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Total phenols, ascorbic acid, β-carotene and lycopene in Portuguese wild edible mushrooms and their antioxidant activities

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Abstract

The antioxidant activities of three Portuguese wild edible mushroom species, *Leucopaxillus giganteus, Sarcodon imbricatus,* and *Agaricus arvensis*, were evaluated. Methanolic extracts were screened for their reducing power, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging capacity, inhibition of erythrocytes hemolysis and antioxidant activity using the β-carotene linoleate model system. The amounts of ascorbic acid, β-carotene and lycopene found in the mushroom extracts were very low. Otherwise, the high contents of phenolic compounds might account for the good antioxidant properties found in all species. *L. giganteus* had the highest content of phenols and proved to be the most active, presenting lower EC_{50} values in all the antioxidant activity assays.

Keywords: Mushrooms; Antioxidant; Phenolics; Ascorbic acid; Beta-carotene; Lycopene

1. Introduction

Free radical formation is associated with the normal natural metabolism of aerobic cells. The oxygen consumption inherent in cell growth leads to the generation of a series of oxygen free radicals. The interaction of these species with molecules of a lipidic nature produces new radicals: hydroperoxides and different peroxides (Aust & Sringen, 1982; Pryor, Lightsey, & Prier, 1982; Torel, Cillard, & Cillard, 1986). This group of radicals (superoxide, hydroxyl and lipoid peroxides) may interact with biological systems in a clearly cytotoxic manner. In this respect, flavonoids and phenols have been shown to possess an important antioxidant activity toward these radicals, which is principally based on the redox properties of their phenolic hydroxyl groups and the structural relationships between different parts of their chemical structure (Bors & Saran, 1987; Visioli, Bellomo, & Galli, 1998a, Visioli, Bellosta, & Galli, 1998b). Free radicals and their uncontrolled production, in fact, are responsible for several pathological processes, such as certain tumours (prostate and colon cancers) (Keys, 1995) and coronary heart disease (Lipworth, Martinez, Angell, Hsien, & Trichopoulos, 1997).

In the past few years, the suspected toxicity of some synthetic compounds used in food has raised interest in natural products (Fukushima & Tsuda, 1985; Stone, Leclair, Ponder, Bagss, & Barret-Reis, 2003). Some industries, such as those related to food additive production, cosmetics, and pharmaceuticals, have increased their efforts in preparing bioactive compounds from natural products by extraction and purification. Antioxidant compounds can scavenge free radicals and increase shelf life by retarding the process of lipid peroxidation, which is one of the major reasons for deterioration of food products during processing and storage (Halliwell, 1997; Halliwell & Gutteridge, 1999). Thus a need for identifying alternative natural and safe sources of food antioxidants has been created, and

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the search for natural antioxidants, especially of plant origin, has notably increased in recent years (Skerget et al., 2005).

Vegetables and fruits are rich sources of antioxidants, such as vitamin A, vitamin C, vitamin E, carotenoids, polyphenolic compounds and flavonoids (Diplock et al., 1998), which prevent free radical damage, reducing risk of chronic diseases. Thus, the consumption of dietary antioxidants from these sources is beneficial in preventing cardiovascular diseases, especially atherosclerosis (Hu, 2000).

Mushrooms have been used for traditional foods and medicines in Asia (Chang, 1996). Mushrooms contain various polyphenolic compounds recognized as an excellent antioxidant due to their ability to scavenge free radicals by single-electron transfer (Hirano et al., 2001). Some common edible mushrooms, which are widely consumed in Asian culture, have currently been found to possess antioxidant activity, which is well correlated with their total phenolic content (Cheung & Cheung, 2005; Cheung, Cheung, & Ooi, 2003; Lo & Cheung, 2005; Mau, Chang, Huang, & Chen, 2004, 2002; Yang, Lin, & Mau, 2002; Yen & Hung, 2000).

Recently, we described the first study on the antioxidant activity of Portuguese wild edible mushrooms (*Lactarius deliciosus* and *Tricholoma portentosum*), comparing the entire mushroom, the cap and stipe DPPH$^{\cdot}$-scavenging capacities and reducing powers (Ferreira, Baptista, Vilas-Boas, & Barros, 2007). Herein, we report the antioxidant activity of three new Portuguese wild edible mushroom species (*Leucopaxillus giganteus, Sarcodon imbricatus, Agaricus arvensis*), and their correlation with phenol, ascorbic acid, beta-carotene and lycopene contents. For the screening of mushroom antioxidant properties, we evaluated their reducing power, DPPH radical scavenging activity and inhibition of erythrocyte hemolysis, and we also used the β-carotene linoleate model system.

