Portuguese wild mushrooms at the "Pharma-Nutrition" interface: Nutritional characterization and antioxidant properties

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Abstract

The search for foods that might improve health or reduce disease risk, has been progressively gaining interest. Mushroom could be examples of these foods, presenting the additional advantage of being recognized as a delicacy. This feature might place mushrooms in the pharma-nutrition interface. Herein, eight different mushroom species were characterized in terms of nutrients (proteins, carbohydrates, fat, individual sugars, fatty acids) and bioactive compounds (tocopherols, carotenoids, organic acids and phenolic compounds) with recognized antioxidant properties. These medicinal properties are often related with the antioxidant potential presented by mushroom extracts. *Boletus regius* was the species with the highest levels of carbohydrates (88.79 g/ 100 g dw) and PUFA (56.55%), bioactive compounds such as tocopherols (763.80 µg/100 g dw), citric acid (3.32 g/ 100 g dw) and phenolic compounds (23.49 mg/ 100 g dw), including two chrysin derivatives, presenting also the highest antioxidant activity. The identified bioactive compounds might be used as nutraceuticals to prevent chronic diseases related with oxidative stress. Furthermore, all tested species are edible, and could be incorporated directly in diet acting as functional foods.

Keywords: Wild edible mushrooms; Nutrients; Antioxidants; Pharma-nutrition

1. Introduction

Diplock et al. (1999) define functional food as a food that improves health or wellbeing, or reduces disease risk, through beneficially targeting the body's functions. A functional food is similar in appearance to, or may be, a conventional food; is consumed as part of an usual diet and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions (Walji & Boon, 2008; Falguera, Aliguer & Falguera, 2012). Functional foods represent one of the most interesting areas of research and innovation in the food industry (Jones & Jew, 2007; Sirò, Kapolna, Kapolna & Lugasi, 2008). In Europe, functional foods sales have increased significantly; Germany, France, United Kingdom and the Netherlands represent the most important countries within this market. However, many other European markets are experiencing high growth rates (Annunziata & Vecchio, 2011).

Traditionally, pharmaceuticals have been used to cure diseases or to alleviate the symptoms of disease. Nutrition, on the other hand, is primarily aimed to prevent diseases by providing the body with the optimal balance of macro- and micronutrients needed for good health. Due to the emerging knowledge of disease, medicines are now increasingly being used to lower risk factors, and thereby to prevent chronic diseases. The appearance of functional foods and dietary supplements on the market has further blurred the distinction between pharma and nutrition (Eussen et al., 2011).

Mushrooms might be in the pharma-nutrition interface. Since ancient times mushrooms have been consumed by humans not only as a part of the normal diet, but also as a delicacy due to their highly desirable taste and aroma. In addition, the nutritional, tonic and medicinal properties of mushrooms have been recognized for a long time (Mattila, Suonpaa & Piironen, 2000). Mushrooms are quite high in protein (including all the essential amino acids) and low in fat. Mushrooms also contain relatively large amounts

of carbohydrates and fiber and significant amounts of vitamins, namely thiamin, riboflavin, ascorbic acid and vitamin D2, as well as minerals (Kalac, 2009). In addition to their nutritional value, some mushrooms may also have a medicinal value; antitumor, antiviral and hypolipidemic effects have been reported (Lindequist, Niedermeyer & Jülich, 2002; Poucheret, Fons & Rapior, 2006; Ferreira, Vaz, Vasconcelos & Martins, 2010).

Although functional foods are currently in fashion, the majority of people are unsure of their benefits. The use of functional foods may offer opportunities to reduce health risk factors and risk of diseases, both as monotherapy and in combination with prescription drugs. For example, under stress, our bodies produce more reactive oxygen species (ROS) (*e.g.*, superoxide anion radicals, hydroxyl radicals and hydrogen peroxide) than enzymatic (*e.g.*, superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase) and non-enzymatic antioxidants (*e.g.*, glutathione). This imbalance leads to cell damage and health problems (Ferreira, Barros & Abreu, 2009; Krishnaiah, Sarbatly & Nithyanandam, 2011). In this context, foods with antioxidants (*e.g.*, ascorbic acid (vitamin C), α-tocopherol (vitamin E), carotenoids or flavonoids) may reduce the oxidative stress in cells and be, therefore, useful in the treatment of many human diseases, including cancer, cardiovascular diseases and inflammatory diseases.

