisted of an integrated system with a pump (Knauer, Smartline system 1000), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco) and a RI detector (Knauer Smartline 2300). Data were analysed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6 × 250 mm, 5 mm, Knauer) operating at 30ºC (7971 R Grace oven). The mobile phase was acetonitrile/deionized water, 70:30 (v/v) at a flow rate of 1 ml/min. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was performed using the internal standard method and sugar contents were further expressed in g per 100 g of dry weight (dw).

2.3.3. Fatty Acids. Fatty acids were determined by gas-liquid chromatography with flame ionization detection (GC-FID)/capillary column as described previously by the authors (Heleno et al., 2009). The analysis was carried out with a DANI model GC 1000 instrument equipped with a split/splitless injector, a flame ionization detector (FID at 260 ºC) and a Macherey-Nagel column (30 m × 0.32 mm ID × 0.25 μm d_f). The oven temperature program was as follows: the initial temperature of the column was 50 ºC, held for 2 min, then a 30ºC/min ramp to 125 ºC, 5ºC/min ramp to 160 ºC, 20ºC/min ramp to 180 ºC, 3ºC/min ramp to 200 ºC, 20ºC/min ramp to 220 ºC and held for 15 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. 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 in relative percentage of each fatty acid.

2.4. Micronutrients

2.4.1. Tocopherols. Tocopherols content was determined following a procedure previously described by Heleno et al. (2010), using tocol as IS. The analysis was carried out in the HPLC system described above connected to a fluorescence detector (FP-2020; Jasco) programmed for excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a Polyamide II normal-phase column (250 × 4.6 mm; YMC Waters) operating at 30ºC. The mobile phase used was a mixture of n-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 ml/min. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response, using the internal

standard method, and tocopherols contents were further expressed in mg per 100 g of dry weight (dw).

2.4.2. Ascorbic acid. Ascorbic acid was determined following a procedure previously described by the authors (Grangeia, Heleno, Barros, Martins, & Ferreira, 2011) with 2,6-dichloroindophenol, and measuring the absorbance at 515 nm (spectrophotometer AnalytikJena). Content of ascorbic acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid (0.006-0.1 mg/ml), and the results were expressed as mg of ascorbic acid per 100 g of dry weight (dw).

2.4.3. Carotenoids. β-carotene and lycopene were determined following a procedure previously described by the authors (Grangeia et al., 2011), measuring the absorbance at 453, 505, 645, and 663 nm. Contents were calculated according to the following equations: β-carotene (mg/100 ml) = $0.216 \times A_{663} - 1.220 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453}$; Lycopene (mg/100 ml) = $- 0.0458 \times A_{663} + 0.204 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453}$, and further expressed in mg per 100 g of dry weight (dw).

2.5. Non-nutrients and in vitro antioxidant properties

2.5.1. Extraction procedure. A fine dried powder (20 mesh; ~1g) stirring with 50 ml of methanol at 25 ºC at 150 rpm for 1 h and filtered through Whatman No. 4 paper. The residue was then extracted with one additional 50 ml portion of methanol. The combined methanolic extracts were evaporated at 35ºC under reduced pressure (rotary evaporator Büchi R-210), re-dissolved in methanol at 50 mg/ml, and stored at 4 ºC for further analysis of bioactive compounds and antioxidant properties according to procedures described by Heleno et al. (2010).

2.5.2. Phenolics. Phenolics were determined by Folin-Ciocalteu assay. The extract solution (1 ml) was 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 colour development. Absorbance was then measured at 765 nm. Gallic acid was used to obtain the standard curve (0.0094-0.15 mg/ml), and the results were expressed as mg of gallic acid equivalents (GAE) per g of extract.

2.5.3. Flavonoids. For flavonoids quantification, the extract sample concentrated at 2.5 mg/ml (0.5 ml) was mixed with distilled water (2 ml) and $NaNO_2$ solution (5%, 0.15 ml). After 6 min, $AlCl_3$ solution (10%, 0.15 ml) was added and allowed to stand further 6 min. NaOH solution (4%, 2 ml) was added to the mixture, followed by distilled water until a final volume of 5 ml. The mixture was properly mixed and allowed to stand for 15 min. The intensity of pink colour was measured at 510 nm. (+)-Catechin was used to calculate the standard curve (0.015-1.0 mM) and the results were expressed as mg of (+)-chatequin equivalents (CE) per g of extract.

2.5.4. DPPH radical-scavenging activity. This methodology was performed using an ELX800 Microplate Reader (Bio-Tek). The reaction mixture in each one of the 96-wells consisted of one of the different concentrations of the extracts (30 μl) and aqueous methanolic solution (80:20 v/v, 270 μl) containing DPPH radicals (6×10^{-5} mol/l). The mixture was left to stand for 60 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation: %

RSA = [(A_{DPPH}-A_S)/A_{DPPH}] × 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.

2.5.5. Reducing power. This methodology was performed using the Microplate Reader described above. The different concentrations of the extracts (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) was added. The mixture (0.8 ml) was poured in the 48-wells, as also deionised water (0.8 ml) and ferric chloride (0.1% w/v, 0.16 ml), and the absorbance was measured at 690 nm. 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.

2.5.6. Inhibition of β-carotene bleaching. A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 ml). Two millilitres of this solution were pipetted into a round-bottom flask. After the chloroform was removed at 40ºC under vacuum, 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 different test tubes containing different concentrations of the extracts (0.2 ml). The tubes were shaken and incubated at 50ºC in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm. β-Carotene bleaching inhibition was calculated using the following equation:

(β-carotene content after 2h 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.

2.6. Statistical analysis

For each sample three extracts were obtained 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 α = 0.05. This treatment was carried out using SPSS v. 16.0 program.

