Tocopherols composition of Portuguese wild mushrooms with antioxidant capacity

Sandrina A. Heleno[a], Lillian Barros[a], Maria João Sousa[b], Anabela Martins[b], Isabel C.F.R. Ferreira[a,*]

[a]Mountain Research Centre (CIMO), Instituto Politécnico de Bragança, *Campus* de Santa Apolónia, Apartado 1172, 5301-855 Bragança, Portugal.

[b]Escola Superior Agrária, Instituto Politécnico de Bragança, *Campus* de Santa Apolónia, Apartado 1172, 5301-855 Bragança, Portugal.

* Author to whom correspondence should be addressed (e-mail: iferreira@ipb.pt telephone +351-273-303219; fax +351-273-325405).

ABSTRACT

The antioxidant composition and properties of eighteen Portuguese wild mushrooms (*Clitocybe alexandri*, *Cortinarius glaucopus*, *Fistulina hepatica*, *Hydnum repandum*, *Hygrophoropsis aurantiaca*, *Hypholoma capnoides*, *Laccaria amethystina*, *Laccaria laccata*, *Lactarius aurantiacus*, *Lactarius salmonicolor*, *Lepista inversa*, *Lepista sordida*, *Mycena rosea*, *Russula delica*, *Russula vesca*, *Suillus collinitus*, *Suillus mediterraneensis*, *Tricholoma sulphureum*) were evaluated, in order to contribute to the overall characterization of these products. Their radical scavenging capacity, reducing power and inhibition of lipid peroxidation measured in liposome solutions was fully studied. Furthermore, the tocopherols composition was determined by HPLC-fluorescence. The analysed mushrooms contain powerful antioxidants such as phenols (0.51-7.90 mg/g) and tocopherols (0.02-8.04 μg/g). β-Tocopherol was the vitamer detected in higher amounts, while δ-tocopherol was not detected in the majority of the samples. All the species proved to have antioxidant activity being more significant for *Hygrophoropsis aurantiaca* (EC_{50} values lower than 1.35 mg/ml) due to the contribution of antioxidants such as phenols (7.90 mg/g) and tocopherols (0.02-1.94 μg/g). The ongoing research states the nutraceutical potential of all these unique species, making the information available for a better management and conservation of mushrooms and related habitats.

Keywords: Wild mushrooms; Tocopherols; HPLC; Antioxidant activity.

1. Introduction

Reactive oxygen and nitrogen species (ROS and RNS) seem to be implicated in the oxidative deterioration of food products as well as in the pathogenesis of several human diseases such as atherosclerosis, diabetes mellitus, chronic inflammation, neurodegenerative disorders and certain types of cancer (Halliwell, 1996). There is a great interest in finding natural antioxidants for use in food to retard lipid oxidative rancidity or in pharmaceutical applications for chronic diseases related to the production of free radicals (Prior, 2003). Macrofungi (mushrooms) are rich sources of antioxidant compounds such as phenolic compounds (phenolic acids and flavonoids) and tocopherols (Ferreira, Barros, & Abreu, 2009). In the last years nineteen different mushroom species from the Northeast Portugal, one of the European regions with higher wild mushrooms diversity, were evaluated for their antioxidant properties, in order to valorise them as a source of nutraceuticals (Barros, Correia, Ferreira, Baptista, & Santos-Buelga, 2008; Barros, Venturini, Baptista, Estevinho, & Ferreira, 2008; Ferreira et al., 2009). Nevertheless, assuming that the proportion of mushrooms used among the undiscovered and unexamined ones is only 5%, there are thousands of macrofungi species potentially beneficial for mankind. Even among the already known species the proportion of well investigated mushrooms is very low. Therefore, our research group intends to go on studying this matrix, documenting the nutraceutical potential of all unique species and making the information available for a better management and conservation of this natural resource and related habitats. Particularly, data on *Clitocybe alexandri*, *Cortinarius glaucopus*, *Hydnum repandum*, *Hygrophoropsis aurantiaca*, *Hypholoma capnoides*, *Laccaria amethystina*, *Laccaria laccata*, *Lactarius aurantiacus*, *Lactarius salmonicolor*, *Lepista inversa*, *Lepista sordida*, *Mycena rosea*, *Russula vesca*, *Suillus collinitus*, *Suillus mediterraneensis* and

62 *Tricholoma sulphureum* have not yet been reported. Tocopherol compositions of
63 *Russula delica* from Turkey and *Hydnum repandum* from Spain were studied but there
64 are no studies on Portuguese samples.
65 Vitamin E is a term frequently used to designate a family of chemically related
66 compounds, namely tocopherols and tocotrienols, which share a common structure
67 having a chromanol head and isoprene side chain. Due to its role as a scavenger of free
68 radicals, vitamin E is also believed to protect our bodies against degenerative
69 malfunctions, mainly cancer and cardiovascular diseases (Burton & Traber, 1990;
70 Kamal-Eldin & Appelqvist, 1996). In the past α-tocopherol was considered the most
71 active form of vitamin E in humans and it was reported to exhibit the highest biological
72 activity. However, many recent publications have been focused on the health effects of
73 the other vitamin E isoforms (Traber, 1999; Schwenke, 2002).
74 Some reports have been published on mushrooms tocopherols contents (Mau, Chao, &
75 Wu, 2001; Mau, Lin, & Chen, 2002; Mau, Lin, & Song, 2002; Yang, Lin, & Mau, 2002;
76 Mau, Chang, Huang, & Chen, 2004; Elmastas, Isildak, Turkekul, & Temur, 2007; Lee,
77 Kim, Jang, Jung, & Yun, 2007; Tsai, Tsai, & Mau, 2007; Barros, Correia et al., 2008;
78 Barros, Venturini et al., 2008; Lee, Jian, Lian, & Mau, 2008; Jayakumar, Thomas, &
79 Geraldine, 2009). They all described the same methodology including saponification in
80 the extraction process and analysis by HPLC coupled to UV detector. Our research
81 group (Barros, Correia et al., 2008) was the only describing an extraction process
82 without saponification, by adding an antioxidant to avoid tocopherols oxidation, while
83 protecting the samples from light and heat, followed by HPLC-fluorescence analysis.
84 Using this process, α-, β-, δ- and γ-tocopherols were identified and quantified in wild
85 mushrooms, while tocotrienols were not detected in any of the cited studies.
86

2. Materials and methods

2.1. Mushroom species

Samples of *Clitocybe alexandri* (Gillet) Konrad, *Cortinarius glaucopus* (Schaeff), *Fistulina hepatica* (Schaeff.: Fr.), *Hydnum repandum* (L.: Fr.), *Hygrophoropsis aurantiaca* (Wulf.: Fr.) Mre., *Hypholoma capnoides* (Fr.) Quel., *Laccaria amethystina* (Bolt. ex Fr.) R.Maire, *Laccaria laccata* (scop.: Fr.) Berk. & Broome, *Lactarius aurantiacus* (Fr.), *Lactarius salmonicolor* (Heim y Leclair), *Lepista inversa* (Scop.: Fr.) Pat., *Lepista sordida* (Fr.) Singer, *Mycena rosea* (Schumach.) Gramberg, *Russula delica* (Fr.), *Russula vesca* (Fr.), *Suillus collinitus* (Fr.) Kuntz, *Suillus mediterraneensis* (Jacquetant & Blum) Redeuilh, *Tricholoma sulphureum (Bull.: Fr.) Kumm.* were collected under *Quercus pyrenaica* Willd. and mixed stands of *Quercus sp. and Pinus sylvestris* Ait., in Bragança (Northeast Portugal), in autumn 2008. *Mycena rosea* and *Tricholoma sulphureum* are not edible species and *Cortinarius glaucopus* is a species of unknown edibility. Taxonomic identification of sporocarps was made according to several authors (Marchand, 1971-1986; Moser, 1983; Breitenbach & Kränzlin, 1984-2000); Alessio, 1985; Bon, 1988; Noordeloos (1988-2001); Candusso & Lanzoni, 1990; Courtecuisse, 1999; Courtecuisse & Duhem, 2005) 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 the samples were lyophilised (Ly-8-FM-ULE, Snijders) and reduced to a fine dried powder (20 mesh).

