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The antiviral potency of *Fagus sylvatica* 4*O*Me-glucuronoxylan sulfates

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ABSTRACT

Herpes simplex virus belongs to Herpesviridae family and causes infection of humans from ancient times. 4*O*Me-glucuronoxylans as the renewable biopolymers can be promising glycomaterials for various applications in pharmacy. Control enzymatic degradation of the native 4*O*Me-glucuronoxylan (GX1) followed by targeted sulfation procedure afforded a range of 4*O*Me-glucuronoxylan sulfates differed in the degree of sulfation (10–16%) and molecular mass (21,000–5000 g/mol; GXS1 > GXS2 > GXS3 > GXS4). Antiviral activity tests on GXS1–4 against herpes simplex virus (HSV) types 1 and 2 revealed the positive effect of all compounds against strains of herpes virus. Of them, the compounds GXS1 and GXS4 were shown to be the most active for both HSV serotypes. The antiviral activity of GXS1 and GXS4 was similar to those of heparin or dextran sulfate, used as reference compounds. It was found that GXS1 and GXS4 were active as well against Polio and dengue viruses, however, on a smaller scale. The mode of antiviral action of 4*O*Me-glucuronoxylan sulfates is due to inhibition of the virus binding to the cell receptors.

1. Introduction

4*O*Me-glucuronoxylans are after cellulose the second most widespread natural polymers of hardwoods. These acidic pentosans with methyl groups at *O*-4 of glucuronic acid consist of β(1,4) linked xylopyranose backbone carrying the monomeric glucuronic acid at *O*-2 as a side-chain, randomly distributed along the backbone. On average, every ninth or tenth xylose unit can be branched by 4*O*Me-glucuronic acid [1]. These renewable biopolymers represent a promising group of glycomaterials for various industrial applications, however, in economic terms, these polymers are less attractive than cellulose or starch. It seems that less attractiveness of 4*O*Me-glucuronoxylans derives from different carbohydrate composition, lower molecular mass, extraction conditions or their ionic character, compared with those of hexosans, i.e. cellulose and starch which are essential mainly for paper and food industries. For a longer time the attention has been focused on the xylan and xylan-related biopolymers to enhance their application possibilities. Trends have led to the identification of new biological activities of native or functionally modified xylan-related polymers such as antioxidant, antitumor, antimicrobial, anti-inflammatory, antitussic, anticoagulant and antithrombotic activities [1–4]. Among the functional derivatives, the sulfated xylan, known as pentosan polysufate (PPS), Elmiron, etc., prepared from beech 4*O*Me-glucuronoxylan, reached the widest application in pharmacy and medicine as an anticoagulant, antithrombotic and anti-inflammatory agent, as well as the remedy to treat an interstitial cystitis and osteoarthritis [5,6].

In view of the above findings regarding the pharmaceutical effects of beech 4*O*Me-glucuronoxylan derivatives, the aim of our study was to verify the possible impact of 4*O*Me-glucuronoxylan sulfates, differing in molecular weight and degree of sulfation, against certain human viruses. The antiviral activity was tested against herpes simplex virus (HSV) types 1 and 2, agents of oral, genital and neurological infections [7], dengue virus (DENV), a flavivirus agent of dengue fever and dengue hemorrhagic fever in humans [8], and Polio virus (PV), a neurotropic enterovirus causing poliomyelitis [9].

2. Materials and methods

2.1. Isolation and depolymerization of 4-O-methylglucuronoxylan

Sawdust, prepared from the trunk of *Fagus sylvatica* L., Male Karpaty (Slovakia), were used for isolation of 4*O*Me-

