Fruiting body, spores and *in vitro* produced mycelium of *Ganoderma lucidum* from Northeast Portugal: A comparative study of the antioxidant potential of phenolic and polysaccharidic extracts

Sandrina A. Heleno[a,b], Lillian Barros [a,c], Anabela Martins[a], Maria João R.P. Queiroz[b], Celestino Santos-Buelga [c], Isabel C.F.R. Ferreira[a]

[a]*Centro de Investigação de Montanha, Escola Superior Agrária, Campus de Santa Apolónia, apartado 1172, 5301-854 Bragança, Portugal*

[b]*Centro de Química, Universidade do Minho, Campus de Gualtar 4710-057 Braga, Portugal*

[c]*GIP-USAL, Facultad de Farmacia, Universidad de Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain*

* Author to whom correspondence should be addressed (e-mail: iferreira@ipb.pt telephone +351-273-303219; fax +351-273-325405).

Abbreviations

CAT Catalase

DAD Diode array detector

DPPH 2,2-Diphenyl-1-picrylhydrazyl

EC_{50} Extract concentration providing 50% antioxidant activity or 0.5 absorbance in the reducing power assay

FB Fruiting body

GAE Gallic acid equivalents

GPx Glutathione peroxidase

GSH Reduced glutathione

HPLC High-performance liquid chromatography

IS Internal standard

M Mycelium

MMN Melin-Norkans medium

MS Mass spectrometry

PDA Potato dextrose agar medium

PE Polysaccharides equivalents

Ph Phenolic extract

Ps Polysaccharidic extract

RI Refraction index

RSA Radical scavenging activity

S Spores

SOD Superoxide dismutase

TBARS Thiobarbituric acid reactive substances

v/v ml/100 ml

w/v g/100 ml

ABSTRACT

Ganoderma lucidum is one of the most extensively studied mushrooms due to its medicinal properties. Herein, a systematic study was carried out in order to compare the antioxidant activity of phenolic and polysaccharidic extracts from fruiting body, spores and mycelium, obtained in three different culture media, of *G. lucidum* from Northeast Portugal. Phenolic extracts were characterized using high-performance liquid chromatography coupled to photodiode array detection, while polysaccharidic extracts were hydrolysed and further characterized using HPLC and refraction index detection. In general, the phenolic extracts (Ph) proved to have higher antioxidant potential than their corresponding polysaccharidic extracts (Ps). Amongst phenolic extracts, FB-Ph provided the highest antioxidant activity ($EC_{50} \leq 0.6$ mg/ml) and the highest content in total phenolics (~29 mg GAE/g extract) and phenolic acids (*p*-hydroxybenzoic and *p*-coumaric acids). S-Ps was the polysaccharidic extract with the best antioxidant activity ($EC_{50} \leq 2$ mg/ml); nevertheless, the highest levels of total phenolics were obtained in FB-PS (~56 mg GAE/g extract), while the highest levels of total polysaccharides (~14 mg PE/g extract) and individual sugars were observed in mycelia obtained from solid culture media, M-PDA-Ps and M-sMMN-Ps. The free radical scavenging properties, reducing power and lipid peroxidation inhibition of *G. lucidum* seemed to be correlated with phenolic compounds mostly in a free form, but also linked to polysaccharides.

Keywords: *Ganoderma lucidum*; fruiting body/spores/mycelium; phenolic/polysaccharidic extracts; antioxidant properties

1. Introduction

Ganoderma lucidum (Curtis) P. Karst. is a woody Basidiomycota mushroom from the Polyporales order and Ganodermataceae family; it could be a parasitic species of living hardwoods (especially oaks) or saprobic of deadwood from hardwoods. It has been used in functional food and preventive medicines in the Far East for more than 2000 years and becomes a popular dietary supplement ingredient in Western countries, with an annual global market value of over $1.5 billion for *G. lucidum* extracts (Sullivan, Smith, & Rowan, 2006).

Some pharmacological properties have been related to its capacity to lower the risk of cancer, liver, and heart diseases and to boost the immune system (Paterson, 2006). The beneficial health properties of Ganoderma species are attributed to a wide variety of bioactive components such as polysaccharides, triterpenes, sterols, lectins and some proteins (Ferreira, Vaz, Vasconcelos, & Martins, 2010).

There are some reports regarding antioxidant properties of *G. lucidum* methanolic (Mau et al., 2002) and aqueous (Lin, Lin, Chen, Ujiie, & Takada, 1995) extracts, but also of its polysaccharides (Jia et al., 2009; Ping, Yan, Bing, Guo, Yun, & Ping, 2009; Liu, Wang, Pang, Yao, & Gao, 2010; Kozarski et al., 2011) and phenolic compounds (Kim et al., 2008). Phenolic compounds possess an established antioxidant capacity and their antiradical mechanism has almost completely revealed (Ferreira, Barros, & Abreu, 2009). Regarding polysaccharides, their antioxidant properties have been evaluated by the free radical scavenging abilities of 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl radical, superoxide radical, conjugated diene method, reducing power and chelating effects on ferrous ions (Ping et al., 2009; Liu et al., 2010; Kozarski et al., 2011). Their radical scavenging activity seems to be related to an increase in the antioxidant enzyme activities: superoxide dismutase (SOD) which catalyzes dismutation of superoxide

anion to hydrogen peroxide, catalase (CAT) which detoxifies hydrogen peroxide and converts lipid hydroperoxides to nontoxic substances, and glutathione peroxidase (GPx) which maintains the levels of reduced glutathione (GSH) (Guo et al., 2009; Ping et al., 2009). Besides the increase in the enzymes activity, some authors described an increase in the mRNA expression of SOD and GPx (Jia et al., 2009).

In the search for active compounds, studies have been performed using extracts from the fruiting body, while mycelium has been dismissed (Saltarelli et al., 2009). The benefits of *in vitro* cultivation include the ability to manipulate the culture medium to optimise mycelia growth and a shorter cultivation time (Paterson, 2006). It is known that media composition and their constituent's concentrations also influence fungi and plant cells metabolism and production of metabolites *in vitro* (Chattopadhyay, Farkya, Srivastava, & Bisaria, 2002; Nigam, Verma, Kumar, Kundu, & Ghosh, 2007). The production of fruiting bodies includes a long cultivation in a plastic bag and, therefore, mycelia are alternative or substitute products of mature fruiting bodies, for use in the formulation of nutraceuticals and functional foods (Saltarelli et al., 2009).

