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Agaricus bisporus fucogalactan: Structural characterization and pharmacological approaches

Andrea C. Ruthes[a], Yanna D. Rattmann[a,b], Simone M. Malquevicz-Paiva[a], Elaine R. Carbonero[c], Marina M. Córdova[d], Cristiane H. Baggio[e], Adair R.S. Santos[d], Philip A.J. Gorin[a], Marcello Iacomini[a,*]

[a] Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, CP 19046, CEP 81531-980, Curitiba, PR, Brazil
[b] Departamento de Saúde Comunitária, Universidade Federal do Paraná, Rua Padre Camargo 280, CEP 80060-240, Alto da Glória, Curitiba, PR, Brazil
[c] Departamento de Química, Universidade Federal de Goiás, Campus Catalão, 75704-020 Catalão, GO, Brazil
[d] Laboratório de Neurobiologia da Dor e Inflamação, Departamento de Ciências Fisiológicas, Universidade Federal de Santa Catarina, Campus Universitário, Trindade, 88040-900 Florianópolis, SC, Brazil
[e] Departamento de Farmacologia, Universidade Federal do Paraná, CP 19031, CEP 81531-980, Curitiba, PR, Brazil

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ABSTRACT

The fucogalactan from *Agaricus bisporus* (EFP-Ab) obtained on aqueous extraction followed by purification had M_w 37.1×10^4 g mol^{-1} relative to a (1→6)-linked α-D-Gal*p* main-chain partially methylated at HO-3, and partially substituted at O-2 by nonreducing end-units of α-L-Fuc*p* or β-D-Gal*p*. EFP-Ab also inhibited significantly the neurogenic and inflammatory phases of formalin-induced licking, however, the antinociceptive effect was more pronounced against the inflammatory phase with ID_{50} of 36.0 (25.8–50.3) mg kg^{-1}. In addition, EFP-Ab decreased the lethality induced by CLP. Its administration reduced the late mortality rate by 40%, prevented neutrophil accumulation in lungs and markedly decreased iNOS and COX-2 protein expression by ileum cells. These data show for the first time that EFP-Ab has significant anti-sepsis, antinociceptive and anti-inflammatory actions, which seems to be related to the decreased iNOS and COX-2 expression. Collectively, the present results demonstrate that EFP-Ab could constitute an attractive molecule of interest for the development of new drugs.

1. Introduction

Mushrooms are currently evaluated for their nutritional value and acceptability as well as for their pharmacological properties. The benefits of mushroom compounds on different clinical conditions have attracted the interest of the scientific community in the last decade in order to understand the molecular mechanisms responsible for their actions (Wasser, 2010). Polysaccharides and other mushroom compounds such as proteins, lypopolysaccharides, glycoproteins and secondary metabolites have been classified as molecules that have potent effects on the immune system (Wasser, 2010). In pursuit of such molecules, several polysaccharides have been isolated from basidiomycetes, such as linear or branched glucans and heterogalactans, which can contain *O*-methyl groups or a variety of side chains (Wasser, 2002; Zhang, Cui, Cheung, & Wang, 2007). Most heterogalactans have a main chain composed of (1→6)-linked α-D-Gal*p* units with mainly fucose or mannose as substituent (Carbonero, Gracher, Komura, et al., 2008; Carbonero, Gracher, Rosa, et al., 2008; Rosado et al., 2003; Smiderle et al., 2008; Wasser, 2002; Zhang et al., 2007), although there are few studies relating their detailed structure and biological activity.

Some of these polysaccharides have been evaluated as biological response modifiers, especially for their antitumor activity (Moradali, Mostafavi, Ghods, & Hedjaroude, 2007; Wasser & Weis, 1999; Zhang et al., 2007). Some reports have shown that extracts from mushrooms can have other effects, such as anti-inflammatory and antinociceptive activity (Dore et al., 2007; Komura et al., 2010; Moro et al., 2012; Queiroz et al., 2010; Ruthes, Rattmann, Carbonero, Gorin, & Iacomini, 2012; Smiderle et al., 2006, 2008). However, it is not possible to attribute a relation between structure and activity, because most of the investigations were carried out using crude polysaccharide extracts (Lindequist, Niedermeyer, & Julich, 2005; Poucheret, Fons, & Rapior, 2006). Recent studies concerned anti-inflammatory and antinociceptive effects of fucogalactans, fucomannogalactans, and mannogalactans isolated from *Agaricus brasiliensis* and *Agaricus bisporus* var. *hortensis* (Komura et al., 2010), *Lentinus edodes* (Carbonero, Gracher, Komura, et al., 2008), and *Pleurotus pulmonarius* (Smiderle et al., 2008), respectively.

A. bisporus white button mushroom constitutes the bulk of all mushrooms consumed and contains bioactive compounds that have been shown to exhibit immunomodulating and anticancer

* Corresponding author. Tel.: +55 41 3361 1655; fax: +55 41 3266 2042.
E-mail address: iacomini@ufpr.br (M. Iacomini).

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properties (Adams, Phung, Wu, Ki, & Chen, 2008; Jeong, Koyyalamudi, & Pang, 2012; Jeong, Sundar, Jeong, Song, & Gerald, 2012; Zhihong, Zhuyan, Nikbin, & Dayong, 2008), besides have presented an effect against sepsis, possibly related to anti-inflammatory potential of its polysaccharide (heterogalactan) (Ruthes et al., 2012). Until this moment, there are very few reports dealing with the ability of mushroom polysaccharides in reducing mortality caused by sepsis in mice. Sepsis is a considerable health problem and a leading cause of morbidity and mortality in many intensive care units. It represents a state of overproduction of pro-inflammatory mediators which frequently occurs after various noxious injuries, especially bacterial infection, as a consequence of abdominal surgery, appendicitis, perforated ulcers, or an ischemic bowel (Angus et al., 2001).

