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Isolation, chemical investigation and antiviral activity of polysaccharides from *Gracilaria corticata* (Gracilariaceae, Rhodophyta)

Sutapa Mazumder [a], Prodyut K. Ghosal [a], Carlos A. Pujol [b], María J. Carlucci [b], Elsa B. Damonte [b], Bimalendu Ray [a,*]

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

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Abstract

Polysaccharides were sequentially extracted from the agarophyte *Gracilaria corticata*. Chemical analysis combined with infrared spectroscopy showed that the cold water extracted material consists mainly of a high molecular weight sulfated galactan. Most of the sulfate groups are alkali labile and are located at C-4 of the 1,3-linked D-galactose units and C-6 of the 1,4-linked L-galactose residues. The autoclaved extracts contain agar type polysaccharide having a high pyruvate content and a variable degree of methylation, but were contaminated with floridean starch. ^{1}H-NMR studies indicate that methoxyl groups, when present, occur at C-6 of the 1,3-linked D-galactose units and C-2 of the 1,4-linked L-galactose residues of agar polymer. Bioassays showed that a high molecular weight galactan sulfate, exhibited selective antiviral activity against herpes simplex virus types 1 and 2, likely due to an inhibition of the initial virus attachment to the host cell.

Keywords: *Gracilaria corticata*; Galactan sulfate; Agar; Starch; Anti-herpes simplex virus activity; Anticoagulant activity

1. Introduction

Naturally occurring sulfated carbohydrate polymers from various sources can have numerous physiological activities [1–4]. In recent years, screening assays of the antiviral activity of extracts from a number of marine algae have led to the identification of a number of carbohydrate polymers having potent inhibitory effects against herpes simplex virus (HSV) types 1 and 2, human cytomegalovirus, human immunodeficiency virus type 1, respiratory syncytial virus and influenza virus [5–15]. These polysaccharides include fucoidans, sulfated galactans, ulvans and mannans. Thus, the antiviral potential of sulfated polysaccharides extracted from algae became of considerable interest, although there is a lack of information about their chemical structures and physiological activities.

A number of red alga are found near the coast of India, with *Gracilaria corticata* as a predominant species. The purpose of the present work is to determine the chemical structures and physiological activities of water-extracted polymers from *Gracilaria corticata* of Indian origin and to investigate the effect of an alkaline treatment on the above mentioned parameters.

2. Method

2.1. Plant material and preliminary treatments

Samples of *Gracilaria corticata* were collected from the Gujrat coast of Western India in December 1994. The gathered material was sorted, washed and dried by

* Corresponding author. Current address: UMR CNRS 6037, SCUEOR, CCME, Rouen University, 76821 Mont Saint Aignan, France. Tel.: +33-2-35-14-67-68; fax: +33-2-35-14-66-15
E-mail address: bimalendu_ray@yahoo.co.uk (B. Ray).

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forced air circulation at 35–40 °C. After pulverization in a commercial grinder, the seaweed (120 g) was extracted sequentially in a Soxhlet apparatus with benzene (40 h) and acetone (20 h) to leave a de-pigmented algal powder (DAP, 80 g).

2.2. Polysaccharide extraction

Extraction of polysaccharides was performed at 35–40 °C for 4 h by continuous stirring of the algal powder (DAP, 10 g) in 500 ml of distilled water. The pH of the suspension was adjusted to 6.5 prior to extraction. The extract formed was of high viscosity, and was, therefore, diluted with hot water (500 ml) before centrifugation ($14\,000 \times g$, 30 min). The supernatant was filtered through a sintered glass filter (G-2) and the filtrate was concentrated, allowed to cool, and then frozen at −10 °C. The frozen sample was thawed and centrifuged to separate the soft-gelling fraction (SGF) from the non-gelling fraction (NGF). After dissolving the gel in a minimum volume of boiling water, the freeze-thaw process was repeated once. Polysaccharides from the SGF and NGF were recovered by precipitation with 4 volumes of isopropyl alcohol and centrifugation ($8000 \times g$, 15 min). After dehydration by solvent exchange the residues obtained were dried in vacuum over silica gel to obtain water extractable SGF (WE1SGF; yield 0.275 g) and NGF (WE1NGF; yield 1.97 g). The algal residues were extracted once more in the same way, giving the fractions WE2SGF (yield 0.33 g) and WE2NGF (yield 0.63 g).

Subsequently, the cold-water insoluble residue was autoclaved in distilled water (750 ml, 1.1 kg/cm^2) for 2.5 h. The supernatant solution recovered by centrifugation was filtered through a G-2 sintered glass filter, and the soluble material was recovered from the filtrate by precipitation with isopropanol as described above. The recovered material is referred to as Au1 fraction (yield 0.9 g). The insoluble debris was extracted twice more under similar condition (fractions Au2, yield 0.6 g; and Au3, yield 0.32 g). The remaining residue was then dehydrated by solvent exchange and dried as described above to yield the insoluble residue (INS; yield 1.4 g).

