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Structural characterization and anti-inflammatory activity of a linear β-D-glucan isolated from *Pleurotus sajor-caju*

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ABSTRACT

Glucans comprise an important class of polysaccharides present in basidiomycetes with potential biological activities. A (1 → 3)-β-D-glucan was isolated from *Pleurotus sajor-caju via* extraction with hot water followed by fractionation by freeze-thawing and finally by dimethyl sulfoxide extraction. The purified polysaccharide showed a ^{13}C-NMR spectrum with six signals consisting of a linear glucan with a β-anomeric signal at 102.8 ppm and a signal at 86.1 ppm relative to O-3 substitution. The other signals at 76.2, 72.9, 68.3, and 60.8 ppm were attributed to C5, C2, C4, and C6, respectively. This structure was confirmed by methylation analysis, and HSQC studies. The β-D-glucan from *P. sajor-caju* presented an immunomodulatory activity on THP-1 macrophages, inhibited the inflammatory phase of nociception induced by formalin in mice, and reduced the number of total leukocytes and myeloperoxidase levels induced by LPS. Taken together, these results demonstrate that this β-D-glucan exhibits a significant anti-inflammatory activity.

1. Introduction

Mushrooms of the genus *Pleurotus* are edible and must be one of the most cultivated genera in several countries because of their high adaptability. The annual production of these species reaches more than 900,000 t (Synytsya et al., 2009).

Besides their culinary importance, mushrooms have been appreciated by their medicinal properties and they have been used for different health care purposes, especially in the oriental countries (Chen & Seviour, 2007; El Enshasy & Hatti-Kaul, 2013; Zhang, Cui, Cheung, & Wang, 2007). One class of molecules that are in evidence are the polysaccharides that have emerged as important biologically active polymers (Synytsya & Novák, 2013). Basidiomycete polysaccharides have been showed to modulate the immune system, and to inhibit tumor growth, inflammation, nociception and other health problems (Chen & Seviour, 2007; Smiderle et al., 2008; Dalonso et al., 2010; Maji et al., 2012; Kanagasabapathy, Chua, Malek, Vikineswary, & Kuppusamy, 2014).

The mechanisms by which the mushroom polysaccharides exhibit their bioactivities are still unknown, although some authors have showed that these molecules can bind to immune cell receptors, and initiate immune reactions, as the production of pro- and anti-inflammatory cytokines (Chen & Seviour, 2007; El Enshasy & Hatti-Kaul, 2013). The affinity ligand-receptor depends mainly on the chemical structure of the ligand, that can be determined by its chemical formulae, conformation, and molecular weight (Zhang et al., 2007). Mushroom polysaccharides vary greatly on these three characteristics, and some studies have shown that different polysaccharide structures may exhibit different biological effects. However, the research data presented up to now are not enough to determine which molecule is responsible for each bioactivity. Therefore, the therapeutic application of a polysaccharide requires the careful knowledge of its chemical structure (El Enshasy & Hatti-Kaul, 2013).

The branched (1 → 3), (1 → 6)-linked β-D-glucans, similar to lentinan are the most common homopolysaccharides present in Basidiomycetes (Maji et al., 2012; Palacios, García-Lafuente, Guillamón, & Villares, 2012; Smiderle et al., 2008; Carbonero et al., 2006; Santos-Neves et al., 2008). The lentinan was firstly isolated from *Lentinus edodes* and has attracted much attention because of

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its pronounced anti-tumor activity, which is probably related to the activation of T-cells, natural-killer cells, and macrophages (Zhang, Li, Wang, Zhang, & Cheung, 2011).

Besides branched lentinan-type polysaccharides, linear glucans have also been isolated from Basidiomycetes. As example, it can be mentioned linear α-D-glucans (1 → 3)-linked (from *Pleurotus ostreatus* and *Pleurotus eryngii*), or (1 → 4)-linked (from *Agaricus blazei* and *P. ostreatus*) (Synytsya & Novák, 2013; Palacios et al., 2012), and (1 → 6)-β-D-glucan (from *Agaricus bisporus* and *Agaricus brasiliensis*) (Smiderle et al., 2013).

In this study, a linear (1 → 3)-β-D-glucan was isolated, for the first time, from *Pleurotus sajor-caju*. We have not found any studies showing the presence of this polysaccharide in other *Pleurotus* species. It was chemically characterized, and its biological properties were evaluated *in vitro*, using THP-1 macrophages, and *in vivo*, through formalin and peritonitis tests in mice.

2. Materials and methods

2.1. Microorganisms and maintenance

P. sajor-caju CCB019 was obtained from the Center for Basidiomycete Cultivation of the São Paulo University, Brazil, and maintained in Petri dishes containing WDA (1 L of wheat extract, 20 g of dextrose, and 15 g of agar) at 4 °C (Furlan et al., 1997).

2.2. Cultivation conditions of the fruiting bodies

P. sajor-caju fruiting bodies cultivation was conducted at the Biotechnology Laboratory of UNIVILLE University, Joinville (SC) Brazil, using banana straw.

Banana straw was packed in polypropylene bags, supplemented with rice bran, sterilized, and inoculated using 10% solid inoculum. The first step of the process, the mycelial growth, was carried out at 25 °C, with 60% relative air humidity, artificial light, for 20 days. The induction of the fruiting body formation (second step) was achieved by perforating the plastic bags to increase air exchange, and by exposing them to light for a period of 12 h a day while increasing relative air humidity to 90%. After 20 days, the fruiting bodies were harvested, frozen, and freeze-dried.

