Antioxidant properties and phenolic profile of the most widely appreciated cultivated mushrooms: a comparative study between *in vivo* and *in vitro* samples

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Abstract

The present study reports a comparison of the antioxidant properties and phenolic profile of the most consumed species as fresh cultivated mushrooms and their mycelia produced *in vitro*: *Agaricus bisporus* (white and brown), *Pleurotus ostreatus* (oyster), *Pleurotus eryngii* (king oyster) and *Lentinula edodes* (shiitake). The antioxidant activity was evaluated through reducing power (Folin-Ciocalteu and Ferricyanide/Prussian blue assays), free radical scavenging activity (DPPH assay) and lipid peroxidation inhibition (β-carotene/linoleate and TBARS assays). The analysis of phenolic compounds was performed by HPLC/PAD. The mushroom species with the highest antioxidant potential was *Agaricus bispous* (brown). However, concerning to the species obtained *in vitro*, it was *Lentinula edodes* that demonstrate the highest reducing power. Generally, *in vivo* samples revealed higher antioxidant properties than their mycelia obtained by *in vitro* techniques. About the phenolic compounds researched, they were detected both in mushrooms and mycelia without any particular abundance. Results showed that there is no correlation between the studied commercial mushrooms and the corresponding mycelia obtained *in vitro*. Nevertheless, this study contributes to the rise of data relatively to the species consumed as fresh mushrooms and the possibility of their *in vitro* production as a source of bioactive compounds.

Keywords: Worldwide cultivated mushrooms; Mycelium; Antioxidant activity; Phenolic compounds

INTRODUCTION

Free radicals are produced in the normal natural metabolism of aerobic cells, mostly in the form of oxygen reactive species (ROS). Once produced, most of the free radicals are neutralized by cellular antioxidant defenses (enzymes and non-enzymatic molecules). Maintenance of equilibrium between free radicals production and antioxidant defences is an essential condition for normal organism functioning (Valko et al., 2007; Ferreira et al., 2009). In fact, the non-controlled production of free radicals has been related to more than one hundred diseases including several kinds of cancer, diabetes, among others (Valko et al., 2007; Ferreira et al., 2009).

Although almost all organisms are equipped with antioxidant defense and repair systems that have evolved to protect them against oxidative damage, these systems are often inadequate to completely prevent oxidative stress-induced damage. Hence, antioxidant supplements, or natural products containing bioactive compounds, may be used to help reduce oxidative damage to the human body (Kanter, 1998). Indeed, natural products with antioxidant activity, in particular mushrooms, are used to aid the endogenous protective system, increasing interest in the antioxidative role of functional foods or nutraceutical products (Ferreira et al., 2009; Jayakumar et al., 2011; Barros et al., 2008; Reis et al., 2011).

Mushrooms have been part of the normal human diet for thousands of years and, in recent times, the amounts consumed have risen greatly, involving a large number of species. Mushrooms are considered, all over the world, valuable health foods since they are poor in calories, fat, and essential fatty acids, and rich in proteins, vitamin and minerals (Agrahar-Murugkar and Subbulakshmi, 2005; Manzi et al., 2004; Pereira et al., 2012; Sanmee et al., 2003; Reis et al., 2012) . Moreover, their medicinal properties have been reported such as anti-tumor and immunomodulating effects (Ferreira et al., 2010),

inhibition of platelet aggregation (Hokama and Hokama, 1981), reduction of blood cholesterol concentrations (Jeong et al., 2010), prevention or alleviation of heart disease and reduction of blood glucose levels (Jeong et al., 2010), and antimicrobial activity (Hirasawa et al., 1999). Some of the mentioned properties are attributed to bioactive products with antioxidant activity such as phenolic compounds (Ferreira et al., 2009; Barros et al., 2009).

The most cultivated mushroom worldwide is *Agaricus bisporus*, followed by *Lentinula edodes*, *Pleurotus* spp. and *Flammulina velutipes* (Chang and Miles, 2004). Despite several studies focused on the therapeutic effects of these commercial mushrooms, a few reports are available on antioxidant properties of samples from Brazil (Kitzberger et al., 2007), China (Cheung et al., 2003), India (Jayakumar et al., 2009), Spain (Palacios et al., 2011), Taiwan (Yang et al., 2002; Tsai et al., 2009) and Thailand (Chirinang and Intarapichet, 2009). Although these species are available throughout the year, it may be useful to keep their *in vitro* culture to produce compounds with biological interest. Thus, it becomes important the study of the antioxidant potential of the most commonly consumed mushrooms, their *in vitro* maintenance and the comparative study between samples obtained under *in vivo* and *in vitro* conditions.

In the present work, the main objective was to evaluate the antioxidant properties and phenolic profile of the most popular cultivated edible mushroom species marketed in Portugal and all over the world as fresh mushrooms: *Agaricus bisporus* (white and brown mushrooms), *Pleurotus ostreatus* (oyster mushroom), *Pleurotus eryngii* (king oyster mushroom), and *Lentinula edodes* (shiitake). This study was carried out in fresh samples and in the mycelia obtained by *in vitro* culture allowing, for the first time, a comparison between them.

MATERIAL AND METHODS

Samples. The mushroom species were obtained in local supermarkets (Bragança, Northeast Portugal) in March and April 2011. From each package, 2-3 samples of mushrooms were selected for *in vitro* isolation and culture. An internal part of the mushroom mycelium was excised and isolated in a Petri dish containing Melin-Norkrans culture medium (MMN) (Marx, 1969). After the isolation procedure and growth in solid medium (**Figure 1**), inocula of each mushroom species were introduced in flasks (7.5 diameter; 8.5 height) containing the same medium but in liquid form, and lead until obtain the necessary mass for the analysis: 25 days for *Pleurotus ostreatus* and *Pleurotus eryngii*, 48 days for *Lentinula edodes* and 53 days for *Agaricus bisporus*. The mycelium was further recovered from the medium.