2. Materials and methods

2.1. Standards and reagents

Standards BHA (2-*tert*-butyl-4-methoxyphenol), TBHQ (*tert*-butylhydroquinone), L-ascorbic acid, α-tocopherol and gallic acid were purchased from Sigma (St. Louis, MO, USA). 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). Methanol was obtained from Pronalab (Lisbon, Portugal). Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

2.2. Samples

Samples of *L. giganteus* and *A. arvensis* were colleted under grassland whereas *S. imbricatus* was collected under live pine trees (*Pinus* sp.), in Bragança (northeast of Portugal), in autumn 2005. After collection, the mushrooms were grouped by taxon and were air-dried in a liophylizator (Ly-8-FM-ULE, Snijders, HOLLAND) before analysis. Taxonomic identification was done according to several authors (Bon, 1988; Courtecuisse, 1999; Courtecuisse & Duhem, 1995; Marchand, 1971–1986; Moser, 1983) and representative voucher specimens were deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança.

2.3. Sample preparation

A fine dried mushroom powder (20 mesh) sample (5 g) was continuously extracted with methanol in a Soxhlet apparatus for 24 h. The methanolic extract was evaporated to dryness at 40 °C and redissolved in methanol at a concentration of 5 mg/ml, and stored at 4 °C prior to further use.

2.4. Determination of antioxidant components

Phenolic compounds in the mushroom methanolic extracts were estimated by a colorimetric assay, based on procedures described by Singleton and Rossi with some modifications (Singleton & Rossi, 1965). Briefly, 1 ml of sample was mixed with 1 ml of Folin and Ciocalteu's phenol reagent. After 3 min, 1 ml of saturated sodium carbonate solution was added to the mixture and it was adjusted to 10 ml with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm (Analytikijena 200-2004 spectrophotometer). Gallic acid was used to calculate the standard curve (0.01–0.4 mM). Estimation of the phenolic compounds was carried out in triplicate. The results were mean values ± standard deviations and expressed as mg of gallic acid equivalents (GAEs) per g of extract.

Ascorbic acid was determined according to the method of Klein and Perry (1982). The dried methanolic extract (100 mg) was extracted with 10 ml of 1% metaphosphoric acid for 45 min at room temperature and filtered through Whatman No. 4 filter paper. The filtrate (1 ml) was mixed with 9 ml of 2,6-dichlorophenolindophenol and the absorbance was measured within 30 min at 515 nm against a blank. Content of ascorbic acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid (0.020–0.12 mg/ml). The assays were carried out in triplicate; the results were mean values ± standard deviations and expressed as mg of ascorbic acid/g of extract.

β-Carotene and lycopene were determined according to the method of Nagata and Yamashita (1992). The dried methanolic extract (100 mg) was vigorously shaken with 10 ml of acetone–hexane mixture (4:6) for 1 min and filtered through Whatman No. 4 filter paper. The absorbance of the filtrate was measured at 453, 505, 645 and 663 nm. Contents of β-carotene and lycopene were calculated according to the following equations: lycopene (mg/100 ml) $= -0.0458\ A_{663} + 0.372\ A_{505} - 0.0806\ A_{453}$; β-car-

otene (mg/100 ml) = 0.216 A_{663} − 0.304 A_{505} + 0.452 A_{453}. The assays were carried out in triplicate; the results were mean values ± standard deviations and expressed as mg of carotenoid/g of extract.

2.5. Reducing power assay

The reducing power was determined according to the method of Oyaizu (1986). Various concentrations of mushroom methanolic extracts (2.5 ml) were mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After 2.5 ml of 10% trichloroacetic acid (w/v) were added, the mixture was centrifuged at 1000 rpm for 8 min (Centorion K24OR-2003 refrigerated centrifuge). The upper layer (5 ml) was mixed with 5 ml of deionised water and 1 ml of 0.1% of ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm. The assays were carried out in triplicate and the results expressed as mean values ± standard deviations. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 700 nm against extract concentration. BHA and α-tocopherol were used as standards.