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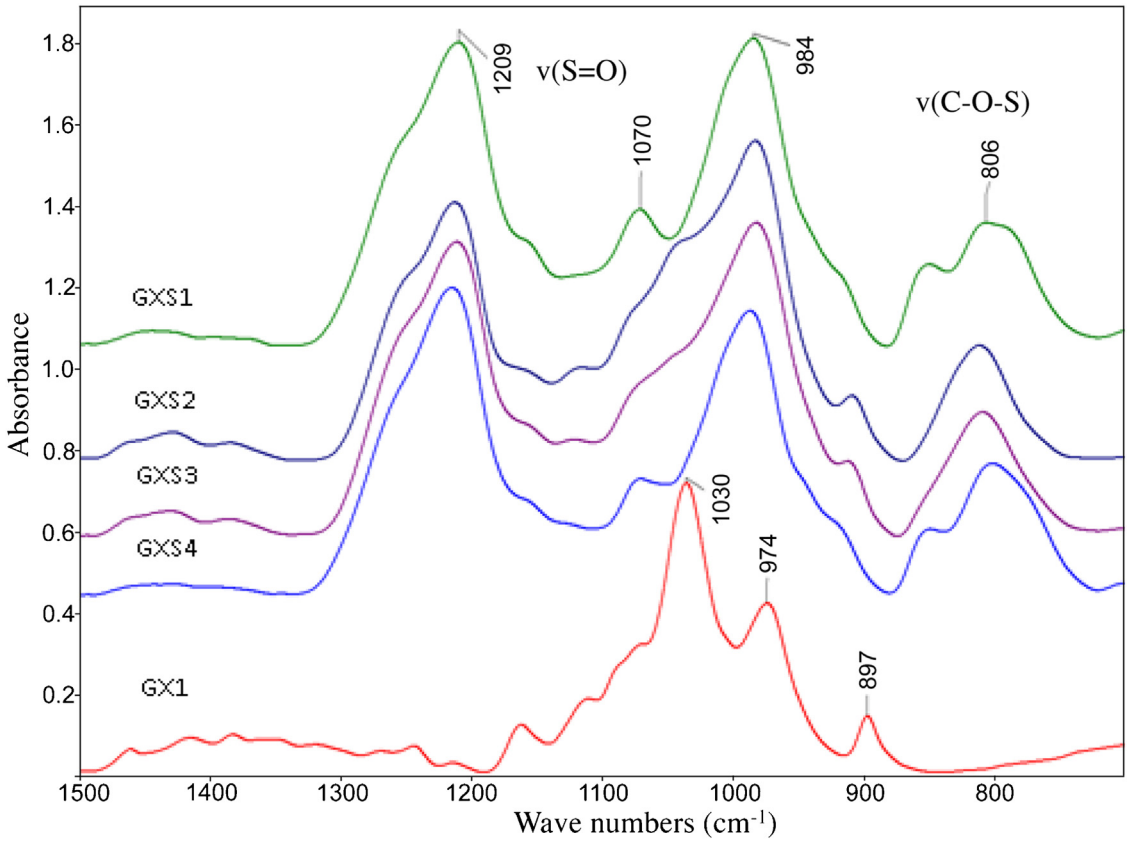

Fig. 1. The FT-IR spectra of beech 4*O*Me-glucuronoxylan sulfate (GXS1) and its degraded fractions GXS2, GXS3 and GXS4 in the range of 1500–700 cm^{-1}.

glucuronoxylan. Isolation was performed according to already described procedures [10,11]. Enzymatic depolymerization of the native 4*O*Me-glucuronoxylan (GX1) with microbial *endo*-1,4-beta-xylanase afforded three lower molecular weight fractions marked as GX2, GX3 and GX4 [4].

2.2. Sulfation of 4-O-methylglucuronoxylans

To prepare water soluble 4*O*Me-glucuronoxylans for biological tests, dried native polymer (GX1) and its three depolymerized fractions (GX2, GX3 and GX4) were suspended in dry DMF and sulfated with oleum in DMF at laboratory temperature for 24–48 h [11]. Reaction mixtures were poured on ice and neutralized with sodium hydroxide solution, dialyzed and freeze-dried to give 4*O*Me-glucuronoxylan sulfates marked as GXS1, GXS2, GXS3 and GXS4 [4].

2.3. General methods

Organic elemental analyzer FLASH 2000 (Thermo Fisher Scientific, USA) for the determination of the sulpur content was used. Fourier-transform infrared spectroscopy (FT-IR) of samples were obtained on a NICOLET Magna 750 spectrometer with DTGS detector and OMNIC3.2 software, where 128 scans were recorded with 4 cm^{-1} resolution. Samples were pressed into KBr pellets with a sample/KBr ratio ~1/200 mg. ^{1}H and ^{13}C NMR spectra of samples were recorded in D_2O at 25 °C on Varian 400 NMR spectrometer on direct 5 mm PFG AutoX probe. Samples were twice freeze-dried from D_2O before measurements. For ^{1}H and ^{13}C NMR spectra, chemical shifts were referenced to internal standard-acetone (δ 2.22 and 31.07, respectively).

