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Tocopherols composition of Portuguese wild mushrooms with antioxidant capacity

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ABSTRACT

The antioxidant composition and properties of 18 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 characterisation 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 *H. 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.

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*, *L. sordida*, *Mycena rosea*, *Russula vesca*, *Suillus collinitus*, *Suillus mediterraneensis* and *Tricholoma sulphureum* have not yet been reported. Tocopherol compositions of *Russula delica* from Turkey and *H. repandum* from Spain were studied but there are no studies on Portuguese samples.

Vitamin E is a term frequently used to designate a family of chemically related compounds, namely tocopherols and tocotrienols, which share a common structure having a chromanol head and isoprene side chain. Due to its role as a scavenger of free radicals, vitamin E is also believed to protect our bodies against degenerative malfunctions, mainly cancer and cardiovascular diseases (Burton & Traber, 1990; Kamal-Eldin & Appelqvist, 1996). In the past α-tocopherol was considered the most active form of vitamin E in humans and it was reported to exhibit the highest biological activity. However, many recent publications have been focused on the health effects of the other vitamin E isoforms (Schwenke, 2002; Traber, 1999).

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Some reports have been published on mushrooms tocopherols contents (Barros, Correia et al., 2008; Barros, Venturini et al., 2008; Elmastas, Isildak, Turkekul, & Temur, 2007; Jayakumar, Thomas, & Geraldine, 2009; Lee, Jian, Lian, & Mau, 2008; Lee, Kim, Jang, Jung, & Yun, 2007; Mau, Chang, Huang, & Chen, 2004; Mau, Chao, & Wu, 2001; Mau, Lin, & Chen, 2002; Mau, Lin, & Song, 2002; Tsai, Tsai, & Mau, 2007; Yang, Lin, & Mau, 2002). They all described the same methodology including saponification in the extraction process and analysis by HPLC coupled to UV detector. Our research group (Barros, Correia et al., 2008) was the only describing an extraction process without saponification, by adding an antioxidant to avoid tocopherols oxidation, while protecting the samples from light and heat, followed by HPLC-fluorescence analysis. Using this process, α-, β-, δ- and γ-tocopherols were identified and quantified in wild mushrooms, while tocotrienols were not detected in any of the cited studies.

2. Materials and methods

2.1. Mushroom species

Samples of *C.* alexandri (Gillet) Konrad, *C.* glaucopus (Schaeff), *F.* hepatica (Schaeff.: Fr.), *H.* repandum (L.: Fr.), *H.* aurantiaca (Wulf.: Fr.) Mre., *H.* capnoides (Fr.) Quel., *L.* amethystina (Bolt. ex Fr.) R.Maire, *L.* laccata (scop.: Fr.) Berk. & Broome, *L.* aurantiacus (Fr.), *L.* salmonicolor (Heim y Leclair), *L.* inversa (Scop.: Fr.) Pat., *L.* sordida (Fr.) Singer, *M.* rosea (Schumach.) Gramberg, *R.* delica (Fr.), *R.* vesca (Fr.), *S.* collinitus (Fr.) Kuntz, *S.* mediterraneensis (Jacquetant & Blum) Redeuilh, *T.* 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. *M.* rosea and *T.* sulphureum are not edible species and *C.* glaucopus is a species of unknown edibility. Taxonomic identification of sporocarps was made according to several authors (Alessio, 1985; Bon, 1988; Breitenbach & Kränzlin, 1984; Courtecuisse, 1999; Candusso & Lanzoni, 1990; Courtecuisse & Duhem, 2005; Marchand, 1971–1986; Moser, 1983–2000; Noordeloos, 1988–2001) 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 gram of extract.