Thus, in order to compare chemical structure with biological properties of mushroom polysaccharides, another *A. bisporus* fucogalactan was characterized. Its antinociceptive and anti-inflammatory effects on neurogenic and inflammatory phases of formalin-induced pain, besides of activity against murine sepsis, namely its effects on lethality, neutrophil migration and pro-inflammatory proteins expression were also investigated.

2. Materials and methods

2.1. Biological material

Dried fruiting bodies of *A. bisporus* (*champignon de Paris*) were provided by Makoto Yamashita Company (Miriam Harumi Yamashita), Sao José dos Pinhais, State of Paraná, Brazil.

2.2. Extraction and purification of fucogalactans

Extraction of crude polysaccharides and their purification was carried out as in Fig. 1A. Wiley-milled powder fruiting bodies from *A. bisporus* (100 g) were extracted with H_2O at 4 °C for 6 h (6×; 2000 ml). The combined aq. extracts were added to excess ethanol (EtOH, 3:1, v/v) to precipitate polysaccharides, which were collected by centrifugation (8000 rpm at 5 °C for 20 min). Polysaccharide fraction was then dissolved in H_2O, dialyzed against tap water for 24 h to remove low-molecular-weight carbohydrates, giving rise to a solution containing a fraction, denominated CW-Ab. This was frozen and then allowed to thaw slowly (Gorin & Iacomini, 1984), resulting in an insoluble fraction ICW-Ab, which was separated by centrifugation as described above. The supernatant fraction SCW-Ab, was treated with Fehling solution (Jones & Stoodley, 1965), giving precipitated Cu^{2+} complex (FP-Ab), which was separated by centrifugation. The precipitate was neutralized with acetic acid (HOAc), dialyzed against tap water (48 h), deionized with mixed ion exchange resins, and then freeze-dried.

FP-Ab fraction was further purified by closed dialysis through a membrane with a 100 kDa M_r cut-off (Spectra/Por® Cellulose Ester), giving rise to a retained (RFP-Ab) and an eluted (EFP-Ab) material (Fig. 1A).

2.3. Monosaccharide composition of polysaccharides

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 $NaBH_4$ (1 mg) and acetylated with Ac_2O-pyridine (1:1, v/v; 200 μl) at 100 °C for 30 min following the method of Sassaki et al. (2008). 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.

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

The homogeneity and molecular mass (M_w) of the purified fucogalactan EFP-Ab were determined by high performance steric exclusion chromatography (HPSEC), using a refractive index (RI) detector. The eluent was 0.1 M $NaNO_3$, containing 0.5 g l^{-1} NaN_3. The polysaccharide 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.

2.5. Methylation analysis of fucogalactan

Per-*O*-methylation of isolated polysaccharide (EFP-Ab; 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% aqueous formic acid (HCO_2H, 1 ml) for 6 h at 100 °C, followed by NaB_2H_4 reduction and acetylation as above (Section 2.3), to give a mixture of partially *O*-methylated alditol acetates, which was analyzed by GC–MS using a Varian model 4000 gas chromatograph, equipped with fused silica capillary columns of CP-Sil-43CB. The injector temperature was maintained at 220 °C, with the oven starting at 50 °C (hold 2 min) to 90 °C (20 °C min^{-1}, then held for 1 min), 180 °C (5 °C min^{-1}, then held for 2 min) and to 220 °C (3 °C min^{-1}, then held for 5 min). Helium was used as carrier gas at a flow rate of 1.0 ml min^{-1}. Partially *O*-methylated alditol acetates were identified from m/z of their positive ions, by comparison with standards, the results being expressed as a relative percentage of each component (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005).

2.6. Nuclear magnetic resonance (NMR) spectroscopy

Mono- (^{13}C, ^{1}H and DEPT) and bidimensional NMR spectra (HMQC, COSY, and coupled HMQC) were prepared using a 400 MHz Bruker model DRX Avance spectrometer incorporating Fourier transform. Analyses were carried out at 70 °C on samples dissolved in D_2O. Chemical shifts are expressed in δ relative to acetone at δ 32.77 (^{13}C) and 2.21 (^{1}H), based on DSS (2,2-dimethyl-2-silapentane-5-sulfonate-d_6 sodium salt; $\delta = 0.0$ for ^{13}C and ^{1}H).

2.7. Biological activity

2.7.1. Experimental animals

Male Swiss mice (25–35 g) provided from Universidade Federal do Paraná (UFPR) and Universidade Federal de Santa Catarina (UFSC) facilities were kept in a temperature controlled room (20 ± 2 °C) on a 12-h light–dark cycle (light on from 6:00 h). Food and water were freely available. The experiments were performed after approval of the protocol by the Institutional Ethics Committee of the UFPR (sepsis model, CEUA/BIO-UFPR, protocol number 529) and the UFSC (formalin model, CEUA-UFSC, protocol number PP00682). All experiments were conducted in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for the investigation of experimental pain in conscious animals (Zimmermann, 1983). The numbers of animals and

