



## $\beta$ -(1 $\rightarrow$ 3,1 $\rightarrow$ 6)-D-glucans produced by *Diaporthe* sp. endophytes: Purification, chemical characterization and antiproliferative activity against MCF-7 and HepG2-C3A cells



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### ARTICLE INFO

#### Article history:

Received 12 July 2016

Received in revised form 10 October 2016

Accepted 16 October 2016

Available online 17 October 2016

#### Keywords:

Endophytic fungi

Exopolysaccharide

MTT assay

Spectroscopic analysis

### ABSTRACT

This study reports the characterization and antiproliferative activity of exopolysaccharides (EPS) produced by submerged cultures of the endophytes *Diaporthe* sp. JF766998 and *Diaporthe* sp. JF767007 isolated from the medicinal plant *Piper hispidum* Sw. Both strains secreted a crude EPS that, upon size exclusion chromatography, showed to contain a heteropolysaccharide (galactose, glucose and mannose) and a high-molecular weight glucan. Data from methylation analysis, FTIR and NMR spectroscopy (<sup>1</sup>H, COSY, TOCSY and HSQC-DEPT) indicated that the purified glucan consisted of a main chain of glucopyranosyl  $\beta$ -(1  $\rightarrow$  3) linkages substituted at O-6 by glucosyl residues. According to MTT assay, some treatments of both  $\beta$ -glucans have antiproliferative activity against human breast carcinoma (MCF-7) and hepatocellular carcinoma (HepG2-C3A) cells after 24 and 48 h of treatment, exhibiting a degree of inhibition ratio that reached the highest values at 400  $\mu$ g/mL: 58.0% (24 h) and 74.6% (48 h) for MCF-7 cells, and 61.0% (24 h) and 83.3% (48 h) for HepG2-C3A cells. These results represent the first reports on the characterization and antiproliferative effect of  $\beta$ -glucans from *Diaporthe* species and also expand the knowledge about bioactive polysaccharides from endophytic sources.

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### 1. Introduction

The World Health Organization ranked the hepatocellular carcinoma as the second most common cancer-related cause of death worldwide in 2012. On the other hand, breast carcinoma represented the fifth cause of death from cancer overall, being the first and second causes of cancer- death for women in less developed and more developed countries, respectively [1]. It is well known that the use of synthetic drugs as chemotherapeutic agents implies some limitations as the relatively severe side-effects in patients; thus, efforts to find new antitumor drugs with fewer undesirable effects are of great importance for human health.

In this context, fungal glucans are relevant bioactive molecules for their antimicrobial [2], anticancer [3,4], and glucose-lowering [5] activities. In particular,  $\beta$ -glucans are known to interact with several receptors of immune cells triggering innate and adaptive responses, and are considered as potent modifiers of the immune response [6,7]. Structurally, fungal  $\beta$ -(1,3)-glucans are made up of a linear backbone of  $\beta$ -(1,3)-glucopyranose randomly branched, generally at O-6 positions, by side chains of variable sizes. Factors like branching degree, molecular mass and tertiary structure affect the bioactive properties of  $\beta$ -glucans and, for example, polymers of high molecular weight seem to exert better antitumor action than smaller ones [8]. These polysaccharides are components of fungal cell walls but some of them, as scleroglucan or schizophyllan, are produced as extracellular polymers, and are of especial interest for being easily recovered from the culture broths.

The medicinal plant *Piper hispidum* Sw. (called “cordoncillo” in Mexico and “falso-jaborandi” in Brazil) harbors a diversity of endo-

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phytes [9], which include isolates that secrete compounds with antimicrobial and enzymatic activity [10–12]. In a previous paper we identified two of these strains as two different *Diaporthe* sp. isolates. Both are exopolysaccharides (EPS) producers and one of them secretes a glucose-rich exopolysaccharide (EPS) when incubated for 96 h in submerged cultures [13]. This study reports the production and characterization of  $\beta$ -glucans from these endophytic strains and the results from evaluation of their antiproliferative activity.

## 2. Materials and methods

### 2.1. Reagents and culture media

Potato dextrose agar medium (PDA) was purchased from HiMedia Labs. (Mumbai, MH, India). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco-Invitrogen Co. (Carlsbad, CA, USA). Analytical standards, dimethyl sulfoxide (DMSO), dimethyl sulfoxide- $d_6$  (DMSO- $d_6$ ), trifluoroacetic acid (TFA), methyl methanesulphonate (MMS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemicals were of analytical grade. The Vogel's minimal salts medium (VMSM) was prepared according to Vogel [14].

