**β-(1→3,1→6)-ᴅ-glucans produced by *Diaporthe* sp.endophytes: purification, chemical characterization and cytotoxic 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. JF767007isolated 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 (1H, 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 knowledgeabout bioactive polysaccharides from endophytic sources.

**Keywords:** endophytic fungi, exopolysaccharide, MTT assay, spectroscopic analysis.

**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 moleculesfor their antimicrobial [2], anticancer [3,4], and glucose-lowering[5] activities. In particular, -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 -(1,3)-glucans are made up of a linear backbone of -(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 -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 endophytes [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 -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 ºC.

*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 ºC at 180 rpm for 96 h), fungal biomass was removed by vacuum filtration. The cell-free fluid was extensively dialyzed (Mw cut-off 12,000 Da) against distilled water for 24 h, concentrated in a rotary evaporator (< 39 ºC) and treated with 3 volumes of absolute ethanol. The precipitated crude EPS was recovered by centrifugation (5000 × *g* for 15 min at 4 ºC) 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 ᴅ-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 ºC until used.

*2.6. Homogeneity and molecular weight (Mw)*

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 Mw.

*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 ºC 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-Psamples (2-3 mg) were deuterium-exchanged by two successive lyophilization steps in D2O. 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 ºC. The experiments were carried out using the pulse programs supplied with the Bruker manual. Proton chemical shifts refer to residual HDO at δ = 2.45 ppm (30 ºC) and carbon chemical shifts to internal acetone at δ = 40.00 ppm (50 ºC). 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 ºC, 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 ºC at 2 ºC/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) cells were acquired from the Rio de Janeiro Cell Bank, Brazil. Cells were cultured in 25 cm2-culture flasks containing DMEM supplemented with 10% FBS, in a humidified atmosphere of 5% CO2 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 g/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 × 104cells/well) were seeded in 96-wellplates with 100 L of DMEM plus 10% FBS and incubated (37◦C and 5% CO2) overnight for adhesion. Then, culture medium was removed and cells were treated (eight replicates) with: EPS-P solution (5–400 g/mL), cytotoxic agent MMS (final concentration of 150 mM) or DMEM plus 1% DMSO (control group) for 24 hand 48 h. After, the medium was discarded and the MTT solution (0.167 mg/mL DMEM) was added. Plates were incubated for4 h, then MTT solution was removed and the formazan crystals were dissolved in DMSO (100 L/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):

(1)

where: AC is the mean of absorbance measured for the control group and AT 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 Dunnet 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-CD1) and *Diaporthe* sp. JF767007 (EPS-CD2) contained ≥90% carbohydrate (Table 1). The yield of EPS-CD1 (0.08 g/L) was remarkably higher than that of EPS-CD2 (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 Mw were detected in both crudes, suggesting the presence of at least two EPS. In parallel, EPS-C 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-SD1 and EPS-SD2 fractions eluted at 52.8 and 53.4 min, respectively, being homogeneous on HPSEC/RID (Mw/Mn 1.2), with Mw of 46.6×103 and 39.4×103 g/mol, respectively. These fractions were composed of galactose, glucose and mannose in molar ratios of 2:1.5:1 (EPS-SD1) and 3:1:1.5 (EPS-SD2). The EPS-PD1 and EPS-PD2 eluted at 42.1 and 46.2 min, with Mw of 5.2×106 and 5.2×105 g/mol, respectively. The polydispersity of EPS-PD1 was 1.35 indicating a relative narrow distribution of Mw, while the EPS-PD2 showed more heterogeneous distribution (Mw/Mn 1.7). Water-insoluble fractions were mainly composed of glucose (99%) with traces of galactose and mannose.