Furthermore, biomedical investigations have been conducted mainly in China, Korea, Japan and USA (Paterson, 2006); only in the last few years some experiments demonstrating medicinal properties of local *Ganoderma*, have been performed in Europe as well (Saltarelli et al., 2009). Portuguese *Ganoderma* species have not been characterized so far and it will now make part of the chemical, nutritional and bioactive inventory of potentially interesting species (and not yet characterized in literature) from different Portuguese habitats. Being a potential source of important antioxidants this wild species can be used as a nutraceutical and/or functional food and, its biotechnological production *in vitro* could be an interesting approach for large scale production. Different media can account for differential growth rates and mycelia

biomass production at the same time that they can interfere in oxidative stress metabolism and antioxidants production.

In the present work *Ganoderma lucidum* from Northeast Portugal was used to perform a chemical characterization and antioxidant activity evaluation of phenolic and polysaccharidic extracts from its fruiting body, spores and mycelium produced in different culture media. Phenolic extracts were characterized using high-performance liquid chromatography coupled to photodiode array detection (HPLC-DAD-MS), while polysaccharidic extracts were hydrolysed and further characterized using HPLC and refraction index (RI) detection. The bioactive compounds were related to free radical scavenging properties, reducing power and lipid peroxidation inhibition.

2. Materials and methods

2.1. Standards and Reagents

Acetonitrile 99.9%, *n*-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Lab-Scan (Lisbon, Portugal). Phenolic standards (gallic, *p*-hydroxybenzoic, *p*-coumaric, and cinnamic acids), sugars (D-fructose 98%, D-glucose, D-sucrose 99.0%, D-mannitol 98% and D-trehalose di-hydrate 99.5%), starch and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma (St. Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). All other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.2. Preparation of samples

Samples of *Ganoderma lucidum* (Curtis) P. Karst. were collected in Bragança (Northeast Portugal) in July 2011. After taxonomic identification of the sporocarps (Phillips, 1981; Hall, Stepherson, Buchanan, Yun, & Cole, 2003; Oria de Rueda, 2007), specimens were deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança. Fruiting bodies (FB) were further separated from spores (S) using a scalpel. Mycelium (M) was isolated from sporocarps on:

i) solid Melin-Norkans medium (sMMN) pH 6.6 (NaCl 0.025 g/l; malt extract 5 g; casamino acids 1 g; $(NH_4)_2HPO_4$ 0.25 g/l; KH_2PO_4 0.50 g/l; $FeCl_3$ 0.0050 g/l; $CaCl_2$ 0.050 g/l; $MgSO_4.7H_2O$ 0.15 g/l; thiamine 0.10 g/l; glucose 10 g/l; agar 20 g/l, in tap water) (Marx, 1969); ii) incomplete solid MMN (sMMNi) pH 6.6 (NaCl 0.025 g/l; $(NH_4)_2HPO_4$ 0.25 g/l; KH_2PO_4 0.50 g/l; $FeCl_3$ 0.0050 g/l; $CaCl_2$ 0.050 g/l; $MgSO_4.7H_2O$ 0.15 g/l; thiamine 0.10 g/l; glucose 10 g/l; agar 20 g/l, in tap water) (Marx, 1969); iii) liquid MMN (lMMN); pH 6.6 (NaCl 0.025 g/l; $(NH_4)_2HPO_4$ 0.25 g/l; malt extract 5 g; casamino acids 1g; KH_2PO_4 0.50 g/l; $FeCl_3$ 0.0050 g/l; $CaCl_2$ 0.050 g/l; $MgSO_4.7H_2O$ 0.15 g/l; thiamine 0.10 g/l; glucose 10 g/l) (Marx, 1969); iv) Potato Dextrose Agar medium (PDA) pH 5.6 ± 0.2; potatoes infusion from 200 g/l; dextrose 20 g/l; agar 15 g/l (Biolab).

Mycelia were grown in Petri dishes (9 cm diameter) with 8 ml of solid media covered with cellophane and in flasks (250 ml) with 20 ml of MMNc liquid medium, 25 ºC in the dark. After 15 days of growth (when mycelium reached maximum radial growth in PDA medium) the mycelium was recovered from the medium. All samples were lyophilised (FreeZone 4.5 model 7750031, Labconco, Kansas, USA), quantified and reduced to a fine dried powder (20 mesh). The codes attributed to each sample are given in **Table 1**.

2.3. Preparation and characterization of phenolic extracts

2.3.1. Preparation of phenolic extracts

The lyophilized samples (~1 g) were extracted with methanol:water (80:20, v/v; 30 ml) at -20 °C for 2 h. After sonication for 15 min, the extract was filtered through Whatman nº 4 paper. The residue was then extracted with two additional 30 ml portions of the methanol:water mixture. Combined extracts were evaporated at 40 ºC under reduced pressure (rotary evaporator Büchi R-210) to remove methanol. The aqueous phase was washed with *n*-hexane, and then submitted to a liquid-liquid extraction with diethyl ether (3 × 30 ml) and ethyl acetate (3 × 30 ml). The organic phases were evaporated at 40 ºC to dryness, re-dissolved in water:methanol (80:20, v/v) for the antioxidant activity assays, and further chemically characterized.

2.3.2. Quantification of total phenolics

The extracts solutions (1 ml) were mixed with *Folin-Ciocalteu* reagent (5 ml, previously diluted with water 1:10, v/v) and sodium carbonate (75 g/l, 4 ml). The tubes were vortex mixed for 15 s and allowed to stand for 30 min at 40 °C for colour development. Absorbance was then measured at 765 nm (Analytikjena spectrophotometer). Gallic acid was used to obtain the standard curve (0.0094 – 0.15 mg/ml), and the results were expressed as mg of gallic acid equivalents (GAE) per g of extract.

2.3.3. Analysis of phenolic compounds

The extracts solutions were (1 ml) were filtered through a 0.22 µm disposable LC filter disk for HPLC analysis. The analysis was performed using a Hewlett-Packard 1100 series liquid chromatograph (Agilent Technologies) as previously described (Barros, Dueñas, Ferreira, Carvalho, & Santos-Buelga, 2011). The phenolic compounds were

characterised according to their UV and MS spectra, and retention times compared with commercial standards. For the quantitative analysis of phenolic compounds, a calibration curve was obtained by injection of known concentrations (5-100 µg/ml) of different standards compounds, and the results were expressed as mg per 100 g of dry weight (dw). MS detection was performed using an Agilent Technologies MSD Trap XCT detector (Santa Clara, CA) equipped with an ESI source and an ion trap mass analyser, following the conditions described by Barros et al. (2011b).