Molecular mass (Mp) was performed with HPLC Shimadzu apparatus (Vienna, Austria) equipped with a differencial refractometer RID-6A and a UV–vis detector SPD-10AV using the tandem of columns HEMA-BIO 40 and 100 (8 mm × 250 mm) of particle size 10 μm (Tessek, Prague, Czech Republic). The mobile phase 0.02 M phosphate buffer pH 7.2 containing 0.1 M NaCl was used at a flow rate 0.8 mL/min. Dextran standards were used for calibration of the columns (Gearing Scientific, Hertfordshire, UK). The peak molecular mass (Mp) was calculated from a calibration curve using formula $\log Mp = K^{+}a$. Ve (Parameters K and a are calculated from calibration curve, and Ve represents an elution volume of sample measured).

2.4. Cells and viruses

Vero (African green monkey kidney) cells were grown in minimum essential medium (MEM) supplemented with 5% bovine serum. For maintenance medium (MM), serum concentration was reduced to 1.5%. HSV-1 strain *F* and HSV-2 strain *G* were obtained from the American Type Culture Collection (Rockville, USA). Field is a HSV-1 TK^{-} acyclovir-resistant strain obtained from Professor Dr. E. De Clercq (Rega Institute, Belgium). DENV-2 (strain NGC) was provided by Dr. A. S. Mistchenko (Hospital de Niños Dr. Ricardo Gutiérrez, Buenos Aires, Argentina). Polio virus type 1 (Sabin strain).

2.5. Cytotoxicity assay

Vero cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma–Aldrich) method [12]. Confluent cultures (2 × 104 cells/well) in 96-well plates were exposed to different concentrations of the polysaccharides (ranging from 31.25 to 1000 μg/mL), with three wells for each concentration, using incubation condi-

