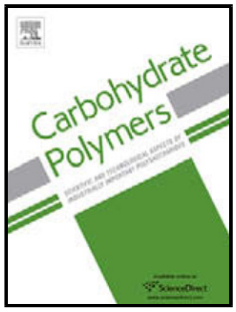

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Structure characterization of the mannofucogalactan isolated from fruit bodies of Quinine conk *Fomitopsis officinalis*

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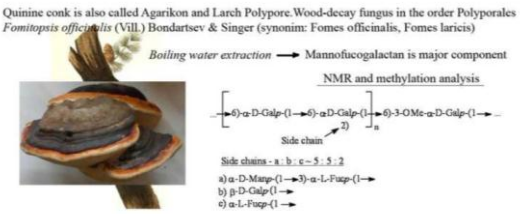
Graphical abstract



Highlights

- The mannofucogalactan was extracted from *Fomitopsis officinalis* by hot water.
- Backbone of mannofucogalactan composed from 1,6-linked D-Gal*p* residues.
- 3-*O*-D-Man*p*-L-Fuc*p*/β-D-Gal*p*/L-Fuc*p* units substitute Gal*p* residues of backbone at *O*-2.
- Part of the Gal*p* residues of backbone is methylated at 3-O-position.

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Abstract

The mannofucogalactan as a major component of water extract was obtained from fruit bodies of *Fomitopsis officinalis* by extraction with boiling water followed by deproteination, decoloration, and purification using anion-exchange chromatography and size exclusion chromatography. Its structure was characterized using the data of monosaccharide composition, methylation analysis, one- and two-dimensional NMR spectroscopy. The studied polysaccharide was a branched mannofucogalactan with a backbone composed of partially 3-*O*-methylated 1,6-*O*-linked α-D-galactopyranosyl residues. Almost

every second residue in the backbone was substituted at *O*-2 by 3-*O*-α-D-mannopyranosyl-α-L-fucopyranosyl and β-D-galactopyranosyl residues. The non-reducing terminal α-L-fucopyranosyl units, which were identified by GC–MS analyses, appeared to be the part of mannofucogalactan side chains also.

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Keywords: medicinal fungi; *Fomitopsis officinalis;* mannofucogalactan; spectroscopy NMR; structure.

1. Introduction

Macrofungi (mushrooms) have been used as food component and medicine from ancient times. The mushrooms are extensively applied in traditional Oriental medicine for many centuries, in which knowledge and practice of use of mushroom and of their water-soluble fractions (decoctions and essences) were primarily originated. However, mushrooms were used in folk medicine as curatives in other countries also (Chang, 1999; Hobbs, 2000; Wasser, 2014). Ancient traditions revealed the importance of several mushroom species, namely, Shiitake mushrooms [*Lentinus edodes* (Berk.) Sing], Lingzhi (in China)/Reishi (in Japan) [*Ganoderma lucidum* (W.Curt.:Fr.) P. Karst.], Split gill mushroom [*Schizophyllum commune* (Fr.:Fr.)], Chaga/birch fungus [*Inonotus obliquus* (Pers.:Fr.) Pilát], Turkey tail [*Trametes versicolor* (L.: F) Pilát], Quinine conk [*Fomitopsis officinalis* (Vill.:Fr.) Bond. et Sing.], Tinder bracket mushrooms [*Fomes fomentarius* (L.: Fr.) Fr.], Birch bracket fungus/Razor strop fungus [*Piptoporus betulinus* (Bull.:Fr.) P. Karst.] (Chang & Wasser, 2012; Grienke, Zöll, Peintner & Rollinger, 2014; Shikov et al., 2014; Wasser, 2002). Nowadays mushrooms are promoted as natural product-based pharmaceuticals with lower toxic effects and providing high potential for the treatment of different pathological processes.

The polysaccharides and polysaccharide complexes have been shown to be bioactive components of mushrooms. Primarily they attracted considerable attention due to their efficient immunomodulatory, anti-cancer (Meng, Liang, & Luo, 2016; Moradali, Mostafavi, Ghods, & Hedjaroude, 2007; Ren, Perera, & Hemar, 2012; Zhang, Cui, Cheung, & Wang, 2007) and anti-inflammatory effects (Li, Zhang, & Ma, 2014; Muszyńska, Grzywacz-Kisielewska, Kała, & Gdula-Argasińska, 2018; Smiderle et al., 2008a; Taofiq, Martins, Barreiro, & Ferreira, 2016). The structure of a vast amount of glucans and different heteropolysaccharides have been precisely defined recently. The immunomodulating and anti-tumor

activity of fungi were associated mainly with β-D-glucans, whereas heteropolysaccharides also presented in mushrooms revealed the pronounced biological activities (Ruthes, Smiderle, & Iacomini, 2016; Villares, Mateo-Vivaracho, & Guillamón, 2012).

The investigation of structure of fungi polysaccharides is highly desirable, due to their potential biological activity. Although, studies including clinical trials need to be carried out to ascertain the safety of these compounds as adequate alternatives to conventional drugs (Corrêa, Brugnari, Bracht, Peralta, & Ferreira, 2016), component composition of many fungi used in folk medicine has not been studied yet. This study is a part of our complex elucidation of structure and biological activity of polysaccharides of fruit bodies of the Quinine conk/Agarikon [Fomitopsis officinalis (Vill.) Bondartsev & Singer], which belong to a group of xylotroph basidiomycetes growing on stems of larch trees. These fungi attracted interest due to extensive use of its basidiocarps for medical purposes, which even resulted in a decrease of fungi population (Chlebicki, Mukhin, & Ushakova, 2003). Nevertheless, the structural characteristics of its polysaccharides have not been investigated yet. Here we report the isolation and structure analysis of a mannofucogalactan as major component of water extract of these fungi. Further our experiments aimed to investigate structure of polysaccharides obtained by alkaline extraction, and to study the cytotoxic activity of all polysaccharides isolated from these fungi in order to reveal the relations between chemical structures with biological properties.

2. Materials and methods

2.1. Materials and chemical reagents

Chemical reagents: ethyl alcohol, ethanol, C_2H_5OH (96%, JSC Kirov Pharmaceutical Factory, Russia); methyl alcohol, methanol, CH_3OH (99.9%, Reakhim, Russia); sodium hydroxide, NaOH (98%, Fluka, Germany); sodium chloride, NaCl (99%, Sigma-Aldrich, USA); hydrogen peroxide solution, H_2O_2 (29.0-32.0% %, Sigma-Aldrich, USA); lead (II) acetate trihydrate, $Pb(CH_3COO)_2 \times 3H_2O$ (99.999%, Sigma-Aldrich, USA); (R)-(−)-2-butanol (Aldrich, USA); dichloromethane, CH_2Cl_2 (≥99.8%, Sigma-Aldrich, USA), chloroform, $CHCl_3$ (>99.9%, Ekos-1, Russia); ammonium hydroxide solution, NH_4OH (≥ 25 NH_3 in H_2O, >99.9%, Ekos-1, Russia); 1-methylimidazole, $C_4H_6N_2$ (99%, Sigma-Aldrich, USA); acetyl chloride (≥98%, Merck, Germany);

iodmethane, CH_3I (99%, Merck, Germany); trifluoroacetic acid, CF_3COOH (99%, Acros organics, USA); pyridine, C_5H_5N (99%, Ekos-1, Russia); acetic acid, CH_3COOH (99.9%, Khimreactive, Russia); sodium borohydride, $NaBH_4$ (>98.5%, Sigma-Aldrich, USA); dimethylsulfoxide, DMSO (≥99.9 %, PanReac Quimica, Spain); D_2O (99.9 atom % D, Sigma-Aldrich, USA); sodium sulphate, Na_2SO_4 (99%, Ekros, Russia); toluene, C_6H_5-CH_3 (99 %, Ekos-1, Russia); sulfuric acid, H_2SO_4 (>99.9%, Vekton, Russia); phenol, C_6H_5OH (99%, Reakhim, Russia).

