Anti-herpes simplex virus activity of sulfated galactans from the red seaweeds *Gymnogongrus griffithsiae* and *Cryptonemia crenulata*

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Abstract

This study presents the chemical composition and antiviral activity against herpes simplex virus type 1 (HSV-1) and 2 (HSV-2) of sulfated galactan crude extracts and main fractions obtained from two red seaweeds collected in Brazil, *Gymnogongrus griffithsiae* and *Cryptonemia crenulata*. Most of the eighteen tested products, including homogeneous kappa/iota/nu carrageenan and dl-galactan hybrid, exhibited antiherpetic activity with inhibitory concentration 50% (IC50) values in the range 0.5–5.6 μg/ml, as determined in a virus plaque reduction assay in Vero cells. The galactans lacked cytotoxic effects and showed a broad spectrum of antiviral activity against HSV-1 and HSV-2. No direct virus inactivation was observed after virion treatment with the galactans. The mode of action of these compounds could be mainly ascribed to an inhibitory effect on virus adsorption. Most importantly, a significant protection against a murine vaginal infection with HSV-2 was afforded by topical treatment with the sulfated galactans.

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Keywords: Herpes simplex virus; Antiviral activity; Sulfated polysaccharide; Kappa/iota/nu carrageenan; d,l-Galactan hybrid; Red seaweed

1. Introduction

Sulfated polysaccharides are known to present a broad range of biological activities comprising antiviral and antitumoral action, variable effects on the immune system and anticoagulant activity. In particular, the antiviral effect of naturally occurring and chemically synthesized polysulfates against a variety of enveloped viruses, such as herpes simplex virus type 1 (HSV-1) and 2 (HSV-2), human immunodeficiency virus (HIV), human cytomegalovirus, respiratory syncytial virus and influenza virus, has been reported [1,2].

At present, the availability of safe and potent antiviral agents against herpesviruses is far from ideal. Acyclovir (ACV) is the compound of choice for clinical use against HSV-1 and HSV-2, in systemic or topical therapy [3]. Other ACV-related nucleoside analogs, all targeted against viral DNA synthesis, have been more recently licensed for human use [4]. However, the emergence of viral resistant variants after prolonged treatment in immunocompromised patients is one of the main reasons for the continuous search of novel antiviral agents. In this context, the mentioned sulfated polysaccharides represent an interesting type of compounds to pursue as promising virus inhibitors.

Red seaweeds contain large amounts of polysaccharides, and sulfated galactans represent the main matrix polysaccharide. They consist of linear chains of alternating 3-linked d-galactopyranosyl residues (A units) and 4-linked galactopyranosyl (or 3,6-anhydrogalactosyl) residues (B units). The latter residues are from the d-series in the case of carrageenans and l-series for agarans, and with d- and l-galactose interspersed on the same molecule in the dl-galactan hybrids [5,6]. These structures are usually substituted with sulfate esters, pyruvic acid ketals, methoxyl groups and may have different degrees and kinds of branching [5].

Previous reports have described the effective inhibitory action of this type of natural polysulfates against HSV-1, HSV-2 and HIV [7–14].

This study presents the chemical composition and in vitro and in vivo antiviral properties of sulfated galac-
2. Materials and methods

2.1. Extraction and fractionation of polysaccharides

G. griffithsiæ (Turner) Martius was collected in Caiobá, Paraíba State, Brazil, and C. crenulata (J. Agardh) J. Agardh was obtained at Cupe’s beach, Pernambuco State, Brazil. A voucher of each specimen has been deposited in the herbarium of Departamento de Botânica, Universidade Federal do Paraná, with the herbarium number UPCB-46433 and 46432, respectively. The samples were washed with running water, sun-dried and milled.

G. griffithsiæ was extracted twice with water (3.0% w/v) at 25 °C with mechanical stirring for 16 h. After centrifugation the supernatant was dialyzed, concentrated and freeze-dried to give the crude extract C1. C2, G1 and G2. The algal residue was submitted to aqueous extraction at 100 °C with mechanical stirring for 2 h, and treated as described above to afford the crude extract G3. C. crenulata was extracted with water (1.5% w/v) at 25 °C for 5 h, with mechanical stirring, and then twice with phosphate buffer (NaH2PO4, 0.025 M, pH 6.5) at 80 °C for 6 h. The extracts obtained at each step were concentrated and lyophilized to produce C1, C2 and C3.

G3, C1 and C2 were dissolved in water (0.25% w/v) and submitted to KCl fractionation according to Pujol et al. [14]. Solid KCl was added with constant agitation to increase the concentration in steps of 0.1 M to an upper limit of 2.0 M. The precipitates obtained by centrifugation, as well as the material soluble in 2.0 M KCl, were dialyzed sequentially against water, 2.0 M NaCl and water, and freeze-dried. G3 rendered the fractions G3a, G3b, G3c and G3d, precipitated at 0.1, 0.3, 0.1 and 1.2 M KCl, respectively, and G3S, soluble in 2.0 M KCl. C1 and C2 were treated with KCl in the same way and 94.0% (C1S) and 97.5% (C2S) were recuperated in the supernatant after addition of 2.0 M KCl whereas the precipitated fractions were discarded.