3. Results

The results of the macronutrients composition obtained for the studied wild edible mushrooms are shown in **Table 2**. Moisture ranges between 72 g/100 g fw in *Boletus armeniacus* and 92 g/100 g fw in *Hygrophorus chrysodon*. The highest levels of protein were found in *Agaricus lutosus* (23 g/100 g dw). *Lycoperdon umbrinum* (33 g/100 g dw) and *Bovista aestivalis* (32 g/100 g dw) revealed the highest ash contents without significant statistical differences ($p<0.05$). Otherwise, these two mushrooms gave the lowest fat levels (< 0.2 g/100g dw). Carbohydrates, calculated discounting protein and fat levels, were the most abundant macronutrients and the highest levels were found in *Ramaria aurea* (77 g/100 g dw). Carbohydrates content includes also fibre. Information on dietary fibre content in wild mushrooms ranged between 4.2–9.2% and 22.4–31.2% of dry matter for soluble and insoluble fibre, respectively. In fact, mushrooms contain structural polysaccharides such as chitin, hemicelluloses and pectic substances (Kalac,

2009). Herein, we focused in the analysis of individual molecules that could supply energy to the human body (such as sugars and fatty acids) in order to recommend the use of mushrooms from a nutritional point of view. Particularly sugars, mainly mannitol and trehalose, are abundant carbohydrates in the wild edible mushrooms (**Table 2**). The species where fructose was found are all mycorrhizal, which is in agreement with our previous results (Grangeia et al., 2011). *Cortinarius praestans* revealed the highest total sugars content (61 g/100 g dw), with the highest levels of trehalose (60 g/100g dw).

The results of the main fatty acids found in the studied wild mushrooms, as also their saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) percentages are shown in **Table 3**. Up to twenty-seven fatty acids were detected in most of the samples (data not shown). The major fatty acid found was linoleic acid (C18:2n6) (prevalence of PUFA), unless for *Hygrophorus chrysodon*, *Suillus variegatus*, *Clavariadelphus pistillaris*, *Ramaria aurea*, *Amanita umbrinolutea*, *Clavariadelphus truncatus* and *Clitocybe costata* where oleic acid (C18:1n9) predominated, contributing to the prevalence of MUFA in those species. The studied species also revealed palmitic acid (C16:0) as a major fatty acid. *Agaricus comtulus*, *Agaricus lutosus*, *Leucoagaricus leucothites* and *Chlorophyllum rhacodes* gave the highest levels of PUFA (72-75%), while *Hygrophorus chrysodon* gave the highest levels of MUFA (63%).

Micronutrients such as vitamins and carotenoids contents were determined and the results are given in **Table 4**. Ascorbic acid was not found in *Boletus armeniacus*, *Lycoperdon umbrinum*, *Agaricus comtulus*, *Bovista aestivalis*, *Bovista nigrescens*, *Chlorophyllum rhacodes* and *Clitocybe costata*. Nevertheless, in the other mushroom species it was more abundant than tocopherols. *Agaricus lutosus* and *Hygrophorus chrysodon* revealed the highest ascorbic acid concentration (~30 mg/100 g dw).

Lycoperdon umbrinum presented the highest content of tocopherols (1.7 mg/100 g dw) with the highest levels of α- (1.5 mg/100 g dw) and β- (0.1 mg/100 g dw) isoforms. *Suillus variegatus* revealed the highest concentration of γ-tocopherol (1.4 mg/100 g dw). Carotenois were found in low amounts; the highest levels of β-carotene and lycopene were observed in *Agaricus campestris* and *Ramaria aurea*, respectively (< 1 mg/100 g dw).

The composition in non-nutrients and *in vitro* antioxidant activity of the studied wild mushrooms is shown in **Table 5**. *Suillus variegatus* gave the best results in all the antioxidant activity assays (DPPH scavenging activity, reducing power and β-carotene bleaching inhibition) with EC_{50} values ≤ 1 mg/ml. This is in agreement to its highest levels of phenolics (58 mg GAE/g extract) and flavonoids (33 mg CE/g extract). Otherwise, *Hygrophorus chrysodon* revealed the lowest antioxidant properties (EC_{50} values ranging from 6 to 20 mg/ml) and also the lowest phenolics (5 mg GAE/g extract) and flavonoids concentrations (2 mg CE/g extract).

Besides *Suillus variegatus* from *Pinus* sp. habitat, there are other mushrooms with high antioxidant activity and that revealed increasing effects with the increase of extract concentration, as can be observed in **Figure 1**: *Boletus armeniacus* (*Castanea sativa* habitat), *Clavariadelphus pistillaris* (Quercus sp. habitat), *Agaricus lutosus* (fields) and *Bovista aestivalis* (mixed stands).

4. Discussion

Mushrooms are widely appreciated for their unique taste and flavour, but also for their chemical and nutritional properties (Kalač, 2009). We proved that wild mushrooms from different habitats have high moisture, proteins and carbohydrates contents, in contrast to low fat levels, which make them suitable to incorporate low caloric diets.

These results are in agreement to different studies reported by us (Barros et al., 2007a; Barros et al., 2007b; Barros, Venturini, Baptista, Estevinho, & Ferreira, 2008; Heleno et al., 2009; Grangeia et al., 2011) and by other authors (Ouzouni et al., 2009; Kalač, 2009).

The proximate composition of Croatian *Agaricus campestris* and *Flammulina velutipes* was recently reported by Beluhan & Ranogajec (2011). Despite the similar energetic contribution, the Portuguese samples studied by us revealed lower protein and fat contents but higher ash and carbohydrate levels. The observed results could be due to differences in the maturity stage of Croatian and Portuguese fruiting bodies as demonstrated by us in a previous study, where it was observed an increase in protein levels and a decrease in carbohydrate contents with the increase of the maturity (Barros et al., 2007b).

