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Bioactives of fruiting bodies and submerged culture mycelia of *Agaricus brasiliensis* (*A. blazei*) and their antioxidant properties

Anne Elise S.S. Carvajal, Eloá A. Koehnlein, Andréia A. Soares, Gabrieli J. Eler, Alika T.A. Nakashima, Adelar Bracht, Rosane M. Peralta*

Department of Biochemistry, Universidade Estadual de Maringá, 87020-900 Maringá, PR, Brazil

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ABSTRACT

Agaricus brasiliensis is a mushroom native from Brazil largely studied due to its polysaccharide contents, particularly β-glucans. In this study, the phenolics and organic acids contents as well as the antioxidant activities of its fruiting bodies and its mycelia obtained from submerged cultivation were compared. The hydroalcoholic extracts obtained from the fruiting bodies, early stationary mycelia and late stationary mycelia contain at least ten phenolic compounds and ten organic acids. Three phenolic compounds were identified as gallic acid, syringic acid and pyrogallol. Eight organic acids were identified as benzoic, oxalic, malic, acetic, alpha-ketoglutaric, citric, fumaric and *trans*-aconitic acids. All extracts presented antioxidant properties. The latter were evaluated by four assays: DPPH and ABTS radical scavenging activities, chelating ability for ferrous ions and inhibition of lipid peroxidation. The fruiting body extracts were more effective in the DPPH radical scavenging activity and lipid peroxidation inhibition that the mycelia extracts ($P \leq 0.05$). The mycelia extracts were more effective in the ABTS radical scavenging activity and ferrous ion chelating ability ($P \leq 0.05$). In conclusion, our results show that the mycelia of *A. brasiliensis* obtained in submerged cultivation can also be, as its fruiting bodies, valuable sources of antioxidant compounds.

1. Introduction

Free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS), are constantly produced by cells during normal and pathological energy metabolism. Both ROS and RNS have been associated with many diseases and degenerative processes in aging (Halliwell, 2000). Almost all organisms are well protected against free radical damage by antioxidant enzymes such as superoxide dismutase and catalase. However, these systems are frequently insufficient to totally prevent the damage, resulting in diseases and accelerated aging. Natural products obtained through the diet, such as tocopherol, ascorbic acid, carotenoids and phenolic compounds with antioxidant activity can be useful to reduce oxidative damage in the human body. Many fruits, vegetables, herbs, cereals, sprouts, seeds and edible mushrooms have been investigated for their antioxidant activities in the last years (Babar, Oberoi, Uppal, & Palil, 2011; Cheung, Cheung, & Ooi, 2003; Dimitrios, 2006; Mau, Chang, Huang, & Chen, 2004; Mau, Lin, & Chen, 2002; Mau, Lin, & Song, 2002; Ramirez-Anguiano, Santoyo, Reglero, & Soler-Rivas, 2007; Sowndhararajan, Siddhuraju, & Manian, 2011; Wong & Chye, 2009).

Mushrooms are world wide appreciated for their taste and flavor and are consumed both in fresh and processed form. Their biochemical composition, with significant contents of proteins, carbohydrates, lipids, enzymes, minerals, vitamins and water, has attracted attention also as functional health promoters (Chang, 2008). Mushrooms have also become an attractive source for the development of drugs and nutraceuticals (Lakhanpal & Rana, 2008). The growth of an edible mushroom, however, is a lengthy and complex process involving the use of solid composts or lignocellulosic beds, such as straw or cotton, and a long cultivation period. In addition to dried mushrooms, alternative or substitute mushroom products are their mycelia, mainly derived from submerged cultures. Growing mushroom mycelia in liquid culture on a defined nutrient medium has long been a simple and fast alternative method to produce fungal biomass (Zhong & Tang, 2004). These mycelia could be used as food and food-flavoring material, or in the formulation of nutraceuticals and functional foods. For using the mycelial biomass of mushrooms, it is necessary to prove that they possess nutritional and medicinal values comparable to those of

* Corresponding author. Tel./fax: +55 4430111372.
E-mail addresses: rosanemperalta@gmail.com, rmperalta@uem.br (R.M. Peralta).

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mushroom fruiting bodies. Some studies have already shown that the mycelial biomass of different medicinal mushrooms possess pharmacologic properties comparable to those of mushroom fruiting bodies (Asatiani, Elisashvili, Wasser, Reznick, & Nevo, 2007; Barros, Ferreira, & Baptista, 2008; Kalyoncu, Oskay, & Kayalar, 2010; Mao, Eksriwong, Chauvatcharin, & Zhong, 2005; Mau et al., 2004).

Agaricus brasiliensis Wasser & Didukh, formerly known as *Agaricus blazei* Murril ss. Heinemann, is a basidiomycete popularly known in Brazil as Cogumelo do Sol and Cogumelo Piedade. It is widely used today in several Oriental countries both as an edible mushroom, considered as functional food, and as natural therapy in the form of a medicinal extract used mostly for prevention and treatment of cancer. In Brazil it is consumed as concentrated extract or tea and popularly used against a variety of diseases such as diabetes, atherosclerosis, hypercholesterolemia and heart disease (Firenzuoli, Gori, & Lombardo, 2007). The major bioactive molecules of *A. brasiliensis* are polysaccharides and protein–polysaccharide complexes containing beta-glucan obtained from fruiting body, liquid-cultured mycelium or liquid medium filtrate after submerged cultivation (Firenzuoli et al., 2007). These molecules have been demonstrated to possess anti-tumor, antiproliferative, anti-genotoxic, and anti-mutagenic activities. Concerning small bioactive molecules in *A. brasiliensis,* recent studies have demonstrated that the mushroom contains significant amounts of tocopherol (Tsai, Tsai, & Mau, 2007), ergosterol (Gao & Gu, 2007), agaritine (Endo et al., 2010), phenolic compounds (Soares et al., 2009) and nucleotides and nucleosides (Oliveira, Eler, Bracht, & Peralta, 2010). These molecules are possibly involved in the therapeutic and physiological properties of *A. brasiliensis*.