Tocopherols content was determined following a procedure previously optimised 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 homogenised 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 homogenised (1 min), centrifuged (Centurion K24OR-2003 refrigerated centrifuge, 5 min, 6185 rpm) and the clear upper layer was carefully transferred to a vial. The sample was re-extracted twice with hexane. The combined extracts were taken to dryness under a nitrogen stream, redissolved in 1 ml of *n*-hexane, dehydrated with anhydrous sodium sulphate, filtered through a 0.22 μm disposable LC filter disk, transferred into a dark injection vial and analysed by HPLC. The HPLC equipment consisted of an integrated system with a Smartline pump 1000 (Knauer, Germany), a degasser system Smartline manager 5000, an AS-2057 auto-sampler and a 2500 UV detector at 295 nm (Knauer, Germany) connected in series with a FP-2020 fluorescence detector (Jasco, Japan) programmed for excitation at 290 nm and emission at 330 nm. Data were analysed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Polyamide II (250 × 4.6 mm) normal-phase column from YMC Waters (Japan) operating at 30 °C (7971 R Grace oven). 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, and the injection volume was 20 μl. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response, using the internal standard method. Tocopherol contents in mushroom samples are expressed in μg per gram of dry mushroom.

2.4. Antioxidant activity

The samples (1.5 g) were extracted by stirring with 40 ml of methanol (25 °C at 150 rpm) for 12 h and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with 20 ml of methanol (25 °C at 150 rpm) for 4 h. The combined methanolic extracts were evaporated at 40 °C to dryness and redissolved in methanol at a concentration of 50 mg/ml, and stored at 4 °C 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.

2.4.1. 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 discolouration using the equation: $\%\ \mathrm{RSA} = [(A_{\mathrm{DPPH}} - A_S)/A_{\mathrm{DPPH}}] \times 100$, 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.

2.4.2. 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 °C for 20 min. After addition of trichloroacetic 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.

2.4.3. 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 at 40 °C under vacuum, linoleic acid (40 mg), Tween® 80 emulsifier (400 mg), and distilled water (100 ml) were added to the flask and vigorously shaken. Aliquots (4.8 ml) of this emulsion were transferred into different test tubes containing different concentrations (0.2 ml) of the extracts. The tubes were shaken and incubated at 50 °C in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance at 470 nm was measured. Absorbance readings were then recorded at 20 min intervals until the control sample changed colour. A blank, devoid of β-carotene, was used for background subtraction. Lipid peroxidation inhibition was calculated using the following equation: (β-carotene content after 2 h of assay)/(initial β-carotene content) × 100. The extract concentration providing 50% antioxidant activity (EC_{50}) was calculated by interpolation from the graph of antioxidant activity percentage against extract concentration. TBHQ was used as standard.

2.5. Statistical analysis

For each one of the mushroom species three samples were analysed and also all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD) or standard errors (SE). The results were analysed 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.16.0 software. The ANOVA results were classified using letters (different letters mean significant differences among results). The letters are alphabetically ordered according to the decrease of the result values (e.g. letter "a" represents the best result for phenol content and extraction yields, and the worst results for antioxidant activity assays).

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:

$$RO_2^{\cdot} + ArOH \rightarrow ROOH + ArO^{\cdot}$$

To be effective ArO· must be a relatively stable free radical, so that it reacts slowly with substrate RH but rapidly with $RO_2^{\cdot}$, 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 influence of the physicochemical environment on the antioxidant effectiveness and the occurrence of other biological activities (Faria, Calhau, Freitas, & Mateus, 2006).

Plants are among the most important sources of phenolic compounds, providing high quantities of these phytochemicals, but mushrooms should also be considered as possible sources. In the present report we conclude that *H. aurantiaca* and *S. mediterraneensis* presented the highest phenols contents (higher than 7 mg/g), while *L. aurantiacus* and *H. repandum* revealed the lowest levels (lower than 0.6 mg/g) (Table 1). Regarding phenol content, the ANOVA analysis indicated high heterogeneity among the different mushroom species. In fact, the phenolic composition in mushrooms might be affected by a number of factors, namely mushroom strain/species, composition of growth media (for *in vitro* cultured species), time of harvest, management techniques, handling conditions, and preparation of the substrates (in case of cultivated species) and soil/substrate composition or host associated species (in case of wild species either saprotrophic or mycorrhizal). All these factors might have an influence in the secondary metabolism of fungi, including shikimate and acetate pathways, and therefore in their phenols production. The higher levels of phenols found in *H. aurantiaca*, *S. mediterraneensis* and *R. vesca* could be related to higher stress conditions in their growth that could stimulate the secondary metabolism.