Mannitol and trehalose were the main sugars in the studied mushrooms. Beluhan & Ranogajec (2011) reported also the presence of mannose and glucose in Croatian *Agaricus campestris* and *Flammulina velutipes*. Nevertheless, we could not find these sugars in our samples, as can be observed in **Figure 2** for *Agaricus campestris*.

The alcohols derivatives of sugars, mostly mannitol, are responsible for the support and expansion of mushrooms fruiting bodies (Barros et al., 2008). In fact, sugars are central in cellular energetic metabolism and can also be used in the synthesis of storage or structural polysaccharides (Lehninger, Nelson, & Cox, 2008). Sugars are only a small part of total carbohydrates, being wild mushrooms rich in polysaccharides such as glycogen and β-glucans (Kalač, 2009).

The main fatty acids found in the studied wild mushrooms, linoleic and oleic acids, are common in eukaryotic organisms such as fungi. Otherwise, palmitic acid is common to different organisms. Linoleic acid is an essential fatty acid to mammals, and therefore,

could be supplied in diet through mushrooms. It is precursor of arachidonic acid and of prostaglandins biosynthesis, which play important physiologic activities (Lehninger et al., 2008). Linoleic acid is also precursor of 1-octen-3-ol, known as "fungi alcohol", the main aromatic component in fungi (Maga, 1981). As stated by us and by other authors, UFA were higher than SFA levels (Yilmaz, Solmaz, Turkekul, & Elmastas, 2006; Kalač, 2009; Heleno et al., 2009; Ouzouni et al., 2009; Grangeia et al., 2011; Lee et al., 2011). In particular, the UFA oleic and linoleic acids were also reported as main fatty acids in *Agaricus campestris* from Turkey (Yilmaz et al., 2006) and *Flammulina velupites* from Korea (Lee et al., 2011). As it was observed for the samples of *Agaricus campestris* and *Flammulina velupites* herein studied, those authors also observed higher amounts of linoleic acid than oleic acid.

Besides macronutrients, the studied wild mushrooms have also important micronutrients (e.g. vitamins) and non-nutrients (e.g. phenolics) with bioactive properties such as antioxidant potential. Those molecules seem to play a protective role in diseases related to oxidative stress, such as cancer and cardiovascular diseases (Ferreira et al., 2009; Ferreira et al., 2010). In fact, the studied species with relevance for *Suillus variegatus* (*Pinus* sp. habitat), *Boletus armeniacus* (*Castanea sativa* habitat), *Clavariadelphus pistillaris* (Quercus sp. habitat), *Agaricus lutosus* (fields) and *Bovista aestivalis* (mixed stands), demonstrated capacity to scavenge free radicals such as DPPH, high reducing power and capacity to inhibit lipid peroxidation in a β-carotene-linoleate system, after neutralization of the linoleate-free radical and other free radicals formed in the system which attack the highly unsaturated β-carotene models. As far as we know, the antioxidant potential of the studied species was not previously reported, unless *Flammulina velutipes* (in hydrophilic extracts of a sample from Japan; Bao, Ochiai, & Ohshima, 2010). The medicinal potential of this particular mushroom has been

demonstrated due to the antitumor properties of different compounds: the protein flammulin, the polysaccharides galactomannoglucan and riboglucan, the isoflavone genistein and selenium (Ferreira et al., 2010).

In conclusion, wild mushrooms from different habitats are nutritionally well-balanced foods (high carbohydrate and protein levels and low fat concentration), and based on their antioxidant potential and bioactive compounds (vitamins and phenolics) they might find applications in the prevention of free radical-related diseases. Despite being collected in different habitats, the studied mushrooms revealed similar profiles of macronutrients, micronutrients and non-nutrients. The differences observed in their concentrations are certainly due to the species rather than to the habitat. To understand the specific influence of habitat, the same species should be collect in different habitats and further analysed.

The present study contributes to the nutritional and chemical characterization of wild species, making available an inventory to be disseminated in order to promote the consumption of wild edible mushrooms and to conserve their habitats. Furthermore, being a source of important antioxidants the wild species can be used in diet as nutraceuticals and/or functional foods maintaining and promoting health, longevity and life quality.

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Table 1. Information about the wild edible species analysed.

Scientific name	English name	Habitat	Ecology	Date of collection
Boletus armeniacus Quél.	None	*Castanea sativa*	Mycorrhizal	November 2010
Clitocybe gibba (Pers.) Kumm	Common funnel cap	Pinus sp.	Saprotrophic	November 2009
Hygrophorus chrysodon (Fr.) Fr.	None	Pinus sp.	Saprotrophic	November 2010
Lycoperdon umbrinum Pers.	Umber-brown puffball	Pinus sp.	Saprotrophic	October 2010
Suillus variegatus (Sw.) Kuntze	Velvet bolete	Pinus sp.	Mycorrhizal	October 2010
Boletus impolitus Fr.	Iodine bolete	Quercus sp.	Mycorrhizal	November 2010
Clavariadelphus pistillaris (L.:Fr.) Donk	Pestle-shaped coral	Quercus sp.	Mycorrhizal	October 2010
Ramaria aurea (Schaeff.) Quél.	Golden coral	Quercus sp.	Mycorrhizal	November 2010
Agaricus campestris (L.)	Field mushroom	Fields	Saprotrophic	October 2010
Agaricus comtulus Fries	None	Fields	Saprotrophic	October 2010
Agaricus lutosus (Møller) Møller	None	Fields	Saprotrophic	November 2010
Leucoagaricus leucothites Vittad. Wasser	Smooth parasol mushroom	Fields	Saprotrophic	October 2010
Amanita umbrinolutea (Secr. ex Gillet)	Unknown	Mixed stands	Mycorrhizal	October 2010
Bovista aestivalis (Bonord.) Demoulin	None	Mixed stands	Saprotrophic	November 2010
Bovista nigrescens (Pers.)	Brown puffball	Mixed stands	Saprotrophic	November 2010
Chlorophyllum rhacodes (Vittadini) Vellinga	Shaggy parasol	Mixed stands	Saprotrophic	October 2010
Clavariadelphus truncatus (Quel.) Donk	Club Coral	Mixed stands	Mycorrhizal	November 2010
Clitocybe costata Kühner & Romagn	None	Mixed stands	Saprotrophic	October 2010
Cortinarius Praestans Cordier	Goliath webcap	Mixed stands	Mycorrhizal	October 2010
Flammulina velutipes (Curtis) Singer	Golden needle mushroom	Mixed stands	Saprotrophic	November 2010

Table 2. Macronutrients composition of the wild edible mushrooms.

