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Structure and antiviral activity of sulfated fucans from *Stoechospermum marginatum*

Utpal Adhikari [a,1], Cecilia G. Mateu [b,1], Kausik Chattopadhyay [a], Carlos A. Pujol [b], Elsa B. Damonte [b], Bimalendu Ray [a,*]

[a] *Natural Products Laboratory, Department of Chemistry, The University of Burdwan, Golapbag, Burdwan, West Bengal 713 104, India*
[b] *Laboratorio de Virología, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales UBA, Ciudad Universitaria-Pabellón 2 Piso 4, 1428 Buenos Aires, Argentina*

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Abstract

A sulfated fucan containing fraction (SmWE) was isolated from water extract of the brown seaweed *Stoechospermum marginatum* collected from the Arabian Sea. Anion exchange chromatography of the crude fraction results in the production of a sulfated fucan (F3) having a molecular mass of 40 kDa and specific rotation $[\alpha]_D^{30}$ − 124° (*c* 0.5, H_2O). NMR spectroscopic studies and methylation analysis suggested that the polymer consists of a backbone of (1 → 4)- and (1 → 3)-linked-α-L-fucopyranosyl residues that are substituted at C-2 and C-3, and that fucosyl residues are sulfated mostly at C-2 and/or C-4. SmWE and F3 were selective inhibitors of herpes simplex virus type 1 (strain F, thymidine kinase-deficient strains field and B2006 and syncytial variants arising after selection with a natural carrageenan syn 13-8 and 14-1) and type 2 (strain MS) in Vero cells, with antiviral effective concentration 50% (EC_{50}) values in the range 0.63–10.0 μg/ml. The compounds were highly selective due to the lack of cytotoxicity. The antiviral activity was dependent on the presence of the sulfated fucans during the adsorption period. No direct inactivating effect on virions was observed in a virucidal assay. The absence of anticoagulant activity at concentrations near EC_{50} confirmed that there was no correlation between the antiviral and anticoagulant properties.

Keywords: Antiviral agent; Herpes simplex virus; Anticoagulant activity; *Stoechospermum marginatum*; Sulfated fucan; Molecular mass; Methylation analysis; NMR spectroscopy

1. Introduction

Human herpes simplex virus (HSV) infections cause one of the most important life-threatening diseases, particularly for immunocompromised individuals such as patients with AIDS and renal transplant recipients (Whitley and Gnann, 1993). Although acyclovir has gained a notable success in reducing the severity of HSV infection (Brady and Bernstein, 2004), the fact that viral mutants, which acquire resistance to acyclovir, have arisen during the prolonged drug treatment is one of the reasons for a continuous search for new antiviral compounds (Bacon et al., 2003; Eizuru, 2003).

Recent observations have accumulated evidence about the *in vitro* activity of sulfated polysaccharides against animal viruses including HSV types 1 and 2, human cytomegalovirus (HCMV), human immunodeficiency virus type-1 (HIV-1), respiratory syncytial virus (RSV), influenza virus as well as bovine viral diarrhea virus, probably by competing with cell surface heparan sulfate for binding to the virus (Duarte et al., 2004; Ghosh et al., 2004a,b; Ponce et al., 2003; Mazumder et al., 2002; Franz et al., 2000; Iqbal et al., 2000; Gunay and Linhardt, 1999; Witvrouw and De Clercq, 1997). The possibility of an interaction of these

* Corresponding author. Tel.: +91 342 26 57 790; fax: +91 342 2564452.
E-mail addresses: bimalendu_ray@yahoo.co.uk, dgp_konica2@sancharnet.in (B. Ray).
[1] The authors contributed equally to this work.

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compounds with the target cell to block virus entry was also suggested (McClure et al., 1992). Thus, the antiviral potential of polysaccharides extracted from marine algae becomes of considerable interest.

Like many other sulfated polysaccharides, fucans possess a wide spectrum of activity in biological systems. They play an important role in the anticoagulant and antithrombotic activity, act on the immune system and inflammatory process, have antiproliferative and antiadhesive effect on cells, interfere with mechanisms involved in fertilization and protect cells from viral infection (Boisson-Vidal et al., 1995). Furthermore, sulfated fucans appear to play important natural roles concerning the algal cell wall organization and the morphogenesis of algae embryos (Bisgrove and Kropf, 2001).

