Food Chemistry 129 (2011) 1667–1675

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Antioxidative and immunomodulating activities of polysaccharide extracts of the medicinal mushrooms *Agaricus bisporus*, *Agaricus brasiliensis*, *Ganoderma lucidum* and *Phellinus linteus*

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ARTICLE INFO

Article history:
Received 9 December 2010
Received in revised form 19 April 2011
Accepted 16 June 2011
Available online 24 June 2011

Keywords:
Antioxidant activity
Immunomodulating activity
Medicinal mushrooms
Polysaccharides

ABSTRACT

Partially purified polysaccharides were obtained from four medicinal mushroom species, *Agaricus bisporus*, *Agaricus brasiliensis*, *Phellinus linteus* and *Ganoderma lucidum* by hot water extraction, followed by ethanol precipitation. The four samples contained varying amounts of both α- and β-glucans as determined by FT-IR and by quantitative estimation after prior partial hydrolysis (Megazyme β-glucan assay kit). EC_{50} values of the DPPH· scavenging activity of the polysaccharides from *G. lucidum* spores and *P. linteus* fruiting bodies were found to be particularly low, i.e. $EC_{50} < 0.1$ mg/ml. For *A. brasiliensis* and *A. bisporus*, EC_{50} values were 0.27 and 2.0 mg/ml. EC_{50} values of the antioxidant activity were 7.07 mg/ml for *G. lucidum*, 13.25 mg/ml for *A. brasiliensis* and >20 mg/ml for *A. bisporus* polysaccharide, respectively. EC_{50} values of the chelating activity of ferrous ions ranged from 0.59 mg/ml for *G. lucidum* to 7.80 mg/ml for *A. bisporus*. The EC_{50} values of the extracts in the reducing power assay ranged from 0.47 to 14.83 mg/ml. A correlation was found between EC_{50} values of the chelating and reducing power abilities and the amount of total glucans content in the extracts. *In vitro* measurements of immunomodulatory capacity of polysaccharide extracts showed that *A. bisporus*, *A. brasiliensis* fruiting bodies and *G. lucidum* spores extracts express an immunostimulating effect on activated human PBMCs and induce synthesis of IFN-γ. The polysaccharide extract of *P. linteus* fruiting bodies showed an immunosuppressive effect.

1. Introduction

Free radicals play an important positive physiological role and, at the same time, they may exert toxic effects (Papas, 1999). They are generated during normal cellular metabolism as a side product of oxidative phosphorylation. Free radicals participate in the regulation of signal transduction and gene expression, activation of receptors and as nuclear transcription factors (Ajith & Janardhanan, 2007; De la Fuente, 2002). They also play a vital role in phagocytosis (Papas, 1999). On the other hand, there is increasing evidence that free radicals may play a causative role in a variety of diseases including heart disease, cancer, Parkinson's and Alzheimer's disease, impairment of immune function, cataracts and muscular degeneration in elderly people (Papas, 1999; Shin, Lim, Lee, Lee, & Cho, 2001). All organisms are protected against free radical damage by defence systems involving superoxide dismutase and catalase or ascorbic acid, tocopherol and glutathione (Mau, Chao, & Wu, 2001; Shin et al., 2001). Improved antioxidant status may have an immunostimulatory effect (Mau et al., 2001; Shin et al., 2001).

The antioxidant status of humans reflects the dynamic balance between the antioxidant defence and prooxidant conditions. When the mechanism of antioxidant protection becomes unbalanced by factors such as ageing, impaired physiological functioning may occur, resulting in diseases and accelerated ageing (Tiwari, 2004). Antioxidant supplements or foods containing high concentrations of antioxidants may help to reduce oxidative damage. Identification of new antioxidants remains a highly active research area.

Polysaccharides are potentially useful biologically active ingredients for pharmaceutical use, such as for immune regulation, for anti-radiation, anti-blood coagulation, anti-cancer, anti-HIV and hypoglycemic activities (Lee et al., 2002; Yang et al., 2005; Yoon et al., 2003). The mushroom-derived polysaccharides lentinan, schizophyllan, and krestin have been accepted as immunoceuticals in Japan, Korea and China (Zhenga, Jiea, Hanchuanb, & Mouchenga, 2005).

The activity of polysaccharides is determined by their conformation, composition and size (Bohn & Be Miller, 1995). Among

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doi:10.1016/j.foodchem.2011.06.029

the large resources of fungi, medicinal higher *Basidiomycetes* are unlimited sources of biologically active polysaccharides, as well as from fruit bodies and from cultured *mycelia* (Wasser, 2002). Most bioactive mushroom polysaccharides are β-(1→3)(1→6)-glucans; some are heteropolysaccharides. Active glucans containing both α- and β-configurations are found in Basidiomycetes. Some glucans are bound to protein or peptide residues and form proteoglucans (Wasser, 2002).

The objective of the present study was to evaluate and compare antioxidant properties and immunomodulatory effects of hot water extracted polysaccharides from the medicinal mushroom species *Agaricus bisporus* (J. Lge) Imbach, *Agaricus brasiliensis* S. Wasser et al. (syn. *Agaricus blazei* Murill), *Ganoderma lucidum* (W. Curt.: Fr.) P. Karst., and *Phellinus linteus* (Berk. et Curt.) Teng. The polysaccharide profiles of the extracts were studied by FT-IR and sugar composition was determined after acid hydrolysis and HPLC analysis. Their antioxidant potentials were studied by the conjugated diene method, reducing power, free radical scavenging abilities of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals and chelating ability of ferrous ions. Immunomodulation was tested *in vitro*, by measuring the synthesis of cytokines by enzyme linked immunosorbent assay (ELISA).

2. Materials and methods

2.1. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), ferrous sulphate, potassium ferricyanide, ferric chloride, linoleic acid, bovine serum albumin (BSA), Coomassie Brilliant Blue G-250, trifluoroacetic acid (TFA), Tween 20, ferrous chloride, ferrozine, standards such as α-tocopherol, ascorbic acid, synthetic antioxidant butylated hydroxytoluene (BHT), citric acid and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Merck Co. (Darmstadt, Germany). Absolute methanol (Methanol Optigrade) was provided by LGC Promochem, Germany. Interferon-gamma (IFN-γ), Streptavidin–HRP, phorbol 12-myristate 13-acetate (PMA) and Ca-ionophore were obtained from BioSource™ (USA). RPMI-1640 medium with L-glutamine and sodium bicarbonate was from Sigma Chemicals Co. (St. Louis, MO, USA). An analytical mushroom β-glucan kit was obtained from Megazyme Int. (Wicklow, Ireland).