The production of EPSs inspecies 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* 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 ᴅ-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 β-glucans*

The EPS-PD1 and EPS-PD2 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*-acetyl-glucose, but also 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl-glucose from terminal glucopyranose and 2,4-di-*O*-methyl-1,3,5,6-tetra-O-acetyl-glucose 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-PD1 and 18% for EPS-PD2. The two FT-IR spectra (not shown) showed bands at 1040, 1110, 1150, 1558 cm-1, corresponding to the (1→3) glycosidic linkages. In addition, the absence of bands at 850 and 920 cm-1 showed the inexistence of α linkages, and the presence of others at 890 and 1371 cm-1 characteristic of the β configuration indicated that the polysaccharides were -(1,3)-glucans [15, 26-28].

The solubility of the two -glucans resulted to be quite different, and the trials to achieve the complete solubilization of EPS-PD1 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-PD2 (10% *vs.* 18%). The low solubility of EPS-PD1 avoided obtaining a reasonable NMR spectrum, but the 1H NMR analysis from EPS-D2 (Figure 1a) was analyzed, showing the signals for two anomeric protons at 4.55 ppm that corresponded to the anomeric protons of →3)-Glc*p*-(1→ and →3,6)-Glc*p*-(1→units,whilethe signal at 4.22 ppm corresponded to the anomeric proton of Glc*p*-(1→ units. On the basis of their observed chemical shifts and 3*J*H1, H2 (~7.1 Hz) all the residues were assigned as β-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-PD2 (Figure 1b) containing C1/H1 cross peaks at 102.8/4.22 ppm was assigned to the reducing end unit Glc*p*-(1→ and all the values from C-2 to C-6 of that residue corresponded nearly to the standard values of metyl glucoside [26]. The C1/H1 cross peak at 102.9/4.55 ppm was assigned to (1→3,6)- and (1→3)-D-Glc*p* 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 α-effect of glycosylation. The C-6 linkage from (1→3,6)-D-Glc*p* unit as well as free C-6 from reducing end units and (1→3)-D-Glc*p* were confirmed from the HSQC-DEPT spectrum (Figure 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 β 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-Glc*p* 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-PD2 consists of a backbone of (1→3)-linked β-D-glucopyranosyl units, where 18% of them are substituted at *O*-6 positions by a single β-D-glucopyranosyl residue. Although EPS-PD1 could not be analyzed by NMR, the results from the other analysis suggest that EPS-PD1 displays similar structural features but has a less branched chain (10%).

*3.3. Antiproliferative activity of β-glucans*

The present study shows, for the first time, the antiproliferative effects of β-glucans from *Diaporthe* strains on MCF-7 and HepG2-C3A cells. For MCF-7 cells (Figure 2a), no inhibitory action was observed for the lowest concentrations tested, but doses of ≥ 300 and ≥ 200 µg/mL of EPS-PD1 and EPS-PD2,respectively, had significant (p < 0.05) inhibitory activity. After 24 h of incubation, the highest inhibition of tumor cells reached 47.9% (EPS-PD1) and 58.0% (EPS-PD2) at 400 µg/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-PD1, EPS-PD2 and MMS, respectively. These values were higher than those described for 400 µg/mL of β-(1→3,1→4)-ᴅ-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 ≥ 75 (EPS-PD1) and ≥ 25 µg/mL (EPS-PD2); both glucans exhibited significant action within the dose range of 125-400 µg/mL and reached inhibition ratios of 41.9% (EPS-PD1) and 61.0% (EPS-PD2) at 400 µg/mL, while the cytotoxic agent MMS reduced cell growth by 31.9% . After 48 h, except for 5 µg/mL of EPS-PD1, all EPS-P treatments decreased cell proliferation. Interestingly, both β-glucans exhibited an inhibition ratio of 83.8% at 400 µg/mL, whereas MMS inhibited 79% of HepG2-C3A growth. The highest inhibition ratio (~84%) was similar to that reported for the β-(1→3,1→6)-ᴅ-glucan from the fruiting bodies of *Lactarius rufus*, where a dose of 400 µg/mL promoted almost complete cell death [8]. Although the mechanisms related to the antiproliferativeactivity of polysaccharides are not yet fully known, the presence of a main chain of (1→3)-β-glucopyranose more or less substituted at *O*-6 positions by β-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 β-glucans from *Diaporthe* genus. Two polysaccharides were purified from the culture liquids of two isolates of this genus: a heteropolysaccharide composed of galactose, glucose and mannose, and a high-molecular weight, *O*-6 branched β-(1→3)-ᴅ-glucan. The β-glucans of both endophytes had antiproliferative action against the tumor cell lines, although this effect is observed at different doses for each polysaccharide and each cell line.