### 2.2. *Diaporthe* strains

*Diaporthe* sp. JF766998 and *Diaporthe* sp. JF767007, isolated as endophytes from healthy leaves of *P. hispidum*, belong to the fungal culture collection of the Laboratory of Microbial Biotechnology, State University of Maringá, Brazil. Molecular identification was based on sequencing of the ITS1-5.8S-ITS2 region of rDNA and sequences were deposited in the GenBank database [9]. Fungi were maintained on PDA at 4 °C.

### 2.3. EPS production by endophytic fungi

The fungal isolates were grown in submerged culture conditions for EPS production in the conditions described by Steluti et al. [15] and outlined by Orlandelli et al. [13], except that 16-mL aliquots of standardized mycelial suspension were transferred to 2000-mL Erlenmeyer flasks containing 400 mL of VMSM and glucose (50 g/L) as carbon source. After incubation (28 °C at 180 rpm for 96 h), fungal biomass was removed by vacuum filtration. The cell-free fluid was extensively dialyzed ( $M_w$  cut-off 12,000 Da) against distilled water for 24 h, concentrated in a rotary evaporator (<39 °C) and treated with 3 vols of absolute ethanol. The precipitated crude EPS was recovered by centrifugation (5000  $\times$  g for 15 min at 4 °C) and dissolved in deionized water.

### 2.4. Sugar and protein content

Total sugars were determined by the phenol-sulfuric acid method [16] and reducing sugars were measured by the dinitrosalicylic acid method [17], using D-glucose as standard. Protein was determined using the Bradford method [18] using bovine serum albumin as standard.

### 2.5. Purification of crude EPS

Each crude EPS (EPS-C) was dissolved in deionized water, followed by centrifugation at 12,900  $\times$  g for 10 min. The supernatant fraction (EPS-S) was removed and reserved, while the precipitate (EPS-P) remained being washed until no soluble sugar was detected by the phenol-sulfuric acid method. Aliquots of EPS-C, EPS-S and EPS-P were used for the determination of sugars and protein con-

tent. The rest of material was lyophilized and stored at –20 °C until used.

### 2.6. Homogeneity and molecular weight ( $M_w$ )

Lyophilized samples (1 mg of total sugar) of EPS-C, EPS-S and EPS-P were dissolved in aqueous DMSO solution (1:1), filtered through a Millipore® membrane (0.22- $\mu$ m pore size) and injected (200  $\mu$ L) in high performance size exclusion chromatography (HPSEC) coupled to a refractive index (RI) detector model RID 10A, and UV-vis detector (Shimadzu Co., Kyoto, KYT, Japan). Analysis conditions were described by Orlandelli et al. [13]. A standard curve of dextran with MW of 1400, 1100, 670, 500, 410, 266, 150, 77.8, 72.2, 50, 40.2, and 9.4 kDa was made to determine the  $M_w$ .

### 2.7. Monosaccharide composition analysis

Lyophilized samples (50  $\mu$ g of total sugar) of EPS-C, EPS-S and EPS-P were hydrolyzed with 2 M TFA (300  $\mu$ L) in a sealed tube at 121 °C for 2 h, followed by evaporation (three water dissolution-evaporation cycles). The final residue was dissolved in 500  $\mu$ L water and 25- $\mu$ L diluted aliquots were analyzed by high performance detection (HPAEC/PAD) on a DX 500 Chromatograph (Dionex Co., Sunnyvale, CA, USA) following the protocol described by Orlandelli et al. [13]. Monosaccharide quantification was carried out by measuring the peak area using response factors obtained with standard neutral monosaccharide standards.

### 2.8. Fourier-transform infrared (FT-IR) and nuclear magnetic resonance (NMR) spectroscopies

FT-IR spectroscopy of EPS-P was performed using an IRAffinity-1 spectrometer (Shimadzu Co., Kyoto, KYT, Japan) on 0.8 mg freeze-dried samples of EPS-P in 250 mg KBr discs. Scans were conducted within 4000–500  $\text{cm}^{-1}$  at a resolution of 4  $\text{cm}^{-1}$ .

For the mono- ( $^1\text{H}$  NMR) and bi-dimensional (HSQC-DEPT) spectra, the EPS-P samples (2–3 mg) were deuterium-exchanged by two successive lyophilization steps in  $\text{D}_2\text{O}$ . The final lyophilized sample was dissolved in 250  $\mu$ L of DMSO- $d_6$ . The spectra were obtained on an Avance 500 MHz (500/125 MHz, H/C) spectrometer (Bruker Corp., Ettlingen, BW, Germany) equipped with 5-mm wide bore probe, operating at 30 °C. The experiments were carried out using the pulse programs supplied with the Bruker manual. Proton chemical shifts were recorded relative to the resonance of DMSO- $d_6$  at  $\delta$  = 2.45 ppm and carbon chemical shifts were also recorded relative to resonance of DMSO- $d_6$  at  $\delta$  = 40.00 ppm. Data were analyzed using the Bruker TopSpin 2.1 software.

