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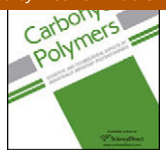

Chemical and biological properties of a highly branched β-glucan from edible mushroom *Pleurotus sajor-caju*

Elaine R. Carbonero[a], Andrea Caroline Ruthes[b], Cristina Setim Freitas[c], Pillar Utrilla[c], Júlio Gálvez[c], Estefânia Viano da Silva[a], Guilherme Lanzi Sassaki[b], Philip Albert James Gorin[b], Marcello Iacomini[b,*]

[a] *Departamento de Química, Universidade Federal de Goiás, Campus Catalão, 75704-020 Catalão GO, Brazil*
[b] *Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, CP 19046, 81531-980 Curitiba PR, Brazil*
[c] *Departamento de Farmacología, Facultad de Farmacia, Universidad de Granada, 18071 Granada, Spain*

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ABSTRACT

Hot aqueous extraction of the basidiocarps of the mushroom *Pleurotus sajor-caju* provided a cold water-soluble, gel-like glucan, which was characterized chemically, and its effects on RAW 264.7 cell line (mouse leukaemic monocyte macrophage) activation were determined. NMR spectroscopy, HPSEC, methylation analysis, and a controlled Smith degradation showed it to have a branched structure with a (1→3)-linked β-Glc*p* main-chain, substituted at O-6 by single-unit β-Glc*p* side-chains, on the average of two to every third residues of the backbone, with a molar mass of 9.75×10^5 g mol^{-1}. In macrophage cell culture, the β-glucan induced production of NO and the cytokines TNF-α, IL-1β, these effects being very similar as those of *Escherichia coli* serotype 0111:B4 Sigma–Aldrich lipopolysaccharide (LPS), although not modifying the response of LPS-activated macrophages. The results suggest that the (1→3), (1→6)-linked β-glucan from *P. sajor-caju* may have potential for immunological activities, although additional experiments are necessary for a better understanding of the mechanisms involved.

1. Introduction

The popularity of edible mushrooms of the genus *Pleurotus* has increased, since they present good nutritional values, ready cultivation and are excellent sources of molecules such as polysaccharides that can act as biological response modifiers (Gonzaga, Ricardo, Heatly, & Soares, 2005; Lavi, Friesem, Geresh, Hadar, & Schwartz, 2006; Smith, Rowan, & Sullivan, 2002).

Among these polymers, homo- and heteroglucans, with β-(1→3), β-(1→4) and β-(1→6) glycosidic linkages, are supposed to play a key role in some health aspects of mushrooms (Manzi & Pizzoferrato, 2000). The most frequently described β-glucans have the same main-chain (1→3), substitution (O-6) and a degree of branching of 1:3:1, as the scleroglucan (Tabata, Ito, & Kojima, 1981); lentinan (Sasaki & Takasura, 1976) and grifolan (Kato, Mutoh, Egashira, Hiura, & Ueno, 1978), among others (Yoshioka, Tabeta, Saito, Uehara, & Fukuoka, 1985; Santos-Neves et al., 2008; Smiderle et al., 2006, 2008). Most of them had been shown significant anti-tumor, anti-inflammatory and immunomodulating effects (Wasser, 2011; Zhang, Cui, Cheung, & Wang, 2007).

Several studies have been carried out to confirm the immunomodulatory activity of some *Pleurotus* spp. (Paulík, Mojzisová, Durove, Benísek, & Húska, 1996; Sedaghat & Ghazanfari, 2011; Sun & Liu, 2009).

The immune body response is mediated by a diverse group of leukocytes. The mononuclear phagocyte system is a subgroup of leukocytes that circulate in the blood as monocytes and populate tissues as macrophages in the steady state and during inflammation (van Furth & Cohn, 1968). Macrophages are resident phagocytic cells in lymphoid and non-lymphoid tissues and are believed to be involved in steady-state tissue homeostasis, promoting destruction of apoptotic cells, and the production of growth factors, as Insulin-like Growth Factor I (IGF-I), Transforming Growth Factor-α and β (TGF-α and TGF-β), Epidermal Growth Factor (EGF), Platelet-Derived Growth Factor (PDGF), basic Fibroblast Growth Factor (FGF) (Gordon, 2002; Rom et al., 1988; Rappolee, Mark, Banda, & Werb, 1988).