2.2. Sample preparation

Dried hot water extract of wild type *P. linteus* was kindly provided by Amazing Grace Health Industries (Bangkok, Thailand). *G. lucidum* spore extract Grade A was obtained from Fujian Xianzhilou Biological Sciences and Technology Co., Ltd. (PR China). *A. brasiliensis and A. bisporus* Horst U1 fruiting bodies were obtained from the Mushroom Experimental station (Horst, The Netherlands). Crude polysaccharide extracts were prepared by hot water extraction as described before (Song, Helsper, & Van Griensven, 2008). Polysaccharides were semi-purified by precipitation in 65% ethanol and repeated washing to remove the excess mannitol and ethanol-soluble phenolic compounds. The preciptitate was dried at 42 °C, in vacuum and stored for further use.

2.3. Polysaccharide analyses

The FT-IR spectra of polysaccharide extracts were recorded on a Thermo-Nicolet Model 6700 spectrophotometer (Thermo Scientific, USA). The spectrophotometer was equipped with DTGS TEC detector and OMNIC 7.3 software. Spectra were recorded in the 400–4000 cm^{-1} range using KBr disc technique.

2.4. Measurement of glucan content

Contents of total and α-glucans were determined in the polysaccharide extracts using the Mushroom and Yeast β-glucan Assay Procedure K-YBGL 09/2009 (Megazyme Int.) The enzyme kit, contains exo-1,3-β-glucanase, β-glucosidase, amyloglucosidase, invertase, glucose determination reagent (GOPOD-glucose oxidase, peroxidase, 4-aminoantipyrine) and glucose standard solution. To estimate the total glucan content polysaccharide samples were hydrolysed with ice-cold 60% sulphuric acid (v/v) for 1 h and for 2 h at 100 °C. After neutralization, hydrolysis was proceeded to glucose using a mixture of exo-β-(1→3)-D-glucanase plus β-glucosidase in sodium acetate buffer (pH 4.5) for 1 h at 40 °C. To measure total glucan content, glucose oxidase/peroxidase reagent was added. The absorbance of all solutions was analysed spectrophotometrically at 510 nm. α-Glucan contents were estimated upon enzymatic hydrolysis with amyloglucosidase plus invertase. Chromophore development employing glucose peroxidase reagent was conducted according to the total glucan content assay. The β-glucan content was calculated by subtracting the α-glucan from the total glucan content. All values of glucan contents were expressed as g of glucose equivalents per 100 g of dry matter.

2.5. Determination of polysaccharide content and composition

The total polysaccharide content of the extracted polysaccharides was determined using the phenol–sulphuric acid method with D-glucose as a reference (Du Bois, Gilles, Hamitton, Reders, & Smith, 1956). Analysis of monosaccharide composition was performed as published (Smiderle et al., 2010). Lyophilized samples were hydrolysed with 2 M TFA at 100 °C overnight, followed by evaporation to dryness. Residual TFA was removed by two evaporation cycles with 0.5 ml of methanol, and the final residue was dissolved in 0.5 ml of water. After 100-fold dilution monosaccharides were determined using a Dionex HPLC system (Dionex Co., Sunnyvale, Cal. USA) fitted with a Carbo Pac PA-1 column (4–250 mm), and a 25 μl sample loop with 20 mM NaOH isocratic solution (1 ml/min) as the mobile phase. An ED40 electrochemical detector fitted with a pulsed amperometric cell was used. Glucose and galactose were used as standards.

2.6. Protein determination

Protein concentrations were determined by Bradford (1976), using BSA as the standard. Total protein content of the extracts is expressed as g of BSA equivalents per 100 g of dry weight.

2.7. Inhibition of lipid peroxidation

The antioxidant activity was determined by the conjugated diene method with slight modifications (Lingnert, Vallentin, & Eriksson, 1979). Each polysaccharide powder (0.1 to 20 mg/ml, 100 μl) in Milli-Q water was mixed with 2 ml of 10 mM linoleic acid emulsion in 0.2 M sodium phosphate buffer (pH 6.5). Then 6.5 mM Tween 20 was added to provide a stable emulsion which was shaken in darkness at 37 °C to accelerate oxidation. After incubation for 15 h in 0.2 ml of the antioxidant mixture 6 ml absolute methanol was added. The absorbance of the supernatant mixture was measured at 234 nm against a blank using a UV/Vis spectrophotometer (Shimadzu UV-1650 PC, Japan). The blank was the solution with all reagents but without extract. The proportional antioxidant activity was calculated from the equation $[(A_0 - A_1)/A_0] \times 100$, where A_0 was the absorbance of the control reaction and A_1 the absorbance in the presence of sample. Ascorbic acid and α-tocopherol were used as positive controls. A value of 100% indicated the strongest inhibitory ability.

2.8. Ferric-reducing antioxidant power assay

Reducing power was determined according to Oyaizu (1986). Each polysaccharide powder (0.1 to 20 mg/ml, 2.5 ml) in Milli-Q water was mixed with 2.5 ml 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was vortexed and incubated at 50 °C for 20 min. Then, 2.5 ml of 10% trichloroacetic acid (w/v) was added and the mixture was centrifuged at 2000*g* for 10 min. The upper layer (5 ml) was mixed with 5 ml of Milli-Q water and 1 ml of 0.1% ferric chloride. The absorbance was measured at 700 nm against a blank using a UV/Vis spectrophotometer (Shimadzu UV-1650 PC, Japan). The blank was the solution with all reagents but without extract. A higher absorbance indicates a higher reducing power. Ascorbic acid was used as the positive control.

2.9. DPPH radical scavenging activity

The assay was carried out according to the modified method of Bilos (1958). In the first series each polysaccharide powder (0.1–10 mg/ml, 2 ml) in Milli-Q water was mixed with 1 ml freshly prepared DMSO solution containing 0.2 mM DPPH. In the second series each sample was mixed with 1 ml DMSO solution. The reaction mixture was vortexed vigorously for 1 min and kept in the dark at 20 °C for 40 min. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm against the blank using a UV/Vis spectrophotometer (Shimadzu UV-1650 PC, Japan). DPPH free radical scavenging activity was calculated from the equation $[1-(A_i-A_j)/A_c]\times 100$, where A_i was the absorbance of 2 ml extract mixed with 1 ml DPPH solution, A_j was the absorbance of 2 ml extract mixed with 1 ml DMSO solution and A_c was the absorbance of blank–2 ml of DMSO mixed with 1 ml of DPPH solution. Ascorbic acid, BHT and α-tocopherol dissolved in DMSO were used as the positive control. The EC_{50} value (milligrammes of extract per millilitre) is the effective concentration at which DPPH radicals were scavenged by 50% and was obtained by interpolation from linear regression analysis.