### 2.9. Methylation analysis

EPS-P samples (1–3 mg) were methylated (3 times) using the procedure described by Ciucanu and Kerek [19]. The methylated products were hydrolyzed (3 M TFA, 120 °C, 1 h), reduced (sodium borodeuteride) and acetylated (acetic anhydride-pyridine 1:1) according to Ahrazem et al. [20]. The products were analyzed by gas chromatography-mass spectrometry (GC-MS) in an Agilent 7980A-5975C instrument (Agilent Technol., Palo Alto, CA, USA) with He as the carrier gas at a flow rate of 1.0 mL/min. A HP5-MS capillary column (30 m  $\times$  0.25 i.d.) was used applying a temperature gradient of 160 (1 min) to 210 °C at 2 °C/min.

### 2.10. Tumor cell lines and antiproliferative assay

Human breast carcinoma (MCF-7 cell line, BCRJ code 0162) and hepatocellular carcinoma (HepG2-C3A cell line, BCRJ code 0291)

**Table 1**  
Production, molecular mass and monosaccharide composition of EPS produced by *Diaporthe* sp. endophytes.

<i>Diaporthe</i> sp. isolate	EPS fractions	EPS yields (g/L)	HPSEC/RID			Monosaccharide composition (HPAEC/PAD)		
			RT (min)	M <sub>w</sub> (g/mol)	M <sub>w</sub> /M <sub>n</sub>	Glc	Gal	Man
JF766998	EPS-C <sub>D1</sub>	0.08	45.2	5.2 × 10 <sup>6</sup>	1.20	43	39	18
			52.8	46.6 × 10 <sup>3</sup>	1.35	33	45	22
	EPS-S <sub>D1</sub>	0.04	52.8	46.6 × 10 <sup>3</sup>	1.35	99	*	*
JF767007	EPS-P <sub>D1</sub>	0.04	42.1	5.2 × 10 <sup>6</sup>	1.20	99	*	*
			44.7	5.2 × 10 <sup>5</sup>	1.22	56	26	18
	EPS-C <sub>D2</sub>	0.04	53.1	39.4 × 10 <sup>3</sup>	1.70	17	55	28
			53.4	39.4 × 10 <sup>3</sup>	1.22	99	*	*
EPS-S <sub>D2</sub>	0.02	46.2	5.2 × 10 <sup>5</sup>	1.70				
		46.2	5.2 × 10 <sup>5</sup>	1.70				

EPS-C = crude EPS, EPS-S = EPS purified from the water-soluble fraction, EPS-P = EPS purified from the precipitated fraction (water-insoluble). M<sub>w</sub> = molecular weight; M<sub>w</sub>/M<sub>n</sub> = polydispersity index (number-average molecular mass). Glc = glucose, Gal = galactose, Man = mannose.

**Table 2**  
Linkage types and their proportions deduced from GC-MS analysis of the partially methylated alditol acetates obtained from EPS-P fractions from two *Diaporthe* sp. endophytes.

EPS	Rt (min)	Deduced linkage types	Molar ratio (%)	Major mass fragments (m/z)
EPS-P <sub>D1</sub>	7.52	Glc-p-(1→	11	71, 88, 102, 118, 145, 162, 205
	9.72	→3)-Glc-p-(1→	79	87, 101, 118, 129, 161, 203, 234
	13.28	→3,6)-Glc-p-(1→	10	87, 101, 118, 129, 139, 160, 189, 234
EPS-P <sub>D2</sub>	7.51	Glc-p-(1→	20	71, 88, 102, 118, 145, 162, 205
	9.71	→3)-Glc-p-(1→	62	87, 101, 118, 129, 161, 203, 234
	13.28	→3,6)-Glc-p-(1→	18	87, 101, 118, 129, 139, 160, 189, 234

**Table 3**  
<sup>1</sup>H NMR and <sup>13</sup>C NMR chemical shifts for the EPS-P<sub>D2</sub> isolated from *Diaporthe* sp. JF767007, in DMSO-*d*<sub>6</sub> at 30 °C.

Residue	C-1 H-1	C-2 H-2	C-3 H-3	C-4 H-4	C-5 H-5	C6/H-6a and H-6b
β-D-Glcp-(→	102.8 4.22	73.6 3.00	76.8 3.11	69.9 3.06	76.1 3.17	60.6 3.54 <sup>a</sup> /3.44 <sup>b</sup>
→3,6)-β-D-Glcp-(→	102.9 4.55	72.4 3.33	86.8 3.49	68.5 3.24	74.4 3.54	68.4 4.11 <sup>a</sup> /3.49 <sup>b</sup>
→3)-β-D-Glcp-(→	102.9 4.55	72.5 3.29	86.0 3.50	68.5 3.24	75.9 3.29	60.8 3.69 <sup>a</sup> /3.44 <sup>b</sup>

<sup>a</sup> Interchangeable.

