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# Chemical structure and antiviral activity of carrageenans from *Meristiella gelidium* against herpes simplex and dengue virus

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**Abstract**

Chemical and spectroscopic methods showed that the major KCl-precipitated galactans from *Meristiella gelidium* (Solieriaceae) are iota/kappa/nu-hybrid carrageenans with the former one in higher proportion. These carrageenans showed, by HPSEC-MALLS analysis, unimodal symmetrical peaks with MW of 425.6–956.7 kDa. The effectiveness of the crude extracts from *M. gelidium* against HSV-2 was higher than the corresponding extract from *G. griffithsiae*, previously determined. However, when considering the homogeneous carrageenans, the fractions obtained from both seaweeds showed the same level of activity. The extracts and carrageenan derived from *M. gelidium* were more effective inhibitors of DENV-2 if compared with *G. griffithsiae* samples and reference polysaccharides. The most active fraction obtained from *M. gelidium* showed a selectivity index against HSV-2 of 25,000, a value high enough to consider this carrageenan as a promising agent to be evaluated for the treatment of genital HSV-2 infections.


*Keywords:* Iota/kappa-carrageenan; Chemical structure; Antiviral activity; Herpes simplex virus; Dengue virus; *Meristiella gelidium*; Solieriaceae

## 1. Introduction

Sulfated galactans are the major extracellular polysaccharides produced by red seaweeds (Rhodophyta). They are constituted by a repetitive backbone of alternating 3-linked β-D-galactopyranosyl (G units) and 4-linked α-galactopyranosyl residues, belonging to the D-series (D unit) in carrageenans and to the L-series (L unit) in agarans. In these galactans, the D and L units may exist in the 3,6-anhydrogalactopyranosyl form (DA and LA, respectively) (Usov, 1998). A third group of galactans, namely the DL-hybrids (Estevez, Ciancia, & Cerezo, 2001), contains G units linked to both, D and L units (Chopin, Kerin, & Mazerolle, 1999; Estevez et al., 2001; Stortz, Cases, & Cerezo, 1997; Takano, Shiomoto, Kamei, Hara, & Hirase, 2003; Zibetti, Noseda, Cerezo, & Duarte, 2005).

Some sulfated galactans such as kappa-, iota- and lambda-carrageenans show commercial interest (Renn, 1997). These polysaccharides are constituted by the following repetitive disaccharide units, G4S-DA, G4S-DA2S and G2S-D2S,6S, respectively (Knutsen's, Knutsen, Myslabodski, Larsen, & Usov, 1994). Kappa- and iota-carrageenans frequently contain variable amounts of their biological precursors, namely mu- (G4S-D6S) and nu- (G4S-D2S,6S) carrageenans, respectively (Van de Velde, Knutsen, Usov, Rollema, & Cerezo, 2002).

Red seaweeds of the family Phyllophoraceae (Gigartinales) are known to be carrageenophytes, producing kappa/iota- and lambda-carrageenans (Furneaux & Miller, 1986; McCandless, West, & Guiry, 1982; Saito & Oliveira, 1990; Talarico, Zibetti, Faria, Scolaro, Duarte and Noseda, 2004). The Solieriaceae family includes species of great industrial importance, such as *Eucheuma denticulatum* (iota-carrageenan producer) and *Kappaphycus alvarezii* (kappa-carrageenan producer) (Anderson, Dolan, & Rees, 1973) and others producing carrageenans with unusual high levels of substituents (methoxyl and/or pyruvic acid acetal). Among these carrageenans, those from species of the genera *Callophycus* (Chiovitti, Bacic, Craik, Munro, Kraft and Liao, 1997), (Falshaw,

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Furneaux, & Wong, 2003), *Rhabdonia* (Chiovitti, Liao, Kraft, Munro, Craik and Bacic, 1996), *Sarconema* (Chiovitti, Bacic, Craik, Kraft, Liao and Falshaw, 1998a), and *Claviclonium* (Chiovitti, Bacic, Craik, Kraft, & Liao, 2004) are worth mentioning.

The diverse structural types of natural carrageenans have shown a potent antiviral activity against enveloped viruses, including the human pathogens herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and dengue virus (DENV) (Carlucci, Ciancia, Matulewicz, Cerezo, & Damonte, 1999; Talarico et al., 2004, 2005). Since these viruses interact with the glycosaminoglycan heparan sulfate (HS) for their initial binding to the host cell (Chen, Maguire, Hileman, Fromm, Esko and Linhardt, 1997; WuDunn & Spear, 1989), these polysulfates may interfere with the early events leading to virus entry.