Because it usually takes several months to cultivate the fruiting body of *A. brasiliensis* and because it is also difficult to control the product quality during its cultivation, there is a great need to regularly supply the market with enough high-quality *A. brasiliensis* products. Submerged fermentation of *A. brasiliensis* is viewed as a promising alternative for the production of mycelial biomass and endo- and exo-polysaccharides (Lin & Yang, 2006). This strategy has merits because the fruiting body, the mycelium, and the liquid broth of *A. brasiliensis* are comparable in their anti-carcinogenic actions as well as in some other beneficial biological activities (Lindequist, Niedermeyer, & Julich, 2005). Some recent work has described the antioxidant properties of *A. brasiliensis* fruiting bodies (Kim et al., 2008; Soares et al., 2009; Tsai et al., 2007), but until now, no study has been done using its mycelia. Taking this into consideration, the objectives of this study were to compare the contents in phenolics and organic acids as well as the antioxidant activities of the fruiting bodies and mycelia of *A. brasiliensis.* The mycelia were obtained in submerged cultures during the early (young mycelia) as well as during the late stationary phases (old mycelia).

2. Materials and methods

2.1. Fruiting body selection

Fruiting bodies (basidiocarps) of *A. brasiliensis* were obtained from a local producer in Maringá, PR, Brazil, in Spring 2008. The fruiting bodies were selected in accordance with the commercial requirements in Brazil, i.e., before the rupture of the veil (closed cap). This is mainly due to sensory characteristics and enhanced firmness. The latter makes cropping easier and reduces fragmentation during processing (Soares et al., 2009).

2.2. Production of the A. brasiliensis mycelia

The stock culture was maintained on malt extract-dextrose-agar (MDA) slants and sub-cultured every 3 months. The slants were incubated at 28 °C for 4 days and then stored at 4 °C in a refrigerator. The inocula were prepared by adding actively growing mycelia from a newly prepared slant culture (5 mycelial agar discs with 0.5 cm of diameter) into 50 mL medium in a 250 mL Erlenmeyer flask. The culture was incubated for 4 days on static conditions at 28 °C. The medium used for *A. brasiliensis* cultivation was based in the composition of some media used for production of biomass and polysaccharides by *A. brasiliensis* in submerged cultures (Liu & Wang, 2007; Shu, Wen, & Lin, 2003) and had the following composition (g/L): glucose, 40; peptone, 3; yeast extract, 3; $KH_2PO_4 \cdot H_2O$ 0.5; and $MgSO_4 \cdot 7H_2O$, 0.3. For the submerged culture, 100 mL of the same medium were prepared in a 500 mL flask, and pre-culture broth was inoculated (at 1.0 mL/L). All experiments were carried out at 28 °C on a rotary shaker at 160 rpm for up to 10 days. The mycelia were recovered from the liquid medium by filtration, washed with distilled water and dried at 40 °C during 24 h to obtain the yield of biomass. The residual amount of reducing sugars in the filtrates was evaluated by using the dinitrosalicylic method (Miller, 1959).

2.3. Extraction of bioactive compounds

Fruiting bodies and the mycelia were dried and milled to a fine powder (40 mesh). The samples (5 g) were extracted by stirring with 100 mL of ethanol 70:30 (in water) at 25 °C and at 130 rpm for 3 h and filtered through Whatman n° 1 paper. Ethanol was chosen because of its abundance and low cost. The extractions were repeated two times. No increases in yield were achieved by further extractions. The combined filtrates were concentrated with a rotary vacuum evaporator at 40 °C to eliminate ethanol, freeze-dried and weighted. The freeze-dried powders were stored in freezer until use.

2.4. Chemical analytical procedures

The reducing and total carbohydrates of extracts were determined by using the dinitrosalicylic (Miller, 1959) and anthrone (Morse, 1947) methods, respectively, and expressed as glucose equivalents. Evaluation of the reducing and non-reducing sugars present in the extracts was also carried out using thin layer chromatography. The samples were spotted onto Silica Gel 60 plates (Merck, Darmstadt, Germany) and developed with butanol–pyridine–water (15:30:20, vol/vol) as the mobile phase. The spots were detected by spraying with 5 g/L $KMnO_4$ in 1 mol equi/L NaOH. Glucose, mannitol and trehalose at 20 g/L were used as standards. Total phenolic compounds were measured using Lowry reagent (Singleton & Rossi, 1956) and expressed as gallic acid equivalents. The determination of flavonoids was done by means of a colorimetric assay (Zhishen, Mengcheng, & Jianming, 1999) and expressed as catechin equivalents. Compounds bearing free amino groups were measured using the ninhydrin method with L-alanine as standard (Starcher, 2001). Amino acids were evaluated also using paper chromatography (Moffat & Lytle, 1959).