	Moisture (g/100 g fw)	Ash (g/100 g dw)	Proteins (g/100 g dw)	Fat (g/100 g dw)	Carbohydrates (g/100 g dw)	Fructose (g/100 g dw)	Mannitol (g/100 g dw)	Trehalose (g/100 g dw)	Total Sugars (g/100 g dw)
Boletus armeniacus	71.50 ± 0.43	12.09 ± 0.35 h	18.25 ± 0.06 edf	1.56±0.42 ih	68.10 ± 0.51 cd	10.46 ± 0.91 a	23.56 ± 2.43 c	5.62 ± 0.35 fe	39.64 ± 1.17 c
Clitocybe gibba	72.66 ± 0.99	20.68 ± 0.15 f	14.59 ± 0.27 k	4.29 ± 0.00 b	60.45 ± 0.23 gh	nd	0.63 ± 0.02 h	1.04 ± 0.11 jk	1.67 ± 0.08 ji
Hygrophorus chrysodon	92.09 ± 1.01	26.91 ± 1.99 cb	15.11 ± 0.18 kji	3.48 ± 0.09 cd	54.51 ±1.28 kj	nd	nd	7.27 ± 1.40 e	7.27 ± 1.40 hg
Lycoperdon umbrinum	71.98 ± 0.32	33.14 ± 1.06 a	14.53 ± 0.07 k	0.37 ± 0.00 k	51.96 ± 0.70 kl	nd	0.28 ± 0.04 h	0.18 ± 0.11 jk	1.46 ± 0.07 ji
Suillus variegates	90.77 ± 0.76	15.36 ± 2.10 g	17.57 ± 0.56 egf	3.31 ± 0.49 cd	63.76 ± 2.17 ef	nd	nd	4.85 ± 0.28 fgh	4.85 ± 0.28 hi
Boletus impolitus	88.90 ± 1.45	24.43 ± 0.84 ed	16.01 ± 0.02 hji	2.94 ± 0.33 ed	56.63 ± 0.84 ij	0.31 ± 0.01 e	8.08 ± 0.08 g	1.84 ± 0.05 ji	10.23 ± 0.02 g
Clavariadelphus pistillaris	84.22 ± 1.78	20.77 ± 0.86 f	16.27 ± 0.24 hji	0.59 ± 0.07 kj	62.37 ± 0.48 gf	0.93 ± 0.22 c	24.43 ± 3.25 c	nd	25.36 ± 3.47 d
Ramaria aurea	88.52 ± 0.12	5.68 ± 0.74 j	14.60 ± 0.10 k	2.26 ± 0.05 gf	77.47 ± 0.61 a	1.53 ± 0.02 b	15.11 ± 0.30 ef	0.95 ± 0.11 jk	17.59 ± 0.21 fe
Agaricus campestris	88.17 ± 0.44	23.16 ± 0.00 e	18.57 ± 0.00 ed	0.11 ± 0.00 k	58.16 ± 0.00 ih	nd	16.94 ± 2.71 ed	3.62 ± 0.33 gh	20.56 ± 3.03 e
Agaricus comtulus	87.94 ± 0.77	28.14 ± 0.18 cb	21.29 ± 0.83 b	0.46 ± 0.00 kj	50.11 ± 0.89 ml	nd	15.39 ± 0.73 edf	3.60 ± 0.06 gh	18.99 ± 0.78 fe
Agaricus lutosus	87.04 ± 2.01	25.96 ± 2.64 cd	23.24 ± 0.44 a	1.10 ± 0.04 ij	49.71 ± 1.72 ml	nd	16.42 ± 0.62 edf	3.35 ± 0.19 ih	19.77 ± 0.43 fe
Leucoagaricus leucothites	85.29 ± 1.00	26.46 ± 0.01 cd	20.51 ± 0.47 cb	1.10 ± 0.15 ij	51.93 ± 0.53 kl	nd	13.33 ± 2.77 f	3.21 ± 0.70 ih	16.54 ± 3.47 f
Amanita umbrinolutea	73.60 ± 0.17	28.86 ± 0.00 cb	16.78 ± 0.00 hgi	6.77 ± 0.00 a	47.59 ± 0.00 m	nd	31.83 ± 0.69 b	10.06 ± 0.58 d	41.89 ± 1.27 cb
Bovista aestivalis	73.23 ± 0.93	31.86 ± 0.20 a	15.59 ± 1.23 ji	0.18 ± 0.02 k	52.37 ± 1.31 kl	nd	nd	0.38 ± 0.08 k	0.38 ± 0.08 j
Bovista nigrescens	76.41 ± 0.18	3.24 ± 0.17 k	20.94 ± 0.31 b	3.64 ± 0.96 cb	72.18 ± 0.76 b	nd	0.93 ± 0.01 h	5.09 ± 0.29 fg	6.02 ± 0.30 h
Chlorophyllum rhacodes	88.28 ± 0.33	12.10 ± 0.31 h	19.32 ± 0.04 cd	3.29 ± 0.33 cd	65.29 ± 0.48 ed	nd	18.43 ± 0.45 d	25.57 ± 0.38 b	44.00 ± 0.07 b
Clavariadelphus truncatus	90.97 ± 1.29	12.86 ± 0.33 h	15.98 ± 0.15 ji	1.54 ± 0.25 ih	69.62 ± 0.37 cb	0.40 ± 0.04 e	43.34 ± 2.76 a	nd	43.74 ± 2.79 b
Clitocybe costata	76.92 ± 2.11	10.87 ± 1.36 ih	17.27 ± 0.25 hgf	1.50 ± 0.00 ih	70.36 ± 1.10 cb	nd	15.53 ± 0.85 edf	10.99 ± 0.54 d	26.65 ± 1.40 d
Cortinarius praestans	89.16 ± 0.19	18.89 ± 0.01 f	14.56 ± 0.24 k	2.58 ± 0.28 ef	63.98 ± 0.22 ef	nd	0.37 ± 0.01 h	60.51 ± 2.31 a	60.88 ± 2.30 a
Flammulina velutipes	90.68 ± 0.58	9.42 ± 0.66 i	17.89 ± 0.02 egf	1.84 ± 0.14 gh	70.85 ± 0.36 cb	nd	5.98 ± 1.19 g	15.08 ± 1.60 c	24.61 ± 3.05 d