2.3. Extraction and purification of the polysaccharide

Fruiting bodies were milled in a blender and the powder was defatted by addition of a chloroform–methanol (2:1 v/v) mixture at 50 °C for 24 h. Then, the residue was submitted to successive aqueous extraction at 100 °C for 24 h (10×, 800 mL each). The hot-water extract (HW) was centrifuged (6000 rpm, 30 min, 10 °C) and reduced to a small volume, by concentration under reduced pressure in a rotary-evaporator, and the polysaccharides were recovered from this extract by precipitation with excess of ethanol (4 volume). The precipitated polysaccharides were then dialyzed against tap water for 24 h (2 kDa MWCO membrane), concentrated under reduced pressure, and submitted to freeze-thawing process. The insoluble fraction (PHW) obtained from freeze-thawing was recovered by centrifugation (12,000 rpm, 30 min, 5 °C), and submitted to one extraction with 50 mL dimethyl sulfoxide (Me_2SO; 80 °C for 5 h). The Me_2SO-extract (M-PHW) was dialyzed against tap water for 24 h (2 kDa MWCO membrane) and then resubmitted to the freeze-thawing process, giving rise to a water-insoluble fraction (G-PHW) recovered by centrifugation (12,000 rpm, 30 min, 5 °C). The chemical structure of this fraction was evaluated as well as its antinociceptive and anti-inflammatory activities.

2.4. Monosaccharide analysis

The monosaccharide composition was determined after total acid hydrolysis of a polysaccharide sample (~1 mg) with 1 M TFA at 100 °C for 12 h. The remaining acid was evaporated to dryness, and the hydrolysis product was submitted to reduction with $NaBH_4$ (Wolfrom & Thompson, 1963b) and acetylation with Ac_2O-pyridine (1:1, v/v; 300 μL) for 12 h at room temperature (Wolfrom & Thompson, 1963a). The resulting alditol acetates were analyzed by gas chromatography–mass spectrometry (GC–MS) using a Varian Saturn 2000R-3800 gas chromatograph coupled to a Varian Ion-Trap 2000R mass spectrometer with He as the carrier gas. A DB-225 capillary column (30 m × 0.25 mm i.d.), which was maintained at 50 °C during injection and then programmed to increase to 220 °C at a rate of 40 °C min^{-1}, was used for the quantitative analysis of the alditol acetates (Ruthes et al., 2013). The products were identified by their typical retention times and electron impact profiles.

2.5. Methylation analysis of the polysaccharide

The isolated polysaccharide was per-*O*-methylated according to the method described by Ciucanu and Kerek (1984), with slight modifications. The sample (10 mg) was dissolved in Me_2SO (0.5 mL), followed by addition of iodomethane (0.5 mL), and powdered NaOH (200 mg). After vigorous stirring for 30 min, the mixture was maintained overnight at room temperature. The reaction was interrupted by the addition of water, neutralized with HOAc, dialyzed against distilled water (2 kDa MWCO membrane) and freeze-dried. The product was submitted to one more cycle of methylation, however in the second cycle, after neutralization with HOAc, the per-*O*-methylated polysaccharide was recovered by partition between $CHCl_3$ and water. The per-*O*-methylated derivatives were hydrolyzed with 45% (v/v) formic acid (HCO_2H; 1 mL) at 100 °C for 15 h, followed by evaporation to dryness. The resulting mixture of *O*-methylated monosaccharides was reduced with $NaBH_4$ and acetylated with Ac_2O-pyridine (1:1, v/v; 300 μL) for 12 h at room temperature to obtain a mixture of *O*-methyl-alditol acetates. These derivatives were analyzed by GC–MS using the same conditions as described for alditol acetates (Section2.4), with the exception that the final temperature was 215 °C. The derivatives were identified by their typical retention times and electron impact spectra (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005).

2.6. Nuclear magnetic resonance spectroscopy

The NMR spectra mono-(^{13}C) and bidimensional (HSQC) were obtained using a 400-MHz Bruker Avance III spectrometer with a 5 mm inverse probe. The ^{13}C-NMR (100.6 MHz) and ^{1}H-NMR (400.13 MHz) analyses were performed at 70 °C, and the samples were dissolved in D_2O or Me_2SO-d_6. The chemical shifts are expressed in ppm (δ) relative acetone (for samples in D_2O) at δ 30.2 and δ 2.22 or to Me_2SO-d_6 at δ 39.7 and δ 2.40 for ^{13}C and ^{1}H signals, respectively.

2.7. Homogeneity and average molecular mass (M_w) analysis

The homogeneity and average molecular mass (M_w) of the β-D-glucan were determined by high-performance size-exclusion chromatography (HPSEC) coupled to refractive index and multi-angle laser light-scattering detectors (MALLS). Four gel-permeation Ultrahydrogel columns, with exclusion sizes of 7×10^6, 4×10^5, 8×10^4, and 5×10^3 Da, were used in series. The eluent was 0.1 M aq. $NaNO_2$ containing 200 ppm aq. NaN_3 at 0.6 mL min^{-1}. The sample was dissolved in the same solution used as eluent at a concentration of 1 mg mL^{-1}, filtered through a membrane (0.22 μm), and injected

using a 100 μL loop. The specific refractive index increment (d*n*/d*c*) was determined, and the results were processed with software provided by the manufacturer (ASTRA 4.70.07, Wyatt Technologies).

2.8. Cell culture

The human monocytic cell line THP-1 (Rio de Janeiro Cell Bank, Rio de Janeiro, Brazil) was grown in RPMI 1640 culture medium (Sigma–Aldrich, cat. R8758) supplemented with 10% heat-treated newborn calf serum (Gibco, cat. 161010159) and penicillin/streptomycin (100 U mL^{-1}; 100 g mL^{-1}, respectively) (Sigma–Aldrich), at 37 °C in 5% CO_2 in a humidified incubator. The medium was renewed twice a week.

2.9. Macrophage differentiation and stimulation

The mature macrophage-like state was induced by treating THP-1 monocytes (500,000 cells mL^{-1}) for 48 h with 30 ng mL^{-1} phorbol 12-myristate 13-acetate (PMA; Sigma–Aldrich) in 24-wells polystyrene tissue culture plates (Costar) with 1 mL cell suspension in each well. The medium was then removed and replaced by fresh medium containing the isolated β-D-glucan (G-PHW) at 10, 50, and 250 μg mL^{-1}; phosphate buffered saline (PBS; 50 μL), or lipopolysaccharide (LPS; 1 μg mL^{-1}) as negative and pro-inflammatory controls, respectively. Cells were harvested at 0 h, 3 h, and 6 h after addition of treatment and kept in lysis buffer at −20 °C for the next step. Time point 0 h was used to normalize the calculations. All experiments were performed with the same amount of cells (0.5×10^6 per mL). Total RNA was isolated from the cells as follows.