It has been described that the main phenolic compounds found in mushrooms are phenolic acids (Ferreira et al., 2009).

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

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

Particularly, the presence of *p*-coumaric, caffeic and ellagic acids in *F. hepatica* was already reported in literature, as also the presence of protocatechuic, gallic, gentisic, vanillic, syringic, cinamic and tanic acids in *H. repandum*.

Vitamin E reacts with peroxyl radicals produced from polyunsaturated fatty acids in 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).

$$LH + \text{Oxidantinitiator} \rightarrow L^{\cdot}$$
$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$$
$$LOO^{\cdot} + \text{Tocopherol} \rightarrow LOOH + \text{Tocopherol}$$

In the literature, there are studies on tocopherols composition of mushrooms from India: *Cantharelus cibarius* and *Pleurotus ostreatus* (Jayakumar, Thomas, & Geraldine, 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, Chang, Huang, & Chen, 2004; Mau, Chao, & Wu, 2001; Mau, Lin, & Chen, 2002; Mau, Lin, & Song, 2002; Tsai, Tsai, & Mau, 2007; Yang et al., 2002) and Turkey: *Agaricus bisporus, Boletus badius, Lepista nuda, Pleurotus ostreatus, Polyporus squamosus, R. delica* and *Verpa conica* (Elmastas, Isildak, Turkekul, & Temur, 2007). The reports on Portuguese mushrooms (*Agaricus arvensis, A. 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 18 different Portuguese mushrooms. *L. inversa* had 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 *L. laccata* (7.06 μg/g). *C. alexandri* contained the highest content of γ-tocopherol (1.34 μg/g). δ-Tocopherol was only found in three species (*L. inversa*, *H. aurantiaca* and *L. 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, *L. laccata* presented the highest concentration of total tocopherols (8.04 μg/g). The presence of α-tocopherol in

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

Mushroom species	α-Tocopherol	β-Tocopherol	γ-Tocopherol	δ-Tocopherol	Total
Clitocybe alexandri	$0.04 \pm 0.00^{f,g,h}$	2.17 ± 0.22^{c}	1.34 ± 0.11^{a}	n.d	3.55 ± 0.33^{c}
Cortinarius glaucopus	$0.11 \pm 0.01^{d,e}$	$0.29 \pm 0.04^{f,g,h,i}$	$0.52 \pm 0.04^{d,e}$	n.d	$0.92 \pm 0.09^{e,f,g}$
Fistulina hepatica	0.12 ± 0.01^{d}	1.73 ± 0.09^{d}	$0.41 \pm 0.03^{d,e,f}$	n.d	2.26 ± 0.13^{d}
Hydnum repandum	n.d	n.d	$0.51 \pm 0.08^{d,e}$	n.d	0.51 ± 0.08^{h}
Hygrophoropsis aurantiaca	$0.20 \pm 0.01^{b,c}$	$0.44 \pm 0.03^{f,g}$	1.08 ± 0.04^{b}	0.21 ± 0.02^{b}	1.94 ± 0.10^{d}
Hypholoma capnoides	0.17 ± 0.00^{c}	$0.19 \pm 0.01^{g,h,i,j}$	0.35 ± 0.02^{f}	n.d	$0.71 \pm 0.03^{g,h}$
Laccaria amethystina	$0.05 \pm 0.00^{f,g}$	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	$0.03 \pm 0.00^{f,g,h}$	n.d	$1.21 \pm 0.10^{a,b}$	n.d	1.24 ± 0.10^{e}
Lactarius salmonicolor	$0.04 \pm 0.00^{f,g,h}$	$0.11 \pm 0.02^{i,j}$	n.d	n.d	0.15 ± 0.03^{i}
Lepista inversa	0.28 ± 0.06^{a}	$0.25 \pm 0.03^{f,g,h,i}$	n.d	0.64 ± 0.03^{a}	$1.18 \pm 0.06^{e,f}$
Lepista sordida	$0.02 \pm 0.00^{f,g,h}$	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	$0.01 \pm 0.00^{g,h}$	$0.16 \pm 0.02^{h,i,j}$	0.57 ± 0.07^{d}	n.d	$0.74 \pm 0.06^{g,h}$
Russula vesca	$0.02 \pm 0.00^{f,g,h}$	0.49 ± 0.04^{f}	$0.36 \pm 0.04^{e,f}$	n.d	$0.88 \pm 0.05^{f,g}$
Suillus collinitus	$0.06 \pm 0.00^{e,f}$	$0.22 \pm 0.01^{g,h,i,j}$	0.55 ± 0.01^{d}	n.d	$0.83 \pm 0.03^{f,g,h}$
Suillus mediterraneensis	$0.04 \pm 0.00^{f,g,h}$	$0.07 \pm 0.00^{i,j}$	n.d	n.d	0.11 ± 0.01^{i}
Tricholoma sulphureum	$0.06 \pm 0.00^{f,g}$	$0.40 \pm 0.03^{f,g,h}$	0.07 ± 0.01^{g}	n.d	0.52 ± 0.03^{h}