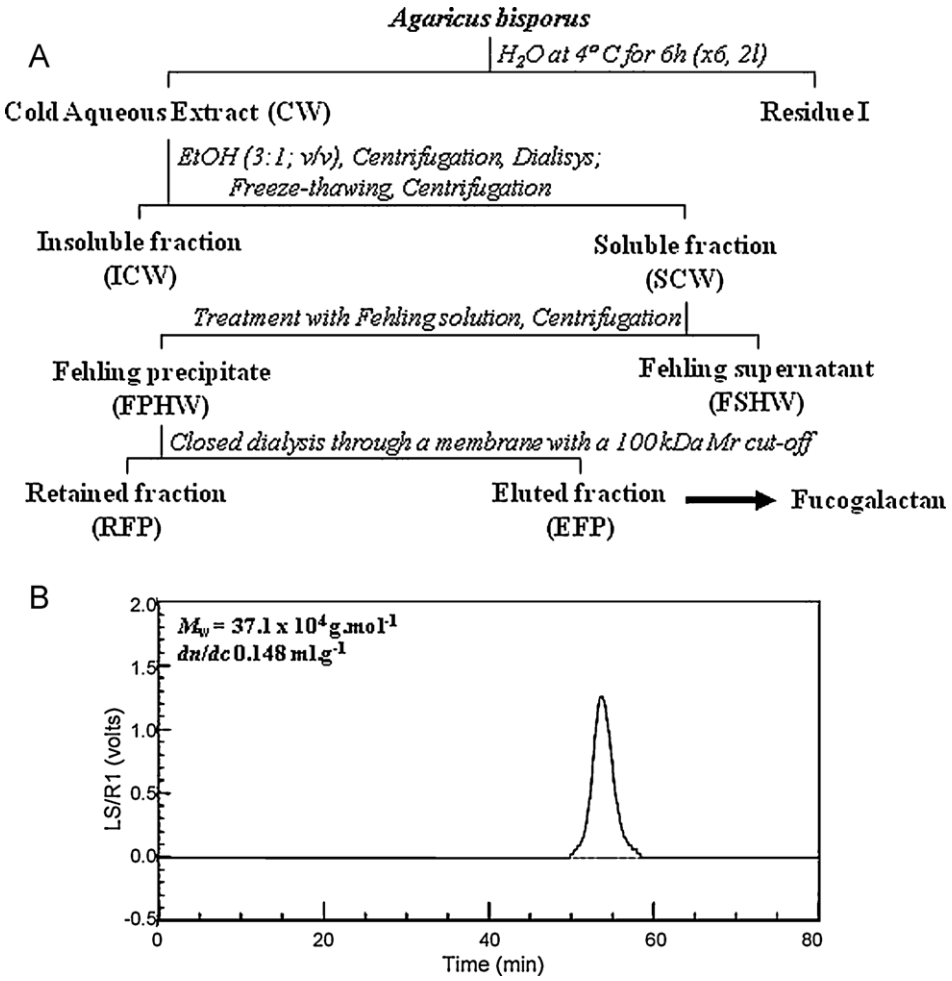

Fig. 1. (A) Scheme of extraction and purification of *A. bisporus* (Ab) fucogalactan. (B) Elution profile of fraction EFP-Ab determined by HPSEC using light scattering (···) and refractive index detectors (–).

intensities of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the drug treatments.

2.7.2. Nociception induced by intraplantar injection of formalin

The procedure used was similar to a previously described protocol (Santos & Calixto, 1997). The mice received 20 μl of a 2.5% formalin solution (0.92% formaldehyde) in saline via an intraplantar (i.pl.) injection in the ventral surface of the right hindpaw. Animals were pretreated with vehicle (saline, 10 ml kg^{-1}, i.p.), EFP-Ab (10, 30 and 100 mg kg^{-1}) or dexamethasone (0.5, 5 and 10 mg kg^{-1}) 30 min before the formalin injection. Following the intraplantar injection of formalin, the mice were immediately placed in a glass cylinder (20 cm diameter), and the time spent licking the injected paw was recorded with chronometer for both the early neurogenic phase (0–5 min) and late inflammatory phase (15–30 min) of this model. These values were considered measures of nociception.

2.7.3. Procedure to induce sepsis by cecal ligation and puncture (CLP)

Mice were randomly divided into five groups with 10 mice/group: sham-operation, CLP plus saline (10 ml kg^{-1} s.c.), CLP plus EFP-Ab (30 mg kg^{-1} s.c.), CLP plus EFP-Ab (100 mg kg^{-1} s.c.) and CLP plus dexamethasone (0.5 mg kg^{-1} s.c.). Saline was used as vehicle for dissolving the polysaccharide; and dexamethasone was commercially purchased. It was administered 60 μl of each treatment solution, regarding the corporeal weigh of the animals (~30 g). Ketamine (80 mg kg^{-1}) and xylazine (20 mg kg^{-1}) were injected intraperitoneally to anesthetize the mice before the surgical procedures. Polymicrobial sepsis was induced by CLP as previously described (Rittirsch, Huber-Lang, Flierl, & Ward, 2009). A midline incision about ~1.5 cm was performed on the abdomen. The cecum was carefully isolated and the distal 50% was ligated. The cecum was then punctured twice with a sterile 16-gauge needle and squeezed to extrude the fecal material from the wounds. The cecum was replaced and the abdomen was closed in two layers. Sham-control animals were treated identically, but no cecal ligation or puncture was carried out. Each mouse received subcutaneous sterile saline injection (1 ml) for fluid resuscitation after surgery. The mice were then placed on a heating pad until they recovered from the anesthesia. Food and water ad libitum were provided throughout the experiment. Survival was monitored for 7 days, each 12 h. During this period, saline and drugs were subcutaneously administered daily.

In other experiments, during the surgery, mice were treated subcutaneously with saline, EFP-Ab or dexamethasone. After 6 h post-operation, mice were sacrificed. Their tissues from lungs and small intestines (ileum) were collected and frozen for later use to determine the myeloperoxidase (MPO) activity and iNOS and COX-2 expression.