nd- not detected. In each column, different letters mean significant differences between species (p<0.05).

Table 3. Main fatty acids (percentage) found in the wild edible mushrooms.

	C16:0	C18:0	C18:1n9	C18:2n6	SFA	MUFA	PUFA
Boletus armeniacus	15.68 ± 0.34 ef	2.92 ± 0.20 g	27.61 ± 0.42 f	48.95 ± 0.06 f	21.01 ± 0.27 gh	29.67 ± 0.36 f	49.32 ± 0.09 e
Clitocybe gibba	13.81 ± 0.16 g	7.89 ± 0.03 a	4.91 ± 0.18 lk	64.45 ± 0.15 c	27.82 ± 0.14 d	6.16 ± 0.10 ml	66.02 ± 0.24 c
Hygrophorus chrysodon	25.95 ± 0.61 a	3.88 ± 0.01 e	57.26 ± 0.57 a	1.23 ± 0.06 m	35.32 ± 0.67 c	63.05 ± 0.55 a	1.63 ± 0.13 k
Lycoperdon umbrinum	19.92 ± 0.12 c	7.14 ± 0.5 b	22.83 ± 0.33 g	29.36 ± 0.11 j	42.48 ± 0.49 a	24.79 ± 0.77 g	32.74 ± 0.19 h
Suillus variegates	12.71 ± 0.29 hgi	3.47 ± 0.08 f	42.00 ± 0.26 d	37.44 ± 0.13 h	18.09 ± 0.29 j	44.24 ± 0.16 d	37.67 ± 0.12 g
Boletus impolitus	16.77 ± 0.40 d	1.10 ± 0.16 l	14.21 ± 1.45 ji	60.95 ± 1.10 d	23.19 ± 0.41 f	15.48 ± 1.42 j	61.33 ± 1.01 d
Clavariadelphus pistillaris	16.76 ± 0.81 d	3.99 ± 0.07 ed	49.11 ± 0.23 b	24.74 ± 0.82 k	24.86 ± 0.84 e	50.11 ± 0.02 c	25.03 ± 0.86 i
Ramaria aurea	7.32 ± 0.04 k	4.07 ± 0.09 ed	56.92 ± 0.49 a	25.60 ± 0.17 k	15.27 ± 0.23 k	58.47 ± 0.40 b	26.26 ± 0.17 i
Agaricus campestris	12.48 ± 0.01 hi	2.73 ± 0.01 g	6.09 ± 0.01 k	68.97 ± 0.07 b	20.91 ± 0.05 gh	9.05 ± 0.03 k	70.04 ± 0.02 b
Agaricus comtulus	12.98 ± 0.35 hgi	2.66 ± 0.03 g	3.50 ± 0.01 l	72.88 ± 0.57 a	22.04 ± 0.63 gf	4.42 ± 0.04 m	73.55 ± 0.59 a
Agaricus lutosus	12.03 ± 0.01 i	2.26 ± 0.22 h	6.11 ± 0.85 k	74.40 ± 0.19 a	18.49 ± 0.53 ij	6.63 ± 0.83 l	74.88 ± 0.30 a
Leucoagaricus leucothites	12.16 ± 0.20 i	1.81 ± 0.11 ij	6.27 ± 0.39 k	74.72 ± 1.32 a	18.00 ± 0.84 j	6.74 ± 0.43 l	75.25 ± 1.27 a
Amanita umbrinolutea	15.10 ± 0.13 f	3.87 ± 0.01 e	58.82 ± 0.08 a	18.81 ± 0.02 l	21.18 ± 0.10 gh	59.82 ± 0.12 b	19.00 ± 0.02 j
Bovista aestivalis	21.43 ± 1.70 b	4.32 ± 0.24 d	12.63 ± 0.13 j	41.51 ± 3.75 g	41.80 ± 2.72 a	15.53 ± 1.20 j	42.68 ± 3.92 f
Bovista nigrescens	17.39 ± 0.07 d	4.19 ± 0.26 ed	21.01 ± 0.24 h	38.28 ± 0.17 h	37.78 ± 0.47 b	23.16 ± 0.26 hg	39.06 ± 0.21 g
Chlorophyllum rhacodes	16.35 ± 0.31 ed	1.59 ± 0.03 kj	5.68 ± 0.06 k	72.61 ± 0.51 a	20.11 ± 0.35 ih	6.91 ± 0.02 l	72.98 ± 0.36 a
Clavariadelphus truncatus	14.80 ± 0.18 f	2.11 ± 0.01 ih	47.26 ± 0.02 c	29.77 ± 0.12 j	21.43 ± 0.06 gh	48.31 ± 0.07 c	30.26 ± 0.02 h
Clitocybe costata	12.76 ± 0.07 hgi	5.99 ± 0.18 c	37.27 ± 0.20 e	34.68 ± 0.92 i	22.34 ± 0.41 gf	38.02 ±0.31 e	39.64 ± 0.87 g
Cortinarius praestans	13.44 ± 0.03 hg	1.78 ± 0.41 j	20.76 ± 3.04 h	59.95 ± 3.33 d	17.93 ± 0.67 j	21.49 ± 3.09 h	60.59 ± 3.76 d
Flammulina velutipes	10.31 ± 0.39 j	1.38 ± 0.08 kl	15.08 ± 0.47 i	56.33 ± 0.14 e	14.36 ± 0.34 k	17.56 ± 0.51 i	68.08 ± 0.17 cb