n.d. – not detected.

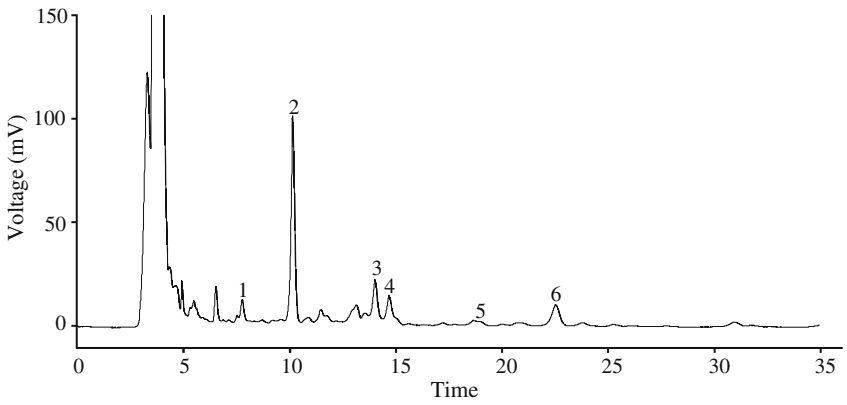

Fig. 1. HPLC fluorescence chromatogram of *Hygrophoropsis aurantiaca*. Peaks: (1) α-tocopherol; (2) BHT (butylated hydroxytoluene); (3) β-tocopherol; (4) γ-tocopherol; (5) δ-tocopherol; (6) I.S. – internal standard (tocol).

a sample of *R. 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 18 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 (Fig. 2). The reducing power was evaluated measuring the conversion of a Fe^{3+}/ferricyanide complex to the ferrous form by the samples (Fig. 3). The lipid peroxidation inhibition was measured by the inhibition of β-carotene bleaching, by neutralising the linoleate-free radical and other free radicals formed in the system which attack the highly unsaturated