2.4. Preparation and characterization of polysaccharidic extracts

2.4.1. Preparation of polysaccharidic extracts

The lyophilized mushrooms (~1.5 g) were extracted with water at boiling temperature (50 ml) for 2 h and agitated (150 rpm; Velp Are magnetic stirrer) and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with two more portions of boiling water, in a total of 6 h of extraction. The combined extracts were lyophilized, and then 95% ethanol (10 ml) was added and polysaccharides were precipitated overnight at 4 ºC. The precipitated polysaccharides were collected after centrifugation (Centorion K24OR refrigerated centrifuge) at 3100 × *g* for 40 min followed by filtration, and then were lyophilized, resulting in a crude polysaccharidic sample (Cheng et al., 2008). The crude polysaccharidic samples were re-dissolved in water for the antioxidant activity assays, and further hydrolysed for chemical characterization.

2.4.2. Quantification of total polysaccharides

The extracts solutions were (1 ml) added to 80% phenol (25 µl) and conc. sulphuric acid (1 ml). The mixture was shaken and allowed to stand at 30 ºC for 30 min. The

absorbance was measured at 490 nm (Dubois et al., 1956). Starch (although being glycogen the storage polysaccharide in mushrooms, starch is the most available polysaccharide) was used to obtain the standard curve (0.625-40 mg/ml), and the results were expressed as mg of polysaccharides equivalents (PE) per g of extract.

The total content in phenolics (following the procedure described above) was also determined in the polysaccharidic extracts, as they might contain linked and co-extracted phenols.

2.4.3. Characterization of polysaccharidic extracts

The polysaccharidic extracts were hydrolyzed with 0.05 M trifluoroacetic acid (TFA, 2 ml), maintained at 90 ºC for 16h and then centrifuged, following a procedure described by Cheng et al. (2008) with some modifications. The supernatant was lyophilized, redissolved in distilled water (1 ml) and filtered through 0.2 µm nylon filters for HPLC-RI analysis, using the conditions previously described by the authors (Grangeia, Heleno, Barros, Martins, & Ferreira, 2011). Sugars identification was made by comparing the relative retention times of sample peaks with standards. For the quantitative analysis of sugars, a calibration curve was obtained by injection of known concentrations (0.5-40 mg/ml) of different standards compounds, and the results were expressed in g per 100 g of dry weight (dw).

For a comparison, free sugars were also determined. The lyophilized samples (1 g) were spiked with raffinose as internal standard (IS, 5 mg/ml) and were extracted with 40 ml of 80% aqueous ethanol at 80 ºC for 30 min. The resulting suspension was centrifuged at 15,000 *g* for 10 min. The supernatant was concentrated at 60 ºC under reduced pressure and defatted three times with 10 ml of ethyl ether, successively. After concentration at 40 ºC, the solid residues were dissolved in water to a final volume of 5

ml and filtered through 0.2 μm nylon filters for HPLC-RI analysis using the same procedure described above (Grangeia at al., 2011).

2.5. Evaluation of antioxidant activity

The antioxidant activity of the phenolic and polysaccharidic extracts was evaluated using four different *in vitro* assays already described by the authors (Barros, Carvalho, & Ferreira, 2011): DPPH radical-scavenging activity and reducing power (performed in an ELX800 Microplate Reader, Bio-Tek Instruments, Inc), inhibition of β-carotene bleaching in the presence of linoleic acid radicals, and inhibition of lipid peroxidation using TBARS (thiobarbituric acid reactive susbtances) assay in brain homogenates (performed using the spectrophotometer already mentioned). The extract concentrations providing 50% of antioxidant activity or 0.5 of absorbance (EC_{50}) were calculated from the graphs of antioxidant activity percentages (DPPH, β-carotene bleaching and TBARS assays) or absorbance at 690 nm (reducing power assay) against extract concentrations. Trolox was used as standard.

2.6. Statistical analysis

For each sample assayed three replicates were made and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SPSS v. 18.0 program.

3. Results and Discussion

Phenolic (Ph) and polysaccharidic (Ps) extracts were prepared from *Ganoderma*

lucidum fuiting body (FB), spores (S) and its mycelium (M) obtained in three different culture media: solid (s) and liquid (l) MMN and PDA. It was also performed an experiment of mycelium production in solid MMN incomplete medium (sMMNi), but it showed a very poor and insufficient mycelium development due to the stress caused by the absence of some nutrients in the culture medium, which induced the fungus fructification. Among the mycelia obtained in solid culture media, the mycelium isolated and grown in PDA showed a faster radial growth compared to mycelium isolated and grown in sMMN (solid MMN complete medium) (**Figure 1**). This growth doesn´t correspond to biomass production since sMMN produced higher biomass than PDA. Nevertheless, the lMMN (liquid MMN) revealed the greater mycelia biomass production after 15 days of inoculation, showing a rapid and an abundant growth (**Figure 2**). It is known that agar gel may restrict the diffusion of large molecules (Romberger & Tabor, 1971) as well as inorganic substances (Faye, David, & Lamant, 1986). Compared to the solid medium, lMMN has a better distribution and availability of nutrients and oxygen that allows the fungus a better absorption of nutrients, resulting in a faster growth and a higher mycelia biomass. Liquid biomass production can be important for the direct production of suitable compounds *in vitro,* easy recovery and for the scaling up of this production. Alternatively, if the mycelium itself can not produce the compounds detected in the sporophore, the liquid inoculum can be used for spawn production and sporophore production with the advantage of its uniform distribution in the substrate and growth time reduction (Song, Cho, & Nair, 1987; Hassegawa et al., 2005).

