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Lentinus edodes heterogalactan: Antinociceptive and anti-inflammatory effects

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ABSTRACT

Cold aqueous extraction of basidiocarps (fruiting bodies) of the edible mushroom *Lentinus edodes* (*shiitake*) gave rise to a heteropolysaccharide, whose chemical structure, antinociceptive and anti-inflammatory properties were determined. Its chemical structure was based on monosaccharide composition, methylation analysis, and NMR spectroscopy (^{1}H, ^{13}C, HSQC, HSQC-TOCSY, HSQC-NOESY, and coupled HMQC). It was found to be a fucomannogalactan with a main chain of (1 → 6)-linked α-D-galactopyranosyl units, partially substituted at O-2 by single-unit β-D-Man*p* or α-L-Fuc*p* side chains. The polysaccharide produced a marked and dose-related effect when assessed against acetic acid-induced visceral nociception. Prevention of peritoneal capillary permeability and leukocyte infiltration caused by the acetic acid was similar in potency and effectiveness.

1. Introduction

Mushrooms are rich in dietary fiber, minerals, and vitamins and low in fat (Manzi, Aguzzi, & Pizzoferrato, 2001). They have recently become attractive as food (physiologically functional) and as sources for the development of drugs (Manzi & Pizzoferrato, 2000). Some mushroom components can lower cholesterolemia, modulate the immune system, and inhibit tumor growth (Smith, Rowan, & Sullivan, 2002; Wasser, 2002). Extracts of many mushrooms, including *Lentinus edodes*, *Agaricus blazei*, *Ganoderma lucidum*, and *Grifola frondosa*, suppress tumor growth by controlling the immune system of the host (Schepetkin & Quinn, 2006). In the global market, *L. edodes* (*shiitake*) is the second most popular edible mushroom, its importance being attributed to both its nutritional value and medical application (Hatvani, 2001). Several important components, including biologically active polysaccharides (lentinan), dietary fiber, ergosterol, vitamin B1, B2 and C, and minerals have been isolated from its basidiocarp, mycelium, and culture medium (Choi et al., 2006). Lentinan, a β-glucan, is the most important polysaccharide isolated from *L. edodes,* because of its immunomodulatory and antitumor effects. Related structures that also have antitumor activity have been isolated from *G. frondosa* (grifolan) (Kato et al., 1983) and *Schizophyllum commune* (schizophyllan) (Tabata, Ito, & Kojima, 1981). These polysaccharides are now used in clinics in Japan, Korea, China, and other Asian countries (Zaidman, Yassin, Mahajna, & Wasser, 2005). Other important polysaccharides isolated from mushrooms are heteropolymers, such as heterogalactans, which, usually, have a main chain of (1→6)-linked α-D-galactopyranose that can be substituted at O-2 by L-Fuc*p*, 3-*O*-D-Man*p*-L-Fuc*p* or D-Man*p* groups. These structures occur in several basidiomycetes, namely *Flammulina velutipes*, *Ganoderma applanatum, Laetiporus sulphureus, Coprinus comatus*, among others (Alquini, Carbonero, Rosado, Cosentino, & Iacomini, 2004; Fan et al., 2006; Mukumoto & Yamaguchi, 1977; Usui, Iwasaki, & Mizuno, 1983).

Besides antitumor activity, anti-inflammatory effects have been reported. Glucans of *Trametes gibbosa* and *Dictyophora indusiata* were active *in vivo* against inflammation induced by carrageenan (Hara, Kiho, Tanaka, & Ukai, 1982; Poucheret, Fons, & Rapior, 2006). However, most of the investigations on anti-inflammatory action of mushrooms were carried out with crude polysaccharide extracts (Lindequist, Niedermeyer, & Jülich, 2005; Poucheret et al., 2006). We now determine the detailed chemical structure of a pure fucomannogalactan from basidiocarps of *L. edodes*, and

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investigate its antinociceptive and potential anti-inflammatory properties, using a model of inflammatory pain in mice.

2. Materials and methods

2.1. General experimental procedures

Gas liquid chromatography–mass spectrometry (GC–MS) was performed using a Varian (model 3300) gas chromatograph linked to a Finnigan Ion-Trap model 810 R-12 mass spectrometer, with He as carrier gas. A capillary column (30 m × 0.25 mm i.d.) of DB-225, held at 50 °C during injection and then programmed at 40 °C min^{-1} to 220 °C or 210 °C (constant temperature) was used for quantitative analysis of alditol acetates and partially *O*-methylated alditol acetates, respectively.