Our research group has been devoted, in the last years, to the study of wild mushrooms from Northeast Portugal as products that may be included in the pharma-nutrition interface (Barros, Venturini, Baptista, Estevinho & Ferreira, 2008; Vaz et al., 2010; Grangeia, Heleno, Barros, Martins & Ferreira, 2011; Pereira, Barros, Martins & Ferreira, 2012; Vaz, Martins, Almeida, Vasconcelos & Ferreira, 2012). Herein, eight different mushroom species have been characterized, for the first time, in terms of nutrients and bioactive compounds mainly with antioxidant properties.

2. Materials and methods

2.1. Samples

Eight wild edible mushroom species were collected in Bragança (Northeast Portugal) in different habitats in the Spring of 2011, 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; 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 (FreeZone 4.5, Labconco, Kansas, USA), 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 and standards of sugars (D-(-)-fructose, D-(+)-mannitol, D-(+)-trehalose), tocopherols (α-, β-, γ- and δ-isoforms), organic acids (citric acid, malic acid, oxalic acid, fumaric acid and quinic acid), phenolic compounds (chrysin, , *p*-coumaric, *p*-hydroxybenzoic, protocatechuic acids), cinnamic acid and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Racemic tocol, 50 mg/mL, was purchased from Matreya (Chalfont, PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA,

USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Nutritional compounds

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. Energy was calculated according to the following equation: Energy (kcal) = 4 × (g protein + g carbohydrate) + 9 × (g fat).

2.3.2. Sugars. Free sugars were determined by a High Performance Liquid Chromatography (HPLC) system consisted of a pump (Knauer, Smartline system 1000), degasser system (Smartline manager 5000) and auto-sampler (AS-2057 Jasco), coupled to a refraction index detector (RI detector Knauer Smartline 2300) as previously described by the authors (Pereira et al., 2012). Sugars identification was made by comparing the relative retention times of sample peaks with standards. Data were analyzed using Clarity 2.4 Software (DataApex). Quantification was based on the RI signal response of each standard, using the internal standard (IS, raffinose) method and through calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g of dry weight (dw).

2.3.3. Fatty Acids. Fatty acids were determined after a transesterification procedure as described previously by the authors (Pereira et al., 2012), using a gas chromatographer (DANI 1000) equipped with a split/splitless injector and a flame ionization detector (GC-FID). 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). The results were expressed in relative percentage of each fatty acid.

2.4. Bioactive compounds

2.4.1. Tocopherols. Tocopherols were determined following a procedure previously optimized and described by the authors (Pereira et al., 2012). Analysis was performed by HPLC (equipment described above), and a fluorescence detector (FP-2020; Jasco) programmed for excitation at 290 nm and emission at 330 nm. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response of each standard, using the IS (tocol) method and calibration curves obtained from commercial standards of each compound. The results were expressed in μg 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). A fine dried powder (500 mg) was vigorously shaken with 10 mL of acetone–hexane mixture (4:6) for 1 min and filtered through Whatman No. 4 filter paper. The absorbance of the filtrate was measured at 453, 505, 645 and 663 nm. Content of β-carotene and lycopene 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 × A_{663} + 0.204 × A_{645} − 0.304 × A_{505} + 0.452 × A_{453}; and further expressed in mg per 100 g of dry weight (dw).

2.4.4. Organic acids. Organic acids were determined following a procedure previously optimized and described by the authors (Barros, Pereira & Ferreira, 2012). Analysis was performed by ultra fast liquid chromatograph (UFLC) coupled to photodiode array detector (PDA), using a Shimadzu 20A series UFLC (Shimadzu Corporation). Detection was carried out in a PDA, using 215 nm and 245 as preferred wavelengths. The organic acids were quantified by comparison of the area of their peaks recorded at 215 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g of dry weight (dw).