2.2. Anion-exchange chromatography of G3S and C2S

A column (30 cm × 4.5 cm) was filled with DEAE-Sephalac and samples from G3S and C2S were dissolved in water. Fractions were first eluted with water (G3S-1 and C2S-1) and then sequentially with increasing concentrations of NaCl: 0.25 M (G3S-2, C2S-2), 0.5 M (G3S-3, C2S-3), 0.75 M (G3S-4, C2S-4), 1.0 M (G3S-5, C2S-5), 1.5 M (C2S-6) and 4.0 M (G3S-7, C2S-7). C2S-2 was refractionated using the same column and elution system with water and increasing concentrations of NaCl: 0.3 M (C2S-2a), 0.35 M (C2S-2b), 0.45 M (C2S-2c), and 1.0 M (C2S-2d).

2.3. Analytical methods

Total carbohydrate was estimated by the phenol–sulfuric acid method [15] using α-galactose as standard. Sulfate content was determined by the turbidimetric method of Dodgson and Price [16]. Protein was determined by the method of Lowry et al. [17]. Optical rotations of 0.2% aqueous solution of the polysaccharide samples were measured at 20 °C, using a 10-cm cell and sodium d line (589.3 nm) with a Rudolph Autopol III automatic polarimeter.

2.4. Monosaccharide composition

Monosaccharide composition was determined by the reductive hydrolysis method [18]. Extra reducing agent (N-methylmorpholine borane) was used to quantify the sugar components of the galactans from C. crenulata [19]. The alditol products of the hydrolysis were acetylated and analysed by GC and GC-MS as alditol acetates. To distinguish between co-eluting derivatives by GC-MS, partially methylated alditol acetates were also generated by hydrolysis in aqueous formic acid (45%) followed by NaBD4 reduction and acetylation. GC analyses were carried out on a HP-5890 gas–liquid chromatograph equipped with a flame ionization detector (FID), using a fused silica capillary column (30 m × 0.25 mm) coated with DB-225 (Durowax) and eluted isothermically at 210 °C. Nitrogen was used as carrier gas at flow rate of 1 ml/min and a split ratio of 100:1. GC-MS analysis was performed using a Varian 3900 chromatograph and a Finnigan Mat ITD spectrometer, equipped with two different columns, DB-225 or a DB-23. Helium was used as carrier gas at 1 ml/min.

2.5. Spectroscopic analysis

Fourier-transform infrared spectra (FTIR) of the fractions C2S-3 and G3M in the form of KBr pellets were recorded in a Bommen, series MB-100 FTIR spectrophotometer.

For nuclear magnetic resonance (NMR) spectroscopy analysis the freeze-dried sample was dissolved in D2O (30 mg/ml). The NMR spectrum of G3M was recorded at 50 °C using a Bruker Avance DRX 400 NMR spectrometer. Chemical shifts are expressed in ppm using acetone as the internal standard at 30.2 ppm.

2.6. 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 strain G were obtained from the American Type Culture Collection (Rockville, USA) and used as reference strains. B2006 was an HSV-1 TK-ACV-resistant strain obtained from Prof. Dr. E. De Clercq (Rega Institute, Belgium). HSV-2 strain MS was provided by
Dr. F. Benencia (Immunochimistry Lab., UBA, Argentina). The syncytial variants of HSV-1 IC3-syn 13-8 and IC3-syn 14-1 were obtained by serial passage in the presence of the mu/mu-cartagenenan IC3 as previously described [20]. Virus stocks were propagated and titrated by plaque formation in Vero cells.

2.7. Cytotoxicity assay

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 compounds. After 2 h of incubation at 37°C, residual inoculum was replaced by medium containing unadsorbed formazan crystals. After vigorous shaking, absorbance was measured in a microplate reader at 595 nm. The cytotoxic concentration 50% (CC50) was calculated as the compound concentration required to reduce cell viability by 50%.

2.8. In vitro antiviral assays

Antiviral activity was evaluated by a virus plaque reduction assay. Vero cell monolayers grown in 24-well plates were infected with about 50 PFU per well in the absence or presence of various concentrations of the compounds. After 1 h of incubation 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% (IC50) was calculated as the compound concentration required to reduce virus plaques by 50%. All determinations were performed twice and each in duplicate.

To test the effect of the incubation time on the antiviral activity of the sulfated galactans, Vero cells grown in 24-well plates were infected with 50 PFU of HSV-1, strain F, in MM containing 0.7% methylcellulose, 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% (CC50) was calculated as the compound concentration required to reduce cell viability by 50%.

2.9. In vitro virucidal assay

A virus suspension containing 4 × 10^5 PFU of HSV-1, strain F, was incubated with an equal volume of MM with or without various concentrations of the compounds for 1.5 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% (VC50), defined as the concentration required to inactivate virions by 50%, was then calculated.