2.10. Gene expression kinetics by real-time PCR

Total RNA was isolated by using RNeasy mini kit (Qiagen, USA) with a RNase-free DNase (Qiagen) treatment for 15 min according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from isolated RNA (1 μg) with High Capacity RNA-to-cDNA kit (Applied Biosystems, USA). Expression levels of each gene were measured in triplicate reactions, performed with the same cDNA pool (1:5 diluted), in the presence of the fluorescent dye (iQ SYBR Green Supermix) using a StepOne Plus™ instrument (Applied Biosystems, USA). The experiments were performed in a 20 μL reaction volume with specific primer pairs (Chanput, Mes, Vreeburg, Savelkoul, & Wichers, 2010), and the conditions of real-time quantitative PCR were performed as follows: denaturation at 95 °C for 10 min and amplification by cycling 40 times at 95 °C for 15 s and 60 °C for 60 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), actin (ACTB), and β-2-microglobulin were used as endogenous controls, and GAPDH was chosen for normalization. The PCR of all products were subjected to a melting curve analysis to verify the single amplification product. The relative messenger RNA (mRNA) expression was presented as described in Chanput et al. (2010): values were expressed as fold change relative to the value at time point zero, calculated as ΔΔCt [$\Delta\Delta Ct = 2(Ct_{GAPDH} - Ct_{Sample})$] (Livak & Schmittgen, 2001). The q-PCR analyses were performed twice on each sample (in triplicate), to evaluate the mRNA expression level of pro-inflammatory cytokine genes IL-1β and TNF-α and also the inflammation-related enzyme COX-2.

2.11. Experimental animals

Experiments were conducted using female Swiss mice (25–35 g), provided by the Federal University of Paraná colony. The animals were kept under standard laboratory conditions (12 h light/dark cycles, temperature 22 ± 2 °C) with food and water provided *ad libitum*. The animals were acclimatized to the laboratory for at least 12 h before testing and were used only once for experiments. All the experiments were performed after approval of the respective protocols by the Committee of Animal Experimentation of Federal University of Paraná (CEUA/BIO—UFPR; approval number 657). The study was conducted in accordance with the "Principles of Laboratory Animal Care" (NIH Publication 85–23, revised 1985) and with the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983). The number of animals and intensity of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the drug treatments.

2.12. Nociception induced by intraplantar injection of 2.5% formalin

The procedure used was similar to previously described (Hunskaar, Fasmer, & Hole, 1985). The mice received 20 μL of a 2.5% formalin solution (0.92% formaldehyde, in saline) intraplantarly under the ventral surface of the right hind paw. The animals were observed from 0 to 5 min (early phase) and 15 to 30 min (late phase) and the time that they spent licking the injected paw was considered as indicative of nociception. The animals were treated with vehicle [saline plus 5% Me_2SO, 10 mL kg^{-1}, intraperitonealy (i.p.)] or β-D-glucan (G-PHW) (1, 3 and 10 mg kg^{-1}, i.p), 30 min before the formalin injection.

2.13. Peritonitis induced by intraperitoneal injection of LPS

Peritonitis was induced with LPS according to Borges et al. (2014) with modifications. The mice were pre-treated with vehicle (saline plus 5% Me_2SO, 10 mL kg^{-1}), dexamethasone (DEXA, a synthetic glucocorticoid, 0.5 mg kg^{-1}) or β-D-glucan (G-PHW) (1, 3 and 10 mg kg^{-1}) by i.p. route, 30 min before LPS injection (2 μg kg^{-1}, i.p.). Naive group received only sterile saline solution (0.9% NaCl, 10 mL $kg,^{-1}$ i.p.). Four hours after the peritonitis induction, the mice were sacrificed and the peritoneal cavity was opened and washed with 1 mL of sterile saline containing heparin (25 IU mL^{-1}). Then, the peritoneal fluid was collected to determine the total number of leukocytes and levels of myeloperoxidase (MPO).

2.14. Quantification of total leukocytes and levels of myeloperoxidase (MPO)

An aliquot of the peritoneal fluid was diluted with Türk solution (1:20) and the total leukocyte counts were performed in a Neubauer chamber. For the measurement of MPO levels, samples of the peritoneal fluid were added to 80 mM potassium phosphate buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB), and centrifuged at 11,000 × *g* for 20 min at 4 °C. MPO levels of supernatants were determined in the presence of 0.017% H_2O_2 and 3,3′,5,5′-tetramethylbenzidine in dimethylformamide (TMB, 18.4 mM). The reaction was incubated at 37 °C for 3 min, and then stopped by the addition of sodium acetate (1.46 M, pH 3.0). The absorbance was measured using a microplate reader at 620 nm and MPO levels were expressed as units of optic density (O.D.) mL^{-1} (Da Silva et al., 2011).

2.15. Statistical analysis

The results of the cultured cell are expressed as mean ± standard deviation of duplicate cultures of two representative experiments. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Bonferroni's test, selected pairs. *P* values < 0.05 were considered statistically significant.