The Folin & Ciocalteu's phenol reagent (Sigma-Aldrich, USA) was used in Lowry assay.

For Ion Exchange chromatography we used DEAE-cellulose (Sigma-Aldrich, USA), for gel permeation chromatography – Sephacryl S-300 high resolution (Albersham Bioscientes, Sweden).

The pullulans: 1.3, 6, 12, 22, 50, 110, 200, 400, and 800 kDa) (Fluka, Germany); bovine serum albumin (≥96%, Sigma-Aldrich, USA); L-(–)-fucopyranose (≥99%, Sigma-Aldrich, USA), D-(+)-mannopyranose (≥99%, Sigma-Aldrich, USA) and D-(+)-galactopyranose (≥99%, Sigma-Aldrich, USA); *myo*-inositol (≥99%, Sigma-Aldrich, USA) were used as standards.

Biological material: the fruit bodies of *Fomitopsis officinalis* (Vill.: Fr.) Bond. et Sing. were collected in November, 2012 from trees grown in Eruu soum, Selenge province, Mongolia. They were identified by Prof. Ch. Sanchir (Botany Institute of Mongolian Academy of Sceinces, Mongolia). The fruit bodies were washed with distilled water, cut into small pieces, and dried in a thermostat (Jouan, France) at 45 °C for 24 h.

2.2. Isolation of polysaccharide FoHWE, it's separation and purification

Dry fruit bodies of *F. officinalis* (200 g) were milled in a blender, and the powder was defatted with 96% ethanol at 60°C for 5 h (2 × 350 mL) to remove soluble lipids, sugars or phenolic compounds. The supernatant was removed by filtration. The polysaccharides were extracted twice with boiling water under reflux for 4 h (3 × 1000 mL). The residue was separated by centrifugation and the extracts were collected, combined, concentrated by rotary evaporator to 100 – 200 mL. For the deproteinization 2% lead acetate was added to the extract to its final concentration of 0.4 – 0.5 % (Chen, 2012). The extract

was decolorized by addition of 30 % H_2O_2 to its final concentration of 5 %, and then 10 % aqueous NH_4OH was added to achieve pH 7, and the mixture was kept in the dark for 15 h at room temperature. The insoluble precipitate was removed by centrifugation. The aqueous solution of polysaccharide was dialyzed against distilled water during 2 days at 10 °C and concentrated on a rotary evaporator. Then polysaccharides were precipitated with 95 % ethanol, dissolved in water after centrifugation and lyophilized, the traces of ethanol was removed on a rotary evaporator. A fraction FoHWE was obtained as a result of the extraction. The yield of the fraction FoHWE and its monosaccharide composition are presented in Table 1.

The yield of polysaccharide FoHWE was calculated as follows:

$$\omega(\text{FoHWE}) = \frac{\text{m(FoHWE)}}{\text{m(dry biological material)}} \times 100\%,$$

where m (FoHWE) (g) – weight of polysaccharide FoHWE obtained after extraction, m (dry biological material) (g) – weight of dried fruit bodies of *F. officinalis* used for the extraction.

Anion-exchange chromatography and gel permeation chromatography were used for separation and purification of polysaccharide FoHWE. Polysaccharide FoHWE (50 mg) was dissolved in 5 mL of distilled water and solution was applied to a column (2.5 cm × 40 cm) of DEAE-cellulose (OH^--form). The column was stepwise eluted with distilled water, 0.01, 0.1, 0.2, 0.3 and 0.5M NaCl solutions (400 mL of each eluent) at a flow rate of 1 mL/min. The fractions were collected at 10 min intervals using low-pressure system Pharmacia Biotech (Sweden) with a fraction collector F RAC-100, pump P-50, Uvicord SII, Recorder REC 101. The carbohydrates content of each tube was determined by phenol-sulfuric acid method (Dubois et al., 1956). One major fraction, eluted with water, was collected, concentrated, dialyzed, and further purified by gel permeation chromatography on a column of Sephacryl S-300 (1.6 cm×100 cm, V_0=27 mL), which was eluted with 0.15M NaCl solution included NaN_3 (0.02%) at a flow rate of 0.3 mL/min. The eluate (2.5 mL/tube) was collected and detected as described above. As a result, one major purified fraction FoHWE-1 (kav 0.43) was collected, dialyzed and lyophilized with a yield 37.35 mg (Table 1). The elution curves for anion-exchange column and gel filtration column are presented in Supplementary Data (Fig. 1S).

The yield polysaccharide FoHWE-1 was calculated as follows:

$$\omega(\text{FoHWE-1}) = \frac{\text{m(FoHWE-1)}}{\text{m(FoHWE)}} \times 100\%,$$

where m (FoHWE-1) – weight of purified polysaccharide FoHWE-1 obtained after anion-exchange and gel permeation chromatography, m (FoHWE) – weight of crude polysaccharide FoHWE.

2.3. General analytical methods

Specific optical rotation was measured in 1 mL cell on a Polatronic MHZ polarimeter (Germany) at 20°C. The absorbance was measured using Ultrospec 3000 spectrophotometer (Pharmacia Biotech, England). The solutions were concentrated with a Laborota 4002 rotary evaporator (Heidolph, Germany) under reduced pressure at 40 °C. The samples were centrifuged with a 6 K 15 centrifuge (Sigma, Germany) at 11000 rpm at 4 °C for 20 min, lyophilized using an Edwards lyophilizer (England) under a constant vacuum of <10 mTorr at – 65 °C.

The monosaccharide composition of polysaccharides was determined as described earlier in details (Popov et al., 2014). The content of monosaccharides was detected by gas–liquid chromatography (GLC) after the polysaccharides hydrolysis and transformation into their alditol acetates. The calculation of monosaccharides content (weight %) was made as follows:

$$\omega(\text{i}) = \frac{\text{S(i)}\times\text{m(In)}}{\text{S(In)}\times\text{k(i)}\times\text{m}} \times 100\%,$$

where S(i) – area of each monosaccharide peak on GLC chromatogram; S(In) – area of *myo*-inositol (as internal standard) peak on GLC chromatogram; m (mg) – weight of polysaccharide used for hydrolysis; k(i) – considering weight response-factors for each monosaccharide. k(i) was detected as S(i) (standard monosaccharide)/S(In) (*myo*-inositol), which were used in equal weight ratios.

Table 1 presented data calculated as a relative percentage of sum of all monosaccharides and protein, which were determined in simples, as follows:

$$\text{a(i)} = \frac{\omega(\text{i})}{\sum_{\text{i}=1}^{\text{n}} \omega(\text{i}) + \omega(\text{protein})} \times 100\%,$$

where a(i) – calculated content of monosaccharide (weight %), ω (i) – content of each monosaccharide (weight %), ω (protein) – content of protein detected by Lowry method (weight %).

Protein concentration was determined using Lowry's procedure (Lowry, Roserbourgh, Farr, & Randall, 1951) with bovine serum albumin as a standard.

The monosaccharides were determined at 490 nm using the phenol-sulphuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

The homogeneity and molecular weight of the polysaccharide samples were determined by high performance gel-permeation chromatography (HPGPC). Pullulans (1.3, 6, 12, 22, 50, 110, 200, 400, and 800 kDa) were used as standards. The details of procedure were described earlier (Golovchenko, Khramova, Ovodova, Shashkov, & Ovodov, 2012).