2.6. DPPH radical-scavenging assay

The capacity to scavenge the "stable" free radical DPPH· was monitored according to the method of Hatano, Kagawa, Yasuhara, and Okuda (1988). Various concentrations of methanolic extracts from mushrooms (0.3 ml) were mixed with 2.7 ml of methanolic solution containing DPPH radicals (6×10^{-5} mol/l). The mixture was shaken vigorously and left to stand for 60 min in the dark (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: $\%RSA = [(A_{DPPH}\text{-}A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The assays were carried out in triplicate and the results expressed as mean values ± standard deviations. The extract concentration providing 50% inhibition (EC_{50}) was calculated from the graph of RSA percentage against extract concentration. BHA and α-tocopherol were used as standards.

2.7. Assay for erythrocyte hemolysis mediated by peroxyl free radicals

The antioxidant activity of the mushroom methanolic extracts was measured as the inhibition of erythrocyte hemolysis (Miki, Tamia, Mino, Yamamoto, & Niki, 1987). Blood was obtained from a male ram (churra galega transmontana) of body weight ~67 kg. Erythrocytes, separated from the plasma and the buffy coat, were washed three times with 10 ml of 10 mM phosphate buffer saline (PBS) at pH 7.4 (prepared by mixing 10 mM NaH_2PO_4 and Na_2HPO_4, and 125 mM NaCl in 1 l of distilled water) and centrifuged at 1500 *g* for 5 min. During the last washing, the erythrocytes were obtained by centrifugation at 1500 *g* for 10 min. 0.1 ml of a 20% suspension of erythrocytes in PBS was added to 0.2 ml of 200 mM 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) solution (in PBS) and 0.1 ml of mushroom methanolic extracts of different concentrations. The reaction mixture was shaken gently (30 rpm) while being incubated at 37 °C for 3 h. The reaction mixture was diluted with 8 ml of PBS and centrifuged at 3000 *g* for 10 min; the absorbance of its supernatant was then read at 540 nm by a spectrophotometer, after filtration with a syringe filter (cellulose membrane 30 mm, 0.20 μm, Titan). The percentage hemolysis inhibition was calculated by the equation: % hemolysis inhibition = $[(A_{AAPH}\text{–}A_S)/A_{AAPH}] \times 100$, where A_S is the absorbance of the sample containing the mushroom extract, and A_{AAPH} is the absorbance of the control sample containing no mushroom extract. The assays were carried out in triplicate and the results expressed as mean values ± standard deviations. The extract concentration providing 50% inhibition (EC_{50}) was calculated from the graph of hemolysis inhibition percentage against extract concentration. L-ascorbic acid was used as standard.

2.8. Antioxidant assay using the β-carotene linoleate model system

The antioxidant activity of mushroom extracts was evaluated by the β-carotene linoleate model system (Mi-Yae, Tae-Hun, & Nak-Ju, 2003). A solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 ml of chloroform. Two millilitres of this solution were pipetted into a 100 ml round-bottom flask. After the chloroform was removed at 40 °C under vacuum, 40 mg of linoleic acid, 400 mg of Tween 80 emulsifier and 100 ml of distilled water were added to the flask with vigorous shaking. Aliquots (4.8 ml) of this emulsion were transferred into different test tubes containing 0.2 ml of different concentrations of the mushroom 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 was measured at 470 nm using a spectrophotometer. Absorbance readings were then recorded at 20 min intervals until the control sample had changed colour. A blank, devoid of β-carotene, was prepared for background subtraction. Antioxidant activity was calculated using the following equation: antioxidant activity = (β-carotene content after 2 h of assay/initial β-carotene content) × 100. The assays were carried out in triplicate and the results expressed as mean values ± standard deviations. The extract concentration providing 50% antioxidant activity (EC_{50}) was calculated from the graph of antioxidant activity percentage against extract concentration. TBHQ was used as standard.