2.2. Standards and reagents

The eluents n-hexane 95% and ethyl acetate 99.98% were of HPLC grade from Lab-Scan (Lisbon, Portugal). Methanol was of analytical grade purity and supplied by

Pronalab (Lisbon, Portugal). Tocopherol standards (α, β, γ and δ), and the standards used in the antioxidant activity assays: BHA (butylhydroxyanisole**),** TBHQ (tert-butylhydroquinone), L-ascorbic acid, α-Tocopherol and gallic acid were purchased from Sigma (St. Louis, MO, USA). Racemic tocol, 50 mg/ml, was purchased from Matreya (PA, USA). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Determination of phenols and tocopherols

For phenols content determination, the sample (1 ml) was mixed with Folin-Ciocalteu phenol reagent (1 ml). After 3 min, saturated sodium carbonate solution (1 ml) was added to the mixture and adjusted to 10 ml with distilled water. The reaction was kept in the dark for 90 min (following Barros, Venturini et al., 2008, after which the absorbance was read at 725 nm (Analytikijena 200-2004 spectrophotometer). Gallic acid was used to calculate the standard curve of absorbance vs. concentration (10^{-5}-4×10^{-4} mol·l^{-1}; Y = 2.8557X–0.0021; R^2 = 0.9999) and the results were expressed as mg of gallic acid equivalents (GAEs) per g of extract.

Tocopherols content was determined following a procedure previously optimized and described by Barros, Correia et al. (2008). BHT (butylhydroxytoluene) solution in hexane (10 mg/ml; 100 µl) and internal standard (IS) solution in hexane (tocol; 2.0 µg/ml; 250 µl) were added to the sample prior to the extraction procedure. The 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. After that, saturated NaCl aqueous solution (2 ml) was added, the mixture was homogenized (1

138 min), centrifuged (Centurion K24OR- 2003 refrigerated centrifuge, 5 min, 6185 rpm)
139 and the clear upper layer was carefully transferred to a vial. The sample was re-
140 extracted twice with hexane. The combined extracts were taken to dryness under a
141 nitrogen stream, redissolved in 1 ml of n-hexane, dehydrated with anhydrous sodium
142 sulphate, filtered through a 0.22 µm disposable LC filter disk, transferred into a dark
143 injection vial and analysed by HPLC. The HPLC equipment consisted of an integrated
144 system with a Smartline pump 1000 (Knauer, Germany), a degasser system Smartline
145 manager 5000, an AS-2057 auto-sampler and a 2500 UV detector at 295 nm (Knauer,
146 Germany) connected in series with a FP-2020 fluorescence detector (Jasco, Japan)
147 programmed for excitation at 290 nm and emission at 330 nm. Data were analysed
148 using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved
149 with a Polyamide II (250 × 4.6 mm) normal-phase column from YMC Waters (Japan)
150 operating at 30 ºC (7971 R Grace oven). The mobile phase used was a mixture of n-
151 hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 ml/min, and the injection
152 volume was 20 µl. The compounds were identified by chromatographic comparisons
153 with authentic standards. Quantification was based on the fluorescence signal response,
154 using the internal standard method. Tocopherol contents in mushroom samples are
155 expressed in µg per g of dry mushroom.
156
157 *2.4. Antioxidant activity.*
158 The samples (1.5 g) were extracted by stirring with 40 ml of methanol (25 ºC at 150
159 rpm) for 12 h and subsequently filtered through Whatman No. 4 paper. The residue was
160 then extracted with 20 ml of methanol (25 ºC at 150 rpm) for 4 h. The combined
161 methanolic extracts were evaporated at 40 ºC to dryness and redissolved in methanol at
162 a concentration of 50 mg/ml, and stored at 4 ºC until analysis.

Chemical assays already described by the authors in previous studies (Barros, Venturini et al., 2008), were applied to evaluate the antioxidant activity of all samples.

DPPH radical-scavenging activity. Various concentrations of the extracts (0.3 ml) were mixed with 2.7 ml of a methanolic solution of DPPH radicals (6×10^{-5} mol/l). The mixture was shaken vigorously and left to stand in the dark for 60 min (until stable absorption values were obtained). The reduction of the DPPH radical was determined by measuring the absorption at 517 nm. The 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 and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% of radicals scavenging activity (EC_{50}) was calculated by interpolation from the graph of RSA percentage against extract concentration. BHA and α-tocopherol were used as standards.

Reducing power. Various concentrations of the extracts (2.5 ml) were mixed with sodium phosphate buffer (2.5 ml of 0.2 mol·l^{-1}, pH 6.6) and potassium ferricyanide (1% w/v, 2.5 ml). The mixture was incubated at 50 ºC for 20 min. After addition of tricloroacetic acid (10%, 2.5 ml) the mixture was centrifuged at 1000 rpm for 8 min. The upper layer (5 ml) was mixed with deionised water (5 ml) and ferric chloride (0.1%, 1 ml), and the absorbance was measured spectrophotometrically at 700 nm: higher absorbances indicate higher reducing powers. BHA and α-tocopherol were used as standards.

Inhibition of β-carotene bleaching. The antioxidant activity of the extracts was evaluated by the β-carotene linoleate model system. A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 ml). Two millilitres of this solution were added to a 100 ml round-bottom flask. After the chloroform was removed

188 at 40 ºC under vacuum, linoleic acid (40 mg), Tween® 80 emulsifier (400 mg), and
189 distilled water (100 ml) were added to the flask and vigorously shacked. Aliquots (4.8
190 ml) of this emulsion were transferred into different test tubes containing different
191 concentrations (0.2 ml) of the extracts. The tubes were shaken and incubated at 50 ºC in
192 a water bath. As soon as the emulsion was added to each tube, the zero time absorbance
193 at 470 nm was measured. Absorbance readings were then recorded at 20 min intervals
194 until the control sample changed colour. A blank, devoid of β-carotene, was used for
195 background subtraction. Lipid peroxidation inhibition was calculated using the
196 following equation: (β-carotene content after 2 h of assay)/(initial β-carotene content) ×
197 100. The extract concentration providing 50% antioxidant activity (EC_{50}) was calculated
198 by interpolation from the graph of antioxidant activity percentage against extract
199 concentration. TBHQ was used as standard.
200
201 *2.5. Statistical analysis*
202 For each one of the mushroom species three samples were analysed and also all the
203 assays were carried out in triplicate. The results are expressed as mean values and
204 standard deviation (SD) or standard errors (SE). The results were analyzed using one-
205 way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This
206 treatment was carried out using SPSS v.16.0 software. The ANOVA results were
207 classified using letters (different letters mean significant differences among results). The
208 letters are alphabetically ordered according to the decrease of the results values (e.g.
209 letter "a" represents the best result for phenols contents and extraction yields, and the
210 worst results for antioxidant activity assays).
211

3. Results and discussion

Natural phenolic compounds accumulate as end-products from the shikimate and acetate pathways and can range from relatively simple molecules (phenolic acids, phenylpropanoids, flavonoids) to highly polymerised compounds (lignins, melanins, tannins), with flavonoids representing the most common and widely distributed sub-group (Bravo, 1998). The method of Folin-Ciocalteu's is used to evaluate total phenols despite all the interferences of this assay since the reagent (a mixture of phosphotungstic acid and phosphomolybdic acid) also reacts with other non-phenolic reducing compounds (ascorbic acid and other reducing substances such as some sugars and amino acids) leading to an overvaluation of the phenolic content (George, Brat, Alter, & Amiot, 2005). In fact, there are a large number of studies reporting determination of total phenols in mushrooms by Folin Ciocalteu's assay (Ferreira et al., 2009 and references cited).