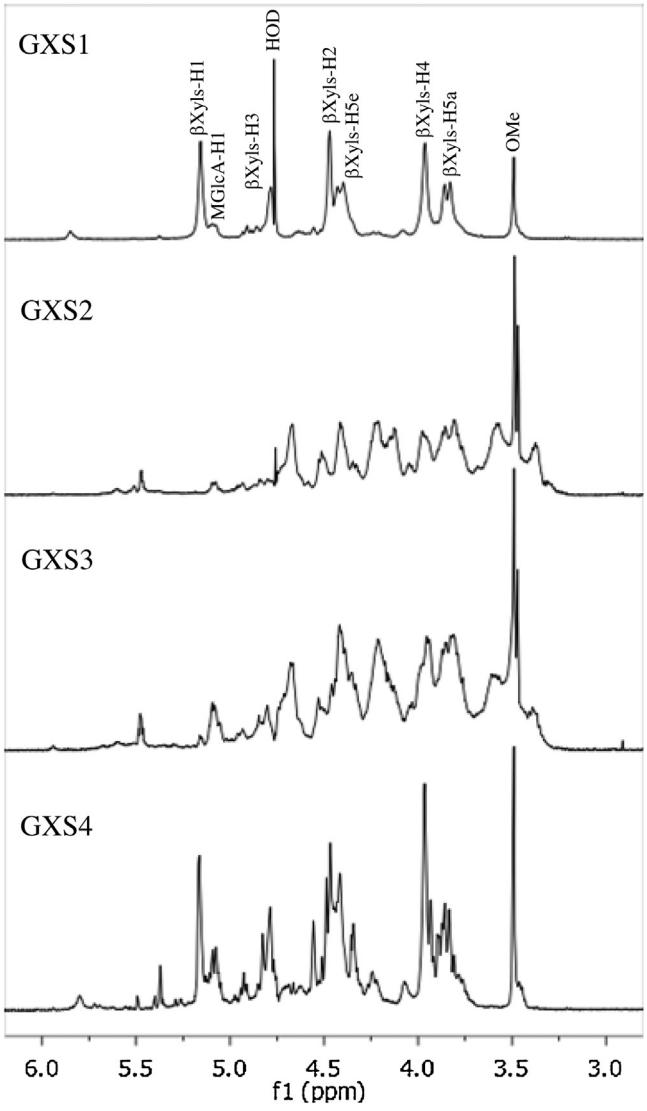

Fig. 2. The ^{1}H NMR spectra of beech 4*O*Me-glucuronoxylan sulfate (GXS1) and its degraded fractions GXS2, GXS3 and GXS4. βXyls = 1,4-linked β-D-xylopyranose unit sulfated at *O*-2 and *O*-3, MGlcA = 4-*O*-methyl-α-D-glucuronic acid.

tions equivalent to those used in the antiviral assays. Then, 10 μl of MM containing MTT (final concentration 0.5 mg/mL) was added to each well. After 2 h of incubation at 37 °C, the supernatant was removed and 200 μL of ethanol was added to each well to solubilize the formazan crystals. After vigorous shaking, absorbance was measured in a microplate reader at 595 nm. The cytotoxic concentration 50% (CC_{50}) was calculated as the compound concentration required to reduce cell viability by 50%.

2.6. Antiviral assays

The antiviral activity against HSV-1, HSV-2, DENV-2 and Polio I was determined by a virus plaque-reduction assay. Vero cell monolayers (1 × 105 cells/well) grown in 24-well plates at 37 °C and 4% CO_2 atmosphere were infected with about 50 plaque forming units per well (PFU/well) in the absence or presence of various concentrations of the compounds (ranging from 0.625 to 40 μg/mL). After 1 h of adsorption at 37 °C, the residual inoculum was replaced by MM containing 0.7% methylcellulose and the corresponding dose of each compound. Plaques were counted after 2 days of incubation at 37 °C for HSV-1 and HSV-2, or after 1 or 7 days for Polio and DENV-2, respectively. The inhibitory concentration 50% (IC_{50}) was calculated as the compound concentration required to reduce virus plaques by 50%. All determinations were performed twice and each in duplicate.

2.7. Virucidal assay

A virus suspension of HSV-2 strain *G* containing 5×10^7 PFU/mL was incubated with an equal volume of MM with or without compound for 2 h at 37 °C. The samples were then diluted in cold MM to determine residual infectivity in a plaque formation assay using Vero cells (1×10^5 cells/well). The sample dilution effectively reduced the compound concentration to be incubated with the cells at least 100-fold to assess that titer reduction was only due to cell-free virion inactivation. The virucidal concentration 50% (VC_{50}), defined as the concentration required to inactivate virions by 50%, was then calculated.

2.8. Effect of treatment period on the antiviral activity

Vero cells (1×10^5 cells/well) grown in 24-well plates were infected with 50 PFU/well of HSV-2 strain G following different treatment conditions, as follows. Adsorption: cells were exposed

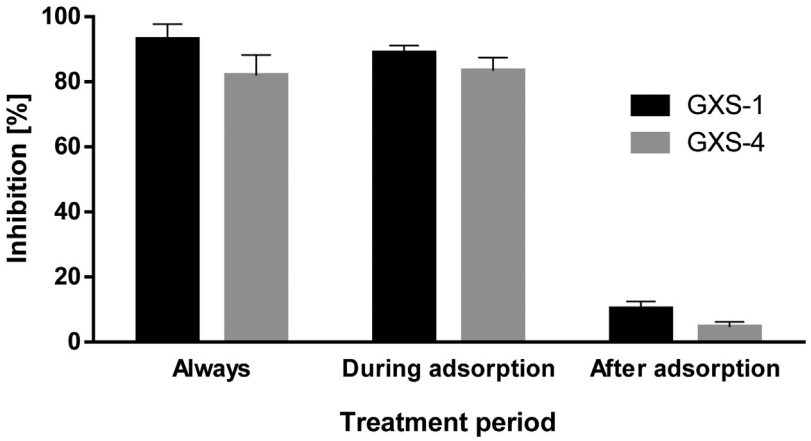

Fig. 3. Inhibitory effect of GXS1 and GXS4 against HSV-2(G) during and after virus adsorption. Vero cells were infected with 50 PFU of virus in a plaque assay under different treatment conditions. Always: GXS1 and GXS4 (10 μg/mL) were present both during and after the adsorption period; during adsorption: the 4*O*Me-glucuronoxylan sulfates were present only during adsorption; after adsorption: the 4*O*Me-glucuronoxylan sulfates were added in the plaquing medium after adsorption. Viral plaques were counted after 2 days of incubation at 37 °C. Results are expressed as % inhibition respect to untreated infected control. Each value is the mean of duplicate assays ± SD.