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# Conflict of interest

The authors declare that there is no conflict of interests.

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

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| ***Diaporthe* sp.**  **isolate** | **EPS**  **fractions** | **EPS yields (g/L)** | **HPSEC/RID** | | |  | **Monosaccharide composition (HPAEC/PAD)** | | |
| **RT (min)** | **Mw (g/mol)** | **Mw/Mn** |  | **Glc** | **Gal** | **Man** |
| JF766998 | EPS-CD1 | 0.08 | 45.2 | 5.2×106 | 1.20 |  | 43 | 39 | 18 |
|  |  |  | 52.8 | 46.6×103 | 1.35 |  |  |  |  |
|  | EPS-SD1 | 0.04 | 52.8 | 46.6×103 | 1.20 |  | 33 | 45 | 22 |
|  | EPS-PD1 | 0.04 | 42.1 | 5.2×106 | 1.35 |  | 99 | \* | \* |
|  |  |  |  |  |  |  |  |  |  |
| JF767007 | EPS-CD2 | 0.04 | 44.7 | 39.4×103 | 1.22 |  | 56 | 26 | 18 |
|  |  |  | 53.1 | 5.2×105 | 1.70 |  |  |  |  |
|  | EPS-SD2 | 0.02 | 53.4 | 39.4×103 | 1.22 |  | 17 | 55 | 28 |
|  | EPS-PD2 | 0.02 | 46.2 | 5.2×105 | 1.70 |  | 99 | \* | \* |

EPS-C = crude EPS, EPS-S = EPS purified from the water-soluble fraction, EPS-P = EPS purified from the precipitated fraction (water-insoluble). Mw = molecular weight; Mw/Mn = 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-PD1 | 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-PD2 | 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 |