The role of phenolic antioxidants (ArOH) is to interrupt the chain reaction according to:

$$RO_2^{\bullet} + ArOH \longrightarrow ROOH + ArO^{\bullet}$$

To be effective $ArO^{\bullet}$ must be a relatively stable free radical, so that it reacts slowly with substrate RH but rapidly with $RO_2^{\bullet}$, hence the term "chain-breaking antioxidant" (Wright, Johnson, & DiLabio, 2001). Apart from their direct antioxidant properties, phenolic compounds can exert other activities that may not be related to their radical scavenging capacity. These activities, mostly mediated by receptor-ligand interactions, include antiproliferation, cell cycle regulation, and induction of apoptosis. It is becoming evident that the activity of phenolic compounds as preventive agents must be evaluated from different angles to cover not only their antioxidant capacity but also the

236 influence of the physicochemical environment on the antioxidant effectiveness and the
237 occurrence of other biological activities (Faria, Calhau, Freitas, & Mateus, 2006).
238 Plants are among the most important sources of phenolic compounds, providing high
239 quantities of these phytochemicals, but mushrooms should also be considered as
240 possible sources. In the present report we conclude that *Hygrophoropsis aurantiaca* and
241 *Suillus mediterraneensis* presented the highest phenols contents (higher than 7 mg/g),
242 while *Lactarius aurantiacus* and *Hydnum repandum* revealed the lowest levels (lower
243 than 0.6 mg/g) (**Table 1**). Regarding phenols contents, the ANOVA analysis indicated
244 high heterogeneity among the different mushroom species. In fact, the phenolic
245 composition in mushrooms might be affected by a number of factors, namely mushroom
246 strain/species, composition of growth media (for in vitro cultured species), time of
247 harvest, management techniques, handling conditions, and preparation of the substrates
248 (in case of cultivated species) and soil/substrate composition or host associated species
249 (in case of wild species either saprotrophic or mycorrhizal). All these factors might have
250 influence in the secondary metabolism of fungi, including shikimate and acetate
251 pathways, and therefore in their phenols production. The higher levels of phenols found
252 in *H. aurantiaca*, *S. mediterraneensis* and *R. vesca* could be related to higher stress
253 conditions in their growth that could stimulate the secondary metabolism.
254 It was described that the main phenolic compounds found in mushrooms are phenolic
255 acids (Ferreira et al., 2009). Particularly, the presence of *p*-coumaric, caffeic and ellagic
256 acids in *Fistulina hepatica* was already reported in literature, as also the presence of
257 protocatechuic, gallic, gentisic, vanillic, syringic, cinamic and tanic acids in *Hydnum*
258 *repandum*.
259 Vitamin E reacts with peroxyl radicals produced from polyunsaturated fatty acids in
260 membrane phospholipids or lipoproteins to yield a stable lipid hydroperoxide. They act

as antioxidants by donating a hydrogen atom to peroxyl radicals of unsaturated lipid molecules, forming a hydroperoxide and a tocopheroxyl radical, which reacts with other peroxyl or tocopheroxyl radicals forming more stable adducts (Lampi, Kataja, Kamal-Eldin, & Vieno, 1999).

$$LH + \text{Oxidant initiator} \longrightarrow L^{\bullet}$$

$$L^{\bullet} + O_2 \longrightarrow LOO^{\bullet}$$

$$LOO^{\bullet} + \text{Tocopherol} \longrightarrow LOOH + \text{Tocopherol}^{\bullet}$$

In the literature, there are studies on tocopherols composition of mushrooms from India: *Cantharelus cibarius* and *Pleurotus ostreatus* (Jayakumar et al., 2009), Taiwan: *Agaricus blazei, Agrocybe cylindracea, Auricularia mesenterica, Auricularia fuscosuccinea* (brown), *Auricularia fuscosuccinea (white), Auricularia polytricha, Boletus edulis, Ganoderma lucidum, Ganoderma tsugae, Grifola frondosa, Hericium erinaceus, Hypsizigus marmoreus, Lentinula edodes, Morchella esculenta, Pleurotus cystidiosus, Pleurotus ostreatus, Termitomyces albuminosus, Tremella fuciformis* and *Trichloma giganteum* (Mau et al., 2001; Mau, Lin, & Chen, 2002; Mau, Lin, & Song, 2002; Yang et al., 2002; Mau et al., 2004; Tsai et al., 2007) and Turkey: *Agaricus bisporus, Boletus badius, Lepista nuda, Pleurotus ostreatus, Polyporus squamosus, Russula delica* and *Verpa conica* (Elmastas et al., 2007). The reports on Portuguese mushrooms (*Agaricus arvensis, Agaricus bisporus, Agaricus romagnesii, Agaricus silvaticus, Agaricus silvicola, Boletus edulis, Calocybe gambosa, Cantharelus cibarius, Craterellus cornucopioides, Hypholoma fasciculare, Lepista nuda, Lycoperdon molle, Lycoperdon perlatum, Marasmius oreades, Ramaria botrytis* and *Tricholoma acerbum*) are from our research group (Ferreira et al., 2009).

Table 2 presents the tocopherols composition of eighteen different Portuguese mushrooms. *Lepista inversa* revealed the highest content of α-tocopherol (0.28 μg/g). β-

Tocopherol was the most abundant vitamer in the analysed species, and the highest contents were obtained for *Laccaria laccata* (7.06 μg/g). *Clitocybe alexandri* revealed the highest content of γ-tocopherol (1.34 μg/g). δ-Tocopherol was only found in three species (*Lepista inversa, Hygrophoropsis aurantiaca* and *Laccaria laccata*). Up to now, this vitamer had only been found in species from Taiwan, but not in Portuguese, Indian or Turkish species (Ferreira et al., 2009). In general, *Laccaria laccata* presented the highest concentration of total tocopherols (8.04 μg/g). The presence of α-tocopherol in a sample of *Russula delica* from Turkey was already reported (Elmastas et al., 2007), but in the present study the four vitamers (α, β, γ and δ-tocopherols) were detected and quantified.

Since there is no universal method that can measure the antioxidant capacity accurately and quantitatively for all samples, the antioxidant properties of eighteen different mushrooms species were evaluated through different assays: scavenging activity on DPPH radicals, reducing power and lipid peroxidation inhibition by β-carotene-linoleate system**.** The radical scavenging effects of the samples were examined by the capacity to decrease the absorbance at 517 nm of DPPH solution (**Figure 2**). The reducing power was evaluated measuring the conversion of a Fe^{3+}/ferricyanide complex to the ferrous form by the samples (**Figure 3**). The lipid peroxidation inhibition was measured by the inhibition of β-carotene bleaching, by neutralizing the linoleate-free radical and other free radicals formed in the system which attack the highly unsaturated β-carotene models (**Figure 4**). From the analysis of **Figures 2-4** we can conclude that the antioxidant effects increase with the concentration, being different for each mushroom sample.

For an overview of the results, **table 3** presents the EC_{50} values (mg/ml) obtained for the radical scavenging activity and lipid peroxidation inhibition assays. The results are

given per mg of extracts, however, when the extraction yields are considered (**Table 1**), and the results expressed per mg of mushroom, the activities would be different. Nevertheless, in the present study we opted to compare the results per mg of extracts since not all the mushrooms are edible or traditionally consumed.