All the samples (mushrooms and mycelia) were lyophilised (FreeZone 4.5 model 7750031, Labconco, Kansas, USA), reduced to a fine dried powder (20 mesh) and mixed to obtain homogenate samples for further analysis.

Standards and Reagents. Acetonitrile 99.9% was of HPLC grade from Lab-Scan (Lisbon, Portugal). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Phenolic standards (gallic, *p*-hydroxybenzoic, *p*-coumaric, protocatechuic and cinnamic acids) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma (St. Louis, MO, USA). Methanol and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

Evaluation of Antioxidant Activity

General. The lyophilized powder (1.5 g for mushrooms and 0.5 g for mycelia) was stirred with methanol (40 ml and 20 ml, respectively) 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 (Reis 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.

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.

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 50ºC 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 ELX800 Microplate Reader (Bio-Tek Instruments, Inc; Winooski, United States). The reducing power was obtained directly from the absorbances.

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: % 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 DPPH solution.

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 40ºC 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 50ºC in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm. β-Carotene bleaching inhibition was calculated using the following equation: (β-carotene content after 2h of assay/initial β-carotene content) × 100.

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* for10 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 37ºC for 1 h. The reaction was stopped by the addition of trichloro acetic 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.

Analysis of phenolic compounds

Extraction procedure. The lyophilized samples (~1.5 g) were extracted with methanol:water (80:20, v/v; 30 ml) at -20°C for 1.5h. After sonication for 15 min, the extract was filtered through Whatman nº 4 paper. The residue was then re-extracted with an additional 30 ml portion of the methanol:water mixture. Combined extracts were evaporated under reduced pressure to remove the methanol. The aqueous phase was washed with diethyl ether (2 × 20 ml) and ethyl acetate (2 × 20 ml). To the combined aqueous phases, anhydrous sodium sulphate was added and the extracts were filtrated though Whatman nº 4 paper, evaporated to dryness and then re-dissolved in

methanol:water (80:20, v/v) (Barros et al., 2009). The extracts (1 ml) were filtered through a 0.22 µm disposable LC filter disk for HPLC analysis.

Chromatographic analysis. The analysis was performed using a Shimadzu 20A series ultra fast liquid chromatograph (UFLC, Shimadzu Coperation, Kyoto, Japan). Separation was achieved on an AQUA® (Phenomenex) reverse phase C18 column (5 µm, 150 mm × 4.6 mm i.d) thermostatted at 35 ºC. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was 10% B to 15% B over 5 min, 15–25% B over 5 min, 25–35% B over 10 min, isocratic 50% B for 10 min, and re-equilibration of the column, using a flow rate of 0.5 ml/min. Detection was carried out in a photodiode array detector (PDA), using 280 nm as the preferred wavelength. The phenolic compounds were characterised according to their UV and retention times compared with commercial standards. For the quantitative analysis of phenolic compounds, a calibration curve was obtained by injection of known concentrations (5-80 µg/ml) of different standards compounds: cinnamic acid ($y=266.32x+45.75$; $R^2=0.9997$); *p*-coumaric acid ($y=23.75x+1.85$; $R^2=1$); gallic acid ($y=18.05x-36.93$; $R^2=0.9981$); *p*-hydroxybenzoic acid ($y=358.92x+47.14$; $R^2=0.9999$); protocatechuic acid ($y=263.87x+29.85$; $R^2=0.9999$). The results were expressed as µg per g of dry weight (dw).

Statistical analysis. For each one of the mushroom species three samples were used 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 analysis was carried out using SPSS v. 18.0 program.

RESULTS AND DISCUSSION

The response of antioxidants to different radical or oxidant sources may be different. Therefore, no single assay accurately reflects the mechanism of action of all radical sources or all antioxidants in a complex system (Prior et al., 2005). This way, the antioxidant activity of the samples was assessed through five different methods.

To evaluate the reducing power of the studied species, two methodologies were performed: Folin-Ciocalteu and Ferricyanide/Prussian blue assays. *Folin-Ciocalteu* reagent contains, in its constitution, phosphomolybdic/phosphotungstic acid complexes. The assay is based on the transfer of electrons in alkaline medium from phenolic compounds and other reducing species to molybdenum, forming blue complexes that can be monitored spectrophotometrically at 750–765 nm (Magalhães et al., 2008), according to the following equations:

$$Na_2WO_4/Na_2MoO_4 \rightarrow (\text{phenol-}MoW_{11}O_{40})^{-4}$$

$$\text{Mo (VI) (yellow)} + e^- \rightarrow \text{Mo(V) (blue)}$$

The reducing power assay is based on the ability of phenolics to reduce yellow ferric form to blue ferrous form by the action of electron-donating antioxidants (Benzie et al., 1999). When the antioxidant species is either Fe (III) or $Fe(CN)_6^{3-}$ in the composite ferricyanide reagent, either Fe(II) or $Fe(CN)_6^{4-}$ is formed as the reduction product with the antioxidant, and combines with the other reagent component to produce Prussian blue, $KFe[Fe(CN)_6]$, as the colored product. Thus, when Fe^{3+} is used along with $Fe(CN)_6^{3-}$ as the oxidizing agent, either one of the two reaction pairs occur, both ending up with the same colored product (Berker et al., 2007):

$$Fe^{3+} + \text{antioxidant} \leftrightarrow Fe^{2+} + \text{oxidized antioxidant},$$

$$Fe^{2+} + Fe(CN)_6^{3-} \leftrightarrow Fe[Fe(CN)_6]^-$$

or

$$Fe(CN)_6^{3-} + \text{antioxidant} \leftrightarrow Fe(CN)_6^{4-} + \text{oxidized antioxidant},$$

$$Fe(CN)_6^{4-} + Fe^{3+} \leftrightarrow Fe[Fe(CN)_6]^-$$

The resulting blue color could be measured spectrophotometrically at 700 nm and it is taken as linearly related to the total reducing capacity of electron-donating antioxidants (Huang et al., 2005).

