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Anti-herpetic activity of a sulfated xylomannan from *Scinaia hatei*

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ABSTRACT

Many viruses display affinity for cell surface heparan sulfate proteoglycans with biological relevance in virus entry. This raises the possibility of the application of sulfated polysaccharides in antiviral therapy. In this study we have analyzed polysaccharide fractions isolated from *Scinaia hatei*. The crude water extract (ShWE) as well as one fraction (F1) obtained by size exclusion chromatography had potent anti-HSV activity. Their inhibitory concentration 50% (IC_{50}) values ranging from 0.5 to 4.6 μg/ml were much lower than the cytotoxic concentration 50% (CC_{50}) values (⩾1000 μg/ml). These fractions had very low anticoagulant activity. Furthermore, they had a weak inactivating effect on virions in a virucidal assay at concentrations in the range of 60–100 μg/ml. Chemical, chromatographic and spectroscopic methods showed that the major polysaccharide, which had 0.4 sulfate group per monomer unit and an apparent molecular mass of 160 kDa, contained a backbone of α-(1 → 3)-linked D-mannopyranosyl residues substituted at C-6, C-4 and C-2 with single stub of β-D-xylopyranosyl residues. Sulfate groups, when present, are located at C-4 of α-(1 → 3)-linked D-mannopyranosyl units, and appeared to be very important for the anti-herpetic activity of this polymer.

1. Introduction

The *Herpesviridae* family includes several pathogenic viruses that are able to cause a variety of inapparent, mild or severe human infections. A common feature of this group is the establishment of long-term latent infections, with periods of recurring viral replication. A very effective treatment for herpesvirus is available since the introduction of acyclovir in the 1970s and it is still the most commonly used chemotherapy (Brady and Bernstein, 2004). However, this compound is not always well tolerated and drug-resistant strains are rapidly emerging, particularly in immunocompromised patients. Therefore, the demand for antiviral drugs with novel mode of action is great (Bacon et al., 2003; Eizuru, 2003).

The observation that herpes simplex virus (HSV) and human immunodeficiency virus bind heparan sulfate provided the rationale for the development of sulfated polymers as topical agents. The first reports of the antiviral activity of polysaccharides appeared almost fifty years ago (Ginsberg et al., 1947; Green and Wooley, 1947). Seventeen years later, it was demonstrated that heparin and other sulfated polysaccharides can act as HSV inhibitors (Nahmias et al., 1964; Takemoto and Fabisch, 1964). In recent years, screening assays of the antiviral activity of extracts from a number of marine algae and cyanobacteria have lead to the identification of carbohydrate polymers with potent inhibitory effects against several enveloped viruses (Arad et al., 2006; Cos et al., 2003; Damonte et al., 2004; Luescher-Mattli, 2003; Smit, 2004; Witvrouw and De Clercq, 1997; Zjawiony, 2004). In deed some of these macromolecules are in various phases of clinical trails as microbicide (Balzarini and Van Damme, 2007; Kilmarx et al., 2006; Kleymann, 2005; McReynold and Gervay-Hague, 2007; Nikolic et al., 2007). Thus, evaluating the potential of sulfated polysaccharide extracted from marine algae as anti-HSV drug candidate will be of considerable interest.

In previous studies, we have analyzed the structural characteristics and antiviral properties of diverse types of sulfated polysaccharides isolated from seaweeds collected in Indian and South American coasts, including galactans, agarans, carrageenans and fucans (Adhikari et al., 2006; Carlucci et al., 2002; Chattopadhyay et al., 2007; Duarte et al., 2004; Ghosh et al., 2004; Mandal et al., 2007; Mazumder et al., 2002; Ponce et al., 2003). We now report the structural characteristics and the antiviral activity against HSV-1 and HSV-2 of sulfated xylomannans present in the red seaweed *Scinaia hatei*.