Hygrophoropsis aurantiaca, *Suillus mediterraneensis*, *Russula vesca* and *Tricholoma sulphureum* presented radical scavenging effects higher than 85% at 20 mg/ml (**Figure 2**) with EC_{50} values lower than 5 mg/ml (**Table 3**). *Russula delica*, *Hypholoma capnoides*, *Laccaria laccata*, *Clitocybe alexandri*, *Lactarius aurantiacus* and *Hydnum repandum* presented high EC_{50} values (higher than 20 mg/ml) with RSA percentages lower than 48% at 20 mg/ml. *Hygrophoropsis aurantiaca*, *Suillus mediterraneensis* and *Russula vesca* were the species with higher reducing power (absorbance higher than 2.5 at 10 mg/ml; **Figure 3**). *Clitocybe alexandri*, *Lactarius aurantiacus* and *Hydnum repandum* presented the lowest reducing powers with absorbances lower than 0.7 at 10 mg/ml. Concerning the lipid peroxidation inhibition, *Hygrophoropsis aurantiaca*, *Suillus mediterraneensis*, *Russula vesca*, *Tricholoma sulphureum* and *Fistulina hepatica* were the species with higher capacity (higher than 73% at 20 mg/ml; **Figure 4**) with EC_{50} values lower than 1 mg/ml (**Table 3**). *Hydnum repandum* presented the highest EC_{50} value (29 mg/ml) with 33% of lipid peroxidation inhibition capacity at 20 mg/ml. The antioxidant properties are in agreement with the phenols contents found in the mushrooms species (**Table 1**). *Hygrophoropsis aurantiaca* was the most promissory species presenting the highest levels of phenols and the best antioxidant activity (lowest EC_{50} values). It also has all the tocopherol isoforms (**Figure 1**). *Hydnum repandum* presented the lowest phenols levels and highest EC_{50} values.

From the eighteen species only two had been previously evaluated for their antioxidant properties: *Hydnum repandum* and *Russula delica*. Nevertheless, those species were not

337 from Portugal, but from Spain (Murcia, Martinez-Tome, Jimenez, Vera, Honrubia, &
338 Parras, 2002) and Turkey (Elmastas et al., 2007), respectively.
339 Overall, this kind of studies is important to increase the proportion of investigated
340 mushrooms and contributes to the establishment of the nutraceutical potential of wild
341 mushrooms, which proved to have good radical scavenging properties and lipid
342 peroxidation inhibition capacity. The studied samples are sources of powerful
343 antioxidants such as phenols and tocopherols, which could be used against diseases
344 related to oxidative stress, dermatological applications, cosmetics, as also as
345 supplements in food industry.
346

347 **Acknowledgements**

348 The authors are grateful to Foundation for Science and Technology
349 (PPCDT/AGR/56661/2004 project and BPD/4609/2008 grant) for financial support of
350 this work.
351

352 **References**

353 Alessio, C.L. (1985). *Boletus Dill. ex L. Fungi Europaei*. Vol. 2. Libreria Editrice
354 Giovanna Biella, Saronno.
355 Barros, L., Correia, D.M., Ferreira, I.C.F.R., Baptista, P., & Santos-Buelga, C. (2008).
356 Optimization of the determination of tocopherols in *Agaricus* sp. edible mushrooms by a
357 normal phase liquid chromatographic method. *Food Chemistry*, *110*, 1046-1050.
358 Barros, L., Venturini, B.A., Baptista, P., Estevinho, L.M., & Ferreira, I.C.F.R. (2008).
359 Chemical Composition and Biological Properties of Portuguese Wild Mushrooms: A
360 comprehensive study. *Journal of Agricultural and Food Chemistry*, *56*, 3856-3862.
361 Bon, M. (1988). *Guia de campo de los hongos de Europa*. Ediciones Omega, Barcelona.

362 Bravo, L. (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional
363 significance. *Nutrition Reviews, 56*, 317-333.
364 Breitenbach, J., & Kränzlin, F. (1984-2000). *Champignons de Suisse.* Vols. 1-5.
365 Mykologia Lucern, Lucern.
366 Burton, G.W., & Traber, M.G. (1990). Vitamin E: antioxidant activity, biokinetics, and
367 bioavailability. *Annual Review of Nutrition*, *10*, 357-382.
368 Candusso, M., Lanzoni, G. (1990). *Lepiota s.l. Fungi Europaei*. Libreria Editrice
369 Giovanna Biella, Saronno.
370 Courtecuisse, R. (1999). *Mushrooms of Britain and Europe*. HarperCollins Publishers,
371 London.
372 Courtecuisse, R., & Duhem, B. (2005). *Guía de los hongos de la Península Ibérica*,
373 Europa y Norte de África. Ediciones Omega, Barcelona.
374 Elmastas, M., Isildak, O., Turkekul, I., & Temur, N. (2007). Determination of
375 antioxidant activity and antioxidant compounds in wild edible mushrooms. *Journal of*
376 *Food Composition and Analysis*, *20*, 337-345.
377 Faria, A., Calhau, C., Freitas, V., & Mateus, N. (2006). Procyanidins as antioxidants and
378 tumor cell growth modulators. *Journal of Agricultural and Food Chemistry*, *54*, 2392-
379 2397.
380 Ferreira, I.C.F.R., Barros, L., & Abreu, R.M.V. (2009). Antioxidants in wild
381 mushrooms. *Current Medicinal Chemistry*, *16*, 1543-1560.
382 George, S., Brat, P., Alter, P., & Amiot, M.J. (2005). Rapid determination of
383 polyphenols and vitamin C in plant-derived products. *Journal of Agricultural and Food*
384 *Chemistry*, *53*, 1370-1373.
385 Halliwell, B. (1996). Antioxidants in human health and disease. *Annual Review of*
386 *Nutrition*, *16*, 33-50.

Jayakumar, T., Thomas, P.A., & Geraldine, P. (2009). In-vitro antioxidant activities of an ethanolic extract of the oyster mushroom, *Pleurotus ostreatus*. *Innovative Food Science & Emerging Technologies, 10,* 228-234.

Kamal-Eldin, A., & Appelqvist, L.A. (1996). The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids, 31*, 671-701.

Lampi, A.M., Kataja, L., Kamal-Eldin, A., & Vieno, P. (1999). Antioxidant activities of alpha- and gamma-tocopherols in the oxidation of rapeseed oil triacylglycerols. *Journal of the American Oil Chemists' Society*, *76*, 749-755.

Lee, Y.-L., Jian, S.-Y., Lian, P.-Y., & Mau, J.-L. (2008). Antioxidant properties of extracts from a white mutant of the mushroom *Hypsizigus marmoreus*. *Journal of Food Composition and Analysis, 21*, 116-124.

Lee, I.K., Kim, Y.S., Jang, Y.W., Jung, J.Y., & Yun, B.S. (2007). Antioxidant properties of various extracts from *Hypsizigus marmoreus*. *Food Chemistry*, *104*, 1-9.

Marchand, A. (1971-1986). *Champignons du Nord et du Midi*. Vols.1-9. Société Mycologique des Pyrénées Méditerranéennes, Perpignan.

Mau, J.-L., Chang, C.-N., Huang, S.-J., & Chen, C.-C. (2004). Antioxidant properties of methanolic extracts from *Grifola frondosa*, *Morchella esculenta* and *Termitomyces albuminosus* mycelia. *Food Chemistry*, *87*, 111-118.

Mau, J.L., Chao, G.R., & Wu, K.T. (2001). Antioxidant properties of methanolic extracts from several ear mushrooms. *Journal of Agricultural and Food Chemistry*, *49*, 5461-5467.

Mau, J.L., Lin, H.C., & Chen, C.C. (2002). Antioxidant properties of several medicinal mushrooms. *Journal of Agricultural and Food Chemistry*, *50*, 6072-6077.

Mau, J.-L., Lin, H.-C., & Song, S.-F. (2002). Antioxidant properties of several specialty mushrooms. *Food Research International*, *35*, 519-526.