In the present work, the mushroom species with the highest reducing power, measured by total phenolics content, was *Agaricus bisporus* (brown) (37.33 mg GAE/g extract), while *Pleurotus eryngii* showed the lowest value (7.14 mg GAE/g extract). Concerning the mycelia, *Lentinula edodes* presented the highest reducing power (12.53 mg GAE/g extract), while *Agaricus bisporus* (white) gave the lowest one (4.22 mg GAE/g extract) (**Table 1**). Regarding the Ferricyanide/Prussian blue assay, the results were similar as *Agaricus bisporus* (brown) revealed the highest reducing power (lowest EC_{50} value 1.47 mg/ml), and *Pleurotus eryngii* the lowest one (highest EC_{50} value 3.72 mg/ml). Concerning the mycelia, the highest reducing power was revealed by *Lentinula edodes* (2.26 mg/ml), while *Agaricus bisporus* (white) showed the lowest reducing capacity (8.12 mg/ml) (**Figure 2**, **Table 1**).

The result obtained for *P. ostreatus* mushroom in Folin Ciocalteu assay (12.54 mg GAE/g extract; **Table 1**) was lower than the one reported for methanolic extracts from Chinese samples (15.7 mg GAE/g extract; Yang et al., 2002), but much higher than the results described by Oke and Aslim (2011) for water (1.42 mg GAE/g extract) and methanol (0.93 mg GAE/g extract) extracts prepared with wild Turkish samples. The studied sample of *L. edodes* mushroom also gave a slightly higher result (8.84 mg GAE/g extract; **Table 1**) than the one obtained for methanolic extracts of Chinese samples (8.51 mg GAE/g extract, calculated from the result expressed in μmol GAE/mg

extract; Cheung et al., 2003). Nevertheless, the result described by Yang et al. (2002) for methanolic extracts from Chinese samples (9.11 mg GAE/g extract) was higher than the one obtained herein. The studied methanolic extract of *P. ostreatus* gave higher reducing power, measured by the Ferricyanide/Prussian blue assay (EC_{50} value 3.31 mg/ml; **Table 1**), than ethanolic extracts prepared with samples from India (~4 mg/ml) (Jayakumar et al., 2011). The opposite was observed when compared to methanolic extracts obtained with samples from China (~2.5 mg/ml) (Yang et al., 2002). The studied *L. edodes* mushroom also gave higher reducing power (EC_{50} value 2.62 mg/ml; **Table 1**) than those extracts (~5 mg/ml) (Yang et al., 2002).

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical is a long-lived organic nitrogen radical with a deep purple color. In the performed method, the purple chromogen radical is reduced by antioxidant/reducing compounds to the corresponding pale yellow hydrazine, according to the following equation:

$$Z^{\bullet} + AH \rightarrow ZH + A^{\bullet}$$

where $Z^{\bullet}$ represents the DPPH radical and the donor molecule is represented by AH. In this reaction, ZH is the reduced form and $A^{\bullet}$ is the free radical produced in this first step. This latter radical will then undergo further reactions which control the overall stoichiometry, that is, the number of molecules of DPPH reduced (decolorised) by one molecule of the reductant (Molyneux, 2004).

This reduction could be monitored measuring the absorbance decrease at 515-528 nm until the absorbance remains stable in organic media (Karadag et al., 2009), and free radical scavenging activity can be determined by the discoloration of the DPPH solution (Ndhlala et al., 2010).

Agaricus bisporus (brown) was the species that presented the highest radical scavenging activity both fruiting body and mycelium (2.29 and 8.71 mg/ml, respectively), despite statistical similarities with other samples such as *Agaricus bisporus* (white) fruiting body (3.13 mg/ml) and *Lentinula edodes* mycelium (7.82 mg/ml). *Pleurotus eryngii* was the mushroom species with the lowest radical scavenging activity (8.67 mg/ml), while among mycelia *Pleurotus ostreatus* gave also the lowest activity (58.13 mg/ml) (**Figure 3**, **Table 1**).

The studied methanolic extract of *P. ostreatus* gave a higher DPPH scavenging activity (EC_{50} value 6.54 mg/ml; **Table 1**) than water (~11.25 mg/ml) and ethanol (~22.5 mg/ml) extracts of samples from Thailand (Chirinang and Intarapichet, 2009), and hot-water extracts of samples from Taiwan (24.8 mg/ml) (Tsai et al., 2009). Nevertheless, the results obtained herein were worse than the ones reported for ethanolic extracts of samples from Taiwan (5.58 mg/ml) (Tsai et al., 2009), methanolic extracts of samples from China (~4.5 mg/ml) (Yang et al., 2002), and even water (0.545 mg/ml) and methanolic (0.909 mg/ml) extracts from wild Turkish samples (Oke and Aslim, 2011). Regarding *L. edodes* mushroom, it gave much higher DPPH scavenging activity (50% at 6.43 mg/ml; **Table 1**) than methanolic extracts of Chinese samples studied by Cheung et al. (2003) (29.4% at 9 mg/ml), but lower than methanolic extracts of Chinese samples studied by Yang et al. (2002) (50% at ~4.75 mg/ml).