3. Results and discussion

3.1. Determination of antioxidant components

Table 1 shows the phenol, ascorbic acid, β-carotene and lycopene concentration in the mushroom extracts. Whereas total phenols were the major antioxidant components found in the mushroom extracts, ascorbic acid was found in small amounts (0.13–0.35 mg/g), and β-carotene and lycopene were only found in vestigial amounts (<3 μg/g), which is in agreement with other authors (Mau, Lin, & Song, 2002). These antioxidants were determined in different mushrooms but ascorbic acid and β-carotene were not detected by spectrophotometry and HPLC, respectively. *L. giganteus* extracts showed the highest phenolic content (6.29 ± 0.20 mg/g); the amount found in *A. arvensis* extracts (2.83 ± 0.09 mg/g) was slightly lower than the content found in *S. imbricatus* (3.76 ± 0.11 mg/g). The highest content of total phenols in the *L. giganteus* extracts might account for the better results found for their antioxidant activity. In fact, it had been reported that the antioxidant activity of plant materials is well correlated with the content of phenolic compounds. Polyphenols, such as BHT (butylated hydroxytoluene) and gallate, are known to be effective antioxidants (Velioglu, Mazza, Gao, & Oomah, 1998).

3.2. Reducing power assay

Fig. 1 shows the reducing power of mushroom methanolic extracts as a function of their concentration. In this assay, the yellow colour of the test solution changes to various shades of green and blue, depending on the reducing power of each compound. The presence of reducers (i.e. antioxidants) causes the conversion of the Fe^{3+}/ferricyanide complex used in this method to the ferrous form. Therefore, by measuring the formation of Perl's Prussian blue at 700 nm, we can monitor the Fe^{2+} concentration; a higher absorbance at 700 nm indicates a higher reducing power.

The reducing power of the mushroom methanolic extracts increased with concentration. Reducing powers obtained for all the mushrooms were excellent (Fig. 1); at 5 mg/ml they were above 0.67 and in the order: *L. giganteus* > *S. imbricatus* ~ *A. arvensis*. At 5 mg/ml, reducing powers of methanolic extracts from wild edible mushrooms were 0.67–1.47, and at 1 mg/ml were 0.072–0.26. Reducing powers of BHA at 3.6 mg/ml and α-tocopherol at 8.6 mg/ml were only 0.12 and 0.13, respectively. Methanolic extracts from *A. arvensis* showed only slightly lower reducing power values than did those from *S. imbricatus* (Fig. 1). It was reported that the reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Shimada, Fujikawa, Yahara, & Nakamura, 1992). Accordingly, *L. giganteus* might contain higher amounts of reductone, which could react with free radicals to stabilise and block radical chain reactions.

Table 1
Contents of total phenols, ascorbic acid, β-carotene and lycopene in the mushroom extract

	L. giganteus	*S. imbricatus*	*A. arvensis*
Total phenols (mg/g)	6.29 ± 0.20	3.76 ± 0.11	2.83 ± 0.09
Ascorbic acid (mg/g)	0.13 ± 0.0069	0.16 ± 0.0072	0.35 ± 0.0015
β-Carotene (μg/g)	1.88 ± 0.090	2.53 ± 0.11	2.97 ± 0.12
Lycopene (μg/g)	0.69 ± 0.034	1.3 ± 0.070	1.0 ± 0.049

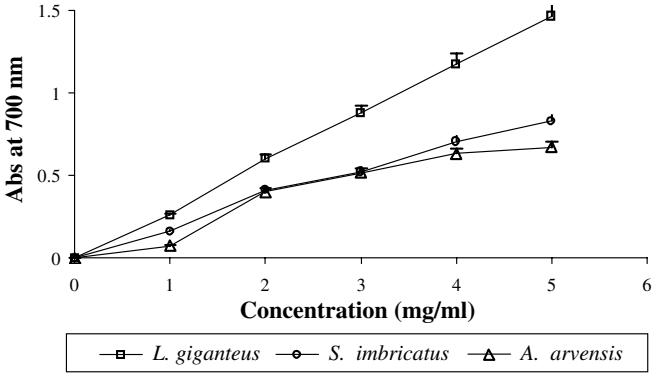

Fig. 1. Reducing power of mushroom methanolic extracts (higher absorbance indicates higher reducing power). Each value is expressed as mean ± standard deviation ($n = 3$).

3.3. Radical-scavenging activity (RSA) assay

The free radical DPPH· possesses a characteristic absorption at 517 nm (purple in colour), which decreases significantly on exposure to radical-scavengers (by providing hydrogen atoms or by electron donation). A lower absorbance at 517 nm indicates a higher radical-scavenging activity of the extract. Free radical-scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation. This test is a standard assay in antioxidant activity studies and offers a rapid technique for screening the RSA of specific compounds or extracts (Amarowicz, Pegg, Rahimi-Moghaddam, Barl, & Weil, 2004).