2.5. Phenolic compounds analysis by HPLC

The hydroalcoholic extracts from both mycelia and fruiting bodies of *A. brasiliensis* were fractionated by means of high-performance liquid chromatography (HPLC). The HPLC system (Shimadzu, Japan) consisted of a system controller SCL-10AVP, two pumps, model LC10ADVP, a column oven, model CTO-10AVP, and a UV–Vis detector, model SPD-10AVP. A reversed-phase C18 CLC-ODS column (5 μm; 250 × 6 mm i.d.; Shimadzu) protected with a GHRC-ODS pre-column (5 μm; 10 × 4 mm i.d.; Shimadzu) was used. The absorbance of each sample solution was measured at

280 nm. The mobile phase was distilled water:glacial acetic acid (99.9:0.1) (solvent A) and acetonitrile:glacial acetic acid (99.9:0.1) (solvent B). The gradient was 0 min, 92:8 (A:B); 0–2 min, 90:10 (A:B); 2–27 min, 70:30 (A:B); 27–50 min, 10:90 (A:B); 50–60 min, 0:100 (A:B); 60–63 min, 92:8 (A:B). Run time was 63 min using a flow rate of 1 mL/min (Kim et al., 2008). The temperature was kept at 35 °C, and the injection volume was 20 μL. The freeze-dried extract was dissolved in distilled water. Identification of the peaks of the investigated compounds was carried out by comparison of their retention times with those obtained by injecting standards in the same conditions, as well as by spiking the samples with stock standard solutions. The concentrations of the identified compounds in the extract samples were calculated by means of the regression parameters obtained from calibration curves. All standard calibration curves showed high degrees of linearity ($r^2 > 0.99$). The following standards of flavonoids, phenolic acids and aromatic compounds were used as standards: gallic acid, protocatechuic acid, syringic acid, caffeic acid, cinnamic acid, *p*-coumaric acid, benzoic acid, pyrogallol, catechin, myricetin and quercetin. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.6. Organic acids analysis by HPLC

A HPLC system (Shimadzu, Tokyo) with a LC-20AT Shimadzu system controller, Shimadzu SPD-20 A UV–Vis detector, equipped with a reversed-phase Shimpak C18 column (4.6 × 250 mm), maintained at 30 °C, was used for analysis of organic acids. All samples in duplicate were filtered through a 0.22 μm filter unit (Millex® – GV, Molsheim, France) before injection and the solvents were filtered through a 0.45 μm filter (Whatman, Maidstone, England). A solvent system consisting of Milli-Q water:-phosphoric acid (99.9:0.1) was used as mobile phase at a flow rate of 1 mL/min and the injection volume was 20 μL. Run time was 10 min and detection of organic acids was carried out at 230 nm. Identification of the peaks of the investigated compounds was carried out by comparison of their retention times with those obtained by injecting standards in the same conditions, as well as by spiking the samples with stock standard solutions. The concentrations of the identified compounds in the extract samples were calculated by means of the regression parameters obtained from calibration curves. All standard calibration curves showed high degrees of linearity ($r^2 > 0.99$) (data not shown). The following standards of organic acids were used: citric, ascorbic, oxalic, succinic, tartaric, malic, malonic, lactic, fumaric, *trans*-aconitic, oxaloacetic, acetic, propionic, butyric and α-ketoglutaric acids. Water was treated in a purification system (TGI Pure Water Systems, USA).

2.7. Evaluation of antioxidant properties

2.7.1. DPPH radical scavenging activity

The 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay was done as described previously (Soares et al., 2009). Briefly, the stock solution was prepared by dissolving 24 mg DPPH in 100 mL methanol and then stored at −20 °C until needed. The working solution was obtained by mixing 10 mL stock solution with 45 mL methanol to obtain an absorbance of 1.1 ± 0.02 units at 515 nm. A volume of 150 μL of each extract (final concentrations ranging from 50 to 800 μg/mL) was allowed to react with 2850 μL of the DPPH solution (final concentration of 0.1 mmol/L), vigorously shaken and maintained for 1 h at room temperature in the dark. Distilled water was used instead of extract as a control. Then the absorbance was measured at 515 nm. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging activity } (\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100,$$

where A_{control} was the absorbance of the reaction in the presence of water and A_{sample} the absorbance of the reaction in the presence of the extract. The extract concentration producing 50% inhibition (EC_{50}) was calculated from the graph of the DPPH scavenging effect against the extract concentration. Gallic acid, syringic acid and pyrogallol were used as standards.

2.7.2. ABTS radical scavenging activity

The 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS)) assay was done as previously described (Soares et al., 2009). Briefly, the stock solutions were 7.4 mmol/L $ABTS^{\bullet+}$ and 2.6 mmol/L potassium persulfate. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 mL $ABTS^{\bullet+}$ solution with 60 mL methanol to obtain an absorbance of 1.1 at 734 nm. A fresh $ABTS^{\bullet+}$ solution was prepared for each assay. A volume of 150 μL of each extract (final concentrations from 5 to 100 μg/mL) was allowed to react with 2850 μL of the $ABTS^{\bullet+}$ solution (final concentration of 0.02 mmol/L) for 2 h in the dark. Finally, the absorbance at 734 nm was measured. Distilled water was used instead of mushroom extracts as a control. The capability to scavenge the ABTS radical was calculated using the following equation:

$$\text{ABTS scavenging activity } (\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100,$$

where A_{control} was the absorbance of the reaction in the presence of water and A_{sample} the absorbance of the reaction in the presence of the extract. The extract concentration producing 50% inhibition (EC_{50}) was calculated from the graph of the ABTS scavenging effect against the extract concentration. Gallic acid, syringic acid and pyrogallol were used as standards.