Ultrafiltration was performed on a filter holder (Sartorius – Model 16249), with compressed air at 10 psi as carrier gas.

NMR spectra (^1H, ^{13}C, DEPT, HSQC, HSQC-TOCSY, HSQC-NOESY, and coupled HMQC) were obtained using a 600 MHz Bruker Avance spectrometer incorporating Fourier transform. Analyses were performed at 40 °C on a polysaccharide sample dissolved in D_2O. Chemical shifts are expressed in δ relative to acetone at δ 32.77 (^{13}C) and 2.21 (^1H), referred to DSS (2,2-dimethyl-2-silapentane-3,3,4,4,5,5-d_6-5-sulfonate sodium salt; δ = 0.0 for ^{13}C and ^1H), in accordance with IUPAC recommendations.

2.2. Biological material

Fresh *L. edodes* (2 kg) was furnished by Makoto Yamashita Company (Miriam Harumi Yamashita), located in São José dos Pinhais, State of Paraná, Brazil. The basidiocarps were grown on sweet chestnut (*Castanea sativa*) logs.

2.3. Extraction and purification of the polysaccharide

Fresh basidiocarps of *L. edodes* (2 kg) were freeze-dried, resulting in 162 g of material, which were pulverized and their polysaccharides were extracted with water at 4 °C for 6 h (×5, 2000 ml). Each extract was filtered; the filtrate was collected, and centrifuged at 9000 rpm at 20 °C for 20 min, giving a clear solution. The combined aqueous extracts were evaporated to a small volume, followed by addition of excess EtOH (3:1; v/v). The polysaccharide precipitate was collected by centrifugation at 8500 rpm at 10 °C for 20 min, and was dissolved in H_2O, dialyzed against distilled water for 20 h to remove low-molecular-weight material, and freeze-dried to give fraction CW. This was then dissolved in distilled water and the solution submitted to freezing followed by mild thawing at 4 °C, which furnished cold water-soluble (SCW) and insoluble fractions (ICW), which were separated by centrifugation (8500 rpm at 4 °C for 20 min). The soluble portion (SCW) was treated with Fehling solution and precipitated Cu^{2+} complex was centrifuged off. This was neutralized with aq. HOAc, dialyzed against tap water, deionized with mixed ion exchange resins, and freeze-dried. The product was further purified by ultrafiltration through a 300 kDa M_r cut-off membrane (Millipore; polyethersulfone), giving rise to retained (EFPCW) and eluted material (FMG).

2.4. Monosaccharide composition of polysaccharide fractions

Monosaccharide components of the polysaccharide fractions were identified and their ratios were determined following hydrolysis with 1 M TFA for 8 h at 100 °C. The resulting aldose mixtures were converted to alditol acetates (GC–MS) by successive $NaBH_4$ and/or NaB^2H_4 reduction, and acetylation with Ac_2O-pyridine (1:1, v/v) for 12 h at room temperature.

2.5. Determination of homogeneity of polysaccharide fractions and the molecular weight of FMG

Determination of homogeneity and molar mass (M_w) 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 detector (MALLS). Waters Ultrahydrogel columns (2000, 500, 250 and 120) were connected in series and coupled with multidetection equipment, using a $NaNO_2$ solution (0.1 M) as eluent, containing 0.5 g/l NaN_3. The polysaccharide solutions (1 mg/ml) were dissolved in the same solvent and filtered through a nitrocellulose membrane (Millipore), with pores of 0.22 or 0.45 μm. HPSEC data were collected and analyzed by the Wyatt Technology ASTRA program. The specific refractive index increment (d*n*/d*c*) was determined using a Waters 2410 detector. All experiments were carried out at 25 °C.

2.6. Methylation analysis

Per-*O*-methylation of the fucomannogalactan (FMG) was carried out by the method of Ciucanu and Kerek (1984). The sample (10 mg) was dissolved in dimethyl sulfoxide (1 ml), and powdered NaOH (20 mg) and iodomethane (CH_3I) (1 ml) were added. After 30 min at 25 °C with vigorous stirring, the mixture was maintained overnight at 25 °C. The reaction was interrupted by addition of water, neutralization with HOAc, dialysis against distilled water and freeze-drying. The products were submitted to one more cycle of methylation, and the products were isolated by partition between $CHCl_3$ and water. The per-*O*-methylated derivatives from the lower layer were hydrolyzed with 45% aqueous formic acid (1 ml) for 6 h at 100 °C, followed by NaB^2H_4 reduction and acetylation as above (item 2.4), to give a mixture of partially *O*-methylated alditol acetates, which was analyzed by GC–MS.