In this paper, we report the chemical structure and the antiviral activity against HSV-2 and DENV-2 of the gelling polysaccharides obtained from the Brazilian red seaweed *Meristiella gelidium* (Solieriaceae).

## 2. Material and methods

### 2.1. Extraction and purification of polysaccharides

The specimens of *M. gelidium* (J. Agarth) D.P. Cheney & P.W. Gabrielson were collected in Pitimbu, Paraíba State (northeastern coast of Brazil). A voucher specimen was deposited in the herbarium of Departamento de Botânica da Universidade Federal do Paraná, Brazil with the herbarium number UPCB-46434. The algae were cleaned to remove undesirable material, washed with tap water and sun-dried. The milled seaweed was extracted twice with water (1% w/v) at 25 °C with mechanical stirring for 15 h. The extracts were individually dialyzed and freeze-dried yielding the crude extracts M1 and M2. Sequentially the algal residue was extracted three more times with water at 100 °C for 3 h, and treated as described above yielding M3, M4 and M5 (Table 1).

### 2.2. Fractionation with potassium chloride

The crude extracts (M1, M2 and M3) were individually dissolved in water (0.25% w/v) and solid, finely divided KCl was added in small portions with constant and violent mechanical agitation so that the concentration was increased by 0.1 M each time. After each addition, the material was maintained at low temperature (~4 °C) for 15 h; the upper limit of KCl concentration was 2.0 M. The precipitates as well as the respective soluble 2 M fractions were dialyzed (molecular-weight cut-off 14,000), concentrated and freeze-dried (Table 2).

### 2.3. Alkaline treatment

The KCl-precipitated fractions M1a and M3a were dissolved in water (0.2% w/v) and $NaBH_4$ (10% w/w) was added. After 17 h at 25 °C, 3.0 M NaOH was added (to give a final concentration of 1 M NaOH) along with a further quantity of $NaBH_4$. The solution was heated at 80 °C for 2 h (Ciancia, Noseda, Matulewicz, & Cerezo, 1993). 3,6-Anhydrogalactose content was measured by the resorcinol method (Yaphe, 1960). The solution was cooled to room temperature, dialyzed, concentrated and freeze-dried, originating the fractions named M1a-T and M3a-T, respectively (Table 2).

### 2.4. General analysis

Total carbohydrate was estimated by the phenol-sulfuric acid method of Dubois, Gilles, Hamilton, Rebers, and Smith (1956), using galactose as standard. Sulfate content was measured by Dodgson & Price (1962) method. Protein content was measured by the method of Lowry, Rosebrough, Farr, and Randall (1951) using Folin-Ciocalteau reagent and BSA (bovine serum albumin) as the standard. The specific optical rotation ($(\alpha)_D$) was measured at 20 °C, at a concentration of 0.1% (w/v).

The D- and L-galactose content was estimated by the method of Cases, Cerezo, and Stortz (1995) through the diastereomeric acetylated 1-deoxy-1-(2-hydroxypropylamino)alditols. The D- and L- 3,6-anhydrogalactose content was estimated by the method of Errea, Ciancia, Matulewicz, and Cerezo (1998) using an oxidative hydrolysis to obtain the aldonic acids, which are further converted to the acetylated diastereomeric *sec*-butyl esters.

Table 1
Yield, optical rotation, analyses and monosaccharide composition of the crude extracts from *M. gelidium* (M1–M5)

| Fraction[a] | Yield[b] (%) | Sulfate[c] (%) | Protein (%) | $[\alpha]_D$ (°) | Monosaccharide[d] (mol%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Gal | AGal | 6-Gal | Xyl | Glc |
| M1 | 12 | 29 | 17 | +48 | 57 | 26 | 4 | 3 | 10 |
| M2 | 6 | 32 | 15 | +36 | 66 | 24 | 3 | 1 | 6 |
| M3 | 25 | 33 | 8 | +45 | 46 | 37 | 4 | 1 | 11 |
| M4 | 3 | 20 | 19 | +50 | 35 | 9 | 3 | 2 | 51 |
| M5 | 1 | 11 | 20 | +61 | 13 | 2 | – | 1 | 84 |

–, Not detected.
[a] Fractions as defined in text.
[b] Percentage based on milled seaweed.
[c] Expressed as $SO_3Na$.
[d] AGal corresponds to 3,6-anhydrogalactosyl, 6-Gal to 6-*O*-methylgalactosyl, etc.