2.10. Protection assay in a mouse model of HSV-2 intravaginal infection

Fourty-six to eight-week-old female BALB/c mice were used for a protection assay against vaginal inoculation with HSV-2, strain MS. Prior to virus inoculation, animals were injected subcutaneously with 20 μl of medroxyprogesterone acetate (Medrosterona; Gador, Argentina), prepared as 25 mg/ml solution in PBS, a treatment known to increase the susceptibility of mice to HSV-2 infection [21]. Five days later, animals were divided in four groups of 10 animals each: 50 μl of C2 (8 mg/ml in PBS) were instilled into the vagina of mice from groups 1 and 2, animals from group 3 received 50 μl of C2 (0.8 mg/ml in PBS), and the group 4 was inoculated with PBS. One minute after receiving C2 or placebo, mice from groups 2–4 were inoculated intravaginally with 1 × 10^5 PFU of HSV-2, strain MS, whereas the animals in group 1 were inoculated with PBS, as polysaccharide toxicity control. A second dose of C2, at the corresponding concentration, was administered 2 h after PBS (group 1) or HSV-2 (groups 2 and 3) inoculation, whereas group 4 was treated with PBS. Animals were examined for morbidity and mortality during 20 days.

To study virus shedding, samples of vaginal secretions were collected at 3 days post-infection, by washing the vagina with 100 μl of PBS, and frozen at −70°C until titration of HSV-2 infectivity by plaque assay. Data were analysed by using the chi-square method. A P value of 0.005 or less was considered significant.

3. Results and discussion

3.1. Extraction, fractionation and chemical characterization of the polysaccharides

Table 1 shows yield, chemical analysis and composition of crude extracts and fractions obtained from G. griffithsi. The extraction of water-soluble polysaccharides was carried out at 25°C (G1–G2) and 100°C (G3). G2, the major extract, was submitted to gradient KCl precipitation giving rise to four insoluble fractions (G3a–G3d) and the soluble G3s. The galactan G3d was homogenous by HPSEC-MALLS (data not shown), and contained galactose, 3,6-anhydrogalactose and 6-O-methylgalactose. The FTIR spectrum of G3d had strong absorption at 846 and 803 cm⁻¹ that are characteristic of 4-sulfated and 2-sulfated ester on galactosyl and 3,6-anhydrogalactosyl residues, respectively. The peak at 930 cm⁻¹ confirmed the presence of 3,6-anhydrogalactosyl units [22,23]. The anomeric region of the 13C NMR spectrum of G3d showed two major sig-
Table 1

Yields, chemical analysis, specific rotation and monosaccharide composition of polysaccharide fractions obtained from *G. griffithsiae* (G)

<table>
<thead>
<tr>
<th>Fractiona</th>
<th>Yield (%)</th>
<th>Carbohydrate (%)</th>
<th>Sulfate b (%)</th>
<th>Protein (%)</th>
<th>[α]D 20</th>
<th>Monosaccharides (mol %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3</td>
<td>24.0</td>
<td>55.2</td>
<td>33.1</td>
<td>3.6</td>
<td>+29.2</td>
<td>67.5 25.4 3.7</td>
</tr>
<tr>
<td>G3a</td>
<td>1.5</td>
<td>39.2</td>
<td>16.1</td>
<td>31.0</td>
<td>n.d.</td>
<td>64.5 15.6 3.0</td>
</tr>
<tr>
<td>G3b</td>
<td>5.5</td>
<td>52.2</td>
<td>25.5</td>
<td>14.1</td>
<td>n.d.</td>
<td>47.4 13.5 3.2</td>
</tr>
<tr>
<td>G3c</td>
<td>7.0</td>
<td>54.0</td>
<td>26.3</td>
<td>6.5</td>
<td>n.d.</td>
<td>70.0 30.0 –</td>
</tr>
<tr>
<td>G3d</td>
<td>68.6</td>
<td>52.0</td>
<td>29.4</td>
<td>1.7</td>
<td>+53.0</td>
<td>57.0 41.0 2.0</td>
</tr>
<tr>
<td>G3S</td>
<td>20.2</td>
<td>60.0</td>
<td>26.0</td>
<td>2.3</td>
<td>–60.0</td>
<td>66.3 9.1 13.6</td>
</tr>
<tr>
<td>G3S-1</td>
<td>24.0</td>
<td>47.4</td>
<td>4.2</td>
<td>6.5</td>
<td>–46.0</td>
<td>62.8 – 13.9 3.4</td>
</tr>
<tr>
<td>G3S-2</td>
<td>32.0</td>
<td>59.6</td>
<td>12.1</td>
<td>3.3</td>
<td>–50.0</td>
<td>72.9 – 14.0 10.8</td>
</tr>
<tr>
<td>G3S-3</td>
<td>5.5</td>
<td>52.2</td>
<td>25.5</td>
<td>21.1</td>
<td>14.1</td>
<td>47.4 13.5 3.2</td>
</tr>
<tr>
<td>G3S-4</td>
<td>7.0</td>
<td>54.0</td>
<td>28.3</td>
<td>6.5</td>
<td>n.d.</td>
<td>70.0 30.0 –</td>
</tr>
<tr>
<td>G3S-5</td>
<td>3.5</td>
<td>49.8</td>
<td>21.0</td>
<td>6.0</td>
<td>n.d.</td>
<td>88.7 7.3 –</td>
</tr>
<tr>
<td>G3S-6</td>
<td>9.5</td>
<td>36.6</td>
<td>35.0</td>
<td>5.3</td>
<td>+40.0</td>
<td>84.6 13.8 –</td>
</tr>
<tr>
<td>G3S-7</td>
<td>7.6</td>
<td>29.6</td>
<td>20.6</td>
<td>8.0</td>
<td>–52.0</td>
<td>67.6 13.7 6.8</td>
</tr>
</tbody>
</table>