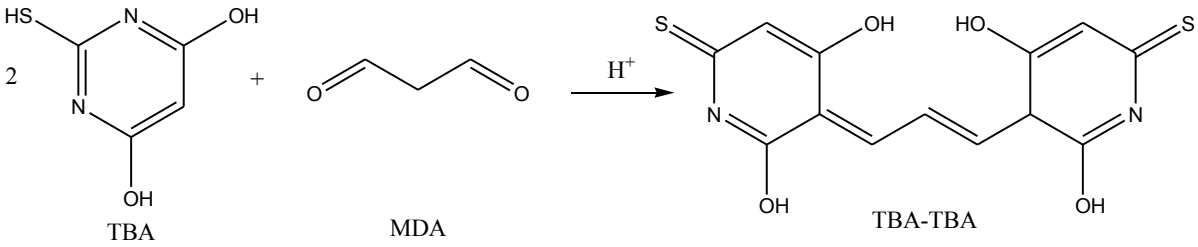
The evaluation of the lipid peroxidation inhibition of the studied species was carried out through two different methods: β-carotene/linoleate and TBARS assays. In the first method, the antioxidant capacity is determined by measuring the inhibition of the production of volatile organic compounds and the formation of conjugated diene hydroperoxides due to linoleic acid oxidation, which bleach the β-carotene in the emulsion. The reaction mechanism involves the bleaching of carotenoids via heat-

induced oxidation and the resultant discoloration being inhibited or diminished by antioxidants that donate hydrogen atoms to quench radicals. Absorbance of β-carotene is measured at 470 nm (Ndhlala et al., 2010). Thus, the reaction mechanism can be described as follows (Kaur and Geetha, 2006):

$$\beta\text{-carotene-H (orange)} + ROOH \rightarrow \beta\text{-carotene}^{\cdot} \text{ (discoloured)} + ROOH$$

$$\beta\text{-carotene-H (orange)} + ROOH + AH \rightarrow \beta\text{-carotene-H (orange)} + ROOH + A^{\cdot}$$

A frequently used marker for lipid peroxidation consists in the quantification of the loss of reactants, the formation of radicals and the formation of primary and secondary oxidation products, depending on the stage of oxidation. Lipid oxidation involves concomitant formation and degradation of several products. Thiobarbituric acid (TBA) reactive substances (TBARS) methodology is based on formation of secondary oxidation products. It measured the formation of malondialdehyde (MDA) and other oxo-compounds that can be measured spectrophotometrically at 532 nm (Ndhlala et al., 2010):



As previously described, the lipid peroxidation inhibition was assessed through two different methods. Relatively to β-carotene/linoleate assay, all the samples revealed similar β-carotene bleaching inhibitions (**Figure 4**; **Table 1**), unless *Pleurotus ostreatus* mycelium that gave a lowest capacity (highest EC_{50} value 16.95 mg/ml; **Table 1**).

The studied *Pleurotus ostreatus* mushroom methanolic extract gave lower β-carotene bleaching inhibition (50% at 2.74 mg/ml; **Table 1**) than water (85.94% at 2 mg/ml) and methanol (78.12% at 2 mg/ml) extracts obtained from Turkish samples (Oke and Aslim, 2011). The opposite was observed for the studied sample of *L. edodes* mushroom that gave much higher β-carotene bleaching inhibition (50% at 3.92 mg/ml; **Table 1**) than methanolic extracts of Chinese samples (45.8% at 20 mg/ml; Cheung et al., 2003).

Agaricus bisporus (brown) and *Lentinula edodes* were the mushroom species that revealed the highest lipid peroxidation inhibition by TBARS assay (1.45 and 1.64 mg/ml, respectively). The lowest results were obtained by the species *Pleurotus eryngii* both *in vivo* (fruiting body) and *in vitro* (mycelium) samples (3.95 and 21.03 mg/ml, respectively). The mycelia that shown the highest lipid peroxidation inhibition were from *Agaricus bisporus* (white), *Pleurotus ostreatus* and *Lentinula edodes* with no statistical difference between them (0.87, 1.00 and 1.66 mg/ml, respectively) (**Figure 5**, **Table 1**). The studied methanolic extract of *Pleurotus ostreatus* gave higher lipid peroxidation inhibition, measured by the TBARS assay (EC_{50} value 2.58 mg/ml; **Table 1**), than ethanolic extracts prepared with samples from India (~6 mg/ml) (Jayakumar et al., 2011).

In this work, some phenolic compounds namely gallic, protocatechuic, *p*-hydroxybenzoic and *p*-coumaric acids, as well as a related compound, cinnamic acid, were also searched in the studied species. All these compounds were found both in the samples obtained under *in vivo* and *in vitro* conditions (**Table 2**). Gallic acid was detected in *Agaricus bisporus* (white) mushroom (62.76 µg/g dw) and its corresponding mycelium (30.77 µg/g dw), but also in *Pleurotus eryngii* mycelium (50.91 µg/g dw) (**Table 2**). Protocatechuic acid was mainly found in *Pleurotus ostreatus* mushroom (0.77 µg/g dw) but also in *Agaricus bisporus* (brown) mycelium (0.06 µg/g dw),

Pleurotus eryngii mushroom (0.06 µg/g dw) and in both *Lentinula edodes* samples (0.36 and 0.73 µg/g dw) (**Table 2**). *p*-Hydroxybenzoic acid was found in almost all samples except in *Agaricus bisporus* mushrooms (white and brown); however, it was quantified in these species obtained *in vitro* (0.02 and 0.21 µg/g dw, respectively) (**Table 2**). *p*-Coumaric acid was detected mainly in *Agaricus bisporus* (white) mushroom and its mycelium (2.31 and 3.66 µg/g dw, respectively) and cinnamic acid was specially found in *Pleurotus ostreatus* mycelium (9.65 µg/g dw) and undetected in *Agaricus bisporus* (brown) and *Lentinula edodes* mycelia (**Table 2**). Despite some contribution to the antioxidant properties of the studied samples, it was not observed a correlation between the identified phenolic acids and the antioxidant activity of those samples. In fact it is well known that non-flavonoid phenolic compounds present less antioxidant activity than flavonoids.