2.10. Chelating ability on ferrous ions

Chelating ability was determined according to the method of Dinis, Madeira, and Almeida (1994). Each polysaccharide powder (0.1 to 20 mg/ml, 1 ml) in Milli-Q water was mixed with 3.7 ml of methanol and 0.1 ml of 2 mM ferrous chloride. The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine. After 10 min at room temperature, the absorbance of the mixture was determined at 562 nm against the blank. Blank was the solution with all reagents but without extract. A lower absorbance indicates a higher chelating ability. The EC_{50} value (mg extract/ml) is the effective concentration at which ferrous ions were chelated by 50%. Citric acid and (EDTA) were used for comparison.

2.11. Measuring immunomodulatory effects in vitro

2.11.1. Cells

Human peripheral blood mononuclear cells (PBMC) were prepared from buffy coats of human blood, supplied by the Netherlands Blood Transfusion Service (Sanquin, Nijmegen, The Netherlands). Buffy coats were diluted 1:1 with phosphate buffered saline (PBS, pH 7.2) and centrifuged for 15 min at 2500*g* over a layer of Histopaque 1077 (Sigma). PBMCs were carefully collected at the interphase and washed with PBS. Cells were counted and resuspended at $5–10\times 10^6$/ml in RPMI-1640 containing 10% fetal calf serum (FCS), 1% penicilin and 1% streptomycin (Song et al., 2008).

2.11.2. Immunomodulatory activity

Polysaccharide solutions (50 μg/ml) were heated before application at 95 °C for 20 min. Immunomodulatory activity of the various polysaccharides extracts were tested in PBMCs stimulated with 1 ng/ml PMA and 0.5 μg/ml Ca-ionophore. After incubation for 48 and 72 h at 37 °C in 5% CO_2 atmosphere, the culture medium was tested for IFN-γ concentration by sandwich ELISA using a Duo Set® ELISA Kit DY 285 (R&D Systems, Inc.) according to the manufacturer's protocol. The detection range for IFN-γ was 7.81–1000 pg/ml.

2.12. Statistical analysis

All determinations for antioxidant activities were carried out in triplicate and for immunomodulating activity in quadruplicate. The results were reported as mean ± standard error of the mean (SEM). For antioxidant activities one-way analysis of variance (ANOVA) was done for the comparison of mean values, which were further separated using Fisher's least significant difference (LSD) at $p = 0.05$. Comparisons of IFN-γ mean values were performed by ANOVA procedure, a Dunnett test was then used to compare all treatment groups vs. the control group ($p = 0.05$). Statistical analyses were done using MS Excel (Microsoft Office 2007 Professional). Calculation of EC_{50} values was done by linear regression analyses using free statistical regression calculations online (http://easycalculation.com/statistics/regression.php). The correlation coefficient, *r*, between polysaccharides, proteins and antioxidant activity was determined using MS Excel (Microsoft Office 2007 Professional).

3. Results and discussion

3.1. Polysaccharide analyses

3.1.1. General

The FT-IR spectrum of hot water extracted polysaccharides of *A. brasiliensis* showed a typical carbohydrate pattern (Fig. 1). The characteristic strong broad band between 3000 and 3500 cm^{-1} region indicates the presence of OH stretching in hydrogen bonds which is indicative of strong inter- and intramolecular interaction of the polysaccharide chains. The band at ca. 1028 cm^{-1} evidenced (C–O) stretching, absorption at 1153 cm^{-1} indicated C–O–C stretching, and the band between 1000 and 1100 cm^{-1}, i.e. 1079 cm^{-1} was characteristic for the presence of β-glucans due to O-substituted glucose residues (Mathlouthi & Koenig, 1986; Stone & Clarke, 1992). A small amount of proteins was also observed with characteristic absorptions at 1635, 1540 and 1412 cm^{-1}. Typical N–H vibration at about 3400 cm^{-1} could be overlapped by OH stretch vibration at 3000–3500 cm^{-1} (Carey, 1992).

The FT-IR spectrum of polysaccharides isolated from spores of *G. lucidum* displayed a characteristic peak at ca. 850 cm^{-1} indicating the α-linked glycosyl residues (Fig. 1). The absorption band at 1080 cm^{-1} indicated that the glucosidic linkages of the glucosyl residues were β-linked. Characteristic bands for OH stretching at 3000–3500 cm^{-1}, and for C–O–C stretching at 1153 cm^{-1} were also present, as well as bands at 1639 and 1242 cm^{-1}, characteristic for a small amount of protein.

The FT-IR spectrum of polysaccharides isolated from *P. linteus* showed, among bands characteristic of glycosidic structures; a wide band at 3000 cm^{-1} and above, a band at 1155 cm^{-1} corresponding to C–O–C stretching, and a weak band at 890 cm^{-1}, revealing α-linked glycosyl residues of the main chain, i.e. an axial C_1–H. The band at 1074 cm^{-1} also indicated the presence of β-linkages in the glucosidic chain (Fig. 1).

The FT-IR spectrum of glucan from *A. bisporus* showed characteristic bands for polysaccharides *v* (OH) at 3000–3500 cm^{-1}, *v*

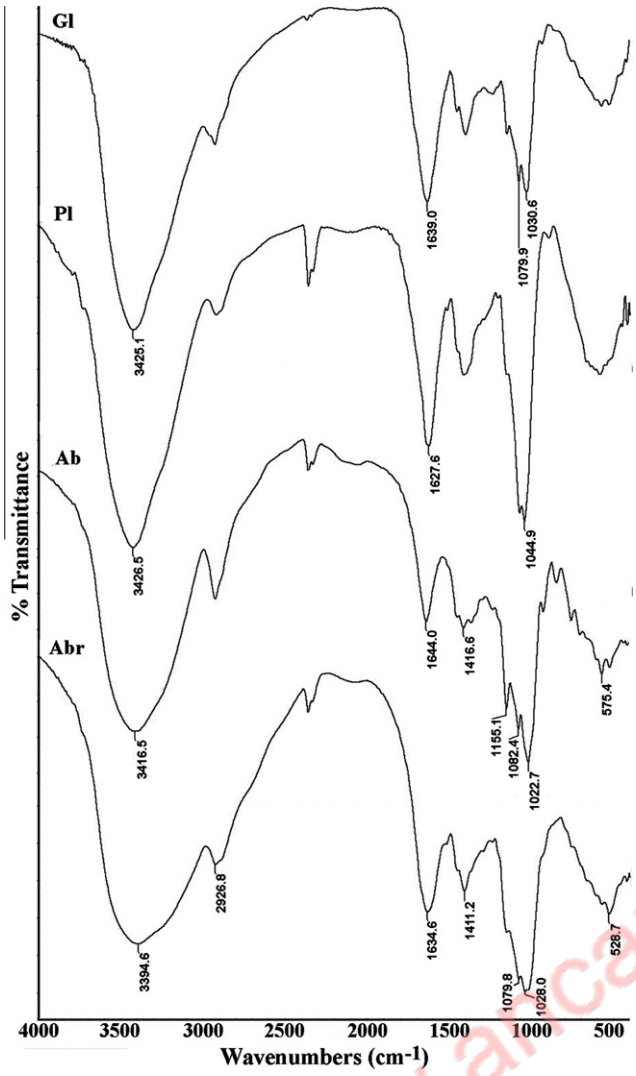

Fig. 1. FTIR spectra of polysaccharide extracts obtained from fruiting bodies of *A. bisporus* (Ab); *A. brasiliensis* (Abr); *P. linteus* (Pl); and spore of *G. lucidum* (Gl).

