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Galactan sulfate of *Grateloupia indica*: Isolation, structural features and antiviral activity

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Abstract

Natural compounds offer interesting pharmacological perspectives for antiviral drug development with regard to broad-spectrum antiviral properties and novel modes of action. In this study, we have analyzed polysaccharide fractions isolated from *Grateloupia indica*. The crude water extract (GiWE) as well as one fraction (F3) obtained by anion exchange chromatography had potent anti-HSV activity. Their inhibitory concentration 50% (IC_{50}) values (0.12–1.06 μg/ml) were much lower than cytotoxic concentration 50% values (>850 μg/ml). These fractions, which were effective antiviral inhibitors if added only during the adsorption period, had very low anticoagulant activity. Furthermore, they had no direct inactivating effect on virions in a virucidal assay. Chemical, chromatographic and spectroscopic methods showed that the active polysaccharide, which has an apparent molecular mass of 60 kDa and negative specific rotation $[\alpha]_D^{32}$ −16° (*c* 0.2, H_2O), contains α-(1 → 4)- and α-(1 → 3)-linked galactopyranose residues. Sulfate groups, if present, are located mostly at C-2/6 of (1 → 4)- and C-4/6 of (1 → 3)-linked galactopyranosyl units, and are essential for the anti herpetic activity of this polymer.

Keywords: *Grateloupia indica*; Red algae; Galactan sulfate; Herpes simplex virus; Antiviral activity

1. Introduction

Seaweeds have been widely used as food for centuries in Asia (Darcy-Vrillon, 1993; Indergaard and Minsaas, 1991), but in western countries they are generally employed for the production of valuable chemicals. The main compounds agars, aligns and carrageenans are used as ingredients in food, pharmaceuticals and diverse consumer products and industrial processes (Mazumder, 2006; Renn, 1997; Stephen, 1995; Zilinskas and Lundin, 1993; Skjak-Braek and Martinsen, 1991; Lewis et al., 1988). Recent observations have accumulated evidence about the *in vitro* activity of algal sulfated polysaccharides against animal viruses including herpes simplex virus types 1 (HSV-1) and 2 (HSV-2), human cytomegalovirus (HCMV), human immunodeficiency virus type-1 (HIV-1), respiratory synytial virus (RSV) and influenza virus (Tischer et al., 2006; Rechter et al., 2006; Matsuhiro et al., 2005; Schaeffer and Krylov, 2000; Franz et al., 2000; Gunay and Linhardt, 1999; Witvrouw and De Clercq, 1997). Thus, the potential of polysaccharides extracted from seaweed as antiviral agents becomes of considerable interest.

We have previously analyzed the structural characteristics and antiviral properties of sulfates polysaccharides isolated from green and red seaweeds collected on Indian and South American coasts (Adhikari et al., 2006; Duarte et al., 2004; Ghosh et al., 2004; Ponce et al., 2003; Carlucci et al., 2002; Mazumder et al., 2002). This paper describes the isolation, purification, structural features

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and antiviral activity of a sulfated galactan present in the red seaweed *Grateloupia indica* against HSV-1 and HSV-2.

2. Results and discussion

2.1. Isolation, purification and structural analysis of the sulfated galactan from the red seaweed Grateloupia indica

2.1.1. Isolation and composition of polysaccharide fractions

The depigmented algal powder (DAP) from *G. indica*, which contained galactose as dominant monosaccharide (Table 1), was extracted with water as described in the experimental section. Purification of the water-extracted fraction was then achieved by repeated precipitation of the macromolecule from solution with dehydrated ethanol (4 vol.). This fraction (GiWE), which amounted for 13% of DAP dry weight, had negative specific rotation $[\alpha]_D^{32}$ −5° (*c* 0.2, H_2O). The total sugar content of GiWE was 43% with galactose as the major sugar (Table 1). No methylated sugars were detected during GLC-MS analysis of the derived alditol acetates. Both TLC analysis of the sugar released during hydrolysis and GLC analysis of the TMS-derivatives of the generated methyl glycosides confirmed the presence of glucuronic acid. The uronide content of GiWE was 3% and this fraction contained sulfate (Table 1). The FT-IR spectrum of GiWE showed an intense absorption band in the region 1253 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, but the band at 930 cm^{-1} characteristic of 3,6-anhydrogalactosyl residues was not observed.

