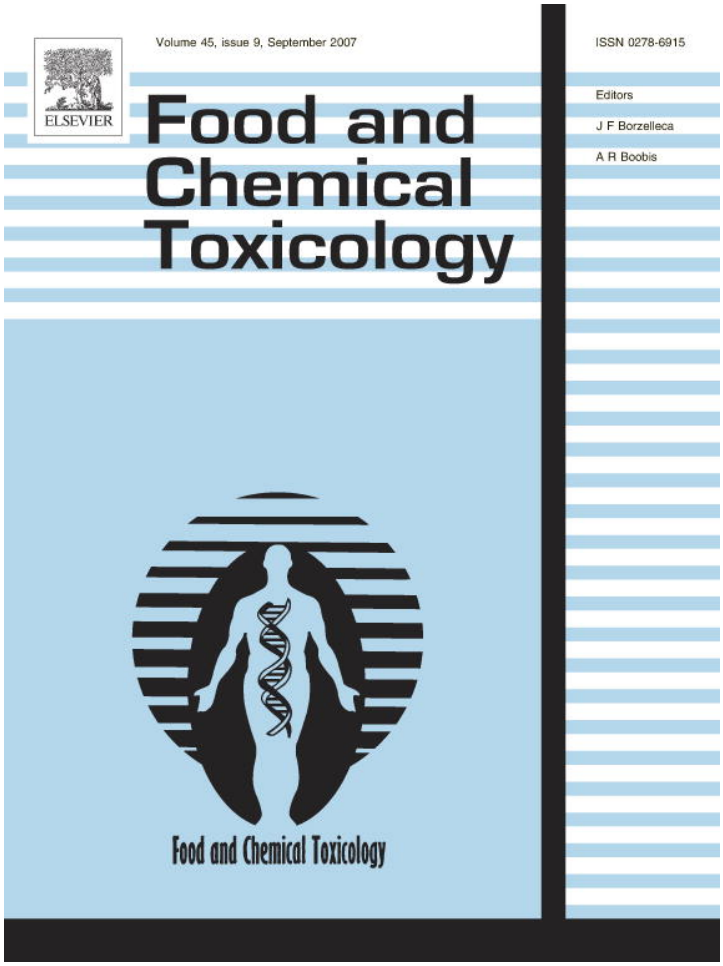

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Effect of *Lactarius piperatus* fruiting body maturity stage on antioxidant activity measured by several biochemical assays

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Abstract

The effects of fruiting body maturity on antioxidant activity and antioxidants production of the wild mushroom, *Lactarius piperatus*, were evaluated. Several biochemical assays were used to screen the antioxidant properties: reducing power, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity, inhibition of erythrocytes hemolysis mediated by peroxyl radicals and inhibition of lipid peroxidation using the β-carotene linoleate model system. The amounts of phenols, flavonoids, ascorbic acid, β-carotene and lycopene present in the immature, mature and degraded fruiting bodies were also determined. The highest antioxidant contents and the lowest EC_{50} values for antioxidant activity were obtained in the mature stage with immature spores.

Keywords: Wild mushroom; Fruiting body maturity; Antioxidant activity; Antioxidant components

1. Introduction

Oxidation is essential to many living organisms for the production of energy to fuel biological processes, proceeding in lipids with polyunsaturated fatty acids, and generating reactive oxygen species (ROS) such as hydroxyl radicals (Halliwell and Gutteridge, 1989). However, oxygen-centred free radicals and other ROS species, that are continuously produced in vivo as by products, result in cell death and tissue damage. The oxidative damage caused by free radicals may be related to aging and diseases, such as atherosclerosis, diabetes, cancer and cirrhosis (Halliwell and Gutteridge, 1984). Antioxidant compounds reduce the action of reactive oxygen species (ROS) in tissue damage.

Although humans and other organisms possess antioxidant defences (enzymes, such as superoxide dismutase and catalase, or compounds such as ascorbic acid, tocopherols and glutathione) and repair systems that have evolved to protect them against oxidative damage, these systems are insufficient to totally prevent the damage (Simic, 1988; Niki et al., 1994; Mau et al., 2002).

