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In vitro anti-herpetic activity of sulfated polysaccharide fractions from *Caulerpa racemosa*

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Abstract

A sulfated polysaccharide fraction was isolated from the hot water extract of the green alga *Caulerpa racemosa* and designated HWE. This polymer, which contained galactose, glucose, arabinose and xylose as the major component sugars, had $[\alpha]_D^{30} + 46.2^\circ$ in water and contained 9% sulfate hemiester groups. Sugar linkage analysis indicates that HWE was branched and mainly contained 1,3- and 1,3,6-linked galactose, 1,3,4-linked arabinose, 1,4-linked glucose and terminal- and 1,4-linked xylose residues. Sulfation was deduced from infrared spectroscopy and methylation analysis to occur on *O*-6 of galactose and *O*-3 of arabinose. The native polysaccharide could be fractionated by size exclusion chromatography into two overlapping fractions and the major fraction has a hydrodynamic volume similar to that of 70 kDa dextran. HWE was a selective inhibitor of reference strains and TK^- acyclovir-resistant strains of herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) in Vero cells, with antiviral effective concentration 50% (EC_{50}) values in the range of 2.2–4.2 μg/ml and lacking cytotoxic effects. Furthermore, HWE did not exhibit anticoagulant properties at concentrations near the EC_{50}.

Keywords: *Caulerpa racemosa*; Caulerpaceae; Sulfated heteropolysaccharide; Herpes simplex virus; Antiviral activity

1. Introduction

The antiviral properties of sulfated polysaccharides have been recognised several years ago (Andersson et al., 1979). In recent years, screening assays of the antiviral activity of extracts from a number of marine algae and cyanobacteria such as *Acanthophora spicifera* (Duarte et al., 2004), *Gracilaria corticata* (Mazumder et al., 2002), *Bostrychia montagnei* (Duarte et al., 2001), *Spirulina platensis* (Lee et al., 2001), *Sargassum horneri* (Preeprame et al., 2001), *Monostroma latissimum* (Lee et al., 1999), *Ulva lactuca* (Ivanova et al., 1994), among others, has led to the identification of a number of carbohydrate polymers with potent inhibitory effects against several animal viruses, including important human pathogenic agents (Franz et al., 2000; Gunay and Linhardt, 1999; Witvrouw and De Clercq, 1997). These polysaccharides include carrageenans (Carlucci et al., 1997), fucans (Preeprame et al., 2001), mannans (Kolender et al., 1997), rhamnan sulfates (Lee et al., 1999), and sulfated galactans (Mazumder et al., 2002; Duarte et al., 2001; Haslin et al., 2001; Witvrouw et al., 1994). Thus, the antiviral potential of sulfated polysaccharides extracted from algae becomes of considerable interest, although the structure–activity relationships are not always fully elucidated.

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A number of green algae is grown along Indian coastal line, with *Caulerpa racemosa* as a predominant species (Wealth of India, 1985). Mackie and Percival (1959, 1960, 1961) reported the presence of water-soluble polysaccharides in *Caulerpa filiformis*, whereas Rao and Rao (1986) studied the structural features of a sulfated polysaccharide of *Caulerpa taxifolia*. However, no studies on the antiviral activitiy of the polysaccharides present in *C. racemosa* have, to the best of our knowledge, yet been reported.

The purpose of the present study was to isolate polysaccharide fractions from the green alga *C. racemosa* and to investigate their chemical nature and antiviral activity against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2).

2. Results and discussion

2.1. Isolation and chemical composition

Polysaccharide fractions were extracted from depigmented algal powder (DAP) of *C. racemosa* as described in Section 3. The yields of the different extracted fractions are given in Table 1. The hot water extracted fraction (designated HWE) which had positive specific rotation $[\alpha]_D^{30} + 46.2^\circ$ (*c* 0.2, H_2O), was soluble in water. Sugar composition analysis of HWE fraction showed the presence of galactose, glucose, arabinose and xylose, together with smaller amounts of mannose and rhamnose and traces of fucose residues (Table 1). In addition to neutral sugars, 4% of uronic acid residue, 9% of sulfate hemiester group and 5% (w/w) protein were found. The protein present in this fraction contained leucine, aspartic acid, glutamic acid, serine, valine, glycine, alanine, isoleucine, lysine and phenylalanine in the molar percentage of 13, 13, 13, 12, 12, 11, 11, 7, 4 and 4, respectively. No other amino acids were detected.