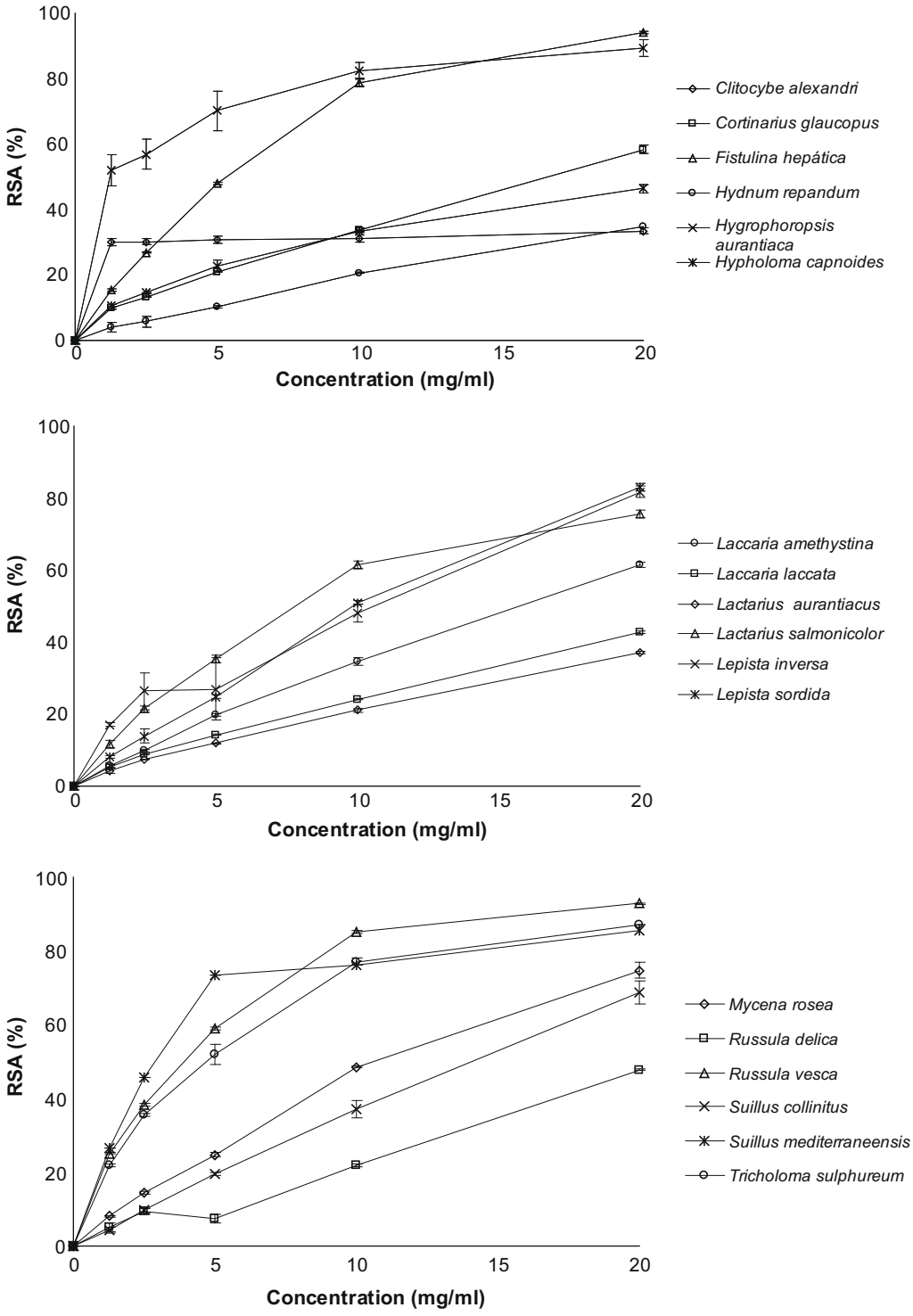

Fig. 2. Radical-scavenging activity on DPPH radicals (%) of the mushroom samples. Each value is expressed as mean ± SE (n = 3).

β-carotene models (Fig. 4). From the analysis of Figs. 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.