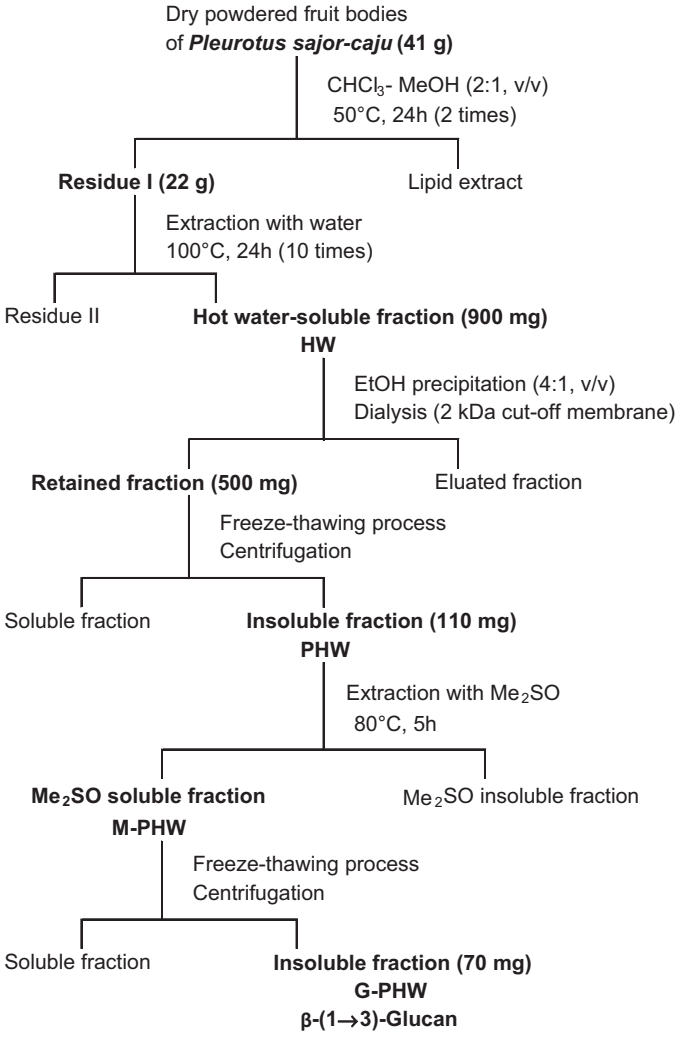

Fig. 1. Scheme of extraction and purification of β-D-glucan obtained from fruiting bodies of *Pleurotus sajor-caju*.

The results of the animal experiments are presented as the mean ± standard error of the mean (SEM), except for the ID_{50} values (*i.e.*, the dose of polysaccharide necessary to reduce the nociceptive response by 50% relative to the control value), which were reported as geometric means accompanied by their respective 95% confidence limits. The ID_{50} value was determined by nonlinear regression from individual experiments using GraphPad software (San Diego, CA, USA). The statistical significance of differences between groups was detected by ANOVA followed by Newman–Keuls' test. P values < 0.05 were considered indicative of significance.

3. Results and discussion

3.1. Purification process and chemical evaluation

After cultivation, the fruiting bodies (41 g) were freeze-dried, milled, and defatted with chloroform-methanol. Fig. 1 represents the scheme of extraction and purification of the β-D-glucan obtained from *P. sajor-caju*.

Defatted fruiting bodies (residue I) were submitted to extraction with hot water, to give the hot-water extract (HW). Polysaccharides present in HW were precipitated by addition of ethanol and recovered by centrifugation. The crude polysaccharide fraction (550 mg) was submitted to freeze and thaw mildly resulting in a cold-water soluble fraction and a cold-water insoluble fraction (PHW, 110 mg). The latter was evaluated for its monosaccharide composition, presenting mannose (15.2%) and glucose (75.1%) as the main sugars. With the aim to purify this fraction, PHW was submitted to an extraction with Me_2SO. The Me_2SO-extract (M-PHW) was dialyzed and submitted to the freeze-thawing process to completely separate any water-soluble contaminant. The water-insoluble fraction (G-PHW, 70 mg) obtained yielded 1.7 mg g^{-1} of dry mushroom. Me_2SO extraction followed by freeze-thawing procedure was effective to obtain a purified glucan, since the glucose content increased of 75.1% (PHW) to 98% (G-PHW). This procedure seems to be successful to purify β-D-glucans, considering that linear (1 → 6)-linked β-D-glucans were isolated from *A. bisporus* and *A. brasiliensis* mushrooms using the same method (Smiderle et al., 2013).

The methylation analysis of G-PHW indicated the presence of 2,4,6-tri-*O*-methyl-Glc*p* (99.9%) and 2,3,4,6-tetra-*O*-methyl-Glc*p* (0.1%), relatives to a linear (1 → 3)-linked glucan structure. This D-glucan was analyzed by HPSEC-MALLS and its molecular weight was estimated at 6.0×10^4 g mol^{-1}. The fractions PHW, M-PHW and G-PHW were analyzed by NMR spectroscopy and their ^{13}C-NMR spectra are depicted in Fig. 2A–C, respectively. By comparison of the spectra, it was possible to observe the reduction of minor signals and the evidence of six main signals, typical of a linear D-glucan. HSQC spectrum of G-PHW (Fig. 3) showed typical signals of a β-D-glucan (1 → 3)-linked observed at 102.8/4.41; 86.1/3.37; 76.2/3.13; 72.9/3.18; 68.3/3.12 and 60.8/3.59,3.34, from C1/H1, C3/H3, C5/H5, C2/H2, C4/H4, and C6/H6, respectively. Similar signals were observed by Santos-Neves et al. (2008), and Synytsya and Novák (2013).

Linear β-D-glucans have been isolated from several mushrooms, but have not been reported in the *Pleurotus* genus yet. A (1 → 3)-β-D-glucan was isolated from a water-insoluble fraction from *Poria cocos* sclerotium (Hoffmann, Simson, & Timell, 1971), and from an aqueous 0.5 M NaOH/0.2 M urea solution (Chen, Xu, Zhang, & Kennedy, 2009) of the same mushroom. A similar β-D-glucan was isolated from the hot alkaline extract of fruiting bodies of *Termitomyces eurhizus* (Chakraborty, Mondal, Rout, & Islam, 2006), and from water-insoluble fraction from *Ganoderma lucidum* after extraction with aq. NaOH solution (Wang & Zhang, 2009).

3.2. Evaluation of the treatment with β-D-glucan in vitro

Considering that many studies have shown that β-D-glucans exhibit biological properties, the linear (1 → 3)-β-D-glucan from *P. sajor-caju* was tested on THP-1 cells. Therefore, the fraction G-PHW was added to the THP-1 macrophages at 10, 50, and 250 μg mL^{-1} and the expression of pro-inflammatory genes (IL-1β, TNF-α, COX-2) was evaluated. For both incubation periods (3 h and 6 h), the β-D-glucan significantly stimulated the production of IL-1β and COX-2 mRNAs, while the TNF-α mRNA was significantly produced after 3 h of treatment (Fig. 4).