The hot water insoluble residue was then successively extracted with strong alkali (1- and 4M-KOH) to isolate hemicellulosic polysaccharides. Monosaccharide composition analysis of the isolated fractions (1OH and 4OH) shows them to be enriched in xylose and glucose, the Glc/Xyl ratio being ~1.4 (Table 1). The hemicellulosic polysaccharide of *Ulva perisuta*, of the order Ulvales, is somewhat similar, except that it does not contain galactose residues (Ray and Lahaye, 1995a). The major sugar of the alkali insoluble residue is xylose, indicating the presence of a xylan. Mackie and Percival (1959) have reported the presence of a xylan in *C. filiformis* (see Fig. 1).

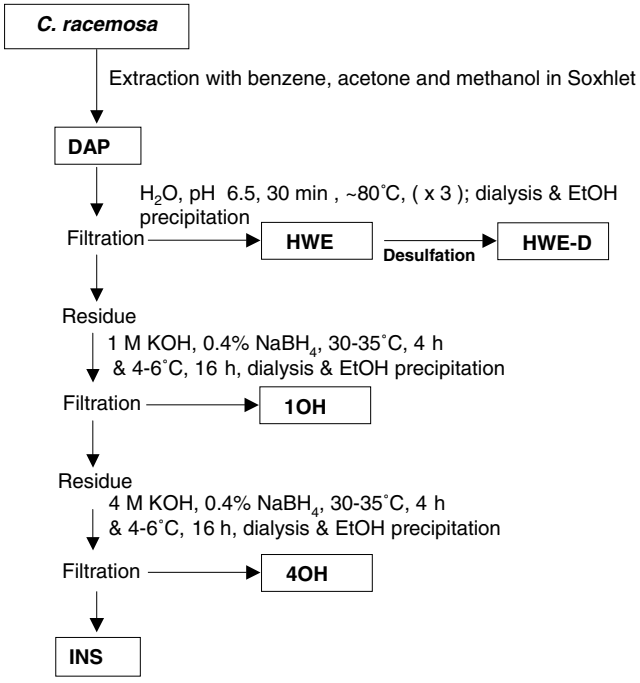

Fig. 1. Scheme for the extraction of polysaccharides from the green seaweed *Caulerpa racemosa* by sequential extraction with inorganic solvents.

Table 1
Yields and sugar composition of fractions obtained from *Caulerpa racemosa* (see text for the identification of fractions)

	DAP	HWE	1OH	4OH	INS	HWE-D	F1	F2
Yield[a]	100	20	12	16	40			
NS[b]	34	33	35	20	25	52	34	41
UA[b]	3	4	6	5	4	3	nd	nd
Rha[c]	Tr	1	Tr	Tr	Tr	–	–	–
Fuc[c]	–	Tr	Tr	1	–	–	–	–
Ara[c]	6	19	Tr	Tr	Tr	16	21	29
Xyl[c]	67	15	37	34	77	21	18	26
Man[c]	1	5	1	2	1	4	7	6
Gal[c]	5	31	11	14	6	30	23	39
Glc[c]	20	30	51	48	15	29	31	Tr

–, not detected; nd, not determined; Tr, trace.
[a] Percentage weight of DAP dry weight.
[b] Percentage weight of the fraction.
[c] Percentage mol.

