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Structural investigation and comparative cytotoxic activity of water-soluble polysaccharides from fruit bodies of the medicinal fungus quinine conk

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ABSTRACT

The structures and cytotoxic activities of water-soluble polysaccharides were investigated to search for biologically active polysaccharides from the fruit bodies of quinine conks (*Fomitopsis officinalis*). The decoctions of this medical fungus are actively used in folk medicine in many countries and traditional Chinese medicine. From the fungal extract we prepared, only branched β-glucan had cytotoxic activity among all the water-soluble polysaccharides. This glucan is characterized by a regular structure. Its backbone is formed by 1,3-linked β-D-Glc*p* residues, of which every third residue is substituted at O-6 by a single β-D-Glc*p* residue. It has a triple helix conformation according to the data obtained from a colorimetric assay with Congo red dye and is characterized by a high-weight average molar mass (Mw > 800 kDa). β-Glucan possessed cytotoxic activity against HeLa cells (IC_{50} = 318 ± 47 μg/mL) and induced the formation of apoptotic bodies around most cancer cells at a concentration of 200 μg/mL. It should be noted that extraction with boiling water, which is usually used to obtain extracts and decoctions, is unable to isolate active β-glucan. Active β-glucan can be obtained in an individual state by cold alkali extraction after dehydration of the fruit bodies and removal of the components extractable by boiling water.

1. Introduction

Basidiomycota fungi (mushrooms) have a huge impact on ecosystem functioning and are also used as food and sources of different bioactive compounds. The water-soluble fractions (extracts, decoctions) of Basidiomycota, both edible and medical, have been widely used in the treatment of diseases in traditional Chinese medicine for many centuries. The knowledge of Basidiomycota usage has been extensively applied in various folk medicines in Asia, Russia, the USA, Canada, Mexico, and Venezuela and in Traditional Chinese Medicine. Basidiomycotal fungi are incorporated into the pharmacopeia and medicine of indigenous populations worldwide, are considered important natural resources of biologically active substances for medicinal applications and provide a rich variety of active secondary metabolites and polysaccharides (Grienke et al., 2014). The immunomodulatory and antitumour activities of fungal polysaccharides are associated with β-glucans and their derivatives (Ferreira et al., 2015; Moradali et al., 2007; Paterson, 2006; Bohn and BeMiller, 1995), but biologically active heteropolysaccharides have also been found in fungi (Ruthes et al., 2016; Smiderle et al., 2008). The triple helical conformation of β-glucans and the presence of hydrophilic groups located on the outer surface of the triple helix are regarded as important structural features for their biological immunostimulatory activity (Zhang et al., 2005; Yanaki et al., 1986).

Fomitopsis officinalis (Batsch) Bondartsev and Singer (1941) (synonyms: *Boletus officinalis* Vill. (1789); *Polyporus officinalis* (Batsch) Fr. (1821); *Laricifomes officinalis* (Batsch) Kotl. and Pouzar (1957)), common names – conks of larch and quinine conk – is a genus of bracket fungi belonging to the family Fomitopsidaceae, order Polyporales (Han et al., 2016a). The name *Fomes officinalis* (Batsch) Bres. (1931) is also widely used for this fungus (Han et al., 2016b). *F. officinalis* belongs to a group of xylotrophic Basidiomycota growing on the stems of larch trees. This fungus is known for its medicinal properties, and its fruit bodies are used as a tea to stimulate the

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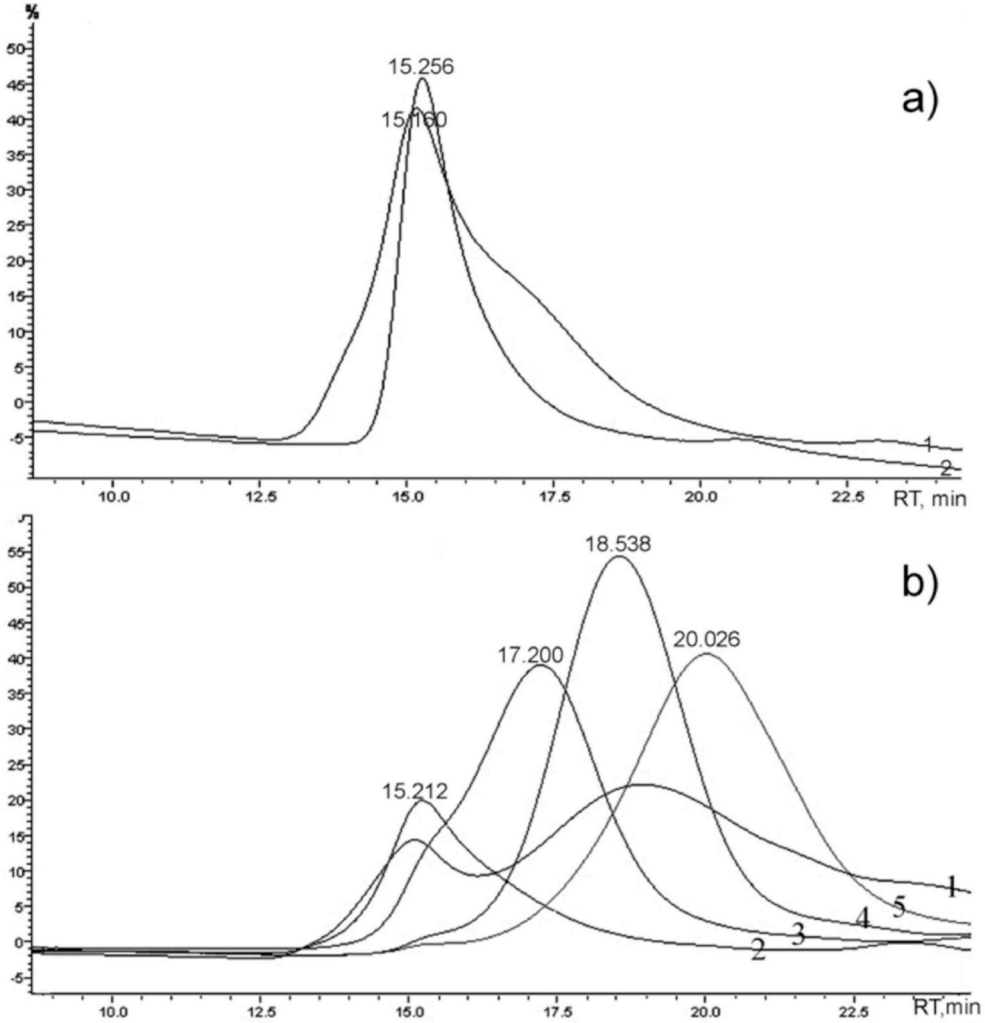


Fig. 1. HPSEC profiles of polysaccharides obtained (a) from cold alkali extraction: [1] crude polysaccharide (Mw > 800 kDa, MDD 1.7) and [2] polysaccharide FoCA obtained by SEC on a column of Sepharose CL-4B (Mw > 800 kDa, MDD 1.3); (b) from hot alkali extraction: [1] crude polysaccharide (Mw 742 kDa, MDD 19.0) and polysaccharides obtained by SEC on a column of Sepharose CL-4B: [2] FoHA1, kav 0.02; [3] FoHA2, kav 0.18; [4] FoHA3, kav 0.42; [5] FoHA4, kav 0.66.

immune system, as an anticancer agent, as antipyretic and analgesic drugs, as an anti-swelling agent or a sedative and as a medicine for the treatment of digestive system disorders. Very slow growth and the active gathering of *F. officinalis* fruit bodies has resulted in a decrease in its polypore population (Pietka, 2004). Artificial mycelium cultivation attempts and artificial inoculation of larch trees by mycelia of *F. officinalis* have been successful (Gregori, 2013). Various structural types of physiologically active triterpenes (Naranmandakh et al., 2018; Han et al., 2016b; Anderson et al., 1972) and coumarins (Hwang et al., 2013) have been previously isolated from *F. officinalis*. Nevertheless, the structural characteristics and biological activity of the polysaccharides from *F. officinalis* have not yet been investigated. The structure of mannofucogalactan extracted with water from fruit bodies of this fungus were reported recently (Golovchenko et al., 2018). Here, we present the results of the structural determination of alkali-extractable water-soluble polysaccharides and cytotoxicity data of all the water-soluble polysaccharides extracted from fruit bodies of *F. officinalis*.