(–) Not detected. n.d.: not determined.

a Fractions are defined in text.
b Expressed as SO3 Na.
c Gal corresponds to galactosyl, AG to 3,6-anhydrogalactosyl, 4Gal to 4-O-methylgalactosyl, 6Gal to 6-O-methylgalactosyl, Xyl to xylosyl and Glc to glucosyl residues.
d Percentages based on milled seaweed.
e Percentages based on material recovered from KCl fractionation (79.1%).
f Percentages based on material recovered from anion-exchange chromatography (89.2%).

nals at 101.7 and 91.6 ppm, corresponding to β-D-galactose 4-sulfate linked to 3,6-anhydro-α-D-galactose 2-sulfate. Additionally, the 13C NMR spectra showed C1 signals at 94.8 ppm (3,6-anhydro-α-D-galactose) and at 104.3 ppm, corresponding to β-D-galactose 4-sulfate linked to α-D-galactose 2,6-sulfate (97.7 ppm). These results together with the monosaccharide content and high positive specific rotation suggest that G3d has a carrageenan type backbone typical of the *kappapolyalgalacturonan* (Fig. 1, Ia, Ib, IIc) [24], which was also observed in other species of the genus *Gymnogongrus* [6,25,26].

Fraction G3S showed a negative specific rotation, suggesting principal agaran structures. Galactose, 4-O-methylgalactose and xylose were the principal monosaccharide components. G3S was fractionated by anion-exchange chromatography on DEAE-Sephacel to give seven fractions (G3S-1–G3S-7). The fractions G3S-1–G3S-3 (71.4% of total recovery) were mainly composed of galactose, 4-O-

Table 2

Yields, chemical analysis, specific rotation and monosaccharide composition of polysaccharide fractions obtained from *C. crenulata* (C)

<table>
<thead>
<tr>
<th>Fractiona</th>
<th>Yield (%)</th>
<th>Carbohydrate (%)</th>
<th>Sulfate b (%)</th>
<th>Protein (%)</th>
<th>[α]D 20</th>
<th>Monosaccharides (mol %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>4.0</td>
<td>44.6</td>
<td>22.0</td>
<td>8.4</td>
<td>+71.5</td>
<td>70.7 7.3 5.9</td>
</tr>
<tr>
<td>C2</td>
<td>64.8</td>
<td>59.3</td>
<td>28.0</td>
<td>4.8</td>
<td>+42.5</td>
<td>66.4 9.5 7.9</td>
</tr>
<tr>
<td>C3</td>
<td>1.1</td>
<td>49.2</td>
<td>42.0</td>
<td>6.9</td>
<td>+32.0</td>
<td>65.0 10.8 8.3</td>
</tr>
<tr>
<td>C1S</td>
<td>94.0</td>
<td>63.8</td>
<td>26.0</td>
<td>4.5</td>
<td>+25.5</td>
<td>62.7 10.2 7.3</td>
</tr>
<tr>
<td>C2S</td>
<td>97.5</td>
<td>63.6</td>
<td>27.7</td>
<td>1.5</td>
<td>+27.0</td>
<td>72.6 9.9 6.4</td>
</tr>
<tr>
<td>C3S-1</td>
<td>3.2</td>
<td>67.3</td>
<td>20.1</td>
<td>4.8</td>
<td>+11.0</td>
<td>72.8 8.5 6.9</td>
</tr>
<tr>
<td>C3S-2</td>
<td>24.1</td>
<td>69.2</td>
<td>25.1</td>
<td>1.9</td>
<td>–6.5</td>
<td>71.5 6.3 6.7</td>
</tr>
<tr>
<td>C3S-3</td>
<td>54.2</td>
<td>61.0</td>
<td>28.3</td>
<td>0.5</td>
<td>+23.5</td>
<td>68.4 13.8 8.6</td>
</tr>
<tr>
<td>C3S-4</td>
<td>3.3</td>
<td>69.3</td>
<td>17.5</td>
<td>2.1</td>
<td>+4.0</td>
<td>73.9 9.6 9.5</td>
</tr>
<tr>
<td>C3S-5</td>
<td>55.0</td>
<td>62.0</td>
<td>20.4</td>
<td>1.0</td>
<td>n.d.</td>
<td>76.7 9.0 6.2</td>
</tr>
</tbody>
</table>

(–) Not detected. n.d.: not determined.

a Fractions are defined in text.
b Expressed as SO3 Na.
c Gal corresponds to galactosyl, AG to 3,6-anhydrogalactosyl, 2AG to 3,6-anhydro-2-O-methyl-D-galactosyl, 4Gal to 4-O-methylgalactosyl, 6Gal to 6-O-methylgalactosyl, Xyl to xylosyl and Glc to glucosyl residues.
d Percentages based on milled seaweed.