2.7.3. Ferrous ion chelating ability

The ferrous ion chelating ability of extracts was determined as described previously described (Soares et al., 2009). Briefly, a sample (0.7 mL) of each extract was diluted in 0.7 mL of distilled water and mixed with 0.175 mL of $FeCl_2$ (0.5 mmol/L) and the absorbance (A_0) was measured at 550 nm. After, the reaction was initiated by the addition of 0.175 mL ferrozine (0.5 mmol/L). The mixture was shaken vigorously for 1 min and left standing at room temperature for 20 min when the absorbance (A_1) was again measured at 550 nm. The percentage of inhibition of the ferrozine–Fe^{2+} complex formation was calculated as follows:

$$\text{chelating ability } (\%) = \frac{A_0 - A_1}{A_0} \times 100.$$

A lower absorbance indicates higher chelating ability. The extract concentration producing 50% chelating ability (EC_{50}) was calculated from the graph of antioxidant activity percentage against the extract concentration. Gallic acid, syringic acid and pyrogallol were used as standards.

2.7.4. β-Carotene–linoleic acid assay (lipid peroxidation inhibition assay)

The antioxidant activity of extracts was evaluated by the β-carotene–linoleate model system as previously described (Soares et al., 2009). Firstly, β-carotene (0.2 mg) was dissolved in 1.0 mL chloroform. After, 0.02 mL linoleic acid plus 0.2 mL Tween 80 were added and the mixture was left standing at room temperature for 15 min. After evaporation of chloroform, 50 mL of oxygenated

distilled water was added and the mixture was shaken to form an emulsion (β-carotene–linoleic acid emulsion). Aliquots of 3.0 mL of this emulsion were transferred into test tubes containing 0.2 mL of different concentrations of 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 (A_0) was measured at 470 nm. A second absorbance (A_1) was measured after 120 min. A blank, without β-carotene was prepared for back-ground subtraction. Lipid peroxidation (LPO) inhibition was calculated using the following equation:

$$\text{LPO inhibition}\ (\%) = \frac{A_0 - A_1}{A_0} \times 100.$$

The assays were carried out in triplicate and the results expressed as mean values ± standard deviations. The extract concentration producing 50% antioxidant activity (EC_{50}) was calculated from the graph of antioxidant activity percentage against the extract concentration. Gallic acid, syringic acid and pyrogallol were used as standards.

2.8. Chemicals

DPPH, ABTS, potassium persulfate, β-carotene, linoleic acid, phenolic acids, flavonoids, aromatic compounds, organic acids, Folin–Ciocalteu's phenol reagent and were obtained from Sigma Chemical Co. All other chemicals were of analytical grade.

2.9. Statistical analysis

All analyses were performed in triplicate. The data were expressed as means ± standard deviations and one-way analysis of variance (ANOVA) and Tukey test were carried out to assess for any significant differences between the means. Differences between means at the 5% ($P < 0.05$) level were considered significant.

3. Results

3.1. Production of A. brasiliensis mycelia in submerged cultures

Fig. 1 shows the curve of growth of *A. brasiliensis* in submerged cultures. Maximum production of biomass (10.2 ± 1.10 g/L) was obtained after 4 days of cultivation in the beginning of stationary growth phase. After that, the analysis of residual reducing sugars showed depletion of glucose and a decline in dry weight owed to autolysis of the fungi (late stationary growth phase). To evaluate the main chemical components as well as the antioxidant properties, mycelia obtained at two times of cultivation were collected, one after 4 days of cultivation (designated in this work as young mycelia) and another after 8 days (here designated as old mycelia).

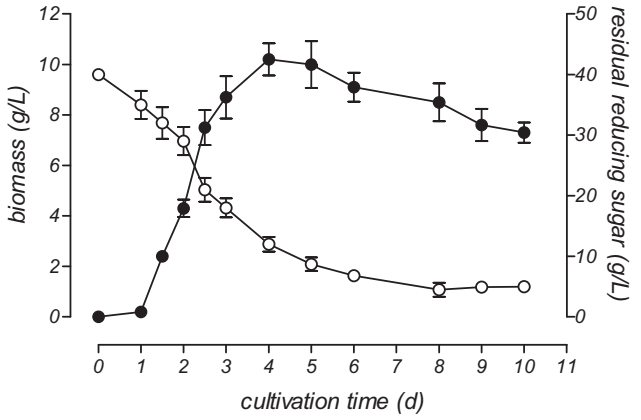

Fig. 1. Time courses of production of mycelia and consumption of sugar by *A. brasiliensis*. The cultures were developed at 28 °C on a rotary shaker at 160 rpm for up to 10 days.