Seaweeds grow abundantly along Indian coastal line and represent an important biomass poorly exploited (Wealth of India, 1985). Among these, *Stoechospermum marginatum* (Ag) Kutz is a very abundant brown seaweed, rich in sulfated fucans, and known to possess spasmogenic activity (Wealth of India, 1985). But the polysaccharides present in this seaweed have not been thoroughly investigated. Since most studies of biological activity have been carried out using a relatively crude sulfated fucan preparation, it is not easy to determine the relationships between activity and structure. Thus, in order to get more information in this respect, we isolated and purified a sulfated fucan from water extract of *S. marginatum* and analyzed its chemical and biological properties.

2. Results and discussion

2.1. Isolation of fucan sulfate

In order to study the chemical structure and antiviral activity of polysaccharides present in *S. marginatum*, we have extracted the algal powder sequentially with hexane and acetone using a Soxhlet apparatus and then extracted the resulting depigmented algal powder (DAP) with water as shown in Fig. 1. Purification is then achieved by repeated precipitation of the macromolecule from solution with dehydrated ethanol (4 vols.). The water extracted fraction SmWE amounted to 9% of the starting algal dry weight and contained 43% total sugar on the basis of fraction dry weight. This polymeric material, which had negative specific rotation $[\alpha]_D^{30} - 93°$ (*c* 0.3, H_2O), was soluble in water and contained sulfate group. Compositional analysis revealed that it consists of a polysaccharide containing high amount of fucose together with smaller amount of galactose, glucose, xylose and mannose (Table 1). Thin layer chromatographic analysis of the monosaccharides present in the hydrolysate indicates the presence of, *inter alia*, an uronic acid with R_f value similar to that of galacturonic acid.

Anion exchange chromatography on a DEAE–Sepharose FF column chromatography separated the crude fucan sulfate into three fractions (I, II and III). The two peaks (I and II) eluted with 0.2- and 0.6-M NaOAc, containing uronic acid, correspond to heterogeneous sulfated polysaccharides

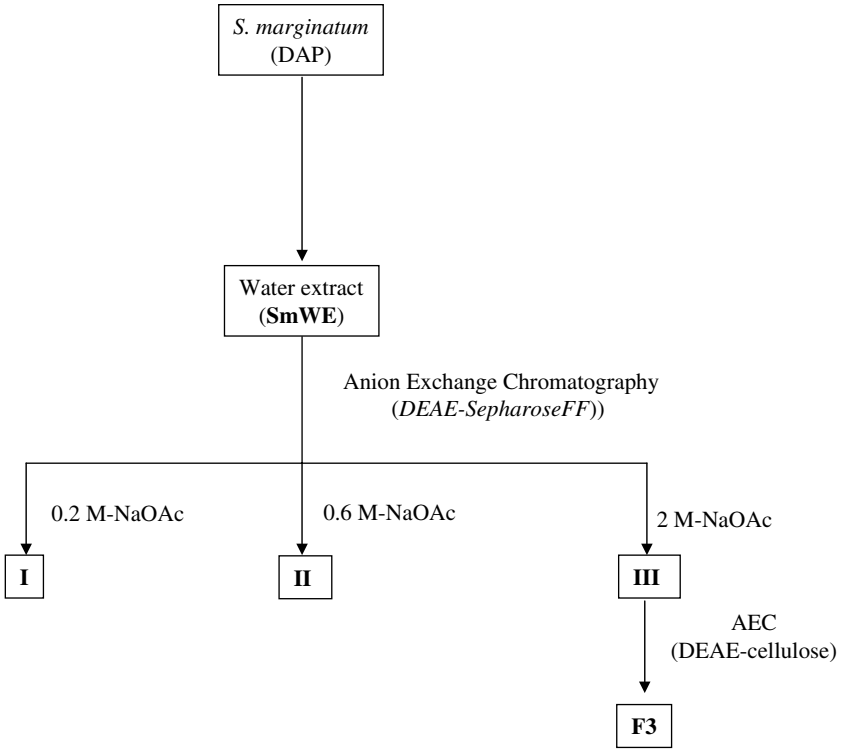

Fig. 1. Scheme for the isolation of fucan sulfate from the brown seaweed *S. marginatum*.

Table 1
Sugar composition and optical rotation of polysaccharides containing fractions from *Stoechospermum marginatum* (see text for the identification of fractions)

Fractions	SmWE	SmWE-D	I	II	III	F3	F3D
$[\alpha]_D$ 30 °C	−93°	–	–	–	–	−124°	–
NS[a]	39	60	29	36	35	37	66
Galacturonic acid[a]	4	7	3	5	1	–	–
Sulfate[a]	10	1	–	–	12	13	–
Fucose[b]	91	93	84	83	95	96	97
Xylose[b]	3	3	10	9	2	2	2
Mannose[b]	1	tr	3	4	nd	nd	nd
Galactose[b]	3	3	2	2	3	2	1
Glucose[b]	2	1	1	1	nd	nd	nd

–, not determined; nd, not detected; tr, trace; NS, neutral sugar.
[a] Percent weight of fraction dry weight.
[b] Mol percent of neutral sugars.