<sup>b</sup> Interchangeable.

cells were acquired from the Rio de Janeiro Cell Bank, Brazil. Cells were cultured in 25 cm<sup>2</sup>-culture flasks containing DMEM supplemented with 10% FBS, in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. Aliquots of 1 mg of EPS-P samples were dissolved in DMSO and diluted with the DMEM (final DMSO concentration ≤1%), generating doses of 5, 25, 50, 75, 100, 125, 150, 200, 300 and 400 μg/mL. The antiproliferative action of EPS was determined by a modified version of the MTT assay [21]. This test consists in the cleavage of MTT by all living and metabolically active cells, but not by dead cells that lost this ability of conversion. These properties are consistent with the cleavage of MTT only by active mitochondria and could reflect the dose-related toxicity [21,22].

Briefly, tumor cells (1 × 10<sup>4</sup> cells/well) were seeded in 96-well plates with 100 μL of DMEM plus 10% FBS and incubated (37 °C and 5% CO<sub>2</sub>) overnight for adhesion. Then, culture medium was removed and cells were treated (eight replicates) with: EPS-P solution (5–400 μg/mL), cytotoxic agent MMS (final concentration of 150 mM) or DMEM plus 1% DMSO (control group) for 24 h and 48 h. After, the medium was discarded and the MTT solution (0.167 mg/mL DMEM) was added. Plates were incubated for 4 h, then MTT solution was removed and the formazan crystals were dissolved in DMSO (100 μL/well). Absorbance was measured (550 nm) in a FlexStation microplate reader (Molecular Devices, Sunnyvale, CA, USA), and data were normalized to the control group. Results were expressed as percentage of inhibition of cell proliferation, calculated as (1):

$$\text{Inhibition ratio (\%)} = \left( \frac{A_C - A_T}{A_C} \right) \times 100 \quad (1)$$

where: A<sub>C</sub> is the mean of absorbance measured for the control group and A<sub>T</sub> is the mean of absorbance detected for each treated group.

### 2.11. Statistical analysis

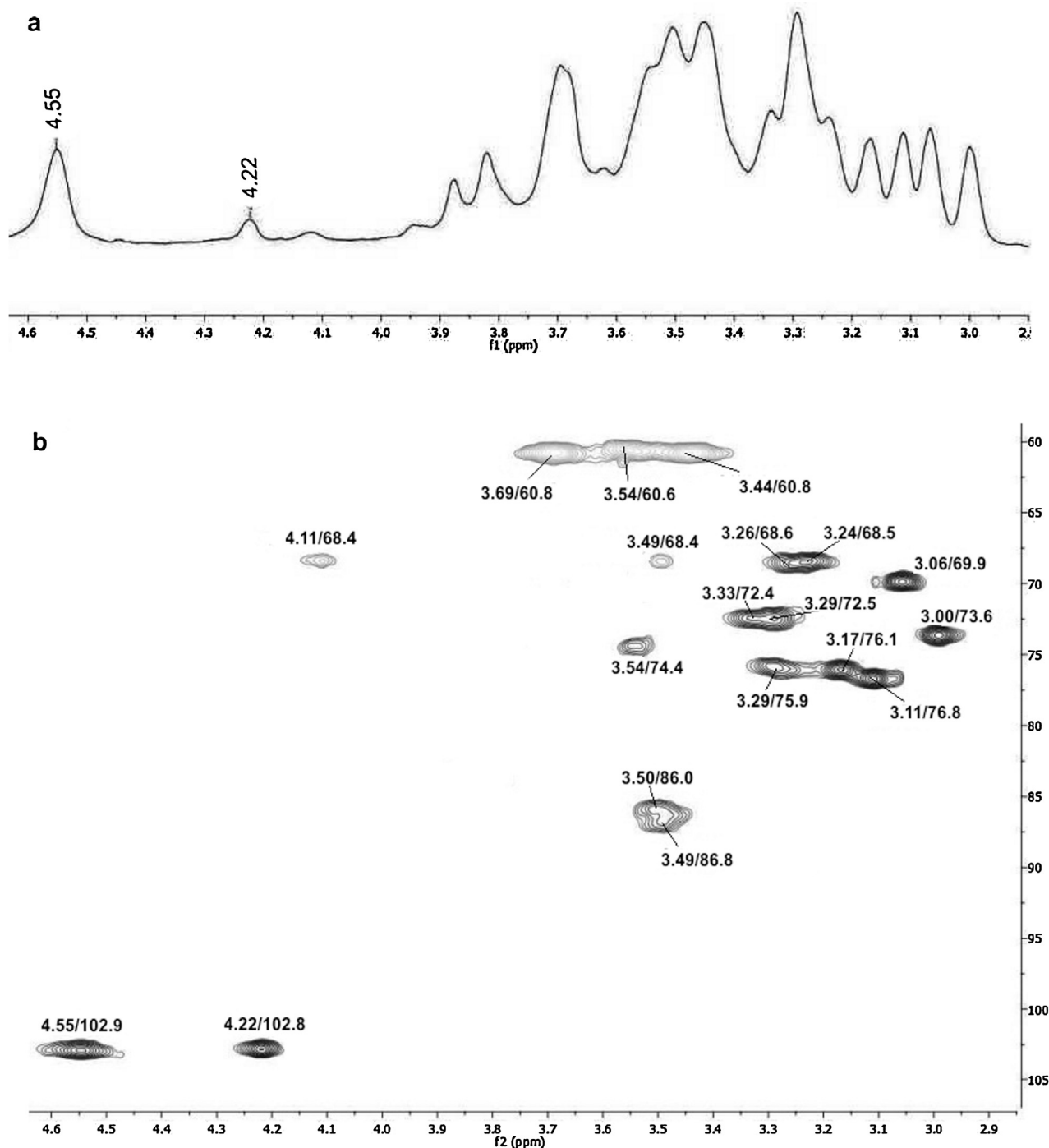
The results of the MTT assay were expressed as the mean ± standard deviation of inhibition ratio. The significant differences between groups (n=8) was analyzed through one-way ANOVA followed by a Dunnett test (p<0.05) performed using the GraphPad InStat Program version 3.02.