Anion exchange chromatography on a DEAE Sepharose column separated the water-extracted polymers of *G. indica* into three sub-fractions (F1, F2 and F3). F1, which accounted for 7% of the total sugars recovered from the anion exchanger, was the minor component of GiWE. It contained mostly galactose (>92%) together with small amounts of xylose, glucose and fucose residues (Table 1). Sugar composition of F2 was very similar to F1. The differential elution of F1 and F2 was due to the level of uronic acid and sulfate. In the major sub-fraction F3, galactose accounted for more than 99% of the neutral sugars. This sub-fraction amounted to 73% of the total carbohydrates recovered from the column and contained 2% (w/w) of uronic acid. It is, therefore, essentially a galactan that might contain high amount of sulfate group, as indicated by its late elution. Indeed, the high charge density of this polysaccharide was confirmed by its high sulfate content (16%, w/w). This purified galactan sulfate had negative specific rotation $[\alpha]_D^{32}$ −16° (*c* 0.2, H_2O) and was used for further analysis.

Table 1
Sugar composition (mol %) of fractions obtained from *Grateloupia indica* (see text for the identification of fractions)

	DAP	GiWE	GiWE-D	F1	F2	F3	F3D
Sulfate[a]	nd	11	1	9	12	16	1
NS[a]	39	40	48	38	37	41	50
UA[a]	4	3	4	3	5	2	nd
Fuc[b]	1	1	2	1	1	tr	–
Xyl[b]	1	2	3	4	3	tr	–
Gal[b]	84	94	93	92	93	99	100
Glc[b]	14	3	2	3	3	tr	–

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

2.1.2. Molecular mass

Size exclusion chromatography of F3 on Sephacryl S-300 suggests that the polymer is homogeneous. Based on calibration with standard dextrans, the apparent molecular weight of the galactan present in F3 would be 60 kDa. It should, however, be noted that polysaccharides containing sulfate groups, due to intramolecular electrostatic repulsions by charge effects, may have a different hydrodynamic volume than dextrans and, therefore, elute at a different rate than expected on the basis of their molecular weight.

2.1.3. Desulfation

The crude (GiWE) and the purified (F3) galactan sulfates were desulfated by solvolysis in dimethyl sulfoxide (Falshaw and Furneaux, 1998). Preliminary experiments (data not shown) showed a higher recovery with this method compared to methanol–HCl and auto-desulfation methods (Percival and Wold, 1963). Desulfation of GiWE and F3 had a recovery yield of 49% and 43%, respectively. Notably the sugar composition of GiWE and F3 and their desulfated derivatives (GiWED and F3D) were nearly similar (Table 1). The IR spectrum of F3 was similar to that of GiWE, with bands at 1253 and 830 cm^{-1}, whereas in the IR spectrum of the desulfated galactan F3D these bands became weak.

2.1.4. Linkage analysis

Methylation analysis of desulfated F3D revealed the presence of 2,4,6- and 2,3,6-tri-*O*-methyl galactose in the ratios of 44.9:49.7, indicating the presence of (1 → 3)- and (1 → 4)-linked galactopyranosyl residues, respectively (Table 2). Small proportions of 1,3,4-linked galactose residues (2.7%) as well as terminal galactose residues (2.7%) have also been detected, indicative of a branched polysaccharide. Possibly these derivatives came from non desulfated units and/or from minor structural components.

Linkage analysis of F3 yielded a variety of mono-, di- and trimethylated products (Table 2). The results of this study also suggest that sulfate groups, when present, reside mostly at *O*-2/6 of (1 → 4)- and *O*-4/6 of (1 → 3)-linked galactopyranosyl units. This result is similar to those obtained from sulfated galactans of red algae and seagrasses (Aquino et al., 2005; Farias et al., 2000; Painter, 1983), but differs from the report of Sen et al. (1994) where

Table 2
Partially methylated galactitol acetates derived from sulfated galactan (F3) of *Grateloupia indica* and its desulfated derivative (F3D)

Methylation products	Peak area[a]	
	F3	F3D
2,3,4,6-Gal[b]	–	2.7
2,4,6-Gal	24.3	44.9
2,3,6-Gal	8.3	49.7
2,6-Gal	20.0	2.7
3,6-Gal	7.2	–
2,3-Gal	17.7	–
2,4-Gal	8.0	–
2-Gal	3.1	–
4-Gal	3.4	–
Gal	7.8	–

–, not detected.
[a] Percentage of total area of the identified peaks.
[b] 2,3,4,6-Gal denotes 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, etc.

it had been claimed that the galactan of *G. indica* is predominantly (1 → 3)-linked.