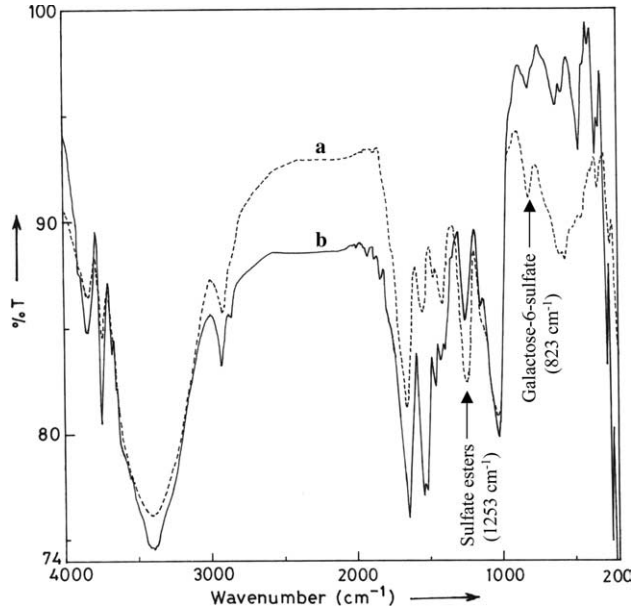

Fig. 2. FT-IR spectra of (a) hot water soluble polysaccharide fractions (HWE) obtained from *Caulerpa racemosa* and (b) their partially desulfated derivatives (HWE-D).

2.2. IR spectroscopy

Infrared spectroscopy provides useful information on the position of sulfate groups of polysaccharides. The IR spectrum of HWE showed an absorption band at 1253 cm^{-1}, indicating the presence of sulfate ester (Fig. 2). An absorption band at 823 cm^{-1} characteristic for galactose-6-sulfate (Mazumder et al., 2002) was also observed in this spectrum. However, the position of IR bands between 810 and 860 cm^{-1} could not be used with certainty to predict the position of sulfate substitution (Ray and Lahaye, 1995b).

2.3. Desulfation

It has been reported that the antiviral activities of polysaccharides are linked to the anionic features of the molecule (Witvrouw and De Clercq, 1997; Franz et al., 2000; Gunay and Linhardt, 1999). To investigate the effect of sulfate groups, the native polysaccharide was desulfated by the methanol/DMSO method (Nagasawa et al., 1977). Preliminary experiments (data not shown) showed that this method gives better recovery and the lowest sulfate content compared to auto-desulfation and the methanol–HCl method (Percival and Wold, 1963). Dry weight recovery of 51% was obtained but the desulfated derivative which still contained 3% of sulfate groups. IR spectrum of HWE-D showed a band at 1253 cm^{-1} confirming incomplete desulfation. The sugar composition of the native (HWE) and the partially desulfated material (HWE-D) are nearly similar (Table 1).

Table 2
Methylation analysis of hot water soluble polysaccharides fraction (HWE) isolated from *Caulerpa racemosa* and its desulfated derivative (HWE-D)

Methylation product[a]	Mol% of		Linkage pattern
	(HWE)	(HWE-D)	
2,3,4,6-Gal	2	1	Gal(1→
2,4,6-Gal	9	18	→3)Gal(1→
2,4-Gal	14	5	→3,6)Gal(1→
2-Gal	5	4	→3,4,6)Gal(1→
Gal	3	2	→2,3,4,6)Gal(1→
Total Gal	33	30	
2,3-Ara	6	16	→4)Ara(1→
2-Ara	17	12	→3,4)Ara(1→
Ara	5	2	→2,3,4)Ara(1→
Total Ara	28	30	
2,3,4-Xyl	10	12	Xyl(1→
2,3-Xyl	8	9	→4)Xyl(1→
Xyl	2	2	→2,3,4)Xyl(1→
Total Xyl	20	23	
2,3,4,6-Glc	1	1	Glc(1→
2,3,6-Glc	16	15	→4)Glc(1→
2,3-Glc	4	1	→4,6)Glc(1→
Total Glc	21	17	

[a] 2,3,4,6-Gal denotes 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, etc.

2.4. Linkage analysis

The glycosidic linkages and the positions of sulfate groups in the heteropolysaccharide fraction (HWE) were determined by methylation analysis of the native polysaccharide fraction and its partially desulfated derivative (HWE-D, Table 2). Permethylated native sample demonstrated the presence of mainly 1,3-, 1,3,6- and 1,3,4,6-linked galactose; 1,4 and 1,3,4-linked arabinose; terminal- and 1,4-linked xylose, and 1,4- and 1,4,6-linked glucose residues. Desulfation of HWE fraction increased the proportion of 1,3-linked galactose and 1,4-linked arabinose residues in HWE-D (Table 2). On the other hand proportions of 1,3,6-linked galactose as well as 1,3,4-linked arabinose residues reduced in HWE-D. These data demonstrate that *O*-6 of galactose and *O*-3 of arabinose were sites of sulfation.