3.2. Extraction yield and chemical characterization of A. brasiliensis extracts

High extraction yields were obtained from three materials using ethanol:water (70:30): 42.5 ± 1.4 g/100 g, 48.3 ± 1.8 g/100 g and 44.9 ± 1.2 g/100 g, for *A. brasiliensis* fruiting bodies, young mycelium and old mycelium, respectively. Table 1 shows the chemical characterization of the *A. brasiliensis* hydroalcoholic extracts obtained from three materials. The extracts presented high amounts of carbohydrates, mostly of the non-reducing type. Thin layer chromatography revealed that the main non-reducing sugar present in the extracts was mannitol (data not shown). Significant amounts of ninhydrin-reactive amines were also observed in all extracts, according to the following decreasing order: old mycelium > young mycelium > basidioma. The evaluation of extracts by paper chromatography (not shown) has shown that these ninhydrin positive compounds are predominantly, but not exclusively, amino acids. As the antioxidant activity appears to be related with the phenolic content of mushrooms, the extracts were also evaluated for their content in total phenols and flavonoids. The amounts of total phenolics were high in all extracts, being the old mycelia extract much richer in total phenolics than young mycelia and fruiting bodies extracts (Table 1). When compared with the total phenolic contents, the flavonoid values obtained in all extracts were very low.

3.3. Identification of chemical components of A. brasiliensis extracts by HPLC analysis

The analysis by HPLC/UV allowed the identification of three phenolics in the extracts: gallic acid, syringic acid and pyrogallol (Fig. 2, Table 2). It must be mentioned that the running time was

Table 1
Chemical composition of the *A. brasiliensis* hydroalcoholic extracts.

	Fruiting bodies	Young mycelia (4 day-cultivation)	Old mycelia (8 day-cultivation)
Total carbohydrates[a]			
μg/mg extract	121.2 ± 9.20[a]	174.2 ± 2.70[b]	65.2 ± 0.90[c]
μg/mg dried fungus	51.5 ± 3.91[a]	84.1 ± 1.30[b]	29.3 ± 0.40[c]
Reducing carbohydrates[a]			
μg/mg extract	25.2 ± 2.10[a]	36.0 ± 2.80[b]	28.8 ± 1.90[a]
μg/mg dried fungus	10.7 ± 0.89[a]	17.4 ± 1.35[b]	12.9 ± 0.85[a]
Non-reducing carbohydrates (by difference)			
μg/mg extract	96.0	138.2	36.4
μg/mg dried fungus	40.8	66.6	16.3
Amino acid and other ninhydrin-reactive amine[b]			
μg/mg extract	115.0 ± 7.07[a]	225.0 ± 7.07[b]	299.0 ± 7.07[c]
μg/mg dried fungus	48.9 ± 3.00[a]	108.7 ± 3.42[b]	134.2 ± 3.17[c]
Total phenolic[c]			
μg/mg extract	20.0 ± 0.70[a]	20.8 ± 0.90[a]	38.7 ± 0.89[b]
μg/mg dried fungus	8.5 ± 0.30[a]	10.0 ± 0.43[a]	17.4 ± 0.40[b]
Flavonoids[d]			
μg/mg extract	1.8 ± 0.16[a]	2.1 ± 0.20[a]	2.3 ± 0.20[a]
μg/mg dried fungus	0.8 ± 0.07[a]	1.0 ± 0.10[a]	1.0 ± 0.10[a]

[a] μg of glucose equivalents.
[b] μg of alanine equivalents.
[c] μg of gallic acid equivalents.
[d] μg of catechin equivalents. Means with different letters within a row are significantly different ($P \leq 0.05$).

63 min, but no significant peak appeared after 10 min. For this reason only the first 12 min of the total chromatogram are shown. A significantly higher amount of pyrogallol was found in the mycelial extracts when compared to the amount found in the fruiting body extracts. On the other hand, the highest amounts of gallic acid were found in the fruiting body extracts.

The HPLC/UV analysis allowed also the identification of eight organic acids in the extracts: benzoic, oxalic, aconitic, citric, malic, acetic, fumaric and α-ketoglutaric acids (Fig. 2 and 3; Table 3). Citric acid was the most abundant, followed by malic, acetic and oxalic acids. Several other non-identified organic acids were present in all extracts (Fig. 3, Table 3).

3.4. Determination of antioxidant activities of A. brasiliensis extracts

The antioxidant activities are summarized in Table 4. EC_{50} (in μg/mL) for all extracts are shown, as well as the corresponding values for 3 standard phenolic compounds and for 2 standard organic acids. Two methods for evaluating the free radical scavenging properties of *A. brasiliensis* extracts were used: DPPH radical and ABTS radical cation assays. The EC_{50} values obtained using the ABTS assay were lower than those obtained using the DPPH method for both, extracts and standards. Besides, the results obtained with the two methods are pointing to different directions. Using the DPPH assay, the order of antioxidant efficiency was basidioma extract > old mycelium extract > young mycelium extract. Using the ABTS assay the order of antioxidant efficiency was old mycelium extract > young mycelium extract > basidioma extract. The third method used to evaluate the antioxidant activities of the *A. brasiliensis* extracts was the β-carotene–linoleate model. With this method, the order of antioxidant efficiency was the same obtained with the DPPH assay. Finally, the antioxidant activity of the *A. brasiliensis* extracts was evaluated through the ferrous ion chelating activities. In this case the order of antioxidant efficiency was old mycelium extract > basidioma extract > young mycelial extract. Among the organic acids, the order of efficiency in chelating the ferrous ions was citric acid > oxalic acid > α-ketoglutarate acid. The capability of chelating ferrous ions of the standard phenolics was weak (EC_{50} values higher than 2000 μg/mL).