2.4.5. Phenolic compounds. Each sample (≈1 g) was extracted with 30 mL of methanol:water 80:20 (*v/v*) at room temperature, 150 rpm, for 1h. The extract was filtered through Whatman nº 4 paper. The residue was then re-extracted twice with additional 30 mL portions of methanol:water 80:20 (*v/v*). The combined extracts were evaporated at 35 ºC (rotary evaporator Büchi R-210) to remove methanol. The aqueous phase was lyophilized and re-dissolved in 20% aqueous methanol at 5 mg/mL and filtered through a 0.22-µm disposable LC filter disk. Phenolic compounds were determined by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, USA) as previously described by the authors (Vaz et al., 2011). Double online detection was carried out in the diode array detector (DAD) using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (API 3200 Qtrap, Applied Biosystems, Darmstadt, Germany) connected to the HPLC system via the DAD cell outlet. The phenolic compounds were characterized according to their UV and mass spectra and

retention times, and comparison with authentic standards when available. For quantitative analysis, calibration curves were prepared from different standard compounds. The results were expressed in mg per 100 g of dried sample.

2.5. Antioxidant activity

2.5.1. General. The lyophilized samples (~1 g) was stirred with methanol (40 mL) at 25 ºC at 150 rpm for 1 h and filtered through Whatman No. 4 paper. The residue was then extracted with an additional portion of methanol. The combined methanolic extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210; Flawil, Switzerland), re-dissolved in methanol at 20 mg/mL (stock solution), and stored at 4 ºC for further use. Successive dilutions were made from the stock solution and submitted to *in vitro* assays already described by the authors (Grangeia et al., 2011) to evaluate the antioxidant activity of the samples. The sample concentrations providing 50% of antioxidant activity or 0.5 of absorbance (EC_{50}) were calculated from the graphs of antioxidant activity percentages (DPPH, β-carotene/linoleate and TBARS assays) or absorbance at 690 nm (reducing power assay) against sample concentrations. Trolox was used as standard.

2.5.2. Folin-Ciocalteu assay. One of the extract solutions (5 mg/mL, 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 (Analytikjena spectrophotometer; Jena, Germany). Gallic acid was used to obtain the standard curve (0.0094 – 0.15 mg/mL), and the reduction of *Folin-Ciocalteu* reagent by the samples was expressed as mg of gallic acid equivalents (GAE) per g of extract.

2.5.3. Ferricyanide/Prussian blue assay. The extract solutions with different concentrations (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 plate, 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 in an ELX800 Microplate Reader (Bio-Tek Instruments, Inc; Winooski, United States). The reducing power was obtained directly from the absorbances.

2.5.4. DPPH scavenging activity assay. This methodology was performed using the Microplate Reader mentioned above. The reaction mixture on 96 wells plate consisted of a solution by well of the extract solutions with different concentrations (30 µL) and methanolic solution (270 µL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 30 min in the dark, and the absorption was measured at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation: $\% \text{ RSA} = [(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample, and A_{DPPH} is the absorbance of the control (DPPH solution- 270 µL and methanol- 30 µL).

2.5.5. β-carotene/linoleate assay. 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. The chloroform was removed at 40 ºC 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 extract solutions with different concentrations (0.2 mL). The tubes were shaken and incubated at 50 ºC 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.

2.5.6. TBARS assay. 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 (100 μL) of the supernatant was incubated with the different concentrations of the samples solutions (200 μL) in the presence of $FeSO_4$ (10 mM 100 μL) and ascorbic acid (0.1 mM, 100 μL) at 37 ºC for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% *w/v*, 500 μL), followed by thiobarbituric acid (TBA,2%, w/v, 380 μL), and the mixture was then heated at 80ºC 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. 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 sample solution, respectively.

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 $\alpha = 0.05$. This treatment was carried out using SPSS v. 18.0 program.

3. Results

The results of the nutritional composition obtained for the studied wild edible mushrooms are shown in **Table 2**. Moisture ranged between 65.57 g/100 g fw in *Boletus poliporus* and 92.49 g/100 g fw in *Russula virescens*, which also presented the highest protein levels (21.85 g/100 g dw). *Gyromitra esculenta* revealed the highest ash content (32.10 g/100 g dw). Otherwise, this mushroom gave the lowest fat levels (0.73 g/100g dw), with the lowest energetic value (275.23 Kcal/100 g dw), while *Amanita mairei* revealed the highest fat content (8.30 g/100g dw). Carbohydrates were the most abundant macronutrients and the highest levels were found in *Boletus regius* (88.79 g/100 g dw). Mannitol and trehalose were abundant sugars found in the studied species (**Table 2**), but only *Boletus* species (*B. poliporus and B. regius*) presented fructose (**Figure 1A**). *Boletus poliporus* revealed the highest total sugars content (41.26 g/100 g dw), with the highest levels of fructose and mannitol (~20 g/100 g dw, each).