2.5. Size exclusion chromatography

The hot water extracted polysaccharides were separated by size exclusion with Sephacryl S-1000 into two overlapping fractions, F1 and F2 (Fig. 3). Based on calibration with dextrans, the apparent molecular weight of the major peak (F2) of HWE would be 70 kDa while that of minor fraction (F1) might be estimated to 120 kDa. It should be noted however that polysaccharides containing sulfate groups, due to intramolecular electrostatic repulsions by charge effects, may have a different

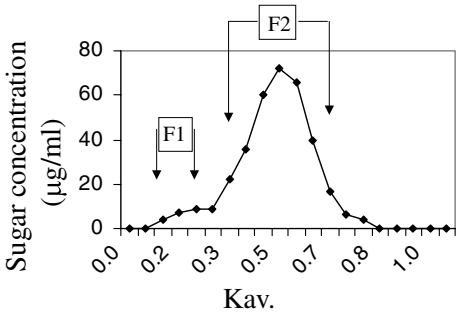

Fig. 3. SEC elution profile of hot water soluble polysaccharides fraction (HWE) obtained from *Caulerpa racemosa*. Collected fractions were analysed for total sugar content by phenol–sulfuric acid. Elution of polysaccharide was expressed as a function of the partition coefficient K_{av} [$K_{av} = (V_e - V_0)/(V_t - V_0)$ with V_t and V_0 being the total and void volume of the column determined as the elution volume of glucose and dextran (500 kDa), respectively, and V_e is the elution volume of the sample].

hydrodynamic volume than dextrans and, therefore, elute at a different rate than expected on the basis of their molecular weight. Recovery yield from the column was 72% on the total sugar basis. Fraction F1, which consists of 5% of the total sugar eluted from the column, contained most of the glucose, whereas fraction F2 contained high amounts of galactose, arabinose, xylose together with a smaller amount of mannose (Table 1). F2 contained 11% (w/w) sulfate.

2.6. Antiviral and cytotoxic activities

The HWE fraction was initially evaluated for cytotoxicity on preformed monolayers of Vero cells by MTT method. No cytotoxic effects were observed in the range of concentrations assayed up to 1000 μg/ml. The antiherpetic activity of HWE was then determined by a plaque reduction assay against reference strains of HSV-1 and HSV-2. The polysaccharide was effective to inhibit both serotypes of HSV to approximately the same extent in a dose-dependent manner (Table 3). Due to the lack of toxicity for cultured cells of the polysaccharide obtained from *C. racemosa*, high values of selectivity index (ratio between cytotoxic CC_{50} and antiviral EC_{50}) against HSV-1 and HSV-2 were obtained. To extend the spectrum of antiherpetic activity of this natural polysaccharide, HWE was evaluated against two TK^- strains of HSV-1 resistant to acyclovir, the antiviral compound presently in clinical use against human herpesvirus infections. HWE was inhibitor of these drug-resistant viruses, with EC_{50} and SI values comparable to those obtained against the reference strains (Table 3).

Table 3
Antiviral activity of HWE isolated from *C. racemosa* against herpes viruses

Virus	EC_{50}(μg/ml)[a]	SI[b]
HSV-1 (F)	4.2 ± 1.5	>238
TK^- HSV-1 (B2006)	2.4 ± 0.7	>417
TK^- HSV-1 (field)	2.2 ± 0.1	>454
HSV-2 (G)	3.0 ± 1.0	>333

[a] EC_{50} (effective concentration 50%): concentration required to reduce plaque number in Vero cells by 50%. Each value is the mean of two determinations ± standard deviation. The CC_{50} (cytotoxic concentration 50%: concentration required to reduce 50% the number of viable cells) was >1000 μg/ml.

[b] SI (selectivity index): ratio CC_{50}/EC_{50}.