3.1. Characterization of phenolic extracts

The phenolic extracts were analyzed by HPLC-DAD-MS and the results are given in

Table 2. Two phenolic acids, *p*-hydroxybenzoic and *p*-coumaric acids, and one related compound, cinnamic acid, were found. *p*-Hydroxybenzoic was not found in spores, while *p*-coumaric acid was not detected in none of the mycelia. The fruiting body (FB-Ph) revealed the highest total amount (1.23 mg/100 g dw, dry weight), while the mycelium obtained in liquid MMN culture medium (M-lMMN-Ph) provided the lowest concentration (0.25 mg/100 g dw). Kim et al. (2008) reported a higher amount of phenolic compounds in a sample of *G. lucidum* from Korea (16.2 mg/100 g dw), as also the presence of different phenolic compounds, such as other phenolic acids and derivatives (gallic acid, protocatechuic acid, 5-sulfosalicylic acid and pyrogallol), flavan-3-ols (catechin), flavanones (naringin and hesperetin), flavonols (myricetin, quercetin and kaempherol), and isoflavones (formononetin and biochanin A). The absence of these compounds in the sample herein studied was confirmed using mass spectrometry (MS).

3.2. Characterization of polysaccharidic extracts

The polysaccharidic extracts were hydrolyzed with trifluoroacetic acid and analyzed by HPLC-RI. Furthermore, the sugars obtained after the hydrolysis process were also compared with the free sugars presents in the samples (**Table 3**). In general, the same sugars were obtained after polysaccharide's hydrolysis or as free sugars and were the monosaccharides fructose and glucose, the disaccharides sucrose and trehalose, and the alcohol-sugar derivative mannitol. Nevertheless, the amounts found in each process varied as it can be observed in **Figure 3** for the example of mycelium obtained in PDA (M-PDA-Ps), in which free fructose and sucrose were higher than the corresponding sugars obtained by polysaccharide's hydrolysis, while the opposite was observed for glucose and trehalose (**Figure 3**, **Table 3**). Unless for the mentioned sample, the highest

amounts of sugars were obtained after polysaccharide's hydrolysis. Free mannitol was not detected in none of the mycelia samples, as also it was not obtained after hydrolysis of the M-PDA-Ps extract. In the other cases, mannitol was the most abundant sugar. Free sucrose was found in fruiting body and spores (FB-Ps and S-Ps), but it disappeared after polysaccharide's hydrolysis giving glucose (it was not present as free sugar but appeared after hydrolysis) and fructose (increased levels after hydrolysis) (**Table 3**). Mycelia samples provided higher sugars concentration than fruiting body and spores, probably due to its incorporation from the culture media, as sugars are more available in this situation (*in vitro* growth) than in *in vivo* conditions (Kitamoto & Gruen, 1976; Barros, Ferreira, & Baptista, 2008). It can also be observed that the acid hydrolysis performed in polysaccharidic extracts of mycelia samples was not complete, as sucrose (disaccharide) is still present despite a significant decrease. Particularly, M-PDA-Ps provided the highest levels of free sugars (44.25 g/100 g dw), while M-sMMN-Ps revealed the highest sugars amount after polysaccharide's hydrolysis (**Table 3**).

3.3. Evaluation of antioxidant activity

Four different assays were carried out for the *in vitro* evaluation of the antioxidant properties of the phenolic and polysaccharidic extracts: scavenging activity on DPPH radicals, reducing power and inhibition of lipid peroxidation in a β-carotene-linoleate system and in brain homogenates through the TBARS assay. Total phenolic and total polysaccharides contents were obtained by *Folin-Ciocalteu* and phenol/sulphuric acid assays, respectively.

Among the phenolic extracts, the highest antioxidant values were found in the fruiting body (FB-Ph), which is in agreement to its highest total phenolic content (measured by *Folin-Ciocalteu* assay; 28.64 mg GAE/g extract, **Table 4**), and phenolic acids +

cinnamic acid content (measured by HPLC-DAD-MS; 1.23 mg/100 g dw; **Table 2**). This sample gave the highest DPPH scavenging activity (EC_{50} 0.14 mg/ml), reducing power (EC_{50} 0.62 mg/ml), β-carotene bleaching inhibition (EC_{50} 0.26 mg/ml) and lipid peroxidation inhibition through TBARS formation inhibition (0.10 mg/ml). Otherwise, culture medium samples revealed the lowest antioxidant properties and also the lowest phenolic concentrations, particularly sMMN-Ph sample that showed the lowest DPPH scavenging activity (EC_{50} 9.65 mg/ml), reducing power (EC_{50} 5.39 mg/ml), β-carotene bleaching inhibition (EC_{50} 69.64 mg/ml) and TBARS formation inhibition (EC_{50} 32.99 mg/ml), as also the lowest phenolic content (3.17 mg GAE/g extract, **Table 4**).

The studied sample of *G. lucidum* fruiting body revealed higher reducing power than a sample from Taiwan (~50% at 0.75 mg/ml; Mau et al., 2002) and higher DPPH scavenging activity than samples from Korea (~74% at 10 mg/ml; Kim et al., 2008) and from Taiwan (~50% at 0.5 mg/ml; Mau et al., 2002). Furthermore, the antioxidant activity of mycelium ethanolic extract of *G. lucidum* from Central Italy was previously reported (Saltarelli et al., 2009), and the EC_{50} value for DPPH scavenging activity (~0.3 mg/ml; mycelium obtained in PDA culture medium) was lower than the one reported in the present study (0.90 mg/ml). Nevertheless, herein it was in fact demonstrated that PDA is the most indicate media to increase the antioxidant potential of *G. lucidum* mycelium (M-PDA-Ph with the lowest EC_{50} values among mycelia samples). An analysis of the three culture media where mycelia were obtained was also carried out, and once more, PDA-Ph provided the highest antioxidant activity (**Table 4**).

The correlations between the antioxidant activity of Ph extracts and total phenolic contents were demonstrated by the high linear correlation coefficients obtained: from 0.7211 in TBARS assay to 0.9012 in DPPH scavenging activity (**Table 5**). In fact, the chemoprotective effects of phenolic antioxidants against oxidative stress-mediated

disorders stem mainly from their free radical scavenging and metal chelating properties, although their effects on cell signaling pathways and on gene expression (Soobrattee, Neergheen, Luximon-Ramma, Aruoma, & Bahorun, 2005).