H. aurantiaca, *S. mediterraneensis*, *R. vesca* and *T. sulphureum* presented radical-scavenging effects higher than 85% at 20 mg/ml (Fig. 2) with EC_{50} values lower than 5 mg/ml (Table 3). *R. delica*, *H. capnoides*, *L. laccata*, *C. alexandri*, *L. aurantiacus* and *H. repandum* presented high EC_{50} values (higher than 20 mg/ml) with RSA

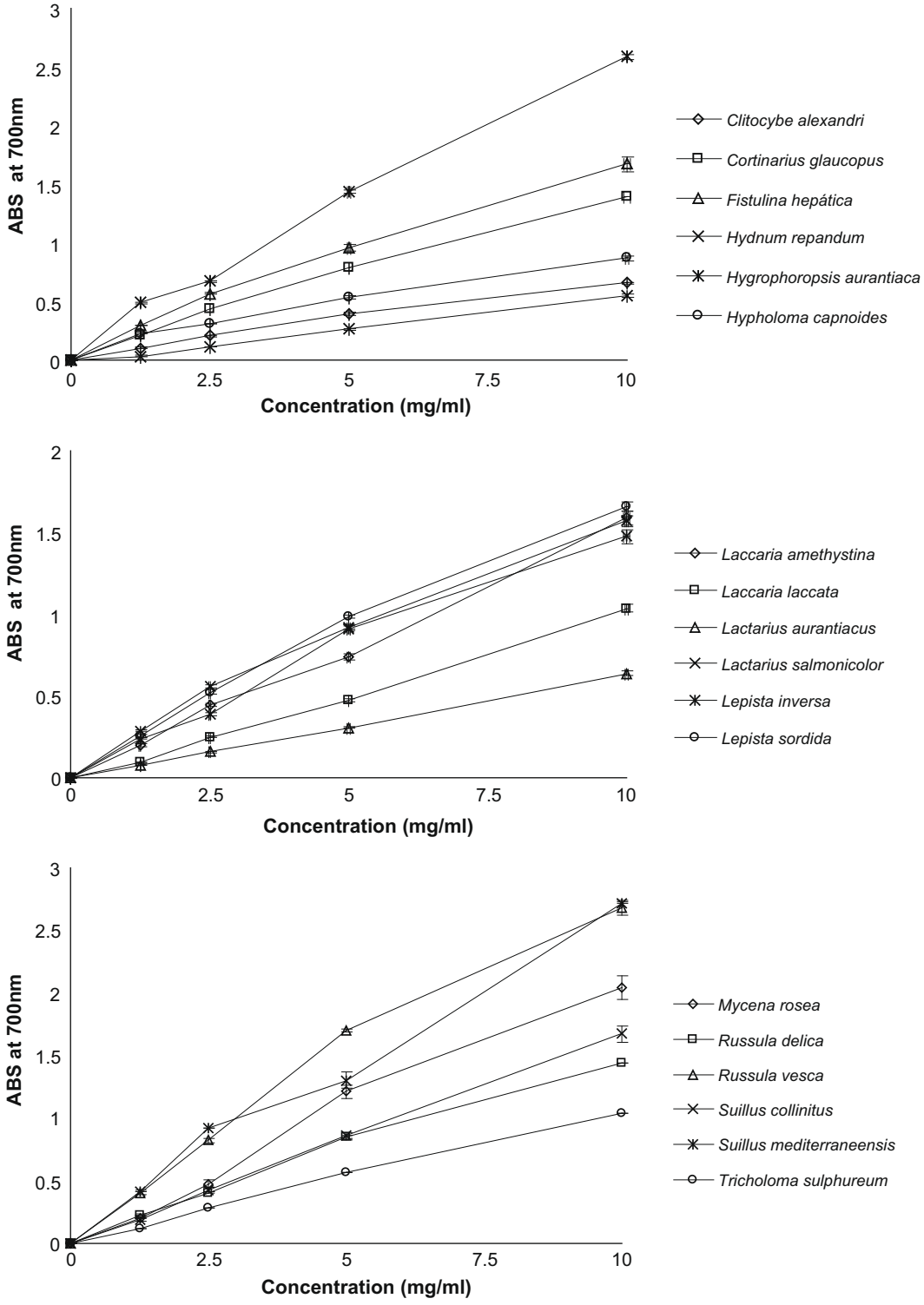

Fig. 3. Reducing power of the mushroom samples. Each value is expressed as mean ± SE ($n = 3$).