2.1.5. NMR spectroscopy

The native sulfated galactan (F3) has a very complex ^{1}H NMR spectrum (Fig. 1a). The presence of at least 16 distinguishable anomeric signals suggests heterogeneous sulfation pattern. The desulfated galactan F3D showed three anomeric resonances, one at 5.2 and two others around 5.7–5.8 ppm (Fig. 1b). These chemical shift values suggest that the galactose residues present in the sulfated galactan

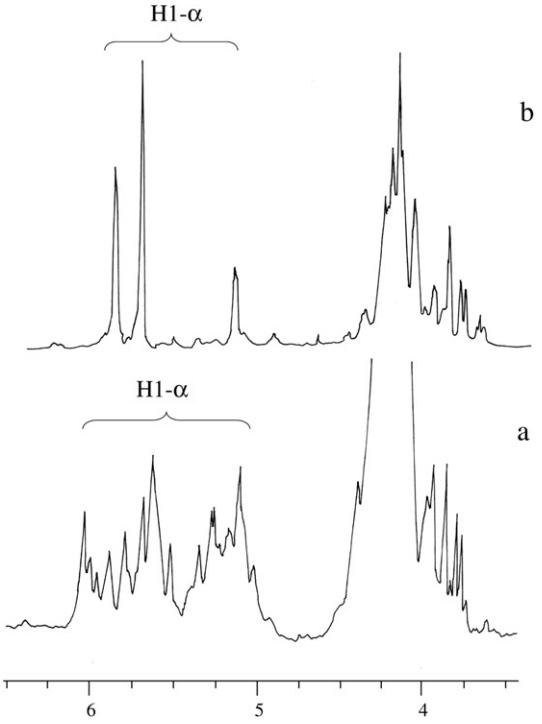

Fig. 1. ^{1}H NMR spectra at 500 MHz of (a) the native galactan sulfate of *Grateloupia indica* and (b) its desulfated derivative (F3D). The spectrum was recorded at 70 °C for samples in D_2O solution.

of this study are α-linked. Normally, the (1 → 3)-linked galactosyl residues present in the sulfated galactan of marine red macroalgae are β-linked (Matsuhiro et al., 2005; Farias et al., 2000; Knutsen et al., 1994; Painter, 1983). Even the sulfated D-galactan of the seagrass Ruppia maritima is made up of the following regular tetrasaccharide repeating unit → 3)-β-D-Gal-2(OSO_3)-(1 → 4)- α-D-Gal-(1 → 4)-α-D-Gal-(1 → 3)- β-D-Gal-4(OSO_3)-(1 → (Aquino et al., 2005). In the ^{1}H NMR spectrum of this galactan there is a signal at 5.2 ppm for the anomeric proton of the α-(1 → 4)-units and two signals around 4.8 ppm for the β-(1 → 3)-units. In contrast, the (1 → 3)-linked galactopyranosyl residues present in the sulfated galactan of *G. indica* are α-linked. Notably, the presence of a 2-sulfated, 3-linked α-galactan in marine invertebrates has already been reported (Pereira et al., 2002).

2.2. Pharmacological activities of the sulfated galactans from the red seaweed G. indica

2.2.1. Antiherpetic activity

Table 3 summarizes the results of the antiviral activity and selectivity indices of the sulfated galactans of *G. indica*, and the corresponding desulfated derivatives in a plaque reduction assay. GiWE and F3 may be considered potent inhibitors of HSV-1 (F) and HSV-2 (MS), with values of IC_{50} ranging from 0.25 to 0.31 μg/ml. On the other hand, the desulfated derivatives GiWE-D and F3D were inactive against these viruses up to a concentration of 50 μg/ml. The conclusion that can be drawn is that the antiviral activity of these polysaccharides is linked to the anionic features of the molecules, given mainly by the high amount of sulfate groups.

In order to increase the antiviral activity spectrum, the sulfated compounds were also tested against two TK^- acyclovir-resistant variants (B2006 and Field) and two syncytial variants (1C3-syn 13-8 and 1C3-syn 14-1) of HSV-1. In both cases, GiWE appeared to be more active than F3 for the TK^- and syncytial strains. In spite of the small differences observed in the IC_{50} values of the compounds, GiWE and F3 exhibited high selectivity indices (943–7083) due to the low toxicity on Vero cells. According to these values, the sulfated galactans from *G. indica* represents a very potent antiherpetic compound among the diverse types of natural sulfated polysaccharides tested for antiviral activity (Damonte et al., 2004). They also showed a higher inhibitory effect when compared with reference sulfated polysaccharides such as dextran sulfate 8000 and heparin (Table 3).