* Percentage based on soluble material recovered using 2 M KCl.

† Percentage based on material recovered from anion-exchange chromatography.
agaran sulfate from
ride units IIa, IId and III. The last disaccharide unit is present in the
was extracted with water at 25
◦

G. griffithsiae
lambda
2-
mainly composed of galactose, 3,6-anhydrogalactose plus its
dl
G. torulosus
contains significant amounts of
dl
(a) R1 = R2 = SO3
(b) R1= H, R2 = SO3
(c) R1 = R2 = SO3, R3 = SO3
(d) R1 = SO3, R2 = H, R3 = SO3, R4 = H

G3S
methylgalactose, xylose and glucose. The major subfrac-
tions obtained from
G3S
were shown to have positive ("+23"), in agreement with the presence of both agaran (Fig. 1, III) and
carrageenan (Fig. 1, Ila, IId) chains, suggesting that
C2S-3
is a \( \mu \)-hybrid galactan [27] as observed in species of var-ious families [26,28] including Haliomniaceae [29].

Further purification of
C2S-2
on DEAE-Sephacel yielded a major fraction
C2S-2d
composed of galactose, 3,6-
anhydrogalactose, 3,6-anhydro-2-O-methylgalactose, 2-O-
methylgalactose and glucose (Table 2). Xylose was not
detected in this purified product.

Fig. 1. Schematic representation of the different repeating units of car-
rageeans and agars and disaccharide units of galactan sulfates isolated
from G. griffithsiae and C. creulata. Repeating unit of \( \lambda \)-Ia, \( \lambda \)-IIa, \( \mu \)-Ia, \( \mu \)-IIa, \( \nu \)-Ia, \( \nu \)-IIa. (a) \( \lambda \)-Ia, \( \mu \)-IIa, \( \nu \)-Ia.

methylgalactose, xylose and glucose. The major subfrac-
tions obtained from
G3S
had negative specific rotations, except
G3S-6,
and in agreement with the presence of agaran structures (Table 1). Similarly, the soluble fraction isolated from
G. torulosus
was composed of various polysaccharides showing positive ("kappaluota-carrageean" \( \mu \)-hybrids containing significant amounts of l-galactose) or negative (agaran and agaran \( \mu \)-hybrids) specific rotations [6].

The yields and chemical properties of galactans isolated from
C. creulata
are shown in Table 2. This seaweed was extracted with water at 25°C (C1) and at 80°C (C2 and C3). C1 and C2 were submitted to a KCl gradient precipitation giving the soluble fractions C1S and C2S. Both fractions showed positive specific rotations, suggesting major carrageenan structures. Fractionation of
C2S
on DEAE-Sephacel gave four fractions (C2S-1-C2S-4), mainly composed of galactose, 3,6-anhydrogalactose plus its 2-O-methyl derivative, and 2-O-methylgalactose. The FTIR spectrum of the C2S-3 fraction showed a peak at 832 cm\(^{-1}\)
which indicates the presence of equatorial sulfate groups on C-2 of galactosyl residues. A shoulder at 805–820 cm\(^{-1}\)
suggests the presence of 3,6-anhydrogalactosyl residues.

2-sulfated and sulfate group on C-6 of galactosyl residues.

The characteristic absorption of 3,6-anhydrogalactosyl units (932 cm\(^{-1}\)) was also observed [22,23]. HPSEC-MALLS analysis (data not shown) demonstrated that
C2S-3
is a homogeneous fraction. These results, together with the low positive value of the specific rotation ("+23"), are in agree-
ment with the presence of both agaran (Fig. 1, III) and
carrageenan (Fig. 1, Ila, IId) chains, suggesting that
C2S-3
is a \( \mu \)-hybrid galactan [27] as observed in species of var-ious families [26,28] including Haliomniaceae [29].

The results obtained for galactan
C2S-3
are strikingly different from those previously presented [30].

3.2. Cytotoxicity and in vitro antiviral activity

The crude extracts as well as the \( \kappa \)appa/\( \iota \)/\( \nu \)-carrageean, \( \mu \)-galactan hybrid and other main products, in yield, obtained from
G. griffithsiae
and
C. creulata
were initially evaluated for cytotoxicity by assessing their effects on cell viability. No effect on cell viability was ob-
served with any of these compounds at concentrations up
to 1000 µg/ml (Table 3).

Thereafter, the compounds were screened for antiviral ac-
tivity against HSV-1 strain F by a virus plaque reduction assay. Dextran sulfate and heparin were used as reference sub-
stances. As shown in Table 3, most tested products exhib-
ted antiviral activity. The initial polysaccharide mix-
tures of the aqueous extracts of both seaweeds (G3, C1, C2 and C3) were very active against HSV-1 with IC50 values in the range 0.5–2.5 µg/ml. After fractionation with KCl, a similar level of antiviral activity was retained in the hetero-
genous soluble fractions (G3S, C1S and C2S) as well as in the pure insoluble \( \kappa \)appa/\( \iota \)/\( \nu \)-carrageean (G3d). In the last case, only the insoluble fraction of
G. griffithsiae
was tested for biological activity because the yield of the insoluble product obtained from
C. creulata
was very low. By DEAE-Sephacel chromatography of the soluble fraction
G3S
of
G. griffithsiae,
four active galactans (G3S-1, G3S-
4, G3S-5, and G3S-6) were obtained with antiviral efficacy at concentrations similar to G3S, whereas in the case of
C. creulata
two active galactans (C2S-3 and C2S-2) were isolated. The compound C2S-2d, obtained by further purifi-
cation of C2S-2, showed a lower IC50 than the precedent
fraction.