Among the polysaccharidic extracts, the highest antioxidant values were found in the spores (S-Ps), despite the highest polysaccharides contents found in mycelia samples (M-Ps) without statistically significant differences (13.23 to 14.50 mg PE/g extract; **Table 4**). Therefore, the correlations between the antioxidant activity and total polysaccharides content provided low linear correlation coefficients: from 0.3412 in β-carotene-linoleate assay to 0.6831 in reducing power (**Table 5**). Nevertheless, some polysaccharides are linked to phenols, and might be co-extracted using polar solvents (Galanakis, Tornberg, & Gekas, 2010a; 2010b). Therefore, phenolic contents in polysaccharidic extracts were also determined, and fruiting body provided the highest levels (55.53 mg GAE/g extract), even higher than the content obtained in the corresponding phenolic extract. As this was also observed for the other samples, it might indicate that a significant amount of the phenolic compounds present in *G. lucidum* are linked to polysaccharides. Furthermore, considering phenolic contents obtained both in phenolic and polysaccharidic extracts, the linear correlation with antioxidant activity increased (**Table 5**). Phenolic extracts (Ph) of fruiting body (FB) and mycelia (M) proved to have higher antioxidant potential than their corresponding polysaccharidic extracts (Ps) (**Table 4**), highlighting the higher contribution of free phenolic compounds than the ones linked to polysaccharides.

Overall, *G. lucidum* fruiting body, spores and mycelium obtained in three different culture media proved to have antioxidant properties in different extensions, considering phenolic or polysaccharidic extracts. Regarding phenolic extracts, fruiting body was the

most potent and revealed the highest content in total phenolics and phenolic acids such as *p*-hydroxybenzoic and *p*-coumaric acids. Among the polysaccharidic extracts, spores provided the best antioxidant activity, but the highest phenolics content was also obtained in fruiting body, while the highest levels of total polysaccharides and individual sugars were observed in mycelia obtained from solid culture media (PDA and MMN). The free radical scavenging properties, reducing power and lipid peroxidation inhibition of *G. lucidum* seemed to be correlated with phenolic compounds mostly in a free form, but also linked to polysaccharides.

Acknowledgements

The authors are grateful to Fundação para a Ciência e a Tecnologia (FCT, Portugal) and COMPETE/QREN/UE (research project PTDC/AGR-ALI/110062/2009) for financial support. S.A. Heleno (BD/70304/2010) and L. Barros (BPD/4609/2008) thank to FCT, POPH-QREN and FSE for their grants. The GIP-USAL is financially supported by the Spanish *Ministerio de Ciencia e Innovación* through the *Consolider-Ingenio 2010* Programme (FUN-C-FOOD, CSD2007-00063), and *Junta de Castilla y León* (Grupo de Investigación de Excelencia, GR133).

References

Barros, A., Carvalho, A.M., & Ferreira, I.C.F.R. (2011a). Exotic fruits as a source of important phytochemicals: Improving the traditional use of *Rosa canina* fruits in Portugal. *Food Research International*, *44*, 2233-2236.

Barros, L., Dueñas, M., Ferreira, I.C.F.R., Carvalho, A.M., & Santos-Buelga, C. (2011b). Use of HPLC-DAD-ESI/MS to profile phenolic compounds in edible wild greens from Portugal. *Food Chemistry*, *127*, 169-173.

Barros, L., Ferreira, I.C.F.R., & Baptista, P. (2008). Phenolics and antioxidant activity of the mushroom *Leucopaxillus giganteus* mycelium at different carbon sources. *Food Science and Technology International*, *14*, 47-55.

Chattopadhyay, S., Farkya, S., Srivastava A.K., & Bisaria, V.S. (2002). Bioprocess considerations for production of secondary metabolites by plant cell suspension cultures. *Biotechnology and Bioprocess Engineering*, *7*, 138-149.

Cheng, J.-J., Lin, C.-Y., Lur, H.-S., Chen, H.-P., & Lu, M.-K. (2008). Properties and biological functions of polysaccharides and ethanolic extracts isolated from medicinal fungus, *Formitopsis pinicola*. *Processes in Biochemistry*, *43*, 829-834.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., & Smith, F. (1956). Colorimetric method for the determination of sugars and related substances. *Analytical Chemistry*, *28*, 350-356.

Hall, I.R., Stepherson, L.S., Buchanan, P.K., Yun, W., & Cole, A.L.J. (2003). Edible and poisonous mushrooms of the world. Timber Press, Portland, Cambridge.

Faye, M., David, A., & Lamant, A. (1986). Nitrate reductase activity and nitrate accumulation in in vitro produced axillary shoots, plantlets and seedlings of *Pinus pinaster*. *Plant Cell Reports*, *5*, 368-371.

Ferreira, I.C.F.R., Barros, L., & Abreu, R.M.V. (2009). Antioxidants in wild mushrooms. *Current Medicinal Chemistry*, *16*, 1543-1560.

Ferreira, I.C.F.R., Vaz, J.A., Vasconcelos, M.H., & Martins, A. (2010). Compounds from wild mushrooms with antitumor potential. *Anti-cancer Agents in Medicinal Chemistry*, *10*, 424-436.

Galanakis, C.M., Tornberg, E., & Gekas, V. (2010a). A study of the recovery of the dietary fibres from olive mill wastewater and the gelling ability of the soluble fibre fraction . *LWT - Food Science and Technology*, *43*, 1009-1017.

Galanakis, C.M., Tornberg, E., & Gekas, V. (2010b). Recovery and preservation of phenols from olive waste in ethanolic extracts. *Journal of Chemical Technology and Biotechnology*, *85*, 1148-1155.

Grangeia, C., Heleno, S.A., Barros, L., Martins, A., & Ferreira, I.C.F.R. (2011). Effects of trophism on nutritional and nutraceutical potential of wild edible mushrooms. *Food Research International*, *44*, 1029-1035.

Guo, C.Y., Ji, S.Z., & Ping, C.X. (2009). Modulatory effect of *Ganoderma lucidum* polysaccharides on serum antioxidant enzymes activities in ovarian cancer rats. *Carbohydrate Polymers*, *78*, 258–262.

Jia, J., Zhang, X., Hua, Y.-S., Wua, Y., Wang, O.-Z., Li, N.-N., Guo, Q.-C., & Dong, X.-C. (2009). Evaluation of in vivo antioxidant activities of *Ganoderma lucidum* polysaccharides in STZ-diabetic rats. *Food Chemistry*, *115*, 32–36.

Kim, M.-Y., Seguin, P., Ahn, J.-K., Kim, J.-J., Chun, S.-C., Kim, E.-H., Seo, S.-Y., & Kang, E.-Y., Kim, S.-L., Park, Y.-J., Ro, H.-M., Chung, I.-M. (2008). Phenolic compound concentration and antioxidant activities of edible and medicinal mushrooms from Korea. *Journal of Agricultural and Food Chemistry*, *56,* 7265–7270.