## 3. Results and discussion

### 3.1. Production, purification and characteristics of EPS

The crude material secreted by *Diaporthe* sp. JF766998 (EPS-C<sub>D1</sub>) and *Diaporthe* sp. JF767007 (EPS-C<sub>D2</sub>) contained ≥90% carbohydrate (Table 1). The yield of EPS-C<sub>D1</sub> (0.08 g/L) was remarkably higher than that of EPS-C<sub>D2</sub> (0.04 g/L), corroborating the marked differences obtained in our previous study [13]. As reported before, the synthesis of fungal polysaccharides is a strain-dependent process [23], what could explain the differences in EPS secreted by closely related species cultured under the same conditions.

Sugar composition of the crudes was analyzed upon acid hydrolysis, revealing glucose as their main component (≥43%), although galactose and mannose were also detected. The plots from HPSEC/RID analysis of these samples exhibited similar profiles, as two peaks of different M<sub>w</sub> were detected in both crudes, suggesting the presence of at least two EPS. In parallel, EPS-C



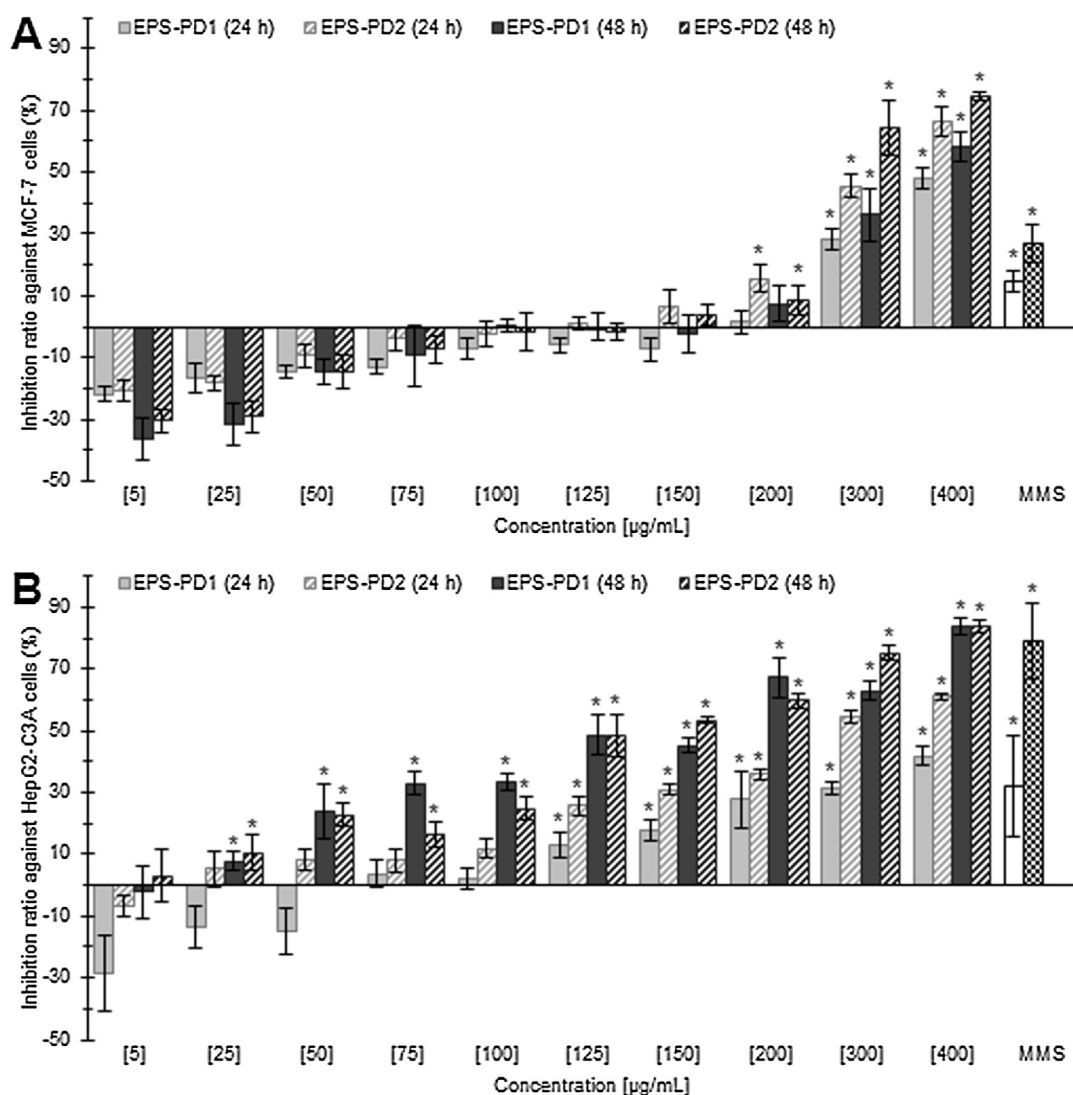
**Fig. 1.** Structural characterization of glucans: (a)  $^1\text{H}$  NMR spectrum of EPS- $\text{P}_{\text{D}1}$  in  $\text{DMSO}-d_6$  at  $30^\circ\text{C}$ . (b) HSQC-DEPT spectrum of EPS- $\text{P}_{\text{D}2}$  in  $\text{DMSO}-d_6$  at  $30^\circ\text{C}$ , where light gray contour represents free and substituted C-6; other C/H correlations were represented by black contour.