2.3. Alkali pretreatment and extraction of polysaccharides from the modified residues

Algal powder was pretreated with alkali as described by Lahaye et al. [16]. To a suspension of DAP (5 g) in 50 ml of water at 80 °C was added 0.5 g of $NaBH_4$ to prevent β elimination. After 15 min, 50 ml of 2 M NaOH solution was added. The suspension was heated for 1.5 h in a hot water bath at 80 °C and then cooled in an ice bath, neutralized by slow addition of acetic acid and dialyzed against water. The algal residues isolated by centrifugation were extracted twice with distilled water (35–40 °C, 500 ml) for 4 h. As the chemical compositions of the various SGF and NGFs of the first water extract from untreated DAP were similar to those of the second extracts, the two cold-water extracts obtained from the alkali pre-treated de-pigmented algal residues were mixed and then filtered through a sintered glass filter (G-2). The combined filtrate was treated as for the native cold-water extract and the recovered materials are referred to as AWESGF (yield 0.4 g) and AWENGF (yield 0.7 g). The residue was then autoclaved (1.1 kg/cm^2) twice for 2.5 h each. The two polysaccharide extracts were recovered separately as for the autoclaved extract described above and are referred to in the text as AAu1 (yield 0.43 g) and AAu2 (yield 0.07 g), respectively. The insoluble residues, after dehydration by solvent exchange, were then dried in vacuum to leave the insoluble residues (AINS; yield, 0.6 g).

2.4. Analytical methods

2.4.1. General

Chemicals used were analytical grade or best available. Except otherwise stated data presented in tables are mean values of at least three independent analyses. Experiments were repeated when the standard deviation (S.D.) was greater than 5%.

2.4.2. Protein analysis

Proteins present in soluble fractions were estimated by the method of Lowry et al. [17] using bovine serum albumin as standard. Amino acids were released by hydrolysis with 6 M HCl at 110 °C for 22 h in a sealed tube and analysed as described [18].

2.4.3. Sulfate estimation

Samples (90–100 mg) were hydrolysed in 5 ml of 2 N hydrochloric acid in sealed glass tubes at 100 °C. Sulfate was then determined using a modified [19] turbidometric barium chloride method of Tabatabai [20].

2.4.4. Sugar analysis

Amounts of neutral sugars were estimated by the phenol–sulfuric acid assay [21] using galactose as standard. The uronic acid content was determined by the *m*-hydroxydiphenyl assay as described by Ahmed and Labavitch [22] using galacturonic acid as standard. The content of 3,6-anhydro-L-galactose was determined colorimetrically by using a resorcinol assay, with fructose as the standard sugar [23]. Neutral sugars were released by hydrolysis in 1 M H_2SO_4 or according to Saeman and analyzed as their alditol acetates [24] by GLC on columns of SGE BP 225 and DB-225 (JW) as described [25]. Myo-Inositol was used as internal

standard, and hydrolytic losses were accounted for by using an external standard.

2.4.5. Mass spectrometry

EI mass spectra were recorded with Shimadzu QP5050A GLC/MS instrument at 70 eV. The characterisation of sugars, including 6-*O*-methyl galactose, was done on the basis of previously reported mass spectra [26,27] and relative retention times as described elsewhere [28].

2.4.6. Thin layer chromatography

The sugars released by acid hydrolysis (as described above) were also analyzed by TLC as described by Mondal et al. [29].

2.5. Molecular weight

Intrinsic viscosities [η] were determined at 32 °C using an Ostwald viscometer with a flow time 5.11 min for water. Viscosity average molecular weights were calculated from the intrinsic viscosity using the Mark–Houwink equation for agarose

$$[\eta] = 0.07\ M^{0.72}$$

where [η] is ml/gm as described by Rochas and Lahaye [30].

2.6. Infra red spectroscopy

Infrared spectra of agar films were recorded on a JASCO FT/IR 420 spectrophotometer.

2.7. ^{1}H and ^{13}C-NMR spectroscopy

Samples were dissolved in D_2O and nuclear magnetic resonance (NMR) spectra were acquired on a Bruker DRX-300 NMR spectrometer at 70 °C. ^{1}H chemical shifts were measured relative to external DSS. The starch content of the extracted polymers was estimated by determining the ratio between the integral of H-1 of glucose at 5.353 ppm [31] and of H-1's of 4-linked-L-galactose in the region from 5.152 to 5.286 ppm [32,33] and assuming a perfect alternating agar backbone. The pyruvate content was determined in the same way by using one third of the area of the methyl resonance at 1.467 ppm [34]. Methyl concentration was determined from the ratio of the integrations of the signals at 3.429 and 3.517 ppm for the methyl ether protons located on C-6 of D-galactose and C-2 of 3,6-anhydro-L-galactose, respectively, to the mean integral values for L-galactose or D-galactose, depending on the location of the methyl ether [33].