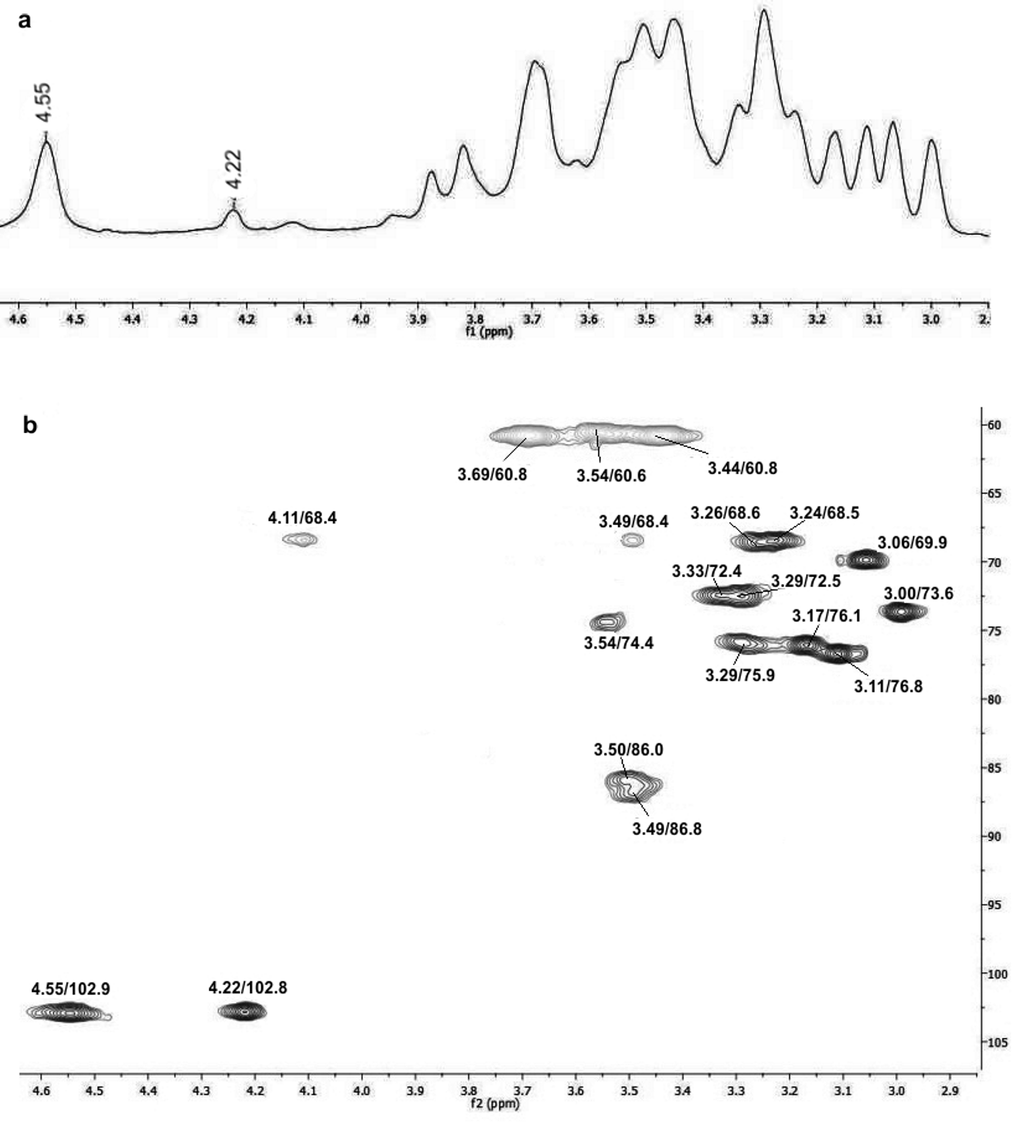
# Table 3.

1H NMR and 13C NMR chemical shifts for the EPS-PD2 isolated from *Diaporthe* sp. JF767007, in DMSO-*d*6 at 30 ºC.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **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-Glc*p*-(→** | 102.8  4.22 | 73.6  3.00 | 76.8  3.11 | 69.9  3.06 | 76.1  3.17 | 60.6  3.54a/3.44b |
| **→3,6)-β-D-Glc*p*-(→** | 102.9  4.55 | 72.4  3.33 | 86.8  3.49 | 68.5  3.24 | 74.4  3.54 | 68.4  4.11a/3.49b |
| **→3)-β-D-Glc*p*-(→** | 102.9  4.55 | 72.5  3.29 | 86.0  3.50 | 68.5  3.24 | 75.9  3.29 | 60.8  3.69a/3.44b |

a,bInterchangeable

**Figure 1.** Structural characterization of glucans: (a) 1H NMR spectrum of EPS-PD1 in DMSO-d6 at 30 ºC. (b) HSQC-DEPT spectrum of EPS-PD2 in DMSO-d6 at 30 ºC, where light gray contour represents free and substituted C-6; other C/H correlations were represented by black contour.



**Figure 2.** Antiproliferative assay of glucans: Inhibition ratio (%) on (a) MCF-7 and (b) HepG2-C3A cells incubated with 5-400 µg/mL of EPS-PD1 and EPS-PD2. DMSO = control group (1% DMSO plus DMEM); MTT = cytotoxic agent (0.167 mg/mL DMEM). \*The inhibition ratio (%) was significant when compared to the control group according to the Dunnet test (p<0.05).