With the results obtained, it can be concluded that phenolic acids and cinnamic acid were detected both in mushrooms and mycelia, mainly in *Agaricus bisporus* (white), *Pleurotus ostreatus* and *Lentinula edodes* species. Other authors also compared phenolic acids contents in some cultivated mushrooms, *Agaricus bisporus* (white and brown) and *Lentinula edodes* (Mattila et al., 2001), detecting higher amounts of cinnamic acid in *Agaricus bisporus* (white) (2.69 µg/g dw) and higher contents of *p*-hydroxybenzoic and protocatechuic acids in *Lentinula edodes* (7.90 and 1.39 µg/g dw). Nevertheless, the results reported by those authors are consistent with our achievements since the highest values of cinnamic acid were detected in *Pleurotus ostreatus* followed by *Agaricus bisporus*, and the lowest ones in *Lentinula edodes*. Higher content of *p*-hydroxybenzoic and protocatechuic acids were also verified in *Pleurotus ostreatus* and *Lentinula edodes* than in *Agaricus bisporus* species.

Overall, in this study, *Agaricus bisporus* (brown) was the mushroom species with highest reducing power and antioxidant potential, since it revealed the lowest EC_{50} values in all the assayed methodologies. Regarding mycelia produced *in vitro*, *Lentinula edodes* was the species with highest reducing power and antioxidant activity. Thus, there is not a coherency between the commercial mushrooms (*in vivo* samples) and the corresponding mycelium obtained *in vitro*. The different conditions of growth certainly influenced their chemical constitution which leads to divergent antioxidant effects and phenolic acids profile and amounts. Nevertheless, comparing mushrooms with the corresponding mycelium, in general, the species *in vivo* gave highest reducing power, free radical scavenging activity and lipid peroxidation inhibition properties than the *in vitro* samples (after the studied times of growth). The main exceptions were *Pleurotus eryngii* and *Lentinula edodes*.

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Table 1. Reducing power, scavenging activity and lipid peroxidation inhibition of the studied edible mushrooms.

Species	Sample	Reducing power		Scavenging activity	Lipid peroxidation inhibition	
		Folin-Ciocalteu assay (mg GAE/g extract)	Ferricyanide/Prussian blue assay (EC_{50}; mg/ml)	DPPH scavenging activity assay (EC_{50}; mg/ml)	β-carotene/linoleate assay (EC_{50}; mg/ml)	TBARS assay (EC_{50}; mg/ml)
Agaricus bisporus (white)	Mushroom	23.34 ± 0.36 b	1.80 ± 0.03 i	3.13 ± 0.09 f	3.42 ± 1.35 b	2.87 ± 0.50 cd
	Mycelium	4.22 ± 0.04 f	8.12 ± 0.06 a	39.68 ± 1.60 b	2.38 ± 0.60 b	0.87 ± 0.76 f
Agaricus bisporus (brown)	Mushroom	37.33 ± 0.23 a	1.47 ± 0.06 j	2.29 ± 0.06 f	4.85 ± 0.17 b	1.45 ± 0.08 f
	Mycelium	8.06 ± 0.53 ed	4.02 ± 0.05 c	8.71 ± 0.19 d	0.15 ± 0.00 b	3.64 ± 0.86 cb
Pleurotus ostreatus	Mushroom	12.54 ± 0.18 c	3.31 ± 0.03 f	6.54 ± 0.16 e	2.74 ± 0.16 b	2.58 ± 0.86 ed
	Mycelium	5.19 ± 0.14 f	4.73 ± 0.18 b	58.13 ± 3.02 a	16.95 ± 21.95 a	1.00 ± 0.28 f
Pleurotus eryngii	Mushroom	7.14 ± 2.01 e	3.72 ± 0.09 e	8.67 ± 0.12 d	4.68 ± 0.60 b	3.95 ± 0.58 b
	Mycelium	9.11 ± 0.23 d	3.81 ± 0.02 d	25.40 ± 0.33 c	1.43 ± 0.60 b	21.03 ± 0.45 a
Lentinula edodes	Mushroom	8.84 ± 0.91 d	2.62 ± 0.05 g	6.43 ± 0.66 e	3.92 ± 0.32 b	1.64 ± 0.40 ef
	Mycelium	12.53 ± 0.30 c	2.26 ± 0.03 h	7.82 ± 0.56 ed	4.65 ± 0.68 b	1.66 ± 0.87 ef

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.

Table 2. Phenolic acids and cinnamic acid of the studied edible mushrooms.

Species	Sample	Gallic acid (µg/g dw)	Protocatechuic acid (µg/g dw)	*p*-Hydroxybenzoic acid (µg/g dw)	*p*-Coumaric acid (µg/g dw)	Cinnamic acid (µg/g dw)
Agaricus bisporus (white)	Mushroom	62.76 ± 12.55 a	nd	nd	2.31 ± 0.06 b	0.38 ± 0.02 cb
	Mycelium	30.77 ± 4.09 c	nd	0.02 ± 0.00 d	3.66 ± 0.18 a	0.76 ± 0.03 b
Agaricus bisporus (brown)	Mushroom	nd	nd	nd	nd	0.09 ± 0.00 c
	Mycelium	nd	0.06 ± 0.01 d	0.21 ± 0.00 c	nd	nd
Pleurotus ostreatus	Mushroom	nd	0.77 ± 0.02 a	1.56 ± 0.06 a	0.81 ± 0.03 d	0.23 ± 0.02 cb
	Mycelium	nd	nd	0.05 ± 0.00 d	nd	9.65 ± 0.86 a
Pleurotus eryngii	Mushroom	nd	0.06 ± 0.03 d	0.10 ± 0.01 dc	1.04 ± 0.04 c	0.20 ± 0.01 c
	Mycelium	50.91 ± 6.01 b	nd	vestigial	nd	0.05 ± 0.04 c
Lentinula edodes	Mushroom	nd	0.36 ± 0.01 c	1.57 ± 0.16 a	nd	0.02 ± 0.00 c
	Mycelium	nd	0.73 ± 0.01 b	0.36 ± 0.00 b	nd	nd

In each row, different letters mean significant differences between species (p<0.05).