^{13}C NMR spectra of 4–5% (w/v) solutions in D_2O were recorded at 80 °C. Proton decoupled ^{13}C NMR chemical shifts were measured in parts per million relative to internal dimethylsulfoxide, set at 39.6 ppm.

2.8. Cells and viruses

Vero (African green monkey kidney) cells were grown as monolayers in minimum essential medium (MEM) (GIBCO, USA) supplemented with 5% inactivated calf serum. For maintenance medium (MM), 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). Virus stocks were propagated and assayed by plaque formation in Vero cells.

2.9. Antiviral assay

Antiviral activity was evaluated by reduction of virus plaque formation. Vero cell monolayers grown in 24-well plates were infected with about 50 plaque-forming units (PFU) of virus per well in the absence or presence of various concentrations of the compounds (two wells per concentration). After 1 h of adsorption at 37 °C, residual inoculum was replaced by MM containing 0.7% methylcellulose and the corresponding dose of each compound. Plaques were counted after 2 days of incubation at 37 °C. The inhibitory concentration 50% (IC_{50}) was calculated as the compound concentration required to reduce virus plaques by 50%. All tests were performed twice, in duplicate, and results are expressed as mean value ± S.D.

2.10. Virucidal assay

A virus suspension of HSV-2 containing 4×10^5 PFU was incubated with an equal volume of MM with or without compound for 1 h at 37 °C. The samples were then diluted in cold MM and residual infectivity was determined by plaque formation in Vero cells. Virucidal concentration 50% (VC_{50}) was calculated and results are expressed as mean value of two determinations ± S.D.

2.11. Influence of various treatment periods on the anti-HSV activity of the compounds

Vero cells grown in 24-well plates were infected with 50 PFU of HSV-1 or HSV-2 under different treatment conditions: (1) the cells were exposed to the virus in the presence of the compound (10 μg/ml) and after 1 h of virus adsorption, both compound and unadsorbed virus were removed, the cells were washed three times with PBS and were further incubated with MM containing 0.7% of methylcellulose. (2) The cells were exposed to the virus and after the adsorption period, unadsorbed virus was removed and the cells were further incubated with medium containing 10 μg/ml of the compound. (3) The compound was present both during and after the

adsorption period. After 2 days of incubation at 37 °C, virus plaques were counted.

2.12. Cytotoxicity assay

Vero cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich, USA) method. Confluent cultures in 96-well plates were exposed to different concentrations of the test compounds, 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 5 μg/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 micro plate reader at 595 nm. The cytotoxic concentration 50% (CC_{50}) was calculated as the compound concentration required to reduce cell viability by 50%. Results are expressed as mean value of two determinations ± S.D.

2.13. Assay for anticoagulant activity

The thrombin time (TT) was measured using pooled human plasma and various concentrations of compounds. Human plasma (170 μl) and test sample (30 μl) were mixed and the mixture was incubated at 37 °C for 2 min. Bovine thrombin (Sigma Chemical Co., USA; 7.5 U/ml, 100 μl) was added to the mixture and the time to clot formation was recorded.

3. Results and discussion

In order to study the chemical structures and physiological activities of polymers present in *Gracilaria corticata*, we have extracted them from DAP as shown in Fig. 1. The yields of the different extracted fractions are given in Table 1. Cold water extracted the highest amount (32.1%) of material from DAP. An additional fraction (18.2%) was then recovered by autoclaving the residual material. Attempts were made to purify the cold-water soluble polymers by a freeze-thaw process. Two fractions WE1SGF and WE1NGF were obtained from the first cold-water extract in the proportion of 28:19.8 (Table 1) and similarly, two other fractions WE2SGF and WE2NGF were obtained (Fig. 1) from the second cold-water extract. The overall SGF and NGFs correspond respectively to 9.1% and 23% of the starting algal material. Extraction of polymers from *Gracilaria corticata* at room temperature with water suggests the presence of loosely associated materials. In addition to these fractions, three further fractions Au1–Au3 (Fig. 1) were obtained by sequential extraction of