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 2**. Up to twenty-three fatty acids were detected in almost all of the samples (see for example **Figure 1B**). The major fatty acid found was oleic acid (C18:1n9) (prevalence of MUFA), except for *Boletus* species and *Gyromitra esculenta* where linoleic acid (C18:2n6) predominated, contributing to the prevalence of PUFA in those species. The studied species also revealed palmitic acid (C16:0) as a major saturated fatty acid. *Boletus regius* and *Gyromitra esculenta*

gave the highest levels of PUFA (~56%), while *Amanita* species gave the highest levels of MUFA (~54%).

Bioactive compounds such as tocopherols, carotenoids, organic acids and phenolic compounds were determined and the results are given in **Table 3**. Tocopherols were found in all the studied mushrooms being higher in *Boletus regius* (763.80 μg/100 g dw), that also gave the highest levels of α- (360.73 μg/100 g dw) and γ- (403.07 μg/100 g dw) isoforms. The profile of tocopherols in the mentioned species can be observed in **Figure 2A**. *Russula virescens* was the only mushroom that revealed β-tocopherol (21.30 μg/100 g dw), while *Gyromitra esculenta* and *Helvella lacunosa* were the only species that present δ-tocopherol.

β-carotene was not detected in any of the studied species, while lycopene was found in all the samples, being higher in *Helvella lacunosa* (0.53 mg/100 g dw).

Table 3 shows the results of organic acids composition, and as an example **Figure 2B** shows the organic acids profile obtained for *Gyromitra esculenta*. *Helvella lacunosa* and *Russula aurea* revealed the highest organic acids content (6.93 and 7.19 g/100 g dw, respectively) and particularly oxalic acid (2.98 μg/100 g dw) for the first case, and malic acid (4.53 g/100 g dw) and fumaric acid (0.38 g/100 g dw) for the second species. The highest levels of quinic and citric acids were found in *Boletus poliporus* (1.93 g/100 g dw) and *Boletus regius* (3.32 g/100 g dw), respectively (**Table 3**). Citric acid was the most abundant organic acid in half of the species, while quinic acid predominated in *Boletus poliporus*, oxalic acid was the most abundant in *Helvella lacunosa*, and malic acid was the major compound in *Russula* species.

The composition of the phenolic compounds of the studied mushrooms is shown in **Table 3.** Phenolic acids (protocatechuic, *p*-hydroxybenzoic and *p*-coumaric acids) and a related compound (cinnamic acid) were found in the studied species. *Boletus regius*

revealed the highest content in total phenolic compounds (23.49±0.38 mg/100 g dw), mainly due to the presence of two possible flavonoids, tentatively identified as chrysin derivatives 1 (chrysin hexoside) and 2 (11.16±0.54 and 7.33±0.06 mg/100 g dw, respectively). Those two compounds eluted at different retention times (24.9 min and 27.3 min), but they showed identical UV spectrum with λ_{max} at 268 nm (**Figure 3A**) and presented in their mass spectra a majority signal at *m/z* 253 (**Figure 3B**), both of them similar to a standard of chrysin. In the mass spectrum of the compound 1 another minority signal appeared at *m/z* 461 (**Figure 3B)**, which might be interpreted as related to the pseudo molecular ion $[M-H]^-$ of the compound. This ion, 208 mu higher than chrysin's (*m/z* at 253), could correspond to one hexosyl moiety and a formic acid molecule (162+46 mu, respectively), suggesting that the compound might be a formic acid (used as HPLC solvent) adduct of a chrysin hexoside produced in the ionization source. A similar compound was already described in *Cytisus multiflorus* (Barros, Dueñas, Carvalho, Ferreira & Santos-Buelga, 2012). The mass spectrum of the derivative 2 (**Figure 3C**) was similar to that of chrysin, and showed no signal that could be assigned to a possible molecular ion. The compound was, however, discarded to correspond to the chrysin aglycone, as this elutes at 38 min in the same HPLC conditions. Even though flavonoids have been occasionally reported in other mushroom species (Kim et al., 2008; Oke & Aslim, 2011), their presence in this type of products is controversial and must be, therefore, adequately confirmed.