The enantiomeric configuration of monosaccharides was determined by the method described earlier (Gerwig, Kamerling, & Vliegenthart, 1978) with minor modification. The polysaccharide (3.0 mg) was hydrolyzed with CF_3COOH at 100°C for 8 h, and then the acid was removed by co-distillation with water. Mixture of monosaccharides was dissolved in 0.1mL (R)-(−)-2-butanol and added 15 μL of acetyl chloride; the mixture was heated at 80 °C for 16 h. Then the solution was evaporated; butylglycosides was dissolved in 0.2 mL pyridine and 0.2 mL acetic anhydride was added; the mixture was heated at 100°C for 1 h. The solution was evaporated, O-acetylated butylglycosides were dissolved in 0.1 mL in $CHCl_3$ and analyzed by GLC using a capillary column VF-5 ms (Varian, USA; 0.25 mm, 30 m), a temperature program (3 °C/min) raised from 150 (1 min) to 210 °C (3 min). The (R)-(−)-2-butyl 2,3,4,6-tetra-*O*-acetyl-glycosides were identified by the comparison with those prepared from the D-mannopyranose, D-galactopyranose and L-fucopyranose.

2.4. Methylation analysis

The polysaccharide fraction FoHWE-1 was activated with powdered NaOH and methylated with CH_3I (Ciucanu & Kerek, 1984) as described earlier (Cerqueira et al., 2011) with minor modifications. The sample (4 mg) was dispersed in dried DMSO (2mL) and sonicated occasionally until it was fully dispersed.

NaOH pellets (80 mg) powdered under argon were added to the solution. The mixture was stirring using a magnetic stirrer at 250 rpm (Heidolph MR 3001, Germany) for 2 h at 23 °C. The mixture was cooled and 1 mL of methyl iodide was added under continuous stirring at 700 rpm in an ice bath for 1 hours. The reaction was stopped with water (2 mL) and the resulting partially methylated derivatives were extracted with chloroform (3 mL). The organic layer was collected and washed with water (4 × 2mL), dried with Na_2SO_4 and evaporated to dryness and remethylated. The methylation process was repeated three times. The fully methylated polysaccharides were then hydrolyzed with 2 M CF_3COOH (1 mL) at 121 °C for 1 h using thermostat (Jouan, France), cooled and rotary evaporated at 35 °C. The partially methylated sugars were dissolved in 2M NH_4OH (0.3 mL), reduced with $NaBH_4$ (20 mg) (1 h at 23°C) and with *conc.* CH_3COOH (0.2 mL) to decompose the excess of the reducing agent and finally rotary evaporated to dryness. The acetylation of the partially methylated alditols was performed by adding of 1-methylimidazole (0.5 mL) and acetic anhydride (2 mL) for 30 min at 23 °C. Water (2 mL) was added to the solution to decompose the excess of acetic anhydride. The partially methylated alditol acetates were extracted with dichloromethane (3 mL). The dichloromethane layer was washed with water (2 × 3 mL), dried with Na_2SO_4, and evaporated to dryness. The partially methylated alditol acetates were dissolved in dichloromethane (100 μL) and analyzed by GC–MS.

Partially acetylated, partially methylated alditols were analyzed by Agilent 7890A/5977A GC-MS mass spectrometer (Agilent, Germany) equipped with a HP-5ms capillary column (Agilent Technologies). Helium was used as a carrier gas (flow rate 1.8 mL/min). Injector temperature 280 °C, injection volume 1 μL (autosampler injection mode), split ratio 1 : 10. Temperature program started at 150 °C (hold time 1 min), linear increase at 10 °C/min to 280 °C then linear increase at 35 °C/min to 325 °C. EI energy was fixed at 70 eV, scanning range from *m*/*z* 50 to *m*/*z* 400, ion source temperature 230 °C.

The content of methylated monosaccharides (molar %) was detected as follows:

$$\mathrm{C(i)} = \frac{\mathrm{S(i)} \times \mathrm{Rf(i)}}{\sum_{\mathrm{i}=1}^{\mathrm{n}} \mathrm{S(i)} \times \mathrm{Rf(i)}} \times 100\%,$$

where S(i) – area of each methylated monosaccharide peak on GC–MS chromatogram; R*f*(i) – considering molar response-factors previously determined (Sweet et al., 1975).

2.5. Nuclear magnetic resonance (NMR) spectroscopy and NMR spectra analyses

The samples (25 mg) were deuterium-exchanged by freeze-drying from D_2O for three times and then dissolved in 500 μL D_2O with 0.5 μL of acetone (99.96 atom % D, Sigma Aldrich) as a reference for the calibration of the chemical shift (δ_H 2.225 ppm, δ_C 31.45 ppm). All homo- and heteronuclear NMR experiments of the samples were carried out on a Bruker DRX Avance II 600 MHz spectrometer (Germany) at a probe temperature that providing a minimum overlap of the signal of deuterated water with the polymer signals (313 K). 2D NMR spectra were obtained using a standard Bruker software.

3. Results and discussion

3.1. Isolation, purification and composition of mannofucogalactan

Using boiling water the fraction FoHWE (yield 3.68 % of the air-dried material) was isolated from defatted with hot 96 % ethanol fruit bodies of *F. officinalis*. The extract was centrifuged, deproteinizated by lead acetate (Chen et al., 2012), and decolorized with H_2O_2. After removing of insoluble precipitate by centrifugation, the extract was dialyzed against distilled water, and polysaccharides were precipitated from the extract with an excess of ethanol and the solution was freeze-dried. The monosaccharide composition of polysaccharide fractions (Table 1) was identified by GLC after the complete acid hydrolysis of polysaccharides with 2 M aqueous CF_3COOH and transformation of monosaccharides to corresponding alditol acetates. The polysaccharides of fraction FoHWE contained fucose, mannose, glucose, galactose residues as major components of carbohydrate chains, but had a heterogeneous HPGPC elution profile (Fig.1a). Therefore, the fraction FoHWE was successively separated by anion-exchange chromatography using a DEAE-cellulose (OH^--form) column and gel permeation chromatography on a column of Sephacryl S-300 by water elution. As a result, polysaccharide fraction FoHWE-1 was obtained (yield 74.7 %) as a dominant component.

The fraction FoHWE-1 exhibited an asymmetrical single peak on HPGPC with Mw/Mn 1.4, however, homogeneity FoHWE-1 was significantly higher than FoHWE (Mw/Mn 13.6). Its molecular weight (M_W) was equal to 17.4 kDa (Fig. 1b), and its specific rotation was equal to $[\alpha]^{D}_{20}$ + 77.4° (*c* 0.155, H_2O). The monosaccharide composition of the fraction FoHWE-1 was typical for mannofucogalactans (Table 1).

There was no absorption peak at 280 nm in the UV spectrum of the fraction FoHWE-1 and the Lowry assay confirmed that it was free of protein.

3.2. Structural characterization of mannofucogalactan

3.2.1. Interpretation of NMR data

In order to confirm the types of linkages in the mannofucogalactan of fraction FoHWE-1, NMR spectroscopy was used. The sugar residues were assigned applying $^{1}H/^{1}H$ correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), rotating frame Overhauser effect spectroscopy (ROESY), $^{1}H/^{13}C$ heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) experiments. The assignments of the protons and carbons (by HSQC measurements) of the residues are listed in Table 2.

The five anomeric protons were observed in ^{1}H NMR spectrum of the fraction FoHWE-1 (Fig. 2a), but $^{1}H/^{13}C$ HSQC spectrum (Fig. 2b) contained an anomeric region with signals for six spin systems C-1/H-1 at δ 103.64/5.14, 102.56/5.13, 102.56/5.09, 99.23/5.05, 99.23/5.00, 104.63/4.47. The sugar residues were designated (**A–F**) according to the decreasing chemical shifts of the anomeric protons (Table 2). As judged by the absence from the ^{13}C NMR spectrum of signals within δ 82–88 ppm, all sugar residues were in the pyranose form (Buchmarinov et al., 2004). The integrated ratio of the H-1 signals in the ^{1}H NMR spectra showed that the peak area ratio of A+B:C:D:E:F of the fraction FoHWE-1 was approximately 1.5:0.3:1.2:1.0:0.4.