Table 2
Yield, analyses, optical rotation and monosaccharide composition of the fractions obtained by KCl precipitation and of the alkaline treatment (M1a-T and M3a-T) of the crude extracts M1–M3 from *M. gelidium*

| Fraction[a] | Range[b] (M) KCl | Yield[c] (%) | Sulfate[d] (%) | Carbohydrate (%) | Protein (%) | $[\alpha]_D$ (°) | Monosaccharide[e] (mol%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Gal | AGal | 4-Gal | 6-Gal | Xyl | Glc |
| M1a | 1.0–1.2 | 76 | 24 | 58 | 4 | +64 | 51[f] | 41[f] | – | 5 | – | 3 |
| M1a-T | | 77 | nd | nd | nd | nd | 47 | 45 | – | 4 | – | 3 |
| M1S | 2.0[g] | 14 | 13 | 46 | 2 | +22 | 73 | 7 | 2 | 2 | 5 | 11 |
| M2a | 0–0.1 | 7 | nd | 64 | 3 | nd | 28 | 15 | – | 8 | 2 | 47 |
| M2b | 1.0–1.2 | 79 | 25 | 54 | 6 | +56 | 55 | 37 | – | 6 | – | 2 |
| M2S | 2.0[g] | 8 | 18 | 47 | 7 | −38 | 86 | 3 | – | 2 | 3 | 6 |
| M3a | 1.0–1.2 | 62 | 29 | 43 | 9 | +51 | 53[f] | 41[f] | – | 6 | – | – |
| M3a-T | | 75 | nd | nd | nd | nd | 50 | 44 | – | 6 | – | – |
| M3S | 2.0[g] | 11 | 13 | 70 | 10 | +47 | 45 | 3 | 2 | – | 7 | 43 |

nd, Not determined; –, not detected.
[a] Fractions are defined in text.
[b] Range, Range of fractionation.
[c] Percentage of material recovered from KCl precipitation or after alkaline treatment.
[d] Expressed as $SO_3Na$.
[e] AGal corresponds to 3,6-anhydrogalactosyl, 4-Gal to 4-*O*-methylgalactosyl, 6-Gal to 6-*O*-methylgalactosyl, etc.
[f] Only D-isomer.
[g] Soluble in 2.0 M KCl.

### 2.5. Monosaccharide composition

The monosaccharide composition of red seaweed galactans (Tables 1 and 2) was obtained by reductive hydrolysis (Stevenson & Furneaux, 1991). After acetylation these alditol acetate derivatives were analysed by gas liquid chromatography (GC) and GC–mass spectrometry (GC–MS) (Stevenson & Furneaux, 1991). To distinguish between co-eluting derivatives (naturally methylated 3- and 4-*O*-methylgalactose), alditol acetates were also generated by hydrolysis in aq $HCO_2H$ (45%) for 16 h at 100 °C, followed by $NaBD_4$ reduction, acetylation and analysed by GC–MS (Table 2).

### 2.6. Methylation analysis

The polysaccharides were converted to their triethylammonium salts by anion- exchange with Dowex-50 resin (Stortz & Cerezo, 1993). The dry salt of the polysaccharides (20–50 mg) was dissolved in $Me_2SO$ (1.2 ml) with stirring and submitted to methylation analysis using the Ciucanu method (Ciucanu & Kerek, 1984). Powdered NaOH (~40 mg) was added, and the mixture was stirred for 30 min at 25 °C, then $CH_3I$ (0.08 ml) was added. The addition of NaOH and $CH_3I$ was repeated two more times. Water was added to stop the reaction, and the solution was neutralized with AcOH, dialyzed against distilled water, and freeze-dried to give the permethylated polysaccharides. GC and GC–MS analyses of methylated products were carried out as partially methylated alditol acetates after reductive hydrolysis and acetylation as described by Stevenson & Furneaux (1991). To distinguish between co-eluting derivatives (3- and 4-*O*-methylgalactose) the partially methylated alditol acetates were also generated by hydrolysis in aq $HCO_2H$ (45%) for 16 h at 100 °C, followed by $NaBD_4$ reduction, acetylation and GC–MS analysis (Table 3).