Natural products with antioxidant activity are used to aid the endogenous protective system, increasing interest in the antioxidative role of nutraceutic products (Kanter, 1998). Concerning this, the antioxidants in human diets are of great interest as possible protective agents to help human body reduce oxidative damage. Recently, a multitude of natural antioxidants have already been isolated from different kinds of plant materials such as oilseeds, cereal crops, vegetables, fruits, leaves, roots, spices, and herbs (Ramarathnam et al., 1995). Mushrooms have also become attractive as a functional food and as source for the development of drugs and nutraceuticals (Yanga et al., 2002; Barros et al., 2007, in press).

Mushrooms accumulate a variety of secondary metabolites, including phenolic compounds, polyketides, terpenes and steroids. The quality of a nutraceutical is dependent on the chemical composition of the fruiting body, particularly in relation to the phenols and flavonoids content. Phenolic compounds were found to have antioxidant activity in the inhibition of LDL oxidation (Teissedre and Landrault, 2000). Phenolics are one of the major groups

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of nonessential dietary components that have been associated with the inhibition of atherosclerosis and cancer (Williams and Iatropoulos, 1997). Flavonoids have been proven to display a wide range of pharmacological and biochemical actions, such as antimicrobial, antithrombotic, antimutagenic and anticarcinogenic activities (Cook and Samman, 1996; Kandaswami and Middleton, 1997; Sahu and Green, 1997).

Several studies described by us (Barros et al., 2007, in press; Ferreira et al., 2007) and also by other authors (Yen and Hung, 2000; Mau et al., 2002, 2004; Yanga et al., 2002; Cheung et al., 2003; Cheung and Cheung, 2005; Lo and Cheung, 2005; Turkoglu et al., 2007) report a correlation between the mushrooms antioxidant activity and their phenolic content. However, none of the existent reports on mushrooms antioxidants composition and antioxidant activity indicated the stage of development of the fruiting bodies selected for the studies. Furthermore, no studies have been developed to evaluate the antioxidant activity at different stages of fruiting body maturity for potential use on the preparation of nutraceutical.

In this study, we examined the evolution of antioxidant components and antioxidant activity of a *Basidiomycete* fungus, *L. piperatus*, in four stages of fruiting body maturity. The antioxidant properties were evaluated though reducing power, DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, inhibition of oxidative hemolysis in erythrocytes induced by 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) and inhibition of lipid peroxidation by β-carotene-linoleate system. These assays have been extensively studied as models for the peroxidative damage in biomembrane. All these antioxidant activity parameters were correlated to the phenolic, flavonoidic, ascorbic acid, β-carotene and lycopene contents.

2. Materials and methods

2.1. Chemicals

Standards BHA (2-*tert*-butyl-4-methoxyphenol), TBHQ (*tert*-butylhydroquinone), L-ascorbic acid, α-tocopherol, gallic acid and (+)-catechin were purchase 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. Fruiting body selection

Wild fruiting bodies of *Lactarius piperatus* (L.) Pers. were obtained under live oak trees (*Quercus pyrenaica*), in Bragança (northeast of Portugal), in spring 2006. Taxonomic identification was made according to several authors (Marchand, 1971-1986; Moser, 1983; Bon, 1988; Courtecuisse and Duhem, 1995; Courtecuisse, 1999) and a representative voucher specimen was deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança. The fruiting bodies were harvested and dried in different stages of maturity: immature (cap closed) and mature (cap opened). The mature stage was further characterized into immature spores and mature spores as seen on Fig. 1.

2.3. Sample preparation

The fruiting bodies were air-dried in a liophylizator (Ly-8-FM-ULE, Snijders) and powdered before analysis. The dried samples (~5 g) were extracted by stirring with 100 mL of methanol at 25 °C at 150 rpm for 24 h and filtered through Whatman No. 4 paper. The residue was then extracted with two additional 100 mL portions of methanol, as described earlier. The combined methanolic extracts were evaporated at 40 °C to