2. Results and discussion

2.1. Extraction, purification and characterization of polysaccharides

The fruit bodies *F. officinalis* were defatted with hot 96% ethanol and then polysaccharides extractable with boiling water were removed. Then, the remaining polysaccharides were successively extracted with cold (at 4 °C) and hot (at 80 °C) 5% aqueous NaOH. The alkali extracts were neutralized with 36% CH_3COOH as described previously (Surenjav et al., 2006). The precipitates were removed by centrifugation. Water-insoluble polysaccharides that precipitated during neutralization were not considered here. The supernatants were treated with lead acetate to remove the proteins as described earlier (Chen et al., 2012) and decolourized with H_2O_2. The polysaccharides were precipitated from the corresponding dialyzed and evaporated extracts with an excess of ethanol.

High-performance size-exclusion chromatography (HPSEC) of the crude polysaccharides showed that both polysaccharides had a high weight average (Mw) and number average (Mn) molar mass and were characterized by a wide molar mass distribution (MMD). The crude polysaccharide obtained by hot alkali extraction was significantly more polydisperse (MMD 19.0) than that obtained by cold alkali extraction (MMD 1.7). Therefore, the crude polysaccharides were separated by size-exclusion chromatography (SEC) on a Sepharose CL-4B column. As a result, polysaccharides with a lower polydispersity were obtained (Fig. 1). The single polysaccharide FoCA was obtained from the crude polysaccharide extractable by cold alkali (Fig. 1S), and its monosaccharide composition was typical for glucans (Table 1). Four polysaccharide fractions were obtained from the crude polysaccharide extracted with hot aqueous alkali (Fig. 2S); these monosaccharide compositions were similar, and second and third fractions (FoHA2 and

Table 1
Characterization of the polysaccharides obtained by SEC on a Sepharose CL-4B.

PC	Yield, %[a]	Mw, kDa	MMD	Content, %						
				Protein[b]	Fuc[c]	Xyl[c]	Man[c]	Glc[c]	Gal[c]	Σ(sugar)[b]
FoCA	2.17 (77.4)	>800	1.3	3.18	0.96	0.11	0.61	97.29	1.03	98.19
FoHA1	0.11 (9.0)	>800	1.6	0.91	2.16	2.50	6.48	86.51	2.36	96.76
FoHA2	0.14 (11.5)	>800	1.7	1.00	4.73	5.85	14.41	73.95	1.05	89.12
FoHA3	0.41 (33.5)	411	1.7	1.98	4.76	5.81	14.44	73.94	1.06	92.47
FoHA4	0.27 (22.5)	219	2.8	2.51	1.31	2.53	5.13	90.88	0.14	79.84

[a] per weight of dry fruit bodies of fungus (per weight of crude polysaccharides taken for SEC, are given in parentheses).
[b] wt %.
[c] mol %.

FoHA3) had identical monosaccharide compositions (Table 1). Glucose and mannose residues were the major components of all these fractions. FoHA3 (from the obtained polysaccharides) was characterized by greater homogeneity, and due to its highest yield, it was chosen for biological and structural studies.

The polysaccharides contained small amounts of strongly bound protein; we failed to remove protein after treatment with lead acetate and SEC.

NMR spectroscopy was used to determine the structure of polysaccharides from FoCA and FoHA3. The signals were assigned using the $^{1}H,^{1}H$ 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 absolute D-configurations of the Man*p* and Glc*p* residues were adopted from genetic considerations since L-Man*p* and L-Glc*p* have not been found so far in fungi. The absolute L-configuration of Fuc*p* was found based on the regularities reported previously (Lipkind et al., 1988; Shashkov et al., 1988).

The β-configurations of Glc*p* and Xyl*p* residues were determined from the up-field signals of H-1 (Figs. 3S and 4S) and down-field signals of C-1 (Figs. 2 and 5S) (Oshima et al., 2006; Jansson et al., 1989; Hall and Johnson, 1969). The β-configuration of Glc*p* was confirmed by the chemical shift of C-5 at approximately δ_C 77 ppm (Jansson et al., 1989; Lipkind et al., 1988). The down-field signals of H-1 (Fig. 4S) and up-field signals of C-1 (Fig. 5S) confirmed the α-configuration of Fuc*p* and Man*p* residues (Nyman et al., 2016; Jansson et al., 1989). The mannose residues behave differently than other hexoses due to the equatorial orientation of H-2 (Jansson et al., 1989). However, according to our observations, the position of H-1 of β-sugars can not be more down-field then δ_H 5.00 ppm for any type of substitution. Additionally, the α-configuration of Man*p* was confirmed by chemical shifts of C-5 at δ_C 74 ppm (for β-configuration it would be about 77 ppm) (Jansson et al., 1989).

2.2. Structural studies of β-D-glucan FoCA

Two intense signals at δ_C 102.83 and 102.76 ppm were detected in the anomeric region of the ^{13}C spectrum (Fig. 2). The HSQC spectrum (Fig. 3) of glucan FoCA contains two distinct cross peaks in the anomeric region of $_{/H}$ 102.83/4.54 ppm, which includes anomeric signals of two different residues: →3)-β-Glc*p*-1→3 (**A**) and →3,6)-β-Glc*p*-1→3 (**B**); and at $\delta_{C/H}$ 102.83/4.23 ppm corresponding to β-Glc*p*-1→6 (**C**). Two 1,3-*O*-β-D-Glc*p* residues (**A**) with different positions of C-2 and C-3 (Table 2, Figs. 2 and 3) were identified in the NMR spectra. These differences in the chemical shifts of C-2 and C-3 occurred due to their different environments of the 1,3-*O*-β-D-Glc*p* or 3,6-*O*-β-D-Glc*p* residues, respectively.

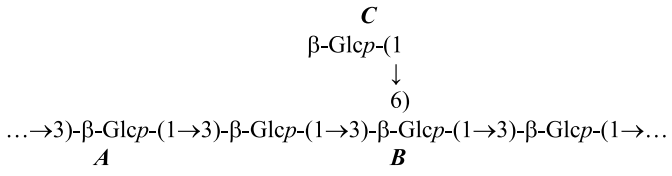
Assignments of the protons of the individual carbohydrate residues were determined by the TOCSY and COSY spectra. On the basis of the proton assignments, a computerized approach of the structural analysis of polysaccharides (Lipkind et al., 1988) and comparison with data on glucan structures previously reported (Samuelsen et al., 2019; de Jesus et al., 2018; Yan et al., 2018; Hreggvidsson et al., 2011; Smiderle et al., 2006; Tabata et al., 1981), the correlations of carbons and protons were identified in the $^{1}H,^{13}C$ HSQC spectrum (Fig. 3), and the chemical shifts are presented in Table 2.

Additionally, β-glycosidic configuration of the residue **C** was confirmed by a coupling constant $J_{C\text{-}1,H\text{-}1}$ 160.3 Hz observed in the HMBC spectrum and a large coupling constant value $^{3}J_{1\text{-}2}$ 7.6 Hz.