Figure 1. Information about the studied edible mushrooms produced in Portugal.

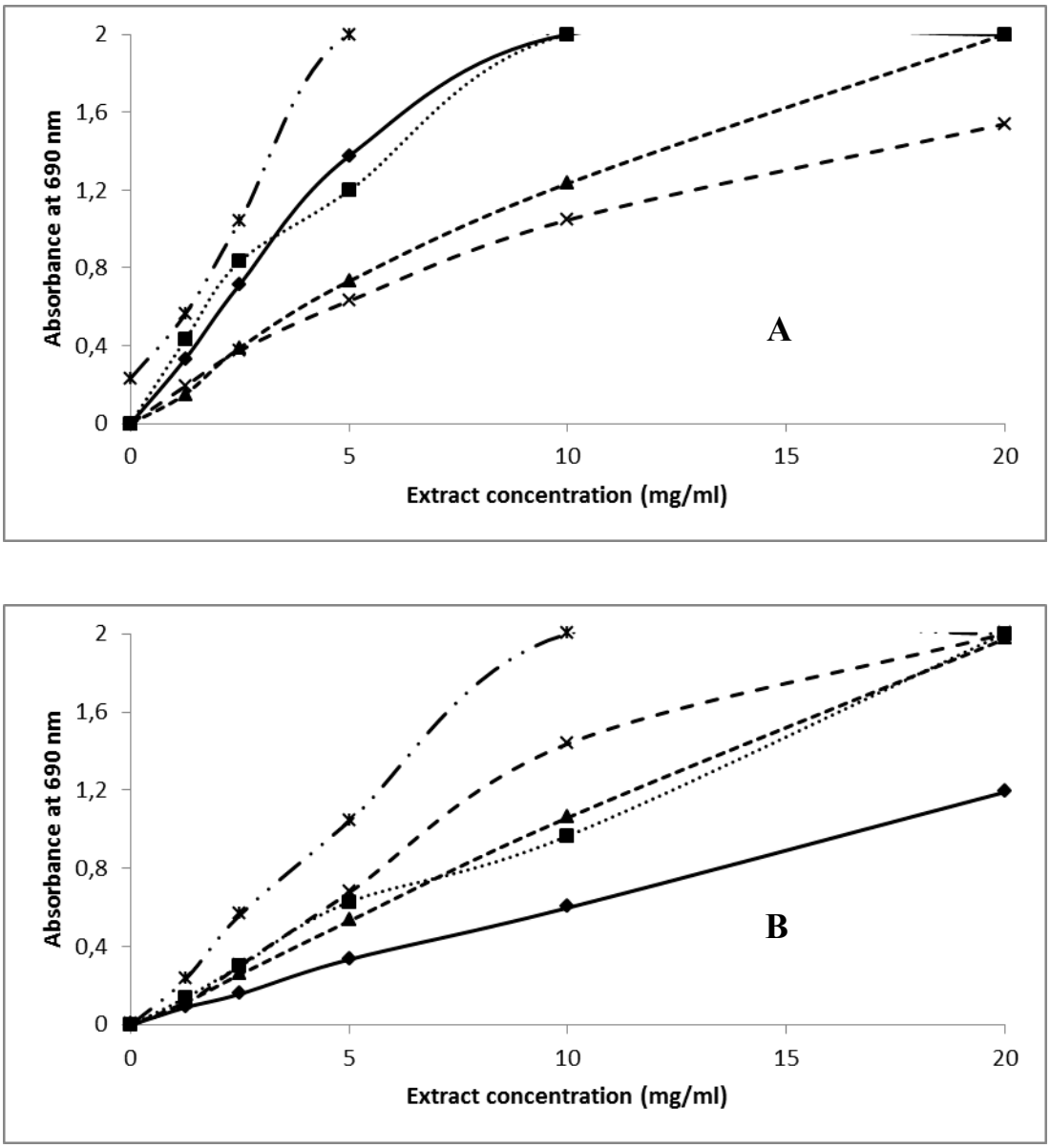
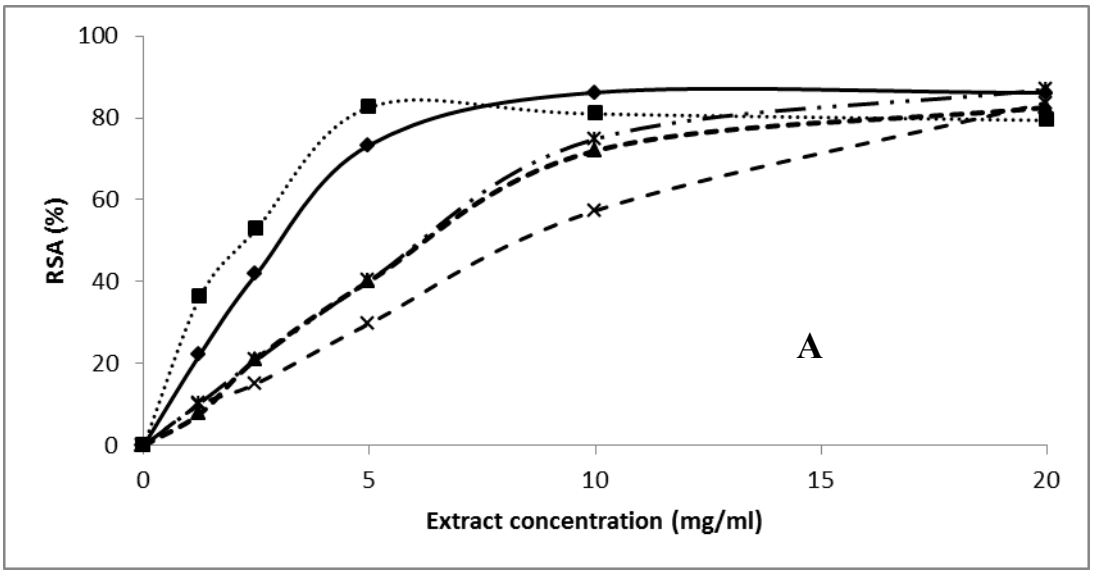


Figure 2. Reducing power of the mushroom samples (**A**) and the corresponding mycelia (**B**). Each value is expressed as mean ± SE (n = 9). *Agaricus bisporus* (white) ( ), *Agaricus bisporus* (brown) ( ), *Pleurotus ostreatus* ( ), *Pleurotus eryngii* ( ), Lentinula edodes ( ).