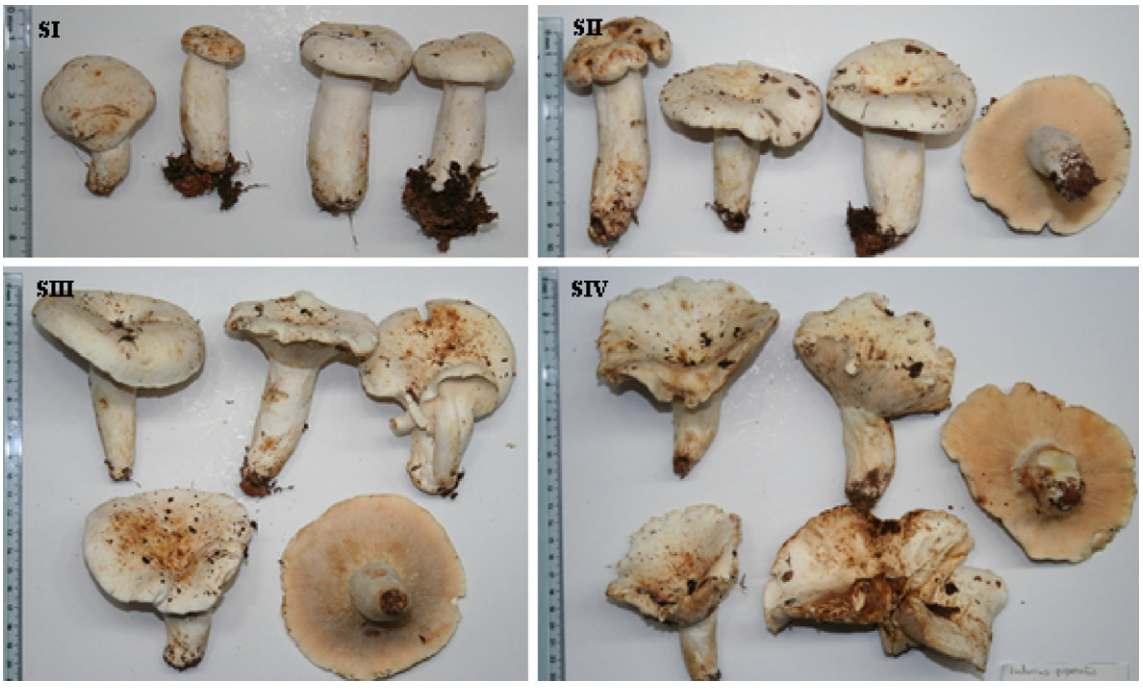

Fig. 1. *Lactarius piperatus* fruiting bodies in different stages of maturity: (SI) immature (cap closed), (SII) mature (cap opened) with immature spores, (SIII) mature with mature spores, and (SIV) degraded.

dryness and redissolved in methanol at a concentration of 50 mg/mL, and stored at 4 °C for further use.

2.4. Determination of total antioxidant components

Phenolic compounds in the mushroom extracts were estimated by a colorimetric assay, based on procedures described by Singleton and Rossi with some modifications (Singleton and 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 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; $Y = 2.8557X - 0.0021$; $R^2 = 0.9999$) and the results were expressed as mg of gallic acid equivalents (GAEs) per g of extract.

Flavonoid contents in the extracts were determined by a colorimetric method described by Jia et al. (1999) with some modifications. The mushroom extract (250 μL) was mixed with 1.25 mL of distilled water and 75 μL of a 5% $NaNO_2$ solution. After 5 min, 150 μL of a 10% $AlCl_3 \cdot H_2O$ solution was added. After 6 min, 500 μL of 1 M NaOH and 275 μL of distilled water were added to the mixture. The solution was mixed well and the intensity of pink colour was measured at 510 nm. (+)-Catechin was used to calculate the standard curve (0.022–0.34 mM; $Y = 0.9629X - 0.0002$; $R^2 = 0.9999$) and the results were expressed as mg of (+)-catechin equivalents (CEs) 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; $Y = 3.4127X - 0.0072$; $R^2 = 0.9905$) and the results were 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 and 663 nm. Contents of β-carotene and lycopene were calculated according to the following equations: lycopene (mg/100 ml) = −0.0458 A663 + 0.372 A505 − 0.0806 A453; β-carotene (mg/100 ml) = 0.216 A663 − 0.304 A505 + 0.452 A453. The results were expressed as μg of carotenoid/g of extract.

2.5. DPPH radical-scavenging activity

The capacity to scavenge the "stable" free radical DPPH was monitored according to the method of Hatano et al. (1988). Various concentrations of mushroom extracts (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 discolouration 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 at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. 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.

2.6. Reducing power

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 mmol/L sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 2 min. After 2.5 mL of 10% tricloroacetic 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 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.