2.7. Absolute configuration of monosaccharides

The enantiomeric configuration of monosaccharides in FMG was determined by reductive amination with chiral 1-amino-2-propanol in the presence of sodium cyanoborohydride, followed by acetylation and GC analysis of the resulting 1-deoxy-1-(2′-hydroxypropylamino)–alditol mixture (Ultra-2 column, Hewlett–Packard) (Cases, Cerezo, & Stortz, 1995).

2.8. Experimental animals

Male Swiss mice (25–35 g) were kept in an automatically controlled temperature room (23 ± 2 °C) in 12 h light–dark cycles, with water and food freely available. Animals were acclimatized to the laboratory for at least 2 h before testing and were used only once. The experiments were performed following the protocol, approved by the Institutional Ethics Committee of the Federal University of Santa Catarina (UFSC), carried out in accordance with current protocols for the care of laboratory animals and ethical guidelines for investigation of experimental pain in conscious animals (Zimmermann, 1983). The numbers of animals and intensities of noxious stimuli were the minimum necessary to demonstrate consistent effects of the drug treatments.

2.9. Abdominal constriction, peritoneal capillary permeability, and leukocyte infiltration caused by intraperitoneal injection of 0.6% acetic acid

Abdominal constrictions in mice were induced according to a previously described procedure (Lucena et al., 2007), which in response to the intraperitoneal injection (i.p.) of acetic acid (0.6%),

resulted in contraction of the abdominal muscle and stretching of the hind limbs.

This was preceded by intravenous treatment with a 2.5% Evans blue solution (10 ml/kg), used as a peritoneal capillary permeability marker. One hour later, they received FMG (3–100 mg/kg) by the intraperitoneal route 30 min prior to the acetic acid injection. Control animals received a similar volume of saline solution (10 ml/kg, i.p.) used to dilute the FMG. After the challenge, the mice were placed individually into glass cylinders of 20 cm diameter, and the abdominal constrictions were counted cumulatively over a period of 20 min. Antinociceptive activity is expressed as the reduction in the number of abdominal constrictions [i.e., the difference between control mice (mice pre-treated with saline) and animals pre-treated with FMG]. Immediately after the test, mice were sacrificed by cervical dislocation and the peritoneal cavity was washed with 1 ml of sterile saline plus heparin (25 IU/ml) and the volume collected with automatic pipettes. Total leukocyte counts were performed using a Neubauer chamber via optical microscopy after diluting a sample of the peritoneal fluid with Türk solution (1:20). A sample of the collected fluid (700 μl) was centrifuged at 1000 rpm for 10 min and the absorbance of the supernatant was read at 610 nm with an ELISA analyzer. The peritoneal capillary permeability induced by acetic acid is expressed in terms of dye (μg/ml), which leaked into the peritoneal cavity according to the standard curve of Evans blue dye (Lucena et al., 2007).

2.10. Statistical analysis

Results are expressed as means ± SEM, except that the ID_{50} values (i.e. the dose of FMG reducing the abdominal constriction, peritoneal capillary permeability, and leukocyte infiltration responses by 50%, relative to the control value), are presented as geometric means accompanied by their respective 95% confidence limits. ID_{50} values were determined by linear regression from individual experiments using linear regression GraphPad software (Graph Pad software, San Diego, CA, USA). The statistical significance of differences between groups was detected by ANOVA followed by Newman–Keuls' test. *P*-values less than $P < 0.05$ were considered to be significant.

3. Results and discussion

The basidiocarps of *L. edodes,* a widely popular edible and highly nutritious mushroom, was shown to contain 91.9% moisture on desiccation in a freeze dryer, and the product was submitted to aqueous extraction at 4 °C. Polysaccharides were recovered by ethanol precipitation, and were dialyzed against tap water, and the solution freeze-dried (fraction CW; 10.2% yield based of dried fungus).