The *in vitro* antioxidant activity of the studied wild mushrooms is shown in **Table 4**. *Boletus regius* gave the best results in all the antioxidant activity assays, with the highest reducing power measured by Folin-Ciocalteu (30.21 mg GAE/ g extract) and Ferricyanide/Prussian blue assay (lowest EC_{50} value=0.49 mg/mL), highest scavenging activity (lowest EC_{50} value=2.06 mg/mL) and highest lipid peroxidation inhibition

measured by β-carotene/linoleate (lowest EC_{50} value=3.81 mg/mL) and TBARS (lowest EC_{50} value=0.51 mg/mL) assays.

4. Discussion

As far as we know, there are no reports on nutritional characterization and antioxidants analysis of these eight wild edible species: *Amanita crocea*, *Amanita mairei*, *Boletus poliporus*, *Boletus regius*, *Gyromitra esculenta*, *Helvella lacunosa*, *Russula aurea* and *Russula virescens*. Some of these species have been studied for other purposes such as metals analysis in *Boletus regius* (Figueiredo, Soares, Baptista, Castro & Bastos, 2007) and *Russula virescens* (Chen, Zhou & Qiu, 2009; Busuioc, Elekes, Stihi, Lordache & Ciulei, 2011), protease isolation from *Helvella lacunosa* (Zhang, Wang, Zhang & Ng, 2010) and toxicity of raw *Gyromitra esculenta* (Toth, Patil, Pyysalo, Stessman & Gannett, 1992; Leathem & Dorran, 2007).

In a nutritional point of view, the studied mushrooms are rich in water, carbohydrates and proteins (in a dehydrated form), and present low content in fat. Mannitol and trehalose were the main sugars, being the first one (alcohol derivative of mannose) responsible for the support and expansion of mushroom fruiting bodies (Barros et al., 2008). Linoleic, oleic and palmitic acids were major fatty acids in the studied species, being the first one precursor of 1-octen-3-ol, known as "fungi alcohol", the main aromatic component in fungi (Maga, 1981). UFA's were higher than SFA levels. All these data are in agreement with other studies reporting nutrient analysis of different mushroom species from all over the world (Kalač, 2009; Ouzouni, Petridis, Koller & Riganakos, 2009), including from Northeast Portugal, one of the European regions with higher biodiversity in wild mushrooms, most of them with great gastronomic importance (Barros et al., 2008; Grangeia et al., 2011; Pereira et al., 2012).

In addition to being nutrient-rich foods and quite tasty, edible mushrooms have been known to be a source of bioactive products. The studied species revealed to possess powerful antioxidants such as tocopherols, lycopene, and phenolic compounds (mainly phenolic acids). Furthermore, some organic acids may have a protective role against various diseases due to their antioxidant activity (such as the case of tartaric, malic, citric or succinic acids), being able to chelate metals or to delocalize the electronic charge coming from free radicals (Seabra et al., 2006). Besides antioxidant properties, citric acid has been widely used as acidulant in pharmaceutical and food industries, due to its low toxicity (Anastassiadis, Morgunov, Kamzolova, & Finogenova, 2008).

All the mentioned molecules are certainly involved in the antioxidant properties observed for the different species, either as reducing power, scavenging effects or lipid peroxidation inhibition. Particularly, cinnamic acid (a compound found in interesting amounts in *Russula* species) was found to inhibit the growth of a tumour cell line (NCI-H460), and combined with protocatechuic and *p*-hydroxybenzoic acids provided a strongest decrease in the viable cell number, suggesting a possible concomitant effect of those compounds (Vaz et al., 2012).

Boletus regius was the species with the highest levels of carbohydrates and PUFA, bioactive compounds such as tocopherols, citric acid and phenolic compounds, including two possible chrysin derivatives, presenting also the highest antioxidant activity.

Overall, the bioactive compounds identified in the studied wild mushrooms could be extracted for the purpose of being used as nutraceuticals namely against chronic diseases related with oxidative stress. Being edible species, they can also be incorporated directly in diet acting as functional foods.

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Table 1. Information about the wild edible species analysed.