Determination of protons position of **residue (A)** revealed stepwise connectivities from H-1 (δ_H 5.14) to H-2 (δ_H 4.09), H-3 (δ_H 3.91), H-4 (δ_H 3.69), H-5 (δ_H 3.81) in the COSY spectrum, and the TOCSY spectrum showed the correlation of H-1(δ_H 5.14) with H-2 (δ_H 4.09), and H-5 (δ_H 3.81) with H-6-s (δ_H 3.79 and 3.90). The HMBC spectrum demonstrated the correlation of C-5 (δ_C 74.67) with the H-4 (δ_H 3.69) and H-6 (δ_H 3.79).

The single correlation peak of H-1/H-2 ($\delta_{H/H}$ 5.13/3.95) was detected in the COSY spectrum for **residue (B**), and the TOCSY spectrum showed connectivities from H-1 (δ_H 5.13) to H-3 (δ_H 3.98). The positions H-4 and H-5 were detected from cross peaks between methyl group protons (δ_H 1.25) and H-4 (δ_H 3.99) and H-5 (δ_C 4.17) in ROESY spectrum (Fig.3a). Additionally the cross peaks between methyl group protons (δ_H 1.25) and C-4 (δ_C 72.72) and C-5 (δ_C 68.17) were identified in HMBC spectrum, and confirmed in HSQC spectrum (Fig. 2c).

The positions of H-2 (δ_H 3.87) and H-3 (δ_H 4.09) in the COSY spectrum for **residue (D**) were detected, in TOCSY spectrum the positions of H-2 (δ_H 3.87), H-3 or/and of H-4 (δ_H 4.09), and H-5 (δ_H 4.16) were detected from correlations with H-1 (δ_H 5.05). In addition, the ROESY spectrum included correlation peaks of H-1/H-2 at $\delta_{H/H}$ 5.05/3.87; H-1/H-5 at $\delta_{H/H}$ 5.05/4.16; H-1/H-6 at $\delta_{H/H}$ 5.05/3.70 and two correlation peaks of low intensity of H-1/H-4 or H-1/H-3 at $\delta_{H/H}$ 5.05/4.09 and H-1/H-6' at $\delta_{H/H}$ 5.05/3.98 (Fig. 3b). The positions of H-6; H-6' were detected in TOCSY spectrum from correlations with H-5 ($\delta_{H/H}$ 4.16/3.70; 3.98) (Fig. 4).

The positions of H-2 (δ_H 3.87) and H-3 (δ_H 3.89) from correlation with H-1 (δ_H 5.00) were detected in the COSY and TOCSY spectrum for **residue (E**). Additionally position of H-4 (δ_H 4.04) was detected from correlation with H-1 in the TOCSY spectrum too. In the ROESY spectrum correlation peaks

of H-1/H-2 at $\delta_{H/H}$ 5.00/3.87 and H-1/H-6 at $\delta_{H/H}$ 5.00/3.72 and two correlation peaks of low intensity of H-1/H-4 at $\delta_{H/H}$ 5.00/4.04 and H-1/H-5 at $\delta_{H/H}$ 5.00/4.20 were observed (Fig. 3b). The positions of H-6-s were detected in TOCSY spectrum from correlations of H-5/H-6; H-6' ($\delta_{H/H}$ 4.20/3.72; 3.92) (Fig. 4).

The COSY spectrum showed the connectivities from H-1 (δ_H 4.47) to H-5 (δ_H 3.71) in **residues (F).** The TOCSY spectrum showed the connectivities from H-1 (δ_H 4.47) to H-4 (δ_H 3.95), and from H-5 (δ_H 3.71) to H-6-s (δ_H 3.80 and 3.91). The HMBC spectrum demonstrated correlation of C-5 (δ_C 76.42) with the H-6 (δ_H 3.80). The ROESY spectrum included the correlation signals of H-1 (δ_H 4.47) with H-2 (δ_H 3.58), H-3 (δ_H 3.67), H-5 (δ_H 3.71) and H-6-s (δ_H 3.80 and at δ_H 3.91) (Fig. 3c).

On basis of the proton assignments, data of HMBC experiments, computerized approach to the structural analysis of polysaccharides (Lipkind, Shashkov, Knirel, Vinogradov, & Kochenkov, 1988), and comparison with data reported earlier (Carbonero, Mellinger, Sassaki, Gorin, & Iacomini, 2008b; Cho, Yun, Yoo, & Koshino, 2011; Smiderle, Alquini et al., 2004) the correlations of carbons and protons were detected in the HSQC spectrum (Fig. 2c), and chemical shifts from C-1 to C-6 are listed in Table 2 and Fig. 5.

The α-configuration for sugar residues **(A–E**) and β-configuration for sugar residue **(F**) were detected from chemical shifts of C-5-s and H-5-s. Chemical shift of H-5 at δ 3.81 ppm and C-5 at δ 74.67 ppm for Man*p* residues are characteristic of α-configuration of Man*p* residues (Jansson, Kenne & Widmalm, 1989; Lipkind et al., 1988). Chemical shift of C-5 at δ 68.17 ppm is typical for α-configuration of Fuc*p* residues (Jansson et al., 1989). Chemical shifts in the range δ 70-72 ppm are characteristic of C-5 of Gal*p* with α-configuration of Gal*p* residues, but chemical shift at δ 76.42 ppm is typical for β-configuration of Gal*p* residues (Lipkind et al., 1988).

Positions of signals of residues **(A)** and **(F)** were identified as corresponding to the α-mannopyranose and β-galactopyranose residues located at non-reducing terminal positions, respectively. The positions of signals of C-6-s atoms at δ_C 62.42 ppm evidently point to the absence of substitutes at these carbons (Lipkind et al., 1988).

The residue **(B)** was identified as the 3-*O*-substituted fucopyranosyl residue. The $^{1}H/^{13}C$ HSQC spectrum contained signals at δ 16.95/1.25 ppm, both carbon and proton chemical shifts were typical for methyl group signals of 6-deoxyhexopyranose; here fucopyranose is the only monosaccharide of this type identified in carbohydrate chains of polysaccharide. The down-field shift of C-3 (δ_C 78.88) and highfield shift of C-2 (δ_C 68.83) with respect to standard values for glycopyranoses (Lipkind et al., 1988) indicated the presence of 3-*O*-substitution.

Positions of signals of residues **(D)** and **(E)** and downfield shift of C-6-s (δ_C 68.44 and 67.86, respectively) and additionally of C-2 (δ_C 78.75) of residue (D) with respect to standard values for glycopyranoses (Lipkind et al., 1988) indicated these residues as the 2,6-di-*O*-substituted and 1,6-linked α-galactopyranose residues, respectively.

The residue **(C)** was not identified and the assignment of each signal was not detected, due to the presence of only one cross peak of H-1/H-2 with low intensity at $\delta_{H/H}$ 5.09/3.86 ppm in the COSY spectrum, which overlapping with intensive signals of H-2 of residues **(D)** and **(E)**. The TOCSY spectrum also failed to be useful because of overlapping and/or no proton correlation. The residue (C) appeared to be the non-reducing end α-L-Fuc*p* residues. The anomeric region of HSQC spectrum presented in Fig. 2a was similar to anomeric region of HSQC spectrum of mannofucogalactan from edible mushroom *Grifola frondosa* (Oliveira et al., 2018), where the authors identified it as non-reducing end α-L-Fuc*p* residues. Moreover, the positions of H-1 and H-2 of residue **(C)** conformed to positions of H-1 and H-2 of non-reducing end α-L-Fuc*p* residues of mannofucogalactan *G. frondosa* (Oliveira et al., 2018).