Important inter-residual correlations in β-D-glucan along with some intra-residual contacts were revealed from the ROESY (Fig. 6S) and HMBC (Fig. 4) spectra.

The NMR data were supported by methylation analysis. The polysaccharide FoCA was methylated by the Ciucanu and Kerek procedure (Ciucanu and Kerek, 1984) and converted into mixtures of partially *O*-methylated alditol acetates, which were analysed by GC–MS using characteristic retention times (T-values) (Sims et al., 2018; Bjorndal et al., 1970) and molar response factors (Rf) previously determined for the partially methylated alditol acetates of the individual constituents (Sweet et al., 1975). Three units were identified in FoCA as a non-reducing terminal, a 1,3-linked and a 3,6-di-O-substituted glucose residue, and according to their peak areas, the ratio of these units was found to be 1:2:1, respectively. The methylation analysis showed that the glucose residues were pyranoses.

Thus, the NMR and methylation analysis data revealed that the water-soluble polysaccharides from the cold alkali extract of the fruit bodies of the quinine conk represent branched β-glucans with a 1,3-*O*-β-Glc*p* backbone in which every third residue is substituted with a single β-Glc*p* residue at *O*-6 as given below:



Branched β-D-glucans with a backbone formed with 1,3-*O*-β-D-Glc*p* residues and partially substituted at *O*-6 by single β-D-Glc*p* residues have been found for most fungal cell walls, and the degree of substitution varies depending on the species (Chen and Seviour, 2007; Kogan, 2000). Structures of glucans with side chains formed by oligosaccharide chains have also been found in several fungi (Amaral et al., 2008; Methacanona et al., 2005). The degree of backbone substitution and the length of side chains appear to be very important factors in determining the conformation and biological activities of the glucans (Bao et al., 2001).

2.3. Structural studies of polysaccharides of FoHA3

The broad peaks from anomeric protons were observed in the ^{1}H NMR spectrum (Fig. 4S) of fraction FoHA3 for which spin systems C-1/

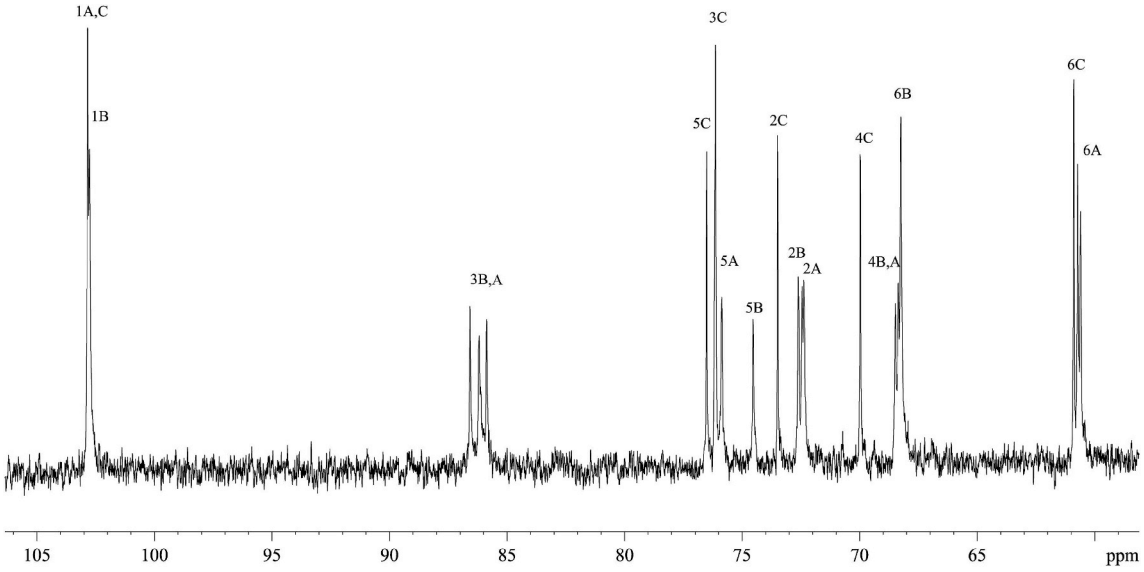


Fig. 2. The ^{13}C NMR spectrum of β-glucan FoCA.

H-1 were detected in the ^{1}H,^{13}C HSQC spectrum (Fig. 5). The cross peaks at $\delta_{C/H}$ 103.8/4.78 and 104.1/4.55 ppm belongs to Glc*p* residues of β-glucan, which similar β-glucan FoCA. The assignments of cross peaks other residues were as follows: $\delta_{C/H}$ 102.5/5.17 ppm, 1,3-*O*-α-D-Man*p* (residues **D**); $\delta_{C/H}$ 103.2/5.14, 3,4-*O*-α-D-Man*p* (residues **E**); $\delta_{C/H}$ 102.7/4.46 ppm, 1,2-*O*-Xyl*p* (residues **F**); and $\delta_{C/H}$ 100.6/5.32, non-reducing terminal α-Fuc*p* (residues **G**) (Table 3). In addition, in the anomeric region of the HSQC spectrum, the signal of low intensity at $\delta_{C/H}$ 105.0/4.43 ppm of the non-reducing terminal of β-Xyl*p* residues was detected, for which only the correlation of H-1,H-2 at $\delta_{H,H}$ 4.43/3.30 ppm was detected in the TOCSY spectrum.

On the basis of the proton assignments of the individual sugar residues determined by the TOCSY and COSY spectra, the computerized approach to the structural analysis of polysaccharides (Lipkind et al., 1988; Shashkov et al., 1988), and comparison with the data reported earlier (Samuelsen et al., 2019; Hreggvidsson et al., 2011), the C/H correlations were detected in the HSQC spectrum (Fig. 5) and their assignments are listed in Table 3.

The single correlation peak of H-1, H-2 at $\delta_{H,H}$ 5.32/3.84 ppm was detected in the TOCSY and COSY spectra for residue **G**. The positions C-4, C-5 were identified from their cross peaks with protons of methyl group in the HMBC spectrum. The position of H-3 failed to be detected in the COSY, TOCSY and HMBC spectra, but according to the regularities reported previously (Lipkind et al., 1988; Shashkov et al., 1988) for non-reducing terminal α-D-Fuc residues, the chemical shift of C-3 should be equal to δ_C 71.2 ppm.

The type substitution of residues **D**, **E**, and **F** were identified from the down-field positions of the signals of C-3 of residue **D** at δ_C 79.6 ppm, C-3 and C-4 of residue **E** at δ_C 77.0 and δ_C 73.9 ppm, as respectively, and C-2 of residue **F** at δ_C 79.5 ppm, compared with