samples were dissolved in deionized water, separating a soluble fraction (EPS-S) from a precipitate (EPS-P). Aliquots of the soluble and insoluble fractions were analyzed for homogeneity by HPSEC and for monosaccharide's identification by HPAEC/PAD and the results obtained are presented in Table 1. The EPS- $\text{S}_{\text{D}1}$  and EPS- $\text{S}_{\text{D}2}$  fractions eluted at 52.8 and 53.4 min, respectively, being homogeneous on HPSEC/RID ( $M_w/M_n$  1.2), with  $M_w$  of  $46.6 \times 10^3$  and  $39.4 \times 10^3$  g/mol, respectively. These fractions were composed of galactose, glucose and mannose in molar ratios of 2:1.5:1 (EPS- $\text{S}_{\text{D}1}$ ) and 3:1:1.5 (EPS- $\text{S}_{\text{D}2}$ ). The EPS- $\text{P}_{\text{D}1}$  and EPS- $\text{P}_{\text{D}2}$  eluted at 42.1 and 46.2 min, with  $M_w$  of  $5.2 \times 10^6$  and  $5.2 \times 10^5$  g/mol, respectively.

The polydispersity of EPS- $\text{P}_{\text{D}1}$  was 1.35 indicating a relative narrow distribution of  $M_w$ , while the EPS- $\text{P}_{\text{D}2}$  showed more heterogeneous distribution ( $M_w/M_n$  1.7). Water-insoluble fractions were mainly composed of glucose (99%) with traces of galactose and mannose.