### 2.7. Gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) analyses

GC analyses were carried out with a HP-5890 gas chromatograph equipped with a flame ionization detector (FID), using different columns. A fused silica capillary column (30 m × 0.25 mm) coated with DB-225 was used to analyse the mixtures of alditol acetates and partially methylated alditol acetates. Chromatography was run isothermally at 210 °C. Both injector and FID temperatures were 250 °C. Nitrogen was used as the carrier gas at a flow rate of 1 ml/min and a split ratio of 100:1. The acetylated diastereomeric *sec*-butyl esters were analysed using a SP-2330 column (30 m × 0.25 mm) in

Table 3
Methylation analysis of fractions M1a and M3a

| Derivative[a] | Fraction (mol%) | | Deduced unit and substitution pattern[b] |
|---|---|---|---|
| | M1a | M3a | |
| 3-*O-Linked residues* | | | |
| 2,4,6-Gal | 2 | 2 | G and/or G6M |
| 2,6-Gal | 47 | 46 | G4S + G6M,4S |
| 4-*O-Linked residues* | | | |
| 2,3,6-Gal | 2 | 1 | D |
| 3-Gal | 4 | 3 | D2S,6S |
| 2-AGal | 2 | 2 | DA |
| AGal | 42 | 42 | DA2S |
| *Undefined residues* | | | |
| 2-Gal | 1 | 3 | G4S,6S and/or D3S, 6S |
| 6-Gal | – | 1 | G2S,4S (6M) and/or D2S,3S (6M) |

–, Not detected.
[a] Mol% of monosaccharide having methyl groups at the positions indicated. 2,4,6-Gal analyzed as 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol, etc.
[b] Knutsen's nomenclature (Knutsen et al., 1994).

the same conditions described by Errea et al. (1998). The acetylated 1-deoxy-1-(2-hydroxypropylamino)alditols were analysed using a Ultra-2 column (50 m × 0.2 mm) as previously described by Cases et al. (1995).

GC–MS analysis was performed using a Varian 3300 chromatograph equipped with a DB-225 (30 m × 0.25 mm) column interfaced to a Finnigan Mat ITD mass spectrometer. Helium was used as carrier gas (1 ml/min).

### 2.8. HPSEC-MALLS analysis

The determination of the homogeneity was performed on a Waters high-performance size exclusion chromatography (HPSEC) apparatus coupled to a differential refractometer (RI) and a Wyatt Technology Dawn-F multi-angle laser light scattering detector (MALLS). Four Waters Ultrahydrogel columns (2000, 500, 250 and 120) were connected in series and coupled with multi-detection equipment, using a $NaNO_2$ solution (0.1 M) as eluent, containing $NaN_3$ (200 ppm) as preservative. The samples (1 mg/ml) were dissolved in the same solvent under magnetic stirring for 2 h and filtered through a 0.45 and 0.22 μm nitrocellulose membrane (GSWP, Millipore). HPSEC data were collected and analysed by the Wyatt Technology ASTRA program. The light scattering signal was detected simultaneously at 11 scattering angles, $\theta$, ranging from 35 to 132°. All experiments were carried out at 25 °C.

### 2.9. Spectroscopic methods

Fourier-transform infrared (FTIR) spectra of KBr pellets of polysaccharides were recorded in a Perkin–Elmer Series 2000 FTIR spectrophotometer (eight scans, at a resolution of 4 $cm^{-1}$) scanning between 4000 and 400 $cm^{-1}$. For nuclear magnetic resonance (NMR) spectroscopy analysis, the lyophilised samples were dissolved in $D_2O$ (15–30 mg/ml). NMR spectra of solutions were recorded at 50 °C using a Bruker Advance DRX 400 NMR spectrometer. $^{13}C$ NMR spectra were obtained in base frequency of 100.63 MHz and chemical shifts were referenced to internal acetone (30.20 ppm).

### 2.10. Cells and viruses

Vero (African green monkey kidney) (ATCC, USA) cells were grown in Eagle's minimum essential medium (MEM) (GIBCO, USA) supplemented with 5% calf serum. For maintenance medium (MM), the serum concentration was reduced to 1.5%. The C6/36 HT mosquito cell line from *Aedes albopictus* (adapted to grow at 33 °C) was provided by the Instituto Nacional de Enfermedades Virales Humanas (INEVH) Dr J. Maiztegui (Pergamino, Argentina) and cultured in L-15 Medium (Leibovitz) supplemented with 0.3% tryptose phosphate broth, 0.02% glutamine, 1% MEM non-essential amino acids solution and 5% fetal calf serum.