(Table 1). The peak (III) eluted at highest NaOAc concentration (2-M) contains fucose and has high sulfate content. The purity of the major fraction (III) was confirmed by re-chromatography on DEAE–cellulose column (Fig. 2). The final sulfated fucan (F3), which contained mostly fucose and sulfate, was subjected to structural analysis. This fucan sulfate, which had negative specific rotation $[\alpha]_D^{30}$ − 124° (*c* 0.5, H_2O), is soluble in water. The high negative rotation of the polysaccharide revealed that fucose belongs to the L-series, like in other sulfated fucans from brown seaweed.

2.2. Molecular mass

To evaluate the relationship between the polysaccharide molecular weight and the inhibitory capacity, we have determined the apparent molecular mass of fucan sulfate (F3) by gel permeation chromatography. The result suggested that it is homogeneous and has an apparent molecular mass of 40 kDa.

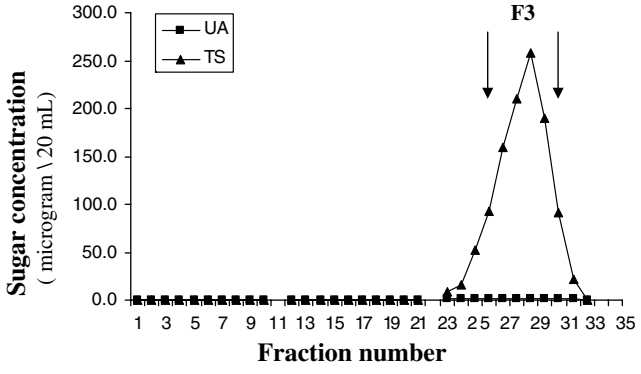

Fig. 2. Anion exchange chromatography of fraction III isolated from *S. marginatum* on DEAE–cellulose column. F3: Fraction eluted with 2 M NaOAc buffer (pH 5.0).

2.3. Desulfation

Antiviral activities of polysaccharides are linked to the anionic features of the macromolecules (Franz et al., 2000; Gunay and Linhardt, 1999; Witvrouw and De Clercq, 1997). To investigate the effect of sulfate group and to determine the location of this group, we have desulfated the crude fucan sulfate (SmWE) and the purified fucan sulfate (F3) by methanol/DMSO method (Nagasawa et al., 1977). Preliminary experiments (data not shown) showed that this method gives lowest sulfate content compared to auto-desulfation and methanol–HCl method (Percival and Wold, 1963). Desulfated derivative of SmWE (SmWE-D) had a recovery yield of 46%, whereas desulfation of F3 gave lesser recovery yield (41%). The sugar composition of SmWE and F3 and their desulfated derivatives SmWE-D and F3-D, respectively, were close (Table 1) indicating that sugar backbone remained almost unaffected by chemical modification.

2.4. IR analysis

The IR spectrum of F3 contained an intense absorption band at 1251 cm^{-1} common to all sulfate esters. An additional sulfate absorption band at 821 cm^{-1} (C–O–S, secondary equatorial sulfate) indicated that majority of sulfate groups occupy positions C-2 and/or C-3 of fucopyranose residues as in other sulfated fucans (Bilan et al., 2004). Desulfation of F3 resulted in the disappearance of these absorbances demonstrating that they were associated with sulfate groups.

2.5. Methylation analysis

F3 and F3-D were subjected to methylation analysis to determine the linkage positions of fucose branches and sulfate groups. Glycosyl linkage analysis of the desulfated fucan sulfate gave, *inter alia*, 2,3,4- tri-*O*-methylfucitol, 2,3-di-*O*-methylfucitol, 2,4-di-*O*-methylfucitol, 3-*O*-methylfucitol and 2-*O*-methylfucitol residues, suggesting that the fucan sulfate has (1 → 4)- and (1 → 3)-linked backbone together with branches at C-2 and C-3 (Table 2). In addition, the presence of 3,4-di-*O*-methylfucitol indicates the presence of 2-linked fucosyl residues. F3 gave 10 different partially methylated alditol acetates (PMAA) indicating that the structure of the sulfated fucans is highly complex. 3-*O*-methyl fucitol and unmethylated fucitol were the abundant products of methylation analysis of the native polymer. It should, however, be noted that complete methylation of the sulfated polysaccharide, because of the steric hindrance by the sulfate esters, is very difficult (Patankar et al., 1993; Pereira et al., 1999). The increase in the proportions of 2,3-di-*O*-methyl fucitol, 2,4-di-*O*-methyl fucitol after desulfation, as a consequence of decreased proportions of fucitol (unmethylated fucose), 2-*O*-methyl fucitol and 3-*O*-methyl fucitol residues, suggests that sulfate esters when present are located at C-2, or C-4, or C-2 and C-4 of