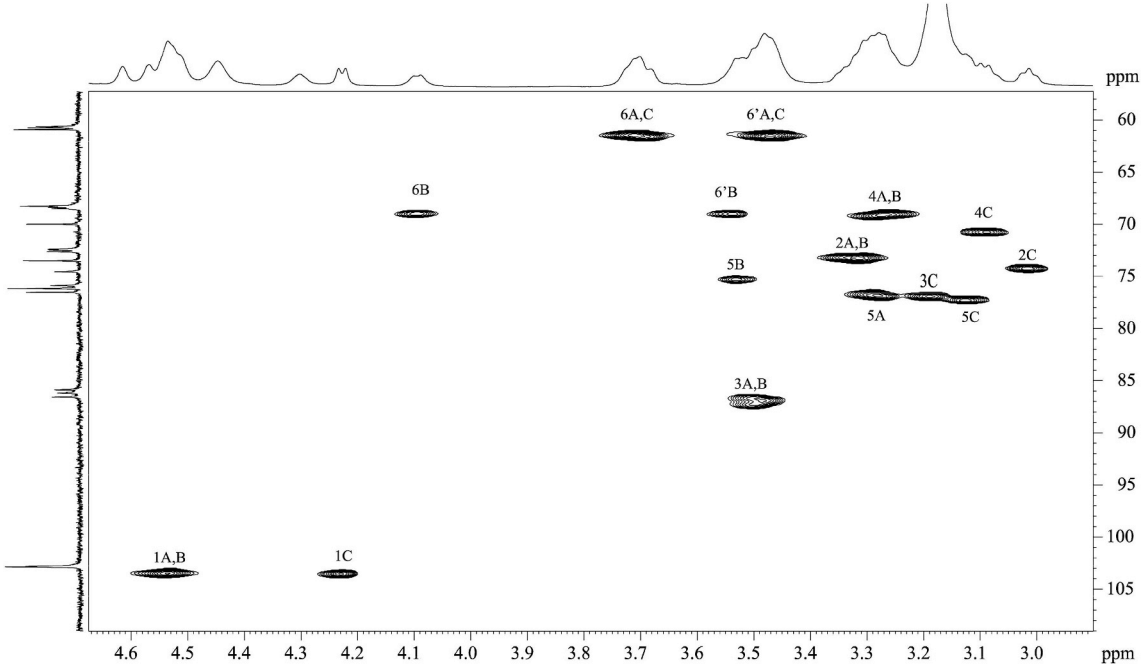


Fig. 3. The ^{1}H,^{13}C HSQC spectrum of β-glucan FoCA. The corresponding parts of the ^{1}H and ^{13}C NMR spectra shown at the top and left of the spectrum, respectively. Arabic numerals belong to carbon numbers in residues indicated by Latin letters in accordance with Table 2.

Table 2
Chemical shifts of the signals in the ^{1}H and ^{13}C NMR spectra of the FoCA (333 K, DMSO-d_6, δ_H 2.5, δ_C 39.5).

Residue	Position	δ_C, ppm	δ_H, ppm	ROESY	HMBC[b]
→3)-β-Glc*p*-(1→3 **(A)**	1	102.83	*4.54*	3 A, 3 B, 5 A	3 B
	2	72.47 (72.37)	*3.31*		
	3	85.87;86.57[a]	*3.50*		1 B
	4	68.37	*3.30*		
	5	75.88	*3.28*		
	6; 6′	60.74 (60.61)	*3.47;3.71*	6′A, 5 A	
→3,6)-β-Glc*p*-(1→3 **(B)**	1	102.76	*4.51*	3 B, 3 A, 5 B	3 A
	2	72.62	*3.26*		
	3	86.18;86.57[a]	*3.49*		1 A
	4	68.49	*3.29*		
	5	74.54	*3.53*		
	6; 6′	68.27	*3.54;4.10*	6′ B	1C
β-Glc*p*-(1→6 **(C)**	1	102.83	*4.23*	2C, 3C, 5C, 6' B	6 B
	2	73.50	*3.02*		
	3	76.15	*3.19*		
	4	70.00	*3.09*		
	5	76.52	*3.12*	5C, 6′C	
	6	60.90	*3.47;3.69*		1C

[a] Depending on substitution in the neighboring residues.
[b] Inter-residual correlation peaks.

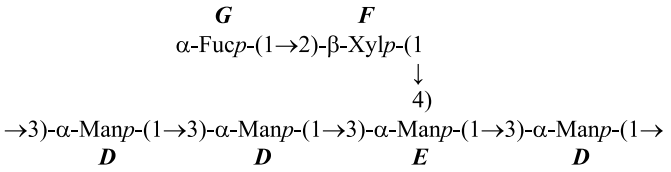
corresponding unsubstituted residues (Fig. 5 and 5S).

The ROESY spectrum (Fig. 7S) was used also to confirm type substitution of residues and to establish the following sequences of glycosyl residues of the fucosylated xylomannan from FoHA3. The high intensity of the correlation peak of H-1/H-3 at $\delta_{H/H}$ 5.15/4.03 ppm indicates inter-residual contacts between 1,3-*O*-α-D-Man*p* residues and confirms the substitution of 1,3-*O*-α-D-Man*p* residues at *O*-3 by 3,4-*O*-α-D-Man*p* residues. The correlation peak at $\delta_{H/H}$ 4.46/3.90 ppm identifies the substitution of 3,4-O-α-D-Man*p* residues at *O*-4 by 1,2-O-β-Xyl*p* residues, whose substitution by terminal α-Fuc*p* residues is confirmed correlation peak at $\delta_{H/H}$ 5.32/3.46 ppm.

Evidence that mannan chains are incorporated into β-D-glucan chains has not been obtained. FoHA3 is evidently a mixture of β-glucan (or β-glucans) and fucosylated 1,2-β-xylo-1,3-α-mannan. Similar compositions and the presence of substantial amounts of Man*p* and Glc*p* units in all four fractions obtained by SEC on Sepharose (Table 1) confirmed the presence of complex polymer associations, and SEC appeared unable to separate this mixture.

NMR spectroscopy analyses revealed the presence of not only 1,3-linked β-D-Glc*p* residues but also the presence of oligomers with 1,6-linked of β-D-Glc*p*, while β-D-glucan FoCA included only 1,3-linked β-D-Glc*p* residues. Methylation analysis data confirmed the presence of 1,6-linked β-D-Glc*p* residues and other residues identified by NMR spectroscopy. Additionally methylation analysis data confirmed and expanded the NMR spectroscopy analysis data. It showed that all sugar components of FoHA3 were pyranoses and were mainly present as non-reducing terminal Xyl*p*, Fuc*p*, Hex*p* residues; 1,2-linked Xyl*p* residues; and 1,3-, 1,6-linked and 3,6- and 3,4-di-*O*-substituted Hex*p* residues (Table 4). GC analysis of partially *O*-methylated alditol acetates obtained from FoHA3 spiked with 2,6-Me_2-Glc allowed us to identify the problematic peak due to lack of a complete match as 2,6-Me_2-Man, so the corresponding residue of 3,4-di-*O*-substituted Hex*p* is Man*p*.

Thus, the proposed structure of fucosylated 1,2-β-xylo-1,3-α-D-mannan is as follows:



The similar fucoxylomannan has been isolated earlier from the alkali extract of fruit bodies of the *Fomes annosus*. However, configuration of the glycosidic linkage between Man*p* residues (was determined from optical rotation) was identified as β (Axelsson et al., 1971). Recently, similar structural units were detected in the water-soluble fraction