2.7.4. Lung MPO activity

MPO activity, assessment measure of neutrophil influx, was determined according to established protocols (Bradley, Priebat, Christensen, & Rothstein, 1982). Briefly, lung tissue was

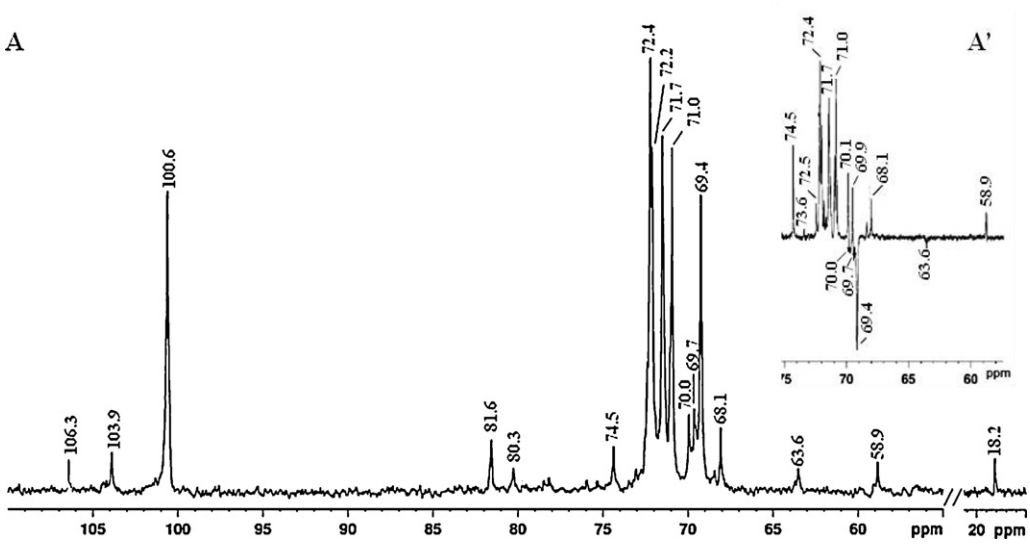

Fig. 2. ^{13}C NMR spectrum of the fucogalactan EFP-Ab (A) with insert of its DEPT —CH_2 inversion (A′), in D_2O at 70 °C (chemical shifts are expressed in δ ppm).

homogenized in 0.5 ml of 50 mM potassium buffer pH 6.0 with 0.5% hexadecyltrimethylammonium bromide, sonicated on ice, and then centrifuged at 14,000 rpm at 4 °C for 15 min. Supernatants were then assayed at a 1:20 dilution in reaction buffer (9.6 mM 3,3,5,5-tetramethylbenzine, 150 nmol l^{-1} H_2O_2 in 50 mM potassium phosphate buffer), and read at 620 nm. Results are expressed as change in optical density per milligram of protein (measured by Bradford assay).

2.7.5. Western blot analysis

The ileum samples were washed twice with PBS and then homogenated and lysed in extraction buffer [composition in mM: Tris/HCl 20 (pH 7.5; QBiogene), NaCl 150, Na_3VO_4, sodium pyrophosphate 10, NaF 20, okadaic acid 0.01 (Sigma), a tablet of protease inhibitor (Roche) and 1% Triton X-100 (QBiogen)]. Total protein (20 μg) was separated on 8% SDS–polyacrylamide (Sigma) gels at 80 V for 2 h. Isolated proteins were transferred electrophoretically onto polyvinylidine difluoride membranes (Bio-Rad) at 100 V for 120 min. Membranes were blocked with blocking buffer containing 3% low fat milk powder, Tris-buffered saline solution (Bio-Rad) and 0.1% Tween 20 (Sigma) (TBS-T) for 1 h. Membranes were then incubated with the primary antibodies of either iNOS or COX-2 (dilution of 1:1000), overnight at 4 °C. After washing, membranes were incubated with the secondary antibody (peroxidase-labeled anti-mouse IgG, dilution of 1:5000) at room temperature for 60 min. Detection of β-tubulin proteins was used for normalization and quantification of iNOS and COX-2. Prestained markers (Invitrogen) were used for molecular mass determinations. Immunoreactive bands were detected by enhanced chemiluminescence (Bio-Rad).

2.7.6. Statistical analysis

Data are presented as means ± standard error of the mean (S.E.M.), except for the ID_{50} values (i.e., the dose of EFP-Ab necessary to reduce the nociceptive response by 50% relative to the control value), which are reported as geometric means accompanied by their respective 95% confidence limits. The ID_{50} values were determined by nonlinear regression from individual experiments using linear regression with GraphPad software (GraphPad software, San Diego, CA, USA). Comparisons between experimental and control groups were performed by one-way analysis of variance (ANOVA) followed by Bonferroni's or Newman–Keuls' tests. *P* values less than 0.05 were considered as indicative of significance.

3. Results and discussion

3.1. Fucogalactans structural characterization

In order to obtain pure heteropolysaccharides from the fruiting bodies of *A. bisporus*, they were submitted to aqueous extraction at 4 °C. The extracted polysaccharides were precipitated with excess ethanol, obtained as sediments on ultracentrifugation, dialyzed against tap water, and the solution freeze-dried to give CW-Ab (55.3 g) (Fig. 1).