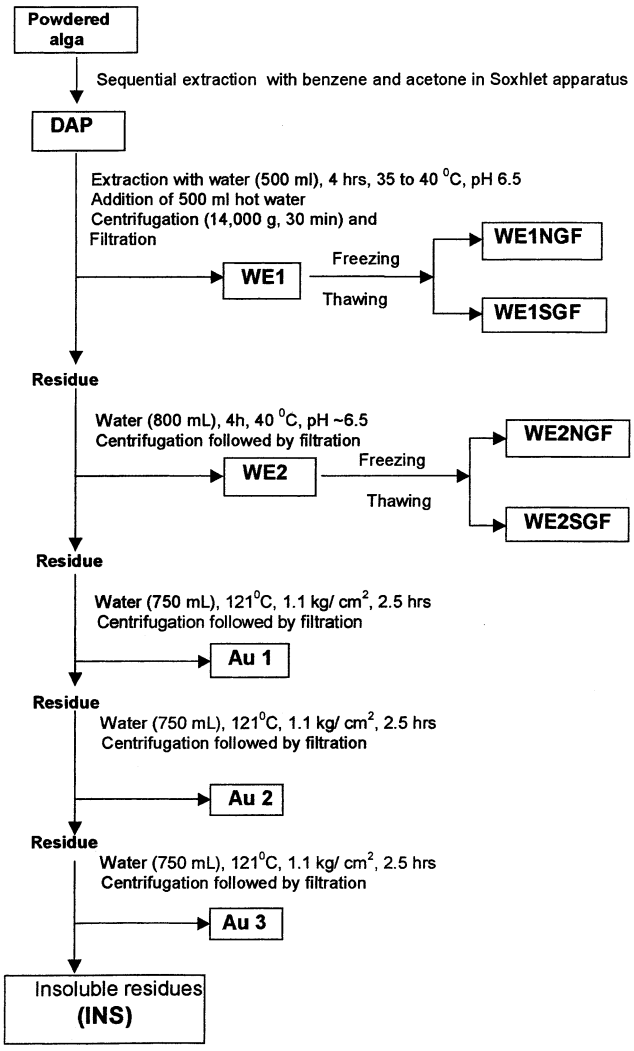

Fig. 1. Flow chart for the extraction of polymers from *Gracilaria corticata* with water under various conditions.

the cold-water un-extractable residue with hot water (121 °C).

The extraction procedure was repeated but following a pretreatment of DAP with alkali as described by Lahaye et al. [16]. The fractions AWESGF, AWENGF, AAu1, AAu2 and AINS were obtained from the alkali pretreated material as shown in Fig. 2. Comparison of the yield of soluble polymers from native *Gracilaria corticata* with those of the alkali pretreated material showed that alkali modification significantly reduced the yield of the extracted polymers.

GLC and GLC-MS analysis of the alditol acetates of the fractions showed that galactose was the major monosaccharide in all cold-water extracted fractions (Table 1). In addition to galactose, a large amount of 6-*O*-methyl-galactose was present in the hot-water extracted fractions. Some galactose was still left in the residues (INS) even after exhaustive extraction with cold and hot water. The presence of 2-*O*-methyl-3,6-anhy-

Table 1
Yields and chemical composition of fractions obtained by sequential extraction with aqueous solvents from native and alkali modified *Gracilaria corticata*

	Yield[a]	TS[b]	UA[b]	3,6-AG[b]	Sulfate[b]	Fuc[c]	Rha[c]	Ara[c]	Xyl[c]	Me–Gal[c]	Man[c]	Gal[c]	Glc[c]
DAP	100	nd	nd	nd	nd	3.2	–	2.6	6.3	15.0	2.1	23.4	47.3
WE1SGF	2.8	51.4	3.4	3.2	10.5	–	–	1.8	1.1	0.8	–	96.4	–
WE1NGF	19.7	54.4	4.3	2.1	11.7	–	–	1.1	1.0	0.8	–	96.8	–
WE2SGF	6.3	48.0	6.4	4.2	9.0	–	–	2.0	1.0	0.8	–	93.4	2.8
WE2NGF	3.3	50.0	5.3	3.3	11.5	–	–	1.3	1.3	0.8	–	94.3	2.3
Au1	9.0	44.0	6.6	8.8	2.1	0.9	–	1.8	–	43.4	–	25.6	28.3
Au2	6.0	41.8	6.3	17.8	2.6	2.0	–	2.5	tr	45.3	1.1	45.5	4.6
Au3	3.2	32.6	5.2	20.4	2.5	1.9	–	2.2	tr	49.1	–	44.6	2.2
INS	14.0	34.6	4.6	–	–	0.3	–	2.1	tr	1.3	3.7	5.1	87.6
AWESGF	8.0	65	nd	nd	2.7	3.0	–	4.8	9.2	4.8	3.7	17.1	57.4
AWENGF	14	70	nd	nd	3.1	5.3	tr[d]	2.6	4.0	19.5	–	14.1	54.5
AAu1	8.6	49	nd	16	2.4	6.5	–	2.3	4.2	22.9	6.1	9.6	48.3
AAu2	1.4	51	nd	18	2.5	10.1	–	3.7	9.2	14.4	tr	19.9	42.6
AINS	12	47	nd	nd	nd	–	3.9	4.3	15.0	1.8	1.3	63.6	10.1

See text for the identification of fractions. nd, not determined; –, not detected.
[a] Percentage weight of alga (DAP) dry weight.
[b] Percentage weight of the fraction dry weight.
[c] Mole% of anhydro sugar.
[d] tr, trace.