Palmitic acid (C16:0); Stearic acid (C18:0); Oleic acid (C18:1n9c); Linoleic acid (C18:2n6c); SFA-saturated fatty acids; MUFA- monounsaturated fatty acids; PUFA- polyunsaturated fatty acids. The results are expressed in percentage. The difference to 100% corresponds to other 23 less abundant fatty acids (data not shown). In each column different letters mean significant differences between species ($p < 0.05$).

Table 4. Micronutrients composition of the wild edible mushrooms.

	α-tocopherol	β-tocopherol	γ-tocopherol	δ-tocopherol	Total tocopherols (mg/100 g dw)	Ascorbic acid (mg/100 g dw)	β-carotene (mg/100 g dw)	Lycopene (mg/100 g dw)
Boletus armeniacus	0.01 ± 0.00 c	0.03 ± 0.00 c	0.03 ± 0.00 fe	nq	0.07 ± 0.00 hg	nd	0.15 ± 0.00 k	nd
Clitocybe gibba	0.03 ± 0.00 c	nd	0.19 ± 0.01 b	nd	0.22 ± 0.02 d	19.47 ± 1.62 d	nd	0.09 ± 0.00 d
Hygrophorus chrysodon	nq	0.01 ± 0.00 d	nd	0.01 ± 0.00 ed	0.02 ± 0.00 i	33.16 ± 0.57 a	0.43 ±0.01d	nd
Lycoperdon umbrinum	1.48 ± 0.01 a	0.10 ± 0.00 a	0.07 ± 0.00 c	0.02 ± 0.00 c	1.67 ± 0.01 a	nd	0.17 ± 0.00 j	nd
Suillus variegatus	0.02 ± 0.00 c	nd	1.44 ± 0.03 a	nq	1.45 ± 0.03 b	6.39 ± 0.22 f	nd	0.27 ± 0.00 c
Boletus impolitus	nq	0.01 ± 0.00 d	0.06 ± 0.01 dc	nq	0.07 ± 0.01 hg	1.99 ± 0.54 h	0.29 ± 0.01 g	nd
Clavariadelphus pistillaris	nq	0.02 ± 0.00 dc	nd	0.01 ± 0.00 e	0.03 ± 0.00 hi	3.45 ± 0.20 g	0.08 ± 0.00 m	0.04 ± 0.00 g
Ramaria aurea	0.01 ± 0.00 c	0.10 ± 0.00 a	0.03 ± 0.01 fe	0.01 ± 0.00 e	0.15 ± 0.01 fe	0.66 ± 0.07 i	nd	0.51 ± 0.00 a
Agaricus campestris	0.01 ± 0.00 c	0.03 ± 0.01 c	0.04 ± 0.00 de	0.03 ± 0.00 cb	0.11 ± 0.01 fg	18.74 ± 1.09 d	0.60 ± 0.00 a	nd
Agaricus comtulus	0.01 ± 0.00 c	0.06 ± 0.00 b	0.03 ± 0.00 fe	0.07 ± 0.01 a	0.17 ± 0.01 e	nd	0.59 ± 0.01 b	nd
Agaricus lutosus	nq	0.01 ± 0.00 dc	0.01 ± 0.00 f	0.04 ± 0.00 b	0.07 ± 0.01 hg	32.18 ± 0.20 a	nd	0.44 ± 0.00 b
Leucoagaricus leucothites	nq	nd	0.01 ± 0.00 f	0.03 ± 0.00 c	0.04 ± 0.00 hi	18.87 ± 0.26 d	0.40 ± 0.01e	nd
Amanita umbrinolutea	0.01 ± 0.00 c	0.03 ± 0.00 c	nd	0.01 ± 0.00 ed	0.05 ± 0.00 hi	22.73 ± 1.91 c	0.56 ± 0.00 c	nd
Bovista aestivalis	nq	nq	0.04 ± 0.00 de	nq	0.04 ± 0.00 hi	nd	0.05 ± 0.00 n	nd
Bovista nigrescens	nq	0.02 ± 0.00 dc	nd	nd	0.02 ± 0.00 i	nd	0.21 ± 0.00 i	nd
Chlorophyllum rhacodes	nq	0.02 ± 0.00 dc	nd	0.01 ± 0.00 ed	0.03 ± 0.00 hi	nd	0.15 ± 0.00 k	nd
Clavariadelphus truncatus	0.02 ± 0.00 c	nq	0.04 ± 0.01 de	0.01 ± 0.00 ed	0.07 ± 0.01 hg	7.14 ± 0.13 f	0.11 ± 0.01 l	0.07 ± 0.00 e
Clitocybe costata	0.48 ± 0.06 b	0.03 ± 0.01 c	nd	nd	0.51 ± 0.07 c	nd	0.07 ± 0.00 m	0.03 ± 0.00 h
Cortinarius praestans	nq	0.02 ± 0.00 dc	0.03 ± 0.01 fe	0.01 ± 0.00 e	0.06 ± 0.01 hi	9.06 ± 1.33 e	0.26 ± 0.00 h	0.06 ± 0.00 f
Flammulina velutipes	0.01 ± 0.00 c	0.03 ± 0.00 c	nd	0.02 ± 0.00 cd	0.06 ± 0.00 hgi	23.87 ± 0.38 b	0.34 ± 0.00 f	0.02 ± 0.00 i

nd- not detected; nq- not quantifiable. In each column different letters mean significant differences between species (p<0.05).