Table 2
Partially methylated fucitol acetates derived from fucan sulfate (F3) of *Stoechospermum marginatum* and its desulfated derivative (F3D)

Methylation products	*m/z* Values	Peak area[a]	
		F3	F3D
2,3,4-Fuc[b]	43, 72, 89, 102, 115, 118, 131, 162 and 175	1	8
2,3-Fuc	43, 102, 118, 143, 162 and 203	5	38
2,4-Fuc	43, 89, 118, 131,173, 187, 215 and 234	6	25
3,4-Fuc	43, 88, 89, 115, 130, 131,175, 190 and 234	4	5
2-Fuc	43, 118, 215 and 275	14	7
3-Fuc	43, 88,101, 130, 143, 190 and 203	28	12
Fuc	43, 103, 115, 128, 145, 157, 171, 187, 218, 260 and 290	37	3
2,3,4-Xyl	43, 101, 102, 117, 118, 161 and 162	1	
3,4-Xyl	43, 87, 102, 118, 129, 173, 189 and 233	2	1
2,3,6-Gal	43, 45, 87, 101, 102, 113, 118, 129, 162 and 233	2	1

[a] Percentage of total area of the identified peaks.
[b] 2,3,4-Fuc denotes 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylfucitol, etc.

the fucose residues. IR analysis showed that sulfate groups when present are located at C-2/3 of fucosyl residues. It was, however, reported that the position of IR peaks at 815–850 cm^{-1} could not be used with certainty to predict the position of sulfate groups (Ray and Lahaye, 1995).

2.6. NMR Spectroscopy

NMR spectroscopy is a convenient method to give valuable structural information of polysaccharides. We employed NMR analysis to determine the anomeric configuration and the sulfation pattern of the fucan sulfate. The native polysaccharide has a very complex ^{1}H NMR spectrum (Fig. 3) due to its heterogeneous sulfation pattern. At least 13 separate spin systems attributable to anomeric protons of α-fucose residues were distinguishable in the spectrum of the pure fucan sulfate. It also include resonances characteristic of sulfated α-fucans such as signals from ring protons (H-2–H-5) between 3.2 and 4.8 ppm, and intense signals from the methyl protons H-6, one at