2.7. Inhibition of erythrocyte hemolysis mediated by peroxyl free radicals

The antioxidant activity of the mushroom extracts was measured as the inhibition of erythrocyte hemolysis (Miki et al., 1987). Blood was obtained from male ram (churra galega transmontana) of body weight ~67 kg. Eryhrocytes 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 of NaH_2PO_4 and Na_2HPO_4, and 125 mM of 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. A 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 = $[(A_{AAPH} - A_S)/A_{AAPH}] \times 100$, 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 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. Inhibition of lipid peroxidation using the β-carotene linoleate model system

The antioxidant activity of mushroom extracts was evaluated by the β-carotene linoleate model system (Mi-Yae et al., 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. Lipid peroxidation (LPO) inhibition was calculated using the following equation: LPO inhibition = (β-carotene content after 2 h of assay/initial β-carotene content) × 100. 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.

2.9. Statistical analysis

All the determinations were carried out in triplicate and the results are expressed as mean values and standard deviations (SD). Differences among means were done by analysis of variance (ANOVA), using SAS v.

9.1.3, and averages were compared using Tukey test ($p < 0.05$). A regression analysis, using Excel for Windows Software, was established between phenolic and flavonoid contents of different mushroom samples and EC_{50} values obtained by different antioxidant assays.

3. Results and discussion

Table 1 shows phenol, flavonoid, ascorbic acid and carotenoids concentrations obtained in *L. piperatus* in different stages of fruiting body maturity. Whereas total phenols were the major antioxidant components found in the extracts, ascorbic acid was found in small amounts (0.03–0.16 mg/g), and β-carotene and lycopene were only found in vestigial amounts (<34 μg/g), which is in agreement with other reports concerning ascorbic acid and β-carotene quantification in different mushrooms (Mau et al., 2002; Barros et al., 2007, in press). Curiously, the stages where the fruiting bodies presented immature spores (stage I and II) revealed a higher content in phenol and flavonoid compounds. The amount found in stages III (with mature spores) and IV (degraded) significantly ($p < 0.05$) decreased when compared with the content found in the first stages. Probably, the aging process elicits the formation of reactive oxygen species, which are neutralised by the polyphenolic compounds, resulting in the lowering of their contents and antioxidant capacities in the most advanced stages. The highest content of antioxidant compounds in the first stages might account for the better results found in their antioxidant activity (Velioglu et al., 1998; Ferreira et al., 2007). Furthermore, Cheung et al. (2003) found a direct correlation between mushrooms antioxidant activity and total phenolic content, although the antioxidant action is raised by other substances such as tocopherols and β-carotene. The bioactivity of phenolics may be related to their ability to chelate metals, inhibit lipoxygenase and scavenge free radicals (Decker, 1997). Also, in food systems, flavonoids can act as free radical scavengers and terminate the radical chain reactions that occur during the oxidation of triglycerides. Therefore, they present antioxidative efficiency in oils, fats and emulsions (Madhavi et al., 1996; Roedig-Penman and Gordon, 1998).

Fig. 2 shows the antioxidant activity of *L. piperatus* in different stages of fruiting body maturity, measured by different biochemical assays: reducing power, scavenging activity on DPPH radicals, inhibition of the erythrocyte hemolysis mediated by peroxyl free radicals and LPO inhibition by β-carotene-linoleate system.

The radical scavenging activity (RSA) values were 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 fruiting body extracts on DPPH radicals increased with the concentration increase and were very good for stage II (84.9% at 20 mg/mL), but lower than the scavenging effects of the standards BHA (96% at 3.6 mg/ml) and α-tocopherol (95% at 8.6 mg/ml). The RSA values at 20 mg/mL were moderate for the other stages (stage I – 64.2%, stage III – 49.4%, stage IV – 43.4%) and, particularly stage III and IV revealed a very similar scavenging activity.

The reducing power also increased with concentration, and the values obtained for all the extracts were excellent (Fig. 2); at 20 mg/mL were higher than 1.3 and in the order of stage II > stage I > stage III > stage IV. Reducing power of BHA at 3.6 mg/mL and α-tocopherol at 8.6 mg/mL was only 0.12 and 0.13, respectively. The extracts obtained in stages III and IV showed similar reducing power values. 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 et al., 1992). Accordingly, in stage II, it was produced higher amounts of reductone, which could react with free radicals to stabilize and block radical chain reactions.