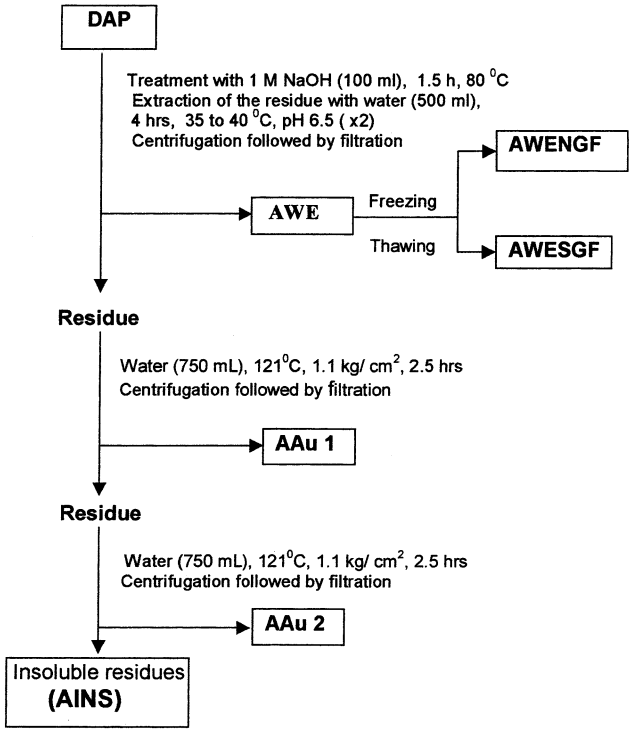

Fig. 2. Flow chart for the extraction of polymers from alkali pre-treated *Gracilaria corticata* with water under various conditions.

dro-galactose and 6-*O*-methyl-galactose was confirmed by NMR spectroscopy (Fig. 4). Arabinose, mannose, fucose and rhamnose were also present at lower levels. Glucose contents exhibit a broad range from trace amounts in the cold-water extracted fraction to 28.3% in the autoclaved fraction. The presence of a high amount of glucose in the acid hydrolysate of autoclave-extracted fractions suggested the presence of floridean starch granules in *Gracilaria corticata*. The uronic acid contents of the fractions varied between 3.4 and 6.6%. However, no uronic acid was found by TLC analysis and IR spectroscopic data (no band for carbonyl group was observed in the acid form of the polymers).

Chemical analysis and IR studies indicated the presence of sulfate ester group in all fractions, whose amount varied significantly from 2.1% to over 11.7% (Table 1). The cold-water extracted materials, particularly the NGFs extracts, contain very high amount of sulfate ester groups. There is a little difference in the chemical composition between the SGF and NGFs. The differences in behavior between these two fractions may be based on molecular size or probably some other chemical differences that could not be determined by the methods used for analysis. The low yield of the sugar was probably due to the extreme labiality of 3,6-anhydrogalactose to acid hydrolysis [35].

The neutral sugar compositions of alkali pre-treated fractions are shown in (Table 1). Glucose is the predominant sugar in all fractions (except AINS). Galactose and 6-*O*-methyl galactose were the next most abundant sugars. Small amounts of fucose, mannose, xylose and arabinose residues were also present. The protein content of the WE2NGF fraction was very small (3.2%, w/w). These proteins consist of leucine, aspartic acid, glutamic acid, serine, valine, glycine, alanine, isoleucine, lysine and phenylalanine in the molar percentage of 13.2, 13.1, 12.6, 11.8, 11.7, 11.4, 10.4, 7.5, 4.5, 3.6 respectively.

Infrared spectroscopy yields useful information on the sulfate and 3,6-anhydrogalactose contents in agars. The band at 1250 cm^{-1} for sulfate ester group [36] was present in the IR spectra of all the fractions (data not shown). The sulfate content, measured by considering the ratio of the intensity of the 1250 cm^{-1} band to the intensity of the 2920 cm^{-1} band [37], varied from 3.8 to 5.6 for the native fractions and from 0.1 to 0.6 for the alkali pretreated ones. The absorption band at 823 cm^{-1} characteristics for galactose-6-sulfate [38] was observed in the spectrum of WE2NGF fraction (Fig. 3a), but was absent in the spectrum of AWENGF (Fig. 3b). In addition, the later fraction showed a significant increase in the 3,6-anhydrogalactose content as evidenced by an increase in IR band at 936 cm^{-1} [39]. This means that band at 823 cm^{-1} was due to 6-sulfate on L-galactose unit, which is converted to 3,6-anhydrogalactose after alkali treatment. The 6-sulfate-α-L-galactose is known to be the precursor of the 3,6-anhydro-α-L-galactose [40]. The absorption band at 850 cm^{-1} for D-galactose-4-sulfate [36] was also observed in the spectra of alkali-pretreated fractions. Therefore, it can be concluded that only some of the sulfate groups are alkali labile. Besides, it seems that the sulfate groups, when present, occurs at C-6 of L-galactose and C-4 of D-galactose units.