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2. Results and discussion

2.1. Chemical characterization of sulfated xylomannan from Scinaia hatei

2.1.1. Preliminary characterization

The depigmented algal powder (DAP) from *S. hatei* which on hydrolysis with 2 M TFA yielded xylose, mannose and galactose as dominant neutral monosaccharide (Table 1), was extracted with water as described in the Section 4. Purification of the water extracted fraction was then achieved by repeated precipitation with ethanol (4 volumes). The aqueous 80% ethanol soluble polymeric fraction (named as ShWE-S) contained galactose as the major constituent sugar, whereas the water extracted ethanol insoluble fraction (ShWE), which amounted to 14% of DAP dry weight, contained mannose and xylose as the glycosyl residues (Table 1). This fraction did not contain galactose residue. Neither any methylated sugars were detected during GLC–MS analysis of the derived alditol acetates. The FT-IR spectrum of ShWE showed an intense absorption band in the region 1250 cm^{-1} related to >S═O stretching vibration of the sulfate group (Lloyd et al., 1961; Turvey and Williams, 1962), and another band at 830 cm^{-1} arising from secondary equatorial sulfate groups of polysaccharides (Rees, 1963). Notably, the sugar composition of the desulfated derivative (ShWE-D), obtained by solvolytic desulfation (Falshaw and Furneaux, 1998) of ShWE is similar to the later fraction.

2.1.2. Size exclusion chromatography (SEC)

The water extracted polysaccharides (ShWE) were separated by SEC with Sephacryl S-400 using 0.5 M sodium acetate buffer (pH 5.0) as eluant into two peaks (Fig. 1A). The first peak ($K_{av.}$ 0.19–0.98) accounted for 78% of the total sugar eluted from the column. The distribution of this peak tailed to lower molecular weights. Sub fractions, which appeared at $K_{av.}$ 0.26–0.61 were mixed and the recovered material has been named as F1 fraction. This fraction by re-chromatography eluted as a symmetrical peak (Fig. 1B). Based on calibration with standard dextrans, the apparent molecular mass of F1 would be 160 kDa. It contained mannose and a small amount of xylose, whereas fraction F2 contained xylose only (Table 1). The xylomannan containing fraction (F1), which contained 8% (w/w) sulfate, was subjected to further structural analysis. This polymer had a positive specific rotation $[\alpha]_D^{30}$ +42° (*c* 0.2, H_2O) and is soluble in water. The sulfated polysaccharide of the red alga *Nothogenia fastigiata*, a member of the family Liagoraceae contained D-mannonose and D-xylose residues (Kolender et al., 1997; Kolender et al., 1995; Matulewicz and Cerezo, 1987). Assuming that the genetic information for the synthesis of D-mannopyranosyl and D-xylopyranosyl units, already menifest in *Nothogenia* (Liagoraceae) xylomannan, has not been lost in this red alga it may be concluded that the configuration of both mannose and xylose is D. The similarity in the optical rotation value of the polymer of present study and the xylomannan of *N. fastigiata* also support this hypothesis.

2.1.3. Chemical modifications

Two modified polysaccharide preparations were obtained as the result of desulfation (F1D) and both desulfation and Smith degradation (F1D-Sm) of F1. Solvolytic desulfation (Falshaw and Furneaux, 1998) of the purified (F1) xylomannan sulfate (as pyridinium salt) produces a desulfated derivative (F1D). Preliminary experiments (data not shown) showed a higher recovery with this method compared to methanol–HCl and auto-desulfation methods (Percival and Wold, 1963). Notably the sugar composition of F1 and its desulfated derivative (F1D) were comparable (Table 1). The FT-IR spectrum of F1 showed an intense absorption band in the region 1250 cm^{-1} related to >S═O stretching vibration of the sulfate group (Lloyd et al., 1961). An additional sulfate absorption band at 830 cm^{-1} (C–O–S, secondary equatorial sulfate) indicated that the sulfate group is located at position 4 or 3 of the mannopyranose residue. Desulfation of F1 resulted in the disappearance of these absorbances demonstrating that they were associated with sulfate groups.

Smith degradation of F1D was used to simplify further the structure of the polysaccharide. The oxidized polymer was reduced with sodium borohydride and subjected to mild acid hydrolysis according to the usual Smith degradation conditions. After separation from low-molecular-mass fragments, a Smith degraded material (F1D-Sm) containing mannose as the only component sugar was obtained in 50% yield.

2.1.4. Glycosidic linkage analysis

The native (F1), desulfated (F1D), and desulfated-degraded (F1D-Sm) polysaccharide preparations were methylated to localize the positions of sulfate groups and glycosidic linkages. Methylation analysis of desulfated F1D yielded 2,4,6-tri-*O*-methyl mannitol, indicating the presence of (1 → 3)-linked mannopyranosyl residues (Table 2). Smaller proportions of 2,6- and 2,4-di-*O*-methyl mannitol, 4-mono-*O*-methyl mannitol and 2,3,4-tri-*O*-methyl xylitol, indicative of a branched polysaccharide were also generated. The native xylomannan sulfate (F1), on the other hand, gave seven different partially methylated alditol acetates (PMAA) showing that the structure of this polymer is complex. Notably, the amount of sulfate groups in the native polysaccharide as calculated from partially methylated alditol acetates was not in good agreement with the experimentally determined sulfate. But it is well known that methylation of sulphated polysaccharides does not always yield reliable proportions of methylated alditol acetates (Patankar et al., 1993; Pereira et al., 1999; Ray, 2006). The percentage of xylose in F1 as obtained from sugar composition analysis (Table 1) and methylation analysis (Table 2) are in quite good agreement.