In this study, the protective effect of the fruiting body extracts on hemolysis by peroxyl radical scavenging activity was also investigated. 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. The extracts inhibited hemolysis, as a result of protection against the oxidative damage of cell membranes of erythrocytes from ram induced by AAPH, in a concentration-dependent manner (Fig. 2). Once more, in stage II there was a higher protective effect against erythrocytes hemolysis (82.4% at 50 mg/mL) than in other stages (stage I – 57.5%, stage III – 54.2%, stage IV – 41.0%). However, the inhibition percentage of the standard L-ascorbic acid on hemolysis of red blood cell was much higher (94.6% at 1 mg/mL) than those of mushroom extracts.

The lipid peroxidation inhibition (LPO), measured by the bleaching of β-carotene, is presented in Fig. 2. The

Table 1
Phenols, flavonoids, ascorbic acid, β-carotene and lycopene contents (mean ± SD) of *Lactarius piperatus* in different stages of maturity

	Stage I	Stage II	Stage III	Stage IV
Phenols (mg/g)	5.52 ± 0.14 b	5.76 ± 0.09 a	3.09 ± 0.12 c	2.03 ± 0.11 d
Flavonoids (mg/g)	1.26 ± 0.09 b	1.58 ± 0.02 a	0.35 ± 0.03 c	0.19 ± 0.01 d
Ascorbic acid (mg/g)	0.15 ± 0.01 a	0.16 ± 0.01 a	0.13 ± 0.01 b	0.03 ± 0.01 c
β-carotene (μg/g)	26.08 ± 0.05 b	33.78 ± 0.05 a	17.22 ± 0.00 c	15.11 ± 0.02 d
Lycopene (μg/g)	8.14 ± 0.03 b	13.04 ± 0.02 a	5.80 ± 0.01 c	5.41 ± 0.01 d

In each row different letters mean significant differences ($p < 0.05$).