(CO) at 1023 cm^{-1}, *v* (C–O–C) at 1155 cm^{-1}. This spectrum showed a weak band at 850 cm^{-1} characteristic for equatorial C_1–H i.e. α-D-linkages, and an absorption band of β-glucans at 1082 cm^{-1}.

Based on the FT-IR spectra it was concluded that all four polysaccharide extracts showed absorption bands characteristic for the configurations of both glycosidic linkages, i.e. α-D- and β-D-types.

3.1.2. Polysaccharide and glucan content

Total polysaccharides and glucans (total, α- and β-) contents were measured using the Megazyme β-glucan assay kit. The results are presented in Table 1. Total polysaccharide contents of the extracts varied considerably, i.e. from 27.6 ± 1.2 g/100 g for *G. lucidum* spores to 74.4 ± 2.1 g/100 g for *A. bisporus* fruiting bodies. The low yield from *G. lucidum* may be explained by the hard nature of its spores.

The different polysaccharide extracts showed a varying glucan content. Total glucan contents of *A. bisporus*, *A. brasiliensis*, *P. linteus* and *G. lucidum* were 63.8 ± 0.9, 40.1 ± 1.0, 20.8 ± 0.5 and 24.5 ± 1.3 g/100 g, respectively. The differences in total glucan content of the samples were significant ($p < 0.05$). α-Glucan content ranged from 2.7 ± 0.6 to 19.6 ± 0.4 g/100 g. High content of α-glucan was found in *A. brasiliensis* fruiting bodies (17.3 ± 0.3 g/100 g) and *G. lucidum* spore (19.6 ± 0.4) extracts. Lee and Kim (2005) found 10.88 ± 1.52% α-glucan in dried fruiting bodies of *A. blazei* Murill (syn. *A. brasiliensis*). Amounts of confirmed α-glucans in *G. lucidum* spore polysaccharides extract were significantly higher than amounts of β-glucan (Table 1). In most mushrooms α-glucan was found at levels below 1% (Lee & Kim, 2005). Smiderle et al. (2010) recently published high values for the α-glucans in *A. bisporus* polysaccharide extracts, i.e. of up to 90%. Contrary to our extraction from fresh mushroom fruiting bodies caps, theirs were obtained from highly concentrated hot water extracts that had been industrially prepared from mostly *A. bisporus* stipe remnants.

β-Glucan content was determined by substracting α-glucan content from total glucan content. For tested samples, *A. bisporus*, *A. brasiliensis*, *P. linteus*, *G. lucidum*, β-glucan contents were 58.2 ± 0.9, 22.8 ± 0.4, 21.8 ± 1.7 and 1.2 ± 0.4 g/100 g, respectively. In a previous study, also using a Megazyme β-glucan assay kit it was found that levels of β-glucans ranged from 4.71 ± 0.59 to 46.20 ± 0.27% on a dry weight basis, in various edible mushrooms (Lee & Kim, 2005). Using the tungsten carbide nano-particle extraction and the Mushroom β-glucan kit, β-glucan contents from *Sparassis crispa* and *Phellinus linteus* were 70.2 ± 5.4% and 65.2 ± 5.9% (Park, Shim, Choi, & Park, 2009). Enormous variations in α- and β-glucan concentrations have been reported for all mushrooms studied (Lee & Kim, 2005; Manzi & Pizzoferrato, 2000; Park et al., 2009). This is not surprising as the major storage carbohydrate of fungi is glycogen, i.e. α-(1→4) glucan. In *Phycomyces blakesleanus* it was found that mycelial glycogen content depended greatly on the environmental and nutritional conditions of fungal growth, spores contained almost no glycogen (Rua, Busto, De Arriaga, & Soler, 1993). As α-glucans are intracellular compounds they will be easily extracted; β-glucans, being structural components of the fungal cell wall, are more difficult to extract. No functional explanation can therefore be given for the variability in α-glucan content of our different extracts.

3.1.3. Monosaccharide composition

The monosaccharide composition of *A. bisporus, A. brasiliensis* and *P. linteus* polysaccharide extracts are shown in Fig. 2. The glucans in the *A. bisporus* and in *P. linteus* contain mainly (>85%) glucose (Glc) with some minor proportions of galactose (Gal) and xylose (Xyl). *A. brasiliensis* (Fig. 2) glycan is rather complex; it contains, in addition to Glc (58%), considerable proportions of Gal (28%), mannose (Man) and Xyl (not separated, together 7%), fucose (Fuc) 4%, and some rhamnose (Rha), arabinose (Ara), fructose (Fru) and glucosamine (GlcN), each about 1%. GlcN is probably present in the native oligosaccharide as *N*-acetylglucosamine (GlcNAc), which is degraded by TFA-hydrolysis.

3.2. Protein content

The total protein content of polysaccharide extracts of *A. bisporus*, *G. lucidum*, *P. linteus* and *A. brasiliensis* was found to be 0.9 ± 0.1, 2.6 ± 0.1, 5.7 ± 0.3 and 7.3 ± 0.4 g/100 g, dw, respectively. The differences in total protein content of the samples were significant ($p < 0.05$). Content of protein to total polysaccharide content of *A. bisporus*, *G. lucidum*, *P. linteus* and *A. brasiliensis* was 1.3 ± 0.1, 9.5 ± 0.3, 9.0 ± 0.4 and 15.9 ± 0.6 g/100 g, respectively.

3.3. Antioxidant activity

3.3.1. General

For measuring antioxidant activity *in vitro*, different methods have been used corresponding to different levels of antioxidant action. The radical absorbance ability of polysaccharide extracts in aqueous medium using the DPPH free radical-scavenging capability, inhibition of lipid peroxidation and ferric reducing antioxidant

Table 1
Total polysaccharide and glucan contents of hot-water extracted polysaccharides.