Numerous studies have shown that various β-glucans activate cellular and humoral components of the host immune system and increase functional activity of macrophages, suggesting that they are able to serve as immunostimulants (Chang et al., 2009; Dellinger et al., 1999; Liu et al., 2007; Moradali, Mostafavi, Ghods, & Hedjaroude, 2007; Ross, Větvička, Yan, Xia, & Vetvicková, 1999).

Accordingly, the edible mushroom *Pleurotus sajor-caju*, known in Japan as Houbitake and whose therapeutic properties are attributed to its carbohydrates, were now extracted and resulting polysaccharides were purified for further structural characterization. A branched β-glucan was isolated and characterized and its

* Corresponding author. Tel.: +55 41 33611655; fax: +55 41 3266 2042.
E-mail address: iacomini@ufpr.br (M. Iacomini).

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biological effects were evaluated to determine its immunomodulatory properties using a RAW 264.7 macrophage cell culture.

2. Materials and methods

2.1. Biological material

Fresh basidiocarps (fruiting bodies) of *P. sajor-caju* (2 kg) were furnished by Makoto Yamashita Company (Miriam Harumi Yamashita), São José dos Pinhais, State of Paraná, Brazil.

2.2. Reagents

All chemicals were purchased from Sigma–Aldrich Chemical (and cytokine ELISA kits from R&D Systems (Europe, Abingdon, UK).

2.3. Extraction and purification of β-glucan

Dried and powdered fruiting bodies of *P. sajor-caju* (222 g) were treated with successive extraction with water at 4 °C (6×, 2000 mL each) and 100 °C (6×, 2000 mL each). Polysaccharides were separately recovered from the respective aqueous extracts by addition to excess EtOH, giving fractions named CW, for Cold Water aqueous extract obtained at 4 °C, and HW, for Hot Water aqueous extract obtained at 100 °C. Crude HW was submitted to a freeze–thawing process (Gorin & Iacomini, 1984), furnishing cold water-soluble (SHW) and soluble gel-like fractions (SGHW), which were separated by centrifugation (9000 rpm for 30 min. at 4 °C).

2.4. Monosaccharide composition

Each polysaccharide fraction (1 mg) was hydrolyzed with 2 M TFA at 100 °C for 8 h, followed by evaporation to dryness. The residues were successively reduced with excess of $NaBH_4$ and acetylated with Ac_2O–pyridine (1:1, v/v; 400 μL) at room temperature for 12 h (Wolfrom & Thompson, 1963a, 1963b). The resulting alditol acetates were analyzed by gas chromatography–mass spectrometry (GC–MS), using a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap, Model 810-R12 mass spectrometer. Incorporated was a DB-225 capillary column (30 m × 0.25 mm i.d.) programmed from 50 to 220 °C at 40 °C min^{-1}, then hold, and the alditol acetates identified by their typical retention times and electron impact profiles.

2.5. Determination of homogeneity of polysaccharides and their molecular weight (M_w)

The determination of the homogeneity and molar mass (M_w) of the fractions were performed on a Waters high-performance size-exclusion chromatography (HPSEC) apparatus coupled to a differential refractometer (RI) and a Wyatt Technology Dawn-F Multi-Angle Laser Light Scattering (MALLS) detector. The eluent was 0.1 M $NaNO_3$, containing 0.5 g L^{-1} NaN_3. The polysaccharides solutions were filtered through a membrane with 0.22 μm diameter pores (Millipore). The specific refractive index increment (dn/dc) was determined using a Waters 2410 detector, the samples being dissolved in the eluent, five increasing concentrations, ranging from 0.2 to 1.0 mg mL^{-1} being used to determine the slope of the increment.