In addition, the 3-O-methylated 1,6-linked α-Gal*p* residues (namely **(G)** residue) were determined. The $^{1}H/^{13}C$ HSQC spectrum in high-field contained the signal at $\delta_{C/H}$ 57.6/3.49 ppm distinctive for methyl group. The $^{1}H/^{13}C$ HMBC indicated on the correlation between proton at δ_H 3.49 ppm and carbon at δ_C 80.4 ppm and carbon of methyl group at δ_C 57.6 ppm with proton at δ_H 3.58 ppm. The ROESY spectrum revealed the correlation between the proton of methyl group at δ_H 3.49 ppm and proton at δ_H 3.58 ppm. The presence and position of cross peak at δ_C/δ_H 80.4/3.58 ppm in HSQC spectrum (Fig. 2c) confirmed the presence of 3-*O*-Me-α-Gal*p* residues as noted previously (Carbonero et al., 2008; Oliveira

et al., 2018, Ruthes, Rattmann, Carbonero, Gorin, & Iacomini, 2012; Smiderle et al., 2008c). In the TOCSY spectrum (Fig. 4) in the region of correlation of signals of H-5-s with H-6-s presented three spin systems for three different galactose residues substituted at 6-O position, two of which belonged to **(D)** and **(E)** residues, the third apparently – to **(G)** residue. In the $^{1}H/^{13}C$ HSQC spectrum three spin systems of C-6/ H-6; 6' at δ_C/δ_H 68.44/3.70; 3.98 and 68.20/3.72; 3.92 and 67.86/3.72; 3.92 characterizing three different galactose residues substituted at 6-O position were detected also. Position of H-5 for **(G)** residue was detected from cross peak at $\delta_{H/H}$ 4.24/3.92; 3.72 in the TOCSY spectrum (Fig. 4). The correlation of C-4/H-4 at $\delta_{C/H}$ 70.15/4.24 ppm for **(G)** residue was detected in HSQC spectrum (Fig. 2c). The assignments of C-6 for **(D), (E)** and **(G)** residues were assigned as δ_C 68.44, 68.20 and 67.86 ppm, respectively, similar to the positions of C-5 signals of these residues (Fig. 2c) and data presented for analogical mannofucogalactan of *Grifola frondosa* (Oliveira et al., 2018). The presence of cross peaks of C-6 **(D)** with H-3 **(G)** at $\delta_{C/H}$ 68.44/3.58 and with H-6 **(E)** and **(G)** at $\delta_{C/H}$ 68.44/3.92 and with H-3 **(D)** at $\delta_{C/H}$ 68.44/4.09 in the HMBC spectum indicated the neighborhood of these residues.

The sequences of glycosyl residues of the polysaccharide along with some intra-residual contacts were determined from ROESY experiments (Fig. 3). As a result, the following inter-residual correlations were detected:

H-1(A)/H-3(B) at $\delta_{H/H}$ 5.14/3.98 demonstrated the presence of

α-D-Man*p*-(1→3)-α-L-Fuc*p*-(1→;

...→6)-α-D-Gal*p*-(1→ ...
...→3)-α-L-Fuc*p*-(1↗2)

H-1(B)/H-2(D) at $\delta_{H/H}$ 5.13/3.87 and proton of methyl group (B)/H-1(D) at $\delta_{H/H}$ 1.25/5.05 demonstrated the presence of

...→6)-α-D-Gal*p*-(1→ ...
β-D-Gal*p*-(1↗2)

H-1(F)/H-2(D) at $\delta_{H/H}$ 4.47/3.87 demonstrated the presence of

The substitution of fucopyranose residues by non-reducing terminal mannose residues and the substitution of galactose residues by fucose residues were confirmed by the presence of correlation peaks at $\delta_{C/H}$ 103.5/3.98 and 102.56/3.87 in HMBC spectrum, respectively.

Absolute D-configuration of Man*p* residues was adopted from genetic considerations since natural L-Man*p* has not been found so far. Absolute L-configuration of α-Fuc*p* was found in α-D-Man*p*-(1→3)-α-Fuc*p* fragment based on the regularities reported previously (Lipkind et al., 1988; Shashkov, Lipkind, Knirel, & Kochetkov, 1988). Correspondingly, absolute D-configuration of α-Gal*p* was determined in α-L-Fuc*p*-(1→2)-α-Gal*p* fragment. The absolute configuration of the sugars was confirmed by the method described earlier (Gerwig et al., 1978). These are in agreement with previously published data (Carbonero et al., 2008a; Usui, Iwasaki, & Mizuno, 1981).

3.2.2. Interpretation of methylation analysis

The NMR data were supported by the methylation analysis (the original GC/MS spectra are presented in Supplementary Data (Fig. 2S)). The polysaccharide FoHWE-1 was methylated by the Ciucanu & Kerek method (Ciucanu & Kerek, 1984) and converted into the mixtures of partially *O*-methylated alditol acetates, which were analyzed by GC–MS with use of characteristic retention times (T-values) (Bjorndal, Hellerqvist, Lindberg, & Svensson, 1970; Sims, Carnachan, Bell, & Hinkley, 2018) and molar response factors (Rf) previously determined for the partially methylated alditol acetates individual constituents (Sweet, Shapiro, & Albersheim, 1975). The methylation analysis showed that all sugar components were pyranoses.

According to the peak areas, five major components were identified by their retention times and typical fragmentation patterns: non-reducing terminal units: Man*p*-(1→; Gal*p*-(1→, linear units: →6)-

Gal*p*-(1→; →3)-Fuc*p*-(1→; and 2,6-di-*O*-substituted galactose residues as points of branching (Table 3), which were detected in NMR spectra (Table 2). Results of methylation and NMR analysis indicated that 1,6-linked and 2,6-di-O-substituted galactose residues presented in almost equal amounts (Table 3). Other residues were identified as structural units of side chains.

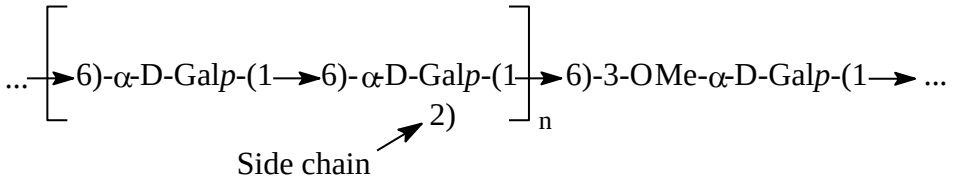
The presence of non-reducing terminal Fuc*p* units was confirmed by GC–MS analyses. As shown earlier for mannofucogalactans from *Flammulina velutipes* (Mukumoto & Yamaguchi, 1977; Smiderle et al., 2008b) and edible mushroom *Grifola frondosa* (Oliveira et al., 2018), and fucomannogalactan (Carbonero et al., 2008a) and heterogalactan (Shida, Haryu, & Matsuda, 1975) from *Lentinus edodes*, the α-L-Fuc*p* residues appeared to form side chains of *F. officinalis* mannofucogalactan.

In addition, the following unusual substituents for mannofucogalactan were identified by GC–MS analyses as minor components: 3,6-substituted residues of Man*p* and Gal*p*, 1,2- and 1,6-linked residues of Hex*p* (Glc*p* and/or Man*p*), 1,4-linked residues of Hex*p* (Man*p* and/or Gal*p*), 1,2- and 1,3-linked residues of Glc*p* and Gal*p*, respectively. Accordingly to polydespersity of polysaccharide fraction FoHWE-1 (Mw/Mn 1.4), we can assume presence of minor amount of glucomannan and/or glucan and/or mannan in FoHWE-1. The attachment of these substituents to the side chains to the mannofucogalactan appeared to be unlikely, but can not be completely excluded.