Mushroom species	Total polysaccharide content (g/100 g)	Glucan content (g/100 g)[a]		
		Total	α	β
A. bisporus	74.4 ± 2.1 A[b]	63.8 ± 0.9 A	5.6 ± 0.2 C	58.2 ± 0.9 A
A. brasiliensis	45.9 ± 0.7 C	40. 1 ± 1.0 B	17.3 ± 0.3 B	22.8 ± 0.4 B
G. lucidum	27.6 ± 1.2 D	20.8 ± 0.5 D	19.6 ± 0.4 A	1.2 ± 0.7 C
P. linteus	62.6 ± 0.3 B	24.5 ± 1.3 C	2.7 ± 0.6 D	21.8 ± 1.7 B

[a] All percentages are on a dry weight basis. Data represent mean ± SEM ($n = 3$).
[b] Means with different letters within a column are significantly different ($p < 0.05$).

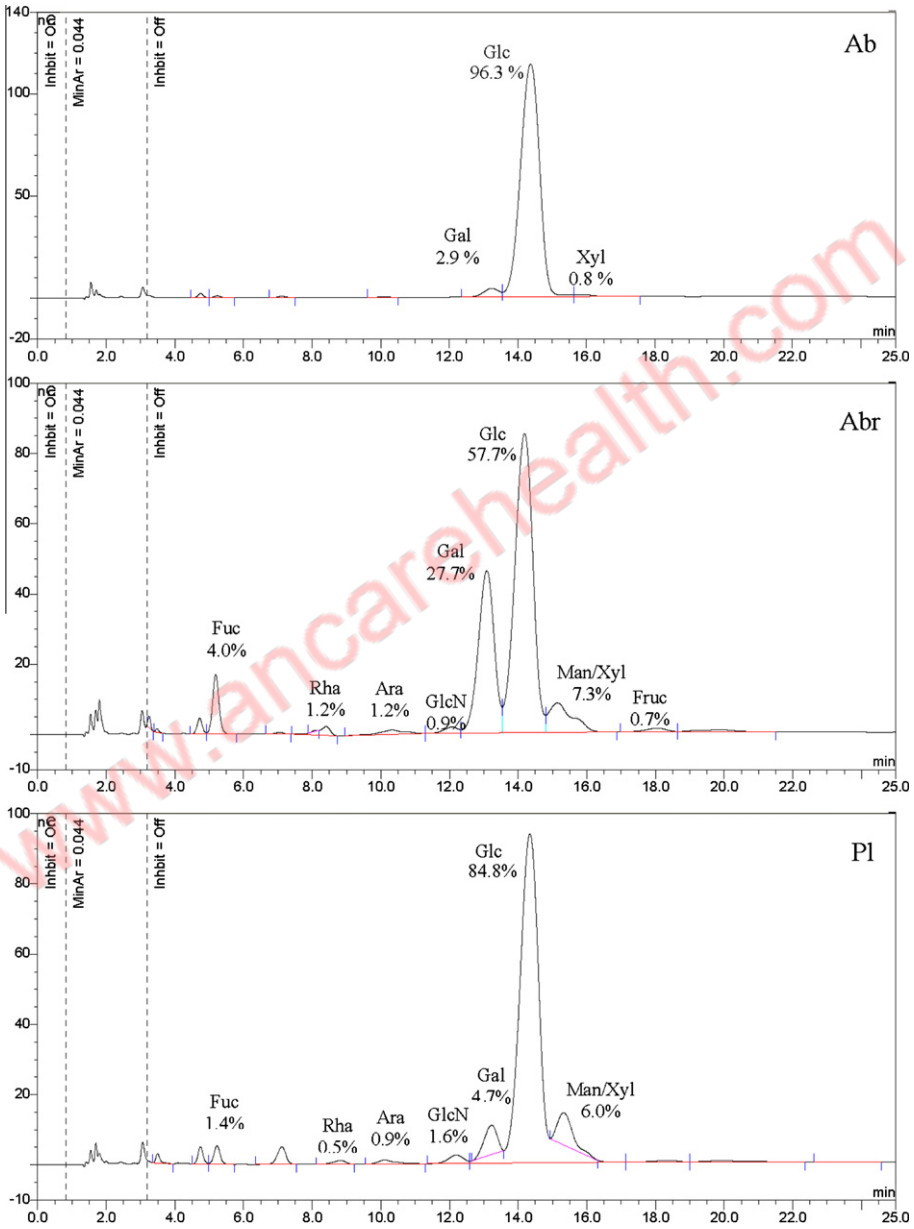

Fig. 2. HPLC patterns of the acid (TFA) hydrolysates of polysaccharides from *A. bisporus* (Ab); *A. brasiliensisi* (Abr) and *P. linteus* (Pl).

power assay were compared. Also, the chelating ability on ferrous ions of polysaccharide extracts was observed. Reduction of Fe^{2+} concentrations in the Fenton reaction protects against oxidative damage (Meir, Kanner, Akiri, & Hadas, 1995).

Tsiapali et al. (2001) proposed a model for the antioxidant ability of carbohydrate polymers. They demonstrated that glucan and nonglucan polymers are significantly better free radical scavengers than the monosaccharides. The antioxidant effect of the carbohydrate polymers do not correlate with the type of intrachain linkages, molecular weight, or degree of polymer branching. It was stated that the activity appears to correlate with the monosaccharide composition of the polymer. They proposed that the weak free

radical scavenging activity of monosaccharides is due to abstraction of the anomeric hydrogen. The enhanced antioxidant activity of the polymers over the monomeric form may be due to the greater ease of abstraction of the anomeric hydrogen from one of the internal monosaccharide units rather than from the reducing end. They also stated that the antioxidant effect of the carbohydrates is concentration dependent.

3.3.2. Scavenging ability on DPPH

DPPH is a free radical compound that has been widely used to determine the free radical-scavenging ability of various samples (Su, Shyu, & Chien, 2008). This method is based on the reduction of DPPH in solution, in the presence of a hydrogen-donating antioxidant and is dependent on the formation of the nonradical form DPPH–H in the reaction. The colour of the DPPH radical solution becomes lighter and its absorbance goes down in the presence of an antioxidant compound (Gulcin, Oktay, Kirecci, & Kufrevioglu, 2003).

At concentrations of 0.1–10 mg/ml, the scavenging abilities of *A. bisporus*, *A. brasiliensis*, *G. lucidum* and *P. linteus* polysaccharides on DPPH radicals were between 12.3–75.5%, 44.7–66.6%, 58.8–94.5%, and 77.9–86.9% (Fig. 3). At 0.1–10 mg/ml, the radical scavenging ability of the positive controls BHT, ascorbic acid and α-tocopherol were between 1.1–55.2%, 80.6–87.6% and 79.9–78.4%, respectively. The radical scavenging ability at of the extracts and positive controls, at 5 mg/ml decreased in the following order: *G. lucidum* > *P. linteus* > ascorbic acid > α-tocopherol ≈ *A. bisporus* > *A. brasiliensis* > BHT. Tseng, Yang, and Mau (2008) found that at 5 mg/ml, the hot water polysaccharides extracts from *Ganoderma tsugae* (mature and baby fruit bodies, mycelia and fermentation filtrate from a submerged culture) showed scavenging ability between 36.4% and 58.4%. At 20 mg/ml, scavenging abilities increased to 93.7–100%, except for that of the hot water extracted polysaccharide from filtrate, which was 74.9% (Tseng et al., 2008).