2.6. Methylation analysis of β-glucan

Per-*O*-methylation of the isolated polysaccharide (10 mg) was carried out using NaOH-Me_2SO-MeI (Ciucanu & Kerek, 1984). The process, after isolation of the products by neutralization (HOAc), dialysis, and evaporation was repeated, and the methylation was found to be complete. The per-*O*-methylated derivatives were hydrolyzed with 45% (v/v) aq. formic acid (1 mL) for 15 h at 100 °C, followed by NaB^2H_4 reduction and acetylation as in item 2.4, to give a mixture of partially *O*-methylated alditol acetates, which was analyzed by GC–MS using a DB-225 capillary column (30 m × 0.25 mm i.d.) programmed from 50 to 215 °C at 40 °C min^{-1}, then hold, and identified from m/z profiles of their positive ions, by comparison with standards, the results being expressed as a relative percentage of each component.

2.7. Controlled Smith degradation

The purified β-glucan (42.8 mg) was submitted to oxidation with 0.05 M aq. $NaIO_4$ (15 mL) at room temperature for 72 h in the dark. Ethylene glycol was then added to stop the reaction, the solution dialyzed, and the resulting polyaldehydes were reduced with $NaBH_4$ for 24 h, neutralized with HOAc, dialyzed, and concentrated (Goldstein, Hay, Lewis, & Smith, 2005). The residue was partially hydrolyzed with aq. TFA at pH 2.0 (30 min at 100 °C, under reflux) (Gorin, Horitsu, & Spencer, 1965) followed by dialysis against tap water using membranes with a size exclusion of 2 kDa and the solution containing retained material was freeze-dried to give polymeric fraction SM-SGHW (31.0 mg), and analyzed by ^{13}C NMR spectroscopy.

2.8. Nuclear magnetic resonance (NMR) spectroscopy

^{13}C, ^1H and HSQC NMR spectra were obtained using a 400 MHz Bruker model DRX Avance spectrometer, incorporating Fourier transform, by procedures described in the Bruker manual. Analyses were performed at 70 °C on polysaccharide samples dissolved in Me_2SO-d_6 (30 mg in 0.4 mL for ^{13}C, and 2 mg in 0.6 mL for ^1H NMR analysis). Chemical shifts of samples are expressed in δ (ppm) relative to Me_2SO-d_6 at δ 39.7 and 2.40 for ^{13}C and ^1H signals, respectively.

2.9. Cell line and culture conditions

The murine macrophage cell line, Raw 264.7 (obtained from the Cell Culture Unit of the University of Granada, Granada, Spain), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS, high glucose (4.5 g L^{-1}) plus 1% of streptomycin–penicillin, 1% amphotericin, 1% glutamine at 37 °C under 5% CO_2. Cells were seeded onto 24-well culture plates at a density of 5×10^5 cells/well and grown until confluence, and were incubated in media containing β-glucan (0.1–300 μg mL^{-1}), for 1 h. Cells were then incubated with or without LPS (0.1 μg mL^{-1}; Lipopolysaccharides from *Escherichia coli* serotype 0111:B4 Sigma–Aldrich) for 24 h (37 °C under 5% CO_2). Their viability was then determined by the Crystal Violet assay (Gilles, Didier, & Denton, 1986). Levels of nitric oxide (NO) were measured using the Griess reagent (Park, Quinn, Wright, & Schuller-Levis, 1993), and the concentration of pro-inflammatory cytokines (TNF-α, IL-1β) was finally investigated using ELISA kits following the manufacturer's protocol.

3. Results and discussion

3.1. Structural characterization of (1→3), (1→6) β-D-glucan

The basidiocarps (fruiting bodies) of *P. sajor-caju*, after desiccation in a freeze dryer gave 11.1% of their original weight. They were then treated with successive extractions with water at 4 °C and 100 °C. The extracted polysaccharides were recovered via ethanol precipitation, giving fractions CW (12.4 g) and HW (11.4 g), respectively.