In order to test the ability of the β-D-glucan to reduce the effects caused by LPS stimulation, 1 μg mL^{-1} of LPS plus 10, 50 or 250 μg mL^{-1} of G-PHW was added to the cells concomitantly (Fig. 5). After 3 h and 6 h of incubation, the cells showed a significant lower expression of TNF-α, with an inhibition of 61.8 ± 5.74% (50 μg mL^{-1}, 3 h) and 77.5 ± 0.99% (250 μg mL^{-1}, 6 h), respectively. IL-1β and COX-2 mRNAs were also significantly inhibited after 3 h of incubation. At the concentration of 250 μg mL^{-1}, IL-1β mRNA was inhibited at 37.0 ± 0.67%, while COX-2 mRNA was inhibited at 63.6 ± 2.76%.

These results suggest that the linear (1 → 3)-β-D-glucan from *P. sajor-caju* exhibits an immunomodulatory effect on THP-1 macrophages, stimulating the production of pro-inflammatory

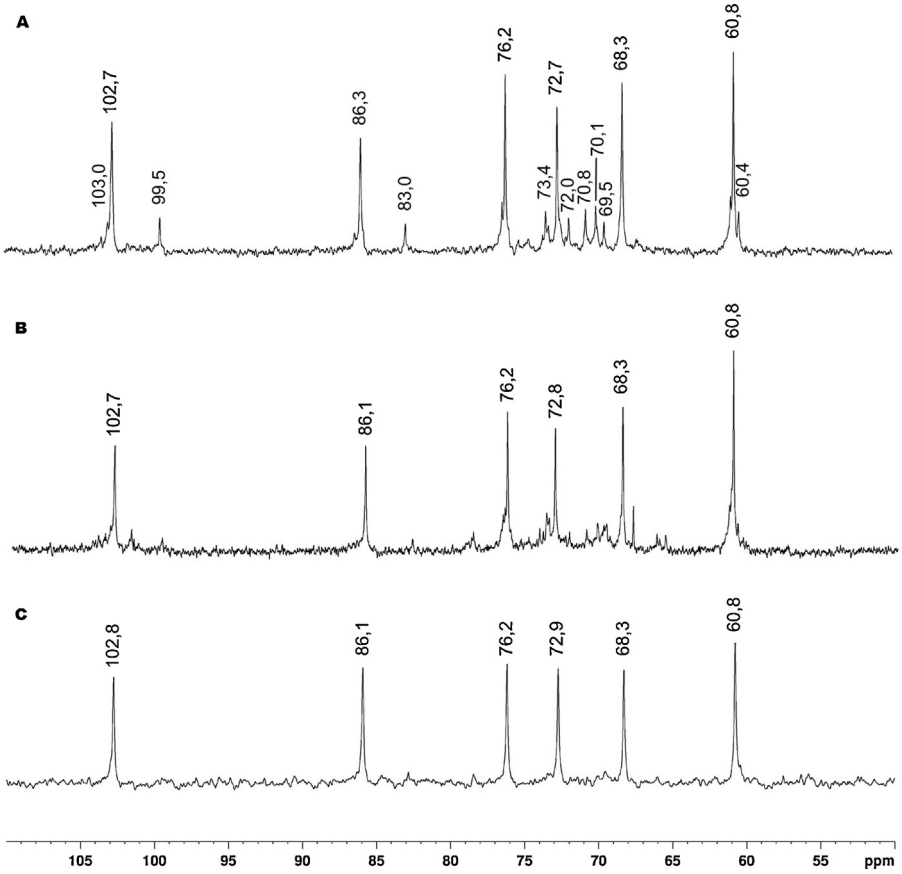

Fig. 2. ^{13}C-NMR spectrum of β-D-glucan obtained from fruiting bodies of *Pleurotus sajor-caju*, in D_2O at 70 °C: (A) PHW, and in Me_2SO-d_6 at 70 °C: (B) M-PHW, and (C) G-PHW (purified β-D-glucan).

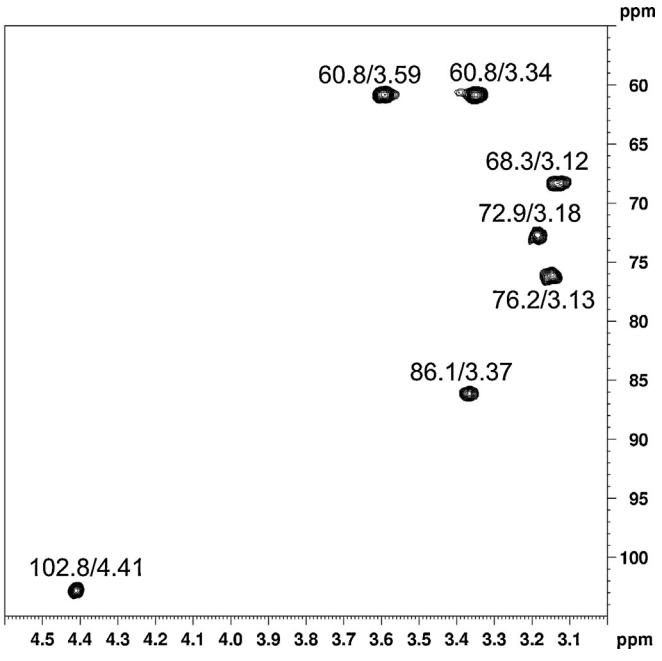

Fig. 3. HSQC spectrum of the β-D-glucan obtained from *Pleurotus sajor-caju*, in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in ppm).

genes when incubated alone, although it inhibits their production when administered with LPS, a strong inflammatory agent. The (1 → 6)-linked β-D-glucans isolated from *A. bisporus* and *A. brasiliensis* also exhibited an immunomodulatory effect when incubated with THP-1 derived macrophages (Smiderle et al., 2013). Both glucans were able to stimulate the expression of IL-1β, TNF-α, and COX-2, when incubated with that cells. When the macrophages were incubated with LPS + β-(1 → 6)-D-glucan, the same inhibitory effect was observed only for IL-1β and COX-2 genes. The curdlan, a β-(1 → 3)-D-glucan from Sigma (M_w not provided), activated the production of IL-1β by macrophages, through binding to dectin-1 receptor (Kankkunen et al., 2010). Researchers have observed that pattern recognition receptors (PRRs) of immune cells, such as dectin-1, complement receptor 3 (CR3), scavenger receptors, lactosylceramide (LacCer), and toll-like receptors (TLRs), recognize the β-D-glucans and initiate immune responses (Chen & Seviour, 2007). Although more information is required to understand how these molecules act after binding to the PRRs and which receptor is the preferable.

