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β-(1 → 3,1 → 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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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 (^{1}H, COSY, TOCSY and HSQC-DEPT) indicated that the purified glucan consisted of a main chain of glucopyranosyl β-(1 → 3) linkages substituted at *O*-6 by glucosyl residues. According to MTT assay, some treatments of both β-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 μg/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 β-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, β-glucans are known to interact with several receptors of immune cells triggering innate and adaptative responses, and are considered as potent modifiers of the immune response [6,7]. Structurally, fungal β-(1,3)-glucans are made up of a linear backbone of β-(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 β-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 β-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-il)-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 × *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 × *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 content. 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-μm pore size) and injected (200 μL) 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 μg of total sugar) of EPS-C, EPS-S and EPS-P were hydrolyzed with 2 M TFA (300 μL) 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 μL water and 25-μL 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 cm^{-1} at a resolution of 4 cm^{-1}.

For the mono- (^1H NMR) and bi-dimensional (HSQC-DEPT) spectra, the EPS-P samples (2–3 mg) were deuterium-exchanged by two successive lyophilization steps in D_2O. The final lyophilized sample was dissolved in 250 μL 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 × 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)

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