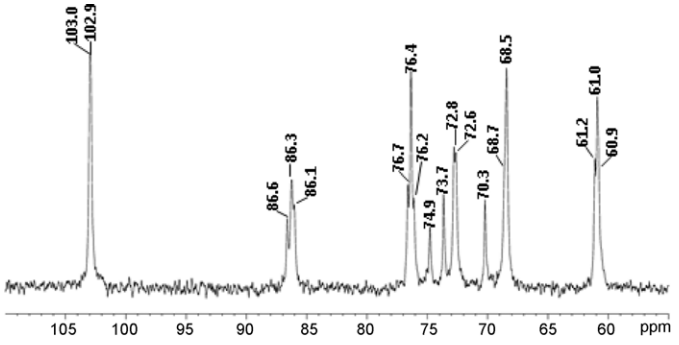

Fig. 1. ^{13}C NMR spectrum of fraction SGHW from *P. sajor-caju*, in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in ppm).

Fraction HW was shown to be heterogeneous by HPSEC and was composed of units of glucose (89.0%), besides rhamnose (4.5%), arabinose (2.2%), mannose (2.2%), and galactose (2.1%). Fractionation and purification of HW was carried out by a freeze–thawing procedure (Gorin & Iacomini, 1984), which resulted in respective cold water-soluble (SHW, 8.3 g) and in a soluble gel-like fractions (SGHW, 3.1 g).

Fraction SGHW was shown to be homogeneous on HPSEC, and had M_w of 9.75×10^{-5} g mol^{-1} (dn/dc 0.159 mL g^{-1}).

The purified polysaccharide fraction (SGHW) contained mainly glucose (97.8%) as its monosaccharide component (GC–MS). Methylation-GC–MS analysis gave rise to partially *O*-methylated alditol acetates, corresponding to a branched (1→3), (1→6)-linked β-D-glucan with nonreducing end-(40.1%), 3-*O*- (19.4%) and 3,6-di-*O*-substituted units (40.5%) in a molar ratio of 2:1:2, respectively.

The ^{13}C NMR of fraction SGHW contained only one anomeric C-1 signal at δ 103.0 (Fig. 1), while in its HSQC spectrum (Fig. 2) two distinct signals (C-1/H-1) could be observed, one at δ 103.0/4.21 corresponding to nonreducing end-units, and other at δ 102.9/4.51 from 3-*O*- and 3,6-di-*O*-substituted residues (Fig. 2). The β-configuration was shown by low frequency H-1 (δ 4.51 and 4.21) and high-frequency C-1 signals (δ 103.0 and 102.9) (Figs. 1 and 2A and B) (Hall & Johnson, 1969). All the signals were assigned using the literature values for similar polysaccharides (Chauveau, Talaga, Wieruszeski, Strecker, & Chavant, 1996; Santos-Neves et al., 2008; Tabata et al., 1981; Yoshioka et al., 1985) (Table 1).

The positions of the glycosidic linkages of the glucan were shown by the presence of 3-*O*-substituted signals at δ 86.6, 86.3, and 86.0 (Figs. 1 and 2) and an *O*-substituted $\underline{C}H_2$-6 signal at δ 68.3, which appeared as doublet in its HSQC spectrum at δ 68.3; 4.06/3.52 (Fig. 2).

The main-chain structure of fraction SGHW was shown by a controlled Smith degradation, its final polymeric product (SM-SGHW) being analyzed by ^{13}C NMR spectroscopy. Its ^{13}C NMR spectrum (not shown) showed it to be a linear (1→3)-linked β-D-glucan with typical ^{13}C signals at δ 102.9 (C-1); 86.1 (C-3); 76.3 (C-5); 72.8 (C-2); 68.4 (C-4), and 60.9 (C-6) (Gorin, 1981).

Analysis of fraction SGHW, based on monosaccharide composition, methylation data, NMR spectroscopic analysis, and controlled Smith showed it to be a β-D-glucan with a (1→3)-linked main chain, partially substituted at O-6 by side chains of nonreducing end-units of β-D-Glc*p*, on the average of two to every third unit of the backbone (Fig. 3).