Scientific name	English name	Habitat	Ecology	Date of collection
Amanita crocea (Quél. in Bourd.) Singer ex Singer	Saffron Ingless Amanita	Mixed stands	Mycorrhizal	May 2011
Amanita mairei (Foley)	René Maire's Ringless Amanita	Quercus sp.	Mycorrhizal	June 2011
Boletus porosporus (Imler ex Bon & G. Moreno)	Sepia bolete	*Castanea sativa*	Mycorrhizal	September 2011
Boletus regius (Krombh.)	Butter Bolete	Quercus sp.	Mycorrhizal	May 2011
Gyromitra esculenta (Pers. ex Pers.) Fr.	False morel, Rain mushroom, Beefsteak morel	Pinus sp.	Mycorrhizal	April 2011
Helvella lacunosa (Afzel.)	Slate grey saddle, Fluted black elfin saddle	Mixed stands	Mycorrhizal	April 2011
Russula aurea Pers.	Gilded brittlegill	Quercus sp.	Mycorrhizal	May 2011
Russula virescens (Schaeff.) Fr.	Green-cracking Russula, Quilted green Russula, Green brittlegill	Quercus sp.	Mycorrhizal	June 2011

Table 2. Nutritional value and nutritional compounds of the wild edible mushrooms (mean ± SD).

	Amanita crocea	*Amanita mairei*	*Boletus poliporus*	*Boletus regius*	*Gyromitra esculenta*	*Helvella lacunosa*	*Russula aurea*	*Russula virescens*
Moisture (g/100 g fw)	89.04 ± 0.00 a	76.82 ± 8.50 ba	65.57 ± 7.94 b	79.15 ± 9.43 ba	85.68 ±8.39 ba	82.37 ±3.43 ba	79.99 ± 9.13 ba	92.49 ± 4.81 a
Ash (g/100 g dw)	25.73 ±1.75 b	11.21 ± 0.09 d	4.20 ± 0.05 e	4.40 ± 0.29 e	32.10 ±3.55 a	21.70 ± 1.10 c	12.75 ± 0.38 d	11.04 ± 0.19 d
Carbohydrates (g/100 g dw)	49.64 ± 1.34 e	62.75 ± 0.74 d	79.11 ± 1.82 b	88.79 ± 0.44 a	52.43 ± 2.22 e	71.50 ± 1.02 c	75.68 ± 0.79 b	62.27 ± 0.83 d
Proteins (g/100 g dw)	20.02 ±1.33 ba	17.74 ± 0.79 bc	15.74 ± 1.78 dc	5.22 ± 0.22 f	14.74 ±0.79 d	4.40 ± 0.36 f	10.33 ± 10.33 e	21.85 ± 0.79 a
Fat (g/100 g dw)	4.62 ± 0.16 b	8.30 ± 0.00 a	0.96 ± 0.06 g	1.59 ± 0.11 e	0.73 ± 0.01 h	2.40 ± 0.01 c	1.24 ± 0.02 f	1.85 ± 0.09 d
Energy (kcal/100 g dw)	320.19 ± 4.39 c	396.67 ± 0.26 a	388.00 ± 0.09 a	390.36 ± 0.42 a	275.23 ± 10.07 d	325.21 ± 3.05 c	355.18 ± 1.01 b	365.09 ± 0.87 b
Fructose (g/100 g dw)	nd	nd	19.94 ± 0.10 a	14.04 ± 0.72 b	nd	nd	nd	nd
Mannitol (g/100 g dw)	3.57 ± 0.33 e	1.47 ± 0.05 f	19.33 ± 0.90 a	6.25 ± 0.35 d	4.17 ± 0.21 e	4.13 ± 0.69 e	9.56 ± 0.14 c	10.90 ± 0.13 b
Trehalose (g/100 g dw)	4.54 ± 0.37 a	1.31 ± 0.05 c	1.99 ± 0.01 b	0.66 ± 0.03 d	1.96 ± 0.04 b	0.23 ± 0.07 e	2.29 ± 0.33 b	0.20 ± 0.01 e
Total Sugars (g/100 g dw)	8.11 ± 0.69 d	2.78 ± 0.00 g	41.26 ± 1.00 a	20.95 ± 1.04 b	6.13 ± 0.24 e	4.36 ± 0.62 f	11.85 ± 0.19 c	11.10 ± 0.13 c
C16:0	17.66 ± 1.21 c	18.08 ± 0.11 c	15.32 ± 0.36 d	15.94 ± 0.83 d	19.29 ± 0.16 b	29.05 ± 0.06 a	9.28 ± 0.14 e	17.31 ± 0.19 c
C18:0	3.68 ± 0.23 c	3.47 ± 0.02 c	2.93 ± 0.17 c	1.63 ± 0.03 d	1.64 ± 0.01 d	5.51 ± 0.03 b	5.29 ± 1.24 b	7.16 ± 0.12 a
C18:1n9	54.46 ± 0.65 a	53.02 ± 0.12 a	34.02 ± 0.06 d	21.84 ± 0.45 e	13.44 ± 0.06 f	43.82 ± 0.03 b	40.63 ± 2.42 c	40.27 ± 0.04 c
C18:2n6	20.14 ± 0.08 d	22.02 ± 0.04 d	41.90 ± 0.67 b	56.11 ± 0.60 a	55.30 ± 0.24 a	12.21 ± 0.14 e	40.32 ± 3.31 b	29.18 ± 0.04 c
SFA (relative percentage)	24.21 ± 0.59 d	23.64 ± 0.15 d	21.54 ± 0.58 e	19.50 ± 0.93 f	25.76 ± 0.21 c	39.87 ± 1.16 a	17.25 ± 1.02 g	28.78 ± 0.08 b
MUFA (relative percentage)	55.42 ± 0.65 a	53.94 ± 0.11 a	36.31 ± 0.06 d	23.96 ± 0.30 e	17.86 ± 0.01 f	46.44 ± 0.04 b	42.09 ± 2.45 c	41.51 ± 0.01 c
PUFA (relative percentage)	20.37 ± 0.06 d	22.42 ± 0.04 d	42.15 ± 0.64 b	56.55 ± 0.64 a	56.38 ± 0.22 a	13.70 ± 1.12 e	40.66 ± 3.46 b	29.71 ± 0.09 c