Fractionation and purification of CW-Ab was carried out by a freeze–thawing procedure, resulting in a respective cold-water soluble SCW-Ab (43.4 g) and insoluble fraction (11.9 g). Fraction SCW-Ab contained fucose, xylose, galactose, glucose and 3-*O*-methyl-galactose (confirmed by the presence of the fragments *m*/*z* 130 and 190, after hydrolysis, reduction with NaB_2H_4 and acetylation) (Ruthes et al., 2012), but gave a heterogeneous HPSEC elution profile, so it was then treated with Fehling solution. Its respective insoluble Cu^{2+} complex FP-Ab (2.1 g) was further purified by closed dialysis through a 100 kDa M_r cut-off membrane. This procedure gave rise to a retained RFP-Ab (0.2 g) and an eluted EFP-Ab (1.9 g) fraction, respectively (Fig. 1A). The EFP-Ab fraction was homogeneous on HPSEC, and had M_w 37.1 × 10^4 g mol^{-1} (*dn*/*dc* 0.148 ml g^{-1}) (Fig. 1B).

As well as previously described for fraction RFP-Ab (Ruthes et al., 2012), EFP-Ab also consists of a fucogalactan, composed of fucose (11.2%), galactose (74.2%) and 3-*O*-methyl-galactose (14.6%).

EFP-Ab was submitted to methylation analysis, where GC–MS of resulting *O*-methylalditol acetates showed the presence of a branched structure, containing mainly, nonreducing end-units of Fuc*p* (2,3,4-Me_3Fuc; 13.1%), besides the 6-*O*-(2,3,4-Me_3Gal; 65.6%) and 2,6-di-*O*-substituted units (3,4-Me_2Gal; 17.4%) of D-galactopyranose. Small amounts of non-reducing end-units of Gal*p* (2,3,4,6-Me_4Gal; 3.9%) was also observed in the EFP-Ab fraction. The ratio of units of Fuc*p*: 2,6-di-*O*-Gal*p*: 6-*O*-Gal*p* was ~1:1:5 for EFP-Ab from *A. bisporus*. This relation was also confirmed by the integration of H-1 signals (5.10:5.06:5.01) present in the ^{1}H NMR spectrum (data not shown).

In order to elucidate the fucogalactan (EFP-Ab) structure, additional NMR spectroscopy [^{1}H, ^{13}C and DEPT (Fig. 2A and A′,

Table 1
^{1}H and ^{13}C NMR chemical shifts [expressed as δ (ppm)] of *A. bisporus* fucogalactan (EFP-Ab).[a]

Units		1	2	3	4	5	6		*O*—CH_3
							6a	6b	
α-Fuc*p*-(1→	^{13}C	103.9	72.0	71.2	74.5	69.9	18.2	–	–
	^{1}H	5.10	3.85	3.84	3.85	4.20	1.27	–	–
β-Gal*p*-(1→	^{13}C	106.3	73.6	76.1	71.1	78.3	63.7	–	–
	^{1}H	5.01	3.90	3.79	4.08	3.58	3.80	–	–
2,6→)-α-Gal*p*-(1→	^{13}C	100.7	80.3	71.1	71.2	71.7	69.7	69.7	–
	^{1}H	5.06	3.84	4.08	4.09	4.21	3.70	4.01	–
6→)-α-Gal*p*-(1→	^{13}C	100.7	71.1	72.4	72.4	71.7	69.4	69.4	–
	^{1}H	5.01	3.84	3.89	4.03	4.21	3.72	3.93	–
6→)-3-*O*-Me-α-Gal*p*-(1→	^{13}C	100.6	70.1	81.6	68.2	71.7	69.4	69.4	58.9
	^{1}H	5.01	3.88	3.58	4.30	4.21	3.72	3.93	3.43

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

respectively), HMQC (Fig. 3), COSY and coupled HMQC (data not shown)] analysis was also useful (Table 1). ^{13}C NMR (Fig. 2A) and HMQC spectrum (Fig. 3), both obtained using D_2O as solvent at 70 °C, have shown C-1/H-1 signals at δ 106.3/4.64, 103.9/5.10, 100.7/5.06, 100.7/5.01, 100.6/5.01, which could be attributed to β-Gal*p*, Fuc*p*, Gal*p* 2,6-di-*O*- and 6-*O*-substituted, and 3-*O*-Me-Gal*p* units, respectively.

The glycosidic configuration was confirmed by the coupling constants values $J_{C\text{-}1/H\text{-}1}$ found in $^{1}H/^{13}C$-coupled HMQC spectrum. The nonreducing end- of Fuc*p* and the main-chain units (Gal*p* or 3-*O*-Me-Gal*p* or both) had the α-configuration due to respective $J_{C\text{-}1/H\text{-}1}$ 162.6 Hz and 172.6 Hz, while those of nonreducing end-units of Gal*p* from EFP-Ab had β-configuration, consistent with $J_{C\text{-}1/H\text{-}1}$ 161.3 Hz (Perlin & Casu, 1969).

The presence of 6-*O*- and 2-*O*-substituted Gal*p* units as indicated by methylation analysis was confirmed by NMR spectroscopy. *O*-Substituted C-2 signal could be observed at δ 80.3 (Figs. 2 and 3), while signals of —CH_2 groups of the 6-*O*-(Gal*p* and 3-*O*-Me-Gal*p*) and 2,6-di-*O*-subst. (Gal*p*) units of the main chain appears at δ 69.4/69.7 and 70.0, respectively, giving rise to inverted signals in the DEPT spectrum (Fig. 2A′). The presence and position of *O*-methyl groups of the *A. bisporus* fucogalactan was confirmed by the presence of signal at δ 58.9/3.43 and δ 81.6/3.58 (C/H) corresponding to —OCH_3 and *O*-substituted C-3 substituted/H-3, respectively (Figs. 2 and 3 and Table 1).