Table 1
Sugar composition of fractions obtained from the red alga *Scinaia hatei* (see text for identification of fractions)

	DAP	ShWE	ShWE-D	F1	F1D	F1D-Sm
Sulfate[a]	nd[c]	9	tr	8	tr	nd
Neutral sugar[a]	25	39	59	46	68	nd
Xyl[b]	54	38	39	23	21	tr
Man[b]	25	62	61	77	79	99
Gal[b]	17	tr[d]	tr	–[e]	–	–
Glc[b]	4	tr	tr	–	–	–

[a] Percent weight of the fraction.
[b] Mol% of anhydro sugar.
[c] nd, not determined.
[d] tr, trace.
[e] –, not detected.

Table 2
Partially methylated alditol acetates derived from sulfated xylomannan (F1) of *Scinaia hatei*, its desulfated (F1D) and desulfated-degraded (F1D-Sm) derivatives

Methylation products	Peak area[b]		
	F1	F1D	F1D-Sm
2,4,6-Man_p[a]	6	58	100
2,6-Man_p	40	9	–
2,4-Man_p	12	11	trace
4-Man_p	16	7	–
2-Man_p	4	–[c]	–
Man_p	3	–	–
2,3,4-Xyl_p	19	15	–

[a] 2,4,6-Man_p denotes 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylmannitol, etc.
[b] Percentage of total area of the identified peaks.
[c] –, not detected.

4-*O*-Methyl mannitol and unmethylated mannitol were among the products of methylation analysis of the native polymer. The increase in the proportion of 2,4,6-tri-*O*-methyl mannitol after desulfation, together with decreased proportion of 2,6-di-*O*-methyl mannitol suggests that sulfate esters, when present, are located at C-4 of (1 → 3)-linked mannopyranosyl units. The backbone of this polymer is, therefore, similar to the sulfated xylomannans of the red alga *N. fastigiata* except the position of sulfate group (Kolender et al., 1995; Matulewicz and Cerezo, 1987).

The results obtained from the methylation analysis of the Smith degraded polymer (F1D-Sm) showed (Table 2) the degraded polysaccharide to be a linear mannan built up of 3-linked mannopyranose residues. The disappearance of terminal-xylitol, and 2,4-, 2,6- and 4-*O*-methyl mannitol residues suggest the presence of single stub of xylose residues at 6, 4 and 2 positions of the mannose units of native xylomannan sulfate.

2.1.5. NMR spectroscopy

NMR spectroscopy is a convenient method that gives valuable information on polysaccharide structures. We employed NMR analysis to determine the anomeric configuration and the overall structure of the xylomannan sulfate. The presence of a number of broad signals in the anomeric region of ^{1}H NMR spectrum of the native xylomannan sulfate (F1) suggests that its structure is very complex. The desulfated xylomannan (F1D), on the other hand, showed only two broad anomeric resonances, one at 5.119 ppm and the other at 4.426 ppm (Fig. 2A). Therefore, the sulfation pattern of native xylomannan is complex. The anomeric configuration of mannose and xylose residues of a structurally related sulfated xylomannan isolated from *N. fastigiata* have been reported to be α- and β-, respectively (Kolender et al., 1995; Matulewicz and Cerezo, 1987). Therefore, signals at 5.119 and 4.426 ppm were tentatively assigned to anomeric protons of α-(1 → 3)-linked mannopyranosyl and β-linked terminal xylose residues, respectively. The disappearance of the later signal in the NMR spectrum of the Smith degraded polymer (F1D-Sm) confirms this finding (Fig. 2B). Like desulfated xylomannan (F1D) this degraded material (F1D-Sm) also include resonances characteristic of polysaccharides such as signals from ring protons (H-2–H-6) between 3.2 and 4.2 ppm. The disappearance of the signals at 3.307, 3.441 and 3.631 ppm in the ^{1}H NMR spectrum of the Smith degraded polymer (F1D-Sm) suggests that they originated from the ring protons of