For purification, CW was submitted to a freezing/thawing process, which furnished cold water-soluble (SCW; 2.8% yield) and insoluble (ICW; 7.4% yield) polysaccharide fractions, which were separated by centrifugation. SCW contained fucose (6%), xylose (2%), mannose (22%), galactose (35%) and glucose (35%), and HPSEC–MALLS analysis showed it to be heterogeneous. It was then treated with Fehling solution, giving rise to an insoluble Cu^{2+} complex, which was converted to polysaccharide (FPCW; 1.0% yield), which was further fractionated by ultrafiltration (300 kDa M_r cut-off membrane). The eluted fraction (0.9% yield) was homogeneous on HPSEC–MALLS, and had M_w 16.2×10^3 g/mol ($dn/dc = 0.167$ ml/g) and contained fucose (11%), mannose (21%) and galactose (68%) as monosaccharide components, consistent with a fucomannogalactan, named FMG. The enantiomeric configuration of each monosaccharide was determined by GC–MS examination of a mixture of acetylated 1-deoxy-1-(2́-hydroxyamino)-alditols, derived

Table 1
Glycosidic linkages in fucomannogalactan (FMG), shown by methylation analysis

Partially *O*-methylated alditol acetate	Linkage type[a]	R_T (min)	% Area	Ion fragments (*m*/*z*)
2,3,4-Me_3-Fuc	Fuc*p*-(1→	7.80	11	89,101,115, 117,131,161,175
2,3,4,6-Me_4-Man	Man*p*-(1→	9.18	21	87,101,117,129,145,161,205
2,3,4,6-Me_4-Gal	Gal*p*-(1→	9.80	1	87,101,117,129,145,161,205
2,3,4-Me_3-Gal	6→)-Gal*p*-(1→	14.91	34	87,101,117,129,161,173,189,233
3,4-Me_2-Gal	2,6→)-Gal*p*-(1→	21.50	33	87,99,129,159,173,189,233

[a] Based on derived *O*-methylalditol acetate.

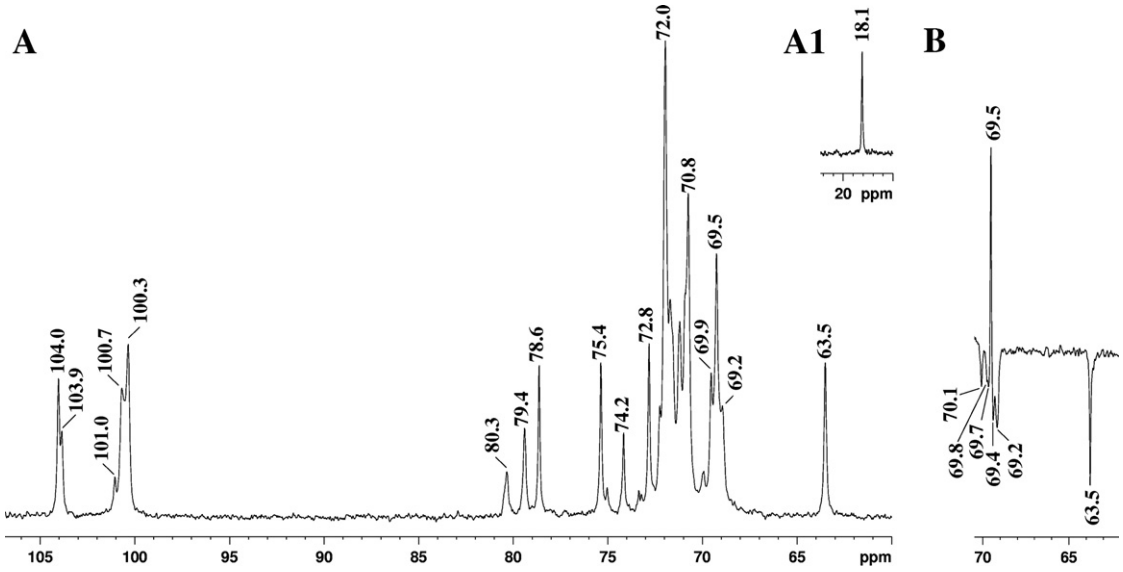

Fig. 1. ^{13}C NMR (A) and 6-CH_2 region of DEPT (B) spectra, obtained at 40 °C, of fucomannogalactan (FMG) in D_2O. Insert of 6-CH_3 region of ^{13}C NMR spectrum (A1).

by reductive amination with (S)-l-amino-2-propanol (Cases et al., 1995). Galactose and mannose residues had the D- and that of fucose an L-configuration.

In order to characterize the glycosidic linkages of FMG, it was submitted to methylation analysis, which showed a highly branched structure, containing non-reducing end units of Fuc*p* (11%), Man*p* (21%), and Gal*p* (1%), besides 6-*O*-substituted (34%) and 2,6-di-*O*-substituted units (33%) of Gal*p* (Table 1).