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

Table 3. Bioactive compounds of the wild edible mushrooms (mean ± SD).

	Amanita crocea	*Amanita mairei*	*Boletus poliporus*	*Boletus regius*	*Gyromitra esculenta*	*Helvella lacunosa*	*Russula aurea*	*Russula virescens*
α-tocopherol	30.57 ± 5.65 b	21.40 ± 4.23 b	4.99±0.79 b	360.73 ± 14.52 a	2.19 ± 0.35 b	1.38 ± 0.51 b	7. 39 ± 0.51 b	20.00 ± 0.31 b
β-tocopherol	nd	nd	nd	nd	nd	nd	nd	21.30 ± 2.43 a
γ-tocopherol	131.62±8.16 b	37.19 ± 2.55c	23.05 ± 2.16 c	403.07 ± 4.98 a	11.51±2.11c	nd	15.39 ± 1.42 c	8.00 ± 0.83 c
δ-tocopherol	nd	nd	nd	nd	99.07±10.68 a	15.09 ± 3.15 b	nd	nd
Total tocopherols (μg/100 g dw)	162.19±2.51 b	58.59 ± 1.68 cd	28.04 ± 2.96 d	763.80 ± 19.50 a	112.83 ± 8.92 cb	16.47 ± 2.64d	22.78 ± 2.27d	49.30 ± 1.91 cd
β-carotene (mg/100 g dw)	nd	nd	nd	nd	nd	nd	nd	nd
Lycopene (mg/100 g dw)	0.23 ± 0.00 c	0.11 ± 0.00 f	0.04 ± 0.00 g	0.17 ± 0.00 e	0.43 ± 0.00 b	0.53 ± 0.01 a	0.05 ± 0.00 g	0.19 ± 0.00 d
Oxalic acid	0.28 ± 0.01 d	0.26 ± 0.00 d	0.34 ± 0.03 d	0.17 ± 0.03 ef	0.13 ± 0.03 f	2.98 ± 0.11 a	1.09 ± 0.05 b	0.78 ± 0.00 c
Quinic acid	0.23 ± 0.01 c	nd	1.93 ± 0.16 a	0.18 ± 0.02 c	1.43 ± 0.20 b	0.24 ± 0.04 c	nd	nd
Malic acid	0.94 ± 0.00 d	nd	nd	nd	0.69 ± 0.10 d	1.96 ± 0.39 c	4.53 ± 0.75 a	2.71 ± 0.04 b
Citric acid	2.07 ± 0.21 c	2.63 ± 0.19 b	0.21 ± 0.02 f	3.32 ± 0.23 a	1.46 ± 0.02 d	1.42 ± 0.13 d	1.20 ± 0.08 d	0.55 ± 0.00 e
Fumaric acid	0.33 ± 0.01 b	0.30 ± 0.02 c	0.07 ± 0.00 e	0.07 ± 0.00 e	0.36 ± 0.03 ba	0.33 ± 0.02 b	0.38 ± 0.01 a	0.23 ± 0.01 d
Total organic acids (g/100 g dw)	3.86 ± 0.21 cb	3.19 ± 0.19 cd	2.56 ± 0.16 d	3.74 ± 0.23 cb	4.06 ± 0.37 b	6.93 ± 0.36 a	7.19 ± 0.71 a	4.26 ± 0.03 b
Protocatechuic acid	21.33±1.46 a	nd	nd	1.15±0.18 c	3.74±0.26 b	0.77±0.00 cd	nd	nd
p-hydroxybenzoic acid	0.74±0.08 c	5.94±0.65 b	0.31±0.05 c	1.77±0.23 c	nd	0.13±0.01 c	1.02±0.23 c	22.59±4.24 a
p-coumaric acid	nd	nd	nd	2.08±0.27 a	nd	0.17±0.00 b	nd	nd
Chrysin derivative 1	nd	nd	nd	11.16±0.54	nd	nd	nd	nd
Chrysin derivative 2	nd	nd	nd	7.33±0.06	nd	nd	nd	nd
Total phenolic compounds (mg/100 g dw)	22.07±1.54 a	5.94±0.65 b	0.31±0.05 d	23.49±0.38 a	3.74±0.26 bc	1.07±0.00 cd	1.02±0.26 cd	22.59±4.24 a
Cinnamic acid (mg/100 g dw)	tr	6.87±0.03 c	Tr	6.05±0.57 d	tr	nd	17.15±0.33 a	15.75±0.54 b