In general, the most active galactans extracted from
C. creulata
exhibited a more effective antiviral action and
The content of 

\(\text{Gigartina skottsbergii}\)

\(\lambda\)-carrageenans (Fig. 1, Ib/Ia, IIb/IIc and IIa, respectively). Considering that structurally different galactan sulfates from marine algae display similar antitherpetic activity, the derivation from the original polymers of oligosaccharides with variable degree of polymerization may be a useful tool to provide new information to fully elucidate the structure–activity relationship.

The most active galactans representative of each step of the extraction and purification procedure for both seaweeds (G3, G3S, G3d and G3S-6 from \(G. griffithsiae\) and C2, C2S, C2S-3 and C2S-2d from \(C. crenulata\)) were chosen for further characterization. The spectrum of antitherpetic activity of these galactans was evaluated (Table 4). All the compounds were effective inhibitors against B-2006, a TK− strain of HSV-1 resistant to ACV, with IC\(_{50}\) values in a range similar to those observed against the reference F strain. Two strains of the other serotype, HSV-2, were also screened for susceptibility to the galactans by the plaque reduction assay. As can be seen in Table 4, IC\(_{50}\) values found against HSV-2 strain G were comparable to those obtained against HSV-1, whereas the MS strain of HSV-2 was found to be significantly more susceptible to the inhibitory action of the galactans, and to a lesser extent, to dextran sulfate and heparin. The inhibitory effect of the galactans was also assayed against the syncytial variants of HSV-1 (IC3-syn 13 and IC3-syn 14), isolated after serial passages in Vero cells of HSV-1 strain F in the presence of the antiviral carrageenan IC3 [20]. These two variants acquired the syncytial phenotype with large plaque size after the continuous selective pressure exerted by the carrageenan but differed in their susceptibility to IC3. IC3-syn 13 was as sensitive to IC3 as the parental F strain, but IC3-syn 14 showed a partial level of resistance exhibiting an IC\(_{50}\) 10-fold higher than the value obtained with the original virus [20]. As seen in Table 4, the behavior of the galactans isolated from \(G. griffithsiae\) and \(C. crenulata\) against these two syncytial variants was similar to that described for IC3.

Polysaccharonic compounds might be expected to interact with the positively charged amino acids in the surface proteins of virus envelope [1]. In our system, the interaction between sulfated galactans and the external glycoprotein of HSV may lead to virus inactivation and/or blockade in virion binding to cell receptor during adsorption, the first step of the replicative cycle. To elucidate these two possibilities, a virucidal assay against HSV-1 was first carried out. Seven galactans were unable to inactivate HSV-1 virions at concentrations up to 20 \(\mu\)g/ml, and the remaining tested product, G3, showed a very weak virucidal effect with a VC\(_{50}\) highly exceeding the corresponding antiviral IC\(_{50}\), 8.5 \(\mu\)g/ml versus 1.1 \(\mu\)g/ml (Table 3). Thus, we can conclude that these sulfated compounds do not produce virion inactivation.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>(\text{CC}_{50b} (\mu\text{g/ml}))</th>
<th>(\text{IC}_{50b} (\mu\text{g/ml}))</th>
<th>(\text{VC}_{50a} (\mu\text{g/ml}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3</td>
<td>&gt;1000</td>
<td>1.1</td>
<td>&gt;909</td>
</tr>
<tr>
<td>G3S</td>
<td>&gt;1000</td>
<td>4.3</td>
<td>&gt;233</td>
</tr>
<tr>
<td>G3S-1</td>
<td>&gt;1000</td>
<td>1.0</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>G3S-2</td>
<td>&gt;200</td>
<td>4.1</td>
<td>&gt;244</td>
</tr>
<tr>
<td>G3S-3</td>
<td>&gt;1000</td>
<td>&gt;200</td>
<td>Inactive n.d.</td>
</tr>
<tr>
<td>G3S-4</td>
<td>&gt;1000</td>
<td>100</td>
<td>&gt;10</td>
</tr>
<tr>
<td>G3S-5</td>
<td>&gt;1000</td>
<td>100</td>
<td>&gt;10</td>
</tr>
<tr>
<td>G3S-6</td>
<td>&gt;1000</td>
<td>100</td>
<td>&gt;20</td>
</tr>
<tr>
<td>G3S-7</td>
<td>&gt;1000</td>
<td>100</td>
<td>&gt;20</td>
</tr>
<tr>
<td>C1</td>
<td>&gt;1000</td>
<td>2.5</td>
<td>&gt;400</td>
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<tr>
<td>C2</td>
<td>&gt;1000</td>
<td>5.0</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>C3</td>
<td>&gt;1000</td>
<td>1.0</td>
<td>&gt;902</td>
</tr>
<tr>
<td>C1S</td>
<td>&gt;1000</td>
<td>0.8</td>
<td>&gt;1250</td>
</tr>
<tr>
<td>C2S</td>
<td>&gt;1000</td>
<td>0.9</td>
<td>&gt;1111</td>
</tr>
<tr>
<td>C2S-2</td>
<td>&gt;1000</td>
<td>2.8</td>
<td>&gt;357</td>
</tr>
<tr>
<td>C2S-3</td>
<td>&gt;1000</td>
<td>0.5</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>C2S-2d</td>
<td>&gt;1000</td>
<td>1.0</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Heparin</td>
<td>&gt;1000</td>
<td>3.0</td>
<td>&gt;333</td>
</tr>
<tr>
<td>DS8000</td>
<td>&gt;1000</td>
<td>2.8</td>
<td>&gt;357</td>
</tr>
</tbody>
</table>