The RSA values of mushroom methanolic extracts are presented in Fig. 2; results are expressed as the ratio percentage of sample absorbance decrease and the absorbance of DPPH solution in the absence of extract at 517 nm. From the analysis of Fig. 2, we can conclude that the scavenging effects of mushrooms methanolic extracts on DPPH radicals increased with the concentration increase and were excellent for *L. giganteus* (100% at 5 mg/ml), even higher than the scavenging effects of BHA (96% at 3.6 mg/ml) and α-tocopherol (95% at 8.6 mg/ml). The RSA values were good for *S. imbricatus* (80% at 5 mg/ml) and moderate for *A. arvensis* (68.3% at 5 mg/ml).

3.4. Assay for erythrocyte hemolysis mediated by peroxyl free radicals

The oxidative hemolysis in erythrocytes induced by AAPH has been extensively studied as a model for perox-

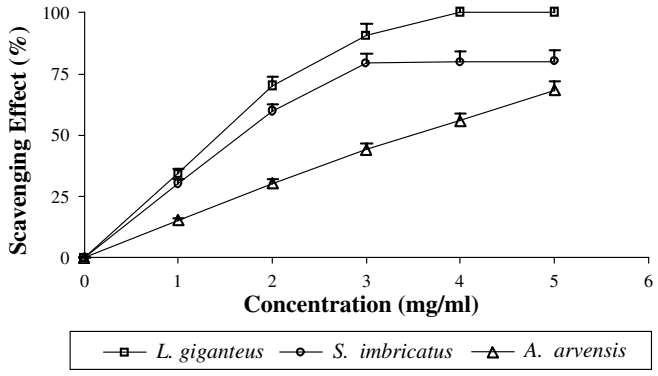

Fig. 2. Scavenging activity (%) on DPPH radicals of mushroom methanolic extracts. Each value is expressed as mean ± standard deviation ($n = 3$).

idative damage in biomembranes (Zhang et al., 1997). AAPH is a peroxyl radical initiator that generates free radicals by its thermal decomposition and will attack the erythrocytes to induce the chain oxidation of lipid and protein, disturbing the membrane organization and eventually leading to hemolysis. In this study, the protective effect of the mushroom extracts on hemolysis by peroxyl radical-scavenging activity was investigated. Fig. 3 shows inhibition percentage of hemolysis, as a result of protection against the oxidative damage of cell membranes of erythrocytes from ram, induced by AAPH. The mushroom extracts inhibited hemolysis of erythrocytes in a concentration-dependent manner. Once more, *L. giganteus* showed higher protective effect against erythrocytes hemolysis (72.8% at 5 mg/ml) than did the other mushrooms (34.2% for *S. imbricatus* and 31.8% for *A. arvensis*). However, the inhibition percentage of the standard L-ascorbic acid on hemolysis of red blood cells was much higher (94.6% at 1 mg/ml) than those of mushroom extracts.

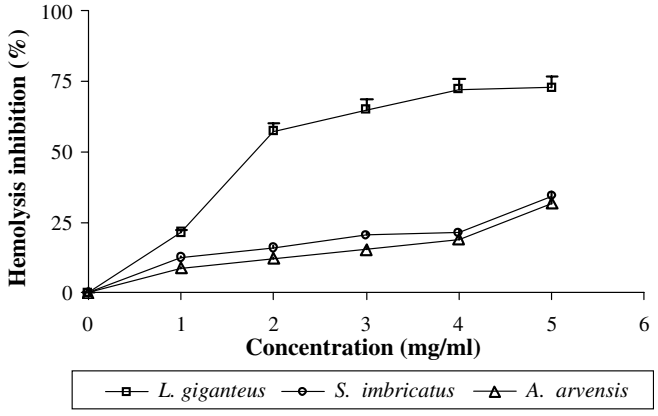

Fig. 3. Hemolysis inhibition (%) of the mushroom methanolic extracts. Each value is expressed as mean ± standard deviation ($n = 3$).