To study the inhibition of GiWE on virus replication an additional experiment was performed employing immunofluorescence staining of viral proteins. In this experiment, the reduction in the number of fluorescent viral foci observed on HSV-1 (F) infected Vero cells and treated with GiWE was notorious when compared with the viral foci counted in infected cells in the absence of the compound. A reduction of 60% and 87% in virus positive foci forma-

Table 3
Antiviral activity against herpes simplex virus and selectivity indices of galactan sulfate isolated from *Grateloupia indica* and the desulfated derivatives

Virus	IC_{50} (μg/ml)[a]					
	GiWE	GiWE-D	F3	F3D	DS 8000	Heparin
HSV-1	0.27 ± 0.03	>50	0.27 ± 0.02	>50	2.12 ± 0.4	1.20 ± 0.48
(F)	(3148)[b]	(–)	(>3703)	(–)	(>472)	(>833)
HSV-2	0.25 ± 0.05	>50	0.31 ± 0.1	>50	0.57 ± 0.01	0.53 ± 0.12
(MS)	(3400)	(–)	(>3226)	(–)	(>1754)	(>1887)
HSV-1	0.18 ± 0.01	nd	0.89 ± 0.16	nd	2.5 ± 0.11	4.29 ± 0.99
B2006	(4722)		(>1123)		(>400)	(>233)
HSV-1	0.12 ± 0.05	nd	0.87 ± 0.19	nd	2.19 ± 0.75	4.1 ± 1.28
Field	(7083)		(>1149)		(>457)	(>244)
HSV-1	0.32 ± 0.08	nd	1.06 ± 0.26	nd	3.6 ± 1.8	7.4 ± 0.5
1C3-syn 13-8	(2656)		(>943)		(>278)	(>135)
HSV-1	0.42 ± 0.14	nd	0.81 ± 0.06	nd	10.9 ± 3.8	9.1 ± 2.7
1C3-syn 14-1	(2024)		(>1234)		(>92)	(>110)

nd, not determined.

[a] IC_{50} (Inhibitory concentration 50%): concentration required to reduce plaque number in Vero cells by 50%. Mean of two determinations ±SD.

[b] SI (Selectivity index): CC_{50}/IC_{50}. CC_{50} (Cytotoxic concentration 50%): concentration required to reduce 50% the number of viable Vero cells after 48 h of incubation with the compounds. The values of CC_{50} (μg/ml) were 850 for GiWE, 958 for GiWE-D and >1000 for F3, F3D, DS 8000 and heparin. DS 8000 (Dextran sulfate MW 8000) and heparin are included as reference substances.

tion was achieved when GiWE was present at a concentration of 0.5 and 2 μg/ml, respectively.

The virucidal concentration (VC_{50}) was also estimated for GiWE and F3 against HSV-1 (F) in order to elucidate the possibility that these polysaccharides may act directly on the virus particles. Preincubation of the virus with the compounds had no significant direct inactivating effect on HSV-1 virions up to 40 μg/ml that was the maximum concentration assayed (data not shown). Thus, the inhibitory effect of these sulfated polysaccharides appears to be based mainly on their ability to interfere with the replication cycle of HSV-1. The lack of virucidal activity for the galactans from *G. indica* is in accordance with previous studies that found most algal sulfated galactans are not able to produce significant virion inactivation (Mazumder et al., 2002; Talarico et al., 2004; Matsuhiro et al., 2005). The λ-carrageenan form *Gigartina skottsbergii*, which possesses potent inactivating properties against HSV-1 (Carlucci et al., 1999) is, however, an exception.

In order to establish the stage of the virus replication cycle at which the compounds exert their antiviral activity, a virus plaque reduction assay for HSV-1 (F) in Vero cells upon different treatment periods was employed (Fig. 2). A high level of efficacy was attained if the compounds (2 μg/ml) were present either only during HSV-1 adsorption or during the whole period of the plaque assay. When present only after adsorption, they were no longer effective despite the fact that their concentration was ten fold higher (20 μg/ml) for this treatment condition. This result is in agreement with previous studies that stated that the mode of antiviral action of the polysaccharides was attributed predominantly to inhibition of virus binding to the cells (Carlucci et al., 1997, 1999; Talarico et al., 2004; Matsuhiro et al., 2005) or, less frequently, to either inhibition of virus-cell fusion or inhibition of both virus-cell binding and fusion (Hosoya et al., 1991).