HSV-2 strain MS was provided by Dr F. Benencia (Immunochemistry Lab., UBA, Argentina). DENV-2 strain NGC was provided by Dr A.S. Mistchenko (Hospital de Niños Dr Ricardo Gutiérrez, Buenos Aires, Argentina). HSV-2 and DENV-2 stocks were prepared in Vero and C6/36 HT cells, respectively, and titrated by plaque formation in Vero cells.

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

### 2.12. Antiviral assay

Antiviral activity was evaluated by a virus plaque reduction assay. Vero cell monolayers grown in 24-well plates were infected with about 50 PFU/well in the absence or presence of serial twofold dilutions of the compounds. After 1 h of virus adsorption at 37 °C, residual inoculum was replaced by MM containing 0.7 or 1% methylcellulose, for HSV or DENV experiments, respectively, and the corresponding dose of each compound. Plaques were counted after 2 or 6 days of incubation at 37 °C, according to the virus. 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. Results and discussion

The water-soluble polysaccharides from *M. gelidium* (M) were extracted at 25 °C (twice) rendering M1–M2, respectively. The algal residues were extracted at 100 °C (three times) yielding the crude extracts M3–M5. Yields, analyses, specific rotations and monosaccharide composition of the extracts are given in Table 1. The major fraction (M3, 25% yield) was obtained from the first extraction at 100 °C and contains galactose and 3,6-anhydrogalactose as the principal monosaccharide constituents. Xylose and 6-*O*-methylgalactose were present in low amounts (Table 1). The presence of galactose as the major monosaccharide constituent of fractions M1–M3 together with the positive values of specific optical rotation (+36 to +48°), suggest that these extracts are constituted principally by carrageenans. Fractions M4 and M5 contain significative amounts of glucose (51 and 84%, respectively). The high percentage of this monosaccharide was attributed to the presence of floridean starch as determined by NMR spectroscopy. $^{13}C$-NMR spectra of these crude extracts contained characteristic signals at 100.1, 71.7, 73.4, 78.0,

71.4, and 60.7 ppm, corresponding to C-1(C-6 of the (1→4)-linked α-D-glucopyranose units of floridean starch (Chiovitti, Bacic, Craik, Munro, Kraft, Liao, 1998b). $^{13}$C-NMR spectra of M1–M3 contain only signals corresponding to iota-carrageenans (Usov & Shashkov, 1985).

In order to fractionate these extracts they were submitted to potassium chloride precipitation. Four fractions were precipitated from *M. gelidium* extracts (M1–M3) between 0 and 0.1 M (M2a), 1.0–1.2 M (M1a and M3a) and at 1.0–1.4 M (M2b). This treatment also originated KCl-soluble fractions (M1S–M3S), which showed relatively low sulfate contents (13–18%) compared to those of the precipitated fractions (Table 2). Constituent sugar analysis of M1S and M2S fractions showed galactose as the major constituent (73–86 mol%) besides minor amounts of 3,6-anhydrogalactose, 6-*O*- and/or 4-*O*-methylgalactose, xylose and glucose (Table 2). The floridean starch content was exceptionally high in M3S as shown by its glucose percentage (43 mol%).

The negative optical rotation of the soluble fraction M2S (−38°), (Table 2) suggests that *M. gelidium* biosynthesise agarans and/or DL-hybrid galactans, as observed for other red seaweeds considered typical carrageenophytes (Chopin et al., 1999; Ciancia et al., 1993; Estevez, Ciancia, & Cerezo, 2000; Estevez et al., 2001; Usov & Klochkova, 1992; Takano, Iwane-Sakata, Hayashi, Hara, & Hirase, 1998). The chemical analyses of this fraction will be published in a separate paper.