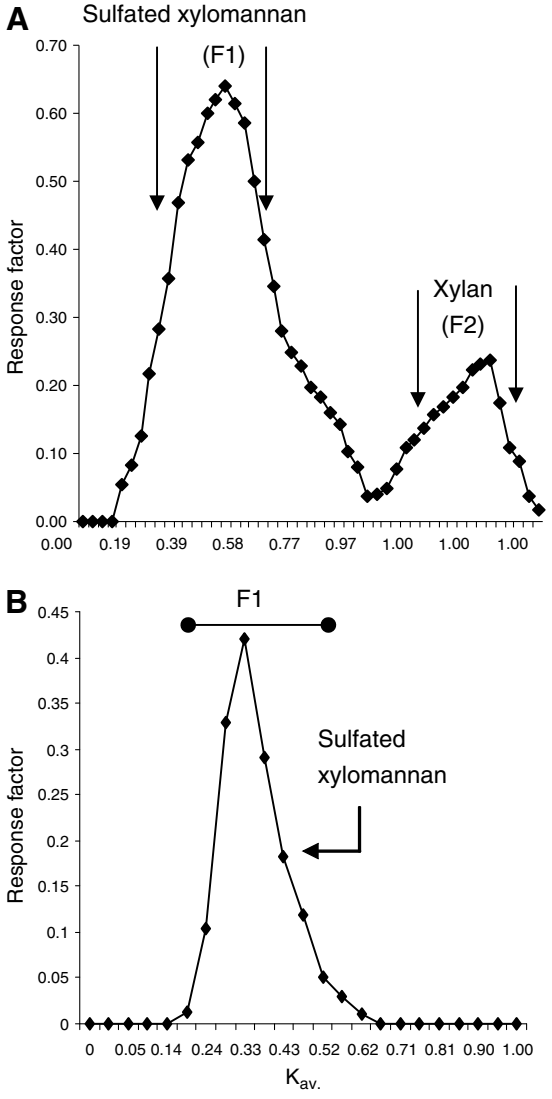

Fig. 1. Purification of the sulfated xylomannan from the marine alga *Scinaia hatei* by size exclusion chromatography on Sephacryl S-400 column. (A) the crude water extracted polymeric fraction (ShWE) was purified as described in the Section 4. (B) the partly purified sulfated xylomannan was then re-chromatographed on the same column under the same experimental conditions.

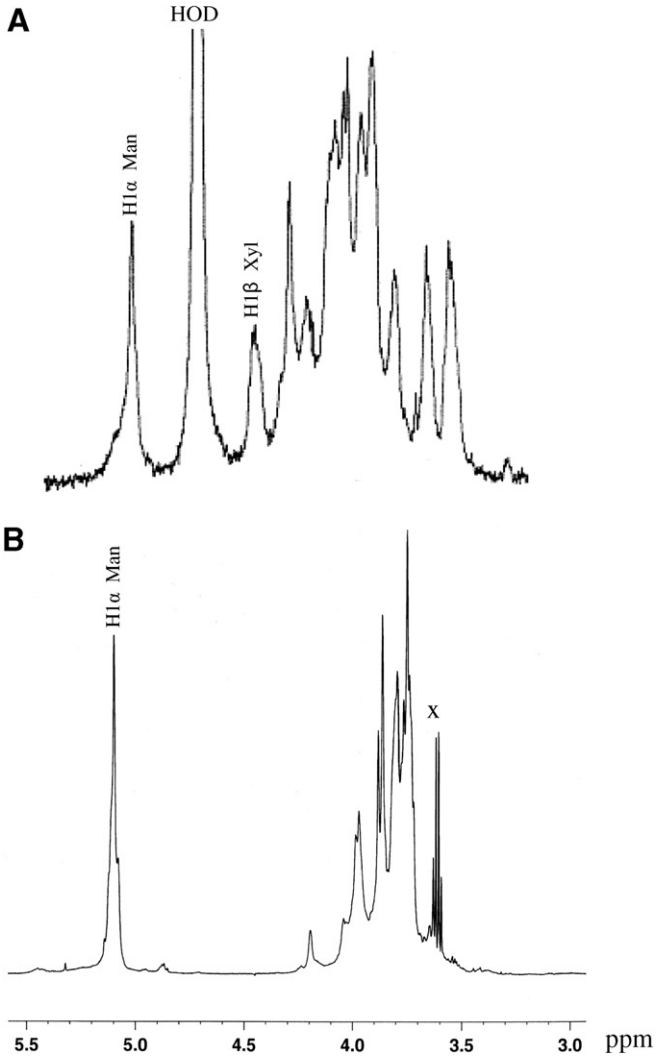

Fig. 2. ^{1}H NMR spectra of (A) the desulfated xylomannan (F1D) of *Scinaia hatei* and (B) its Smith degraded derivative (F1D-Sm). The spectrum for samples was recorded in D_2O solution. The signal for the residual water was designated as HOD. X, Impurity.