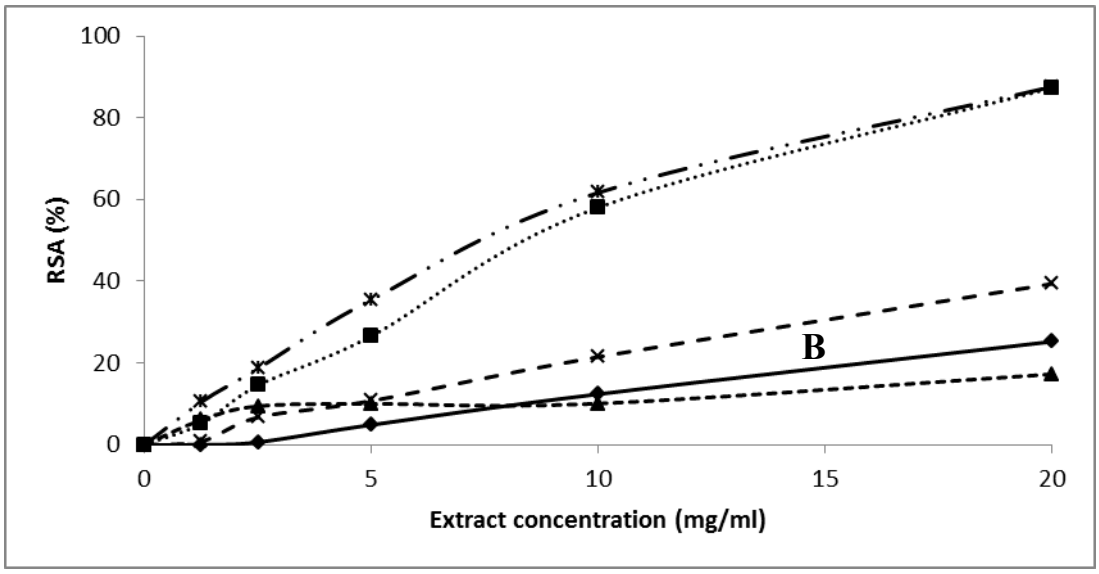


Figure 3. Radical scavenging activity (%) of the mushroom samples (**A**) and the corresponding mycelia (**B**). Each value is expressed as mean ± SE (n = 9). *Agaricus bisporus* (white) (—◆—), *Agaricus bisporus* (brown) (···■···), *Pleurotus ostreatus* (--▲--), *Pleurotus eryngii* (– × –), *Lentinula edodes* (—✳·).