This type of β-glucan is a common component of basidiomycetes, with the degree of substitution varying with the fungus. They have mainly been found to have the same main chain and O-6 substitution with a degree of branching of 1–3. β-Glucans containing the same proportion of branching to those now described, have been isolated from *Auricularia auricula judae* basidiomycetes (Sone, Kakuta, & Misaki, 1978). However, for *P. sajor-caju*

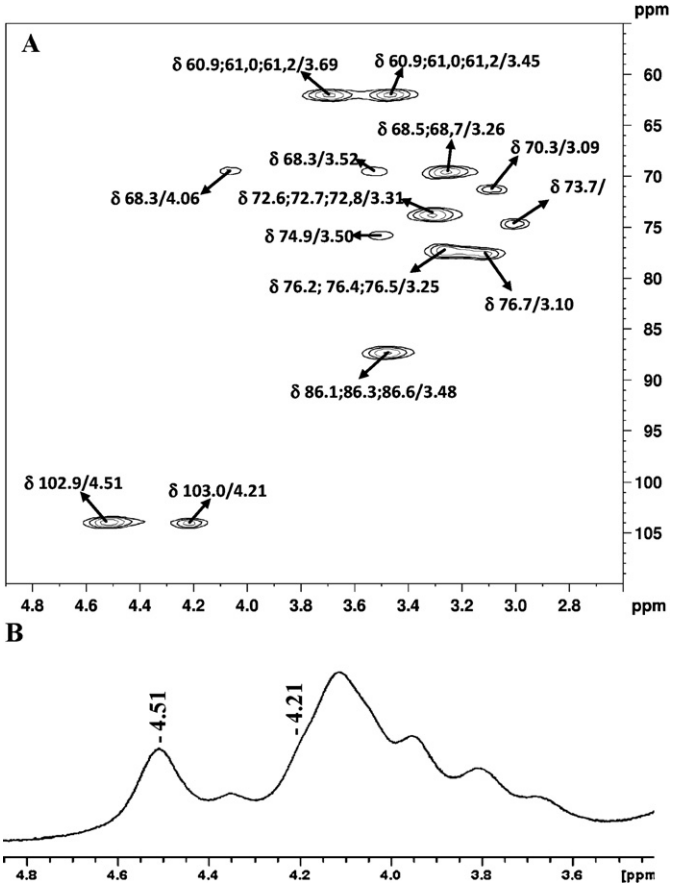

Fig. 2. HSQC (A) and ^{1}H NMR (B) spectra of fraction SGHW from *P. sajor-caju*, in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in ppm).

only other polysaccharides containing glucose have been characterized (Pramanik, Mondal, Chakraborty, Rout, & Islam, 2005; Pramanik, Chakraborty, Mondal, & Islam, 2007; Roy, Maiti, Mondal, Das, & Islam, 2008). Two of them refer to heteropolysaccharides (Pramanik et al., 2005; Roy et al., 2008) and one to a glucan with an unusual structure, which contain α- and β-Glc*p* units, besides of Glc*p* (1→2)-linked units (Pramanik et al., 2007). In this way, no polysaccharide already described for *P. sajor-caju* resembles the branched β-D-glucan described in this report.

Previous investigations have described the antitumor and immunostimulating properties of homopolymers (Borchers, Keen, & Gershwin, 2004; Zhang et al., 2007), depending on the structural characteristics as solubility, degree of branching, molecular weight, among others. Studies have indicated that the

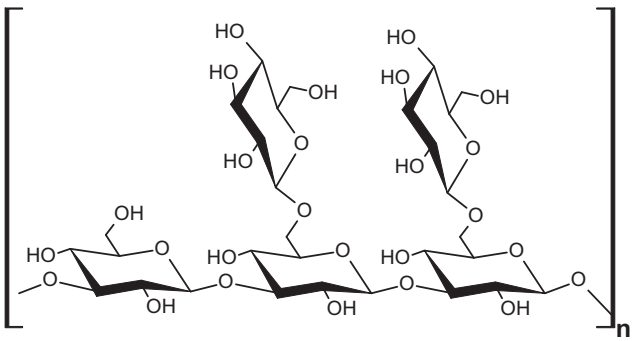

Fig. 3. Structure of water-soluble gel-like β-glucan (SGHW) obtained from *P. sajor-caju*.

Table 1
^{1}H and ^{13}C NMR chemical shifts [expressed as δ (ppm)] of β-D-Glucan (1→3), (1→6) from *P. sajor-caju*[a].