xylose residues. Therefore, NMR analysis confirmed the results of methylation analysis and indicated the presence of α-linked mannopyranosyl and β-linked terminal xylopyranosyl residues.

2.2. Biological activities of the sulfated xylomannan from S. hatei

2.2.1. Cytotoxic activity

The crude water extract from *S. hatei* (ShWE), the major purified sulfated xylomannan F1 and the desulfated derivative F1D were initially evaluated for cytotoxicity by assessing their effects on Vero cell viability. For comparative purposes, heparin was simultaneously assayed as known reference polysaccharide. No effect on cell viability was observed with any of these compounds at concentrations up to 1000 μg/ml.

2.2.2. Antiherpetic activity

The three polysaccharide fractions were then screened for antiviral activity against the reference strains F of HSV-1 and G of HSV-2, by a virus plaque reduction assay on Vero cells. As shown in Table 3, ShWE and F1 exhibited potent *in vitro* antiherpetic activity with inhibitory concentration 50% (IC_{50}) values in the range of 0.5–1.4 μg/ml. The purified xylomannan F1 was more active than ShWE for both tested strains. On the other hand, the antiviral effect of the respective desulfated sample F1D was lost (IC_{50} > 100 μg/ml) as an apparent consequence of the desulfation process. This result is in agreement with previous reports indicating that the antiviral activity of polysaccharides is linked to the anionic features of the macromolecules (Damonte et al., 2004; McReynold and Gervay-Hague, 2007; Witvrouw and De Clercq, 1997). For comparative purposes, heparin was simultaneously assayed as a known reference polysaccharide and it was found less effective than F1 to inhibit the multiplication of HSV-1 and HSV-2 (Table 3).

To determine the spectrum of antiherpetic activity of the sulfated xylomannans, both ShWE and F1 were evaluated against several HSV strains, including two TK^- strains of HSV-1 resistant to acyclovir (B2006 and Field strains), two syncytial HSV-1 variants named 1C3-syn 13-8 and 1C3-syn 14-1 and the MS strain of HSV-2. As shown in Table 4, both compounds were effective inhibitors of all tested strains with IC_{50} values ranging from 0.6 to 4.6 μg/ml. Again, F1 was more active than the crude extract ShWE and it also exhibited higher effectiveness than heparin against all HSV strains.

Given the lack of cytotoxicity exhibited by the sulfated xylomannans, and the very good antiviral activity against the wide spectrum of HSV strains tested, these type of polysaccharides presented very high selectivity indices (SI: ratio CC_{50}/IC_{50}). Particularly, the values of SI for F1 against all the spectrum of HSV-1 and HSV-2 variants analyzed were in the range of >2000–>654 (calculated from data in Tables 3 and 4), indicating the specificity of the inhibitory effect of this type of polysaccharides against herpesviruses.

Although the efficacy of sulfated polysaccharides against diverse enveloped viruses is well known (Damonte et al., 2004), most studies have been focused predominantly on naturally occurring galactans and carrageenans. Only, a few reports performed the evaluation of antiviral activity of mannose polysulfates. The complex xylomannan synthesized by the red seaweed *N. fastigiat* was one of the most extensively analyzed systems of this type of polysaccharides, reporting a selective inhibitory activity against HSV-1, HSV-2 and HCMV, attributable to a sulfate group distribution mimicking the negative binding site of the primary virus receptor heparan sulfate (Damonte et al., 1994; Kolender et al., 1997). Other studies have also shown inhibition of HIV by mannan sulfate (Ito et al., 1989) and a synthetic α-(1 → 6)-D-mannopyranan sulfate (Hatanaka et al., 1991). In a more recent study Ono et al. (2003) found *in vitro* and *in vivo* antiviral properties against yellow fever virus and dengue virus in two galactomannans isolated from seeds of *Mimosa scabrella* and *Leucaena leucocephala*. However the very high effectiveness and selectivity exhibited by the purified sulfated xylomannan F1 against both serotypes of HSV (Table 3) in comparison with the other sulfated mannans of previous study is noteworthy.