nd- not detected; tr- traces. In each row different letters mean significant differences between species (p<0.05).

Table 4. *In vitro* antioxidant properties of the wild edible mushrooms (mean ± SD).

		Amanita crocea	*Amanita mairei*	*Boletus poliporus*	*Boletus regius*	*Gyromitra esculenta*	*Helvella lacunosa*	*Russula aurea*	*Russula virescens*
Reducing Power	Folin-Ciocalteu assay (mg GAE/g extract)	22.27 ± 0.38 c	8.94 ± 0.15 e	20.15 ± 1.68 c	30.21 ± 1.45 a	27.16 ± 5.29 b	13.66 ± 0.84 d	12.23 ± 0.21 d	14.05 ± 0.27 d
	Ferricyanide/Prussian blue assay (EC_{50}; mg/mL)	1.08 ± 0.23 ef	2.00 ± 0.02 ed	1.58 ± 0.01 e	0.49 ± 0.00 f	6.82 ± 0.74 c	17.60 ± 2.09 a	2.91 ± 0.16 d	8.24 ± 0.06 b
Scavenging activity	DPPH scavenging activity (EC_{50}; mg/mL)	7.94 ± 0.08 f	13.81± 0.18 c	6.97 ± 0.25 f	2.06 ± 0.07 g	12.66 ± 0.22 d	26.92 ± 1.37 b	11.34 ± 0.22 e	30.21 ± 1.36 a
Lipid peroxidation inhibition	β-carotene/linoleate (EC_{50}; mg/mL)	50.44 ± 7.65 a	14.10 ± 1.50 b	17.08 ± 0.97 b	3.81± 0.32 d	9.15 ± 0.67 c	6.53 ± 0.38 dc	9.70 ± 0.24 c	4.28 ± 0.42 d
	TBARS assay (EC_{50}; mg/mL)	1.44 ± 0.48 dc	0.66 ± 0.10 d	5.49 ± 2.80 a	0.51 ± 0.01 d	3.95 ± 0.72 ba	1.13 ± 0.31 d	2.98 ± 0.25b c	0.23 ± 0.03 d

In each row different letters mean significant differences between species (p<0.05). Concerning the Folin-Ciocalteu assay, higher values mean higher reducing power; for the other assays, the results are presented in EC_{50} values, what means that higher values correspond to lower reducing power or antioxidant potential. EC_{50}: Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance for the Ferricyanide/Prussian blue assay.