The signals at δ 72.0/3.85, 71.2/3.84, 74.5/3.85, 69.9/4.20 and *d* 18.2/1.27 could be attributed to C-2/H-2 to C-6/H-6 of Fuc*p* units, respectively, while those at δ 70.1/3.88, 81.6/3.58, 68.2/4.30, 71.7/4.21 and *d* 69.4/69.7/3.72/3.93 were from C-2/H-2 to C-6/H-6 of 3-*O*-Me-Gal*p* units (Figs. 2 and 3 and Table 1). β-Gal*p* units also have their C-2/H-2 to C-6/H-6 signals attributed to those at δ 73.6/3.90, 76.1/3.79, 71.1/4.08, 78.3/3.58 and 63.7/3.80, respectively (Table 1).

EFP-Ab fucogalactan analysis showed it to consist essentially of a (1→6)-linked α-D-galactopyranosyl main-chain, partially substituted at O-2 by nonreducing end-units of α-L-Fuc*p*. Although its main chain showed to be partially *O*-methylated and had some β-D-Gal*p* nonreducing end-units as substituents.

Fucogalactans similar to those now described have been isolated from cultivated mycelium of *Coprinus comatus* (Fan et al., 2006), fruiting bodies of *Sarcodon aspratus* (Mizuno et al., 2000), *Hericium erinaceus* (Zhang et al., 2006), *A. brasiliensis* and *A. bisporus* var. *Hortensis* (Komura et al., 2010) and from *Lactarius rufus* and *A. bisporus* (Ruthes et al., 2012). The fucogalactana described in the present work shows a great similarity to the previously one described for the same mushroom (Ruthes et al., 2012). However, it has a lower content of nonreducing end-units of β-Gal*p*. In this way, EFP-Ab fucogalactan now characterized appears more similar with that from *A. bisporus* var. *hortensis* (Komura et al., 2010).

Since *A. bisporus* var. *hortensis* represents *A. bisporus* when mature, commonly known as Portobello mushroom, one may suggest that the fucogalactan as described in this paper is present in both the young stage of the mushroom, and in its mature phase, i.e., its structure is not affected by *A. bisporus* fruiting body maturation.

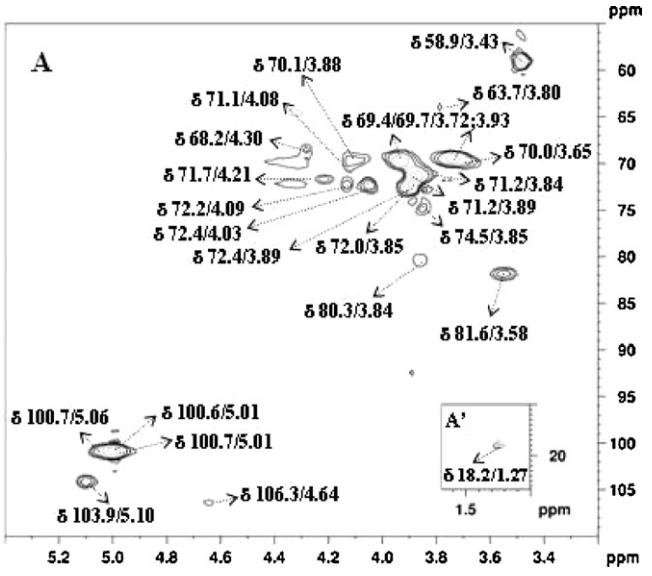

Fig. 3. ^{1}H (obs.)/^{13}C HMQC spectrum of *A. bisporus* (A) fucogalactan EFP-Ab, in D_2O at 70 °C. Insert of CH_3-6 region from Fuc*p* units (A′) (chemical shifts are expressed in δ ppm).

3.2. Biological activities of EFP-Ab

It was previously demonstrated that fucogalactans exhibited protective effects against sepsis and antinociceptive and anti-inflammatory activities in mice (Komura et al., 2010; Ruthes et al., 2012). In this study, we evaluated if the fucogalactan isolated from *A. bisporus* also presented these properties.

In the present study, we demonstrated that EFP-Ab has a significant antinociceptive effect on formalin-induced pain in mice, a classical chemical model of nociception. The results reported here indicate that intraperitoneal administration of EFP-Ab produced significant inhibition of both the neurogenic (0–5 min) and inflammatory (15–30 min) phases of formalin-induced licking. However, the antinociceptive effect was significantly more pronounced against the second phase of this model of pain (Fig. 4A and B). The mean ID_{50} values calculated for these effects were >100