Kitamoto, Y., & Gruen, H.E. (1976). Distribution of cellular carbohydrates during development of the mycelium and fruitbodies of *Flammulina velutipes*. *Plant Physiology*, *58*, 485-491.

Kozarski, M., Klaus, A., Niksic, M., Jakovljevic, D., Helsper, J. P.F.G., & Griensven, L.J.L.D.V. (2011). Antioxidative and immunomodulating activities of polysaccharide extracts of the medicinal mushrooms *Agaricus bisporus*, *Agaricus brasiliensis*, *Ganoderma lucidum* and *Phellinus linteus*. *Food Chemistry*, *129*, 1667–1675.

Lin, J.-M, Lin, C.-C., Chen, M.-F., Ujiie, T., & Takada, A. (1995). Radical scavenger and antihepatotoxic activity of *Ganoderma formosanum, Ganoderma lucidum* and *Ganoderma neo-japonicum. Journal of Ethnopharmacology*, *47*, 33-41.

Liu, W., Wang, H., Pang, X., Yao, W., & Gao, X. (2010). Characterization and antioxidant activity of two low-molecular-weight polysaccharides purified from the fruiting bodies of *Ganoderma lucidum*. *International Journal of Biological Macromolecules, 46*, 51–457.

Mau, J.-L., Lin, H.-C., & Chen, C.-C. (2002). Antioxidant properties of several medicinal mushrooms. *Journal of Agricultural and Food Chemistry*, *50,* 6072-6077.

Nigam, V.K., Verma, R., Kumar, A., Kundu, S., & Ghosh, P. (2007). Influence of medium constituents on the biosynthesis of cephalosporin-C. *Electronic Journal of Biotechnology*, *10*, 2.

Oria de Rueda, J.A. (2007). Hongos y setas. Tesoro de nuestros montes. Ediciones Cálamo, Palencia, Spain.

Paterson, R.R.M. (2006). *Ganoderma*- A therapeutic fungal biofactory. *Phytochemistry*, *67*, 1985–2001.

Phillips, R. (1981). Mushrooms and other fungi of Great Britain and Europe. Macmillan Publishers Ltd, London, United Kingdom.

Ping, C.X., Yan, C., Bing, L.S., Guo, C.Y., Yun, L.J., & Ping, L.L. (2009). Free radical scavenging of *Ganoderma lucidum* polysaccharides and its effect on antioxidant enzymes and immunity activities in cervical carcinoma rats. *Carbohydrate Polymers*, *77*, 389–393.

Romberger, J.A., & Tabor, C.A. (1971). The Picea abies shoot apical meristem in culture. I. Agar and autoclaving effects. *American Journal of Botany*, *58*, 131-140.

Saltarelli, R., Ceccaroli, O., Iotti, M., Zambonelli, A., Buffalini, M., Casadei, L., Vallorani, L., & Stocchi, V (2009). Biochemical characterization and antioxidant activity of mycelium of *Ganoderma lucidum* from Central Italy. *Food Chemistry*, *116*, 143–151.

Song, C.H., Cho, K.Y., & Nair, N.G. (1987). A synthetic medium for the production of submerged cultures of *Lentinus edodes*. *Mycologia*, *79*, 866-876.

Soobrattee, M.A., Neergheen, V.S., Luximon-Ramma, A., Aruoma, O.I., & Bahorun T. (2005). Phenolics as potential antioxidant therapeutic agents: mechanism and actions. *Mutation Research*, *579*, 200–213.

Sullivan, R, Smith, J.E., & Rowan, N.J. (2006). Medicinal mushrooms and cancer therapy: Translating a traditional practice into Western medicine. *Perspectives in Biology and Medicine*, *49*, 159-170.

Table 1. Identification of the samples: Phenolic and polysaccharidic extracts of *Ganoderma lucidum* fruiting body, spores and mycelium obtained in three different culture media, and phenolic extracts from the three culture media.

	Samples	Extract	Code
In vivo	Fruiting body	Phenolic	FB-Ph
		Polysaccharidic	FB-Ps
	Spores	Phenolic	S-Ph
		Polysaccharidic	S-Ps
In vitro	Mycelium grown in solid MMN	Phenolic	M-sMMN-Ph
		Polysaccharidic	M-sMMN-Ps
	Solid MMN culture medium	Phenolic	sMMN-Ph
	Mycelium grown in liquid MMN	Phenolic	M-lMMN-Ph
		Polysaccharidic	M-lMMN-Ps
	Liquid MMN culture medium	Phenolic	lMMN-Ph
	Mycelium grown in PDA	Phenolic	M-PDA-Ph
		Polysaccharidic	M-PDA-Ps
	PDA culture medium	Phenolic	PDA-Ph

Table 2. Composition in phenolic acids and related compounds of the phenolic extracts from *Ganoderma lucidum* fruiting body, spores and mycelium obtained in three different culture media. In each line different letters mean significant differences (p<0.05).

	FB-Ph	S –Ph	M-sMMN-Ph	M-lMMN-Ph	M-PDA-Ph
p-Hydroxybenzoic acid (mg/100 g dw)	0.58 ± 0.04 a	nd	0.63 ± 0.05 a	0.21 ± 0.02 b	0.68 ± 0.07 a
p-Coumaric acid (mg/100 g dw)	0.38 ± 0.03 a	0.28 ± 0.03 b	nd	nd	nd
Cinnamic acid (mg/100 g dw)	0.28 ± 0.03 b	0.33 ± 0.02 a	0.02 ± 0.00 d	0.04 ± 0.00 d	0.09 ± 0.00 c
Total (mg/100 g dw)	1.23 ± 0.04 a	0.61 ± 0.05 b	0.65 ± 0.05 b	0.25 ± 0.02 c	0.77 ± 0.07 b

nd- not detected

Table 3. Composition in sugars (after polysaccharide hydrolysis and free sugars) of the polysaccharidic extracts from *Ganoderma lucidum* fruiting body, spores and mycelium obtained in three different culture media. In each line different letters mean significant differences (p<0.05).