412 Moser, M. (1983). *Keys to Agarics and Boleti (Polyporales, Boletales, Agaricales,*
413 *Russulales)*. Mad River Pr Inc, London.
414 Murcia, M.A., Martinez-Tome, M., Jimenez, A.M., Vera, A.M., Honrubia, M., & Parras,
415 P. (2002). Antioxidant activity of edible fungi (truffles and mushrooms): losses during
416 industrial processing. *Food Protection, 65*, 1614-1622.
417 Noordeloos, M.E. (1988-2001). *Flora Agaricina Neerlandica: Critical Monographs on*
418 *Families of Agarics and Boleti occurring in the Netherlands*. Vols. 1-5. A.A. Balkema
419 Publishers.
420 Prior, R.L. (2003). Fruits and vegetables in the prevention of cellular oxidative damage.
421 *American Journal of Clinical Nutrition*, *78*, 570S-578S.
422 Schwenke, D.C. (2002). Does lack of tocopherols and tocotrienols put women at
423 increased risk of breast cancer? *Journal of Nutritional Biochemistry, 13*, 2-20.
424 Traber, M.G. (1999). Vitamin E. In *Modern Nutrition in Health and Disease*. Shils,
425 M.E., Olson, J.A., Shike, M., Ross, A.C., Eds.; Baltimore: Williams and Wilkins (10th
426 ed.), pp 347-362.
427 Tsai, S.Y., Tsai, H.L., & Mau, J.L. (2007). Antioxidant properties of *Agaricus blazei*,
428 *Agrocybe cylindracea*, and *Boletus edulis*. *Lwt-Food Science and Technology, 40*, 1392-
429 1402.
430 Wright, J.S., Johnson, E.R., & DiLabio, G.A. (2001). Predicting the activity of phenolic
431 antioxidants: theoretical method, analysis of substituent effects, and application to major
432 families of antioxidants. *Journal of the American Chemical Society, 123*, 1173-1183.
433 Yang, J.-H., Lin, H.-C., & Mau, J.-L. (2002). Antioxidant properties of several
434 commercial mushrooms. *Food Chemistry*, *77*, 229-235.

435 **Table 1.** Extraction yields and phenolic contents in the mushroom samples. The results
436 are expressed as mean ± SD (n = 3 for yield and n = 9 for phenols). In each column
437 different letters mean significant differences between results ($p< 0.05$).
438

Mushroom species	η (%)	Phenolics (mg GAE/g)
Clitocybe alexandri	47.74 ± 5.34 b	1.53 ± 0.06 j
Cortinarius glaucopus	34.00 ±3.24 cd	2.80 ± 0.36 gh
Fistulina hepatica	71.00 ± 5.76 a	4.44 ± 0.06 cd
Hydnum repandum	32.57 ± 2.67 cde	0.51 ± 0.02 k
Hygrophoropsis aurantiaca	31.90 ± 2.98 cde	7.90 ± 0.29 a
Hypholoma capnoides	38.96 ± 3.08 bc	1.71 ± 0.01 ij
Laccaria amethystina	23.53 ± 2.14 ef	2.85 ± 0.24 gh
Laccaria laccata	21.04 ± 1.97 f	1.59 ± 0.02 ij
Lactarius aurantiacus	21.29 ± 1.67 f	0.58 ± 0.03 k
Lactarius salmonicolor	26.42 ± 0.98 def	4.14 ± 0.26 cde
Lepista inversa	38.98 ± 1.89 bc	3.60 ± 0.07 ef
Lepista sordida	38.34 ± 2.56 bc	4.10 ± 0.20 de
Mycena rosea	25.13 ± 2.40 def	3.56 ± 0.37 ef
Russula delica	44.87 ± 3.61 b	2.23 ± 0.18 hi
Russula vesca	25.71 ± 1.32 def	6.61 ± 0.36 b
Suillus collinitus	77.41 ± 5.78 a	3.16 ± 0.14 fg
Suillus mediterraneensis	26.96 ± 2.07 def	7.46 ± 0.21 a
Tricholoma sulphureum	38.28 ± 3.05 bc	4.76 ± 0.19 c

439 **Table 2.** Tocopherol composition (μg/g) of the mushroom samples (mean ± SD; n = 9).
440 In each column different letters mean significant differences between results ($p < 0.05$).
441

Mushroom species	α-tocopherol	β-tocopherol	γ-tocopherol	δ-tocopherol	Total
Clitocybe alexandri	0.04 ± 0.00 fgh	2.17 ± 0.22 c	1.34 ± 0.11 a	n.d	3.55 ± 0.33 c
Cortinarius glaucopus	0.11 ± 0.01 de	0.29 ± 0.04 fghi	0.52 ± 0.04 de	n.d	0.92 ± 0.09 efg
Fistulina hepatica	0.12 ± 0.01 d	1.73 ± 0.09 d	0.41 ± 0.03 def	n.d	2.26 ± 0.13 d
Hydnum repandum	n.d	n.d	0.51 ± 0.08 de	n.d	0.51 ± 0.08 h
Hygrophoropsis aurantiaca	0.20 ± 0.01 bc	0.44 ± 0.03 fg	1.08 ± 0.04 b	0.21 ± 0.02 b	1.94 ± 0.10 d
Hypholoma capnoides	0.17 ± 0.00 c	0.19 ± 0.01 ghij	0.35 ± 0.02 f	n.d	0.71 ± 0.03 gh
Laccaria amethystina	0.05 ± 0.00 fg	1.09 ± 0.11 e	0.83 ± 0.07 c	n.d	1.98 ± 0.04 d
Laccaria laccata	0.22 ± 0.01 b	7.06 ± 0.17 a	0.57 ± 0.03 d	0.19 ± 0.01 b	8.04 ± 0.20 a
Lactarius aurantiacus	0.03 ± 0.00 fgh	n.d	1.21 ± 0.10 ab	n.d	1.24 ± 0.10 e
Lactarius salmonicolor	0.04 ± 0.00 fgh	0.11 ± 0.02 ij	n.d	n.d	0.15 ± 0.03 i
Lepista inversa	0.28 ± 0.06 a	0.25 ± 0.03 fghi	n.d	0.64 ± 0.03 a	1.18 ± 0.06 ef
Lepista sordida	0.02 ± 0.00 fgh	n.d	n.d	n.d	0.02 ± 0.00 i
Mycena rosea	0.11 ± 0.01 d	3.86 ± 0.15 b	0.92 ± 0.07 c	n.d	4.89 ± 0.21 b
Russula delica	0.01 ± 0.00 gh	0.16 ± 0.02 hij	0.57 ± 0.07 d	n.d	0.74 ± 0.06 gh
Russula vesca	0.02 ± 0.00 fgh	0.49 ± 0.04 f	0.36 ± 0.04 ef	n.d	0.88 ± 0.05 fg
Suillus collinitus	0.06 ± 0.00 ef	0.22 ± 0.01 ghij	0.55 ± 0.01 d	n.d	0.83 ± 0.03 fgh
Suillus mediterraneensis	0.04 ± 0.00 fgh	0.07 ± 0.00 ij	n.d	n.d	0.11 ± 0.01 i
Tricholoma sulphureum	0.06 ± 0.00 fg	0.40 ± 0.03 fgh	0.07 ± 0.01 g	n.d	0.52 ± 0.03 h

442 *n.d*.- not detected

443

444 **Table 3.** Antioxidant activity EC_{50} values (mg/ml) of the mushroom samples. The
445 results are expressed as mean ± SD (n = 9). In each column different letters mean
446 significant differences between results ($p < 0.05$).
447

Mushroom species	DPPH scavenging activity	β-carotene bleaching inhibition
Clitocybe alexandri	28.72 ± 3.21 a	4.45 ± 0.20 a
Cortinarius glaucopus	16.59 ± 2.82 bcd	1.73 ± 0.11 efg
Fistulina hepatica	5.32 ± 0.70 ghij	0.94 ± 0.01 fg
Hydnum repandum	30.00 ± 3.05 a	28.72 ± 2.04 a
Hygrophoropsis aurantiaca	1.20 ± 0.22 j	0.71 ± 0.12 g
Hypholoma capnoides	20.85 ± 2.23 bc	2.90 ± 0.21 de
Laccaria amethystina	15.72 ± 1.80 cde	1.23 ± 0.22 fg
Laccaria laccata	21.95 ± 2.02 b	3.69 ± 0.30 cd
Lactarius aurantiacus	30.00 ± 3.13 a	7.48 ± 0.50 b
Lactarius salmonicolor	7.80 ± 0.52 ghi	1.01 ± 0.14 fg
Lepista inversa	10.57 ± 1.14 efg	1.08 ± 0.11 fg
Lepista sordida	9.82 ± 0.84 fgh	1.03 ± 0.01 fg
Mycena rosea	10.58 ± 0.92 efg	1.15 ± 0.12 fg
Russula delica	20.53 ± 1.81 bc	2.28 ± 0.22 def
Russula vesca	3.91 ± 0.32 ij	0.91 ± 0.01 fg
Suillus collinitus	14.05 ± 1.24 def	1.20 ± 0.11 fg
Suillus mediterraneensis	2.90 ± 0.11 ij	0.81 ± 0.01 fg
Tricholoma sulphureum	4.69 ± 0.32 hij	0.93 ± 0.01 fg