NMR spectroscopy is a convenient method to follow the composition and to give valuable structural information of agars. The ^{1}H NMR spectrum of AAu2 fraction is given in Fig. 4. The anomeric resonances at 4.566 and 5.152–5.286 ppm originated from β-(1,3)-linked D-galactopyranose and α-(1,4)-linked-L-galactopyranose units, respectively [32,33,41]. Low intensity signals at 5.243 and 5.286 ppm were attributed to the anomeric proton of L-galactopyranose residue adjacent to a pyruvate substituted galactose residue and H-1 of the precursor 4-O-linked 6-sulfated-α-L-galactose, respectively [33,41,42]. Signal appearing at 5.353 ppm was attributed to the anomeric proton of the floridean starch [31]. The sharp signal at 1.467 ppm was due to the methyl group of pyruvic acid ketal in the R-form corresponding to the equatorial disposition of the methyl group [34,43,44]. This fraction shows a high content of pyruvate residues. The later accounted for 12% of the fraction as estimated from the integral of the anomeric signals. The very high intensity sharp signals appearing at 3.517 and 3.429 ppm in the ^{1}H-Spectrum were attributed to the methoxy group of 2-*O*-methyl-3,6-anhydro-L-galactose and 6-*O*-methyl-D-galactose, respectively [16,42]. The type and degree of methylation may provide useful information in taxonomic studies of *Gracilaria* sp. [33]. The hot water (121 °C) extracted polymers have high contents of pyruvate and high degrees of methylation as evidenced by their peak area.

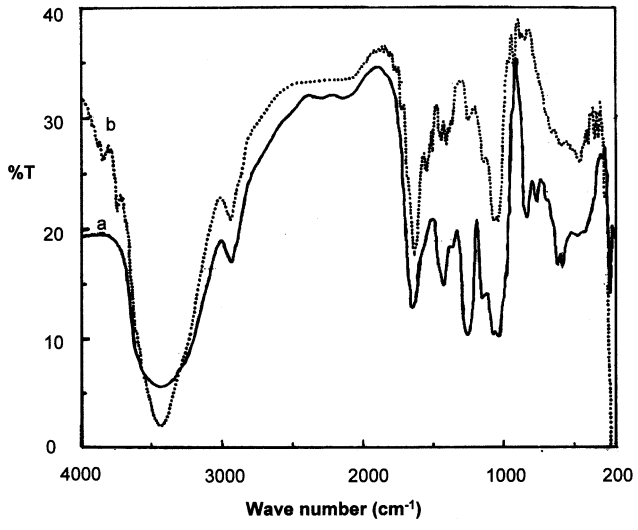

Fig. 3. FT-IR spectra of (a) WE2NGF and (b) AWENGF fractions isolated from *Gracilaria corticata* with water (see text for the identification of fractions).

The ^{13}C NMR spectrum of autoclaved fraction from *Gracilaria corticata* contained signals at 103.7 and 100.2-ppm characteristics of β-D-galactopyranosyl and 6-*O*-sulfate-L-galactopyranosyl residues corresponding to the repeating unit of the agarose biological precursors [45,46]. A series of signals (C-1, 100.1; C-2, 72.2; C-3, 73.8; C-4, 78.1; C-5, 71.9; C-6, 61.3 ppm) were detected in the ^{13}C NMR spectrum of the autoclave-extracted polymers. The presence of these signals demonstrates the presence of floridean starch in this fraction [16,47]. The characteristic chemical shifts due to 6-*O*-methyl-D-galactose (73.6 and 71.8 ppm) and 2-*O*-methyl-3,6-anhydro-L-galactose (82.7; 98.8; 78.9 and 78.6 ppm) [16] were also observed.

The isolated polysaccharides showed viscosity -average molecular weights between 165 197 and 52 404. These values, except for WE2NGF fraction, are notably lower than the values recorded for commercial samples but comparable to laboratory extracted agar type polymers [29]. The value for polymers in WE2NGF fraction is much lower than that of the native agar from *Gracilaria dura* [42]. The alkali pretreatment significantly reduced the molecular weight of the polymers, as seen in Table 2. This is clearly due to alkali promoted degradation of the polysaccharide chain, even using $NaBH_4$ to prevent alkaline peeling reaction during alkali pretreatment.