Table 3
Antiviral activity and selectivity indices of xylomannan fractions from *Scinaia hatei* (See text for abbreviations)

Compound	IC_{50} (μg/ml)[a]		SI[b]	
	HSV-1 (F)	HSV-2 (G)	HSV1 (F)	HSV-2 (G)
ShWE	1.4 ± 0.3	1.3 ± 0.5	>714	>769
F1	0.5 ± 0.1	0.5 ± 0.2	>2000	>2000
F1D	>100	>100	I[c]	I[c]
Heparin	1.3 ± 0.1	2.1 ± 0.1	>769	>476

[a] IC_{50} (inhibitory concentration 50%): concentration required to reduce plaque number in Vero cells by 50%. Each value is the mean of two determinations ± SD.
[b] SI (selectivity index): CC_{50}/IC_{50}. CC_{50} (cytotoxic concentration 50%): concentration required to reduce the number of viable Vero cells by 50% after 48 h of incubation with the compounds. This concentration was ⩾1000 μg/ml for all the compounds.
[c] I: inactive.

Table 4
Spectrum of antiherpetic activity of sulfated xylomannans from *Scinaia hatei* (See text for abbreviations)

Virus strain	IC_{50} (μg/ml)[a]		
	ShWE	F1	Heparin
HSV-1 (B2006)	4.2 ± 1.2	1.1 ± 0.2	4.3 ± 1.0
HSV-1 (Field)	2.8 ± 0.4	0.7 ± 0.1	4.1 ± 1.3
HSV-1 (1C3-syn 13-8)	2.8 ± 0.4	0.6 ± 0.2	7.4 ± 0.5
HSV-1 (1C3-syn 14-1)	2.7 ± 0.8	1.53 ± 0.01	9.1 ± 2.7
HSV-2 (MS)	4.6 ± 0.6	1.2 ± 0.4	0.5 ± 0.1

[a] IC_{50} (inhibitory concentration 50%): concentration required to reduce plaque number in Vero cells by 50%. Each value is the mean of two determinations ± SD.

2.2.3. Virucidal activity

In order to analyze the possibility that these polysaccharides may act directly on the virus particle leading to infectivity inactivation, a virucidal assay against HSV-1 (F) virions was carried out. ShWE and F1 had a very weak inactivating effect on HSV-1 virions at concentrations far from the antiviral IC_{50}. The values of virucidal concentration 50% (VC_{50}) were 100 and 61 μg/ml for ShWE and F1, respectively. The comparison of these data with the IC_{50} values (Table 3) indicated that the concentrations of ShWE and F1 required to inactivate HSV-1 virons by pretreatment before infection were 72- and 122-fold higher, respectively, than the concentrations required to reduce virus plaques when compounds were added at the time of virus adsorption. These results confirmed that the inhibitory effect detected by the plaque reduction assay was mainly due to interference with some step of the HSV-1 multiplication cycle. In agreement with these data, previous studies stated that the mode of antiviral action of the polysaccharides was attributed predominantly to inhibition of virus binding to the cells (Adhikari et al., 2006; Chattopadhyay et al., 2007; Mandal et al., 2007).

2.2.4. Anticoagulant activity

Activated partial thromboplastine time (APTT) was measured to evaluate the anticoagulant activity of the sulfated xylomannans. The APTT value of the blood treated with saline was 38.0 s. Treatment of the blood with F1 and ShWE at concentrations near the antiviral IC_{50} did not produce an important change in the APTT value, which was only 1.2–1.4-fold enhanced at concentrations of 1–2 μg/ml (Table 5). It was required a concentration highly exceeding the IC_{50} for both xylomannans to produce a significant anticoagu-

Table 5
Anticoagulant activity of sulfated xylomannans from *Scinaia hatei*

Compound	Concentration (μg/ml)	APTT (s)[a]
ShWE	200	>180
	20	95.1 ± 8.6
	2	51.0 ± 3.7
	1	45.8 ± 2.9
F1	200	>180
	20	>180
	2	51.8 ± 7.6
	1	51.7 ± 2.8
Heparin	1	± 180

[a] APTT: activated partial thromboplastine time. The data are the mean values of two experiments ± SD. For control sample with PBS: 38 s.

lant effect. Furthermore, it must be noted that both compounds had a marked reduced anticoagulant activity when compared with heparin. In fact, the APTT of F1 and ShWE were of the same order of that of heparin only at concentrations 20–200 times higher, respectively, (Table 5).