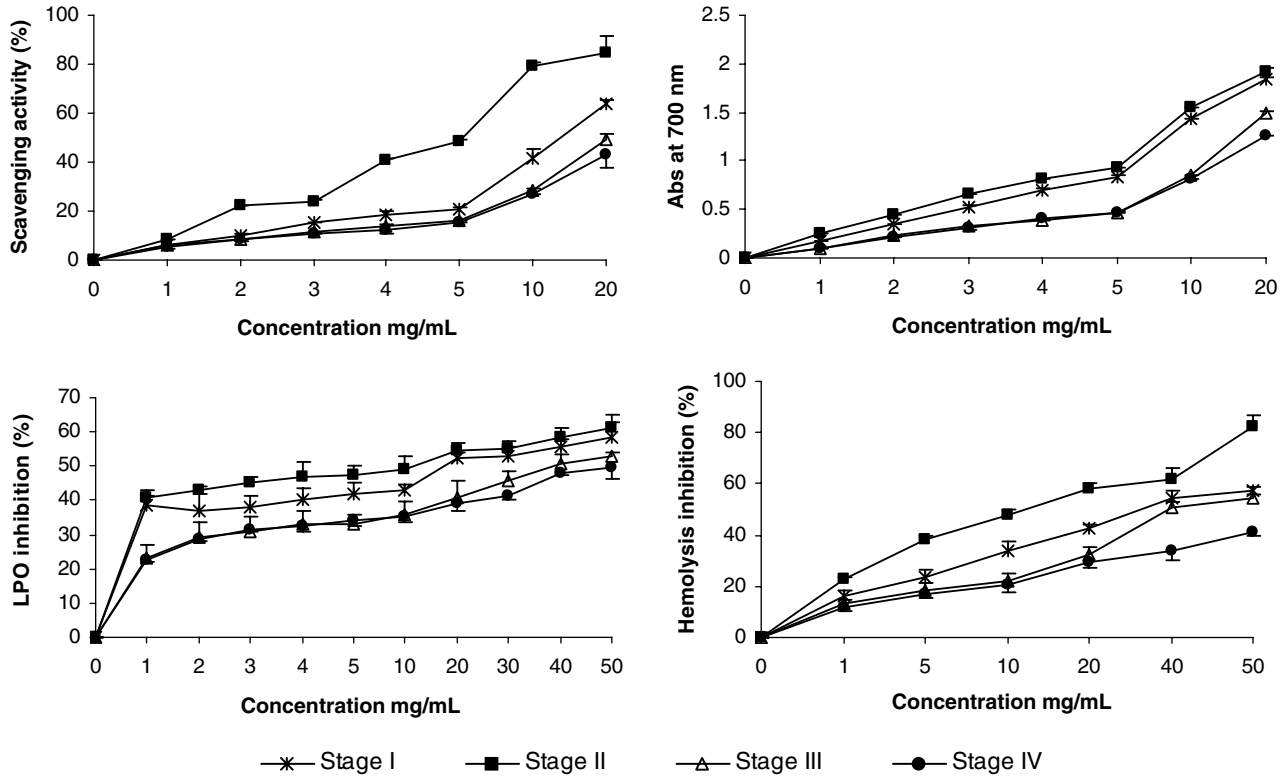

Fig. 2. Antioxidant activity of *Lactarius piperatus* in different stages of maturity: Scavenging activity on DPPH radicals (%), reducing power, hemolysis inhibition (%) and lipid peroxidation (LPO) inhibition (%). Each value is expressed as mean ± standard error ($n = 3$).

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 et al., 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. LPO inhibition in the presence of *L. piperatus* in different stages of fruiting body maturity increased with their increasing concentration, and the values at 50 mg/mL for each one of the stages were 58.3%, 61.2%, 52.9% and 49.72%. 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, stage II was the most effective for antioxidant activity. The antioxidant activity of TBHQ standard reached 82.2% at 2 mg/mL and was more efficient than the samples. Nevertheless, this and other synthetic antioxidants applied in fat and oily foods to prevent oxidative deterioration were found to be anticarcinogenic as well as carcinogenic in experimental animals (Loliger, 1991).

In Table 2 we present EC_{50} values obtained in the antioxidant activity assays of *L. piperatus* in different stages of fruiting body maturity. Overall, the mushroom in stage II revealed better antioxidant properties (significantly lower EC_{50} values; $p < 0.05$) than in other stages, which is in agreement with the higher content of antioxidants found in the first case. The EC_{50} values obtained for reducing power and scavenging effects on DPPH radicals were better than for LPO inhibition and for hemolysis inhibition mediated by peroxyl free radicals.

Significantly negative linear correlations were established between the phenols and flavonoids content, and EC_{50} values of DPPH scavenging activity (determination coefficient 0.806 for phenols and 0.868 for flavonoids; $p < 0.001$), reducing power (determination coefficient 0.946

Table 2
EC_{50} values obtained in the antioxidant activity assays of *Lactarius piperatus* in different stages of maturity

EC_{50} values (mg/L)	Stage I	Stage II	Stage III	Stage IV
Scavenging effect	13.92 ± 0.73 c	5.19 ± 0.03 d	20.24 ± 0.78 b	23.44 ± 3.33 a
Reducing power	2.83 ± 0.01 c	2.29 ± 0.02 d	5.40 ± 0.05 b	5.50 ± 0.03 a
Hemolysis inhibition	32.92 ± 0.72 c	12.14 ± 0.03 d	38.91 ± 0.18 b	>50.0 a
LPO inhibition	15.26 ± 4.15 c	12.08 ± 3.15 c	34.25 ± 5.46 b	45.06 ± 2.21 a

In each row different letters mean significant differences ($p < 0.05$).