Thus, the three types of side chains were identified in obtained mannofucogalactan: 3-*O*-D-mannopyranosyl-L-fucopyranosyl, β-D-Gal*p*, α-L-Fuc*p* residues. The structural elements of two types of side chains were uniquely identified by NMR spectroscopy: 3-*O*-D-mannopyranosyl-L-fucopyranosyl and β-D-Gal*p*, therefore, the approximate ratio of side chains was calculated from data of methylation analysis.

The NMR and methylation analysis data revealed that the major component in water soluble polysaccharide of fruit bodies of Quinine conk *F. officinalis* was partially 3-*O*-methylated branched mannofucogalactan, in which almost every second residue was substituted at *O*-2 by 3-*O*-D-

mannopyranosyl-L-fucopyranosyl or β-D-Gal*p* or α-L-Fuc*p* residues, the mentioned units presented in an approximate ratio of 5 : 5 : 2. We propose the following repetitive unit of mannofucogalactan:



Side chains - a : b : c ~ 5 : 5 : 2

a) α-D-Man*p*-(1→3)-α-L-Fuc*p*-(1→
b) β-D-Gal*p*-(1→
c) α-L-Fuc*p*-(1 →

This structure was similar to those previously proposed for the mannofucogalactans obtained from other fungi of the *Basidiomycetes* group (Ruthes et al., 2016), and contained 1,6-*O*-galactan as backbone, in which almost every second residue was substituted at *O*-2 by side chains. 3,6-substituted residues of Gal*p* detected by GC–MS analyses in mannofucogalactans of *F. officinalis* also might be branching points of backbone mannofucogalactans of *F. officinalis*. 3-*O*-Me-α-D-Gal*p* residues were detected in mannofucogalactans as structural units of backbone. 3-*O*-Me-α-D-Gal*p* residues have been early determined in the backbone of fucogalactans (Ruthes et al., 2012, 2013b; Samanta et al., 2015) and mannogalactans (Carbonero et al., 2008b; Maity et al., 2014; Rosado et al., 2003; Smiderle et al., 2008; Silveira et al., 2015; Zhang, Xu, & Sun, 2013). Recently, 3-*O*-Me-α-D-Gal*p* residues were also determined in the structure of mannofucogalactan obtained from edible mushroom *Grifola frondosa* (Oliveira et al., 2018).

The side chains of mannofucogalactan of *F. officinalis* included single L-fucopyranosyl and/or 3-*O*-α-D-mannopyranosyl-L-fucopyranosyl residues as it was found for mannofucogalactans of other *Basidiomycetes* (Alquini et al., 2004; Cho, Koshino, Yu, & Yoo, 1998; Cho et al., 2011; Mukumoto & Yamaguchi, 1977; Ruthes et al., 2013; Shida et al., 1975; Smiderle et al., 2008b; Usui et al., 1981; Wang et al., 2014; Zhang, Xiao, Deng, He, & Sun, 2012). However some peculiar features in the structure of the side chains of mannofucogalactan of *F. officinalis* were found, specifically non-reducing terminal β-D-Gal*p*

residues were detected as side chains of this mannofucogalactan. They were found to substitute of Gal*p* residues of backbone at O-2 position. Earlier these units were detected only in fucogalactan isolated from *Agaricus bisporus*, and authors suggested that β-D-Gal*p* residues substituted of 1,6-linked α-D-Gal*p* residues of backbone at O-2 position (Komura et al., 2010; Ruthes et al., 2012, 2013b).

4. Conclusions

This study presents structure of branched partially 3-*O*-methylated mannofucogalactan as a major component of water extract. The extract was obtained by boiling water from fruit bodies of medicine fungi Quinine conk or Agarikon *Fomitopsis officinalis* (Vill.) Bondartsev & Singer, which belong to a group of xylotroph basidiomycetes, growing on stems of larch trees. Generally the structure of backbone of mannofucogalactan from *F. officinalis* was composed of 1,6-*O*-linked D-galactopyranosyl residues, in which almost every second residue was substituted at *O*-2 by 3-*O*-D-mannopyranosyl-L-fucopyranosyl, non-reducing terminal β-D-galactopyranosyl and fucopyranosyl units (approximate ratio of 5 : 5 : 2.).

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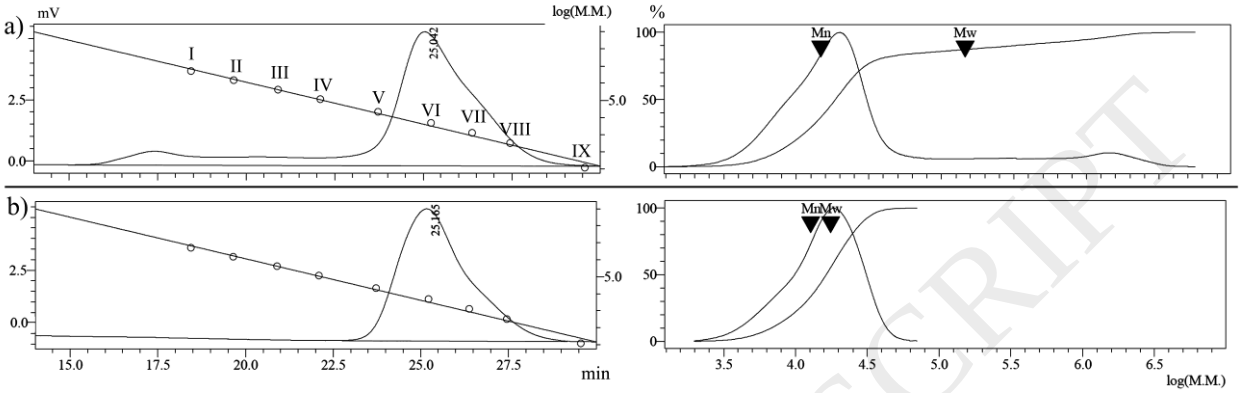

Fig. 1. HPGPC profile of fractions FoHWE (a) and FoHWE-1 (b) with calibration curve. The pullulans with Mw 800, 400, 200, 110, 50, 22, 12, 6 and 1.3 kDa were used as standards, which were designated I-IX, respectively.