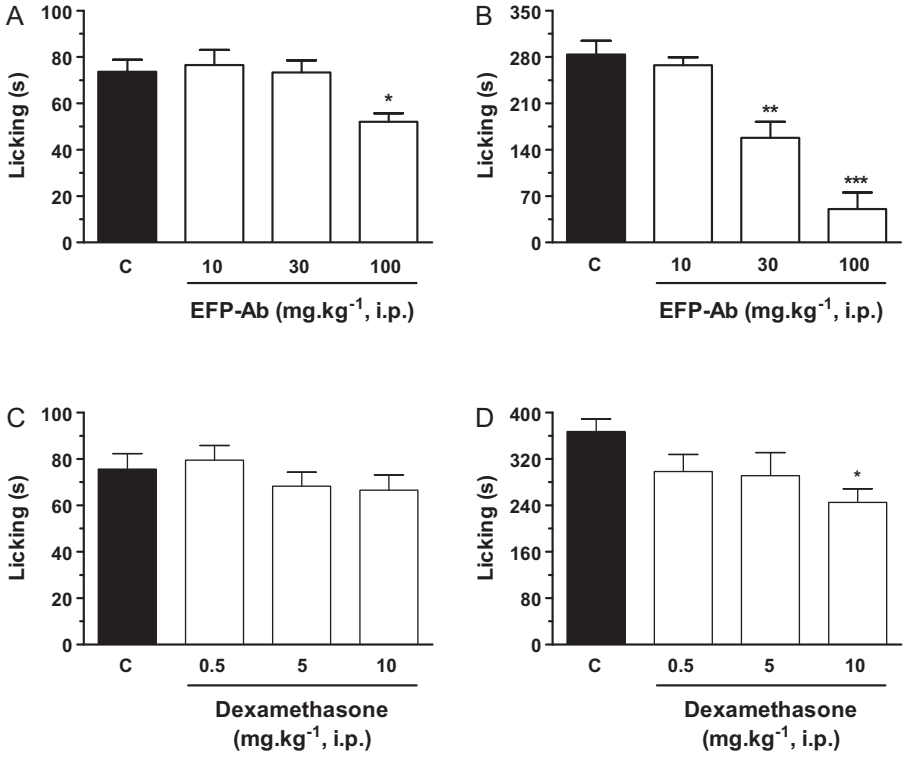

Fig. 4. Effect of EFP-Ab (panels A and B) or dexamethasone (panels C and D) administered by intraperitoneal (i.p.) injection against formalin-induced nociception (neurogenic phase, panels A and C, and second phase, panels B and D) in mice. Each group represents the mean of 6–8 animals and the error lines indicate the S.E.M. The control group (C, black bars), indicates the mice treated by intraperitoneal (i.p.) injection with vehicle (10 ml kg^{-1}) before formalin. The symbols $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ denote the significance level when compared to the control group. One way ANOVA followed by the Newman–Keuls' test.

and 36.0 (25.8–50.3) mg kg^{-1} and the inhibitions observed were 29 ± 5% and 82 ± 9% at a dose of 100 mg kg^{-1}, for neurogenic and inflammatory phases, respectively (Fig. 4A and B).

The formalin test is a satisfactory and comprehensive model for evaluating the antinociceptive activity of drugs. The intraplantar injection of formalin activates nociceptive nerve terminals and produces neurogenic pain, whereas inflammatory pain is mediated by a combination of peripheral input and spinal cord sensitization (Hunskaar & Hole, 1987; Tjølsen, Berge, Hunskaar, Rosland, & Hole, 1992). Moreover, it has been demonstrated that the intraplantar injection of formalin in rodents increases spinal levels of excitatory amino acids, PGE_2, nitric oxide, tachykinin, kinins, among other peptides (Malmberg & Yaksh, 1995; Santos & Calixto, 1997; Santos, Vedana, & De Freitas, 1998; Tjølsen et al., 1992). Experimental data indicate that formalin predominantly evokes activity in C-fibers peroxidase (Tjølsen et al., 1992), although Aδ-fibers are thought to be responsible for fast nociceptive transmission in the first phase of the pain response (Julius & Basbaum, 2001). It is notable that the nociception produced by formalin (neurogenic phase) is quite resistant to the majority of NSAIDs, such as acetylsalicylic acid, indomethacin, paracetamol, and diclofenac. However, these drugs can dose-dependently attenuate the inflammatory phase of formalin-induced licking (Hunskaar & Hole, 1987; Malmberg & Yaksh, 1992; Santos et al., 1998). Here, we also demonstrated that dexamethasone, a synthetic glucocorticoid, was only able to significantly inhibit (33 ± 6%) inflammatory pain induced by formalin in high dose (10 mg kg^{-1}) (Fig. 4C and D). Thus, our study provides evidence that EFP-Ab inhibited both phases of formalin test but was more effective against the inflammatory pain, which is different from the effect induced by dexamethasone; this data led us to investigate its anti-sepsis effect.

It is believed that sepsis may lead to aberrant host inflammatory responses, causing cell injury and organ dysfunction. Furthermore, neutrophil infiltration is an important pathophysiologic alteration associated with sepsis and these cells cause directly damage to the tissues by releasing pro-inflammatory mediators, as cytokines, superoxide-derived free radicals and lysosomal enzymes, such as MPO, which amplify the systemic inflammatory response and cause multiple organ failure (Landry & Oliver, 2001). In the present study, a polymicrobial sepsis was induced by cecal ligation and puncture (CLP) in mice to investigate the effects of EFP-Ab. The results of the survival experiments are shown in Fig. 5A. Mice treated with vehicle started to die between 12 h and 24 h after CLP, with a death rate reaching 37.8% and 87.5% after 24 h and 96 h post-CLP, respectively. The overall mortality in this group, at the end of the observation period, was 100%, and the corresponding area under the curve was 3.720 (arbitrary units). The lethality was markedly delayed in mice treated subcutaneously with EFP-Ab. Their areas under the curve were increased to 9.060 and 11.160 after treating with EFP-Ab 30 and 100 mg kg^{-1}, respectively. At the end of the study, the overall survival in these EFP-Ab groups was 30% and 40%. Dexamethasone-treated mice showed a significant improvement in survival (area under curve 10.100), with an overall survival rate of 16.7% at the end of the observation period. No death occurred in the sham-operated mice and its corresponding value for area under the lethality curve was 16.800 (arbitrary units). This result could be attributable to an anti-inflammatory activity, which is consistent with previous study provided by our group (Komura et al., 2010; Ruthes et al., 2012).