Table 1
Chemical characteristics of 4*O*Me-glucuronoxylans.

Compounds	Xyl[c] (wt%)	4*O*Me-GlcA[d] (wt%)	Gal/Glc/Ara[e] (wt%)	Sulpur[b] (wt%)	Mp[f] (g/mol)
GX1[a]/GXS1[b]	84	10	6	15.2	21 500[b]
GX2/GXS2	89	11	–	9.8	10 700[b]
GX3/GXS3	88	12	–	11.8	6300[b]
GX4/GXS4	89	11	–	15.5	4700[b]

[a] GX1-4: 4*O*Me-glucuronoxylans.
[b] GXS1-4: 4*O*Me-glucuronoxylan sulfates.
[c] Xyl: xylose.
[d] 4*O*Me-GlcA: 4*O*-methyl-glucuronic acid.
[e] Gal/Glc/Ara: galactose, glucose and arabinose content.
[f] Mp: peak molecular weight.

to HSV-2 in the presence of 10 μg/mL of the compound. After 1 h at 4 °C, both compound and unadsorbed virus were removed, the cells were washed with cold phosphate-buffered saline (PBS) and overlaid with plaquing medium. After adsorption: cells were infected with HSV-2 in the absence of the compound and after adsorption at 4 °C the unadsorbed virus was removed, the cells were washed twice with cold PBS and further incubated with plaquing medium containing 10 μg/mL of the compound. Always: the compound was present during HSV-2 adsorption at 4 °C and in the plaquing medium added after adsorption. For all treatments, virus plaques were counted after 2 days of incubation at 37 °C and results were expressed as percent inhibition for each treatment with respect to untreated infected cell control.

3. Results and discussion

3.1. Characteristics of 4-O-methylglucuronoxylan sulfates

The 4*O*Me-glucuronoxylan (GX1) was isolated from beech sawdust (*F. sylvatica*) with sodium hydroxide solution and hydrogen peroxide [10,11]. It was composed of Xyl (84%) and 4*O*Me-GlcA (10%), and a small amount (~5–6%) of other sugars as contaminants. Molecular mass (Mp) of GX1 was determined to be 16,000 g/mol [4]. Its chemical structure has been already described. Beech hemicellulose polymer is composed of β(1,4) linked xylopyranose residues carrying radomly at *O*-2 of xylose units (on average, every ninth or tenth unit) monomeric 4*O*Me-GlcA residues as a side chain [1]. To verify the biological effect of lower molecular mass 4*O*Me-glucuronoxylans, the native polymer (GX1) was partly depolymerized to give fractions GX2, GX3, and GX4 of molecular mass ~3000, 2600 and 1800 g/mol, respectively [4].

To check the influence of negative charge and molecular size on the biological activity, the functional sulfate groups were introduced into the native GX1 and its degraded fractions GX2, GX3 and GX4. Final products contained ~10% (GXS2), ~12% (GXS3), ~15% (GXS1) and ~16% (GXS4) of the sulfur content, determined by elemental analysis. Besides, 4*O*Me-glucuronoxylan sulfates differed as well in molecular mass (21,000–5000 g/mol; GXS1 > GXS2 > GXS3 > GXS4). The presence of sulfate groups was verified as well by FT-R spectroscopy (Fig. 1). As can be seen from Fig. 1, the FT-IR spectra of sulfated glucuronoxylans showed intensive bands appeared at 1209 and 806 cm^{-1} characteristic for stretching vibrations of $\nu(S{=}O)$ and $\nu(C{-}O{-}S)$ groups, respectively.