Table 2
Quantification of phenolic compounds in *A. brasiliensis* hydroalcoholic extracts by HPLC.

Compound	Fruiting bodies	Young mycelia	Old mycelia
Gallic acid			
μg/mg extract	4.50 ± 0.10^a	1.40 ± 0.10^b	4.00 ± 0.20^a
μg/mg dried fungus	1.91 ± 0.04^a	0.68 ± 0.04^b	1.80 ± 0.09^a
Syringic acid			
μg/mg extract	5.70 ± 0.10^a	8.10 ± 0.30^b	4.30 ± 0.30^a
μg/mg dried fungus	2.42 ± 0.04^a	3.91 ± 0.14^b	1.94 ± 0.13^a
Pyrogallol			
μg/mg extract	3.50 ± 0.10^a	10.0 ± 0.3^b	19.0 ± 0.90^c
μg/mg dried fungus	1.49 ± 0.04^a	4.83 ± 0.14^b	8.55 ± 0.40^c

Each value is expressed as mean ± standard deviation ($n = 3$). Means with different letters within a row are significantly different ($P \leq 0.05$).

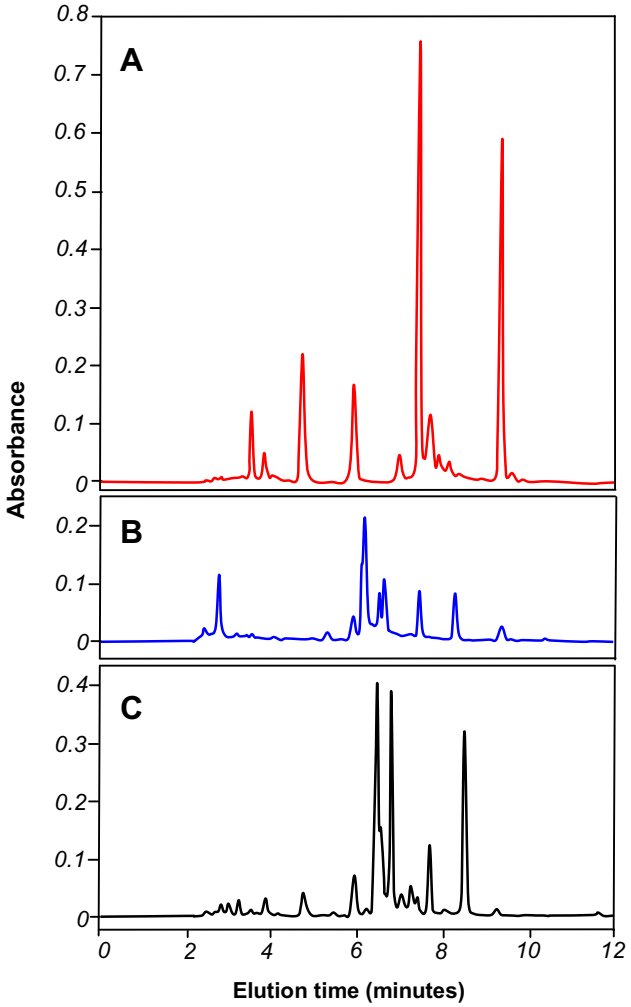

Fig. 2. Chromatogram of phenolic and aromatic compounds by HPLC. A = old mycelia extract; B = fruiting body (basidioma) extract; C = young mycelia extract. The following standards with their respective retention times in parenthesis were used to identify the components of *A. brasiliensis* extracts: gallic acid (2.8 min), syringic acid (6.1 min), benzoic acid (6.3 min), and pyrogallol (6.6 min).

4. Discussion

Although current research mainly focuses on the fruiting body of *A. brasiliensis*, cultured mycelia can also be considered potent sources of bioactive substances such as exo- and endo-polysaccharides (Lin & Yang, 2006; Liu & Wang, 2007; Shu et al., 2003) and ergosterol (Gao & Gu, 2007). However, until now, no

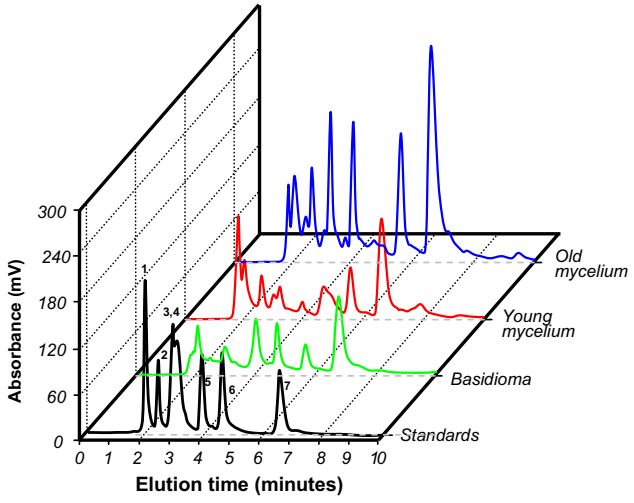

Fig. 3. HPLC organic acid profile of *A. brasiliensis* hydroalcoholic extracts. Detection at 230 nm. Standards: 1. oxalic acid; 2. malic acid; 3. acetic acid; 4. α-ketoglutarate; 5. citric acid; 6. fumaric acid; 7. *trans*-aconitic acid.