448

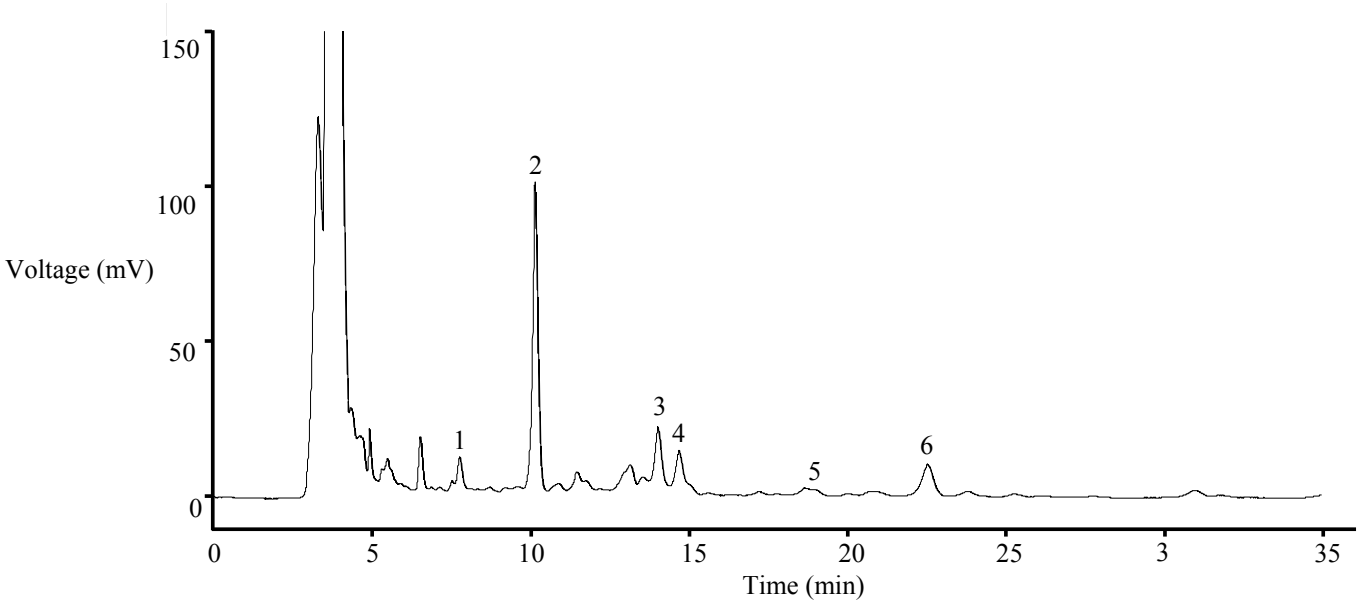
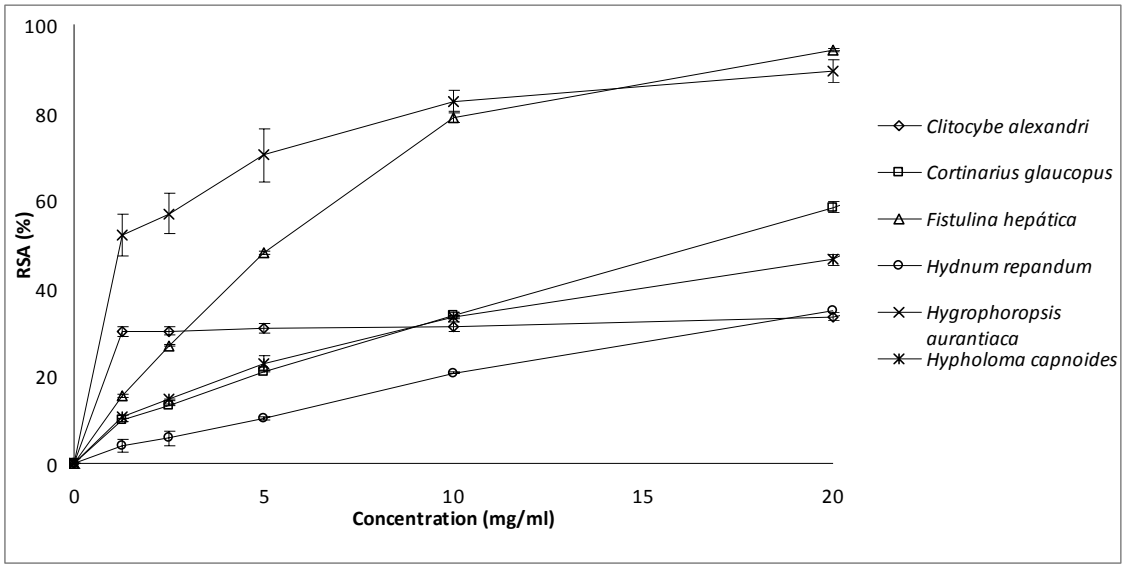
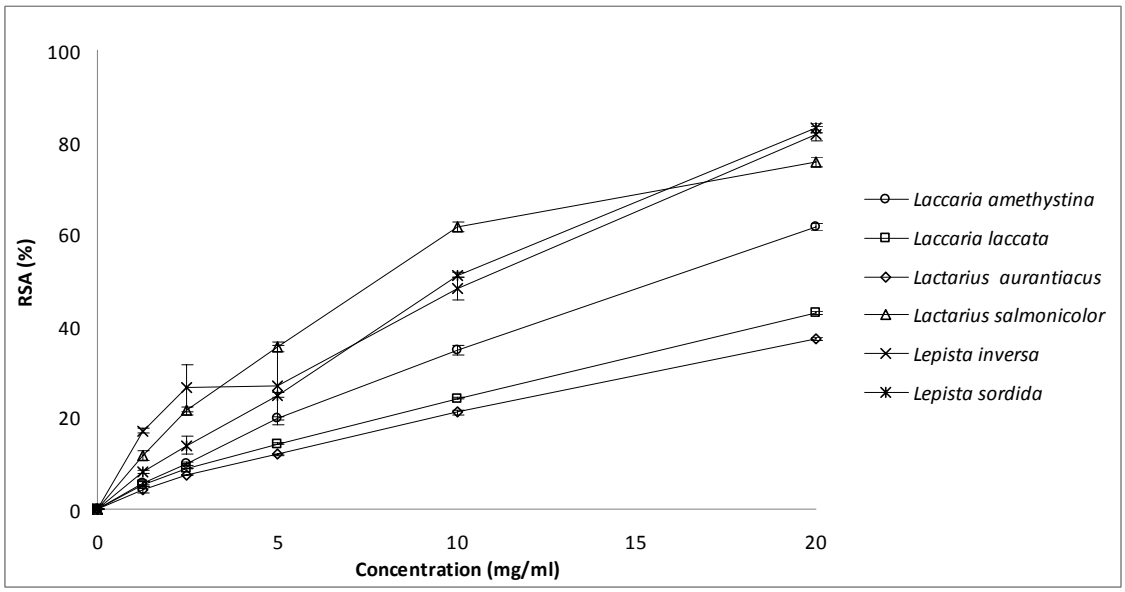