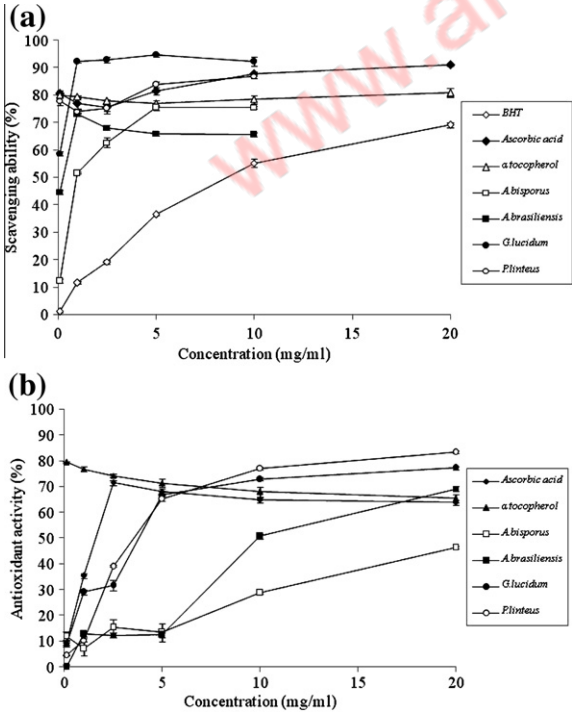

Fig. 3. Scavenging ability on DPPH (a); antioxidant activity evaluated in the linoleic acid model system (b) of polysaccharide extracts from of *A. bisporus*, *A. brasiliensis*, *P. linteus* and *G. lucidum*. Each value is expressed as mean ± SEM ($n = 3$).

3.3.3. Inhibition of lipid peroxidation

Lipid peroxidation is a major cause of food deterioration, which affects colour, flavour, texture, and nutritional value. The oxidative modification of low-density lipoproteins (LDLs) may play a role in the development of atherosclerosis. Oxidative modification depends on a common initiating step in the peroxidation of polyunsaturated fatty acid components in the LDLs (Puttaraju, Venkateshaiah, Dharmesh, Urs, & Somasundaram, 2006). Using the conjugated diene method, all tested extracts showed different patterns of antioxidant activities at 0.1–20.0 mg/ml (Fig. 3). The antioxidant activities of the polysaccharide extracts from *G. lucidum* and *P. linteus* increased as the concentration increased from 0.1 to 10.0 mg/ml and reached a plateau of 72.9–77.2% and 77.0–83.4% at 10.0–20.0 mg/ml, respectively. In contrast, polysaccharide extracts from *A. bisporus* and *A. brasiliensis* showed steadily increasing antioxidant activities as the concentrations increased to 46.3% and 69.2% at 20.0 mg/ml, respectively. Antioxidant activities of ascorbic acid and α-tocopherol were 63.8% and 65.4% at 20 mg/ml.

3.3.4. Reducing power

Fe^{3+} reduction is often used as an indicator of electron-donating activity. In the reducing power assay, the presence of antioxidants in the samples would result in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. This reducing capacity of compounds could serve as an indicator of potential antioxidant properties and the increasing absorbance suggests an increase in reducing power (Adesegun, Fajana, Orabueze, & Coker, 2009). The reducing power of the polysaccharide extracts increased in two patterns with an increase of concentration (Fig. 4). Polysaccharide extracts from *A. bisporus* and *A. brasiliensis* showed steadily increasing reducing activities as concentrations increased to 0.66 and 2.76 at 20.0 mg/ml, respectively. The reducing power of polysaccharide extracts from *G. lucidum* and *P. linteus* increased as the concentration increased from 0.1 to 5.0 mg/ml and reached a plateau of 3.01–3.14 and 3.14–3.11 at 5.0–20.0 mg/ml, respectively. Ascorbic acid, used as a positive control, had a reducing power of 3.92 at 5.0 mg/ml. High reducing power of *G. lucidum* and *P. linteus* polysaccharide extracts at 5 mg/ml suggest a high potential in hydrogen-donating ability (Adesegun et al., 2009).

3.3.5. Chelating ability on ferrous ions

Iron chelators mobilize tissue iron by forming soluble, stable complexes that are then excreted from the body (Ebrahimzadeh, Nabavi, & Nabavi, 2009). Chelation therapy may possibly reduce iron-related free radical damage and increase overall survival in human diseases as thalassemia major (Shinar & Rachmilewitz, 1990) and hereditary hemochromatosis. Testing chelating ability on ferrous ions, our polysaccharide extracts showed different patterns of chelating ability at 0.1–20.0 mg/ml (Fig. 4). The polysaccharide extract from *G. lucidum* achieved its maximum chelating ability of 85.1% at 1 mg/ml. Extracts from *P. linteus* and *A. brasiliensis* reached their maximum chelating ability of 77.3% and 91.3% respectively at 5 mg/ml, Above the maximum, at higher concentrations of polysaccharides extracts (till 20 mg/ml) a declining tendency of chelating ability was noted. Ker et al. (2005) reported that the concentration of available hydroxyl groups is responsible for the chelating ability of polysaccharides from *A. blazei* mycelia. They stated that declining tendency with higher dosages of polysaccharides is probably caused by the limitation of solubility and by increased hydrogen bonding, once the concentration of polysaccharide is increased. The diminished solubility would result in the aggregation of the polysaccharide molecules to form small colloidal particles, while the inter- and intramolecular hydrogen bonding can cause reduction of available hydroxyl groups; otherwise they can be active for ferrous ions chelation at a reasonably low concentration (Ker et al., 2005). These authors reported that the

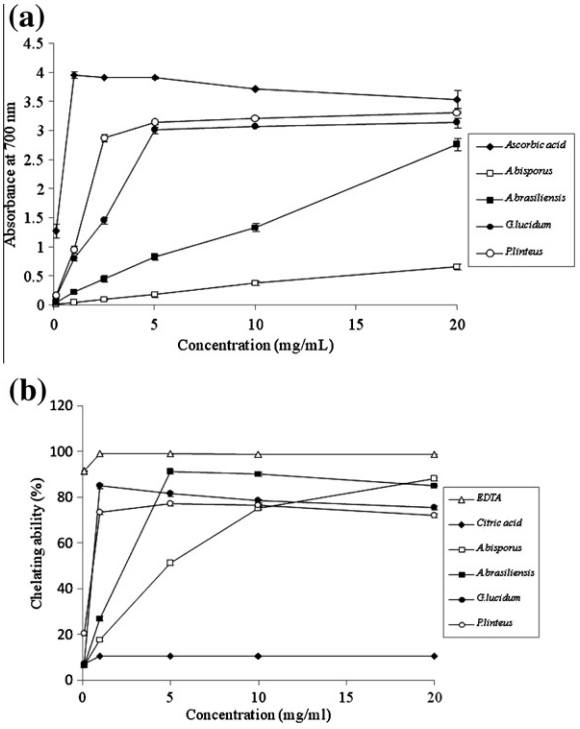

Fig. 4. Reducing power (a); and chelating ability on ferrous ions (b) of polysaccharide extracts from of *A. bisporus*, *A. brasiliensis*, *P. linteus* and *G. lucidum*. Each value is expressed as mean ± SEM ($n = 3$).