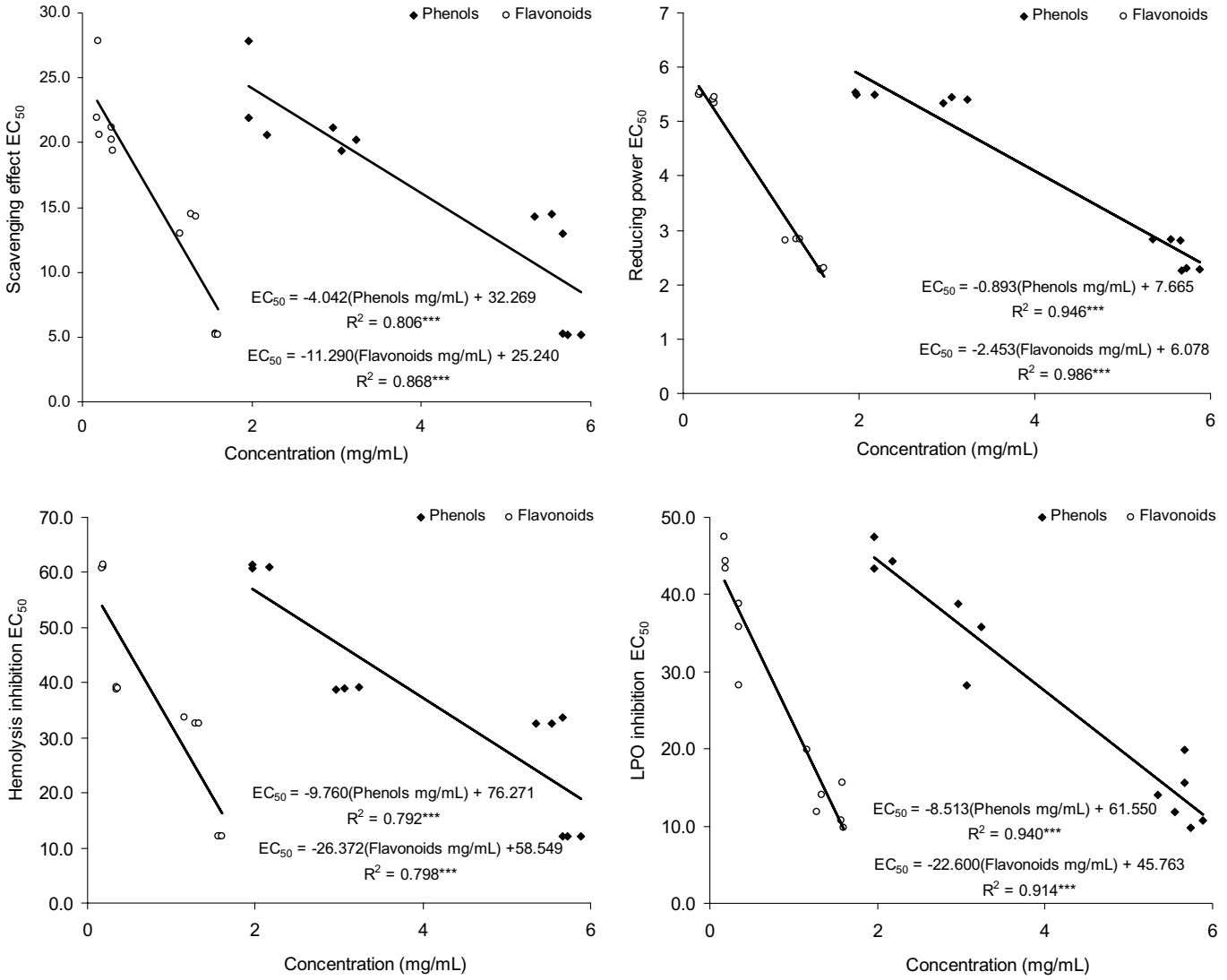

Fig. 3. Correlation established between total phenol and flavonoid contents, and scavenging effect on DPPH radicals, reducing power, hemolysis inhibition, and LPO inhibition.

for phenols and 0.986 for flavonoids; $p < 0.001$), hemolysis inhibition (determination coefficient 0.792 for phenols and 0.798 for flavonoids; $p < 0.001$) and LPO inhibition (determination coefficient 0.940 for phenols and 0.914 for flavonoids; $p < 0.001$) (Fig. 3). These negative linear correlations prove that the sample with highest antioxidant contents shows higher antioxidant activity and lower EC_{50} values (stage II, mature with immature spores), while the sample with lowest antioxidant contents presents lower antioxidant activity and higher EC_{50} values (stage IV, degraded). The correlations also support that the mechanism of action of the extracts for the antioxidant activity may be identical, being related with the content in phenols and flavonoids, and their free-radical scavenging activity.

Over two-thirds of cancer-related death could be prevented through lifestyle modification, particularly through dietary means and, mushrooms consumption, could contribute to minimize cancer risks trough antioxidants input. Nevertheless, it is important to know the best maturity stage to collect wild mushrooms concerning antioxidants production and, to our best knowledge, the present study was the first report to demonstrate that the antioxidative components production by wild mushrooms and their antioxidant properties depends on the stage of fruiting body maturity.

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