3.5. Antioxidant assay using the β-carotene linoleate model system

Fig. 4 shows the antioxidant activity of the mushroom extracts as measured by the bleaching of β-carotene. The antioxidant activity of carotenoids is based on the radical adducts of carotenoids with free radicals from linoleic acid. The linoleic acid free radical attacks the highly unsaturated β-carotene models. The presence of different antioxidants can hinder the extent of β-carotene-bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system (Jayaprakasha, Singh, & Sakariah, 2001). Accordingly, the absorbance decreased rapidly in samples without antioxidant whereas, in the presence of an antioxidant, they retained their colour, and thus absorbance, for a longer time. Antioxidant activities of *L. giganteus*, *S. imbricatus* and *A. arvensis* extracts increased with their increasing concentration. Their antioxidant activities were 61.4%, 54.3% and 46.7% at 5 mg/ml, but antioxidant activity of TBHQ standard reached 82.2% at 2 mg/ml. It is probable that the antioxidative components in the mushroom extracts can reduce the extent of β-carotene destruction by neutralizing the linoleate free radical and other free radicals formed in the system. Again, *L. giganteus* was the most effective, with an EC_{50} value of 2 mg/ml.

Table 2 shows the EC_{50} values for the antioxidant activity assays obtained from each mushroom methanolic extract.

Overall, *L. giganteus* revealed better antioxidant properties (lower EC_{50} values) than did either *S. imbricatus* or *A. arvensis*, which is in agreement with the higher content of phenols found in the first species. The EC_{50} values obtained for reducing power and scavenging effects on DPPH radicals were better than those for hemolysis inhibition mediated by peroxyl free radicals and for the antioxidant activity using the linoleate-βcarotene system. A relationship between the reducing power, DPPH·-scavenging activity, hemolysis inhibition and β-carotene-bleaching extent was found, indicating that the mechanisms of action of the extracts for the antioxidant activity may be identical,

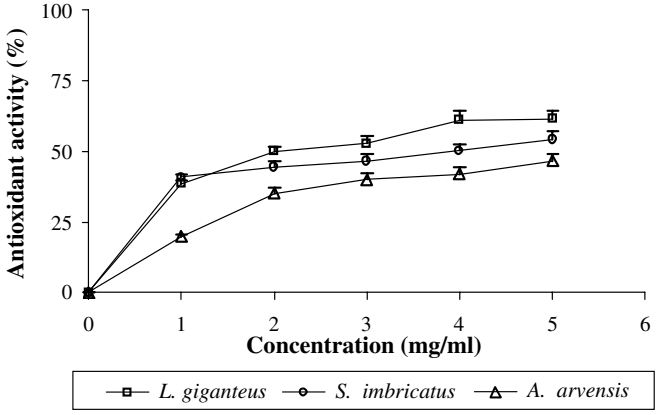

Fig. 4. Antioxidant activity (%) of the mushroom methanolic extracts by β-carotene bleaching method. Each value is expressed as mean ± standard deviation ($n = 3$).

Table 2
EC_{50} values (mg/ml) of mushroom extracts in the antioxidant activity evaluation assays

Samples	Reducing power (EC_{50}[a])	DPPH (EC_{50}[b])	Hemolysis inhibition (EC_{50}[c])	β-Carotene bleaching (EC_{50}[d])
L. giganteus	1.71	1.44	1.80	2.00
S. imbricatus	2.79	1.67	>5	3.97
A. arvensis	2.86	3.50	>5	>5

[a] EC_{50} (mg/ml): effective concentration at which the absorbance is 0.5.
[b] EC_{50} (mg/ml): effective concentration at which 50% of DPPH radicals are scavenged.
[c] EC_{50} (mg/ml): effective concentration at which 50% of the erythrocytes hemolysis are inhibited.
[d] EC_{50} (mg/ml): effective concentration at which the antioxidant activity is 50%.

being related to the content of total phenols. Though other antioxidants were probably present in these mushroom extracts, the amounts of ascorbic acid, β-carotene and lycopene found in the three Portuguese mushroom extracts were very low, which emphasises the idea that phenolic compounds could make a significant contribution to the mushrooms' antioxidant activity. To our knowledge, the present study is to demonstrate that the antioxidative components in the Portuguese mushrooms extracts can protect the membranes of erythrocytes incubated with AAPH, and can reduce the extent of β-carotene destruction by neutralizing the linoleate free radicals and other free radicals formed in the system.

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