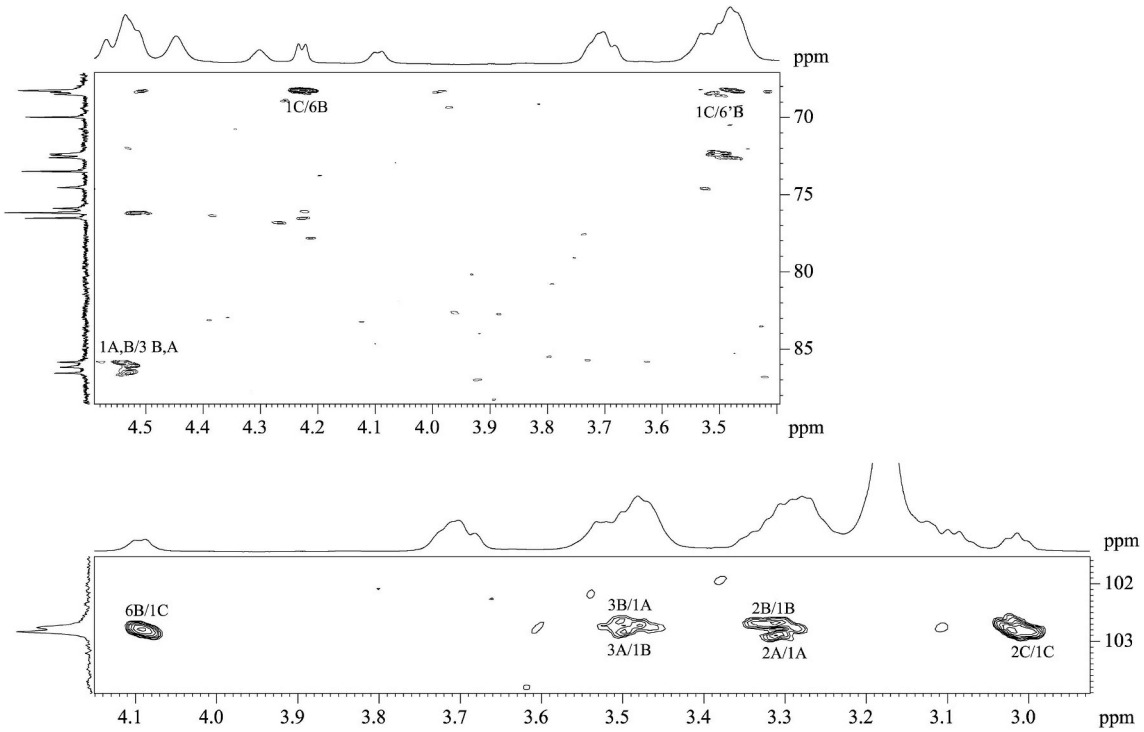


Fig. 4. The ^{1}H,^{13}C HMBC spectrum of β-glucan FoCA. The corresponding parts of the ^{1}H and ^{13}C NMR spectra shown at the top and left of the spectrum, respectively. Arabic numerals before slash belong to proton, and Arabic numerals after slash belong to carbon in the residues indicated by Latin letters in accordance with Table 2.

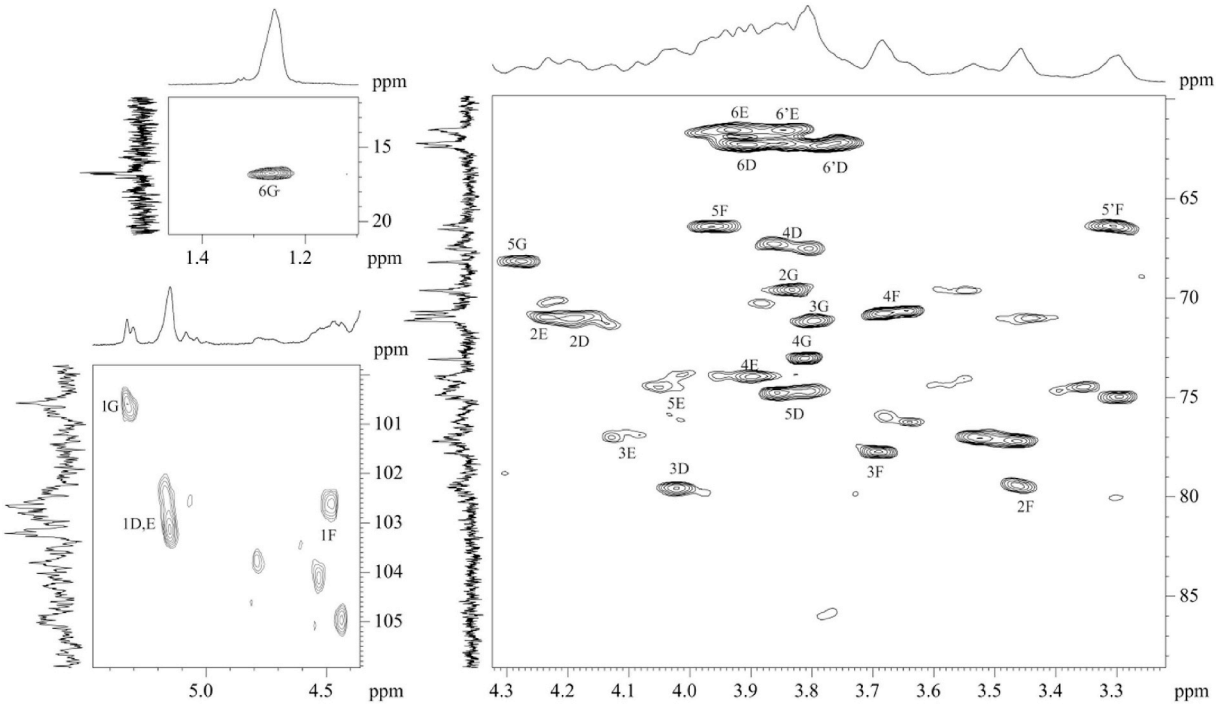


Fig. 5. The $^{1}H,^{13}C$ HSQC spectrum of β-glucan FoHA3. The corresponding parts of the ^{1}H and ^{13}C NMR spectra shown at the top and left of the spectrum, respectively. Arabic numerals belong to carbon in residues indicated by Latin letters in accordance with Table 3. Non-designated correlation peaks belong to β-glucan.

Table 3
Chemical shifts of the signals in the ^{1}H and ^{13}C NMR spectra of the fucosylated xylomannan in FoHA3 (340 K, D_2O, TSP, δ_H 0.0, δ_C −1.6).

Residue	Position	δ_C, ppm	δ_H, ppm	ROESY
→3)-α-Man*p*-(1→	1	102.5	5.17	2D, 3D
(***D***)	2	71.0	4.20	
	3	79.6	4.03	
	4	67.3; 67.5[a]	3.86; 3.82	
	5	74.8	3.86; 3.81	
	6; 6′	62.4	3.91; 3.78	
→3)-α-Man*p*-(1→	1	103.2	5.14	2 E, 3D
4)	2	71.0	4.23	
↑	3	77.0	4.13	
(***E***)	4	73.9	3.90	
	5	74.4	4.05	
	6; 6′	61.6	3.93; 3.85	
↑	1	102.7	4.46	5′F, 2 F, 3 F, 4 E, 3 E
→2)-β-Xyl*p*-(1	2	79.5	3.46	
(***F***)	3	77.8	3.69	
	4	70.7	3.65	
	5; 5′	66.3	3.97; 3.31	
α-Fuc*p*-(1→	1	100.6	5.32	2G, 2 F
(***G***)	2	69.6	3.84	
	3	71.2	3.80	
	4	73.1	3.81	
	5	68.2	4.28	
	6	16.8	1.27	

[a] Depending on substitution in the neighboring residues.

obtained in re-dissolved polysaccharides isolated from alkali extracts of the edible fungus *Albatrellus ovinus*. β-D-Glc*p*, 1,3-O-α-D-Man*p*, and a small amount of terminal and 1,2-O-β-D-Xyl*p* were also found in the structure of the polysaccharide fraction from *A. ovinus*. However, authors failed to reveal the relation of the xylan chains to mannan and/or glucan (Samuelsen et al., 2019). It is worth noting that mannans are not usual for basidiomycetes in contrast to yeasts, where mannans are an indispensable component of cell walls (Križková et al., 2001; Kogan et al., 1990). Xylomannans have been identified in the alkali-soluble fractions of only some Basidiomycetes: *Armillaria mellea* (Bouveng et al., 1967), *Polyporus tumulosus* (Angyal et al., 1974), *Coprinus macrorhizus* var. *microsporus* (Bottom and Siehr, 1979), and *Flammulina velutipes* (Smiderle et al., 2006). They have similar structures, and their backbone is composed of 1,3-linked α-Man*p* residues partially substituted at O-4 with single Xyl*p* residues and/or chains formed by Xyl*p* residues with different linkages. It should be noted that both in the previous studies and in the present study, xylomannans were coextracted with glucans. These polymers appeared to be associated with the cell wall (as in yeast cell walls) and may have a structural role in fungi (Bouveng et al., 1967). It is not clear why mannans (xylomannans) are found only in some Basidiomycetes.