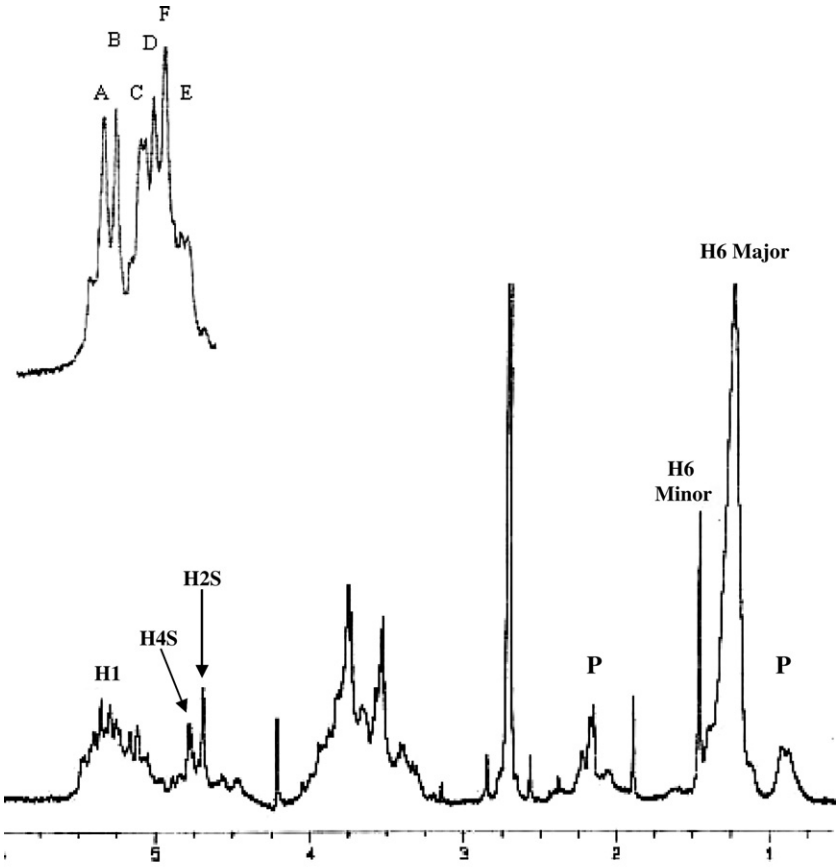

Fig. 3. ^{1}H NMR spectrum at 500 MHz of α-fucan from *S. marginatum*. The spectrum was recorded at 70 °C for samples in D_2O solution. P = signals of protein. Expansion of the 4.9–6.1 ppm region of the ^{1}H NMR spectrum of the desulfated fucan is shown in the inset.

about 1.42 ppm (minor) and a major envelope of signals at around 1.23 ppm. The residues with H-6 signals at 1.23 ppm may be 4-linked (Mulloy et al., 2000). Broad signals between 2.4 and 3.5 ppm, and at about 0.8 ppm, can be attributed to a small proportion of protein molecule in the sample. The signal appearing at 4.78 ppm attributed to the H-4 of 4-*O*-sulfated residues (Bilan et al., 2004; Kariya et al., 2004; Pereira et al., 1999) confirmed the results of methylation analysis. By comparison with the NMR spectra of with model compounds, the sharp signal at 4.72 ppm was tentatively assigned to the H-2 of 2-*O*-sulfated residues (Vilela-Silva et al., 2002; Pereira et al., 1999). Interestingly, even after desulfation at least six prominent signals were appeared in the anomeric region of the ^{1}H NMR spectrum (Fig. 3; inset) of the desulfated fucan (F3D), each consistent with α-fucosyl residues. Therefore, it is apparent that the NMR spectrum of this fucan sulfate is complex as observed for sulfated fucans from other sources (Kariya et al., 2004; Vilela-Silva et al., 2002; Pereira et al., 1999; Murao et al., 1996; Patankar et al., 1993).

2.7. Biological activity

The antiviral activity of SmWE and F3 against HSV-1 (F) and HSV- 2 (MS) was evaluated in Vero cells by a virus plaque reduction assay. As shown in Table 3, the two compounds were very active against both serotypes of herpes virus, being SmWE more active than F3 for HSV-1 (EC_{50} 1.15 and 3.55 μg/ml, respectively) and equally active for HSV-2 (EC50 0.78 μg/ml for SmWE and 0.63 μg/ml for F3). Furthermore, a very good antiviral activity was observed when the assay was performed against the TK^- strains B2006 and Field of HSV-1, with values of the EC_{50} in the range 0.95–2.26 μg/ml. The compounds were also active against the syncytial strains syn 13-8 and syn 14-1, although the EC_{50} values were slightly higher (in the range 4.52–10 μg/ml) (Table 4). For comparative purposes of the antiviral potential of these compounds, commercial samples of dextran sulfate 8000 and heparin were used as reference substances. In general, as seen in Tables 3 and 4, the compounds obtained from *S. marginatum* were similarly active than the commercial samples. On the contrary, the desulfated polysaccharides SmWE-D and F3-D showed a significant reduction in their antiviral activity, with an increment of 14–64-fold in the EC_{50} values (Table 3).

Table 3
Antiviral activity and selectivity indices of fucan sulfate isolated from *Stoechospermum marginatum* (Ag) Kutz and the desulfated derivatives against herpes simplex virus type 1 and 2

Compound	EC_{50} (μg/ml)		SI (CC_{50}/EC_{50})	
	HSV-1 (F)	HSV-2 (MS)	HSV-1 (F)	HSV-2 (MS)
SmWE	1.15 ± 0.09	0.78 ± 0.2	>869	>1282
SmWE-D	31.8 ± 2.1	>50	>31	–
F3	3.55 ± 0.63	0.63 ± 0.01	>281	>1587
F3-D	50 ± 1.0	40.77 ± 5.21	>20	>24
DS 8000	2.12 ± 0.4	0.57 ± 0.01	>472	>1754
Heparin	1.20 ± 0.48	0.53 ± 0.12	>833	>1887

EC_{50} (Inhibitory concentration 50%): concentration required to reduce plaque number in Vero cells by 50%. Mean of two determinations ± SD.
CC_{50} (cytotoxic concentration 50%): concentration required to reduce 50% the number of viable Vero cells after 48 h of incubation with the compounds. This concentration was >1000 μg/ml for all the compounds tested.
SI (selectivity index): CC_{50}/IC_{50}.
Dextran sulfate MW 8000 (DS 8000) and heparin are included as reference substances.