Taking into account that of all the fractions obtained through the isolation process, some were present in very small quantities and some others became difficultly soluble in water, the biological properties of three fractions that were highly soluble in water and were present in considerable amounts were evaluated. So, the fractions WE2NGF, Au2 and AAu2, were evaluated for their antiviral activity against HSV-1 and HSV-2 by a virus plaque reduction assay (Table 3). A dose-dependent inhibition of plaque formation was observed. The

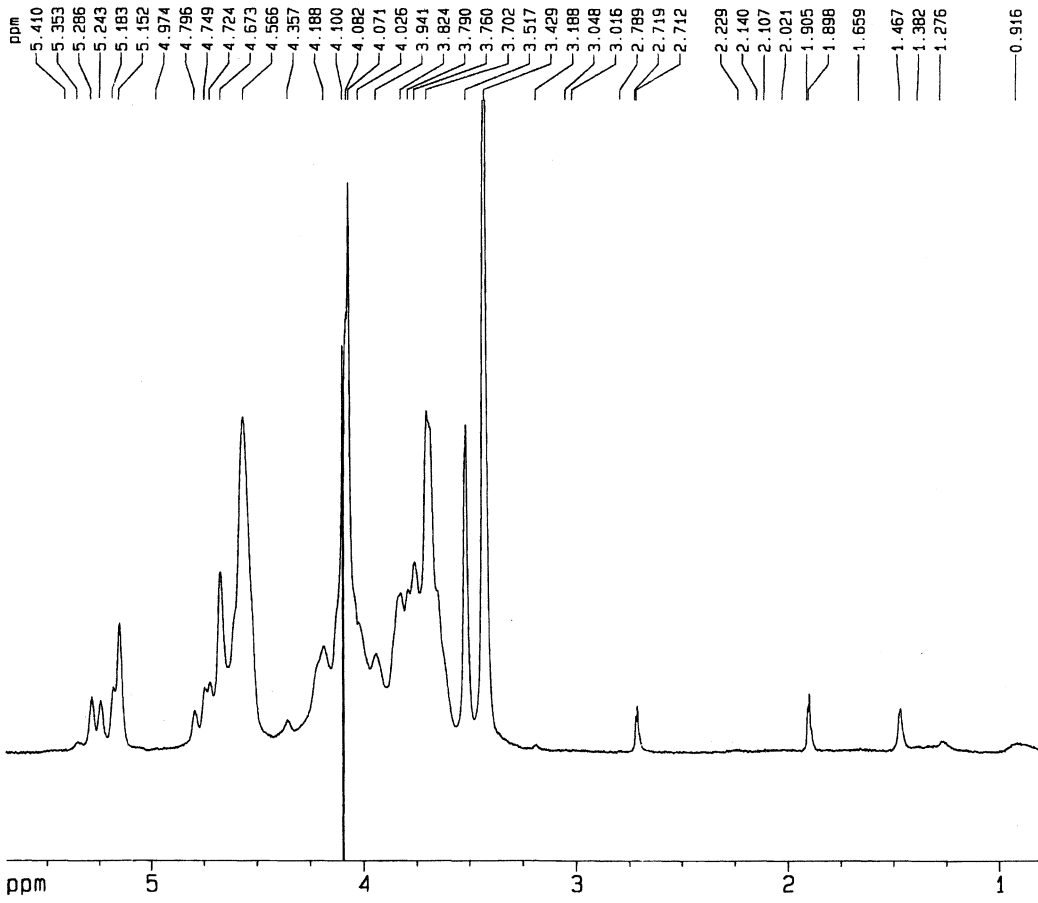

Fig. 4. ^{1}H-NMR spectrum of agar polymer extracted with hot water (121 °C) from *Gracilaria corticata* with alkali pre-treatment.

Table 2
Intrinsic viscosities [η] in ml/gm and viscosity average molecular weight (M) of the commercial agar and water-soluble polymers of *Gracilaria corticata*

Sample	Intrinsic viscosities [η]	Molecular weight (M)
WE2NGF	400.0	1 65197
AWE NGF	175.0	52 404
Au 2	197.5	61 990
AAu 2	180.0	54 494
Commercial agar	245.0	83 621

See text for the identification of fractions.

cold-water soluble non-gelling galactan WE2NGF elicited a marked antiviral activity, with IC_{50} values of 0.19 and 0.24 μg/ml for HSV-1 and HSV-2, respectively. This compound was more active than heparin (IC_{50}: 1.3 and 2.1 μg/ml for HSV-1 and HSV-2, respectively), used as a reference substance. The fractions Au2 and AAu2 were less active for both serotypes of herpes virus, with IC_{50} ranging from 27.5 to 50.0 μg/ml. An explanation for these results may be the fact that WE2NGF has a higher amount of sulfate ester groups and a higher molecular weight than the two other fractions tested. In general, the antiviral activity of sulfated polysaccharides increases with the degree of sulfation and molecular weight of the macromolecule [3]. A next point of interest is the influence of the distribution of sulfate groups along polymer chain and the conformational flexibility of this chain for adopting a definite shape that might be required during the formation of polysaccharide-virus complex [48].

No cytotoxicity was observed with any of the compounds when viability was evaluated on Vero cell monolayers in the presence of concentrations up to 1000 μg/ml. The selectivity index revealed high values for all the fractions (Table 3). Thus, they can be considered as selective inhibitors of HSV.