To obtain more information on how the β-(1 → 3)-D-glucan from *P. sajor-caju* can act in a biological system, the effects of this polysaccharide were evaluated *in vivo*.

3.3. Evaluation of the treatment with β-D-glucan in vivo

Previous studies have demonstrated that branched (1 → 3), (1 → 6)-β-D-glucans isolated from *P. sajor-caju* exhibit antineoplasic and immunostimulatory effects, and modulate the expression of

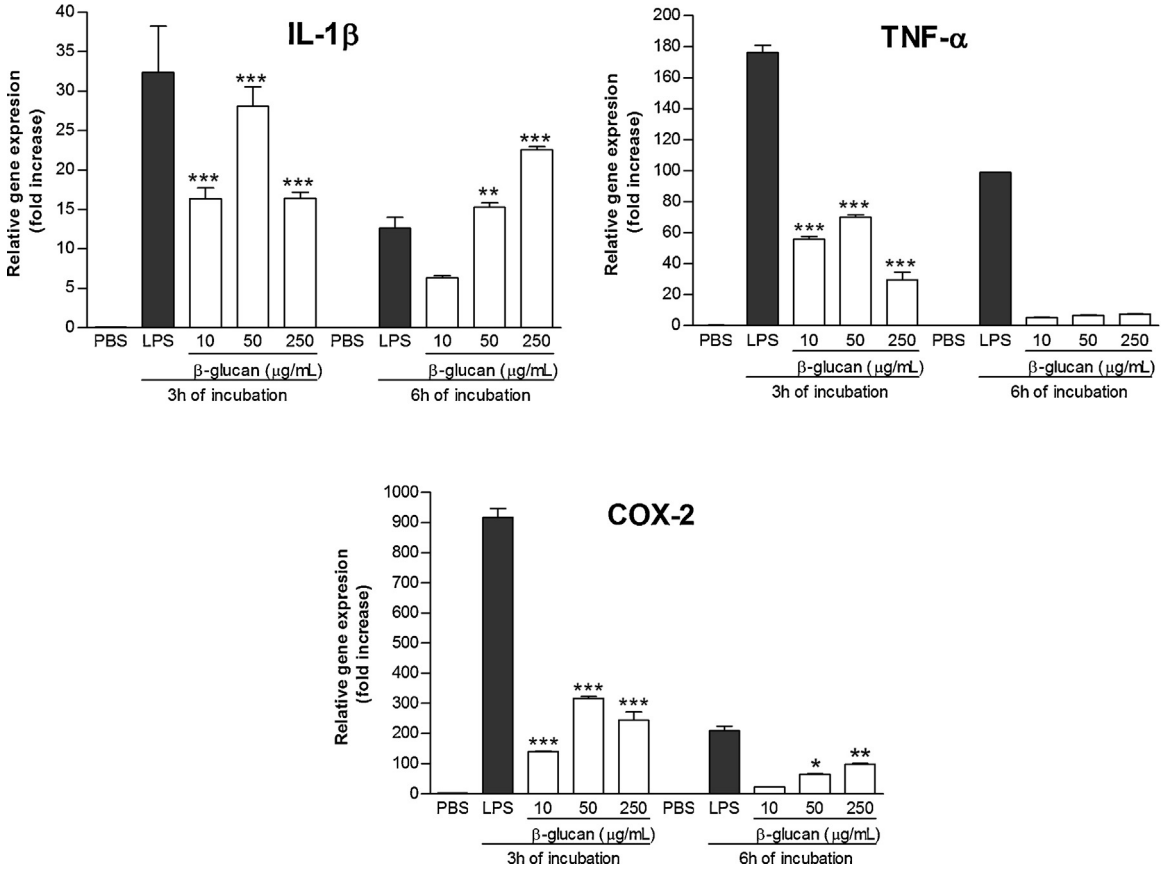

Fig. 4. mRNA expression level of genes IL-1β, TNF-α, and COX-2 after treatment with G-PHW for 3 h and 6 h. *Footnote:* Negative control (PBS) and positive control (LPS; 1 μg mL^{-1}). G-PHW (β-D-glucan) was added at concentrations of 10, 50, and 250 μg mL^{-1}. Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by Bonferronis' test, selected pairs. The results represent the mean ± SD of duplicate cultures of two representative experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus negative control.

some inflammatory markers (Carbonero et al., 2012; Dalonso et al., 2010; Satitmanwiwat et al., 2012). However, there is no information about the biological properties of linear (1 → 3)-β-D-glucan from any species of *Pleurotus*. Besides, it was observed that (1 → 3), (1 → 6)-β-D-glucans from *P. pulmonarius* and *Lactarius rufus* exhibited marked anti-inflammatory and antinociceptive effects (Ruthes et al., 2013; Smiderle et al., 2008). Taking into account that the linear (1 → 3)-β-D-glucan showed an anti-inflammatory effect *in vitro*, a study was performed to evaluate its possible anti-inflammatory and antinociceptive properties *in vivo*. For these experiments, the formalin test was used in mice, which is considered a classical chemical model of inflammatory pain. Results showed that intraperitoneal administration of the β-D-glucan did not reduce the nociceptive response of neurogenic pain (first phase) (Fig. 6A) but significantly inhibited the nociception of inflammatory pain (second phase) induced by formalin, with an ID_{50} value of 2.09 (1.71–2.56) mg kg^{-1} and inhibition of 98 ± 2% at a dose of 10 mg kg^{-1} (Fig. 6B). Ruthes et al. (2013) produced a linear (1 → 3)-linked β-D-glucan as a product of controlled Smith degradation from a (1 → 3), (1 → 6)-linked β-D-glucan isolated from *L. rufus*. The linear polysaccharide (M_w not determined) inhibited the nociceptive behavior of the neurogenic phase by 58 ± 4%, and of the inflammatory phase by 80 ± 9%, at a dose of 30 mg kg^{-1}. Considering the dose used, it was less potent in reducing the inflammatory phase than the β-D-glucan isolated in the present study. The molecular weight of polysaccharides may influence their bioactivity (El Enshasy & Hatti-Kaul, 2013), although in this case it was not possible to confirm this hypothesis. The branched (1 → 3), (1 → 6)-β-D-glucan (11.3 × 10^4 g mol^{-1}) (Ruthes et al., 2013) showed similar inhibition of the inflammatory phase (96 ± 3%) in comparison with the linear β-D-glucan from *P. sajor-caju* (98 ± 2%). However, the lentinan-type glucan was less potent, considering that it was administered at a dose 3× higher than the linear β-D-glucan.