NMR analysis [^{1}H-, ^{13}C- (Fig. 1A), DEPT (Fig. 1B), HSQC (Fig. 2), HSQC-TOCSY (Fig. 3) and HSQC-NOESY (Fig. 4)] contributed to elucidation of the fucomannogalactan (FMG), since the coupling of protons of the units made possible assignments of their respective

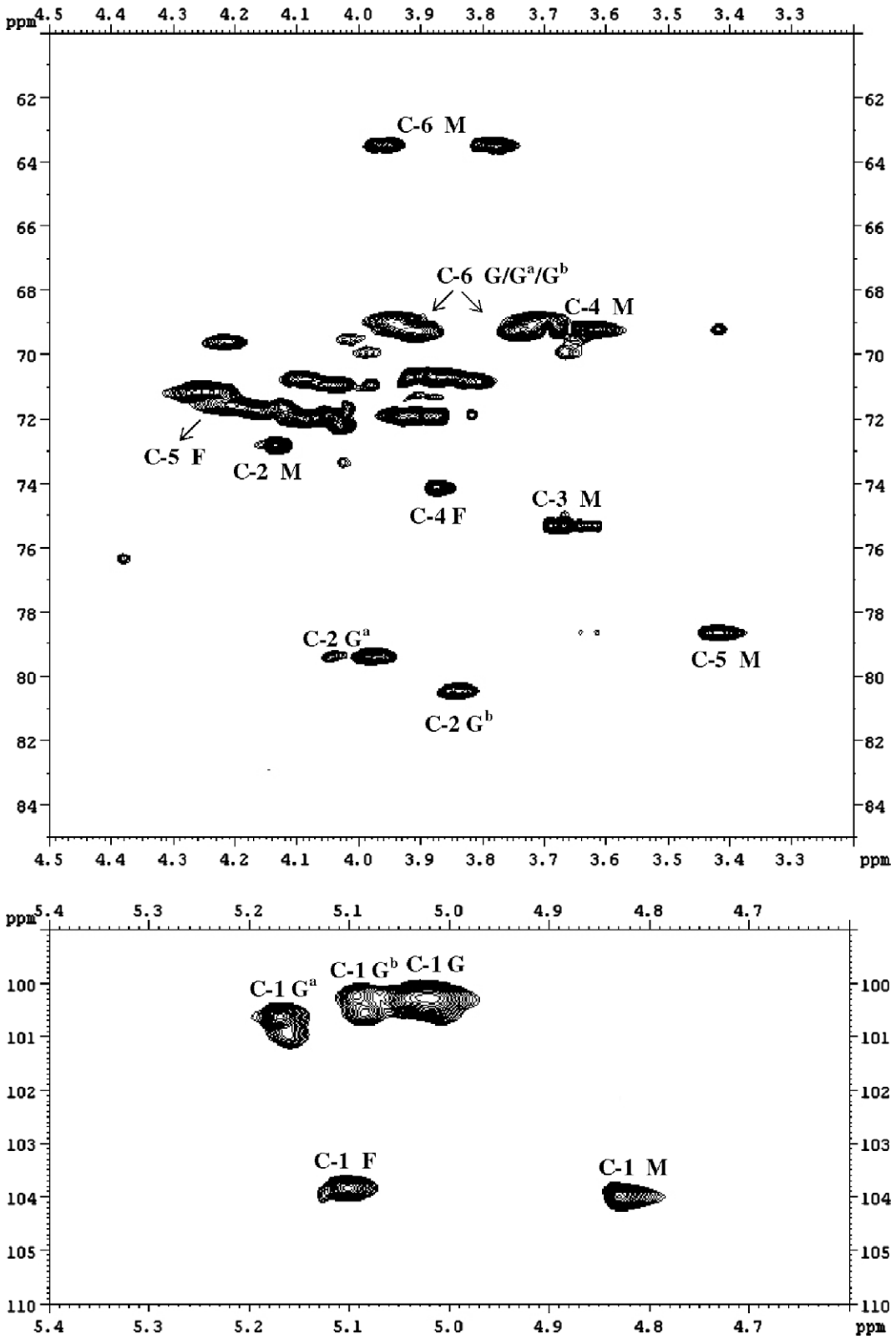

Fig. 2. HSQC spectrum of fucomannogalactan (FMG) in D_2O at 40 °C.