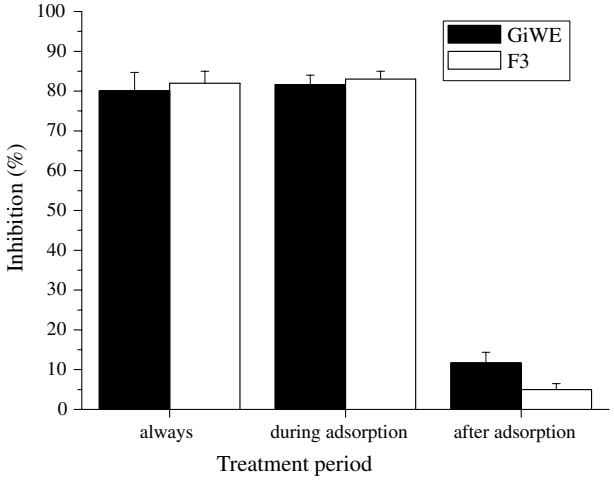

Fig. 2. Inhibitory effect of GiWE and F3 against HSV-1 (F) during and after virus adsorption. Vero cells were infected with 50 PFU of virus in a plaque assay under different treatment conditions. Always: GiWE and F3 (2 μg/ml) were present both during and after the adsorption period; during adsorption: GiWE and F3 (2 μg/ml) were present only during adsorption; after adsorption: GiWE and F3 (20 μg/ml) were added in the plaquing medium after adsorption. After 2 days of incubation at 37 °C, plaques were counted. Results are expressed as % inhibition respect to untreated infected control. Each value is the mean of duplicate determinations ± SD.

2.2.2. Anticoagulant activity

To evaluate the anticoagulant activity of the sulfated galactans, the activated partial thromboplastin time (APTT) was measured. The APTT value of plasma treated with PBS was 37 s. For GiWE and F3 a value of 38 s was recorded when they were tested at a concentration of 2 μg/ml. On the contrary, heparin at 2 μg/ml showed APTT value higher than 180 s. These results indicated that both natural sulfated polysaccharides have no anticoagulant activity at concentrations 2–20-fold higher than the IC_{50}, showing a negative correlation between antiviral properties

and intrinsic coagulation pathways. Several natural polysulfates present this independence between antiviral and anticoagulant activities, supporting the therapeutic perspectives of this class of compounds (Damonte et al., 2004). However it is noticeable that a preliminary communication has reported anticoagulant activity of a sulphated galactan isolated from *G. indica* (Sen et al., 1994). To our knowledge, there are no further studies on the biological properties of *G. indica* polysaccharides until the present study. As above mentioned, the structural features of polysaccharide preparation used by Sen et al. (1994) for studying anticoagulant activity is different from the galactan of the present study (see Section 2.1.4). In addition, the dissimilar results can also be explained considering the parameters analyzed to assay the anticoagulant properties: in present study the APTT was measured, whereas Sen et al. (1994) determined the prothrombin time and the clotting time.

3. Experimental

3.1. Isolation and chemical characterization of sulfated galactan from the red algae Grateloupia indica

3.1.1. Plant material and preliminary treatments

Grateloupia indica (Grateloupiaceae, Rhodophyta), collected from Okha coast of Gujrat, India, in August 1995, was freed from attached impurities, washed thoroughly with tap water, dried by forced air circulation (35–40 °C) and ground to a flour in a Waring Blender. This powdered seaweed (260 g) was extracted sequentially with benzene (20 h) and acetone (20 h), in a Soxhlet apparatus to leave a depigmented algal powder (DAP, yield 164 g).

3.1.2. Extraction of sulfated galactan

Extraction of DAP with water (pH 6.0) at a solute to solvent ratio of 1:120 (w/v) was conducted at 30–38 °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 solution was dialyzed against water and lyophilized. The recovered polymer was re-dissolved in water and then precipitated with ethanol (4 vol.). This process of dissolution of the polymers in water and their precipitation with ethanol was repeated twice. The final pellet was dissolved in water and lyophilized to yield water-extracted fraction, namely GiWE.