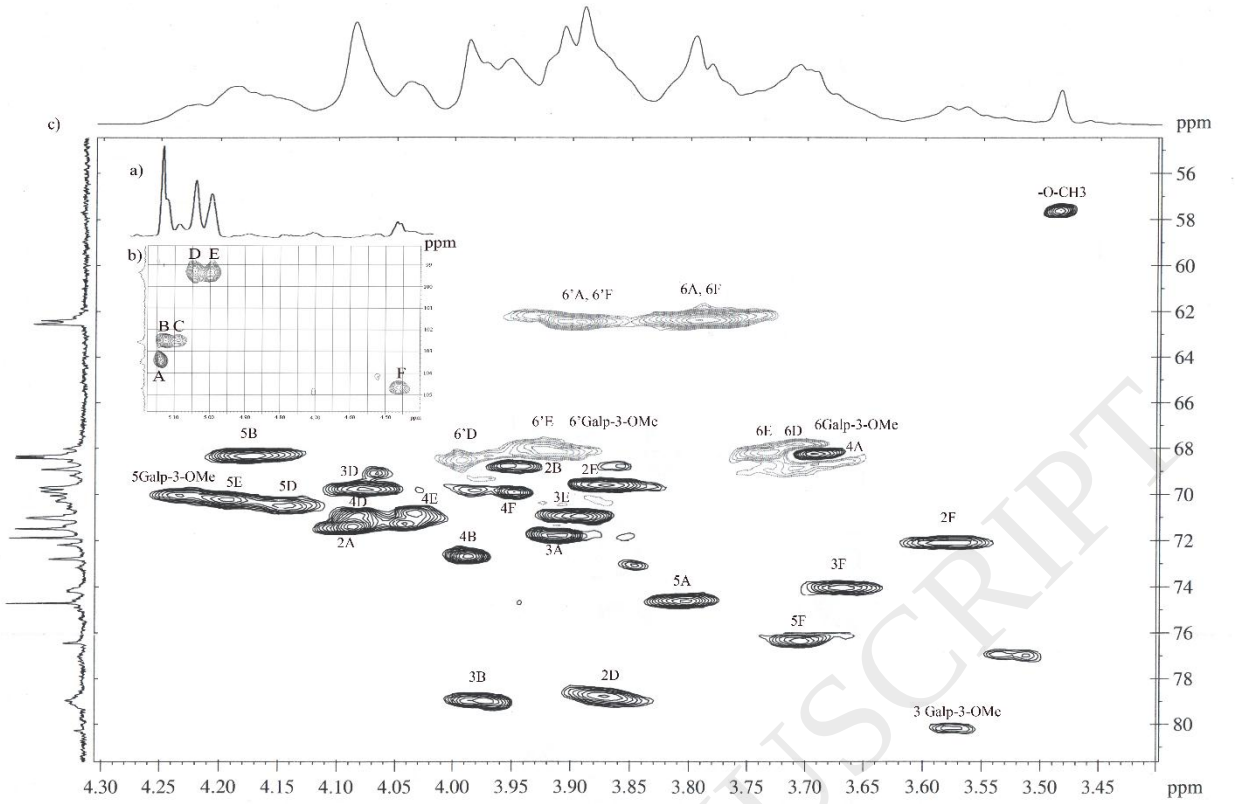

Fig. 2. The anomeric region of ^{1}H NMR spectrum (a), anomeric region (b) and C-2 – C-6 region (c) of $^{1}H/^{13}C$ HSQC spectrum of mannofucogalactan FoHWE-1. The cross-peaks were labeled by letters A–F as explained in the text.

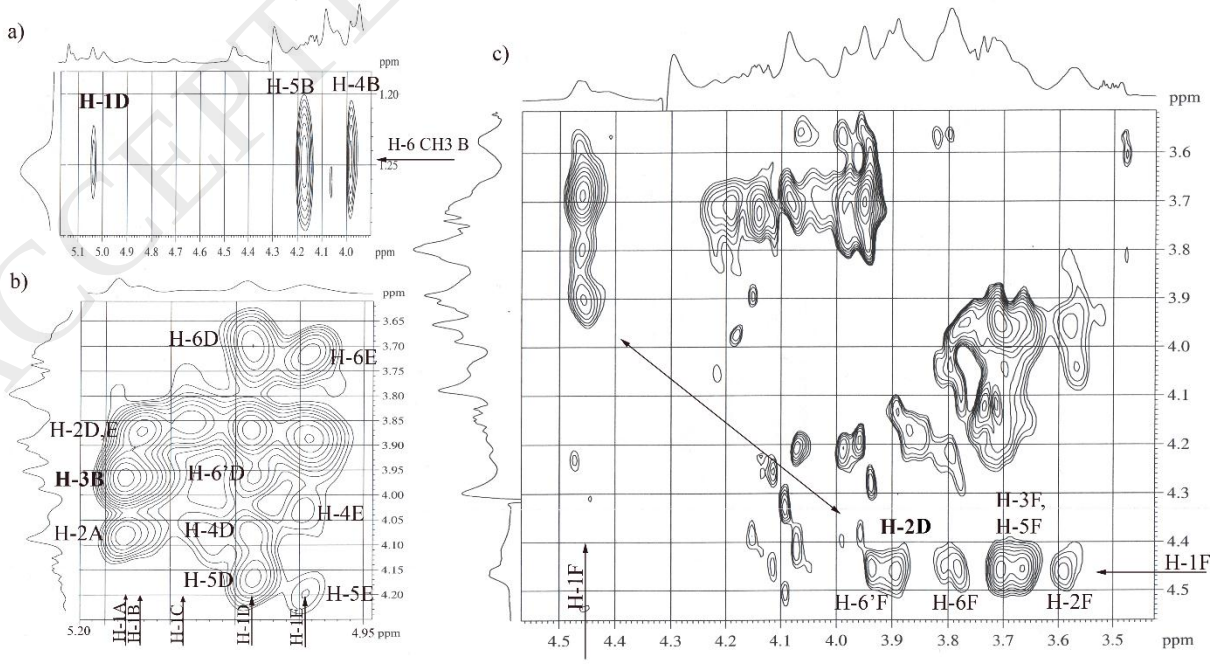

Fig. 3. The parts of anomeric region of $^{1}H/^{1}H$ ROESY spectrum of mannofucogalactan FoHWE-1 differently enlarged for better visibility of the cross-peaks showing relevant connections.

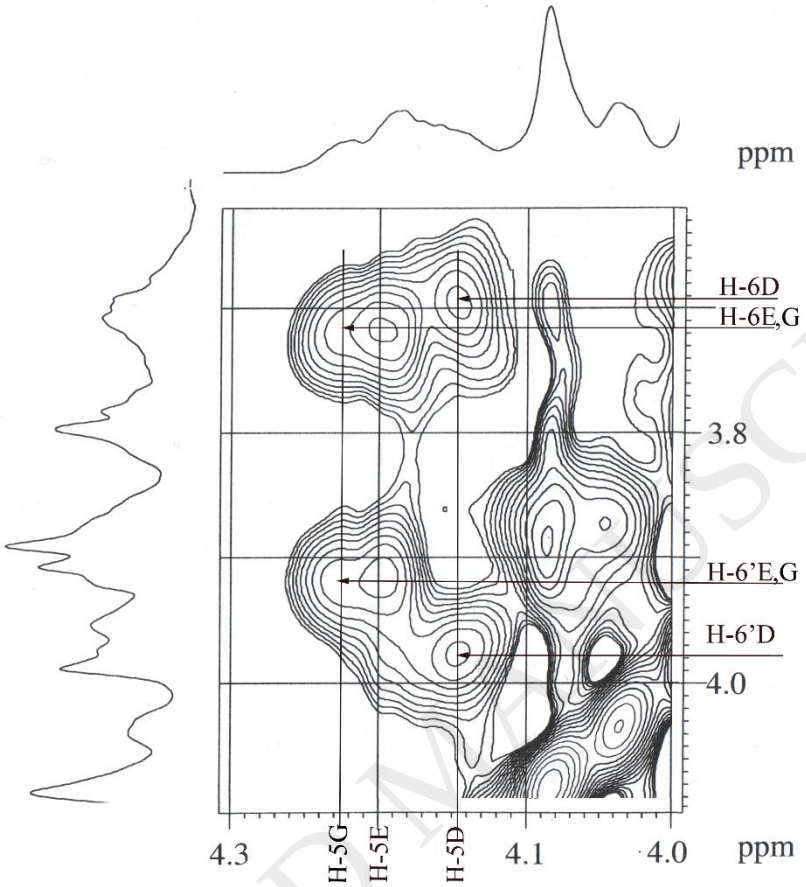

Fig. 4. $^{1}H/^{1}H$ TOCSY spectrum of cross-connections of H-5-s/H-6-s α-Gal residues region in mannofucogalactan FoHWE-1.