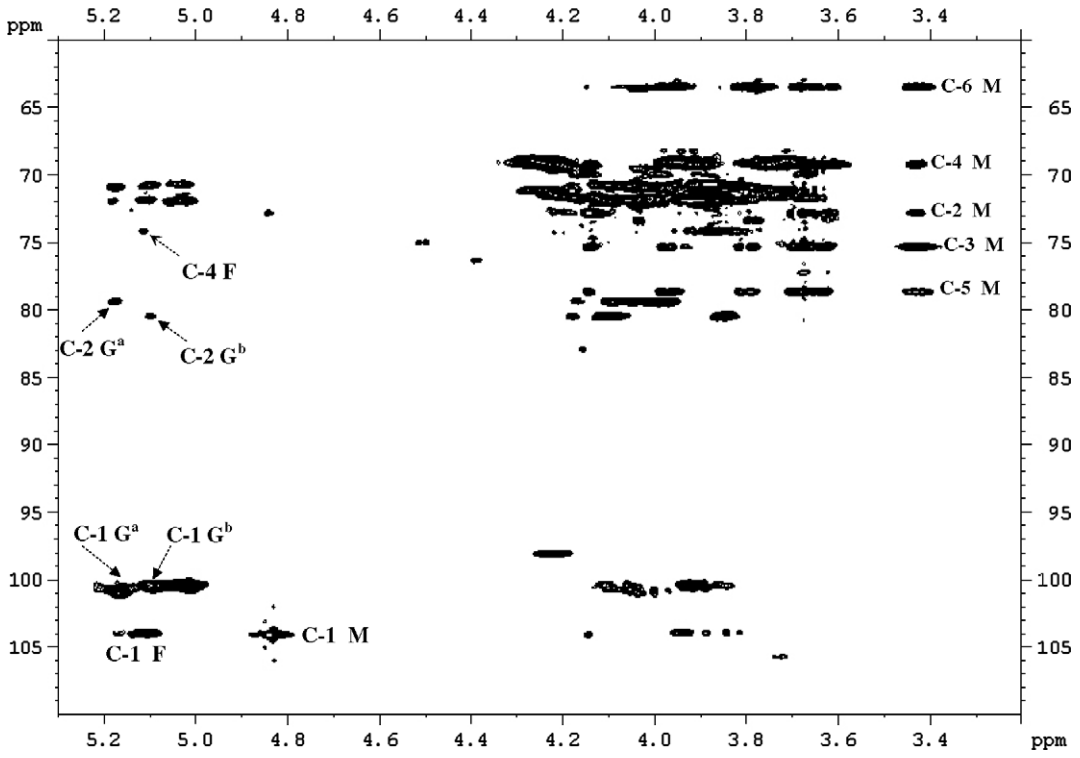

Fig. 3. HSQC-TOCSY spectrum of fucomannogalactan (FMG) in D_2O at 40 °C.