Various sulpur contents, determined by elemental analysis, are also reflected in the ^{1}H NMR spectral profiles of 4*O*Me-glucuronoxylan sulfates GXS1–GXS4 (Fig. 2). Compounds GXS1 and GXS4 with the highest sulpur contents (15% and 16%, respectively) showed only one dominant signal for anomeric Xyl units at δ 5.20 indicating the persulfation of this sugar at positions *O*-2 and *O*-3 [13]. Besides, a low intensity signal at around δ 5.09 and the intensive one at δ 3.49 could be assigned to H1 and 4-*O*-methyl group of GlcA, respectively [11]. Glucuronoxylans GXS2 and GXS3 with sulpur contents ~10% and 12%, respectively, do not show H1 signals at δ 5.20 characteristic for persulfated Xyl residues. In the anomeric region of GXS2 were observed signals at δ 4.72, 4.67 and 4.50 derived from H1 of Xyl3S (sulfate group at position O3), Xyl2S (sulfate group at position O2), and non-substituted Xyl residues, respectively [4,12]. The ^{1}H region of GXS3 contains two signals at δ 4.72 and 4.67 assigned to H1 of Xyl3S and Xyl2S, respectively. NMR

Table 2
Antiviral activity of beech 4*O*Me-glucuronoxylan sulfates (GXS1-4) against herpes simplex virus and selectivity indices.

Compound	[a]CC_{50} (μg/mL)	[b]IC_{50} (μg/mL)			[c]SI (CC_{50}/IC_{50})		
		HSV-1 (F)	HSV-2 (G)	HSV-1 (Field)	HSV-1 (F)	HSV-2 (G)	HSV-1 (Field)
GXS-1	>1000	1.92 ± 0.07	1.4 ± 0.11	0.66 ± 0.04	>521	>714	>1515
GXS-2	>1000	22.00 ± 0.14	30.65 ± 7.28	nd	>45	>33	nd
GXS-3	>1000	12.90 ± 0.44	18.40 ± 1.76	nd	>77	>54	nd
GXS-4	>1000	4.30 ± 0.12	1.94 ± 0.78	2.18 ± 0.45	>232	>515	>459
DS 8000	>1000	2.12 ± 0.4	1 ± 0.03	2.19 ± 0.75	>472	>1,000	>457
Heparin	>1000	1.20 ± 0.48	2.1 ± 0.3	4.1 ± 1.28	>833	>476	>244

nd: not determined.
[a] CC_{50} (Cytotoxic concentration 50%): concentration required to reduce cell viability by 50% after 48 h of incubation with the compounds.
[b] IC_{50} (Inhibitory concentration 50%): concentration required to reduce plaque number in Vero cells by 50%. Mean of two determinations ± SD.
[c] SI (Selectivity index): (CC_{50}/IC_{50}). DS 8000 (Dextran sulfate, Mw 8000 g/mol) and heparin are included as reference substances.

measurements revealed the persulfation of Xyl residues in GXS1 and GXS4, partial sulfation of Xyl at positions O2 or O3 in GXS3, and partial sulfation of Xyl residues at O2 or O3 as well non-substituted xylose residues in 4*O*Me-glucuronoxylan GXS2.

Highly sulfated glucuronoxylan, named as Pentosan, Elmiron, PPS, etc., is used for a long time in treating various clinical conditions, notably as an anticoagulant [14]. Recently, it was found that also partially sulfated 4*O*Me-glucuronoxylans may have remarkable antitussive activity [4,8]. To verify the effect of molecular weight and sulpur content, 4OMe-glucuronoxylan sulfates (GXS1-4) were tested on the antiviral potency against HSV-1 strain F, HSV-2 strain G and the TK mutant strain Field of HSV-1. The effect was evaluated in Vero cells by a virus plaque reduction assay. As shown in Table 1, all the compounds were active against all herpesviruses at concentrations ranging from 30.6 to 0.66 μg/mL. GXS1 and GXS4 were the most active compounds for both HSV serotypes. These results are in agreement with the higher degree of sulfation that presents these compounds (15% for GXS1 and 16% for GXS4) with respect to GXS2 (10%) and GXS3 (12%). Besides, although the degree of sulfation of GXS1 and GXS4 was similar, GXS1 resulted more active than GXS4 and this could be attributable to its higher molecular weight. Furthermore, GXS1 and GXS4 elicited a strong antiviral activity against HSV-1 Field with IC_{50} values of 0.66 ± 0.04 and 2.18 ± 0.45 μg/mL, respectively. Dextran sulfate 8000 and heparin, used as reference compounds, showed similar level of antiviral potency than GXS1 and GSX4. Polysaccharides active against herpesvirus were also obtained from plant extracts like *Prunella vulgaris* [15] and *Cedrela tubiflora* [16]. Nevertheless, from all the wide spectrum of polysulfates, the red seaweed-derived polysaccharides represent the most potent and selective antiviral agents able to block HSV replication at concentrations as low as 0.1–1 μg/mL [17,18].