percentages lower than 48% at 20 mg/ml. *H. aurantiaca*, *S. mediterraneensis* and *R. vesca* were the species with higher reducing power (absorbance higher than 2.5 at 10 mg/ml; Fig. 3). *C. alexandri*, *L. aurantiacus* and *H. repandum* presented the lowest reducing powers with absorbances lower than 0.7 at 10 mg/ml. Concerning the lipid peroxidation inhibition, *H. aurantiaca*, *S. mediterraneensis*, *R. vesca*, *T. sulphureum* and *F. hepatica* were the species with higher capacity (higher than 73% at 20 mg/ml; Fig. 4) with EC_{50} values lower than 1 mg/ml (Table 3). *H. repandum* presented the highest EC_{50} value (29 mg/ml) with 33% of lipid peroxidation inhibition capacity at

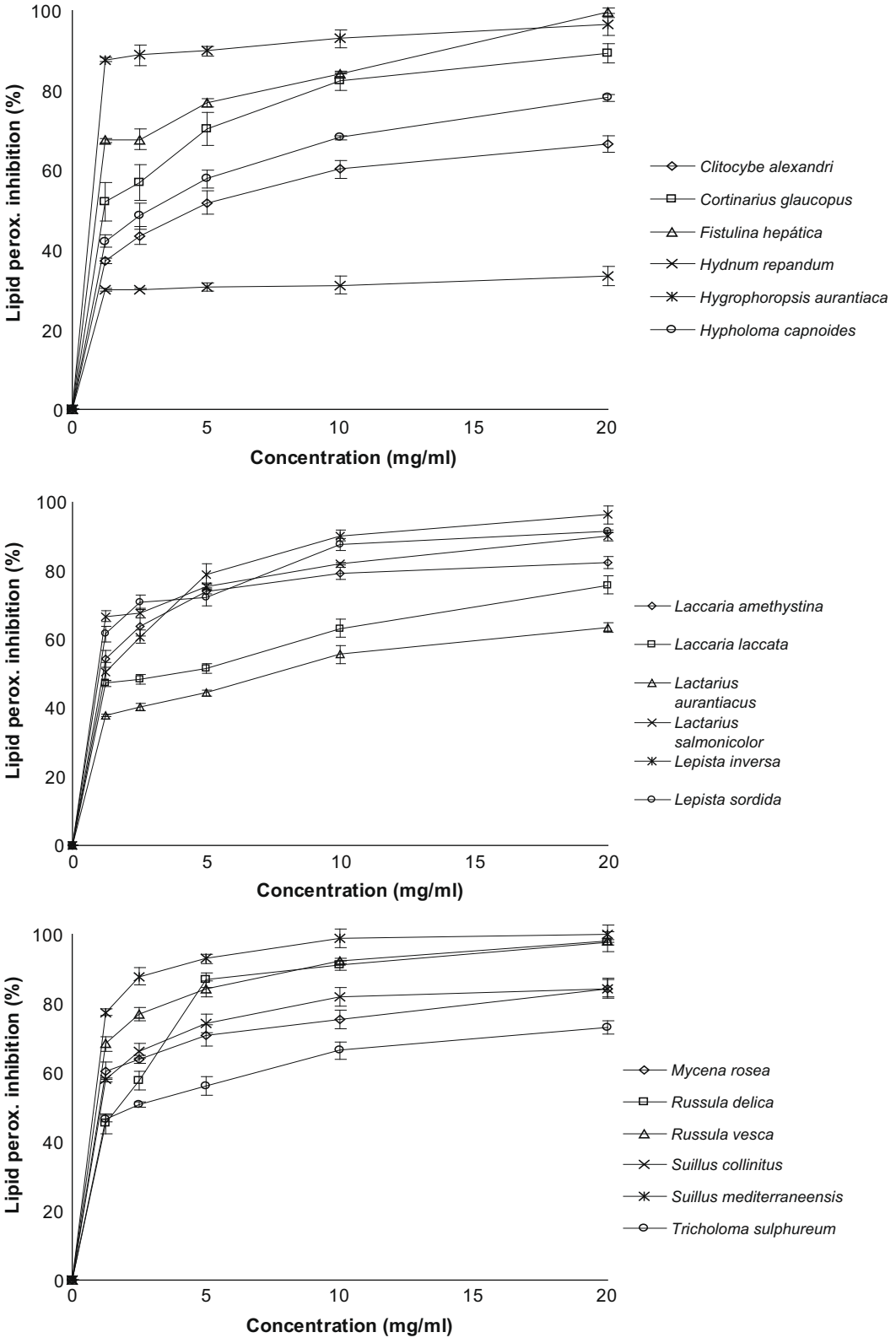

Fig. 4. Lipid peroxidation inhibition measured by the β-carotene bleaching inhibition of the mushroom samples. Each value is expressed as mean ± SE ($n = 3$).

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

Mushroom species	DPPH scavenging activity	β-carotene bleaching inhibition
Clitocybe alexandri	28.72 ± 3.21^{a}	4.45 ± 0.20^{a}
Cortinarius glaucopus	$16.59 \pm 2.82^{b,c,d}$	$1.73 \pm 0.11^{e,f,g}$
Fistulina hepatica	$5.32 \pm 0.70^{g,h,i,j}$	$0.94 \pm 0.01^{f,g}$
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	$20.85 \pm 2.23^{b,c}$	$2.90 \pm 0.21^{d,e}$
Laccaria amethystina	$15.72 \pm 1.80^{c,d,e}$	$1.23 \pm 0.22^{f,g}$
Laccaria laccata	21.95 ± 2.02^{b}	$3.69 \pm 0.30^{c,d}$
Lactarius aurantiacus	30.00 ± 3.13^{a}	7.48 ± 0.50^{b}
Lactarius salmonicolor	$7.80 \pm 0.52^{g,h,i}$	$1.01 \pm 0.14^{f,g}$