Table 5. Non-nutrients composition and *in vitro* antioxidant properties (EC_{50} values) of the wild edible mushrooms.

	Phenolics (mg GAE/g extract)	Flavonoids (mg CE/g extract)	DPPH scavenging activity (mg/ml)	Reducing power (mg/ml)	β-carotene bleaching inhibition (mg/ml)
Boletus armeniacus	44.66±1.65 c	8.59 ± 0.28 d	1.74 ± 0.10 kl	0.63 ± 0.02 lk	0.77 ± 0.09 h
Clitocybe gibba	25.26 ± 1.15 ed	3.56 ± 0.79 ih	10.61 ± 1.08 cb	1.46 ± 0.27 g	4.00 ± 0.51 cb
Hygrophorus chrysodon	4.58 ±1.12 j	1.78 ± 0.08 i	20.02 ± 1.27 a	7.82 ± 0.03 a	5.95 ± 0.50 a
Lycoperdon umbrinum	27.02 ± 0.17 d	3.82 ± 0.24 ihg	3.45 ± 0.09 hg	1.27 ± 0.06 ih	3.24 ± 0.70 cd
Suillus variegatus	58.14 ± 4.51 a	33.00 ± 4.98 a	0.86 ± 0.02 m	0.52 ± 0.01 l	1.00 ± 0.15 h
Boletus impolitus	15.50 ± 0.53 g	3.72 ± 0.22 ih	5.81 ± 0.17 ed	2.04 ± 0.01 e	2.04 ± 0.44 f
Clavariadelphus pistillaris	48.10 ± 0.76 cb	18.61 ± 0.85 b	1.30 ± 0.07 ml	0.70 ± 0.00 k	1.94 ± 0.02 fg
Ramaria aurea	8.46 ± 0.41 ji	2.44 ± 0.46 i	3.70 ± 0.11 g	0.99 ± 0.02 j	2.46 ± 0.40 fde
Agaricus campestris	20.94 ± 4.98 ef	5.59 ± 0.29 ehgf	5.48 ± 0.08 ed	2.70 ± 0.23 c	4.59 ± 1.30 b
Agaricus comtulus	24.13 ± 7.98 ed	3.76 ± 0.94 ihg	2.22 ± 0.05 kji	1.29 ± 0.01 ih	1.08 ± 0.05 hg
Agaricus lutosus	46.56 ± 4.16 cb	7.67 ± 0.90 ed	2.54 ± 0.44 kji	0.91 ± 0.02 j	0.90 ± 0.10 h
Leucoagaricus leucothites	15.75 ± 1.98 g	2.43 ± 0.69 i	11.33 ± 1.05 b	3.28 ± 0.05 b	1.00 ± 0.21 h
Amanita umbrinolutea	9.22 ± 0.16 hji	6.54 ± 0.25 edf	10.02 ± 0.34 c	2.71 ± 0.04 c	3.69 ± 0.70 c
Bovista aestivalis	50.91 ± 1.97 b	8.51 ± 0.43 d	2.05 ± 0.10 kjl	0.51 ± 0.01 l	0.61 ± 0.02 h
Bovista nigrescens	26.50 ± 1.18 d	14.10 ± 0.70 c	4.62 ± 0.44 f	1.21 ± 0.02 i	1.91 ± 0.22 fg
Chlorophyllum rhacodes	22.77 ± 5.26 edf	2.63 ± 0.13 i	5.32 ± 0.06 ef	2.22 ± 0.01 d	2.33 ± 0.41 fe
Clavariadelphus truncatus	7.66 ± 1.37 j	5.82 ± 0.36 egf	2.74 ± 0.04 hji	1.33 ± 0.05 h	2.35 ± 0.20 fde
Clitocybe costata	13.71 ± 1.30 hg	4.80 ± 0.35 hgf	10.56 ± 0.55 cb	1.66 ± 0.01 f	3.22 ± 0.60 cd
Cortinarius praestans	17.81 ± 0.83 gf	5.46 ± 0.52 hgf	3.04 ± 0.08 hgi	1.70 ± 0.01 f	2.04 ± 0.44 f
Flammulina velutipes	12.98 ± 0.32 hgi	2.46 ± 0.20 i	6.19 ± 0.17 d	1.94 ± 0.01 e	1.12 ± 0.23 hg