No cytotoxicity was observed with the water extract SmWE and the fraction F3 when cell viability was evaluated in preformed monolayers of Vero cells in the presence of concentrations up to 1000 μg/ml. Thus, the evaluation of the selectivity index (ratio CC_{50}/IC_{50}) revealed very high values for the compounds (range of selectivity indices 100–1587) (Tables 3 and 4).

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. SmWE and F3 were unable to inactivate HSV-1 (F) virions at the maximum concentration tested of 40 μg/ml. This concentration is far from the antiviral EC_{50}, indicating that the inhibitory effect detected by the plaque reduction assay was really due to interference with some step of the HSV-1 replication cycle.

The inhibitory effect of SmWE and F3 against HSV-1 (F) was totally dependent on the presence of the compounds during the initial virus adsorption to the cell. When the plaque reduction assay was performed omitting the presence of the compounds in the adsorption period and including them only in the plaquing medium after

Table 4
Antiviral activity and selectivity indices of fucan sulfate isolated from *Stoechospermum marginatum* (Ag) Kutz against thymidine kinase-deficient strains and syncytial variants of HSV-1 (F)

Compound	EC_{50} (μg/ml)				SI (CC_{50}/EC_{50})			
	TK^-		Syncytial		TK^-		Syncytial	
	B2006	Field	13–8	14–1	B2006	Field	13–8	14–1
SmWE	2.26 ± 0.33	1.75 ± 0.03	5.48 ± 0.2	10 ± 0.5	>442	>571	>182	>100
F3	0.95 ± 0.14	1.52 ± 0.38	4.52 ± 1.67	5.67 ± 0.94	>1053	>658	>221	>176
DS 8000	2.5 ± 0.2	2.19 ± 0.75	3.61 ± 1.82	10.88 ± 3.85	>400	>457	>277	>92
Heparin	4.29 ± 0.99	4.1 ± 1.28	7.37 ± 0.52	9.09 ± 2.76	>233	>244	>136	>110

For references see Table 3.

adsorption, no significant reduction in the number of virus plaques was detected (Fig. 4). By contrast, the presence of these compounds only during virus adsorption was as effective to reduce plaque number as the treatment during the whole incubation period, during and after adsorption, suggesting that the main target of the sulfated polysaccharides is the binding of virus to the host cell receptor. The effects of the sulfated fucans were not reversible, since the withdrawal of the compounds after adsorption did not affect the antiviral activity.

No anticoagulant activity was observed when the sulfated fucans were tested at concentrations near the antiviral EC_{50} (2 μg/ml) (Table 5). Furthermore, when the concentration was raised to 20 μg/ml only a slight increase in the APTT value was seen for SmWE, while the value for F3 was similar to the control in which PBS was used.

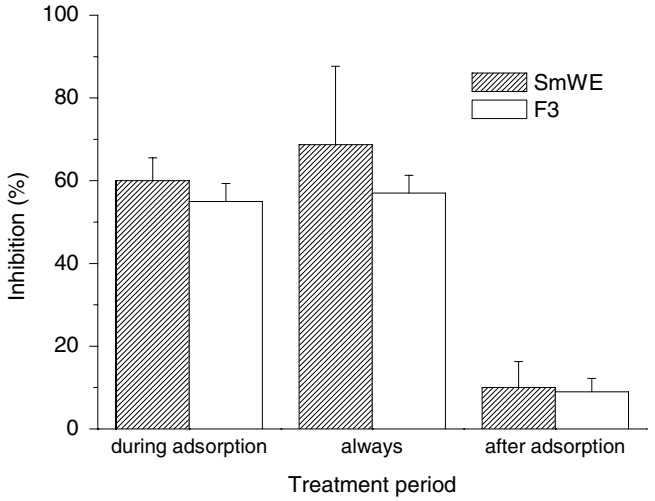

Fig. 4. Influence of various treatment periods on the anti HSV-1 (F) activity of SmWE and F3. Vero cells were infected with 50 PFU/ml of the virus. After 1 h of virus adsorption at 4 °C in MEM with or without the compounds (5 μg/ml), unabsorbed virus and the compounds were removed. The cells were overlaid with medium containing 0.7% methylcellulose with or without the compounds and further incubated at 37 °C for 2 days, where after the number of plaques was determined. Each value represents the mean of duplicate assays.