Data are the average of duplicate assays, n.d.: not determined.

\(\%\) 50 Cytotoxic concentration, or concentration required to reduce by 50% the number of viable Vero cells after 48h of incubation with the fractions.

\(\%\) 50 Inhibitory concentration, or concentration required to reduce plaque number in Vero cells by 50%.

\(\%\) Selectivity index or ratio CC\(_{50b}\)/IC\(_{50b}\).

\(\%\) Virucidal concentration, or concentration required to inactivate virions by 50% after 1.5 h of incubation with the fractions.

Higher selectivity indices than the reference compounds heparin and dextran sulfate, whereas for \(G. griffithsiae\) the differences in effectiveness were not so notorious, with the exception of G3 and GM.

It has been reported that an increasing antiviral activity of polysulfates is related to an increasing degree of sulfation [1, 2]. In our case, although GM and C2S-3 fractions have high content of sulfate groups (Tables 1 and 2) and high antitherpetic activity (Table 3), other galactans with similar degree of sulfation, as G3S-6 or C2S-2, are less active whereas the fractions G3S-1, G3S-4, with lower amount of sulfate groups, were inactive or with very low antiviral effect. Therefore, other structural factors, such as the degree of polymerization and the position of sulfate groups on the polymer backbone, could be also correlated with the antiviral activity. Despite the differences in the position of the sulfate groups on \(\beta\)-d-galactose residues, C-4 in G4d and C-2 in C2S-3, in both fractions this unit is linked to \(\alpha\)-l-galactose 6-sulfate (Fig. 1, Ic and IIa, respectively). Additionally, in C2S-3 there are links to \(\beta\)-d- and \(\alpha\)-l-galactose 6-sulfate (Fig. 1, IIb and III) and to \(\alpha\)-l-galactose 2,6-sulfate (Fig. 1, III). Antitherpetic activity of kappa, partially cyclized marine and lambdas-carrageenans (Fig. 1, Ib/la, Ib/la and IIa, respectively) from \(Gigartina skottsbergii\) was directly correlated to the content of \(\alpha\)-l-galactose 2,6-sulfate residues [11]. The presence of \(\beta\)-d-galactose 2-sulfate linked to \(\alpha\)-l-galactose 6-sulfate (Fig. 1, IIIb) in agars from \(Acanthophora spicifera\) [31] is structurally analog to the minimal binding sequence necessary for the interaction of heparan sulfate with the glycoprotein gC of HSV-1 [32], and therefore, it may be responsible for the antitherpetic activity of these polysaccharides. Considering that structurally different galactan sulfates from marine algae display similar antitherpetic activity, the derivation from the original polymers of oligosaccharides with variable degree of polymerization may be a useful tool to provide new information to fully elucidate the structure–activity relationship.

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Table 4

<table>
<thead>
<tr>
<th>Spectrum of antiherpetic activity of the sulfated galactans from <em>G. griffithsiae</em> (G) and <em>C. crenulata</em> (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractions</td>
</tr>
<tr>
<td>G3</td>
</tr>
<tr>
<td>G3S</td>
</tr>
<tr>
<td>G3d</td>
</tr>
<tr>
<td>G3S-6</td>
</tr>
<tr>
<td>C2</td>
</tr>
<tr>
<td>C2S</td>
</tr>
<tr>
<td>C2S-3</td>
</tr>
<tr>
<td>C2S-2d</td>
</tr>
<tr>
<td>C2S-2d</td>
</tr>
</tbody>
</table>

Data are the average of duplicate assays.

\( ^{a} \) 50% Inhibitory concentration, or concentration required to reduce plaque number in Vero cells by 50%.

Next, the inhibitory effect of the galactans on virus adsorption was studied performing a virus plaque reduction assay under different treatment conditions. When the antiviral assay was performed omitting the presence of the galactans in the adsorption period and including the compounds only in the plaquing medium after adsorption, no significant reduction in the number of virus plaques was detected, even at a high concentration of 10^4 g/ml (Table 5).

By contrast, the presence of the galactans only at virus adsorption was as effective as the treatment throughout the whole incubation period, at and after adsorption (Table 5), indicating that HSV adsorption is the main target for the antiviral effect of these polysaccharides. Both serotypes of HSV use the heparan sulfate residues of cellular proteoglycans as the primary receptor for virion binding and adsorption [33,34], and thus, the sulfated galactans seem to mainly interfere with the interaction virion glycoprotein-heparan sulfate.