3. Conclusions

In conclusion, the xylomannan sulfate of the red seaweed *S. hatei*, which contained a backbone of α-(1 → 3)-linked mannopyranosyl residues substituted at position 2, 4 and 6 positions with single stubs of β-linked terminal xylose residues, exhibited potent antiviral activity against reference strains, syncytial variants and TK^- ACV resistant strains of HSV-1 and HSV-2. The inhibition of *in vitro* HSV replication was observed at concentrations, which did not have any effect on cell viability. Moreover, this polymer did not exhibit significant anticoagulant properties.

Several sulfated polysaccharides are currently undergoing clinical evaluation and perspectives for finding antiviral drugs with novel mode of action are promising. Given the very interesting chemical characteristics of the xylomannan sulfate of *S. hatei* and the promising *in vitro* anti-herpetic properties here reported, this compound represents a good candidate for further clinical research.

4. Experimental

4.1. General Experimental Procedures

Chemicals used were analytical grade or best available. All determinations were done at least in duplicate. Evaporations were performed under diminished pressure at ~45 °C (bath) and small volume of aqueous solutions was lyophilized. Recording of IR spectra and optical rotation measurements were carried out as described previously (Ray, 2006). Total sugars and uronic acids were determined by the phenol–sulfuric acid (Dubois et al., 1956) and *m*-hydroxydiphenyl (Ahmed and Labavitch, 1977) assay, respectively. For the determination of sugar composition, the monosaccharide residues released by acid hydrolysis were converted into their alditol acetate (Blakeney et al., 1983) and analyzed by gas–liquid chromatography (GLC; Shimadzu GC-17A, Shimadzu, Kyoto, Japan). Monosaccharides were identified by thin-layer chromatography and gas–liquid chromatography–mass spectrometry (GLC–MS; Shimadzu QP 5050 A, Shimadzu) as described (Mazumder et al., 2005). Alternatively, TMS-derivatives of methyl glycosides were analyzed by GLC (York et al., 1985).

4.2. Plant material and preliminary treatments

Samples of *S. hatei* (Rhodophyta) were collected from the Okha coast of Gujarat, India in August 1995. The seaweeds were washed thoroughly with tap water, dried by forced air circulation and pulverized in a blender. Algal powder (270 g) was depigmented using sequential extraction with petroleum ether (24 h) and acetone (24 h) as solvent in a Soxhlet apparatus. The unextracted material was placed in a plastic beaker and air dried to yield depigmented algal powder (DAP; 166 g).

4.3. Extraction of sulfated xylomannan

Extraction of DAP with water (pH 6.0) at a solute to solvent ratio of 1:100 (w/v) was conducted at 25–32 °C for 12 h under constant stirring for three times. Separation of the residue from the extract was performed by filtration through glass filter (G-3). The residue was briefly washed with additional distilled water and the wash was collected to maximize polysaccharide recovery. The liquid extract was dialyzed extensively against water and lyophilized. The recovered material was dissolved in water; the polysaccharides were precipitated twice with ethanol (4 volumes) and then collected by centrifugation. The final pellet was dissolved in water and lyophilized to yield the water extracted polysaccharide, named ShWE. The aqueous 80% ethanol soluble fractions were combined, desalted on Sephadex™ G-10 column (90 × 2.6 cm; Amersham Pharmacia biotech AB) and finally lyophilized to produce ShWE-S fraction.

4.4. Purification of sulfated xylomannan by size exclusion chromatography

The water extracted fraction ShWE was chromatographed on a Sephacryl S-400 column (90 × 2.6 cm; Amersham Biosciences AB) using 0.5 M sodium acetate buffer (pH 5.0) as eluant. The flow rate of the column was 0.5 ml/min, and fractions of 7 ml were collected and checked by the phenol–sulfuric acid reaction (Dubois et al., 1956). The column was calibrated with standard dextrans (2000, 500, 70, 40 and 10 kDa). In separate experiments, the first fraction F1 was re-chromatographed on the same column under experimental condition described above except that factions of 10 ml were collected. The final xylomannan sulfate preparation recovered after lyophilization of dialyzed eluate has been designated as F1.