The antiviral activity against HSV-1, strain F, and HSV-2, strain G, of the partially desulfated derivative HWE-D was also evaluated. Surprisingly, the values of EC_{50} for HWE-D were similar to those obtained for the original fraction HWE (data not shown). Because of the great diversity in chemical structure among sulfated polysaccharides with antiviral activity, the structure–activity relationship for this class of polyanions has not been clearly established. However, different investigators have reported that the antiviral activity of sulfated polysaccharides increases with the degree of sulfation and the molecular weight of the macromolecule (Witvrouw and De Clercq, 1997; Lüscher-Mattli, 2000; Haslin et al., 2001). In order to exhibit antiviral activity, the molecular weight of the polymers must be at least 5–10 kDa and the molecule must contain more than two SO_3^- groups per sugar residue. It must, however, be emphasised that structural and conformational aspects of the polymer, as well as charge density and/or distribution also play an important role for antiviral properties. The behavior of HWE-D may be due to the only partial desulfation produced in the polysaccharide by the method here employed. The remaining degree of sulfation in HWE-D, together with other structural characteristics of the polysaccharide not yet elucidated, may be enough to maintain the antiviral activity of the compound.

2.7. Anticoagulant activity

The activated partial thromboplastine time (APTT) and the thrombine time (TT) were measured to evaluate the anticoagulant activity of the antiviral polysaccharide HWE. The APTT and TT values of the blood treated with saline were 45 and 20 s, respectively. Treatment of the blood with HWE at a concentration near the antiviral EC_{50}, such as 2 μg/ml, did not significantly alter both coagulation parameters since the APTT and TT values were not duplicated compared to control samples (Table 4). Concentrations as high as 20 or 200 μg/ml, which are about 9–90 times higher than the EC_{50} against HSV-1 and HSV-2, were necessary to affect the APTT or TT values, respectively. However, it must be noted that HWE had a markedly reduced anticoagulant activity when compared with heparin. In fact, the APTT and TT of HWE were of the same order of those of heparin only at concentrations 10–100 times higher, respectively (Table 4). These results allow to conclude that the antiviral properties of HWE are not correlated with anticoagulant activity.

Table 4
Anticoagulant activity of HWE isolated from *C. racemosa*

Compound	Concentration (μg/ml)	TT[a] (s)	APTT[b] (s)
HWE	200	>180	>180
	20	43	>180
	2	34	65
Heparin	20	>180	>180
	2	>180	>180

[a] TT: thrombin time. TT for control sample with PBS: 20 s.
[b] APTT: activated partial thromboplastine time. APTT for control sample with PBS: 45 s.

In conclusion, this study describes, for the first time, the antiviral activity against reference strains and TK^- acyclovir-resistant strains of HSV-1 and HSV-2 of hot water extracted polysaccharide fractions from the green alga *C. racemosa*. Here it is shown that: (1) three families of polysaccharides namely, sulfated heteropolysaccharides, polymers containing xylose and glucose (xyloglucans) and skeletal xylans are present in *C. racemosa*, and (2) the sulfated heteropolysaccharide fractions present in HWE are highly branched and selective in vitro inhibitor of HSV-1 and HSV-2, without undesirable anticoagulant properties at the antivirally active concentrations.

HSV-1 and HSV-2 are responsible for a wide range of human diseases, particularly seriously in immunocompromised individuals (Whitley and Roizman, 2001). Prolonged therapies with acyclovir in this kind of patients have resulted in some undesirable complications and also induced the emergence of drug-resistant virus. Therefore, there is a real need for new compounds directed to a different target in the viral replicative cycle. To this end, the polysaccharide from *C. racemosa* may represent an interesting alternative to be considered against herpes virus infections.

3. Experimental

3.1. Plant material

Samples of *C. racemosa* were collected from the Gujrat coast of Western India in August 1995. The gathered material was sorted, washed and dried immediately by forced air circulation at 35–40 °C. After grinding in a warring blender, the seaweed (130 g) extracted sequentially with benzene (20 h), acetone (20 h) and methanol (20 h) in a Soxhlet apparatus to leave a DAP (yield 85 g).

3.2. General

All determinations were done at least in duplicate. IR spectra (KBr disc) were obtained with a JASCO FTIR 420 spectrophotometer.

Total sugars and uronic acids were determined by the phenol–sulfuric acid (Dubois et al., 1956) and *m*-hydroxydiphenyl (Ahmed and Labavitch, 1977) assay, respectively. All fractions were hydrolysed in 1 M sulfuric acid (3 h, 100 °C) for measurement of individual neutral sugar, with an additional pre-treatment of 72% sulfuric acid (1 h, 30 °C) for insoluble residues. The individual sugars were reduced, acetylated and analysed as their alditol acetate (Blalkeney et al., 1983) by GLC and GLC/MS (Shimadzu QP5050A) on columns of SGE BP 225 and DB-225 (JW). *myo*-Inositol was used as internal standard.