### 3.3. In vivo antiherpetic activity

The in vivo efficacy of these algal compounds was evaluated in a murine model of vaginal infection with HSV-2 strain MS. To this end, the crude galactan preparation from *C. crenulata* C2 was chosen to be tested since it was very active in vitro and was available in considerable amount. Given the polymeric structure and the mode of antiviral action of the galactans above demonstrated, preventing HSV entry to the host cell, a topical prophylactic use of the compound was assayed, treating the animals with a dose of compound before infection and then a second dose after infection. Results obtained are shown in Table 6. No irritation or toxicity was observed in C2-treated animals (group 1). BALB/c mice inoculated with 10^3 PFU of HSV-2 developed vulvovaginal lesions, starting at day 4–5 after infection with redness, then swelling and hair loss, some animals showed hind limb paralysis and, finally, all mice died between 7 and 14 days post-infection (group 4). All animals shed virus in vaginal secretions at high infectious titers, in the range 10^3 to 10^5 PFU/ml. When animals were treated with C2 at a concentration of 8 mg/ml, a highly significant level of protection was observed in comparison with infected untreated mice (30% mortality in group 2 versus 100% mortality in group 4, \( P < 0.005 \)), associated to a lower virus shedding and a delay in the mean day of death. The level of protection was reduced when a 10-fold lower concentration of compound

Table 5

<table>
<thead>
<tr>
<th>Influence of various drug treatment periods on the anti-HSV-1 activity of galactans from <em>G. griffithsiae</em> (G) and <em>C. crenulata</em> (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractions</td>
</tr>
<tr>
<td>G3</td>
</tr>
<tr>
<td>G3S</td>
</tr>
<tr>
<td>G3d</td>
</tr>
<tr>
<td>G3S-6</td>
</tr>
<tr>
<td>C2</td>
</tr>
<tr>
<td>C2S</td>
</tr>
<tr>
<td>C2S-3</td>
</tr>
<tr>
<td>C2S-2d</td>
</tr>
</tbody>
</table>

\(^{a}\) 50% Inhibitory concentration, or concentration required to reduce plaque number in Vero cells by 50%.
Table 6
Effect of C2 treatment on vaginal mouse infection with HSV-2.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Mortality</th>
<th>Virus shedding (PFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Number of dead mice/total (%)</td>
<td>Mean day of death ± S.D.</td>
</tr>
<tr>
<td>1</td>
<td>C2 (8 mg/ml) (−1 min) PBS (0 min)</td>
<td>0/10 (0)</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>C2 (8 mg/ml) (−1 min) HSV-2 (0 min)</td>
<td>3/10 (30)</td>
<td>12.3 ± 0.6 &lt;10^2 to 6.6 × 10^4</td>
</tr>
<tr>
<td>3</td>
<td>C2 (0.8 mg/ml) (−1 min) HSV-2 (0 min)</td>
<td>8/10 (80)</td>
<td>10.8 ± 2.3 1.4 × 10^3 to 7.8 × 10^4</td>
</tr>
<tr>
<td>4</td>
<td>PBS (−1 min) HSV-2 (0 min)</td>
<td>10/10 (100)</td>
<td>9.7 ± 1.8 1.4 × 10^3 to 1.2 × 10^5</td>
</tr>
</tbody>
</table>

a BALB/c mice were intravaginally inoculated with PBS (group 1) or 10^5 PFU of HSV-2 (groups 2–4) at time 0. Doses of 50 µl of PBS or C2 (8 or 0.8 mg/ml solution) were instilled into the vagina at the indicated times pre- (−) or post-infection (+). Animals were monitored daily for morbidity and mortality.
b Vaginal lavages were collected at day 3 post-infection and titrated by plaque formation on Vero cells. Results are expressed as the range of virus titers for individual mice in each group.
c P<0.005.

was administered (group 3), confirming the dose-dependent in vivo antiviral activity of C2.

4. Conclusions

The results presented here allow to conclude that both G. griffithsiae and C. crenulata represent an interesting source of carrageenans and/or m-m-hybrid galactans with selective and potent antiviral action against reference strains, syncytial variants and ACV-resistant strains of HSV-1 and HSV-2. Most of the eighteen tested products, including homogeneous kappa/iota/nu carrageenan and m-m-galactan hybrid, exhibited in vitro antiviral activity with IC50 values in the range 0.5–5.6 µg/ml, and lacking cytotoxicity. The crude galactan preparation C2, obtained from C. crenulata, showed also a significant protective effect in vivo against HSV-2 vaginal infection in a murine model, suggesting the potential use of this low cost product, easy to obtain in large quantities, for prophylaxis of virus infections. Considering the characteristic of herpetic infections as a sexually transmitted disease, there is a real need for safe topical agents to reduce the spread of infection [35–37]. These preliminary in vivo results are encouraging to further assay topical formulations of the algal extract in a gel vehicle to prolong the presence of the active agent in the vagina and improve its efficacy.

Acknowledgements

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