Lepista inversa	$10.57 \pm 1.14^{e,f,g}$	$1.08 \pm 0.11^{f,g}$
Lepista sordida	$9.82 \pm 0.84^{f,g,h}$	$1.03 \pm 0.01^{f,g}$
Mycena rosea	$10.58 \pm 0.92^{e,f,g}$	$1.15 \pm 0.12^{f,g}$
Russula delica	$20.53 \pm 1.81^{b,c}$	$2.28 \pm 0.22^{d,e,f}$
Russula vesca	$3.91 \pm 0.32^{i,j}$	$0.91 \pm 0.01^{f,g}$
Suillus collinitus	$14.05 \pm 1.24^{d,e,f}$	$1.20 \pm 0.11^{f,g}$
Suillus mediterraneensis	$2.90 \pm 0.11^{i,j}$	$0.81 \pm 0.01^{f,g}$
Tricholoma sulphureum	$4.69 \pm 0.32^{h,i,j}$	$0.93 \pm 0.01^{f,g}$

20 mg/ml. The antioxidant properties are in agreement with the phenols contents found in the mushrooms species (Table 1). *H. 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 (Fig. 1). *H. repandum* presented the lowest phenols levels and highest EC_{50} values.

From the 18 species only two had been previously evaluated for their antioxidant properties: *H. repandum* and *R. delica*. Nevertheless, those species were not from Portugal, but from Spain (Murcia et al., 2002) and Turkey (Elmastas et al., 2007), respectively.

Overall, this kind of study is important in increasing the proportion of investigated mushrooms and contributes to the establishment of the nutraceutical potential of wild mushrooms, which have been proved to have good radical-scavenging properties and lipid peroxidation inhibition capacity. The studied samples are sources of powerful antioxidants such as phenols and tocopherols, which could be used against diseases related to oxidative stress, dermatological applications, cosmetics, as well as supplements in food industry.

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