3.3. Extraction of polysaccharides

The DAP (4 g) was extracted sequentially with: (i) water (200 ml, pH 6.5) at 80 °C for 30 min, three times (HWE); (ii) 500 ml of 1 M KOH + 20 mM $NaBH_4$ at 30–35 °C for 4 h and then 16 h at 4–6 °C (1OH) and (iii) 500 ml of 4 M KOH + 20 mM $NaBH_4$ at 30–35 °C for 4 h followed by 16 h at 4–6 °C (4OH). Alkaline extracts were acidified with acetic acid to pH 6 and all extracts dialysed extensively against water using membrane having molecular weight cut-off of 12 kDa. Soluble materials were recovered by diluting the retentate with ethanol to a final concentration of 80% (v/v). The final residue was suspended in water, acidified to pH 5.2, dialysed and lyophilised to produce the insoluble residue (INS).

3.4. 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 turbidometric barium chloride method (Craigie et al., 1984).

3.5. Protein and amino acid analysis

Proteins present in soluble fractions were estimated by the method of Lowry et al. (1951) 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. The liberated amino acids were analysed by Pharmacia LKB ALPHA PLUS amino acid analyser.

3.6. Desulfation

Desulfation of the pyridinium salt of the polysaccharide with dimethyl sulphoxide (DMSO) containing 10% methanol was carried out at 100 °C as described (Nagasawa et al., 1977) to yield partially desulfated polymeric fractions (HWE-D).

3.7. Size exclusion chromatography

Solutions (5 ml) of HWE in 500 mM sodium acetate buffer (pH 5.0) was loaded to a column of (90 cm × 2.6 cm) Sephacryl S-1000 equilibrated with the same buffer. The column was eluted ascendingly with the same buffer

at 20 ml h^{-1} and the temperature was 30–35 °C. Elution of polysaccharide was expressed as a function of the partition coefficient K_{av} [$K_{av} = (V_e - V_0)/(V_t - V_0)$ with V_t and V_0 being the total and void volume of the column determined as the elution volume of glucose and dextran (500 kDa), respectively and V_e is the elution volume of the sample]. The column was calibrated with standard dextrans within a molecular weight range of 10,000–500,000. Fractions (7.5 ml) were collected and analysed for total sugar content using glucose as standard by phenol–sulfuric acid. Standard dextrans were a gift from Dr. Tapani Vuorinen.

3.8. Methylation analysis

Methylation was carried out by the method of Harris et al. (1984). Permethylated alditol acetates were analysed by GLC and GLC/MS (Shimadzu QP 5050A GLC/MS) as described (Ray et al., 2004).

3.9. Cells and viruses

Vero (African green monkey kidney) cells were grown in minimum essential medium (MEM) supplemented with 5% bovine serum. For maintenance medium (MM), serum concentration was reduced to 1.5%.

HSV-1 strain F and HSV-2 strain G were obtained from the American Type Culture Collection (Rockville, USA) and used as reference strains; B2006 and Field were HSV-1 TK^- strains received from Prof. Dr. E. De Clercq (Rega Institute, Leuven, Belgium). Virus stocks were propagated and titrated by plaque formation in Vero cells.

3.10. Cytotoxicity test

Vero cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma–Aldrich) method (Mosmann, 1983). Confluent cultures in 96-well plates were exposed to different concentrations of the polysaccharide, with three wells for each concentration, using incubation conditions equivalent to those used in the antiviral assays. Then 10 μl of MM containing MTT (final concentration 0.5 mg/ml) was added to each well. After 2 h of incubation at 37 °C, the supernatant was removed and 200 μl of ethanol was added to each well to solubilise the formazan crystals. After vigorous shaking, absorbance was measured in a microplate reader at 595 nm. The cytotoxic concentration 50% (CC_{50}) was calculated as the compound concentration required to reduce cell viability by 50%.

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

3.12. Assays for anticoagulant activity

Anticoagulant activity was determined using the activated partial thromboplastine time (APTT) assay (Andersson et al., 1979) and thrombine time (TT) (Carlucci et al., 1997), using heparin as the standard reference and polysaccharide in various concentrations (2-200 μg/ml).

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