The injection of formalin evokes two distinct phases: a first phase (1–5 min after injection, approximately) and a second phase (15–60 min approximately) of pain sensation in humans and nociceptive behavior in animals (Porro & Cavazzuti, 1993; Tjølsen, Berge, Hunskaar, Rosland, & Hole, 1992). The first phase of the formalin response is due to the excitation of spinal cord neurons by impulses from primary afferent fibers. The second phase is due to an increase in the spinal cord concentration of neuropeptides, excitatory amino acids, and pro-inflammatory mediators (Hunskaar & Hole, 1987; Porro & Cavazzuti, 1993; Tjølsen et al., 1992). Taking into account that the β-D-glucan inhibited inflammatory pain, its anti-inflammatory effect *in vivo* was evaluated.

For this evaluation, bacterial lipopolysaccharide (LPS, 2 μg kg^{-1}) was administered in mice, by intraperitoneal route, to promote an acute inflammatory response. This inflammation model is characterized by recruitment and activation of leukocytes (both mononuclear cells and neutrophils), which are responsible of releasing pro-inflammatory mediators (Ni et al., 2010). After 4 h of LPS application, an increase of total leukocyte number was observed in the peritoneal fluid when compared to animals treated

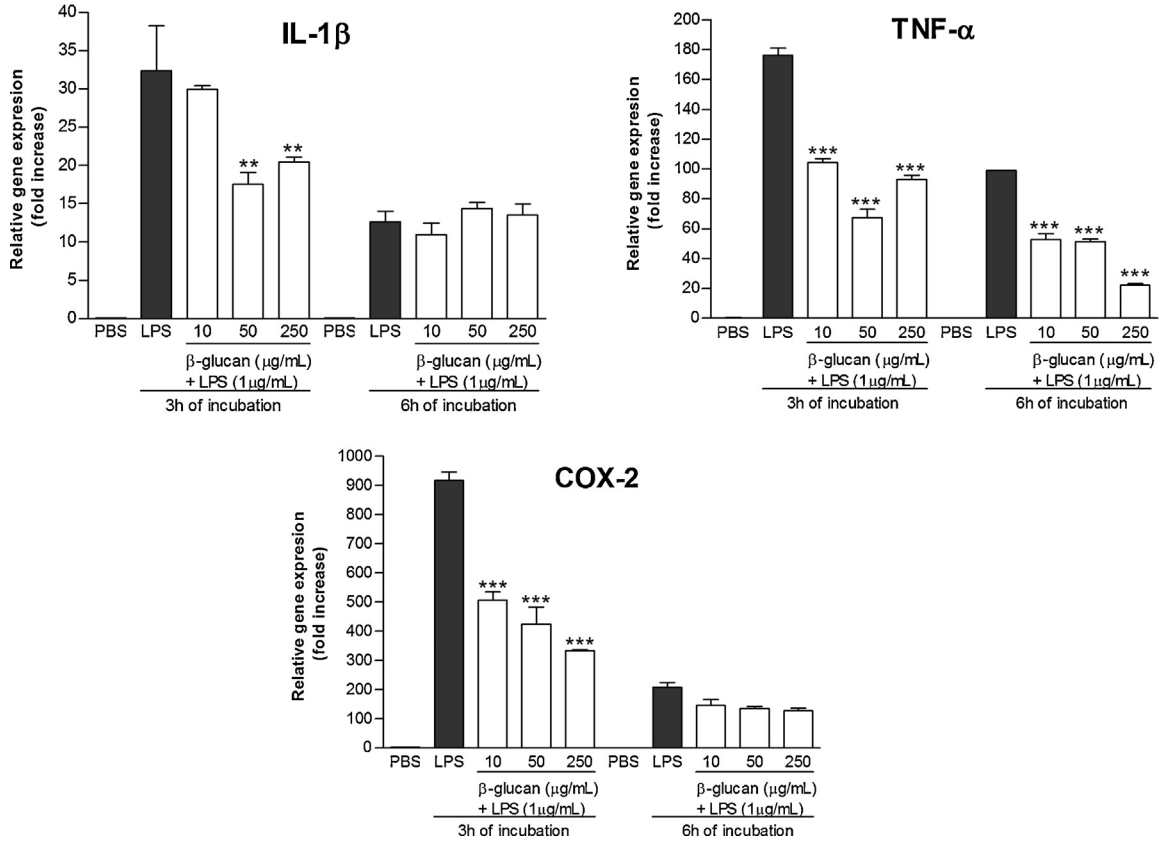

Fig. 5. mRNA expression level of genes IL-1β, TNF-α, and COX-2 after treatment with LPS + G-PHW for 3 h and 6 h. *Footnote:* Negative control (PBS) and positive control (LPS; 1 μg mL^{-1}). G-PHW (β-D-glucan) was added at concentrations of 10, 50, and 250 μg mL^{-1}. Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by Bonferronis' test, selected pairs. The results represent the mean ± SD of duplicate cultures of two representative experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus positive control.