mole numbers of polysaccharide required to chelate the mole numbers of ferrous (Fe^{2+}) ions (ACP), are inversely related with the mean molecular mass. The lower the values of ACP, the more powerful chelating capability the polysaccharide can possess. However, chelating effects of the polysaccharide extract from *A. bisporus* on ferrous ion increased with the increased concentrations (Fig. 4). At 0.1–20 mg/ml, the chelating ability of *A. bisporus* polysaccharide extract was between 6.6% and 88.2%. The chelating effect of the synthetic metal chelator EDTA was between 91.6% and 99% at 0.1–20 mg/ml, while citric acid was not a good chelating agent for ferrous ions in this assay and its chelating ability was 10.7% at 20 mg/ml. Since ferrous ions are the most effective pro-oxidants in the food system (Yamaguchi, Tatsumi, Karo, & Yoshimitsu, 1988), the high ferrous-ion chelating abilities of polysaccharides from *A. bisporus*, *A. brasiliensis*, *G. lucidum* and *P. linteus* could add to food quality.

3.4. EC_{50} values in antioxidant properties

The results of DPPH free radical scavenging activity, antioxidant activity, reducing power and chelating effect on ferrous ion were normalized and expressed as EC_{50} (mg/ml) values which are the effective concentrations of each mushroom extract that are required to show 50% antioxidant properties (Table 2). A lower EC_{50} value corresponds to higher antioxidant activity of the mushroom's extract.

With regard to scavenging ability on DPPH radicals, the polysaccharides from *G. lucidum* and *P. linteus* showed very good scavenging ability as evidenced by their particularly low EC_{50} values (<0.1 mg/ml). For *A. brasiliensis* and *A. bisporus*, EC_{50} values of polysaccharides extracts were 0.27 and 2.0 mg/ml, respectively. Ascorbic acid and α-tocopherol are both confirmed as excellent scavenger of DPPH radicals ($EC_{50} < 0.1$ mg/ml). These are accepted food additives, and used at mg levels in foods. BHT is also a good DPPH radicals scavenger ($EC_{50} = 11.86$ mg/ml).

EC_{50} values of the antioxidant activity for *G. lucidum*, *P. linteus* and *A. brasiliensis* were 7.07, 7.11 and 13.25 mg/ml, respectively. Significant difference was not found at $p < 0.05$ for EC_{50} values between *G. lucidum* and *P. linteus* polysaccharide extracts. The EC_{50} value for *A. bisporus* was higher than 20 mg/ml. α-Tocopherol showed excellent antioxidant activity ($EC_{50} < 0.1$ mg/ml), whereas also ascorbic acid had a good activity as shown by its low EC_{50} value (1.64 mg/ml).

For reducing power of *G. lucidum*, *P. linteus*, *A. brasiliensis* and *A. bisporus* EC_{50} values were found of 0.67, 0.47, 3.13 and 14.83 mg/ml, respectively. Reducing capacities of *G. lucidum* and *P. linteus* polysaccharides extracts were good and no significant difference was found between these two at $p < 0.05$. Ascorbic acid showed excellent reducing activity ($EC_{50} < 0.1$ mg/ml). Regression analysis revealed a very strong and significant correlation between EC_{50} (reducing power) and total glucan ($r = 0.963$, $p < 0.05$). An increase of EC_{50} values correlated with a higher content of total glucans. There was a significant negative correlation between the EC_{50} of reducing activity and the total protein content of moderate strength ($r = -0.635$, $p < 0.05$). This could be due to the presence of the amino acids cysteine, methionine and tyrosine in the protein remnants of the extracts.

EC_{50} values of the chelating abilities on ferrous ions for *G. lucidum*, *P. linteus*, *A. brasiliensis* and *A.bisporus* extracts were 0.59, 0.91, 2.04 and 7.80 mg/ml, respectively. For comparison, the chelator EDTA showed a higher activity ($EC_{50} < 0.1$ mg/ml). No significant difference, at $p < 0.05$ was found between EC_{50} values of *G. lucidum* and *P. linteus* polysaccharide extracts. Correlation between EC_{50} values of the chelating abilities and total glucan content was very strong and significant ($r = 0.967$, $p < 0.05$). *G. lucidum* extract, with the lowest content of total glucans (Table 1) showed the best chelating ability (minimum EC_{50}). *A. bisporus* extract had the highest content of total glucans and the lowest chelating ability (maximum EC_{50}). Regression analysis revealed a moderate negative correlation between EC_{50} and total protein contents ($r = -0.606$, $p < 0.05$). Remnants of mushroom tyrosinase could have catalysed Fe^{2+} oxidation and hence have led to a small increase of chelation.

Many scientists have reported various antioxidant effects of hot water extracted mushroom polysaccharides (Liu, Wang, Pang, Yao, & Gao, 2010; Ramirez-Anguiano, Santoyo, Reglero, & Soler

Table 2
EC_{50} values of polysaccharide extracts from *A. bisporus*, *A. brasiliensis*, *G. lucidum* and *P. linteus* in antioxidant properties.

	EC_{50}[a] (mg extract/ml)			
	A. bisporus	*A. brasiliensis*	*G. lucidum*	*P. linteus*
Scavenging ability on DPPH radicals	2.00 ± 0.18 A[b]	0.27 ± 0.02 B	<0.1	<0.1
Antioxidant activity	>20	13.25 ± 0.30 A	7.07 ± 0.23 B	7.11 ± 0.13 B
Reducing power	14.83 ± 1.16 A	3.13 ± 0.14 B	0.67 ± 0.01 C	0.47 ± 0.03 C
Chelating ability on ferrous ions	7.80 ± 0.21 A	2.04 ± 0.58 B	0.59 ± 0.01 C	0.91 ± 0.18 C

[a] EC_{50} value: the effective concentration at which the DPPH radicals were scavenged by 50%; the antioxidant activity was 50%; the absorbance was 0.5 for reducing power; and ferrous ions were chelated by 50%, respectively. EC_{50} value was obtained by interpolation from linear regression analysis.
[b] Each value is expressed as mean ± SEM ($n = 3$). Means with different letters within a row are significantly different ($p < 0.05$).