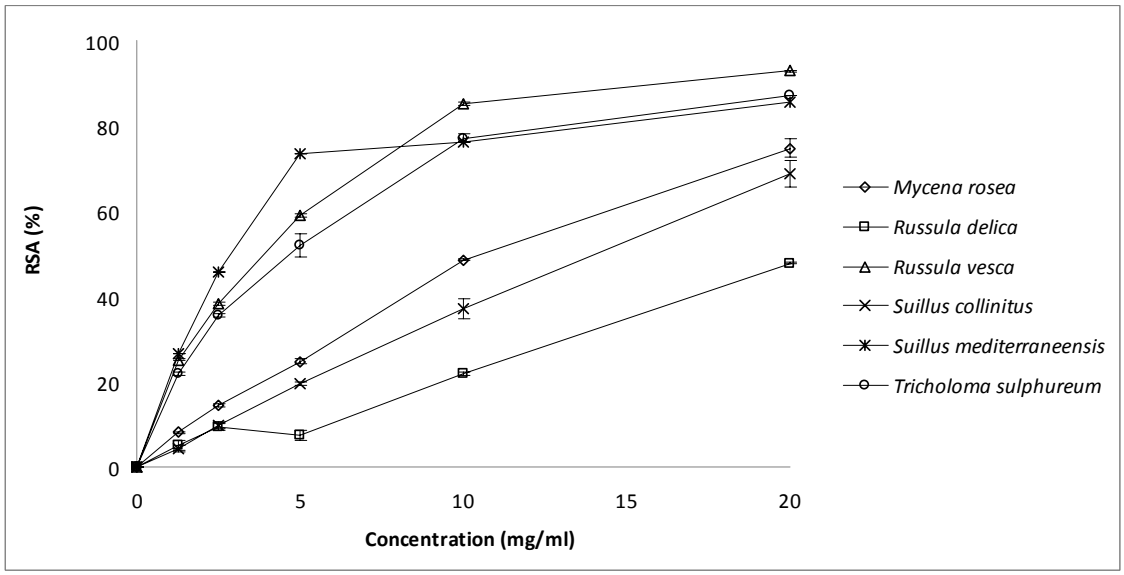
Figure 1. HPLC fluorescence chromatogram of *Hygrophoropsis aurantiaca*. Peaks: 1-α-tocopherol; 2-BHT (butylated hydroxytoluene); 3-β-tocopherol; 4- γ-tocopherol; 5- δ-tocopherol; 6-I.S.- internal standard (tocol).



462

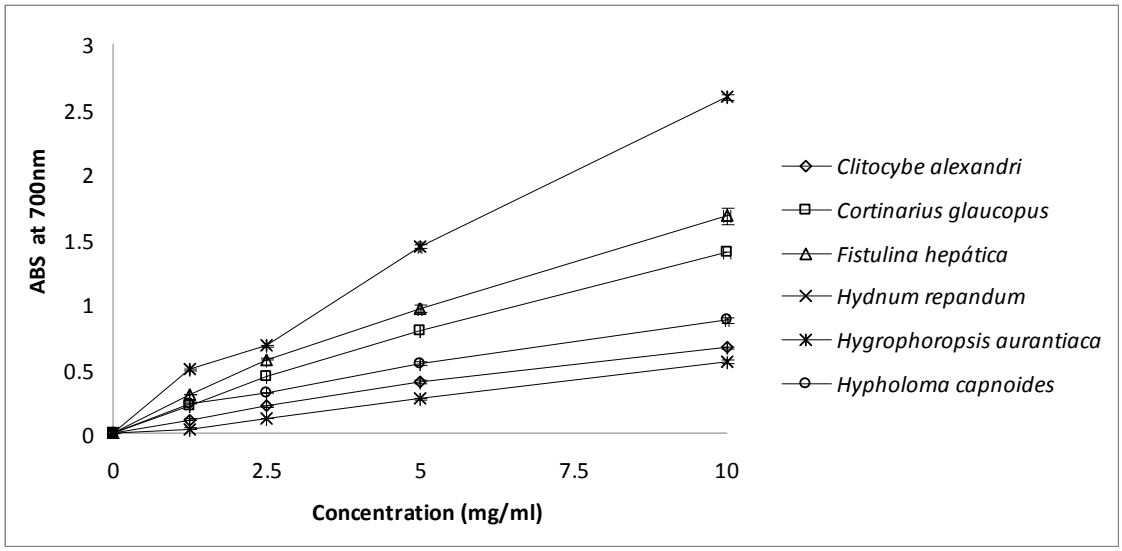


463

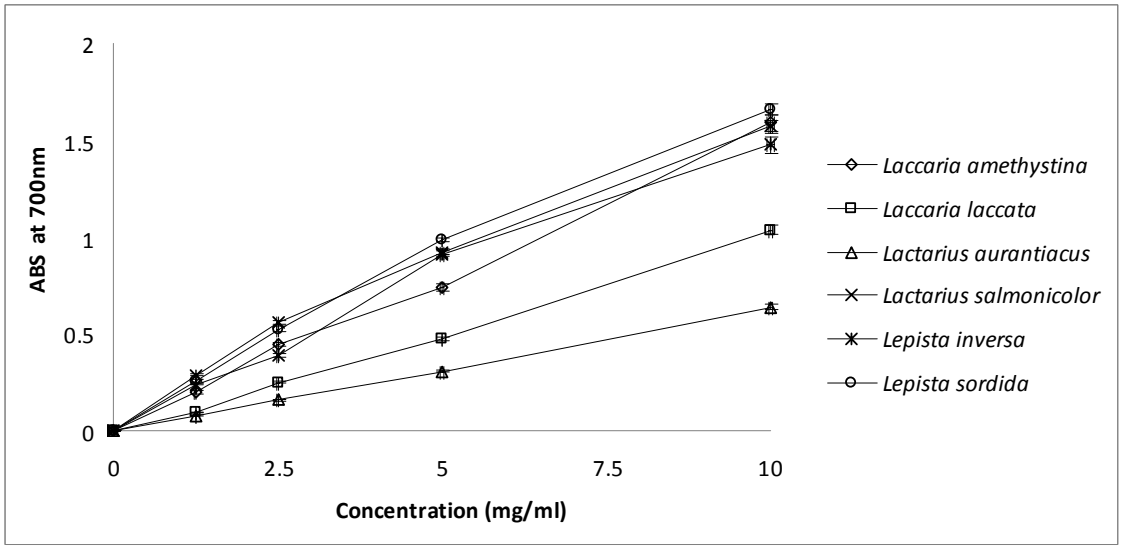


464

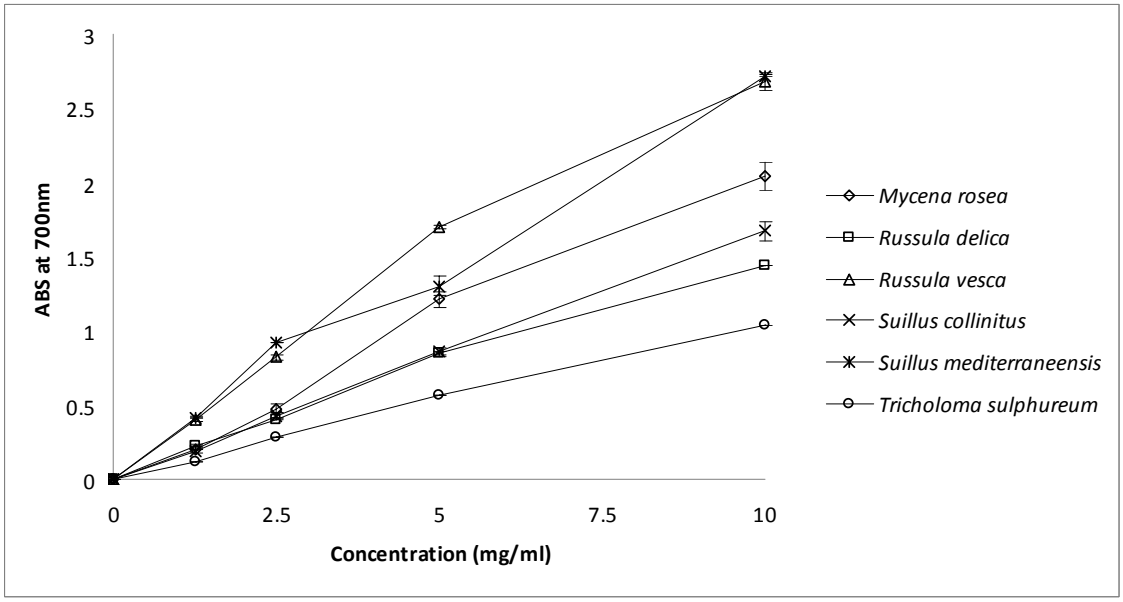
465 **Figure 2**. Radical scavenging activity on DPPH radicals (%) of the mushroom samples.
466 Each value is expressed as mean ± SE (n = 3).