4.5. Smith degradation of xylomannan

Periodate oxidation of polysaccharide was carried out as described by Fry (1988). Briefly, a solution of 100 mg of polysaccharide in 50 ml of reagent (50 mM $NaIO_4$ made up in 0.25 M formic acid, pH adjusted to 3.7 with 0.5 M NaOH) was incubated in the dark for 144 h at 4–6 °C. Next, the excess of periodate was decomposed with 10 ml ethane-1,2-diol, and the solution stirred for a further 1 h period at room temperature. To the vial containing oxopolysaccharide a solution (50 ml) of 950 mg of $NaBH_4$ in 1 M NaOH was added and the mixture was kept at room temperature for 12 h. The solution was subsequently neutralized with HOAc, dialysed, and lyophilsed to give the oxidized and reduced polysaccharide. This preparation was hydrolysed with TFA (pH 2) for 10 min at 100°C and the resulting hydrolysate was desalted by passing through a column (90 × 2.6 cm) of Sephadex™ G-10. Fractions (5 ml) were collected and analyzed for their total sugar (Dubois et al., 1956) contents. Appropriate polymeric fractions were lyophilized to afford a Smith degraded xylomannan, F1D-Sm (50 mg).

4.6. Sulfate estimation and desulfation

Estimation of sulfate by the modified barium chloride method (Craigie et al., 1984) and IR-spectrometry (Rochas et al., 1986),

and solvolytic desulfation by the method of Falshaw and Furneaux (1998) were carried out as described (Ghosh et al., 2004).

4.7. Linkage analysis

The triethylamine form (Stevenson and Furneaux, 1991) of native (F1), desulfated (F1D) and desulfated Smith degraded (F1D-Sm) xylomannan (~3 mg of each) was subjected to two rounds of methylation (Blakeney and Stone, 1985). Permethylated samples were hydrolysed, converted into their partially methylated alditol acetates and analyzed by GLC and GLC–MS as described (Ray and Lahaye, 1995).

4.8. NMR Spectroscopy

The ^{1}H NMR spectra of the F1, F1D and F1D-Sm were recorded on a Bruker 600 spectrometer (Bruker Biospin AG, Fallanden, Switzerland) operating at 600 MHz for ^{1}H. The sulfated xylomanan was converted into sodium salt by passage through a column (7 mL, Bio-Rad, Hercules, CA, USA) of Amberlite IR 120 (H^+), and all samples were deuterium-exchanged by lyophilization with D_2O and then examined as 1% solutions in 99.8% D_2O.

4.9. Cells and viruses

Vero (African green monkey kidney) cells were grown in Eagle's minimum essential medium (MEM) supplemented with 5% calf serum. For maintenance medium (MM), the serum concentration was reduced to 1.5%.

HSV-1 strain F and HSV-2 strains G and MS were used as reference strains. B2006 and Fields are HSV-1 thymidine kinase negative (TK^-) acyclovir-resistant strain obtained from Prof. Dr. E. De Clercq (Rega Institute, Belgium). The syncytial variants of HSV-1 1C3-syn 13-8 and 1C3-syn 14-1 were obtained by serial passage in the presence of the *mu*/*nu*-carrageenan 1C3 as previously described (Carlucci et al., 2002). Virus stocks were propagated and titrated by plaque formation in Vero cells.

4.10. Cytotoxicity test

Vero cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma–Aldrich, St. Louis, MO, USA) method. Confluent cultures in 96-well plates were exposed to different concentrations of the polysaccharides, with three wells for each concentration, using incubation conditions 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%.

4.11. Virus plaque reduction assay

Antiviral activity was evaluated by a virus plaque reduction assay. Vero cell monolayers grown in 24-well plates were infected with about 50 PFU/well in the absence or presence of various concentrations of the compounds. After 1 h of adsorption at 37 °C, 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. 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.

4.12. Virucidal assay

A virus suspension of HSV-1 (F) containing $4x10^6$ PFU was incubated with an equal volume of MM with or without various concentrations of the polysaccharides for 2 h at 37 °C. The samples were then diluted in cold MM to determine residual infectivity by plaque formation. The sample dilution effectively reduced the drug 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.

4.13. Assay for anticoagulant activity

Anticoagulant activities of the sulfated xylomannans were determined using the activated partial thromboplastine time (APTT) assay. Briefly, 30 μl of test solution were added to 100 μl of pooled human plasma and 100 μl of APTT reagent (Wiener lab, Rosario, Argentina). The mixture was incubated for 1 min at 37 °C. After the incubation, 70 μl of $CaCl_2$ 0.025 M were added and the time to clot formation was recorded.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2008.05.004.

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