As commented above, MPO is a lysosomal enzyme, produced by polymorphonuclear leukocytes, and related to the production of hypochlorous acid (powerful oxidant). For this reason, the effects of EFP-Ab treatment on MPO activity were also investigated. Sepsis CLP surgery markedly increased lung tissue MPO levels compared with Sham group (45%) (Fig. 5B). This increasing in tissue MPO was significantly prevented by EFP-Ab 30 and 100 mg kg^{-1}, with an inhibition of 17.5% and 39.8%, respectively, vs. vehicle group.

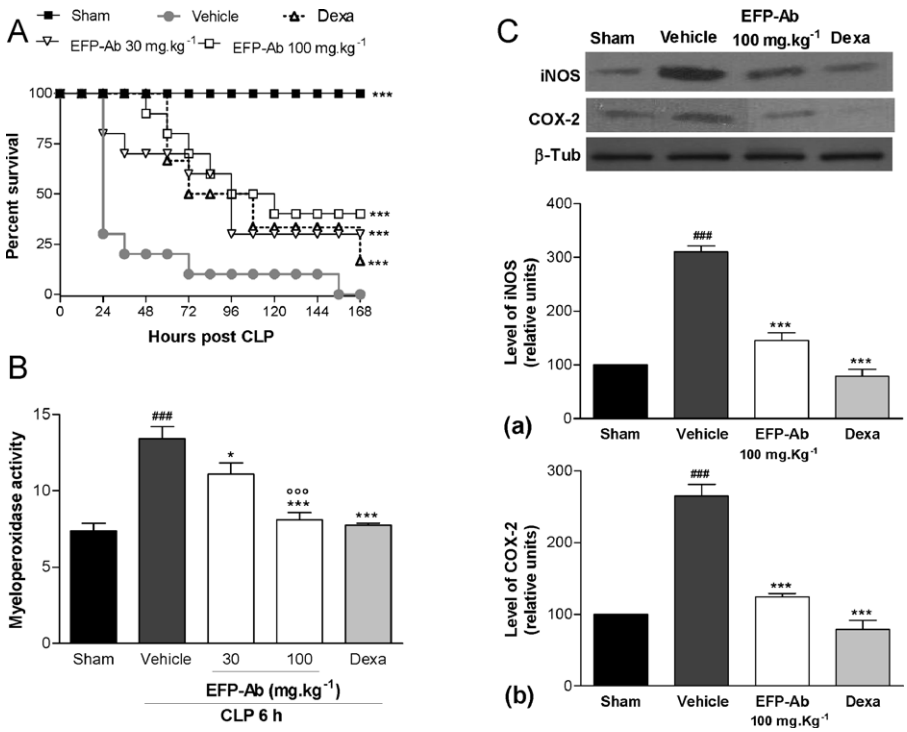

Fig. 5. EFP-Ab protects against sepsis-induced lethality (A), inhibits myeloperoxidase activity (B) and reduces iNOS and COX-2 tissue expression, determined by Western blot (C). The panels (a) and (b) are corresponding to the representative immunoblots iNOS and COX-2, respectively. Mice were subcutaneously administered EFP-Ab (30 and/or 100 mg kg^{-1}), vehicle (saline) or dexamethasone (0.5 mg kg^{-1} s.c.). Values represent means ± S.E.M. of 4–10 animals/group. $^{*}p < 0.05$ and $^{***}p < 0.001$ versus vehicle; $^{###}p < 0.001$ versus sham; $^{\circ\circ\circ}p < 0.001$ versus EFP-Ab 30 mg kg^{-1}. ANOVA followed by Bonferroni's test.

Dexamethasone, the anti-inflammatory drug used as positive control, strongly inhibited the MPO activity in lungs (42.3%). Therefore, it was observed that EFP-Ab prevented the elevation of MPO activity, indirectly indicating reductions in both neutrophil recruitment to lungs and in oxidative tissue damage. This is of particular relevance because oxidative stress is known as a probable mechanism for gut mucosal barrier dysfunction during sepsis condition, amplifying and perpetuating the initial systemic inflammatory responses (Pastores, Katz, & Kvetan, 1996).

In this study, we also examined the effects of EFP-Ab on iNOS and COX-2 expression in ileum of septic mice by immunoblotting. EFP-Ab 100 mg kg^{-1} decreased both iNOS and COX-2 expression by 53% and 54%, respectively [Fig. 5C, panels (a) and (b)]. Dexametasone also affected both iNOS and COX-2 expression, reducing by 74.5% and 71.4%, respectively. These results strongly confirmed the anti-inflammatory activity of EFP-Ab.

The enzymes iNOS and COX-2 are both upper expressed during pro-inflammatory events including sepsis. The iNOS (inducible nitric oxide synthase), once expressed, produces high amounts of NO over long periods of time, which causes cellular damage. It is also associated with the septic shock, considered the main cause of mortality among the septic patients (Landry & Oliver, 2001). Compounds that inhibit iNOS expression or iNOS activity have anti-inflammatory properties (Tinker & Wallace, 2006). In turn, COX-2 is the inducible isoform of the cyclooxygenase enzyme that catalyzes the production of inflammatory prostanoids. Systemic COX-2 is increasingly recognized as an important player in sepsis-induced inflammation. In fact, COX-2-deficient mice are protected from sepsis-induced inflammation and death (Ejima et al., 2003).

4. Conclusions

In conclusion, we observed that EFP-Ab presented antinociceptive and anti-inflammatory activities against formalin-induced nociception with more pronounced effect on inflammatory phase. In addition, EFP-Ab can prevent the lethality caused by polymicrobial sepsis in mice. This beneficial effect seems to be, at least in part, due to a reduction in neutrophil infiltration with consequent protection against tissue damage, and also due to an inhibition on iNOS and COX-2 tissue expression.

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