Table 5
Anticoagulant activity of fucans isolated from *Stoechospermum marginatum*[a]

Compound	APTT (s)[b]		
	Concentration (μg/ml)		
	2	20	200
SmWE	36.5	43	109
F3	36.5	37.5	143
Heparin	>180	nd[c]	nd

[a] The data are the mean values of two experiments.
[b] APTT: activated partial thromboplastine time. For control sample without compound 36.5 s.
[c] nd: not done.

The anticoagulant activities of the sulfated fucans were evident only when the highest concentration of 200 μg/ml was employed, showing a significant increase in the APTT value (more than 100 s). On the other hand, when 2 μg/ml of heparin was used as a reference substance with well proved anticoagulant activity, the time to clot formation was >180 s.

3. Experimental

3.1. Plant materials

Samples of *S. marginatum* were collected from the Gujarat coast (India) of Arabian Sea in August 1995. The gathered material was cut, washed, dried immediately by forced air circulation at 35–40 °C and grinded in a warring blender.

3.2. Isolation of fucoidan containing fraction

The milled algal biomass (130 g) was treated sequentially with petroleum ether and acetone in a Soxhlet for 2×28 h to remove pigments, filtered and vacuum dried to yield 85 g of depigmented algal powder (DAP). Extractions of DAP (10 g) with water at pH 6.5–7 (w/v: 1:10) were conducted at 30–35 °C for 3.5 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 solution was dialyzed extensively against water and lyophilized. The recovered material was re-dissolved in water and then precipitated with ethanol (4 vols.). This process of dissolution of the macromolecules in water and their precipitation with ethanol was repeated twice. The final pellet dissolved in water and lyophilized to yield the water-extracted polysaccharide (SmWE, 0.9 g).

3.3. Purification of fucoidan by anion exchange chromatography

System a. Water extracted sulfated polysaccharides were applied to a DEAE–Sepharose FF column (20×2 cm) equilibrated with 50 mM NaOAc buffer (pH 5.0) and washed with 200 mL of same buffer. Thereafter, the column was then eluted successively with 0.2 M (fraction I), 0.6 M (fraction II) and 2 M NaOAc (fraction III) buffer in a stepwise manner. The flow rate of the column was 0.5-ml/min and elution with each buffer was carried out up to the absence of a positive reaction for carbohydrates with phenol and sulfuric acid. The collected fractions were analyzed for their total sugar and uronic acid contents. All the solutions obtained were dialyzed and then lyophilized.

System b. In a separate experiment, the isolated fucan sulfate (III) was re-chromatographed on a DEAE–cellulose

column under experimental condition described above. The final fucan sulfate preparation recovered after lyophilization of dialyzed eluate has been designated as F3.

3.4. General methods

Recording of IR spectra and optical rotation measurements were carried out as described previously (Ghosh et al., 2004a,b).

3.5. Sugar analysis

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 neutral sugar composition, the acid hydrolyzed glycoses were converted into their alditol acetates and separated by gas liquid chromatography (Blakeney et al., 1983). Monosaccharides were identified by thin layer chromatography and gas liquid chromatography-mass spectrometry as described (Ghosh et al., 2005).

3.6. Sulfate estimation

Samples were hydrolyzed in 3 ml concentrated hydrochloric acid in sealed glass tubes at 100 °C. Sulfate was then determined using a modified turbidometric barium chloride (Craigie et al., 1984). The sulfate content was also measured by considering the ratio of the intensity of the 1250 cm^{-1} band to the intensity of the 2920 cm^{-1} band as described by Rochas et al. (1986).

3.7. Desulfation

Desulfation of the sulfated fucan was performed by solvolysis in dimethyl sulfoxide by the methods of Nagasawa et al. (1977) as described previously for desulfation of other types of polysaccharides (Ray and Lahaye, 1995; Ghosh et al., 2004a,b). Alternatively, this sulfated polysaccharide was desulfated by auto-desulfation and the methanol–HCl method (Percival and Wold, 1963).

3.8. Methylation analysis

The native (F3) and desulfated (F3D) fucans (2 mg of each) were subjected to two rounds of methylation (Blakeney and Stone, 1985), with the modifications suggested by Stevenson and Furneaux (1991). Permethylated alditol acetates were analyzed by GLC and GLC/MS (Shimadzu QP 5050 A GLC/MS) as described (Ghosh et al., 2004a,b).