2.4. Cytotoxic activities of glucan and heteropolysaccharides and determination of their tertiary structures

The cytotoxic effects of the polysaccharides extracted from fruit bodies of *F. officinalis* were assessed *in vitro* for their anticancer activities against human cancer cells. The activities of three isolated polysaccharides FoCA, FoHWE or FoHA3 were examined by MTT assay using HeLa cells with 50-fold range of polysaccharide concentrations (50–1000 μg/mL). The results showed that only FoCA was cytotoxic and had an IC_{50} = 318 ± 47 μg/mL and IC_{90} of 520 ± 42 μg/mL (Fig. 6). In contrast, neither FoHWE nor FoHA3 failed to inhibit the viability of HeLa cells at any concentration used.

Cell viability after treatment with the polysaccharides FoCA, FoHWE and FoHA3 was examined by fluorescence microscopy using simultaneous staining of the cells with propidium iodide and acridine orange. Fig. 7a-d shows the results of the cell incubation with the test substances at 500 μg/mL. Only FoCA was adverse to the cells. Most cells were detached from the substrate, and the nuclei of the remaining cells showed a red color, indicating the cell death. The differences from the control failed to be detected by fluorescence microscopy, and when the concentration of FoCA was lowered to 200 μg/mL (Fig. 7e) we observed apoptotic bodies (vesicles) around most of the cells (Fig. 7f). The other two tested polysaccharides did not produce any changes in the HeLa

Table 4
Retention times and molar % of partially *O*-methylated, *O*-acetylated alditol acetates prepared from the hydrolyzate of permethylated polysaccharide fraction FoHA3.

RT[a], min	Methylated monosaccharide[b]	Linkage	%[c]	Abundant fragments, *m/z*
3.976	2,3,4-Me_3-Xyl	Xyl*p*-(1→	4	71,101,117,161
4.236	2,3,4-Me_3-Fuc	Fuc*p*-(1→	9	72,89,101,115,131,161,175
4.871	3,4-Me_2-Xyl	→2)-Xyl*p*-(1→	7	71,87,101,117,129,189
5.228	2,3,4,6-Me_4-Hex	Hex*p*-(1→	21	71,87,101,117,129,145,161,205
6.115	2,4,6-Me_3-Hex	→3)-Hex*p*-(1→	50	71,87,99,101,117,129,161,233
6.387	2,3,4-Me_3-Hex	→6)-Hex*p*-(1→	3	71,87,101,117,129,161,189,233
6.763	2,6-Me_2-Hex	→3,4)-Hex*p*-(1→	7	87,101,117,129,139,159,189,233
7.291	2,4-Me_2-Hex	→3,6)-Hex*p*-(1→	10	71,87,99,101,117,129,139,159,189,233

[a] retention time.
[b] determined as alditol acetates.
[c] molar %, calculated as a relative percentage of all derivatives, based on peak areas corrected with corresponding response factors (Rf) taken from Sweet et al. (1975).

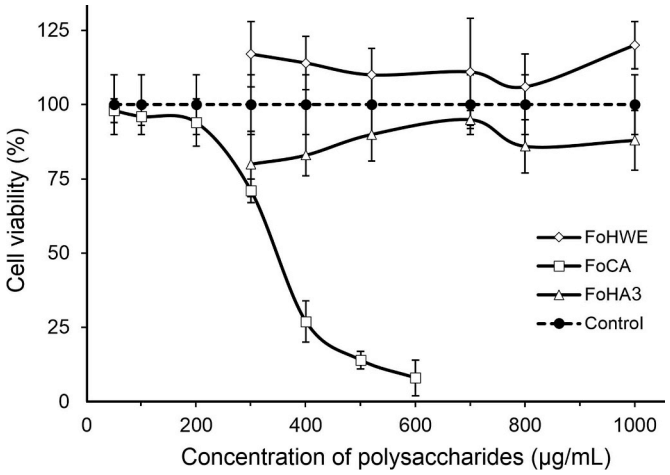


Fig. 6. Results of the MTT assay for HeLa cells after 24-h incubation with polysaccharides from fruit bodies of *F. officinalis* (FoHWE, FoHA3, and FoCA) at different concentrations.

cells either at 500 μg/mL or at 800 μg/mL (data not shown). The IC_{50} value for FoCA glucan was 318 ± 47 μg/mL, which indicated high activity of this polysaccharide compared to that of the 1,3-β-glucans of other fungi. 1,3-β-Glucans have the greatest biological activity among fungal polysaccharides (Villares et al., 2012; Kitamura et al., 1994), and the activity of other fungal glucans towards cancer cell cultures varies greatly (IC_{50} = 165–2000 μg/mL) (Khan et al., 2019).

The tertiary structure of polysaccharide molecules was detected by the interaction with Congo red dye (Semedo et al., 2015) because the triple helix conformation of β-glucans is regarded as an important structural feature for their activity (Zhang et al., 2005; Bohn and BeMiller, 1995).

The incorporation of Congo red dye into the triple helix, possibly owing to hydrogen bond formation with free hydroxyls, leads to a bathochromic shift (Ogawa et al., 1972). As a result, the maximum absorption wavelength (λ_{max}) shifted from 492 to 505 nm for FoCA, to 501 nm for FoHA3, and FoHWE did not produce a bathochromic shift with Congo red (Fig. 8S). Thus, polysaccharides containing FoCA and FoHA appeared to have triple helix conformations according to the data obtained from the colorimetric assay with Congo red dye. However, only 1,3-β-glucan FoCA was cytotoxic against HeLa cells at a concentration of 300 μg/mL and higher. 1,3-β-D-Glucan was identified in both FoHA3 and FoCA. The difference in FoHA3 from FoCA exhibited