The production of EPSs in species belonging to the genus *Diaporthe* has only been previously described by Corsaro et al. [24] for the phytopathogen *Phomopsis* (= *Diaporthe*) *foeniculi*. This strain, isolated from fennel, secreted a mixture of a soluble polysaccharidic fraction made up of galactose and mannose (1.0:2.2) and an insoluble mannan. Then, to the best of our knowledge, this constitutes the first report of insoluble, extracellular glucans from *Diaporthe*





**Fig. 2.** Antiproliferative assay of glucans: Inhibition ratio (%) on (a) MCF-7 and (b) HepG2-C3A cells incubated with 5–400 µg/mL of EPS-P<sub>D1</sub> and EPS-P<sub>D2</sub>. DMSO = control group (1% DMSO plus DMEM); MMS = cytotoxic agent (0.167 mg/mL DMEM). \*The inhibition ratio (%) was significant when compared to the control group according to the Dunnett test ( $p < 0.05$ ).

species. Most studies dealing with the biological activity of fungal glucans have been performed with crude extracts or polysaccharides' mixtures that are mistakenly considered as pure D-glucans. Indeed, the complete purification and chemical characterization of the molecules to be tested are necessary for a correct correlation between chemical structure and biological activity [25], and for this reason, the two glucose-rich insoluble fractions were purified and their chemical structure elucidated before evaluation of their antiproliferative properties.

### 3.2. Chemical characterization of $\beta$ -glucans

The EPS-P<sub>D1</sub> and EPS-P<sub>D2</sub> fractions were submitted to methylation analysis. GC-MS analysis of the partially O-methylated alditol acetates (Table 2) and FT-IR spectra revealed that both polysaccharides were branched (1,3)-glucans similar to other fungal [26–28]. The major chromatographic peak from GC-MS analysis was identified as 2,4,6-tri-O-methyl-1,3,5-tri-O-acetylglucose, but also 2,3,4,6-tetra-O-methyl-1,5-di-O-acetylglucose from terminal glucopyranose and 2,4-di-O-methyl-1,3,5,6-tetra-O-acetylglucose from branching points in units substituted at positions O-3 and O-6 were detected. The major difference between

the glucans from both isolates was their substitution degree that amounted to 10% for EPS-P<sub>D1</sub> and 18% for EPS-P<sub>D2</sub>. The two FT-IR spectra (not shown) showed bands at 1040, 1110, 1150, 1558  $\text{cm}^{-1}$ , corresponding to the (1  $\rightarrow$  3) glycosidic linkages. In addition, the absence of bands at 850 and 920  $\text{cm}^{-1}$  showed the inexistence of  $\alpha$  linkages, and the presence of others at 890 and 1371  $\text{cm}^{-1}$  characteristic of the  $\beta$  configuration indicated that the polysaccharides were  $\beta$ -(1,3)-glucans [15,26–28].

The solubility of the two  $\beta$ -glucans resulted to be quite different, and the trials to achieve the complete solubilization of EPS-P<sub>D1</sub> were unsuccessful. This fact can be explained taking into account that, as explained above, this polymer has much higher Mw (one order of magnitude) and less branching points than EPS-P<sub>D2</sub> (10% vs. 18%). The low solubility of EPS-P<sub>D1</sub>, avoided obtaining a reasonable NMR spectrum, but the  $^1\text{H}$  NMR analysis from EPS-P<sub>D2</sub> (Fig. 1a) was analyzed, showing the signals for two anomeric protons at 4.55 ppm that corresponded to the anomeric protons of  $\rightarrow$ 3)-Glc-(1 $\rightarrow$  and  $\rightarrow$ 3,6)-Glc-(1 $\rightarrow$  units, while the signal at 4.22 ppm corresponded to the anomeric proton of Glc-(1 $\rightarrow$  unit. On the basis of their observed chemical shifts and  $^3J_{\text{H1,H2}}$  ( $\sim 7.1$  Hz) all the residues were assigned as  $\beta$ -hexapyranosyl residues. All

the proton chemical shifts were assigned using COSY, TOCSY and HSQC-DEPT NMR spectra (Table 3).

The HSQC-DEPT spectrum of EPS-P<sub>D2</sub> (Fig. 1b) containing C1/H1 cross peaks at 102.8/4.22 ppm was assigned to the reducing end unit Glcp-(1→ and all the values from C-2 to C-6 of that residue corresponded nearly to the standard values of methyl glucoside [26]. The C1/H1 cross peak at 102.9/4.55 ppm was assigned to (1→3,6)- and (1→3)-D-Glcp moieties. The chemical shift at 68.4 ppm was assigned to C-6 of branched (1→3,6)-D-glucosyl moiety which corresponded to its proton peaks at 4.11 and 3.49 ppm in HSQC spectrum. The chemical shift of C-6 to lower field was due to  $\alpha$ -effect of glycosylation. The C-6 linkage from (1→3,6)-D-Glcp unit as well as free C-6 from reducing end units and (1→3)-D-Glcp were confirmed from the HSQC-DEPT spectrum (Fig. 1b). C5 from 3,6-di-O substituted units (74.4 ppm), C2 (72.4 and 72.5 ppm) and C4 (68.5 ppm) from 3,6-di-O and 3-O-substituted units were changed to higher field when compared to the corresponding methyl glycoside due to  $\beta$  effect (Table 3). The C3/H3 cross peak at 86.7/3.49 and 86.0/3.50 ppm corresponded to C-3 linkages of (1→3,6)-D-glucosyl and (1→3)-D-Glcp moieties belonging to the main chain.