only with saline (S: $2.99 \pm 0.44 \times 10^6$ cells) (Fig. 7A) Furthermore, the treatment of mice with β-D-glucan (1, 3 and 10 mg kg^{-1}, i.p.) and dexamethasone (positive control of the test, 0.5 mg kg^{-1}, i.p.) markedly reduced the migration of total leukocytes by 73 ± 7, 94 ± 15, 100 and 100%, respectively, compared to control group (C: $6.90 \pm 0.62 \times 10^6$ cells) (Fig. 7A). Similarly, the MPO level, an indirect marker of neutrophils, was increased by LPS treatment from 0.10 ± 0.01 to 0.29 ± 0.07 O.D. mL^{-1} (Fig. 7B). The β-D-glucan (1, 3 and 10 mg kg^{-1}, i.p.) and dexamethasone treatments reduced the MPO levels by 84 ± 11, 76 ± 16, 84 ± 12 and 94 ± 4%, respectively, compared to the control group (Fig. 7B). The results confirm the anti-inflammatory activity of the linear (1 → 3)-β-D-glucan from *P. sajor-caju*, reducing the migration of total leukocytes, mainly of neutrophils.

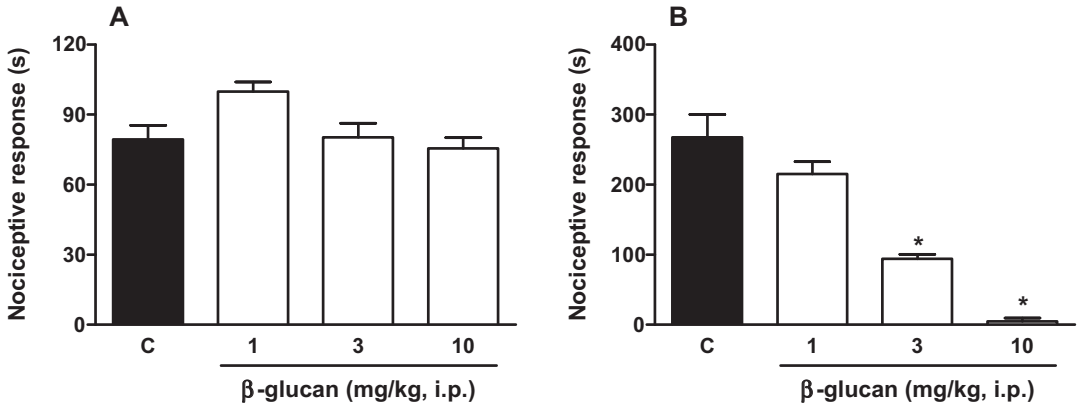

Fig. 6. Effect of β-D-glucan on neurogenic (panel A) and inflammatory phase (panel B) of nociception induced by formalin in mice. *Footnote:* The animals received vehicle (C: saline plus 5% Me_2SO, 10 mL kg^{-1}, i.p.) or G-PHW (β-D-glucan) (1, 3 and 10 mg kg^{-1}, i.p.) 30 min before formalin administration. Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by Newman–Keuls' test. The results represent the mean ± SEM of 6–8 animals. * $P < 0.05$ versus control group.

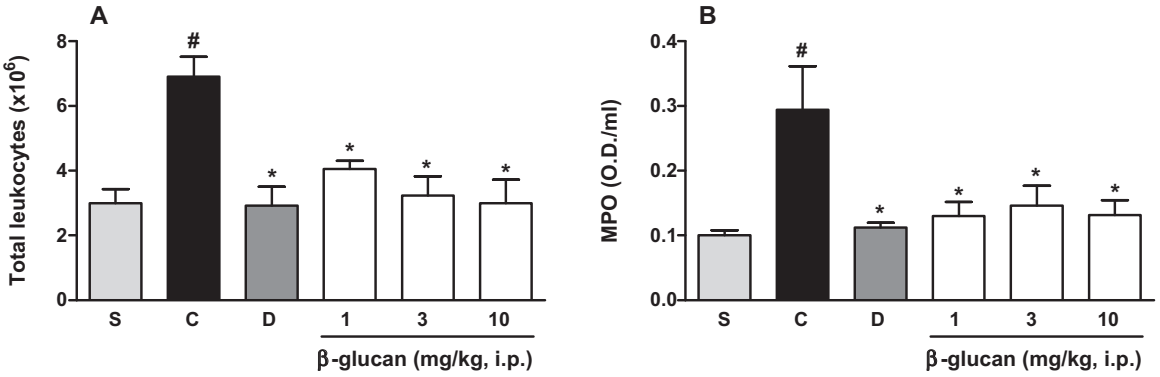

Fig. 7. Effect of β-D-glucan on number of total leukocytes (panel A) and myeloperoxidase levels (panel B) induced by LPS in mice. *Footnote:* The animals received vehicle (C: saline plus 5% Me_2SO, 10 mL kg^{-1}, i.p.), dexamethasone (D: 0.5 mg kg^{-1}, i.p.) or G-PHW (β-D-glucan) (1, 3 and 10 mg kg^{-1}, i.p.) 30 min before LPS administration. Naive group received only sterile saline (S: 10 mL kg^{-1}, i.p.). Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by Newman–Keuls' test. The results represent the mean ± SEM of 6–8 animals. $^{\#}$ $P < 0.05$ versus naive group; * $P < 0.05$ versus control group.

4. Conclusion

P. sajor-caju fruiting bodies cultivated in banana straw, produced a linear β-D-glucan (1 → 3)-linked. This is the first report of such structure isolated from *Pleurotus* genus. An immunomodulatory effect was observed when THP-1 macrophages were treated with the β-D-glucan, which stimulated the production of pro-inflammatory genes when incubated alone. However, the β-D-glucan inhibited production of pro-inflammatory genes when administered with LPS, suggesting an anti-inflammatory effect. The linear β-D-glucan was able to inhibit the inflammatory phase of nociception induced by formalin in a low dose and reduced the number of total leukocytes and myeloperoxidase (MPO) levels induced by LPS. The results observed reinforce the importance of mushroom polysaccharides, as biological response modifiers, and particularly for anti-inflammatory applications.

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