The major KCl-precipitated fractions, M1a, M2b and M3a showed positive specific rotation (51–64°) in agreement with those published for kappa/iota-carrageenans (56.1–66.5°, Stortz & Cerezo, 1993). These fractions were eluted as symmetric unimodal peaks (HPSEC-MALLS), showing MW of 425.6, 950.8 and 956.7 kDa, respectively. Constituent monosaccharide analyses showed that galactose and 3,6-anhydrogalactose were the dominant sugars, accounting for 92–98% of the total monosaccharides (Table 2). Small amounts of glucose (2–3%) and 6-*O*-methylgalactose (5–6%) were also present. $^{13}$C-NMR spectrum of these fractions showed two major anomeric signals at 101.8 and 91.6 ppm corresponding to G4S linked to DA2S (iota-carrageenan diad) (Usov & Shashkov, 1985). M1a, M2b and M3a spectra have very low intensity signals at 104.6, 97.7 and 95.0 ppm that could be attributed to nu- (Stortz, Bacon, Cherniak, & Cerezo, 1994) and kappa-diads (Usov & Shashkov, 1985). Therefore, these results showed that these fractions are constituted by iota-/nu-/kappa-hybrid carrageenans with the first one in higher proportion. FTIR spectra of these fractions were very similar and showed an intense band of absorption at 1260 cm$^{-1}$ indicative of sulfate ester in agreement with the degree of sulfation (Table 1). The diagnostic region (940–800 cm$^{-1}$) showed sharp absorption bands at, 848–847 cm$^{-1}$ characteristic of axial sulfate ester located at O-4 of β-D-galactopyranosyl, 805 cm$^{-1}$ corresponding to axial sulfate ester located at O-2 of 3,6-anhydrogalactopyranosyl, and the band at 931 cm$^{-1}$, due to the presence of this anhydro sugar. These FTIR spectra are characteristic of iota-carrageenan (Craigie & Leigh, 1978; Stancioff & Stanley, 1969). Although methylation analysis (Table 3) of M1a and M3a showed the presence of iota-precursor units (α-galactopyranose 2,6-sulfate), the correspondent FTIR absorptions (830 and 820 cm$^{-1}$ for sulfate esters at O-2 and O-6, respectively) were not observed in their spectra. In the same way these absorption bands were not detected in the FTIR spectrum of the iota-/nu-carrageenan isolated from *E. denticulatum* even do this polysaccharide contains 10.3% of precursor units (Viana, Noseda, Duarte, & Cerezo, 2004).

In order to determine the percentage of precursors units (galactose 2,6-sulfate and/or galactose 6-sulfate), fractions M1a and M3a were submitted to alkaline treatment (Ciancia et al., 1993) giving rise to the modified fractions M1a-T and M3a-T, respectively. The sugar compositional analysis of these modified polysaccharides showed an increased of the 3,6-anhydrogalactose content (∼5 and ∼3%, respectively) with a similar decreased of galactose (Table 2).

$^{13}$C-NMR spectra of the alkali-modified fractions M1a-T and M3a-T showed only the iota-carrageenan chemical shifts (C-1-C6 of G4S: 101.8, 69.0, 76.7, 71.8, 74.4, 60.9 ppm and C-1-C6 of DA2S: 91.6, 74.6, 77.5, 77.9, 76.4, 69.0 ppm) (Usov & Shashkov, 1985).

Methylation analysis of M1a and M3a is given in Table 3. The principal derivatives were 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylgalactitol (2,6-Gal) and 1,2,4,5-tetra-*O*-acetyl-3,6-anhydrogalactitol (AGal). They indicate that the 3-linked β-D-galactopyranosyl units are highly 4-*O*-sulfated and the 4-linked anhydrogalactopyranosyl units are mainly 2-*O*-sulfated. These results are in accordance with those of FTIR and $^{13}$C-NMR, showing the iota-diad as the principal repeating disaccharide structure of these fractions. The presence of 1,2,4,5,6-penta-*O*-acetyl-3-mono-*O*-methylgalactitol (3-Gal) showed that these fractions also contain iota-carrageenan precursors (D2S, 6S), 4 and 3 mol%, respectively. These percentages are in agreement with the 3,6-anhydrogalactose increases in the alkali-treated polysaccharides (Table 2), showing that the alkaline condition used during the methylation procedure did not produce the cyclization of the precursor units. Similar results were described for the iota/nu-carrageenan isolated from *E. denticulatum* (Viana et al., 2004). Besides iota-carrageenan and its biological precursor, methylation analysis showed that these carrageenans contain very low (∼2 mol%) amounts of 2AGal that could arise from kappa-carrageenan structures. M1a and M3a carrageenans contain minor amounts of 2,4,6-Gal and 2,3,6-Gal corresponding to unsulfated 3-linked and 4-linked residues, respectively. Additionally, ambiguous derivatives (2-Gal and 6-Gal) were also detected and could be originated from different units as shown in Table 3, or alternatively from undermethylation.