		FB-Ps	S –Ps	M-sMMN-Ps	M-lMMN-Ps	M-PDA-Ps
Polysaccharide hydrolysis	Fructose (g/100 g dw)	0.65 ± 0.07 c	2.15 ± 0.00 b	2.43 ± 0.07 a	2.11 ± 0.07 b	0.34 ± 0.02 d
	Glucose (g/100 g dw)	0.55 ± 0.06 d	0.83 ± 0.00 d	16.66 ± 0.57 a	4.52 ± 0.02 c	7.73 ± 0.30 b
	Mannitol (g/100 g dw)	7.36 ± 0.00 c	8.24 ± 0.11 c	33.43 ± 1.28 a	14.54 ± 0.38 b	nd
	Sucrose (g/100 g dw)	nd	nd	1.49 ± 0.02 b	1.05 ± 0.00 c	4.96 ± 0.02 a
	Trehalose (g/100 g dw)	2.76 ± 0.00 c	3.27 ± 0.16 c	27.96 ± 0.27 a	17.44 ± 0.23 b	17.72 ± 0.25 b
	Total sugars (g/100 g dw)	11.31 ± 0.01 e	14.50 ± 0.05 d	81.97 ± 1.07 a	39.65 ± 0.10 b	30.74 ± 0.59 c
Free sugars	Fructose (g/100 g dw)	0.56 ± 0.11 c	0.63 ± 0.06 bc	0.48 ± 0.04 c	0.83 ± 0.03 a	0.76 ± 0.06 ba
	Glucose (g/100 g dw)	nd	nd	8.96 ± 0.11 a	7.53 ± 0.34 b	5.37 ± 0.34 c
	Mannitol (g/100 g dw)	6.43 ± 0.47 a	4.65 ± 0.09 b	nd	nd	nd
	Sucrose (g/100 g dw)	0.84 ± 0.07 c	0.47 ± 0.03 c	7.04 ± 0.22 b	4.34 ± 0.24 b	28.00 ± 2.25 a
	Trehalose (g/100 g dw)	2.46 ± 0.37 d	2.91 ± 0.41 dc	12.66 ± 0.50 a	3.98 ± 0.01 c	10.11 ± 0.87 b
	Total sugars (g/100 g dw)	10.29 ± 0.88 d	8.65 ± 0.46 d	29.14 ± 0.56 b	16.68 ± 0.16 c	44.25 ± 1.10 a

nd- not detected

Table 4. Antioxidant activity (EC_{50} values), total phenolics and total polysaccharides of the *Ganoderma lucidum* fruiting body, spores and mycelium obtained in different culture media, and of the three culture media. In each column different letters mean significant differences (p<0.05).

	DPPH scavenging activity (mg/ml)	Reducing power (mg/ml)	β-carotene bleaching inhibition (mg/ml)	TBARS inhibition (mg/ml)	Phenolics (mg GAE/g extract)	Polysaccharides (mg PE/g extract)
FB-Ph	0.14 ± 0.01 j	0.62 ± 0.02 i	0.26 ± 0.03 i	0.10 ± 0.01 g	28.64 ± 0.28 c	nd
FB-Ps	0.22 ± 0.03 i	0.81 ± 0.03 h	9.03 ± 0.56 c	1.21 ± 0.15 e	55.53 ± 0.74 a	4.19 ± 0.01 c
S-Ph	0.58 ± 0.04 h	1.25 ± 0.04 g	1.61 ± 0.21 h	0.77 ± 0.01 fe	14.94 ± 0.22 e	nd
S-Ps	0.15 ± 0.00 j	0.69 ± 0.02 i	2.02 ± 0.29 hg	1.07 ± 0.49 e	43.17 ± 2.0 b	6.06 ± 0.03 b
M-sMMN-Ph	0.91 ± 0.01 g	1.90 ± 0.03 f	2.80 ± 0.21 fg	1.10 ± 0.02 e	14.22 ± 0.29 e	nd
M-sMMN-Ps	1.37 ± 0.05 e	3.17 ± 0.03 c	4.01 ± 0.09 e	3.33 ± 0.31 c	16.36 ± 0.83 d	13.98 ± 1.77 a
sMMN-Ph	9.65 ± 0.06 a	5.39 ± 0.18 b	69.64 ± 0.73 a	32.99 ± 0.30 a	3.17 ± 0.26 h	nd
M-lMMN-Ph	1.32 ± 0.03 e	2.82 ± 0.00 d	3.20 ± 0.39 fe	2.52 ± 0.18 d	6.03 ± 0.98 g	nd
M-lMMN-Ps	1.78 ± 0.08 d	2.73 ± 0.01 d	2.53 ± 0.40 fg	2.82 ± 0.19 dc	11.38 ± 0.21 f	13.23 ± 2.33 a
lMMN-Ph	6.57 ± 0.07 b	2.09 ± 0.12 e	6.22 ± 0.96 d	14.33 ± 0.86 b	5.95 ± 0.09 g	nd
M-PDA-Ph	0.90 ± 0.03 g	1.32 ± 0.01 g	1.47 ± 0.40 h	0.45 ± 0.04 fg	15.19 ± 0.59 ed	nd
M-PDA-Ps	0.99 ± 0.02 f	1.79 ± 0.03 f	2.12 ± 0.07 hg	2.29 ± 0.11 d	14.74 ± 0.34 e	14.50 ± 0.26 a
PDA-Ph	4.93 ± 0.05 c	7.12 ± 0.21 a	24.79 ± 0.99 b	33.17 ± 0.65 a	5.06 ± 0.02 g	nd

Phenolic content was also determined in the polysaccharidic extracts regarding the linked and co-extracted phenols; nd- not determined.

Table 5. Correlations established between total phenolics, total polysaccharides and antioxidant activity EC_{50} values of the *Ganoderma lucidum* fruiting body, spores and mycelium obtained in the three different culture media.