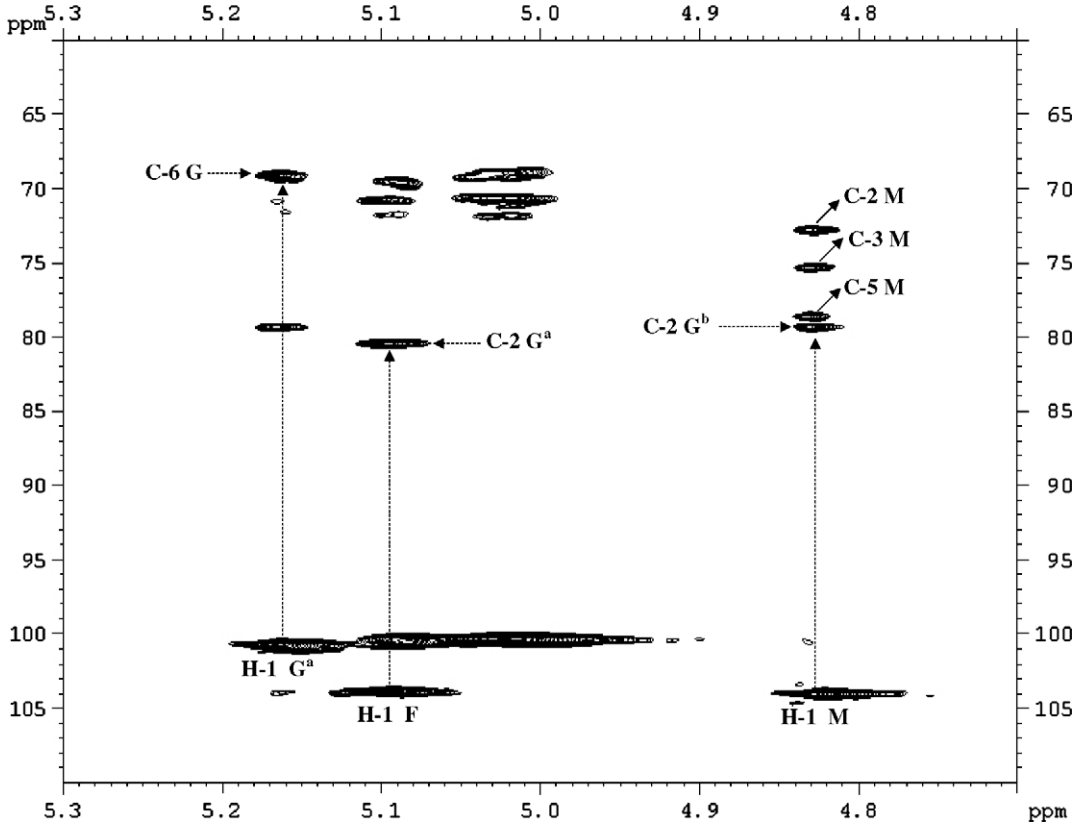

Fig. 4. HSQC-NOESY spectrum of fucomannogalactan (FMG) in D_2O at 40 °C.

carbons using HSQC analysis (Fig. 3). In addition, HSQC-NOESY experiment was carried out to determine the sequence of units in the polymer.

The ^{1}H NMR spectrum of FMG contained H-1 signals corresponding to non-reducing end groups of Man*p* (δ 4.83) and Fuc*p* (δ 5.11), and units of 6-*O*-(δ 5.02) and 2,6-di-*O*-substituted Gal*p*

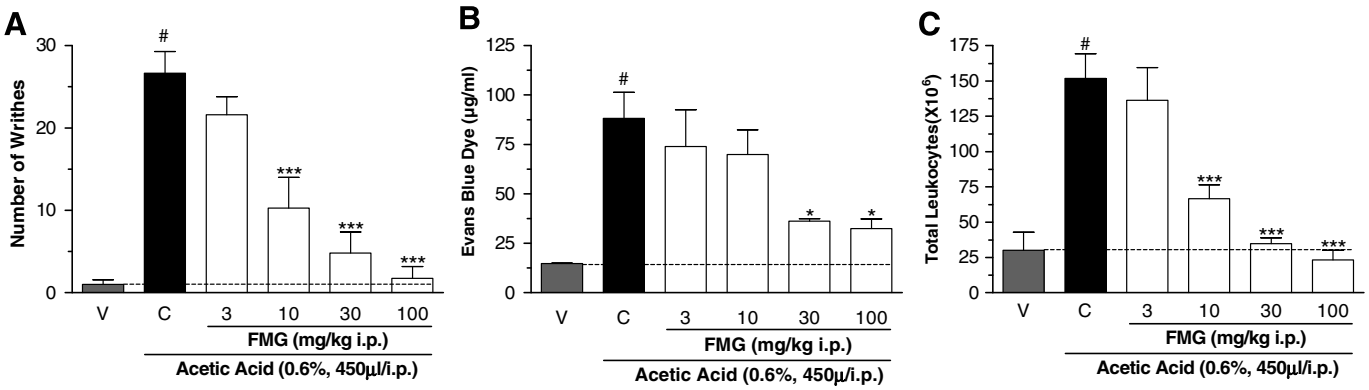

Fig. 5. Effect of i.p. administration to mice of fucomannogalactan (FMG) (3–100 mg/kg) on acetic acid-induced abdominal constriction (A), Evans blue leakage (B), and leukocyte infiltration (C).

(δ 5.17, 5.16, and 5.08). ^{13}C NMR and HSQC spectra of FMG (Figs. 1A and 2, respectively) had signals (C-1/H-1) at δ 104.0/4.83 and 103.9/5.11 corresponding to Man*p* and Fuc*p* units, respectively. Anomeric signals at δ 101.0/5.16, 100.7/5.17 and 100.7/5.08, were from 2,6-di-*O*- and these at δ 100.3/5.02 was from 6-*O*-substituted Gal*p* residues. The glycosidic configurations were confirmed by values of the coupling constants $J_{C\text{-}1,H\text{-}1}$ found in $^{1}H/^{13}C$ coupled HMQC spectra (Perlin & Casu, 1969). The non-reducing ends of Man*p* have a β-configuration consistent with the value of 162.6 Hz. The residues of Gal*p* and Fuc*p* had α-configuration, in agreement with a 172.2 Hz coupling constant.