Therefore, KCl-precipitated carrageenans from *M. gelidium* are constituted by iota- (88–90%), nu- (6–8%) and kappa- (4%) disaccharide repeating units.

The more abundant compounds of the initial extraction and the KCl-fractionation from *M. gelidium*, the crude extracts M1 and M3 and the M3a fraction, respectively, were chosen for characterization of their biological properties. The G3 extract and the G3d fraction from *G. griffithsiae*, partially characterized in their structure and antiherpetic properties in a previous report (Talarico et al., 2004), were also included in this study

for comparison with *M. gelidium* samples. Further analyses of chemical structure showed that G3d is constituted by iota- (70%), nu- (17%) and kappa- (13%) diads with MW of 845.0 kDa (unpublished results). In addition, heparin and dextran sulfate 8000 (DS 8000) were considered as reference antiviral polysaccharides. No cytotoxicity was observed with any of the compounds when viability was evaluated by MTT assay on Vero cell monolayers incubated in the presence of up to 1000 μg/ml of the polysaccharides. Then, antiviral activity was analysed by a plaque reduction assay against HSV-2 and DENV-2. As shown in Table 4, all the compounds obtained from *M. gelidium* exhibited antiherpetic activity, with $IC_{50}$ values in the range 0.04–0.06 μg/ml. The effectiveness of the crude extracts from *M. gelidium* was higher than the corresponding extract from *G. griffithsiae*: the $IC_{50}$ for G3 was twofold higher than the values for M1 and M3. However, when considering the KCl-fractionated samples, the carrageenans obtained from both seaweeds showed the same level of activity. Given the lack of cytotoxicity of these polysaccharides, their selectivity indices (ratio $CC_{50}/IC_{50}$) were very high (25,000 for M3a, the most active fraction). Noticeably, the compounds obtained from *M. gelidium*, as well as those from *G. griffithsiae*, showed a higher antiviral action and selectivity index than the reference substances heparin and DS 8000.

Thereafter, the antiviral activity of these carrageenans was evaluated against DENV-2. In general, the inhibitory action against DENV-2 was lower than that detected against HSV-2 (Table 4). The range of $IC_{50}$ against DENV-2 was 0.14–1.6 μg/ml. Again, the extracts and the fraction derived from *M. gelidium* were more effective inhibitors of DENV-2 if compared with *G. griffithsiae* samples and reference polysaccharides.

In conclusion, the carrageenans isolated from *M. gelidium* were found to be among the most potent sulfated polysaccharides obtained from red seaweeds according to their inhibitory activity against herpesvirus (Damonte, Matulewicz, & Cerezo, 2004). For M3a, the most active fraction, the selectivity index against HSV-2 is 25,000, a value high enough to consider this carrageenan as a promising agent to be evaluated for the treatment of genital HSV-2 infections.

Table 4
Antiviral activity against herpes simplex and dengue virus in fractions from *M. gelidium* (M1, M3, M3a)

| Fraction | $CC_{50}$[a] (μg/ml) | $IC_{50}$[b] (μg/ml) | |
|---|---|---|---|
| | | HSV-2 (MS) | DENV-2 (NGC) |
| M1 | >1000 | 0.06±0.01 | 0.79±0.04 |
| M3 | >1000 | 0.05±0.02 | 0.14±0.01 |
| M3a | >1000 | 0.04±0.01 | 0.21±0.04 |
| G3 | >1000 | 0.11±0.01[c] | 1.6±0.1 |
| G3d | >1000 | 0.05±0.02[c] | 0.9±0.4[d] |
| Heparin | >1000 | 0.6±0.1[c] | 1.9±0.2[d] |
| DS 8000 | >1000 | 0.6±0.1[c] | 0.9±0.1[d] |

*Gymnogongrus griffithsiae* fractions (G3, G3d) added for comparison.
[a] Cytotoxic concentration 50%: compound concentration required to reduce Vero cell viability by 50% after 48 h incubation.
[b] Inhibitory concentration 50%: compound concentration required to reduce virus plaque number in Vero cells by 50%. Data are the mean of duplicate assays±standard deviation.
[c] Talarico et al., 2004.
[d] Talarico et al., 2005.

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