Table 3
Quantification of organic acid compounds in *A. brasiliensis* hydroalcoholic extracts by HPLC.

Compound	Fruiting bodies	Young mycelia	Old mycelia
Oxalic acid			
μg/mg extract	2.70 ± 0.50^{a}	3.50 ± 0.30^{b}	6.60 ± 0.50^{c}
μg/mg dried fungus	1.15 ± 0.21^{a}	1.69 ± 0.14^{b}	2.97 ± 0.22^{c}
Malic acid			
μg/mg extract	5.10 ± 1.00^{a}	19.50 ± 0.40^{b}	11.30 ± 0.80^{c}
μg/mg dried fungus	2.17 ± 0.42^{a}	9.42 ± 0.20^{b}	5.09 ± 0.36^{c}
Acetic acid			
μg/mg extract	17.80 ± 1.20^{a}	10.60 ± 1.1^{b}	9.90 ± 1.40^{c}
μg/mg dried fungus	7.57 ± 0.51^{a}	5.12 ± 0.53^{b}	4.46 ± 0.63^{c}
α-Ketoglutaric acid			
μg/mg extract	nd	0.90 ± 0.20^{a}	6.90 ± 0.50^{b}
μg/mg dried fungus	nd	0.43 ± 0.10^{a}	3.11 ± 0.22^{b}
Citric acid			
μg/mg extract	27.70 ± 2.20^{a}	49.8 ± 0.40^{b}	41.00 ± 0.30^{c}
μg/mg dried fungus	11.79 ± 0.94^{a}	24.06 ± 0.19^{b}	18.47 ± 0.13^{b}
Fumaric acid			
μg/mg extract	0.20 ± 0.10^{a}	0.10 ± 0.10^{b}	0.10 ± 0.1^{b}
μg/mg dried fungus	0.08 ± 0.04^{a}	0.05 ± 0.05^{b}	0.04 ± 0.04^{b}
trans-Aconitic acid			
μg/mg extract	0.50 ± 0.10^{a}	0.50 ± 0.1^{a}	1.00 ± 0.10^{b}
μg/mg dried fungus	0.21 ± 0.04^{a}	0.24 ± 0.05^{a}	0.45 ± 0.04^{b}
Benzoic acid[a]			
μg/mg extract	2.50 ± 0.1^{a}	4.30 ± 0.20^{b}	2.4 ± 0.10^{a}
μg/mg dried fungus	1.06 ± 0.04^{a}	2.08 ± 0.10^{b}	1.08 ± 0.04^{a}

Each value is expressed as mean ± standard deviation ($n = 3$). Means with different letters within a row are significantly different ($P \leq 0.05$). nd = Not detected.
[a] Benzoic acid was quantified together with phenolic compounds (Fig. 2).

efforts have been expended to compare the antioxidant bioactives of fruiting bodies and mycelia of *A. brasiliensis*. This was the main focus of this work, in which the antioxidant properties of hydroalcoholic extracts of commercial *A. brasiliensis* fruiting bodies and mycelia produced in laboratory under submerged conditions were compared. The option was to use a soluble medium based on glucose–peptone–yeast extract. With this medium it was possible to obtain a considerable mycelial biomass comparable to those obtained by other authors using several types of culture media (Gao & Gu, 2007; Lin &Yang, 2006; Liu & Wang, 2007).

To extract small molecules from mushrooms, including antioxidant molecules, methanol is the most common solvent, with yields ranging from 3.97 to 47.7 g/100 g (Mau, Lin, & Chen, 2002; Vaz et al., 2010). However, several investigations have shown that different bioactives, particularly phenolic compounds found in mushrooms, present high polarity (Jayakumar, Thomas, & Geraldine, 2009; Mau, Lin, & Chen, 2002; Mau, Lin, & Song, 2002; Wong & Chye, 2009). For this reason, in this work a mixture of ethanol and water was used, what allowed high extraction yields for both, fruiting body and mycelia of *A. brasiliensis*. The hydroalcoholic extracts were rich in reducing and non-reducing carbohydrates and free amino acids. Polysaccharides, including β-glucan, can be excluded because they are not extracted by solvents containing high proportions of ethanol. Contrary to a previous study (Kim et al., 2009), mannitol was present in all *A. brasiliensis* extracts. Mannitol is one of the most abundant polyols occurring in filamentous fungi. In the button mushroom *Agaricus bisporus* it is reported to account for up to 20 g/100 g of the dry weight of the mycelium and up to 50 g/100 g of the dry weight of the fruiting body (Horer, Stoop, Mooibroek, Baumann, & Sassoon, 2001). Several explanations have been put forward in order to explain the physiological significance of mannitol in filamentous fungi. These roles include carbohydrate storage, a reservoir of reducing power, stress tolerance and spore dislodgement and/or dispersal (Solomon, Waters, & Oliver, 2007). However, none of these explanations has received experimental support until now.

The detection of free amino acids in our extract corroborates previous observations about the presence of these compounds and other amines in several mushroom species including *A. brasiliensis* (Kim et al., 2009; Oliveira et al., 2010). The evaluation of extracts by paper chromatography has shown that these ninhydrin positive compounds are predominantly, but not exclusively, amino acids and can include polyamines and biogenic amines as already described for other mushrooms (Nishibori, Fujihara, & Akatuki, 2007).