Based on the results from monosaccharide composition, methylation analysis, FTIR and NMR experiments it was possible to conclude that the structure of EPS-P<sub>D2</sub> consists of a backbone of (1→3)-linked  $\beta$ -D-glucopyranosyl units, where 18% of them are substituted at O-6 positions by a single  $\beta$ -D-glucopyranosyl residue. Although EPS-P<sub>D1</sub> could not be analyzed by NMR, the results from the other analysis suggest that EPS-P<sub>D1</sub> displays similar structural features but has a less branched chain (10%).

### 3.3. Antiproliferative activity of $\beta$ -glucans

This present study shows, for the first time, the antiproliferative effects of  $\beta$ -glucans from *Diaporthe* strains on MCF-7 and HepG2-C3A cells. For MCF-7 cells (Fig. 2a), no inhibitory action was observed for the lowest concentrations tested, but doses of  $\geq 300$  and  $\geq 200$   $\mu\text{g/mL}$  of EPS-P<sub>D1</sub> and EPS-P<sub>D2</sub>, respectively, had significant ( $p < 0.05$ ) inhibitory activity. After 24 h of incubation, the highest inhibition of tumor cells reached 47.9% (EPS-P<sub>D1</sub>) and 58.0% (EPS-P<sub>D2</sub>) at 400  $\mu\text{g/mL}$ , indicating the potent action of these doses when compared with MMS (25.5%). These effects on cell proliferation were more pronounced when the incubation was carried out for 48 h, confirming that glucans were more effective than the cytotoxic agent: inhibition ratios of 66.5, 74.6 and 33.3%, measured for EPS-P<sub>D1</sub>, EPS-P<sub>D2</sub> and MMS, respectively. These values were higher than those described for 400  $\mu\text{g/mL}$  of  $\beta$ -(1→3,1→4)-D-glucan of *Poria cocos*, which reduced 50% of MCF-7 cell proliferation after 72 h of treatment [29].

Upon 24 h-incubation of HepG2-C3A cells, the inhibitory effects initiated at  $\geq 75$  (EPS-P<sub>D1</sub>) and  $\geq 25$   $\mu\text{g/mL}$  (EPS-P<sub>D2</sub>); both glucans exhibited significant action within the dose range of 125–400  $\mu\text{g/mL}$  and reached inhibition ratios of 41.9% (EPS-P<sub>D1</sub>) and 61.0% (EPS-P<sub>D2</sub>) at 400  $\mu\text{g/mL}$ , while the cytotoxic agent MMS reduced cell growth by 31.9%. After 48 h, except for 5  $\mu\text{g/mL}$  of EPS-P<sub>D1</sub>, all EPS-P treatments decreased cell proliferation. Interestingly, both  $\beta$ -glucans exhibited an inhibition ratio of 83.8% at 400  $\mu\text{g/mL}$ , whereas MMS inhibited 79% of HepG2-C3A growth. The highest inhibition ratio (~84%) was similar to that reported for the  $\beta$ -(1→3,1→6)-D-glucan from the fruiting bodies of *Lactarius rufus*, where a dose of 400  $\mu\text{g/mL}$  promoted almost complete cell death [8]. Although the mechanisms related to the antiproliferative activity of polysaccharides are not yet fully known., the presence of a main chain of (1→3)- $\beta$ -glucopyranose more or less substituted at O-6 positions by  $\beta$ -glucopyranose seems to be required for an inhibitory activity, since branches may favor the interaction with

cell receptors, more effectively in tumor cells, playing an important role for induction of cell death [8,25].

## 4. Conclusions

The current study is the first report on the production, chemical characterization and evaluation of antiproliferative properties of  $\beta$ -glucans from *Diaporthe* genus. Two polysaccharides were purified from the culture liquids of two fungal strains of this genus: a heteropolysaccharide composed of galactose, glucose and mannose, and a high-molecular weight, O-6 branched  $\beta$ -(1→3)-D-glucan. The  $\beta$ -glucans of both endophytes had antiproliferative action against the tumor cell lines, although this effect is observer at different doses for each polysaccharide and each cell line.

## Conflict of interest

The authors declare that there is no conflict of interests.

## Acknowledgments

The authors are grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; 311534/2014-7 and 447265/2014-8), Fundação Araucária (276/2014) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; 05/53879-3) for financial support. R.C. Orlandelli thanks Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the doctoral scholarship.

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