In order to analyze the possibility that these polysaccharides may directly inactivate the virus particle, a virucidal assay was carried out. Preincubation of virus with the compounds showed a virucidal effect on HSV-2 virions at concentrations 10–100 fold higher than the IC_{50} (Table 3). The lack of virucidal effect at concentrations near the IC_{50} confirms that the reduction observed in virus plaques was due to an interference with the virus replication cycle. Similar results were observed when the assay was performed against HSV-1 (data not shown).

As a first approach to establish the stage of the virus replication cycle at which the compounds exert their

Table 3
Antiviral and virucidal activities of the fractions obtained from *Gracilaria corticata*

Compound	IC_{50} (μg/ml)[a]		SI[b]		VC_{50} (μg/ml)[c]
	HSV-1	HSV-2	HSV-1	HSV-2	HSV-2
WE2NGF	0.19±0.010	0.24±0.03	> 5263	> 4166	19.18±0.58
Au2	27.5±3.2	38.4±2.28	> 36	> 26	472.5±15.5
Aau2	50.0±4.3	45.9±5.83	> 20	> 22	500.0±10.0
Heparin	1.3±0.1	2.1±0.1	> 769	> 476	ND

See text for the identification of fractions.
[a] IC_{50} (inhibitory concentration 50%): concentration required to reduce plaque number in Vero cells by 50%. Each value is the mean of two determinations ± S.D.
[b] SI (selectivity index): CC_{50}/IC_{50}. CC_{50} (cytotoxic concentration 50%): concentration required to reduce the number of viable Vero cells by 50% after 48 h of incubation with the compounds. This concentration was > 1000 μg/ml for all the compounds.
[c] VC_{50} (virucidal concentration 50%): concentration required to inactivate an HSV-2 suspension by 50%. Each value is the mean of two determinations ± S.D.

antiviral activity, a virus plaque reduction assay for HSV-1 and HSV-2 in Vero cells upon different treatment periods with WE2NGF was set up. As can be concluded from the data presented in Table 4, WE2NGF lost virtually all-antiviral activity when not present during the virus adsorption period. The presence of the compound only during virus adsorption was as effective as when present during the whole incubation period (during and after adsorption). The mechanism of action of the compound could thus be attributed to inhibition of virus adsorption, interfering with the interaction of viral glycoproteins with the negatively charged heparan sulfate receptors present in the cell membrane.

To evaluate the anticoagulant activity of WE2NGF, Au2 and AAu2, the TT was measured (Table 5). Treatment of blood with the different compounds at concentrations near the IC_{50}, did not significantly change the normal TT value. On the other hand, 0.5 μg/ml of heparin raised the TT to > 180 s. These results allowed us to conclude that the antiherpetic activity is not related to their anticoagulant properties, an important topic to be taken into account for the potential use of these compounds as antiviral drugs in vivo.

Table 4
Influence of various treatment periods on the antiherpetic activity of 'WE2NGF' fraction

Compound present	Inhibition (%)	
	HSV-1	HSV-2
During adsorption	88.6	87.9
During and after adsorption	89.7	91.3
After adsorption	0.5	3.4

See text for the identification of fraction and method.

Table 5
Anticoagulant activity of the fractions from *Gracilaria corticata*

Compound	Concentration (μg/ml)	TT (s)[a]
WE2NGF	200	118.5
	50	114.0
	0.5	32.5
Au2	200	23.5
	50	17.3
	0.5	14.4
AAu2	200	19.8
	50	19.0
	0.5	14.0
Heparin	20	> 180
	5	> 180
	0.5	45.4

See text for the identification of fractions.
[a] TT, thrombin time. TT for control sample without compound: 14.6 s.

4. Conclusions

Three main types of soluble polysaccharides were extracted from *Gracilaria corticata* collected from the shorelines of India. The major population that was extracted with water at room temperature was a high molecular weight galactan having sulfate group at C-6 of L-galactose and C-4 of D-galactose units. A second category of polysaccharide, solubilised by hot water under pressure and characterised by NMR was floridean starch. The third type of polysaccharide isolated with hot water is agar polymer having high degree of methylation and being pyruvated.

Alkali pretreatment significantly reduced the molecular weight of the cold water extracted polymers. This was due to alkali promoted degradation of the polysaccharide backbone. Moreover, alkali treatment converted L-galactose-6-sulfate residues of the polymer into 3,6-anhydro-α-L-galactose residues.

The high molecular weight galactan sulfate exhibits strong antiviral activity against HSV-1 and HSV-2. Unlike heparin and other known anionic polysaccharides, the *Gracilaria* polysaccharide contains no detectable anticoagulant activity. These results are in agreement with previous observations on the perspectives as antiviral compounds of galactan sulfates isolated from other red seaweeds [13,49,50]. Finally, this polysaccharide lacks cytotoxicity and therefore may be a potential anti HSV drug candidate.

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