The 6-*O*-substituted signals of Gal*p* units were present at δ 69.2, 69.4, 69.7, 69.8, and 70.1 in ^{13}C NMR (Fig. 1A) and HSQC spectra (Fig. 2), being confirmed from inverted signals in its DEPT spectrum (Fig. 1B). Assignments of the *O*-substituted C-2 signals from Gal*p* units of the main chain were determined using the inter-residue connectivities observed in HSQC-NOESY. The units of β-Man*p* have an inter-residue correlation from H-1 (δ 4.83) to C-2 (δ 79.4) of Gal*p* residues that had signals of C-1/H-1 at δ 101.0/5.16 and 100.7/5.17 assigned from HSQC-TOCSY (Fig. 3). The H-1 signal (δ 5.11) from Fuc*p* residues showed inter-residue correlation (HSQC-NOESY spectrum) with C-2 linked signal at δ 80.3 corresponding to Gal*p* units with C-1/H-1 at δ 100.7/5.08, on the basis of the crosspeaks of C-2/C-1,H-1 present in HSQC-TOCSY.

The signals at δ 72.8/4.21, 75.4/3.67, 69.5/3.63, 78.6/3.41, and 63.5/3.96;3.78 arose from C-2/H-2 to C-6/H-6 of Man*p* units, respectively, while those at δ 72.3/3.82, 71.3/3.84, 74.2/3.88, 72.2/4.16, and 18.1/1.27 were from similar C-2/H-2 to C-6/H-6 correlations of Fuc*p* residues. All these signals were confirmed by 2D-NMR spectroscopy (Figs. 2–4).

The results of monosaccharide composition, methylation data, and NMR analysis of FMG, thus show it to be a fucomannogalactan containing a (1→6)-linked, α-D-galactopyranosyl main chain, partially substituted at O-2 by single side chain units of β-D-mannopyranose or α-D-fucopyranose.

A polysaccharide resembling FMG has been previously found in an extract of basidiocarps of *L. edodes*, using a different method of isolation (Shida, Haryu, & Matsuda, 1975). The present study reports enriched structural details based on NMR spectroscopy, particularly spectra which can serve as fingerprints.

For the other object of our investigation, the evaluation of the antinociceptive and anti-inflammatory properties of FMG, it was administrated (3–100 mg/kg, i.p.), 0.5 h prior to irritant injection, and caused a marked and dose-dependent inhibition of the acetic acid-induced nociceptive response, with ID_{50} of 13.8 (7.8–23.5) mg/kg and inhibition of 97% at a dose of 100 mg/kg (Fig. 5A). Interestingly, FMG (3–100 mg/kg) also caused a significant and dose-dependent inhibition of both peritoneal capillary permeability (Evans Blue dye exudation) and leukocyte infiltration (total cell migration), induced by acetic acid. The calculated mean ID_{50} value for these effects were 13.9 (8.2–23.7) and 6.5 (1.5–28.2) mg/kg and inhibition of 76% and 100%, for peritoneal capillary permeability and leukocyte infiltration, respectively (Figs. 5B and C).

The acetic acid-induced writhing reaction in mice, as a reaction to novel agents, has long been used as a screening tool for determination of analgesic or anti-inflammatory properties, and is described as a model for visceral inflammatory pain (Tjølsen & Hole, 1997; Vinegar, Truax, Selph, & Jonhston, 1979). The most important transmission pathways for inflammatory pain are those involving peripheral polymodal nociceptors sensitive to protons, such as ASICs (acid sensitive ion channel) and to algogens, such as bradykinin, prostaglandin, and cytokines. These receptors signal to the central nervous system (CNS) via sensory afferent C-fibers entering the dorsal horn (Julius & Basbaum, 2001; Reeh & Kress, 2001). Moreover, it is well established that the nociceptive response caused by acetic acid is also dependent on the release of some cytokines, such as TNF-α, interleukin-1β and interleukin-8 via modulation of macrophages and mast cells localized in the peritoneal cavity (Ribeiro et al., 2000). More recently, Feng, Cui, & Willis, 2003 demonstrated that intraperitoneal injection of acetic acid-induced an increase in the concentration of glutamate and aspartate in the cerebrospinal fluid. Thus, our present results could indicate that the antinociceptive and anti-inflammatory action of FMG in the acetic acid-induced writhing test could be due to inhibition of cytokine release (such as TNF-α, interleukin-1β and interleukin-8 by resident peritoneal cells), glutamate in the cerebrospinal fluid, or by COX activity and consequently prostaglandin synthesis.

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