Units		1	2	3	4	5	6	
							a	b
→3)-β-D-Glc*p*-(1→	^{13}C	102.9	72.8	86.6/86.3	68.4	76.5/76.4	61.0/60.9	61.0/60.9
	^{1}H	4.51	3.31	3.48	3.26	3.25	3.69	3.45
→3,6)-β-D-Glc*p*-(1→	^{13}C	103.0	72.7	86.1	68.7	74.9	68.3	68.3
	^{1}H	4.51	3.31	3.48	3.26	3.50	4.06	3.52
β-D-Glc*p*-(1→	^{13}C	103.1	73.7	76.7	70.3	76.2	61.2	61.2
	^{1}H	4.21	3.01	3.10	3.09	3.25	3.69	3.45

[a] Assignments are based on ^{13}C, ^{1}H and HSQC examination.

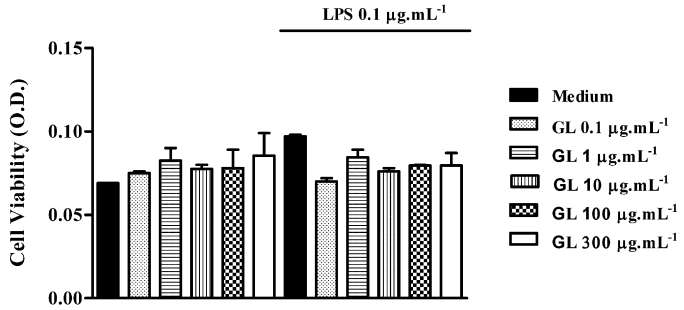

Fig. 4. Effects of SGHW β-glucan and lipopolysaccharide (LPS) on the viability of RAW264.7 macrophages, measured by Crystal Violet assay. Data represent mean ± SEM. * Significant difference ($p < 0.05$) compared to control group (Dulbecco's modified Eagle's medium).

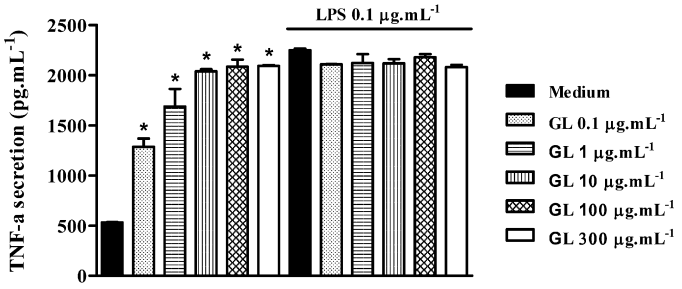

Fig. 6. Effects of SGHW β-glucan and lipopolysaccharide (LPS) on TNF-α secretion, measured by ELISA in RAW264.7 macrophage medium. Data represent mean ± SEM. * Significant difference ($p < 0.05$) compared to control group (Dulbecco's modified Eagle's medium).

distribution of the single glycosyl units along the main chain confers their immunomodulating activity, their solubility in water also being important for biological applications (Bohn & BeMiller, 1995; Wasser, 2011).

Consequently, water-soluble gel-like β-D-glucan of *P. sajor-caju* might be considered as good candidate to be tested for its potential as biological response modifiers, although biological investigations are necessary in order to attribute a relation between structure and activity of its highly branched structure, since most of the previous investigations were on β-glucans having a lower degree of branching.

3.2. Biological properties of the β-glucan

To investigate the effects of *P. sajor-caju* β-glucan on macrophages, it was first confirmed that it was not toxic to RAW 264.7 macrophages, since it did not affect the cell viabilities within the range of concentrations tested (Fig. 4).

After 24 h of β-glucan exposure (0.1–300 μg mL^{-1}), RAW 264.7 cells showed an increase in NO production (Fig. 5). The concentration of cytokines was also investigated with RAW 264.7 macrophages, which were exposed to β-glucan. It increased the amount of TNF-α from 532.5 ± 3.6 from the medium group to 1287 ± 81, 1686 ± 178, 2038 ± 21, 2084 ± 71 and 2092 ± 9 pg mL^{-1}, with the 0.1, 1, 10, 100 and 300 μg mL^{-1} of β-glucan, respectively (Fig. 6). The concentration of IL-1β also increased after incubation with β-glucan from 434 ± 76 (medium) to 760 ± 54, 1976 ± 3, 2147 ± 25 and 2102 ± 41 with 0.1, 1, 10, 100 and 300 μg mL^{-1} of β-glucan, respectively (Fig. 7).