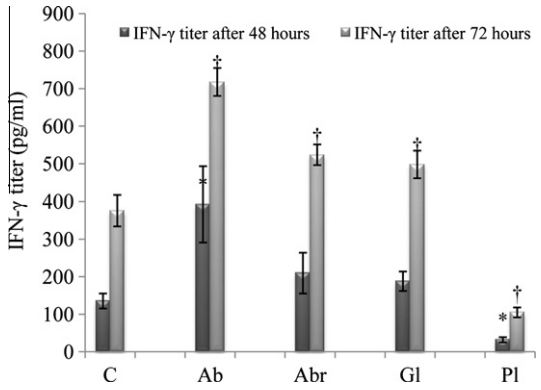

Fig. 5. IFN-γ production of stimulated PBMCs incubated with *A. bisporus* (Ab), *A. brasiliensis* (Abr), *G. lucidum* (Gl) and *P. linteus* (Pl) polysaccharides extracts. Suspension of PBMCs in PBS was used as control (C), same conditions. IFN-γ levels were measured by ELISA after 48 and 72 h of stimulation. Data are expressed as mean ± SEM ($n = 4$). Significantly different from the control, $^{*}p < 0.05$ after 48 h and $^{\dagger}p < 0.05$ after 72 h.

Rivas, 2007; Yang, Lin, & Mau, 2002), without proven evidence for purity of extracts. Song and Van Griensven (2008) demonstrated that many if not all commercially available hot water extracted polysaccharides of medicinal mushrooms contain phenolic compounds. They showed a statistically high significant linear relationship between the scavenging activity of the polysaccharide extracts and their content of phenols. Clearly, hot water treatment and ethanol precipitation are not sufficient to remove all phenols from the polysaccharide extracts. It remains possible that polysaccharide-phenol complexes or phenolic compounds have contributed to the antioxidant properties that were observed for the polysaccharide extracts of *A. bisporus, A. brasiliensis, G. lucidum* and *P. linteus*.

3.5. Immunomodulating capability

IFN-γ has antiviral, immunoregulatory, and anti-tumour properties (Schroder, Hertzog, Ravasi, & Hume, 2004). It alters transcription in up to 30 genes producing a variety of physiological and cellular responses (Schroder et al., 2004). IFN-γ is used to treat chronic granulomatous disease and osteopetrosis (Key, Ries, Rodriguiz, & Hatcher, 1992; Todd & Goa, 1992).

In these experiments all four extracts expressed an immunomodulatory effect on activated PBMCs (Fig. 5). The IFN-γ titer measured after 48 h of incubation showed an increased value for the polysaccharide extracts from *A. bisporus* and *A. brasiliensis* fruiting bodies and *G. lucidum* spores. Titer of IFN-γ produced by activated PBMCs for *A. bisporus*, *A. brasiliensis* and *G. lucidum* extracts was 392.2, 209.6 and 187.8 pg/ml, respectively. *P. linteus* fruiting bodies polysaccharide extract showed immunosuppressive effect, 32.6 pg/ml. IFN-γ titer for positive control, suspension of PBMCs in PBS, was 135.2 pg/ml. The differences in IFN-γ contents of *A. bisporus* and *P. linteus* extracts vs. model control were strongly significant ($p < 0.05$).

After 72 h of incubation immunomodulating effects of extracts were also detected (Fig. 4). For *A. bisporus*, *A. brasiliensis*, *G. lucidum*, *P. linteus*, the IFN-γ titers were 717.7, 523.8, 497.9 and 104.8 pg/ml, respectively. *P. linteus* polysaccharide extract kept its characteristic immunosuppressive effect on activated PBMCs and synthesis of IFN-γ. The difference between IFN-γ content produced by control and PBMCs stimulated with *P. linteus* polysaccharides extract was approximately fourfold, after 48 and 72 h of incubation. *A. bisporus*, *A. brasiliensis* and *G. lucidum* tested extracts showed immunostimulatory effect vs. control. The differences in IFN-γ content of four tested extracts vs. control were significant ($p < 0.05$).

4. Conclusions

Measurements of antioxidant properties of hot water polysaccharide extracts of fruiting bodies of *A. bisporus*, *A. brasiliensis*, *P. linteus* and spores of *G. lucidum* showed relatively high antioxidant activities. The activity was in descending order: *G. lucidum* ≈ *P. linteus* > *A. brasiliensis* > *A. bisporus*. For all four polysaccharides, a positive correlation was observed between the EC_{50} values of the chelating abilities and reducing power on the one hand and the amount of total glucans on the other. A darker colour of the extracted polysaccharide was associated with a higher antioxidant activity. Given the latter's structural analogy to the oxidized monophenol derivatives that occur as intermediary products in the tyrosinase driven browning of mushroom extracts, it seems that during extraction of polysaccharides tissue is damaged, and tyrosinase has access to the phenolic compounds of the extracts; oxidations leading to browning then occur (Fenoll, Rodriguez-Lopez, Varon, et al., 2000; Fenoll, Rodriguez-Lopez, Garcia-Sevilla, et al., 2000). Tyrosinase, widely distributed throughout the phylogenetic scale, catalyses the hydroxylation of monophenols to *o*-diphenols and the oxidation of the latter to *o*-quinones which polymerize to form brown-coloured pigments (Prota, 1992; Van Gelder & Flurkey, 1997). This suggests that oxidized polyphenols were still present in the extracts in spite of ethanol precipitation and wash of the polysaccharides suggesting a covalent linkage between these components (Renard, Baron, Guyot, & Drilleau, 2001; Sun, Payne, Moas, Chu, & Wallace, 1992).

In vitro measurements of the immunomodulatory activity showed that *A. bisporus*, *A. brasiliensis* fruiting bodies and *G. lucidum* spores polysaccharides extracts cause an increased synthesis of IFN-γ in human PBMCs, suggesting proinflammatory effects. Polysaccharide extracts from *P. linteus* fruiting bodies showed a characteristic decrease of IFN-γ synthesis suggesting an immunosuppressive activity.

The results of the present study suggest that polysaccharide extracts of medicinal mushrooms act as natural antioxidants and possess immunomodulatory properties. Polysaccharide extracts may be good sources for the development of antioxidant food additives. In addition, the possible immunosuppressive effect of *P. linteus* polysaccharide extract is particularly interesting and may find application in suppression of autoimmune diseases such as rheumatoid arthritis. Further investigations are necessary to verify these activities *in vivo*.

Acknowledgements

This study was financed by grants from the Federation of European Microbiological Societies (FEMS) and the Serbian Ministry of Science and Technological Development.

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