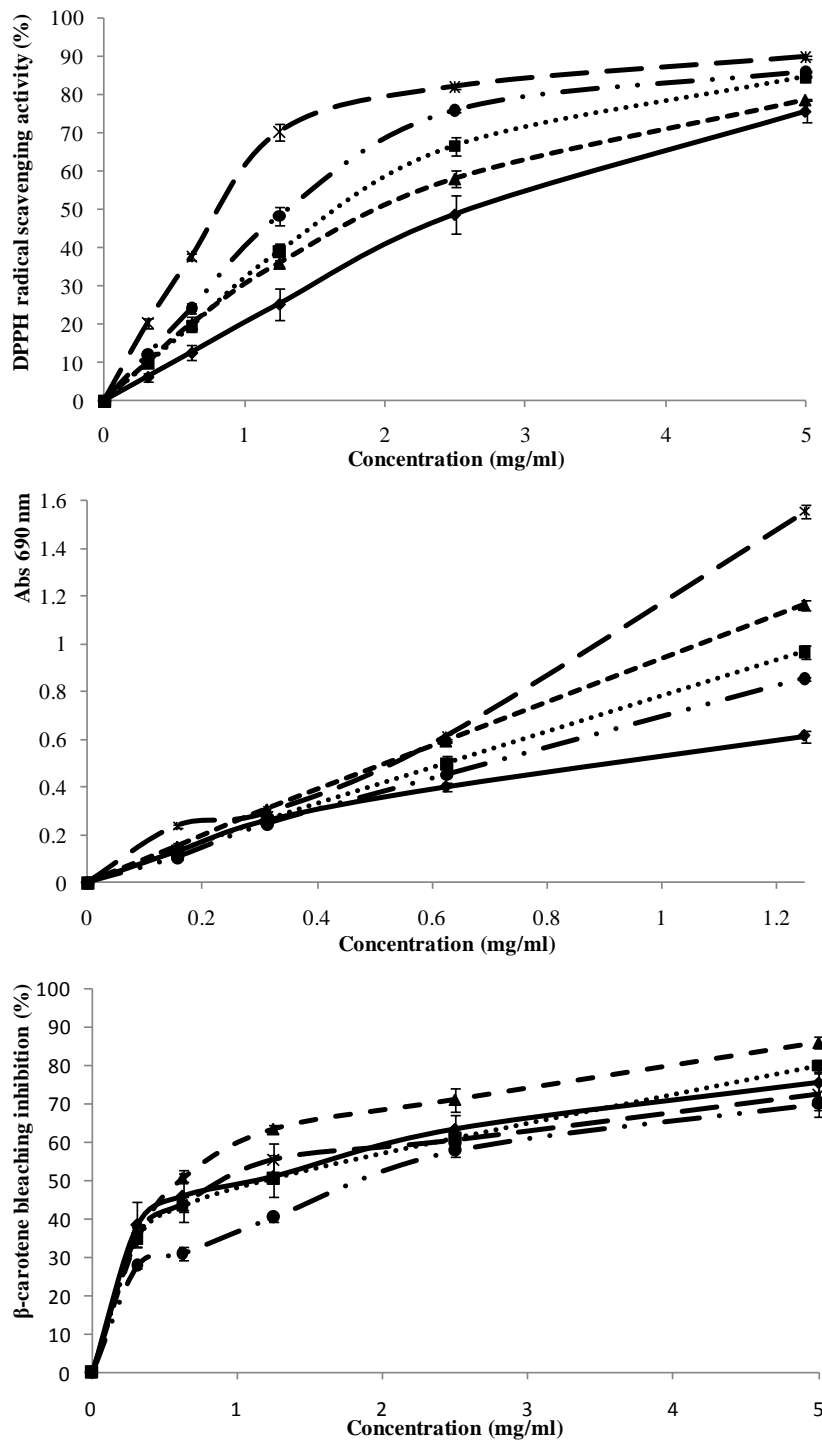In each column different letters mean significant differences between species ($p < 0.05$).

Figure 1. DPPH scavenging activity, reducing power and β-carotene bleaching inhibition of the five mushrooms with lowest EC_{50} values (highest antioxidant properties): ▬▲▬ *Boletus armeniacus* (*Castanea sativa* habitat), ▬● *Clavariadelphus pistillaris* (Quercus sp. habitat), ▬◆▬ *Agaricus lutosus* (fields), ∙∙∙■∙∙ *Bovista aestivalis* (mixed stands) and ▬✱ *Suillus variegatus* (Pinus sp).
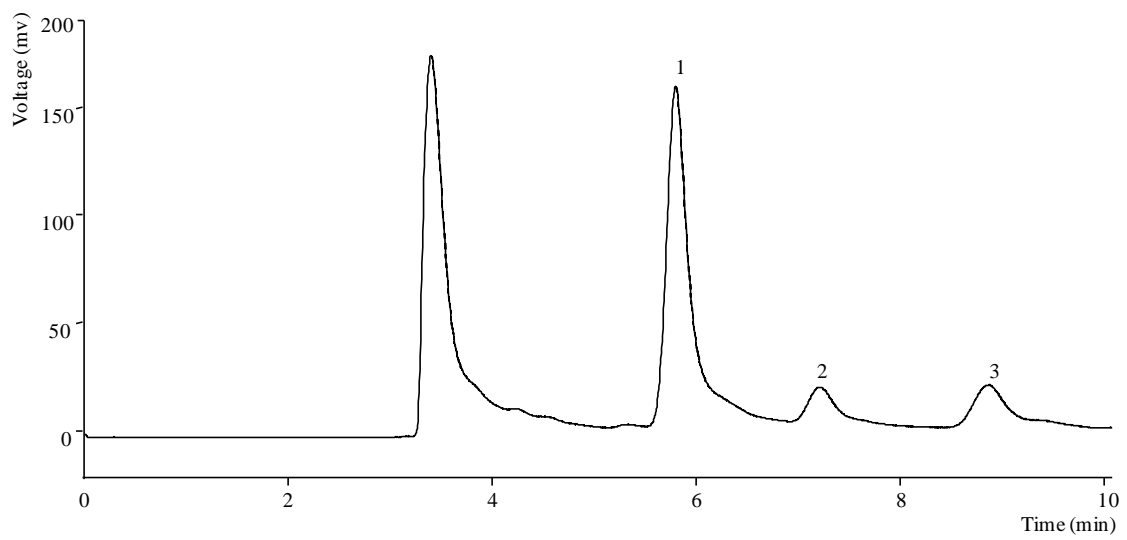
Figure 2. Individual sugars chromatogram of *Agaricus campestris*: 1-mannitol; 2-trehalose; 3-raffinose (IS).