Cytotoxic effects of polysaccharides were observed at a concentration much higher than the IC_{50}, with CC_{50} values above 1000 μg/ml for Vero cells. Thus, all the compounds possess good selectivity indices (Table 1). In order to broaden the spectrum of action of the most active compounds, they were tested against other unrelated viruses such as PV-1 and DENV-2. It was observed that the compounds were also active, although in a lesser extent to these viruses (Table 2).

As regards the possibility that sulfated 4*O*Me-glucuronoxylans may act directly on the virus particles, a virucidal assay was carried out. Preincubation of the virus with GXS-1 or GXS-4 had no significant direct inactivating effect on HSV-2 (G) virions with a VC_{50} value higher than 100 μg/mL. To establish the stage of the virus replication cycle at which the 4OMe-glucuronoxylan sulfates exert their antiviral activity, a virus plaque reduction assay for HSV-2 (G) in Vero cells upon different treatment periods was employed. A high level of efficacy was attained if the 4OMe-glucuronoxylan sulfates were present either only during HSV-2 adsorption or during the whole period of the plaque assay. When present only after adsorption, they were no longer effective, thus confirming that the tested compounds interfere with a very early stage of virus replication (Fig. 3). This result confirms that the mode of antiviral action of the 4OMe-glucuronoxylan sulfates is predominantly due to inhibition of virus binding to the cell receptors (Table 3).

Table 3
Antiviral activity of beech 4*O*Me-glucuronoxylan sulfates (GXS1and GXS4) against Polio virus type 1 and dengue virus type 2.

Compound	IC_{50}(μg/mL)		SI (CC_{50}/IC_{50})	
	Polio I	DENV-2	Polio I	DENV-2
GXS-1	14.35 ± 1.54	17.40 ± 3.39	>70	>57
GXS-4	25.00 ± 4.14	27.00 ± 5.25	>40	>37
DS 8000	>200	0.9 ± 0.1	Inactive	>1.111
Heparin	112.00 ± 6.5	1.9 ± 0.2	>8.9	>526

For references see legend of Table 2.

4. Conclusions

The impact of molecular mass and sulfate contents of 4*O*Me-glucuronoxylan sulfates on the antiviral potency against HSV-1, HSV-2, DENV-2 and PV-1 was investigated. Enzymatic depolymerization of beech 4*O*Me-glucuronoxylan afforded a series of lower molecular mass fractions which were further sulfated to give final 4*O*Me-glucuronoxylan sulfates differing in molecular mass and degree of sulfation. Biological tests on GXS1-4 against HSV-1 and HSV-2 revealed the positive effect of all compounds against strains of herpes virus. It has been found that GXS1 and GXS4, with the highest content of sulfate, were the most active compounds against both HSV serotypes. Their antiviral activity was similar to those of heparin or dextran sulfate. Moreover, highly sulfated 4*O*Me-glucuronoxylans (GXS1 and GXS4) were active as well against PV-1 and DENV-2, but with higher IC_{50} values. Cytotoxic effects of the polysaccharides were not observed at a concentration up to 1000 μg/mL. Preliminary results showed that the mode of antiviral effect of 4*O*Me-glucuronoxylan sulfates is due to inhibition of the virus binding to the cell receptors. It can be concluded that sulfated 4*O*Me-glucuronoxylans are in vitro selective antiviral agents against herpes virus.

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