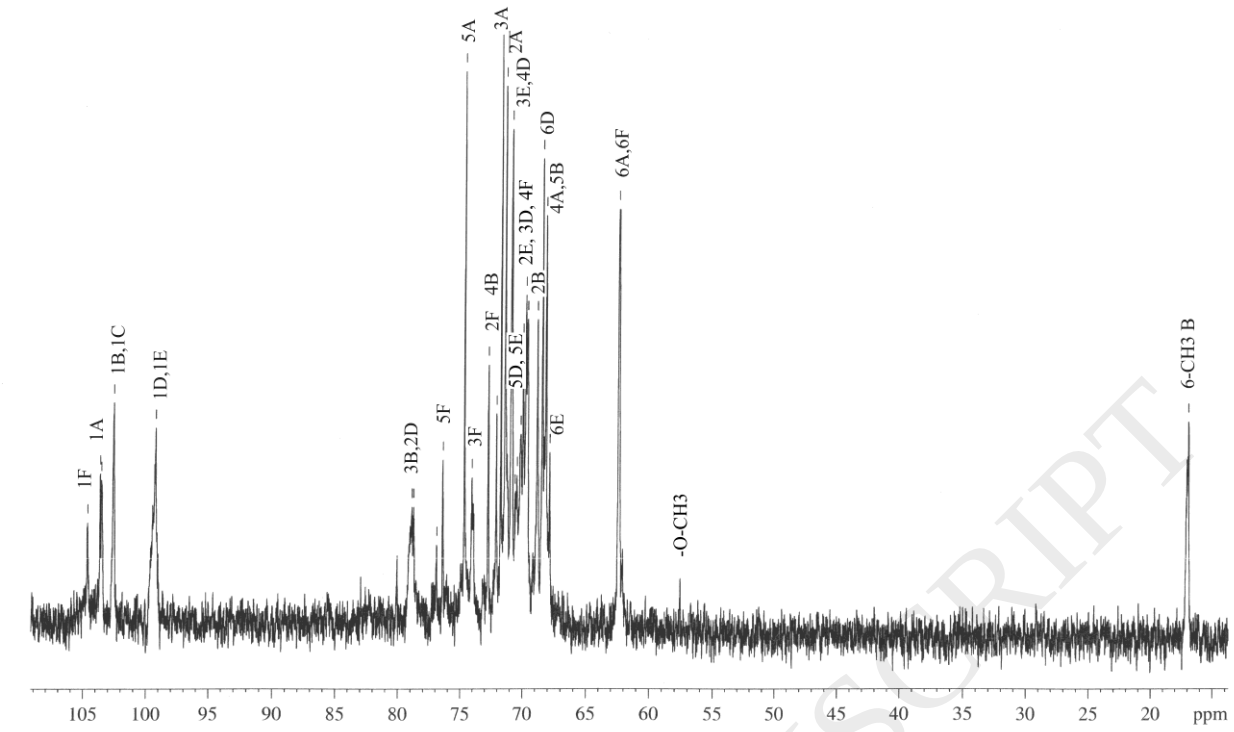

Fig. 5. ^{13}C NMR spectrum of mannofucogalactan FoHWE-1.

Table 1

Chemical characterization of the polysaccharide fractions.

PC	Yield, %	Content, % [c]						Total sugar content, %[d]	Mw, kDa	Mn, kDa	Mw/Mn
		Protein	Fuc	Xyl	Man	Glc	Gal				
FoHWE	3.68 [a]	4.2	13.7	2.0	20.2	26.3	33.5	85.3	151.7	11.2	13.6
FoHWE-1	74.7 [b]	0.0	13.1	1.4	18.6	4.1	62.8	89.7	17.4	12.1	1.4

[a] – yield of the dried material.

[b] – yield calculated from the amount of FoHWE applied to the column.

[c] – calculated as a relative percentage of sum of all monosaccharides and protein, which were detected in simples.

[d] - data were calculated as weight %.

Table 2

Chemical shifts of the signals in the ^{1}H and ^{13}C NMR spectra of the polysaccharide FoHWE-1.

Residue	^{13}C NMR chemical shifts (δ_C acetone 31.45) and ^{1}H (*italic*, δ_H acetone *2.225* ppm)						
	C-1	C-2	C-3	C-4	C-5	C-6	O-CH_3
	H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-6; H-6'*	
α-Man-(1→	103.64; 103.55	71.38	71.72	68.17	74.67	62.42	
(A)	*5.14*	*4.09*	*3.91*	*3.69*	*3.81*	*3.79;3.90*	
→3)-α-Fuc*p*-(1→	102.56	68.83	78.88	72.72	68.17	16.95 *	
(B)	*5.13*	*3.95*	*3.98*	*3.99*	*4.17*	*1.25*	
→2,6)-α-Gal*p*-(1→	99.23	78.75	69.73	70.88	70.59	68.44	
(D)	*5.05*	*3.87*	*4.09*	*4.09*	*4.16*	*3.70; 3.98*	
→6)-α-Gal*p*-(1→	99.23	69.60	70.88	70.88	70.42	68.20	
(E)	*5.00*	*3.87*	*3.89*	*4.04*	*4.20*	*3.72; 3.92*	
β-D-Gal-(1→	104.63	72.08	74.07	69.97	76.42	62.42	
(F)	*4.47*	*3.58*	*3.67*	*3.95*	*3.71*	*3.80;3.91*	
→6)-α-D-Galp-3-OMe(1→	n.d.	n.d.	80.4	n.d.	70.15	67.86	57.6
(G)			3.58		*4.24*	*3.72; 3.92*	*3.49*

n.d. – not determined;

* – for methyl group.

Table 3

Retention times and Molar % of partially *O*-methylated sugars, in the form of their alditol acetates from the hydrolyzate of permethylated mannofucogalactan FoHWE-1

RT[a], min	Methylated monosaccharide[b]	Linkage	%[c]	Abundant fragments, *m*/*z*
4.237	2,3,4-Me_3-Fuc	Fuc*p*(1→	4	71, 89, 101, 115, 117, 131, 161, 175
5.079	2,4-Me_2-Fuc	→3)Fuc*p*(1→	10	85, 89, 101, 117, 131, 159, 173, 233
5.248	2,3,4,6-Me_4-Man	Man*p*(1→	13	71, 87, 101, 117, 129, 145, 161, 205
5.434	2,3,4,6-Me_4-Gal	Gal*p*(1→	10	71, 87, 101, 117, 129, 145, 161, 205
6.083	3,4,6-Me_3-Hex	→2)Hex*p*(1→[d]	2	71, 87, 99, 101, 129, 149, 161, 189
6.116	2,4,6-Me_3-Glc	→3)Glc*p*(1→	3	71, 87, 101, 117, 129, 143, 161, 233
6.220	2,3,6-Me_3-Hex	→4)Hex*p*(1→[e]	2	71, 85, 87, 99, 101, 113, 117, 129, 131, 143, 161, 173, 233
6.298	2,4,6-Me_3-Gal	→3)Gal*p*(1→	3	71, 87, 99, 101, 117, 129, 145, 161, 189, 233
6.394	2,3,4-Me_3-Hex	→6)Hex*p*(1→[d]	2	71, 87, 99, 102, 118, 129, 161, 173, 189, 233
6.693	2,3,4-Me_3-Gal	→6)Gal*p*(1→	21	71, 87, 99, 101, 117, 129, 159, 161, 173, 189, 233
7.296	2,4-Me_2-Man	→3,6)Man*p*(1→	3	71, 87, 99, 101, 117, 127, 129, 139, 159, 173, 189, 233, 261
7.531	3,4-Me_2-Gal	→2,6)Gal*p*(1→	24	71, 87, 99, 113, 129, 159, 189, 233
7.570	2,4-Me_2-Gal	→3,6)Gal*p*(1→	3	85, 87, 97, 99, 101, 117, 127, 129, 139, 159, 173, 189, 201, 233, 261, 305

[a] – Retention time.

[b] – Determined as alditol acetates.

[c] – Molar %, calculated as a relative percentage of all present derivatives, based on the peak area with considering molar response-factors (Rf) previously determined (Sweet et al., 1975).

[d] – Hex*p*: Glc*p* or Man*p*.

[e] – Hex*p*: Man*p* or Gal*p*.