The values obtained for *A. brasiliensis* extracts indicate that both fruiting body and mycelium are rich in phenolic compounds and its contents are similar or higher than those found in other edible and medicinal mushrooms including *Grifola frondosa*, *Pleurotus ostreatus*, *Ganoderma lucidum* and *Lentinula edodes* (Asatiani et al., 2007; Barros et al., 2008; Jayakumar et al., 2009; Kalyoncu et al., 2010; Mau, Lin, & Chen, 2002; Mau, Lin, & Song, 2002; Tsai et al., 2007; Wong & Chye, 2009). The evaluation of the amount of total phenolic compounds as well as the identification of the main phenolics in mushrooms, have both great importance in their nutritional and functional characterization. Phenolics are secondary metabolites commonly found in plants, mushrooms and fungi and have been reported to exert multiple biological effects including antioxidant activity (Dimitrios, 2006; Kim et al., 2008). It is well-known that phenolics are antioxidants with redox properties, which allow them to act as reducing agents, hydrogen donors, free radical scavengers, and singlet oxygen quenchers (Dimitrios, 2006). Unfortunately, only three from ten phenolic detected in HPLC experiments were identified in this work.

The flavonoid content, as indicated by the chemical identification procedure utilized in the present work, is very low. The HPLC analysis failed to identify any of these compounds. Although flavonoids such as quercetin and myricetin have been putatively identified in mushrooms including *A. brasiliensis* (Kim et al., 2008), these findings are still demanding confirmation by more sensitive and specific methods.

Because different antioxidant compounds may act *in vivo* through different mechanisms, no single method can fully evaluate the total antioxidant capacity of materials. For this reason, in this work, four complementary test systems were used for evaluating the antioxidant activities of the extracts. Two tests, DPPH

Table 4
Antioxidant activities (EC_{50} values) of *Agaricus brasiliensis* extracts and phenolic standards evaluated by different methods.

	A. brasiliensis extracts EC_{50} (μg/mL)			Phenolic standards EC_{50} (μg/mL)				
	Fruiting bodies	Young mycelia	Old mycelia	Gallic acid	Syringic acid	Pyrogallol	Citric acid	Oxalic acid
DPPH scavenging activity	305 ± 14^{a}	1413 ± 52^{b}	599 ± 35^{c}	1.42 ± 0.02	3.29 ± 0.16	1.31 ± 0.03	>500	>500
ABTS scavenging activity	84 ± 4.3^{a}	49.0 ± 5.4^{b}	20 ± 1.7^{c}	0.65 ± 0.03	1.84 ± 0.01	0.55 ± 0.01	58.5 ± 0.71	>500
LPO inhibition	44 ± 3.5^{a}	278 ± 15^{b}	129 ± 14.1^{c}	75.6 ± 5.30	34.1 ± 2.21	465.9 ± 0.16	12.0 ± 1.15	6.25 ± 0.64
Fe^{++} chelating ability	1002 ± 67^{a}	$1,325 \pm 89^{b}$	438 ± 34^{c}	>2000	>2000	>2000	684 ± 35	1190 ± 57

EC_{50} values for DPPH were obtained by interpolation from linear regression analysis. EC_{50} values for ABTS, LPO inhibition and chelating ability were obtained by interpolation from nonlinear regression analysis. Each value is expressed as mean ± standard deviation ($n = 3$). For extracts, means with different letters within a line are significantly different ($P \leq 0.05$).

scavenging activity and LPO inhibition, indicated stronger antioxidant activity for the fruiting bodies extracts when compared to the mycelial extracts. The other tests, ABTS scavenging activity and ferrous ion chelating activity, indicated the opposite. The cause for these apparently discrepant results could be partly related to the fact that different extracts may contain different types of polyphenolics with quite different reactivities. It should also be pointed out that the antioxidant activity of fungal extracts is not solely given by phenolics. Several types of organic acids are also reactive in the various antioxidant methods, especially in ferrous ion chelation (Kayashima & Katayama, 2002; Valentão et al., 2005). The high amounts of organic acids found in the mycelial extracts, especially citric acid, suggest that these compounds could be responsible, partly at least, for their high ABTS scavenging and ferrous ion chelating activities. Furthermore, possible synergistic effects involving phenolics and organic acids should not be ruled out and deserves future investigations.

To our best knowledge, the present study was the first report to demonstrate the antioxidant activity of the *A. brasiliensis* mycelia. These results suggest that the consumption of the fruiting body and the mycelial mass of *A. brasiliensis* can be beneficial for health, since they presumably offer antioxidant protection against oxidative damage. The same can be said about their use as a food supplement or in the pharmaceutical industry. However, only a single condition culture was used to obtain the mycelia. In a recent work the importance of the carbon sources in the production of antioxidant molecules by *Leucopaxillus giganteus* in submerged cultivation it was demonstrated (Barros et al., 2008). It is probable that different culture conditions such as temperature, carbon and nitrogen sources, among a series of other factors, may be related with the production of antioxidant compounds by *A. brasiliensis*. Studies to verify these variables in the production of antioxidant molecules by *A. brasiliensis* are under progress in our laboratory.

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