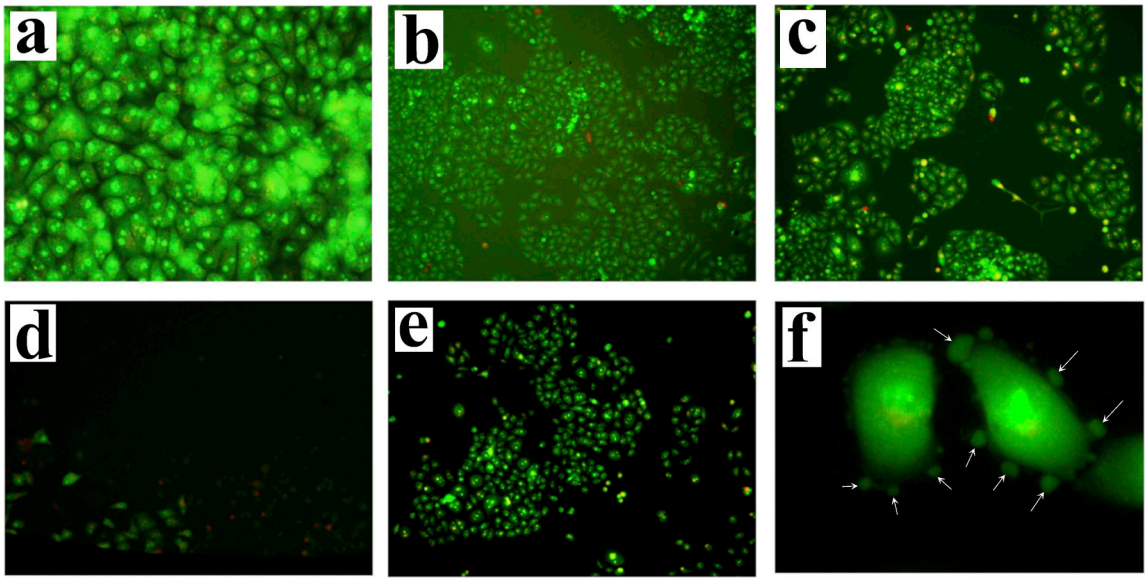


Fig. 7. HeLa cells stained with acridine orange and propidium iodide after 24-h incubation: a, control; b, with FoHWE at 500 μg/mL; c, with FoHA3 at 500 μg/mL; d, with FoCA at 500 μg/mL; e, with FoCA at 200 μg/mL (× 50 magnification); f, cells with membrane vesicles (apoptotic bodies) after incubation with FoCA at 200 μg/mL (× 200 magnification). Apoptotic bodies are shown by arrows (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

by the inclusion 1,6-β-D-glucan and 1,3-α-D-mannan in the former. Notably, 1,3-α-mannan, having a backbone conformation similar to that of 1,3-β-glucan, has shown antitumour action comparable to that of 1,3-β-glucan (Zhang et al., 2007). High molecular weight FoCA (Mw > 800 kDa) (in comparison with FoHA3, Mw 411 kDa) appeared to play an important role in the anticancer activity. The activity of β-glucans strongly depends on their molecular weight. Generally, high molecular weight glucans (ranging from 500 to 2000 kDa (Mizuno et al., 1996)) are more effective in suppressing tumour cell growth of low molecular weight glucans (Meng et al., 2016; Zong et al., 2012; Zhang et al., 2007).

The mechanisms of action of fungal polysaccharides on cancer cells is pleiotropic (Khan et al., 2019) and includes cell cycle arrest, depolarization of the mitochondrial membrane, nitric oxide biosynthetic pathways, and the activation of immune processes (production of TNF-α, cytokines, and other immunomodulators) (Meng et al., 2016). For example, a *Pleurotus nebrodensis* polysaccharide acts via cell cycle arrest at the G2/M phase and the produces cytokines (Cui et al., 2014). In our experiments, exposure of HeLa cells to 200 μg/mL FoCA 1,3-β-glucan gave rise to apoptotic bodies around the cells (Fig. 7f). We suppose that this effect is related to the ability of 1,3-β-glucan to arrest the cell cycle. The formation of apoptotic bodies at concentrations below the IC_{50} has been described earlier for three *Cordyceps militaris* polysaccharides and explained by cell cycle arrest at the G_0/G_1 phase (Chen et al., 2015).

Our results suggested that the fruit bodies of *F. officinalis* are a source of a high molecular weight, water-soluble 1,3-β-glucan possessing cytotoxic activity. However, extraction with boiling water (used for the extract and decoction preparation) appeared to be inappropriate, and β-glucan can be obtained in its pure form by cold alkali extraction after the removal of components extractable by boiling water. In our opinion, β-glucan from *F. officinalis* fruit bodies is a promising substance for use in anticancer therapy. However, further *in vitro* and *in vivo* studies are needed to determine of the biochemical and physiological mechanisms of its anticancer effects and its ability to stimulate the immune system.

3. Experimental

3.1. Biological material

The fruit bodies of *F. officinalis* 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.

3.2. Isolation and purification of polysaccharides

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 the soluble lipids, low molar. weight sugars, and phenolic compounds. The supernatant was removed by filtration and the remaining residue was air-dried. The polysaccharides were extracted successively with boiling water under reflux for 4 h, cold (at 4 °C) and hot (at 80 °C) 5% (w/v) aqueous solution NaOH containing 0.5% (w/v) $NaBH_4$ for 24 h and 6 h, respectively. Treatment with each extractant was performed twice. The residue after each extraction was separated by centrifugation and the corresponding extracts were collected and combined. Finally, the aqueous extract (3 L), cold alkali extract (1 L) and hot alkali extract (1 L) were obtained. The alkali extracts were neutralized with 36% (v/v) aqueous CH_3COOH and the precipitate was removed by centrifugation as described previously (Surenjav et al., 2006). Aqueous lead acetate (2%, w/v) was added to the extracts to a final concentration of 0.4–0.5% (w/v) for deproteinization (Chen et al., 2012). The extract was decolourized by the addition of 30% H_2O_2 to a final concentration of 5%, and 10% (w/v), aqueous NH_4OH was added to increase the pH to 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 for 48 h at 10 °C and concentrated on a Heidolph 4002 rotary evaporator (Germany) under reduced pressure at 40 °C. Then, the polysaccharides were precipitated with 95% ethanol, dissolved in water after centrifugation and lyophilized on a VirTis freeze-drier (USA) under a constant vacuum of < 10 mTorr at −65 °C. All precipitates were isolated/obtained by centrifugation at 7000 rpm and 4 °C for 10–20 min on a Sigma 6 K 15 centrifuge with rotor N 12,256 (USA). Finally, three water-soluble crude polysaccharide fractions were obtained by hot water extraction, cold alkali extraction and hot alkali extraction. The yields of the polysaccharide fractions obtained by cold alkali extraction and hot alkali extraction were 5.60 g and 2.42 g, or 2.8% and 1.21%, from the dry fruit bodies, respectively. The structure of mannofucogalactan as the main component of the hot water extract was reported in our previous paper (Golovchenko et al., 2018).

SEC on Sepharose CL-4B was performed for the separation and purification of polysaccharides obtained by alkali extraction. Polysaccharide (20 mg) was dissolved in 1 mL of 0.15 M NaCl, and the solution was applied to a column (1.5 cm × 72 cm, V_0 = 23 mL, V_t = 73 mL) with Sepharose CL-4B. The gel bed was equilibrated with degassed elution of 0.15 M NaCl used as an eluent. Elution was performed at a flow rate of 0.25 mL/min at room temperature. The eluate was collected in 3.5 mL tubes and the carbohydrate content of each tube was determined by the phenol-sulfuric acid method (Dubois et al., 1956). Fractions corresponding to the separated peaks obtained on the elution curve were combined, concentrated, dialyzed, and lyophilized. Finally, one main polysaccharide fraction FoCA (K_{av} 0.02, yield 15.48 mg) was obtained after purification of the crude polysaccharide from the cold alkali extract, and four polysaccharide fractions were obtained after purification of crude polysaccharide of hot alkali extract as follows: FoHA1 polysaccharide with K_{av} 0.02 (yield 1.8 mg), polysaccharide FoHA2 with K_{av} 0.18 (yield 2.3 mg), polysaccharide FoHA3 (K_{av} 0.42, yield 6.7 mg) further designated FoHA and polysaccharide FoHA4 with K_{av} 0.66 (yield 4.5 mg). The procedure was repeated several times affording substantial amounts of fractions FoCA and FoHA3, which were used for further investigations.