	DPPH scavenging activity		Reducing power		β-carotene bleaching inhibition		TBARS inhibition	
EC_{50} value (mg/ml)	Linear equation	R^2	Linear equation	R^2	Linear equation	R^2	Linear equation	R^2
Phenolics in phenolic extracts (mg GAE/g extract)	Y=-17.518x+29.2720	0.9012	Y=-8.9400x+29.9660	0.8264	Y=-5.8862x+26.7970	0.7609	Y=-7.3613x+23.062	0.7211
Polysaccharides in polysaccharidic extracts (mg PE/g extract)	Y=-5.7084 x+5.3419	0.6526	Y=-3.7406x+3.5158	0.6831	Y=-0.9884x+14.2870	0.3412	Y=-3.9983x+1.8264	0.6774
Phenolics in phenolic and polysaccharidic extracts (mg GAE/g extract)	Y=-24.666x+50.052	0.7815	Y=-15.268x+56.297	0.7298	Y=-4.445x+10.716	0.4426	Y=-16.064x+62.645	0.7014

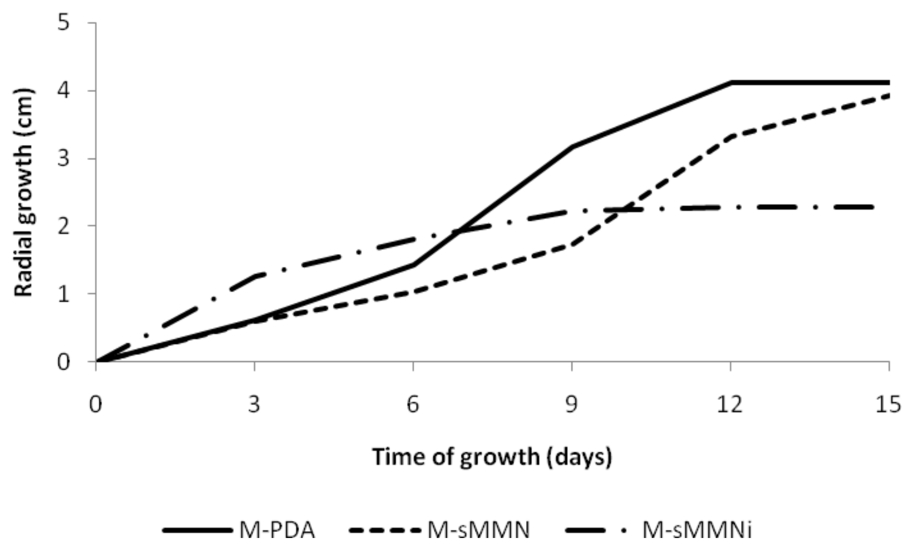

Figure 1. Radial growth of the mycelia obtained in solid culture media: incomplete (M-sMMNi) and complete (M-sMMN) MMN and PDA (M-PDA).

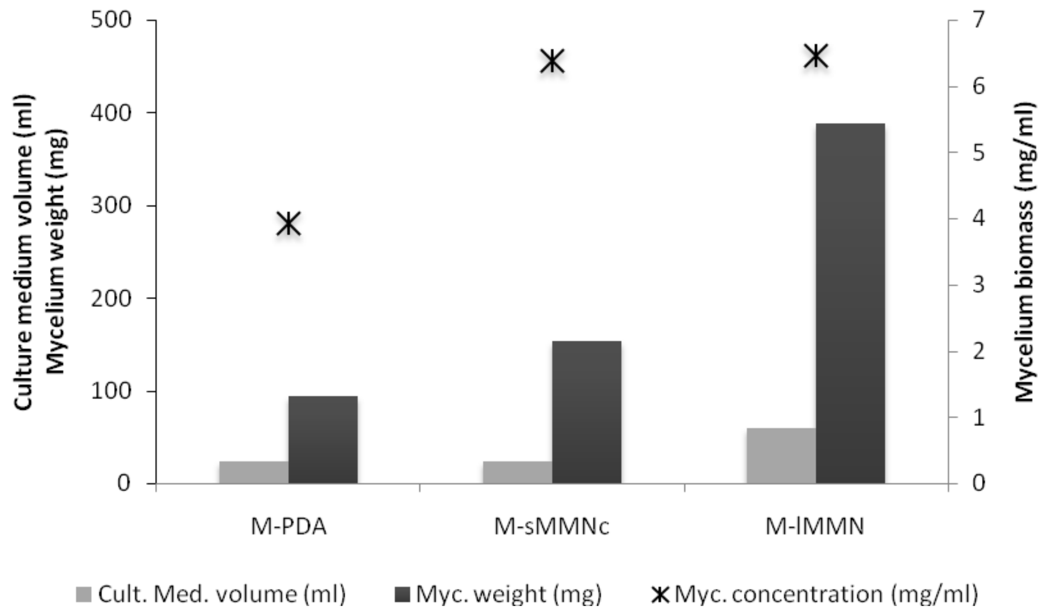

Figure 2. Biomass production of mycelia in solid and liquid MMN, and in solid PDA culture media.

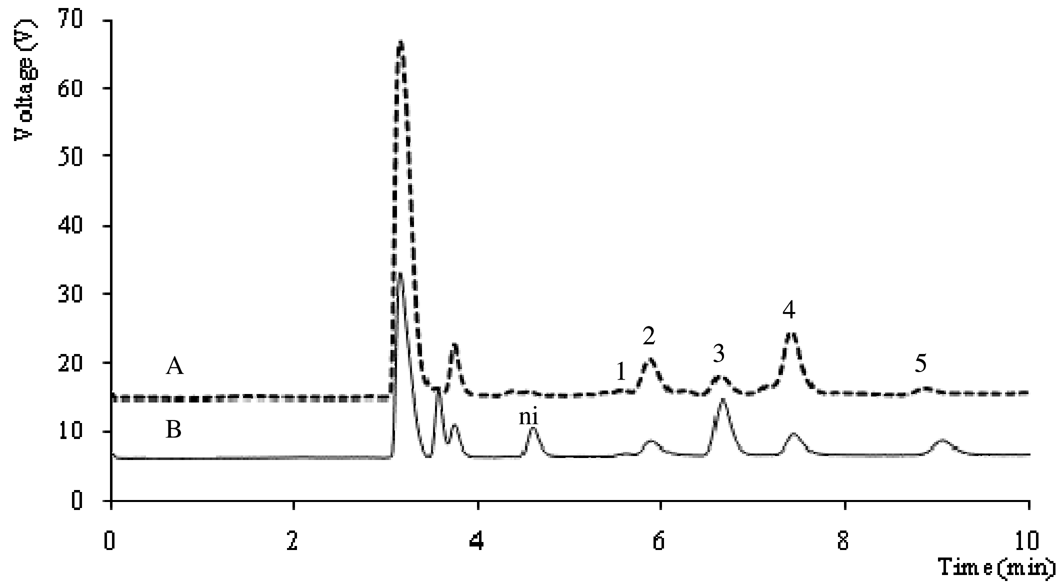

Figure 3. Chromatogram of the sugars obtained after polysaccharide hydrolysis (A) and free sugars (B) of the mycelium obtained in solid PDA culture medium (M-PDA-Ps). 1-fructose; 2- glucose; 3-sucrose; 4-trehalose; 5-melezitose (IS); ni- not identified.