The primary effect of polysaccharides is to enhance and/or activate macrophage immune responses. In particular, they have been shown to activate phagocytic activity, increase reactive oxygen species and NO synthesis, and enhance secretion of cytokines and chemokines, such as TNF-α, IL-1β, IL-6, IL-8, IL-12, IFN-γ and IFN-β_2 (Schepetkin & Quinn, 2006).

Polysaccharides isolated from several mushrooms (Kim, Choi, Lee, & Park, 2004; Lee et al., 2008; Son et al., 2006;) and higher plants (Schepetkin & Quinn, 2006) have been shown to be effective inducers of NO synthesis in macrophages.

In the present study, β-glucan from *P. sajor-caju* activated RAW 264.7 macrophages and induced synthesis of NO and cytokines TNF-α, IL-1β, these effects being very similar to that of LPS.

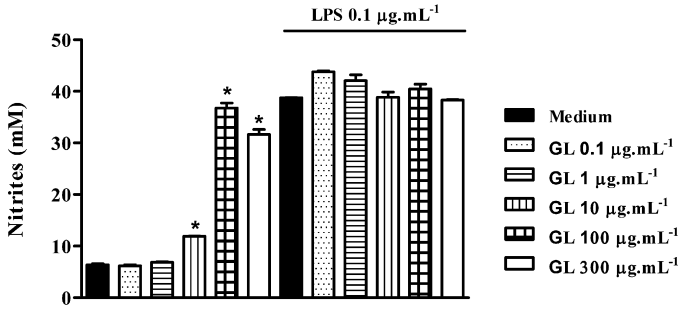

Fig. 5. Effects of SGHW β-glucan and lipopolysaccharide (LPS) on the amount of nitrite measured by Griess reaction in RAW264.7 macrophage medium. Data represent mean ± SEM. * Significant difference ($p < 0.05$) compared to the control group (Dulbecco's modified Eagle's medium).

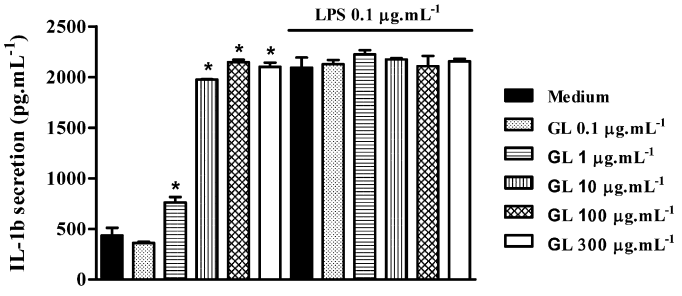

Fig. 7. Effects of SGHW β-glucan and lipopolysaccharide (LPS) on IL-1β secretion, measured by ELISA in RAW264.7 macrophage medium. Data represent mean ± SEM. * Significant difference ($p < 0.05$) compared to control group (Dulbecco's modified Eagle's medium).

However, the β-glucan did not modify release of NO and the cytokines, when the macrophages were activated with LPS.

Activated macrophages contribute to inflammatory responses, nonseptic hypersensitivity reactions and diseases, and even infection-associated tissue injury (Molloy, Mannick, & Rodrick, 1993; Stout, 1993). During inflammation, macrophages can produce and release NO and cytokines into the general circulation to exert systemic effects (West, Seatter, Bellingham, & Clair, 1995).

TNF-α and IL-1β are two typical pro-inflammatory cytokines, which are produced by activated macrophages and are involved in the immune responses and host defense (Beutler & Cerami, 1986; Carswell, Old, & Kassel, 1975).

In conclusion, the present results suggest that the β-glucan of *P. sajor-caju* has an immunostimulatory effect, although further studies are necessary for a better understanding of the mechanisms involved.

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