3.9. Molecular mass

The purified F3 fraction (100 mg) was chromatographed on a Sephacryl S-300 column (90 cm × 2.6 cm) using 500 mM sodium acetate buffer (pH 5.0) as eluant. The flow rate of the column was 0.3 ml/min, and fractions of 5 ml were collected and checked by the phenol–sulfuric acid reaction (Dubois et al., 1956). The column was calibrated with standard dextrans (500, 70, 40 and 10 kDa). Standard dextrans were a gift from Dr. Tapani Vuorinen.

3.10. Spectroscopy

3.10.1. GC-MS

EI mass spectra were recorded with a Shimadzu OP5050A GC-MS instrument at 70 eV.

3.10.2. NMR spectroscopy

The ^1H spectra of native and desulfated fucans were recorded using Bruker DRX-500 NMR spectrometer. Samples were deuterium-exchanged by lyophilization with D_2O and then examined as 0.7% solutions in 99.8% D_2O. All spectra were recorded at 70 °C with HOD suppression by pre-saturation. ^1H chemical shifts were measured relative to external DSS.

3.11. Biological activity

3.11.1. 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 MS were used as reference strains. B2006 and Field were HSV-1 TK^- strains received from Prof. Dr. E. De Clercq (Rega Institute, Leuven, Belgium). Syn 13-8 and syn 14-1 were HSV-1 syncytial variants arising after serial passages on Vero cells in the presence of a natural carrageenan obtained from the red seaweed *Gigartina skottsbergii* (Carlucci et al., 2002). Virus stocks were propagated and titrated by plaque formation in Vero cells.

3.11.2. Cytotoxicity test

Vero cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma–Aldrich) method (Mosmann, 1983). Confluent cultures in 96-well plates were exposed to different concentrations of the polysaccharide, 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%.

3.11.3. 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 plaque-forming units (PFU) of virus/well in the absence or presence of various concentrations of the polysaccharide. After 1 h of adsorption at 4 °C, residual inoculum was replaced by MM containing 0.7% methylcellulose and the corresponding dose of compound. Plaques were counted after 2 days of incubation at 37 °C. The effective concentration 50% (EC_{50}) was calculated as the compound concentration required to reduce virus plaques by 50%. All determinations were performed twice and each in duplicate.

3.11.4. Virucidal assay

A virus suspension of HSV-1 (F) containing 4×10^6 PFU was incubated with an equal volume of MM with or without various concentrations of the compounds 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.

3.11.5. Effect of the incubation time on the antiviral activity

Vero cells grown in 24 well plates were infected with 50 PFU of HSV-1 (F) under different treatment conditions: exposure to 5 μg/ml of the compounds was restricted to the virus adsorption phase only (compound in the inoculum), or to adsorption and post-adsorption (compound in the inoculum and in the plaquing medium) or to the post-adsorption period only (in the plaquing medium). After 2 days of incubation at 37 °C, plaques were counted and the EC_{50} values were calculated for each treatment.

3.11.6. Assay for anticoagulant activity

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

4. Conclusions

In conclusion, this is the first time that antiherpetic activities are reported in products derived from the marine algae *S. marginatum*. The inhibition of *in vitro* HSV replication was observed at concentrations, which do not have any effect on the cell viability. These results encourage the use of the compounds as antiviral agents in alternative therapies.

Besides, it is well known that antiviral and anticoagulant activities increase with increasing molecular weight and sulfate content, but perhaps these activities are not simply a function of charge density but depends critically on the conformation and dynamic stereochemistry (time and solvent dependent aspect of conformation) of the polysaccharide.

The complete structure of the sulfated fucan isolated by extraction with water was not obtained, but some important structural features were established. We have found that the purified polymer is (1 → 3)- and (1 → 4)-linked and sulfated at C-2 and/or C-4. But nothing is known about their conformations and dynamic stereochemistry or how their spatial patterns of sulfate groups determine their biological properties. The structural basis for biological and physiological properties of sulfated fucans depends crucially on the presence of sulfate groups. Both their charge density and their specific positions influence biological properties, as discussed. To date the only way to assess the importance of position of sulfate groups is to try to compare related fucans.

The availability of fucans with well-defined structures and of enzymes able to modify these polysaccharides will bring clarity to analysis of their biological properties and allow the design of new experiments. Future conformational studies of well-defined sulfated fucan structures should lead to better understanding of the biological properties of fucoidans.

The complexity and heterogeneity of sulfated fucans, and their wide range of activities in mammalian systems, are not attractive within current paradigms of drug development. The combination of structural elucidation and assignment of biological activity to specific structural features can only improve the potential of sulfated fucans and fucoidans both as therapeutic agents and as research reagents.

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