467

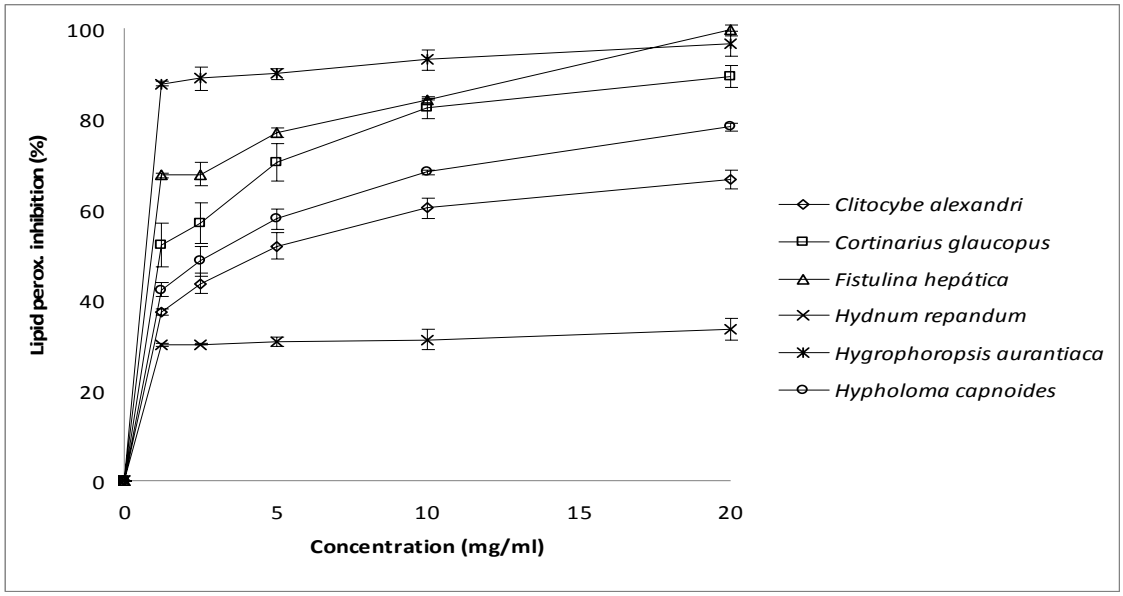


468

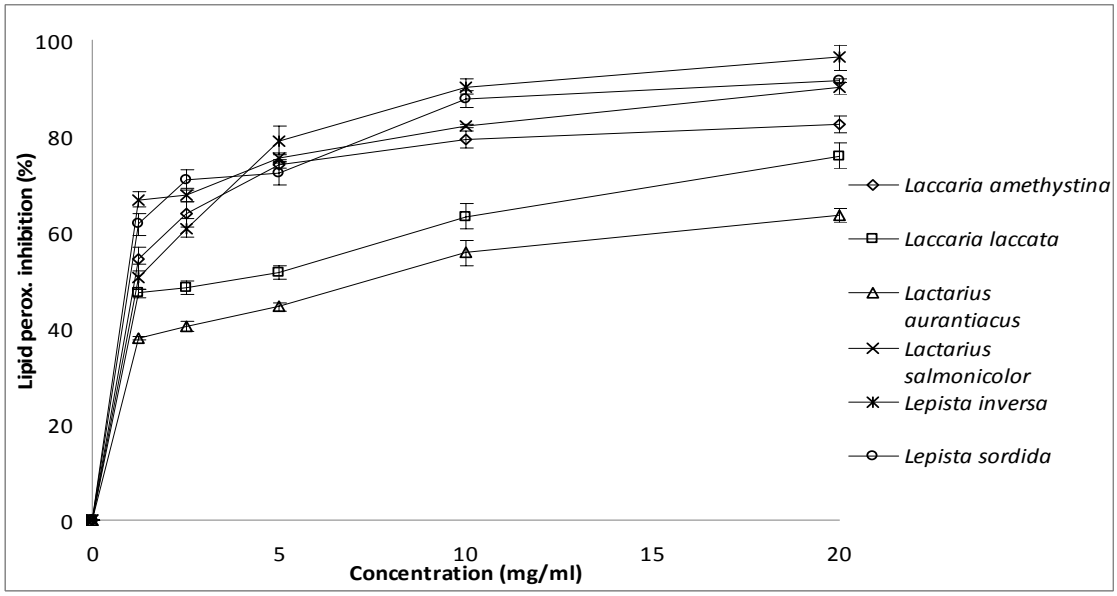


469

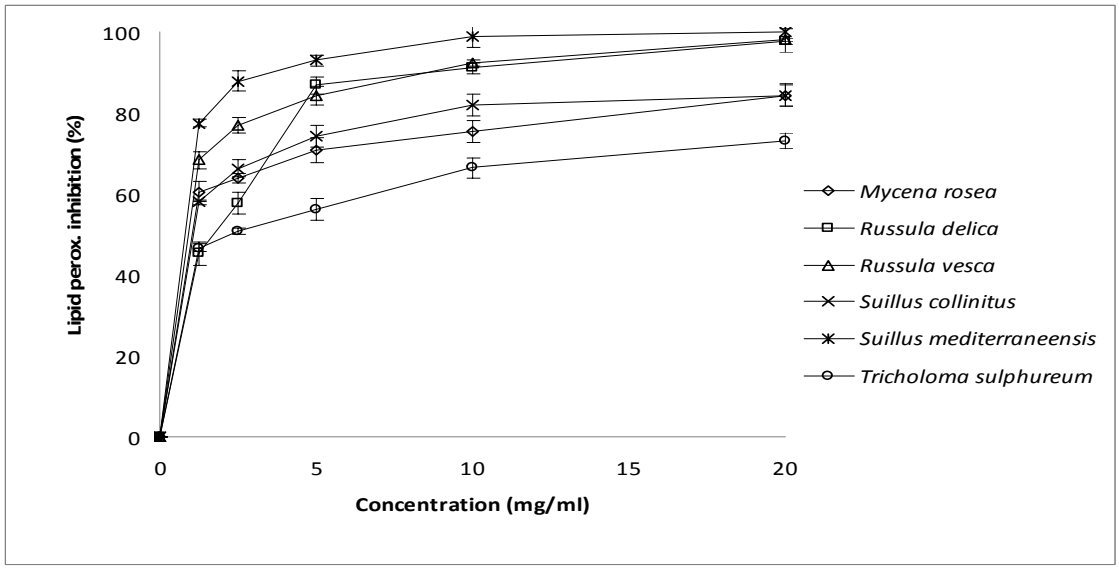
470 **Figure 3**. Reducing power of the mushroom samples. Each value is expressed as mean
471 ± SE (n = 3).



472



473



474

475 **Figure 4**. Lipid peroxidation inhibition measured by the β-carotene bleaching inhibition
476 of the mushroom samples. Each value is expressed as mean ± SE (n = 3).