3.1.3. Purification of sulfated galactan by anion exchange chromatography

A solution (20 mL) of the crude water extract (GiWE, 75 mg) in 50 mM sodium acetate (pH 5.5) was applied to a column (2.6 × 25 cm) of DEAE-Sepharose FF (AcO^-). Thereafter, the column was eluted (0.6 mL min^{-1}) successively with 0.05-, 0.15-, 0.75- and 2.0-M NaOAc buffer (pH 5.5) in a stepwise manner. Fractions (20 ml) were collected and analyzed for their total sugar (Dubois et al., 1956) and uronic acid (Ahmed and Labavitch, 1977) contents. Appropriate fractions were pooled, dialyzed and lyophilized.

3.1.4. Size exclusion chromatography

Size exclusion chromatography of the sulfated galactan (F3) on Sephacryl S-300 column (90 × 2.6 cm) using 0.5-M sodium acetate buffer (pH 5.0) as eluant was done as described (Adhikari et al., 2006). The column was calibrated with standard dextrans (500, 70, 40 and 10 kDa).

3.1.5. Chemical analysis

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 GLC (Shimadzu GC-17 A). Monosaccharides were identified by thin-layer chromatography and gas liquid chromatography–mass spectrometry (Shimadzu QP 5050 A) as described (Mazumder et al., 2005). Alternatively, TMS-derivatives of methyl glycosides were analyzed by gas chromatography (York et al., 1985).

3.1.6. 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).

3.1.7. Linkage analysis

The triethylamine form (Stevenson and Furneaux, 1991) of native and desulfated galactan (~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 analysed by GLC and GLC/MS (Shimadzu QP 5050 A) as described (Ray and Lahaye, 1995).

3.1.8. NMR spectroscopy

The ^1H NMR spectra of the native and desulfated galactan were recorded on a Bruker ARX 500 spectrometer operating at 500 MHz for ^1H. The sulphated galactan was converted into sodium salt by passage through a column (7 mL, Bio-RAD) 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. ^1H NMR spectra were recorded at 70 °C with HOD suppression by pre-saturation.

3.2. Evaluation of biological activities

3.2.1. Cells and viruses

Vero (African green monkey kidney) cells were grown in minimum essential medium (MEM) supplemented with 5% fetal 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). 1C3-syn 13-8 and 1C3-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.

3.2.2. Cytotoxicity test

Vero cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma–Aldrich) method. 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%.

3.2.3. 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 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.

3.2.4. 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.

3.2.5. Effect of the incubation time on the activity of GiWE and F3 against HSV-1(F)

Vero cells grown in 24 well plates were infected with 50 PFU of HSV-1 (F) under different treatment conditions: exposure to 2 μg/ml of the compounds at 4 °C 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 (20 μg/ml of the compound in the plaquing medium). After 2 days of incubation at 37 °C, plaques were counted and the IC_{50} values were calculated for each treatment.

3.2.6. Indirect immunofluorescence staining

Vero cells grown on glass coverslips were infected with HSV-1 (F) (multiplicity of infection 0.1), in the absence or in the presence of 0.5 or 2 μg/ml of GiWE. At 24 h p.i., cells were washed with PBS and fixed with methanol for 15 min at −20 °C. Then, cells were washed with PBS and reacted with DEAE purified anti-HSV-1 IgG from hyperimmune rabbit serum for 30 min at 37 °C, followed by incubation with fluorescein-conjugated goat anti-rabbit IgG (Sigma–Aldrich, USA) for 30 min at 37 °C. After a final washing with PBS, the cells were counterstained with Evans blue at a dilution of 1:10,000 for 3 min at room temperature and mounted in a glycerol solution containing 1,4-diazabicyclo[2,2,2]octane (DABCO). The fluorescent virus foci in each preparation were counted.

3.2.7. Assay for anticoagulant activity

Anticoagulant activity of the galactans was 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, 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.

4. Conclusion

In conclusion, this is the first report of the antiherpetic activity of a sulfated polysaccharide derived from the red seaweed *G. indica*. The isolated galactan exhibited potent antiherpetic activity against reference strains, syncytial variants and TK^- ACV resistant strains, mainly affecting virus adsorption to the host cells. The inhibition of *in vitro* HSV replication was observed at concentrations which do not have any effect on the cell viability. Therefore, sulfated galactan of *G. indica* is a good candidate for further clinical research.

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