3.3. General analytical methods

The monosaccharide composition was determined after hydrolysis of the polysaccharides, where 2 M aqueous CF_3COOH (1 mL) containing myo-inositol (0.5 or 1 mg/mL) was added to a weighed portion of polysaccharide (2–3 mg). The mixture was incubated for 5 h at 100 °C. The excess acid was removed by repeated evaporation to dryness of the hydrolysate with methanol. The mixture of monosaccharides was transformed into alditol acetates by using $(CH_3CO)_2O$ followed by reduction with $NaBH_4$ in 1 M aqueous NH_4OH and monosaccharides were identified by gas–liquid chromatography (GLC) on a Varian 450-GC chromatograph (Varian, USA) equipped with a flame-ionization detector. GLC was run on a VF-5 ms capillary column (Varian, USA; 0.25 mm, 30 m) using the following temperature program: 175 °C (isotherm, 1 min), ramp to 250 °C (isotherm, 2 min), at a rate of 3 °C/min.

Protein concentration was determined using Lowry's procedure (Lowry et al., 1951) with bovine serum albumin as a standard. The sugar concentration was determined at 490 nm using the phenol-sulfuric acid assay (Dubois et al., 1956). Absorbance was measured using an Ultrospec 3000 spectrophotometer (Pharmacia Biotech, England).

The Mw, Mn and MMD of the polysaccharide samples were determined by an SEC separation of polysaccharides with high-performance liquid chromatography (HPLC). The chromatographic system consisted of an LC-20AD pump, a DGU-20A3 degasser, a CTO-10AS

thermostat, an RID-10 A refractometric detector, a Shodex OH-pak SB-804 HQ column (7.6 mm × 30 cm) and a Shodex GS-2G 7 B precolumn (7.6 mm × 5 cm) (all Shimadzu, Japan). The HPLC experiments were performed at 40 °C with a flow rate of 0.3 mL/min. The column was equilibrated with 0.15 M sodium chloride containing 0.02% NaN_3 as a preservative, and elution was carried out with the same solution. Deionised water supplied by the Simplicity 185 Millipore water purification system (France) was used to prepare the eluents and samples. Pullulans ((Fluka, Germany) Mw 800 (RT 16.725 min), 400 (RT 18.118 min), 200 (RT 19.406 min), 110 (RT 20.666 min), 50 (RT 22.162 min), 22 (RT 23.719 min), 12 (RT 24.827 min), 6 (RT 25.945 min) and 1.3 kDa (RT 28.288 min)) were used as standards. The molar mass characteristics (Mw, Mn, MMD) were calculated by the LCsolution GPC program (LCsolution, version 1.24 SP1). The samples and standards were injected twice.

3.4. Nuclear magnetic resonance (NMR) spectroscopy

The FoCA (20 mg) and FoHA3 (25 mg) samples were deuterium-exchanged by freeze-drying from D_2O twice. NMR spectra were recorded using a Bruker Avance 600 spectrometer for solution FoCA in 700 µL of DMSO-d_6 (the signals in the spectra were calibrated by the chemical shifts of residual **D** ($\delta_{H\text{-}2}$ 3.02 ppm, $\delta_{C\text{-}2}$ 73.05 ppm and $\delta_{H\text{-}4}$ 3.09 ppm, $\delta_{C\text{-}4}$ 70.00 ppm) at 333 K, and for solution FoHA3 (25 mg) in 700 µL of 99.96% D_2O (TSP δ_H 0.00 ppm, δ_C −1.6 ppm was used as internal standard for ^1H and ^{13}C spectra) at 340 K. The 2D NMR spectra were recorded and treated according to the standard Bruker (Germany) procedures. The mixing time of 100 ms was used in the TOCSY experiments and a spin lock time of 150 ms was used in the ROESY experiments. The HMBC experiments were optimized for coupling constants $J_{H,C}$ 8 Hz.

3.5. Methylation analysis

The polysaccharide fractions were activated with powdered NaOH and methylated with CH_3I (Ciucanu and Kerek, 1984) as described earlier (Cerqueira et al., 2011) with minor modifications. A sample (4 mg) was dispersed in dried DMSO (2 mL) until it was fully dispersed. Then powdered NaOH (80 mg) under argon was added to the solution. The mixture was stirred using a magnetic stirrer (Heidolph MR 3001, Germany) at 250 rpm for 2 h at 23 °C. The mixture was cooled in an ice bath, and methyl iodide (1 mL) was added slowly under continuous stirring at 700 rpm. The mixture stirred in an ice bath for 1 h. The reaction was stopped by the addition of water (2 mL), and the resulting partially methylated derivatives were extracted with ethyl acetate (3 mL). The organic layer was separated and evaporated to dryness, and the residue was remethylated. The methylation process was repeated twice. The permethylated polysaccharides were then hydrolysed with 2 M CF_3COOH (1 mL) at 121 °C for 1 h using a thermostat (Jouan, France), cooled and rotary evaporated at 35 °C. The partially methylated sugars were dissolved in 2 M NH_4OH (1.0 mL), reduced with $NaBH_4$ (5 mg) (4 h at 23 °C) and then concentrated CH_3COOH (0.4 mL) was added to decompose the excess reducing agent and the solution was rotary evaporated to dryness. Acetylation of the partially methylated alditols was performed by adding pyridine (0.2 mL) and acetic anhydride (0.2 mL) for 12 h at 23 °C. Water (2 mL) was added to the solution to decompose the excess acetic anhydride. The partially methylated alditol acetates were extracted with ethyl acetate and analysed by GC–MS.

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

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

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

where S(i) is an area of each methylated monosaccharide peak on GC–MS chromatogram and R*f*(i) is a corresponding molar response factor previously determined (Sweet et al., 1975).

3.6. Congo red test

The conformational structures of the three polysaccharides were determined by the Congo red test (Semedo et al., 2015). Each reaction mixture contained equal volumes of polysaccharide (1 µg/mL in 15 mM phosphate buffered saline (PBS), pH 7.4) and 163 µM Congo red in the same buffer. Absorption spectra were recorded with an Ultrospec 3000 spectrophotometer (Pharmacia, England), with the reaction mixture preliminarily diluted twice with buffer. Absorbance was measured from 300 to 600 nm. The spectra were compared with those of a blank reaction mixture, which contained buffer instead of the sample.

3.7. Cytotoxicity assay

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) media supplemented with 10% (v/v) foetal bovine serum and 1% (v/v) 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were cultured in an incubator at 37 °C in a 5% CO_2 humidified atmosphere. The cytotoxic effect of the polysaccharides was measured using the MTT assay. Cells were seeded in 96-well microtiter plates at 1.0×10^4 per well at 37 °C in a 5% CO_2 humidified atmosphere. After a 24-h incubation, the medium was removed, and the cells were treated with medium containing the test polysaccharide samples at different concentrations (from 50 to 1000 µg/mL). Control cells were incubated without a test sample. After a 24-h incubation with the polysaccharide samples, the medium was carefully removed and replaced with fresh medium containing MTT dissolved in PBS (5 mg/mL) and incubated at 37 °C for 4 h as described previously (Mosmann, 1983). Absorbance values were measured with a Tecan Spark 10 M microplate reader (Tecan, Austria) at 540 nm, with the reference at 690 nm. All assays were performed in triplicate and repeated in three independent experiments. The average absorbance $A_{540} - A_{690}$ and the standard deviation were determined. The cell viability was calculated by comparing the absorbance values of the control wells and those of the samples, both represented as % viability to the control. The IC_{50} value was determined from a plot of the relative respiratory activity versus test substance concentration in the medium.

3.8. Fluorescence microscopy

After the cells were grown in the wells with the test substances, the medium was decanted and the wells were washed three times with PBS. Cells were then stained for 10 min in PBS in an incubator. The final stain concentrations were 6 µM for propidium iodide and 50 µM for acridine orange. The stained cells were washed three times with PBS, after which 100 µl of PBS was added. The preparations were viewed with a Leica DMI 3000 B (Leica Microsystems, Germany) at × 50 and × 200 magnification.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2020.112313.

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