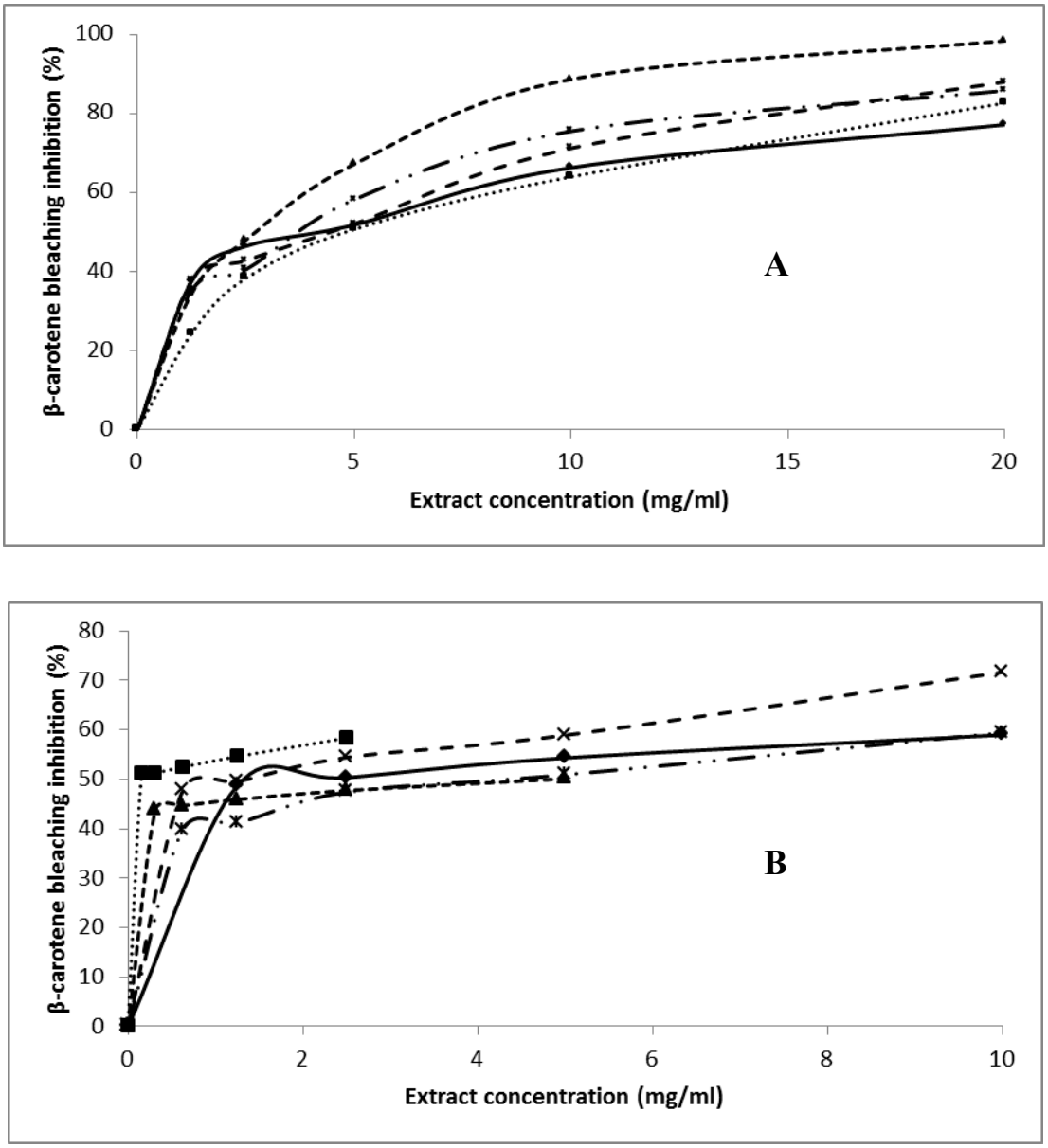


Figure 4. β-carotene bleaching inhibition of the mushroom samples (**A**) and the corresponding mycelia (**B**). Each value is expressed as mean ± SE (n = 9). *Agaricus bisporus* (white) ( ), *Agaricus bisporus* (brown) ( ), *Pleurotus ostreatus* ( ), *Pleurotus eryngii* ( ), *Lentinula edodes* ( ).

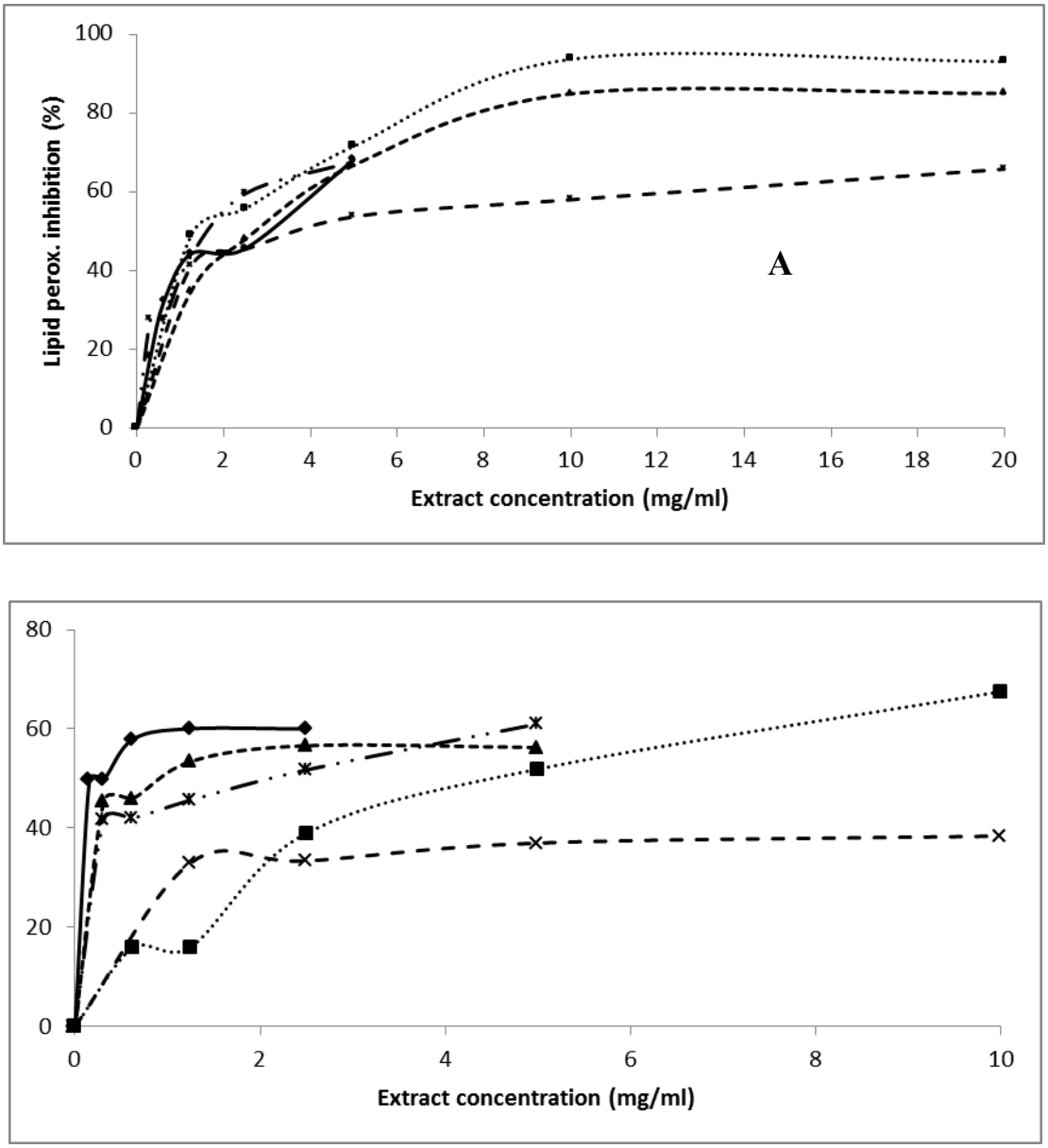


Figure 5. Lipid peroxidation (%) measured by the TBARS assay of the mushroom samples (1) and the corresponding mycelia (2). Each value is expressed as mean ± SE (n = 9). *Agaricus bisporus* (white) (—◆—), *Agaricus bisporus* (brown) (···■···), *Pleurotus ostreatus* (--▲--), *Pleurotus eryngii* (– ✱ –), *Lentinula edodes* (—✱· ·).